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INDEX TO AUTHORS
INDEX TO SUBJECTS

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REVIEW ARTICLE

Hormones and Resistance

H. SELYE

Keyphrases ☐ Hormones and resistance—literature review ☐ Catatoxic, syntoxic steroids—classification ☐ Protective steroids—identification, assessment ☐ Steroids—protective spectrum ☐ Detoxication activity—steroids

CONTENTS

INTRODUCTION 1
HISTORY 3
CURRENT INVESTIGATIONS ON PROTECTIVE STEROIDS 7
Procedure for *In Vivo* Identification of Protective Steroids and Their Substrates 8
First Step: Protection against Digitoxin and Indomethacin. 17
Second Step: Determination of "Protective Spectrum" 18
Third Step: Identification of Damaging Agents Amenable to Prophylaxis 21
Special Studies: Unusual Effects of Certain Protective Steroids 23
RETROSPECT AND PROSPECT 24
Reevaluation of Methodology 24
Possible Clinical Applications 24
SUMMARY 26
REFERENCES 26

INTRODUCTION

The purpose of this article is to review our knowledge concerning the hormones that influence the resistance of the body to changes in its external and internal environment. Special attention will be given to the natural steroids and their synthetic derivatives, because they have been most systematically investigated in connection with the humoral control of resistance.

We became interested in this matter when we began to realize the decisive role played by hormones in the "*General Adaptation Syndrome*" (*G.A.S.*), the stereotyped response to stress as such, which develops whenever exposure to any kind of stimulus requires acute or chronic adaptive readjustment.

In the earliest stages of the G.A.S., the instantly acting epinephrine and norepinephrine, later the corticoids, appear to be more important for defense. Among the latter, the glucocorticoids play a particularly crucial part in the regulation of nonspecific resistance. They are secreted under the influence of ACTH, whose discharge from the pituitary is in turn regulated by a hypothalamic releasing factor. These observations showed that a whole chain of endocrine messengers is concerned with the maintenance of resistance to environmental changes.

It is not yet clear to what extent hormones, other than those of the hypothalamus–pituitary–adrenal axis, influence adaptability to stress in general, but numerous accidental observations have suggested that resistance to many pathogens can also be greatly enhanced or diminished by an excess or deficiency of thyroid, gonadal, and pancreatic hormones. These may be secreted in response to a need, or they may modify reactivity merely through their continuous presence in the body, irrespective of requirements.

Originally, the principal functions of hormones were seen in the regulation of metabolism in general, differentiation, growth, and sex. Analysis of the mechanism of the G.A.S. called attention to the fact that at least pituitary and adrenal hormones participate in a natural adaptive mechanism. Occasional observations on changes in resistance to certain agents caused by other hormones were usually brushed aside as mere curiosities

or incidental "pharmacologic actions" having no fundamental biologic importance.

The time has come to question this view. Resistance to many drugs is influenced by the removal of endocrine glands or the administration of physiologic amounts of their hormones which could hardly be said to act as "drugs." To facilitate work on the role of endocrine factors in resistance, this review was designed to accomplish a dual task: (*a*) to survey and correlate the relevant observations scattered throughout the literature, many of which are hard to find, since they are often recorded incidentally in publications on other topics; and (*b*) to describe numerous (partly unpublished) personal observations on the effect of various hormones upon adaptation to exogenous stimuli.

At this stage, it is still not easy to detect much lawfulness in the hormonal control of nonspecific resistance apart from the G.A.S.; yet, it is now, when pertinent systematic studies are just beginning, that an inventory of the established facts is most urgently needed.

Meanwhile, it is even difficult to see how this kind of research should be planned. In the past, relevant facts were obtained mainly by chance, but we are not likely to succeed in unravelling the complex hormonal regulatory system of resistance by mere empiricism.

Of course, the great question is: Through what mechanisms do hormones affect resistance? We have learned that some of them, the "*syntoxic hormones*," merely adjust the body's response, so that it tolerates pathogens without attacking them; others, the "*catatoxic hormones*," actually destroy the aggressor, mostly through the induction of drug-metabolizing enzymes. That much has been found more or less by chance.

Because of their antistress effect, the glucocorticoids proved to be highly efficient in normalizing the otherwise low resistance of adrenalectomized animals to virtually all types of damage. However, our hopes of raising stress resistance above normal did not materialize. Treatment with neither corticoids nor with any other hormones succeeded in increasing nonspecific resistance much above the level assured by a normally functioning endocrine system. Yet, the experiments which established this disappointing fact, quite unexpectedly, also showed that certain hormones or hormone derivatives possess an extraordinary protective effect at least against certain types of intoxications. Thus, we saw that thyroxine, long known to antagonize acetonitrile, also protects against such diverse lesions as the nephrocalcinosis produced by dietary excess of NaH_2PO_4, the skeletal changes elicited by lathyrogenic amines, and intoxication with elementary yellow phosphorus. Later we found that ethylestrenol prevents digitoxin poisoning and shortens the anesthetic effect of various barbiturates and steroid hormone derivatives. It was particularly instructive to learn that a steroid need not possess any classic hormonal properties to protect against drugs. The first typical catatoxic steroid of this kind, "CS-1" (the antimineralocorticoid compound bearing the factory code number SC-11927), protected against acute intoxication with dihydrotachysterol as well as against the infarctoid cardiac necroses produced by various combinations of corticoids, electrolytes, lipids, and stress. Conversely, thyroxine proved to increase sensitivity to various insecticides, anticoagulants, and indomethacin. These, and numerous other observations which shall be discussed here, showed that resistance to many agents is decisively influenced by the endocrine system.

We still had no way of predicting which hormones would increase or decrease the effect of a given drug, but it became clear that animals are endowed with a complex, comparatively nonspecific *general hormonal defense system comparable in its scope to those based upon nervous or immune reactions.* When faced with situations that require adaptation, the organism can respond essentially through three distinct pathways:

1. The nervous system: conscious planning of defense, development of appropriate conditioned reflexes (Pavlov), and autonomic "emergency reactions" (Cannon).

2. Antibody formation: immunity (Pasteur), including even such derailed, actually pathogenic, defensive responses as anaphylaxis (Richet) or allergy (v. Pirquet).

3. The adaptive hormonal system: the syntoxic hormones, which permit tolerance of the pathogen and the catatoxic substances that attack the aggressor.

It was on the basis of these considerations that we decided to initiate systematic investigations on the possible resistance-modifying effect of a carefully selected series of hormones and hormone derivatives with vastly different endocrine properties. These compounds were tested against numerous drugs, chosen more or less at random; yet, the toxicity of most of them was decisively influenced by one or the other compound in our series. Random fact gathering is not a very elegant way of scientific investigation; yet, in the beginning, all we could do was to test many hormones for their possible protective effect against many agents. At this stage our work was not guided by any logically conceived theory concerning underlying mechanisms; it rested merely on the hope that the adaptive hormones could be properly classified on the basis of their defensive actions as manifested by simple observations *in vivo*. If so, the individual members of each class thus identified could then be subjected to a more profound pharmacokinetic analysis.

In other words, we had to determine first which hormone protects against which drug, before we could explore how it did this. We had to know first that a hormone has adaptive value before we could ask whether this is due to a syntoxic or a catatoxic mechanism. Such observations as the fact that an indomethacin-induced intestinal ulcer can be prevented by ethylestrenol, or that cortisol aggravates certain infections, reveal nothing about how these hormones work; but only findings of this type can tell us where further research would be rewarding.

Of course, scientists can rarely identify by direct observation the things that they are looking for; most of the time they have to be guided by indirect indexes. The chemist often first detects a compound, or even a particular functional group in its molecule, by inference from a color reaction, a melting point, or the formation of a characteristic precipitate. The physician must suspect the presence of a microbe by noticing certain clinical signs and symptoms before he can verify his

diagnosis by looking for a particular organism. It is perhaps not too daring to hope that in our first efforts to clarify the role of hormones in resistance, simple, directly visible indicators might also serve us best.

These thoughts have guided the experimental investigations and the selection of the literature discussed in this review. Therefore, major emphasis will be placed on such immediately detectable manifestations of activity as morphologic and functional changes or mortality rates, these being most suitable for the large-scale experimentation on many compounds that is required to obtain material for meaningful generalizations. Unfortunately, it will not be possible to present an equally complete picture of the much more fundamental biochemical changes responsible for the observed phenomena. Besides, in most cases, these have not yet been elucidated; but where such information is available, key references will be given, especially to the most important data on enzyme induction.

It would have been redundant to burden the bibliography of this review by the repetition of literature surveys on related phenomena that were previously published in other monographs. These data helped us considerably in the evaluation of the topics presented here and will be incorporated in the discussions, but without specific reference to individual papers. In particular, it would have served no useful purpose to dilute our account with the voluminous literature on the antistress, antiphlogistic, immunosuppressive, ulcerogenic, and other well-known actions of corticoids, or the specific interactions between various sex hormones. The same is true of the bibliographies on the restoration of resistance by corticoids in adrenal insufficiency and on the hormonal control of various "pluricausal lesions." For details on all of these subjects, the reader is referred to the corresponding sections of several monographs (1–13).

HISTORY

The idea that the body possesses inherent mechanisms for the restoration of health after exposure to pathogens is very old; it was clearly recognized by Hippocrates (460–377 B.C.) as the remarkable "*vis medicatrix naturae*." However, this concept gained much in precision when Bernard (14) pointed out that the internal medium of living organisms is not merely a vehicle for carrying nourishment to cells far removed from contact with the outside world, but that "it is the fixity of the '*milieu intérieur*' which is the condition of free and independent life." The English physiologist Haldane (15) said of this phrase that "no more pregnant sentence was ever framed by a physiologist." Certainly, few if any statements about life have been more frequently quoted, but one wonders whether its great impact was not largely due to what has been intuitively read into it. Naturally, the fixity of any system is what makes it independent of changes in its surroundings—indeed the independence, the resistance of any system, is what we call its fixity—but many inanimate objects are more independent of their atmosphere than living beings. The salient feature of life, the secret of its resistance, is adaptability to change, not rigid fixity.

A much greater merit of Bernard was to call attention to the importance of mechanisms safeguarding the immutability of the "*milieu intérieur*." Thereby he stimulated innumerable investigators throughout the world to follow him in his classic investigations on the adaptive changes responsible for the "steady state."

Pflüger (16) pointed to the relationship between active adaptation (the "*vis medicatrix naturae*") and the "steady state" by his famous dictum: "The cause of every need of a living being is also the cause of the satisfaction of that need."

A similar thought was expressed by the physiologist Fredericq (17) when he said: "The living being is an agency of such sort that each disturbing influence induces by itself the calling forth of compensatory activity to neutralize or repair the disturbance."

Richet (18) wrote as a commentary about the steady state that: "By an apparent contradiction it (living matter) maintains its stability only if it is excitable and capable of modifying itself according to external stimuli and adjusting its response to the stimulation. In a sense it is stable because it is modifiable—the slight instability is the necessary condition for the true stability of the organism."

The great physiologist Cannon (19) has spent some 20 years of his life studying various mechanisms that help the organism to maintain its steady state, which he first called "*homeostasis*." Cannon's most important contribution was to show that there exist numerous highly specific homeostatic mechanisms for protection against hunger, thirst, hemorrhage, or agents that tend to disturb the normal body temperature, the blood pH, or the plasma level of sugar, protein, fat, or calcium. He placed special emphasis upon the stimulation of the sympathetic nervous system, with the resulting catecholamine discharge, which occurs during acute emergencies, such as pain or rage.

He taught us that this autonomic response induces metabolic and cardiovascular changes that prepare the body for fight or flight. Cannon's classic studies revealed many valuable facts about the mechanism through which the steady state of the "milieu intérieur" can be maintained in the face of agents that tend to alter one or the other of its constituents selectively. It soon became evident also that, in addition to the nervous system, hormones play an important part in such specific adaptive responses, *e.g.*, adrenal medullary catecholamines and pancreatic hormones in the maintenance of carbohydrate metabolism, parathyroid hormone in calcium homeostasis, and thyroid hormones in temperature regulation.

Stimulated by all these earlier findings, we became interested in the possible nonspecific adaptive function of hormones against what we called "*biologic stress*," that is, the nonspecific response of the organism to any demand made upon it. In 1936, we observed "a syndrome produced by diverse nocuous agents" (20), which was essentially the same irrespective of the evocative agent and later became known as the "stress syndrome." It was characterized, among other things, by manifestations of adrenocortical hypertrophy and increased production of those steroids for which we recommended the terms "glucocorticoids" or "antiphlogistic corti-

coids" because of their characteristic effects on sugar metabolism and inflammation.

The principal antistress and antiphlogistic actions of these adrenocortical hormones depend upon their syntoxic effects; they help to tolerate pathogens, not to destroy them.

As time went by, it became evident that many of the manifestations of "*nonspecific resistance*" (or "cross-resistance") induced by stress, especially those that offer protection against inflammatory lesions, are due to the activation of the hypothalamus–pituitary–adrenocortical axis. However, by 1961, we had seen that "certain types of cross-resistance are demonstrable even in adrenalectomized animals, and in some cases, increased thyroid-hormone activity appears to be the cause of the induced tolerance. The bulk of evidence now available suggests that all forms of cross-resistance cannot be attributed to any single biochemical mechanism. This is true even of those very nonspecific types that are induced by stress itself. We must remember that, although the response to stress is essentially stereotyped and largely independent of the evocative agent, it represents a mosaic of numerous local and systemic, humoral and nervous reactions, some of which may protect against one pathogen, others against another" (21).

Thus, it became clear that there exist adaptive mechanisms that are nonspecific both as regards their causation and their effects: they can be activated by many agents and they can protect against numerous pathogens. Some of these nonspecific adaptive phenomena are undoubtedly regulated through the hypothalamus–pituitary–adrenocortical axis; these largely depend upon the resulting suppression of inflammatory lesions by an excessive production of glucocorticoids. However, we had to conclude that there must exist additional mechanisms which raise nonspecific resistance through other means, since they are manifest even following removal of the adrenals. Little was known at that time about the nature of these additional resistance phenomena, except that: (*a*) unlike glucocorticoid-dependent reactions, they are not directed particularly against stress or inflammatory changes; (*b*) their protective effect, although not specifically opposing any one agent, is not as general as that of glucocorticoids; and (*c*) they often raise resistance far above normal and do not merely restore the low stress resistance of hypocorticoid individuals toward the norm.

The effects of stress and of the hormones produced during stress have been extensively discussed in several earlier monographs (2–7, 22). Here we shall place major emphasis upon those adaptive hormones that act either by accelerating the metabolic degradation of pathogens or through unknown mechanisms, as long as they increase resistance nonspecifically to many agents and do not merely rectify one particular homeostatic derangement.

For many among these adaptive hormones, it has not yet been clearly shown that they can be secreted in response to a need; nevertheless, they undoubtedly represent decisive factors in disease susceptibility, since their concentration in the "milieu intérieur" can determine whether a stimulus will or will not be pathogenic.

These were the main facts and speculations that guided our research on the hormonal regulation of resistance. However, in addition to this concise introductory sketch, designed to trace the outlines of our own approach, a historic survey of the field should mention some independently made findings which, undoubtedly, have also influenced our thinking and are likely to stimulate further research in this domain.

The *thyroid gland* was probably the first endocrine organ whose role in detoxication could be shown by objective animal experiments. At the beginning of this century, Hunt (23) demonstrated that mice given thyroid powder in their food become unusually resistant to acetonitrile; this increased drug tolerance has even been used as a basis for the bioassay of thyroid preparations.

The role of the *adrenals* as organs of detoxication has been suspected for a long time, but at first only on the basis of very indirect evidence. There was much discussion about whether poisonous substances brought to the glands by the blood are destroyed locally, or whether the adrenals increase resistance by remote action through their hormones. Either interpretation appeared to be equally compatible with the striking diminution of drug resistance seen in adrenalectomized animals and in patients with Addison's disease (24, 25).

The problem was further complicated by the fact that some investigators violently denied that bilateral adrenalectomy decreases tolerance to toxicants (*e.g.*, morphine), stating that the positive results of earlier workers were merely due to postoperative shock. Rats that survived the first few days of the postoperative period showed normal resistance despite the absence of their adrenals (26). In the light of our subsequent work, it seems highly likely that the particular strain of rats that recovered its drug resistance had accessory adrenals, or that the adrenalectomy was incomplete, and that the restoration of drug resistance was due to compensatory hypertrophy of cortical remnants. This question was subsequently settled when numerous investigators showed that after adrenalectomy, resistance to various drugs can be restored by adrenal extracts. In this respect, adrenocortical preparations proved to be much more efficient than medullary catecholamines. Still later, the extraction, followed by the synthesis, of pure corticoids made it possible to establish certain relationships between the resistance-increasing effect of these compounds and their chemical structure.

By 1940, it became evident that whereas cortical extracts are highly efficient in elevating the low stress resistance of adrenalectomized animals, they rarely raise it above the normal level, either in the presence or in the absence of the adrenals. Indeed, even as late as 1960, it had been claimed that "in only two situations have adrenocortical hormones been shown to be protective to the host: the replacement of hormone in hypoadrenalism, and the protective action against the lethal toxicity of bacterial lipopolysaccharides" (27). Yet, as early as 1940, it had been noted that the great sensitivity to surgical shock and other stressors, that is induced by partial hepatectomy even in nonadrenalectomized rats,

could be combated by cortical extracts; hence, at least in this condition, endogenous corticoids were not optimally efficacious (28). These findings called attention to the existence of close relationships between the liver and the resistance-increasing effect of corticoids. The subsequent observation that desoxycorticosterone could not, whereas corticosterone could, replace the adrenocortical extracts first demonstrated the importance of an 11-oxygen for antistress activity (28).

The claim that specific *defensive enzymes* ("*Abwehrfermente*") are produced against various compounds and tissues—including endocrine organs, following their parenteral administration—could not be substantiated by the techniques available to Abderhalden (29), who first enunciated this concept. On the other hand, there can be no doubt that protein extracts of heterologous endocrine glands can gradually induce resistance through the development of *antihormones* (30, 31).

It seemed unlikely that the *body could be made insensitive to its own hormones*, since this type of resistance would be expected to interfere with the physiologic activity of endocrine glands. Still, the observation that partial hepatectomy sensitizes to the anesthetic effect of natural steroid hormones suggested that the liver does possess a mechanism for the inactivation of these compounds. The question arose whether this defensive activity could be stimulated by very large amounts of those substrates which the inactivating mechanism is designed to metabolize.

Experiments performed in rats to check this possibility revealed that, following repeated massive overdosage with progesterone, desoxycorticosterone, or testosterone, the anesthetic effect of these hormones gradually diminishes. In fact, this type of resistance is not strictly substrate specific, since pretreatment with any one of these natural steroids also induced resistance to the others (32).

Apparently, at near-physiologic dose levels, the natural steroids do not markedly activate this defense mechanism (a phenomenon which would interfere with their normal function); yet they may accelerate their own degradation more intensely when given in abnormally high and potentially pathogenic amounts. It is difficult to explain this dose dependence of the inactivating mechanism, and available data do not justify far-reaching speculations. However, it may be pertinent that at near-physiologic concentrations, the steroid hormones circulate mainly as protein complexes, which are perhaps unable to reach the inactivating receptors. Conversely, after sudden flooding of the body with very large amounts of them, a certain portion of the injected steroid may enter the inactivating sites (*e.g.*, the smooth endoplasmic reticulum) before being thus protected by coupling to large carrier molecules (33).

Still, even at physiologic concentrations, gonadal steroid hormones do appear to affect drug sensitivity to some extent, as shown by sex differences in the susceptibility to various intoxications. It remained to be seen, however, whether this physiologic difference in sensitivity and the induction of resistance by excessive amounts of exogenous steroids depend upon the same mechanism.

Several earlier observers noted *sex differences* in drug sensitivity, which are apparently due to steroid hormones produced by the gonads and not to genetically determined resistance factors inherent in the somatic cells. Thus, it was found that adult male rats are less sensitive to barbiturates than are females. This difference disappears after gonadectomy, but the resistance characteristic of intact males can be induced by treatment with testosterone and related compounds in females or gonadectomized rats of either sex (34–37). Furthermore, female rats proved to be more sensitive than males to progesterone anesthesia, but this sex difference became obvious only after maturity. By 1941, we concluded that the "normal endocrine activity of the testis is largely, if not entirely, responsible for this comparative resistance of the male, since castration increases sensitivity in males but is without effect in female rats. Conversely, the resistance of castrate males and females may be raised by methyltestosterone administration" (38, 39).

A similar *sex difference* in susceptibility had also been noted, in 1957, with regard to cardiovascular calcification elicited in rats by overdosage with dihydrotachysterol (DHT). This form of calcinosis was aggravated by orchidectomy; hence, we concluded that "some testicular factor exerts a protective effect against this type of intoxication" (40).

These findings were the first to suggest that the steroids of the gonads, like those of the adrenal cortex, can increase resistance, although not necessarily against the same agents and through the same mechanism.

The liver, as the "central laboratory of the body," has long been suspected of playing an important part in the inactivation of exogenous and endogenous toxic substances. However, large-scale systematic studies, designed to identify the compounds subject to hepatic detoxication, were virtually impossible because of the lack of appropriate techniques. Comparisons between the drug resistance of intact and *hepatectomized* animals were difficult to interpret; complete removal of the liver causes severe shock rapidly terminating in death, especially when toxic substances are given, irrespective of whether or not these are amenable to hepatic detoxication. Animals in which partial hepatic insufficiency was created (*e.g.*, by ligature of the bile duct, hepatotoxic drugs, or the establishment of an Eck fistula) likewise yielded variable results, often complicated by damage to extrahepatic tissues. Finally, the search for presumed drug metabolites in hepatic vein-blood *in vivo* did not lend itself to the screening of many drugs, whereas similar studies on liver perfusates *in vitro* often failed to reflect *in vivo* conditions.

To test hepatic participation in the detoxication of numerous compounds, a screening test became necessary. It was to answer this need that, in 1931, we devised a simple surgical technique for the ablation of the left lateral and median lobes of the liver in mice (41). This operation removes about 70% of the hepatic tissue and markedly reduces resistance only with respect to drugs detoxified by the liver. For such tests it is best to use the animals about 24 hr. after the intervention, when they have recovered from the surgical insult but hepatic regeneration is still negligible. With this

technique we showed, for example, that the partially hepatectomized mouse is extremely sensitive to the anesthetic effect of tribromoethanol,[1] which is detoxified by the liver, but not to that of an equally anesthetic dose of $MgCl_2$, which is not subject to hepatic detoxication. Almost at the same time, Higgins and Anderson (42) recommended an essentially similar operation for the stimulation of hepatic regeneration in the rat. However, they, like the earlier investigators, did not attempt to use partial hepatectomy for detoxication studies.

About 10 years later, the site of steroid hormone detoxication became a major subject of controversy, difficult to solve with the chemical methods then available. However, in the meantime, we had observed that sudden overdosage with steroid hormones and their derivatives produces profound anesthesia in the rat (43). This indication of activity was clear-cut, common to virtually all steroid hormones, not particularly damaging, and almost immediately evident; thus, it was applicable to acute experiments on partially hepatectomized rats before regeneration could become important. Hence, we injected threshold doses of desoxycorticosterone (DOC), progesterone, testosterone, and estradiol into intact and partially hepatectomized rats. The latter proved to be unusually sensitive to all these steroids, whereas their resistance to several other anesthetics remained uninfluenced. Even overdosage with the nonsteroidal folliculoid, stilbestrol (which normally causes only a very mild hypnotic effect), produced prolonged narcosis after partial hepatectomy. These observations lead us to conclude that "it appears most probable that the liver is the site at which all the above-mentioned compounds are normally detoxified" (44).

Subsequent investigations have amply confirmed the importance of the liver as the organ principally responsible for the detoxication of steroid hormones and the value of partial hepatectomy as a simple screening test for compounds whose actions largely depend upon the speed of their hepatic detoxication. Furthermore, we have seen recently (in agreement with our expectations) that those drugs against which catatoxic steroids can offer protection through hepatic microsomal enzyme induction become particularly toxic following partial resection of the liver. However, the steroidal enzyme inducers themselves are also subject to hepatic detoxication and, consequently, their catatoxic activity likewise increases when their metabolic degradation is impeded by partial hepatectomy. Thus, in the rat, this operation facilitates both the production of perforating intestinal ulcers by indomethacin (a substrate for hepatic detoxication) and the prevention of these lesions by small doses of a catatoxic steroid such as spironolactone (45).

In evaluating the results of partial hepatectomy upon drug toxicity, it must be kept in mind, however, that the liver may also participate in the defense against toxic substances through mechanisms unrelated to the induction of microsomal enzymes [*e.g.*, synthesis of energy-yielding metabolites, elimination of pathogens through the bile, or their storage in the reticulo-endothelial system (RES)]. Hence, in any one case, aggravation of drug toxicity by partial hepatectomy merely suggests, but does not prove, that resistance may be increased by catatoxic steroids.

Some of the earliest work on the hepatic detoxication of steroids was performed *in vitro* by incubation with *liver slices or fractions*. Soon after it had been observed that partial hepatectomy increases sensitivity to the anesthetic action of steroid hormones, Zondek *et al.* (46) showed that both estrone and stilbestrol can be inactivated by rat liver pulp *in vitro*, and that "in rats treated with large amounts of stilbestrol, the capacity of the liver to inactivate stilbestrol is increased."

Subsequently, a group of investigators at our school undertook an extensive study of the relationship between sex differences in barbiturate resistance and the inactivation of barbiturates by liver tissue. They noted that pentobarbital anesthesia lasts much longer in female than in male rats and that the high resistance of the male is abolished by castration but restored to normal by testosterone. In ovariectomized rats, estradiol was virtually ineffective, but testosterone raised resistance to the male level. All these *in vivo* effects were found to run parallel with the pentobarbital detoxifying power of hepatic tissue *in vitro* (47). Liver homogenates of intact adult male rats destroyed pentobarbital *in vitro* more rapidly than those of castrate males. Furthermore, pretreatment of the castrates with testosterone enhanced the detoxication process, whereas estradiol pretreatment had an opposite effect (37).

The possible participation in the G.A.S. of "*adaptive enzymes*," similar to those previously demonstrated in microorganisms, was suggested in the first monograph on stress (2), as early as 1950, but at that time we had no precise ideas about the localization of these enzymes or the mechanisms involved in their activation.

The most recent development in the field of hormonal regulation of resistance is the recognition that *hepatic microsomal enzyme* induction may play a decisive role here. The fact that many drugs and certain hormones can induce the formation of hepatic enzymes has been well established by the fundamental biochemical observations of J. Axelrod, W. F. Bousquet, B. B. Brodie, A. H. Conney, K. P. DuBois, J. R. Fouts, H. V. Gelboin, R. J. Gillette, R. Kato, F. T. Kenney, W. E. Knox, R. Kuntzman, G. J. Mannering, E. C. and J. A. Miller, H. Remmer, and others. For example, it was found that the liver of the mouse and rat possesses an enzyme system which *N*-demethylates 3-methyl-4-monomethylaminoazobenzene. The activity of this system depends upon the diet, being highest in rodents kept on aged or otherwise treated animal products such as an old cholesterol preparation, liver extracts, and peptones. A variety of pure sterols were inactive but could be activated by peroxidation (48).

There followed a large number of publications suggesting that the induction of this type of resistance depends upon corticoid (49–54), folliculoid (55), testoid, or anabolic (56–62) activity.

The first observation that showed that *the catatoxic action is independent of all classic hormonal properties* was the demonstration in 1960 that a nonhormonal steroid—then identified merely by the factory code designation SC-11927—protects the rat against the particularly severe cardiovascular calcification produced

[1] Avertin, K & K Laboratories.

by DHT + Na_2HPO_4. This effect of the compound, subsequently called "catatoxic steroid No. 1" or "CS-1," could not even have been ascribed specifically to its antimineralocorticoid activity since here the substrate, namely DHT, is not a mineralocorticoid (63). However, it was only quite recently that the independence of the enzyme-inducing capacity from all known steroid hormone actions could be definitely proven (64, 65). It was found that in the rat, pretreatment with a variety of catatoxic steroids, such as spironolactone, norbolethone, and ethylestrenol, increases the oxidation of pentobarbital by hepatic microsomes and enhances its disappearance from the blood proportionally to their ability to shorten the depth of anesthesia *in vivo* (66). Norbolethone and ethylestrenol possess strong anabolic properties but little or no antimineralocorticoid effect, whereas spironolactone is a strong antimineralocorticoid devoid of anabolic actions. Since none of these steroids exhibits glucocorticoid, mineralocorticoid, or folliculoid effects, the catatoxic enzyme-inducing property appears to be independent of the former.

Another early approach to the problem of hepatic hormone metabolism was based on the demonstration that the *portal route of administration* is unfavorable for the obtention of various physiologic effects by steroids. For example, the implantation of functional ovaries or of pellets of folliculoid and testoid compounds into the spleen or mesenteries of gonadectomized rats produces much less stimulation of the accessory sex organs than if the same hormone sources are introduced subcutaneously or elsewhere into the systemic circulation. These findings, and the fact that many steroids are more active when given parenterally than enterally, strongly suggested that sex steroids are presumably inactivated by hepatocytes when brought directly to the liver through the portal vein. Of course, such observations do not distinguish between hepatic degradation, storage, or biliary excretion of hormones and, in any event, are not concerned with the hormonal stimulation of resistance but merely with the site of hormone inactivation.

Essentially the same is true of what was perhaps the first clear-cut demonstration of hepatic inactivation of a steroid, in which a *liver perfusion* technique was used (67).

Many investigators believe that the induction of hepatic microsomal enzymes by various drugs and steroids is associated with a marked proliferation of the *smooth endoplasmic reticulum (SER) in hepatocytes* (61, 68–72). This effect is also independent of the known steroid hormone actions and can be demonstrated in rats after treatment with such typical catatoxic steroids as spironolactone (73) or norbolethone (74). However, more recent investigations showed that the relationship between catatoxic activity and the proliferation of the SER is much less constant than had been originally thought (75).

Our attempts to distinguish between *syntoxic and catatoxic* actions go back to the earliest studies (1937–1944) on the role of corticoids in inflammatory responses, such as the acute anaphylactoid reaction (76), and in the myocarditis, nephritis, periarteritis, and arthritis that are elicited under certain conditions by mineralocorticoids (77). The fact that the antiphlogistic glucocorticoids are truly syntoxic was first demonstrated in 1953, using the granuloma pouch technique. Cortisol inhibits inflammation produced by croton oil in this test. However, if this irritant is removed after 14 days of sojourn in the pouch of a cortisol-treated rat and injected into the paw of an untreated control, it still produces an intense inflammatory response in the latter. It was concluded that glucocorticoids act by depressing the inflammatory potential of tissues, not by destroying the irritant (77*a*). By contrast, typical catatoxic steroids such as ethylestrenol, norbolethone, or spironolactone do not significantly modify the direct response of tissues to potential pathogens but they attack the latter, usually through increased enzymatic degradation.

The extraordinarily *broad activity spectrum* of catatoxic steroids is illustrated by the following partial list of toxicants against which they offer protection: numerous digitalis alkaloids (78–82), indomethacin (83), various anesthetics and hypnotics including many barbiturates and steroids (81, 84–86), dimethylbenzanthracene or DMBA (87, 88) and its highly active metabolite 7-OHM-MBA (89), nicotine (90), mephenesin (91), picrotoxin (92), phenindione (93), bishydroxycoumarin (94), hypervitaminosis A (95), cycloheximide (96), cyclophosphamide (97), meprobamate (98), colchicine (99), methyprylon (100), and a great variety of pesticides (101). Catatoxic compounds also protect against the diverse forms of infarctoid cardiopathies which are produced by steroids or digitoxin on certain diets (102). Even the fatal renal damage produced by $HgCl_2$ can be prevented by certain catatoxic steroids but, in this respect, only those (spironolactone, isoxasone, and emdabol) containing thioacetyl groups are active (103, 104). Evidently, the protection offered by catatoxic steroids extends to compounds of vastly different chemical structure and pharmacologic activity.

CURRENT INVESTIGATIONS ON PROTECTIVE STEROIDS

The most important tasks of current research on protective steroids are: (*a*) the clarification of pharmacopharmacologic interrelations, that is, the relationships, if any, between syntoxic, catatoxic, and "classical" hormonal actions; and (*b*) the elucidation of the structural prerequisites for the protective effects, that is, pharmacochemical interrelations.

This type of research presupposes many types of bioassays on numerous steroids, a truly monumental undertaking, but the only one that holds much promise of leading us to compounds with high protective and yet little, if any, undesirable effects. Such compounds would be of great value, not only in the treatment of disease but also for the study of the intimate mechanism of defensive enzyme induction.

On the basis of our present knowledge—as outlined in the preceding pages—no simpler approach to the problem appeared possible. Still it may be asked whether it is justified to invest a great deal of labor and money into an enormous screening program in which countless potential inducers and substrates are tested, with little rationale for their selection, and no immediate

attempt to determine the ultimate mechanism of their action. It is certainly not desirable to undertake blind screening projects based merely on the hope for chance.

However, our attempts to identify protective steroids and their substrates were not totally blind. When we embarked on this project, we already knew that:

1. Numerous steroids offer protection against diverse toxicants.
2. Certain steroids offer considerable protection but only against a few toxicants. Indeed, some steroids may protect against one toxic substance and actually diminish resistance to another. Thus, we knew that there exists a considerable "substrate specificity."
3. Some steroids have a very broad spectrum of protective effects, in that they increase resistance against many toxicants. In other words, resistance is largely nonspecific.
4. Many of the protective steroids, especially those with catatoxic properties, are singularly devoid of undesirable side effects.
5. We had no evidence of a close parallelism between the protective effect of steroids, on the one hand, and their classic pharmacologic actions or chemical structure on the other.

In view of these considerations, our first tasks were to identify the protective (syntoxic or catatoxic) steroids and to determine the toxicants against which they offer protection.

Procedure for *In Vivo* Identification of Protective Steroids and Their Substrates

Outline of Procedure—Even the very first exploratory investigations have shown great differences in the "resistance spectrum" of various steroids. For example, some steroids induce considerable resistance against digitoxin but not against indomethacin or vice versa; other steroids protect against both or neither one of these toxicants. Hence, the protective value of steroids could not be properly assessed by testing them against any one toxic agent.

It was obviously not possible to arrive at a reasonable classification of these substances by testing all potentially protective steroids against every type of toxicant; we had to make a selection. Preliminary experiments had shown that digitoxin and indomethacin are readily detoxified by many steroids and, almost invariably, those steroids that did not raise resistance against these two drugs were also ineffective in offering protection against others.

As the *first step* in our screening procedure, it seemed reasonable therefore to test, even if only against these two substrates, as many steroids as we could obtain. Thereby, we immediately eliminated the large group of steroids that offered little hope of being valuable inducers of resistance.

The *second step* was designed to appraise the "protective spectra" of those steroids that showed a high degree of prophylactic potency against digitoxin, indomethacin, or both. These compounds were tested (together with some nonsteroidal compounds) against a heterogeneous set of 10 pathogens, widely differing in their chemical structure and in the changes (motor disturbances, anesthesia, cardiac necroses, or calcification) that they produce.

In the *third step* of screening, we attempted to identify the substrates that can be detoxified by steroids. For this purpose, we used a representative set of natural or synthetic steroidal compounds, purposely selected to include proven syntoxic or catatoxic substances, as well as compounds that had never been shown to protect against any toxic agent. This standard set of steroids was examined for its ability to induce resistance against a great variety of toxicants. For purposes of comparison, we also tested two nonsteroidal compounds (thyroxine and phenobarbital) known to influence resistance to many drugs.

Finally, a few steroids (*e.g.*, folliculoids, luteoids, compounds containing thioacetyl, nitrile, or quaternary ammonium radicals) were subjected to more extensive special studies because of the unusual nature of their detoxicating mechanisms. Similarly, certain toxicants [*e.g.*, endotoxins, ganglionic blocking agents, lathyrogens, and octamethyl pyrophosphoramide (OMPA)] had to be examined by specially devised tests because of some particular characteristics which distinguish them from the majority of substrates.

Experimental Animals—For all the experiments of this series, we used female Sprague-Dawley rats of the ARS or Holtzman Farms, with an initial body weight of 100 g. (90–110 g.).

Assessment and Tabulation of the Results—With each toxicant we registered the characteristic functional (motor disturbances) or structural (intestinal ulcers, cardiac necrosis, and calcinosis) changes in terms of an arbitrary scale in which 0 = no change, 1 = just detectable, 2 = moderate, and 3 = maximal change, as previously described (13, 105). However, for statistical evaluation we recognized only two grades: minor and sometimes dubious degrees of lesions (between 0 and 1 in our scale) were rated as negative, while all others were rated as positive. For groups comprised of 5–10 rats, these data as well as the mortality rates were then arranged in a 2×2 contingency table, and their statistical significance was determined by the "exact probability test" of Fisher and Yates (106, 107). For groups comprised of 10 rats or more, we used the same procedure of grading, but the statistical evaluation was performed by the chi-square test using the 2×2 table. The severity of all functional disturbances was listed at the time, when the difference between the pretreated and not pretreated animals was most evident.

Only in the case of anesthesia or paralysis did we assess the results by the time (in minutes) necessary to regain the righting reflex. Here, the significance of the apparent differences between the sleeping or paralysis time of the controls and the experimental animals was computed by Student's t test. When one of the two results was 0, we calculated the statistical significance on the basis of confidence limits.

In all tables, $3 = p < 0.005$, $2 = p < 0.01$, $1 = p < 0.05$, and the results are summarized on the basis of the degree of this significance rating.[2] The figures in-

[2] According to a system developed by Mrs. I. Mécs of this Institute.

dicate the means of the statistical significance grades of the changes (functional or structural) used as indicators plus that of the mortality rates divided by 2. Thus, in an experiment in which the protection against intestinal ulcers had a significance rating of 0 (no protection), and the significance of the protection against mortality was 3 (perfect protection), the figure given in the tables would be 1.5. Only in the case of compounds that normally cause no mortality (*e.g.*, anesthetics and muscle relaxants) do the grades correspond to structural or functional lesions alone. Aggravation of toxicity is indicated by figures preceded by a minus sign.

The individual statistical results are listed in all tables. However, in Tables I and II, we also list the "Overall Protective Index" computed according to a procedure closely related to the "Simplified Activity Grading" system previously described (105). This Index represents the sum of all the individual activity gradings for a certain protective substance divided by the number of toxicants against which it was tested. Thus, if the activity gradings of a steroid employed against 10 toxicants add up to 10 (irrespective of the individual values) the "Overall Protective Index" will be 1; but if the steroid has been tested only against 5 toxicants, it will be 2. In addition, we computed the "Protective Spectrum Index" which is the percentage of those toxicants tested against which significant protection is obtained (irrespective of the degree of significance). Thus, if a steroid offers significant protection against 6 out of 10 toxicants examined, its "Protective Spectrum Index" is 60%.

In Tables IIA and IIB, these Indexes have also been computed for the amenability to protection of the various toxicants (two bottom horizontal lines). In other words, here the figures indicate the mean degree and the percent frequency of protection offered by the entire series of conditioners against any one toxicant.

Since the large number of experiments to be reported here was performed over a considerable period of time, in each case a group of unpretreated controls received the same toxicant simultaneously with the rats that had been pretreated with potentially protective substances. The statistical significance of the resulting changes in the pretreated animals was always calculated in comparison with the corresponding group of unpretreated controls handled under identical circumstances, at the same time and by the same technician.

The Protective Compounds—All *steroids* to be assayed for possible protective effects were given in 1 ml. water (*p.o.*) by stomach tube twice daily, as pretreatment and treatment, usually from the 1st day until the termination of the experiment. Only in the digitoxin series was steroid treatment limited to the period between the 1st and 5th day, and in the groups given navadel or anesthetics to between the 1st and 4th day, because with these rapidly acting toxicants the outcome was already clearly evident by that time. Since most of the steroids used are poorly soluble in water, they were given in the form of microcrystal suspensions prepared with the addition of a trace of polysorbate 80 (Tween 80).

In the case of *nonsteroidal* protective compounds, the technique of administration was much less uniform, since it had to be adjusted to the very different chemical and pharmacologic properties of the drugs examined. Therefore, the technique of administration is given with each of the compounds in Table II.

The Damaging Agents (Toxicants)—The following model intoxications were used to explore the specificity of the protection offered by various prophylactic agents. Only the toxicants marked with an asterisk were employed in the second step (determination of "Protective Spectrum" of various protective agents). They were selected because they differ widely, both in their chemical structure and in the changes (motor disturbances, anesthesia, cardiac necroses, or calcification) that they produce. However, these same model intoxications, as well as all the others listed, were employed in the third step of screening, designed to determine which drugs are amenable to prophylaxis by members of a representative set of steroidal and nonsteroidal protective compounds. Unless otherwise indicated, the doses are expressed per 100 g. body weight. However, all animals had a mean initial body weight of 100 g.; hence, the doses marked "per rat" were fairly closely adjusted to body weight in any case, except in a few animals which gained or lost much more weight than the mean.

The term "dyskinesia" is used here to indicate all types of motor disturbances including simple prostration and tremor, which often appear in combination and are usually difficult to express more precisely. Only clear-cut convulsions (*e.g.*, after digitoxin), anesthesia (*e.g.*, after barbiturates or steroids), or muscular paralysis (*e.g.*, after zoxazolamine) are specifically so identified.

The cumulative mortality was always registered on the day on which the experiment was terminated. As previously stated, with agents causing no significant mortality (*e.g.*, anesthetics and muscle relaxants) the grading is based exclusively on *in vivo* changes. Accordingly, these experiments were terminated after the final reading, and mortality is not mentioned in the description of the procedures. The reference numbers cited with some of the techniques refer to earlier publications in which pertinent additional details can be found.

The following is a list of the procedures used to produce and appraise various types of damage.

Aminoacetonitrile "AAN" (Abbott Laboratories)—20 mg. in 1 ml. water *p.o.* twice daily from the 4th to the last day of the experiment. Osteolathyrism was assessed, and mortality was listed on the 16th day.

dl-*Amphetamine (K & K Laboratories)*—12 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was assessed 4 hr. after administration of the drug, and mortality was listed 24 hr. later.

Barbital (Brickman)—20 mg. in 2 ml. water i.p. once on the 4th day. The sleeping time (min.) was determined.

Bile Duct Ligature—As conditioner on 1st day (Table IIB) and as toxicant (p. 22) on the 4th day. Final body weights of eviscerated rats were measured (g.), and mortality was listed on the 10th day.

Bishydroxycoumarin (Abbott Laboratories)—13 mg. in 1 ml. water *p.o.* daily from the 4th to the last day of the experiment. The animals developed multiple hemorrhages mainly in the gastrointestinal tract, but these

Table I—First Screening of Steroids against Digitoxin and Indomethacin

Group	Steroids	Dose, mg.	Digitoxin[a]	Indomethacin[a]	Overall Protective Index
1	17β-Hydroxy-3-oxo-5α-androstane-1α-carbonitrile acetate SC-16027 (Searle)	0.5	0	0	0
2	17β-Hydroxy-3-oxo-5α-androstane-1α-carbonitrile SC-16026 (Searle)	0.5	0	0	0
3	17β-Hydroxy-4,4,17-trimethyl-3-oxoandrost-5-ene-2α-carbonitrile, trimethylandrostenolone carbonitrile, "TMACN" (Winthrop)	10 0.5 0.1 0.03	3 2.5 2 0	3 3 2 0	3 2.8 2 0
4	17β-Hydroxy-5α-androst-2-ene-3-carbonitrile (Lepetit)	10 0.5	0.5 0	2 0	1.3 0
5	17-Oxo-5α-androst-2-ene-3-carbonitrile (Lepetit)	10 0.5	2 0	2 0	2 0
6	20,20-(Ethylenedioxy)-5β-pregn-2-ene-3-carbonitrile (Lepetit)	10	0	0	0
7	20,20-(Ethylenedioxy)-5α-pregn-2-ene-3-carbonitrile (Lepetit)	10 0.5	0.5 0	2 2	1.3 1
8	17β-Hydroxy-3ξ-amino-5α-androstane-3-carbonitrile SC-13265 (Searle)	0.5	0	0	0
9	17β-Hydroxy-3-oxo-5ξ-androstan-5-carbonitrile SC-13389 (Searle) same as SC-13269 : 5 epimers	0.03 0.015 0.005 0.001	0 0 0 0	0 0 0 0	0 0 0 0
10	17β-Hydroxy-3-oxo-5ξ-androstane-5-carbonitrile SC-13269 (Searle) same as SC-13389 : 5 epimer	0.5	0	0	0
11	3α,17-Dihydroxy-5α,17α-pregn-20-yne-5-carbonitrile SC-13675 (Searle)	0.5	0	0	0
12	17-Hydroxy-3,20-dioxo-5α-pregnane-5-carbonitrile acetate 13795 (Searle)	0.5	0	0	0
13	17-Hydroxy-22-methyl-3-oxo-19,21,24-trinor-5ξ,17α-chol-22-ene-5-carbonitrile SC-13969 (Searle)	0.5	0	0	0
14	17-Hydroxy-22-methyl-3-oxo-19,21,24-trinor-5β,17α-chol-22-ene-5-carbonitrile SC-14373 (Searle)	0.5	0	0	0
15	17-Hydroxy-3-oxo-19-nor-5β,17α-pregn-20-yne-5-carbonitrile SC-13823 (Searle)	0.5	0	0	0
16	17β-Hydroxy-17-methyl-3-oxo-5β-androstane-5-carbonitrile SC-13754 (Searle)	0.5	0	0	0
17	17β-Hydroxy-17-methyl-3-oxo-5α-androstane-5-carbonitrile SC-13503 (Searle)	0.5	0	0	0
18	17β-Hydroxy-3-oxo-5β-androstane-5-carbonitrile propionate SC-14175 (Searle)	0.5	0	0	0
19	17β-Hydroxy-3-oxo-5α-androstane-5-carbonitrile propionate SC-14174 (Searle)	0.5	0	0	0
20	3β,5α-Dihydroxy-20-oxopregnane-6β-carbonitrile (Syntex)	0.5	0	0	0
21	α-Cyano-3β-hydroxy-20-oxopregn-5-ene-16α-acetic acid ethyl ester (SK & F)	10	0	0	0
22	3-Methoxy-16-methyl-17-oxoestra-1,3,5(10)-triene-16ξ-carbonitrile (Roussel)	0.5	0	0	0
23	17β-Hydroxy-3-methoxy-16-methylestra-1,3,5(10)-triene-16β-carbonitrile acetate (Roussel)	0.5	0	0	0

Table I—*(Continued)*

Group	Steroids	Dose, mg.	Digitoxin[a]	Indomethacin[a]	Overall Protective Index
24	20,20-(Ethylenedioxy)-3β-hydroxypregn-5-ene-16α-carbonitrile U-19553 (Upjohn)	0.5 0.1 0.03 0.015	2.5 2 0.5 0	2 2 1.5 0	2.3 2 1 0
25	3β-Hydroxy-7,20-dioxopregn-5-ene-16α-carbonitrile acetate SC-6703 (Searle)	0.5 0.03	3 0	2 1.5	2.5 0.8
26	3β-Hydroxy-7,20-dioxopregn-5-ene-16α-carbonitrile SC-6813 (Searle)	0.5 0.03	3 0	1.5 0.5	2.3 0.3
27	3,3-(Ethylenedioxy)-11,20-dioxopregn-5-ene-16α-carbonitrile U-35006 (Upjohn)	0.5 0.1 0.03 0.015	2.5 2.5 0 0	2 2 1.5 1.5	2.3 2.3 0.8 0.8
28	3,3,20,20-Bis(ethylenedioxy)-11-oxopregn-5-ene-16α-carbonitrile U-35910 (Upjohn)	0.5 0.1 0.03 0.015	2.5 0 0 0	2 2 1.5 0	2.3 1 0.8 0
29	3β-Hydroxy-20-oxo-5-pregnene-16α-carbonitrile SC-4674 (Searle), U-14975 (Upjohn) Pregnenolone carbonitrile "PCN"	10 1 0.5 0.2 0.1 0.03 0.015	3 3 3 3 3 1.5 0	3 3 3 3 3 1.5 0	3 3 3 3 3 1.5 0
30	3β-Hydroxy-20-oxopregn-5-ene-16α-carbonitrile acetate U-34889 (Upjohn) Pregnenolone carbonitrile acetate "PCN-ac"	0.5 0.1 0.03 0.015	2.5 0.5 0 0	2 2 1.5 0	2.3 1.3 0.8 0
31	3β-Hydroxy-11,20-dioxo-5β-pregnane-16α-carbonitrile acetate U-34575 (Upjohn)	0.5 0.1 0.03 0.015	2.5 2.5 0 0	2 2 1.5 1.5	2.3 2.3 0.8 0.8
32	3β-Hydroxy-20-oxopregn-5-ene-16α-carbonitrile acetate (Syntex)	0.5 0.03	3 2	2 1.5	2.5 1.8
33	3β,20-Dihydroxypregn-5-ene-16α-carbonitrile (Syntex)	0.5 0.1 0.03	1.5 1 0	3 1.5 —	2.3 1.3 0
34	3β-Hydroxy-20,20-ethylenedioxypregn-5-ene-16α-carbonitrile acetate (Syntex)	0.5 0.1 0.03	1.5 0 0	3 1.5 0	2.3 0.8 0
35	17-Cyano-3β-hydroxyandrost-5-ene-17β-malononitrile U-28406 (Upjohn)	0.5	0	0	0
36	3β,17-Dihydroxy-16β-methyl-5β-androstane-17ξ-carbonitrile (Rousse!)	0.5	0	0	0
37	17-Hydroxy-3-oxoandrost-4-ene-17ξ-carbonitrile acetate (Roussel)	0.5	0	0	0
38	17-Hydroxy-3,11-dioxoandrost-4-ene-17ξ-carbonitrile acetate (Roussel)	0.5	0	0	0
39	3α,17-Dihydroxy-5β-androstane-17ξ-carbonitrile 3-acetate (Roussel)	0.5	0	0	0
40	17-Hydroxy-3,11-dioxoandrost-4-ene-17ξ-carbonitrile (Roussel)	0.5	0	0	0
41	3α-Hydroxy-5β-androst-16-ene-17-carbonitrile acetate (Roussel)	0.5	0	0	0
42	17-Cyano-3α-hydroxy-11-oxo-5β-androstane-17β-malononitrile (Roussel)	0.5	0	0	0
43	3-Methoxyestra-1,3,5(10),16-tetraene-17-carbonitrile acetate (Roussel)	0.5	0	0	0
44	3α-Hydroxy-5β-androstane-17β-acetonitrile (Roussel)	0.5	0	0	0
45	3-Hydroxyestra-1,3,5(10),16-tetraene-17-carbonitrile (Roussel)	0.5	0	0	0

Table I—(*Continued*)

Group	Steroids	Dose, mg.	Digitoxin[a]	Indomethacin[a]	Overall Protective Index
46	3β-Hydroxyandrosta-5,16-diene-17-carbonitrile acetate (Roussel)	0.5	0	0	0
47	3,3-(Ethylenedioxy)-17-hydroxypregn-5-ene-17β-carbonitrile (Roussel)	0.5	0	0	0
48	3-Oxoandrosta-4,16-diene-17-carbonitrile (Roussel)	0.5	0	0	0
49	3α-Hydroxy-5β-androstane-17β-carbonitrile acetate (Roussel)	0.5	0	0	0
50	3α-Hydroxy-5β-androstane-17β-acetonitrile (Roussel)	0.5	0	0	0
51	3α-Hydroxy-11-oxo-5β-androstane-17β-acetonitrile acetate (Roussel)	0.5	0	0	0
52	3β-(Tetrahydropyran-2-yloxy)-5β-androstane-17β-acetonitrile (Roussel)	0.5	0	0	0
53	3α-Hydroxy-11-oxo-5β-androstane-Δ17-acetronitrile acetate (Roussel)	0.5	0	1.5	0.8
54	3α-Hydroxy-11-oxo-5β-androstane-Δ17-malononitrile (Roussel)	0.5	0	0	0
55	3-(3α-Hydroxy-5β-androstan-17β-yl)glutaronitrile acetate (Roussel)	0.5	0	0	0
56	3α,20-Dihydroxy-5β-pregnane-20ξ-carbonitrile 3-acetate (Roussel)	0.5	0	0	0
57	20-Cyano-3α-hydroxy-5β-pregn-17(20)-en-21-oic acid acetate ethyl ester (Roussel)	0.5	0	0	0
58	20-Cyano-3α-hydroxy-11-oxo-24-nor-5β-cholan-21-oic acid amide (Roussel)	0.5	0	0	0
59	3β,20,21-Trihydroxy-5β-pregnane-20ξ-carbonitrile 3,21-diacetate (Roussel)	0.5	0	0	0
60	20-Cyano-3β-hydroxypregna-5,17(20)-dien-21-oic acid acetate ethyl ester (Roussel)	0.5	0	0	0
61	3α,20ξ-Dihydroxy-11-oxo-5β-pregnane-20ξ-carbonitrile 3-acetate (Roussel)	0.5	0	0	0
62	20-Hydroxy-3-oxo-19-norpregna-4,9,11-triene-20ξ-carbonitrile (Roussel)	0.5	0	0	0
63	20-Cyano-3α-acetoxy-11-oxo-24-norcholan-21-oic acid ethyl ester (Roussel)	0.5	0	0	0
64	20-Cyano-3α-hydroxy-5β-pregnan-21-oic acid (Roussel)	0.5	0	0	0
65	20-Cyano-3α-hydroxy-11-oxo-5β-pregn-17(20)-en-21-oic acid acetate ethyl ester (Roussel)	0.5	0	0	0
66	3α-Hydroxy-11-oxo-5β-pregn-17(20)-ene-20-carbonitrile acetate (Roussel)	0.5	0	0	0
67	20-Cyano-3α-hydroxy-11-oxo-5β-pregn-17(20)-en-21-oic acid (Roussel)	0.5	0	0	0
68	3α-Hydroxy-11-oxo-5β-pregn-17(20)-ene-20-carbonitrile acetate (Roussel)	0.5	0.5	1.5	1
69	20-Cyano-3β-hydroxypregn-5-en-21-oic acid acetate ethyl ester (Roussel)	0.5	0	0	0
70	21-Cyano-3β-hydroxy-5β-pregn-20-ene-21-carboxylic acid ethyl ester (Roussel)	0.5	0	0	0
71	21-Cyano-3α-hydroxy-5β-pregn-20-ene-21-carboxylic acid ethyl ester (Roussel)	0.5	0	0	0

Table I—(*Continued*)

Group	Steroids	Dose, mg.	Digitoxin[a]	Indomethacin[a]	Overall Protective Index
72	20,20-(Ethylenedioxy)-3β-acetoxypregn-5-ene-16α-carboxylic acid U-35939 (Upjohn)	0.5	0	0	0
73	3,20-Dioxopregn-4-ene-16α-carboxylic acid methyl ester U-35258 (Upjohn)	0.5	0	0	0
74	20,20-(Ethylenedioxy)-3β-hydroxypregn-4-ene-16α-carboxylic acid U-12872E (Upjohn)	0.5	0	0	0
75	20,20-(Ethylenedioxy)-3β-hydroxypregn-4-ene-16α-carboxylic acid methyl ester U-36548 (Upjohn)	0.5	0	0	0
76	3β-Hydroxy-20-oxo-17α-pregn-5-ene-16β-carboxylic acid (Syntex)	0.5	0	0	0
77	3β-Hydroxy-20-oxo-5α,17α-pregnane-16β-carboxylic acid (Syntex)	0.5	0	0	0
78	3β,20-Dihydroxy-17α-pregn-5-ene-16β-carboxylic acid (Syntex)	0.5 0.1 0.03	1.5 0.5 0	3 0 0	2.3 0.3 0
79	17β-Hydroxy-3-oxoandrost-4-ene-7β-carboxamide (Syntex)	0.5	0	0	0
80	3β-Hydroxy-20-oxo-17α-pregn-5-ene-16β-carboxamide (Syntex)	0.5 0.1	0 0	1.5 0	0.8 0
81	3α-Hydroxy-11,20-dioxo-5β-pregnane-16α-carboxamide U-35827 (Upjohn)	0.5	0	0	0
82	17α-Methyl-3β,17-dihydroxy-5α-androstane-2α-hydroxymethyl (Syntex)	0.5	0	0	0
83	17α-Methyl-17-hydroxy-3-oxo-5α-androstane-2-aminomethylene (Syntex)	0.5	0	0	0
84	17β-Hydroxy-3-oxo-5α-androstane-2-hydroxymethylene (Syntex)	0.5	0	0	0
85	Thiocyanic acid 3α,17β-dihydroxy-17-methyl-5α-androstan-2-yl ester SC-12697 (Searle)	0.5	0	0	0
86	17-Hydroxy-4-aza-17α-pregnan-3-one (Organon)	10 0.5	1.5 0	3 0	2.3 0
87	1α,2α-Epoxyandrosta-4,6-diene-3,17-dione (Linet)	10	0	0	0
88	Mestranol 3-Methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17-ol (Lilly)	10 0.5	0 —	3 0	1.5 0

[a] For details, see *Digitoxin* and *Indomethacin* in the list of techniques used to produce and appraise various types of damage (pp. 9–17).

did not lend themselves well even to semiquantitative appraisal. Hence, only mortality was listed on the 9th day.

Bromobenzene (*J. T. Baker Chemical Co.*)—50 mg./day from the 4th to the 6th day and 75 mg. once on the 7th day and twice on the 8th day, always in 1 ml. oil *p.o.* Hepatic steatosis was estimated on the day of death; prostration and mortality were listed on the 9th day.

Brompheniramine Maleate (*Robins Research Lab.*)—30 mg. in 0.5 ml. dimethyl sulfoxide (DMSO) *p.o.* twice daily from the 4th to the last day of the experiment. Dyskinesia was estimated on the 5th day 3 hr. after administration of the drug, and mortality was listed on the 6th day.

Cadmium Chloride, $CdCl_2$ (*Fisher*)—700 mcg. in 1 ml. water i.v. once on the 4th day. The characteristic hemorrhages in the Gasserian ganglia were estimated on the day of death in animals that survived at least 5

Table IIA—"Protective Spectra" of Some Steroidal Agents

Conditioning Agent	Dose, mg.	Toxicant: Digitoxin	Navadel	Parathion	Nicotine	Hexobarbital	Progesterone	Zoxazolamine	Indomethacin	Fluorocortisol NaH_2PO_4 Corn Oil	Dihydrotachysterol (DHT)	Overall Protective Index	Protective Spectrum Index, %
3β-Hydroxy-20-oxo-5-pregnene-16α-carbonitrile SC-4674 PCN (Searle)	10	3	3	2	2.5	3	3	3	3	1.5	2	2.6	100
	1	3	3	2.5	0	1	3	3	3	0.5	—		
	0.5	3	3	1.5	0	1	3	3	3	0	2		
	0.2	3	—	3	—	—	2	3	3	—	1.5		
	0.1	3	3	—	—	—	—	—	3	—	—		
	0.03	2	0.5	—	—	0	—	—	3	—	—		
	0.015	0	—	—	—	—	—	—	0	—	—		
9α-Fluoro-11β,17-dihydroxy-3-oxo-4-androstene-17α-propionic acid potassium salt CS-1, SC-11927, (Searle)[a]	10	3	1.5	1.5	3	3	3	2	3	1.5	3	2.5	100
	0.5	3	3	0.5	0	0	3	1	3	0	1		
	0.1	1	1.5	—	—	—	2	—	1.5	—	—		
	0.03	0	—	—	—	—	0	—	—	—	—		
17-Hydroxy-7α-thioacetyl-3-oxo-4-androstene 17α-propionic acid γ lactone Spironolactone SC-9420 (Searle)	10	3	1.5	2	3	3	3	1	3	2	2	2.4	100
	0.5	0	0	0	0	0	2	0	1.5	0	0		
	0.1	—	—	—	—	—	0	—	0	—	—		
6-Chloro-17-hydroxy-1β,2β-dihydro-2′H-cyclopropa[1,2]-4,6-pregnadiene 3,20-dione acetate Cyproterone acetate (Schering)	10	3	2	3	3	3	3	3	3	0	1.5	2.5	90
	1	2.5	3	1.5	0	1	3	—	3	—	—		
	0.5	2	2	0.5	—	0	3	2	3	—	0		
	0.1	0	0	—	—	—	2	—	0.5	—	—		
	0.03	—	—	—	—	—	0	—	—	—	—		
17α-Ethyl-4-estren-17-ol Ethylestrenol (Organon)	10	3	1.5	2	3	3	3	2	3	0	3	2.4	90
	0.5	0	0	0	0.5	1	2	0	0	—	0.5		
	0.1	—	—	—	0	0	3	—	—	—	—		
	0.03	—	—	—	—	—	0	—	—	—	—		
13,17α-Diethyl-17-hydroxy-4-gonen-3-one Norbolethone Wy-3475 (Wyeth)	10	3	1.5	1.5	3	3	3	0	3	1	3	2.2	90
	0.5	0	2	0	0	0	2	—	0.5	0	1		
	0.1	0	0	—	—	—	—	—	—	—	—		
17β-Hydroxy-4,4,17-trimethyl-3-oxoandrost-5-ene-2α-carbonitrile TMACN (Winthrop)	10	3	2.5	2	0	3	3	3	3	2	2	2.1	90
	0.5	2.5	3	0.5	0	1	3	1	3	—	1.5		
	0.1	2	1.5	—	—	0	3	—	2	—	—		
	0.03	0	—	—	—	—	3	—	0	—	—		
16β-Methyl-16,17-epoxy-3β,11α-dihydroxy-5α-pregnan-20-one (Lepetit)	10	3	3	2	0	1	3	3	3	0.5	1.5	2.0	90
	0.5	0	0	1	—	0	1	0	0.5	0	—		
	0.1	—	—	—	—	—	0	—	—	—	—		
6α-Methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione "Medrol" 6α-Methylprednisolone (Upjohn)	10	3	3	0.5	0	3	3	2	3	0	1.5	1.9	80
	0.5	0	0	0	—	2	1	1	0	—	0.5		
	0.1	—	—	—	—	2	0	—	—	—	—		
9α-Fluoro-17α-methyl-11β,17-dihydroxy-4-androsten-3-one Fluoxymesterone U-6040 (Upjohn)	10	3	3	0	1	2	3	3	2	0	2	1.9	80
	0.5	3	2	—	0	3	3	0	0	—	1		
	0.1	—	3	—	—	3	3	—	—	—	0		
	0.03	—	0.5	—	—	—	0	—	—	—	—		
7α-Thioacetyl-(17R)-spiro-[4-androsten-17,2′-(furan)]-3-one Spiroxasone (Merck Sharp & Dohme)	10	3	3	1.5	0	2	3	0	3	2	1.5	1.9	80
	0.5	0	0	0	—	1	1	—	1.5	0.5	0.5		
	0.1	—	—	—	—	0	0	—	—	—	—		
11β,17,21-Trihydroxy-1,4-pregnadiene-3,20-dione 21-acetate Prednisolone acetate (Schering)	10	2	1.5	0	0.5	3	3	0	3	0.5	1.5	1.5	80
	0.5	0	0	—	0	0	0	—	0	0	0		
	0.1	—	—	—	—	0	—	—	—	—	—		
17α-Methyl-17-hydroxy-2-oxa-4-androsten-3-one Oxandrolone (Searle)	10	3	1.5	0	3	3	2	0	3	0	3	1.9	70
	0.5	0	0	—	0	0	2	—	0	—	0.5		
	0.1	—	—	—	—	—	0	—	—	—	—		
9α-Fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione 21-acetate Dexamethasone acetate (Schering)	10	3	—	—	—	—	—	—	0	—	—	1.3	60
	1	3	3	−0.5	0	2	3	0	1.5	0	1.0		
	0.5	2.5	3	0	—	3	3	—	0.5	—	0		
	0.1	3	0	0	—	1	0	—	1	—	0		
	0.03	3	—	—	—	2	—	—	0	—	0		
	0.015	0	—	—	—	—	—	—	—	—	—		
17α-Methyl-17-hydroxy-1α,7α-dithio-4-androsten-3-one 1,7-diacetate Emdabol (Merck)	10	2	2	0	0	3	3	0	1	0	2	1.3	60
	0.5	0.5	0	—	—	0	0	—	0	—	0		
	0.1	0	—	—	—	—	—	—	—	—	—		
11β,21-Dihydroxy-4-pregnene-3,20-dione Corticosterone, Kendall "Cpd. B" (Merck Sharp & Dohme)	10	3	0.5	0.5	0	0	3	0	3	0	2	1.2	60
	0.5	0	—	—	—	—	1	—	0	—	0		
	0.1	—	—	—	—	—	0	—	—	—	—		
17,21-Dihydroxy-4-pregnene-3,11,20-trione 21-acetate Cortisone acetate (Upjohn)	10	3	0.5	0	0	3	2	3	0	−1	1	1.2	60
	0.5	0	0	0	−0.5	0	0	0	0	0	0		
17β-Hydroxy-4-androsten-3-one Testosterone (Roussel, NBC)	10	0	2.5	0	0	2	2	1	0.5	0	2.5	1.1	60
	0.5	—	0	—	—	0	0	0	0	—	0		
3β-Hydroxy-5-androsten-17-one Dehydro-iso-androsterone (Ayerst)	10	2	0.5	1	0	2	2	0	3	0	0	1.1	60
	0.5	0	—	0	—	2	0	—	0.5	—	—		
	0.1	—	—	—	—	0	—	—	—	—	—		
17α-Methyl-5-androstene-3β,17-diol Methylandrostenediol "MAD" Organon	10	0	3	0	0	3	3	3	0	0	2	1.4	50
	0.5	—	0	—	—	0	0	0	—	—	0		

Table IIA—*(Continued)*

Conditioning Agent	Dose, mg.	Toxicant: Digitoxin	Navadel	Parathion	Nicotine	Hexobarbital	Progesterone	Zoxazolamine	Indomethacin	Fluorocortisol Na_2HPO_4 Corn Oil	Dihydrotachysterol (DHT)	Overall Protective Index	Protective Spectrum Index, %
17α-Hydroxy-4-pregnene-3,20-dione acetate 17-Acetoxyprogesterone U-5533 (Upjohn)	10	3	1	0	0	0	2	0	3	0	2	**1.1**	**50**
	0.5	0	0.5	—	—	—	0	—	0.5	—	0		
9α-Fluoro-16β-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione 21-acetate Betamethasone acetate (Schering)	10	2	—	—	—	—	—	—	0	—	—		
	2	3	—	—	—	—	—	—	3	—	—		
	1	2	1	−1.5	0	0	3	0	1.5	0	1.5	**0.8**	**50**
	0.5	1.5	0.5	0	—	—	3	—	1.5	—	0.5		
	0.1	0	0	0	—	—	1	—	−1	—	0		
	0.03	—	—	—	—	—	0	—	—	—	—		
4-Pregnene-3,20-dione Progesterone (Roussel, Organon)	10	1.5	0.5	0	1	0	0	0	3	0	1.5	**0.8**	**50**
	0.5	0	0.5	—	0	—	—	—	0	—	0		
11β,17,21-Trihydroxy-4-pregnene-3,20-dione 21-acetate Cortisol acetate (Roussel)	10	3	0	−0.5	−0.5	3	0	3	0	−0.5	1.5	**0.9**	**40**
	0.5	0	0	0	0	0	3	2	0	0.5	1		
5β-Pregnane-3,20-dione Pregnanedione (Searle)	10	0	1	2.5	0	2	0	0	2	0	0	**0.8**	**40**
	0.5	—	0	0	—	0	—	—	0	—	—		
21-Hydroxy-5β-pregnane-3,20-dione hemisuccinate sodium salt Hydroxydione Sodium (Schering, Pfizer)	10	3	1.5	0.5	0.5	0	0	0	0	0	0	**0.6**	**40**
	0.5	0	—	0	0	—	—	—	—	—	—		
21-Hydroxy-4-pregnene-3,20-dione acetate Desoxycorticosterone acetate "DOC-Ac" (Schering)	10	0	1.5	0	2	0	0	0	1	0	0.5	**0.5**	**40**
	0.5	—	0	—	0	—	—	—	—	—	—		
1,3,5(10)-Estratriene-3,17β-diol Estradiol (Roussel)	10	0	—	—	0	—	—	0	—	0.5	0		
	1	0	1.5	−1.5	0	1	1	0	0.5	0	0	**0.3**	**40**
	0.5	—	0.5	0	—	0	3	—	—	—	—		
	0.1	—	—	—	—	0	2	—	—	—	—		
	0.03	—	—	—	—	—	0	—	—	—	—		
11α-Hydroxy-4-pregnene-3,20-dione 11α-Hydroxyprogesterone (SKF, Ayerst)	10	1.5	0	0	0	0	2	0	3	0	0	**0.7**	**30**
	0.5	0	—	—	—	—	0	—	0	—	—		
9α-Fluoro-11β,16α,17,21-tetra-hydroxy-1,4-pregnadiene-3,20-dione Triamcinolone (Lederle)	10	—	—	—	0	—	—	0	—	0.5	1.5		
	2	0	−1.5	−0.5	0	1	0	—	0	1	1.5	**0.2**	**30**
	0.5	—	0	0	—	0	—	—	—	—	0		
9α-Fluoro-11β,17,21-trihydroxy-4-pregnene-3,20-dione 21-acetate 9α-Fluorocortisol (F-COL) acetate U-4845 (Upjohn)	2	0	0	0	−0.5	2	0	0	0.5	—	0	**0.2**	**20**
	0.5	—	—	—	—	0	—	—	0	—	—		
	0.1	—	—	—	—	0	—	—	—	—	—		
Overall protective index		**2.1**	**1.5**	**0.6**	**0.8**	**1.9**	**2.1**	**1.1**	**2.0**	**0.4**	**1.6**		
Protective spectrum index, %		**80**	**90**	**50**	**40**	**80**	**80**	**40**	**80**	**30**	**80**		

[a] For the sake of simplicity, we referred to this compound as "CS-1" since it was the first steroid devoid of classic hormonal actions that could be shown to possess catatoxic activity (63).

days, and mortality was listed on the 7th day. Only in the group pretreated with thyroxine is the grading based on mortality alone, since here all animals died before lesions in the Gasserian ganglia could have developed.

Cinchophen (K & K Laboratories)—35 mg. in 0.2 ml. DMSO s.c. once on the 4th day. The characteristic dyskinesia was measured by the "Flick test" (9) 4 hr. after injection of the toxicant.

Cocaine Hydrochloride (Mallinckrodt Chemical Works)—6 mg. in 1 ml. water i.p. once on the 4th day. Dyskinesia was measured 30 min. after administration of the drug, and mortality was listed 24 hr. later (108).

Colchicine (Abbott Laboratories)—200 mcg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated, and mortality was listed on the 7th day (99).

DL-*Coniine Hydrochloride (K & K Laboratories)*—5 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 2 hr. after injection, and mortality was listed 24 hr. later.

Croton Oil (AMEND Drug and Chemical Co. Inc.)—Per rat, 1 ml. of a 1% solution in corn oil injected into the lumen of a 25-ml. air pouch under the shaved dorsal skin (granuloma pouch technique) on the 4th day. The accumulated exudate was measured (ml.) on the 11th day.

Cyclobarbital (Sterling Winthrop)—7.5 mg. in 2.5 ml. water i.p. once on the 4th day. Sleeping time (min.) was determined.

Cycloheximide (The Upjohn Co.)—100 mcg. in 0.2 ml. physiologic NaCl solution s.c. twice daily from the 4th to the 6th day. Only mortality was listed on the 8th day. In a second experiment, 800 mcg. in 1 ml. of water was given *p.o.* on the 4th day. Dyskinesia was estimated, and mortality was listed on the 5th day (96).

Cyclophosphamide (Frank W. Horner Ltd.)—10 mg. in 0.4 ml. water s.c./day from the 4th to the last day of the experiment. The final body weight was measured (g.), and mortality was listed on the 15th day (97).

DDT [1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane] (Eastman Organic Chemicals)—25 mg. in 1 ml. corn oil *p.o.* once on the 4th day. Dyskinesia was measured 24

Table IIB—"Protective Spectra" *of Some Nonsteroidal Agents*

Conditioning Agent	Dose[a]	Toxicant: Digitoxin	Navadel	Parathion	Nicotine	Hexobarbital	Progesterone	Zoxazolamine	Indomethacin	Fluorocortisol Na_2HPO_4 Corn Oil ++	Dihydrotachysterol	Overall Protective Index	Protective Spectrum Index, %
Phetharbital (Burroughs Wellcome) in	10	2	3	2	2.5	3	3	3	3	0	2.5	**2.4**	**90**
1 ml. water, *p.o.*, twice daily	0.5	0	1.5	1	0	0	0	0	0	—	0		
	0.1	—	0	0	—	—	—	—	—	—	—		
Phenobarbital sodium (BDH) in 1 ml.	6	0	3	2.5	3	3	3	1	3	0	2	**2.1**	**80**
water, *p.o.*, twice daily	0.5	0	2	1	0	3	3	0	3	—	0		
	0.1	0	0	0.5	—	1	1	0	0	—	—		
	0.03	—	—	—	—	0	—	—	—	—	—		
Phenylbutazone (Geigy) in 1 ml. water,	10	0	3	1	3	3	3	0	3	0	0	**1.6**	**60**
p.o., twice daily	0.5	—	0	0	0	0	1	—	0	—	—		
	0.1	—	—	—	—	—	0	—	—	—	—		
Tolbutamide (Hoechst Pharmaceutical)	50	0	3	1	0	3	3	0	2	0	0.5	**1.3**	**60**
in 1 ml. water, *p.o.*, twice daily	10	—	0.5	0	—	0	2	—	0	—	—		
W-1372 (Wallace) in 1 ml. corn oil,	10	0	3	0	0	3	3	3	3	0	0	**1.5**	**50**
p.o., twice daily	5	0	3	1	0	0	2	3	0	0	—		
	1	0	1.5	0.5	0	0	0	0	0	0	—		
	0.5	—	0.5	—	—	—	—	0	—	—	—		
Vitamin E (Distillation Products Ind.)	50	0	1	0	0	1	1	0	0	0	1.5	**0.5**	**40**
in 1 ml. corn oil, *p.o.*, twice daily	10	—	—	—	—	0	—	—	—	—	0		
Bile duct ligature	—	0	0.5	0	0	−3	−3	−3	1.5	1.5[b]	1.5	**−0.4**	**40**
Acetylsalicylic acid (Merck) in 2 ml.	10	0	0	0	0	0	3	1	3	0	0	**0.7**	**30**
water, *p.o.*, twice daily	0.5	—	—	—	—	—	0	0	0	—	—		
Sodium Salicylate (Fisher) in 1 ml.	10	0	0	1	0	0	1	0	3	0	0	**0.5**	**30**
water, p.o., twice daily	0.5	—	—	0	—	—	0	—	0	—	—		
Nicotine (Eastman Organic Chemical)	3	1	0.5	0	0	0	0	0	1.5	0	0	**0.3**	**30**
in 1 ml. water, *p.o.*, twice daily	0.15	0	—	—	—	—	—	—	0	—	—		
ACTH (Nordic Biochemical Ltd.) in	50 I.U.	0	0	0	0	0	0	2	0	0	1	**0.3**	**20**
0.2 ml. water, s.c., twice daily	2.5 I.U.	—	—	—	—	—	—	1	—	—	0		
Vitamin D_2 (Wander) in 0.5 ml. corn	0.025	0.5	0	0.5	0	0	0	0	0	0	0	**0.1**	**20**
oil, *p.o.*, twice daily													
Vitamin A (Hoffmann-La Roche) in	2500 I.U.	0	0	0	0	0	0	0	0	0	0.5	**0.1**	**10**
0.5 ml. corn oil, *p.o.*, twice daily													
Indomethacin (Merck Sharp & Dohme)	0.15	0	0	0	0	0	0	0	0	0	0	**0**	**0**
in 0.2 ml. water, s.c., twice daily													
STH (C. H. Li) in 0.2 ml. water, s.c.,	1	0	0	0	0	0	0	0	0	0	0	**0**	**0**
twice daily	0.05	—	—	—	—	—	2	0	—	—	—		
	0.01	—	—	—	—	—	0	—	—	—	—	—	
Vitamin C (Fisher) in 1 ml. water,	50	0	0	0	0	0	0	−1	0	0	0	**−0.1**	**0**
p.o., twice daily													
Digitoxin (Roussel) in 1 ml. water,	0.15	0	0	0	0	0	0	0	0	0	−0.5	**−0.1**	**0**
p.o., twice daily													
L-Thyroxine (BDH) in 0.2 ml. water,	0.2	0	−1.5	−2	0	0	0	0	0	0	0	**−0.4**	**0**
s.c., daily	0.01	—	0	0	—	—	—	—	—	—	—		
Phentolamine (Ciba) in 0.2 ml. water,	3	2	1	—	—	0	—	—	0.5	—	—		
s.c., twice daily	0.5	0	0	—	—	—	—	—	—	—	—		
Overall protective index		**0.3**	**0.9**	**0.3**	**0.5**	**0.7**	**0.9**	**0.3**	**1.2**	**0.1**	**0.5**		
Protective spectrum index, %		**20**	**50**	**30**	**20**	**30**	**40**	**30**	**50**	**10**	**40**		

[a] Doses in international units (I.U.) are so indicated; all other dosages are expressed in milligrams. [b] This inhibition may be spurious since all rats were moribund or dead by the end of the experiment, although they showed no cardiac necrosis.

hr. after administration of the toxicant, and mortality was listed 24 hr. later.

Desoxycorticosterone Acetate (Schering)—2 mg. in 0.2 ml. water s.c. daily; and *sodium phosphate monobasic (Merck Sharp & Dohme)*—2 mmoles in 2 ml. water *p.o.* twice daily from the 4th day until the end of the experiment. Nephrocalcinosis was estimated on day of death in animals that lived at least 9 days, and mortality was listed on the 19th day.

**Digitoxin (Roussel)*—Per rat, 2 mg. in 1 ml. water *p.o.* on 4th and 5th days. The severity of the convulsions was estimated on the 7th day, and mortality was registered on the 9th day. Only in a few groups of Table II, which are taken from an earlier experimental series (105), did we deviate slightly from this technique in that we gave 1 mg. of digitoxin daily, beginning on the 4th day and continuing until the end of the experiment. The convulsions were estimated on the 6th day, and mortality was listed on the 9th day.

Digitoxin (Roussel)—Per rat, 0.4 mg. in 2 ml. water mixed with *sodium phosphate dibasic anhydrous (Fisher)* —1 mmole and *corn oil (Canada Starch Ltd. "Mazola")* —1 ml. *p.o.* twice daily from the 4th day until the end of the experiment. Cardiac necrosis was estimated on day of death in animals that lived at least 6 days, and mortality was listed on the 9th day (102).

**Dihydrotachysterol (DHT) (Wander)*—Per rat, 3 mg. in 0.5 ml. corn oil *p.o.* once on the 4th day. Cardiovascular

calcinosis was estimated on the day of death (but only in animals that survived at least until the 7th day, by which time the lesions were clearly visible). Mortality was listed on the 9th day (109).

Diisopropyl Fluorophosphate (DFP) (Merck Sharp & Dohme)—250 mcg. in 0.2 ml. corn oil s.c. once on the 4th day. Dyskinesia was estimated on the 5th day, and mortality was listed on the 6th day.

Dimercaprol (BAL) (J. T. Baker Chemical Co.)—0.2 ml. of a 5% solution in corn oil s.c. once on the 4th day. Dyskinesia was estimated 4 hr. after administration of the drug, and mortality was listed 24 hr. later.

Dinitrophenol (Brickman)—3.2 mg. in 0.2 ml. DMSO s.c. once on the 4th day. Dyskinesia was estimated 1 hr. after injection of the toxicant, and mortality was listed 24 hr. later.

Diphenylhydantoin (Eastman Organic Chemicals)—30 mg. in 1 ml. water i.p. once on the 4th day. Dyskinesia was measured 3 hr. after injection of the toxicant.

Dipicrylamine (Eastman Organic Chemicals)—10 mg. on the 4th day and 15 mg. on the 5th day in 0.2 ml. DMSO s.c. Prostration was measured on the 5th day, 2 hr. after dipicrylamine injection, and mortality was listed 24 hr. later.

Escherichia coli *endotoxin No. 08 (Difco Laboratories)*—Per rat, 800 mcg. in 0.8 ml. water i.v. once on the 4th day Dyskinesia was measured 3 hr. after injection of the endotoxin, and mortality was listed on the 7th day.

Edrophonium Chloride (Hoffmann-La Roche)—5 mg. in 0.1 ml. water s.c. once on the 4th day. Dyskinesia was estimated 15 min. after injection, and mortality was listed 24 hr. later.

Emetine Hydrochloride (S. B. Penick & Co.)—3 mg. in 1 ml. water *p.o.* on the 4th day and 4 mg. on the 5th day. Prostration, adrenal necrosis, and mortality were determined on the 6th day.

Ephedrine Sulfate (Brickman)—50 mg. in 0.2 ml. water s.c. twice on the 4th day with an interval of 5.5 hr. between the injections. Dyskinesia was measured 4.5 hr. after the first injection of the toxicant, and mortality was listed on the 7th day.

Epinephrine Bitartrate (Brickman)—1.5 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 5 hr. after injection, and mortality was listed 24 hr. later.

EPN (Phenylphosphonothioic Acid O-Ethyl O-p-Nitrophenyl Ester) (Dupont)—0.7 mg. in 0.2 ml. DMSO i.p. once on the 4th day. Dyskinesia was estimated 1 hr. after injection, and mortality was listed 24 hr. later.

Estradiol (Schering)—500 mcg. in 0.2 ml. water s.c. daily; and NaH_2PO_4 *(Merck Sharp and Dohme)*—2 mmoles in 2 ml. water *p.o.* twice daily, from the 4th day until the end of the experiment. Nephrocalcinosis was estimated on day of death in animals that lived at least 9 days, and mortality was listed on the 19th day.

Ethion (Niagara Brand Chemicals)—0.5 ml. of a 1.2% corn oil solution *p.o.* once on the 4th day. Dyskinesia was estimated 4 hr. after ethion administration, and mortality was listed 24 hr. later.

Ethyl Alcohol—2 ml. of a 50% aqueous solution *p.o.* daily from the 4th to 6th day. Dyskinesia was estimated on the 6th day 3 hr. after ethyl alcohol administration, and mortality was listed on the 8th day.

Ethylene Chlorohydrin, 2-Chloroethanol (Eastman Organic Chemicals)—0.2 ml. of a 4% aqueous solution on the 4th day and 0.3 ml. on the 5th day *p.o.* Dyskinesia was estimated on the 5th day 1 hr. after administration of the drug, and mortality was listed 24 hr. later.

Ethylene Glycol (Fisher)—0.8 ml. of a 100% solution *p.o.* daily from the 4th day until the end of the experiment. Dyskinesia was estimated on the 5th day 5 hr. after ethylene glycol administration, and mortality was listed 24 hr. later (110).

Ethylmorphine Hydrochloride (May & Baker)—20 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 3 hr. after injection, and mortality was listed 24 hr. later.

Fluorocortisol Acetate (The Upjohn Co.)—750 mcg. in 0.2 ml. water s.c. once daily; *sodium perchlorate (Fisher)*—per rat, 1 mmole in 2 ml. water *p.o.* twice daily; and *corn oil (Canada Starch Ltd. "Mazola")*—1 ml. *p.o.* twice daily from the 4th day until the end of the experiment. Cardiac necrosis was estimated on day of death in animals that lived at least 8 days, and mortality was listed on the 9th day (102).

Fluorocortisol Acetate (The Upjohn Co.)—750 mcg. in 0.2 ml. water s.c. daily; Na_2HPO_4 *(Fisher)*—1 mmole in 2 ml. water *p.o.* twice daily from the 4th day until the end of the experiment; and *restraint*—during 17 hr. from the 6th day. Cardiac necrosis was estimated on day of death in animals that lived at least 7 days, and mortality was listed on the 8th day (102).

**Fluorocortisol Acetate (The Upjohn Co.)*—750 mcg. in 0.2 ml. water s.c. once daily; Na_2HPO_4—1 mmole in 2 ml. water; and *corn oil*—1 ml., the latter two by stomach tube twice daily. All three agents were applied from the 4th day to the end of the experiment. The severity of the cardiac necroses was estimated on the day of death (but only in animals that survived at least until the 7th day, by which time the lesions become visible). Mortality was listed on the 11th day when the experiment was terminated (13).

Fasting—The mean survival after total fasting (with drinking water *ad lib.*) beginning on 4th day was listed, and the body weight was measured on the 6th day.

Glycerol (Fisher)—0.8 ml. of a 100% solution s.c. once on the 4th day. Nephrocalcinosis was estimated on the day of death in animals that lived at least 6 days, and mortality was listed on the 9th day. Only in groups pretreated with triamcinolone is the grading based on mortality alone, because all animals died before the development of nephrocalcinosis.

Griseofulvin (Ayerst)—Per rat, 7.5 mg. in 0.1 ml. DMSO i.v. once on the 4th day. Dyskinesia was estimated 30 min. after injection, and mortality was listed 24 hr. later.

Hexamethonium Chloride (Matheson Coleman & Bell)—8 mg. in 0.2 ml. water s.c. once on the 4th day. Prostration was estimated 30 min. after injection.

**Hexobarbital (Sterling Winthrop)*—7.5 mg. in 1 ml. water i.p. once on the 4th day. The sleeping time was determined immediately.

Homatropine Hydrobromide (Brickman)—80 mg. on the 4th day and 100 mg. on the 5th day in 1 ml. water *p.o.* Dyskinesia was estimated on the 5th day, 1 hr. after administration of the drug; mortality was listed on the 6th day.

Hydroquinone (*Baker*)—15 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 15 min. after injection.

Indium Trichloride (*Brickman*)—800 mcg. in 1 ml. water i.v. once on the 4th day. Hepatic lipidosis was estimated on day of death from the 6th day, and mortality was registered on the 9th day when experiment was terminated.

**Indomethacin* (*Merck Sharp & Dohme*)—Per rat, 1 mg. in 0.2 ml. water s.c. once daily from the 4th day until the end of the experiment. Intestinal ulcers were appraised on day of death, but only in animals that survived at least 6 days; mortality was listed on the 9th day (105).

Mechlorethamine Hydrochloride (*Merck Sharp & Dohme*)—Per rat, 100 mcg. in 0.2 ml. water s.c. daily from the 4th to 6th day. Mortality was listed on the 8th day.

Mephenesin (*Squibb*)—30 mg. on the 4th day and 60 mg. on the 5th day in 0.2 ml. propylene glycol s.c. Paralysis was estimated on the 5th day, 2.5 hr. after injection (91).

Meprobamate (*Wallace*)—50 mg. in 0.2 ml. DMSO s.c. once on the 4th day. Prostration was estimated 3 hr. after injection (98).

Mercuric Chloride (*May & Baker*)—400 mcg. in 1 ml. water i.v. once on the 4th day. Nephrocalcinosis was estimated on day of death from the 5th day, and mortality was registered on the 7th day.

Mercuric Chloride (*May & Baker*)—300 mcg. in 1 ml. water i.v. once on the 1st day, 1 hr. after the last steroid treatment. Nephrocalcinosis was estimated on day of death from the 2nd day, and mortality was registered on the 4th day.

Mersalyl (*K & K Laboratories*)—In the first series, 4 mg. in 1 ml. water i.v.; in the second series, 10 mg. in 0.2 ml. s.c. once on the 4th day. Nephrocalcinosis was estimated on day of death in animals that lived at least 5 days, and mortality was listed on the 7th day.

Methadone Hydrochloride (*Baker*)—1.5 mg. in 1 ml. water i.p. once on the 4th day. Motor disturbance was estimated 2 hr. after injection, and mortality was registered 24 hr. later.

Methylphenidate (*Ciba*)—10 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 2 hr. after injection, and mortality was listed 24 hr. later.

Methyprylon (*Hoffmann-La Roche*)—20 mg. in 0.2 ml. water s.c. once on the 4th day. The sleeping time was determined immediately (100).

Morphine Sulfate (*Merck Sharp & Dohme*)—20 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 2 hr. after injection, and mortality was listed 24 hr. later.

**Navadel* (*Hercules Inc.*)—4 mg. in 1 ml. corn oil *p.o.* once on the 4th day. Dyskinesia was estimated 5 hr. after administration of the toxicant, and mortality was listed 48 hr. later.

Nephrectomy—On the 8th day. Body weight (g.) and mean survival were listed on the 7th day.

**Nicotine* (*Eastman Organic Chemicals*)—1 ml. of a 1% aqueous solution *p.o.* daily from the 4th day until the end of the experiment. Dyskinesia was estimated on the 6th day, 30 min. after nicotine administration; mortality was listed on the 9th day.

Octamethyl Pyrophosphoramide (*K & K Laboratories*)—In the first experiment 1 mg. in 0.2 ml. corn oil s.c. once on the 4th day. Dyskinesia was estimated 3 hr. after injection, and mortality was listed 24 hr. later.

Pancuronium Bromide (*Organon*)—10 mg. in 1 ml. water *p.o.* twice daily on the 4th and 5th days. Dyskinesia was estimated on the 5th day, 30 min. after the second injection; mortality was listed on the 6th day.

**Parathion* (*Niagara Brand Chemicals*)—1 mg. in 0.5 ml. DMSO i.p. daily from the 4th to the last day of the experiment. Dyskinesia was estimated on the 6th day, 4 hr. after injection; mortality was listed on the 7th day.

Pentylenetetrazol (*Knoll Pharmaceutical*)—8.5 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 2 hr. after injection, and mortality was listed 24 hr. later.

Perchlorate Sodium (*Fisher*)—1 mmole in 1 ml. water *p.o.* twice on the 4th day and 2 mmoles twice daily from the 5th day. Flick test was estimated on the 6th day, 1 hr. after the first perchlorate sodium administration; mortality was listed on the 11th day.

Phenindione (*Schieffelin*)—10 mg. in 0.2 ml. DMSO s.c. daily from the 4th day until the end of the experiment. Intestinal hemorrhage was estimated on day of death, and mortality was listed on the 8th day (93).

Phosphorodithioic Acid O,O-*Dimethyl Ester*, S-*Ester with 3-(Mercaptomethyl)-1,2,3-benzotriazin-4(3*H)-*one*[3] (*Bayer A.G.*)—1 mg. in 0.2 ml. propylene glycol s.c. once on the 4th day. Dyskinesia was estimated 2 hr. after injection.

Physostigmine Sulfate (*Brickman*)—1 mg. in 1 ml. water *p.o.* once on the 4th day. Dyskinesia was estimated 1 hr. after physostigmine sulfate administration, and mortality was listed 24 hr. later.

Picrotoxin (*BDH*)—350 mcg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 30 min. after injection, and mortality was listed 24 hr. later (92).

Piperidine (*Matheson Coleman & Bell*)—50 mg. in 1 ml. water *p.o.* once on the 4th day. Dyskinesia was estimated 5 hr. after piperidine administration.

Pipradrol HCl (*Wm. S. Merrell Co.*)—30 mg. in 1 ml. corn oil *p.o.* once on the 4th day. Dyskinesia was estimated 3 hr. after pipradrol administration, and mortality was listed 24 hr. later.

Pralidoxime Chloride (*Ayerst*)—16 mg. in 0.2 ml. water s.c. on the 4th and 5th days. Dyskinesia was estimated on the 4th day, 30 min. after injection; mortality was listed on the 6th day.

**Progesterone* (*Roussel*)—10 mg. in 1 ml. oil i.p. once on the 4th day. Sleeping time was determined immediately.

Propionitrile (*Baker*)—15 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 4 hr. after injection, and mortality was listed 24 hr. later.

Propylthiouracil (*C. E. Frosst Co.*)—30 mg. in 0.15 ml. DMSO i.p. once on the 4th day. Dyskinesia was estimated 3 hr. after injection, and mortality was listed 24 hr. later.

[3] Guthion, *O,O*-dimethyl-*S*-(4-oxobenzotriazino-3-methyl)phosphorodithioate.

Pyrilamine Maleate (*Poulenc*)—8 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 30 min. after injection, and mortality was listed 24 hr. later.

SKF 525-A (*β-Diethylaminoethyldiphenylpropyl Acetate*) (*Smith Kline & French*)—15 mg. in 1 ml. water i.p. once on the 4th day. Dyskinesia was estimated 2 hr. after injection.

Strychnine Hydrochloride (*BDH*)—150 mcg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 15 min. after injection, and mortality was listed on the same day.

Tetraethylammonium Chloride (*Eastman Organic Chemicals*)—10 mg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 30 min. after injection, and mortality was listed 24 hr. later (117).

Thallium Chloride (*Fisher*)—16 mg. in 0.5 ml. corn oil s.c. once on the 4th day. Nephrocalcinosis was estimated on day of death in animals that lived at least 6 days, and mortality was listed on the 7th day.

Thiopental Sodium (*Abbott*)—5 mg. in 1 ml. water *p.o.* once on the 4th day. Sleeping time was determined immediately.

Triamcinolone (*Lederle*)—1 mg. in 1 ml. water *p.o.* twice daily from the 4th to 14th day and 2 mg. from the 15th day. Final body weight was listed on the 30th day.

Tribromoethanol (*K & K Laboratories*)—25 mg. in 1 ml. water and amylene hydrate s.c. once on the 4th day. Sleeping time was determined immediately.

Trichloroethanol (*K & K Laboratories*)—1 ml. of a 5% aqueous solution *p.o.* once on the 4th day. Sleeping time was determined immediately.

3,3,5-Triiodo-L-thyronine (*K & K Laboratories*)—200 mcg. in 0.2 ml. water s.c. twice daily from the 4th day until the end of the experiment. Final body weight and mortality were listed on the 12th day.

Tri-o-cresyl Phosphate (*K & K Laboratories*)—Per rat, 50 mg. in 1 ml. corn oil *p.o.* daily from the 4th to 11th day and twice on the 12th day with 1-hr. interval. Dyskinesia was estimated on the 7th day, 3 hr. after tri-*o*-cresyl phosphate administration; mortality was listed on the 8th day. (With thyroxine on the 12th day, the rats were alive.)

d-*Tubocurarine Chloride* (*Mann Research Laboratories Inc.*)—20 mcg. in 0.2 ml. water s.c. once on the 4th day. Dyskinesia was estimated 30 min. after injection.

L-*Tyrosine* (*N. B. C.*)—10% Alam diet (111) from the 2nd day until the end of the experiment. Eye lesions were estimated on day of death from the 8th day, and mortality was registered on the 15th day (112).

W-1372 (N-γ-*Phenylpropyl*-N-*benzyloxy Acetamide*) (*Wallace*)—In first experiment, 30 mg.; in second, 40 mg., in 1 ml. corn oil *p.o.* twice daily on 4th and 5th days. Hepatic lipidosis was estimated on day of death, and mortality was listed on the 7th day.

Warfarin (*K & K Laboratories*)—10 mg. in 1 ml. water *p.o.* twice daily from the 4th day until the end of the experiment. Mortality was listed on the 9th day.

**Zoxazolamine* (*K & K Laboratories*)—10 mg. in 1 ml. water i.p. once on the 4th day. Paralysis time was determined immediately.

First Step: Protection against Digitoxin and Indomethacin

The first systematic investigations designed to identify protective steroids consisted of a series of bioassays in which 304 natural and synthetic steroidal compounds were tested for their ability to protect the rat against digitoxin and indomethacin, under the experimental conditions outlined in the preceding section. Since these results have been the subject of an extensive review (105), we shall limit ourselves here to a brief description of the principal conclusions derived from this work.

First, it must be stated that the steroids that protect against digitoxin or indomethacin actually accelerate the disappearance of these toxicants from the blood (113). This fact, in conjunction with many other biochemical observations (114–116), strongly supports the view that the prophylactic action of these toxicants is due to their catatoxic properties (an acceleration of drug clearance) rather than to an increased tissue tolerance. It is not yet quite clear, however, to what extent protection is achieved through increased drug destruction or through an acceleration of excretion (*e.g.*, in bile or urine) of the unchanged drug or of its metabolites. Indeed, until the mechanism of protection by each steroid against each toxicant has been fully clarified, even the possibility of increased tissue tolerance (that is, a syntoxic effect) cannot be excluded in all cases.

In any event, protective activity was widespread among the 304 steroids tested; it was demonstrable among gonanes, estranes, androstanes, androstenes, and 5β- and 5α-pregnanes, as well as among pregnenes, with one or more double bonds, and with or without halogen substitution in the ring system. On the other hand, cholanes, cholestanes, and genins were uniformly inactive, with the sole exception of methylnordeoxycholanate (3α,12α-dihydroxy-24-nor-5β-cholan-23-oic acid methyl ester).

Because of this widespread distribution of anti-indomethacin and antidigitoxin activity throughout various classes of steroids, it is very difficult to formulate any clear-cut rules about pharmacochemical correlations in this field. It does appear, however, that although catatoxic activity is not strictly dependent upon any single structural prerequisite, in general the 17α-propionic acid-γ-lactone side chain is advantageous for both antidigitoxin and antiindomethacin activity. It is perhaps also not purely coincidental that a very large number of active catatoxic steroids are found among the 1,4-androstadienes, as well as among halogenated androstene and pregnene derivatives. It is likewise noteworthy that several of the most active catatoxic steroids are 19-nor compounds; hence, the angular methyl group at C_{10} is not only dispensable but often detrimental. The most striking observation in this series of tests was that among all 304 steroids tested, the most active against both substrates proved to be a cyano-compound, namely 5-pregnenolone-16α-carbonitrile (PCN).

This first systematic screening series also revealed that the catatoxic activity is not strictly dependent upon any other known pharmacologic property, although most of the highly potent antidigitoxin and antiindomethacin

steroids also exhibit antimineralocorticoid or anabolic properties.

Because of the comparatively small number of animals that could be used for the bioassay of the many not readily available steroids, only the lowest and the highest activity grades were given serious consideration. However, even on this rigid basis of appraisal, we found that at a 10-mg. dose level among 304 steroids tested, there were:

Active only against indomethacin	42
Active only against digitoxin	32
Active against both substrates	24

the remainder being inactive or of doubtful activity.

At the 0.5-mg. dose level, we found:

Active only against indomethacin (Compounds 255 and 277)	2
Active only against digitoxin (Compound 85)	1
Active against both substrates (Compound 233)	1

These compounds correspond to the following structures:

Compound 85 = 9α-fluoro-11β,17-dihydroxy-3-oxo-4-androstene-17α-propionic acid potassium salt (SC-11927) (CS-1).

Compound 233 = 3β-hydroxy-2-oxo-5-pregnene-16α-carbonitrile (SC-4674) (PCN).

Compound 255 = 9α-fluoro-16β-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione 21-acetate (betamethasone acetate).

Compound 277 = 21-hydroxy-3-oxo-1,4,9(11)-pregnatrieno-[17α,16α-*d*]-2′-methyloxazoline acetate.

It is especially noteworthy that several of the active catatoxic steroids are naturally occurring hormones, hormone precursors, or hormone metabolites such as: progesterone, 17α-hydroxyprogesterone, 5-pregnenolone, and dehydro-*iso*-androsterone (105).

Encouraged by these first observations, we then proceeded to repeat some of the key observations at lower dose levels. We also performed similar tests on many additional steroids, especially carbonitriles and other compounds related to the most active members in the preliminary series. This work was done under experimental conditions exactly corresponding to those of the first screening tests, and the results are summarized in Table I.

The carbonitriles have been arranged according to the increasing number of the skeletal carbon atoms to which the —CN groups or —CN-bearing side chains are attached. At the end of Table I, a few additional steroids, other than nitriles (tested or retested at reduced dose levels since our last publication), are listed in arbitrary order. A glance at the overall protective indexes of these compounds shows that the most potent catatoxic steroids against both substrates used in this preliminary test were those bearing a 2α- or 16α-carbonitrile group. Among these, several showed potency against both substrates at individual dose levels as low as 100 mcg. (Compounds 3, 24, 27, 29, 30, and 31). Several of these compounds, as well as Compound 28, protected against indomethacin even at the individual dose level of 30 mcg., indicating that protection against indomethacin is more readily obtained than against digitoxin.

Compound 23, the only 16β-carbonitrile of our series, as well as Compound 22, in which the steric position of the 16-carbonitrile is unknown, were inactive in protecting against either substrate, even at the dose level of 500 mcg. Compound 21, in which the —CN group is attached to a 16α-side chain rather than to the C_{16} carbon of the steroid skeleton itself, showed no protective activity, even at the dose of 10 mg. On the other hand, it is hardly coincidental that all 11 16α-carbonitriles tested (Compounds 24–34) were active and most of them even at very low dose levels. This suggests that the attachment of a —CN group in the 16α-position directly to the steroid skeleton is very favorable for this type of protective effect; the configuration of the rest of the steroid molecule, although capable of influencing the degree of activity, is of lesser importance.

It is known from our previous work that a carbonitrile group in position 2α (*e.g.*, Compound 3, TMACN) is also compatible with high catatoxic activity against a variety of substrates; additional evidence justifying this conclusion is given in Tables I and IIA.

Carbonitrile groups in position 3 were found to convey some potency in the present series (Compounds 4, 5, and 7), but steroids with carbonitriles attached to C_1 (Compounds 1 and 2) or C_5 (Compounds 9–19) were uniformly inactive at all dose levels tested. Compound 9 was tested at many small dose levels, because at the standard initial dose of 500 mcg. it caused a coagulation defect with a 100% mortality from diffuse bleeding into various organs. This complication could have masked a catatoxic effect, but no such effect could be demonstrated even when the steroid was given in amounts causing no serious damage as a consequence of bleeding. Besides, subsequent samples of this steroid failed to prevent blood coagulation even at the 30-mcg. dose level.

Carbonitrile substitution at C_{17}, C_{20}, C_{21}, or in side chains resulted in no remarkable catatoxic potency at the dose levels tested, with the exception of Compounds 53 and 68 which were moderately effective in this respect at the dose level of 500 mcg.

Among steroids other than carbonitriles in this list, special interest attaches to Compound 86, an azasteroid, and Compound 88 (mestranol), a strong folliculoid (used in anticonceptional pills), both of which showed some catatoxic activity at least at the high dose of 10 mg. This degree of activity is of little practical significance, but it is interesting that a heterocyclic aza-compound and a folliculoid can possess some catatoxic potency.

Finally, it is noteworthy that (except for the moderate potency of Compound 78) all 16-carboxylic acids (Compounds 72–77) are devoid of catatoxic potency against both substrates. *A priori*, the possibility could not have been excluded that nitriles are metabolized *in vivo* into the corresponding carboxylic acids and that the latter would be responsible for catatoxic activity, but this does not appear to be the case.

Second Step: Determination of "Protective Spectrum"

Having selected the most promising protective substances by first screening them for activity against

digitoxin and indomethacin, we proceeded to appraise the "Protective Spectra" of the most potent ones among them. These compounds were now tested against a heterogeneous set of 10 pathogens, widely differing in their chemical structure and in the organ changes that they elicit.

The statistical significance of the results was computed (as outlined on pp. 8–9) for the inhibition or aggravation of the changes produced by each of the 10 model toxicants. After this, the "Overall Protective Index" and the "Protective Spectrum Index" were calculated as rough indications of the mean degree and the specificity of protection, that is, of the quantitative and qualitative prophylactic potencies, respectively. These data are summarized in Table II.

The 10 damaging agents enumerated in the caption of the table have been marked with an asterisk in the list of procedures used to produce and appraise various types of damage (pp. 9–17); hence it will not be necessary here to describe either the techniques of administration of these toxicants or the manner in which protection is expressed. Suffice it to recall that the highest possible degree of protection corresponds to grade 3, the "Overall Protective Index" expresses the mean grade of protection, and the "Protective Spectrum Index" of a compound gives the percentage of those among the toxicants tested against which significant protection is obtained (irrespective of the degree of significance). These two indexes—given in the last two vertical columns of Tables IIA and IIB—do not run strictly parallel, but the various compounds tested for protective potency are listed roughly in decreasing order of their Protective Spectrum Indexes. Whenever the material at our disposal permitted it, compounds active at a certain dose were retested at a lower dose level; but, of course, in calculating the two indexes, dose levels at which a protective compound could not be tested (marked with a dash) were excluded. In any event, the indexes are listed only for the highest, but still well-tolerated, dose of each protective compound and only if the latter could be tested against all 10 standard toxicants. The last two horizontal columns in Tables IIA and IIB list the indexes for the amenability of the toxicants to detoxication by the conditioning agents.

Perusal of Table IIA indicates that all steroids were active in offering protection, at least against some of the damaging agents; but this is so merely because only those steroids that had shown some potency in preliminary tests were included in this study.

It is noteworthy that among all steroids tested, PCN again exhibited the highest catatoxic activity as judged by both indexes. It must be remembered, however, that this was the only 16α-carbonitrile available in sufficient amounts to test against 10 substrates. CS-1, cyproterone acetate, ethylestrenol, spironolactone, norbolethone, and TMACN were almost equally efficacious at the highest dose (10 mg.); but at the 500-mcg. dose level, activity fell rapidly, roughly in the order in which the compounds are mentioned here. Indomethacin and digitoxin are most readily detoxified—in the case of PCN even at the dose level of 30 mcg. However, the general indexes of activity would not be meaningful at the low dose levels at which efficacy against other toxicants has not been examined; hence, they are listed only for the optimal protective dose. It will be noted that usually this is 10 mg. (the highest dose tested); but in the case of such compounds as the strong glucocorticoids or estradiol, inherent toxicity of heavy overdosage counteracts the protective effect against drugs by causing severe mortality. In these instances, lower dose levels were selected for the computation of the Overall Protective Index and the Protective Spectrum Indexes of the steroids.

On the other hand, pregnanedione and the steroids listed after it in Table IIA exhibit only negligible, if any, activity with the exception of occasional strong inhibitory effects (grade 2 or 3) against individual toxicants (*e.g.*, pregnanedione against parathion, hexobarbital, and indomethacin; progesterone against indomethacin; 11α-hydroxyprogesterone against indomethacin; hydroxydione against digitoxin; and DOC against nicotine). This singular specificity of protection among compounds having a very low, if any, protective effect against other substrates may well depend upon specific so-called "physiologic antagonisms" (*e.g.*, the anesthetic effect of hydroxydione or the mineralocorticoid action of DOC), but further experiments will be necessary to prove this.

A glance at the Overall Protective Indexes and the Protective Spectrum Indexes of the toxicants (last two horizontal lines at the bottom of Table IIA) shows that digitoxin, navadel, hexobarbital, progesterone, indomethacin, and DHT were most readily detoxified by the largest number of conditioning agents; parathion, nicotine, zoxazolamine, and especially the infarctoid cardiopathy produced by fluorocortisol + Na_2HPO_4 + corn oil were most resistant.

Among the nonsteroidal agents of Table IIB, rather specific antagonisms of this type were quite common. For example, ACTH increased resistance to DHT and to the neuromuscular blocking action of zoxazolamine, although this pituitary hormone had little, if any, effect against other agents.

Vitamin E offered some protection against navadel, hexobarbital, and DHT; whereas acetylsalicylic acid protected against progesterone anesthesia, zoxazolamine paralysis, and indomethacin ulcers. Yet, these compounds offered no noteworthy protection against other toxicants.

Bile duct ligature offered complete protection against DHT-induced calcinosis and indomethacin ulcers, but the mortality was not completely prevented and hence the grade of protection—which reflects the mean inhibition of lesions plus mortality—is comparatively moderate. It is very likely that occlusion of the choledochus acts by preventing bile secretion, thereby interfering with the enterohepatic circulation.

Digitoxin, indomethacin, and vitamin D, all of which are readily detoxified under the influence of catatoxic steroids, do not act as inducers of protective enzymes against any of the substrates tested. Obviously there is no relationship between amenability to detoxication by steroid-induced enzymes and the power to induce such enzymes.

As with the steroidal protective agents, indomethacin intoxication appears to be particularly easy to prevent,

Table III—Toxicants Amenable to Conditioning

Toxicant	Conditioning Agent: PCN	Ethyl-estrenol	CS-1 (SC-11927)	Spirono-lactone	Norbo-leth-one	Oxan-drolone	Predni-solone-Ac	Proges-terone	Triam-cino-lone	DOC-Ac	Hy-droxy-dione	Estra-diol	L-Thy-roxine	Pheno-barbital	Overall Protective Index	Protective Spectrum Index, %
Aminoacetonitrile	0	0	0	0	0	0	3[b]	1	3[d]	1	0	1[b]	3	0	**0.9**	**40**
Barbital	0	0	−1	0	−1	0	2	0	0[a]	−3	−1	−3[b]	0	−2	**−0.6**	**10**
Bishydroxycoumarin	0	1	1	2	1	2	0	0	0[a]	0	0	3	0	0	**0.7**	**40**
Cadmium chloride	1.5	1	0	1	0.5	0	2.5	0	1.5	0	0	2.5	1	0	**0.8**	**60**
Cinchophen	3	2	0	3	2	0	3	0	0[a]	0	0	0[b]	0	3	**1.1**	**40**
Cocaine HCl	3	3	3	3	2.5	2	3	1.5	0	0	1	3	0	3	**2.0**	**80**
Colchicine	3	1.5	3	3	0	2	0	0.5	0[a]	0.5	0	0[b]	0	2	**1.1**	**60**
DL-Coniine HCl	2	0	0	0	0	0	1.5	0	2	−0.5	0	2	0	0	**0.5**	**30**
Croton oil	2	0	0	0	0	0	2	0	2[a]	0	0	0[b]	0	0	**0.4**	**20**
Cyclobarbital	3	3	3	3	3	3	3	0	3[a]	0	0	0[b]	0	3	**1.9**	**60**
Cycloheximide																
100 mcg.	3	3	3	3	3	3	0	3	0[a]	1	3	0	0	3	**2.0**	**70**
800 mcg.	3	3	3	3	1.5	2	1.5	2	0[a]	0.5	0	0[b]	0	3	**1.6**	**70**
Cyclophosphamide	3	1	3	3	1.5	0	−1.5	1.5	−1.5	0	0	−1.5	−0.5	0	**0.6**	**40**
Desoxycorticosterone-Ac + NaH_2PO_4	0	0	2	0	0	0	−3	0	−3[a]	0	0	0[b]	1.5	0.5	**−0.1**	**20**
Digitoxin + Na_2HPO_4 + corn oil	1.5	2	2.5	2	2.5	1.5	0	2	0	0	0	0	0	0.5	**1.0**	**60**
Diisopropyl fluorophosphate "DFP"	0.5	0.5	0.5	0.5	0	0	0.5	0	0[a]	0	0	0[b]	0	0	**0.2**	**40**
Diphenylhydantoin	0	0	1	1	0	0	−3	0	−3[a]	0	0	0[b]	−1	2	**−0.2**	**20**
Dipicrylamine	2	0	2	0	0	0	0	0	−1.5	0	0	0	−1.5	0	**0.1**	**10**
E. coli, endotoxin No. 08	0	0	−0.5	0	−0.5	0	1.5	0	3[a]	0	0	0[b]	−1	0	**0.2**	**10**
Emetine HCl	1.5	0	1.5	0.5	0	−0.5	−0.5	−0.5	0	−0.5	0	0	−0.5	0	**0.1**	**20**
Epinephrine	0	0	0	0	0	0	−0.5	0	−1.5	0	−0.5	−1	−3	−0.5	**−0.5**	**0**
EPN	3	3	3	3	3	3	0.5	0.5	0[a]	1.5	2	2[b]	−1	2	**1.8**	**90**
Estradiol + NaH_2PO_4	−0.5	0	0	0	−0.5	0	−1.5	0	−1.5[a]	0	0	0[b]	0.5	0	**−0.2**	**10**
Ethion	3	3	3	3	3	3	2.5	3	0[a]	0	0	1[b]	0	3	**2.0**	**70**
Ethyl alcohol	0	0	0	0	0	0	−1.5	0	−0.5[a]	0	0	0[b]	0	0	**−0.1**	**0**
Ethylene glycol	0	0	0	−0.5	0	0	1.5	0	2[a]	0	0	−2	2.5	0	**0.3**	**20**
Ethylmorphine HCl	3	3	3	3	2	2	1	2	0	1.5	0	3	0	3	**1.9**	**80**
Fluorocortisol + corn oil + $NaClO_4$	1.5	0	2	2	0.5	0	−0.5	0	−1	0	0	0	0	1	**0.3**	**40**
Fluorocortisol + Na_2HPO_4 + restraint	2	0	1.5	2	0	0	−0.5	0	−0.5	0	1	0	0	1	**0.5**	**40**
Glycerol	0	1.5	1	0	1	0.5	1.5	1	−2	0	0	0	1.5	0.5	**0.5**	**60**
Griseofulvin	1.5	−0.5	0	−1.5	0	0	0	−1	−1[a]	0	−1.5	−3[b]	−1	0.5	**−0.5**	**10**
Hexamethonium Cl	0	0	0	0	0	0	3	0	3	0	0	0	0	0	**0.4**	**10**
Hydroquinone	2	3	1	0	0	0	−1	0	−3	0	0	0	0	2	**0.3**	**30**
Mephenesin	3	3	3	3	3	0	1	0	0[a]	1	0	0	0	0	**1.2**	**50**
Meprobamate	3	3	3	3	3	3	3	0	0[a]	0	0	0[b]	0	3	**1.7**	**60**
Mercuric chloride																
300 mcg.	0	0	0	3[c]	0	0	0	0	0	0	0	0	0	0	**0.2**	**10**
400 mcg.	0	0.5	0	3	0	0	0.5	2	0	0	0	1	−0.5	0	**0.5**	**40**
Mersalyl																
10 mg.	0	2.5	0	2.5	0.5	0.5	2	0	0	0	0.5	1	0	2.5	**0.9**	**60**
4 mg.	0	2	0		1.5	0.5	3	0.5	1	0	0	0	0	0	**0.8**	**50**
Methadone	0	1.5	0	0	0	0	0	−1	0.5	0	0	0	−0.5	0	**0.04**	**10**
Methylphenidate	0	0	0	0	0	0	0	0	0	0	0	0	−1.5	0	**−0.1**	**0**
Methyprylon	3	3	3	3	3	3	3	3	3	0	1	2	0	3	**2.4**	**90**
Morphine	0	0	0	0	0	0	0	0	0	0	0	0	−0.5	0	**−0.04**	**0**
OMPA (1 mg.)	0	0	0	0	0	0	0	0	0	0	0	−3	0	0	**−0.2**	**0**

Phenindione	1.5		3	3	3	3	3	0	1	0[a]	0.5	1	0.5[b]	0	3	1.6	80
Phosphorodithioic acid *O,O*-dimethyl ester, *S*-ester with 3-(mercaptomethyl)-1,2,3-benzotriazin-4(3*H*)-one	3	3	3	3	3	3	1	0	0[a]	0	0	0	0[b]	0	3	1.6	60
Physostigmine sulfate	0.5	1	1	0.5	0	0	0.5	0	0[a]	0	0	0	−1[b]	−2	0	0.04	40
Picrotoxin	3	3	3	3	3	3	3	3	0.5[a]	2	1	0[b]	0	2	2.1	90
Piperidine	0	3	3	0	3	3	0	3	3	1	3	0	1	3	1.9	70
Pralidoxime Cl	0	1	0	0	0	0	2.5	0	2	0.5	0	1	0	0.5	0.5	40
Propionitrile	−2.5	0	0	0	0	0	−2.5	0	−3	0	0.5	0.5	−3	0	−0.7	10
Propylthiouracil	0	1.5	2	1.5	1.5	2	0	0.5	−0.5	0	0	0	−0.5	1.5	0.7	50
SKF 525-A	2	1	3	3	1	3	3	3	3[a]	0	1	0[b]	0	1	1.7	80
Sodium perchlorate	0	0	−1.5	0	0	0	−1	0	0[a]	0	0	0[b]	1.5	0	−0.1	10
Strychnine HCl	0.5	0.5	0	0.5	0	0	3	0	3	0	0	3	−0.5	3	0.9	50
Tetraethylammonium Cl	0	0	0	0	0	0	2.5	0.5	3	0	0	1	0.5	1.5	0.6	40
Thiopental-Na	3	3	3	0	2	0	3	0	0[a]	0	0	0[b]	0	3	1.4	50
Triamcinolone	3	0	3	2	2	0	−3	0	−3[a]	−1	0	−3	0	−1	−0.1	30
Tribromoethanol	3[b]	3	2	3	1	0	3	0	0[a]	0	0	0[b]	1	3	1.4	60
Trichloroethanol	0	3	2	3	2	0	3	0	0	0	0	−2	1	3	1.1	50
Tri-*o*-cresyl phosphate	0	0	0	0	−0.5	0	−0.5	0	−1	0	0	0	1	0	−0.1	10
d-Tubocurarine	3	3	3	3	2	0	3	2	3	0	0	0	0	0	1.6	60
L-Tyrosine	3	3	1.5	2	1	0	1.5	1	1.5[a]	0	0	0.5	−1	3	1.1	70
W-1372																
30 mg.	0	0	0.5	0	0	0	−1.5	0	−1.5	0	0	0	0	0	−0.2	10
40 mg.	3	1.5	3	3	1.5	1	0	1.5	0	1	0.5	2	0	2	1.4	80
	1.3	**1.3**	**1.3**	**1.4**	**0.9**	**0.8**	**0.8**	**0.6**	**0.2**	**0.1**	**0.2**	**0.2**	**−0.05**	**1.1**		
Overall protective index	**60**	**60**	**60**	**60**	**50**	**30**	**60**	**40**	**30**	**20**	**20**	**30**	**20**	**50**		
Protective spectrum index, %																

[a] Conditioning agent administered at 2-mg. dose level; [b] at 1-mg. dose level; [c] at 5-mg. dose level; [d] at 0.5-mg. dose level.

but digitoxin poisoning (which is likewise combated by virtually all catatoxic steroids) is singularly resistant to protection by nonsteroidal agents, with the exceptions of nicotine and phentolamine.

Among the nonsteroidal agents, the highest general protective indexes are exhibited by phenobarbital, phetharbital, and phenylbutazone; but at high dose levels, tolbutamide and compound W-1372 are also efficacious.

The infarctoid cardiopathy produced by fluorocortisol + Na_2HPO_4 + corn oil, which is inhibited by several steroids (particularly spironolactone and spiroxasone, among those listed in Table IIA), was consistently resistant to prophylaxis by any of the nonsteroidal agents in Table IIB. Of course, potassium salts (*e.g.*, KCl) or potassium-sparing agents (*e.g.*, amiloride and triamterene) offer excellent protection against this cardiopathy, as shown by our previous investigations; since spironolactone and spiroxasone likewise retain potassium, it is probable that here they also act primarily through this mechanism.

The relative amenability of the other toxicants to protection by nonsteroidal conditioning agents can be most readily appraised on the basis of the indexes listed in the last two horizontal lines of Table IIB. Indomethacin has the highest Overall Protective Index and, in general, the overall protective effect of these nonsteroidal agents falls far short of that of the steroids listed in Table IIA. Indeed, whatever overall protective values can be ascribed to the set of nonsteroidal agents are mainly due to the comparatively high efficacy of phenobarbital, phetharbital, and phenylbutazone, and to a lesser extent of tolbutamide and W-1372, whereas the other agents in this list are either inactive or offer protection only against very few toxicants.

Third Step: Identification of Damaging Agents Amenable to Prophylaxis

As explained in the *Introduction*, this third step of the screening procedure was designed primarily to identify the types of compounds that can be detoxified by steroids. However, for comparative purposes, we have also tested thyroxine and phenobarbital under identical conditions, as examples of nonsteroidal agents previously shown to influence resistance against many toxicants. The steroids included in this battery of tests were purposely selected to comprise proven syntoxic or catatoxic substances, as well as compounds which had never been shown to protect against any toxic agent.

The prophylactic steroids were administered as outlined previously in 1 ml. water by a stomach tube twice daily from the 1st day until termination of the experiment, unless otherwise stated in the footnotes. Thyroxine was administered at the dose of 0.2 mg. in 0.2 ml. water s.c. once daily and phenobarbital at the dose of 6 mg. in 1 ml. water *p.o.* twice daily as described in Table IIB. The treatment with the toxicants and the assessment of the lesions they produce were again expressed as outlined previously. The results are summarized in Table III.

In this series of experiments, the Overall Protective Index and Protective Spectrum Index refer to the

amenability of the individual damaging agents to the protective effect of the compounds listed in the caption of Table III. In other words, whereas in the two last vertical columns of Tables I, IIA, and IIB, these indexes were computed to express the protective action of many agents against a standard set of toxicants, in Table III (as in the two last horizontal lines of Tables IIA and IIB) they are meant to reflect the amenability of diverse toxicants to inactivation by a standard set of potential prophylactic agents.

In Table III the damaging agents are listed merely in alphabetic order, but a glance at the Overall Protective Index column reveals that the toxicants most amenable to prophylaxis by diverse agents are cocaine; cyclobarbital; cycloheximide; EPN; ethion; ethylmorphine; phosphorodithioic acid *O,O*-dimethyl ester, *S*-ester with 3-(mercaptomethyl)-1,2,3-benzotriazin-4(3*H*)-one; mephenesin; meprobamate; methyprylon; phenindione; picrotoxin; piperidine; SKF 525-A; tubocurarine; W-1372; and the infarctoid cardiopathy produced by digitoxin + Na_2HPO_4 + corn oil. To these may be added, from Tables I and II: digitoxin (given alone for the production of convulsions), navadel, parathion, hexobarbital, progesterone (in anesthetic doses), and indomethacin, which have been tested against an even greater number of prophylactic agents. The Protective Spectrum Index of these toxicants runs roughly parallel to their Overall Protective Indexes; that is, the agents whose toxicity is most significantly impeded by various prophylactics are in general also detoxified by the largest number of potentially prophylactic substances.

Several substrates in Table III are very amenable to detoxication but only by very few compounds; hence, despite their great activity in one or two respects, they have extremely low general protective indexes. For example, mercuric chloride is almost completely detoxified by spironolactone, yet its overall amenability to protection is very low, because its detoxication depends upon a steroid-borne thioacetyl group. In this series, such a substituent occurs only in this particular steroid. Similarly, the intoxications amenable to protection by glucocorticoids only give comparatively low overall protective indexes, because only two of the prophylactic substances tested possess strong glucocorticoid potency.

Reading the columns in Table III vertically, we confirmed furthermore that the best protection against the largest number of toxicants is offered by the typical catatoxic steroids: PCN, ethylestrenol, CS-1, spironolactone, norbolethone, and oxandrolone. However, in addition to the inflammation produced by croton oil (granuloma pouch technique), many systemic toxicants (*e.g.*, aminoacetonitrile, cyclobarbital, *E. coli* endotoxin, ethylene glycol, hexamethonium, methyprylon, pralidoxime, SKF 525-A, strychnine, tetraethylammonium, and *d*-tubocurarine) are equally well and, in some cases, even more efficiently combated by prednisolone and triamcinolone, the two glucocorticoids included in this series. Several others (*e.g.*, barbital, cinchophen, cocaine, ethion, meprobamate, picrotoxin, thiopental, tribromoethanol, and trichloroethanol) are well detoxified by prednisolone but not by triamcinolone, although the latter is the more potent glucocorticoid. Presumably, prednisolone possesses both catatoxic and syntoxic properties, yet here again we must remember that such *in vivo* tests can only determine whether a compound is or is not amenable to detoxication by steroids having syntoxic or catatoxic actions with respect to other substrates; further investigations will be required to identify the underlying mechanism.

The indexes of protection are not very meaningful in the interpretation of heterogeneous groups of toxicants such as are included in Table III. Yet, it is interesting that among these toxicants—selected more or less at random—PCN, ethylestrenol, CS-1, spironolactone, and prednisolone proved to be most active, both as regards the degree and the spectrum of protection offered. However, in the case of prednisolone, it must be kept in mind that the compound possesses both catatoxic and syntoxic effects; hence, it improves resistance against toxicants in which either one or both of these resistance mechanisms would be useful. On the other hand, DOC, hydroxydione, estradiol, and thyroxine are comparatively ineffective, both as regards the intensity and the spectrum of protection. In fact, in many cases, pretreatment with thyroxine results in toxication rather than detoxication. In the computation of the protective indexes, the results of toxication are not deducted from those of detoxication but merely considered as 0; hence, the aggravating effect of thyroxine does not emerge clearly from the indexes listed.

The damaging effects of the following agents were not significantly influenced by any of the potentially conditioning compounds listed in Table III; these negative results are not included in that tabulation but they should be listed for comparative purposes:

- Amphetamine
- Bile duct ligature
- Bromobenzene
- Brompheniramine maleate
- Dimercaprol (BAL)
- Dinitrophenol
- DDT
- Edrophonium chloride
- Ephedrine sulfate
- Ethylene chlorohydrin
- Fasting (mean survival)
- Homatropine hydrobromide
- Indium trichloride
- Mechlorethamine hydrochloride
- Nephrectomy (mean survival)
- Pancuronium bromide
- Pentylenetetrazol
- Pipradrol hydrochloride
- Pyrilamine maleate
- Thallium chloride
- 3,3,5-Triiodo-L-thyronine
- Warfarin

A comparison of this short list with the much larger number of toxicants in Table III that are, at least to some extent, amenable to prophylaxis or aggravation may give the erroneous impression that the actions of most compounds are subject to conditioning by steroids, thyroxine, or phenobarbital. This is not the case. We were guided in the selection of the toxicants to be tested by preliminary assays and gave preference to compounds that proved to be amenable to prophylaxis or aggravation by one or the other conditioning compound, as well as those that were closely related to such toxicants in their chemical structure or pharmacologic activity. Hence, we are not dealing with a completely random group of damaging agents. Yet it is noteworthy that such a large number of chemically and pharmacologi-

cally diverse toxic agents is amenable to this type of conditioning.

Morphine appears to be completely resistant to conditioning by any of the agents tested, yet we have included a detailed report of our pertinent findings in Table III for comparison with ethylmorphine, which, on the contrary, is readily detoxified by most of the catatoxic steroids as well as by phenobarbital. This point is of special significance since it suggests that a toxicant resistant to prophylaxis by catatoxic agents may be rendered susceptible by the addition of a side chain. The phenomenon is reminiscent of the "opsonization" of bacteria which renders them amenable to destruction by phagocytes. It is conceivable that a chemical structure that is not attacked by certain enzymes becomes amenable to enzymatic degradation by the addition of an appropriate radical.

Special Studies: Unusual Effects of Certain Protective Steroids

In the course of the systematically planned experimental work described in the preceding pages, several chance observations were made which required special tests for the clarification of the protective or sensitizing mechanisms involved. It would be beyond the scope of this review to discuss each of these special cases at length, but at least brief mention should be made of those that illustrate interesting facets of the role of hormones in resistance.

The Detoxication of Ganglioplegics—Glucocorticoids, unlike many other steroids tested, protect the rat against certain ganglioplegics [tetraethylammonium (TEA), hexamethonium, and pentolinium] but not against others (trimethaphan, mecamylamine, trimethidinium, and pempidine). Under identical conditions, various stressors such as bone fractures, fasting, spinal cord transection, and formalin offer excellent protection against TEA, whereas restraint or exposure to cold does not. Even large doses of ACTH are ineffective in this respect; hence, the anti-TEA action of stressors cannot be ascribed merely to increased corticoid secretion. Since glucocorticoids protect only against some of the ganglioplegics, this effect presumably depends upon the chemical structure and not upon the pharmacologic action of the substrate. By contrast, the protective effect of the steroids does not depend upon their chemical structure but upon their glucocorticoid activity, since all seven glucocorticoids tested for this effect proved to protect against ganglioplegics (117).

The comparable protective effect of certain stressors furnishes an additional example of the induction of "cross-resistance" by stress against certain toxicants. It is difficult to see, however, why ACTH itself does not share this effect of stressors and glucocorticoids and why only certain stressors are effective. Possibly, the glucocorticoids secreted under the influence of ACTH and of the inactive stressors are not as potent in inhibiting ganglioplegics as are the synthetic glucocorticoids with which they were compared. Another explanation that might be considered is that the effect of ACTH and of the ineffective stressors is associated with metabolic phenomena which nullify this particular protective action. Comparatively few other systemic intoxications can be easily inhibited by glucocorticoids but not by potent catatoxic steroids. Yet, this special responsiveness to glucocorticoids is also characteristic of endotoxins, lathyrogens, and anaphylactoid reactions (117) and several other toxicants listed in Table III.

Sensitization to the Toxic Effect of Octamethyl Pyrophosphoramide (OMPA)—In rats, typical catatoxic steroids (*e.g.*, ethylestrenol, CS-1, spironolactone, and norbolethone) previously shown to induce hepatic microsomal enzymes against other pesticides do not alter sensitivity to OMPA. Yet, estradiol, estrone, and stilbestrol, which fail to protect against the classic substrates of catatoxic steroids, considerably increase the toxicity of OMPA. Apparently, steroids can influence resistance to drugs in a very selective manner. As we have pointed out repeatedly in this review, the protective effect of catatoxic steroids is presumably due to their structural characteristics and is independent of other pharmacologic actions. However, sensitization to OMPA depends more upon the folliculoid action as such than upon chemical structure, since stilbestrol, a nonsteroidal folliculoid, is also highly effective in this respect (65).

Protection by Steroids that Function as Carriers of Active Groups—The fatal renal damage normally produced by acute mercurial intoxication in the rat is more effectively prevented by thioacetyl-containing steroids (*e.g.*, spironolactone, spiroxasone, and emdabol) than by inorganic sodium thioacetate. Steroids possessing sulfur in forms other than thioacetyl, as well as steroids devoid of sulfur, do not protect against acute $HgCl_2$ intoxication under the same experimental conditions (103, 104).

Apparently, the steroid molecule is a particularly suitable carrier of thioacetyl. This may be because it makes sulfur more gradually available for combination with Hg than thioacetyl given as the Na-salt or because, if bound to a steroid, the thioacetyl reaches the receptor site more readily. The structure of the steroid to which thioacetyl is bound apparently plays a comparatively minor role here. The cyclic side chain of spironolactone contains two oxygens, that of spiroxasone possesses only one, and emdabol has no cyclic side chain; yet all these thioacetylated steroids are highly potent, nontoxic antagonists of $HgCl_2$.

Several other observations suggest that steroids can function as useful carriers of pharmacologically active groups. For example, the antitumor activity of certain drugs has been claimed to be increased in this manner.

A series of steroid esters of *p*-[*N*,*N*-bis(2-chloroethyl) amino]phenylacetic acid (BCAPAA), steroidal sulfides of *p*-(*N*,*N*-bis-2-chloroethylamino)thiophenol, and a variety of steroidal ethylenimine derivatives were synthesized and tested for antitumor potency. "Activity was found only in those instances in which the steroid and potential oncolytic agent were connected by ester or heterocyclic ether linkages. The steroidal BCAPAA esters were of particular interest showing excellent inhibition of a DMBA-induced and transplantable mammary adenocarcinoma, and marked increase in survival when tested on a variety of rat leukemias .... The steroidal BCAPAA esters were judged to be less

toxic than some of the well-known nitrogen mustards in general use" (118).

The introduction of two quaternary ammonium bases in positions 2β and 16β into the molecule of 5α-androstane-3α,17β-diol diacetate led to the compound pancuronium, which is now in clinical use in the form of its bromide as a potent *neuromuscular blocker* (119, 120). Apparently, here the steroid molecule serves as a vector enhancing the neuromuscular blocking action of the quaternary ammonium bases.

As shown by Table I, steroids are also suitable carriers for nitrile groups, endowing the latter with extraordinarily intense catatoxic activity which cannot be duplicated by inorganic cyanides or nitriles of organic compounds other than steroids (*e.g.*, acetonitrile, propionitrile, and acrylonitrile). Here, the structure of the steroid and the position of the —CN group can considerably modify the quality of the effect produced by the latter.

For example, whereas the 16α-carbonitrile (PCN) is one of the most active catatoxic compounds known (105), the 2α-cyano derivative of 17β-hydroxy-4,4,17-trimethyl-3-oxoandrost-5-ene (Win-19578) produces adrenal hyperplasia with sexual anomalies (121, 122).

Finally, according to some of our hitherto unpublished observations, some of the cyanosteroids exert strong goitrogenic actions. It is not yet clear to what extent the position of the —CN group in the steroid molecule affects this action.

Interruption of Pregnancy and Lactation—In experiments on rats designed to explore the possible changes produced by catatoxic steroids upon the embryo and neonate, we found that several of them (ethylestrenol, spironolactone, and CS-1) have an abortifacient effect when administered in otherwise nontoxic doses *p.o.* during early pregnancy. If given after delivery, they interfere with lactation.

It is tempting to assume that these effects result from the induction by the catatoxic steroids of microsomal enzymes that metabolize hormones necessary for the maintenance of gestation and milk production. However, other possible mechanisms must also be considered, and a more detailed account of pertinent studies will be published.

RETROSPECT AND PROSPECT

Reevaluation of Methodology

Looking back upon the research on hormones and resistance outlined in this review, it may be constructive to reexamine the justification of the path followed and to assess the future possibilities of this field.

It was not without hesitation that we embarked upon this project some 35 years ago; we realized to start with that it would be a lifelong undertaking with virtually no background data for logical planning. On the basis of what we had learned just then about the role of the adrenal cortex in defense against stress, no other course seemed to be open to us but that of a purely empirical, large-scale screening of many steroids (more or less closely related to the corticoids) for protective effects against many toxicants.

However, the possibility of finding highly potent and comparatively nontoxic protective steroids appeared to hold considerable promise of practical applicability and—as outlined in the beginning of this review—it did not have to be based on chance alone. To some extent we could be guided by the pharmacologic and chemical characteristics of compounds previously shown to have protective potency against certain substrates. We used the same guidelines for the selection of toxicants amenable to prophylaxis by steroids. It is on the basis of this kind of empirical research that we are now beginning to see at least the vague outlines of a classification which permits us to predict, with some degree of probability, what compounds are likely to possess protective effects against what types of toxicants.

Because of the large number of experiments required to explore the many possible combinations of such interactions, we had to rely on simple *in vivo* observations in which directly visible (functional or structural) changes and mortality rates were our principal indexes of activity. Yet, in the early days we were encouraged by the knowledge that similar screening efforts did prove to be eminently successful in many other fields. The classification of bacteria on the basis of their ability to grow on certain media or to take up the Gram stain, the screening of antibiotics on plates inoculated with various bacteria, and the blind testing of 606 chemotherapeutic agents that led Ehrlich to the discovery of "Salvarsan" are but a few examples to illustrate this point.

In our own work, we could demonstrate the nonspecificity of the pituitary-adrenal response only by countless *in vivo* tests with many stressors; only the screening of numerous calciphylactic sensitizers and challengers permitted us eventually to induce localized tissue calcification in a predictable and highly specific manner.

Naturally, as soon as any new protective phenomenon was discovered, molecular biologic studies became necessary to clarify the underlying mechanisms, for example, after we noted the prevention by spironolactone of digitoxin and mercurial intoxication, or the extraordinary degree and spectrum of protective effects that can be induced in steroids by the introduction of a nitrile group. Much of this work is still to be done, but before we could even think about elucidating the manner in which a protective phenomenon works, we first had to know that the phenomenon exists. So much for self-justification.

Now the principal tasks before us are the elucidation of the biochemical, particularly enzymatic, mechanisms through which hormones condition (that is, raise or diminish resistance) to toxicants and the determination of the practical applicability of our animal experiments to problems of clinical medicine.

Possible Clinical Applications

The therapeutic implications of the syntoxic glucocorticoids have been reviewed in the first monograph on stress (2) and it would be far beyond the scope of this review to discuss the extensive literature on their manifold uses in inflammatory diseases, in allergies, as immunosuppressants, as adjuncts in cancer therapy, *etc.* The initial enthusiasm for systemic glucocorticoid therapy, especially in chronic rheumatic diseases, has been greatly dampened by their undesirable side effects,

but there is no doubt that these hormones have come to represent a very important group of therapeutic agents.

As this manuscript goes to press, we are just beginning to explore the possible clinical applications of catatoxic compounds. There is no doubt that hepatic microsomal enzyme induction can be useful in the treatment of certain spontaneous diseases of man. This had first been shown in 1966. In an infant with congenital unconjugated hyperbilirubinemia, the serum bilirubin was considerably lowered by phenobarbital (123), presumably as a result of the induction of a glucuronide-conjugating enzyme system. By now the value of barbiturate treatment in similar cases has been well established by many investigators (124–128).

Since we now know that man possesses the same mechanism for enzyme induction by catatoxic drugs as do experimental animals, he would presumably also respond to catatoxic steroids in a similar manner. If so, we may hope to obtain favorable results by such pretreatment in patients suffering from the most varied forms of endogenous or exogenous intoxications with steroids, digitalis compounds, pesticides, carcinogens, *etc.*

It is more debatable whether this type of steroid treatment would also be effective in already established morbid conditions, since the induction of defensive enzymes often takes several days. However, under certain circumstances, enzyme activation has been demonstrated within hours in mice; the speed of its development appears to depend upon many factors including genetic predisposition, the type of inducer used, and the route of its administration. To what extent the speed of induction would limit the practical applicability of catatoxic steroids as therapeutic agents remains to be seen. In any event, beneficial results may be expected in patients suffering from chronic diseases in which even the gradual activation of defensive mechanisms over several days would suffice. It is not inconceivable, furthermore, that defensive enzymes induced in the livers of large animals could be extracted and used for treatment, thus obviating the time necessary for the induction of these enzymes in the patient. Of course, here several technical problems would have to be overcome (*e.g.*, solubilization of the enzymes and prevention of possible antigenicity), but none of these is necessarily insurmountable. It is reassuring to note in this connection that the antigenicity of vaccines or antisera, also containing foreign proteins, does not prevent us from using them successfully in man; besides, concurrent treatment with immunosuppressives may help to overcome this drawback.

The time necessary for the induction of protection by catatoxic compounds is of special interest for the elucidation of the underlying mechanism. The first pertinent experiments showed that, at least in the rat, the development of optimal resistance usually takes several days; hence most of our observations were performed by applying the toxicants after 3–4 days of continuous treatment with the potentially protective substances. However, recently we found that even a single dose of a catatoxic steroid (*e.g.*, CS-1) or drug (*e.g.*, phenobarbital) given 30 min. before exposure to most of the "standard toxicants" (listed in Tables IIA and IIB) offers considerable protection. It would be hazardous to ascribe a virtually immediate induction of resistance to neosynthesis of defensive enzymes. This phenomenon may be due to altogether different mechanisms (*e.g.*, an increase in biliary or urinary excretion and the activation of preexisting enzymes). However, in connection with possible future clinical applications, it is encouraging to know that at least in many instances catatoxic compounds can induce immediate protective effects.

What diseases might be amenable to catatoxic steroid treatment? It has long been known that glucocorticoids, folliculoids, and thyroxine can protect the rabbit against cholesterol atheromatosis (13). Recently it was shown that pretreatment with phenobarbital diminishes hypercholesterolemia and atheromatosis in cholesterol-fed rabbits (129), although this barbiturate also augments the synthesis of cholesterol from ^{14}C-acetate in rats and hamsters (130). Furthermore, several catatoxic steroids are highly potent in protecting the rat against many other types of cardiovascular disease (13). Yet, only clinical trials will be able to show whether any of these agents exerts comparable effects in spontaneous cardiovascular diseases of man.

Numerous observations have established beyond doubt that microsomal drug-metabolizing enzyme activity can be induced in man as it can in animals, and essentially by the same type of hormonal and nonhormonal compounds. The pertinent literature has been the subject of several excellent reviews (131–133). It had been thought at first that these drug-metabolizing enzymes attack only substrates foreign to the body; yet, by now there is ample evidence that they participate in the synthesis or degradation of steroid hormones, cholesterol, fatty acids, thyroxine, bilirubin, and sympathomimetic amines (133). As we have shown in the preceding pages, protective steroids can also increase resistance *in vivo* to intoxication with such natural dietary ingredients as tyrosine, vitamin A, or vitamin D.

Thus, work with experimental models suggests that the protective steroids may find clinical applications not only against intoxications with drugs (*e.g.*, digitalis compounds, barbiturates, and hormones) causing iatrogenic diseases and against environmental pollutants (*e.g.*, carcinogens, pesticides, and mercury compounds), but also against spontaneous diseases caused by endogenous intoxications.

Furthermore, we shall have to think about the changes in responsiveness to drugs that are induced in patients receiving hormonal treatment. This consideration is of special importance in respect to steroids, such as spironolactone, which are prescribed for many patients with cardiovascular disease. The problem is also pertinent to the even larger number of women who use contraceptive pills. These have been shown to affect liver function (134) with the induction of typical hepatic drug-metabolizing enzymes (135) as well as of tyrosine, tryptophan, and alanine-metabolizing enzymes (136, 137).

Conversely, the induction of steroid-metabolizing enzymes by phenobarbital may also have unexpected clinical implications. As Mowat and Arias (135) pointed out, "we can only speculate as to what this may mean for the insomniac on phenobarbital who relies on the pill for contraception."

SUMMARY

This review attempts to outline the history and present status of research on the regulation of resistance by hormones. Special attention is given to the effect of natural and artificial steroids upon comparatively nonspecific resistance phenomena.

The protective agents are classified according to their mechanism of action into two main groups: (*a*) "syntoxic" compounds which improve tissue tolerance by permitting a symbiotic type of coexistence with the pathogen (*e.g.*, by suppressing inflammatory reactions) and (*b*) "catatoxic" substances which actually destroy the aggressor (*e.g.*, through the induction of hepatic microsomal enzymes).

The syntoxic effects are virtually limited to glucocorticoids. Since these have received sufficient attention in the past, this review deals primarily with recent studies on catatoxic steroids.

Several hundred steroids have been tested for their possible protective effect against numerous toxicants. The results of these studies are tabulated, and a procedure for the pharmacologic analysis of the catatoxic effect is described in detail.

The following are the highlights of the observations made possible by this type of analysis:

1. The catatoxic effect can manifest itself independently of all classic hormonal actions, although it is frequently associated with anabolic, antimineralocorticoid, or glucocorticoid properties.

2. Some of the most potent catatoxic steroids are carbonitriles; these also have the broadest "spectrum of activity" against many toxicants.

3. The 16α-position of the —CN group appears to be particularly advantageous for this activity. Its introduction into a virtually ineffective steroid, *e.g.*, 5-pregnenolone, endows the latter with sufficient catatoxic potency to protect a rat against fatal digitoxin or indomethacin intoxication at dose levels as low as 300 mcg./kg.

4. Steroids may serve as especially favorable carriers of pharmacologically active groups, for example, of thioacetyl (for the detoxication of mercury), quaternary ammonium bases (for the induction of a neuromuscular block), and oncolytic agents.

5. Certain catatoxic steroids possess abortifacient properties and interfere with lactation.

6. Certain catatoxic steroids (*e.g.*, CS-1) or drugs (*e.g.*, phenobarbital) can induce a high degree of resistance almost immediately, when given as a single dose 30 min. before a toxicant. This rapid induction of resistance may be due to mechanisms other than neosynthesis of drug-metabolizing hepatic enzymes (*e.g.*, to the activation of enzyme precursors, accelerated excretion, and temporary storage in tissue depots).

7. It is not yet proven that catatoxic steroids can be secreted in response to a need (as glucocorticoids are during stress). However, it is now well established that they represent basic "soil-factors" determining normal resistance. For example, normal amounts of testosterone are sufficient to induce resistance far above that of females or gonadectomized animals of either sex. Corticosterone, a natural life-maintaining steroid, possesses catatoxic activity against several substrates.

8. Attention is called to the fact that certain substrates, which are not subject to inactivation by steroidal or nonsteroidal catatoxic compounds, can be "opsonized"—made amenable to this type of detoxication—by the addition of a radical. Thus, morphine is resistant whereas ethylmorphine is highly sensitive to the prophylaxis by various catatoxic steroids as well as by phenobarbital.

9. A review of the literature suggests that catatoxic steroids may have important clinical applications in a variety of diseases caused by endogenous or exogenous toxicants that are amenable to biotransformation by hepatic microsomal enzymes.

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RESEARCH ARTICLES

Synthesis and Properties of Some Hypotensive *N*-Alkylaminopropionic Esters and *N,N*-Dialkylaminopropionic Esters and Their Hydroxamic Acids

R. T. COUTTS, J. W. HUBBARD, KAMAL K. MIDHA, and K. PRASAD

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Keyphrases □ 3-(*N*-Alkylamino)propionic esters and hydroxamic acids—synthesis □ 3-(*N,N*-Dialkylamino)propionic esters and hydroxamic acids—synthesis □ Hypotensive activity—3-(*N*-alkylamino)propionic esters and 3-(*N,N*-dialkylamino)propionic esters, hydroxamic acids □ IR spectrophotometry—identification □ PMR spectroscopy—identification □ Mass spectroscopy—identification

In a previous communication (1), the preparation was reported of β-aminopropionohydroxamic acids and β-aminopropionic esters of general structure I which possessed hypotensive properties in rats and cats. These hydroxamic acids (I, R_3 = NHOH) and esters (I, R_3 = OMe) had R_2 = H or Me, and R_1 was a substituted piperidine ring or related ring structure. By changing

$$R_1CH_2CHR_2COR_3$$
I

the nature of the basic group R_1 in I, the magnitude and duration of the fall in blood pressure were affected significantly. This observation prompted the authors to prepare a large number of compounds and to evaluate them as hypotensive agents. In this communication, the synthesis, some physical properties, and the hypotensive properties of selected 3-(*N*-alkylamino)propionic esters and hydroxamic acids, 3-(*N,N*-dialkylamino)propionic esters and hydroxamic acids, and a few additional representative compounds, in which the basic center is a ring structure, are reported. The aliphatic esters are listed in Table I. All were prepared by the interaction of methyl acrylate or methyl methacrylate and an appropriate amine. Their hydrochlorides were obtained by passing dry hydrogen chloride through ether solutions of each ester.

With two exceptions, monoalkylamines and dialkylamines with unbranched alkyl chains reacted readily with methyl acrylate and methyl methacrylate in the absence of basic or acidic catalysts to give good yields of β-aminoesters. The exceptions were di-*n*-propylamine and phenethylamine, which reacted only slowly with methyl methacrylate. Attempts to react diisopropylamine with methyl methacrylate in boiling methanol or *n*-butanol for lengthy periods of time were unsuccessful, and only starting materials were recovered. These results are in agreement with Hughes' (2) findings but contrast with those of Suminov (3) who reported that he obtained methyl 2-methyl-3-(diisopropylamino)propionate by the interaction of diisopropylamine and methyl methacrylate under rela-

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RESEARCH ARTICLES

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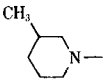
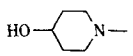
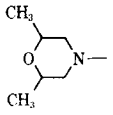
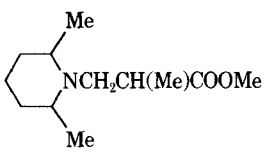
Table I—β-Aminopropionates

$R_1CH_2CHR_2CO_2R_3$
II

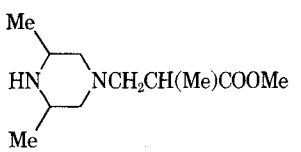
	R_1	R_2	R_3	Yield, %	B.p./mm.	Lit. b.p./mm.	Lit. Ref.	Formula	Anal., % Calcd.	Anal., % Found	Hydrochloride m.p.
a	CH_3NH—	H	CH_3	40	70–72°/20	43.3–43.8°/0.8	(8)	$C_5H_{11}NO_2$	C, 51.26 H, 9.47 N, —	C, 51.48 H, 9.58 N, —	Hygroscopic
b	CH_3CH_2NH—	H	CH_3	73	28°/0.5	27°/0.3	(9)	$C_6H_{13}NO_2$	C, 54.94 H, 9.99 N, 10.68	C, 54.72 H, 9.89 N, 10.84	70–71°
c	*n*-C_3H_7NH—	H	CH_3	70	86–88°/16	57°/3	(10)	$C_7H_{15}NO_2$	C, 57.48 H, 10.41 N, 9.65	C, 57.48 H, 9.94 N, 8.80	130–132°
d	*n*-C_3H_7NH—	CH_3	CH_3	58	—	—		$C_8H_{18}ClNO_2$	C, 49.10 H, 9.27 N, 7.16	C, 48.87 H, 9.51 N, 7.36	130–131°
e	*n*-C_4H_9NH—	H	CH_3	75	48–50°/0.5	75°/5	(11)	$C_8H_{17}NO_2$	C, 60.34 H, 10.76 N, —	C, 60.22 H, 10.53 N, —	174–176°
f	*n*-$C_6H_{13}NH$—	H	CH_3	62	80–84°/0.5	—		$C_{10}H_{21}NO_2$	C, 64.13 H, 11.30 N, 7.48	C, 64.13 H, 11.50 N, 7.11	190–192°
g[a]	$C_6H_5CH_2CH_2NH$—	CH_3	CH_3	65	124–126°/1	116–118°/0.6	(12)	$C_{13}H_{19}NO_2$	C, 70.55 H, 8.65 N, —	C, 70.77 H, 8.54 N, —	116–118°
h	$(CH_3)_2N$—	H	C_2H_5	—	—	—		—	C, — H, — N, —	C, — H, — N, —	137–138°[b]
i	$(CH_3)_2N$—	CH_3	CH_3	75	66–68°/30	70–71°/24	(13)	$C_7H_{15}NO_2$	C, 57.90 H, 10.41 N, 9.65	C, 57.81 H, 10.33 N, 9.33	123–125°
j	$(C_2H_5)_2N$—	CH_3	CH_3	68	98–99°/35	77–79°/15	(4)	$C_9H_{19}NO_2$	C, 62.39 H, 11.05 N, 8.09	C, 62.52 H, 10.61 N, 8.23	168–170°
k	$(n\text{-}C_3H_7)_2N$—	CH_3	CH_3	60	58–60°/1	—		$C_{11}H_{23}NO_2$	C, 65.63 H, 11.52 N, —	C, 65.81 H, 11.74 N, —	175–176°
l		CH_3	CH_3	80	122–124°/25	—		$C_{11}H_{21}NO_2$	C, 66.29 H, 10.62 N, —	C, 65.81 H, 10.81 N, —	Hygroscopic
m		CH_3	CH_3	90	58–60°/0.5	—		$C_{11}H_{21}NO_2$	C, 66.29 H, 10.62 N, —	C, 66.61 H, 10.71 N, —	129–130°
n		CH_3	CH_3	14	70°/0.1	—		$C_{12}H_{23}NO_2$	C, 67.56 H, 10.87 N, 6.57	C, 67.77 H, 10.68 N, 6.55	Hygroscopic
o		CH_3	CH_3	18	92°/0.03	—		$C_{13}H_{25}NO_2$	C, 68.68 H, 11.08 N, 6.16	C, 68.52 H, 10.84 N, 5.82	Hygroscopic
p		CH_3	CH_3	65	—	—		$C_{10}H_{20}ClNO_3$	C, 50.52 H, 8.48 N, 5.89	C, 49.53 H, 8.23 N, 6.55	71–73°
q		H	CH_3	89	—	—		$C_{10}H_{19}NO_3$	C, 59.68 H, 9.52 N, 6.96	C, 59.74 H, 9.59 N, 6.51	231–233°

[a] Fourfold molar excess of amine used in preparation (*Reference 14*). [b] Sample obtained commercially.

tively mild conditions. Suminov also reported a successful preparation of methyl 2-methyl-3-(2,6-dimethylpiperidino)propionate (III) in 70% yield from methyl methacrylate and 2,6-dimethylpiperidine. Attempts to repeat this reaction under the same and then more vigorous conditions failed to give any III. Similarly, when 2 moles of methyl methacrylate and 1 mole of 2,6-dimethylpiperazine were heated under reflux in methanol, the only product obtained was methyl 2-methyl-3-(3,5-dimethyl-1-piperazinyl)propionate (IV). It seems probable that steric hindrance prevented reaction at the nitrogen atom that had the two methyl groups on adjacent carbon atoms.



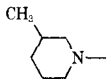
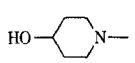
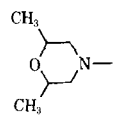
III



IV

Table II—β-Aminopropionohydroxamic Acids

$$R_1CH_2CHR_2\overset{\overset{O}{\|}}{C}NHOH\cdot HCl$$
XI

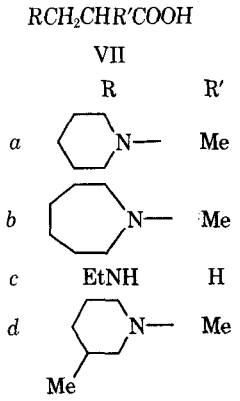
	R_1	R_2	Reaction Time, hr.	Method	Yield, %	M.p.	Formula	Anal., % Calcd.	Anal., % Found
a	CH_3NH—	H	60	A	65	114–115°	$C_4H_{11}ClN_2O_2$	C, 31.07 H, 7.17 N, 18.12	C, 31.10 H, 7.19 N, 17.91
b	C_2H_5NH—	H	40	B	64	44–45°	$C_5H_{13}ClN_2O_2\cdot H_2O$	C, 32.17 H, 8.10 N, 15.01	C, 32.22 H, 7.99 N, 15.36
c	*n*-C_3H_7NH—	H	48	A	72	78–79°	$C_6H_{15}ClN_2O_2$	C, 39.45 H, 8.27 N, 15.34	C, 39.36 H, 8.62 N, 15.34
d	*n*-C_3H_7NH—	CH_3	40	A	65	125–127°	$C_7H_{17}ClN_2O_2$	C, 42.74 H, 8.71 N, 14.24	C, 42.53 H, 8.45 N, 14.05
e	*n*-C_4H_9NH—	H	48	B	61	108–109°	$C_7H_{17}ClN_2O_2\cdot 1/2H_2O$	C, 40.87 H, 8.81 N, 13.62	C, 40.49 H, 8.68 N, 13.95
f	*n*-$C_6H_{13}NH$—	H	40	B	79	134–135°	$C_9H_{21}ClN_2O_2$	C, 48.10 H, 9.42 N, 12.47	C, 47.72 H, 9.49 N, 12.72
g	$C_6H_5CH_2CH_2NH$—	CH_3	48	B	75	126–127°	$C_{12}H_{19}ClN_2O_2$	C, 55.70 H, 7.40 N, 10.83	C, 55.77 H, 7.69 N, 10.94
h[a]	$(CH_3)_2N$—	H	10	A	71	86–88°	$C_5H_{13}ClN_2O_2$	C, 35.61 H, 7.77 N, 16.62	C, 35.59 H, 7.94 N, 16.77
i	$(CH_3)_2N$—	CH_3	10	A	77	140–142°	$C_6H_{15}ClN_2O_2$	C, 39.45 H, 8.27 N, 15.34	C, 39.29 H, 7.91 N, 15.32
j	$(C_2H_5)_2N$—	CH_3	10	A	73	118–119°	$C_8H_{19}ClN_2O_2$	C, 45.59 H, 9.08 N, 13.29	C, 45.82 H, 9.28 N, 13.09
k	$(n\text{-}C_3H_7)_2N$—	CH_3	10	A	62	152–153°	$C_{10}H_{23}ClN_2O_2$	C, 50.30 H, 9.71 N, 11.74	C, 50.60 H, 9.93 N, 11.30
l		CH_3	48	B	69	170–172°	$C_{10}H_{21}ClN_2O_2$	C, 50.73 H, 8.94 N, 11.84	C, 50.45 H, 9.31 N, 11.63
m		CH_3	18	B	86	190–193°	$C_{10}H_{21}ClN_2O_2$	C, 50.73 H, 8.94 N, 11.84	C, 50.23 H, 8.93 N, 11.81
n		CH_3	48	B	70	124–125°	$C_{11}H_{23}ClN_2O_2$	C, 52.68 H, 9.25 N, 11.17	C, 52.62 H, 9.10 N, 11.03
o		CH_3	48	B	67	167–168°	$C_{12}H_{25}ClN_2O_2$	C, 54.43 H, 9.52 N, 10.58	C, 54.34 H, 9.33 N, 10.60
p		CH_3	20	B	65	154–155°	$C_9H_{19}ClN_2O_3$	C, 45.28 H, 8.02 N, 11.74	C, 45.38 H, 8.02 N, 11.95
q		H	20	B	48	170–171°	$C_9H_{19}N_2O_3$	C, 45.28 H, 8.02 N, 11.74	C, 45.56 H, 8.24 N, 11.50

[a] Lit. (15) m.p. 90–91°.

When the duration of the reaction between some of the amines and the α,β-unsaturated esters was prolonged, amides were formed. Thus, 3-ethylaminopropionic ethylamide (V) was the major product of the reaction between ethylamine and methyl acrylate when the reactants were allowed to react at room temperature for 10 days. 2-Methyl-3-*n*-propylaminopropionic *n*-propylamide (VI) was prepared in a similar manner. Amides were also observed as being by-products of

$EtNHCH_2CH_2CONHEt$ (V) $\quad$ $nPrNHCH_2CH(CH_3)CONHnPr$ (VI)

the reaction between other primary amines (methylamine, *n*-propylamine, *n*-butylamine, and *n*-hexylamine) and methyl acrylate, especially when the reaction period was prolonged. Their presence was detected in the

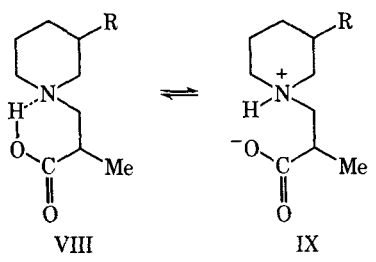


less volatile portion of the reaction mixture by means of IR spectra (strong carbonyl absorption in the 1645–1690 cm.$^{-1}$ region), but they were not isolated. In contrast, no amides were detected in the reactions between methyl methacrylate and secondary amines or cyclic amines.

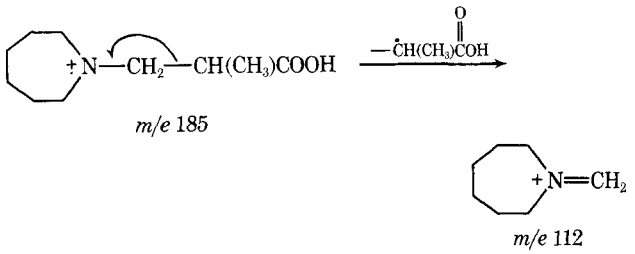
When some of the esters prepared in this and in the previous study (1) were stored for 6–8 weeks at room temperature, colorless crystalline solids slowly deposited. These products were characterized as 3-amino acids by means of elemental analysis, IR and proton magnetic resonance (PMR) spectra, and, in one instance (VII*b*), by mass spectrometry. In this way, 2-methyl-3-piperidinopropionic acid (VII*a*), 2-methyl-3-homopiperidinopropionic acid (VII*b*), 3-ethylaminopropionic acid (VII*c*), and 2-methyl-3-(3-methylpiperidino)propionic acid (VII*d*) were obtained from the corresponding esters, presumably the result of hydrolysis by trace quantities of moisture present. Bieber (4) has already commented on the ease with which methyl 2-methyl-3-piperidinopropionate hydrolyzes when shaken with cold water.

The IR spectra of the three tertiary amino acids (VII*a*, *b*, and *d*) showed broad bands between 2120 and 2400 cm.$^{-1}$ (ν $\overset{+}{\text{N}}$H), whereas broad bands in the region 2660–2740 cm.$^{-1}$ (ν $\overset{+}{\text{N}}$H$_2$) were observed in the spectrum of VII*c*. Strong bands near 1620 and 1450 cm.$^{-1}$ (ν CO_2^-) were present in all four IR spectra. The PMR spectrum of VII*a* and VII*d* had deuterium-exchangeable singlets at 843 and 887 Hz., respectively, *i.e.*, considerably further downfield than is usually observed for either intermolecularly hydrogen-bonded carboxylic acid protons (5) or $\overset{+}{\text{N}}$H protons. These two acids apparently exist in tautomeric forms VIII $\rightleftharpoons$ IX (Scheme I).

The mass spectrum of the homopiperidino compound VII*b* was simple and consistent with its structure. The



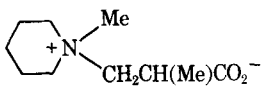
Scheme I



Scheme II

molecular ion, *m/e* 185, was present but of relatively low intensity. The only fragment ion of relative abundance greater than 30% was the base peak at *m/e* 112 which undoubtedly was formed in the manner illustrated in Scheme II (6).

In 1950, Vystrcil and Hudecek (7) reported their studies on the addition of amines to methyl methacrylate and the effect of the presence of water on the nature of the reaction products obtained. According to this study, aliphatic amines such as diethylamine, piperidine, and morpholine add to methyl methacrylate to form *N*-substituted-3-amino esters in nonaqueous media; but in an aqueous medium, a shift of the ester methyl group to the amino group was postulated which resulted in the formation of *N*-methylbetaines (*e.g.*, X) in very good yields. This particular betaine (X) was reported to melt at 106–108°, which is suspiciously close to the melting point of 2-methyl-3-piperidino-



X

propionic acid (VII*a*) (109–110°). For this reason, methyl methacrylate was reacted with piperidine in the presence of water, using the conditions described by Vystrcil and Hudecek (7). A betaine was not obtained; the product was VII*a*. It is probable that none of the products described by these workers was a betaine. None was isolated in the present study.

The hydroxamic acids (Table II) were prepared from the amino esters by the action of hydroxylamine and were converted to hydrochlorides for pharmacological evaluation. Some of the hydrochlorides were appreciably hygroscopic. All the hydroxamic acids gave a violet color with alcoholic ferric chloride, and each was characterized by elemental analysis and IR and PMR spectroscopy. In their IR spectra, all showed a carbonyl absorption band attributable to the hydroxamic acid group between 1635–1675 cm.$^{-1}$. The PMR study showed that acyclic hydroxamic acids of general structure XI (Table II), when examined in DMSO-d_6, gave three characteristic 1-proton broad signals within the 500–700 Hz. range, all of which exchange in D_2O. One of these signals was located between 521 and 540 Hz. and another between 603 and 635 Hz. These are due to the two protons of the NHOH group. The third signal was observed in all spectra between 645 and 663 Hz. and is ascribable to the $\overset{+}{\text{N}}$H proton; this signal was absent when the spectra of bases rather than hydrochlorides were examined.

Table III—Effect of Methyl β-Aminopropionate Hydrochlorides and β-Aminopropionohydroxamic Acid Hydrochlorides on the Arterial Blood Pressure of Anesthetized Cats

Compound	Dosage, mg./kg.	Percent Fall in Blood Pressure[a]	Duration,[b] min.
II *a*	25	62	5
b	25	48	10
c	25	(27)[c]	50
d	25	21	10
e	25	13	10
f	25	87	80
g	5	18	8
	10	35	30
h	25	38	5
i	10	72	90
	25	67	115
j	25	39	5
k	25	28	5
l	—	—	—
m	25	43	5
n	25	58	3
o	25	60	3
p	25	31	2
q	25	13	3
XI *a*	25	(33)[c]	10
b	25	53	5
c	25	30	15
d	25	16	5
e	25	39	15
f	25	40	120
g	5	17	60
	10	39	80
	25	50	120
h	25	56	3
i	25	58	30
j	25	50	15
k	25	41	20
l	25	33	5
m	25	39	5
n	25	72	4
o	25	44	3
p	25	44	4
q	25	45	5

[a] Average of two experiments. [b] Time for blood pressure to return to control level. [c] Rise in blood pressure.

PHARMACOLOGY

A preliminary study was made of the effect of the β-aminopropionohydroxamic acid hydrochlorides (XI) and β-aminopropionate hydrochlorides (II) on the blood pressure of anesthetized cats.

Cats of either sex, weighing 2–3 kg., were anesthetized with pentobarbital sodium (35 mg./kg.). The blood pressure (carotid artery) was recorded by means of a force displacement transducer (E & M model 3000) coupled to an E & M physiograph (model IV). All drugs were dissolved in distilled water and administered *via* a cannula inserted into a femoral vein.

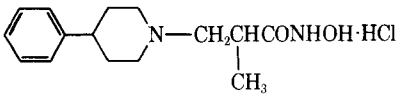
The results are summarized in Table III. The majority of the β-aminopropionohydroxamic acids and β-aminopropionates produced a fall in blood pressure. In certain cases, the hypotensive effect was prolonged (hydroxamic acids XI*f* and XI*g* and ester II*i*), but none of the compounds was as active as the 2-methyl-3-(4-phenylpiperidino)propionohydroxamic acid hydrochloride(XII) reported earlier (1, 16).

The four aminopropionic acids (VII*a*–VII*d*) were also tested and found to have no effect on blood pressure in anesthetized cats.

EXPERIMENTAL[1]

Melting and boiling points are uncorrected. IR spectra were measured with a Beckman IR-10 spectrophotometer. Mass spectra were measured at an ionizing potential of 70 ev. with an A.E.I. MS-9 spectrometer, using the direct insertion technique, and PMR spectra were recorded on a Varian A-60 spectrometer using trimethylsilane (TMS) as standard.

[1] Microanalyses were performed by Mr. W. Dylke, and Mrs. S. K. Li recorded the PMR spectra.



XII

Esters—The methods used to prepare and characterize the esters described in Table I were essentially the ones reported previously (1). The reaction time varied from 2 hr. to 15 days, depending on the amine used. Reaction was continued until the C=C stretching band near 1630 cm.$^{-1}$ (due to the acrylate starting material) disappeared from the IR spectrum of the crude product.

Ester hydrochlorides were prepared and characterized as described previously (1).

Methyl 2-Methyl-3-(3,5-dimethyl-1-piperizinyl)propionate (IV)—In an attempt to synthesize 1,4-bis(2-methoxycarbonylpropyl)-2,6-dimethylpiperazine, 2,6-dimethylpiperazine (14.25 g.) was dissolved in anhydrous methanol (50 ml.), and to this solution was added methyl methacrylate (25 g.). The mixture was boiled under reflux for 2 weeks. The methanol was removed *in vacuo*, and the resulting oil was distilled under reduced pressure. The first fraction collected was methyl methacrylate. The fraction b.p. 88–90°/4 mm. (19.1 g.) was the title compound. IR (film): 3320 (ν NH), 1740 (ν C=O) cm.$^{-1}$. PMR (CCl_4), methyl group characteristics: 60 Hz. [t, 9 (overlap of two doublets), ring methyl groups and side-chain methyl group]; 211 Hz. (s, 3, $COOCH_3$).

Anal.—Calcd. for $C_{11}H_{22}N_2O_2$: C, 61.6; H, 10.3. Found: C, 61.1; H, 9.9.

Hydroxamic Acids—They were prepared by two methods.

Method A—To a constantly stirred solution of hydroxylamine hydrochloride (0.18 mole in 50 ml. of anhydrous methanol) at 0° was added a cold methanolic solution of potassium hydroxide (0.12 mole in 40 ml. of anhydrous methanol); after 3 min., the precipitated potassium chloride was filtered off. To the filtrate was added a solution of the appropriate ester (0.03 mole) in anhydrous methanol (20 ml.). The temperature of the stirred solution was maintained at 0 to −5° for 1 hr. and then allowed to come to room temperature. Stirring was continued for 18–48 hr., and then the methanol was removed *in vacuo*. To the resulting viscous oil, sufficient acetone was added to give a turbidity. On refrigeration, the hydroxamic acid hydrochloride separated as a colorless solid which was recrystallized from absolute ethanol.

Method B—This method has been described previously (1).

*3-Ethylaminopropionic Acid (VII*c)—When methyl 3-ethylaminopropionate (II*b*) was stored at room temperature for 4 weeks, a white crystalline solid slowly separated. It was collected, recrystallized from ethanol–acetone, and characterized as 3-ethylaminopropionic acid, m.p. 188° [lit. (17) m.p. 174–176°]. IR (KBr): 2740–2660 and 2500–2420 (broad bands, ν $\overset{+}{N}H_2$); 1630, 1450 (ν CO_2^-) cm.$^{-1}$. PMR (D_2O): 78 Hz. (t, 3, $J=7$, CH_3CH_2); 154 Hz. (t, 2, $J=6.6$, CH_2CO); 177–201 Hz. (m, 4, CH_2NHCH_2).

Anal.—Calcd. for $C_5H_{11}NO_2$: C, 51.3; H, 9.5. Found: C, 51.0; H, 9.3.

*2-Methyl-3-(3-methylpiperidino)propionic Acid (VII*d)—This acid, m.p. 122–124°, was obtained from the corresponding ester (II*m*) in a manner similar to that just described. IR (KBr): 2260–2120 (broad, ν $\overset{+}{N}H$); 1610, 1450 (ν CO_2^-) cm.$^{-1}$. PMR (D_2O): 57 Hz. (d, 3, $J=6$, ring CH_3); 69 Hz. (d, 3, $J=7$, CH_3CHCO); 93–230 Hz. (m, 12, remaining protons). PMR ($CDCl_3$): 887 Hz. (s, 1, COOH, exchanged in D_2O).

Anal.—Calcd. for $C_{10}H_{19}NO_2$: C, 64.8; H, 10.3. Found: C, 64.6; H, 10.2.

*2-Methyl-3-piperidinopropionic Acid (VII*a)—This acid, m.p. 109–110° [lit. (4) m.p. 108°], was obtained from the corresponding ester (1) in the manner described for the previous two acids. IR (KBr): 2300–2120 (broad, ν $\overset{+}{N}H$); 1610, 1450 (ν CO_2^-) cm.$^{-1}$. PMR ($CDCl_3$): 67 Hz. (d of d, 3, CH_3CHCO); 98 Hz. (m, 6, three ring CH_2 groups remote from N atom), 130–190 Hz. (m, 7, $\begin{matrix}CH_2\\CH_2\end{matrix}\!\!>\!NCH_2CH$), 843 Hz. (s, 1, COOH, exchanged in D_2O).

Anal.—Calcd. for $C_9H_{17}NO_2$: C, 63.2; H, 9.9. Found: C, 63.4; H, 9.9.

2-Methyl-3-homopiperidinopropionic Acid (*VII*b)—This acid, m.p. 100°, was prepared in the same way as the acids just mentioned. IR (KBr): 2400–2120 (broad, ν $\overset{+}{N}H$); 1620, 1450 (ν CO_2^-) cm.$^{-1}$. PMR (D_2O): 69 Hz. (d, 3, $J=6.5$, CH_3CHCO); 105 Hz. (s, 8, four ring CH_2 groups remote from N atom); 138–181 Hz. (m, 7, $\begin{matrix}CH_2\\CH_2\end{matrix}\!\!>\!NCH_2CH$). Mass spectrum: (percent relative abundance) *m/e* 185 (6.9%), 112 (100%).

Anal.—Calcd. for $C_{10}H_{19}NO_2 \cdot 0.5\ H_2O$: C, 61.8; H, 10.3. Found: C, 61.7; H, 10.6.

3-Ethylaminopropionic Ethylamide (*V*)—In an attempt to prepare methyl 3-ethylaminopropionate by reacting ethylamine and methyl methacrylate at room temperature for 10 days, using the general method for the preparation of esters, the reaction mixture was fractionally distilled and yielded the title compound (46% yield), b.p. 113–114°/2 mm. IR (film): 3300 (ν NH); 1645 (ν C=O) cm.$^{-1}$. PMR (CCl_4): 63 Hz. (t, 3, $J=7$, $CH_3CH_2NHCH_2$) overlapping with 66 Hz. (t, 3, $J=7$, $CONHCH_2CH_3$); 88 Hz. (s, 1, CH_3CH_2NH); 498 Hz. (s, 1, CONH), both singlets exchanged in D_2O.

Anal.—Calcd. for $C_6H_{14}N_2O$: C, 55.3; H, 10.8. Found: C, 54.9; H, 10.8.

*2-Methyl-3-*n*-propylaminopropionic* n-*Propylamide* (*VI*)—The title compound was obtained in 40% yield in a manner similar to that described for the preparation of V.

This compound was characterized as the hydrochloride, m.p. 130–131.5°. IR (mineral oil) 3270 (amide ν NH); 2900–2410 (ν $\overset{+}{N}H_2$); 1660 (C=O).

Anal.—Calcd. for $C_{10}H_{23}ClN_2O$: C, 53.92; H, 10.41. Found: C, 53.83; H, 10.41.

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Keyphrases □ Tetracyclines—structure–activity relationships □ Structure–activity relationships, tetracyclines—*E. coli* inhibition □ Rate constants, *E. coli* cell division—tetracyclines □ Protein, nucleic acid syntheses, *E. coli*—tetracycline effect □ Viable, total cell counts—*E. coli* division rates □ Concentration, tetracyclines—*E. coli* inhibition

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Table I—First-Order Generation-Rate Constants, sec.$^{-1}$ × 10^4, for *E. coli* W Cultures Inhibited by Various Tetracycline Antibiotics, $M \times 10^6$

M	k_v	k_p	$k_{n.a.}$
	Tetracycline		
0.000	5.57	5.85	5.07
0.599	3.80	4.46	3.85
0.898	3.23	2.97	2.89
1.198	2.27	1.96	2.10
1.497	1.49	1.76	1.45
1.796	1.10	1.25	1.27
	7-Chlortetracycline		
0.000	5.41	5.94	5.80
0.246	4.82	4.51	4.97
0.492	3.48	3.33	3.53
0.788	2.33	2.17	2.14
0.985	2.16	1.84	2.16
1.231	0.52	0.93	0.51
	5-Hydroxytetracycline		
0.000	5.75[a]	5.79	5.97
0.537	4.71	3.49	3.75
0.878	4.04	3.47	—
0.878	3.75[b]	—	—
1.073	2.25	2.24	2.14
1.376	2.23	2.24	2.40
1.376	2.20[b]	—	—
	7-Chloro-dm-tetracycline[c]		
0.000	5.42	5.02	5.90
0.221	4.99	4.41	5.26
0.441	3.37	3.05	3.68
0.706	1.46	1.13	1.50
0.882	0.55	0.68	1.06
1.103	—	0.55	0.59
	dm-do-Tetracycline[d]		
0.000	5.89	5.05	5.25
0.978	4.58	4.55	4.77
1.955	3.65	3.42	3.50
2.972	2.76	2.57	2.47
3.910	1.46	1.57	1.65
4.888	0.84	1.07	0.97

M	k_t[e]	k_p	$k_{n.a.}$
	7-Bromo-dm-do-tetracycline		
0.000	5.89	5.41	5.89
2.468	4.29	3.78	3.52
4.938	2.70	2.59	2.29
7.406	1.26	1.50	1.50
9.876	—	0.85	0.55
	7-Chloro-isotetracycline		
0.00	5.71	5.26	5.40
163.49	5.81	4.74	5.39
322.30	5.71	4.86	5.53
	12*a*-Deoxytetracycline		
0.00	5.71	5.26	5.40
79.14	3.90	3.69	3.59
156.03	1.59	1.57	0.95
	5*a*(11*a*)-Dehydrochlortetracycline		
0.00	5.71	5.26	5.40
150.68	5.23	4.26	4.55
297.06	4.64	4.03	4.66
	2-Cyano-2-decarboxamidotetracycline		
0.00	5.71	5.26	5.40
33.16	5.94	4.92	5.44
128.88	5.88	4.63	4.72

[a] An average value, determined on several different days. [b] Values of k_t which were used in the calculation of the viable inhibitory rate constant (Table III). [c] dm- is 6-demethyl-. [d] dm-do- is 6-demethyl-6-deoxy-. [e] Values of k_t were obtained with 0.2% HCHO in the diluting solution (see *Experimental*).

that may be different than activity at the receptor site. Different activities were reported for several analogs under various test conditions. The present work was undertaken to establish activities suitable for the study of structure–activity relationships, insofar as possible, for a series including both clinically active and "inactive" tetracycline antibiotics.

Activities were obtained by three types of kinetic measurements on exponentially growing *Escherichia coli* inhibited by the antibiotics. Rate constants for cell division, $k_{c.d.}$, in a high peptone broth were measured by the viable plate count method of Brown and Garrett (2) and by the total cell count method of Garrett and Miller (3). A filter technique using detergent-extracted cellulose ester membranes was developed to isolate trichloroacetic acid (TCA)–ethanol precipitates of the *E. coli* as a function of time of growth. The hot TCA-soluble extract of these precipitates was used to measure nucleic acids by the orcinol reaction. The hot TCA-insoluble material remaining on the filter was trypsin-digested, and the protein content was estimated by the Folin–Lowry assay. These data were used to calculate rate constants for protein synthesis, k_p, and nucleic acid synthesis, $k_{n.a.}$.

Inhibition of protein synthesis and nucleic acid synthesis, rather than inhibition of cell division alone, was studied so that the activities might be more directly related to the mode of action. Gale and Folkes (4) showed that protein synthesis in washed suspensions of *Staphylococcus aureus* is inhibited at concentrations of tetracyclines similar to those causing inhibition of growth.

Nucleic acid synthesis was reported to be inhibited only at higher antibiotic concentrations. Hash *et al.* (5) reported that incorporation of several radioactive amino acids into cytoplasmic protein was greatly inhibited by chlortetracycline and tetracycline at 1 mcg./ml. Chemical analysis of inhibited cells revealed that the cells contained increased cell wall content and hexosamine, as well as ribonucleic acid (RNA), but had decreased protein and deoxyribonucleic acid (DNA) content as compared with control cells. Cerny and Habermann (6) made kinetic measurements of the inhibition of protein and nucleic acid syntheses in cultures of *E. coli* C at tetracycline concentrations of 20–50 mcg./ml. They found an immediate cessation of protein synthesis followed by a later but complete inhibition of nucleic acid synthesis. Holmes and Wild (7) recently discussed the nature of an RNA synthesized in unaerated *E. coli* K12 cultures whose protein synthesis was inhibited by 15 mcg./ml. of chlortetracycline. These authors felt that ribosomal precursor RNA accumulates under such circumstances in a manner similar to that seen in chloramphenicol-treated cells.

Eagle and Saz (8) believed that the inhibition of protein synthesis observed by Gale and Folkes (4) was caused by a primary inhibition of a nitro reductase system. Colaizzi *et al.* (9) summarized the evidence for this theory which suggested that the site of inhibition was at the level of metalloflavoenzymes. Kremery and Kellen (10) proposed that this property may be more directly related to the development of resistance, although Franklin and Godfrey (11) disagreed with their proposal. Snell and Cheng (12) indicated that several modes of action may exist; Jones and Morrisson

Table II—First-Order Generation-Rate Constants, sec.$^{-1}$ × 10^4, for *E. coli* W Cultures Inhibited by Tetracyclines, $M \times 10^6$, and the Fraction of Viable to Total Cells Present at the Start, ϕ_1, and at the End, ϕ_5, of the Experiments

M	k_v	k_t[a]	k_p	$k_{n.a.}$	ϕ_1[b]	ϕ_5[b]
7-Amino-dm-do-tetracycline[c]						
0.000	5.84	5.87	5.46	5.93	0.84	0.81
0.493	4.93	4.80	4.32	4.72	0.80	0.96
0.985	3.71	3.83	3.36	3.66	0.75	0.63
1.478	2.98	3.05	2.74	2.77	0.68	0.62
1.971	2.41	2.41	1.96	2.02	0.72	0.71
9-Amino-dm-do-tetracycline						
0.000	5.84	5.87	5.46	5.93	0.84	0.81
0.474	5.27	5.27	4.61	4.86	0.74	0.74
0.948	4.06	4.59	3.91	4.08	0.87	0.40
1.421	3.79	3.73	3.38	3.44	0.72	0.80
1.895	—	3.06	3.02	3.17	—	—
5*a*(6)-Anhydrotetracycline						
0.000	5.82	5.62	5.40	5.90	0.91	1.21
4.477	5.00	5.46	4.70	4.90	0.69	0.35
8.953	4.38	4.67	3.79	3.87	0.79	0.52
13.430	4.12	3.88	3.25	3.45	0.64	0.90
17.907	—	3.32	2.56	2.77	—	—
7-Nitro-dm-do-tetracycline						
0.000	5.82	5.62	5.40	5.90	0.91	1.21
0.111	5.68	5.50	4.86	5.44	0.67	0.87
0.222	4.20	4.75	3.88	4.32	0.74	0.33
0.333	3.17	3.82	3.11	3.52	0.72	0.28
0.444	2.45	2.82	2.19	2.59	1.00	0.61
9-Nitro-dm-do-tetracycline						
0.000	6.10	5.86	5.81	6.14	0.74	1.05
2.337	4.77	5.37	5.07	5.15	0.97	0.41
4.674	3.93	4.11	3.48	3.54	0.68	0.53
7.011	2.93	2.96	2.82	2.55	0.82	0.77
9.348	1.95	2.02	1.92	1.85	0.69	0.62
4-Dedimethylaminotetracycline						
0.000	6.10	5.86	5.81	6.14	0.74	1.05
3.030	5.27	5.39	4.63	5.01	0.79	0.67
6.060	4.22	4.50	3.96	4.30	0.80	0.53
9.091	3.43	3.80	3.58	3.51	0.86	0.50
12.121	3.17	3.12	2.97	3.04	0.65	0.70
9-Dimethylamino-dm-do-tetracycline						
0.000	6.25	6.09	5.45	5.40	0.66	0.83
3.467	5.60	5.71	5.01	5.00	0.87	0.74
6.935	4.32	4.69	3.92	3.99	0.70	0.41
10.402	3.34	3.47	3.09	3.51	0.60	0.50
13.870	2.83	2.94	2.40	2.56	0.74	0.63
Tetracycline methiodide						
0.00	6.25	6.09	5.45	5.40	0.66	0.83
63.48	4.87	5.14	4.03	4.26	0.83	0.56
126.95	3.02	3.39	2.78	2.91	0.71	0.42

[a] Values of k_t were obtained with 0.2% HCHO in the diluting solution see *Experimental*). [b] ϕ was calculated from Eq. 1 using the values $k_{e.a.}$ and N_0, obtained by regression analysis of the original data. [c] dm-do- is 6-demethyl-6-deoxy-.

(13, 14) and Benbough and Morrisson (15) provided evidence for this view but believed that the primary action is a direct inhibition of protein synthesis.

Franklin (16), in experiments with cell-free extracts designed to prove that an inhibition of protein synthesis was a direct effect of the tetracyclines, showed that protein synthesis is directly inhibited at the level of incorporation of aminoacyl-sRNA. Laskin and Chan (17) reached similar conclusions from studies of synthetic messenger-directed protein-synthesizing extracts. Hierowski (18) and Suarez and Nathans (19) showed that inhibition is caused by preventing the binding of aminoacyl-sRNA to ribosome-mRNA complexes; their experiments were confirmed by other workers (20–23). Lucas-Lenard and Haenni (24) showed that

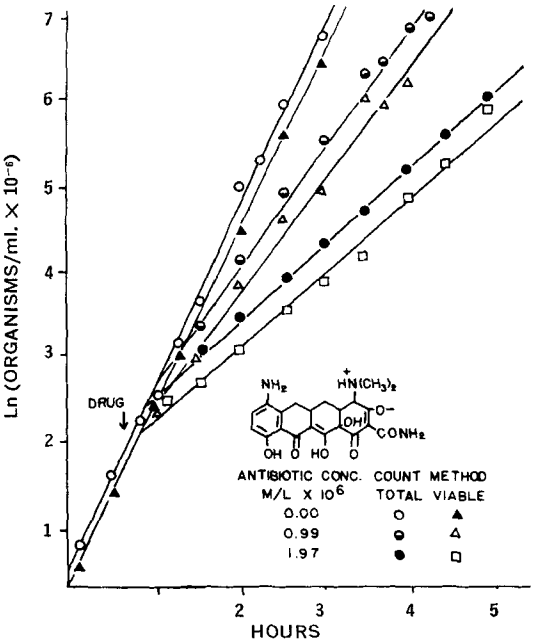

Figure 1—*Generation-rate curves for cell division in the presence of various concentrations of 7-amino-6-demethyl-6-deoxytetracycline. Samples obtained from each culture were counted by a viable and a total count method. The slopes,* k_v *and* k_t, *are given in Table II.*

the GTP-dependent binding of aminoacyl-sRNA at low magnesium-ion concentrations is dependent upon the presence of the T-factor of Nishizuka and Lipmann (25). The effect of chlortetracycline is to prevent binding of aminoacyl-sRNA at a level beyond that involving the formation of an initial aminoacyl-sRNA–GTP–T complex (24). Connamacher and Mandel (20) and Day (26–28) studied the binding of tetracycline to ribosomes and believed that the binding is related to the prevention of aminoacyl-sRNA binding. Maxwell (29–31) investigated this binding as well as other factors and was led to similar conclusions. Cundliffe (32) described tetracycline effects on polyribosomes in growing protoplasts of *Bacillus megaterium* which are consistent with this hypothesized mode of action. Rifino *et al.* (33) studied the effects of several tetracycline analogs on cell-free protein-synthesizing systems in addition to those studied by Laskin and Chan (17) and Hierowski (18), and they reported preliminary correlations of antibacterial activity in synthetic messenger-directed systems but not in those directed by natural messengers.

EXPERIMENTAL

Materials—*E. coli* W, obtained as a frozen paste,[1] was cultured over several growth cycles in broth and on agar plates. An isolate from a single colony was used to inoculate a nutrient agar slant, which was maintained by monthly transfers. The culture broth was a high peptone media containing, in a volume of 1 l., 40 g. casein hydrolysate (GBI No. 20), 15 g. yeast autolysate (GBI No. 120), 3 g. K_2HPO_4, and 0.7 g. KH_2PO_4. The pH was maintained constant at 6.7 throughout the experiments. For experiments at a pH value of 6.2, the broth contained 0.5 g. K_2HPO_4 and 2.5 g. KH_2PO_4, while 21.85 g. of K_2HPO_4 and 1.7 g. of KH_2PO_4 were used in addition to the peptones for those experiments at pH 7.4. The dextrose-salts broth (pH 6.7) used contained, in a volume of 1 l., 10 g. dextrose, 3 g. $(NH_4)_2SO_4$, 0.1 g. $MgSO_4$, 6 g. K_2HPO_4, and 8 g. KH_2PO_4. Broth was filtered through cellulose ester membranes[2] (0.22-μ diameter

[1] General Biochemicals, Chagrin Falls, Ohio.
[2] Millipore Corp., Bedford, Mass.

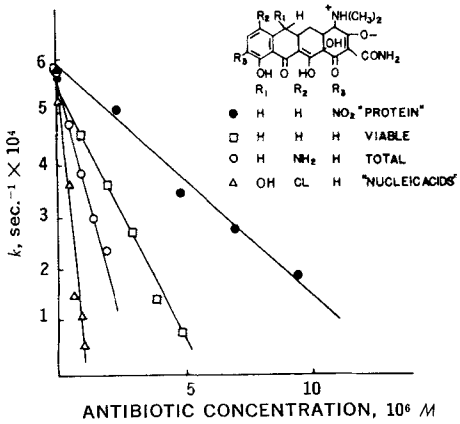

Figure 2—*The linear dependence of generation-rate constants*—k_v, k_t, k_p, *and* $k_{n.a.}$—*upon antibiotic concentration for several different tetracyclines. The lines shown are the average inhibitory rate constants,* $k^1_{avg.}$, *for each compound and are given in Table III.*

pores) and autoclaved prior to use. Samples of various tetracycline analogs were supplied by the manufacturers.[3] The nomenclature used was described previously (9).

Growth—A broth culture was allowed to grow for 12 hr. at 37.5°. A dilution of this culture, whose growth was measured turbidimetrically, was allowed to grow into the logarithmic phase. When a concentration of 1×10^8 organisms/ml. was attained, 2-ml. samples were used to inoculate nine replicate 100-ml. volumes of broth in loosely capped 500-ml. flasks. The flasks were shaken in a constant-temperature water bath (37.5°). Freshly prepared antibiotic solutions were added in 1-ml. volumes to eight of the nine replicate cultures after 2700 sec. of growth. Experiments designed to study time of onset of inhibition were performed with cultures of either 200 ml. /1-l. flask or 350 ml./2-l. flask.

Viable Count Method—One-milliliter samples of the culture were diluted with 0.9% saline solution so that a concentration of 100–200 organisms/ml. would result. One milliliter of this suspension was pipeted into each of five Petri dishes containing solidified peptone–casein agar USP[4] and then covered with melted agar. The plates were incubated for 36–48 hr. at 37.5°, and the colonies were counted (2).

Total Count Method—One-milliliter samples of the cultures were placed in bottles containing appropriate volumes of a saline–

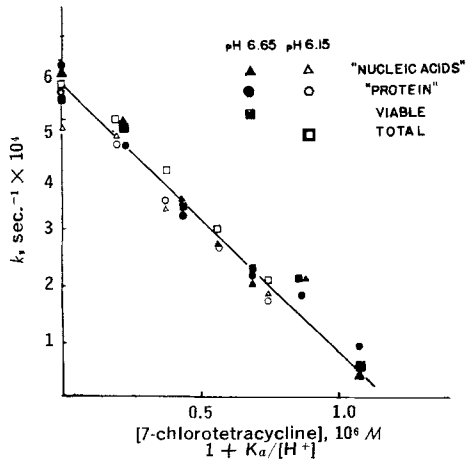

Figure 3—*The linear dependence of generation-rate constants obtained at two broth pH values upon the concentration of the zwitterionic form of 7-chlorotetracycline. The slope is −470 l./mole-sec.*

[3] The authors are especially grateful to Dr. James H. Boothe, Lederle Laboratories, Pearl River, N. Y., and Dr. Charles R. Stephens, Charles Pfizer Laboratories, Groton, Conn., for their assistance in providing the tetracycline analogs employed in this study.

[4] Fisher Scientific, Fairlawn, N. J.

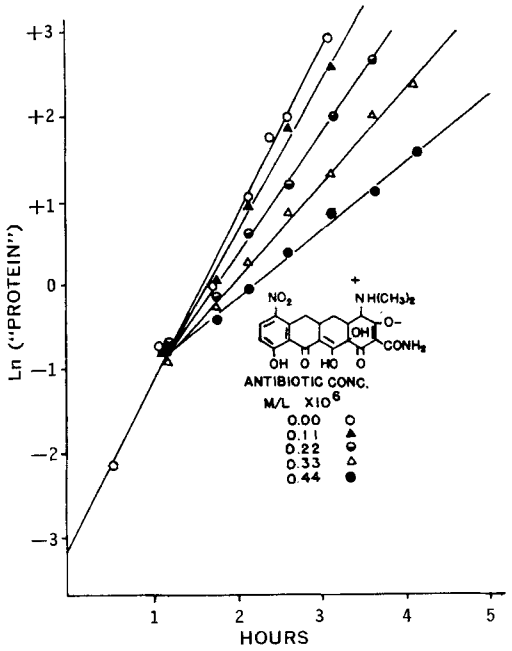

Figure 4—*Generation-rate curves for protein synthesis in the presence of various concentrations of 7-nitro-6-demethyl-6-deoxytetracycline. The slopes,* k_p, *are given in Table II.*

formaldehyde solution so that a concentration of 10,000–30,000 organisms/50 μl. was obtained. This solution had previously been filtered to remove particulate contamination. The HCHO concentration was either 0.2 or 1%; the higher concentration gave values of total cells which remained constant for 24 hr., while the lower concentration allowed some growth (1–5%) in the first 10 min. after samples were obtained. Diluted samples were counted with a Coulter counter model B[6] equipped with a 30-μ orifice (3). Instrument settings that gave satisfactory results were: 1/aperture current, 0.707; 1/amplification, 1/4; matching switch, 32H; gain, 100; lower

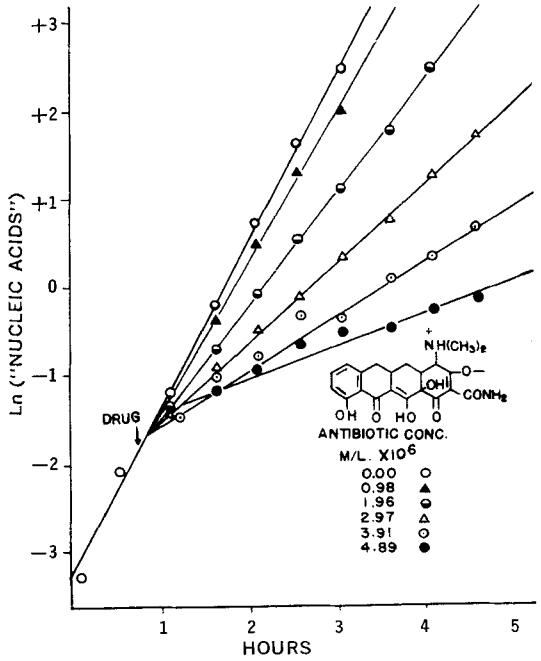

Figure 5—*Generation-rate curves for nucleic acid synthesis in the presence of various concentrations of 6-demethyl-6-deoxytetracycline. The slopes,* $k_{n.a.}$, *are given in Table I.*

[5] Coulter Electronics, Inc., Hialeah, Fla.

Table III—Inhibitory Rate Constants, l./mole-sec., for Inhibition of *E. coli* W by Various Tetracyclines[a]

Tetracycline Analog	$-k_v^I$	$-k_t^I$	$-k_p^I$	$-k_{n.a.}^I$	$-k_{avg.}^I$
7-Nitro-dm-do-tetracycline[b]	833.51	656.76	735.68	769.19	748.78[c]
7-Chloro-dm-tetracycline	591.86	—[d]	460.25	536.48	517.21
7-Chlorotetracycline	387.77	—	396.14	420.20	401.37
Tetracycline	267.84	—	279.97	267.95	271.50
5-Hydroxytetracycline	275.19	—	254.79	251.83	257.15
7-Amino-dm-do-tetracycline	179.01	175.90	174.08	197.95	181.73[c]
9-Amino-dm-do-tetracycline	155.53	151.23	128.82	146.13	145.03[e]
dm-do-Tetracycline	103.62	—	86.70	92.93	94.42[e]
7-Bromo-dm-do-tetracycline	—	62.68	46.09	51.42	51.79
9-Nitro-dm-do-tetracycline	43.40	43.15	42.91	47.86	44.33
4-Dedimethylaminotetracycline	25.39	23.29	22.24	25.45	24.09
9-Dimethylamino-dm-do-tetracycline	26.22	24.62	23.11	20.73	23.67[c]
5*a*(6)-Anhydrotetracycline	12.75	13.81	15.91	17.23	15.48[c]
12*a*-Deoxytetracycline	—	2.63	3.85	2.37	2.62
Tetracycline methiodide	2.54	2.12	1.96	2.10	2.18
5*a*(11*a*)-Dehydrochlortetracycline	—	0.36	0.25	0.42	0.34
7-Chloro-isotetracycline			No activity observed		
2-Cyano-2-decarboxamido-tetracycline			No activity observed		

[a] Tested by analysis of covariance to determine if significant differences existed between the inhibitory rate constant determined using all values of generation-rate constants collectively, $k_{avg.}^I$, and individual inhibitory rate contants determined separately. [b] dm-do- is 6-demethyl-6-deoxy-. [c] Significant at $p > 0.975$. See, for example, R. Steel and J. Torrie, "Principles and Procedures of Statistics," McGraw-Hill, New York, N. Y., 1960, p. 174. [d] Insufficient data available for testing by analysis of covariance. [e] Indicates significant differences at $p > 0.95$.

threshold, 10; and upper threshold, off. The mean of four total counts per sample was obtained and corrected for background noise (200–600 counts).

Nucleic Acid and Protein Determinations—Appropriate volumes of culture (0.5–25 ml.) were filtered under vacuum through 25-mm. diameter cellulose ester membranes[2] (0.22-μ diameter pores), which had previously been treated to extract detergent. Detergent extraction was carried out by placing filters in cold distilled water—4 l./100 filters—and boiling the suspension for 15 min. This process was repeated three times with fresh water (34). Filters treated in this manner gave minimal and constant blank reactions with the orcinol reagent and were able to retain *E. coli* quantitatively. The retained *E. coli* were washed with 0.9% saline solution under vacuum, after which the bacteria were precipitated by layering, without vacuum, 2 ml. of 5% TCA over the filters for 5 min. TCA-soluble material was removed by application of vacuum, and 2 ml. of 95% ethanol was layered over the filters for 5 min. After removal of the ethanolic extract, the filters with retained nucleic acids and proteins were placed in screw-cap test tubes. Two milliliters of 5% TCA was added, and the tubes were placed in a boiling water bath for 20 min.

Nucleic Acids—One-milliliter aliquots of the hot TCA-soluble material were removed from the cooled tubes. Three milliliters of the orcinol reagent (35) was added; after heating in a boiling water bath for 20 min., the absorption at 650 nm. of the cooled solution was measured. This absorption was corrected for that produced when an equal volume of sterile broth instead of culture was applied to the filters. It was necessary to prepare separate blank reactions for each lot of filters and broth. The quantity reported as "nucleic acids" is the corrected absorbance due to a 12.5-ml. culture sample in an orcinol reagent volume of 4 ml.

Proteins—The TCA solution was removed completely by vacuum aspiration from the tubes containing the filters. The filters with retained protein precipitate were covered with 1.5 ml. of a trypsin solution [10 mcg./ml. in 0.05 *M* $(NH_4)_2CO_3$ and 0.005 *M* NH_4OH]. The usual 0.1 *N* alkali protein digestant hydrolyzed the filters and gave rise to high blank values. After the precipitate was mixed vigorously, it was allowed to digest for 2 hr. (up to 48 hr. was not harmful). The resulting solution in the presence of the filters was used as a sample for protein determination by the Folin–Lowry method. The reagents, prepared according to the method of Layne (36), were added directly to the tubes, and the absorbance of the solutions was measured at 750 nm. The resulting absorbance was corrected for that occurring in the blank reactions, and the absorbance due to a 25-ml. sample of culture contained in a final reaction volume of 6.5 ml. is reported as "protein."

The protein and nucleic acid content of *E. coli* cultures collected on cellulose membranes and treated as described in this report was comparable to that observed when bacteria were collected by centrifugation (5). The method reported here appears to be more reproducible and more convenient when handling large numbers of samples.

RESULTS AND DISCUSSION

Effect of Tetracyclines on Rate Constants for Cell Division—It was previously shown (3) that the effect on the culture population of tetracycline at low concentrations is a reduction in the generation-rate constant; that is:

$$N = N_0 e^{k_{c.d.}t} \qquad k_0 < k_{c.d.} > 0 \qquad \text{(Eq. 1)}$$

where N is the concentration of cells, $k_{c.d.}$ is the generation-rate constant for cell division in the presence of antibiotic, k_0 is the rate constant in the absence of antibiotic, and t is the time. The present results show that Eq. 1 satisfactorily describes the equilibrium inhibition of *E. coli* W cultures produced by low concentrations of 16 tetracycline derivatives. F tests for the fit of experimental data to Eq. 1 were found to be highly significant in cases where data obtained for times greater than 30 min. but less than 4 hr. after antibiotic addition were used. Rate constants obtained in this way are given in Tables I and II. Two experimental methods were used to obtain $k_{c.d.}$. The constant k_v is from viable plate counts, and the constant k_t is from total cell counts obtained with the Coulter counter. Rate constants obtained by the two methods were identical within experimental error (Table II). However, in *E. coli* W cultures—in contrast to previous work with *E. coli* B/r (3)—the concentrations of cells at any time, t, as determined by the two methods, were quite different (Fig. 1). It is, therefore, not possible in the present work to conclude with certainty that the antibiotics act solely as growth inhibitors. However, the fraction of viable cells to total cells, φ, remained relatively constant in most cultures before and after addition of antibiotics (Table II). In the cases where it did vary, it did not appear to be consistently related to antibiotic concentration. At higher antibiotic concentrations, this fraction, ϕ, had values of 0.1 or 0.01. The authors believe that there is no large antibiotic-induced death of cells, at these concentrations, but feel that a constant fraction of colonies formed on plates is the result of more than one viable cell. Inclusion of a surfactant in the diluting solution did not, however, greatly affect this fraction. The use of a higher HCHO concentration in the diluting fluid for total counts did make this fraction more constant.

Rate constants for cell division were found to obey the simple equation:

$$k_{c.d.} = k_0 - k_{c.d.}^I \{Tc\} \qquad \text{iff } k_{c.d.} > 0 \qquad \text{(Eq. 2)}$$

where $k_{c.d.}$ may be either k_v or k_t and $\{Tc\}$ is the antibiotic concentration. Figures 2 and 3 are examples of the fit of experimental data to this equation. The experimentally observed rate constants do not

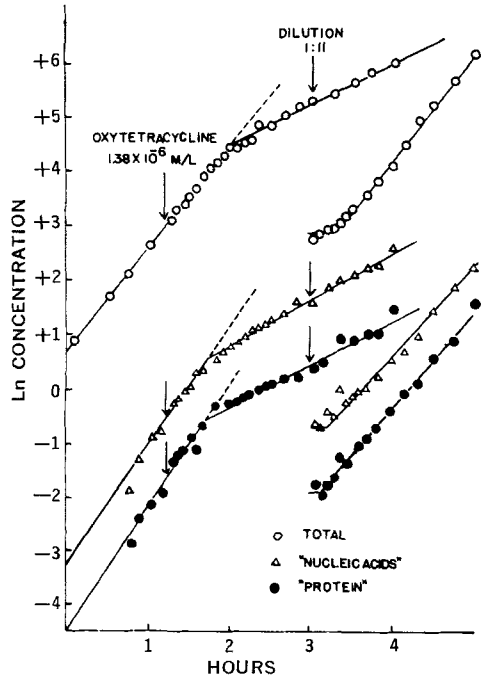

Figure 6—*Generation-rate curves for cell division, by the total count method, nucleic acid synthesis, and protein synthesis in the presence of 5-hydroxytetracycline and a 1:11 dilution of the inhibited culture. The generation-rate curve for protein synthesis has been transposed by subtracting 0.5 from the actual value of* Ln_e *"protein." The generation-rate constants, sec.*$^{-1} \times 10^4$*, for the undiluted culture are given in Table I, except for culture turbidity (not shown) which had values of 5.63 and 2.19 in the absence and presence of antibiotic, respectively. The values in the diluted culture were:* $k_t = 5.43$, $k_p = 5.09$, $k_{n.a.} = 4.61$, k_v *(not shown)* $= 5.33$ *and for turbidity 4.76. The time of antibiotic addition was 4470 sec., and the time of dilution was 10,850 sec. The times of onset of inhibition and the times of onset of recovery calculated as the intersection of the regression lines, respectively, in seconds were: for protein synthesis, 6200 and 11,800; for turbidity (not shown), 6400 and 11,500; for nucleic acid synthesis, 6400 and 12,400; and for cell division—total, 7100 and 12,500, and viable (not shown), 6500 and 12,200.*

fit the more complete expression:

$$\frac{1}{k_0 - k_{c.d.}} = k^I\,(1/\{Tc\} + k) \qquad \text{(Eq. 3)}$$

where k^I and k are constants. Equation 3 was derived by Garrett *et al.* (37).

Effect of Tetracyclines on Rate Constants for Protein and Nucleic Acid Syntheses—As noted, many workers (4–7) reported inhibition of protein synthesis at low tetracycline concentrations. Many of these workers also reported different effects on nucleic acid synthesis, either a stimulation (4, 5) or a lag time before onset of inhibition (6, 7). The present work shows that an equation similar to Eq. 1, which described the concentration of cells, can be written for both protein and nucleic acid concentrations (Figs. 4 and 5). Values of $k_{n.a.}$ and k_p derived from this equation are given in Tables I and II. In addition, these generation-rate constants are related to antibiotic concentration in a manner similar to the relationship of cell division to antibiotic:

$$k_p = k_0 - k_p{}^I\,\{Tc\} \qquad \text{(Eq. 4)}$$

$$k_{n.a.} = k_0 - k_{n.a.}{}^I\,\{Tc\} \qquad \text{iff } k_{c.d.} > 0 \qquad \text{(Eq. 5)}$$

Values of the inhibitory rate constants, $k_v{}^I$, $k_t{}^I$, $k_p{}^I$, and $k_{n.a.}{}^I$, obtained for 18 antibiotics are listed in Table III. The hypothesis was tested that these values are experimentally indistinguishable, and no statistically significant differences were found between the individual values and an average for seven of these antibiotics. Statistically significant differences were found for the six other antibiotics tested (Table III). This simple test did not consider the error involved in the determination of the individual generation-rate constants. The authors believe that within experimental accuracy, the four parameters are the same for all antibiotics. That is, a one-to-one relationship is found among these parameters not only in control cultures but also in antibiotic-inhibited cultures. The inhibited cultures are growing in balanced growth, and the rate of cell division is proportional to the total rate of protein synthesis rather than to a net rate as was previously postulated (37). The net rate theory assumed that a minimum rate of protein synthesis was necessary to maintain viability. Additional protein synthesis was needed for cell division and, it was believed, the tetracycline selectively inhibited the protein synthesis needed for cell division.

Time of Onset of Inhibiton—The fact that inhibited cultures exhibit parallel rates of cell division and protein and nucleic acid syntheses is not in accord with the interpretation of previous experiments (4, 5). These experiments were single-point measurements and noted decreased protein/cell and increased nucleic acid content/cell. However, Cerny and Habermann (6), in a kinetic experiment, showed parallel rates of protein and nucleic acid syntheses with different times of onset of action. Their work was done at antibiotic concentrations 15 times higher than those presently employed, which caused complete inhibition and undoubtedly cell death. Experiments of the type shown in Figs. 1, 4, and 5 gave indications that at these low concentrations the effect of antibiotics was also not immediate.

Figure 6 shows an experiment designed to document this observation. It can easily be seen that the onset of inhibition for all parameters is considerably beyond the time of antibiotic addition. Viable plate counts (not shown) paralleled those obtained by the Coulter counter. This experiment was repeated at a different oxytetracycline concentration with similar results. In addition, Fig. 6 shows that the time of onset of recovery from inhibition upon dilution of the

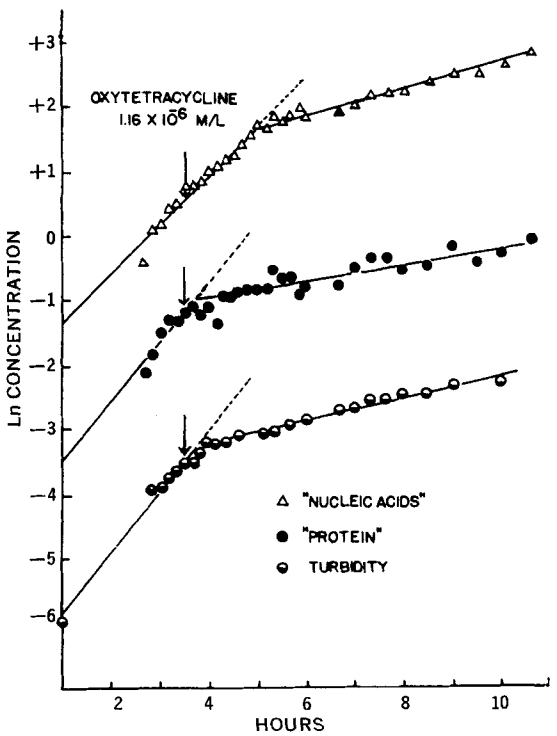

Figure 7—*Generation-rate curves in a dextrose–salts broth for nucleic acid synthesis, protein synthesis, and culture turbidity in the presence of 5-hydroxytetracycline. The generation-rate curve for nucleic acid synthesis has been transposed by adding 2.0 to the actual value of* Ln_e *"nucleic acids." Values of generation-rate constants, sec.*$^{-1} \times 10^4$*, in the absence and presence of antibiotic, respectively, were:* $k_{n.a.} = 2.13$ *and 0.56,* $k_p = 2.62$ *and 0.37,* k_t *(not shown)* $= 2.44$ *and 0.12 and for turbidity 2.54 and 0.37. The time of antibiotic addition was 8900 sec. The times of onset of inhibition, in seconds, calculated as the intersection of the regression lines were: for protein synthesis, 9900; for turbidity, 10,300; for cell division, 10,750; and for nucleic acid synthesis, 14,200.*

Table IV—First-Order Generation-Rate Constants, sec.$^{-1}$ × 10^4, and Inhibitory Rate Constants, l./mole-sec., for *E. coli* W in Various Broth Cultures Inhibited by Several Tetracyclines

10^6 *M*	k_t	k_p	$k_{n.a.}$	$k^I_{avg.}$
7-Chlorotetracycline, peptone broth, pH 6.2				
0.000	5.56	5.50	4.88	
0.192	4.95	4.55	4.66	
0.384	4.06	3.50	3.38	
0.575	3.01	2.69	2.71	
0.767	2.10	1.78	1.86	
k^I	−461.11	−484.15	−417.52	−454.28[a]
5a(6)-Anhydrotetracycline, peptone broth, pH 6.2				
0.00	5.56	5.50	4.88	
4.67	4.71	4.68	3.90	
9.34	3.94	3.23	3.45	
14.01	3.35	2.57	2.92	
18.68	1.73	1.99	2.35	
k^I	−19.27	−19.54	−12.95	−17.25[a]
4-Dimethylaminotetracycline, peptone broth, pH 7.4				
0.00	4.57	5.22	4.76	
3.34	3.87	5.01	3.96	
6.65	3.33	3.73	3.48	
9.96	2.71	3.03	3.00	
13.24	2.09	2.13	2.54	
k^I	−18.48	−24.70	−16.32	−19.83[a]
7-Chlorotetracycline, pH 6.7 dextrose–salts broth				
0.000	2.39	2.30	2.18	
0.163	2.07	1.92	2.01	
0.327	0.97	0.84	0.79	
0.490	0.39	0.51	0.50	
0.655	0.22	0.38	0.37	
k^I	−368.34	−315.81	−312.59	−332.25

[a] Significant differences between inhibitory rate constants determined by analysis of covariance as explained in Table III.

cultures into antibiotic free media is not immediate either but also exhibits a lag time.

Inhibition and recovery of protein synthesis seemed to occur slightly before the other parameters were affected. To test this observation, an experiment was performed in a dextrose–salts broth where the rate of cell division in control cultures was half that in the peptone broth. This single experiment (Fig. 7) indicates that protein synthesis measured by the Folin–Lowry technique and cell mass measured by turbidity are most likely inhibited sooner than nucleic acid synthesis. Cell division (not shown) was inhibited at an intermediate time. The small number of data points obtained in this experiment before the addition of antibiotic, necessitated by the culture conditions, makes it difficult to calculate the times of onset of inhibition with a high degree of confidence. However, it seems most likely that the rates of increase for all parameters in the absence of antibiotic are the same in this broth, just as they were in the peptone broth (38). Therefore, the conclusion of different times of onset of inhibition calculated from this experiment (Fig. 7) seems valid.

The existence of different growth rate-dependent lag times, as observed in this experiment, would explain the discrepancy between parallel equilibrium inhibition rates and nonparallel amounts of protein and nucleic acid per cell. This order of inhibition and recovery is consistent with the hypothesized primary inhibition of protein synthesis followed by a secondary, indirect inhibition of nucleic acid synthesis and cell division due to a lack of protein. In addition to the growth rate-dependent difference in time of onset of inhibition of the various culture parameters, a lag time is observed before any parameter is inhibited (Fig. 7). Two general explanations are possible for this lag time. First, a finite time may be required to establish equilibrium concentrations within the cells. Second, antibiotic may permeate the cell and arrive at the receptor site but require finite time to exert a measurable effect. Since this lag time is not growth-rate dependent and does vary with broth conditions, which do not change the growth rate, the first explanation is preferred.

Effect of Broth pH Changes on Inhibition by Tetracyclines—Jones and Morrisson (13) and Benbough and Morrisson (15) reported that when the pH of culture media of known free metal-ion concentration is varied, the inhibition produced is proportional only to the "molecular," presumably zwitterionic, species of several tetracyclines. Because of the slow growth rate characteristics of *E. coli* W in dextrose–salts broth, it was not possible to verify these results for other tetracyclines. However, experiments in peptone broth where the free metal-ion concentration is not precisely known are consistent with this result.[6] Figure 3 shows a plot of Eq. 2 for experiments at two pH values, where the concentration of antibiotic is corrected so that it reflects the concentration of zwitterionic species present, assuming the fraction of total tetracycline chelated to metal ions of the medium is the same at both pH values. Activities obtained at a broth pH of 7.4, where a much lower free metal-ion concentration would be expected, do not fit the single line plotted in Fig. 3. Results obtained with 5a(6)-anhydrotetracycline (pH 6.2 and 6.7) and 4-dedimethylaminotetracycline (pH 6.7 and 7.4) (Table IV) do not fit such a simple concept either. Connamacher *et al.* (39) recently reported data on uptake of tetracycline in *Bacillus cereus* cultures at pH 6.7 and 7.3 and at two magnesium-ion concentrations which are in qualitative agreement with the hypothesis of Jones and Morrisson (13, 14).

In addition to causing a change in the degree of equilibrium inhibition produced by a given total antibiotic concentration, pH changes are also capable of changing the observed lag time for onset of inhibition. Figure 8 shows an experiment identical to that presented previously (Fig. 6), except that the pH of the broth was 7.4 instead of 6.7. The lag for onset of inhibition of protein synthesis (measured as cell mass) and cell division is seen to be shortened by two-thirds. The equilibrium amount of inhibition achieved was the same in both experiments. If the hypothesis of Jones and Morrisson (13, 14) is correct, then the concentration of zwitterionic species

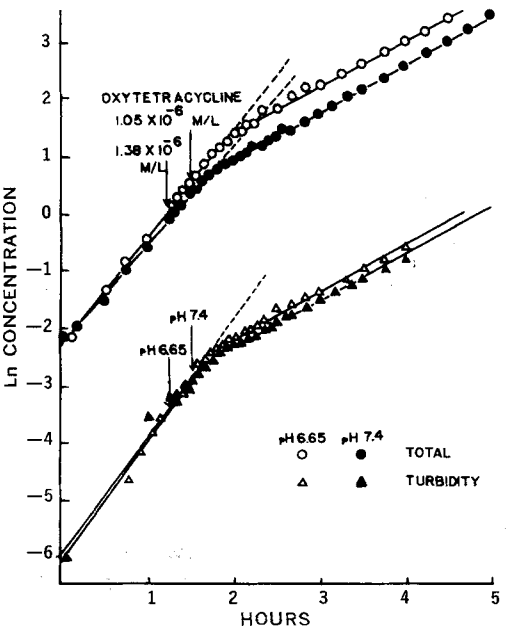

Figure 8—*Generation-rate curves, done on separate days at two peptone broth pH values, for cell division by the total count method and culture turbidity in the presence of 5-hydroxytetracycline. The generation-rate curves for cell division have been transposed by subtracting 3.0 from the actual value of* Ln_e *organisms/ml. The generation-rate constants, sec.*$^{-1}$ × 10^4, *in the absence and presence of antibiotic, respectively, for cell division were 5.16 and 2.20 at pH 6.7 and 4.75 and 2.30 at pH 7.4, and for turbidity were 5.63 and 2.19 at pH 6.7 and 5.56 and 2.21 at pH 7.4. The times of onset of inhibition, in seconds, calculated as the intersection of the regression lines were: for cell division, 7100 and 6200; and for turbidity, 6400 and 5900 at pH 6.7 and 7.4, respectively.*

[6] The divalent metal-ion concentration, expressed as calcium, in the broths has been estimated with a divalent metal-ion electrode to be approximately 10^{-4} *M* (Orion Research Inc., Cambridge, MA 02139). The calcium-ion concentration in these broths has been determined by Dr. Bartholomew van't-Riet, Department of Chemistry, Medical College of Virginia (titration with ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid) to be 1.0 to 1.1 × 10^{-4} *M*.

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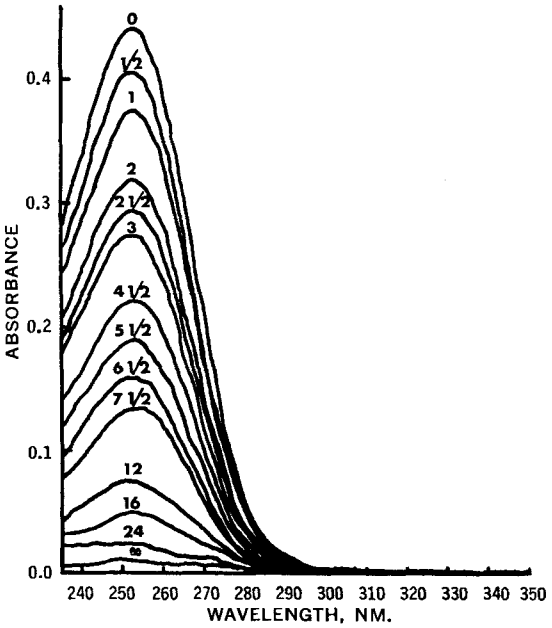

Figure 1—*Curve for absorbance diminution of itobarbital (2.2 × 10⁻⁴ M) as a function of time, hr., for 0.6 N NaOH at 60°. The number of hours after initiation of the reaction is indicated on the curves. Each sample was diluted to four times its initial volume, and these readings are shown in the graph.*

neutral and alkaline solvolysis of the compound.

Papers dealing with phenobarbital breakdown (1, 2) and other barbiturates are available (3), as well as an excellent review of barbiturate kinetics (4).

EXPERIMENTAL

Kinetic Procedures—A stock solution of sodium itobarbital[1] containing 271 mg. (0.0011 mole)/100 ml. was prepared using distilled water. Aliquots (4 ml.) were taken from the stock solution and placed in volumetric flasks (200 ml.) containing the appropriate buffer or NaOH solution (196 ml.). These flasks were previously equilibrated at the selected temperatures (60, 70, and 80 ± 0.1°), and their final concentration with regard to barbiturate was 2.2 × 10^{-4} *M*. These solutions were sampled periodically (10 ml.), and their UV spectra were read between 210 and 250 nm. on a Cary model 14 recording spectrophotometer.

Buffer solutions, unless otherwise specified, were run at an ionic strength of 0.1. Alkali solutions were prepared by dilution of standardized NaOH solution with freshly boiled distilled water and were studied at ionic strength of 0.1 wherever possible (NaCl added to bring up ionic strength).

The pH values of the buffer solutions were determined on a Metrohm pH meter standardized with borate and phthalate buffers (5) at the pertinent temperatures. The pH's of the NaOH solutions were calculated from activity coefficient data available in the literature (6). The pKa_1 values were determined at the temperature of the kinetic runs by titration of 0.005 mole 5-allyl,5-isobutylbarbituric acid with 5 ml. 0.1 *N* KOH (5).

RESULTS AND DISCUSSION

The decomposition of itobarbital over the pH range from neutrality to 13 was found to follow first-order kinetics for at least three half-lives under the experimental conditions employed. The degradation was monitored by absorbance loss of the UV chromophore as a function of time. The $\lambda_{max.}$ was located between 237 and 255 nm., depending on hydrogen-ion concentration, and a small residual absorbance remained at $t \sim \infty$. Peak height varied with pH. A linear absorbance–concentration (Lambert–Beer) relationship was exhibited at the various acidities.

The absorbance at $\lambda_{max.}$ for the pH values and temperatures studied disappears by apparent first-order processes with no perceptible change in location of the absorption band. A typical example of absorbance diminution with time may be seen in Fig. 1 and is illustrative of the general picture at the pH values and temperatures considered.

The observed first-order rate constants were calculated from the slopes of plots of the logarithm absorbance *versus* time using the expression:

$$\log(A - A_\infty) = \log(A_0 - A_\infty) - kt/2.303 \qquad \text{(Eq. 1)}$$

where A_0 is the initial absorbance; A_∞ is the residual absorbance; A is the absorbance at any time, t; and k is the observed or apparent first-order rate constant. Figure 2 is a characteristic plot of the data utilizing Eq. 1 at 60°; the rate constants for the various conditions are listed in Table I.

Rate Constant–pH Relationship—The rate–pH profile (Fig. 3) for 5-allyl,5-isobutylbarbituric acid is similar in shape to those of the closely related 5-halouridines and 5-halouracils (7, 8). Data used to construct the log *k*–pH profile are given in Table I.

Barbituric acids being diprotic, H_2A, ionize in a two-step process, producing HA^- and A^{-2} as well as two protons. This makes plausible the decomposition of the three species over the pH range 6.7–13 (pKa_1 about 7.8, 70°; pKa_2 about 11.8, 70°). However, water or hydrogen-ion attack on the neutral molecule may be generally considered negligible (7).

Degradation at pH values in the region of the pKa_2 (Figs. 3 and 4) may be accounted for by attack of water on the dianion, using the equation:

$$k''_{A^{-2}} = k''_{H_2O} f_{A^{-2}} = k''_{H_2O} \frac{Ka_2}{[H^+] + Ka_2} \qquad \text{(Eq. 2)}$$

where k''_{H_2O} is the specific catalytic constant for attack of water on the dianion; and the fraction of dianion present, $f_{A^{-2}}$, may be written in terms of hydrogen-ion concentration and the Ka_2. Equation 3 relates to Eq. 2 and expresses the equivalent hydroxyl-ion attack on the monoanion:

$$k''_{A^{-2}} = k_{OH^-}[OH^-] f_{HA^-} = k_{H_2O} f_{A^{-2}};$$
$$k'_{OH^-} = k''_{H_2O} f_{A^{-2}}/f_{HA^-}[OH^-] \qquad \text{(Eq. 3)}$$

This is a possibility because of observed ionic strength effects (Table II) in the alkaline region. However, the high ionic strengths which necessarily must be employed (Table II) give inconclusive results concerning the ion–ion interaction (9).

The observed rate constant may be expected to be reasonably constant in the pH 9.2–9.5 range (pKa_1 about 7.8), which is the area of predominant HA^- concentration with little H_2A and almost no A^{-2} (pKa_2 about 11.8). The contribution to the apparent rate constant, as seen in Fig. 3, may be described by the relationship:

$$k_{HA^-} = k'_{H_2O} f_{HA^-} = k'_{H_2O} \frac{[HA^-]}{[HA^-] + [H_2A] + [A^{-2}]} =$$
$$\frac{k'_{H_2O}}{[H^+]/Ka_1 + 1 + Ka_2/[H^+]} \qquad \text{(Eq. 4)}$$

where k'_{H_2O} is the specific constant for the attack of water on the monoanion. Equation 4 reduces to

$$k_{HA^-} = \frac{k'_{H_2O}[H^+]}{[H^+] + Ka_2} \qquad \text{(Eq. 5)}$$

when $Ka_1 \gg [H^+]$ and essentially no undissociated barbiturate is present, and it may be expressed as

$$k_{HA^-} = \frac{k'_{H_2O} Ka_1}{[H^+] + Ka_1} \qquad \text{(Eq. 6)}$$

where $[H^+] \gg Ka_2$. These two equations are demonstrative of water attack on the monoanion, and Eq. 6 is kinetically equivalent to (8)

$$k_{HA^-} = k_{OH^-}[OH^-] f_{H_2A} = \frac{k_{OH^-} k_w}{[H^+] + Ka_1} \qquad \text{(Eq. 7)}$$

Equation 7 describes the attack of hydroxyl ion on the undissociated molecule.

[1] Sandoptal Sod., Sandoz Pharmaceuticals (Ganes Chemical Works, New York, N. Y).

Table I—Observed First-Order Rate Constants[a] (k, hr.$^{-1}$) for Breakdown of Itobarbital (2×10^{-4} M) in Aqueous Solution[b]

Buffers[c]		60°			70°			80°		
$[NaH_2PO_4]$	$[Na_2HPO_4]$	pH[d,e]	Exptl.[f]	Calcd.[g]	pH[d,e]	Exptl.[f]	Calcd.[g]	pH[d,e]	Exptl.[f]	Calcd.[g]
0.025	0.025	6.80	0.00055	—	6.75	0.0017	—	6.75	0.0056	—
0.006	0.031	7.42	0.0011	—	7.40	0.0036	—	7.43	0.010	—
0.002	0.033	7.90	0.0016	—	7.90	0.0048	—	8.01	0.013	—
$[H_3BO_3]$	[NaOH]									
0.05	0.004	7.92	0.0016	—	7.88	0.0048	—	8.02	0.014	—
0.05	0.012	8.25	0.0016	—	8.15	0.0050	—	8.24	0.014	—
0.05	0.021	8.75	0.0017	0.0018	8.73	0.0055	0.0059	8.65	0.017	0.018
0.05	0.037	9.25	0.0021	0.0020	9.20	0.0069	0.0071	9.21	0.021	0.021
0.05	0.044	9.60	0.0023	0.0021	9.48	0.0078	0.0080	9.45	0.022	0.023
—	0.001[h]	9.94	0.0027	0.0037	9.73	0.0077	0.0092	9.52	0.023	0.025
—	0.003[h]	10.47	0.0058	0.0077	10.27	0.0128	0.0150	10.08	0.029	00.41
—	0.005	10.68	0.0081	0.0102	10.46	0.0179	0.0233	10.28	0.039	0.054
—	0.007	10.83	0.0092	0.0149	10.62	0.0211	0.0299	10.41	0.047	0.066
—	0.010	11.02	0.0131	0.0206	10.80	0.0271	0.0407	10.50	0.053	0.076
—	0.030	11.47	0.031	0.046	11.38	0.084[i]	0.095	10.99	0.121	0.171
—	0.050	11.61	0.046	0.060	11.51	0.111[i]	0.132	11.20	0.189	0.237
—	0.070	11.76	0.061	0.074	11.62	0.149[i]	0.157	11.33	0.244	0.288
—	0.100	11.90	0.078	0.089	11.70	0.149	0.171	11.49	0.315	0.342
—	0.200	12.18	0.114	0.116	11.97	0.230	0.231	11.75	0.498	0.468
—	0.400	12.45	0.137[i]	0.139	12.26	0.309	0.287	12.05	0.635	0.576
—	0.600	12.62	0.145[i]	0.149	12.44	0.335	0.317	12.23	0.657	0.634
—	0.800	12.74	0.157[i]	0.156	12.53	0.348	0.325	12.35	0.690	0.665
—	1.000	12.83	0.159	0.158	12.64	0.351	0.351	12.50	0.696	0.695

[a] Supplementary results in the following order: temperature; NaOH normality (k, hr.$^{-1}$) 40°; 0.75 (0.0229), 0.50 (0.0231), 0.25 (0.0223), and 0.10 (0.0145); 30°; 0.75 (0.00760), 0.50 (0.00759), 0.25 (0.00709), 0.10 (0.00524), and 0.05 (0.00384). [b] Ionic strength constant at 0.1 (NaCl). [c] $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ hydrated forms used in preparation of buffer. [d] pH values for phosphate and borate buffers obtained from Metrohm pH meter calibrated with phthalate and borate buffers at indicated temperatures. [e] pH values of NaOH solutions calculated from: $pK_w - p\text{OH} = \text{pH}$ where $p\text{OH} = -\log a$ [NaOH]. The activity coefficient and pK_w values were obtained from the literature (6). [f] Rate constants reproducible within 10%. [g] Points below pH of third borate buffer not calculated. Values calculated from equations in *Results and Discussion* section and rounded off by authors. [h] First-order plots not strictly linear past two half-lives due to OH^- consumption. [i] Values for 0.04, 0.06, and 0.08 N NaOH, respectively. [j] Other runs gave values of 0.157, 0.151, and 0.123 for 0.8, 0.6, and 0.4 N NaOH, respectively, at 60°.

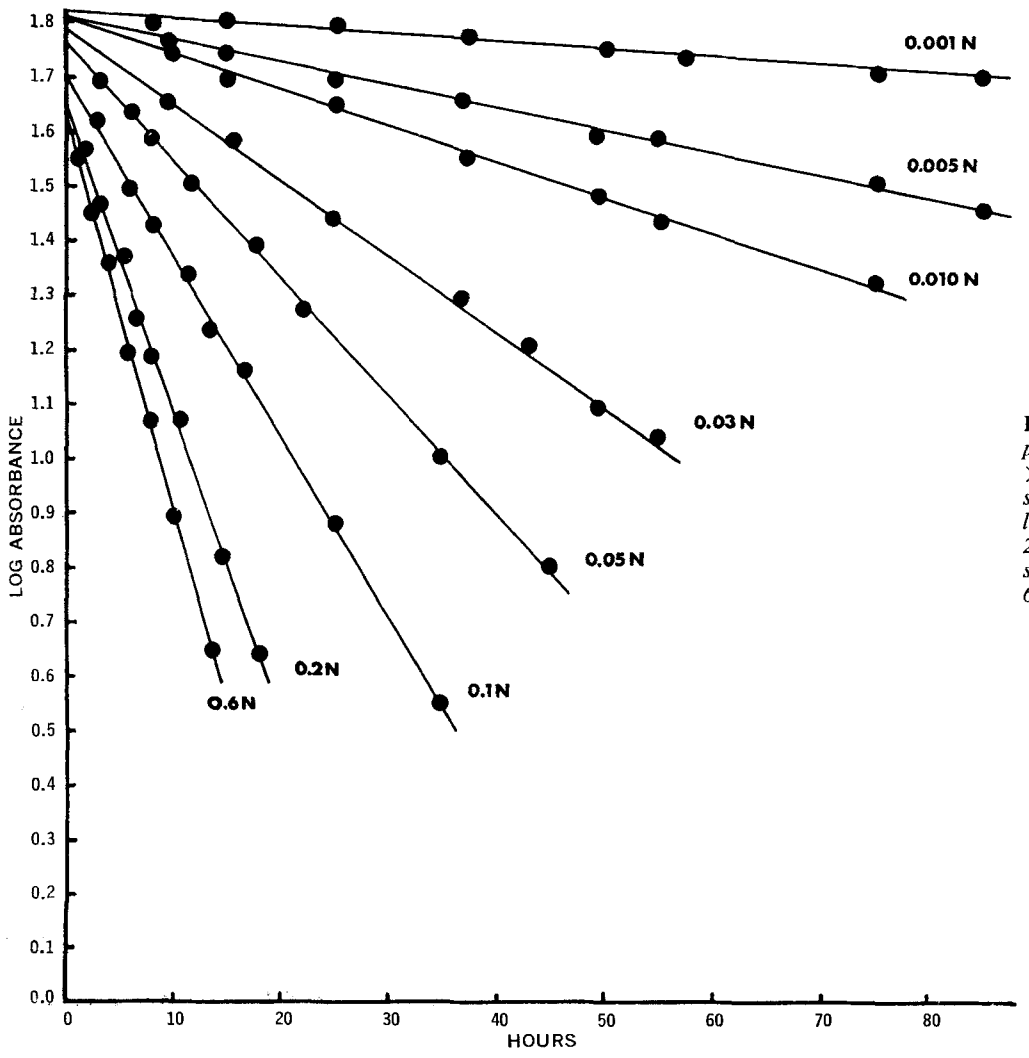

Figure 2—*Apparent first-order plots for the decomposition of 2.2 × 10^{-4} M itobarbital in NaOH solutions. Reaction followed by loss of absorbance in the range of 240–255 nm., depending on the sample normality; temperature 60°*

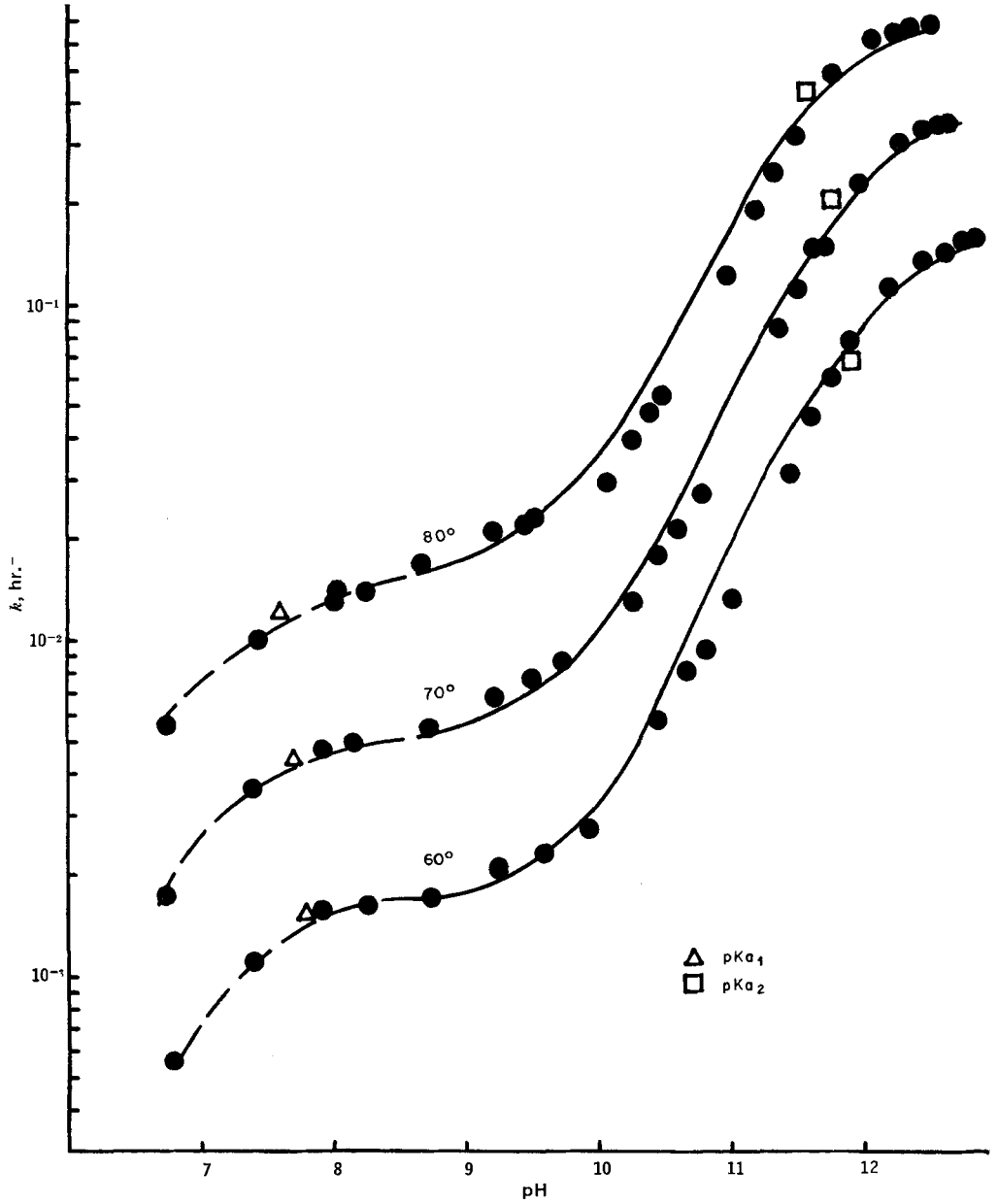

Figure 3—*The log* k–*pH profile for the solvolysis of 5-allyl,5-isobutylbarbituric acid at the indicated temperatures. Reaction monitored by loss of absorbance as a function of time.*

Figure 4 illustrates the construction of the theoretical rate–pH curve for itobarbital at 70°. The overall contribution of Eq. 2 to the log k–pH profile may be seen in Fig. 4, Curve A, and is derived in the following manner. The k''_{H_2O} is evaluated using Eq. 2 and the velocity constant data at the highest pH attainable at a given temperature—12.82 for 70° (6). It is evident in Eq. 2 that as $[H^+]$ approaches zero, $k''_{A^{-2}} \sim k''_{H_2O}$. The pKa_2 is obtained from Eq. 2 and experimental results where $[H^+] = Ka_2$; $k''_{A^{-2}} = k''_{H_2O}/2$.

The k'_{H_2O} may be similarly obtained using Eq. 4 from experimental data around pH 9.2. Since the pKa values are widely separated, Eq. 5 may be used to synthesize the right-hand limb of Curve B above pH 9 and Eq. 6 may be used for the left-hand portion with pH 6.6–9 (8). The k'_{H_2O} could also be chosen as the observed rate constant at pH 9.2 where there should be no other significant kinetic contributions.

Curve C represents the theoretical value for the rate constants and is a summation of the contributions of Curves A and B in the pH region above 8.5.

The experimental data points in Figs. 3 and 4 are listed in Table I. These data may be seen to approximate closely the theoretical rate–pH profiles described by solid lines.

The experimental results below pH 8.5 do not coincide with the left-hand portion of Curve B in Fig. 4 and are represented by dashed lines. This may be attributable to solvent or buffer attack on the undissociated species (8).

The expression describing the observed rate constant above pH 8.5 is

$$k_{obs.} = k''_{H_2O} f_{A^{-2}} + k'_{H_2O} f_{HA^-} \qquad \text{(Eq. 8)}$$

or the kinetically equivalent:

$$k_{obs.} = k'_{OH^-}[OH^-] f_{HA^-} + k_{OH^-}[OH^-] f_{H_2A} \qquad \text{(Eq. 9)}$$

A tabulation of these catalytic constants is shown in Table III.

Buffer Effects—Neither phosphate nor borate buffers exhibited appreciable catalytic activity on 5-allyl,5-isobutylbarbituric acid decomposition in the ranges and conditions employed (Table IV).

A small difference in the observed velocity constants may be noted in Table IV; however, these can be ascribed to the minor pH differences of the buffers.

Ionic Strength Effects—Table II seems to imply this phenomenon to be taking place. The question as to whether Eq. 2 or its kinetic equivalent holds for these data cannot be resolved from the results (Table II). The reason for this is that the Debye–Hückel theory and, consequently, the Brönsted–Bjerrum equation are only valid for solutions below 0.01 molal of 1-1 electrolytes (9).

Activation Energies—Estimates of the apparent activation energies were evaluated from the Arrhenius equation:

$$\log k_{obs.} = \log P - E_a/2.303RT \qquad \text{(Eq. 10)}$$

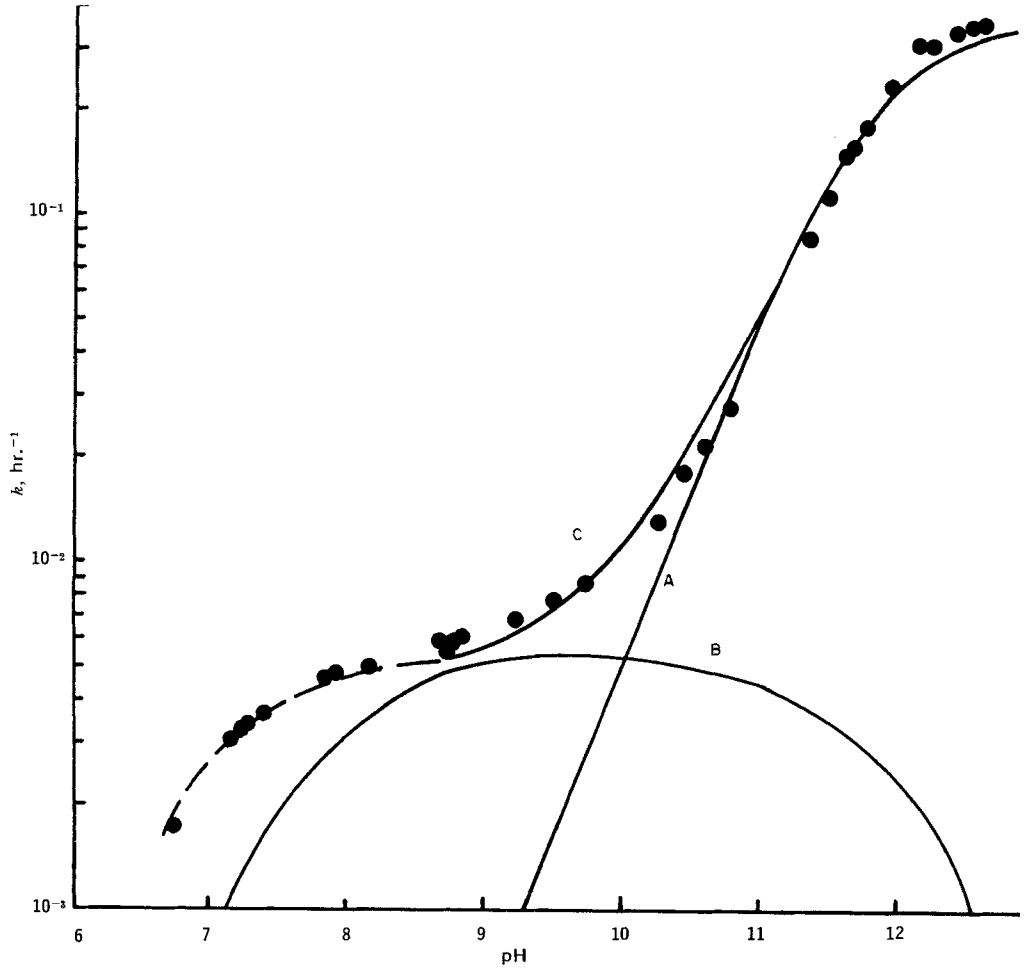

Figure 4—*Synthesis of log* k–*pH profile for itobarbital at 70°. Curve A represents water attack on dianion, and Curve B is indicative of water attack on monoanion. Curve C is a summation of A and B. See* Results and Discussion *section for a detailed explanation.*

where log $k_{obs.}$ plotted against $1/T$ absolute produces a straight line, with slope $\times$ 2.303R equal to the apparent activation energy, E_a. Values derived vary from 17.3 and 17.6 kcal./mole at 1.0 and 0.8 N NaOH to 26.6 and 27.1 kcal./mole at pH's 9.6 and 8.75, respectively.

Table II—Effect of Ionic Strength on Observed First-Order Rate Constants for Degradation of 2 $\times$ 10^{-4} M Itobarbital in 0.1 N NaOH at 70.0°

[NaOH]	[NaCl]	u	$u^{1/2}$	10^3 k, hr.$^{-1}$
0.1	0	0.1	0.32	162
0.1	0.39	0.49	0.70	187
0.1	0.90	1.00	1.00	194
0.1	1.59	1.69	1.30	194

Table III—Specific Catalytic Rate Constants (k, hr.$^{-1}$) and pKa Values for Degradation of Itobarbital in Aqueous Solution

	60°	70°	80°
pKa_1[a]	7.75	7.70	7.60
pKa_2[b]	11.90	11.80	11.60
k''_{H_2O}[c]	0.178	0.386	0.785
k'_{H_2O}[d]	0.0020	0.0063	0.020
$k_{OH^-} \times 10^2$[e]	3.70	8.41	21.49
k'_{OH^-}[f]	2.34	4.15	8.35

[a] Determined by titration of 0.005 M itobarbital at the specified temperature on a pH meter standardized by phthalate and borate buffers at specified temperature. [b] Obtained from best fit of log k–pH profiles. [c] k''_{H_2O} found using Eq. 2; pKa_2 values listed in Table III and experimental data for highest pH values of each temperature, Table I. [d] k'_{H_2O} derived from Eq. 4 using pKa values, Table III, and experimental data k_{HA^-} at various temperatures, Table I, pH 9.2. [e] Values calculated from the expression: $k'_{H_2O} f_{HA^-} = k_{OH^-} [OH^-] f_{H_2A}$ (Eqs. 4 and 7 combined). [f] Obtained from the kinetically equivalent relationship: $k''_{H_2O} f_{A^{-2}} = k_{OH^-} [OH^-] f_{HA^-}$ (Eq. 3).

SUMMARY

Itobarbital, 5-allyl,5-isobutylbarbituric acid, breaks down in aqueous solution by apparent first-order processes to produce nonchromophoric reaction products. The decomposition is easily followed in the UV by loss of absorbance as a function of time. Little buffer catalysis was witnessed under the conditions studied, and ionic strength effects in the highly alkaline range are of questionable validity.

Table I lists both experimental and calculated results, and a reasonable agreement may be noted down to pH 8.5. Below this pH, experimental and calculated values deviate, possibly due to small amounts of buffer catalysis of water attack on undissociated barbiturate. The observed rate constant may be described in the

Table IV—Influence of Buffers on Observed First-Order Rate Constants for Breakdown of 2 $\times$ 10^{-4} M Itobarbital at 70.0°[a]

pH[b,c]	Buffers: [$NaH_2PO_4 \cdot H_2O$]	Buffers: [$Na_2HPO_4 \cdot 7H_2O$]	NaCl	10^3k, hr.$^{-1}$
7.18	0.030	0.155	0	3.4
7.15	0.018	0.093	0.20	3.4
7.10	0.012	0.062	0.30	3.2
7.07	0.006	0.031	0.40	3.0
	[NaH_4BO_4]	[H_5BO_4]		
8.85	0.30	0.03	0	6.3
8.80	0.20	0.20	0.1	5.9
8.75	0.10	0.10	0.2	5.9
8.75	0.05	0.05	0.25	5.9

[a] Ionic strength constant at 0.5 for phosphate buffers and 0.3 for borate buffers. [b] pH values measured at 70° by pH meter standardized at this temperature with phthalate and borate buffers and are $\pm$ 0.05. [c] pH's of solutions at 25° were 9.30 and 7.45 ($\pm$0.05), respectively.

following manner to fit the experimental findings:

$$k_{obs.} = k''_{H_2O} f_{A^{-2}} + k'_{H_2O} f_{HA^-} \quad \text{(Eq. 8)}$$

Or it may be written in the kinetically equivalent:

$$k_{obs.} = k'_{OH^-}[OH^-] f_{HA^-} + k_{OH^-}[OH^-] f_{H_2A} \quad \text{(Eq. 9)}$$

The specific catalytic rate constants for Eqs. 8 and 9 are given in Table III along with other pertinent data.

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H. ZIA*, R. H. COX, and L. A. LUZZI

Abstract □ NMR spectroscopy was employed to examine the competitive binding of sulfamerazine, sulfacetamide, and penicillin G to bovine serum albumin. Variations in spin-spin relaxation rates were measured, and evidence for the displacement of penicillin by sulfa drugs was obtained.

Keyphrases □ Sulfonamide binding—bovine serum albumin □ Penicillin binding—bovine serum albumin □ Bovine serum albumin—sulfonamide displacement, bound penicillin □ NMR spectroscopy—analysis

The phenomena of drug–protein binding and of the competitive binding of drugs for available protein sites have been the subjects of many investigations (1). In this respect the antibiotics and sulfonamides have received considerable attention. Certain of these medicinal drugs have been shown to bind to bovine serum albumin (BSA). However, the methods of investigation have varied, and the data from competitive binding in certain instances have been inconclusive. Recent studies established the value of nuclear magnetic resonance (NMR) spectroscopy as a powerful tool for conformational determinations of pharmacologically active molecules in solution (2) and for elucidation of possible interactions between small molecules (3, 4). Furthermore, NMR is invaluable in assessing the extent to which various functional groups on small drug molecules participate in drug–protein interactions (5).

Fischer and Jardetzky (6) and Jardetzky and Wade-Jardetzky (7) utilized the extreme sensitivity of spin-spin ($1/T_2$) and spin-lattice ($1/T_1$) relaxation rates to small variations in the molecular environment of a proton species to show clearly how measurement of these parameters reveals formation of intermolecular complexes. In studies on penicillin and sulfa drug binding to BSA, they demonstrated that there were changes in relaxation rates large enough to be measured. This was true in spite of the fact that the concentration of bound molecules did not exceed 1% of the total drug concentration. The ability to detect the differences in relaxation rates upon binding, especially those differences due to diminished molecular motion, particularly rotational motion, made these studies possible. Observation of the relatively large increments in relaxation rate of the phenyl protons of penicillin and the *p*-aminobenzenesulfonamide moiety of sulfonamides lead them to conclude that these portions of the drug molecules are involved in binding.

In another report, Jardetzky and Wade-Jardetzky (8) indicated that there was no displacement of sulfa drugs bound to BSA by penicillin at reasonable concentrations of penicillin. This led them to postulate that the sites of binding on BSA for sulfa drugs and penicillin were different. However, there was no indication of concentration ratios used nor was there any indication that ionic strength and/or pH were controlled. Later, Kunin (9), using dialysis studies, showed that sulfa drugs in general displace certain BSA-bound penicillins.

Jardetzky and coworkers (8) reported that no displacement of sulfa drugs bound to BSA by penicillin occurs; Kunin (9), using different techniques, observed that the reverse displacement does take place. Thus, it appeared desirable to examine both displacement avenues while, at the same time, controlling factors such as ionic strength and pH which are known to be of significance in protein-binding studies. In this paper, the authors report the results of investigations of competitive binding to BSA between sulfamerazine (I) and penicillin G (II) and between sulfacetamide (III) and penicillin G (II) while ionic strength and pH are controlled.

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Table I—Effect of Variations of Solution Contents on the Relaxation Rate (sec.$^{-1}$) of Selected[a] Protons on the Penicillin G and Sulfamerazine Molecules

Compounds	Merazine[b]	CH_2[c]	CH-COO^-[d]	PABS[e]
0.1 *M* potassium penicillin G alone	—	4.71	1.88	—
0.5 *M* sodium sulfamerazine alone	1.57	—	—	2.51
0.1 *M* potassium penicillin G + 2.5% BSA	—	17.77	4.84	—
0.5 *M* sodium sulfamerazine + 2.5% BSA	3.38	—	—	3.71
0.1 *M* potassium penicillin G + 0.5 *M* sodium sulfamerazine + 2.5% BSA	3.03	10.5	3.45	3.34

[a] Those protons whose $1/T_2$ changes would best elucidate binding characteristics were chosen. [b] Proton 6 on the pyrimidine ring of sulfamerazine. [c] Methylene protons adjacent to the phenyl ring in penicillin G. [d] Methine proton on some carbon as carboxyl group in penicillin G. [e] Protons *ortho* to the sulfonyl group in sulfamerazine.

EXPERIMENTAL

Materials—The following materials were used: crystallized BSA,[1] 99.8% deuterium oxide,[2] potassium penicillin G,[3] sodium sulfacetamide, and sodium sulfamerazine.[4] All chemicals were used without further purification.

Methods—Solutions for NMR studies were made in deuterium oxide (D_2O). Drug concentrations are all expressed as molar (*M*), and protein concentrations are given in percent weight per volume (w/v). Ionic strengths of the solutions were adjusted using reagent grade KCl. The ionic strengths of the final solutions were kept 1 unit in excess of the ionic strength contributed by albumin, phosphate buffer, and potassium penicillin G at pH 7.15 ± 0.05. The pH's of the sample solutions were kept constant using 0.1 *M* phosphate buffer in D_2O to supplement the buffer capacity contributed by albumin. All pH measurements were made on a Radiometer titrator (TTT_{1c}) with microelectrodes. The pH values given are actual meter readings and are uncorrected for deuterium isotope effects.

Spectra—All proton NMR spectra were taken on a Varian Associates HA-100 spectrometer operating in the field-sweep mode with a probe temperature of 29°. Tetramethylsilane (TMS) was used as an external reference. Spectra were calibrated by the usual frequency difference techniques. A scan width of 50 Hz. was employed with a sweep time of 500 sec. Each reported value of $1/T_2$ is the mean of at least four, and usually eight, separate measurements. The values of $1/T_2$ given for the various protons were obtained from the spectral line-widths using Eq. 1:

$$\Delta V = (\pi T_2)^{-1} \qquad \text{(Eq. 1)}$$

where ΔV is the line-width at one-half maximum peak height, and T_2 is the spin-spin relaxation time (10).

RESULTS

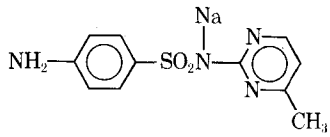
Displacement of Bound Potassium Penicillin G from BSA by Sodium Sulfamerazine—The drug sulfamerazine, which is known to bind rather strongly to serum albumin, was used to displace bound penicillin G from BSA. Spectra of the following five solutions were obtained: (*a* and *b*) potassium penicillin G, 0.1 *M*, alone, and sodium sulfamerazine, 0.5 *M*, alone: these served as controls for the combined solutions; (*c*) penicillin G, 0.1 *M*, and BSA, 2.5%, as a control for the effect of sodium sulfamerazine; (*d*) sodium sulfamerazine, 0.5 *M*, and BSA, 2.5%, as a control for the effect of potassium penicillin G; and (*e*) the combination of all three substances to detect displacement of penicillin G, if any. For penicillin G, $1/T_2$ was calculated for the methylene protons on the side chain and for the methine proton adjacent to the carboxylic group. The phenyl protons were not used since, at 100 MHz., a rather complex pattern is observed for these protons. The two *p*-aminobenzenesulfonamide protons adjacent to the sulfonyl group and a proton (H6) on the pyrimidine ring were used for the calculation of $1/T_2$ values for sodium sulfamerazine. The results obtained from these experiments are given in Table I.[5]

Table II—Effect of Various Concentrations of Sulfacetamide on Relaxation Rates (sec.$^{-1}$) of 0.1 *M* Penicillin G–5% BSA Solution at pH 7.15 ± 0.05 and Constant Ionic Strength

Compounds	PABS[a]	CH_2[b]	CH-COO^-[b]	C_6H_5[c]
0.1 *M* potassium penicillin G + 5% BSA only	—	25.75	6.63	16.64
0.1 *M* potassium penicillin G + 0.1 *M* sulfacetamide + 5% BSA	3.17	23.14	6.65	15.13
0.1 *M* potassium penicillin G + 0.3 *M* sulfacetamide + 5% BSA	3.60	21.38	5.37	15.07
0.1 *M* potassium penicillin G + 0.5 *M* sulfacetamide + 5% BSA	4.34	20.72	5.97	15.57
0.1 *M* potassium penicillin G + 1.0 *M* sulfacetamide + 5% BSA	4.24	20.47	6.37	15.39

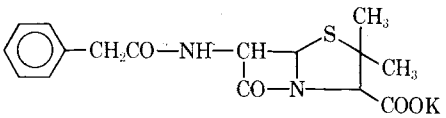
[a] Protons *ortho* to the sulfanyl group in sulfacetamide. [b] The same as in Table I. [c] Phenyl protons of penicillin G.

Displacement of Potassium Penicillin G from BSA by Sodium Sulfacetamide—Since it has been reported that the relaxation rates of penicillin and sulfa drugs are both pH and ionic strength dependent (6, 8), further experiments were designed to show that the decrease in $1/T_2$ obtained in potassium penicillin G with the addition of sodium sulfamerazine was not due to these factors. To measure relaxation rates in the pH region where pH effects on the $1/T_2$ of penicillin G are minimum (6), it was necessary to choose another sulfa drug that did not have the limitations of solubility inherent with sulfamerazine. In this series of experiments, the intent was to displace potassium penicillin G (0.1*M*) from BSA solutions (2.5 and 5%) by adding various concentrations of sodium sulfacetamide. The pH and ionic strength of these sample solutions were adjusted



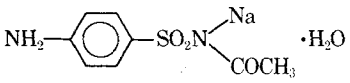
sodium sulfamerazine

I



potassium penicillin G

II



sodium sulfacetamide

III

[1] Nutritional Biochemical Corp.
[2] Mallinckrodt.
[3] Bristol Laboratories.
[4] Abbott Laboratories.

[5] To measure accurately the relaxation times for a proton, one must be able to observe an isolated single signal due to a transition from that proton. At 100 MHz., the phenyl protons of penicillin G give rise to a complex AA'BB'C NMR spectrum. Since there is considerable overlap of the signals from the phenyl protons, a single transition, due to the phenyl protons, could not be measured with accuracy. Therefore, the methylene protons, adjacent to the phenyl ring, of penicillin G were used for calculating relaxation times.

Table III—Effect of Various Concentrations of Sulfacetamide on Relaxation Rates (sec.$^{-1}$) of 0.1 *M* Potassium Penicillin G–2.5% BSA Solution at pH 7.15 ± 0.05 and Constant Ionic Strength

Compounds	PABS[a]	CH_2[a]	CH-COO^-[a]	C_6H_5[a]
0.1 *M* potassium penicillin G + 2.5% BSA only	—	15.70	4.36	14.62
0.1 *M* potassium penicillin G + 0.1 *M* sulfacetamide + 2.5% BSA	2.54	14.79	3.83	13.85
0.1 *M* potassium penicillin G + 0.3 *M* sulfacetamide + 2.5% BSA	2.91	13.84	3.96	13.63
0.1 *M* potassium penicillin G + 0.5 *M* sulfacetamide + 2.5% BSA	3.47	13.41	4.21	13.19
0.1 *M* potassium penicillin G + 1.0 *M* sulfacetamide + 2.5% BSA	3.58	13.22	4.18	13.47

[a] The same as in Table II.

according to the method reported in the *Experimental* section.

The data obtained with these experiments are given in Tables II and III. The relaxation times for the phenyl protons of penicillin G are given in these tables. However, since it cannot be ascertained with certainty that the values were calculated from a single transition, no conclusions could be drawn from the $1/T_2$ value for the phenyl protons. The values for the phenyl protons are given here to demonstrate the inconclusiveness of these data.[5] A typical plot of $1/T_2$ against concentration of sodium sulfacetamide is in Fig. 1.

DISCUSSION

The results presented in Table I show that the relaxation rates of both the methylene and methine protons of potassium penicillin G are increased by approximately two to threefold in the presence of 2.5% BSA as compared to the same protons of potassium penicillin G without BSA. Furthermore, addition of 0.5 *M* sodium sulfamerazine decreases significantly the $1/T_2$ value of the potassium penicillin protons. This decrease in relaxation rate indicates displacement of potassium penicillin G from BSA. Although the relaxation times reported in this investigation are smaller than those reported previously for potassium penicillin G (6), the increase in relaxation rates upon addition of BSA is approximately the same. This difference in relaxation rates in the two investigations is thought to arise from the differences in resolution of the two instruments used.

Although sodium sulfacetamide has less affinity for BSA than sodium sulfamerazine (11), addition of increasing amounts of sodium sulfacetamide to potassium penicillin G–BSA solutions results in a displacement of potassium penicillin G from BSA (Tables II and III). The decrease in relaxation rates for the penicillin protons is smaller than the decrease observed in the experiments with sulfamerazine, and this reflects the relative affinities of the two sulfa drugs for BSA. The results in Tables II and III show, however, that higher concentrations of sulfacetamide do not yield proportional increases in penicillin G displacement.

The results obtained from this investigation (Tables I–III) indicate that both sulfamerazine and sulfacetamide are able to displace penicillin G from BSA. It is also apparent from these results that both the concentration and affinity of sulfa for BSA seem to be important factors in the degree of displacement of penicillin G from BSA, except at higher sulfa concentrations where displacement is not proportional to concentration. This may be interpreted, in part, on the basis that addition of a high concentration of the sulfa drug (0.5 *M* or more) increases the viscosity of the solution to the extent that decreases in relaxation rates due to displacement are offset with increases in the relaxation rate produced by the viscosity of the solution.

Although the addition of sulfa drugs to the system may change the conformation of the BSA molecules and this change may in part be

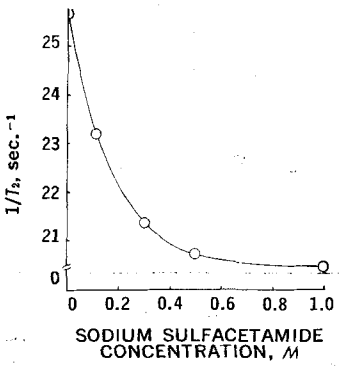


Figure 1—*Effect of concentration of sulfacetamide on relaxation rate of methylene protons for 0.1* M *potassium penicillin G–5% BSA solution at pH 7.15 ± 0.05 and constant ionic strength.*

responsible for the decrease in penicillin–BSA binding, it does not preclude that penicillin is displaced from its protein-bound position.

The possibility that penicillin G may displace sulfacetamide at constant pH and ionic strength was also examined. A 10-fold excess of penicillin G resulted in only a small decrease in the relaxation rates for the sulfacetamide protons compared to the relaxation rate for sulfacetamide–BSA solutions. However, due to the narrow linewidths obtained for the sulfa protons compared to that of the methylene protons from penicillin G and due to the experimental error incurred with relaxation rates, a fair statement concerning whether or not penicillin G displaces sulfacetamide or sulfamerazine cannot be made. It was an aim of this experimentation to establish a definite relationship in this case; however, it was not possible within the limitation of the instrumentation. The results should not be taken to indicate that penicillin is binding to the same site on BSA as the sulfa drugs. The sulfa drugs could be binding to a site adjacent to the penicillin-binding site on BSA and inhibit the binding of penicillin. It is also true that the binding or presence of sulfa drugs could cause a change in the conformation of BSA which could inhibit the binding of penicillin. The data do not allow one to distinguish between these possibilities.

The results of the studies reported here are in agreement with the dialysis data reported by Kunin (9) and, in this respect, further substantiate the usefulness of NMR in studying drug–protein binding. The major advantage of this method compared with dialysis methods is the rapidity with which reliable results may be obtained. No conclusive evidence indicating that penicillin inhibits the binding of sulfa drugs to BSA was found, and this is in agreement with previous studies.

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Effect of Three Liquid Additives on Diffusion of Atropine from Ointment Bases

CLYDE W. WHITWORTH and ROBERT E. STEPHENSON*

Abstract □ Water, alcohol, and dimethyl sulfoxide were each incorporated at 1, 2, and 5% concentrations into four ointment bases containing atropine. The diffusion of drug from five samples of each base was determined at 20-min. intervals for 2 hr. The diffusion process was zero order after 20 min. In the majority of instances, diffusion was significantly enhanced by the presence of the liquids. The concentration of liquid additive had significant effect in some instances; however, this effect was dependent on the type of base. Rate constants were calculated, and data were subjected to statistical analyses. The viscosity of each mixture was determined and, in some instances, appeared to correlate with the diffusion rates.

Keyphrases □ Atropine diffusion—ointment bases □ Ointments, atropine diffusion—water, alcohol, dimethyl sulfoxide effects □ Membrane permeation—atropine from ointments □ UV spectrophotometry—analysis

Percutaneous absorption of drugs from lotions, creams, and ointments is of considerable importance from the standpoint of toxicity, sensitivity, and effectiveness. The widespread use of such external preparations has led to increased interest in factors that may influence absorption through the skin. *In vitro* experiments usually involve the measurement of drug release from bases. Such measurements of drug release may detect interactions between drugs and vehicles which, in turn, may influence drug absorption through the skin.

It was previously found that the diffusion of salicylic acid from various ointment bases was influenced by the presence of low concentrations of certain liquids (1). It was subsequently suggested that other drugs be tried, and the weak base atropine was selected. This drug is extensively used in ophthalmic ointments. Coincidentally the water solubility of atropine is very near that of salicylic acid. Diffusion techniques have been used extensively to measure drug release from heterogeneous preparations such as ointments (2) and emulsions (3). The absorption of atropine from ophthalmic ointments probably involves the diffusion of the drug from the base into tear fluid.

Table I—Bases Used in This Study

Base	Percent
Water-in-oil base	
White petrolatum USP	64
Sorbitan monostearate[a]	6
Distilled water	30
Oil-in-water base	
Stearyl alcohol	25
White petrolatum	25
Glycerin	12
Polyoxyethylene (20) sorbitan monooleate[b]	5
Distilled water	33
Hydrogenated cottonseed oil base	
Partially hydrogenated cottonseed oil[c]	80
Completely hydrogenated cottonseed oil[d]	15
Sorbitan monooleate[e]	5
PEG base	
PGE 400	40
PEG 4000	40
Stearyl alcohol	20

[a,b] Trademarked as Span-60 and Tween-80, respectively, by Atlas Chemical Industries, Wilmington, Del. [c,d] Trademarked as Cotmar and Cotoflakes, respectively, by Proctor and Gamble Co., Cincinnati, Ohio. [e] Trademarked as Span-80 by Atlas Chemical Industries, Wilmington, Del.

Table II—Diffusion Rate Constants, K, for Atropine in moles/l./min. and Viscosity, N, of Ointments in Brookfield Viscometer Units

Additives	K	N	K	N
	Water-in-Oil		**Oil-in-Water**	
None	0.019	12.0	0.042	24.0
1% Alcohol	0.025	13.5	0.049	22.6
2% Alcohol	0.033	12.4	0.048	22.5
5% Alcohol	0.036	11.4	0.057	20.2
1% Water	0.037	19.0	0.055	24.0
2% Water	0.034	19.0	0.067	22.0
5% Water	0.044	14.5	0.069	20.6
1% DMSO	0.047	11.0	0.081	21.4
2% DMSO	0.042	9.5	0.066	20.6
5% DMSO	0.041	9.2	0.054	15.5
	Cottonseed Oil		**PEG**	
None	0.030	4.4	0.056	100+
1% Alcohol	0.035	4.5	0.067	100+
2% Alcohol	0.051	4.2	0.074	98.0
5% Alcohol	0.044	5.0	0.076	73.4
1% Water	0.053	5.7	0.069	100+
2% Water	0.037	3.2	0.073	100+
5% Water	0.035	4.5	0.075	86.0
1% DMSO	0.039	4.5	0.060	98.7
2% DMSO	0.038	3.7	0.066	89.8
5% DMSO	0.035	3.2	0.069	63.7

EXPERIMENTAL

Three liquids—water, alcohol, and dimethyl sulfoxide (DMSO)—were incorporated at 1, 2, and 5% concentrations into four different ointment bases. Two of the bases used in this study differ from those used in the previous investigation (1). The petrolatum base and the hydrogenated cottonseed oil base used in the first study did not release atropine. Consequently, a polyethylene glycol (PEG)

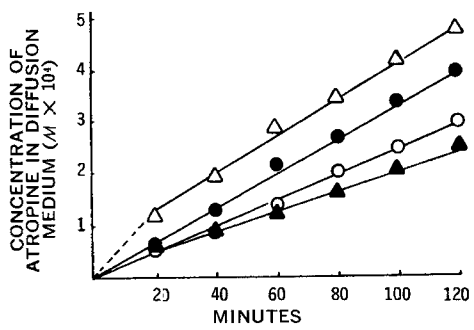

Figure 1—*Diffusion of 5% atropine from water-in-oil base* (▲) *and from water-in-oil base containing: 1% alcohol* (○), *2% alcohol* (●), *and 5% alcohol* (△).

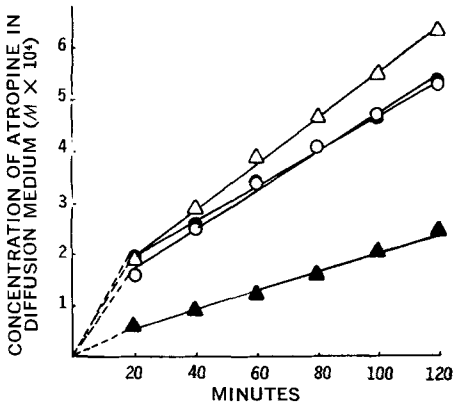

Figure 2—*Diffusion of 5% atropine from water-in-oil base* (▲) *and from water-in-oil base containing: 1% water* (○), *2% water* (●), *and 5% water* (△).

mixture was substituted for petrolatum, and 5% sorbitan monooleate was incorporated into the formula composed of hydrogenated cottonseed oil. The ingredients of the bases are given in Table I.

Atropine was incorporated into the emulsion bases by mixing it with the melted white petrolatum phase at low temperature prior to mixing the aqueous and oleaginous phases. For other ointments, the drug was added to the melted bases and stirred until the bases became firm. The concentration of atropine was 5% in all bases. To minimize evaporation, the liquids were each incorporated into the ointments just prior to use. All ointments including controls were thoroughly spatulated.

Five samples of each ointment were packed into uniform polyethylene cups which had a top (open end) diameter of 40 mm., a bottom diameter of 30 mm., and a depth of 25 mm. The open end was covered by a membrane[1] and sealed by a rubber band, so the entire exposed surface of the ointment was in contact with the membrane. The membranes used were of animal origin and during processing had been defatted and subjected to light density tests to ascertain their uniform character and thickness. The cups were then inverted in 60 ml. of distilled water contained in 4-oz. ointment jars, and the tops were replaced on the jars to prevent evaporation. The jars were then placed in a constant-temperature shaker-type bath at 37.5° for the diffusion to proceed. At 20-min. intervals for 2 hr., aliquots of the diffusion medium of each sample were assayed spectrophotometrically for drug content and returned to the container to maintain volume.

Diffusion of atropine from samples without additives was measured and taken as the control. A set of samples containing no drugs was run which furnished diffusion media for the reference cell. Atropine showed a spectrophotometric absorbance peak at 247.6 nm. Plots of concentration against absorbance showed conformity to Beer's law.

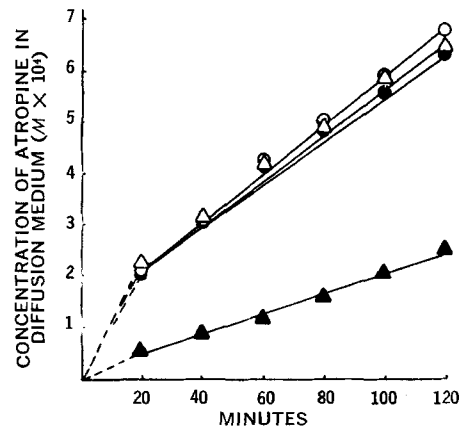

Figure 3—*Diffusion of 5% atropine from water-in-oil base* (▲) *and from water-in-oil base containing: 1% DMSO* (○), *2% DMSO* (●), *and 5% DMSO* (△).

[1] Supplied by Young Drug Co.

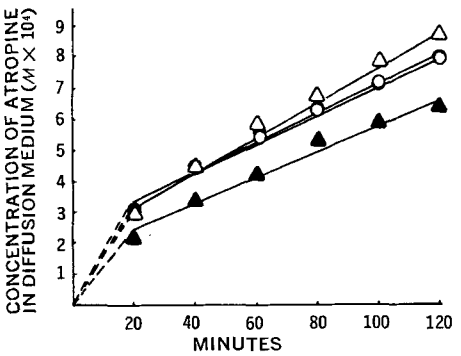

Figure 4—*Diffusion of 5% atropine from oil-in-water base* (▲) *and from oil-in-water base containing: 1% alcohol* (○), *2% alcohol* (●), *and 5% alcohol* (△).

Viscosity measurements were made with a Brookfield Synchro-Lectic viscometer model RVT mounted on a Helipath stand. A t-shaped spindle was used in all the measurements.

The influence of the additives on the permeability of the membrane was considered. It was determined that 1, 2, and 5% concentrations of alcohol or DMSO had no effect in 2 hr. on the passage of atropine through the membrane from aqueous solutions. This was found to be true with salicylic acid in the previous study (1). It was, therefore, assumed that the liquids used in this study exerted their influence on diffusion by means other than their action on the membrane.

Statistical methods were used to compare samples containing additives with controls. The *t* test at the 95% confidence level was employed (4).

The diffusion process was zero order between 20 and 120 min., with straight lines obtained when the concentration of the drug was plotted against time. Correlation coefficients confirmed these findings. All points on the graphs represent the average of five runs. The straight-line plots were calculated by the method of least squares. Rate constants were equal to the slopes of the lines and are shown in Table II along with viscosities.

RESULTS

Water-in-Oil Base—All liquids except 1% alcohol significantly increased the diffusion of atropine from this base. Figure 1 shows the effect of alcohol on diffusion. Although little effect was noted on viscosity (Table II), diffusion increased as the concentration of alcohol increased, as seen in the figure and in the rate constants of Table II.

In Fig. 2, water is seen to increase greatly the rate of diffusion of atropine from the water-in-oil base. It also increased the viscosity of the ointment over that of the control.

DMSO had the greatest effect in increasing the diffusion of atropine from this base (Fig. 3). Little difference was noted between

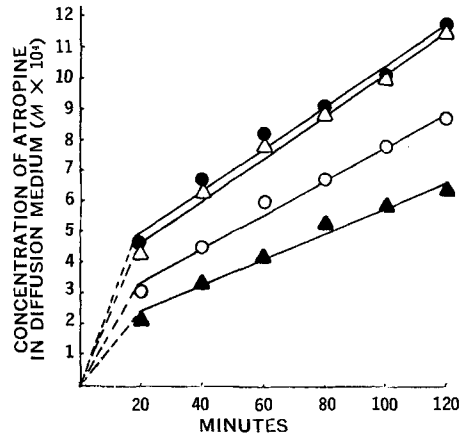

Figure 5—*Diffusion of 5% atropine from oil-in-water base* (▲) *and from oil-in-water base containing: 1% water* (○), *2% water* (●), *and 5% water* (△).

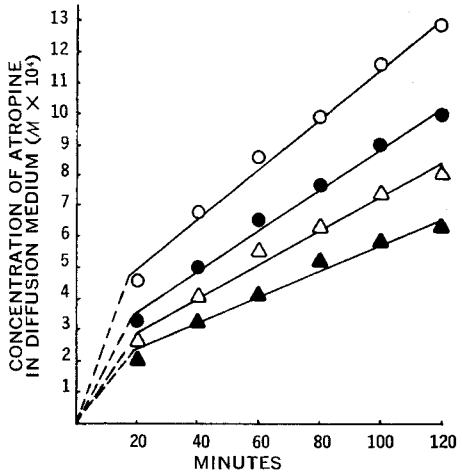

Figure 6—*Diffusion of 5% atropine from oil-in-water base* (▲) *and from oil-in-water base containing: 1% DMSO* (○), *2% DMSO* (●), *and 5% DMSO* (△).

the three concentrations of DMSO. The viscosities of the ointments containing DMSO were slightly lower than the viscosity of the control, but they do not vary greatly from each other.

Oil-in-Water Base—All concentrations of alcohol significantly increased the diffusion of atropine from the oil-in-water base (Fig. 4). Little difference was noted between 1 and 2% alcohol, either in effect on diffusion or on viscosity. The viscosity of this ointment was lowered slightly by all concentrations of alcohol, with the 5% giving the greatest reduction.

In Fig. 5, water is seen to increase the rate of diffusion of atropine from this base and a slight decrease in viscosity was observed. The diffusion results with 2 and 5% were almost identical.

Again, DMSO (Fig. 6) showed the greatest ability to increase the diffusion of atropine. The 1% concentration of DMSO showed

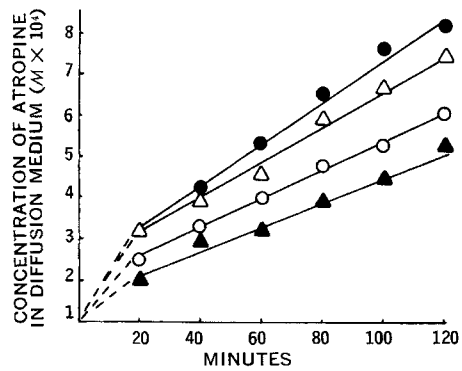

Figure 7—*Diffusion of 5% atropine from cottonseed oil base* (▲) *and from cottonseed oil base containing: 1% alcohol* (○), *2% alcohol* (●), *and 5% alcohol* (△).

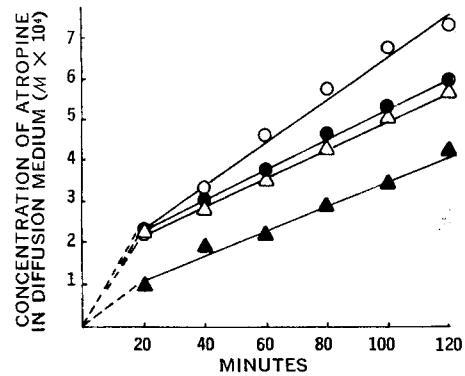

Figure 8—*Diffusion of 5% atropine from cottonseed oil base* (▲) *and from cottonseed oil base containing: 1% water* (○), *2% water* (●), *and 5% water* (△).

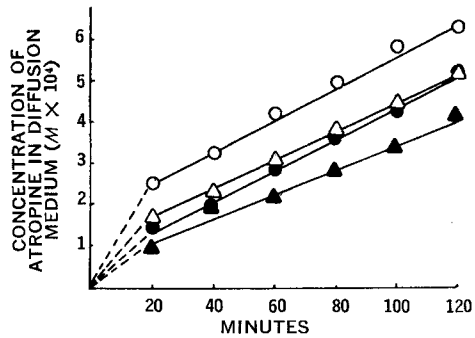

Figure 9—*Diffusion of 5% atropine from cottonseed oil base* (▲) *and from cottonseed oil base containing: 1% DMSO* (○), *2% DMSO* (●), *and 5% DMSO* (△).

the greatest effect followed by 2 and 5%, although the viscosities were reduced by increasing the concentration of DMSO.

Hydrogenated Cottonseed Oil Base—All liquids except 1% alcohol (Fig. 7) significantly increased the diffusion of atropine from the hydrogenated cottonseed oil base. The results with water (Fig. 8) and DMSO (Fig. 9) were very similar, with the greatest increase in diffusion produced by 1% concentrations of each. The viscosities were not changed greatly by the additives.

PEG Base—All liquids except 1% DMSO significantly increased the release of atropine from the PEG base (Figs. 10–12). Water had the greatest effect, as seen in Fig. 11; in general, an increase in concentration of liquid produced an increase in diffusion. Viscosity decreased with increasing concentrations of each liquid. The diffusion curves with this base are zero order in many instances over the entire 2-hr. period, indicating a constant rate of diffusion. This differed from the other bases which showed a greater rate during the first 20 min. and a steady rate thereafter.

DISCUSSION AND CONCLUSIONS

Higuchi (2) devised a modified mathematical statement of Fick's law to predict the amount of drug diffusing from certain types of ointment bases. The results, such as straight-line plots, of this study preclude the utilization of this equation. Certain assumptions are also made by Higuchi (2) that are not met by the conditions of this study.

Except in three instances involving 1% concentrations of liquids, the liquids increased the diffusion of atropine from the bases tested.

The PEG base gave the greatest release of atropine of the bases tested, both from the control and from the bases containing liquids. The average rate constant for the PEG base was 0.685. Next, in descending order, the average rate constants were: oil-in-water base, 0.588; cottonseed oil base, 0.397; and water-in-oil base, 0.358.

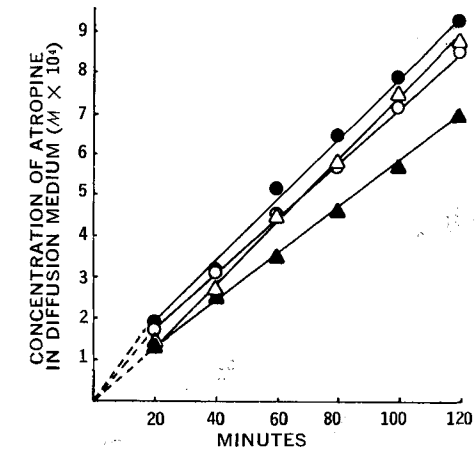

Figure 10—*Diffusion of 5% atropine from PEG base* (▲) *and from PEG base containing: 1% alcohol* (○), *2% alcohol* (●), *and 5% alcohol* (△).

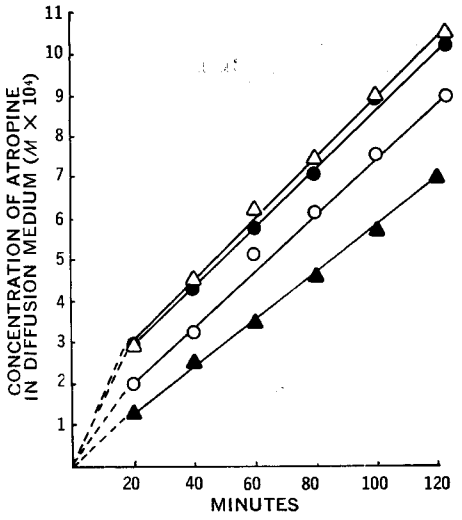

Figure 11—*Diffusion of 5% atropine from PEG base (▲) and from PEG base containing: 1% water (○), 2% water (●), and 5% water (△).*

Referring to the rate constants listed in Table II, one can see that DMSO was the most effective in increasing diffusion from both the water-in-oil and the oil-in-water bases followed by water and alcohol, respectively, in these bases.

The order was reversed in the case of the cottonseed oil base, with alcohol giving the greatest increase followed by water and DMSO.

The PEG base showed little difference between the results with alcohol and water. However, the rate constants for both of these were greater than those for DMSO.

The concentration of liquids had an effect on the diffusion patterns. As the concentration of DMSO was increased, the diffusion rates decreased for the water-in-oil, oil-in-water, and cottonseed oil bases and increased slightly for the PEG base.

As the concentration of both alcohol and water increased, the diffusion rate increased from all bases except dehydrogenated cottonseed oil which showed a decrease in the case of water and an increase and then a decrease as the alcohol concentration went from 1 to 2 to 5%.

In most cases, the viscosity of the ointments decreased, if only slightly, as the concentration of liquid was increased. With the exception of DMSO, this corresponded in most instances to an increase in diffusion.

The solubility of a drug in an ointment base undoubtedly plays a major role in its diffusion from that base. In other studies, liquids were used to simulate the single-component ointment bases and to correlate solubility with release (5) and *in vivo* absorption (6). It is not possible to determine accurately the solubility of drugs in the intact ointment base in studies in which the bases are composed of several components such as the emulsion bases. In some cases, it is possible to make assumptions and draw conclusions concerning solubility from the physical and chemical properties of both the drug and base. In this study the results of the diffusion measurements show no apparent correlation to the solubility that atropine might be assumed to have in the bases. The bases that

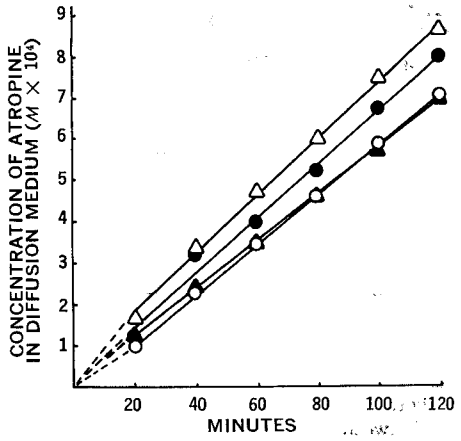

Figure 12—*Diffusion of 5% atropine from PEG base (▲) and from PEG base containing: 1% DMSO (○), 2% DMSO (●), and 5% DMSO (△).*

were more like the diffusion medium, *i.e.*, PEG and oil-in-water emulsion, gave the greatest release.

It is apparent that the incorporation of even small concentrations of certain liquids into certain types of ointment bases may increase the diffusion of some drugs from these bases. This is true for certain emulsion bases which themselves already contain appreciable amounts of water and oil. It should be remembered that the liquids were incorporated uniformly after the emulsion bases were prepared.

Some researchers have suggested that the rate of dissolution of suspended drugs in ointment bases as well as methods of preparation are quite likely to influence drug release. Some of these factors are currently under investigation in these laboratories.

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Determination of Rate Constants for *In Vitro* Three-Compartment Transfer Using a Two-Phase System

MARTIN H. YUNKER and SAUL BORODKIN

Abstract □ The use of two-phase studies to obtain the four rate constants involved in the transfer of drug between three compartments is described. The systems studied include the transfers of salicylic acid between pH 2 buffer, cyclohexane, and pH 7.4 buffer; erythromycin between pH 6.8 buffer, toluene, and pH 7.4 buffer; and erythromycin between pH 6.8 buffer, cyclohexane, and pH 7.4 buffer. The rate constants derived were shown to be independent of interfacial areas and phase volumes by studies in which these parameters were varied. Three phase curves showing the amount of drug in each phase *versus* time were drawn by an analog computer. The analog computer program included as parameters the four rate constants, the volumes of each of the three phases, and two interfacial areas. Thus, volumes and areas more likely to exist in biological systems, but impractical experimentally, can be selected to show drug transfer kinetics. Experimental points obtained from three-phase studies followed the computer-drawn curves closely.

Keyphrases □ Erythromycin transfer rate—two-phase system □ Salicylic acid transfer rate—two-phase system □ Transfer rate constants—salicylic acid, erythromycin, two-phase systems □ Distribution coefficients—salicylic acid, erythromycin, two-phase systems □ Interfacial area, phase volume, stirring rate, effects—transfer-rate constants

Absorption of drug from the gastrointestinal tract into the bloodstream is believed to occur by passive diffusion through a lipoidal membrane (1, 2). A number of workers used three-phase *in vitro* models to simulate the *in vivo* system to study the factors influencing drug transfer (3–9). Doluisio and Swintosky (4) devised an inverted rocking Y-tube, with aqueous phases in each of the two arms overlaid with a connecting layer of an immiscible organic liquid. Perrin (5) suggested a rectangular cell with a partition separating the two aqueous phases and the organic phase on top. These investigators demonstrated that drug transfer between three compartments can be described by four first-order rate constants.

In selecting a model for studying *in vitro* transport characteristics of erythromycin, it appeared that the four rate constants involved in transfer between three layers could most easily and accurately be determined by two-phase systems. Simulated three-phase curves can be obtained by using the rate constants in an analog computer program. This is in contrast to the approach taken by Khalil and Martin (6) who determined the rate constants from experimental curves by means of an analog computer.

This paper reports studies with salicylic acid and erythromycin using the described approach. Salicylic acid transfer between pH 2 and 7.4 buffers was selected as representative of gastrointestinal absorption of an acidic drug. Erythromycin transfer between pH 6.8 and 7.4 was studied primarily to develop *in vitro* data that could be related to systemic absorption of this drug from milk in the treatment of bovine mastitis. This system can also be considered representative of absorption of a basic drug from the small intestine.

EXPERIMENTAL

Apparatus—The vessel chosen for the two-phase transfer studies consisted of a 2-l. round-bottom, three-necked, standard taper flask fitted with a ground-glass sealed stirrer in the center opening. A three-blade propeller was attached to the bottom of the stirring shaft which was driven by a variable speed stirrer.[1] A second three-blade propeller was placed on the stirring shaft 7.62 cm. (3 in.) above the first. When 1 l. of each phase was added to the flask, the stirrer was positioned so that the propellers were 3.81 cm. (1.5 in.) above and below the interface. Two glass tubes were inserted through a rubber stopper in one of the necks and extended into each of the liquid phases through which samples were withdrawn. The other opening served for introducing the liquid phases and was stoppered during the test. This flask gave an interfacial area of 194 cm.^{2}. For the purpose of studying the effect of phase volumes and interfacial area on transfer rate, a 2-l. graduated beaker was converted into a three-necked container with 24 × 40 taper joints. This vessel permitted changing volumes without affecting interfacial area. The interfacial area using this flask was 96 cm.^{2}.

The apparatus used for three-phase studies was a modification of the two-phase apparatus. Two 2-l. round-bottom flasks were placed next to each other, and the organic liquid phase was circulated between the two flasks by means of a tubing pump[2] and a siphon tube. Stirrers identical to those used for the two-phase study were used in each flask.

Reagents and Buffers—All chemicals were reagent grade unless specified otherwise. Salicylic acid, erythromycin, monobasic sodium phosphate, dibasic sodium phosphate, potassium chloride, hydrochloric acid, cyclohexane, and toluene were used.

The pH 2 buffer was prepared by adding 65 ml. of 0.2 *M* hydrochloric acid and 250 ml. of 0.2 *M* potassium chloride to a 1-l. volumetric flask and adding water to volume. The pH 6.8 buffer was prepared by dissolving 28.9 g. of dibasic sodium phosphate (heptahydrate) and 8.05 g. of monobasic sodium phosphate (monohydrate) in sufficient water to make 1 l. The pH 7.4 buffer was prepared by dissolving 37.4 g. of dibasic sodium phosphate and 3.7 g. of monobasic sodium phosphate in sufficient water to make 1 l. The final pH of the 6.8 and 7.4 buffers was adjusted with hydrochloric acid. All pH measurements were made with a Corning model 7 pH meter.

Procedure—In the two-phase experiments, the drug was dissolved in 1 l. of either the organic liquid or the aqueous buffer, depending on which procedure was most satisfactory for measuring the transfer. One-half gram of salicylic acid and 2.0 g. of erythromycin were used in all of the studies. One liter of the aqueous phase was added to the vessel which was then immersed in a constant-temperature bath at 37 ± 0.05° and allowed to reach temperature. One liter of the organic liquid was heated to 37° and then added slowly onto the top of the aqueous phase so as not to disturb the interface. It was not considered necessary to preequilibrate the phases prior to the transfer studies because of the marked difference in polarity between the aqueous and organic phases employed. The system was stirred at 80 r.p.m., samples (2 ml.) of each phase were withdrawn at various time intervals, and the drug concentration in one or both

[1] Servodyne Mixer, model 4420, Cole-Parmer Instrument and Equipment Co., Chicago, Ill.

[2] Masterflex Pump, model 7015, Cole-Parmer Instrument and Equipment Co., Chicago, Ill.

Table I—Rate Constants and Distribution Coefficients from Two-Phase Transfer Studies

Drug	pH of Aqueous Phase	Organic Phase	Phase to which Drug Was Added	K_C	k_1, cm. hr.$^{-1}$	k_{-1}, cm. hr.$^{-1}$
Salicylic acid	2	Cyclohexane	Organic	0.157	0.246	1.57
Salicylic acid	2	Cyclohexane	Aqueous	0.157	0.248	1.58
Salicylic acid	7.4	Cyclohexane	Organic	<0.002	<0.04	1.84
Erythromycin	6.8	Cyclohexane	Organic	0.0436	0.0309	0.670
Erythromycin	7.4	Cyclohexane	Organic	0.153	0.0959	0.625
Erythromycin	6.8	Toluene	Aqueous	1.679	0.573	0.341
Erythromycin	7.4	Toluene	Aqueous	6.52	0.699	0.107
Erythromycin	7.4	Toluene	Organic	6.52	0.710	0.109

of the two phases was measured. The volume of samples removed had an insignificant effect on the interfacial area.

In the three-phase experiments, 1 l. of the aqueous buffered drug solution (0.5 g./l. salicylic acid or 2.0 g./l. erythromycin) was added to one of the flasks and brought to 37°. One liter of the other aqueous buffer and one-half of the organic liquid were added to the other flask and brought to 37°. The remainder of the organic liquid was heated to 37° and then added to the flask containing the drug solution. Circulation of the organic liquid between the two flasks was maintained at 100 ml./min. with the pump. Samples (2 ml.) of each of the three phases were withdrawn at various time intervals, and the drug concentrations in each phase were determined.

Distribution coefficients (K_C) of the drugs between the organic liquids and the aqueous buffers, as well as the equilibrium amounts in the two phases, were determined in separate experiments by rapidly mixing the two phases (100 ml. of each) in a 250-ml. conical flask for 2 hr. at 37°, allowing the phases to separate, and determining the drug concentrations in each phase. Studies showed that 2 hr. of stirring was sufficient to reach equilibrium.

Assay—Salicylic acid was determined spectrophotometrically with a Beckman DU-2 spectrophotometer at 297 nm. after an appropriate dilution with 0.1 *N* sodium hydroxide. Erythromycin was determined by the arseno-molybdate method (10). The cyclohexane and toluene samples were evaporated to dryness and reconstituted with water.

RESULTS AND DISCUSSION

Evaluation of Rate Constants—Two-phase transfer can be represented by the kinetic model:

$$C_A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_B \qquad \text{(Eq. 1)}$$

where C_A and C_B represent the concentration of drug in the two layers A and B, respectively, and k_1 and k_{-1} are the rate constants for the forward and reverse transfers, respectively. By considering the transfers to be first-order processes, the net rate of decrease in quantity of drug in layer A is given by the rate at which drug enters layer B less the rate at which drug returns from B to A. This is represented by

$$-\frac{dQ_A}{dt} = k'_1 C_A - k'_{-1} C_B \qquad \text{(Eq. 2)}$$

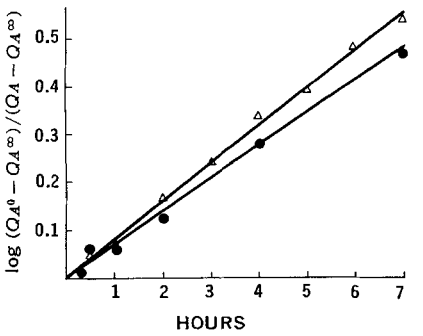

Figure 1—*Rate of attaining equilibrium in the transfer of erythromycin from aqueous buffers (1 l.) to toluene (1 l.). Interfacial area equals 194 cm.*^{2}*. Key: △, pH 6.8; and ●, pH 7.4.*

where Q_A is the quantity of drug in A, and k'_1 and k'_{-1} are apparent rate constants. Since the transfer rate will be directly proportional to the interfacial area, S, and concentrations may be expressed as quantity divided by volume, Eq. 2 may be written:

$$-\frac{dQ_A}{dt} = k_1 \frac{SQ_A}{V_A} - k_{-1} \frac{SQ_B}{V_B} \qquad \text{(Eq. 3)}$$

where k_1 and k_{-1} are rate constants independent of interfacial area and phase volumes with the dimensions of length per time (cm. hr.$^{-1}$).

It can be shown that Eq. 3 is related to Fick's law of diffusion by assuming that equilibrium is always present at the interface and the concentration gradient in phase A is rate determining (11). Thus, Eq. 4 may be written:

$$-\frac{dQ_A}{dt} = \frac{DS}{\Delta X}\left(C_A - \frac{C_B}{K_C}\right) \qquad \text{(Eq. 4)}$$

where D is the diffusion constant, ΔX is the thickness of the diffusion layer, and K_C is the drug-distribution coefficient. Howard *et al.* (12) derived a similar equation to describe the transfer of a drug from an aqueous phase to an unstirred lipid sink. By expressing concentrations as quantities divided by volumes, Eq. 4 may be written:

$$-\frac{dQ_A}{dt} = \frac{DSQ_A}{\Delta X V_A} - \frac{DSQ_B}{\Delta X K_C V_B} \qquad \text{(Eq. 5)}$$

where $D/\Delta X$ and $D/\Delta X K_C$ are equivalent to k_1 and k_{-1} in Eq. 3. Since D and ΔX have the dimensions of area per time and length, respectively, $D/\Delta X$ and $D/\Delta X K_C$ have dimensions identical to k_1 and k_{-1}, length per time.

Integration of Eq. 3 yields

$$\ln \frac{Q_A^0 - Q_A^\infty}{Q_A - Q_A^\infty} = S\left(\frac{k_1}{V_A} + \frac{k_{-1}}{V_B}\right)t \qquad \text{(Eq. 6)}$$

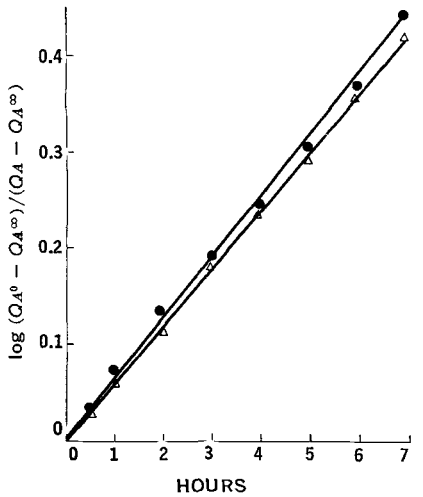

Figure 2—*Rate of attaining equilibrium in the transfer of erythromycin from aqueous buffers (1 l.) to cyclohexane (1 l.). Interfacial area equals 194 cm.*^{2}*. Key: △, pH 6.8; and ●, pH 7.4.*

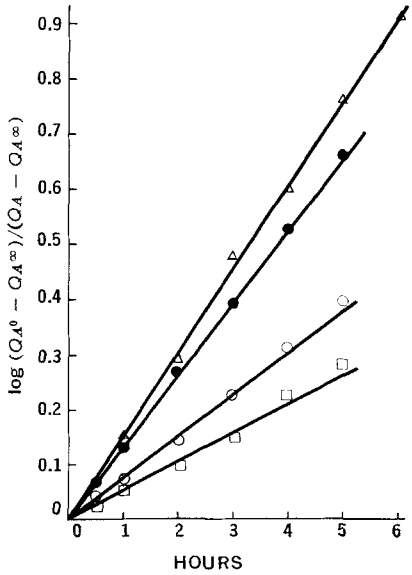

Figure 3—*Rate of attaining equilibrium in the transfer of salicylic acid from cyclohexane* (B) *to pH 2 aqueous buffer* (A). *Key:* △, S = *194 cm.*², V_B = *1 l.*, V_A = *1 l.*; ●, S = *96 cm.*², V_B = *0.5 l.*, V_A = *1.5 l.*; ○, S = *96 cm.*², V_B = *1.0 l.*, V_A = *1.0 l.*; *and* □, S = *96 cm.*², V_B = *1.5 l.*, V_A = *0.5 l.*

where Q_A^0 is the quantity of drug in layer A at zero time, and Q_A^∞ is the quantity of drug in layer A at equilibrium. A plot of log $(Q_A^0 - Q_A^\infty)/(Q_A - Q_A^\infty)$ *versus* t should yield a straight line with an intercept of zero and a slope defined by Eq. 7:

$$\text{slope} = \frac{S}{2.303}\left(\frac{k_1}{V_A} + \frac{k_{-1}}{V_B}\right) \qquad \text{(Eq. 7)}$$

Since

$$K_C = \frac{C_B}{C_A} = \frac{k_1}{k_{-1}} \qquad \text{(Eq. 8)}$$

where K_C is the apparent distribution coefficient, substitution into Eq. 7 will allow evaluation of k_1 by the following expression:

$$k_1 = \frac{2.303}{S\left(\frac{1}{V_A} + \frac{1}{K_C V_B}\right)} \times \text{slope} \qquad \text{(Eq. 9)}$$

Then k_{-1} can be obtained from Eq. 8.

The half-life equation for attaining equilibrium may be derived from Eq. 7:

$$t_{1/2} = \frac{\ln 2}{S\left(\frac{k_1}{V_A} + \frac{k_{-1}}{V_B}\right)} \qquad \text{(Eq. 10)}$$

All experimental results obtained with the erythromycin and salicylic acid two-phase system followed the preceding relationships. Figures 1–3 are representative of the linearity obtained when the data are plotted according to Eq. 6. Rate constants and distribution coefficients for the two-phase systems used are shown in Table I.

The results show that the rate of salicylic acid transfer from pH 2 to cyclohexane is about 70 times faster than from pH 7.4. Although the faster rate would be expected, the magnitude of the difference is much less than would be predicted on the basis of transfer of only unionized species. Based on a pKa of 3, the concentration of unionized salicylic acid at pH 2 would be more than 20,000 times greater than at pH 7.4. The closeness of the two reverse rate constants indicates that the pH of the aqueous phase has little effect on transfer rate out of cyclohexane. For the erythromycin–cyclohexane system, the rate constant from pH 7.4 buffer is 3.1 times greater than at pH 6.8. This compares with a ratio of 3.86 for the fraction of unionized species at the two pH levels (0.99% at pH 6.8 and 3.83% at pH 7.4 based on a pKa of 8.8 for erythromycin). The rate constants

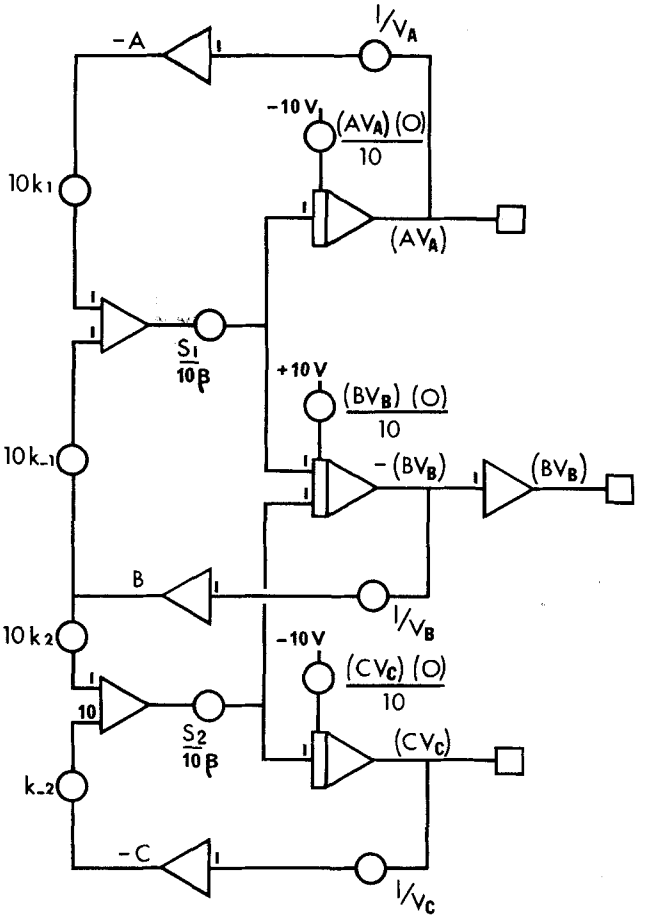

Figure 4—*Analog computer program for obtaining three-phase drug transfer curves.*

for the transfer of erythromycin from the aqueous phases into toluene are much greater than for cyclohexane. However, there is no correlation between the rate constants and the fraction of unionized species. The rate constant for the transfer from pH 7.4 buffer into toluene was only 1.2 times that from pH 6.8 *versus* the expected 3.86 ratio. A possible explanation for this lack of correlation might be the adsorption of the unionized erythromycin at the interface.

Distribution coefficients for unionized erythromycin were calculated from the apparent distribution coefficients by assuming that the drug in the organic phase was in the unionized form. In this manner, distribution coefficient values of 170 are derived for the toluene–pH 6.8 and toluene–pH 7.4 systems. The distribution coefficients of unionized erythromycin in the cyclohexane–pH 6.8

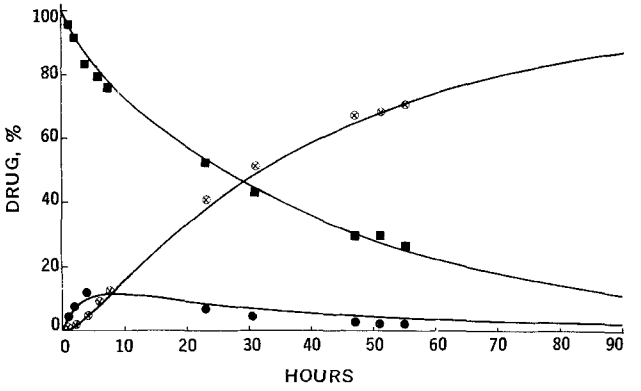

Figure 5—*Transfer of salicylic acid from pH 2 buffer (1 l.) through cyclohexane (2 l.) to pH 7.4 buffer (1 l.). Lines were obtained from the analog computer program and points are experimental. Interfacial areas equal 194 cm.*². *Key:* ■, *pH 2.0 layer;* ●, *cyclohexane layer; and* ⊗, *pH 7.4 layer.*

Table II—Effect of Phase Volumes and Interfacial Area on Rate Constants in the Transfer of Salicylic Acid from Cyclohexane into pH 2 Buffer

Experiment	Volume of pH 2 Buffer, l.	Volume of Cyclohexane, l.	Interfacial Area, cm.^{2}	$t_{1/2}$, hr.	k_1, cm. hr.$^{-1}$	k_{-1}, cm. hr.$^{-1}$
1	1	1	194	1.97	0.246	1.57
2	1	1	96	3.92	0.249	1.59
3	0.5	1.5	96	4.77	0.250	1.52
4	1.5	0.5	96	2.24	0.252	1.53

and cyclohexane–pH 7.4 systems are 4.4 and 4, respectively. The fact that the distribution coefficients are nearly the same at both pH's for toluene and cyclohexane indicates that essentially only the unionized species is transferring into these solvents.

Table I shows that the same rate constants are obtained regardless of whether the drug is added to the aqueous or the organic phase. This is shown for the transfer of salicylic acid between pH 2 and cyclohexane and erythromycin between pH 7.4 and toluene. Therefore, the drug can be dissolved in either phase to determine the rate constants. Generally, it is most advantageous to add the drug to the phase in which the drug is least soluble.

Toluene and cyclohexane were selected for the lipoidal phases after studying distribution coefficients with a number of organic solvents. Although these solvents have similar delta values, they gave quite different rate constants. The magnitude of the difference was similar to that reported by Khalil and Martin (6) for the transfer of salicylic acid into these two solvents. Although both toluene and cyclohexane have a lower density than water, rate constants may be as easily determined with solvents heavier than water. In most previously described three-compartment models, only solvents lighter than water could be used.

Effects of Stirring Rate, Phase Volumes, and Interfacial Area—Since the transfer-rate constants are dependent on the ΔX in Eq. 5, it is important that this parameter does not vary. The most likely cause of change in the diffusion layer would be variations in the stirring rate. The effect of stirring rate on salicylic acid transfer was studied over a range of 50–130 r.p.m. The rate constants were found to be constant over the range 70–90 r.p.m. Increasing rates observed from 110 to 130 r.p.m. can be explained by a decrease in diffusion layer thickness, while a slight decrease in rate obtained at 50 r.p.m. can be attributed to insufficient stirring of the layers. On the basis of these results, a stirring speed of 80 r.p.m. was used in all experiments.

Several experiments were run to demonstrate that the rate constants defined in Eq. 3 are independent of phase volumes and interfacial area. Figure 3 and Table II show the results obtained when these parameters were varied in the transfer of salicylic acid from cyclohexane into pH 2 buffer. Experiments 2, 3, and 4 demonstrate that changes in phase volumes affect the rates of achieving equilibrium but not the rate constants. Experiments 1 and 2 (Table II) show that when the interfacial area is approximately halved, the half-life is doubled while k_1 and k_{-1} remain constant. The fact that the rate constants did not change when two vessels of different shape were used would indicate that the diffusion layer thickness remained constant in these experiments. However, Augustine and Swarbrick (13) showed that the design of the transfer cell can be important. The agitation intensity and thus the resulting diffusion layer thickness achieved at a given stirring rate may be dependent on the shape of the vessel.

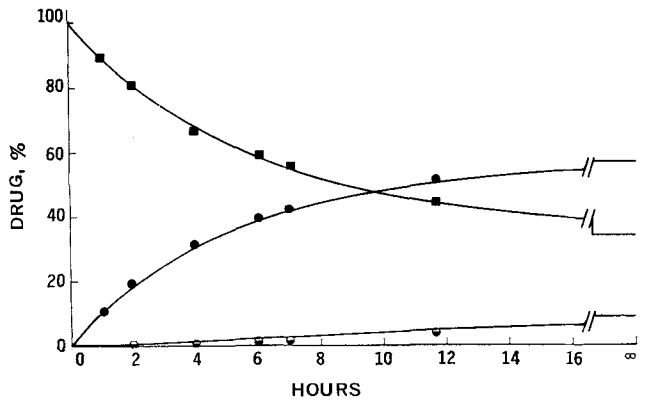

Figure 6—*Transfer of erythromycin from pH 6.8 buffer (1 l.) through toluene (1 l.) to pH 7.4 buffer (1 l.). Interfacial areas equal 194 cm.^{2}. Lines were obtained from the analog computer program and points are experimental. Key: ■, pH 6.8; ●, toluene; and ◒, pH 7.4.*

Use of Rate Constants in Three-Phase Systems—Three-phase transfer can be represented by the model:

$$C_A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C_C \qquad \text{(Eq. 11)}$$

where k_1 and k_2 are the rate constants for the forward transfer, and k_{-1} and k_{-2} are the corresponding reverse transfer constants. C_A and C_C represent the drug concentration in the two aqueous phases, and C_B is the drug concentration in the organic liquid. Rate equations for the transfer of drug from each of the three phases can be written as extensions of Eq. 3:

$$-\frac{dQ_A}{dt} = k_1 \frac{SQ_A}{V_A} - k_{-1} \frac{SQ_B}{V_B} \qquad \text{(Eq. 12)}$$

$$-\frac{dQ_B}{dt} = -k_1 \frac{SQ_A}{V_A} + k_{-1} \frac{SQ_B}{V_B} + k_2 \frac{SQ_B}{V_B} - k_{-2} \frac{SQ_C}{V_C} \qquad \text{(Eq. 13)}$$

$$-\frac{dQ_C}{dt} = -k_2 \frac{SQ_B}{V_B} + k_{-2} \frac{SQ_C}{V_C} \qquad \text{(Eq. 14)}$$

These equations differ from those presented by Perrin (5) and Khalil and Martin (6) for transfer of drug between three phases. Their equations show that the rate of transfer of total drug is a function of volume times concentration (quantity) rather than concentration in each phase. In their relationship, different values for the rate constants would be obtained each time the volumes are changed.

An analog computer[3] was programmed to represent three-phase transfer based on Eqs. 12–14. The program is given in Fig. 4. The four rate constants can be determined by the two-phase studies described. Rate constants k_1 and k_{-1} are from a two-phase experiment employing phases A and B, while k_2 and k_{-2} are determined similarly from

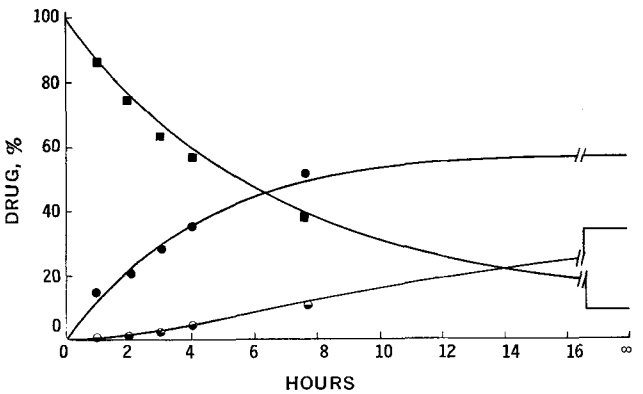

Figure 7—*Transfer of erythromycin from pH 7.4 buffer (1 l.) through toluene (1 l.) to pH 6.8 buffer (1 l.). Lines were obtained from the analog computer program and points are experimental. Interfacial areas equal 194 cm.^{2}. Key: ■, pH 7.4 layer; ●, toluene layer; and ◒, pH 6.8 layer.*

[3] Pace TR-48, EAI, Long Branch, N. J.

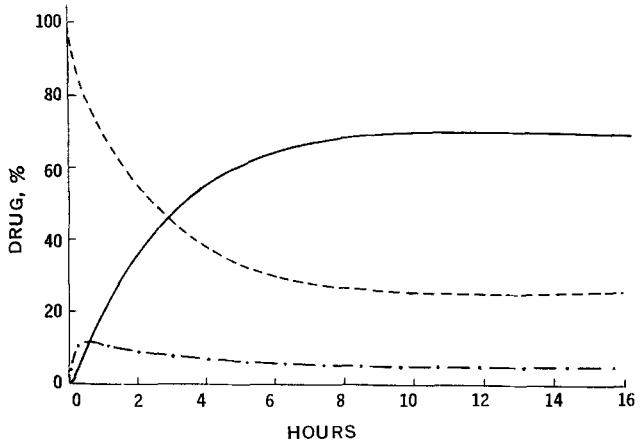

Figure 8—*Transfer of erythromycin from pH 6.8 buffer (1 l.) through toluene (0.1 l.) to pH 7.4 buffer (10 l.). Interfacial areas equal 194 cm.². Curves were obtained from the analog computer program. Key: - -, pH 6.8 buffer; –·–, toluene; and ——, pH 7.4 buffer.*

another two-phase experiment using phases *B* and *C*. Curves showing the amount of drug in each phase can then be obtained by putting these rate constants, along with desired phase volumes and interfacial areas, into the computer fitted with an X–Y recorder.

To demonstrate that rate constants from two-phase systems can be extended to this model, several three-phase runs were made and the experimental points were plotted against computer-drawn curves. The results are shown in Figs. 5–7. The experimental points followed the curves closely in all cases.

An advantage of the proposed method is that different volumes and interfacial areas can be fitted into the analog computer program. Thus, values more characteristic of those existing in the body can be used to simulate drug transport. For example, with the conditions described in Fig. 6, the percent of erythromycin in phase *C* at equilibrium is less than 10 and its $t_{1/2}$ is about 12 hr. However, if the volumes of phases *A*, *B*, and *C* are assigned 1, 0.1, and 10 l., respectively, and the interfacial area is increased 10-fold, the curves in Fig. 8 are obtained. Under these conditions, the percent of erythromycin in phase *C* at equilibrium is increased to 73 and its half-life is decreased to 2 hr. These latter conditions would be impractical to use experimentally with the three-phase apparatus reported.

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Solubility of Alkyl Benzoates II: Effect of Dielectric Constant on the Solubility of Substituted Alkyl Benzoates

F. SHIHAB, J. SPROWLS, and J. NEMATOLLAHI

Abstract □ The solubility of a homologous series of alkyl parabens, ranging from methyl to butyl together with benzyl paraben and methyl *p*-methoxybenzoate, were evaluated in solvents consisting of polyethylene glycol and water in various proportions. The relationship between dielectric constant value and mutual solubilizing effect, which was observed upon combining two parabens (benzyl paraben with any one of the parabens in the series), was evaluated. Decreasing the dielectric constant value of the solvent induced solubility of these esters. The dielectric constant, however, was observed to be not the sole factor, and there seems to exist a concentration effect of one paraben on the solubility of the other.

Keyphrases □ Solubility—substituted alkyl benzoates □ Dielectric constants, parabens—solubility relationship □ Parabens—mutual solubilizing effect □ Oscillometry—dielectric constant determination

In an earlier communication (1), the authors reported some solubility features of alkyl *p*-hydroxybenzoates (parabens). The data of these previous experiments, obtained as a result of determining solubility of a combination of each paraben in a homologous series (methyl, ethyl, *n*-propyl, *n*-butyl paraben) and benzyl paraben, were indicative of the existence of a mutual solubilizing phenomenon among parabens. As compared to the solubility of a single paraben in polyethylene glycol (PEG) and water, there was an enhancement of solubility when a combination of two different parabens was allowed to dissolve.

Table I—Dielectric Constant of PEG–H_2O Mixtures

PEG, %	*k*
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20	68.52
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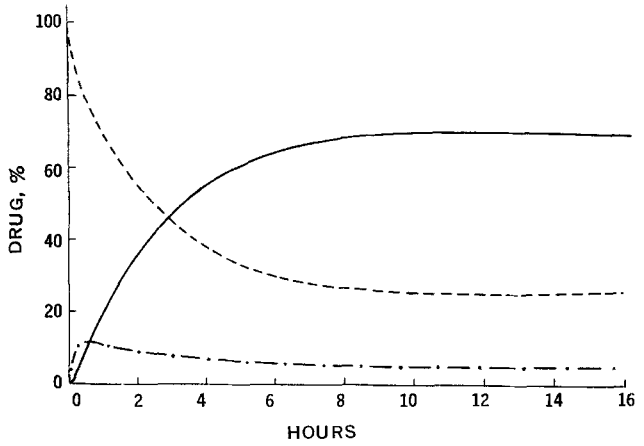

Figure 8—*Transfer of erythromycin from pH 6.8 buffer (1 l.) through toluene (0.1 l.) to pH 7.4 buffer (10 l.). Interfacial areas equal 194 cm.². Curves were obtained from the analog computer program. Key: - -, pH 6.8 buffer; –·–, toluene; and ——, pH 7.4 buffer.*

another two-phase experiment using phases *B* and *C*. Curves showing the amount of drug in each phase can then be obtained by putting these rate constants, along with desired phase volumes and interfacial areas, into the computer fitted with an X–Y recorder.

To demonstrate that rate constants from two-phase systems can be extended to this model, several three-phase runs were made and the experimental points were plotted against computer-drawn curves. The results are shown in Figs. 5–7. The experimental points followed the curves closely in all cases.

An advantage of the proposed method is that different volumes and interfacial areas can be fitted into the analog computer program. Thus, values more characteristic of those existing in the body can be used to simulate drug transport. For example, with the conditions described in Fig. 6, the percent of erythromycin in phase *C* at equilibrium is less than 10 and its $t_{1/2}$ is about 12 hr. However, if the volumes of phases *A*, *B*, and *C* are assigned 1, 0.1, and 10 l., respectively, and the interfacial area is increased 10-fold, the curves in Fig. 8 are obtained. Under these conditions, the percent of erythromycin in phase *C* at equilibrium is increased to 73 and its half-life is decreased to 2 hr. These latter conditions would be impractical to use experimentally with the three-phase apparatus reported.

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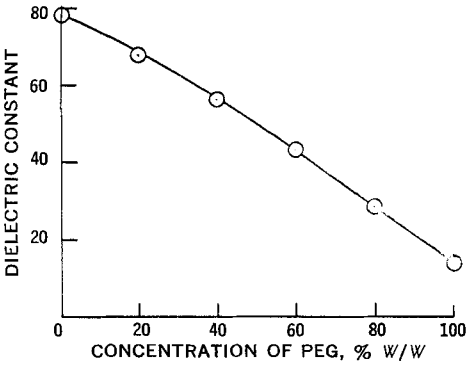


Figure 1—*Dielectric constant values of PEG–H_2O mixtures.*

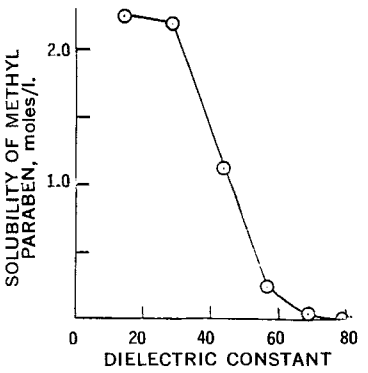


Figure 2—*Solubility of methyl paraben as a function of dielectric constant (PEG–H_2O mixtures).*

Envisioning the plausibility of physicochemical and biological applicability of solubility properties of parabens, in general, and the solubilizing potential of one paraben on the other, in particular, the authors attempted to elucidate the mechanism of solubility of parabens.

Two major influencing factors were considered to be involved in the solubility of parabens, irrespective of their use singly or in combination: (*a*) the dielectric constant of solvents or solutions in which one or two parabens were to be dissolved, and (*b*) hydrogen bonding between the ester molecules and between the solute and solvent molecules.

This communication is concerned with the evaluation of the solubility of parabens as a function of the dielectric constant. The experiments were designed to determine the dielectric constants of a number of solvents and solutions. These data were assumed to provide information leading to the elucidation of influencing factors responsible for the solubility enhancement of parabens.

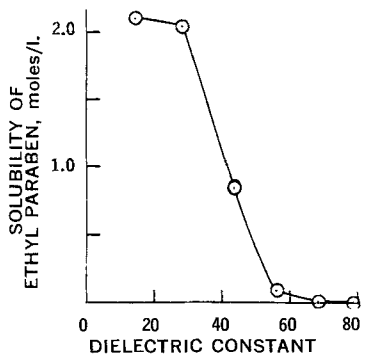
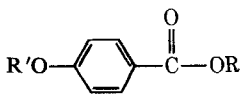


Figure 3—*Solubility of ethyl paraben as a function of dielectric constant (PEG–H_2O mixtures).*

Table II—Dielectric Constant of Various Concentrations of Paraben Solutions in 60% PEG–H_2O



Paraben R	Paraben R'	Concentration of Paraben, mole/liter 0.0	0.1	0.2	0.3	0.4	0.5
CH_3	H	43.86	43.36	43.01	42.65	42.22	41.57
C_2H_5	H	43.86	43.23	42.50	42.12	41.77	41.16
n-C_3H_7	H	43.86	43.12	42.18	41.71	40.59	40.12
n-C_4H_9	H	43.86	43.08	41.92	41.53	40.67	39.90
CH_3	CH_3	43.86	43.20				

EXPERIMENTAL

The dielectric constants of the solvents and the solutions were determined by using a Sargent chemical oscillometer, model V.

The solvents and solutions, whose dielectric constants were to be determined, were prepared by the previously reported procedure (1). The dielectric constants were determined according to the manual (2). The 10-ml. sample cell was washed with water, alcohol, and ether and dried thoroughly. The empty cell was then installed in position in the holder, and the instrument was adjusted to zero balance. Without removing the cell, the liquid under investigation was poured into the cell (about 1 cm. above the electrode). The instrument was again balanced, and the scale reading was recorded. The cell was then emptied, cleaned, and dried, and the procedure was repeated in the same manner for the next sample.

From the scale readings for two pure solvents with known dielectric constants, the cell constant, C_g, and the ratio of constants, C_g/C_0, were determined by employing the following formulas:

$$\frac{C_0}{C_g} = \frac{S_2(k_1 - 1) - S_2(k_2 - 1)}{k_1S_1(k_2 - 1) - k_2S_2(k_1 - 1)} \qquad \text{(Eq. 1)}$$

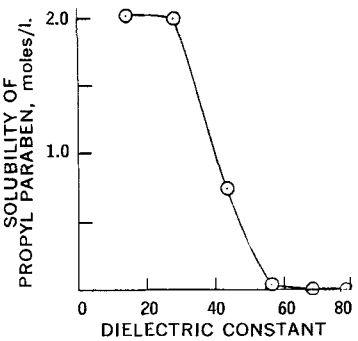


Figure 4—*Solubility of propyl paraben as a function of dielectric constant (PEG–H_2O mixtures).*

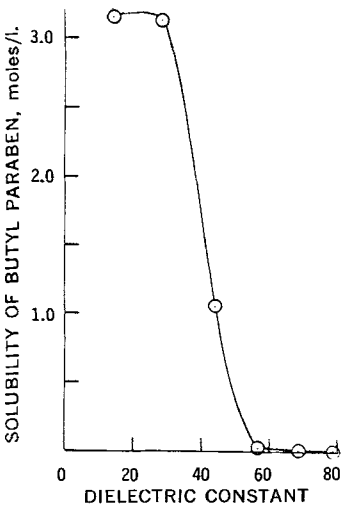


Figure 5—*Solubility of butyl paraben as a function of dielectric constant (PEG–H_2O mixtures).*

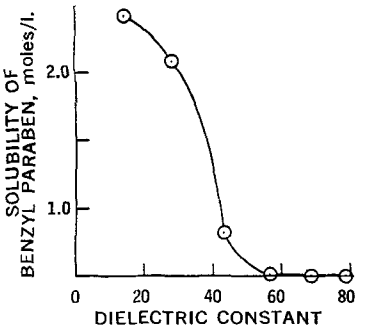


Figure 6—*Solubility of benzyl paraben as a function of dielectric constant (PEG–H_2O mixtures).*

$$C_g = \frac{S(C_g/C_0 + 1) + kS(C_0/C_g + 1)}{k - 1} \qquad \text{(Eq. 2)}$$

where C_0 = capacitance of the empty cell, C_g = capacitance of the cell with liquid, S = scale reading of a pure solvent, and k = dielectric constant.

The dielectric constant of each sample was then determined from its scale reading, using the following equation:

$$k = \frac{C_g + S(C_g/C_0 + 1)}{C_g - S(C_0/C_g + 1)} \qquad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

The dielectric constant values of various concentrations of PEG–H_2O mixtures are depicted in Table I and Fig. 1. As expected, the dielectric constant of the solvent was inversely proportional to the concentration of PEG and gives a nearly linear relationship. The linearity is exact between 20 and 60% PEG, but beyond this amount the line bends slightly toward the x-axis (concentration axis). This could possibly be due to concentration effect.

Figures 2–6 depict the solubility of five parabens (methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl paraben) individually as a function of the dielectric constant. The results coincide with those reported for other solvent systems (3, 4). All the curves have the shape of an inverted S. There seems to be an optimum solubility at the dielectric constant of about 30, beyond which there is a sharp drop in the solubility. Except for benzyl paraben, decreasing the dielectric constant value below 30 does not contribute to an increase in solubility.

The solubility features of methyl, ethyl, and *n*-propyl parabens are somewhat similar. The solubility of butyl paraben, however, differs from these three because it affords a significant increase in solubility upon adjusting the dielectric constants of the solvent to a value below 40. This difference in solubility property between butyl paraben and its lower homologs can be ascribed to the property of a long alkyl chain of the butyl group.

The change in the dielectric constant values as a function of concentration of parabens in 60% PEG–H_2O is depicted in Table II and Fig. 7. Methyl, ethyl, *n*-propyl, and *n*-butyl parabens, together with methyl *p*-methoxybenzoate, in concentrations ranging from 0.1 to 0.5 *M* were employed. The dielectric constant values decrease as the concentration values increase. The dielectric constant-lowering potential increases in an ascending order of the alkyl chain of the parabens.

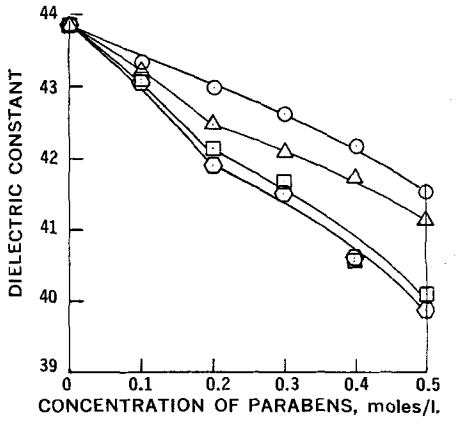


Figure 7—*Dielectric constant values of alkyl parabens in concentrations ranging from 0.1 to 0.5* M. *Key:* ⊙, *methyl;* △, *ethyl;* ⊡, n-*propyl; and* ○, n-*butyl.*

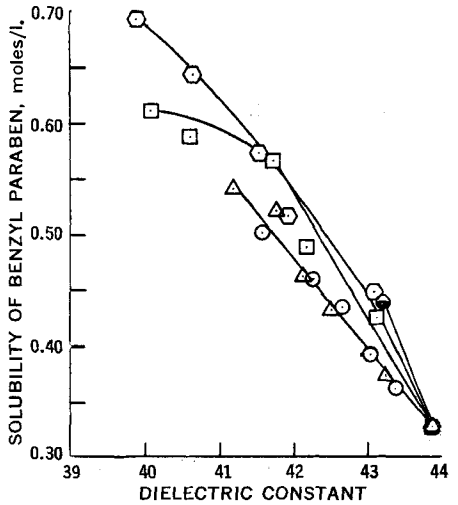
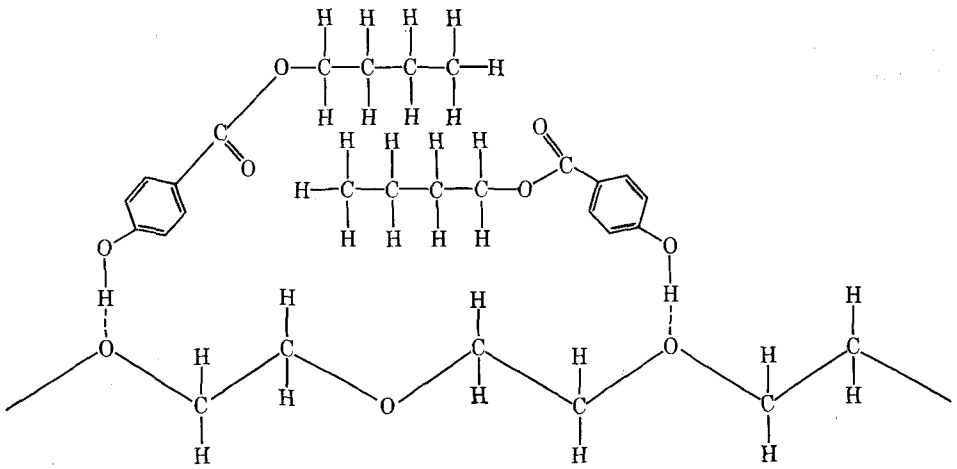


Figure 8—*Solubility of benzyl paraben in solutions of various concentrations (various dielectric constants) of alkyl parabens. Key:* ⊙, *methyl;* △, *ethyl;* ⊡, n-*propyl;* ○, n-*butyl; and* ◒, *methyl* p-*methoxy.*

The difference between the dielectric constant-lowering potential of two distant paraben homologs, methyl and butyl, is small at 0.1 *M* but increases significantly at 0.5 *M* (see Table II). This is probably



I

due to a difference in the length of the alkyl chain. Considering the preferred conformation of polyethylene glycol, the long alkyl chains of butyl parabens, whose OH are hydrogen bonded to alternate oxygens of the polyethylene glycol, can overlap and become stabilized by van der Waals' force. The semirigid structure illustrated in Structure I and/or analogously crosslinked structures, formed as a result of overlap between alkyl chains of butyl paraben, hydrogen bonded to two different polyethylene glycol molecules, can contribute to the lowering of the polarity of the medium upon increasing the concentration more than with methyl paraben, in which such linking is not possible.

The solubility of benzyl paraben in various solutions of each alkyl paraben (methyl, ethyl, *n*-propyl, and *n*-butyl) with known dielectric constants were determined. The plots are depicted in Fig. 8. The results indicate that the solubility of benzyl paraben in a solution of alkyl paraben is not solely dependent on the dielectric constant-lowering potential of the alkyl paraben. Reference to Fig. 8 and solubility values of benzyl parabens in various concentrations of alkyl parabens, reported in the previous communication (1), clarifies this contrast. The 0.4 *M* concentrations of butyl paraben and propyl paraben have dielectric constants $k = 40.59$ and $k = 40.67$, respectively. The solubility of benzyl paraben in these solutions is 0.52 and 0.59 *M*, respectively, obviously opposite of what would have been expected from their dielectric constant values. The most conspicuous results were obtained from 0.1 *M* ethyl paraben and 0.1 *M* methyl *p*-methoxybenzoate, both with $k = 43.2$, in whose solutions the solubility of benzyl paraben was found to be 0.37 and 0.44 *M*, respectively.

In summary, there exists a relationship between the dielectric constant and solubility of parabens. The solubility data of combinations of two parabens, however, indicate the involvement of factors other than dielectric constants. A theoretical consideration of these will be given in a future communication.

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Inhibited Dissolution of Drug Crystals by Certified Water-Soluble Dyes II

J. PICCOLO* and R. TAWASHI

Abstract □ Experiments on dissolution inhibition of poorly water-soluble drug crystals (sulfathiazole and diethylstilbestrol) in the presence of low concentrations of certified water-soluble dyes are presented. The effect of the degree of undersaturation and the dependence of dissolution rate on the inhibitor concentration of powder systems were investigated. Data obtained are consistent with previous findings and suggest preferential adsorption at the primary dissolution sources on the crystal surface. Further dissolution studies in the presence of 0.04 *M* sodium cholate showed that the dye caused substantial reduction in surface dissolution of the sulfathiazole single crystal. Biological implications in these systems are considered.

Keyphrases □ Dyes, water-soluble—drug crystal dissolution, inhibition □ Dissolution rates, crystals—water-soluble dye effect □ Crystals, sulfathiazole, diethylstilbestrol—dissolution rates, dye effect □ Sodium cholate effect, dissolution—crystals–dyes

In previous communications, the effect of low concentrations of FD&C Blue No. 1 on the dissolution rate of certain crystalline drugs was reported (1, 2). Concentrations of 5–10 mcg./ml. were sufficient to exert a remarkable inhibition on the surface dissolution of sulfathiazole, sulfaguanidine, and phenobarbital monohydrate single crystals. Additional dissolution experiments on compressed disks of the pure drug gave similar results. Studies made on a powder system showed a marked inhibition of the dissolution rate; however, the results of only one powder system were insufficient to draw definite conclusions.

The present work was undertaken to: (*a*) determine the influence of the dye incorporated in a compressed disk rather than dissolved in the test media; (*b*) define the manner in which undersaturation of the solution and concentration of the dye affect dissolution in powder systems; and (*c*) investigate the influence of the dye on micellar solubilization.

EXPERIMENTAL

Materials—Sulfathiazole Form I crystals were obtained using the method previously described (1). Diethylstilbestrol,[1] sodium chlorate,[1] and 0.90% NaCl solution[2] were USP grade; all other solvents were purified before use.

Dissolution-Rate Studies—Dissolution experiments to determine the influence of FD&C Blue No. 1 incorporated in a compressed disk were carried out using sulfathiazole Form I as a test material. Tablets of drug crystals alone and of drug crystals containing 5 mg. of the dye were compressed under the same pressure (Fig. 1). The dissolution rate was determined using a method described in a previous communication (1).

To substantiate earlier results obtained in the surface dissolution studies, a series of experiments was carried out on a powder system using diethylstilbestrol as a model substance. The Coulter counter method, described by Higuchi and Saad (3, 4) and by Edmundson and Lees (5), was used in these experiments. A suspension of diethylstilbestrol was prepared using the following method: 1 ml. of ethyl alcohol containing 10 mg. of diethylstilbestrol was added to 150 ml. of 0.90% NaCl solution. Magnetic stirring was maintained for 2 hr. The suspended crystals were then sonified for 2 hr. (using a Sonifier Cell Disruptor model W, 140 D)[3] to get a particle size

[1] Obtained from Matheson Coleman & Bell Inc., East Rutherford, N. J.

[2] Normal saline for injection. Abbott Laboratories, Montreal, Canada.

[3] Ultrasonic Inc., Plainview, N. J.

due to a difference in the length of the alkyl chain. Considering the preferred conformation of polyethylene glycol, the long alkyl chains of butyl parabens, whose OH are hydrogen bonded to alternate oxygens of the polyethylene glycol, can overlap and become stabilized by van der Waals' force. The semirigid structure illustrated in Structure I and/or analogously crosslinked structures, formed as a result of overlap between alkyl chains of butyl paraben, hydrogen bonded to two different polyethylene glycol molecules, can contribute to the lowering of the polarity of the medium upon increasing the concentration more than with methyl paraben, in which such linking is not possible.

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Abstract □ Experiments on dissolution inhibition of poorly water-soluble drug crystals (sulfathiazole and diethylstilbestrol) in the presence of low concentrations of certified water-soluble dyes are presented. The effect of the degree of undersaturation and the dependence of dissolution rate on the inhibitor concentration of powder systems were investigated. Data obtained are consistent with previous findings and suggest preferential adsorption at the primary dissolution sources on the crystal surface. Further dissolution studies in the presence of 0.04 *M* sodium cholate showed that the dye caused substantial reduction in surface dissolution of the sulfathiazole single crystal. Biological implications in these systems are considered.

Keyphrases □ Dyes, water-soluble—drug crystal dissolution, inhibition □ Dissolution rates, crystals—water-soluble dye effect □ Crystals, sulfathiazole, diethylstilbestrol—dissolution rates, dye effect □ Sodium cholate effect, dissolution—crystals–dyes

In previous communications, the effect of low concentrations of FD&C Blue No. 1 on the dissolution rate of certain crystalline drugs was reported (1, 2). Concentrations of 5–10 mcg./ml. were sufficient to exert a remarkable inhibition on the surface dissolution of sulfathiazole, sulfaguanidine, and phenobarbital monohydrate single crystals. Additional dissolution experiments on compressed disks of the pure drug gave similar results. Studies made on a powder system showed a marked inhibition of the dissolution rate; however, the results of only one powder system were insufficient to draw definite conclusions.

The present work was undertaken to: (*a*) determine the influence of the dye incorporated in a compressed disk rather than dissolved in the test media; (*b*) define the manner in which undersaturation of the solution and concentration of the dye affect dissolution in powder systems; and (*c*) investigate the influence of the dye on micellar solubilization.

EXPERIMENTAL

Materials—Sulfathiazole Form I crystals were obtained using the method previously described (1). Diethylstilbestrol,[1] sodium chlorate,[1] and 0.90% NaCl solution[2] were USP grade; all other solvents were purified before use.

Dissolution-Rate Studies—Dissolution experiments to determine the influence of FD&C Blue No. 1 incorporated in a compressed disk were carried out using sulfathiazole Form I as a test material. Tablets of drug crystals alone and of drug crystals containing 5 mg. of the dye were compressed under the same pressure (Fig. 1). The dissolution rate was determined using a method described in a previous communication (1).

To substantiate earlier results obtained in the surface dissolution studies, a series of experiments was carried out on a powder system using diethylstilbestrol as a model substance. The Coulter counter method, described by Higuchi and Saad (3, 4) and by Edmundson and Lees (5), was used in these experiments. A suspension of diethylstilbestrol was prepared using the following method: 1 ml. of ethyl alcohol containing 10 mg. of diethylstilbestrol was added to 150 ml. of 0.90% NaCl solution. Magnetic stirring was maintained for 2 hr. The suspended crystals were then sonified for 2 hr. (using a Sonifier Cell Disruptor model W, 140 D)[3] to get a particle size

[1] Obtained from Matheson Coleman & Bell Inc., East Rutherford, N. J.

[2] Normal saline for injection. Abbott Laboratories, Montreal, Canada.

[3] Ultrasonic Inc., Plainview, N. J.

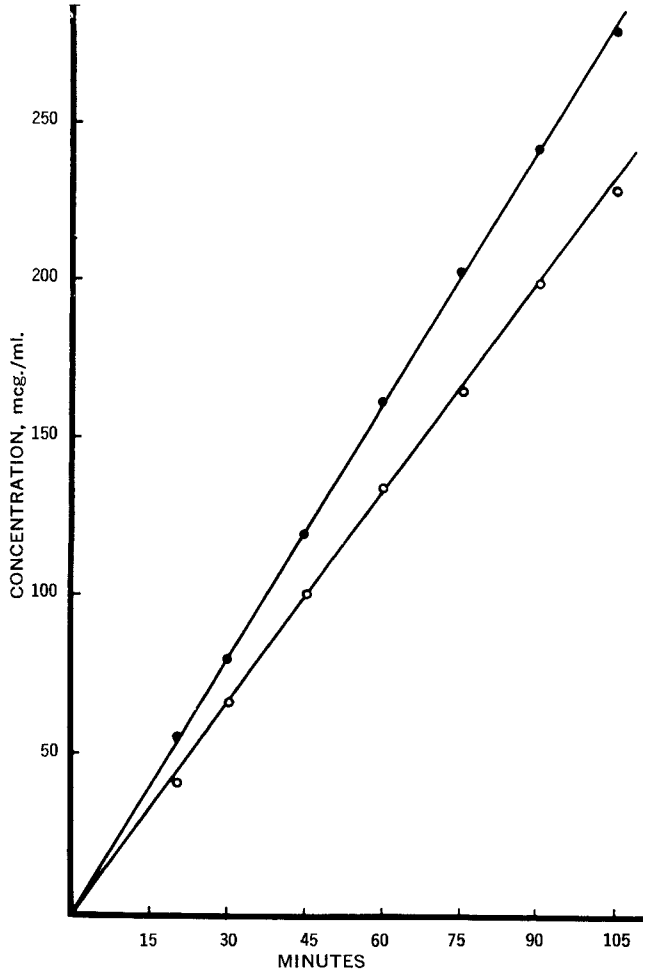

Figure 1—*Dissolution behavior of sulfathiazole compressed disks. Key: ●, compressed disks of drug crystals alone; and ○, compressed disks of drug crystals containing 5 mg. FD&C Blue No. 1.*

suitable for the Coulter counter technique. The resulting crystals were in the range of 1–2 μ in diameter.

The Coulter counter was calibrated with polystyrene latex, having a diameter of 1.305 μ, using a 30-μ aperture tube. For each experiment, 15 ml. of the diethylstilbestrol suspension was added to 135 ml. of 0.90% NaCl solution. Stirring was provided during and after addition of the aliquot. The data were plotted as number of particles *versus* time at various threshold settings, each threshold setting corresponding to a certain diameter (Fig. 2). From these graphs, other sets of curves were constructed, showing the number of particles above given size *versus* the diameter for different times (Fig. 3). The rate of change in radius (dr/dt) was then determined for the powder system in the absence and in the presence of the dye.

The influence of Blue No. 1 on the solubilizing effect of sodium cholate was studied on sulfathiazole single crystals. The dissolution rate of the single crystals was determined in distilled water, in a 0.04 *M* solution of sodium cholate, and in a 0.04 *M* solution of sodium cholate containing 5 mcg./ml. of FD&C Blue No. 1. The procedure used to determine the dissolution rate of the single crystals was identical to that described in a previous communication (1).

RESULTS AND DISCUSSION

Figure 1 shows the dissolution behavior of compressed disks of sulfathiazole crystals alone and of sulfathiazole crystals containing 5 mg. of FD&C Blue No. 1. A significant dissolution inhibition by the action of the incorporated dye is present, as in the previous study where the dye was present in the dissolution media.

Since most pharmaceutical systems contain a large number of solutes that should act in a similar manner, in varying degrees, toward dyes, the study of the effect of the dye on a powder system with high surface area would be of major interest. Figures 2 and 3 summarize the data obtained with diethylstilbestrol microcrystals suspended in normal saline. A concentration of 10 mcg./ml. of Blue No. 1 (triphenylmethane dye) was sufficient to reduce the dissolution rate of diethylstilbestrol from 6.00×10^{-8} cm./sec. to 2.4×10^{-8} cm./sec.

Additional experiments using Red No. 3 (a fluorescein dye) gave a marked inhibition of the dissolution rate in the same system. Figure 4 permits a comparison between the relative dissolution rates obtained with FD&C Blue No. 1 and Red No. 3. The marked decrease in dissolution rate at such a low concentration strengthens

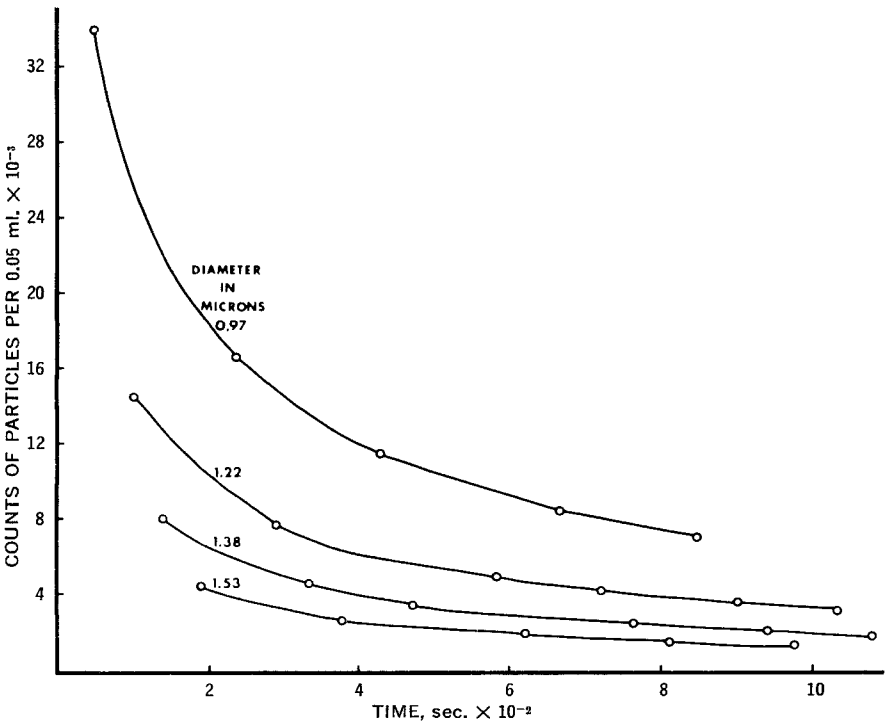

Figure 2—*Coulter counter data showing the decrease in cumulative counts with time at various threshold settings as diethylstilbestrol particles dissolve in 0.9% NaCl solution.*

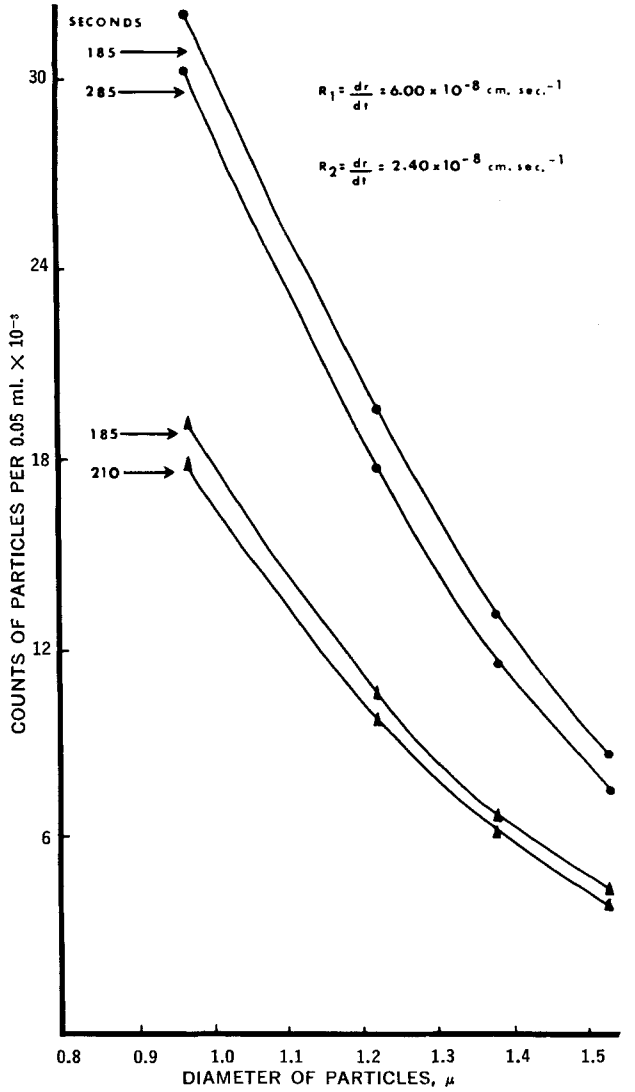

Figure 3—*Cumulative particle-size distribution curves constructed from Coulter counter data. Key: ▲, in 0.90% NaCl solution; and ●, in 0.90% NaCl solution containing 10 mcg./ml. FD&C Blue No. 1.*

the possibilities that these dyes play an important part in the dissolution step of the drug-absorption process.

Studies confined to surface dissolution of the single crystal in the presence of dyes gave results that might appear dramatic to the user of polycrystalline material. The application of the Coulter counter method to study crystal poisoning of diethylstilbestrol microcrystals at different saturation ratios and with different inhibitor concentrations gave good agreement with surface dissolution measurements. For instance, Fig. 5 shows the influence of undersaturation on the dissolution inhibition of diethylstilbestrol. A concentration of 10 mcg./ml. of the dye reduced the dissolution rate to an unmeasurable value at a fraction of the saturation condition. This result agrees well with those of Ives and Plewes (6) in their study on lithium fluoride surface dissolution using ferric ion as an inhibitor.

The study of dissolution-rate dependence on inhibitor concentration gave similar kinetics to single-crystal work. Data in Fig. 6 show the effect of dye concentration on the dissolution rate of diethylstilbestrol microcrystals in normal saline. The curve tends to stabilize after a concentration of 50 mcg./ml. of Blue No. 1, and it agrees well with previous findings on sulfaguanidine single crystal (1). Those results were discussed on the basis of the kink mechanism, proposed by Gilman *et al.* (7) and Ives (8), which involves preferred adsorption of the dye molecules on the primary dissolution sources of the crystal surface. Such selective adsorption could also explain the dissolution inhibition of diethylstilbestrol microcrystals in the

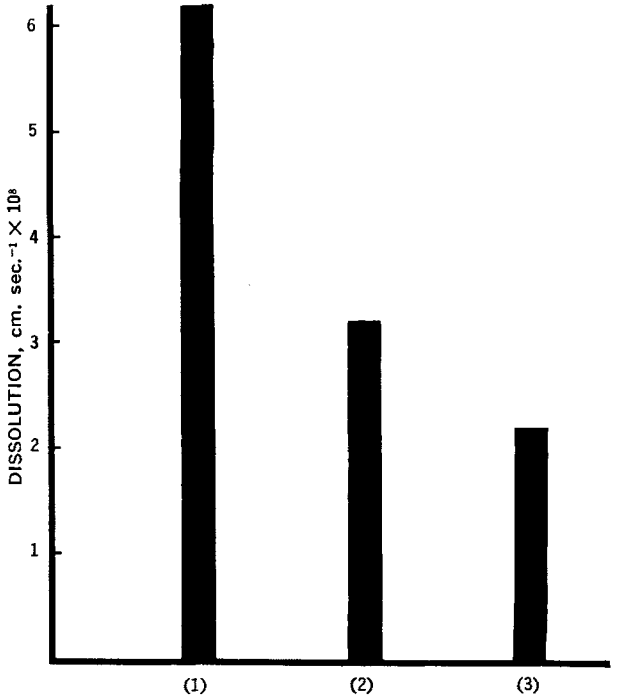

Figure 4—*Influence of water-soluble dyes on the dissolution rate of diethylstilbestrol. Key: (1) dissolution in 0.90% NaCl solution; (2) dissolution in 0.90% NaCl solution containing 10 mcg./ml. FD&C Red No. 3; and (3) dissolution in 0.90% NaCl solution containing 10 mcg./ml. FD&C Blue No. 1.*

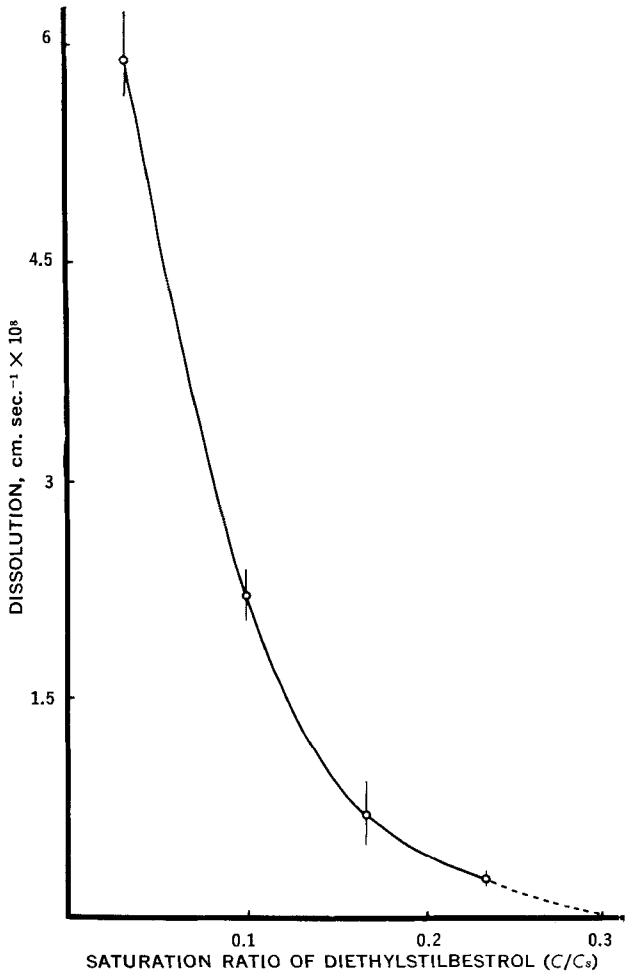

Figure 5—*Effect of undersaturation on the dissolution rate of diethylstilbestrol in the presence of 10 mcg./ml. FD&C Blue No. 1.*

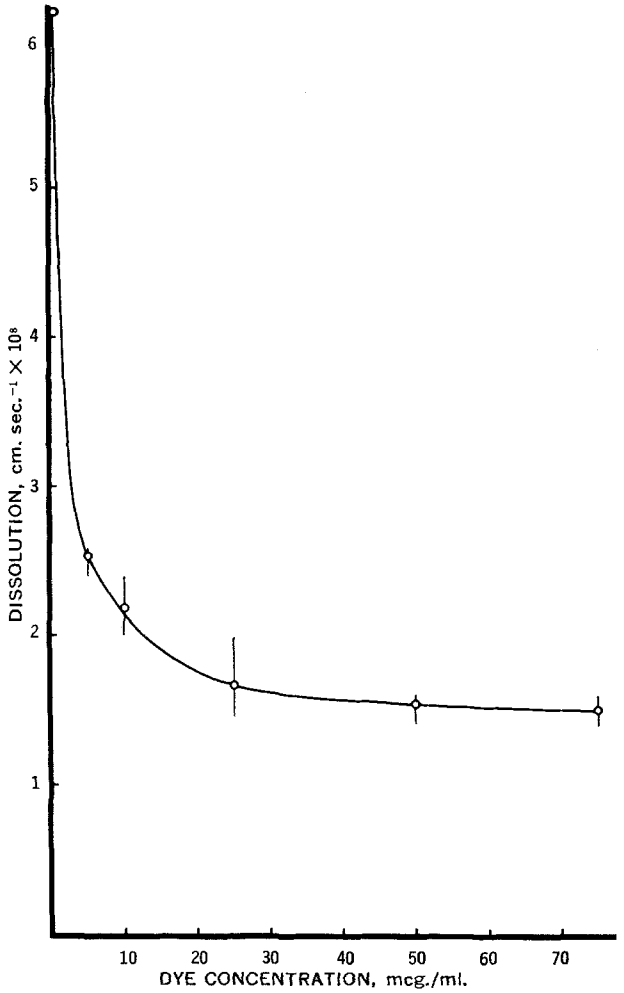

Figure 6—*Effect of FD&C Blue No. 1 concentration on the dissolution rate of diethylstilbestrol crystalline powder.*

presence of low concentrations of the dye. By applying the relationship between the relative differences in dissolution rates and the inhibitor concentration, a correlation can be suggested for this situation. If it is assumed that: (*a*) the fractional rate reduction caused by the dye is proportional to the fraction of the surface covered by the adsorbed dye, and (*b*) the dye adsorption can be represented by a simple Langmuir isotherm (9), the dissolution rate can be expressed as a simple function of the dye concentration in solution:

$$R = R_0\left(1 - \frac{KbC}{1 + bC}\right) \qquad \text{(Eq. 1)}$$

where R = dissolution rate with dye, R_0 = dissolution rate without dye, K = fraction of surface covered when the surface is "saturated" with dye, C = dye concentration in bulk solution, and b = Langmuir isotherm constant.

This relationship was applied to diethylstilbestrol, using the data in Fig. 5, and to sulfaguanidine single crystals reported in a previous communication (1). These results can be represented by such a correlation within the limits of experimental accuracy (Fig. 7). The values of the constants K and b are given in Table I.

The b values are indicative of adsorption intensity, and K is a measure of the saturation capacity of the surface for FD&C

Table I—Adsorption and Dissolution Inhibition Constants for FD&C Blue No. 1 on Diethylstilbestrol and Sulfaguanidine

Material	K	b
Diethylstilbestrol (microcrystalline powder)	0.77	4.79
Sulfaguanidine (single crystal)	0.61	0.76

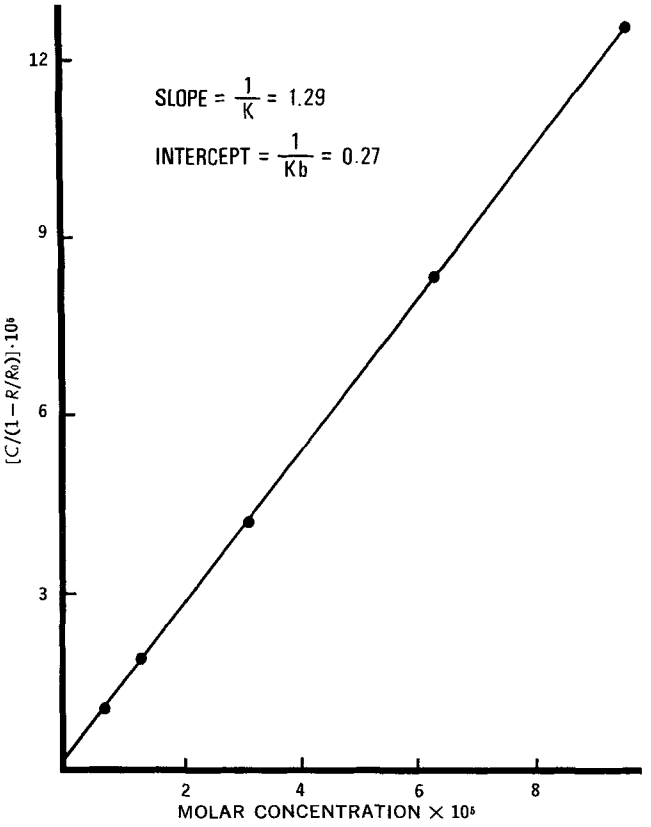

Figure 7—*Determination of adsorption and dissolution-inhibition constants for diethylstilbestrol in the presence of FD&C Blue No. 1.*

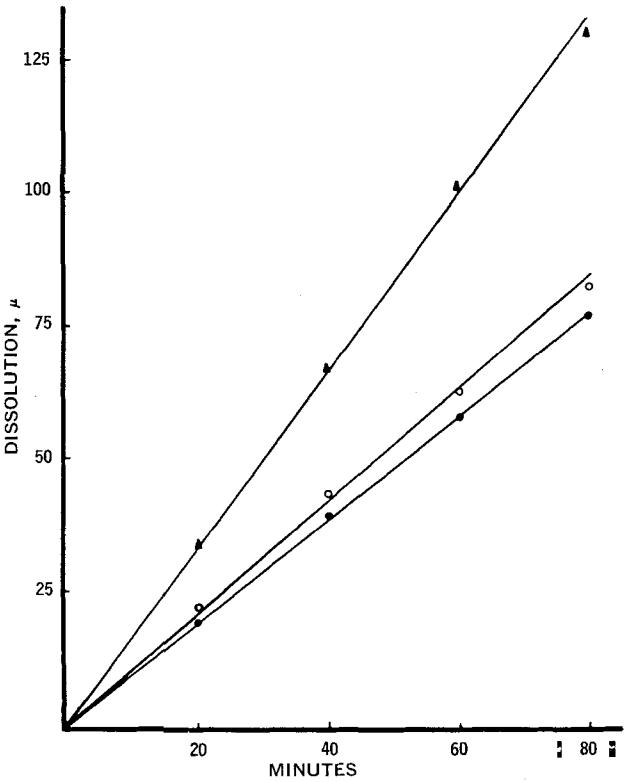

Figure 8—*Single-crystal dissolution of sulfathiazole as a function of time. Key: ●, in distilled water; ▲, in 0.04 M solution of sodium cholate; and ○, in 0.04 M solution of sodium cholate containing 5 mcg./ml. FD&C Blue No. 1.*

Blue No. 1. The fact the K values are smaller than 1.0 for both drugs supports a selective adsorption mechanism rather than complete coverage of the crystal surfaces by the dye.

It has been reported (10–12) that bile salts increase the solubility of poorly soluble drugs by micellar solubilization. Consequently, the dissolution rate is expected to increase in the presence of sodium cholate. The influence of Blue No. 1 on the solubilizing effect by 0.04 M sodium cholate was studied using sulfathiazole Form I (single crystal) as the model substance. Data obtained in Fig. 8 show that Blue No. 1 modified markedly the dissolution kinetics of sulfathiazole in sodium cholate solution. A concentration of 5 mcg./ml. brought the dissolution rate to a value very close to that in distilled water. The solubility of sulfathiazole at 25° was found to be 0.85 mg./ml. in both 0.04 M sodium cholate and in 0.04 M sodium cholate containing 5 mcg./ml. Blue No. 1. In distilled water, the solubility was 0.47 mg./ml. Therefore, the dissolution of sulfathiazole in the cholate–dye system does not follow the laws predicted by simple diffusion kinetics and suggests an interfacial mechanism.

This observation is extremely interesting in relation to the intestinal absorption process in the presence of water-soluble dyes. More studies are necessary for complete characterization of this problem from the physical-chemical standpoint. Such studies will be generally important to the eventual understanding of the role of water-soluble dyes in dissolution and drug transport.

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* MRC graduate research studentship.

Survey of a Laboratory Building for Airborne Antibiotics

MARY ANN GARTH, HENRY BRYANT, JULIAN KRAMER, and AMIEL KIRSHBAUM

Abstract □ Antibiotics are routinely examined by laboratory analysis in a six-story building which houses the laboratories of the National Center for Antibiotics and Insulin Analysis (NCAIA) on the second floor. A survey was performed to determine if antibiotic dust was being disseminated throughout the building. Air throughout the building was analyzed both quantitatively and qualitatively for residues of penicillin and tetracyclines. The microbiological assay plates used were sensitive enough to detect 0.001 unit of penicllin and 0.0004 mcg. of chlortetracycline in the air. Results of this study showed that, outside of the actual laboratories of NCAIA, the incidence of antibiotic contamination of the air was negligible. In the immediate areas where antibiotics were physically handled, 0.012 unit of penicillin and 0.0185 mcg. of chlortetracycline per cubic foot of air were detected.

Keyphrases □ Antibiotic, airborne dissemination—laboratory building □ Cultures, plate—antibiotic detection □ Air sampling—vacuum collection through membrane □ Laboratory contamination—antibiotics, airborne

As the use of chemicals becomes more diversified in modern life, the extent to which these substances contaminate the environment becomes a matter of increasing concern. Similarly, for some years, effort has been directed toward the study of the incidence of microbial contamination of the environment, especially in hospitals (1, 2), and the control of this contamination. Recently, attention has been directed toward microbe-free environments for the assembly of aerospace equipment (3, 4).

Work in the laboratories of the National Center for Antibiotics and Insulin Analysis (NCAIA) involves the possibility of a special kind of contamination. Approximately 100 chemists and microbiologists perform laboratory analyses of antibiotic drugs. There is little information concerning the extent to which these antibiotics may permeate the atmosphere as the result of manipulations in the course of analysis. Welch *et al.* (5) demonstrated that a group of antibiotics analysts carried nasal staphylococci possessing a significantly higher incidence of antibiotic resistance than did control groups. The antibiotic resistance was attributed to antibiotic dusts and aerosols, but the amount of antibiotics in the air of the laboratory was not determined. The authors, therefore, surveyed the atmosphere of laboratories and of the entire building in which they are housed for the presence of two of the most frequently tested groups of antibiotics: penicillins and tetracyclines.

NCAIA is located mainly on the second floor of the U. S. Food and Drug Administration (FDA) Building in Washington, D. C.; at the time of this study, it also had ancillary laboratory space on the first and third floors, used for penicillin contamination and for turbidimetric assays, respectively. This modern well-equipped laboratory building consists of six floors, a basement, and a subbasement. All air enters by way of five separate air-conditioning systems using 15 induction fans

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Figure 1—*Vacuum system used in the air sampling techniques.*

and leaves with the aid of 56 exhaust fans. There is no recirculation of air. Roll-type filters are used for filtering incoming air. Each room is equipped with induction and exhaust systems and is closed off from adjoining rooms by swinging doors. The total average airflow for the entire building is 683,000 cu. ft./min. for the air conditioning and 700,000 cu. ft./min. for the exhaust system. The average relative humidity is approximately 47% throughout the building.

The study was divided into three phases. The first was a passive fallout test intended to determine the incidence, if any, of antibiotic dust in representative areas of the building. The second was a vacuum filtration of air in areas in which positive results were found in the first phase, other than NCAIA laboratories. In the third phase, every room of NCAIA was monitored for antibiotic fallout, and then quantitative determinations were made of antibiotics per volume of air in those rooms found positive.

MATERIALS AND METHODS

Test Organisms—Since the penicillins and tetracyclines constitute the largest proportion of drugs tested and were therefore the largest source of possible contamination, the test organisms selected were those most sensitive to these antibiotics, namely, *Sarcina lutea* (ATCC 9341) and *Bacillus cereus* var. *mycoides* (ATCC 11778). Zones of inhibition are produced when as little as 0.001 unit of penicillin or 0.0004 mcg. of chlortetracycline (CTC) comes in contact with an agar surface inoculated with test organisms. CTC was selected as a representative of the tetracycline group of antibiotics.

Identification of Penicillin—Penicillinase, an enzyme, will reduce or completely inactivate the antibacterial activity of penicillin. This enzyme was incorporated in some of the test systems to determine if any antibacterial activity observed was, indeed, penicillin.

Tests Plates—*Fallout Plates*—Plates were prepared by adding 8 ml. of agar inoculated with one test organism into each of 90 plastic Petri dishes, 100 × 15 mm. Some plates of each type were modified by the addition of 0.5 ml. of penicillinase to the melted inoculated agar.

Penicillin Plates (*P Plates*): Yeast-Beef Agar (Medium 4), prepared according to CFR Title 21, Section 141.103, was inoculated with 0.1% of an *S. lutea* suspension, prepared according to CFR Title 21, Section 141.104.

CTC Plates (*T Plates*): Base Agar, pH 5.9 (Medium 8), prepared according to CFR Title 21, Section 141.103, was inoculated with 0.1% of a *B. cereus* spore suspension prepared according to CFR Title 21, Section 141.104.

Fallout Plates for Sterile Areas—To avoid microbial contamination of sterile areas, uninoculated fallout plates were used for these locations. Six milliliters of the appropriate uninoculated agar was placed in Petri dishes. These plates were exposed in the respective sterile areas and then covered and returned to the assay laboratory. There the base agar was overlaid with 4 ml. of inoculated agar.

Cylinder Plates—Cylinder plate assays were performed as described in CFR Title 21, Part 141, Subpart B, using the following buffers: (*a*) for penicillin assay, 1% pH 6.0 potassium phosphate buffer (CFR Title 21, Section 141.102, Solution 1); and (*b*) for CTC assay, 0.1 *M* pH 4.5 potassium phosphate buffer (CFR Title 21, Section 141.102, Solution 4).

Air Sampling Equipment—A vacuum system (Fig. 1) was used with a membrane filter with a porosity of 0.22 μ. The filter was placed in a clean support system connected to a vacuum pump through an airflow meter.

Controls—*Negative Control of Fallout Plates*—Each time plates were exposed, a plate prepared in the same way as the exposed plate but left covered was used as a negative control.

Positive Control of Fallout Plates—Each day that plates were exposed, tests were performed to determine their sensitivity to penicillin and tetracycline, as follows:

1. Sodium penicillin G working standard, prepared according to CFR Title 21, Section 141.110(b), was diluted in water to make concentrations of 0.2, 0.1, and 0.05 unit/ml.
2. Chlortetracycline hydrochloride working standard, prepared according to CFR Title 21, Section 141.110(b), was diluted in water to make concentrations of 0.08, 0.04, and 0.02 mcg./ml.

Next, 0.01-ml. portions of each of the penicillin standard solutions were placed directly on the surfaces of duplicate *S. lutea* plates. Similarly, CTC standard solutions were placed on the surface of two *B. cereus* plates. The amounts used were thus 0.002, 0.001, and 0.0005 unit of penicillin and 0.0008, 0.0004, and 0.0002 mcg. of CTC.

Negative Filtration Control—As a negative control, an area was selected that was negative for activity in Phase I. The filtration system was used exactly as for the other sites.

Positive Filtration Control—An area where antibiotic drugs were actively handled was used as the positive control.

Recovery Controls—Membrane filters were spotted with solutions containing varying known amounts of penicillin or CTC. These filters were extracted and the extracts assayed.

EXPERIMENTAL

Phase I—Passive Fallout Phase—The presence of antibiotics in the atmosphere of the building was first determined by exposing the surface of a number of agar plates to fallout from the air. Plates were exposed for 1 hr. in at least five different locations on each of seven floors of the building excluding the subbasement. One plate of each type was exposed at each site. On each of 4 days, one set of plates was exposed in the late morning after work had commenced and another set was exposed in the late afternoon. Figure 2 shows the typical location of plates on one floor. In some rooms where antibiotics were handled, additional plates containing agar with penicillinase added were exposed. After the exposure, all plates were incubated overnight at 30°.

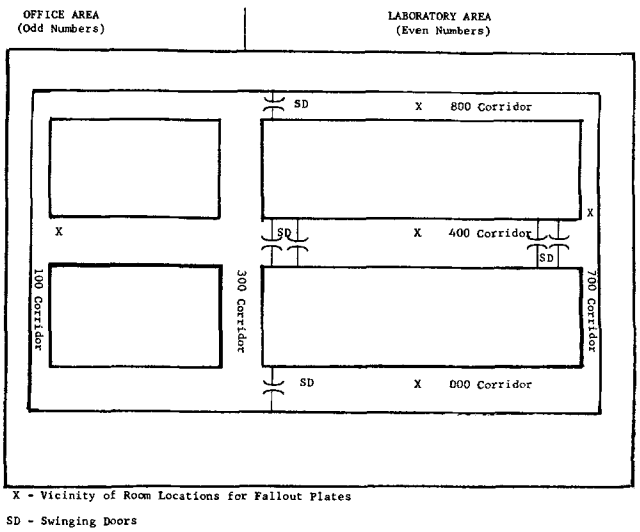

Figure 2—*Location of fallout plates.*

Table I—Antibiotic Fallout: Number of Zones of Inhibition per Plate (Phase I)[a]

Location	Oct. 26 A.M. P	Oct. 26 A.M. T	Oct. 26 P.M. P	Oct. 26 P.M. T	Oct. 31 A.M. P	Oct. 31 A.M. T	Oct. 31 P.M. P	Oct. 31 P.M. T	Nov. 7 A.M. P	Nov. 7 A.M. T	Nov. 7 P.M. P	Nov. 7 P.M. T	Nov. 14 A.M. P	Nov. 14 A.M. T	Nov. 14 P.M. P	Nov. 14 P.M. T	Total
Basement																	
B305	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
B433A	1	0	0	0	1	0	—	—	5	0	3	0	2	0	2	0	14
B042), B672), B756)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
First floor																	
1056	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1758	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1464), 1820), 1823)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Second floor																	
2018	N	2	3	3	0	2	N	0	6	0	4	2	1	1	0	0	24 + 2N
2034	—	—	—	—	N	2	N	10	9	3	N	4	4	4	N	4	38 + 4N
2034[b]	—	—	—	—	4	N	0	0	—	—	2	0	0	6	1	5	18 + 1N
2090	0	2	0	0	1	2	3	0	0	0	0	0	0	0	3	6	17
2159	0	0	0	0	0	0	0	0	0	0	0	0	3	0	4	0	7
2417	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2
2426	0	0	1	1	0	0	1	1	0	0	0	1	0	0	0	0	5
2756	—	—	—	—	0	14	0	2	1	0	0	1	0	9	0	4	31
2756[b]	—	—	—	—	—	—	—	—	—	—	—	—	0	4	0	3	7
2804	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	2
2884	1	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	7
Third floor																	
3470	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2
3052), 3321), 3736), 3832)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fourth floor																	
4434	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
4762	0	0	0	0	0	0	0	0	—	—	0	0	1	0	0	0	1
4048), 4415), 4834)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fifth floor																	
5417	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	2
5046), 5758), 5807), 5852)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sixth floor																	
6415	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
6426	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	3
6840	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	2
6018), 6747)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] P = *S. lutea* plates (for detecting penicillin), T = *B. cereus* plates (for detecting antibiotics of the tetracycline group), N = numerous zones, unable to count, and — = not done. [b] Penicillinase plates.

Phase II—Vacuum Sampling of Air at Sites Outside of NCAIA—The locations selected for this part of the study were those which had shown positive results in the first phase, other than NCAIA laboratories. Measured volumes of air were sampled. Air was allowed to flow through the filter for approximately 1 hr. Flow meter and vacuum pump meter readings were recorded at the beginning and end of each sampling period. The length of the sampling period was noted and used with the airflow data to calculate the total volume of air filtered.

Two filters were exposed at each test site, one immediately fol-

Table II—Antibiotics Found by Air Filtration (Phase II)

Date	Room	Penicillin cu. ft. Tested	Penicillin Number of Zones	Tetracyclines cu. ft. Tested	Tetracyclines Number of Zones
11/29	B433A	34	Numerous	35	1
11/29	B305	34	Negative	33	Negative
12/7	1056	33	Negative	32	Negative
12/8	1758	32	Negative	29	Negative
12/8	3470	33	Negative	32	Negative
12/12	4762	33	Negative	32	Negative
12/14	4434	33	Negative	33	Negative
12/14	5417	32	Negative	33	Negative
12/18	6426	31	Negative	30	Negative
12/18	6415	30	Negative	30	Negative
12/19	6840	32	Negative	31	Negative
11/22	2018 (positive control)	32	Numerous	32	Negative
11/24	4415 (negative control)	35	Negative	32	Negative

Table III—Number of Antibacterial Spots Found in Fallout Plates in NCAIA Area (Phase III)[a]

Room	Day 1 P	Day 1 T	Day 2 P	Day 2 T	Room	Day 1 P	Day 1 T	Day 2 P	Day 2 T
1016	0	0	2	0	2468	1	0	1	0
2010	N	N	N	N	2472	2	0	0	0
2012	N	N	N	N	2474	0	0	0	0
2014	N	3	10	4	2476	7	1	0	0
2016	1	2	4	0	2720	3	3	4	N
2018	2	0	2	0	2732	0	0	0	0
2024	6	4	4	2	2740	2	1	N	0
2026	N	0	N	0	2756	0	2	0	1
2032	12	7	3	2	2766	3	1	0	0
2034	9	10	N	12	2768	0	0	1	0
2042	5	6	N	11	2772	0	0	0	0
2046	0	0	0	0	2803	0	0	0	0
2058	0	1	0	0	2804	1	1	4	4
2062	1	0	4	0	2813	0	0	4	1
2066	1	0	N	0	2819	0	0	1	0
2074	8	1	6	0	2821	2	0	0	0
2082	0	2	1	0	2825	0	0	0	0
2086	1	2	1	2	2826	0	0	0	1
2090	0	0	0	1	2827	0	0	0	0
2159	0	0	0	0	2831	0	1	2	0
2161	0	0	0	0	2846	0	1	0	0
2169	0	0	0	0	2850	2	17	0	0
2410	1	0	0	0	2858	1	0	3	0
2414	1	3	1	0	2874	0	0	0	0
2415	N	8	N	5	2884	0	0	0	0
2420	0	0	0	0	3054	0	9	0	0
2422	10	1	2	0	3058	0	0	0	0
2426	0	0	0	0	3062	0	0	1	1
2427	0	0	0	0	3068	0	0	8	0
2432	0	0	0	0	3070	0	1	3	2
2434	0	0	0	0	3074	0	0	0	0
2438	1	0	0	0	Totals	84	88	74	49
2448	1	0	2	0		+5N	+2N	+8N	+3N

[a] P = *S. lutea* plates, T = *B. cereus* plates, and N = numerous, unable to count.

lowing the other. The setup was changed after each exposure. The filter was removed for testing and the rest of the equipment was thoroughly washed before reuse. Each filter was placed directly on the agar surface of the appropriate test plate. One filter from each location was placed on a P plate; the other from the same location was placed on a T plate. The plates were then incubated overnight at 30°. Negative and positive filtration controls were also processed.

Phase III—Measurements in Every NCAIA Room—Phase III was divided into two tests. First, fallout plates, prepared as in Phase I, were placed in every NCAIA room and laboratory. Then a quantitative air-filtration study was undertaken as a followup to the fallout results in areas where positive results were most consistently obtained.

Two air filtrations were done at each test site, one to determine penicillin and the other to determine tetracycline activity. After filtration the membrane filter was removed from the holder and extracted with 4.0 ml. of the appropriate buffer by shaking with a wrist-action shaker for 30 min. If the resultant solution was to be assayed for tetracycline activity, penicillinase was added to inactivate any penicillin that might have been picked up in the filtration. If the solution was to be tested for penicillin, the solution was divided into two aliquots; 0.5 ml. of penicillinase was added to one aliquot and the other was left untreated. Both aliquots were then tested. A positive test result for the untreated aliquot and a negative result for the aliquot treated with penicillinase identified the positive result as penicillin.

Table IV—Amounts of Antibiotics Found in Areas with Most Spots of Fallout Plates in NCAIA (Phase III)

Date	Room	Penicillin cu. ft.	Penicillin unit/cu. ft.	Tetracyclines cu. ft.	Tetracyclines mcg./cu. ft.
1/29	2010	35	0.009	32	0.0185
1/30	2012	31	0.003	32	0.003
1/30	2014	33	0.012	33	0.002
2/6	2026	32	0.004	33	0
2/6	2034	33	0.012	32	0
2/8	2042	32	0.009	32	0
2/8	2415	34	0	32	0
2/9	2720	32	0.0025	32	0
2/9	2740	33	0	32	0

RESULTS

In Phase I, as shown in Table I, numerous zones of inhibition were found in plates exposed in laboratories where antibiotics were regularly tested (second floor).

To determine qualitatively the incidence of airborne penicillin in a room where large amounts of antibiotics were handled, some plates were exposed with penicillinase added to the agar. On plates exposed in Room 2032, a reduction in the number of zones was observed in plates containing penicillinase. Of 10 plates without penicillinase exposed in Room 2032, four had so much antibiotic fallout that no bacterial growth occurred, and the other six had a total of 38 zones. In the 10 plates with the enzyme added, one plate had too many zones to count, but a total of only 18 zones was found in the other nine plates.

Zones were found only infrequently in plates exposed in other areas of the building, even in those where antibiotics were also handled (first and third floors). The only exception to this was Room B433A, a cleanup area servicing the entire building. Soap powders, antiseptics, and antibiotic-contaminated glassware were continuously processed there. Both test organisms are, to some degree, susceptible to antiseptics and most antibiotics.

The sensitivities to the test antibiotics were uniform throughout the testing periods. *S. lutea* sensitivity to penicillin was 0.001 unit, and *B. cereus* sensitivity to CTC was 0.0004 mcg.

In Phase II, recovery experiments indicated that when 0.06 unit of penicillin or 0.024 mcg. of CTC activity was placed on a membrane, 99% could be recovered. Table II shows that all areas in which some antibiotic fallout had been detected were negative when tested by air filtration, with the exception of Room B433A. Further testing was performed near this room. Air was sampled for 2.25 hr. with 72 cu. ft. of air passing through the membrane filter. The solution obtained after extraction of this filter was divided into two portions, and penicillinase was added to one portion. The solution was negative for the tetracyclines but positive for penicillin, as demonstrated by inactivation with penicillinase. The total activity on the filter was equivalent to 0.08 unit of penicillin G.

As shown in Table III, some air contamination was detected as fallout in areas where antibiotics were actively handled in operations such as weighing of antibiotic powders and blending of solutions. Table IV lists the results of filtration sampling of the areas indicated by fallout plates to have antibiotics in the air. The highest concentrations detected were equivalent to 0.012 unit of penicillin and 0.0185 mcg. of CTC per cu. ft.

In Phases I and III, a total of 378 plates was exposed on the second floor. Of these, 153 were positive and showed 426 countable particles, with 25 plates containing too many spots to count. Of 504 plates exposed in other areas, 28 were positive with a total of 56 particles detected. Of these, half were detected in the area of Room B433A.

DISCUSSION

Over 260,000 analytical determinations were made by NCAIA during the course of testing more than 20,000 batches of antibiotics over a 12-month period. These tests involved weighing, blending, and other handling of antibiotic powders and solutions that tend to release particles and aerosols. This study was undertaken to determine whether the handling and testing of antibiotic drugs constitute a source of contamination to the environment of the building.

The air-filtration system selected for this study is more stringent than that specified for monitoring airborne particulate contamination levels in open atmospheres, clean rooms, and other manufacturing and assembly areas, as described in the requirements of American Society for Testing and Materials Specification F25-63T and Society of Automobile Engineers Specification ARP743 for microscopic particle counting as referenced in the *USAF Technical Order 00-25-203 Revised;* a 0.8-μ porosity filter is satisfactory for these requirements. The 0.22-μ porosity filter used in this study is superior to that requirement.

Only small amounts of antimicrobial activity were found outside NCAIA except in the area of Room B433A. However, when these areas were subjected to quantitative assay of the air, the results

showed that, outside of NCAIA, antibiotics were detected only in the neighborhood of Room B433A. This basement location is in the hallway between the cage-washing operation and the glassware-washing area of the Laboratory Services Branch.

The antibiotic activity found in the air on the second floor, the largest area occupied by NCAIA, frequently appeared to be due to antibiotics other than penicillin. This conclusion is based on a comparison of results from assay plates with and without penicillinase. Although two spots of penicillin were found on one plate of four exposed in Room 1016 where penicillin contamination testing was performed, the many actual and control tests performed in that room have given no indication that environmental penicillin is ever detected by these tests. Most of the air coming into this room is filtered through Cambridge absolute filters to remove particles down to 0.3 μ in diameter.

This study shows that even in rooms occupied by NCAIA, there is no widespread heavy contamination of antibiotics in the air. Significant amounts occurred only in rooms where antibiotic powders were opened for weighing or where the drugs were extracted from dosage forms by blending. However, the quantities were not great in relation to the volume of air. The highest concentration of penicillin was 0.012 unit/cu. ft. and that of CTC was 0.0185 mcg./cu. ft. These small amounts would not affect the outcome of assays being performed as part of the certification program.

CONCLUSIONS

The incidence of contamination by antibiotic dust is negligible outside of the actual laboratory rooms where testing is performed. Therefore, persons in this building not testing antibiotics are not exposed to them by way of the atmosphere. Analysts testing antibiotics are exposed to a minimal degree.

REFERENCES

(1) U. S. Public Health Service, "Sampling Microbiological Aerosols," Communicable Disease Center, Technical Development Laboratories, and the Physical Defense Division, U. S. Army Chemical Corps; Public Health Service Publication No. 686, April 1959, pp. 1–53.

(2) G. Shaffer and J. McDade, *J. Amer. Hosp. Ass.*, **38**, 16(1964).

(3) Federal Standard No. 209, Clean Room and Work Station Requirements, Controlled Environment, December 16, 1963.

(4) National Aeronautics and Space Administration, "NASA Standards for Clean Rooms and Work Stations for the Microbially Controlled Environment," NHB 5340.2, August 1967.

(5) H. Welch, W. W. Wright, R. J. Reedy, E. J. Oswald, and D. M. Wintermere, *Antibiot. Annu.*, **1958–1959**, 942.

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Conformation of Some Acetylcholine Analogs as Solutes in Deuterium Oxide and Other Solvents

A. F. CASY, M. M. A. HASSAN, and E. C. WU

Abstract □ The proton magnetic resonance spectra of α- and β-methylacetylcholines in deuterium oxide are described and analyzed. Chemical shift and coupling constant data derived from these analyses and from the spectra of model compounds establish that β-methylacetylcholine shows a preference for *gauche* $^{+}$N/O conformers, while the α-isomer displays a lack of conformational preference in the solute condition. Additional IR and proton magnetic resonance studies of acetylcholine and some analogs of acetylcholine, in which one *N*-methyl is replaced by a bulkier group, uphold the preferred nature of *gauche* $^{+}$N/O conformers in the parent compound as a solute and support an intramolecular $^{+}$N. . . OCOMe interaction as a factor governing conformational preferences in these esters. The significance of the results in terms of the influence of conformational isomerism upon the duality of action of acetylcholine is discussed.

Keyphrases □ Acetylcholine analogs—conformation determination in deuterium oxide □ α-, β-Methylcholine—conformational analysis □ Conformation—acetylcholine analogs □ PMR spectroscopy—structure □ IR spectrophotometry—analysis

Knowledge of the conformation of acetylcholine (Ach) and related agonists has been increasingly sought during the last few years. Such information, while of potential value to the delineation of receptor characteristics and agonist–receptor interactions (1), is of additional importance in the cholinergic field because of recent postulates associating muscarinic and nicotinic effects of Ach with different conformations of the Ach molecule, recently reviewed (2). Reports upon the conformation of Ach and its congeners chiefly relate to the solid sate. Canepa *et al.* (3) showed that Ach bromide has a *gauche* $^{+}$NCCO conformation; this arrangement also occurs in many related compounds such as α-glycerophosphorylcholine and muscarine iodide (4), all in the solid state. Solute conformations of Ach in ethanol (5) and heavy water (6) have been advanced on the basis of infrared (IR) and proton magnetic resonance (PMR) spectroscopic data, respectively, the latter study in particular providing good evidence for a preferred *gauche* conformation. Molecular orbital calculations for Ach lead to the same prediction of preferred conformation (7).

This article reports some spectroscopic studies relating to the solute conformations of α- and β-methylacetylcholines analogs which retain, respectively, the nicotinic and muscarinic properties of Ach (8); the solid-state conformations of both compounds recently were described (9). Some studies of Ach analogs in which *N*-methyl is replaced by a bulkier substituent are also described.

PMR SPECTROSCOPY

PMR evidence of the conformation of substituted ethanes requires knowledge of the magnitude of spin-spin coupling between

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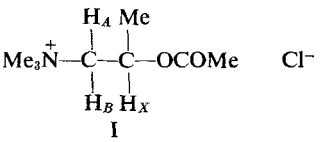
PMR SPECTROSCOPY

PMR evidence of the conformation of substituted ethanes requires knowledge of the magnitude of spin-spin coupling between

vicinal protons (10, 11). In the case of β-methylacetylcholine (β-MeAch), this information may be obtained by analysis of the 100 MHz. resonance signal due to the methylene protons (H_A and H_B in I) by Bible's method (12); use of the methine signal (due to H_X)

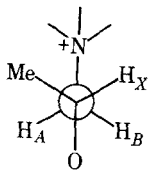


I

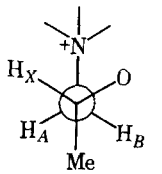
was not attempted since this formed an unresolved multiplet as a result of additional coupling with the β-methyl protons and ^{14}N (13).

Unless otherwise stated, the solvent used in the PMR work was deuterium oxide (D_2O), and the reference standard was sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The methylene signal, treated as the *AB* part of an *ABX* system, was composed of two partially overlapping quartets (Fig. 1), each being identified by the separation 14.4 Hz. (J_{AB}) which occurs four times within the 8-line signal. The distance between the centers of the two quartets is 5.6 Hz. where $|(J_{AX} + J_{BX})| = 11.2$ Hz. Construction of right-angled triangles (12), using the quantities J_{AB} (14.4 Hz.), $2D_+$ (26.8 Hz.), and $2D_-$ (20.8 Hz.) (the distances between the 1st and 3rd peaks of the two *AB* quartets), gives a $\Delta\nu_{AB}$ value of 4 or 19 Hz. and a $|(J_{AX} - J_{AB})|$ value of 37.2 or 8.2 Hz. The intensity ratio of peaks in the *X* signal was not available, so the correct alternatives could not be selected in this way; the values $\Delta\nu_{AB} = 19$ and $|(J_{AX} - J_{BX})| = 8.2$ Hz. were chosen as the more probable, however, and on the basis of comparisons with calculated spectra (14). Combination of the quantities $|(J_{AX} - J_{BX})| = 8.2$ Hz. and $|(J_{AX} + J_{BX})| = 11.2$ Hz. gives the values $J_{AX} = 9.7$ and $J_{BX} = 1.5$ Hz.

From the order of magnitude of these values (15, 16), it is clear that the preferred rotamer(s) of β-MeAch involves *trans* and *gauche* vicinal coupling constants; this conclusion supports either II or III as the preferred conformation. (In these and other Newman



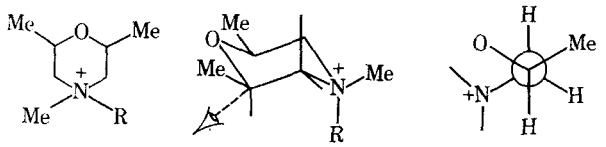
II



III

projections, the N—Me and O—COMe groups are omitted for clarity.)

The fact of a chemical shift difference between H_A and H_B of 19 Hz. (at 100 MHz.) further indicates that β-MeAch displays a marked conformational preference but does not aid in the choice of the preferred conformation because environments of the two protons differ in both conformers II and III. The morpholino derivatives, IV*a* and IV*b*, serve as rigid models for the *gauche* $^+$N/O conformer III. Data on their spectra generally support the latter as a preferred conformation, because vicinal coupling constants (for IV*a* and IV*b*) of similar orders of magnitude as those found for β-MeAch are derived from their spectra. Thus, analysis of the *X* mul-



IV

(*a*) R = H, $\bar{X} = \bar{Cl}$

(*b*) R = Me, $\bar{X} = \bar{I}$

tiplet of IV*a* gives the value $|(J_{AX} + J_{BX})| = 13.3$ Hz., where $J_{AX} = 11.2$ and $J_{BX} = 2.1$ Hz. by first-order treatment, following Booth and Gidley (17) who examined the 2,6-dimethylmorpholine base. Similar values were obtained by the same analysis of the methine signal of IV*b* (the *AB* and *X* signals of IV*a* and IV*b* virtually over-

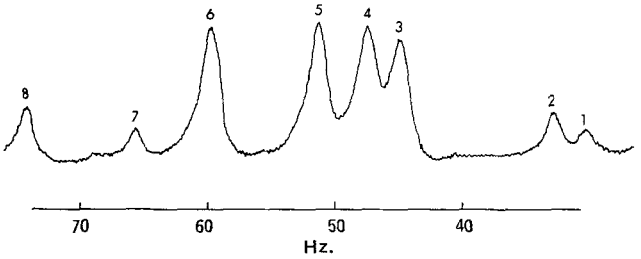


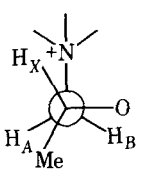
Figure 1—*100 MHz. PMR methylene signal of β-methylacetylcholine chloride in D_2O; sweep width 100 Hz. and sweep offset 370 Hz. Lines 8, 6, 4, and 2, and 7, 5, 3, and 1 form the two* AB *quarters, respectively.*

lapped); details are given in the *Experimental* section. Surprisingly, HCCN coupling was negligible in the cyclic quaternary salt IV*b*; this point is being studied further. Chemical shift data upon the secondary methyl group of β-MeAch and IV*b* also support the preferred nature of conformer III. Although the two values differ (ν_{Me}; III δ 1.33, IV*b* δ 1.23), allowance for the acylation shift (ν_{Me} in V is δ 1.24) shows that β-methyl has a similar environment in both I and IV*b*.

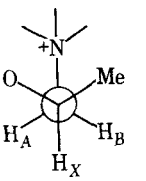
$Me_3\overset{+}{N}CH_2CHMeOH\ \bar{I}$

V

The small, but significant, differences observed between the J_{AX} and J_{BX} values of IV*b* and those of β-MeAch are best accounted for in terms of a *gauche* $^+$N/O conformer in which the NCCO dihedral angle is increased from 60° (as in III) to about 90° (as in VI); dihedral angles of this magnitude occur in solid-state conformations of Ach, α-MeAch, and β-MeAch (9) and are probably the result of a compromise between steric and electronic interactions of the nitrogen and oxygen functions. In VI, the H_XCCH_A and H_XCCH_B dihedral angles exceed and fall below, respectively, corresponding angles in the fully staggered conformation III. On the basis of the Karplus $\cos^2 \phi/J$ relationship (16), both these changes will lead to a reduction in vicinal coupling constants; hence, values lower than those in models such as IV*b*, in which dihedral angle deviations from 60° are less likely on account of constraints imposed by the cyclic structure, are to be anticipated. A significant population of the sterically unfavorable all-*gauche* conformer VII would also account for the lower J_{AX} and J_{BX} values of I compared with those of



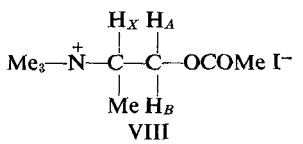
VI



VII

IV*b*, but the β-methyl chemical shift values would not be expected to correspond so closely because methyl is deshielded by charged nitrogen in VII. The population of the *trans* $^+$N/O conformer II is likely to be small since its contribution, although providing a lower J_{BX} coupling (J_{BX} is *trans* in III but *gauche* in II), would enhance the J_{AX} value.

In the case of α-MeAch, knowledge of the J_{AX} and J_{BX} coupling constant magnitudes was obtained by analysis of the H_X signal of the $ABXK_3$ system (see VIII), since this exhibited negligible coupling with ^{14}N (*cf.*, H_X signal of the β-isomer). The *AB* signal (a broad multiplet low field of the methine signal) was unsuitable for analysis because of coupling with ^{14}N and its overlap with the HDO peak; ^{14}N coupling effects were particularly clear in the α-methyl signal which formed a sharp doublet of triplets (*J* 7.0 and 2.0 Hz.)



VIII

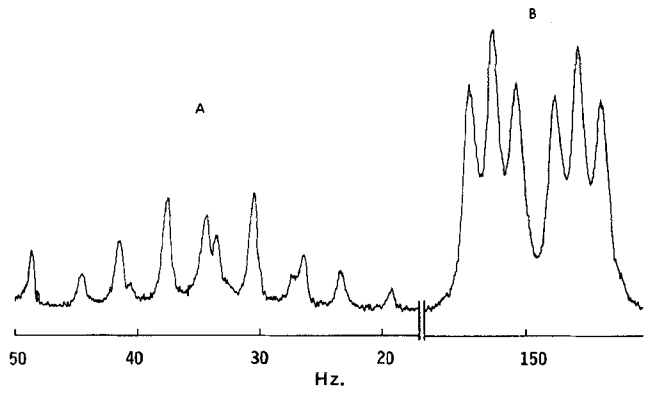
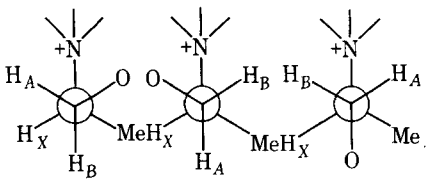


Figure 2—*100 MHz. PMR methine (A) and secondary methyl (B) signals of α-methylacetylcholine iodide in D_2O; sweep width 100 Hz. and sweep offset 420 Hz. for A and zero for B.*

(Fig. 2B). Identification of the four XK_3 quartets among the 12-line methylene signal (Fig. 2A) by the Booth and Gidley procedure (17), taking $J_{XK} = 7.0$ Hz. from the α-methyl signal, gives the quantities $|(J_{AX} + J_{BX})| = 8.8$ Hz. and the approximate values $J_{AX} = 4.2$ and $J_{BX} = 4.6$ Hz.; the latter values could not be refined by data from the *AB* portion of the spectrum because this signal was poorly resolved. The first-order values are of sufficient accuracy, however, for the present purpose. The geminal H_A and H_B protons formed a multiplet of five broad lines (partially obscured by the HDO peak) about 1.1 p.p.m. downfield of the methine signal. The overall narrowness of the signal ($W_H \sim 10$ Hz.) shows that the methylene protons have very similar chemical shifts, and the appearance of the *ABX* spectrum obtained closely approaches that calculated for the values $\Delta\nu_{AB}2$, $J_{AB}10$, and $J_{AX} = J_{BX} = 4$ Hz. (14).

The near-coincidence of the vicinal coupling constants derived by the analysis indicates that conformational preferences among α-MeAch conformers are low. *Gauche* and *trans* coupling constants for IX–XI are unknown; but values predicted on the basis of data



IX	X	XI
$J_{AX} \sim 1.5$	~ 1.5	~ 10 Hz.
$J_{BX} < 1.5$*	~ 10	~ 1.5 Hz.

* This value is judged the lowest because both coupling protons are *trans* to highly electronegative groups (18).

for β-MeAch and the models IV*a*, IV*b*, XII (the methiodide of phendimetrazine), and XIII (derived from ephedrine) should give reasonable estimates of their orders of magnitude. The probable values are listed beneath each conformer.

Similar populations of IX, X, and XI should lead to average J_{AX} and J_{BX} coupling constants of just over 4 Hz., *e.g.*, (1.5 + 1.5 + 10)/3 = 4.3 Hz., as obtained by analysis. Conformer distributions of this order would also result in similar averaged environments (and hence like chemical shifts) for the H_A and H_B protons, again in agreement with spectral observations.

The α-methyl chemical shift (δ 1.5) is lower field than that of methyl in XIV (δ 1.33) (19); this result is evidence for significant populations of IX and XI in which methyl is close to the deshielding

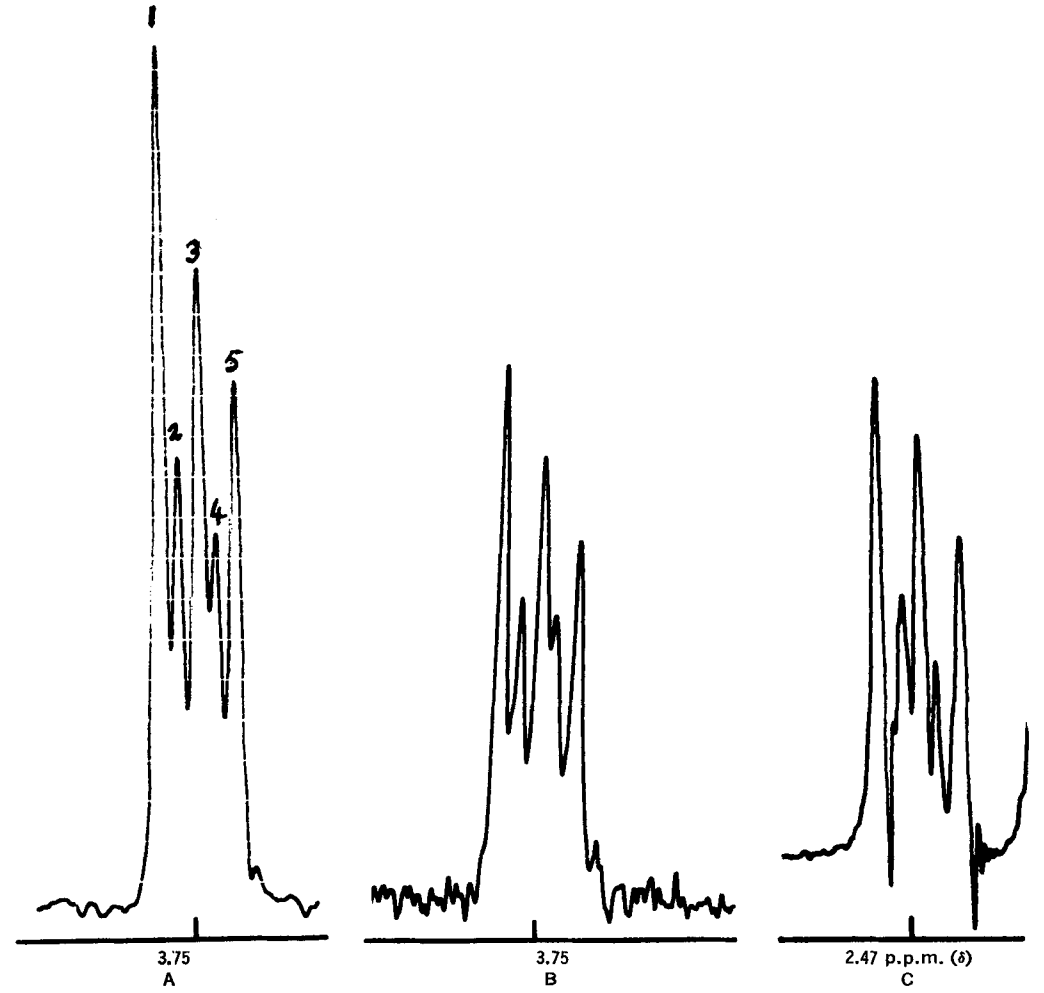
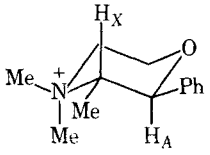


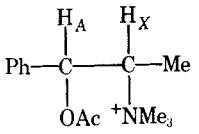
Figure 3—*60 MHz. PMR N-methylene signals of acetylcholine (A), (β-acetoxyethyl)benzyldimethylammonium bromide (B), and N-methylmorpholine (C) in D_2O.*

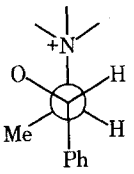
Table I—PMR Characteristics of the *N*-Methylene Protons of Some Acetylcholine Analogs in Deuterium Oxide ($RNMe_2CH_2CH_2OR'$ X^-)

Compound No.	R	R′	X	NCH_2 PMR Characteristics[a] Chemical Shift[b]	Line 1–5 Separation	Line 2–4 Separation
1	Me	COMe	Cl	3.75	9.5	5.0
				3.8	9.5	5.0[c]
				4.13	9.5	4.8[d]
2	Me	H	Cl	3.52	10.0	5.0
3	CH_2:$CHCH_2$	COMe	Br	3.68	9.8	4.8
4	CH_2:$CHCH_2$	H	Br	3.48	10.0	5.0
5	$PhCH_2$	COMe	Br	3.75	10.0	4.5
				4.2	10.0	5.8[d]
6	$PhCH_2$	H	Br	3.52	10.0	5.0
7	Me_2CH[e]	COMe	Br	3.72	10.0	4.0
8	Me_2CH	H	Br	3.48	9.5	4.0
9	*tert*-Bu[f]	COMe	I	3.67	10.0	4.0
10	*tert*-Bu	H	I	3.43	10.0	4.0
11	Morpholine based[g]			2.43	10.0	4.5
12	*N,N*-Dimethyl-morpholinium iodide			3.50	9.5	4.5

[a] Chemical shifts in p.p.m. from external tetramethylsilane; line separations in Hz. Spectra recorded at 60 MHz. at the normal operating temperature. [b] Center of multiplet. [c] In DMSO-d_6. [d] In $CDCl_3$. [e] Me signal: broad doublet (No. 7), doublet of triplet (J = 2 and 7 Hz.) (No. 8). [f] *tert*-Bu signal: broad singlet (No. 9), triplet (J = 1.5 Hz.) (No. 10). [g] Smith and Shoulders (39).

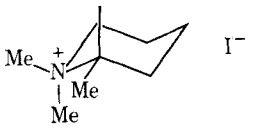






J_{AX} = 10.4 Hz. (*trans*) J_{AX} = 1.5–2.0 Hz. (*gauche*)

XII XIII XIII



XIV

oxygen function. IR evidence supports the *gauche* $^+N/O$ conformers IX and X.

To conclude the PMR studies, data are given for some analogs of Ach in which one *N*-methyl is replaced by a bulkier group. These derivatives were examined to determine whether the preference for *gauche* $^+N/O$ conformers, established for Ach itself in solvent D_2O (6), is maintained when the destabilizing steric interaction between the oxygen and nitrogen functions is increased. The original PMR analysis of Ach rests upon the extraction of the sum (N) and difference (L) of the vicinal coupling constants from the bimethylene signal treated as an $A_2'B_2'$ system. The analysis concerns, in particular, the higher field methylene multiplet (due to $^+NCH_2$), because the lower field signal (OCH_2) is complicated by coupling with ^{14}N. In the present case, no complete analysis (involving first estimation of the L and N values from line separations and then refinement by means of an iterative calculation) is attempted. Instead, the $^+NCH_2$ signal appearance and line separations are compared with those of Ach, and conformational conclusions are drawn from observed similarities or differences. Where 5-line multiplets result, separations of Lines 1 and 5 and 2 and 4 (Fig. 3) give the approximate N and L values, respectively. These data are given in Table I and the following points are made.

1. The data show that the $^+NCH_2$ methylene proton characteristics of the Ach analogs 3, 5, 7, and 9 closely mirror those of Ach itself and the model compounds morpholine and *N,N*-dimethylmorpholine (Compounds 11 and 12) which are constrained to *gauche* N/O conformations. Three examples are shown in Fig. 3. These results, following the analysis of Culvenor and Ham (6), support preferred *gauche* conformations for the analogs 3, 5, 7, and 9 in D_2O and show that replacement of an *N*-methyl group of Ach by a bulkier group has no major effect upon conformational equilibria in these quaternary salts. The same conclusions follow for the corresponding quaternary alcohols (Compounds 2, 4, 6, 8, and 10).

2. The appearance of the $^+NCH_2$ signal of Ach shows little variation when D_2O is replaced by DMSO-d_6 or $CDCl_3$ (Table I) or by D_2O–pyridine or trifluoroacetic acid (6). Hence the conformation of Ach appears to be little influenced by the solvent. This result suggests that the conformation of Ach is goverened primarily by interactions within the molecule.

3. The A_2 and B_2 methylene signals of 3,3-dimethyl-*n*-butanol and its acetoxy ester (carbocholine) in CCl_4, the tertiary base analogs of choline and Ach in D_2O, and 2-(*N*-*tert*-butyl-*N*-methylamino) ethanol (the base related to Compound 10, Table I) in $CDCl_3$ form near-symmetrical triplets of separation, 5–6 Hz., characteristic of an absence of conformational preference (11). These results indicate that *gauche* conformers are preferred in β-substituted ethanols only when the substituent is highly electronegative as in the case for a halogen (11) or a charged nitrogen atom. The spectrum of the hydrochloride of the tertiary base related to Ach in D_2O also shows a $^+NCH_2$ signal characteristic of the quaternary salts (N ~10 Hz., L ~3.8 Hz.).

4. The spectrum of acetylthiocholine (thio-Ach) was markedly different from that of Ach, the NCH_2 and SCH_2 signals being much

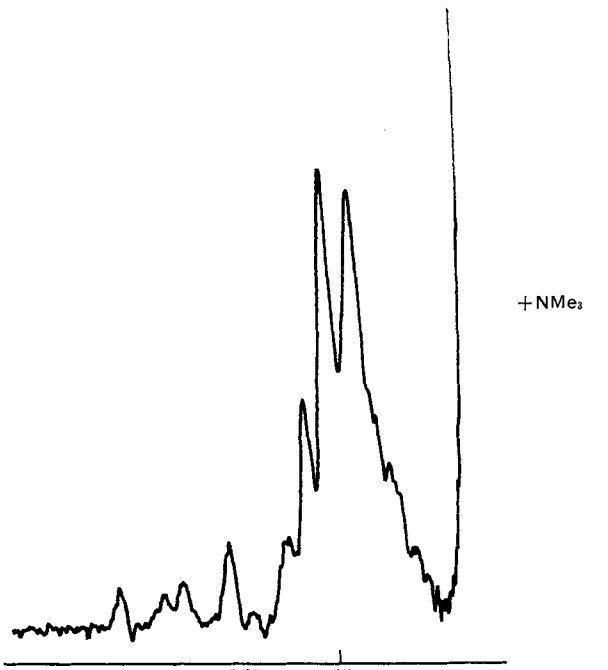


Figure 4—*60 MHz. PMR* N- *and* O-*methylene signals of acetylthiocholine chloride in* D_2O *(sweep width 250 Hz.).*

Table II—Carbonyl Stretching Frequencies of Acetylcholine and Related Compounds in Chloroform

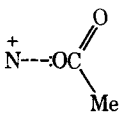
No.	Compound	$\nu_{C=O}$ (cm.$^{-1}$) 10%	$\nu_{C=O}$ (cm.$^{-1}$) 0.005 mole/l.	$\Delta\nu_{C=O}$[a]
1	$Me_3\overset{+}{N}(CH_2)_2OCOMe\ \overline{Cl}$	1750 1757[b]	1755	+20
2	$Me_2N(CH_2)_2OCOMe$	1732	1732	−3
3	EtOCOMe	1735 1745, 1725[b]	1735	—
4	$Me_2N(CH_2)_2OCOMe\cdot HCl$	1752	1757	+22
5 (β-)	$Me_3\overset{+}{N}CH_2CHMeOCOMe\ \overline{Cl}$	1745	1746	+11
	$Me_3\overset{+}{N}CH_2CHMeOCOMe\ \overline{I}$	1745	1748	+13
6	$Me_2NCH_2CHMeOCOMe$	1732	1732	−3
7 (α-)	$Me_3\overset{+}{N}CHMeCH_2OCOMe\ \overline{I}$	1757	1757	+22
8	$t\text{-}Bu\overset{+}{N}Me_2(CH_2)_2OCOMe\ \overline{I}$	1749	1753	+18
9	$CH_2{:}CHCH_2\overset{+}{N}Me_2(CH_2)_2OCOMe\ \overline{Br}$	1750	1755	+20
10	$PhCH_2\overset{+}{N}Me_2(CH_2)_2OCOMe\ \overline{Br}$	1750	1757	+22
11	$Me_2CH\overset{+}{N}Me_2(CH_2)_2OCOMe\ \overline{Br}$	1750	1750	+15
12	$Me_3C(CH_2)_2OCOMe$	1732	1732	−3
13	$Me_3\overset{+}{N}(CH_2)_2SCOMe$	1695	1695	−40

[a] $\nu_{C=O}$ (compound) − $\nu_{C=O}$ ethyl acetate. [b] In ethanol.

closer together and forming a complex multiplet approximately symmetrical about its midpoint and obscured on the high field side by the $^+NMe_3$ signal (Fig. 4). No assignment of *L* and *N* values was possible. This result is evidence that conformational preferences in the thio-derivative are different from those in Ach and its congeners; it is significant in this respect that X-ray analysis of thio-Ach bromide shows that the crystalline salt has a *trans* N/S conformation (20). Mautner (21), quoting unpublished results, recently proposed a *trans* conformation for this compound in solution.

IR SPECTROSCOPY

Several IR studies have established that the carbonyl stretching mode ($\nu_{C=O}$) of Ach is higher than that of the corresponding tertiary amine and of ethyl acetate (5, 22, 23). The shift to higher wave numbers that occurs when a β-hydrogen in ethyl acetate is replaced by trimethylammonium may be interpreted in terms of an electronic interaction between charged nitrogen[1] and a lone pair orbital of the alkyl oxygen atom of the ester function (XV); relay of the consequent drift of electrons toward nitrogen to carbonyl carbon



XV

would depress the polarization of the carbonyl group, with a consequent rise in its absorption frequency (2).[2] An interaction of the type shown in XV requires close proximity of the charged nitrogen and ester functions; demonstration of its operation thus provides support for *gauche* $^+$N/O conformers of Ach and its congeners.

The published IR data have two limitations: (*a*) interpretation is complicated by solvent effects ($\nu_{C=O}$ values were measured in hydrogen bonding solvents such as ethanol and water), and (*b*) dilution studies, necessary to differentiate between intermolecular and intramolecular effects, were not reported. For these reasons, further IR studies of Ach and its analogs (some novel) were undertaken in this work. Spectroscopic chloroform was chosen as the reference solvent, since solvent effects should then be negligible. Esters were examined over the concentration range from 10% to 0.1 or 0.05%, the lowest concentration being 0.005 mole/l. At this dilution, intermolecular effects may be discounted (25). IR data are given in Table II, where only $\nu_{C=O}$ values at the concentration extremes are recorded. The following points are made:

1. In all cases, little change in the $\nu_{C=O}$ values of the salts occurs on dilution; this result shows that intermolecular interactions do not significantly influence the carbonyl stretching frequency of these esters.

2. The $\nu_{C=O}$ wavenumber of Ach in chloroform is similar to that of the hydrochloride of 2-dimethylaminoethyl acetate, but 23 and 20 cm.$^{-1}$ higher, respectively, than those of the corresponding tertiary base and ethyl acetate (Table I, Compounds 1–4). Carbonyl stretching frequency shifts relative to the ethyl acetate value are also seen for α- and β-MeAch (Table II, Compounds 7 and 5); the α-shift (22 cm.$^{-1}$) is similar to that of Ach, but the β-value (11 cm.$^{-1}$) is lower (a possible reason for this is given later). The corresponding β-tertiary base has a $\nu_{C=O}$ value close to that of ethyl acetate (Table II, Compounds 6 and 3). The $\nu_{C=O}$ (and $\Delta\nu_{C=O}$) values of the Ach analogs (Table II, Compounds 8 and 11), in which one of the *N*-methyl groups of the onium head is replaced by a bulkier group, are close to that of the parent compound.

3. The similar $\nu_{C=O}$ values of 3,3-dimethyl-*n*-butyl acetate (carbocholine) and ethyl acetate (Table II, Compounds 3 and 12) show that the $\nu_{C=O}$ elevation seen in Ach and related compounds is not steric in origin. In addition, results upon the free tertiary amine esters (Table II, Compounds 2 and 6) establish that the basic nitrogen function has no significant influence on the ester carbonyl stretching frequency.

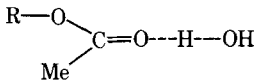
4. The very low $\nu_{C=O}$ value of thio-Ach (Table II, Compound 13) reflects the different influences of oxygen and the substantially less electronegative atom, sulfur, upon the carbonyl stretching vibration; release of a sulfur lone pair of electrons is responsible for lowering $\nu_{C=O}$ of thiol esters below the values found for ketones (26). Although the tertiary amine corresponding to thio-Ach was not available for assessing $N^+\cdots S$ interactions, the fact that the $\nu_{C=O}$ value falls within the range 1698–1690 cm.$^{-1}$ quoted for dialkyl thiolesters (26) suggests that such interactions are absent, as would be the case if a *trans* $^+$N/S conformer was preferred (*PMR Spectroscopy* section).

5. The IR evidence for favored *gauche* $^+$N/O conformers of Ach and its congeners relates to solute molecules in chloroform and is not necessarily relevant when D_2O or water is the solvent. The close approximation of the PMR features of Ach in $CDCl_3$ and D_2O (previously described), however, strongly suggests that Ach

[1] A reviewer points out, on the basis of molecular orbital calculations on the ammonium ion (24), that the positive charge of the onium group may be distributed over the atoms attached to the nitrogen atom. If such be the case, the interaction depicted in XV is more correctly represented as between the onium group as a whole and the ether oxygen atom; this modification does not affect the IR interpretations which follow.

[2] Fellman and Fugita (5) considered that a direct $^+$N/O interaction was unlikely on the grounds of the smaller $\nu_{C=O}$ shifts seen in 3- and 4-carbon chain analogs of Ach; their use of planar rather than three-dimension models, however, invalidates this view. Canepa and Mooney (23) criticized their proposal of the onium influence upon the ester function being transmitted inductively through the two-carbon chain.

conformer populations are alike in polar and feebly polar solvents. The influence of the onium group upon the carbonyl stretching frequency in polar solvents may be obscured by solute–solvent interactions. Thus the lower $\nu_{C=O}$ values of Ach obtained in water (1727 cm.$^{-1}$) and 50% ethanol in water (1737 cm.$^{-1}$) by attenuated total reflectance (5, 23) show that the hydrogen bonding interaction (XVI) (which reduces $\nu_{C=O}$) is the predominant influence (2).



XVI

6. The carbonyl stretching frequency of the ester methiodide XIII, derived from ephedrine and for which there is clear PMR evidence for a preferred *gauche* $^+$N/O conformation, is 1760 cm.$^{-1}$ (0.5 and 0.1%), a value close to that of Ach and its congeners.

IR and PMR evidence is thus complementary in supporting the existence or absence of conformational preferences among quaternary esters such as Ach and the tertiary bases from which they are derived. The lack of conformational preference in the β-methyl tertiary base XVII, for example, is shown both by its having a $\nu_{C=O}$ value close to that of ethyl acetate (Table II, Compound 6) and by

$Me_2NCH_2CHMeOCOMe$
XVII

the averaged values of its CH_2CH coupling constants (J = 6 Hz. in $CDCl_3$).[3]

The small, but consistently obtained, differences between the $\nu_{C=O}$ values of Ach, α-, and β-MeAch (Table II, Compounds 1, 5, and 7) may be tentatively explained in terms of the methyl substituent aiding or opposing the close approach of the nitrogen and oxygen functions. A methyl substituent would be expected to oppose the approach of the $^+$N and OCOMe functions through the generation of unfavorable Me–H interactions (as in β-III) or assist the same approach by a buttressing effect which causes the C—$\overset{+}{N}Me_3$ bond to bend away from a geminally attached methyl group (as in α-X) and by relief of an Me–O interaction (as in α-IX). The halide ion has a negligible influence, as seen by the identical spectral properties of the chloride and iodide of β-MeAch.

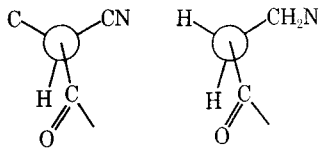
The following conclusions may be drawn from the spectroscopic evidence:

1. The additional IR and PMR studies of Ach and its *N*-substituent congeners uphold the preferred nature of the *gauche* $^+$N/O conformers of the parent compound in deuterium oxide and, by analogy, in water. The $^+$N- - -OCOMe interaction is a probable stabilization factor, since it appears capable of compensating for destabilizing steric interactions of $^+NMe_3$ and other onium groups of even greater bulk. If the positive charge on the onium function is distributed around the periphery of the group (24), the interaction might be represented more correctly by NC—$\overset{\delta+}{H}$- - -O, as proposed earlier (5), even though there is no precedent or evidence for the existence of formal hydrogen bonds of this nature (27, 28).

2. β-MeAch shows a pronounced preference for *gauche* $^+$N/O conformers and there is good evidence for a high population of the skewed form VI; the α-isomer displays a lack of conformation preference. These results correlate closely with the solid-state conformations by these analogs established by X-ray studies (9); that of the β-isomer is similar to VI, and the α-isomer exists in two forms that are related to IX and XI, respectively. There is no simple explanation for these conformational differences. It is evident from models, however, that steric interactions of the Me and $^+NMe_3$ groups in the β-conformers are of a higher order than those of Me and OCOMe in the α-forms. Hence, II and VII (β) are probably much more destabilized relative to III (or VI) than are the comparable α-conformers, IX and XI, relative to X. The preferred *gauche* conformer β-III (or VI) thus combines operation of the $^+$N- - -O interaction with minimum steric interactions of the methyl substituent.

[3] Details of the analysis of the 100 MHz. spectrum of XVII as neat liquid, which gave similar results, are given under *Experimental*. Surprisingly, the base XVII displays a pronounced conformational preference in D_2O, as is evident from the broad nature of its methine signal.

Culvenor (29) proposed use of acylation shift data (HO-CH resonance moves downfield when the OH group is acylated) as evidence of ester conformation; he suggested that normal values (1.0–1.15 p.p.m. for secondary and 0.45–0.6 p.p.m. for primary esters) are indicative of a preferred conformation in which the ester carbonyl group is eclipsed by a methine or methylene proton. Acylation shifts for α-MeAch (~0.5 p.p.m.) and β-MeAch (~1 p.p.m.) fall in the normal ranges; hence, C—C—O—C=O and H—C—O—C=O dihedral angles of about 60° are probable, as in XVIII and XIX.



XVIII XIX

While work upon rigid and semirigid analogs of Ach is subject to certain limitations, *e.g.*, fairly large deviations from the molecular dimensions of the natural agonist in the case of decalin derivatives (30) and from fully staggered *gauche* and *trans* O/N dihedral angles in the case of cyclopropane analogs (31) [a recent X-ray study established that the NCCO dihedral angle is 137° in crystalline (+)-*trans*-2-acetoxycyclopropyltrimethylammonium iodide (32)], potency variations among the various isomers provide reasonable evidence that muscarinic properties are most pronounced in isomers that approach a *trans* $^+$N/O geometry. If work on these rigid analogs is valid in terms of the acyclic agonists acetylcholine and β-methylacetylcholine, then the preferred and "active" (muscarinic) conformations of these two compounds must differ. The fact that β-methylacetylcholine is only feebly active at nicotinic sites might be taken as evidence that *gauche* conformers cannot be the active form of acetylcholine at nicotinic receptors, thereby discounting the proposal of the duality of action of Ach being related to conformational isomerism. A firm conclusion cannot be drawn, however, since the influence of the β-methyl substituent upon drug–receptor uptake at nicotinic sites is unknown.

EXPERIMENTAL

Commercial samples of acetylcholine chloride, choline chloride, β-methylacetylcholine chloride, acetylthiocholine chloride, and 2-dimethylaminoethyl acetate were used. The last ester formed a hydrochloride, m.p. 126–128°.

Anal.—Calcd. for $C_6H_{14}ClNO_2$: C, 43.0, H, 8.4. Found: C, 42.7; H, 8.4.

2-Acetoxy-1-dimethylaminopropane hydrochloride separated when acetyl chloride (15.7 g.) was added to 1-dimethylaminopropan-2-ol (10.3 g.) in toluene. The base, recovered from the hydrochloride, was distilled to give 2-acetoxy-1-dimethylaminopropane (10 g.), b.p. 47°/14 mm.; hydrobromide, m.p. 113–115°, from ethanol—ether [Tammelin (33) gave b.p. 53°/13 mm. for the base and m.p. 121° for the hydrobromide]; and methiodide, m.p. 138–139°.

Anal.—Calcd. for $C_8H_{18}INO_2$: C, 33.5; H, 6.3. Found: C, 33.5; H, 6.1.

The PMR methine signal of the base [MeCH(OCOMe)CH_2-NMe_2] formed a near sextet (J = 6.4 Hz.), symmetrical about its midpoint, near δ 4.45 (neat) and 5.1 (in $CDCl_3$); in D_2O the signal was composed of 14 lines centered near δ 5.1 and gave J values 4.6 and 7.8 Hz. by first-order analysis.

The (±)-methiodides of 2-dimethylaminopropan-1-ol, m.p. 299–300°, and 1-acetoxy-2-dimethylaminopropane (α-methylacetylcholine), m.p. 128–130° [Beckett *et al.* (34) gave 299–300° for the former and 131–132° for the latter salt] were prepared by the literature procedure.

Morpholine Derivatives—*N,N*-Dimethylmorpholinium iodide, m.p. 246–248°, from ethanol [Knorr (35) gave 251°] was obtained from *N*-methylmorpholine and methyl iodide. Methylation of a commercial mixture of *cis*- and *trans*-2,6-dimethylmorpholine with 40% formaldehyde and 88% formic acid, followed by acidification of the basic product with concentrated hydrochloric acid, gave a crude mixture of the isomeric *N*-methyl hydrochlorides. Fractional crystallization of the mixture from ethanol–ether gave pure *N*-methyl-*cis*-2,6-dimethylmorpholine hydrochloride, m.p. 222–223°.

Anal.—Calcd. for $C_7H_{16}ClNO$: C, 50.75; H, 9.7. Found: C, 50.85; H, 9.6.

The derived base with methyl iodide gave *N,N*-dimethyl-*cis*-2,6-dimethylmorpholinium iodide, m.p. 230.5–231.5°.

Anal.—Calcd. for $C_8H_{18}INO$: C, 35.4; H, 6.9. Found: C, 35.7; H, 6.8.

trans-(3-Me/4-Ph) 3,4-dimethyl-2-phenylmorpholine (phendimetrazine) (36) formed a methiodide, m.p. 175–177°, from methanol–ether.

Anal.—Calcd. for $C_{13}H_{20}INO$: C, 46.85; H, 6.05. Found: C, 46.6; H, 6.1.

Erythro-3-acetoxy-2-dimethylamino-3-phenylpropane Methiodide—The dimethylamino analog of ephedrine, obtained by treating ephedrine with excess of methyl iodide, gave 2-dimethylamino-3-hydroxy-3-phenylpropane methiodide, m.p. 198–200°, from methanol–ether.

Anal.—Calcd. for $C_{12}H_{20}INO$: C, 44.9; H, 6.3. Found: C, 44.6; H, 6.2.

This salt with acetic anhydride gave the acetate methiodide (XIII), m.p. 225–226° (from methanol–ether).

Anal.—Calcd. for $C_{14}H_{22}INO_2$: C, 46.3; H, 6.1. Found: C, 46.5; H, 5.9.

Quaternary Salts of 2-Dimethylaminoethanol—2-Dimethylaminoethanol (8.9 g.) in acetone was added dropwise to benzyl bromide (17.1 g.) in the same solvent; the solid, which separated on storage, was collected and recrystallized from ethanol–ether to give benzyldimethyl(β-hydroxyethyl)ammonium bromide (21 g.), m.p. 129–130° [Jones (37) gave 130–131°]. Allyldimethyl(β-hydroxyethyl)-ammonium bromide monohydrate, m.p. 63–65° (*Anal.*—Calcd. for $C_7H_{16}BrNO \cdot H_2O$: C, 36.85; H, 7.95. Found: C, 36.5; H, 8.35) and dimethyl(β-hydroxyethyl)*iso*-propylammonium bromide, m.p. 275–276° [Robinson (38) gave 274°] were similarly prepared. A mixture of 2-*tert*-butylaminoethanol (11.7 g.), 40% formaldehyde in water (11.2 ml.), and 88% formic acid (7.8 g.) was heated under reflux for 12 hr. and then acidified with concentrated hydrochloric acid and evaporated. The residue was made alkaline with aqueous sodium hydroxide and extracted with ether. The extract was dried (Na_2SO_4), and evaporated, and the residue was distilled to give 2-(*N*-*tert*-butyl-*N*-methylamino)ethanol (11 g.), b.p. 68–70°/17 mm. It formed a hydrochloride, m.p. 113–114°, from ethanol–ether (*Anal.*—Calcd. for $C_7H_{18}ClNO$: C, 50.1; H, 10.8. Found: C, 50.2; H, 10.8) and a methiodide, m.p. 200–202°, from methanol–ether (*Anal.*—Calcd. for $C_8H_{20}INO$: C, 35.2; H, 7.4. Found: C, 35.3; H, 7.2). The methiodide was also obtained directly from 2-*tert*-butylaminoethanol after several treatments with methyl iodide and sodium methoxide in methanol at the reflux temperature.

Quaternary Salts of 2-Dimethylaminoethyl Acetate—Allyl bromide (1.2 g.) in acetone was added to 2-dimethylaminoethyl acetate (1.3 g.) in the same solvent when (β-acetoxyethyl)allyldimethylammonium bromide (2.4 g.), m.p. 101–103°, from ethanol–ether, separated.

Anal.—Calcd. for $C_9H_{18}BrNO_2$: C, 42.9; H, 7.2. Found: C, 42.9; H, 7.2. A mixture of benzyldimethyl(β-hydroxyethyl)ammonium bromide (5.2 g.), acetic anhydride (3.1 g.), and pyridine (5 ml.) was stirred for 10 hr. at room temperature and then concentrated under reduced pressure. The residue was recrystallized from ethanol–ether to give (β-acetoxyethyl)benzyldimethylammonium bromide (5.5 g.), m.p. 125.5–126.5°.

Anal.—Calcd. for $C_{13}H_{20}BrNO_2$: C, 51.65; H, 6.7. Found: C, 51.7; H, 6.8. (β-Acetoxyethyl)dimethylisopropyl ammonium bromide, m.p. 122–124°, from ethanol–acetone–ether (*Anal.*—Calcd. for $C_9H_{20}BrNO_2$: C, 42.5; H, 7.9. Found: C, 42.6; H, 8.1) and (β-acetoxyethyl)-*tert*-butyldimethylammonium iodide, m.p. 173–174°, from methanol–ether (*Anal.*—Calcd. for $C_{10}H_{22}INO_2$: C, 38.1; H, 7.0. Found: C, 38.1; H, 7.1.), were similarly prepared.

IR spectra were recorded with a Beckman (R-10) spectrophotometer. Cells of pathlength 0.05 mm. (10–5%), 0.5 mm. (5–3%), and 2 mm. (1.0–0.1%) were used for solution studies in $CHCl_3$; spectra were calibrated with polystyrene. PMR spectra were recorded with Varian H60-D and HA-100 instruments with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the standard in D_2O and tetramethylsilane in all other solvents.

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Synthesis and Characterization of Some 3-Nitroso-2-arylimidazo(1,2-*a*)pyrimidines

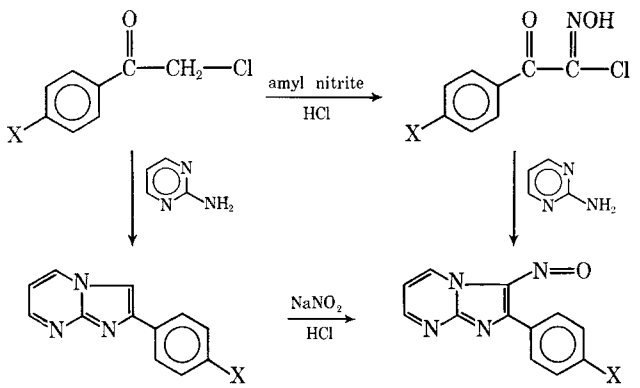
JOSEPH P. LaROCCA, C. A. GIBSON, and B. BLACKBURN THOMPSON*

Abstract □ While attempting to prepare amides derived from 2-aminopyrimidine and arylglyoxylohydroxamyl chlorides, deeply colored, highly insoluble products were obtained. Both IR and NMR data proved that the desired amides had not been obtained. This manuscript details the information used to identify the products as 3-nitroso-2-arylimidazo(1,2-*a*)pyrimidines.

Keyphrases □ 3-Nitroso-2-arylimidazo(1,2-*a*)pyrimidines—synthesis □ Antimicrobial activity—3-nitroso-2-arylimidazo(1,2-*a*)-pyrimidines □ NMR spectroscopy—structure □ IR spectrophotometry—structure

While attempting to condense arylglyoxylohydroxamyl chlorides with 2-aminopyrimidine to obtain the corresponding *N*-(2-pyrimidinyl)arylglyoxylohydroxamamides, nearly insoluble, highly colored compounds were obtained instead which, according to data detailed in this report, were identified as 3-nitroso-2-arylimidazo(1,2-*a*)pyrimidines.

The route for the synthesis of the title compounds and an alternative route involving direct nitrosation of 2-phenylimidazo(1,2-*a*)pyrimidine are given in Scheme I.



Scheme I

EXPERIMENTAL[1]

The arylglyoxylohydroxamyl chlorides required for this project were synthesized according to previously described procedures (1, 2). The α-chloroketones, which served as starting materials, were obtained from Distillation Products Industries as was 2-aminopyrimidine. All melting points were taken on a Thomas–Hoover melting-point apparatus and are uncorrected.

Synthesis of 3-Nitroso-2-phenylimidazo(1,2-*a*)pyrimidine (Compound 1, Table I)—*Method A: Ring Closure*—Phenylglyoxylohydroxamyl chloride (1.8 g., 0.01 mole) was dissolved in 50 ml. of anhydrous ether and treated with 1.9 g. (0.02 mole) of 2-aminopyrimidine. Since the aminopyrimidine is only sparingly soluble in dry ether, it was added in small portions with stirring. The reaction progressed slowly and, when all the amine had been added, the flask was stoppered tightly and stirred continuously at room temperature for 2 days. The product and amine hydrochloride coprecipitated as a chalky green solid. After filtration, the solid was washed with ether to remove unreacted starting materials and then with water to remove the by-product amine hydrochloride salt. The crude product was recrystallized twice from boiling isopropyl alcohol (175 ml./g. of crude product) and gave 1.17 g. (52.1% yield) of fine, emerald-green needles melting at 223.5–224.5°.

Anal.—Calcd. for $C_{12}H_8N_4O$: C, 64.27; H, 3.59; N, 24.99. Found: C, 64.20; H, 3.70; N, 24.59.

NMR and IR spectral data are given and discussed in context with structural proof in the *Discussion* section.

Method B: Nitrosation of 2-Phenylimidazo(1,2-a)pyrimidine—1. *Synthesis of 2-Phenylimidazo(1,2-a)pyrimidine (Compound 5, Table I)*—Preparation of this intermediate was accomplished by a modification of the procedures of Buu-Hoi and Xuong (3) and Almirante *et al.* (4).

Thus, phenacyl chloride (2-chloroacetophenone) (0.05 mole, 773 g.) and 2-aminopyrimidine (0.05 mole, 476 g.) in 50 ml. of absolute ethanol in a round-bottom flask was heated at 60° with stirring (magnetic stirring apparatus) for 3 hr.,[2] followed by 18 hr. of stirring at room temperature. The reaction mixture was chilled and filtered. The resultant salt was recrystallized once from hot 10% hydrochloric acid solution, collected on a sintered-glass funnel, suspended in 100 ml. of water, and neutralized with 10% sodium hydroxide. The free base was then extracted repeatedly with 50-ml. portions of chloroform[3] until no further base was obtained. The combined chloroform extracts were freed from chloroform in a rotary evaporator, and the solid base recrystallized (charcoal treatment) from isopropyl alcohol. Yields varied from about 40 to 60% theory on repeated runs. The product appeared as broad, flat needles melting at 200.5–201.5° [lit. (3) m.p. 202°].

2. *Nitrosation of 2-Phenylimidazo(1,2-a)pyrimidine*—Nitrosation of 2-phenylimidazo(1,2-*a*)pyrimidine has not previously been reported. Since procedures have been reported for nitrosation of 2-phenylimidazo(1,2-*a*)pyridine (5–7) and 2-phenylindolizine (2-phenylpyrrocoline) (8, 9), the probability of success was considered favorable. Although some difficulties were encountered,[4] fine emerald-green needles were obtained according to the following procedure, a modification of the procedure of Pentimalli and Bozzini (7).

Thus, 1.54 g. (0.0075 mole) of 2-phenylimidazo(1,2-*a*)pyrimidine was warmed with 6 ml. of glacial acetic acid until dissolved. Then, 9 ml. of distilled water was added, the solution was quickly cooled to about 5°, and 0.75 g. (0.19 mole) of sodium nitrite was added portionwise with cooling and stirring. The formation of a green coloration followed by precipitation of a green solid rapidly ensued.[4] After 24 hr. of periodic stirring, 1.6 g. (95.2% of theory) of crude, green product was obtained by filtration. Recrystallization once

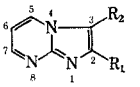
[1] Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

[2] The red coloration which resulted during the heating period proved to be very difficult to remove except by direct charcoal treatment of the hydrochloride salt.

[3] Due to the poor solubility, a large quantity of chloroform is required. The procedure is not satisfactory for obtaining sizable quantities of this compound but it is more satisfactory than other procedures (3, 4) for obtaining a pure product. Direct recrystallization of the free base from isopropyl alcohol also proved superior to literature procedures.

[4] Neutralization of the reaction mixture with base, as would be customary when aqueous hydrochloric acid serves as the reaction solvent, resulted in loss of the nitroso group in early experiments. The use of aqueous acetic acid as the reaction solvent circumvents the necessity of adding base prior to filtration.

Table I—NMR Data of H-3, H-5, H-6, H-7, and Aromatic Protons [2-Aryl-3-nitrosoimidazo(1,2-*a*)pyrimidines and 2-Phenylimidazo(1,2-*a*)pyrimidine][a-c]



Compound No.	R_1	R_2	Chemical Shift, p.p.m. H-3	H-5	H-6	H-7	Aromatic[c]		Coupling Constant, Hz. $H_{5,6}$	$H_{5,7}$	$H_{6,7}$
1	Phenyl	−N = O	—	9.98	7.28	8.92	7.6(3)	8.8(2)	6.9	2.1	4.3
2	4-Methoxyphenyl[d]	−N = O	—	(10.0)	(7.5)	(9.1)	(7.0(2))	(8.8(2))	(6.5)	(2.0)	(4.5)
3	4-Chlorophenyl	−N = O	—	9.96	7.28	8.93	7.5(2)	8.7(2)	6.5	2.5	4.5
4	4-Methylphenyl	−N = O	—	10.01	7.3[e]	8.90	7.4(2)	8.7(2)	7.0	2.0	4.0
5	Phenyl	−H	8.41	9.1	7.07	8.59	7.5(3)	8.3(2)	6.8	2.0	4.2

[a] Similar spectra have been reported (12, 13). [b] Solutions were in $CDCl_3$ (saturated) and generally were less than 1%; Compounds 2 and 5 were run in DMSO-d_6 (saturated). TMS served as the reference compound. [c] Aromatic protons for these compounds appeared as two multiplets. These data are given in the table with the number of protons, in parentheses, immediately following. [d] Resolution and sensitivity were inadequate, due to low concentration, for more than rough approximation of chemical shifts and coupling constants. For this compound in saturated solution, resolution required settings of 1000 sec. sweep time, sensitivity of 400, time constant of 1 sec., and power of 5×10^3. [e] A portion of the aromatic proton multiplet overlapped this pattern. The H-6 multiplet, in part, appeared as shoulders on the aromatic proton pattern.

from isopropyl alcohol gave 1.25 g. (74.4% of theory) of fine emerald-green needles melting at 223.5–224.5°. The IR and NMR spectra were identical in every regard to those obtained for the same compound prepared by reaction of phenylglyoxylohydroxamyl chloride with 2-aminopyrimidine.

Synthesis of 3-Nitroso-2-(4-methoxyphenyl)imidazo(1,2-*a*)pyrimidine (Compound 2, Table I)—4-Methoxyphenylglyoxylohydroxamyl chloride (2.0 g., 0.01 mole), prepared by previously described procedures (1, 2), was dissolved in 50 ml. of anhydrous ether and treated with 0.95 g. (0.01 mole) of 2-aminopyrimidine according to the previously described procedure (Method A). Thus, fine green crystals, melting at 247–248°, were obtained.

Anal.—Calcd. for $C_{13}H_{10}N_4O_2$: C, 61.40; H, 3.96; N, 22.04. Found: C, 60.78, 60.85; H, 4.22, 4.18; N, 21.87, 21.60.

Spectral data are summarized in the *Discussion* section.

Synthesis of 3-Nitroso-2-(4-chlorophenyl)imidazo(1,2-*a*)pyrimidine (Compound 3, Table I)—In a similar manner, 3.6 g. (0.02 mole) of 4-chlorophenylglyoxylohydroxamyl chloride and 1.90 g. (0.02 mole) of 2-aminopyrimidine gave 2.0 g. (41.7% of theory) of a green compound melting at 228°.

Anal.—Calcd. for $C_{12}H_7ClN_4O$: C, 55.71; H, 2.72; N, 21.66. Found: C, 56.32, 56.19; H, 2.90, 3.03; N, 21.63, 21.48.

Spectral data are given in the *Discussion* section.

Synthesis of 3-Nitroso-2-(4-methylphenyl)imidazo(1,2-*a*)pyrimidine (Compound 4, Table I)—In a similar manner, 3.58 g. (0.02 mole) of 4-methylphenylglyoxylohydroxyamyl chloride and 1.90 g. (0.02 mole) of 2-aminopyrimidine gave 1.5 g. (31.3% of theory) of green crystals melting at 253–254°.

Anal.—Calcd. for $C_{13}H_{10}N_4O$: C, 65.53; H, 4.23; N, 23.52. Found: C, 65.27, 65.08; H, 4.37; 4.17; N, 23.29, 23.48.

Spectral data are summarized in the *Discussion* section.

DISCUSSION

LaRocca and Gibson (10) reported the conversion of arylglyoxylohydroxamyl chlorides and *N,N*-bis-(2-chloroethyl)amine into the corresponding *N,N*-bis-(2-chloroethyl)arylglyoxylohydroxamamides. The present project was begun as an attempt to prepare the similar *N*-2-pyrimidinylarylglyoxylohydroxamamides from a condensation of arylglyoxylohydroxamyl chlorides with 2-aminopyrimidine. Instead of the expected products, highly colored, difficultly soluble products were obtained. The deep color and poor solubility in most solvents suggested the possible presence of a nitroso compound.

For example, the phenyl analog was prepared as described in Method A and was found to be a chalky green, amorphous powder upon initial recovery from the reaction mixture. After washing with water and recrystallizing the residue to constant melting point (185–189°) from toluene, the compound gave 23.13 and 23.50% *N* (semimicro Kjeldahl procedure) compared to a calculated value of 23.13% for the "amide," $C_{12}H_{10}N_4O_2$. On attempting to dissolve a sample of this product in deuterochloroform for NMR studies, however, the presence of two compounds was clearly indicated. TLC studies further verified this observation. A complete study by TLC further suggested that effective separation of the relatively abundant green compound from its colorless contaminant might be effected from 1- or 2-propanol, the only solvents in which the contaminant was more soluble (larger R_f value) than the principal product.

Recrystallization of a 1.0-g. sample of the crude, water-washed product required approximately 175 ml. of 2-propanol. After two recrystallizations, a pure product was obtained which amounted to 52.1% of theory and, based on recovery percentages, constituted about 80% by weight of the original, crude product. Similarly, about 20% of the original sample appeared to be composed of the white solid.

The IR spectra[5] of the crude product, when compared with that of the purified green compound, revealed that the impurity possessed a prominent amide carbonyl band at 1695 cm.$^{-1}$ and a strong C=N stretch frequency at 1655 cm.$^{-1}$. It cannot be stated that this colorless impurity is, indeed, the expected *N*-2-pyrimidinylphenylglyoxylohydroxamamide, since complete purification and characterization were not effected (11).

The purified principal product of this reaction was identified as 3-nitroso-2-phenylimidazo(1,2-*a*)pyrimidine in the following way. 2-Phenylimidazo(1,2-*a*)pyrimidine, whose synthesis previously was described (3), was prepared and nitrosated as described using Method B. The nitrosation would therefore have taken place at one of the unoccupied ring sites, *i.e.*, carbons 3, 5, 6, or 7. The NMR spectrum[6] showed three typical 1:1:1:1 quadruplet patterns with *J* values of >2.0 Hz., thus establishing the 3-nitroso isomer. Differences observed in the chemical shifts of the aromatic protons and loss of the one-proton singlet (H-3) on nitrosation gave further confirmation of the site of substitution. The H-3 singlet, observed at 8.4 p.p.m. in the spectrum of 2-phenylimidazo (1,2-*a*) pyrimidine, disappeared on nitrosation. Furthermore, the phenyl protons, which in the starting material appeared as two complex multiplets centered at about 7.5 and 8.3 p.p.m. and integrating for three and two protons, respectively, were found centered at 7.6 and 8.7 p.p.m. after nitrosation.

The NMR and IR absorption spectra for the phenyl analog prepared by nitrosation (Method B) and those for the same compound prepared by ring closure (Method A) were in every measurable respect identical. Table I gives the NMR data for the compounds prepared.

The IR spectra for all four of the nitroso-containing products were devoid of any absorption bands in the 1800 to 1650 cm.$^{-1}$ region where carbonyl and C=N frequencies normally are expected. Furthermore, all four of these compounds possess a moderately strong absorption at 1530 cm.$^{-1}$, which probably can be assigned as the C—N=O stretch frequency with reasonable confidence (11).

In conclusion, the products isolated from the condensation of 2-aminopyrimidine with arylglyoxylohydroxamyl chlorides have been identified as 3-nitroso-2-arylimidazo(1,2-*a*)pyrimidines.

PHARMACOLOGICAL RESULTS

Almirante *et al.* (4) investigated a series of 2-arylimidazo(1,2-*a*)-pyrimidine analogs for analgesic, anti-inflammatory, antipyretic,

[5] Perkin-Elmer model 237-B.
[6] Varian HA-100.

Table II—Antimicrobial Activity (*In Vitro*) of 3-Nitroso-2-arylimidazo(1,2-*a*)pyrimidines[a,b]

Compound	Organisms[c]			
	S. aureus[d]	*E. coli*	*C. albicans*	*C. neoformans*
2-Phenyl	1	1	1	2
2-*p*-Methylphenyl	1	0	1	1
2-*p*-Chlorophenyl	1	2	2	2
2-*p*-Methoxyphenyl	0	0	0	0

[a] Data for only four organisms are included. A total of 22 organisms was tested, however. [b] Activity ranges were as follows: 0, inactive at the 200 mcg./ml. level; 1, active in the 100–200 mcg./ml. level; and 2, active in the 40–99 mcg./ml. level. [c] *S. aureus* ranked fourth of four Gram-positive organisms tested in susceptibility, *i.e.*, poorest response; *E. coli* ranked third of four Gram-negative bacteria; *C. albicans* ranked third (tie) of five fungi tested; and *C. neoformans* ranked first of two yeasts tested. [d] Resistant strain.

and anticonvulsant properties. Their investigation revealed significant activity of the analgesic and anti-inflammatory types. Several of the compounds were active as antipyretic and hypothermic agents. One compound afforded protection against strychnine convulsions, while several of the compounds protected somewhat against electroshock and/or pentamethylenetetrazole seizures. Almirante *et al.* (4) concluded that while interesting pharmacological activity had been uncovered, no satisfactory structure–activity relationships could be formulated. Further, they noted that none of these compounds was as active as the previously reported imidazo(1,2-*a*)-pyridines (5).

Collins *et al.* (14) studied the hydrobromide salt of 2-phenylimidazo(1,2-*a*)pyrimidine (U-13,376B) as a strychnine antagonist. They reported that while this compound is a potent strychnine antagonist, it also shows strychninelike toxicity, albeit only 1/1000th as potent. This suggests that the compound functions as a partial agonist of the strychnine type (14).

The compounds prepared in this study were evaluated as potential antimicrobial and antiviral agents.[7] A total of 22 microbial agents, two helminths, and three viruses were used in the *in vitro* screening. All of the nitroso derivatives were inactive as anthelmintics and as antiviral agents. Using the results of tests *versus Staphylococcus aureus* (resistant strain), *Escherichia coli*, *Candida albicans*, and *Cryptococcus neoformans* as examples of Gram-positive bacteria, Gram-negative bacteria, fungi, and yeast, respectively, the spectrum of activity of these compounds was tabulated (Table II). 3-Nitroso-2-(4-chlorophenyl)imidazo(1,2-*a*)-pyrimidine was also examined *in vivo* using *Klebsiella pneumoniae*-infected Dierolf mice (18–20-g. males). A 20% survival at 250 mg./kg. *versus* 20% survival with kanamycin at 1 mg./kg. was observed. The compound was administered in water by the subcutaneous route. The general rank of activity seems remarkably parallel to the solubility of these compounds, all of which are difficultly soluble at best.

[7] Antimicrobial and antiviral screens were performed courtesy of Smith Kline & French Laboratories. The authors are indebted to Dr. Paul N. Craig of SKF for providing the screening data cited in this section.

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Enhancement of Fatty Acid Oxidation and Medium-Chain Fatty Acyl Coenzyme A Synthetase by Adenine Nucleotides in Rat Heart Homogenates

V. GENE ERWIN, A. DUANE ANDERSON, and GRETHE JURGENSEN EIDE

Abstract □ Cyclic 3′,5′-adenosine monophosphate, 5′-adenosine monophosphate, or 2′-adenosine monophosphate markedly enhanced the rate of oxidation of medium-chain fatty acids by rat heart homogenates, as measured by oxygen utilization and carbon dioxide formation from ^{14}C-labeled substrate. These nucleotides did not alter the rate of oxidation of medium-chain acylcoenzyme A derivatives. The activity of a medium-chain fatty acyl coenzyme A synthetase from rat heart homogenates was increased by these nucleotides, and it was suggested that the ability of the nucleotides to enhance fatty acid oxidation by heart homogenates was due to activation of acyl coenzyme A synthetase.

Keyphrases □ Adenine nucleotides—rat heart homogenates □ Oxygen-uptake enhancement, rat heart homogenates—adenine nucleotides □ $^{14}CO_2$-evolution, 1-^{14}C-octanoate—adenine nucleotide enhancement □ Acyl coenzyme A synthetase—rat heart nucleotide effect □ Scintillometry—analysis

Recent observations suggest that various nucleotides, including 5′-adenosine monophosphate (5′-AMP) and cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP), may be involved in the regulation of energy metabolism (1). The ability of 5′-AMP and 5′-adenosine diphosphate (5′-ADP) to enhance liver glutamate dehydrogenase activity has been well established (2, 3). In addition, these nucleotides have been found to alter the catalytic activity of isocitric dehydrogenase (4) and phosphofructokinase (5). Mansour (6) showed that 5′-AMP and cyclic 3′,5′-AMP activate phosphofructokinase isolated from guinea pig heart and from a parasitic liver fluke.

Studies by many investigators (7–16) indicated that cyclic 3′,5′-AMP, which is formed from adenosine triphosphate (ATP) by the enzyme adenyl cyclase, is involved in the regulation of various metabolic pathways including glycogenolysis, lipolysis, and steroidogenesis. Recently Harada *et al.* (17) reported that starvation of animals on alloxan treatment produced a marked increase in acylcoenzyme A synthetase activity. In addition, Bar-Tana and Rose (18, 19) observed heterotropic cooperative effects with 5′-ATP or 5′-AMP on a partially purified medium-chain acyl coenzyme A synthetase from bovine liver. Inasmuch as the heart can derive much of its required energy from fatty acid oxidation (20–22), the actions of various nucleotides, including cyclic 3′,5′-AMP, on the metabolism of fatty acids were investigated.

MATERIALS AND METHODS

All nucleotides[1] used in this study were found to be 99% pure when assayed spectrophotometrically. They were demonstrated to be homogeneous by a descending paper chromatographic technique with a solvent system consisting of 3 parts 1 *M* ammonium acetate (adjusted to pH 7.5 with ammonium hydroxide) and 7 parts 95% ethanol. Molar absorbances and R_f values were virtually identical to values reported previously (23).

Adult Sprague-Dawley rats of either sex were decapitated, and the hearts were homogenized in sufficient 0.25 *M* sucrose to make a 10% suspension. Homogenization was performed in a tight-fitting glass homogenizer by several passes of a Teflon pestle, driven at approximately 1200 r.p.m. The homogenate (0.5 ml.) was then added to Warburg flasks containing 0.1 m*M* ATP; 0.03 m*M* CoA; 0.03 m*M* $MgCl_2$; 0.3 m*M* nicotinamide; 1 m*M* theophylline; 0.1 m*M* carnitine; 4 m*M* phosphate buffer, pH 7.4; and 0.17 m*M*

[1] Obtained from Sigma Chemical Co., St. Louis, Mo.

Table I—Effects of Adenine Nucleotides on the Rates of Oxidation of Various Substances by Heart Homogenates

Substrate	Nucleotide	Q_{O_2}[a]	Percent Stimulation
Octanoate[b]	None	1.92 ± 0.21	—
Octanoate[b]	3′,5′-AMP	5.82 ± 0.40	203
Octanoate[b]	5′-AMP	5.97 ± 0.42	211
Octanoate[b]	2′-AMP	5.34 ± 0.39	178
Butyrate	None	2.08 ± 0.25	—
Butyrate	3′,5′-AMP	5.74 ± 0.38	176
Butyrate	5′-AMP	6.10 ± 0.42	193
Butyrate	2′-AMP	5.00 ± 0.40	140
β-Hydroxybutyrate	None	2.96 ± 0.29	—
β-Hydroxybutyrate	3′,5′-AMP	5.20 ± 0.38	75
β-Hydroxybutyrate	5′-AMP	6.89 ± 0.39	133
β-Hydroxybutyrate	2′-AMP	5.55 ± 0.35	87
Pyruvate	None	4.42 ± 0.26	—
Pyruvate	3′,5′-AMP	8.00 ± 0.37	81
Pyruvate	5′-AMP	8.30 ± 0.40	88
Pyruvate	2′-AMP	8.25 ± 0.37	86
α-Ketoglutarate	None	3.52 ± 0.30	—
α-Ketoglutarate	3′,5′-AMP	9.22 ± 0.41	162
α-Ketoglutarate	5′-AMP	9.46 ± 0.46	169
α-Ketoglutarate	2′-AMP	7.70 ± 0.39	119
Glutamate	None	3.98 ± 0.20	—
Glutamate	3′,5′-AMP	4.88 ± 0.40	22
Glutamate	5′-AMP	6.85 ± 0.36	72
Glutamate	2′-AMP	4.91 ± 0.30	23
Succinate	None	9.28 ± 0.31	—
Succinate	3′,5′-AMP	10.79 ± 0.21	16[c]
Succinate	5′-AMP	10.53 ± 0.35	13
Succinate	2′-AMP	10.01 ± 0.40	7
Malate	None	3.25 ± 0.19	—
Malate	3′,5′-AMP	3.45 ± 0.23	—
Malate	5′-AMP	3.18 ± 0.31	—
Malate	2′-AMP	3.85 ± 0.26	—
Citrate	None	2.44 ± 0.18	—
Citrate	3′,5′-AMP	2.12 ± 0.17	—
Citrate	5′-AMP	2.17 ± 0.22	—
Citrate	2′-AMP	2.00 ± 0.20	—

[a] Q_{O_2} values represent microliters of oxygen uptake per milligram protein per 22.5 min. at 37° and represent the average of four–five experiments with the standard error of the mean. Corrections were made for oxidation of endogenous substrates in the presence or absence of nucleotide. The average value from 10 separate experiments for the endogenous rate of oxygen uptake in the presence or absence of nucleotide was 1.30 μl./22.5 min. [b] Octanoate concentration was 0.17 m*M*, which was found to give optimal rates of oxygen uptake. [c] These results are not significant at the 95% probability level as shown by the Student *t* test. All other results are significant at this level.

Table II—Effect of Theophylline on Conversion of Cyclic 3′,5′-AMP to 5′-AMP by Heart Homogenates

Minutes	Theophylline, m*M*	5′-AMP, c.p.m.[a]	Cyclic 3′,5′-AMP, c.p.m.	% 3′,5′-AMP Hydrolyzed
15	0	269	4466	5.6
15	1.0	116	4857	2.3
30	0	585	4428	11.6
30	1.0	319	4340	6.8
45	0	900	4225	17.5
45	1.0	497	4353	10.2

[a] Cyclic 3′,5′-AMP (Schwartz) contained approximately 3% 5′-AMP (representing about 200 c.p.m. in these experiments), and the values in this column were appropriately corrected. Experimental details are given in the text.

nucleotide or water. The side arms contained substrate and malate (24). The total volume was 3.0 ml., and the pH of each added solution was 7.4. All reagents used were the highest quality available and were prepared in triple-distilled water. After an equilibration period of 10 min., the flasks were closed to the atmosphere, the substrate was tipped from the side arms, and oxygen uptake was measured at 7.5-min. intervals for 45 min. at 37° (25). All rates of oxygen uptake were linear for 22.5 min. Each determination of oxygen uptake was performed in triplicate and repeated at least three times.

Octanoyl coenzyme A and butyryl coenzyme A were prepared by the method of Stadtman (26). The reaction of butyric anhydride or octanoic anhydride with coenzyme A was conducted under an atmosphere of N_2, and completion of the reaction was determined by the nitroprusside test for free sulfhydryl groups. Octanoyl coenzyme A or butyryl coenzyme A was then separated from the reaction mixture on a 1 × 50-cm. synthetic polysaccharide[2] column. The concentration of the thioester was determined by the method of Lipmann and Tuttle (27) with sodium butyrohydroxamate as a standard. The yield of butyryl coenzyme A was 90% based on the amount of coenzyme A used in the reaction. The yield of octanoyl coenzyme A was slightly less than 90%. The preparations were not contaminated with free unoxidized coenzyme A, as indicated by an absence of color formation with nitroprusside or with Ellman (28) reagent [5,5′-dithiobis-(2-nitrobenzoic acid)].

Studies with 1-^{14}C-octanoate were performed in double side-arm Warburg flasks closed with glass stoppers. The main compartment of each flask contained the necessary reagents as previously described. At zero time, 0.1 ml. of 5.0 m*M* 1-^{14}C-octanoate (specific activity 0.57 mc./mmole) was tipped from the first side arm, and the flasks were allowed to shake gently for 45 min. at 37° in a Dubnoff metabolic shaker. The reaction was stopped by tipping H_2SO_4 from the second side arm of each flask, followed by a 30-min. incubation period to allow the $^{14}CO_2$ to be trapped in the center wells. The center wells contained 0.2 ml. of a mixture of 0.01 mmole NaOH and 1.5 mmoles piperidine. After incubation, aliquots were transferred to vials containing scintillation fluid, which consisted of 7 g. PPO and 0.42 g. POPOP/l. of toluene.[3] Determination of radioactivity was performed with a Packard liquid scintillation spectrometer, model 3310. In all studies, values for background radioactivity were obtained by using a boiled enzyme preparation; values reported in this paper were corrected for this activity.

The activity of a medium-chain fatty acyl coenzyme A synthetase from rat heart homogenates with butyrate or octanoate as substrates was determined by the method of Kornberg and Pricer (29), using hydroxylamine to trap the acylthioesters. The incubation medium was as described with 0.3 *M* hydroxylamine and 0.1 *M* tromethamine buffer, pH 8.0. All components were adjusted to pH 8.0 except coenzyme A. Reaction mixtures without fatty acid substrates or ATP served as controls, and the reactions were initiated by addition of homogenate. After incubation at 37° for 30 min., the reaction was stopped by addition of 1 ml. of a freshly prepared solution of 10% $FeCl_3$ in 0.2 *N* HCl with 7% perchloric acid. Following centrifugation to sediment protein, the optical density of the supernatant was determined at 520 nm. All protein determinations were performed by the biuret procedure with bovine serum albumin as a standard.

[2] Sephadex G-10, Pharmacia Fine Chemicals, Piscataway, N. J.

[3] PPO refers to 2,5-dimethylphenyloxazole, and POPOP represents 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene.

Table III—Enhancement of Octanoate Oxidation by Cyclic 3′,5′-AMP or 5′-AMP in Heart Homogenates Aged for Various Times at 0°

Minutes after Preparation of Homogenate	Nucleotide	Q_{O_2}[a]	Increase, %
10	None	1.73 ± 0.21	—
10	5′-AMP	5.70 ± 0.45	229
10	3′,5′-AMP	5.61 ± 0.39	224
20	None	0.35 ± 0.05	—
20	5′-AMP	2.93 ± 0.17	737
20	3′,5′-AMP	2.58 ± 0.20	637
30	None	0.11 ± 0.03	—
30	5′-AMP	2.82 ± 0.33	2463
30	3′,5′-AMP	2.73 ± 0.29	2381

[a] Experimental conditions were as described in Table I and the text. Corrections were made for endogenous oxygen uptake as in Table I, except Q_{O_2} values for the 10-, 20-, and 30-min. times were 1.27, 1.23, and 0.64 μl. oxygen uptake/22.5 min., respectively. Sufficient nucleotide was added to give final concentrations of 0.17 m*M*. Ten percent heart homogenates were aged at 0° for the times indicated before being added to the flasks. Q_{O_2} values represent microliters of oxygen uptake per milligram protein/22.5 min. at 37° and represent the average of four experiments with the standard deviation. Results are significant at the 95% probability level.

The effect of theophylline on the conversion of cyclic 3′,5′-AMP to 5′-AMP by heart homogenates was determined by incubating 0.33 m*M* 8-^{14}C-cyclic 3′,5′-AMP (specific activity 1.0 μc./μmole) with a complete reaction mixture as already described. The reaction mixture was incubated for various times at 37° in the presence or absence of 1 m*M* theophylline. The reaction was stopped by placing the tubes in boiling water for 10 min. Following centrifugation, aliquots were applied along with unlabeled nucleotides to polyethylene imin-impregnated cellulose thin-layer plates. After the plates were developed with 0.1 *M* formate buffer, pH 3.3, spots observed with a UV lamp were removed from the plates, placed in PPO and POPOP scintillation fluid, and counted. Each determination was performed in duplicate; values represent the averages of two experiments.

RESULTS

Enhancement of Oxygen Uptake in Rat Heart Homogenates by Adenine Nucleotides—As shown in Table I, 0.17 m*M* cyclic 3′,5′-AMP, 5′-AMP, or 2′-AMP enhanced the rate of oxidation of octanoate, butyrate, or DL-β-hydroxybutyrate by rat heart homogenates approximately two or threefold. The rates of oxidation of the fatty acids were linear for 22.5 min. in the absence or presence of nucleotide. Data presented in Table I show that these nucleotides did not markedly alter the rate of oxidation of citrate, succinate, malate, or glutamate by heart homogenates. However, the rates of oxidation of pyruvate or α-ketoglutarate were enhanced by the nucleotides.

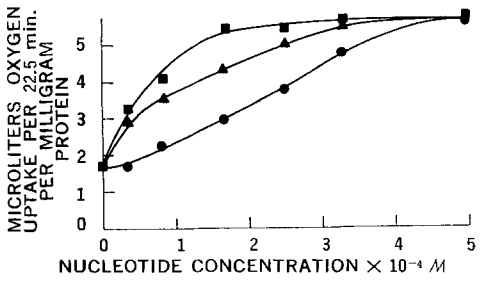

Figure 1—*Enhancement of octanoate oxidation by various concentrations of nucleotides. Conditions are as described in Table I and the text; each point represents the average of four experiments. Final concentrations of nucleotides are as shown. Key: ■, cyclic 3′,5′-AMP; ▲, 2′-AMP; and ●, 5′-AMP.*

Table IV—Enhancement of Carbon Dioxide Formation in Rat Heart Homogenates by Cyclic 3′,5′-AMP and 5′-AMP with 1-^{14}C-Octanoate as Substrate

Nucleotide	c.p.m.[a]	Stimulation, %
None	1105	—
Cyclic 3′,5′-AMP	3896	253
5′-AMP	3481	215

[a] For procedures, see text. Values are the average of three separate experiments.

Since it has been demonstrated that the presence of carnitine may influence the rate of oxidation of fatty acids (30, 31), the effects of this compound on the rate of fatty acid oxidation in the presence or absence of nucleotides were examined. Carnitine did not affect the rate of oxidation of butyrate or DL-β-hydroxybutyrate, but this compound produced a slight increase in the rate of oxygen uptake with octanoate as the substrate. The presence of carnitine did not alter the enhancement of oxygen uptake produced by the nucleotides with octanoate as the substrate. However, to assure that the concentration of carnitine would not be rate limiting in the oxidation of the fatty acids, all experiments were conducted with 0.1 m*M* carnitine in the reaction mixture. This concentration of carnitine was above optimal under these conditions.

It is known that heart contains the enzyme cyclic 3′,5′-phosphodiesterase, which catalyzes the conversion of cyclic 3′,5′-AMP to 5′-AMP (32). Therefore, all results presented in this paper were obtained with 1 m*M* theophylline, previously reported to be an inhibitor of cyclic 3′,5′-phosphodiesterase (33), in the reaction mixture. However, it has been reported that 1 m*M* theophylline is ineffective in completely inhibiting phosphodiesterase activity (33). Therefore, the possibility that the enhanced oxygen produced by 3′,5′-AMP may be due to conversion of this nucleotide to 5′-AMP was investigated. To examine this possibility, 8-^{14}C-cyclic 3′,5′-AMP[4] in a final concentration of 0.33 m*M* was incubated at 37° for various times with 0.5 ml. of 10% heart homogenate in a complete reaction mixture in the presence or absence of 1 m*M* theophylline. As shown in Table II, after 45 min. approximately 17.5% of the cyclic nucleotide was converted to 5′-AMP in the absence of theophylline. In the presence of 1 m*M* theophylline, 10% of the cyclic 3′,5′-AMP was converted to 5′-AMP. Inasmuch as only 10% of the added cyclic 3′,5′-AMP was hydrolyzed, the enhancement of oxygen uptake by the cyclic nucleotide probably was not due to 5′-AMP. In addition, if conversion of cyclic 3′,5′-AMP to 5′-AMP was involved in increasing octanoate oxidation, it is unlikely that linear rates of oxygen uptake would have been observed.

Enhancement of Oxygen Uptake in Rat Heart Homogenates by Various Concentrations of Nucleotides—As shown in Fig. 1 with heart homogenate, which was used 10 min. after preparation, the minimum concentration of cyclic 3′,5′-AMP or 2′-AMP that was effective in significantly augmenting octanoate oxidation by rat heart homogenates was 0.033 m*M*. A maximum enhancement was obtained with approximately 0.5 m*M* final concentration of the nucleotides. At low concentrations, 5′-AMP produced less enhancement of oxygen uptake than cyclic 3′,5′-AMP, but results obtained with 5′-ADP were virtually identical to those found with the cyclic nucleotide. When 5′-AMP and cyclic 3′,5′-AMP were added to the same reaction mixture, the percent stimulation of octanoate oxidation was additive at total nucleotide concentrations less than 0.5 m*M*.

The data presented in Table III show that the rate of oxidation of octanoate by heart homogenates in the absence of nucleotide was greatly diminished if the preparations were allowed to stand for 30 min. at 0° before use. Since the rate of oxygen uptake in the presence of nucleotide was not as markedly altered, the enhancement of octanoate oxidation produced by the nucleotides was increased from 3.3 to 24-fold for homogenates aged at 0° for 10 min. compared to those aged for 30 min.

To provide further indication of the ability of 5′-AMP or cyclic 3′,5′-AMP to enhance octanoate oxidation, the rate of $^{14}CO_2$-evolution from 1-^{14}C-octanoate was examined. As shown in Table IV, the amount of $^{14}CO_2$-evolution was increased 253% by 0.33 m*M* cyclic 3′,5′-AMP. When 5′-AMP was added to the incubation mixture, a 215% increase in $^{14}CO_2$-formation was observed.

[4] Schwartz Bio Research, Inc.

Table V—Effect of Various Nucleotides on Oxygen Uptake by Rat Heart Homogenates with Octanoate as Substrate

Nucleotide	Q_{O_2}[a]	Increase, %
None	1.90 ± 0.20	—
3′,5′-AMP	5.41 ± 0.36	185
5′-AMP	5.15 ± 0.27	171
5′-GDP	3.84 ± 0.29	102
5′-GTP	5.30 ± 0.39	179
5′-ADP	5.54 ± 0.41	192
3′,5′-Dibutyryl AMP	5.38 ± 0.37	183
2′-AMP	4.27 ± 0.16	125
2′,3′-AMP	1.90 ± 0.10	—
3′-AMP	2.60 ± 0.23	37
Adenosine	2.06 ± 0.12	—
Adenine	1.93 ± 0.20	—
3′,5′-GMP	2.70 ± 0.19	42

[a] Experimental conditions are as described in the text, and values are expressed as in Table I. Results are significant at the 95% probability level as shown by the Student *t* test.

Specificity of Action of Nucleotides—As shown in Table V, cyclic 3′,5′-dibutyryl AMP, 5′-ADP, or 5′-guanosine triphosphate (5′-GTP) was as effective as cyclic 3′,5′-AMP, 5′-AMP, or 2′-AMP in enhancing oxygen uptake by rat heart homogenates with octanoate as the substrate. However, cyclic 2′,3′-AMP, 3′-AMP, adenosine, or adenine produced little change in the rate of octanoate oxidation. In addition, cyclic 3′,5′-guanosine monophosphate (3′,5′-GMP) or 5′-guanosine diphosphate (5′-GDP) was not as effective in increasing the rate of oxygen uptake as the corresponding adenine nucleotides.

Effects of Nucleotides on Rat Heart Acyl Coenzyme A Synthetase—To determine the site of action of the nucleotides on fatty acid oxidation, studies were performed on the effects of cyclic 3′,5′-AMP or 5′-AMP on oxidation of octanoyl coenzyme A, butyryl coenzyme A, or acetoacetyl coenzyme A. As shown in Table VI, the rates of oxidation of these thioesters were not altered by the nucleotides. The results presented in Table VI are of particular interest when the rates of oxidation of the thioesters are compared with the rates of oxidation of the corresponding fatty acids. In the absence of the nucleotides, 5′-AMP or cyclic 3′,5′-AMP, octanoyl coenzyme A and butyryl coenzyme A were oxidized at rates appreciably faster than their corresponding fatty acids, indicating that under these conditions acyl coenzyme A synthetase activity is rate limiting.

Since it is likely that cyclic 3′,5′-AMP or 5′-AMP enhances the rate-limiting step in the oxidation of fatty acids, the effects of these nucleotides on a medium-chain fatty acyl coenzyme A synthetase from rat heart homogenates were investigated. As shown in Table VII, the activity of the enzyme with butyrate as the substrate was increased 100, 66, or 60% by 0.33 m*M* cyclic 3′,5′-AMP, 5′-AMP, or 2′-AMP, respectively. Nucleotide specificity similar to

Table VI—Effects of Nucleotides on the Rate of Oxidation of Acyl Coenzyme A Derivatives

Substrate[a]	Nucleotide	Q_{O_2}
Acetyl coenzyme A	None	0.90 ± 0.11
Acetyl coenzyme A	Cyclic 3′,5′-AMP	0.95 ± 0.13
Acetyl coenzyme A	5′-AMP	1.05 ± 0.09
Butyryl coenzyme A[a]	None	3.82 ± 0.19
Butyryl coenzyme A[a]	Cyclic 3′,5′-AMP	3.60 ± 0.37
Butyryl coenzyme A[a]	5′-AMP	3.80 ± 0.41
Octanoyl coenzyme A[a]	None	4.55 ± 0.26
Octanoyl coenzyme A[a]	Cyclic 3′,5′-AMP	4.61 ± 0.35
Octanoyl coenzyme A[a]	5′-AMP	4.32 ± 0.42
Acetoacetyl coenzyme A	None	2.00 ± 0.39
Acetoacetyl coenzyme A	Cyclic 3′,5′-AMP	2.12 ± 0.40
Acetoacetyl coenzyme A	5′-AMP	2.04 ± 0.39

[a] Of a number of concentrations used, 1.0 m*M* was found to give optimal rates of oxygen uptake. After 22.5 min., at least 70% of the added acylthioesters was present as measured by hydroxamate formation (25). Experimental procedures are as described in the text.

Table VII—Activation of Medium-Chain Fatty Acyl Coenzyme A Synthetase from Rat Heart Homogenate by Various Nucleotides

Substrate	Nucleotides	Thioester,[a] nanomoles
Butyrate	None	4.8
Butyrate	3′,5′-AMP	9.6
Butyrate	5′-AMP	8.0
Butyrate	2′-AMP	7.6
Butyrate	3′-AMP	5.2
Butyrate	2′,3′-AMP	4.0
Octanoate	None	10.8
Octanoate	3′,5′-AMP	20.0
Octanoate	5′-AMP	15.2
Octanoate	2′-AMP	14.8
Octanoate	3′-AMP	11.2
Octanoate	2′,3′-AMP	11.6

[a] Represents nanomoles of butyrohydroxamate or octanoylhydroxamate formed per 30 min. per milligram protein, based on a molar absorbance coefficient of 1000 for the acyl hydroxamates (29).

that obtained with oxygen uptake was observed in that 3′-AMP or cyclic 2′,3′-AMP was without effect on hydroxamate formation. It has been reported that the high concentrations of hydroxylamine used to determine the rate of thioester formation in crude tissues may inhibit the acyl coenzyme A synthetase reaction (29). The inhibitory effect of hydroxylamine might also prevent maximal activation of the enzyme by the nucleotides, which might account for the differences in enhancement of hydroxamate formation compared to the increase in oxygen uptake produced by the nucleotides.

Inasmuch as Bar-Tana and Rose (18, 19) observed that high concentrations of ATP activated a medium-chain fatty acyl coenzyme A synthetase from bovine liver and since it appeared that the activity of this enzyme was rate limiting in the overall rate of fatty acid oxidation by heart homogenates, the rate of oxygen uptake at various concentrations of ATP in the presence or absence of cyclic 3′,5′-AMP with octanoate as substrate was determined. As shown in Fig. 2, when the rate of oxygen uptake by heart homogenates is plotted as a function of ATP concentration, a sigmoidal curve is obtained with a maximum rate at 0.33 m*M* ATP. At this concentration of ATP, 0.33 m*M* cyclic 3′,5′-AMP was without effect; however, in the presence of the cyclic nucleotide the curve obtained was hyperbolic, showing that at concentrations of 0.02–0.1 m*M* ATP, cyclic 3′,5′-AMP markedly enhanced octanoate oxidation.

DISCUSSION

The initial step in the oxidation of fatty acids requires "activation" of the fatty acid by acyl coenzyme A synthetase to give an acyl coenzyme A thioester. This reaction is followed by β-oxidation of the acyl group, apparently requiring carnitine transferase and the β-oxidative enzymes (24). Since 5′-AMP, 5′-ADP, and cyclic 3′,5′-AMP enhanced the oxidation of fatty acids but were without effect on the more rapidly oxidized thioesters, it is suggested that these nucleotides increase the rate of the acyl coenzyme A synthetase enzyme reaction. Direct evidence in support of this proposal was obtained by the observation that 5′-AMP or cyclic 3′,5′-AMP increased the rate of formation of acyl hydroxamates in rat heart homogenates.

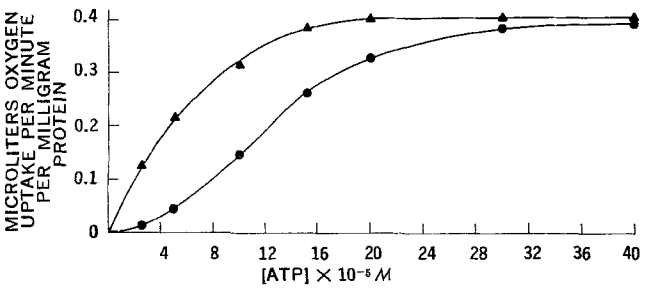

Figure 2—*Effects of cyclic 3′,5′-AMP on octanoate oxidation by rat heart homogenates as a function of ATP concentration. Conditions are as described in Table I. All rates were linear for 22.5 min., and the values of oxygen uptake per minute per milligram protein were calculated from the respective slopes. The final concentration of cyclic 3′,5′-AMP (▲) was 0.33 m*M*, and each value represents the average of three experiments; ● represents values obtained in the absence of cyclic nucleotide.*

As shown in the results, a sigmoidal curve was obtained when the rate of oxygen uptake was plotted as a function of ATP concentration in the absence of cyclic 3′,5′-AMP; in the presence of the cyclic nucleotide, the curve was hyperbolic. It would be interesting to speculate that these results may be due to a cooperative interaction of the nucleotides with acyl coenzyme A synthetase, particularly since Bar-Tana and Rose (18, 19) showed that ATP and 5′-AMP functioned as heterotropic cooperative ligands for the partially purified enzyme from bovine liver. This speculation is possible, because it appears that the rate of oxygen uptake is dependent upon the rate of the acyl coenzyme A synthetase reaction. However, inasmuch as crude heart homogenates were used in these studies, other explanations for the data are possible. Studies on the mechanism of activation of medium-chain acyl coenzyme A synthetase from heart by nucleotides are in progress, but the extreme lability of the enzyme makes such studies difficult. Also, reliable kinetic data are difficult to obtain due to the presence of acyl coenzyme A deacylase, even in the partially purified preparations, which interferes with the standard assay procedures.

Robison *et al.* (34) reported the normal levels of cyclic 3′,5′-AMP in rat heart to be approximately 0.4 μ*M;* after perfusion with epinephrine, this level rapidly increased to 1.4 μ*M*. The concentrations of 5′-AMP and 5′-ADP in heart tissues have been reported to be approximately 0.2 and 0.4 m*M*, respectively (35). The results reported here show that 0.2 m*M* 5′-AMP or 5′-ADP appreciably enhanced fatty acid oxidation and that higher concentrations (Fig. 1) were required for maximal activation. However, the concentration of cyclic 3′,5′-AMP in heart tissue is much lower than that required to augment octanoate oxidation in this study. To what extent these nucleotides are involved in the regulation of heart fatty acid oxidation remains to be elucidated.

SUMMARY

Cyclic 3′,5′-AMP, 5′-AMP, or 2′-AMP enhanced the rate of oxygen uptake by rat heart homogenates with octanoate, butyrate, or DL-β-hydroxybutyrate as the substrate approximately 3.0-, 3.1-, or 2.8-fold, respectively. In studies performed with 1-^{14}C-octanoate as the substrate, 5′-AMP or cyclic 3′,5′-AMP produced a 2.5-fold increase in the rate of $^{14}CO_2$-evolution by heart homogenates. However, these nucleotides did not alter the rate of oxidation of octanoyl coenzyme A, butyryl coenzyme A, or acetoacetyl coenzyme A. Similarly, these nucleotides produced little effect on the oxidation of various citric acid cycle intermediates or glutamate by rate heart homogenates. The rate of butyrate or octanoate oxidation by rat heart homogenates was not altered by cyclic 2′,3′-AMP, 3′-AMP, adenosine, or adenine. However, 5′-ADP, 3′,5′-dibutyryl-cyclic AMP, 5′-GDP, or 5′-GTP enhanced fatty acid oxidation. Under the conditions used, 5′-AMP, 2′-AMP or cyclic 3′,5′-AMP was effective in augmenting fatty acid oxidation at concentrations as low as 0.025 m*M*.

The nucleotides increased the activity of a medium-chain fatty acyl coenzyme A synthetase from rat heart homogenates, and it is suggested that the ability of the nucleotides to enhance fatty acid oxidation by heart homogenates is due to activation of acyl coenzyme A synthetase.

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Abstract □ Working under the hypothesis that sympathetic nerve stimulation is involved in the degranulation of enterochromaffin cells in the duodenum of the guinea pig, a series of experiments utilizing both chemical and immunological sympathectomy was performed. Once sympathectomy was established, histological examination of the duodenum revealed enterochromaffin cell granulation was unaffected by peripheral vagal stimulation. This would tend to suggest adrenergic, and not cholinergic, fibers are implicated in the degranulation process in the guinea pig. This does not conflict with previous reports that vagal stimulation induces degranulation, because it has been shown that sympathetic fibers run in the vagal trunk of the guinea pig.

Keyphrases □ Enterochromaffin cell system, guinea pig—vagal adrenergic degranulation □ Sympathectomy, chemical, immunological—guinea pig □ Serotonin release—vagal stimulation effect □ Vagal stimulation effect—enterochromaffin cells

It is well known that the duodenum is the predilection site of the enterochromaffin cell (EC) and serotonin (5-HT) occurrence in the alimentary canal of the guinea pig (1). On the other hand, the exact physiologic release mechanism of liberated 5-HT from the EC in the intestinal tract is still largely unknown. Previously, the authors reported (2) that adequate electrical stimulation of the cervical vagus peripherally produced a reduction in argentaffin-positive EC granularity in guinea pig duodena.

Although no synaptic contacts to EC have been detected in mouse colon glands, in cells containing granules characteristic of EC, continuity has been established in a few cases between their basal processes and mucosal nerve fasciculi (3). By fluorescent microscopy, Gabella and Costa (4) traced adrenergic fibers to epithelial structures in the guinea pig gut. Jacobowitz (5) also claimed to follow some fluorescent fibers from perivascular plexuses to the surface epithelium in the mucosa of the cat and monkey small intestine. Whether the EC system is directly influenced by these nerves or affected only by sympathetic transmitter released from adjacent perivascular nerves remains to be seen.

Data from a previous study (2) seemed to indicate that EC granulation may have occurred *via* a noncholinergic mechanism. This suggestion, plus the fact that sympathetic fibers within the vagus were reported for the cat (6), the dog (7), and others (8), supports the contention that adrenergic fibers present in the guinea pig vagus may have been stimulated. Therefore, the present study is designed to provide further information on the nature of vagal degranulation of the EC system in the guinea pig duodenum.

METHODS

Animals—Albino male guinea pigs of the same strain and about 400 g. were used.[1] After an appropriate acclimatization period and a 24-hr. fast from a commercial pellet food (Purina), each guinea pig

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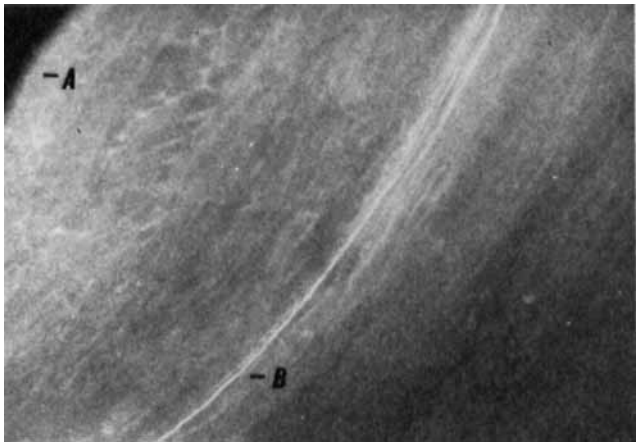

Figure 1—*Longitudinally sectioned vagus nerve of guinea pig. Key: A, edge of cervical trunk (X160); and B, fluorescent slender fiber seen in the nerve trunk 48 hr. before vagectomy.*

was intraperitoneally anesthetized with a preparation[2] containing 100 mg. of urethan and 400 mg. of diallylbarbital/ml. in a dosage of 0.8 ml./kg. of body weight.

Design—One hundred fifteen animals were randomly divided into 18 sham control and eight test groups of approximately 12 animals each subjected to: (*a*) peripheral vagal stimulation (P.V.S.); (*b*) immunosympathectomy (I.M.S.); (*c*) chemical sympathectomy with 6-hydroxydopamine (6-OH-DA); (*d*) P.V.S. after I.M.S.; (*e*) P.V.S. after 6-HO-DA; (*f*) central vagal stimulation (C.V.S.); (*g*) norepinephrine infusion; and (*h*) epinephrine infusion.

Procedures—The surgical, stimulation, histologic, and cell-counting techniques employed in this study were detailed in a previous publication (2). In short, the animals to be stimulated were vagotomized bilaterally, and either the central or peripheral ends were prepared for electrical stimulation. P.V.S. was accomplished with a Grass stimulator using parameters of 10 v. and 25 Hz. with a 1-msec. duration. Central ends of the vagi were alternately stimulated with monophasic squarewave impulses using "pressor" parameters described by Feldman (9). After stimulation the duodenum of each animal was excised, and its most proximal portion was saved for histologic examination. The tissue was immediately fixed in 10% formalin, embedded in paraffin, and sectioned at 6 μ. Sections were stained by the Fontana Masson method for argentaffin cells. Counts were made of the number of argentaffin cells seen in 30 or more high power fields (H.P.F.) at ×430, and the mean number of cells per H.P.F. was taken as a measure of density.

Fluorescent Histochemistry—The histochemical method developed by Falck *et al.* (10), as modified by Csillik and Kalaman (11), was used in these fluorescence studies to determine if the guinea pig vagus contains sympathetic fibers. Under the conditions used, primary catecholamines condense with formaldehyde without diffusion and are readily converted to 6,7-hydroxy-3,4-dihydroisoquinolines, which show an intense green to yellow fluorescence. In each of six guinea pigs under ether anesthesia, both vagus nerves were atraumatically exposed in the neck but only one was tightly ligated with silk thread as far caudal as the tissues would permit. Forty-eight hours after ligation, the neck was surgically reentered, and a 1-cm. segment of nerve most cranial to the ligature was removed for histochemical examination. The isolated nerve specimens were immediately placed on solid CO_2 until completely frozen. The nerve preparations were attached to cryostat holders, and 20–30 16-μ sections were cut at approximately −30°. The sections were transferred to a precooled sealed container and dried over 20 g. anhydrous phosphorus pentoxide for approximately 25 hr. at −30°. After equilibration at room temperature, the tissue was transferred to a 1-l. desiccator containing paraformaldehyde. The desiccator was warmed to 80°, thereby allowing the evolved formaldehyde gas to saturate the tissue sections. The sections were mounted on nonfluorescent glass with nonfluorescent mounting media and cover slip. The monoamine fluorescence, developed by the exposure to formaldehyde gas, was observed under a Zeiss fluorescence microscope and photographed on Panatomic X film with ASA 32. Exposure time depended on fiber intensity and ranged from 4 to 8 min. per exposure. The exciting light was delivered from the Osram HBO 200 high-pressure mercury lamp and was filtered through Schott BG 12 and Zeiss 53 as the primary and secondary filters.

Immunosympathectomy—Salivary gland protein was reported to contain a protein, nerve growth factor (N.G.F.), with a stimulatory effect on the sympathetic nervous system (12). Antiserum to this factor (anti-N.G.F.) destroys most sympathetic nerve cells in newborn animals (13). Guinea pig salivary glands[3] were homogenized in phosphate buffered saline (P.B.S.), pH 7.2, and centrifuged to remove the insoluble residue. Protein was precipitated with 5% trichloroacetic acid and centrifuged, and the precipitate was redissolved in 1 *N* NaOH. This solution was dialyzed for 24 hr. against P.B.S. at 4°. The final protein concentration of this extract was 3.84 mg./ml., using the method of Lowry (14).

Antiserum to guinea pig salivary protein was prepared in rabbits; 1 ml. of extract was mixed with an equal volume of Freund adjuvant and injected subcutaneously in two sites on the shaved flank of the rabbit. Two additional intravenous injections of 1.0 ml. of extract were given at weekly intervals, starting one after the initial injections. The rabbit was bled from the ear vein 2 weeks after the last injection. Several precipitin lines were noted when the resulting antiserum was tested against the salivary gland extract using the Ochterlony method (15). Newborn guinea pigs were injected intraperitoneally with 0.01 ml. anti-N.G.F. serum/g. body weight within 36 hr. of birth and again 24 hr. later or, in some instances, during the first 5 days of postnatal life. Control guinea pigs received normal rabbit serum (N.R.S.).

Chemical Sympathectomy—It was reported that a single injection of 6-hydroxydopamine (6-OH-DA) markedly reduces the norepinephrine content in various sympathetically innervated organs of different species for several days or weeks (16–18). For destruction of adrenergic nerve endings in this study, guinea pigs were given 20 mg./kg. of 6-OH-DA intraperitoneally for 3 consecutive days; on the 4th day they were given 30 mg./kg. i.v. and were sacrificed 4 hr. after the last injection. In some animals, P.V.S. was performed at that time. The purpose of this part of the study was to investigate the action of 6-OH-DA on the adrenergic nerve endings in the guinea pig duodenum, employing, in principle, the histochemical method developed by Falck *et al.* (10).

Drugs—Each of 18 guinea pigs received an intravenous infusion of 1/100 norepinephrine[4] over 15 min. at a rate of 0.1 ml./min. Seven others received 1/100 epinephrine[5] comparably infused. The 6-hydroxydopamine (2,4,5-trihydroxyphenethylamine) was obtained as the hydrobromide salt.[6]

RESULTS

Whole-mount preparations of the guinea pig vagus under fluorescence microscopy revealed the presence of adrenergic fibers (Fig. 1). An abundance of fluorescent material was also found in Auerbach's and Meissner's plexus. However, no fluorescent fibers were found to innervate the EC scattered throughout the duodenal mucosa. Most of the fluorescent nerves in the mucosal connective tissue seemed to be associated with the vasculature. Conversely, in animals injected with anti-N.G.F. or 6-hydroxydopamine, there was little nerve fluorescence in the duodenal preparations.

In certain of the experiments, comparable EC granulation was found in immunosympathectomized and chemical sympathectomized animals as compared to normal guinea pigs. In subsequent experiments, adequate P.V.S. at the cervical level was unable to reduce 5-HT stores in both chemical (Fig. 2) and immunosympathectomized guinea pigs, but the reduction occurred as reported (2) in normal animals (Fig. 3).

Data presented here show that with C.V.S., EC granulation also disappeared from the duodena. But the magnitude of the fall produced by C.V.S. was not as great as that produced by P.V.S. or norepinephrine infusion. Following epinephrine infusion, similar 5-HT stores were found in an equivalent number of fields as compared to the controls. A summary analysis of this data is exhibited in Fig. 4.

[2] Dial Urethane, Ciba Pharmaceutical Co.

[3] Pel-Freez Biologicals Inc., Rogers, Ark.
[4] Levophed, Winthrop.
[5] Adrenalin, Parke Davis.
[6] Regis Chemical Co., Chicago, Ill.

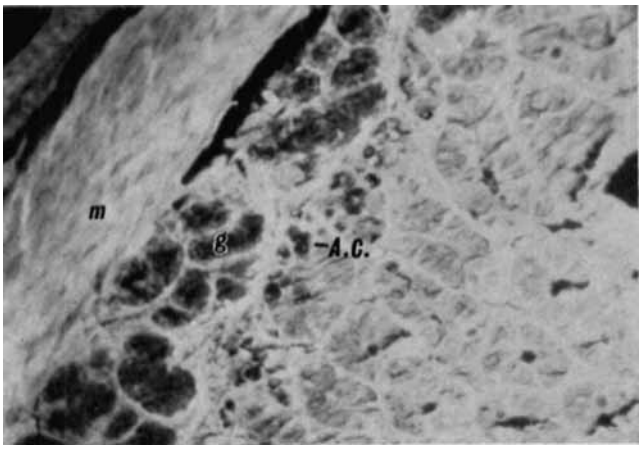

Figure 2—*Photomicrograph of a section of duodenum from a peripheral vagally stimulated animal after pretreatment with 6-OH-DA. Note the numbers of argentaffin cells (A.C.) apparent adjacent to Brunner's glands (g); m indicates the tunica muscularis (fluorescent microscopy, X160).*

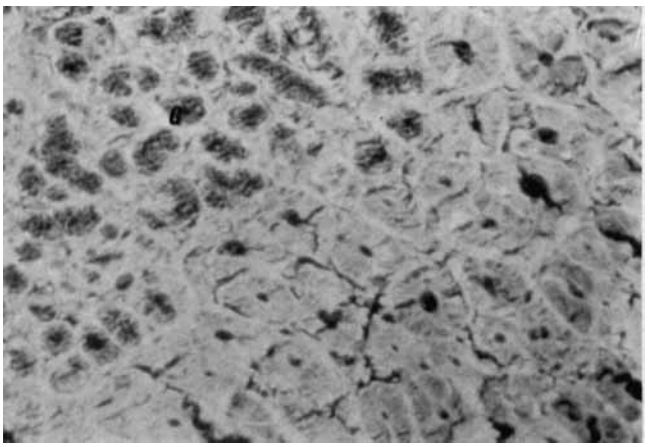

Figure 3—*Photomicrograph of a representative section from a peripheral vagally stimulated animal. Note the complete absence of argentaffin cell granulation in the area adjacent to Brunner's glands (g)(fluorescent microscopy, X160).*

DISCUSSION

The present experiments confirm 5-HT release by vagal stimulation, at least as measured by argentaffin cell granulation, in the guinea pig duodenum. An interesting supposition made in the initial study was that vagal degranulation occurs *via* a noncholinergic mechanism. In support of this, the data presented here show EC granulation practically disappearing with P.V.S., but pretreatment of the guinea pig with 6-hydroxydopamine or anti-N.G.F. completely abolishes the response. These findings are perhaps less surprising in view of the fact that numerous adrenergic fibers were detected in the guinea pig vagus.

Although the role of histologically demonstrated sympathetic axons in the vagus is unknown, it seems quite plausible that these fibers could have been excited by the stimulus parameters used in these experiments and, in turn, could have been responsible for degranulating EC. Unfortunately, because the present experiments were designed primarily to ascertain the presence of adrenergic fibers in the guinea pig vagus, no attempt was made to determine their origin or ultimate course. Nevertheless, while no experimental data support the presumption, it is to be expected that some of the adrenergic fibers in the cervical vagus proper terminate in the duodenum.

In certain experiments with animals injected with anti-N.G.F., there was little nerve fluorescence in the duodenal preparations and occasionally a few scattered fibers remained. Curiously, all of the guinea pigs did not react alike in this respect to the I.M.S. On the other hand, pretreatment of all guinea pigs with 6-OH-DA led to ultramorphological alterations of adrenergic nerve terminals similar to those previously described in detail for cats (16). The superiority over I.M.S. is evident. In this connection, an informative series of experiments was recently conducted by McGregor and Phelan (19) who tried to discover if the ability to evoke vasoconstrictor responses returns to the damaged but regenerating nerve endings before tissue noradrenaline stores are fully replenished. Essentially, they found vasoconstrictor responses of mesenteric arteries to sympathetic nerve stimulation were virtually abolished at 2, 3, and 4 days after administration of 6-OH-DA; by 6 days the response had partially returned and by 8 days was almost normal. From the standpoint of this study's data and these morphological and physiological observations, the possibility of a direct effect of the vagal adrenergic transmitter on the EC system in the intestinal mucosa of the guinea pig is a very real one. Experimental evidence has shown that a decrease in the granularity of these cells can be obtained with C.V.S.

Recent work by Ben-Ishay *et al.* (20) identified the pressor agent released from the sympathetic nerve terminals in response to C.V.S. as norepinephrine. Therefore, it should only follow that norepinephrine infusion can also degranulate EC in the guinea pig small intestine. While norepinephrine is taken up better than epinephrine in the rat adrenergic nerves, the reverse holds true for frog adrenergic nerves which contain adrenaline as the transmitter (21). This, plus the fact that Singh (22) earlier suggested that vagal stimulation results in release of 5-HT from the frog's stomach, prompted a study on the effects of epinephrine infusion on EC granularity. Following epinephrine infusion, however, the concentration of granulated argentaffin cells in the guinea pig duodena numerically remained the same.

Analyses of the data also reveal that 5-HT granulation persists in the apparent absence of sympathetics. From this observation, one might presume that adrenergic innervation has no influence on 5-HT metabolism in the guinea pig. Yet, 5-HT levels were reported to be increased in certain intestinal tissues following I.M.S. in mice (23). According to Thompson (24), this increase was probably not due to a greater population of argentaffin cells but to augmented amine storage. From the denervated preparations, it becomes evident that the elevated 5-HT levels observed in the gastrointestinal mucosa of the immunosympathectomized mouse were undoubtedly the result of an increased concentration of 5-HT per argentaffin cell rather than due to a larger population of cells.

In sum, it is the opinion of the authors that sufficient attention has not been paid to the physiologic release of 5-HT from EC. The complexity of the results reported preclude an explanation on the basis of any very simple theory. The mechanism whereby norepinephrine and adrenergic but not cholinergic nerve stimulation degranulate the EC system in the guinea pig duodenum is beyond the scope of this paper. However, the correlation between morphological and biochemical data, together with the failure of atropine to

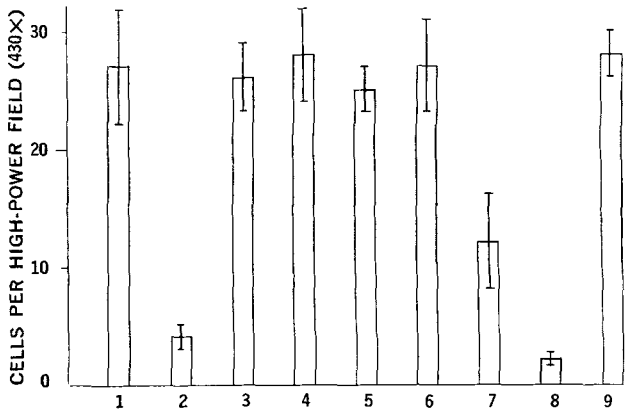

Figure 4—*Bar 1, controls; Bar 2, peripheral cervical vagal stimulation; Bar 3, immunosympathectomized animals; Bar 4, peripheral cervical vagal stimulation after immunosympathectomy; Bar 5, chemical sympathectomy with 6-hydroxydopamine; Bar 6, peripheral cervical vagal stimulation after chemical sympathectomy; Bar 7, central cervical vagal stimulation; Bar 8, norepinephrine infusion; Bar 9, epinephrine infusion. Each bar represents the mean value ± standard deviation.*

block vagal degranulation, provides a basis for a hypothesis on adrenergic release of 5-HT from the bowel.

CONCLUSION

The experiments were designed to provide additional information on the effects of vagal stimulation on the granulation of the EC system as a link to the release of 5-HT in the bowel. As evidenced by the data, it was confirmed that peripheral electrical stimulation of the cervical vagus produced a reduction of argentaffin-positive EC granulation. This effect was not blocked by atropinization of the animal. C.V.S. and infusion of norepinephrine but not epinephrine produced a similar degranulation. This effect was blocked by both chemical and immunological sympathectomy. Finally, catecholamine-fluorescent fibers were found to be present in the trunk of the cervical vagus in the guinea pig. In short, the mutual agreement in the morphological, histochemical, and physiological observations tested provides a basis for the hypothesis of the noradrenergic release of 5-HT from EC of the guinea pig small bowel.

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Topical Mosquito Repellents III: Carboxamide Acetals and Ketals and Related Carbonyl Addition Derivatives

P. TSAKOTELLIS*, H. L. JOHNSON†, W. A. SKINNER†, D. SKIDMORE‡, and H. I. MAIBACH‡

Abstract □ Slow release of topically applied mosquito repellents was attempted *via* formation of low volatility carbonyl addition derivatives of repellent alcohols and carbonyl compounds. Sufficiently rapid hydrolytic release of volatile, repellent moieties on the skin surface did not occur. Vapor repellency was due to the intact derivatives and was found to be dependent upon volatility characteristics. It was concluded that optimum volatility for prolonged topical repellency varies with each homologous series. Compounds incorporating both an acetal and an amine function, although not comparable to diethyltoluamide in topical repellency, appear to be worthy of additional study.

Keyphrases □ Mosquito repellants, topical—synthesis □ Carboxamide acetals, ketals—synthesis □ Repellency, mosquito—volatility relationship

In previous studies of mosquito repellency among structural analogs of diethyltoluamide (DEET), the dominant significance of volatility was demonstrated as a factor in both potency and duration of topical repellent action (1, 2). A limitation is imposed on the concept that duration is maximized by decreased volatility owing to a concomitant decline in potency (1). In the present study an alternative means of prolongation of repellent action through slow release was examined. In addition, the authors examined the effect on repellency of functional group alteration of various repellent carbonyl compounds and extended studies on the relationship between volatility and repellency to classes of compounds other than amides.

Attempts at slow release were based on the assumption that hydrolysis (chemical or enzymatic) of acetal-type derivatives on the skin surface could result in a prolonged release of the parent alcohol and carbonyl moieties as repellent agents. Choice of repellent moieties was based partly on preliminary estimations of "in-

block vagal degranulation, provides a basis for a hypothesis on adrenergic release of 5-HT from the bowel.

CONCLUSION

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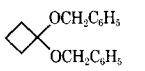
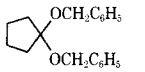
Attempts at slow release were based on the assumption that hydrolysis (chemical or enzymatic) of acetal-type derivatives on the skin surface could result in a prolonged release of the parent alcohol and carbonyl moieties as repellent agents. Choice of repellent moieties was based partly on preliminary estimations of "in-

Table I—Repellency of Intermediates and Standards

Compound	B.p.	Duration of Topical Repellency, hr.[a]
Acetophenone	202°	<0.25
Benzyl alcohol	205°	<0.25
n-Butanol	118°	<0.25
p-Chloroacetophenone	236°	1.0
Cycloheptanone	180°	2.0
Cyclohexanone	156°	1.0
Cyclopentanone	131°	<0.25
N,N-Dimethylacetamide	165°	1.0[b]
N,N-Dimethylformamide	153°	1.0[b]
N,N-Diethyltoluamide	300°	6–10[b]

[a] Time of first confirmed bite; application to human forearm at 2 mg./cm.^{2} except where otherwise noted. [b] Application at 0.35 mg./cm.^{2}.

Table II—Comparative Repellency on Skin and Cloth

Compound[a] Number	Structure	Duration of Repellency, hr.[b] Skin	Cloth
22		5	>4
23		2	8
N,N-Diethyl-toluamide[c]		12–16	—

[a] See Table V. [b] Application to skin and to cheesecloth at 2.0 mg./cm.^{2}; cheesecloth was positioned 0.5–1.0 cm. above skin surface. In all cases, repellency was apparent on initial test (0.25 hr.) and thereafter for indicated duration. [c] Obtained from Eastman Organic Chemicals.

trinsic repellency" of various functional classes. These estimations were made by a rapid screening method comparing olfactometric repellency of compounds of similar volatility on exposure of approximately identical evaporative surface areas to carrier airstreams. Two volatility ranges were examined (b.p. ~100° and ~200°). These preliminary observations indicated that compounds which would hydrolyze to volatile alcohols, aldehydes, ketones, and amides might be suitable compounds for slow-release studies. Topical duration was not of primary concern with regard to intermediates because of intended slow release. In fact, poor duration was expected due to selection of high volatility for optimization of repellent potency (1, 2). This was verified in a few cases by skin testing where only the less volatile compounds tested exhibited repellency more than 15 min. after application (Table I).

In contrast to the poor topical repellency of the alcohol and carbonyl intermediates, somewhat enhanced topical repellency was observed for some derivatives incorporating these relatively volatile moieties (Table II). However, the lack of greatly extended repellency duration or of latent appearance of repellency in tests conducted over periods of several hours suggested that sufficiently rapid hydrolytic release on the skin surface was not occurring with the candidate compounds. This conclusion was substantiated in parallel tests with selected compounds in which the substances were applied both on cloth and directly to skin and the protection times compared (Table II). Since protection was immediate and protection times were comparable in the two methods (or prolonged on cloth), it was concluded that repellency may be due primarily to the intact derivative and not to hydrolysis on the moist, acidic skin surface. The compounds reported herein appear to be relatively stable under the test conditions; all subsequent discussion refers to the intact structures listed in Tables III–VIII.

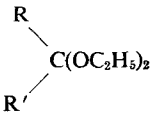
As expected on the basis of previous findings, all of the highly volatile diethyl ketals (Table III) were ineffective topically. This was presumably due to rapid evaporative loss during solvent evaporation (1). Only Compound 3, whose boiling point is comparable to that of DEET, showed significant protection. Similar findings apply to the di-*n*-butyl ketals (Table IV). Furthermore, topical repellency could only be demonstrated with application of very large amounts. Only with the dibenzyl ketals (Table V) was volatility sufficiently low to allow significant durations of repellency at an application rate of 2 mg./cm.^{2}. However, very low volatility resulted in inactive compounds (Compounds 20, 21, 25, and 27) due to insufficient vapor concentration (1). In this series, a volatility corresponding to a boiling point of 150° (0.5 mm.) appears to be approximately optimal.

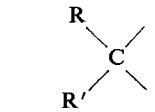
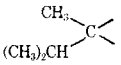
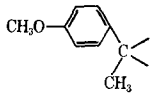
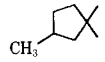
The acetals examined (Table VI) exhibited poor activity at 2.0 mg./cm.^{2} in spite of a seemingly favorable volatility range. On the other hand, the carboxamide acetals and ketals (Table VII) exhibited some repellency characteristics over a wide range of volatility. The orthoesters examined (Table VIII) were ineffective, presumably because of their volatility characteristics.

The present results emphasize the generality of volatility considerations in topical repellent design. Thus, the decline in topical effectiveness with increasing volatility (b.p. <100°, 0.5 mm.) seen previously with various amides (1) is apparent with other compounds as well. Likewise, the loss of effectiveness with decreasing volatility beyond a rather narrow optimal range is apparent, as in the case of the amides (1). However, while the optimum volatility for various amides corresponds approximately to a boiling point of 100° (0.5 mm.) (1), that for the ketals and acetals seems to correspond to approximately a boiling point of 150° (0.5 mm.). Since many factors, such as spreading characteristics, absorption rates, and binding to protein, can affect rates of topical vaporization, this difference is difficult to interpret. It is apparent, however, that optimum volatility characteristics for prolonged topical repellency are different for different classes of compounds and must be determined experimentally for each homologous series.

As a group, acetals and ketals do not represent a particularly promising repellent class. Even optimal members of the series exhibited protection times comparable to amides such as DEET only at concentrations five times greater than those of the latter substances (1). However, the addition of an amine function, as in the case of acetal and ketal derivatives of amides, appears to improve repellency characteristics. Unfortunately, initial attempts to prepare carboxamide acetals and ketals of lower volatility in pure form were unsuccessful, but the class appears worthy of additional study.

Table III—Diethylketals



Compound Number		Yield, %	Formula	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.^{2})
1		41	$C_9H_{20}O_2$	50–51° (21)[a]	<25°	0.25
2		69	$C_{12}H_{18}O_2$	58–60° (0.45)[b]	59°	1.0
3		70	$C_{13}H_{20}O_3$[c]	77–78° (0.20)	95°	2.0
4		92	$C_9H_{18}O_2$	55–56° (24)[d]	<25°	0.25
5		83	$C_8H_{16}O_2$	140–141°[e]	<25°	0.25
6		88	$C_9H_{18}O_2$	62–64° (20)[f]	<25°	0.25
7		93	$C_{10}H_{20}O_2$[g]	63–64° (12)	<25°	0.25
8		59	$C_9H_{17}ClO_2$	81–83° (12)[h]	35°	0.25
9		65	$C_{10}H_{20}O_2$	30–32° (0.45)[i]	31°	0.25
10		85	$C_{11}H_{22}O_2$	79–81° (13)[i]	31°	0.25
11		94	$C_{11}H_{22}O_2$	125–127° (57)[i]	45°	0.25

[a] Lit. (3) b.p. 52.4° (20 mm.). [b] Lit. (4) b.p. 110–112° (12 mm.). [c] Calcd. for C, H: 69.6, 9.0; Found: 69.7, 9.0. [d] Lit. (5) b.p. 55° (23 mm.). [e] Lit. (6) b.p. 138–141°. [f] Lit. (7) b.p. 63–65° (20 mm.). [g] Calcd. for C, H: 69.7, 11.7; Found: 69.1, 11.7. [h] Lit. (8) b.p. 93–95° (15 mm.). [i] Lit. (9) b.p. 72° (9 mm.) for Compound 9, b.p. 76–77° (15 mm.) for Compound 10, and b.p. 88–89° (15 mm.) for Compound 11.

EXPERIMENTAL[1]

Except where otherwise noted, compounds were purified and boiling points (uncorrected) were determined by vacuum distillation through a 10-cm. Vigreux column.

Diethylketals—All the diethylketals, except that from cyclobuaneone, were prepared from ethanol, triethyl orthoformate, and the corresponding ketone, using a trace of mineral acid as a catalyst. The diethylketal of cyclobutanone (boiling point similar to that of triethyl orthoformate) was prepared from ethanol and the diethylketal of cyclopentanone. The diethylketal of cyclobutanone was only distilled at atmospheric pressure.

Di-*n*-butylketals—All the di-*n*-butylketals were prepared by the method of MacKenzie and Stocker (10) from *n*-butanol, triethyl orthoformate, and the corresponding ketone, using a catalytic amount of *p*-toluenesulfonic acid. The mixture was slowly heated at such a rate that the ethyl formate removal through a 10-cm. Vigreux column started after 1 hr.; the heating was continued until all the ethyl formate and the ethanol formed were removed. The mixture was cooled, neutralized with a dilute solution of sodium in *n*-butanol, washed with water, dried, and fractionally distilled.

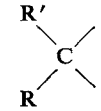
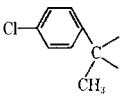
Dibenzylketals and Acetals—These compounds were prepared by transketalization of the corresponding diethylketals and diethylacetals using a trace of mineral acid, according to the method of Lorette and Howard (18). The mixture was heated at 130–140°, and the ethanol was removed from the reaction mixture through a 10-cm. Vigreux column. Two or more moles of benzyl alcohol per mole of diethylketal gave predominately the dibenzylketal with small amounts of the ethylbenzylketal, depending on the completeness of removal of the ethanol. The mixture was cooled, neutralized with a dilute solution of sodium in benzyl alcohol, and fractionally distilled except for the dibenzylketals of acetophenone, *p*-methoxyacetophenone, cyclohexanone, and cycloheptanone which, on distillation, decomposed to the unsaturated ether and benzyl alcohol. Pentane solutions of these compounds were purified by rapid filtration through basic aluminum oxide.

Carboxamide Acetals—These were synthesized (15) by adding dropwise under cooling an excess of thionyl chloride to the corresponding *N,N*-dialkylformamide. After stirring at 20°, the mixture was heated at 40° *in vacuo* to remove sulfur dioxide. The off-white crystals of the corresponding dichloroamine were dissolved in chloroform and were added dropwise to an ice-cold solution of sodium in the corresponding alcohol. The mixture was stirred 3–4 days at 20° or heated. The sodium chloride was removed and the filtrate was fractionally distilled.

[1] Elemental analyses are by the Microanalytical Laboratory, Department of Chemistry, Stanford University, Stanford, Calif.

Table IV—Di-*n*-butylketals



Compound Number		Yield, %	Formula	Anal., % Calcd.	Anal., % Found	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
12		59	$C_{11}H_{24}O_2$			71–72° (8)[a]	30°	0.25
13		63	$C_{16}H_{26}O_2$			67–69° (0.2)[a]	80°	0.25
14		75	$C_{16}H_{25}ClO_2$	C, 67.4 H, 8.9 Cl, 12.4	C, 66.9 H, 8.6 Cl, 12.1	113–115° (2.4)	90°	0.25[b]
15		63	$C_{12}H_{24}O_2$	C, 71.9 H, 12.0	C, 71.7 H, 12.1	108–109° (16)	55°	0.25
16		79	$C_{13}H_{26}O_2$	C, 72.8 H, 12.2	C, 72.6 H, 12.1	82–84° (3)	60°	0.25[c]
17		80	$C_{14}H_{28}O_2$			98–100° (5)[a]	65°	0.25
18		80	$C_{15}H_{30}O_2$	C, 74.3 H, 12.5	C, 74.0 H, 12.3	69–70° (0.2)	80°	1.0

[a] Lit. (10) b.p. 64° (2.3 mm.) for Compound 12, b.p. 85° (0.3 mm.) for Compound 13, and b.p. 89° (1.9 mm.) for Compound 17. [b] 4 mg./cm.²; 1.0 hr. [c] 4 mg./cm.²; 0.25 hr.

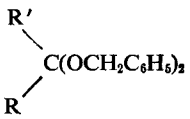
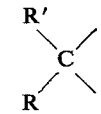
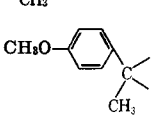
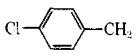


Table V—Dibenzylketals

Compound Number		Yield, %	Formula	Anal., % Calcd.	Anal., % Found	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
19		42	$C_{17}H_{20}O_2$			124–126° (0.45)[a]	125°	0.25
20		58	$C_{22}H_{22}O_2$	C, 82.9 H, 7.0	C, 82.7 H, 6.7	dec.[b]	dec.	0.25
21		47	$C_{23}H_{24}O_3$	C, 79.2 H, 6.9	C, 79.5 H, 6.6	dec.[b]	dec.	0.25
22		67	$C_{18}H_{20}O_2$	C, 80.5 H, 7.5	C, 80.0 H, 7.5	124–125° (0.15)	146°	2.5[c] 4.0 5.0
23		61	$C_{19}H_{22}O_2$	C, 80.8 H, 7.9	C, 80.1 H, 8.2	140° (0.20)	150°	5.5[c] 2.0 2.0
24		45	$C_{20}H_{24}O_2$	C, 81.0 H, 8.2	C, 80.9 H, 8.1	140–142° (0.15)	160°	2.0
25		60	$C_{20}H_{24}O_2$	C, 81.0 H, 8.2	C, 80.4 H, 7.6	dec.[b]	dec.	0.25
26		83	$C_{21}H_{26}O_2$	C, 81.2 H, 8.4	C, 81.8 H, 8.0	160–162° (0.07)	190°	1.0
27		71	$C_{21}H_{26}O_2$	C, 81.2 H, 8.4	C, 80.8 H, 8.3	dec.[b]	dec.	0.25

[a] Lit. (11) b.p. 125° (1 mm.). [b] Pentane solution was purified by rapid filtration through basic aluminum oxide. [c] Duration of repellency varies considerably when material is applied at the same levels to different subjects.

Table VI—Acetals $(RCHOR')_2$

Compound Number	R	R′	Yield, %	Formula	Anal., % Calcd.	Anal., % Found	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
28	$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	79	$C_{13}H_{28}O_2$			86–88° (2.5)[a]	60°	0.25
29	$CH_3(CH_2)_3$	$C_6H_5CH_2$	61	$C_{19}H_{24}O_2$	C, 80.2 H, 8.5	C, 80.0 H, 8.6	136–138° (0.20)	152°	1.0
30	$CH_3(CH_2)_3$		44	$C_{19}H_{22}Cl_2O_2$[b]	C, 64.4 H, 6.3	C, 64.3 H, 6.3	174–175° (0.30)	180°	0.25
31	$CH_3(CH_2)_3$		58	$C_{19}H_{32}O_2$	C, 78.0 H, 11.0	C, 78.1 H, 10.9	119° (0.30)	130°	0.25
32	$CH_3(CH_2)_3$		49	$C_{21}H_{36}O_2$	C, 78.7 H, 11.3	C, 78.9 H, 11.3	135° (0.15)	155°	0.25
33	$CH_3(CH_2)_3$		60	$C_{23}H_{40}O_2$	C, 79.2 H, 11.5	C, 79.5 H, 11.5	146–148° (0.15)	170°	0.25
34	C_6H_5	$C_6H_5CH_2$	7	$C_{21}H_{20}O_2$			170–171° (0.15)[c]	195°	0.25
35			41	$C_{24}H_{40}O_2$	C, 79.9 H, 11.1	C, 79.5 H, 11.1	150–152° (0.15)	175°	0.25

[a] Lit. (12) b.p. 87° (2.5 mm). [b] Analysis, Cl: calcd., 20.0; found, 20.0. [c] Lit. (13) b.p. 170° (0.15 mm.).

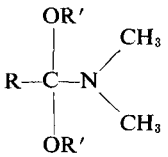


Table VII—Carboxamide Acetals and Ketals

Compound Number	R	R′	Yield,[a] %	Formula	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
36	H	$CH_3(CH_2)_3$	26	$C_{11}H_{25}NO_2$	101–103° (11)[b]	50°	0.25
37	H	$(CH_3)_3CCH_2$	—[c]	$C_{13}H_{29}NO_2$	85–87° (10)	40°	0.25
38	H		—[c]	$C_{15}H_{29}NO_2$	115–116° (0.75)	110°	1.00
39	C_6H_5	C_2H_5	48	$C_{13}H_{21}NO_2$	57–58° (0.4)[d]	58°	1.00
40	C_6H_5	$CH_3(CH_2)_3$	79	$C_{17}H_{29}NO_2$[e]	86–87° (0.15)	100°	1.00

[a] Yields were calculated as to the starting amide. [b] Lit. (14) b.p. 96–98° (16 mm.). [c] Compounds were obtained from Aldrich Chemical Company. [d] Lit. (15) b.p. 60° (0.5 mm.). [e] Calcd. for C, H, N: C, 73.0, H, 10.4, N, 5.0; Found: C, 72.9, H, 10.4, N, 5.0.

Carboxamide Ketals—Dry phosgene was bubbled through an ice-cold benzene solution of *N,N*-dimethylbenzamide (15). During the addition, white crystals precipitated; the mixture was stirred 16 hr. at 20° and then was evaporated to dryness. The hygroscopic dichloramine was dissolved in chloroform and was added dropwise to an ice-cold solution of sodium in the corresponding alcohol. The mixture was stirred 2 days at 20° and was worked up as for the carboxamide acetals.

Orthoesters—Tribenzyl orthoformate was prepared from triethyl orthoformate by removal of the ethanol formed, according to the method of Alexander and Buch (16). Triethyl orthobutyrate was prepared by alcoholysis of 1,1-dichlorobutyldiethylamine, which was prepared from the *N,N*-diethylbutyramide and phosgene.

Biological Evaluation—Repellency duration testing was conducted by uniform application of repellent in EtOH to an exposed area of the arm of a human subject as described previously (1). Female *Aedes aegypti* (nonblood fed) mosquitoes (5–7 days old) were used in all tests.

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(4) L. Claisen, *Chem. Ber.*, **31,** 1012(1898); *ibid.*, **40,** 3908, 3913 (1907); H. E. Carswell and H. Adkins, *J. Amer. Chem. Soc.*, **50,** 240(1928).

Table VIII—Orthoesters

Compound Number	Structure	Yield, %	Formula	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
41	$HC(OCH_2CH_2CH_2CH_3)_3$	—[a]	$C_{13}H_{28}O_3$	75° (0.5)	75°	0.25
42	$HC(OCH_2C_6H_5)_3$	72	$C_{22}H_{22}O_3$	dec.[b]	dec.	0.25
43	$CH_3(CH_2)_2C(OC_2H_5)_3$	69	$C_{10}H_{22}O_3$	62–63° (10)[c]	<25°	0.25

[a] The compound was obtained from Eastman Organic Chemicals Co. [b] Lit. (16). [c] Lit. (17) b.p. 58–59° (7 mm.).

(5) S. Julia, M. Julia, S. Tchen, and P. Graffin, *Compt. Rend.*, **253** (4), 678(1961).
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In Vitro Binding Study of Epinephrine and Bovine Serum Albumin

H. ZIA*, R. H. COX, and L. A. LUZZI

Abstract □ The binding of epinephrine to bovine serum albumin and to acetylated bovine serum albumin was studied with respect to evaluation of the active site on the small molecule and to the exchange rate. The effects of varying concentrations of bovine serum albumin on the relaxation rate and chemical shift of 0.1 M (molal) epinephrine were examined. Temperature and pH effects on the relaxation rate of various epinephrine lines were examined. A comparison of relaxation rates between epinephrine–bovine serum albumin and epinephrine–acetylated bovine serum albumin was made. NMR instrumentation was used to follow the reactions. It was found that binding between epinephrine and bovine serum albumin was detectable with this instrumentation and that the most probable active site on the small molecule was the alkyl side chain. It also appeared that there was a fast exchange rate between bound and unbound epinephrine.

Keyphrases □ Epinephrine binding—bovine serum albumin □ Bovine serum albumin, acetylated bovine serum albumin—epinephrine binding □ Temperature, pH effects—epinephrine–bovine serum albumin relaxation rates □ NMR spectroscopy—analysis

The interaction of hormones with plasma proteins is attracting increasing interest because of the involvement of binding in events that are of considerable physiological and pharmacological importance. The numerous studies in this area have been the subjects of several extensive review articles (1–4). During the last decade, there have been a number of conflicting reports concerning whether or not the endogenous hormones, epinephrine and norepinephrine, are bound to plasma proteins. In 1958, Antoniades *et al.* (5), employing dialysis, cationic exchange-resin techniques, and chemical and biological assays, concluded that although epinephrine was completely bound to plasma proteins an appreciable amount of norepinephrine seemed to be unbound, and that the plasma protein responsible for the binding and transport of these hormones was albumin.

In 1962, Bickel and Bovet (6) utilized the method of "crossing paper electrophoresis" and postulated that there was no interaction between these hormones and dog serum albumin. However, during the same year, Litt (7) published the results of his equilibrium dialysis studies and indicated that there was binding between epinephrine and bovine serum albumin (BSA).

In 1968, Cohen *et al.* (8), using paper electrophoresis, showed that isotopically labeled epinephrine and norepinephrine were bound by rabbit serum albumin and also by α- and β-globulin. Neither this nor any of the previous studies has defined the active site of binding on the adrenergic molecules.

Recent studies have established the value of NMR spectroscopy as a tool for conformational determinations of pharmacologically active molecules in solution (9–11), for the elucidation of interactions between small molecules (10, 11), for studying protein small molecule interactions, and in assessing the extent to which various functional groups on the small molecule participate in the interaction (12–16).

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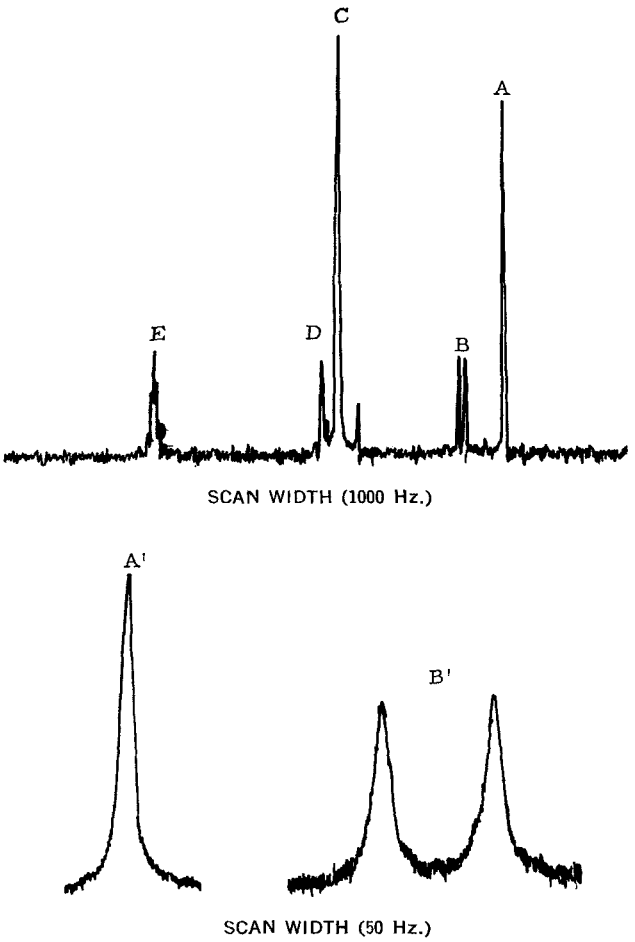

Figure 1—*Proton magnetic resonance spectrum of 0.1 M epinephrine at pH 5.8. At 1000* Hz., *A* = *N—CH₃, B* = *CH₂, C* = *HOD, D* = *CH, and E* = *phenyl protons. At 50 Hz., A'* = *N—CH₃, and B'* = *CH₂ (obtained at different instrument settings).*

EXPERIMENTAL

Materials and Methods—The following materials were used: crystallized BSA,[1] *l*-epinephrine,[2] and 99.8% deuterium oxide.[3] All chemicals were used without further purification.

Acetylated BSA was prepared according to the procedure of Fraenkel-Conrat *et al.* (17). Preliminary purification of acetylated BSA from ions and excess reagents was immediately followed by dialysis of the reaction products at 4° against 8 l. of deionized distilled water for 36 hr. with four changes of distilled water. The purified preparation was then lyophilized.

The extent of acetylation was estimated by treating the sample when *p*-toluene sulfonic acid and distillation of the acetic acid released, followed by titration of the acetic acid with barium hydroxide. The percentage of acetylation was estimated to be 2.5%.

Adjustments of pH were made with DCl and NaOD. All measurements were made on a Radiometer titrator (TTT_1c), with microelectrodes calibrated with standard buffer solutions. All pH values given are actual meter readings and are uncorrected for deuterium isotope effects. All samples were run within 2–3 hr. of preparation.

SPECTRA

Solutions for NMR studies were made in deuterium oxide. Drug concentrations are expressed as molal (M), and protein concentrations are given in percent weight per volume (w/v). All spectra were obtained on a Varian Associates HA-100 spectrometer operating in the field-sweep mode. A probe temperature of 29° and a tetramethylsilane (TMS) external reference were used. A scan width of 50 Hz. was employed with a sweep time of 500 sec. Each reported value of $1/T_2$ is the mean of at least four, and usually six, separate measurements reliable within ± 0.3 sec.$^{-1}$. The values of $1/T_2$ for the various protons were obtained from the spectral line widths using the formula

$$\Delta V = (\pi T_2)^{-1} \qquad \text{(Eq. 1)}$$

where ΔV is the line width at one-half maximum peak height, and T_2 is the spin-spin relaxation time (18). Chemical shifts are reported in cycles per second downfield from TMS and are uncorrected for bulk susceptibility differences.

RESULTS

Effect of Various Concentrations of BSA on Spin-Spin Relaxation Rates of 0.1 M Epinephrine—The proton magnetic resonance spectrum of 0.1 M epinephrine in D_2O at pH 5.8 is shown in Fig. 1. The aromatic protons are easily identified by their characteristic low-field position (736.5 Hz.) and the single proton on the β-carbon by a triplet (563.3 Hz.) close to the DOH peak (516.0 Hz.). The methylene protons are identified by a doublet (370.5 Hz.), and the N—CH_3 protons by a sharp single peak (320.0 Hz.). A typical example of the effect of albumin on the line width of 0.1 M epinephrine and 5% BSA is shown in Fig. 2. It may be seen from this figure that all lines are broadened to some extent. However, the effect is maximum for the methylene protons. The results of several concentrations of BSA on the relaxation rates of 0.1 M epinephrine at pH 5.8 are summarized in a plot of $1/T_2$ of the side-chain protons *versus* percent (w/v) of BSA (Fig. 3).

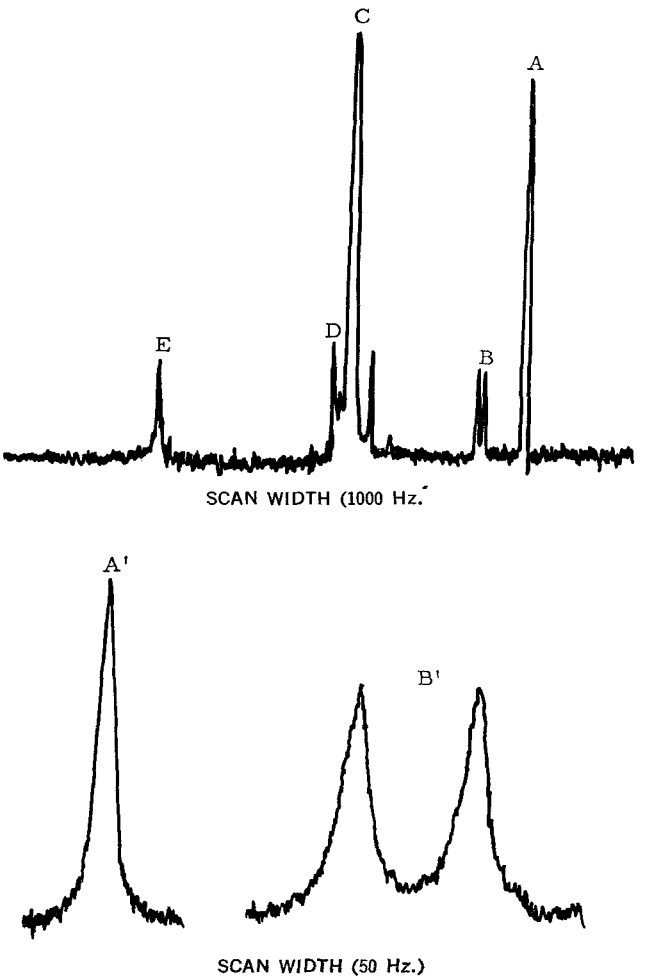

Figure 2—*Proton magnetic resonance of 0.1 M epinephrine–5% BSA in D_2O at pH 5.8. A, A', B, B', C, D, and E bear the same designations as in Fig. 1.*

[1] Nutritional Biochemical Corp.
[2] Wilson Laboratories.
[3] Mallinckrodt.

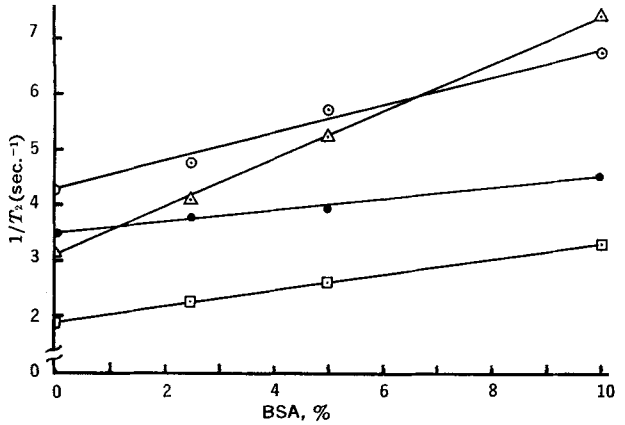

Figure 3—*Effect of BSA on the relaxation rates of 0.1 M epinephrine spectral lines at pH 5.8. Key:* ⊙ = *CH*, △ = CH_2, ⊡ = CH_3, *and* ● = *phenyl protons.*

It can be seen from Fig. 3 that the addition of BSA to a solution of epinephrine increased the relaxation rates of all peaks. However, the effect was more pronounced in the case of methylene protons.

For solutions in which oxidized epinephrine was present, an increase in $1/T_2$ values for the phenyl protons was found which was similar to the increase in the methylene protons. Therefore, it was important to maintain the pH of the epinephrine solutions below 5.8 to prevent oxidation. At pH 5.8, no oxidation of the drug occurred during the experiment.

Effect of BSA on Chemical Shifts of Epinephrine Lines—Chemical shifts of 0.1 M epinephrine for several concentrations of BSA showed no marked differences. In the range of concentrations used here, only a small fraction of epinephrine molecules can be in the bound form and, consequently, changes in chemical shift may not be detectable. Findings such as these have been discussed by Fisher and Jardetzky (14).

Effect of Temperature on Relaxation Rates of Epinephrine–BSA Solution—To confirm the existence of a specific interaction between epinephrine and BSA, several corollary experiments, including temperature studies, were conducted. The effect of variations in temperature on the relaxation rates of 0.1 M epinephrine–2.5% BSA over a temperature range of 10–39° and pH 5.8 is given in Table I. A plot of these data is shown in Fig. 4. Although there were no significant changes in the chemical shifts of epinephrine–BSA solution, it may be seen from Table I and Fig. 4 that the relaxation rates were increased two to threefold as the temperature was lowered. Since the effect of temperature on the relaxation of free and bound molecules has been reported to be small (14), it seems reasonable to assume that the major difference in the relaxation rate observed here as temperature was decreased was due to an increase in the fraction of epinephrine bound.

Effect of pH on the Relaxation Rates of Epinephrine–BSA Solution—To study the effect of changes in pH on the relaxation rates of epinephrine–BSA, a solution of 0.05 M epinephrine and 5% BSA was chosen. The pH range over which such a study could be carried out was restricted due to oxidation of epinephrine at pH values above 5.8 and a reversible configurational change below pH 4.2 (19).

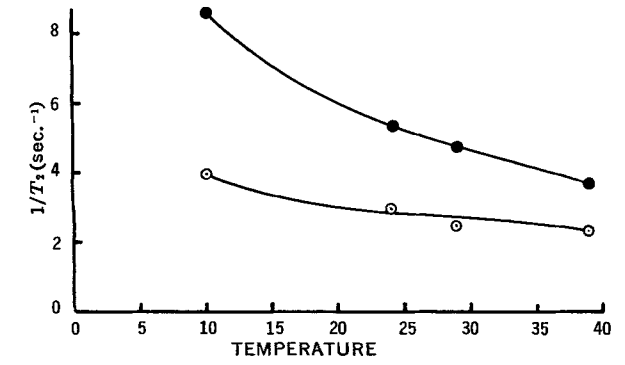

Figure 4—*Effect of variations in temperature on relaxation rates of 0.1 M epinephrine–2.5% BSA at pH 5.8. Key:* ● = CH_3, *and* ⊙ = CH_2.

Table I—Effect of Temperature on Relaxation ($1/T_2$ sec.$^{-1}$) Rates of 0.1 M Epinephrine–2.5% BSA at pH 5.8

Temperature	Phenyl[a] Ring	CH_3	CH[b]	CH_2
10°	4.05	3.99	Overlap	8.67
24°	3.77	3.11	5.81	5.34
29°	3.77	2.51	5.34	4.71
39°	3.61	2.30	4.87	3.77

[a] Indicates measurement taken on the center of the multiplet. [b] Indicates measurement taken on the first line of triplet due to overlap of other peaks in the triplet with the HOD peak.

A plot of pH *versus* $1/T_2$ for methylene protons is shown in Fig. 5. A general increase of relaxation rates was observed, with the methylene peak again exhibiting the greatest change. With the present data, it is difficult to determine the exact mechanism leading to the increase in relaxation rates. However, it seems likely that an increase in pH alters the conformational characteristics of the protein so as to favor formation of the complex and, thus, increase the $1/T_2$ values.

Effect of Temperature on Relaxation Rates of Epinephrine–Acetylated BSA Solution—To elucidate further the nature of the epinephrine–BSA interaction, BSA was acetylated in order to partially block ε-ammonium side chains of the BSA and, thereby, increase the net negative charge on the protein.

As the results in Table II indicate, there was a larger fraction of epinephrine bound to the protein in the 0.1 M epinephrine–2.5% acetylated BSA system than in the 0.1 M epinephrine–2.5% BSA system. This finding is consistent with the proposed mechanism that epinephrine binds to BSA through its side chain by an electrostatic force that may, or may not, be reinforced by the hydrogen bonding of hydroxyl groups.

DISCUSSION

The spin-spin relaxation rate measurements obtained using concentrations of 0.1 M and lower of epinephrine, both with and without varying the BSA concentration, demonstrated that addition of BSA increased the $1/T_2$ of the methylene protons to a greater degree than it did for other epinephrine protons (Fig. 3). Fischer and Jardetzky (14), upon examination of a system consisting of penicillin and BSA, postulated that such findings indicate a specific type of interaction as opposed to one in which nonspecific mechanisms, such as viscosity and intermolecular interaction, may operate. Nonspecific mechanisms, such as those described, should increase the $1/T_2$ of all lines in a given molecule to the same extent. However, if binding is the cause for the increase in relaxation rates, then that part of the molecule that is bound will exhibit the greatest change. Therefore, the findings reported here indicate that not only does a specific interaction take place between epinephrine and BSA, but they also suggest that the side chain of epinephrine is directly involved in the binding process.

The speculation that the results shown in Fig. 3 represent a specific interaction between the epinephrine side chain and BSA is further strengthened by data gathered in the temperature-variation experiments. It can be seen from Table II and Fig. 4 that temperature variations affected the $1/T_2$ of all lines to some degree but that the

Table II—Effect of Temperature on Relaxation Rates ($1/T_2$ sec.$^{-1}$) of 0.1 M Epinephrine–2.5% Acetylated BSA at pH 5.8

Temperature	Phenyl[a] Ring	CH_3	CH	CH_2
3°	—	11.24	Overlap	Not measurable
29°	4.71	5.84	Overlap	15.20
38°	4.55	6.03	Overlap	13.25
46°	4.71	5.93	Overlap	11.68

[a] Indicates measurement taken on the center of the multiplet.

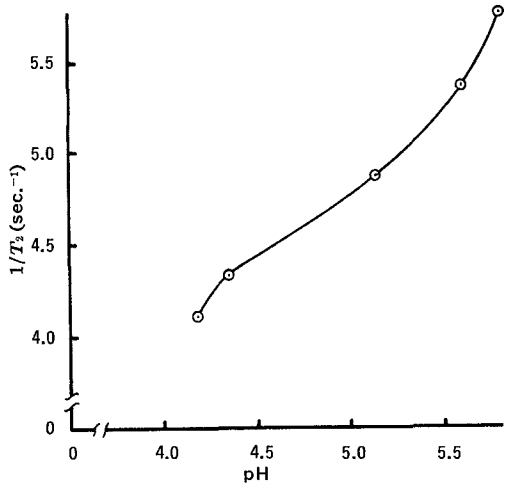

Figure 5—*Effect of pH on relaxation rate of methylene protons for 0.05 M epinephrine–5% BSA solution.*

effect was more pronounced in the case of the methylene lines. These results seem to lend further credence to the previous position that the binding of epinephrine to BSA occurs through the side chain portion of the epinephrine molecule. These results also tend to rule out nonspecific mechanisms as a major factor. Thus, the major increase observed in the relaxation rate, when temperature was the variable, may be attributed to an increase in the fraction of bound epinephrine molecules.

The postulated mechanism that a predominately electrostatic force (which may or may not be reinforced by H-bonding of the hydroxyl group) was operating tends to be supported by the data obtained from the acetylated BSA studies. One would expect that the interaction between BSA and epinephrine would be enhanced by the increased negative charge on the protein if the proposed mechanism is correct. This indeed seems to be the case; the results shown in Table II indicate that BSA showed greater affinity for the drug when acetylated.

A question which remains unanswered is why should the relaxation rate involving epinephrine and BSA increase when pH is increased over the limited range of 4.2–5.8. One possibility would be a change in the conformation of the BSA molecule so as to favor formation of the complex. This reasoning is based on the supposition that in the range of concentration used here, the limiting factor is the number of protein binding sites and not the quantity of ionized drug. At the pH values indicated, ionized epinephrine molecules should be in sufficient supply since the pKa of the amine function has been reported to be at a value in excess of 8.0 (20).

Under the experimental conditions used here, there seemed to be no measurable contribution from the aromatic portion of the epinephrine molecule to the binding process. This may or may not be true if one is considering the binding of epinephrine to serum albumin in a biological system. It is well known that this class of compounds has outstanding chelate-forming properties (21). Trace metal ions in serum and their biological importances have been known for many years. Thus, one may not be able to rule out the possible formation of a three component (epinephrine–metal–albumin) complex. However, as Klotz and Loh-Ming (22) pointed out, such mediation of metal may not be true in all systems.

In 1957, Zimmerman and Brittin (23), while studying absorption of water on silica gel, developed a theory to explore the characteristic relaxation rates of small molecules existing in equilibrium in two or more phases (*i.e.*, bound and free). The observed relaxation rates for such molecules were found to be dependent upon the rate of exchange of molecules between the bound and free forms, as well as on the equilibrium binding constant. Therefore, considering a system where small molecules can exist in only two forms, bound and free, and assuming that the relaxation rate for the bound state is much faster than for the free, three possible cases may be described: slow exchange, intermediate exchange, and fast exchange. Fischer and Jardetzky (14) discussed each case in relation to NMR studies.

The results obtained in this study seem to demonstrate a fast exchange mechanism for the epinephrine–BSA system. The slow exchange mechanism is not acceptable, since no double lines were observed. Neither does intermediate exchange seem to be indicated, since all protons do not have the same relaxation rate. Thus, the only remaining mechanism which explains the nature of epinephrine–BSA interaction is fast exchange. Since the broadening of the lines is not as large as is found in the case of penicillin–BSA (14), one may speculate that the fraction of bound molecules in the penicillin–BSA system is greater than in the epinephrine–BSA system.

In conclusion, it has been shown in the work reported here that binding does occur between epinephrine and BSA. Furthermore, the results obtained suggest that the alkyl side chain is the active site for binding to BSA. Finally, it appears that bound epinephrine is exchanging rapidly with epinephrine in the unbound state.

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Induction and Inhibition of *In Vivo* Glucuronidation of Apomorphine in Mice

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Abstract □ The effect of various inducers and inhibitors of drug-metabolizing enzyme systems was studied on the kinetics of apomorphine disappearance and of the appearance of apomorphine glucuronides. The generally claimed inducers, phenobarbital and testosterone, and inhibitors, β-diethylaminoethyl diphenylpropylacetate and actinomycin D, were found to have no significant effect on the metabolism of apomorphine. Estradiol, however, produced a significant inhibition of the metabolism. Apomorphine pretreatment for 3 days exhibited a definite induction of its own metabolism.

Keyphrases □ Apomorphine glucuronidation—induction, inhibition □ Polyestradiol phosphate—apomorphine glucuronidation inhibition □ Metabolism, apomorphine—self-induced □ Pentobarbital sleeping time—apomorphine metabolism determination

Glucuronide conjugation is a major pathway of metabolism of numerous drugs containing phenolic hydroxyl groups (1, 2). In recent years, evidence has accumulated regarding the ability of certain compounds either to stimulate or inhibit various drug-metabolizing enzyme systems (3, 4). Induction with phenobarbital in microsomal oxidase and dealkylating systems and in glucuronidation of several compounds has been recently reviewed (3). This paper presents evidence that *O*-glucuronide conjugation, at least in the case of apomorphine, may not necessarily be induced by phenobarbital or inhibited by substances such as β-diethylaminoethyl diphenylpropyl acetate (SKF 525-A).

Apomorphine was chosen as a substrate in these studies, because this compound was shown previously to be metabolized and excreted in rabbits and rats primarily as a 7:3 mixture of 3,*O*- and 4,*O*-glucuronides (5). By using analogous criteria of characterization in the present study, mice were also found to metabolize apomorphine into 3,*O*- and 4,*O*-glucuronides in a proportion similar to that observed in rabbits.

EXPERIMENTAL

Method of Assay—The rate of glucuronide conjugation in terms of apomorphine disappearance and the appearance of conjugated apomorphine in intact Swiss mice of mixed sexes weighing 20 ± 4 g. was determined by the method described by Kaul *et al.* (6), with the following modifications: (*a*) the dose of apomorphine hydrochloride used in all kinetic studies was 40 mg./kg. i.v., and (*b*) ethyl acetate (A.R.) was used for final extraction of the colored oxidation product of apomorphine prior to spectrophotometric determination. The specificity of the assay method for apomorphine was already established (7).

Glucuronidation by Mice—The details of the procedure for isolation of apomorphine metabolites and evidence that apomorphine in rabbits is primarily metabolized into a 7:3 mixture of 3,*O*- and 4,*O*-glucuronides was presented earlier (5). Mice were also found to metabolize apomorphine into glucuronides by using the following selective ion-exchange fractionation.

Groups of Swiss mice were given 30 mg./kg. apomorphine hydrochloride intraperitoneally, and 24-hr. pooled urine samples were collected. The urine was passed through a column of ion-exchange resin[1] (H^+). The column was washed with water followed by methanol, and the conjugated apomorphine was eluted with 4 *N* ammoniacal methanol. The eluate was concentrated under reduced pressure and passed through an ion-exchange resin[2] formate column. The column was washed with water and then eluted with 1 *N* formic acid. The eluate on hydrolysis gave positive test for apomorphine. It was further purified, as described previously (5), and found to consist of two compounds in 7:3 proportion. The purified conjugated apomorphine, when hydrolyzed by mild acid conditions and tested as described before (5), gave positive tests for apomorphine as well as glucuronic acid. Based on this evidence, rabbits, like mice, also seem to metabolize apomorphine into 3,*O*- and 4,*O*-glucuronides.

Pretreatment—The inducers tested were phenobarbital sodium (50 mg./kg. i.p.), testosterone propionate in oil (2 mg./kg. s.c. in female mice only), and apomorphine hydrochloride (20 mg./kg. i.p.). SKF 525-A (50 mg./kg. i.p.) and polyestradiol phosphate[3] (4 mg./kg. s.c.) represented the inhibitors of the drug-metabolizing enzyme systems tested in this work.

Although actinomycin D was reported (3) to block only the phenobarbital-induced induction of drug-metabolizing enzymes, preliminary experiments indicated that it also directly inhibited the pentobarbital metabolism. Therefore, actinomycin D (dactinomycin, 100 mcg./kg. i.p.) was included as a possible direct inhibitor of apomorphine metabolism.

As expected, animals receiving actinomycin D showed a considerable weight loss in the treatment period. To dissect the effects of the antibiotic on apomorphine metabolism due to: (*a*) its inhibition of protein synthesis, and (*b*) weight loss, a study was carried out wherein two groups of animals were control-fed orally with 1:1 diluted Infant Standardized Food[4] once daily but with *ad libitum* water supply. Actinomycin D (100 mcg./kg. i.p.) was given to one group; the other group served as the control.

All drugs were given once daily for 3 days before determining the kinetics of apomorphine conjugation 36 hr. later, with the exceptions of polyestradiol which was given only once 4 days prior to the kinetic study and of SKF 525-A which was given 30 min. prior to the kinetic study. Each treatment group was accompanied by a control group of mice receiving saline injections.

Pentobarbital Sleeping Time—Pentobarbital sleeping time in mice was used as a comparison control for assessing the enzyme induction and inhibition produced by the agents tested on apomorphine metabolism. Four randomly selected groups of mice were given four different treatments. One group each received daily doses of actinomycin D (100 mcg./kg. i.p.), apomorphine hydrochloride (20 mg./kg. i.p.), and saline (0.2 ml./mouse i.p.) for 3 days. These groups were subjected to pentobarbital sleeping-time determinations 36 hr. after the last treatment. The fourth group, however, received 0.25 ml./mouse saline i.p. daily for 3 days and, 35 hr. after the last saline injection, SKF 525-A (50 mg./kg. i.p.) was given. Pentobarbital sleeping time was determined 30 min. later. Pentobarbital sodium (75 mg./kg. i.p.) was given to all groups; sleeping time, according to the method of Westfall *et al.* (8), was noted as the interval from the time of injection to the time when the animal showed a righting reflex in response to tail pinching carried out every 5 min.

Student's *t* test was employed to determine the values of significance.

[1] Dowex 50-X2, Dow Chemical Co., Midland, Mich.
[2] Dowex 1-X8.
[3] Estradurin.
[4] SMA, Wyeth Laboratories, Philadelphia, Pa.

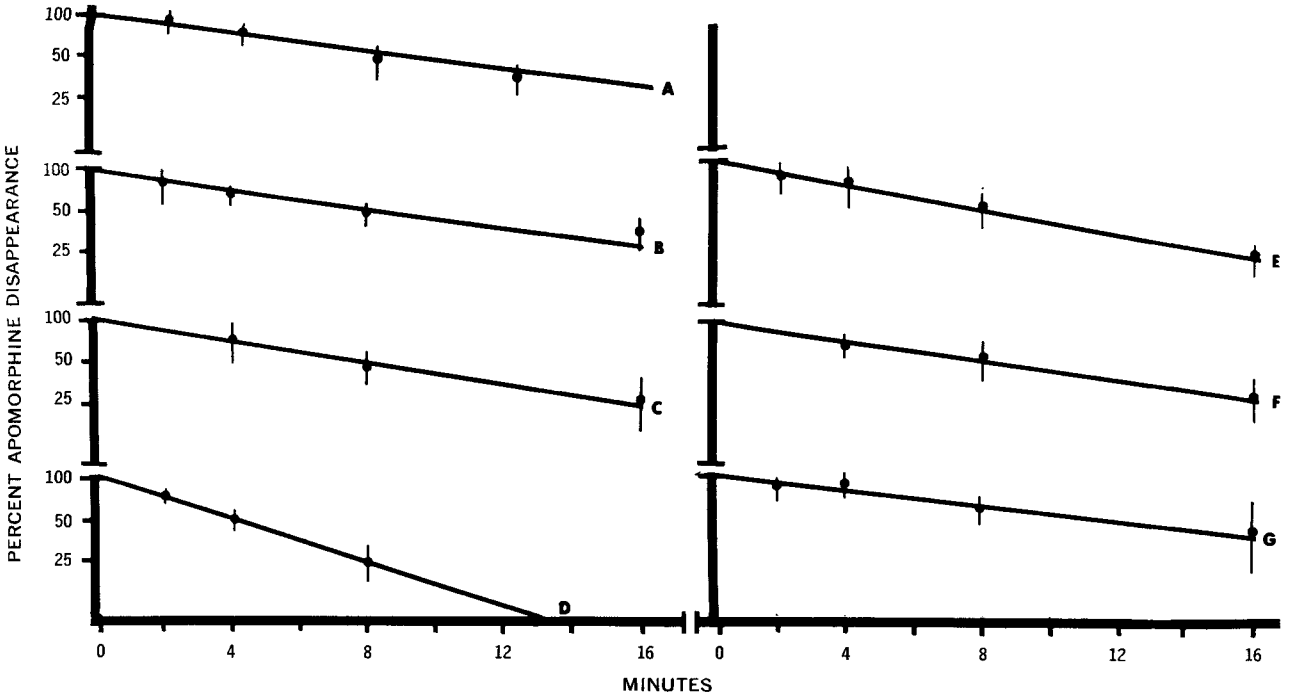

Figure 1—*Percent disappearance of apomorphine with time in whole mice given apomorphine intravenously. Each point represents the mean of 5 to 10 animals* ±SD. *Key: A, controls, no pretreatment,* $t_{1/2}$ *8 min.; B, phenobarbital treated,* $t_{1/2}$ *8 min.; C, testosterone treated,* $t_{1/2}$ *8.3 min.; D, apomorphine pretreated,* $t_{1/2}$ *5 min.; E, SKF 525-A treated,* $t_{1/2}$ *7.2 min.; F, actinomycin D treated,* $t_{1/2}$ *9 min.; and G, polyestradiol treated,* $t_{1/2}$ *10.8 min.*

RESULTS AND DISCUSSION

Determination of free and conjugated drug in the whole animal at any fixed time eliminates the need to consider influence of factors such as organ distribution, volume of distribution, and biliary and renal clearance, which all affect the drug and metabolite concentrations present in the blood. After apomorphine injection, each mouse was kept in a beaker until the time of homogenization. The washings of the beaker were added to the homogenate, including any urine or feces that may have been excreted by the animal. The assay, therefore, represents the total free as well as conjugated form (in this case, glucuronide) of apomorphine at any particular time.

Figure 1 shows the effect of various inducers and inhibitors on the rate of apomorphine disappearance in mice. Phenobarbital and testosterone did not show any induction ($p > 0.1$), but apomorphine significantly induced ($p < 0.05$) its own metabolism (Curve D). Of the inhibitors, actinomycin D and SKF 525-A did not show any reduction ($p > 0.05$) in the disappearance rate, whereas polyestradiol produced a significant inhibition ($p < 0.02$) of the kinetics (Curve G). Inhibition of apomorphine glucuronidation by polyestradiol is in line with the finding of Inscoe and Axelrod (9) that estradiol inhibits the glucuronidation of *o*-aminophenol in rats. In the case of SKF 525-A, however, no inhibition of glucuronidation was observed in the present study. This is in contrast to the finding of Ikeda *et al.* (10) that *o*-aminophenol glucuronidation in rats and guinea pigs was inhibited by this compound.

Table I includes the first-order kinetic constants of apomorphine disappearance after pretreatment of animals with the various drugs and the amounts of apomorphine glucuronides formed in the body at 8 min. (normal $t_{1/2}$) after intravenous injection of apomorphine hydrochloride. Table II gives the effect of various agents on pentobarbital sleeping time in mice, confirming the literature reports that the drugs tested on apomorphine metabolism did have definite effects on the rate of pentobarbital metabolism under the

Table I—Disappearance-Rate Constants of Apomorphine and the Amounts of Glucuronides Formed in Mice Pretreated with Various Drugs

Treatment	Apomorphine Disappearance Constants, $K \times 10^{-2}$ min.$^{-1}$	Appearance of Apomorphine Glucuronides at 8 min. Percent of total ±*SE*	Effect[a]	*n*	*p* Value
Saline	8.66	73.0 ±2.5		9	
Phenobarbital	8.66	70.6 ±3.2	0	10	>0.1
Testosterone	8.31	73.3 ±3.3	0	5	>0.1
Apomorphine	13.86	93.8 ±4.7	+	4	<0.01
Actinomycin D	7.70	63.9 ±3.5	0	5	>0.05
SKF 525-A	8.66	75.6 ±3.3	0	12	>0.1
Polyestradiol	6.49	61.2 ±3.4	−	9	<0.02
Starvation	5.54	47.6 ±9.0	−	3	<0.01

[a] Insignificant effect = 0; induction = +; and inhibition = −.

Table II—Effect of Various Drugs on Pentobarbital Sleeping Time in Mice

Treatment	n/ Sex	Sleeping Time ± SE, min.	Effect[a]	Data Source
Saline	9	67.9 ± 7.8		This study
Actinomycin D	9	202.2 ± 23.9	− ($p < 0.01$)	This study
Apomorphine	11	78.9 ± 3.0	0 ($p > 0.05$)	This study
SKF 525-A	10	>400	− ($p < 0.001$)	This study
Phenobarbital			+	(12)
Testosterone	♀		−	(8)
	♂		+	(8)
Estradiol[b]	♂		—	(13)

[a] Effect on the metabolism of pentobarbital; insignificant effect = 0; induction = +; and inhibition = −. [b] Effect on hexobarbital metabolism.

present experimental conditions.

Studies on actinomycin D generally have supported that it only blocks the enzyme induction initiated by inducers such as phenobarbital (3). In the present experiments, however, this compound showed a significant prolongation of the pentobarbital sleeping time, suggesting a direct inhibitory effect on the metabolism of pentobarbital. However, on apomorphine metabolism, actinomycin D showed no significant effect (Fig. 1).

In the experiments involving restricted oral feeding, the actinomycin D group showed nearly the same degree of inhibition of apomorphine metabolism as did the control group (Fig. 2), suggesting that the inhibition ($p < 0.01$) was a result of partial starvation due to restricted feeding. Dixon *et al.* (11) also showed that weight loss associated with starvation generally causes a decrease in the animal's ability to metabolize drugs.

Actinomycin D appears to bind with deoxyribonucleic acid (DNA) and thus blocks the DNA-dependent synthesis of nuclear ribonucleic acid (RNA) which is required for protein synthesis (14). This indirect inhibition of protein synthesis was postulated to be the mechanism by which actinomycin D inhibits or blocks the enzyme induction produced by phenobarbital (3). However, enhancement of protein synthesis or its inhibition need not necessarily reflect an induction or inhibition of a particular enzyme. For example, Wada *et al.* (15) showed that phenobarbital may increase the cytochromes P-450 and b_5, and also *N*-demethylase, but it had no effect on the delta-aminolaevulinic acid (ALA) synthetase. Likewise, another compound, 3,5-dicarbethoxy-1,4-dehydrocollidine (DDC), decreased the cytochromes P-450 and b_5 whereas it enormously induced the ALA synthetase.

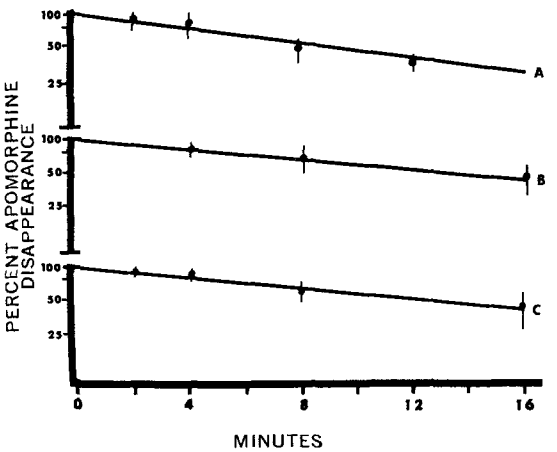

Figure 2—*Rate profiles of apomorphine disappearance with time in intact mice control-fed orally, showing the effect of partial starvation on apomorphine metabolism. Each point represents the average of 5 to 10 animals* ±SD. *Key: A, control,* ad lib. *feed,* $t_{1/2}$ *8 min.; B, control-fed, saline treated,* $t_{1/2}$ *12.5 min.; and C, control-fed, actinomycin D treated,* $t_{1/2}$ *12 min.*

Species such as horse, rabbit, and rat metabolize apomorphine into a mixture of two *O*-monoglucuronides (5, 16). The present studies show that the mouse also excretes apomorphine predominately as a mixture of 3,*O*- and 4,*O*-glucuronides. The uridine 5′-diphosphate (UDP)-glucuronyl transferase, which catalyzes the glucuronidation of most foreign and endogenous compounds, was partially purified by Isselbacher *et al.* (17). In fact, there is some evidence that more than one glucuronyl transferase may be present in the body (17, 18), but their localization in respect to the smooth and rough surfaced endoplasmic reticulum has yet to be demonstrated. Phenobarbital causes proliferation of the smooth endoplasmic reticulum but has little effect on the tough surfaced component of the membrane (19). It was suggested that the glucuronyl transferase for the synthesis of ester and ethereal glucuronides may be localized in this rough component (17) and, subsequently, it was shown to be so, at least in the case of *o*-nitrophenol (20). The apomorphine case apparently would seem to substantiate this belief, because phenobarbital did not have any effect on its glucuronidation. However, induction of ethereal glucuronidation by phenobarbital reported in the rat (21) and man (22, 23) raises a question of whether or not there is any species variation in regard to this suggested glucuronyl transferase distribution in the microsomal surfaces.

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Drug-Absorption Analysis from Pharmacological Data I: Method and Confirmation Exemplified for the Mydriatic Drug Tropicamide

VICTOR F. SMOLEN and RONALD D. SCHOENWALD

Abstract □ A theoretical basis is developed for the performance of drug-absorption analysis from data obtained from the observation of the time course of pharmacological response intensity following single, multiple, or continuous dosing of a drug by any route of administration. The results of such analyses permit an evaluation of the physiological availability characteristics of drugs from dosage forms and the elucidation of the kinetics and mechanisms operative in the passage of drugs across biological barriers. It is demonstrated that through the suitable use of intravenous dose–effect curves, the postulation of hypothetical models for pharmacon–receptor site interaction is obviated. The dose–effect curve graphically provides the relationship between the quantity of drug in body compartments (biophase) and the observed intensity of drug response. The use of pharmacological data in contrast to the use of time course of drug level in body fluid data has the advantage, in addition to not requiring a method of direct assay for the drug, of being applicable to cases where the drug first penetrates from a local site of administration to a vicinal site of action prior to reaching the systemic circulation. The method is confirmed and illustrated for tropicamide, with results derived from the slow intravenous infusion of the drug to rabbits and the measurement of pupillary diameters. Agreement was observed between the quantities of drug known to be infused at any time and the amounts calculated wholly from pharmacological data on the basis of an assumed single-compartment model. The rapid appearance of the maximum mydriatic response following rapid intravenous dosing allows approximation of the biophase as a compartment kinetically indistinguishable from the central systemic compartment.

Keyphrases □ Drug-absorption analysis—pharmacological data □ Pharmacological response intensity—biophasic drug level relationship □ Tropicamide-produced mydriasis—drug-absorption analysis □ Dose–pharmacological effect relationship determination—pharmacological data □ Model, one compartment—tropicamide dose–effect relationship

A biokinetic method of analysis of *in vivo* data that does not involve *a priori* assumptions of kinetic models for the absorption of the drug from an administration site has the distinct advantage of allowing the kinetics and mechanisms of the biological availability of the drug to be elucidated under *in vivo* conditions. The principal purposes of this report are to describe a basis for the use of temporal pharmacological response intensity data for drug-absorption analysis and to determine its applicability to the description of the biokinetic behavior of a mydriatic drug, tropicamide.

Generally, the ability of the drug to reach its biological site(s) of action (contained in a body compartment termed the biophase) is either dosage form rate limited by its release from the dosage form or biological factor rate limited by biotransformation, permeation of biological barriers, and/or unavailability due to partitioning into body depots of relatively large solubility volume (1). Information concerning some of these processes *in vivo* can be gained from a mathematical analysis of temporal blood level and/or urinary excretion data through the application of the now familiar treatments first described by Wagner and Nelson (2) and further developed by others (3). Since these treatments rely on the periodic determination of the drug by direct chemical or radiological assay techniques, they may not be practical when the detection of very small quantities of very labile or potent drugs is required. In addition, these treatments have only been described for cases where the drug is absorbed into a central systemic compartment prior to, or coincident with, inducing its biological response. This is most often the intent of oral and parenteral administration, for example.

In contrast, the study of drug availability to a biophase vicinal to the site of administration following topical or parenteral use of the drug for local effects has not been described. Provided the appropriate tissue locations could be unambiguously identified, their sampling for the periodic determination of drug levels could present intractable technical difficulties. If drug-absorption analysis could be performed using temporal pharmacological response intensity measurements, the problems associated with the direct assay for drug levels in such locations could also be obviated. The development and confirmation of a basis for the utilization of pharmacological data for the absorption analysis of tropicamide are the subjects of the present report. Tropicamide was chosen as one of the model drugs in these studies because of the relative ease of following its mydriatic activity. Moreover, due to its nonionic character at physiological pH, it was anticipated to possess relatively uncomplicated biological transference properties.

Prior to the utilization of the presently described pharmacological method of drug-absorption analysis, several procedures should be performed to confirm its unmodified applicability to a particular drug system of interest. When the method is applicable, it becomes entirely unnecessary to assay for drug concentration levels in body fluids or tissue compartments in order to perform analyses of drug absorption and availability into the systemic circulation or directly into the biophase. The areas under the curves of the results derived from the transformation of pharmacological measurements and plotted *versus* time have exactly the same interpretation with respect to physiological drug availability as similar curves obtained from blood level or urinary excretion rate *versus* time plots (2–3). In addition, when the sites of drug action are located in a compartment, termed the biophase, separate from the systemic circulation, the pharmacological method permits the determination of the "biophasic drug availability" as the fractional amount of the dose absorbed systemically that has entered and traversed the biophasic compartment. Analogous calculations of the

relative quantity of a topically administered drug that has penetrated directly to its vicinal biophase and the amount that is dissipated from the administration site by other routes, *e.g.*, systemic absorption, can also be performed. These and other aspects of the applicability of the pharmacological method to the performance of drug-absorption analysis for all routes of administration will be treated fully in subsequent reports.

THEORETICAL

Relationship of Pharmacological Response Intensity to Biophasic Drug Levels—The kinetic behavior of a drug in the body (*e.g.*, as regards its distribution, metabolism, and excretion) can most concisely be characterized in terms of compartment models. Data derived from the determination of the time course of variation of drug levels in a body compartment provide the necessary input for the construction of such biomathematical models. By treating the biophase as a compartment, its level of drug at any time following dosing is by definition uniform throughout its volume and is responsible for the observed intensity of the response to the drug. Since the biophase is a hypothetical concept, it is seldom possible to sample directly its drug content in the manner of blood, for example.

However, if a functional relationship between the level of drug in the biophase at any time and the observable intensity of drug response is known, the use of pharmacological data can, in appropriate cases, obviate the use of direct analytical methods for detecting the presence of the drug. Previous reports concerning the kinetics of pharmacological effects (4–10) invariably assumed that the intensity of a pharmacological effect is directly or otherwise simply related (*e.g.*, through hypothetical models for drug–receptor interaction) to the drug level in the biophase. In the present approach, the functional relationship between the pharmacological response intensity and biophasic drug levels is defined by the experimental intravenous dose–effect curve instead of through the postulation of hypothetical mechanisms for the dose–effect relationship. The intravenous dose–effect curve is utilized in the manner of a calibration curve to transform observed values of pharmacological response intensity (I) at any time into values of $f(I)$,[1] which are variables directly proportional to the quantity of drug in the biophase.

The present approach is developed on the consideration that the biokinetic behavior of many drugs is describable in terms of linear compartment models (11). The quantity of drug in the biophase following an intravenous dose, Q_{BS},[2] at any time, t, is given by the product of the dose, D, and a summation of exponential terms, as expressed by Eq. 1:

$$Q_{BS} = D \sum_{i=1}^{n} A_i e^{-m_i t} \qquad \text{(Eq. 1)}$$

The A_i's and m_i's are equation parameters evaluated from the data; n denotes the number of compartments included in the model. An intravenous dose–effect curve may be constructed by plotting the intensity of the pharmacological response, which has been consistently recorded at a fixed time, t_r, following rapid intravenous dosing, as a function of the dose. Substituting the symbol $f(I)_{t_r}$ for D and the constant, β_{t_r}, for the summation, $\sum_{i=1}^{n} A_i e^{-m_i t_r}$, Eq. 1 can be rewritten at t_r as Eq. 2:

$$Q_{BSt_r} = f(I)_{t_r}\beta_{t_r} \qquad \text{(Eq. 2)}$$

Assuming Eq. 2 to hold at times other than t_r allows it to be rewritten as Eq. 3, which now provides a general relationship between the quantity of drug in the biophase and the intensity of response, where $Q_B = Q_{BS}$ and/or Q_{BB}:

$$Q_B = f(I)\beta_{t_r} \qquad \text{(Eq. 3)}$$

[1] This symbol is read as "function of I"; I is not multiplied by f.

[2] The subscript, S, signifies that the quantity of drug, Q_{BS}, has reached the biophase *via* the systemic compartment. A subscript B, in place of S, indicates that the drug has entered the biophase directly from the site of administration without first being absorbed systemically.

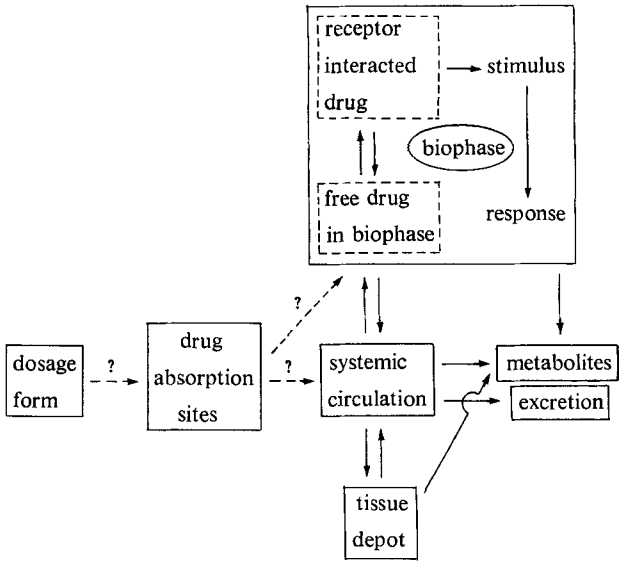

Figure 1—*Simplified compartmental illustration of the processes generally operative in affecting the pharmacological response characteristics of drugs. The purposes of drug absorption analysis are to elucidate the processes (designated by - - →) by which the drug is systemically absorbed, gains access to the biophase (which may or may not be a compartment distinct from the systemic circulation), and to determine the extent to which the absorption is efficient.*

The relationship between I and Q_B given by Eq. 3 applies at any time and irrespective of the route of administration. Substituting from Eqs. 2 and 3 into Eq. 1 allows the time course of change in the relative drug levels in the biophase following intravenous dosing to be described by Eq. 4. Equation 4 also applies to routes of administration other than the intravenous, provided the drug is absorbed from the site of its administration by an apparent first-order process, *i.e.*, passive diffusion. When this is known to be the case, data obtained from dosing by such routes can also be used in the construction of the dose–effect curve.

$$f(I) = \frac{f(I)_{t_r}}{\beta_{t_r}} \sum_{i=1}^{n} A_i e^{-m_i t} \qquad \text{(Eq. 4)}$$

Confirming the Applicability of the Approach—By referring to Fig. 1, it is implicit in the treatment that, first of all, the rate-limiting process in the manifestation of the pharmacological response at any time is the drug's ability to gain access to its biological sites of action within the compartment designated as the biophase. The subsequent interaction of the drug with the receptor sites and the induction of the stimulus resulting in the observed response are sufficiently rapid that their extents are representative of instantaneous reversible equilibrium with the levels of drug in the biphase at any time. Second, all processes are entirely reversible, *i.e.*, nonhysteretic. And, third, the biokinetic behavior of the drug is describable in terms of a linear compartment model.

When these three conditions are not satisfied, the direct use of the dose–effect curve may not be appropriate and it may be necessary to derive other mathematical models to relate intensities of response to biophasic drug levels which are based on postulated mechanisms of drug–receptor site interaction. A difficulty in deriving such relations becomes evident when it is considered that an equilibrium between the density of drug-affected receptor sites and the quantity of drug in the biophase cannot be expected. In fact, this precisely is the cause of the complication. However, some insight into the nature of the kinetic processes may again be gained from consideration of the intravenous dose–effect curve. If the curve is constructed using maximum values of the response intensity ($I_{max.}$), then considering I to be a monotonic increasing function of the equilibrium concentration (*i.e.*, concentration of drug in equilibrium with the receptor sites), the most propitious time to assume this equilibrium to be approached would be such that the rate of change of the concentration of drug in the biophase is minimal (zero). This will occur when a steady-state drug concentration in the biophase

is achieved as, for example, through a slow intravenous infusion of a drug at a constant rate. Following a rapid intravenous dose, this time is obviously $t_{max.}$, *i.e.*, the time at which the maximal response, $I_{max.}$, is observed. A dose–effect curve constructed using $I_{max.}$ values corresponding to the plateaus of I *versus* time plots observed for differing rates of intravenous infusion or, if justified, to $t_{max.}$ following rapid intravenous dosing may then be assumed to characterize the equilibrium between drug-affected and free receptor sites with free drug. This information may, in turn, be used to provide the insight necessary for the construction of a kinetic model to apply at other times besides $t_{max.}$ and to provide the needed relationship between the observed intensity of response and quantity of drug in the biophase. Such a final relation is then valid irrespective of the path by which the drug gains entry to the biophase and can be used for drug-absorption analysis in lieu of the dose–effect curve which would, in this case, not be applicable for this purpose. As will be shown, the introduction of such complications into the model for tropicamide is not necessary.

When the previously stated conditions are satisfied, the functional relationship between the quantity of drug in the biophase and the observed response intensity is accurately given by Eq. 3. Obviously, in all cases a clearly defined graded intensity of pharmacological response must be measurable.

It is not generally sufficient to assume arbitrarily that the described conditions are met for any given drug and biological system. The applicability of the approach in any given case should be confirmed. The most obvious manner to accomplish this is through a comparison of results obtained by the actual measurement of drug levels (in, for example, plasma) with similar results calculated from pharmacological data. However, the greatest utility of the use of the pharmacological method is its applicability to systems where direct assay methods for the drug are not practical. However, other equally valid means may be applied to confirm the method. Two of these means are suggested by the form of Eqs. 3 and 4 and involve the comparison of the behavior of the data as actually observed with that predicted by the equations.

Congruency Test—The first test derives directly from inspection of Eq. 4, which predicts that plots of dose normalized values of $f(I)$ as a function of time should be independent of the dose. The values of $f(I)$ are normalized by division by the dose, which by definition equals $f(I)_{t_r}$. The curves so obtained for various doses should be congruent. This provides a test of the assumption of a linear compartment model.

$f(I)$ and β Ratio Tests—The second test becomes obvious from the consideration that the value of Q_B must be the same for any given value of I, irrespective of the dose or the time at which the value of I is observed. Since the choice of t_r for the selection of I values for use in the construction of intravenous dose–effect curves is arbitrary, any number of such curves can be constructed merely by selecting values of I corresponding to any number of values of t_r and plotting them as a function of dose. For two such curves using values of t_r and t_r', Eq. 3 will be $Q_B = f(I)_{t_r}\beta_{t_r}$ and $Q_B = f(I)_{t_r'}\beta_{t_r'}$ respectively. The values of Q_B for any given value of I are the same; therefore, the ratio of the two equations will equal unity and the result given by Eq. 5 is obtained:

$$\frac{f(I)_{t_r}}{f(I)_{t_r'}} = \frac{\beta_{t_r'}}{\beta_{t_r}} \qquad \text{(Eq. 5)}$$

It is, therefore, predicted that the ratio of $f(I)$ values, for any given value of I, obtained from the abscissas of any two intravenous dose–effect curves—differing only in the selection of the time of recording of the I values used in their construction—should be constant and equal to the inverse ratio of their β values. The observation of this predicted behavior of the data may be considered as an affirmation of the assumptions that the response to the drug is nonhysteretic and the instantaneous result of a continuous equilibrium between the free and receptor bound drug in the biophase.

A_t to CAD Comparison Test—The performance of this test of the applicability of the mathematical treatment to any given system involves the direct comparison of values for the amount, A_t, of drug absorbed calculated wholly from pharmacological data with actual known amounts, CAD, of drug cumulatively administered by slow intravenous infusion. Satisfactory agreement between the known and calculated quantities may be considered as a confirmation of all the assumptions implicit in the treatment as well as of the compartment model on which the calculation of A_t is based.

Table I—Steps in the Procedure, and Their Purposes, for the Application of Pharmacological Data to Drug Absorption Analysis

Step 1	
Procedure:	Experimental determination of the relationship between I and time for several intravenous doses
Purpose:	Collection of data needed: a. For the construction of intravenous dose-effect curves b. For the congruency test c. To fit a compartment model to simulate the biokinetics of the drug
Step 2	
Procedure:	Construct two intravenous dose–effect curves
Purpose:	a. Transformation of observed I values into $f(I)$ b. Performance of $f(I)$ ratio test
Step 3	
Procedure:	Derive a linear compartment model from a computerized, constrained, sums of exponentials fit to the $f(I)$/dose to time relationship
Purpose:	a. To obtain values of transfer constants (K_{iJ}'s) needed for the computation of the amount (A_t) of drug absorbed at any time following dosing by systemic routes; not needed for direct biophasic absorption[a] b. Calculation of β ratios for comparison to $f(I)$ ratios c. Performance of A_t to CAD comparison test (CAD = known cumulative amount of drug administered by slow intravenous infusion)
Step 4	
Procedure:	Apply the method to any route of administration
Purpose:	Elucidation of the kinetics and mechanisms operative in drug bioavailability

[a] The procedure in this case will be treated in a subsequent report in the present series.

Fitting a Compartment Model to the Data—Although desirable, a biomathematical, compartment model of the biokinetic behavior of a drug does not necessarily need to have a counterpart in biophysical reality in order to possess utility. Such models are merely mathematical abstractions of the actual biological processes. For the purposes of the present application to drug-absorption analysis, the questions of whether the model chosen to describe the data is unique and whether the compartments possess physiological reality are irrelevant. In fact, it is seldom possible to determine uniquely a compartment model since any model that is compatible with the data can always be interpreted as a degenerate case of a higher order model, *i.e.*, one possessing an additional number of compartments. Therefore, the simplest model consistent with the data to which physically realizable values of the model parameters can be assigned (*i.e.*, transfer constants between compartments, K_{iJ}'s, must be real and nonnegative) is chosen.

For the present purposes, the general procedure for choosing a compartment model to describe the biokinetic behavior of the system is initiated by fitting a sum of exponential terms to a composite plot of $f(I)$/dose values as a function of time. The familiar graphical techniques (12, 13) of "peeling off" the exponentials may be used for this purpose to obtain values for the A_i's and m_i's constituting the equation parameters. Each exponential term in the summation corresponds to a compartment in the model. These hand-calculated values of the A_i's and m_i's are used as initial estimates in computer programs which are used to calculate least-square values of the A_i's and m_i's consistent with physically realizable values of the model parameters, *i.e.*, the K_{iJ}'s. The authors developed digital computer programs, using a method of systematized iterative guessing (14) for the fitting of data to a wide variety of multicompartment models. This method of curve fitting is advantageous because it converges rapidly and is devoid of the danger of merely reaching a local minimum in the sums of squares (11). The derivation of equations for relating the A_i's and m_i's to the K_{iJ}'s necessary for the computer programs is well described by Rescigno and Segre (15) as well as other authors (16, 17). In the present communication, the biokinetic behavior of tropicamide is simply described in terms of a single-compartment model in which the biophase is assumed to be kinetically indistinguishable from the central systemic compartment. A subsequent report will present results obtained from testing several

multicompartment models. Various criteria on which to base the selection of a model and judge its adequacy with regard to describing the drug biotransference behavior of pharmacologically responding systems will also be presented at that time.

Calculation of A_t for Systemic Absorption—The form of the equations for the calculation of the amount, A_t, of drug absorbed systemically from the site of administration at any time following dosing depends on the compartment model adopted to describe the distribution and elimination of the drug from the system (18, 19). When the ability of the drug to permeate tissue barriers is rapid, it may occur that the body can be treated as a single homogeneous compartment in which the biophase (as shown in Fig. 1) and other peripheral tissue depot compartments are kinetically indistinguishable from the central systemic compartment. In this case the value of A_t is simply given by Eq. 6 which resembles that of Wagner and Nelson (2). If t_r is chosen as zero, the value of β_{t_r} will be unity:

$$A_t = \beta_{t_r}\left[f(I) + K_e\int_0^t f(I)dt\right] \qquad \text{(Eq. 6)}$$

The transfer constant, K_e, represents the overall elimination constant from the system and is obtained from the slope of a linear semilogarithmic plot of $f(I)$ values as a function of time.

The procedural steps and their purposes in the application of the pharmacological method of drug-absorption analysis are summarily presented in Table I.

MATERIALS AND METHODS

Materials—Isotonic, phosphate-buffered, pH 7.4, tropicamide[3] solutions were prepared from double-distilled water for intravenous administration to rabbits.

To minimize intersubject variation, 3–4 month old, male, New Zealand white rabbits were screened with regard to: (*a*) the sensitivity of their pupillary response to varying light intensities; (*b*) the magnitude of their maximum mydriatic response to opththalmic and intravenous doses of tropicamide; and (*c*) the clarity of definition of their pupils. Four rabbits were ultimately selected from this group on the basis of observed similarities in their pupillary response behavior. These four rabbits were used throughout the remainder of the study.

Methods—Intravenous dosing of the rabbits was performed into their marginal ear veins. The doses ranged from 13.7 to 416.0 mcg./kg. and were contained in fluid volumes of 0.3 to 0.5 ml. which were rapidly injected.

The results obtained from the rapid intravenous dosing were utilized in the construction of the dose–effect curves. Additional measurements of mydriatic response were performed on the four rabbits during the time course of a slow intravenous infusion of a 0.015% w/v pH 7.4 phosphate-buffered solution of tropicamide to the rabbit at a zero-order rate. The solution was infused using a Sigmamotor[4] model T8SH pump. The rate of infusion was found to fluctuate between 0.15 and 0.19 ml./min. The variable flow rate precluded the cumulative amount of drug infused at any given time to be accurately gauged from the duration of the experiment. To circumvent this difficulty, the infusion was monitored gravimetrically. A 10-ml. covered flask containing the tropicamide solution was placed on a Mettler (p20) balance. Polyethylene tubing[5] was inserted into the solution, led through the finger projections of the pump, and finally affixed to the rabbit's ear vein *via* a 27 gauge 1.27 cm. (0.5 in.) hypodermic needle. The balance was tared, and the time course of weight change of the solution was followed simultaneously with variations of pupillary diameters.

Figure 2 describes the experimental arrangement utilized for the measurement of the rabbit's pupillary diameters. The rabbits were restrained in a box. The measurements were performed using a vernier caliper accurate to 0.1 mm. The caliper was held against the cheekbone of the rabbit at a nearly constant distance from the eye. The experimental procedure was routinely initiated by adjusting the light intensity, provided by two Bausch and Lomb microscope illuminators used in conjunction with rheostats, to obtain constant predetermined, standard pupil diameters. This procedure was found to decrease significantly both intersubject and intrasubject variations in observed mydriatic responses. When measurements were performed on both eyes, an additional light source was arranged symmetrically to that shown in Fig. 2. The control of lighting conditions was found to be a very important factor, affecting the reproducibility of the experimental results. Prolonged illumination of the eye was found to cause the pupil to dilate, following an initial constriction, and was therefore avoided. Consequently, the eye was illuminated only prior to a measurement. The minimum values of the pupillary diameters observed within a 30–45-sec. period following illumination were found to correspond to full accommodation and were recorded along with the time of their observation.

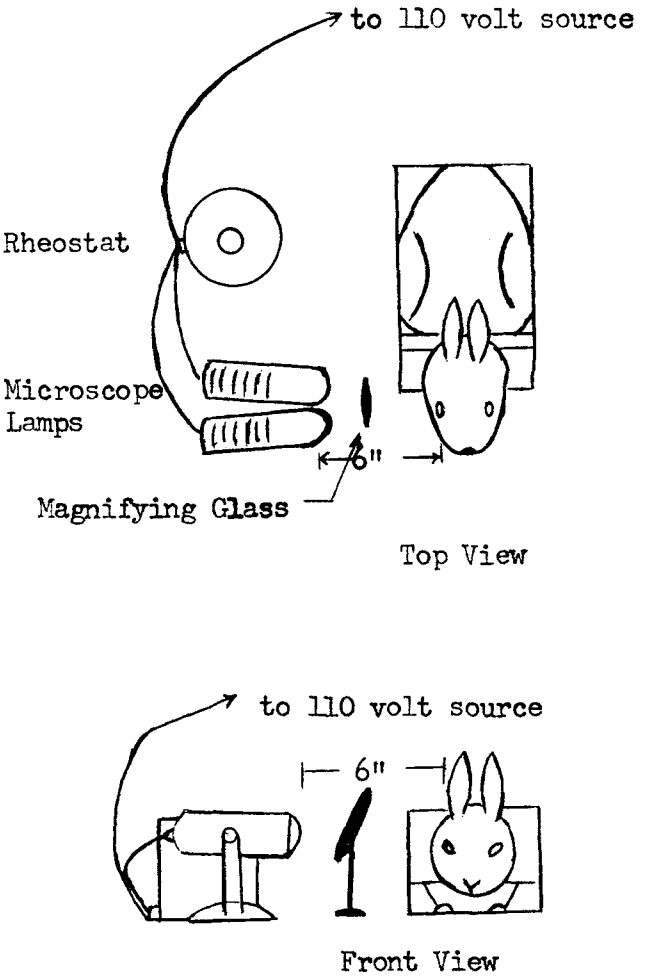

Figure 2—*Diagram of the experimental arrangement for the measurement of rabbit pupillary diameters.*

The mydriatic response intensity, I, was related to measured pupillary diameters at any time following the administration of the drug (d_t) and at time zero (d_0) by Eq 7:

$$I = \frac{d_t - d_0}{d_0} \qquad \text{(Eq. 7)}$$

An average error of precision of the $f(I)$ values, obtained through the conversion of observed I values *via* the $I_{max.}$ dose–effect curve, was calculated as less than 12%. Since all the $f(I)$ values were included in this estimate of error, it represents the overall average for the experiment. However, due to the constant error involved in the measurement of pupillary diameters, the percent average error of any value decreases with its magnitude. Mydriatic responses corresponding to drug-induced changes of less than 0.1 mm. in the diameter of the pupil could not be conveniently monitored. Therefore, responses of less than this magnitude were considered as subthreshold and recorded as zero.

[3] Tropicamide, Lot No. 2092, was supplied by Alcon Laboratories, Fort Worth, Tex.
[4] Middleport, N. Y.
[5] The tubing was animal tested PE 190: i.d. 0.119 cm. (0.047 in.), o. d. 0.170 cm. (0.067 in.); supplied by Clay-Adams, Inc., New York, N. Y.

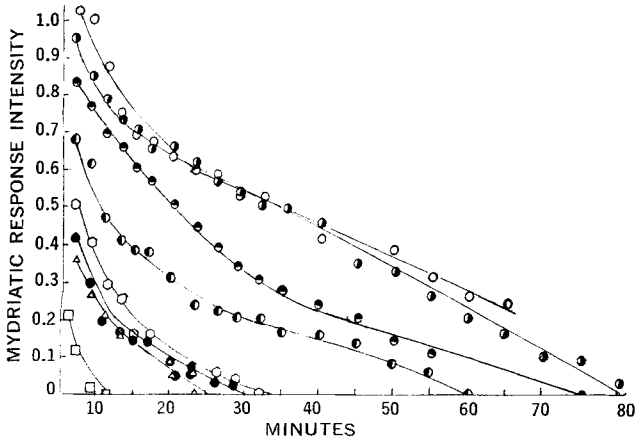

Figure 3—*Time course of mydriatic response intensity for rapidly administered intravenous doses of tropicamide. Each point is the average of a minimum of four determinations on four rabbits. The doses represented are 13.7 (□), 25.0 (△), 50.0 (●), 66.7 (○), 167 (◐), 250.5 (◓), 334 (◑), and 416.5 (○) mcg./kg. Continuous monitoring immediately following the dose revealed that, on the average, the maximum response occurred at* $t_{max.} = 1.88 \pm 0.72$ *min.*

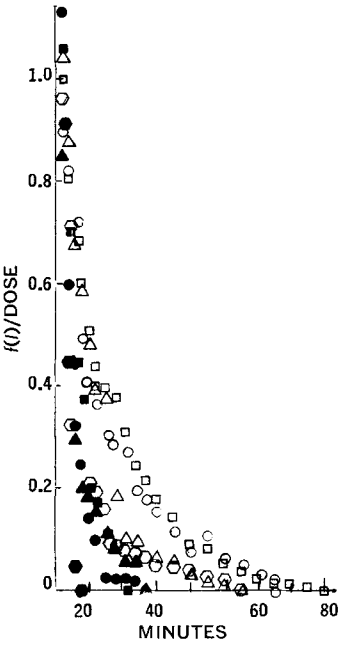

Figure 5—*The temporal variation of dose normalized relative quantities of drug in the biophase,* f(I). *Each value is the average of a minimum of four replications on four rabbits for doses of 13.7 (●), 25.0 (■), 50.0 (▲), 66.7 (●), 167.0 (○), 250.5 (△), 344.0 (□), and 416.0 (○) mcg/kg.*

RESULTS AND DISCUSSION

Mydriatic Response to Rapid Intravenous Dosing—Figure 3 graphically demonstrates the time and rapid intravenous dose dependency of the mydriatic response. The results are averages of four replications performed on the four rabbits screened for use in the study. The maximum values on the curves were recorded in each case following the continuous monitoring of the pupillary diameters immediately after dosing until the maximum response was observed to develop. The rapid appearance of the maxima, within 2 min. following dosing, precluded the accurate observation of submaximal values at prior times. The prompt development of the maximal responses is reflective of the rapidity with which the *de facto* unionized tropicamide ($pk_B \simeq 9.0$) can traverse tissue barriers to enter the biophase from the blood. Morphologically the biophase is likely constituted by some location in the iris.

Intravenous Dose–Effect Curves and the *f(I)* Ratio Test—The precision of observed values of the mydriatic response intensity increases with their magnitude. Therefore, dose–effect curves constructed by plotting the maximum observed values of I as a function of the dose produce the most precise relationship. The time, $t_{max.}$, corresponding to each $I_{max.}$ value is constant and independent of dose; this can readily be verified by differentiating Eq. 1 and setting the derivative equal to zero at $t = t_{max.}$. When $t_{max.}$ is experimentally observed to be constant, as in the present case with tropicamide, this observation may be taken as a further indication that a linear compartment model is adequate for simulating the biokinetic behavior of the drug. The choice of a constant time, t_r, at which to observe values of I for use in the construction of intravenous dose–effect curves is, therefore, consistent with the use of $I_{max.}$ values. The value of $t_{max.}$ observed for either I *versus* t or $f(I)$ *versus* t plots will of necessity be the same, provided the relationship between I and Q_B—as demonstrated by the intravenous dose–effect curve—is a single-valued, continuously increasing function. The $t_{max.}$ dose–effect curve was used for the routine conversion of I values into $f(I)$ values.

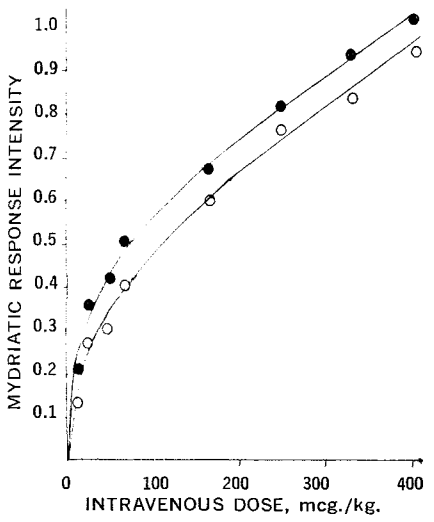

Figure 4—*Dose–effect relationships resulting from the intravenous dosing of rabbits with tropicamide. The upper curve (●) represents the maximum intensities. The lower curve (○) is a plot of the intensities observed at a time corresponding to twice the observed time of the maximum response. Each point is the average of a minimum of four replications using four animals.*

The choice of a time for the selection of I values for the construction of a second intravenous dose–effect curve may conveniently be taken as a multiple of $t_{max.}$. Figure 4 contains dose–effect curves constructed with I values corresponding to values of $t_{max.}$ and $2t_{max.}$ of 2 and 4 min., respectively. The values used in the preparation of these curves are listed in Table II.

The average ratio of $f(I)$ values, taken from the $t_{max.}$ and $2t_{max.}$ curves for I values arbitrarily chosen on the $2t_{max.}$ curve corresponding to the doses, is 1.52 ± 0.20 average error. Similar results are obtained when $f(I)$ ratios are computed from dose–effect curves constructed with I values observed at other times. Considering the exaggeration of experimental errors which occurs in the conversion of I values into $f(I)$ as well as in the calculation of the ratios, the agreement of the $f(I)_{2t_{max.}}/f(I)_{t_{max.}}$ ratios to constancy is considered acceptable. These results of the $f(I)$ ratio test are, therefore, considered as confirming the assumed nonhysteretic and reversible nature of the relationship between biophasic drug levels and the mydriatic response.

Congruency Test—A plot of average $f(I)$ values, normalized by dividing by their corresponding dose, is contained in Fig. 5. Ideally, curves drawn through each set of points corresponding to each dose should be congruent. However, the smaller values, which derive from the lowest doses, fail to coincide closely with the results corresponding to the higher doses. In addition to these particular values being derived from the smallest and least precise of the measurements, their deviant behavior may be attributed to the high sensitivity of the iris in response to low biophasic drug levels, as evidenced by the relatively large initial slopes of the dose–effect curves. This characteristic of the mydriatic response behavior becomes manifest at low dosage as an apparent premature disappearance of the effect relative to high doses. In reality the response has merely rapidly declined to below the limits of detection. Therefore, the apparent deviant behavior of the smallest doses is attributed to the experimental limitations that preclude the mensura-

Table II—Mydriatic Response Results for Rapid Intravenous Dosing of Rabbits with Tropicamide

Dose, mcg./kg.	$t_{max.}$[a]	$I_{max.}$[b]	I at $2t_{max.}$[b]	$\frac{f(I)_{2t_{max.}}}{f(I)_{t_{max.}}}$
13.7	2,2,2,1	0.215 ± 0.015	0.119 ± 0.063	1.52
25.0	2,2,1,1	0.364 ± 0.016	0.274 ± 0.060	1.72
50.0	2,2,2,2	0.420 ± 0.031	0.306 ± 0.060	1.92
66.7	2,2,4,2	0.510 ± 0.050	0.409 ± 0.044	1.67
167.0	2,1,1,2	0.671 ± 0.065	0.609 ± 0.120	1.39
250.5	1,2,1,1	0.831 ± 0.038	0.765 ± 0.012	1.17
334.0	2,1,2,2	0.940 ± 0.064	0.837 ± 0.080	1.47
416.0	1,2,4,4	1.017 ± 0.131	0.955 ± 0.251	1.16
Averages	1.88 ± 0.72			1.52 ± 0.20[b]

[a] Values, corresponding to individual rabbits, were recorded to the nearest minute. [b] Recorded ± average error.

tion of the subthreshold intensities of response which correspond to these lowest biophasic drug levels. Excluding these points, curves drawn through the remaining values would appear decidedly similar. The random scatter of points around the single curve shown in Fig. 6 may be taken as a further indication that the conditions of the congruency test are satisfied.

Single-Compartment Model for Tropicamide—Figure 6 is a semilogarithmic plot of average $f(I)$ values, which have been normalized for dose, as a function of time. A linear correlation coefficient of 0.990 was calculated for the points plotted in Fig. 6. The least-squares regression line is plotted in the figure. This line represents a fit to a single-compartment model, *i.e.*, $B + C \rightarrow$, where B and C denote the biophase and systemic circulation, respectively. Even though the convexity of the curve toward the abscissa indicates that additional compartments communicating with the biophase are operative, the single-compartment model may in some cases be preferred for its simplicity, provided the results derived on its assumption are reasonably accurate. Adoption of the linear single-compartment model in effect assumes that all kinetic processes are apparent first order and nonsaturable as well as that mixing of the drug between the systemic circulation, the biophase, and all other regions of the body is sufficiently rapid that these regions are kinetically indistinguishable. Ideally, in accordance with this model the distribution of the drug throughout the body should occur instantaneously. In adopting the model, the observed lag time of approximately 2 min. between the time of rapid intravenous dosing and the appearance of the maxima in the I and $f(I)$ *versus* time curves is subtracted from real time. On the corrected time scale, $I_{max.}$ now occurs at time zero; the value of the constant β_{t_r}, appropriate for use with the $I_{max.}$ dose–effect curve, is unity.

A comparison of the ratio of $f(I)$ values, derived from dose–effect curves constructed for two different times with the inverse ratio of their corresponding β values in accordance with Eq. 5, provides a criterion for judging the accuracy of a compartment model in simulating the change in biophasic drug levels in the period between the times corresponding to the values of I used in the construction of the dose–effect curves. The relatively poor agreement of the β ratio value of 1.17, computed on the basis of the single-compartment model, to its $f(I)$ ratio, previously noted as 1.52, reflects the inaccuracy of the model in simulating the biokinetic behavior of the system in the period of 2–4 min. Inspection of Fig. 6 reveals that the values of $f(I)$ occurring prior to 6 min. are among the largest of the values that systematically deviate from the least-squares regression line representing the fit of the data to the model. The utility of performing a comparison for an expanded time period is vitiated by the magnification of errors in the $f(I)$ ratio which occurs from the necessary use of smaller, and less precise, values of $f(I)$. The β ratio test was found to be a more useful criterion in its application to a cationic mydriatic drug (18, 19) having a longer onset and duration of action.

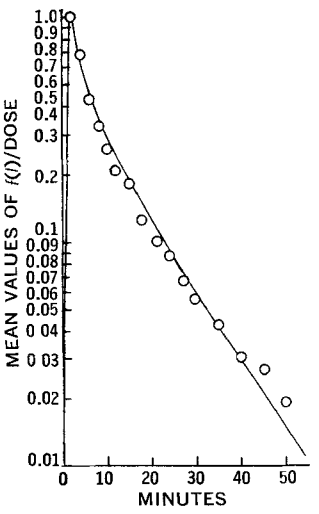

Figure 6—*Relationship of mean, normalized for dose, values of* f(I) *to time following intravenous dosing of rabbits with tropicamide. The points represent the average of four replications on different animals for each intravenous dose of 13.7, 25.0, 60.0, 66.7, 167.0, 250.5, 334.0, and 416.0 mcg./kg. The straight line is a least sums of squares regression line representing a fit of the data to a single-compartment model; the half-life for the overall elimination of the drug from the animal is 9.73 min.*

Resolution of the Dose–Effect Curve—Figure 7 demonstrates the resolution of the $I_{max.}$ intravenous dose–effect curve into apparent linear and hyperbolic components. The separation was performed by the extrapolation of the latter linear segment of the experimental curve to the ordinate and its subsequent transposition through the origin. The hyperbolic curve is obtained as the difference between the linear and the experimental curves.

The hyperbolic component of the curve is suggestive of Langmuirian adsorption and conforms to the fundamental tenets of the familiar drug–receptor occupation theory (20) for the operation of a single type of receptor (21). In accordance with these views, the hyperbolic contribution to the intensity of response (I_h) is directly related to the extent of receptor occupation and approaches a maximum, equal to αR, with the saturation of the receptor sites; this is expressed mathematically by Eq. 8, where Q_B is the quantity of drug in the constant volume of the biophase that is responsible for the total intensity of effect. The symbols α, R, and K_D refer to the intrinsic activity, total quantity of receptor sites, and the dissociation constant of the drug–receptor complex, respectively.

$$I_h = \frac{\alpha R Q_B}{K_D + Q_B} \qquad \text{(Eq. 8)}$$

The linear component of the dose–effect curve may be interpreted as a result of an apparently nonsaturable process such as could

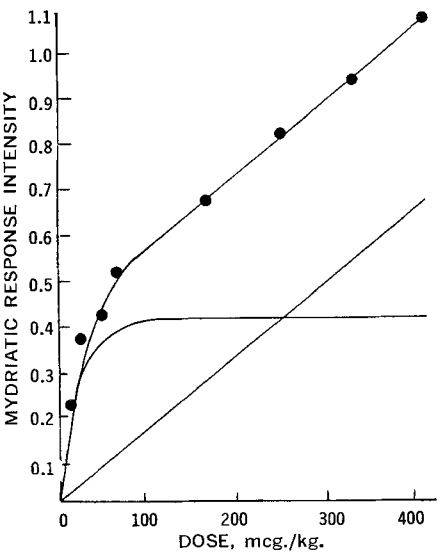

Figure 7—*Resolution of the* $I_{max.}$ *intravenous dose–effect curve for tropicamide into linear and hyperbolic components.*

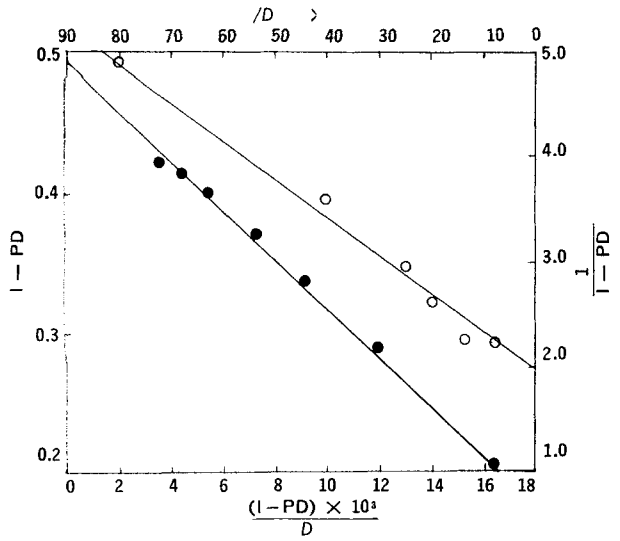

Figure 8—*Scatchard-* (●) *and reciprocal-* (○) *type linearized plots of the hyperbolic component resolved from the* I_{max}. *intravenous dose–effect curve of tropicamide for rabbits.*

conceivably involve the partitioning of the drug into some "intrabiophasic" location. The partitioning would be required to occur at rates sufficiently rapid for the continuous maintenance of distribution equilibrium throughout the volume of the biophase. Based on this surmise, the direct proportionality constant, P, relating the linear contribution to the response intensity (I_l) in Eq. 9 has some connotation both as a distribution coefficient and an intrinsic activity:

$$I_l = PQ_B \qquad \text{(Eq. 9)}$$

By combining Eqs. 8 and 9, the observed response intensity can be expressed by Eq. 10, in which Q_B is substituted by the equivalent product βD as defined by Eq. 2:

$$I = \frac{\alpha R \beta D}{K_D + \beta D} + P\beta D \qquad \text{(Eq. 10)}$$

Since β equals unity, P can be directly evaluated from the slope of the linear component curve in Fig. 7. Equations 11 and 12 are linearized forms of Eq. 10, which permit the evaluation of the equation parameters from the slopes and intercepts of the straight lines shown in Fig. 8. The curves in Fig. 8 result from plots of $I - P\beta D$ *versus* $(I - P\beta D)/D$ and $1/(I - P\beta D)$ *versus* $1/D$ and are analogous to Scatchard and reciprocal plots, respectively.

$$I - P\beta D = \alpha R - \frac{K_D}{\beta}\left(\frac{I - P\beta D}{D}\right) \qquad \text{(Eq. 11)}$$

$$\frac{1}{I - P\beta D} = \frac{1}{\alpha R} + \frac{K_D}{\alpha R \beta}\left(\frac{1}{D}\right) \qquad \text{(Eq. 12)}$$

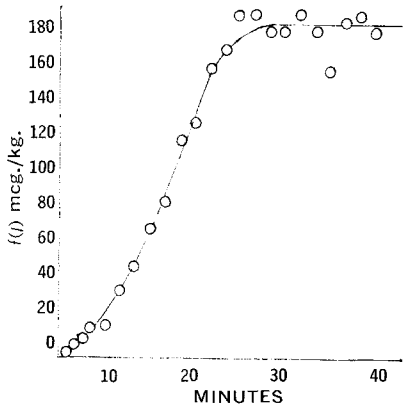

Figure 9—*The variation of* f(I), *the relative quantity of drug in the biophase, during the slow intravenous infusion of 0.015% tropicamide in an isotonic pH 7.4 solution at an average rate of 0.17 ml./min. The results are the average of four determinations on different rabbits.*

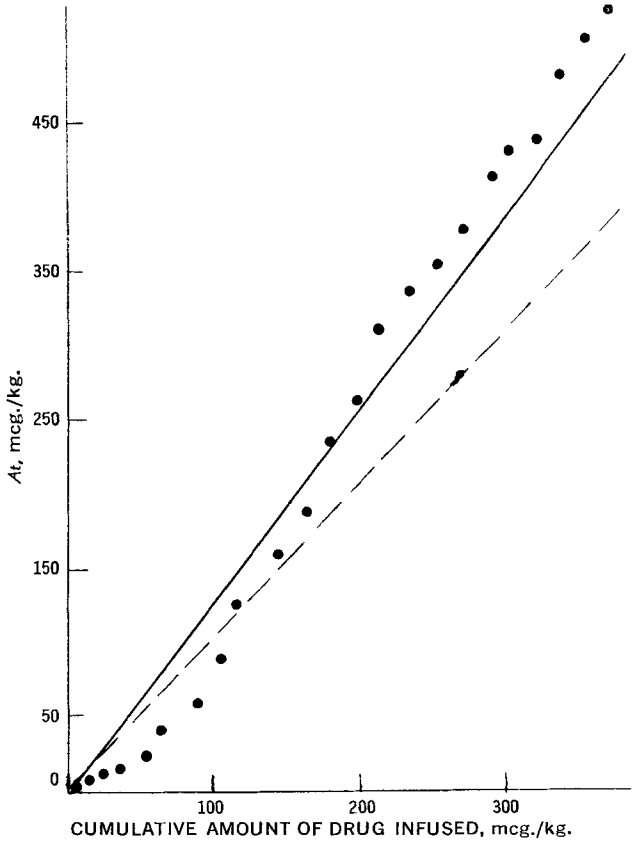

Figure 10—*A comparison of actual cumulative amounts of tropicamide administered to rabbits by slow continuous intravenous infusion to amounts,* A_t, *calculated from pharmacological data on the basis of a single-compartment model. The slope of the least-squares regression line (solid) forced through the origin is 1.29. The linear correlation coefficient is 0.994. The dotted line represents the theoretically perfect correlation with a slope of unity.*

The values of the model parameters, as computed from least-squares regression values of slopes and intercepts, are listed in Eqs. 13–15.

$$K_D = 17.33 \text{ mcg./kg.} \qquad \text{(Eq. 13)}$$

$$\alpha R = 0.496 \text{ mcg./kg.} \qquad \text{(Eq. 14)}$$

$$P = 1.57 \times 10^{-3} \text{ kg./mcg.} \qquad \text{(Eq. 15)}$$

Although the analysis of the intravenous dose–effect curve for tropicamide suggests some interesting aspects relative to the mechanisms of the pharmacological action of the drug, such information is not required and is in fact irrelevant to the use of the pharmacological method of drug-absorption analysis when the conditions of the $f(I)$ ratio and congruency tests are satisfied.

Comparison of Known and Calculated Amounts of Slow Intravenously Infused Drug—Figure 9 graphically demonstrates the variation of $f(I)$ values during the time course of the slow intravenous infusion of tropicamide at a zero-order rate of approximately 10 mcg. $kg.^{-1}$ $min.^{-1}$. A steady state was attained after approximately 20 min. The infusion was terminated at the end of 40 min.

Using the $f(I)$ values in Fig. 9 and the previously obtained value of 0.0713 for K_e, Eq. 8 was implemented for the calculation of A_t for each time corresponding to the known values of the cumulative amount of drug infused. Figure 10 compares normalized values of the known cumulative amounts of drug administered by slow intravenous infusion with the similar amounts of drug calculated from the pharmacological data. Ideally the plot in Fig. 10 should be linear with a zero intercept and a slope of unity as represented by the dashed line. The least-squares regression line plotted in the figure has been forced through the origin and has a slope of 1.29 The linear correlation coefficient for the relationship between the calculated and experimentally known values of A_t is 0.994. Although

there are obvious deviations, considering that the calculated values were derived entirely from the measurement of pupillary diameters, these results may be considered as providing additional confirmation of the applicability of the pharmacological method of analysis to this system. The majority of the results indicate that the single-compartment model represents a fair estimation of the kinetic behavior of tropicamide. However, the appearance in some instances of apparent systematic deviations of the data from values predicted on the basis of the single-compartment model suggests that the simulation of the biokinetic behavior of tropicamide might be improved with the adoption of a multicompartment model. The results obtained from the development and testing of 12 two-compartment and 9 three-compartment models will be the subject of a subsequent report.

SUMMARY AND CONCLUSIONS

The principal purpose of the present report has been the introduction of a method, and the means by which it may be confirmed, for the performance of drug-absorption analysis entirely from pharmacological data. A direct means of confirming the applicability of the described method to any particular pharmacologically responding system can most obviously be obtained through a comparison of results derived by its use with similar results based on the detection of the drug in corporeal fluids. However, the greatest utility of the pharmacological method obtains when such direct measurements are not convenient or practicable. As exemplified for tropicamide, confidence in the applicability of the techniques may alternatively be obtained by comparing the behavior of the observed pharmacological results with that predicted by a postulated model. In the present case, the conformancy of the results for tropicamide was concluded as sufficient for verity in the application of the method to the absorption analysis of this drug.

Unmodified, the presently described method may be expected to fail for cases where: (*a*) the biological response to the drug is not a continuous single-valued function of the equilibrium drug level in the biophase, *e.g.*, it may exhibit hysteresis; (*b*) a continuously graded intensity of response is not detectable; (*c*) the effects of the drug are not immediately reversed by the removal of the drug from the biophase; and (*d*) the biokinetic behavior or the drug at some stage following its absorption into the central compartment or the biophase from its site of administration occurs by processes not describable by linear compartment modeling. Some of these conditions can be corrected for by suitable and sometimes simple modifications of the mathematical relations derived to describe the system behavior. In other cases, the innate behavior of the systems will preclude the use of pharmacological data for drug-absorption analysis. In every case, indirect or direct verification of the pharmacological method should be obtained prior to its application to new drugs or biological systems.

Generally, results derived from the use of pharmacological data corresponding to the sought clinical response to the drug are of particular interest. However, when the sought therapeutic response cannot be quantitated or is otherwise not amenable to treatment by the methods described, data obtained from the monitoring of a side effect of the drug may be utilized instead. In this manner the same information that is commonly derived from blood level and urinary excretion data can still be obtained from pharmacological results. In this case the utilization of pharmacological data merely serves as an analytical tool which can be applied in lieu of directly assaying for the drug in body fluids or tissues. Since confidence in the applicability of the pharmacological data for this purpose is obtainable from the pharmacological data itself, there may be no necessity for performing direct assays, even in those instances with drugs whose direct detection in the body is practicable. The choice between the use of direct assay or pharmacological data may in each case depend upon the expected relative accuracy, reproducibility, and ease with which experimental results can be obtained.

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Metabolism of Methocarbamol in the Rat, Dog, and Human

ROBERT B. BRUCE, LENNOX B. TURNBULL, and JACK H. NEWMAN

Abstract □ Methocarbamol-^{14}C has been shown to be metabolized in the dog, rat, and man by dealkylation, hydroxylation, and conjugation with glucuronide and sulfate. Two metabolites have been isolated and identified in the urine of the three species. Blood levels and urinary excretion are also described.

Keyphrases □ Metabolism, methocarbamol, ^{14}C and unlabeled—rat, dog, human □ Methocarbamol-^{14}C—synthesis □ Blood levels—methocarbamol □ Urinary excretion—methocarbamol □ TLC—separation □ Scintillometry—analysis □ IR spectrophotometry—identification □ UV spectrophotometry—identification □ NMR spectroscopy—identification

Methocarbamol was first described by Murphey (1), and its effectiveness as a muscle relaxant was shown by a number of investigators. A study of its distribution and metabolism in dogs and humans was reported by Campbell *et al.* (2). The metabolites were not identified. This study reports the findings of an investigation to determine the metabolic products excreted by the rat, dog, and human.

EXPERIMENTAL

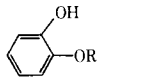
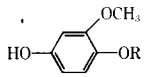
Synthesis—*1,2,3-^{14}C-Glycerol-1,2-isopropylidine (3)*—A 921-mg. sample of uniformly labeled glycerol (10.0 mmoles; 10 mc.) was shaken overnight at room temperature in 30 ml. acetone and 1.5 g. sodium sulfate. Magnesium oxide (750 mg.) was added, and the mixture was shaken until a neutral pH resulted. The solids were removed by filtration, and the concentrated residue was partitioned between chloroform and water, producing 815 mg., 6.18 mmoles, of product; yield 62%. The TLC pattern (10% methanol–chloroform) of the product indicated one major zone of radioactivity.

1,2,3-^{14}C-Glycerol-1,2-isopropylidine Tosylate—The 815 mg. (6.18 mmoles) of isopropylidine derivative was dissolved in 8 ml. of pyridine containing 1.16 g. (6.18 mmoles) of *p*-toluene sulfonyl chloride, and the solution was allowed to stand overnight. After removing the pyridine at reduced pressure, the residue was partitioned between bicarbonate solution and chloroform and then between dilute HCl and water. The chloroform extract crystallized and amounted to 1.37 g. (4.9 mmoles), 81% yield. TLC (5% methanol–benzene) indicated a major zone and one minor radioactive zone.

1,2,3-^{14}C-Glyceryl Guaiacolate (4)—The crystalline tosylate (1.30 g., 4.55 mmoles) was dissolved in 8 ml. of *n*-butanol and refluxed with stirring with 0.209 g. (9.10 mmoles) of sodium and 1.13 g. (9.10 mmoles) guaiacol for 5 hr. The reaction mixture was partitioned between water and ether. After evaporating the solvent, the extract was refluxed 1 hr. in 35 ml. of 1 *N* H_2SO_4. After the solution was made alkaline and extracted several times with chloroform, solvent distillation produced 0.527 g. and 2.2 mc. radioactivity. TLC in 10% methanol–chloroform gave a single spot of radioactive material.

1,2,3-^{14}C-Methocarbamol[1]—The glyceryl guaiacolate sample was refluxed for 1.5 hr. in 15 ml. of diethylcarbonate containing 4 drops of a 30% methanolic solution of sodium methoxide, during which time about two-thirds of the solvent was boiled off. The remaining reaction mixture was taken to dryness on a rotating evaporator. Traces of the reagent were removed from the product by adding two portions of xylene and evaporating on the rotating evaporator. This residue was then dissolved in 3 ml. of toluene, and the solution was shaken in a small vial with 1.4 ml. of concentrated ammonium hydroxide overnight. After removing the reagents under reduced pressure, the crystalline residue was recrystallized from 8 ml. benzene containing a trace of acetic acid. The 408 mg. obtained was recrystallized from 6 ml. of ethyl acetate to give 242 mg., m.p. 94–96.5°. This fraction had a specific activity of 0.995 mc./mmole, and TLC (15% isopropanol–chloroform) indicated complete homogeneity. Unlabeled methocarbamol (432 mg.) was added to the mother liquor from the recrystallization, and a fraction of lower specific activity was crystallized from ethyl acetate, 447 mg., m.p. 93.5–95°, specific activity 0.272 mc./mmole or 2.50×10^6 d.p.m./mg. The overall radioyield from 1,2,3-^{14}C-glycerol was 15%. IR curves of the two samples were identical to authentic samples of methocarbamol.

Table I—Percentages of Total Urinary Radioactivity Excreted by the Rat and Dog after Receiving Methocarbamol-^{14}C Orally

Fraction	^{14}C in Urine, %: Rat[a]	Dog[a]
Extract of fresh urine[b]	8.3	—
Extract of β-glucuronidase-treated urine[b]	28	30
Extract of glusulase-treated urine[b]	78	77
R_f 0.85 component[c]	2	0
Methocarbamol	10	46
 [c,d]	31	10
 [c,d]	24	17

[a] Dose: Rat and dog: 100 mg./kg. orally; 0–24 hr. urine. [b] Extract: three double volumes of ethyl acetate at pH 5.0. [c] Individual component assay obtained by TLC scrape-off, extraction, and counting. [d] R = $-CH_2CHOHCH_2OCONH_2$.

Animal Studies—*Dogs*—The ^{14}C-labeled drug was administered to a mongrel dog as a crystalline solid in a gelatin capsule at a level of 100 mg./kg. Unlabeled methocarbamol was dosed similarly at a level of 200 mg./kg. The urine was collected by means of a runoff tray at 24-hr. intervals.

Rats—Rats were dosed by means of a stomach tube with 100 mg./kg. of methocarbamol in 5 ml. of aqueous solution. They were placed in metabolism cages, and urine and feces were collected at 24-hr. intervals.

Human Studies with Unlabeled Methocarbamol—Two male subjects received oral doses of 2.0 g. of methocarbamol (four 500-mg. tablets).[2] Urine was collected at 2-hr. intervals.

Human Studies with Labeled Methocarbamol—Two normal male subjects were administered the drug.[3] One subject received a single oral dose of 200 mg. of methocarbamol containing 113 μc., and the other subject received 1.0 g. of drug also containing 113 μc. of methocarbamol-^{14}C. Samples of urine and blood were collected at intervals and submitted to these laboratories for analysis.

Analytical—*Radioactivity*—Samples were analyzed for radioactivity using a Packard liquid scintillation spectrometer, series 314E. Aliquots of urine and whole blood were counted directly. Fecal specimens were dried in a vacuum oven prior to grinding and combustion to $^{14}CO_2$, which was trapped in β-phenethylamine for counting. Corrections for quenching were carried out by the internal

[1] AHR-85.

[2] Robaxin.

[3] By Dr. Albert J. Wasserman at the Medical College of Virginia.

Table II—Urinary Excretion of Methocarbamol and Metabolites following Oral Doses of 200 and 1000 mg. Containing 113 μc. of Methocarbamol-^{14}C

Time after Dose, hr.	Total-^{14}C, mg.	Metho-carbamol, mg.	Metabolite 1, mg.	Metabolite 2, mg.
	Subject 1–200 mg.			
2	109.0	14	32	32
4	43.8	3	15	13
6	18.6	1	7	7
8	8.2	0.3	2	3
10	3.8	—	—	—
12	2.4	—	—	—
24	4.8	—	—	—
48	2.2	—	—	—
72	1.2	—	—	—
	Subject 2–1000 mg.			
2	259.0	57	57	57
4	316.0	52	66	66
6	158.0	18	32	37
8	118.0	14	40	52
10	51.0	4	11	14
12	29.0	2	1	10
24	41.0	—	—	—
48	11.0	—	—	—
72	4.0	—	—	—

standard method with addition of a calibrated standard solution of benzoic acid-^{14}C.

Urine Fractionation—Urine samples were incubated at pH 5 and 38° for 48 hr. with glusulase and extracted with three double volumes of ethyl acetate. The extract was concentrated and chromatographed on thin-layer silica gel with isopropanol–chloroform (15:88). Radioactive spots were located by exposing the chromatograms to X-ray film.

RESULTS

TLC of the extract of glusulase-hydrolyzed human, dog, and rat urine showed three major radioactive components at R_f 0.50, 0.35, and 0.27. The rat urine also showed a minor component at R_f 0.85 and several minor components between this and the spot at R_f 0.50.

The residual aqueous phase of unextractable material containing 20–30% of the radioactivity was tested in various ways to obtain additional information. Gel filtration, ion exchange, and TLC were run. Several components were evident from TLC, including what appears to be one major polar component. However, none of these components has been identified.

To identify the three major components, 300 ml. of dog urine was treated with glusulase and extracted with ethyl acetate. The extract was concentrated, and preparative TLC was run on 20 × 20-cm. silica GF plates. The zones were scraped off and extracted with methanol, and the extract was partitioned between water and ethyl acetate. The ethyl acetate was evaporated to dryness, giving 151 mg. at R_f 0.50, 18 mg. at R_f 0.35, and 28 mg. at R_f 0.27. The R_f 0.50 fraction was compared with authentic methocarbamol and proved to be identical according to NMR, IR, and UV. It also cochromatographed with methocarbamol.

The UV, NMR, and IR data of the R_f 0.35 component corresponded exactly with a sample of 3-(2-hydroxyphenoxy)-1,2-propanediol-1-carbamate (5). A small sample of component R_f 0.35 in methanol–ether solution was treated with diazomethane. The R_f of the resulting product by TLC was the same as methocarbamol, further proving its identity.

The spectral data, NMR, IR, and UV, of the component at R_f 0.27 were consistent with the structure: 3-(4- or 5-hydroxy-2-methoxyphenoxy)-1,2-propanediol-1-carbamate. The diacetate was prepared, and its mass spectrum was in complete agreement with the two possible structures. The probable assignment of the hydroxyl group is to position 4 of the guaiacol ring. This assignment is based on a similar metabolic conversion of 4′-fluoro-4-3-(2-methoxyphenoxy)-1-pyrrolidinyl butyrophenone,[4] which is both demethylated and hydroxylated in the 4-position of the guaiacol ring (6). The two possible metabolites of 4′-fluoro-4-3-(2-methoxyphenoxy)-1-pyrrolidinyl butyrophenone were synthesized, and comparison with the metabolite from urine showed definitely that the 4-position was hydroxylated.

[4] AHR-1900.

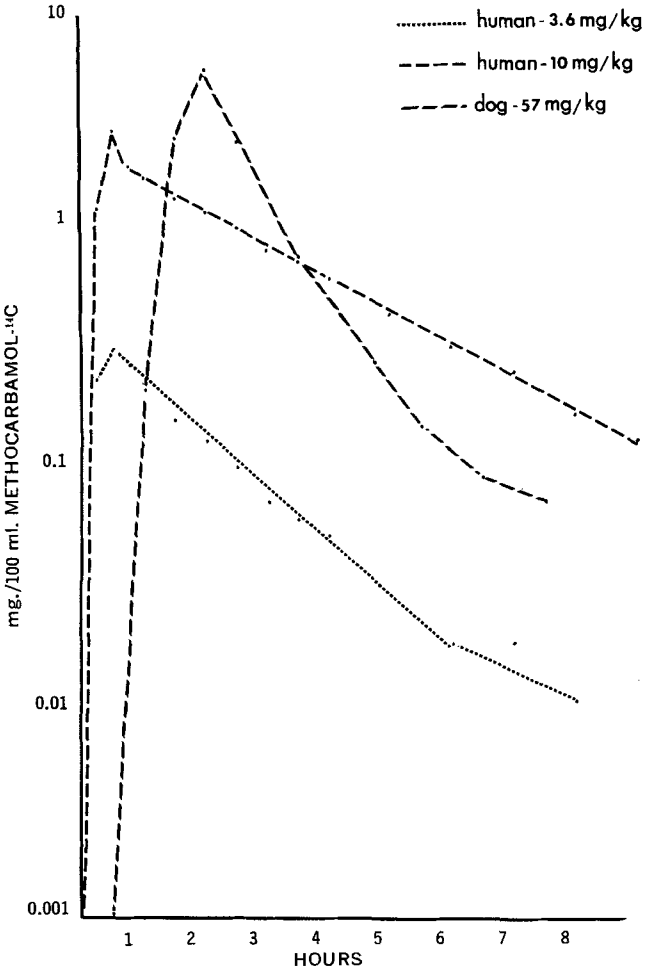
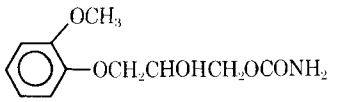
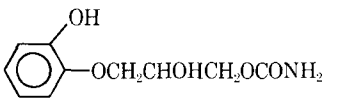


Figure 1—*Blood levels of methocarbamol-^{14}C following its administration to humans and dogs.*



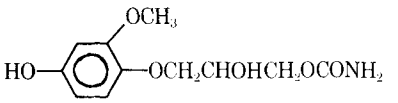
methocarbamol

I



metabolite 1
3-(2-hydroxyphenoxy)-1,2-propanediol-1-carbamate

II



metabolite 2
3-(4-hydroxy-2-methoxyphenoxy)-1,2-propanediol-1-carbamate

III

Table III—Excretion of Methocarbamol-^{14}C by a Female Mongrel Dog following Administration of 57 mg./kg. Orally

Time after Dose, hr.	Methocarbamol-^{14}C, % of Dose
Urinary Excretion	
0–24	85.01
24–51	2.97
51–74	0.80
74–98	0.07
Total	88.85
Fecal Excretion	
0–51	1.64
51–74	0.46
74–98	0.04
Total	2.14

Table IV—Excretion of Methocarbamol-^{14}C by the Rat following an Oral Dose of 100 mg./kg.

Time after Dose, hr.	Rat No. 1	Rat No. 2	Rat No. 3
	% of Dose		
Urinary Excretion			
0–26	90	90	83
26–47	2.0	1.5	1.0
47–71.5	0.5	1.0	0.5
Total	92.5	92.5	84.5
Fecal Excretion			
0–26	—	5.4	13
26–47	—	5.9	0.25
47–71.5	—	0	0
Total	0	11.3	13.25

Blood Levels—The results from the blood level studies in the dog and man are shown as a logarithmic plot in Fig. 1. These results represent the total radioactivity expressed as mg. of methocarbamol per 100 ml. of blood and therefore include both methocarbamol and its metabolites.

DISCUSSION

The blood level curves shown in Fig. 1 indicate that methocarbamol was absorbed slower by the dog than by the human subjects. The maximum concentration in the humans occurred at 0.5 hr., whereas the maximum in the dog occurred at 2.0 hr. The half-lives calculated from these data indicate more rapid elimination by the dog than by the humans. The calculated estimates for half-lives were 2.15 hr. for the 1000-mg. dose, 1.6 hr. for the 200-mg. dose, and 0.6 hr. for the dog. These results agree with those previously found (2), indicating that the drug is readily absorbed by both species.

The results indicate that methocarbamol (I) and its metabolites (II and III) are extensively conjugated before excretion in the urine. The primary paths of metabolism are through dealkylation and hydroxylation.

Conjugation could occur at a number of sites on these compounds and they could be conjugated as glucuronides or as sulfates.

A sample of fresh, unhydrolyzed rat urine was extracted directly at neutral pH. Only 8.3% of the counts present in the urine was extracted, indicating that most of the components were conjugated. A TLC run of this extract indicated that most of the material was unchanged methocarbamol. It is obvious from the studies that the majority of the material excreted is conjugated. Attempts to separate the conjugates were unsuccessful. When a sample of the rat urine was incubated with Sigma bacterial type II β-glucuronidase at pH 6.8 and 37° for 2 days and then extracted at neutrality with ethyl acetate, 28% of the urine counts was extracted. A similar experiment with dog urine gave 30% of urine radioactivity, indicating similar conjugations in both species. The results in Table I indicate that this is, however, less than half the total conjugated material and that the major portion excreted must be as the sulfate, since glusulase contains both glucuronidase and sulfatase.

Chlorphenesin carbamate (7) has been found to be metabolized in the dog to neutral and acidic components. The major metabolite was the glucuronide of chlorphenesin carbamate, and a smaller amount was conjugated as the sulfate. Some 19% of the drug was excreted as free or conjugated *p*-chlorophenol, *p*-chlorophenoxylactic acid, and *p*-chlorophenoxyacetic acid.

Urine from rats given methocarbamol was, therefore, investigated for acid metabolites. A sample of the radioactive glusulase-hydrolyzed rat urine was extracted three times at pH 3.0 with a double volume of ethyl acetate. Back-extraction with sodium bicarbonate solution gave 8% of the original urine counts. After acidifying the bicarbonate fraction, reextracting the radioactivity into ethyl acetate, and removing the solvent under reduced pressure, the residue was chromatographed in a formic acid–ethanol–chloroform system (5:10:75). One major component at R_f 0.20 and several minor components resulted.

None of these components cochromatographed with *o*-methoxyphenoxyacetic acid or β-(*o*-methoxyphenoxy)lactic acid.

Since simple methods were available, it was of interest to study the pharmacodynamics of the urinary excretion of methocarbamol and its metabolites. Following TLC of the extracts, the spots were eluted and the radioactivity in each was determined. The results are shown in Table II. The column headed "Total-^{14}C" shows the total radioactivity excreted and includes not only the compounds shown in the next two columns but also all other radioactive material excreted. This would include one, two, and three carbon fragments resulting from hydrolysis of the glyceryl ether linkage. Such small molecules were not identified. The maximum excretion rates occurred at the 2nd to 4th hours for all the material quantitated, indicating that excretion and metabolism are rapid. The total percentages of radioactivity excreted in the urine was 97% for Subject 1 and 98.7% for Subject 2 in 72 hr. Extremely small amounts were found in the feces.

The excretion of methocarbamol and related materials by the dog and rat is shown in Tables III and IV, respectively. In both cases, excretion was again rapid, with most material being excreted in the first 24 hr. Only 2% was excreted by the dog in the feces, whereas some 11–13% was excreted in the feces by the rat.

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Colorimetric Assay of Tetracycline Antibiotics

L. G. CHATTEN and S. I. KRAUSE

Abstract □ A sensitive colorimetric method of analysis for seven compounds of the tetracycline series was developed, based on the formation of a colored complex between the tetracyclines and quadrivalent thorium. The effects of pH and time upon the stability of the complexes were observed. The method was applied to 29 different pharmaceutical dosage forms, and satisfactory results were obtained for most of these.

Keyphrases □ Tetracycline dosage forms—analysis □ Thorium nitrate–tetracyclines, complex formation—tetracycline analysis □ pH, time, effects—thorium nitrate–tetracycline complex □ Colorimetric analysis—spectrophotometer

Maintained by the BP 1968 (1) and the USP XVII (2) as the official method for the assay of antibiotics, the microbiological assay serves as the standard method employed by pharmaceutical manufacturers to ascertain the potency of their products. However, numerous shortcomings of this method have stimulated many attempts to develop more rapid and precise techniques for the analysis of tetracyclines. Among these have been several paper chromatographic procedures (3–5) and, more recently, a column chromatographic technique using edetic acid (EDTA)-impregnated earth (6).

In addition to chromatographic procedures, numerous other methods including nonaqueous titrimetry (7, 8), UV spectroscopy (9), and fluorometry (10) have exhibited some usefulness.

Colorimetry is yet another technique that lends itself to the quantitative analysis of tetracyclines. In 1955, Sakaguchi and Taguchi (11) noted the formation of a yellow chelate between chlortetracycline and thorium and subsequently developed a colorimetric method for the determination of chlortetracycline in an acetate buffered system of pH 4.0. This method was later applied also to the quantitative estimation of tetracycline and oxytetracycline.

In the present study, efforts were made to investigate the nature and stability of the complexes formed between the tetracyclines and quadrivalent thorium. The purpose of this work was to develop a simple but efficient colorimetric procedure for the quantitative analysis of all the tetracyclines presently marketed and their dosage forms.

EXPERIMENTAL

Apparatus—The following were used: conventional laboratory glassware, Beckman model B spectrophotometer, Beckman zeromatic II pH meter fitted with glass-calomel electrode system, magnetic stirring apparatus, and Büchner funnels (5 cm. diameter).

Reagents and Solutions—All chemicals employed were ACS or reagent grade in quality. The following were used: ethanol, chloroform, glacial acetic acid, 0.05 *N* perchloric acid in dioxane (standardized against primary standard potassium acid phthalate), 0.5% crystal violet in glacial acetic acid, 0.5% thorium nitrate (aqueous), and 0.5% thorium nitrate (ethanolic).

The tetracycline reference compounds used in this investigation were as follows: tetracycline HCl, chlortetracycline HCl, oxytetracycline HCl, demeclocycline HCl, tetracycline phosphate complex, doxycycline monohydrate, and doxycycline HCl.

Methods—*Determination of Reference Standard Purity*—The purities of the crystalline reference compounds were determined by the nonaqueous titrimetric method of Sideri and Osol (7). The progress of all titrations was followed potentiometrically with a Beckman zeromatic II pH meter. In all instances the purity of the standard was found to be not less than 94.8%. This information was taken into account in subsequent calibration curve preparation for the colorimetric assays.

Preparation of Calibration Curves for Colorimetric Procedure—A 25-mg. sample of the crystalline reference compound was dissolved in 100 ml. of distilled water. Into five separate 10-ml. volumetric flasks, aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 ml. of the 0.25-mg./ml. stock solution were pipeted. To each of these was added 0.5 ml. of 0.5% thorium nitrate. The content of each flask was

Table I—Data Pertaining to Tetracyclines Employed in Study

Name of Drug	Supplier	$\lambda_{max.}$ Complex, nm.	Molar Absorbance of Complex	Time Required for Stable Color, min.
Tetracycline HCl	Upjohn	395	23,404	20
Oxytetracycline HCl	Pfizer	395	14,784	20
Chlortetracycline HCl	Cyanamid	405	12,714	20
Demeclocycline HCl	Cyanamid	405	12,709	20
Tetracycline phosphate complex	Bristol	395	—	20
Doxycycline HCl	Pfizer	395	19,936	35
Doxycycline monohydrate	Pfizer	395	14,506	35

Table II—Results of Thorium Nitrate Colorimetric Method

Commercial Product	Manufacturer	Labeled Drug Content	Labeled Strength, %: Colorimetric	Labeled Strength, %: Manufacturer's Result[a]
Achromycin capsules	Lederle	250 mg. Tetracycline HCl/ capsule	99.6	99.84
Albamycin T capsules	Upjohn	125 mg. Tetracycline HCl/ capsule	99.9	100.00
Aureomycin capsules	Lederle	Chlortetracycline HCl 250 mg./capsule	101.1	Not available
Declostatin capsules	Lederle	Demeclocycline HCl 250 mg./capsule	97.7	99.90
Resteclin capsules	Squibb	250 mg. Tetracycline HCl/ capsule	101.5	118.00[b] 101.20[c]
Signemycin capsules	Roerig	167 mg. Tetracycline HCl/ capsule	99.5	108.90[c]
Vibramycin Scripak capsules	Pfizer	Doxycycline hyclate equivalent to 100 mg. doxycycline base/capsule	100.7	Not available
Achrocidin tablets	Lederle	Tetracycline HCl 125 mg./tablet	100.1	102.91
Aureomycin soluble tablets	Lederle	Chlortetracycline HCl 50 mg./tablet	100.2	Not available
Declomycin tablets	Lederle	Demeclocycline HCl 300 mg./tablet	96.3	100.79
Achrocidin Compound syrup	Lederle	Tetracycline HCl 25 mg./ml.	99.7	111.72
Declomycin syrup	Lederle	Demeclocycline HCl 15 mg./ml.	98.4	121.83
Resteclin syrup	Squibb	25 mg. Phosphate-potentiated tetracycline/ml.	99.4	116.80[b] 136.80[c]
Signemycin syrup	Roerig	Tetracycline HCl 16.6 mg./ml.	101.2	119.70[c]
Aureomycin ear solution	Lederle	Chlortetracycline HCl 50 mg./vial	99.7	Not available
Terra-Cortril eye/ear suspension	Pfizer	Oxytetracycline HCl equivalent to 5 mg. oxytetracycline base/ml.	98.9	100.00[c]
Resteclin aqueous drops	Squibb	Tetracycline HCl 100 mg./ml.	100.00	109.10[b]
Terra-Cortril ointment	Pfizer	Oxytetracycline HCl equivalent to 30 mg. oxytetracycline base/g.	91.6	Not available
Aureomycin ophthalmic ointment	Lederle	Chlortetracycline HCl 10 mg./g.	89.6	Not available
Vibramycin oral suspension	Pfizer	Doxycycline monohydrate equivalent to 5 mg. doxycycline base/ml. (when reconstituted)	105.7	Not available
Aureomycin surgical powder	Lederle	Chlortetracycline HCl 200 mg./g.	99.6	Not available
Terra-Cortril spray	Pfizer	Oxytetracycline HCl equivalent to 60 mg. oxytetracycline base/ml. (300 mg./can)	88.2	106.33[c]
Tetrex capsules	Bristol	Tetracycline phosphate complex = 250 mg. tetracycline HCl/capsule	107.1	104.40[c]
Tetrex-bid capsules	Bristol	Tetracycline phosphate complex = 500 mg. tetracycline HCl/capsule	106.7	104.00[c]
Tetrex-APC capsules	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/capsule	110.8	110.40[c]
Azotrex capsules	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/capsule	107.7	112.00[c]
Azotrex syrup	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/5 ml.	96.8	108.00[c]
Tetrex syrup	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/5 ml.	103.9	100.00[c]
Tetrex-AP syrup	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/5 ml.	99.3	104.00[c]

[a] Data supplied by manufacturer. [b] Microbiological assay. [c] Chemical assay method of manufacturer.

then brought to volume with distilled water, and the flasks were well shaken. After 20–35 min., absorbances were determined in a Beckman model B spectrophotometer against a blank prepared identically to the samples but with the exclusion of the thorium nitrate. The wavelengths employed varied from 395 to 405 nm., depending upon the particular tetracycline under investigation. This information, together with the time required to develop a stable color, is given in Table I. For doxycycline hyclate, a calibration curve was

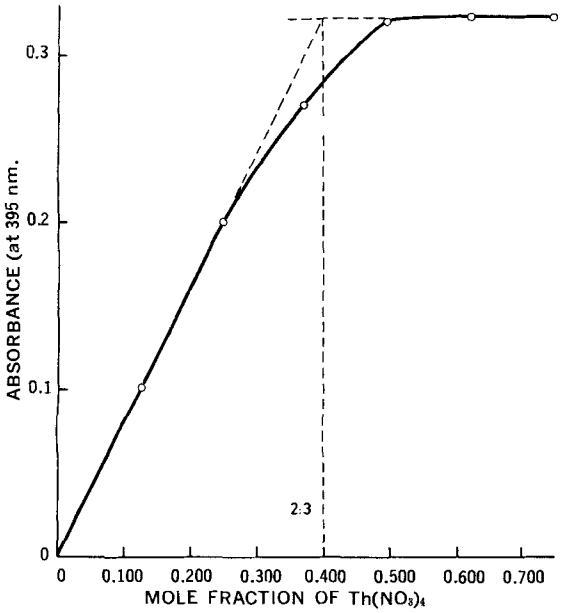

Figure 1—*Molar ratio study of tetracycline–thorium complex system.*

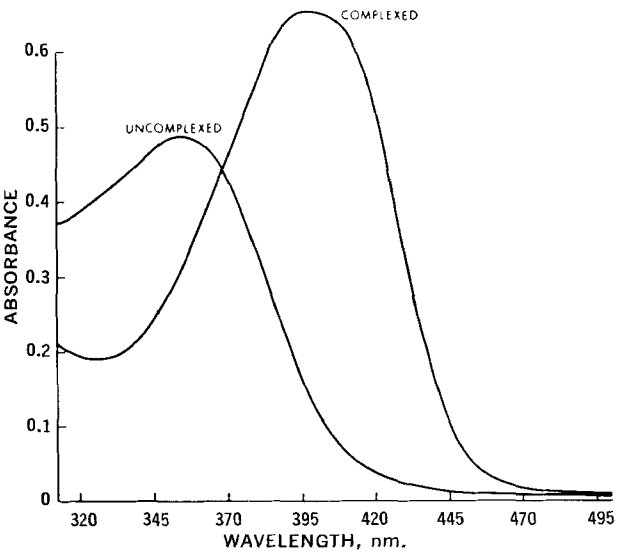

Figure 2—*Absorption spectrum of tetracycline hydrochloride before and after complexation with thorium.*

derived by extrapolation of the data obtained with doxycycline HCl. A calibration curve for an oxytetracycline–thorium complex in chloroform was prepared for the assays of ear/eye suspensions, ointments, and sprays. Good linear curves of absorbance *versus* concentration were obtained in all instances.

Assays of Tablets, Capsules, Syrups, Solutions, and Surgical Powder—Where tablets and capsules were being assayed, samples were taken from the contents of 10 capsules or from the powder obtained by crushing 10 tablets. Solutions containing approximately 25 mg./100 ml. of the particular tetracycline were prepared by placing an approximate quantity of the powdered material or syrup in 100 ml. of distilled water. For tablets and capsules, filtration through Whatman No. 1 filter paper was required. Aliquots of 0.6 ml. of this solution were placed in each of two 10-ml. volumetric flasks. To one of these was added 0.5 ml. of 0.5% thorium nitrate, and the contents of both flasks were brought to volume with distilled water. After a suitable period of time (Table I), the absorbance of the solution containing tetracycline and thorium nitrate was read against a blank (the solution to which thorium nitrate had not been added) at the optimum wavelength.

Assay of Ear/Eye Suspensions—The same procedure was used except that chloroform was employed as the solvent and 0.5% thorium nitrate (ethanolic) was the color-inducing reagent.

Assay of Ointments—The initial 0.025 mg./ml. solution was prepared by heating and stirring a suitable weight of ointment with 90 ml. of distilled water prior to filtration and dilution to an accurate volume. The previous procedure for solutions was then applicable.

Assay of Antibiotic Sprays—A short length of Tygon tubing was attached to the spray nozzle of the can, and the entire contents were expelled into a beaker. The propellant was boiled away, and the remaining liquid was brought to volume with chloroform in a 1000-ml. volumetric flask. Suitable aliquots were than transferred to 10-ml. volumetric flasks for subsequent treatment as with eye/ear preparations.

RESULTS

A large variety of pharmaceutical dosage forms were assayed for their tetracycline content. All figures given in Table II for the colorimetric assay are averages of five determinations. From these data it can be seen that results obtained by the thorium complexation method agree favorably with the manufacturer's results for all tablets and capsules investigated. However, a few discrepancies appear in the comparison of results for some syrups and drops. These differences can be attributed generally to a small degree of chemical alteration and degradation of the constituents between the time of the manufacturers' assays and the thorium complexation assays.

Attempts to achieve good agreement with the manufacturer's results for the antibiotic spray investigated were unsuccessful, probably due to the extreme difficulty in expelling the entire contents of the spray can. Since the method employed was based upon the use of the total can contents, any residual liquid in the can would necessarily lead to low results.

DISCUSSION

The electronic structure of thorium has not yet been fully established to the satisfaction of all physical chemists concerned. Dissent has arisen in the elucidation of the ground-state electronic configuration in the levels outside the radon structure. The two most generally accepted theories are that these outer levels are either occupied as $6d^2$, $7d^2$ or as 5f, 6d, $7s^2$. In which of the actinide elements the first development of a 5f level occurs is not known.

Thorium has been shown to be capable of valences of 2, 3, or 4. In the present study, Th^{+4} is made available for complexation with the tetracyclines as a result of ionic release in aqueous solution from its salt complex $Th(NO_3)_4$. The complexing ability of the tetracyclines is generally attributed to the availability of oxygen for bond formation. The presence of hydroxyl groups in the ligand leads to the ready formation of a species containing thorium as a complex cation.

The stoichiometry of the complex formed between thorium and the tetracyclines is uncertain. However, data obtained from a molar ratio study (Fig. 1) suggest two possibilities: (*a*) that a 2:3 thorium–tetracycline complex is formed, and (*b*) that in the presence of ex-

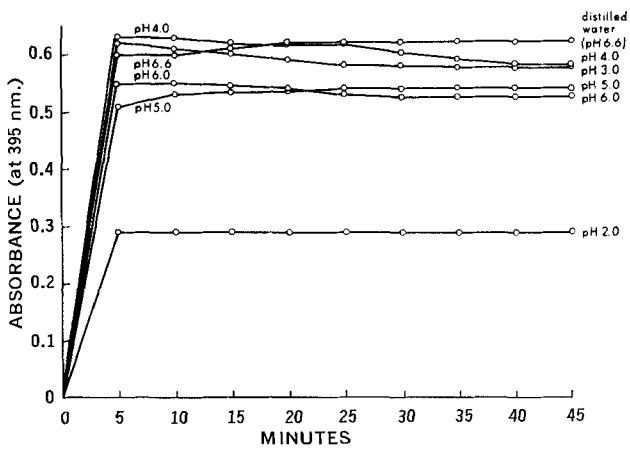

Figure 3—*Effect of pH on color development for tetracycline HCl.*

cess thorium, an equilibrium mixture is formed containing a constant proportion of complexes having differing stoichiometry. If, in fact, only one complex is being formed, its formation constant can be derived from the same data and is 7.7×10^4.

In the series of tetracyclines investigated, absorption maxima of the uncomplexed drugs in aqueous solution were found to range from 345 to 365 nm. Complexation with thorium invariably resulted in a bathochromic shift of 40–50 nm. This can be attributed to the formation of single covalent and dative bonds between oxygen and thorium in the complexed species, leading to further delocalization of electrons from the conjugated portion of the tetracycline molecules and, hence, to increased stability of the system.

In all experimental work conducted, a large excess of thorium nitrate was used so that, theoretically, the tetracycline compound in solution should be 100% complexed. Figure 2 shows only the effect of complexation with thorium on the absorption spectrum of tetracycline itself. However, similar observations were made for the remainder of the compounds studied. It is apparent that if any uncomplexed antibiotics were present, a slight error might be introduced owing to a small absorbance by this material at the optimum wavelength for the complex. To compensate for this possible error, all blanks contained the uncomplexed tetracycline at a suitable concentration. This procedure deviates from the usual method of blank preparation.

Studies undertaken with two of the degradation products of tetracycline, epianhydrotetracycline and anhydrotetracycline, showed that complexation with thorium led to color development in the same manner as with undegraded tetracycline. However, the complexed tetracycline derivatives exhibited maximal absorbance at 485 nm. and, in fact, showed no absorbance at the wavelength used in the colorimetric assay procedure.

In determining the most suitable pH and minimum time for stable color development, it was found that near-maximum absorbances, accompanied by a high degree of stability, were obtained within 20–35 min. using a nonbuffered aqueous system. A large number of buffers appeared to interfere in the complexation process. When attempts were made to obtain quantitative readings in systems above pH 7.0, results were adversely affected by the appearance of a white, flocculent precipitate. Although complexation between thorium and tetracycline still occurs above this point, the presence of $Th(NO_3)_4$ in large excess at this pH leads to the formation of insoluble hydroxides, primarily in the form of $Th(OH)_4$. Thus, distilled water ($pH \simeq 6.6$) was selected as a very convenient and suitable experimental medium. Figure 3 illustrates the effect of pH on color development for tetracycline HCl. Similar curves were obtained for the other tetracyclines in this study, although the levels of absorbance were not always identical. Once maximum color development was achieved, the color of the complexes remained stable for at least 30 min.

Good linear relationships between absorbance and concentration were obtained for all tetracyclines studied.

CONCLUSIONS

1. The proposed colorimetric procedure exhibits a high degree of accuracy and precision which makes it superior to many of the procedures presently being employed for the analysis of the tetracyclines.

2. The structure of the complex formed between the tetracyclines and quadrivalent thorium cannot be precisely postulated at this time. Experimental evidence suggests that a coordination compound containing two thorium to three tetracycline moieties may be formed.

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Spectrophotometric Determination of Pyrantel in Pyrantel Pamoate Bulk Samples and Pharmaceutical Formulations

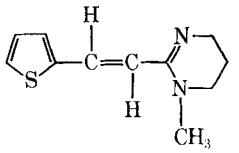
GEORGE A. FORCIER, ROBERT F. MUSHINSKY, and RICHARD L. WAGNER, Jr.

Abstract □ A spectrophotometric method for the determination of the pyrantel content of pyrantel pamoate bulk, oral suspension, and chewable tablets is described. This method involves the following sequence of steps: (*a*) dissolution of the sample in ammoniacal 1,4-dioxane solution; (*b*) removal of pamoic acid by precipitation with perchloric acid; (*c*) isolation of the pyrantel content by a two-stage extraction procedure; and (*d*) measurement of the pyrantel content of the final extract by UV absorption spectrophotometry. This analytical procedure for pyrantel circumvents any interference from pamoic acid and the potential degradation products [*i.e.*, (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide and the *cis*-isomer of pyrantel] of this anthelmintic agent.

Keyphrases □ Pyrantel pamoate, bulk, dosage forms—analysis □ Degradation products presence—pyrantel determination □ UV spectrophotometry—analysis

Pyrantel pamoate is an effective anthelmintic agent for human use (1–3). The chemical name of this drug is 1,4,5,6-tetrahydro-1-methyl-2-[*trans*-2-(2-thienyl)vinyl]-pyrimidine pamoate (1:1). A highly specific UV absorption spectrophotometric assay method has been developed for the determination of the pyrantel content of pyrantel pamoate bulk, oral suspension, and chewable tablets.

Pyrantel (I) exhibits UV absorption maximum at 311 nm. in aqueous 0.02 *N* hydrochloric acid. Pamoic acid, as well as two potential contaminants of pyrantel [the *cis*-isomer of pyrantel and (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide (II)], also exhibits absorption at this wavelength. These potential contaminants may be generated from pyrantel by photodecomposition and alkaline degradation, respectively. The UV absorption spectra of pyrantel, its *cis*-isomer, and (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide are illustrated in Fig. 1. The UV absorption spectrum of the *cis*-isomer of pyrantel is sufficiently different from that of pyrantel to permit the measurement of pyrantel in the presence of its *cis*-isomer by utilizing a two-component spectrophotometric technique (4, 5). Both (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide and pamoic acid would represent significant interferences in the spectrophotometric measurement of pyrantel unless previously removed.



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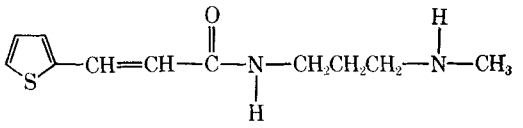

II

The assay procedure involves the following sequence of steps: (*a*) the sample is dissolved in aqueous ammonium hydroxide–1,4-dioxane (1:1, v/v); (*b*) pamoic acid is removed from this solution by precipitation with 0.5 *N* perchloric acid; (*c*) the cationic form of pyrantel is separated from the cationic form of the (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide by extraction into chloroform of the ion pair formed between the pyrantel cation and the perchlorate anion; (*d*) pyrantel is back-extracted into aqueous 0.02 *N* hydrochloric acid; and (*e*) the pyrantel content of this aqueous solution is determined by UV absorption spectrophotometry.

EXPERIMENTAL

Apparatus—A Varian Aerograph ultrasonic cleaner was utilized to facilitate the dissolution of pyrantel pamoate. A Cary spectrophotometer (model 11, 14, or 15) was used for recording the UV absorption spectra.

Reagents—Ammonium hydroxide, 1,4-dioxane, perchloric acid, hydrochloric acid, and chloroform were used.[1] In addition, reference samples of pyrantel tartrate, pyrantel pamoate, pamoic acid, the *cis*-isomer of pyrantel pamoate, the *cis*-isomer of pyrantel tartrate, and (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide hydrochloride were prepared in this laboratory.

Treatment of Sample—*Bulk Samples*—Approximately 100 mg. of sample was accurately weighed into a 200-ml. volumetric flask. This sample was dissolved in 10 ml. of 1,4-dioxane plus 10 ml. of 0.05 *N* ammonium hydroxide by placing the flask and its contents in the ultrasonic cleaner for approximately 5 min. This solution was then diluted to 200 ml. with 0.5 *N* perchloric acid, whereupon pamoic acid was precipitated. This suspension was clarified by the filtration procedure described later.

Oral Suspension—Approximately 1 ml. of the oral suspension was accurately weighed into a 20-ml. beaker. This sample was quantitatively transferred into a 250-ml. volumetric flask using 10 ml. of 1,4-dioxane and 10 ml. of 0.05 *N* ammonium hydroxide. The sample was completely dissolved by placing the flask and its contents into the ultrasonic cleaner for approximately 5 min. This solution was then diluted to 250 ml. with 0.5 *N* perchloric acid. The precipitated pamoic acid was removed by the filtration procedure described later.

Chewable Tablets—Each pyrantel pamoate tablet was reduced to a homogeneous powder using a mortar and pestle. The resulting powder was transferred to a 100-ml. volumetric flask using 0.05 *N* ammonium hydroxide–1,4-dioxane (1:1, v/v) as the wash solvent. The content of the flask was diluted to volume with this same solvent. Pyrantel pamoate was dissolved by placing the flask and its content in the ultrasonic cleaner for 10 min. The content of the flask was swirled several times during this period to ensure complete dissolution of the pyrantel pamoate. Only a white, insoluble precipitate remained after this treatment. A 15-ml. aliquot of the medium was diluted to 200 ml. with 0.5 *N* perchloric acid. The resulting suspension was clarified by filtration as described.

Filtration and Extraction—The suspension obtained from the previously described step was filtered through Whatman No. 2 filter paper. A 5-ml. aliquot of the clear filtrate was diluted to 50

[1] "Baker Analyzed" reagents, J. T. Baker, Phillipsburg, N. J.

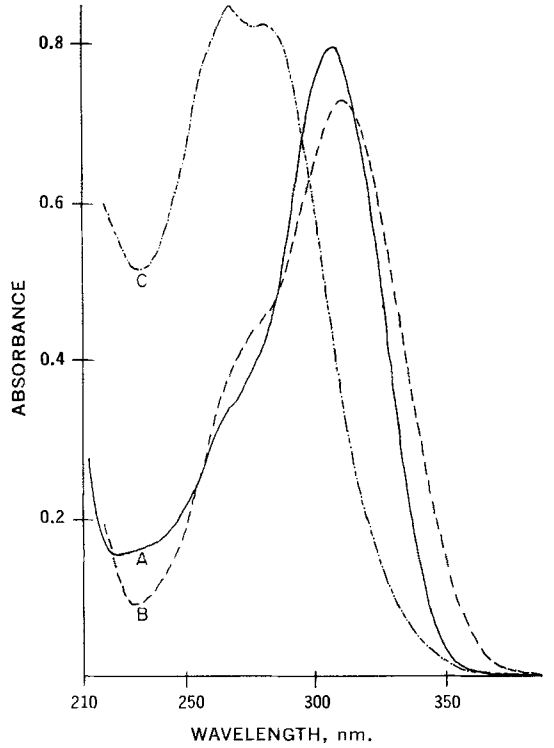

Figure 1—*UV absorption spectra in 0.02* N *hydrochloric acid. Key: A,* (E)-N-(*3-methylaminopropyl*)-*2-thiopheneacrylamide* (*4.3 mcg./ml.*), ——; *B, pyrantel* (*4.0 mcg./ml.*), - - -; *and C,* cis*-isomer of pyrantel* (*8.7 mcg./ml.*), —·—.

ml. with 0.5 *N* perchloric acid. A 25-ml. aliquot of this solution was transferred to a 250-ml. separator and extracted with two 100-ml. portions of chloroform. The chloroform phases were combined in a second separator and back-extracted with two 40-ml. portions of aqueous 0.02 *N* hydrochloric acid. The aqueous 0.02 *N* hydrochloric acid phases were combined in a 100-ml. volumetric flask. The separator was rinsed several times using 0.02 *N* hydrochloric acid as the wash solvent. These rinses were added to the volumetric flask and diluted to the mark with 0.02 *N* hydrochloric acid. The UV absorption spectrum of this solution was recorded *versus* 0.02 *N* hydrochloric acid as the reference solvent.

Determination of Fractional Recovery of Pyrantel—*Bulk Samples*—Synthetic mixtures of the reference samples of pyrantel tartrate and pamoic acid were taken through the assay procedure. The ratio of the pyrantel (milligrams) found (calculated from a Beer's law relationship) to pyrantel (milligrams) added represents the fractional recovery (RF^B) of pyrantel in the bulk assay procedure.

Oral Suspension and Chewable Tablets—The reference sample of pyrantel pamoate was taken through the assay procedure for the determination of pyrantel in oral suspension and chewable tablets. The ratio of the pyrantel (milligrams) found (calculated from a Beer's law relationship) to pyrantel (milligrams) added represents the fractional recovery (RF^{OS} or RF^{CT}) of pyrantel in the assay procedure for pyrantel in the oral suspension or chewable tablet formulations.

Calculation of Results—The pyrantel content of the final aqueous solution was determined using a two-component spectrophotometric technique (4, 5). This technique involves the measurement of the absorbances of a binary mixture of pyrantel and its *cis*-isomer at the isoabsorptive wavelength (267 nm.) and at the wavelength (311 nm.) of maximum absorbance of pyrantel. Using this technique, the pyrantel content of the final aqueous solution was calculated from the following equation:

$$C = \frac{[A^S_{311\text{ nm.}} \times a_{(267\text{ nm.})} - a^{(C)}_{(311\text{ nm.})} \times A^S_{267\text{ nm.}}]}{[a^{(T)}_{(311\text{ nm.})} - a^{(C)}_{(311\text{ nm.})}] \times L \times a_{(267\text{ nm.})}} \quad \text{(Eq. 1)}$$

where C is the pyrantel concentration in grams per liter; $A^S_{311\text{ nm.}}$ is the absorbance of the solution at 311 nm.; $A^S_{267\text{ nm.}}$ is the absorbance of the solution at 267 nm.; $a^{(C)}_{(311\text{ nm.})}$ is the absorptivity of the *cis*-isomer of pyrantel at 311 nm.; $a^{(T)}_{(311\text{ nm.})}$ is the absorptivity of pyrantel at 311 nm.; $a_{(267\text{ nm.})}$ is the absorptivity of pyrantel and its *cis*-isomer at the isoabsorptive point (267 nm.); and L is the cell path in centimeters.

The pyrantel content of bulk samples of pyrantel pamoate was then calculated from the following equation:

$$\%\text{ pyrantel} = \frac{C \times 8 \times 10^5}{\text{wt. (mg.) of sample} \times RF^B} \quad \text{(Eq. 2)}$$

In a similar manner, the pyrantel content of the oral suspension was calculated as follows:

$$\text{mg. pyrantel per ml. oral suspension} = \frac{C \times 10^4 \times \text{density}}{\text{wt. (g.) of sample} \times RF^{OS}} \quad \text{(Eq. 3)}$$

where the density of the oral suspension was expressed as grams per milliliter.

Finally, the weight of pyrantel per chewable tablet was calculated using the following equation:

$$\text{wt. (mg.) of pyrantel per chewable tablet} = \frac{C \times 5.33 \times 10^4}{RF^{CT}} \quad \text{(Eq. 4)}$$

Table I—Evaluation of Blanks in Oral Suspension Assay Procedure

No. of Determinations	[*A*(Blank)/*A*(Sample)] × 100 311 nm.	267 nm.
14	0.2 ± 0.1%[a]	1.9 ± 0.4%[a]

[a] Average ± *SD*.

Table II—Fractional Recovery of Pyrantel from Bulk and Pharmaceutical Formulations

No. of Determinations	Assay Procedure	Fractional Recovery (Average ± *SD*)
19	Bulk samples	$RF^B = 0.963 \pm 0.009$
19	Oral suspensions	$RF^{OS} = 0.967 \pm 0.008$
10	Chewable tablets	$RF^{CT} = 0.974 \pm 0.009$

RESULTS AND DISCUSSION

The blanks associated with the inactive ingredients of the chewable tablets and the oral suspension were evaluated. There was no significant blank associated with the chewable tablet assay proce-

Table III—Pyrantel Content of Pyrantel Pamoate Bulk Samples

Sample	Percent Pyrantel Found[a] (Average ± *SD*)	No. of Determinations
1	34.5 ± 0.1[b]	6
2	33.8 ± 0.3	3
3	34.5 ± 0.3	3
4	34.5 ± 0.1	4
5	34.6 ± 0.3	15
6	34.5 ± 0.3	3
7	34.4 ± 0.3	3
8	34.7 ± 0.2	3
9	34.3 ± 0.3	3
10	34.5 ± 0.3	3
11	33.7 ± 0.1	3
12	34.5 ± 0.2	6
13	34.7 ± 0.3	6
14	34.5 ± 0.2	6
15	34.5 ± 0.3	3
16	34.4 ± 0.2	4
17	34.6 ± 0.1	3
18	34.5 ± 0.1	3
19	35.0 ± 0.2	3

[a] Theoretical pyrantel content = 34.7%. [b] Average *SD* = ±0.2%.

Table IV—Pyrantel Content of Pyrantel Pamoate Oral Suspension Lots

Sample	Pyrantel Content[a] Found, mg./ml. (Average ± *SD*)	No. of Determinations
1	53.2 ± 0.4[b]	20
2	53.2 ± 0.5	5
3	53.7 ± 0.8	5
4	52.3 ± 0.7	6
5	51.2 ± 0.3	3
6	53.3 ± 0.3	6
7	53.1 ± 0.6	10
8	49.9 ± 0.6	3
9	51.7 ± 0.1	3
10	51.7 ± 0.2	3
11	52.5 ± 0.3	3

[a] Intended pyrantel content = 51.0 mg./ml. [b] Average *SD* = ±0.4 mg./ml.

Table V—Pyrantel Content of Pyrantel Pamoate Chewable Tablet Lots

Sample	Pyrantel Content[a] Found, mg./Tablet (Average ± *SD*)	No. of Determinations
1	264 ± 4.9[b]	10
2	268 ± 5.8	10
3	253 ± 4.8	10
4	260 ± 2.1	10
5	256 ± 2.7	10
6	260 ± 3.2	10
7	249 ± 3.6	10
8	254 ± 5.2	10

[a] Intended pyrantel content = 255 mg./tablet. [b] Average *SD* = ±4.0 mg./tablet.

dure. The blanks associated with the oral suspension procedure were both low and reproducible. A summary of the blank values for the oral suspension procedure is shown in Table I.

The fractional recovery values for pyrantel in the pyrantel pamoate bulk, oral suspension, and chewable tablet assays were both high and reproducible. Results of experiments designed to evaluate the fractional recovery values are listed in Table II. These data also indicate that the inert components of the oral suspension and the chewable tablets exert no significant effect on the fractional recovery of pyrantel in the assay procedure.

The fractional recovery values for the *cis*-isomer of pyrantel in the pyrantel pamoate bulk, oral suspension, and chewable tablet assays were evaluated by taking synthetic mixtures through the assay procedures. The fractional recovery values for the *cis*-isomer of pyrantel in the bulk, oral suspension, and chewable tablet assays were 0.91 ± 0.01, 0.90 ± 0.01, and 0.91 ± 0.01, respectively. These values indicate that approximately 90% of any *cis*-isomer present in the sample to be assayed would be carried through the assay procedure along with the pyrantel. The pyrantel content was calculated using a two-component spectrophotometric technique which served to correct for the presence of the *cis*-isomer of pyrantel.

The fractional recovery values for (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide were evaluated in the bulk, oral suspension, and chewable tablet assays. These fractional recovery values were 0.09 ± 0.01, 0.09 ± 0.01, and 0.07 ± 0.01, respectively. Consequently, greater than 90% of any (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide present in pyrantel pamoate bulk, oral suspension, and chewable tablets is removed from the pyrantel by the extraction procedure.

The separation of the pyrantel from the (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide was based on the fact that the cationic form of pyrantel and the perchlorate anion are capable of forming an ion pair which is extracted from an aqueous solution into chloroform (5–8). Under the conditions described in this assay, the (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide was only partially extracted (<10%) into the chloroform. Consequently, the ion-pair extraction served as a convenient method of eliminating the interference due to the presence of the (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide in the sample.

The interference due to the presence of the *cis*-isomer of pyrantel in the assay procedures for bulk, oral suspension, and chewable tablets was evaluated by taking synthetic mixtures of the pyrantel pamoate, its *cis*-isomer, and the inactive ingredients of the formulation through the assays. When these synthetic mixtures were taken through the bulk, oral suspension, and chewable tablet assay procedures, the percent of pyrantel found was 99.8 ± 0.9, 100.2 ± 0.9, and 100.9 ± 1.0, respectively. The data indicate that the presence of the *cis*-isomer of pyrantel does not interfere in the assay for pyrantel in pyrantel pamoate bulk, oral suspension, and chewable tablets.

The potential interference due to (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide was evaluated in an analogous manner. When synthetic mixtures of pyrantel pamoate and (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide were taken through the bulk, oral suspension, and chewable tablet assay procedures, the percent of pyrantel found was 100.2 ± 1.0, 100.9 ± 0.7, and 101.6 ± 1.4, respectively. These data indicate that contamination of pyrantel pamoate with up to 12% of this potential degradation product causes no significant interference in the assay procedures.

The assay procedure for pyrantel in pyrantel pamoate bulk samples was applied to 19 samples. The pyrantel contents found for these samples are listed in Table III. The percent pyrantel found for these 19 samples ranged from 33.7 to 35.0%. The theoretical content of pyrantel in pyrantel pamoate is 34.7%.

In addition, the assay for pyrantel in pyrantel pamoate oral suspension was applied to 11 formulated lots of the oral suspension. The average concentration found for these 11 lots ranged from 49.9 to 53.7 mg. of pyrantel per milliliter of oral suspension. The intended concentration of pyrantel in these formulated lots was 51 mg. of pyrantel per milliliter of oral suspension. These results are listed in Table IV.

Finally, the assay for pyrantel in pyrantel pamoate chewable tablets was applied to eight formulated lots of the chewable tablets. The pyrantel content found for these eight lots ranged from 249 to 268 mg. of pyrantel/tablet. The intended pyrantel content of these tablets was 255 mg./tablet. These results are reported in Table V.

SUMMARY

The recovery, blanks, reproducibility, and specificity of the assay procedure for the determination of the pyrantel content of pyrantel pamoate bulk samples and dosage forms were evaluated. This assay was applicable to the determination of pyrantel in pyrantel pamoate bulk samples, oral suspension, and chewable tablets in the presence of its potential degradation products [the *cis*-isomer of pyrantel and (*E*)-*N*-(3-methylaminopropyl)-2-thiopheneacrylamide].

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Fluorometric Determination of Salicylic Acid in Buffered Aspirin Products

NORMAN SHANE and ROBERT STILLMAN

Abstract □ A rapid fluorometric method for the determination of total salicylic acid in buffered aspirin products has been developed and compared with existing methods. The new procedure is essentially a one-step operation; the complete analysis, exclusive of calibration, requires less than 10 min. The results obtained are in close agreement with those found using two alternative methods. All tedious operations (*e.g.*, extractions and liquid column chromatographic separations) are eliminated.

Keyphrases □ Salicylic acid in buffered aspirin products—determination □ Formic acid–chloroform solution—salicylic acid fluorescence □ Aspirin hydrolysis—salicylic acid assay effect □ Quinine sulfate—standard fluorescent species □ Fluorometry—analysis

Salicylic acid (SA) can be determined in the presence of acetylsalicylic acid (ASA) by measuring the fluorescence intensity of a chloroformic solution (1). In the application of this technique to commercial buffered aspirin products, the SA must be freed from the buffering components on which it may be physically adsorbed, and the chloroform-insoluble salicylate salts formed by free SA in the presence of buffer materials must be solubilized. Both of these objectives must be accomplished without appreciable hydrolysis of the ASA.

In a recent paper, Guttman and Salomon (2) reported the desorption of SA and aspirin by triturating powdered tablet mass with citric acid monohydrate (3). The SA and aspirin were dissolved in chloroform, separated by liquid column chromatography, and determined by UV absorption spectroscopy. The chloroform-insoluble residue was treated with an aqueous solution of strong mineral acid and extracted with chloroform. The chloroform solution was purified chromatographically and analyzed by UV spectroscopy. Thus, free SA and salicylate salts were determined separately.

A procedure for total nonaspirin salicylates was described by Levine and Weber (4). This method requires an initial treatment with 98–100% formic acid, which displaces SA from its salts, followed by the chromatographic separation and analysis.

The time-consuming chromatographic operation is circumvented in the fluorometric procedure of Shane and Miele (5). Release of SA and its salts was accomplished by treatment with a dilute HCl–citric acid solution, which caused minimal hydrolysis during the period specified for contact. The aqueous solution was then extracted into chloroform and reextracted with pH 4 buffer. Under these conditions, the aspirin hydrolysis was slow, and the SA concentration could be determined as a function of fluorescence intensity.

The present study was undertaken to determine the feasibility of combining the formic acid procedure with fluorescence assay, thus eliminating the chromatographic step and all extraction procedures. This has been accomplished by measuring the fluorescence intensity of the formic acid–chloroform solution directly. Since 98–100% formic acid is miscible with chloroform, no extractions are required. The direct fluorometric examination of the chloroform–acid solutions is permissible, because: (*a*) the reagent blank has no interfering fluorescence under the conditions specified in the procedure, and (*b*) the fluorescence emission spectra of synthetic mixtures of aspirin and SA in chloroform containing formic acid are essentially identical to those obtained in chloroform alone.

The validity of the formic acid procedure with respect to hydrolysis of aspirin was examined by Levine and Weber (4). If, after brief exposure of the powdered tablet mass to a small volume of formic acid, the sample was immediately diluted with chloroform, the hydrolytic action of the acid was sharply diminished (approximately 0.03%/hr.). Thus, hydrolysis was negligible in the 5 min. specified for dissolving the SA and the aspirin. During the period the sample was in contact with the formic acid (45 sec.), the total extent of hydrolysis was found to be on the order of 0.01% (4). These results were confirmed in this laboratory, and the extent of hydrolysis in chloroform was found to be appreciably less than reported.

As a check on the completeness of solution, the residual tablet mass was examined for the presence of SA and aspirin. The total residual salicylate was less than 0.03%, which is in close agreement with the published results of Levine and Weber (4).

EXPERIMENTAL

Instrument Calibration—An Aminco-Bowman spectrophotofluorometer (SPF), model 4-8202, with a solid-state photomultiplier was employed in these studies. The advantages of instruments of this type compared with the more common filter fluorometers may be summarized briefly.

1. The use of a continuous xenon arc source and activating monochromator provides high-intensity activating energy at the exact wavelength of maximum absorption, in contrast to the few wavelengths corresponding to the lines of the mercury spectrum available in conventional fluorometers.
2. The detection of fluorescence is not limited to the visible region, but is extended into the UV.
3. Specificity and elimination of interference from the fluorescence of impurities are enhanced by the greater resolving power or sharper bandwidths of monochromators as compared to filters.
4. The ability to scan the full fluorescence spectrum provides a convenient means of detecting the presence of extraneous materials which may cause errors due to light scattering, blank fluorescence, or absorption of either activating or fluorescent energy (quenching).

To utilize effectively the spectrophotofluorometer, careful attention to several factors that can vary the instrument sensitivity (*e.g.*, changes in ambient temperature, photomultiplier instability, and deterioration of the xenon arc source) is required. These factors are accounted for by standardizing the instrument sensitivity from day-to-day (and morning-to-afternoon) by means of

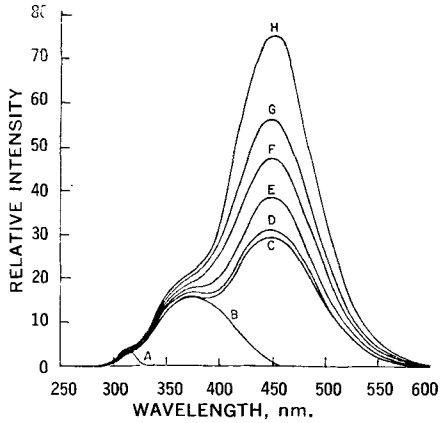

Figure 1—*Fluorescence emission spectra of: (A) formic acid–chloroform solvent; (B) pure ASA; (C) buffered aspirin tablets with no added SA; (D) buffered aspirin tablets with 0.1 mg. SA added; (E) buffered aspirin tablets with 0.5 mg. SA added; (F) buffered aspirin tablets with 1.0 mg. SA added; (G) buffered aspirin tablets with 1.5 mg. SA added; and (H) buffered aspirin tablets with 2.5 mg. SA added.*

a standard solution of a stable fluorescent species. In these experiments, quinine sulfate, 1.0 mcg./ml., in 0.1 *N* H_2SO_4 was used. This solution is stable for at least 1 month if stored in the dark to avoid photodecomposition. Quinine sulfate is intensely fluorescent, and its maximum emission at 450 nm. coincides with that of SA in chloroform. (This is desirable since the responses of light sources and detectors are not uniform over the entire spectral range.)

Reagents—The following were used: chloroform (Merck spectrophotometric grade); formic acid, 98–100% (Matheson Coleman & Bell); and salicylic acid USP standard.

Tablets—Most of the data reported here pertain to commercial buffered aspirin tablets which had been stored in plastic containers for 1 year prior to analysis. For comparison purposes, a synthetic mixture to simulate the tablets was formulated as follows: 325 mg. ASA (Monsanto, 80 mesh), 97 mg. magnesium carbonate, and 49 mg. aluminum glycinate.

ANALYTICAL PROCEDURE

Calibration—In the initial calibration experiments with production tablets, 20 tablets from a given batch were finely powdered and an amount equivalent to 325 mg. aspirin was weighed into each of seven 100-ml. volumetric flasks. A 5-ml. aliquot of 98–100% formic acid was added to the first flask, and the contents were swirled for 45 sec. Afterwards, 70 ml. chloroform was added, and the flask was placed on a mechanical shaker. This process was repeated for the remaining samples, and the assembly was shaken until the final flask had been on the shaker for 5 min. During this period, known increments of SA were added in 5-ml. aliquots from appropriate standard solutions. The standards were as follows: 0 (no added SA; run in duplicate, *i.e.*, no SA was added to the first and last samples), 0.1, 0.5, 1, 1.5, and 2.5 mg. The stock solution was 50 mg. SA dissolved in 100 ml. chloroform (0.5 mg./ml.), 5 ml. of which delivered 2.5 mg. SA. A 30-ml. aliquot diluted to 50 ml. with chloroform (0.3 mg./ml.) served for the 1.5-mg. addition, 20 ml./50 ml. (0.2 mg./ml.) for the 1-mg. addition, 20 ml./100 ml. (0.1 mg./ml.) for the 0.5-mg. addition, and 2 ml./50 ml. (0.2 mg./ml.) for the 0.1-mg. addition.

After the volume was adjusted with chloroform, the contents of each flask were filtered through a loose plug of glass wool, and 1-ml. aliquots of the filtrates were diluted to 100 ml. with chloroform. The relative fluorescence intensities were obtained (excitation, 310 nm., emission, 450 nm.).

Routine Analysis—For single-tablet assays, a tablet was crushed in a double thickness of glassine weighing paper and transferred to a 100-ml. volumetric flask. The previously described procedure was followed, except that addition to standards was omitted.

Analysis of Residual Tablet Mass—The residue from the formic acid–chloroform procedure was retained on a fine sintered-glass funnel; the material remaining in the flask was rinsed onto the funnel with 1 *N* HCl, and the residue was washed with a total volume of

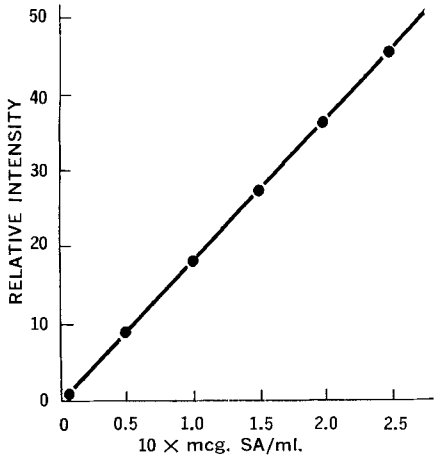

Figure 2—*Fluorescence of SA as a function of concentration in formic acid (5×10^{-5} %)–chloroform.*

50 ml. 1 *N* HCl. The filtrate was transferred to a 125-ml. separator and extracted three times with 25-ml. portions of chloroform. The chloroform extracts were drawn into a 100-ml. volumetric flask, brought to volume with chloroform, and examined for SA with the SPF at the same sensitivity used in the tablet analysis.

Determination of Extent of Hydrolysis—The increase of fluorescence intensity of the SA emission peak at 450 nm. provides a convenient means to monitor the hydrolysis of ASA. For a tablet treated with formic acid and chloroform, the initial intensity is noted and the increase with time observed. Typically, no increase in fluorescence can be detected until at least 2 hr. have elapsed.

RESULTS AND DISCUSSION

The fluorescence emission spectrum of a sample of powdered buffered aspirin tablets equivalent to 325 mg. ASA and treated with formic acid and chloroform is shown in Fig. 1, Curve C, together with the spectra obtained by adding known increments of SA to samples similarly treated (Curves D–H). The excitation wavelength, 310 nm., is evident as a small Raman scattering peak; the constant background due to ASA appears as a shoulder (maximum emission, 370 nm.) on the broad SA band (maximum emission, 450 nm.). That the fluorescence at 450 nm. of Curve C arises entirely from SA present in the tablet is confirmed by the spectrum of 325 mg. ASA (USP Standard) in formic acid–chloroform solution, Curve B, which has negligible fluorescence at 450 nm. (However, almost all samples of unpurified ASA do show a peak at 450 nm., indicating the presence of SA in varying amounts.)

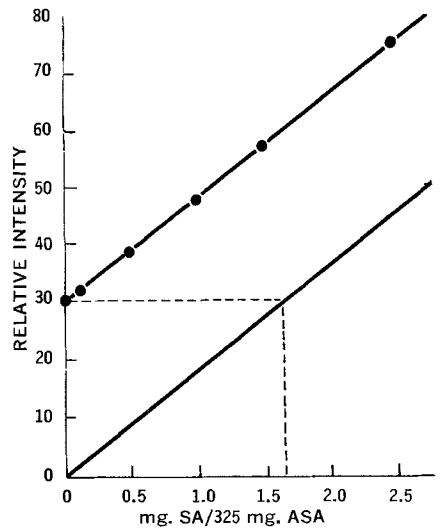

Figure 3—*Calibration curve for SA in buffered aspirin tablets.*

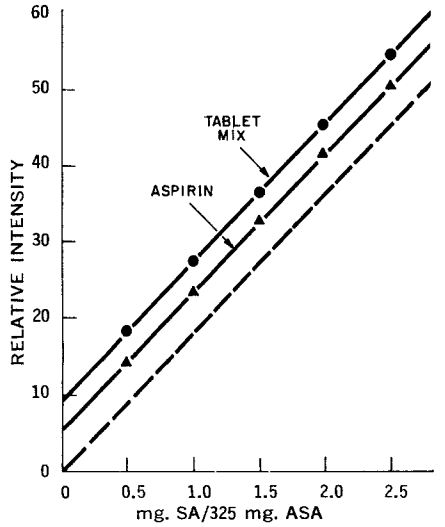

Figure 4—*Calibration curves for SA in ASA and synthetic tablet mixture.*

The linear proportionality of fluorescence to concentration for pure SA in formic acid–chloroform solutions at the dilutions used in the analysis (0.01–0.25 mcg./ml.) is shown in Fig. 2. Thus, a calibration curve can be obtained from the data in Fig. 1 (Curves C–H) by plotting fluorescence intensity at 450 nm. *versus* milligrams of added SA and extrapolating as shown in Fig. 3 to obtain the amount of SA corresponding to the initial reading (no added SA, Curve C, Fig. 1).

In a region of concentration where fluorescence is proportional to concentration, the measurement of fluorescence is made, essentially, in the absence of any significant absorption. This represents a limiting case of the Beer–Lambert law, where the energy available for excitation is uniformly distributed throughout the solution (6). At higher concentrations, an increasing proportion of energy is absorbed by that part of the solution nearest to the excitation monochromator. The resulting nonuniform distribution of fluorescence, in turn, leads to the failure of Beer's law. The effect, known as concentration quenching, is evidenced by marked deviations from linearity, especially as concentration is increased. Thus, for amounts of SA exceeding 3 mg./325 mg. aspirin, additional dilutions are required to obtain the linear range.

The results obtained with the synthetic tablet mix are shown in Fig. 4, together with the results for a concurrent run with pure aspirin (the same material used to prepare the mixture). The value for pure aspirin, expressed as mg. SA/325 mg. aspirin, is in accord with previous experience (7, 8) and incidentally demonstrates that the extent of aspirin hydrolysis resulting from the formic acid procedure is not significant. (In an actual analysis of pure aspirin or aspirin tablets not containing buffering materials, the initial treatment with formic acid is superfluous, because the aspirin can be dissolved directly in chloroform.) The slightly higher SA level in the tablet mix probably results from hydrolysis induced by atmospheric moisture during the period of preparation and grinding. This value for SA in the synthetic mix compared quite closely with that found for freshly prepared tablets of the same formulation, indicating that the tableting process does not contribute to aspirin decomposition in this preparation.

The formic acid procedure has been used to assay several additional aspirin products, both buffered and unbuffered. Satisfactory results are obtained with tablets containing noninterfering fluorescent substances, such as phenylephrine HCl, chlorpheniramine maleate, phenylpropanolamine HCl, phenyltoloxamine citrate, glyceryl guaiacolate, caffeine, and acetaminophen.

Table I—Results of SA Determination by Three Methods[a]

Run	SA Found, mg.					$\bar{x}$[b]	SD[c]
Guttman and Salomon (2)							
1	1.6	1.8	1.5	1.7	1.9	1.7	0.15
2	1.9	2.0	1.8	1.8	1.5	1.8	0.19
3	1.9	2.0	2.3	1.8	1.7	1.9	0.23
Shane and Miele (5)							
1	1.5	1.8	1.7	1.7	1.6	1.7	0.12
2	1.7	1.9	1.8	1.7	1.9	1.8	0.10
3	1.8	1.9	1.6	1.9	2.0	1.8	0.15
Formic Acid							
1	1.7	1.7	1.8	1.9	1.9	1.8	0.10
2	1.6	1.9	2.0	1.8	1.9	1.8	0.15
3	2.0	1.7	1.8	1.7	1.9	1.8	0.13

[a] Results obtained on the analysis of SA content on buffered aspirin composed of 325 mg. of aspirin, 97 mg. of magnesium carbonate, and 49 mg. of aluminum glycinate. The different runs were made on three successive days. [b] $\bar{x}$ = means of results. [c] SD = standard deviation.

To confirm the accuracy of the results obtained with the formic acid procedure, SA in buffered aspirin tablets was determined by two independent methods. The results are summarized in Table I for the Guttman and Salomon (2) and Shane and Miele (5) procedures as well as for the formic acid method. There is good agreement among results obtained with all three methods.

The desirability of employing the formic acid method on a routine or control basis is apparent from considerations of convenience, speed, accuracy, and repeatability. Once a calibration curve is established, a single tablet can be analyzed in less than 10 min., half of which is consumed by mechanical shaking; the analyst is spared the laborious extractions of existing procedures and can run many tablets concurrently. The suitability of the method is limited only by the requirement of an expensive monochromatic instrument (which does not lend itself to careless handling and which demands periodic calibration and maintenance) and the scrupulous attention that must be given to instrumental factors affecting sensitivity. A filter instrument for quantitative comparison of an unknown to a standard would probably be more appropriate for routine use. However, the band filters conventionally employed are not suitable, since in chloroformic solutions aspirin is weakly fluorescent and the broad emission band contributes an undesirable background fluorescence. The feasibility of having narrow band interference filters prepared to specification is under consideration.

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Improved Thermoelectric Osmometer

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Abstract □ The original osmometer described by Goyan and Johnson was improved, resulting in a 10-fold increase in sensitivity and greater convenience in use. A concentration change of 0.001 molal is easily detected in aqueous solutions; however, the instrument may be used without modification for nonaqueous solvents. A new measuring circuit and a new temperature control system are described. The original features for introduction and removal of samples are retained. Solutions contact only glass and platinum. Valuable compounds may be recovered unchanged by evaporation. The instrument may be used in various analytical applications, ranging from the study of complex formation to the determination of concentrations of known solutes. It is useful for the determination of molecular weights and for direct measurement of osmotic properties of solution.

Keyphrases □ Osmometer, thermoelectric—improved design □ Thermoelectric osmometer, improved—diagram □ Temperature control system, osmometer—diagram □ Sucrose standard—osmometer calibration

Goyan and Reck (1) used thermocouples to show that the reference junction and sample junction of the conventional thermoelectric osmometer need not occupy the same air chamber. Each may occupy separate cells positioned in a constant-temperature bath so both are subjected to the same minor temperature fluctuations. Goyan and Johnson (2) developed a circuit which permitted the substitution of thermistors for thermocouple junctions in separate chambers saturated with solvent vapor. They showed that the slight heating caused by relatively high battery voltages used to power the bridge did not produce a significant error. A 6-v. battery is now used routinely for this purpose.

Later, the same workers (3) developed a cell or chamber for the sample thermistor that permitted the introduction and removal of sample with fitted medicine droppers without exposing the thermistor to outside air or allowing solvent or sample to contact any material other than platinum and glass. They showed how this instrument could be used with the bridge and reference thermistor previously described for molecular weight determinations in aqueous and nonaqueous solvents. Solvents may be changed by appropriate rinsing without modification of the instrument because of the platinum–glass construction. Johnson *et al.* (4) used the same equipment to measure isotonicity values and osmotic coefficients of aqueous solutions of several univalent electrolytes.

In concluding this phase of the work, Johnson and Goyan (5) showed that the calibration data for several different solvents related to their latent heats of vaporization approximately as predicted for boiling-point elevation. This established the hypothesis that when the sample is in contact with air saturated with solvent vapor, its temperature is elevated sufficiently to compensate for the vapor pressure lowering caused by the solute. Later experiments with better temperature control showed that this elevated temperature is established in minutes and remains essentially unchanged for hours with the same drop of sample solution, refuting the commonly held belief that all instruments of this type operate on the principle of competitive evaporation rather than an approach to an equilibrium temperature. However, the fact that the shape, size, and position of the sample drop is important in work of the highest accuracy indicates that true equilibrium is not fully attained, although it is approached closely enough to prevent significant change in the concentration of the sample solution during the usual period of observation.

When Borazan (6) elected to use this apparatus to study possible complex formation and self-association of compounds of biological interest, he discovered that higher precision was needed and recommended a program of development designed to accomplish this. The essential changes made by Goyan and Borazan (7) are published in outline form, but it remains for this paper to make them clear and to present additional refinements mentioned briefly in the previous paper. All of the essential features of the Goyan–Johnson instrument are retained while achieving improved accuracy and ease of operation.

DISCUSSION

Measuring Circuit—The bridge circuit described by Goyan and Johnson (2) was used by Borazan (6) and Goyan and Borazan (7) in their work, after achieving a 10-fold increase in the sensitivity of the recorder, bringing its sensitivity to 0–1 mv. Measurement shows that, at this higher sensitivity, one small division on the paper used (2.5 mm.) is equivalent to a 0.7-ohm change in resistance of the thermistor at 25°. Random noise may be estimated to be of the order of 0.1 small division under good conditions so that the sensitivity approaches 0.1 ohm. The thermistors used approximate 100 K ohms with 4% temperature coefficient; therefore, the limit of temperature sensitivity is of the order of 2×10^{-5}° or a concentration discrimination of 1×10^{-4} molal, based on the slope of nearly 1000 ohms/molal for sucrose.

Although there is no need to change the bridge circuit, the modified circuit shown in Fig. 1 offers the advantage of requiring only one decade resistance box. This circuit can be switched so that the box may be substituted for either thermistor when it is desired to follow both temperatures. Another switching mode allows the box to be connected in series with either thermistor. This is an advantage when experimenting with different thermistors because it avoids

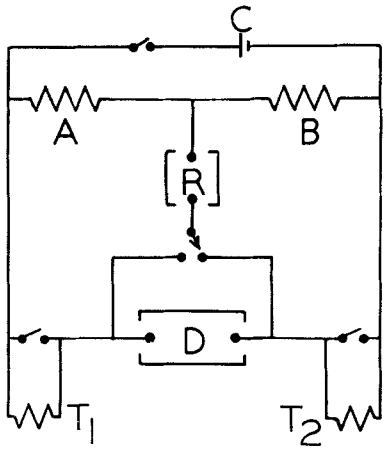

Figure 1—*Measuring bridge. Key: A and B, ratio arm box, 10K ohms each; C, battery (6 v. normally used); D, General Radio Decade Resistance Box Type 7433G. Ser. 543, 7 dials; T_1 and T_2, thermistors (Victory Engineering 51 A 1); and R, Leeds and Northrup Speedomax Recorder Type G with regulated power supply (part No. 099012) and range card (part No. 650999), 0–1- and 0–10-mv. range.*

the need to select the one with slightly lower resistance for use with the sample, as was required before. No attempt was made to calibrate the decade boxes used in either circuit or the resistors used in the ratio arm box. For this reason, minor differences in the slopes of calibration curves may be expected when comparing results from two different bridges and when using the new bridge with interchanged components. This is of little importance because all results in ohms are related to the calibration data obtained for the thermistors and bridge circuit in current use.

Sample Compartment—Figure 2 shows how the sample may be delivered to the platinum spiral surrounding the measuring thermistor. The sample passes through the capillary and continues down the glass body of the thermistor where it is held by capillary action to the loosely fitting spiral of platinum wire (F of Fig. 2 made from 0.33 mm. wire). The spiral is fastened firmly at one point by a second much finer piece of platinum wire. The most important improvement over the earlier design (3) is the short length of glass rod (H of Fig. 2) sealed onto the lower portion of the platinum spiral. This serves to draw away larger drops into the retrieval area so that the spiral and thermistor expose a nearly uniform sample each time the sample is changed.

Another innovation is the air vent (C of Fig. 2). It has always been possible to lift the thermistor holder (B of Fig. 2) for the purpose of renewing the solvent on the platinum gauze fitted against the inner surface of the chamber. When this is done without the vent, a liquid seal often develops, causing unwanted changes in pressure within the chamber.

All of the advantages of the sample compartment described by Goyan and Johnson (3) are retained. Less than 1 ml. of solution is sufficient for rinsing and establishing a new concentration, although the usual practice is to rinse and fill several times with 0.5-ml. portions. After each filling, most of the excess is removed from the bottom of the cell by means of a medicine dropper with a tip ground to fit the top of the waste tube. It is easy to save the waste solution without contamination when the solute is worth reclaiming by evaporation.

Best results are obtained when the sample compartment is cleaned from time to time with a suitable cleaning solution so that the inner glass surfaces wet easily. Other obvious precautions associated with precise analytical work are important.

Water Bath—Figure 3 shows a schematic diagram of the water bath built around a 4-l., wide-mouthed, vacuum flask. A brass cylinder fitted around the outside of the mouth of the flask provides a secure anchor for a brass top insulated on the underside with about 2 cm. of plastic foam. This heavy brass cover is provided with four large holes to accommodate the corks used to mount the cells. The stirring shaft passes through a bearing attached to the cover, which also provides a hole for the thermometer. The retaining cylinder extends 1 cm. above the brass cover. This extension serves to hold an inverted cover made of plastic foam and designed to protect the cells from air currents. This cover is split so the front half is easily removed when changing samples. The brass cover also serves as a firm foundation for attaching aluminum rods to support the cover when it is desired to remove the vacuum flask for cleaning or adjusting the attached frame holding the cells in a vertical position. Uniform stirring is an essential part of the water bath.

Temperature Control—Goyan and Borazan (7) mentioned the occasional use of a servo system to control temperature but were forced to resort to manual control. Either system requires an adequate air bath. The constant-temperature air bath has served well for several years and consists of a chamber 63 cm. deep, 65 cm. wide, and 120 cm. high. It is constructed with double walls packed with insulation on a table 75 cm. high. Access to the interior is provided in front by a sliding double-pane glass window 44 cm. wide. The window slides up and down and closes against a board 41 cm. high inserted in the lower part of the window frame. This arrangement enables the operator to open the window a crack at the level of the top of the water bath. Cloth draped on the lower part of the sliding window further discourages the entrance of outside air while changing samples.

The temperature of the air bath is maintained by means of a special Melabs proportional temperature controller modified to use thermistors as transducers. The bath also contains the cooling coil of a Kelvinator (model 4D2CR) designed as a portable cooler. The refrigeration unit is operated so that the cooling coil is coated with ice and maintained in a plastic bucket secured in the upper part of the bath. The bucket is equipped with a drain to the outside and can be raised and lowered with respect to the coil so as to expose more or less cold surface. A 10-cm. Muffin fan was recently installed to replace the old fan. Either one keeps the air stirred sufficiently so that the cooling unit compensates for heat generated

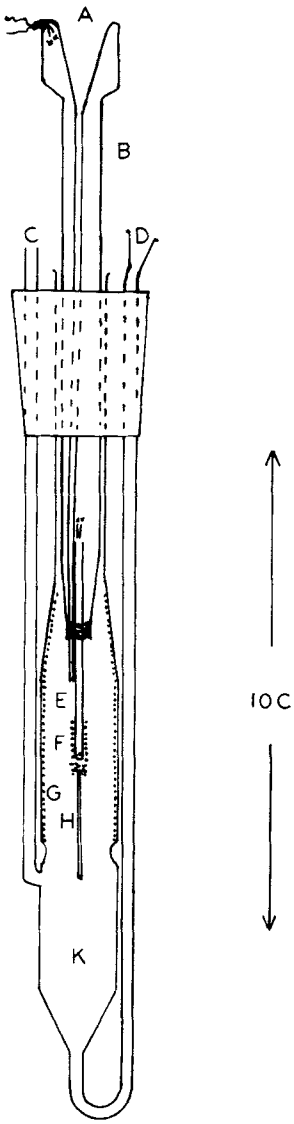

Figure 2—*Sample compartment. Key: A, ground-glass socket for fitted dropper; B, inner tube holding thermistor and capillary; C, air vent; D, ground-glass socket for withdrawing samples after rinsing; E, thermistor (Victory Engineering 51 A1) and tip of capillary sealed at the bottom of tube B with dental amalgam; F, platinum coil for holding sample; G, platinum gauze on inner surface for holding solvent; H, glass rod; and K, lower compartment for collecting rinse.*

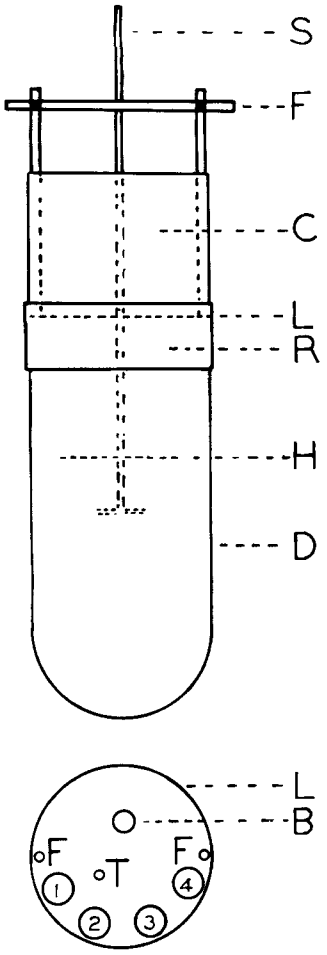

Figure 3—*Water bath (in regulated air bath). Key: S, stirrer rotated by a 300 r.p.m. 2.0 in-oz. Haydon general duty synchronous motor; F, part of standard frame; C, cover made of plastic foam; L, brass cover; R, felt-covered brass cylinder soldered to cover; H, metal cell holder attached to cover; B, bearing for stirrer; T, thermometer; and holes 1–4, location of thermistors,* etc., *mounted in corks.*

by motors in the air bath as well as for heat coming in from outside. The air temperature inside the bath is adjusted so as to require a minimum of help from the servo system or from manual control of current flowing through the heater in the water bath. At present the air is maintained at 25.1° and the bath at 25.00°. In spite of the apparently tight cover, there is sufficient evaporation of water from the water bath to compensate for stirring and a very small amount of heating current (about 1 v. a.c. across a 17-ohm heater). The heating current varies and is controlled by a servo system or manually, based upon the resistance of a thermistor *versus* a decade resistance box. Even when the control systems are in perfect order, any large change in temperature of the water bath must be made manually by using hot and cold water. The servo system is not designed to handle large amounts of heating current.

Figure 4 is a diagram of the servo system used to control the temperature of the water bath. A separate bridge circuit balances a decade box against a thermistor maintained in the water of the bath. Any off-balance signal of this bridge is amplified and presented to a typical recording potentiometer circuit. However, instead of a mechanical link to a recording device, the servo motor is geared to a variable resistor which controls the current going through the heater. The mechanical link to the servo motor uses a large gear attached to both resistors through a clutch mechanism that allows the servo motor to continue after the resistors have reached their limit. The large gear is meshed with the output of the servo motor. When operating normally, this circuit holds the temperature within 1×10^{-4}°, with changes of this magnitude occurring slowly enough that the reference thermistor compensates for these small changes.

Reference Thermistors—The reference thermistor used in previous studies (2–5) nearly matched the one used for the sample. The resistance of the sample thermistor plus a selected value on the decade resistance box equaled the resistance of the reference thermistor and balanced the bridge. A drop of solvent was placed on a platinum loop in contact with the reference and the whole kept in a tube with fitted platinum gauze on the inner surface also wet with solvent. It soon became apparent that when changing solvents, there

Table I—Standardization with Aqueous Solutions of Sucrose

Molal Concentration	ΔR (ohms), av. of 3	ΔR Calculated from Slope	Difference, ohms	Molal Concentration Calculated
0.01	9.6	9.3	0.3	0.0103
0.02	18.5	18.6	0.1	0.0199
0.03	27.5	27.8	0.3	0.0296
0.05	46.1	46.4	0.3	0.0497
0.1	92.7	92.8	0.1	0.0999
0.2	185.6	185.6	0.0	0.2000

was no need to change the solvent in the reference compartment since its only function was to follow slight variations in the temperature of the water bath. Later it was found that the reference thermistor worked about as well without the platinum when the tip of the thermistor was submerged about 1 cm. below the surface of water contained in the same tube. Care was taken to avoid keeping the amalgam seal under water. In all cases the reference tube is mounted in one of the holes of the cover (Fig. 3) and extends well under the surface of the water. Theoretically, it would seem that the reference thermistor should be arranged in a tube as nearly the equivalent of the sample tube as possible to follow temperature changes at the same rate. However, with the new servo control, this consideration has little practical importance.

Operation—Solutions are prepared and stored in small bottles in the air bath along with a sample of the pure solvent. A medicine dropper, with its tip ground to fit the top of the capillary (A of Fig. 2), is used to transfer about 0.5 ml. of solvent or solution to the

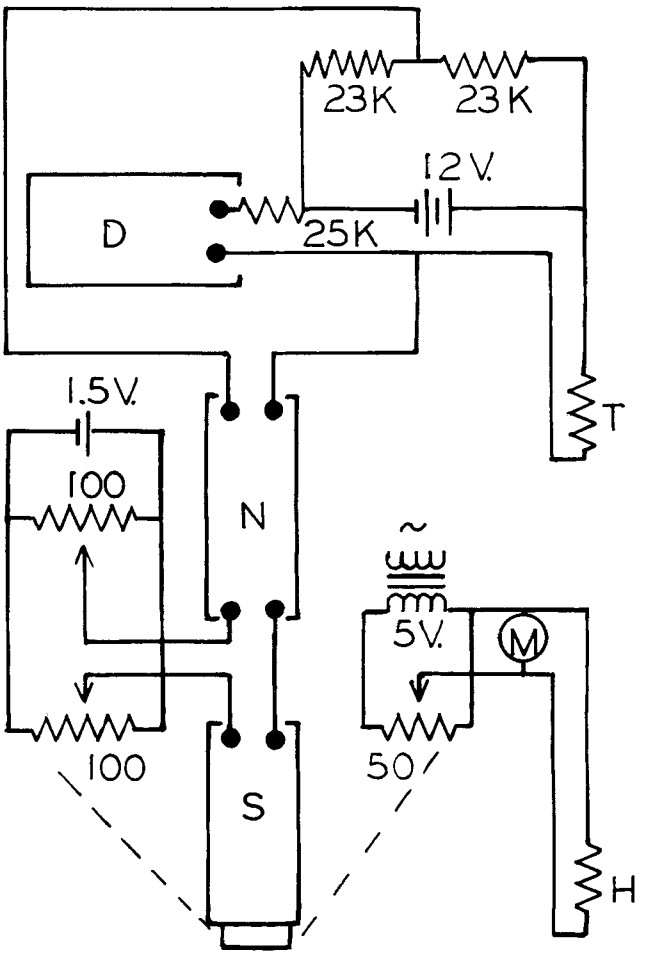

Figure 4—*Servo system. Key: D, Heathkit Decade Resistor model DR-1 (to 1 ohm); N, Leeds and Northrup D.C. Null Detector Cat. 9834; S, Honeywell Brown Electronic continuous balance amplifier and servo motor; M, a.c. milliampmeter converted to a voltmeter; T, thermistor (Victory Engineering 51 A1); and H, heater (approx. 17 ohms).*

capillary and to pass slowly down the thermistor and over the platinum coil (F of Fig. 2). The first portion of this sample serves to rinse the coil, and the last portion is automatically retained by capillary action as the next sample. Before replacing the front half of the cover (C of Fig. 3), all of the fluid accumulated at the bottom of the tube is withdrawn with a different medicine dropper fitted to the waste tube (D of Fig. 2). The waste fluid is collected so far below the working sample that its complete removal is not a matter of concern, although a large accumulation should be avoided.

Within 5 min. after the sample is placed on the coil, the resistance box may be adjusted to approximately its final reading. Any line on the recorder, preferably near zero, may be selected as a null position for the purpose of this initial adjustment. When the recorder shows a vertical line (10–30 min., depending upon solvent and concentration), the resistance box may be changed so as to bring the pen exactly to the selected null position. The new resistance value thus established is recorded for the sample. Exactly the same results are obtained by calculation based on the pen displacement from the null position compared to the displacement caused by changing the decade box by 10 ohms.

Several samples of the same solution are read in this way to test the adequacy of the rinsing when highest accuracy is sought. The second and third measurements, and often the first, agree within 0.4 ohm or better, and undisturbed samples tend to retain their established position within 0.1 ohm. The average values may be plotted directly against concentration, but it is more convenient to obtain ΔR values by subtracting the average for the pure solvent from the resistance reading for each sample (or vice versa if box and reference are in series). These ΔR values may be treated as colligative properties of the various solutions studied (3–5).

Calibration—Sucrose is chosen as a standard substance when working with aqueous solutions. Although not an ideal substance up to 0.2, molal the osmotic coefficients given by Robinson and Stokes (8) may be used to make slight corrections. Table I shows the results obtained using all of the new equipment described. The first column gives the molal concentration, and the second column shows the corresponding ΔR values calculated directly from the measurements, with the exception that for 0.1 molal the tabulated value shows the effect of dividing by 1.008 and the 0.2 molal reading is divided by 1.017 according to the values given by Robinson and Stokes (8) for osmotic coefficients, ϕ, represented in this case by the equation, $\phi = \Delta R_m/\Delta R_i$. The subscripts m and i represent measured and ideal, respectively. The more dilute sucrose solutions studied may be assumed to be ideal without correction.

When ΔR values for an ideal solute are plotted against molal concentration, a straight line results within the limit of concentration studied. In this case, the points fall on the line so closely that a simple plot on a small scale fails to show error. When the data given in the first two columns of Table I are treated by the method of least squares, a slope of 928 ohms molal^{-1} is found. Without including the origin as an experimental point, the intercept is found to be 0.08 ohm. A further test of precision is displayed in the third and fourth columns where, for each solution, a ΔR value is calculated by multiplying concentration by the slope of the line. The difference between the calculated and experimental values is shown in column four. Since, with corrections, sucrose is an ideal substance, any difference between molal and osmolal concentration may be attributed to experimental error. The osmolal concentration for each solution is calculated by dividing the ΔR value by the slope to obtain the values shown in the last column. The average error is 2×10^{-4} molal in concentration units or 0.2 ohm for ΔR.

Nonaqueous Solvents—Earlier work (5) showed that the calibration slopes for nonaqueous solvents are much steeper, as would be predicted from the lower latent heat of vaporization. While this factor alone may be expected to improve accuracy when converting ΔR values to osmolal concentration, the lower heat of vaporization and larger size of the molecules require a longer time to approach equilibrium. It is assumed that solvent molecules must diffuse through the air space and condense on the sample to elevate the temperature to nearly equilibrium value. Since this source of heat in calories per minute is less than with water, it takes longer to read each sample. As a result, the work with nonaqueous solvents is more difficult, especially if manual temperature control is required continuously. With the servo system described, this problem is eliminated, with the result that the recorder draws out smooth curves ending in vertical lines. Another difficulty is encountered with some nonaqueous systems. The solvent is essentially held in place by capillary action. Careful inspection of the cell from time to time is desirable to avoid attempting to work when the platinum gauze is not holding a sufficient amount of solvent. The gauze should be adjusted to press tightly against the glass.

CONCLUSIONS

The new features described have greatly improved the use of the instrument, both from the standpoint of accuracy and of ease of operation without diminishing any of the original advantages.

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Desoxypodophyllotoxin, the Cytotoxic Principle of *Callitris columellaris* F. Muell

Y. AYNEHCHI

Abstract □ An alcoholic extract of *Callitris columellaris* F. Muell was found to show significant inhibitory activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (KB). Systematic fractionation of the extract led to the isolation and characterization of desoxypodophyllotoxin as the active principle.

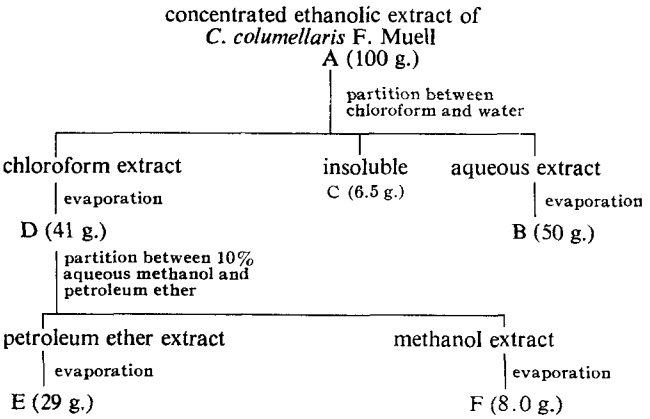
Keyphrases □ Desoxypodophyllotoxin—isolation from *Callitris columellaris*, cytotoxicity evaluation □ *Callitris columellaris*—isolation of cytotoxic principle, desoxypodophyllotoxin, evaluation □ TLC—separation, identification

It was reported earlier (1) that leaves of several junipers possess tumor-necrotizing activity for a number of transplant mouse tumors. In several species the active material was podophyllotoxin (2); in one species, a previously undescribed but related lignin, silicicolin (3), now called desoxypodophyllotoxin (4), was the active material. Further investigation of the leaves of four additional genera (*Libocedurs*, *Podocarpus*, *Chamacyparis*, and *Callitris*) revealed the presence of tumor-necrotizing substances in these plants.

Previous examination of *Callitris columellaris* indicated the presence of *l*-citronellic acid, eudesmol or cryptomeridol, guaiol, and azulene (5). The identification of these compounds was confirmed by GLC and IR and NMR spectroscopy (6). Furthermore, it has been shown that the extract from *C. columellaris* has fungistatic activity. The antifungal property is mainly due to the presence of *l*-citronellic acid, which is an antifungal agent toward *Conifora olivocea* and cryptomeridol, which are antifungal agents toward *C. sanguineus* (7).

In the course of a continuing search for tumor inhibitors from plant sources, an alcoholic extract of *C. columellaris* F. Muell[1] (Pinaceae) was found to have significant inhibitory activity against cells derived from human carcinoma of the nasopharynx in tissue culture (KB). Consequently, a systematic study aimed at isolation of the KB inhibitory principle of *C. columellaris* was undertaken.

The preliminary fractionation of the alcohol extract is summarized in Scheme I. Fraction F material dissolved in chloroform was subjected to chromatography on magnesia–silica gel,[2] whereupon the total activity



Scheme I—Flow Sheet for Fractionation of Cytotoxic Extract from C. columellaris

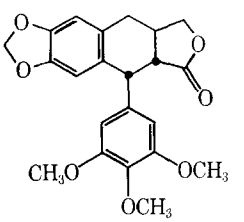
was eluted by using chloroform and 2% methanol in chloroform as eluent.

Further careful chromatography on a silica gel column, collecting fractions which are analyzed by TLC, led to the isolation of desoxypodophyllotoxin (I) from Fraction G.

Results of the tissue culture assays are given in Table I. Material was considered active if the ED_{50} (dose inhibiting to 50% of control growth) was equal or less than 1 mcg./ml. The fractions containing desoxypodophyllotoxin were found to show the highest cytotoxicity. The results indicate that desoxypodophyllotoxin is the major principle of the extract of *C. columellaris* F. Muell.

EXPERIMENTAL

Melting points were obtained on a Hoover Uni-Melt capillary melting-point apparatus. IR spectra were obtained in chloroform solution on a Beckman IR5-A spectrophometer. TLC was carried out on silica gel G and H plates (E. Merck), and the chromatograms were sprayed with a $Ce(SO_4)_3$–H_2SO_4 solution followed by heating until brown spots appeared. Petroleum ether was used,[3] b.p. 60–68°. Evaporation was carried out at less than 40°.



I

[1] Leaves and stems gathered in Australia in June 1965 by the U. S. Department of Agriculture (USDA). The author acknowledges the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., USDA, Beltsville, Md., in accordance with the program developed with USDA by Cancer Chemotherapy National Service Center. A voucher specimen has been kept by USDA, Beltsville, Md.

[2] Florisil, Floridin Co., Pittsburgh, Pa.

[3] Skellysolve B; Skelly Oil Co., Kansas City, Mo.

Table I—Cytotoxicity of Fractions from *C. columellaris*[a]

Fractions	ED_{50}, mcg./ml.
A	0.40
B	64.0
C	100.0
D	0.57
E	32.0
F	3×10^{-2}
G	2.4×10^{-2}
Desoxypodophyllotoxin	5.8×10^{-7}

[a] Assays were performed under the auspices of the Cancer Chemotherapy National Service Center. The procedures were those described in *Cancer Chemother. Rep.*, **25**, 1(1962).

Extraction and Preliminary Fractionation of *C. columellaris*— Coarsely ground leaves and stems of *C. columellaris* (5 kg.) were twice extracted continuously with 95% ethanol for 10 hr., and the ethanol extract was concentrated under water pump pressure to a thick dark syrup (A, 1690 g.). A portion of the crude extract (100 g.) was partitioned between water (1.0 l.) and chloroform (2.0 l.), and the two solutions were evaporated under reduced pressure (B, 59.0 g.; and D, 41 g.). The insolubles (C, 6.50 g.) were collected separately.

The chloroform-soluble portions were partitioned between petroleum ether (2.0 l.) and 10% aqueous methanol (1750 ml.), and the two solutions were evaporated under reduced pressure (E, 29.10 g.; and F, 8.0 g.). The 10% aqueous methanol-soluble portions were dissolved in chloroform and chromatographed on a column (60 × 4 cm.) containing 800.0 g. magnesia–silica gel (100–200 mesh, F-101); then they were eluted with 2.0 l. chloroform followed with 1.0 l. 2% methanol in chloroform. The eluents (chloroform and 2% MeOH in chloroform solutions) were combined and evaporated under reduced pressure (G, 4.10 g.).

Isolation of Desoxypodophyllotoxin—A large batch of Fraction G (55.0 g.) was prepared from crude extract (5.0 kg.), dissolved in benzene, added to a column (75 × 6 cm.) of silica gel (1200 g.), and chromatographed, using 20% chloroform in benzene as the solvent. Fractions (30 ml.) were collected and examined by TLC on silica gel G and H, using 1% MeOH in chloroform as eluent and ceric sulfate (3% in 3 *N* sulfuric acid) spray reagent. The fractions richest in desoxypodophyllotoxin were combined, evaporated to dryness (4.20 g.), and crystallized from ethanol. The colorless crystalline product (3.10 g.) was characterized as desoxypodophyllotoxin by mixed melting point, m.p. 166–168°, mixed TLC, and IR spectral comparison with the authentic sample.

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3-Substituted-2-thiohydrothymines as Potential Antitumor Agents

A. C. GLASSER* and JOHN TRIPLETT

Abstract □ A series of 3-substituted-2-thiohydrothymines was synthesized through the cyclization of the corresponding 1-(2-methyl)-carboxyethyl-3-substituted-2-thioureas which were formed from the appropriate isothiocyanate and β-aminoisobutyric acid. The cyclized products were screened for antitumor activity against lymphoid leukemia L-1210. Results of the screening indicated the compounds have little significant activity in the test system employed.

Keyphrases □ 2-Thiohydrothymines, 3-substituted—synthesis, antitumor testing □ Antitumor agents, potential—2-thiohydrothymines, synthesized, screened □ IR spectrophotometry—structure, identity

Most chemical agents that prevent cancerous growth do so by affecting nucleotide or nucleic acid metabolism. This is both a conclusion of screening (empirical) and a vindication of the search for such agents on purely theoretical reasons (1). Uncontrolled growth is a hereditary property of the neoplastic cell, and this heritable nature of cancer is one reason for considering interference with nucleic acid metabolism in the search for new agents (2).

An earlier report from these laboratories on the synthesis and antituberculous and antitumor activities of a series of 3-substituted-2-thiohydrouracils showed a degree of antitumor activity slightly above the level of inhibition needed for further experimental testing according to the Cancer Chemotherapy National Service Center (CCNSC) Protocol for tumor weight inhibition (3). Compounds in the thiohydrouracil series, such as 3-*p*-ethoxyphenyl- and 3-*p*-(*n*-butoxyphenyl)-, showed test/control (T/C) % values of 62 and 63, respectively, against Sarcoma 180 as tested by CCNSC.

On further consideration of the general class of compounds, it was felt that modifying the structures to correspond to that of thymine, which has a methyl

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On further consideration of the general class of compounds, it was felt that modifying the structures to correspond to that of thymine, which has a methyl

Table I—1-(2-Methyl)carboxyethyl-3-substituted-2-thioureas $CO_2HCHCH_3CH_2NHCSNHR$

Number	R	M.p.[a]	RS[b]	Yield, %	Formula	Analysis, %[c] Calcd.	Found
1	C_6H_5—	137–138°	E–H	74	$C_{11}H_{14}N_2O_2S$	C, 55.19 H, 5.99 N, 11.71	C, 54.91 H, 6.02 N, 11.74
2	*p*-$CH_3OC_6H_4$	142–143°	E–H	80	$C_{12}H_{16}N_2O_3S$	C, 53.71 H, 6.01 N, 10.44	C, 53.90 H, 5.89 N, 10.65
3	*p*-$C_2H_5OC_6H_4$—	157–158°	E–H	92	$C_{13}H_{18}N_2O_3S$	C, 55.49 H, 6.09 N, 9.96	C, 55.28 H, 6.37 N, 9.90
4	*p*-(*n*-C_3H_7O)C_6H_4—	136–137°	E–H	44	$C_{14}H_{20}N_2O_3S$	C, 56.73 H, 6.80 N, 9.45	C, 56.74 H, 6.73 N, 9.40
5	*p*-(*iso*-C_3H_7O)C_6H_4—	116–117°	E–H	74	$C_{14}H_{20}N_2O_3S$	C, 56.73 H, 6.80 N, 9.45	C, 56.88 H, 6.97 N, 9.63
6	*p*-(*n*-C_4H_9O)C_6H_4—	99–101°	E–H	77	$C_{15}H_{22}N_2O_3S$	C, 58.04 H, 7.14 N, 9.03	C, 58.14 H, 7.19 N, 9.03
7	*p*-(*n*-$C_5H_{11}O$)C_6H_4—	105–106°	E–H	67	$C_{16}H_{24}N_2O_3S$	C, 59.23 H, 7.45 N, 8.64	C, 59.76 H, 7.33 N, 9.00
8	*p*-FC_6H_4—	157–158°	E–H	63	$C_{11}H_{13}FN_2O_2S$	C, 51.55 H, 5.11 N, 10.93	C, 51.39 H, 4.80 N, 10.68
9	1-$C_{10}H_7$—	141–142°	E–H	54	$C_{15}H_{16}N_2O_2S$	C, 62.16 H, 5.59 N, 9.37	C, 62.24 H, 6.14 N, 9.63
10	*tert*-C_4H_9—	100–101°	B	75	$C_9H_{18}N_2O_2S$	C, 49.51 H, 8.26 N, 12.85	C, 49.39 H, 8.45 N, 12.89

[a] Melting points on Fisher-Johns block—corrected. [b] Recrystallization solvents: E, ethanol; B, benzene; and H, water. [c] Microanalyses by Dr. Kurt Eder, Geneva, Switzerland.

group in the 5-position of the pyrimidine ring, would be worthy of study. Support for this reasoning can be found in consideration of the pathway by which thymine is catabolized, where it has been shown that normal and leukemic leucocytes catabolize thymine to dihydrothymine in the presence of $TPNH_2^+$ (4). Furthermore, thiopyrimidines have been shown to be normal minor constituents of sRNA (5), and the anticytogenic activity of 2-thiothymine on *N. crassa* was determined to meet the criteria of CCNSC in horizontal proliferation tests (6).

DISCUSSION

The compounds of the series being reported were prepared in order to examine the effect of altering the structures in such a manner as to make them coincide with the thiohydrothymine structure and thus to allow some degree of comparison with the antitumor activities of an analogous series of thiohydrouracils reported previously (3). The thiohydrothymines summarized in Tables II and III were prepared through the dehydration and cyclization of the corresponding 1,3-disubstituted thioureas of Table I.

The thioureas of Table I were prepared from the required isothiocyanates and an aqueous solution of the sodium salt of β-aminoisobutyric acid in a general reaction through stirring at room temperature for 1–4 days. Completion of the reaction was indicated by the disappearance of the immiscible isothiocyanate and the loss of the marked odor of the isothiocyanate. *tert*-Butyl isothiocyanate, the only alkyl isothiocyanate used, was reacted with the amino acid in a solution of sodium ethoxide at reflux. Treatment of the reaction mixtures with hydrochloric acid was the procedure used to release the intermediate 1-(2-methyl)carboxyethyl-3-substituted-2-thioureas.

A previous study of thiohydrouracils (3) indicated that polyphosphoric acid was a useful cyclization agent. Indeed, the substituted thioureas of Table I, with the exception of No. 10 which is quite hindered, were cyclized in good yields using polyphosphoric acid at 140–150°. The required isothiocyanates were prepared using thiophosgene and the appropriate amine in a water–chloroform media (7) and were isolated by vacuum distillation of the isothiocyanate.

Spectral correlations of the structures were good in that the IR spectra[1] of both the thioureas and thiohydrothymines showed good agreement in a comparison of spectra of related thioureas and thiohydrouracils (3). The compounds of Tables I and II show IR absorption in general agreement with the —NC=S bands I, II, and III of Rao and Venkataraghaven (8). The spectra of the aromatic substituted, cyclized thiohydrothymines showed the same characteristic loss of the sharp 2.95-μ aromatic NH—stretching band after cyclization of the thioureas as was seen for the thiohydrouracils. The thiohydrothymines could be further identified following cyclization through the appearance of strong absorption around 8.5 μ, which was usually resolved into sharp bands at 8.4, 8.5, and 8.7 μ. Absorption in this area of the spectrum can be assigned to the C=S stretching of the thioamide bond, which was displaced from the normal area of 8.9 μ by the substituted aromatic ring attached to the No. 3 nitrogen of the heterocyclic ring.

The thiohydrothymines were tested under the direction of CCNSC for antitumor activity in mice at dosage levels of 400 mg./kg., with the exception of Compound 5 which proved toxic at this level. The compounds were tested against L-1210 lymphoid leukemia and displayed no significant activity. In this respect, the T/C % values compared quite closely to the analogous thiohydrouracils.

EXPERIMENTAL

Aryl Isothiocyanates—Phenyl, *p*-fluorophenyl, naphthyl, and *tert*-butyl isothiocyanates were used.[2] The remaining isothiocyanates were prepared from thiophosgene and the appropriate amine according to the typical procedure used for *p*-*n*-amoxyphenylisothiocyanate. A solution of 17.9 g. (0.1 mole) of *p*-*n*-amoxyaniline (9) in 50 ml. of chloroform was added with stirring to 11.5 g. (7.3 ml.) of thiophosgene in 80 ml. of water in a 300-ml. flask under a reflux condenser while held at 0° by an ice–salt bath in an efficient hood. The ice was allowed to melt, and the temperature was allowed

[1] Beckman model IR-8 using KBr disks.
[2] Commercial products, Eastman Kodak.

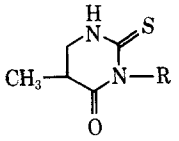

Table II—3-Substituted-2-thiohydrothymines

Number	R	m.p.[a]	RS[b]	Yield, %	Formula	Analysis, %[c] Calcd.	Found
1	C_6H_5—	197–198°	A–B	78	$C_{11}H_{12}N_2OS$	C, 60.00 H, 65.45 N, 12.73	C, 60.13 H, 5.57 N, 12.77
2	*p*-$CH_3OC_6H_4$—	186–187°	A	69	$C_{12}H_{15}N_2O_2S$	C, 57.61 H, 5.62 N, 11.21	C, 57.64 H, 5.69 N, 11.23
3	*p*-$C_2H_5OC_6H_4$—	192–194°	A	70	$C_{13}H_{17}N_2O_2S$	C, 59.10 H, 6.06 N, 10.60	C, 58.91 H, 6.27 N, 10.60
4	*p*-(*n*-C_3H_7O)C_6H_4—	184–185°	A–B	54	$C_{14}H_{19}N_2O_2S$	C, 60.41 H, 6.52 N, 10.14	C, 60.40 H, 6.57 N, 10.17
5	*p*-(*iso*-C_3H_7O)C_6H_4—	208–209°	A–B	84	$C_{14}H_{19}N_2O_2S$	C, 60.41 H, 6.52 N, 10.14	C, 60.48 H, 6.49 N, 10.21
6	*p*-(*n*-C_4H_9O)C_6H_4—	179–180°	A–B	71	$C_{15}H_{21}N_2O_2S$	C, 61.64 H, 6.85 N, 9.59	C, 61.79 H, 6.99 N, 9.56
7	*p*-(*n*-$C_5H_{11}O$)C_6H_4—	211–213°	A–B	63	$C_{16}H_{23}N_2O_2S$	C, 62.73 H, 7.19 N, 9.15	C, 62.96 H, 7.21 N, 9.16
8	*p*-FC_6H_4—	214–215°	A–B	83	$C_{11}H_{11}FN_2OS$	C, 55.51 H, 4.62 N, 11.76	C, 55.58 H, 4.90 N, 11.73
9	1-$C_{10}H_7$—	208–210°	A–B	74	$C_{15}H_{14}N_2OS$	C, 66.73 H, 5.42 N, 10.32	C, 66.72 H, 5.35 N, 10.37

[a] See Table I. [b] Recrystallization solvents: A, acetone; and B, benzene. [c] See Table I.

to rise to that of the room. Two layers developed upon standing overnight. The upper layer consisted of a white, milky emulsion which was broken by the addition of a saturated sodium chloride solution. Following the cracking of the emulsion, the layer was separated and dried over sodium sulfate, and the chloroform evaporated. The residue was subjected to vacuum distillation at 1 mm. to yield 14 g. (63%) of *p*-*n*-amoxyphenylisothiocyanate, b.p. 142–144°. Reported 147–150° (0.8 mm.) (10). IR (film) 4.8 μ.

1-(2-Methyl)carboxyethyl-3-*p*-(*n*-amoxy)phenyl-2-thiourea—A 0.03 *M* solution of *p*-*n*-amoxyphenylisothiocyanate (6.8 g.) and β-aminoisobutyric acid (3.2 g.) in 35 ml. of 1 *N* aqueous sodium hydroxide solution was stirred on a magnetic stirrer in a stoppered 125-ml. extraction flask for 4 days at room temperature. The reaction mixture was worked up in an analogous manner to that used by Glasser and Doughty (3) to yield 7.3 g. of product, m.p. 94–103°, which, after recrystallization from alcohol–water, gave 6.5 g. of 1-(2-methyl)carboxyethyl-3-(*p*-*n*-amoxy)phenyl-2-thiourea as a white crystalline solid, m.p. 105–106°. IR (KBr) 2.95, 3.15, 3.40, 5.96, 6.52, 6.68, 8.08, 9.81, 12.02 μ. (See Table I, No. 7.)

Table III—Antitumor Screening Results for 3-Substituted-2-thiohydrothymines[a]

Number[b]	Dose[c]	Survivors	Animal Wt. Diff., T/C	Average Evaluation[d] Test	Control	T/C, %
1	400	6/6	−1.8	8.8	9.1	96
2	400	5/6	−0.2	8.4	9.1	92
3	400	6/6	−1.7	9.0	9.1	98
4	400	6/6	−1.2	9.3	9.1	102
5	25	6/6	−1.5	9.0	9.3	96
6	400	6/6	−1.3	8.5	9.1	93
7	400	6/6	−2.1	8.5	9.1	93
8	400	6/6	−2.1	8.5	9.1	93
9	400	6/6	−1.0	9.3	9.1	102

[a] CCNSC according to protocol using lymphoid leukemia L-1210. [b] Table II. [c] Milligrams/kilogram in BDF mice. [d] Tumor weight in grams.

3-*p*-(*n*-Amoxy)phenyl-2-thiohydrothymine—A sample of 3.8 g. of 1-(2-methyl)carboxyethyl-3-(*p*-*n*-amoxy)phenyl-2-thiourea was cyclized using 1 g. of polyphosphoric acid as previously described (3) to yield 3.6 g. of product, m.p. 199–208°. Recrystallization from acetone–benzene gave 2.3 g. (64%) of 3-*p*-(*n*-amoxy)phenyl-2-thiohydrothymine as a white crystalline solid, m.p. 212–213°. IR(KBr) 3.14, 3.38, 5.82, 6.40, 6.62, 7.26, 8.05, 8.40, 8.51, 8.68 μ. (See Table II, No. 7.)

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Solid Dispersion Approach to the Formulation of Organic Liquid Drugs Using Polyethylene Glycol 6000 as a Carrier

WIN LOUNG CHIOU and LLOYD D. SMITH

Abstract □ Five poorly water-soluble liquid compounds—benzonatate, clofibrate, methyl salicylate, benzyl benzoate, and *dl*-α-tocopheryl acetate—were solid dispersed in a polyethylene glycol 6000 carrier by the melting method. The solidified mass, containing up to 5 or 10% (w/w) of liquid compounds, could be pulverized, encapsulated, and tableted. In the *in vitro* dissolution studies, the active ingredients were completely dissolved in 4–14 min. The preparations were highly reproducible, and the active ingredients were shown to distribute homogeneously within the carrier matrix. This approach represents a new method in the formulation of liquid medicinal compounds. The application of the solid dispersion principle in formulating a prolonged or sustained-release dosage regimen is also discussed.

Keyphrases □ Dispersion, solid, using polyethylene glycol 6000—in formulating organic liquid drugs □ Medicinals, liquid—solid dispersed using polyethylene glycol 6000 as carrier □ Polyethylene glycol 6000—carrier for solid dispersion of benzonatate, clofibrate, methyl salicylate, benzyl benzoate, *dl*-α-tocopheryl acetate □ Sustained-release dosage—solid-dispersion technique potential

Most liquid drugs used for oral and rectal administrations are prepared in solution or emulsion forms. In certain circumstances, it may be advantageous in terms of the ease of formulating, packing, handling, and administering if one can convert the liquid dosage form into a solid dosage form of the same drug. This can be seen in drugs like vitamins A and E. This is usually accomplished by the mechanical mixing of the liquid drugs with other solid components. This conventional method of mixing, however, often may cause serious nonhomogeneity problems, especially when the concentration of the liquid ingredient is very low. Therefore, it appears worthwhile to explore an alternative method for the formulation of liquid compounds.

In the past, a number of poorly water-soluble drugs were studied (1–11) for their solid-state dispersions in physiologically inert, water-soluble carriers as a potential means to increase their dissolution and oral absorption. They, however, are limited to solid drugs at room temperature. The purpose of this preliminary investigation was to study the feasibility of such a technique for the formulation of liquid compounds or drugs by employing polyethylene glycol (PEG) 6000 as a carrier. Furthermore, the reproducibility and the homogeneity of the distribution of active drugs in the carrier were studied.

EXPERIMENTAL

Materials—The following were used: benzonatate,[1] clofibrate,[2] methyl salicylate (synthetic),[3] *dl*-α-tocopheryl acetate NF,[4] benzyl benzoate,[5] and PEG 6000.[6]

[1] Supplied by Ciba Pharmaceutical Co., Summit, N. J.
[2] Supplied by Ayerst Laboratories Inc., New York, N. Y.
[3] J. T. Baker Chemical Co., Phillipsburg, N. J.
[4] Supplied by Hoffmann-La Roche Inc., Nutley, N. J.
[5] Matheson Coleman & Bell, Norwood, Ohio.
[6] Union Carbide Corp., New York, N. Y.

Table I—Homogeneity Studies of 5% (w/w) of Benzyl Benzoate and α-Tocopheryl Acetate Dispersed in PEG 6000

Sample	Percent Recovery—Benzyl Benzoate—Lot I	Benzyl Benzoate—Lot II	α-Tocopheryl Acetate Lot I	α-Tocopheryl Acetate Lot II
1	99.6	98.1	97.6	97.0
2	100.4	98.6	97.8	98.9
3	98.6	100.7	99.3	98.2
4	98.9	100.2	98.5	97.4
Mean	99.4	99.4	98.3	98.0

Preparation of Solid Dispersions—Solid dispersion forms of the five poorly water-soluble liquid compounds in PEG 6000 were prepared by the melting method. Mixtures of various compositions of the drug and PEG 6000 (2.5, 5, and 10% w/w of drugs in the mixtures) were accurately weighed into beakers, heated directly on a hot plate with stirring until all melted, and then spread in a thin layer onto a stainless steel plate. The mixtures were cooled and solidified rapidly. The solid or semisolid masses could be removed easily from the plates and were kept in desiccators containing silica gel for 1 or 2 days for further hardening. They were then pulverized at room temperature (about 27°) in a mortar, and the 20–40 mesh particles were collected for studies reported in this communication.

Dissolution-Rate Studies—Dissolution-rate studies were conducted in 500 ml. distilled water in a water-jacketed beaker maintained at 37°. The dissolution medium was stirred by a stainless steel paddle, 5.5 × 2.7 cm., placed at the center and maintained at a rate of 100 r.p.m. by an overhead motor. About 2.5 ml. sample was taken rapidly through a washed pledget of glass wool every 2 min. for 14 min. After measurement of its absorbance by a Beckman DBG spectrophotometer, the sample was immediately poured back into the dissolution bath. The amount of the powder used for each dissolution study was equivalent to 5 mg. methyl salicylate, 5 mg. benzonatate, 2.5 mg. clofibrate, and 2.0 mg. benzyl benzoate. The wavelengths used for measuring their absorbances were 297, 310, 225, and 230 nm., respectively. They all obeyed Beer's law at the concentrations studied.

All dissolution studies were run at least in duplicate. Generally the results were quite reproducible. The recycling and flow cell system (3) was not employed in this investigation because the silicone tubing would sometimes adsorb a significant amount of drug from the solution. For example, the absorbance of 500 ml. of aqueous benzyl benzoate solution was reduced from 0.24 (4.5 mcg./ml.) to 0.18 after a few minutes of circulation.

Homogeneity Studies—Ten grams of solid dispersion forms of 5% (w/w) benzyl benzoate and α-tocopheryl acetate in PEG 6000 was prepared in duplicate. After pulverization, four samples, each containing about 0.5% of the total preparation, were taken from various parts of the container. They were dissolved in 95% alcohol and the concentrations were determined spectrophotometrically.

RESULTS AND DISCUSSION

Solid Dispersions in PEG—It was quite surprising that all the solidified masses from the drug–PEG melt mixtures containing up to 5% liquid compounds (benzonatate, clofibrate, methyl salicylate, *dl*-α-tocopheryl acetate, and benzyl benzoate) could be easily pulverized into fine particles at room temperature. This might be due to the formation of solid solutions of the drugs in the PEG 6000. Recently, Chiou and Riegelman (9) theorized that a great variety of small organic compounds with molecular weight less than 1000 could form extensive interstitial solid solutions in the high molecular weight of PEG polymers. Such contention also is

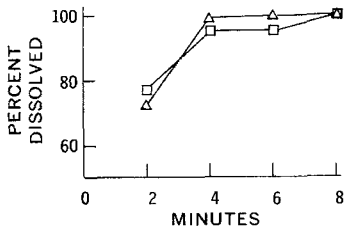

Figure 1—*Cumulative plots of dissolution from clofibrate, solid dispersed in PEG 6000. Key:* △, *2.5%; and* □, *5%.*

supported by experimental data on the indomethacin–PEG 6000 system (11) in which up to 10% of indomethacin was believed to be molecularly dispersed in the PEG 6000.

The pulverization of the 10% benzyl benzoate system also posed no difficult problem, while preparations containing 10% of other liquid compounds at the ambient temperature were not hard and brittle enough to be subdivided easily. However, they could be powdered more easily when maintained at a lower temperature such as 5°. This may be attributed to the higher viscosity of the fine liquid drops of the drugs at lower temperature when the concentrations of the drugs exceed their solubility in the carrier.

Although only PEG 6000 was tested as a carrier, it is expected that similar results can be obtained when other solid PEG polymers are used as carriers. The preparation of the solid dispersions of the five selected liquid compounds is not intended to advocate their use as dosage forms for therapeutic purpose but is rather aimed at the demonstration of such a technique for the general liquid drugs. Since only about 10% of a drug can be solid dispersed in PEG polymers, this new approach of formulation is limited to liquid drugs with low therapeutic doses, for example, below 50–100 mg. The resultant solid dispersion can be encapsulated or tableted.

One big advantage of using PEG for the solid dispersion of liquid compounds over other water-soluble polymers or inert carriers with smaller molecular weight is their low melting points (all below 67°) and their high thermal stability. Therefore, the chemical stability of the drugs generally can hardly be affected because they can be incorporated to the melt of PEG below 67°. Moreover, the preparation can be rapidly accomplished by the simple fusion process rather than by the more laborious solvent methods suggested (5, 6) for the preparation of the solid dispersions of drugs in polyvinylpyrrolidone (PVP).

Fast *versus* Slow-Release Solid Dispersion—Since the dissolution rate of a component may be affected by the second component in the multiple-component mixture (11), the selection of a water-soluble, readily dispersible carrier might lead to a formulation with fast-release characteristics, as discussed by various authors (1–10), and the selection of a poorly soluble or insoluble carrier might result in a preparation with slow, prolonged, or sustained-release property. It is then logical to assume that one could obtain desired release characteristics of a dosage form through the control of the carrier system by using different solubilities of the carrier and/or different concentrations of the carrier in the same dosage regimen. Such possibility is currently under investigation in this laboratory.

Dissolution Studies—The plots of cumulative dissolution from four poorly soluble drugs are shown in Figs. 1–4. Except for benzyl benzoate, more than 50% of the drugs dispersed in PEG 6000 dissolved in the first 2 min. and 100% in 4–8 min. However, it required 14 min. to dissolve all the solid dispersed benzyl benzoate. The dissolution rates from the pure liquid compounds were not studied because of difficulties of control of the liquid particle size and their accurate weight (less than 5 mg. used). In spite of the lack of comparison with pure compounds, the demonstrated fairly fast *in vitro* release of the compounds from dispersion systems in the moderately stirred medium indicated their high potential application for the formulation of liquid compounds. The bioavailability of such dosage forms as compared to the conventional forms, however, remains to be studied further.

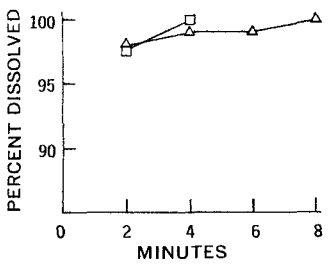

Figure 2—*Cumulative plots of dissolution from methyl salicylate, solid dispersed in PEG 6000. Key:* □, *2.5%; and* △, *5%.*

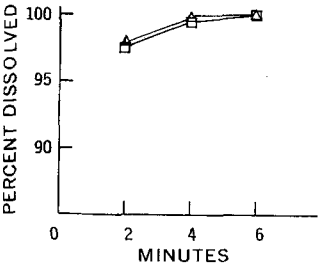

Figure 3—*Cumulative plots of dissolution from benzonatate, solid dispersed in PEG 6000. Key:* □, *2.5%; and* △, *5%.*

In these dissolution-rate studies, all the solid dispersion systems of each drug containing 2.5, 5, and 10% drug in the polymer exhibited almost the same dissolution rate within experimental error. It is not intended, however, in this preliminary report to construct a mathematical model describing quantitatively the drug-release rates from the fine powders of solid dispersion systems. Due to many similar physical properties between the PEG and the PVP polymers, it is believed that the theoretical model for the dissolution rates of high-energy PVP sulfathiazole coprecipitates from a constant-tablet surface, proposed in a recent excellent article by Simonelli *et al.* (8), may provide a possible qualitative explanation to this interesting phenomenon. If one assumes that the dissolution rate from the powder surface is rate limited by the dissolution of the controlling PEG external layer, as in the analogy to the controlling PVP layer in the high PVP weight fractions, then the dissolution rate of the drug from the same drug–PEG dispersion surface will be proportional to the drug–PEG ratio of the solid dispersion. In other words, the dissolution rate of a drug from a 5% solid dispersion will be almost twice as fast as from a 2.5% solid dispersion if their dissolution surface is the same. The total initial surface of the powders containing 5% drug will be only one-half of that containing 2.5% drug for a given amount of the active drug if they have the same particle-size distribution. If one further assumes that the dissolution rates are also proportional to the surface area of the powders under the sink condition as employed in this study, the dissolution rates of the solid dispersion powders containing 2.5, 5, and 10% drug will be expected to be all the same, as shown in Figs. 1–4. Other factors such as the possible complexation between the drug and PEG and the conversion of the molecular dispersion to particulate droplets of pure compounds may also affect their dissolution.

Homogeneity Studies—Although the solid dispersion approach has been known for almost a decade, it seems that no reproducibility and homogeneity of distribution have been quantitatively reported. It is believed that they are among the important factors to be considered. The results of the studies on benzyl benzoate and α-tocopheryl acetate are summarized in Table I. It is clear, within the experimental error, that the preparations were highly reproducible and the active ingredients were homogeneously distributed in the PEG carrier. The consistent, slightly lower recovery of α-tocopheryl acetate might be due to the lower extinction coefficient of the possible complexation with PEG, thereby resulting in lower calculated recovery values. It might also be due to the partial evap-

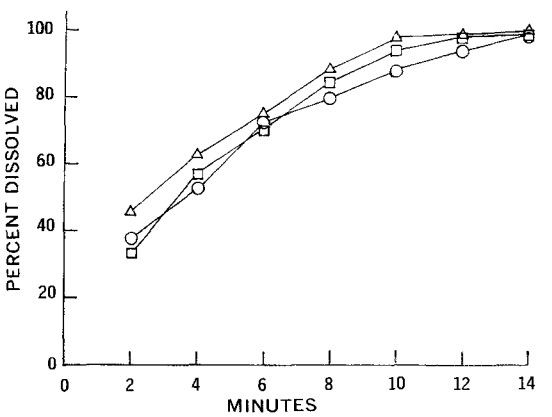

Figure 4—*Cumulative plots of dissolution from benzyl benzoate solid dispersed in PEG 6000. Key:* □, *2.5%;* △, *5%; and* ○, *10%.*

oration of the tocopheryl acetate or its adsorption to the mortar and pestle during the grinding process.

CONCLUSIONS

The solid dispersion principle was tested on five poorly soluble liquid compounds by using PEG 6000 as a carrier. They were prepared by the melting method. Below the 10% concentration, they could be pulverized, encapsulated, and tableted. The preparations were reproducible, and the active ingredients were distributed homogeneously in the solid matrix. The active ingredients were found to be released rapidly from the matrix, with complete dissolution time ranging from 4 to 14 min. This approach represents a new method for the formulation of liquid medicinal compounds, either water soluble or insoluble. It is proposed that one can formulate a prolonged or sustained-release dosage form of drugs by the careful control of the carriers.

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Synthesis and Anticonvulsant Activity of Substituted 2-Thioquinazolin-4-ones I: Preliminary Studies

ARTHUR C. GLASSER*, LOUIS DIAMOND, and GEORGE COMBS

Abstract □ A series of 2-thioquinazolin-4-ones with varying substituents on position 2, 3, or 6 was synthesized and studied for their ability to prevent maximal electroshock and chemoshock seizures in mice within the dosage range of 10–600 mg./kg. The 2-ethylthio-3-(2-phenyl)ethylquinazolin-4-one exhibited full protection against electroshock at the 100-mg./kg. level. The compound 2-carboxymethylthio-3-(2-phenyl)ethylquinazolin-4-one showed full protection against electroshock at the 600-mg./kg. level and partial activity at the 100-mg./kg. level. Other than these examples of activity, there was little significant activity in the remaining members of the series, with 2-ethylthio-3-phenylquinazolin-4-one showing only partial protection at both the 600- and 100-mg./kg. levels. With one exception within the dosage range studied, none of the compounds appeared to be active in preventing pentylenetetrazole seizures, thus indicating that the compounds possibly act in an analogous manner to diphenylhydantoin and related molecules.

Keyphrases □ 2-Thioquinazolin-4-ones, substituted—synthesis, anticonvulsant activity evaluated □ Anticonvulsants, synthesized, evaluated—substituted 2-thioquinazolin-4-ones □ Pentylenetetrazole-induced seizures—evaluation of substituted 2-thioquinazolin-4-ones anticonvulsant activity

Despite the many significant advancements that have occurred in recent years, newer, safer, and more effective CNS depressant drugs are needed for therapeutic application as anticonvulsant, sedative, or hypnotic agents. In approaching this problem, it was thought that the broad spectrum of physiological activities of the 4-quinazolones would make them good candidates for study; indeed, CNS depressant drugs have been developed from this class. The history of the development of 2-methyl-3-*o*-tolyl-4-quinazolone, methaqualone, was reviewed recently by Cain (1), and Boissier *et al.* (2) showed the *ortho*-chloro compound to be the most active from a study of the *ortho*-, *meta*-, and *para*-isomers. Hayao *et al.* (3) reported on the synthesis of a series of 3-substituted-2,4-(1*H*,3*H*)-quinazolinediones. In their examination for CNS depressant effects, one of the series was reported to approach chlorpromazine in its activity upon experimental animals (3). Burkhalter and Scarborough (4) previously reported that 2,4-(1*H*,3*H*)-quinazolinedione and the 1,3-dimethyl derivative showed protective action against electroshock and pentylenetetrazole-induced seizures in mice. Gujral *et al.* (5, 6) also noted hypnotic and anticonvulsant properties in a series of 2-alkyl-3-aryl-4-(3*H*)-quinazolones. This work was confirmed by Boissier (7). The substance 2-methyl-3-*p*-bromophenyl-4-quinazolone was reported by Bianchi and David (8) to have strong anticonvulsant properties against pentylenetetrazole-induced seizures. Gupta *et al.* (9) continued their study of substituted-4-quinazolones as CNS depressants in a report on some 2,3-disubstituted derivatives, and Chaurasia (10) reported the synthesis of potential antimalarial and ataractic thioquinazolone derivatives.

The present series of thioquinazolin-4-ones resulted from the observation of a marked decrease in coordination (11), as shown by the rota-rod test (12) and narrow strip (horizontal and inclined) test (13) when a suspension of 3-phenyl-2-thioquinazolin-4-one, which was prepared as a part of another study, was administered intraperitoneally to mice at a dosage level of 100 mg./kg. As an expansion of this observation, it was of interest to determine the effect of substituting the thio group, the aromatic ring, and altering the substituent at the 3-nitrogen to gain information as to potential anticonvulsant activity in the general structure. The results of the syntheses are summarized in Tables I and II.

oration of the tocopheryl acetate or its adsorption to the mortar and pestle during the grinding process.

CONCLUSIONS

The solid dispersion principle was tested on five poorly soluble liquid compounds by using PEG 6000 as a carrier. They were prepared by the melting method. Below the 10% concentration, they could be pulverized, encapsulated, and tableted. The preparations were reproducible, and the active ingredients were distributed homogeneously in the solid matrix. The active ingredients were found to be released rapidly from the matrix, with complete dissolution time ranging from 4 to 14 min. This approach represents a new method for the formulation of liquid medicinal compounds, either water soluble or insoluble. It is proposed that one can formulate a prolonged or sustained-release dosage form of drugs by the careful control of the carriers.

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Synthesis and Anticonvulsant Activity of Substituted 2-Thioquinazolin-4-ones I: Preliminary Studies

ARTHUR C. GLASSER*, LOUIS DIAMOND, and GEORGE COMBS

Abstract □ A series of 2-thioquinazolin-4-ones with varying substituents on position 2, 3, or 6 was synthesized and studied for their ability to prevent maximal electroshock and chemoshock seizures in mice within the dosage range of 10–600 mg./kg. The 2-ethylthio-3-(2-phenyl)ethylquinazolin-4-one exhibited full protection against electroshock at the 100-mg./kg. level. The compound 2-carboxymethylthio-3-(2-phenyl)ethylquinazolin-4-one showed full protection against electroshock at the 600-mg./kg. level and partial activity at the 100-mg./kg. level. Other than these examples of activity, there was little significant activity in the remaining members of the series, with 2-ethylthio-3-phenylquinazolin-4-one showing only partial protection at both the 600- and 100-mg./kg. levels. With one exception within the dosage range studied, none of the compounds appeared to be active in preventing pentylenetetrazole seizures, thus indicating that the compounds possibly act in an analogous manner to diphenylhydantoin and related molecules.

Keyphrases □ 2-Thioquinazolin-4-ones, substituted—synthesis, anticonvulsant activity evaluated □ Anticonvulsants, synthesized, evaluated—substituted 2-thioquinazolin-4-ones □ Pentylenetetrazole-induced seizures—evaluation of substituted 2-thioquinazolin-4-ones anticonvulsant activity

Despite the many significant advancements that have occurred in recent years, newer, safer, and more effective CNS depressant drugs are needed for therapeutic application as anticonvulsant, sedative, or hypnotic agents. In approaching this problem, it was thought that the broad spectrum of physiological activities of the 4-quinazolones would make them good candidates for study; indeed, CNS depressant drugs have been developed from this class. The history of the development of 2-methyl-3-*o*-tolyl-4-quinazolone, methaqualone, was reviewed recently by Cain (1), and Boissier *et al.* (2) showed the *ortho*-chloro compound to be the most active from a study of the *ortho*-, *meta*-, and *para*-isomers. Hayao *et al.* (3) reported on the synthesis of a series of 3-substituted-2,4-(1*H*,3*H*)-quinazolinediones. In their examination for CNS depressant effects, one of the series was reported to approach chlorpromazine in its activity upon experimental animals (3). Burkhalter and Scarborough (4) previously reported that 2,4-(1*H*,3*H*)-quinazolinedione and the 1,3-dimethyl derivative showed protective action against electroshock and pentylenetetrazole-induced seizures in mice. Gujral *et al.* (5, 6) also noted hypnotic and anticonvulsant properties in a series of 2-alkyl-3-aryl-4-(3*H*)-quinazolones. This work was confirmed by Boissier (7). The substance 2-methyl-3-*p*-bromophenyl-4-quinazolone was reported by Bianchi and David (8) to have strong anticonvulsant properties against pentylenetetrazole-induced seizures. Gupta *et al.* (9) continued their study of substituted-4-quinazolones as CNS depressants in a report on some 2,3-disubstituted derivatives, and Chaurasia (10) reported the synthesis of potential antimalarial and ataractic thioquinazolone derivatives.

The present series of thioquinazolin-4-ones resulted from the observation of a marked decrease in coordination (11), as shown by the rota-rod test (12) and narrow strip (horizontal and inclined) test (13) when a suspension of 3-phenyl-2-thioquinazolin-4-one, which was prepared as a part of another study, was administered intraperitoneally to mice at a dosage level of 100 mg./kg. As an expansion of this observation, it was of interest to determine the effect of substituting the thio group, the aromatic ring, and altering the substituent at the 3-nitrogen to gain information as to potential anticonvulsant activity in the general structure. The results of the syntheses are summarized in Tables I and II.

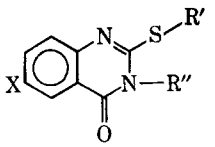

Table I—Substituted 2-Thio-(3*H*)-quinazolin-4-ones

Number	R′	R″	X	M.p.[a]	Yield, %	Recrystallization Solvent[b]	Anal., %[c] Calcd.	Found
1	—Et	—$CH_2CH_2\phi$	—Cl	101–103°	74	EtOH	C, 62.70 H, 5.01 N, 8.10	C, 62.82 H, 4.95 N, 8.08
2	—Et	—ϕ	—H	105–107°	66	EtOH	C, 68.10 H, 5.01 N, 9.90	C, 68.21 H, 5.11 N, 10.12
3	—Et	—$CH_2CH_2\phi$	—H	81–83°	85	EtOH	C, 69.70 H, 5.80 N, 9.01	C, 69.62 H, 5.93 N, 8.94
4	—CH_2CO_2H	—$CH_2CH_2\phi$	—H	151–153°	45	Ac	C, 63.50 H, 4.70 N, 8.20	C, 63.11 H, 4.84 N, 7.91
5	—CH_2CO_2H	—ϕ	—H	273–274°	37	Ac	C, 61.50 H, 3.86 N, 8.99	C, 61.26 H, 3.91 N, 9.01

[a] Mel-Temp apparatus, corrected. [b] Ac = acetone; DMF = dimethylformamide. [c] Microanalyses performed by Dr. Kurt Eder, Geneva, Switzerland, and Galbraith Laboratories, Knoxville, Tenn.

PHARMACOLOGY

The anticonvulsant activity of each compound was evaluated by means of maximal electroshock seizure (MES) and pentylenetetrazole seizure threshold (PST) tests (13). A group of three male albino mice was used for each test, and the results were compared with saline-treated control groups. The animals were stimulated with 60-cycle alternating current for 200 msec. *via* a pair of earclip electrodes. A variable transformer was used to regulate the applied voltage. The desired stimulus duration was obtained by the inclusion of a relay in the circuit, which was operated by a Grass S-4 square-wave stimulator. A cathode ray oscilloscope (CRO) was used to monitor the voltage drop across a 10-ohm resistor coupled in series with the animal; thus the corresponding current could be calculated. The sweep on the CRO screen was triggered by the stimulator, so accurate readings could be obtained with each shock. Preliminary studies showed that 12–16 mamp. was required to produce a convulsive seizure pattern having a tonic flexor phase, a tonic extensor phase, and a clonic phase.

After adequate control data were obtained, each group of animals received, intraperitoneally, 10, 100, or 600 mg./kg. of the drug under study; the MES tests were repeated 1, 2, and 3 hr. after drug administration. All drugs were administered as suspensions in 0.5% methylcellulose (4000 cps.). An animal was considered to be protected if the tonic extensor phase of the seizure pattern was absent.

A similar experimental design was used for conducting the PST tests. Animals received pentylenetetrazole, 85 mg./kg. s.c., 2 hr. after administration of a test compound. Animals that showed no convulsive activity of any kind during a 30-min. observation period were considered protected.

The ability of the selected experimental testing procedures to detect anticonvulsant activity was assessed by including in the study three agents known to provide significant protection against either electrically or chemically induced seizures—*viz.*, diphenylhydantoin, phenobarbital, and trimethadione.

The neurologic status of each animal was evaluated prior to each test for anticonvulsant activity. Toxicity was considered present if the animal failed to exhibit a righting reflex or a hind-limb placing reaction or if the animal's equilibrium, gait, or stance deviated from normal. The results of the MES and PST tests are summarized in Tables III and IV.

CHEMISTRY

The compounds studied were prepared by reaction of the appropriate anthranilic acids with phenethyl isothiocyanate or phenyl isothiocyanate in ethyl alcohol or dimethylformamide (DMF) at reflux. The thiosubstituted compounds were prepared by the action of ethyl iodide or chloroacetic acid upon the quinazolones in alcoholic sodium hydroxide solution (14). Selective thioethylation of quinazolones under these conditions can be confirmed through desulfurization with Raney nickel (14). Phenethyl isothiocyanate was prepared in 63% yield by a modified Kaluza synthesis according to

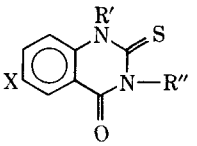

Table II—Substituted 2-Thio-(1*H*,3*H*)-quinazolin-4-ones

Number	R′	R″	X	M.p.[a]	Yield, %	Recrystallization Solvent[b]	Anal., %[c] Calcd.	Found
1	—H	—$CH_2CH_2\phi$	—H	244–245°	33	Ac	C, 68.10 H, 5.00 N, 9.90	C, 68.20 H, 5.15 N, 9.81
2	—H	—$CH_2CH_2\phi$	—Cl	242–244°	53	Ac	C, 60.70 H, 4.10 N, 8.80	C, 60.77 H, 4.02 N, 8.96
3	—H	—ϕ	—H	304–305°[d]	85	DMF	C, 66.10 H, 3.97 N, 11.01	C, 65.91 H, 4.13 N, 11.17

[a–c] The same as in Table I. [d] Literature 304–306°, B. Pawlewski, *Chem. Ber.*, **38**, 131(1905).

Table III—Maximal Electroshock Seizure Tests

Compound Number	Dose, mg./kg.	—Number of Animals Protected— 1 hr.	2 hr.	3 hr.	—Number of Animals Showing Toxicity— 1 hr.	2 hr.	3 hr.
1	600	0/3	0/3	0/3	0/3	0/3	0/3
2	600	2/3	2/3	2/3	3/3	3/3	3/3
	100	1/3	1/3	1/3	0/3	0/3	0/3
3	600	3/3	3/3	3/3	3/3	3/3	3/3
	100	3/3	3/3	3/3	0/3	0/3	0/3
	10	0/3	0/3	0/3	0/3	0/3	0/3
4	600	0/3	1/3	1/3	0/3	0/3	0/3
5	600	3/3	3/3	3/3	0/3	0/3	0/3
	100	1/3	1/3	1/3	0/3	0/3	0/3
6	600	3/3	3/3	3/3	0/3	2/3	2/3
	100	0/3	0/3	0/3	0/3	0/3	0/3
7	Not tested						
8	600	0/3	0/3	0/3	0/3	0/3	0/3
Diphenylhydantoin	25	3/3	3/3	3/3	0/3	0/3	
Phenobarbital	25	3/3	3/3	3/3	0/3	0/3	0/3
Normal saline	1 ml.	0/3	0/3	0/3	0/3	0/3	0/3

Table IV—Pentylenetetrazole Seizure Threshold Tests

Compound Number	Dose, mg./kg.	Number of Animals Protected 2 hr. after Administration
1	600	0/3
2	100	0/3
3	100	0/3
4	600	0/3
5	600	0/3
6	600	3/3
	100	0/3
7	Not tested	
8	600	0/3
Trimethadione	600	3/3
Normal saline	1 ml.	0/3

Hodgkins and Reeves (15). The anthranilic acids and phenyl isothiocyanate were commercial products purified as necessary before use.

EXPERIMENTAL[1]

Phenethyl Isothiocyanate—Into a 1000-ml. three-necked flask equipped with a dropping funnel, mechanical stirrer, and reflux condenser, there were added 68.3 g. (0.9 mole) of carbon disulfide and 80 ml. of 11 *M* sodium hydroxide solution. The flask was cooled to 0° with an ice–salt bath. 2-Phenethylamine, 109 g. (0.9 mole), was added dropwise over a period of 30 min. with stirring. The reaction was allowed to warm to room temperature and then was heated on a water bath for 1.5 hr. The solution was cooled to room temperature, and 87.5 ml. (0.9 mole) ethylchloroformate was added dropwise to the solution with stirring; the stirring was continued for 30 min. The isothiocyanate appeared as a lower layer and was separated and dried over magnesium sulfate overnight. The product was distilled under reduced pressure for a yield of 92.5 g. (63%) of a colorless liquid, b.p. 146–148° at 15 mm., lit. 141–144° at 11 mm. (16).

Anal.—Calcd. for C_9H_9NS: C, 66.20; H, 5.60; N, 8.60. Found: C, 66.17; H, 5.76; N, 8.39.

2-Thio-3-phenethyl-6-chloroquinazolin-4-one—Into a 250-ml. boiling flask equipped with a reflux condenser, there were added 16.3 g. (0.1 mole) phenethyl isothiocyanate, 17.2 g. (0.1 mole) 2-amino-5-chlorobenzoic acid, and 50 ml. of DMF. The solution was refluxed 2.5 hr. and allowed to stand overnight in a refrigerator. Filtration of the cooled reaction mixture yielded a crystalline solid, m.p. 231–235°, which, upon recrystallization from acetone, yielded 16.7 g. (53%) of a crystalline solid, m.p. 242–244°.

Anal.—Calcd. for $C_{16}H_{13}ClN_2OS$: C, 60.70; H, 4.10; N, 8.81. Found: C, 60.77; H, 4.02; N, 8.96.

2-Ethylthio-3-phenethyl-6-chloroquinazolin-4-one—2-Thio-3-phenethyl-6-chloroquinazolin-4-one (5.7 g., 0.02 mole) was added to a solution of 5 g. of sodium hydroxide in 85 ml. of an ethyl alcohol–water (50:50) solution. The solution was mixed, and 6 g. (4 ml.) of ethyl iodide was added; then the solution was stirred for 1.5 hr. The product separated out and was filtered, washed with EtOH–H_2O, and dried. Upon recrystallization from alcohol, 4.6 g. (74%) of colorless crystals, m.p. 101–103°, was obtained.

Anal.—Calcd. for $C_{18}H_{17}ClN_2OS$: C, 62.70; H, 5.01; N, 8.10. Found: C, 62.82; H, 4.95; N. 8.08.

[1] Melting points were taken on a Mel-Temp block and are corrected. Structural correlations by means of IR were made using a Beckman IR-8.

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Entropic Factor in the Solubility of Small Particles

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Abstract □ A mechanistic basis for the dependence of solubility on particle size was investigated. The solubility difference between a crystalline and microcrystalline solid was determined; the microcrystalline solid had a greater solubility than the crystalline. The heat of solution, as determined from a Van't Hoff plot, was found to be independent of particle size. The observed solubility difference was interpreted to be due to a relatively greater entropy of solution for the microcrystalline solid as compared to the crystalline solid.

Keyphrases □ Entropic factor—small particle solubility □ Particle size—solubility dependence □ Griseofulvin, crystalline, microcrystalline—Van't Hoff plot of relative solubilities

Wollaston (1) first noted the relationship between particle size and solubility when he observed that finely divided solids dissolved to a greater extent than relatively coarse particles. Ostwald (2) derived a mathematical relationship between particle size and solubility, based on the Kelvin relationship between vapor pressure and curvature, which was later corrected by Freundlich (3). Recently, the role of entropy in this phenomenon was postulated (4). The present experiment was undertaken to obtain predictive evidence for this hypothesis.

THEORETICAL

Equation 1 can readily be derived (4) from a consideration of the Gibbs free energy change accompanying the transfer of solid material from a flat surface, having a solubility[1] C_o, to spherical particles of radius r, having a solubility C, where M and ρ are the molecular weight and density of the substance. The interfacial tension between the particles and their saturated solution is symbolized by γ.

$$\ln \frac{C}{C_o} = \frac{2\gamma M}{rRT\rho} \qquad \text{(Eq. 1)}$$

Since γ must be positive, $C > C_o$ and increases with the decreasing radius of the spherical phase. Thus a solution saturated with respect to large particles is not saturated with respect to small particles. In a mixture, small particles will dissolve and subsequently precipitate, causing the growth of larger particles. This is a general phenomenon independent of the specific chemical nature of the solute or the solvent. The larger solubility of the smaller particles is a consequence of a larger free energy of solution, $\Delta G_{sol.}$, which can be composed of an enthalpic, $\Delta H_{sol.}$, and an entropic, $\Delta S_{sol.}$, contribution. The difference in the free energies of solution from a spherical particle of radius r_2 and a spherical particle of radius r_1 can be written as:

$$\Delta(\Delta G_{sol.}) = \Delta(\Delta H_{sol.}) - T\Delta(\Delta S_{sol.}) \qquad \text{(Eq. 2)}$$

$$= \frac{2\gamma M}{r\rho}\left(\frac{1}{r_2} - \frac{1}{r_1}\right) \qquad \text{(Eq. 3)}$$

$$= RT \ln \frac{C_2}{C_1} \qquad \text{(Eq. 4)}$$

The specific purpose of the present study was to determine the relative contributions of entropy and enthalpy to the increased solubility of small particles.

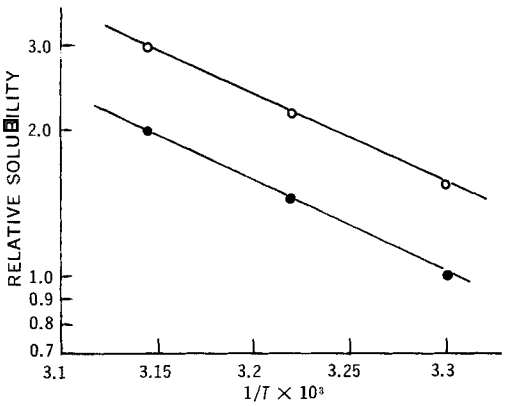

Figure 1—*Van't Hoff plot of the relative solubilities of crystalline* (●) *and microcrystalline* (○) *griseofulvin.*

[1] It is assumed that C and C_o are sufficiently similar that the ratio of activity coefficient which should appear in Eq. 1 is equal to unity.

Table I—Relative Solubilities of Griseofulvin in Water[a] at Various Temperatures

Temperature	Crystalline	Microcrystalline
29.5°	1.00	1.55
37.6°	1.45	2.15
44.7°	2.01	3.00

[a] Referred to the absorbance reading of crystalline griseofulvin at 29.5°.

EXPERIMENTAL

Griseofulvin[2] was chosen for this study because of its availability in two distinct particle-size ranges.

The solubility of griseofulvin, crystalline and microcrystalline, was determined at 29.5, 37.6, and 44.7 ± 0.1° by stirring 1.000 g. in 150 ml. of twice-distilled water until a constant absorbance reading was obtained on a Bausch and Lomb spectronic 505 spectrophotometer at 298.5 nm. The relationship between absorbance and concentration was obtained by diluting the saturated solution of highest absorbance and plotting absorbance *versus* the dilution factor. The linear relationship obtained eliminated the necessity of determining absolute concentration values.

RESULTS

The relative solubilities of crystalline and microcrystalline griseofulvin at different temperatures are shown in Table I. A Van't Hoff plot of these results is presented in Fig. 1. The slopes of the lines, $\Delta H_{sol.}/2.303R$, are equal. Therefore, the enthalpy of solution, having a value of 8.6 kcal./mole for griseofulvin, is independent of particle size and does not contribute to the increased solubility of the smaller particles. This has previously been postulated to be the case in general (4). The phenomenon may, therefore, be attributed to a comparatively greater entropy of solution for smaller particles as described by Eq. 5, where $\Delta(\Delta H_{sol.}) = 0$.

$$\Delta(\Delta S_{sol.}) = R \ln \frac{C_1}{C_2} \qquad \text{(Eq. 5)}$$

DISCUSSION

Once the substance is in solution, its kinetic microparticles, *i.e.* molecules or ions, have the same properties irrespective of the physical properties of the pure phase from which they derived. It is, therefore, reasonable to consider the difference in solution entropy as attributable to some fundamental and general difference in the process of removal of microparticles from a pure phase which depends upon its geometry and dimensions. The configurational entropy of R ln 2 (5), which a surface microparticle possesses relative

[2] Supplied by McNeil Labs, Fort Washington, PA 19034

Table II—Differences in Molar Entropies of Solution of Microcrystalline and Crystalline Griseofulvin Observed at Three Temperatures

Temperature	$\Delta(\Delta S_{sol.})$,[a] cal./degree/mole
29.5°	0.87
37.6°	0.78
44.7°	0.80
Average	0.82

[a] Calculated using Eqs. 5 and 7.

to the bulk solid, is independent of the geometry and dimensions of the solid and cannot be responsible for the phenomenon.

Consider a microparticle in a box and the fact that entropy is related to probability and can be envisaged as increasing with the randomness of a system. A system consisting of a microparticle in a box will have a maximum randomness when the probability of finding the particle in any volume element of the box at any instant is uniform. This would be the case when the resultant of all forces acting on the microparticle is zero. The microparticle in a box is analogous to a molecule or ion located in the interior of a pure phase, in which the symmetric forces to which it is subjected by its nearest neighbors cancel. If an asymmetric electrical field is acting upon a polar or polarizable microparticle, it would cause it to move in a preferred direction, tending to locate it in a preferred volume element. This is the case when the particle is at the surface of the phase, where there is an unbalance of forces, and the particle is not acted upon equally in all directions but has a preferential direction of motion and a preferred location within the confines of its allotted space. As a consequence, there is a diminished entropy due to a decrease in the space in which the particle is preferentially confined.

It can be seen from these considerations that ions and molecules dissolving from a surface enjoy a greater increase in entropy than those that hypothetically could be transferred from the interior of the material into the solution. However, in reality, only the surface microparticles dissolve, irrespective of the dimensions and geometry of the material from which they are derived. For the dissolution of n microparticles from the surface leaving m microparticles exposed in a new surface, the entropy change per mole of microparticles can be written as:

$$\Delta S_{n-m} = \Delta S_1 + \frac{m}{n}\Delta S_2 \qquad \text{(Eq. 6)}$$

where ΔS_1 is the entropy change per mole of microparticles dissolving from the surface and ΔS_2 is the negentropic change per mole of microparticles upon being exposed at the surface. Assuming that ΔS_1 and ΔS_2 are the same for both flat and spherical surfaces, it can be seen that the smaller the ratio of microparticles left exposed (m) to microparticles dissolving (n), the greater is the overall entropy of the dissolution process. The key to the problem then is the relative number of microparticles of decreased entropy that are exposed when a monolayer of surface microparticles dissolves.

For every microparticle removed from a flat surface, there is exposed another underlying microparticle, and the ratio of exposed to removed microparticles, m/n, is unity. However, the number of underlying microparticles exposed by the dissolution of n surface microparticles will decrease with decreasing radius. It is apparent that the molar overall entropy of solution from a small spherical surface will be greater than from a flat surface; therefore, the negative free energy change is greater.

The entropy difference accompanying the transfer of dN moles of solute to a pure phase having a spherical radius, r_2, in equilibrium with its saturated solution from a similar pure phase of radius, r_1, where $r_1 < r_2$, will be given by Eq. 7:

$$\Delta(\Delta S_{sol.}) = \Delta S_2\left(\frac{1}{N_1} - \frac{1}{N_2}\right) \qquad \text{(Eq. 7)}$$

The symbols N_2 and N_1 denote the number of moles of microparticles that are covered and exposed, respectively, at surfaces accompanying the transfer of 1 mole microparticles between the pure spherical phases. Assuming that γ, the interfacial tension, does not appreciably differ for the two pure phases in equilibrium with their saturated solution, multiplying Eq. 7 by T renders it equivalent to Eqs. 2–4. The values of $\Delta(\Delta S_{sol.})$ calculated for the crystalline and microcrystalline griseofulvin studied in the present experiment are listed in Table II. These values appear approximately constant.

CONCLUSIONS

Predictive experimental evidence has been obtained for implicating the entropy of solution as the dominant factor responsible for the increased solubility of small particles. Although the results apply strictly only to the systems studied, it may be expected that the conclusions reached in rendering these phenomena conceptually explicable in terms of geometry and probability are of general significance.

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The Dog as a Quantitative Model for Evaluation of Nondisintegrating Sustained-Release Tablets

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Abstract □ Beagle dogs were evaluated as a possible model for the preliminary evaluation of nondisintegrating sustained-release tablets. A quantitative comparison of the rate and extent of absorption of the drug from sustained- and nonsustained-release dosage forms in man and dog was obtained. The data suggest that the dog is a satisfactory model to screen dosage forms for possible human testing. The specific limitations of the model are discussed.

Keyphrases □ Dog, quantitative model—nondisintegrating sustained-release tablet evaluation □ Sustained-, nonsustained-release dosage form, rate, extent of absorption—studied, compared, man, dog □ Absorption, sustained-, nonsustained-release dosage forms—rate, extent studied in man, dog, comparison

It is an accepted practice to screen dosage forms of a new drug in animals, whose general physiology approaches that of man, before submission of an Investigational New Drug (IND) Application permits use of these dosage forms in humans. The literature contains little information comparing data on the rate and extent of drug absorption obtained in animals during the development of a dosage form to similar data obtained in humans. This is especially true when one looks for reports on the development of sustained-release dosage forms. Rosen *et al.* (1) reported the plasma levels of radioactivity obtained in man and beagle dogs following administration of dextroamphetamine-^{14}C. The compound was administered in a pelleted sustained-release form and as a t.i.d. dosage regimen in nonsustained-release pellets. From a comparison of the similarities in plasma levels and urinary excretion obtained in the two species, they concluded that dogs could be used to assist in the selection of pelleted sustained-release formulations worthy of objective human testing.

With any sustained-release dosage form, the production of a sustained, essentially unchanging, plasma level of drug is desired. The mechanism for attaining this condition is assumed to be the development of a steady state, in which the amount of drug absorbed per unit of time is essentially equivalent to the amount of drug eliminated in the same period of time. The condition might also be described as zero-order absorption and equivalent pseudo-zero-order elimination, where the product of the clearance rate for plasma and the steady-state plasma concentration are represented by a zero-order rate constant for elimination.

The relationship between the first-order rate constant for plasma loss and clearance rate is:

$$\text{clearance rate} = (k_e)(V_D) \qquad \text{(Eq. 1)}$$

where k_e is the first-order rate constant for plasma loss (elimination), and V_D is the apparent volume of distribution of the drug. Most pharmacokinetic models use weight of drug rather than concentrations. Therefore, for the case of a steady-state plasma or body-compartment level:

$$A \xrightarrow{k_0} B \xrightarrow{k_e} C \qquad \text{(Eq. 2)}$$

where A is the amount of drug in the gut, B is the amount of drug in the plasma or body compartment, C is the amount of drug eliminated, k_0 is a zero-order absorption-rate constant, and k_e is a first-order elimination-rate constant;

$$\frac{dB}{dt} = 0 = k_0 - k_e B \qquad \text{(Eq. 3)}$$

One can relate this to plasma concentration by

$$k_0 = k_e (V_D)(C_B) \qquad \text{(Eq. 4)}$$

where C_B is the plasma concentration of drug, and V_D is its apparent volume of distribution. Absorption under these conditions must equal the product of the elimination-rate constant (k_e) and the amount of drug in the plasma or the product of the clearance rate ($k_e \cdot V_D$) and the concentration of drug in the plasma, *i.e.*, C_B. The first-order rate constant obtained from a semilog plot of C_B *versus* t is k_e, since the equation used:

$$\log (C_B) = \frac{-k_e t}{2.303} + \log C_{B0} \qquad \text{(Eq. 5)}$$

can be rewritten as

$$\log B = \frac{-k_e t}{2.303} + \log B_0 \qquad \text{(Eq. 6)}$$

Table I—Plasma Levels of Radioactivity Obtained following Oral Administration of Various Dosage Forms of Aminorex Fumarate to Dogs

Hours	3.93 mg. Aminorex Base (as Fumarate Salt) in a Capsule	20-mg. Sustained-Release Tablet of Aminorex Base (as Fumarate Salt)[a]
0.5	6[b] (2)[c]	50 (8)
1	214 (11)	164 (6)
1.5	—	228 (11)
2	202 (19)	281 (10)
3	202 (35)	320 (22)
4	188 (33)	368 (22)
5	175 (40)	408 (54)
6	164 (29)	416 (50)
7	146 (33)	—
8	128 (35)	364 (60)
9	118 (28)	—
24	29 (17)	—
Average wt., kg.	7.5	12.1
No. of animals	2	3

[a] Dosage form H of *Reference 2*. [b] Values given as micrograms aminorex (base)/liter (± *SD*). [c] Included only to give indication of the range of values obtained.

This rearrangement affects the intercept of the line described by Eq. 5 but not the slope. The equation obtained, Eq. 6, is identical to that obtained on integration of the rate equation for Eq. 2 after absorption is complete.

It should be realized from this discussion that in any animal species, one could not, *a priori*, expect the plasma level profile obtained following administration of a sustained-release dosage form to be identical to the profile expected in humans. Even if one assumes equivalent absorption rates (since this would hopefully be determined by the dosage form), the rate constants for elimination would have to be similar in the animal and man in order to obtain similar plasma level profiles.

It was hoped that an animal model, specifically the purebred beagle dog, could be used as a rapid, concise means of evaluating sustained-release formulations of various drug entities. Dosage forms for *in vivo* screening would be selected on the basis of having dissolution characteristics (2) similar to those observed for sustained-release tablets of aminorex fumarate (*Reference 2*, dosage form H). The aminorex tablet had been shown (2) to produce prolonged, relatively stable plasma levels of total drug and satisfactory clinical response.

Because the aminorex dosage form had never been evaluated in dogs, the following question arose. Is the beagle dog an acceptable model for the evaluation of this type (3) of nondisintegrating sustained-release dosage form? To test the proposed animal model for its ability to mimic the absorption profiles observed for nondisintegrating sustained-release tablets in human subjects, this study was designed. In this study, the plasma-level profiles and sustained-release properties of tablets of aminorex fumarate (*Reference 2*, dosage form H) in beagle dogs are compared with results obtained several years ago in human subjects (2).

EXPERIMENTAL

Human Protocol (2)—Four to five subjects were each administered one 20-mg. sustained-release tablet of aminorex-^{14}C base (as the fumarate salt) (*Reference 2*, dosage form H), or 7.5 mg. of aminorex-^{14}C base (as the fumarate salt) dissolved in 200 ml. of water and followed by a 100-ml. water wash. The drug was administered before breakfast, following an overnight fast. No food was consumed for at least 2 hours after dosing, but water was permitted *ad lib.* Blood samples were withdrawn at time intervals dictated by the dosage form.

Animal Protocol—Three male, 1-year-old, purebred beagle dogs were each administered one 20-mg. sustained-release tablet of aminorex-^{14}C base (as the fumarate salt) (*Reference 2*, dosage form H). Two dogs were given capsules containing 3.93 mg. of aminorex-^{14}C base (as the fumarate salt). The same procedure as described under *Human Protocol* was followed.

Assay Method—The plasma samples were assayed by dissolving 100 μl. of plasma in a toluene scintillator containing 10% Beckman[1] Biosolve B. The samples were counted directly in a Beckman[1] LS 200 B ambient temperature scintillation spectrometer. Efficiencies were determined by the external standard method.

RESULTS

The plasma levels of radioactivity, expressed as their equivalent in micrograms per liter of aminorex, are given in Table I. The data in humans have been presented previously (2) but are repeated here for comparison. These data are plotted in Fig. 1.

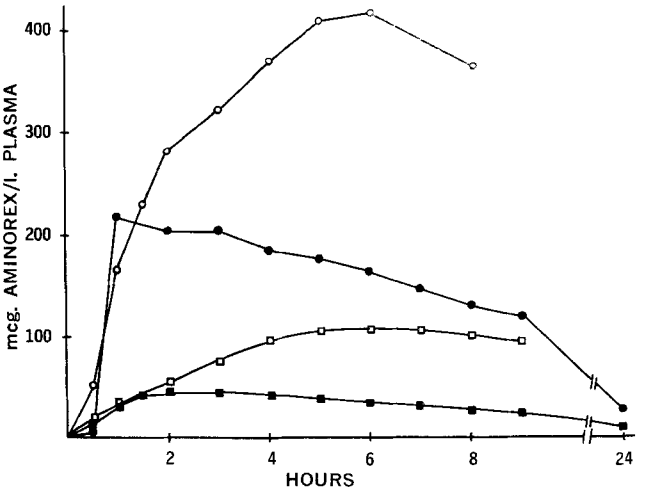

Figure 1—*Plasma levels of radioactivity, expressed as micrograms of aminorex, obtained following oral administration of aminorex fumarate. Key: ■, 7.5 mg. as a solution to human subjects; □, 20-mg. sustained-release tablet to human subjects; ●, 3.93-mg. capsule to dogs; and ○, 20-mg. sustained-release tablet to dogs.*

The data were evaluated using the method of Wagner and Nelson (4) and the approach reported previously (2). The use of plasma levels of total radioactivity instead of intact drug is compatible with the proposed model if the plasma loss of radioactivity is first order over the time period of interest. Application of this model (2) permits one to calculate the absorption rate of the drug from various dosage forms.

The plasma half-life of radioactivity in human subjects (2) was 7.7 hr. The plasma half-life of radioactivity in dogs was calculated from Fig. 2. The half-life, determined by linear-regression analysis, was 7.4 hr. By using these values for plasma half-lives, the absorption rates of the various dosage forms in human subjects and dogs could be calculated. The equation used to generate the data was:

$$A_t/V = k \int_0^t C_p\, dt + C_p \qquad \text{(Eq. 7)}$$

where A_t is the amount of the dose absorbed at time t, V is the apparent volume of distribution of the drug, k is the plasma elimination-rate constant for radioactivity, $\int_0^t C_p\, dt$ is the area under the

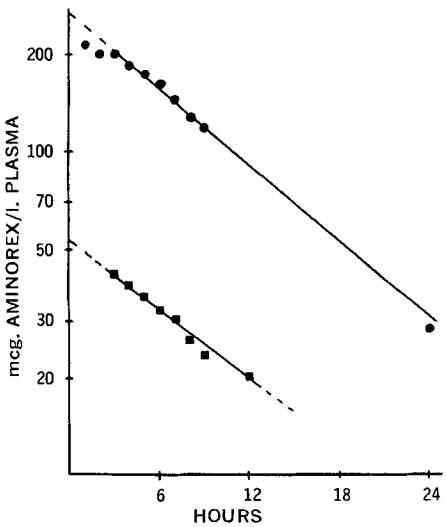

Figure 2—*First-order plot of plasma levels of radioactivity, expressed as micrograms of aminorex, obtained following oral administration of 7.5 mg. as a solution (■) to human subjects or 3.93 mg. in a capsule (●) to dogs.*

[1] Beckman Instruments Inc., Fullerton, CA 92634.

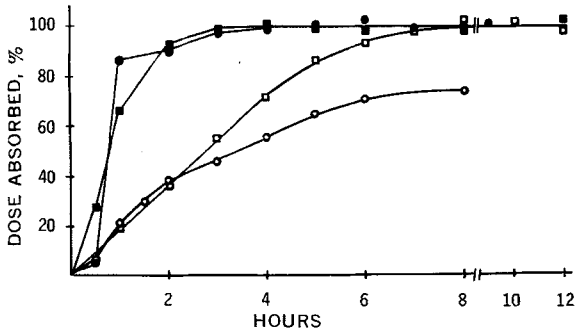

Figure 3—*Absorption of aminorex fumarate following administration to human subjects and to dogs. Key:* ■, *7.5 mg. as a solution to human subjects;* □, *20-mg. sustained-release tablet to human subjects;* ●, *3.93-mg. capsule to dogs; and* ○, *20-mg. sustained-release tablet to dogs.*

plasma level curve up to time t, and C_p is the plasma concentration at time t. The percent of the dose absorbed (Fig. 3) is calculated relative to the most readily available and (assumed) completely absorbed dosage form, in this case the solution or gelatin capsule of the drug.

DISCUSSION

From the data given in Fig. 2, it can be seen that the half-life of radioactivity for aminorex is very similar in dog and man. The values are 7.4 and 7.7 hr., respectively. The rate of absorption of aminorex fumarate (from readily available dosage forms) by man and dog is very similar (Fig. 3). A slight (15–30 min.) lag time in absorption is seen for the capsules in the dog. This lag is attributed to the time required for the capsule to disintegrate and release its contents.

Although the sustained-release tablets prolonged the absorption period of aminorex in both man and dog, the rates of absorption differed. The rates were equivalent for the first 2 hr. Following this time, absorption in man continued at a steady rate of ~18% of the dose/hour. The absorption by dogs did not continue at a steady rate but instead decreased. The data of Fig. 3 also indicate that the dog only absorbed 70–80% of the administered dose of aminorex from the sustained-release tablets. Human subjects absorbed 100% of the administered dosage form.

The reason for the reduced rate of absorption in dogs has not been established. However, in a subsequent experiment with nondisintegrating sustained-release tablets, an examination of the dogs' feces provided interesting information. In two of the four dogs used in the study, the tablet was expelled in the 6th hr. In the remaining two dogs, the tablets were found in the 8–12-hr. fecal sample. These results suggest that the dosage form may traverse the gut faster in dogs than in man. If, in the present study, rapid intestinal transit (6–8 hr.) occurred, this would account for the termination of absorption of aminorex at approximately 6–8 hr. A reduced rate of absorption or dissolution in the lower portions of the gut would account for the reduced absorption rate observed in the dog after the 2nd hr.

Although the rate and extent of absorption of aminorex were reduced in the dog, coincidentally the plasma level profile produced (Fig. 1) was not as divergent from man as might first appear. The plasma level curves obtained for the sustained-release tablet in man and dog are similar in their general shape. The rate of change in plasma level is amplified in the dog because of the much higher levels obtained. Such a result would not have been expected if the elimination constants in man and dog had not been similar.

CONCLUSIONS

The use of purebred beagle dogs as model animals for the preliminary evaluation of nondisintegrating sustained-release dosage forms would appear to be pharmacokinetically justifiable with certain limitations. The model should only be used to determine if a given dosage form has affected the rate of absorption, relative to some readily available dose, of the drug under study. The model should not be used to extrapolate the rate and/or extent of absorption observed to that expected in humans. Decisions as to the ability of the sustained-release product to provide satisfactory plasma levels in human subjects should not be made solely on the basis of the results of studies using the animal model. The authors have shown that both the rate and extent of absorption differed when the same dosage form was studied in the animal model and in man. The aminorex dosage form was 100% absorbed by human subjects; however, only 70–80% absorption was observed in the animal model. Clearly, incomplete absorption by the model system cannot be considered to reflect, with any accuracy, similar incomplete absorption in man.

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Thermolysis of 1,4-Benzodiazepines during Gas Chromatography and Mass Spectroscopy

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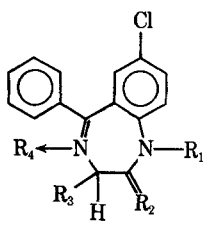
Abstract □ Diazepam (I), desmethyldiazepam (II), oxydiazepam (III), medazepam (IV), and oxazepam (V) were analyzed gas chromatographically using flame-ionization detection, electron-capture detection, and total ion-current detection (gas chromatograph connected to a mass spectrometer). 3-Hydroxy substitution of III and V induced a partial decomposition of III within the column, whereas V quantitatively rearranged to the quinazoline carboxaldehyde (X). This rearrangement occurred also under the conditions of electron impact. *N*-4-Oxides of benzodiazepines were thermally labile under the gas chromatography conditions. Thus, VII was converted partially to I by desoxygenation. The corresponding diazepam, 4,5-epoxide (XI), did not give the expected thermal conversion to the nitrone (VII), but rearranged to the 4-benzoylquinoxalinone (XII) as a major product.

Keyphrases □ 1,4-Benzodiazepines, thermolysis, rearrangement—during GC, mass spectroscopy analysis □ Thermolysis, 1,4-benzodiazepines—during GC, mass spectroscopy analysis □ Gas chromatography—thermolysis, rearrangement of 1,4-benzodiazepines, on column □ Mass spectroscopy—analysis

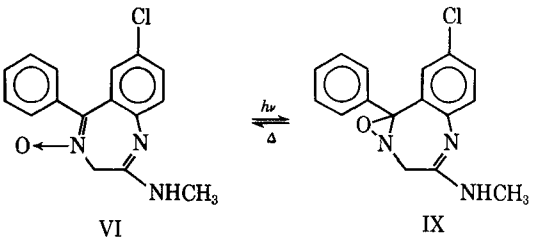
The group of 1,4-benzodiazepines is widely used as ataractics, hypnotics, central muscle relaxants, and anticonvulsants. Sensitive assays have been evaluated using gas chromatographic detection of these compounds in extracts of biological fluids. Primarily, the methods were based on hydrolysis to 2-aminobenzophenones (1–3). However, in recent publications it was shown that the intact benzodiazepines can also be separated by gas chromatography (GC) with reproducible results (4, 5). The detection in the low nanogram range was made possible using the ^{63}Ni-electron-capture detector (ECD).

Equimolar amounts of diazepam (I), desmethyldiazepam (II), oxydiazepam (III), and medazepam (IV) do not yield equal responses using flame-ionization detection (FID) and total ion-current detection (TICD). Preliminary investigations suggested that rearrangements of the diazepine ring can occur at the GC temperatures. Oxazepam (V) appeared in the GC spectrum between I and IV with the same retention time as desmethylmedazepam, although it possessed the highest polarity and should give the longest retention time. Chlordiazepoxide (VI), as well as the *N*-4-oxides of



	R_1	R_2	R_3	R_4
I	CH_3	O	H	-
II	H	O	H	-
III	CH_3	O	OH	-
IV	CH_3	H_2	H	-
V	H	O	OH	-
VII	CH_3	O	H	O
VIII	H	O	H	O

structures of benzodiazepines



VI IX

Scheme I

diazepam (VII) and of desmethyldiazepam (VIII), proved to be thermally labile. Compound VIII represents a major metabolite of VI. These nitrones were reported to be in photolytic and thermolytic equilibrium with the corresponding oxaziranes (*e.g.*, IX) (Scheme I) (6–8). The oxazirane–nitrone relation under the conditions of electron impact was discussed recently (9).

It is conceivable that there will be a correlation between the thermolysis during GC and some of the fragmentations observed during mass spectrometry (MS). The problem was approached by comparing mass spectra of directly inserted samples to mass spectra of samples subjected to GC analysis prior to MS. A GC–MS combination proved to be especially valuable for the present investigation.

MATERIALS AND METHODS

Mass spectra with high resolution (direct insertion) were taken on a MS-9, using the peak matching technique. The Finnigan system was used for the GC–MS analysis (a Varian GC model 1700 connected to a Finnigan quadrupole model 1015 with a Gohlke separator). The D/A model 150 data acquisition system (Digital Equipment Corp.) was used. The mass spectra were taken at 70 ev., 450 amp. ionization current, and the column bleeding spectra were automatically substracted. Mass ranges of 50–200, 200–300, and 300–400 were taken with a relative integration time of 1, 4, and 16. Mass spectra were normalized to the largest peak (=100). The resolution of the quadrupole instrument was about 500. The reconstructed gas chromatograms were based on the registration of the TICD in the MS. In some cases, a limited mass range was selected to get a clear gas chromatogram (*m/e* 250–310), which was normalized to the largest peak (=100).

GC Conditions—*TICD*—The following were used: glass columns, 0.9 m. × 0.32 cm. (3 ft. × 0.125 in.), 1.5% OV-1 on HP chrom G; temperature, 200–280°, linearly programmed; and carrier gas helium, 20 ml./min.

FID—The following were used: Varian hi-fi III series 1200; ss column, 1.8 m. × 0.32 cm. (6 ft. × 0.125 in.), 3% OV-17 on gas chrom W (AW); temperature, 245°; and carrier gas, nitrogen (70 ml./min.), hydrogen (40 ml./min.), and oxygen (700 ml./min.).

ECD—The following were used: Varian hi-fi III series 1200; ss column, 1.8 m. × 0.32 cm. (6 ft. × 0.125 in.), 3% OV-17 on gas chrom W (AW); temperature, 245°; and carrier gas, argon–methane (5%), 70 ml./min. The compounds were prepared by known methods or were provided.[1]

[1] By Hoffmann-La Roche Inc., N. J. (Compound X by Dr. L. H. Sternbach and Compounds XI and XIII by Dr. R. Y. Ning), and Wyeth Labs., Inc. (Compound XIV by Dr. S. C. Bell).

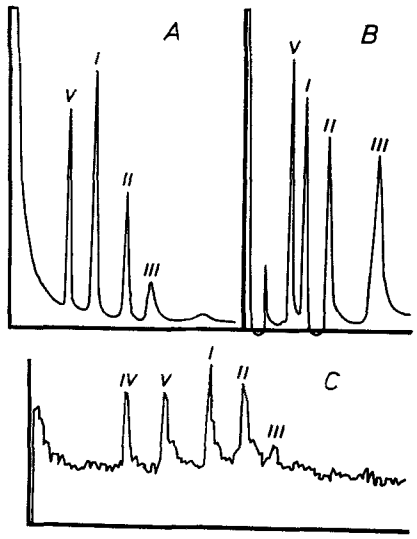


Figure 1—*Gas chromatograms of an equimolar mixture of I–V. Key: A, FID (8 × 10⁻⁹ M); B, ECD (16 × 10⁻¹¹ M); and C, TICD (8 × 10⁻⁹ M).*

RESULTS AND DISCUSSION

Figures 1 A, B, and C show the gas chromatograms of I–III, V, and, in one case (C), of IV, using FID, ECD, and TICD. Compound III gave only about 20% of the expected peak area with FID and TICD, whereas the response was about equal to the II area with ECD. This suggests a partial decomposition of III and an increased sensitivity to ECD.

The retention time of V was shorter than expected on the basis of its relative polar properties. It is conceivable that under the GC conditions, V will rearrange in a high yield to a product of lower polarity to give a well-resolved peak with a relatively short retention time. Indeed, the mass spectrum demonstrated that a thermolytic rearrangement occurred by loss of water.

Figure 2 shows the mass spectrum of oxazepam (V) and of the reaction product to which the structure of the known quinazoline carboxaldehyde (X) was assigned. An equivalent reaction was shown to occur in solution upon alkali treatment (10, 11). The authentic sample of X proved to give an identical retention time in the gas chromatogram and the same mass spectrum as the rearrangement product of V. The yield of X was practically 100%. The mechanism of the thermolysis can be described as shown in Scheme II.

The proposed mechanism is supported by the mass spectrum

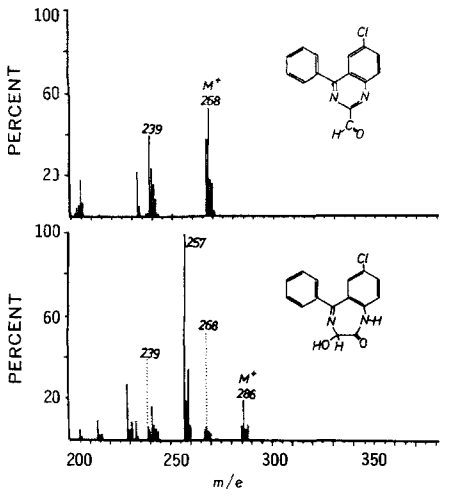


Figure 2—*Mass spectra of the quinazoline carboxaldehyde (X) (mol. wt. 268) and of oxazepam (V) (mol. wt. 286) on the MS-9 mass spectrometer (direct insertion).*

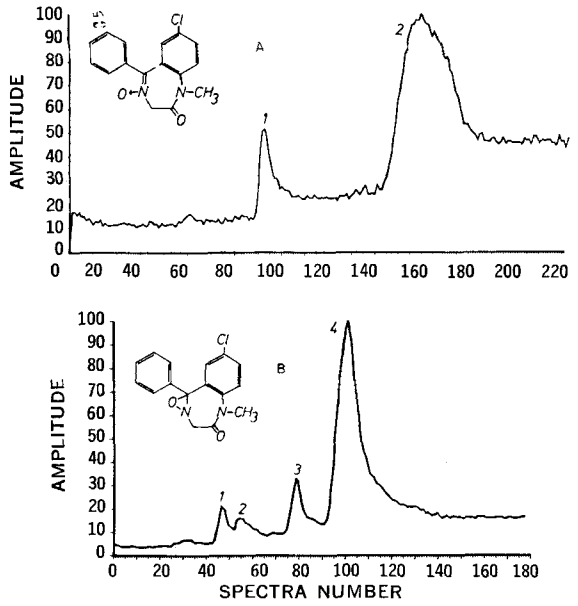
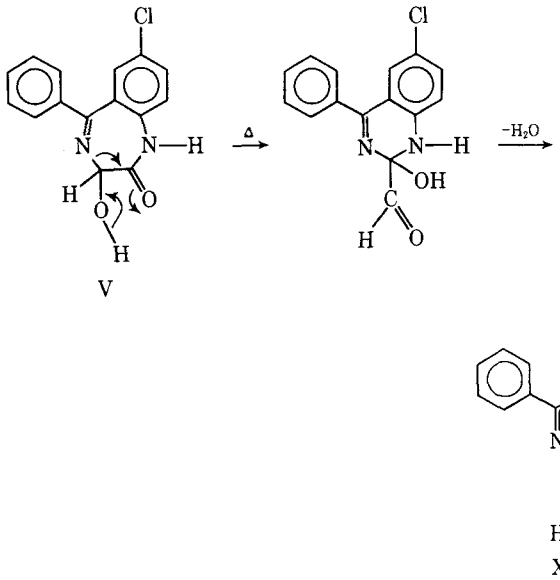


Figure 3—*Reconstructed gas chromatograms (TICD) of VII (A) and XI (B).*

of III, with the 0–18 label in position 3 (12). The peak at M^+-18 (loss of H_2O) is derived from the loss of the carbonyl oxygen rather than from the loss of the 3-OH-oxygen.

It can be postulated that the peaks at M^+-18 (*m/e* 268) as well as *m/e* 239 (M^+-H_2O, HCO) in the mass spectrum of V resulted from an initial thermolytic rearrangement to X and not from electron impact.

Another thermolytic and photolytic equilibrium was found with the nitrones (*e.g.*, VII) and oxaziranes (*e.g.*, XI) of benzodiazepines (6–8). Under the conditions of electron impact, the nitrones were reported to rearrange to oxaziranes and further to quinoxalinones such as XII (9). The same reaction occurred upon prolonged irradiation of oxaziranes (2, 4). Treatment of benzodiazepine *N*-4-oxides with $POCl_3$ also led to quinoxalinones (11). Therefore, VII and the corresponding oxazirane XI were analyzed with the GC–MS combination. Figure 3A shows the gas chromatogram of VII. The two peaks were readily identified as I and VII. Loss of oxygen was also observed during MS (9), and it appears that this reaction is at least partially thermolytic. No thermal rearrangement of the nitrone (VII) to the oxazirane (XI) could be detected.



Scheme II

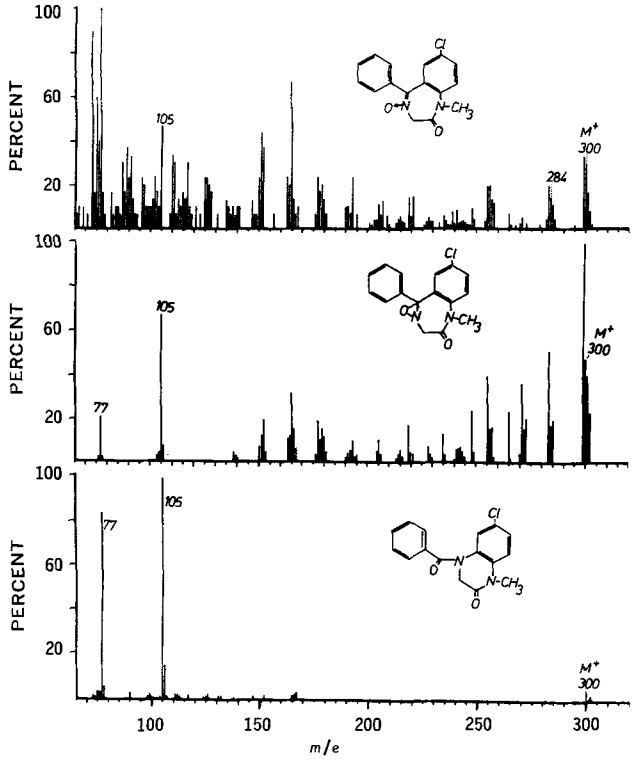
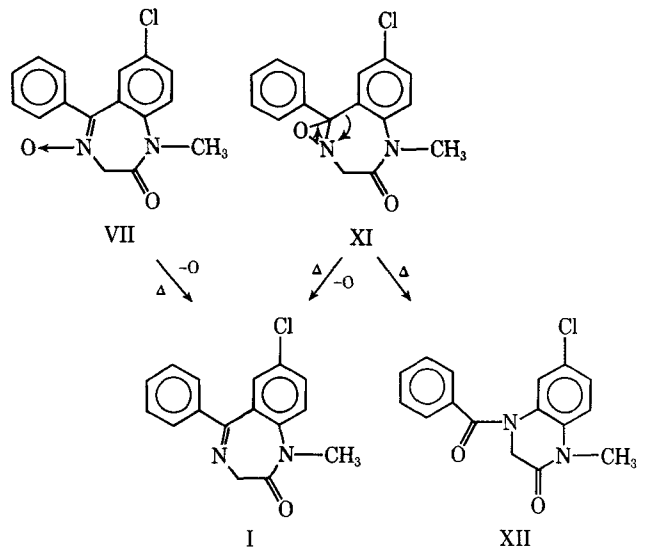


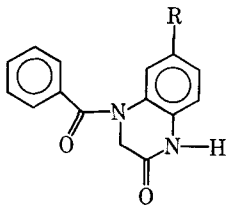
Figure 4—*Mass spectra of: A, VII (Finnigan system); B, XI (MS-9, direct insertion); and C, XII (Finnigan system).*

The gas chromatogram of the oxazirane (XI) proved to be more complex (TICD, Fig. 3B). At least four peaks were detected, of which 1 and 2 were not identified. Again, one peak was found to be I (3, ~10%), while the larger peak (4, ~85%) gave a mass spectrum with major fragments at *m/e* 51, 77, and 105 and a very small molecular ion peak at *m/e* 300. This suggested the formation of the 4-benzoylquinoxalinone (XII) in a Beckmann-type rearrangement (Scheme III).



Scheme III

The peak at *m/e* 105, which stands for the benzoyl ion, increased in relative abundance in the spectra of the nitrone (VII) to the oxazirane (XI) and to the 4-benzoylquinoxalinone (XII) (Fig. 4). This pattern supported the recently postulated rearrangement to quinoxalinones under electron impact, which was based on the presence of the peak at *m/e* 105 (9). This peak was present only in the spectrum of the nitrones and not in the spectrum of *N*-4-unsubstituted benzodiazepines. The mass spectra of the quinoxalinones (XIII and XIV) proved to possess the same fragmentations as XII. The thermolytic rearrangement of the benzodiazepin-2-one



XIII; R = Cl
XIV; R = H

4,5-epoxide (XI) was initiated by N—O bond fission. This is in contrast to the known formation of the nitrone (VI) from the corresponding oxazirane (IX) upon heating (7), which implied a C—O bond fission.

CONCLUSIONS

The benzodiazepines I, II, and IV and desmethylmedazepam were found to be thermally stable as judged by the mass spectra. 3-Hydroxy derivatives, *N*-4-oxides, and the corresponding 4,5-epoxides reacted by desoxygenation or by rearrangements to rather stable 1,3-quinazolines and quinoxalines at the GC temperatures. The formation of quinoxalines from *N*-4-oxides during MS is presumably only a result of electron impact, since this rearrangement did not occur at the GC conditions.

For the GC analysis of the nitrone (VII), the reaction of VII with acetic anhydride would result in a high yield to a 3-acetoxy benzodiazepine in a Polonovski-type rearrangement (13) which proved stable at the GC temperatures.

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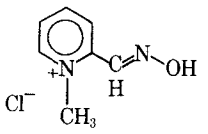
Aryl-*N*-methyl-2-pyridiniumketoxime Acetylcholinesterase Reactivators

C. F. BARFKNECHT, J. P. LONG, and F. W. BENZ

Abstract □ *syn*- and *anti*-Phenyl-*N*-methyl-2-pyridiniumketoximes (II and III) were synthesized as reactivators of organophosphate-inhibited acetylcholinesterase, and the *anti*>*syn* potency relationship was confirmed. 2′-Pyridyl-*N*-methyl-2-pyridiniumketoxime (IV) was synthesized and found to be more potent than II or III and equipotent with *syn*-4-pyridiniumaldoxime methochloride. IV was more potent than II but less potent than III as an inhibitor of acetylcholinesterase. NMR evidence indicates that the phenyl groups of II and III are in different conformations.

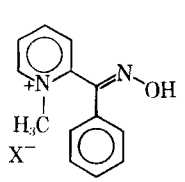
Keyphrases □ Phenyl-*N*-methyl-2-pyridiniumketoximes (*syn* and *anti*)—acetylcholinesterase reactivators, synthesis, potency relationship □ Acetylcholinesterase reactivators—synthesis, potency relationship, *syn*- *anti*-phenyl-*N*-methyl-2-pyridiniumketoximes □ NMR spectroscopy—structure

The standard of comparison for compounds intended as acetylcholinesterase (AChE) reactivators is 2-pyridiniumaldoxime methochloride (2-PAM) (I). Some controversy has existed about which configurational isomer (*syn* or *anti*) is more potent, but there is conjecture that the *syn* is more potent and is the isomer sold as an antidote for AChE inhibition (1–3). It has been proposed that in *syn*-I, the oxime hydroxyl group is positioned to attack the phosphorylated (or carbamylated) esteratic site when the pyridinium nitrogen binds to the anionic site of AChE.



I

In the study of other pyridinium-2-oximes, one might expect the configurational isomers corresponding to *syn*-2-PAM to be more potent than their *anti*-isomers. It must be assumed that these pyridinium-2-oximes can attain the same conformation as *syn*-2-PAM when interacting with AChE. Kitz *et al.* (4) reported the synthesis and *in vitro* biochemical evaluation of *syn*- and *anti*-phenyl-*N*-methyl-2-pyridiniumketoxime iodides (II and III, respectively). Their data showed the *anti*-isomer (III) to be 20 times more potent than the *syn* (II) and approximately equipotent to *syn*-4-PAM (Table I). These results are the opposite to what is expected for 2-PAM-like compounds. Examination of molecular models suggests difficulty for II and III to attain some conformations of I and thus supports the results obtained by Kitz *et al.* (4). Efforts to verify their data and extend the series to the 2-pyridyl analog (IV) are reported in this communication.



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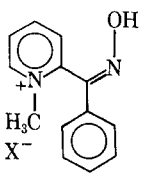
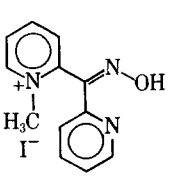


III



IV

Table I—Biochemical Data of Aryl-*N*-methyl-2-pyridiniumketoximes

Compound	ID_{50} (*M*)	k_2, l./mole/min.	Relative Potency (95% Fiducial Limits)
2-PAM	8.1×10^{-4}	3.3×10^{4}[a]	1.00
syn-4-PAM	16.4×10^{-4}	4.9×10^{2}	1.4×10^{-2} (1.2–1.7)
II Iodide	4.3×10^{-4}	2.9×10^{1}	9.4×10^{-4}[b] (8.8–10.0)
II Bromide	4.4×10^{-4}	3.0×10^{1}	9.9×10^{-4}[b] (9.5–10.3)
III Bromide	1.2×10^{-4}	2.6×10^{2}	8.1×10^{-3} (6.8–9.8)
IV Bromide	2.3×10^{-4}	6.8×10^{2}	2.0×10^{-2} (1.8–2.3)

[a] Mean value of 24 determinations. [b] Relative rate calculated by dividing k_2 test/k_2 standard in *paired* experiments.

RESULTS

The phenyl-2-pyridylketoximes (*syn* and *anti*) were synthesized and separated by the method of Huntress and Walker (5). Completeness of separation was demonstrated by TLC. The correctness of structural assignments, made by classical methods, was confirmed by the NMR spectra. While Kitz *et al.* (4) had succeeded in quaternizing both isomers utilizing methyl iodide, in the present study only II could be prepared. Both II and III were synthesized by reaction with methyl bromide in a sealed tube, demonstrating that configurational isomerization does not occur under these conditions. The NMR data (Table II) are consistent with the configurational assignments (6).

2,2′-Dipyridylketoxime was readily prepared by standard methods and easily quaternized with methyl iodide to form IV. All attempts to form the diquaternary compound (*N*-methyl-*N*′-methyl) with methyl iodide failed. A sealed-tube reaction with methyl bromide also yielded the monoquaternary compound. The configurational assignment of IV (*syn*-2′-pyridyl) is based upon the inability to diquaternize with methyl iodide, the NMR data, and examination of molecular models which indicated that an interaction between the oxime hydroxyl and the 2′-pyridyl nitrogen is likely.

The pH stat method was used for the biochemical evaluation of the compounds. Diethylphosphoryl thiocholine-inhibited acetylcholinesterase from the electric eel was used for the reactivation experiments. Detailed methodology appears in the *Experimental* section. Results of the reactivation and inhibition experiments are summarized in Table I.

DISCUSSION

The reactivation data demonstrate that in aryl-*N*-methyl-2-pyridiniumketoximes, the *anti*-phenyl isomer is more potent than the *syn*-phenyl isomer and thus *anti*-2-PAM may be more active than *syn*-2-PAM. This is the conclusion reached by Kitz *et al.* (4), and the present authors are now in full agreement with it. The inhibition data reinforce the notion that the phenyl group of II and III is interacting with AChE in a detrimental fashion. That the

phenyls of II and III are not in the same conformation is demonstrated by the NMR spectra. The protons of the phenyl group of II are observed as a multiplet, while the protons of the III phenyl group are observed as a singlet. To be observed as a singlet, all protons must be influenced equally by the remainder of the molecule. This could be accomplished by rotating the phenyl ring of III out-of-plane. The phenyl protons of both *syn*- and *anti*-phenyl-2-pyridylketoxime are observed as a singlet. From examination of space-filling models of these compounds, it is clear that free rotation of the phenyl groups is possible. It is not clear why, when quaternized, the conformation of the phenyl groups of II and III should differ.

That IV interacts differently than II and III is established by the reactivation and inhibition data. Compound IV exhibits higher reactivation than II and III and higher inhibition than II but lower inhibition than III. Compound IV's increased potency as a reactivator is undoubtedly linked to its favorable pKa. Whether the conformations of I and IV are identical cannot be determined from the available information. The 2′-pyridyl-2-pyridiniumketoxime group (present in IV) warrants further investigation with diquaternary systems that should enhance its reactivator potency.

EXPERIMENTAL

Phenyl-2-pyridylketoximes—The method of Huntress and Walker (5) was used for the synthesis and separation of the isomers. *syn*-Phenyl-2-pyridylketoxime (m.p. 151–152°) and *anti*-phenyl-2-pyridylketoxime (m.p. 166–167°) were used.

***syn*-Phenyl-*N*-methyl-2-pyridiniumketoxime Iodide and Bromide (II)**—The method of Kitz *et al.* (4) was followed for the preparation of the II iodide, m.p. 206–208° (lit. m.p. 212–213°).

The II bromide was synthesized by reacting the phenyl-2-pyridylketoxime, dissolved in the minimum isopropyl alcohol, with a slight excess of methyl bromide in a sealed tube on a steam bath for 2 days. The product was obtained in near quantitative yield and recrystallized from absolute ethanol–ethyl acetate, m.p. 212–214°.

Anal.—Calcd. for $C_{13}H_{13}BrN_2O$: C, 53.44; H, 4.11; N, 9.59. Found: C, 53.60; H, 4.01; N, 9.67.

***anti*-Phenyl-*N*-methyl-2-pyridiniumketoxime Bromide (III)**—The procedure employed for the synthesis of the II bromide was used. A nearly quantitative yield of product, m.p. 190–193°, was obtained (absolute ethanol–ethyl acetate).

Anal.—Calcd. for $C_{13}H_{13}BrN_2O$: C, 53.44; H, 4.11; N, 9.59. Found: C, 53.32; H, 3.99; N, 9.72.

2,2′-Dipyridylketoxime—The procedure of Case and McMenamin (7) was employed; yield 83%, m.p. 141–143° [lit. (8) m.p. 141–143°].

2′-Pyridyl-*N*-methyl-2-pyridiniumketoxime Iodide (IV)—A nearly quantitative yield of IV was obtained by stirring the 2,2′-dipyridylketoxime with a slight molar excess of methyl iodide in anhydrous diethyl ether for 2 hr. at room temperature, m.p. 194–196° (ethanol–ethyl acetate).

Anal.—Calcd. for $C_{12}H_{12}IN_3O$: C, 42.40; H, 3.52; N, 12.34. Found: C, 42.16; H, 3.51; N, 12.27.

Enzyme—Acetylcholinesterase [acetylcholine, acetylhydrolase (3·1·1·7)] from the electric eel was used.[1] The enzyme (0.58 mg. = 1000 μM units) was dissolved in 5 ml. of 1% bovine albumin[1] and stored frozen. It had a specific activity of 2.5 mmole acetylcholine hydrolyzed per min./mg.

Inhibition—The enzyme was inhibited by reacting 0.1 ml. (20 μM units) of the enzyme solution with 0.1 ml. (2×10^{-6} M) diethylphosphoryl thiocholine iodide at pH 7.0 and 20°. After 2 hr. the inhibited enzyme was diluted to 4 μM units/ml. with a solution containing 0.03 M NaCl and 0.02 M $MgCl_2$ and was stored at 4°. This stock solution of inhibited enzyme could be used in reactivation experiments for 2–3 days without loss of activity.

Reactivation—The reactivation was carried out at 37° at pH 7.4 in a medium consisting of 20 ml. of 0.03 M NaCl and 0.02 M $MgCl_2$. The final concentrations of enzyme and acetylcholine were 8 μM units/l. and 10^{-3} M, respectively. A Radiometer titrator-type TTT1d with SBR2c recorder and syringe buret SBU1a were used to monitor the reaction rate.

Pseudo-first-order rate constants, k, were calculated from a plot of $\log E_\infty - E_t$ *versus* t, where E_∞ is the total amount of enzyme available and E_t is the total amount of enzyme at any time t. A linear relationship between velocity of hydrolysis (v) and enzyme concentration (E) was verified. The first-order rate constants were proportional to the reactivator concentrations, revealing that the reactivations were bimolecular and that the reactivator concentrations were less than the dissociation constants for the intermediate complexes. Relative potencies were calculated by a 2×2 parallel line bioassay (9). First-order rate constants for two concentrations of the test reactivator were compared with first-order rate constants for two concentrations of the standard reactivator, 2-PAM. All criteria for a valid bioassay were met. In those cases where a 2×2 bioassay was unfeasible, because the reactivator acted as a reversible inhibitor of the enzyme during the reactivation, a relative rate of reactivation as compared to 2-PAM was calculated by the formula, relative rate = k_2 test/k_2 2-PAM, where k_2 is the second-order rate constant obtained by dividing k/[R]. Relative rates are identical to relative potencies if the plots of k *versus* [R] for the test reactivator and the standard reactivator, 2-PAM, are parallel.

ID_{50} Determinations—The ID_{50} was determined from plots of percent inhibition *versus* log inhibitor concentration. At least three concentrations between 30 and 70% inhibition were used.

[1] Obtained from Sigma Chemical Co., St. Louis, Mo.

Table II—NMR and pKa Data on Aryl-*N*-methyl-2-pyridiniumketoximes[a]

Configuration		Aryl Group	Oxime Hydroxyl	*N*-Methyl Group	pKa[b]
II Iodide	*syn*	Phenyl 7.62	13.05	4.23	8.9
II Bromide	*syn*	Phenyl 7.62	13.08	4.22	—
III Bromide	*anti*	Phenyl 7.50[c]	12.83	4.23	8.7
IV Iodide	*syn*	2-Pyridyl-	13.26	4.23	7.9

[a] All samples were run in DMSO-d_6 with tetramethylsilane as the internal standard; chemical shifts are expressed in parts per million. [b] Determined by potentiometric titrations; literature value for 2-PAM (pKa = 8.0) was duplicated. [c] All aryl groups exhibited a complex multiplet except III; both *syn*- and *anti*-phenyl-2-pyridylketoximes showed a singlet for the phenyl group at 7.33 and 7.42 p.p.m., respectively.

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Further Investigation of Reserpine and Deoxycholic Acid Potentiation

LOUIS De CATO, Jr.*, ROGER G. STOLL†, THEODORE R. BATES†, and MARVIN H. MALONE*

Abstract □ Intravenous administration of reserpine acetate and oral deoxycholic acid showed no increase in blepharoptotic potency relative to intravenous reserpine acetate alone. Since this experiment eliminates physicochemical interactions within the gastrointestinal tract, it suggests that the potentiation of reserpine taken orally as reserpine–bile acid coprecipitates is by physicochemical rather than by physiopharmacological means.

Keyphrases □ Reserpine–deoxycholic acid—potentiation □ Potentiation—reserpine by deoxycholic acid □ Interactions—possible potentiation of reserpine acetate by deoxycholic acid

The oral administration of reserpine as a deoxycholic acid coprecipitate was shown by Malone *et al.* (1) to increase its relative blepharoptotic potency as well as to hasten its onset of action and prolong its duration. This phenomenon was also seen using coprecipitates of reserpine with other cholanic acid derivatives (2). No attempt was made by these authors to elucidate the mechanism of action for this potentiation.

Gibaldi *et al.* (3) studied the dissolution properties of varying molar ratios of reserpine–deoxycholic acid coprecipitates and attributed the correlation of increased dissolution rates with the *in vivo* ptotic activities to a decrease in the particle size of the administered reserpine. Recently, Stoll *et al.* (4) studied such mixtures in regard to micellar solubilization, reduction of interfacial tension, and particle size, all of which could increase the dissolution rate of the insoluble reserpine particles. Surface-tension determinations of the coprecipitates in a pH 6.4 buffer eliminated micellar formation as a factor, since they did not exhibit a break in the surface tension *versus* concentration curve typical of the CMC for micelle formation. There was, however, a qualitative relationship between the compounds' abilities to lower interfacial tension and to potentiate ptotic activity. A near rank correlation was found for increased dissolution rates of the coprecipitates and the 4–10-hr. postadministration rise in potency. This was felt to be due to the decreased particle size achieved during the coprecipitation procedure and the surface tension-lowering abilities of the bile acids which facilitate wetting of the insoluble reserpine particles. Possible physiological effects of the cholanic acid derivatives such as changes in gastric motility and emptying, competition for protein binding sites, changes in membrane permeability, and involvement with enterohepatic circulation were considered by these authors to play a minor role, if any, in the potentiation phenomena, since a physical mixture of reserpine with deoxycholic acid was shown to produce only a slight rise in ptotic activity compared to the coprecipitates (1). Physiological factors, however, were not eliminated.

The present paper investigates the effect of intravenous reserpine after the oral dosing of deoxycholic acid. This experiment should eliminate physicochemical interactions within the gastrointestinal tract but not affect any physiopharmacologic mechanisms.

Table I—Apparent Intravenous Potencies of Reserpine Acetate in Mice Receiving Orally Administered Deoxycholic Acid Relative to Intravenous Reserpine Acetate in Mice Receiving Dosage Vehicle Only Orally

Hours after Dosage				
2	4	6	10	24
1.01[a] (0.80–1.28)	0.98 (0.82–1.22)	1.02 (0.83–1.22)	1.11 (0.86–1.18)	1.62 (0.45–2.22)

[a] The range of figures within parentheses indicates the calculated 95% confidence limits for the listed potency.

EXPERIMENTAL

Materials—Deoxycholic acid[1] was suspended for dosing in 0.25% agar.[2] Reserpine acetate was prepared by solubilizing reserpine[1] in glacial acetic acid and diluting with distilled water to a final acetic acid concentration of 0.029%.

Assay Procedure—Male albino mice[3] (15–26 g.) were maintained in this laboratory on Purina laboratory chow and tap water *ad libitum* for at least 1 week prior to testing. The animals were removed from food 18 hr. before dosing and placed back on food after the +6-hr. ptotic readings had been completed. At −1 hr. the test mice were dosed orally with the deoxycholic acid suspension (30 ml./kg.), while the reference animals received only 0.25% agar vehicle (30 ml./kg.). This was followed at 0 hr. with a 20-sec. intravenous injection of reserpine acetate (10 ml./kg.). The intravenous dosages used for the 3 × 3 assay (0.125, 0.25, and 0.50 mg./kg., calculated as reserpine base) are ptotically equivalent to the oral dosages of reserpine base (3.0, 6.0, and 12.0 mg./kg.) originally used (1) to document the reserpine–deoxycholic acid potentiation. The oral deoxycholic acid dosages used in the present study (20.7, 41.5, and 82.9 mg./kg.) correspond to the dosages used in the original studies with the coprecipitates. Therefore, the 1:257 *M* ratio of reserpine to deoxycholic acid in this study should be pharmacologically equivalent to the 1:16 *M* ratio in the coprecipitate studies (1, 2). In all other respects, the blepharoptotic assays were carried out exactly as described by Malone *et al.* (1).

RESULTS AND DISCUSSION

Reserpine had to be administered intravenously as the acetate salt due to its low solubility in aqueous media. It was shown previously that acetate salt formation does not significantly alter the slope of oral dose–response curves as compared to reserpine base (2). The statistical treatment for the 3 × 3 assays (132 mice, 22 animals/dosage level) involved analysis of variance, factorial analysis, and calculation of potency with its 95% confidence limits as described by Bliss and Calhoun (5).

As illustrated in Table I, the intravenous potencies of reserpine acetate in combination with oral deoxycholic acid when compared to intravenous reserpine acetate as a standard ranged from 0.98 to 1.11 for the first 10 hr. of the experiment. The average λ value (s/b) for these four assays is 0.25, which agrees well with the assay char-

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Difco Laboratories, Detroit, Mich.
[3] Obtained from E. G. Steinhilber Inc., Oshkosh, Wis.

acteristics of 0.27 and 0.28 reported earlier (1, 2). Waning of ptotic activity at +24 hr. decreased the slope of the dose–response curves, causing a wide range in the 95% confidence limits of the potency and an increase in the calculated λ value (0.96) for this assay. From these results, it can be seen that the potency of the reserpine–deoxycholic acid combination was equivalent to 1.0 over the entire time-course spectra of activity.

To detect if there was any acceleration of activity, ptotic readings were also collected at the +1-hr. interval. However, because of the characteristic slowness of the reserpine response, ptotic activity was seen only with the top doses of both the test and standard treatments. The mean responses were equivalent. Controls receiving a 20-sec. intravenous injection of 0.029% acetic acid, with and without orally administered deoxycholic acid, were without ptotic activity.

The absence of reserpine potentiation with the concomitant administration of reserpine acetate and deoxycholic acid by different routes lends further support to the theory that the mechanism of potentiation with orally administered reserpine–bile acid coprecipitates is by physicochemical rather than by pharmacological means. A physical mixture of 1:16 *M* reserpine and deoxycholic acid was shown to cause a slight but significant rise in potency (maximum of 1.8×) when given orally (1). Although such a mixture does not allow the intimate combination of ingredients found in a coprecipitate and does preclude reduction in particle size during precipitation, there still exists the possibility of local surface-tension lowering by the bile acids. As both drugs enter the circulation in the present experiment, actual physical contact in the gastrointestinal tract is eliminated, yet any true pharmacologic potentiation should occur. The only chance for physical interaction in the gastrointestinal tract is the possibility of enterohepatic excretion of the intravenous reserpine into the intestine. The absence of potentiation, therefore, also precludes an enterohepatic recirculation phenomenon as a potentiation mechanism for the reserpine–deoxycholic acid coprecipitates.

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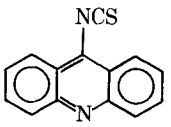
Fluorescent Analysis of Primary Aliphatic Amines by Reaction with 9-Isothiocyanatoacridine

J. E. SINSHEIMER, D. D. HONG*, J. T. STEWART†, M. L. FINK‡, and J. H. BURCKHALTER

Abstract □ Primary aliphatic amines are reacted with 9-isothiocyanatoacridine to yield the corresponding thiourea derivatives. These derivatives, after reaction in base, yield new compounds of intense fluorescence. Conditions have been developed so that it is possible to relate the fluorescence of the final reaction mixture to the concentration of original amine or amine salts without separation of products, even in the presence of excess fluorescent reagent. This method of analysis is successful at the microgram level and can be extended to the nanogram level by TLC separation and direct TLC fluorimetry of the reaction product.

Keyphrases □ 9-Isothiocyanatoacridine reagent—fluorescent analysis, primary aliphatic amines □ Amines, primary aliphatic—fluorescent analysis using 9-isothiocyanatoacridine □ TLC fluorimetry—analysis

These laboratories are interested in the development and evaluation of fluorescent isothiocyanate compounds as new fluorescent protein-labeling agents (1). Such compounds are also being evaluated as functional group reagents for the analysis of trace amounts of amines by formation of fluorescent thiourea derivatives. For example, 9-isothiocyanatoacridine (I) has been found useful for the detection of traces of penicillin (2).



I

In the course of developing assays based upon fluorescent thiourea compounds from 9-isothiocyanatoacridine, it was observed that base treatment of the initial reaction product resulted in an increase in fluorescence of the reaction mixture. This increase was also accompanied by a change in the activation and fluorescent wavelengths of the mixture. These conditions are advantageous for assay of traces of amines based upon the measurement of a reaction product, even in the presence of excess original fluorescent reagent. For example, when the procedure is applied to *n*-butylamine, activation for the final reaction mixture is at 300 nm. with fluorescence at 490 nm. These are compared to an activation wavelength of 410 nm. and fluorescence at 440 and 460 nm. for the butylthiourea derivative of 9-isothiocyanatoacridine and also for the reagent *per se*. The purpose of this paper is to report the development and results of such an assay.

EXPERIMENTAL

Development of Reaction Conditions—Fluorescence intensity as a function of the period of heating was studied. Samples containing 1.5012 mg. of 9-isothiocyanatoacridine (I) and 0.0339 mg. of *n*-butylamine in 10 ml. of absolute alcohol were shaken in a 60°

acteristics of 0.27 and 0.28 reported earlier (1, 2). Waning of ptotic activity at +24 hr. decreased the slope of the dose–response curves, causing a wide range in the 95% confidence limits of the potency and an increase in the calculated λ value (0.96) for this assay. From these results, it can be seen that the potency of the reserpine–deoxycholic acid combination was equivalent to 1.0 over the entire time-course spectra of activity.

To detect if there was any acceleration of activity, ptotic readings were also collected at the +1-hr. interval. However, because of the characteristic slowness of the reserpine response, ptotic activity was seen only with the top doses of both the test and standard treatments. The mean responses were equivalent. Controls receiving a 20-sec. intravenous injection of 0.029% acetic acid, with and without orally administered deoxycholic acid, were without ptotic activity.

The absence of reserpine potentiation with the concomitant administration of reserpine acetate and deoxycholic acid by different routes lends further support to the theory that the mechanism of potentiation with orally administered reserpine–bile acid coprecipitates is by physicochemical rather than by pharmacological means. A physical mixture of 1:16 *M* reserpine and deoxycholic acid was shown to cause a slight but significant rise in potency (maximum of 1.8×) when given orally (1). Although such a mixture does not allow the intimate combination of ingredients found in a coprecipitate and does preclude reduction in particle size during precipitation, there still exists the possibility of local surface-tension lowering by the bile acids. As both drugs enter the circulation in the present experiment, actual physical contact in the gastrointestinal tract is eliminated, yet any true pharmacologic potentiation should occur. The only chance for physical interaction in the gastrointestinal tract is the possibility of enterohepatic excretion of the intravenous reserpine into the intestine. The absence of potentiation, therefore, also precludes an enterohepatic recirculation phenomenon as a potentiation mechanism for the reserpine–deoxycholic acid coprecipitates.

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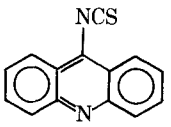
Fluorescent Analysis of Primary Aliphatic Amines by Reaction with 9-Isothiocyanatoacridine

J. E. SINSHEIMER, D. D. HONG*, J. T. STEWART†, M. L. FINK‡, and J. H. BURCKHALTER

Abstract □ Primary aliphatic amines are reacted with 9-isothiocyanatoacridine to yield the corresponding thiourea derivatives. These derivatives, after reaction in base, yield new compounds of intense fluorescence. Conditions have been developed so that it is possible to relate the fluorescence of the final reaction mixture to the concentration of original amine or amine salts without separation of products, even in the presence of excess fluorescent reagent. This method of analysis is successful at the microgram level and can be extended to the nanogram level by TLC separation and direct TLC fluorimetry of the reaction product.

Keyphrases □ 9-Isothiocyanatoacridine reagent—fluorescent analysis, primary aliphatic amines □ Amines, primary aliphatic—fluorescent analysis using 9-isothiocyanatoacridine □ TLC fluorimetry—analysis

These laboratories are interested in the development and evaluation of fluorescent isothiocyanate compounds as new fluorescent protein-labeling agents (1). Such compounds are also being evaluated as functional group reagents for the analysis of trace amounts of amines by formation of fluorescent thiourea derivatives. For example, 9-isothiocyanatoacridine (I) has been found useful for the detection of traces of penicillin (2).



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In the course of developing assays based upon fluorescent thiourea compounds from 9-isothiocyanatoacridine, it was observed that base treatment of the initial reaction product resulted in an increase in fluorescence of the reaction mixture. This increase was also accompanied by a change in the activation and fluorescent wavelengths of the mixture. These conditions are advantageous for assay of traces of amines based upon the measurement of a reaction product, even in the presence of excess original fluorescent reagent. For example, when the procedure is applied to *n*-butylamine, activation for the final reaction mixture is at 300 nm. with fluorescence at 490 nm. These are compared to an activation wavelength of 410 nm. and fluorescence at 440 and 460 nm. for the butylthiourea derivative of 9-isothiocyanatoacridine and also for the reagent *per se*. The purpose of this paper is to report the development and results of such an assay.

EXPERIMENTAL

Development of Reaction Conditions—Fluorescence intensity as a function of the period of heating was studied. Samples containing 1.5012 mg. of 9-isothiocyanatoacridine (I) and 0.0339 mg. of *n*-butylamine in 10 ml. of absolute alcohol were shaken in a 60°

Table I—Analysis of Amines and Amine Salts

Compound	Range,[a] mcg./10 ml.	Fluorescence: Activation, nm.	Fluorescence: Fluorescent, nm.	Intensity/ Concentration[b]
1. *n*-Propylamine	2–13	300	490	5.6
2. *n*-Butylamine	9–18	300	510	4.7
3. *iso*-Butylamine	26–60	300	510	1.28
4. *tert*-Butylamine	30–60	300	490	1.01
5. 2-Ethylhexylamine	6–20	295	490	1.0
6. Allylamine	7–10	295	490	3.46
7. *n*-Butylamine HCl	10–20	295	490	1.76
8. Adamantamine HCl	5–20	295	490	0.8
9. Cyclohexylamine HCl	5–20	295	490	1.08
10. *n*-Butylamine HCl (in 0.5 ml. of water)	20–30	295	510	0.75

[a] Range observed for a linear response extending through the origin. [b] Slope of linear range: given as fluorescence (calculated for a meter multiplier reading of 1) and divided by concentration (in mcg. per ml.).

water bath.[1] After reaction periods from 0 to 30 min., 1 ml. of 0.2 *N* sodium ethoxide was added to each sample and its corresponding reagent control; heating in a bath was continued for 2 hr. Samples and controls were transferred to 25-ml. volumetric flasks, which contained 1 ml. of 10% HCl and were made to volume with absolute alcohol. Fluorescence was read on a spectrophotofluorometer,[2] with an activation wavelength of 340 nm. and emission wavelength of 490 nm., and compared to the corresponding control solution. From this study, 0.5 hr. was selected as the initial reaction period.

In a similar manner, the optimal period of heating with base, after the 0.5-hr. initial reaction, was found to be 1 hr. The effect of temperature during the initial 0.5-hr. reaction period was examined by reacting samples at different temperatures in the 30–80° range. The optimal temperature was found to be 60°. To study the effect of acid quenching, the fluorescences of samples with and without the addition of 1 ml. of 10% HCl were compared.

General Analytical Procedure—The quantities of amines indicated in Table I, dissolved in 4 ml. of absolute alcohol and 2 ml. of an absolute alcohol solution of 9-isothiocyanatoacridine (0.8 mg./ml.), were placed in 10-ml. volumetric flasks. Samples, together with control solutions of reagent, were heated for 0.5 hr. at 60° before 1 ml. of 0.2 *N* sodium ethoxide was added; heating was continued for an additional hour at 60°. After the flasks were cooled, 1 ml. of 10% HCl was added and solutions were diluted to 10 ml. with absolute alcohol. The increase in fluorescence of the reaction mixtures compared to their corresponding reagent blanks was measured. Activation and fluorescent wavelengths for each compound were selected so that there was a maximum difference between the control and sample.

The analysis of amine salts was conducted in the same manner, except that 1 ml. of absolute alcohol in the original amine solution was replaced by 1 ml. of 0.5% absolute alcohol solution of sodium acetate before reaction with the reagent solution.

TLC Fluorimetry—Cellulose plates, scored into 0.5-cm. channels, were spotted in alternate channels with reaction mixture material and developed for a distance of 13 cm. The cellulose plates, after air drying, were turned upside down and scanned with a thin-layer scanner[3] connected to the spectrophotofluorometer[2] and a recorder[4] with expanded range attenuation (2).

The following conditions were used in all analyses: (*a*) spectrophotofluorometer—sensitivity, 45; meter multiplier, 0.003; scanner motor, 1 r.p.m.; (*b*) recorder—recorder speed, 36 in./hr.; damping, 1/4, and (*c*) expanded range attenuation—zero offset to chart reading of 10%; sensitivity of 0.4 of maximum sensitivity.

The assay of *n*-butylamine serves as a typical example of the procedure; it was performed in the following manner. To each of a series of 5-ml., glass-stoppered, pear-shaped boiling flasks were added 50 μl. of an absolute alcohol solution of Reagent I (9 mcg.) and 10–250 μl. of an absolute alcohol solution of amine (0.12–3.06 ng.). The mixture was shaken in a 60 ± 1° water bath for 30 min., 50 μl. of 0.2 *N* sodium ethoxide was added, and the mixture was heated for an additional hour. The mixtures were cooled, 50 μl. of 10% HCl (aqueous) was added to each flask, and the contents were lyophilized. A control containing no amine was prepared in a similar manner. The lyophilized products and control were dissolved in 100 μl. of absolute alcohol, and 1 μl. of each was spotted on a cellulose thin-layer plate. Development was with a DMF–$CHCl_3$–28% NH_4OH (5:13:3) system with measurement at $\lambda_{act.}$ 270 and $\lambda_{fluor.}$ 485 nm.

[1] Research Specialties No. 2156.
[2] Aminco-Bowman 4-8106 with slit arrangement 4.
[3] Aminco No. 4-8221A.
[4] Heath No. EU-20B.

RESULTS AND DISCUSSION

The general procedure for the synthesis of isothiocyanates has been to react the corresponding amine with thiophosgene (1). However, in the case of 9-isothiocyanatoacridine, this approach was unsatisfactory in yield and purity. That is, the HCl liberated during this synthesis appeared to promote the well-recognized (3) attack upon 9-aminoacridine derivatives. Therefore, the procedure of Kristan (4) employing an S_N2 displacement of the chloro group in 9-chloroacridine by the thiocyanato group from silver thiocyanate was used.

The analytical procedure involves essentially three steps: (*a*) reaction of amine and 9-isothiocyanatoacridine to form a thiourea; (*b*) reaction of the thiourea in sodium ethoxide to form compounds which change the fluorescence activation and emission wavelengths of mixture; and (*c*) quenching of this reaction with acid. Optimal conditions were sought for the amine–reagent reaction and the subsequent treatment with base. As indicated in Fig. 1, a temperature of 60° was most satisfactory for the reaction of an absolute alcohol solution of amine and excess 9-isothiocyanatoacridine. The evaluation of this part of the procedure also depends upon the subsequent addition of base to convert any thiourea formed into the fluorescent compounds capable of detection in the presence of excess reagent.

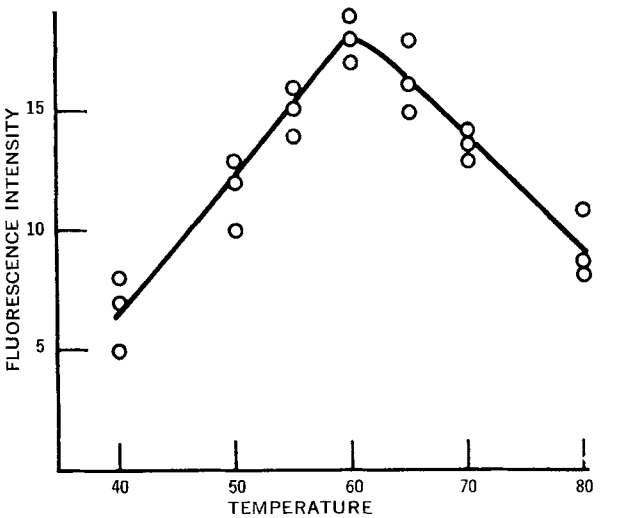

Figure 1—*Fluorescence of the reaction of 9-isothiocyanatoacridine with butylamine as a function of temperature.*

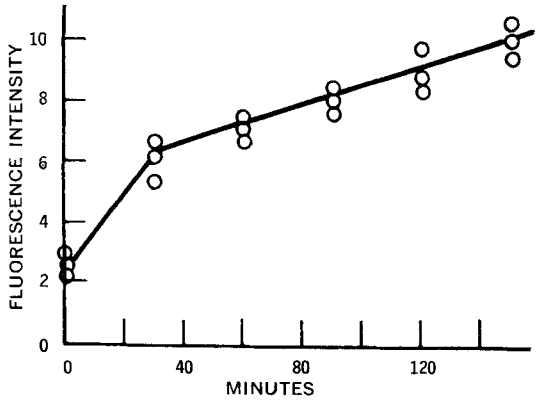

Figure 2—*Fluorescence of the reaction mixture of 9-isothiocyanatoacridine and* n*-butylamine as a function of the time of reaction in sodium ethoxide solution.*

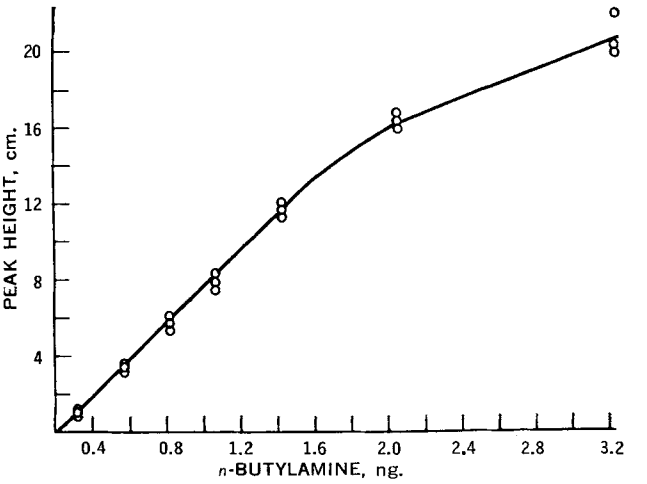

Figure 3—*TLC fluorimetry procedure applied to* n*-butylamine.*

The initial reaction proceeds rapidly at 60°, reaching a maximum plateau of fluorescence within 15 min. so that a reaction time of 0.5 hr. proved to be more than adequate. The increase in fluorescence of the thiourea product over that of its corresponding reagent control upon reaction with sodium ethoxide solution, however, proceeds more slowly and does not plateau (Fig. 2). Therefore, a reaction period of 1 hr. was selected for this step in the procedure. Acid was then added to quench the sodium ethoxide-activated reaction and, thus, to make the procedure less dependent upon the time required in determining the fluorescence of samples. The addition of acids increased the sensitivity of the procedure. That is, the quenched samples showed a difference in fluorescence between the final reaction mixture and a reagent blank about double that of a corresponding nonquenched set of samples.

These parameters were incorporated into a general procedure as described in the *Experimental* section. In the order of 7×10^{-4} moles of 9-isothiocyanatoacridine was employed in this procedure, which amounts to an excess of dye to sample varying from 10- to 70-fold, depending upon the concentration of amine involved. The procedure permits detection of fluorescence of the final reaction product and correlation of fluorescence to concentration of amine, even in the presence of the large excess of fluorescent reagent and its reaction products.

The general procedure is also suitable for salts of aliphatic primary amines. However, amine salt analysis requires addition of 1 ml. of 0.5% solution of sodium acetate in absolute alcohol to yield free amine for reaction with the isothiocyanate reagent.

Analytical results for amines and amine salts successfully examined by this procedure are summarized in Table I. The assay of an unknown concentration of amine by this procedure requires a direct comparison of fluorescence of the unknown to that of a known concentration under the same experimental conditions. The linear fluorescence to concentration range and slope of this response indicates the range over which the sample could be compared and the sensitivity of the procedure. Reproducibility of the technique is indicated from fluorescent data for nine sets of three duplicate samples of propylamine. The average maximum range for these sets was a fluorescent reading equivalent to 2 mcg. of amine.

While water interferes with the method, limited concentrations of water can be tolerated to permit analysis of aqueous solutions of amine. It was found that 0.5 ml. of water could be present if the total final reaction volume was increased to 25 ml. and if a 2-hr. heating period after base addition (instead of the usual 1-hr. period) was employed. The reagent blank must also contain an equivalent amount of water. Results for *n*-butylamine HCl in 0.5 ml. of water under these conditions are listed in Table I.

The general procedure is limited to aliphatic primary amines and their salts. An increase in fluorescence over the corresponding reagent control was not detected when dibutylamine, diethylamine, or aniline was employed in the general procedure. While alcoholic ammonia solutions did yield an increase in fluorescence, results were difficult to duplicate, probably because of a loss of ammonia during the initial heating periods. Also, conditions were not established for an analysis of ammonium chloride or benzylamine. Ammonium chloride showed no indication of reaction when either conditions used successfully for amine salts were tried or when stronger bases were employed. The procedure with benzylamine showed only a limited increase in fluorescence and was not suitable for its assay.

Sensitivity of the procedure was extended by separation of the final fluorescent product from excess reagent and by-products using TLC and measurement of fluorescence directly from the plate. A solvent system of DMF–$CHCl_3$–28% NH_4OH (5:13:3) was employed for separation of the reaction product on cellulose plates. Analogous to the solution procedure, a thiourea was first formed and then reacted with base, followed by quenching with acid as described in the *Experimental* section. Minimal quantities of reaction solutions were employed so that it was possible to assay amines at nanogram levels. Figure 3 depicts the results obtained for the TLC fluorimetry of *n*-butylamine.

The same TLC system developed for TLC fluorimetry was also applicable for investigating compounds present in the reaction mixture. As expected, spots corresponding in R_f value and fluorescence characteristics to the usual hydrolysis products of 9-aminoacridine derivatives (3), *i.e.*, acridone and 9-aminoacridine, were observed. Also, as anticipated, a spot consistent with the presence of *N*-(9-acridinyl)thiourethan was detected. These spots were obtained for both reagent control and reaction mixtures and did not bear a linear relationship to the amount of amine originally present. However, an additional spot (R_f 0.25 for the butylamine reaction mixture) was observed for the reaction mixtures only and could be related to the concentration of amine under investigation. Work is in progress concerning the identification of this highly fluorescent compound.

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Identification of Polymeric 5,7,3′,4′-Tetrahydroxyflavan-3,4-diol from Tannin Extract of Powdered Cassia Bark, Saigon, Cinnamon USP, *Cinnamomum loureirii* Nees, Family Lauraceae

LEONARD BUCHALTER†

Abstract □ A phytochemical investigation of a tannin extract from powdered cinnamon USP showed the presence of flavanoidal-type condensed tannins. The tannin extract consisted of polymeric 5,7,3′,4′-tetrahydroxyflavan-3,4-diol units. No monomeric leucoanthocyanidins or polyphenolic derivatives of benzoic or cinnamic acid were present in the tannin extract. Identification was accomplished by paper chromatography and visible and IR spectrophotometry.

Keyphrases □ 5,7,3′,4′-Tetrahydroxyflavan-3,4-diol (polymeric)—identification □ Tannin extract—identification of polymeric 5,7,3′,4′-tetrahydroxyflavan-3,4-diol □ Paper chromatography—identification □ IR and visible spectrophotometry—identification

The presence of tannins in several plant extracts has been reported but, in many cases, an exact phytochemical investigation of the tannins was not carried out. In previous papers (1, 2), the author phytochemically evaluated tannin fractions from the tubers of *Rumex hymenosepalus*, Family Polygonaceae, and wild cherry bark USP, *Prunus serotina* Erhart, Family Rosaceae. Tannin content has been reported for the powdered cassia bark of *Cinnamomum loureirii* Nees, Family Lauraceae (3). A phytochemical investigation of this material was undertaken as part of a continuing investigation into the chemical nature of the reported tannin content of drug extracts.

EXPERIMENTAL

Materials—Paper chromatography was carried out on Whatman No. 1 filter paper in glass tanks. IR spectra were made on a Beckman IR 8 spectrophotometer using a KBr pellet. Visible spectra were obtained from a Beckman DB spectrophotometer. Commercial samples of cyanidin[1] and powdered cinnamon bark USP[2] were used.

Preparation of Tannin Extract—Five hundred grams of powdered bark of cinnamon USP, *Cinnamomum loureirii* Nees, was defatted with successive washings of petroleum ether and chloroform. The marc was extracted with 500 ml. of water–ethanol (1:1) and then filtered. The resulting brown solution was evaporated *in vacuo*, yielding a dark-brown amorphous residue. This material was tested for tannin content with 5% alcoholic ferric chloride solution. Gelatin and alkaloidal precipitation tests were also used. A deep-green color was obtained with the ferric chloride reagent, and the gelatin and alkaloidal precipitation tests proved positive for tannins.

Hydrolysis Tests—A portion of the extract was subjected to hydrolytic procedures in both acid and alkaline media. The extract was found to be of nonhydrolyzable tannins.

Paper Chromatography—A portion of the tannin extract, dissolved in a 1:1 mixture of ethanol–water, was spotted on Whatman No. 1 filter paper and exposed to paper chromatography in the following solvent systems: Solvent 1, butanol–acetic acid–water (4:1:5), organic phase; Solvent 2, butanol–acetic acid–water (4:1:5), aqueous phase; and Solvent 3, 10% acetic acid. In all solvent systems, only a long very light-brown streak running from the origin to very high R_f values was apparent. No single, visible, or UV fluorescent materials were apparent on the chromatograms. All three papers, when sprayed with 5% alcoholic ferric chloride and heated in an oven, showed only a solid gray-green streak from the origin to high R_f values. All papers were sprayed with 5% ethanolic vanillin HCl reagent and then heated in an oven. A pink-red streak developed on all papers extending from the origin to very high R_f values. No single spot was apparent. This paper chromatography ruled out the presence of polyphenolic materials related to benzoic or cinnamic acid. In addition, the paper showed that no polyphenolics of the trihydroxy gallic acid type were present by the absence of a deep-blue color when the ferric chloride reagent was used. The streak indicated polymeric flavanoidal material in the tannin extract.

Leucoanthocyanidin Tests—The tannin extract was soluble in a 1:1 mixture of ethanol–water but was insoluble in water alone. Those natural chromagens that are insoluble in water, or give only colloidal solutions, correspond to condensed polymers (4). A portion of the tannin extract was boiled for 20 min. in a solution of ethanol–HCl. The solution turned from brown to deep red. A spot of an alcoholic solution of the tannin extract was placed on filter paper, and a drop of 5% ethanolic vanillin HCl was added. The paper was heated in an oven, and the spot developed a persistent pink color. Attempts to extract the tannin with ethyl acetate from aqueous solution proved negative. These tests indicated the presence of polymeric flavanoidal 3,4-diols or leucoanthocyanidins in the tannin extract (5, 6).

IR Spectra—An IR spectrum of the tannin extract was made using a KBr pellet. This spectrum was superimposable with one obtained from polymeric leucoanthocyanidins in a tannin extract of wild cherry bark, *Prunus serotina* Erhart. Broad absorption occurred in the 2.9–3.3 μ region. Other major peaks occurred at 6.35 and 6.6 μ.

Anthocyanidins—Condensed tannins consisting of polymeric leucoanthocyanidin units retain the ability to be converted to the individual anthocyanidin units. By this method the polymeric material may be identified. Several grams of the tannin extract was dissolved in ethanol and boiled in a solution of ethanol–HCl for 20 min. The solution changed from brown to deep red. After cooling, the solution was extracted with amyl alcohol. Amyl alcohol selectively extracts anthocyanidins. The amyl alcohol was evaporated *in vacuo* and resulted in a dark-red semicrystalline powder. The red semicrystalline material was dissolved in methanol, and several drops of basic lead acetate were added. A blue precipitate resulted, which was extractable by glacial acetic acid, yielding a deep-red color. A spot of an alcoholic solution of the red material was placed on a filter paper and held over ammonia fumes. A deep-blue color resulted and changed to red when exposed to HCl fumes. These tests were positive for the presence of anthocyanidins (7).

Paper Chromatography of Anthocyanidins—Paper chromatography of the anthocyanidins produced from the polymeric leucoanthocyanidins was carried out on Whatman No. 1 filter paper. The following solvent systems were used: Solvent 1, acetic acid–concentrated HCl–water (10:30:3); Solvent 2, acetic acid–concentrated HCl–water (5:1:5); and Solvent 3, formic acid–3 *N* HCl (1:1). In all three solvent systems, only one red pigment appeared. The R_f values were: Solvent 1, R_f 0.5; Solvent 2, R_f 0.34; and Solvent 3, R_f 0.22. The red pigment in all solvent systems turned deep blue when exposed to ammonia fumes. The R_f values in all three solvent systems were those for 3,5,7,3′,4′-pentahydroxyflavylium chloride (8–10). A commercial sample of the material was obtained,[1] and the paper chromatography was repeated in all three solvent systems.

[1] Obtained from K & K Labs, Plainview, N. Y.
[2] Obtained from S. B. Penick & Co., New York, N. Y.

The R_f values obtained in all solvent systems were identical with that of the commercial sample.

IR and Visible Spectra of Anthocyanidin—An IR spectrum was made of the red pigment and was found to be superimposable with that of a commercial sample of cyanidin chloride. Visible spectrum analysis of the pigment in ethanol–0.1% HCl solution was 545 nm. (11).

DISCUSSION

A tannin extract obtained from powdered cinnamon USP was examined phytochemically. The tannin extract consisted of polymeric leucoanthocyanidin units. Based on the conversion of the polymer to 3,5,7,3′,4′-pentahydroxyflavylium chloride and the identification of the same, it was concluded that the polymer consisted of polymeric units of 5,7,3′,4′-tetrahydroxyflavan-3,4-diol. No monomeric leucoanthocyanidins or other polyphenolic derivatives of benzoic and cinnamic acid were found in the tannin extract.

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Physiologic Surface-Active Agents and Drug Absorption VIII: Effect of Bile Flow on Sulfadiazine Absorption in the Rat

CHARLES H. NIGHTINGALE*, JAMES E. AXELSON, and MILO GIBALDI

Abstract □ The absorption of sulfadiazine was determined in rat intestinal loops *in situ* under four experimental conditions—*viz.*, control, bile duct ligation, sham bile duct ligation, and sodium dehydrocholate-stimulated bile flow. Enhanced bile flow increased the absorption of the drug about 50%, apparently by increasing the solubility and dissolution rate of sulfadiazine. This possibility is supported by the results of *in vitro* solubility studies. Rats with a ligated bile duct, on the average, showed significantly reduced absorption compared to control levels. The results suggest that bile plays an important, although not critical, role in the absorption of sulfadiazine under these experimental conditions.

Keyphrases □ Sulfadiazine—absorption, bile-flow effect, rats □ Absorption, sulfadiazine—effect of bile flow, rats □ Bile—role in sulfadiazine absorption, rats

Numerous studies have shown that surface-active agents may increase the gastrointestinal absorption of poorly water-soluble drugs by enhancing dissolution *via* an effect on the effective surface area or apparent solubility (1). Since bile manifests considerable surface activity, it is reasonable to consider that bile salts and certain phospholipids, which are normally present in the small intestine, solubilize and enhance the dissolution rate of poorly soluble drugs and should thereby promote the absorption of these compounds. It follows that where there is a diminished bile salt concentration in the proximal intestine, such drugs will be poorly absorbed. This possibility is supported by the findings of Bernhard *et al.* (2) who showed that in the biliary fistula of the rat, less than 1% of a dose of vitamin A was absorbed and that bile administration increased absorption significantly.

Greaves (3) also reported that bile salts are essential for the intestinal absorption of vitamin K in the rat. Adequate absorption of vitamin D by the rat was shown to require bile by Greaves and Schmidt (4). While the bile duct was anastomosed into the colon, the animals absorbed little or none of the vitamin. Oral administration of deoxycholic acid greatly improved absorption of the vitamin in these rats. Taylor *et al.* (5) confirmed the need of bile for adequate vitamin D absorption in dogs. Heymann (6) also found that dogs did not absorb crystalline vitamin D_2 when bile was not present in the small intestine.

Pekanmaki and Salmi (7) studied the gastrointestinal absorption of phenolphthalein and its glucuronide in the cat. Peak blood levels (portal vein) of 50 mcg./ml. were observed in control animals, in contrast to peak levels of 23 mcg./ml. in test animals (ligation of common bile duct) 1 hr. after gastric intubation of phenolphthalein. There was a marked decrease in the absorption of the poorly soluble drug when drainage of bile into the intestine was prevented. However, the absence of bile from the intestine had no effect on the absorptions of the highly water-soluble glucuronide.

More recently, Meli *et al.* (8) reported that endogenous bile influences the rate of intestinal absorption of ethynylestradiol-6,7-^{3}H-3-cyclopentyl ether in rats. The rate of absorption of the estrogen was considerably lower in bile duct-cannulated rats than in control

The R_f values obtained in all solvent systems were identical with that of the commercial sample.

IR and Visible Spectra of Anthocyanidin—An IR spectrum was made of the red pigment and was found to be superimposable with that of a commercial sample of cyanidin chloride. Visible spectrum analysis of the pigment in ethanol–0.1% HCl solution was 545 nm. (11).

DISCUSSION

A tannin extract obtained from powdered cinnamon USP was examined phytochemically. The tannin extract consisted of polymeric leucoanthocyanidin units. Based on the conversion of the polymer to 3,5,7,3′,4′-pentahydroxyflavylium chloride and the identification of the same, it was concluded that the polymer consisted of polymeric units of 5,7,3′,4′-tetrahydroxyflavan-3,4-diol. No monomeric leucoanthocyanidins or other polyphenolic derivatives of benzoic and cinnamic acid were found in the tannin extract.

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Physiologic Surface-Active Agents and Drug Absorption VIII: Effect of Bile Flow on Sulfadiazine Absorption in the Rat

CHARLES H. NIGHTINGALE*, JAMES E. AXELSON, and MILO GIBALDI

Abstract □ The absorption of sulfadiazine was determined in rat intestinal loops *in situ* under four experimental conditions—*viz.*, control, bile duct ligation, sham bile duct ligation, and sodium dehydrocholate-stimulated bile flow. Enhanced bile flow increased the absorption of the drug about 50%, apparently by increasing the solubility and dissolution rate of sulfadiazine. This possibility is supported by the results of *in vitro* solubility studies. Rats with a ligated bile duct, on the average, showed significantly reduced absorption compared to control levels. The results suggest that bile plays an important, although not critical, role in the absorption of sulfadiazine under these experimental conditions.

Keyphrases □ Sulfadiazine—absorption, bile-flow effect, rats □ Absorption, sulfadiazine—effect of bile flow, rats □ Bile—role in sulfadiazine absorption, rats

Numerous studies have shown that surface-active agents may increase the gastrointestinal absorption of poorly water-soluble drugs by enhancing dissolution *via* an effect on the effective surface area or apparent solubility (1). Since bile manifests considerable surface activity, it is reasonable to consider that bile salts and certain phospholipids, which are normally present in the small intestine, solubilize and enhance the dissolution rate of poorly soluble drugs and should thereby promote the absorption of these compounds. It follows that where there is a diminished bile salt concentration in the proximal intestine, such drugs will be poorly absorbed. This possibility is supported by the findings of Bernhard *et al.* (2) who showed that in the biliary fistula of the rat, less than 1% of a dose of vitamin A was absorbed and that bile administration increased absorption significantly.

Greaves (3) also reported that bile salts are essential for the intestinal absorption of vitamin K in the rat. Adequate absorption of vitamin D by the rat was shown to require bile by Greaves and Schmidt (4). While the bile duct was anastomosed into the colon, the animals absorbed little or none of the vitamin. Oral administration of deoxycholic acid greatly improved absorption of the vitamin in these rats. Taylor *et al.* (5) confirmed the need of bile for adequate vitamin D absorption in dogs. Heymann (6) also found that dogs did not absorb crystalline vitamin D_2 when bile was not present in the small intestine.

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More recently, Meli *et al.* (8) reported that endogenous bile influences the rate of intestinal absorption of ethynylestradiol-6,7-^{3}H-3-cyclopentyl ether in rats. The rate of absorption of the estrogen was considerably lower in bile duct-cannulated rats than in control

Table I—Sulfadiazine Absorption from Rat Intestinal Loops

Experimental Condition[a]	Volume of Intestinal Contents[b] (Range, ml.)	pH of Intestinal Contents (Range)	Length (cm.) of Loop (Mean ± *SD*)	Percent Absorbed[c] (Mean ± *SD*)
Normal (6)	0.5–1.0	7.2–7.6	23.0 ± 2.3	43 ± 8
Sham ligated (6)	—	—	24.9 ± 4.0	44 ± 7
Stimulated bile flow (6)	1.0–1.2	6.6–6.8	24.5 ± 2.9	63 ± 8[d]
Bile duct ligated (10)	0.1–0.2	7.2–7.4	22.3 ± 3.8	26 ± 17[d]

[a] Parenthetical values denote the number of rats. [b] Recovered from the loop 3 hr. after administration. [c] Calculated from residual levels in tissue and loop contents 3 hr. after administration of a 10-mg. dose. [d] Values are significantly different from those obtained with either normal or sham ligated rats, $p < 0.05$, one-tail t test.

animals. Since the drug is relatively water insoluble, it is reasonable to consider that the presence of bile increases the solubility of the drug in the intestinal lumen and thereby enhances the dissolution and absorption rate.

In view of the potential influence of endogenous bile on drug absorption, the purpose of this investigation was to study the role of bile flow on the absorption of a poorly water-soluble sulfa drug, sulfadiazine. Absorption was determined in the rat under four experimental conditions—*viz.*, in normal bile flow animals, in sham ligated animals, in bile duct ligated animals, and in bile flow stimulated animals.

EXPERIMENTAL

Sulfadiazine USP,[1] sodium dehydrocholate,[2] and Marshall's reagent [*N*-(1-naphthyl)-ethylenediamine dihydrochloride][3] were used. All other reagents and chemicals were of certified grade.[3] All compounds were used as received without further purification.

Male rats, Sprague-Dawley strain,[4] were deprived of food for 24 hr. prior to the absorption experiment but allowed water *ad libitum*. The rats used weighed approximately 260 g. (range 226–293 g.). They were divided into four groups (Table I): normal, bile flow stimulated, bile flow deprived, and sham ligated.

Normal Animals—The rats were anesthetized with ether, and the abdominal cavity was opened with a midline incision. A loop, approximately 25 cm. in length, was prepared by ligating the intestine approximately 2 cm. proximal and about 23 cm. distal to the bile duct. Prior to completing the ligation, a 20-gauge hypodermic needle was inserted into the proximal portion of the duodenum so that when the ligature was tightened the tip of the hypodermic needle was approximately 1 cm. proximal to the bile duct. At this time, a 10-mg. dose of sulfadiazine suspended in water was injected into the duodenum, the needle was removed, and the ligature was tightened simultaneously so that backflow of the drug suspension did not occur. The animal's abdominal cavity was closed with wound clips, and the anesthetic was removed so that the animal regained consciousness within 10–15 min. Three hours after administration of the drug, the animal was sacrificed by placing it in an ether tank.

Bile Stimulated Animals—These animals were treated in the same manner as the normal rats, except that 1 ml. of a 100 m*M* solution of sodium dehydrocholate (SDHC) was administered immediately prior to injecting the drug suspension. The SDHC was administered by placing the solution into the exposed peritoneal cavity, directly beneath the portal vein.

Bile Flow Deprived Animals—The abdominal cavity was opened as previously described and the bile duct exposed. Two ligatures were placed on the bile duct approximately 0.5 cm. apart and tightened to prevent bile flow or backflow from the intestine. The bile duct was then cut between the two ligatures. The abdominal cavity was closed by suture, and the animals were allowed to recover in individual cages for 48 hr. with food and water *ad libitum*. After 48 hr. the food was removed, and 24 hr. later the animal was anesthetized and treated in the same manner as described for the normal rats.

Sham Ligated Animals—These animals were treated in the same manner as the bile flow deprived rats, except that the bile duct was not ligated nor cut.

Preparation of Drug Suspension—To ensure minimum loss of drug in the preparation and transfer of the dosage form, the suspension was prepared *in situ* by placing the drug into the barrel of a dry 1-ml. hypodermic syringe, adding 0.5 ml. water, and injecting the resulting suspension. An additional 0.5 ml. water was added to the syringe in small increments and injected to ensure complete delivery of the drug from the syringe. Delivery of the suspension in this manner resulted in an approximate drug loss of 3–5%. Accordingly, a 5% excess of drug was added in each case.

Analytical Procedure—After sacrifice of the animal, the intestinal loop was excised and its contents were carefully milked into a collecting vessel to estimate volume and determine pH. The loop and the contents were then homogenized with about 10 ml. of 0.1 *N* NaOH in a Waring blender for 2 min. The blender was washed with 10-ml. increments of water until a total volume of 50 ml. was obtained. The homogenate and washings were mixed by inversion in a 50-ml. centrifuge tube and centrifuged for 20 min. at 2000 r.p.m. in an International centrifuge, model CS. An aliquot of the supernatant liquid was removed, treated with 30% (w/v) trichloroacetic acid to precipitate protein, and recentrifuged. The supernatant liquid was filtered, and an aliquot was assayed for drug content using the Bratton and Marshall method (9). All analyses were performed at 545 nm. using a Gilford model 222 photometer and power supply of the optical system of a Beckman DU spectrophotometer.

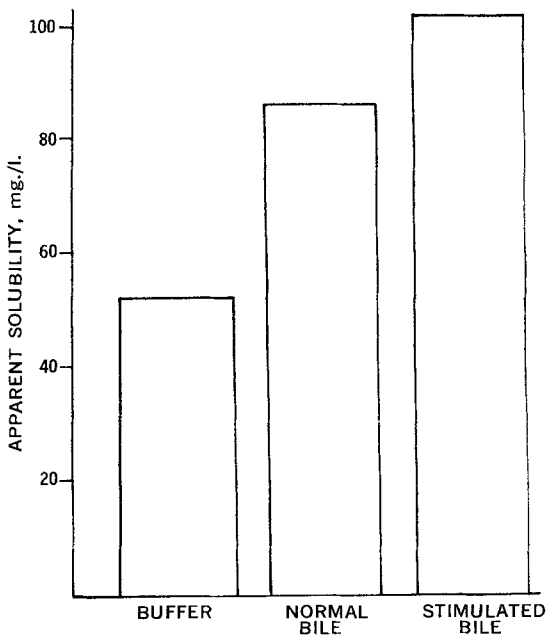

Figure 1—*Apparent solubility of sulfadiazine at 26° in pH 5.8 phosphate buffer and in buffer containing 40% (v/v) normal or SDHC stimulated bile.*

[1] Obtained from Rexall Drug Co., St. Louis, Mo.
[2] Obtained from General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.
[3] Obtained from Fisher Scientific Co., Fair Lawn, N. J.
[4] Obtained from Blue Spruce Farms, Altamont, N. Y.

Tissue blanks were studied in animals using distilled water as the injected substance, and measurable absorbance values could not be detected.

Drug Recovery—Recoveries were performed by removing a 20-cm. segment of intestine from a freshly sacrificed animal, homogenizing in the presence of a known amount of drug (5–9 mg.), and extracting as previously described. A mean recovery (6 trials) of 87.8% was obtained. Further recovery studies were performed in a manner more analogous to experimental conditions. In these studies the abdominal cavity of the animal was opened and a loop was prepared as described for the control animals. Immediately prior to drug administration, the animals were sacrificed by ether. The drug was administered from suspension and allowed to remain in the loop for 3 hr. At this time, the loop was removed and assayed for drug content as described. A mean recovery of 83.9% (10 trials) was obtained using this technique. No statistical difference existed between the results of the two recovery studies. Accordingly the data were combined, which resulted in a mean recovery of 85.3%. All experimental results were corrected for this recovery value.

Solubility Study—To obtain an indication of the ability of bile to solubilize sulfadiazine, the following study was performed. An excess of sulfadiazine was placed in a 10-ml. graduated test tube. Either 2 ml. of distilled water, normal rat bile, or stimulated rat bile was added. Sufficient 0.5 *M* phosphate buffer (pH 5.8) was added to bring the system to a total volume of 5 ml. The suspension was vigorously agitated for 5 min. at room temperature. Agitation was accomplished with a Vortex-Genie model K-550-G mixer. After mixing, the suspension was filtered and an aliquot was diluted 10-fold and assayed according to the method of Bratton and Marshall (9). Blanks were negligible in all cases.

Normal or stimulated rat bile was obtained immediately prior to the solubility determination by the methods outlined previously (10). Bile was stimulated by intraperitoneal injection of 100 μmoles of SDHC. In each case, the bile of several rats was pooled to obtain a sufficient amount of material for the study.

RESULTS AND DISCUSSION

Results of the various absorption studies are summarized in Table I. It is apparent that sulfadiazine was rather poorly absorbed from this preparation. In control rats, only about 40% of a 10-mg. dose was absorbed over a 3-hr. period. It is also evident that the surgical procedure *per se*, to which bile duct ligated animals were subjected, had no influence on the absorption of the drug. Sham ligated rats absorbed sulfadiazine to the same extent as control animals.

Modification of bile flow produced several interesting findings. When bile flow was stimulated by coadministering SDHC, the absorption of sulfadiazine was increased about 50% compared to control values. In addition, the fluid recovered from the loop after the absorption period was of somewhat greater volume and distinctly more acidic than that observed in control rats.

The mean absorption of sulfadiazine in bile duct ligated rats was reduced to about 60% of control levels, but the results were highly variable. The coefficient of variation of percent drug absorbed in bile duct ligated animals was about 65%, in contrast to coefficients of variation of 13–19% observed under the other experimental conditions. Furthermore, in rats where bile flow was terminated, there was a marked decrease in the volume of fluid in the loop at the end of the experiment. The pH of the intestinal fluid, however, was comparable to control values.

The influence of rat bile on the solubility of sulfadiazine is shown in Fig. 1. As expected from the results of previous studies with components of bile, whole bile from both normal and stimulated (SDHC) rats exerted a solubilizing effect on sulfadiazine, presumably *via* micelle formation. Stimulated bile was a somewhat more effective solubilizing agent than unstimulated bile, producing a two-fold increase in the solubility of the drug.

The solubilizing effect of bile on sulfadiazine may well be responsible for the decreased absorption of the drug observed in bile duct ligated rats and for the enhanced absorption found upon stimulation of bile flow. While a strong argument can be developed for the latter observation, a more equivocal position must be taken for the former. The absence of bile in the proximal intestine would decrease the solubility of the drug in the intestinal fluids, and one would anticipate that dissolution-rate-limited absorption would also diminish. However, the volume change in ligated rats resulting from water absorption and the lack of replacement by bile introduces a complicating factor. Conceivably, the small volume present in the gut permitted the buildup of a high concentration of sulfadiazine in solution which would inhibit the dissolution rate of the drug.

The enhanced absorption of sulfadiazine upon coadministration of the hydrocholeretic (SDHC) appears to be more clearly related to the solubilization phenomenon. Although a difference in pH was noted when comparing intestinal fluid from rats where bile flow was stimulated with that from control rats, this difference cannot account for the increased absorption since the direction of the shift would result in a decreased solubility and dissolution rate of the weakly acidic drug. On the other hand, the significant solubilizing effect of stimulated bile on sulfadiazine, observed *in vitro*, could readily account for an enhancement in dissolution-rate-limited absorption.

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Isolation and Characterization of Urinary Metabolites of Nalorphine in Dogs and Cats

S. Y. YEH and L. A. WOODS*

Abstract □ Nalorphine-3-glucuronide dihydrate and nalorphine-6-glucuronide were isolated as urinary metabolites of nalorphine in dogs, and nalorphine-3-ethereal sulfate and nalorphine-3-glucuronide dihydrate were isolated in cats. These metabolites were characterized by UV and IR spectra, phenolic test, nalorphine and glucuronic acid determinations, elemental analyses, and enzymatic hydrolysis.

Keyphrases □ Nalorphine, urinary metabolites—isolation, characterization, in dogs, cats □ TLC—separation □ Paper chromatography—separation □ UV spectrophotometry—identification □ IR spectrophotometry—identification

Nalorphine is *N*-dealkylated to normorphine *in vitro* by rat liver microsomes (1) and *in vivo* in rats (2) and cats (3), and it is conjugated in rats (4) and dogs (5). Recently, nalorphine-3-glucuronide and nalorphine-3-ethereal sulfate were isolated from the urine of rabbits and cats (6), respectively. The present studies describe the isolation and characterization of these and other nalorphine metabolites from the urine of dogs and cats injected with nalorphine.

METHODS

Urine of female dogs injected with 20 mg./kg. s.c. of nalorphine HCl (total of 2.1 g.) was collected with a catheter up to 24 hr. after the last injection and frozen immediately after each collection. Urine of two male cats injected three times daily, each with 50 mg./kg. s.c. of nalorphine HCl (total of 1.0 g.), was cage-collected up to 48 hr. after the last injection and frozen immediately after each collection.

Isolation and Purification of Nalorphine Metabolites in Dogs—The procedure used for the isolation and purification of nalorphine metabolites was similar to that used for isolation of morphine-3-glucuronide from the urine and bile of rats injected with codeine (7); only the details essential to the method are given here. The urine sample (450 ml.), separated from insoluble substances, was loaded onto an ion-exchange (Amberlite XAD-2) resin[1] column. The column was washed with water (250 ml.) and eluted with methanol (400 ml.). A portion of the methanolic eluate (150–400 ml.) was passed through an ion-exchange (Amberlite IRC-50) resin[1] column, and the column was washed with methanol (50 ml.). Free alkaloids were adsorbed by the resins, whereas conjugated metabolites passed through them.

Isolation of Conjugated Metabolites—The effluent and washing from the Amberlite IRC-50 column were concentrated at 55° under a stream of N_2. White crystals of nalorphine conjugate were separated from the methanolic solution, which was finally evaporated to a viscous residue. The residue was triturated with methanol and recrystallized from an aqueous methanol solution. These crystals and those separated from methanolic effluent during concentration were chromatographed on instant thin-layer chromatography (ITLC)-type silica gel[2] and paper and showed the same R_f values in different solvent systems. Therefore, these crystals were combined, recrystallized from boiling water, washed with methanol, and dried at 100° *in vacuo* for 24 hr. to yield snow-white crystals (1.02 g., 55% of injected nalorphine). The purity of these crystals, called nalorphine Metabolite I from dogs, was established by paper chromatography and ITLC.

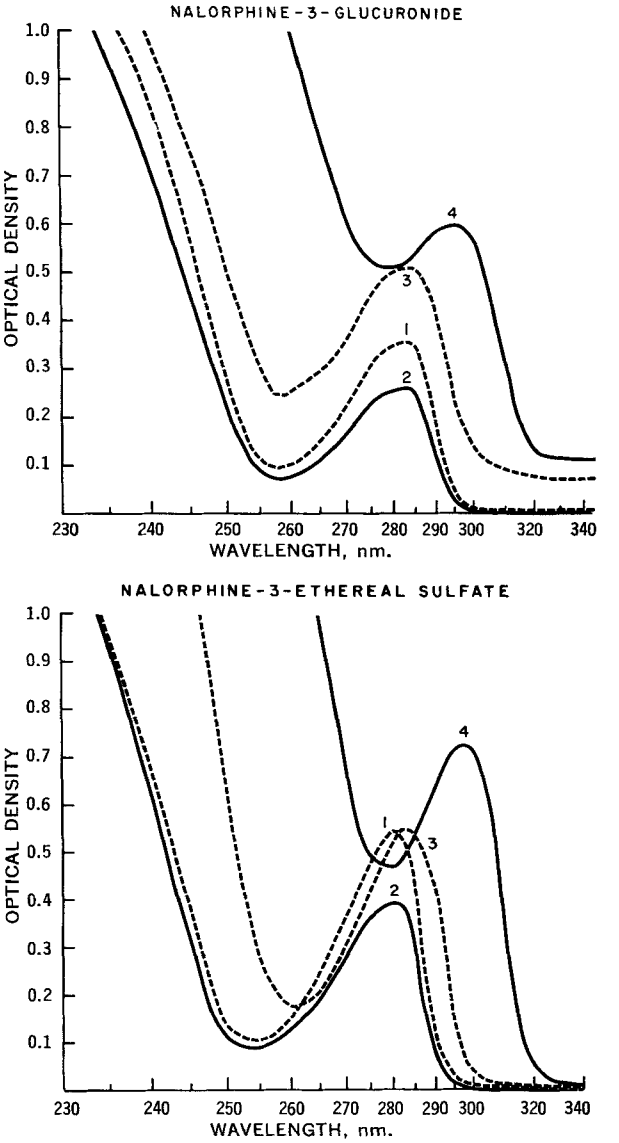

Figure 1—*UV spectra of nalorphine-3-glucuronide and nalorphine-3-ethereal sulfate. Key: Curve 1, 2.5 ml. of nalorphine-3-glucuronide or nalorphine-3-ethereal sulfate in H_2O (1 mg./10 ml.) and 0.5 ml. of 1 N HCl; Curve 2, 2.0 ml. of the metabolite solution and 1.0 ml. of 1 N NaOH solution; Curve 3, 3 ml. of the acid-hydrolyzed metabolite in HCl solution; and Curve 4, the acid-hydrolyzed metabolite in NaOH solution.*

Paper Chromatographic Separation of Conjugated Metabolites—The aqueous methanolic mother liquor remaining after crystallization of Metabolite I was evaporated to dryness. This residue was dissolved in about 10 ml. of water and passed through a neutral aluminum oxide column (1 by 10 cm.). The column was eluted with water (300 ml.), colored impurities being held on the column. Evaporation of the eluate to dryness gave a slight yellowish residue,

[1] Rohm & Haas Co., Philadelphia, Pa.
[2] Gelman Instrument Co., Ann Arbor, Mich.

Table I—R_f Values of Nalorphine Metabolites Isolated from Dog and Cat Urine[a]

Substance	Paper Chromatography[b]		TLC[b]		
	n-BuOH–AcOH–H_2O (4:1:2)	*n*-BuOH–H_2O(9:1)[c]	*n*-BuOH–AcOH–H_2O (35:3:10)	EtOAc–MeOH–NH_4OH-(30%NH_3) (17:2:1)	*n*-Butyl Ether–*n*-BuOH–NH_4OH(30%NH_3) (70:25:2)
Nalorphine-3-glucuronide (Metabolite I)	0.36	—	0.41		
Nalorphine-6-glucuronide (Metabolite II)	0.40	—	0.62		
Nalorphine-3-ethereal sulfate(Metabolite III)	0.50	—	0.69		
Residue of free alkaloid fraction					
a, from the dog urine	0.58; 0.68	0.13; 0.95	0.9	0.95	0.24; 0.81
b, from the cat urine	0.58; 0.68	0.13; 0.95	0.9	0.95	0.24; 0.81
Authentic normorphine	0.58	0.13	0.9	0.83	0.24
Authentic nalorphine	0.68	0.95	0.9	0.98	0.81

[a] Chromatograms were sprayed with potassium iodoplatinate solution or 3% ninhydrin in methanol. [b] *n*-BuOH = *n*-butanol; AcOH = acetic acid; EtOAc = ethyl acetate; MeOH = methanol. [c] Paper buffered with citrate phosphate solution at pH 6.8.

which was dissolved in 1 ml. of water and chromatographed (5 μl./spot) on Whatman 3 MM paper (19 by 22 in.) with *n*-butanol–acetic acid–water (4:1:2 v/v) as the solvent system. The chromatograms showed two clearly separated zones having R_f values: Zone A, 0.36; and Zone B, 0.40. These bands were separated and extracted with 100–200 ml. of boiling water. Each extract was evaporated to dryness, and the residue was recrystallized from an aqueous methanol solution. The substance extracted from Zone A, obtained in crystalline form, was identical with Metabolite I, whereas the substance from Zone B (called Metabolite II) could not be crystallized.

The Amberlite IRC-50 column was eluted with 200 ml. of ammoniacal (30% NH_3)–methanol (1:1), and the eluate was evaporated to dryness. Free nalorphine and normorphine were identified in the residue by ITLC, using *n*-butyl ether, *n*-butanol, and NH_4OH (30% NH_3) as the solvent system, and by paper chromatography on Whatman 3 MM paper buffered with citrate phosphate solution at pH 6.8 (8) and developed with *n*-butanol and water (Table I).

Isolation of Nalorphine Metabolites from Cat Urine—By application of a procedure similar to that used for dog urine, the major nalorphine metabolite (205 mg.) was obtained as colorless crystals (called Metabolite III, 23% of injected nalorphine).

The minor metabolite (called Metabolite IV, 20 mg., 2.3% of injected nalorphine) was isolated by paper chromatography from the methanolic mother liquor as described previously. Metabolite IV was identical with Metabolite I and characterized as nalorphine-3-glucuronide.

Free nalorphine and free normorphine were identified in the free alkaloid fraction as already described.

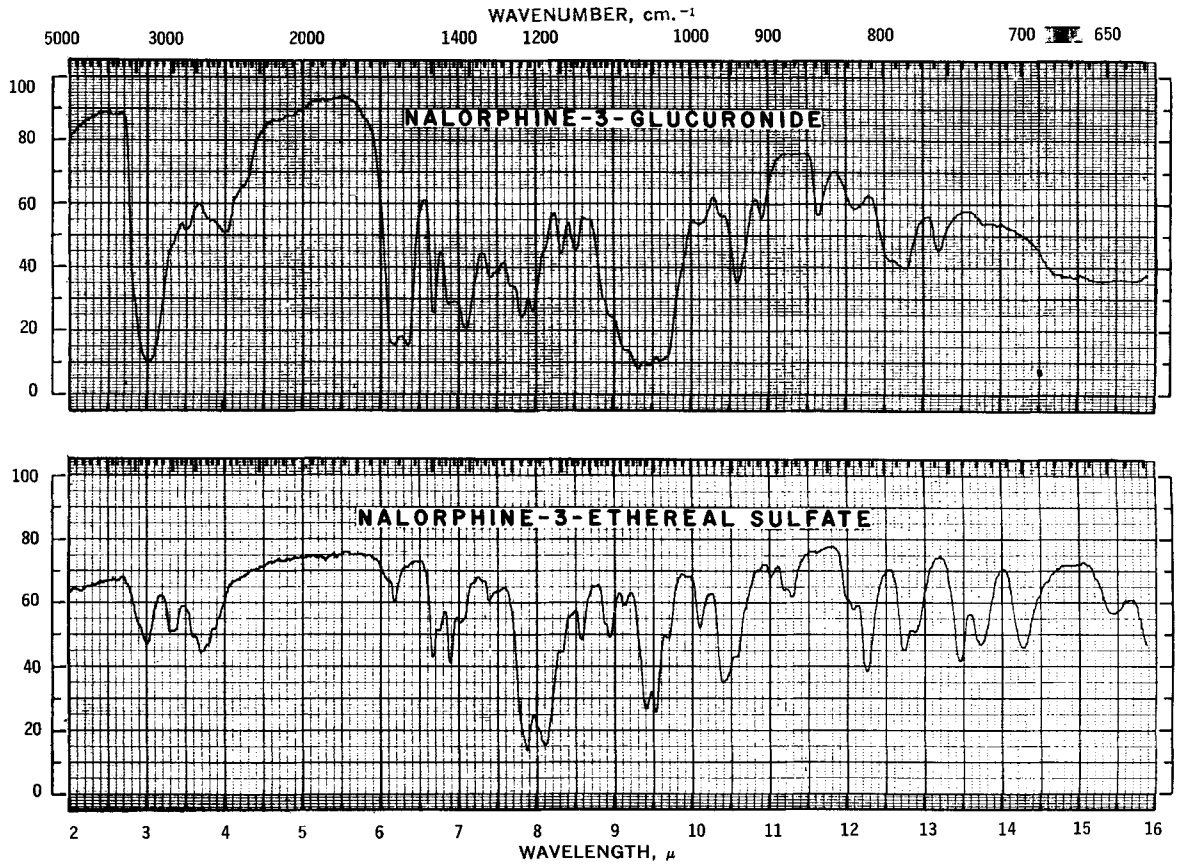

Figure 2—*IR spectra of nalorphine-3-glucuronide and nalorphine-3-ethereal sulfate.*

Table II—Physicochemical Characteristics of Urinary Nalorphine Metabolites

Characteristic	Metabolite I	Metabolite II	Metabolite III
Solubility	Sparingly soluble in H_2O; insoluble in usual organic solvents		Sparingly soluble in H_2O; insoluble in usual organic solvents
Melting point	237–238° dec.		>300°
Phenolic test[a]	Negative	Positive	Negative
UV spectra(Fig. 1):			
In HCl and H_2O, max. nm.	285	285	280
In NaOH, max. nm.	285	295	280
IR spectra (Fig. 2)	3.0 μ (polyhydroxy), 6.1 μ (ionized carboxylic acid)		7.8 and 8.1 μ (ethereal sulfate)
Acid hydrolysis	+[b]	+	+
Enzymatic hydrolysis:			
With β-glucuronidase[c]	+	+	−
With glusulase[d]			+
With phenol sulfatase[c]			+
Elemental analyses[e]	Found (%) Calcd. for $C_{25}H_{29}NO_9 \cdot 2H_2O$	Found Calcd.	Found (%) Calcd. for $C_{19}H_{21}NO_6S$
	C, 57.36 C, 57.35		C, 58.6 C, 58.29
	H, 6.54 H, 6.35		H, 5.13 H, 5.13
	N, 2.57 N, 2.57		N, 3.47 N, 3.58
			S, 8.09 S, 8.20
Content of nalorphine[f]	60.0 59.47	62.0 63.87	78.5 79.4
Glucuronic acid[g]	38.5 37.08	38.5 39.82	

[a] Sequential treatment with alkaline ferricyanide and acid ferric chloride solution. [b] + indicates nalorphine obtained on hydrolysis. [c] Obtained from Sigma Chemical Co., St. Louis, Mo. [d] Obtained from Endo Lab., Garden City, N.Y. [e] Performed by the Department of Chemistry, University of Iowa, and Huffman Lab., Wheatridge, Colo. [f] By methyl orange method (9). [g] By carbazole method (10).

RESULTS AND DISCUSSION

Chromatographic Studies—Chromatographic data on ITLC and paper chromatograms of nalorphine Metabolites I, II, and III are presented in Table I. These R_f values varied somewhat on repeated chromatography, but the basic pattern of separation remained the same.

Characterization of Nalorphine Metabolites—The physicochemical characteristics of the nalorphine metabolites are listed in Table II. Based on these data, nalorphine Metabolite I, isolated from the urine of dogs after subcutaneous injection of nalorphine, was characterized as nalorphine-3-glucuronide dihydrate. Metabolite II was identified as nalorphine-6-glucuronide. Nalorphine Metabolite II on ITLC and paper chromatograms had higher R_f values than nalorphine-3-glucuronide. Previous studies (11, 12) also reported that morphine-6-glucuronide had higher R_f values than morphine-3-glucuronide. It is probable that an unknown nalorphine metabolite observed by Fujimoto *et al.* (6) in the urine of rabbits is nalorphine-6-glucuronide on the basis of similarity of chromatographic behavior observed in the present study.

Nalorphine Metabolite III, identified as nalorphine-3-ethereal sulfate, was reported to be a metabolite of nalorphine in the cat (6). Nalorphine-3-glucuronide, a minor metabolite of nalorphine in the cat, was also observed in the cat urine by Fujimoto *et al.* (6) but was not isolated.

Chromatographic evidence for the presence of a small amount of normorphine (0.1% of injected dose) was obtained in the urine of dogs and cats.

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Metal-Binding Abilities of Radioprotective Organic Thiosulfates

WILLIAM O. FOYE and CHOON MI KIM

Abstract □ Ionization constants and stability constants for the complexation of Cu(II), Al(III), and Fe(III) ions by several aminoalkane and cyanoalkane thiosulfates were determined. Metal-binding avidities of the aminoalkane thiosulfates were found to be significantly less than those for the amino (or guanidino) thiols or for dithiocarbamates having radioprotective properties, but the possibility exists that organic thiosulfates may stabilize cuprous ion-containing macromolecules against radiation damage.

Keyphrases □ Metal-binding abilities, radioprotective organic thiosulfates—with Cu(II), Al(III), Fe(III), ionization, stability constants calculated □ Thiosulfates, radioprotective organic—metal-binding abilities, ionization, stability constants calculated □ Potentiometric titrimetry—determinations

The aminoalkane thiosulfates, or Bunte salts, have been recognized as a particularly effective class of radiation-protective agents (1, 2). All Bunte salts with appreciable protective properties observed so far have had the amino and thiosulfate functions separated by two or three carbons, a molecular arrangement conducive to metal-ion chelation. The role of metal binding in cellular radiation protection has not been adequately explained, but a variety of observations has shown that metal ions are involved (3, 4). For this reason it was considered desirable to determine metal-binding avidities of aminoalkane thiosulfates as well as of other organic thiosulfates having complexing functions. Although the thiosulfate ion has metal-binding properties, and stability constants have been recorded (5), no stability constants for metal binding of organic thiosulfates were found in the literature.

Possible roles for metal-binding agents in radiation protection lie in the complexation of the metal constituents of metalloenzymes and prevention of radiation-induced conversion to an unfavorable oxidation state. A correlation between extent of complexation of catalase by a series of radiation-protective agents and their relative protective abilities in mice has been found (6). All compounds in this series had the ability to bind metals; the sole exception to the correlation was the Bunte salt of 2-mercaptoethylamine, which stimulated rather than inhibited the enzyme. It was supposed that the Bunte salt must have a much different affinity for metal ions than the other compounds of the series, *i.e.*, amino (or guanidino) thiols, trithiocarbonates, and dithiocarbamates; therefore, the metal-binding abilities of aminoethane and aminopropane thiosulfates, as well as of cyanoethane and cyanomethane thiosulfates, were determined.

METHODS

Materials—Melting points were taken in capillaries using a Mel-Temp apparatus. Elemental analyses were obtained.[1] 2-Aminoethanethiosulfuric acid[2] was recrystallized from methanol; m.p. 207–209° dec. The preparations of sodium 1-cyanomethanethiosulfate monohydrate and sodium 2-cyanoethanethiosulfate monohydrate were previously described (7).

3-Aminopropanethiosulfuric Acid—The method of Kaluszyner *et al.* (8) was used. A solution of 15.3 g. (0.07 mole) of 3-bromopropylamine hydrobromide[3] and 17.4 g. (0.07 mole) of sodium thiosulfate pentahydrate in 50 ml. of water was refluxed for 75 min. The solution was cooled, diluted with 150 ml. of ethanol, and chilled overnight. The product crystallized in long needles, and 6.4 g. (53%) was obtained and recrystallized from methanol; m.p. 189–190° dec.

Anal.—Calcd. for $C_3H_9NO_3S_2$: C, 21.05; H, 5.26; N, 8.19. Found: C, 21.51; H, 5.58; N, 8.12.

Analytical reagent grade $CuCl_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, and $Fe(NO_3)_3 \cdot 9H_2O$ were used, and carbonate-free 0.01 *M* potassium hydroxide was prepared according to Armstrong (9). The solutions were stored in polyethylene bottles under nitrogen and diluted quantitatively with boiled distilled water just prior to use. Normalities were checked against potassium biphthalate.

The purity of the organic ligands was ascertained by TLC. Eastman chromatogram sheets were spotted with approximately 0.1% solutions of the compounds in water. The developing solvent contained water and methanol (1:9). When the solvent front was 1.27 cm. (0.5 in.) from the top of the sheet, the sheet was air-dried and placed in a jar saturated with iodine vapor. The presence of one spot confirmed the absence of contaminating compounds.

Ionization Constants—The method employed was that of Albert and Serjeant (10), using a Beckman research pH meter with glass and calomel electrodes. It consisted of titrations of 0.001 *M* aqueous solutions of the compounds with acid and base of such strength that 5 ml. of the titrant contained an equivalent. The titrant was added in 0.5-ml. or 0.25-ml. portions, and the pH was recorded after each addition. Each titration yielded nine pH values, giving nine values for the pKa which were averaged. Where pH values fell outside the range of 5–9, corrections were made for hydrogen- or hydroxyl-ion concentration. The pKa values obtained are listed in Table I.

Stability Constants—Potentiometric titrations were carried out under nitrogen in freshly boiled distilled water at 25° using the described equipment. Fifty-milliliter volumes containing 0.001 mole of the thiosulfates were titrated with 0.01 *N* potassium hydroxide in 0.5-ml. portions, first in the absence of metal ions and then in the presence of 0.0005 mole of divalent metal salt or 0.00033 mole of trivalent metal salt. Titration with 0.01 *N* hydrochloric acid also was carried out with the thiosulfate–$Fe(NO_3)_3$ solutions, since all the necessary values were not uncovered by titration with alkali. Fifty-milliliter volumes of the same quantities of the metal salts were also titrated with 0.01 *N* potassium hydroxide. The pH readings were recorded 2 min. after each addition of titrant to allow equilibrium to be reached. Some values of K_2 or K_3 were not obtained because of precipitation of the metal complexes.

Calculations were done as previously described (11); values for log K_1, log K_2, log K_3, and either log β_2 or log β_3 are recorded in Table II. Values for K_1, K_2, and K_3 were obtained from Eqs. 1–3

Table I—Ionization Constants (25°)

Acid	pKa_1	pKa_2
2-Aminoethanethiosulfuric	9.15	3.12
3-Aminopropanethiosulfuric	9.76	3.16
1-Cyanomethanethiosulfuric	3.53	
2-Cyanoethanethiosulfuric	3.44	

[1] Weiler and Strauss, Oxford, England.

[2] Supplied by Dr. T. R. Sweeney, Walter Reed Army Institute of Research, Washington, D. C.

[3] Aldrich Chemical Co.

Table II—Stability Constants of Metal-Ion Complexes (25°)

Acid	Cu (II) Complexes: Log K_1	Log K_2	Log β_2[a]	Log β_2[b]
2-Aminoethanethiosulfuric	5.58			
3-Aminopropanethiosulfuric	6.34	6.07	12.41	12.21
	Al (III) Complexes			
	Log K_1	Log K_2	Log K_3	Log β_3[a]	Log β_3[c]
2-Aminoethanethiosulfuric	7.50	7.20	7.08	21.78	22.28
3-Aminopropanethiosulfuric	8.10	7.97	7.84	23.91	24.91
1-Cyanomethanethiosulfuric	1.68	1.20			
2-Cyanoethanethiosulfuric	1.88	1.71	1.68	5.23	5.07
	Fe (III) Complexes			
	Log K_1	Log K_2	Log K_3	Log β_3[a]	Log β_3[c]
2-Aminoethanethiosulfuric	9.65	8.91	8.69	27.25	27.49
3-Aminopropanethiosulfuric	10.15	9.66	9.49	29.30	29.25
1-Cyanomethanethiosulfuric	2.56	2.40			
2-Cyanoethanethiosulfuric	3.25	2.90	2.77	8.92	8.69

[a] Determined from the sum of the log K values. [b] Determined from Eq. 4. [c] Determined from Eq. 5.

according to Flood and Loras (11) and Albert (12):

$$K_1 = \frac{n}{(1 - \bar{n})(L^-)} \quad \text{(Eq. 1)}$$

$$K_2 = \frac{(\bar{n} - 1)}{(2 - \bar{n})(L^-)} \quad \text{(Eq. 2)}$$

$$K_3 = \frac{(\bar{n} - 2)}{(3 - \bar{n})(L^-)} \quad \text{(Eq. 3)}$$

Values for log β_2 and log β_3 were obtained from the sum of the log K values. In cases where the difference between values of log K_1 and log K_2, or between log K_2 and log K_3, was less than 0.5, log β_2 and log β_3 were obtained from Eqs. 4 and 5 (13):

$$\log \beta_2 = \log \bar{n} - \log (2 - \bar{n}) - 2 \log (L^-) \quad \text{(Eq. 4)}$$

$$\log \beta_3 = \log \bar{n} - \log (3 - \bar{n}) - 3 \log (L^-) \quad \text{(Eq. 5)}$$

Formation curves were plotted ($\bar{n}$ *versus* $-\log [L^-]$) to show whether stepwise complexation had taken place (13). Figure 1 shows formation curves for 3-aminopropanethiosulfuric acid with Cu (II), Al (III), and Fe (III) ions, which are also typical of the other systems reported here. Since the stability constants for the binding of successive molecules of ligand are not greatly different, no steps are shown in the plots.

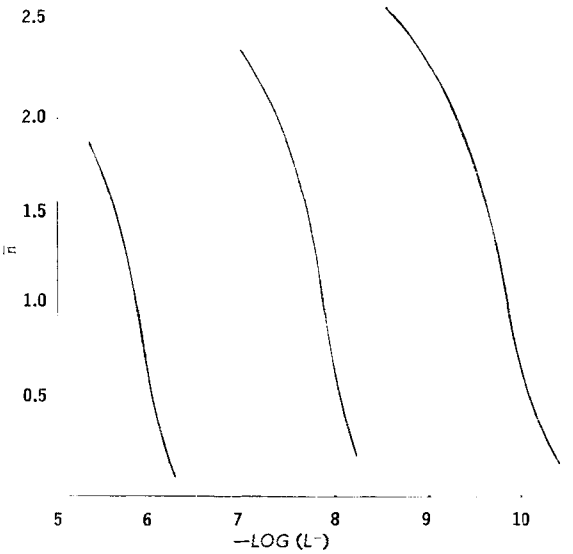


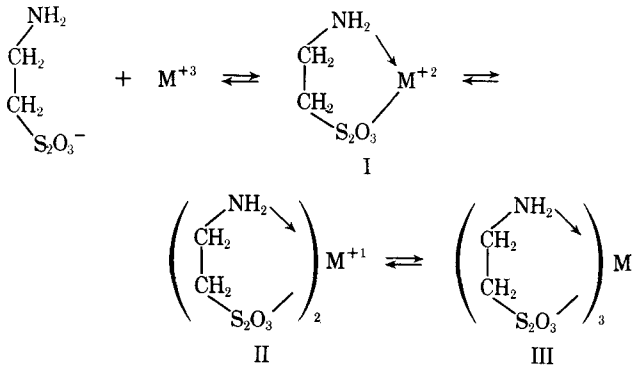
Figure 1—*Formation curves for the 3-aminopropanethiosulfuric acid systems with Cu (II), Al (III), and Fe (III) ions at* $-\log$ (L^-) *values of 6, 8, and 10, respectively.*

RESULTS AND DISCUSSION

The sequence of metal-binding abilities of the organic thiosulfates for cupric, aluminum, and ferric ions in order of decreasing strength was: 3-aminopropanethiosulfuric acid > 2-aminoethanethiosulfuric acid > 2-cyanoethanethiosulfuric acid > cyanomethanethiosulfuric acid. An appreciable difference between the stability constants for the aminoalkane derivatives and those of the cyanoalkane derivatives indicates that the amino groups are involved in the metal binding with the formation of chelates, whereas the cyano groups are probably not involved. Comparison with the metal-binding strength of thiosulfate ion for ferric ion shows a comparable value (log K_1 = 2.65) (14) to that for the binding of ferric ion by the cyanoalkane derivatives (log K_1 = 3.25, 2.56), which indicates little or no contribution from the cyano group.

A similar comparison for the aluminum and cupric ions cannot be made, since no values are recorded for the thiosulfate–Al(III) system, and cupric ion is reduced by thiosulfate ion. Recorded values for the thiosulfate–Cu(I) system, however, in terms of log βn (11.69–14.30) (5), are very similar to those (log β_2 = 12.41) for the 3-aminopropanethiosulfuric–Cu(II) system. This suggests that the amino group is not participating in the binding of cupric ion, at least, and that the Cu(II) is being reduced to Cu(I) in the complex. Stability constants for Cu(I) generally have been higher than those for Cu(II) for a given ligand, where such a comparison has been possible (5), so the close similarity in constants may be taken as a good indication that the organic thiosulfate–copper complexes include Cu(I) ions. Sufficient values for the cyanoalkanethiosulfuric–Cu(II) systems were not obtained to give reliable constants.

Thiosulfate ion is usually unidentate, but it may also be bidentate (15). When unidentate, it is believed to be S-bonded to metal ions, but coordination through both oxygen and sulfur is assumed for bidentate thiosulfate ion (16). Because of the relatively strong binding of thiosulfate ion for Cu(I) and the similarity in binding strengths between thiosulfate ion and the 3-aminopropanethiosulfuric acid, it may be concluded that binding with copper ion by the organic thiosulfates is wholly through the anionic thiosulfate group rather than the central sulfur. With Al(III) and Fe(III) ions, however, the amino function of the organic thiosulfates can be assumed to contribute to the binding; chelate structures (I–III), involving both coordination and ionic bond formation, may be proposed for these complexes (Scheme I).



Scheme I

Comparison of log K_1 values for binding of Cu(II) by a dithiocarbamate, aminoalkanethiol, and aminoalkanethiosulfuric acid shows the latter to be much weaker in binding strength. Diethyldithiocarbamate had a log K_1 value for Cu(II) of 14.9 (17), 2-mercaptoethylamine had a value of 10.05 for Ni(II) [generally comparable to Cu(II) in binding ability] (18), whereas 2-aminoethanethiosulfuric acid had a value of 5.58 for Cu(II). This relatively lower binding ability could account for the failure of 2-aminoethanethiosulfuric acid to inhibit catalase (6), whereas the dithiocarbamate and amino thiol were strong inhibitors.

No indication that organic thiosulfates act as radiation-protective agents through metal-binding ability may be drawn from this study.

However, 2-aminoethanethiosulfuric acid has good protective ability (2), whereas the cyanomethanethiosulfuric acid has none (19). It may be more significant that the thiosulfates can stabilize Cu(I) ion by complex formation; it has been proposed that one mechanism of radiation protection involves stabilization of the valence state of copper in copper-containing enzymes (20) or other macromolecules (21).

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Serum Levels of Chloramphenicol in Children, Rhesus Monkeys, and Cats after Administration of Chloramphenicol Palmitate Suspension

SACHCHIDANANDA BANERJEE, ASOK BANDYOPADHYAY, RAMESH CHANDRA BHATTACHARJEE, ARUN KUMAR MUKHERJEE, and ARUP KUMAR HALDER

Abstract □ The absorption of amorphous and polymorph A forms of chloramphenicol palmitate suspensions made with polysorbate 80 was investigated in children, rhesus monkeys, and cats. In children, both forms were absorbed. While polymorph A was absorbed at a slower rate, maintaining the blood level of chloramphenicol for 6 hr., the amorphous form was absorbed maximally at 2 hr. followed by a profound fall in the blood level. Although the absorption of the amorphous form was definitely superior, the polymorph A form in suspension with a surface-active agent was also absorbed to a considerable extent in children, maintaining blood levels of chloramphenicol for a prolonged period. In experiments with cats, polysorbate 80 increased the absorption of both forms of chloramphenicol palmitate, with the amorphous variety having superior absorption. In rhesus monkeys, there was little absorption of the polymorph A form of chloramphenicol palmitate in suspension, possibly indicating the absorption as species specific.

Keyphrases □ Chloramphenicol serum levels—following chloramphenicol palmitate suspension administration, in children, rhesus monkeys, cats □ Polysorbate 80—effects on absorption of chloramphenicol palmitate (amorphous and polymorph A forms), in cats □ Serum levels, chloramphenicol, amorphous *versus* polymorph A forms—comparison in children, rhesus monkeys, cats

Three polymorphic forms of chloramphenicol palmitate have been described; two are crystalline, termed α and β, and one is amorphous. In the solid state, transition occurs from α to β and is irreversible (1). These crystalline forms differ in their physicochemical properties (2). The IR spectrum of the amorphous form is identical to that of the α-type of crystals; but that of the β-type crystals, also termed polymorph A, is different. By using a relative absorbancy ratio of 862–864 $cm.^{-1}$, characteristic bands for α- and β-type crystals and the relative amounts of both types in a mixture can be determined (3).

Contradictory reports appear about the absorption of the different forms of chloramphenicol palmitate or stearate. Altmann *et al.* (4) determined blood levels of chloramphenicol in human adults after feeding chloramphenicol and chloramphenicol palmitate, as large or small crystals, and chloramphenicol palmitate incorporated with surface-active agents. They observed that both the administration of chloramphenicol and chloramphenicol palmitate with surface-active agents yielded the highest blood levels of chloramphenicol, whereas the blood levels following chloramphenicol palmitate as large crystals were definitely lower for the first 4 hr. There was no appreciable difference in the blood levels of chloramphenicol at 6- and 8-hr. intervals following administration of chloramphenicol and the various preparations of chloramphenicol palmitate.

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Table I—Serum chloramphenicol (mcg./100 ml.) of Children and Rhesus Monkeys Administered Suspensions of Chloramphenicol Palmitate Equivalent to 250 mg. Chloramphenicol

Chloramphenicol Palmitate Suspension Used[a]	Hours after Feeding Chloramphenicol Palmitate Suspension			
	2	4	6	8
Children				
Amorphous, 100%	1018 ± 127	604 ± 39	417 ± 43	263 ± 16
Range of values	793–1607	429–776	258–518	240–296
	(7)	(7)	(7)	(4)
Polymorph A, 100%	344 ± 22	347 ± 56	567 ± 66	234 ± 24
Range of values	263–481	207–484	422–762	180–337
	(10)	(4)	(7)	(7)
Rhesus Monkeys				
Amorphous, 100%	675 ± 120	389 ± 83	183 ± 12	
Range of values	454–1243	245–806	132–227	
	(7)	(7)	(7)	
Polymorph A, 100%	223 ± 38	173 ± 21	170 ± 44	
Range of values	108–394	108–276	108–282	
	(7)	(7)	(7)	

[a] Values = mean ± standard error. Figures in parenthesis indicate the number of subjects or animals.

Menachenoff (5) observed that crystals of chloramphenicol palmitate that could be hydrolyzed within 45 min. were absorbed to give blood levels comparable with chloramphenicol *per se* at equivalent dosage. Dony and de Roeck (6) reported that certain suspensions where the palmitate appeared under the microscope as fine needles or submicro crystals were hydrolyzed completely by pancreatin in 4 hr. and were well absorbed by the rabbit. In experiments with rats, Almirante *et al.* (7) observed that only the amorphous form of chloramphenicol stearate was hydrolyzed in the organism, liberating the antibiotic, whereas the crystalline form was therapeutically inert. They also reported that blood levels obtained in man after administration of a syrup containing chloramphenicol stearate with polysorbate 80 as emulsifier were equal to those obtained with a tablet of chloramphenicol. Aguiar *et al.* (8) reported that suspensions containing only α or amorphous chloramphenicol palmitate gave higher blood levels in adult male human volunteers following a single oral dose than did suspensions containing only β crystals of chloramphenicol palmitate (termed polymorph A). They did not find any influence of surface-active agents on the absorption of chloramphenicol palmitate. They observed that increasing the particle size of chloramphenicol palmitate to approximately 25 μ had little effect on the blood levels. According to the U. S. Food and Drug Administration Regulations (9), the limit of polymorph A (β-crystals) in chloramphenicol palmitate suspension should not exceed 10%.

In view of the conflicting reports about the absorption of chloramphenicol palmitate and in the absence of any report on such studies in children where palmitate suspensions are ordinarily used due to the absence of the bitter taste of chloramphenicol, this study was conducted on the absorption of amorphous and polymorph A forms of chloramphenicol palmitate in children. Comparative studies were also undertaken in rhesus monkeys and cats.

EXPERIMENTAL

Materials—Amorphous chloramphenicol palmitate was prepared from commercial chloramphenicol palmitate (10). Polymorph A chloramphenicol palmitate was prepared by dissolving commercial chloramphenicol palmitate in benzene and recrystallizing in the cold condition (11). The purity of the substance was determined by IR spectrophotometry. Suspensions of both 100% amorphous and 100% polymorph A forms of chloramphenicol palmitate were prepared using polysorbate 80 at the 2% level as the dispersing agent. The particle-size ranges of the various drug lots were 5–10 μ.

Children—Suspensions of chloramphenicol palmitate, equivalent to 250 mg. chloramphenicol, were administered orally to children aged 5–7 years in the basal condition. Blood samples were collected at 2-hr. intervals for 8 hr. after the administration of the suspension.

Rhesus Monkeys—Rhesus monkeys of 5 kg. average weight were fed suspensions of chloramphenicol palmitate equivalent to 250 mg. chloramphenicol in the early hours of the morning after a fast for 16 hr. Blood samples were withdrawn from the femoral vein at intervals of 2 hr. for 6 hr. after the administration of chloramphenicol.

Cats—Six cats, weighing 2.5–3 kg., were anesthetized by intramuscular injection of sodium phenobarbital, 150 mg./kg., to facilitate the feeding of the drug and the collection of blood samples. Through a stomach tube they were fed chloramphenicol, chloramphenicol palmitate amorphous or polymorph A, and suspensions of amorphous or polymorph A forms of chloramphenicol palmitate prepared with polysorbate 80 at the 2% level. The dose of chloramphenicol or chloramphenicol palmitate was equivalent to 42 mg. chloramphenicol/kg. body weight. Blood samples were withdrawn from the femoral vein at intervals of 2 hr. for 6 hr. after drug administration.

Serum of the blood samples was used for the estimation of chloramphenicol (12).

RESULTS

Children—The rise in the blood levels of chloramphenicol after administration of suspensions containing the polymorph A form of chloramphenicol palmitate was much less at 2 and 4 hr. as compared to blood levels with amorphous palmitate suspension. At 6 hr. the blood level of chloramphenicol was higher with the polymorph A suspension. At 8 hr. there was no difference between the blood levels with either form of chloramphenicol palmitate. The results are shown in Table I.

Monkeys—There was little rise in the blood levels of chloramphenicol when suspensions of polymorph A chloramphenicol palmitate were fed to monkeys (Table I).

Cats—The rise in the blood levels of chloramphenicol was less marked and of comparable degree after the feeding of raw amorphous or polymorph A forms of chloramphenicol palmitate. Suspensions of these preparations with polysorbate 80 produced higher blood levels of chloramphenicol. Suspensions of amorphous chloramphenicol palmitate produced blood levels of chloramphenicol much higher than those produced by either suspension of polymorph A chloramphenicol palmitate or chloramphenicol itself. The results are given in Fig. 1.

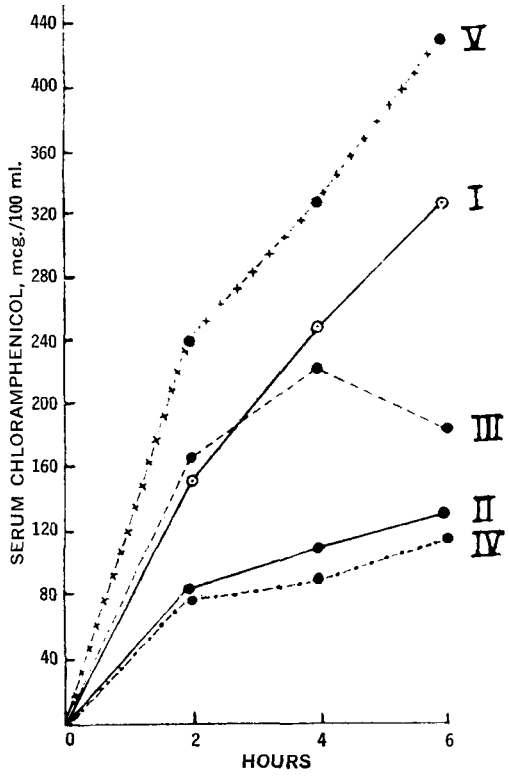

Figure 1—*Serum chloramphenicol after administration of chloramphenicol or its equivalent of chloramphenicol palmitate in cats. Key: I, animals fed chloramphenicol; II, animals fed chloramphenicol palmitate, polymorph A crystals; III, animals fed chloramphenicol palmitate, polymorph A suspension with polysorbate 80; IV, animals fed chloramphenicol palmitate, amorphous; and V, animals fed chloramphenicol palmitate, amorphous suspension with polysorbate 80.*

DISCUSSION

There were biological variations in the individual blood levels of chloramphenicol in both children and animals studied. But these variations were not inordinately high or low, as indicated by the standard error of the mean values and the range of values given in Table I.

The presence of chloramphenicol in blood of children after administration of suspensions of either amorphous or polymorph A varieties of chloramphenicol palmitate indicated that both these forms were absorbed. With polymorph A the rise in the blood chloramphenicol was gradual, and the maximum was reached 6 hr. after administration. With the amorphous suspension the rise was maximum at 2 hr. followed by a gradual decline. Due to practical difficulties in the collection of urine from children, urinary excretion of chloramphenicol could not be studied. This would have furnished further information about the absorbability of different forms of chloramphenicol.

Experiments with cats indicated that surface-active agents had a profound influence on the absorption of different forms of chloramphenicol palmitate. While the amorphous form was superior to crystalline polymorph A in raising the blood level of chloramphenicol, the latter was absorbed considerably when administered with a surface-active agent such as polysorbate 80.

Observations with children and cats were contrary to those of Aguiar *et al.* (8) in adult human males. Possibly the mechanism of absorption of chloramphenicol palmitate was different for adult males (8) and for the adult rhesus monkeys in this study where chloramphenicol palmitate polymorph A crystal administration with surface-active agents resulted in little absorption.

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Synthesis of Azetidines

J. N. WELLS and O. REED TARWATER

Abstract □ The synthesis of 3-amino-2-phenylazetidine and the *p*-chloro analog is reported. The reduction of azetidinones with diborane was investigated and found successful with *N*-unsubstituted systems. 3-Amino-2-phenylazetidine was found active *in vitro* against monoamine oxidase.

Keyphrases □ Azetidines (3-amino-2-phenylazetidine, *p*-chloro analog)—synthesis, monoamine oxidase inhibitory activity evaluated *in vitro* □ Monoamine oxidase inhibitors—azetidines, synthesized, evaluated *in vitro* □ IR spectrophotometry—structure, identification □ NMR spectroscopy—structure, identification

It has been postulated that the potent monoamine oxidase (MAO) inhibitory activity exhibited by 2-phenylcyclopropylamine might be explained in terms of the electronic and steric properties of the cyclopropane ring system (1, 2). Larger cycloalkyl analogs including 2-phenylcyclobutylamine are much less active MAO inhibitors, presumably for steric reasons and because of a change of electronic properties of the ring system. Relative to 2-phenylcyclopropylamine, 2-phenylcyclobutylamine is only $^1/_{1000}$ times as active by *in vitro* testing for MAO inhibition (3). Since the geometrical factors are approximately constant, this difference in activity is presumably due to the loss of electronic delocalization.

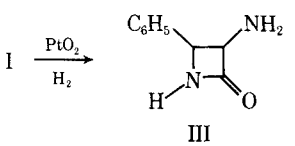
To investigate this theory, it appeared that heterocyclic analogs of 2-phenylcyclobutylamine, in which the hetero atom could provide the electron density, would merit study for MAO inhibitory activity.

As a continuation of a study of small ring heterocycles (4–6) as potential MAO inhibitors, several azetidines were prepared by reductive methods. This article reports the use of lithium aluminum hydride to reduce 3-amino-4-phenyl-2-azetidinone to the azetidine without ring cleavage. It also reports the first use of diborane to reduce 2-azetidinones to the corresponding azetidines.

As previously reported (4), *cis*-3-azido-4-phenyl-2-azetidinone (I) may be prepared from azidoacetyl chloride and hydrobenzamide. When treated with lithium aluminum hydride, I gave rise to a mixture of 3-amino-2-phenylazetidine (II, Scheme I) and *N*-benzylethylenediamine.



Scheme I



Scheme II

Catalytic reduction converted I into 3-amino-4-phenyl-2-azetidinone (III, Scheme II) in good yield.

Reaction of III with lithium aluminum hydride in ether gave 3-amino-2-phenylazetidine (II) in 84% yield. No evidence of *N*-benzylethylenediamine was found in the reaction mixture. The NMR spectrum of II showed a five-proton aromatic signal at δ 7.32, a one-proton doublet at 5.0 (methine), a two-proton doublet at 3.98 (NH—C$\underline{H}_2$), a one-proton doublet at 3.20 (C$\underline{H}$NH$_2$), and a three-proton singlet at 1.88 (NH and NH$_2$) which was exchangeable with D_2O. This spectrum is in agreement with the deduced spectrum of II in a mixture with *N*-benzylethylenediamine (4).

Only *N*-benzylethylenediamine was isolated when tetrahydrofuran (THF) was used in the hydride reduction. The downfield doublet at δ 5.0, characteristic of II, was absent in the NMR of the crude reaction mixture. The only apparent difference in these two systems is that III is soluble in THF and relatively insoluble in ether.

Diborane has been used to reduce various primary, secondary, and tertiary amides (7), pyrrolidinones (8), and azides (9); thus it seemed germane to attempt the reduction of both III and I with diborane. When III was reacted with excess diborane in THF, followed by hydrolysis with dilute hydrochloric acid, a 66.5% yield of II was obtained. When I was reacted with excess diborane, a 65% yield of II was obtained, both the azido and the carbonyl functions being reduced in one step. The NMR spectrum of the product of each of these diborane reductions was identical with that of II produced by lithium aluminum hydride reduction of III. The mass spectrum showed a parent peak at *m/e* 148 and a fragmentation pattern consistent with 2-phenylazetidines (10). This reaction also proved successful in the preparation of 3-amino-2-(*p*-chlorophenyl)-azetidine.

To examine the diborane reduction of other azetidinones, 4-phenyl-2-azetidinone (11) was reacted with diborane. 2-Phenylazetidine was isolated in 81% yield. The IR spectrum of the concentrated reaction mixture showed absence of the carbonyl, indicating that the reduction was complete.

Examination of the literature reveals that there is no method available for reduction of *N*-substituted azetidinones to the corresponding azetidines. Reduction of *N*-substituted azetidinones with lithium aluminum hydride, Raney nickel, lithium aluminum hydride–aluminum chloride, and sodium borohydride–aluminum chloride all result in cleavage of the 1–2 bond to give the substituted 3-aminopropanols (12). It was hoped that diborane could be used to reduce *N*-substituted

azetidinones to the azetidine without ring cleavage. Thus, 1,4-diphenyl-2-azetidinone (13) was reacted with diborane to give a 53% yield of 3-anilino-3-phenylpropanol. Authentic 3-anilino-3-phenylpropanol was prepared (14). Mixed melting point showed no depression, and the IR and NMR spectra were identical. The diborane reduction of 1,4-diphenyl-2-azetidinone was repeated and hydrolyzed with water to avoid the acidic workup. This system also gave rise to 3-anilino-3-phenylpropanol as the only isolated product.

Inhibition of MAO *in vitro* was assessed by the method of Wurtman and Axelrod (15). The potential MAO inhibitor and the substrate (2-^{14}C-tryptamine) were incubated with rat liver homogenate. After a suitable time, the deaminated product (^{14}C-indolacetic acid) was extracted and counted for its radioactive content. This preliminary evaluation showed 3-amino-2-phenylazetidine to be 62% as active as iproniazid which was used as a standard in this screen.

EXPERIMENTAL[1]

3-Amino-4-phenyl-2-azetidinone (III)—3-Azido-4-phenyl-2-azetidinone (I) (6.0 g., 32.3 mmoles) was dissolved in 300 ml. 95% ethyl alcohol and catalytically reduced over platinum at 50 p.s.i. for 72 hr. at room temperature. The catalyst was removed by filtration, and the solvent was removed *in vacuo*. Recrystallization from methylene chloride–petroleum ether gave 4.13 g. (80% yield) of white crystalline solid, m.p. 128–130°; NMR ($CDCl_3$), δ 7.30 (s, 5, Ar), 7.2–6.9 (bs, 1, NH), 4.82 (d, 1, methine), 4.42 (d, 1, CH-CHNH$_2$), and 2.01 (s, 2, NH$_2$); signals at 7.2–6.9 and 2.01 were exchangeable with D_2O; IR ($CHCl_3$), 5.65 (C=O), and complete absence of azide.

Anal.—Calcd. for $C_9H_{10}N_2O$: C, 66.59; H, 6.21. Found: C, 66.34; H, 6.36.

3-Amino-2-phenylazetidine (II) by Lithium Aluminum Hydride Reduction of III—III (780 mg., 4.81 mmoles) was stirred in 150 ml. absolute ether at 0° under nitrogen, while lithium aluminum hydride (910 mg., 24 mmoles) was added through Gooch tubing over a 1-hr. period. After the addition was complete, the ice bath was removed and the reaction was allowed to warm to room temperature and then was refluxed for 12 hr. The reaction mixture was cooled to 0° and hydrolyzed with 1.75 ml. of water. The solid was removed by filtration and washed several times with ether. The ether extracts were combined and dried. The ether was removed *in vacuo* to give 600 mg. (84% yield) of liquid. The IR showed complete absence of carbonyl. The dihydrochloride was formed and recrystallized from absolute ethanol, m.p. 154–156°.

3-Amino-2-phenylazetidine (II) by Diborane Reduction of III—To 50 ml. of THF was added 95 ml. of 1 *M* diborane in THF. To this solution was added (at 0° under nitrogen) 3.0 g. (18.5 mmoles) of III dissolved in 150 ml. THF over a 2-hr. period. After the addition was complete, the reaction was allowed to warm to room temperature and then was refluxed for 16 hr. The reaction mixture then cooled to room temperature and was hydrolyzed with 100 ml. of water followed by 50 ml. of 3 *N* HCl. The mixture was stirred for 1–2 hr., and then most of the THF was distilled off at atmospheric pressure and the remainder removed *in vacuo*. The aqueous phase was made basic with aqueous sodium hydroxide and extracted with chloroform. Removal of the dried chloroform *in vacuo* gave 1.8 g. (66.5%) of II; the NMR was identical to II produced by lithium aluminum hydride reduction of III. Recrystallization of the dihydrochloride from absolute ethyl alcohol gave a white solid, m.p. 155–158°.

3-Amino-2-phenylazetidine (II) by Diborane Reduction of 3-Azido-4-phenyl-2-azetidinone (I)—The reaction of 4.0 g. (21.4 mmoles) I in 225 ml. THF with 130 ml. 1 *M* diborane in THF, using the procedure for diborane reduction of III, gave a 63% yield of II; NMR was identical with II produced in both cases previously described. Recrystallization of the dihydrochloride gave a white solid, m.p. 158–159°.

Anal.—Calcd. for $C_9H_{14}Cl_2N_2$: C, 48.89; H, 6.38. Found: C, 48.64; H, 6.43.

3-Amino-2-(*p*-chlorophenyl)azetidine by Diborane Reduction of 3-Azido-4-(*p*-chlorophenyl)-2-azetidinone—The reaction of 2.0 g. (9.0 mmoles) 3-azido-4-(*p*-chlorophenyl)-2-azetidinone in 200 ml. THF with 80 ml. 1 *M* diborane in THF, using the procedure for diborane reduction of III, gave a 73% yield of 3-amino-2-(*p*-chlorophenyl)azetidine; NMR ($CDCl_3$), δ 7.35 (s, 4, Ar), 5.0 (d, 1, ArCH), 3.98 (d, 2, NHCH$_2$), 3.15 (d, 1, CHNH$_2$), and 2.34 (s, 3, NH and NH$_2$); exchangeable with D_2O. Recrystallization of the dihydrochloride from absolute ethyl alcohol gave a white solid, m.p. 130–133°.

Anal.—Calcd. for $C_9H_{13}Cl_3N_2$: C, 42.29; H, 5.13. Found: C, 41.99; H, 5.40.

2-Phenylazetidine—The reaction of 1.47 g. (10 mmoles) of 4-phenyl-2-azetidinone (11) in 60 ml. THF with 40 ml. 1 *M* diborane in THF gave an 81% yield of 2-phenylazetidine; NMR ($CDCl_3$), δ 7.3 (m, 5, Ar), 4.88 (t, 1, methine), 3.5 (bm, 2, NCH$_2$), 2.9 (s, 1, NH), and 2.45 (m, 2, CHCH$_2$); the signal at 2.9 exchanged with D_2O. The picrate was prepared, m.p. 122–123° [lit. (11) 128–129°]. The picrate of authentic 2-phenylazetidine was prepared (11), and mixed melting point of the two picrate derivatives showed no depression.

Diborane Reduction of 1,4-Diphenyl-2-azetidinone—The reaction of 2.27 g. (10 mmoles) 1,4-diphenyl-2-azetidinone (13) in 150 ml. THF with 40 ml. of 1 *M* diborane in THF gave a 53.8% yield of 3-anilino-3-phenylpropanol, m.p. 88.5–89.5 [lit. (14) 87–88°]. IR showed complete absence of carbonyl. NMR ($CDCl_3$), δ 7.46–6.4 (m, 10, Ar), 4.55 (t, 1, methine), 3.7 (t, 2, CH$_2$OH), 3.27 (s, 2, OH and NH), and 2.05 (q, 2, CHCH$_2$); the signal at δ 3.27 was exchangeable with D_2O.

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[1] Melting points were determined on a Buchi apparatus with open capillary tubes and are uncorrected. NMR spectra were obtained with a Varian Associates A-60-A instrument in $CDCl_3$ with tetramethylsilane as an internal standard. IR spectra were determined on Perkin-Elmer model 21 and model 237B spectrophotometers. Combustion analyses were conducted by Midwest Microlab, Inc., Indianapolis, IN 46226. The mass spectrum was determined with a Hitachi RMU-6A at 75 ev. THF was dried by distillation from lithium aluminum hydride immediately before use.

New Compounds: Synthesis of Certain *p*-Alkoxyphenylacetamide Derivatives as Potential Analgesic Agents

A. A. ABOU OAUF, M. A. ELDAWY*, and H. H. FARAG

Abstract □ A recent pharmacological screening of certain *p*-alkoxyphenylacetamides revealed their analgesic activity. This communication outlines the synthesis of some related compounds involving certain analgesically active pharmacophores which are thought to increase potency and decrease toxicity.

Keyphrases □ *p*-Alkoxyphenylacetamide derivatives—synthesized as potential analgesic agents □ Analgesic agents, potential—*p*-alkoxyphenylacetamide derivatives synthesized □ IR spectrophotometry—structure, identification

Nguyen *et al.* (1) investigated a series of *p*-alkoxyphenylacetohydroxamic acids. They found that the *p*-butoxyphenylacetohydroxamic acid (I) has anti-inflammatory activity and that its high analgesic potency exceeds that of aspirin, aminopyrine, and phenacetin.

In an extension of their investigation (2), it was found that the replacement of the hydroxamic acid moiety by an amide group abolishes the anti-inflammatory activity and enhances the analgesic potency. Experimental pharmacometrics proved that the *p*-*n*-butoxyphenylacetamide (II) is the derivative of choice.

This report describes the synthesis of certain new compounds designed to have the recently reported phenylacetamide moiety embodied together with other analgesically active pharmacophores in one and the same molecule.

The described compounds were prepared by condensation of the properly substituted acyl chlorides with the appropriate amines (3). The corresponding parent acids were synthesized by application of Willgerodt's reaction (4) on the respective *p*-alkoxyphenylketones. The latter were obtained *via* the Fries rearrangement (5) of the corresponding acyl phenols. The original conditions of the Fries rearrangement were modified to increase the yield of the desired *p*-substituted phenols. The synthesis was carried out as shown in Scheme I.

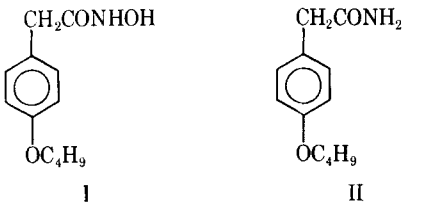
The structures of the prepared compounds (Table I) were assigned on the bases of reaction mechanisms, microanalytical data, and IR spectra (6).

EXPERIMENTAL[1]

4-Hydroxyacetophenones—These compounds were prepared according to the method of organic reactions (7), increasing the amount of nitrobenzene solvent and controlling the temperature throughout the reaction time so that it did not exceed 40°. This method led to a higher percentage of the desired *p*-isomers.

4-Alkoxyacetophenones—These compounds were prepared by Williamson's etherification synthesis (8) of the corresponding phenols with the appropriate alkyl halides.

4-Alkoxyphenylacetic Acids—In a 250-ml. round-bottom flask, 0.013 mole of 4-alkoxyacetophenone was mixed with 0.2 mole of morpholine and 0.19 mole of sulfur. The mixture was refluxed on an oil bath at 130° for 14 hr. and then poured slowly as a thin stream on cold water while being stirred until the yellowish mass, which was initially formed (crude *p*-alkoxyphenylthioacetomorpholide), solidified.



I II

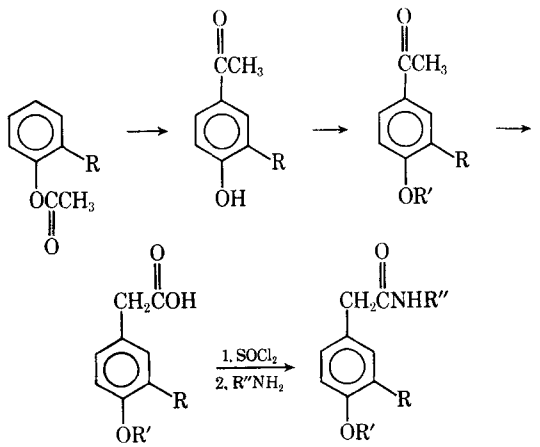
The crude product was isolated and triturated in a mortar with 20 ml. of water, filtered, and then refluxed with 250 ml. of 10% alcoholic sodium hydroxide in a 500-ml. round-bottom flask for 10 hr. Alcohol was then distilled over, and the residue was gradually acidified with dilute hydrochloric acid. The crude *p*-alkoxyphenylacetic acid was extracted with ether and obtained in a crude form after recovery of ether; it was then crystallized from *n*-hexane.

IR spectral analysis showed a broad peak in the range of 3300–2900 cm.$^{-1}$, which is usually assigned to hydrogen-bonded O—H stretching of —COOH, and an acid —CO— band around 1700 cm.$^{-1}$, and an ether —C—O—C band around 1250 cm.$^{-1}$.

4-Alkoxyphenylacetamide—(*A*) *Acid Chlorides*—In a 250-ml. round-bottom flask, fitted with a reflux condenser and a gas trap, 0.048 mole of 4-alkoxyphenylacetic acid was treated with 0.065 mole of thionyl chloride. Effervescence immediately occurred, and the mixture slowly changed into a greenish liquid. After gases ceased to evolve, the mixture was warmed gently and then heated on a water bath for about 1 hr. or until no more gases evolved. Excess thionyl chloride was removed by distillation under vacuum.

The produced acid chloride was vacuum distilled before being used in condensation with the appropriate amines.

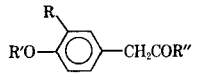
(*B*) *Amides*—In a 100-ml. round-bottom flask, 0.01 mole of the desired amine was dissolved in 10 ml. of ether in which 2.4 g. anhydrous potassium carbonate was suspended. A 0.01-mole solution of *p*-*n*-alkoxyphenylacetyl chloride in ether was added slowly, with constant shaking, to the amine solution. The mixture was then boiled under reflux for 30 min. Ether was distilled, 30 ml. of water was added to the residue, and the mixture was shaken vigorously. The solid was filtered and washed on the filter paper successively with cold dilute sodium hydroxide, water, warm dilute sulfuric acid,

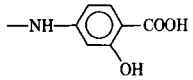
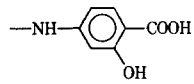


Scheme I

[1] Melting points are uncorrected. Elemental analyses were conducted by Janssen Pharmaceutica, Beerse, Belgium. IR absorption spectra were determined in a Beckman IR spectrophotometer model IR-4 at the Drug Research and Control Center, Cairo, U.A.R.

Table I—*p*-Alkoxyphenylacetamide Derivatives



No.	R	R′	R″	Anal., % Calcd.	Anal., % Found	Yield, %	M.p.[a]
1	H	C_4H_9	—NHC_6H_5	C, 76.32 H, 7.42 N, 4.9	C, 76.40 H, 7.61 N, 5.1	53.0	106–108°
2	H	C_4H_9	*p*-$NHC_6H_4CH_3$	C, 76.76 H, 7.74 N, 4.7	C, 76.75 H, 7.66 N, 4.8	55.2	130–131°
3	H	C_4H_9	*o*-$NHC_6H_4CH_3$	C, 76.76 H, 7.74 N, 4.7	C, 76.82 H, 7.85 N, 4.66	55.0	104–106°
4	H	C_4H_9	*p*-$NHC_6H_4OC_2H_5$	C, 73.39 H, 7.64 N, 4.28	C, 73.45 H, 7.57 N, 4.54	62.5	139–140°
5	H	C_4H_9	*p*-$NHC_6H_4COOC_2H_5$	C, 70.98 H, 7.04 N, 3.94	C, 70.49 H, 6.81 N, 3.97	57.0	132–133°
6	H	C_4H_9		C, 69.31 H, 8.30 N, 5.05	C, 69.53 H, 8.25 N, 5.10	50.0	67–68°
7	H	C_4H_9	—NH—$NHCONH_2$	C, 58.86 H, 7.17 N, 15.80	C, 58.83 H, 7.12 N, 15.58	40.0	178–180°
8	H	C_4H_9	*p*-NHC_6H_4COOH	C, 69.72 H, 6.42 N, 4.25	C, 70.01 H, 6.49 N, 4.45	58.0	264–266°
9	H	C_4H_9		C, 66.46 H, 6.12 N, 4.08	C, 66.22 H, 6.09 N, 3.86	15.5	228–230°
10	H	C_5H_{11}	*p*-NHC_6H_5	C, 76.76 H, 7.74 N, 4.71	C, 76.76 H, 7.70 N, 4.66	54	100–101°
11	H	C_5H_{11}	*p*-NHC_6H_4COOEt	C, 71.54 H, 7.31 N, 3.79	C, 71.60 H, 7.31 N, 3.68	42.0	129–130°
12	H	C_5H_{11}	*p*-$NHC_6H_4CH_3$	C, 77.17 H, 8.03 N, 4.50	C, 77.10 H, 8.08 N, 4.53	45.0	98–99°
13	H	C_5H_{11}	*p*-$NHC_6H_4CH_2COOH$	C, 70.98 H, 7.04 N, 3.90	C, 70.72 H, 7.01 N, 4.00	43.0	179–181°
14	H	C_5H_{11}	*p*-NHC_6H_4COOH	C, 70.38 H, 6.74 N, 4.10	C, 70.21 H, 16.73 N, 4.20	58.0	260–262°
15	H	C_5H_{11}		C, 67.22 H, 6.44 N, 3.92	C, 67.33 H, 7.47 N, 3.70	11.0	227–228°
16	CH_3	C_4H_9	—NH_2	C, 70.58 H, 8.59 N, 6.33	C, 70.16 H, 8.47 N, 6.66	46.0	131–132°
17	CH_3	C_4H_9	*p*-NHC_6H_4COOEt	C, 71.54 H, 7.31 N, 3.80	C, 71.19 H, 7.19 N, 4.00	37.9	135–136°

[a] All melting points are uncorrected.

and water again. The solid was then dried, dissolved in 95% alcohol, decolorized with charcoal, and finally filtered. The alcoholic solution was then concentrated and left to cool. The pure amide separated.

IR spectra showed amide frequency in the range of 3500–3200 cm.$^{-1}$, which was assigned to symmetric and asymmetric N—H stretching of the amide NH_2 group, a —C—O—C— band at 1300–1200 cm.$^{-1}$, and an amide —CO— band at 1675–1575 cm.$^{-1}$.

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Algebraic Extrapolation of Tie Lines in Ternary Phase Diagrams

Keyphrases ☐ Phase diagrams, ternary—algebraic extrapolation of tie lines ☐ Solid–liquid system—solid determination, phase diagrams ☐ Tie lines—extrapolation

Sir:

Binary and ternary phase diagrams are being increasingly applied to pharmaceutical systems to represent the phase relationships of solid–liquid systems (1–3). Most ternary systems contain both solids and liquids. Schreinemakers developed an experimental procedure, called the method of wet residues (4), for determining the composition of solid phases; this method eliminates the necessity of removing all of the adhering mother liquor. It consists of preparing two or more ternary mixtures of known composition or of analyzing for the compositions of the corresponding liquid phases. The composition of the *wet* solids is also determined by analysis. The composition of the dry, pure solid phase is then determined by the point of convergence of the extrapolated tie lines, each passing through the composition of the liquid phase and/or the composition of the initial mixture, plus the composition of the wet solid.

The composition of the pure solid phase common to all systems can be determined by graphical extrapolation or, with higher accuracy, by algebraic extrapolation. An existing procedure for the algebraic extrapolation of tie lines useful in calculating the results for the method of wet residues is described. Its limitations are discussed and the procedure is extended to yield an explicit equation, which can be used for a general case and eliminates the necessity of making any assumptions regarding the composition of the solid phase.

According to the Hill and Ricci method (5), the amount of one of the three components in the solid phase must be known or assumed. For instance, in a system containing solids A and B plus water, the solid must be known to be a dihydrate or to be anhydrous. A simple proportion between the differences in the amounts of two components at the two known points, and the differences in the amounts of the same components at one of those points and at the composition of the solid phase, results in the composition of the solid phase (5). For instance, if a mixture made up of components A and B plus water, containing 80% of water and 6.5% of A, gives a solution containing 90% of water and 7% of A, one can calculate the content of A in the solid phase, p_A, provided the solid is anhydrous, by the following proportion:

$$\frac{(7 - 6.5 = 0.5)}{(90 - 80 = 10)} = \frac{x}{(90 - 0 = 90)} \qquad \text{(Eq. 1)}$$

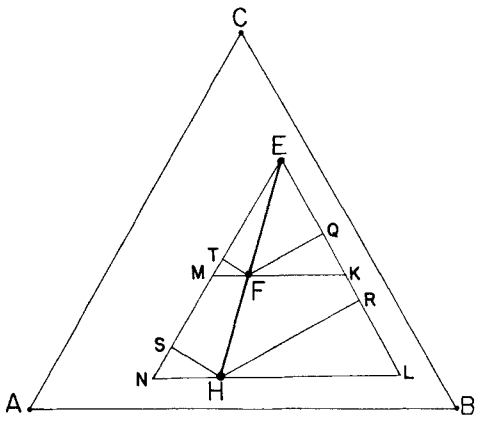

Figure 1—*Tie line* EFH *in the ternary phase diagram of components* A, B, *and* C.

Hence, $x = 4.5\%$ and $p_A = 7 - 4.5 = 2.5\%$.

Assuming the solid to be anhydrous eliminates one concentration variable; the composition of the solid phase can be determined from a single tie line. The concordance of the results can be assessed by departure of the data from the assumed composition (5).

This procedure has the following shortcomings. It becomes cumbersome for higher solvates and for liquid–liquid systems. It does not cover the case of several converging tie lines, nor does it give an explicit equation for the representation of a single tie line (6). The advantage of using explicit equations for converging tie lines is the possibility of calculating their common intersection by the method of least squares, thereby minimizing the errors of chemical analysis. Since a set of tie lines based on experimental data seldom converges sharply on a single point, the applicability of statistical methods is a desirable feature.

Such an equation is derived, following the considerations of Hill and Ricci (5). In Fig. 1, consider the tie line EFH in the Gibbs triangle ABC (3, 4), where E represents the composition of the solution in equilibrium with a solid phase of composition represented by H, and F is made to represent either the composition of the original mixture or of the wet residue. The contents of the two components, A and B, usually expressed as weight percentages, are p_{A1} and p_{B1} at E, p_{A2} and p_{B2} at F, and p_A and p_B at H. It is desired to calculate p_A and p_B, knowing the other four p values.

Through point E are drawn EKL parallel to the side BC, and EMN parallel to the side AC, so that MK and NL are parallel to the third side AB. The line FQ is drawn perpendicular to EL and to BC and represents, therefore, $p_{A2} - p_{A1}$ according to the properties of the Gibbs ternary phase triangle (3, 4). Likewise, HR is perpendicular to EL and to BC and represents $p_A - p_{A1}$; HS and FT are drawn perpendicular to EN and to AC and represent $p_B - p_{B1}$ and $p_{B2} - p_{B1}$, respectively. The four triangles FQK, HRL, NSH, and MTF are similar because they have the same angles; one

is 90° and a second 60° according to the construction of Fig. 1, so the third angle is 30°. This results in the proportion

$$(p_A - p_{A1})/(p_{A2} - p_{A1}) = (p_B - p_{B1})/(p_{B2} - p_{B1}) \quad \text{(Eq. 2)}$$

Multiplying out and simplifying result in

$$p_A p_{B2} - p_{A1} p_{B2} - p_A p_{B1} - p_{A2} p_B + p_{A1} p_B + p_{A2} p_{B1} = 0 \quad \text{(Eq. 3)}$$

This is identical with

$$\begin{vmatrix} p_A & p_B & 1 \\ p_{A1} & p_{B1} & 1 \\ p_{A2} & p_{B2} & 1 \end{vmatrix} = 0 \quad \text{(Eq. 4)}$$

This last expression is equivalent to those of another method of extrapolation (7), as it should be since both are solutions of the same problem. The present method has the advantage of being more direct. By applying Eq. 4 to two or more systems having convergent tie lines, the composition of the pure solid phase is obtained. If the data for several tie lines are available, the best fit for the composition corresponding to their common intersection can be calculated by least-squares analysis (8, 9). This minimizes the effect of random analytical errors.

(1) A. H. Goldberg, M. Gibaldi, and J. L. Kanig, *J. Pharm. Sci.*, **54,** 1145(1965).

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Received May 22, 1970.
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Compaction Effect on Flow Property Indexes for Powders

Keyphrases ☐ Flow property indexes—compaction effect ☐ Compaction effect—flow indexes accuracy

Sir:

Powder technologists are reluctant to suggest a single figure to describe the flow properties of a powder since it is recognized that many factors such as moisture, temperature, degradation, and time affect flow properties. Manufacturers, on the other hand, require a simple measure of the flow properties of their powders for process control—hence, the prevalent use of the much criticized angle of repose tests.

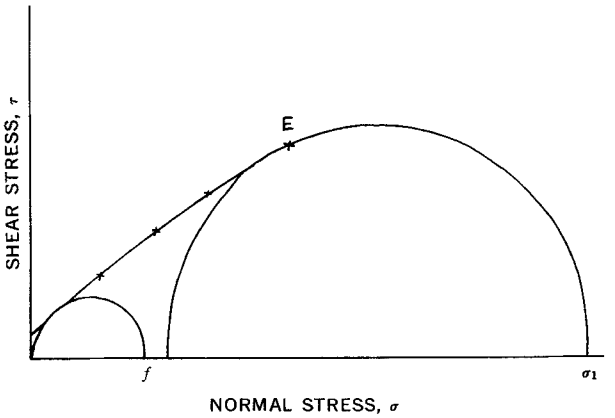


Figure 1—*Idealized Jenike yield locus.*

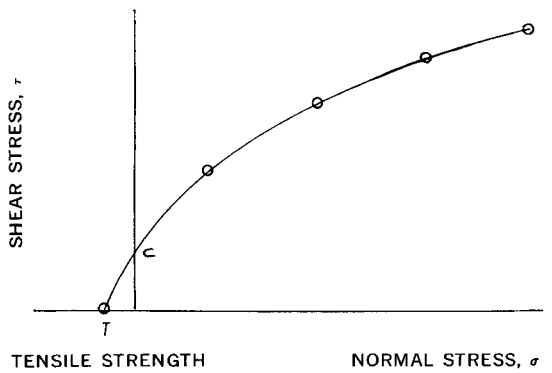
Two methods, both based on results obtained from a shear cell, have been suggested for characterizing powders with regard to their flow properties. The method of Jenike *et al.* (1) is called the flow factor, while that of Ashton *et al.* (2, 3) is termed the shear index.

Consider a yield locus, as shown in Fig. 1, which is obtained in the normal manner using a shear cell (1). Two Mhor circles are drawn, one passing through the origin and tangential to the locus and the other tangential to and passing through the end-point of the locus.

The basis of the Jenike flow factor, *ff*, is that the unconfined yield strength, f, of a powder is a function of the consolidating stress, σ_1, applied during the sample preparation. Small values of f in relation to the value of σ_1 will indicate a free-flowing material. The flow factor, *ff*, is taken as the ratio σ_1/f. It is found that values of *ff* generally range between 1 for highly cohesive powders and 10 for free-flowing powder.

If a family of yield loci are produced at a series of compaction levels and the values of f and σ_1 are measured for each locus, it is found that the flow factor usually increases with increasing compaction.

When the shear cell is used in conjunction with a tensile tester, the end-point of the locus on the negative portion of the normal stress axis (the tensile strength T) may be plotted. Thus the complete locus can be drawn as shown in Fig. 2. It was shown by Ashton *et al.* (2)



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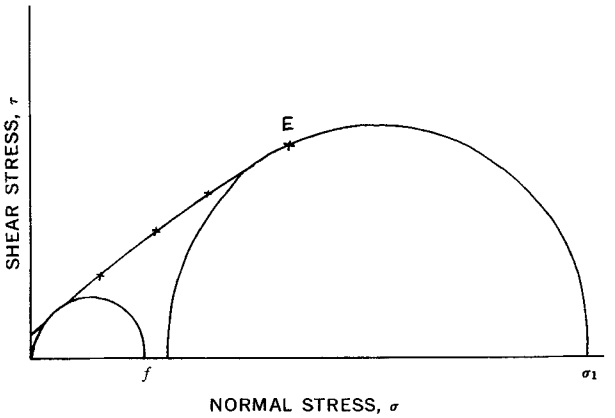

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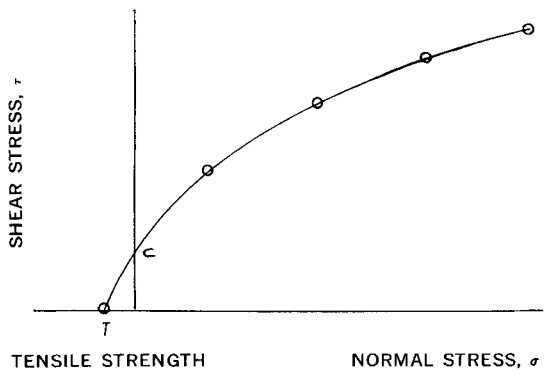

Figure 2—*Complete yield locus including tensile strength.*

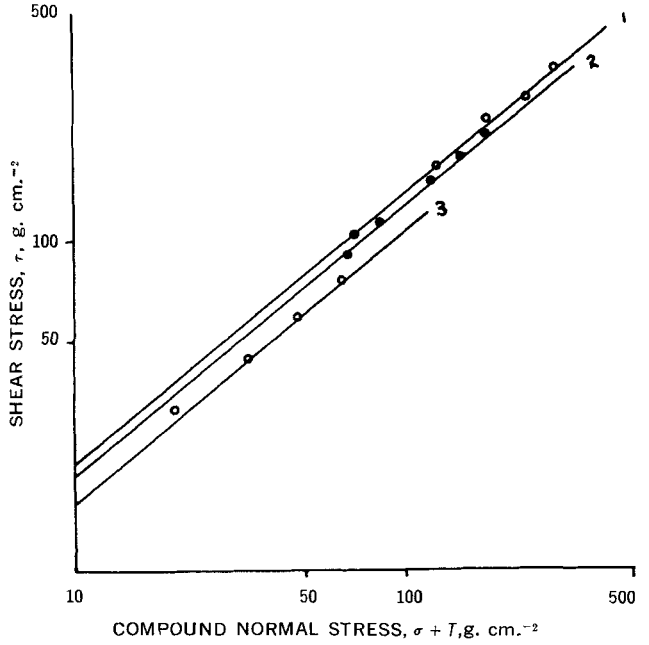

Figure 3—*Logarithmic plot of shear stress* versus *compound normal stress for griseofulvin. Bulk density: 1., 0.567 g. cm.$^{-3}$; 2., 0.542 g. cm.$^{-3}$; and 3., 0.488 g. cm.$^{-3}$.*

that the yield locus can be described by the equation:

$$\left(\frac{\tau}{c}\right)^n = \frac{\sigma + T}{T} \qquad \text{(Eq. 1)}$$

where τ = shear stress, g./cm.^{2}; σ = normal stress, g./cm.^{2}; c = cohesion, g./cm.^{2}; T = tensile strength, g./cm.^{2}; and n = shear index.

For a great many tests on a particular material, the value of n was reported to be independent of the bulk density of the sample. The value of n was generally found to be between 1 for free-flowing powders and 2 for very cohesive powders.

Certain differences are apparent in the results found between these authors. Jenike *et al.* (1) found that the flow potential may improve with increasing compaction,

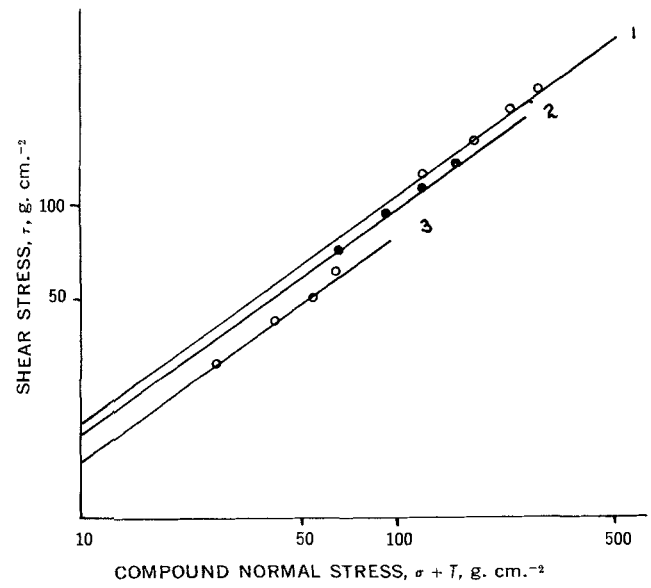

Figure 4—*Logarithmic plot of shear stress* versus *compound normal stress for lactose.*

Table I—Shear Index Value at Various Bulk Densities for Griseofulvin and Lactose

Griseofulvin		Lactose	
Bulk Density, g. cm.$^{-3}$	Shear Index, n	Bulk Density, g. cm.$^{-3}$	Shear Index, n
0.488	1.43	0.787	1.46
0.542	1.26	0.835	1.28
0.567	1.16	0.885	1.26

and this finding was substantiated by Walker (4) and the present author (5). Conversely, the results reported by Ashton *et al.* (2) suggest that the shear index is a constant, for a given powder, irrespective of the bulk density to which it is compacted.

The purpose of this communication is to point out that, in fact, the index of flow as measured by both methods does improve with increasing compaction.

Of the numerous papers on the use of the shear cell, the results of Williams and Birks (6) are particularly interesting because the normal load is reduced to the compound normal stress, that is $\sigma + T$. This results in the yield loci taking the form where all the loci have a common origin. If n were to remain constant, then all the loci would follow the same curve. It is obvious from the results of Williams and Birks (6) that the curvature is reduced with increasing compaction; thus, n must approach 1 and the flow properties must improve.

The point can be further made by considering the evidence of the measurements on a grade of lactose (average particle size 12.5 μ) and a fine grade of griseofulvin (average particle size 4 μ). Both samples were dried at 70° for 24 hr. in an air oven before being stored in vacuum desiccators until use.

The shear cell data were measured in the manner recommended by Jenike (7).

Equation 1 may be rearranged to give

$$\tau = \frac{c}{T^{1/n}}(\sigma + T)^{1/n} \qquad \text{(Eq. 2)}$$

By taking logarithms and plotting log τ *versus* log $(\sigma + T)$, the slope of the curves is $1/n$ and the intercept $\log c/T^{1/n}$.

The points have been plotted in Figs. 3 and 4 where it can be seen that, to a first approximation, all the curves are parallel and thus have the same value of n. However, on subjecting the values to computer analysis to find the best straight-line fit by the method of least squares, it is seen from Table I that the values of n indicate a more free-flowing condition with an increase of bulk density. This is the same result as is implicit in the treatment of Williams and Birks (6).

Thus, from the implicit data of other workers and from the experimental evidence presented here, it can be seen that both the flow factor and the shear index are in agreement in predicting an increase in the flow properties with an increase of bulk density. No explanation is offered in this brief communication, since it has merely been the purpose to point out the agreement between the two indexes. Further work is being undertaken to provide an explanation for the observed facts.

Finally, it is not suggested that a powder will be more free flowing when compacted than it is at its normal

bulk density. This is patently not true. However, its flow potential, as a relative measure, does improve as measured by the mentioned procedures.

(1) A. W. Jenike, P. J. Eysey, and R. H. Woolley, *Proc. ASTM*, **60**, 1168(1960).
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GLC Analysis of the Trimethylsilyl Derivative of 2,4-Dihydroxy-3,3-dimethylbutyric Acid γ-Lactone in Pantothenyl Alcohol

Keyphrases □ Pantothenyl alcohol—analysis □ 2,4-Dihydroxy-3,3-dimethylbutyric acid γ-lactone—determination in pantothenyl alcohol □ GLC—analysis

Sir:

The production of *d*-pantothenyl alcohol involves the reaction between 2,4-dihydroxy-3,3-dimethylbutyric acid γ-lactone (*l*-lactone) and 2-amino-*l*-propanol (1). For manufacturing purposes, it became desirable to know the amount of residual lactone present in *d*-pantothenyl alcohol. Initial attempts to quantitate the lactone content utilized a TLC method supplied by the manufacturer[1] (2). However, the method was not completely satisfactory.

The relative volatility of the lactone (sublimes) (3) favored the investigation of GLC as a means of analysis. The initial work involved injecting pyridine solutions of known amounts of *l*-lactone and the internal standard (2,6-dimethylphenol) directly onto the GLC columns. The resulting plot of concentration *versus* peak height ratio gave a negative *y*-intercept, which suggests irreversible adsorption of the lactone. In addition, analysis of a sample of pantothenyl alcohol produced an extraneous interfering peak, which we assumed to result from thermal degradation of the polyalcohol.

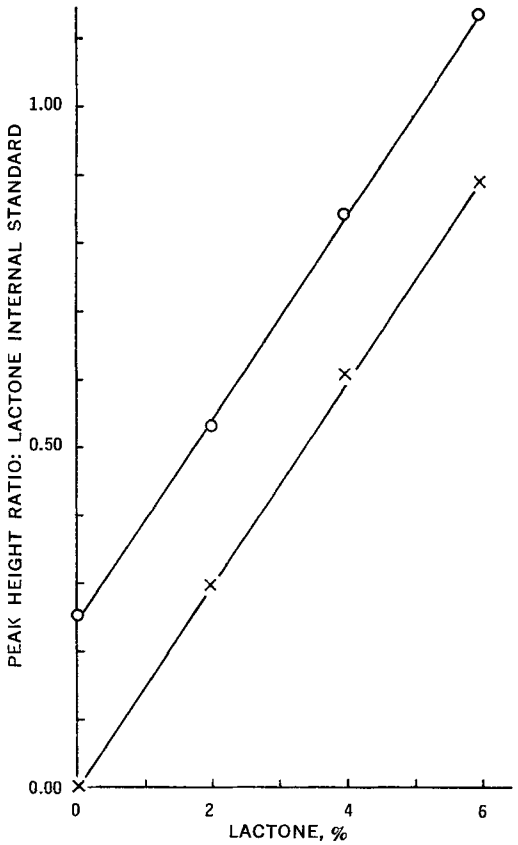

Figure 1—*Key:* ○, *lactone added to* d-*pantothenyl alcohol; and* ×, *lactone standards.*

In an attempt to obtain a suitable chromatographic moiety, the trimethylsilyl ether derivatives of known amounts of *l*-lactone were prepared and subsequently analyzed. 2,6-Dimethylphenol was used as the internal standard. The preparation of the silyl ethers is discussed. The resulting curve of concentration *versus* peak height ratio passed through the origin and was linear, as indicated in Fig. 1.

A series of samples was run in which known amounts of *l*-lactone were added to a sample of pantothenyl alcohol, and the percent recovery was determined using the recommended procedure. In Fig. 1, the slope of the curve for *l*-lactone added to panthenol is essentially the same as the slope of the curve obtained in the standard calibration method. This indicates that the recovery is linear and essentially 100% relative to the normal calibration procedure.

An indication of the precision was determined by assaying three aliquots of a given sample of panthenol. The mean percent lactone was found to be 2.88% w/w with a standard deviation of ±0.03% w/w. The 2.88% is within our specifications for the sample of panthenol studied. The method cannot be used for resolving the *l*- and *d*-isomers of the lactone.

Basically, the method consists of weighing exactly 100 mg. of pantothenyl alcohol into a suitable vial and smearing the sample around the lower inside portion of the vial with a glass rod. A 50-μl. aliquot of internal standard solution, prepared by dissolving 1.00 g. of

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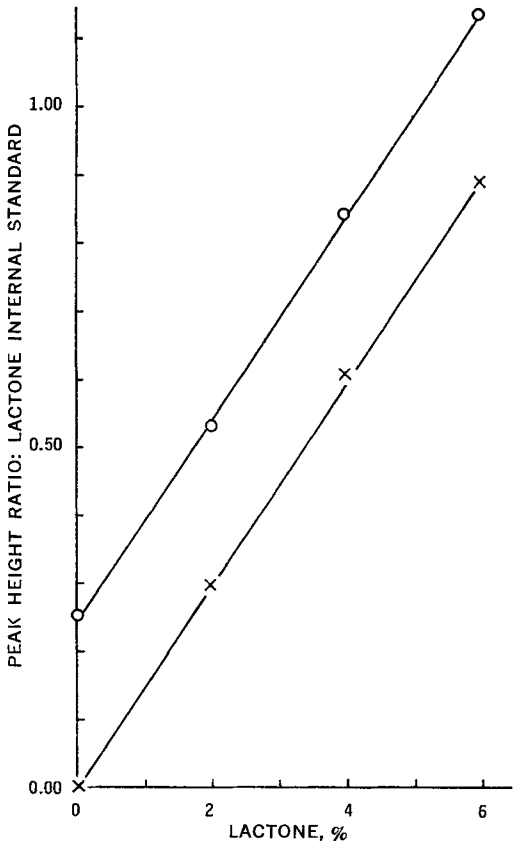

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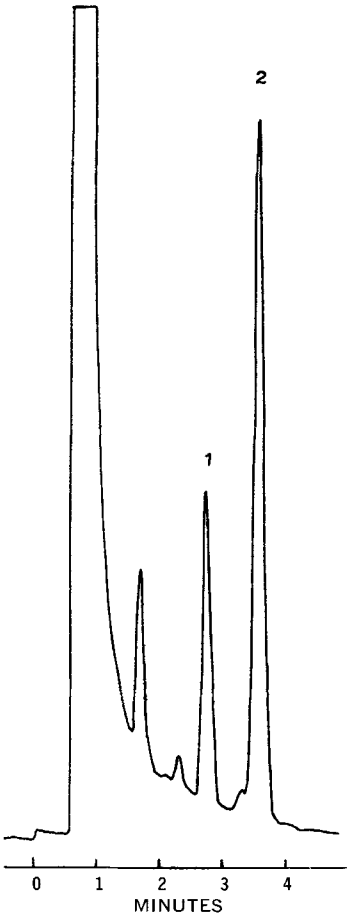

Figure 2—*Key: 1, lactone derivative* (R_T = *2.7 min.*); *and 2, internal standard* (R_T = *3.6 min.*).

2,6-dimethylphenol[2] in 10 ml. of benzene (A.R. grade), and 1 ml. (1 ampul) of Sil-Prep[3] reagent is added to the vial which is then stoppered, and the contents are shaken vigorously for at least 30 sec. Simultaneously, 2 and 6% w/w (in terms of pantothenyl alcohol) lactone standards are prepared by pipeting 0.2 ml. of the *l*-lactone standard solution, which is prepared by dissolving 100 mg. of *l*-lactone[4] in dichloromethane (A.R. grade) and diluting to 10.0 ml., into one vial and 0.60 ml. into a second vial. The solvent is evaporated from each sample by using a steady stream of dry air.

A 50-μl. aliquot of internal standard solution and 1 ml. (1 ampul) of Sil-Prep are added to each standard. The vials are stoppered, and the contents are shaken vigorously for at least 30 sec.

Each sample is chromatographed by injecting approximately 0.5 μl. of the specific sample into a GLC unit[5] containing a 1.8 m. × 0.32 cm. (6 ft. × 0.125 in.) column packed with 5% SE-30 on 80/100 mesh Chromosorb G (AW-DMCS), and a hydrogen flame-ionization detector. The column temperature is maintained at 170° and the injection port temperature at 180°. Helium is used as the carrier gas at a flow rate of 15 ml./min. The detector temperature could not be independently adjusted, but the design of the unit is such that the temperature is equal to or up to 50° in excess of the column temperature. Figure 2 shows an example of a typical chromatograph.

(1) U. S. pat. 2,413,077.
(2) Private communication.
(3) "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N. J., 1968, pp. 780, 781.

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The authors acknowledge the cooperation of their colleagues in the Quality Control Laboratory.

[2] Eastman Kodak.
[3] Sil-Prep is available from Applied Science Laboratories and is a 9:3:1 mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane.
[4] Supplied by the manufacturer of pantothenyl alcohol. We assumed 100% purity for the lactone.
[5] Perkin-Elmer model 800.

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The highlight of this book is a totally unrelated but thoroughly engrossing autobiographical presentation by Mr. Jonas Carol, who reviews his experiences at FDA in a paper entitled "Thirty-Nine Years of Change as Seen from Within FDA."

Reviewed by Durward F. Dodgen
National Academy of Sciences
Washington, DC 20018 ■

REVIEWS

Remington's Pharmaceutical Sciences. 14th Edition. Edited under the direction of ARTHUR OSOL and JOHN E. HOOVER. Mack Publishing Co., 20th and Northampton Streets, Easton, PA 18042, 1970. xii + 2074 pp. 21 × 29 cm. Price $25.00.

This, the fourteenth edition, could be called a truly *enlightened* edition. It has been extensively revised to reflect progress in the profession of pharmacy; to this end, thirteen new chapters have been added. And, it is three pounds trimmer than the thirteenth edition!

In the section on Pharmaceutics, seven new chapters have been added including Computer Science, Solution and Phase Equilibria, Interfacial Phenomena, and Coarse Dispersion.

Drug nomenclature and structure–activity relationships are topics of two new chapters in the section on Pharmaceutical Chemistry. The last section, General Practice, has been enlarged, incorporating new material on drug incompatibilities, and a table on drug interactions has been added.

The use of lighter weight paper to reduce the weight of the new edition should be appreciated by all those who utilize this essential reference source on a regular basis.

For those readers who are not already familiar with "Remington," the volume is highly recommended as a comprehensive and accurate source of information on all facets of the pharmaceutical sciences and their application to the pharmacy practice. For those who are already familiar with previous editions, it will suffice to say that this latest compilation exceeds all past efforts; the extensive additions and revisions now make the preceding edition completely outdated.

Staff Review ■

Textbook of Pharmacognosy. Fifth Edition. By T. E. WALLIS. J. & A. Churchill Ltd., London, England, 1967. xi + 652 pp. 14.5 × 22 cm. Price $9.00.

This is the most recent revision of a pharmacognosy textbook which has been used in England for approximately a quarter of a century. The author has retained both the monographic approach for presenting individual drugs of natural origin and the morphologic organization for grouping these drugs. Significant discussions of the chemical nature and properties of drug constituents are excluded from the textbook, and such information is stated to be outside the scope of the book.

The current emphasis in the United States on chemical and biochemical aspects of pharmacognosy essentially precludes consideration of using this book as a text. A further indication of the limited educational utility of the book in this country is obtained by noting that penicillin is the only antibiotic mentioned and that the coverage of this important natural product is restricted to early observations and developments.

The primary value of this book to persons in the United States will probably be as a reference for a few specialists who have a need for macro- and micromorphologic details and for information on cultivation and collection of plant drugs.

Reviewed by Lynn R. Brady
College of Pharmacy
University of Washington
Seattle, WA 98105 ■

Common Sense Lives Here: A Community Guide to Drug Abuse Action. National Coordinating Council on Drug Abuse Education and Information, Inc., 1211 Connecticut Ave., Washington, DC 20036, 1970. i + 104 pp. 21 × 21.5 cm. Price $2.00.

This very attractively designed and illustrated booklet is aimed at community groups or individuals within a community who wish to become better informed about drug abuse action. It is intended to aid in the development of community programs to handle problems associated with drug abuse. A great deal of information is presented in a straight-forward, easily understandable form.

Staff Review ■

British Medical Bulletin: Drugs: Development and Use. Vol. 26, No. 3. Edited by D. R. LAURENCE. Medical Department, The British Council, 97 & 99 Park Street, London W1Y 4HQ, England, 1970. pp. 185–266. 22 × 28.5 cm. Price $6.50.

This edition contains fifteen articles on selected aspects of drug development and drug use. Among the topics covered are how drugs have changed the general pattern of disease in the community, the value of animal studies in predicting the safety of drugs in man, and how to ensure that manufactured drugs are of known and consistent purity.

Staff Review ■

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Reviewed by Durward F. Dodgen
National Academy of Sciences
Washington, DC 20018 ■

Micromethods for the Clinical and Biochemical Laboratory. By HERMANN MATTENHEIMER. Ann Arbor Science Publishers, Inc., P. O. Box 1425, Ann Arbor, MI 48106, 1970. xii + 232 pp. 15.5 × 24 cm. Price $18.75.

The advantages of using microliter techniques—less time consumed, less money spent, and less space required—have produced increased interest in this area of analysis. This book brings together for biochemists and clinical chemists current information, techniques, and instrumentation available and in use. The book covers chemical and enzymatic methods used in a modern clinical laboratory. Included is a chapter on the determination of enzymes with ultramicromethods.

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NOTICES

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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REVIEW ARTICLE

Experimental Modification of the Chemistry and Biology of the Aging Process

CHARLES G. KORMENDY* and A. DOUGLAS BENDER

Keyphrases ☐ Gerontology, experimental—review of literature ☐ Aging process modification—biology, chemistry ☐ Tissue cultures—*in vitro* study, aging process ☐ Cellular systems—aging changes ☐ Biological consequences—cellular system aging

CONTENTS

INTRODUCTION 167
AGING IN VITRO 168
EXPRESSIONS OF AGING 169
DNA 169
RNA and Protein Synthesis 171
Lysosomes 173
Accumulation of Waste Products 173
Changes in the Immune System 175
Collagen 175
Hormones 176
Miscellaneous Agents 177
CONCLUSIONS 177

INTRODUCTION

Throughout his history, man has sought answers to why he grows old, and he has continually advanced theories and tested treatments designed to extend his life (1). According to recent technological forecasts, it is possible that, within the next 20 to 30 years, the average human lifespan will be extended significantly through an alteration of the basic aging process (2, 3). Thus, the ancient wish of man to attain longevity appears about to move from fantasy into reality. This review will consider the basic question: Is there any sound scientific evidence to support such bold optimism? Perhaps it is too early to propose rational or productive research which would retard the aging process and prolong life through pharmacological control because of the lack of experimental evidence indicating the causes of aging *per se*. What seems likely, however, is that, in the near future, progress in age research will lead to therapies and procedures that will permit a more comfortable and productive life for man in his later years. It is our hope that the material presented here will: (*a*) enable those considering future research in this field to draw their own conclusions regarding anticipated research progress, and (*b*) help them design the experiments they feel are most meaningful and relevant.

We believe that this review is particularly timely. For many years, age research has suffered from unfortunate circumstances created, in part, by Brown Sequard's ill-reputed experiment, youth doctors, and self-proclaimed rejuvenators (4). In recent years, however, the tide has turned; age research is becoming a respectable discipline and has attracted national and international attention, with the result that many significant and important contributions have been made. This situation is evident from the fact that one-third of the papers cited in this review appeared in 1969 and 1970. Verzár (5) recently described this change in interest and expansion of age research.

A major stimulus to this increasing interest in experimental gerontology has come from social as well as economic considerations. Actuarial statistics place the current average life expectancy in the United States at nearly 75 years, while the lifespan (that is, the maximum attainable age) is estimated to be around 110 years (1). If we achieve no further increase in lifespan,

the number of individuals in this country over 65 years of age will grow from the current 20 million figure to 30 million in the year 2000. The ability to improve the quality of life in later years for this growing segment of the population could do much to relieve many financial burdens and free resources for other social problems. Thus, the conservation or restoration of vigor in old age presents a practical challenge to those engaged in age research.

As noted, we shall summarize those experiments that demonstrate an ability to alter expressions of aging or to extend the lifespan itself. No attempt has been made to review extensively, highlight, or list the various theories that have been proposed to explain why aging occurs. This subject has been dealt with in a more extensive review (6). We have also excluded clinical studies, such as those with procaine and hormones, since they belong more appropriately to geriatrics. A recent review may fill the gap for the interested reader (6). Nor shall we discuss the vast literature on intermediary metabolism, since it could add little to our objectives; a recently published book (7) provides detailed information on this subject. Likewise, the following related subjects have been adequately covered elsewhere: environmental factors (8), nutritional aspects (9, 10), and modification of drug activity with aging (11–14).

AGING *IN VITRO*

With the advancement of tissue culture techniques, it has become possible to study the aging process of mammalian cells *in vitro*. A clonal aging phenomenon, discovered by Hayflick and Moorhead (15), in a human diploid cell line has received considerable attention and has provided age research with a convenient experimental model (16–20). When trypsinized human embryonic fibroblasts are cultivated serially in a ratio of 2:1 in a basal medium supplemented with 10% calf serum and standard antibiotics, the cells survive only 50 ± 10 doublings (15). At this point the cells rapidly lose their dividing ability and ultimately die. Cellular proliferation occurs in a three-phase pattern: adaptation (Phase I), logarithmic growth (Phase II), and mitotic arrest followed by death (Phase III). An important feature of the system is that the cells maintain their normal diploid karyotype throughout the entire growth cycle. Subsequently, Hayflick (21) showed that the doubling capacity correlates with the age of the donor; fibroblasts from adults have a significantly shorter lifetime than those of embryos.[1] Furthermore, cell populations of different doubling levels when mixed together maintain their individual clonal doubling capacity (21).

Both intrinsic and extrinsic factors have been implicated in limiting the number of cell doublings. However, Hayflick's (21, 22) experience suggests that neither medium artifacts nor infection nor depletion of some nonreplicating component from the cells is responsible for cell death. He attributed the finite number of doublings to a manifestation of cellular aging. This suggestion is consistent with the results obtained by others employing human fibroblasts (23–25).

Several intrinsic factors have been suggested as responsible for limiting the number of cell divisions; however, each suggestion lacks sufficient experimental verification. These possibilities range from the accumulation of errors in DNA during Phase III (21) to possibly an age-dependent increase in cell volume which could conceivably compensate for cellular disorganization (26). In addition, defects in cellular regulatory mechanisms are also prime suspects and will be discussed later.

Certain, yet unknown, cytoplasmic elements have also been cited as potential factors causing mitotic arrest. For instance, Muggleton and Danielli (27) transformed "immortal" clones of *amoebae* to "mortal" (spanned) ones by injecting them with the cytoplasm of spanned cells. A puzzling question is whether the Szent-Györgyi *et al.* (28) retine, believed to be a glyoxal-containing mitotic inhibitor and present in the cells, plays a role in the events leading to cellular senescence. Recently, it was suggested that retine might be identical with dehydroascorbic acid, a substance structurally related to glyoxal (29).

Although, as mentioned earlier, Hayflick (21, 22) ruled out certain extrinsic factors as possible causes of cell death, general observations (30) and specific experimental results of others (20, 26, 31) indicate that culturing techniques and the composition of the media may have a direct bearing on the number of generations produced in normal diploid cells. Thus, it could be dangerous to overinterpret tissue culture data and attach a dogmatic meaning to the number of doublings obtained with a particular cell type in an artificial milieu. It would be equally erroneous to extrapolate from the properties of one cell type to that of another, since the proliferative behavior depends on the level of differentiation. This point is perhaps best illustrated by LeBlond's (32) classification of normal postnatal cells on the basis of their mitotic capacity: *static cells* do not divide and compose the nervous system and retina; *expanding cell populations* such as the parenchymal and supporting cells of the kidney, pancreas, adrenal, liver, thyroid, skeletal, and cardiac muscle fibers show only scattered mitosis; *renewing cells*, which include the epithelial, alveolar, and red-blood cells and the lymphocytes, proliferate abundantly through the entire lifespan. From the point of view of age research, it would be specially important to investigate static cells, particularly those of the nervous system, which are likely to suffer most from age-inflicted damage (33). The techniques of culturing nervous and muscle tissues are in fact available (34–36). Postmitotic cells, which are believed to approximate a senescent state, have been suggested as a suitable tool for age research (17, 37). In this regard the works of Chang (38) and Yuan *et al.* (39, 40), who used postmitotic human amnion cultures, are noteworthy. Their results will be discussed later.

An inherent limitation of tissue culture ought to be

[1] *Note added in proof:* Extending Hayflick's (21) observations, a recently published study [G. M. Martin, C. A. Sprague, and C. J. Epstein, *Lab. Invest.*, 23, 86(1970)] established that the replicative capacity of cultured human skin fibroblast cells inversely correlates with the age of the donor.

Table I—Biological Expressions of Aging

Target (Cell Component)	Observed Change	Possible Consequence
DNA	Covalent (?) binding of histones	
Chromatin	Increased nucleoprotein binding; decreased template activity	
RNA	Decreased *m*-RNA content (?); decreased ribosomal (polysomal) content	Impaired protein synthesis
Protein	Decreased protein synthesis; delayed response to enzyme induction	
Lysosomes ↕?	Increased enzyme activity (*e.g.*, RNase)	Breakdown of essential cellular components due to enzyme leakage
Lipofuscin	Increased accumulation in postmitotic cells (*e.g.*, brain and heart)	Impaired cellular function
Amyloid ↕?	Increased accumulation; "senile plaques"	Product of autoimmune response
Immune system ↕?	Decreased number of antigen-sensitive and antibody-forming cells; increased autoimmune factors	Reduced immunocompetence; Progressive autoimmunity
Collagen	Increased stability *via* crosslinks; increased Ca-binding	Loss of plasticity; autoantigenic
Hormonal regulation	Decreased tissue sensitivity to hormones	Impaired homeostatic control

considered, namely, that the cells are in an artificial environment detached from the intricate physiological regulation. Consequently, a tissue culture approach cannot substitute, but only complement, whole organ or *in vivo* experiments. There is no agreement, however, concerning which species or strains are most appropriate for gerontological research. A recent symposium (41) dealt with this problem in considerable detail.

EXPRESSIONS OF AGING

Changes associated with age may occur in a number of cellular systems and may be expressed in DNA, RNA, protein synthesis and metabolism, lysosomes, waste-product accumulation, structural substances such as collagen, hormonal function, and immunological competence. These expressions of aging are summarized in Table I, along with the possible biological consequences of these changes. The subsequent discussion deals with a further consideration of these biochemical and chemical aspects of aging, with particular emphasis on those studies that have dealt with experimental approaches to modifying these expressions of advancing age.

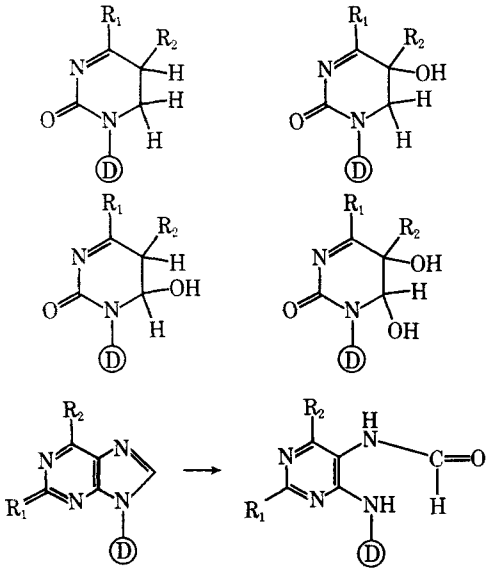
DNA—Today many regard aging as some sort of information loss on the molecular level (42). Hence, DNA, the key matrix of the genetic and regulatory information, could be a potential locus for such a functional loss. Specifically, a programmed sequential mechanism, random somatic mutation, and aging of DNA through protein binding have been considered as possibilities.

The theory of programmed aging (43) proposes an epigenetic switching-on and switching-off of certain regulatory genes which determine the rate of aging of a given species. Such mutational alterations would introduce error factors in DNA, RNA, and protein synthesis leading to impaired cellular function and eventual death. If such a mechanism would prove to be correct, then the elucidation of the genetic program could provide the basis for a rational approach to alter the aging process. However, with the rare exception of the so-called premature aging syndromes (44, 45) such as progeria and Werner's syndrome, and perhaps Alzheimer's disease, a type of presenile dementia (45), there is no convincing evidence to support a gene-programmed aging.

Considerable experimentation and debate have surrounded the question of somatic mutations as a primary cause of aging (44, 45). In essence, it was assumed that gradual accumulation of random mutations in DNA impairs an increasing number of somatic cells through faulty RNA and protein synthesis. The mutagenic agents could be extrinsic factors, *e.g.*, low grade radiation, or intrinsic factors, such as free radicals or destructive enzymes. In an attempt to test the validity of this hypothesis, the relationship between radiation and aging has been investigated (44–48). Animals, who in early life receive sublethal doses of X-irradiation, become prematurely senile and develop fatal diseases. Mutations increase steadily with age, as indicated by chromosomal aberrations in liver cells.

From a chemical point of view, radiation damage of DNA arises from free radical reactions (49–51) and from strand breaks (50, 52). The known mechanisms involve the addition of $H^{\cdot}$ and $OH^{\cdot}$ radicals to the base components (Scheme I), breaks of the water–hydrogen bonds in the secondary structure leading to local separation of the double helix, and "direct hits" (Scheme II), which can result in complete scission of the twin strands. It should be noted here that besides radiation, normal metabolic pathways also can produce free radicals. According to Harman (53), free-radical reactions, regardless of the source of the free radicals, promote the aging process. To probe this hypothesis, he tested a variety of free-radical scavengers, including antiradiation agents, for their effects on lifespan in mice. These results will be discussed in the section dealing with waste products.

Not only ionizing but also UV radiation can catalyze transformations in the DNA structure (50). Photochemical attack takes place along the 5,6-double bond of pyrimidine bases, yielding water adducts (*a*) and cyclobutane-bridged dimers (*b*) as the UV radiation products (50).

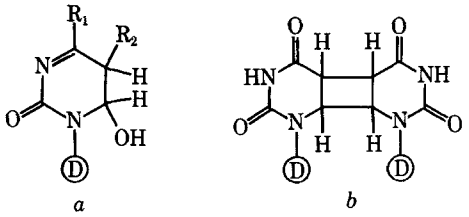


Scheme I—Ionizing radiation products of pyrimidine and purine bases. D = deoxyribophosphate; uridine: R_1 = OH, R_2 = H; thymidine: R_1 = OH, R_2 = CH_3; cytidine: R_1 = NH_2, R_2 = H; adenosine: R_1 = H, R_2 = NH_2; and guanosine: R_1 = NH_2, R_2 = OH.

In lower organisms, as well as in mammalian cells, direct and enzymatic photoreactivation restores the UV-damaged DNA (54). While there are no experimental data about the effects of age on the efficiency of the repair mechanism in mammals, the degree of photoreactivation in old macroconidia of *Neurospora crassa* does not differ from that of young ones (55).

In addition to radiation, DNase, a lysosomal enzyme, could conceivably produce DNA strand breaks. Allison and Paton (56) demonstrated experimentally in human diploid cell cultures that photosensitized lysosomes readily release DNase which can enter the intact nucleus. It remains to be seen whether actual strand scission can occur under these conditions.

The relevancy of somatic mutations in general and nonlethal radiation effects specifically to aging is debatable on a number of points. Mammalian cells and, in particular, the postmitotic types have a remarkable ability to rejoin enzymatically the broken strands, to replenish the altered base units in DNA (33, 50, 52, 57), or to excise unphysiological inclusions in the chain. Electron-spin resonance studies (51) also showed that the mitochondrial electron-transport system is capable of inactivating radiation-induced free radicals. When CBA mice, a strain known to have a low incidence of neoplasia, were X-irradiated, their rate of aging as measured by the tail tendon test (*cf.*, collagen) remained unchanged although their average lifespan decreased (33). If radiation-induced life shortening is, indeed, a result of somatic mutations, then powerful mutagens, such as monofunctional alkylating agents, would be expected to decrease the average lifespan of treated animals. However, the results with ethyl methanesulfonate (EMS) are conflicting. In one set of experiments (33), even very high doses of EMS did not alter the lifespan of mice. In another experiment (58), EMS significantly increased the rate of mortality in mice.



a *b*

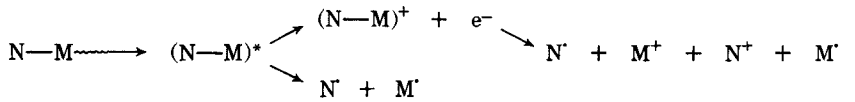
While somatic mutation may occur and increase with time in mammalian cells, it is not possible now to correlate it directly with aging. Since the shortening of the lifespan does not necessarily indicate an acceleration of aging, the observed effects of radiation do not seem to support a mutational theory.

Cellular aging was also attributed to an irreversible nuclear protein–DNA interaction (59). It was found that DNA isolated from bovine thymus (59) and rat liver (60) of old animals contains strongly bound residual histones, and that this DNA–protein complex is denatured at higher temperatures than comparable DNA preparations from young animals. Similarly, whole nucleoprotein (chromatin) isolated from the brain of older mice (61) and from adult bovine thymus (62) showed an increase in the mean temperatures of denaturation.

Examination of histone patterns indicates a distinct loss of the arginine-rich fraction from "old" nucleoprotein (63–65). The nature of the histone–DNA bond and how it differs from young to old have yet to be determined. It is believed (59) that in "young" nucleoprotein, histones are attached to DNA mainly by loose ionic bonds, while in "old" nucleoprotein, covalent bonds are formed between histones and phosphate end-groups or base substituents of DNA. Recent observations suggest that perhaps divalent cations, such as Mg(II) or Mn(II), mediate the ionic bonds; the depletion of these cations, for instance by treatment with EDTA, a strong chelating agent, renders the "young" nucleoprotein preparation similar to an "old" one (65).

The firm binding of histones to DNA, if it accumulates progressively with age, could permanently repress an increasing number of genes which normally code for specific proteins; hence, cellular function could be irreversibly impaired (59).

The priming ability of lung and prostatic chromatin decreases with age (66), which may reflect an age-dependent deficiency in DNA-directed RNA synthesis. There is no age-associated change in template activity of bovine thymus chromatin (62). The observed template activities of "old" liver chromatin are conflicting. Mainwaring (66) and Samis and Wulff (67) did not detect age variations at all. On the other hand, Devi



Scheme II—"Direct hit" of ionizing radiation on the nucleic acid strand

et al. (68) reported a significant decrease in RNA synthesis when primed with liver chromatin from old rats. They attributed this finding to a failure of the DNA double helix to unwind. For instance, a tight DNA–histone complex as suggested (59) could hinder unwinding. However, other possibilities that do not involve DNA, such as RNA polymerase inactivation by arginine-rich histones (69), should also be considered.

The metabolic fate of DNA during aging has been monitored *via* radioactive thymidine incorporation. Chang (38) found a decreased incorporation in postmitotic human amnion cells. On the other hand, cultured human fibroblasts in their senescent state (Phase III) continued to synthesize DNA, even though most cells ceased to divide (22). The turnover of liver DNA has been shown to increase in aging rats (70). The active DNA synthesis in senescent fibroblasts and in the liver of old rats might be interpreted as a compensatory mechanism for age-inflicted cellular damage. However, Chang's (38) findings do not fit this interpretation.

RNA and Protein Synthesis—The cellular events that control protein synthesis, such as the synthesis of enzymes, are transcription and translation. The former involves DNA-directed RNA synthesis; in the latter, messenger RNA directs the assembly of polypeptides which requires ribosomal and transfer RNA. Thus, theoretically, it is conceivable that aging may affect the protein-synthesizing machinery either at the DNA level, due to mutation or structural changes, or at subsequent stages. Such an impairment could cause a serious deficit in vital proteins (59) or generate errors in the protein structure (71).

Cultured diploid fibroblasts in their senescent stage (Phase III) seem to continue to synthesize RNA and protein at normal or near normal rates (72, 73). When fibroblast cells are near death, degradation exceeds the biosynthesis of RNA (74). In postmitotic cells, such as in human amniotic cells, RNA and protein synthesis do not change appreciably with age (38).

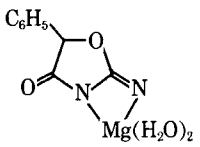
Wulff *et al.* (75) reviewed the metabolism of RNA of aging rodents and concluded that the muscle, liver, and kidney of old animals incorporate more cytidine in nuclear and cytoplasmic RNA and have a more rapid turnover in nuclear RNA than the same tissues of young animals. From the characteristics of liver nuclear RNA, Wulff *et al.* (75) suggested that perhaps the increased RNA turnover during aging is associated primarily with *m*-RNA. However, sedimentation patterns of ribonucleoprotein particles from the prostate and liver of aging mice seem to indicate an age-associated depletion of *m*-RNA (66, 76). According to recent reports, total RNA, polysomal content (77), and uridine incorporation into RNA (78) decrease with increasing age in the mouse skeletal muscle.

The metabolism of brain RNA is different from that of the other tissues investigated, inasmuch as total RNA levels remain fairly constant during the adult and senescent state of rodents (75, 79). On the other hand, polysome-bound ribosomal RNA decreases in aging rats (80–83). These findings, together with the finding that an *r*-RNA fraction may mediate learning and memory in the rat brain (84), might help to uncover the biochemical processes involved in the mental function and its deterioration in senility.

The search for agents that enhance learning and memory received considerable attention in recent years. Weissman's (85) critical review covered this subject adequately; therefore, we shall mention here only those drugs that supposedly act on brain RNA or are derivatives of RNA.

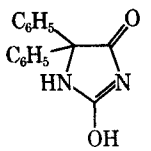
Yeast RNA—Cook *et al.* (86) demonstrated that yeast RNA administered chronically to young rats potentiates the acquisition of conditioned response and enhances its retention. Subsequently, other investigators also studied the psychopharmacological properties of yeast RNA by various test methods and obtained mixed results. Cook and Davidson (87) extended their studies and discussed them in detail along with the results of others. Gardner (88) reviewed the effects of yeast RNA on aging. Mice and rats fed daily with yeast RNA showed an increase in lifespan of 7–8.4 and 25%, respectively. It is not known how yeast RNA exerts its beneficial effect on performance or lifespan.

5-Phenylpseudohydantoin [*Pemoline* (*I*)]—This mild CNS stimulant, complexed with magnesium hydroxide, was reported to enhance the conditioned avoidance response in young rats (89). According to Glasky and Simon (90), magnesium pemoline stimulates brain RNA polymerase and the synthesis of brain nuclear RNA (91). However, the results of independent investigators support neither the claimed memory and learning-enhancement activity (85) nor the biochemical properties (85, 92). Whatever effect magnesium pemoline may have on performance is probably attributable to its CNS-stimulant activity (85). Similar conclusions were drawn from avoidance acquisition experiments with old rats (93).



I

5,5-Diphenylhydantoin (*II*)—This known antiepileptic drug was shown to improve the avoidance response and retention in old and, to a lesser extent, in middle-aged rats (82, 94). Others observed similar performance-enhancing effects (95). This drug supposedly stimulates polyribosome formation in the aging brain tissue (83).



II

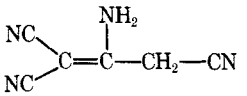
Procaine Amide (*III*)—This drug is used in the treatment of cardiac arrhythmias. Gordon *et al.* (82) tested it for learning-enhancement activity in rats of different ages. While it improved acquisition and retention in old animals, it did not affect young adults nor was it as active as diphenylhydantoin. Procaine amide was shown to increase brain RNA content in the microsome-plus-cell-sap fraction and to redistribute the intracellular

amino acids from the free pool to an RNA-bound form (94). However, lysine incorporation into brain protein decreased, which is inconsistent with the observed RNA-bound amino acid profile.



III

Tricyanoaminopropene (IV)—This drug is reported to enhance retention of an avoidance response and "consolidation" of memory in the rat (85). Biochemically, it increases RNA content of the nerve cell and it also seems to alter the RNA-base makeup (85).



IV

Ribaminol—This complex of RNA and diethylaminoethanol (96) was claimed to enhance learning and to stimulate protein synthesis in the brain (85). Despite the wide publicity this agent received, more definitive scientific data are needed for its objective evaluation.

Of course, the ultimate test of these agents must come from objective clinical studies in senile conditions. Since the discussion of clinical results is beyond the scope of this paper, the interested reader is referred to recent reviews that deal with this subject (6, 85). However, the clinical trials conducted so far prove, at best, marginal and perhaps only subjective improvement in the elderly suffering from senility.

Protein Synthesis—One fashionable theory of aging is currently that of Orgel's (71), which proposes a cytoplasmic "error catastrophe" in protein synthesis. More precisely, Orgel suggested that, while low-level errors introduced into certain metabolic enzymes can be washed out by rapid degradation and *de novo* synthesis, errors in the synthesis of information-handling enzymes, such as RNA polymerase or *t*-RNA acylating enzymes, can accumulate progressively. Hence, the accuracy of the entire protein-synthesizing machinery might ultimately be jeopardized.

Recent findings may be taken as conjectural evidence. Earlier, we mentioned the experiments of Muggleton and Danielli (27) in which they transferred an unspecified cytoplasmic factor from "mortal" *amoebae* to "immortal" ones and thus transformed them to a "mortal" state. This transformation might have involved the transfer of an error-producing component such as a misspecified regulatory enzyme. Harrison and Holliday (97) were able to shorten the lifespan of adult fruit flies by administering nontoxic amounts of amino acid or RNA-base analogs. They attributed the life-shortening effect of such analogs to the synthesis of faulty proteins. However, more recently Dingley and Smith (98) found that the lifespan of adult *Drosophila subobscura* fed with amino acid analogs was not reduced. Holliday (99) reported an irreversible clonal senescence of certain fungi, which he ascribed to errors in protein synthesis. Finally, it was shown that error frequency in *poly-U* transcription increases when RNA polymerase is X-irradiated (100).

These results, however, do not prove the validity of Orgel's (71) theory, nor can one extrapolate from insects and fungi to a mammalian system. Until direct and unambiguous evidence is presented such as the isolation and characterization of a regulatory enzyme (*e.g.*, RNA polymerase) which embodies age-dependent errors, Orgel's concept remains in doubt.

Protein synthesis, as demonstrated by incorporation studies, declines with increasing age. Hrachovec (101) compared the incorporation of amino acids of microsomes isolated from the livers of young and old rats by incubating the microsomes with a uniformly labeled yeast protein hydrolysate. Microsomes from old animals incorporated significantly less radioactivity into protein than microsomes from young animals, but maximum incorporation occurred at about the same time in both age groups. Guanosine triphosphate (GTP) stimulated amino acid uptake which, in the case of "old" microsomes, approached the level of the GTP-free "young" microsomes. Liver cell-sap, on the other hand, inhibited the incorporation of amino acids, but the cell-sap prepared from the liver of old animals had an increased inhibitory effect. In Hrachovec's (101) view, these results indicate an age-dependent increase of two inhibitory factors of protein synthesis; one is antagonized by an excess of GTP, while the other is not.

Leucine incorporation also decreases in the homogenate and cellular fractions of mouse skeletal muscle during aging (78). This decrease was attributed to a loss of polysomes (77, 78). Liver microsomes from older mice exhibited a decreased ability to synthesize proteins, probably because of reduced *m*-RNA content (76).

Enzyme concentration and activity are known to fluctuate with environmental stress. Recently, several examples appeared in the literature suggesting that age, too, may have an effect. Singhal (102) demonstrated an age-dependent decrease in the activity of glucose-6-phosphatase and fructose-1,6-diphosphatase in rat liver following hormonal induction. He obtained similar results with testosterone-induced phosphofructokinase in the prostate and seminal vesicle of the rat (103). However, in both cases, enzyme activities were measured only at one time point after induction; therefore, possible changes in enzyme levels cannot be ascertained as a function of time. More recently, the kinetics of several enzymes were analyzed in relation to aging. Haining and Correll (104) determined the rate of synthesis and degradation for liver tryptophan pyrrolase stimulated by tryptophan in rats ranging in age from 1 month to 2 years. The rate constants for synthesis and degradation corresponded in each age group, decreasing between 1 and 6 months of age, then increasing around 1 year, and, finally, decreasing again in the 2-year-old group.

These observations indicate that age-dependent correlations between enzyme (protein) depletion and repletion are important to consider for further investigation. Finch *et al.* (105) and Adelman (106, 107) carried out kinetic analyses of enzyme inductions in stressed young and old animals. Finch *et al.* (105) induced liver tyrosine aminotransferase (TAT) in fasted young and old mice with exposure to cold and measured the rate of TAT activity at different time intervals. Adelman (106)

studied the kinetics of liver glucokinase as a function of age in rats after fasting the animals for 72 hr. and subsequently refeeding them with glucose. The common element of these experiments is that, while in young animals the respective enzyme activities begin to rise shortly after induction, the activities in old animals start to rise only after a significant lag period of several hours but eventually reach levels comparable to those of the young animals. Adelman (108) demonstrated a similar age-dependent pattern in the phenobarbital-induced NADPH/cytochrome-c reductase system.

The additional finding that newly regenerated liver cells in aging animals respond to glucokinase induction like old cells (107) raises some interesting questions. For instance, since the response of old animals to exogenous hormones parallels that of the young animals in liver TAT (109) and liver glucokinase activities (106), an age-associated somatic mutation of liver cells is not likely the cause of the delayed adaptive response (106, 108). Other possible explanations that have been considered are humoral or neural factors, alterations in membranes, and deficiencies in protein synthesis and degradation (105, 106, 108).

Lysosomes—Lately, the possible role of lysosomal enzymes in cellular aging has received considerable attention. Lysosomes are membrane-encased cytoplasmic organelles which store within their lumen acid hydrolytic enzymes such as acid phosphatase, ribonuclease, DNase, collagenase, and esterases. Normally, lysosomes dispose of intracellular waste and debris and phagocytose alien particles, but they can also cause or contribute to certain pathological conditions. DeDuve and Wattiaux (110) reviewed the functions of lysosomes in detail. Specific agents or stresses, such as free radicals (111); vitamin A, diethylstilbestrol, testosterone, endotoxins (112); viruses (113); ischemia, starvation, anoxia (112); and UV or gamma radiation (111) can labilize the lysosomal membrane which engenders the leakage of lytic enzymes into the cytoplasm. Rampant lysosomal enzyme activity was suggested as a cause of cellular death in aging (37). Increased lysosomal enzyme activity was demonstrated in old human leukocytes (114), in senescent human fibroblasts (Phase III generation) (73), in skeletal muscle of old mice (115), and in the liver of aged mice, hamsters, and guinea pigs (116). Goto *et al.* (116) specifically identified a rise in acid ribonuclease with increasing age which might account, at least in part, for the age-associated loss of cellular RNA. As yet, it is not known whether lysosomal DNase activity (56) increases with age. Collagen, which manifests aging in its greater stability due to crosslink formation, is unaffected by collagenase from rat liver lysosomes; other types of collagen, presumably having fewer crosslinks, are hydrolyzed (117). A positive, age-dependent correlation was established between the increase of acid phosphatase activity and collagen denaturation (118). Although lysosomal enzyme activity is apparently aggravated during aging, a causal relationship cannot be corroborated at this time.

Certain agents [*e.g.*, corticosteroids, chloroquine, aspirin (119), chlorpromazine (120), and stilbamidines (121, 122)] are known to stabilize lysosomal membranes. Treatment with hydrocortisone (39), prednisolone, and other 11-hydrocorticosteroids (123) increased cell density and decreased disintegration in X-irradiated human amniotic cells. Hydrocortisone was also shown to be beneficial to the recovery of amnion cells from their degenerative phase (124). According to a subsequent study (123), hydrocortisone seems to interfere in amnion cells with the age-associated increase of the lipid/CO_2 ratio and the incorporation of cholesterol into lipids. On the other hand, ketocorticosteroids such as cortisone, prednisone, and sex hormones, like testosterone and estradiol (123), had no effect on primary human amnion cultures with or without X-irradiation.

Cortisone, however, prolonged the mitotic capacity of human embryonic fibroblasts by about 15 doublings, if treatment commenced at the 21st passage (125); this finding was confirmed by others (123). Similarly, hydrocortisone added to the culture medium of diploid fibroblasts extended the number of generations significantly, provided treatment began before Phase III (73). Contrary to expectations, hydrocortisone did not suppress lysosomal enzyme activity which increases in fibroblast cells of late passages. Cristofalo (73) suggested that hydrocortisone might mediate the mitotic recovery of Phase III cells by inducing protein synthesis. Such a mode of action agrees with the idea that corticosteroids might stimulate transcription of RNA *via* the activation of regulatory genes which are thought to become strongly repressed in the process of aging (126).

The question whether corticosteroids in aging systems stimulate protein synthesis or stabilize lysosomal membranes, or perhaps both, merits thorough investigation.

Accumulation of Waste Products—A discernible concomitant of aging is the progressive accumulation of lipofuscin pigments in the cytoplasm and deposition of amyloids in the interstitial space of tissues. Both lipofuscin and amyloids are believed to be biologically degenerate substances—hence their name waste products.

Lipofuscin, also known as age-pigment or ceroid, tends to accumulate with advancing age, especially in postmitotic cells such as those of the brain, heart muscle, and adrenals. Even though its origin and role are poorly understood, lipofuscin, based on circumstantial evidence, might be an important indicator of the degree of cellular insufficiency in aging. Earlier reviews discussed in considerable detail the histochemistry and ultrastructure (127, 128), isolation and chemical composition (18, 129), and origin (18, 130, 131) of lipofuscin.

Lipofuscin is a basophilic autofluorescent, chemically heterogeneous substance. It consists of 30–40% lipids, a substantial amount of protein, and traces of inorganic elements. The protein component seems to be relatively abundant in glycine and valine, whereas the lipids resemble those of the source tissue (18, 129).

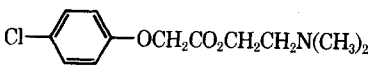
The possible origin of lipofuscin is still a much debated question. The lipid component is most likely derived from the peroxidation of phospholipids and unsaturated fatty acids (132, 133). Growing evidence suggests (133, 134) that irreversible autoxidation of lipids can generate free radicals, which are not inactivated by the mitochondrial electron-transport chain. Hence, these free radicals can attack membranes (*e.g.*, mitochondrial

and lysosomal) as well as proteins, yielding eventually the lipofuscin conglomerate of degraded and polymerized cellular debris.

Cytochemical assays indicated the presence of acid hydrolytic enzymes, which would support a lysosomal origin of lipofuscin (130). Membranous fragments also were found in the pigment (131). Some investigators detected mitochondrial enzymes such as DPN-diaphorase and succinic dehydrogenase around, but not inside, the lipofuscin granules, which suggested a mitochondrial origin (130). Others proposed the endoplasmic reticulum of the Golgi apparatus as possible loci of lipofuscin formation (130). Although lipofuscin can occur early in life (135, 136), aging elicits a significant increase in number, size, and distribution. Such an increase was clearly demonstrated in the different topological areas of brain neurons of rodents (136–138), guinea pigs (139–141), dogs, pigs (142), rhesus monkeys (143), and humans (144); in the heart muscle of rats (145), dogs (146), and humans (147); and in the kidneys, adrenals, testes, and ovaries of rats (145). Excessive lipofuscin deposits were also observed in certain mental disorders of children (148–150) and in a case of presenile dementia (151).

However, the relationship between lipofuscin accumulation and age-associated dysfunction, such as in presenile and senile CNS deficiencies, needs to be investigated under well-controlled experimental conditions. In this regard, experimental lipofuscinosis induced in young animals by various means of stress (152, 153) or chronic vitamin E deficiency (152, 154, 155) could provide useful methods to study this problem in greater detail.

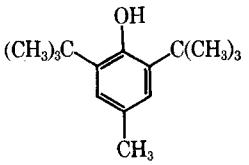
Meclofenoxate [centrophenoxine (V)], a drug of reported benefit in presenile and senile mental disorders, was shown to reverse the accumulation of lipofuscin pigments in old guinea pigs (140, 141).



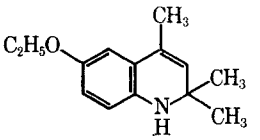
V

Nandy and Bourne (140) observed that lipofuscin, although present in neurons of young and old guinea pigs, begins to increase steadily at the age of 4 years until it occupies a considerable segment of the cytoplasm. Guinea pigs, 4–6 years of age, treated with meclofenoxate daily for 4–8 weeks showed a significant decrease in pigmentation in most areas of the CNS. The degree of activity depends upon the duration of the treatment, giving rise to the greatest reduction after 10 or more weeks (141). At the same time, meclofenoxate reduced the activities of succinic and lactic dehydrogenase and cytochrome oxidase, all predominantly present in the pigmented zone. The decrease in succinic acid dehydrogenase activity seems to indicate a drug-induced reduction in glucose metabolism *via* the Krebs cycle, while the decrease in lactic dehydrogenase activity reflects a reduction in glycolysis. On the other hand, glucose-6-phosphate dehydrogenase activity increased, which, according to Nandy (141), may suggest the diversion of glucose into the pentose cycle. The lipofuscin-depleting activity of meclofenoxate, however, has not been correlated with its claimed efficacy in confusional and demented states.

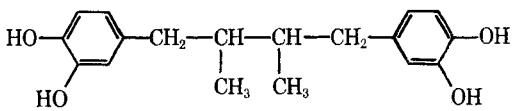
Harman (53, 156) suggested that free-radical production is responsible for many degenerative changes associated with the pathogenesis of aging. While the actual mechanism by which free radicals may produce their effects is as yet undetermined, there is sufficient support for the observation that they tend to react with various cellular materials and produce bonding of adjacent macromolecules, resulting in degradation of the involved substances. Support for Harman's hypothesis comes primarily from a series of experiments (157–160) which he conducted using various inhibitors of free-radical reactions. The results of these studies indicate that many antioxidants and free-radical scavengers, such as cysteine, 2-mercaptoethylamine, 2,2′-diaminodiethyldisulfide, hydroxylamine, butylated hydroxytoluene (VI), ethoxyquin (VII), and ammonium diethyldithiocarbamate, when added to the daily diet, produce an increase in the mean lifespan of mice. Beneficial effects of free-radical inhibitors have been shown for female C3H mice, male AKR mice, and male LAF_1 mice. The first two strains died prematurely of lymphatic leukemia and mammary carcinoma, respectively, while the LAF_1 mouse is a low tumor incidence strain. No effect was found when antioxidants were added to the diet of male Swiss mice. Another study, conducted in Wistar rats, showed that an increase in lifespan may be produced with the antioxidant nordihydroguaiaretic acid (VIII) (161).



VI

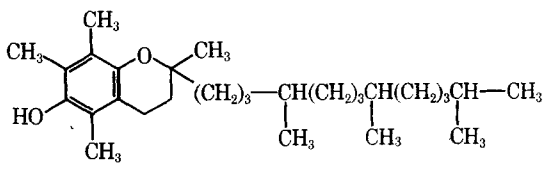


VII



VIII

To counteract antioxidant decay, Tappel (133) suggested vitamin E, glutathione, cysteine, and selenium compounds as potential free-radical scavengers. Among these agents, vitamin E (IX) might have particular significance since its deficiency is known to enhance the production of lipofuscin in rodents (152, 154, 155).



IX

Amyloids, in contrast to lipofuscin, deposit extracellularly in various organs of old mice, hamsters, rabbits, dogs, apes, birds, horses, and humans (162, 163). While the exact chemical composition of amyloid has not yet been established, its major constituent is protein, consisting in part of γ-globulins and collagen, which is complexed with a mucopolysaccharide (132, 164). Amyloidosis can be induced experimentally, for example, with Freund's adjuvant, casein-rich diet fed to mice (132), hyperimmunization (163), *Leishmania* injection, and parabiosis (162). These inducing conditions, as well as the presence of serum globulin components in amyloids, strongly suggest that an autoimmune mechanism might be involved in the genesis of amyloidosis (162, 163, 165).

Amyloids accumulate most severely with increasing age in the brain, heart, aorta, and pancreatic islets (163). The occurrence of cerebral amyloids and "senile plaques," a form of amyloidosis, seems to correlate with senile and Alzheimer's presenile dementia (163, 164, 166, 167). Thus far, any attempt to prevent or mitigate senile amyloidosis has not been reported in the literature.

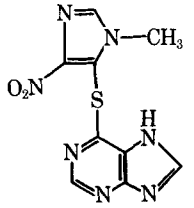
Changes in the Immune System—Age-associated deterioration of immunocompetence is manifested in two ways: (*a*) the responsiveness to extrinsic antigens decreases, and (*b*) several autoimmune factors increase with age.

The primary antibody response of the mouse to heterologous erythrocytes declines significantly with increasing age (168, 169). This deficiency, however, is not caused by a decrease in the amount of antibody produced by the individual antibody-forming cells (169) but rather by the progressive loss of the number of antigen-sensitive cells (168, 169). According to a recent report (170), mixed polynucleotide complexes such as *poly A:U*, *poly C:G*, and *poly I:C* can restore the impaired antigen–antibody response of aging mice.

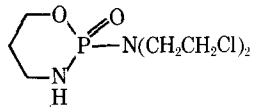
Walford (165, 171–173) elaborated on the idea of an autoimmune mechanism of aging. He regards it as a mild but chronic autorejection phenomenon involving the progressive failure of the organism's own cells to distinguish self from foreign cells. Observed rises of several indicators which may reflect autoimmunity are serum γ-globulins; rheumatoid, antinuclear, antithyroid, and antiparietal factors; and renal lysozyme, which usually increases after immunization and transplantation. According to Walford (165, 171–173), senile amyloidosis is, to date, one of the most specific expressions of an age-associated autoimmune reaction in which the amyloids are the visible end-products. This, however, does not exclude the possibility that amyloids themselves could further act as antigens.

Recently, Albright *et al.* (174) reported that the spleen of old mice harbors life-shortening factors, since splenectomy almost doubled their survival, while spleen cells from old animals injected into younger ones reduced lifespan. Although the factor(s) has not been identified, it is tempting to speculate on its immunologic nature.

Based on the autoimmune theory of aging, Walford (165) tested the effect of azathioprine (X), an immunosuppressive agent, on the lifespan of aging mice. A daily dose of 100 mg./kg. extended the 50% survival score of the treated group by 10 weeks as compared to controls. However, the lifespan of the oldest survivors in the control and treated groups was alike, which Walford (165) attributed to the high toxicity of the drug. Similar results were obtained with cyclophosphamide (XI), another toxic immunosuppressant (173).



X

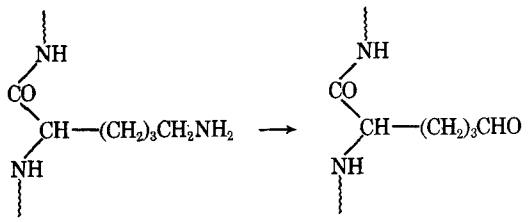


XI

From the foregoing, it is evident that attrition of the immunosurveillance mechanism does accompany aging, but the principal elements involved in cause and effect have yet to be identified.

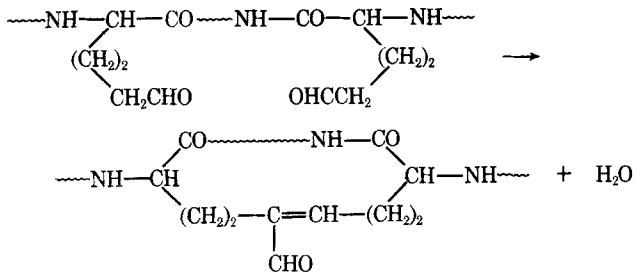
Collagen—A considerable amount of research has been devoted to describing the chemical and physical changes that occur in collagen with age (175, 176) and, in more general terms, has contributed to the understanding of the role of molecular crosslinking as it determines both the structure and plasticity of connective tissue (177). The changes noted in collagen with age include a decreased susceptibility to enzymatic digestion, a decrease in solubility and swelling capacity, and an increase in stability of rat tail tendon subjected to a breaking load. These changes have been attributed to an increase in the formation of covalent crosslinkages which, in a sense, immobilize these large macromolecules. Evidence cited by Bailey (175) confirms that the proportion of intermolecular and intramolecular crosslinking increases with age. Current evidence, however, suggests that the physical changes in collagen with age are mainly a reflection of increased intermolecular crosslinking.

The collagen fiber has a triple helical structure composed of three similar polypeptide chains. While intramolecular crosslinking occurs within the same polypeptide strand, intermolecular networks are formed between the three chains. The initial step in both intramolecular and intermolecular crosslink formation seems to involve the same enzymatic deamination of free ε-amino groups of lysyl residues yielding the corresponding aldehyde (Scheme III) (177, 178). Intra-



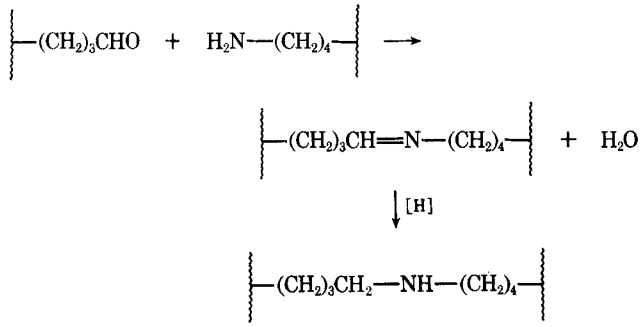
Scheme III

molecular crosslinks arise from aldol condensation of the spacially close aldehydes (Scheme IV). Less certain



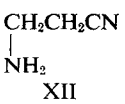
Scheme IV

is the nature of the intermolecular crosslinks; but according to cursory evidence (177, 178), most likely the aldehyde residues couple with free ε-amino groups of lysine and hydroxylysine to a Schiff base, which is further stabilized by reduction to the secondary amine (Scheme V). However, there is no direct proof that such a reductive step takes place *in vivo*.



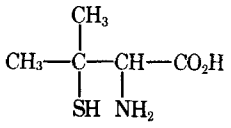
Scheme V

A number of studies have been directly concerned with the inhibition or reversal of crosslink formation within collagenous tissue. The most dramatic of these situations deals with the condition known as lathyrism. This condition is characterized by increased tissue fragility and extractability of collagen and is the result of an inhibition in the formation of normal crosslinkages. Lathyrism was first observed in animals that had ingested sweet pea, *Lathyrus odoratus;* the toxic principle has been identified as β-aminopropionitrile (BAPN) (XII). This substance, when fed to animals, produces a distinct pathological syndrome characterized by a defective maturation of collagen. Levene (179) noted that other substances, in addition to the nitriles, possess lathyrogenic properties. Some urea derivatives such as semicarbazide and certain hydrazides and hydrazines will produce effects similar to that seen with BAPN, but they are not as potent as BAPN. Kohn and Leash (180) failed to show any influence of BAPN on the survival of pathogen-free rats, despite the appearance of characteristic lathyrism at high doses.



XII

Inhibition of intermolecular crosslinks was also demonstrated for penicillamine (XIII) (175, 177). It has been shown that D-penicillamine, when fed to rats, tends to produce a reversal in the degree of stability exhibited by rat tail tendons in older animals. This observation apparently occurs because penicillamine is capable of inhibiting the formation of crosslinkages. Other studies showed that when penicillamine is removed from the diet, the inhibition of crosslinkage is reversed (177). Recently, Ruiz-Torres (181) indicated that penicillamine disturbs the collagen maturation process.



XIII

Several other studies on the reversibility of crosslink formation in collagen are of interest. Wynn (117) showed that rat liver lysosomes are capable of degrading undenatured insoluble collagen (*cf.*, lysosomes). Spichtin and Verzár (182) showed that extraction of calcium from skin collagen leads to a decrease in the stability of collagen as measured by its solubility. According to some studies (183–185), collagen also has antigenic properties. However, it appears that antigenicity decreases with increased structural stability which characterizes aging (184).

Hormones—From the early days of gerontological research, probably no other area has received as much attention as the endocrine system. The literature has recorded countless experiments with glandular extracts and hormones, ranging from naive rejuvenation attempts to palliative replacement therapies of the elderly. Despite the long-standing interest in the relationship of hormonal function and aging, the problem is relatively little understood; a serious deficiency is the lack of comprehensive biochemical studies.

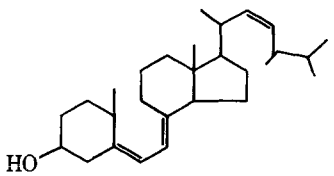
Bellamy (186) reviewed in detail the recently available information on the hormonal states of the aging mammal. Therefore, we shall summarize here only the general observations. Hormone output as a function of age cannot be measured adequately, since there are no techniques available to assay plasma hormone levels accurately. Age-associated sex hormone deficiencies have only been inferred from the decreased urinary excretion of 17-ketosteroids. Thyroid and growth hormone, adrenocorticotrophin, and thyrotrophin levels do not seem to change with age. Generally, tissue sensitivity to hormones declines with increasing age, as in the case of insulin, the antidiuretic activity of exogenous vasopressin, and the renal activity of aldosterone and hydrocortisone. In contrast, exogenous thyroid hormone elicits greater response in older than in young animals. Hormonal regulation in age-related tissue involution has not been clearly demonstrated.

The electrolyte distribution undergoes marked changes during aging, whereby water shifts from the intracellular to the extracellular milieu and is accompanied by potassium depletion and sodium retention. With this in mind, Friedman and his coworkers (187–189) studied the effect of whole posterior hypophysal extract, vasopressin, and their combination with corticosteroids on aging rats. The pituitary extract alone and in combination with hydrocortisone extended the lifespan significantly of the chronically treated group. Adrenocorticotrophin did not potentiate the effect of the pituitary preparation, nor did aldosterone. Vasopressin, alone and together with aldosterone (190),

ameliorated the electrolyte balance and the function of the *gastrocnemius* muscle of old rats without affecting the lifespan. However, both hormones proved to be toxic to old animals (187, 188). Independent investigators (191) related the life-prolonging effect of the posterior pituitary extract to oxytocin. Further experimentation is needed to ascertain whether the posterior pituitary has a *de facto* control over the rate of aging.

Corticosteroids are known to enhance the viability of cells cultured *in vitro* (*cf.*, lysosomes); furthermore, chronic dietary administration of prednisolone to a short-lived, highly inbred strain of mice sharply increased their lifespan (192). At the same time, prednisolone treatment minimized the age-associated rise in the calcium–collagen ratio and the loss of liver nucleic acids. This activity of prednisolone might be related to its immunosuppressive properties, since the short life expectancy of the strain used in these experiments might be due to an autoimmune condition.

Dihydrotachysterol (XIV) administered chronically to rats induces a progerialike syndrome, a form of premature aging (193, 194). The syndrome is accom-



XIV

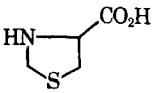
panied by arteriosclerosis, loss of weight and skin elasticity (193, 195), deposition of calcium (195, 196), and loss of collagen (197). Anabolic steroids, such as testosterone and methyltestosterone (194), conjugated equine estrogens (198), and ferric dextran (193) prevent the adverse effects of dihydrotachysterol. Adminstration of pituitary growth hormone (199), thyroxine (200), and the plant growth hormone, indole-3-acetic acid (201), did not influence the lifespan of the rat.

Miscellaneous Agents—This category includes substances and preparations that have been claimed to hinder the aging process, but their mode of action is either unknown or different from those described earlier.

Panax Ginseng—A crude aqueous extract of this plant was shown to mitigate cellular degeneration of X-irradiated and unirradiated cultures of postmitotic human amnion cells (40). The chemical composition of the active principle has not been identified.

Thiazolidinecarboxylic Acid (*XV*)—Experimental evidence indicates (202–204) that disulfide groups tend to accumulate in proteins during aging. Furthermore, serum proteins of old rats were shown to contain a decreased amount of free carboxylic and amino groups (205).

The relative number of the free carboxyl and amino groups increased when aged rats were treated with a mixture of folic acid and 4-thiazolidinecarboxylic acid (XV), a precursor of *N*-formylcysteine (205). This mixture, as well as cysteine, also extended the lifespan of male mice and female guinea pigs but not of female rats (206).



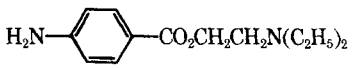
XV

Pantothenic Acid (*XVI*)—Given daily as a dietary supplement to mice, this drug increased their mean lifespan from about 550 to 653 days and increased the body weight (207).



XVI

Procaine (*XVII*)—As a rejuvenating agent, this drug has created one of the greatest controversies in gerontology. Here, it will suffice to say that other controlled human trials (6, 208, 209) could not substantiate clinical claims made by Aslan (210). Hence the gerontological usefulness of this drug is in great doubt. From subsequent animal studies, Aslan *et al.* (211) reported that chronic parenteral administration of procaine to rats increased the lifespan of the males but not of females, improved maze-running efficiency, and mitigated age-associated morphological changes in various organs. In contrast, Verzár (212) obtained only negative results in rats treated with procaine and its hydrolytic components, *p*-aminobenzoic acid and diethylaminoethanol.



XVII

There is widespread belief among the laity that the Rumanian preparation, which in addition to procaine contains *p*-aminobenzoic acid, benzoic acid, diethylaminoethanol, and potassium salts of dibasic phosphate and metabisulfite, is effective, and despite the dubious value of procaine, it might merit a stringently controlled, double-blind clinical battery test such as Comfort (213) outlined in his experimental model.

CONCLUSIONS

In this review we have attempted to focus the attention on a relatively new science, experimental gerontology, which set out to learn the causes of aging and, hopefully, to mitigate them on the basis of a sound rationale. The problem is by no means near to a solution since we do not know what causes us to age. With the current state of the art, we can only define *expressions* of aging which do not necessarily equate with *causes*. Agents that were shown to subdue one or another expression of aging or that prolonged the lifespan of experimental animals should not be looked at as cures of senility but rather as useful models for future research. At the present, one vexing problem of gerontology is the lack of sound measures of biological aging as opposed to chronological age. Lifespan alone has serious liabilities as a measure of aging inasmuch as the rate of mortality is its only critical parameter and does not take into account

the gradual loss of function with the increase of age. Until we learn more about the intricate mechanisms of the aging process, the short-range objectives of age research might be best geared toward ways of improving the quality of life of the elderly, that is, improving their general mental and physical conditions by pharmacological measures.

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RESEARCH ARTICLES

Absolute Configuration of *cis*- and *trans*-2-(*o*-Bromophenyl)cyclohexanols and (+)-*cis*-2-(*o*-Bromophenyl)cyclohexylamine

TODD G. COCHRAN*, DARRYL V. WAREHAM, and ALAIN C. HUITRIC

Abstract □ The absolute configurations of (−)-*trans*-2-(*o*-bromophenyl)cyclohexanol (V), (+)-*cis*-2-(*o*-bromophenyl)cyclohexanol (VII), and (+)-*cis*-2-(*o*-bromophenyl)cyclohexylamine (X) have been chemically related to that of (2S)-(−)-2-(*o*-bromophenyl)-cyclohexanone (VI), whose absolute configuration can be reliably assigned from its optical rotatory dispersion and circular dichroism spectra.

Keyphrases □ *cis*- and *trans*-2-(*o*-Bromophenyl)cyclohexanols and (+)-*cis*-2-(*o*-bromophenyl)cyclohexylamine—synthesis, absolute configuration □ Absolute configuration—cyclohexanol and cyclohexylamine derivatives □ IR spectrophotometry—identity □ NMR spectroscopy—identity □ Optical rotatory dispersion—structure □ Circular dichroism—structure

Concurrent with the study of the optical rotatory dispersion (ORD) and circular dichroism (CD) of the aromatic chromophore, it became necessary to synthesize, resolve, and assign the absolute configuration to a number of substituted 2-arylcyclohexanols and cyclohexylamines. Galpin and Huitric discussed the resolution (1) and absolute configurations (2) of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol and (1R,2R)-(−)-*cis*-2-*o*-tolylcyclohexanol. The assignment of absolute configuration was based on the sign of the Cotton effect of the (2R)-(+)-2-*o*-tolylcylcohexanone obtained upon oxidation of the *cis*- and *trans*-alcohols, and it has recently been confirmed by single-crystal X-ray diffraction analysis of the 3-nitro-4-bromobenzoate of the (+)-*trans*-alcohol (3). In this paper, the authors report on the synthesis and optical resolution of (−)-*trans*-2-(*o*-bromophenyl)cyclohexanol (V) and its conversion to (−)-2-(*o*-bromophenyl)cyclohexanone (VI), (+)-*cis*-2-(*o*-bromophenyl)cyclohexanol (VII), and (+)-*cis*-2-(*o*-bromophenyl)cyclohexylamine (X). The absolute configuration of this series of compounds can be related to that of the ketone VI, whose absolute configuration is assigned from its ORD and CD spectra.

The synthesis and optical resolution of *trans*-2-(*o*-bromophenyl)cyclohexanol (V) are depicted in Scheme I. Racemic 2-(*o*-bromophenyl)cyclohexanone (II) was obtained from the permanganate oxidation (4, 5) of the potassium aci-nitro salt of *trans*-2-(*o*-bromophenyl)-nitrocyclohexane (I), which was previously prepared as an intermediate in the synthesis of the *cis*- and *trans*-2-(*o*-bromophenyl)cyclohexylamines (6). Reduction of II with lithium aluminum hydride gave *trans*-2-(*o*-bromophenyl)cyclohexanol (III) as the predominant isomer. The racemic mixture of alcohols III was resolved by fractional crystallization of its diastereomeric (−)-menthoxyacetate esters, in the manner described by Galpin and Huitric for the resolution of *trans*-2-*o*-tolylcyclohexanol (1). The resolution was conveniently followed by NMR spectroscopy, utilizing the magnetic nonequivalence of the geminal methylene protons (hydrogens a and a′) on the acetate portion of the esters.[1] This nonequivalence is sufficiently different in the two diastereomeric esters to allow the progression of the separation of the two diastereomers to be readily followed by NMR.

Figure 1 shows the pertinent portions of the NMR spectra of a 50:50 mixture of the diastereomers (A) and

[1] For a general discussion of the use of NMR spectroscopy as a monitor for optical purity, see Raban and Mislow (7). For the specific application to (−)-menthoxyacetates, see Galpin and Huitric (1).

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The synthesis and optical resolution of *trans*-2-(*o*-bromophenyl)cyclohexanol (V) are depicted in Scheme I. Racemic 2-(*o*-bromophenyl)cyclohexanone (II) was obtained from the permanganate oxidation (4, 5) of the potassium aci-nitro salt of *trans*-2-(*o*-bromophenyl)-nitrocyclohexane (I), which was previously prepared as an intermediate in the synthesis of the *cis*- and *trans*-2-(*o*-bromophenyl)cyclohexylamines (6). Reduction of II with lithium aluminum hydride gave *trans*-2-(*o*-bromophenyl)cyclohexanol (III) as the predominant isomer. The racemic mixture of alcohols III was resolved by fractional crystallization of its diastereomeric (−)-menthoxyacetate esters, in the manner described by Galpin and Huitric for the resolution of *trans*-2-*o*-tolylcyclohexanol (1). The resolution was conveniently followed by NMR spectroscopy, utilizing the magnetic nonequivalence of the geminal methylene protons (hydrogens a and a′) on the acetate portion of the esters.[1] This nonequivalence is sufficiently different in the two diastereomeric esters to allow the progression of the separation of the two diastereomers to be readily followed by NMR.

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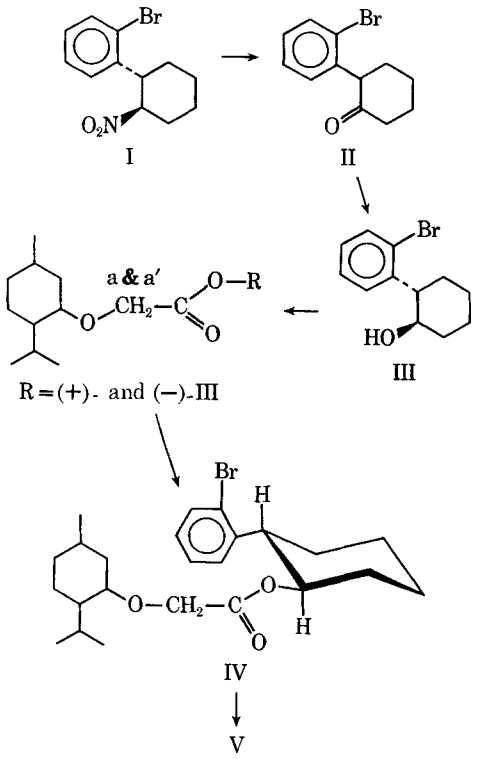


Scheme I

the pure menthoxyacetate IV (B). The signals of the methylene hydrogens (a and a′) appear as highly skewed doublets with geminal coupling of 15.5 Hz.; but the nonequivalence is not the same in the two diastereomers, thus making them readily distinguishable. The NMR spectrum of the pure (−)-*trans*-2-(*o*-bromophenyl)-cyclohexyl (−)-menthoxyacetate (IV) shows no indication of the presence of the other diastereomer, indicating that this ester is of at least 90% optical purity (95% diastereomeric purity).[2] Basic hydrolysis of IV afforded the (−)-*trans*-alcohol V.

The chemical conversion of V to the other compounds in this series is depicted in Scheme II. All conversions involve chemical modification at C-1; thus the absolute configuration of the benzylic carbon, C-2, is conserved. Chromic acid oxidation of V in a two-phase ether–aqueous system afforded (−)-2-(*o*-bromophenyl)cyclohexanone (VI). Reduction of VI with triisobutylaluminum (8) yielded (+)-*cis*-2-(*o*-bromophenyl)cyclohexanol (VII) as the predominant isomer. The *cis*-isomer VII was obtained in pure form by column chromatography on neutral alumina, followed by crystallization from hexane. The relative stereochemistry of the C-1 hydroxyl group of the *cis*- and *trans*-alcohols V and VII can be readily determined from the NMR spectra, which are consistent with the conformations shown in Scheme II.[3] The *trans*-alcohol V was converted to its tosylate (VIII) by the usual procedure and then to its C-1 epimeric azide by the stereospecific displacement of the tosylate with sodium azide in dry *N*-methylpyrroli-

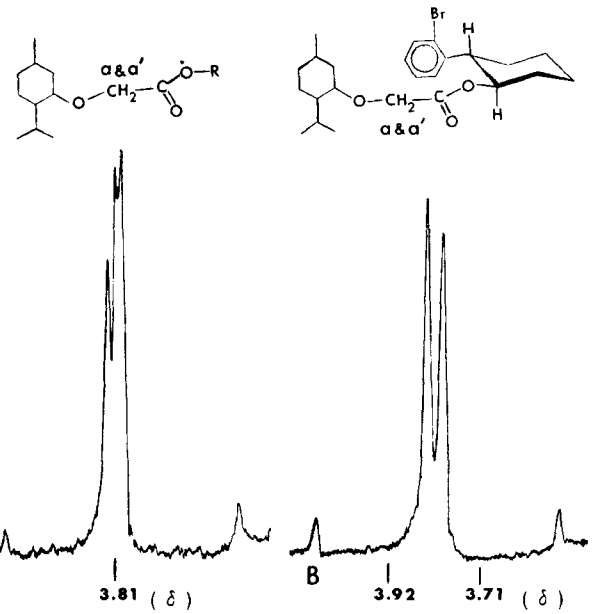
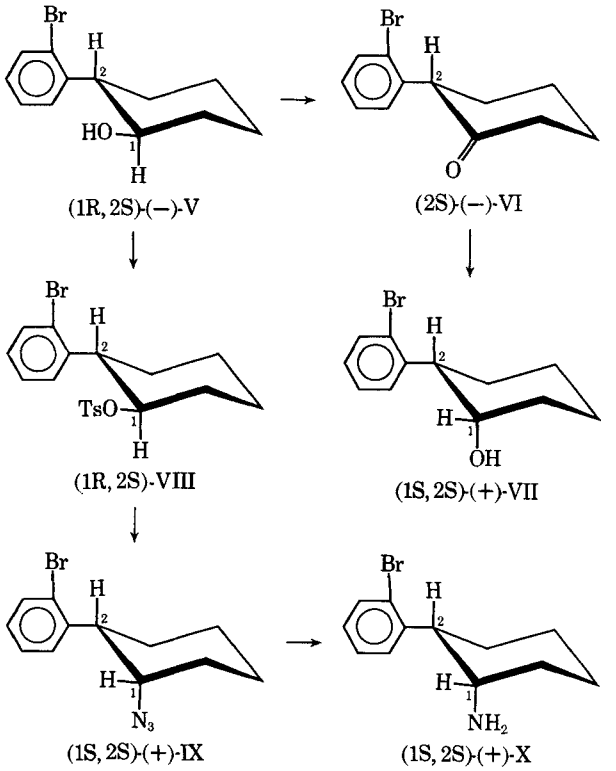


Figure 1—*Portions of the 60-MHz. NMR spectra (250 Hz. sweep width) of the* trans-*2-(o-bromophenyl)cyclohexyl (−)-menthoxyacetates measured in chloroform-*d *at 37°. Key: A, 50:50 mixture of diastereomers [R = (+)- and (−)-III]; and B, pure IV.*

done (10). The (+)-*cis*-azide (IX) was obtained as the exclusive displacement product from this reaction.[4]

The *cis*-configuration of this azide and the *trans*-configuration of the tosylate VIII are confirmed from their NMR spectra, parts of which are shown in Fig. 2. Spectrum A shows the signal of H-1 at δ 4.68 and of



Scheme II

[2] This NMR method has an estimated sensitivity of at least 95% in determining diastereomeric purity. This is discussed further by Raban and Mislow (7).

[3] Determination of the conformation and relative configuration of *cis*- and *trans*-2-arylcyclohexanols by NMR was discussed by Huitric *et al.* (9).

[4] Djerassi *et al.* (11) demonstrated that the similar displacement of steroidal mesylates with sodium azide in dimethyl sulfoxide proceeds with a high degree of stereospecificity.

H-2 at δ 3.33 for the tosylate VIII. The width of about 25 Hz. and the apparent six-peak multiplet for each signal are consistent with both H-1 and H-2 having axial orientations and being adjacent to two axial and one equatorial hydrogens. The signal of H-1 gives apparent coupling of $J_{aa} \simeq 10.5$ and $J_{ae} \simeq 4.0$ Hz. Spectrum B shows the signal of H-1 at δ 4.10 and H-2 at δ 3.24 for the azide IX. The narrow signal of H-1 (half-weight width of about 7 Hz.) is consistent with H-1 having an equatorial orientation.The six-peak multiplet of H-2 indicates that this hydrogen has an axial orientation and is adjacent to one axial and two equatorial hydrogens. The apparent coupling constants are $J_{aa} \simeq 11.5$ and $J_{ae} \simeq 3$ Hz. Thus, the NMR spectra also establish that in chloroform both VIII and IX have a very high time-average population of the chair conformation with the *o*-bromophenyl group in an equatorial orientation.

Lithium aluminum hydride reduction of the azide IX afforded (+)-*cis*-2-(*o*-bromophenyl)cyclohexylamine (X) in good yield. Formylation of this amine in a toluene–formic acid mixture produced (+)-*cis*-2-(*o*-bromophenyl)cyclohexyl formamide (XI), which was physically and spectrally identical to the formamide of the (+)-*cis*-2-(*o*-bromophenyl)cyclohexylamine previously resolved through its (−)-menthoxyacetamide (6).

Although these reactions can be expected to proceed with a high degree of stereospecificity, a small amount of racemization cannot be ruled out. Therefore, the optical purity of the compounds subsequent to ester IV cannot be stated with certainty.[5] However, since the specific rotation of the formamide XI agrees within 1% of that of the identical formamide obtained from the *cis*-amine resolved as its (−)-menthoxyacetamide (6), the optical purity of the entire series would appear to be quite high.

The absolute configuration of the ketone VI and of the benzylic carbon, C-2, of the compounds that have been related to it can be reliably obtained from the sign of the Cotton effect of the $n \rightarrow \pi^*$ transition of the carbonyl chromophore, as determined from ORD and CD spectra. The NMR spectrum of VI is consistent with a conformation in which the cyclohexane ring is in a chair form with the aryl group in an equatorial orientation, as seen for other 2-arylcyclohexanones (13). This equatorial orientation of the aromatic ring prevents the direct application of the octant rule (14) for assignment of absolute configuration. However, Galpin and Huitric (2) were able to assign the absolute configuration of (+)-2-*o*-tolylcyclohexanone as (2R) by comparison with the ORD curve of 3-β-phenyl-2-cholestanone, and their assignment has recently been proven correct by X-ray crystallographic studies (3). The ORD and CD spectra of (−)-2-(*o*-bromophenyl)cyclohexanone VI, shown in Fig. 3, are almost exact mirror images, both qualitatively and quantitatively, of those reported for (2R)-(+)-2-*o*-tolylcyclohexanone (2); thus the (−)-ketone VI can be reliably assigned a (2S) configuration. This establishes a (2S) configuration for the benzylic carbon of the compounds that were related chemically with VI.

[5] Brown and Garg (12) showed that the two-phase oxidation procedure used for obtaining VI from V proceeds without racemization of asymmetric centers adjacent to a carbonyl.

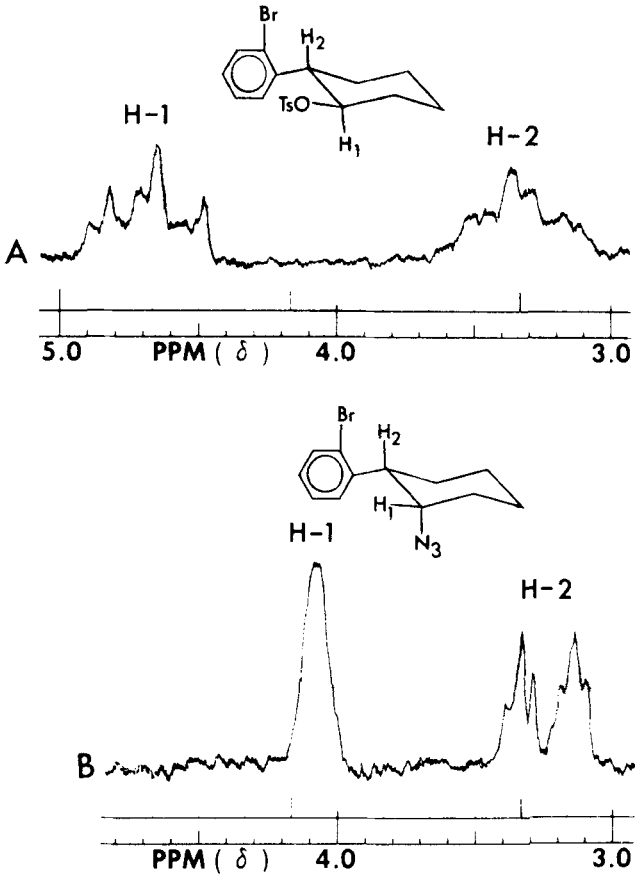


Figure 2—*Portions of the 60-MHz. NMR spectra of VIII (A) and IX (B) measured in chloroform-*d *at 37°.*

The ORD and CD spectra of the *cis*-alcohol VII and *cis*-amine X show Cotton effects of the same sign for the ^1L_a and ^1L_b transitions of the aromatic chromophore. This similarity is also true between the *trans*-alcohol V and (+)-*trans*-2-(*o*-bromophenyl)cyclohexylamine of the same chirality, whose resolution (6) and absolute configuration (15) were reported. This indicates a similarity in the nature of the dissymmetric perturbation of the aromatic chromophore by the amino and hydroxyl substituents. The ORD and CD spectra of the *cis*- and *trans*-amines and alcohols will be discussed in detail in a subsequent publication.

EXPERIMENTAL[6]

2-(o-Bromophenyl)cyclohexanone (II)—To a stirred solution of 10.5 g. (0.037 mole) of *trans*-2-(*o*-bromophenyl)nitrocyclohexane (I) (6) in 735 ml. of dioxane was slowly added 735 ml. (0.145 mole) of 0.2 *M* aqueous potassium hydroxide. The resulting clear solution was stirred at room temperature for 40 min., cooled in an ice–salt bath to 0°, and then brought to a pH of approximately 7.5 by the rapid addition of 295 ml. (0.59 mole) of 2.0 *M* aqueous magnesium sulfate. A solution of 4.2 g. (0.026 mole) of potassium permanganate in 100 ml. of water was added with stirring over a period of 30

[6] Melting points were determined on a Kofler micro hotstage and are corrected. Specific rotations were measured at ambient room temperature with a Rudolph polarimeter using a sodium lamp and a 2-dcm. tube. IR spectra were obtained on a Beckman IR5-A spectrophotometer. All NMR spectra were measured in the solvent specified on a Varian A-60 spectrometer using tetramethylsilane (TMS) as an internal reference. The ORD and CD spectra were obtained on a Cary 60 spectropolarimeter equipped with a model 6001 CD accessory. All VPC analyses were done on an F&M 5750 gas chromatograph equipped with hydrogen-flame detectors. Elemental analyses were performed by Huffman Laboratories, Wheatridge, Colo.

min. at 0–5°. This wine-colored mixture was stirred for an additional 20 min. and then extracted with 2 l. of ether in four portions. The combined ether solutions were washed with water, 10% hydrochloric acid, and water until neutral and then dried over anhydrous sodium sulfate. Filtration and removal of solvent afforded a pale-yellow oil, which was taken up in a small amount of ether and crystallized from hexane–ether (10:1). Decolorization with charcoal and recrystallization afforded 8 g. (87%) of colorless crystals, m.p. 58.2–59°, IR (mineral oil) 5.85 μ (C=O); NMR ($CDCl_3$) δ 4.08 (broad mult., half-width 19 Hz., 1 H, axial H on C-2).

Anal.—Calcd. for $C_{12}H_{13}BrO$: C, 56.94; H, 5.18. Found: C, 56.83; H, 5.28.

***trans*-2-(*o*-Bromophenyl)cyclohexanol (III)**—A solution of 7.8 g. (0.031 mole) of II in 150 ml. of anhydrous ether was added over a period of 1 hr. to a stirred slurry of 1.18 g. (0.031 mole) of powdered lithium aluminum hydride in 250 ml. of anhydrous ether. The mixture was stirred for an additional 1 hr.; then the excess lithium aluminum hydride was destroyed by the dropwise addition of an aqueous 40% Rochelle salts solution. This mixture was diluted with water and acidified with 10% hydrochloric acid. The ether layer was separated, and the aqueous phase was extracted twice with ether. The combined ethereal solutions were washed with water, 10% sodium carbonate, and water until neutral and then dried over anhydrous sodium sulfate. Filtration and removal of solvent afforded 7.64 g. (97%) of a colorless oil, which solidified upon standing. Analysis of this solid by VPC on an Apiezon column at 200° showed this material to be composed of 88% of the *trans*-alcohol III, 6% of its *cis*-isomer, and 6% of the debrominated 2-phenylcyclohexanols in the same *trans*/*cis* ratio. Repeated fractional crystallization of this mixture from hexane at room temperature yielded the pure *trans*-alcohol III, m.p. 73-73.5°; IR (mineral oil) 3.0 μ (O—H).

Anal.—Calcd. for $C_{12}H_{15}BrO$: C, 56.49; H, 5.93. Found: C, 56.62; H, 6.01.

(+) and (−)-*trans*-2-(*o*-Bromophenyl)cyclohexyl (−)-Menthoxyacetates—To a solution of 6.7 g. (0.026 mole) of III and 23 ml. (0.29 mole) of dry pyridine in 100 ml. of anhydrous ether was slowly added a solution of 6.7 g. (0.028 mole) of (−)-menthoxyacetyl chloride[7] (14) in 30 ml. of anhydrous ether. This mixture was allowed to stand at room temperature for 2 days and was then diluted with 100 ml. of ether. This ethereal solution was washed with cold 10% hydrochloric acid, water, cold 10% sodium carbonate, and water until neutral and then dried over anhydrous sodium sulfate. Filtration and removal of solvent yielded 11.8 g. (99%) of viscous oil.

(−)-*trans*-2-(*o*-Bromophenyl)cyclohexyl (−)-Menthoxyacetate (IV)—This compound was obtained by fractional crystallization of the mixture of diastereomeric menthoxyacetates from hexane at room temperature. The crude ester mixture was dissolved in 100 ml. of hot hexane and allowed to cool to room temperature. Compound IV crystallizes first in rocklike crystals. The most soluble diastereomer crystallizes in needle-shaped crystals. The rocklike crystals were recrystallized from hexane to a constant melting point, affording 7.8 g. from 28 g. of ester of colorless crystals, m.p. 111.7–112.7°; $[\alpha]_D = -43°$ (c 6, chloroform); IR (mineral oil) 5.70 μ (C=O). The NMR spectrum indicated the presence of only one diastereomer.

(−)-*trans*-2-(*o*-Bromophenyl)cyclohexanol (V)—To a solution of 2 g. of IV in 30 ml. of 95% ethanol was added 15 ml. of aqueous 25% sodium hydroxide, and the resulting mixture was refluxed for 3 hr. The solution was cooled, diluted with 100 ml. of cold water, and extracted with three 50-ml. portions of ether. The ethereal extracts were washed with water until neutral and then dried over anhydrous sodium sulfate. Filtration and removal of solvent afforded a pale-yellow oil, which was vacuum distilled to give an essentially quantitative yield of colorless oil, b.p. 104–106° at 0.1 mm.; $[\alpha]_D = -68°$ (c 5, methanol); IR (neat) 2.95 μ (O—H). The NMR spectrum was identical to that of its racemic modification III.

(−)-2-(*o*-Bromophenyl)cyclohexanone (VI)—To a stirred solution of 510 mg. (2.0 mmoles) of V in 15 ml. of ether was added a solution of 1.0 g. (3.3 mmoles) of sodium dichromate dihydrate and 0.8 ml. of 96% sulfuric acid in 15 ml. of water; the resulting mixture was stirred for 5 hr. and then diluted with 15 ml. of ether. The two phases were separated, and the aqueous layer was extracted with two 15-ml. portions of ether. The combined ethereal solutions were washed with water, 10% sodium carbonate, and water until neutral and then dried over anhydrous sodium sulfate. Filtration and removal of solvent afforded an oil which was shown by VPC analysis on an XE-60 column at 200° to contain less than 5% of the starting alcohol V. This material was crystallized from hexane to give 400 mg. (80%) of colorless crystals, m.p. 82.5–83.5°; $[\alpha]_D = -16°$ (c 3, methanol); IR (mineral oil) 5.86 μ (C=O). The NMR spectrum was identical to that of its racemic modification II. ORD in methanol (c 0.102 $[M]_{400}$ −248°, $[M]_{307}$ −2830° (trough), $[M]_{274}$ +4120° (peak), $[M]_{272}$ +3600° (trough), $[M]_{268}$ +4170° (peak), $[M]_{258}$ +3380° (trough), $[M]_{240}$ +4750°; CD in methanol (c 0.102) $[\theta]_{325}$ 0.0°, $[\theta]_{286}$ −5060°, $[\theta]_{246}$ 0.0°, $[\theta]_{240}$ +500°.

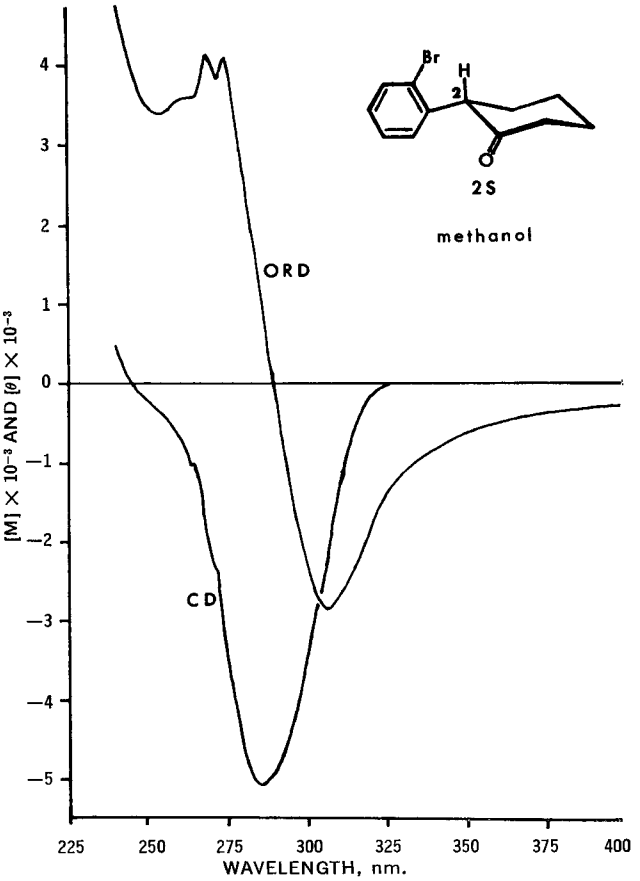


Figure 3—*ORD and CD curves of* (−)-*2*-(o-*bromophenyl*)*cyclohexanone VI.*

(+)-*cis*-2-(*o*-Bromophenyl)cyclohexanol (VII)—A solution of 0.48 g. (1.9 mmoles) of VI in 15 ml. of dry benzene was added over 15 min. to a stirred solution of 1.2 g. (6.1 mmoles) of triisobutylaluminum[8] in 25 ml. of dry benzene under nitrogen, and the resulting solution was stirred for an additional 45 min. This mixture was decomposed by the careful addition of 10 ml. of cold water while stirring and cooling in an ice bath, acidified with 10% hydrochloric acid, and extracted with 75 ml. of ether in three portions. The ethereal solution was washed with water until neutral and dried over anhydrous sodium sulfate. Filtration and removal of solvent yielded 0.47 g. of an oil, which was shown by VPC analysis on an Apiezon column at 200° to be a mixture of the *cis*- and *trans*-alcohols in a ratio of 4:1. Column chromatography of this mixture on alumina (Merck, neutral), using hexane and hexane–benzene mixtures as eluents, yielded the pure *cis*-alcohol VII, which was crystallized from hexane to give 250 mg. of colorless crystals, m.p. 63–63.5°; $[\alpha]_D = +123°$ (c 5, methanol); IR (mineral oil) 2.90 and 2.98 μ (free and bonded O—H).

Anal.—Calcd. for $C_{12}H_{15}BrO$: C, 56.49; H, 5.93. Found: C, 56.50; H, 6.03.

Tosylate of (−)-*trans*-2-(*o*-Bromophenyl)cyclohexanol (VIII)—To an ice-cold solution of 1.72 g. (6.75 mmoles) of V in 25 ml. of dry pyri-

[7] For an improved synthetic method for the preparation of this reagent, see Cochran and Huitric (6).

[8] K & K Laboratories.

dine was added 1.72 g. (9.0 mmole) of *p*-toluenesulfonyl chloride; the resulting solution was allowed to stand at refrigerator temperature for 2 days and was then diluted with 100 ml. of ice water and extracted with three 50-ml. portions of ether. The ethereal solution was washed with ice cold water, 10% hydrochloric acid, 10% sodium carbonate, and water until neutral and then dried over anhydrous sodium sulfate. The solution was filtered and concentrated to a volume of 5 ml., diluted with 10 ml. of hexane, and cooled in an ice bath to give 1.65 g. (60%) colorless fluffy crystals, m.p. 85–86°; IR (mineral oil) 8.55 μ (—SO_3—); NMR ($CDCl_3$) δ 2.32 (s, 3H, arom. —CH_3).

(+)-*cis*-2-(*o*-Bromophenyl)-1-azidocyclohexane (IX)—A vigorously stirred slurry of 1.5 g. (23 mmole) of dry sodium azide and 0.95 g. (2.3 mmole) of VIII in 50 ml. of dry *N*-methylpyrrolidone under nitrogen was slowly heated in an oil bath (4 hr.) at 90° and maintained at that temperature for 20 hr. The reaction mixture was cooled, diluted with 250 ml. of water, and extracted with three 100-ml. portions of petroleum ether (b.p. 40–50°) followed by three 100-ml. portions of ether. The combined petroleum ether and ether solutions were washed with water and dried over anhydrous sodium sulfate. Filtration and evaporation of solvent using a 40° water bath afforded 0.63 g. (98%) of an oil, which was chromatographed on silica gel, eluting with petroleum ether. A small amount of colorless oil, containing no azide stretching band in the IR, eluted first, followed by pure IX as a clear oil, IR (neat) 4.72 μ (—N_3); $[\alpha]_D = +123°$ (c 4, chloroform).

(+)-*cis*-2-(*o*-Bromophenyl)cyclohexylamine (X)—An ether solution of 0.33 g. of IX, which had not been chromatographed, was stirred with 0.15 g. of powdered lithium aluminum hydride for 2 hr., decomposed by the addition of a few drops of water, and filtered through a sintered-glass funnel. Evaporation of solvent yielded 0.27 g. (90%) of colorless oil, $[\alpha]_D = +95°$ (c 4, methanol); IR (neat) 2.98 and 3.05 μ (N—H).

(+)-*cis*-2-(*o*-Bromophenyl)cyclohexyl Formamide (XI)—A mixture of a solution of 0.25 g. of X in 10 ml. of toluene and 3 ml. of 99% formic acid was heated at gentle reflux for 1 hr. and then at a slightly higher temperature for 18 hr. so that water and formic acid azeotroped into a Dean-Stark tube. The reaction mixture was cooled, diluted with 25 ml. of ether, and washed twice with water, twice with 10% sodium carbonate, and with water until neutral; then it was dried over anhydrous sodium sulfate. Filtration and removal of solvent afforded a yellow oil, which was crystallized from a benzene–hexane mixture to give 150 mg. of off-white crystals, m.p. 159–160°; $[\alpha]_D = +119°$ (c 2, methanol); IR (mineral oil) 3.07 μ (N—H), 5.97 and 6.08 μ (C=O).

The formamide prepared in the same manner from (+)-*cis*-2-(*o*-bromophenyl)cyclohexylamine, which was resolved as its (−)-menthoxyacetamide (6), had the following physical properties: m.p. 158–159°; $[\alpha]_D = +120°$ (c 3, methanol). The IR and NMR spectra of the two formamides were identical.

Anal.—Calcd. for $C_{13}H_{16}BrNO$: C, 55.33; H, 5.72; N, 4.96. Found: C, 55.20; H, 5.72; N. 5.15.

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PETER H. DOUKAS* and TULLY J. SPEAKER

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Keyphrases □ Procainamide azulene analogs—synthesis □ Azulene analogs, procainamide—synthesis □ Column chromatography—separation □ IR spectrophotometry—structure □ UV spectrophotometry—structure

It is recognized that the presence of aromatic moieties in pharmacodynamic entities of varying specific activity may be prerequisite for optimal activity. These aromatic functions are, for the most part, benzenoid in nature. Several cyclic systems exist which show aromaticity but which are not benzenoid in character (1). Among these, azulene (isomeric to naphthalene), by virtue of its totally hydrocarbon nature, closely resembles benzene and its derivatives but differs in that it has a dipole moment, is a nonalternant hydrocarbon, has a resonance energy

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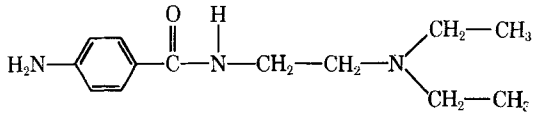
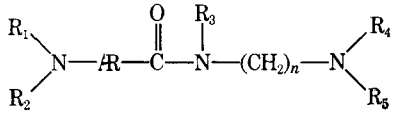
intermediate between that of benzene and naphthalene, and is considerably more reactive (2). The authors anticipate that these differences might alter the association of a pharmacophore with the biophase. Replacing the benzene moiety with the azulyl structure in pharmacodynamic systems requiring aromaticity in their pharmacophore affords the opportunity to study what effects this nonphysiologic, nonbenzenoid aromatic system might have upon the activity of a pharmacologically active molecule.

As a part of a continuing study (3) of the effects of nonbenzenoid aromatic systems on the activity of pharmacodynamic agents, a series of azulene analogs of procainamide (I), a known local anesthetic and antifibrillatory agent, was prepared. The synthesis of these materials is reported in this paper. Their biological evaluation is in process and will be reported in the near future.

Structure I may be reduced to the general formula II,



I

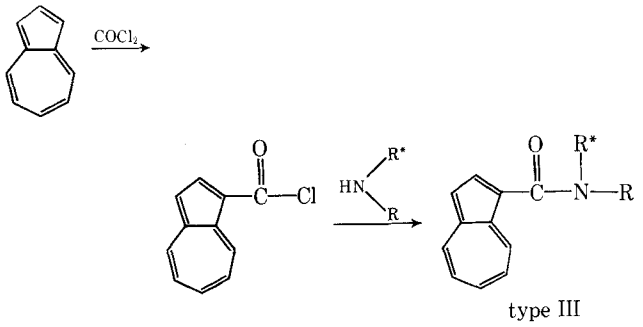


II

where AR indicates the presence of an aromatic group. In this series of 1,3-disubstituted azulenes (III, V, and VI), the structures and physical properties of which are given in Table I, the azulene nucleus would satisfy the aromatic prerequisite, R_2 would be a function altering the electronic distribution of the already polar azulene nucleus, and R_1 would be a substituted aminoalkyl side chain. Alteration of the electronic character of the aromatic system would be expected to impart different electrostatic properties to the variously substituted compounds and perhaps thereby alter affinity and/or intrinsic activity. Although resonance interactions may not occur between the electron-rich 1- and 3-positions of azulene, as they occur in *para*-substituted benzenoid pharmacologic agents, the inductive interactions are quite strong, and substitution at either position markedly affects the electron density of the other.

Type III compounds (azulene-1-carboxamides) were prepared *via* carboxychlorination of azulene, after the method of Treibs *et al.* (4), followed by reaction of the azulene acid chloride with the appropriate amine (Scheme I).

3-Nitroazuloic acid (IV), which would easily serve as a common intermediate in the synthesis of compounds of Types V and VI, has not been previously reported in the literature. The preparation of its methyl ester, *via* cupric nitrate nitration in low yield and a nonreproducible tetranitromethane nitration in good yield, was reported by Anderson *et al.* (5). A method requiring critical attention to reaction conditions was developed in this laboratory for the synthesis of 3-nitroazuloic acid in satisfactory yields (about 45–65%). The synthesis



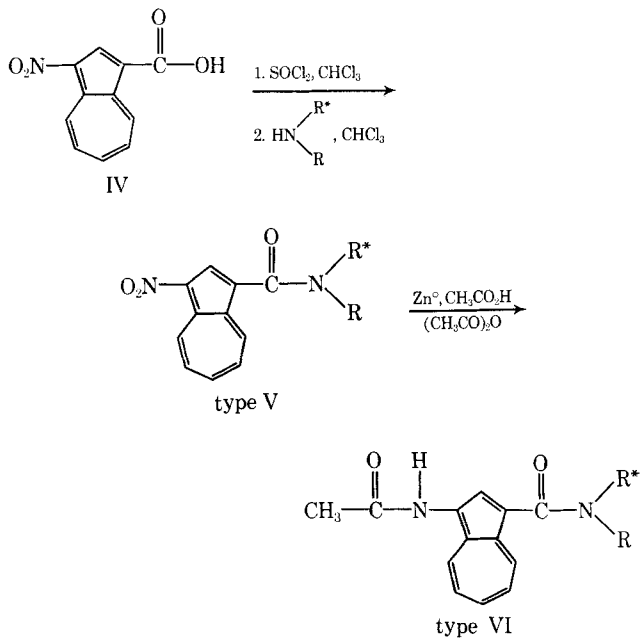
type III

R* may be H or R

Scheme I

requires a very slow addition of tetranitromethane in ethanol to a solution of azuloic acid in freshly distilled pyridine. The purity of the pyridine and the addition rate are essential, since alteration of either condition results in drastic reduction of yield and the production of intractable tars. The product displays a visible absorption maximum hypsochromic shift of 40–50 nm. from that of azuloic acid. It has been shown that a shift of this type occurs when a known azulene is substituted in the 3-position with a nitro group (5). Further structural proof was obtained by conversion of the acid to two known derivatives, methyl-3-nitroazuloate and methyl-3-acetamidoazuloate, for which physical data were reported previously. The preparation of 3-nitroazuloic acid affords an intermediate that will facilitate the synthesis of several 1,3-disubstituted azulyl systems, heretofore unattainable or attainable only through extended syntheses with poor yields.

Reacting the 3-nitro acid chloride with appropriate amines gave 3-nitroazulene-1-carboxamides (Type V compounds), as shown in Scheme II.



IV

type V

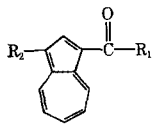
type VI

R* may be H or R

Scheme II

The 3-nitro group provided a strong electron-withdrawing substituent; in the interest of including a

Table I—Azulene Carboxamides



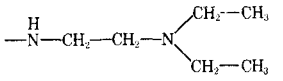
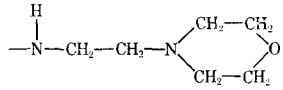
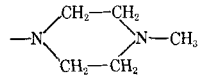
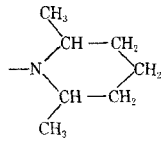
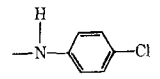
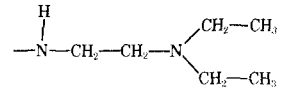
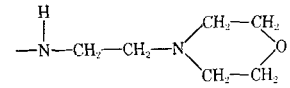
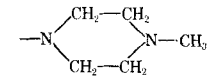
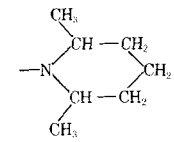
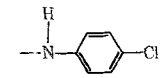
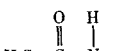
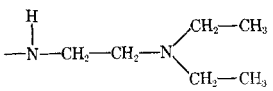
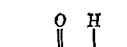
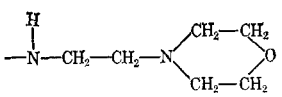
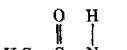
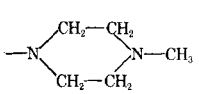
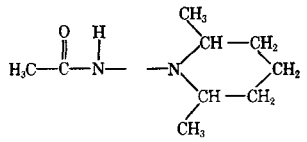
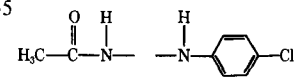
No.	R_2	R_1	M.p.	Formula	Anal., % Calcd.	Anal., % Found	Spectral Data IR Max., $cm.^{-1}$	Spectral Data UV-Vis. Max., nm.	Spectral Data Log ϵ
III-1	H—		Oil	$C_{17}H_{22}N_2O$	C, 75.47 H, 8.20 N, 10.36	C, 75.23 H, 7.97 N, 10.17	3240 1651	545 370 353 290 232	2.48 3.78 3.70 4.63 4.40 sh
III-2	H—		112–114°	$C_{17}H_{20}N_2O_2$	C, 71.80 H, 7.09 N, 9.85	C, 71.69 H, 7.13 N, 9.85	3250 1625	550 365 350 290 230	2.60 3.89 3.74 4.66 4.46 sh
III-3	H—		94.5–95.5°	$C_{16}H_{18}N_2O$	C, 75.56 H, 7.13 N, 11.02	C, 75.30 H, 7.17 N, 11.10	1610	560 360 342 285 230	2.54 3.61 3.65 4.61 4.39
III-4	H—		96°	$C_{17}H_{21}NO$	C, 79.95 H, 8.29 N, 5.48	C, 80.17 H, 8.01 N, 5.21	1615	570 355 340 286 282 230	2.34 3.29 3.45 3.40 sh 4.42 4.18
III-5	H—		182–183.5°	$C_{17}H_{12}ClNO$	C, 72.47 H, 4.29 N, 5.68	C, 72.35 H, 4.05 N, 5.54	3200 1625	543 365 300 260 235	2.67 4.17 4.67 4.21 4.35
V-1	O_2N—		99–100°	$C_{17}H_{21}N_3O_3$	C, 64.74 H, 6.71 N, 13.32	C, 64.57 H, 6.90 N, 13.11	3250 1640	495 382 310 285 235	3.01 4.03 4.18 sh 4.35 4.27
V-2	O_2N—		210–212°	$C_{17}H_{19}N_3O_4$	C, 61.99 H, 5.81 N, 12.76	C, 62.25 H, 5.69 N, 12.40	3250 1640	490 387 305 285 230	3.07 4.14 4.34 4.48 4.38
V-3	O_2N—		197–198°	$C_{16}H_{17}N_3O_0$	C, 64.20 H, 5.73 N, 14.04	C, 64.15 H, 5.94 N, 13.99	1610	502 395 308 280 235 220	2.95 3.04 3.23 3.34 3.28 sh 3.36
V-4	O_2N—		156–158°	$C_{18}H_{22}N_2O_3$	C, 69.21 H, 6.45 N, 8.97	C, 69.21 H, 6.56 N, 8.81	1635	510 398 310 278 220	2.92 4.01 4.20 4.30 4.35
V-5	O_2N—		>270°	$C_{17}H_{11}ClN_2O_3$	C, 62.49 H, 3.39 N, 8.57	C, 62.47 H, 3.57 N, 8.28	1650	390 295 240	0.55[a] 1.48 0.82
VI-1			125–128°	$C_{19}H_{25}N_3O_2$	C, 69.69 H, 7.70 N, 12.83	C, 69.40 H, 7.45 N, 12.72	3215 1640	598 375 298 238	2.60 3.76 4.57 4.39
VI-2			186–187°	$C_{19}H_{23}N_3O_3$	C, 66.84 H, 6.79 N, 12.31	C, 66.56 H, 6.86 N, 12.02	3200 1650	600 380 300 240	2.43 3.65 4.54 4.42
VI-3			157–159°	$C_{18}H_{21}N_3O_2$	C, 69.43 H, 6.80 N, 13.49	C, 69.30 H, 6.67 N, 13.27	3200 1650	615 378 295 235	2.53 3.71 4.54 4.34

Table I—*(Continued)*

No.	R_2	R_1	M.p.	Formula	Anal., % Calcd.	Anal., % Found	Spectral Data: IR Max., $cm.^{-1}$	Spectral Data: UV–Vis. Max., nm.	Spectral Data: Log ϵ
VI-4			144–145°	$C_{20}H_{24}N_2O_2$	C, 72.04 H, 7.56 N, 8.40	C, 71.99 H, 7.47 N, 8.40	3250 1675	620 383 370 295 236	2.56 3.76 3.74 sh 4.62 4.38
VI-5			144–146° dec.	$C_{19}H_{15}ClN_2O_2 \cdot H_2O$	C, 63.96 H, 4.80 N, 7.85	C, 64.28 H, 4.98 N, 7.82	3245 1650	590 373 305 240	2.50 3.80 4.07 4.00

[a] $OD_{max.}$

strong electron-donating group so as to encompass as wide a range of electronic effects as possible, the preparation of 3-amino-1-azulene carboxamides was investigated. The reduction of the 3-nitro compounds seemed to be a facile route to the production of the desired 3-amino compounds. In attempting to prepare these compounds, it was found that both the free amines and several of their acid salts were very unstable and decomposed readily. This property of azulyl amines was reported previously (6), but the extreme degree of instability was unexpected. In effect, the 3-amino compounds, although perhaps often prepared, could not be isolated in this study.

By contrast, the acetamidoazulenes are known to be stable systems and, recognizing that the acetamido function exerts a mild positive inductive effect, it was decided that this group might well be substituted for the apparently unavailable 3-amino function.

The 3-acetamidoazulene-1-carboxamides (Type VI compounds) were prepared from the 3-nitro carboxamides *via* a dissolving metal reduction utilizing zinc in acetic acid in the presence of acetic anhydride (Scheme II).

EXPERIMENTAL[1]

Materials and Instrumentation—All melting points were taken on a Thomas-Hoover Uni-Melt apparatus and are uncorrected. UV and visible absorption spectra were obtained with a Cary model 15 recording spectrophotometer utilizing ethanol as the solvent. IR spectra were obtained with a Perkin-Elmer Infracord model 127 recording spectrophotometer utilizing potassium bromide disks unless otherwise indicated. Azulene,[2] phosgene,[3] tetranitromethane,[4] and ethanol[5] were used. Other solvents and other reagents were of reagent grade.[6] When necessary, solvents were freshly distilled prior to use.

General Chromatographic Procedure—Chromatographic separations employed Merck Reagent Aluminum Oxide (Alumina) No. 71707 in the following procedure: concentrated solutions containing starting materials, reaction products, and decomposition products were adsorbed on a small amount of alumina (5–15 g.). The alumina adsorbate was air dried with frequent stirring. The resulting free-flowing adsorbate was packed above a 2 × 10, 4 × 20, or 4 × 40-cm. column of fresh alumina and eluted with solvents of increasing polarity. The following solvents proved useful: petroleum ether, benzene, toluene, dichloromethane, chloroform, ethanol, and combinations of these.

General Method for the Preparation of Azulene-1-carboxamides (Type III Compounds)—A slow stream of gaseous phosgene was added with stirring to a solution of 0.500 g. (0.004 mole) of azulene in 70 ml. of freshly distilled toluene contained in a 300-ml. three-necked flask equipped with a gas inlet tube and gas trap. After an addition time of 30–40 min., during which the color of the mixture changed from blue to wine-red, the gas supply was removed, and the mixture was allowed to stir for another 40 min. At the end of this interval, the reaction mixture was flushed of phosgene. This was conveniently done at this scale by placing the reaction mixture in a 100-ml. sintered-glass funnel secured atop a filter flask into which gaseous nitrogen was being introduced (at a very low pressure) *via* the side arm. The nitrogen escaped through the sinter into the toluene in the form of numerous small bubbles. The system was flushed in this way with nitrogen for 3–4 hr. to remove excess HCl and phosgene. At the end of this interval the nitrogen flow was stopped, 0.02 mole of the appropriate amine in 10 ml. of dry toluene was placed at the bottom of the filter flask, and the acid chloride solution was added *via* suction through the sintered-glass funnel. Upon contact with the amine solution, the wine-red color of the acid chloride solution turned to dark blue with the liberation of HCl. After complete addition, the reaction mixture was stirred for 10 min., filtered to remove insoluble amine hydrochloride, and then worked up to give the azulene-1-carboxamides. The purification of the individual azulene-1-carboxamides is reported later. Unless otherwise indicated, the reaction mixture was extracted with dilute hydrochloric acid, and the aqueous phase was chilled in an ice bath and made alkaline with dilute aqueous ammonia. The aqueous phase was extracted with chloroform, and the resultant blue organic layer was dried over sodium sulfate, filtered, concentrated, and chromatographed on alumina. Typical yields ranged from 75–90%.

1-(2-Diethylaminoethyl)-azulenecarboxamide (III-1)—This material was prepared and isolated according to the general procedure for the preparation of azulene-1-carboxamides. Chloroform eluted a single blue band. Evaporation of the chloroform yielded a blue oil which could not be induced to crystallize. IR: 3240, 1650 $cm.^{-1}$; $\lambda_{max.}$ (log ϵ): 545(2.48), 370(3.79), 353(3.70), 290(4.63), 232(4.40 sh).

Anal.—Calcd. for $C_{17}H_{22}N_2O$: C, 75.47; H, 8.20; N, 10.36. Found: C, 75.23; H, 7.97; N, 10.17.

1-(2-Morpholinoethyl)-azulenecarboxamide (III-2)—This material was prepared and isolated according to the general procedure for the preparation of azulene-1-carboxamides. Chloroform eluted a single blue band. Partial evaporation of the chloroform at room temperature gave successive crops of blue crystals which were collected, washed with petroleum ether, and air dried, m.p. 112–114°. IR: 3250, 1625 $cm.^{-1}$; $\lambda_{max.}$ (log ϵ): 550(2.60), 365(3.89), 350 (3.74), 290(4.66), 230(4.46 sh).

Anal.—Calcd. for $C_{17}H_{20}N_2O_2$: C, 71.80; H, 7.09; N, 9.85. Found: C, 71.69, H, 7.13; N, 9.85.

1-(*N*-Methylpiperazine)-azulenecarboxamide (III-3)—This material was prepared and chromatographically isolated according to the general procedures already described. The chloroform eluate

[1] Analyses were performed by the Schwarzkopf Microanalytical Laboratories, Woodside, N. Y.
[2] Purchased from Henley & Co., Inc., New York, N. Y.
[3] Purchased from the Union Carbide Corp., Linde Div.
[4] Purchased from Aldrich Chemical Company as were all the amines utilized in the syntheses.
[5] Publiker Industries.
[6] B. A. Baker & Co. or Fisher Scientific Co.

was allowed to evaporate at room temperature to yield a blue oil. The oil was triturated with petroleum ether in a dry ice–acetone bath to yield a dark-blue solid, which was collected, air dried, and redissolved in chloroform. Slow evaporation of the chloroform gave dark-blue crystals, m.p. 94.5–95.5°. IR: 1610 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 560(2.54), 360(3.61), 342(3.65), 285(4.61), 230(4.39).

Anal.—Calcd. for $C_{16}H_{18}N_2O$: C, 75.56; H, 7.13, N, 11.02. Found: C, 75.30; H, 7.17; N, 11.10.

1-(2,6-Dimethylpiperidine)-azulenecarboxamide (III-4)—This material was prepared according to the general procedure for the preparation of azulene-1-carboxamides. The reaction mixture was washed with 5% hydrochloric acid and dilute aqueous ammonia. The toluene layer was then dried over sodium sulfate, filtered, concentrated, and chromatographed on alumina. Petroleum ether eluted unreacted azulene and left a large blue band. The blue band was immobile in the usual solvents, so it was extruded and extracted in a continuous extraction apparatus with a mixture of equal parts of chloroform and carbon tetrachloride for 10 hr. The solvent was evaporated at room temperature to yield a blue solid which, upon recrystallization from hot aqueous ethanol, gave blue crystals, m.p. 96°. IR: 1615 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 570(2.34), 355(3.29), 340(3.45), 286 (3.40 sh), 282 (4.42), 230(4.18).

Anal.—Calcd. for $C_{17}H_{21}NO$: C, 79.95; H, 8.29; N, 5.48. Found: C, 80.17; H, 8.01; N, 5.21.

1-(*p*-Chloroaniline)-azulenecarboxamide (III-5)—This material was prepared according to the general procedure for the preparation of azulene-1-carboxamides. During this reaction, most of the product precipitated out of the reaction mixture. The resultant blue solid was taken up in chloroform, and the organic layer was washed with 5% hydrochloric acid and dilute aqueous ammonia; it was dried over sodium sulfate and allowed to evaporate at room temperature to give dark-blue crystals, m.p. 182–183.5°. IR: 3200, 1625 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 543(2.67), 365(4.17), 300(4.67), 260(4.21), 235(4.35).

Anal.—Calcd. for $C_{17}H_{12}ClNO$: C, 72.47; H, 4.29; N, 5.68. Found: C, 72.35; H, 4.05; N, 5.54.

Preparation of Azuloic Acid—This material was prepared according to the method of Treibs *et al.* (4).

Preparation of 3-Nitroazuloic Acid (IV)—At room temperature a solution of 1.345 ml. (0.0112 mole, 40% molar excess) tetranitromethane in 3 ml. anhydrous ethanol was added dropwise to a solution of 1.360 g. (0.0079 mole) azuloic acid in 15 ml. of recently distilled pyridine contained in a 100-ml. three-necked flask equipped with dropping funnel, mechanical stirrer, and drying tube. Addition time was 2.5 hr. The reaction mixture was stirred for 16 hr., during which the color of the mixture changed from blue to reddish-brown, and then was filtered in a sintered-glass funnel. The yield of orange solid was repeatedly washed with petroleum ether until the washings showed no bluish color, indicative of unreacted azuloic acid. The solid was then washed with anhydrous ether, which removed all but the last traces of pyridine, and was allowed to air dry. The yield of crude 3-nitroazuloic acid was 1.10 g. (65.0% yield). When recrystallized from hot ethanol and water, the crude product gave orange needles, m.p. >270°. IR: 1690 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 483(2.87), 328(3.90), 312(4.17), 285(4.36), 215 (4.36).

Anal.—Calcd. for $C_{11}H_7NO_4 \cdot H_2O$: C, 56.17; H, 3.86; N, 5.96. Found: C, 56.65; H, 3.45; N, 6.22.

Preparation of Methyl Ester of 3-Nitroazuloic Acid—The acid chloride of 3-nitroazuloic acid was prepared according to the *in situ* method described later and used without further purification. The acid chloride was dissolved in 30 ml. of chloroform and then stirred at room temperature with an excess of methanol. The solvent mixture was allowed to evaporate at room temperature overnight to yield 0.320 g. of red crystals, m.p. 144–146° [lit. (5) 146°].

Preparation of Methyl Ester of 3-Acetamidoazuloic Acid—Powdered zinc, 0.500 g. (0.0077 g. atom), was added in divided portions over a 0.5-hr. interval to a solution of methyl-3-nitroazuloate, 0.150 g. (6.4 mmoles), and sodium acetate, 1.00 g. (0.012 mole), in a mixture of 2 ml. acetic acid and 15 ml. acetic anhydride. The reaction mixture was stirred for 0.5 hr. after complete addition of the zinc, during which the color of the solution changed from red to blue. The mixture was decanted from any unreacted zinc, diluted with 50 ml. of ice water, and extracted with chloroform. The blue chloroformic extract was dried over sodium sulfate, filtered, concentrated, and chromatographed on alumina. Benzene eluted a yellow band and chloroform eluted a brown band, closely followed by a blue band. The blue eluate was evaporated at room temperature to yield 0.105 g. of blue needles, m.p. 204–206° [lit. (5) 206°].

General Method for the Preparation of 1-(3-Nitroazulene)-carboxamides (Type V Compounds)—In Situ *Preparation of 3-Nitroazuloic Acid Chloride*—A solution of 0.960 g. (0.0081 mole) of thionyl chloride in 10 ml. of recently distilled chloroform was added dropwise to a refluxing suspension of 0.600 g. (0.0027 mole) 3-nitroazuloic acid in 100 ml. of recently distilled chloroform in a 250-ml. three-necked flask equipped with a heating mantle, dropping funnel, reflux condenser, and mechanical stirrer. Addition time was 30 min., after which the mixture was refluxed with stirring for 2.5 hr. During this interval, most of the solids dissolved. The mixture was filtered hot, and the filtrate was evaporated to dryness under reduced pressure and with the aid of gentle heat (50°) to yield a reddish-orange solid. This solid was suitable for use without additional purification.

Conversion of Acid Chloride to Amide—The solid acid chloride was dissolved in 100 ml. of recently distilled chloroform. A 3–5 molar excess of the appropriate amine in 10 ml. of chloroform was added dropwise to the resultant solution, over a period of 15 min., at room temperature. The mixture was stirred for an additional 30 min. at room temperature and then was successively washed with dilute aqueous ammonia and water. The chloroformic solution, after drying over sodium sulfate, was filtered and concentrated *in vacuo* to about one-tenth volume. The concentrate was then adsorbed onto alumina and chromatographed. The elution and crystallization procedures for the individual 1-(3-nitroazulene)-carboxamides are given later. The typical yields were 75–80%.

1-(2-Diethylaminoethyl)-3-nitroazulenecarboxamide (V-1)—This compound was prepared by the general method of preparation of 1-(3-nitroazulene)-carboxamides described above and chromatographed on alumina. Elution with petroleum ether separated a minor orange band. Dichloromethane–petroleum ether (50:50) eluted a minor orange band and a closely following greenish-brown band. Dichloromethane slowly eluted a large red band, leaving several bands yet uneluted. The dichloromethane eluate was allowed to evaporate spontaneously to yield a red oil. The red oil was dissolved in a chloroform–*n*-heptane mixture (50:50) which, on slow partial evaporation at room temperature, gave red needles, m.p. 99–100°. IR: 3250, 1640 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 495(3.01), 382(4.03), 310(4.18 sh), 285(4.35), 235(4.27).

Anal.—Calcd. for $C_{17}H_{21}N_3O_3$: C, 64.74; H, 6.71; N, 13.32. Found: C, 64.57; H, 6.90; N, 13.11.

1-(2-Morpholinoethyl)-3-nitroazulenecarboxamide (V-2)—This compound was prepared by the general method described for 1-(3-nitroazulene)-carboxamides and was chromatographed on alumina. Elution with toluene gave a minor yellow band, and dichloromethane–chloroform (50:50) separated a large orange band from a faintly yellow band. The orange band was extruded, packed atop a fresh alumina column, and eluted with chloroform. The chloroform eluate was diluted with an equal amount of *n*-heptane, and the mixture was allowed to evaporate spontaneously to yield reddish-orange crystals, m.p. 210–212°. IR: 3250, 1640 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 490(3.07), 387(4.14), 305(4.34), 285(4.48), 230(4.38).

Anal.—Calcd. for $C_{17}H_{19}N_3O_4$: C, 61.99; H, 5.81; N, 12.76. Found: C, 62.25; H, 5.69; N, 12.40.

1-(N-Methylpiperazine)-3-nitroazulenecarboxamide (V-3)—This compound was prepared by the general method described for 1-(3-nitroazulene)-carboxamides and was chromatographed on alumina. Elution with toluene produced a faintly yellowish-orange band. Chloroform slowly eluted a major reddish-orange band, leaving a weak yellow band. The chloroform eluate was allowed to evaporate spontaneously to yield a red solid which was recrystallized from chloroform–*n*-heptane (50:50) to give reddish-maroon crystals, m.p. 197–198°. IR: 1610 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 502(2.95), 395(3.04), 308(3.23), 280(3.34), 235 (3.28 sh), 220(3.36).

Anal.—Calcd. for $C_{16}H_{17}N_3O_3$: C, 64.20; H, 5.73; N, 14.04. Found: C, 64.15; H, 5.94; N, 13.99.

1-(2,6-Dimethylpiperidine)-3-nitroazulenecarboxamide (V-4)—This compound was prepared by the general method described for 1-(3-nitroazulene)-carboxamides and was chromatographed on alumina. Dichloromethane eluted first a minor orange band and then a closely following major red band. Because these bands were difficultly resolved, this separation required a long column. The red eluate was collected and allowed to evaporate spontaneously, yielding a red glass. The glass was taken up in the minimum amount of chloroform, and the resultant solution was diluted with an equal

amount of *n*-heptane. Slow evaporation yielded reddish-maroon crystals, m.p. 156–158°. IR: 1635 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 510(2.92), 398(4.01), 310(4.20), 278(4.30), 220(4.35).

Anal.—Calcd. for $C_{18}H_{22}N_2O_3$: C, 69.21; H, 6.45; N, 8.97. Found: C, 69.21; H, 6.56; N, 8.81.

1-(p-Chloroaniline)-3-nitroazulenecarboxamide (V-5)—This material separated out of the reaction mixture and was collected by filtration. It was found to be poorly soluble in most solvents. To purify the compound, 50-mg. aliquots were dissolved in 100 ml. of boiling anhydrous ethanol. The resultant solution was allowed to cool to room temperature to give red needles, which were collected by filtration and washed with cold ethanol, m.p. >270°. IR: 1650 cm.$^{-1}$; $\lambda_{max.}$ ($OD_{max.}$):[7] 390(0.55), 295(1.48), 240(0.82).

Anal.—Calcd. for $C_{17}H_{11}ClN_2O_3$: C, 62.49; H, 3.39; N, 8.57. Found: C, 62.47; H, 3.57; N, 8.28.

General Method for the Preparation of 1-(3-Acetamidoazulene)-carboxamides (Type VI Compounds)—Over an interval of 1 hr., 0.600 g. (0.0090 g. atom) of powdered zinc was gradually added to a stirred solution of 0.001 mole of the appropriate 1-(3-nitroazulene)-carboxamide and 0.600 g. (0.0074 mole) of sodium acetate in 30 ml. of a mixture of equal volumes of acetic acid and acetic anhydride. During the addition, the color of the solution gradually turned from red to blue. After complete addition, the mixture was stirred an additional 0.5 hr. and then decanted from any unreacted zinc into 100 ml. of ice water. The resulting aqueous solution was slowly made alkaline with 10% sodium hydroxide solution in an ice water bath and extracted with chloroform. The greenish-blue chloroform extracts were dried over sodium sulfate, filtered and concentrated. The purification procedures for the individual materials follow. Typical yields were 60–70%.

1-(2-Diethylaminoethyl)-3-acetamidoazulenecarboxamide (VI-1)—This material was prepared according to the general procedure for 1-(3-acetamidoazulene)-carboxamides and was chromatographed on alumina. Benzene eluted yellow and blue bands. A mixture of ethanol and toluene (5:95) eluted the product. Evaporation of the ethanol–toluene eluate gave a bluish-green solid, m.p. 125–128°. IR: 3215, 1640 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 598(2.60), 375(3.76), 298(4.57), 238(4.39).

Anal.—Calcd. for $C_{19}H_{25}N_3O_2$: C, 69.69; H, 7.70; N, 12.83. Found: C, 69.40; H, 7.45; N, 12.72.

1-(2-Morpholinoethyl)-3-acetamidoazulenecarboxamide (VI-2)—This material was prepared according to the general procedure for 1-(3-acetamidoazulene)-carboxamides and was chromatographed on alumina. Dichloromethane eluted a light-orange band. Ethanol–toluene (10:90) eluted a brown band closely followed by a greenish-blue band. The greenish-blue eluate was allowed to stand at room temperature, and partial evaporation gave green crystals, m.p. 186–187°. IR: 3200, 1650 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 600(2.43); 380(3.65), 300(4.54), 240(4.42).

Anal.—Calcd. for $C_{19}H_{23}N_3O_3$; C, 66.84; H, 6.79; N, 12.31. Found: C, 66.56; H, 6.86; N, 12.02.

1-(N-Methylpiperazine)-3-acetamidoazulenecarboxamide (VI-3)—This material was prepared according to the general procedure for 1-(3-acetamidoazulene)-carboxamides and was chromatographed on alumina. Dichloromethane eluted a small green band. A mixture of ethanol and toluene (10:90) eluted a greenish-blue band which, upon evaporation, gave a green solid (m.p. 154–156°) that could not be satisfactorily recrystallized. The crude solid was taken up in the minimum amount of chloroform and chromatographed on alumina. A mixture of ethanol and toluene (15:85) eluted the product and yielded greenish-blue needles upon slow evaporation, m.p. 157–159°. IR: 3200, 1650 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 615(2.53), 378(3.71), 295(4.54), 235(4.34).

Anal.—Calcd. for $C_{18}H_{21}N_3O_2$: C, 69.43; H, 6.80; N, 13.49. Found: C, 69.30; H, 6.67; N, 13.27.

1-(2,6-Dimethylpiperidine)-3-acetamidoazulenecarboxamide (VI-4)—This material was prepared according to the general procedure for 1-(3-acetamidoazulene)-carboxamides and chromatographed on alumina. Toluene eluted yellow and red bands. A mixture of ethanol and toluene (10:90) eluted the product. Slow evaporation of the ethanol–toluene eluate yielded a blue solid which was recrystallized from hot aqueous ethanol, m.p. 144–146°. IR: 3250, 1675 cm.$^{-1}$: $\lambda_{max.}$(log ϵ): 620(2.56), 383(3.76), 370(3.74 sh), 295(4.62), 236(4.38).

Anal.—Calcd. for $C_{20}H_{24}N_2O_2$: C, 72.04; H, 7.56; N, 8.40. Found: C, 71.99; H, 7.47; N, 8.40.

1-(p-Chloroaniline)-3-acetamidoazulenecarboxamide (VI-5)—This material was prepared according to the general procedure for 1-(3-acetamido)-azulenecarboxamides and chromatographed on alumina. The nitroamide was initially insoluble in the reduction mixture, but as the zinc was added (and reduction began to take place) the material gradually dissolved. Petroleum ether eluted a yellow band, and toluene–chloroform (50:50) eluted a brown band. Chloroform slowly eluted the product. Evaporation of the chloroform yielded a crude solid which was recrystallized from hot aqueous ethanol to give yellowish-green crystals, m.p. 144–146° dec. IR: 3245, 1650 cm.$^{-1}$; $\lambda_{max.}$ (log ϵ): 590(2.50), 373(3.80), 305(4.07). 240(4.00).

Anal.—Calcd. for $C_{19}H_{15}ClN_2O_2 \cdot 1H_2O$: C, 63.96; H, 4.80; N, 7.85. Found: C, 64.28; H, 4.98; N, 7.82.

[7] A sufficient quantity of this material to give log ϵ data could not be dissolved; therefore, optical density values ($OD_{max.}$) are given.

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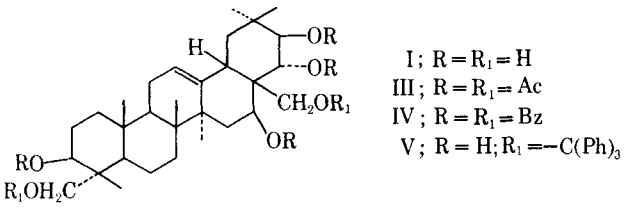
Constituents from *Gymnema sylvestre* Leaves VIII: Isolation, Chemistry, and Derivatives of Gymnemagenin and Gymnestrogenin

G. SUBBA RAO* and JOSEPH E. SINSHEIMER

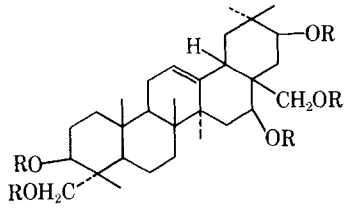
Abstract □ Isolation of crystalline gymnemagenin and gymnestrogenin directly from the leaves of *Gymnema sylvestre*, together with preparation of various derivatives of the two aglycones, is described.

Keyphrases □ *Gymnema sylvestre* leaves—constituents □ Gymnestrogenin, gymnemagenin—isolation, identification □ IR spectrophotometry—structure □ UV spectrophotometry—structure

In an earlier paper (1) on the constituents of gymnemic acid, the antisweet principle of *Gymnema sylvestre* R. Br. (Asclepiadaceae) leaves, the authors showed gymnemagenin (I) and gymnestrogenin (II) to be the aglycones of gymnemic acids A and B and C and D, respectively. The antiviral activity of acids A and B was also noted (2). To elucidate the structure of gymnemagenin and to obtain material for antiviral structure–activity studies, it became necessary to develop an isolation procedure for securing substantial quantities of this aglycone directly from the leaves of *G. sylvestre*. The present paper describes: (*a*) procedures for the isolation of crystalline gymnemagenin and gymnestrogenin; (*b*) the chemistry of gymnemagenin to support Structure I, deduced recently on the basis of spectral data (3–5); and (*c*) the synthesis of derivatives of the aglycones and of gymnemic acid A for antiviral activity studies.



I; R = R₁ = H
III; R = R₁ = Ac
IV; R = R₁ = Bz
V; R = H; R₁ = —C(Ph)₃



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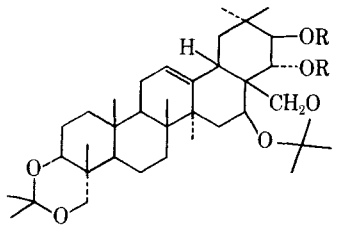
VI; R = Ac



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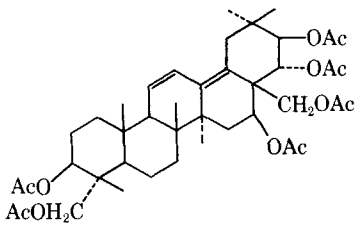
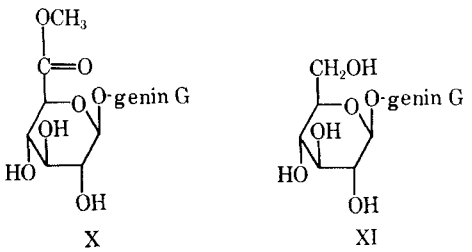
VIII; R = Ac



IX



X XI

Acidic and basic hydrolyses of the crude gymnemic acid mixture, obtained by mineral acid precipitation (6, 7) from the aqueous extracts of *G. sylvestre* leaves, gave gymnemagenin containing a trace amount of gymnestrogenin. Resolution of this genin mixture was carried out by silicic acid chromatography. Recrystallization from chloroform–methanol gave gymnemagenin (I) as stout, white needles, m.p. 329.5–331° [lit. (8), m.p. 328–335°], and gymnestrogenin (II) as fine needles, m.p. 286–288° [lit. (9), m.p. 288–289°]. Identity of gymnemagenin and gymnestrogenin with reference samples (8, 9) was established through undepressed mixed melting point, superimposable IR spectra, and identical TLC behavior. Similarly, the hexaacetate of gymnemagenin (III) and the pentaacetate of gymnestrogenin (VI) were found to be identical to the corresponding reference compounds.

Gymnemagenin formed the hexaacetate (III), m.p. 290–291° [lit. (8), m.p. 290–291°], as well as an hexabenzoate (IV), m.p. 186–188°, under mild conditions. This indicated the presence of six sterically unhindered primary and/or equatorially oriented secondary hydroxyl groups (10). Further confirmation for these six hydroxyl groups was attained by spectrophotometric determination (11). Of these six hydroxyl groups, two were found to be primary since gymnemagenin gave rise to a dityrityl derivative (V), m.p. 268–269°, upon treatment with triphenyl chloromethane (10, 12). While reactions of gymnemagenin with lead tetraacetate (13) and sodium periodate (14) were suggestive of a 1,2-glycol system, as shown by Stöcklin (4), its *trans*-configuration was inferred in the present investigation by the reversible reaction of gymnemagenin with monopotassium triacetylosmate (15).

Copper pyrolysis (12) of gymnemagenin at 270–290° generated formaldehyde, which was characterized as its dimedone derivative. Treatment of gymnemagenin with acetone–sulfuric acid at 0° for 48 hr. gave a di-*O*-isopropylidene derivative (VII),[1] m.p. 301–303°. Acet-

[1] Structure elucidation of the isopropylidene derivatives, VII and VIII, is reported separately (16).

ylation of VII yielded a diacetate (VIII),[1] m.p. 275–276°. These results were indicative of two 1,3-glycol systems in gymnemagenin (17, 18).

The presence of the β-amyrin skeleton in gymnemagenin was substantiated by refluxing its hexaacetate (III) with selenium dioxide in acetic acid for 17 hr. (12). Dehydrogenation occurred to yield the heteroannular 11,13(18)-diene (IX). The diene (IX) was characterized by triple maxima at 241, 250, and 259 nm. (log ε 4.16, 4.21, and 4.00, respectively). This facile dehydrogenation has been considered characteristic of the β-amyrin derivatives (10, 17, 19).

Thus, the chemical evidence obtained in the present investigation confirms gymnemagenin (I) to be a derivative of β-amyrin, containing one 1,2-*trans*-glycol and two 1,3-glycol systems with a total of six sterically unhindered primary and/or equatorially oriented secondary hydroxyl groups. This is consistent with the structure, I, proposed for gymnemagenin on the basis of spectral data (3, 5).

The derivatives were also of interest as part of an antiviral structure–activity study of triterpenoid compounds. In addition, two derivatives of gymnemic acid A (6), its methyl ester and its diborane reduction product, were prepared for this study. Thus, the free carboxyl function of the glucuronic acid group of gymnemic acid A was reacted with diazomethane to yield the methyl ester (X); treatment of acid A with diborane (20, 21) resulted in the reduction of the glucuronic acid moiety to yield a glucoside (XI). Formation of the glucoside (XI) was confirmed by hydrolysis with ethanolic hydrochloric acid and identification of β-D-glucose in the hydrolysate by paper chromatography and the glucostat test (6).

EXPERIMENTAL[2]

Dry leaves of *G. sylvestre* were employed in this investigation.[3] Melting points were taken on the Kofler micro hot stage and are uncorrected. Spectra were recorded on Perkin-Elmer 137B and 337 IR and Beckman DK-2A UV spectrophotometers. A Zeiss model PMQ II spectrophotometer was employed for a quantitative determination of hydroxyl groups in gymnemagenin (I) based upon the method of Umbreit and Houtman (11).

Silica gel G TLC analysis was carried out as described previously (6). Solvent systems employed were as follows: benzene–methanol (7:3), chloroform–methanol (9:1) (8), benzene–chloroform–methanol (5:8:3), and benzene–methanol–acetic acid (45:8:4) (22) for I and II; and benzene–ethyl ether (1:1) (8) and chloroform–ethyl acetate–methanol (97:2:1) (8) for acetate and isopropylidene derivatives. Visualization of the compounds on chromatograms was carried out by spraying with either modified Liebermann–Burchard (23) or ceric sulfate–sulfuric acid (24) reagents.

Isolation of Gymnemagenin (I)—A crude gymnemic acid mixture was obtained from the aqueous extracts of *G. sylvestre* leaves by precipitation with 10% hydrochloric acid as described earlier (6). A 115-g. quantity of crude gymnemic acid mixture was extracted with 3.5 l. of hot acetone (in seven batches of about 15 g. each, with 500 ml. of acetone per batch) in a large beaker and filtered; the acetone extract was concentrated to a volume of about 200 ml. Addition of 200 ml. of chloroform to this concentrated acetone extract caused copious precipitation which settled as a gummy mass. The clear supernatant liquid was decanted; the gummy precipitate, upon drying, yielded 40.17 g. of a light-brown residue.

A 30-g. quantity of this residue was dissolved in 500 ml. of methanol and refluxed with 80 ml. of concentrated hydrochloric acid for 20 hr. The reaction mixture was concentrated *in vacuo* to about 100 ml. and then poured into 2 l. of ice water with vigorous stirring to cause precipitation. After allowing the precipitate to settle for 1 hr., it was filtered, washed with water until washings were neutral (pHydrion paper), and dried in a vacuum desiccator for 18 hr. to yield 16.69 g. of a light-yellow residue.

The acid-hydrolyzed fraction (16.37 g.) was dissolved in 500 ml. of methanol, with 20 g. of potassium hydroxide pellets added, and refluxed for 20 hr. The reaction mixture was then concentrated *in vacuo* to about 100 ml. and poured into 2 l. of ice water with stirring. The resulting fine precipitate was centrifuged, washed repeatedly with water until washings were neutral, and then dried to yield 9.77 g. of a pale-yellow residue.

Since the mother liquor decanted off during centrifugation was not clear, it was extracted with 1 l. of ethyl acetate for 48 hr. continuously in a liquid–liquid extractor. The ethyl acetate extract, after removal of the solvent, gave 0.75 g. of a light-yellow residue. This residue was combined with the main precipitate obtained during centrifugation to give a total yield of 10.5 g. (9.1% of crude gymnemic acid mixture).

The acid- and base-hydrolyzed fraction, obtained as described, was dissolved in 300 ml. of methanol and decolorized twice with activated charcoal.[4] The residue obtained after removal of the solvent was recrystallized from chloroform–methanol to yield 5.68 g. of a white crystalline product, sintering at 298° and melting at 314–316°. This crystalline material was observed to be comprised of gymnemagenin (I) and a trace amount of gymnestrogenin (II) by TLC.

A 2-g. quantity of this genin mixture was subjected to column (1.2 × 95 cm.) chromatography on 50 g. of silicic acid. Elution was carried out with chloroform–methanol (9:1). The solvent flow rate was maintained at 10 ml./hr., and 150 fractions (5 ml. each) were collected. Fractions 50–80 were pooled based upon TLC results, and evaporation of the solvent gave 1.12 g. of a white, homogeneous residue. Three recrystallizations of this residue from chloroform–methanol gave 0.87 g. of gymnemagenin (I) as stout, white needles, m.p. 329.5–331° [lit. (8), m.p. 328–335°]; $\nu_{max.}^{KBr}$ 3450 (O—H), 2900 (C—H), 1635 (trisubstituted C=C), 1250 (O—H), 1070 and 1040 cm.$^{-1}$ (C—OH).

Anal.—Calcd. for $C_{30}H_{50}O_6$: C, 71.11; H, 9.95. Found: C, 70.95; H, 9.91.

Isolation of Gymnestrogenin (II)—A 17.8-mg. quantity of gymnestrogenin (II) was isolated from Fractions 28–31 obtained during silicic acid column chromatographic purification of gymnemagenin (I). Upon recrystallization from chloroform–methanol, II was obtained as fine needles, m.p. 286–288° [lit. (9) m.p. 288–289°]; $\nu_{max.}^{KBr}$ 3470 (O—H), 2945 (C—H), 1635 (trisubstituted C=C), 1070 and 1040 cm.$^{-1}$ (C—OH).

3β,16β,21β,23,28-Penta-*O*-acetylolean-12-ene (VI)—A 10-mg. quantity of gymnestrogenin (II) was treated with 1 ml. of dry pyridine and 0.8 ml. of acetic anhydride and left at 37° for 18 hr. The reaction mixture was evaporated *in vacuo*, and the residue obtained was extracted with chloroform (5 × 10 ml.). The chloroform extract, after washing with 20-ml. portions of 2 *N* hydrochloric acid, 10% potassium bicarbonate, and water, was dried over anhydrous sodium sulfate. Removal of the solvent from the chloroform extract yielded 11.4 mg. of a white residue. Recrystallization of this residue from petroleum ether–chloroform gave the pentaacetate (VI) as thin flakes, m.p. 121–123°; $\nu_{max.}^{KBr}$ 1740 (C=O, acetoxyl), 1460 and 1375 cm.$^{-1}$ (side-chain methylene and methyl of —CH_2—O—CO—CH_3, respectively). This pentaacetate was found to be identical by IR and TLC in two systems to a reference sample of noncrystalline material previously reported by Stöcklin (9).

3β,16β,21β,22α,23,28-Hexa-*O*-acetylolean-12-ene (III)—A 500-mg. quantity of gymnemagenin (I) was acetylated with 10 ml. of acetic anhydride and 16 ml. of dry pyridine at 0° for 18 hr. The reaction product (785.3 mg.), processed as in the case of VI, was found to be a mixture by TLC. It was chromatographed on a column (1.2 × 28 cm.) of 15 g. of neutral alumina using benzene–ether (2:1) for elution to yield 314.4 mg. of hexaacetate (III). Recrystallization from petroleum ether–chloroform afforded III as fine needles, m.p. 290–291° [lit. (4) m.p. 290–291°]; $\nu_{max.}^{KBr}$ 1740

[2] Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[3] Prachi Gobeson Co., Calcutta, India, and the Himalaya Drug Co., Bombay, India.

[4] Norit, American Norit Co., Jacksonville, Fla.

(C=O, acetoxyl), 1250 (asymmetric stretching of acetate —C—O—C), 1460 and 1375 cm.$^{-1}$, the intensity of the 1375 band being much stronger than the 1460 band (side-chain methylene and methyl, respectively, of —CH_2—O—CO—CH_3).

Anal.—Calcd. for $C_{42}H_{60}O_{12}$: C, 66.46; H, 8.23. Found: C, 66.33; H, 8.57.

3β,16β,21β,22α,23,28-Hexa-*O*-benzoyloolean-12-ene (IV)—A 100-mg. quantity of gymnemagenin (I) in 5 ml. of dry pyridine was heated with 2 ml. of benzoyl chloride on a water bath (70–74°) for 1 hr. The cooled reaction mixture was poured into 25 ml. of water, and the precipitate formed was filtered. This precipitate was stirred with 5 ml. of 5% sodium carbonate, filtered, washed with water, and dried to yield 148.2 mg. of a white residue. Recrystallization of this residue from chloroform–methanol gave hexabenzoate (IV) as white prisms, m.p. 186–188°; $\nu_{max.}^{KBr}$ 1700, 1730 (C=O stretching of benzoyloxy group), 1600 (phenyl nucleus), 1135 and 1295 cm.$^{-1}$ (C—O stretching of benzoates).

Anal.—Calcd. for $C_{72}H_{74}O_{12}$: C, 76.44; H, 6.59. Found: C, 76.72; H, 6.81.

3β,16β,21β,22α-Tetrahydroxy-23,28-ditrityloolean-12-ene (V)—A mixture of 100 mg. of gymnemagenin (I) and 300 mg. of triphenyl chloromethane in 5 ml. of dioxane–pyridine (1:1) was heated on a steam bath for 16 hr. The reaction mixture was then evaporated *in vacuo*, and the residue obtained was extracted with ether (3 × 10 ml.). The ether extract, after removal of the solvent, was chromatographed on a column (1.2 × 36 cm.) of neutral alumina (20 g.). Elution with benzene (100 ml.) gave triphenyl carbinol (108.6 mg.), while benzene–methanol (7:1) (300 ml.) eluted the dityrityl derivative (V). Recrystallization from benzene–methanol gave 38.8 mg. of V as colorless needles, m.p. 268–269°; $\nu_{max.}^{KBr}$ 3540 (O—H), 1600, 1585, 1495 (phenyl nucleus), 750, 705 cm.$^{-1}$ (5 adjacent H on phenyl nucleus).

Anal.—Calcd. for $C_{68}H_{78}O_6$: C, 82.39; H, 7.93. Found: C, 82.53; H, 8.19.

3β,16β,21β,22α,23,28-Hexa-*O*-acetyloolean-11,13(18)-diene (IX)—A 50-mg. sample of hexaacetate (III) in 5 ml. of glacial acetic acid was refluxed with 30 mg. of selenium dioxide for 18 hr.; the red metallic selenium that formed was filtered over a bed of diatomaceous earth[5] using methanol for transfer and washing. The filtrate was evaporated *in vacuo* to yield 29.8 mg. of a white residue. Recrystallization of this residue from petroleum ether–methanol afforded the diene (IX) as white crystals, m.p. 286–288°; $\lambda_{max.}^{EtOH}$ 241, 250, and 259 nm. (log ε 4.16, 4.21, and 4.00, respectively).

Anal.—Calcd. for $C_{42}H_{60}O_{12}$: C, 66.65; H, 7.99. Found: C, 66.78; H, 8.16.

Copper Pyrolysis of Gymnemagenin (I)—A 200-mg. quantity of gymnemagenin (I) was mixed with finely powdered copper (1.4 g.) and heated to 270–290° for 1 hr. in a stream of nitrogen, passing the evolved gases into a saturated aqueous solution of dimedone. The precipitate formed in dimedone solution was collected (12.4 mg.) and recrystallized from methanol to give colorless fine needles, m.p. 188–189°. This was identified as a formaldehyde dimedone derivative [lit. (25), m.p. 189°] upon comparison with an authentic sample, undepressed mixed melting point and superimposable IR spectra being the criteria for identity.

Gymnemic Acid A Methyl Ester (X)—A solution of 100 mg. of gymnemic acid A (6) in 30 ml. of an ether–methanol mixture was treated with excess of diazomethane (26) at 0°. The reaction product was recrystallized twice from ethyl acetate to yield 87.1 mg. of methyl ester X, m.p. 250–255° dec.; $\nu_{max.}^{KBr}$ 3450 (O—H), 2950 (C—H), 1720 (C=O), 1635 (trisubstituted C=C), 1440, 1360 (side-chain methylene and methyl, respectively, of —CH_2—O—CO—CH_3), 1260 (H-bonded O—H), 1070, 1040 (C—OH), and 915 cm.$^{-1}$ (β-pyranose ring vibration).

Anal.—Calcd. for $C_{50}H_{76}O_{16}$: C, 64.36; H, 8.21. Found: C, 63.98; H, 8.38.

Compound X was found to be homogeneous in the TLC Systems III and IV previously reported for the gymnemic acids (6).

Diborane Reduction Product of Gymnemic Acid A (XI)—Diborane (4 mmoles) was generated (20) over a period of 1 hr. by the addition of a solution of 1.9250 g. of boron trifluoride etherate (10 mmoles) in 3.3 ml. of diglyme to 0.2550 g. of sodium borohydride (6.75 mmoles) in 6.25 ml. of diglyme and was passed into a flask containing 100 mg. of gymnemic acid A (10 mmoles) in 7.5 ml. of diglyme. The reaction mixture was allowed to stand at room temperature for 1 hr. About 5 ml. of ethanol was then added to destroy excess diborane and evaporated *in vacuo* to yield 89.6 mg. of a white residue. This residue was found upon TLC analysis [silica gel G, chloroform–methanol (10:3)] to be a mixture of unreacted gymnemic acid A and its reduction product. Isolation of reduction product XI was achieved by chromatography on a column (12 × 250 mm.) of activated silica gel G (25 g., activated at 105° for 1 hr.) with elution by chloroform–methanol (10:3). A yield of 38.6 mg. of XI was obtained. Recrystallization from ethyl acetate gave XI as white microcrystals, m.p. 173–176° dec.; $\nu_{max.}^{KBr}$ 3450 (O—H), 2980 (C—H), 1720 (C=O), 1640 (trisubstituted C=C), 1440, 1380 (side-chain methylene and methyl, respectively, of —CH_2—O—CO—CH_3), 1070 and 1045 cm.$^{-1}$ (C—OH).

Anal.—Calcd. for $C_{49}H_{76}O_{15}$: C, 65.02; H, 8.46. Found: C, 64.76; H, 8.71.

Reduction of the glucuronic acid moiety in gymnemic acid A to glucose in the diborane reduction product (XI) was confirmed by its acidic hydrolysis (3 *N* ethanolic hydrochloric acid, 12 hr.) and detection of glucose in the hydrolysate by paper chromatography and by glucostat test. Method 1-A of the reagent kit[6] was employed.

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[5] Celite 535, Johns-Manville Products Corp., New York, N. Y.

[6] Worthington Biochemical Corp., Freehold, N. J.

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Reactions of Germine-3,16-diacetate in Aqueous Solution

EDWARD M. COHEN and REZSO ACZEL

Abstract □ Germine-3,16-diacetate degrades rapidly in aqueous solution above pH 7 at room temperature ($t_{90\%} \sim 1$ hr. at pH 7.2 and $t_{1/2} < 1$ hr. at pH 9.5). The compound undergoes ester hydrolysis to form germine monoacetate and germine, as well as conversion to two as yet unidentified germine acetate esters. TLC was used to monitor these changes. A kinetic model describing the degradative behavior is proposed. Directions are given for preparing an aqueous solution stable for at least 3 days at room temperature.

Keyphrases □ Germine-3,16-diacetate reactions—aqueous solution □ Degradation—germine-3,16-diacetate in aqueous solution □ Stability profile and pH effect—germine-3,16-diacetate □ TLC—degradation monitoring □ IR spectrophotometry—identification

Recent work in this laboratory has revealed that germine-3,16-diacetate (GDA-3,16) degrades rapidly in aqueous solution at its natural pH, 9–10. The relatively rapid rate of hydrolysis of GDA-3,16 was first detected when titrating the compound as a base with standard acid. Consistently low results were obtained as the result of liberation of acetic acid during the dissolution of the compound in water prior to titration (about 40 min.). A concurrent observation of significance, reflecting this hydrolysis, was that the pH of a 0.1% GDA-3,16 solution decreased from an initial value of about 9.5 to about 6.5 in 3.5 days at room temperature.

These findings are of considerable importance because several publications have described the pharmacology and clinical experience obtained with unbuffered aqueous solutions of the drug, presumed to be GDA-3,16 (1). The significance of GDA-3,16 instability in connection with previously reported biological data is given by Torchiana *et al.* (2). The purposes of this publication are fourfold:

1. To alert investigators using GDA-3,16 about the chemical instability of this compound and recommend a method for preparing solutions that avoid the initial decomposition.
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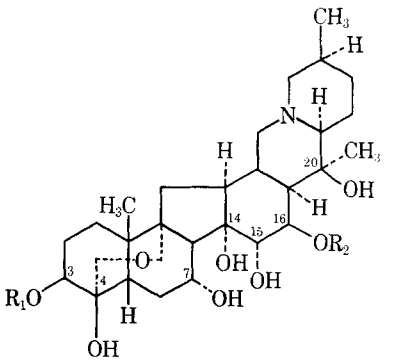

structure of germine compounds

I

Compound	R_1	R_2
G	—H	—H
GDA-3,16	—$COCH_3$	—$COCH_3$
GMA-3	—$COCH_3$	—H
GMA-16	—H	—$COCH_3$

EXPERIMENTAL

Chemicals—GDA-3,16, germine-3-monoacetate (GMA-3), and germine-16-monoacetate (GMA-16) were synthesized,[1] starting from commercially available germine (G). The structure of germine and the acetate esters discussed is given in Structure I. The physical and chemical constants obtained for these compounds were in accord with either literature or expected values (3, 4). All of the germine compounds were reduced in particle size to less than 100-mesh to facilitate dissolution of the solids. All other chemicals used in these studies were reagent grade or better and were not further purified.

TLC—Glass plates were coated by standard techniques (5) with a 250-μ thick layer of a commercial adsorbent.[2] The TLC plates were air dried for about 15 min. and then further dried at 105° for 30 min.; they were stored in a desiccator prior to use.

An ethyl acetate–methanol–concentrated ammonia (80:15:5) mobile solvent resolved all of the germine compounds. Some typical R_f values observed by ascending TLC in a vapor-saturated developing chamber follow:

Compound	R_f
Unknown 1	0.80
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GMA-3	0.60
Unknown 2	0.45
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Germine	0.25

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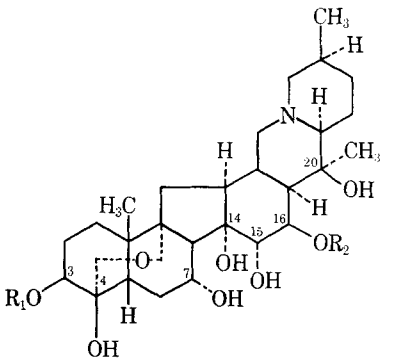

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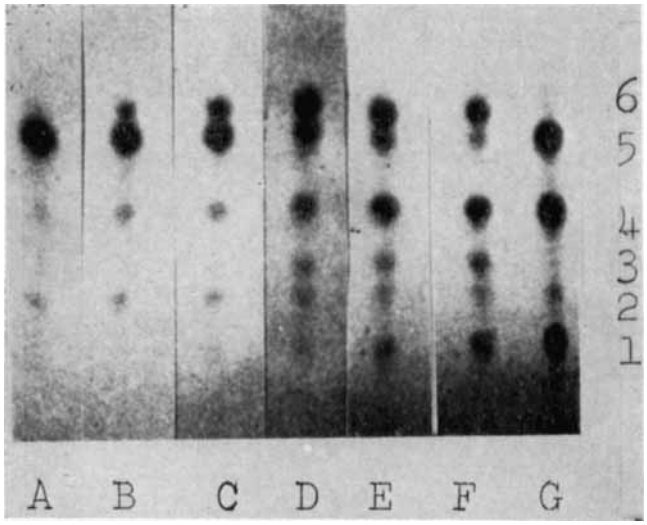


Figure 1—*TLC pattern obtained for solution of GDA-3,16 in water* versus *time. A, B, C, D, E, and F are solutions at 1, 5, 30, and 90 min. and 3 and 24 hr., respectively. G is a mixture of GDA-3,16 (5), GMA-3 (4), and germine (1). Spot 2 = GMA-16; Spot 3 = Unknown 2; and Spot 6 = Unknown 1.*

The exact R_f's obtained varied slightly from time to time, but the relative R_f's observed for these compounds were reasonably constant throughout a number of experiments.

The separated germine compounds were identified on the TLC plate by comparison of their R_f values with those exhibited on the same plate by the available authentic specimens—*viz.*, GDA-3,16, GMA-3, GMA-16, and germine, after visualization with iodine vapor. Semiquantitative estimates of the amounts of separated germine compounds were made by visual comparison of the size and intensity of the sample spots with the spots given by known amounts of the reference compounds.

No significant *in situ* degradation of GDA-3,16 occurred during TLC development when two-dimensional TLC was done as described by Penner *et al.* (6).

Aqueous Stability Profiles—A 1-mg./ml. solution of GDA-3,16 was prepared by dissolving the compound in water. One-milliliter aliquots were taken periodically and "quenched" by adding 1 drop of 0.1 *N* HCl to each sample. This lowered the pH to between 2 and 3, and at that pH no change in solution composition occurred during the subsequent TLC experiments. Fifty microliters of each "quenched" solution was spotted on the TLC plate, and the solvent front was allowed to ascend 12 cm. from the origin (about 45 min. for a 20 × 20-cm. TLC plate). The plate was then removed from the tank, dried until the mobile solvent odor dissipated, and then visualized with iodine vapor. The pH of the stock GDA-3,16 solution was monitored occasionally during the run. Similar experiments were done with aqueous solutions of GMA-3, GMA-16, and germine.

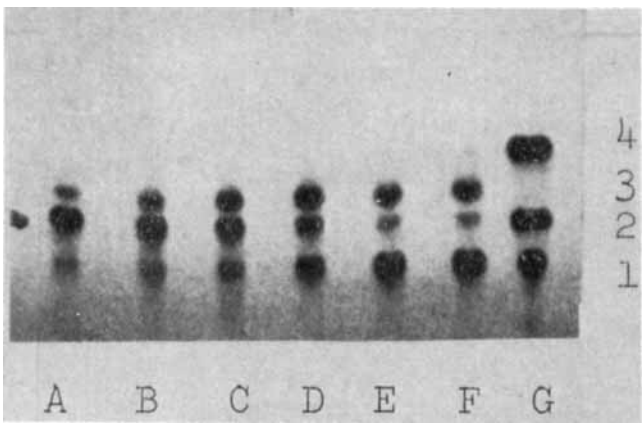


Figure 2—*TLC pattern obtained for solution of GMA-16 in water* versus *time. A, B, C, D, E, and F are solutions at 1, 10, and 30 min. and 2, 20, and 24 hr., respectively. G is a mixture of GMA-3 (4), GMA-16 (2), and germine (1). Spot 3 = Unknown 2.*

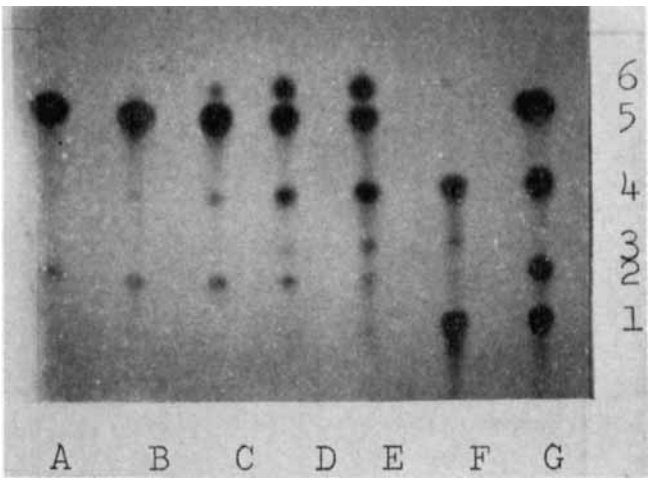


Figure 3—*TLC pattern obtained for GDA-3,16 solutions* versus *time at pH 4.6 (A = 1 hr., B = 24 hr.), pH 7.2 (C = 1 hr., D = 24 hr.), and pH 9.5 (E = 1 hr., F = 24 hr.). G is a mixture of GDA-3,16 (5), GMA-3 (4), GMA-16 (2), and germine (1). Spot 6 = Unknown 1, and Spot 3 = Unknown 2. GMA-16 occurs as an impurity in the GDA-3,16 sample used in these studies.*

Conversion of Unknowns 1 and 2 to Germine—A degraded aqueous solution of GDA-3,16 (initial concentration 1 mg./ml.), which had a pH of 8 and contained both Unknown 1 and Unknown 2, was adjusted to above pH 13 with sodium hydroxide and stored at room temperature for 2 hr. After this alkaline treatment, only one spot was detected in the solution by TLC, corresponding in R_f to germine. The alkaline solution was saturated with sodium chloride and extracted with several portions of chloroform. A white solid was obtained after evaporating the combined chloroform extracts to dryness with a nitrogen stream. The IR spectrum of the solid (KBr mull) was essentially the same as the spectrum of an authentic sample of germine.

Stability of GDA-3,16 *versus* pH and Solvent Composition—For the pH studies, 0.1% solutions of GDA-3,16 were prepared by directly dissolving the solid in the following solvents: 0.1 *N* HCl, pH 4.6 (0.1 *M* acetate buffer), pH 7.2 (0.1 *M* phosphate buffer), pH 9.5 (0.1 *M* ammonia buffer), and 0.1 *N* NaOH. The solutions were stored at room temperature and samples were removed periodically and evaluated by TLC.

The stability of GDA-3,16 in the following solvents was assessed in a similar fashion: methanol, methanol–water (1:1 by volume), alcohol USP, alcohol–water (1:1 by volume), chloroform, acetone, acetonitrile, pyridine, and methylene chloride.

RESULTS AND DISCUSSION

Aqueous Stability Profiles—Figure 1 shows the degradation profile *versus* time for GDA-3,16 in water. An examination of the TLC plate revealed the following:

1. There was very rapid conversion of GDA-3,16 to Unknown 1 (R_f > GDA-3,16). The amount of Unknown 1 continued to increase while the GDA-3,16 continued to decrease with time. At some point (90 min. *versus* 3 hr.), the amount of Unknown 1 decreased.
2. The formation of GMA-3 also occurred very rapidly, and its level increased with time.
3. There was a gradual buildup of Unknown 2 and germine with time.
4. There did not appear to be any significant change in the GMA-16 level with time. As is evident in the reference migration zone, GMA-16 occurred as an impurity of GDA-3,16.

The corresponding solution stability profile for GMA-3 is very simple, with the only observed degradation product being germine. Unknowns 1 and 2 were not detected by TLC.

Figure 2 gives the solution composition as a function of time for a water solution of GMA-16. The salient features of this chromatogram are:

Table I—Stability of GDA-3,16 (0.1%) *versus* pH at Room Temperature

Degradation Products Observed	Solution Composition[a]									
	Concentrated HCl, 2 hr.	0.1 *N* HCl, 1 hr.	0.1 *N* HCl, 10 days	pH 4.6, 1 hr.	pH 4.6, 24 hr.	pH 7.2, 1 hr.	pH 7.2, 24 hr.	pH 9.5, 1 hr.	pH 9.5, 24 hr.	0.1 *N* NaOH, 1 hr.
G	++	−	−	−	−	−	−	−	+++	+++[b]
GMA-16	+++	−	++	−	−	+	+	+	−	−
Unknown 2	−	−	−	−	−	−	+	+	+	−
GMA-3	+	−	−	−	−	+	+	++	++	−
Unknown 1	−	−	−	−	−	+	++	++	−	−
Intact GDA-3,16	10%	100%	90%	100%	100%	90%	60%	20%	0	0

[a] Amount of degradate present estimated as −(<1%), +(<10%), ++(<50%), and +++(>50%). [b] Solution completely degraded in less than 1 hr.

1. There was rapid conversion of GMA-16 into a compound whose R_f is identical with Unknown 2 noted in Fig. 1.
2. Accompanying the buildup of Unknown 2 was a somewhat slower buildup of germine. A slight amount of germine was present initially as an impurity in the GMA-16 sample used for these studies.
3. After 2 hr., germine buildup was accompanied by a decline in the amount of Unknown 2, suggesting conversion of the latter to the former.

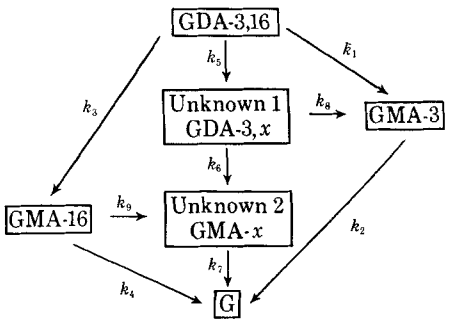
The data from the GMA-16 experiment indicate that Unknown 2 can be made from GMA-16 at a rate that is comparable to the rate of formation of Unknown 1 in GDA-3,16 solutions (Fig. 1). Furthermore, since Unknown 1 was not observed here (Fig. 2), it almost certainly is derived exclusively from GDA-3,16.

An additional experiment conducted in this series was to observe the behavior of germine itself in water. No change in the TLC pattern or solution pH was observed after 24 hr. Thus, as expected, neither Unknown 1 nor 2 was produced from germine.

In all of these experiments, with the exception of those starting with germine, the pH of the stock solutions decreased with time, reflecting the hydrolysis of the acetate ester linkage.

Structures of Unknowns 1 and 2—A consideration of the relative R_f's on the TLC plates shows that Unknown 1 lies near GDA-3,16 and that Unknown 2 lies near GMA-3, suggesting a germine diacetate structure for the former and a germine monoacetate structure for the latter. The observations that Unknown 1 was derived exclusively from GDA-3,16 and Unknown 2 was derived from GMA-16, coupled with the fact that both Unknowns 1 and 2 were converted to germine in alkaline solution, lend further support to a GDA structure for Unknown 1 and a GMA structure for Unknown 2. Experiments are currently in progress to isolate and characterize both Unknowns 1 and 2.

Proposed Kinetic Model for GDA-3,16 Degradation—The observed aqueous stability profiles (Figs. 1 and 2) suggest the following model (Scheme I) for the degradation of GDA-3,16 in water.



Scheme I

The rate constants in the model reflect the following chemical changes:

$$\frac{\text{hydrolysis of } C_3\text{-acetoxy}}{k_2,\ k_3,\ k_6} \qquad \frac{\text{hydrolysis of } C_{16}\text{-acetoxy}}{k_1,\ k_4}$$

$$\frac{\text{hydrolysis of } C_x\text{-acetoxy}}{k_7,\ k_8} \qquad \frac{\text{conversion of GDA-3,16 to GDA-3,}x}{k_5}$$

$$\frac{\text{conversion of GMA-16 to GMA-}x}{k_9}$$

The continual decrease in pH with time in the aqueous stability profile precluded any attempt to estimate rate constants at this time, since each rate constant described is very likely to be pH dependent (Table I).

Influence of pH on Stability of GDA-3,16—Table I summarizes the stability patterns obtained for GDA-3,16 in buffered aqueous solutions at room temperature; Fig. 3 shows the TLC chromatograms obtained for pH's 4.6, 7.2, and 9.5.

The overall stability of GDA-3,16 increases with decreasing pH and apparently reaches a maximum below pH 7, since the degradation occurs faster in both concentrated HCl and pH 7.2 than it does in 0.1 *N* HCl and pH 4.6. Not enough data were obtained in these studies to ascertain the pH of maximum stability.

Above pH 7, conversion to Unknowns 1 and 2 as well as hydrolysis occurred in solutions of GDA-3,16 (1 hr. stability data, Table I). Moreover, both types of reactions are evidently base catalyzed; in 0.1 *N* NaOH, GDA-3,16 degraded completely to germine within 1 hr. In 0.1 *N* HCl and concentrated HCl, GMA-16 was the initial degradation product obtained from GDA-3,16. This observation suggests that the conversion reaction is not acid catalyzed and, in addition, that the C_3-acetoxy hydrolysis (k_3) is faster than the C_{16}-acetoxy hydrolysis (k_1) in strongly acidic media (3). As time permits, the individual rate constants will be evaluated in more detail and the data reported.

Recommended Preparation of Extemporaneous GDA-3,16 Solutions—Germine-3,16-diacetate solutions, which are stable for several days at room temperature, can be conveniently prepared by dissolving the compound in water containing a quantity of organic or inorganic acid such that the final pH of the solution is between 4.5 and 6. A typical solution preparation is as follows. Dissolve 200 mg. of GDA-3,16 (0.34 mmole) in 100 ml. of water containing 27 mg. of anhydrous citric acid (0.12 mmole). This solution had a final pH of 5.8. Of the several acids tried in this laboratory (hydrochloric acid, phosphoric acid, citric acid, and lactic acid), citric acid was found to be most convenient. It can be weighed out as needed and, because of its overlapping ionization constants in the desired pH range, will not result in drastic pH changes for small variations in the ratio of GDA-3,16 to citric acid.

Table II—GDA-3,16 Stability *versus* Solvent Composition

Solvent	Storage Time at Room Temperature	Observations
Chloroform	1 month	Stable[a]
Methylene chloride	1 month	Stable[a]
Acetone	1 day	Stable[a]
Acetonitrile	1 day	Stable[a]
Pyridine	1 day	Stable[a]
Alcohol USP	4 days	Unstable[b]
Alcohol USP–water, 1:1 by volume	<3 hr.	Unstable[b]
Methanol	3 hr.	Stable[a]
Methanol–water, 1:1 by volume	3 hr.	Unstable[b]

[a] No extraneous spots detected by TLC. [b] TLC pattern similar to that observed in aqueous stability profile. The instability of GDA-3,16 in alcohol is particularly important since alcohol might be used by some workers to prepare GDA-3,16 stock solutions for subsequent dilution with water prior to use.

Stability of GDA-3,16 *versus* Solvent Composition—Some data accumulated to date on short-term stability of GDA-3,16 in some solvents are given in Table II. These observations were obtained during the course of experiments to devise efficient reaction media for the conversion of GDA-3,16 to either Unknown 1 or GMA-16.

SUMMARY

GDA-3,16 degrades rapidly in water at its natural pH (about 9.5). In addition to the expected ester hydrolysis to germine monoacetate and germine, rapid conversion of GDA-3,16 to another as yet unidentified GDA occurred. Degradation of GDA-3,16 solution was accompanied by a drop in pH, reflecting the ester hydrolysis. A kinetic model describing these changes was proposed. TLC was used to monitor the chemical changes of GDA-3,16.

Overall stability of GDA-3,16 increases with decreasing pH ($t_{1/2}$ <1 hr. at pH 9.5, $t_{90\%}$ ~ 1 hr. at pH 7.2) and reaches a maximum near pH 4.6 (no instability detected after 24 hr.). Directions are given for preparing an aqueous GDA-3,16 solution stable for several days at room temperature.

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M. J. CHO*, A. G. MITCHELL, and M. PERNAROWSKI†

Abstract □ The binding of bishydroxycoumarin to human serum albumin was studied by means of spectrophotometry, solubility analysis, and equilibrium dialysis. The data were interpreted on the basis of the theory of multiple equilibria. The human serum albumin–bishydroxycoumarin association is exothermic and occurs spontaneously under the experimental conditions. The α,β-unsaturated lactone structure in bishydroxycoumarin is involved in the complexation. A heterogeneity of binding sites on human serum albumin was observed. The average number of the first-type binding sites is approximately three. The corresponding intrinsic association constant is equal to 1.7×10^5 l./mole at 40° and 3.5×10^5 l./mole at 20°. The binding sites are believed to be hydrophobic regions, probably located in the interior of the human serum albumin molecule whose van der Waals' contour approximates and thus, in a sense is selective for, the bishydroxycoumarin molecule. The selectivity of the binding sites was supported by the large contribution of $\Delta H°$ to $\Delta G°$. The main binding energy is derived from nonionic sources.

Keyphrases □ Bishydroxycoumarin–human serum albumin—interaction □ Serum albumin, human—bishydroxycoumarin binding □ Solubility analysis—human serum albumin □ Equilibrium dialysis—analysis □ UV spectrophotometry—analysis

The binding of bishydroxycoumarin to human serum albumin was first reported about 20 years ago (1). However, the mechanism of interaction has not been extensively studied. The present work was designed to evaluate the mechanism.

EXPERIMENTAL

Materials—Bishydroxycoumarin USP[1] and human serum albumin Fraction V[2] were used in this study. Tris(hydroxymethyl)aminomethane was obtained commercially. Cellophane dialysis tubing[3] was cut to the size of the dialysis cell, washed thoroughly with distilled water, and stored in the refrigerator.

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In the biologically significant pH range, there is little choice of buffer system. Phosphate buffer interferes with the protein binding of small molecules (6). Tris(hydroxymethyl)aminomethane has intramolecular hydrogen bonds (7) and, hence, is expected to be relatively inert. Chloride ion may interfere with the human serum albumin–bishydroxycoumarin interaction. However, its competitive

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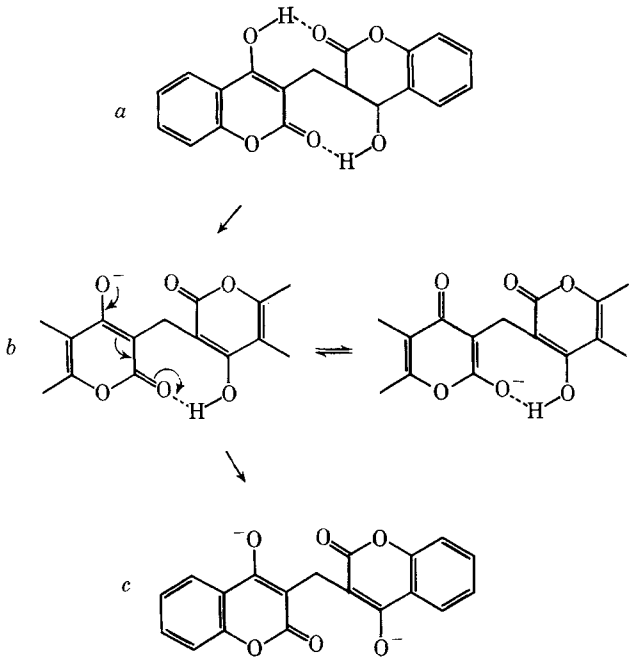

Scheme I—Chemical structure of bishydroxycoumarin showing two intramolecular hydrogen bonds (a), *resonance after the first ionization* (b), *and completely ionized bishydroxycoumarin* (c)

interaction was neglected since the intrinsic affinity of chloride ion for human serum albumin (8) would be far less than that observed for human serum albumin–bishydroxycoumarin.

On the basis of the preliminary experiments, a tris(hydroxymethyl)aminomethane–hydrochloric acid buffer system was selected having a pH of 7.4 and an ionic strength of 0.15. The pKa of tris(hydroxymethyl)aminomethane is 8.075 (9), and the buffer capacity of the system was 0.021. The ionic strength is sufficiently high to prevent the Donnan effect (10) in dialysis experiments.

Solubility Analysis—A series of solutions containing various amounts of human serum albumin was prepared in tris(hydroxymethyl)aminomethane buffer. Excess bishydroxycoumarin was added to 25.0 ml. of each solution in a 30-ml. centrifuge tube. The tubes were sealed and tumbled in a water bath at 20 ± 0.1°. After 40 hr., they were centrifuged for 20 min. at 2500 r.p.m. The temperature increase after centrifugation was always less than 1.0°. The bishydroxycoumarin content of the supernatant was determined spectrophotometrically.

Equilibrium Dialysis—Dialysis cells similar to those described by Patel and Foss (12) were used, and the procedures of Yang and Foster (13) and Pollansch and Briggs (14) were followed to cover a wide range of free drug concentration. The volume ratio of human serum albumin to human serum albumin-free compartments was varied by adding an extra spacer on either side of the membrane.

The cells were tumbled at 30 r.p.m. in a constant-temperature bath until equilibrium was reached (approximately 40 hr.). The human serum albumin-free compartment was analyzed for free bishydroxycoumarin. Experiments were carried out at 20 and 40° using 0.1, 0.2, and 0.4% human serum albumin.

Spectrophotometric Analysis—Bishydroxycoumarin in samples from the solubility and dialysis experiments was analyzed spectrophotometrically[4] at 304 nm. using tris(hydroxymethyl)aminomethane buffer as a blank. Corrections were made, where appropriate, for both human serum albumin content and depression of absorbance due to complex formation. The percent depression of the absorbance readings in the presence of excess human serum albumin was used to estimate the molar absorptivity value of bound bishydroxycoumarin. The absorptivity ratio of bound to free drug was 0.899 ± 0.012. The correction procedure and the calculation of binding parameters from the spectrophotometric data will be described in a subsequent paper (11).

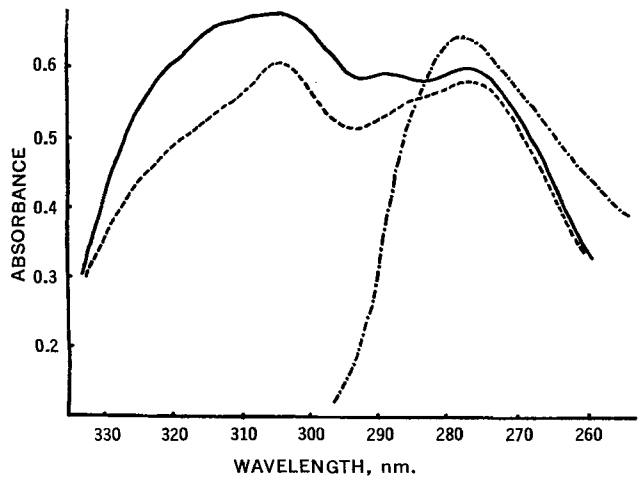

Figure 1—*Absorption spectra for 0.1% human serum albumin* (···) *and for bishydroxycoumarin in the presence* (- - -) *and absence* (——) *of human serum albumin at pH 7.4.*

RESULTS AND DISCUSSION

Apparent pKa Values of Bishydroxycoumarin—Recently, Hutchinson and Tomlinson (15) proposed a hydrogen-bonded structure for bishydroxycoumarin (Scheme I*a*). Two ionizable hydrogen atoms are involved in the internal hydrogen bonds. As shown in Scheme I*b*, the negative charge on the oxygen atom produced by the first ionization is expected to make the oxygen atom in the carbonyl group more negative by an electronic shift through the conjugated chain. This increase in electronegativity apparently increases its ability to form a hydrogen bond with the remaining hydrogen atom. The strengthened hydrogen bond will, therefore, make it more difficult for the second hydrogen atom to ionize. Only one ionization constant has been reported in the literature (16, 17).

Bishydroxycoumarin is very insoluble in acidic solution (0.5 mg./l. or 1.5×10^{-6} mole/l.), and the basic titrimetric approach in aqueous media cannot be utilized to estimate the pKa value. By use of precision spectrophotometry (18), the apparent pKa value was determined in dimethylformamide (DMF)–water mixtures (2). Extrapolation to 0% DMF resulted in pKa values of 4.4 and 8.0 for the first and second ionization constants, respectively. At pH 7.4, therefore, 80% bishydroxycoumarin exists as the monoionized species and 20% bishydroxycoumarin exists as the diionized species.

Spectrophotometric Analysis—As shown in Fig. 1, the spectral characteristics of bishydroxycoumarin are changed by human serum albumin. It is most likely that the functional group producing the absorption peak at 304 nm., which is depressed in the presence of human serum albumin, is involved in the complexation process. The same absorption peak is shifted to a lower wavelength as bishydroxycoumarin undergoes the second ionization (2).[5] The predominating chromophore on bishydroxycoumarin is probably the α,β-unsaturated lactone structure, which shows an electronic shift as the —OH group in the β-carbon ionizes (Scheme I*b*). The changes in electronic configuration which accompany the ionization process are believed to cause the shift of the absorption peak.

An attempt was made to evaluate the spectral changes due to complexation in a quantitative way following the method of Klotz (19). Agreement between binding data calculated from the spectrophotometric and equilibrium dialysis methods was poor. However, the dialysis method indicates a heterogeneity of binding sites on human serum albumin. Hence, it is likely that the estimated molar absorptivity value of bound bishydroxycoumarin is not a constant but varies according to the proportion of bishydroxycoumarin on each type of site.

Solubility Analysis—Many investigators, particularly Higuchi and Connors (20), have used phase-solubility analysis to study a wide variety of molecular interactions. Under favorable conditions, this method yields quantitative information about complexation. The method deals with a single free drug concentration and, there-

[4] Beckman DU-2 spectrophotometer.

[5] It was not possible to determine the changes in absorption spectrum associated with the first ionization of bishydroxycoumarin because of the extremely low solubility of bishydroxycoumarin in acidic solutions.

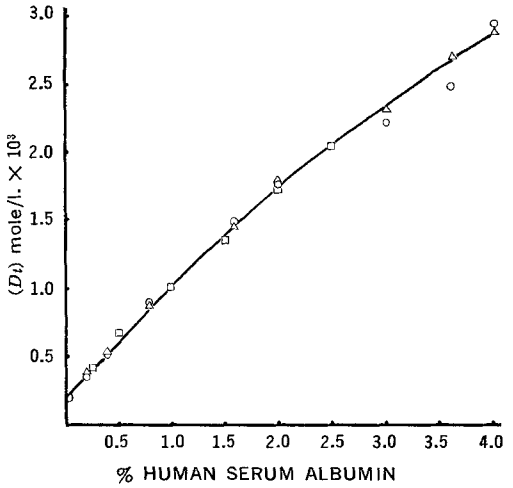

Figure 2—*Effect of human serum albumin on the apparent solubility of bishydroxycoumarin at 20° in tris(hydroxymethyl)aminomethane buffer. Different symbols represent separate experiments.*

fore, gives only one point on any binding curve based on the theory of multiple equilibria. However, this method is important since it provides information on binding at a high free drug concentration.

The effect of human serum albumin on the apparent solubility of bishydroxycoumarin is illustrated in Fig. 2. The solubility increases linearly with human serum albumin up to a concentration of 1.0%. Over this concentration range, the r value in Eq. 1 (the slope of the line) is a constant.

Equilibrium Dialysis—On the basis of preliminary experiments, a correction factor of 4% of the total bishydroxycoumarin[6] was used to correct for losses due to binding to the membrane and possibly to the Plexiglas of the dialysis cell.

Human serum albumin has a net negative charge of 18 at pH 7.4 (22). The ionic strength of the tris(hydroxymethyl)aminomethane buffer was 0.15, and the highest human serum albumin concentration used was 0.4% (5.8×10^{-5} mole/l.). Under these conditions, the distribution ratio of univalent anions across the membrane is equal to 1.0035 (10). It is possible, therefore, to neglect the abnormal distribution of bishydroxycoumarin anion due to the Donnan effect.

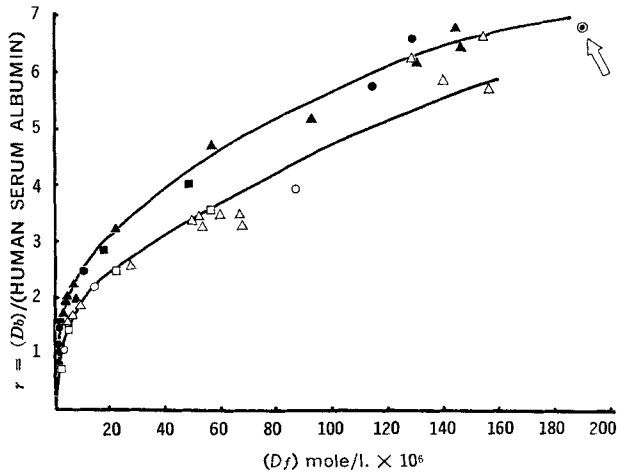

Figure 3—*Langmuir-type plot for human serum albumin–bishydroxycoumarin interaction in tris(hydroxymethyl)aminomethane buffer at 20° (closed symbols) and 40° (open symbols). Human serum albumin concentration: 0.1%, △; 0.2%, ○; and 0.4%, □. Data for solubility method indicated by an arrow.*

[6] Meyer and Guttman (21) suggested that corrections should be based on the amount of free drug only. Neither approach is strictly correct, and the membrane should be treated as a separate binding site. It is believed that the procedure described in this article will have a smaller error for a system in which most drug molecules exist in the bound form. The approach of Meyer and Guttman will have a smaller error for a system in which the extent of binding is low.

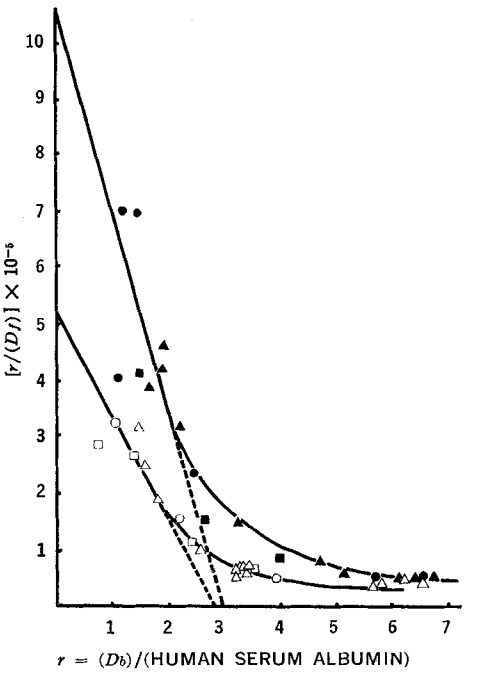

Figure 4—*Scatchard plot for human serum albumin–bishydroxycoumarin interaction in tris(hydroxymethyl)aminomethane buffer at 20 and 40°. Symbols as in Fig. 3.*

Binding data obtained using equilibrium dialysis are shown in Figs. 3 and 4.

Theory of Multiple Equilibria—The principles and concepts fundamental to an understanding of protein binding are found in *References 23–28.*

The molar ratio of bound drug to total protein, r, is a measure of binding. The value of r is expressed in terms of the intrinsic association constant, k, the maximum number of binding sites on a protein molecule, n, and the concentration of free drug (D_f).

$$r = \frac{nk(D_f)}{1 + k(D_f)} \qquad \text{(Eq. 1)}$$

The intrinsic association constant, k, is defined as the equilibrium constant for the association reaction between 1 mole of free drug and 1 mole of unoccupied binding site to form 1 mole of complex.

Equation 1 is similar to the equation derived by Langmuir (29) to describe a certain type of adsorption isotherm. Although they are similar, it is not always correct to assume that binding and adsorption are identical processes. The equations are similar because both have been derived from the law of mass action.

The so-called Scatchard equation (25) is derived from Eq. 1:

$$r/(D_f) = kn - kr \qquad \text{(Eq. 2)}$$

A plot of $r/(D_f)$ *versus* r is a straight line, where the intercept on the abscissa yields n and the intercept on the ordinate is equal to kn. Equation 1 is valid only if there is one type of binding site between which no electrostatic interaction exists. Most binding systems fail to obey these conditions.

Interpretation of Binding Data—The simplest way to treat the binding data is with a Langmuir-type plot of r value *versus* (D_f). Equation 1 is a segment of a rectangular hyperbola passing through the origin. If (D_f) in Eq. 1 becomes infinite, the r value approaches n as a limit:

$$\lim_{(D_f) \to \infty} r = n \qquad \text{(Eq. 3)}$$

and at $r = n/2$:

$$(D_f) = 1/k \qquad \text{(Eq. 4)}$$

These two equations show the importance of a wide concentration range of free drug in any binding study.

Figure 3 shows that, at low concentrations, bishydroxycoumarin is more easily bound to human serum albumin than at high concentrations. Only the data obtained from the solubility method show saturation of the binding sites. Quantitative information derived from Eqs. 3 and 4 is valid only if there is a single type of independent binding site. Therefore, binding parameters were not derived from this plot.

The binding data are replotted on the basis of the Scatchard equation (Eq. 2) in Fig. 4. The sharp bend near the abscissa indicates either that more than one type of binding site is present in the human serum albumin molecule or that there is a significant electrostatic interaction between the binding sites. The "screening" effect of high ionic strength in the buffer system rules out the latter possibility. Therefore, using the procedure of Rosenthal (28), each curve was resolved into two straight lines, each of which represents a different type of binding site. The values of n of the first type of binding site (3.0 at 20° and 2.8 at 40°) were rounded off to the nearest whole integer, 3. The intercepts on the ordinate, nk, are 10.5×10^5 and 5.1×10^6, which yield k values of 3.5×10^5 and 1.7×10^5 l./mole at 20 and 40°, respectively. The second type of binding site will not be considered because the intrinsic affinity of the bishydroxycoumarin molecule for these sites is much smaller than for the first type.

Thermodynamic Analysis and Mechanism of Interaction—Assuming that there is no significant temperature dependence of enthalpy change within the temperature range in which the interaction was carried out, it is possible to estimate the standard enthalpy change, $\Delta H°$, for the association of 1 mole of bishydroxycoumarin with 1 mole of the binding site from Eq. 5:

$$\log \frac{k \text{ at } 20°}{k \text{ at } 40°} = \frac{-\Delta H°}{2.303R}\left(\frac{1}{293} - \frac{1}{313}\right) \qquad \text{(Eq. 5)}$$

The standard free energy, $\Delta G°$, for complex formation is estimated from

$$\Delta G° = -RT \ln k \qquad \text{(Eq. 6)}$$

and the entropy change, $\Delta S°$, is obtained by substituting $\Delta H°$ and $\Delta G°$ into the Gibbs–Helmholtz equation:

$$\left(\frac{\partial \Delta G°}{\partial T}\right)_p = -\Delta S° \frac{\Delta G° - \Delta H°}{T} \qquad \text{(Eq. 7)}$$

Thermodynamic parameters for the association reaction are reported in Table I. The decrease in binding strength of human serum albumin for bishydroxycoumarin with increasing temperature is characteristic of an exothermic reaction and has been reported for many protein interactions (*Reference 30* gives some examples). The negative sign for $\Delta G°$ means that the binding process is spontaneous. The $\Delta S°$ is positive, in agreement with observations for other albumin–anion interactions (31).

A small temperature dependence of binding strength is a characteristic of an interaction between oppositely charged species (32). If the nature of the human serum albumin–bishydroxycoumarin interaction was largely electrostatic, *i.e.*, if the ionic part of the bishydroxycoumarin molecule combined with the cationic parts of the human serum albumin molecule, the main source of the $-\Delta G°$ value would be a large contribution of $+\Delta S°$ term with little contribution from the $\Delta H°$ factor. Thermodynamic changes for the human serum albumin–bishydroxycoumarin interaction indicate a large contribution to the $-\Delta G°$ value by the $-\Delta H°$ factor. It is very unlikely, therefore, that the association is ionic in nature. A similar interpretation for the interaction between human serum albumin and warfarin has been suggested by O'Reilly and Kowitz (30).[7]

In the absence of information about attendant conformational changes, most interpretations of binding processes make the assumption that preexisting binding sites are involved. On the other hand, Karush (33) postulated that serum albumin possesses "configurational adaptability" for a variety of small molecules and that binding sites might form during the binding process. Using this hypothesis, Karush (34) interpreted the differences in thermodynamic parameters observed in nonspecific albumin–anion and specific antibody–hapten interactions.

Table I—Thermodynamic Data for Human Serum Albumin–Bishydroxycoumarin Interaction

Temperature	$k \times 10^{-5}$ l./mole	$-\Delta H°$ kcal./mole	$\Delta G°$	$\Delta S°$, e.u.
20°	3.5	−6.58	−7.43	+2.90
40°	1.7	−6.58	−7.49	+2.91

The positive $\Delta S°$ associated with many reactions involving proteins is usually attributed to disorientation and unfolding of the protein molecule. This does not appear to be a satisfactory explanation for the human serum albumin–bishydroxycoumarin interaction, because the enthalpy changes observed are very negative. A process of unfolding presumably requires the breaking or bending of several bonds and should result in an endothermic reaction of appreciable magnitude (35). It is postulated, therefore, that the human serum albumin molecule has some kind of preexisting sites for the bishydroxycoumarin molecule.

The binding sites are probably hydrophobic regions whose van der Waals' contour is closely complementary to, and thus in a sense is selective for, the bishydroxycoumarin molecule. The selectivity is explained by a large contribution of enthalpy to the free energy (approximately 88% at both 20 and 40°). In this respect, the human serum albumin–bishydroxycoumarin interaction is in between the human serum albumin–warfarin interaction, in which the $\Delta H°$ contribution to $\Delta G°$ is approximately 50% (30), and specific interactions between antibody and hapten, in which the free energy term is due almost entirely to the enthalpy term (34). However, the human serum albumin–bishydroxycoumarin interaction is presumably quite different from antibody–hapten interactions because the site on human serum albumin is not completely rigid and the "key to lock" concept cannot be applied. It is suggested that hydrophobic interaction acts as the driving force for binding and that the sites are located in the interior of the protein molecule. The binding site cannot resist the disruptive tendencies either of thermal energy or of intramolecular electrostatic repulsion to which the human serum albumin molecule is subject at extreme values of pH. Hence, as Nagashimi *et al.* (17) observed, the human serum albumin–bishydroxycoumarin interaction is much less at pH 3.0–3.5 where a configurational change of the protein from a "compact form" to an "expandable form" takes place. Displacement of bishydroxycoumarin by another drug molecule from the protein (36) may arise in a similar way. It can be postulated that van der Waals' contour is disturbed by the third molecule resulting in a drastic decrease in binding affinity to the protein.

The human serum albumin–bishydroxycoumarin interaction involves the transfer of a hydrophobic molecule from an aqueous environment to a region with a lower dielectric constant. After binding occurs, a hypothetical hole previously occupied by the bishydroxycoumarin molecule remains and will be filled with an equal volume of hydrogen-bonded water molecules (34). The number of hydrogen bonds formed in this way will exceed the number of hydrogen bonds that the free bishydroxycoumarin molecule previously formed with its neighboring water molecules. It is believed from the intramolecularly hydrogen-bonded structure of bishydroxycoumarin (Scheme I*a*) that the number of hydrogen bonds between water and bishydroxycoumarin molecules is practically nil. On the basis of this explanation, complex formation will be exothermic and associated with a substantial decrease in the enthalpy of the system.

The "iceberg" concept of water structure (37) assumes that hydrocarbon groups, such as those present both in human serum albumin and bishydroxycoumarin molecules, are surrounded in aqueous solution with one or more layers of water molecules which are more highly ordered than the molecules in ordinary liquid water. The number of "structured" water molecules around the bishydroxycoumarin–human serum albumin complex will be less than those around the two unbound entities. Consequently, heat will be required (endothermic) for the fusion of the "icebergs." The exothermic nature of the binding, caused by an increase in the number of hydrogen bonds accompanying complexation, is believed to exceed the endothermic melting of the "icebergs," resulting in net negative $\Delta H°$ (Table I).

[7] In this paper, there is an error in the calculation of r (21). However, this error should not affect the thermodynamic data.

The melting of "icebergs" around bishydroxycoumarin and human serum albumin molecules will result in an increase in randomness (*i.e.*, positive $\Delta S°$) (4, 5, 38–40). The bishydroxycoumarin molecule will lose rotational and translational degrees of freedom after binding to yield a higher ordering (*i.e.*, negative $\Delta S°$). Formation of a bonded water cluster at the hypothetical hole left by the bishydroxycoumarin molecule will give an ordering effect (41). The disordering effect due to the melting of "icebergs" probably exceeds the ordering effects, resulting in the net entropy change of +2.9 (Table I). In more specific interactions, the net entropy change will be smaller than the value reported here and may become negative because of a significant loss of degrees of freedom.

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Substituted Tetralins III: Synthesis and Analgesic Activities of Some Substituted 2-Methyl- and 2-Benzyl-4,4-dimethyl-2-aminotetralins

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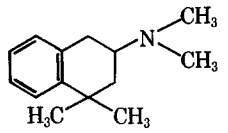
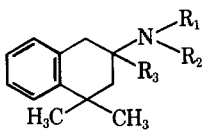
Abstract □ The synthesis of five 2-methyl- and 2-benzyl-4,4-dimethyl-2-aminotetralins is described and their analgesic potencies are reported. The introduction of methyl or benzyl substituents in the 2-position does not appear to significantly affect analgesic potency in the 2-aminotetralin series.

Keyphrases □ 2-Methyl- and 2-benzyl-4,4-dimethyl-2-aminotetralins, substituted—synthesis □ Analgesic activity—substituted tetralins □ IR spectrophotometry—identification □ NMR spectroscopy—identification

In a recent publication the synthesis and analgesic potency of some derivatives of 2-aminotetralin were reported (1). The observation that one of the series, *N,N*-dimethyl-1,2,3,4-tetrahydro-4,4-dimethyl-2-naphthylamine (I), has an analgesic potency about two and one-half times that of meperidine encouraged the authors to prepare 2-substituted-2-aminotetralins (II) as potential analgesics.

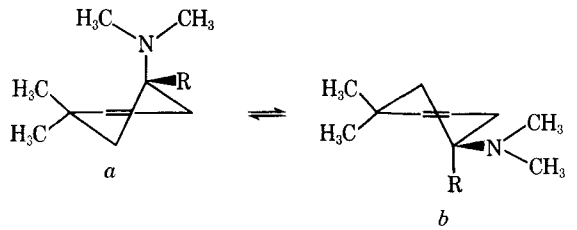


I



II

The decision to prepare 2-substituted derivatives was based on conformational considerations. Because the half-chair conformation is considered the most energetically favored in the tetralin system (2), it is fair to assume that the 2-aminotetralins reported exist predominantly as an equilibrium mixture of the half-chair conformers shown in Scheme I.



a *b*

Scheme I

If the 2-amino group was oriented axially in the half-chair conformation *a*, the molecules would be capable of fitting the analgesic receptor site proposed by Beckett and Casy (3) without using the cavity postulated to exist between an anionic site and a flat surface for an aromatic ring. However, it is likely that the active analgesic I exists predominantly as conformer *b* (R = H), wherein the 2-amino function is equatorial. A nonbonding interaction between the bulky 2-amino group and a quasiaxial methyl group in the 4-position is thereby avoided. The introduction of a second bulky group in the 2-position should increase the preference for conformer *a*, wherein the amino group is oriented axially.[1]

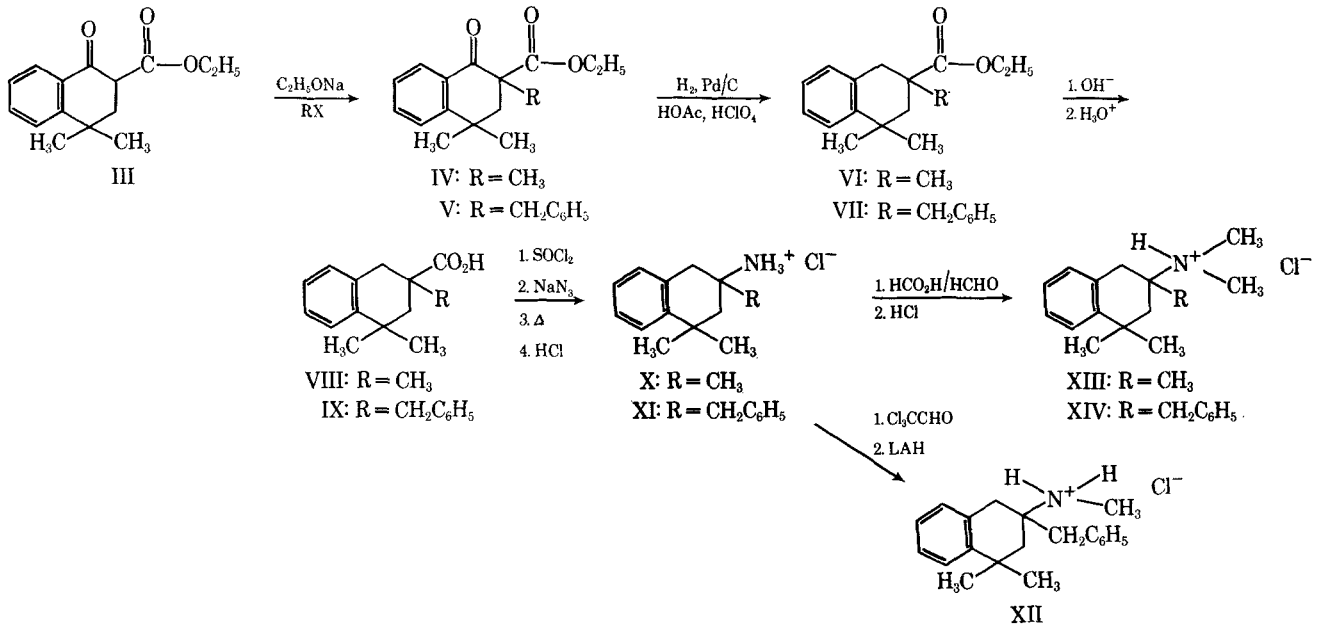
The synthesis of the desired amines was accomplished by the procedures outlined in Scheme II. Methylation and benzylation of ethyl 1,2,3,4-tetrahydro-4,4-dimethyl-1-oxo-2-naphthoate (III) were accomplished by the classical procedure of Kotz and Michaels (5), giving IV and V, respectively. Confirmation that *C*-alkylation had occurred in both instances was demonstrated by the NMR spectra of the alkylated products. Compound IV exhibited three methyl group singlets in the (relatively) shielded region of 1.2–1.4 p.p.m. and lacked the absorption at 3.5–4.0 p.p.m. anticipated for a methyl ether. The methylene protons associated with the 2-benzyl group of V appeared as an *AB* quartet centered at 3.21 p.p.m. The nonequivalence of the methylene protons can be ascribed to the presence of an asymmetric center in V.[2]

Hydrogenolysis of the 2-methyl- and 2-benzyl-2-carbethoxytetralones, IV and V, using palladium-on-charcoal catalysts in glacial acetic acid to which a small amount of perchloric acid had been added, gave the reduced esters, VI and VII. Esters VI and VII were hydrolyzed to the corresponding 1,2,3,4-tetrahydro-2-naphthoic acids, VIII and IX. The Curtius procedure effected the conversion of VIII and IX to the primary amines X and XI, respectively. Eschweiler–Clarke methylation of X and XI gave the corresponding dimethyl amino derivatives, XIII and XIV. The secondary amine, XII, was obtained by treatment of XI with chloral, followed by lithium aluminum hydride reduction of the intermediate formyl derivative.

The analgesic potencies of the amines (Table I) were determined by the mouse hot plate assay of Eddy and Leimbach (6). For purposes of comparison, the ED_{50}'s are expressed as millimoles per kilogram since a variety of salts were employed. The 2-alkyl substituted compounds, XII, XIII, and XIV, although less active than the parent compound, I, retain significant analgesic activity comparable to that of meperidine and codeine. At the outset, it was anticipated that substitution of a bulky group at the 2-position of I would

[1] A similar objective was sought by Shelver and Burger (4) who reported the synthesis of certain 2-substituted-2-aminotetralins. Their compounds, which were inactive as analgesics, lacked a highly substituted central atom, and the 2-substituents possessed highly polar groups.

[2] Numerous examples of magnetic nonequivalence of methylene and isopropyl groups in asymmetric compounds have been reported. For examples, see E. I. Snyder, *J. Amer. Chem. Soc.*, **85**, 2624(1963); G. M. Whitesides, D. Holtz, and J. D. Roberts, *J. Amer. Chem. Soc.*, **86**, 2628 (1964); and references cited therein.



Scheme II

increase the conformational preference for the otherwise disfavored axial orientation of the dimethyl amino group. Since the amino functions of morphine and other established conformationally restricted analgesics are held in an axial orientation, substitutions increasing the preference for that orientation in the 2-aminotetralins were anticipated to increase analgesic activity in the series. However, the essentiality of an axial orientation of the amino function has recently been disputed by the identification of a number of analgesic compounds in which the amino group is oriented in a position comparable to the equatorial position of the half-chair tetralin system (7, 8). In light of these reports and of the concept of Portoghese *et al.* (9) that differing modes of binding of various narcotic analgesics to analgesic receptors may occur, activity may reside in either half-chair (or some other) conformation of the 2-aminotetralins. The reduction in activity caused by the introduction of a 2-alkyl group may be the result of an unfavorable steric interaction of the group with the receptor. The role that stereochemistry may play in the relative activity of the 2-aminotetralins is under investigation.

EXPERIMENTAL[3]

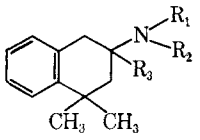
Ethyl 1,2,3,4-Tetrahydro-2,4,4-trimethyl-1-oxo-2-naphthoate (IV)—In a dry, 1-l., two-necked, round-bottom flask, equipped with a reflux condenser and an addition funnel, was placed 340 ml. of absolute ethanol, in which was dissolved 16.1 g. (0.7 g. atom) of sodium in small portions. To this solution of sodium ethoxide were added 34.65 g. (0.141 mole) of ethyl 1,2,3,4-tetrahydro-4,4-dimethyl-1-oxo-2-naphthoate (III) and 175 ml. of anhydrous benzene. After refluxing the reaction mixture for 2.5 hr., it was cooled to 25° and treated with 49 ml. (0.77 mole) of methyl iodide. The reaction mixture was maintained at 25° for 2 hr. with continuous stirring and then treated with a second 49-ml. portion of methyl iodide. After stirring continuously for 2 hr., the reaction mixture was warmed slowly, refluxed gently for 90 min., cooled, and neutralized with acetic acid. After being evaporated nearly to dryness, the mixture was treated with 200 ml. of benzene and 20 ml. of water. The benzene layer was separated, washed once with 5% sodium hydroxide solution, washed twice with distilled water, and dried over anhydrous sodium sulfate. The benzene was removed, and two distillations yielded 26.0 g. (71%) of a product, b.p. 113°/0.28 mm. The viscous oily product turned into a waxy solid within a short period. Recrystallizations from a mixture of warm ethanol and water yielded large rhomboid crystals, m.p. 50–50.4°. The IR spectrum (KBr) exhibited maxima of μ: 3.26, aromatic CH; 3.40–3.49, aliphatic CH; 5.75, ester carbonyl; and 5.85, ketone. The NMR spectrum exhibited values of δ: 1.12, triplet, 3H, ethyl CH_3; 1.25, 1.32, 2 singlets, 2 × 3H, gem-CH_3s; 1.39, singlet, 3H, αCH_3; 2.19, *AB* quartet, 2H, ring CH_2; 4.02, quartet, 2H, ethyl CH_2; 7.3, multiplet, 3H, aromatic; and 7.9, multiplet, 1H, aromatic.

Anal.—Calcd. for $C_{16}H_{20}O_3$: C, 73.81; H, 7.74. Found: C, 73.96; H, 7.41.

Ethyl 1,2,3,4-Tetrahydro-2-benzyl-4,4-dimethyl-1-oxo-2-naphthoate (V)—In a dry, 500-ml., two-necked, round-bottom flask, equipped with a reflux condenser and an addition funnel, was placed 100 ml. of absolute ethanol; in it was dissolved 2.6 g. (0.11 g. atom) of sodium in small portions. To this solution of sodium ethoxide was added 24.6 g. (0.1 mole) of ethyl 1,2,3,4-tetrahydro-4,4-dimethyl-1-oxo-2-naphthoate (III) dissolved in 100 ml. of anhydrous benzene. The reaction mixture was refluxed for 2 hr. and cooled. To this reaction mixture, 14 g. (0.115 mole) of benzyl chloride was added through the addition funnel over a period of 15 min. After the addition of benzyl chloride was completed, the reaction mixture was refluxed for another 3 hr., cooled, and neutralized with glacial acetic acid. After most of the solvent was removed from the reaction mixture, a solid was separated. To this, 100 ml. of water was added and the reaction mixture was extracted with ether (3 × 75 ml.). The combined ether extract was washed once with 5% sodium hydroxide solution and then with distilled water until it was neutral. After drying the extract over anhydrous sodium sulfate, the ether was removed by distillation, causing separation of a soild. Recrystallization of this solid from a mixture of ethanol and water yielded 24.0 g. (71.8%) of white hexagonal crystals, m.p. 100–100.5°. The IR spectrum (KBr) exhibited maxima of μ: 3.25, 3.30, aromatic CH; 3.37–3.54, aliphatic CH; 5.80, ester carbonyl; and 5.93, ketone. The NMR spectrum gave values of δ: 1.06, triplet, 3H, ethyl CH_3; 1.21, 1.28, 2 singlets, 2 × 3H, gem-CH_3s; 2.12, *AB* quartet, 2H, ring CH_2; 3.21, quartet, 2H, benzyl CH_2; 3.94, quartet, 2H, OCH_2; and 9 aromatic protons.

[3] Melting points were determined on a calibrated Fisher-Johns melting-point block. Microanalyses were performed by the Weiler and Strauss Microanalytic Laboratories, Oxford, England, and by the Galbraith Laboratories, Knoxville, Tenn. IR spectra were obtained with Beckman IR-5 and Beckman IR-8 spectrophotometers. NMR spectra were obtained with a Varian A-60 spectrometer, using approximately 15% concentrations of compound in CCl_4 with tetramethylsilane as an internal standard.

Table I—Analgesic Activity of Substituted 2-Methyl- and 2-Benzyl-4,4-dimethyl-2-aminotetralins



Compound	R_1	R_2	R_3	ED_{50}[a]
I[b]	CH_3	CH_3	H	0.02
X[b]	H	H	CH_3	0.10
XI[b]	H	H	$CH_2C_6H_5$	0.13
XII[b]	H	CH_3	$CH_2C_6H_5$	0.06
XIII[b]	CH_3	CH_3	CH_3	0.05
XIV[b]	CH_3	CH_3	$CH_2C_6H_5$	0.06
Meperidine[b]				0.05
Codeine[c]				0.05
Morphine[d]				0.005

[a] Expressed in millimoles per kilogram. [b] HCl salt. [c] Phosphate salt. [d] Sulfate salt.

Anal.—Calcd. for $C_{22}H_{24}O_3$: C, 78.54; H, 7.19. Found: C, 78.37; H, 7.21.

Ethyl 1,2,3,4-Tetrahydro-2,4,4-trimethyl-2-naphthoate (VI)—In a 500-ml. pressure bottle were placed 19.5 g. (0.075 mole) of ethyl 1,2,3,4-tetrahydro-2,4,4-trimethyl-1-oxo-2-naphthoate (IV), 175 ml. of glacial acetic acid, 30 ml. of a 1.25% solution of perchloric acid in glacial acetic acid prepared according to Rosenmund and Karg (10), and 5.0 g. of 10% palladium-on-carbon catalyst. The bottle was attached to a Parr hydrogenation apparatus equipped with a heater; after evacuation and heating the contents to 60°, hydrogenation was carried out with an initial pressure of 48 p.s.i. The temperature was maintained between 60–63°, with the theoretical amount of hydrogen being taken up during a 2-hr. period. The contents were cooled and the catalyst was removed by filtration and washed with hot alcohol (3 × 10 ml.). The washings and the filtrates were combined and transferred to a distillation assembly, and most of the acetic acid was removed *in vacuo* (aspirator). To the residue, 200 ml. of distilled water was added, and the mixture was extracted with ether (4 × 150 ml.). The combined ethereal extract was washed once with 10% sodium bicarbonate solution, followed by washing with water until the aqueous washings were neutral to short-range pH paper. The ether extract was dried overnight over anhydrous sodium sulfate; after removal of the solvent, the residue was distilled to yield 15.0 g. (82%) of a clear, colorless liquid, b.p. 91°/0.4 mm. The liquid immediately crystallized in the receiver while the distillation was still in progress, yielding transparent rhomboid crystals. Recrystallizations from an ethanol–water mixture yielded large rhomboid crystals, m.p. 59.5–60°. The IR spectrum (KBr) showed maxima of μ: 3.26, aromatic CH; 3.40–3.48 aliphatic CH; and 5.76, ester carbonyl. The NMR spectrum exhibited values of δ: 1.13, triplet, 3H, ethyl CH_3; 1.20, 1.32, 2 singlets, 2 × 3H, gem-CH_3s; 1.24, singlet, 3H, αCH_3; 1.92, *AB* quartet, 2H, "aliphatic" ring CH_2; 2.87, *AB* quartet, 2H, benzylic ring CH_2; 3.97, quartet, 2H, ethyl CH_2; and 7.02, multiplet, 3H, aromatic.

Anal.—Calcd. for $C_{16}H_{22}O_2$: C, 78.00, H, 9.00. Found: C, 77.85; H, 9.10.

Ethyl 1,2,3,4-Tetrahydro-2-benzyl-4,4-dimethyl-2-naphthoate (VII)—In a 500-ml. pressure bottle were placed 20.0 g. (0.065 mole) of ethyl 1,2,3,4-tetrahydro-2-benzyl-4,4-dimethyl-1-oxo-2-naphthoate (V), 150 ml. of glacial acetic acid, 30 ml. of a 1.25% solution of perchloric acid in glacial acetic acid, and 3.0 g. of 5% palladium-on-carbon catalyst. The bottle was attached to a Parr hydrogenator equipped with a heater. After evacuation and heating the contents to about 50°, the hydrogenation was carried out with an initial pressure of 50 p.s.i. over a period of 5 hr., after which no more hydrogen was consumed. The contents were cooled, and the catalyst was removed by filtration and washed with hot alcohol (3 × 10 ml.). The washing and the filtrate were combined, and most of the acetic acid was removed by distillation under vacuum (aspirator). To the residue, 80 ml. of distilled water was added and the mixture was extracted with ether (3 × 75 ml.). The ether extract was washed with 10% sodium bicarbonate solution followed by washing with distilled water until the aqueous washings were neutral. After drying over anhydrous sodium sulfate, the solvent was removed from the ether extract and a solid separated. Recrystallization from an alcohol–water mixture gave 16.5 g. (86.4%) of white rhomboid crystals, m.p. 81.5°. The IR spectrum (KBr) exhibited maxima of μ; 3.25, 3.30, aromatic CH; 3.37–3.48, aliphatic CH; and 5.76, ester carbonyl. The NMR spectrum gave values of δ: 0.95, triplet, 3H, ethyl CH_3; 1.13, 1.27, 2 singlets, 2 × 3H, gem-CH_3s; 1.96, *AB* quartet, 2H, "aliphatic" ring CH_2; 2.80, 2 overlapping *AB* quartets, 4H, benzyl CH_2s; 3.84, quartet, 2H, ethyl CH_2; and 9 aromatic protons.

Anal.—Calcd. for $C_{22}H_{16}O_2$: C, 81.94; H, 8.12. Found: C, 81.61; H, 8.16.

1,2,3,4-Tetrahydro-2,4,4-trimethyl-2-naphthoic Acid (VIII)—A solution of 10.8 g. (0.043 mole) of ethyl 1,2,3,4-tetrahydro-2,4,4-trimethyl-2-naphthoate (VI) in 190 ml. of ethanol was refluxed for 2 hr. with 170 ml. of 10% sodium hydroxide solution contained in a 500-ml., one-necked, round-bottom flask equipped with a reflux condenser. At the end of the reflux period, the clear and colorless solution was made slightly acidic to a pH of 6.6 with acetic acid. The condenser was replaced with a Claisen distilling head with the condenser turned downward, and the alcohol was removed by distillation *in vacuo* (aspirator). After a small amount of ethanol was removed, a white solid started to separate and continued to increase in amount as the alcohol was being removed, leading to some troublesome bumping. This solid was filtered off, dried, and weighed (7.2 g.). From the filtrate, the rest of the ethanol was distilled off and the flask was kept in a refrigerator overnight. The white solid, which separated out as small granular crystals, was filtered off, dried, and weighed (2.0 g.). When combined with the previously collected crop, the total yield was 9.2 g. (96%). Recrystallizations from a saturated solution of the compound in ethanol, to which a few drops of water were added, led to large, transparent, six-sided crystals, m.p. 151.5–152°. The IR spectrum exhibited maxima of μ: 3.75 (broad), bonded OH; 5.86, carbonyl.

Anal.—Calcd. for $C_{14}H_{18}O_2$: C, 77.02; H, 8.31. Found: C, 76.60; H, 8.39.

1,2,3,4-Tetrahydro-2-benzyl-4,4-dimethyl-2-naphthoic Acid (IX)—A solution of 15.0 g. (0.046 mole) of ethyl 1,2,3,4-tetrahydro-2-benzyl-4,4-dimethyl-2-naphthoate (VII) in 100 ml. of 95% ethanol was refluxed with 200 ml. of 10% sodium hydroxide solution for 15 hr. At the end of this period, the alcohol was removed by distillation *in vacuo* and the mixture was acidified with dilute hydrochloric acid and cooled. The crystals formed were filtered and recrystallized from a mixture of ethanol and water, giving 13.5 g. (97.4%) of white needle-shaped crystals, m.p. 148–148.5°. The IR spectrum (KBr) exhibited maxima of μ: 3.6–4.0 (continuous series), bonded OH; 5.89, carbonyl.

Anal.—Calcd. for $C_{20}H_{22}O_2$: C, 81.59; H, 7.53. Found: C, 81.47; H, 7.70.

1,2,3,4-Tetrahydro-2,4,4-trimethyl-2-naphthylamine Hydrochloride (X)—To 4.36 g. (0.02 mole) of 1,2,3,4-tetrahydro-2,4,4-trimethyl-2-naphthoic acid (VIII) contained in a 500-ml., two-necked, round-bottom flask, equipped with a drying tube and an inlet tube with a stopcock, was added 17 ml. of thionyl chloride. The mixture was warmed to 40° and maintained at that temperature for 5 min. with simultaneous stirring. The flask was flushed with dry nitrogen gas, and the mixture was allowed to stand at room temperature overnight. The drying tube was replaced with a glass stopper, and the unreacted thionyl chloride was removed *in vacuo* (aspirator), producing a buff-colored solid. The acid chloride thus formed was dissolved in 40 ml. of anhydrous reagent grade acetone, and the solution was cooled in an ice bath. To it was added, over a period of 5 min., 1.37 g. (0.021 mole) of sodium azide dissolved in 4 ml. of water *via* an addition funnel. The solution was stirred for 1 hr., maintaining the temperature between 6–10°, and then was allowed to warm slowly to room temperature (23°) over a period of 45 min. At the end of that period, a white granular solid settled out and the supernatant liquid became turbid. To this mixture, 90 ml. of water was added, and the mixture was extracted with xylene (4 × 60 ml.). The xylene extracts were combined, washed with 20 ml. of 10% sodium bicarbonate solution, and dried over anhydrous sodium sulfate. The acidified sodium bicarbonate solution yielded 0.66 g. of starting material. The xylene solution was filtered into a 500-ml. round-bottom flask, maintaining anhydrous conditions.

The rearrangement of the azide to the isocyanate was accomplished in the usual manner by applying vacuum (aspirator) until

no further evolution of nitrogen was in evidence. The IR spectrum showed a maximum at 4.44 μ, which is consistent with an isocyanate.

The isocyanate was converted to the amine hydrochloride by adding the xylene solution dropwise, during a period of about 30 min., to 20 ml. of concentrated hydrochloric acid saturated with hydrogen chloride while being heated to about 80° and stirred in a 1-l. round-bottom flask. A copious evolution of gas was noted during the addition. The stirring and heating were continued for an additional 2 hr., during which time a white solid separated out and remained dispersed in the xylene phase. The solid was filtered off, dried, and weighed, yielding 2.3 g. (53%) of X. The aqueous acidic phase was separated, and the xylene phase was washed with 10% hydrochloric acid solution (3 × 300 ml.). The combined acidic extracts were evaporated nearly to dryness to obtain additional amine hydrochloride.

The solid obtained was dissolved in hot water, and the solution was made alkaline to a pH 12 and extracted with ether (4 × 70 ml.). The ether extracts were washed with dilute acetic acid followed by water and thoroughly dried over anhydrous sodium sulfate. Dry hydrogen chloride gas was bubbled through the filtered ethereal solution to produce a white precipitate of the amine hydrochloride. Recrystallizations from a mixture of hot absolute alcohol and anhydrous ether yielded a white amorphous solid, m.p. 295–296° (*in a nitrogen-filled, sealed, capillary tube*). The IR spectrum (KBr) showed maxima of μ: 3.5–4.3 (continuous), amine salt; 4.83 (NH_3^+).

Anal.—Calcd. for $C_{13}H_{20}ClN$: C, 69.15; H, 8.92; Cl, 15.70; N, 6.20. Found: C, 69.17; H, 8.92; Cl, 15.45; N, 6.31.

1,2,3,4-Tetrahydro-2-benzyl-4,4-dimethyl-2-naphthylamine Hydrochloride (XI)—To 2 g. (0.0067 mole) of 1,2,3,4-tetrahydro-2-benzyl-4,4-dimethyl-2-naphthoic acid (IX) contained in a 200-ml., two-necked, round-bottom flask, equipped with a drying tube and an inlet tube with a stopcock, was added 6 ml. of freshly purified thionyl chloride. The mixture was stirred and kept overnight. The drying tube was replaced with a glass stopper, and the unreacted thionyl chloride was removed *in vacuo*, giving a white waxy solid. The resulting acid chloride was dissolved in 80 ml. of anhydrous toluene; to this was added 3.0 g. of freshly activated sodium azide. The suspension was refluxed for 24 hr. while protected from moisture by a calcium chloride tube. The cooled suspension was filtered with suction directly into a 200-ml. round-bottom flask, maintaining anhydrous conditions. The flask was rinsed with a little toluene which was poured through the filter. The IR spectrum of the unisolated intermediate in toluene solution showed a maximum at 4.44 μ, which is consistent with an isocyanate.

To the filtrate in the round-bottom flask was added 60 ml. of concentrated hydrochloric acid, and the mixture was heated to about 80° when a vigorous reaction was observed. The reaction mixture was refluxed for another 2 hr. and cooled; 100 ml. of distilled water was added, and the acid layer separated from the toluene layer. The toluene layer was extracted with dilute hydrochloric acid (2 × 25 ml.). The combined acid solution was made alkaline by 10% sodium hydroxide solution and extracted with ether (4 × 50 ml.). The ether extract, after washing with distilled water until neutral, was dried over anhydrous sodium sulfate. To this dried ethereal solution was added ether saturated with dry hydrogen chloride gas to yield 1.46 g. (72.6%) of the amine hydrochloride (XI) as a heavy, white precipitate. Recrystallization from water and a few drops of ethanol gave a white solid, m.p. 256°. The IR spectrum (KBr) gave maxima of μ: 2.8–3.0 (broad) and 3.6–4.2 (continuous), amine salt; 4.9, NH_3^+.

Anal.—Calcd. for $C_{19}H_{24}ClN$: C, 75.6; H, 8.01; N, 4.64. Found: C, 75.6; H, 8.08; N, 4.65.

***N*-Methyl-1,2,3,4-tetrahydro-2-benzyl-4,4-dimethyl-2-naphthylamine Hydrochloride (XII)**—A solution of 2 g. of XI in 50 ml. of water was made alkaline by the addition of 5% sodium hydroxide solution, and the free amine formed was extracted with ether (4 × 50 ml.). The ether extract was washed with distilled water until neutral; after drying over anhydrous sodium sulfate, the ether was removed by distillation. To the residue of the free amine dissolved in 50 ml. of chloroform in a 100-ml. round-bottom flask, equipped with an addition funnel and a thermometer, 1.0 g. of chloral was added dropwise while the reaction mixture was kept between 0–5° with the aid of an ice bath. After the addition of the chloral was completed, the reaction mixture was stirred for 6 hr. at room temperature and refluxed for another 2 hr. over a steam bath. The chloroform was removed by distillation, and the solid was recrystallized once from a mixture of ether and chloroform to give 1.2 g. of the formyl derivative, m.p. 170–173°. The IR spectrum showed a strong absorption at 6.02 μ assignable to the C=O group of the formyl derivative.

The formyl derivative, without further purification, was dissolved in 150 ml. of dry ether. The solution was added little by little, with constant stirring, to an ice-cold suspension of 0.3 g. of lithium aluminum hydride in 50 ml. of ether in a 500-ml., three-necked, round-bottom flask. After the addition was completed, the reaction mixture was stirred and refluxed for 6 hr. At the end of this period, the reaction mixture was cooled, and 10 ml. of distilled water was added dropwise to it to destroy the unreacted lithium aluminum hydride. The reaction mixture was filtered through a sintered-glass funnel, and the inorganic matter was washed with ether. After the combined ether solution was dried over anhydrous sodium sulfate, ether saturated with dry hydrogen chloride gas was added to it. The white precipitate formed was filtered and recrystallized from an ethanol–ethyl acetate–ether mixture, giving 0.7 g. (33.5%) of the amine hydrochloride XII, m.p. 208°.

Anal.—Calcd. for $C_{20}H_{26}ClN$; C, 76.04; H, 8.29; N, 4.43. Found: C, 75.96; H, 8.12; N, 4.36.

***N,N*-Dimethyl-1,2,3,4-tetrahydro-2,4,4-trimethyl-2-naphthylamine Hydrochloride (XIII)**—To 5.46 g. (0.025 mole) of 1,2,3,4-tetrahydro-2,4,4-trimethyl-2-naphthoic acid (VI) in a 250-ml. two-necked flask, equipped with a drying tube and an inlet tube with a stopcock, was added 15 ml. of thionyl chloride. The mixture was warmed to 40° with stirring for 5 min. The flask was then flushed with dry nitrogen and allowed to stand at room temperature overnight. The excess thionyl chloride was removed *in vacuo* (aspirator), leaving a white solid. To the solid was added 50 ml. of acetone (A.R.) previously dried over calcium chloride. The resulting solution was cooled to 0–5°; 1.63 g. (0.026 mole) of sodium azide, dissolved in 5 ml. of distilled water, was added *via* an addition funnel over a period of 5 min. The solution was stirred at 6–10° for 1 hr. and then allowed to warm to 25° over a period of 45 min. During this period, a white solid separated. Water (75 ml.) was added, and the mixture was extracted with toluene (3 × 75 ml.). The toluene extracts were combined, washed with sodium bicarbonate (2 × 25 ml.) and 50 ml. of water, and filtered through anhydrous sodium sulfate. The azide in the filtrate was rearranged to the isocyanate with the aid of vacuum (aspirator) over a 2-hr. period, and the filtrate then concentrated to about 20 ml. Twelve milliliters of 98% formic acid was added, the mixture was refluxed for 2 hr., and the volatile constituents were removed *in vacuo*. To the residue were added 25 ml. of 88% formic acid and 25 ml. of 37% formaldehyde solutions; then the mixture was refluxed for 12 hr. Twenty-five milliliters of concentrated hydrochloric acid was added, and the excess formaldehyde and formic acid were removed with the aid of the aspirator. The residue was made alkaline to pH 12 with cold 20% sodium hydroxide solution and then extracted with ether (3 × 100 ml.). The ether extracts were washed with water and filtered through anhydrous sodium sulfate. To the ether solution was added 200 ml. of anhydrous ether saturated with anhydrous hydrogen chloride, causing the amine hydrochloride (XIII) to precipitate as a white solid. The solid was collected on a filter and then recrystallized from absolute ethanol and ether to give 5 g. (78.9%) of white needles, m.p. 249–250° (*sealed tube*). The IR spectrum (KBr) exhibited maxima of μ: 3.7–4.8 (continuous), amine salt.

Anal.—Calcd. for $C_{15}H_{24}ClN$: C, 70.98; H, 9.51; Cl, 13.97; N, 5.52. Found: C, 70.70; H, 9.56; Cl, 13.77; N, 5.77.

***N,N*-Dimethyl-1,2,3,4-tetrahydro-2-benzyl-4,4-dimethyl-2-naphthylamine Hydrochloride (XIV)**—A solution of 2.5 g. (0.008 mole) of 1,2,3,4-tetrahydro-2-benzyl-4,4-dimethyl-2-naphthylamine hydrochloride in 50 ml. of water was made alkaline by adding 5% sodium hydroxide solution to it. The free amine formed was extracted with ether (4 × 50 ml.). The ether extract was washed with distilled water until the washings were neutral and then dried over anhydrous sodium sulfate. The ether was removed by distillation; to the milky residue obtained was added 4 ml. of 98% formic acid. A vigorous reaction was noticed. After the reaction subsided, the excess formic acid was removed *in vacuo* to yield a white solid. To this solid were added 5 ml. of 35% formaldehyde solution and 5 ml. of 88% formic acid solution, and the mixtue was refluxed for 12 hr. on a water bath. After the evolution of carbon dioxide ceased, the formaldehyde and formic acid were removed *in vacuo*. To this residue, 20 ml. of concentrated hydrochloric acid was added, and the mixture was refluxed on a water bath for 2 hr. The acid solution was

cooled, made alkaline with sodium hydroxide, and extracted with ether (3 × 75 ml.). The combined ether extract, after washing with distilled water and drying over anhydrous sodium sulfate, was added to an ethereal solution of dry hydrogen chloride to yield a white precipitate. Recrystallization from a mixture of ethanol–ethyl acetate–ether yielded 2.1 g. (76%) of white needle-shaped crystals, m.p. 210°. The IR spectrum (KBr) exhibited maxima of μ: 3.9–4.9 (continuous), amine salt.

Anal.—Calcd. for $C_{21}H_{28}ClN$: C, 75.93; H, 9.17; N, 4.21. Found: 76.09; H, 8.97; N, 4.34.

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ALLEN I. KAY and THOMAS H. SIMON

Abstract □ An analog computer program was developed to simulate drug decomposition induced during linear nonisothermal stability studies. From the analog computer values used to generate the simulation curve best-fitting experimental data, the activation energy, the reaction rate constant at any temperature, and the predicted shelflife at that temperature can be determined. By using the decomposition data reported for the first-order, linear nonisothermal degradation of *N*-acetyl-*p*-aminophenol and the analog computer program presented here, kinetic parameters identical with published values were obtained. The analog computer program can be easily modified to accommodate nonlinear heating regimens as well as reactions of any order.

Keyphrases □ Linear nonisothermal stability studies—simulation □ Analog computer simulation—stability studies □ Shelflife prediction—kinetic parameters □ Diagram—analog computer program

The customary procedure for predicting the shelflife of a drug involves the determination of the degradation rate constant at a few elevated temperatures, the determination of the activation energy, and finally the extrapolation of this information to room temperature. This requires large numbers of samples and ultimately a great deal of personnel time. By means of a rapid and simple concentration–time–temperature study in which temperature is changed at a preselected rate, it is possible to obtain these parameters in 1 or 2 days.

The concept of using programmed elevated temperatures to arrive at the kinetic parameters required to predict shelflife is not new. Borchardt and Daniels (1) obtained the rate, order, and activation energy for the decomposition of benzenediazonium chloride by analysis of the slope, height, and area of a differential thermal analysis curve. Davies (2) followed a chemical reaction by observing the change in optical density of one reactant with time as its temperature was progressively raised. Reaction kinetics were also followed using differential scanning calorimetry (3) and thermogravimetric analysis (4).

Various heating regimens have been employed. Cole and Leadbeater (5) studied the reaction rate of several compounds. They employed the temperature program designed by Rogers (6) in which the inverse of the temperature was varied logarithmically with time. Eriksen and Stelmach (7) obtained the necessary kinetic parameters from a single experiment, using the following time–temperature relationship:

$$\frac{1}{T} = \frac{1}{T_0} - at \qquad \text{(Eq. 1)}$$

where a is a reciprocal heating constant, and T_0 and T are the absolute temperatures at the initial time and at time t, respectively. Zoglio *et al.* (8) followed the first-order decomposition of *N*-acetyl-*p*-aminophenol and procainamide hydrochloride as the temperature was raised linearly with time according to the expression

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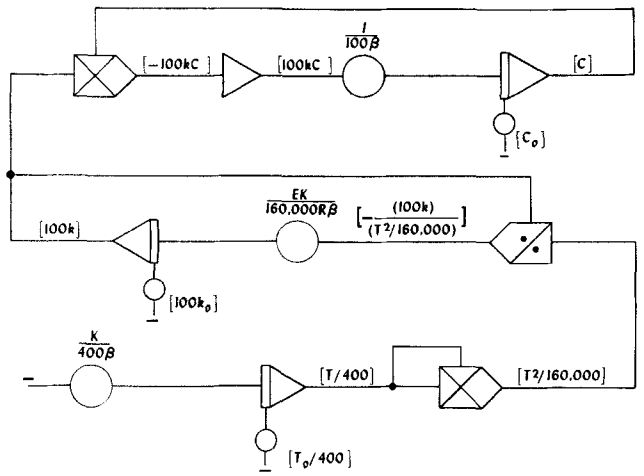

Figure 1—*Scaled computer program for simulation of first-order decomposition induced during linear nonisothermal stability studies.*

data were superimposed over the model curves to obtain the energy of activation for the reaction. The use of digital computation eliminates the need for manual slope estimation, which is generally regarded as a major drawback of some of the previous methods (1, 2).

An analog computer program was developed which is designed to simulate drug decomposition induced during nonisothermal stability studies. The method is illustrated for the case of first-order degradation induced during a linear nonisothermal experiment. The data given by Zoglio *et al.* (8) were plotted, and analog computation was used to obtain the degradation curve best fitting the experimental data points. The rate constant at 35° and the energy of activation, obtained directly from the computer values, were in excellent agreement with those reported by Zoglio *et al.* (8).

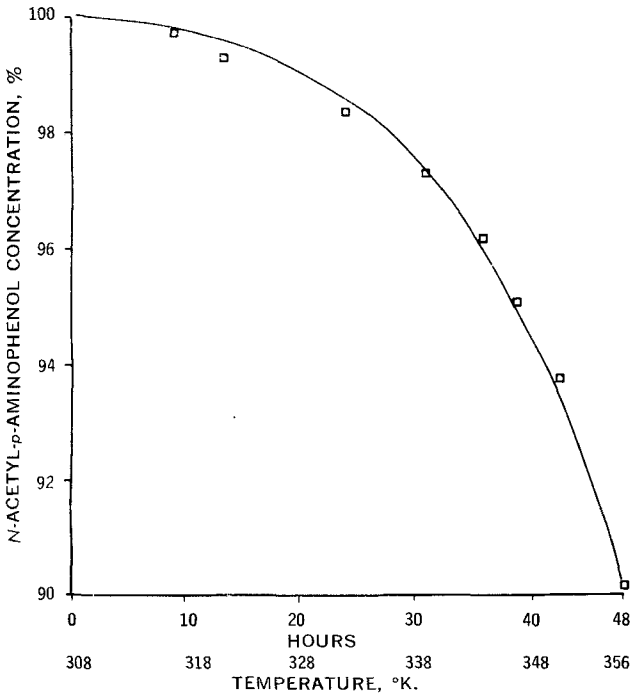

Figure 2—*Analog computer simulation of the nonisothermal hydrolysis of* N-*acetyl*-p-*aminophenol in aqueous solution (pH = 2).*

Table I—Analog Computer Scaling Table

Problem Variable	Rounded-Up Maximum	Computer Variable
C	1 (normalized)	$[C]$
k	0.01	$[100k]$
T	400	$[T/400]$
T^2	160,000	$[T^2/160,000]$

THEORY

The basic equations employed are as follows:

$$-\frac{dC}{dt} = kC^a \qquad \text{(Eq. 3)}$$

where a is the order of the reaction, C is the drug concentration at time t, and k is the observed rate constant which can be obtained from the expression

$$k = Ae^{-E/RT} \qquad \text{(Eq. 4)}$$

where E is the energy of activation, A is the frequency of molecular collisions, T is the absolute temperature, and R is the molar gas constant (1.987 cal. deg.$^{-1}$ mole^{-1}).

Differentiating with respect to temperature yields

$$\frac{dk}{dT} = \frac{kE}{RT^2} \qquad \text{(Eq. 5)}$$

If temperature is varied linearly with respect to time, the following relationship exists:

$$T = T_0 + Kt \qquad \text{(Eq. 6)}$$

where T_0 is the starting temperature and K is the heating rate.

Differentiating with respect to time yields

$$\frac{dT}{dt} = K \qquad \text{(Eq. 7)}$$

Thus

$$\frac{dk}{dt} = \frac{KkE}{RT^2} \qquad \text{(Eq. 8)}$$

EXPERIMENTAL

Based on the rounded-up maximum values given in Table I and a time scaling factor[1] of $\beta = 0.1$, the following scaled differential equations were used to construct the analog computer program shown in Fig. 1.

$$\frac{d[C]}{d\tau} = -\left\{\left(\frac{1}{100\beta}\right)[100k][C]\right\} \qquad \text{(Eq. 9)}$$

$$\frac{d[100k]}{d\tau} = -\left\{\left(\frac{EK}{160{,}000R\beta}\right)\left[-\frac{[100k]}{[T^2/160{,}000]}\right]\right\} \qquad \text{(Eq. 10)}$$

$$\frac{d[T/400]}{d\tau} = -\left(\frac{-K}{400\beta}\right) \qquad \text{(Eq. 11)}$$

The equations were solved using an Electronic Associates Inc. Analog/Hybrid Computing System equipped with a series 1140 Variplotter recorder.

Equation 9 is a special case of Eq. 3 and describes first-order degradation. The analog computer can be programmed to solve Eq. 3 for reactions other than first order by incorporating additional analog computer components into the program which will raise the concentration term to an appropriate power. Equation 11 describes a linear time–temperature relationship. Other heating regimens can be simulated. If the equation describing the relationship between temperature and time is known, it can be incorporated directly into the computer program. If the equation for the time–

[1] $\beta = \tau/t$ where τ is computer time in seconds and t is problem time in hours.

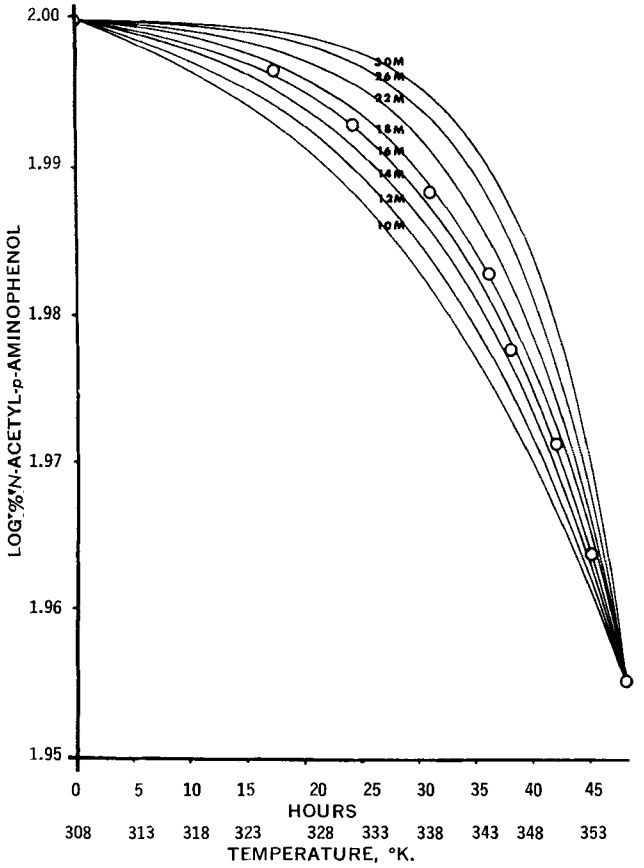

Figure 3—*Nonisothermal hydrolysis of* N-*acetyl*-p-*aminophenol in aqueous solution (pH = 2). Curves were generated using digital computation (Ref. 8).*

temperature relationship is unknown, the temperature of the system can be measured at various time intervals and a function generator can be employed to provide temperature as a function of time.

The usefulness of the analog computer program for prediction of shelflife based on evaluation of kinetic data obtained from nonisothermal stability studies was investigated utilizing data reported by Zoglio *et al.* (8) for the linear nonisothermal hydrolysis of *N*-acetyl-*p*-aminophenol. These authors obtained their experimental data in only 48 hr. Ampuls initially containing a solution of 0.15% *N*-acetyl-*p*-aminophenol in 0.2 *M* phosphate buffer were heated in an oven at the rate of 1°/hr. from 35–83°. Ampuls were removed at convenient time intervals and analyzed for intact drug.

The percentage of intact drug remaining was plotted as a function of time (temperature), and the analog computer program was employed to simulate first-order decomposition of a drug according to the time–temperature relationship consistent with the experiment. The decomposition curve best fitting the experimental data points was obtained by varying the initial rate constant (rate constant at 35°) and the energy of activation until the degradation curve matched the data points (Fig. 2). This was easily accomplished since the analog computer program was constructed to allow isolation of these kinetic parameters on separate potentiometers. The values of the initial rate constant, k_0, and the energy of activation were obtained directly from their potentiometer settings.

RESULTS AND DISCUSSION

The degradation curve best fitting the experimental data corresponded to that having an activation energy of 17 kcal./mole and an initial rate constant of 2×10^{-4} hr.$^{-1}$. Both values are in excellent agreement with those reported by Zoglio *et al.* (8).

The basis for the approach of Zoglio *et al.* (8) is that a degradation curve can be described by a series of slopes or rate constants and that the arithmetic average of these instantaneous rates of change can be assumed to be equal to the total drug degradation during the experiment divided by the time span required for the experiment.

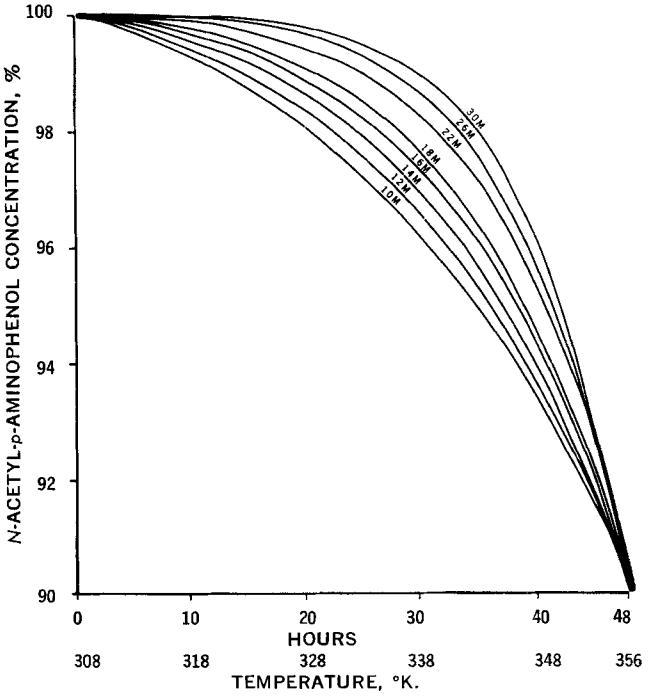

Figure 4—*Degradation curves generated using analog computation. Activation energies were selected and* k_0 *was varied so that curves were forced through initial and final concentration levels.*

Since a large number of rates (384) was obtained for each particular activation energy, this assumption is valid. These rates were then used to synthesize a series of degradation curves on a first-order scale (Fig. 3). The experimental data were then plotted on the same graph containing the family of curves, and the energy of activation was obtained by inspection and interpolation.

Figure 4 shows a series of decomposition curves generated using the analog computer. An activation energy ranging from 10–30 kcal./mole was arbitrarily selected for each curve, and k_0 was varied so that each curve was forced through the initial and final concentration levels. This method is similar to the approach followed

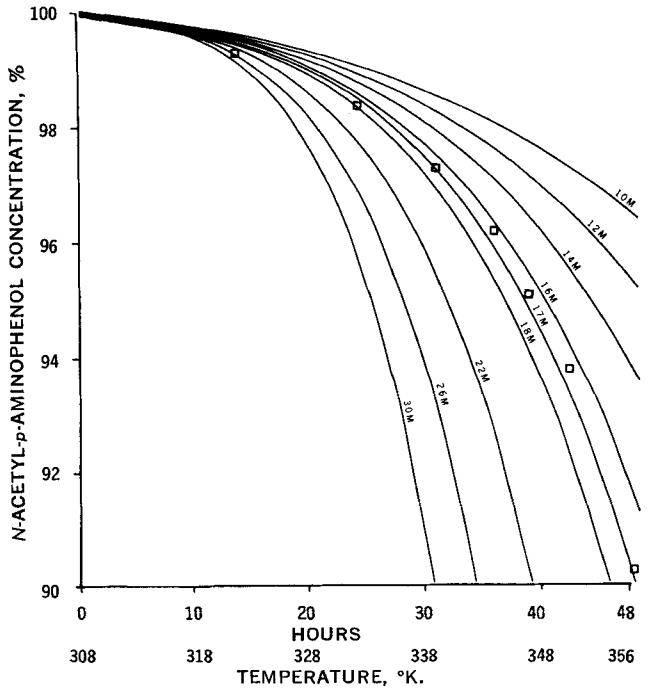

Figure 5—*Degradation curves generated by setting* k_0 *at* 2×10^{-4} *hr.*$^{-1}$ *and varying the energy of activation.*

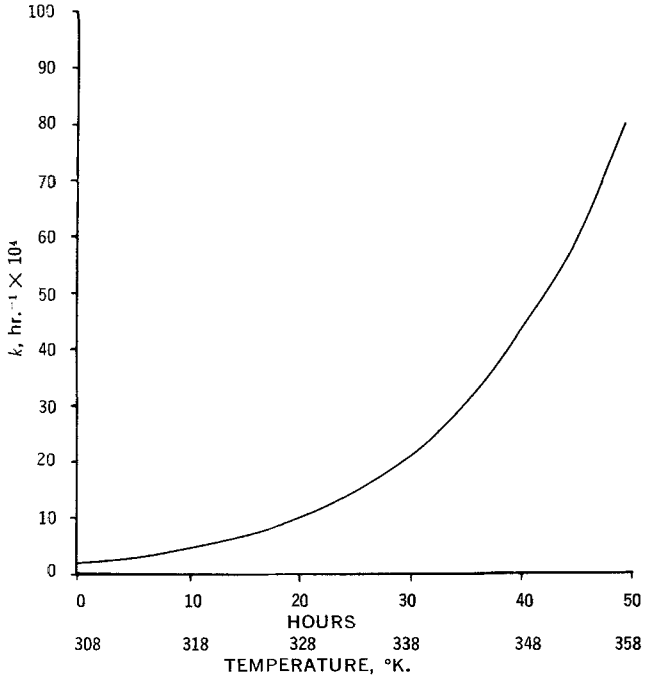

Figure 6—*Degradation rate constants generated as a function of temperature (time).*

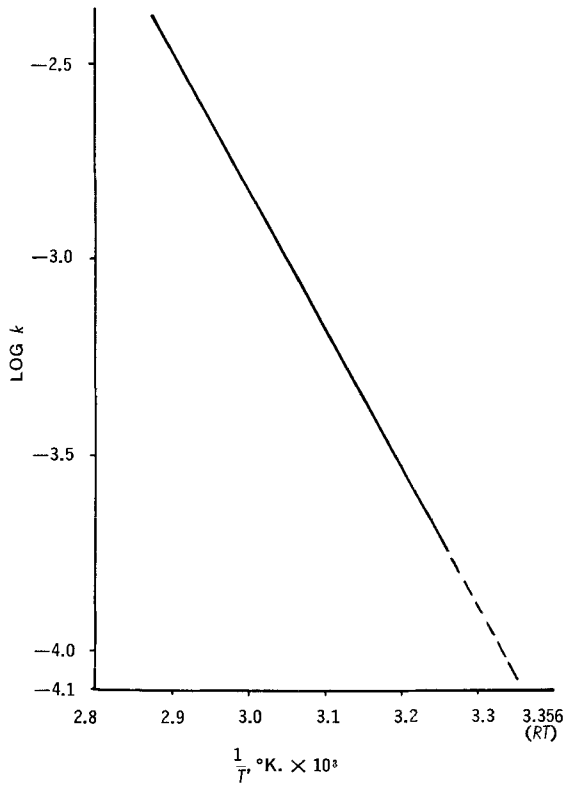

Figure 7—*Arrhenius plot which can be extrapolated to obtain the degradation rate constant at room temperature.*

by Zoglio *et al.* (8); the similarity between the curves obtained using analog simulation and those obtained employing digital computation (Fig. 3) indicates that either method can be used ultimately to predict shelflife. However, since only one curve can be correct for a particular experiment, this curve can be obtained simply and rapidly using the analog computer program presented here, which allows simultaneous adjustment of k_0 and activation energy. All experimental data points are used in the simulation, which should increase accuracy and reduce the possibility of a serious mistake should one experimental point be incorrect.

If the rate constant at the initial temperature was experimentally determined by analysis of a few samples under isothermal conditions, then the value of k_0 could be inserted into the analog computer program and degradation curves could be drawn varying only the energy of activation. This method is illustrated in Fig. 5. The initial rate constant was taken as 2×10^{-4} hr.$^{-1}$. This method sacrifices time but increases sensitivity. Comparison of Figs. 4 and 5 indicates that the same change in activation energy results in a greater displacement of the concentration–time–temperature curve when k_0 is fixed. However, such an approach is seldom necessary since the value of the initial rate constant obtained using analog simulation should be sufficiently sensitive to allow confident prediction of shelflife.

Either the experimentally determined or the computer-generated k_0 value can be incorporated into the program, which is designed to generate degradation rate constants as a function of time (temperature) so that k values can be graphically displayed (Fig. 6), and by means of a log function generator directly yield an Arrhenius plot. Alternately, these values can be taken for subsequent plotting (Fig. 7). By extrapolating to 25°, the rate constant for the hydrolysis of *N*-acetyl-*p*-aminophenol at room temperature was found to be 8.07×10^{-5} hr.$^{-1}$, corresponding to a shelflife of 55 days.

By using the analytical data obtained from a single, linear, nonisothermal stability study and the analog computer program presented here, kinetic parameters required to predict shelflife were obtained. Manual analysis of heights, slopes, or areas of curves is not required, since the analog computer provides instantaneous graphical solutions to the differential equations employed. Although the method was illustrated for first-order degradation induced using a linear heating program, it is applicable to other heating regimens and for reactions other than first order.

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Drug Release from Methyl Acrylate–Methyl Methacrylate Copolymer Matrix I: Kinetics of Release

B. FARHADIEH, S. BORODKIN, and J. D. BUDDENHAGEN

Abstract □ The release rates of four commonly used drugs incorporated into a methyl acrylate–methyl methacrylate copolymer matrix were studied. The quantity released per unit surface area in single-surface release experiments was found to be proportional to the square root of time. The hardness of the tablet had little effect on the release-rate constant, although some tablets swelled and broke open before complete elution. Heating the tablets at 70° or exposing them to acetone vapor prevented the breaking and increased the release-rate constant. The effects of drug, drug concentration, compressional force, heating, and exposure to acetone vapor on the drug release-rate constant were analyzed in terms of matrix porosity and tortuosity. Drug release from tablets in which the total surface was exposed showed little deviation from linearity during the first 75% of the release when plotted against the square root of time.

Keyphrases □ Drug release—methyl acrylate–methyl methacrylate matrix □ Kinetics, drug release—copolymer matrix □ Release rate, drug–copolymer matrix—factors affecting □ Linearity—drug release from matrix *versus* square root of time

Higuchi (1) suggested that the release of drug from a planar system having an insoluble granular matrix should follow the equation:

$$Q = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_S)C_S t\right]^{1/2} \qquad \text{(Eq. 1)}$$

where Q = the amount of drug released after time t per unit exposed area, D = the diffusivity of the drug in the permeating fluid, τ = the tortuosity, A = the amount of drug present in the matrix per unit volume, C_S = the solubility of the drug in the permeating fluid, and ϵ = porosity of the matrix.

Desai *et al.* (2–5) studied this relationship extensively, using polyvinyl chloride and polyethylene as the insoluble matrixes. Drug release from tablets compressed using the latter plastic was found to be linear with the square root of time, as predicted by Eq. 1. Singh *et al.* (6, 7) extended the study to solid drug mixtures using the same two plastics.

The purpose of this investigation was to examine drug release from a methyl acrylate–methyl methacrylate copolymer matrix. The use of this plastic as the matrix substance in tablets suitable for oral administration has been described (8, 9). Four commonly used drugs were employed in this evaluation. Release rates from one surface were obtained to determine whether the described relationship is applicable. Release rates from tablets in which all surfaces were exposed to the release medium were obtained for comparison.

EXPERIMENTAL

Chemicals—The plastic used for all tablets was a powdered methyl acrylate–methyl methacrylate copolymer.[1] This plastic is insoluble and inert in aqueous media at all pH values. The drugs used were either USP or NF grade.

[1] Rohm & Haas Co., Philadelphia, Pa.

Table I—Physical Constants of Drugs Used

Drug	$\lambda_{max.}$, nm.	ρ, g. cm.$^{-3}$	Solubility at 37°, g./ml.	$D \times 10^6$, cm.^{2}-sec.$^{-1}$	Predominant Particle-Size Range, μ[a]
Sodium pentobarbital	240[b]	1.342	0.750[c]	18.5	149
Methapyrilene HCl	312[d]	1.295	0.676[d]	19.2	149
Ephedrine HCl	257[d]	1.203	0.325[d]	21.9	177–420
Dextromethorphan HBr	278[d]	1.368	0.036[d]	18.3	149

[a] Range containing greater than 75% by weight of the drug. [b] In 0.1 *N* NH_4OH. [c] In water. [d] In 0.1 *N* HCl.

Determination of Physical Constants of Drugs—The particle-size ranges were determined using U.S. Standard sieves with a Ro-Tap mechanical shaker. Solubilities were obtained by adding an excess of drug to the solvent, shaking the mixture in a 37° water bath for 24 hr. with a Burrell wrist-action shaker, filtering, and assaying by UV spectrophotometry. Densities were determined with a Beckman air compression pycnometer, model 930. Diffusion coefficients were obtained using the procedure described by Desai *et al.* (3). This method involves measurement of the solute transfer rate through a sintered-glass disk located between two conical flasks. The cell constant, using a saturated sodium salicylate solution at 30°, was found to be 0.380 cm. For the drugs used in this study, the diffusion coefficients were measured at 37° using saturated solutions. In all cases the diffusion of drug across the sintered glass was linear with time after 1 hr.

The physical constants determined for the drugs used are listed in Table I.

Tablet Compression—All tablets were compressed on a Manesty layer press using 1.03-cm. ($^{13}/_{32}$-in.) flat-face punches and dies. Uniform mixtures of drug and plastic were used. For each drug–plastic mixture, tablets were compressed at several different hardnesses ranging from 12 to 27 Strong-Cobb hardness units. In all cases, tablets weighing approximately 242 mg. were made. Exact thickness and weight measurements were then obtained on 20 tablets to establish the averages for each lot. All individual values were within 3% of the mean.

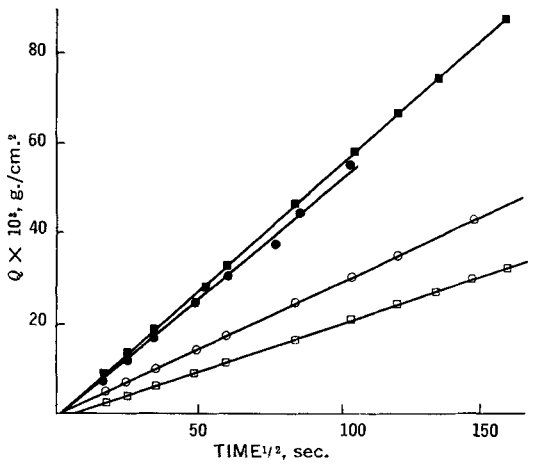

Figure 1—*Single-surface release from tablets containing 100 mg. of drug. Key:* ●, *sodium pentobarbital;* ■, *methapyrilene HCl;* ○, *ephedrine HCl; and* □, *dextromethorphan HBr.*

Table II—Release-Rate Constants and Fractional Release Times from Single-Surface Leach Tests on Tablets

Drug	Concentration, mg./Tablet	$k \times 10^4$, g. cm.$^{-2}$ sec.$^{-1}$	Time (hr.) Required for Release of 25%	50%	75%
Sodium pentobarbital	50	2.25	1.21	4.83	10.86
	100	5.38	0.84	3.38	7.60
Methapyrilene HCl	50	2.05	1.45	5.81	13.08
	100	5.48	0.81	3.25	7.32
Ephedrine HCl	50	1.46	2.87	11.46	25.79
	100	2.94	2.83	11.31	25.44
Dextromethorphan HBr	30	0.606	5.99	23.95	53.88
	100	2.14	5.33	21.34	48.01

Release-Rate Determinations—Release rates from a single tablet surface were determined using a resin-kettle flask with four ground-glass openings in the cover. The flask contained 600 ml. of solvent maintained at 37° in a constant-temperature water bath. Water was used in the sodium pentobarbital leaches, while 0.1 *N* HCl was used for methapyrilene hydrochloride, ephedrine hydrochloride, and dextromethorphan hydrobromide. Tubes containing one tablet each, imbedded in wax with only one flat face exposed, were inserted into three of the openings, so that the tablets were 2.54 cm. (1 in.) below the solvent surface. The fourth opening was equipped with a motor-driven stainless steel stirrer run at 100 r.p.m. Results from experiments using 200 and 400 r.p.m. indicated that the stirring rate was not critical.

Release rates from the total tablet surface were determined by putting three tablets into a basket of 40-mesh screen attached to a stirring rod. The chamber was then inserted into a resin-kettle flask containing 600 ml. of solvent at 37° and rotated at 100 r.p.m.

In both test methods, samples were withdrawn periodically through a small drilled hole which was otherwise closed with a stopper. The samples were assayed spectrophotometrically after the required dilutions.

RESULTS AND DISCUSSION

For a particular drug with solubility C_S, diffusivity D, and a given drug matrix concentration A, if τ and ϵ remain constant, drug release from a single surface should follow the relationship

$$Q = kt^{1/2} \qquad \text{(Eq. 2)}$$

where k is the release-rate constant and

$$k = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_S)C_S\right]^{1/2} \qquad \text{(Eq. 3)}$$

All tablets formulated with methyl acrylate–methyl methacrylate copolymer were found to follow the time dependency predicted by Eq. 2. Linearity was obtained when Q was plotted *versus* $t^{1/2}$ in single-surface release experiments from tablets compressed at maximum hardnesses (Figs. 1 and 2). Table II shows the release-rate constants obtained from these tablets. The table also includes the time required for 25, 50, and 75% drug release which is calculated from the following expression:

$$t_F = \left(\frac{Q_\infty F}{k}\right)^2 = \left(\frac{IF}{kS}\right)^2 \qquad \text{(Eq. 4)}$$

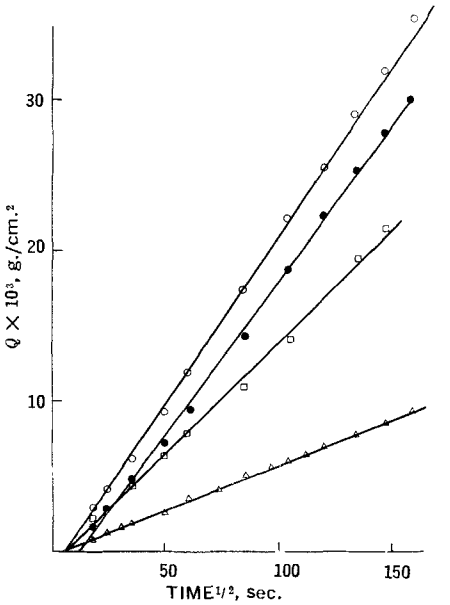

Figure 2—*Single-surface drug release from tablets. Key:* ○, *sodium pentobarbital, 50 mg.;* ●, *methapyrilene HCl, 50 mg.;* □, *ephedrine HCl, 50 mg.; and* △, *dextromethorphan HBr, 30 mg.*

where t_F is the time required to release F fraction of the total amount of drug I in the tablet through surface area S.

The differences in release-rate constants obtained for the four drugs at the same concentration can be attributed to the parameters appearing in Eq. 3. However, because of the physicochemical characteristics of the drugs, these are dependent variables and therefore difficult to study individually. Thus, in a comparison of different drugs there would be not only changes in solubility and diffusivity, but differences in the tortuosity and porosity of the matrix would result. This is exemplified by the solubility effect on the release-rate constant obtained from 100-mg. tablets. Although the release-rate constant increased with greater drug solubility, the increases were not quantitatively identical to those predicted if solubility was the predominant effect. Keeping all variables except solubility constant, the calculated release-rate constants for ephedrine hydrochloride, methapyrilene hydrochloride, and sodium pentobarbital were 2.77, 3.56, and 3.64 times that of dextromethorphan hydrobromide. However, the experimental rate constants obtained for these three drugs were 1.37, 2.56, and 2.51 times dextromethorphan hydrobromide.

The effect of the diffusion coefficient on the release-rate constant appears to be minimal. This is due both to the small differences observed in the values of D for the four drugs and their square root relationship to k. A 12.5% change in the diffusion coefficient (maximum difference observed between the experimental and average values) would change the release-rate constant by only 5.7%.

The effect of drug concentration A on the release-rate constant was tested by studying two different concentrations of each of the four drugs. In comparing the release-rate constants for different concentrations of a single drug, the solubility and diffusion coefficient remain constant. Furthermore, the expression proposed by Higuchi (1)

$$\epsilon = \epsilon_0 + kA \qquad \text{(Eq. 5}$$

Table III—Evaluation of Porosity and Tortuosity Factors

Drug	Concentration, mg./Tablet	Average Weight, g.	Average Thickness, cm.	A, g. cm.$^{-3}$	ϵ_0	ϵ	τ
Sodium pentobarbital	50	0.243	0.294	0.202	0.238	0.389	12.0
	100	0.240	0.264	0.450	0.169	0.505	12.6
Methapyrilene HCl	50	0.243	0.286	0.208	0.210	0.370	18.8
	100	0.248	0.257	0.462	0.109	0.466	12.2
Ephedrine HCl	50	0.244	0.274	0.216	0.164	0.343	36.5
	100	0.240	0.250	0.475	0.084	0.478	31.3
Dextromethorphan HBr	30	0.245	0.280	0.127	0.193	0.286	12.5
	100	0.243	0.251	0.473	0.127	0.472	6.3

Table IV—Effect of Tablet Hardness on Release Rate of 100-mg. Sodium Pentobarbital Tablets

Hardness, Strong-Cobb Units	$k \times 10^4$, g. cm.$^{-2}$ sec.$^{-1/2}$	ϵ_0	ϵ	τ
13.1	5.21	0.254	0.554	11.1
16.7	5.57	0.235	0.541	10.2
20.0	5.56	0.219	0.536	10.8
23.2	5.30	0.188	0.515	12.6
26.2	5.38	0.169	0.505	12.7

Table V—Effect of 70° Heating and Acetone Vapor Exposure on the Release Rate

Tablet	Treatment	$k \times 10^4$, g. cm.$^{-2}$ sec.$^{-1/2}$	ϵ	τ
Sodium pentobarbital, 100 mg. (hardness = 13.1)	None	5.21	0.554	11.1
	70°	5.87	0.554	8.77
	Acetone	5.50	0.557	10.2
Methapyrilene HCl, 100 mg. (hardness = 14.4)	None	5.10	0.502	13.3
	70°	5.95	0.524	9.29
	Acetone	5.57	0.499	11.1

where ϵ_0 is the initial porosity and k is the specific volume of the drug, indicates that porosity is proportional to the drug concentration A when ϵ_0 is negligible. Assuming this along with relative constancy in the tortuosity, Eq. 2 indicates that k would be proportional to A. Calculations show that increasing the amount of drug per tablet from 50 to 100 mg. with sodium pentobarbital, methapyrilene hydrochloride, and ephedrine hydrochloride and from 30 to 100 mg. in the case of dextromethorphan hydrobromide should increase the k by factors of 2.23, 2.22, 2.20, and 3.72, respectively. The increases obtained experimentally were 2.39, 2.67, 2.01, and 3.53, respectively. The small differences between the calculated and observed values can be attributed to a significant ϵ_0 and tortuosity changes accompanying changes in drug concentrations.

The porosities and tortuosities for these tablets are shown in Table III. The porosity values were calculated from Eq. 5. For these tablets, ϵ_0 was obtained by dividing the calculated volume of air by the tablet volume. The tablet volume was calculated from its dimension, while the air volume was obtained by subtracting the calculated volumes of drug and plastic (density = 1.275 g./ml.) from the volume of the tablet.

The tortuosity values were calculated from Eq. 3. Generally, the tortuosity was found to decrease with increasing concentration of drug in the tablet. This decrease, however, varied from practically zero in the case of sodium pentobarbital to a factor of 2 when dextromethorphan hydrobromide concentration was increased 3.33 times. The higher tortuosities obtained with ephedrine hydrochloride *versus* the other drugs may be attributed to its larger particle-size distribution (177–420 μ *versus* less than 149 μ). Desai *et al.* (2) observed that larger particle sizes of solute exhibit considerably higher tortuosities than smaller particle sizes.

The hardness of the tablet was found to have little effect on the leach rate. When a sodium pentobarbital–plastic blend was compressed to five different hardnesses, from 13 to 26 Strong-Cobb units, the release-rate constants varied randomly from 5.21 to 5.57 $\times$ 10^{-4} g. cm.$^{-2}$ sec.$^{-1/2}$. The results are shown in Table IV. Similar results were obtained in a study using methapyrilene hydrochloride tablets with varying hardnesses. Although a lower compression force increases the tablet porosity, it also decreases the tortuosity. Thus, it appears that the changes in the release-rate constant caused by these two parameters are compensating to some extent.

During release-rate testing, tablet breakage occurred with all 100-mg. sodium pentobarbital tablets before completion of drug release. This resulted in positive deviation from Eq. 2. This breakage came after 50% release at the higher hardnesses and 25% release at hardness 13. No such problem was encountered with the other three drugs, where tablets with hardnesses as low as 14.4 remained intact throughout the leach.

Heating the tablets at 70° for 24 hr. was found to overcome the rapid sodium pentobarbital tablet breakage observed in the softest tablet. However, such treatment increased the release-rate constant substantially. Likewise, this heating condition increased the methapyrilene hydrochloride release-rate constant from the softest tablet to the same extent. These results are given in Table V. It appears that the rate increase caused by heating can be attributed to a marked decrease in tortuosity.

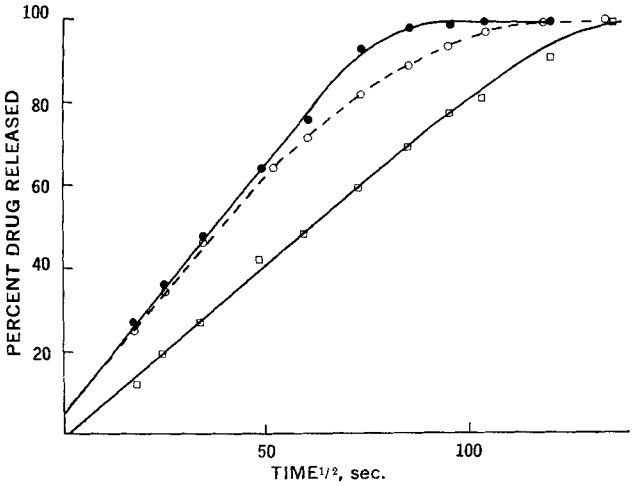

Figure 3—*Drug release from tablets in which all surfaces are exposed. Key:* ●, *sodium pentobarbital, 100 mg.;* ○, *methapyrilene HCl, 100 mg.; and* □, *ephedrine HCl, 50 mg.*

Exposure of sodium pentobarbital tablets to acetone vapor (9) was also found to overcome the tablet breakage problem. In this treatment the tablets were kept in a desiccator under 200 mm. Hg acetone vapor for 2 hr. at 33°. The residual acetone was then eliminated from the tablets at 50°. The results of such treatment are given in Table V. As in the case of the 70° heating, the tortuosity decreased and the release-rate constant increased, but to a lesser extent.

The leach rate of drug from the total tablet surface was also studied. Higuchi's evaluation (1) of drug release from a spherical pellet would indicate that initial release should follow the square root of time relationship, but that significant deviation would occur toward the end of the leach. Figure 3 shows the results obtained from three tablets when the percent of drug released was plotted against the square root of time. In all cases there appeared to be little deviation from linearity during the release of the first 75% of the drug. Apparently, the flat cylindrical shape of the tablet gives more prolonged linearity than a sphere.

SUMMARY AND CONCLUSIONS

The results of this study show that drug release from tablets compressed directly from mixtures of drug and methyl acrylate–methyl methacrylate copolymer follow the time dependency suggested by Higuchi's theoretical relationship (1). This was observed to be the case with all four drugs tested. The release rate for any given tablet may be expressed by a constant k, equal to the slope of the Q *versus* $t^{1/2}$ plot, or by a fractional life term. The magnitude of this release-rate constant is dependent on the matrix porosity and tortuosity as well as the solubility, diffusivity, and concentration of the drug.

Deviation from linearity occurs only when tablets break open before completion of drug release. This breakage may be prevented by heat or exposure to acetone vapor.

Tablet hardness appears to have little effect on the release-rate constant. This can be attributed to compensating effects from tortuosity and porosity.

Drug release from tablets in which all surfaces are exposed shows little deviation from the Q *versus* $t^{1/2}$ linearity during the first 75% of the release. Greater deviations in the later stages can be attributed to geometric effects.

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Drug Release from Methyl Acrylate–Methyl Methacrylate Copolymer Matrix II: Control of Release Rate by Exposure to Acetone Vapor

B. FARHADIEH, S. BORODKIN, and J. D. BUDDENHAGEN

Abstract □ The rate of drug release from tablets made with a methyl acrylate–methyl methacrylate copolymer matrix containing dispersed solid drug can be decreased by exposure to acetone vapor. As in the case of untreated tablets, the quantity of drug released per unit surface area is proportional to the square root of time. An apparatus was developed to study the treatment variables. The extent of reduction in release rate is dependent on the amount of acetone absorbed. This reduction is primarily due to an increase in the tortuosity of the matrix. Generally, the release-rate constant is lowered by decreasing the temperature or increasing the acetone vapor pressure. Thus, exposure of tablets to acetone vapor under controlled conditions is an added means for regulating the drug-release rate.

Keyphrases □ Drug release—methyl acrylate–methyl methacrylate matrix □ Acetone vapor effect—drug release rates, copolymer matrix □ Temperature, pressure effects—acetone absorption, copolymer matrix □ Tortuosity, copolymer matrix—drug release-rate relationship

In the first paper of this series (1), it was shown that the release of drug from a methyl acrylate–methyl methacrylate copolymer matrix follows the relationship proposed by Higuchi (2), in which the amount of drug released per unit surface area is linear with the square root of time. Desai *et al.* (3–6) studied this theoretical relationship extensively, using polyethylene and polyvinyl chloride as plastic matrixes. Endicott (7) demonstrated that the release of drug from tablets compressed with methyl acrylate–methyl methacrylate copolymer can be prolonged by exposure to acetone vapor.

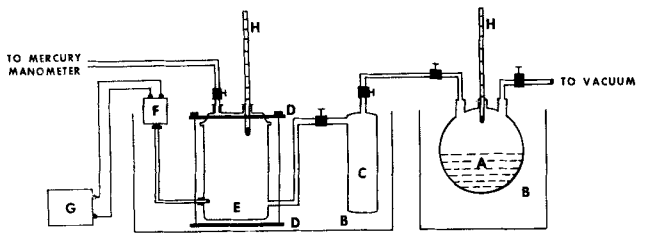

Figure 1—*Acetone vapor treatment apparatus. Key: A, acetone reservoir; B, constant-temperature water bath; C, acetone vapor trap; D, holder plastic plate; E, acetone treatment chamber; F, pressure sensor; G, amplifier and recorder; and H, thermometer.*

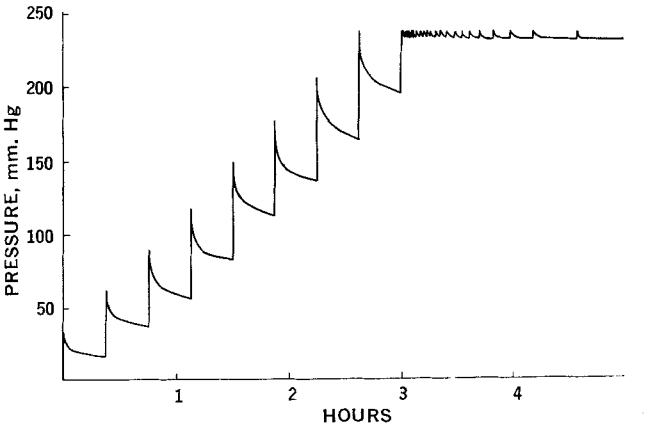

Figure 2—*Recorder tracing of acetone vapor pressure* versus *time at 29°.*

The purposes of this study were: (*a*) to determine whether drug release from tablets treated with acetone vapor would continue to follow the square root of time relationship, and (*b*) to use this relationship to evaluate the effect of different exposure conditions. The tablets used were primarily those made with sodium pentobarbital, although other drugs were examined. The principal acetone exposure variables were temperature and acetone vapor pressure.

EXPERIMENTAL

Chemicals—The plastic used in all tablets was a powdered methyl acrylate–methyl methacrylate copolymer (8).[1] This polymer is insoluble and inert in aqueous media at all pH values. The acetone was reagent grade. All drugs were USP or NF. The densities, diffusion constants, solubilities, and particle-size ranges of these drugs were determined previously (1).

Tablet Compression—All tablets were compressed on a Manesty

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Keyphrases □ Drug release—methyl acrylate–methyl methacrylate matrix □ Acetone vapor effect—drug release rates, copolymer matrix □ Temperature, pressure effects—acetone absorption, copolymer matrix □ Tortuosity, copolymer matrix—drug release-rate relationship

In the first paper of this series (1), it was shown that the release of drug from a methyl acrylate–methyl methacrylate copolymer matrix follows the relationship proposed by Higuchi (2), in which the amount of drug released per unit surface area is linear with the square root of time. Desai *et al.* (3–6) studied this theoretical relationship extensively, using polyethylene and polyvinyl chloride as plastic matrixes. Endicott (7) demonstrated that the release of drug from tablets compressed with methyl acrylate–methyl methacrylate copolymer can be prolonged by exposure to acetone vapor.

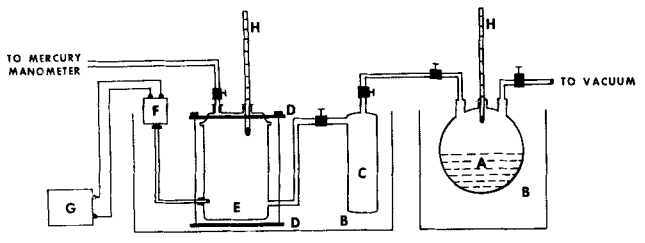

Figure 1—*Acetone vapor treatment apparatus. Key: A, acetone reservoir; B, constant-temperature water bath; C, acetone vapor trap; D, holder plastic plate; E, acetone treatment chamber; F, pressure sensor; G, amplifier and recorder; and H, thermometer.*

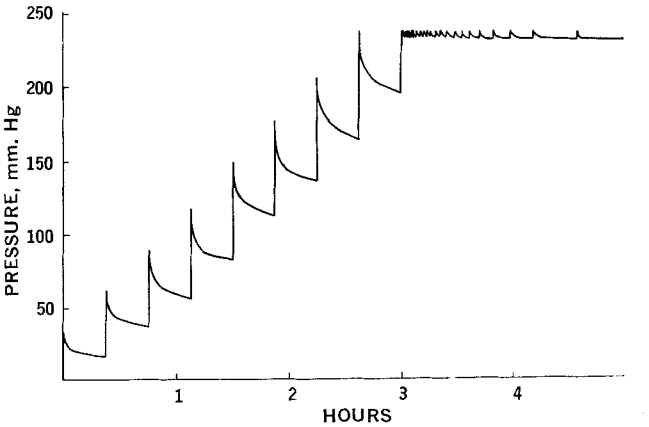

Figure 2—*Recorder tracing of acetone vapor pressure* versus *time at 29°.*

The purposes of this study were: (*a*) to determine whether drug release from tablets treated with acetone vapor would continue to follow the square root of time relationship, and (*b*) to use this relationship to evaluate the effect of different exposure conditions. The tablets used were primarily those made with sodium pentobarbital, although other drugs were examined. The principal acetone exposure variables were temperature and acetone vapor pressure.

EXPERIMENTAL

Chemicals—The plastic used in all tablets was a powdered methyl acrylate–methyl methacrylate copolymer (8).[1] This polymer is insoluble and inert in aqueous media at all pH values. The acetone was reagent grade. All drugs were USP or NF. The densities, diffusion constants, solubilities, and particle-size ranges of these drugs were determined previously (1).

Tablet Compression—All tablets were compressed on a Manesty

[1] Rohm & Haas Co., Philadelphia, Pa.

Table I—Effect of Acetone Vapor Exposure on Release Rate of Drug from Tablets Treated at 28°

Drug	Concentration, mg./Tablet	Acetone Vapor Pressure, mm. Hg	Acetone Absorbed, mg./Tablet	ϵ	τ	$k \times 10^4$, g. cm.$^{-2}$ sec.$^{-1/2}$	$t_{1/2}$, hr.
Sodium pentobarbital	100	Untreated	0	0.575	9.24	5.38	3.38
Sodium pentobarbital	100	244	36.2	0.538	82.2	1.96	25.4
Methapyrilene HCl	100	Untreated	0	0.487	12.2	5.48	3.25
Methapyrilene HCl	100	227	18.1	0.463	37.7	3.08	10.3
Ephedrine HCl	50	Untreated	0	0.375	36.2	1.46	11.5
Ephedrine HCl	50	170	17.1	0.388	46.7	1.28	14.9
Dextromethorphan HBr	30	Untreated	0	0.376	14.2	0.606	23.9
Dextromethorphan HBr	30	172	19.4	0.367	57.6	0.299	98.4

layer press equipped with 1.04-cm. ($^{13}/_{32}$-in.) flat surface punches and dies. Except where stated, tablets used weighed 242 mg. and had an average hardness of 12.0 Strong-Cobb units.

Acetone Vapor Treatment Apparatus—The basic apparatus used consists of an acetone vapor generator, a trap, a treatment chamber, a pressure-sensing device, and a recorder (Fig. 1). The acetone vapor generator consists of a round-bottom, three-necked flask equipped with a thermometer, a stopcock to a vacuum line, and a second stopcock leading to the treatment chamber. The acetone vapor is generated by placing 200 ml. of acetone in the flask, submerging it into a constant-temperature bath maintained about 6° above the treatment temperature, and flushing the air from the flask by drawing a vacuum several times. The acetone vapor is then introduced through the trap into the treatment chamber, both immersed in a constant-temperature bath. The treatment chamber is a resin kettle flask with a removable top and has openings for a thermometer, a mercury manometer, a stopcock to the trap, and connection to the pressure sensor. This sensor, a Statham[2] temperature-compensated absolute pressure transducer, is immersed in the constant-temperature bath and connected through an Ellis[3] bridge amplifier to a model SR Sargent[4] recorder.

Acetone Treatment Procedure—The sensor response is calibrated into pressure from 0–500 mm. Hg, at the temperature of the run by introducing increments of air into the totally evacuated treatment chamber and reading the pressure on the mercury manometer. The treatment chamber is removed from the bath and opened, and 22.0 ± 0.1 g. of tablets is introduced. The chamber is then returned to the bath without the connection to the mercury manometer, and the system is evacuated with a vacuum pump. Acetone vapor is introduced slowly in increments until the desired vapor pressure is attained. Additional portions of acetone are then injected to maintain the vapor pressure within 3 mm. Hg of the desired level. These injections are continued until the rate of acetone absorption by the tablets becomes negligible. At the end of the run the tablets are removed from the treatment chamber, and the acetone is removed from the tablets by evaporation.

Release-Rate Studies—All release-rate studies were on a single surface, using the method described in the first paper of this series (1).

RESULTS AND DISCUSSION

Acetone Absorption—Figure 2 shows a typical recorder tracing of acetone vapor pressure *versus* time. The pressure decreases observed at each interval were due to acetone absorption by the tablets. That these truly reflected tablet absorption was demonstrated by observing no pressure changes in a run in which the tablets were omitted. The amount of acetone absorbed by the tablets at each increment was calculated from the recorded pressure drop. By assuming ideality, the pressure decrease was converted to milligrams of acetone absorbed per tablet from the following equation:

$$W = \frac{1000\,PVM}{760\,RTN} \qquad \text{(Eq. 1)}$$

where W = milligrams acetone absorbed per tablet, P = vapor pressure decrease expressed in mm. Hg, V = volume of treatment chamber (1.46 l.), M = molecular weight (58.08), R = gas law constant (0.082 l. atm. deg.$^{-1}$ mole^{-1}), T = absolute temperature, and N = number of tablets.

The amount of acetone absorbed was dependent on the conditions used. In the cases studied, this varied from 16 to 45 mg./tablet. Weight loss studies with a Cahn Electrobalance[5] on tablets immediately after completion of treatment confirmed the accuracy of the acetone weight absorption.

Drug-Release Rate—Except for very mild conditions, exposure to acetone vapor was found to decrease the drug-release rate from the

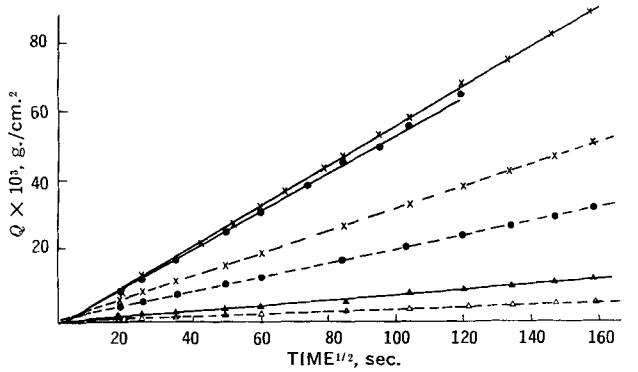

Figure 3—*Comparison of release rates between acetone vapor-treated and untreated tablets. Key: -x—x-, sodium pentobarbital untreated; -●—●-, methapyrilene HCl untreated; -▲—▲-, dextromethorphan HBr untreated; -x- - -x-, sodium pentobarbital treated; -●- - -●-, methapyrilene HCl treated; and -△- - -△-, dextromethorphan HBr treated.*

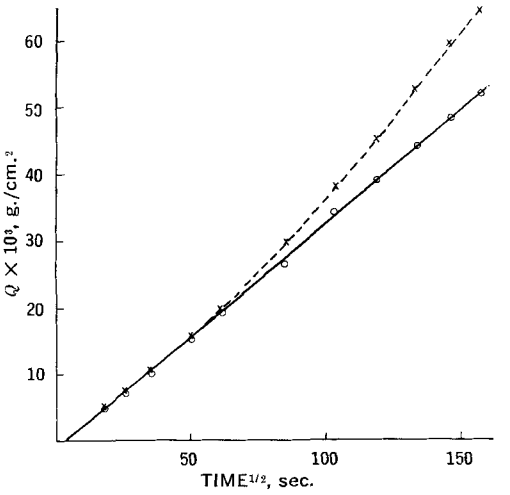

Figure 4—*Effect of hardness on the release of sodium pentobarbital from tablets treated at 30° and 240 mm. Hg. Key: ×, hardness > 27; and ○, hardness = 13.0.*

[2] Statham Instruments, Inc., Los Angeles, Calif.
[3] Ellis Associates, Pelham, N. Y.
[4] E. H. Sargent and Co., Chicago, Ill.
[5] Cahn Instrument Co., Paramount, Calif.

Table II—Effect of Temperature in the Acetone Vapor Treatment of 100-mg. Sodium Pentobarbital Tablets at Two Different Vapor Pressures

Acetone Vapor Pressure, mm. Hg	Temperature	Acetone Absorbed, mg./Tablet	ϵ	τ	$k \times 10^4$, g. cm.$^{-2}$ sec.$^{-1/2}$	$t_{1/2}$, hr.
Untreated tablets		0	0.575	9.24	5.38	3.38
217	25°	33.9	0.516	195.4	1.27	60.6
217	28°	31.1	0.536	50.6	2.48	15.9
217	31°	26.1	0.551	18.6	4.01	6.08
217	34°	18.4	0.560	12.2	4.79	4.26
217	37°	16.2	0.567	11.5	4.93	4.02
244	28°	36.2	0.538	82.2	1.96	25.4
244	31°	29.3	0.540	36.4	2.91	11.5
244	34°	22.5	0.551	16.6	4.19	5.57
244	37°	19.0	0.559	13.3	4.64	4.54

tablets. As with untreated tablets, drug release continued to follow Higuchi's (2) theoretical relationship:

$$Q = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_S)C_S t\right]^{1/2} \qquad \text{(Eq. 2)}$$

where Q = amount of drug released after time t per unit exposed area, D = diffusivity of the drug in the permeating fluid, τ = tortuosity, A = amount of drug present in the matrix per unit volume, C_S = solubility of the drug in the permeating fluid, and ϵ = porosity of the matrix. This was found to be the case with all drugs tested. Plots of Q *versus* $t^{1/2}$ for both acetone-exposed and untreated tablets are shown in Fig. 3. The graph demonstrates both the continued linearity and the reduced release rate resulting from acetone vapor treatment.

The calculated values for porosity, tortuosity, release-rate constant, and half-life for both acetone-treated and untreated tablets are compared in Table I. The method of calculation was previously described (1). It is apparent from an examination of the data that reduction in the release-rate constant is primarily due to increased tortuosity of the matrix.

As in the case of untreated tablets, the release-rate constants after acetone treatment did not show significant change with hardness. However, when sodium pentobarbital tablets with hardness greater than 22 Strong-Cobb units were treated, deviation from linearity was observed. This deviation increased with hardness. Figure 4 compares the release rates from tablets with hardnesses of 13 and 27 treated under the same conditions. Although the initial rates were identical, the release from the harder tablet soon began to approach that of untreated tablets. Apparently, with high hardnesses, acetone vapor

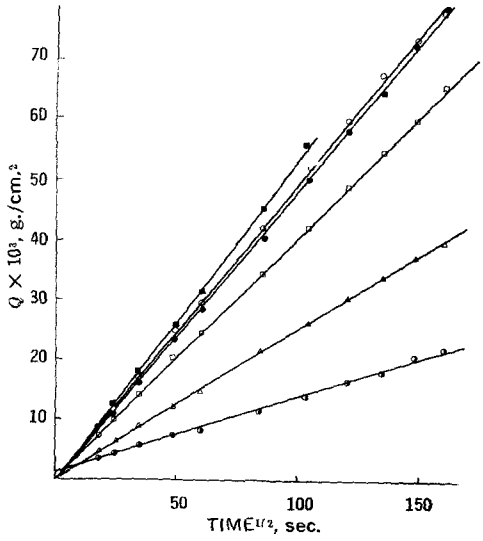

Figure 5—*Effect of temperature on the release rate from 100-mg. sodium pentobarbital tablets at 217 mm. Hg. Key:* ■, *untreated;* ○, *37°;* ●, *34°;* □, *31°;* △, *28°; and* ◑, *25°.*

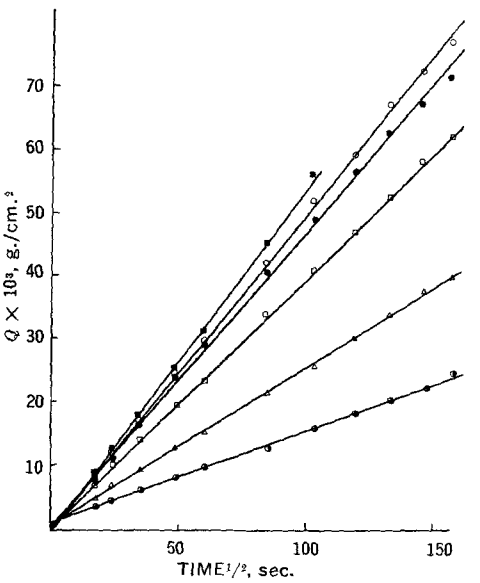

Figure 6—*Effect of acetone vapor pressure on the release rate from 100-mg. sodium pentobarbital tablets treated at 37°. Key:* ■, *untreated;* ○, *217 mm. Hg;* ●, *244 mm. Hg;* □, *267 mm. Hg;* △, *307 mm. Hg; and* ◑, *347 mm. Hg.*

penetration is reduced, resulting in a decrease in tortuosity with increasing distance from the tablet surface.

Effect of Treatment Temperature and Pressure—A series of 15 experiments was run with sodium pentobarbital tablets to study the effects of treatment temperature and acetone vapor pressure on the drug release rate.

Figure 5 and Table II summarize the effects of treatment temperature. The data show that reducing the temperature increases the acetone absorption and lowers the release-rate constant. The maximum rate reduction was achieved when condensation temperature was approached. Table II shows that rate reduction can be attributed primarily to increased matrix tortuosity. However, a decrease in porosity accompanies the treatment temperature decrease.

The effects of acetone vapor pressure on sodium pentobarbital tablets at three temperatures are summarized in Table III. Figure 6 shows the slopes obtained at 37°. The results demonstrate that increasing the acetone vapor pressure increases the acetone absorption and reduces the release-rate constant. As in the case of temperature, the maximum reduction is obtained as condensation pressure is approached. Again, higher tortuosity and lower porosity accompany the increased vapor pressure, with the tortuosity having the major effect on drug release.

An analysis of the data reveals that the extent of release-rate reduction by temperature and pressure effects is governed solely by how far away the selected treatment is from condensation condi-

Table III—Effect of Acetone Vapor Pressure on the Release Rate of Drug from 100-mg. Sodium Pentobarbital Tablets at Three Different Temperatures

Temperature	Acetone Vapor Pressure, mm. Hg	Acetone Absorbed, mg./Tablet	ϵ	τ	$k \times 10^4$, g. cm.$^{-2}$ sec.$^{-1/2}$	$t_{1/2}$, hr.
Untreated tablets		0	0.575	9.24	5.38	3.38
37°	217	16.2	0.567	11.5	4.93	4.02
37°	244	19.0	0.559	13.3	4.64	4.54
37°	267	25.3	0.559	19.7	3.87	6.52
37°	307	30.7	0.535	52.2	2.44	16.4
37°	347	44.9	0.530	154.9	1.43	47.8
34°	217	18.4	0.560	12.2	4.79	4.26
34°	244	22.5	0.551	16.6	4.19	5.57
34°	267	27.9	0.539	43.2	2.67	13.7
34°	307	39.7	0.534	198.2	1.26	61.5
31°	217	26.1	0.551	18.6	4.01	6.08
31°	244	29.3	0.540	36.4	2.91	11.5
31°	267	32.8	0.528	86.2	1.93	26.2

tions. Thus, the desired reduction of k could be achieved at any temperature by adjusting the appropriate acetone vapor pressure. Conversely, at any vapor pressure the optimum release rate could be attained by manipulation of the treatment temperature. The extent of rate reduction would be limited only by condensation.

SUMMARY AND CONCLUSIONS

An apparatus was developed to study the variables involved in the acetone vapor treatment of tablets made from a methyl acrylate–methyl methacrylate copolymer matrix containing dispersed solid drug. This apparatus allows close control of temperature and acetone vapor pressure. It also allows monitoring of the amount of acetone absorbed throughout the run.

Release from the acetone-treated tablets followed the Higuchi relationship in which the quantity of drug released per unit surface area was proportional to the square root of time. Except under very mild conditions, the drug release rate was reduced. The extent of reduction was related to the amount of acetone absorbed. Generally, increasing the vapor pressure or decreasing the treatment temperature increased acetone absorption. Thus, by manipulation and control of these two variables, the drug release rate may be regulated in a reproducible fashion. The decrease in release rate can be explained primarily on the basis of an increase in matrix tortuosity.

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Drug Biotransformation Interactions in Man III: Acetaminophen and Salicylamide

GERHARD LEVY and HIDEO YAMADA*

Abstract □ Acetaminophen (1 and 2 g.), salicylamide (1 g.), and 1 g. acetaminophen followed 1.5 hr. later by 1 or 2 g. salicylamide were administered in aqueous solutions to healthy human subjects. Urine was collected every 15–30 min. for the first 4–5 hr. and then at longer intervals. The urine samples were analyzed for acetaminophen, acetaminophen sulfate, acetaminophen glucuronide, salicylamide, salicylamide sulfate, and salicylamide glucuronide. The excretion rates of acetaminophen sulfate and acetaminophen glucuronide decreased immediately after administration of salicylamide. Acetaminophen decreased the formation of salicylamide sulfate. Coadministration of L-cysteine (a source of sulfate) prevented this effect and also increased the excretion of acetaminophen sulfate. The results indicate a competitive inhibition in the formation of acetaminophen sulfate and salicylamide sulfate and suggest also a competitive inhibition in the formation of acetaminophen glucuronide and salicylamide glucuronide.

Keyphrases □ Acetaminophen, salicylamide—biotransformation interaction, human □ Biotransformation interaction—acetaminophen, salicylamide □ Metabolites, acetaminophen, salicylamide—isolated, identified □ Colorimetric analysis—spectrophotometer

Previous reports from this laboratory have shown that the formation of salicylamide sulfate from salicylamide is easily saturated in man (1) and that there occurs a mutual inhibition of glucuronide formation by salicylamide and salicylate when these drugs are administered concomitantly (2). Since acetaminophen, like salicylamide, is eliminated primarily by glucuronide and sulfate formation, and since these mild analgesic and antipyretic drugs are often taken together, the effect of each on the biotransformation of the other has been investigated.

EXPERIMENTAL

Healthy, male, ambulatory human subjects received orally 1 or 2 g. acetaminophen in one test, 1 g. salicylamide in the second test, and 1 g. acetaminophen followed 1.5 hr. later by 1 or 2 g. salicylamide in the third test. Intervals between tests were at least 1 week. The drugs were administered in aqueous solution in the morning on an empty stomach. Food was withheld for at least 2 hr. thereafter. Urine was collected every 15–30 min. for the first 4–5 hr. and then at longer intervals for at least 24 hr. About 50 ml. of water was ingested after each urine collection to assure adequate urine output.

Determination of Acetaminophen and Its Metabolites in Urine—Free acetaminophen was extracted from urine by a method similar to that of Brodie and Axelrod (3). Acetaminophen glucuronide and acetaminophen sulfate were hydrolyzed enzymatically and then extracted as acetaminophen. The concentration of this drug was determined by a modification of the colorimetric method of Welch and Conney (4).

Specifically, free acetaminophen was extracted by adding 4 g. sodium chloride and 15 ml. ether[1] to 10 ml. urine. This mixture was shaken mechanically for 15 min. and centrifuged to separate the phases. Ten milliliters of the ether phase was extracted with 3 ml. 0.1 *N* sodium hydroxide solution by shaking 5 min. and centrifuging. One-half milliliter of the aqueous phase was pipeted into a graduated test tube, and 0.5 ml. 4 *N* hydrochloric acid was added. The test tube was then placed in a boiling water bath for 30 min. The solution was then cooled to room temperature, and 10 ml. phenol–hypobromite mixture (4) and sufficient distilled water to yield a total volume of 11 ml. were added. Forty minutes later, the absorbance of the solution at 625 nm. was determined spectrophotometrically. Reagent blank determinations were made by using water instead of urine in the procedure. A urine sample containing 2 mg. acetaminophen/100 ml. will yield a reagent phase with an absorbance of about 0.21/cm. pathlength by this method.

[1] Ether for Anesthesia, Merck, purified by successive washings with 1 *N* NaOH, 1 *N* HCl, and three washings with distilled water.

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[1] Ether for Anesthesia, Merck, purified by successive washings with 1 *N* NaOH, 1 *N* HCl, and three washings with distilled water.

Table I—Verification of Assay Method for Acetaminophen and Its Metabolites and Salicylamide and Its Metabolites in Urine Samples Containing Both Groups of Substances

Metabolite[a]	Sample[b]	Concentrations, as mg. % APAP or SAM Equivalent: A — Urine after Acetaminophen Administration	B — Urine after Salicylamide Administration	Equal Parts, A and B: Assay	Equal Parts, A and B: Theoretical
APAP	I	1.6	0.0	0.8	0.8
	II	1.8	0.0	0.9	0.9
APAPG	I	10.4	0.0	5.0	5.2
	II	40.0	0.0	21.1	20.0
APAPS	I	9.2	0.0	5.0	4.6
	II	22.8	0.0	10.0	11.4
SAMG	I	0.0	8.6	4.3	4.3
	II	0.0	49.2	24.6	24.6
SAMS	I	0.0	10.2	5.1	5.1
	II	1.0	12.8	6.9	6.9

[a] Abbreviations: APAP, acetaminophen; APAPG, acetaminophen glucuronide; APAPS, acetaminophen sulfate; SAMG, salicylamide glucuronide; and SAMS, salicylamide sulfate. [b] Roman numerals refer to a specific urine sample obtained after either acetaminophen or salicylamide administration.

Table II—Recovery of Acetaminophen and Its Metabolites in Man[a]

Subject	Sex	Age, yr.	Weight, kg.	Dose, g.	Urinary Excretion Products,[b] % of Dose: APAP	APAPG	APAPS
Y	M	36	62	1	3.2	57.9	31.1
Y	M	36	62	2	3.4	65.3	29.3
J	M	26	85	1	3.1	62.5	20.5
K	M	24	66	1	3.7	63.7	25.8
K	M	24	66	2	3.5	63.4	24.1

[a] Acetaminophen in solution administered orally. [b] Abbreviations: APAP, acetaminophen; APAPG, acetaminophen glucuronide; and APAPS, acetaminophen sulfate.

Acetaminophen glucuronide was assayed as free acetaminophen after incubating 2 ml. urine with 1 ml. (5000 units) beef liver β-glucuronidase[2] and 3 ml. distilled water at 37° for 4 hr. Free acetaminophen was then extracted and assayed as described in the preceding paragraphs. Control experiments with pH 5.0 acetate buffer (0.2 *M*) instead of beef liver β-glucuronidase showed that nonenzymatic hydrolysis of acetaminophen conjugates was negligible (<1%) under the experimental conditions. Acetaminophen glucuronide concentrations were determined by subtracting the acetaminophen concentration obtained in the assay for free acetaminophen from the acetaminophen concentration obtained in the present assay.

Acetaminophen sulfate was determined as free acetaminophen after hydrolysis with sulfatase. Glusulase[3] containing 50,000 units of sulfatase and 100,000 units β-glucuronidase/ml., was diluted 10-fold with pH 5.0 acetate buffer (0.2 *M*). One milliliter of this solution and 3 ml. distilled water were added to 2 ml. urine, and this mixture was incubated at 37° for 4 hr. Acetaminophen was then extracted and assayed as described previously. Acetaminophen sulfate was calculated as the difference in acetaminophen concentration obtained in this assay and in that for acetaminophen glucuronide. Similar results were obtained with another enzyme preparation,[4] but this required a longer (>10 hr.) period of incubation.

Determination of Acetaminophen and Its Metabolites in the Presence of Salicylamide Metabolites—Gentisamide, a metabolite of salicylamide, interfered in the acetaminophen assay and had to be removed. Urine samples containing acetaminophen and salicylamide metabolites were adjusted to pH 9.0 with borate buffer, saturated with sodium chloride, and extracted with ether. Acetaminophen, but not gentisamide, is thereby extracted. Specifically, 2 g. sodium chloride, 5 ml. pH 9.0 borate buffer (0.2 *M*) saturated with sodium chloride, and 15 ml. washed ether were added to 5 ml. urine. The mixture was shaken for 15 min. and centrifuged. Ten milliliters of the organic phase was extracted with 1 ml. 0.1 *N* sodium hydroxide, and the subsequent procedures were as described for acetaminophen alone.

Acetaminophen glucuronide was assayed as acetaminophen after incubating 1 ml. urine with 2 ml. (10,000 units) β-glucuronidase and 2 ml. distilled water at 37° for 16 hr. To 2 ml. of this solution were added 1 g. sodium chloride, 8 ml. borate buffer saturated with sodium chloride, and 15 ml. washed ether. Acetaminophen was then extracted and assayed as described previously.

Acetaminophen sulfate was assayed as acetaminophen after incubating 1 ml. urine with 2 ml. of 10-fold diluted glusulase and 2 ml. distilled water at 37° for 16 hr. To 2 ml. of this solution were added 8 ml. borate buffer saturated with sodium chloride, 1 g. sodium chloride, and 20 ml. washed ether. Acetaminophen was then extracted and assayed as described previously.

Determination of Salicylamide and Its Metabolites—These determinations were carried out as described by Levy and Matsuzawa (1).

Individual urine samples were diluted with distilled water where necessary. All assays were carried out at least in duplicate. The sum of the assay results obtained from individual samples in a given experiment was compared to the results obtained by assaying a composite sample prepared by combining aliquots of the individual urine samples. These agreed within 5% or less. All samples were stored in a freezer until analyzed. Urine samples obtained from nonmedicated subjects produced no measurable blank values in the acetaminophen assay (<1 mg. per day).

RESULTS

The assay methods for acetaminophen, salicylamide, and their respective metabolites were checked for possible interference by the other substances. Thus, urine samples obtained after either acetaminophen or salicylamide administration were assayed, combined in equal volumes, and reassayed. This revealed an interference by gentisamide, a minor metabolite of salicylamide, in the acetaminophen assay. The assay procedure was therefore modified by adjusting the pH for extraction to 9.0, thereby separating the interfering substance from acetaminophen. Table I shows a representative example of analytical results which verify the assay methods employed in this study. The recovery of acetaminophen and its metabolites after oral administration of 1- and 2-g. doses of acetaminophen is summarized in Table II. The average recovery of drug in all forms was 92%, mostly as acetaminophen glucuronide and acetaminophen sulfate. Only about 3.4% of the dose was recovered as free acetaminophen.

[2] Ketodase, Warner-Chilcott, Morris Plains, N. J.
[3] Endo Laboratories, Garden City, N. Y.
[4] Mylase P, Nutritional Biochemicals Corp., Cleveland, Ohio.

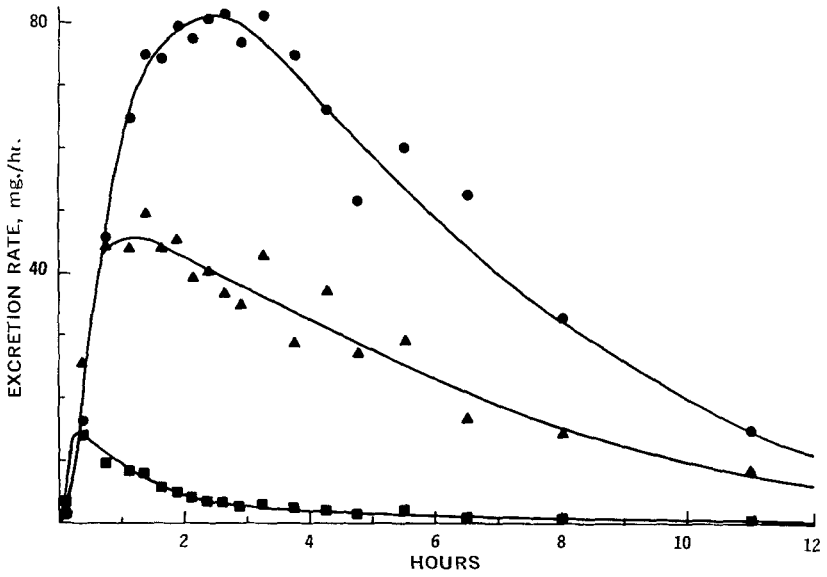

Figure 1—*Excretion rate of acetaminophen (■), acetaminophen glucuronide (●), and acetaminophen sulfate (▲) following oral administration of 1 g. acetaminophen to Subject Y. The excretion rate in this figure and in the subsequent figures is expressed in mg./hr. acetaminophen equivalent.*

Figure 1 shows the time course of urinary excretion of acetaminophen and its metabolites by Subject Y after oral administration of 1 g. of the drug. A semilogarithmic plot of acetaminophen excretion rate *versus* time could be resolved into two postabsorption exponential phases, suggesting an initial distributive phase (Fig. 2). Administration of 2 g. acetaminophen to the same subject resulted in the excretion of acetaminophen sulfate at an essentially constant rate for about 5 hr., this rate being only slightly higher than the peak excretion rate of acetaminophen sulfate after the 1-g. dose (Fig. 3). On the other hand, the maximum excretion rate of acetaminophen glucuronide after 2 g. acetaminophen (200 mg. acetaminophen equivalent per hour) was more than twice as high as the maximum rate after the 1-g. dose (80 mg./hr.). Subject J excreted acetaminophen sulfate at a much lower rate than Subject Y and showed an excretion rate plateau of over 4 hr. even with a 1-g. dose of acetaminophen (Fig. 4). No such plateau in the excretion of acetaminophen sulfate was apparent after administration of 2 g. acetaminophen to Subject K (Fig. 5).

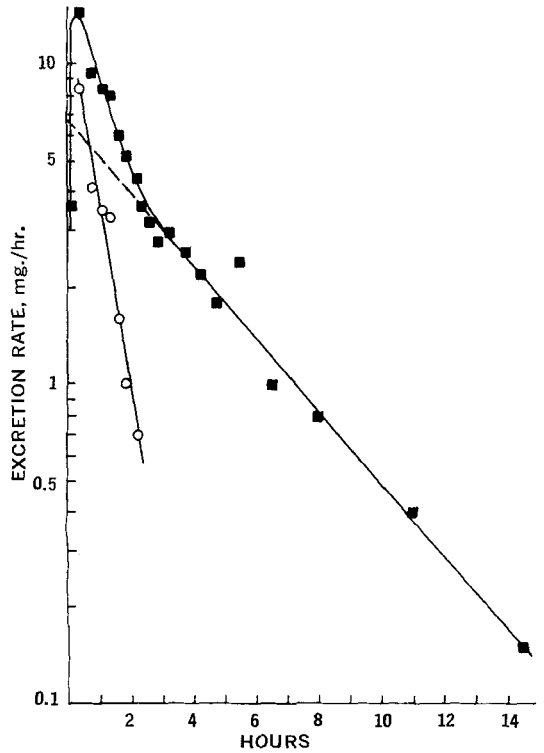

Figure 2—*Semilogarithmic plot of excretion rate of acetaminophen (■) following oral administration of 1 g. acetaminophen to Subject Y. The open circles represent the differences between the experimental values and the hypothetical values obtained by back-extrapolation of the terminal linear segment.*

One gram salicylamide, given 1.5 hr. after 1 g. acetaminophen, decreased the urinary excretion of acetaminophen glucuronide and acetaminophen sulfate for some time (Fig. 6). A more pronounced effect was obtained when the dose of salicylamide was increased to 2 g. (Fig. 7). The excretion rate of acetaminophen sulfate during the 1.5 hr. before salicylamide administration in these experiments was in good agreement with similar data obtained during the control experiments. This is particularly significant in the case of Subject J, whose excretion rate of acetaminophen sulfate was consistently low (Fig. 8). Concomitant administration of L-cysteine increased the excretion rate of acetaminophen sulfate and partly counteracted the effect of salicylamide (Fig. 9).

Acetaminophen decreased the formation of salicylamide sulfate from salicylamide, resulting in a somewhat increased formation of salicylamide glucuronide (Fig. 10, Table III). Concomitant administration of L-cysteine with salicylamide increased salicylamide sulfate formation and prevented the inhibition of salicylamide sulfate formation by acetaminophen (Table IV).

Table III—Effect of Acetaminophen on the Metabolic Fate of Salicylamide in Man[a]

	Recovery of Salicylamide Metabolites,[b] % of Dose			
	SAMG		SAMS	
Subject	Control	With APAP	Control	With APAP
Y	50.9	63.6	33.4	27.2
J	45.3	56.3	36.5	28.0
K	48.0	53.8	41.3	26.7

[a] One gram salicylamide administered orally alone or 1.5 hr. following 1 g. acetaminophen. [b] Abbreviations: SAMG, salicylamide glucuronide; SAMS, salicylamide sulfate; and APAP, acetaminophen.

Table IV—Effect of L-Cysteine on the Biotransformation Interaction of Acetaminophen and Salicylamide[a]

	Percent of Dose Recovered in Urine			
Metabolite	Control	With APAP	With L-Cysteine	With APAP and L-Cysteine
SAMG	50.9	63.6	40.2	41.8
SAMS	33.4	27.2	47.5	47.9

[a] One gram SAM alone or 1.5 hr. after 1 g. APAP. Two grams L-cysteine every hour from −1.5 to +2.5 hr. Abbreviations as in Table I, Subject Y.

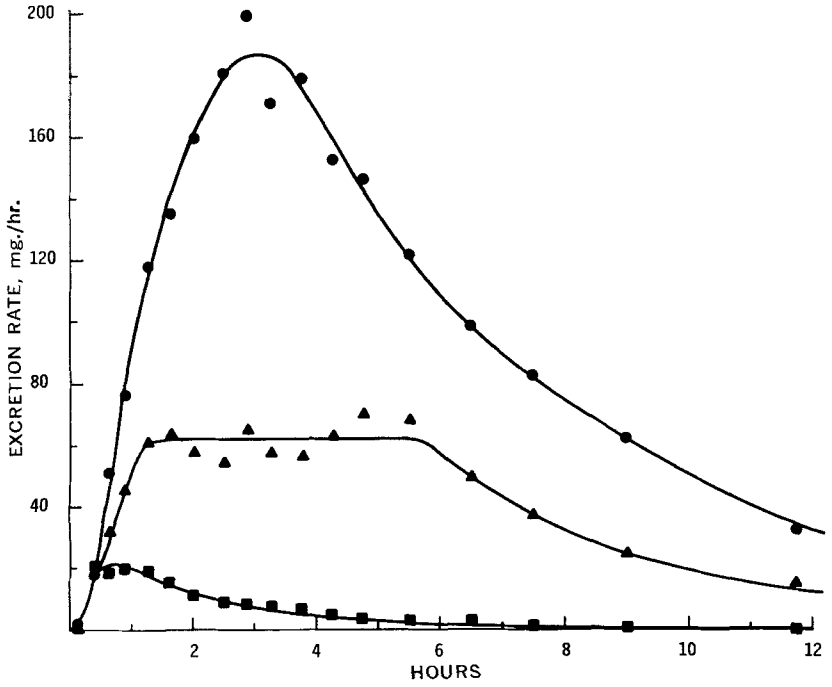

Figure 3—*Excretion rate of acetaminophen* (■), *acetaminophen glucuronide* (●), *and acetaminophen sulfate* (▲) *following oral administration of 2 g. acetaminophen to Subject Y.*

DISCUSSION

The total recovery of acetaminophen and its metabolites in this study (92% of the dose on the average) is similar to the average recoveries of 82 and 95% reported by Prescott *et al.* (5) and Nelson and Morioka (6), respectively. Cummings *et al.* (7) showed that acetaminophen is eliminated mainly as the glucuronide and sulfate and to a smaller extent as free drug. They identified the two metabolites by comparing their chromatographic characteristics and IR absorption spectra with those of pure acetaminophen glucuronide and acetaminophen sulfate. The composition of urinary excretion products of acetaminophen found in the present study (Table II) is very similar to that reported by Cummings *et al.* (7) (4% acetaminophen, 26% acetaminophen sulfate, and 49% acetaminophen glucuronide).

It appears that the formation of acetaminophen sulfate in man may be capacity limited in the 1–2-g. dose range. This is indicated by the acetaminophen sulfate excretion-rate plateaus following acetaminophen administration (Figs. 3 and 4) and by the less than proportional increase in acetaminophen sulfate excretion rates but more than proportional increase in acetaminophen glucuronide excretion rates when the dose of acetaminophen was increased from 1 to 2 g. (Figs. 1 and 3). While there were appreciable intersubject differences in acetaminophen sulfate formation, the results in any one subject were quite reproducible (Figs. 6–8). The fraction of acetaminophen excreted as acetaminophen sulfate decreased only slightly with increasing dose (Table II) in the dose range tested. This may be due to the fact that acetaminophen sulfate formation accounts for less than one-third of the acetaminophen elimination process and that deviation from apparent first-order acetaminophen sulfate formation may occur only when the amount of drug in the body is a relatively large fraction of the doses used in this study. There is also the possibility that acetaminophen glucuronide formation becomes capacity limited upon administration of the 2-g. dose of acetaminophen. In that case, the composition of the major urinary metabolites of acetaminophen would not change much with dose. Saturation of acetaminophen sulfate and acetaminophen glucuronide formation was observed in the rat by Büch *et al.* (8). They found that the same amount of acetaminophen sulfate was excreted within 4 hr. after administration of 300 and 600 mg./kg. acetamino-

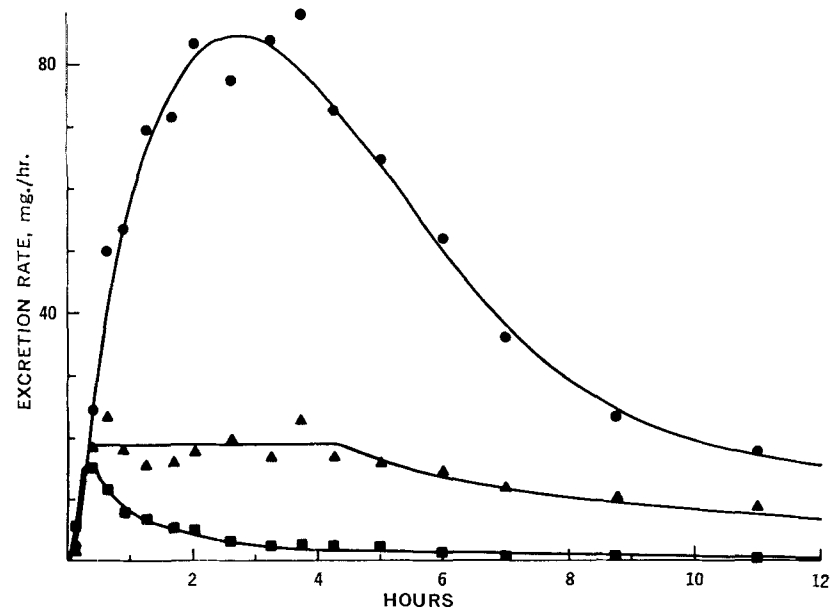

Figure 4—*Excretion rate of acetaminophen* (■), *acetaminophen glucuronide* (●), *and acetaminophen sulfate* (▲) *following oral administration of 1 g. acetaminophen to Subject J.*

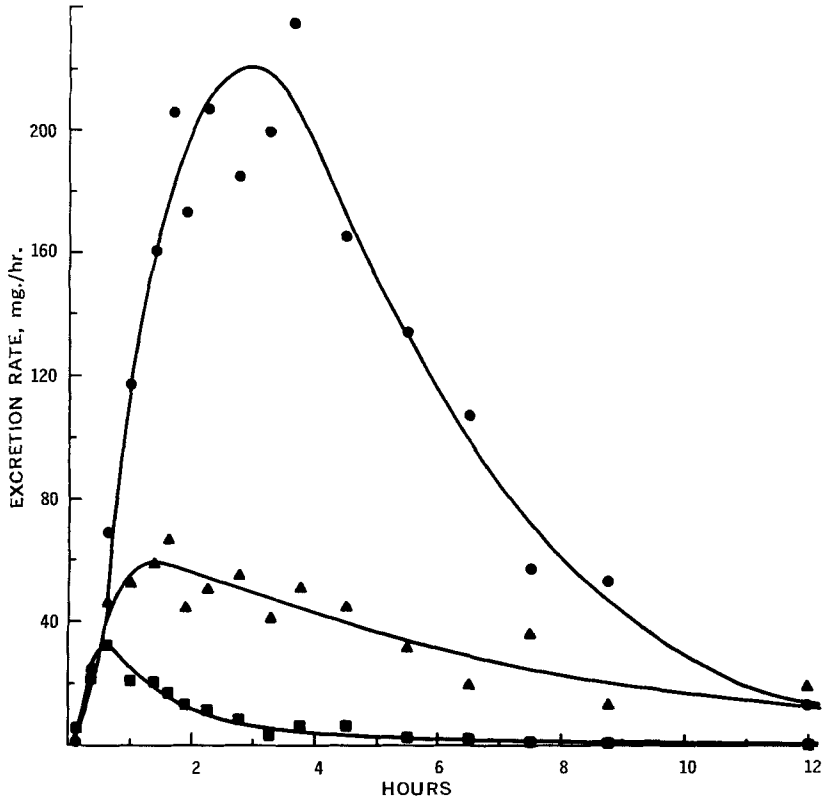

Figure 5—*Excretion rate of acetaminophen (■), acetaminophen glucuronide (●), and acetaminophen sulfate (▲) following oral administration of 2 g. acetaminophen to Subject K.*

phen and that acetaminophen glucuronide excretion increased by only 25%. The formation rate of acetaminophen sulfate could be increased by intravenous injection of sodium sulfate (9).

Concomitant administration of acetaminophen and salicylamide resulted in a mutual inhibition of sulfate formation (Figs. 6–8 and 10 and Table III). This effect was counteracted by L-cysteine (Fig. 9 and Table IV), a source of inorganic sulfate (10), which unlike inorganic sulfate is well absorbed upon oral administration. The formation of organic sulfate is a biphasic process: activation of inorganic sulfate to 3′-phosphoadenosine-5′-phosphosulfate followed by the transfer of sulfate to the acceptor molecule (11). The transfer reaction is mediated by a number of substrate-specific sulfokinases, while the activation step is common to each of these transfer reactions. Since the capacity-limiting step in the formation of salicylamide sulfate and acetaminophen sulfate appears to be the availability of sulfate, salicylamide and acetaminophen may be expected to inhibit competitively not only the formation of other phenolic sulfates but also the conjugation with sulfate of nonphenolic compounds such as various steroids which utilize different sulfokinases.

Based on the previously demonstrated competitive inhibition of glucuronide formation by salicylamide and salicylate (2), the decreased excretion of acetaminophen glucuronide after salicylamide administration (Figs. 6–8) is believed to be due to a similar competitive effect. However, the possibility that the renal clearance (rather than the formation) of acetaminophen glucuronide was inhibited by salicylamide or its metabolites cannot be excluded on the basis of the experimental data. The simultaneous inhibition of the parallel and competing process, sulfate formation, actually results in increased conversion of both acetaminophen and salicylamide to their respective glucuronides. Even the magnitude of a temporary inhibition of acetaminophen glucuronide formation rate would be "dampened" therefore by the more pronounced inhibition of the concurrent sulfate formation process.

This study showed not only that acetaminophen elimination may become capacity limited and that there occurs a mutual inhibition in

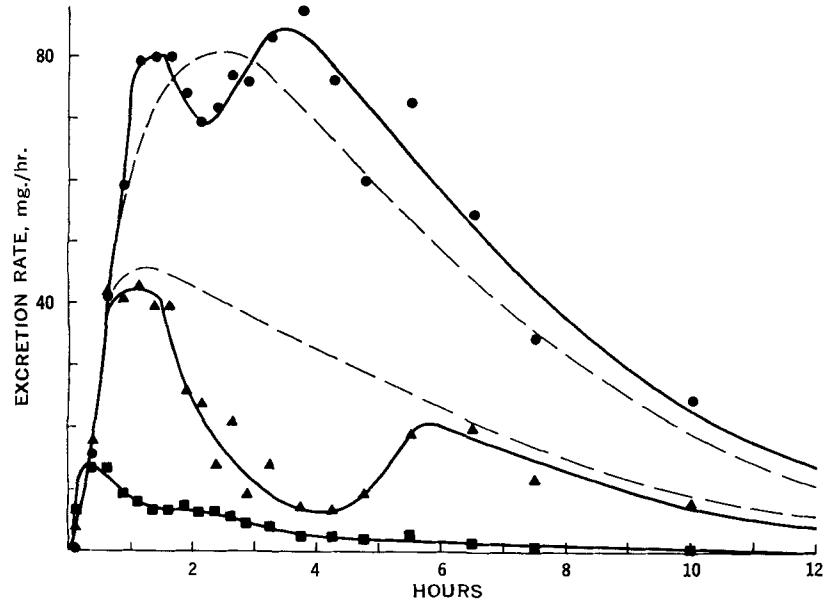

Figure 6—*Effect of 1 g. salicylamide, given at 1.5 hr., on the elimination of 1 g. acetaminophen by Subject Y. Key: ■, acetaminophen; ●, acetaminophen glucuronide; and ▲, acetaminophen sulfate. The broken line in this and the subsequent figures represents the excretion-rate curves of the corresponding substances following oral administration of 1 g. acetaminophen alone.*

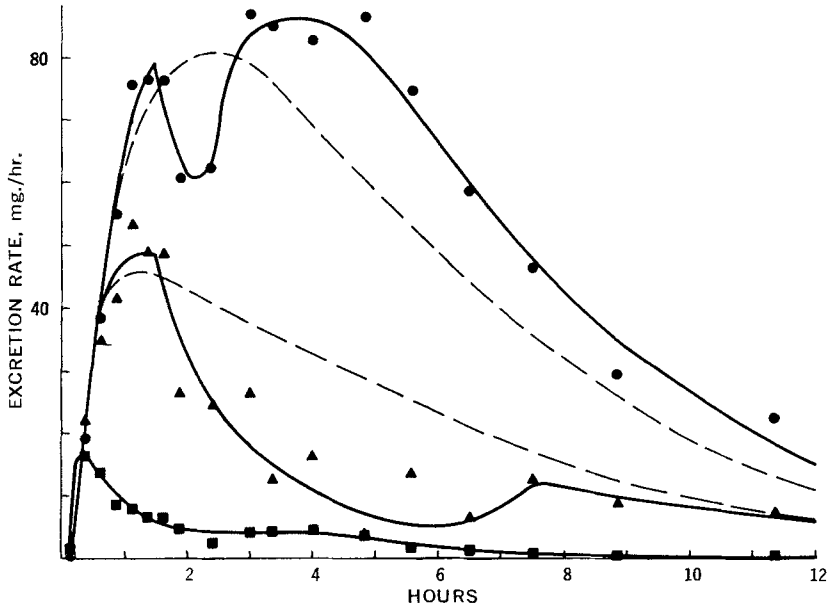

Figure 7—*Effect of 2 g. salicylamide, given at 1.5 hr., on the elimination of 1 g. acetaminophen by Subject Y. Key:* ■, *acetaminophen;* ●, *acetaminophen glucuronide; and* ▲, *acetaminophen sulfate.*

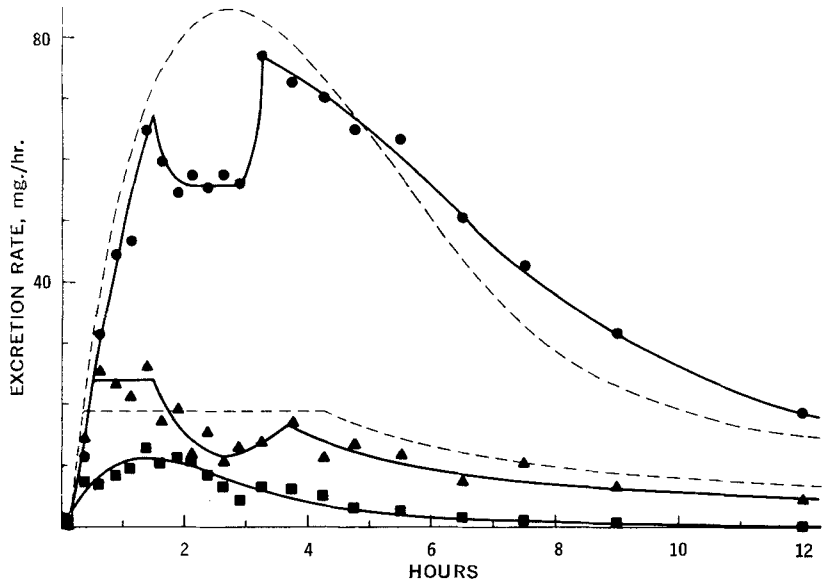

Figure 8—*Effect of 1 g. salicylamide, given at 1.5 hr., on the elimination of 1 g. acetaminophen by Subject J. Key:* ■, *acetaminophen;* ●, *acetaminophen glucuronide; and* ▲, *acetaminophen sulfate.*

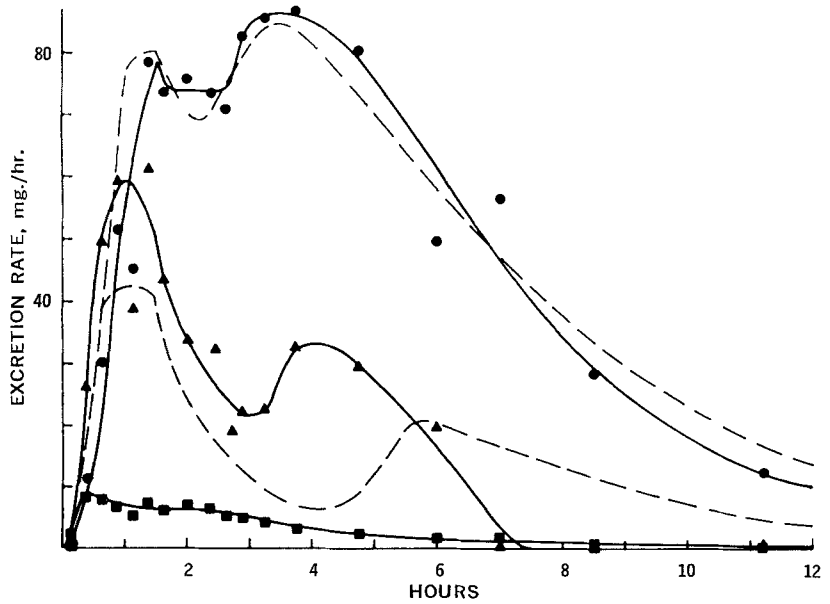

Figure 9—*Effect of a sulfate precursor* (L-*cysteine*) *on the influence of salicylamide on the elimination of acetaminophen by Subject Y. Two-gram doses of cysteine were administered at 0, 1, 2, 3, and 4 hr. The 1 g. acetaminophen was administered at zero time and 1 g. salicylamide was given 1.5 hr. later. Symbols are as in Fig. 1. The broken line is from Fig. 7 (acetaminophen and salicylamide).*

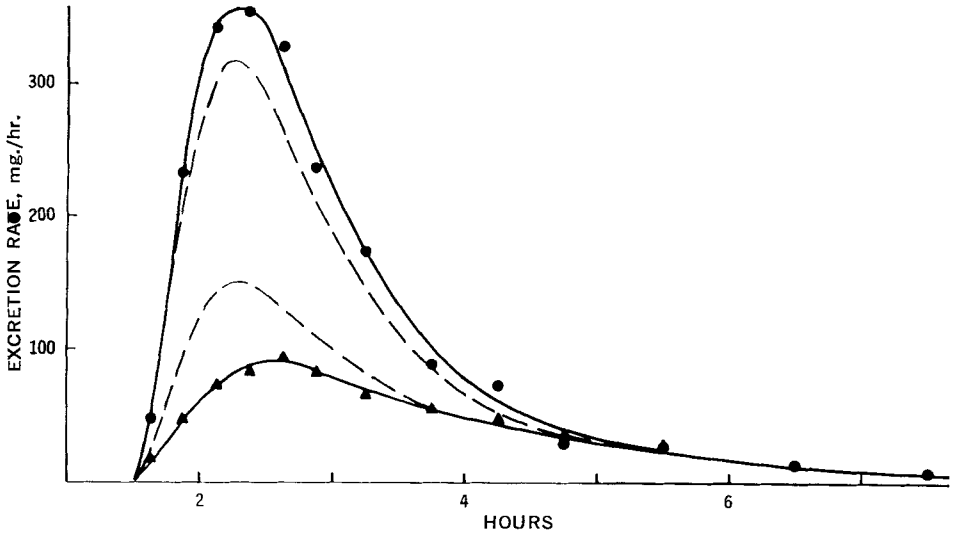

Figure 10—*Excretion rate of salicylamide glucuronide (●) and salicylamide sulfate (▲) following oral administration of 1 g. salicylamide at 1.5 hr. after 1 g. acetaminophen (Subject Y). The excretion rates are expressed as mg./hr. salicylamide equivalent. The broken lines represent the excretion-rate curves following oral administration of 1 g. salicylamide alone.*

the elimination of acetaminophen and salicylamide. It also revealed that, in the absence of noticeable saturation effects, acetaminophen elimination is a biexponential process. This feature of acetaminophen pharmacokinetics became apparent when the drug was administered in a rapidly absorbed form (solution) and when urine was collected every 15 min. (Fig. 2). Previous investigators, who administered the drug in solid form and collected urine less frequently, characterized acetaminophen elimination in terms of a one-compartment model (6, 7). If the results in Fig. 2 are reexpressed by combining several data points and thereby simulating results that would be obtained by collecting urine at greater intervals, the kinetics of acetaminophen elimination would agree perfectly with those obtained by other investigators (Fig. 11).

In this study, characterization of the previously reported biotransformation interaction between salicylamide and salicylate (2) has been extended to a description of the interaction of salicylamide and acetaminophen. The biotransformation of salicylate and acetaminophen in the presence of one another will be the subject of a subsequent report (12). These interactions between widely used non-narcotic analgesics have important pharmacokinetic and therapeutic implications (2, 13–15).

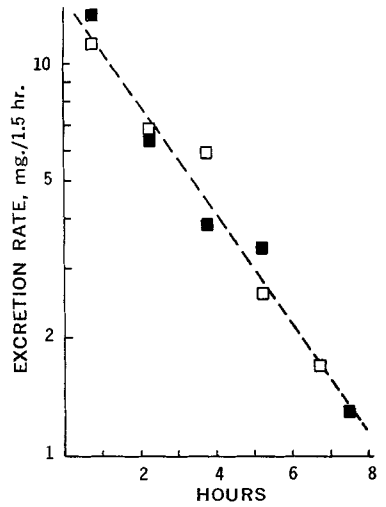

Figure 11—*Comparison of excretion rates of acetaminophen reported by Cummings* et al. *(Subject D, in Table I,* Reference 7*) with those of Subject Y in the present study. Key:* □, *Subject D from the study of Cummings* et al.; *and* ■, *data from Subject Y from Fig. 1 of the present study, but presented at greater intervals.*

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Determination of Ethylene Oxide in Surgical Materials by Vacuum Extraction and Gas Chromatography

J. A. MOGENHAN, J. E. WHITBOURNE, and R. R. ERNST

Abstract □ A method for the determination of ethylene oxide sterilized plastic is described. The ethylene oxide is extracted from the plastic by vacuum and condensed in a cold trap. The gas is subsequently analyzed by GLC. Other methods are compared.

Keyphrases □ Ethylene oxide in plastics—determination □ Vacuum extraction—ethylene oxide □ GLC—analysis

Ethylene oxide (EO) gaseous sterilization has found increased use since the first patent was applied for by Schrader and Bossert in 1933 (1). Its advantages such as low temperature, good penetration, effectiveness against all organisms, and effectiveness at low humidities are well known. At present, many items made of temperature-sensitive materials, which should not be exposed to heat, are sterilized by means of EO. One disadvantage of sterilization with EO is the residual EO that remains in the plastic. Attempts to determine these residual amounts have resulted in a variety of analytical methods.

One early method was used to determine residual EO in fumigated grain (2). Later, a colorimetric method (3) for determining EO in spices was used. After this, in succession, appeared a distillation and titration method (4), a water extraction method (5), and an acetone extraction method (6, 7).

The authors have developed a method by which residual EO is removed from the EO sterilized plastic or silastic material by vacuum. The eluted gas is liquified and collected in a cold trap under vacuum, using liquid nitrogen as the coolant. The condensate is subsequently revaporized by mild heat and injected into the helium stream of a gas chromatograph.

EXPERIMENTAL

Apparatus—The gas chromatograph was a Perkin-Elmer model 154 equipped with a gas sampling valve. A 0.95-cm. (0.375-in.) o.d. × 20.32-cm. (8-in.) long condenser U-tube was connected to the gas sampling valve, which was further modified by removing the metal hose connectors, in place of which two stainless steel valves were fitted. To each valve was connected a 7.62-cm. (3-in.) piece of 0.635-cm. (0.25-in.) o.d. stainless steel tubing. A union (Swagelok) to adapt glass to steel ended in a 24/40 ground-glass fitting, connected to a 250-ml. round-bottom flask. The upper stainless steel tubing was connected to a Welch duo-seal pump. A McLeod vacuum gauge was located in front of the pump by means of a T-connection. The apparatus is shown in Fig. 1.

A 1.8288-m. (6-ft.) × 0.635-cm. (0.25-in.) stainless steel standard B-column (Perkin-Elmer) consisted of a 25% di(2-ethylhexyl) sebacate on a GC-22 super support (60/80 mesh) operated at 70° at a power setting of 70. The operating parameters were: helium flow rate, 165 ml./min. at 25 psig.; and thermal conductivity detector, 8 v. The indium tubing was 0.1524 cm. (0.06 in.) o.d. × 0.0762 cm. (0.03 in.) i.d.[1]

[1] Catalog No. 37-000094-00; purchased from Wilkins Instrument and Research, Inc., a division of Varian Aerograph.

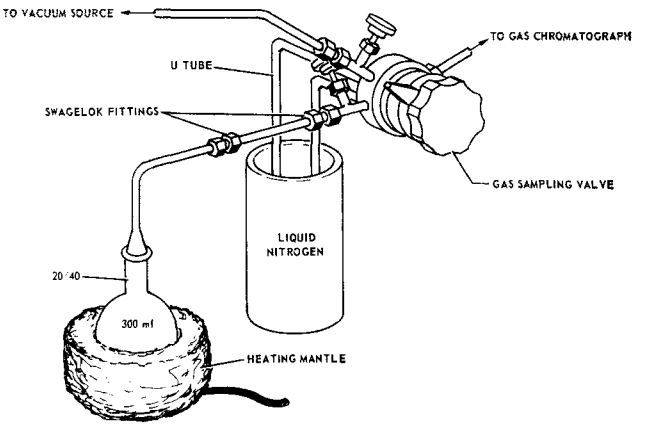

Figure 1—*Test apparatus.*

For comparing acetone, water, and chloroform extraction methods, determinations of EO were accomplished using the model 800 flame-ionization gas chromatograph (Perkin-Elmer). The columns, 1.8288 m. (6 ft.) × 0.3175 cm. (0.125 in.) o.d., were 10% polyethylene glycol[2] on Teflon (35 mesh) solid support. The operating parameters were: helium flow rate, 29 ml./min.; 40 psig. air, zero gas; 17 psig., hydrogen; temperature block, 200°; and detector temperature, 160°.

Procedure—The plastic and rubber tested were exposed in a commercial sterilizer to 750–800 mg./l. EO for 4 hr.

After sterilization, samples were cut, weighed, and placed in a round-bottom flask. Plastic tubing and other thicker materials were first chilled in liquid N_2 and crushed to increase surface area. The flask was attached to a U-collection tube *via* the glass tube and fitting. The Dewar flask, with liquid nitrogen in it, was raised into position so that the condenser tube was partially immersed in the cooling liquid. When the condenser tube was cooled, the valves connecting the system to the vacuum were opened, and the sample flask was subjected to mild heat (80–90°). Condensation proceeded for 1 hr. The valves were closed at the end of the sampling period, and the cold trap was removed and replaced by a water bath of 50–60° for 3 min. Then the gas sampling valve was turned to the sampling position, allowing the carrier gas to sweep the sample into the gas chromatograph. The average retention time was 1.4 min. for EO.

A calibration curve was made by determining the instrument response to various amounts of EO by the following procedure. A short piece of indium tubing was cut and crimped at one end and then weighed. The tube was cooled along with a syringe and needle. Cold EO was drawn into the syringe, and the needle of the syringe was inserted into the open end; the cold EO was gently forced into the tubing as the needle was gradually withdrawn. The remaining opening of tubing was quickly crimped. The tubing was allowed to warm to ambient temperature and was again weighed. The net weight was EO.

Subsequently, the indium tubing with the EO in it was placed in the round-bottom flask, which was positioned in the vacuum system, and vacuum was applied. The bottom of the flask was heated with a plastic welding torch until the small piece of indium tubing softened and the EO escaped. This was noted by a slight movement of the tube when the gas escaped. The sampling then proceeded as described. The round-bottom flask was always covered with a safety shield. A second vacuum of 0.5 hr. was applied to the same

[2] Carbowax 1540, Union Carbide Corp., New York, N. Y.

Table I—Comparison of Three EO Determination Methods[a]

Sample	Distillation and Titration	Vacuum Extraction	H_2O Extraction
1	20,651 p.p.m. EO	27,005 p.p.m. EO	22,107 EO; 3043 p.p.m. EG
2	21,213 p.p.m. EO	26,960 p.p.m. EO	23,662 EO; 3082 p.p.m. EG

[a] Nearly identical samples were used, all exposed 2 hr. to 1200 mg./l. EO at 54° with no poststerilization vacuum. The plastic was 0.051-cm. (0.020-in.) PVC squares.

Table II—Recovery of EO after Aeration

Sample	Aeration, hr.	EO, p.p.m. Vacuum Extraction	EO, p.p.m. Acetone Extraction	Ratio, Vacuum/Acetone
1	0	14,598	12,029	1.21
2	18.5	7,481	3,117	2.40
3	72	1,653	1,559	1.06

sample to assure that no EO remained in the flask. The procedure was repeated with different weights of EO. A plot of peak areas in millivolts *versus* concentration at an attenuation of ×128 produced a straight-line graph. The areas were determined on a Hewlett Packard 3370A integrator.

In the comparative acetone extraction method, the volume of the extracting liquid varied. The extraction at the zero aeration time used 100 ml. acetone and 25 ml. at other times. The duration of extraction was 18 hr. for both acetone and chloroform.

Samples of 0.2 μl. were injected into the gas chromatograph, and the amount of EO in the extracting liquid was determined by referring to a standard curve. This standard curve, comparing areas to milligrams of EO, was prepared by injecting into the gas chromatograph various known levels of EO in acetone.

RESULTS

A summary of the data is given in Tables I–IV. Table I shows a comparison of the Gunther (4), Bartak and Kulkarni (5), and vacuum methods. The same plastic and the same sterilization cycles were used in all cases. The plastic was 20-mil polyvinyl chloride (PVC) sheeting. The extraction solvent used in the Bartak and Kulkarni method (5) was also analyzed for ethylene glycol (EG), using a modification of the Critchfield–Johnson (3) method. If the EO had not hydrolyzed to EG during the 3-day H_2O extraction, this method would conceivably recover amounts equivalent to the vacuum method. However, the extraction time is severely long.

Table II shows the EO residuals as determined by vacuum and acetone extraction methods over an aeration period. Aeration is defined as the desorption in time of the sterilant gas from polymeric materials. All aeration for this work was performed at ambient temperature. The plastic was a 60-mil plastic vinyl sheet. No wrap was used when sterilizing and aerating these samples. The first two samples in Table II were compounded from Formula A. The third sample was compounded from Formula B. The plastic formulations were as follows:

Sample	Epoxy Content	Dioctyl Phthalate (DOP)	Total
A	10	50	60
B	10	30	40

Table III—Determination of EO by Weight Difference

Sample	Sample Wt., g., Presterilization	Sample Wt., g., Poststerilization	EO Determination Method	EO, p.p.m.
A	0.1044	0.1062	Weight	17,241
B	0.0875	—	Bartak and Kulkarni (5)	27,143
C	0.1143	0.1143	Weight	—

Table IV—Cycle Parameters *versus* Recovery and Recovery Ratio

Plastic Material	Cycle[a]	EO Recovered, p.p.m. Vacuum	Acetone	Ratio, Vacuum/Acetone
A	1	26,570	21,670	1.25
A*[b]	1	14,712	10,835	1.36
A	2	18,519	13,124	1.4
B	3 (100% RH)	75,599	69,696	1.10
B	2	24,732	22,526	1.10
B	3 (excess of 100%)	116,762	111,053	1.05
B	3 (50% RH)	42,636	28,991	1.47
C	4	13,232	8,740	1.50
C	5	18,352	10,364	1.76
C**[b]	4	384	273	1.41

[a] Cycle 1: 60°, 1200 mg./l. EO, 50% RH, 3 hr. of exposure. Cycle 2: similar to 1; 2 hr. of exposure. Cycle 3: similar to 1; 25.5° (78°F.), 2 hr. of exposure. Cycle 4: 63.5 cm. (25 in.) prevacuum, 50% RH, 30 min. humidification under vacuum, 8 psig. 12/88 EO F_{-12} mix., 4 hr. of exposure, 63.5 cm. (25 in.) postvacuum. Cycle 5: similar to 4; 50 psig., 12/88 EO F_{-12} mix., 1 hr. of exposure. [b] (*) 20 hr. or (**) 30 hr., respectively, elapsed prior to sampling. Plastic material without asterisks was sampled at zero aeration time.

Sample A was a phthalate epoxy combination at the 60 per hundred resin (phr.) level and was typical of commercially used compounds. Sample B was similar to A, but the total plasticizer level was lowered to only 40 phr. to determine the effect of using harder compounds.

Further results are portrayed graphically in Figs. 2 and 3. Figure 2 shows the elution rates obtained when various extraction methods were used. Samples [0.95-cm. (0.375-in.) o.d. × 0.711-cm. (0.281-in.) i.d.; 0.1188-cm. (0.047-in.) wall] of the same PVC plastic tubing were sterilized under identical conditions in the commercial sterilizer, using 750–800 mg./l. EO concentration for 4 hr. of exposure.

Extraction with water was for a period of 1 day. The data indicate that the vacuum method gives superior results in the determination

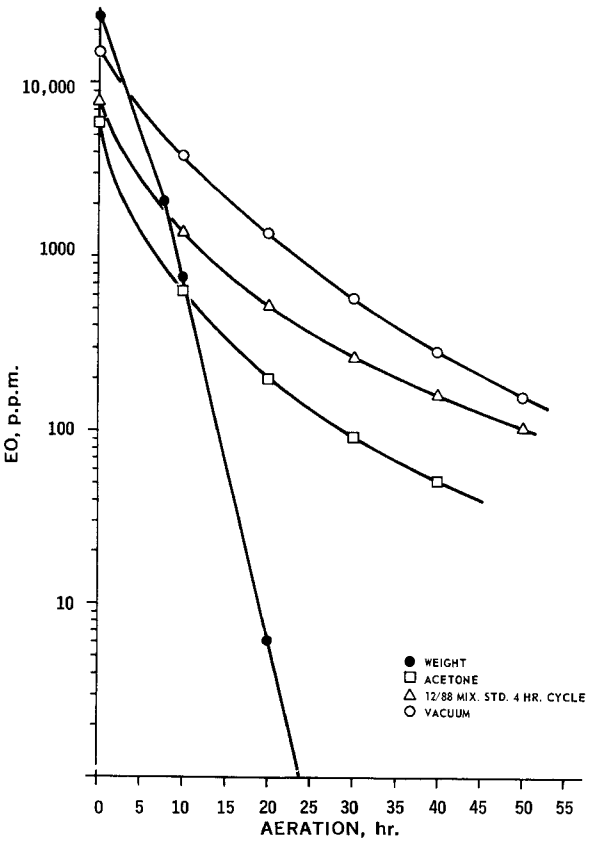

Figure 2—*Comparison of EO extraction methods for similar PVC formulations over extended periods, including EO retention values for pure EO* versus *12/88 EO/freon 12 mixture determined by acetone extraction.*

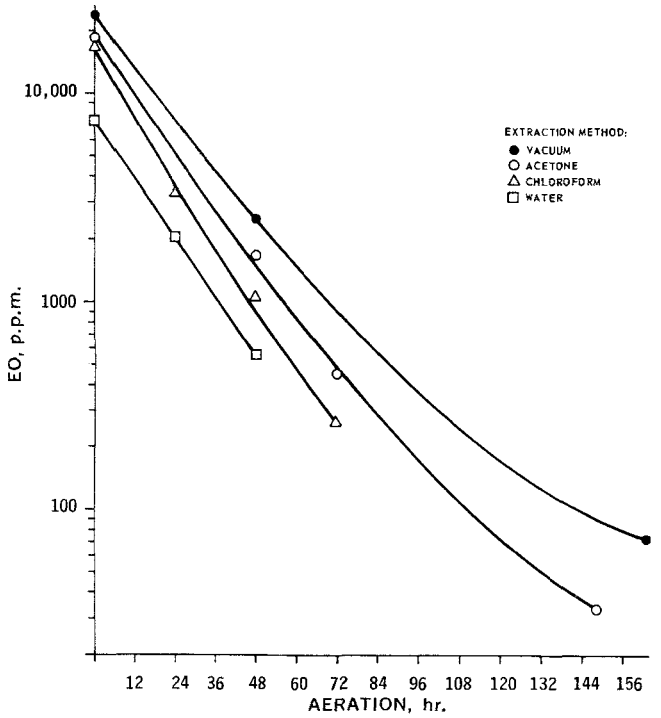

Figure 3—*Comparison of EO extraction methods for similar PVC formulations over extended aeration periods.*

of EO residuals in this material. Data in Table III on 5-mil polyurethan (Thermoplastic MP 1485) show the difficulty of determining by weight analysis the total amount of EO absorbed. This is also graphically portrayed in Fig. 3. Samples A and B were weighed and placed in a sterilizer chamber at 55°. Undiluted EO (1200 mg./l.) was added and the cycle was run for 2 hr. A control sample, C, was run later in the same chamber with no EO at 55° for 2 hr. In this test, the sample was weighed immediately after removal from the sterilizer. The other sample was then treated by the Bartak and Kulkarni (5) water extraction method of analysis. A PVC tubing of 0.237-cm. (0.094-in.) wall gave similar values for determinations by weight and by chloroform extraction. This was not true of all PVC samples. A PVC plastic obtained from one company lost weight following a long aeration period.

Table IV shows additional results of comparing the vacuum and acetone extraction methods using PVC. Plastic A is compared at 0-hr. aeration and at 20-hr. aeration by both methods. Plastic A is also compared at 0-hr. aeration where the duration of the EO exposure in the sterilizer was reduced from 3 to 2 hr. The humidity in the sterilizing cycle was varied for Sample B. Zero-aeration residual values are shown. Plastic C is compared at 0-hr. aeration and at 30-hr. aeration. The plastics used had the following dimensions:

Plastic	o.d.	i.d.	Wall
A	1.106 cm. (0.438 in.)	0.790 cm. (0.313 in.)	0.158 cm. (0.063 in.)
B	0.95 cm. (0.375 in.)	0.711 cm. (0.281 in.)	0.118 cm. (0.047 in.)
C	1.004 cm. (0.4 in.)	0.632 cm. (0.25 in.)	0.079 cm. (0.031 in.)

The vacuum-to-acetone ratio for Plastic A is approximately 1.3–1.4; for Plastic B, it is 1.0; and for Plastic C, it is 1.5.

SUMMARY

Many methods have appeared in the literature for the determination of EO and other toxic residues (reaction products of EO such as EG and ethylene chlorohydrin) in polymeric materials, rubber, and food. In most of these methods, the residue is extracted and then analyzed using GLC.

The data indicate that the vacuum method extracts as large or larger amounts of EO in almost all cases, as compared with other extraction techniques. The vacuum method has additional advantages: (*a*) large samples may be used to determine low p.p.m. EO residual; (*b*) fluorocarbons used as diluents for EO are absorbed by plastic and may also be determined, eluting from the column in 0.58 min.; and (*c*) extraction time and, consequently, the entire determination are faster and more reliable. Determinations based on weight determinations alone would be very unreliable because of water absorption; the absorption or elution of other volatile components such as freon, CO_2, air, and plasticizers. These effects become more pronounced, as indicated in Fig. 2, at lower retention levels.

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Drug Transport III: Influence of Various Sugars on Passive Transfer of Several Drugs across the Everted Rat Intestine

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Abstract □ Depending upon the sugar (monosaccharide) present in the drug–buffer solution (isotonic Krebs bicarbonate, pH 7.4), one can effectively alter the passive transfer of several drugs across the everted rat intestine. A similar phenomenon was noted in a previous study concerned with the influence of various cations on transfer. Thus, the transfer of riboflavin, salicylate, and sulfanilamide is significantly decreased in the presence of glucose. Concentrations of glucose as low as 25 m*M* were found to inhibit riboflavin transfer significantly. Phlorizin (1 m*M*), which is known to inhibit active glucose transport, abolishes the glucose-stimulated inhibition of riboflavin transfer. A similar inhibition of riboflavin transfer was noted in the presence of xylose. Mannitol, on the other hand, a nonpenetrating sugar, had no effect on the cumulative transfer of these compounds over a 2-hr. period. As noted in the previous study, there appears to be a good correlation between the extent of tissue-fluid uptake under various experimental conditions and the inhibition of riboflavin transfer. Thus, materials causing tissue-fluid uptake (*e.g.*, glucose and xylose) inhibit riboflavin transfer; materials causing no tissue-fluid uptake (mannitol and glucose plus phlorizin) have no effect on drug transfer. Several plausible mechanisms are suggested to account for the observed decrease in passive drug transfer.

Keyphrases □ Drug transport, everted rat intestine—monosaccharide effect □ Glucose, xylose—passive-transport inhibition □ Phlorizin inhibition—glucose-retarded riboflavin transport □ Tissue fluid uptake relationship—drug transport

Much of the past work concerned with solute movement across biological membranes has assumed that the membrane is inert and behaves simply as a barrier to diffusion. This approach has served quite well for formulating basic physiocochemical laws describing the transfer process. However, it has become increasingly apparent that considering a biological membrane as a simple barrier to diffusion may be an undesirable oversimplification. For example, Barr and Riegelman (1, 2), showed that salicylamide is metabolized and stored by several intestinal tissue preparations; they suggested that a compartmental model rather than a barrier model should be used to describe transfer (or absorption) of certain drugs. A similar conclusion may be reached based on the work of Doluisio *et al.* (3), who suggested membrane distribution and storage during the absorption of several drugs in an *in situ* intestinal preparation.

Research efforts in this laboratory have been concerned with the evaluation of various factors that may influence the passive drug-transfer process. Previously reported results (4, 5) suggested that one is able to alter the membrane effectively and thereby influence the transfer process of drug molecules. Using an isolated intestinal preparation (everted rat intestine), the authors showed that the passive transfer of several drugs may be significantly influenced by the ionic content of the drug–buffer solution. For example, the transfer of several drugs is decreased when the major cation of the buffer solution is K^+ or NH_4^+. There appears to be a relationship between the extent to which these cations inhibit transfer and their ability to increase water uptake by the tissue. Recent literature reports supported these initial observations. Caldwell *et al.* (6, 7) noted that there is a significant decrease in the passive transfer of ouabain and digoxin across the everted rat intestine in the presence of K^+. In addition, there was direct verification of these observations concerning the influence of K^+ on salicylate transfer by Benet *et al.* (8).

Since several reports in the literature suggested that various sugars may alter membrane water uptake (9, 10), and since Nogami *et al.* (11) gave preliminary evidence that glucose decreased sulfanilamide transfer, it was decided to examine the influence of several of these sugars on the drug-transfer process and hopefully to elucidate further the nature of the inhibitory effects noted previously.

EXPERIMENTAL

Determination and Treatment of Drug-Transfer Data—Male, Sprague-Dawley strain rats,[1] weighing approximately 250 g., were fasted 20–24 hr. prior to the experiment. Water was allowed *ad libitum*. The animals were anesthetized with diethyl ether, and a midline abdominal incision was made. The intestine was isolated and severed at the ileo-cecal junction. The entire length of the intestine was removed and freed by cutting the intestine at the pyloric juncture. The intestine was placed immediately into normal saline at room temperature, and the lumen was washed with normal saline to remove any solid matter. An initial 15-cm. portion of the proximal intestine was discarded to ensure use of the jejunal region of the intestine. The intestine was then everted, and two 10-cm. segments were attached to individual glass cannulas, according to the method of Crane and Wilson (12). The 10-cm. segments were measured after stretching the entire intestine with an 11-g. weight. The initial, proximal segment was designated Segment 1 and the distal portion was designated Segment 2. A weight (7 g.) was attached to each segment and maintained during the course of the experiment. Both segments were then placed into test tubes containing 100 ml. of the mucosal drug solution, previously equilibrated at 37°. Due to the large solution volume, the mucosal concentration of drug remained essentially constant throughout the experiment. Two milliliters of buffer solution (devoid of drug) was then placed into the serosal compartment. A gas mixture of O_2–CO_2 (95:5, v/v) was constantly bubbled through the mucosal solution.

The serosal compartment was sampled every 10 min. during the entire 2-hr. experiment. The entire serosal volume was removed at the sampling time. Two milliliters of buffer solution was then introduced into the serosal compartment as a rinse, immediately removed, and added to the previous sample. Finally, another 2-ml. portion of buffer was placed into the serosal compartment and withdrawn at the next sampling interval.

Results of the transfer experiments are generally expressed in terms of the cumulative amount of drug transferred in 2 hr. How-

[1] Blue Spruce Farms, Altamont, N. Y.

Table I—Influence of Various Glucose Concentrations on the Transfer of Riboflavin across the Everted Rat Intestine

Glucose Concentration, m*M*	Segment 1 Amount Transferred in 2 hr., mcg. ± *SD*[a]	Segment 1 Inhibition, %	Segment 2 Amount Transferred in 2 hr., mcg. ± *SD*[a]	Segment 2 Inhibition, %	Level of Significance,[b] Control *versus* Glucose
0 (control)	14.2 ± 2.5	—	11.8 ± 0.8	—	—
25	12.3 ± 1.4	14	10.3 ± 0.7	13	$p < 0.05$
50	11.3 ± 1.7	21	8.6 ± 2.0	27	$p < 0.01$
100	8.1 ± 1.0	43	6.3 ± 0.6	47	$p < 0.01$
150	8.3 ± 1.3	41	6.2 ± 1.0	47	$p < 0.01$
200	7.1 ± 1.3	50	4.4 ± 1.5	63	$p < 0.01$
250	8.3 ± 2.3	41	6.0 ± 2.2	49	$p < 0.01$

[a] Represents the mean of five experiments ± standard deviation of the mean. [b] Using a 2 × 2 ANOVA. *F*-ratio values generated for segmental differences were significant in all cases ($p < 0.01$). *F*-ratio values for interaction were not significant ($p > 0.05$) in all cases.

ever, cumulative transfer for 0.5 and 1 hr. was also evaluated; usually, identical results were obtained when these intervals were chosen for comparison of effects. The statistical test employed to ascertain the significance of differences in experimental results was a twoway analysis of variance (ANOVA).

The data were also interpreted in terms of the percent of control transfer of drug as a function of time in the presence of various sugars or in terms of the percent inhibition of normal transfer as a function of time produced by the sugars. Percent of control transfer is the ratio of the cumulative amount of drug transferred to a given time under a test condition to that observed under control conditions to the same time, multiplied by 100. Percent inhibition is simply 100 minus the percent of control transfer. This method of analysis offers the advantage of canceling out all changes in the membrane that might be produced by any of a number of factors, except those changes resulting from the test material itself.

Determination of Tissue Fluid Uptake—The intestine was everted and the first 15 cm. discarded. The next 30 cm. of intestine was used to prepare six 5-cm. segments. Twelve segments (from two rats) were used per buffer solution. A ligature was attached to each segment; the segment was briefly and carefully blotted on Whatman No. 40 filter paper to remove any excess adhering fluid and then weighed. The weight of the ligature was subtracted from this initial weight of tissue. Each segment was then placed into 50 ml. of buffer solution in individual conical flasks maintained at 37°. A gas mixture of O_2–CO_2 (95:5, v/v) was then constantly bubbled through this solution. At the end of 20 min. the segments were removed, drained, and placed into weighing cups. Blotting the tissue at this point caused a significant loss of mucosal material. For this reason, blotting was considered inappropriate. Once the segment was placed into the weighing dish, there was formation of a small amount of fluid (*i.e.*, fluid adhering to the membrane). This fluid was wiped up, and the final tissue weight was determined. Fluid uptake was expressed in milligrams fluid per gram initial wet tissue. It was calculated by subtracting the initial tissue weight from the final tissue weight after 20 min. of incubation in the buffer solution, and dividing by the initial tissue weight.

Buffer Solutions—The buffer solution used was a modified Krebs bicarbonate buffer,[2] pH 7.4, after gassing with O_2–CO_2 (95:5). The pH never varied by more than ±0.2 pH unit before and after each experiment. In all cases, the total cation or cation equivalent concentration was 154 m*M*. Except when stated otherwise, the mucosal and serosal solutions were identical except for drug content. As a result, there was never an electrochemical or osmotic gradient existing between the mucosal and serosal solutions.

Whenever a buffered sugar solution was prepared, the sugar quantitatively replaced NaCl in terms of osmotic equivalents in the control buffer. Thus, a 250-m*M* sugar solution is equivalent (in osmotic terms) to 125 m*M* NaCl, since the sugar is unionized. The sugars examined were glucose,[3] xylose,[4] and mannitol.[5] In certain experiments, phlorizin[6] (1 m*M*) was added to the mucosal and serosal solutions.

Drug Solutions and Assay Procedure—The drugs examined and the concentrations used were riboflavin[7] (20.0 mcg./ml.), salicylate[8] (2.0 mg./ml.), and sulfanilamide[9] (0.1 mg./ml.). At pH 7.4, salicylate is completely ionized and sulfanilamide and riboflavin essentially nonionized. These compounds were chosen for investigation because they have a wide divergence in structure, physical–chemical properties, and presumably permeability characteristics. In addition, these materials are also presumed to be passively transferred across biological membranes. The passive nature of the transfer process for the compounds investigated here was inferred previously [sulfanilamide (11), riboflavin (13–15), and salicylate (16)].

Riboflavin was assayed fluorometrically using the Turner fluorometer, model 110,[10] based on the procedure reported by Levy and Jusko (17). Salicylate was assayed spectrophotometrically with the Hitachi-Perkin-Elmer model 139 spectrophotometer[11] by the method of Trinder (18). Sulfanilamide was assayed spectrophotometrically using the method of Bratton and Marshall (19).

RESULTS AND DISCUSSION

Influence of Glucose on Transfer—The effect on transfer rate of essentially replacing Na^+ from the buffer with glucose is shown in Fig. 1. The plot shows the percent of inhibition of transfer across Segment 1 for riboflavin and sulfanilamide in the presence of 250 m*M* glucose and for salicylate in the presence of 219 m*M* glucose. The influence of glucose on the transfer of salicylate and sulfanilamide is maximal within 10–20 min. and tends to remain essentially constant at approximately 18–20% inhibition. Glucose produces a much greater decrease in riboflavin transfer (40% inhibition), although a longer period of time is required to obtain a maximal effect, compared to the other two drugs. Similar differences between riboflavin transfer and salicylate and sulfanilamide transfer

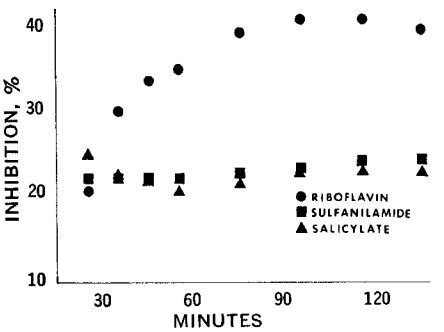

Figure 1—*Percent inhibition of drug transfer across intestinal Segment 1 as a function of time in the presence of glucose (250 m*M *for riboflavin and sulfanilamide, 219 m*M *for salicylate). Each data point represents the mean of five experiments with riboflavin (●) and salicylate (▲) and four experiments with sulfanilamide (■).*

[2] In m*M* concentrations: NaCl, 122; KCl, 5; KH_2PO_4, 1; and $NaHCO_3$, 26. With no additives, this solution is referred to in the text as the control buffer solution.
[3] Fisher Scientific Co., Fair Lawn, N. J., lot no. 791360.
[4] Fisher Scientific Co., Fair Lawn, N. J., lot no. 791758.
[5] Nutritional Biochemicals Corp., Cleveland, Ohio, lot no. 4654.
[6] Nutritional Biochemicals Corp., Cleveland, Ohio, lot no. 6398.
[7] General Biochemicals, Chagrin Falls, Ohio, lot no. 84134.
[8] Sodium salicylate, Fisher Scientific Co., Fair Lawn, N. J., lot no. 762784.
[9] Eastman Organic Chemicals, Rochester, N. Y.
[10] G. K. Turner Associates, Palo Alto, Calif.
[11] Perkin-Elmer Corp., Palo Alto, Calif.

Table II—Effect of Phlorizin (1 m*M*) on the Glucose Inhibition of Riboflavin Transfer

Experiment	Segment 1 Amount Transferred in 2 hr., mcg. ± *SD*[a]	Segment 1 Inhibition, %	Segment 2 Amount Transferred in 2 hr., mcg. ± *SD*[a]	Segment 2 Inhibition, %	Comparison and Level of Significance[b]
1. Control buffer	14.2 ± 2.5	—	11.8 ± 0.8	—	—
2. Control buffer + 1 m*M* phlorizin	12.9 ± 0.8	9	11.6 ± 1.1	2	2 *vs.* 1: n.s.
3. 25 m*M* Glucose	12.3 ± 1.4	14	10.3 ± 0.7	13	3 *vs.*1: $p < 0.05$
4. 25 m*M* Glucose + 1 m*M* phlorizin	13.4 ± 1.1	5	11.3 ± 1.4	4	4 *vs.*3: $p < 0.05$ 4 *vs.*1: n.s.
5. 25 m*M* Glucose—mucosal only	9.3 ± 1.0	35	8.2 ± 1.6	31	5 *vs.*1: $p < 0.01$
6. 25 m*M* Glucose—mucosal only + 1 m*M* phlorizin	12.7 ± 0.7	11	11.2 ± 1.1	5	6 *vs.*5: $p < 0.01$ 6 *vs.*1: n.s.

[a] Represents the mean of five experiments ± standard deviation of the mean. [b] Using a 2 × 2 ANOVA. *F*-ratio values for interaction for all comparisons were not significant (n.s.). *F*-ratio values for segmental differences were significantly different ($p < 0.05$).

were noted in the cation inhibition study (5). Although the data presented in the figures represent results from Segment 1, identical trends and similar values were obtained using Segment 2. When comparing the results of the glucose experiments shown in Fig. 1 to the control values, using an analysis of variance for the data obtained with both segments, there is a significant reduction in transfer ($p < 0.01$) for all drugs at each time interval evaluated.

Since glucose had the greatest influence on the transfer of riboflavin, additional experiments were conducted at lower glucose concentrations to examine further this inhibitory effect. The results of these experiments are summarized in Fig. 2 and Table I. Figure 2 is a plot of percent inhibition of cumulative riboflavin transfer over a 2-hr. period as a function of glucose concentration. An increase in inhibition is observed as the glucose concentration increases from 25 to 100 m*M*. Above 100 m*M* glucose, there appears to be a rather constant level of inhibition (40–50%). Table I summarizes all of the data for this series of experiments. At all glucose concentrations, there is a significant reduction in riboflavin transfer compared to the control. The reduction in cumulative transfer after 30 and 60 min. is also significant ($p < 0.01$) at all glucose concentrations.

After observing that 25 m*M* glucose on both sides of the membrane causes a decrease in riboflavin transfer, it was of interest to examine the influence of 25 m*M* glucose present initially only in the mucosal solution. In these studies the serosal solution was the normal control buffer, whereas the mucosal solution contained 25 m*M* glucose. Consequently, there was a concentration gradient with respect to glucose between the mucosal and serosal solutions. When glucose was initially present only on the mucosal side of the intestinal preparation, an even greater decrease in riboflavin transfer was noted compared to the experiment where glucose was present on both sides of the membrane. This can be seen in Fig. 3 and in Table II by comparing Experiments 3 and 5.

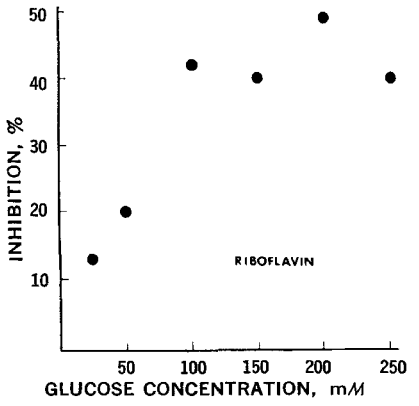

Figure 2—*Percent inhibition of riboflavin transfer as a function of glucose concentration* (mM). *Each point represents the mean of five experiments, based on the 2-hr. cumulative transfer across intestinal Segment 1.*

Influence of Phlorizin on Glucose Inhibition of Transfer—Since several reports in the literature (*e.g.*, 10 and 20) demonstrate that phlorizin can block the active uptake and transfer of glucose across the isolated rat intestine, it was decided to examine the influence of phlorizin on the glucose inhibition of riboflavin transfer. It was reasoned that if the uptake or transfer of glucose, or some function related to this process, was responsible for the decrease in drug transfer, one would expect that inhibition would be abolished or at least diminished in the presence of phlorizin. Thus, the influence of phlorizin (1 m*M* on both sides of the membrane) on the transfer of riboflavin in the presence of the lowest glucose concentration producing a significant decrease in transfer was evaluated. These results are summarized in Table II and Fig. 3. The data clearly demonstrate that when phlorizin is added to the glucose solutions, the inhibition of riboflavin transfer is markedly reduced. When phlorizin is added to the system containing glucose on both sides of the membrane, inhibition of transfer is reduced from 13–14% to 4–5% (Experiment 3 *versus* 4). The influence of phlorizin is even more striking when it is added to the system in which glucose is present initially only on the mucosal side. Whereas in the absence of phlorizin the glucose solution results in a 30–35% inhibition of transfer, in its presence inhibition is decreased to 5–10% (Experiment 5 *versus* 6).

Thus, it appears that phlorizin (1 m*M*) effectively prevents the glucose inhibition of riboflavin transfer, at least at the glucose concentration examined (25 m*M*). In addition, it may be inferred from these findings that with respect to active transport of glucose the tissue is apparently functional for the entire 2-hr. duration of the experiment.

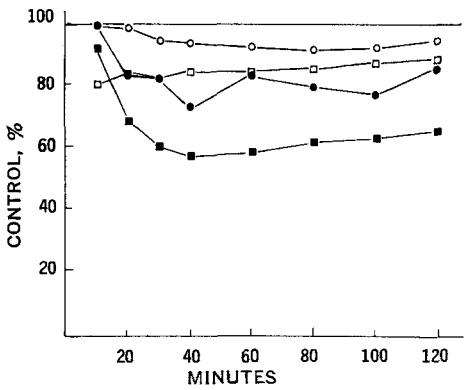

Figure 3—*Percent of control transfer of riboflavin as a function of time in the presence of 25* mM *glucose in the absence and presence of 1* mM *phlorizin. Each point represents the mean of five experiments for Segment 1. Solid horizontal line represents the control level. Key:* ●, *glucose present in both mucosal and serosal solutions;* ○, *glucose present in both mucosal and serosal solution + 1* mM *phlorizin;* ■, *glucose initially in mucosal solution only; and* □, *glucose initially in mucosal solution only + 1* mM *phlorizin.*

Table III—Influence of Xylose and Mannitol on Drug Transfer

Experimental Condition	Segment 1 Amount Transferred in 2 hr. ± SD[a]	Control, %	Segment 2 Amount Transferred in 2 hr. ± SD[a]	Control, %	Comparison and Level of Significance[b]
Riboflavin (mcg.)					
1. Control buffer	14.2 ± 2.5	—	11.8 ± 0.8	—	—
2. 250 m*M* Xylose	8.5 ± 0.9	60	7.6 ± 1.0	65	2 *vs.* 1: $p < 0.01$
3. 250 m*M* Mannitol	13.9 ± 0.9	98	11.5 ± 1.5	97	3 *vs.* 1: n.s.
Salicylate (mg.)					
1. Control buffer	3.6 ± 0.7	—	3.7 ± 0.5	—	—
2. 219 m*M* Mannitol	3.5 ± 0.2	97	3.3 ± 0.2	88	2 *vs.* 1: n.s.
Sulfanilamide (mcg.)					
1. Control buffer	131.2 ± 6.2	—	126.4 ± 23.7	—	—
2. 250 m*M* Mannitol	136.9 ± 17.6	104	134.3 ± 9.3	106	2 *vs.* 1: n.s.

[a] Represents the mean of five experiments ± standard deviation of the mean in the case of riboflavin and salicylate, and the mean of four experiments in the case of sulfanilamide. [b] Using a 2 × 2 ANOVA. *F*-ratio values for interaction were not significant (n.s.) ($p > 0.05$). *F*-ratio values for segmental differences were significant at $p < 0.05$ for riboflavin but not significant for salicylate and sulfanilamide.

Influence of Xylose and Mannitol on Transfer—The influence of two other sugars, xylose and mannitol, on drug transfer was also evaluated. Table III and Figs. 4 and 5 summarize the results. It is apparent that 250 m*M* xylose causes a significant decrease ($p < 0.01$) in the transfer of riboflavin compared to the control. The extent of inhibition of riboflavin transfer in the presence of xylose is identical to the inhibitory effect seen with glucose concentrations of 100 m*M* or higher.

Mannitol, on the other hand, had no effect on the cumulative transfer of riboflavin, sulfanilamide, and salicylate over a 2-hr. period, as shown in Table III. As can be seen in Fig. 5, which is a plot of percent control *versus* time in the presence of mannitol, there is initially (0–30 min.) a rather marked increase in the transfer of riboflavin over the control (horizontal line) but a decrease in the transfer of salicylate and sulfanilamide compared to control levels. After about 30 min., however, the values become quite constant and are maintained essentially at control levels. This unusual enhancement in transfer of riboflavin during the first 30 min. was not noted with any of the other sugars examined. However, examination of riboflavin transfer data obtained when either tromethamine[12] or Li^+ replaced Na^+ in the buffer also reveals an initial potentiation of riboflavin transfer over control levels. This potentiation essentially disappears in all cases after 30 min.

Tissue Fluid Uptake Study—Table IV lists the tissue fluid uptake values under various experimental conditions. As pointed out in a previous report (5), there is a great deal of variability associated with measurements of fluid uptake as is apparent from the standard deviation values shown in the table. Chauvenets' criterion (21) was applied to the values in each series of determinations; for this reason the mean may represent the average of 11 rather than 12 segments. At each of the glucose concentrations studied, there is a large increase in the mean fluid uptake compared to the control buffer (150–210 mg./g. *versus* 50 mg./g.). A similar effect is apparent when comparing the uptake in the presence of xylose to the control. Although the authors' studies have shown xylose to increase tissue fluid uptake by the rat intestine, Csaky and Esposito (22) suggested that xylose does not cause water uptake in the intestine of the bullfrog. Species differences as well as possible concentration differences with respect to xylose could account for this apparent discrepancy.

Several reports in the literature demonstrate that glucose can increase tissue hydration, *i.e.*, tissue water uptake (23, 24). Csaky and Esposito (22) suggested that active accumulation of a sugar in the intestinal tissue is associated with epithelial cellular swelling and discussed the possible correlation of this swelling with transmural electric potential. Jackson and Cassidy (25) measured gut fluid uptake using everted sacs of rat small intestine and demonstrated experimentally that this fluid uptake can be accounted for, at least in part, by epithelial cellular swelling. Thus, measurements of tissue fluid uptake in this and the previous study probably reflect swelling of the epithelial cells.

Since the method used in this study to determine tissue fluid uptake utilizes open intestinal segments, another approach was necessary to examine the influence of 25 m*M* glucose present initially only on the mucosal side. When using closed everted sacs of intestine, essentially no difference in tissue fluid uptake was noted when glucose was present either on both sides of the membrane or on the mucosal side alone. This observation was supported by the work of Lifson and Parsons (26), who found no difference in water absorption, which is related to tissue fluid uptake (9), when 29 m*M* glucose is present either in both serosal and mucosal solutions or only in the mucosal solution.

Of particular interest is the fact that when phlorizin is present in the 25 m*M* glucose solution, the fluid-uptake value is reduced from 210 mg./g. to about 50 mg./g., a level essentially identical to that observed under control conditions. Moreover, phlorizin also appears to inhibit tissue fluid uptake from the normal Na^+ control

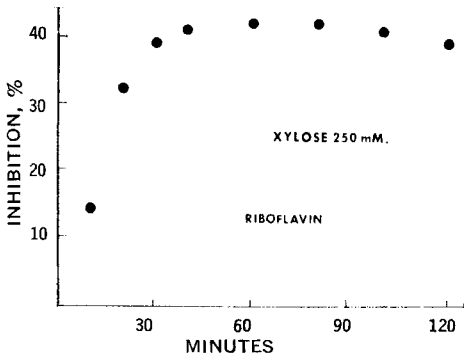

Figure 4—*Percent inhibition of riboflavin transfer as a function of time in the presence of 250 m*M *xylose. Each point represents the mean of five experiments for Segment 1.*

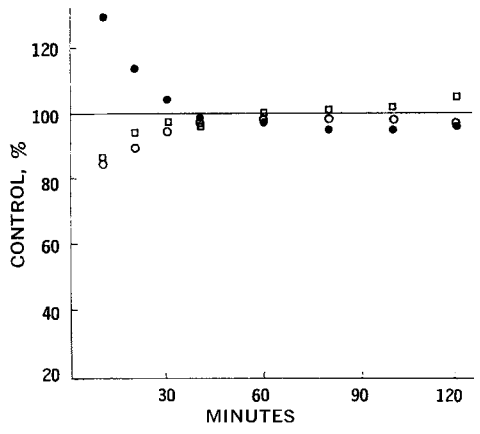

Figure 5—*Percent control transfer across intestinal Segment 1 as a function of time in the presence of mannitol (250 m*M *for riboflavin and sulfanilamide, 219 m*M *for salicylate). Each point represents the mean of five experiments with riboflavin (●) and salicylate (○) and four experiments with sulfanilamide (□).*

[12] TRIS.

Table IV—Tissue Fluid Uptake by Segments of Everted Rat Intestine under Various Experimental Conditions

Solution	Tissue Fluid Uptake, mg. H_2O/g. Tissue ± SD[a]
Control	52 ± 40 (11)
Control + 1 m*M* phlorizin	10 ± 29 (11)
Glucose 25 m*M*	209 ± 43 (12)
Glucose 25 m*M* + 1 m*M* phlorizin	50 ± 35 (11)
Glucose:	
50 m*M*	196 ± 48 (12)
100 m*M*	184 ± 50 (12)
150 m*M*	210 ± 47 (12)
200 m*M*	172 ± 40 (11)
250 m*M*	149 ± 39 (12)
Xylose 250 m*M*	148 ± 64 (12)
Mannitol 250 m*M*	27 ± 27 (11)

[a] The number of segments used are in parentheses.

buffer. In the presence of 1 m*M* phlorizin, there is a reduction in water uptake from 50 mg./g. to 10 mg./g. Parsons *et al.* (10) showed that 1 m*M* phlorizin can significantly decrease tissue fluid uptake by sacs of everted rat intestine, in addition to its ability to block glucose transfer. These authors suggested that phlorizin interferes with fluid uptake by disturbing glucose uptake and metabolism.

Since mannitol is known not to penetrate membranes to any significant extent (27), in contrast to glucose and xylose, it was expected that its presence would not cause swelling of the intestinal tissue. The important fact to be borne in mind here is that although the overall solution may be isoosmotic with respect to another solution, the penetrating ability of the various materials in the solution determines whether there will be a net flow of water across a membrane. The data in Table IV clearly support this hypothesis because the mean value for water uptake in the presence of mannitol is actually less than the corresponding value for the control.

Relationship between Tissue Fluid Uptake and Drug Transfer—The results of the present investigation clearly support the initial observation (5) that experimental conditions that modify the water content of intestinal tissue also modify drug-transfer rate. Thus, in the presence of glucose, there is a large increase in the mean tissue fluid uptake as well as a significant decrease in the transfer rate of each drug studied. Similarly, xylose significantly increases tissue fluid uptake and produces a marked inhibition of riboflavin transfer across the everted intestine. Mannitol, on the other hand, decreases gut fluid uptake and has little effect on the transfer of the drugs examined. Probably of greatest significance is the observation that concentrations of phlorizin that effectively block glucose-stimulated tissue fluid uptake also abolish the glucose inhibition of riboflavin transfer.

In the previous report (5), an excellent correlation was noted between the extent of inhibition of riboflavin transfer by various cations and the effect of these cations on tissue fluid uptake. By using these data as well as results from the present study, this correlation was extended and is presented in Fig. 6. The data points (representing both segments) were fitted by least-squares regression, and the equation of the line is $y = -0.41(x) + 115.19$, with a correlation coefficient of -0.940, which is highly significant ($p < 0.001$). If a correlation is attempted using the results of the mannitol, xylose, glucose (200 and 250 m*M*), and control experiments only, the equation of the line is $y = -0.38\ (x) + 113.30$, with a correlation coefficient of -0.966 ($p < 0.001$). A considerably poorer quantitative correlation is observed when one attempts to include the data from experiments using <200 m*M* glucose (*i.e.*, >50 m*M* Na^+). In view of the degree of inhibition of riboflavin transfer produced by these glucose solutions, their effect on fluid uptake is notably greater than predicted by the regression line.

Several plausible mechanisms might be suggested to explain the effects of cations and sugars on drug transport. The first of these considers the effective drug concentration in the intestinal tissue (*i.e.*, the epithelial cellular compartment). There is undoubtedly an increase in the compartmental cellular volume of the intestinal tissue in the presence of the various inhibitors. If this increase in cellular volume results in a decrease in the drug concentration in

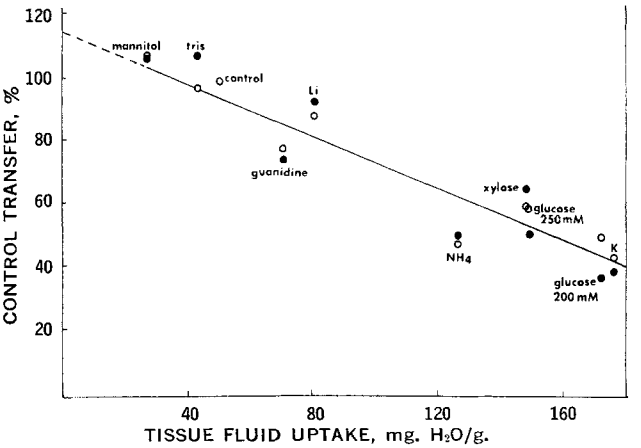

Figure 6—*Relationship between tissue fluid uptake (mg. H_2O/g. tissue) in the presence of various materials and percent of control transfer for riboflavin. Each point represents the mean of five experiments. Regression equation,* $y = -0.41\ (x) + 115.91$. *Correlation coefficient,* -0.940 ($p < 0.001$). *Key:* ○, *Segment 1; and* ●, *Segment 2.*

the cell, one expects that the concentration gradient existing between this compartment and the serosal solution would be decreased. If the transfer from the tissue to the serosal compartment is rate limiting, there would be a decrease in the driving force and a decreased rate of appearance of drug in the serosal compartment. Support for this mechanism can be sought only by a kinetic analysis of the changes of concentration and amount of drug in the tissue as a function of experimental conditions. Thus, if one finds that the same amount of drug is present in the tissue compartment when drug transfer is measured from the control buffer or any of the sugar or cation solutions examined, then there must be a concomitant change of the drug concentration due to volume changes of this compartment. Schultz *et al.* (28) showed that in the presence of K^+, which causes swelling of the intestinal tissue of rabbits, there is a large decrease in intracellular L-alanine concentration but virtually no change in the amount of this amino acid in the tissue. This effect was not a result of a specific inhibitory effect of K^+ on the transfer of L-alanine.

A second possibility requires one to consider the so-called pore route for solute movement across biological membranes. For example, Crone and Keen (29) suggested that pyridinium aldoximes traverse the isolated rat intestine by movement through aqueous channels or pores which exist between cells in the tissue. Kipnis and Parrish (30) reported that cellular swelling of rat diaphragm tissue in the presence of K^+ takes place at the expense of the extracellular volume. Thus, as the cells swell, there is a concomitant decrease in the space between the cells, which should result in a decrease in the effective pore or channel diameter. Two pathways may be available for the diffusion of drugs across the everted intestine: a cellular route which is associated with a lipoid barrier and an extracellular or pore route. If this is the case, then the degree of inhibition of drug transfer caused by cellular swelling is dependent upon the absorption pathway. For example, the transfer of a drug that diffuses essentially *via* the extracellular space would be markedly inhibited by cellular swelling.

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Dissolution of Slightly Soluble Powders under Sink Conditions II: Griseofulvin Powders

ISMAT ULLAH* and DONALD E. CADWALLADER†

Abstract □ Dissolution studies were carried out on micronized and regular milled griseofulvin powders. A three-compartment dissolution apparatus previously developed by the authors was used. A barrier was used in the dissolution medium to prevent the floating masses from entering directly into the sink phase. The individual, wetted particles of these powders could pass through the barrier pores. It was demonstrated that these particles do not enter and dissolve in the sink phase due to the presence of the aqueous layer around them. The presence of a sink phase and the introduction of samples in the form of suspensions were considered necessary to obtain appropriate rank order in the dissolution rates. A modified Hixon and Crowell cube root law was applied to obtain dissolution-rate constants for two different particle-size griseofulvin powders.

Keyphrases □ Griseofulvin powders—dissolution □ Dissolution, griseofulvin powders—sink conditions □ Particle-size effect—griseofulvin dissolution □ UV spectrophotometry—analysis

The importance of dissolution studies as an index of drug availability from solid dosage forms is widely accepted. The need to maintain appropriate sink conditions to conduct suitable dissolution studies for slightly soluble drugs was mentioned in a previous paper (1). In drugs with very low solubility, the dissolution process is controlled by the interfacial rate constant. Since the interfacial rate constant is the same for each drug species, the reduction of particle size is the common approach to increase the drug availability from drug formulations. Several investigators (2–5) showed an increased absorption rate for griseofulvin when the particle size of the drug was reduced. Similar effects from particle-size reduction were noted for chloramphenicol (6) and various salts of tetracycline (7). The relationship between particle size and drug availability is well recognized and, as a result, several excellent review articles were published in the last 5 years (8–11).

The general problems (flocculation, flotation, agglomeration, and wetting) encountered in the *in vitro* dissolution-rate studies of slightly soluble powders under sink conditions were discussed in the first article (1) of this series. Although dissolution studies under sink conditions might be generally desirable, they are almost necessary for poorly soluble drugs to obtain meaningful results. Rapid saturation of the dissolution medium may not allow the visualization of even major formulation differences. The limitations of other available sink methods (12, 13) for dissolution studies of powders were mentioned earlier (1). Although the use of a diffusion cell (14) or a large volume of dissolution medium (15, 16) could be used to avoid concentration build up, a method that utilizes an organic sink phase might be of value in the dissolution studies.

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The objectives of this investigation were to demonstrate the use of a previously developed dissolution apparatus (1) to conduct dissolution studies of a very poorly soluble compound, griseofulvin, and to show

an application of the modified Hixon and Crowell cube root law for such studies.

THEORY

Parrott *et al.* (17) modified the Hixon and Crowell cube root law (18) for a single spherical particle. Niebergall and Goyan (19) extended this law to apply it to a multiparticulate system. In its integrated form, the cube root law is

$$Wo^{1/3} - Wt^{1/3} = kt \qquad \text{(Eq. 1)}$$

where Wo is the initial weight of powder, Wt the weight of the undissolved powder at time t, and k is the rate constant. This cube root law has been derived on the basic assumption that the amount of the solute needed to saturate the dissolution medium is much greater than the solute concentration in the dissolution medium, $Cs>>>>Ct$; therefore, $Cs - Ct$ can be taken as constant. If dissolution of a very poorly soluble material is carried out under appropriate sink conditions, the concentration of such a drug in the dissolution medium would essentially remain constant. Under these conditions, the modified cube root law for a multiparticulate system could be applied. A plot of $Wt^{1/3}$ *versus* t should give a straight line with a slope of k. Given various samples of the same drug with different particle sizes, the number of drug particles and conversely the surface area would differ for the same weight of powder. This difference in surface area should result in a difference in the dissolution-rate constant, k. Such a rate constant could be used an an index of drug availability from such powders and as an aid in formulation development and control.

EXPERIMENTAL

Chemicals and Materials—Regular milled and micronized griseofulvin powders were used without further sizing.[1] The *n*-butyl acetate employed was reagent grade.[2] FD&C Orange No. 2,[3] isopropyl myristate,[4] and polysorbate 80[5] were also used.

Transport Studies of FD&C Orange No. 2—The dissolution apparatus described earlier (1) was used to conduct these studies. A 200-mesh screen was used as a barrier in the dissolution medium. In the preliminary studies on the dissolution of regular and micronized griseofulvin powders, it was observed that both forms of griseofulvin formed partial suspensions in the dissolution medium under the force of agitation. These fine particles in suspension were capable of passing through the 200-mesh screen barrier. It was, therefore, necessary to determine if hydrophobic particles that can pass through this barrier can dissolve directly in the organic sink phase.

A very fine[6] FD&C Orange No. 2 powder was selected for these experiments. This dye is insoluble in water even in the presence of polysorbate 80 (0.05%) and is quite soluble in isopropyl myristate (33.75 mg./ml. at 37°). Four propellers were attached to the stirrer located in Compartment A, as described in the previous communication (1). Six hundred milliliters of distilled water containing 0.05% polysorbate 80 which was previously equilibrated to 37° was placed below the barrier in Compartment A with the help of a long-stem separator. Seven hundred and eighty milliliters of isopropyl myristate was placed in Compartments B and C (two-compartment system). Stirrers for Compartments A and B, which were previously set at 100 and 150 r.p.m., respectively, were started and the system was allowed to equilibrate for 45 min. A suspension of 250.0 mg. of FD&C Orange No. 2 was made using 1.5 ml. of dissolution medium within the sample injector by stirring with a capillary tube. A Whatman No. 1 filter paper disk was used to cover the injector opening. This suspension was injected into the dissolution medium over a period of 30 sec. One-milliliter samples were removed from the organic phase at regular intervals, and the sample volume was replaced immediately with isopropyl myristate. The samples were diluted with isopropyl myristate and assayed spectrophotometrically at 482 nm., using a Beckman DU-2 spectrophotometer.

[1] Supplied by McNeil Laboratories, Inc.
[2] Fisher Scientific Co.
[3] Magnus, Mabee and Raynard, Inc.
[4] S. B. Penick and Co.
[5] Atlas Chemical Ind.
[6] Under microscopic examination, the average size of the individual particles was found to be under 1 μ.

Table I—Transport of FD&C Orange No. 2 from Suspension in Aqueous Phase to the Organic Phase Using 200-Mesh Screen at 100 r.p.m. and 37°

Hours	Weight[a] of Dye Dissolved in Organic Phase, mg.
0.5	2.3
1.0	3.1
2.0	4.8
3.0	6.0
4.0	7.1
5.0	7.7

[a] Each value is an average of two experiments.

Dissolution of Griseofulvin Powders under Nonsink Conditions—These experiments were carried out in Compartment A. Six hundred milliliters of distilled water containing 0.05% polysorbate 80 was used as the dissolution medium. A 250-mg. sample of griseofulvin was introduced into the dissolution medium either as a dry powder or as a suspension. About 1.5 ml. of dissolution medium was removed from the top of the screen while filtering through a 0.45-μ Acropor filter[7] using a Swinnex-13 filter unit. One milliliter of this filtrate was diluted with distilled water and assayed spectrophotometrically at 295 nm., using a Beckman DU-2 spectrophotometer. The remaining filtrate was returned to the dissolution medium, and 1.0 ml. of fresh dissolution medium was forced through the filter unit to flush back the griseofulvin that might have deposited on the back of the filter.

Dissolution of Griseofulvin Powders under Sink Conditions—These experiments were carried out as described under transport of FD&C Orange No. 2, except that the organic phase used was *n*-butyl acetate. The samples were introduced either as dry powder or as a suspension. A glass cover was used to prevent evaporation of the organic phase. Samples from the aqueous phase were withdrawn from the top of the screen as described under nonsink conditions by introducing the needle through the organic phase while injecting air, filtering the sample, diluting with distilled water, and assaying as described earlier. Samples from the organic phase were removed, diluted with methanol, and assayed spectrophotometrically at 290 nm., using a Beckman DU-2 spectrophotometer. A volume of each phase equal to the sample volume was replaced immediately with appropriate solvent. The volume of the aqueous phase removed was replaced while backflushing the filtration unit.

RESULTS AND DISCUSSION

Transport of FD&C Orange No. 2 from Aqueous Suspension to Organic Phase—In transport studies on FD&C Orange No. 2, it was observed that the dye particles moved across the screen barrier immediately after the introduction of the sample. The data in Table I show that even after 5 hr., only 3.1% of the dye dissolved in the organic phase. This surprisingly low concentration in the organic phase is probably due to the fact that most of the dye particles are completely wetted in the aqueous phase, and there is a thin aqueous layer around the dispersed dye particles. These wetted particles cannot come in contact with the organic phase and therefore are not dissolved in it.

A comparison of the solubilities of FD&C Orange No. 2 in the isopropyl myristate (33.75 mg./ml. at 37°) and that of griseofulvin in *n*-butyl acetate (6.3 mg./ml. at 37°) shows that the dye has a greater solubility in its sink phase than does griseofulvin. Since there is a direct relationship between the solubility of a compound and its dissolution rate (17), there would be less tendency for griseofulvin to dissolve directly in the organic phase than for the FD&C Orange No. 2 dye.

Since griseofulvin has some solubility in the dissolution medium (18.4 mg./l. at 37°),[8] there is a greater probability for the griseofulvin

[7] The Acropor filters are reinforced with nylon cloth and can allow a two-way filtration without rupture.
[8] Solubility in dissolution medium saturated with *n*-butyl acetate is 54.0 mg./l. at 37°.

Table II—Dissolution of Micronized and Regular Griseofulvin Powders under Nonsink Conditions Using Samples as Dry Powders and Suspensions at 100 r.p.m. and 37°

Dissolution Time, min.	Weight[a] of Griseofulvin in Solution, mg.			
	Dry Powder Samples		Suspension Samples	
	Micronized	Regular	Micronized	Regular
5	8.80	8.40	10.06	10.06
10	10.03	10.05	10.07	10.06
20	10.06	10.04	10.06	10.07
30	10.06	10.06	10.06	10.07
40	10.07	10.06	10.07	10.06
60	10.06	10.06	10.06	10.06

[a] Each value is an average of two experiments.

particles to be wetted and therefore less chance for the particles that pass through the barrier to dissolve directly in the organic phase. Because of these arguments, it is felt that when dissolution studies are conducted with griseofulvin under sink conditions, using samples as dry powders or suspensions, the error involved in the dissolution data due to the direct contact of griseofulvin particles with the organic phase would be insignificant.

The experiments with the dye show that particles in suspension do not behave in the same manner as floating masses of powders. In the case of floating masses, the particles are not completely wetted; in the absence of some barrier in the dissolution medium, these masses enter the organic phase and dissolve directly in that phase. Wetted particles in suspension, even if they pass through the barrier pores, still do not dissolve in the organic phase and probably will not be a problem in dissolution studies.

The dye studies could also explain why dissolution of a drug in the gastrointestinal contents is necessary before any absorption can take place. Due to the presence of physiologic surfactant in the gastrointestinal contents (20–22) and its motility, the drug would be expected to be wetted rapidly. The wetted particles would be in contact with the gastrointestinal membranes through the wetting aqueous layer around the particles. Thus, in spite of the very fine particle size of the drug or its great solubility for the lipoid membrane, the drug would not dissolve directly into the lipoid membrane.

Dissolution of Griseofulvin Powders under Nonsink Conditions—The dissolution studies of micronized and regular griseofulvin powders under nonsink conditions (Table II) show that a rank order in the dissolution rates of the two powders was not determinable under these conditions when the samples were introduced as dry powders or as suspensions. This failure was probably due to rapid saturation of the dissolution medium in the absence of appropriate sink conditions. The data show that when dry powder samples are used, there is less dissolution in the beginning for regular powder than for the micronized powder; however, the difference is very small and probably not significant. In the case of samples introduced as suspensions, there seems to be rapid saturation of the dissolution medium for both powders, and no noticeable difference in the dissolution rates of these powders could be detected.

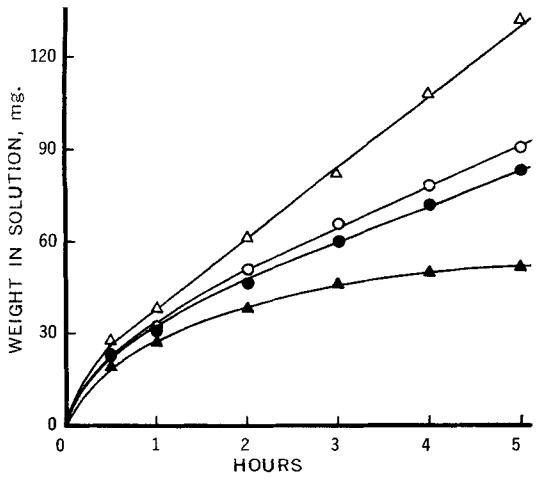

Figure 1—*Dissolution profiles of micronized and regular griseofulvin powders under sink conditions, using samples as dry powders and suspensions at 100 r.p.m. and 37°. Key: △, micronized suspension; ○, regular suspension; ●, regular dry powder; and ▲, micronized dry powder.*

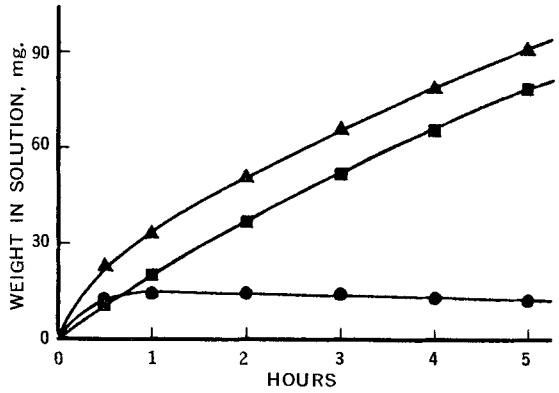

Figure 2—*Dissolution and partitioning of regular milled griseofulvin powder. Key: ●, concentration in dissolution medium; ■, concentration in n-butyl acetate; and ▲, cumulative dissolution.*

Dissolution of Griseofulvin Powders under Sink Conditions—The dissolution profiles of micronized and regular griseofulvin powders introduced as dry powders and suspensions are shown in Fig. 1. These studies show that when the griseofulvin samples were introduced as dry powders, there was a higher dissolution rate for regular griseofulvin compared to the micronized form. These results are in contrast with *in vivo* findings (2–5). This discrepancy is probably due to the micronized powder being in the form of glomerates because of the presence of greater static charge and higher surface energies. The regular powder was almost free of glomerates. When both powders were introduced into the dissolution medium as dry powders, a major portion of the regular powder was able to break up into individual particles, whereas even the presence of the lower impeller did not break up the glomerates of the micronized powder. These glomerates settled at the bottom of the dissolution vessel, giving a lower effective surface area and, therefore, a lower dissolution rate than with the regular powder.

Dissolution profiles for griseofulvin powders, when the samples were introduced in the form of a suspension (Fig. 1), show a good rank order for the two particle-size powders. Dissolution and partitioning profiles are shown in Figs. 2 and 3. These figures show almost constant concentration of griseofulvin in the dissolution medium. Under these conditions, the cumulative dissolution profiles should follow the modified Hixon and Crowell cube root law (19).

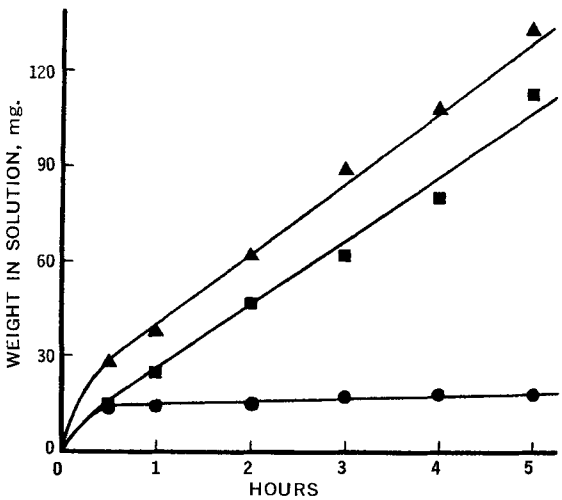

Figure 3—*Dissolution and partitioning of micronized griseofulvin powder. Key: ●, concentration in dissolution medium; ■, concentration in n-butyl acetate; and ▲, cumulative dissolution.*

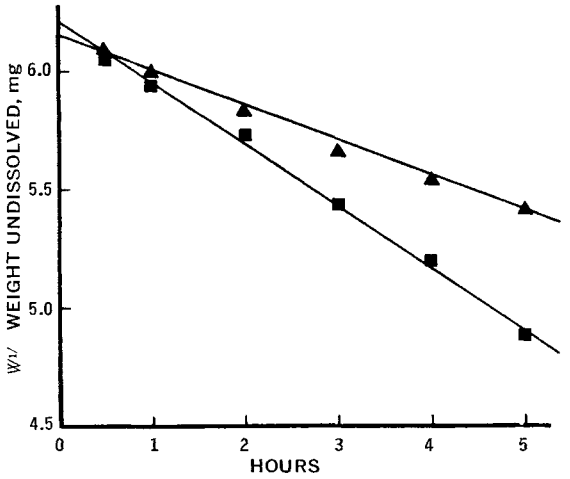

Figure 4—*Plots of* $Wt^{1/3}$ versus *time. Key:* ▲, *regular milled griseofulvin powder; and* ■, *micronized griseofulvin powder.*

Figure 4 shows good straight-line plots for the dissolution of griseofulvin powders under sink conditions when the samples were introduced in the form of suspensions. The ratio of k values is 0.56. The authors did not conduct any *in vivo* studies for these powders. Other investigators have, however, reported that the micronized griseofulvin is twice as well absorbed as the regular milled powder (2). The ratio of rate constant from present studies is in general agreement with these *in vivo* studies. This was possible due to two factors. First, while making the suspensions, the glomerates were broken up and both sized powders were readily dispersed in the dissolution medium, thus making relative surface areas available for dissolution. Second, there was no saturation of the dissolution medium due to the presence of sink conditions.

In the present studies, a 200-mesh screen was used as a barrier in the dissolution medium. Although the wetted particles of the powders under study could pass through the barrier pores, the barrier was needed to prevent the direct dissolution of the powders in the sink phase. Preliminary studies without this screen barrier showed that the powders could enter directly into the organic phase when introduced as dry powders or wetted suspensions. This was due to the entrapment of air in glomerates in the case of dry powders and to a slight foam formation while making suspensions of the samples. The use of a screen barrier for suspension samples was also needed to keep the geometry of the dissolution system the same throughout the study.

These studies demonstrate a successful use of the previously developed apparatus (1) for dissolution studies of very sparingly soluble fine powders under sink conditions. The studies show a need for sink conditions to differentiate the dissolution patterns of different powders such as griseofulvin. These studies also demonstrate a technique to overcome the problems due to excessive agglomeration in the dissolution of such fine powders. In general, a rate constant would more fully describe the dissolution process than do dissolution profiles or some points on the profile (such as t_{30} or t_{50}). Dissolution profiles can only give a qualitative rank order but not a single quantitative value which could be used for correlation with *in vivo* data. Single points on the dissolution profile would not describe the dissolution behavior on either side of such points. Although a dissolution rate constant is often difficult to obtain for such multiparticulate systems, these studies show that such a rate constant is possible to obtain for such powders when studies are conducted under appropriate sink conditions. The rate constant obtained in these studies using the modified Hixon and Crowell cube root law (19) might be a useful parameter for comparing the dissolution characteristics of the powders. The apparatus developed by the authors (1) allows a great flexibility and versatility for such studies.

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Effect of Buffer Constituents and Time on Drug Transfer across *In Vitro* Rat Intestine

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Abstract □ The *in vitro* absorption kinetics for salicylate ion and acetanilide were followed in six different buffer solutions over 2-hr. periods. Both everted and noneverted absorption rates were measured during four consecutive periods of 30-min. duration. Comparisons were made between initial rates, rates over the 4–30-min. periods, total amounts of drug transferred, lag time of initial runs, and increase of rates over the 2-hr. experiment (as percent of initial rate). By using this latter statistic as a measure of the loss in integrity of the intestinal tissue, Krebs bicarbonate pH 7.4 buffer was found to provide the best environment for maintenance of the *in vitro* membrane in a constant, viable condition. A sodium phosphate buffer and a tromethamine buffer were also found usable, although less effective than the Krebs buffer. In isotonic sodium chloride, serosal transfer rates of salicylate ion increased sharply while mucosal transfer rates remained constant over the 2-hr. experiment. Acetanilide, a nonionized drug, showed little increase in either mucosal or serosal transfer rates in this medium.

Keyphrases □ Salicylate, acetanilide transfer—rat intestine, *in vitro* □ Buffer, time, effects—drug transfer, rat intestine □ Intestine, everted, noneverted—absorption kinetics □ UV spectrophotometry—analysis

Recent studies by Turner *et al.* (1) and Turner and Benet (2) pointed out that drug ions show a difference in directional permeability coefficients in the *in vitro* rat intestine, with mucosal to serosal transfer occurring at faster rates than serosal to mucosal transfer. It appears that the difference in directional permeability coefficients might be related to a sodium-ion coupled transport; when sodium was completely replaced by potassium in phosphate buffer solutions, the difference in permeability coefficients tended to disappear. Nonionized drugs did not show a difference in directional permeability coefficients in either sodium or potassium phosphate buffers.

Mayersohn and Gibaldi (3, 4) showed that replacement of sodium ion by potassium ion in Krebs bicarbonate buffer significantly reduced the passive transfer of several water-soluble drugs (both ionized and nonionized) across the everted rat intestine. They also showed that: (*a*) ouabain (10^{-3} *M*) had no effect on the transfer process; (*b*) replacing sodium ion by ammonium ion or lithium ion in the buffer solution produced effects on selected drugs similar to those seen with potassium ion; and (*c*) the positively charged tromethamine[1] ion did not affect the rate of transfer. The inhibition of transfer with the various cations appeared to correlate well with the degree of tissue fluid uptake in the intestine.

Mayersohn and Gibaldi (3, 4) employed a modified Crane and Wilson technique (5), measuring the total amount of drug transferred in a 2-hr. period. The work in this laboratory measured the initial rates of absorption (during a 35-min. period) using a perfusion apparatus (1).

[1] Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol].

The present study was undertaken to examine time effects on the rates of transfer through everted and noneverted segments of the rat intestine, and the effect of different buffer constituents on these rates.

EXPERIMENTAL

Materials—The following USP, NF, or reagent grade chemicals were employed: salicylic acid, acetanilide, tromethamine, monobasic sodium phosphate with one water of hydration, dibasic sodium phosphate anhydrous, monobasic potassium phosphate anhydrous, dibasic potassium phosphate anhydrous, sodium chloride, potassium chloride, calcium chloride, magnesium sulfate with seven waters of hydration, sodium bicarbonate, potassium hydroxide, a 100% oxygen gas, and oxygen–carbon dioxide gas mixture (95:5).

Procedure—Full strength Krebs–Henseleit buffer (Krebs bicarbonate buffer), pH 7.4, was prepared as described in the literature (6). The two modified Krebs bicarbonate buffers utilized were: (*a*) ½ Na^+ replaced by K^+, and (*b*) all of the Na^+ replaced by K^+. The pH 7.4 sodium phosphate buffer was prepared as previously described (1). The 0.154 *M* tromethamine buffer (pH 7.4) and the 0.9% sodium chloride (pH 6.6) solutions were prepared in the usual manner. The pH values of the solutions used were routinely checked at the beginning and end of each experiment on a Beckman research pH meter.

Male Sprague-Dawley rats, weighing 220–250 g., were fasted for 24 hr. prior to the experiment but water was allowed *ad libitum*. The rats were rendered unconscious with carbon dioxide in an airtight chamber and were then sacrificed by a sharp blow at the base of the skull. The small intestine (from the pyloric junction to about the middle of the jejunum) was removed at once *via* a midline abdominal incision and flushed with 40 ml. of the buffer (warmed) to be used in the absorption study. The first 15-cm. segment was discarded and the next 2–12.5-cm. segments (stretched length using a 20-g. weight) were employed, with the proximal segment being everted alternately. The *in vitro* absorption kinetics for salicylic acid and acetanilide (in various buffers) were followed using perfusion devices and techniques previously described (1), except that each intestinal segment was used for four consecutive experiments with the solutions changed after each run. At appropriate intervals, 0.1-ml. samples were removed for assay.

For salicylate-ion transfer, the time for the experiment was 2 hr. and 5 min. For acetanilide transfer in 0.9% sodium chloride, rates were measured during four consecutive 15-min. periods. In the Krebs bicarbonate buffers, however, absorption measurements were made during the following time periods: initial rate, 0–15 min.; rate 2, 30–45 min.; rate 3, 60–75 min.; and rate 4, 105–120 min. Fifteen-minute transfer experiments were necessary in the acetanilide studies to correspond with requirements described previously (1) so that transfer would appear to follow unidirectional zero-order kinetics.

Assay Procedures—The 0.1-ml. samples were diluted and assayed as follows. Acetanilide was diluted with 1.0 ml. pH 7.4 isotonic buffer, and the concentration was determined by UV measurement at 239 nm. on a Beckman DB-G spectrophotometer. Salicylic acid was diluted with 5.0 ml. of 0.1 *N* sodium hydroxide or potassium hydroxide, and the drug concentration was determined on an Aminco-Bowman spectrophotofluorometer at an excitation wavelength of 300 nm. and an emission wavelength of 408 nm. (uncorrected). The choice of diluent depended upon the presence or absence of sodium ion in the environment. Standard curves for each drug were made using five known concentrations of the drug in the appropriate diluent. As in previous experiments (1, 5), no significant difference in transfer rate was observed between the first and second segments of intestine.

Table I—Summary of Transfer Data ± Standard Deviation for Salicylate Ion in Krebs Bicarbonate pH 7.4 Buffers Containing Varying Amounts of Sodium Ion

	No.	Everted (E)	Noneverted (NE)	Ratio	Level of Significance E *versus* NE
Full strength sodium ion					
A. Average rates—all runs (mcg./ml./min.)	7	1.65 ± 0.50	0.92 ± 0.28	1.90 ± 0.83	$p < 0.01$
B. Average initial rates (mcg./ml./min.)	7	1.59 ± 0.59	1.01 ± 0.30	1.62 ± 0.72	$p < 0.05$
C. Average rate increase (4th rate/initial rate) as %	6	109 ± 20	82 ± 19		N.S.[a]
D. Average lag time—initial runs (min.)	7	5.2 ± 1.7	6.5 ± 0.8		N.S.
F. Total amounts transferred —average (mg.)	6	3.12 ± 0.97	1.84 ± 0.59	1.82 ± 0.85	$p < 0.05$
One-half sodium replaced					
A.	12	1.26 ± 0.34	1.07 ± 0.24	1.17 ± 0.24	$p < 0.05$
B.	12	0.87 ± 0.36	0.64 ± 0.20	1.52 ± 1.02	N.S.
C.	12	201 ± 98	283 ± 179		N.S.
D.	12	7.6 ± 1.9	8.8 ± 1.4		N.S.
F.	12	2.36 ± 0.64	2.05 ± 0.43	1.16 ± 0.21	$p < 0.05$
All sodium replaced					
A.	8	0.62 ± 0.53	0.52 ± 0.27	1.26 ± 1.08	N.S.
B.	8	0.28 ± 0.40	0.22 ± 0.20	1.14 ± 0.55	N.S.
C.	8	660 ± 531	697 ± 446		N.S.
D.	8	—[b]	—[b]		
F.	8	1.55 ± 1.07	1.29 ± 0.99	1.42 ± 0.97	N.S.

[a] Not significant. [b] Because some of the initial rates approached zero, the lag times approached $-\infty$, and the averages appear meaningless.

Plots were made of concentration *versus* time; rates of transfer, intercepts, and ratios were obtained by the use of an unweighted least-squares program executed on a Hewlett-Packard model 9100A desk calculator. Although there was often an increase in the absorption rate (slope of concentration–time plot) during the four consecutive runs on each intestinal segment, the regression coefficients for all slopes were as high as those reported previously (1) in the single-run study, that is >0.980 in every case, with the majority >0.995. Using the everted (E) and noneverted (NE) data, the following calculations were made: A, the average E and NE rates and the E/NE ratios for all determinations on a particular drug in a particular buffer; B, the average initial rates (E and NE) and ratios (E/NE), *i.e.*, the first rate measured on each intestinal segment; C, the average increase in rates over the time of the experiment as a percent of the initial rate [*i.e.*, (4th rate/1st rate) × 100]; D, the average *X*-intercept or lag time observed with initial rate measurements; and F, the average total amount of drug transferred across the gut during the time for the experiment (four runs).

RESULTS AND DISCUSSION

The summary of the data is presented in Tables I and II for salicylate ion and in Table III for nonionized acetanilide. In one of the Krebs bicarbonate (full strength sodium ion) studies in Table I, the gut leaked during the fourth consecutive time period, thereby resulting in only six values for C (average rate increase) and F (total amounts transferred). The values for D (average lag time–initial runs) in the "all sodium replaced" studies in Table I have been omitted, since some of the initial rates yielded zero transfer of drug, resulting in an apparent lag time of $-\infty$, and, consequently, a meaningless average. The ratios reported in Tables I–III do not exactly correspond to the everted average values divided by non-

Table II—Summary of Transfer Data ± Standard Deviation for Salicylate Ion in Three Isotonic Solutions

	No.	Everted (E)	Noneverted (NE)	Ratio	Level of Significance E *versus* NE
Sodium phosphate pH 7.4 buffer					
A. Average rates—all runs (mcg./ml./min.)	9	2.13 ± 0.47	1.82 ± 0.47	1.20 ± 0.22	$p < 0.05$
B. Average initial rates (mcg./ml./min.)	9	1.79 ± 0.39	1.37 ± 0.25	1.32 ± 0.19	$p < 0.005$
C. Average rate increase (4th rate/initial rate) as %	9	140 ± 20	157 ± 27		N.S.
D. Average lag time for initial runs (min.)	9	6.1 ± 1.6	6.8 ± 3.1		N.S.
F. Average total amounts transferred (mg.)	9	4.04 ± 0.74	3.52 ± 0.82	1.17 ± 0.18	N.S.
0.154 *M* Tromethamine buffer, pH 7.4					
A.	5	1.33 ± 0.25	1.25 ± 0.20	1.09 ± 0.29	N.S.
B.	5	1.03 ± 0.22	0.83 ± 0.16	1.26 ± 0.21	$p < 0.05$
C.	5	160 ± 20	183 ± 55		N.S.
D.	5	4.9 ± 0.2	7.4 ± 1.7		$p < 0.05$
F.	5	2.51 ± 0.37	2.42 ± 0.42	1.07 ± 0.24	N.S.
0.9% Sodium chloride, pH 6.6					
A.	5	1.68 ± 0.19	1.20 ± 0.45	1.58 ± 0.59	N.S.
B.	5	1.68 ± 0.30	0.74 ± 0.36	2.68 ± 1.14	$p < 0.01$
C.	5	113 ± 21	242 ± 104		$p < 0.05$
D.	5	4.9 ± 3.0	6.7 ± 3.6		N.S.
F.	5	3.32 ± 0.45	2.41 ± 0.78	1.50 ± 0.50	N.S.

Table III—Summary of Transfer Data ± Standard Deviation for Acetanilide in Three Isotonic Solutions

	No.	Everted (E)	Noneverted (NE)	Ratio	Level of Significance E *versus* NE
0.9% Sodium chloride, pH 6.6					
A. Average rates—all runs (mcg./ml./min.)	3	4.42 ± 0.65	4.11 ± 0.65	1.08 ± 0.09	N.S.
B. Average initial rates (mcg./ml./min.)	3	4.26 ± 0.75	3.94 ± 0.55	1.08 ± 0.07	N.S.
C. Average rate increase (4th rate/initial rate) as %	3	115 ± 25	101 ± 6		N.S.
D. Average lag time for initial runs (min.)	3	0.74 ± 0.36	0.87 ± 0.49		N.S.
F. Average total amounts transferred (mg.)	3	4.16 ± 0.58	3.93 ± 0.62	1.06 ± 0.05	N.S.
Krebs bicarbonate—full strength sodium ion					
A.	5	3.88 ± 0.75	3.49 ± 0.34	1.11 ± 0.19	N.S.
B.	5	3.79 ± 0.64	3.66 ± 0.42	1.05 ± 0.27	N.S.
C.	5	111 ± 10	107 ± 17		N.S.
D.	5	0.93 ± 2.0	0.53 ± 2.1		N.S.
F.	5	4.48 ± 0.65	4.19 ± 0.53	1.08 ± 0.21	N.S.
Krebs bicarbonate—all sodium replaced					
A.	4	3.82 ± 0.37	4.79 ± 1.23	0.83 ± 0.16	N.S.
B.	4	3.08 ± 0.26	4.22 ± 0.92	0.75 ± 0.15	N.S.
C.	4	148 ± 32	124 ± 42		N.S.
D.	4	0.45 ± 1.32	−1.30 ± 2.94		N.S.
F.	4	4.45 ± 0.60	6.07 ± 1.10	0.74 ± 0.06	$p < 0.02$

everted average values, since the ratio values reported are the average of ratios calculated for each individual run. As explained previously (1, 2), it is hoped that this method of treatment will cancel out the biological variability of differences in absorption rates due to the individual physiological characteristics seen in a number of different rats.

The level of significance for each of the five averages (A through F) used to compare transfer through everted and noneverted segments is also reported in Tables I–III. It can be seen that significant differences (at 95% or greater confidence levels using Student's *t* test for paired data) are observed for salicylate ion in Krebs bicarbonate (full strength sodium ion), 0.9% sodium chloride, and sodium phosphate buffers. No significant difference is seen between everted and noneverted rates for the nonionized acetanilide. These results are in accord with those reported previously (1, 2), where differences in directional permeability coefficients were observed in initial rate studies for ionized drugs in the presence of sodium ions. However, in this study, a significant difference in directional transport for salicylate ion was also seen in the initial rate studies with tromethamine buffer, suggesting that a sodium couple is not involved in the difference in directional transport and that the results seen previously (1) with sodium and potassium phosphate buffers might be better explained in terms of the effects of phosphate on the intestinal mucosa (2).

Qualitatively, it can be seen that when the values in row C are close to 100% (*i.e.*, no change between initial and fourth rate), the ratios calculated by all three methods (A, B, and F) are almost identical. If one considers the change in rates (percent of initial) over the four experiments as a measure of the loss of biologic integrity of the intestinal tissue, it would appear that the best buffer for maintenance of the membrane in a constant condition is the Krebs bicarbonate (full strength sodium) buffer. In comparison, the sodium phosphate and tromethamine buffers appear to have less ability to maintain gut viability but are superior to unbuffered 0.9% sodium chloride solutions or the modified Krebs buffers employed. In unbuffered 0.9% sodium chloride solution, there is a sharp rise in the serosal (NE) transfer of salicylate ions as compared to mucosal (E) transfer (242 and 113% of initial, respectively, as shown in Table II), while for acetanilide, a nonionized drug, there is very little change in the rate of passage in either direction (Table III).

The lag time before absorption of salicylate ion begins falls into the range of 5–7 min. for all of the buffers, with the exception of the Krebs bicarbonate buffers where potassium replaced sodium. However, the nonionized acetanilide yields lag times that are not significantly different than zero (at 95% confidence levels) for all three of the buffers studied. The results indicate that the lag time for salicylate-ion absorption is 7 to 8 times greater than that for acetanilide.

The difference between average initial rates (B) and the average of all rates (A) is presented in Table IV. A good correlation may be seen between the values taken as representing intestinal integrity (C values in Tables I–III) and the level of significance of the differences between the two sets of measurements compared in Table

Table IV—Comparison of Levels of Significance for Differences between Average Initial Rates and Average Rates for Four Consecutive Runs

Drug	Buffer	Level of Significance: Between Everted Rates	Level of Significance: Between Noneverted Rates
Salicylate	Krebs bicarbonate—full strength sodium ion	N.S.	N.S.
	Krebs bicarbonate—one-half sodium ion replaced	$p < 0.001$	$p < 0.001$
	Krebs bicarbonate—all sodium ion replaced	$p < 0.01$	$p < 0.001$
	Sodium phosphate, pH 7.4 buffer (1)	$p < 0.002$	$p < 0.005$
	0.154 *M* Tromethamine buffer, pH 7.4	$p < 0.002$	$p < 0.05$
	0.9% Sodium chloride, pH 6.6	N.S.	N.S.
Acetanilide	Krebs bicarbonate—full strength sodium ion	N.S.	N.S.
	Krebs bicarbonate—all sodium replaced	$p < 0.05$	N.S.
	0.9% Sodium chloride, pH 6.6	N.S.	N.S.

Table V—Level of Significance for Differences between Average Initial Everted Transfer Rates and between Total Amounts Transferred for Salicylate Ion and Acetanilide in Different Buffers

Drug	Buffer 1	Buffer 2	Average Initial Everted Rates	Total Amounts Transferred (E)
Salicylate	Krebs bicarbonate—full strength sodium	Krebs bicarbonate—one-half sodium replaced	$p < 0.01$	$p < 0.1$
	Krebs bicarbonate—full strength sodium	Krebs bicarbonate—all sodium replaced	$p < 0.001$	$p < 0.02$
	Krebs bicarbonate—full strength sodium	Sodium phosphate, pH 7.4	N.S. ($p < 0.8$)	$p < 0.1$
	Krebs bicarbonate—full strength sodium	0.154 *M* Tromethamine, pH 7.4,	$p < 0.05$	N.S. ($p < 0.2$)
	Krebs bicarbonate—full strength sodium	0.9% Sodium chloride, pH 6.6	N.S. ($p < 0.5$)	N.S. ($p < 0.5$)
	Sodium phosphate, pH 7.4	0.154 *M* Tromethamine, pH 7.4	$p < 0.001$	$p < 0.001$
	Sodium phosphate, pH 7.4	0.9% Sodium chloride, pH 6.6	N.S. ($p < 0.5$)	$p < 0.05$
	0.154 *M* Tromethamine, pH 7.4	0.9% Sodium chloride, pH 6.6	$p < 0.005$	$p < 0.02$
Acetanilide	Krebs bicarbonate—full strength sodium	Krebs bicarbonate—all sodium replaced	$p < 0.02$	N.S. ($p < 0.5$)
	Krebs bicarbonate—full strength sodium	0.9% Sodium chloride, pH 6.6	N.S. ($p < 0.2$)	N.S. ($p < 0.2$)
	Krebs bicarbonate—all sodium replaced	0.9% Sodium chloride, pH 6.6	$p < 0.05$	N.S. ($p < 0.5$)

IV. For example, in Krebs bicarbonate (full strength sodium ion) where intestinal membrane integrity is assumed to be the best, there is no difference between average rates obtained from initial rate–30-min. experiments or from 2-hr. experiments. However, whenever the sodium ion is replaced (in the buffers studied here), the rates increase with time, and the absolute values of the rates differ significantly between the 30-min. and 2-hr. experiments. A significant difference is also observed in the comparison of everted rates for acetanilide in Krebs bicarbonate buffer where all the sodium was replaced, indicating that the presence of potassium has a deteriorating effect on the membrane which affects the transport of the unionized acetanilide as well as the ionized salicylate.

Table V presents a comparison between two methods utilized to determine the effect of buffer constituents on *in vitro* drug absorption [*i.e.*, initial rates used in this laboratory (1, 2) and total amounts transferred as used by Mayersohn and Gibaldi (3, 4)]. From the data in Table V, it appears that under the conditions used in this work, the average of initial rates is a more sensitive measure than the total amounts transferred, since the average initial rate is not influenced to the same degree by the *in vitro* degradation of the gut membrane. When there is a significant difference between rates in any two buffers, the level of significance is generally greater for the average initial rate data (values of $p < 0.1$ considered significant here). The cases where there is no significant difference also seem to be better demonstrated by the average initial rate data, as seen by comparison of the values in parentheses in Table V. One particularly interesting comparison is the rate of salicylate-ion transfer in Krebs bicarbonate (full strength sodium-ion buffer) *versus* the rate in 0.154 *M* tromethamine buffer. Mayersohn and Gibaldi (4) reported no significant difference between total amounts of salicylate transferred in these two buffers. Similarly, in the present work, no significant difference was found using total amounts transferred. However, there was a difference between the two buffers at the 95% confidence level when initial transfer rates were employed. It must be emphasized, however, that these discrepancies may be a result of the methodology employed in this work, since relative values of the standard deviations reported for total amounts transferred in this work are greater than those reported by Mayersohn and Gibaldi (3, 4).

An interesting comparison can be made with respect to the effects of replacing sodium by potassium on the transport of the two drugs studied. In Table I it can be seen that the replacement of sodium with potassium in Krebs bicarbonate causes a 60–80% reduction (depending on whether the A or B values are compared) in the transport of salicylate ion, while in Table III only a 15–30% reduction is observed for nonionized acetanilide. As was pointed out initially by Bosackova and Crane (7) and recently by Mayersohn and Gibaldi (4), the uptake of water by the intestinal membrane increases as a function of the potassium ion causing a marked increase in the swelling of the tissues. In the present work, this led to a very significant decrease in the transport of the ionized salicylate but only to a slight decrease in the transport of the nonionized acetanilide. These results might be intepreted as indicating that the two drugs were transported through different pathways. Possibly the salicylate ion diffuses through aqueous pores, as is suggested by Crone and Keen (8) for the pyridinium aldoximes, and that in the presence of potassium where the membrane is swollen, these pores are effectively blocked. However, acetanilide might diffuse through the lipid portions of the membrane and be affected to a lesser extent by the amount of water in the tissues. In either case, an increase in transfer rates with time is seen as the membrane deteriorates in the presence of potassium. These suggestions are made with some hesitation, however; although Mayersohn and Gibaldi (4) found a similar percent inhibition of the salicylate transfer in the presence of large amounts of potassium as was found in this work for the 2-hr. experiments, they found even greater inhibition for the nonionized drugs, riboflavin and sulfanilamide. Work is continuing in this area in an attempt to explain these differences.

Recently, Levine *et al.* (9) pointed out that everted intestinal rat sacs are morphologically intact after inversion but that they progressively lose structural integrity in an oxygenated Krebs–Henseleit buffer, pH 7.4, containing 1 g. glucose/l. Histological studies indicate that there is total disruption of the epithelial border after 1 hr. Since no significant increase in the everted transfer rates of either salicylate or acetanilide was observed in this buffer (without glucose) in the present work, it would appear that the epithelial border may not be the rate-limiting barrier to transport across the *in vitro* rat intestinal segment. If this was true, the use of the everted intestinal sac as a screen for the possible absorption characteristics of a new drug would appear questionable. This was pointed out in the previous report (1) with respect to some ionized drugs.

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CHIMIN SUNWOO* and HENRY EISEN

Abstract □ This investigation was primarily concerned with the application of Hildebrand's solubility equation to the determination of the solubility parameter of selected sulfonamides. The vapor pressure of several selected solvent systems was determined by an isoteniscopic method. These data were used to calculate the solubility parameter of the mixed solvents. The heat of fusion of each sulfonamide was determined by differential thermal analysis. The solubility of the sulfonamides was measured in a series of solvent blends varying in polarity, and the solubility parameter of the sulfonamides was determined from their solubility data. An attempt was made to correlate the solubility parameter with the dielectric constant. The results were found to be linear with respect to the solvent blends of alcohol–water, alcohol–water–propylene glycol, water–glycerin, and dimethylacetamide–water–glycerin. Hildebrand's solubility concept appears to be a useful tool for predicting drug solubility where the solubility parameters of drug and solvent system are close to each other.

Keyphrases □ Sulfonamides—solubility parameter □ Solubility, solubility parameter—sulfonamides □ Isoteniscopic method—solvent vapor pressure determination □ Differential thermal analysis—heat of fusion, sulfonamides □ UV spectrophotometry—analysis

The wide application of liquid dosage forms used in pharmaceuticals is demonstrable proof of the importance of solutions in formulation. A convenient and reliable means of determining enhanced solubility of pharmaceutical substances has long been sought by formulation workers. The concept of solubility parameters has been found to be useful, particularly in guiding the selection of solvents for film formers and in the formulation of paints, varnishes, and printing inks (1). Hildebrand's solubility equation (2) was applied by Chertkoff and Martin (3) and by Restaino and Martin (4) to determine the solubility of benzoic acid

Table I—Heat of Vaporization and Solubility Parameter of Solvents Employed

Solvent	Slope	ΔH_v, kcal./mole at 25°, Determined	ΔH_v, kcal./mole at 25°, Reported Value	δ at 25°, Determined	δ at 25°, Reported Value
Water, double-distilled	−2250 ± 6	10.296	9.730[a]	23.2	23.4[c]
Absolute alcohol	−2136 ± 5	9.765	9.22[a]	12.5	—
Absolute alcohol, distilled	−2233 ± 5	10.217	10.08[b]	12.8	12.7[c] 13.0[d]
Propylene glycol	−2700 ± 7	12.357	—	12.6	—
Dimethyl-acetamide	−2297 ± 4	10.454	—	10.3	10.6[e]
2-Ethoxy-ethanol	−2516 ± 7	11.748	—	10.7	9.9[c]

[a] *Reference 13.* [b] *Reference 14.* [c] *Reference 15.* [d] *Reference 4.* [e] *Reference 16.*

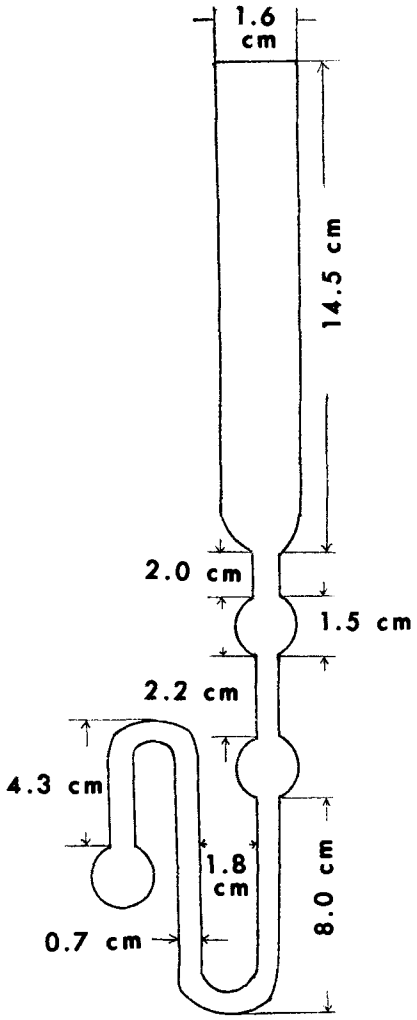

Figure 1—*Schematic diagram of the isoteniscope used for vapor pressure determination.*

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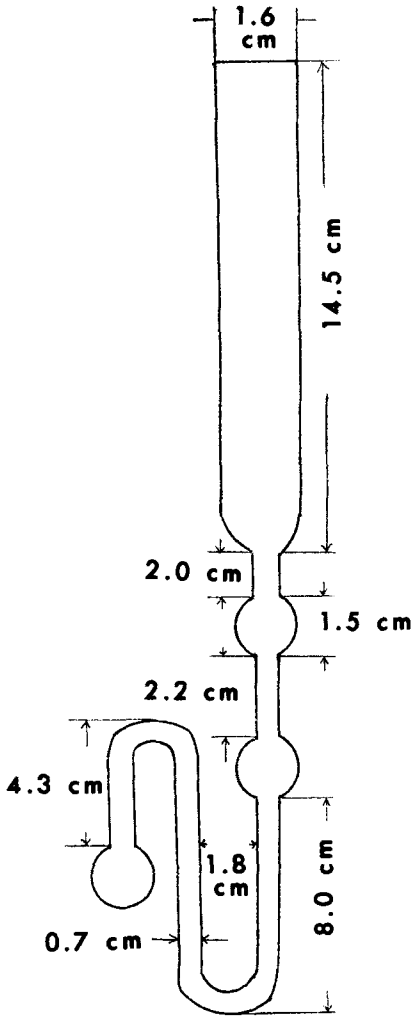

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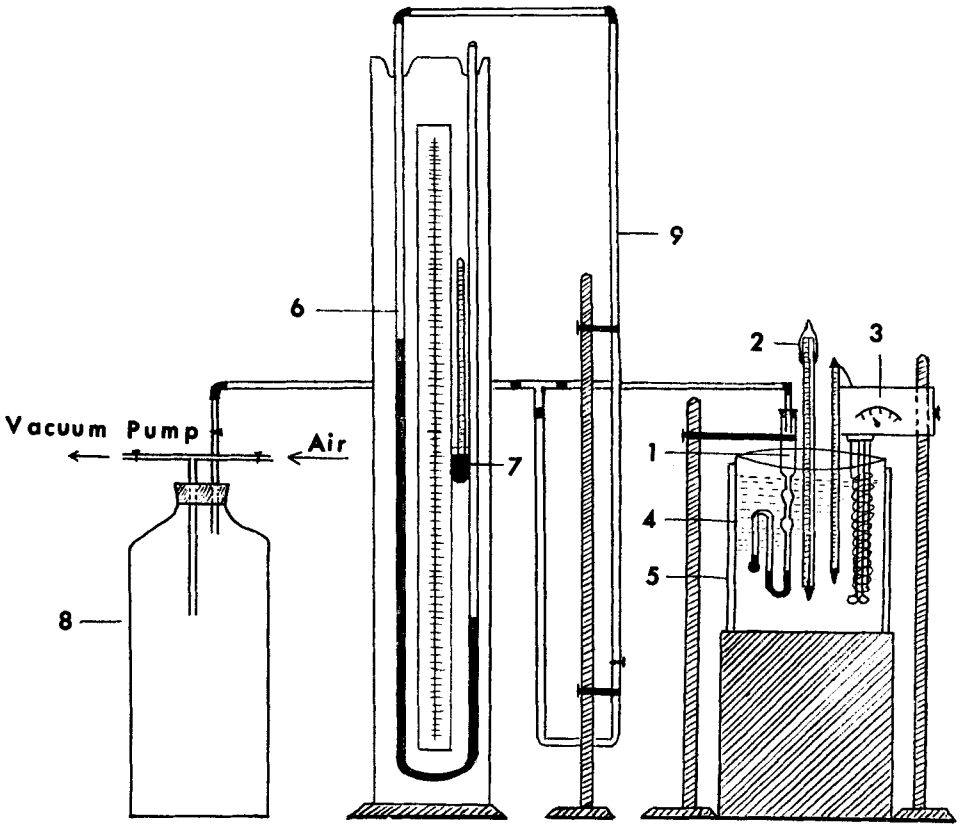

Figure 2—*Schematic diagram of the apparatus used for vapor pressure determination. Key: 1, isoteniscope; 2, differential thermometer; 3, constant-temperature circulator; 4, crystallization jar; 5, metal bath jacket; 6, open-tube manometer; 7, differential thermometer; 8, ballast bottle; and 9, glass tubing.*

and the esters of *p*-hydroxybenzoic acid in a series of *n*-alkanols. Gorman and Hall (5) studied the relationship of the dielectric constant with solubility, solubility parameters, and the application of an approximate dielectric constant to cosolvent systems (6).

This study was also concerned with the applicability of Hildebrand's solubility parameter concept. The solubilities of several sulfonamides in polar solvent blends were determined.

To apply the equation, the heat of vaporization of the solvents, the heat of fusion, the molar volumes of the sulfonamide, and the solubility of each solid were also determined.

EXPERIMENTAL

Solvents—The following were used: water, double-distilled; alcohol USP;[1] absolute alcohol USP[1] (distilled and treated with calcium oxide); propylene glycol USP;[2] dimethylacetamide[3] (DMAC, technical grade); 2-ethoxyethanol[4] (industrial grade); and glycerin USP.[5]

Sulfonamides—Sulfonamides used were recrystallized from supersaturated solution: sulfathiazole[6] form I (7, 8) from distilled water; sulfathiazole[6] form II (7, 8) and sulfadiazine USP[6] from warm acetone; and sulfamerazine USP,[6] sulfamethazine USP,[6] sulfisomidine,[7] sulfameter[8] (8), sulfamethoxazole,[9] and sulfisoxazole[9] from warm alcohol.

[1] U. S. Industrial Chemical Co., New York, N. Y.
[2] Ruger Chemical Co., Irvington, N. Y.
[3] E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.
[4] Cellosolve solvent, Union Carbide Corp., New York, N. Y.
[5] Fisher Scientific Co., Fair Lawn, N. J.
[6] American Cyanamid Co., Pearl River, N. Y.
[7] Ciba Pharmaceutical Co., Summit, N. J.
[8] A. H. Robins Co., Richmond, Va.
[9] Hoffmann-La Roche, Inc., Nutley, N. J.

Procedure—*Determination*[10] *of Heat of Vaporization of Solvents from Vapor Pressures*—The modified static isoteniscope (Fig. 1) (9) was used to determine vapor pressures of all solvents except glycerin. The liquid to be determined was placed in the isoteniscope shown in Fig. 2 so that the bulb was almost full. The U-tube was filled with about 3 cm. of liquid. The two bulbs above the U-tube prevent the ascent of the confining liquid by suction in the U-tube. The isoteniscope was connected to the ballast bottle which regulates the pressure.

The manometer was then filled with mercury. For all connections, glass tubing and high pressure rubber were used. They were tightened with the aid of copper wire. To determine the vapor pressure, the isoteniscope was immersed in the heating bath filled with water (10) for solvents boiling below 100°. For solvents boiling above 100°, light mineral oil was used as the bath liquid. A differential thermometer was placed in the mercury tube and suspended along the gauge of the barometer and between the arms of the manometer tube to read the nearest temperature of the mercury in the manometer indirectly.

A very thin wire was attached to the outside of the glass thermostat vessel to eliminate any error due to parallax in reading. Air was removed from the bulb by lowering the pressure in the ballast bottle until the liquid in the bulb boiled at a reasonable rate. After approximately 3 min., the pressure was raised until the boiling stopped and the liquid levels in both arms of the U-tube were equal. Then the temperature of the system and the ascending and descending pressures from the zero point were recorded. The barometric pressure was recorded at the same time. This procedure was repeated to ensure complete removal of air in the system. By heating the thermostat bath, new pressures and temperatures were recorded at approximately 5° intervals. The liquid levels in the U-tube were kept approximately equal by manipulating two stopcocks on the ballast bottle. All manometer and barometer readings were corrected to 0° by multiplying the factor $(1 - 1.8 \times 10^{-4})$ (11), where it is the centigrade temperature of the manometer and barometer.

[10] All experimental values reported in this work represent the mean of three determinations.

Table II—Heat of Fusion of Selected Sulfonamides Employed

Sulfonamide	Molecular Weight	Fusion Temperature	Heat of Fusion, ΔH_f, cal./mole
Sulfathiazole			
Form I	255.33	164°[a]	(1420 ± 40)[c]
		(161°)[b]	1552 ± 25[d]
Form II	255.33	201.5°	6247 ± 220
		(200°)[b]	(5970 ± 230)[b]
Sulfamerazine	264.31	242.0°	7541 ± 70
Sulfamethazine	278.33	198.5°	7438 ± 170
Sulfisomidine	278.34	250.4°	10,194 ± 170
Sulfadiazine	250.28	265.6°	7464 ± 180
Sulfameter	280.32	213.5°	8255 ± 14
Sulfamethoxazole	253.31	166.3°	6852 ± 110
Sulfisoxazole	267.30	195.0°	6990 ± 13

[a] Temperature of transition. [b] Reported value (19). [c] Heat of transition reported (19). [d] Heat of transition.

By employing the Clausius-Clapeyron equation, the slopes were calculated by the linear least-squares treatment of these data using a library program, STAT 9A, available on the Control Data Multi-Access Computer (System 420). From these slopes the heat of vaporization for each solvent was calculated. The compressibility factor was taken as unity for these calculations. Solubility parameters for the solvents (Table I) were calculated, using the following equation (12):

$$\delta = \frac{H_v - RT^{1/2}}{V^1} \qquad \text{(Eq. 1)}$$

where δ = solubility parameter, H_v = molar heat of vaporization, R = gas constant, T = absolute temperature, and V^1 = molar volume.

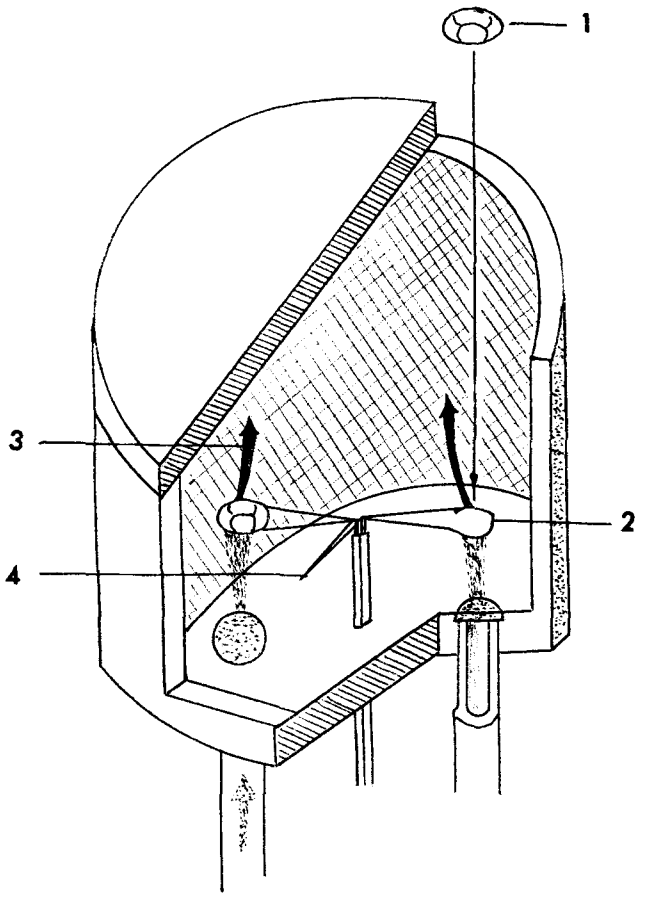

Figure 3—*Schematic diagram of the sample holder of differential thermal analyzer. Key: 1, sample dish; 2, differential thermocouple, bead; 3, flooding gas; and 4, reference thermocouple, bead.*

Table III—Apparent Molar Volumes at 25°

Sulfonamide	V_2, ml./mole
Sulfathiazole, form II	162
Sulfamerazine	186
Sulfamethazine	197
Sulfisomidine	195
Sulfadiazine	156
Sulfameter	183
Sulfamethoxazole	176
Sulfisoxazole	188

The solubility parameter value (δ = 16.5) for glycerin was taken from Burrell (15). Equation 2 (15) was employed to calculate the solubility parameter for mixed solvents:

$$\delta_{mix} = (x_1V_1\,\delta_1 + x_2V_2\delta_2)/(x_1V_1 + x_2V_2) \qquad \text{(Eq. 2)}$$

where x = mole fraction and V = molar volume.

Determination of Heat of Fusion—The determinations of the heat of fusion of the sulfonamides were made using a differential thermal analyzer.[11]

The sample holder assembly consists of two separate platinel differential thermocouples and a platinel furnace couple which is utilized for programming the furnace (Fig. 3). The instrument was calibrated by the method reported by David (17) utilizing a linear programming rate of 10°/min.; the K values, where K is the heat transfer coefficient of the system in cal./mm.^{2}, were determined using the heat of fusion of tin, 14.0 cal./g. (18).

K was determined employing the following equations:

$$\frac{\text{area}}{\text{g.}} = \frac{\text{area of sample transition} \times \text{range setting of interest } (\mu\text{v.})}{\text{sample wt. (g.)}} \qquad \text{(Eq. 3)}$$

$$\frac{H_f \text{ of standard (cal./g.)}}{\text{area/g.}} = \frac{\text{cal.}}{\text{area}} = \frac{\text{cal.}}{\text{mm.}^2} = K \qquad \text{(Eq. 4)}$$

The K for flattened tin was found to be 6.1×10^{-7} cal./mm.^{2}. Samples of each sulfonamide were weighed on a Cahn Electrobalance[12] and ranged from approximately 1.6 to 3.0 ± 0.002 mg.

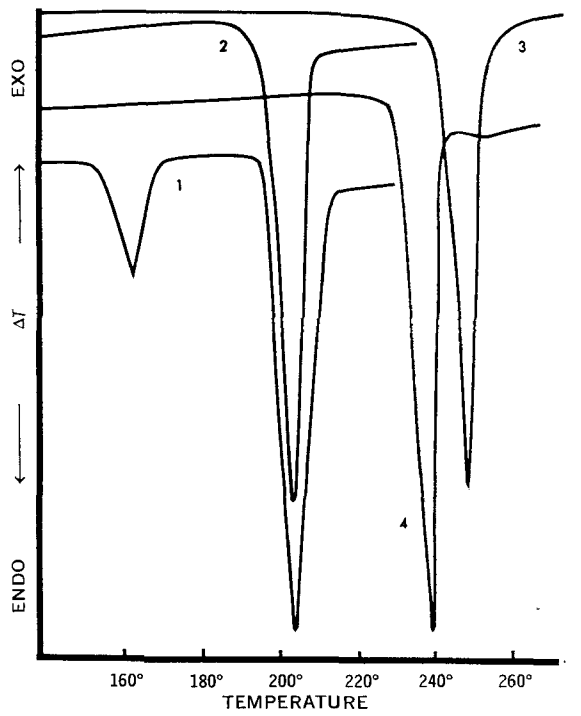

Figure 4—*Thermograms of sulfathiazole form I (1) and form II (2), sulfisomidine (3), and sulfamerazine (4).*

[11] Model KA-2H, Robert L. Stone Co., Austin, Tex.
[12] Model M-10, Cahn Instrument Co., Paramount, Calif.

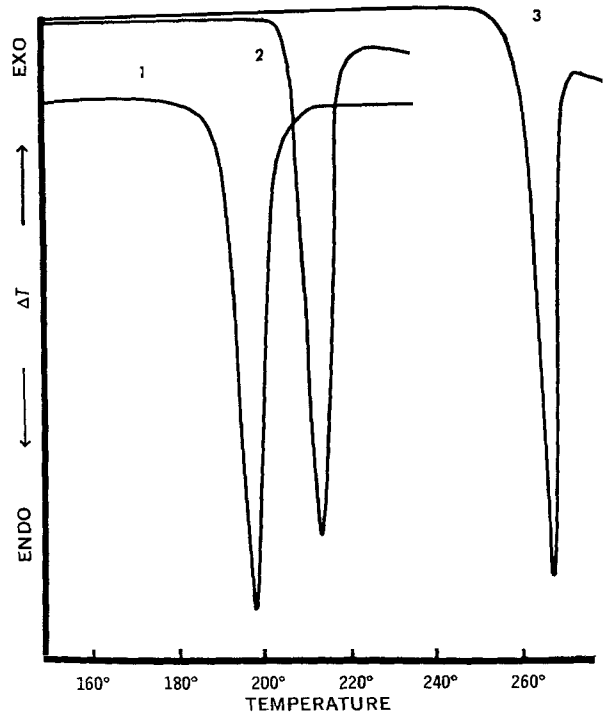

Figure 5—*Thermograms of sulfamethazine (1), sulfameter (2), and sulfadiazine (3).*

The areas encompassed by endothermic peaks were determined utilizing a Keuffel and Esser compensating polar planimeter. The heat of fusion was calculated from the following equation:

$$H_f \text{ or } H_v = \frac{K \times \text{area in mm.}^2 \times \text{range setting of interest } (\mu\text{v.})}{\text{g.}} \qquad \text{(Eq. 5)}$$

Thermograms and heats of fusion for the selected sulfonamides are presented in Figs. 4–6 and Table II, respectively.

Solubility Studies—The solubility was determined by the method reported by Restaino and Martin (4). Saturated solutions of sulfonamides at 25 ± 1.0° were prepared in 20 different cosolvent systems varying in polarity. Spectrophotometric assays were carried out with the supernatant liquid on a Coleman-Hitachi 124 double-beam spectrophotometer at predetermined wavelengths [sulfathiazole,

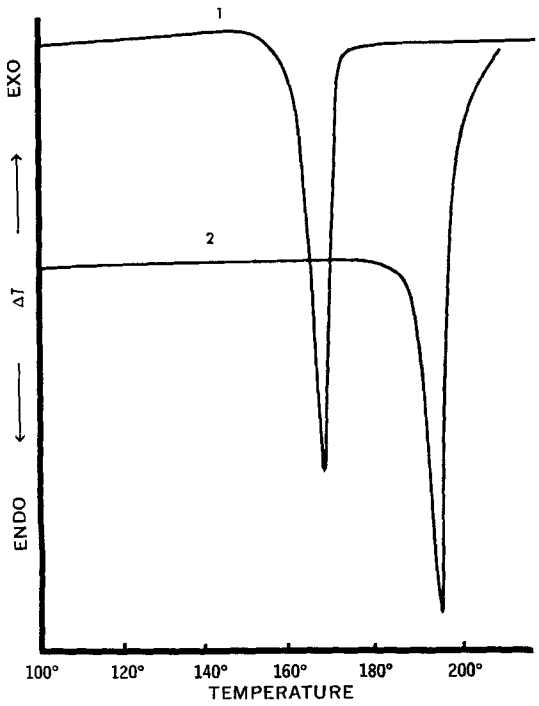

Figure 6—*Thermograms of sulfamethoxazole (1) and sulfisoxazole (2).*

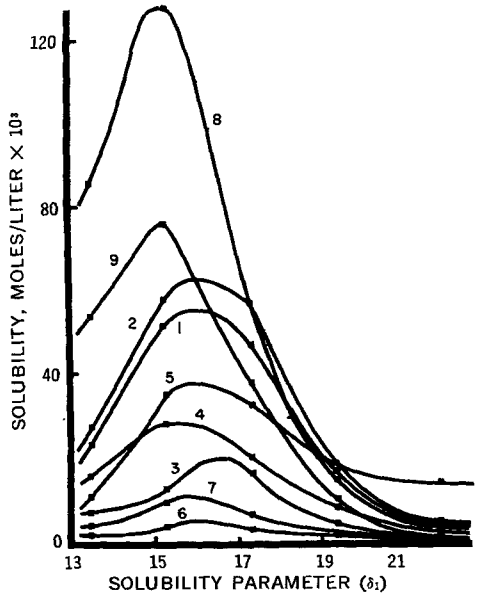

Figure 7—*Solubility in alcohol–water system. Key: 1, sulfathiazole form I; 2, sulfathiazole form II; 3, sulfamerazine; 4, sulfamethazine; 5, sulfisomidine; 6, sulfadiazine; 7, sulfameter; 8, sulfamethoxazole; and 9, sulfisoxazole.*

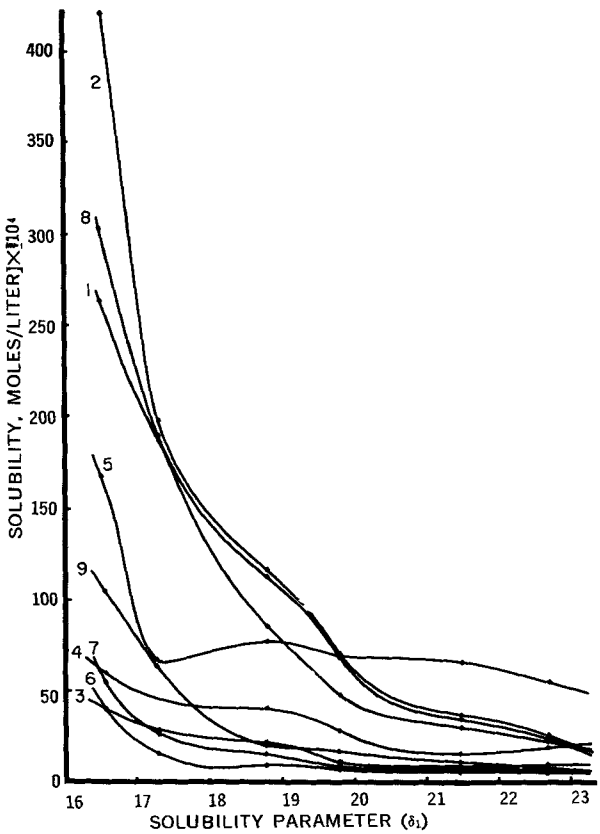

Figure 8—*Solubility in water–glycerin system. Key: 1, sulfathiazole form I; 2, sulfathiazole form II; 3, sulfamerazine; 4, sulfamethazine; 5, sulfisomidine; 6, sulfadiazine; 7, sulfameter; 8, sulfamethoxazole; and 9, sulfisoxazole.*

Table IV—Density of Selected Sulfonamides

Sulfonamide	Density at 25 ± 0.02°
Sulfathiazole	1.5375
Sulfamerazine	1.3390
Sulfamethazine	1.4038
Sulfisomidine	1.4005
Sulfadiazine	1.4703[a]
Sulfameter	1.4819
Sulfamethoxazole	1.4777
Sulfisoxazole	1.4136

[a] Reported value 1.50 (23).

280 nm. (20); sulfameter, 271 nm.; sulfamerazine, sulfamethazine, sulfadiazine, and sulfisoxazole, 270 nm.; and sulfamethoxazole, 271 nm.], diluting with 95% alcohol or water.

Determination of Solubility Parameter of Sulfonamides—The modified Hildebrand's equation (2) was used to determine the solubility parameter of each sulfonamide:

$$\log x_2 = \frac{-\Delta H_m^f (Tm - T)}{4.575(TmT)} - \frac{V_2}{4.575T}(\delta_1 - \delta_2)^2\phi_1^2 \quad \text{(Eq. 6)}$$

where x_2 = mole fraction solubility of solute, ΔH_m^f = heat of fusion of solute at melting point, Tm = absolute melting temperature of solute, T = experimental absolute temperature, V_2 = molar volume of supercooled solute, δ_1 and δ_2 = solubility parameter of solvent and solute, and ϕ_1 = volume fraction of solvent.

The apparent molar volume of each sulfonamide was calculated from the solubility data of several solvent systems employing the equation (22) to approximate the molar volume of the supercooled solute which is not ordinarily available (Table III). To substantiate the experimental result of the apparent molar volume, the density of the sulfonamide was determined by a pycnometric method, using the crystalline form of the compounds (Table IV). The volume fraction was calculated utilizing the following equation:

$$\phi_1 = \frac{n_1 v_1}{n_1 v_1 + n_2 v_2} \quad \text{(Eq. 7)}$$

where ϕ_1 = volume fraction of Component 1, n_1 and n_2 = number of moles of Components 1 and 2, and v_1 and v_2 = molar volume of Components 1 and 2.

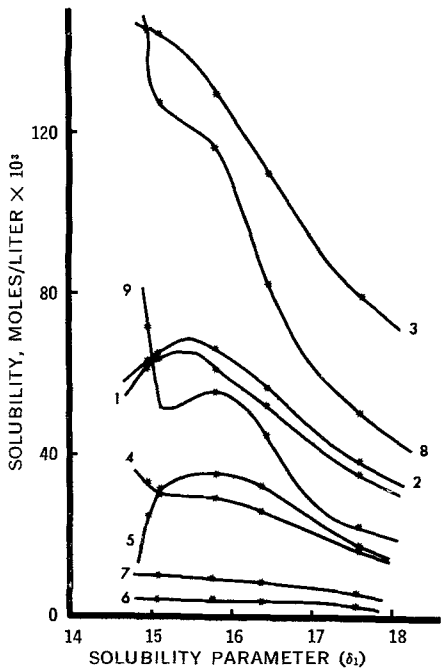

Figure 9—*Solubility in alcohol–water–propylene glycol system. Key: 1, sulfathiazole form I; 2, sulfathiazole form II; 3, sulfamerazine; 4, sulfamethazine; 5, sulfisomidine; 6, sulfadiazine; 7, sulfameter; 8, sulfamethoxazole; and 9, sulfisoxazole.*

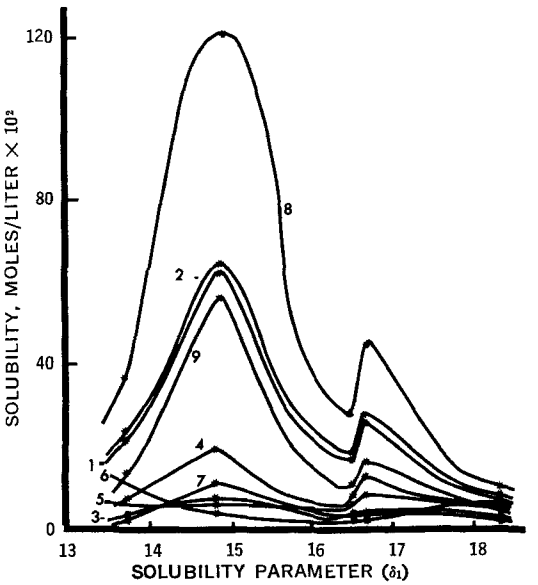

Figure 10—*Solubility in DMAC–water–glycerin system. Key: 1, sulfathiazole form I; 2, sulfathiazole form II; 3, sulfamerazine; 4, sulfamethazine; 5, sulfisomidine; 6, sulfadiazine; 7, sulfameter; 8, sulfamethoxazole; and 9, sulfisoxazole.*

Mole fraction solubilities of all sulfonamides, except sulfadiazine, were determined in 2-ethoxyethanol. For sulfadiazine, the solubility data in DMAC was used (Table V). From knowledge of the reported values experimentally determined, the solubility parameter of each sulfonamide was calculated (Table V). Plots of solubility in moles per liter *versus* δ of each solvent blend are given in Figs. 7–10.

The approximate dielectric constant (A.D.C.) in percent volume-to-volume for each solvent blend was calculated by the method reported by Moore (6):

$$\text{A.D.C.} = \sum (\%\ \text{solvent}_1 \times \text{D.C. solvent}_1) + (\%\ \text{solvent}_2 \times \text{D.C. solvent}_2) \cdots + (\%\ \text{solvent}_n \times \text{D.C. solvent}_n) \div 100 \quad \text{(Eq. 8)}$$

The dielectric constants at 25° for solvents used are: alcohol, 24.3 (24); glycerin, 42.5 (24); water, 78.54 (24); DMAC, 37.78 (25); and propylene glycol, 32.0 (26). A plot was constructed to show the relationship between the solubility parameter and A.D.C. of solvent blends by the linear least-squares method reported previously (Fig. 11).

DISCUSSION

The results in Table I show good agreement between the solubility parameter calculated from the vapor pressure of pure solvent by the isoteniscopic method and the reported value. It is obvious from these results that the purified solvents exhibited closer correlation with reported values. The effect of water content was quite

Table V—Mole Fraction Solubility and Solubility Parameter of Selected Sulfonamides at 25°

Sulfonamide	Mole Fraction Solubility, X_2	δ
Sulfathiazole, form II	0.0224	11.1
Sulfamerazine	0.0109	13.4
Sulfamethazine	0.0184	12.6
Sulfisomidine	0.0111	19.7
Sulfadiazine	0.0239[a]	26.9
Sulfameter	0.0119	13.9
Sulfamethoxazole	0.0911	16.4
Sulfisoxazole	0.0495	15.1

[a] X_2 was obtained from DMAC. All other mole fraction solubilities were obtained from 2-ethoxycthanol.

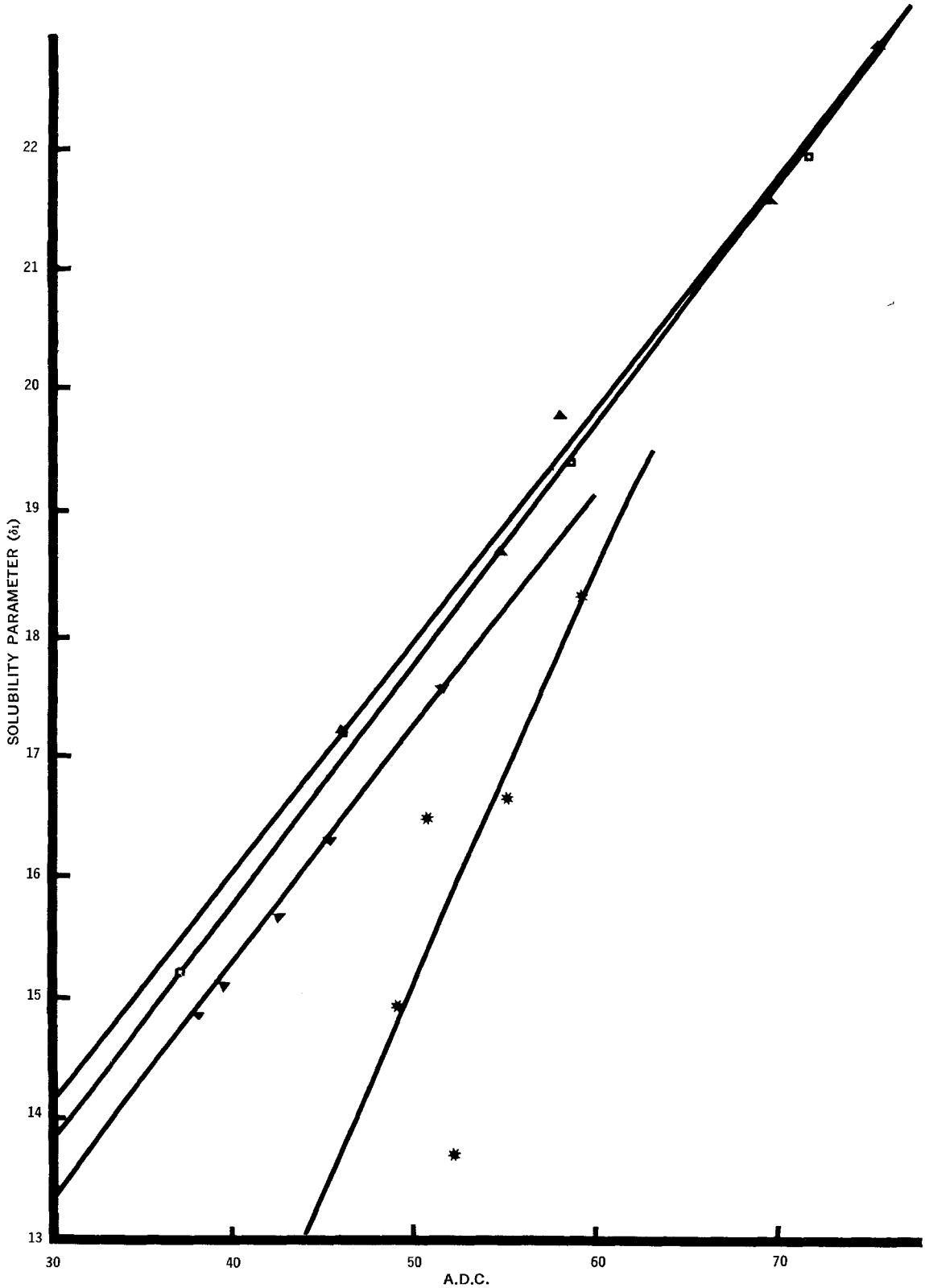

Figure 11—*Plot of solubility parameters of solvent blends used* versus *their respective A.D.C.'s. Key:* □, *alcohol–water;* ▲, *water–glycerin;* ▼, *alcohol–water–propylene glycol; and* *, *DMAC–water–glycerin.*

apparent in the case of absolute alcohol, resulting in a decrease in its heat of vaporization. Because of this effect, it is important to purify each solvent before determining vapor pressure values to calculate the solubility parameter of the solvent indirectly. It is also important to introduce an extremely high vacuum in the system; otherwise there is a considerable decrease in the vapor pressure. All data determined experimentally with a vacuum pump having a capacity of less than 30 psig. gave results much below the reported values. The reproducibility of each determination of solvent vapor pressure was excellent. The sulfonamides were recrystallized to obtain a pure crystal form, employing the same procedures recommended by Mesley and Houghton (7). The existence of polymorphism could only be detected for sulfathiazole by changing the rate of heating employed with the differential thermal analyzer. Sulfathiazole form I could only be produced by recrystallizing from water. The melting point of the polymorphic mixture obtained

from alcohol varied with the ratio of form II to form I, as reported by Miyazaki (8). The presence of a larger ratio of form II gave a form II melting point, while rapid melting between 170 and 175° gave a melting point corresponding to form I. The thermogram produced with a linear programming rate of 10°/min. showed the presence of two polymorphs of sulfathiazole with different melting points at 171–175° and 200–205°. It is conceivable that the polymorphs reported for other sulfonamides can be detected by utilizing a slower rate of heating.

The heat of fusion values obtained by differential thermal analysis proved to be reproducible with the particle-size range and amount of sample used in this study. An average of three determinations showed a deviation of less than 3%. It has been reported that a number of factors affecting differential thermal analysis results are dependent on the particle size and amount of sample used (27). Areas were constructed by drawing a straight line to close the open end of the differential thermal analysis peak. The base line for sulfisoxazole is shifted appreciably. It is believed that another reaction is involved after fusion occurs.

The solubility of the sulfonamides was determined by spectrophotometric assays in 20 different solvent blends of different polarity.

To utilize Hildebrand's solubility equation for the calculation of the solubility parameter of a solid, the molar volume of the supercooled solid must be known. As Hildebrand pointed out, it is ordinarily not available (15). Therefore, the apparent molar volume derived from solubility data in several solvent blends used for solubility determinations was used instead. It is extremely difficult to apply the equation used for molar volume determination to a solid having very limited solubility in the solvent system, yielding an insignificant density differential between solvent and solution.

As can be seen in Fig. 7, when the solubility parameter value of each sulfonamide approaches the δ value of the solvent blend, sulfamethoxazole and sulfisoxazole show peak solubility. However, the remaining sulfonamides do not show this peak solubility in the alcohol–water system.

In the water–glycerin mixture, it can be predicted from Fig. 7 that, except for sulfadiazine, the solubility of the sulfonamides whose δ is much greater than 16.5 will increase (Fig. 8).

In the alcohol–water–propylene glycol system, sulfamerazine, sulfamethoxazole, sulfisoxazole, sulfameter, and sulfamethazine show a tendency for solubility increase as their solubility parameter values approach the solubility parameter value of the solvent blend.

In the DMAC–water–glycerin system (Fig. 10), all the sulfonamides, except sulfadiazine, show peak solubility at the solubility parameter values of 15 and 16.5, regardless of their δ values. Only sulfamethoxazole and sulfisoxazole show the predicted peak point as their δ values approach the δ value of the solvent blend.

An attempt was made to correlate the solubility parameter with A.D.C. (Fig. 11). Excellent linear relationships exist between the solubility parameter and A.D.C. in the solvent systems of the alcohol group and in the DMAC–water–glycerin system. This correlation agrees with that reported by Paruta *et al.* (28).

SUMMARY AND CONCLUSIONS

The static isoteniscopic method was utilized to determine the solubility parameter of several solvents from vapor pressure data. The heat of fusion of several sulfonamides was determined utilizing differential thermal analysis; this technique showed good reproducibility. The solubility of the sulfonamides was determined using a spectrophotometric method in solvent mixtures of varying polarity.

Sulfamethoxazole had the highest solubility and sulfadiazine the lowest solubility in most solvent blends used. The solubility parameter of the sulfonamides was experimentally determined from thermodynamic and solubility data.

Hildebrand's solubility concept appears to be a useful tool for predicting drug solubility where the solubility parameters of drug and solvent system are close to each other.

Finally, an attempt was made to correlate the solubility parameter with the dielectric constant. The results were found to be linear with respect to all solvent blends employed.

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Biopharmaceutical Studies on 4-(Aminoethanesulfonylamino)antipyrine and Related Compounds I

SHUN-ICHI NAITO, YASUKO UENO, HISASHI YAMAGUCHI, and TOSHIO NAKAI

Abstract □ Blood levels of 4-(aminoethanesulfonylamino)antipyrine in rabbits were determined, and binding of the chemical with rabbit serum *in vivo* and *in vitro* was examined. Metabolites of the chemical in rabbit urine were separated into five compounds: rubazonic acid (trace), 4-aminoantipyrine, 4-acetylaminoantipyrine, 4-hydroxyantipyrine (trace), and unchanged chemical. Glucuronide in urine of rabbits administered with 4-(aminoethanesulfonylamino)antipyrine is the conjugated form with 4-hydroxyantipyrine. Some pharmacological activities were investigated; it was observed that the chemical shows analgesic, anti-inflammatory, antihistaminic, and antipyretic actions.

Keyphrases □ 4-(Aminoethanesulfonylamino)antipyrine—biopharmaceutical studies □ Taurinopyrine—rabbit serum protein binding □ Metabolites, taurinopyrine, aminopyrine in urine—separation, analysis □ Pharmacological screening—taurinopyrine

Aminopyrine has been used for many of the same symptoms and conditions as the salicylates, acetophenetidin, and acetaminophen. The relatively infrequent but possibly severe toxicity, however, has led to a gradual decline in its use.

Taurinopyrine [4-(aminoethanesulfonylamino)antipyrine] was synthesized in the hope of obtaining a potent analgesic/antipyretic agent with a metabolic pathway sufficiently different from aminopyrine so as to overcome the toxicities associated with this drug. Aminoethanesulfonic acid, which is expected to separate from taurinopyrine when ingested, is one of the physiological amino acids and is also surface active. Taurinopyrine occurs as colorless needle crystals, m.p. 185° dec., which are fairly soluble in water; sparingly soluble in ethanol, acetone, and benzene; and practically insoluble in ether, petroleum ether, and petroleum benzene.

In a previous paper (1), stabilities of taurinopyrine stored at different temperatures and pH's were reported; it was shown that the chemical is stable, in either solution or powder form, to temperature, pH, or moisture.

The present series of experiments was undertaken to determine the blood level of taurinopyrine in animals, to determine the metabolites of taurinopyrine present in rabbit urine, and to evaluate some of its pharmacological properties.

Table I—TLC of Lyophilized Rabbit Urine[a]

Drug	R_f (Assumed Substance)	Color of Spot
Taurinopyrine (400 mg./kg.)	0.06 (unchanged taurinopyrine)	Black
	0.33 (4-hydroxyantipyrine)	Pale brown
	0.43 (4-acetylaminoantipyrine)	Grey
	0.56 (4-aminoantipyrine)	Pale brown
	0.95 (rubazonic acid)	Brownish black
Aminopyrine (300 mg./kg.)	0.43 (4-acetylaminoantipyrine)	Grey
	0.56 (4-aminoantipyrine)	Pale brown
	0.65 (unchanged aminopyrine)	Black

[a] Adsorbent: diatomite (Kieselgel G), 0.25 mm. in thickness; solvent: acetone–benzene–chloroform (10:10:1); and color developer: 5% silver nitrate solution.

Table II—Glucuronides in Rabbit Urine

Given Drug	Dose per Rabbit, 2 kg. Body Weight	Number of Rabbits Used	Yield of Crude Glucuronide[a]	Component of Glucuronide
Taurinopyrine	800 mg.	2	60 mg.	H[b]
Antipyrine	1000 mg.	6	280 mg.	H
4-Aminoantipyrine	1000 mg.	6	600 mg.	H, A[c]
Methampyrone	800 mg.	6	560 mg.	H, A
Aminopyrine	800 mg.	4	270 mg.	H, A

[a] After hydrolysis of the glucuronides with hydrochloric acid, 4-hydroxyantipyrine and 4-aminoantipyrine were identified through TLC shown in Table I. [b] H: 4-hydroxyantipyrine. [c] A: 4-aminoantipyrine.

EXPERIMENTAL

Binding of Taurinopyrine with Rabbit Serum Protein *In Vitro*— A mixture of 0.1 ml. of taurinopyrine solution (150 mg. %) and 0.9 ml. of normal rabbit serum was treated as reported earlier (2). The modified Folin method (2, 3) was used to quantitate the taurinopyrine.

Binding of Taurinopyrine or Aminopyrine with Rabbit Serum *In Vivo*— Taurinopyrine (300 mg./kg.) was administered orally to four rabbits, and serum samples were removed after 2 hr. One milliliter of this serum was mixed with 2 ml. of absolute ethanol and cooled in ice water for 20 min. This solution was centrifuged to separate the protein precipitate, and 0.5 ml. of the supernatant was removed for analysis. The supernatant was diluted with 0.5 ml. of water, and the modified Folin method was used to assay for free taurinopyrine. Total taurinopyrine was determined after 0.5 ml. of the plasma was mixed with 0.5 ml. of 5 *N* HCl and kept at 37 ± 2° for 2 hr. by using the modified Folin method and *in vitro* procedure with aminopyrine.

Two hours following aminopyrine (200 mg./kg.) administration to four rabbits, blood was collected and 1 ml. of serum was treated according to the *in vitro* procedure employed for taurinopyrine. Both free aminopyrine and total aminopyrine in serum were determined.

Separation and Assay of Metabolites of Taurinopyrine and Aminopyrine in Urine— About 500 ml. of urine collected from each of four rabbits (average body weight, 2 kg.), following the ingestion of 400 mg./kg. of taurinopyrine or 300 mg./kg. of aminopyrine, was lyophilized and assayed as in the case of taurinophenetidine (2) and evaluated by TLC as described previously (1).

To assay the metabolites of taurinopyrine or aminopyrine in urine, a mixture of 1 ml. of 5 *N* HCl and 200 mg. of the freeze-dried residue of urine, obtained from rabbits (average weight, 2 kg.) dosed with either taurinopyrine (400 mg./kg.) or aminopyrine (300 mg./kg.), was stored in an incubator at 37 ± 2° for 1 hr. One milliliter of 5 *N* sodium carbonate solution was added to each sample, and the resulting mixture was centrifuged. Fifty microliters of the supernatant was spotted on a chromatographic plate and submitted to TLC as described in Table I. After development with a mixed solvent of acetone, benzene, and chloroform (10:10:1), the spots corresponding to the unchanged drug, 4-acetylaminoantipyrine and 4-aminoantipyrine, were each scratched off the TLC plate and extracted 3 min. with 3 ml. of water

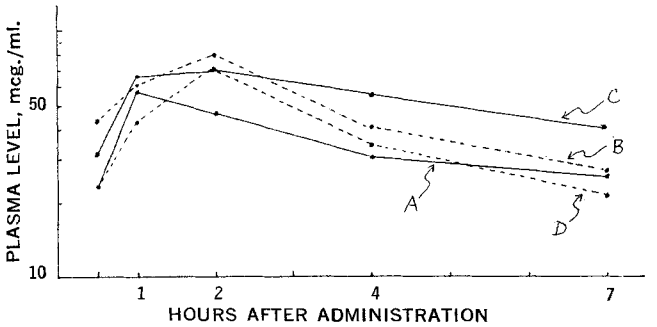

Figure 1—*Mean plasma level of taurinopyrine after oral administration to rabbits at different doses. Each group consisted of four female rabbits weighing 2.3 ± 0.3 kg. Key: A, 200 mg./kg.; B, 300 mg./kg.; C, 400 mg./kg.; and D, 500 mg./kg.*

on a boiling water bath. The mixture was centrifuged, and 1 ml. of the supernatant was treated in the same way to determine the amount of *p*-aminophenol and *p*-acetamidophenol (2), the metabolites of taurinophenetidine in rabbit urine.

A calibration curve for each of the two unchanged drugs and the two major metabolites was prepared, using known concentrations of each and the same quantitative procedure as previously described.

Glucuronides in Rabbit Urine—Taurinopyrine, antipyrine, aminopyrine, 4-aminoantipyrine, or methampyrone was administered to determine the relationship between the chemical structure and metabolites in glucuronide form. The drug was given with water to rabbits through a stomach tube in the form of a solution or suspension (without any suspending agent), depending on the solubility of the drug. Taurinopyrine, 4-aminoantipyrine, and methampyrone were given as solutions, and antipyrine and aminopyrine were given as aqueous suspensions. About 500 ml. of urine was collected during 5 hr. and then treated according to the procedure reported by Williams (4).

The crude glucuronide thus obtained was hydrolyzed with a small amount of 5 *N* HCl at 37 ± 2° for 1 hr. and then neutralized with 5 *N* sodium carbonate solution. The solution was submitted to TLC evaluation as shown in Table I.

Metabolites of Taurinopyrine in Blood—A mixture of 1 ml. of rabbit plasma, taken 2 hr. (peak time of blood level) after oral ingestion of 500 mg./kg. of taurinopyrine, was treated by the same method as described in the previous report (2) and then submitted to TLC (Table I). The experiment was carried out on two rabbits of about 2 kg. in body weight, fasted for 24 hr.

Determination of Taurinopyrine in Plasma—The analysis of taurinopyrine in rabbit and guinea pig plasma was conducted as described previously (2).

Drug to be Tested—4-Hydroxyantipyrine was synthesized by the method of Halberkann and Tretwurst (5). Rubazonic acid was synthesized according to the method of Knorr (6, 7)

Acute Toxicity—Equal numbers of male and female dd strain mice, weighing 17 ± 2 g., were used. The animals were maintained on a commercial diet[1] and drinking water *ad libitum* at the room temperature of 20 ± 2°. Five mice were housed per cage, and from 30 to 50 were used to determine each LD_{50}.

The test solutions of taurinopyrine and aminopyrine were employed for the intraperitoneal and oral administrations. Various doses of the chemical were dissolved in water, and the volume was adjusted to 0.5 ml./10 g. body weight. The animals were fasted 5 hr. prior to drug administration, and they were allowed free access to the diet and drinking water 4 hr. after the administration. The behavioral changes were observed for 72 hr. after drug administration; the LD_{50} of the chemicals was calculated from the number of animals dying within 72 hr., according to the Litchfield–Wilcoxon method (8).

Effect of Taurinopyrine, Aminopyrine, and Related Compounds on Squirming and Capillary Permeability—Male and female dd strain mice, weighing 20 ± 1 g., were given the test compounds by stomach tube. After 20 min., each animal was given an intravenous injection of 0.1 ml. of a 4% solution of pontamine sky blue (Merck). At 30 min. after administration of a test compound, 1 mg. of acetic acid was injected intraperitoneally as 0.4 ml. of a 0.25% (v/v) solution. Each dose group consisted of 12 mice. Animals were primed by intramuscular injection of 0.05 mg. of stilbestrol 48 hr. before the test. Stilbestrol was administered as a solution in ethyl oleate containing 1 mg./ml. The method of Whittle (9) was followed.

Table III—Acute Toxicity of Taurinopyrine and Aminopyrine in Mice

Route	Compound	Dose, mg./kg.	Mortality Male	Mortality Female	LD_{50},[a] mg./kg. (Male and Female)
i.p.[b]	Aminopyrine	180	0/5	0/5	375
		200	0/5	0/5	(295–476)[c]
		230	0/5	1/5	
		260	0/5	3/5	
		280	0/5	2/5	
i.p.	Taurinopyrine	500	0/5	0/5	760
		600	1/5	0/5	(710–813)
		700	1/5	3/5	
		800	2/5	3/5	
		900	5/5	4/5	
p.o.[d]	Aminopyrine	500	1/5	0/5	720
		600	1/5	2/5	(621–835)
		700	2/5	2/5	
		800	3/5	2/5	
p.o.	Taurinopyrine	3000	0/5	1/5	5350
		4000	1/5	1/5	(4180–6848)
		5000	3/5	2/5	

[a] LD_{50} was calculated by the Litchfield–Wilcoxon method (8). [b] Intraperitoneally. [c] 95% confidence limits. [d] *Per os.*

Tail-Pinching Test—The reaction time of mice pinched on the tail with an artery clip was recorded every 15 min. for 1.5 hr. after administration of the compounds by the method described by Haffner (10)

Tail-Withdrawal Reflex in Mice—The same method for analgesimetry described by Ben-Bassat *et al.* (11) was used.

Anti-Inflammatory Activity—Inflammation was produced by injecting carrageenin (0.1 ml. of 2% suspension) into the plantar surface of the rat's hind paw. The test compound was administered as an aqueous solution, and the volume was kept constant at 0.1 ml./100 g. body weight for intraperitoneal dosage. One-half of the total dose was given approximately 6 hr. later. Intraperitoneal injections were given in either caudal quadrant of the abdominal wall. Control animals received injection of the vehicle alone. The results were obtained 24 hr. after administration of carrageenin.

Anaphylactic Shock in Guinea Pigs—Guinea pigs (Hartley strain) were used in the experiment following the procedure of Labelle and Tislow (12). Guinea pigs were challenged exactly 1 hr. after drug administration by rapid injection of 1 ml. of horse serum into the saphenous vein.

Antihistamine Activity—Male and female guinea pigs (Hartley strain), with a body weight of about 200 g., were used in the histamine tests. The compound was administered subcutaneously 0.5 and 1 hr. before histamine injection. Histamine dihydrochloride was injected rapidly into the saphenous vein in a dosage of 1.1 mg./kg. The test was made in fasted animals.

Antipyretic Activity—To rabbits weighing 2.3–2.5 kg., 15 mcg./kg. of nonanaphylactogenic polysaccharides obtained from *Pseudomonas fluorescens*[2] was administered intravenously, and taurinopyrine solution was given subcutaneously 2 hr. after the administration of pyrogen solution. Body temperature was recorded every 15 min. for 6 hr.

RESULTS AND DISCUSSION

Rabbit plasma, taken 2 hr. after oral ingestion of 500 mg. taurinopyrine/kg., contained no detectable metabolites of the chemical. Concentrations of taurinopyrine in blood following the oral administration of 200, 300, 400, and 500 mg./kg. are shown in Fig. 1. Since the peak blood level is not proportional to the dose, the absorption would apparently be active.

[1] CA-1, CLEA Japan, Inc., Tokyo.

[2] T.T.G No. 1, Fujisawa Yakuhin Kogyo Co. Ltd, Osaka.

Table IV—Evaluation of Analgesic Potency in Mice Using the Tail-Pinching Test

Route	Compound	Dose, mg./kg.	Number Not Reacting[a] Male	Number Not Reacting[a] Female	ED_{50},[b] mg./kg. (Male and Female)
i.p.[c]	Aminopyrine	50	2/5	3/5	65
		75	3/5	3/5	(51.1–82.5)[d]
		100	4/5	4/5	
		125	5/5	4/5	
i.p.	Taurinopyrine	50	2/5	0/5	94
		80	2/5	2/5	(73–120)
		90	2/5	2/5	
		100	3/5	3/5	
p.o.[e]	Aminopyrine	50	1/5	2/5	142
		100	2/5	2/5	(90–223)
		150	3/5	2/5	
		200	3/5	3/5	
		300	5/5	3/5	
p.o.	Taurinopyrine	400	1/5	1/5	560
		500	1/5	2/5	(479–655)
		600	3/5	3/5	
		700	4/5	3/5	

[a] The results were obtained 30 min. after administration. [b] ED_{50} was calculated by the Litchfield–Wilcoxon method (8). [c] Intraperitoneally. [d] 95% confidence limts. [e] *Per os.*

Table V—Effect of Test Compounds on Squirming and Capillary Permeability

Compound	Oral Dose, mg./kg.	Reduction of Squirming, %	Inhibition of Squirming, ED_{50}, mg./kg.	Change in Permeability,[a] %
Taurinopyrine	100	8	475	+83
	250	24	(293–770)[b]	−31
	350	36		−41
	500	56		+30
Aminopyrine	50	28	213	0
	100	33	(83–545)	−11
	250	50		−45
4-Aminoantipyrine	50	26	165	−9
	100	35	(79–347)	−32
	250	58		−35
4-Acetylamino-antipyrine	50	12		−12
	100	25		+3
	250	25		−10

[a] Percentages are calculated from the mean of groups consisting of 12 mice compared with controls (12 mice). [b] 95% confidence limits.

Taurinopyrine (300 mg./kg.) produced a peak level of 81.3 ± 15.4 mcg./ml. 2 hr. after oral administration. Eighty-two percent of this amount was bound to serum proteins. Aminopyrine (200 mg./kg.) produced a peak blood level of 117.8 ± 8.7 mcg./ml. 2 hr. after oral administration, and 58% was bound to plasma proteins. On the other hand, the binding ratio of taurinopyrine with rabbit serum *in vitro* was about 52%. These experiments were performed because of the possibility that the binding of a drug with serum protein could be prohibited by the action of hydrochloric acid.

Urine samples collected from rabbits within 48 hr. of the administration of both taurinopyrine (400 mg./kg.) and aminopyrine (300 mg./kg.) were lyophilized, and the residue was examined by TLC for the presence of metabolites (Table I). Five spots representing rubazonic acid, 4-aminoantipyrine, 4-acetylaminoantipyrine, 4-hydroxyantipyrine, and unchanged drug (taurinopyrine or aminopyrine) were separated and identified. A micromelting-point determination of the mixture of evaporated residue of ethanolic extract from 5–10 spots having the same R_f value and an authentic sample was used for identification. Two of the metabolites, rubazonic acid and 4-hydroxyantipyrine, were present only in trace amount, whereas 48.3% was unchanged taurinopyrine, 43.7% was 4-acetylaminoantipyrine, and 8.0% was 4-aminoantipyrine. In the case of aminopyrine, 52.0% was excreted as 4-aminoantipyrine, 32.5% as the unchanged aminopyrine, and 15.5% as 4-acetylaminoantipyrine. The physiological availability of taurinopyrine or aminopyrine in rabbits could not be calculated because of incomplete urine collection.

4-Hydroxyantipyrine was detected from the glucuronides in urine when taurinopyrine was administered orally to rabbits (Table II).

According to Brodie and Axelrod (13), aminopyrine is metabolized in man at the rate of 10–30%/hr., with only about 3% being excreted unchanged. About one-half of the 0.5-g. dose is excreted in 3 days as 4-aminoantipyrine and its acetyl derivative, the latter compound being the major metabolite. Metabolism of taurinopyrine in man has not been studied, but taurinopyrine is metabolized in rabbits in a way similar to aminopyrine; it is dealkylated to 4-aminoantipyrine, which is excreted partly as such and partly acetylated.

Thus, it was clarified that the main substance excreted after ingestion of taurinopyrine is the unchanged taurinopyrine, in contrast to the main metabolite of aminopyrine, that is, 4-aminoantipyrine. Therefore, taurinopyrine may have a characteristic pharmacological action different from aminopyrine.

The details of acute toxicity of various doses of taurinopyrine and aminopyrine are shown in Table III. Although the animals receiving intraperitoneal doses below 500 mg./kg. of taurinopyrine exhibited no behavioral abnormality, the intraperitoneal dose of 700–800 mg./kg. produced a transient ataxic walk with extended hind limbs for 5–10 min. after the administration. Thereafter, the animals showed a marked reduction in spontaneous movements. Within 1 hr. after the administration of 1000 mg./kg. of taurinopyrine, all of the animals died in respiratory failure. No animal exhibited lacrimation, piloerection, or convulsions before death. The righting reflex was usually maintained until death. Absence of convulsions before death in the group receiving taurinopyrine is very different from the manifestation of convulsions before death in the group receiving aminopyrine. No significant difference in the behavioral effect or LD_{50} was observed between the male and female mice.

The results of the tail-pinching test are shown in Table IV. Taurinopyrine shows analgesic action to the tail-pinching test, but its potency is less than that of aminopyrine.

Concurrent changes in the peritoneal capillary permeability during squirming was measured according to the method of Whittle (9). Table V records the values for the ED_{50} for inhibition of squirming; since reduction of the permeability response did not approach 50% over the range of doses that inhibited squirming, there is no

Table VI—Average Pain Reaction Times in Control Mice and Mice Given Intraperitoneal Injections of Test Compounds[a]

Compound	Dose, mg./kg.	Average Reaction as Recorded in Seconds after Injection 15′	30′	60′	90′	120′	150′	PRT[b]
Control		1.3	1.4	1.2	1.2	1.1	1.2	1.2
Aminopyrine	50	1.7	2.0	2.6	2.8	2.9	3.8	2.6
		1/10[c]	4/10	6/10	7/10	6/10	7/10	
	75	1.5	2.6	2.6	2.5	3.0	2.7	2.5
		5/10	8/10	8/10	6/10	7/10	7/10	
	100	2.7	3.9	4.3	4.7	4.7	4.3	4.1
		7/10	9/10	9/10	8/10	8/10	8/10	
Taurinopyrine	50	1.4	2.0	1.8	2.7	2.6	2.9	2.2
		2/10	2/10	3/10	4/10	4/10	4/10	
	100	1.3	1.7	2.4	2.4	2.5	2.2	2.1
		2/10	3/10	4/10	4/10	4/10	6/10	
	150	2.1	2.5	3.0	3.0	3.4	3.7	2.9
		4/10	4/9	5/9	6/9	6/9	6/9	

[a] Each group consisted of 10 mice. [b] PRT: average pain reaction time. [c] Number of effective mice/number of mice used.

Table VII—Effect of the Edema Induced by Subplantar Injection of Carrageenin[a]

Compound	Dose, mg./kg.	Normal Foot Volume ± SE[b]	Mean Foot Volume ± SE	Inhibition, %
Control		0.302 ± 0.056	0.988 ± 0.039	
Aminopyrine	100	0.260 ± 0.071	0.960 ± 0.048	
	200	0.230 ± 0.093	0.910 ± 0.055	
	300	0.305 ± 0.039	0.982 ± 0.036	2
Taurinopyrine	100	0.286 ± 0.042	0.900 ± 0.037	13
	200	0.322 ± 0.056	0.878 ± 0.046	19
	300	0.274 ± 0.075	0.736 ± 0.050[c]	33

[a] Each group consisted of 10 rats, each weighing 150 ± 15 g. [b] Standard error of the mean. [c] Statistically significant effect ($p < 0.05$).

Table VIII—Blood Level of Taurinopyrine (mcg./ml.) following its Subcutaneous Administration to Guinea Pigs[a]

Dose, mg./kg.	Hours after Dosing 0.25	0.5	1.0	2.0	4.0	7.0
100	84 ± 33[b]	35 ± 3	21 ± 8	4 ± 5	0	0
200	95 ± 23	87 ± 11	87 ± 23	52 ± 13	19 ± 12	3 ± 2
300	122 ± 17	101 ± 8	90 ± 7	54 ± 6	16 ± 7	3 ± 4

[a] Each group consisted of three male guinea pigs; the animals of each group were killed for collection of blood every sampling time. At the time of sacrifice, each animal was bled completely, and the blood was reserved for analysis. Average body weight of the guinea pigs was 200 g. [b] Mean value ± standard error.

corresponding ED_{50} for the permeability effect. Although the dose of taurinopyrine at ED_{50} is higher than that of aminopyrine, taurinopyrine does inhibit squirming but not permeability.

Study results of the tail-withdrawal reflex in mice (11) are shown in Table VI. Computation of ED_{50} and its confidence limits was made by probit analysis. This was made by dichotomizing the reactions after 60 and 90 min. into two groups: (*a*) "refractory," *i.e.*, pain reaction time of 1.8 sec. or more; and (*b*) "nonrefractory," for aminopyrine and taurinopyrine. The ED_{50} and 95% confidence limits were 42 (30–59) mg./kg. for aminopyrine and 89 (59–135) mg./kg. for taurinopyrine. Analgesic effect of taurinopyrine seems to be less potent than that of aminopyrine, according to the ED_{50} of the tail-withdrawal reflex in mice.

Table VII shows the anti-inflammatory effects of aminopyrine and taurinopyrine in the carrageenin test. Taurinopyrine was more active than aminopyrine in these tests. However, anti-inflammatory tests using other irritants, such as formaldehyde solution or croton oil, should be performed before definite conclusions are drawn.

Antihistamine and antianaphylactic shock activities of taurinopyrine were evaluated by the method of Labelle and Tislow (12). No difference in antihistamine activity was observed between 300- and 500-mg./kg. doses of taurinopyrine when the compound was administered subcutaneously 1 hr. before histamine injection. As a result, 60% of 10 guinea pigs survived in each group. Therefore, blood levels of taurinopyrine were measured following different doses to determine if some relationship exists between antihistamine activity and blood level of the drug (Table VIII). No definite relationship was observed, although the potency of antihistamine activity seems to be stronger when the chemical is given 30 min. rather than 1 hr. before histamine injection (Table IX). Table IX also presents antianaphylactic results. From these results, it can be concluded that taurinopyrine shows antihistamine activity but its antianaphylactic action is uncertain. The results of antipyretic activity are presented in Fig. 2. Taurinopyrine, at doses of 150 and 250 mg./kg., decreased the elevated body temperature of all rabbits used, not rapidly but gradually.

In conclusion, it was shown that taurinopyrine has some analgesic, anti-inflammatory, antihistamine, and antipyretic activities. More

Table IX—Antianaphylactic and Antihistamine Activities of Taurinopyrine in Guinea Pigs[a]

Antianaphylactic Activity	
Dose, mg./kg.	Survived for 10 min.
Control	0/10
100	2/10
200	1/10
300	1/10
500	4/10

Antihistamine Activity[b]			
	Survived for 24 hr., Administration of Taurinopyrine		
Dose, mg./kg.	30 min. before Histamine Injection	1 hr. before Histamine Injection	ED_{50}, mg./kg.
150	2/10		195
175	4/10		(165–230)[c]
200	5/10		
175		2/10	250
200		4/10	(197–318)
250		4/10	

[a] Guinea pigs weighing 250–300 g. were used. [b] No control animals receiving histamine injection survived for 24 hr. [c] 95% confidence limits.

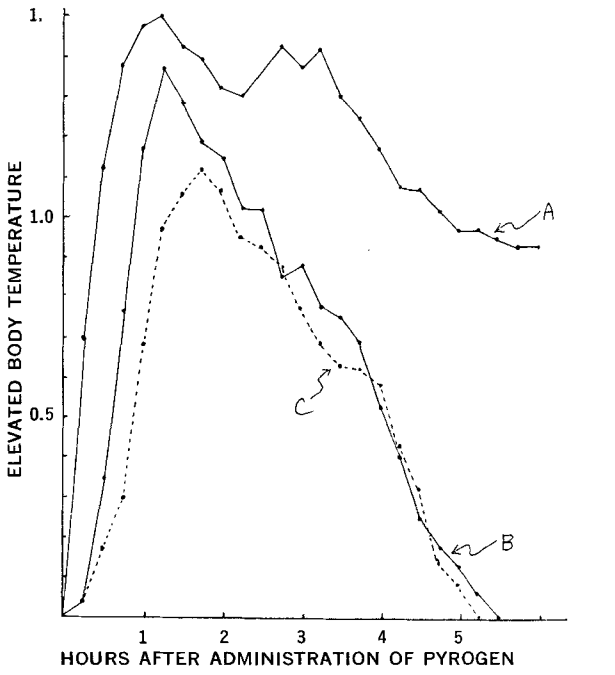

Figure 2—*Mean fall of elevated body temperature of male rabbits receiving taurinopyrine 2 hr. after administration of pyrogen. Each group consisted of three male rabbits weighing 2.4 ± 0.2 kg. and having 39.3 ± 0.3° of basal body temperatures. Key: A, control group; B, 150 mg./kg.; and C, 250 mg./kg.*

extensive investigations are required to determine its practical therapeutic application.

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Biopharmaceutical Studies on Aminoethanesulfonylphenetidine and Related Compounds II

SHUN-ICHI NAITO and KAZUO FUKUI

Abstract □ The binding ratio of taurinophenetidine or nicotinoyltaurinophenetidine with serum protein in rabbits varies according to experimental conditions in dialysis, but the averaged binding ratios of taurinophenetidine and its nicotinate were roughly 40–50% at high doses. Taurinophenetidine and its nicotinate are scarcely excreted in rat feces and in rat and rabbit bile. The amount of both compounds distributed in organs of mice and rats is very small. It was observed that taurinophenetidine has some analgesic and antipyretic activities, and nicotinoyltaurinophenetidine reveals some analgesic and anti-inflammatory activities but no antipyretic action.

Keyphrases □ Aminoethanesulfonylphenetidine, related compounds—biopharmaceutical studies □ Metabolites, taurinophenetidine and derivatives—determination □ Taurinophenetidine and derivatives—distribution, animal organs □ Serum protein binding—taurinophenetidine and derivatives

In a previous work (1), blood levels of aminoethanesulfonylphenetidine (taurinophenetidine) in rabbits were determined and binding of taurinophenetidine with rabbit serum was found to be about 67% of the total amount administered *in vitro* and nil *in vivo*. Metabolites of taurinophenetidine in rabbit urine are separated into four compounds: *p*-aminophenol, *p*-acetamidophenol, *p*-phenetidine, and unchanged taurinophenetidine. It was also recognized that glucuronide in urine of rabbits ingesting taurinophenetidine is the conjugated form of *p*-aminophenol.

The present paper deals with the results obtained from biopharmaceutical studies and with some of the pharmacology on two compounds of *p*-phenetidine derivatives, taurinophenetidine and nicotinoylaminoethanesulfonylaminophenetidine (hereafter abbreviated as nicotinoyltaurinophenetidine).

EXPERIMENTAL

Metabolites of Taurinophenetidine in Blood—Plasma from 10 male mice (dd strain, average weight 20 g., taken at the time of peak blood levels 1.5 hr. after oral administration of 2500 mg./kg. of taurinophenetidine) or plasma from three female rats (Wistar strain, average weight 200 g., taken at the time of peak blood levels 1.5 hr. after oral ingestion of 2000 mg./kg. of taurinophenetidine) was treated by the method described in the previous paper (1).

Metabolites of Nicotinoyltaurinophenetidine in Blood—Plasma from two female rabbits (average weight 2.3 kg., taken at the time of peak blood levels 1.5 hr. after oral ingestion of 1000 mg./kg. of nicotinoyltaurinophenetidine) was treated by the same method (1). Methylene dichloride was used instead of chloroform for extraction.

TLC for separation of the metabolites was carried out under the following conditions: solvent, benzene–acetone–ethyl acetate (1:1:2); adsorbent, diatomite (Kieselgel G); color developer, 0.4% potassium ferricyanide solution followed by spraying with 1% ferric nitrate in 0.7 *N* nitric acid; R_f values in plasma of rabbits ingested the chemical, 0.33; R_f values in control plasma, nil; and R_f value of nicotinoyltaurinophenetidine, 0.33.

Determination of Acetophenetidin in Blood—Four female rabbits (average weight 3.0 kg.) were used. After oral administration of 250 mg./kg. of acetophenetidin, blood samples were taken according to the sampling schedule. To assay the free form of acetophenetidin, a mixture of 1 ml. of plasma and 3 ml. of water was shaken with 7 ml. of chloroform for 15 min. After centrifugation and filtration, a 3-ml. aliquot of the chloroform layer was evaporated to dryness on a water bath, and the residue was treated by the same method as previously reported for the determination of isopropylantipyrine (2).

A mixture of 1 ml. of plasma and 1 ml. of 5 *N* HCl was incubated at 37 ± 2° for 1.5 hr., and 2 ml. of water was added to this mixture. The plasma was treated with the acid due to the consideration that the binding of a chemical with plasma protein can possibly be prohibited by the action of hydrochloric acid. This mixture was shaken with 7 ml. of chloroform and treated by the same procedure as described for determination of total acetophenetidin.

Determination of Nicotinoyltaurinophenetidine in Blood—Each group consisted of four female rabbits (average weight 2.3 kg.).

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Determination of Nicotinoyltaurinophenetidine in Blood—Each group consisted of four female rabbits (average weight 2.3 kg.).

Table I—Experimental Conditions in Distribution of Taurinophenetidine in Animal Organs[a]

Organ	Animal	A, g. ($MV \pm SE$)	B, g.	C, ml.	D, ml.	E, ml.	F, ml.
Heart	Mouse	0.11 ± 0.0	0.3	0.5	0.5	3	2
	Rat	0.7 ± 0.0	2	2	2	5	3
	Rabbit	6.1 ± 0.4	5	3	3	5	3
Lung	Mouse	0.15 ± 0.0	0.5	0.5	0.5	3	2
	Rat	1.2 ± 0.1	3	2	2	5	3
	Rabbit	9.8 ± 0.5	5	3	3	5	3
Liver	Mouse	1.1 ± 0.4	5	5	5	5	3
	Rat	7.6 ± 1.3	15	8	8	10	7
	Rabbit	75.2 ± 8.3	25	10	10	10	7
Kidney	Mouse	0.3 ± 0.2	0.5	0.5	0.5	3	2
	Rat	1.8 ± 0.1	5	3	3	5	3
	Rabbit	14.7 ± 1.4	5	3	3	5	3
Brain	Mouse	0.29 ± 0.1	0.5	0.5	0.5	3	2
	Rat	1.4 ± 0.1	4	3	3	5	3
	Rabbit	6.4 ± 0.2	5	3	3	5	3

[a] A = weight of each organ in animals used; B = weight of the sample of organ used for homogenation; C = volume of 5 *N* HCl; D = volume of supernatant pipeted after centrifugation; E = volume of methylene dichloride for extraction; F = volume of methylene dichloride used for evaporation after extraction; and $MV \pm SE$ = mean value ± standard error.

After oral administration of nicotinoyltaurinophenetidine, blood samples were taken according to the sampling schedule. One milliliter of plasma was treated with cyanogen bromide, using the procedure for determination of guaiacol glycerol ether mononicotinate with ammonia buffer (3). A mixture of 0.9 ml. of normal rabbit plasma and 0.1 ml. of 99.5% ethanolic solution of nicotinoyltaurinophenetidine of known concentration, such as 20, 40, 60, and 80 meg./ml., was treated as described previously to prepare a calibration curve.

Binding of Taurinophenetidine with Rabbit Serum Protein *In Vivo* by Equilibrium Dialysis—The experimental design followed the method of a previous report (4). Taurinophenetidine in the solution outside the bag was determined as free taurinophenetidine by the modified Folin method (1). To assay total taurinophenetidine, blood for dialysis was obtained from the supernatant from centrifugation of a mixture of 1 ml. of plasma and 1 ml. of 5 *N* HCl incubated at 37 ± 2° for 1 hr. and was then treated as described previously.

The stability of taurinophenetidine in phosphate buffer (pH 7.4) was determined for 72 hr.; no decomposition of the compound was observed under the experimental conditions for equilibrium dialysis. A plasma sample was obtained from male rabbits (average weight 2.5 kg.) receiving 200, 280, and 360 mg./kg. of taurinophenetidine; each group consisted of four rabbits.

Binding of Nicotinoyltaurinophenetidine with Rabbit Serum Protein *In Vivo* by Equilibrium Dialysis—The experimental design followed the method of a previous report (4). To assay the compound, a mixture of 0.5 ml. of the solution outside the bag and 1 ml. of phosphate buffer (pH 7.4) was treated with cyanogen bromide, using the same procedure as for the determination of nicotinoyltaurinophenetidine in blood.

The stability of nicotinoyltaurinophenetidine in phosphate buffer (pH 7.4) was determined; no decomposition was observed for 72 hr. A plasma sample was obtained from male rabbits (average weight 2.5 kg.) receiving 240, 320, and 400 mg./kg. of nicotinoyltaurinophenetidine; each group consisted of four rabbits.

Separation of Metabolites of Taurinophenetidine in Mouse Urine—Pooled urine from five male mice (dd strain, average weight 20 g.), ingesting 2500 mg./kg./day of taurinophenetidine for 2 days, was shaken with methylene dichloride for 20 min. The methylene dichloride layer from centrifugation was evaporated *in vacuo* below 50°. The residue was dissolved in a small amount of ethanol and submitted to TLC. The conditions for TLC were: solvent, benzene–acetone (2:1); adsorbent, diatomite (Kieselgel G); and color developer, 0.4% potassium ferricyanide solution followed by spraying with 1% ferric nitrate in 0.7 *N* nitric acid.

Separation of Metabolites of Nicotinoyltaurinophenetidine in Rabbit Urine—About 400 ml. of urine from four male rabbits (average weight 2.2 kg.), ingesting 500 mg./kg. of nicotinoyltaurinophenetidine, was submitted to freezing evaporation. The residue was treated by the method for separation of metabolites of taurinophenetidine in urine described in the previous report (1). The conditions for TLC were: solvent, the same spots obtained from a mixed solvent of acetone–benzene–ethyl acetate (1:1:2), acetone–chloroform–ethyl acetate (1:1:2), or acetone–hexane–ethyl acetate (1:1:2); color developer, iodine vapor, 5% silver nitrate solution, or 0.4% potassium ferricyanide solution followed by spraying with 1% ferric nitrate in 0.7 *N* nitric acid; and adsorbent, diatomite (Kieselgel G).

Assay of Metabolites of Nicotinoyltaurinophenetidine in Urine—A mixture of 1 ml. of 5 *N* HCl and 200 mg. of the frozen evaporate of urine of three male rabbits (mean weight 2.5 kg.), receiving nicotinoyltaurinophenetidine (400 mg./kg.), was treated as previously reported (1) and submitted to TLC, using a mixed solvent of benzene–acetone–ethyl acetate (1:1:2). The spots with R_f values corresponding to nicotinoyltaurinophenetidine and taurinophenetidine were scratched off the glass plate of the TLC and extracted with 3 ml. of ethanol for nicotinoyltaurinophenetidine or 3 ml. of water for taurinophenetidine, using a method similar to one previously reported (1). After centrifugation, 1 ml. of the supernatant was used for assay of the corresponding metabolite. Taurinophenetidine was assayed by the modified Folin method (1), and nicotinoyltaurinophenetidine was determined by the cyanogen bromide method for the determination of nicotinoyltaurinophenetidine in blood.

Assay of Metabolites of Taurinophenetidine in Feces—To three male rats (Wistar strain, average weight 200 g.), 2000 mg./kg. of taurinophenetidine in 0.2% tragacanth solution was administered orally; their feces were collected for 4 days. As a control group, three male rats were given 0.2% tragacanth solution without the compound under the same experimental condition. Total feces for each rat were triturated with 150 ml. of water and shaken vigorously with 150 ml. of methylene dichloride for 2 hr. The supernatant from centrifugation at 12,000 r.p.m. was obtained by decantation and filtered to remove water-insoluble matter. The filtrate was stored in a separator overnight at room temperature, and the layer of methylene dichloride was evaporated *in vacuo* below 50°. A mixture of the residue and 2 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr. and neutralized with about 2 ml. of 5 *N* sodium carbonate solution. This nearly neutralized mixture was extracted with an adequate amount of methylene dichloride, and the solvent was evaporated *in vacuo*. The residue was dissolved in 1 ml. of methylene dichloride, and 20 μl. of this solution was submitted to TLC as described for the assay of metabolites of taurinophenetidine in urine (1).

Distribution of Taurinophenetidine in Animal Organs—Taurinophenetidine used in the experiments was screened through 100 mesh.

Mice—To five male mice (dd strain, average weight 15 g.), 2500 mg./kg. of taurinophenetidine in 0.2% tragacanth solution was administered orally.

Rats—To three female rats (Wistar strain, average weight 200 g.), 2000 mg./kg. of taurinophenetidine in 0.2% tragacanth solution was given orally.

Rabbits—To three female rabbits (average weight 2.5 kg.), 400 mg./kg. of taurinophenetidine in water suspension was administered orally.

Five organs (heart, lung, liver, kidney, and brain) were used for the determination of taurinophenetidine and its metabolites. The sample of each organ after bleeding was taken in equal weight from five mice, three rats, or three rabbits. The sample was homogenized with 5 *N* HCl, and the homogenate was kept at 37 ± 2° for 1 hr. This homogenate was neutralized with 5 *N* sodium carbonate solution, and the supernatant from centrifugation was shaken with methylene dichloride for 20 min. The methylene dichloride solution was evaporated to dryness, and the residue was dissolved in 0.1 ml. of methylene dichloride. Fifty microliters of methylene dichloride solution was spotted on TLC (1), and the metabolites of taurinophenetidine were determined by the method for the assay of metabolites of taurinophenetidine in urine (1). The experimental conditions are shown in Table I.

Distribution of Nicotinoyltaurinophenetidine in Animal Organs—*Mice*—To five male mice (dd strain, average weight 13 g.), 2500 mg./kg. of nicotinoyltaurinophenetidine in 0.2% tragacanth solution was administered orally.

Rats—To three female rats (Wistar strain, average weight 120 g.), 2000 mg./kg. of nicotinoyltaurinophenetidine in 0.2% tragacanth solution was given orally.

Table II—Binding of Taurinophenetidine or Nicotinoyltaurinophenetidine with Serum Protein following Oral Administration to Rabbits[a]

Compound	Dose, mg./kg.	Hours after Dose	Assortment	Cp (*MV* ± *SE*) Buffer (Inside), ml. 0	0.5	1.0
TP	200	1.0	T	29 ± 4	39 ± 1	31 ± 4
			F	24 ± 3	21 ± 5	19 ± 9
			R	18 ± 2	37 ± 3	35 ± 4
		2.0	T	26 ± 7	35 ± 1	20 ± 2
			F	19 ± 2	19 ± 7	15 ± 8
			R	24 ± 2	37 ± 4	34 ± 3
	280	1.0	T	29 ± 1	32 ± 0.4	39 ± 9
			F	31 ± 7	31 ± 4	30 ± 8
			R	5 ± 5	8 ± 8	22 ± 4
		2.0	T	29 ± 4	30 ± 5	28 ± 8
			F	25 ± 5	27 ± 1	28 ± 6
			R	19 ± 2	10 ± 1	2 ± 2
	360	1.0	T	35 ± 1	48 ± 3	49 ± 3
			F	16 ± 8	26 ± 2	27 ± 2
			R	57 ± 7	49 ± 6	60 ± 2
		2.0	T	30 ± 2	36 ± 2	27 ± 5
			F	16 ± 6	24 ± 1	10 ± 2
			R	37 ± 2	35 ± 4	54 ± 9
NTP HCl	240	1.0	T			114 ± 2
			F			91 ± 2
			R			19 ± 2
		2.0	T			94 ± 3
			F			69 ± 1
			R			24 ± 1
	320	1.0	T			123 ± 4
			F			68 ± 1
			R			43 ± 9
		2.0	T			95 ± 3
			F			59 ± 1
			R			37 ± 6
	400	1.0	T	159 ± 1	162 ± 2	158 ± 9
			F	87 ± 1	92 ± 1	91 ± 1
			R	45 ± 6	43 ± 8	42 ± 9
		2.0	T	138 ± 3	144 ± 3	126 ± 2
			F	81 ± 1	81 ± 1	81 ± 1
			R	40 ± 5	44 ± 2	36 ± 1

[a] Cp = concentration of the compound (mcg./ml.) after equilibrium dialysis (outside of bag); T = total compound (mcg./ml.); F = free compound (mcg./ml.); R = binding ratios (%) of the compound with serum protein; *SE* = standard error; TP = taurinophenetidine; and NTP HCl = nicotinoyltaurinophenetidine hydrochloride. Experimental conditions were: rabbit serum/ml. inside the bag, and 8 ml. of the phosphate buffer solution (pH 7.4) outside the bag.

The experimental conditions were the same as described for the distribution of taurinophenetidine in animal organs. After extraction from the homogenate of each organ, the methylene dichloride solution was submitted to TLC; nicotinoyltaurinophenetidine and its metabolites were determined by the method for assay of metabolites of nicotinoyltaurinophenetidine in urine (1).

Collection of Rabbit and Rat Biles—Female rabbits, varying in weight from 2.5 to 3.0 kg., were anesthetized with urethan, 1 g./kg. s.c., following a fasting period of approximately 24 hr.

Blood pressure was recorded by a mercury manometer connected to a cannula inserted in the carotid artery, and respiration was recorded on a kymograph.

The experimental conditions were designed by the method described in a previous paper (5). Taurinophenetidine hydrochloride or nicotinoyltaurinophenetidine hydrochloride, 300 mg./kg. dissolved in 20 ml. of water, was administered orally. Each group consisted of three rabbits. Water (20 ml.) was given to each control rabbit. Male rats (Wistar strain, body weight 190–210 g.) were treated by the same method as for the rabbits. Each group consisted of five rats. Taurinophenetidine hydrochloride or nicotinoyltaurinophenetidine hydrochloride (300 mg./kg.) dissolved in 0.5 ml. of water was given orally. Control rats received 0.5 ml. of water each.

Determination of Taurinophenetidine or Nicotinoyltaurinophenetidine and Its Metabolites in Rabbit and Rat Bile—Rabbit bile from each group was combined and saturated with sodium chloride. The bile was shaken with 150 ml. of methylene dichloride for 2 hr. and the supernatant, after centrifugation at 12,000 r.p.m., was evaporated. The residue (A) obtained was mixed with 2 ml. of 5 *N* HCl and incubated at 37 ± 2° for 1 hr. This solution was neutralized with 2 ml. of 5 *N* sodium carbonate solution and shaken with about 7 ml. of methylene dichloride for 20 min. Residue B, obtained after evaporation of the methylene dichloride layer, was dissolved in 1 ml. of methylene dichloride, and 30 μl. of this solution was submitted to TLC. The component from each spot was determined by the method for assay of metabolites of taurinophenetidine in feces. Other parts of Residue A were submitted to the same TLC as Residue B. Determination of taurinophenetidine and its metabolites in rat bile was carried out by the same method as for rabbit bile, using 50 ml. of methylene dichloride instead of 150 ml. of the solvent for extraction.

Rabbit or rat bile collected after ingestion of nicotinoyltaurinophenetidine hydrochloride was treated in the same way as in the case of administration of taurinophenetidine and submitted to TLC. The component from each spot was determined by the method described for the assay of metabolites of nicotinoyltaurinophenetidine in urine.

Acute Toxicity of Taurinophenetidine, Taurinophenetidine Hydrochloride, Nicotinoyltaurinophenetidine, and Nicotinoyltaurinophenetidine Hydrochloride to Mice—The experiments were designed by the same method described in a previous report (6). Taurinophenetidine and nicotinoyltaurinophenetidine were administered as a suspension in 0.2% tragacanth solution, and the hydrochlorides of both compounds were given to mice as a solution in water. The volume of the test sample was adjusted to keep constant at 0.4 ml./10 g. body weight.

Tail-Pinching Test—The reaction time of mice pinched on the tail with an artery clip was recorded every 15 min. for 1.5 hr. after administration of the compounds.

Effect of Taurinophenetidine, Nicotinoyltaurinophenetidine, and Related Compounds on Squirming and Capillary Permeability—Male dd strain mice, 15 g., were given the test compounds orally. The compounds were screened through 200 mesh and suspended in

Table III—Distribution of Taurinophenetidine (Micrograms per Gram of Each Organ) after Oral Administration to Animals[a]

Organ	Animal	Taurino-phenet-idine	*p*-Amino-phenol	*p*-Phenet-idine	*p*-Acet-amido-phenol
Heart	Mouse	131			
	Rat	42			
	Rabbit	58			
Lung	Mouse	300			
	Rat	55			
	Rabbit	71			
Liver	Mouse	204			
	Rat	5		4	
	Rabbit	6			
Kidney	Mouse	273			Trace
	Rat	74	10		
	Rabbit	256	9	44	
Brain	Mouse	56			
	Rat	1			
	Rabbit	26			

[a] The data were obtained at the time of peak blood levels, 1.5 hr. for mice and rats and 2.0 hr. for rabbits, after oral administration of the compound, 2500 mg./kg. to mice, 2000 mg./kg. to rats, and 400 mg./kg. to rabbits.

0.2% tragacanth solution. The experiments were carried out using a method described in a previous paper (6).

Tail-Withdrawal Reflex in Mice—The same method for analgesimetry described in the paper of Ben-Bassat *et al.* (7) was used.

Anti-Inflammatory Activity—Inflammation was produced by injecting into the plantar surface of the hind paw of a male rat (Wistar strain, average weight 200 g.) carrageenin (0.1 ml. of 2% suspension), formaldehyde solution (0.2 ml. of 0.74% solution), or croton oil (0.1 ml. of 1% in sesame oil). Normal saline solution was given to the other hind paw as a control.

The test compound was administered in an aqueous solution, whose volume was kept constant at 0.1 ml./100 g. body weight for intraperitoneal dosage. One-half of the total dose of the test compound was given immediately after injection of the phlogistic agent, and the remaining half was given approximately 6 hr. later. Intraperitoneal injections were given in either caudal quadrant of the abdominal wall. Control animals received injections of the vehicle.

Each group consisted of five rats. The effect of the test compound on the edema was determined volumetrically 24 hr. after injection of the phlogistic agent. The method reported by Benitz and Hall (8) was used to design the experimental conditions.

Antianaphylactic Activity—Female guinea pigs (Hartley strain, average weight 400 g.) were used. The experiment was carried out by the procedure reported by Labelle and Tislow (9). Guinea pigs were challenged exactly 1 hr. after drug administration by rapid injection of 1 ml. of horse serum into the saphenous vein.

Antihistamine Activity—Female guinea pigs (Hartley strain, average weight 400 g.) were used in the histamine tests. The test was performed under the same condition as described in a previous paper (6).

Antipyretic Activity—To male rabbits weighing 2.2–2.8 kg., 15 mcg./kg. of nonanaphylactogenic polysaccharides obtained from

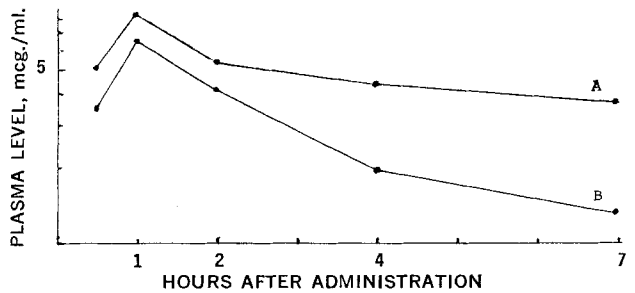

Figure 1—*Mean plasma level of free and total acetophenetidin after oral administration to four rabbits of 250-mg./kg. dose. Key: A, total acetophenetidin; and B, free acetophenetidin.*

Table IV—Distribution of Nicotinoyltaurinophenetidine and Its Metabolites (Micrograms per Gram of Each Organ) after Oral Administration of Nicotinoyltaurinophenetidine to Animals[a]

Organ	Animal	Weight of Organ, g. ($MV \pm SE$)[b]	Nicotinoyl-taurino-phenet-idine, mcg./g.	Taurino-phenet-idine, mcg./g.
Heart	Mouse	0.1 ± 0.0	131	0
	Rat	2.1 ± 0.2	45	34
Lung	Mouse	0.17 ± 0.0	89	26
	Rat	4.2 ± 1.2	66	39
Liver	Mouse	1.0 ± 0.0	139	6
	Rat	15.2 ± 0.3	49	9
Kidney	Mouse	0.2 ± 0.0	103	16
	Rat	3.6 ± 0.1	58	39
Brain	Mouse	0.3 ± 0.0	14	14
	Rat	3.4 ± 0.1	25	0

[a] The data were obtained at the time of peak blood levels, 1.5 hr. after oral administration of the compound, 2000-mg./kg. dose. [b] $MV \pm SE$ = mean value ± standard error.

Pseudomonas fluorescens[1] was administered intravenously; solution of the test compound was given subcutaneously 1.75 hr. after the administration of the pyrogen solution. Body temperature was recorded every 15 min. for 6 hr. by a thermistor-thermometer.[2] Each group consisted of three rabbits.

RESULTS AND DISCUSSION

Plasma of mice and rats, as well as of rabbits (1), after oral ingestion of taurinophenetidine or nicotinoyltaurinophenetidine, contained no detectable metabolites of the chemical in TLC.

Blood level of acetophenetidine, which has a chemical structure similar to taurinophenetidine, was determined in rabbits for the sake of comparison, and the results are shown in Fig. 1. Blood level of taurinophenetidine is clearly higher than that of acetophenetidin at

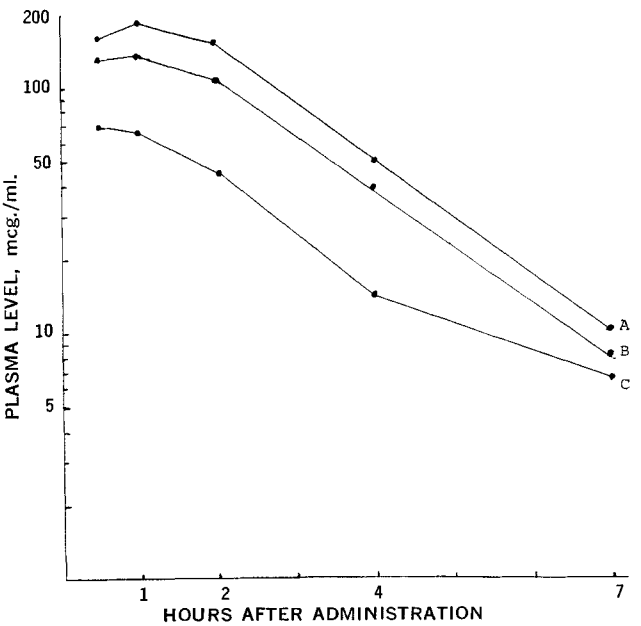

Figure 2—*Mean plasma level of nicotinoyltaurinophenetidine after oral administration of nicotinoyltaurinophenetidine hydrochloride to four rabbits of different doses. Each group consisted of four rabbits. Key: A, 300 mg./kg.; B, 225 mg./kg.; and C, 150 mg./kg.*

[1] T.T.G. No. 1, Fujisawa Yakuhin Kogyo Co. Ltd., Osaka, Japan.
[2] PSM-402, Eiwa Denshi Keiki Seisakusho Co. Ltd., Kyoto, Japan.

Table V—Acute Toxicity of Taurinophenetidine and Its Related Compounds in Mice[a]

Route[b]	Compound[c]	LD_{50}, mg./kg.[d]
p.o.	TP	4500 (3965–5108)
i.p.	TP	1100 (924–1309)
p.o.	TP HCl	1550 (1192–2015)
i.p.	TP HCl	570 (416–781)
s.c.	TP HCl	590 (552–630)
p.o.	NTP	6800 (6326–7310)
i.p.	NTP	1650 (1510–1803)
p.o.	NTP HCl	4650 (3577–6045)
i.p.	NTP HCl	470 (376–588)
p.o.	AP	3100 (2903–3311)

[a] Each group consisted of five male and five female mice (dd strain average weight 17 g.). The LD_{50} was calculated by the Litchfield–Wilcoxon method (12). [b] i.p. = intraperitoneal injection; p.o. = oral administration; and s.c. = subcutaneous injection. [c] TP = taurinophenetidine; TP HCl = taurinophenetidine hydrochloride; NTP = nicotinoyltaurinophenetidine; NTP HCl = nicotinoyltaurinophenetidine hydrochloride; and AP = acetophenetidin. [d] ( ) = 95% confidence limits.

the same dose, and this fact shows that the former is absorbed more easily than the latter.

Blood level of nicotinoyltaurinophenetidine is higher than that of taurinophenetidine at the same dose, as shown in Fig. 2. Therefore, nicotinoyltaurinophenetidine is more desirable than taurinophenetidine for use in pharmacological experiments as far as absorption is concerned.

The binding of taurinophenetidine or nicotinoyltaurinophenetidine with rabbit serum proteins *in vivo* at different concentrations of blood level is shown in Table II. It was confirmed that the binding ratio of taurinophenetidine or nicotinoyltaurinophenetidine with serum proteins varies according to experimental conditions. As a result, the averaged binding ratios of taurinophenetidine and nicotinoyltaurinophenetidine were roughly 40–50% at high doses, such as 360 mg./kg. of taurinophenetidine or 400 mg./kg. of nicotinoyltaurinophenetidine, but the binding ratios of both compounds with serum proteins were questionable at low doses.

Metabolites of taurinophenetidine in mouse urine were examined. The same metabolites were detected in mice as in rabbits (1), namely, unchanged taurinophenetidine, *p*-acetamidophenol, *p*-aminophenol, and *p*-phenetidine, having R_f values of 0.25, 0.35, 0.53, and 0.83, respectively.

Metabolites of nicotinoyltaurinophenetidine in rabbit urine were studied in the freeze-dried residue of the urine within 48 hr. after oral administration. Only two metabolites, unchanged nicotinoyltaurinophenetidine and taurinophenetidine, having R_f values of 0.31 and 0.20, respectively, were detected. *p*-Phenetidine, *p*-aminophenol, and *p*-acetamidophenol, the metabolites of taurinophenetidine, were not detected.

The results of quantitative determination of the metabolites in urine suggested that 9% of taurinophenetidine and 91% of unchanged nicotinoyltaurinophenetidine of the total metabolites were excreted; the ratio of the total metabolites to the dose ingested could not be calculated because of incomplete urine collection. However, 112 mg. of taurinophenetidine and 1200 mg. of nicotinoyltaurinophenetidine were recovered from urine of rabbits within 48 hr. after oral administration of a total of 4 g. of nicotinoyltaurinophenetidine.

Taurinophenetidine in feces of each rat was only 841 ± 132 mcg. when 2000 mg./kg. of the compound was given to a rat weighing about 200 g. The total weight of feces from each rat for 4 days was 39 ± 8 g. No metabolites except unchanged taurinophenetidine were detected in rat feces through TLC.

Table VI—Tail-Pinching Test after Oral Administration to Mice[a]

Compound	ED_{50}, mg./kg.[b]
Taurinophenetidine	580 (523–644)
Taurinophenetidine hydrochloride	365 (294–453)
Nicotinoyltaurinophenetidine	425 (322–561)
Acetophenetidin	750 (684–823)

[a] Each group consisted of five male and five female mice (dd strain, average weight 15 g.). The experiment was performed by the method of Haffner (10), and the results were obtained 30 min. after administration. The ED_{50} was calculated by the Litchfield–Wilcoxon method (12). [b] ( ) = 95% confidence limits.

Table VII—Effect of Test Compounds on Squirming and Capillary Permeability after Oral Administration[a]

Compound	Inhibition of Squirming, ED_{50}, mg./kg.[b]	Inhibition of Permeability, ED_{50}, mg./kg.[b]
Sodium acetylsalicylate	155 (80–301)	
Acetophenetidin	430 (261–710)	825 (453–1502)
Taurinophenetidine	285 (143–560)	
p-Acetamidophenol	970 (485–1940)	
p-Phenetidine hydrochloride	710 (458–1101)	950 (519–1739)
p-Aminophenol	490 (314–764)	
Taurinophenetidine hydrochloride	198 (99–396)	
Nicotinoyltaurinophenetidine	130 (74–229)	
Nicotinoyltaurinophenetidine hydrochloride	128 (82–200)	

[a] Each group consisted of 12 male mice (dd strain, average weight 15 g.). [b] ( ) = 95% confidence limits.

The amounts of taurinophenetidine distributed in the heart, lung, liver, kidneys, and brain of mice were fairly larger than those in rats and rabbits, as shown in Table III. Distribution of taurinophenetidine (micrograms per gram of each organ) showed almost similar values in rats and rabbits, except for the compound in the kidney. A considerable amount of taurinophenetidine was distributed in the kidneys of rabbit, but no metabolites were found in most animal organs except in rat liver and kidneys and in rabbit kidneys. It was also ascertained that taurinophenetidine and its metabolites do not remain in any of the organs of mice, rats, and rabbits 24 hr. after oral administration of the compound.

As shown in Table IV, nicotinoyltaurinophenetidine and taurinophenetidine are distributed in animal organs but none of the metabolites such as *p*-aminophenol, *p*-acetamidophenol, and *p*-phenetidine is found when nicotinoyltaurinophenetidine is administered orally to mice and rats. Nicotinoyltaurinophenetidine and its metabolites do not remain in any of the organs of mice and rats 24 hr. after oral ingestion of the compound, as in the case of taurinophenetidine.

The hydrochloride of taurinophenetidine or nicotinoyltaurinophenetidine at the dose of 300 mg./kg. had no effect on arterial blood pressure, respiration, bile flow, or total bile volume excreted for 7.5 hr. compared with untreated control rabbits. The evaporated residue from methylene dichloride used for extraction of rabbit or rat bile contained no metabolites when the hydrochloride of taurinophenetidine or nicotinoyltaurinophenetidine was administered orally. Even when the evaporated residue was treated with hydrochloric acid since the binding of a drug with bile components can possibly be prohibited by the action of hydrochloric acid, no metabolites were found after oral administration of the hydrochloride of taurinophenetidine or nicotinoyltaurinophenetidine to rabbits or rats. Taurinophenetidine or nicotinoyltaurinophenetidine in rabbit and rat bile, excreted for 7.5 hr. following oral administration of a 300-mg./kg., dose, was almost zero in either rabbits or rats in the former (taurinophenetidine), or 395 and 2325 mcg./kg. in the latter (nicotinoyltaurinophenetidine) at the mean value obtained from three rabbits and five rats.

Acute toxicity according to the number of dead mice 72 hr. after various doses of taurinophenetidine, nicotinoyltaurinophenetidine, or hydrochlorides of both compounds, and intraperitoneal, subcutaneous, and oral LD_{50}'s are shown in Table V. Although the animals receiving intraperitoneal doses below 700 mg./kg. of

Table VIII—ED_{50} for Pain Reaction Times in Mice 60 min. after Intraperitoneal Injection of Test Compounds[a]

Compound	ED_{50}, mg./kg.[b]
Acetophenetidin	435 (300–631)
Taurinophenetidine	310 (238–403)
Taurinophenetidine hydrochloride	127 (81–199)
Nicotinoyltaurinophenetidine	210 (124–357)
Nicotinoyltaurinophenetidine hydrochloride	168 (116–244)

[a] Each group consisted of 10 male mice (dd strain, average weight 15 g.). [b] ( ) = 95% confidence limits.

Table IX—Effect of Test Compounds on the Edema Induced by Subplantar Injection of Phlogistic Agents[a]

Phlogistic Agent	Treatment[b]	Dose, mg./kg.	Mean Volume, $MV \pm SE$[c]	Inhibition, %
Formaldehyde	Control		0.864 ± 0.136	
	NTP HCl	200	0.536 ± 0.087[d]	38
		300	0.582 ± 0.107[d]	33
		400	0.408 ± 0.133[d]	53
		500	0.446 ± 0.130[d]	48
	SA	500	0.735 ± 0.140	
	TP HCl	200	0.872 ± 0.085	
Croton oil	Control		0.868 ± 0.293	
	NTP HCl	200	0.724 ± 0.140	
	TP HCl	200	1.012 ± 0.417	
Carrageenin	Control		0.924 ± 0.131	
	NTP HCl	200	0.562 ± 0.082[d]	39
		300	0.492 ± 0.128[d]	47
		400	0.396 ± 0.153[d]	57
	SA	300	0.870 ± 0.375	
		400	0.864 ± 0.439	
		500	0.832 ± 0.349	
	TP HCl	200	0.904 ± 0.047	
		300	0.772 ± 0.391	
		400	0.710 ± 0.274	

[a] Each group consisted of five male rats (Wistar strain, average weight 200 g.). [b] NTP HCl = nicotinoyltaurinophenetidine hydrochloride; SA = sodium salicylate; and TP HCl = taurinophenetidine hydrochloride. [c] $MV \pm SE$ = mean value ± standard error. [d] Statistically significant effect at 95% confidence limits.

taurinophenetidine, 280 mg./kg. of taurinophenetidine hydrochloride, 1200 mg./kg. of nicotinoyltaurinophenetidine, or 280 mg./kg. of nicotinoyltaurinophenetidine hydrochloride exhibited no behavioral abnormality, intraperitoneal toxic doses of these four compounds produced a transient ataxic walk with extended hind limbs and a marked reduction in spontaneous movements. Within 10 min. after the intraperitoneal administration of 1800 mg./kg. of taurinophenetidine, 800 mg./kg. of its hydrochloride, 2200 mg./kg. of nicotinoyltaurinophenetidine, or 1000 mg./kg. of its hydrochloride, all of the animals were dead. They exhibited no lacrimation, piloerection, or convulsions before death, except that convulsion before death was observed in the cases of intraperitoneal administration of hydrochlorides of both compounds. No significant difference in the behavioral effect or LD_{50} was observed between the male and female

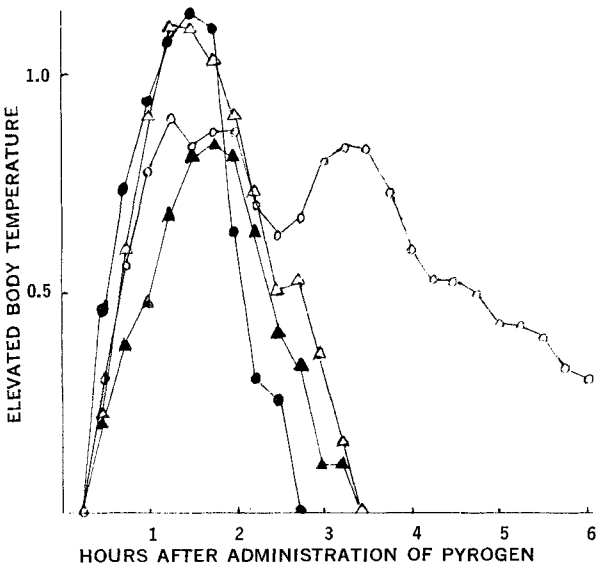

Figure 3—*Mean curve for the fall of elevated body temperature of male rabbits receiving pyrogen. Each group consisted of three rabbits. Key: ○, control group, mean basal body temperature (MBBT) 39.1°; ●, 125 mg./kg. of taurinophenetidine hydrochloride (TP HCl), MBBT 39.2°; ▲, 75 mg./kg. of TP HCl, MBBT 39.1°; and △, 50 mg./kg. of TP HCl, MBBT 39.2°.*

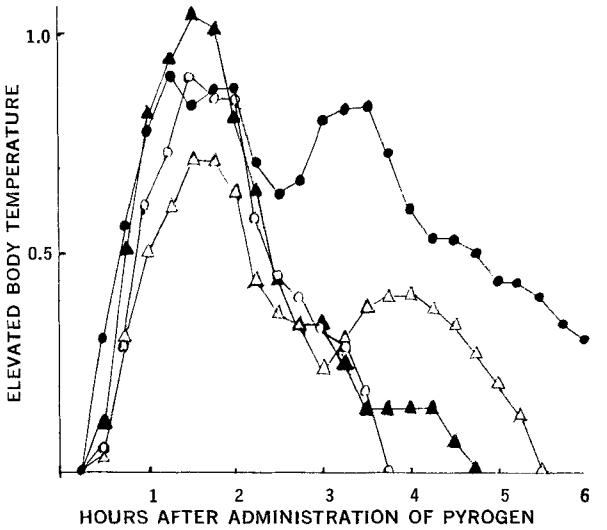

Figure 4—*Mean curve for the fall of elevated body temperature of male rabbits receiving pyrogen. Each group consisted of three rabbits. Key: ○, control group, mean basal body temperature (MBBT) 39.2°; ▲, 75 mg./kg. of nicotinoyltaurinophenetidine hydrochloride (NTP HCl), MBBT 39.3°; △, 50 mg./kg. of NTP HCl, MBBT 39.2°; and ○, 50 mg./kg. of* p*-acetamidophenol, MBBT 39.1°.*

mice. The LD_{50} of acetophenetidin was determined for the sake of comparison.

Results of the tail-pinching test are shown in Table VI. Taurinophenetidine and nicotinoyltaurinophenetidine show analgesic activity in the tail-pinching test and their potency is stronger than that of acetophenetidin.

The purpose of squirming and permeability tests was to obtain some information by measuring the concurrent changes in the peritoneal capillary permeability during squirming through the method of Whittle (11). Table VII records the values for the ED_{50}'s for inhibition of squirming and the ED_{50}'s for reduction of the permeability response. In most of the test compounds, reduction of the permeability response did not approach 50% over the range of doses which inhibited squirming. Sodium acetylsalicylate, acetophenetidin, and the metabolites of taurinophenetidine such as *p*-acetamidophenol, *p*-phenetidine, and *p*-aminophenol were used for comparative examination. None of the metabolites was more potent than taurinophenetidine at the same dose as the ED_{50} of taurinophenetidine for inhibition of squirming.

The results of tail-withdrawal reflex in mice are shown in Table VIII. Computation of ED_{50} was made by dichotomizing the reaction after 60 min. into two groups; "refractory," *i.e.*, pain reaction time of 2.0 sec. or more, and "nonrefractory" for the test compounds. From the above results, it was ascertained that analgesic action of nicotinoyltaurinophenetidine is more potent than that of taurinophenentidine which is more potent than that of acetophenetidin.

Table IX shows some results obtained for anti-inflammatory activity of taurinophenetidine hydrochloride, nicotinoyltaurinophenetidine hydrochloride, and sodium salicylate. It is interesting that the effect of sodium salicylate on the edema could not be found under the present experimental conditions, but nicotinoyltaurinophenetidine hydrochloride did show some anti-inflammatory actions on edema induced by formaldehyde solution and carrageenin. In contrast, taurinophenetidine hydrochloride shows no definite anti-inflammatory action on edema.

Effect of hydrochlorides of taurinophenetidine and nicotinoyltaurinophenetidine on antihistamine activity and antianaphylactic shock in guinea pigs was examined by the method of Labelle and Tislow (9). Neither of the compounds showed any activity at 300-, 500-, and 700-mg./kg. doses.

Taurinophenetidine hydrochloride decreased the elevated body temperature of all rabbits at doses above 75 mg./kg. The antipyretic action of the compound was higher than that of *p*-acetamidophenol even at low doses, such as 75 mg./kg., as shown in Figs. 3 and 4. On the other hand, nicotinoyltaurinophenetidine hydrochloride showed no antipyretic action at 50- and 75-mg./kg. doses. The question remains as to why there is a difference in the

antipyretic effect between taurinophenetidine hydrochloride and nicotinoyltaurinophenetidine hydrochloride; it is hoped that this problem will be solved in the near future.

In conclusion, taurinophenetidine and nicotinoyltaurinophenetidine are scarcely excreted in rat feces and in rat and rabbit bile. The amount of both compounds distributed in organs of mice and rats is very small. Taurinophenetidine has some analgesic and antipyretic activities, and nicotinoyltaurinophenetidine shows some analgesic and anti-inflammatory activities.

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Chromatographic Determination of Benzoic Acid Derivatives for Application to Metabolism Studies

JOSEPH E. SINSHEIMER and GEORGE O. BREAULT*

Abstract □ Thin-layer and gas chromatographic systems were developed in support of metabolism studies to detect 4-hydroxybenzoic, 2-hydroxyanisic, and 3-hydroxyanisic acids in the presence of benzoic acid. Chromatographic systems were also developed for the detection of 4-chlorobenzoic acid in the presence of benzoic and 4-hydroxybenzoic acids. Quantitative results were obtained by gas chromatography and by reverse TLC fluorimetry of the quenching of a fluorescent background.

Keyphrases □ Benzoic acid derivatives—determination as metabolites □ GLC—analysis □ TLC—analysis □ Fluorometry, quenching TLC—analysis

It was found necessary to develop procedures for the separation, identification, and assay of benzoic acid derivatives in support of studies in these laboratories of the importance of cleavage of the ethylenic bond in the metabolism of stilbenes (1). Sensitive methods applicable to rabbit urine extracts containing benzoic acid as a normal metabolite were required.

The initial stilbene under consideration was 4,4′-dimethoxystilbene. It was expected from the reported cleavage of *trans*-stilbene to benzoic acid (2, 3) that the cleavage product of 4,4′-dimethoxystilbene would be anisic acid. Moreover, since *O*-demethylation and aromatic hydroxylation are common metabolic reactions, it was felt that cleavage could also yield 4-hydroxybenzoic, 2-hydroxyanisic, and 3-hydroxyanisic acids. Therefore, it was necessary to develop analytical systems for the detection and estimation of these compounds as possible metabolites.

The major expected cleavage product of 4,4′-dichlorostilbene would be 4-chlorobenzoic acid; emphasis was placed on the analysis of this product in the presence of naturally occurring benzoic and 4-hydroxybenzoic acids.

TLC on a fluorescent background and gas chromatography of trimethylsilyl (TMS) esters met the requirements for the determination of the compounds of interest. The purpose of this paper is to describe the development of these procedures as general methods for the analyses of minor amounts of benzoic acid derivatives.

EXPERIMENTAL

Reagents—All common reagents and solvents utilized were of analytical reagent grade. The following reagents were used: silica gel G;[1] cellulose;[2] fluorescent indicator;[3] SE-52, OV-1, OV-17, and OV-25;[4] Chromosorb G, 100/120 mesh;[5] bis(trimethylsilyl)-acetamide (BSA);[4] benzoic acid;[6] 4-hydroxybenzoic acid;[7] 4-anisic acid;[8] and 2-hydroxy- and 3-hydroxyanisic acids prepared according to the literature (4–6).

TLC—Silica gel G TLC plates were prepared by coating five 20 × 20-cm. glass plates at a thickness of 250 μm. with a slurry composed of 21 g. of silica gel G, 2.1 g. of fluorescent indicator, and 50 ml. of water. Cellulose TLC plates were prepared by similarly coating five 20 × 20-cm. glass plates with a slurry composed of 10 g. of cellulose, 1.0 g. of fluorescent indicator, and 65 ml. of water. After air drying at room temperature, plates were activated at 105° for 30 min. prior to use. Spotting was performed with a 1.0-μl. pipet. Chromatograms were developed to a distance of 15 cm. by the ascending technique. Developing tanks were lined with filter paper and

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EXPERIMENTAL

Reagents—All common reagents and solvents utilized were of analytical reagent grade. The following reagents were used: silica gel G;[1] cellulose;[2] fluorescent indicator;[3] SE-52, OV-1, OV-17, and OV-25;[4] Chromosorb G, 100/120 mesh;[5] bis(trimethylsilyl)-acetamide (BSA);[4] benzoic acid;[6] 4-hydroxybenzoic acid;[7] 4-anisic acid;[8] and 2-hydroxy- and 3-hydroxyanisic acids prepared according to the literature (4–6).

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Table I—TLC of Dimethoxystilbene Reference Acids on Silica Gel G Plates

Acid	R_f Value in Solvent System[a] A	B
Benzoic	0.69	0.86
Anisic	0.56	0.86
2-Hydroxyanisic	0.49	0.86
3-Hydroxyanisic	0.17	0.76
4-Hydroxybenzoic	0.15	0.64

[a] Solvent Systems: A, petroleum ether–acetic acid (4:1); and B, chloroform–acetic acid (4:1).

were saturated with the appropriate solvent mixture. Detection of spots was by the quenching of fluorescence under a UV lamp.

Reverse TLC Fluorimetry—Silica gel G plates, scored in 0.5-cm. channels, were spotted in alternate channels with varying amounts of the compounds to be measured and developed. The developed plates were air dried and placed in the scanning apparatus[9] with the adsorbent side down. Scanning was done with the following conditions: scanner speed, 1 r.p.m.; recorder speed, 60 in./hr.; and sensitivity and meter multiplication, set to give a response of 90–100% for the background fluorescence. Anisic acid was measured with an activation wavelength of 277 nm. and emission wavelength of 530 nm.

Gas Chromatography—Column packings were prepared by coating the liquid stationary phase on the solid support in a rotary evaporator. The dry packing was added to one end of the coiled copper tubing, which was connected at the other end to a water aspirator by means of a glass tube plugged with glass wool. The copper tubing was agitated until fully packed, and the ends were plugged with glass wool.

Qualitative and quantitative gas chromatography was performed with an F&M 700 gas chromatograph equipped with dual flame-ionization detectors. The following conditions were used for all analyses: carrier gas, helium, 60 ml./min. flow; hydrogen flow, 50 ml./min. (16 p.s.i.); air flow, 380 ml./min. (16 p.s.i.); injection port temperature, 250°; detector temperature, 250°; attenuation, 5; range, 10^2; and injection size, 5.0 μl., with a Hamilton 701-N 10-μl. syringe. Other conditions were varied for the determination of different compounds as follows: A, column, 3.7 m. × 0.32 cm. o.d. (12 ft. × 0.125 in. o.d.), 3% OV-17 on Chromosorb G 100/120 mesh; column oven temperature, 190°; B, same as A but with column oven temperature 170°; and C, column, 1.8 × 0.32 cm. o.d. (6 ft. × 0.125 in. o.d.), 3% OV-1 on Chromosorb G 100/120 mesh; column oven temperature, 150°. TMS derivatives were prepared by dissolving the samples in 0.3 ml. tetrahydrofuran and 0.2 ml. BSA at least 15 min. prior to analysis.

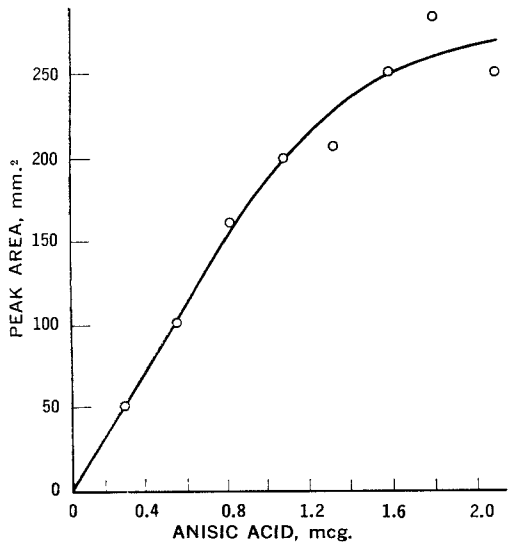

Figure 1—*Reverse TLC fluorimetry of anisic acid.*

[9] Aminco thin-film scanner (No. 4-8221A) connected to an Aminco-Bowman spectrophotofluorometer (SPF 4-8202) and a 100-mv. Beckman recorder (93500).

Table II—TLC of Benzoic, 4-Chlorobenzoic, and 4-Hydroxybenzoic Acids

Acid	R_f Value in Solvent System[a] C	D
4-Chlorobenzoic	0.84	0.75
Benzoic	0.75	0.61
4-Hydroxybenzoic	0.35	0.12

[a] Solvent Systems: C, 2-propanol–ethanol–water–ammonium hydroxide (50:20:10:5); and D, *n*-butanol saturated with ammonium hydroxide.

RESULTS AND DISCUSSION

TLC—Table I lists the R_f values of benzoic and the four reference acids on TLC plates of silica gel G in the solvent systems indicated. While System A yields the better separation of these acids, System B enhances the separation of 3-hydroxyanisic and 4-hydroxybenzoic acids. 4-Chlorobenzoic acid in reference to the naturally occurring urinary metabolites, benzoic and 4-hydroxybenzoic acids, was best analyzed by TLC on cellulose plates in the solvent systems listed in Table II.

Detection of these acids could be performed with bromcresol green solution (7) at a limit of sensitivity of from 10 to 20 mcg. of acid. Except for benzoic acid, this limit of sensitivity could be improved 10–100 times by observing the quenching of a fluorescent indicator added to the TLC plates. The comparatively poor sensitivity of this method for benzoic acid was actually an advantage in these studies since benzoic acid was expected to be present in control urine extracts.

Reverse TLC Fluorimetry—Since fluorescent compounds can be easily analyzed on TLC plates, quantitation of the quenching of background fluorescence was investigated for reference acids by a similar procedure used for direct TLC fluorimetry (8). Plates were scanned perpendicular to the solvent front. Plates were scored in 0.5-cm. channels before development to limit lateral diffusion to the same length as the activation wavelength slit of the fluorescent scanner. Spotting was done in alternate channels. Maximum fluorescence was obtained with activation wavelength 277 nm. and emission wavelength 530 nm. The fluorescence baseline was most stable when the 10% fluorescent indicator was used. Results obtained for

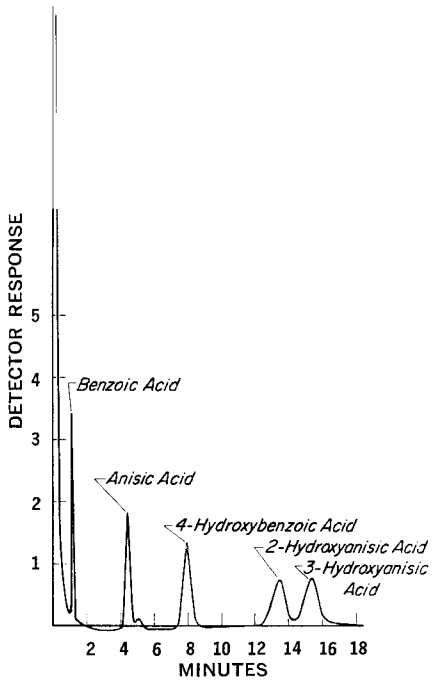

Figure 2—*Gas chromatographic separation of reference acids as their TMS esters.*

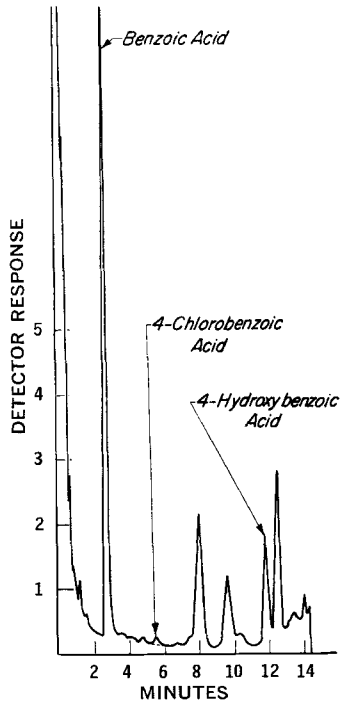

Figure 3—*Gas chromatographic detection (as TMS esters) of 4-chlorobenzoic acid added to rabbit urinary acids.*

anisic acid after development in Solvent System A are shown in Fig. 1. This graph reveals that the method can be used for the quantitative determination of anisic acid below 1 mcg. However, in contrast to direct TLC scanning, baseline stability can be a problem in the reverse technique in a manner analogous to densitometry scanning.

Gas Chromatography—The separation of TMS derivatives of dimethoxystilbene reference acids by gas chromatography was investigated. Good separation was obtained on 3% SE-52, OV-1, OV-17, and OV-25 on Chromosorb G 100/120 mesh columns. The results obtained using Condition C (3% OV-1) are reproduced in Fig. 2. Acid fractions of rabbit urine showed a number of peaks when gas chromatographed, and anisic acid in urine extracts was best analyzed with Condition A (3% OV-17). For quantitative determination of anisic acid, a graph of height or area of the peak *versus* equivalent weight of acid injected was plotted. It was found that using peak height gave a linear relationship only to 15 mcg., whereas peak area gave a linear plot to 150 mcg. The peak area was measured by either a Disc integrator (model 201-B) or by the product of the peak height times the width at half height. The two methods of measuring peak area gave approximately the same results. When anisic acid was added to urine, 90–95% was recovered by extraction and gas chromatographic analysis. The extraction scheme consisted of continuous extraction with ether of acidified urine, back extraction with 5% sodium bicarbonate, acidification of the bicarbonate extract, and final recovery of acids by ether extraction. While this scheme results also in the isolation of a series of naturally occurring acids, they did not interfere with the determination of anisic, 2-hydroxyanisic, or 3-hydroxyanisic acids.

Gas chromatographic analysis of 4-chlorobenzoic acid was performed with Condition B (3% OV-17). 4-Chlorobenzoic acid, when added to a control rabbit urine acid fraction, had a retention time of 5.5 min. and a detection limit of 1 mcg., as shown in Fig. 3. Note also in Fig. 3 that benzoic and 4-hydroxybenzoic acids are indicated on the basis of cochromatography with reference acids. It was attempted to increase the sensitivity by the use of an electron-capture detector (^{3}H). However, the presence of electron-capturing impurities required that the solutions be diluted, which resulted in approximately the same overall sensitivity obtained with flame-ionization detection.

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Solubility of Amino Acids in Mixed Solvent Systems

T. E. NEEDHAM, Jr.*, A. N. PARUTA, and R. J. GERRAUGHTY

Abstract □ The effects of various solvents and pH on the solubility characteristics of glycine, L-alanine, L-valine, L-phenylalanine, and DL-amino octanoic acid were studied in a series of hydroalcoholic solvent systems. The solubility properties of the amino acids studied were found to be dominated by the α-amino carboxylic acid portion of the molecule but also depended to some extent on the nonpolar portion of the molecule and its interaction with each specific solvent system. These interactions gave rise to a rank order of solubility which was characteristic of the specific amino acid rather than the decreasing polarity of the solvent system. Similar solubility profiles were found throughout the concentration spectrum and were defined by using a polynomial regression analysis. As the percent of alcohol was increased in the solvent systems studied, similar increments of acid or base added to the system produced a proportionally greater increase in the magnitude of total amino acid solubility.

Keyphrases □ Amino acid solubility—hydroalcoholic solvent systems □ Hydroalcoholic solvent systems—amino acid solubility □ α-Amino carboxylic acid group domination—amino acid solubility □ Dielectric constant effect—amino acid solubility

In other work the authors studied the solubility of a series of amino acids in pure solvent systems at pH and pH′ above, at, and below the isoelectric point (1). The solubility of the amino acids studied was at a maximum in water and was inversely related to the length of the nonpolar portion of the molecule. Solubility in the series of pure alcoholic solvents was reported to be of the same order of magnitude for the amino acids studied but substantially lower than that found in water. pH variation above and below the isoelectric point was found to increase solubility as a function of acid or base added.

Since this work was originally designed to elucidate the solubility of selected amino acids as a function of pH in various solvents and to correlate this behavior to those properties seen *in vivo*, it was desirable to determine the effect of mixed solvent systems on amino acid solubility.

EXPERIMENTAL

Equipment—A rotating apparatus[1] capable of holding multiple samples was immersed in a water bath, which was kept at constant temperature by a controlled-temperature circulating pump.[2] The apparent pH (pH′) of nonaqueous systems was measured using a combination electrode.

Systems Employed—The amino acids used were glycine, L-alanine, L-valine, L-phenylalanine, and DL-amino octanoic acid. The solubility of each of the amino acids was found in the pure and mixed solvent systems at 0, 10, 30, 50, 70, 90, and 100% (v/v) of methanol–water, ethanol–water, *n*-propanol–water, isopropanol–water, and tertiary butanol–water. The label purity of the manufacturer was accepted, and no pretreatment of the chemicals was deemed necessary. The water was distilled and deionized to minimize the possibility of complex formation. Hydrochloric acid and sodium hydroxide were used to adjust pH. All work was performed at a temperature of 25 ± 0.2°.

Procedure—For each concentration of a solvent system evaluated, six samples of equal volume were prepared from a stock solution, an excess amount of the selected amino acid was added, and the sample bottles were rotated in the constant-temperature bath for 24 hr. or until equilibrium was attained. Either 1- or 5-ml. samples were withdrawn from the sample bottles, using a pipet with a pledget of glass wool as a filtering medium. The size of the sample to be withdrawn was predetermined to minimize error during gravimetric analysis. The sample was immediately transferred to a tared vial, weighed to determine density, placed in an oven, and dried to constant weight. All drying was done at a temperature of 95° or less to avoid decomposition of the amino acids (2). Immediately after the sampling process, the pH or pH′ of each solution was taken. Incremental amounts of acid were added to half of the samples in successive 24-hr. periods, the pH was taken, and a gravimetric analysis was

Table I—Polynomial Regression Data Generated from the Mola Solubility of Glycine as a Function of the Percent Strength of the Solvent System

Solvent System[a]	*Y*-Intercept[b]	Coefficients	
		X	X^2
Methanol	2.6520	−0.0743	0.0005
Ethanol	2.7823	−0.0738	0.0005
n-Propanol	2.7591	−0.0664	0.0004
Isopropanol	2.7915	−0.0721	0.0005
Tertiary butanol	2.7303	−0.0676	0.0004

[a] Molar solubility values of glycine in 0–100% (v/v) of each solvent system were used to generate the polynomial equation. [b] Solubility of glycine (moles/l.) in water.

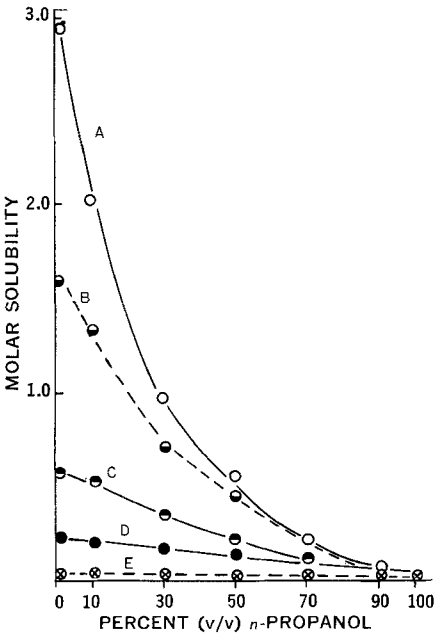

Figure 1—*Molar solubility of amino acid in* n-*propanol–water solvent systems. Key: Curve A, glycine; Curve B,* L-*alanine; Curve C,* L-*valine; Curve D,* L-*phenylalanine; and Curve E,* DL-*amino-octanoic acid.*

[1] Menold rotating apparatus, Lester, Pa.
[2] Porto-Temp, Precision Scientific Co., Chicago, Ill.

Table II—Polynomial Regression Data Generated from the Molar Solubility of L-Alanine as a Function of the Percent Strength of the Solvent System

Solvent System[a]	Y-Intercept[b]	Coefficients	
		X	X^2
Methanol	1.6640	−0.0354	0.0002
Ethanol	1.6238	−0.0366	0.0002
n-Propanol	1.5881	−0.0347	0.0002
Isopropanol	1.6019	−0.0378	0.0002
Tertiary butanol	1.5794	−0.0363	0.0002

[a] Molar solubility values of L-alanine in 0–100% (v/v) of each solvent system were used to generate the polynomial equation. [b] Solubility of L-alanine (moles/l.) in water.

made on the aliquots. Alkali was added to the remaining samples, and a similar analysis was performed.

RESULTS AND DISCUSSION

It has been reported that in pure solvent systems the solubility of certain amino acids was dominated by the α-amino carboxylic acid group, thus producing a high solubility in water and a substantially lower solubility in the alcohols (1). If the attraction that causes the solubility of the amino acid dipoles is defined as being due to the highly polar water molecules interacting with the charged portion of the amino acid, and the semipolar solvents are assumed to have too little polarity to interact with the charged portion of the amino acids, then it would be expected that the addition of a semipolar liquid to the polar aqueous solvent would be reflected by a proportionate decrease in the solubility of the amino acids.

Figure 1 is representative of the variation in molar solubility of the amino acids as the concentration of the semipolar solvents is increased. The decrease in solubility can be seen to be a nonlinear curve rather than the proportional linear decrease expected if the polarity of the solvent system was solely responsible for the solubility behavior. In addition, as the nonpolar–polar ratio of the amino acids is increased, the difference in slope throughout the range of decreasing polarity becomes less, until almost no change is seen for the DL-amino octanoic acid. It would seem that the high charge on the dipolar portion of the molecule is balanced by the nonpolar six-carbon *n*-alkyl chain to produce an invariant solubility. This would seem to correlate with the findings of Cohn (3) in which most α-amino acids were found to be less soluble in ethanol or ethanol–water mixtures than in water, and as the side chain lengthened, a point was reached where the ethanolic solubility was greater than the aqueous solubility.

The overall similarity between the solubility profiles of the amino acids can be illustrated by generating the polynomial regression for each solvent. The equation of the line can be computed using a standard IBM program for an orthogonal polynomial regression (4). By designating the molar solubility as the dependent variable and the percent strength of the alcoholic solution as the independent variable, the polynomial equation of the standard from $Y = A_0 + A_1X + A_2X^2 + A_3X^3 + \cdots A_nX^n$ can be computed.

Table III—Polynomial Regression Data Generated from the Molar Solubility of L-Valine as a Function of the Percent Strength of the Solvent System

Solvent System[a]	Y-Intercept[b]	Coefficients	
		X	X^2
Methanol	0.4581	−0.0089	0.00005
Ethanol	0.4629	−0.0104	0.00006
n-Propanol	0.4732	−0.0080	0.00003
Isopropanol	0.4592	−0.0104	0.00006
Tertiary butanol	0.4578	−0.0104	0.00006

[a] Molar solubility values of L-valine in 0–100% (v/v) of each solvent system were used to generate the polynomial equation. [b] Solubility of L-valine (moles/l.) in water.

Table IV—Polynomial Regression Data Generated from the Molar Solubility of L-Phenylalanine as a Function of the Percent Strength of the Solvent System

Solvent System[a]	Y-Intercept[b]	Coefficients	
		X	X^2
Methanol	0.1646	−0.0019	0.00001
Ethanol	0.1659	−0.0022	0.00001
n-Propanol	0.1602	−0.0021	0.00001
Isopropanol	0.1581	−0.0020	0.00001
Tertiary butanol	0.1609	−0.0022	0.00001

[a] Molar solubility values of L-phenylalanine in 0–100% (v/v) of each solvent system were used to generate the polynomial equation. [b] Solubility of L-phenylalanine (moles/l.) in water.

Tables I–IV show the effect on each of the amino acids in the five solvent systems used in the form of a second-degree equation. The second-degree polynomial equation was considered most representative, since the sum of the squares of the deviation about the regression in the analysis of variants showed little improvement when the third-degree or higher polynomial equation was calculated. The *Y*-intercept in each table is representative of the average solubility in a pure aqueous solvent. The coefficients of the X and X^2 terms define the curve of the line. In Table I, the proximity of the *Y*-intercept values, which range from 2.65204 to 2.79148 with a true solubility of 2.9 *M* in pure water, can be seen. The X and X^2 values also show a similarity. In Table II, L-alanine shows a range of 1.57939–1.66396 in the *Y*-intercepts with a true aqueous solubility of 1.63 *M*. This is a range of only 0.08455 as compared to the range of 0.13944 for glycine, thereby showing a closer approximation of the true solubility. This is expected since the average of the sum of the squares of the deviation about the regression for L-alanine in all solvents is only 0.00538 as compared to 0.11104 for glycine. In Tables III and IV, the computed polynomial equation for L-valine and L-phenylalanine shows an even closer approximation to the experimental points. L-Valine shows a range of only 0.01548 for a solubility of 0.474 *M* and an average sum of squares of the deviation about the regression of 0.00135. L-Phenylalanine has a range of only 0.00788 between its high and low *Y*-intercepts, with a true solubility of 0.172 *M* and an average sum of the squares of the deviation about the regression of only 0.00055.

The closeness of the *Y*-intercepts and the similarity between the coefficients of the X and X^2 terms seem to indicate the existence of a

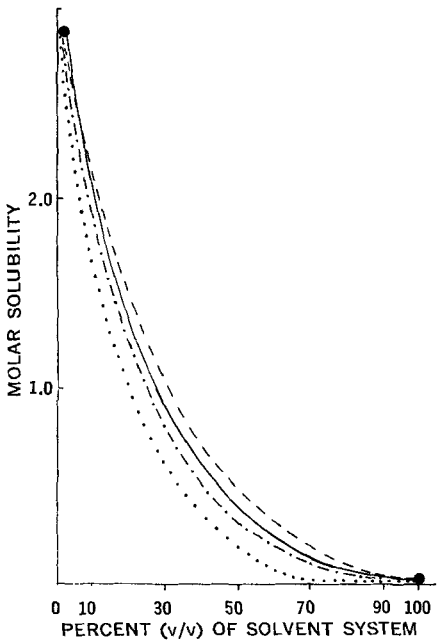

Figure 2—*Molar solubility of glycine in the solvent systems. Key:* ——, *n-propanol;* - - -, *tertiary butanol;* ·-·-, *ethanol; and* ···, *methanol.*

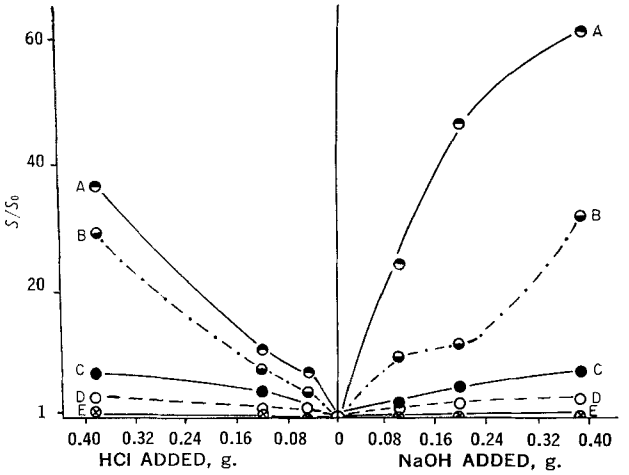

Figure 3—*Ratio of total solubility* (S) *to solubility at the neutral point* (S_0) *for glycine as a function of HCl and NaOH added in an ethanol–water system. Key: Curve A, 100%; Curve B, 90%; Curve C, 70%; Curve D, 50%; and Curve E, 0, 10, and 30%* (v/v) *of ethanol.*

closely related family of curves when the solubilities of each amino acid in the five solvents are compared. Further, it would be expected that the polar amino acids would be the most soluble in the more polar of the mixed solvent systems. By using the dielectric constant as a measure of polarity, the pure solvents can be arranged in this order of decreasing polarity: methanol, ethanol, *n*-propanol, and tertiary butanol. The semipolar solvents in combination with water produce a series of solvent systems with the same order of decreasing polarity. For example, at 30% (v/v) of semipolar solvent–water, the order of decreasing polarity remains methanol–water, ethanol–water, *n*-propanol–water, and tertiary butanol–water. However, it can be seen in Fig. 2 that glycine shows a decreasing solubility in the order of *n*-propanol, tertiary butanol, ethanol, and methanol throughout the concentration scale. The addition of the methyl group to form L-alanine changes the order of binary solubility to what was originally expected; L-valine, with an added isopropyl group, shows its greatest solubility in *n*-propanol followed in decreasing order by methanol, ethanol, and tertiary butanol. The order of solubility of L-phenylalanine does not seem to change appreciably with different solvent systems.

If the solubility of glycine illustrates the behavior of the charged α-amino carboxylic acid portion of the molecule, the variation in order of solubility as methyl, isopropyl, and methyl phenyl groups are added to the α-amino carboxylic acid group serves to illustrate the dependence of solubility on the independent interactions of each nonpolar chain. This appears contrary to the theory that solubility is only a function of the polarity of the solvent system and is not affected by structural variation between systems (5).

The solubility variation when acid or base was added to separate samples of each amino acid in the series of decreasing polarity for 10, 30, 50, 70, 90, and 100% (v/v) of methanol, ethanol, *n*-propanol, isopropanol, and tertiary butanol was similar to that seen previously in pure solvent systems (1). Minimum solubility is seen at the isoelectric point, with a distinct increase in total solubility for each change in pH′ away from the neutral solubility point. However, the increase in total solubility in the semipolar solvents seems to reach a higher magnitude per amount of acid or base added as the polarity of the solvent system is decreased. Figure 3 illustrates this behavior using glycine in seven different ethanol–water solvent systems. The ratio of the total solubility (S) to the solubility at the neutral point (S_0) was plotted as a function of grams of acid or base added. In all cases, the three most polar strengths: 0, 10, and 30% (v/v), merge as one straight line. At 50 and 70% (v/v), a slight increase in salt formation is seen, as evidenced by an increase in the slope of the line. The 90 and 100% (v/v) solutions show a distinct increase in the efficiency of salt formation.

Each amino acid shows this behavior pattern, but there is a difference in the slope of the line for each amino acid which would seem to be due to differences in solute–solvent interaction. It would also seem that these differences in salt formation can be correlated with mole fraction or essentially the number of molecules of water present as compared to the number of molecules of semipolar solvent. In the low percent strengths, where little difference in slope is seen, the water molecules outnumber the ethanol molecules in solution. However, between 70 and 90%, the semipolar solvent molecules begin to outnumber the water molecules and this, in turn, corresponds to the increased efficiency of salt formation for a given solvent system. This can be explained by the increasing importance of interionic forces which make up a greater portion of the attractive charge between oppositely charged ions as the polarity of the solvent system is decreased. Further, since the activity coefficients of the pure HCl and pure NaOH were approximately 0.85 at the molar concentrations in which they were added, the Cl^- and Na^+ ions provided an increasingly greater attractive force for the charged amino acids as the polarity of the solvent system was reduced. Comparison of the ratio of S to S_0 with the quantity of acid or base added seems to indicate reduction of the isoelectric band. Since, by definition, the species present within the isoelectric band are predominately dipolar and have an invariant solubility, an increase in total solubility can probably be attributed to salt formation. The increase in S/S_0 ratio as the concentration of alcohol is increased indicates a reduction in the isoelectric band as a function of decreasing polarity. This reduction is due to an increased affinity of the amino acids for the Na^+ and Cl^- ions.

SUMMARY

1. In the hydroalcoholic solvent systems studied, the solubility of glycine, L-alanine, L-valine, and L-phenylalanine is dominated by the α-amino carboxylic acid portion of the molecule.

2. Each amino acid studied demonstrated an ability to differentiate between the hydroalcoholic solvents used throughout the concentration spectrum, but a similar solubility profile was produced for all molecules.

3. The quantitative order of solubility of each amino acid varied with the change in solvent system. The order of solubility seems to be a function of specific solute–solvent interactions with nonpolar portions of the amino acid molecules.

4. As the concentration of alcohol was increased in the solvent systems, similar increments of acid or base added to the system produced a proportionally greater increase in solubility of the amino acids. This was attributed to an increase in the contribution of the charged species as the polarity of the solvent system was decreased, as well as to an increased affinity of the amino acids for the Na^+ and Cl^- ions present.

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Serum and Urine Levels of Ethchlorvynol in Man

L. M. CUMMINS, Y. C. MARTIN, and E. E. SCHERFLING

Abstract □ Serum and urine levels of ethchlorvynol were determined by gas chromatography using electron-capture detection. The method is sensitive to 0.25 mcg./ml. and reproducible within 2% relative standard deviation. In 25 hr., 0.025% of the total dose is excreted unchanged or as the glucuronide. Peak serum levels of 2.5, 6.5, or 8.0 mcg./ml. are seen at 1 hr. after administration of 200, 500, or 750-mg. capsules, respectively. The decline of the serum levels is biphasic, with disappearance constants of 0.5 and 0.03 $hr.^{-1}$. When the data are interpreted in terms of a two-compartment model, the rate constant for elimination is calculated to be 0.13 $hr.^{-1}$. The ratio of the amount of ethchlorvynol in the tissue compartment to that in the central compartment is 3.5 : 1.

Keyphrases □ Ethchlorvynol levels—urine, serum □ Absorption half-life—ethchlorvynol □ Kinetics—ethchlorvynol absorption, excretion □ GLC, electron-capture detection—analysis

Wallace *et al.* (1) reported therapeutic blood levels and 24-hr. urinary excretion levels of ethchlorvynol,[1] a mild nonbarbiturate hypnotic-sedative, following the ingestion of a 500-mg. capsule. In their method of analysis, ethchlorvynol was destructively hydrolyzed to a carbonyl derivative which was isolated by steam distillation, converted to a semicarbazone, and determined spectrophotometrically. Because of the hydrolysis step, this method could lack specificity and might not differentiate between parent drug and similar metabolites. In addition, blood levels were not determined later than 6 hr. after dosing, which is an insufficient length of time for detailed studies of the kinetics of distribution and metabolism of ethchlorvynol.

Robinson (2) recently published a specific gas chromatographic method which is sensitive enough to determine peak therapeutic and overdosage blood levels of ethchlorvynol. This method, however, can determine only the peak levels with normal dosage and not the lower concentrations observed at later times.

In the study described here, a gas chromatographic method using electron-capture detection (ECD) was developed. The chlorine of ethchlorvynol imparts enough electronegativity to the molecule so that serum levels of the drug are easily detected up to 48 hr. following a single 500-mg. dose. Serum levels on three dosages, 200, 500, and 750 mg., as well as the kinetic interpretation and 24-hr. urinary excretion data, are presented. The urine samples were analyzed both before and after enzymatic hydrolysis in an attempt to determine the extent of conjugation.

EXPERIMENTAL

Apparatus—A model 1200 Varian-Aerograph gas chromatograph equipped with a tritium foil electron-capture detector was used in this study. The column was stainless steel, 1.52 m. × 0.32 cm. (5 ft. × 0.125 in.) o.d., with 5% NPGS on AW-DMCS 100/120 mesh diatomite aggregate.[2] The carrier gas (nitrogen) flow was

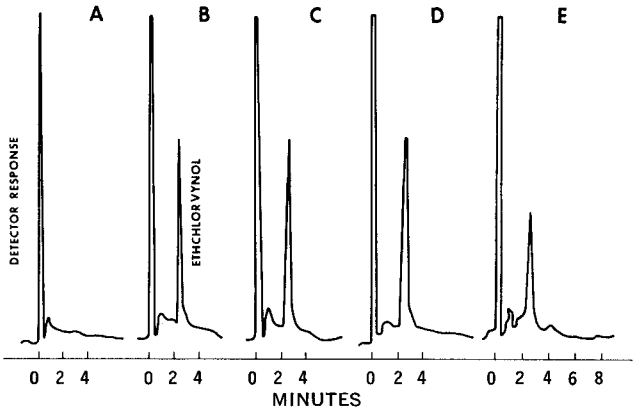

Figure 1—*Chromatograms of: (A) benzene extract from 2.0 ml. of control serum; (B) benzene extract from 2.0 ml. serum containing 3.0 mcg./ml. of ethchlorvynol; (C) benzene extract from 2.0 ml. aqueous solution containing 3.0 mcg./ml. of ethchlorvynol; (D) ethchlorvynol solution at a concentration of 3.0 mcg./ml. in benzene; and (E) benzene extract of serum from a treated subject.*

50–60 ml./min. with injector temperature of 150°, oven temperature of 120°, and a detector temperature of 210°. All quantitation was obtained with a model 480 Varian-Aerograph digital integrator.

Extractions were carried out in 20-ml., 16 × 150-mm. culture tubes fitted with Teflon-lined caps.

Reagents—Spectrograde benzene was used for all extractions. Glusulase enzyme[3] was used for urine hydrolysis.

Drug Administration—The experimental groups consisted of equal numbers of normal adult males and females (24 on the 200-mg. dose and 8 on the 500 and 750-mg. doses). The same subjects were used for the 500 and 750-mg. doses. The weights of the subjects ranged from 100 to 225 lb., and ages ranged from 20 to 51 years. They fasted 12 hr. prior to and 2 hr. subsequent to administration of ethchlorvynol. The drug was given in soft gelatin capsules. Urine was refrigerated as voided and frozen after a 24-hr. collection was completed.

Serum Analysis—Serum was stored frozen until analysis. Four milliliters of serum, obtained from subjects on a 200-mg. dose, or 2.0 ml. of serum, from those on a 500 or 750-mg. dose, was analyzed. In all cases the serum was diluted to 8.0 ml. with distilled water and extracted for 10 min. with 2.0 ml. of benzene. Following centrifugation, 5.0 μl. of the benzene extract was injected into the chromatograph. Standard curves containing the appropriate amount of serum were run with each set of samples analyzed. Typical chromatograms of serum extracts are presented in Fig. 1.

Urine Analysis without Hydrolysis—Five milliliters of urine plus 1.0 ml. of 0.1 *M* acetate buffer (pH 5) was extracted for 10 min. with 2.0 ml. of spectro-benzene. Following centrifugation, 4.0 mcg. was analyzed. As with serum, standard curves were run with ethchlorvynol added to control urine.

Urine Analysis following Enzymic Hydrolysis—Five milliliters of urine plus 1.0 ml. of 0.1 *M* acetate buffer (pH 5) was incubated with 20 μl. of glusulase for 48 hr. at 38° in a Dubnof shaker. To minimize microbial degradation, 0.5 ml. of toluene was added to each tube. Standards of ethchlorvynol added to control urine were also carried through the entire hydrolysis process. These standards indicated minimal degradation of ethchlorvynol during the hydrolysis. Following hydrolysis the samples were extracted and analyzed according to the procedure for the nonhydrolyzed urine samples. A chromatogram of a urine extract is not presented because of its similarity to that of serum.

[1] Placidyl, Abbott Laboratories.
[2] Chromosorb W, Johns-Manville.
[3] Endo Laboratories.

Table I—Total 24-hr. Urinary Excretion of Ethchlorvynol following the Oral Administration of a 500-mg. Capsule

Subject, Sex	Collection Interval, hr.	Volume, ml.	mcg./Total Volume (not Hydrolyzed)	mcg./Total Volume (after Hydrolysis)	Percent Conjugated	Percent Total Dose Excreted
No. 1, male	0–4	224	341	332	0	0.007
	4–8	188	180	259	69	0.005
	8–12	188	128	226	56	0.005
	12–24	204	131	375	35	0.007
Total	0–24	804	780	1192	65	0.024
No. 2, male	0–4	148	314	284	0	0.006
	4–8	150	174	246	71	0.005
	8–12	190	175	403	43	0.008
	12–24	198	83	309	27	0.006
Total	0–24	686	746	1242	60	0.025

Table II—Kinetic Constants Calculated from Serum Levels of Ethchlorvynol following a Single Oral Dose

	Dose, mg. 200	500	750
α (hr.$^{-1}$)	0.530 ± 0.197	0.532 ± 0.043	0.450 ± 0.068
β (hr.$^{-1}$)	0.0274 ± 0.0061	0.0353 ± 0.0017	0.0218 ± 0.0032
V_1 (l)	59.3 ± 10.9	51.4 ± 2.3	60.7 ± 5.1
k (hr.$^{-1}$)	2.80 ± 0.83	2.04 ± 0.16	2.02 ± 0.31
k_{-1} (hr.$^{-1}$)	0.115 ± 0.028	0.132 ± 0.007	0.0962 ± 0.0146
k_1 (hr.$^{-1}$)	0.321	0.293	0.273
k_2 (hr.$^{-1}$)	0.127	0.142	0.102
V_2 (l)	164.	114.	172.

Kinetic Analysis of the Serum Levels—The time course of the rise and fall of the mean serum levels was analyzed by using the computer program NONLIN.[4] This program calculates a statistical "best fit" in the least-squares sense to a nonlinear function. In this case, the equation fit was that for two-compartment distribution following oral absorption (3):

$$C_1 = \frac{kD}{V_1}\left[\frac{(k_{-1}-\alpha)}{(k-\alpha)(\beta-\alpha)}e^{-\alpha t} + \frac{(k_{-1}-\beta)}{(k-\beta)(\alpha-\beta)}e^{-\beta t} + \frac{(k_{-1}-k)}{(\alpha-k)(\beta-k)}e^{-\beta t}\right] \quad \text{(Eq. 1)}$$

In this equation, C_1 is the concentration of drug in the central compartment (serum level), k is the rate constant for absorption, D is the dose, V_1 is the volume of the central compartment, k_{-1} is the rate constant for transfer from the tissue to the central compartment, α is the exponential term for the first phase of decline of serum levels, β is the similar term for the terminal phase of decline of serum levels, and t is the time after dosing at which the sample was taken.

RESULTS AND DISCUSSION

Serum Assay for Ethchlorvynol—From Fig. 1 it is apparent that excellent recovery of ethchlorvynol was obtained with the benzene extraction. Based on integrated values and comparison to a standard benzene solution, it was calculated that 93.8 and 95.8% of the total drug were recovered from serum and aqueous solutions, respectively. From Part E of Fig. 1, it is also apparent that only one peak resulting from ethchlorvynol is detectable in the serum extracts, thus demonstrating the selectivity of ECD.

Linear standard curves were obtained from 0.25 to 5.0 mcg./ml. when 2.0 ml. of serum was analyzed. When 4.0 ml. of serum was analyzed, the range of linearity was from 0.25 to 4.0 mcg./ml. If the responses approached or exceeded the range of linearity, dilutions were made so that responses were obtained in the range of linearity.

Precision of the Assay—Ten replicate analyses of serum samples to which 5.0 mcg./ml. of ethchlorvynol had been added yielded a standard deviation of ±2.13%.

Urine Levels—The data in Table I show that an average of only 0.025% of a dose of ethchlorvynol is excreted unchanged in 24 hr. Approximately 62% of this amount is conjugated to glucuronic acid or sulfate. Thus, ethchlorvynol is apparently metabolized extensively. These recoveries are much lower than those reported by Wallace *et al.* (1).

In this assay, only ethchlorvynol was measured. There were no additional peaks in either serum or urine, which suggests that during metabolism ethchlorvynol is converted to either a benzene-insoluble compound or a nonchlorinated compound. Either type of metabolite could be confused with ethchlorvynol by the Wallace *et al.* method (1). The lack of ECD of any other compound suggests that during metabolism the chlorine atom is lost.

Serum Levels—The mean serum concentrations of ethchlorvynol at various times after dosing are shown in Fig. 2. Twenty-four subjects were averaged for the 200-mg. dose; eight for the 500 and 750-mg. doses. These data show that ethchlorvynol is rapidly absorbed, with the peak serum level occurring 1 hr. after dosing. The initial decline of the serum concentration is also rapid, since at 4 hr. the concentration is less than half that at 1 hr. The second phase of the blood level decline is much slower.

Wallace *et al.* (1) reported whole blood levels, after a 500-mg. capsule, somewhat lower than the serum levels in Fig. 1. They did not see the biphasic decline since their last sample was taken at 6 hr. after dosing.

The data observed do not fit a single-compartment distribution model; the actual serum levels at 24 hr. are 10-fold higher than those predicted by this model.

The parameters calculated by using the two-compartment model are collected in Table II. The terms not previously defined are:

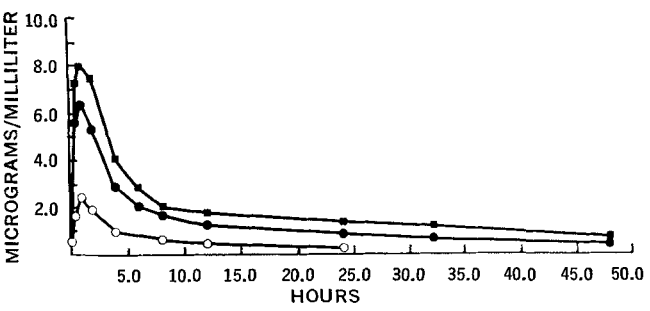

Figure 2—*Human serum levels of ethchlorvynol. Key:* ○, *average value for 24 subjects following a single 200-mg. dose;* ●, *average value for 8 subjects following a single 500-mg. dose; and* ■, *average value for 8 subjects following a single 750-mg. dose.*

[4] Dr. Carl M. Metzler of The Upjohn Co., Kalamazoo, Mich., furnished this program.

k_1, the rate constant for transfer from the central to the tissue compartment; k_2, the rate constant for elimination by metabolism or excretion from the body; and V_2, the volume of the tissue compartment. From the standard deviations of the parameters, it can be seen that the data fit this model rather well. Discussions of the meaning of two-compartment distribution may be found in *References 4–6*. The central compartment is generally thought to consist of blood and highly perfused tissues such as heart, lung, liver, and kidney. The more poorly perfused tissue compartment includes muscle and skin. Because of the oily nature of ethchlorvynol, one would expect that it would be preferentially distributed into fat. Riegelman *et al.* (4) pointed out that, because of direct diffusion from tissues to fat, the effect of distribution of a drug into a fat compartment simply modifies the effective volume of the compartment from which the drug diffused. Thus the identity of the tissue compartment with any anatomical structure cannot be made.

The results point out very clearly that the slow decline of serum levels in the β-phase is not due to a low rate of metabolism but rather to extensive tissue localization of ethchlorvynol since k_2 is at least four times as large as β at each dose.

These data also demonstrate that ethchlorvynol is rapidly absorbed; from the k values, a half-life for absorption of approximately 0.3 hr. is calculated. Likewise the rate of metabolism is rather fast; a half-life of 5.6 hr. is seen. From serum levels in the β-phase, one may calculate f_c, the ratio of the amount of ethchlorvynol in the central compartment to the total amount in the body, and f_T, the similar value for the amount in the tissue compartment, from the following relations:

$$f_c = \frac{k_{-1} - \beta}{k_1 + k_2 - \beta} = 0.22 \qquad \text{(Eq. 2)}$$

$$f_T = 1.0 - f_c = 0.78 \qquad \text{(Eq. 3)}$$

$$f_T/f_c = 0.78/0.22 = 3.5 \qquad \text{(Eq. 4)}$$

This ratio is substantially higher than those for aspirin, griseofulvin, and spectinomycin which were reported by Gibaldi *et al.* (6) to be 1.1, 0.9, and 0.65, respectively.

CONCLUSIONS

Ethchlorvynol is extensively metabolized since only 0.025% of the dose is excreted unchanged or as the glucuronide.

Absorption of ethchlorvynol is very rapid, with an absorptive half-life of 0.3 hr.

The decline of serum levels is biphasic, which suggests a two-compartment model for distribution. The hybrid rate constants are 0.5 and 0.03 $hr.^{-1}$. From these the rate constant for elimination is calculated to be 0.13 $hr.^{-1}$. There is also evidence for extensive tissue localization of ethchlorvynol.

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Keyphrases □ Hydrochlorothiazide tablets—age effect □ Aging, hydrochlorothiazide tablets—accelerated, physical stability □ Tablets, hardness, disintegration, dissolution—aging effect □ Granulating agents effect—physical stability, tablets

The compressed tablet is the most popular dosage form; for many pharmaceutical manufacturers, it comprises the majority of their products. As a part of research and development operations, the chemical stability of the medicinal compound in a tablet is routinely studied. Nevertheless, the manner in which medicinal compounds degrade in solid dosage forms is obscure, and the number of publications reporting degradation in solid dosage forms is not numerous (1–6).

In addition to demonstrating that the chemical nature of the medicinal compound will not change during the recommended life of the product, the manufacturer must consider possible changes in the physical properties of a tablet. A physically stable tablet should retain its original color, disintegration time, friability, hardness, shape, size, weight, and dissolution profile (7, 8).

If the dissolution of the medicinal compound from a tablet is slowed upon aging or storage of the tablet, the biological availability may be seriously affected because the medicinal compound would be less available for gastrointestinal absorption. It cannot be assumed that a rapid release of the medicinal compound from the tablet immediately after its production will be

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If the dissolution of the medicinal compound from a tablet is slowed upon aging or storage of the tablet, the biological availability may be seriously affected because the medicinal compound would be less available for gastrointestinal absorption. It cannot be assumed that a rapid release of the medicinal compound from the tablet immediately after its production will be

Table I—Formulas in Milligrams per Tablet

Ingredients	I	II	III	IV
Hydrochlorothiazide	50.0	50.0	50.0	50.0
Lactose	295.0	282.5	282.5	282.5
Acacia	3.7	—	—	—
Starch	—	1.25	—	—
PVP K 29-32	—	—	7.0	17.5
Magnesium stearate	1.0	1.05	0.9	1.05

maintained upon aging (9). Since it is not economical to test clinically every product at various intervals of time during its storage, a dissolution profile is the best *in vitro* test to detect changes that might affect the release or dissolution of the medicinal compound from the tablet. In general, an unchanged dissolution profile with aging indicates no change of *in vivo* availability.

The purposes of this study were to compare the physical properties of hydrochlorothiazide tablets formulated with three granulating agents and to compare the effect of aging on the physical properties of these formulations. The effect of storage at elevated temperatures on physical properties of tablets was compared to aging at room temperature in order to explore the possibility that valid predictions of changes in physical properties could be made based on a short-term evaluation at elevated temperatures.

EXPERIMENTAL

Materials—Acacia, hydrochlorothiazide, hydrous lactose, and magnesium stearate were USP grade. Boric acid, potassium chloride, and potassium hydroxide were reagent grade. Polyvinylpyrrolidone (PVP)[1] had an average molecular weight of 40,000.

Preparation of Tablets—All tablets were prepared by a wet granulation method. The granulating liquids were 10% acacia, 5% starch paste, and 12.5 and 38.9% PVP in 95% ethanol. The formulas are given in Table I. The tablets were compressed on a Stokes model E single-punch tablet machine using a 1.03-cm. ($^{13}/_{32}$-in.) standard concave punch and die set. The tablet machine was adjusted to prepare satisfactorily appearing tablets, and no attempt was made to maintain a constant pressure during compression of the formulations.

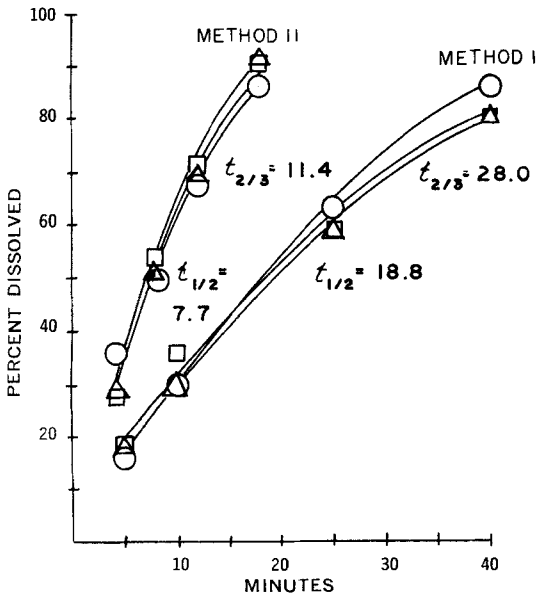

Figure 1—*Dissolution profiles of hydrochlorothiazide at room temperature as determined by dissolution Methods I and II. The symbols are for individual tablets and indicate the variation within a single batch of tablets.*

[1] Plasdone K 29-32, registered trademark of GAF Corporation, New York, N. Y.

Table II—Hardness, Disintegration, and Dissolution Profiles of Tablets of Hydrochlorothiazide Granulated with Acacia, Starch Paste, and PVP after Storage at 80°

Days	Hardness, kg.	Disintegration Time, min.	Dissolution Time, min. —Method I— $t_{1/2}$	—Method I— $t_{2/3}$	—Method II— $t_{1/2}$	—Method II— $t_{2/3}$
			Acacia			
0	5.3	10.8	15.0	20.1	7.0	9.7
1	5.8	20.1	18.3	19.8	10.8	15.9
2	6.8	17.8	11.7	22.1	11.3	15.6
3	6.9	21.9	16.0	22.9	10.7	15.1
4	8.3	18.4	19.8	26.6	10.5	14.4
5	6.8	16.7	22.5	30.8	10.0	13.8
6	6.7	17.0	20.4	28.1	10.0	14.1
7	7.0	16.3	18.8	26.0	10.0	14.1
14	7.8	15.5	21.0	28.3	8.3	12.0
			Starch			
0	5.5	3.5	9.7	11.3	5.3	7.3
1	5.4	3.8	10.3	13.6	4.3	6.3
2	5.3	3.8	11.6	16.0	5.0	6.7
3	5.3	3.2	10.3	12.3	5.7	7.3
4	5.3	4.0	12.3	16.6	5.3	7.3
5	5.5	4.0	9.7	14.2	4.6	6.7
6	5.0	4.4	8.2	10.2	4.9	6.6
7	5.4	3.3	7.0	8.0	6.0	7.7
			2% PVP K 29-32			
0	9.0	14.0	16.9	23.8	7.3	12.1
1	8.0	15.0	16.3	24.5	7.3	11.7
2	8.5	14.4	18.3	23.7	7.8	12.5
3	8.8	12.6	13.4	18.3	7.7	11.0
4	8.0	15.5	16.3	24.0	9.0	15.3
5	9.6	16.3	15.3	24.0	7.7	12.0
6	8.2	13.5	15.3	22.3	8.7	13.3
7	9.2	16.6	15.5	22.0	8.3	12.7
			5% PVP K 29-32			
0	7.2	17.0	19.5	28.8	8.5	13.3
1	7.1	17.3	17.8	22.0	11.7	12.0
3	7.5	17.0	15.5	22.3	9.0	12.5
4	7.0	16.0	14.7	21.7	8.7	12.0
7	7.2	17.3	18.3	25.8	8.7	13.3
14	8.0	19.0	18.8	29.3	7.7	13.7

Hardness—Hardness of the tablets was determined using a Pfizer hardness tester. Each hardness value reported is an average of six tablets.

Disintegration Time—The USP XVIII apparatus and procedure with disks were used to determine the disintegration time of the tablets in distilled water at 37°. The disintegration time reported is the time when all of the six tablets disintegrated and any particles had passed through the 10-mesh lower plate (10).

Dissolution Time—Method I employed in the study was the USP XVIII dissolution apparatus and method (11). The rate of rotation of the basket was 60 r.p.m. The dissolution fluid was a borate buffer at pH 10. The buffer solution was prepared by mixing 250 ml. of a 0.2 *M* boric acid and potassium chloride solution with 220 ml. of a 0.2 *M* sodium hydroxide solution and then adjusting to pH 10 by the addition of 28.0 ml. of the sodium hydroxide solution. The Hydrochlorothiazide Tablet Monograph, which became official after the completion of this study, specifies 150 r.p.m. in dilute hydrochloric acid (1 in 100).

Method II for determining the dissolution was Method II of NF XIII (12).

In determining the dissolution profile of hydrochlorothiazide from the tablet, a 1.0-ml. sample was withdrawn by a filtering pipet at various intervals of time and diluted to 10 ml. with borate buffer. The absorbance was determined by a Beckman DU spectrophotometer at 273 nm. against a blank of borate buffer (13).

Table III—Hardness, Disintegration, and Dissolution Profiles of Tablets of Hydrochlorothiazide Granulated with Acacia, Starch Paste, and PVP after Storage at 50°

Days	Hardness, kg.	Disintegration Time, min.	Dissolution Time, min. — Method I — $t_{1/2}$	Method I — $t_{2/3}$	Method II — $t_{1/2}$	Method II — $t_{2/3}$
			Acacia			
0	5.3	10.8	16.2	22.7	6.8	9.6
1	5.4	15.0	18.4	25.7	7.7	11.6
3	6.6	15.0	20.7	28.0	9.3	14.4
5	6.4	17.5	20.7	24.8	9.3	13.9
7	6.4	17.0	19.0	27.0	9.2	12.3
14	7.2	17.0	14.2	24.5	8.8	11.9
			Starch			
0	5.0	4.1	11.8	—	7.6	9.7
1	5.5	4.6	11.2	—	6.5	8.3
3	4.7	4.1	11.5	—	6.7	8.5
5	5.4	4.1	12.1	—	7.3	9.4
7	4.8	4.4	—	—	6.7	8.2
14	4.7	4.1	9.2	—	5.8	8.7
			PVP K 29-32			
0	7.7	14.2	18.6	26.0	5.4	10.0
1	6.9	14.3	18.0	25.3	7.0	10.8
3	8.7	14.3	15.9	25.0	8.0	12.1
5	8.1	14.1	15.6	25.1	6.7	10.6
7	6.6	13.3	16.4	25.4	6.3	10.3
14	7.3	12.7	15.5	24.8	7.5	11.6

By means of a standard absorption–concentration curve, the amount of hydrochlorothiazide in solution was calculated. Because a total of only 5.0 ml. was withdrawn during the dissolution, no correction was made for a change of volume and no additional dissolution fluid was added to replace the volume of the samples withdrawn.

RESULTS AND DISCUSSION

In determining the dissolution profile, it was realized that some weight variation of tablets was inherent in the tableting operation; therefore, the entire tablet was allowed to dissolve, and the solution was assayed for the total hydrochlorothiazide in each tablet. This procedure permitted the dissolution profile to be plotted in terms of the percent of the total assayed hydrochlorothiazide in each tablet. From the dissolution profile of each tablet, the time required for 50% of the hydrochlorothiazide to dissolve was read and designated as $t_{1/2}$. In a similar manner, the time required for 66.7% of the hydrochlorothiazide to dissolve from the tablet was read and designated at $t_{2/3}$. The value reported for the dissolution time represents the average dissolution time of three tablets. Dissolution profiles, which were obtained by both dissolution methods, are shown in Fig. 1. These are typical of the variation in dissolution of tablets from a single batch of tablets.

The initial values of the physical properties of the four formulations are given in Table II.

The tablets granulated with acacia and starch had the same hardness. The tablets granulated with 5% PVP were not as hard as those granulated with 2% PVP. With a smaller concentration of PVP, it seemed necessary to increase the hardness to form a satisfactory tablet. With a higher concentration of PVP, the polymer provided binding characteristics without as great a pressure during compression. There is no correlation between hardness and dissolution; thus, hardness is of the least significance in evaluating the release of a medicinal compound from a tablet.

The tablet prepared with starch had the fastest disintegration and dissolution. In these formulations, the disintegration and dissolution as determined by both methods followed rank correlation; that is, for both disintegration and dissolution times, the values according to the granulating agent in the formulation were: starch < acacia < 2% PVP < 5% PVP.

Table IV—Hardness, Disintegration, and Dissolution Profiles of Tablets of Hydrochlorothiazide Granulated with Acacia, Starch Paste, and PVP after Storage at 37°

Weeks	Hardness, kg.	Disintegration Time, min.	Dissolution Time, min. — Method I — $t_{1/2}$	Method I — $t_{2/3}$	Method II — $t_{1/2}$	Method II — $t_{2/3}$
			Acacia			
0	5.3	10.8	16.0	21.8	6.9	9.5
1	5.0	13.2	17.6	20.6	8.4	12.0
2	4.3	13.7	13.3	23.3	6.9	9.3
3	7.1	13.0	18.8	26.0	8.6	9.8
4	6.5	14.2	18.3	25.3	7.9	11.4
			Starch			
0	5.0	3.7	10.4	13.3	7.4	10.0
1	4.8	3.9	13.5	14.7	6.1	8.0
2	5.1	3.9	11.7	14.9	6.5	8.8
3	5.2	4.0	15.1	—	6.2	8.4
4	5.0	4.3	13.3	18.1	7.1	9.5
			PVP K 29-32			
0	7.7	13.6	18.7	24.9	6.1	9.8
1	5.9	12.9	16.0	23.7	5.9	11.7
2	7.0	11.9	17.7	25.5	8.3	11.9
3	7.6	14.0	17.9	26.1	8.4	12.5
4	7.4	13.5	15.9	23.1	8.4	12.0

Table V—Hardness, Disintegration, and Dissolution Profiles of Tablets of Hydrochlorothiazide Granulated with Acacia, Starch Paste, and PVP after Storage at Room Temperature

Months	Hardness, kg.	Disintegration Time, min.	Dissolution Time, min. — Method I — $t_{1/2}$	Method I — $t_{2/3}$	Method II — $t_{1/2}$	Method II — $t_{2/3}$
			Acacia			
0	5.3	10.8	14.8	20.2	6.9	9.7
1	5.5	12.7	14.6	24.1	7.3	11.0
2	5.5	12.7	20.3	28.6	7.3	10.3
3	5.9	12.7	19.3	27.1	7.6	10.8
4	5.4	15.0	17.4	24.7	6.9	9.6
5	6.9	16.5	17.5	24.4	8.0	10.9
6	5.8	14.2	22.4	32.9	8.1	12.3
7	5.2	14.7	22.0	31.3	8.5	12.1
8	6.3	14.5	16.8	23.6	7.7	10.8
9	5.4	11.8	16.1	22.4	7.6	11.1
10	8.6	15.0	17.1	24.5	9.0	12.4
11	7.2	14.4	18.9	26.9	6.6	9.1
12	9.1	18.0	19.6	27.8	7.5	10.4
			Starch			
0	6.0	3.7	10.4	—	7.1	—
1	7.0	3.2	6.1	8.8	6.1	7.6
2	8.0	5.0	6.0	8.6	2.4	7.6
3	6.4	4.0	9.8	13.7	2.5	5.2
4	6.5	4.0	8.2	12.2	3.2	4.9
5	8.1	3.6	7.4	10.9	3.5	4.8
6	7.9	3.6	8.5	12.1	3.0	4.5
7	7.8	2.8	7.3	10.4	4.1	6.3
8	8.5	3.4	8.9	12.7	3.8	5.4
9	8.5	3.7	8.4	12.3	4.0	6.1
10	6.8	4.3	5.0	7.7	3.7	5.6
11	9.8	3.5	8.8	13.0	3.9	5.8
12	8.9	3.3	6.6	10.2	3.6	5.3
			PVP K 29-32			
0	8.0	13.6	24.9	24.9	6.1	9.8
1	7.5	13.8	18.3	18.3	5.6	8.3
2	8.2	12.8	19.9	20.9	6.4	8.4
3	8.1	13.3	12.2	18.6	6.5	9.1
4	8.1	13.3	20.1	27.7	10.0	13.2
5	8.0	13.0	17.7	26.9	8.2	12.5
6	7.8	13.6	16.1	25.5	7.5	11.2
7	7.4	13.5	16.0	23.4	8.4	12.7
8	8.1	13.7	19.4	28.9	8.2	12.3
9	7.6	13.8	23.7	35.1	9.2	13.5
10	7.1	13.3	14.5	21.7	7.7	11.4
11	9.4	14.4	16.2	23.4	8.6	12.4
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Although the values of the dissolution times are different as determined by Methods I and II, the relationship of $t_{1/2}$ and $t_{2/3}$ as determined by the two methods is almost constant. In this study the $t_{1/2}$ and $t_{2/3}$ as determined by Method II were approximately half the values obtained by Method I.

When formulating a new tablet, a quick estimate of the physical stability may be obtained by using the concept of chemical kinetics relating to rate and temperature. To obtain stability information in as short a time as possible, it is customary to evaluate samples stored at elevated temperatures for short periods. When using data obtained at elevated temperatures for short times to predict chemical and physical stability in solid dosage forms, one must realize that the mechanisms causing change can be different at higher temperatures than those functioning at room temperature (7). Yet such studies often are indicative of physical stability (14, 15).

Tablets were stored at 80° and their physical properties were measured at various intervals as shown in Table II. Under these conditions, the hardness of tablets granulated with starch and PVP was unchanged. There was no appreciable change in disintegration time of tablets granulated with starch and PVP.

Tablets containing 2 and 5% PVP were stored at 80° to show the influence of concentration of granulating agent on the physical properties of the tablet. The tablet containing 5% PVP had a slightly longer disintegration and dissolution time; however, the dissolution times were unchanged after storage for 14 days at 80°.

The effect of aging at 50 and 37° on the physical properties of hydrochlorothiazide tablets granulated with acacia, starch, and PVP is shown in Tables III and IV, respectively.

The dissolution time determined by Method I for tablets granulated with PVP and stored at 37 and 50° for 4 weeks and 14 days, respectively, appears to decrease slightly; however, the dissolution time as determined by Method II indicates a slight increase in dissolution time. Such data were interpreted as reflecting no change in dissolution time and were probably caused by a variation of a tablet used in the samples.

For tablets granulated with starch paste, the dissolution time after storage at 37 and 50° was unchanged as determined by Methods I and II, respectively. After storage at 37°, Method I indicated an increase in dissolution time; after storage at 50°, Method II indicated a decrease in dissolution time. These results were interpreted as no change in dissolution.

For tablets granulated with acacia, storage at 37 and 50° for 4 weeks and 7 days, respectively, resulted in an increase in dissolution time.

The physical properties of three tablet formulations were determined after storage at elevated temperatures, with the assumption that any changes in properties would occur more rapidly, but in the same manner, than changes would occur at room temperature. To test the validity of this assumption, tablets granulated with acacia, starch, and PVP were stored at room temperature for 1 year during which the physical properties were measured each month. The results are given in Table V.

For tablets granulated with acacia, the hardness, disintegration, and dissolution times were increased during 1 year of aging at room temperature. A similar increase in hardness, disintegration, and dissolution times was observed in tablets stored for 14 days at 50 and 80° and for 4 weeks at 37°.

For tablets granulated with starch, there was an increase in hardness during 1 year of storage at room temperature; no change in hardness was observed in tablets stored at elevated temperatures. For tablets granulated with starch, there was essentially no change in disintegration or dissolution during 1 year of storage at room temperature. Similarly, there was no change in disintegration or dissolution times for tablets stored for 14 days at 50 and 80° and for 4 weeks at 37°.

For tablets granulated with PVP, the hardness and disintegration were essentially unchanged after storage for 1 year at room temperature, for 14 days at 50 and 80°, and for 4 weeks at 37°. The dissolution was unchanged by storage at elevated temperatures, but at room temperature the dissolution time slightly increased after 1 year.

SUMMARY

In this report, changes in physical properties at elevated temperatures were qualitatively related to those at room temperature after 1 year of aging. Thus, one is able to predict from the changes in physical properties after a short time at 37, 50, and 80° that hydrochlorothiazide tablets granulated with acacia would have an increase in hardness, disintegration, and dissolution time as they aged at room temperature. The physical properties of hydrochlorothiazide tablets granulated with starch or PVP were essentially unchanged after short times at elevated temperatures. Based on these accelerated studies, it would be anticipated that starch or PVP would be a more suitable granulating agent for physically stable tablets than acacia. This projection is supported by the stability for tablets aged for 1 year at room temperature.

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Surface Transformation during Dissolution of Aspirin

R. V. GRIFFITHS* and A. G. MITCHELL

Abstract □ A dissolution pattern that indicates a solvent-mediated phase change is observed when aspirin crystals are dissolved in aqueous media. It is suggested that the phase change is caused by the crystallization of a less soluble form on the crystal surface during dissolution. The kinetic and thermodynamic parameters of the two forms were determined using a rotating disk method of dissolution. An isergonic relation was demonstrated for the phase transformations of aspirin and other pharmaceutical compounds reported in the literature.

Keyphrases □ Aspirin dissolution—surface transformation □ Dissolution, aspirin crystals—kinetic, thermodynamic parameters □ Phase change, solvent mediated—aspirin crystal dissolution □ Rotating disk—aspirin dissolution □ Attenuated total reflectance—aspirin crystals □ X-ray analysis—aspirin crystals

Variation in the intrinsic dissolution rates of commercial aspirin was reported previously (1), and polymorphism was suggested as a possible cause (2). Tawashi (3) isolated two polymorphs of aspirin and found a marked difference in the rates of gastrointestinal absorption (4). Attempts to obtain these polymorphs for further study have been unsuccessful so far. However, during the determination of solubility, a pattern of dissolution was observed that suggested that aspirin undergoes a phase change in aqueous media.

Higuchi *et al.* (5) developed a mathematical model for dissolution from a mixture of two polymorphs, and they extended the theory to explain the anomalous behavior of metastable methylprednisolone where a phase change to the stable form occurs during dissolution. Nogami *et al.* (6–10) also developed models for dissolution, including dissolution that involves a simultaneous phase change. The method of Nogami *et al.* (6–10) was used to investigate the nature of the phase change that occurs during the dissolution of aspirin.

EXPERIMENTAL

Aspirin—Aspirin USP grade[1] was used. Melting point and heat of fusion, 131° and 37.9 cal./g., respectively, were determined using a Perkin-Elmer differential scanning calorimeter, DSC-1B, with effluent gas analyzer.

Bulk Dissolution—Excess aspirin (5–10 times the equilibrium solubility) was added to 250 ml. 0.1 *N* hydrochloric acid maintained at a constant temperature and stirred rapidly with a 3-cm. Teflon stirrer. Samples were removed at suitable time intervals, using a pipet fitted with a filter stick, diluted, and analyzed spectrophotometrically at 276 and 305 nm. for aspirin and salicylic acid, respectively. The total amount of aspirin dissolved was calculated using simultaneous equations (11).

Rotating Disk Dissolution—The rotating disk method of Nogami *et al.* (6) was used under the following conditions: 3-cm. diameter disks of aspirin compressed at 3 tons/cm.^{2}; 100 ml. of 0.1 *N* hydrochloric acid at 30, 37, 45, and 50°; and rotation velocities of 100, 200, 300, and 430 r.p.m. checked with an electronic stroboscope. Samples were removed at suitable time intervals and analyzed spectrophotometrically.

[1] British Drug Houses, London, England, Lot 27648, mesh size 40.

IR Attenuated Total Reflectance (ATR)—The ATR spectra (Beckman IR-10 spectrophotometer fitted with TR-9 attachment and a KRS-5 crystal) of the surfaces of aspirin disks were determined before and after exposure to the dissolution medium, using the method of Higuchi *et al.* (12).

X-Ray Analysis—X-ray powder patterns were determined using nickel-filtered copper radiation and a General Electric powder camera.

RESULTS AND DISCUSSION

Bulk Dissolution—Some results of the bulk-dissolution experiments are shown in Fig. 1. The concentration in the bulk increases rapidly and shows an initial peak before the equilibrium solubility, C_s, is reached. Similar curves have been observed both for dissolution that involves polymorphic phase changes (13, 14) and for phase changes due to hydration (10, 13–17). The peak is due to an abrupt transfer of solute from the large surface area of a more soluble form. The bulk liquid becomes supersaturated with respect to the more stable crystalline form which crystallizes out on the surface of the solid.

In previous studies (*e.g.*, 10 and 14), it was possible to identify the nature of the phase change by examination of the solid remaining after equilibrium was attained. However, in the case of aspirin, examination by means of microscopical techniques, melting point, X-ray, ATR, and DSC (heat of fusion and effluent gas analysis for water of hydration) failed to reveal any difference between the final and the initial crystals. In addition, it was possible to reproduce the bulk-dissolution curve using the excess solid filtered from the reaction vessel after equilibrium had been reached; the curve was qualitatively the same whether the excess solid was used after drying under vacuum or as a moist slurry. It is suggested, therefore, that the phase change takes place on the surface of the crystals. The surface layer goes into solution in the initial mass transfer to expose the high solubility form of the remaining crystal, which then yields the usual dissolution pattern.

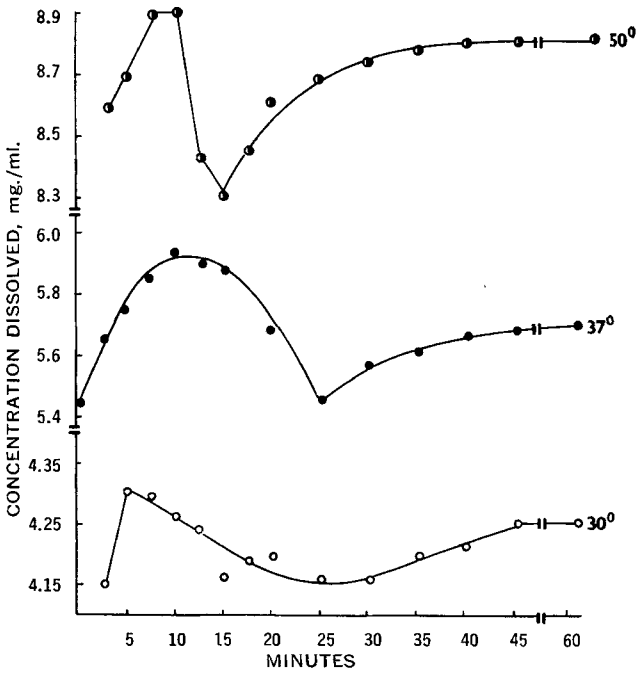

Figure 1—*Bulk dissolution of aspirin in 0.1* N *HCl.*

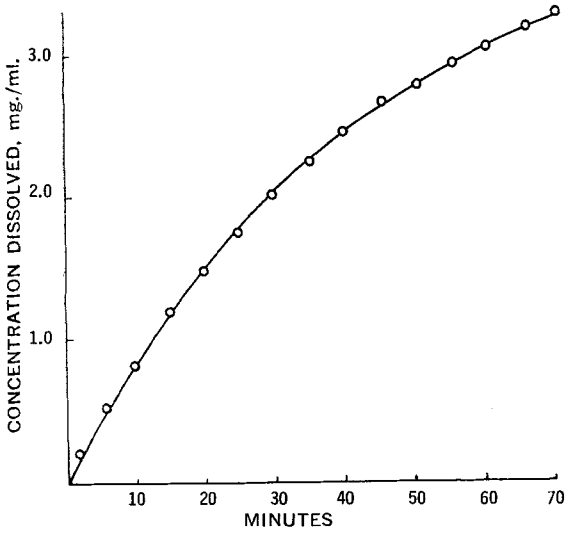

Figure 2—*Rotating disk dissolution of aspirin in 0.1* N *HCl at 30° and 430 r.p.m.*

Rotating Disk Dissolution—Figure 2 shows a typical dissolution curve made by taking samples up to about 80% of the solubility of aspirin. The dissolution curve was used to construct a finite differences diagram (Fig. 3) from which the saturated concentration, C_0, was estimated as described by Nogami *et al.* (6). The equilibrium solubilities, C_s, determined from bulk-dissolution experiments at various temperatures, are in good agreement with C_0 estimated kinetically from the finite differences diagram (Table I).

Figure 4 shows a concentration–time curve for the dissolution of aspirin over a short time interval. The linear curve is preceded by a steeper portion. Where dissolution is accompanied by a simultaneous phase change, Nogami *et al.* (10) showed that the final slope of the dissolution curve is related to the solubility of the stable form (*A*) by

$$\frac{dc}{dt} = k_t C_{sA} \qquad \text{(Eq. 1)}$$

Table I—Saturated Concentrations of Aspirin in 0.1 *N* HCl at Various Temperatures

Temperature	C_{sA}, Bulk Dissolution Method, mg./ml.	C_{0A}, Finite Differences Method, mg./ml.	C_{sB},[a] mg./ml.
30°	4.3	4.3	6.8
37°	5.7	5.8	8.1
45°	7.7	7.8	10.0
50°	8.9	8.9	11.1

[a] Calculated from Eqs. 1 and 2.

Table II—Apparent Dissolution (Transport) Rate Constant, k_t, and Overall Rate Constant, K_T, for Dissolution of Aspirin in 0.1 *N* HCl at Various Temperatures and Rotation Rates

Temperature	100 r.p.m.	200 r.p.m.	300 r.p.m.	430 r.p.m.
		$k_t \times 10^2$, min.$^{-1}$		
30°	0.80	1.15	1.46	1.55
37°	0.98	1.42	1.95	2.39
45°	1.22	1.78	2.40	3.12
50°	1.40	1.99	2.71	3.67
		$K_T \times 10$, cm. min.$^{-1}$		
30°	1.15	1.65	2.06	2.22
37°	1.40	2.03	2.77	3.40
45°	1.73	2.52	3.40	4.41
50°	1.98	2.84	3.83	5.24

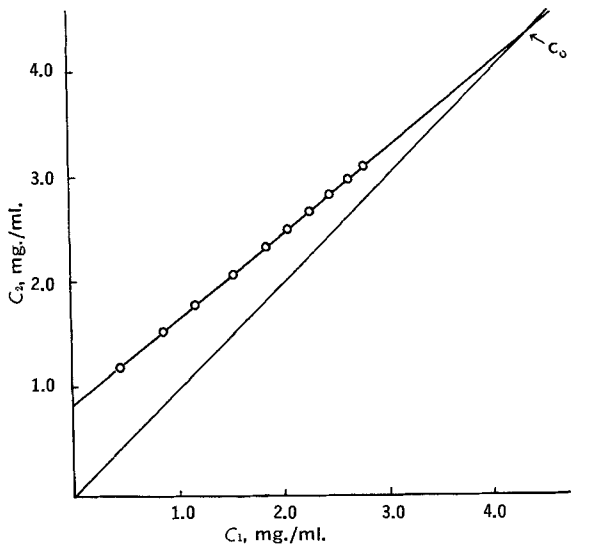

Figure 3—*Finite differences diagram for the dissolution of aspirin in 0.1* N *HCl at 30° and 430 r.p.m. Key:* C_1, *concentration at time* t_1; C_2, *concentration at time* $t_2 = t_1 + 10$ *min.*; -O-, C_2 versus C_1; *and* ——, $C_1 = C_2$.

and the initial slope is related to the solubility of the more soluble form (*B*) by

$$\left(\frac{dc}{dt}\right)_{t=0} = k_t C_{sB} \qquad \text{(Eq. 2)}$$

where k_t is the rate constant of the transport process, and C_{sA} and C_{sB} are the respective solubilities. C_{sA} is known from the bulk-dissolution experiments and the finite differences method; hence, k_t can be calculated from Eq. 1. Using the initial slope and substituting k_t into Eq. 2 give C_{sB}.

From the values of k_t, obtained at different temperatures and rotation rates, the overall dissolution-rate constant, K_T, can be calculated from the Noyes–Nernst equation:

$$\frac{dc}{dt} = k_t(C_{sA} - C_t) = \frac{S}{V} K_T(C_{sA} - C_t) = \frac{S}{V}\frac{D}{h}(C_{sA} - C_t) \qquad \text{(Eq. 3)}$$

where S is the surface area of the disk, V is the volume of the solution, D is the diffusion coefficient, and h the diffusion layer thickness; C_t, the concentration at time t, can be neglected over the short time interval of the experiment.

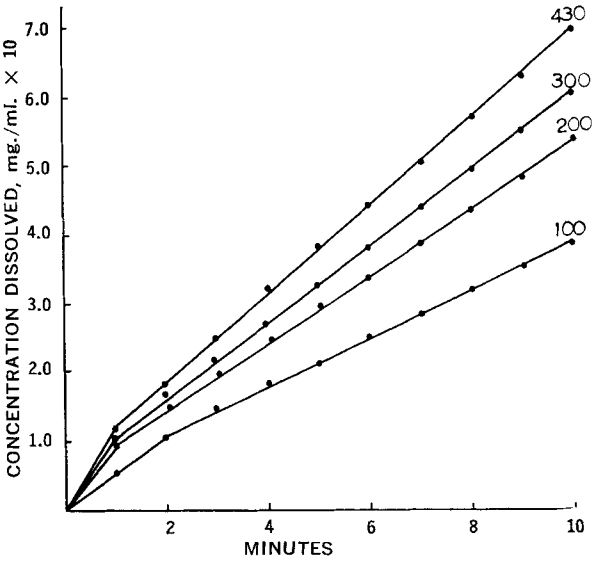

Figure 4—*Initial dissolution curves of aspirin at 30° using rotating disk method at various rotation rates.*

Table III—Diffusion Coefficient, D, and Diffusion Layer Thickness, h, for Aspirin in 0.1 N HCl at Different Temperatures and Rotation Rates

Temperature	$D \times 10^6$, cm.^{2} sec.$^{-1}$	$h \times 10^3$, cm. 100 r.p.m.	200 r.p.m.	300 r.p.m.	430 r.p.m.
30°	8.8	4.60	3.28	2.71	2.37
37°	12.9	4.90	3.51	2.80	2.28
45°	16.4	5.26	3.77	3.24	2.24
50°	21.0	5.51	3.93	3.29	2.41

Table IV—Crystallization Rate Constant, k_r, for Aspirin in 0.1 N HCl at Different Temperatures and Rotation Rates

Temperature	$k_r \times 10^2$, min.$^{-1}$ 100 r.p.m.	200 r.p.m.	300 r.p.m.	430 r.p.m.
30°	57.2	57.5	61.1	59.6
37°	85.5	85.2	85.1	88.2
45°	122.0	124.1	123.3	130.5
50°	144.0	153.0	164.1	153.1

The diffusion coefficient is calculated by substituting K_T into the Levich equation (18):

$$K_T = 0.620 \times D^{2/3} \times v^{-1/6} \times w^{1/2} \qquad \text{(Eq. 4)}$$

where v is the kinematic viscosity of the solvent, and w is the angular velocity of the rotating disk. By knowing K_T and D, the diffusion layer thickness is calculated from Eq. 3. The values of K_T, D, and h are shown in Tables II and III.

The rate constant for the recrystallization of the less soluble phase, k_r, is calculated from Eq. 5 (10):

$$b = \frac{k_t(C_{sB} - C_{sA})}{k_r} \qquad \text{(Eq. 5)}$$

where b is the intercept on the ordinate obtained by extrapolation of the linear portion of the dissolution curve (Fig. 4). The values of k_r (Table IV), are independent of the rotation rate, as expected for a surface-controlled reaction.

The activation energy of the transport process obtained from an Arrhenius plot (Fig. 5) was 5.6 kcal. mole^{-1}. This is within the range 2.8–6.5 kcal. mole^{-1} accepted for transport-controlled processes and agrees with the value of about 5 kcal. mole^{-1} reported by

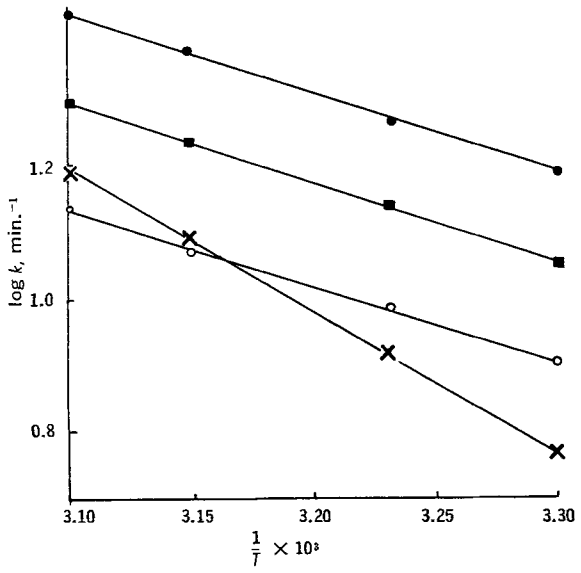

Figure 5—*Arrhenius plot for the effect of temperature on the transport and crystallization rate constants. Key:* ●, ($k_t \times 10^3$) *at 300 r.p.m.;* ■, ($k_t \times 10^3$) *at 200 r.p.m.;* ○, ($k_t \times 10^3$) *at 100 r.p.m.; and* ×, (*mean* $k_r \times 10$).

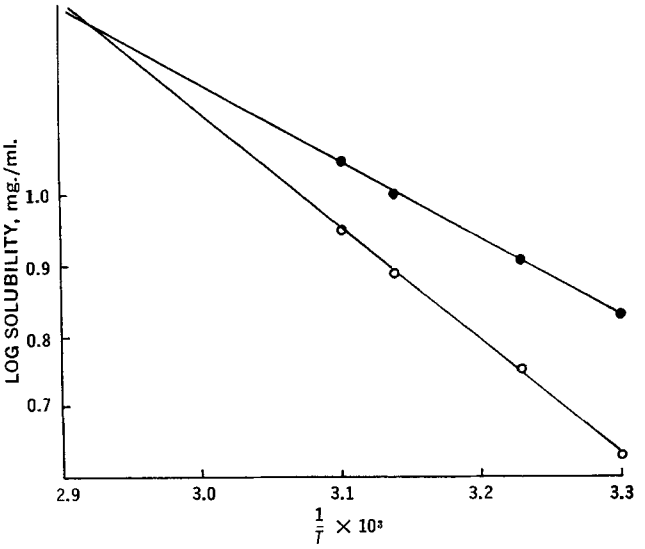

Figure 6—*Effect of temperature on the solubility of aspirin. Key:* ○, *Form* A; *and* ●, *Form* B.

Edwards (19) for the dissolution of aspirin in 0.1 N sulfuric acid. From Fig. 5, the activation energy of the crystallization process was found to be 9.5 kcal. mole^{-1}.

The transition temperature of the two forms of aspirin, obtained from a Van't Hoff-type plot (Fig. 6) was 343°K. The enthalpy change from Form B to Form A, $\Delta H_{B\to A}$, calculated from the difference in slopes was −2.4 kcal. mole^{-1}. The free energy change, ΔG, at any temperature, T, can be calculated from Eq. 6:

$$\Delta G_T = -RT \ln \frac{C_{sB}}{C_{sA}} \qquad \text{(Eq. 6)}$$

Similarly, the entropy change, ΔS_T, at any temperature, T, is given by Eq. 7:

$$\Delta S_T = \frac{\Delta H_{B\to A} - \Delta G_T}{T} \qquad \text{(Eq. 7)}$$

At the transition temperature, ΔG_T is zero and ΔS is −7.0 e.u.

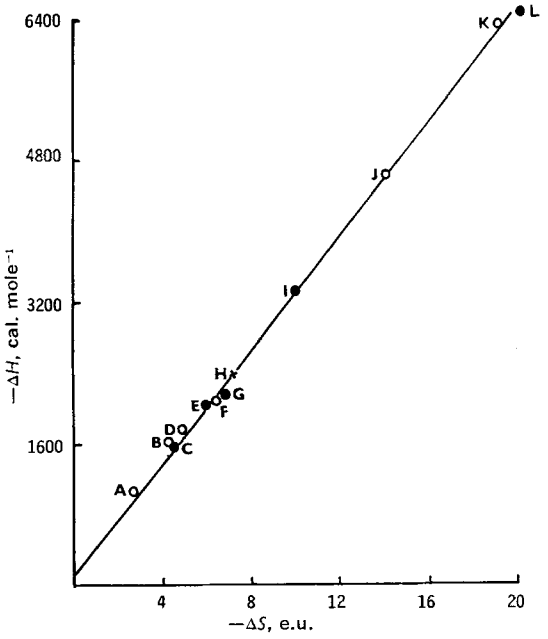

Figure 7—*Isergonic plot of the relation between enthalpy and entropy changes in polymorphic and hydration phase transformations. Key:* ○, *polymorphic changes;* ●, *hydration changes; and* ×, *this work. See Table V for explanation of A–L.*

Table V—Isergonic Relationship between Change of Enthalpy (ΔH) and Change of Entropy (ΔS) for the Phase Transformation of a Number of Pharmaceutical Compounds

Key Fig. 7	Compound	Type of Phase Change	Transition Temperature	ΔH (cal. mole^{-1})	ΔS_{298} (e.u.)	Reference
A	Mefenamic acid	Polymorphic	89°	−1031	−2.6	14
B	Methylprednisolone	Polymorphic	118°	−1600	−4.2[a]	21
C	*p*-Hydroxybenzoic acid	Hydration	84°	−1550	−4.4[a]	10
D	Sulfathiazole	Polymorphic	95°	−1744	−4.7[a]	22
E	Glutethimide	Hydration	52°	−2000	−5.8	15
F	Phenobarbital	Polymorphic	34°	−2300	−6.4[a]	9
G	Phenobarbital	Hydration	37°	−2140	−6.9[a]	10
H	Aspirin	—	70°	−2420	−7.1	This work
I	Theophylline	Hydration	73°	−3300	−10.0	15
J }	Chloramphenicol	Polymorphic $C \rightarrow A$	50°	−4600	−13.8[a]	14
K }	Palmitate	Polymorphic $B \rightarrow A$	88°	−6350	−18.7[a]	14
L	Ampicillin	Hydration	42°	−6400	−20.0	23

[a] Values for ΔS_{298} calculated from data given in the reference.

X-Ray Studies and ATR—X-ray powder patterns on samples of aspirin before and after bulk-dissolution experiments were identical. However, since the crystals were powdered before the X-ray determination, any changes occurring in the surface layer only would not be detected. Samples of powder scraped from the surface of the disk after exposure to the dissolution medium also failed to show any change in the X-ray powder pattern.

ATR is particularly suited to a study of surface layers, since IR energy penetrates to a depth of about 5–10 μ. It was hoped that the method described by Higuchi *et al.* (12) would provide direct evidence of a surface transformation during dissolution. Unfortunately, spectra obtained after exposure of a disk to the solvent were very poor compared with the original spectrum.

Isergonic Relationship in Polymorphic and Hydrate Transformations—A linear relationship between enthalpy and entropy changes of chemical reactions is well known. Such a plot is referred to as an isokinetic or isergonic plot (20). The relationship is normally applied to a series of closely related compounds undergoing an identical reaction. Literature values of enthalpy and entropy changes for polymorphic phase reversions and anhydrate to hydrate changes are shown in Table V. These values and those for aspirin determined in this work can be fitted on a straight-line relationship (Fig. 7):

$$-\Delta H = -0.117 - 0.322\Delta S \qquad \text{(Eq. 8)}$$

with a correlation coefficient of 0.992 compared with a theoretical correlation coefficient ($P' = 0.05$) of 0.58. Although the significance of isergonic plots is not fully understood (20), the relation between ΔH and ΔS for phase transformations in compounds as diverse as aspirin, methylprednisolone, and chloramphenicol palmitate is intriguing.

SUMMARY

A solvent-mediated change to a less soluble form occurs when aspirin is dissolved in 0.1 *N* hydrochloric acid. The "repeatability" of the bulk dissolution experiments suggests that the phase change occurs in the surface layer of the aspirin crystals. This suggestion is supported by the kinetic analysis of the rotating disk dissolution results and the X-ray, ATR, and other physical measurements which show that the bulk of the crystalline material is unchanged.

The nature of the surface phase transformation cannot be established from the kinetic and thermodynamic data. Crystallizations of an hydrate or a more stable polymorph are both possible, and work is being continued on this problem.

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Determination of Ethylene Oxide, Ethylene Chlorohydrin, and Ethylene Glycol by Gas Chromatography

HARVEY D. SPITZ and JOSEPH WEINBERGER

Abstract □ The technique of gas chromatography was employed for the quantitative determination of ethylene oxide, ethylene chlorohydrin, and ethylene glycol. The simultaneous determination of ethylene chlorohydrin and ethylene glycol in an aqueous solution using a single polyethylene glycol column was accomplished under isothermal conditions, while ethylene oxide was determined employing a styrene–divinylbenzene copolymer column. A key step in obtaining useful and reproducible columns for determining trace quantities of ethylene oxide, ethylene chlorohydrin, and ethylene glycol was found to be related to the aging procedure employed. Experimental data indicate that one can quantitatively recover low levels of ethylene chlorohydrin and ethylene glycol from a water-absorbable fabric. The lower limits of detection of ethylene oxide, ethylene chlorohydrin, and ethylene glycol were found to be in the nanogram range.

Keyphrases □ Ethylene oxide, chlorohydrin, and glycol—determination □ Quantitative recovery from fabric—ethylene oxide, chlorohydrin, and glycol □ GLC—analysis

The increasing demand for ethylene oxide as a sterilizing agent has stimulated a great deal of research on the possible toxicological effects of ethylene chlorohydrin (2-chloroethanol) and ethylene glycol, which are associated side products of this sterilant. Although the literature contains a significant number of reports on the toxicity of ethylene oxide (1–12), ethylene chlorohydrin (13–17), and ethylene glycol (13, 18–24), the large variation in the experimental systems employed and the conclusions reached have prompted further investigations.

Several analytical methods have been reported in the literature for the determination of ethylene oxide (25–33), ethylene glycol (27, 34), and ethylene chlorohydrin (35–39). However, the present analytical requirements with respect to sensitivity, specificity, and time of analysis for the quantitative determination of ethylene oxide, ethylene chlorohydrin, and ethylene glycol in water-absorbable fabrics did not readily adapt to these methods.

Some workers (40) advocated a weighing procedure based on the premise that the loss in weight of a sterilized sample with respect to time represents the amount of ethylene oxide lost. The poor sensitivity one obtains with this approach, coupled with other possible concomitant loss of material (such as a loss due to absorbed gases used in diluting the sterilant or a loss of volatile materials from the sample) or even gain in material (such as the formation of ethylene glycol, diethylene glycol, *etc.*), makes this method less attractive as compared to other techniques.

Since low levels of the three residues may be toxicologically significant, the technique of gas chromatography was chosen over other available analytical methods because of its excellent sensitivity and selectivity.

In this paper, emphasis is placed on the quantitative determination of ethylene oxide, ethylene chlorohydrin, and ethylene glycol in the nanogram range, employing two different gas chromatographic columns. One column is capable of determining trace quantities of ethylene oxide, while the second column is capable of simultaneously determining trace amounts of ethylene chlorohydrin and ethylene glycol.

EXPERIMENTAL

Apparatus—F&M model 5750, equipped with a dual-flame ionization detector, connected to a 1-mv. recorder[1] was used.

Column A—A coiled stainless steel column, 1.83 m. (6 ft.) × 0.32 cm. (0.125 in.) i.d., containing a styrene–divinylbenzene copolymer resin (80–100 mesh, 300–400 m.²/g. surface area)[2] was employed for the analysis of ethylene oxide.

Column B—A coiled glass column, 1.83 m. (6 ft.) × 2 mm. i.d., containing 3% polyethylene glycol[3] coated on a styrene–divinylbenzene copolymer resin (80–100 mesh, less than 50 m.²/g. surface area)[4] was used for the analysis of ethylene chlorohydrin and ethylene glycol.

Preparation of Column Packing—To prepare the packing for Column B, the following method was employed. A 1% solution of polyethylene glycol in chloroform was prepared by dissolving 0.6 g. of the polyethylene glycol in 60 ml. of chloroform. To this solution, 19.4 g. of the styrene–divinylbenzene resin[4] was slowly added with gentle stirring. The mixture was allowed to stand for 10 min. before being transferred to a large watchglass. The packing was spread out to a height no greater than 0.64 cm. (0.25 in.). The watchglass was then placed in a ventilation hood with occasional stirring during this drying procedure until all traces of chloroform were removed from the packing.

Column Packing—Column A was vibrated while being packed under vacuum and then coiled to the necessary configuration of the chromatograph oven. The coiled glass column (Column B) was packed in a similar manner.

Column Conditioning—Column B was initially conditioned in the gas chromatograph overnight at 200° with helium flow. The following day, the column was connected to the detector system and 1-μl. injections of distilled water were made approximately every 15 min. for several hours at a column temperature of 180°. Similarly, Column A was aged overnight at 200°, and several 1-μl. injections of acetone were made at this temperature.

With this aging technique, one is able to analyze all three residues in the nanogram range, employing the maximum sensitivity of the instrument.

Instrumental Parameters—For the analysis of ethylene chlorohydrin and ethylene glycol (Column B), the instrument was operated isothermally at a column temperature of 168°, an injector temperature of 195°, and a detector temperature of 220°. Helium was used as the carrier gas with a flow rate of 30 ml./min., while a flow rate of 300 ml./min. was used for air and 30 ml./min. for hydrogen. For the analysis of ethylene oxide (Column A), the instrument was operated at an injector temperature of 125°, a detector temperature of 220°, and a column temperature of 100° for 9 min.; then the instrument was temperature programmed at 50°/min. up to a maximum of 200° and held for 4 min. at this temperature. Flow rates for helium, hydrogen, and air were approximately the same as used for Column B. A chart speed of 0.51 cm. (0.2 in.)/min. was used for the determination of ethylene oxide and 1.3 cm. (0.5 in.)/min. for the simultaneous determination of ethylene chlorohydrin and ethylene glycol.

[1] Sargent recorder, model No. SRG.
[2] Chromosorb 102, Johns-Manville Products Corp.
[3] Carbowax 20M, Union Carbide Corp.
[4] Chromosorb 101, Johns-Manville Products Corp.

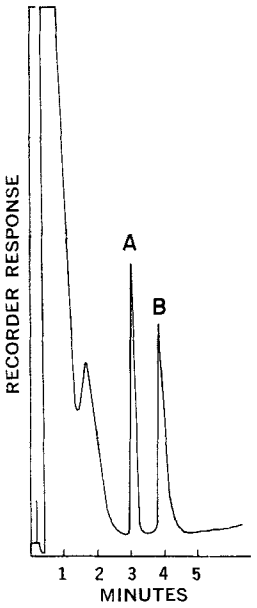

Figure 1—*Typical gas chromatogram of 5.7 ng. of ethylene chlorohydrin (A) and 6.4 ng. of ethylene glycol (B) on Column B. Attenuation range = 1 × 1. Injection volume = 1 μl.*

Recovery Study for the Simultaneous Determination of Ethylene Chlorohydrin and Ethylene Glycol—A water-absorbable fabric was simultaneously spiked with known amounts of ethylene chlorohydrin and ethylene glycol at four different levels of concentration. Approximately 1-g. samples of fabric were placed in 20-ml. serum vials to which 5.0 ml. of different standard solutions was added. These standard solutions were prepared (in water) in such a manner that 5.0 ml. of each standard solution contained ethylene chlorohydrin and ethylene glycol in varying amounts. Sample 1 was simultaneously spiked with 0.022 mg. of ethylene chlorohydrin and 0.099 mg. of ethylene glycol, while succeeding samples were spiked with higher levels of both compounds (Table I). After allowing the samples to stand for 15 min., 5.0 ml. of distilled water was added to each vial. The vials were sealed by crimping an aluminum seal over a rubber septum. The samples were then placed in a mechanical shaker[5] and were shaken for 5 min. Duplicate samples were also prepared in the same manner as previously described. Likewise, corresponding standards were prepared in the same fashion, employing the same standard solutions used to spike the samples except that the vials did not contain any fabric.

One-microliter injections of each of the foregoing solutions were injected into Column B in duplicate, the order of injection being: sample, corresponding standard, and duplicate sample. The peak height method was used to calculate all the experimental data.

A lower concentration range of ethylene chlorohydrin as compared to ethylene glycol was chosen as it is presently considered to be more toxic than ethylene glycol.

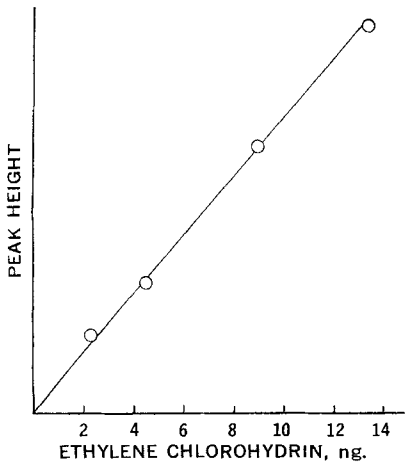

Figure 2—*Plot of the concentration of ethylene chlorohydrin* versus *peak height. Attenuation range = 1 × 1 to 1 × 2. Injection volume = 1 μl.*

[5] Model No. 3800, Pitchford Manufacturing Corp., Pittsburgh, Pa.

Table I—Recovery of Ethylene Chlorohydrin and Ethylene Glycol from Spiked Fabric

Samples	ECH[a] and EG[b] Added to Fabric, mg. ECH[a]	EG[b]	ECH[a] and EG[b] Recovered from Spiked Fabric,[c] mg. ECH[a]	EG[b]
Sample 1	0.022	0.099	0.021	0.112
Duplicate Sample 1	0.022	0.099	0.019	0.099
Sample 2	0.044	0.495	0.046	0.476
Duplicate Sample 2	0.044	0.495	0.047	0.540
Sample 3	0.088	1.48	0.085	1.44
Duplicate Sample 3	0.088	1.48	0.084	1.48
Sample 4	0.133	2.97	0.131	2.93
Duplicate Sample 4	0.133	2.97	0.131	2.98

[a] Ethylene chlorohydrin. [b] Ethylene glycol. [c] Average value of duplicate injections for each sample.

RESULTS AND DISCUSSION

The experimental data shown in Table I indicate that low levels of ethylene chlorohydrin and ethylene glycol can be quantitatively determined in the presence of each other. These results also indicate that no detectable irreversible adsorption takes place on the fabric with either compound.

One should keep in mind that 1 μl. of the final sample and standard solutions, which was injected into the gas chromatograph, contained from 2 to 13 ng. of ethylene chlorohydrin and 10 to 297 ng. of ethylene glycol. To obtain respectable peak heights for the lower concentrations of ethylene chlorohydrin and the lowest concentration of ethylene glycol, the maximum sensitivity of the instrument was required (attenuation 1 × 1). As shown in Fig. 1, the separation of the two compounds is good and baseline noise is negligible. Since the concentration of ethylene glycol was much higher in Samples 2, 3, and 4 (Table I), it was necessary to change the attenuation manually after the elution of the ethylene chlorohydrin peak to accommodate properly the ethylene glycol peak. This very minor inconvenience of manual attenuation can be alleviated by having a recorder or integrator equipped with an automatic attenuator.

This experiment was also designed to obtain linearity curves for both compounds based on the different standard solutions injected into the chromatograph (those used for the recovery study). Since each standard solution contained both compounds, one was able to obtain simultaneously from the data the individual linearity relationships for each compound. Plots of the data are shown in Figs. 2 and 3. Figure 2 covers an attenuation range for ethylene chlorohydrin from 1 × 1 to 1 × 2, while the ethylene glycol plot covers an attenuation range from 1 × 1 to 1 × 32.

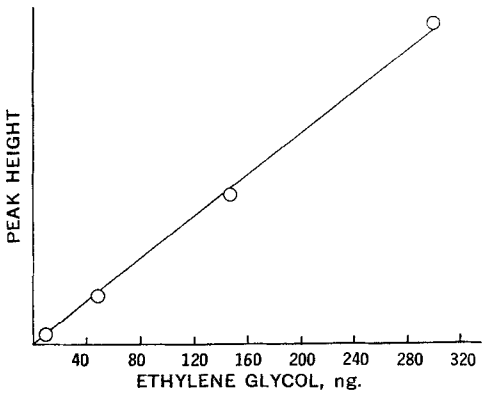

Figure 3—*Plot of the concentration of ethylene glycol* versus *peak height. Attenuation range = 1 × 1 to 1 × 32. Injection volume = 1 μl.*

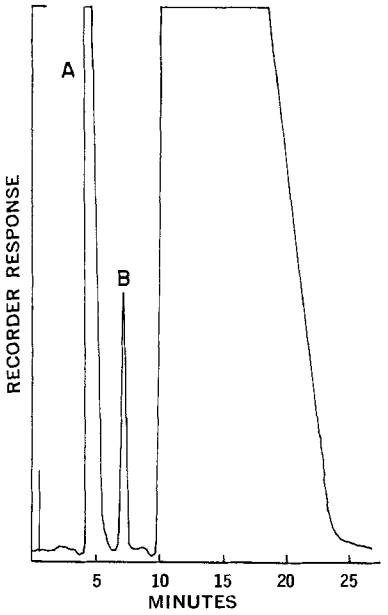

Figure 4—*Typical gas chromatogram of 5.7 ng. of ethylene oxide (B) on Column A. Attenuation range = 1 × 1. Injection volume = 1 μl. (A) is an acetone impurity peak.*

The familiar problem of ghosting (41–43) was observed with ethylene glycol on Column B. Subsequent 1-μl. injections of water after previous injections of the higher concentrations of ethylene glycol produced ethylene glycol peaks that were observable only at the most sensitive attenuations of the instrument. The possible error contributed by this ghosting phenomenon was found to be negligible. Details of this work will be discussed in a future paper.

As in the case of ethylene chlorohydrin and ethylene glycol, a gas chromatographic method was developed for determining trace levels of ethylene oxide. The excellent stability of the styrene–divinylbenzene resin,[2] coupled with its ability to resolve ethylene oxide from a solvent such as acetone (and its impurities), makes Column A highly desirable. A typical chromatogram is shown in Fig. 4. The major acetone impurity, as shown in Fig. 4, was observed in several different brands of acetone only at the most sensitive attenuations of the instrument. In most cases, distillation of the acetone will reduce the concentration of this impurity; however, it is usually separated from the ethylene oxide peak. Temperature programming is required to elute the acetone from the column so that a complete analysis can be accomplished within 25 min. As shown in Fig. 5, a linear relationship is present at low levels of ethylene oxide. The analysis for residual ethylene oxide has been carried out successfully in ethylene oxide sterilized samples, such as fabrics and plastics with acetone or tetrahydrofuran as the solvent. Recent private communications with other workers in the field have informed the authors that the method has performed satisfactorily for the analysis of residual ethylene oxide in their sterilized samples.

The approximate lower limit of detection for ethylene oxide on Column A was found to be 0.7 ng./μl. (representing 7% of the chart paper), employing a 1-μl. injection of acetone. Similarly, the approximate lower limits of detection for ethylene chlorohydrin and ethylene glycol in aqueous solution on Column B were found to be 0.6 ng./μl. and 1.6 ng./μl., respectively (both representing 8% of the chart paper).

Since more than 1 μl. of solution can be injected into the column without any deleterious effects upon the resolution, the limits of detection are further increased for any given sample. Of course, by decreasing the volume of solvent and increasing the sample size and injection volume, the limits of detection can be increased even further.

Recent experiments in this laboratory showed that all three residues can be determined simultaneously on the polyethylene glycol column in the presence of water and/or acetone. The authors hope to present in the near future the optimum conditions and limits of detection for separating and quantitating all three compounds.

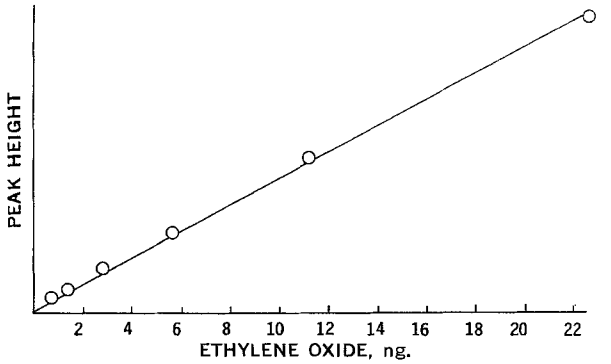

Figure 5—*Plot of the concentration of ethylene oxide* versus *peak height. Attenuation range = 1 × 1. Injection volume = 1 μl.*

Additional work is proposed on separating low molecular weight epoxides and the corresponding glycols and chlorohydrins on the same polyethylene glycol column. Preliminary experiments indicate this is feasible.

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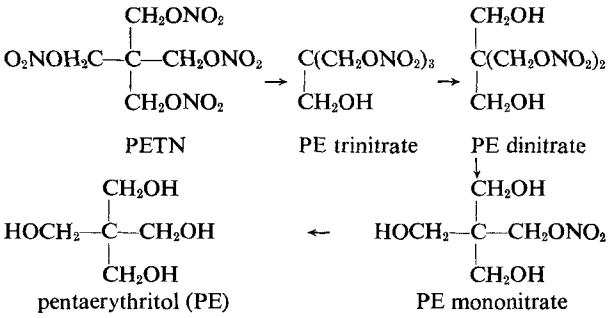
Pharmacodynamics and Biotransformation of Pentaerythritol Tetranitrate in Man

IVAN W. F. DAVIDSON*, HENRY S. MILLER, Jr.*, and FREDERICK J. DiCARLO †‡

Abstract □ The absorption, biotransformation, and excretion of pentaerythritol tetranitrate was studied after oral administration of two dosages, 20 and 40 mg., to patients. The drug was given as ^{14}C-pentaerythritol tetranitrate incorporated into tablets of a type used clinically. The total ^{14}C excretion in 48 hr. was approximately 92% of both doses. However, a greater proportion of the lower dose was excreted in the urine: 60% of the 20-mg. dose and 50% of the 40-mg. dose. Drug radioactivity was detected in the blood within 15 min., and peak levels occurred from 4 to 8 hr. after administration. The only radioactive compounds found in the blood were pentaerythritol, pentaerythritol mononitrate, and pentaerythritol dinitrate. These drug metabolites were also present in the urine and feces. The kinetics of renal excretion of the principal urinary metabolites, pentaerythritol and pentaerythritol mononitrate, were first order. The renal elimination-rate constant, k_e, of pentaerythritol was independent of the dose, but k_e for pentaerythritol mononitrate was dose related and significantly smaller for the higher dose. The ratio of pentaerythritol mononitrate/pentaerythritol excreted in the urine was approximately 1:1 for the lower dose and 3:1 for the higher dose. The findings indicate a rapid deesterification of pentaerythritol tetranitrate by the human to pentaerythritol mononitrate after oral ingestion, but a limited capacity for the conversion of pentaerythritol mononitrate to pentaerythritol.

Keyphrases □ Pentaerythritol tetranitrate and ^{14}C-substituted—human pharmacodynamics, biotransformation □ Biotransformation, pharmacodynamics—pentaerythritol tetranitrate □ Urinary, fecal excretion—pentaerythritol tetranitrate □ TLC—separation □ Scintillometry—analysis

It has been generally assumed that all organic nitrates exert qualitatively similar actions and that the extended duration of action ascribed to the "long-acting" nitrates relates either to differences of absorption and metabolic stability or to specific properties of the drug molecule itself (1). Since little specific information is available on the pharmacodynamics and biotransformation of this group of drugs in man, a study was performed with pentaerythritol tetranitrate (PETN), a "long-acting" organic nitrate in wide clinical use. Biotransformation of PETN (Scheme I) was followed qualitatively and quantitatively by modifying procedures developed earlier using ^{14}C-labeled drug (2). Drug pharmacodynamics were examined at two dose levels, 20 and 40 mg., with a clinical dosage form prepared from ^{14}C-PETN.



Scheme I

EXPERIMENTAL

Subjects—The subjects were 15 male volunteers between the ages of 30 and 68 years who presented no history or evidence of malabsorption, intestinal motility disturbances, or renal disease. For the period of study (4 days), the subjects were restricted to the Clinical Research Unit at Bowman Gray School of Medicine. A complete medical history and physical examination were taken on each subject. Prestudy laboratory data included the serum levels of electrolytes (Na^+, K^+, Cl^-, and CO_2), urea nitrogen, uric acid, blood sugar, cholesterol, inorganic phosphate, lactate dehydrogenase, total protein, calcium, bilirubin, alkaline phosphatase, and glutamic-oxaloacetate transaminase. Additional laboratory tests performed were EKG, chest X-ray, sedimentation rate, hematocrit, CBC, and urinalysis.

Drug Administration—^{14}C-Labeled and nonradioactive PETN were used to prepare compressed tablets, which contained a total of 20 mg. of PETN and 44 μc. each and met the chemical assay and disintegration-time specifications for the manufacture of a commercial product.[1] After an overnight fast, one tablet was administered *per os* to each of 10 subjects and two tablets were administered similarly to each of five subjects. All subjects remained in the fasting state for an additional 2 hr.

Collection of Specimens—Urine was voided directly into plastic bottles stored in a dry-ice chest. The collection periods were 0–2, 2–4, 4–8, 8–12, 12–24, and 24–48 hr. after drug administration.

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Blood specimens (10 ml.) were withdrawn into 15-ml. EDTA-Vacutainers[2] at the following intervals postadministration: 15 and

[1] Peritrate.
[2] Becton, Dickinson Co.

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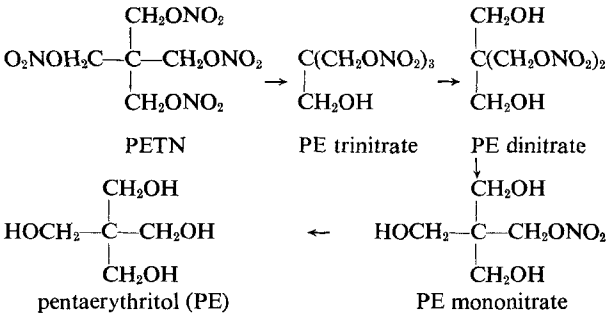

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Collection of Specimens—Urine was voided directly into plastic bottles stored in a dry-ice chest. The collection periods were 0–2, 2–4, 4–8, 8–12, 12–24, and 24–48 hr. after drug administration.

Immediately after defecation into a plastic container, each stool collection was covered with cold dioxane and stored in a dry-ice chest. For each subject the feces were pooled from 0–24, 24–48, and 48–72 hr.

Blood specimens (10 ml.) were withdrawn into 15-ml. EDTA-Vacutainers[2] at the following intervals postadministration: 15 and

[1] Peritrate.
[2] Becton, Dickinson Co.

30 min., 1, 2, 4, 8, 12, 24, and 48 hr. Each blood sample was transferred immediately into a labeled polycarbonate tube and frozen in a dry-ice bath.

Assays for Pentaerythritol (PE) and Its Nitrates—The total radioactivity of blood, urine, and fecal matter from ^{14}C-PETN and its metabolites was measured by liquid scintillation counting with a Packard spectrometer at 7°. Channel ratio and internal standardization methods were used to correct for quenching. The scintillation solution consisted of 8.0 g. of 2-(4-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) and 0.5 g. of 2-(4′-biphenyl)-6-phenylbenzoxazole (PBBO) in 1 l. of toluene to which was added 200 ml. of a solubilizer.[3]

PETN and its metabolites (PE trinitrate, PE dinitrate, PE mononitrate, and PE) were identified in the urine and fecal matter by extraction of total ^{14}C-radioactivity, partial purification, and then separation by TLC using 5 × 20-cm. precoated (250 μ) silica gel G plates.[4] The K solvent system consisted of toluene–ethyl acetate–butanol–water (5:5:1:1, upper phase). The R_f values, with a front movement of 10 cm., were: PE, 0.00; PE mononitrate, 0.16–0.23; PE dinitrate, 0.45–0.55; PE trinitrate, 0.60–0.70; and PETN, 0.82–0.90. The relative amounts of these compounds were estimated by radioscanning with a model 1036 Nuclear-Chicago Actigraph II radioscanner. The areas under the peaks were measured with a compensating polar planimeter, and the relative quantities of each compound were calculated from proportional areas.

The urine from each collection period for a subject was thawed and mixed and the volume was measured. Duplicate 0.5-ml. aliquots were counted for radioactivity by mixing with 15 ml. of the toluene-scintillation cocktail. For chromatography, 5 ml. of urine was evaporated to dryness with an air stream at room temperature. The residue was extracted with 1.0 ml. of methanol–ethyl acetate (1:1, v/v), clarified by centrifugation, and concentrated by evaporation with a nitrogen stream to a minimum volume for spotting on a TLC plate.

The feces of a subject for each collection period were thawed and thoroughly mixed in its container with a Servall Omni-mixer. The feces–dioxane slurry was filtered with vacuum, and the residue was reextracted three times with 50-ml. portions of dioxane. The combined extract was evaporated to 250 ml. with an air stream at room temperature. An aliquot of the extract was diluted 10-fold with dioxane–water (5:1, v/v); duplicate 0.3-ml. aliquots were counted for ^{14}C-radioactivity by mixing with 15 ml. of the toluene-scintillation solution. For chromatography, 10 ml. of the extract was prepared for spotting by the method used previously for urine.

Each blood sample was allowed to thaw in an ice bath and mixed by inversion. A sample of 1.0 ml. was diluted 1:10 with cold distilled water. Duplicate 0.4-ml. aliquots were prepared for radioactivity counting by adding the aliquot to a counting vial containing 1.0 ml. of alcoholic hydrogen peroxide solution (32% aqueous hydrogen peroxide and methanol, 1:3, v/v). The color was allowed to bleach at room temperature for 5 min. before 4 drops were added of a saturated aqueous solution of ascorbic acid. The vials were gently swirled to effect solution, and 15 ml. of the toluene-scintillation solution was added with mixing. The counting samples were virtually colorless and completely dissolved.

The identification and quantitation of the low levels in blood of ^{14}C-PETN and metabolites required pooling of blood samples. For a given time interval after drug administration, 8.0 ml. of blood from each of five subjects was added dropwise to 200 ml. cold dioxane with constant swirling. The homogeneous suspension of blood protein so produced was shaken vigorously for 15 min. and then filtered by vacuum. The residue was washed three times with 50-ml. portions of dioxane. The combined extract and washings were frozen and lyophilized. The dried powder was extracted in the flask four times with 5 ml. of warm methanol. The combined extracts were centrifuged and the supernatant chilled to −20° to induce precipitation of radioactivity-free lipid, which was removed by rapid centrifugation at −5°. The clear supernatant was evaporated to near dryness with an air stream at room temperature. The residue was extracted three times with 1.0 ml. of methanol; the extracts were combined, and 3.0 ml. of distilled water was added to precipitate additional lipid.

After centrifugation, the supernatant was again taken to dryness and the residue extracted with 5.0 ml. of methanol–ethyl acetate (1:1, v/v). The extract was clarified by centrifugation and the supernatant taken to dryness. This residue was extracted three times with 1.0-ml. portions of methanol, and the combined extracts were reduced to approximately 1.0 ml. with a nitrogen stream. The lipid material was again precipitated by adding 1.0 ml. of distilled water. After centrifugation, the supernatant was evaporated to dryness with a nitrogen stream and the residue taken up in 1.0 ml. of the methanol–ethyl acetate solvent. The solution was clarified by centrifugation and reduced to 200 μl. for application to a TLC plate. The sample then was applied in an area of 1.5 by 3.0 cm. The plate was developed first in Solvent System K, with an ascending front movement of 13 cm., and then three successive times in Solvent System D with thorough warm air drying between developments. Multiple development with Solvent System D, which consisted of *n*-butanol–ammonium hydroxide–water (4:1:3, upper phase), moved all of the radioactive components into the upper half of the plate ($R_f > 0.5$) and left the bulk of fatty and other interfering material behind. After radioscanning, the silica gel was scraped from the radioactive area of the plate and extracted successively with 2.5, 2.0, 1.5, and 1.0 ml. of warm methanol. The methanol extracts were combined and evaporated to dryness with a nitrogen stream. The radioactive residue was taken up in 3 ml. of methanol, clarified by centrifugation, and reduced to 50–100 μl. for spotting on a TLC plate. The plate was developed in Solvent System K and scanned to determine the components.

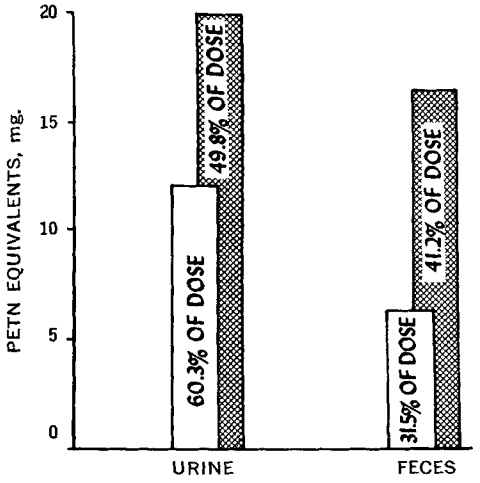

Figure 1—*Total urinary (48 hr.) and fecal (72 hr.) excretion of radioactivity administered as ^{14}C-PETN in 20-mg. (□) and 40-mg. (■) doses.*

RESULTS

Figure 1 shows the recovery of drug radioactivity following the oral administration of 20- and 40-mg. doses of ^{14}C-PETN. Drug radioactivity was excreted in both urine and feces. The total recovery of administered ^{14}C approximated 92% for both doses. In 48 hr., the equivalent of 20 mg. of PETN or 50% of the 40-mg. dose was excreted, whereas 60% or 12 mg. of the 20-mg. dose was so eliminated. PE and PE mononitrate were the major urinary metabolites, together accounting for 98% of urine radioactivity (Fig. 2). No PETN or PE trinitrate was detected in the urine, and PE dinitrate was present only in very small amounts in the urine for the first few hours after drug administration. For the total 48-hr. excretion, the proportion of urinary PE mononitrate to PE was approximately 1:1 for the low dose and about 3:1 for the high dose.

Fecal elimination of drug radioactivity was extensive and corresponded to 32 and 41% of the 20- and 40-mg. doses, respectively (Fig. 1). The feces contained significant quantities of PETN but no PE trinitrate (Fig. 2). PE was the major drug metabolite in the stool and was accompanied by small amounts of PE dinitrate and PE mononitrate.

Figure 3 illustrates the renal elimination of total drug ^{14}C and of the two major metabolites, PE and PE mononitrate. First-order elimination curves were obtained when the time course of elimination (expressed in molar quantities to account for drug metabolism) was plotted as unexcreted drug radioactivity or individual metabolites, according to the kinetic relationship describing postabsorptive

[3] Bio-Solv BBS-3, Beckman Instruments.
[4] Brinkmann Instruments.

Table I—Renal Elimination of PETN Metabolites

Metabolite	Elimination-Rate Constant, $k \times 10^{-2}$ hr.$^{-1}$		Half-Time, hr.	Zero Time Shift, Hr. after Administration
Total ^{14}C				
20-mg. dose	9.77 ± 0.50[a]		7.1	2.0
		$p < 0.02$		
40-mg. dose	8.40 ± 0.22		8.3	2.1
PE				
20-mg. dose	8.55 ± 0.42		8.1	1.1
		$p > 0.10$		
40-mg. dose	7.82 ± 0.26		8.8	0.8
PE mononitrate				
20-mg. dose	11.08 ± 0.55		6.3	2.8
		$p < 0.01$		
40-mg. dose	8.60 ± 0.38		8.1	2.6

[a] k_e ± standard deviation.

and postequilibrative phases of drug excretion and metabolism (3–5):

$$100 \log \left[1 - \frac{Ae}{Ae^\circ}\right] = -k_e t/2.303 \qquad \text{(Eq. 1)}$$

where Ae° is the total amount of drug radioactivity (mole equivalents ^{14}C-PETN) or drug metabolites (moles) excreted after 48 hr., A_e is the cumulative excretion to time t (hr.) after drug ingestion, and k_e is the elimination-rate constant. When absorption, metabolism, and equilibration phases are neglected, the relationship predicts that at $t = 0$ (drug administration) the amount of drug or metabolites distributed in the body equals the drug radioactivity or drug metabolites eventually excreted. Extrapolation of the lines of best fit of the experimental data to $t = 0$ produced values greater than the observed excretion (Fig. 3). Therefore, apparent zero times were calculated from the regression equations for total drug radioactivity and for the PETN metabolites, PE and PE mononitrate. The zero-time shifts were approximately 2.0, 0.9, and 2.7 hr., respectively, after PETN ingestion (Table I). These time periods were not dose dependent.

Table I gives the renal elimination-rate constants and half-times for total PETN radioactivity and also for two constituent major metabolites. Total radioactivity was excreted significantly faster from the 20-mg. than from the 40-mg. dose of ^{14}C-PETN. This finding is attributed to a difference of PE mononitrate excretion, because there was a significant dose-related difference in the excretion-rate constants of PE mononitrate but not of PE. The faster rate of PE mononitrate excretion (20-mg. dose) was 11.1% of the quantity present in the body per hour, with a half-time of 6.3 hr. The slower rate (40-mg. dose) represented an excretion rate of 8.6% per hour of the body content with a half-time of 8.1 hr. Since the rate constants represent the sum of the rate constants of metabolism and renal excretion (5), the dose-dependent difference in PE mononitrate elimination-rate constant indicated a slower rate of conversion of PE mononitrate to PE for the 40-mg. dose.

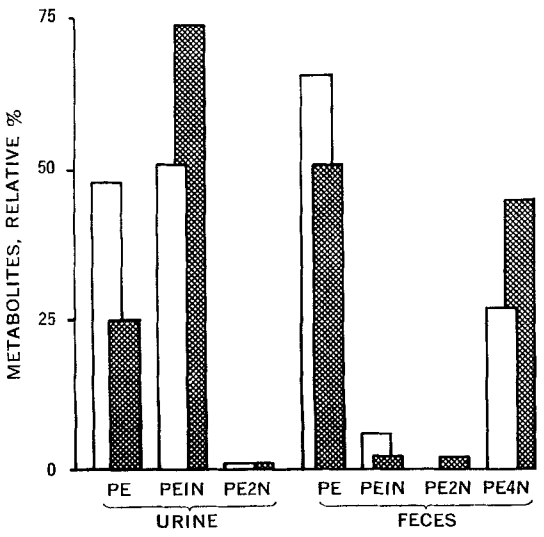

Figure 2—*Drug and drug metabolites excreted into the urine (48 hr.) and feces (72 hr.) from 20-mg. (□) and 40-mg. (■) doses of ^{14}C-PETN. PEIN = PE mononitrate, PN2N = PE dinitrate, and PE4N = PETN.*

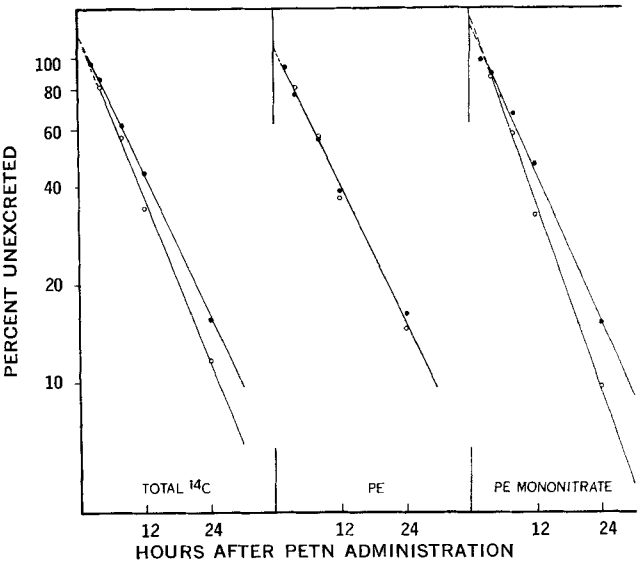

Figure 3—*Renal elimination of drug radioactivity and of the two major metabolites after ^{14}C-PETN administration. The points were calculated from cumulative excretion for 10 subjects (○, 20-mg. dose) and 5 subjects (●, 40-mg. dose). The curves were fitted by the method of least squares.*

Figure 4 shows the blood levels of drug-^{14}C expressed as μmole equivalents of PETN per liter of blood. Significant radioactivity was detected within 15 min. of ingestion of both drug doses. Peak blood levels occurred from 4 to 8 hr., with 60% higher levels after the 40-mg. dose. At 2 hr. postadministration, the blood content of administered ^{14}C accounted for only 4.4 and 3.0% of the 20- and 40-mg. doses, respectively, although approximately 50% of the doses was distributed in the body.

The results obtained by assaying the blood for PETN and its metabolites are also shown in Fig. 4. PETN and PE trinitrate were not detected in the blood. The major metabolite present was PE, with lower levels of PE mononitrate and PE dinitrate. The blood level of these esters was not greatly different for the two doses, and the higher levels of drug-^{14}C observed with the larger dose was accounted for primarily by PE.

DISCUSSION

The absorption, biotransformation, and excretion of oral doses of 20 and 40 mg. of PETN were qualitatively similar but with notable quantitative differences of clinical interest. Comparative results with the two doses indicated that the absorption of PETN from the intestinal lumen and the subsequent deesterification rate were dose related.

The use of commercial-type tablets of PETN led to more extensive absorption than resulted from the administration of encapsulated ^{14}C-PETN (6). This finding emphasizes the advisability of performing human metabolic studies with the clinically used dosage form; but even from this dosage form, relatively large quantities of PETN and its metabolite, PE, were found in the feces. This observation inferred either enterohepatic circulation followed by partial excretion of drug and metabolites in the stool or incomplete drug absorption with some intestinal or fecal deesterification. The latter interpretation is favored, because recirculation of PETN would be expected to result in its reduction and hydrolysis, especially by the blood (7) and liver (8); moreover, PE is excreted principally by the renal route (7). Furthermore, PETN is highly insoluble in water or lipid solvents (9), and the appreciable delay of 1–3 hr. for body distribution and equilibration of its metabolites, as determined by their pharmacokinetics (Table I), suggests a slow

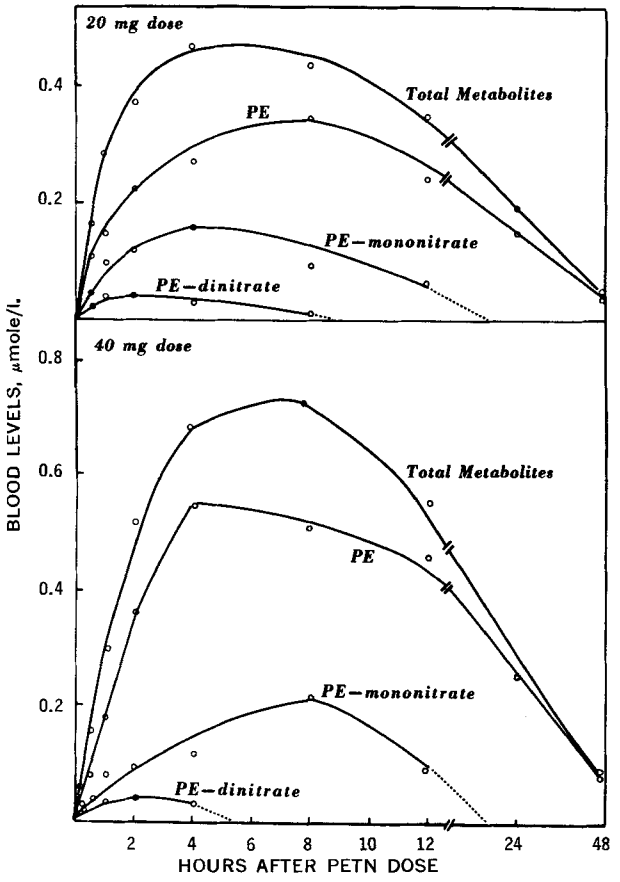

Figure 4—*Mean blood levels of total radioactivity and of drug metabolites after 20- and 40-mg. doses of* ^{14}*C-PETN.*

rate of drug absorption. Therefore, it is thought that 60% (12 mg.) of the 20-mg. dose and 50% (20 mg.) of the 40-mg. dose were absorbed, with the remainder excreted in the feces. These values indicate that the absorption process was not saturated and higher doses would result in correspondingly greater absorption.

As in animal species (10–12), PETN is deesterified extensively in man. The absence of PETN and PE trinitrate in the blood and urine suggests that these compounds are degraded more readily than are PE dinitrate and PE mononitrate, a view supported by observations made earlier *in vitro* (7). However, since the blood content of metabolites did not account for more than a few percent of the body content of drug or metabolites, it is also possible that tissue cells contained substantial amounts of the higher esters which were degraded gradually with the release of metabolites into the circulation.

PE mononitrate and PE were virtually the sole metabolites excreted in the urine. The kinetics of their renal excretion indicate that the absorption, body distribution, and equilibration of PETN, and possibly metabolism to PE mononitrate and PE, were complete essentially within 3 hr. after administration (Table I). At this time, which approximated peak blood levels of drug radioactivity, the body pools for these two metabolites closely accounted for the amount of drug absorbed and excreted into the urine as PE mononitrate and PE (Fig. 3). Perhaps the most significant observation, however, was the contrast between the amounts of PE mononitrate and PE excreted into the urine from the 40- and 20-mg. doses (Fig. 2). The amount of PE, the end metabolite, was nearly the same for the two doses, but the amount of PE mononitrate for the high dose was greater than twice that of the lower dose. This finding demonstrates that, in man, there is a limiting rate of deesterification of PE mononitrate. This limiting rate is reflected also by the apparent decrease of the renal elimination rate of PE mononitrate for the 40-mg. dose *versus* the 20-mg. dose (8.6% hr.$^{-1}$ *versus* 11.1% hr.$^{-1}$), suggesting a decreased rate of metabolism to PE mononitrate. These findings indicate that an accumulation in the body of PE mononitrate, or possibly its precursors, may occur with increasing PETN dosage.

Additionally, if the clinical pharmacology of PETN depends upon the release of nitrite ion, it is unlikely that PE mononitrate contributes significantly because of its slow deesterification. On the other hand, if the PE mononitrate molecule has clinical pharmacological activity, this resistance to degradation may account for the extended duration of action of PETN.

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Intestinal Drug Absorption and Metabolism III: Glycine Conjugation and Accumulation of Benzoic Acid in Rat Intestinal Tissue

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Abstract □ Glycine conjugation of ^{14}C-benzoic acid to ^{14}C-hippuric acid was demonstrated in *in vitro* rat intestinal slices and everted intestinal preparations. Hippuric acid was detected and quantified by two independent analytical techniques, a quantitative TLC method, and a reverse isotope dilution method. Comparison of hippuric acid concentration after 2 hr. in mucosal and serosal fluids when hippuric acid was formed endogenously from benzoic acid, and with mucosal and serosal concentrations when hippuric acid was added exogenously to the mucosal fluid, indicated that hippuric acid is formed in the tissue compartment rather than by bacterial or intestinal enzymes in the mucosal fluid. Hippuric acid is apparently formed enzymatically in the mucosal cell according to a previously proposed cell compartment model and subsequently diffuses to both mucosal and serosal fluids. The net amount of hippuric acid formed under the conditions of the study was small (about 1%) and was found to be due to limited synthesis rather than to extensive hydrolysis. Both exogenously added benzoic acid and hippuric acid accumulated in the intestinal tissue compartment (11.2 and 6.4%, respectively) during transfer from the mucosal fluid.

Keyphrases □ Benzoic acid (carboxyl-^{14}C) absorption, metabolism—intestinal □ Metabolism, transfer, intestinal—everted sac, tissue incubation methods □ Hippuric acid formation—intestinal metabolism, benzoic acid □ Scintillometry—analysis □ Reverse isotope dilution—analysis □ TLC—analysis

The intestinal mucosa has enzyme systems capable of metabolizing many drugs by a variety of metabolic pathways, including sulfate and glucuronide conjugation (1–4). These processes can markedly affect the rate and extent of drug absorption. For example, intestinal glucuronide conjugation was found to affect greatly the rate and extent of transport of free salicylamide in both *in vivo* and *in vitro* preparations (4, 5).

The capability of intestinal tissue to conjugate aryl acids with glycine has not been established. The following studies using benzoic acid as a model substrate were therefore undertaken to determine: (*a*) if glycine conjugation does occur in the intestinal mucosa, and (*b*) if the degree of conjugation of benzoic acid to hippuric acid is sufficient to alter benzoic acid transport and, therefore, require consideration in kinetic studies on mechanisms of absorption. Some interesting observations on *in vitro* accumulation and transport of both benzoic acid (BA) and hippuric acid (HA) are also discussed.

EXPERIMENTAL

Materials—Benzoic acid[1] (carboxyl-^{14}C) had a specific activity of 56.0 mc./mmole and was 99% pure as determined by TLC. Hippuric acid[2] (carboxyl-^{14}C) had a specific activity of 6.66 mc./mmole and was 98% pure as determined by TLC. Periodic analysis of aqueous solutions of the two radioactive chemicals confirmed that degradation in solution was negligible during the period of the experiments. Hippuric acid[3] was recrystallized twice from double-distilled water. All other chemicals and reagents were purchased in the highest grades available and used as such.

Intestinal Transfer and Metabolism Measurements—Transfer and metabolism studies were performed *in vitro* using both an everted sac method, as described by Wilson and Wiseman (6), and a tissue incubation procedure. All experiments were carried out at 37° for a 2-hr. period. In the everted sac method, male Sprague-Dawley[4] rats (175–250 g.) were allowed food and water *ad libitum*. They were anesthetized with ether; the abdomen was opened and the entire small intestine was removed. After discarding the first 5 cm. on both ends, the mucosa was washed well with isotonic saline to remove particulate matter, and the intestine was carefully everted with a glass rod. The everted intestine was then cut into equal segments weighing approximately 4 g., filled with 4.0 ml. of Krebs–Ringer bicarbonate buffer (KRB) at pH 7.4, and tied at both ends. The filled sac was quickly transferred to a 50-ml. conical flask containing 25.0 ml. of a 10^{-2} m*M* solution of either ^{14}C-BA or ^{14}C-HA in KRB. Glycine (1 m*M*), adenosine triphosphate (ATP) (8×10^{-1} m*M*), and cysteine (5×10^{-1} m*M*) were also added, and the mucosal solutions were continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Controls were prepared in a similar manner except for the addition of tissue.

For experiments involving HA transfer (exogenous) from the mucosal side to the serosal side, aliquots of mucosal, serosal, and control sample fluids were assayed after 2 hr. for HA. For experiments involving BA transfer from the mucosal side to the serosal side and HA formation (endogenous) by the everted intestine, aliquots of mucosal, serosal, and control sample fluids were assayed after 2 hr. for both BA and HA. In addition, sections of the everted intestine were assayed for total drug at the end of the 2-hr. incubation period.

In a limited number of trials, the tissue incubation procedure was also used. Sections of everted intestine, 1–2 cm. in length, were randomly selected and placed into 4.0 ml. of KRB containing a 2.5 m*M* solution of ^{14}C-BA. Glycine (10 m*M*), ATP (10 m*M*), and cysteine (5 m*M*) were also added, and the incubation fluid was continually bubbled with the oxygen–carbon dioxide mixture. After incubation, the entire tissue incubation fluid was assayed for HA.

Metabolism by Intestinal Lumen Contents—To determine the amount metabolized by enzymes in lumen fluid, the entire lumen washings (about 5 ml.) containing fluid and fecal debris were collected and filtered through a Whatman No. 1 filter paper; 4 ml. of filtered fluid was incubated with 6 ml. of KRB containing ^{14}C-BA (10^{-2} m*M*). Glycine (1 m*M*), ATP (8×10^{-1} m*M*), and cysteine (5×10^{-1} m*M*) were also added, and the incubation fluid was flushed with the oxygen–carbon dioxide mixture. After incubation, an aliquot of the incubation fluid was assayed for HA.

Assay Procedures—Quantitative assays for ^{14}C were performed by liquid scintillation spectrometry[5] using Snyder's dioxane scintillation solution.[6] External standardization was used for quench corrections.

In transfer studies of BA and HA across the everted intestine, total radioactivity in mucosal and serosal samples was related through the specific activity of the compounds used to the amount or concentration of BA or HA present. Tissue sections were analyzed

[1] Amersham/Searle Corp., Des Plaines, Ill.
[2] New England Nuclear, Boston, Mass.
[3] Eastman Organic Chemicals, Rochester, N. Y.
[4] Blue Spruce Farms, Altamont, N. Y.
[5] Packard Tri-Carb model 3320, Packard Instrument Co., Inc., Downers Grove, Ill.
[6] Snyder's dioxane scintillation solution consists of 0.3 g. POPOP, 7.0 g. PPO, and 100 g. naphthalene in 1 l. of dioxane. To this, 200 ml. distilled water is added.

Table I—Percent HA Formed in Three *In Vitro* Preparations as Determined by Two Analytical Procedures

Preparation[a]	Percent HA Formed/Gram Dry Tissue[b] TLC Assay	Reverse Isotope Dilution Assay
Everted sac		
Mucosal solution (10^{-2})	0.630 ± 0.145 (9)	0.576 (0.655, 0.496)
Serosal solution (0)	0.760 ± 0.134 (9)	—
Tissue incubation (2.5)	1.22 ± 0.385 (3)	1.14 ± 0.367 (3)
Lumen contents (10^{-2}) incubation[c]	0.0443 ± 0.0039 (3)	—

[a] The initial BA concentration (m*M*) is given in parenthesis. [b] Calculated as the amount of HA formed in 2 hr./final total amount of BA and HA and normalized to unit gram dry weight. The number of experiments is given in parenthesis following the standard error of the mean. [c] Lumen contents were obtained from sections of an intestine having a dry weight of 1 g.

for total radioactivity after first dissolving aliquots of tissue into soluene-100.[7]

In studies involving HA formation *in vitro* from BA, two assay procedures were used to measure the amount of ^{14}C-HA formed. The first of these, the quantitative TLC method, involved a slight modification of the extraction procedure developed by Chantrenne (7). After extraction of HA at low pH, the organic solvent [chloroform–*n*-butanol (5:1)] was evaporated to dryness, the residue taken up in hot ethanol, and the ethanol evaporated to approximately 0.5 ml. Then 200 μl. of this solution was spotted on 20 × 20-cm. chromatography plates coated with 250 μ of silica gel HF_{254}.[8] The plates were developed in an ascending technique using the system ether (water saturated)–formic acid (90%)–methanol (98:1:1). This system (8) was chosen because BA (R_f 0.87) and HA (R_f 0.34) were widely separated, while residual amounts of protein remained near the origin. Hippuric acid, easily identified as a dark spot on the fluorescent background when the developed plate was exposed to short UV light, was scraped quantitatively into 20 ml. of the counting solution, and the vial was agitated prior to counting. Controls were treated in an identical manner, and the net ^{14}C-HA formed *in vitro* was expressed as the difference between the experimental and control values. The total recovery of HA by this procedure was 81.3 ± 4.0%.

The second assay procedure, used to confirm results obtained from the TLC procedure, was a reverse isotope dilution technique (9). A known quantity (approximately 200 mg.) of unlabeled HA was dissolved with heat in 10.0 ml. of incubation fluid containing both ^{14}C-BA and ^{14}C-HA. After determining the total amount of radioactive materials in the solution, the solution was then cooled to produce crystals of HA. The crystals were recovered by suction filtration, air dried, and weighed, and an aliquot was counted. Recrystallization was repeated four or five times, or until a constant specific activity of the purified hippuric acid was observed. From the data available, the amount of ^{14}C-HA in the incubation fluid was calculated.

Data Analysis—The significance of differences was determined by Student's *t* distribution. Any value of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Glycine Conjugation by Intestinal Preparations—Evidence that BA is conjugated with glycine to form HA was clearly shown in three different *in vitro* preparations. The percentage of HA formed following 2 hr. of incubation of BA in each of the intestinal preparations is shown in Table I. In all preparations, the percentage of HA formed was quite low. Because the net amounts of HA formed were so small, some difficulties were encountered in using the TLC assay, which required special considerations. To minimize loss of the small amount of ^{14}C-HA during preparation of the sample, it is necessary to add cold carrier HA to each sample. However, as shown in Table II, radioactivity at the HA band from control samples containing ^{14}C-BA and cold carrier HA but no ^{14}C-HA are much greater than the background radioactivity at the HA band obtained when only ^{14}C-BA without cold carrier HA is spotted. The reason for this anomalous effect is thought to be due to complexation of ^{14}C-BA with the cold carrier HA used in the assay. These higher counts from control samples, if not properly accounted for, would indicate the presence of ^{14}C-HA when none is actually present. HA radioactivity from the tissue experiments was always significantly greater than the values obtained from the control samples.

It was necessary, therefore, to run a control sample on each chromatogram and use the differences between the experimental and control samples as a quantitative measure of net ^{14}C-HA formed by the tissue.

To compare the values obtained from the TLC assay with another independent assay method, the more tedious but quite specific reverse isotope dilution technique was used. A representative example of the recovery of ^{14}C-HA utilizing this method is illustrated in Fig. 1. Excellent correlation between the two analytical methods (Table I) validates the values obtained by the TLC method and confirms the presence of ^{14}C-HA following incubation with ^{14}C-BA.

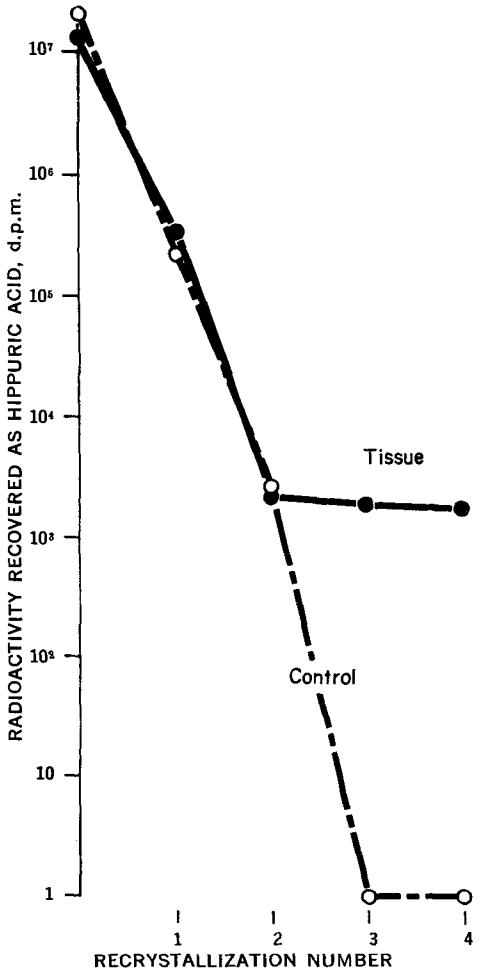


Figure 1—*Demonstration of ^{14}C-HA by isotope dilution technique. The amount of radioactivity after successive recrystallizations of HA initially dissolved in either incubation or control solutions. Key: ●, incubation fluid; and ○, control solution.*

[7] Packard Instrument Co., Inc., Downers Grove, Ill.
[8] E. Merck AG., distributed by Brinkmann Instruments Inc., Westbury, N. Y.

Table II—Recovery of Radioactive Materials at R_f 0.34[a] from the Thin-Layer Plate

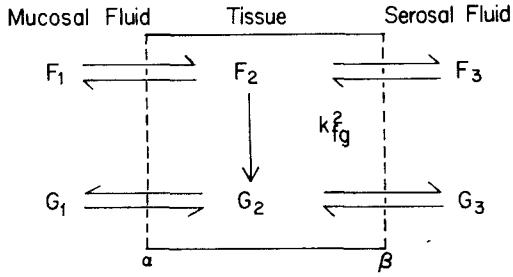
Starting Material	Disintegrations per Minute Recovered × $10^{-3} \pm SE$
^{14}C-BA[b]	0.082 ± 0.0060 (4)[c]
^{14}C-BA + cold carrier HA (before incubation)	0.62 ± 0.090 (6)
^{14}C-BA + cold carrier HA (after incubation	5.72 ± 0.84 (16)

[a] Corresponding to the region where HA is found. [b] Spotted directly as supplied by manufacturer. [c] Number of experiments shown in parenthesis.

Although the tissue incubation procedure appeared to give slightly higher amounts of metabolite than the everted sac preparation, the higher initial concentration of BA used in the tissue incubation procedure precludes direct comparison of the metabolic capacity of these two preparations. The lumen content incubation produced less than one tenth the amount of HA that was produced by the everted sac preparation when both procedures were run under identical incubation conditions. The significance of this finding relative to the site of metabolism is discussed later.

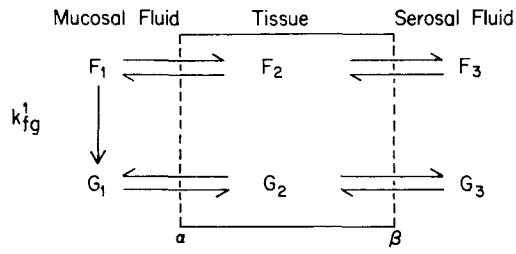
Evidence for Glycine Conjugation by Mucosal Tissue—The presence of ^{14}C-HA following incubation of ^{14}C-BA provided unequivocal evidence for glycine conjugation. Since it is well known that bacterial enzymes can also metabolize drugs (10), two types of experiments were carried out to differentiate between HA formation by the intestinal mucosal cell and by bacterial flora of the intestinal lumen.

These two possibilities can be considered from the previously proposed (4) compartment model which assumes that free drug (F) is conjugated to metabolite (G) in the intestinal mucosal cell (Model I),



Model I

or the alternate case where free drug is converted to metabolite in the lumen by bacterial or other enzymes in the mucosal fluid (Model II), where F_1, F_2, and F_3 are the amounts of free drug



Model II

(BA) in the mucosal fluid, tissue, and serosal fluid, respectively; and G_1, G_2, and G_3 are the amounts of metabolite (HA) in the mucosal fluid, tissue, and serosal fluid, respectively. The rate constants for metabolism in the tissue and in the lumen fluid are represented by $k^2_{f\alpha}$ and $k^1_{f\alpha}$, respectively. The symbols α and β represent the apical (mucosal) and basal (serosal) rate-limiting barriers, as previously defined (4).

The possibility of conjugation in the mucosal fluid (Model II) was evaluated by incubation of the lumen contents with ^{14}C-BA. The entire lumen contents of the rat intestine were collected and filtered with filter paper of sufficient porosity to allow passage of bacteria but exclusion of most cellular and fecal debris. The contents were incubated with ^{14}C-BA under conditions identical to those used for the everted sac studies. The very small amount of HA formed by lumen fluid (0.044%) would, at most, account for only one-tenth the amount of HA formed when tissue is present (Table I).

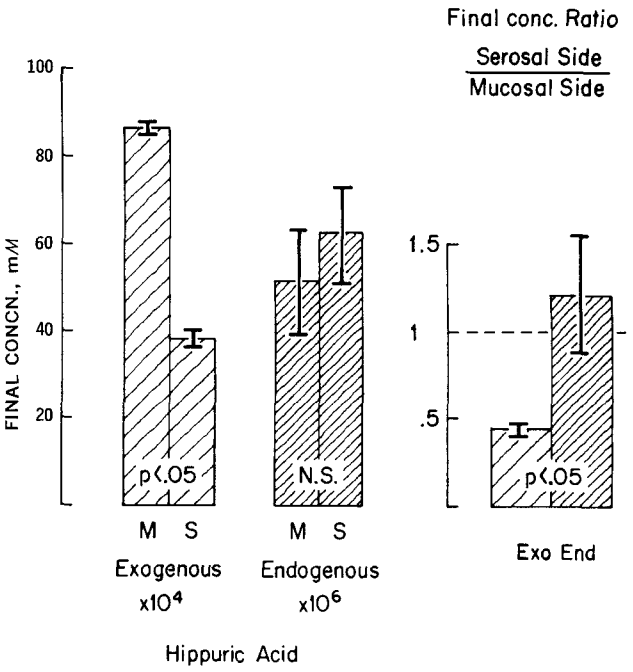


Figure 2—*Comparison of mean final (2 hr.) mucosal and serosal concentrations of HA formed endogenously from BA (8 experiments) or added exogenously to the mucosal fluid (5 experiments) and the corresponding mean of the individual serosal-to-mucosal concentration ratios. Bars indicate standard errors of the mean. Key: M, mucosal solution; and S, serosal solution.*

This study would not rule out HA formation in the lumen compartment by microorganisms that are known to adhere to the villi and microvilli (11). A second experiment was therefore performed to examine this possibility. Exogenous ^{14}C-HA was introduced directly into the mucosal fluid. After 2 hr., the concentrations of ^{14}C-HA in mucosal fluid (C_1), tissue (C_2), and serosal fluid (C_3) were compared to the values obtained when ^{14}C-HA was formed endogenously. If Model II was correct, the observed final concentration gradients of HA should be the same when HA is formed endogenously in the mucosal fluid after incubation with BA as when exogenous HA is added directly to the mucosal fluid—*viz.*, $C_1 \geqslant C_2 \geqslant C_3$.

Figure 2 gives a comparison of the final mucosal[9] and serosal concentrations and serosal-to-mucosal concentration ratios of HA when formed endogenously from BA and when added exogenously to the mucosal fluid. When HA is formed endogenously, the final mucosal and serosal concentrations are not significantly different after 2 hr. incubation. However, when HA (exogenous) is placed initially in the mucosal fluid and allowed to diffuse across the intestine, the final serosal concentration is significantly less than the final mucosal concentration after 2 hr. incubation. The final concentration ratio of HA in the serosal fluid to HA in the mucosal fluid after 2 hr. showed a value of 0.442 for exogenously added HA, which was statistically less than the value of 1.22 for HA formed endogenously after 2 hr. The ratio of 1.22 is not significantly different from unity. It is clear that HA, formed endogenously, shows a different diffusion pattern from HA added exogenously to the mucosal fluid. The observed results are consistent with Model I in which HA is formed in the mucosal cell and diffuses bidirectionally to the mucosal and the serosal fluids, as has been also observed with the glucuronide (5) and sulfate (12) metabolites of salicylamide in *in vitro* rabbit everted intestinal sacs.

Evidence for Limited Glycine Conjugate Hydrolysis—The low net amount of HA formed appears to be due to a low

[9] The word *final* refers to the last sample taken at 2 hr., beyond which the integrity of the tissue becomes questionable. It does not imply that distribution equilibrium within the system has necessarily been achieved.

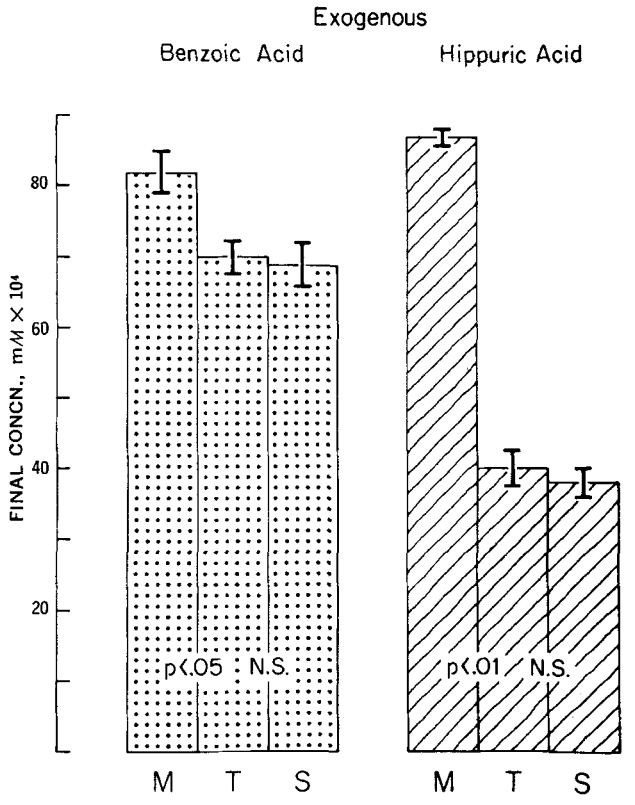


Figure 3—*Comparison of final (2 hr.) concentrations of BA and HA in mucosal fluid (M), tissue (T), and serosal fluid (S) after exogenous drug is placed in mucosal fluid at an initial concentration of 10^{-2} mM.*

synthesis rate rather than extensive hydrolysis after formation, since an insignificant rate of hippuric acid hydrolysis occurs. This was confirmed by comparing total radioactivity in the mucosal fluid, tissue, and the serosal fluid, following addition of exogenous ^{14}C-HA, with the radioactivity determined after organic extraction of an acidified aqueous sample of each compartment. Since exogenous ^{14}C-HA is labeled on the glycine carboxyl moiety, hydrolysis would yield ^{14}C-glycine which, in an acidified solution, would not be extracted into the organic solvent. Appreciable hydrolysis of ^{14}C-HA would, therefore, result in a low fraction of organic extractable ^{14}C. The recovery determined by total radioactivity and organic extractable radioactivity were the same, indicating negligible hydrolysis of HA.

Intestinal Transfer and Accumulation of Exogenous BA and HA—Formation of HA in the intestinal tissue is of biochemical significance in establishing the presence of the glycine conjugating system in the intestinal mucosal tissue; the small amount formed, however, is not significant from a kinetic viewpoint. It will not alter or contribute to the material balance of the system and may be neglected in the kinetics of *in vitro* transfer of BA.

Since neither BA nor HA was appreciably metabolized, it was possible to compare directly the transfer characteristics of ^{14}C-BA and ^{14}C-HA when both were added directly to the mucosal fluid. Under these conditions, both weak acids follow Model I, where k_{fs}^{2} is very small for the conjugation of BA and negligible for the hydrolysis of HA. The amounts of both ^{14}C-BA and ^{14}C-HA in mucosal fluid, tissue, and serosal fluid, 2 hr. after being added exogenously, are shown in Table III.

Accumulation of both BA and HA in the intestinal tissue was quite significant, accounting for 11.2 and 6.40%, respectively, of the initial amount added. Under the conditions of this study, accumulation of these compounds in the tissue must be accounted for if mass balance is to be achieved. The intestinal tissue must be considered as a compartment if either metabolism or accumulation of drug occurs within the tissue at a measurable rate (4, 13). Although metabolism within the tissue is negligible, significant accumulation of drug in the intestinal tissue requires consideration of the tissue as a compartment and a three-compartment model is appropriate.

The data from Table III, based on the compartment volumes indicated, were transformed to concentrations so that the final concentration gradients could be compared as shown in Fig. 3. Comparison of the higher mucosal concentrations to the almost equal concentrations of tissue and serosal fluid demonstates ($p < 0.05$ for both acids) that a gradient between mucosal fluid and tissue, but not between tissue and serosal fluid, exists after 2 hr. The significance of these findings with respect to the rate-limiting steps in aryl acid transfer across the intestine is presently under investigation.

Table III—Comparison of the Amount of Exogenous BA and Exogenous HA in Mucosal, Tissue, and Serosal Compartments

Compartment	Volume, ml.	Percent of Initial Amount ± *SE* BA	HA
Mucosal	25	82.0 ± 2.71 (9)[a]	86.6 ± 0.85 (5)
Tissue	4[b]	11.2 ± 0.22 (9)	6.40 ± 0.36 (5)
Serosal	4	11.0 ± 0.53 (9)	6.12 ± 0.30 (5)
Totals		104.1	99.1

[a] Number of experiments given in parenthesis. [b] Tissue volume assumes a tissue density of 1.0.

SUMMARY AND CONCLUSION

The present study unequivocally established that glycine conjugation can occur during the transfer of BA across the rat everted intestine. The extent of biotransformation was quite small, requiring sensitive tracer techniques for detection. The extent of conjugation may, of course, be greater under other experimental conditions or in other species. The low net amount of conjugate formed is a result of a low synthesis rate rather than to subsequent hydrolysis of the conjugate.

Kinetic evidence indicates that conjugation occurs predominantly in the intestinal tissue, presumably by a cellular enzymatic process. Exit from the mucosal cell is bidirectional, as has also been previously observed with glucuronide and sulfate conjugates.

Although the extent of metabolism is not sufficient to require the intestinal tissue to be considered as a separate compartment, there is significant accumulation of both BA and exogenous HA in the tissue to require the consideration of the intestine as a separate compartment.

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Structural Studies on Molecular Complexes V: Crystal Structures of Sulfathiazole–Sulfanilamide and Sulfathiazole–Theophylline Complexes

ELI SHEFTER and PHYLLIS SACKMAN

Abstract □ The crystal structures of two sulfathiazole complexes, one involving theophylline and the other sulfanilamide, were determined by X-ray diffraction methods. The complexes are 1:1 adducts held together by hydrogen bonding. The hydrogen-bonding schemes in the two complexes are quite different. In the theophylline complex the aromatic amino group of sulfathiazole is involved in the principal intermolecular hydrogen bonds; whereas in the sulfanilamide complex, one of the sulfathiazole oxygens is the dominant participant. Sulfathiazole is present in both structures in the imido-configuration rather than the amido-form.

Keyphrases □ Molecular complexes—crystal structures □ Crystal structures—sulfathiazole–sulfanilamide, sulfathiazole–theophylline complexes □ Complexes, sulfathiazole with sulfanilamide and theophylline—crystal structure □ X-ray diffraction—analysis

The binding of sulfonamides to serum proteins has been extensively studied (1). The affinity of a sulfonamide for the serum proteins is an exceedingly important factor for dosage formulation, since the unbound material is responsible for the bacteriostatic activity (2). The principal protein nemesis in this regard is serum albumin. There appears to be one primary binding site in bovine serum albumin for sulfonamides (3); this site interacts with the *p*-aminobenzene sulfonamide moiety. Details of this interaction on a molecular level have yet to be elucidated.

Sulfonamides have also been reported to form association complexes with a variety of smaller molecules. Higuchi and Lach (4) described the interaction of sulfathiazole with a xanthine. A crystalline sulfathiazole–sulfanilamide complex was isolated and studied by Sekiguchi and Ito (5–7). Since the molecular basis(es) for the complex formation in such simple systems could be related to the principal driving force(s) for the sulfa–protein interactions and possibly for the antibacterial activity of these molecules which is a result of their interaction with a "bioreceptor," structural studies on some of these complexes were initiated.

The first sulfonamide complexes for which crystallographic studies were undertaken were sulfathiazole–theophylline (1:1) and sulfathiazole–sulfanilamide (1:1). These data are reported here.

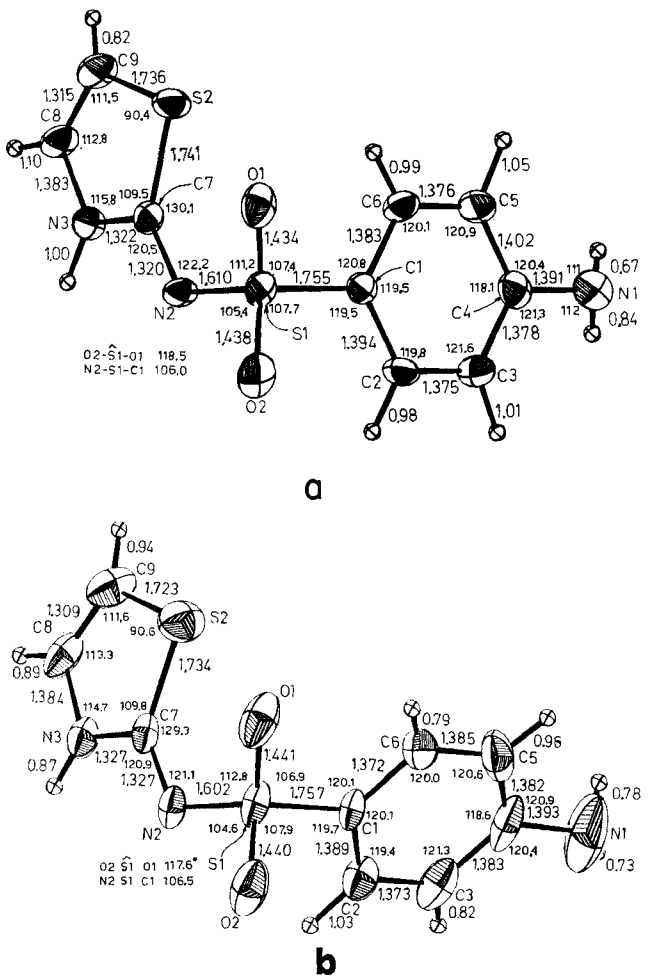


Figure 1—*Intramolecular bond lengths and angles of sulfathiazole found in the theophylline (a) and sulfanilamide (b) complexes. The thermal ellipses for the nonhydrogen atoms are drawn at the 50% probability level in this and all subsequent figures.*

EXPERIMENTAL

Colorless monoclinic prisms of the two complexes were obtained by slow evaporation of alcoholic solutions containing the respective chemical components. The following crystallographic data were measured for these crystals:

Sulfathiazole–Theophylline (1:1)		Sulfathiazole–Sulfanilamide (1:1)
11.020 (0.001) Å	*a*	9.135 (0.001) Å
9.010 (0.001) Å	*b*	5.379 (0.001) Å
20.186 (0.002) Å	*c*	36.633 (0.004) Å
113.68 (0.01)°	*β*	91.17 (0.02)°
1.57 g. cm.$^{-3}$	Density (measured by flotation)	1.47 g. cm.$^{-3}$
1.575 g. cm.$^{-3}$	Density (calculated)	1.431 g. cm.$^{-3}$
4	Z	4
$P2_1/c$	Space group	$P2_1/c$

Data Collection and Structure Determination—Intensity data on the complexes were collected by the stationary crystal-stationary counter technique on a General Electric single crystal orienter, using Cu Kα radiation. Monochromatization was approximately achieved by use of balanced Ross filters (Ni *versus* Co). The crystals, mounted for data collection, were 0.2 × 0.2 × 0.4 mm. for the sulfathiazole–theophylline complex and 0.1 × 0.2 × 0.3 mm. for the sulfanilamide–sulfathiazole complex. Of the 3086 independent reflections measured for the sulfanilamide-containing complex

Table I—Positional and Thermal Parameters[a] with Their Respective Standard Deviations (in Parentheses) × 10^4 for Nonhydrogen Atoms of Both Complexes

Atom	x	y	z	b_{11}	b_{22}	b_{33}	b_{12}	b_{13}	b_{23}
				Sulfathiazole–Theophylline Sulfathiazole					
S(1)	310(1)	2797(1)	1365(1)	65(1)	31(1)	16.1(.3)	−2(2)	20(1)	7(1)
S(2)	245(1)	6281(1)	1856(1)	108(2)	50(1)	14.6(.3)	9(2)	34(1)	−7(1)
O(1)	−245(3)	3151(4)	1878(2)	77(4)	85(5)	22 (7)	30(7)	41(3)	34(4)
O(2)	−212(4)	1544(4)	896(2)	83(4)	32(4)	29 (1)	−26(6)	4(4)	−8(4)
N(1)	6112(4)	1978(5)	2925(2)	78(5)	111(7)	19 (1)	21(9)	22(4)	−9(5)
N(2)	190(4)	4179(4)	837(2)	87(4)	32(4)	14 (1)	0(7)	32(4)	−3(4)
N(3)	230(4)	6672(4)	621(2)	97(5)	32(4)	13 (1)	4(7)	29(4)	10(4)
C(1)	2013(5)	2499(5)	1853(2)	68(5)	33(5)	12 (1)	1(8)	16(4)	3(4)
C(2)	2701(5)	1594(5)	1562(2)	84(5)	56(6)	12 (1)	4(9)	22(4)	−15(5)
C(3)	4051(5)	1449(6)	1915(3)	73(5)	76(6)	16 (1)	24(9)	36(5)	−6(5)
C(4)	4753(5)	2180(5)	2555(3)	68(5)	59(6)	15 (1)	0(9)	23(4)	12(5)
C(5)	4045(5)	3043(5)	2856(3)	89(6)	45(6)	17 (2)	−7(9)	15(5)	−17(5)
C(6)	2694(5)	3211(5)	2505(3)	74(5)	46(6)	18 (1)	9(9)	32(5)	−9(5)
C(7)	218(4)	5568(5)	1049(2)	58(5)	46(5)	14 (1)	8(8)	14(4)	1(4)
C(8)	249(5)	8083(5)	894(3)	99(6)	35(5)	20 (2)	2(9)	39(5)	−5(5)
C(9)	281(6)	8075(5)	1552(3)	119(7)	32(6)	23 (2)	2(10)	36(6)	−11(5)
				Theophylline					
N(1)	6727(4)	1315(4)	452(2)	63(4)	45(5)	20 (2)	12(7)	79(4)	1(4)
C(2)	6729(5)	2084(6)	1053(3)	61(5)	74(7)	21 (2)	10(9)	28(5)	11(5)
N(3)	6703(4)	3603(5)	1016(2)	90(5)	72(5)	16 (1)	12(8)	31(4)	−16(4)
C(4)	6710(5)	4292(5)	416(3)	64(5)	51(6)	19 (2)	6(8)	18(4)	7(5)
C(5)	6717(5)	3520(6)	−162(3)	70(5)	73(6)	15 (1)	−2(9)	21(4)	4(5)
C(6)	6756(5)	1932(6)	−171(3)	64(5)	73(6)	15 (1)	13(9)	6(4)	−2(5)
N(7)	6719(5)	4568(5)	−656(2)	102(5)	103(7)	21 (1)	12(10)	41(5)	28(5)
C(8)	6702(7)	5865(7)	−359(3)	130(8)	78(7)	31 (2)	−1(13)	26(7)	36(7)
N(9)	6702(5)	5785(5)	297(3)	120(6)	64(6)	26 (2)	8(9)	34(5)	6(5)
O(10)	6813(4)	1159(4)	−660(2)	112(5)	115(6)	16 (1)	36(8)	34(4)	−25(4)
C(11)	6801(6)	−320(6)	514(3)	121(7)	51(7)	33 (2)	11(11)	38(6)	11(6)
O(12)	6740(4)	1431(4)	1581(2)	116(5)	108(5)	22 (1)	30(8)	65(4)	32(4)
C(13)	6773(7)	4476(6)	1643(3)	143(8)	105(8)	23 (2)	30(13)	50(6)	−34(6)
				Sulfathiazole–Sulfanilamide Sulfathiazole					
S(1)	−1527(1)	4479(2)	4509.5(.2)	121(1)	209(3)	2.7 (.1)	−30(3)	−6.9(.4)	−6(1)
S(2)	−3737(1)	2867(2)	5154.4(.3)	119(1)	323(4)	7.2 (.1)	121(4)	13(1)	23(1)
O(2)	−164(3)	5219(6)	4352(1)	147(4)	429(12)	4.5 (.2)	−225(12)	−5(1)	16(3)
O(1)	−2452(4)	6407(5)	4651(1)	222(5)	217(9)	4.4 (.2)	100(11)	−6(2)	−9(2)
N(1)	−4880(6)	−660(11)	3334(1)	247(8)	868(29)	6.4 (.3)	−571(27)	−27(3)	5(5)
N(2)	−1072(3)	2453(6)	4812(1)	97(4)	276(11)	2.9 (.2)	1(10)	−7(1)	11(2)
N(3)	−1685(3)	−133(5)	5287(1)	103(4)	237(10)	3.2 (.2)	17(10)	−3(1)	7(2)
C(1)	−2558(4)	2899(6)	4173(1)	93(4)	207(11)	2.4 (.2)	−13(11)	−6(1)	9(2)
C(2)	−2003(4)	753(7)	4017(1)	119(5)	225(12)	4.1 (.2)	15(13)	−1(2)	3(3)
C(3)	−2791(5)	418(7)	3744(1)	168(6)	260(13)	4.1 (.2)	−95(15)	3(2)	−9(3)
C(4)	−4128(5)	497(8)	3622(1)	151(6)	441(18)	2.6 (.2)	−284(17)	−6(2)	10(3)
C(5)	−4659(4)	2647(10)	3777(1)	88(5)	630(23)	5.5 (.3)	−86(17)	−11(2)	57(4)
C(6)	−3876(4)	3843(8)	4052(1)	96(4)	336(14)	4.6 (.2)	64(13)	−1(2)	14(3)
C(7)	−2010(4)	1723(6)	5062(1)	88(4)	216(11)	2.7 (.2)	0(11)	−7(1)	4(2)
C(9)	−3947(5)	657(8)	5490(1)	135(5)	320(15)	6.2 (.3)	11(15)	22(2)	5(4)
C(8)	−2779(5)	−736(7)	5528(1)	149(5)	265(3)	3.3 (.2)	−4(14)	9(2)	11(3)
				Sulfanilamide					
S	1461(1)	4936(2)	3371.9(.2)	82(1)	298(3)	4.4 (.1)	−10(3)	−7.8(.4)	−7(1)
O(1)	2659(3)	5710(6)	3148(1)	79(3)	492(13)	5.9 (.2)	−75(10)	−2(1)	−23(3)
O(2)	1419(3)	2423(5)	3494(1)	130(4)	297(11)	9.3 (.3)	48(10)	−26(2)	10(3)
N(1)	−3841(4)	6795(7)	2460(1)	116(4)	456(16)	6.1 (.3)	−17(13)	−16(2)	20(3)
N(2)	1543(4)	6638(7)	3733(1)	135(14)	389(14)	4.0 (.2)	−73(13)	−3(2)	−4(3)
C(1)	−152(4)	5558(6)	3122(1)	83(4)	240(12)	3.5 (.2)	−12(11)	−6(1)	−2(3)
C(2)	−1317(4)	3931(7)	3130(1)	102(4)	250(13)	5.1 (.3)	−55(12)	−4(2)	25(3)
C(3)	−2542(4)	4338(8)	2911(1)	91(4)	341(15)	6.8 (.3)	−99(14)	−9(2)	18(4)
C(4)	−2626(4)	6374(7)	2681(1)	93(4)	319(14)	3.4 (.2)	31(12)	−3(2)	−3(3)
C(5)	−1467(4)	8064(7)	2686(1)	114(5)	269(13)	4.3 (.2)	12(13)	0(2)	17(3)
C(6)	−235(4)	7655(7)	2901(7)	95(4)	240(12)	4.8 (.2)	−60(12)	2(2)	5(3)

[a] Temperature factors expressed in the form $\exp[-(b_{11}h^2 + b_{22}k^2 + b_{33}l^2 + b_{12}hk + b_{13}hl + b_{23}kl)]$.

(*two theta* limit 130°), 2675 had counts significantly larger than their respective backgrounds. For the theophylline complex, 2580 reflections of the 2718 measured (*two theta* limit 120°) had observable intensities. The intensities were converted to structure factor amplitudes ($|\mathbf{F}|$) by applying corrections for Lorentz-polarization effects, $\alpha_1 - \alpha_2$ splitting, and absorption. The structure factors were placed on an absolute scale by Wilson's method.

The two structures were solved by application of the Sayre relationship (8). The procedure described by Long (9) was utilized to determine the phases of 398 normalized structure factors ($|\mathbf{E}|$), with $\mathbf{E}_{hkl} \geq 1.5$ for the sulfanilamide–sulfathiazole complex, and of 270 reflections with $\mathbf{E}_{hkl} \geq 1.65$ for the theophylline complex. The positions of all the nonhydrogen atoms were recognizable in the E-maps (10) computed with these phases.

Table II—Hydrogen Atom Parameters with Standard Deviations (in Parentheses)

Atom	$x \times 10^3$	$y \times 10^3$	$z \times 10^3$	Biso
		Sulfathiazole–Theophylline Sulfathiazole		
H(N1)1	640(8)	254(9)	315(4)	7.1(1.9) Å²
H(N1)2	648(6)	191(7)	263(3)	4.2(1.3)
H(N3)	28(5)	653(5)	14(3)	2.2(1.0)
H(C2)	237(8)	124(9)	106(4)	7.1(1.9)
H(C3)	462(5)	99(6)	164(3)	2.9(1.1)
H(C5)	440(5)	352(6)	338(3)	2.4(1.0)
H(C6)	210(6)	370(7)	270(3)	3.8(1.3)
H(C8)	34(7)	899(9)	55(4)	6.6(1.8)
H(C9)	38(4)	879(5)	182(2)	1.9(1.0)
		Theophylline		
H(N7)	665(6)	426(7)	−111(3)	4.7(1.4)
H(C8)	656(5)	678(6)	−66(3)	3.1(1.1)
H(C11)1	771(7)	−60(9)	71(4)	6.1(1.8)
H(C13)1	748(9)	391(11)	219(5)	9.8(2.6)
H(C13)2	596(7)	428(8)	178(4)	5.8(1.6)
H(C13)3	669(5)	544(6)	152(3)	3.2(1.1)
H(C11)2	640	−60	80	–[a]
H(C11)3	610	−90	10	–[a]
		Sulfathiazole–Sulfanilamide Sulfathiazole		
H(N1)1	−492(7)	194(13)	327(2)	9.9(1.8)
H(N1)2	−568(5)	−47(10)	339(1)	5.9(1.2)
H(N3)	86(5)	94(9)	471(1)	5.0(1.1)
H(C2)	−101(4)	3(8)	409(1)	3.8(0.9)
H(C3)	−242(4)	−169(8)	366(1)	3.7(0.9)
H(C5)	−553(5)	338(9)	367(1)	5.3(1.1)
H(C6)	−420(4)	501(7)	415(1)	3.3(0.8)
H(C9)	−489(5)	413(9)	558(1)	4.3(1.1)
H(C8)	−259(4)	−191(8)	569(1)	3.5(0.8)
		Sulfanilamide		
H(N1)1	−443(4)	549(8)	245(1)	4.0(0.9)
H(N1)2	−374(5)	761(9)	230(1)	4.8(1.0)
H(N2)1	99(4)	619(7)	390(1)	3.0(0.8)
H(N2)2	157(5)	796(9)	366(1)	4.8(1.0)
H(C2)	−129(4)	262(6)	325(1)	2.3(0.7)
H(C3)	−337(6)	343(12)	295(2)	7.8(1.5)
H(C5)	−152(5)	955(9)	257(1)	5.7(1.1)
H(C6)	53(4)	878(8)	288(1)	3.7(0.8)

[a] Position found in difference electron density map.

The structures were refined by least squares, employing a block diagonal approximation. During the final stages of refinement, difference electron density maps enabled the hydrogen atoms to be located. All but two were refined by least squares. The Cruickshank-type weighting schemes (11) used in the final cycles of refinement were

$$\sigma^2(|F|) = 1.44 - 0.089|F| + 0.0044|F|^2 \quad \text{(Eq. 1)}$$

for sulfanilamide–sulfathiazole, and

$$\sigma^2(|F|) = 1.39 - 0.078|F| + 0.0078|F|^2 \quad \text{(Eq. 2)}$$

for theophylline–sulfathiazole, with the unobserved reflections being given zero weight. The final R values (conventional reliability index $R = \Sigma\,||F_o| - |F_c||/\Sigma\,|F_o|$) were 0.068 (0.070 for all data) and 0.054 (0.080 including "unobserved" data) for the theophylline- and sulfanilamide-containing complexes, respectively. The atomic coordinates and thermal parameters for the complexes are presented in Tables I and II.[1]

The X-ray scattering factors used throughout these calculations were taken from the "International Tables for X-ray Crystallography" (12) with the exception of hydrogen. The form factors published by Stewart *et al.* (13) for hydrogen were utilized.

[1] A tabulation of the structure factors can be obtained from either the authors or the Health Sciences Library of the State University of New York at Buffalo.

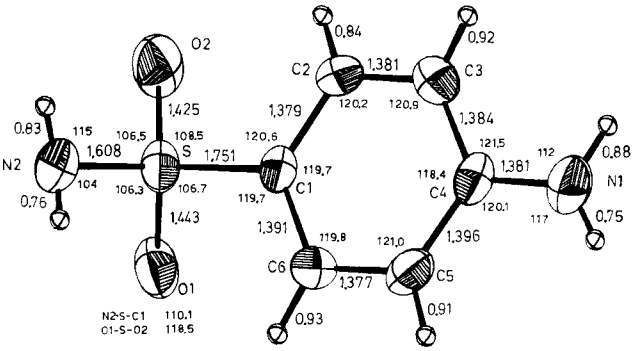


Figure 2—*Bonding parameters observed for sulfanilamide.*

The bond lengths and angles obtained for the molecular entities comprising the two complexes are shown in Figs. 1–3. The uncertainties associated with these parameters are in general about 0.004 Å and 0.2° for bonds and angles involving a sulfur and 0.007 Å and 0.5° for the other nonhydrogen bonds. The errors in the hydrogen positions are such that their bonding parameters are about 10 times as great as those for the nonhydrogen atoms. The high error associated with the hydrogens is not unusual in X-ray structures, especially those containing "heavy" atoms such as sulfur in these structures. For this reason, only those angles involving hydrogens pertinent to the discussion are presented.

DISCUSSION

Sulfathiazole—The sulfathiazole molecules in each complex have similar intramolecular bonding parameters; none of the differences is significant at the $p = 0.001$ level. The bond lengths and angles of the *para*-aminosulfonamide portion of the molecule are in good agreement with these values reported for sulfanilamide (14–17).

The sulfathiazole molecules are present in the imidotautomeric form in both complexes, *i.e.*, the proton is attached to N(3) rather than N(2). The preference of sulfathiazole for the imido-configuration over the amido-form was previously shown by IR studies (18) on this compound. The C(7)—N(3) and C(7)—N(2) bonds have essentially equivalent amounts of double bond character [bond order p is approximately 0.75 when calculated using the formula of Liquori and Vaciago (19)]. Therefore, Formula II would be a better representation of this linkage than I.

Sulfathiazole is capable of attaining a variety of conformation states by rotation about three bonds: C(1)—S(1), S(1)—N(2), and C(7)—N(2). A particular atomic constellation of sulfathiazole can thus be specified by three torsion angles. The three dihedral angles specified are ϕ C(1)—S(1)—N(2)—C(7), ϕ S(1)—N(2)—C(7)—S(2), and either ϕ C(6)—C(1)—S(1)—N(2) or ϕ C(2)—C(1)—S(1)—N(2), the choice being the acute angle. By choosing the smaller of the latter two dihedral angles, the ambiguity resulting

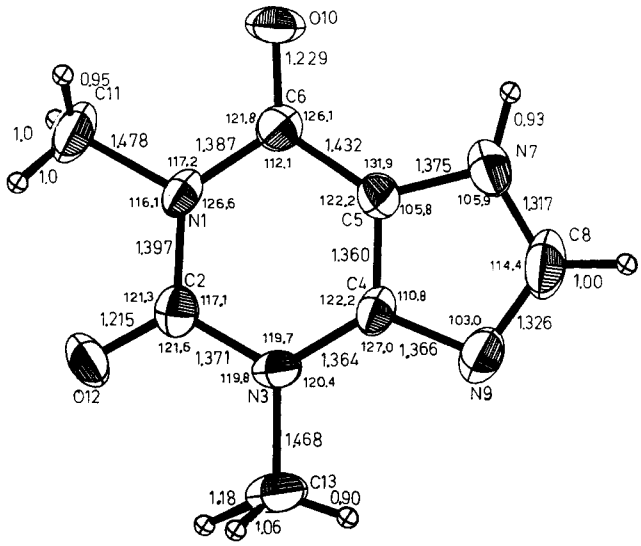
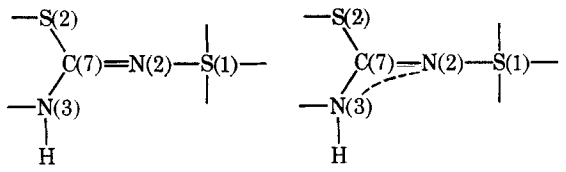


Figure 3—*Bond lengths and angles calculated for theophylline.*

Table III—Conformational Parameters[a]

	Torsion Angles°					
	Sulfathiazole		Sulfanilamide			
	Sulfanilamide Complex	Theophylline Complex	Sulfathiazole Complex	α-Form[b]	β-Form[c]	Hydrate[d]
N(2)—S(1)—C(2)[C(6)]	−52	−87	79	59	71	90
C(1)—S(1)—N(2)—C(7)	−80	−85				
S(1)—N(2)—C(7)—S(2)	−7	−6				

[a] Calculated in the manner prescribed by Klyne and Prelog (24). [b] Reference 15. [c] Reference 16. [d] Reference 14.



I II

from the twofold symmetry of the *p*-aminophenyl residue is resolved. The values obtained for the two sulfathiazole complexes are listed in Table III. The major conformational difference between the sulfathiazoles of the two complexes is the twist of the benzene ring about the C(1)—S(1) bond. Intermolecular packing forces (which are quite different in the two complexes) in all probability influence this conformation. The amide linkage of each sulfathiazole is hydrogen bonded in the same manner; this accounts for the similarity in the other two torsion angles.

The planarity of the benzene and thiazole ring is shown in Table IV. In both complexes, N(1) and S(1) are displaced from the plane of the phenyl ring by small but significant amounts. In the sulfanilamide-containing complex, N(2) is displaced significantly from the thiazole ring; such is not the case in the theophylline complex. These discrepancies are consequences of the intermolecular packing differences.

Sulfanilamide—The intramolecular bond parameters of sulfanilamide in the complexed state correspond quite well with those reported for various crystalline modifications of this molecule (14–17). A comparison of the conformation of sulfanilamide [rotation about the C(1)—S bond] in the various modifications and in the complex can be found in Table III. A molecular model (CPK model) of this compound indicates that there is no highly significant steric hindrance to rotation about this bond, but that torsion angles in the synclinal range (60 ± 30°) should be preferred. The observed angles for sulfanilamide and sulfathiazole all fall in the most favorable range.

Theophylline—In general, the bonding parameters for this molecule in the sulfathiazole–theophylline complex, 5-chlorosalicylic acid–theophylline complex (20), and theophylline monohydrate (21) are similar. The atoms comprising the purine ring in the sulfa complex are essentially coplanar (Table IV), as is the case in theophylline monohydrate. In the 5-chlorosalicylic acid–theophylline complex, "polarization bonding" was implicated as an intermolecular packing force, which resulted in a distinct distortion of the purine ring. Such forces do not appear to be operative in this crystalline complex.

Intermolecular Bonds—Hydrogen bonding appears to be the predominant intermolecular force responsible for the formation of these complexes. The hydrogen bonds in the two structures are illustrated in Figs. 4 and 5.

Although the two hydrogen-bonding schemes are distinctly different, one hydrogen interaction is common to both complexes. This involves the N(3)—H(N3)...N(2) hydrogen bridges between sulfathiazole molecules, which is fairly strong for an N—H...N interaction (22). The centrosymmetric nature of this bond results in a dimeric arrangement of the sulfathiazole molecules.

Theophylline Complex (Fig. 4)—The aromatic amino group [N(1)] is the only functional group of the sulfa that directly participates in the complexation. N(7) of theophylline donates its proton to the sulfa N(1), and the carbonyl oxygen [O(12)] of theophylline accepts an N(1) proton. Although the H(N1)...O(10) length of 2.54 Å is somewhat greater than the accepted maximum for a

Table IV—Displacements from Some Least-Squares Planes[a]

Atoms Comprising Least-Squares Plane	Sulfathiazole–Theophylline			Atoms Comprising Least-Squares Plane	Sulfathiazole–Sulfanilamide		
	Δ, Å	Other Atoms	Δ, Å		Δ, Å	Other Atoms	Δ, Å
	Sulfathiazole					**Sulfathiazole**	
C(1)	−0.012	S(1)	−0.143	C(1)	0.004	S(1)	−0.062
C(2)	0.008	N(1)	0.039	C(2)	−0.002	N(1)	−0.053
C(3)	0.006	O(1)	0.445	C(3)	−0.002	O(2)	−1.280
C(4)	−0.017	O(2)	0.388	C(4)	0.004	O(1)	0.135
C(5)	0.013	N(2)	−1.718	C(5)	−0.003	N(2)	1.103
C(6)	0.001			C(6)	−0.001		
C(7)	0.001	N(2)	0.002	C(7)	−0.012	N(2)	−0.042
S(2)	−0.004	S(1)	0.135	S(2)	0.011	S(1)	−0.232
C(8)	−0.008	H(N3)	0.06	C(8)	0.004	H(N3)	0.03
C(9)	0.008	H(C8)	0.08	C(9)	−0.011	H(C8)	0.06
N(3)	0.004	H(C9)	0.08	N(3)	0.007	H(C9)	−0.19
	Theophylline					**Sulfanilamide**	
N(1)	−0.011	O(10)	0.058	C(1)	0.023	S	−0.090
C(2)	0.006	C(11)	0.048	C(2)	0.034	O(1)	−0.947
N(3)	−0.005	O(12)	0.019	C(3)	0.022	O(2)	−0.449
C(4)	0.001	C(13)	0.084	C(5)	−0.001	N(1)	−0.021
C(5)	−0.008	H(N7)	−0.07	C(6)	0.043	N(2)	1.348
C(6)	0.015	H(C8)	−0.14		0.039		
N(7)	−0.003						
C(8)	−0.002						
N(9)	0.007						

[a] The least-squares planes were calculated according to the method of Schomaker *et al.* (25).

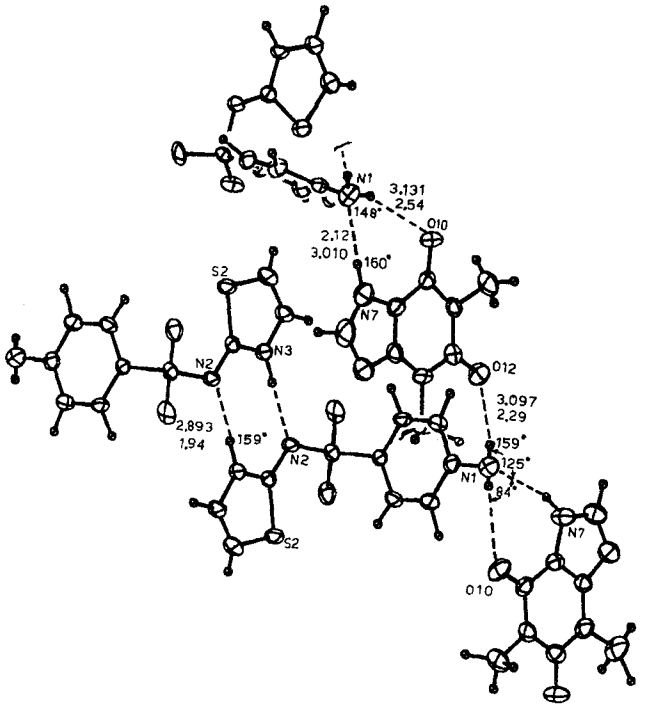


Figure 4—*A general view of the hydrogen bonding (dashed lines) in the theophylline-sulfathiazole complex.*

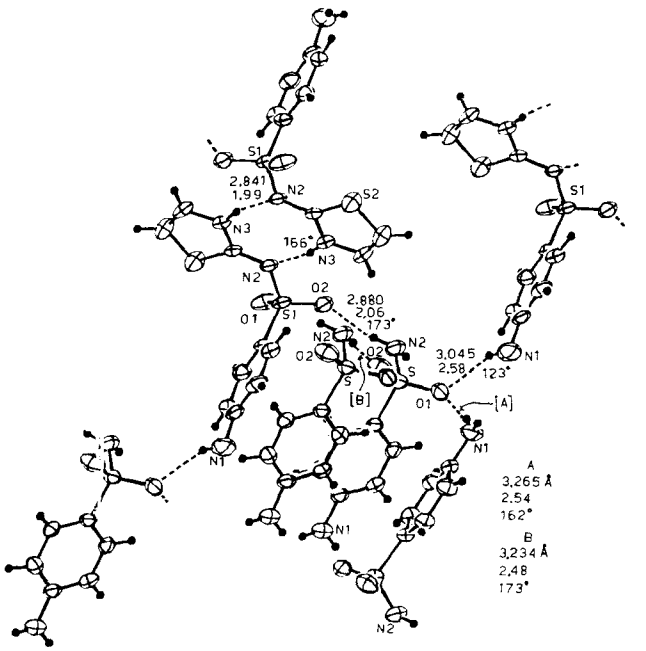


Figure 5—*The proposed hydrogen-bonding scheme (dashed lines) for the crystalline sulfanilamide-sulfathiazole complex, projected down a noncrystallographic axis.*

hydrogen bond [2.4 Å has been proposed as the upper limit for the H to O distance when hydrogen bonding is taking place (23)], the error in the hydrogen position makes it quite conceivable that this is indeed a hydrogen bond and not just a close contact. A consideration of the angles about N(1) and O(10) and the short N(1) — H bond lends credence to the proposed hydrogen-bonding scheme.

The hydrogen bonding about the amino group of sulfathiazole is no doubt responsible to a great extent for the distortion of this functional group from trigonal symmetry. Similar distortions were observed in β-sulfanilamide (16).

Sulfanilamide Complex (Fig. 5)—The relatively high uncertainty in the hydrogen positional parameters puts some doubt on the strength of the proposed hydrogen interactions; but as in the case of the theophylline complex, other molecular parameters were considered in developing the hydrogen-bonding scheme illustrated. The principle intermolecular bond (*i.e.*, greatest strength) between the two molecular entities is the donation of an N(2) hydrogen of sulfanilamide to O(2) of sulfathiazole. Another bridge between the two molecules, but much weaker in strength, involves the acceptance by the sulfanilamide oxygen [H(N1)]. The N to O distance in this hydrogen bond is over 0.16 Å longer than the other interspecies hydrogen bond.

The sulfanilamide molecules are weakly hydrogen bonded to one another through two N—H...O hydrogen bonds. Their N to O lengths (3.234 and 3.265 Å) fall in the upper range of such interactions (22); thus they can be considered extremely weak.

Ito and Sekiguchi (7) proposed a molecular structure for this complex that is quite different from the observed arrangement. The intermolecular bonding found in this crystal structure is consistent with the spectral data used to derive their model.

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Analysis of Binary Mixtures of Pharmaceutical Amines by the Acid Dye Technique

FUMI MATSUI and W. N. FRENCH

Abstract □ A general procedure using the acid dye technique is described for the direct analysis of certain binary mixtures of pharmaceutical amines without prior separation. Benzene (or benzene containing a small amount of isoamyl alcohol) is used to extract selectively the ion-pair formed between the amine and an indicator dye from a suitably buffered aqueous phase. The less-polar amine of the binary mixture is determined using bromcresol purple, while the total of both amines is determined with bromthymol blue. The amount of the more-polar amine is calculated by difference. Specific combinations examined include diphenhydramine–ephedrine, prochlorperazine–amphetamine, promazine–phenmetrazine, promethazine–ephedrine, promethazine–codeine, diphenhydramine–codeine, and diphenylpyraline–codeine. Recoveries for simulated mixtures average 99.76%, with an average coefficient of variation of 0.85 for an individual determination. The method has some limitations in accuracy for binary mixtures containing a large proportion of the less-polar amine. The scope of the technique is demonstrated by application to several commercially available dosage forms.

Keyphrases □ Amine binary mixtures—analysis □ Bromthymol blue—total amine determination □ Bromcresol purple—less-polar amine determination □ Colorimetric analysis—spectrophotometer

The acid dye technique is a general procedure for the quantitative analysis of a variety of pharmaceutical amines. In practice, a buffered aqueous solution containing the amine and a suitable indicator dye is shaken with an organic solvent, and the concentration of the resulting ion-pair in the organic phase is determined spectrophotometrically. Most amines exhibit a high sensitivity while acidic, neutral, and weakly basic compounds, as well as the commonly used excipients, do not interfere. Specific compounds for which methods have been described in the recent literature include thiamine in tablets (1), methapyraline in cough syrups (2), atropine in tablets and elixirs (3), procaine in parenteral preparations (4), ephedrine in tablets (5), imipramine and related compounds in tablets (6), and trifluoperazine in tablets (7). Automated procedures have been described for drugs such as propoxyphene (8), propoxyphene and other amines (9), and for several pharmaceutical amines and quaternary compounds (10). The technique also has been combined with quantitative TLC for the determination of amines in mixtures after separation on the chromatoplate (11).

The majority of publications describe the use of chloroform as the organic solvent. However, for most compounds, benzene is equally good and, in certain cases, has some advantages. With benzene, the experimental technique is simplified since the extraction can be carried out in a centrifuge tube and the organic layer can be clarified by centrifugation and decanted directly into a cell. The use of chloroform sometimes leads to emulsion formation and difficulties in reproducibility when extractions are carried out in a separator.

Table I—Photometric Solvents, Dye Solutions, and Buffers Required for Analysis

Mixture Code	With BCP: Percent IAA in Benzene[a]	With BCP: BCP Concn.[b]	With BCP: Buffer pH	With BTB: Percent IAA in Benzene[a]	With BTB: BTB Concn.[c]	With BTB: Buffer pH
A	0.5	37	5	1.0	40	6
B	0	35	6	0.5	45	6
C	0	35	4	0	45	6
D	0.5	35	4	0.5	42.5	6
E	0	35	4	0	45	6
F	0	37.5	5	0	42.5	6
G	0	37.5	6	0	42.5	6

[a] Percent by volume of isoamyl alcohol (IAA) in benzene. [b] Milligrams/100 ml. in solvent pH 6. [c] Milligrams/100 ml. in solvent pH 8.

Chloroform may be considered as a broad-spectrum solvent for the acid dye technique, since similar sensitivities are realized for different types of amines with a number of indicator dyes. Benzene, on the other hand, may be considered more selective because varying degrees of sensitivity are evident, depending on factors such as the polarity of the amine and type of indicator dye. Sensitivities may range from zero to values approaching those realized by the use of chloroform.

Most pharmaceutical amines fall into two general categories in terms of their sensitivity with different indicator dyes when benzene is used as the photometric solvent. The first category includes relatively polar compounds such as monophenyl amines, dibasic amines, and many alkaloids (*e.g.*, ephedrine, amphetamine, phenmetrazine, chlorpheniramine, and codeine); the second category includes the relatively nonpolar amines such as the diphenylmethane derivatives and compounds that are relatively free from polar structural configurations (*e.g.*, most phenothiazine derivatives, diphenylmethane amine drugs, and compounds such as dextromethorphan and propoxyphene). In general, amines in the more-polar category exhibit good sensitivity only with bromthymol blue (BTB) and little or no sensitivity with bromcresol purple (BCP); amines in the less-polar category display good sensitivity with both BCP and BTB.

Thus, by proper selection of experimental conditions including the choice of indicator dye, binary mixtures containing one amine in each category are amenable to analysis without prior separation. The less-polar amine can be determined with BCP, while the total of

Table II—Concentration of Sample and Reference Solutions[a]

Mixture Code	Components	Stock Sample Soln.	Detn. with BCP: Ref. Soln.	Detn. with BCP: Dil. Sample Solution	Detn. with BTB: Ref. Soln.[b]	Detn. with BTB: Dil. Sample Solution
A	Diphenhydramine HCl	20	2.0	2.0	2.0	1.2
	Ephedrine sulfate	10	—	1.0	2.0	0.6
B	Prochlorperazine[c]	2.5	2.5	2.5	2.5	0.7
	Amphetamine sulfate	5	—	5.0	2.0	1.4
C	Promazine HCl	10	2.0	2.0	2.0	1.0
	Phenmetrazine HCl	10	—	2.0	1.6	1.0
D	Promethazine HCl	10	1.6	1.6	1.6	1.0
	Ephedrine HCl	10	—	1.6	2.0	1.0
E	Promethazine HCl	10	1.6	1.5	2.0	1.0
	Codeine phosphate	20	—	3.0	2.5	2.0
F	Diphenhydramine HCl	32	2.0	1.6	2.0	1.6
	Codeine phosphate	8.0	—	0.4	2.5	0.4
G	Diphenylpyraline HCl	8.0	2.0	1.6	2.0	0.6
	Codeine phosphate	32	—	6.4	2.5	2.4

[a] Milligrams/100 ml. in 1% v/v HCl. [b] Each reference compound in separate reference solutions. [c] Present as the maleate.

both amines is determined with BTB. The amount of the more-polar amine is determined by difference. An investigation of the scope of this concept of analysis was carried out in the present study.

EXPERIMENTAL

Reagents—*Buffer Solutions*—McIlvaine buffers were prepared by mixing appropriate volumes of 0.1 *M* citric acid and 0.2 *M* disodium phosphate.

Bromcresol Purple Dye Solution—BCP (acid form)[1] was dissolved in buffer of pH 6 to give the required concentration (Table I).

Bromthymol Blue Dye Solution—BTB (acid form)[2] was dissolved in buffer of pH 8 to give the required concentration (Table I).

Photometric Solvents—Benzene AR, benzene AR containing 0.5% by volume of isoamyl alcohol AR, or benzene AR containing 1% by volume of isoamyl alcohol AR was used.

Hydrochloric Acid (1% v/v)—Concentrated HCl, diluted 1:100, was used.

Reference Solutions—Appropriate quantities of reference standards were dissolved in 1% v/v HCl to provide the desired concentrations (Table II).

Sample Solutions—Twenty tablets were weighed and finely powdered, or the contents of 20 capsules were combined and weighed. An accurately weighed aliquot of powder containing the weight of drug indicated in Table II was transferred to a 100-ml. volumetic flask. About 80 ml. of 1% v/v HCl was added, and the flask was shaken mechanically for 1 hr. Then 1% v/v HCl was added to volume, and the solution was mixed and filtered, if necessary, through Whatman No. 1 filter paper (the first few milliliters of filtrate were discarded) to give the stock sample solution. Appropriate aliquots of this stock sample solution were diluted with 1% v/v HCl to provide the desired concentrations (Table II).

Assay **Procedure**—The less-polar amine (upper entry for each amine pair in Tables III and IV) in each binary mixture was determined directly with BCP by carrying the appropriate reference solution and diluted sample solution through the following procedure.

Five milliliters of diluted sample solution or reference solution was pipeted into a 42-ml. centrifuge tube,[3] followed by 5 ml. of the appropriate buffer solution, 5 ml. of the appropriate dye solutions, and 10 ml. of the appropriate photometric solvent (as specified in Table I). The tube was shaken for 60 sec. (or alternatively tumbled end-over-end at 100 r.p.m. for 5 min. in a mechanical device designed to process 15 tubes simultaneously) and then centrifuged to effect complete separation and clarification of the organic layer (about 2 min.). The supernatant was decanted directly into a clean dry cell, and its absorbance was measured[4] at 410 nm. against a reagent blank prepared in a similar manner but with 5 ml. of 1% v/v HCl in place of the sample solution.

Comparison of the absorbance values for the reference and sample determination thus gave the amount of less-polar amine in the sample.

The more-polar amine (lower entry for each amine pair listed in Tables III and IV) in each binary mixture was determined indirectly with BTB by carrying the diluted sample solution and separate reference solutions of each amine through the described assay procedure. From the absorbance observed for the reference solution containing the less-polar amine and the amount of this material in the sample as determined by direct analysis with BCP, its absorbance contribution to the total observed for the sample solution with BTB was calculated; hence the absorbance of the more-polar amine was determined by difference. Comparison of this absorbance with the reference solution absorbance then gave the amount of more-polar drug in the sample.

RESULTS AND DISCUSSION

Development of optimum conditions for the acid dye technique involves proper selection of experimental parameters of buffer pH, kind and concentration of indicator dye, and kind of extracting photometric solvent. For application of the technique to binary mixtures of amines, conditions also must be such that the less-polar amine can be determined with BCP without interference from the more-polar amine. Variables examined for the development of the present procedure were the following:

Choice of Buffer pH—The pH of the aqueous phase should be such that any excess dye remains in this phase, thus giving a minimum blank, while formation of the ion-pair in the organic phase

Table III—Analysis of Simulated Mixtures

Mixture Code	Components	Relative Amounts (by wt.)	Recovery, %	CV[a]
A	Diphenhydramine HCl	2	100.9	0.5
	Ephedrine sulfate	1	99.4	1.0
B	Prochlorperazine[b]	1	101.8	0.7
	Amphetamine sulfate	2	98.3	0.5
C	Promazine HCl	1	100.1	1.1
	Phenmetrazine HCl	1	98.3	0.9
D	Promethazine HCl	1	99.0	0.4
	Ephedrine HCl	1	100.3	1.5
E	Promethazine HCl	1	100.1	0.8
	Codeine phosphate	2	99.2	0.5
F	Diphenhydramine HCl	4	101.0	1.3
	Codeine phosphate	1	96.9	1.3
G	Diphenylpyraline HCl	1	102.0	0.5
	Codeine phosphate	4	99.3	0.9

[a] Coefficient of variation based on six determinations. [b] Present as the maleate.

[1] Fisher Scientific.
[2] Fisher Certified Reagent ACS.
[3] Cat. No. 15846, Wilkens-Anderson Co., Chicago, Ill.
[4] Beckman DU-2 spectrophotometer.

Table IV—Analysis of Pharmaceutical Formulations

Product	Components	Label Claim, mg.	Assay Value— Label, %	CV[a]
A	Diphenhydramine HCl	50	95.7	0.8
	Ephedrine sulfate	25	99.5	1.1
B	Prochlorperazine[b]	2.5	102.0	1.5
	Amphetamine sulfate	5	98.9	1.0
C	Promazine HCl	25	95.0	0.6
	Phenmetrazine HCl	25	94.9	0.5
D	Promethazine HCl	25	93.6[c]	0.8
	Ephedrine HCl	25	93.4	0.7

[a] Coefficient of variation based on six determinations. [b] Present as the maleate salt. [c] Assay value of 94.9% of label claim (CV = 0.5) obtained by direct UV absorbance.

is at a maximum, thus giving optimum sensitivity. In practice, the optimum pH values for minimum blank and maximum sensitivity do not always coincide, although for most compounds the variation in sensitivity over several pH units is not appreciable. Table V shows the magnitude of variation encountered for the compounds studied.

With the majority of compounds studied, absorptivities are greater with BTB when buffer pH values are less than 6. However, at these low pH values, the blank is considerable and may be quite variable. This is particularly so when isoamyl alcohol in benzene is used, as in the assay of ephedrine where this photometric solvent is required to enhance the sensitivity. Therefore, a buffer of at least pH 6 is recommended for all analyses. With BCP in the buffer range of pH 3–6, blanks are minimal and reproducible over the entire range while changes in sensitivity are characteristic for the individual amine.

In dealing with mixtures of amines, the determining factor in the selection of buffer pH for the direct analysis of the less-polar amine with BCP is the elimination of any response by the more-polar amine. Most of the polar amines have no response (at the concentrations usually encountered) so the choice of pH is not critical. However, some materials, such as amphetamine, do have a significant sensitivity with BCP at higher pH values (Table VI). Therefore, for example, to eliminate any possibility of interference in the assay of prochlorperazine–amphetamine mixtures (in which the amount of amphetamine in a dosage form is double the amount of prochlorperazine), a buffer of pH 5 was chosen for the direct determination of prochlorperazine with BCP.

In the assay partition system (consisting of 5 ml. sample solution, 5 ml. buffer solution, 5 ml. of buffered dye solution, and 10 ml. of photometric solvent as described in the *Experimental* section), the actual pH of the aqueous phase is lower than the pH of the buffer quoted in Tables I and V because of the influence of the acidic sample solvent. When using BCP made up in a solution of pH 6 along with buffers of pH 4, 5, or 6, the pH in the partition system is 3.3, 3.8, or 4.3, respectively, while with BTB made up in a solution of pH 8 along with buffers of pH 6, 7, or 8, the pH is 5.1, 6.9, or 7.0, respectively. Although it is possible to use one dye–buffer reagent rather than a separate indicator dye solution and buffer solution for

Table VI—Effect of Buffer pH on Absorbance Exhibited by Amphetamine with BCP Using Benzene as the Photometric Solvent

Micrograms of Amphetamine Sulfate in 5-ml. Sample Aliquot for Assay	Absorbance— Buffer pH 5	Buffer pH 6	Buffer pH 7
250	0.003	0.004	0.013
375	0.003	0.006	0.016
500	0.003	0.007	0.018

routine analysis, it is preferable to utilize separate solutions during method development or for the analysis of a variety of compounds. In this way, a fixed stock of dye solution can be used for a series of experiments and only the buffer solution need be changed to obtain optimum conditions.

Choice of Sample and Dye Solvents—The use of dilute acid (1% v/v HCl in the present case) is considered essential for sample dissolution since many amine salts, even though completely soluble in water when in the pure state, are not completely extracted from a powdered tablet mixture with water alone. The use of water alone for the preparation of the dye solution has the disadvantage that many dyes, especially in the acid form, have a low solubility. The use of 0.01 N NaOH overcomes the solubility problem and is satisfactory, providing a good quality dye is available. However, the quality of dye often varies from batch to batch, even from the same manufacturer, giving rise to blanks which are high (*e.g.*, >0.025 absorbance unit) and variable. Prewashing a solution of dye in 0.01 N NaOH with photometric solvent does not overcome the problem. However, if the dye is dissolved in a suitable buffer, prewashing with photometric solvent often reduces the blank to a low and reproducible level (*e.g.*, <0.015 absorbance unit). With some batches of dye, an inordinately large volume of photometric solvent may be required in the prewash to reduce the blank. In such cases, recrystallization of the dye is preferable. This may be readily accomplished by heating about 2 g. of dye with 50 ml. of glacial acetic acid to effect almost complete dissolution, adding a few drops of acetone to clarify the solution, filtering through Whatman No. 2 filter paper, and allowing to crystallize. The residue may then be washed with cold benzene and air dried.

Choice of Dye and Dye Concentration—Although the ion-pair is usually a 1:1 molar ratio of dye to drug in an organic solvent, a higher ratio of dye is required in the partition system used during analysis to achieve maximum absorbance in the organic phase. Typical curves showing the effect of increasing dye concentration on absorbance with a fixed concentration of drug (the relative position of the curves is arbitrary with respect to absorbance) are shown in Fig. 1. Three general types of curve are exemplified in this figure. The first represents relatively less-polar compounds, such as promazine or prochlorperazine with BTB, for which the absorbance increases sharply until a molar ratio of between 2:1 and 4:1 is reached and then becomes independent of dye concentration. The

Table V—Effect of Buffer pH on Absorptivity Values

Drug	Absorptivity[a] With BCP pH 3	pH 4	pH 5	pH 6	With BTB pH 5	pH 6	pH 7	pH 8
Diphenhydramine HCl	57.1	58.9	58.9	56.3	66.9	67.1	66.2	64.5
Diphenylpyraline HCl	—	65.2	64.6	63.7	62.3	61.8	62.2	61.4
Promazine HCl	68.3	68.0	66.9	65.1	—	62.1	61.4	61.0
Promethazine HCl	68.5	67.3	64.4	58.6	—	61.1	60.7	59.2
Prochlorperazine maleate	23.8	30.1	31.4	32.0	—	33.7	32.5	31.6
Phenmetrazine HCl	—	—	—	—	—	75.3	71.3	63.6
Codeine phosphate	—	—	—	—	—	40.2	38.6	34.9
Ephedrine base	—	—	—	—	81.4	80.5	76.4	70.2
Amphetamine sulfate	—	—	—	—	59.8	58.2	52.2	—

[a] Calculated as absorbance/grams/liter based on theoretical weight in 10 ml. of photometric extracting solvent.

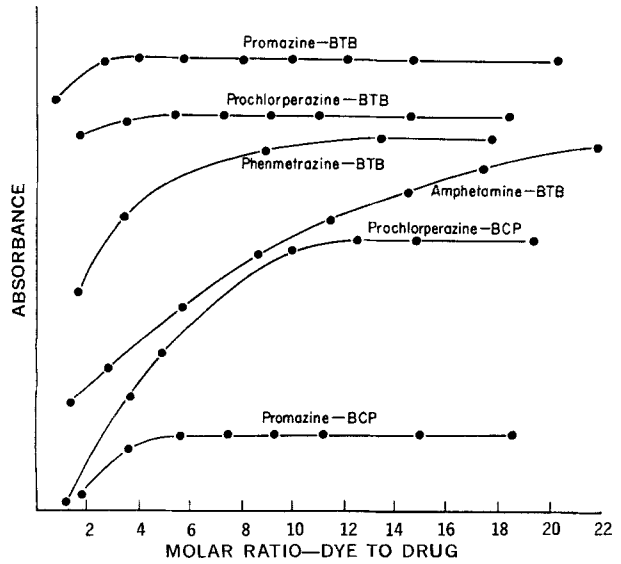

Figure 1—*Effect of dye concentration on absorbance of various drugs (as determined by varying the amount of dye with a fixed concentration of drug).*

second represents more-polar compounds, such as phenmetrazine, for which a molar ratio of 12:1 is required before the absorbance becomes independent of dye concentration. The third represents still more-polar compounds, such as amphetamine, for which the absorbance continuously increases with an increasing ratio of dye to drug.

Under the experimental conditions given in Tables I and II, the concentration of BTB used in the analysis of each amine pair is sufficient to ensure minimal variations in the assay through the effect of variations in molar ratio of dye to drug. For all compounds examined, Beer's law is followed over the concentration range of practical interest. Typical curves are shown in Fig. 2. Even for the amphetamine–BTB system, in which the absorbance varies continuously with a change in dye-to-drug ratio (but using a fixed concentration of drug), Beer's law is followed, providing a fixed concentration of dye is used. Therefore, it is imperative in such a system that the same stock dye solution be used throughout a sequence of analyses.

Choice of Photometric Solvent—With benzene alone as the photometric extracting solvent, many more-polar amines exhibit a much lower sensitivity on a molar basis than the less-polar amines. Inclusion of 0.5–1.0% isoamyl alcohol by volume, however, usually enhances the absorptivity of the more-polar amine to a level comparable to that of the less-polar amine (the absorptivity of the less-polar amine is usually not changed significantly by the inclusion of isoamyl alcohol when using BTB, but there may be some enhancement when using BCP).

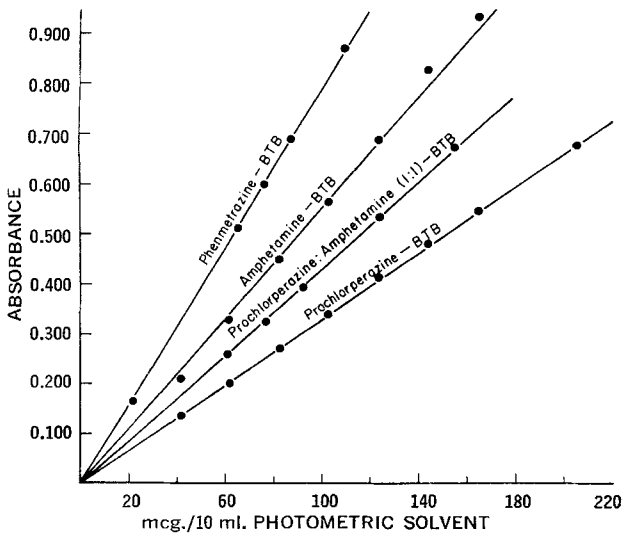

Figure 2—*Relationship of drug concentration and absorbance.*

With ephedrine, for example, the absorbance is increased by 25 and 50% by the addition of 0.5 and 1.0% by volume of isoamyl alcohol; with amphetamine the absorbance is increased 100% by the presence of 1% by volume of isoamyl alcohol. Nevertheless, some precautions must be observed with the use of isoamyl alcohol when dealing with mixtures of amines. With Mixture A (Table I), for example, 0.5% isoamyl alcohol in benzene is used for the determination of diphenhydramine with BCP to increase its sensitivity. Use of isoamyl alcohol is possible in this case since ephedrine causes no interference. With Mixture F, by comparison, benzene alone is used for the determination of diphenhydramine with BCP because of the possibility of interference by codeine. For the determination of both components of Mixture A with BTB, 1% by volume of isoamyl alcohol is included to enhance the sensitivity of ephedrine. By doing so, the sensitivity of ephedrine becomes comparable to that of diphenhydramine, thus minimizing the error that may occur when the amount of ephedrine is obtained by difference.

Analysis of Simulated Mixtures and Commercial Samples—The specific mixtures listed in Table III represent those containing amine pairs in the weight ratio that may be found in commercial formulations. The upper entry for each amine pair falls into the category of a relatively less-polar amine that exhibits good sensitivity with both BCP and BTB. The lower entry for each amine pair falls into the category of a relatively polar amine that exhibits good sensitivity only with BTB. The amount of the less-polar amine (upper entry) is determined directly by analysis with BCP, while the total amount of both amines is determined with BTB. The amount of the more-polar amine (lower entry) is determined by difference.

The analytical results given in Table III demonstrate that the overall accuracy and precision of the method are satisfactory for this type of assay, although a somewhat lower recovery is realized for codeine phosphate in admixture with four times its weight of diphenhydramine hydrochloride (Mixture F). This is not unexpected because the amount of diphenhydramine is obtained directly with BCP whereas the amount of codeine is obtained by difference. Any error in the determination of diphenhydramine would therefore be enhanced in the determination of codeine. The error is even greater if the more-polar compound has a lower sensitivity. In this particular case, an overestimation of 1% with BCP would mean an overcalculation of about 0.005 unit in the absorbance contributed by diphenhydramine in the BTB determination. This would then give an underestimation of 0.005 unit in the absorbance calculated for codeine and would lead to an underestimation in the assay of codeine of about 6.25%. Therefore, the procedure has limitations in accuracy for mixtures with large amounts of the less-polar amine relative to those of the more-polar amine. The optimum situation is that involving a 1:1 weight ratio of the two amines with each having an equal sensitivity, such as Mixture D in Table III. If the sensitivities are not equal, an increase in the proportion of the less sensitive compound (Mixture E) is desirable to maintain precision.

The results of analysis of four commercially available formulations are shown in Table IV. The absolute composition of each formulation was not determined by a secondary method except for the amount of promethazine in Product D. In that case, the assay values of the acid dye technique and those of direct UV measurement agree to within 1.3%. Coupled with the results of application of the method to simulated mixtures, as shown in Table III, the assay values and reproducibility shown in Table IV give an indication of the applicability to dosage forms containing binary mixtures of certain types of pharmaceutical amines.

Application of the technique to an expectorant containing liquid extract of ipecacuanha in addition to the combination of promethazine and codeine did not enable a complete analysis to be carried out. The amount of promethazine could be determined directly by using BCP without interference by codeine or emetine, the major alkaloid component of ipecacuanha. With BTB, emetine formed an ion-pair extractable into benzene, thus interfering with the determination of codeine. Nevertheless, the procedure is of value in determining one component directly in such multicomponent formulations.

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Specificity of Spectrophotometric Determination of Ephedrine and Other Phenalkanolamine Drugs as Benzaldehydes after Periodate Oxidation

LESTER CHAFETZ

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Keyphrases □ Phenalkanolamines—determination □ Periodate oxidation—phenalkanolamines to benzaldehydes □ Specificity, periodate oxidation—phenalkanolamine determination □ UV spectrophotometry—analysis

UV spectrophotometric determination of benzaldehyde or substituted benzaldehydes formed by periodate oxidation of ephedrine and other drugs with vicinal hydroxyl and amine functions has provided simple and sensitive assay methods for them in dosage forms and in biological fluids. Ephedrine HCl, in dilute acid solution, exhibits a benzenoid UV spectrum with a molar absorptivity, ε, of about 190 l./mole cm. at its 258.5 nm. maximum: oxidation of it to benzaldehyde affords an ε-value of about 14,400 at its maximum at about 241 nm. in hydrocarbon solvents, about a 75-fold gain in sensitivity. Although the literature on this reaction provides abundant implications on its specificity, it has not been critically reviewed from this standpoint. The purpose of this article is to provide a synthesis of the relevant literature and of previously unreported observations from these laboratories on the specificity of the method.

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Nicolet and Shinn (5) first reported the use of periodate for oxidation of ethanolamine derivatives. They found that ethanolamines with primary or secondary amine functions were rapidly and quantitatively cleaved to aldehydes and ammonia or a primary amine, while attack on compounds with tertiary amine or acylated amine functions was extremely slow. Wickstrøm (6) studied the rate of periodate oxidation of ephedrine as a function of pH, titrating excess oxidant iodometrically. He found that periodate consumption was too slow to measure at pH 3.0, very slow at pH 6.0, and stoichiometric within 10 min. at pH 7.5 or higher. Overconsumption of periodate, *i.e.*, further oxidation of the aldehyde reaction products, was negligible except at very high pH. [The oxidation potential of the reagent is irrelevant in this reaction. Periodate is a very strong oxidizing agent in acid solution, with the potential of the periodate–iodate couple estimated at −1.6 v. In alkaline solution, however, the potential is only about −0.7 v. (7). Moreover, other oxidizing reagents of comparable redox potentials do not selectively cleave glycols and ethanolamines.] Wickstrøm (6) showed that the ephedrine reaction products are benzaldehyde, acetaldehyde, and methylamine (Scheme

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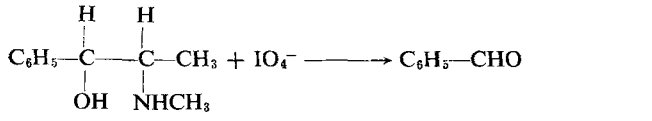


Scheme I

I). He proposed determination of ephedrine *via* colorimetry of acetaldehyde distilled from the reaction mixture.

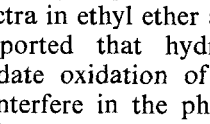
Pisano (8) proposed the determination of metanephrine and normetanephrine, the 3-*O*-methyl metabolites of epinephrine and norepinephrine, respectively, by spectrophotometry. He oxidized the compounds to 3-methoxy-4-hydroxybenzaldehyde (vanillin) with periodate and determined the UV absorption of an oxidized aliquot *versus* an unoxidized reagent blank. Tompsett (9) used the same procedure for the detection of ephedrine and phenylephrine, differentially determining the spectra of benzaldehyde and 3-hydroxybenzaldehyde, respectively.

In work on the pK requirement for the reaction, Chafetz (10) studied the periodate oxidation of ephedrine, phenylpropanolamine, phenyramidol, and the pyrimidyl and *s*-triazinyl analogs of phenyramidol. He found that all of the nonphenolic compounds, except the two phenyramidol analogs, were quantitatively oxidized to benzaldehyde in 10 min. in a bicarbonate medium, and he recommended extraction and spectrophotometry of benzaldehyde in hexane. He ascribed the failure of the reaction with the phenyramidol analogs to the lower availability of electrons on the exocyclic nitrogen, *i.e.*, lesser pKa values. Table I shows the structure of the three compounds and the pKa values attributed to them by Heitmeier and Gray (11). This observation is consistent with the findings of Wickstrøm (6) on the pH dependence of ephedrine oxidation by periodate.

Studying the effect of phenolic phenalkanolamines, several workers (8–10, 12, 13) described spectrophotometric methods for phenylephrine and other monophenolic phenalkanolamines after periodate oxidation. Heimlich *et al.* (13) determined the urinary excretion rate of phenylpropanolamine (norephedrine) by measuring the benzaldehyde spectra in ethyl ether after periodate oxidation. They reported that hydroxybenzaldehydes, formed by periodate oxidation of phenolic alkanolamines, do not interfere in the phenylpropanolamine assay because they are not extracted with ether. Chafetz (10) commented that hexane is preferable as an extraction solvent because its low polarity limits interference by polar chromophores.

The selectivity of the periodate oxidation method for the assay of phenylephrine will be the subject of a forthcoming report; however, it is worthwhile noting that phenylephrine and nonphenolic phenalkanolamines can be determined in the same sample without interference. This is illustrated in a method developed for a nose drop preparation declaring phenylpropanolamine HCl, phenylephrine HCl, thonzylamine HCl, thonzonium bromide, and cetylpyridinium chloride.

Table I—Structure of Phenethanol Derivatives of Aminoheterocycles



Compound	*Het*	pKa (11)
Phenyramidol	2-Pyridyl	6.9
Pyrimidyl analog	2-Pyrimidyl	3.5
Triazinyl analog	2-*s*-Triazinyl	2.9

EXPERIMENTAL

Standard Preparation—Dissolve accurately weighed quantities of about 50 mg. each of phenylephrine hydrochloride reference standard USP and of authentic phenylpropanolamine hydrochloride in water in a 100-ml. volumetric flask and dilute to volume. Dilute 4.0 ml. of the solution to 100 ml. with water to obtain concentrations of about 20 mcg./ml. for each amine.

Assay Preparation—Dilute 4.0 ml. of the solution to 500 ml. with water.

Procedure—Transfer 5.0-ml. portions of the assay preparation and the standard preparation to separate 60-ml. separators. Add 1 ml. of saturated sodium bicarbonate solution and 1 ml. of sodium metaperiodate solution (2 in 100) to each. Swirl, let stand 15 min., and then add 10.0 ml. of 1 *N* hydrochloric acid. After evolution of gas ceases, add exactly 15.0 ml. of hexane, previously shown to be transparent to UV light, shake the mixture for 30 sec., and withdraw most of the lower aqueous phase into a second separator. Retain the aqueous phase for phenylephrine assay. Wash the hexane with two 5-ml. portions of water, and add the washings to the aqueous phase.

Phenylpropanolamine HCl—Filter the washed hexane layers through dry filter paper, and determine their absorbances in 1-cm. cells at the absorption maximum at about 242 nm. in a suitable spectrophotometer, using hexane as the blank. Calculate the concentration, in milligrams per milliliter, of $C_9H_{13}NO \cdot HCl$ from the formula $0.125C(A_U/A_S)$, where C is the concentration of phenylpropanolamine HCl in micrograms per milliliter in the standard preparation, and A_U and A_S are the absorbances of the solutions from the assay preparation and the standard preparation, respectively.

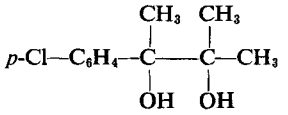
Phenylephrine HCl—Extract the retained aqueous phase with four 15-ml. portions of chloroform, and collect the extracts in a 125-ml. separator. Add exactly 15.0 ml. of 0.1 *N* sodium hydroxide, shake the mixture for 30 sec., and then allow the layers to separate. Discard the chloroform layer, and filter the alkali extract through dry paper. Concomitantly determine the absorbances of the solutions in 1-cm. cells in a suitable spectrophotometer at the wavelength of maximum absorbance at about 237 nm., using sodium hydroxide solution as the blank. Calculate the concentration, in milligrams per milliliter, of $C_9H_{13}NO_2 \cdot HCl$ in the sample taken from the formula $0.125C^1(A_U^1/A_S^1)$, where C^1 is the concentration in micrograms per milliliter of phenylephrine HCl in the standard preparation, and A_U^1 and A_S^1 are the absorbances of the solutions from the assay preparation and the standard preparation, respectively.

Six trials of the method provided a recovery of 101.0 ± 1.6% for phenylpropanolamine HCl and of 99.6 ± 2.6% for phenylephrine HCl. The foregoing procedure is adapted directly from the procedures described by Chafetz (10); recent improvements will be reported.

DISCUSSION

Interference by Other Amine Drugs—Wallace (14) recently described a similar method to that described by Chafetz (10). He described methods for estimation of ephedrine, pseudoephedrine, phenylpropanolamine, and phenyramidol in biological samples. Despite the reports, *inter alia*, by Wickstrøm (6), Heimlich *et al.* (13), and Chafetz (10) showing that the reaction is quantitative within 10 min. at room temperature, Wallace (14) conducted the reaction at the temperature of boiling hexane, about 69°, with a reaction time

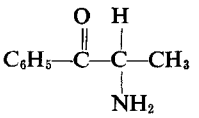
of 30 min. After separating the hexane layer, he washed it with dilute acid and determined benzaldehyde spectrophotometrically either as such or after conversion to its semicarbazone. He reported the absorbance values obtained with more than 80 different drugs, without the phenalkanolamine structure, which had been tested in the procedure; none of them showed any significant degree of interference even under the unnecessarily rigorous oxidation conditions employed. The highest absorbance reported was for phenaglycodol (I), about 10% that obtained with ephedrine; however, phenaglycodol is a 1,2-glycol which could be oxidized to 4-chloroacetophenone and acetone to some extent under Wallace's conditions. Moreover, the intact drug has a strong absorption band in hexane at 222 nm.



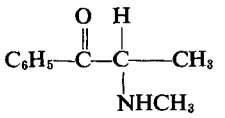
phenaglycodol

I

Possible Interference by Ketone Analogs—Although degradation of ephedrine or phenylpropanolamine *via* oxidation of the secondary alcohol function to the corresponding ketone has never been encountered here or, to the author's knowledge, reported in the literature, the availability of samples of the aminoketones, α-aminopropiophenone and α-methylaminopropiophenone (ephedrone), led the author to study the effect of periodate oxidation on these compounds. These aminoketones (II, III), because they have carbonyl groups in conjugation with the benzene ring, have UV spectra closely similar to that of benzaldehyde. They can easily be distinguished from benzaldehyde, however, by reaction with periodate under the conditions described by Chafetz (10) for ephedrine assay. Benzaldehyde is unaffected, while the spectra of the aminoketones are destroyed. By TLC of benzene extracts of the reaction products of ephedrone and α-aminopropiophenone oxidation, the aromatic reaction product was determined to be benzoic acid. Benzoic acid was identified by comparison with standards on two different thin-layer systems:



II



III

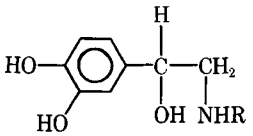
System 1: *n*-butyl alcohol–dehydrated alcohol–concentrated ammonia–water (60:60:60:15) with a 20 × 20-cm. plate consisting of a 0.25-mm. layer of microcrystalline cellulose on glass (Analtech).

System 2: ethyl acetate–dioxane–water (40:90:90) with a 20 × 20-cm. plate consisting of a 0.25-mm. layer of silica gel GF on glass (Analtech).

The reaction mixture was acidified with hydrochloric acid, and the benzene extract was filtered through paper and concentrated to about 1 ml. on the steam bath. The extract and a standard of authentic benzoic acid in benzene, 5 mg./ml., were spotted in 10- and 15-μl. volumes at equidistant points 3 cm. from the bottom of the plates. The chromatograms were developed in standard chambers, lined on three sides with filter paper, and previously equilibrated with the developing solvent mixture at least 30 min. before use. The chromatograms were developed until the solvent front ascended about 12 cm. from the starting line. The plate developed in System 1 was dried at 105° for 1 hr. and then sprayed with a freshly prepared mixture of equal volumes of 8% potassium iodide, 2% potassium iodate, and 1% amylose as described by Chafetz and Penner (15). Benzoic acid was revealed as a dark-blue spot on a light-blue background at about R_f 0.8. The plate developed in System 2 was air dried at room temperature and examined under shortwave UV light. Benzoic acid was revealed as a single spot at R_f about 0.9 along with two spots of lesser intensity. Spraying the plate with 0.1% alcoholic bromcresol purple revealed a single yellow spot for benzoic acid on a purple-brown background. In both systems, using detection reagents selective for acids, the extract of the periodate oxidation products of the aminoketones revealed spots identical in R_f and reaction with authentic benzoic acid.

Benzoic acid is a much weaker UV chromophore than benzaldehyde. Moreover, its partition coefficient between aqueous and hexane phases is much less favorable than with the less polar aldehyde; thus, if present, the corresponding aminoketones will not interfere in periodate assays of ephedrine and related drugs.

Effect of Catecholamines (IV)—Epinephrine (R = CH_3—), norepinephrine (R = H), isoproterenol [R = $(CH_3)_2CH$—], and several other natural and synthetic pressor compounds are 3,4-dihydroxyphenethanolamine derivatives, which might be expected to undergo periodate oxidation to 3,4-dihydroxybenzaldehyde (protocatechualdehyde). These compounds, however, are preferentially



catecholamine structure

IV

oxidized by cyclization to adrenochrome and other indole derivatives by periodate, iodate, and any number of mild oxidizing agents. Under the Chafetz (10) conditions, the compounds are immediately oxidized to red-colored compounds in the aqueous phase, and these are not extractable with hexane. Kaistha (16) reported the periodate oxidation of isoproterenol to protocatechualdehyde at acid pH, using a large excess of periodate and a reaction time of 18 hr. at room temperature. His data indicate that the extent of conversion of isoproterenol HCl to protocatechualdehyde is only about 10% at best.

Acylated Phenalkanolamines—Troup and Mitchner (17) showed that phenylephrine may be acetylated by aspirin in dosage forms. The literature (5) and pK considerations indicate that acylated phenalkanolamines should not be affected by periodate. As a *punctilio*, to demonstrate the lack of interference in the assay by such products of incompatibility, the author prepared *N*-acetylephedrine by the method of Welsh (18) and compared it with ephedrine HCl in the following experiment.

Aqueous solutions were prepared containing, respectively, 20 mcg./ml. of ephedrine HCl and of *N*-acetylephedrine, m.p. 84.0–84.5° (uncorrected). Volumes of 5.0 ml. of each solution were transferred to separate 60-ml. separators and treated with 1.0 ml. of saturated sodium bicarbonate and 1.0 ml. of sodium metaperiodate (2 in 100). The solutions were swirled, allowed to stand for 10 min., and then extracted with exactly 20.0 ml. of hexane. Spectra of the hexane extracts were scanned in 1-cm. cells in a Cary model 14 recording spectrophotometer *versus* hexane from 300 nm., using hexane as the blank. The spectra of the extract of the ephedrine HCl oxidation product showed the typical pattern of benzaldehyde; the solution from the *N*-acetylephedrine showed no absorbance, confirming that acylated compounds do not interfere.

Arylglycolates—Salts and esters of arylglycolates, such as methenamine mandelate, magnesium pemoline, and homatropine HBr, are oxidized to the corresponding carbonyl compounds by periodic acid as illustrated for mandelic acid (Scheme II). Chafetz (19) used



Scheme II

this reaction for determination of these compounds, although he showed that ceric ion is preferable as the oxidant. He showed that acid pH and heat are necessary. The compounds are not oxidized in bicarbonate solution. Thus, they will not interfere, if present, in the determination of ephedrine.

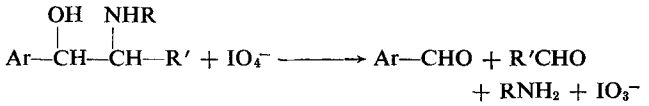
Limitations of the Method—Spectrophotometry of the aromatic aldehyde produced by periodate oxidation of phenalkanolamines will not distinguish between compounds such as ephedrine and phenylpropanolamine, both of which afford benzaldehyde, or phenylephrine and metaraminol, both of which give 3-hydroxybenzaldehyde. It will not discriminate stereoisomers such as ephedrine (*erythro*-configuration) and pseudoephedrine (*threo*-configuration).

These limitations are common to most other methods described for these drugs. Since they are rarely combined with each other in therapy, the limitations are more theoretical than real in dosage form assay. However, ephedrine is metabolized to norephedrine in part, and the method will not distinguish between the two in biological fluids.

A preliminary extraction of the phenalkanolamine drug is necessary in the presence of high concentrations of sugars, as in pharmaceutical syrups. Certain reducing agents, such as iodide, interfere by destroying periodate. In general, the limitations of the method are easily negated by use of suitable adjunctive procedures.

SUMMARY AND CONCLUSIONS

Evidence from the literature and previously unreported observations from these laboratories has been critically examined with respect to the specificity of the periodate method for ephedrine and related phenalkanolamine drugs. The reaction used may be generalized as shown in Scheme III. The method is based on spectrophotometric determination of Ar—CHO after its extraction with an immiscible solvent. Obviously, ethanolamine derivatives that are oxidized to compounds without UV chromophores will not interfere in the method.



Scheme III

The reaction proceeds quantitatively within a few minutes at room temperature and in neutral or alkaline solution where Ar is phenyl or monohydroxyphenyl, R is hydrogen or alkyl, and R′ is hydrogen or alkyl. The reaction does not proceed at any appreciable rate with compounds bearing electron-withdrawing substituents on the amine function, *e.g.*, where R is acyl, 2-pyrimidyl, or 2-*s*-triazinyl.

The accepted mechanism for periodate oxidation of ethanolamines involves formation of a cyclic intermediate comprising the iodine atom of periodate and the amine and hydroxyl functions. Probably because of steric requirements, compounds with tertiary amine functions are not cleaved by periodate under the assay conditions. Preliminary results in these laboratories on periodate oxidations of compounds of the type Ar—O—$CH_2CH(OH)$—CH_2—NHR support this viewpoint; compounds that have the bulky *tert*-butyl substituent for R are not affected by periodate under vigorous reaction conditions, but where R = methyl or isopropyl, the reaction proceeds smoothly.

Specificity in an assay method is a function of both the measurement and isolation procedures. Substitution on the aryl ring will affect both the UV spectra (measurement) and the extraction characteristics of Ar—CHO (isolation).

Because their oxidation products are not extracted by the nonpolar solvents used for the nonphenolic analogs, monophenolic phenalkanolamines do not interfere in the determination of benzaldehyde-producing compounds. A procedure for the determination of phenylpropanolamine and phenylephrine in the same solution has been described where the oxidation products are separately determined after simple solvent extraction procedures. Catecholamines do not interfere in the determination of other phenalkanolamines, because they are preferentially oxidized to adrenochrome derivatives. Aminoketone analogs of phenalkanolamines are oxidized to benzoic acids, which do not interfere in the determination.

Overall, the method requires that a basic compound of circumscribed structural characteristics be oxidized to a nonpolar neutral compound with a characteristic and intense UV spectrum. Since very few drugs or excipient materials satisfy these criteria, one must conclude the method has very high specificity for phenalkanolamines. With respect to specificity, the periodate oxidation procedure is clearly superior to the methods official for ephedrine preparations in the compendia, which imply only the presence of an ether-soluble amine.

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Inclusion Compounds in Pharmaceutical Analysis II: Assay of a Cream following Chemical Dehydration

SUE E. HAYS and LEE T. GRADY

Abstract □ A nitrofurazone cream presented a strong emulsion problem which prevented the drug being isolated from other constituents by simple extraction. Chemical dehydration of the cream by acetone dimethyl acetal made possible the subsequent precipitation of long-chain, emulsifying constituents as channel-type inclusion compounds with urea. Both steps take place in a single tube. Good precision and recoveries of nitrofurazone were obtained using spectrophotometric measurement. Combination of urea inclusion methods with chemical dehydration expands the scope of this analytical methodology for complex dosage forms.

Keyphrases □ Nitrofurazone cream—analysis □ Dehydration, chemical—cream analysis □ Urea inclusion compound—nitrofurazone cream analysis □ UV spectrophotometry—analysis □ Emulsion—analysis □ Acetone dimethyl acetal—dehydration agent

Chemical dehydration based on the known, acid-catalyzed reaction of water with acetals has found practical application in recent years (for example, aviation fuel additives). Application of acetal dehydration to analytical problems also is receiving attention. Acetone dimethyl acetal[1] was used by Erley (1) to remove water from nonvolatile samples for IR analysis. Critchfield and Bishop (2) used the reagent in determining water in organic samples by measuring the IR absorbance of the acetone formed. Other work was done on water determination with acetone dimethyl acetal, but combined with gas-liquid chromatographic measurement of products, by Hager and Baker (3), Badinand *et al.* (4), and Mary (5), whose work was with natural products of pharmaceutical interest. This approach allows water determination using flame-ionization detectors and simplifies sample handling, as was observed by Martin and Knevel (6). Some other applications of acetal dehydration to analytical methodology should be noted. Bousquet *et al.* (7) used acetone dimethyl acetal to dry extracts of biological materials and even reacted it directly with tissues. Incomplete extraction of thyroxine from sera was overcome by Kowal *et al.* (8) by dehydrating the sample with acetone dimethyl acetal prior to radiometric determination.

Acetal dehydration apparently has yet to be applied to the considerable assay problems presented by pharmaceutical emulsions. This article reports that application to a specific problem, nitrofurazone cream NF. The use of inclusion chemistry to facilitate assay schemes for topical dosage forms was developed previously (9) in this laboratory for dienestrol cream NF and, in combination with acetal dehydration, constitutes a new, general approach to the construction of analytical methods for pharmaceutical emulsions and topical dosage forms.

[1] Also called 2,2-dimethoxypropane; each molecule reacts with 1 molecule of water to produce 1 molecule of acetone and 2 molecules of methanol.

METHODS AND MATERIALS

All chemicals were reagent grade. Acetone dimethyl acetal was used.[2] All evaporations were performed at 20–30° at reduced pressure with mixing.[3] Spectrophotometric measurements were made with a recording, double-beam instrument.[4] A sample of commercial cream[5] and reference standard nitrofurazone NF were used.

Standard Preparation[6]—Transfer to a 100-ml. volumetric flask about 25 mg. of reference standard nitrofurazone NF, previously dried at 100° for 1 hr. and accurately weighed. Dilute to volume with methanol and mix (original solution of standard). Transfer a 4-ml. aliquot to a 50-ml. glass-stoppered centrifuge tube and evaporate to dryness under reduced pressure, keeping the temperature below 30°. Add 0.3 ml. of water and continue with the *Procedure*, beginning with "...add 3 ml. of acetone dimethyl acetal."

Procedure—Transfer to a 50-ml. glass-stoppered centrifuge tube an accurately weighed amount of nitrofurazone cream, equivalent to about 1 mg. of nitrofurazone; add 3 ml. of acetone dimethyl acetal; warm in a 30° water bath with occasional swirling until the cream is dispersed. Add 2 drops of 0.1 *N* hydrochloric acid and warm in the water bath for 10 min. with occasional swirling. Add 3 ml. of dehydrated alcohol and, with swirling, add 1.3 g. of urea followed by an additional 2 ml. of dehydrated alcohol. Mix for approximately 2 min. and allow to stand, mixing every 20 min., for at least 2 hr.

Centrifuge and transfer the supernate to a second, similar tube. Wash the residue with one 3-ml. portion and two 2-ml. portions of methyl ethyl ketone, centrifuging each time and adding the supernates to the second tube. Add 25 ml. of ether to this tube, shake well, and centrifuge. Transfer the supernate to a third, similar tube and evaporate to dryness under reduced pressure, keeping the temperature below 30°. Wash the residue in the second tube with one 3-ml. portion and two 2-ml. portions of methyl ethyl ketone, centrifuging each time and adding the supernates to the third tube to be evaporated to dryness.

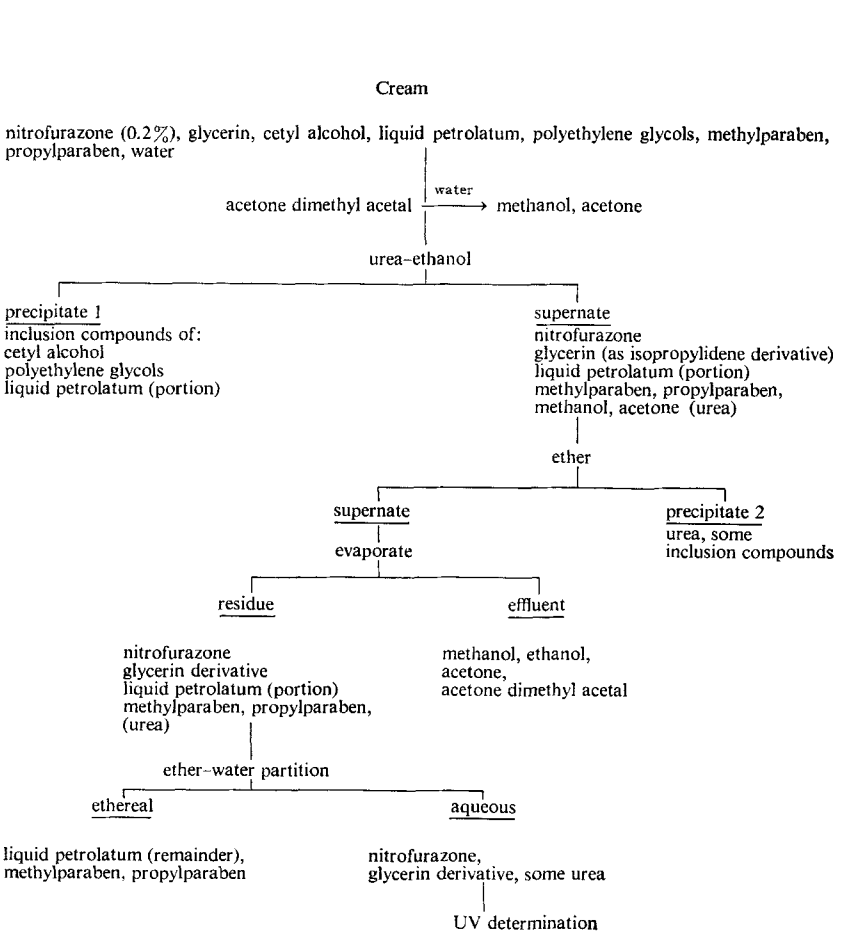
Transfer this residue to a 125-ml. separator with the aid of about 35 ml. of water. Add 20 ml. of ether to the separator and shake carefully. Transfer the aqueous layer to a 100-ml. volumetric flask. Wash the ether with three 15-ml. portions of water, adding these to the volumetric flask. Add water to volume and mix. Filter, if necessary, to obtain a clear solution.

Concomitantly determine the absorbance of the two solutions (sample and standard) in 1-cm. cells with a suitable spectrophotometer at the maximum at about 372 nm. using water as the blank. Calculate the quantity, in milligrams of $C_6H_6N_4O_4$ in the portion of cream taken, by the formula $0.004C \times (A_U/A_S)$, in which C is the exact concentration, in micrograms per milliliter, of the original solution of standard, A_U is the absorbance of the sample solution, and A_S is the absorbance of the standard solution.

RESULTS AND DISCUSSION

The assay in the NF XIII monograph covering nitrofurazone cream provides no isolating procedures. Where possible, compendial procedures require separation of the active ingredient from the other ingredients of the dosage form. A simple, workable assay sequence (Scheme I) was therefore needed which would separate the nitrofurazone from the other ingredients of a typical emulsion formulation. A placebo cream was prepared containing all constituents in common with the labeled ingredients of the commercial

[2] Matheson Coleman & Bell.
[3] Rotary Evapo-Mix, Buchler Instruments, Fort Lee, N. J.
[4] Cary model 14, Monrovia, Calif.
[5] Furacin Vaginal Cream; supplied by Eaton Laboratories, Norwich, N. Y.
[6] Perform assay with minimum exposure to light.



Scheme I—Flow diagram for the assay of nitrofurazone cream

item, although the actual proportions in that item were not known.

The standard (1 mg.) in the presence of this arbitrary placebo mixture yielded a reproducible recovery of 98.1% when compared to the standard put through the procedure (Table I). This 1.9% loss of drug when placebo is present is due in part to the retention of some drug by the large amount of precipitate formed when includable material is present. More rigorous washings of the precipitate are not desirable due to the chance of drawing the included material back into solution. Some drug is also lost during the ether–water partitioning, where sharper interfaces are obtained without the presence of placebo. The arbitrary placebo mixture used consisted of: glycerin, 5%; cetyl alcohol, 20%; liquid petrolatum, 10%; polyethylene glycols (equal amounts of 400, 1600, and 4000), 5%; methyl- and propylparabens, 0.25%; and water, 60%; 0.5-g. portions of the mixture were used.

The coefficients of variation of the standard through the procedure and of the standard plus arbitrary placebo through the procedure were 0.12 and 0.38%, respectively. When four 0.5-g. samples of the commercial cream were assayed, the recovery, based on the standard put through the procedure, was 95.9% of the labeled amount of nitrofurazone; the coefficient of variation was 3.6%. This lesser precision is most likely due to the small amount of sample used. No difficulties were apparent in applying the procedure to the commercial cream formulation. Commercial cream, 2-g. portions, assayed 98.5 and 99.0% by the present NF XIII procedure, in which the cream is dissolved in chloroform and dimethylformamide and the UV absorbance of that solution after a suitable dilution is determined.

As the 98.3% average recovery of standard (1 mg.) through the procedure shows, it is necessary either for standard drug to be carried through the procedure or for a suitable recovery factor to be inserted in the calculation to account for small amounts of drug lost during the washing of precipitates and partitioning. The former, use of a procedural standard, was chosen for this procedure.

Comments—Removal of long-chain compounds, particularly those capable of establishing an emulsion, is a recurrent problem. Dienestrol cream NF (9) presented a several thousandfold ratio of monostearin to dienestrol, which made polarographic assay impossible. An initial ether extraction separated the monostearin and dienestrol from the water and its solutes in the cream. At that point, monostearin was separated from the dienestrol by formation of a crystalline, channel-type inclusion compound with urea.

Nitrofurazone cream formulations present different problems. Nitrofurazone is a water-soluble compound, so an extractive separation of nitrofurazone from these typical water-insoluble cream constituents would appear to be the simplest isolation scheme. However, simple extraction fails, due to the formation of very stable emulsions. Direct partition-column chromatography also offers little hope of reliability in such cases.

Two goals were evident. The first was to destroy the emulsion composition without destroying the labile nitrofurazone. The second was to separate nitrofurazone from the emulsion-forming constituents of the cream. The second goal appeared amenable to the urea inclusion technique, provided the solubility of nitrofurazone in any system was not exceeded. Initial destruction of the emulsion system thus was the primary problem.

The chosen procedure was to dehydrate the emulsion system chemically with acetone dimethyl acetal. This technique does not appear to have been used previously to remove water from pharmaceuticals to facilitate assay. It is especially promising for cream preparations to remove the aqueous phase and thus destroy the emulsion. The resulting system can consist of a single phase, as in the case of this cream. The reaction is endothermic and acid catalyzed and proceeds at room temperature. The methanol and acetone formed and any excess acetone dimethyl acetal are evaporated off easily before further handling. The volume ratio of acetone dimethyl acetal used in this procedure should remove water in amounts up to 90% of the cream formulation.[7]

[7] One milliliter of acetone dimethyl acetal reacts with 0.147 g. of water.

A word of caution should be added. Acetals react with compounds other than water, so the possible effect of the acetal on the analyte should be anticipated. For example, acetals (ketals) react with organic acids to give esters (10) and with alcohols to give a mixture of ketals (11).

Following dehydration, and in the same tube, cetyl alcohol, polyethylene glycol, and a portion of the liquid petrolatum were separated by precipitating them as the urea inclusion compounds. The amount of urea used in this nitrofurazone cream assay is sufficient to react with includable materials of up to 40% of the cream formulation, to saturate the ethanol added as well as methanol formed in the dehydration, and to have some in excess. Methanol is generally used as the solvent from which urea inclusion compounds are formed (9). However, in the case of nitrofurazone cream, ethanol was chosen because at saturation it contains only about one-fourth the amount[8] of urea (12) contained by methanol and thus causes less urea to be carried on to the rest of the procedure by the supernate. Most urea in the supernate is precipitated in the next step of the procedure by addition of ether. This second precipitation also removes includable material not wholly precipitated at first. Of the various solvents tried for washing the precipitates [dioxane, cold ethanol–ether (1:1), ether, methyl ethyl ketone], methyl ethyl ketone seemed most satisfactory. It dissolves[9] the nitrofurazone, dissolves little urea, does not decompose the inclusion compound significantly, and is removed easily by evaporation.

Ether–water partitioning, impossible with this cream in its unmodified condition, is now quickly done after the removal of the emulsifying agents by inclusion. The discarded ether fraction contains the nonincludable branched-chain portion of the liquid petrolatum along with the parabens.[10] The absorbance of the nitrofurazone in the aqueous fraction is then measured. No interference is caused by the glycerin, probably present as its isopropylidene derivative (13) after reaction with the acetal, or by any added urea still present.

Solutions of nitrofurazone are sensitive to light, particularly direct sunlight and strong fluorescent lighting. A 6% loss in UV absorbance value was noted when a 1 in 100,000 solution in ethanol–water was allowed to stand in a fluorescent-lighted room for 6 hr. Heat speeds the decomposition and should be avoided. The 30° temperatures specified in this procedure do not cause observable loss of drug. Solutions of the drug, even at low concentrations, are stable for long periods when kept in complete darkness at room temperature.

Isolation Alternatives—Column chromatography was investigated as an alternative to this ether–water partition as a final separation. Silica gel[11] provided no separation. A diatomite[12] column that worked satisfactorily consisted of a lower bed of diatomite and water topped by a bed of diatomite, water, acid, and nitrofurazone. The drug was retained in the top layer while the column was washed with 50 ml. of ether–hexane (1:1) and was then eluted with 35 ml. of water. Work was done with various types of neutral and basic aluminas and various solvents. If one is willing to specify a definite batch of alumina (not just a definite pH), it is possible to have a procedure with a final column step, based on the NF XIII nitrofurazone ointment assay, which will give a complete separation of the nitrofurazone from every other ingredient of the cream. The column of alumina need be only 4 cm. high and 1.5 cm. in diameter and should be washed with dehydrated alcohol. To the dry residue, after chemical dehydration and urea inclusion, add 5 ml. of dehydrated alcohol and transfer the solution to the prepared column which is then eluted with additional dehydrated alcohol to remove the glycerin derivative, the urea, and the parabens. The drug is eluted with water, leaving any remaining liquid petrolatum behind on the column. Of the five aluminas tried, only one worked at all satisfactorily.[13] If the type of alumina is so critical, the use of an alumina column is not advisable. Chromatographic difficulties with nitrofurans, using other systems, were noted elsewhere (14).

[8] Urea solubilities at 20°/100 g. of solvent are: methanol, 22 g.; ethanol, 5 g.; and ether, 0.6 mg.

[9] The authors determined the solubility of urea as 219 mg./100 g. methyl ethyl ketone at 20° and nitrofurazone as 115 mg./100 g. at 24°.

[10] In the case of methylparaben, 89% transfers to the ether. This percentage would be expected to be higher in the case of propylparaben.

[11] Matheson Coleman & Bell, 60–80 mesh, chromatographic grade.

[12] Celite 545, Supelco, Inc., Bellefonte, Pa.

[13] Merck & Co. reagent aluminum oxide for chromatographic adsorption, 71707.

Table I—Assay Results

	Recovery, %
Standard, 1 mg., and arbitrary placebo, 0.5 g., through procedure. Results based on standard through procedure.	97.7 97.9 97.7 98.5 98.4 98.4
Average	98.1
Coefficient of variation	0.38
Standard alone through procedure, 1 mg.	98.2 98.2 98.2 98.4
Average	98.3
Coefficient of variation	0.12
	Labeled Amount
Cream, commercial, 0.5 g. Results based on standard through procedure.	92.0 95.3 100.4 95.7
Average	95.9
Coefficient of variation	3.6

SUMMARY

Isolation of drugs for assay from complex dosage forms containing large quantities of surfactants and other long-chain compounds in the past has required troublesome steps such as column chromatography or multiple extractions. In the case of the stable emulsions, especially pharmaceutical creams, this problem may be compounded by extreme difficulty in obtaining reliable destruction of the emulsion interface prior to initial isolation of drug. Chemical dehydration was applied to this problem of pharmaceutical emulsion destruction and was combined with subsequent removal of most of the cream-formulation surfactants and long-chain compounds by inclusion chemistry. With these techniques, which facilitate the construction of reliable, workable assay procedures, the authors were able to construct a separation-assay procedure for nitrofurazone cream NF XIII, giving good precision and recovery.

Cream of arbitrary formulation is dehydrated chemically by reacting the water present with acetone dimethyl acetal to yield methanol and acetone. Cetyl alcohol, propylene glycols, and some liquid petrolatum are precipitated as the urea inclusion compounds. These two steps are carried out in a single centrifuge tube. Excess urea is precipitated by adding ether to the supernate in a second tube. The nitrofurazone is isolated from this supernate by means of an ether–water partition and is quantitated spectrophotometrically.

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Rapid Identification and Estimation of Gitoxin in Digitoxin and Digoxin Tablets by TLC

MATTHEW L. DOW, ROSS D. KIRCHHOEFER, and JAMES F. BROWER

Abstract □ A rapid TLC procedure was developed for the identification and estimation of gitoxin in digitoxin and digoxin tablets. A solution of tablet material is spotted on a TLC plate directly, and the gitoxin is estimated by visual comparison of fluorescence with that of standards after spraying with acid–ferric chloride T.S. USP XVII. The method can detect 0.5% of gitoxin in the presence of 1 mcg. of cardiac glycosides.

Keyphrases □ Gitoxin—identification, estimation □ Digitoxin, digoxin tablets—gitoxin identification □ TLC—identification, estimation □ Fluorescence, estimation—UV light

The USP XVII monograph for digitoxin tablets (1) includes separate procedures for the identification of digitoxin and the determination of other digitoxosides. Identification Test A is a color reaction using a version of the Keller–Kiliani reagent (2). The test is neither sensitive nor convenient to use for large numbers of samples. The "Other Digitoxosides" determination is also a colorimetric method based on the Keller–Kiliani reagent. Gitoxin is the principal digitoxoside other than digitoxin occurring in digitoxin tablets (3), and separation on a diatomaceous earth[1] column used in the USP XVII method is based on this premise (4). Gitoxin is also the principal fluorescing substance mentioned in the monograph for digoxin tablets in USP XVII (5). Lanatoside C may also contain gitoxin as an impurity (3), although the NF XII monograph (6) does not include a test for it.

The aglycone of gitoxin with hydroxyl groups in the 14- and 16-positions is readily dehydrated (7, 8) at room temperature to dianhydrogitoxin, which exhibits visible fluorescence when activated by UV light. The anhydro derivative of digitoxin is not visible under the same conditions. The usual dehydrating reagent for this reaction has been either glycerin–HCl or propylene glycol–HCl. Acid–ferric chloride T.S. USP XVII was found to give the same reaction and has the advantage of being volatile. The nonvolatile residues from the acidic glycerin or glycol reagents result in diffusion of spots and lowered sensitivity.

A TLC system using methylene chloride–methanol–formamide on a silica gel plate (9) was found to separate the compounds sufficiently to carry out the tests. The gitoxin content of the sample on the sprayed plate is estimated by visual comparison of its fluorescence with that of standard spots under longwave UV light (3650 Å). The digitoxin chromatogram becomes visible after the plate is heated in a 100° oven for 10 min.

The same technique can be used for the identification of digitoxin, digoxin, acetyldigitoxin, and lanatoside C and for the determination of any gitoxin present in their drug preparations. Digoxin and gitoxin are not completely separated by this chromatographic procedure. Nevertheless, digoxin is not activated to visible fluorescence at room temperature by the acid–ferric chloride. Consequently, any fluorescence present immediately after spraying is due to gitoxin alone. Heating the plate at 100° destroys the gitoxin fluorescence and converts the digoxin to a fluorescent anhydro derivative which may be seen under both UV and visible light.

EXPERIMENTAL

Chromatographic Chamber—A Mitchell tank was used (10).

TLC Plates—Coat a 200–250-μ layer on a clean glass plate, using a 2:1 slurry of water–silica gel G.[2] Air dry; then activate 30 min. at 100°. Store in a desiccator. When several samples are to be run simultaneously, 0.5–1.0-cm. channels may be scored on the plate for convenience. Precoated silica gel GF plates[3] enhance the fluorescence of the spots obtained. A source for long wavelength (3650 Å) UV light was used.

Reagents—*Mobile Solvent*—Methylene chloride, distilled in glass, plus anhydrous acetone-free methanol (Mallinckrodt or

[1] Celite 545, Johns-Manville Products Corp., New York, NY 10016

[2] Catalog No. 7731, Brinkmann Instruments Co., Westbury, NY 11590

[3] Analtech, Inc., Wilmington, DE 19801

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Table I—Summary of Chromatograms of Digitoxosides Obtained by Proposed Method[a]

Drug	R_f	Heat Treatment of Chromatoplate: Room Temp. UV	Room Temp. Vis	100°, 1 min. UV	100°, 1 min. Vis	100°, 10 min. UV	100°, 10 min. Vis
Acetyldigitoxin[b]	0.90	—	—	—	BG	OF	BG
Digitoxigenin	0.83	—	—	—	—	OF	G
Digitoxin	0.83	—	—	—	BG	OF	BG
Gitoxin	0.72	BF	—	—	—	OF	BG
Digoxin	0.70	—	—	BF	BG	BF	BG
Digoxigenin	0.65	—	—	BF	—	BF	Y
Lanatoside C	0.58	—	—	BF	BG	BF	BG

[a] BF = blue fluorescence; OF = orange fluorescence; BG = blue-green; Y = yellow; G = green; UV = visualization under longwave UV light; and Vis = visualization in visible light. [b] The spot obtained from aged acetyldigitoxin standard solution exhibits a blue fluorescent fringe on the lower edge after heating 1 min.

equivalent) plus stabilized formamide (80:19:1) was used. The solvent is stable for 2 days in a glass-stoppered flask.

Spray Reagent—Acid-ferric chloride T.S. USP XVII was used. Mix 60 ml. of glacial acetic acid with 5 ml. of sulfuric acid, add 1 ml. of 9% ferric chloride, mix, and cool. Allow the reagent to age 1 day before use. It is stable at least 1 month.

Reference Standard Solutions—Prepare 1.0 mg./ml. solutions of digitoxin, digoxin, acetyldigitoxin, and lanatoside C, each containing 0.05 mg./ml. of gitoxin in ethanol–formamide (4:1). Spot 1-, 2-, 4-, 5-, and 10-μl. portions.[4]

Assay Procedures—Determine the average weight of not less than 20 tablets and reduce the sample to a fine powder. Weigh a portion of the powdered sample equivalent to 1.0 mg. of drug, and transfer to a suitable covered container. Add 1 ml. of a solution of ethanol–formamide (4:1) to the container. The formamide is necessary to produce compact spots and to reduce tailing. Swirl or stir the container occasionally during a 20-min. period; then allow it to settle for an additional 10 min. Spot 5 μl. of the supernatant liquid on a TLC plate. Spot a series of gitoxin standards containing the cardiac glycoside being identified. Place the plate in an equilibrated chromatographic chamber and develop approximately 100 mm. [Use an unequilibrated chamber without trough for plates that are 5.08 cm. (2 in.) or less in width.] Air dry the plate and spray with the acid-ferric chloride T.S. USP XVII. Ventilate 2–3 min. in a current of air and view under longwave UV light. Estimate gitoxin in the sample by visual comparison of the fluorescence with that of the gitoxin standards. Allow a short period of time for acclimation of the eyes before reading. Place the plate in a 100° oven for 10 min. All of the compounds appear as blue-green spots in visible light and may be identified by comparison of their R_f values with those of concomitant standards. The sequence of observed color changes is summarized in Table I.

RESULTS AND DISCUSSION

The gitoxin content of a series of digitoxin tablet samples was estimated by this procedure and subsequently by the USP XVII method for Other Digitoxosides (1). The results are listed in Table II. Samples that showed no gitoxin spot by TLC were checked periodically by the official procedure; in no case was any found.

A similar comparison made on a series of samples of 10 digoxin tablets each showed no gitoxin present by the TLC procedure, and the samples assayed less than 0.1% "related fluorescent substances" by the USP XVII monograph for Digoxin Tablets (5). A sample prepared to contain 0.5% gitoxin (based on digoxin declaration) assayed 0.5% by the TLC estimate and 0.45% by the USP XVII procedure.

[4] Combined standards were used so that a single spotting would produce a reference for qualitative comparison of the glycoside and quantitative estimation of the gitoxin.

Table II—Comparison of TLC Estimate with USP XVII Determination of Other Digitoxosides

Sample	Other Digitoxosides,[a] %	TLC Estimate,[b] %	Sample	Other Digitoxosides,[a] %	TLC Estimate,[b] %
1	0.5	<1	14	2.0	2.5
2	4.5	5	15	2.0	2.5
3	1.6	1	16	1.6	2
4	0.7	<1	17	5.1	5
5	0.7	<1	18	2.5	3
6	0.7	1	19	5.1	5
7	4.1	2.5	20	6.4	7
8	3.0	2.5	21	4.9	6
9	2.2	2.5	22	4.5	5
10	0.7	1	23	3.4	4
11	2.0	2	24	3.8	5
12	9.0	>5 <10	25	1.0	<1
13	4.0	5	26	0.8	<1
			27	5.7	5

[a] USP XVII monograph for digitoxin tablets; calculated from ratio of the absorbances of sample to standard as digitoxin. [b] As gitoxin.

By this procedure, 15 samples may be analyzed in less than 90 min. The official test for Other Digitoxosides requires 3–4 hr. to complete after digitoxin is eluted from the column.

Solutions of USP Reference Standard Digoxin and USP Reference Standard Digitoxin in ethanol have never given more than one spot when chromatographed. Nevertheless, it is recommended that all reference solutions be prepared at least weekly.

The procedure described in this paper was successfully applied to approximately 850 commercial samples of digitoxin tablets, 600 of digoxin, 100 of acetyldigitoxin, and 100 of lanatoside C, representing most of the formulators in the United States. The correlation between the results by the official determination of Other Digitoxosides and those by the method described in this paper is a good indication that gitoxin is the principal other digitoxoside present in current digitoxin products on the market and confirms the 1948 work of McChesney *et al.* (11).

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Simplified Automatic Determination of *In Vitro* Drug Dissolution Rates Using Direct Concentration Recording

JEFFREY M. BURGER and ROBERT A. NASH

Abstract □ A system is described which permits automatic recording of dissolution rates directly in terms of percentage dissolution *versus* time without the need for an intermediate absorbance–time plot.

Keyphrases □ Automated system—*in vitro* dissolution rates □ Dissolution rates—automated procedure □ Concentration converter—absorbance–time curves □ UV spectrophotometry—analysis

During the past decade, studies of dissolution behavior of tablets and capsules have gained in importance because of the growing awareness of differences among products containing the same drug. The terms "therapeutic equivalency" and "physiological availability" have been employed to highlight the fact that different formulations of the same drug may affect the rate and extent of absorption after oral administration. Schroeter *et al.* (1), Levy *et al.* (2), and Nelson (3) related *in vitro* dissolution behavior of drugs to their *in vivo* absorption.

Various methods for determining *in vitro* dissolution rates were recently reviewed (4). Of the several analytical methods for monitoring drug concentration during a dissolution study, spectrophotometry remains the simplest and most rapid. Determination of dissolution characteristics of a drug preparation requires that measurements be made on a large number of samples. Steadily increasing demands for data on dissolution rates make some degree of automation of obvious benefit.

APPARATUS

The dissolution apparatus employed in this laboratory was previously described (5). The dissolution vessel is a V-shaped, transparent, plastic chamber which oscillates about its center. The frequency of oscillation may be changed by means of a variable speed motor. The rate of oscillation chosen should furnish sufficient agitation to maintain homogeneity in the chamber without imparting unusual mechanical stress to the capsule or tablet. The dissolution medium is maintained at 37° by partially immersing the chamber in a constant-temperature bath.

Fluid is pumped from the dissolution chamber using a Varistaltic[1] pump through polyethylene and Tygon[2] tubing and through a 0.2-cm. silica flowcell in the sample beam of a Beckman model DB spectrophotometer. A glass-wool filter in the pump intake line prevents undissolved solids from entering the cell.

The output signal from the spectrophotometer is 0–100 mv. for 0–100% T (transmittance). This signal is conditioned and recorded using a Beckman model 131902 concentration converter and 100-mv. potentiometric strip chart recorder.

[1] Manostat Corp., New York, N. Y.
[2] U. S. Stoneware Co., Akron, Ohio.

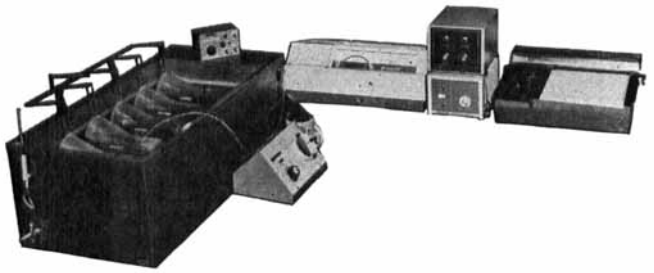

Figure 1—In vitro *drug dissolution apparatus.*

The output signal from the concentration converter can be presented as absorbance over a variety of ranges, and concentration can be presented over a single, adjustable range. Figure 1 is a photograph of the assembled system, and Fig. 2 is a flow diagram of the apparatus.

Previous methods for determination of dissolution rate were based on recording the absorbance of the dissolution medium *versus* time. The percent of drug dissolved at any given time was obtained indirectly by comparing observed absorbance with absorbance of a solution representing total dissolution.

Use of the concentration converter eliminates this intermediate step.

EXPERIMENTAL

Because drug products are marketed in varying strengths, it was found most useful to calibrate the system in terms of a linear relationship between the drug concentration and the converter setting required for fullscale recorder deflection. The equation for this straight line is then used to calculate the setting required to display 0–100% dissolution for any label claim potency directly on the chart paper.

Six solutions of the drug of interest were prepared equivalent to 0–125% of label claim potency of the drug dissolved in a specified volume of dissolution medium.

A wavelength, preferably an absorption maximum, was selected for spectrophotometric monitoring. The zero concentration point was set with dissolution medium in reference and sample cells in the spectrophotometer. Each standard solution was then pumped through the sample cell, and the recorder deflection was adjusted to read 100 chart divisions by means of a calibrated potentiometer on the converter. The dial reading was interpolated to four places. A linear plot was obtained between the dial setting and concentration. The slope and y-intercept of the line were determined by the method of least squares. Abscissa values (x) for the equation were expressed

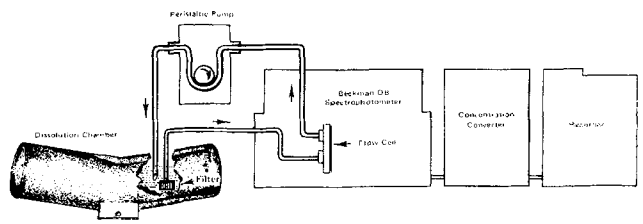

Figure 2—*Flow diagram of* in vitro *dissolution apparatus.*

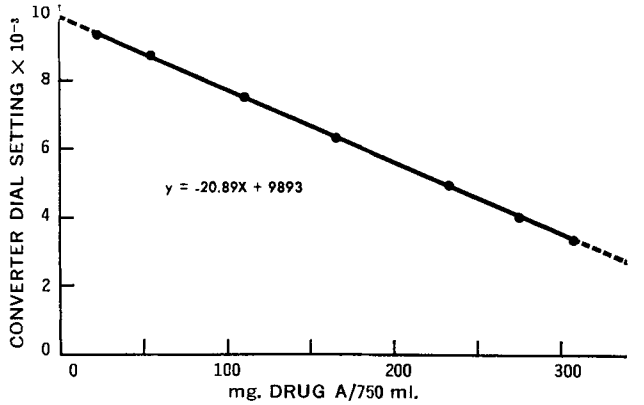

Figure 3—*Calibration curve for Drug A in simulated gastric solution (without pepsin) in 0.2-cm. flowcell at 364 nm.*

as milligrams of drug/volume of dissolution medium. The y-intercept for a given drug is constant regardless of the volume of test medium and y represents the calculated dial setting on the converter required for a given label claim potency in the required volume of dissolution medium. Slope depends on the volume of the dissolution medium and the lightpath of the cell. Thus, the straight-line equation for a particular tablet or capsule potency gives the dial setting required to display percentage dissolution *versus* time directly on the strip chart recorder over the range of 0–100% dissolution.

For example, 300-mg. film-coated tablets of Drug A were studied. Standard solutions were prepared with 0.1 N hydrochloric acid. A plot of concentration *versus* converter dial setting was linear, the least-squares fit yielding the equation $y = -20.89x + 9893$ at a wavelength of 364 nm. in a 0.2-cm. flowcell. The x is the label claim potency, and y is the calculated dial setting. For a label claim potency (x) of 300 mg., a dial setting (y) of 3626 is obtained.

At time zero, one tablet was placed into 750 ml. of simulated gastric solution USP (without pepsin) and agitated at a rate of 25 oscillations/min. This solution was pumped through the flowcell and returned to the chamber; drug concentration, in terms of percent dissolved, was recorded *versus* time.

RESULTS

Calibration plots for Drugs A, B, and C are shown in Figs. 3–5. The graphs were constructed from a least-squares fit of the experimental data. The strip chart recording of time *versus* percent dissolution for a 300-mg. film-coated tablet of Drug A is reproduced in Fig. 6. Prior to the use of a concentration converter, absorbance–time curves, such as that shown in Fig. 7, were obtained for similar tablets. In Fig. 7, the percent dissolved values are indicated at the appropriate absorbance values. One sees from Fig. 7 that percentage dissolution does not necessarily correspond logically to recorder deflection.

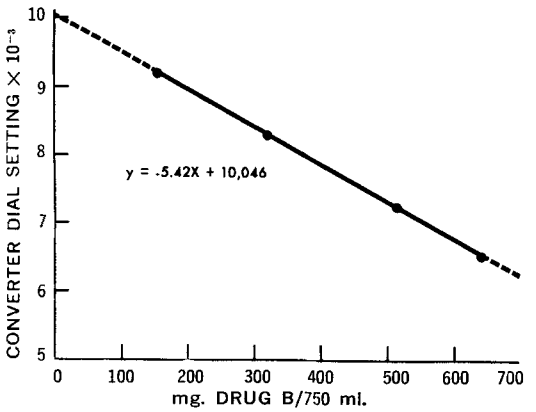

Figure 4—*Calibration curve for Drug B in simulated gastric solution (without pepsin) in 0.2-cm. flowcell at 276 nm.*

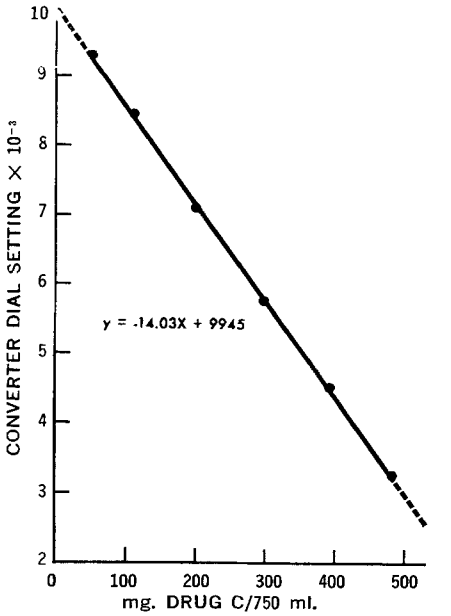

Figure 5—*Calibration curve for Drug C in simulated gastric solution (without pepsin) in 0.2-cm. flowcell at 380 nm.*

DISCUSSION

The calibration plots used here are based upon a specific volume of dissolution medium. It is easily recognized that a change in volume changes the slope of the least-squares line. Because of this, a set of least-squares coordinates and dial settings should be determined for each volume used. Slope also depends on absorptivity of the drug at the selected wavelength. Calibration curves for each of the three drugs studied had different slopes.

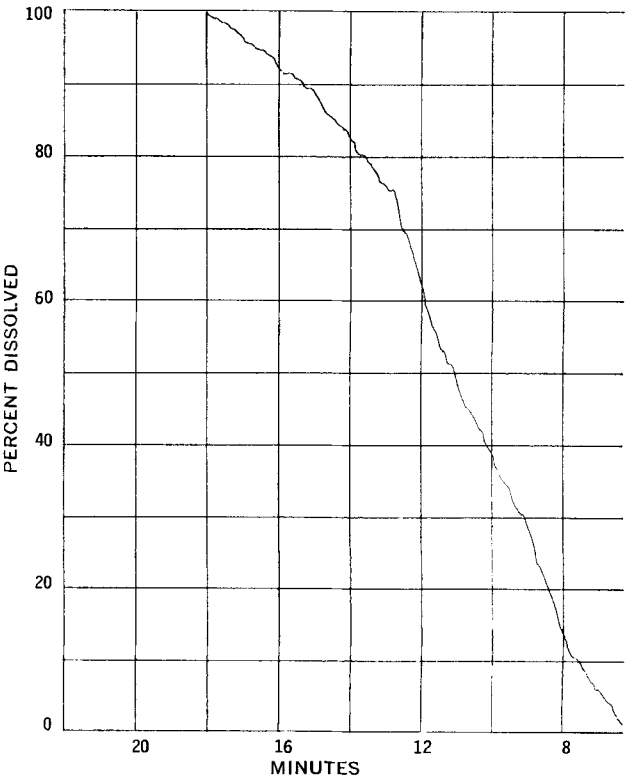

Figure 6—*Percent dissolved* versus *time: 300-mg. film-coated tablets of Drug A in simulated gastric solution (without pepsin); 0.2-cm. flowcell at 364 nm. Converter setting = 3526 for* $y = -20.89x + 9893$.

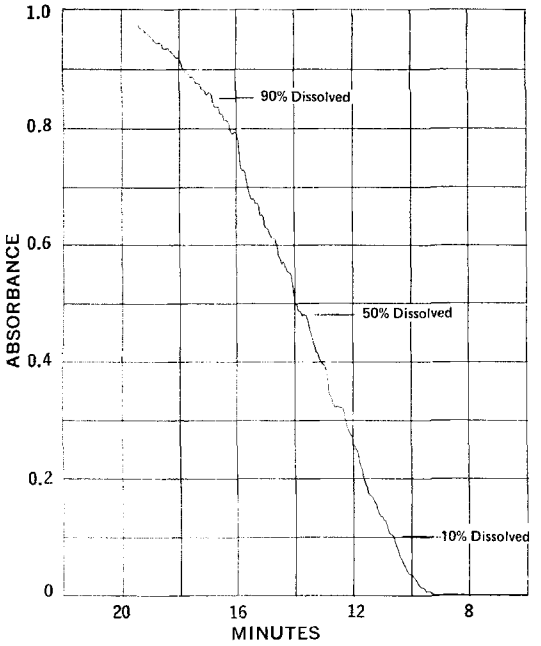

Figure 7—*Absorbance* versus *time: 300-mg. film-coated tablets of Drug A in simulated gastric solution (without pepsin); 0.2-cm. flow-cell at 391 nm.; 100% label claim concentration = 0.950 absorbance units.*

Values for *y*-intercepts appear to approach a limiting value of 9999+. This value is the highest number obtainable on the multipot dial. Although these numbers are arbitrary, 9999+ is analogous to 100% transmittance since the ordinate represents infinite dilution or 0% concentration.

In addition to monitoring concentration dynamics during a dissolution or kinetic study, the concentration converter allows for rapid analysis of solutions assayed by colorimetric or spectrophotometric methods. Samples can be quickly pumped through the flow-cell; their concentrations can be read directly from the chart rather than converting an absorbance value to concentration by means of absorptivity calculations or working curves.

While the primary advantage of the concentration converter in this study was direct display of a concentration parameter, the instrument also permits the presentation of absorbance and percent transmittance data on a recording chart simply by switching the multirange switch on the converter.

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NOTES

Absolute Configuration of (+)-*trans*-2-*o*-Tolyl-*trans*-5-hydroxycyclohexanol: Metabolite of Racemic *trans*-2-*o*-Tolylcyclohexanol

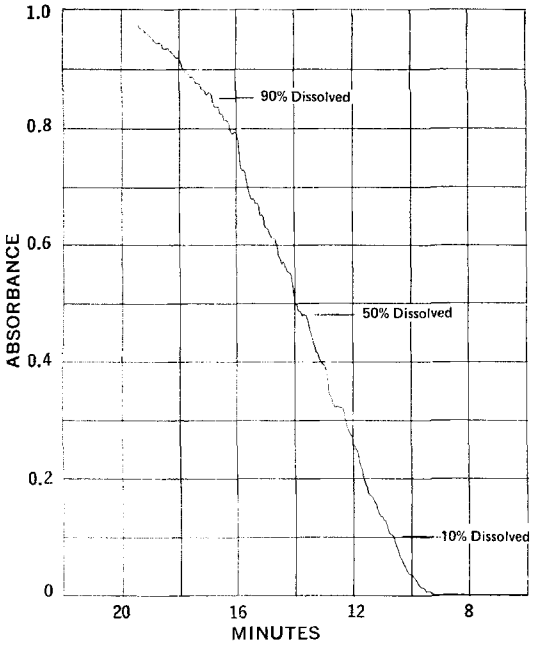
ALAIN C. HUITRIC, N. PETER McGRAW*, and BETTY R. LOWRY

Abstract □ The absolute configuration of the urinary metabolite (+)-*trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol, isolated after administration of racemic *trans*-2-*o*-tolylcyclohexanol in male Holtzman rats, was established by relating it chemically to (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol of known absolute configuration. The results give unequivocal proof that the original tentative assignment of (1S,2R,5R)-(+)-*trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol is correct.

Keyphrases □ (+)-*trans*-2-*o*-Tolyl-*trans*-5-hydroxycyclohexanol, urinary metabolite—absolute configuration □ Urinary metabolites—absolute configuration of (+)-*trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol

In an earlier publication (1), the authors reported the characterization of a major rat urinary metabolite of racemic *trans*-2-*o*-tolylcyclohexanol as the dextrorotatory axial C-5 ring hydroxylated product *trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol (IV). The characterization was done by NMR and by comparison with authentic racemic IV previously synthesized in this laboratory (2). In the same publication, a tentative assignment of absolute configuration of the metabolite was made by comparison of its ORD curve with that of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol (V) (3). The assignment was tentative because of the lack of direct information on the effect of the remote hydroxyl group at C-5 on the Cotton effects of the aromatic chromophore at C-2.

Unequivocal proof is now presented that the original assignment of (1S,2R,5R) is correct. The proof of absolute configuration was obtained by relating (+)-*trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol (IV) chemically to (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol (V), the absolute configuration of which was previously reported (3) and recently confirmed by single crystal X-ray



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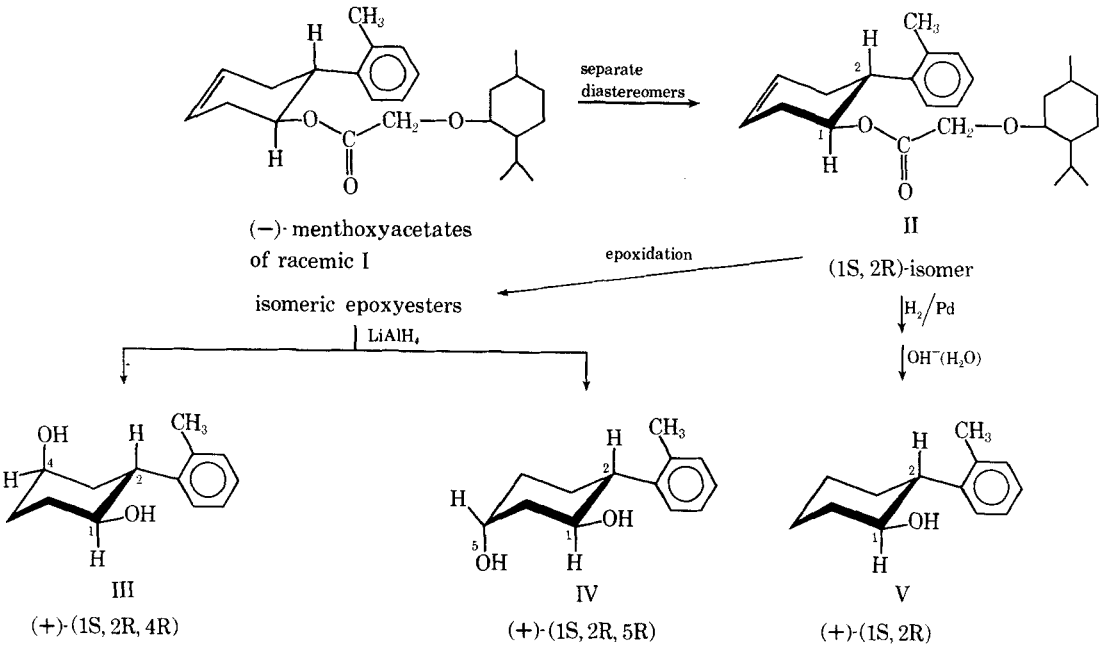

Scheme I

diffraction analysis of its 3-nitro-4-bromobenzoate derivative (4).

EXPERIMENTAL AND DISCUSSION

(+)-*trans*-2-*o*-Tolyl-*trans*-5-hydroxycyclohexanol (IV) was related chemically to V by the procedure depicted in Scheme I.

Known racemic *trans*-5-*o*-tolylcyclohexen-4-ol (I) (2) was converted to the diastereomeric (−)-menthoxyacetate esters by a known method (4). The diastereomeric esters were separated by crystallization, and the separation was monitored by NMR (5). Isomer II {m.p. 73–74°, $[\alpha]_D^{27}$ −25.2° (c 4.3, chloroform)} yielded (+)-(1S, 2R)-2-*o*-tolylcyclohexanol (V) upon hydrogenation of the double bond followed by basic hydrolysis of the ester.[1] Since the absolute configuration of V has been established as (1S,2R) with certainty (4), this also establishes the absolute configuration on carbons 1 and 2 of II as (1S,2R). The introduction of the axial hydroxyl group at position 4 or 5 through epoxidation of the double bond in II, followed by lithium aluminum hydride reduction of the epoxy esters, does not cause any change in the configuration at carbons 1 and 2. Therefore, the optically active diols, III and IV, also have the (1S,2R) configurations. Diols III and IV were separated by chromatography and characterized by comparison of their NMR spectra with those of the known racemic compounds (2). (1S,2R,-5R)-(+)-*trans*-2-*o*-Tolyl-*trans*-5-hydroxycyclohexanol (IV) obtained by Scheme I has $[\alpha]_D^{25}$ + 44.8°, and its ORD curve is qualitatively the same as that reported for the metabolite (1). The metabolite was not purified sufficiently to permit assignment of its optical purity with certainty.

Since metabolite IV, obtained from racemic V, has the absolute configuration (1S,2R,5R), it seems logical to deduce that it has resulted from a stereoselective hydroxylation of the (1S,2R) enantiomer V. This means that the (1R,2S) enantiomer (the mirror image of V) is not metabolized by the same pathway.

[1] The product is identical to authentic V in specific rotation and rotatory dispersion.

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Synthesis and Preliminary Screening of *N*-Ethyltryptamine Derivatives Related to Reserpine and Lysergic Acid

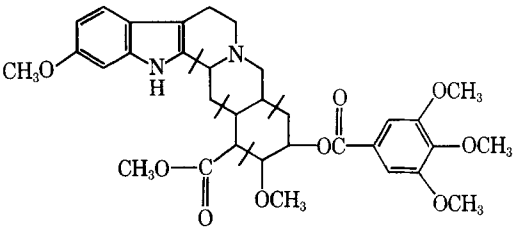
STANLEY SKLAR*, KARL A. NIEFORTH, and MARVIN MALONE

Abstract □ Fourteen derivatives of *N*-ethyltryptamine, structurally related to reserpine and lysergic acid, were synthesized. These compounds plus two intermediates were screened for gross pharmacologic activity in unanesthetized rats. Reserpinelike activity was absent, while LSD-like activity was found in those compounds most closely related to lysergic acid.

Keyphrases □ *N*-Ethyltryptamine derivatives—synthesized, screened for reserpinelike, LSD-like activity □ LSD-like activity—*N*-ethyltryptamine derivatives synthesized, screened □ Reserpinelike activity—*N*-ethyltryptamine derivatives synthesized, screened

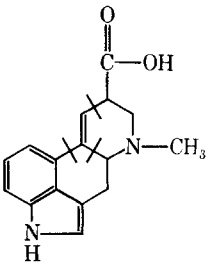
For many years, medicinal chemists have been attempting to determine the active moieties of complex medicinal agents. The first and most important reason for such determinations is to develop newer and more potent drugs with a lower incidence of side effects. The second is to reveal information concerning the mechanism of action of these drugs and possibly their site of action. Reserpine and lysergic acid are typical complex medicinal agents to which these general considerations have already been applied by other investigators with considerable success. By disjunction, both reserpine and lysergic acid can be shown to possess an *N*-alkyl substituted tryptamine fragment and an additional alkyl chain terminating with a carboxy group (III). In the case of reserpine, this alkyl group consists of four methylene groups and terminates with a carbomethoxy group; in the case of lysergic acid, this alkyl group consists of two methylene groups terminating with a carboxyl group.

Examination of the structures of reserpine and lysergic acid diethylamide indicated potential activity of compounds that incorporated certain features of each. The title compounds are aliphatic esters and amides attached at the omega position to the aliphatic nitrogen of tryptamine. Psychotomimetic activity would be anticipated in those compounds that had a two-carbon chain separating the tryptamine and carbonyl function as in lysergic acid diethylamide. Those compounds having a four carbon chain between the two functional groups would be expected to exhibit CNS depression. The length of the chain was varied from two to four carbons. The carbonyl function was a methyl or ethyl ester or an *N*,*N*-dimethyl or diethyl amide. A few compounds were substituted with a 6-methoxy group to resemble more the reserpine molecule.



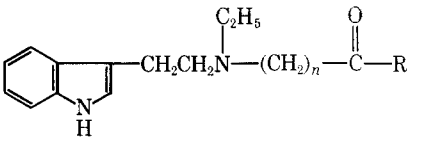
reserpine

I



lysergic acid

II



III

$n = 2,3,4$

$R = OCH_3,\ OC_2H_5,\ N(CH_3)_2,\ N(C_2H_5)_2$

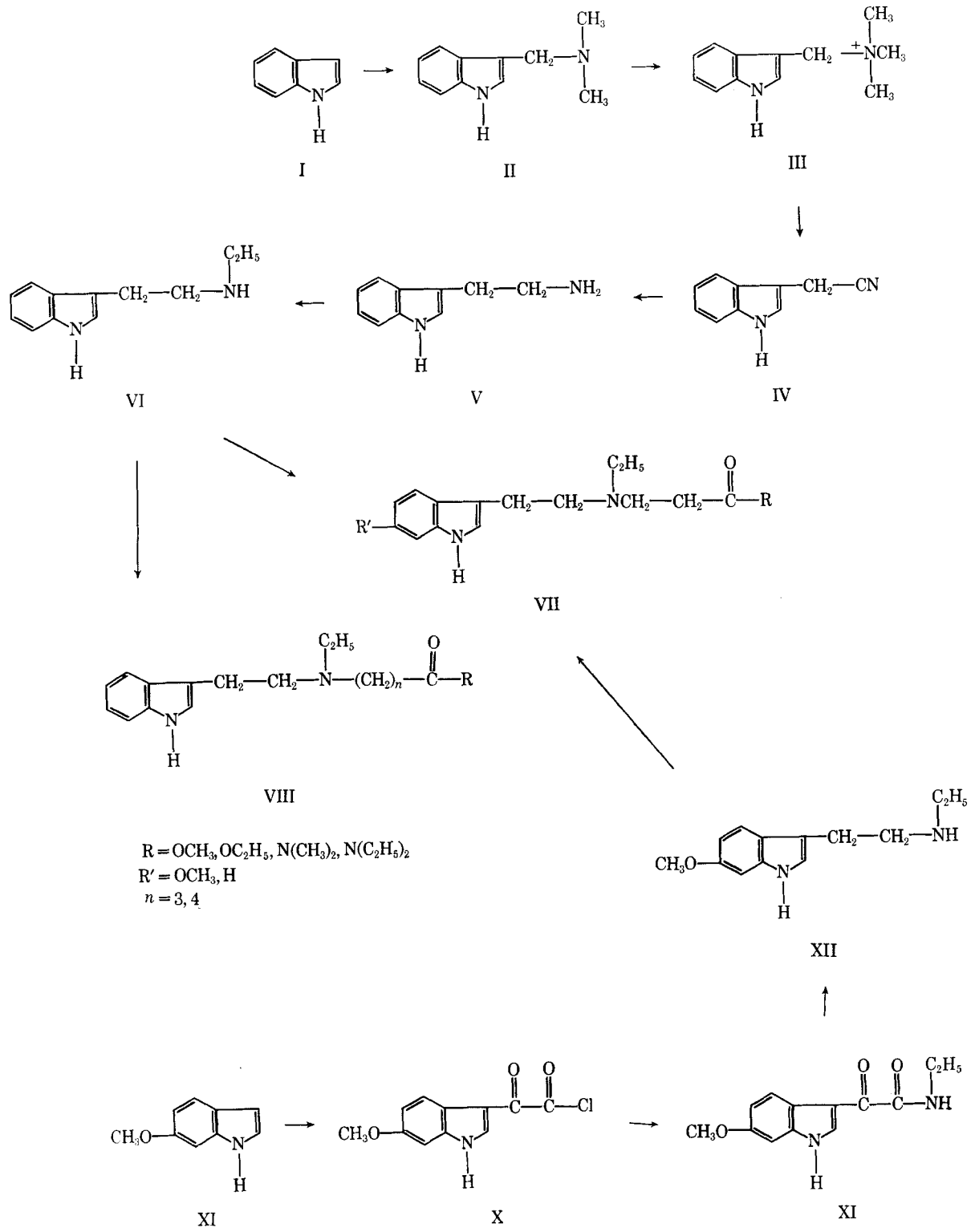
Disjunction of reserpine and lysergic acid

EXPERIMENTAL

N-Ethyltryptamine and *N*-ethylethyl-6-methoxytryptamine were synthesized by the use of published procedures, the former compound by the procedure of Snyder *et al.* (1) and the latter compound by the procedure of Woodward *et al.* (2) (Scheme I). The title compounds were prepared by one of the two following procedures.

Preparation of Amides and Esters of *N*-Ethyl-*N*-(3-indolylethyl)-ω-aminoalkyl Carboxylic Acid—*Procedure A*—The *N*-ethyltryptamine derivative was dissolved in approximately 40 times its weight of absolute methanol. To this solution was added a 10% molar excess of the acrylic acid derivative. The mixture was allowed to stand at room temperature for 4 days. The solvent was removed with reduced pressure and replaced with an equal amount of ether. After the ether solution was washed twice with 10% saline solution, it was dried over anhydrous sodium sulfate. TLC on silica using 5% diethylamine in chloroform and detection using chromic acid or Dragendorff's reagent revealed only one spot with an R_f different than the starting amine. The yields of the reactions ranged between 75 and 90%. Analytical samples were obtained by adding a saturated solution of picric acid in ether to the ethereal solution of the product and recrystallizing the picrate from absolute ethanol.

Procedure B—*N*-Ethyltryptamine (0.3 g.) was dissolved in 10 ml. of dimethyl sulfoxide. To this solution was added an equimolar quantity of the ω-chlorovaleric acid or butyric acid derivative and 0.5 g. of sodium bicarbonate. The suspension was stirred and warmed to 50° (ω-chlorovaleric acid derivatives) or 70° (butyric acid derivatives) and monitored with TLC using the system described previously. After 2 hr. an additional quantity of the chloroacid was added. Further monitoring with TLC was conducted until the reaction reached completion. Water was added to the mixture, and the resulting suspension was extracted with ether. The ethereal extract



Scheme I

was dried and treated with acetic anhydride (2 ml.) at reflux for 30 min. to acetylate any remaining tryptamine derivative. The ethereal solution was extracted with 20% aqueous acetic acid to remove the amines as acetate salts. Hydrochlorides formed from pure amines isolated on preparative TLC were extremely hygroscopic. Analytical samples were obtained by dissolving the hydrochlorides in water to which was added a concentrated solution of disodium pamoate monohydrate (Table I).

Hippocratic Screening—Using the method of Malone and Robichaud (3), a gross observational screen was performed on the compounds using male and female, nonfasted, intact, unanesthetized rats[1] ranging in weight from 210–480 g. Qualitative and semiquantitative symptomatology after intraperitoneal injection was observed at the required time intervals (controls; 5, 10, 15, and 30 min.; 1, 2, 4, and 6 hr.; and 1, 2, 4, and 7 days) using the standardized worksheet (3) to record data. A constant injection volume of 5 ml./kg. was used with 0.25% agar as the diluting and suspending vehicle. All animals surviving the observational period of 1 week

[1] Wistar strain; E. G. Steinhilber, Oshkosh, Wis.

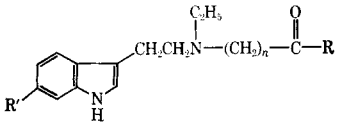


Table I—Amides and Esters of *N*-Ethyl-*N*-(3-indolylethyl)-ω-aminoalkyl Carboxylic Acid

Compound	Free Bases					Derivatives[b]		
	Procedure	*n*	R′	R	R_f Value[a]	M.p.	Calcd., % N	Found, % N
XIII	A	2	H	OCH_3	0.61	148–149.5°	13.95	14.13
XIV	A	2	H	OC_2H_5	0.59	148–149°	13.55	13.29
XV	A	2	H	$N(CH_3)_2$	0.46	166–167°	16.2	16.01
XVI	A	2	H	$N(C_2H_5)_2$	0.54	169–170°	15.45	15.19
XVII	A	2	OCH_3	OC_2H_5	0.61	155–156°	12.8	12.62
XVIII	A	2	OCH_3	$N(C_2H_5)_2$	0.54	174–176°	14.65	14.89
XIX	B	3	H	OCH_3	0.60	>250°	4.01	4.30
XX	B	3	H	OC_2H_5	0.61	>250°	3.94	4.24
XXI	B	3	H	$N(CH_3)_2$	0.53	>250°	5.92	5.99
XXII	B	3	H	$N(C_2H_5)_2$	0.56	>250°	5.79	5.70
XXIII	B	4	H	OCH_3	0.64	>250°	3.93	4.11
XXIV	B	4	H	OC_2H_5	0.66	>250°	3.86	3.75
XXV	B	4	H	$N(CH_3)_2$	0.41	>250°	5.80	5.57
XXVI	B	4	H	$N(C_2H_5)_2$	0.43	>250°	5.68	5.11

[a] R_f values are taken on thin-layer silica plates using 5% diethylamine–chloroform as a developer. [b] Compounds XIII–XVIII are picrates and XIX–XXVI are monosodium pamoates.

were sacrificed by cranio-vertebral dislocation, and a necropsy was performed to detect any grossly apparent latent toxicity. All test compounds as well as the reference materials were coded to conceal identity during screening for activity.

RESULTS AND DISCUSSION

The qualitative nature of the observable symptoms exhibiting dose–response relationships (1–2 rats/dosage level) permits tentative predictions as to possible pharmacologic relationships of the test compounds with reserpine or lysergic acid when these two reference compounds also have been screened by the same procedure. Reserpine- and lysergic acid-like activities can be differentiated easily by Hippocratic screening.

Rats receiving reserpine acetate (0.3–31 mg./kg.) are characterized by the relatively slow onset of miosis, enophthalmos, palpebral ptosis, pilomotor erection, tail grasping, hypothermia, diarrhea, reduction of motor activity, tremors, and passive reactions to both body grasp and head tap challenges (3). Stereotypy and screen grip loss are absent. While reserpinized animals assume statue positions (3), this is associated with profound sedation and decrease of motor activity.

The characteristic symptomatology produced by *d*-lysergic acid diethylamide tartrate (0.1–5.0 mg./kg.) is manifested as a rapid onset of profound mydriasis, pilomotor erection, and hypothermia associated with spontaneous statue positions and stereotypy at doses that do not affect the animal's motor performance significantly. At doses of 1.0–5.0 mg./kg., hyperactivity, hyperreflexia, and tremors are apparent as well as fearful–aggressive reaction patterns to body grasp and head tap challenges. Death following a dosage of 5.0 mg./kg. occurs within 30–45 min. and is associated with cardiac irregularities and general rigidity of musculature, while reserpine-induced lethality is of slow onset (8–24 hr.) and associated with loss of body weight (dehydration due to diarrhea), loss of skeletal muscle tone, and eventually respiratory arrest.

In this series of compounds, reserpinelike activity was totally absent. All methoxy derivatives (XIII, XIX, and XXIII) and ethoxy derivatives (XIV, XX, and XXIV) were essentially inactive, including Compound XVII. However, the diethylamino derivatives appeared to possess some qualitatively similar lysergic acid-like activity, with Compound XVI being the most potent, Compound XXII of intermediate potency, and Compound XXVI possessing only equivocal activity at dosages of 100–178 mg./kg. Compound XVI, in the equivocally effective-to-lethal dosage range of 1.0–10.0 mg./kg., produced dose–response patterns of strong mydriasis, hyperreflexia, tremors, hypothermia, increased motor activity, hyperemia of skin, and evidences for stereotypy and disorientation. Fearful reaction patterns to the head tap and body grasp challenges were observed uniformly. Compound XVII appeared to be active in the same dosage range as XVI and displayed the same qualitative symptomatology except that hyperthermia instead of hypothermia was recorded.

SUMMARY

Fourteen amides and esters of *N*-ethyl-*N*-(3-indolylethyl)-ω-aminoalkyl carboxylic acid were synthesized. These derivatives were formulated to represent a disjunctured reserpine and lysergic acid diethylamide molecule with the hope that the compounds more closely resembling reserpine would possess depressant activity and those resembling LSD would possess LSD-like activity. All synthesized compounds were found to be completely devoid of reserpinelike activity. Each amide showed various degrees of LSD-like activity. The molecule derived directly from the disjuncture of LSD, that is, the diethylamide of the compound where the alkyl chain possesses two carbons, was quantitatively the most potent, showing the desired activity at levels of 1.0–10.0 mg./kg. As the alkyl chain was lengthened, potency decreased. The dimethyl amides were active but to a lesser degree.

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NMR Studies on Acyl Migration in Diglyceride Solutions

R. J. WARREN and J. E. ZAREMBO

Abstract □ NMR spectroscopy has been used to study acyl migration in diglycerides. These studies show that chloroform solutions of 1,2-diglycerides give an equilibrium mixture of 1,2- and 1,3-diglycerides. The process is accelerated with increasing temperature. Under the same conditions, 1,3-diglycerides undergo no significant acyl migration. The NMR method has the advantage of providing qualitative and quantitative data on diglycerides as well as on related side-products such as monoglycerides and triglycerides. The data should be of particular interest to those attempting to synthesize pure isomeric forms of the diglycerides.

Keyphrases □ Acyl migration—in diglycerides, effect of temperature □ Diglycerides—acyl migration, using NMR □ NMR spectroscopy—structure, identity

The migration of acyl groups within the diglyceride molecule was reported by Crossley *et al.* (1) who utilized the technique of differential thermal analysis (DTA). The materials they studied were in the solid state and in solution. With the diglyceride solution, a catalyst in the form of hydrogen chloride was passed through ethereal solutions of the 1,2- and 1,3-diglycerides, and the diglycerides were recovered from solution at various time intervals and examined by DTA. The NMR method proposed here allows one to examine the diglycerides while still in solution, thus eliminating the possibility of change or transformation during the recovery process mentioned in the DTA experiments. The NMR spectrum of the solution also enables one to monitor the system for the formation of side-products such as monoglycerides and triglycerides.

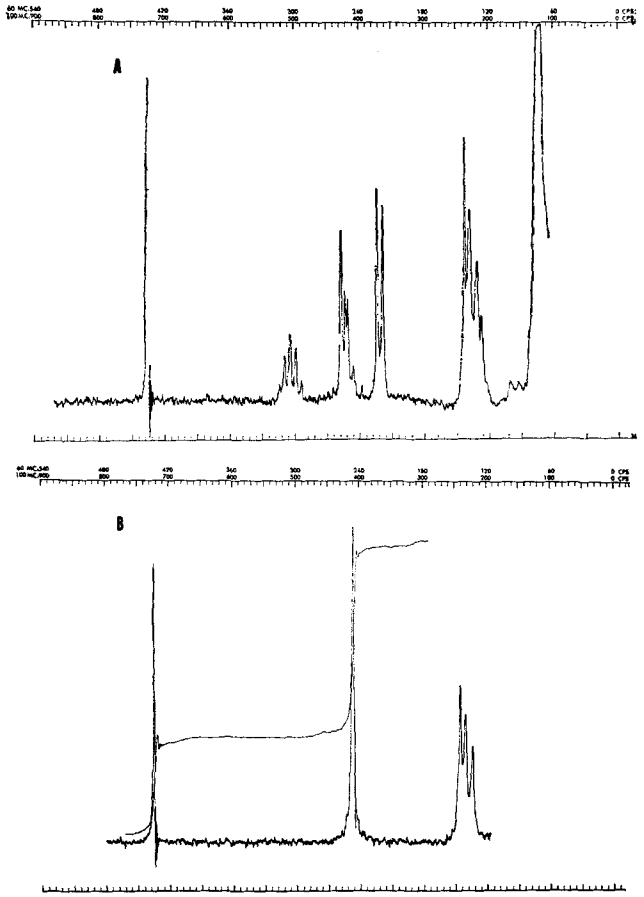

Figure 1—*NMR spectra of: A, 1,2-distearin; and B, 1,3-distearin.*

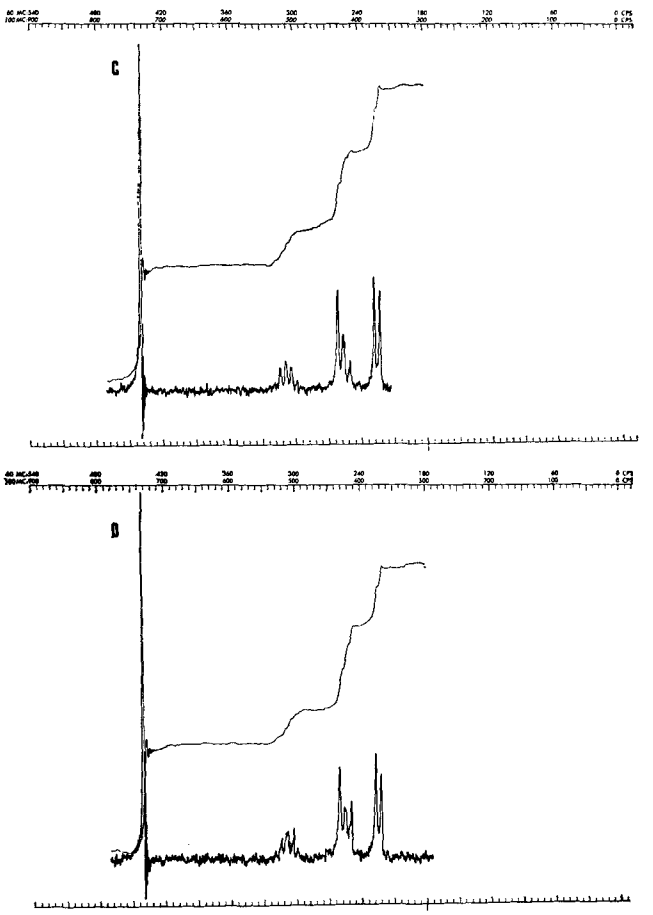

Figure 2—*NMR spectra of 1,2-distearin after being at 60° for several hours.*

In addition, the acyl migration observed in the present experiments took place without the addition of HCl catalyst, and the standard samples used in this study were examined by gas chromatography[1] and found to have no detectable free stearic acid present. These data should be important in the selection of experimental conditions for the synthesis of 1,2- and 1,3-diglycerides.

EXPERIMENTAL

All measurements were carried out with a Jeolco C60H NMR spectrometer equipped with a variable temperature probe.

Deuterated chloroform, with 3% $CHCl_3$ added, was used as

[1] Method to be published separately.

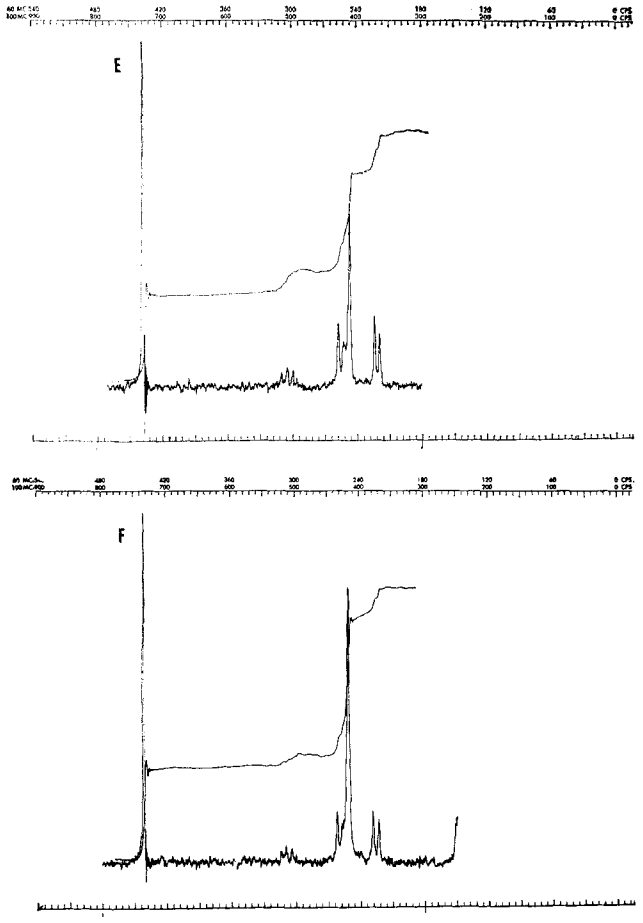

Figure 3—*NMR spectra of: E, 1,2-distearin after storage at 60°; and F, equilibrium mixture of 1,2- and 1,3-distearin.*

solvent. The spectra were recorded on solutions at concentrations of 80 mg./ml.

Samples of 1,2- and 1,3-diglycerides[2] were prepared for NMR analysis by dissolving 80 mg. of the diglycerides in 1 ml. of $CDCl_3$ solution. The spectra of the individual isomers were taken immediately after the samples were dissolved (Fig. 1). The integrated intensity of the band at 259 Hz. relative to the $CHCl_3$ internal standard was recorded. The NMR sample tube containing the 1,2-diglyceride in $CDCl_3$ solution was then transferred to a 60° constant-temperature bath. The sample was withdrawn at selected intervals, and the NMR spectrum was run and integrated.

Thin-layer chromatograms were made using 0.4 *M* boric acid/silica gel GF plates. The solvent system was chloroform–acetone–formic acid (96:4:0.4). The plate was equilibrated in the tank for 15 min. before development. For detection, the plate was sprayed with a 95:5 H_2SO_4–HNO_3 solution and then heated on a hot plate until black spots appeared.

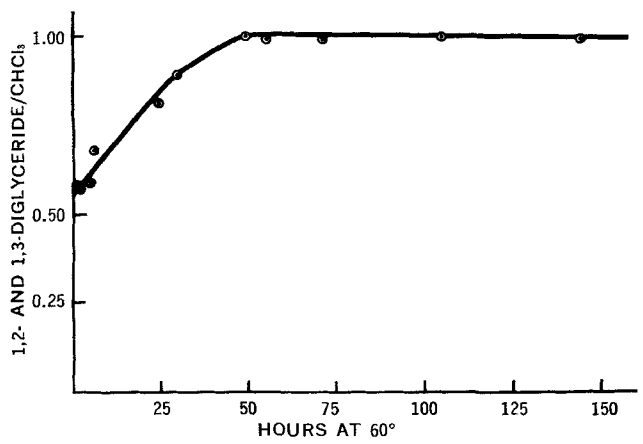

Figure 4—*Plot of amount of diglyceride* versus *time.*

[2] Obtained from the Supelco and Applied Science Laboratories.

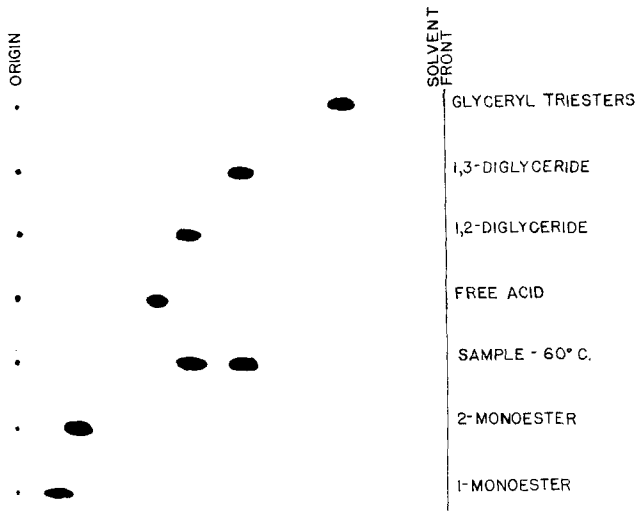

Figure 5—*TLC of glyceride esters.*

RESULTS AND DISCUSSION

The NMR technique has been shown to be applicable in the differentiation and quantitative analyses of 1,2- and 1,3-diglycerides (2). The region of the spectrum used for qualitative and quantitative analysis is 220 to 260 Hz. (3.6 to 4.4 p.p.m.) (Fig. 1). This area contains the absorption signals assignable to the five glyceryl protons in the diglyceride molecules (2). These protons are sensitive to changes within the molecule, such as acyl migration and isomer formation. Therefore, the NMR signals due to these protons afford the basis of a method for following acyl migration in the diglyceride molecules. The technique is quantitative as well as qualitative, and it can be used to calculate the amount of either isomer present at any given time.

The results of these experiments are shown in Figs. 2 and 3. A plot of the amount of 1,2-diglyceride *versus* time shows that, after approximately 50 hr., the solution reaches an equilibrium mixture (Fig. 4). The mixture contains 55% 1,3-distearin and 45% 1,2-distearin. There is no evidence for the formation of any side-products. A thin-layer chromatogram of the equilibrium mixture (Fig. 5) also shows the presence of only two components, namely, the 1,2- and 1,3-distearins. The same experiments at room temperature showed a very slow rate of acyl migration in the same system. The 1,3-diglyceride was found to undergo no significant acyl migration under these conditions.

CONCLUSION

NMR can be used to monitor acyl migration in diglyceride solutions under various conditions. The amount of migration at room temperature is small. Increasing temperature speeds up the migration, and at 60° an equilibrium mixture is attained in approximately 50 hr. The equilibrium mixture contains 55% 1,3-diglycerides and 45% 1,2-diglycerides. No side-products formed under these conditions.

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pKa Determinations Utilizing Solutions of 7-(2-Hydroxypropyl)theophylline

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Abstract □ A method exhibiting applicability in the evaluation of ionization constants of certain difficultly soluble compounds by potentiometric titration is submitted. The neutral xanthine derivative, 7-(2-hydroxypropyl)theophylline, is employed as an aid in dissolution of poorly soluble molecules, allowing their subsequent titration with acid or base in the usual manner. The procedure has been found suitable for compounds belonging to widely diversified chemical and structural classes. The success with which it has been used seems dependent on the tendency of 7-(2-hydroxypropyl)-theophylline to interact in aqueous solutions with compounds leading to their dissolution. This effect is reminiscent of the co-solvent properties of water-miscible agents such as ethanol. Examples of the utility of this treatment are given and pertinent data included as they apply to several structural prototypes.

Keyphrases □ 7-(2-Hydroxypropyl)theophylline solutions—pKa determinations □ pKa determinations—7-(2-hydroxypropyl)theophylline solutions □ Ionization-constant determination—using 7-(2-hydroxypropyl)theophylline, potentiometric titrimetry

The observation that 7-(2-hydroxypropyl)theophylline[1] and several other xanthine analogs have a solubilizing effect on the proteinaceous ergot alkaloids has recently been reported (1–3). When pKa determinations of the ergot alkaloids by conventional methods were unsuccessful, it was decided to attempt utilization of the xanthine effect to keep the alkaloids in solution during titration. The most useful of the xanthine analogs for this purpose was found to be 7-(2-hydroxypropyl)-theophylline (4). This compound has a high degree of water solubility (55%, 25°), is neutral in solution, and exhibits cosolventlike properties.

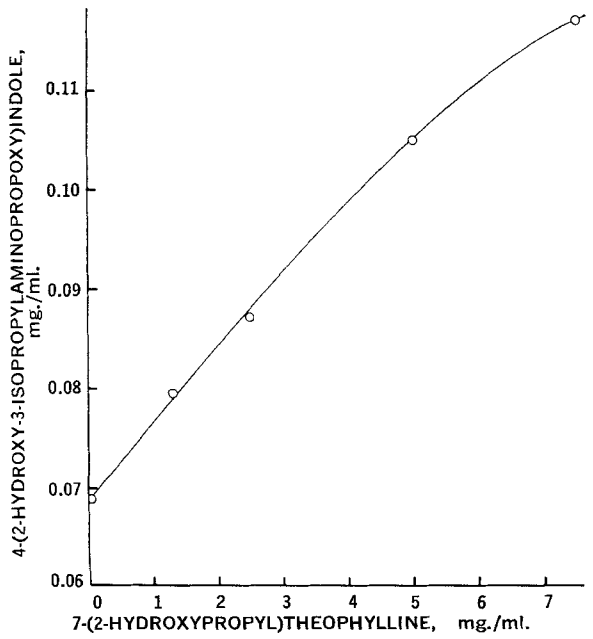

Figure 1—*Phase-solubility diagram for 4-(2-hydroxy-3-isopropylaminopropoxy)indole in distilled water at 30° for 24 hr. employing 7-(2-hydroxypropyl)theopylline as the solubilizing agent. Solubilities determined by the Van Urk procedure (12).*

[1] Referred to as 7-β-hydroxypropyltheophylline in previous reports.

Table I[a]—Approximate pKa Determination for 5-Allyl,5-isobutylbarbituric Acid[b] in 12% 7-(2-Hydroxypropyl)theophylline at 24°

Titrant, ml. (0.1 *N* KOH)	pH	Approximate pKa[c]
0.0	5.25	—
0.5	6.90	7.85
1.0	7.25	7.87
1.5	7.48	7.85
2.0	7.68	7.85
2.5	7.85	7.85
3.0	8.02	7.84
3.5	8.22	7.85
4.0	8.47	7.87
4.5	8.90	7.95
5.0	9.70	—

[a] These data are for titration of itobarbital in the neutral form (HA) with KOH as opposed to titration of the salt form as shown in Fig. 3. [b] 0.005 *M*. [c] Calculated from pKa = pH + log HA/A$^-$.

Several ergot alkaloids were successfully titrated, employing solutions of this xanthine, and their approximate pKa's were determined (4). Although it was assumed that this solubilizing effect was specific for ergot alkaloids, it was later observed that 7-(2-hydroxypropyl)theophylline can interact with a variety of structural types. These include not only compounds containing the lysergic acid nucleus but also derivatives of indole, derivatives of imidazothiazole, molecules containing the barbiturate nucleus, and perhaps others yet to be examined.

This study is concerned with this phenomenon and the scope of its applicability in the measurement of ionization constants of acidic and basic substances of pharmaceutical importance.

EXPERIMENTAL

Crystalline substances to be evaluated were dried 24 hr. at 40–50°, 1 mm. Hg. Solutions were constituted from freshly boiled distilled water (50 or 100 ml.) to which was added 7-(2-hydroxypropyl)theophylline (amounts of 2.5 to 30% w/v) along with 0.005 mole of the compound under study.

Solutions of acidic substances were prepared from 0.005 mole acid[2] in the form of its sodium or potassium salts. Sodium salts were obtained commercially, and potassium salts were produced *in situ* by addition of 5.0 ml. 0.1 *N* KOH to 0.005 mole acid. Amines were dissolved by addition of 0.5 ml. 1 *N* HCl to an equimolar quantity of amine by means of a micropipet.[3]

Titrations of amine hydrochlorides were carried out with 10 equal increments (0.5 ml.) of carbonate-free 0.1 *N* KOH (5). Ten equal portions of 1 *N* HCl (0.05 ml.) were employed in the case of sodium or potassium salts of acids (5). The pH was read initially and after

[2] In several cases, 0.01 *M* was used as well as 0.005 *M*.

[3] Ultraprecision micrometer syringe, Roger Gilmont Instruments, Inc., Great Neck, NY 11021

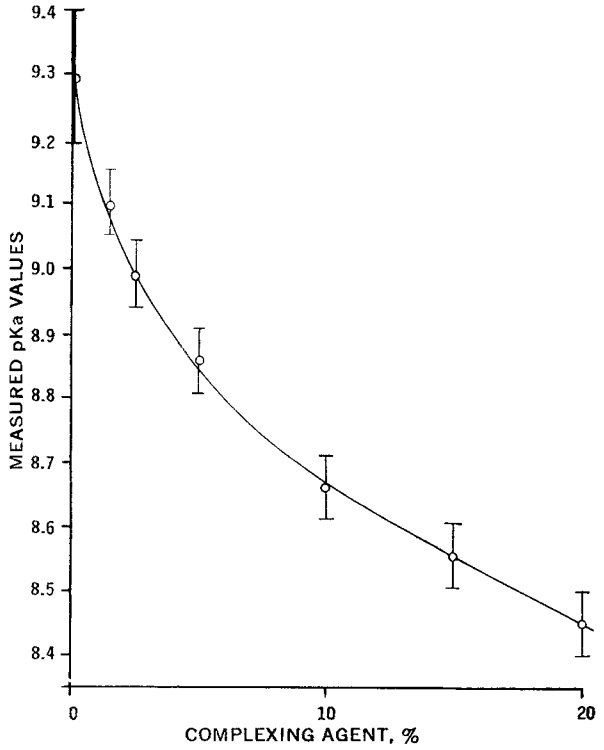

Figure 2—*Plot of pKa values* versus *percentage complexing agent [7-(2-hydroxypropyl)theopylline] for 3-(p-chlorophenyl)-5,6-dihydro-2-ethylimidazo[2,1-b]thiazole at 24°. The thermodynamic pKa value at zero xanthine concentration was determined spectrophotometrically.*

each portion of titrant. Nine values for the ionization constant were calculated.

The pH values were measured on a Metrohm pH meter using electrodes[4] standardized on 0.05 *M* potassium hydrogen phthalate (pH 4.0, 24°) and 0.05 *M* sodium borate (pH 9.20, 24°). Measurements were made at 24°. Electrodes were checked periodically with tris(hydroxymethyl)aminomethane (5).

MATERIALS

Itobarbital, 5-allyl,5-isobutylbarbituric acid,[5] was twice recrystallized from methanol–water and dried 24 hr. (1 mm. Hg), m.p. 137–138°. Phenobarbital[6] was twice recrystallized from methanol–water, m.p. 173–177°, following drying. 8-Bromotheophylline,[7] m.p. 320° dec., was not recrystallized prior to use. 7-(2-Hydroxypropyl)-theophylline,[8] m.p. 135–138°, was used in the form obtained. Tris(hydroxymethyl)aminomethane (THAM), primary standard,[9] was employed as a pKa standard; pKa = 8.18, 20° (5).

Other compounds studied were either analytically pure or used in the condition obtained from the manufacturer.

RESULTS AND DISCUSSION

The use of ethanol as an aqueous cosolvent was first reported by Mizutani (6) and has been utilized since in numerous cases with somewhat dubious success (5). Cellosolves (7) and other mixed solvent systems have been investigated and employed for certain purposes.

The solubilizing effect of xanthines on indole-containing molecules was observed in this laboratory (1–3). It was subsequently shown the dissociation constants could be determined for several

[4] Corning triple-purpose glass electrode No. 476020 and calomel electrode No. 476002, Corning Glass Works, Medfield, MA 02052
[5] Ganes Chemical Works, Inc., New York. N. Y.
[6] Mallinckrodt.
[7] Chemical Procurement Labs., Inc., College Point, NY 11356
[8] Ganes.
[9] Fisher Scientific Co.

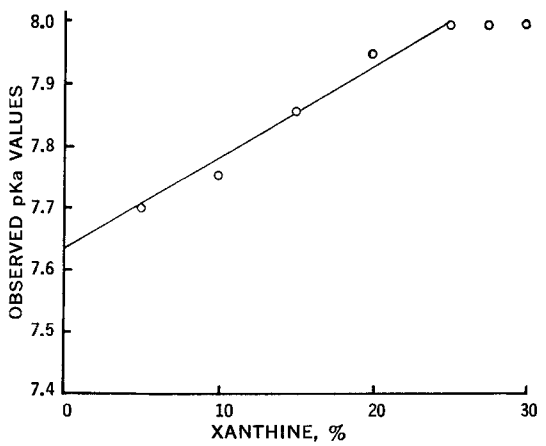

Figure 3—*Plot of pKa values against 7-(2-hydroxypropyl)theophylline concentrations for the titration of itobarbital (0.01 M) in 60-ml. volume at 24°.*

insoluble ergot alkaloids possessing the characteristic cyclic tripeptide moiety by addition of a xanthine for solubilization (4). This procedure has now been found suitable for several other types of compounds.

This method only offers a route toward approximation of ionization constants. The quantities of complexing agent which, of necessity, are added preclude evaluation of thermodynamic constants due to interference with activities of the species in solution.

The basic principle allowing these determinations is increased solubility of the acidic or basic substance in the presence of a complexing agent or cosolvent. The solubility effect as utilized in this procedure is illustrated in Fig. 1, where an almost twofold elevation of 4-(2-hydroxy-3-isopropylaminopropoxy)indole solubility occurs in the presence of 7-(2-hydroxypropyl)theophylline. These phenomena are indicative of an affinity between the two components. The exact chemical nature of this interaction has yet to be defined, but it appears to be a general one between xanthine and other relatively planar molecules.

Ionization constants were evaluated by titration of the hydrochloride salts of amines and sodium or potassium salts of acids in aqueous solutions (0.005–0.010 *M*) containing 7-(2-hydroxypropyl)-theophylline following the outline of Albert and Sergeant (5). The pKa values were calculated using the general Eqs. 1 and 2:

$$\text{pKa} = \text{pH} + \log \frac{\text{BH}+}{\text{B}} \qquad \text{(Eq. 1)}$$

$$\text{pKa} = \text{pH} + \log \frac{\text{HA}}{\text{A}-} \qquad \text{(Eq. 2)}$$

where BH+ and B are concentrations of amine salt and free base, respectively, and HA and A^- are concentrations of undissociated

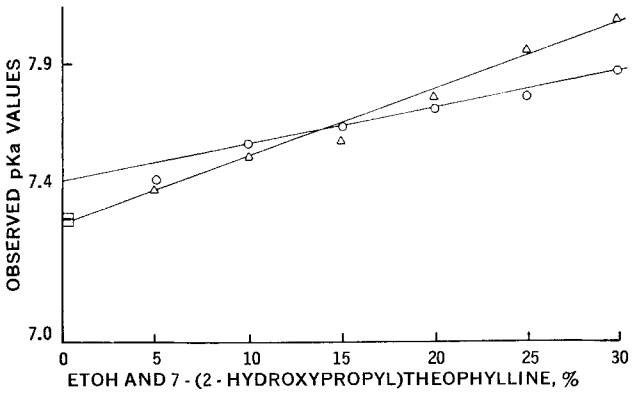

Figure 4—*The observed pKa values are plotted against the percentages of xanthine and ethanol employed for phenobarbital (0.005 M) in the form of the sodium salt titrated with 1.0 N HCl (titrated in 50-ml. volume). Key: O, 7-(2-hydroxypropyl)theophylline; and Δ, ethanol. Actual pKa is 7.41 (9).*

Table II—Ionization Constants[a] Determined by Titration Utilizing Solutions of 7-(2-Hydroxypropyl)theophylline

8-Bromotheophylline	5.45 (±0.1)
Theophylline	8.75 (±0.1)
3-(*p*-Chlorophenyl)-2-ethyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thioazol-3-ol	7.68 (±0.1)
3-(*p*-Chlorophenyl)-5,6-dihydro-2-ethylimidazo[2,1-*b*]thiazole	9.30 (±0.1)
5-Allyl,5-isobutylbarbituric acid	7.63 (±0.1)
4-(2-Hydroxy-3-isopropylaminopropoxy)-indole	9.65 (±0.1)
5-Ethyl,5-phenyl barbituric acid	7.52 (±0.1)
Phenylbutazone	4.70 (±0.2)
8-Chlorotheophylline	5.31 (±0.1)

[a] All values presented are obtained by extrapolation to zero concentration of 7-(2-hydroxypropyl)theophylline.

acid and anion, respectively (5). Nine values were calculated when possible, and the precision was acceptable (usually ±0.05) when pure, dry compounds were used. Table I lists the data from a typical experiment on itobarbital.

Amines and acids were both used in the salt forms in hope of achieving an ease of initial solubilization. Complexation occurs with both ionized and neutral species (1–4). The xanthine prevents precipitation of the relatively insoluble neutral molecule, allowing complete titration. Either the salts were available or prepared *in situ* by addition of equimolar quantities of KOH or HCl. Frequently, warm or hot water was added to the salt along with the complexing agent to attain complete solution. This was especially necessary at the xanthine concentrations below 10%. Titrations were carried out as rapidly as possible to prevent precipitation of supersaturated solutions occurring at lower xanthine concentrations.

Figures 2–4 are plots of pKa *versus* concentration of 7-(2-hydroxypropyl)theophylline and show that both apparently straight and curved lines are obtainable in these experiments, depending on the structure being studied.

Figure 2 gives the limits of precision for a thiazole derivative on a plot of pKa against percent xanthine, while Figs. 3 and 4 illustrate a seemingly linear case with barbituric acid derivatives. Extrapolation of the lines to zero additive concentration should give an approximation of the actual pKa values and does in these cases, with the respective thermodynamic pKa's of Figs. 2–4 being 9.30 (8), 7.70–7.80 (9), and 7.42 (10).

When itobarbital in the form of its sodium salt (0.01 *M*) is titrated with HCl, the xanthine causes a leveling of the observed pKa above 25% concentration; but the lower portion of the data may be extrapolated, and the value obtained gives a good estimation of the actual value (9). The pKa of phenobarbital was previously studied as a function of ethanol percentage (9). Figure 4 gives a comparison of the results when ethanol is used as a cosolvent relative to 7-(2-hydroxypropyl)theophylline. The data are quite similar for the same percentages weight in volume ratio; however, this may be circumstance because the molar ratios of the two are quite dissimilar. Figure 4 indicates that the xanthine gives qualitatively results that are like those of ethanol of equal percentage weight in volume ratio.

Table II summarizes several compounds investigated by this method and gives the extrapolated values as well as the precision of the results. A diversified group of compounds are listed; this procedure lends itself reasonably well to most pKa approximations. Neither diphenylhydantoin or its sodium salt could be treated in this manner due to its failure to dissolve.[10] The same problem arose with alcohol, precluding its use with the hydantoin, although the pKa was reported spectrophotometrically as 8.31 (11).

SUMMARY

The compound, 7-(2-hydroxypropyl)theophylline, is an interesting case of a solid alkaloid substance displaying cosolventlike properties. Its high solubility is unique, and structure modifications might lead to other molecules with exceptional properties, although unusual compounds, with respect to properties, exist in all classes.

The use of this method should be considered for compounds resisting standard procedures for pKa evaluation and as a supplementary method to confirm data obtained by other methods. It is a relatively quick process and shows the ability of this xanthine to interact with a wide variety of substances. No attempt was made to study the scope of these phenomena, and doubtless many other structural types will complex in this manner.

The experimental results do not always extrapolate to zero xanthine concentration in a linear manner, as is seen in Fig. 2. This does not seem to be a limiting factor when enough points are available to describe the general curvature of the line, and acceptable values are produced in the cases examined.

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[10] Diphenylhydantoin sodium would dissolve only at 25% concentration of 7-(2-hydroxypropyl)theophylline.

Effect of Plasticizers on Some Physical Properties of Cellulose Acetate Phthalate Films

R. R. CRAWFORD and O. K. ESMERIAN*

Abstract □ The effects of several plasticizers on the following properties of cellulose acetate phthalate films were studied: water vapor transmission rate, moisture absorption, enteric coating performance (minimum coating required on tablets to prevent gastrodisintegration), tablet-disintegration time, and water permeation. The addition of plasticizers generally lowered the water vapor transmission rate, moisture absorption, and water permeation but had little effect on tablet-disintegration time. However, addition of most plasticizers resulted in poorer enteric coating performance. Significant differences among the plasticizers were observed, and no one plasticizer was found to be superior overall.

Keyphrases □ Cellulose acetate phthalate films, physical properties—effect of plasticizers □ Plasticizers—effect on cellulose acetate phthalate films, physical properties □ Films, cellulose acetate phthalate—effect of plasticizers on physical properties

A polymeric system such as one containing cellulose acetate phthalate usually contains a plasticizer, a component that would be expected to alter one or more physical properties of the polymer. Since cellulose acetate phthalate was first suggested as an enteric coating (1), the material has been widely used in the pharmaceutical industry. However, only a limited amount of work has been reported concerning the effect of plasticizers on cellulose acetate phthalate. Malm *et al.* (2) studied the effect of various plasticizers on ion (sodium chloride) and dilute acid (hydrochloric) permeation and concluded that diethyl phthalate was a preferred plasticizer. Water vapor permeation data were obtained on unplasticized cellulose acetate phthalate (3) and on cellulose acetate phthalate plasticized with polyethylene glycol (4) or with several water-insoluble plasticizers at low levels (5). In the two latter studies, the authors found a decrease followed by an increase in water vapor transmission rate as the plasticizer level was increased.

This study was designed to: (*a*) obtain information on the effect of various plasticizers on some properties of free films of cellulose acetate phthalate, and (*b*) examine the effect of these plasticizers on cellulose acetate phthalate when coated on tablets. Specifically, the following properties were studied: (*a*) water vapor transmission rates of thin films, (*b*) moisture absorption of thin films, (*c*) enteric coating performance, defined as the minimum amount of coating required to protect tablets from disintegrating in gastric fluid, (*d*) disintegration time, *in vitro*, of coated tablets, and (*e*) liquid water permeation of coated tablets.

Seven plasticizers were selected for study: diethyl phthalate, triacetin, fully acetylated monoglycerides,[1] butyl phthalyl butyl glycolate,[2] tributyl citrate, partially acetylated monoglycerides,[3] and castor oil.

[1] Myvacet, distilled acetylated monoglycerides type 9-40, Eastman Chemical Products, Inc., Kingsport, Tenn.
[2] Santicizer B-16, Monsanto Co., St. Louis, Mo.
[3] Myvacet, distilled acetylated monoglycerides type 5-07, Eastman Chemical Products, Inc., Kingsport, Tenn.

Table I—Water Vapor Transmission Rate with Different Plasticizers

Plasticizer[a]	Water Vapor Transmission Rate[b]
None	0.520
Diethyl phthalate, %	
10	0.359
20	0.315
30	0.307
Triacetin, %	
10	0.311
20	0.327
30	0.327
Castor oil, %	
10	0.524
20	0.489
30	0.504
Fully acetylated monoglycerides, %	
10	0.504
20	0.382
30	0.425
Butyl phthalyl butyl glycolate, %	
10	0.288
20	0.307
30	0.276
Tributyl citrate, %	
10	0.386
20	0.351
30	0.386

[a] Plasticizer level as percent of cellulose acetate phthalate. [b] g. × cm./m.²/24 hr.

EXPERIMENTAL

Preparation of Cellulose Acetate Phthalate Solutions—Solutions were prepared in acetone to contain 15% w/w total solids. The appropriate amounts of cellulose acetate phthalate and plasticizers were dissolved in a portion of the acetone. The remainder of the solvent was added to complete the preparation.

Preparation of Thin Films—The solutions were poured onto glass plates, and films were drawn with a doctor blade adjusted so that dried films of about 0.0033-cm. thickness were obtained. After about 1 hr., the films were stripped from the glass and dried for 24 hr. under ambient conditions before use.

Water Vapor Transmission Rates—Two-ounce flint-glass bottles were uniformly filled with a commercial desiccant[4] to within 5 mm. of the top. Disks punched from the cured films (3.2 cm.) were sealed to the bottle lips with silicone grease. Caps, from which 2.5-cm. disks had been removed, were then placed on the bottles.

The bottles were placed in a desiccator containing water saturated with ammonium nitrate at 25° (63% R.H.). The bottles were periodically weighed, and plots were made of weight increase *versus* time. Water vapor transmission rates were calculated from the formula:

$$\text{rate} = \frac{WL}{S} \qquad \text{(Eq. 1)}$$

where W = weight increase/24 hr., g./24 hr., from the straight-line portion of weight gain *versus* time; L = film thickness, centimeters, measured with an Ames dial comparator after W was determined; and S = area of the exposed film in square meters.

Moisture Absorption—Strips, 2.5 × 7.6 cm., were cut from the cured film and dried under vacuum in the presence of the commercial

[4] Drierite, W. A. Hammond Drierite Co., Xenia, Ohio.

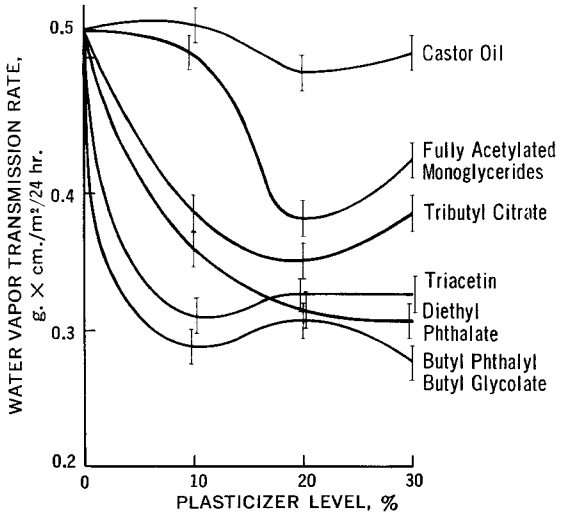

Figure 1—*Water vapor transmission rates of plasticized cellulose acetate phthalate. (Vertical lines represent estimated standard deviation of the measurements.)*

desiccant to constant weight. The strips were placed under 25°/63% R.H. conditions until a constant weight was again obtained. The moisture absorption was calculated as follows:

$$\% \text{ absorption} = \frac{\text{conditioned weight} - \text{dry weight}}{\text{dry weight}} \times 100 \quad \text{(Eq. 2)}$$

Tablet Coating—Fifteen pounds of tablets [dicalcium phosphate, 300 mg., 0.87 cm. ($^{11}/_{32}$ in.) deep concave punches; lactose, 230 mg., 0.87 cm. ($^{11}/_{32}$ in.) deep concave punches] were coated per run in a 40.64-cm. (16-in.) spherical coating pan. Coating solutions were prepared using 10% w/w cellulose acetate phthalate plus plasticizer in acetone. The tablets were spray coated with the solutions, using an airless unit[5] with a 1-sec. spray/10-sec. dry sequence. Samples of the tablets were removed at 5-min. intervals for testing.

Tablet-Disintegration Time—Tests were performed according to the USP XVII Tablet-Disintegration Test, except that the simulated intestinal fluid contained no pancreatin.

Percent Coating Weight—Ten coated tablets were placed in acetone to remove the coatings. The tablets were dried for 16 hr. at 37°, and the average core weight was determined. The coated weights of the tablets were obtained as described under *Liquid Water Permeation*. The percent coating weights were calculated as follows:

$$\% \text{ weight} = \frac{\text{coated weight} - \text{core weight}}{\text{core weight}} \times 100 \quad \text{(Eq. 3)}$$

Liquid Water Permeation—Ten coated tablets (lactose) were dried for 16 hr. at 37°. The tablets were weighed, immersed in distilled water, and reweighed after 1 and 4 hr. Since tablets of identical size were used, water permeation was proportional to percent weight increase, which was calculated as follows:

$$\% \text{ weight increase} = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}} \times 100 \quad \text{(Eq. 4)}$$

RESULTS AND DISCUSSION

Water Vapor Transmission Rate—The results summarized in Table I show that the selection of plasticizer was more important than the level. Castor oil, for example, had essentially no effect on the water vapor transmission rate, but the use of butyl phthalyl butyl glycolate resulted in a rate less than 60% that of unplasticized cellulose acetate phthalate. Conversely, the level of plasticizer (10–30%) had considerably less effect on the rate. These data are illustrated in Fig. 1. Minimum water vapor transmission rates were found for five of the six plasticizers. The minima for three of these

[5] Versa-Spray, Nordson Corp.

Table II—Moisture Absorption with Different Plasticizers

Plasticizer	Moisture Absorption, %
None	2.8
Diethyl phthalate, %	
10	2.1
20	1.6
30	0.3
Triacetin, %	
10	1.2
20	−1.6
30	−6.8
Castor oil, %	
10	1.1
20	1.2
30	1.2
Fully acetylated monoglycerides, %	
20	1.7
30	1.8
Butyl phthalyl butyl glycolate, %	
10	1.0
20	1.3
30	2.0
Tributyl citrate, %	
20	2.4
30	2.8

(castor oil, fully acetylated monoglycerides, and tributyl citrate) occurred at a 20% level and for the remaining two (triacetin and butyl phthalyl butyl glycolate) at 10%. Also, it appeared that the rate for butyl phthalyl butyl glycolate may have had more than one point of inflection.

Moisture Absorption—In addition to the water vapor transmission rate, the moisture absorption of cellulose acetate phthalate was affected by the choice of plasticizer (Table II). The level of plasticizer was also an important factor. Interesting, too, are the different effects of the plasticizer level. As the level of diethyl phthalate, for example, was increased, the moisture absorption, as perhaps expected, decreased (from 2.1% at 10% to 0.3% at 30%). The opposite occurred, however, with butyl phthalyl butyl glycolate; the minimum absorption was found at a level of 10% or lower. The third type of effect, that of insensitivity toward plasticizer level, was noted with castor oil. The unexpected results for triacetin warrant some mention; a loss in weight rather than an increase was observed. This is not understood but it may reflect hydrolysis to, and evaporation of, acetic acid and would predict low plasticizer permanence.

The lowest moisture absorption was found with 30% diethyl phthalate. The low value for unplasticized cellulose acetate phtha-

Table III—Tablet-Coating Performance

Plasticizer	Percent Weight of Coating—Uncorrected[a] Lactose	Uncorrected[a] Dical	Corrected[b] Lactose	Corrected[b] Dical	Disintegration Time (min.) in Intestinal Fluid—Lactose	Intestinal Fluid—Dical
None	7.8	2.3	7.8	2.3	14	6
Diethyl phthalate, 25%	12.3	4.6	9.8	3.7	10	6
Triacetin, 20%	18.8	2.6	15.7	2.2	18	6
Fully acetylated monoglycerides, 20%	5.3	3.7	4.4	3.1	12	6
Partially acetylated monoglycerides						
10%	12.3[c]	1.6	11.2[c]	1.5	—	3
30%	12.7[c]	2.0	10.6[c]	1.5	—	4
Butyl phthalyl butyl glycolate						
10%	5.6	3.6	5.1	3.3	12	6
20%	8.3	3.8	6.9	3.2	14	5
30%	8.5	4.0	6.5	3.1	13	7
Tributyl citrate, 20%	12.7	2.8	11.6	2.3	16	4

[a] Total minimum amount of coating. [b] Amount of cellulose acetate phthalate only. [c] A spray nozzle of reduced capacity was required for the formula containing partially acetylated monoglycerides. This run was discontinued after an inordinately long coating time (5 hr. *versus* 3 hr. in the case of the other runs). These tablets disintegrated in simulated gastric fluid.

Table IV—Liquid Water Permeation of Coated Lactose Tablets

Plasticizer	Percent Weight Gain after 1 hr.	4 hr.
None	1.9	5.9
Diethyl phthalate, 25%	0.73	1.3
Triacetin, 20%	1.9	6.1
Fully acetylated monoglycerides, 20%	0.95	2.4
Butyl phthalyl butyl glycolate		
10%	1.0	2.4
20%	1.1	2.6
30%	0.47	1.0
Tributyl citrate, 20%	0.81	2.9

late, however, suggests that this parameter normally need not be a prime criterion in selecting a plasticizer.

Enteric Coating Performance—Table III summarizes the comparison of tablet coating performance among the plasticizers. The uncorrected columns report the minimum amount of total coating weight required to prevent tablet disintegration for 1 hr. in simulated gastric fluid. The corrected columns report the same data calculated in terms of cellulose acetate phthalate only.

Overall, considerably more coating was required to protect the lactose tablets. While this may be due in part to a difference in tablet porosity, the large differences for most plasticizers suggest that the type of tablet base is indeed of significance.

Greater amounts of coating generally were required when plasticizers were added to cellulose acetate phthalate. Among the six plasticizers tested on lactose, only fully acetylated monoglycerides and butyl phthalyl butyl glycolate performed better than unplasticized cellulose acetate phthalate. On dicalcium phosphate, only partially acetylated monoglycerides performed better.

Furthermore, the plasticizers that promoted good performance on one type of tablet base performed poorly on another base. Partially acetylated monoglycerides, for example, performed excellently on dicalcium phosphate but poorly on lactose. Similar (though opposite) results were found for fully acetylated monoglycerides.

These data show that a plasticizer may be carefully chosen to promote good performance on a given tablet substrate and yet may not perform well on a different substrate.

Tablet-Disintegration Time—Table III also summarizes the disintegration times in simulated intestinal fluid and shows that the plasticizers had little effect.

Liquid Water Permeation—Most enteric coated tablets are finished with sugar coatings. During this operation, the cellulose acetate phthalate film is most likely in contact with water. Transport through the film is therefore by means of liquid water in addition to water vapor permeation. Table IV illustrates the weight increases of lactose tablets after immersion in water (the values again reflect data on tablets coated with the minimum amounts of coating to resist disintegration in simulated gastric fluid for 1 hr.).[6] Varying amounts of water permeation were observed; diethyl phthalate and 30% butyl phthalyl butyl glycolate contributed to the most effective barriers.

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[6] The results would, of course, be inaccurate if some lactose was lost by diffusion through the coating. It was determined by reweighing tablets, dried for 16 hr. at 37° after exposure to water (25°) for 6 hr., that this did not occur.

Facile Isolation of Phenylephrine from Syrups

LESTER CHAFETZ, CHARLES A. GAGLIA, Jr., TSUN-MING CHEN, and CHRISTINA MORAN

Abstract □ Although phenylephrine HCl is easily isolated from most of its dosage forms, the high water solubility of both the base and salt forms often makes its isolation from syrups and elixirs difficult. The partition coefficient for extraction of phenylephrine from water to *n*-butyl alcohol is unfavorable, but the drug can be salted into the organic solvent with an excess of sodium chloride and recovered from butanol by extraction with aqueous alkali. Interference to spectrophotometric determination caused by certain coal tar dyes can be circumvented by preliminary extraction of them as ion pairs with quaternary salts. Recovery of at least 98.0% and excellent precision were obtained in trials of the procedure with syrup USP and an elixir placebo as the test vehicles.

Keyphrases □ Phenylephrine—isolation from syrups □ Syrups—separation, isolation of phenylephrine □ UV spectrophotometry—analysis

Phenylephrine HCl is extensively formulated with other drugs in a variety of liquid and solid dosage forms. Because both the free base and salt forms of the drug are very water soluble, its isolation from excipients and many coformulated drugs often can be accomplished by simple solvent extraction of interferences from a dilute acid or base extract of the product. With syrups and elixirs, however, separation of phenylephrine to provide an unaltered UV spectrum is often difficult.

Several chromatographic methods have been described for isolation of phenylephrine from its dosage forms. Schriftman separated it by paper chromatography (1) and electrophoresis (2). Kelly and Auerbach (3) and Montgomery *et al.* (4, 5) used ion-exchange chromatography. Clark and Rosenberg (6) eluted phenylephrine as its diacetyl derivative, formed on a partition chromatography column, and then determined it spectrophotometrically after hydrolysis of the acetyl functions. Levine and Doyle (7) described the partition chromatographic separation of phenylephrine as its ion pair with di-(2-ethyl)hexylphosphoric acid. Ponder (8) exploited the observation that phenyl-

Table IV—Liquid Water Permeation of Coated Lactose Tablets

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Diethyl phthalate, 25%	0.73	1.3
Triacetin, 20%	1.9	6.1
Fully acetylated monoglycerides, 20%	0.95	2.4
Butyl phthalyl butyl glycolate		
10%	1.0	2.4
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Table I—Partition of Phenylephrine HCl between Butanol and Aqueous Systems

Aqueous phase:	Water[a]	Water	pH 8.4	pH 10.0	pH 11.0	Syrup USP
% in butanol:	7	70	78	84	78	38

[a] All but this sample were saturated with excess sodium chloride.

ephrine base can be extracted with ether from saturated sodium chloride solution, and he described a partition chromatography method which requires elution of 1 mg. of drug with 300 ml. of ether. All of these methods are time consuming and tedious.

Shore and Olin (9) found that epinephrine and norepinephrine can be extracted to the extent of about 65% with 10 vol. of *n*-butyl alcohol from 1 vol. of sodium chloride-saturated aqueous phase, although these catecholamines are poorly extracted into butanol from aqueous solution in the absence of salt. Phenylephrine, the monophenolic analog of epinephrine, has a much better partition coefficient between butanol and aqueous salt solution, 70% of it being extracted by an equal volume. A study of the partition behavior of phenylephrine in this system was undertaken with the objective of providing a simple solvent extraction method for separation of the drug from syrups and elixirs. Such a procedure is reported here.

EXPERIMENTAL

Reagents and Supplies—All of the reagents used were official grades or equivalent in quality. Ammonia buffer was prepared by adjusting the pH of 1 *N* ammonium hydroxide solution to 10.0 ± 0.1 with concentrated hydrochloric acid. Salt-saturated butanol was prepared by equilibrating *n*-butyl alcohol with saturated aqueous sodium chloride solution. Cetylpyridinium chloride was used to remove dye interferences. The method was tested by adding phenylephrine HCl to each of two vehicles to obtain concentrations of about 1 mg./ml. The test vehicles were syrup USP (85% aqueous sucrose) and an elixir vehicle which had been available as a clinical test placebo for an experimental formulation. The latter contained sucrose, sorbitol, glycerin, alcohol, citrate, artificial sweeteners, flavors, FD&C Red No. 2, FD&C Yellow No. 5, and D&C Green No. 5. Spectra were determined in 1-cm. cells in a Cary model 14 recording spectrophotometer.

Procedure—Transfer exactly 2.0 ml. of syrup or elixir to a 125-ml. separator, and add 2 ml. of water. If the preparation is too viscous to afford convenient pipeting, dilute it quantitatively with an equal volume of water and then transfer exactly 4.0 ml. of the dilution to a separator. Add about 2 g. of sodium chloride and 1 ml. of ammonia buffer, and extract the mixture with two 50-ml. portions of salt-saturated butanol. Combine the butanol extracts in a 250-ml. separator, and extract them with successive 35-, 30-, and 30-ml. portions of 1 *N* sodium hydroxide, collecting the alkali extracts in a 100-ml. volumetric flask. Dilute the solution to volume with alkali.

Concomitantly scan the UV absorption spectra of the solution and of a standard preparation containing about 2 mg. of phenylephrine hydrochloride reference standard USP, accurately known, per 100 ml. of 1 *N* sodium hydroxide solution in 1-cm. cells in a suitable spectrophotometer from 350 to 250 nm., using 1 *N* alkali as the blank. If the spectrum of the solution from the assay preparation matches that from the standard preparation, calculate the content of $C_9H_{13}NO_2 \cdot HCl$, in milligrams per milliliter, from the relation $50C(A_U/A_S)$, where C is the concentration in milligrams per milliliter of phenylephrine HCl in the standard preparation, and A_U and A_S are the absorbances of the solution from the assay preparation and the standard preparation, respectively, determined at the absorption maximum at about 292.5 nm.

If the spectrum of the assay preparation shows the presence of interfering colorants, the following expedient has been shown to be effective with most dyes permitted for use in U. S. drug preparations. Add about 10 mg. of cetylpyridinium chloride to a mixture of 2.0 ml. of the elixir or syrup and 2 ml. of water in a 125-ml. separator, and extract the mixture with successive small portions of chloroform until the aqueous layer is colorless. Discard the extracts and follow the method already described beginning with: "Add about 2 g. of sodium chloride. . .."

Table II—Recovery of 1.00 mg./ml. Phenylephrine HCl Added to Two Vehicles

Trial	Sample Found, mg. per ml.	
	Syrup USP	Elixir Placebo
1	0.980	0.985
2	0.980	0.980
3	0.9825	0.9975
4	0.9775	0.9875
5	0.980	0.9875
Mean recovery, %	98.0	98.75
Relative standard deviation, %	0.2	0.6

RESULTS AND DISCUSSION

Partition Behavior of Phenylephrine—The data in Table I were obtained by equilibrating equal volumes of mutually saturated aqueous solutions and butanol containing known amounts of phenylephrine HCl and an excess of sodium chloride. The amount of phenylephrine extracted was determined spectrophotometrically. The phenomenon appears to be simple "salting out," because sodium sulfate and other salts may be substituted for sodium chloride with equivalent results. The relatively low extractability of the drug from syrup USP may be attributed to lower salt solubility in the concentrated sugar solution; however, this effect is minimized in practice since the syrup is diluted 2 in 5 in the procedure.

Interferences—The effect of possible phenylephrine degradation products was not evaluated in this study. (Stability considerations in phenylephrine assay will be the subject of a future communication from these laboratories.) Colorants interfered in the determination of phenylephrine added to the elixir placebo used; this interference was circumvented by extracting the sulfonate dyes as ion pairs with cetylpyridinium chloride.[1] Experiments with the certified dyes permitted in U. S. drug preparations showed that all except FD&C Blue No. 1, FD&C Blue No. 2, FD&C Red No. 4, and D&C Red No. 33 could be removed by this method. Interference by coformulated drugs and excipients in many cases can be circumvented by solvent extraction from acid or basic solution. An unidentified component of a proprietary flavor mixture interfered in application of the method described here to a syrup placebo. In such cases, the highly selective periodate oxidation method described by Chafetz (10) can be used. Although periodate oxidation is inapplicable to many syrup formulations because the sugar reduces the oxidant, such products can be assayed by this method when the drug is isolated by the procedure described in this article.

Precision and Recovery—Application of the method to syrup USP and the elixir placebo resulted in the data shown in Table II. Concomitant extraction of a standard improves the recovery; however, a recovery of not less than 98.0% and not more than 102.0% is this laboratory's acceptance criterion.

SUMMARY

Phenylephrine can be isolated from many elixir or syrup preparations by saturating a diluted sample buffered to pH 10.0 with sodium chloride and extracting with salt-saturated butanol. The drug can be recovered from the butanol phase by extraction with *N* sodium hydroxide for subsequent analysis by spectrophotometry or other means. Certain colorants which interfere in the extraction scheme can be removed by ion-pair formation with a quaternary ammonium salt such as cetylpyridinium chloride.

[1] This technique was demonstrated to Lester Chafetz in 1958 by M. E. Auerbach, who used benzalkonium chloride as the quaternary amine component.

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These observations prompted the present survey of the antibacterial activity of a series of Mannich bases, including those reported by Khullar and Chatten (7).

EXPERIMENTAL

Materials—All Mannich bases employed in this study were synthesized by Method A, as reported earlier (7), and are listed in

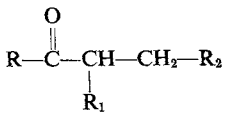

Table I—Mannich Bases Employed in This Study

Compound Number	R	R_1	Melting Ranges, R_2 = 2,6-dimethylmorpholino	R_2 = morpholino	R_2 = piperidino	R_2 = 3-methylpiperidino
1	Me—	H	156–157° (7)[a]	—	175°	—
2	C_6H_5—	H	195–196.5° (7)	186.5° (11)	191–192° (11)	172–174° (11)
3	*p*-Me—C_6H_4—	H	207–208° (7)	224–225° (12)	176.5° (11)	177–178°
4	*p*-MeO—C_6H_4—	H	203–205° (7)	209–210° (11)	209–211° (14)	—
5	*p*-Cl—C_6H_4—	H	203.5–204° (7)	201–202°	187°	186–187.5°
6	*p*-O_2N—C_6H_4—	H	203–204° (7)	206° (13)	193–195° (13)	—
7	*m*-O_2N—C_6H_4—	H	196–197° (7)	186–187° (11)	178–179.5° (15)	—
8	*o*-O_2N—C_6H_4	H	—	192–193°	—	—
9	C_6H_5CH=CH—	H	196–197° (11)	—	—	—
10	C_6H_5—	Me	187–188.5° (7)	—	—	—
11		H	—	200–201.5°	186.5–187.5°	—

[a] Number in parentheses is reference to previously reported melting point.

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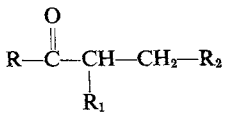

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2	C_6H_5—	H	195–196.5° (7)	186.5° (11)	191–192° (11)	172–174° (11)
3	*p*-Me—C_6H_4—	H	207–208° (7)	224–225° (12)	176.5° (11)	177–178°
4	*p*-MeO—C_6H_4—	H	203–205° (7)	209–210° (11)	209–211° (14)	—
5	*p*-Cl—C_6H_4—	H	203.5–204° (7)	201–202°	187°	186–187.5°
6	*p*-O_2N—C_6H_4—	H	203–204° (7)	206° (13)	193–195° (13)	—
7	*m*-O_2N—C_6H_4—	H	196–197° (7)	186–187° (11)	178–179.5° (15)	—
8	*o*-O_2N—C_6H_4	H	—	192–193°	—	—
9	C_6H_5CH=CH—	H	196–197° (11)	—	—	—
10	C_6H_5—	Me	187–188.5° (7)	—	—	—
11		H	—	200–201.5°	186.5–187.5°	—

[a] Number in parentheses is reference to previously reported melting point.

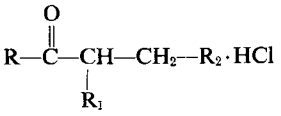


Table II—Antibacterial Activity of Certain Mannich Bases against *E. coli*

Compound Number	R	R_1	Zone of Inhibition, mm. ([a]Dilution, mg./0.1 ml.) R_2 = 2,6-dimethylmorpholino	R_2 = morpholino	R_2 = piperidino	R_2 = 4-methylpiperidino
1	Me—	H	15.8 (1.25)		13.4 (10.0)	
2	C_6H_5—	H	17.5 (1.25)	19.1 (0.625)	14.5 (5.0)	13.5 (5.0)
3	*p*-Me—C_6H_4—	H	25.5 (1.25)	13.9 (0.625)	13.2 (5.0)	11.5 (5.0)
4	*p*-MeO—C_6H_4—	H	—	15.1 (1.25)	Growth (10.0)	—
5	*p*-Cl—C_6H_4—	H	16.0 (0.625)	20.6 (0.625)	13.3 (2.50)	9.6 (0.625)
6	*p*-O_2N—C_6H_4—	H	11.7 (0.625)	—	10.6 (1.25)	—
7	*m*-O_2N—C_6H_4	H	15.6 (5.0)	12.4 (5.0)	8.5 (2.50)	—
8	*o*-O_2N—C_6H_4—	H	—	16.6 (0.625)	11.7 (1.25)	—
9	C_6H_5—CH=CH—	H	11.0 (0.625)	—	—	—
10	C_6H_5—	Me	14.9 (10.0)	—	—	—
11		H	—	15.7 (0.625)	17.9 (10.0)	—

[a] Figures in parentheses represent lowest dilution.

Table I. References to previous literature reports for these substances also are cited wherever available. The following compounds have not been reported previously:

1. Piperidinoethyl methyl ketone HCl—*Anal.*—Calcd. for $C_9H_{18}ClNO$: C, 56.38; H, 9.46; N, 7.31. Found: C, 56.42; H, 9.50; N, 7.31.
2. Morpholinoethyl *o*-nitrophenyl ketone HCl—*Anal.*—Calcd. for $C_{13}H_{17}ClN_2O$: C, 52.00; H, 5.66; N, 9.33. Found: C, 51.30; H, 5.82; N, 9.47.
3. Morpholinoethyl-2-furyl ketone HCl—*Anal.*—Calcd. for $C_{11}H_{16}ClNO_3$: C, 53.76; H, 6.56; N, 5.71. Found: C, 53.60; H, 6.71; N, 5.91.
4. Morpholinoethyl-4-chlorophenyl ketone HCl—*Anal.*—Calcd. for $C_{13}H_{17}Cl_2NO_2$: C, 53.76; H, 5.86; N, 4.82. Found: C, 54.44; H, 6.21; N, 4.83.
5. Piperidinoethyl-4-chlorophenyl ketone HCl—*Anal.*—Calcd. for $C_{14}H_{19}Cl_2NO$: C, 58.33; H, 6.60; N, 4.86. Found: C, 58.39; H, 7.06; N, 4.89.
6. 3-Methylpiperidino-4-chlorophenyl ketone HCl—*Anal.*—Calcd. for $C_{15}H_{21}Cl_2NO$: C, 59.60; H, 6.95; N, 4.64. Found: C, 59.64; H, 7.39; N, 4.50.
7. 3-Methylpiperidino-4-methylphenyl ketone HCl—*Anal.*—Calcd. for $C_{16}H_{24}ClNO$: C, 68.21; H, 8.53; N, 4.97. Found: C, 67.88; H, 8.36; N, 5.19.

The benzalacetone employed for the synthesis of Compound 9 in Tables II and III was obtained from benzaldehyde and acetone in the presence of sodium hydroxide, according to the procedure of Drake and Allen (8).

Microbiological Methods—The test microorganisms used in these experiments were *Staphylococcus aureus* (A.T.C.C. 6538) and *Escherichia coli* (A.T.C.C. 10536). These organisms were chosen because they represent the two principal groups of bacteria commonly causing human infections, namely, the Gram-positive cocci and the Gram-negative bacilli. Furthermore, these particular strains are routinely used in microbiological assays of disinfectants, antiseptics, and antibiotics. The organisms were maintained on nutrient agar[1] slants and transferred once a week between tests.

Qualitative Tests—These were done to determine whether a substance is capable of inhibiting the growth of the test microorganisms.

To ensure that the majority of the cells being tested were of the same physiological age, *i.e.*, in the same growth phase, immediately prior to each experiment the test microorganisms were transferred from the agar slant to 5 ml. of nutrient broth.[1] The inoculated broth was incubated 24 hr. at 37.5°, and a loopful of the culture was transferred to a second tube of broth. The second broth culture was incubated for 24 hr., and 0.1 ml. of this culture was then used to inoculate the surface of a nutrient agar[2] plate in such a manner as to produce confluent growth when the medium was subsequently incubated.

A small amount of each compound in powder form was placed on the inoculated surface of the medium. No attempt was made to measure the amount of the compound used in these tests. Five compounds were tested per plate of medium. Upon incubation for 24 hr. at 37.5°, the presence or absence of a zone of inhibition surrounding the test substances was noted as was any alteration in the character or appearance of the medium. Compounds exhibiting antibacterial activity were set aside for quantitative testing.

Quantitative Tests—These were done to establish the antibacterial potency of compounds that showed any activity in qualitative tests. In this instance the test microorganisms were subcultured twice for 24 hr. at 37.5° in 5 ml. of antibiotic assay broth[3] immediately prior to the test. Preliminary experiments, done in the manner described by Kavanagh (9), established that clearcut zones of inhibition were consistently obtained when 0.1 ml. of the second successive broth subculture was added to 4.0 ml. of seed agar[3] solidified as an even layer on the surface of 21 ml. of hardened base agar (Antibiotic Medium 2).[3]

In the actual tests, five stainless steel cylinders (outside diameter 8 mm., inside diameter 6 mm., length 10 mm.) were placed on the surface of the hardened medium approximately equidistant on the perimeter of a circle of 2.8–3.0-cm. radius (9). Plates with cylinders applied were allowed to stand one-half hour at room temperature to ensure proper seating of the cylinders in the agar surface.

One hundred milligrams of the compound to be tested was dissolved in 1.0 ml. of sterile distilled water (ethanol was used for Compound 9, Table II). The "stock" solution so prepared was diluted 1/10, 1/20, 1/40, 1/80, and 1/160 with the same solvent. Subsequently, 0.1 ml. of each dilution was placed in one cylinder. A control cylinder was prepared using 0.1 ml. of the solvent only. Quantitative test plates prepared as described were incubated 24 hr. at 37.5°. The diameter of the zone of inhibition surrounding each cylinder was then measured using a Fisher–Lilly antibiotic zone reader. Initially, only the dilutions previously mentioned were tested. However, when a compound proved to be very active, additional dilutions were tested in an attempt to determine the greatest dilution that would produce a measurable zone of inhibition.

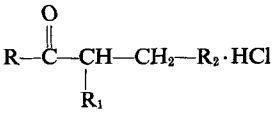
Duplicate samples of each dilution of each compound were tested in each experiment, and at least two complete experiments were done with separately prepared "stock" solutions of each compound.

[1] Difco Laboratories, Detroit, Mich.

[2] Bio-Cert, Fischer Scientific Company (Chemical Manufacturing Division), Fairlawn, N. J.

[3] Baltimore Biological Laboratory (Division of B.D. Laboratories Inc.), Baltimore, Md.

Table III—Antibacterial Activity of Certain Mannich Bases against *S. aureus*



Compound Number	R	R_1	Zone of Inhibition, mm. ([a]Dilution, mg./0.1 ml.) R_2 = 2,6-dimethylmorpholino	R_2 = morpholino	R_2 = piperidino	R_2 = 3-methylpiperidino
1	Me—	H	28.0 (1.25)	—	13.3 (10.0)	—
2	C_6H_5—	H	20.7 (0.625)	25.5 (0.625)	14.8 (5.0)	15.6 (5.0)
3	*p*-Me—C_6H_4—	H	31.0 (0.625)	16.4 (0.625)	13.1 (5.0)	12.8 (2.50)
4	*p*-MeO—C_6H_4—	H	—	10.6 (0.625)	Growth (10.0)	—
5	*p*-Cl—C_6H_4—	H	12.6 (0.078)	14.7 (0.078)	10.3 (0.625)	13.7 (0.625)
6	*p*-O_2N—C_6H_4—	H	11.0 (0.625)	—	10.6 (0.625)	—
7	*m*-O_2N—C_6H_4—	H	14.4 (0.625)	15.2 (1.25)	11.0 (2.50)	—
8	*o*-O_2N—C_6H_4—	H	—	21.7 (0.625)	10.2 (1.25)	—
9	C_6H_5—CH=CH—	H	17.1 (0.625)	—	—	—
10	C_6H_5—	Me	14.1 (10.0)	—	—	—
11		H	—	17.7 (0.625)	11.5 (1.25)	—

[a] Figures in parentheses represent lowest dilution.

RESULTS AND DISCUSSION

Duplicate tests done with a given dilution of a compound gave duplicate inhibition results, and the results obtained in experiments done with separately prepared "stock" solutions were in close agreement.

In no instance did the solvent control produce measurable inhibition of the growth of the test microorganisms.

The test compounds were subdivided according to functional group to permit a study of the effects of each substituent on antibacterial activity. The summarized results of the tests, presented in Tables II and III, are the averages of duplicate determinations.

An examination of Tables II and III reveals that the nature of the R group is not vital for antibacterial activity; those compounds where R is methyl, furyl, phenyl, or substituted phenyl all exhibit measurable activity.

Burckhalter and Johnson (10) synthesized various vinylogs of β-aminopropiophenones with the idea of producing compounds with enhanced antibacterial effect. They noticed that the structural variations failed to improve antibacterial effectiveness. This observation prompted the present investigation of the use of substituted morpholines in the Mannich reaction to determine whether the resulting vinylog of β-2,6-dimethylmorpholinopropiophenone possessed improved antibacterial activity. However, no dramatic change in activity was noted. Again, this finding indicates that the nature of the R group and its distance from the amine moiety are not crucial for antibacterial action.

However, while analysis of the data indicates that the substituents on the phenyl ring do exert some influence, by far the greatest effect results from differences in the amine moiety. In general, a *p*-chloro group exhibits a marked beneficial effect on the antibacterial activity of the compound while a *p*-nitro group generally appears to enhance the activity of that substance when compared to many others in the same series. Those functional groups that have the general effect of reducing the activity of the Mannich bases when tested against both *E. coli* and *S. aureus* are the *p*-methoxy and *m*-nitro. The adverse influence of these two moieties may be entirely due to steric effects. They may simply prevent the respective compounds from making a good contact on the receptor side of either *E. coli* or *S. aureus*. Moreover, differences in potencies observed on introducing the ring substituents at various positions in the benzene ring may be assigned to their influence on transport phenomena (absorption, distribution, excretion, *etc.*).

A comparison of the effects of the amine moiety on activity against *E. coli* (Table II) reveals that there is not much difference between the potency of dimethylmorpholino and morpholino compounds. In addition, the piperidino and 3-methylpiperidino were quite comparable. However, those substances with either a morpholino or dimethylmorpholino group were generally considerably more potent than those in either of the piperidino series. The reason for this situation is not readily apparent but may be due to improved contact with the receptor site because of hydrogen bonding by the oxygen in the morpholino ring. The methyl groups in the dimethylmorpholino may also provide a means of increased contact with the bacterium receptor site through Van der Waals' forces.

Examination of Table III reveals that the same general situation obtains with regard to the potency of the compounds against *S. aureus* as was described for *E. coli*. It is worthy of mention that the *p*-chloro compounds of both the morpholino and dimethylmorpholino series exhibit remarkable activity against *S. aureus* when compared with all other substances included in this study.

Introduction of a methyl group in the α-position to the carbonyl results in a decrease in activity, as noted from Compound 10 in both Tables II and III.

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Ion-Exchange Resins on Cinchona Alkaloids IV: Effect of Resin Particle Size on Equilibrium Exchange and Column Studies

B. R. KAMATH, C. V. BHAT, A. H. VYAS, R. S. SHAH, S. S. KANHERE, and S. L. BAFNA

Abstract □ The effect of particle size of styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins on equilibrium exchange and column studies for cinchona alkaloid sulfates was studied.

Keyphrases □ Ion-exchange resins–cinchona alkaloids—resin particle-size effect on equilibrium exchange, column studies □ Cinchona alkaloids on ion-exchange resins—resin particle-size effect on equilibrium exchange, column studies □ Particle size, styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins—effect on equilibrium exchange, column studies

Earlier (1–3) the equilibrium exchange and rates of exchange of cinchona alkaloid sulfates with styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins were studied; this article discusses the effect of resin particle size on equilibrium exchange and column studies.

EXPERIMENTAL

Resins (4–5)—The following were used: styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins of relative degree of crosslinking, X (percent nominal divinylbenzene content), as 2 and referred to as X2[1] of 200/400 mesh; of X as 4 and referred to as X4[2] of 20/50, 50/100, 100/200, and 200/400 mesh; of X as 8 and referred to as X8[3] of 200/400 mesh; of X as 12 and referred to as X12[4] of 200/400 mesh; and the expanded structure resin (X is presumably about 20), referred to as IR-200,[5] of 20/60 mesh. The resins were conditioned and regenerated into hydrogen form, and moisture content and capacity were estimated (4–6) (Table I). The ammonium form of the resins was prepared by treating the hydrogen form of the resins with an excess of aqueous ammonia (2).

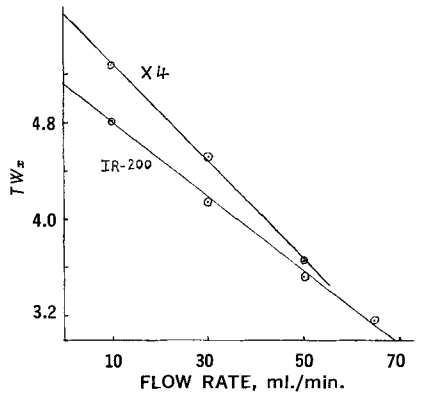

Figure 1—*Plot of* TW_x *against flow rate for resins X4 and IR-200.*

[1] Dowex 50W X2, Dow Chemical Co., Midland Mich.
[2] Dowex 50W X4, Dow Chemical Co., Midland Mich.
[3] Dowex 50W X8, Dow Chemical Co., Midland, Mich.
[4] Dowex 50W X12, Dow Chemical Co., Midland Mich.
[5] Amberlite 200, Rohm & Haas Co., Philadelphia, Pa.

Table I—Capacity of Resins

Resin	Mesh	Capacity, meq./g. Air-Dried Resin	Capacity, meq./g. Oven-Dried Resin
X2	200/400	3.58	4.97
X4	20/50	3.75	5.01
X4	50/100	3.90	5.12
X4	100/200	3.72	4.77
X4	200/400	2.90	4.83
X8	200/400	4.09	4.86
X12	200/400	3.62	4.71
IR-200	20/60	3.56	4.78

Details were given earlier (1–3, 7) regarding the chemicals used, the preparation and estimation of the solutions, the procedure, and the nomenclature.

Column Studies—The known weight of air-dried resin was taken, and the resin was slurried with distilled water and transferred to a column fitted with a sintered-glass disk. The column was backwashed with distilled water and allowed to settle under gravity; column data were then measured. After use the resin was replaced by an equal amount of resin from stock for the next run.

RESULTS AND DISCUSSION

In this study, Resins X2, X4, X8, and X12 of 200/400 mesh were studied for equilibrium exchange with quinine sulfate and cinchonine sulfate to cover a wide range of particle sizes. The results are given in Table II. These may be compared with the corresponding results for these resins of 60/80 mesh given in Tables I and II of an earlier work (1). The results indicate that the average value of P_R for resins of fine mesh (200/400) is higher than that for the same resins of larger mesh (60/80). For the resin with a low degree of crosslinking (X2), P_R increases with a decrease in P_A; for resins of a higher degree of crosslinking (X4, X8, and X12), P_R increases when P_A decreases for the smaller particle size (200/400 mesh) but

Table II—Equilibrium of Aqueous Quinine Sulfate and Cinchonine Sulfate with Cation-Exchange Resins

Resin[a]	Quinine Sulfate $[\bar{A}]_e$	Quinine Sulfate P_A	Quinine Sulfate P_R	Cinchonine Sulfate $[\bar{A}]_e$	Cinchonine Sulfate P_A	Cinchonine Sulfate P_R
X2	0.50	26.2	63.5	0.54	28.6	64.2
	0.92	48.1	57.2	0.77	40.7	57.9
	1.10	57.5	54.2	1.10	58.2	54.2
	1.37	71.3	52.4	1.39	73.5	51.7
X4	0.93	47.9	56.0	0.91	46.0	56.0
	1.11	57.2	55.5	1.06	53.5	53.3
	1.44	74.2	53.7	1.40	70.7	52.4
	1.80	92.0	52.0	1.77	89.4	51.0
X8	0.73	37.8	53.3	0.75	37.2	54.4
	1.02	52.8	50.2	0.90	44.5	53.6
	1.33	68.9	48.2	1.08	53.5	51.2
	1.62	83.9	48.0	1.59	78.7	49.4
X12	0.37	19.3	37.4	0.43	23.0	42.6
	0.58	30.2	37.2	0.83	44.4	42.0
	0.74	38.5	36.3	1.08	57.8	41.1
	1.14	58.6	35.7	1.29	69.0	40.0

[a] Mesh size of the resins = 200/400.

Table III—Total Amount of Alkaloid Exchange (TW_x) for Quinine Sulfate with Resin Column[a]

Run No.	Resin	Mesh	Ionic Form	Bed Length,[b] cm.	Bed Volume,[b] ml.	Flow Rate, ml./min.	Solvent Medium[c]	TW_x
1	IR-200	20/60	H^+	21	9	10	aq.	4.82
2						30		4.15
3						50		3.52
4						65		3.18
5			NH_4^+			50	aq.	2.45
6						50	aq. acid	2.45
7	X4	20/50	H^+	32	14	10	aq.	5.28
8						30		4.52
9						50		3.65
10	X4	50/100	H^+	30	13	10	aq.	6.66
11						30		6.01
12	X4	100/200	H^+	28	12	4	aq.	7.25
13			NH_4^+			4	aq.	7.29
14			NH_4^+			4	aq. acid	7.29

[a] Capacity of resin in column = 16.3 meq. [b] For resin in the hydrogen form. [c] aq. = aqueous; aq. acid = 0.01 *N* aqueous sulfuric acid.

is practically constant with the increase in P_A for the larger particle size (60/80 mesh). The results imply that for a resin of larger particle size, depending on the degree of crosslinking, P_R is practically constant when P_A is decreased; but when the particle size is sufficiently decreased, P_R increases when P_A decreases and the average value of P_R is higher.

Column Studies—Studies were carried out at different flow rates with the resins in hydrogen form for exchange with aqueous quinine sulfate. Four liters of aqueous quinine sulfate solution (concentration = 2 meq./l.) was passed through the column. The effluent was collected in eight samples, each 500 ml., and W_x (milliequivalents of alkaloid sulfate exchanged) for each sample was calculated from UV absorption. For example, the values of the sample number and 100 W_x for Run 1 of Table III were: 1, 100; 2, 100; 3, 93; 4, 76; 5, 54; 6, 28; 7, 19; and 8, 12. The values of TW_x (total amount, in milliequivalents, of alkaloid exchanged) obtained from such data are given in Table III. The data indicate that for the column used, with the flow rate at 10 ml./min., the exchange is close to the effective capacity of Resin IR-200 (1, 2) in the column. Under the same conditions, for Resin X4 in the column, TW_x is less than the effective capacity and increases with decreases in particle size. For both the resins, TW_x decreases almost linearly with an increase in flow rate (Fig. 1).

Runs were then carried out with quinine sulfate from aqueous and 0.01 *N* aqueous sulfuric acid medium with a column of resin in the ammonium form. The procedure was similar to that already described. After each exchange run, for the elution, the column was washed with distilled water and eluted with ammoniacal (0.1 *N*) ethyl alcohol. The rate was 20 ml./min. for Resin IR-200 of 20/60 mesh and 4 ml./min. for Resin X4 of 100/200 mesh. The effluent was collected in 200-ml. samples, and W_1 (milliequivalents of alkaloid base eluted) in each sample was calculated from UV absorption. Complete elution of the alkaloid was obtained. For example, the values of the sample number and 500 W_1 for the elution run after the exchange Run 5 of Table III were: 1, 1096; 2, 85; 3, 17.8; 4, 9; 5, 5.5; 6, 4.1; 7, 3.8; and 8, 3.1; the values of TW_x are given in Table III. With resin in the ammonium form, TW_x was practically the same with aqueous and 0.01 *N* aqueous sulfuric acid as the solvent medium.

The runs suggest that a resin of relatively lower degree of crosslinking such as X4 or of expanded structure such as IR-200 in ammonium form could be suitable for recovery of cinchona alkaloids with aqueous sulfuric acid as solvent for the exchange runs and ammoniacal alcohol as solvent for the elution run.

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Action of Antibiotics on Respiratory Tract IV: Tetracyclines and 4-Epiderivatives

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Abstract □ Tetracyclines increase the motility and/or the tone of the dog bronchial chain *in vitro*, the order of potency in the excitatory activity being rolitetracycline ≫ tetracycline ≫ mepicycline ≥ doxycycline. The time–action relationships *in situ* indicate three distinct phases in tetracycline activity on the respiratory tract: (*a*) a *hyperventilation phase* for a few minutes, (*b*) a *depression of respiratory activity phase* for 60–90 min., and (*c*) a *compensatory phase* with return to the control condition after 90–180 min. The intensity of action and the length of the phases are affected by: (*a*) the used tetracycline, the order of potency being rolitetracycline ≫ tetracycline > doxycycline ≥ mepicycline; (*b*) the dose employed: the larger the level, the greater the maximum effect and the longer the overall duration of action; and (*c*) the 4-epiderivative percentage of the used tetracyclines: the higher the epiderivative concentration, the greater the effect. *In situ*, the antibiotics act, at least in part, on the medullary respiratory center, the action being unaffected by cutting vagi and pretreatment with sympatholytic, parasympatholytic, antihistaminic, antiserotoninic, and ganglion-blocking agents.

Keyphrases □ Tetracyclines—action, mechanism in respiratory tract, time–action relationships □ Antibiotics—effects of tetracyclines on respiratory tract □ Respiratory tract—action of tetracyclines

Research on dog bronchial chains *in vitro* indicates that ampicillin, dicloxacillin, and chloramphenicol antagonize the histamine- or acetylcholine-induced spasm; ampicillin is about 10–20 times and dicloxacillin about 2–3 times less active than ephedrine, while chloramphenicol is about 15–25 times more active than ephedrine. *In situ* the intravenous injection of ampicillin induces an increase of the depth with a moderate decrease in the frequency of respiration, while dicloxacillin decreases the respiratory frequency with a moderate increase in ventilation. In spite of the myolytic activity *in vitro*, chloramphenicol induces *in vivo* a moderate decrease in ventilation and an increase in respiration rate (1–3).

The purpose of this study was to analyze quantitatively the action *in vitro* and *in vivo* of some tetracyclines on the dog's respiratory tract. The percentage of 4-epiderivatives in the employed products was also determined to correlate eventually the degree of these derivates with the pharmacological response. In fact, some syndromes associated with tetracycline toxicity in man were identified. Particularly, a Fanconi-like syndrome characterized by nausea, vomiting, acidosis, proteinuria, glycosuria, and aminoaciduria is associated with degraded tetracycline (4–8). This syndrome may be a result of the products of degradation, anhydrotetracycline, and epianhydrotetracycline. Experimentally, anhydro-4-epitetracycline induces in rats such marked functional changes as proteinuria, glycosuria, cellular debris in the urine sediment, and elevated UGOT activity. The morphologic concomitant of this syndrome in the rat and dog is a necrotic process of the convoluted tubuli in the renal cortex (9).

METHOD

The experiments were performed in the dog both *in vitro* and *in situ*. The following tetracyclines (with the indicated percent of epiderivatives) were used: tetracycline (3.2, 7.4, 13.2, and 39.4), doxycycline (6.8, 12.6, and 37.4), mepicycline (0.8, 7.2, 14.6, and 41.2), and rolitetracycline (7.4, 18.3, and 47.7).

The separation and determination of tetracyclines and the degradation products (4-epiderivatives, anhydro- and 4-epianhydroderivatives) were made according to the methods of Ley (10), Lodi *et al.* (11), and Gyanchandani *et al.* (12). The concentration of the degradation products was calculated as a percentage of the total tetracycline.

Experiments *In Vitro*—Bronchi of 45 mongrel dogs of either sex (weighing 7.8 to 12.5 kg.) were removed immediately after death. All tissues were carefully dissected and cut into rings which were tied together in chains with loops of thread. The mucosa was removed to allow greater freedom of movement of the muscle; the cartilage in each ring was cut so that only the smooth muscle bands were left joining each ring together (13). Four to six rings were suspended in a 50-ml. organ bath containing Tyrode's solution gassed with 95% oxygen and 5% carbon dioxide; the temperature was 36.5–37.3°. The tonus level of the preparations was continuously recorded by a strain-gauge lever, giving a magnification of ×15–20, tension of 200 mg., and writing on a kymograph drum. The preparations were left 2 hr. before any drugs were given. Before any doses of acetylcholine itself or tetracyclines were tested, two submaximal

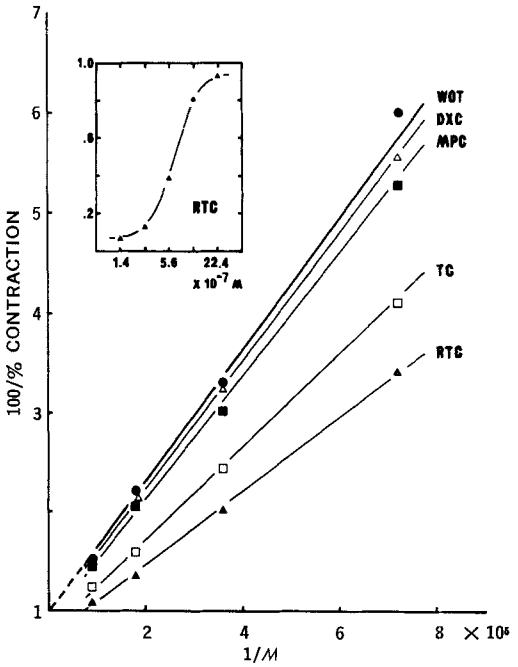

Figure 1—*Lineweaver–Burk plots calculated:* (a) *for the effect of acetylcholine chloride (2 min. of contact) on dog bronchial chain* in vitro *(WOT, ●); and* (b) *for the combinations of varying concentrations of acetylcholine chloride (1/M, in abscissa) with a constant concentration (2.8 × 10⁻⁷ M) of the tested tetracyclines introduced to the bath 2 min. before the acetylcholine doses: doxycycline (DXC, △) with 6.8% of epi-DXC; mepicycline (MPC, ■) with 7.2% of epi-MPC; tetracycline (TC, □) with 7.4% of epi-TC; and rolitetracycline (RTC, ▲) with 7.4% of epi-RTC. The insert shows the dose–response curve of rolitetracycline (for 20 min. of contact) on the dog bronchial chain.*

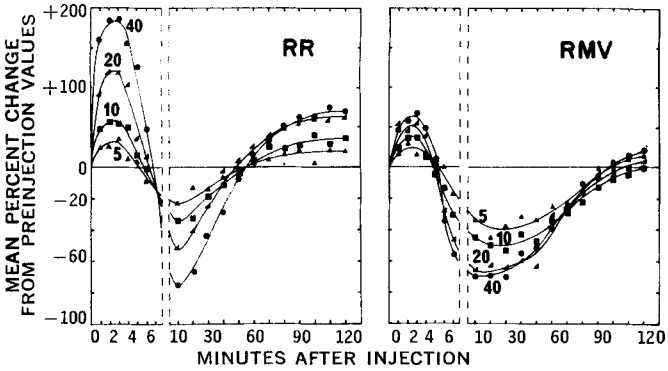

Figure 2—*Time-action curves depicting effects of injected rolitetracycline at four different dose levels (5, 10, 20, and 40 mg./kg. i.v.; 4-epiderivative = 7.4%) upon respiratory rate (RR) and respiratory minute volume (RMV). Plotted points represent average values in six dogs.*

doses of acetylcholine were administered until regular responses were obtained. The action of tetracyclines (0.7 to 22.4 $\times$ 10^{-7} *M*) was evaluated during a 20-min. period of contact.

For comparative assay, the activity of acetylcholine chloride (during 2 min. of contact) was evaluated also after the various tetracyclines (2.8 $\times$ 10^{-7} *M*) were left in contact with the preparation for 2 min.

Experiments *In Situ*—The experiments were carried out on eight beagle dogs of either sex (weighing 11.4–16.0 kg.) and on 34 mongrel dogs (weighing 8.1–12.3 kg.) preanesthetized with urethan (0.4 g./kg. i.p.). Anesthesia was induced and maintained by chloralose (80 mg./kg. i.v.); the arterial blood pressure was measured from a cannula inserted into a femoral artery; the intestinal movement and tone were recorded by a rubber balloon inserted into the jejunum.

During the succinylcholine chloride (1 mg./kg. i.v.) action, an intratracheal Warne tube was set in place; both the respiratory rate and respiratory minute volume were evaluated. All experiments were carried out in a conditioned operating room (26 $\pm$ 1°; R.H. = 55 $\pm$ 3%; pressure = 758 $\pm$ 5 mm. Hg). Immediately after completing the preinjection control measurements, the tetracyclines (5–40 mg./kg.) were administered intravenously by a polystan tube inserted into the femoral vein. The effects of the antibiotics on the respiratory tract were calculated as mean percent change from preinjection values.

To analyze the mechanism of action, tetracyclines were assayed in 18 dogs after: (*a*) cutting the vagi; (*b*) treating with the following drugs: atropine sulfate (2–3 mg./kg. s.c.), dibenamine hydrochloride (3–6 mg./kg. i.v.), D(−)-1-(4-nitrophenyl)-2-isopropylaminoethanol hydrochloride (INPEA: 4–8 mg./kg. i.v.), methysergide maleate (20–40 mcg./kg. s.c. + 10 mcg./kg. i.v.), chlorpheniramine maleate (2–4 mg./kg. s.c.), cyproheptadine hydrochloride (200–400 mcg./kg. i.v.), and hexamethonium bromide (200–400 mcg./kg. i.v.); and (*c*) giving artificial ventilation.

RESULTS AND DISCUSSION

Tetracyclines induce an increase of the motility and the tone of the dog bronchial chain *in vitro*; rolitetracycline and tetracycline act mainly on tone and motility, while doxycycline and mepicycline act mainly on motility, the tone being slightly affected or unaffected.

The order of potency in the excitatory activity on the bronchial muscle is rolitetracycline $\gg$ tetracycline $\gg$ mepicycline $\geq$ doxycycline. In fact, the Lineweaver–Burk plots in Fig. 1 show this order of ability of the mentioned tetracyclines to increase the contracting action of acetylcholine. These data are in agreement with the previous observation that tetracyclines act directly on the bile-duct smooth muscle (14).

In situ, the tetracycline effect changes with time; there are three distinct phases in tetracycline time–action curves. Following the intravenous administration of the antibiotic, a *hyperventilation phase* occurs at first for a few minutes, with time to peak effect from 2 to 3 min. After 4–8 min., a *depression of respiratory activity phase* appears. The length of this second phase is affected by such factors as the tetracycline used, dose, and epiderivative concentration; the duration of action varies from 60 to 90 min. Subsequently, a *compensatory phase* occurs with return to the control condition after 90–180 min.

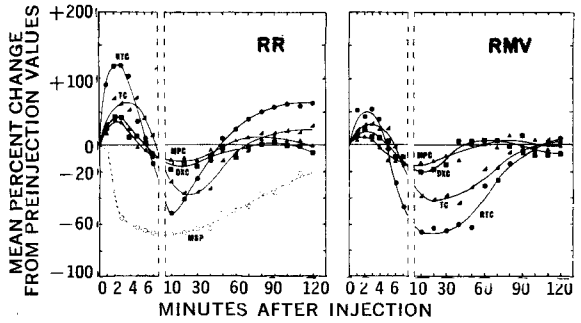

Figure 3—*Time-action curves depicting effects of rolitetracycline (RTC; 4-epiderivative = 7.4%), tetracycline (TC; 4-epiderivative = 7.4%), doxycycline (DXC; 4-epiderivative = 6.8%), and mepicycline (MPC; 4-epiderivative = 7.2%) at the same dose level (20 mg./kg. i.v.) upon respiratory rate (RR) and respiratory minute volume (RMV) and upon mean blood pressure (MBP; only for rolitetracycline). Plotted points represent average values in six dogs.*

As exemplified in Fig. 2 for rolitetracycline, the dosage influences the time–action relationships: the larger the dose, the greater the maximum effect and the longer the overall duration of action.

There is less general uniformity in the characteristics of time–action relationships depending on the tetracycline used: the position of the curve can shift along the abscissa. Nevertheless, in Fig. 3 it is possible to observe the difference in ability of various tetracyclines to induce the change in respiratory activity. The order of potency is rolitetracycline >> tetracycline > doxycycline $\geq$ mepicycline, the epiderivative concentration being quite equal in the used preparations (from 6.8 to 7.4%).

The role of epiderivatives in affecting the respiratory activity is exemplified for mepicycline in Fig. 4; for all tetracyclines, at the same dose level, the higher the epiderivative concentration, the greater is the effect. The increase in response may also result from the increase in total tetracycline, and any of the compounds studied may have caused this effect. The increased percent of the epiderivative may cause the respiratory effect in a way similar to how the increased percentage of the epiderivative causes the Fanconi syndrome.

The action of tetracyclines on the respiratory tract occurred simultaneously with a change in the arterial pressure. As indicated in Fig. 3 for rolitetracycline, a depression of the arterial blood pressure—systolic, diastolic, and mean pressure—occurs. This action was, at least in part, direct on the cardiovascular system, because it occurred also when the dogs were given artificial ventilation. On the other hand, tetracycline activity on the respiratory tract is unaffected by both cutting of the vagi and pretreatment with atropine, dibenamine, INPEA, methysergide, chlorpheniramine, cyproheptadine, and hexamethonium showing an action, at least in part, on the medullary respiratory center.

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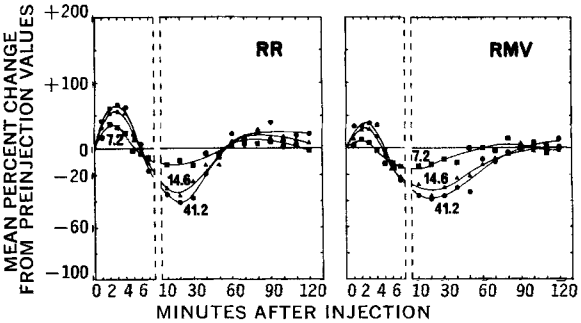

Figure 4—*Time-action curves depicting effects of mepicycline at the same dose level (20 mg./kg. i.v.) but with three different concentrations of 4-epiderivative (7.2, 14.6, and 41.2%) upon respiratory rate (RR) and respiratory minute volume (RMV). Plotted points represent average values in six dogs.*

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Potential Antidiabetics VIII: 4-Arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones, 4-Arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles, and 4-Arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles

H. G. GARG and CHANDRA PRAKASH

Abstract □ A series of 4-arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones was synthesized for evaluation as antidiabetic agents from appropriate ethyl 2,3-dioxobutyrate 2-arylhydrazones and amino guanidine nitrate. Similarly, other series of compounds, *i.e.*, 4-arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles, and 4-arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles were synthesized by the cyclization of 3-arylhydrazono-2,3,4-pentanetriones and 1,3-diphenyl-2-arylhydrazono-1,2,3-propanetriones with amino guanidine nitrate, respectively.

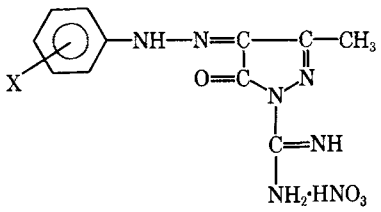
Keyphrases □ Pyrazole congeners—synthesis, screened for antidiabetic activity □ Antidiabetics, potential—pyrazole congeners synthesized, screened □ Hypoglycemic agents—pyrazole congeners synthesized, screened

Prompted by the observation (1) that 3,5-dimethylpyrazoles and isoxazoles lower the blood sugar, numerous congeners of pyrazoles were synthesized and their activity was assessed in these laboratories (2, 3). As a result of the role of *ν*-guanidobutyramide in lowering blood sugar and urea levels (4), the authors prepared several of the pyrazoles (I and II) containing two different biologically active moieties—*viz.*, guanyl and pyrazolyl.

4-Arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones (Table I) were synthesized by the addition of a hot aqueous solution of amino guanidine nitrate to ethyl 2,3-dioxobutyrate 2-arylhydrazones under conditions similar to those used previously (5, 6).

4-Arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles (II*a*) and 4-arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles (II*b*) could not be obtained under these conditions, and a modified procedure was adopted. A hot aqueous solution of amino guanidine nitrate was added to an ethanolic solution of 3-phenylhydrazono-2,3,4-pentanetrione followed by 30% nitric acid until the pH of the reaction mixture became 1. The crystalline 4-phenylazo-*N'*-guanylnitrate-3,5-dimethylpyrazole started precipitating out after the reaction mixture was refluxed for 3 hr. and was allowed to stand for several hours at room temperature. By following similar conditions, other 4-arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles and 4-arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles were obtained. These derivatives are listed in Tables II and III.

All these compounds are highly colored and crystalline substances and are soluble in common organic solvents as well as in hot water.



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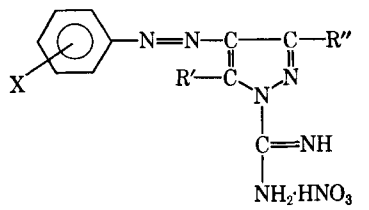

II*a*: R′ = R″ = CH_3

II*b*: R′ = R″ = C_6H_5

X = substituted phenyl

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Potential Antidiabetics VIII: 4-Arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones, 4-Arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles, and 4-Arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles

H. G. GARG and CHANDRA PRAKASH

Abstract □ A series of 4-arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones was synthesized for evaluation as antidiabetic agents from appropriate ethyl 2,3-dioxobutyrate 2-arylhydrazones and amino guanidine nitrate. Similarly, other series of compounds, *i.e.*, 4-arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles, and 4-arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles were synthesized by the cyclization of 3-arylhydrazono-2,3,4-pentanetriones and 1,3-diphenyl-2-arylhydrazono-1,2,3-propanetriones with amino guanidine nitrate, respectively.

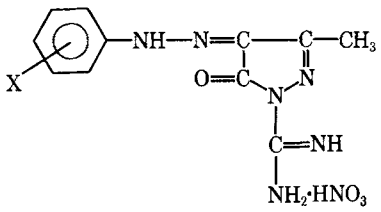
Keyphrases □ Pyrazole congeners—synthesis, screened for antidiabetic activity □ Antidiabetics, potential—pyrazole congeners synthesized, screened □ Hypoglycemic agents—pyrazole congeners synthesized, screened

Prompted by the observation (1) that 3,5-dimethylpyrazoles and isoxazoles lower the blood sugar, numerous congeners of pyrazoles were synthesized and their activity was assessed in these laboratories (2, 3). As a result of the role of *ν*-guanidobutyramide in lowering blood sugar and urea levels (4), the authors prepared several of the pyrazoles (I and II) containing two different biologically active moieties—*viz.*, guanyl and pyrazolyl.

4-Arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones (Table I) were synthesized by the addition of a hot aqueous solution of amino guanidine nitrate to ethyl 2,3-dioxobutyrate 2-arylhydrazones under conditions similar to those used previously (5, 6).

4-Arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles (II*a*) and 4-arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles (II*b*) could not be obtained under these conditions, and a modified procedure was adopted. A hot aqueous solution of amino guanidine nitrate was added to an ethanolic solution of 3-phenylhydrazono-2,3,4-pentanetrione followed by 30% nitric acid until the pH of the reaction mixture became 1. The crystalline 4-phenylazo-*N'*-guanylnitrate-3,5-dimethylpyrazole started precipitating out after the reaction mixture was refluxed for 3 hr. and was allowed to stand for several hours at room temperature. By following similar conditions, other 4-arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles and 4-arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles were obtained. These derivatives are listed in Tables II and III.

All these compounds are highly colored and crystalline substances and are soluble in common organic solvents as well as in hot water.



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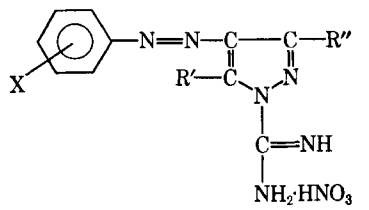

II*a*: R′ = R″ = CH_3

II*b*: R′ = R″ = C_6H_5

X = substituted phenyl

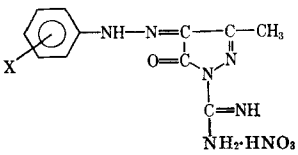

Table I—4-Arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones (I)

No.	X	Yield, %	M.p.	Color[a]	Formula	Anal., % Calcd.	Found
1	2-NO_2	65	211°	DBN	$C_{11}H_{12}N_8O_6$	N, 31.81	N, 31.61
2	3-NO_2	65	233°	YN	$C_{11}H_{12}N_8O_6$	N, 31.81	N, 31.63
3	4-NO_2	70	251°	LtBN	$C_{11}H_{12}N_8O_6$	N, 31.81	N, 31.72
4	3-Cl	73	198°	VP	$C_{11}H_{12}ClN_7O_4$	Cl, 10.39	Cl, 10.29
5	4-Cl	75	245°	YN	$C_{11}H_{12}ClN_7O_4$	Cl, 10.39	Cl, 10.48
6	3-Me	60	221°	YP	$C_{12}H_{15}N_7O_4$	N, 30.52	N, 30.29
7	4-Me	68	223–225°	ON	$C_{12}H_{15}N_7O_4$	N, 30.52	N, 30.27
8	2-MeO	68	216°	ORP	$C_{12}H_{15}N_7O_5$	N, 29.08	N, 28.90
9	3-MeO	65	218°	DRN	$C_{12}H_{15}N_7O_5$	N, 29.08	N, 28.82
10	4-MeO	64	219°	ON	$C_{12}H_{15}N_7O_5$	N, 29.08	N, 28.64
11	4-EtO	70	218° (dec.)	ON	$C_{13}H_{17}N_7O_5$	N, 27.92	N, 27.68
12	4-SO_2NH_2	60	238°	YN	$C_{11}H_{14}N_8O_6S$	N, 29.01	N, 28.82
13	2,4-Me_2	64	235°	BN	$C_{13}H_{17}N_7O_4$	N, 29.25	N, 29.12
14	2,6-Me_2	65	204°	BN	$C_{13}H_{17}N_7O_4$	N, 29.25	N, 29.04
15	2,5-Cl_2	68	221°	GP	$C_{11}H_{11}Cl_2N_7O_4$	Cl, 18.88	Cl, 18.71
16	2,4-$(MeO)_2$	70	227°	BP	$C_{13}H_{17}N_7O_6$	N, 26.70	N, 26.57
17	2,5-$(MeO)_2$	70	189°	ChP	$C_{13}H_{17}N_7O_6$	N, 26.70	N, 26.52
18	2,5-$(EtO)_2$	72	136°	ChP	$C_{15}H_{21}N_7O_6$	N, 24.81	N, 24.61
19	4-Cl-2,5-$(MeO)_2$	60	252°	BP	$C_{13}H_{16}ClN_7O_6$	Cl, 8.84	Cl, 8.63
20	5-Cl-2,4-$(MeO)_2$	60	241°	BP	$C_{13}H_{16}ClN_7O_6$	Cl, 8.84	Cl, 8.59

[a] B, brown; Ch, chocolate; D, dark; G, golden; Lt, light; N, needles; O, orange; P, plates; R, red; V, violet; Y, yellow.

RESULTS

Few compounds of the series *N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones [namely, 4-phenylhydrazono-, 4-(3-nitrophenylhydrazono)-, 4-(3-methylphenylhydrazono)-, and 4-(4-methoxyphenylhydrazono)-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones] and of the series *N'*-guanylnitrate-3,5-dimethylpyrazoles [namely, 4-(2-nitrophenylazo)-, 4-(4-chlorophenylazo)-, 4-(3-methylphenylazo)-, 4-(2,4-dimethylphenylazo)-, and 4-(2,6-dimethylphenylazo)-*N'*-guanylnitrate-3,5-dimethylpyrazoles] have been evaluated for their hypoglycemic activity in CF-1-S mice (Carworth Farms, 25–30 g.). Doses of 1.5 mmoles/kg. compound were administered as carboxymethylcellulose suspensions. Controls received an equal volume of the vehicle. Blood samples (0.05 ml.) obtained from retrobulbar plexuses at 0, 3, and 5 hr. after dosing were analyzed for blood sugar with the aid of a Technician Auto-analyzing unit using the modified method of Hoffman (7). Results showed that these compounds were essentially inactive. The testing on other derivatives is in progress.

EXPERIMENTAL

Melting points were taken with a Kofler hot-stage-type apparatus and are uncorrected.

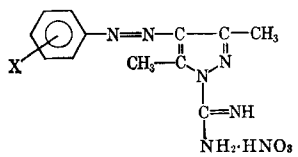

Table II—4-Arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles (II*a*)

No.	X	Yield, %	M.p.	Color[a]	Formula	Anal., % Calcd.	Found
1	2-NO_2	65	186°	OP	$C_{12}H_{14}N_8O_5$	N, 32.00	N, 31.78
2	4-NO_2	68	178°	DYP	$C_{12}H_{14}N_8O_5$	N, 32.00	N, 31.74
3	3-Me	60	189°	YN	$C_{13}H_{17}N_7O_3$	N, 30.72	N, 30.60
4	4-Me	64	193°	DYP	$C_{13}H_{17}N_7O_3$	N, 30.72	N, 30.54
5	3-Cl	60	195°	YP	$C_{12}H_{14}ClN_7O_3$	Cl, 10.45	Cl, 10.27
6	4-Cl	65	166°	OP	$C_{12}H_{14}ClN_7O_3$	Cl, 10.45	Cl, 10.34
7	2-Br	65	190°	YN	$C_{12}N_{14}BrN_7O_3$	Br, 20.83	Br, 20.61
8	4-Br	66	186°	OP	$C_{12}H_{14}BrN_7O_3$	Br, 20.83	Br, 20.64
9	2-MeO	70	195°	YN	$C_{13}H_{17}N_7O_4$	N, 29.25	N, 29.01
10	4-MeO	67	179°	YP	$C_{13}H_{17}N_7O_4$	N, 29.25	N, 29.07
11	4-EtO	68	181°	YP	$C_{14}H_{19}N_7O_4$	N, 28.08	N, 27.94
12	2,3-Me_2	64	196°	YP	$C_{14}H_{19}N_7O_3$	N, 29.42	N, 29.18
13	2,5-Me_2	60	194°	DYN	$C_{14}H_{19}N_7O_3$	N, 29.42	N, 29.29
14	3,4-Me_2	63	217°	YN	$C_{14}H_{19}N_7O_3$	N, 29.42	N, 29.27
15	2,4-$(MeO)_2$	65	185°	YP	$C_{14}H_{19}N_7O_5$	N, 26.84	N, 26.65
16	2,5-Br_2	65	213°	YP	$C_{12}H_{13}Br_2N_7O_3$	Br, 34.55	Br, 34.41
17	3,5-Cl_2	60	209°	LtYP	$C_{12}H_{13}Cl_2N_7O_3$	Cl, 18.98	Cl, 18.70
18	2-Cl-4-NO_2	63	178°	RP	$C_{12}H_{13}ClN_8O_5$	Cl, 9.23	Cl, 9.01
19	2-Cl-6-Me	68	176°	LtON	$C_{13}H_{16}ClN_7O_3$	Cl, 10.04	Cl, 9.78
20	5-Cl-2,4-$(MeO)_2$	65	195°	YP	$C_{14}H_{18}ClN_7O_5$	Cl, 8.88	Cl, 8.55

[a] See footnote *a* of Table I.

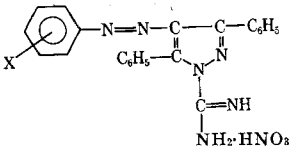

Table III—4-Arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles (II*b*)

No.	X	Yield, %	M.p.	Color[a]	Formula	Anal., % Calcd.	Found
1	2-NO_2	60	184°	YN	$C_{22}H_{18}N_8O_5$	N, 23.62	N, 23.23
2	3-NO_2	62	178°	OP	$C_{22}H_{18}N_8O_5$	N, 23.62	N, 23.32
3	4-NO_2	60	154°	BN	$C_{22}H_{18}N_8O_5$	N, 23.62	N, 23.29
4	2-Br	65	152°	LtOP	$C_{22}H_{18}BrN_7O_3$	Br, 15.74	Br, 15.42
5	4-Cl	60	160°	OP	$C_{22}H_{18}ClN_7O_3$	Cl, 7.65	Cl, 7.30
6	2-Me	55	134°	DYN	$C_{23}H_{21}N_7O_3$	N, 22.12	N, 21.89
7	4-Me	55	167°	LtOP	$C_{23}H_{21}N_7O_3$	N, 22.12	N, 21.81
8	3-MeO	68	186°	YP	$C_{23}H_{21}N_7O_4$	N, 21.35	N, 21.04
9	4-EtO	63	188°	YP	$C_{24}H_{23}N_7O_4$	N, 20.71	N, 20.43
10	4-SO_2NH_2	56	181°	LtRP	$C_{22}H_{20}N_8O_5S$	N, 22.04	N, 21.73
11	2,4-Me_2	55	191°	OP	$C_{24}H_{23}N_7O_3$	N, 21.44	N, 21.14
12	2,5-Me_2	50	112°	BP	$C_{24}H_{23}N_7O_3$	N, 21.44	N, 21.32
13	3,4-Me_2	50	183°	LtRP	$C_{24}H_{23}N_7O_3$	N, 21.44	N, 21.10
14	3,5-Me_2	53	171°	LtOP	$C_{24}H_{23}N_7O_3$	N, 21.44	N, 21.24
15	2,3-Cl_2	55	187°	LtRP	$C_{22}H_{17}Cl_2N_7O_3$	Cl, 14.25	Cl, 14.02
16	2,5-Cl_2	55	163°	YP	$C_{22}H_{17}Cl_2N_7O_3$	Cl, 14.25	Cl, 14.09
17	2-Cl-6-Me	62	185°	DYP	$C_{23}H_{20}ClN_7O_3$	Cl, 7.43	Cl, 7.21
18	4-Cl-2,5-$(MeO)_2$	65	245°	OP	$C_{24}H_{22}ClN_7O_5$	Cl 6.78	Cl, 6.56

[a] See footnote *a* of Table I.

Ethyl 2,3-dioxobutyrate-2-arylhydrazones (5), 2,3,4-pentanetrione 3-arylhydrazones (6), and 1,3-diphenyl-2-arylhydrazono-1,2,3-propanetriones (8) were prepared by coupling diazotized anilines with ethyl acetoacetate, 2,4-pentanedione, and 1,3-diphenyl-1,3-propanedione, respectively.

4-Phenylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-one (I)—Amino guanidine nitrate (0.86 g., 0.005 mole) in water (10 ml.) was added to ethyl 2,3-dioxobutyrate-2-phenylhydrazone (1.17 g., 0.005 mole) in ethyl alcohol (15 ml.). It was refluxed for 2 hr. and then glacial acetic acid (4 ml.) was added. The reaction mixture was again refluxed for 3 hr. On cooling, shining brown crystals of 4-phenylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-one separated out. These were recrystallized from alcohol (70% yield), m.p. 223°.

Anal.—Calcd. for $C_{11}H_{13}N_7O_4$: C, 42.99; H, 4.23; N, 31.92. Found: C, 43.26; H, 4.61; N, 31.80.

The characteristics of other 4-arylhydrazono-*N'*-guanylnitrate-3-methyl-2-pyrazolin-5-ones are given in Table I.

4-Phenylazo-*N'*-guanylnitrate-3,5-dimethylpyrazole (II*a*)—A hot aqueous solution of amino guanidine nitrate (0.005 mole) was added to 2,3,4-pentanetrione-3-phenylhydrazone (1.02 g., 0.005 mole) in ethyl alcohol (15 ml.). To this was added 30% nitric acid until the pH of the reaction mixture became 1. It was then refluxed for 4 hr. On cooling, shining yellow crystals of 4-phenylazo-*N'* guanylnitrate-3,5-dimethylpyrazole separated out. They were recrystallized from ethyl alcohol (75% yield), m.p. 163°.

Anal.—Calcd. for $C_{12}H_{15}N_7O_3$: C, 47.21; H, 4.91; N, 32.13. Found: C, 46.94; H, 5.32; N, 31.89.

The characteristics of other 4-arylazo-*N'*-guanylnitrate-3,5-dimethylpyrazoles prepared similarly are given in Table II.

4-Phenylazo-*N'*-guanylnitrate-3,5-diphenylpyrazole (II*b*)—This was prepared from 1,3-diphenyl-2-phenylhydrazono-1,2,3-propanetrione (1.64 g., 0.005 mole) and amino guanidine nitrate (0.005 mole) by the same procedure used for the preparation of 4-phenylazo-*N'*-guanylnitrate-3,5-dimethylpyrazole (II*a*). The product obtained was recrystallized from alcohol in 65% yield as dark-brown needles, m.p. 101°.

Anal.—Calcd. for $C_{22}H_{19}N_7O_3$: C, 61.53; H, 4.42; N, 22.84. Found: C, 61.18; H, 4.73; N, 22.62.

The characteristics of other 4-arylazo-*N'*-guanylnitrate-3,5-diphenylpyrazoles are given in Table III.

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Drug Transport IV: Influence of Hypotonic and Hypertonic Solutions on Passive Drug Transfer across the Everted Rat Intestine

Keyphrases □ Drug transfer, passive—everted rat intestine □ Hypotonic, hypertonic solutions effect—passive drug transfer □ Everted rat intestine—passive drug transfer

Sir:

Research efforts in our laboratory have been concerned with factors affecting the intestinal absorption of drugs. We showed previously that certain cations may significantly alter drug transport across the isolated everted rat intestine (1, 2). For example, replacing Na^+ with K^+ in a Krebs bicarbonate buffer markedly inhibited the mucosal-to-serosal transfer of a number of ionized (cationic and anionic) and nonionized compounds (1–3). Several recent literature reports (4–6) support our initial observations. In addition, glucose and xylose have also been found to decrease the apparently passive transfer of certain drugs across the everted intestine, whereas mannitol was without effect (7). It appears that the inhibition of drug transfer is related to the degree of intestinal tissue fluid uptake induced by these various inhibitors (2, 7). To examine further the nature of this relationship, experiments were conducted to evaluate the influence of hypotonic and hypertonic solutions on the intestinal transfer of certain compounds.

Sprague-Dawley rats, weighing approximately 250 g., were fasted 20–24 hr. prior to the experiment. Water was allowed *ad libitum*. The experimental method for preparing the everted rat intestine preparation was described previously (2). After severing the intestine at the pyloric junction, the first 15 cm. of intestine was discarded and the following 20 cm. was divided into two 10-cm. segments. The most proximal segment was designated Segment 1, and the distal portion was designated Segment 2.

A modified physiologic Krebs bicarbonate buffer,[1] pH 7.4, was prepared to contain a total cation concentration of 154 m*M*. Buffer solutions were also prepared in which the NaCl concentration was reduced to 18 m*M* (hypotonic solution, about 100 mOsm) or increased to 218 m*M* (hypertonic solution, about 500 mOsm). Two milliliters of buffer was placed inside the sac (serosal solution), and the entire preparation was placed into approximately 100 ml. of buffer solution containing the drug (mucosal solution) which was continually gassed with O_2–CO_2 (95:5, v/v). In any given experiment, the mucosal and serosal solutions were identical in composition except for the presence of drug in the mucosal solution. The serosal compartment was quantitatively sampled every 10 min. during the entire 2-hr. experiment, and the solution was assayed for drug content as previously described (2). The concentrations of the respective compounds in the mucosal solution remained essentially constant (*viz.*, 20 mcg./ml. riboflavin, 2.0 mg./ml. salicylate, and 100 mcg./ml. sulfanilamide) throughout the experiment, due to the large volume of mucosal solution.

Table I—Influence of Hypotonic and Hypertonic Solutions on the 30-Min. Cumulative Transfer of Several Drugs across the Everted Rat Intestine

Drug	Experiment	Segment 1 Amount Transferred in 30 Min. ± *SD*	Segment 1 Percent Control	Segment 2 Amount Transferred in 30 Min. ± *SD*	Segment 2 Percent Control	Comparison and Level of Significance[a]
Riboflavin[b]	Control	1.9 ± 0.2 mcg.	—	1.2 ± 0.1 mcg.	—	
	Hypotonic	0.7 ± 0.2 mcg.	37	0.4 ± 0.1 mcg.	33	2 *vs.* 1: $p < 0.01$[c]
	Hypertonic	2.0 ± 0.4 mcg.	105	1.8 ± 0.3 mcg.	150	3 *vs.* 1: $p < 0.01$[c]
Salicylate[b]	Control	0.7 ± 0.1 mg.	—	0.7 ± 0.1 mg.	—	
	Hypotonic	0.5 ± 0.1 mg.	71	0.4 ± 0.04 mg.	57	2 *vs.* 1: $p < 0.01$
	Hypertonic	0.8 ± 0.04 mg.	114	0.8 ± 0.06 mg.	114	3 *vs.* 1: n.s.
Sulfanilamide[d]	Control	23.5 ± 0.9 mcg.	—	23.2 ± 5.7 mcg.	—	
	Hypotonic	13.9 ± 1.9 mcg.	59	10.6 ± 2.4 mcg.	46	2 *vs.* 1: $p < 0.01$
	Hypertonic	26.2 ± 0.8 mcg.	111	23.4 ± 2.8 mcg.	101	3 *vs.* 1: n.s.

[a] Using a 2 × 2 ANOVA; n.s. = not significant: $p > 0.05$. [b] The values reported represent the mean ± standard deviation of the mean for five determinations under each experimental condition. [c] In these cases, the *F*-ratio values for segments and interactions were also significant ($p < 0.05$). [d] The values reported represent the mean ± standard deviation of the mean for four determinations under each experimental condition.

Table I summarizes the results of these experiments in terms of the cumulative amount of drug transferred in 30 min. In all cases, the hypotonic solution significantly inhibited intestinal transfer. Inhibition was most marked in the case of riboflavin. Statistically significant inhibition of drug transfer in the presence of the hypotonic solution was also observed upon comparison of the cumulative amounts transferred after

[1] KCl, 5 m*M*; KH_2PO_4, 1 m*M*; $NaHCO_3$, 26 m*M*; and NaCl, 122 m*M*.

1 and 2 hr. In contrast, the hypertonic solution increased the amount of drug transferred compared to control values. This increase, however, was statistically significant only in the case of riboflavin.

Measurements of intestinal tissue fluid uptake in the hypotonic and hypertonic buffers were made to test the previously observed relationship between gut fluid uptake and inhibition of drug transfer. Experimental details were reported (2). Essentially, 5-cm. segments of everted rat intestine were incubated for 20 min. in the buffer solution, and fluid uptake was determined as the difference between final and initial tissue weights. The results, in terms of milligrams fluid per gram initial wet weight plus or minus the standard deviation of the mean, were as follows: control buffer (isotonic solution), 52 ± 40 (11); hypotonic buffer, 97 ± 31 (11); and hypertonic buffer, −60 ± 28 (12). The numbers in parentheses represent the numbers of gut segments used. In the case of the hypertonic buffer, a net loss of fluid from the tissue was observed. The results of the gut fluid uptake studies clearly support the apparently general relationship that factors which increase tissue water uptake also inhibit passive intestinal transfer. This has been shown to be the case with glucose, xylose, or K^+ in the buffer medium and now with hypotonic solutions.

Kipnis and Parrish (8) suggested that both hypotonicity and elevated K^+ content of the buffer result in marked expansion of the intracellular volume of rat diaphragm tissue, with concomitant contraction of the extracellular space. The inulin space of the intact tissue, determined after 1 hr. of incubation, was reduced from about 16 ml./100 g. wet weight in control buffer to about 10 ml./100 g. in either high K^+ buffer or hypotonic buffer. Under both conditions, there was a significant increase in total tissue water compared to control levels. Similarly, Fox *et al.* (9), using kidney cortex, and Tews and Harper (10), using rat liver slices, reported a marked decrease in the extracellular space in high K^+ buffer. More recently, Jackson and Cassidy (11) found that glucose-induced fluid uptake by everted sacs of rat intestine was associated with considerable cellular swelling. It is plausible to consider that such swelling occurs at the expense of intercellular space in the mucosal barrier. If this is the case, then intercellular space may play an important role in the transfer of certain compounds across the everted rat intestine.

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Flash Methylation and GLC of Diphenylhydantoin and 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin

Keyphrases ☐ Diphenylhydantoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin—determination ☐ Plasma, urine—diphenylhydantoin and metabolites, determination ☐ GLC, flash methylation—analysis

Sir:

The development of rapid, sensitive, and simple assay methods for determining blood and urine levels of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (major metabolite of diphenylhydantoin) is most imperative for studying the interactions of diphenylhydantoin with other drugs and for more effective control of seizures in patients. We have been able to simplify the GLC analysis of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin by converting them to nonpolar, volatile, methylated derivatives in the injection port of a gas chromatograph with trimethylanilinium hydroxide (1, 2).

The use of trimethylanilinium hydroxide as a methylating reagent offers the following advantages over *N,N* - dimethylformamide – bis(trimethylsilyl)acetamide, a reagent commonly used in preparing the trimethylsilyl derivatives of diphenylhydantoin: (*a*) trimethylanilinium hydroxide is a stable reagent that is easily prepared; (*b*) the methylation reaction with diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin occurs instantaneously in the injection port at temperatures of 300°, producing quantitative yields of methylated derivatives; and (*c*) it is not necessary to dry the purified plasma extracts at high temperatures or take time-consuming measures to exclude water from the extract or derivative-forming reagent *N,N*-dimethylformamide–bis(trimethylsilyl)acetamide.

Normal pooled plasma containing known amounts of drugs and internal standards and the patient's plasma, after adding the internal standards, were purified *via*

1 and 2 hr. In contrast, the hypertonic solution increased the amount of drug transferred compared to control values. This increase, however, was statistically significant only in the case of riboflavin.

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The development of rapid, sensitive, and simple assay methods for determining blood and urine levels of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (major metabolite of diphenylhydantoin) is most imperative for studying the interactions of diphenylhydantoin with other drugs and for more effective control of seizures in patients. We have been able to simplify the GLC analysis of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin by converting them to nonpolar, volatile, methylated derivatives in the injection port of a gas chromatograph with trimethylanilinium hydroxide (1, 2).

The use of trimethylanilinium hydroxide as a methylating reagent offers the following advantages over *N,N* - dimethylformamide – bis(trimethylsilyl)acetamide, a reagent commonly used in preparing the trimethylsilyl derivatives of diphenylhydantoin: (*a*) trimethylanilinium hydroxide is a stable reagent that is easily prepared; (*b*) the methylation reaction with diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin occurs instantaneously in the injection port at temperatures of 300°, producing quantitative yields of methylated derivatives; and (*c*) it is not necessary to dry the purified plasma extracts at high temperatures or take time-consuming measures to exclude water from the extract or derivative-forming reagent *N,N*-dimethylformamide–bis(trimethylsilyl)acetamide.

Normal pooled plasma containing known amounts of drugs and internal standards and the patient's plasma, after adding the internal standards, were purified *via*

liquid–liquid extraction methods similar to those developed by Chang and Glazko (3) and Conard *et al.* (4). Two internal standards,[1] 5-(*p*-tolyl)-5-phenylhydantoin (S-1) and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (S-2), were added to the plasma prior to extraction, carried through the purification procedure, and methylated along with diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin in order to quantitate the drugs and to simplify the extraction procedure by not requiring the transfer of exact volumes during the extraction steps. The standard calibration curves for diphenylhydantoin and free 5-(*p*-hydroxyphenyl)-5-phenylhydantoin were obtained by preparing standard solutions of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin at five concentrations (2.5, 5.0, 7.5, 10, and 20 mcg./ml. plasma) by dissolving the drugs in 0.1 *N* sodium hydroxide and adding a given volume of this solution to the normal pooled plasma. Prior to extraction, a given volume of internal standards S-1 and S-2 (10 mcg./ml. plasma) dissolved in 0.1 *N* sodium hydroxide was also added to the plasma. Six determinations of diphenylhydantoin and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin were run at each concentration level.

The drugs and internal standards were added to 2.0 ml. of normal pooled plasma, adjusted to an acid pH (5–6), and extracted first with cyclohexane and then with isobutyl alcohol–chloroform (1:4). The latter organic layer was then extracted with 1.0 *N* sodium hydroxide and separated; the alkaline aqueous layer was acidified and extracted with ethyl acetate. The ethyl acetate was removed under nitrogen in a constant-temperature bath at 75°. To the residue was added 40 μl. of 0.1 *M* trimethylanilinium hydroxide solution (1), and 1–2 μl. was removed and injected on the gas chromatograph. To determine total 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, the plasma must be hydrolyzed with 3.0 ml. of hydrochloric acid at 100° for 1 hr. (3) to cleave the conjugated sugar form of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, which occurs in plasma and urine primarily as the glucuronide. The hydrolyzed plasma, after neutralization with a saturated aqueous solution of sodium hydroxide, may then be treated similarly to the nonhydrolyzed plasma by liquid–liquid extraction, methylation, and injection on the gas chromatograph.

Gas chromatographic injections were made on a Varian 2100 dual-channel instrument equipped with four hydrogen-flame detectors. The 1.83-m. (6-ft.) long U-shaped glass columns (2 mm. i.d.) were packed with 3% OV-17 on Gas Chrom Q, 100–120 mesh. Operating temperatures were: injector port, 300°; column, 200°; and detector, 300°. Flow rates (milliliters per minute) were: nitrogen, 65; air, 300; and hydrogen, 50. Under these conditions, the retention times (minutes) for the methylated products were: diphenylhydantoin, 7.25; S-1, 10.5; S-2, 16.5; and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, 21.25.

The standard calibration curves were derived by measuring the peak heights and determining the mean peak height ratio of the six determinations at each concentration level [diphenylhydantoin/S-1 and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin/S-2]. The mean peak height ratio of diphenylhydantoin/S-1 was plotted *versus* the weight ratio of diphenylhydantoin/S-1 for the diphenylhydantoin standard calibration curve; the mean peak height ratio of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin/S-2 for free 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (nonhydrolyzed) and total 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (hydrolyzed) was plotted *versus* the weight ratio of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin/S-2. The least-squares method was employed to obtain the best fit for the five concentration levels. The concentration levels were linear over the range studied. Therefore, plasma levels can be determined for diphenylhydantoin, free 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, and total 5-(*p*-hydroxyphenyl)-5-phenylhydantoin by analyzing hydrolyzed and nonhydrolyzed samples.

To determine the concentration levels of unknowns, the peak height ratio is obtained from the chromatogram and the weight ratio is derived from the calibration curve. The micrograms per milliliter of drug, for example, of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin may be calculated as follows:

$$\text{weight ratio} = \frac{\text{5-(p-hydroxyphenyl)-5-phenylhydantoin (mcg./ml. plasma)}}{\text{S-2 (mcg./ml. plasma)}} \qquad \text{(Eq. 1)}$$

$$\text{5-(p-hydroxyphenyl)-5-phenylhydantoin (mcg./ml.)} = [\text{S-2 (mcg./ml. plasma)}]\,(\text{weight ratio from graph}) \qquad \text{(Eq. 2)}$$

Methylation of diphenylhydantoin with a similar reagent, tetramethylammonium hydroxide, was reported by MacGee (5) to form the 1,3-dimethyl-5,5-diphenylhydantoin product. Reaction of diphenylhydantoin with trimethylanilinium hydroxide, considered by Brochmann-Hanssen and Olawuyi Oke (1) to be a better methylating reagent than tetramethylammonium hydroxide, would be expected to form the same 1,3-dimethyl-5,5-diphenylhydantoin derivative. In methylation studies involving phenolic opium alkaloids, Brochmann-Hanssen and Olawuyi Oke (1) reported that morphine, codamine, laudanine, and reticuline gave complete methylation of the phenolic hydroxyl groups with trimethylanilinium hydroxide but not with tetramethylammonium hydroxide. Therefore, it would appear that the flash heater methylation of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin at 300° in the injection port of a gas chromatograph with trimethylanilinium hydroxide would methylate the phenolic hydroxyl group as well as the 1,3 nitrogens of the hydantoin ring, forming 1,3-dimethyl-5(*p*-methoxyphenyl)-5-phenylhydantoin.

Further studies of patients taking only diphenylhydantoin and diphenylhydantoin in combination with other drugs are currently in progress in these laboratories, and the results will be reported at a later date.

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Micelle Formation and Its Significance in Interpretation of NMR Spectra of Phenothiazine Derivatives

Keyphrases ☐ Phenothiazine derivatives—micelle formation ☐ Micelle formation, phenothiazine derivatives—effect on NMR spectra ☐ NMR spectroscopy—micelle formation effect

Sir:

On occasion, we have observed some unusual behavior when evaluating NMR spectra of the salts of phenothiazine derivatives. The spectra of the compounds run in D_2O consisted of a series of broad peaks with little or no resolution and fine structure, which made it impossible to determine J constants. The broadening effect suggested the possibility of a ferromagnetic impurity in the compounds. Accordingly, the phenothiazines were purified as described by Craig *et al.* (1). The spectra were rerun, but the broadening effect previously observed persisted. This effect is illustrated in Fig. 1, using chlorpromazine hydrochloride as the model compound. At this point, the possibility of micelle formation was considered. Micelle formation was reported previously for phenothiazine derivatives (2).

In the previous study, it was noted that a compound in the micellar state showed broad resolution bands; however, when the temperature was raised the micelle was disrupted and the NMR bands became sharper and more distinguishable (3). We obtained similar results when chlorpromazine HCl was run in D_2O at 95°. Figure 1 shows this behavior. The spectrum at the elevated temperature showed increased resolution and sharp absorption peaks, which permitted the determination of J constants. Evidently, the strong association in the micellar state completely masked the NMR spectrum of the compound. A series of phenothiazine derivatives, including promazine HCl, prochlorperazine HCl, trifluoperazine HCl, trifluoromeprazine HCl, and vespazine HCl, were run in the same manner, and all exhibited similar behavior. These studies suggest that the broadening effect observed in the NMR spectra of the compounds in D_2O may be attributable to the formation of micelles, rather than to the presence of ferromagnetic impurities. We feel that this observation should be of general interest to other investigators and should save considerable time and effort when NMR studies of a similar or related nature are involved.

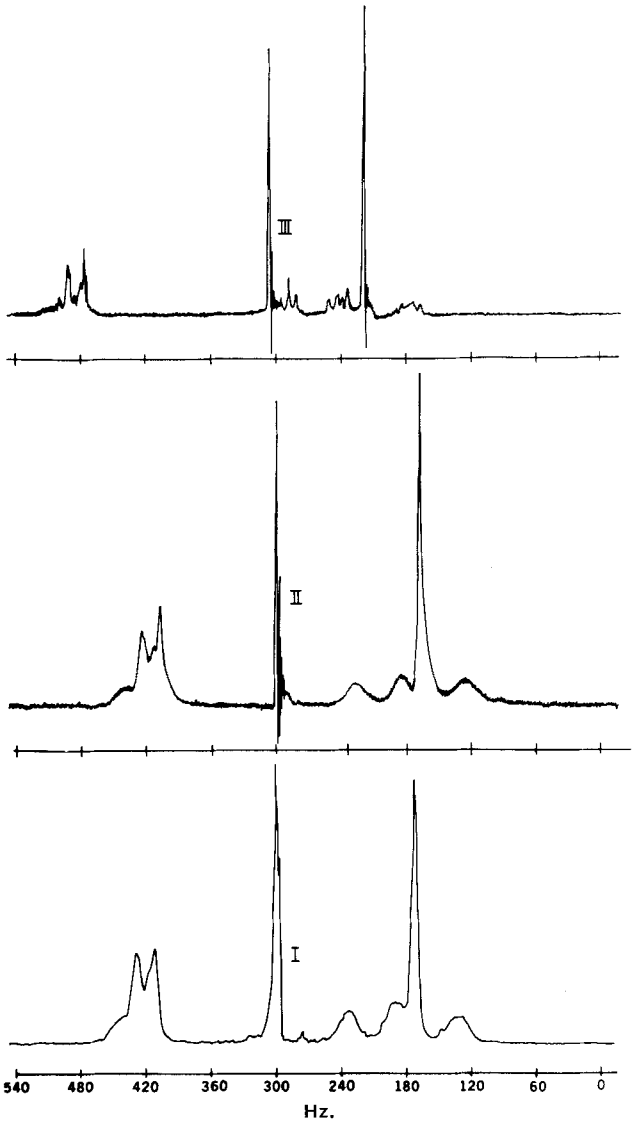

Figure 1—*NMR spectra for 10% solutions of chlorpromazine HCl in D_2O. Key: I, solution containing added amount of known ferromagnetic impurity; II, solution prepared with purified chemical; and III, solution from II run at 95°.*

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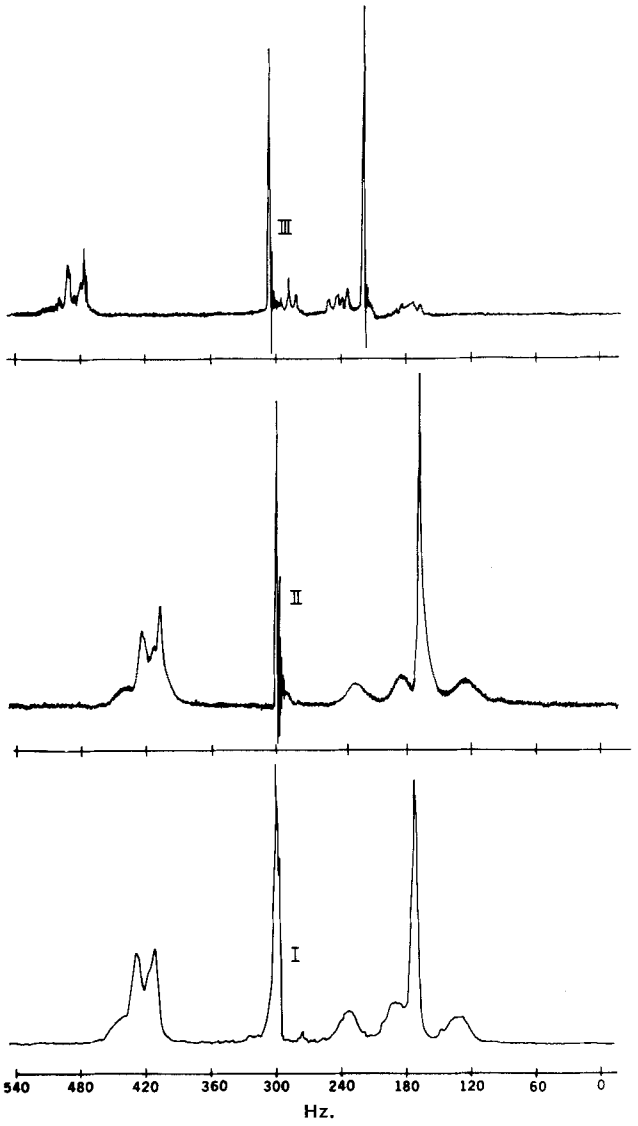

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Linear Polypeptide of a Known Sequence of Amino Acids: Poly-(L-phenylalanyl-L-glutamyl-L-alanylglycyl)glycine Methyl Ester

Keyphrases ☐ Poly-(L-phenylalanyl-L-glutamyl-L-alanylglycyl)glycine methyl ester—synthesis ☐ Polypeptide, linear—known sequence of amino acids ☐ Immunochemical properties—linear polypeptide, known sequence of amino acids

Sir:

Random copolymers containing varying amounts of the amino acid residues, alanine, glutamic acid, and tyrosine, have been shown to be antigenic (1–3). However, due to the unknown primary sequence of these random polymers, it is difficult to describe the locus of the active site of these antigenic polymers. To overcome this difficulty, the use of linear polypeptides with a known sequence of amino acids has been suggested. For this purpose, poly-(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester was recently synthesized (4, 5). This polypeptide was found to be antigenic (6), giving a precipitin reaction with its antiserum. To ascertain the role of the various amino acid residues in this antigenic polypeptide, it was necessary to prepare a series of polymers that differ from each other with respect to the following parameters: primary sequence of amino acids, variations in the steric requirements of the hydrophobic side chains, and the presence or absence of the phenolic hydroxyl group.

To investigate this latter point, we wish to report the synthesis and immunochemical properties of poly-(L-phenylalanyl-L-glutamyl-L-alanylglycyl)glycine methyl ester (Compound 1, Scheme I).

N-tert-Butyloxycarbonyl-L-phenylalanine pentachlorophenyl ester (7) was coupled to γ-*tert*-butyl-L-glutamyl-L-alanylglycine methyl ester hydrochloride (8) to give *N-tert*-butyloxycarbonyl-L-phenylalanyl-γ-*tert*-L-glutamyl-L-alanylglycine methyl ester (Compound 2), m.p. 191–193°, $[\alpha]_D^{29}$ −15.3° (c 1.50 in dimethylformamide).

Anal.—Calcd. for $C_{29}H_{44}N_4O_9$: C, 58.8; H, 7.5; N, 9.5. Found: C, 59.0; H, 7.3; N, 9.5.

Saponification of Compound 2 with *N* NaOH yielded the tetrapeptide free acid *N-tert*-butyloxycarbonyl-L-phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine (Compound 3), m.p. 132.5°, $[\alpha]_D^{29}$ −12° (c 1.46 in dimethylformamide).

Anal.—Calcd. for $C_{28}H_{42}N_4O_9$: C, 58.15; H, 7.3; N, 9.7. Found: C, 58.1; H, 7.25; N, 9.6.

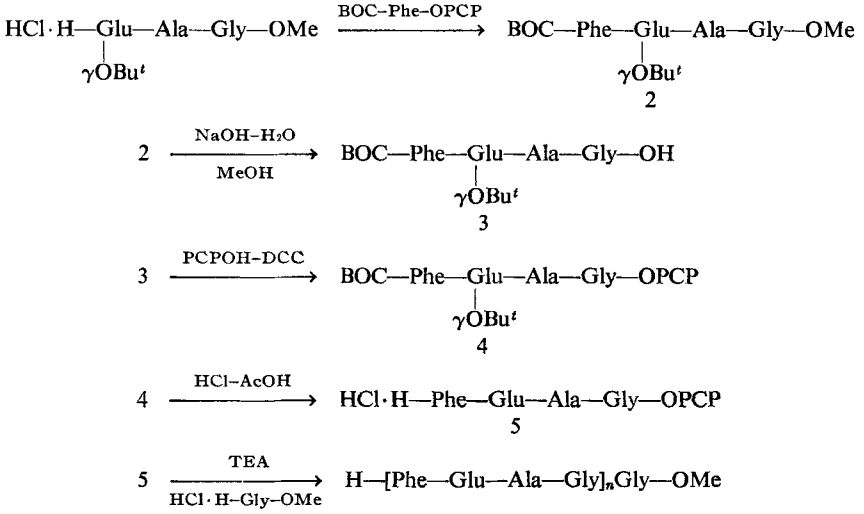
Coupling Compound 3 with pentachlorophenol, using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methotoluene-*p*-sulfonate, yielded the tetrapeptide activated ester, *N-tert*-butyloxycarbonyl-L-phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester (Compound 4), m.p. 210°.

Anal.—Calcd. for $C_{34}H_{41}Cl_5N_4O_9$: C, 49.4; H, 5.0; N, 6.8. Found: C, 49.3; H, 5.0; N, 6.8.

Treatment of Compound 4 with dry hydrogen chloride in glacial acetic acid removed the *N-tert*-butyloxycarbonyl and *tert*-butyl ester protecting groups to give L-phenylalanyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (Compound 5), m.p. 186° dec., $[\alpha]_D^{29}$ 2.5° (c 1.01 in dimethylformamide).

Anal.—Calcd. for $C_{25}H_{26}Cl_6N_4O_7$: C, 42.5; H, 3.5; N, 7.9. Found: C, 42.7; H, 3.8; N, 7.85.

The polymerization of Compound 5 was conducted under dilute conditions in the presence of the preformed monomer glycine methyl ester hydrochloride. This established polymerizing procedure has been shown to yield high molecular weight polypeptides (4, 5, 8–10). Thus, the polymerizing unit, Compound 5, dissolved in dimethyl sulfoxide was added to a solution of glycine methyl ester hydrochloride containing the total amount (3.5 equivalents) of triethylamine such that the final concentration of reactants was never more than 100 mmoles/l. The polymerization was allowed to proceed for a week, after which it was acidified and dialyzed against distilled water. The precipitated polymer, poly-(L-phenylalanyl-L-glutamyl-L-alanylglycyl)glycine methyl ester, was collected by centrifugation, washed with methanol, dried, converted to its sodium salt, and extensively dialyzed. Final purification was achieved by passage through a column of Sephadex G-100 (2.5 ×



Scheme I

40 cm.) using a buffer (0.05 *M* KH_2PO_4 corrected to pH 8.0) as eluent. Acidification and dialysis of the high molecular weight fractions yielded the pure polypeptide, Compound 1.

Anal.—Calcd. for $C_{19}H_{24}N_4O_6$: C, 55.3; H, 6.1. Found: C, 55.0; H, 6.0.

Filtration of the polymer through a calibrated column (11) of Sephadex G-100 (2.5 × 45 cm.), using a solution of 0.05 *M* KH_2PO_4 corrected to pH 8.0 as eluent, indicated a molecular weight of at least 1×10^5.

Two rabbits were immunized with poly-(phe.glu.ala.gly)gly methyl ester (Compound 1) using the same protocol as that previously described (6). To aliquots of the sera obtained from each rabbit was added up to 500 mcg. of the synthetic polypeptide (Compound 1). No precipitin reaction was observed. Thus, substitution of the tyrosyl residue in the antigen poly-(tyr.glu.ala.gly)gly-1-^{14}C ethyl ester with the phenylalanyl moiety caused a loss of antigenicity, as shown by the precipitin reaction. It has been concluded that it is the phenolic hydroxy group of tyrosine that confers antigenicity to the molecule.

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Portal Vein Blood Sampling in Intestinal Drug Absorption Studies

Keyphrases ☐ Portal vein sampling—intestinal drug absorption ☐ Cannulation method—portal vein

Sir:

In an attempt to study the *in situ* absorption of drugs from the rat small intestine, a method of sampling portal vein blood was devised.

Generally, *in situ* drug absorption from the small intestine has been studied by determining the reduction in concentration of drug in the lumen (1–3). To complete the picture of intestinal absorption, simultaneous measurements of drug appearance in the portal blood and in the bile are required.

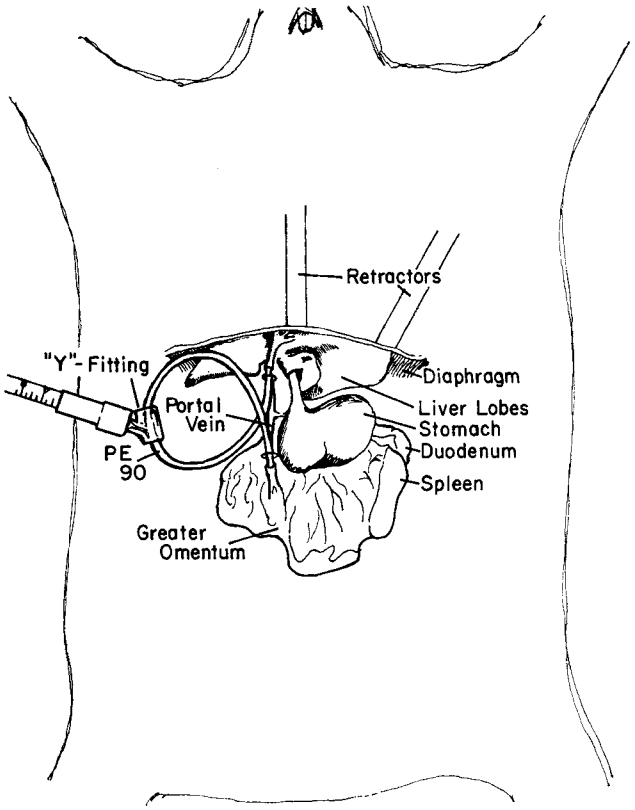

Figure 1—*Portal vein cannulation. Stomach, liver, and mesenteries were retracted to show the cannulation.*

In this communication, we report a method for sampling portal blood. The methods presented in previous work on dogs (4, 5) and rabbits (6) are not practical for the portal vein of a 200-g. rat since this portal vein is quite fragile. We tried direct venous puncture, using a needle point with tubing attached for sampling. The major problems were placing and securing the needle among the liver lobes, the mesenteries, and the small intestine, all of which should remain in their normal positions. The method described here consists of a shunt of polyethylene tubing cannulated in opposite directions in the portal vein. The surgery is delicate, requiring speed and accuracy to prevent excessive engorgement of the intestinal veins and acute loss of blood in the liver.

The viscera were exposed by midline abdominal incision. The portal vein was exposed by gentle retraction of the stomach just above the pyloric sphincter. The distal cannulation was prepared first. A Johns Hopkins bulldog clamp was placed around the portal vein, deep in the greater omentum. Fat and mesentery were included in the clamp to cushion the vein, thus preventing its collapse. The vein should not be cleaned of connective tissue. A cut in the vessel was then made approximately 3 mm. proximal to the clamp, and a slightly beveled length of PE 90 polyethylene tubing [i.d. 0.086 cm. (0.034 in.), o.d. 0.13 cm. (0.050 in.), length 27.9 cm. (11 in.)],[1] containing heparin solution, was inserted

[1] PE 90 Intramedic, Clay Adams.

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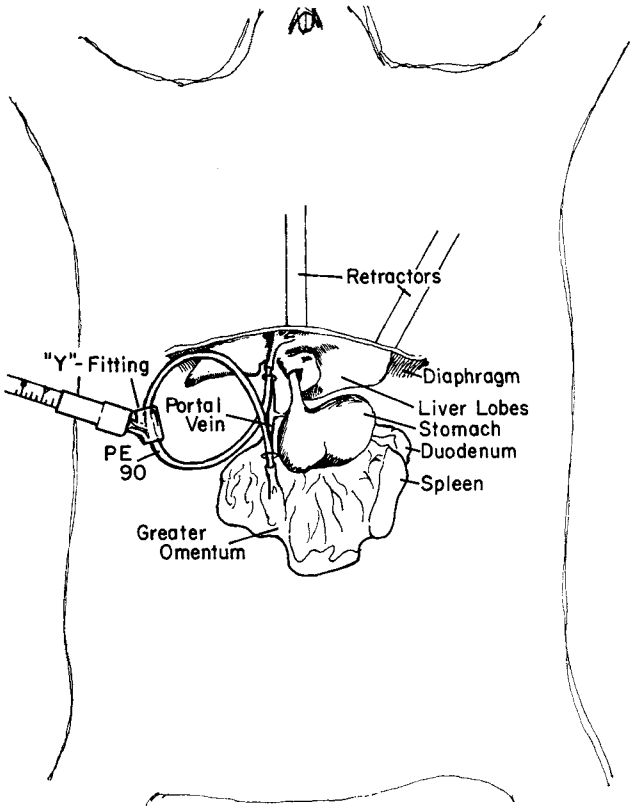

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The glass Y, fitted with a needle support, provided a means of sampling the blood. A 27-gauge needle was placed in the tubing through the support, and the sampling syringe was attached to the needle. Blood samples were withdrawn slowly to prevent embolism. Heparin was occasionally injected through this port to ensure free blood flow.

The bile duct was then cannulated with PE 50 polyethylene tubing, and samples were introduced into the duodenum of the intestine. At this point, the incision was closed by clamp or sutures. If lumen samples are to be taken, the organs are kept warm with a high-intensity lamp and moist with wet gauze.

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Immunochemical Studies on Linear Antigenic Polypeptides of a Known Sequence of Amino Acids

Keyphrases □ Polypeptides, linear, antigenic—immunochemical properties □ Antibodies, polypeptide produced—specificity

Sir:

It has recently been shown that the linear polypeptide poly-(L-tyrosyl-L-glutamyl-L-alanylgycyl)glycine - 1 - C^{14} ethyl ester (1, 2) is antigenic, eliciting antibodies in rabbits (3). It was thought reasonable that if the alanyl residue of this antigen was part of the active site, then substitution of this residue with the sterically larger valyl moiety could possibly alter the immunochemical properties of the polypeptide. For this purpose, poly-(L-tyrosyl-L-glutamyl-L-valylglycyl)glycine-1-C^{14} ethyl ester was synthesized (4), and we wish to report some immunochemical studies using this material.

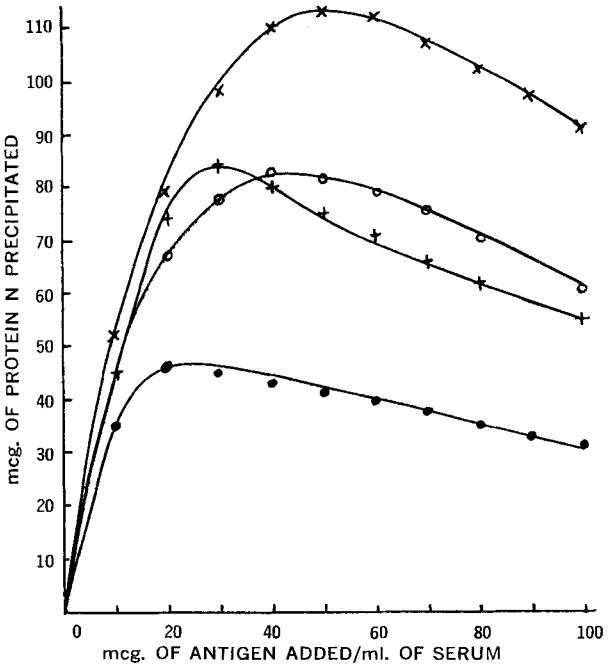

Figure 1—*Precipitin curves. Key:* × = *TGVGly against anti-TGVGly sera,* + = *TGAGly against anti-TGAGly sera,* ○ = *TGVGly against anti-TGAGly sera, and* ● = *TGAGly against anti-TGVGly sera.*

To investigate the antigenicity of poly-(tyr.glu.val.-gly)gly-1-C^{14} ethyl ester, two rabbits were treated at weekly intervals with 500 mcg. of the material. The first 2 weeks, they were injected interdermally using complete Freund's adjuvant as suspension medium; the 3rd week they were injected subcutaneously. The injection on the 4th week was done intravenously using buffered saline. Bleedings were conducted on the following week, and the serum from each animal was found to give a precipitin reaction with the polymer. The preimmunized sera under the same conditions gave a negative precipitin reaction. The quantitative determination of the precipitate was obtained by the addition of dilutions of poly-(tyr.glu.val.gly)gly-1-C^{14} ethyl ester to 1-ml. samples of the pooled rabbit sera. The precipitates were kept at 4° for 48 hr., washed twice with small volumes of buffered saline, and collected by centrifugation. The total amount of protein precipitated was estimated by analysis for nitrogen by the Kjeldahl method. The precipitin curve is shown in Fig. 1, and it would appear that the polymer containing valyl residues is a better antigen than that having alanyl residues.

To investigate the specificity of the antibodies produced by both of these antigenic polypeptides, the following series of crossreactions was performed. To 1-ml. aliquots of rabbit sera immunized against poly-(tyr.glu.ala.gly)gly-1-C^{14} ethyl ester were added dilutions of poly-(tyr.glu.val.gly)gly-1-C^{14} ethyl ester. After incubation at 37° for 1 hr., each aliquot had produced a precipitate. These precipitates were kept at 4° for 48 hr., washed twice with small volumes of buffered

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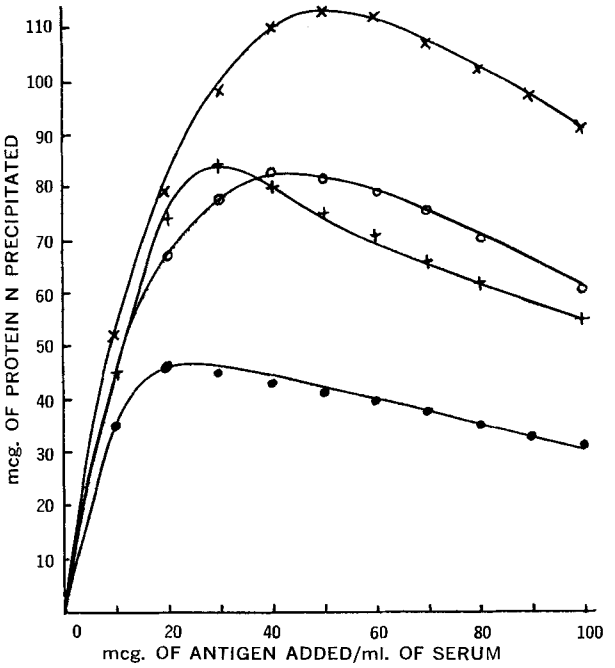

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Antitumor Activity of An Acronycine–Polyvinylpyrrolidone Coprecipitate

Keyphrases ☐ Acronycine–polyvinylpyrrolidone coprecipitate—antitumor activity ☐ Antitumor activity—acronycine–polyvinylpyrrolidone coprecipitate

Sir:

Polyvinylpyrrolidone (PVP) is known to increase the solubility of aromatic compounds (1–3). This has been explained (1) as a result of hydrophobic bonding and an exothermic interaction between the solubilized compound and PVP. Application of this phenomenon to pharmaceuticals has been demonstrated (2, 3), and the advantages of PVP–drug coprecipitates as superior systems for pharmaceutical applications are known (4, 5).

In our work, pharmaceutical grade, 40,000 molecular weight, PVP was coprecipitated with acronycine from ethanol. The ratio of acronycine to PVP was 1:5 (w/w). Solubility of acronycine as the coprecipitate (75 mcg./ml., distilled water) was approximately 15 times that of noncoprecipitated acronycine (5 mcg./ml.). Acronycine,[1] a compound of known experimental antitumor activity (6), was compared with the acronycine–polyvinylpyrrolidone (Ac–PVP) coprecipitate against two of the most sensitive tumors to acronycine, X5563 plasma cell myeloma and C1498 myelogenous leukemia (Table I). These data indicate that PVP coprecipitated acronycine is more active than acronycine itself. Efforts are continuing in this area to optimize acronycine activity by adjusting the acronycine to PVP ratio and by employing other molecular weight grades of PVP.

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[1] The United States Adopted Names Committee (USAN) has approved acronine as the generic name for acronycine.

Table I—Antitumor Activity of Acronycine and an Acronycine–PVP Coprecipitate

Sample	Tumor[a]	mg./kg. × Days Dosed[b]	Route	ATD, mm.[c]	ALS, Days[d]	I, %[e]	PL, %[f]
Acronycine	X5563	30 × 9	i.p.	8.3	—	52	—
Ac–PVP	X5563	180 × 9	i.p.	0.9	—	95	—
Control	X5563	Emulphor × 9	i.p.	17.2	—	0	—
Acronycine	X5563	45 × 9	Oral	6.8	—	46	—
Ac–PVP	X5563	270 × 9	Oral	0.0	—	100	—
Control	X5563	Emulphor + 9	Oral	12.5	—	0	—
Acronycine	C1498	30 × 10	i.p.	—	20.4	—	40
Ac–PVP	C1498	180 × 10	i.p.	—	23.6	—	62
Control	C1498	Emulphor × 10	i.p.	—	14.6	—	0
Acronycine	C1498	45 × 10	Oral	—	19.4	—	24
Ac–PVP	C1498	270 × 10	Oral	—	24.2	—	55
Control	C1498	Emulphor × 10	Oral	—	15.6	—	0

[a] X5563 was implanted by trocar, subcutaneously, in C_3H mice, and therapy was begun 72 hr. later. C1498 was transferred by an intraperitoneal injection of tumor homogenate, and therapy was begun 24 hr. later. [b] Ac–PVP was coprecipitated in a 1:5 w/w basis. Emulphor (General Aniline and Film, New York, N. Y.) was the suspension vehicle for both acronycine and Ac–PVP. [c] ATD = average tumor diameter of 10 animals expressed in millimeters. [d] ALS = average life span of 10 animals expressed in days. [e] % I = ATD of the treated/control expressed as a percent inhibition. [f] % PL = ALS of the treated/control expressed as a percent prolongation of life.

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Antitumor Activity of An Acronycine–Polyvinylpyrrolidone Coprecipitate

Keyphrases ☐ Acronycine–polyvinylpyrrolidone coprecipitate—antitumor activity ☐ Antitumor activity—acronycine–polyvinylpyrrolidone coprecipitate

Sir:

Polyvinylpyrrolidone (PVP) is known to increase the solubility of aromatic compounds (1–3). This has been explained (1) as a result of hydrophobic bonding and an exothermic interaction between the solubilized compound and PVP. Application of this phenomenon to pharmaceuticals has been demonstrated (2, 3), and the advantages of PVP–drug coprecipitates as superior systems for pharmaceutical applications are known (4, 5).

In our work, pharmaceutical grade, 40,000 molecular weight, PVP was coprecipitated with acronycine from ethanol. The ratio of acronycine to PVP was 1:5 (w/w). Solubility of acronycine as the coprecipitate (75 mcg./ml., distilled water) was approximately 15 times that of noncoprecipitated acronycine (5 mcg./ml.). Acronycine,[1] a compound of known experimental antitumor activity (6), was compared with the acronycine–polyvinylpyrrolidone (Ac–PVP) coprecipitate against two of the most sensitive tumors to acronycine, X5563 plasma cell myeloma and C1498 myelogenous leukemia (Table I). These data indicate that PVP coprecipitated acronycine is more active than acronycine itself. Efforts are continuing in this area to optimize acronycine activity by adjusting the acronycine to PVP ratio and by employing other molecular weight grades of PVP.

(1) P. Molyneux and H. Frank, *J. Amer. Chem. Soc.*, **83**, 3169 (1961).
(2) T. Higuchi and R. Kuramoto, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 393(1954).
(3) *Ibid.*, **43**, 398(1954).
(4) M. Mayersohn and M. Gibaldi, *J. Pharm. Sci.*, **55**, 1323(1966).
(5) A. P. Simonelli, S. C. Mehta, and W. I. Higuchi, *ibid.*, **58**, 538(1969).
(6) G. H. Svoboda, G. A. Poore, P. J. Simpson, and G. B. Boder, *ibid.*, **55**, 758(1966).

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[1] The United States Adopted Names Committee (USAN) has approved acronine as the generic name for acronycine.

Table I—Antitumor Activity of Acronycine and an Acronycine–PVP Coprecipitate

Sample	Tumor[a]	mg./kg. × Days Dosed[b]	Route	ATD, mm.[c]	ALS, Days[d]	I, %[e]	PL, %[f]
Acronycine	X5563	30 × 9	i.p.	8.3	—	52	—
Ac–PVP	X5563	180 × 9	i.p.	0.9	—	95	—
Control	X5563	Emulphor × 9	i.p.	17.2	—	0	—
Acronycine	X5563	45 × 9	Oral	6.8	—	46	—
Ac–PVP	X5563	270 × 9	Oral	0.0	—	100	—
Control	X5563	Emulphor + 9	Oral	12.5	—	0	—
Acronycine	C1498	30 × 10	i.p.	—	20.4	—	40
Ac–PVP	C1498	180 × 10	i.p.	—	23.6	—	62
Control	C1498	Emulphor × 10	i.p.	—	14.6	—	0
Acronycine	C1498	45 × 10	Oral	—	19.4	—	24
Ac–PVP	C1498	270 × 10	Oral	—	24.2	—	55
Control	C1498	Emulphor × 10	Oral	—	15.6	—	0

[a] X5563 was implanted by trocar, subcutaneously, in C_3H mice, and therapy was begun 72 hr. later. C1498 was transferred by an intraperitoneal injection of tumor homogenate, and therapy was begun 24 hr. later. [b] Ac–PVP was coprecipitated in a 1:5 w/w basis. Emulphor (General Aniline and Film, New York, N. Y.) was the suspension vehicle for both acronycine and Ac–PVP. [c] ATD = average tumor diameter of 10 animals expressed in millimeters. [d] ALS = average life span of 10 animals expressed in days. [e] % I = ATD of the treated/control expressed as a percent inhibition. [f] % PL = ALS of the treated/control expressed as a percent prolongation of life.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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MARCH 1971
VOLUME 60 NUMBER 3

REVIEW ARTICLE

Stability-Indicating Assay Methods for Drugs and Their Dosage Forms

LESTER CHAFETZ

Keyphrases ☐ Stability-indicating assay methods—literature review ☐ Analytical methods, drugs, dosage forms—stability indicating ☐ Incompatibilities, dosage forms—determination ☐ Labile functional group assays—stability indicating

Several years ago, pharmaceutical scientists used to distinguish between *control* and *stability assay* methods. Batches of pharmaceuticals were released for testing or marketing on the basis of the control assay, whose primary attribute was that it provided a precise and accurate measure of the amount of drug incorporated in a formulation. In some cases, the same method could be used for stability studies, but often a special method, the stability assay, was developed for use in determining the integrity of the drug substance during accelerated shelflife studies. Advances in analytical technology and the legal requirements these have fostered have made the stability-indicating assay the criterion for product acceptance. The dichotomy between assays that measure the amount of drug added and those that measure the amount remaining persists in the use of content uniformity testing.

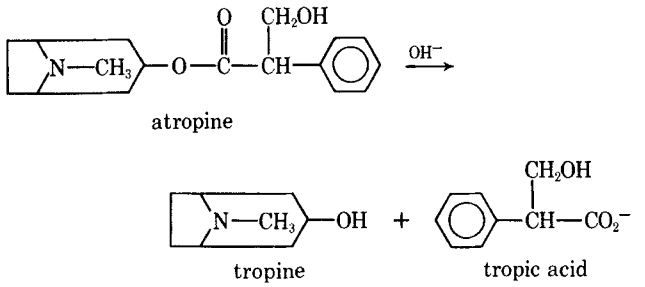
A stability-indicating assay method may be defined as a procedure that affords the selective determination of a drug substance in the presence of its decomposition and reaction products. Drug substances, the majority of which are highly refined organic chemicals, have intrinsic stability under their usual storage conditions. This stability property can often be predicted from inspection of chemical structure and physical properties. Moreover, the environment of a drug substance, *per se*, is easily controllable; there is relatively little limitation on the amount of sample that can be taken for testing; and there is no constraint on the variety of testing techniques and apparatus that can be applied. Most compendial assays for drug substances are relatively nonselective functional group assays chosen for their accuracy; however, the assay is invariably supported by other monograph requirements which provide information on drug quality.

A drug dosage form could be considered as a deliberate contamination of a therapeutic principle, a sophistication which is perpetrated to achieve uniformity and convenience of dosage, product identity, and the physical properties which enable it to be mass produced and shipped in commerce. Depending on its dosage, the active principle in a dosage form may be the major component on a weight basis or a very minor fraction. For example, meprobamate tablets may be 75% drug, while ethinyl estradiol may constitute only 0.007% of the total weight of a tablet. Because a drug dosage form represents a dilution or dispersion of a drug with other substances, the analytical problems with it are more complex and the stability less easily predictable than with the drug substance by itself. A procedure which is stability indicating for a drug in one instance is not necessarily valid for the drug in another environment where reaction possibilities differ. As used here, *stability indicating* is a relative, not an absolute, descriptor in assessing the validity of a method; one method may be more nearly valid than another without either of them being absolutely conclusive. This review provides a critical examination of assay methods for representative drugs with respect to their usefulness in establishing and monitoring chemical stability.

With few exceptions (1, 2), assay methods are combinations of separation and measurement operations. Separation may be as simple as dissolution of a drug from a solid dosage form matrix with filtration of insoluble excipients or as complex as differential migration in a chromatographic system. Measurement could be as simple as weighing a precipitate, or it might require elaborate and expensive instrumentation. The selectivity required may be derived primarily from the separation operation or from the measurement technique used, but it is a function of the method considered as a whole. Obviously, there can be several appropriate approaches to devising stability-indicating assay methods.

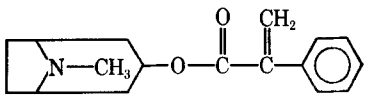
One could determine drug stability or instability, *i.e.*, measure intact drug by a highly selective method, measure total drug content nonselectively and estimate the degradation products, or selectively measure both drug and decomposition products. *A priori*, the third alternative is the most desirable; however, it may not be either possible or practical.

Selective Determination of Active Drug—Determination of intact drug in the presence of decomposition products requires knowledge of degradation routes in most cases. Some investigators (3, 4) considered that the most likely degradation route for atropine would be ester hydrolysis with formation of tropine and tropic acid (Scheme I). They measured hydrolysis rates using a

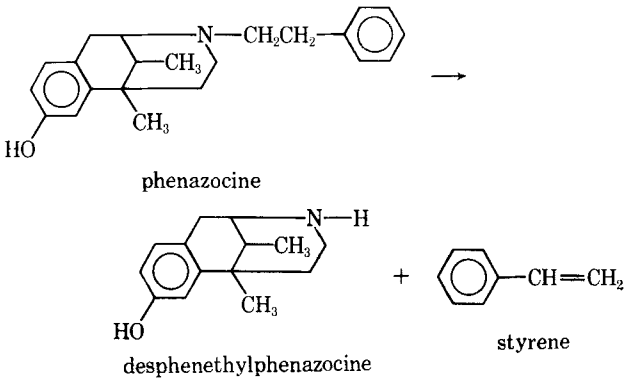


Scheme I

modification of the compendial Salts of Organic Nitrogenous Bases method, where the amine is separated by extraction from alkali into an organic solvent, extracted into dilute aqueous acid, and determined by UV spectrophotometry. Since the UV absorption of atropine is conferred by the benzene ring in the tropic acid moiety, and tropic acid present as a result of hydrolysis would be retained as the anion in the aqueous alkali, the procedure would appear to be valid as a measure of ester hydrolysis. Recently, however, other investigators (5, 6) criticized these studies on the basis that they did not recognize the concomitant formation of apoatropine, which has a much more intense UV spectrum than atropine.



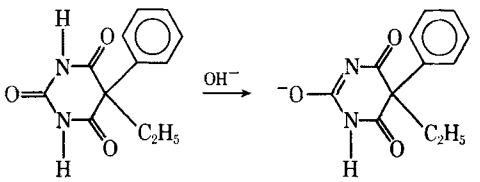
Eisdorfer *et al.* (7) found that phenazocine HBr was unaltered by thermal stress either as the salt or in acid solutions, but the base or neutral or alkaline solutions showed evidence of degradation. They determined that decomposition produced styrene and a secondary amine (Scheme II). They recommended two methods for



Scheme II

determining the drug in the presence of its decomposition products. In one, phenazocine was separated by cyclohexane extraction from alkaline solution and then determined by differential UV spectrophotometry in an extract of the organic solvent. In the other, phenazocine was determined by nonaqueous titration after chloroform extraction of a 0.1 *N* hydrochloric acid solution.

Tishler *et al.* (8) studied assay methods for phenobarbital in relationship to its degradation products. They determined that the UV spectrum of the drug at pH 10, due to the monoanion of the ureide ring, was greatly diminished with cleavage of the ring to decomposition products (Scheme III). Only the intact drug



Scheme III

showed a difference in the UV spectrum with a change in pH from acid to alkaline. They recommended differential UV spectrometry, measurement of an aliquot at pH 10 *versus* an acidified aliquot, as a stability-indicating assay method for phenobarbital. Since the spectra of other 5,5-disubstituted barbiturates are similar to phenobarbital, the method is generally applicable.

In the foregoing examples, the decomposition products were well characterized; however, there are many instances where this situation does not obtain. Khoury and Cali (9) demonstrated the specificity of the fluorometric determination of ethinyl estradiol by TLC of estrogen samples that had been partially degraded by heating or by exposure to light. The chromatograms exhibited several spots when sprayed with phosphomolybdic acid, a general oxidizing agent, but only one fluorescent spot, corresponding in R_f to the intact drug, when the fluorogenic[1] reagent, 90% sulfuric acid, was

[1] As used in this paper, a fluorogenic or chromogenic reagent is a reactant used to produce fluorescence or an absorption band. A fluorophore or chromophore is a molecule that fluoresces or has an absorption band.

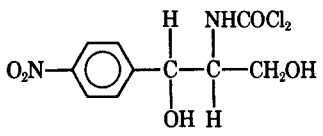
used for detection. Although it might be more satisfying intellectually to know the mechanism of the fluorogenic reaction and the decomposition pathways, this example illustrates the validation of an empirical method where the chemistry of neither has been elucidated. The use of chromatography on paper or thin layers in conjunction with the fluorogenic or chromogenic reagent for spot detection is generally applicable.

Chafetz *et al.* (10) used gas chromatography as the reference method for the methanol–sulfuric acid colorimetric assay for quinestrol, ethinyl estradiol 3-cyclopentyl ether, after intentional degradation of the drug with hydrogen peroxide, dilute acid, and base. They obtained excellent correlation of gas chromatographic and colorimetric assay values for intact drug. Templeton *et al.* (11) used a similar scheme to validate mestranol assays; however, they used intentionally degraded tablets. Use of the dosage form as the model may have advantages in providing additional opportunities for drug decomposition, but excipient decomposition under unrealistic stress conditions may complicate the interpretation of results. It is often possible to correlate actual drug decomposition in dosage forms with stress-induced decomposition of the pure drug substance by comparison of the gas chromatographic or thin-layer patterns produced by the degradation products.

Solubility Analysis as a Reference Method—Comer and Howell (12) evaluated the use of solubility analysis in judging the suitability of routine stability-testing methods for a number of drugs. They pointed out that it is only possible to use this tedious technique on the drug substance free from formulation excipients. In one instance, with propoxyphene, they found no decomposition after thermal stress either by IR spectroscopy, the stability test method, or the reference method. The suitability of the IR method was not established.

Biological Assays as Reference Methods—Since drug assays by physicochemical means are intended to assure the biological activity characteristic of the intact drug, the direct measurement of biological activity would appear to be ideal. In many cases, however, the endpoint of a biological test cannot be measured precisely enough to distinguish between a product that would pass and one that would fail by physicochemical assay. Where the drug is antimicrobial, microbiological assay is often both relatively accurate and convenient.

Higuchi and his students (13–16) performed a classical study on the stability of the antibiotic chloramphenicol. *A priori*, they considered that the most likely



chloramphenicol

degradation routes would be hydrolysis of the amide function or of covalently bound chlorine, and they measured the rates of both reactions. The rate of amide hydrolysis, determined by a column partition chromatography procedure selective for intact drug, was significantly faster than formation of ionic chloride, indicating that the first route was the primary degradative pathway. They inferred from the excellent correlation obtained between the chromatographic assay and a microbiological assay that racemization could not be an important decomposition route.

Garrett (17) showed that a microbiological assay for tetracycline yielded an overestimate of its potency when applied to partially degraded samples, because the decomposition product, although much lower in antibacterial potency, diffused more readily in the culture medium than did the intact antibiotic drug. Here, as is often the case with physicochemical measurements, the sensitivity of the analytical measurement differed markedly for the intact drug and its decomposition products. As with any other method, the specificity of a biological assay should not be taken for granted.

Estimation of Decomposition Products—Using an assay method selective for the intact drug molecule usually entails accepting a relative standard deviation of 1% or more. In stability studies on new drugs or new formulations, it is often difficult to discriminate between normal assay variation and incipient degradation without several data points or an independent measure of degradation products. Accurate measurement of degradation products requires their characterization; however, estimation of their amounts often suffices when they are present in relatively small amounts, *i.e.*, about 1%. In those relatively rare instances where drug degradation leads to a toxic product, as in the formation of nephrotoxic 4-epianhydrotetracycline from tetracycline, the pharmaceutical chemist is obligated to control and limit the amount formed (18–21).

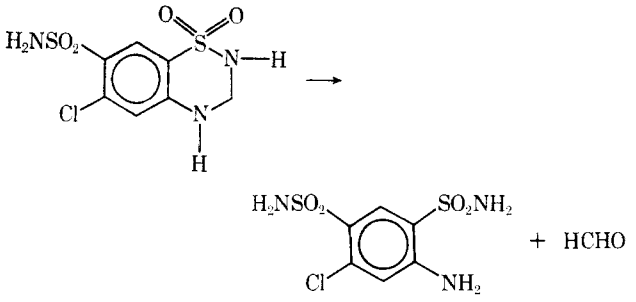
Paper, thin-layer, and gas chromatography techniques have made monitoring decomposition products relatively simple; however, their effective use requires some sophistication. A number of systems of differing polarity should be employed in searching for unknown decomposition products, and the system chosen for routine use should have been demonstrated capable of separating known decomposition products. It is sometimes difficult to discriminate between drug decomposition products and trace impurities present in the drug substance as synthesis contaminants and precursors.

Drug impurities are usually controlled to a level of 1–2%, a concentration below that in which one is generally interested in drug degradation. Since the detectability of spots or peaks on a chromatogram is a function of the loading of the system, the detection technique used, and other factors, the parameters often can be adjusted so that trace impurities are not evident but decomposition above the 1% level is seen. Artifacts from formulation excipients may further confound interpretation of chromatograms, but these can be identified by examination of a blank formulation such as a placebo used in clinical studies.

Quantitation by Means of Decomposition Products—With many drugs, the analytical features of the decomposition product may be more attractive than the intact drug. For example, salicylic acid has a UV absorption band at a long wavelength and more intense absorption than aspirin, and salicylic acid forms an intensely colored chelate with ferric ion. One could measure intact aspirin in a dosage form by the dif-

ference in values of determinations of free salicylic acid and total salicylic acid determined after intentional hydrolysis. (This is rarely done, since relatively simple means for separation of intact aspirin are available.)

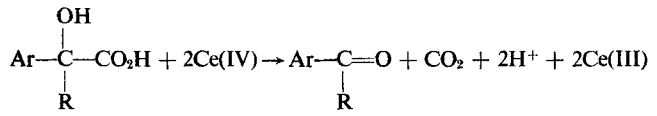
Rehm and Smith (22) studied the stability of hydrochlorothiazide. They found that the drug undergoes a reversible hydrolytic reaction to form 4-amino-6-chloro-1,3-disulfonamide and formaldehyde (Scheme IV). The



Scheme IV

disulfonamide hydrolysis product can be diazotized with nitrous acid and coupled with chromotropic acid or other phenolic compounds to give intensely colored azo dyes. They determined total hydrochlorothiazide by alkaline hydrolysis prior to diazotization and coupling.

Chafetz (23) suggested an approach to the stability analysis of arylglycolate ester drugs, of which eucatropine, glycopyrrolate, homatropine, mepenzolate, oxyphencyclimine, and penthienate are examples recognized in the USP and the NF. The method is based on the selective oxidation of free arylglycolic acids with ceric ion according to Scheme V, where Ar is aromatic

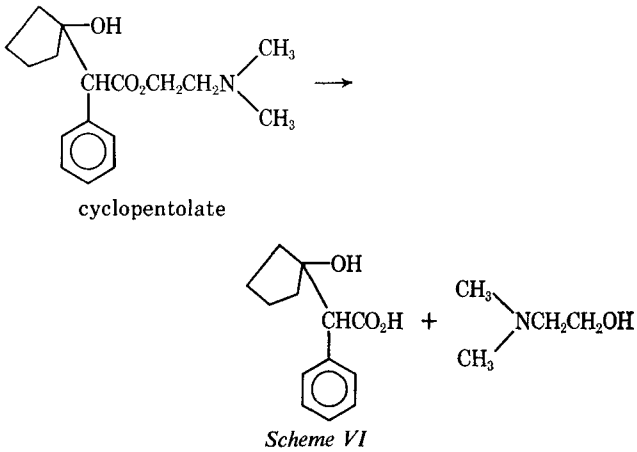


Scheme V

or heterocyclic and R is aromatic, aliphatic, alicyclic, or hydrogen. The formation of a carbonyl group in conjugation with an aromatic ring affords a tremendous increase in UV absorptivity over that of the parent compound, and the chromophore is easily isolated by extraction with a nonpolar solvent. Since intact esters are not oxidized in the reaction, one can: (*a*) determine free arylglycolic acid present as the result of drug hydrolysis; (*b*) determine the total amount of arylglycolate ester by oxidation after saponification; and (*c*) estimate the amount of intact ester by the difference in values. Chafetz and Daly (24) described a variation of this technique for homatropine HBr ophthalmic solutions, determining by titration the amount of ceric ion consumed by saponified and untreated aliquots. Although the titration method eliminates the need for a reference standard, it is less selective than the UV assay.

Solvent Extraction Methods—Extracting the unionized form of an acid or basic drug into an organic solvent and then returning it to aqueous medium for measurement is perhaps the most widely used separation procedure because of its simplicity. Because of its extensive use for compendial drugs, a general method for Salts of Organic Nitrogenous Bases is provided in the USP and the NF. This method uses UV spectrophotometry as the measurement after isolation of the drug by transfers between aqueous base and acid. The method affords separation from acidic and neutral chromophores which might be present in a formulation.

Solvent extraction is often used as a preliminary to titration. The USP XVII assay for Homatropine Hydrobromide Ophthalmic Solution required basification of a measured volume of the preparation, extraction of the base with several portions of chloroform, and titration of it with standard acetous perchloric acid. Chafetz and Daly (24) critized the method, pointing out that the aminoalcohol formed by hydrolysis, tropine, would be extracted and measured as homatropine by this procedure. The USP assay for Cyclopentolate Hydrochloride Ophthalmic Solution is similar, except that prescribed volumes of hexane are used for extracting the base. If one considers hydrolysis as the most probable degradation route, 2-dimethylaminoethanol would be the basic decomposition product formed (Scheme VI).



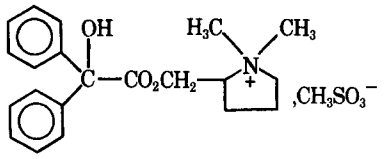
Scheme VI

The partition coefficients for this aminoalcohol between chloroform–water, ether–water, and isooctane–water are 0.22, 0.044, and 0.0043, respectively (25). Hexane should extract very little of the decomposition product; thus the method is stability indicating with respect to hydrolysis. In general, choice of an extraction solvent with the least possible polarity that accomplishes the desired separation provides the greatest selectivity.

Partition chromatography can be considered a special form of solvent extraction; however, the ability to control polarity often affords an extremely high degree of selectivity. Moody (26) described a simple and elegant partition chromatographic method for isolating ephedrine, amphetamine, and other water-miscible amine bases from dosage forms. A portion of the drug dosage form is incorporated with dilute sulfuric acid on chromatographic siliceous earth in a glass column, the column is washed with water-saturated chloroform, and then the internal phase is made alkaline by adding ammonium hydroxide adsorbed on the support. The drug is eluted with wet chloroform, extracted into dilute sulfuric acid, and determined by UV spectrophotometry. Essentially, the method is a highly efficient version of the Salts of Organic Nitrogenous Bases procedure, and it provides the same information.

Dye-Complex and Ion-Pair Formation—Separations by ion-pair formation have been used for many years, although recognition of the phenomenon has been relatively recent. Auerbach (27) used the formation of a solvent-extractable dye complex to measure benzalkonium chloride. In general, ion-pair formation may be considered as an equilibrium process in which oppositely charged ions (neither ion by itself extractable into an organic solvent) form a solvated species in which the hydrophilic ionic moieties are hidden within a hydrophobic envelope. Most of the dye-complex methods use sulfonated dyes as the anion in the pair, and they measure the color of the extracted (ion-paired) dye to provide an indirect measurement of the drug. Since these methods depend on a substituted ammonium function and measure the reagent consumed, they are no more selective than acidimetric titration.

Singleton and Wells (28) reported that dye-complex methods for poldine mesylate did not distinguish between the drug and its hydrolysis product; however, they found that measurement of the chloroform-extracted color of the complex formed by reaction of poldine with ammonium cobaltothiocyanate is selective



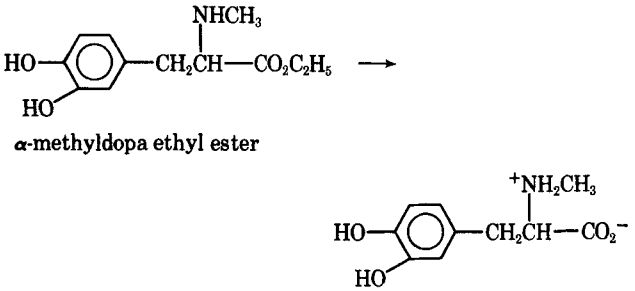
poldine mesylate

for the intact ester. It is likely that the pyrrolidinium alcohol formed by hydrolysis complexes with the cobaltothiocyanate also, but the charged species are not sufficiently well shielded to permit solvent extraction. Higuchi and coworkers (29, 30), Levine and coworkers (31–33), and Temple (34) are among a number of investigators who studied ion-pair techniques for separation of drugs from each other. Relatively little work has been directed toward application of these techniques to separating drugs from their degradation products, although this would seem to be a fruitful direction for exploration. Eisdorfer *et al.* (7) demonstrated that phenazocine could be separated from its desphenethyl decomposition product by extraction with chloroform from dilute hydrochloric acid, undoubtedly an ion-pairing phenomenon. Extraction of lipophilic amines from hydrochloric acid with chloroform is frequently observed. Other anions, such as *p*-toluenesulfonate, trichloroacetate, and cyclamate, can be used; variations in pH, concentration, and solvents can provide very efficient separations.

INCOMPATIBILITIES IN DOSAGE FORMS

Where a pure drug substance may be subject to decomposition by the action of light, heat, adsorbed moisture, and oxygen, incorporation of it in a dosage form extends the possibilities for degradation. Lach and his students (35–39) demonstrated a number of drug–drug and drug–excipient interactions in solid dosage forms which affect drug stability and bioavailability. Solutions of drugs afford much more opportunity for chemical reaction than solid dosage forms; for example, no one has yet provided a stable aspirin elixir, although the preparation of one is a classical problem in pharmaceutics. Solubility and pH requirements for one component of a formulation may provide a hostile environment for another. Talmage *et al.* (40) discovered that cyclamate hydrolyzed to a significant extent in a hydroalcoholic solution used as a vehicle for an antibacterial amine. Although the sweetener is very stable in water, its hydrolysis rate was found to be proportional to the alcohol content of a vehicle and inversely proportional to pH. Considerations of isotonicity and tolerance impose limits on formulation variables for injections and eye solutions, often affording suboptimal conditions for drug stability.

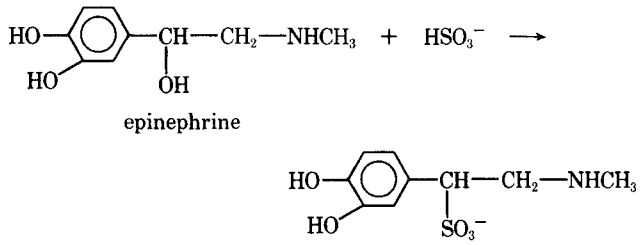
Catecholamines–Bisulfite Reaction—Catecholamines such as epinephrine, isoproterenol, and levarterenol are easily oxidized by air and other oxidants to adrenochromes and products of its further oxidation. Doty (41) showed that catechols form intensely colored derivatives with a ferrocitrate reagent. Since oxidation of epinephrine and its analogs destroys the catechol function on which the Doty reaction depends, the colorimetric method is stability indicating with respect to oxidation. Higuchi *et al.* (42) assayed epinephrine by UV spectrophotometry of its triacetyl derivative following chromatographic isolation of the derivative. The results obtained by this method and the Doty reaction were comparable on solutions degraded by oxidation. Marcus and DeMarco (43) determined α-methyldopa ethyl ester by the Doty reaction after partition chromatography on buffered cellulose. The chromatographic procedure separated the ester from its hydrolysis product (Scheme VII); however, oxidized ester was eluted with the intact drug. Because the measurement technique was selective for the unoxidized compound and interference by the hydrolysis product was eliminated in the procedure; the overall procedure was stability indicating with respect to both routes of degradation.



α-methyldopa ethyl ester

Scheme VII

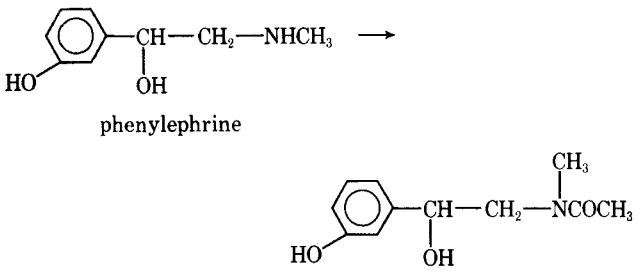
Schroeter *et al.* (44) reported that epinephrine reacts with sodium bisulfite, added to aqueous formulations to inhibit oxidation, to form a biologically inactive sulfonic acid derivative (Scheme VIII). Because the sulfonic acid product retains the catechol group, the Doty reaction does not distinguish between the drug and its reaction product; however, the triacetylation method is applicable. Several years later, Welsh and Sammul (45) reported that isoproterenol, the *N*-isopropyl homolog of epinephrine, reacts with bisulfite in the same way. They found that extraction of the ion pair formed by the catecholamine with bis(2-ethylhexyl)phosphoric acid



Scheme VIII

provides a means for separating isoproterenol from its reaction product, and they used UV spectrophotometry for measurement. These authors cited unpublished work, by E. L. Pratt and M. E. Auerbach of the Sterling-Winthrop Research Institute, which showed that oxidation to the fluorescent adrenolutin also distinguished the drug from its reaction product. Recently, Kaistha (46) proposed two methods for assay of isoproterenol in the presence of decomposition products: a modification of the ion-pairing technique and determination as 3,4-dihydroxybenzaldehyde after periodate oxidation. The latter requires an 18-hr. reaction time in acid solution. The author proposed use of these techniques for tablets as well as solutions, although the rationale for its use with tablets is not clear.

Aspirin Acetylations in Tablets—Troup and Mitchner (47) discovered that phenylephrine reacts with aspirin in tablet formulations, with *N*-acetylation occurring at room temperature and progressive acetylation of the phenolic and secondary alcohol —OH functions taking place during storage at elevated temperatures (Scheme IX). They used a combination of two



Scheme IX

assay methods: (*a*) the 4-aminoantipyrine–ferricyanide reaction (48), selective for a phenol with H or an easily displaceable *para*-substituent, and (*b*) a colorimetric method selective for the secondary amine function, measurement of the cupric-ion complex of the dithiocarbamic acid formed by reaction of the drug with carbon disulfide. One would expect that the Kelly and Auerbach (49) assay method for phenylephrine, colorimetric determination of the azo dye formed by coupling with *p*-nitrobenzenediazonium chloride following separation by ion-exchange chromatography, would be applicable in determining phenylephrine in the presence of *N*-acetylphenylephrine. The Levine and Doyle (50), procedure, partition chromatography of the bis(2-ethylhexyl)phosphoric acid ion pair followed by UV spectrophotometry, should also be stability indicating for this incompatibility. Determination of phenylephrine as 3-hydroxybenzaldehyde as described by Chafetz (51) will distinguish between the drug and its reaction products.

Jacobs *et al.* (52) showed that aspirin can acetylate the alcohol function of codeine at elevated temperature in the presence of moisture; however, the reaction proceeds slowly. They detected the incompatibility by TLC, and they recommended a partition chromatographic method for its separation and determination.

Koshy *et al.* (53) and Boggiano *et al.* (54) found by chromatography that acetaminophen is acetylated at the phenolic function by aspirin in some commercial formulations (Scheme X). Many of the colorimetric methods (55–57) described for acetaminophen require preliminary hydrolysis of it to *p*-aminophenol; none of these would be stability indicating with respect to this incompatibility. The colorimetric method described by Le Perdriel *et al.* (58), measurement of 2-nitro-4-acetamidophenol, formed by nitration of the drug with nitrous acid, would appear to be selective for acetaminophen in the presence of its reaction product, because this reaction is peculiar to phenols.

Some Further Incompatibilities—One may generalize from the aspirin interactions already noted. Of these, the incompatibility with phenylephrine appears the most serious, probably because amides are more stable than esters of alcohols or phenols. One would expect that transacylation reactions in dosage forms may occur with mixtures of esters or other acyl compounds with primary or secondary amines, alcohols, and phenols. Relatively labile esters such as diethylaminoethyl esters of aromatic acids, *e.g.*, tetracaine and procaine, would serve as good sources of acyl groups in such reactions. The ubiquitous use of chromatographic techniques will doubtless yield further examples of transacylation reactions.

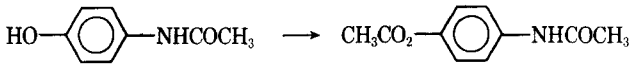
Metal ions have been found to catalyze the decomposition of many drugs. El-Shibini *et al.* (59) found that cupric ion, and lead and iron to a lesser extent, catalyze the decomposition of alkaline phenylephrine solutions. Lewbart and Mattox (60) and Monder (61) reported oxidation of the ketol function of hydrocortisone to a glyoxal by traces of copper ion, and several others (62–64) reported trace metal catalysis of the oxidative degradation of corticosteroids.

Duvall *et al.* (65) attributed the browning of spray-dried lactose to formation of 5-hydroxymethylfurfural. This and other aldehydes in pharmaceutical formulations may react with primary amines to form Schiff bases. Kabasakalian *et al.* (66) showed that benzocaine reacted with citric acid, corn syrup, and natural cherry flavor in a throat lozenge formulation. In each case, the ester group was shown to be intact in the product, and the incompatibility involved the aniline moiety.

MEASUREMENT OF LABILE FUNCTIONAL GROUP

A functional group assay[2] is one that is selective for a particular structural feature in a molecule. Provided that the structural feature determined does not persist in any of the decomposition products, such a method will be stability indicating. Inspection of the structure of chloramphenicol (page 337) shows that its UV

[2] The term is often applied to group-selective reactions. As used here, it applies both to these and to physical methods that are dependent on specific structural features.

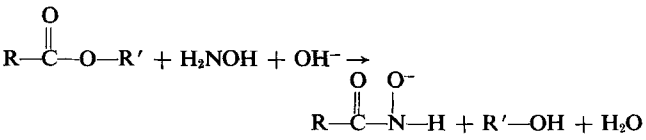


acetaminophen

Scheme X

spectrum derives from its *p*-nitrobenzyl alcohol component. However, several years after publication of the definitive studies by Higuchi *et al.* (13–16), direct UV spectrophotometry was a legally acceptable alternative to microbiological assay for chloramphenicol dosage forms. Schwarm *et al.* (67) criticized this, demonstrating its invalidity by quantitative TLC. Polarographic assay (68) suffers from the same limitation; it measures the nitrobenzene moiety common to the antibiotic and its hydrolysis product. Although neither spectrophotometry nor polarography is a stability-indicating measurement, either could be used in a stability-indicating procedure if provision is made to separate the amine degradation product from the neutral amide intact compound.

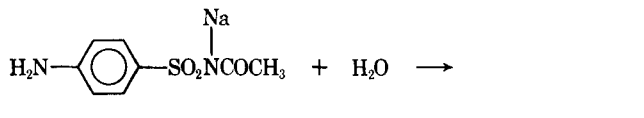
Ferric–Hydroxamate Colorimetry—Alkaline solutions of hydroxylamine react with esters, amides, acyl halides, and other acyl derivatives to form hydroxamic acids (Scheme XI). The red-colored complex formed



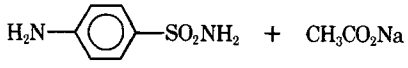
Scheme XI

by treatment of the hydroxamic acid with ferric salts in acid solution serves as the basis of a useful colorimetric method. Carboxylic acids do not react with hydroxylamine under these conditions; thus the method is stability indicating with respect to hydrolysis.

Colorimetric analysis of chloramphenicol has been reported using this procedure (69), and it would appear to be valid. Siegel *et al.* (70) used the method as a means for following the hydrolysis rates of methylphenidate. Schleider *et al.* (71) recommended the method for determining sodium sulfacetamide in the presence of its hydrolysis products, pointing out that the official nitrite titration assay will not distinguish between the drug and its sulfanilamide hydrolysis product, because both bear the primary amine function on which the titration is based (Scheme XII).



sodium sulfacetamide

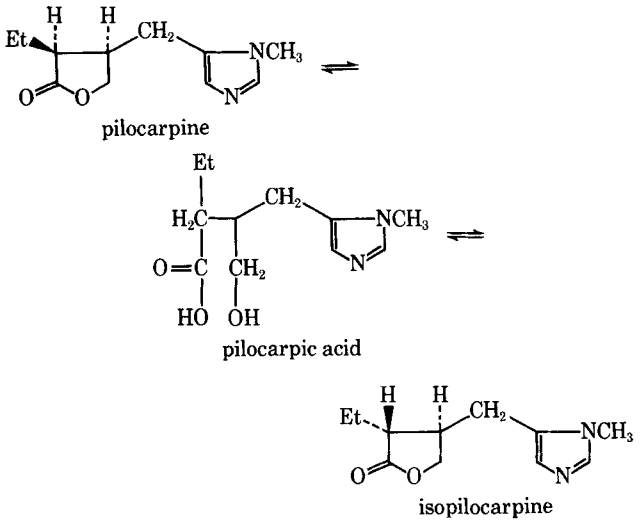


sulfanilamide

Scheme XII

Vincent and Schwal (72) investigated ferric–hydroxamate colorimetry for a number of alkaloids, including pilocarpine, cocaine, physostigmine, atropine, and scopolamine. Of the alkaloids tested, the tropine esters were found to give very weak colors, perhaps because the hydrolysis reaction rate predominates over the rate of hydroxylaminolysis. A number of workers, including Brochmann-Hanssen *et al.* (73), Gibbs and Tuckerman (74), and Baeschlin *et al.* (75–77), recommended this method for analysis of pilocarpine eye solutions.

Baeschlin *et al.* (75–77) used the method in conjunction with TLC and NMR spectroscopy, reporting that it is useful in studying the position of the equilibrium between pilocarpine and pilocarpic acid. Anderson (78) and Anderson and FitzGerald (79), however, used optical rotation measurements to show that an additional equilibrium should be considered, inactivation of pilocarpine through conversion to isopilocarpine (Scheme XIII). Since both stereoisomers have the lac-

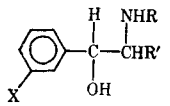


Scheme XIII

tone function measured by the colorimetric assay, it provides an overestimate of solution potency. It would appear that neither the TLC nor NMR method discriminated between the isomers.

Spectrophotometry after Oxidation of Phenalkanolamines—Nicolet and Shinn (80) showed that periodate oxidizes compounds with vicinyl hydroxyl and primary or secondary amine functions to aldehydes and ammonia or a primary amine. Pisano (81) used the reaction as the basis for an assay of metanephrine and normetanephrine in urine, determining the UV absorbance of the vanillin formed by oxidation. Heimlich *et al.* (82) used the reaction to determine phenylpropanolamine in urine as benzaldehyde. Chafetz (51) described dosage form assay methods for phenylpropanolamine, ephedrine, and phenyramidol as benzaldehyde and phenylephrine as 3-hydroxybenzaldehyde. It has been shown (83) that compounds determinable by this method must have the general structure shown in Table I, that the amine function must be basic and unhindered sterically, and that acylated compounds and oxidation products do not interfere. Catecholamines are oxidized to adrenochromes, which do not interfere in methods where the aromatic aldehyde reaction product is separated by solvent extraction before spectrophotometry. The method will not distinguish between optical isomers; however, racemization does not appear

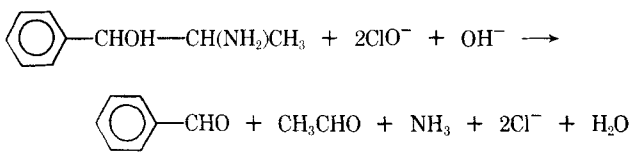
Table I—Structure of Some Phenalkanolamine Drugs



Compound	X	R	R′
Ephedrine	H—	CH_3—	CH_3—
Phenylpropanolamine	H—	H—	CH_3—
Phenylephrine	—OH	CH_3—	H—
Metaraminol	—OH	H—	CH_3—

to be an important degradation route for this class of drugs (84, 85).

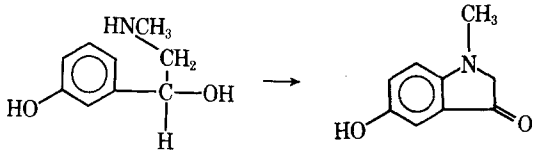
Chafetz *et al.* (86) recently described an alternative method for determination of ephedrine and related compounds; oxidation to benzaldehyde with alkaline sodium hypochlorite (Scheme XIV). Unlike periodate oxidation, this method can be applied directly to the drugs in syrups, and the reaction is significantly faster.



Scheme XIV

However, the selectivity of the method has not been studied as extensively.

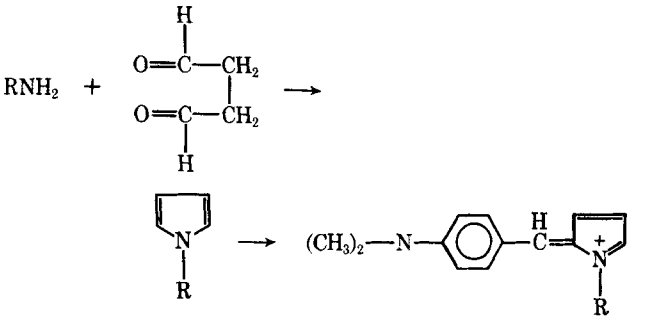
Some Further Methods for Amine Functions—Schriftman (87) used quantitative paper chromatography to study the degradation of phenylephrine HCl solutions in water, 50% glycerin, and 5% dextrose after heating at 75° for 1 week. He found that only intact phenylephrine could be detected with ninhydrin, a reagent applicable to the determination of primary and secondary amines. El-Shibini *et al.* (88) used the cupric dithiocarbamate method described by Troup and Mitchner (47) to follow the rates of phenylephrine decomposition in alkaline solution. The Egyptian workers suggested that phenylephrine cyclizes to form 5-hydroxy-*N*-methylindoxyl; however, they offered no proof of structure (Scheme XV).



phenylephrine

Scheme XV

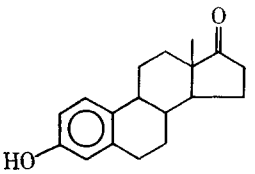
Whatever the structure of decomposition products, selective determination of the secondary amine function would appear to be informative. Sekhon *et al.* (89) and Fujimoto and Ose (90) described photometric methods for ephedrine using ninhydrin. Sawicki and Johnson (91) described a colorimetric method for primary amines, producing an indole by condensation with 2,5-dimethoxytetrahydrofuran, the diacetal of succindialdehyde, and condensing the indole with Ehrlich's reagent (Scheme XVI). This scheme has been used for automated colorimetry of phenylpropanolamine (92) and for the determination of amphetamine (93).



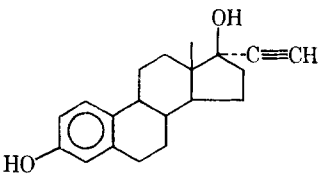
Scheme XVI

Feldmann (94) used the reaction of procaine with 1,2-naphthoquinone-4-sulfonate to determine this primary aromatic amine, reporting no interference from other anesthetics that had no primary aromatic amine function, *e.g.*, meprylcaine, lidocaine, and piperocaine. Auerbach and Angell (95) used this reagent for determining norepinephrine in epinephrine. Pesez and Bartos (96) described a fluorometric method for primary and secondary aralkylamines in which their reaction products with 1,2-naphthoquinone-4-sulfonate are extracted with methylene chloride, reduced with potassium borohydride, and measured after acidification.

Methods for Estrogenic Steroids—Estrogenic steroids have phenolic A-rings and an oxygen function at C-17, as typified by the structures of estrone and ethinyl estradiol. Intentional degradation experiments on



estrone



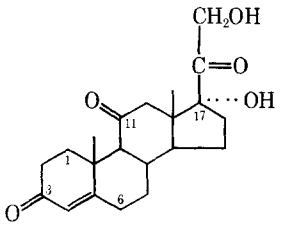
ethinyl estradiol

ethinyl estradiol (9) and two of its 3-ethers (10, 11) monitored by chromatography, showed only small amounts of identifiable products among the several products formed. Empirically, however, the sulfuric acid-induced fluorescence and color have been shown to provide methods selective for these estrogens in the presence of their degradation products and other estrogens. The Kober reaction (97, 98) and its many modifications, production of a pink color by reaction of estrogens with sulfuric acid–hydroquinone or sulfuric acid–phenol–iron, is remarkably sensitive to small changes in structure. Empirical methods based on these reactions have been described for the determination of the components of conjugated estrogens extracted from equine urine (99, 100). Jones and Hähnel (101) studied the specificity and mechanism of the Kober reaction, and they proposed that the Kober chromophores are resonance-stabilized carbonium ions.

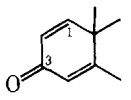
The phenolic moiety of the estrogens provides a UV chromophore, which is similar for most of them, and many of the compounds fluoresce in acid solution. This latter property is used by the British Pharmacopoeia (102) in the assay of ethinyl estradiol tablets. Because they are phenols, they may be coupled with diazonium salts to form azo dyes. Colorimetric methods based on this reaction were described by Urbanyi and Rehm (103)

and others. It has not been established whether, or to what extent, these methods are stability indicating. Most of the attention of investigators of estrogen stability has been directed to development of selective separation procedures for intact drug. Determination of the fate of various functions in the estrogen molecule in its dosage forms or characterization of the principal degradation products would provide valuable insight into the selectivity of these methods.

Corticosteroid Methods—The structure of cortisone and a partial structure of prednisolone, which may be considered prototypes for this important class of drugs, are represented here. The unsaturated ketone system



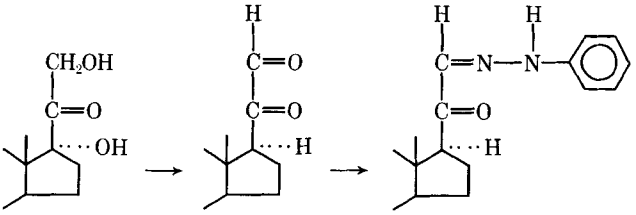
cortisone



prednisolone

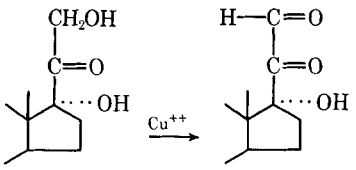
in the A-ring of these steroids is a very good UV chromophore. Görög (104) published a differential UV spectrophotometric method for conjugated ketosteroids. The Δ^4-3-keto chromophore is reduced by sodium borohydride at room temperature to a Δ^4-3-hydroxy structure, which has no spectrum in the near UV. The $\Delta^{1,4}$-3-one system of prednisolone is reduced to the alcohol, but this requires heating. Determination of an untreated aliquot *versus* a reduced aliquot affords a measure of the steroid in the presence of nonspecific UV chromophores. Umberger (105) described a colorimetric method for steroids with the unsaturated ketone structure, formation of an isonicotinic acid hydrazone by reaction with isoniazid. Avdovich *et al.* (106) reported an NMR method for the $\Delta^{1,4}$-3-one corticosteroids. The method is based on the signals derived from the A-ring protons, and it is applicable to their determination in the presence of the cortisone-type structures and dosage form excipients. Although all of these methods based on the properties of the A-ring are useful, and many of them are applicable to the determination of progestins and androgens, they are not stability indicating for corticosteroids.

The most labile group in the corticosteroid structure is the 17α-ketol, which is easily oxidized to a variety of inactive products (60–64), all of which have unaltered A-rings and which would be undetected by the methods described. Mader and Buck (107) exploited the reducing properties of the corticosteroids by measuring the color produced when tetrazolium salts are reduced to formazan dyes in alkaline solution. Tetrazolium methods have been extensively studied, and the blue tetrazolium method has been adopted for corticosteroid dosage form assay by the official compendia. Since the color measured is that of reduced reagent, the method is subject to interference from easily oxidized excipients. Porter and Silber (108) described a method using phenylhydrazine in alcohol and sulfuric acid to produce a yellow color with steroids containing the dihydroxyacetone group at the 17-position. The chromophore has been identified as the 21-phenylhydrazone formed after acid-catalyzed rearrangement (109, 110) (Scheme XVII).



Scheme XVII

One disadvantage of the Porter–Silber reaction for dosage form assay is that heating with the acid reagent often produces nonspecific color by reaction of excipients. Another is that the reaction is inapplicable to corticosteroids that lack a 17-hydroxy function. Lewbart and Mattox (111) described a modification of the Porter–Silber reaction which overcomes these difficulties. The steroid is oxidized to the glyoxal with methanolic cupric acetate; this product reacts immediately with Porter–Silber reagent (Scheme XVIII). Both 17-desoxy-



Scheme XVIII

α-ketols and steroids with the dihydroxyacetone side chain are chromogens in this procedure. Although traces of copper catalyze the oxidation of corticosteroids to glyoxals, these rearrange readily to α-hydroxyacids, which may be further oxidized. Thus this modification would appear to be stability indicating. This method does not appear to have been reported in drug dosage form assay.

Guttman (112) noted that hydrolysis of the ester was a prerequisite for determination of cortisone-21-acetate by the blue tetrazolium method. This consideration is doubtless true for esters in the Porter–Silber reaction as well. One may infer from this that neither procedure is stability indicating with respect to ester hydrolysis.

SUMMATION

It has become widely recognized within the past few years that such factors as crystal form, particle size, and complexation phenomena, undetectable by chemical analysis, may significantly affect drug performance. Nevertheless, a primary requirement for drugs and their dosage forms is that they contain the declared amount of the intact chemical substance within reasonable and predetermined tolerances. A critical review of some methods for determining this fundamental parameter of drug quality has been presented.

The selection of references cited to illustrate principles has been arbitrary, guided by the experience and predilections of the writer. It is conceded that many important drugs and drug classes have been ignored here, and mention of several elegant studies of drug stability has been omitted.

The introduction of the photoelectric UV spectrophotometer and the glass-electrode potentiometer, both of which became commercially available during the 1930's, provided a quantum jump in the capability and productivity of pharmaceutical analysis laboratories. There has since occurred an enormous proliferation of optical, electrochemical, and chromatographic instrumentation and techniques. Computer systems are now available not only for the rapid reduction of the data gushed out by this instrumentation but for controlling their operation as well. The number and compass of the tools available to the pharmaceutical analyst have provided many more alternative solutions to problems and many more means for corroboration. Notwithstanding this sophistication in hardware, the primary asset of the pharmaceutical analyst lies in the creativity of his approach to a problem.

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RESEARCH ARTICLES

Structural Approach to Partitioning: Estimation of Steroid Partition Coefficients Based upon Molecular Constitution

GORDON L. FLYNN

Abstract □ Partition coefficients are directly related to the free energy of transfer of a substance between two immiscible phases and thus have a first principal character rarely ascribed to them. In addition, partition coefficients have been shown to be additive constituitive in character, allowing for their calculation from the individual contributions of the molecular components. These factors, taken together, make partitioning a meaningful and convenient physical phenomenon to match against biological activity. For these reasons, the structural relationships between a large group of steroids and their ether–water parition coefficients were explored. Hansch-like π_e values were estimated for a number of functional groups. These data allow the calculation of partition coefficients of highly substituted steroids from stripped steroid skeletons. The implications of these results from both physical–chemical and biological activity standpoints are discussed.

Keyphrases □ Corticosteroids—partition coefficients □ Partition coefficients—ether–water partitioning, steroids □ Structural group contribution—steroid partition coefficients □ Blue tetrazolium, isonicotinic acid procedures—analysis

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Invariably, activities are compared with partition coefficients obtained in some seemingly arbitrary partitioning system. Hansch and coworkers (3–5) were extremely successful in correlating a spectrum of biological response data with octanol–water partition coefficients. Beckett and Moffat (6) found that *n*-heptane–water partitioning gives excellent rank correlation with buccal absorption for several series of compounds. Other investigators made similar contributions (7, 8).

From the practical standpoint, it is perhaps of equal importance that recent investigators (in particular, Hansch) have provided evidence that the partition co-

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Table I—Partition Coefficients in Ascending Order of Their Experimentally Obtained and Averaged Values

Avg. k_p	Compound	Weight, mg.	Volume Water, ml.	Calcd. Volume Ether, ml.	F	A_E	A_W	k_p Experimental	k_p Calculated
0.757	[1]Triamcinolone	1.0230	100	48.7	1	0.248[a]	0.685[a]	0.743	0.800
		1.0620	50	48.6	1	1.532	2.043	0.771	
1.13	[2]Prednisolone	1.1325	100	48.9	1	0.447	0.801	1.14	Std.
		1.6450	48.5	56.0	1	2.315	1.811	1.11	(1.13)
1.40	[3]Cortisone	0.5040	50	48.2	1	0.938	0.722	1.35	1.55
		1.0245	48	53.7	1	1.792	1.110	1.44	
1.60	[4]Hydrocortisone	0.9850	100	100	2	0.778	0.850	1.83	1.72
		1.5550	50	49.0	1	2.663	1.811	1.50	
		1.0510	50	49.2	1.67	1.688	1.943	1.47	
1.93	[5]6α-Fluoro-prednisolone	1.2795	100	53.3	1.67	1.692	2.740	1.93	2.00
2.32	[6]9α-Fluoro-hydrocortisone	1.3305	50	47.8	2	1.238	1.117	2.32	2.46
3.44	[7]6α-Methyl-prednisolone	0.9948	100	100	2	1.010	0.518	3.90	3.24
		1.2800	100	54.9	1.67	2.210	2.123	3.16	
		1.1155	100	49.6	1.67	1.864	1.923	3.25	
3.87	[8]Dexamethasone	1.3865	100	49.2	1.67	2.379	2.078	3.88	4.09
		1.2950	100	48.5	1.67	2.228	1.982	3.86	
4.17	[9]6α-Methyl-9α-fluoro-prednisolone	2.7510	200	48.7	2	0.839	1.652	4.17	4.64
4.52	[10]Corticosterone[b]	1.8600	100	49.3	4	0.403	0.771	4.24	6.97
		1.4775	100	50.1	4	0.910	1.515	4.79	
4.76	[11]Betamethasone	1.0910	100	49.0	1	3.115	1.332	4.78	5.03
		1.3300	100	48.8	1.67	1.706	1.232	4.73	
7.50	[12]6α-Fluorodexa-methasone	2.1150	200	45.5	5	0.841	2.480	7.36	7.24
		1.1745	150	49.3	2.5	2.242	2.235	7.63	
14.6	[13]Triamcinolone acetonide	2.2150	100	47.9	20	1.047	3.375	13.0	15.5
		1.7010	50	50.1	10	1.803	1.032	17.4	
		1.1600	50	49.3	10	1.251	0.935	13.6	
21.1	[14]Prednisolone-21-acetate	1.6330	100	47.6	5	0.765	1.900	21.1	20.8
25.1	[15]Cortisone-21-acetate	1.0075	100	46.1	10	1.181	1.023	25.1	28.6
25.5	[16]6α-Fluoro-triamcinolone acetonide	~1	100	47.6	10	0.975	0.670	30.6	27.1
		~1	100	47.8	10	0.970	0.925	22.0	
		0.9950	100	49.5	5	2.223	0.901	24.9	
		1.2050	100	49.4	5	2.707	1.111	24.7	
26.0	[17]Hydrocortisone-21-acetate	0.9418	100	100	10	1.184	0.440	26.9	31.6
		~1	100	100	10	1.102	0.735	15.0	
		0.9639	100	100	10	1.180	0.462	25.3	
		0.9935	100	100	10	1.230	0.462	26.6	
		1.0000	100	21.4	10	0.940	1.823	24.1	
		0.9690	100	47.0	10	1.150	0.933	26.2	
		0.9450	100	97.3	10	1.160	0.315	37.8	
32.7	[18]6α-Methyl-9α-fluoro-21-desoxy-prednisolone[b]	1.957	100	48.7	10	1.150	0.725	32.6	36.4
		2.435	100	48.9	10	1.367	0.874	32.0	
		2.243	100	48.9	10	1.312	0.802	33.5	
34.4	[19]6α-Methyl-triamcinolone acetonide	4.8855	750	38.6	40	0.723	1.942[c]	34.9	44.3
		0.9500	100	46.4	10	1.120	0.707	34.1	
		1.1110	100	48.1	10	1.065	0.650	34.1	
36.7	[20]6α-Fluoro-prednisolone-21-acetate	2.454	100	48.7	20	1.372	1.577	35.7	36.8
45.5	[21]6α,16α-Difluoro-prednisolone-21-acetate	1.200	100	46.2	10	0.765	0.408	40.6	44.6
		2.229	100	47.2	20	1.245	1.103	47.8	
		1.5090	100	49.6	10	1.743	0.729	48.2	
45.7	[22]9α-Fluorohydro-cortisone-21-acetate	1.6605	100	46.2	10	1.986	0.940	45.7	45.2
51.5	[23]6α-Methyl-9α-fluoro-21-desoxy-hydrocortisone[b]	2.374	85	45.6	20	0.720	0.515	52.1	56.6
		2.698	100	47.6	20	0.928	0.768	50.8	
52.0	[24]Desoxycorticos-terone[b] (Cortexone)	2.1590	100	49.0	10	1.355	0.533	50.0	80.2
		1.9055	100	50.1	10	1.517	0.561	54.0	
53.2	[25]6α-Methyl-9α-fluoro-16α-hydroxy-hydrocortisone acetonide	~1	100	46.6	10	1.180	0.475	53.3	67.4
		1.9610	100	47.8	20	1.030	0.812	53.0	

Table I—*Continued*

Avg. k_p	Compound	Weight, mg.	Volume Water, ml.	Calcd. Volume Ether, ml.	F	A_E	A_W	k_p Experimental	k_p Calculated
67.1	[26]6α-Methylprednisolone-21-acetate	~1	100	23.6	10	1.150	0.684	71.2	59.7
		~1	100	23.0	10	1.138	0.632	78.2	
		~1	100	48.5	10	1.222	0.390	64.6	
		~1	100	47.4	10	1.202	0.350	72.3	
		2.060	100	22.3	10	2.342	1.775	59.3	
		1.969	100	47.3	20	1.143	0.850	56.8	
80.8	[27]6α,9α-Difluoro-21-desoxyhydrocortisone-17-acetate[b]	3.121	200	49.2	20	0.540	0.587	74.8	97.6
		2.769	150	46.4	20	0.619	0.462	86.7	
82.4	[28]6α-Methyl-9α-fluoroprednisolone-21-acetate	2.504	100	48.7	20	1.390	0.693	82.4	85.4
85.3	[29]6α,9α,16α-Trifluoroprednisolone-21-acetate	1.100	100	47.1	10	1.347	0.365	78.3	78.9
		1.581	200	46.6	10	1.868	0.883	90.7	
		1.8085	200	48.8	10	2.230	1.051	86.9	
87.3	[30]Testosterone	6.133	200	48.3	10	1.231	0.584	87.3	53.9
93.7	[31]6α-Methyl-9α-fluoro-21-desoxyprednisolone-17-acetate	4.750	500	48.2	16	1.152	2.092	91.4	104
		3.356	150	47.5	20	1.811	1.192	95.9	
145	[32]6α-Fluorodexamethasone-21-acetate	3.702	200	45.7	20	2.121	1.250	149	133
		2.5505	200	48.3	12.5	2.425	0.884	142	
222	[33]Hydrocortisone-21-isobutyrate	5.461	200	48.3	100	0.543	0.982	229	312
		5.001	200	49.2	40	1.083	0.824	214	
242	[34]Hydrocortisone-21-butyrate	4.787	300	52.3	40	1.412	1.551	215	334
		6.120	200	51.1	40	1.552	1.083	224	
		8.888	200	50.8	40	2.272	1.245	288	
243	[35]6α,9α-Difluoro-16α-chloroprednisolone-21-acetate	3.363	200	46.8	20	2.029	0.723	240	262
		2.7825	500	46.5	12.5	2.630	1.436	246	
250	[36]6α-Methyl-21-desoxyprednisolone-17-propionate[b]	4.850	750	45.1	40	1.259	3.245	258	237
		4.876	200	45.6	40	1.342	0.978	241	
272	[37]9α-Fluoro-11β-hydroxy-6α-methyl-4-pregnene-3,20-dione[b]	4.950	750	40.7	40	0.977	2.535	284	225
		4.907	200	46.6	40	1.028	0.680	259	
509	[38]Betamethasone-17-valerate	4.458	750	42.1	40	0.650	0.910	509	494
604	[39]Progesterone[b]	2.7960	150	47.6	20	1.108	0.120	582	630
		9.788	250	46.9	50	1.875	0.798	626	
857	[40]6α-Methyl-9α-fluoro-16α-hydroxyprednisolone-16,17-acetonide-21-acetate	6.811	750	25.4	40	1.680	2.320	857	815
960	[41]6α-Methyl-9α-fluoro-16α-hydroxyhydrocortisone-16,17-acetonide-21-acetate	4.829	900	45.2	50	0.985	1.023	960	1240
1380	[42]6α-Methyl-9α-fluoro-16α-hydroxyhydrocortisone-16,17-acetonide-21-propionate	~15	900	42.0	200	0.132	0.410	1380	3550
1530	[43]6α-Fluorodexamethasone-21-butyrate	9.932	900	43.8	100	1.170	1.575	1530	1440
1690	[44]6α-Methyltriamcinolone acetonide-21-propionate	~8.4	900	42.8	100	0.145	0.180	1690	2650
1730	[45]6α-Fluorodexamethasone-21-isobutyrate	10.132	900	44.3	100	1.083	1.275	1730	1320

(*continued*)

Table I—*Continued*

Avg. k_p	Compound	Weight, mg.	Volume Water, ml.	Calcd. Volume Ether, ml.	F	A_E	A_W	k_p Experimental	k_p Calculated
3620	[46]Hydrocortisone-21-caproate (Hexanoate)	4.9695	500	48.8	40	1.345	0.153	3600	3530
		11.01	1000	48.6	50	2.316	0.713	3340	
		13.01	900	48.3	100	1.513	0.720	3910	
	Blank—blue tetrazolium procedure	None	100	47.4	20	0.012[d]	0.017[d]	—	—
	Blank—isonicotinic acid hydrazide procedure	None	100	50	20	0.051[e]	0.042[e]	—	—
						0.000[f]	0.000[f]	—	—

[a] 1-cm. cells. [b] By isonicotinic acid hydrazide method. [c] Required a $^1/_{10}$ dilution with blank. [d] At 525 nm. [e] At 375 nm. [f] At 400 nm.

efficients are of additive-constituitive character (9, 10) and thus can be readily calculated once the functional group contributions are known for a given partitioning system. This has freed the investigator from the tedium of experimentally determining all the partition coefficients in a given drug series. The purpose of this report is to explore in detail the structural approach to partitioning. Cosaturated ether and water phases, henceforth referred to as the ether and the water phases, were used as a system for the determination of the partition coefficients of a large group of polyfunctional steroids, and the π_e values for various groups with varied positioning were determined.

EXPERIMENTAL

The selection of the ether–water partition system was based on partition coefficient data of Katz and Shaikh (11), which showed ether–water corticosteroid partition coefficients in this system to be in an experimentally accessible range. Initial experiments were with their procedure until its limitations became apparent. Shoulders on UV absorption peaks and double peaks were observed in the 240–250-nm. range. Partition coefficients appeared to be dependent on the phase ratio. Partition coefficients starting with a dilute solution of the drug did not remotely agree with those obtained with the filtrate from a saturated solution. Analysis of steroid-free ether-saturated water filtrates through several Whatman filter papers, including No. 41 used by Katz and Shaikh, (11) revealed that significant UV absorbing material is leached out of the filter (Fig. 1). The same problem is observed with other "inert" filters such as the solvent inert Gelman and Millipore types. Because of these problems and the necessity of conserving the compound with many steroids, the following procedures requiring no filtration and more specific assays were employed.

Materials—All steroids tested were used as received[1]. Deionized water and analytical reagent ether were used to prepare the cosaturated phases.

Partitioning Procedure—Water and ether were added to a 2-l. separator and shaken to obtain cosaturation of the phases. The separator was set aside for 24 hr. to allow for temperature and further phase equilibrium. All remaining steps were carried out at room temperature (23 ± 1°). An appropriate amount of steroid (from ~1 to ~10 mg., depending on the steroid) was accurately weighed and placed in a 50-ml. conical flask. This was dissolved in the ether phase, which was maintained at approximately 50 ml. An appropriate amount of water phase (from 50 to 1000 ml., depending on the compound) was placed in an appropriately sized separator (from 125 to 1000 ml.). The steroid solution in the ether phase was added with no attempt to rinse any residual steroid from the flask or funnel used. The phases were shaken intermittently, allowing sufficient time between agitations for the phases to reform.

The system was allowed to stand for 20–30 min. to assure complete phase separation. Then the aqueous phase was transferred to a clean, appropriately sized separator, with great care taken to assure that no ether phase contaminated it at this point. The ether phase was immediately transferred to a 250-ml. round-bottom flask, the phase weight was recorded, and the phase was brought to dryness on a Buchi evaporator. The aqueous phase was extracted with two 50-ml. portions of methylene chloride, and the extracts were collected in a 250-ml. round-bottom flask. The extractions were followed by an additional 10–15 ml. methylene chloride rinse. The combined extracts and rinse were then brought to dryness on a Buchi evaporator. The dried phase residues were reconstituted with 95% ethanol USP (usually 20 ml. of ethanol for the aqueous phase residue and 200 ml. of ethanol for the ether phase residue), appropriately diluted if necessary, and assayed by one of the following procedures.

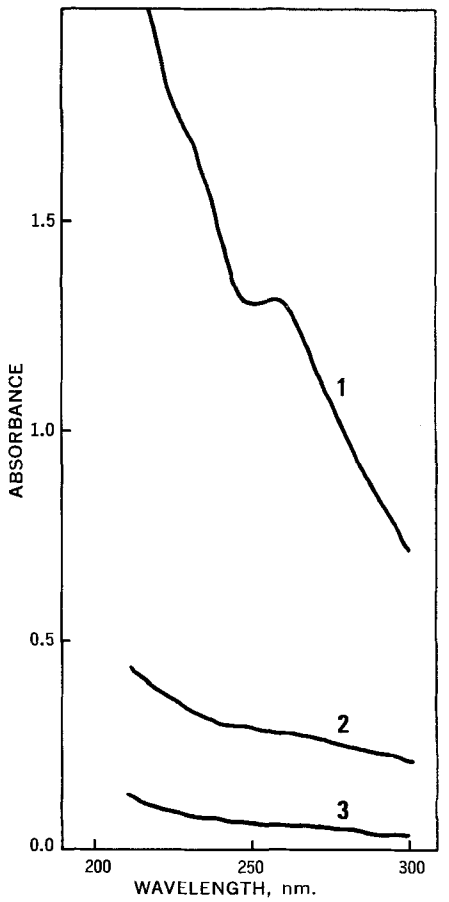

Figure 1—*Absorbance of repetitive 50-ml. water (ether-saturated) filtrates passed through Whatman No. 41 filter paper of approximately 5-cm. diameter. Key: 1, first extract; 2, second extract; and 3, third extract.*

[1] From The Upjohn Company's Biological Screening Office.

Blue Tetrazolium Procedure (12)—This procedure was used on all steroids for which it was applicable. Twenty milliliters of 95% ethanol USP reconstituted ether phase, diluted to contain approximately 0.1 mg., was transferred to a clean 50-ml. glass-stoppered conical flask. Two milliliters of blue tetrazolium reagent (500 mg. of blue tetrazolium in 100 ml. of 95% ethanol USP) and 2 ml. of tetramethylammonium hydroxide reagent (10 ml. of 10% tetramethylammonium hydroxide made to 100 ml. with 95% ethanol USP) were added to this flask and the 250-ml. round-bottom flask containing the aqueous phase residue in 20 ml. of ethanol. A third flask, containing 40 ml. of ethanol to be used as a blank, was similarly and simultaneously prepared. After a lapsed time of 90 min., the solutions were analyzed in the visible range (525 nm.). The Cary 11 used was zeroed at 525 nm. with the blank in both cells. The spectrum was scanned from 650 to 475 nm. Usually, 5-cm. cells were employed, but on occasion other pathlength cells were chosen for analytical convenience.

Isonicotinic Acid Hydrazide Procedure (13)—This procedure was used on compounds for which it was applicable when the blue tetrazolium procedure was not. The procedures are of comparable sensitivity, allowing exact duplication of the blue tetrazolium procedure up to the point of bringing both the ether phase and the methylene chloride extracted aqueous phase residues to dryness. Two hundred milliliters of reagent grade methanol was added to the dried ether extract, the steroid was dissolved, and an appropriately sized aliquot was transferred to a 50-ml. glass-stoppered conical flask. This aliquot ranged from 1 to 20 ml., depending on the original sample size. The methanol aliquot was brought to dryness on a steam bath. The dried aqueous extract, still in its 250-ml. round-bottom flask, was similarly heated to remove any traces of water. Twenty milliliters of isonicotinic acid hydrazide reagent (25 mg. of isonicotinic acid hydrazide and 0.31 ml. of concentrated HCl in 500 ml. of absolute reagent grade methanol) was added to the residues from both the ether and the water phases. After swirling to assure solution, the flasks were tightly sealed and placed in a 47° oven for 1 hr. The flasks were removed from the oven and cooled to room temperature; then the peak absorbance was read between 375 and 405 nm., using cells of appropriate pathlength (usually 5 cm.). A reagent solution that had received identical treatment was used as the blank.

Calculations—In both procedures, the following calculations were used:

$$k_p = F \times \frac{C_E}{C_W} = F \times \frac{W_E/V_E}{W_W/V_W} \quad \text{(Eqs. 1 and 2)}$$

However, in the analytical procedure employed, weight is directly proportional to the absorbance; therefore:

$$k_p = F \times \frac{A_E}{A_W} \times \frac{V_W}{V_E} \quad \text{(Eq. 3)}$$

where:

k_p = ether–water partition coefficient between cosaturated phases
F = dilution factor
C_E = concentration in ether phase
C_W = concentration in water phase
W_E = weight in ether phase
W_W = weight in water phase
V_E = volume of ether phase
V_W = volume of water phase
A_E = absorbance of ether phase
A_W = absorbance of water phase

V_E was determined by dividing the weight of the ether phase by its density, which was independently determined to be 0.712 g./ml.

The dilution factor was calculated for each run from

$$F = \frac{V_s}{A_s} \times \frac{L_W}{L_E} \quad \text{(Eq. 4)}$$

where:

V_s = volume of reconstituted ether phase extract (usually 200 ml.)
A_s = aliquot in milliliters taken from V_s
L_E = cell pathlength ether phase
L_W = cell pathlength water phase

Relatively small amounts of compound were required for the partition coefficient determinations. The amount required for a given compound depended upon the phase volume used and the partition coefficient itself and was calculated from the following equation which assumes that 0.1 mg. must be retained in the water phase:

$$W_T \simeq W_E = 0.1 \text{ mg.} \times \frac{V_E}{V_W} \times k_p \quad \text{(Eq. 5)}$$

The total weight, W_T, is approximately equal to the weight of the ether phase, W_E, for compounds with large partition coefficients (>10). Thus, for a compound with a partition coefficient of 500 and a phase ratio of 0.1 (50 ml. of ether phase to 500 ml. of water phase), one must use approximately 5 mg. to get desired results. Initially, selecting suitable conditions for determining a particular partition coefficient was a matter of trial and error. As facility in their estimation grew, phase ratios and weights of compound were satisfactorily determined in advance with the aid of Eq. 5.

RESULTS

Partition coefficient data for close to 50 steroids are presented in Table I. The compounds are identified according to either their common name (for example, progesterone) or their structural relationship to either hydrocortisone, prednisolone, triamcinolone, dexamethasone, or betamethasone. A knowledge of these few structures will facilitate understanding of the structural dependencies presented. The data in this table include the weight of compound used, the phase volumes, and the dilution factor for each run. In some cases, the weight of compound was approximated and these weights are so indicated.

Hydrocortisone-21-acetate and 6α-methylprednisolone-21-acetate were the compounds used in perfecting the experimental procedure. It was expected that the partition coefficient would be invariant with changing phase volume ratio, and this was the case over a fourfold range for the hydrocortisone ester. The average of the seven hydrocortisone-21-acetate values is 26.0, with a standard deviation of 6.95. As experience was gained with the procedure, experimental conditions were sharpened and points of probable error were eliminated, leading to the likelihood that most of the later data are of better precision and accuracy. In particular, additional care was taken in separating the respective phases. Approximately 0.5 ml. of the aqueous phase was left in the separator to prevent contamination by the ether phase. This was then discarded prior to weighing of the ether phase. This introduced a small error in the value of the water phase (1% or less, depending on volume) but guarded against the far larger error introduced by chance phase contamination.

CALCULATION OF π_e VALUES FOR VARIOUS FUNCTIONAL GROUPS

The estimation of group contribution factors, δ, or their respective log values (π values) is a simple, straightforward process. For example, the group contribution to the partition coefficient for the making of an acetate ester at position 21 would be obtained from

$$\text{group contribution factor} = \delta = \frac{k_p \text{ (21-acetate)}}{k_p \text{ (corresponding alcohol)}} \quad \text{(Eq. 6)}$$

and the π value is then

$$\pi = \log \delta = \log [k_p \text{ (21 acetate)}] - \log [k_p \text{ (corresponding alcohol)}] \quad \text{(Eq. 7)}$$

In the case of the ether–water system, π_e will be used. This will affiliate the ether–water values with the π values of Hansch and at the same time differentiate them with respect to the choice of organic phase.

The partitioning influence of the many functional substitutions for the steroids in Table I in terms of δ and π_e values are listed in Table II. In this table the data are grouped in pairs of compounds,

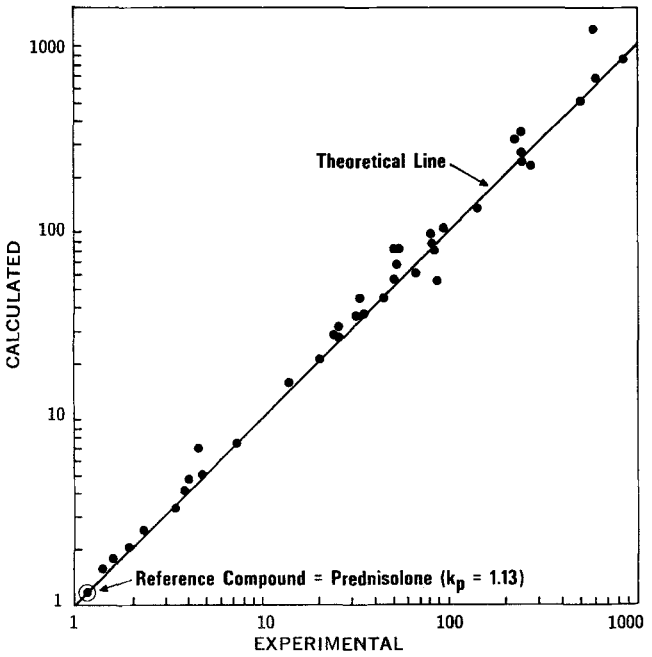

Figure 2—*Log–log plot of π_e value estimated partition coefficients against those determined experimentally. Analysis of the statistical line for all compounds reported in Table I has a correlation coefficient of 0.999.*

one having a particlar substitution relative to the other. For convenience, numbers referring to the order of listing in Table I instead of full compound names are used in Table II. As many as 10 pairs and as few as one pair of compounds have been used to estimate the π_e values. These are given as the log of the averaged group contribution factor for a particular substitution.

Some of the group contributions are themselves based upon calculated partition coefficients. This procedure was employed where no direct comparison and estimation were possible. The value for 9α-fluoroprednisolone used in these estimations was determined by three methods as follows:

prednisolone 1.13	×	9α-fluoro 1.40	=	9α-fluoroprednisolone 1.58	(Eq. 8)
9α-fluorohydrocortisone 2.32	÷	Δ4 to Δ1,4 1.52	=	1.55	(Eq. 9)
6α-methyl-9α-fluoroprednisolone 4.17	÷	6α-methyl 2.87	=	1.45 / Average 1.53	(Eq. 10)

This value (1.53) was then used to calculate the value of 6α,9α-difluoroprednisolone acetate in the following manner:

log 1.53	0.1847
π (6α-fluoro)	0.2480
	0.4327
π (21-acetate)	1.2648
	1.6975

$$\text{antilog } 1.6975 = 49.9 = k_p \qquad \text{(Eq. 11)}$$

These two calculations illustrate the use of both the factor values, δ, or their logs or π values. The agreement between the individual values for 9α-fluoroprednisolone is noteworthy in that they are obtained from the experimentally determined partition coefficients of three different compounds.

A column of calculated partition coefficients using these π_e values appears in Table I. The partition coefficient of prednisolone was arbitrarily chosen as the standard (baseline value) for this series, and all calculated values are based on differences with respect to it. Many of these estimations have built-in redundancies due to the fact that the compounds to which they pertain were also used in π_e value estimation. Numerous compounds in the table were not used in π_e value estimation, however, and these serve to validate the π value system. The agreement between the calculated and the experimental values is good and within experimental error in most cases. The values for the Δ4 compounds are consistently high, and this may indicate that the Δ1,4 to Δ4 π value is slightly larger than it should be. This procedure accurately predicts the value of progesterone (calculated = 630; experimental = 604), a very long extrapolation. Even more interesting is the prediction of 53.9 for testosterone (found 87.3), because it involves the removal of two methylene groups representing C_{20} and C_{21} from progesterone and the correction of a ketone (C_{20}) to a hydroxyl (C_{17}).

The good agreement between experimentally determined and calculated partition coefficients is graphically shown in Fig. 2. For the C_4 and longer esters, the agreement between theory and experiment breaks down some and can only be said to be fair. It can be shown, however, that as little as a 0.1% contamination of a hypothetical long-chain ester (k_p = 3000), with its corresponding alcohol (k_p = 10), will result in a 10% error in the experimental partition coefficient. Since the compounds were not rigorously purified, this represents a potentially substantive source of error. The statistical line generated by all 46 estimations on a log calculated *versus* log experimental basis has an intercept not significantly different from zero, a slope of 1.007, and a correlation coefficient of 0.999.

DISCUSSION

Theoretical Significance of Partition Coefficients—Since partitioning is a process involving molecular equilibria, a partition coefficient is an equilibrium constant of fundamental significance in terms of free energies. It can be seen from Eq. 12:

$$\Delta G = -RT \ln K_{eq.} = RT \ln k_p \qquad \text{(Eq. 12)}$$

that the partition coefficient is directly relatable to the free energy change in the system when a mole of the partitioning agent is transferred between the phases. A mounting body of evidence indicates that when two structurally closely related molecules—such as a corticosteroid free alcohol and its corresponding acetate—are partitioned between the same two immiscible phases, the differences in the free energies of transfer calculated by Eq. 12 are directly relatable to the specific structural modification (7, 9, 10). This generality is at least applicable to solutes that form regular solutions in both phases or for which entropy of mixing is maximized. Thus,

$$\Delta G_{\text{alcohol}} = -RT \ln k_{p\,\text{alcohol}} \qquad \text{(Eq. 13)}$$

$$\Delta G_{\text{ester}} = -RT \ln k_{p\,\text{ester}} \qquad \text{(Eq. 14)}$$

and

$$\Delta G_{\text{acetate moiety}} = \Delta G_{\text{alcohol}} - \Delta G_{\text{ester}} = RT \ln \frac{k_{p\,\text{ester}}}{k_{p\,\text{alcohol}}} = \text{constant} \qquad \text{(Eq. 15)}$$

or in general:

$$\Delta G_{\text{functional group free energy of partitioning}} = \Delta G_{GFE} = -RT \ln \frac{k_{p\,\text{derivative}}}{k_{p\,\text{parent molecule}}} = \text{constant} \qquad \text{(Eq. 16)}$$

If these relationships are valid, it is apparent that by working from the reverse direction one can generate a system whereby partition coefficients can be calculated from a knowledge of the functional

Table II—Effect of Various Functional Groups on Ether–Water Partition Coefficients of Corticosteroids

	Compound	k_p	Corresponding	k_p	δ	π_e
			6α-Methyl			
1.	6α-Methyl group					
	13	14.6	19	34.4	2.35	
	2	1.13	7	3.44	3.04	
	14	21.1	26	67.1	3.18	
					2.87	0.4579
			6α-Fluoro			
2.	6α-Fluoro group					
	13	14.6	16	25.5	1.73	
	8	3.87	12	7.50	1.94	
	2	1.13	5	1.93	1.71	
	14	21.1	20	35.7	1.69	
					1.77	0.2480
			9α-Fluoro			
3.	9α-Fluoro group					
	4	1.60	6	2.32	1.45	
	7	3.44	9	4.17	1.21	
	17	26.0	22	45.7	1.76	
	26	67.1	28	82.4	1.21	
					1.43	0.1553
			16α-Methyl			
4.	16α-Methyl group					
	9α-Fluoroprednisolone	1.53[a]	8	3.87	2.53	0.4031
			16β-Methyl			
5.	16β-Methyl group					
	9α-Fluoroprednisolone	1.53[a]	11	4.76	3.11	0.4928
			16α-Hydroxy			
6.	16α-Hydroxy group					
	9α-Fluoroprednisolone	1.53[a]	1	0.757	0.495	−0.3054
			16α-Fluoro			
7.	16α-Fluoro group					
	20	35.7	21	45.5	1.29	
	6α,9α-Difluoroprednisolone acetate	49.9[a]	29	85.3	1.71	
					1.50	0.1761
			16α-Chloro			
8.	16α-Chloro group					
	6α,9α-Difluoroprednisolone acetate	49.9[a]	35	243	4.97	0.6964
			16,17-Acetonide			
9.	16,17-Diol *versus* the 16,17-acetonide					
	1	0.757	13	14.6	19.3	1.2856
			17-Acetate			
10.	C_{17} Acetate group					
	18	32.7	31	93.7	2.86	0.4564
			21-Acetate			
12.	C_{21} Acetate group					
	19	34.4	41	857	25.3[b]	
	4	1.60	17	26.0	16.3	
	25	53.2	42	960	18.0	
	7	3.44	26	67.1	19.8	
	9	4.17	28	82.4	19.8	
	12	7.50	32	145	19.4	
	3	1.40	15	25.1	17.9	
	5	1.93	20	35.7	18.5	
	2	1.13	14	21.1	18.7	
	6	2.32	22	45.7	19.7	
					18.7	1.2648[c]
			C_{11} Desoxy			
13.	Desoxy at C_{11}					
	10	4.52	24	52.0	11.5	1.0607
			C_{17} Desoxy			
14.	Desoxy at C_{17}					
	23	51.5	37	272	5.28	
	4	1.60	10	4.52	2.83	
					4.06	0.6085

(*continued*)

Table II—*Continued*

	Compound	k_p	Corresponding	k_p	δ	π_e
			C_{21} Desoxy			
15.	Desoxy at C_{21}					
	9	4.17	18	32.7	7.85	0.8949
			C_{21} Desoxy			
16.	C_{21} Acetate *versus* C_{21} desoxy					
	28	82.4	18	32.7	0.397	−0.4012
			Hydroxy			
17.	C_{11} Keto *versus* C_{11} hydroxy					
	15	25.1	17	26.0	1.07	
	3	1.40	4	1.60	1.14	
					1.105	0.0434
			Δ4			
18.	Δ1,4 *versus* Δ4					
	2	1.13	4	1.60	1.42	
	18	32.7	23	51.5	1.57	
	19	34.4	25	53.2	1.57	
					1.52	0.1818
			β			
19.	16α-Methyl *versus* 16β-methyl					
	8	3.87	11	4.96	1.23	0.899
			Branched			
20.	Branching					
	34	242	33	222	0.917	−0.0376
21.	Methylene contribution (calculated):					
	(a) From hydrocortisone butyrate					
	Log (k_p hydrocortisone butyrate)		2.3838			
	− Log (k_p hydrocortisone acetate)		1.4150			
	÷ 2 (—CH_2—CH_2—)		0.9688			
	Antilog = 3.05 = δ					
	(b) From hydrocortisone hexanoate					
	Log (k_p hydrocortisone hexanoate)		3.5587			
	− Log (k_p hydrocortisone acetate)		1.4150			
			2.1437			
	÷ 4 (—CH_2—CH_2—CH_2—CH_2—)		0.5359			
	Antilog = 0.5359 = 3.44 = δ					
	(c) $\sum_n \delta/n$					
	$\frac{\delta_i + \delta_2}{2} = 3.25$					
	π_e = log 3.25 = 0.5119					

[a] Calculated (see text). [b] Not included in average. [c] Corresponds to a previous average with fewer comparisons of 18.4; used in all the calculations.

group free energies of partitioning. This is exactly what Hansch and Steward (3) accomplished with the octanol–water system. The π values obtained from Eq. 7 are directly related to the group free energy change (Eq. 17):

$$\pi = \frac{\Delta G_{GFE}}{2.303\ RT} \qquad \text{(Eq. 17)}$$

Under isothermal conditions, the group free energy of partitioning can be generated by simply multiplying the π value times the constant factor of 2.303 *RT*.

In this study, linear free energy relationships were developed for an as yet uncharacterized group of steroids in a different solvent system; they were found valid within experimental error. The constancy of π or δ values for the conversion of a 21-alcohol to a 21-acetate (Table II) is particularly notable. These values are for corticosteroid alcohols differing by as much as a factor of 50 in partition coefficient. The averaged group free energy of transfer for the 21-acetate at 25° is approximately 1.73 kcal./mole in the ether–water system. A perusal of Table II discloses that not all the π values have been as sharply defined. For instance, δ for the 9α-fluoro group varies up to 19% about the mean of 1.43. Regardless, the consistently close approximation of experimentally determined k_p's by π_e value calculation gives good indication that the group contribution values are within reason.

An interesting conclusion readily drawn from Table II is that there is an inequality of effect for a given functional group located at different positions. The most notable difference is for esters at the 17- and 21-positions, although even the differences in partition coefficient brought about by positioning a methyl group in either the 6α-, 16α-, or 16β-positions are believed to be real. These differences are acceptable, considering the nonsymmetrical environment of the group locations. The marked variance in effect of derivitization at positions 17 and 21 is attributable to the marked difference between a tertiary alcohol enveloped by the huge steroid nucleus and a relatively unhindered, mobile primary alcohol.

The concept of summation of constituent properties to obtain or approximate the properties of the whole molecule is reasonably well established. The work of Hansch and coworkers has already been mentioned (3–5, 10). Cratin (14) recently published on the estimation of HLB values of surfactants using a similar approach. Small (15) and Rheineck and Lin (16) compiled evidence on the additive constitutive character of solubility parameters and related molar attraction constants; these approximations were used in turn by Ostrenga and Steinmetz (17) to characterize successfully the solubility of fluocinolone acetonide and its 21-acetate. These represent but a few examples of the diverse usefulness of this approach.

Interrelationship of Several Common Structure–Activity Parameters–In Fig. 3 the logs of the *experimentally determined* partition coefficients of several selected corticosteroid families are plotted against their respective molecular weights. These families are arbitrarily labeled prednisolone, monofluoroprednisolone, difluoroprednisolone, and triamcinolone. The family membership for

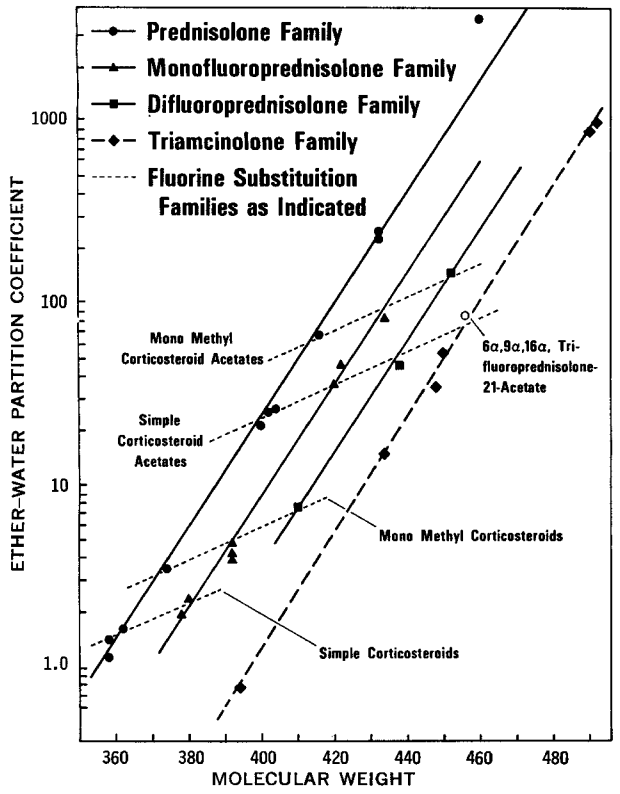

Figure 3—*Plot showing the linear relationship of molecular weight to log partition coefficient for selected families of compounds.*

prednisolone includes hydrocortisone, cortisone, methylprednisolone, and their respective 21-esters. The other families are similarly constituted. The positions of fluoronation are not differentiated from one another. The first point gleaned from this plot is that the very closely related analogs and their 21-acyl derivatives give a linear relationship with log (k_p) (Eq. 18):

$$\log k_p = a(MW) + b \qquad \text{(Eq. 18)}$$

Similar relationships for simple homologous series only were pointed out by several other investigators (18). The significance of this is that good structure–activity correlations with molecular weight are possible for analogs by fortunate (or unfortunate, depending on one's perspective) choice of compounds. Such correlations are by themselves meaningless in terms of any fundamental property of the active species. It is noteworthy that a 17-ester, unlike a 21-ester, would be significantly displaced from the line containing its respective free alcohol. Thus, decreases in relative polarity by acylation are not necessarily directly proportional to molecular weight changes.

The monofluoro and difluoro lines are successively displaced along the molecular weight axis to the right of the unsubstituted series. These lines are drawn parallel, as is the dashed triamcinolone line. This is acceptable within experimental error and a necessary consequence of a working π value system. The dotted intersecting lines represent another group of families, in which the fluorine substitution is varied while the remainder of the steroid nucleus is held relatively constant. The behavior on repetitive substitution of fluorine atoms is thus analogous to the behavior of a homologous series where methylene units are successively added. In an additive-constituitive system, the repetitive addition of any functional unit to positions of comparable molecular environment would be expected to show such correlation. In this case, the correlation is reasonable even for nonsymmetrical substitution. Recently, Pinney and Walters (8) published data correlating the bactericidal activities of certain mono-, di-, and polysubstituted fluorophenols with oleyl alcohol–water partition coefficients, number of fluorine atoms, molar solubility, molecular weight, and surface-tension lowering. Although these authors proposed a nonspecific physical mode of action based on these multiple correlations, they actually better demonstrated the interdependence of various physical–chemical parameters.

In the same paper, it was observed that cyclohexane–water partition coefficients were virtually invariant for the fluorophenols and could not be used to establish a rank-order correlation of bactericidal activity. This leads to the question of how does one choose a partitioning system that will lead to a meaningful relationship with biological activity. The answer lies in closely approximating the biophases involved in transport to the receptor environment or interaction with the receptor site, whichever is activity determining. The choice actually narrows to selecting a lipid phase because water is the obvious choice for the polar phase. Based on knowledge of biological membranes and lipids, it seems reasonable that biophase simulation would be best accomplished with a semipolar solvent such as *n*-octanol or diethyl ether rather than a completely apolar solvent such as cyclohexane. When biophase simulation is good and the activity of the series of compounds is reflected in the free energy of transport into the lipid phase, significant structuring of data can be drawn from distribution coefficients obtained *in vitro*.

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Quantitative Determination of Drug Bioavailability and Biokinetic Behavior from Pharmacological Data for Ophthalmic and Oral Administrations of a Mydriatic Drug

VICTOR F. SMOLEN

Abstract □ A theoretical basis is developed for the performance of drug-absorption analysis from data obtained from the observation of the time course of pharmacological response intensity following a single dose of drug by any route of administration. The results of such analyses permit an evaluation of the physiological availability characteristics of drugs from pharmaceutical dosage forms and the elucidation of the kinetics and mechanisms operative in the passage of drugs across *in vivo* biological barriers. It is demonstrated that through the suitable use of intravenous dose–effect curves, the postulation of hypothetical models for pharmacon–receptor site interaction is obviated. The dose–effect curve graphically provides the relationship between the quantity of drug in body compartments (biophase) and the observed intensity of drug response. The use of pharmacological data, in contrast to the use of time course of drug level in body fluid data, has the advantage, in addition to not requiring a method of direct assay for the drug, of being applicable to cases where the drug first penetrates from a local site of administration to a vicinal site of action prior to reaching the systemic circulation. In the present study, kinetic pharmacological data for tridihexethyl chloride were transformed to obtain the temporal variation of biophasic drug levels, which in turn provided the input for least-squares fits to the linear two- and three-compartment models studied. Digital computer programs, employing an iterative systematized guessing method, were designed to aid in fitting the mathematical models. A triexponential fit was found to be the best with the constraints imposed on the model parameters. The method is further exemplified by its application to the determination of drug absorption following oral and ophthalmic administration.

Keyphrases □ Mydriatic drug—bioavailability, biokinetic behavior □ Tridihexethyl chloride—bioavailability, biokinetics □ Ophthalmic, oral administration—mydriatic drug bioavailability, biokinetics □ Pharmacological response data—biokinetics, mydriatic drug □ Biokinetics, mydriatic drug—pharmacological response data

A biokinetic method of analysis of *in vivo* data that does not involve *a priori* assumptions of kinetic models for the absorption of the drug from the site of administration has the distinct advantage of allowing the kinetics and mechanisms of the biological availability of the drug to be elucidated under *in vivo* conditions. The purpose of this report is to describe a basis for the use of temporal pharmacological response-intensity data for drug-absorption analysis. The method is exemplified by its application to the determination of the absorption of tridihexethyl chloride following oral and ophthalmic administration.

Generally the ability of the drug to reach its biological site(s) of action (contained in a body compartment termed the biophase) is either dosage form rate limited by its release from the dosage form or biological factor rate limited by biotransformation, permeation of biological barriers, and/or unavailability due to partitioning into body depots of relatively large solubility volume (1). Information concerning some of these processes *in vivo* can be gained by absorption analysis of temporal blood level and/or urinary excretion data through the application of the now familiar treatments first described by Wagner and Nelson (2) and further developed by others (3). These treatments, however, have been described only for cases where the drug is absorbed into a central, systemic compartment prior to or coincident with inducing its biological response. This is most often the intent of oral and parenteral administration. In contrast, the study of drug availability to a biophase vicinal to the site of administration following topical or parenteral use of the drug for local effects has not been described. Provided the appropriate tissue locations could even be unambiguously identified, their sampling for the periodic determination of drug levels could present intractable technical difficulties. If drug-absorption analysis could be performed using temporal pharmacological response-intensity measurements, the direct assay for drug levels in such locations could be obviated. The development of a basis for the utilization of pharmacological data for drug-absorption analysis and the determination of a suitable compartment model to describe the biokinetic behavior of tridihexethyl chloride are the subjects of the present report. Tridihexethyl chloride was chosen as one of the model drugs in these studies because of the relative ease of following its mydriatic activity. Furthermore, due to its quaternary ammonium structure, it was anticipated to possess interesting biological transference properties.

Previous investigations (4–10) concerned with the kinetics of pharmacological effects always adopted the sometimes tenuous assumption that the intensity of an observed pharmacological effect at any time is directly or otherwise simply related (*e.g.*, through a hypothetical model for drug–receptor interaction) to the level of drug in a peripheral tissue or other compartment (the biophase) containing the sites at which it acts to induce the observed response. The present approach, when applicable, does not require the postulation of hypothetical mechanisms for dose–effect relationships. It allows biophasic drug levels to be estimated at any time—following drug administration by any route—from the observed intensity of the induced pharmacological effects. The manner in which intravenous dose–effect curves may be applied to this purpose, as well as to obtain confidence in the applicability of the models to any given drug, is presented. Such procedures of indirect, or direct, verification should be performed prior to application of the method to any drug system. By applying the procedures described, it becomes entirely unnecessary to assay for drug concentration levels in body fluids or tissue compartments in order to perform analyses of drug absorption and availability into the systemic circulation or directly into the biophase. The principles developed are generally applicable; however, special

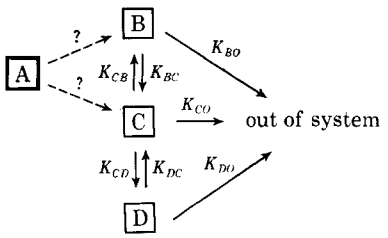
consideration is presently afforded to the treatment of ophthalmic drugs.

THEORETICAL

General—Physiological drug availability has been defined (2) as the ratio of the amount of a drug systemically absorbed from its site of administration when it is presented to the site in a readily available form as compared to other dosage forms. Methods of calculating the physiological drug availability from blood and urine data have become familiar (2, 3). The pharmacological response characteristics of a drug, however, are not necessarily predictable from its concentration in extracellular fluids *versus* time profile (3). A similar profile of drug concentration in its biophase (*i.e.*, the region(s) in the organism containing the primary sites of biological action[1]) could, in many instances, be of greater utility in this respect. The physiological drug availability could then be redefined as a similar ratio with respect to the quantities of drug absorbed into the biophase. Depending upon the specific drug of interest, the biophase may or may not constitute a compartment separate from the extracellular fluids of distribution. For example, Smolen and Schoenwald (11) found that the biophase and central compartment were practically indistinguishable for a nonionic mydriatic drug but constituted separate compartments for the presently studied cationic drug having a similar pharmacological activity. The difference in their behavior may be attributed to the relative rates at which the drugs can penetrate to their sites of action.

The general linear three-compartment model adopted in the present treatment represents the behavior of tridihexethyl chloride. The biophase is treated as a separate compartment; the concentration of drug in the biophase is by definition uniform throughout its volume. It is also precisely the concentration of drug responsible for the corresponding observed intensity of pharmacological response. Since the biophase is a hypothetical concept, it is seldom possible in practice to sample its drug content directly in a manner analogous to blood, aqueous humor, urine, *etc.* However, in appropriate cases (7), the quantity of drug in the biophase can be assumed as being reflected in the intensity of the pharmacological response to the drug at any time following its administration (Appendix I). If the functional relationship between the instantaneous pharmacological response intensity and the corresponding content of drug in the biophase was known, the temporal variation of the pharmacological response intensity could substitute for drug levels and provide the input necessary for a biokinetic analysis of the drug-transport system. Following certain assumptions, the requisite relationship between intensity of pharmacologic response and concentration can be obtained if the intravenous dose–response relationship is known and an appropriate compartment model of drug transport is constructed.

Compartment Model for Ophthalmic Drug Transference—As a first approximation at least, ophthalmic drug transference may be modeled by the linear compartment system shown in Scheme I where



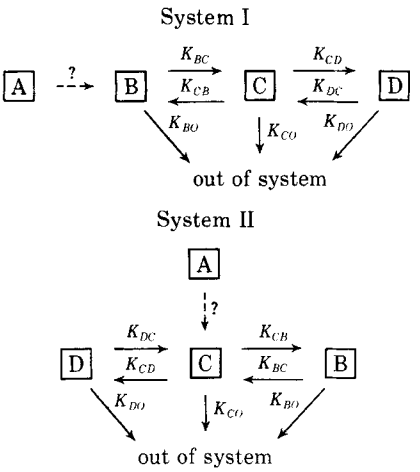
Scheme I—Compartment model for ophthalmic drug transference. Compartments A, B, C, and D correspond to the site of administration, biophase, systemic fluids of distribution, and tissue depot, respectively. The kinetics and mechanisms of drug transference from A to B and A to C are the phenomena to be elucidated from kinetic pharmacological data.

Compartments A, B, C, and D refer to the site of drug administration (*e.g.*, the surface of the eye), the biophase, the systemic fluids of distribution, and a peripheral tissue depot for the drug, respectively.

[1] See Appendix I.

The K_{ij}'s are first-order transfer constants. Only six of the seven constants can be computed from the combined pharmacological data obtained from intravenous dosing and the administration of the drug to a site from which it is directly absorbed into the biophase. The remaining constant must be assigned an arbitrary (*e.g.*, zero) value. This is unnecessary for single and two open-compartment models.

It can be assumed that any of the drug absorbed systemically, rather than directly into the biophase, will have equal access to both eyes of the animal. If the drug is administered only to the experimental eye, the other eye can serve as a control. The level of drug in the biophase which is responsible for the observed intensity of pharmacological response in the experimental eye at any time arises from the sum of the quantities of drug absorbed directly into the biophase and that which has arrived *via* the systemic route. Since this systemic contribution can be assumed to be the same for both the experimental and control eyes, the pharmacological response intensity in the experimental eye which is attributable to the level of drug in the biophase arising through direct absorption from the site of administration can be ascribed, at any time, to the difference in the intensities observed in the experimental and control eyes. However, this is strictly true only if the drug levels and intensities of effect are linearly related. As will be shown, the determination of whether a linear or some other form of functional relationship exists between the intensity of effect and quantity of drug in the biophase can be discovered from inspection of an intravenous dose–effect curve. In any event, the quantities of drug reaching the biophase directly or *via* the systemic circulation can be discerned. These considerations allow the compartment system shown in Scheme I to be resolved into the two systems depicted in Scheme II. By utiliz-



Scheme II—Resolution of the compartment model of ophthalmic drug transference into two systems which is possible through use of a control eye. Each system can be studied individually. System II is also generally appropriate for any other route of administration by which the drug enters the systemic circulation prior to penetrating into the biophase, e.g., *oral, parenteral, and scleral. System I is also applicable to other routes of administration besides ophthalmic when the pharmacological response resulting from drug entering B from C is negligible or can be independently measured.*

ing an experimental and a control eye, each system can be studied individually, although experimentally the data for each study are obtained simultaneously. Appendix II presents a further treatment of these considerations.

If the quantity of drug absorbed systemically relative to that absorbed directly into the biophase is appreciable, it may be an important factor contributing to any observed systemic side effects in addition to the intensity of the sought local pharmacological effect. Under such circumstances, the study of the kinetic processes involved in the systemic absorption route(s) may be as important as those operative in the direct absorption of the drug into the biophase.

A simplified compartmental model for drug absorption directly into the biophase is illustrated in Scheme III. Excluding the volume of the biophase, the remaining volume of distribution for the drug in the animal is assumed very large in comparison to the volume of the biophase. In this case a sink is provided for the drug into which

it is eliminated from the biophase. The backflow into the biophase can then be neglected to simplify the model, as shown in Scheme III. In the event the backflow was appreciable, it can be assumed that the quantity of drug influxed from this source at any time is equal for both the experimental and reference biophases. Therefore, the correction obtained for the control eye also takes account of the backflow of drug from the central compartment. The use of this correction, when necessary, allows the retention of the simplified model even in the event of an appreciable backflow of drug. However, when this is the case, the ability to distinguish between drug absorbed from the site of administration into the biophase and that reaching it by the systemic absorption from the site of administration is vitiated. The absorption analysis then could only be performed for the direct transference of drug into the biophase. Except for the instillation of inordinately high concentrations of drug into the eye, it has thus far been the author's experience with mydriatics that the correction for drug reaching the biophase by a systemic route is negligible.

Based upon these considerations, the fraction, F_{BT}, of the quantity of drug ultimately absorbed into the biophase, $Q_{BT\infty}$, which has been absorbed up to a time, t, can be expressed by Eq. 1, where Q_{BB} refers to the quantity of drug in the biophase at time t that has gained entrance by direct absorption. The elimination constant from the biophase in accordance with Scheme I is given by $K^*_{BO} = K_{BO} + K_{BC}$. It can be evaluated from kinetic data describing biophasic drug levels following the administration of an ophthalmic dose of the drug.

$$F_{BT} = \frac{Q_{BB} + K^*_{BO}\int_0^t Q_{BB}\,dt}{K^*_{BO}\int_0^\infty Q_{BB}\,dt} \qquad \text{(Eq. 1)}$$

In a manner analogous to physiological availability (2), the biophasic drug availability (BDA) and physiological drug availability (PDA) can be defined by Eqs. 2*a* and 2*b*.

$$\text{BDA} = \frac{\left[\int_0^\infty Q_{BB}\,dt\right]_{\text{exptl.}}}{\left[\int_0^\infty Q_{BB}\,dt\right]_{\text{standard}}} \qquad \text{(Eq. 2a)}$$

$$\text{PDA} = \frac{\left[\int_0^\infty Q_{BS}\,dt\right]_{\text{exptl.}}}{\left[\int_0^\infty Q_{BS}\,dt\right]_{\text{standard}}} \qquad \text{(Eq. 2b)}$$

The integrals in the numerator refer to some experimental (*e.g.*, ophthalmic or oral) formulation, while the denominators refer to a standard formulation to which the experimental formulation is being compared. The expression for PDA derives directly from a consideration of Eq. 3*a*.

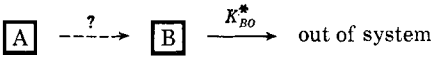
Equation 3*a* is appropriate for drug reaching the biophase by a systemic route (*e.g.*, by oral and parenteral dosing) or through scleral and/or mucosal absorption following instillation into the eye. Equations 3*a*–3*e* exemplify the type of expressions that are applicable to a three-compartment system for which the transfer constants have been determined or assumed. The derivation of an equation for a two-compartment model is presented in Appendix III. A discussion of the properties and application of Eq. 3*a* is presented in Appendix IV. Digital computer programs have been developed by the author for the routine numerical solution of such equations.

$$F_{ST} = \frac{R\frac{d^2Q_{BS}}{dt^2} + S\frac{dQ_{BS}}{dt} + TQ_{BS} + V\int_0^t Q_{BS}\,dt}{V\int_0^\infty Q_{BS}\,dt} \qquad \text{(Eq. 3a)}$$

$$RK_{BC}K_{DC} = 1 \qquad \text{(Eq. 3b)}$$

$$SK_{BC}K_{DC} = [K_{BC} + K_{BO} + K_{CB} + K_{CD} + K_{CO} + K_{DO}] \qquad \text{(Eq. 3c)}$$

$$TK_{BC}K_{DC} = [K_{DC}(K_{CB} + K_{BC} + K_{BO}) - K_{DC}K_{BC}(K_{BC} + K_{BO})(K_{CO} + K_{CO} + K_{CB}) + K_{DO}(K_{BC} + K_{BO} + K_{CB} + K_{CD} + K_{CO}) + K_{DC}K_{CO}] \qquad \text{(Eq. 3d)}$$



Scheme III—Simplified compartment system for the description of ophthalmic drug transference directly into, as well as its removal from, the biophase. In referring to Schemes I and II, the elimination constant from the biophase, K^*_{BO}, is seen to be related to other transfer constants by the equation, $K^*_{BO} = K_{BO} + K_{BC}$.

$$VK_{BC}K_{DC} = [K_{DC}K_{BC}K_{BO} - K_{BC}K_{CB}K_{DD} + (K_{CB} + K_{CD} + K_{CO})(K_{BC} + K_{BO})K_{DO} + K_{CO}K_{DC}(K_{BC} + K_{BO})] \qquad \text{(Eq. 3e)}$$

Through the use of pharmacological data, the cumulative amount of drug that has been absorbed into the biophase from the systemic circulation at any time following dosing by any systemic route can also be computed from Eq. 1. This amount, Q_{BST}, is composed of that quantity present in the biophase at any time and the quantity that has already been eliminated following its absorption from the systemic circulation. The fraction of the drug, F_{BST}, that has been absorbed systemically which has entered the biophase can readily be seen to be given at any time by the ratio of the numerator of Eq. 1 to the numerator of Eq. 3*a*. The limiting value of F_{BST}, *i.e.*, at $t = \infty$, may be termed the systemic biophasic availability, SBA, and be expressed simply by Eq. 4:

$$\text{SBA} = \frac{K^*_{BO}}{K_{BC}K_{DC}V} \qquad \text{(Eq. 4)}$$

Equations 1–4 would be of only academic interest if a means of estimating Q_{BB} and Q_{BS} at any time from the pharmacological response intensity did not exist.

Relationship of Pharmacological Response Intensity to Drug in the Biophase—Following intravenous administration, the quantity of drug in the biophase at any time will be given by Eq. 5 (9) in accordance with the three-compartment model shown in Scheme II. The symbol D refers to the dose of the drug. The relationships between the equation parameters, A_i's and m_i's, and the model parameters, K_{ij}'s, are shown in Appendix IV. Similar relations for biexponential models are given by Riggs (12) and Rescigno and Segre (13, 14).

$$Q_{BS} = D(A_1e^{-m_1t} + A_2e^{-m_2t} + A_3e^{-m_3t}) \qquad \text{(Eq. 5)}$$

A dose-effect relationship may be obtained by observing the intensity of response at a fixed time following rapid intravenous administration. A convenient and most propitious time to observe the response is the time ($t_{\max.}$) corresponding to the peak response intensity, $I_{\max.}$. The time, $t_{\max.}$, is independent of the dose for linear compartment models. This is easily verified by differentiating Eq. 5 and setting the derivative equal to zero.

The determination of the dose-effect relationship allows the dose to be expressed as a function of $I_{\max.}$; *i.e.*, $D = f(I_{\max.})$. Substituting this functional relationship for D into Eq. 5 allows it to be written as Eq. 6:

$$(Q_{BS})_{\max.} = f(I_{\max.})[A_1e^{-m_1t_{\max.}} + A_2e^{-m_2t_{\max.}} + A_3e^{-m_3t_{\max.}}] \qquad \text{(Eq. 6)}$$

The terms on the right-hand side of Eq. 6, other than $f(I_{\max.})$, are constant and will be included in an overall constant, β, as shown by Eq. 7:

$$(Q_{BS})_{\max.} = f(I_{\max.})\beta \qquad \text{(Eq. 7)}$$

Assuming that the relationship between the intensity of pharmacological response and the quantity of drug in the biophase, irrespective of its mode of entrance, given by Eq. 7 is a general relationship and holds at times other than $t_{\max.}$ allows Eq. 7 to be rewritten as Eq. 8 and Eq. 5 to be rewritten as Eq. 9:

$$Q_B = \beta f(I) \qquad \text{(Eq. 8)}$$

$$f(I) = \frac{f(I_{\max.})}{\beta}(A_1e^{-m_1t} + A_2e^{-m_2t} + A_3e^{-m_3t}) \qquad \text{(Eq. 9)}$$

Equation 9 predicts the response behavior of the drug as a function of time following an intravenous dose, while Eq. 8 applies irrespective of the route of administration.

Direct substitution of $f(I)$[2] from Eq. 8 for Q_{BS} or for Q_{BB} in Eqs. 1–4 will allow F_{BT}, BDA, F_{ST}, and PDA to be evaluated from pharmacological data alone. Most often the value of the constant, β, relating Q_B to $f(I)$ in Eq. 8, need not be known, since it cancels between the numerators and denominators and otherwise disappears from Eqs. 1–4 (Appendix IV).

The derivations of Eqs. 1 and 3*a*–3*e* are devoid of any assumptions concerning the transfer processes involved in the penetration of the drug into the biophase or the systemic compartment; they are, therefore, valid irrespective of the nature of these processes and could be utilized to elucidate the nature of their kinetics and mechanisms. Since $f(I)$ can be substituted for Q_{BB} and Q_{BS} in Eqs. 1 and 3*a*, respectively, the biokinetic analysis of drug absorption from the site of administration can be wholly accomplished from pharmacological response-intensity *versus* time data. Subtracting values of F_{BT} and F_{ST} from unity yields values of A_{RB} and A_{RS}, respectively, which refer to fractions of drug ultimately absorbed still remaining to be absorbed at the site of administration at any time. The temporal relationships of A_{RB} and A_{RS} can be utilized to provide insight into the kinetics and mechanisms of the absorption processes (2). This can best be accomplished through the construction of physical models to describe the involved transport processes while making comparisons of predicted behavior with that actually observed. In such physical models, the magnitude of the driving force for the passive transference of the drug across tissue barriers into the biophase or systemic compartment is most closely related to the quantity of drug remaining at the site of administration. This quantity is only equal to A_{RB}, or A_{RS} if there is no peripheral loss of the drug occurring from the absorption site; that is, *all* the drug leaves the administration site by the same route and is 100% available by this route. Generally this is not the case.

The integrals in Eqs. 1 and 3*a* are equal or directly proportional to the areas under curves of plots of $f(I)$ values *versus* time. The areas under curves obtained from pharmacological data can be given precisely the same interpretation and significance as the areas under curves resulting from similar plots of blood levels or urinary excretion rates (2).

MATERIALS AND METHODS

Materials—Crystalline tridihexethyl chloride[3] was dissolved in isotonic, pyrogen-free, sodium chloride solution prior to intravenous administration *via* the rabbit's marginal ear vein.

The rabbits used in the study were New Zealand white males, approximately 3–4 months of age. The rabbits were allowed a minimum of 2 days of recovery time between experiments. A preliminary screening of the rabbits was observed to reduce appreciably intersubject variation in the data. Twelve rabbits out of a total of 40 were chosen for use in the study on the basis of agreement between the maximum intensities of mydriatic response observed when the rabbits were administered the same ophthalmic dose and an approximately ED_{50} intravenous dose of drug. The pupillary response of the rabbits to controlled light intensities was also a criterion in their selection.

Measurement of Pupillary Diameters—The experimental arrangement employed in the present study is illustrated in Fig. 1. When measurements were made on both eyes, an additional light source and mirror were arranged symmetrically to those shown in Fig. 1. The light source(s) consisted of a 100-w. bulb inserted in a microscope lamp. The intensity of the light reflecting from the mirror(s) was measured with a light meter located near the experimental eye of the rabbit. A constant intensity of illumination was maintained by adjusting the light source with a rheostat. The mirror served to minimize the quantity of heat generated by the source that reached the cornea. The constancy of the lighting conditions was found to be an important factor affecting the reproducibility of experimental results. Prolonged illumination of the eye caused the pupillary diameter to dilate, following an initial constriction, and was therefore avoided. Consequently, the eye was illuminated only prior to a measurement. The minimum observed values of the pupillary diameters following illumination were assumed to correspond to full accommodation and were recorded along with the time of their observation.

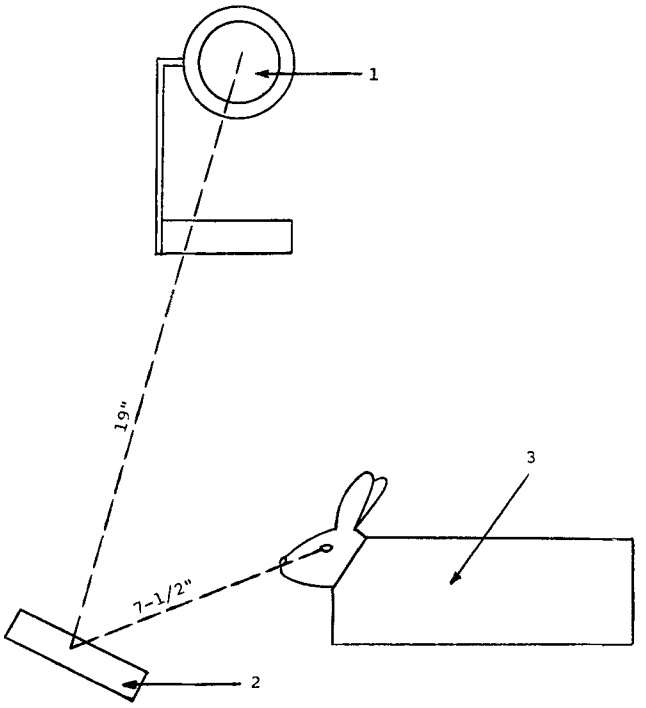


Figure 1—*Experimental arrangement for measurement of pupillary diameters. Key: 1, light source; 2, flat-faced mirror; and 3, rabbit in rabbit box.*

The diameters of the pupils were measured to within 0.1 mm., using a vernier caliper. The precision of the measurements increased with their magnitude, varying from approximately 5 to 55% mean error. The average mean error for the experiment was approximately 20%. The intersubject variation in replicated results was observed to be of approximately the same magnitude as intrasubject variation, therefore permitting the pooling of results obtained with different animals under the same experimental conditions otherwise.

RESULTS AND DISCUSSION

Relationship of Mydriatic Response Intensities to Biophasic Drug Levels—The values of $f(I)$ which are, by Eq. 8, directly proportional

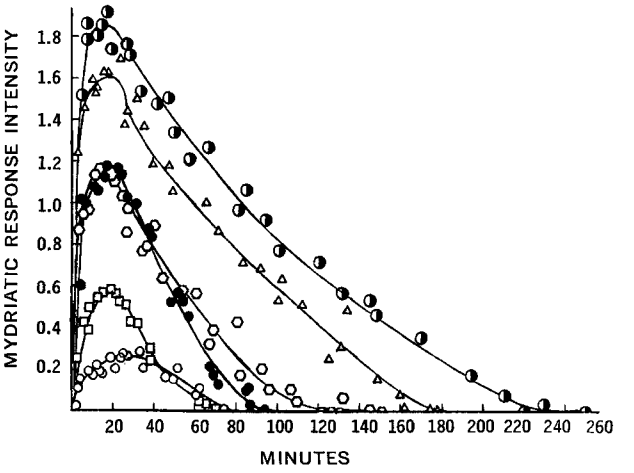


Figure 2—*Time course of mydriatic response intensity for several intravenously administered doses of tridihexethyl chloride to rabbits. Each curve is the result of measurements on a minimum of three separate rabbits. The intravenous doses employed, in mg./kg., were 0.17 (—○—), 0.22 (—□—), 0.30 (—●—), 0.45 (—○—), 0.95 (—△—), and 1.60 (—◑—).*

[2] Read as function of *I*, *not I* multiplied by *f*; the value of $f(I)$ is at all times directly related to the quantity of drug in the biophase; *i.e.*, $f(I) = Q_B/\beta$.

[3] Lot CL 19,813, supplied by Lederle Laboratories.

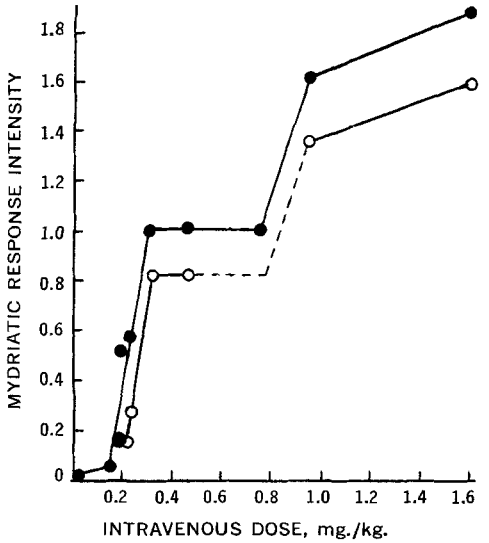


Figure 3—*Relationship of maximum mydriatic response intensity,* —●—●—, *and mydriatic response intensity observed at twice the observed time corresponding to the maximum intensity,* —○—○—, *to the intravenous doses of tridihexethyl chloride employed. The six points on each curve, which correspond to the same value on the abscissa, are the result of a minimum of three determinations on separate rabbits. The remaining points result from single determinations.*

to the quantities of drug in the biophase at any time can be obtained directly from intravenous dose *versus* effect curves. Such curves for tridihexethyl chloride are illustrated in Fig. 3. The experimental curves from which the values of $I_{max.}$ were obtained are shown in Fig. 2. The upper curve in Fig. 3 is a plot of $I_{max.}$ values *versus* dose, while the lower curve was constructed by plotting I values observed at times corresponding to double the experimental $t_{max.}$ values ($2t_{max.}$) as a function of dose. Values of $f(I)$ corresponding to observed values of I at any time may be read directly as the corresponding abscissae using either curve, provided it is used consistently. The choice of the time for the observed I values to be used in the construction of the intravenous dose–effect curve is arbitrary when the drug in the biophase at any time is continually in equilibrium with the processes responsible for the manifestation of the pharmacological response. $I_{max.}$ values corresponding to $t_{max.}$ are best chosen for use because, in addition to theoretical considerations, they often appear to be better defined by the experimental I *versus* time curves.

Indirect Verification for Postulated Use of Pharmacological Data to Reflect Biophasic Drug Levels—A direct means of verifying the use of pharmacological data for the absorption analysis of a particular drug can obviously be obtained through a comparison of the results obtained by its use with similar results based on the detection of the drug in body fluids. As in the present case, due to difficulties encountered in the assay of tridihexethyl chloride in body fluids, such a direct means of confirmation is not always convenient or practical. In fact, the use of pharmacological data possesses the greatest utility when this is indeed the case. However, confidence in the applicability of the techniques may still be obtained by comparing the behavior of the observed pharmacological results with that predicted by the postulated model. A method of direct confirmation using only pharmacological data can be performed through a comparison of the quantities of drug absorbed at any time calculated from the pharmacological data with known quantities administered by slow intravenous infusion. This was not done for tridihexethyl chloride; this test was, however, performed on tropicamide, another mydriatic drug, where exceptionally close agreement was observed between known and calculated quantities. The details of these results are reported (11).

Confidence in the assumption that biophasic drug levels can be monitored by observing the intensity of response, *i.e.*, all processes involved in the manifestation of the response are entirely reversible and nonhysteretic, can be gained by constructing more than one intravenous dose–effect curve. Each curve is constructed by plotting I values observed at a constant arbitrarily chosen elapsed period of

Table I—Mydriatic Response Results Observed for Intravenous Doses of Tridihexethyl Chloride

Dose, mg./kg.	Range of $t_{max.}$[a]	$I_{max.}$[b]	$I_{2max.}$[c]	$[f(I)_{max.}]/[f(I)_{2max.}]$[d]
0.140	—	0.052	—	
0.170	20–25	0.27 ± 0.006	0.18	0.8
0.180	—	0.52	—	—
0.220	15–22	0.58 ± 0.006	0.28	0.8
0.300	15–20	1.10 ± 0.05	0.82	0.8
0.450	14–20	1.14 ± 0.01	0.85	0.6
0.75	—	1.13	—	—
0.95	15–19	1.62 ± 0.01	1.38	0.9
1.60	17–19	1.90 ± 0.03	1.60	0.6
Averages				0.76

[a] The ranges of $t_{max.}$ were visually estimated from plots of I *versus* time for each replication on a different animal. [b] The mean error is indicated for values that are averages of a minimum of three determinations on different rabbits. The remaining values are single determinations. [c] These values were estimated from the curves in Fig. 2 at times corresponding to twice the average $t_{max.}$ [d] The doses in Column 1 were used as values of $f(I)_{2max.}$ for the calculation of the values of the ratios; $(I)_{max.}$ values were read as the abscissae corresponding to the $I_{2max.}$ values in Column 4 using the $I_{max.}$ *versus* dose curve shown in Fig. 3.

time, following the intravenous administration, as a function of the dose; *e.g.*, fractional or multiple values of $t_{max.}$ may be chosen. It can be predicted from Eq. 8 that for any given observed value of I, irrespective of the dose or time of its observation, the ratio of any two $f(I)$ values read from any two of the intravenous dose–effect curves should be constant. This is obvious when it is considered that the quantity of drug in the biophase must be the same for any given value of I. The value of the constant is determined by the ratio of β values corresponding to the times chosen for the construction of the intravenous dose–effect curves. The realization of this predicted behavior is evidenced by the relative constancy of the values listed in the fifth column of Table I. Considering the magnitude of the experimental error and its exaggeration in the computation of the ratios, the agreement of these values is interpreted as very reasonable.

Simulated Curve Fitting without Constraints on Model Parameters—It is obvious from inspection of Eq. 9 that a congruity in curves constructed by plotting normalized values of $f(I)$, *i.e.*, $f(I)/f(I_{max.})$ as a function of time is expected. The independence of such curves of the dose is a necessary condition of the verity of using pharmacological data to describe the biokinetic behavior of a drug. This condition arises from the assumption of linear compartment models. Prior to initiating an extended study of the biophasic availability of tridihexethyl chloride by the presently described methods, a premonitory indication of the applicability of such an approach for use with this first chosen drug was obtained with single determinations of the time course of mydriasis following the administration of intravenous doses of 0.3 and 0.45 mg./kg. to the same animal. The ratio of areas under the two plots of dose normalized values of $f(I)$ *versus* time was found to be 0.97. The average ratio of ordinates compared at 13 separate times was noted as 0.96. The deviation of these values from unity is, therefore, well within experimental error. Further confidence in the applicability of the method to tridihexethyl chloride was obtained from subsequent data obtained with a larger range of doses.

These results are summarily presented by the computer[4]-constructed composite plot of $f(I)/f(I_{max.})$ values *versus* time shown in Fig. 4. The scatter of points can largely be attributed to biological variation and the somewhat unrefined technique of pupillary diameter measurement employed in obtaining this particular set of early results. The use of animals further screened on the basis of their similitude of response to more than one preliminary test dose of the drug, as well as refinements in the method of pupillary diameter measurement, yielded considerably improved data in subsequent studies with tridihexethyl chloride and other mydriatic drugs. Despite the inprecision of the results plotted in Fig. 4, the points appear to scatter around the simulated least-squares biexponential and triexponential curves of best fit.

[4] The curves in Fig. 4 were constructed by a model 563 Calcomp Digital Incremental Plotter, California Computer Products Inc., Anaheim, Calif.

FORTRAN IV programs for use on a CDC 6500 digital computer were developed to obtain the unconstrained biexponential and triexponential curves of best fit to the experimental data. A method of systematized iterative guessing (15) was used in the programs. This method is advantageous because it converges rapidly and is devoid of the danger of merely reaching a local minimum in the sums of squares as can occur with other computational techniques (16). First approximations to the equation parameters—which are necessary to initiate the iterative computer programs—were obtained by the "peeling off" technique (17).

The simulated unconstrained curves in Fig. 4 serve only as references to which other constrained fits are to be compared. The values of the equation parameters do not necessarily correspond to physically allowable (*i.e.*, real and nonnegative) values for the transfer constants. The values of $t_{max.}$, the least sums of squares, and the average values of $\beta_2 t_{max.}/\beta t_{max.}$ for the biexponential fit are 11.6 min., 1.2217, and 0.7724, respectively. In the same order, the values for the triexponential fit are 11.7 min., 1.2227, and 0.7701. On the basis of these criteria alone, it would be difficult to choose between the biexponential and triexponential fits. The expected agreement of the $\beta_2 t_{max.}/\beta t_{max.}$ values with the average value of $f(I_{max.})/f(I_{2max.})$, equal to 0.76, is exceptionally good considering the magnitude of the involved errors.

The biexponential and triexponential equations, respectively, are:

$$f(I)/f(I_{max.}) = 1.1294\,[\exp(-0.014635t) - \exp(-0.26530t)] \quad \text{(Eq. 10)}$$

$$(I)/f(I_{max.}) = 0.39190\exp(-0.01293t) + 0.91438\exp(-0.015713t) - 1.3063\exp(-0.25870t) \quad \text{(Eq. 11)}$$

Biophasic Availability Characteristics of Tridihexethyl Chloride following Ophthalmic Dosing—Figure 5 contains semilogarithmic plots of I, $f(I)$, and A_{RB} as a function of time following ophthalmic dosing with 0.015% tridihexethyl chloride in a pH 7.4 isotonic phosphate buffered solution. The half-life of drug elimination from the biophase ($t_{1/2} = 0.693/K^*_{BO}$) is obtained from the latter linear segment of the log $f(I)$ *versus* time curve as equal to 132 min. This value can be compared to the apparent mydriatic half-life of 165 min., which is similarly obtained from the log I *versus* time curve. The mydriatic half-life corresponds to the rate of decay of the mydriatic response intensity. The mydriatic and elimination from the biophase half-lives, although related, obviously need not necessarily be the same value. For any given drug, the precise nature of the relationship between the decay of I and K^*_{BO} depends upon the form of the dose-effect relationship.

The values of F_{BT} used in the log A_{RB} *versus* time plot were calculated using Eq. 1 and the value of $K^*_{BO} = 0.00524$ corresponding to

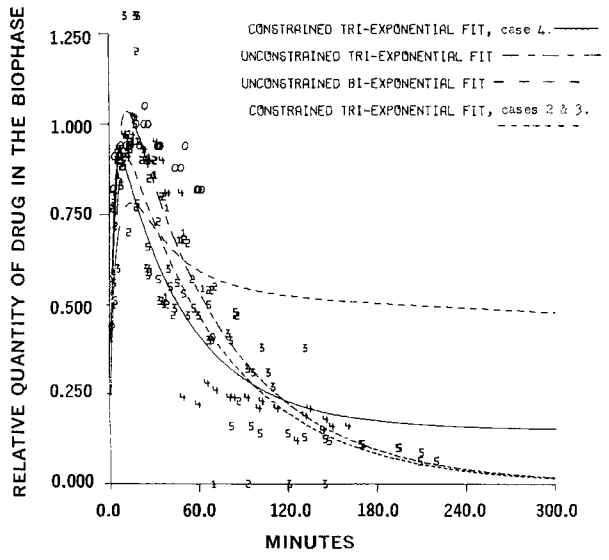


Figure 4—*Computerized least sums of squares fits to a composite plot of* f(I), *normalized for dose, representing the time course of variation of relative levels of tridihexethyl chloride in the biophase. The intravenous doses represented are 0.170 (—0—), 0.220 (—1—), 0.300 (—2—), 0.45 (—3—), 0.95 (—4—), and 1.60 (—5—) mg./kg.*

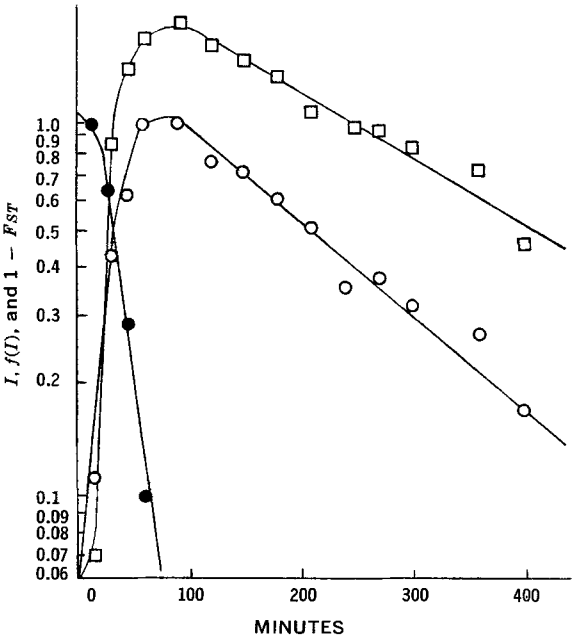
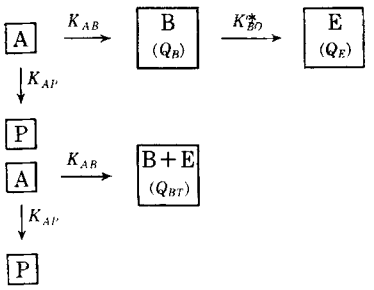


Figure 5—*Semilogarithmic plots of* I, *the mydriatic response intensity* (—□—); f(I), *the relative quantity of drug in the biophase* (—○—); *and* 1 − FST, *the fraction of drug remaining to be transcorneally absorbed into the biophase* (—●—), *as a function of time in minutes, following a 0.02-ml. ophthalmic dose of 0.015% tridihexethyl chloride (in pH 7.4 isotonic buffer) into the cul-de-sac of rabbit eyes. Each point is the average of 12 determinations on different rabbits. The absorption of the drug into the biophase is completed between 60 and 90 min.*

the biophase elimination half-life. The linearity of the log A_{RB} *versus* time plot is indicative of the operation of apparent first-order processes, such as passive diffusion, being responsible for the dissipation of drug from its ophthalmic site of administration. This conclusion justifies the construction of a linear compartment model to describe these processes. Following administration into the cul-de-sac of the eye, the dissipation of the drug from the site of administration occurs through transcorneal absorption into the biophase and through, what will be termed, peripheral loss, which can include scleral absorption and such loss of drug as can occur down the lacrimal nasal canal. By following this consideration, a compartment model such as is shown in Scheme IV can be applied to describe



Scheme IV—Equivalent compartment models pertaining to the dissipation of drug from its site of administration in the eye. The symbol P designates the compartment into which peripheral loss of the drug occurs; B represents the biophase; E is a compartment constituted by the combination of all body compartments other than those shown; A is the site of administration; Q_{BT} *represents the quantity of drug which has been transcorneally absorbed from A into the biophase;* $Q_{BT} = Q_B + Q_E$, *where* $Q_E + Q_B$ *is the sum of the quantities of drug present and having been eliminated from the biophase at any time.*

these processes. On the basis of these models, Eq. 12 can readily be derived:

$$\log A_{RB} = -\left(\frac{K_{AP} + K_{AB}}{2.303}\right)t \quad \text{(Eq. 12)}$$

Table II—Results of Constrained Computerized Fits to Two-Compartment Models

Dose, mg./kg.	Equation Parameters	Model Parameters	$\beta_{2t_{max.}}/\beta_{t_{max.}}$	$t_{max.}$	Sums of Squares
0.450	$A'_1 = 6.205$ $A'_2 = -6.205$ $m_1 = 0.03757$ $m_2 = 0.05010$	$K'_{BO} = 0$ (assigned) $K'_{CB} = 0.0833$ $K'_{CO} = 5.30 \times 10^{-5}$ $K'_{BC} = K^*_{BO}$ $= 0.00524$	0.894	22.7	1.709
0.950	$A'_1 = 5.268$	$K'_{BO} = 0$ (assigned) $K'_{CB} = 0.0939$ $K'_{CO} = 4.91 \times 10^{-6}$ $K'_{BC} = K^*_{BO}$ $= 0.00524$	0.816	20.4	2.033

The sum of the constants, $K_{AP} + K_{AB}$, represents the total proportional rate of loss of the drug from the site of administration. This value is obtained from the slope of the log A_{RB} *versus* t plot. In this manner, a half-life of 17 min. can be calculated to represent the total rate of loss of tridihexethyl chloride from the surface of the eye.

Testing and Selection of Compartment Models—In contrast to the case of drug absorption occurring directly into the biophase, the computation of the cumulative amount of drug absorbed systemically requires the adoption of a biokinetic model, which may not necessarily be constituted of only a single compartment. It is seldom possible in a biokinetic study to determine uniquely a compartment model, since any model that is compatible with the data can always be interpreted as a degenerate case of a higher order model. Generally, for linear models, a model with the smallest number of compartments compatible with the data and physical reality (*i.e.*, the transfer constants, K_{ij}'s, must be real and nonnegative) giving the best fit, as judged by the sums of squares, is chosen (18, 19). Simulated curves should, as far as possible, be devoid of systematic deviations from those experimentally observed (19).

For the purpose of its application to drug-absorption analysis, the questions of whether the model ultimately chosen is unique and whether the compartments have a counterpart in biological reality are irrelevant. For the present application, the utility of a model is limited to its use for the calculation of the relative quantities of drug remaining at the site of administration to be absorbed at any time. For this purpose, any model consistent with the data to which physically real values of the model parameters can be assigned is suitable. The biological and physical reality of models to be used solely for the purpose of drug absorption analysis is further discussed in Appendix IV.

It is apparent from the maximum in the data points, shown in Fig. 4, that the biophase is constituted by a compartment distinguishable from the central systemic compartment; therefore, a minimum of two compartments is necessary to characterize the biokinetic behavior or tridihexethyl chloride. As previously discussed, the sum of K_{BJ}'s should equal the overall elimination constant from the biophase, K^*_{BO}, whose value has been obtained from the results of ophthalmic dosing. With this relationship and the results of intravenous dosing, four transfer constants can be calculated to char-

Table III—Averaged[a] Results for Triexponential Constrained Computerized Fits to Different Cases of Three-Compartment Models

Model Parameters	Equation Parameters	$\beta_{2t_{max.}}/\beta_{t_{max.}}$	$t_{max.}$, min.	Sums of Squares
	Case 1			
$K'_{BO} = 0$ $K'_{BC} = 0.00524$ $K'_{CO} = 0$ $K'_{CB} = 0.3785$ $K'_{CD} = 0.0464$ $K'_{DO} = 0.0200$ $K'_{DC} = 2.79 \times 10^{-4}$	$A'_1 = 0.5391$ $A'_2 = 0.706$ $A'_3 = -1.33$ $m_1 = 3.34 \times 10^{-4}$ $m_2 = 0.032$ $m_3 = 0.417$	0.867	12.2	0.9672 3.288[c]
	Case 2			
$K'_{BO} = 0$ $K'_{BC} = 0.00524$ $K'_{CO} = 0.0462$ $K'_{CB} = 0.379$ $K'_{CD} = 2.069 \times 10^{-4}$ $K'_{DO} = 0$ $K'_{DC} = 0.0203$	$A'_1 = 0.539$ $A'_2 = 0.706$ $A'_3 = -1.33$ $m_1 = 3.33 \times 10^{-4}$ $m_2 = 0.0320$ $m_3 = 0.412$	0.867	12.2	0.9672 3.288[c]
	Case 3[b]			
$K'_{BO} = 0.00519$ $K'_{BC} = 4.55 \times 10^{-5}$ $K'_{CO} = 0.0438$ $K'_{CB} = 0.651$ $K'_{CD} = 2.74$ $K'_{DO} = 0$ $K'_{DC} = 0.0052$	$A'_1 = 6.39 \times 10^{-4}$ $A'_2 = 0.943$ $A'_3 = -0.944$ $m_1 = 0.00520$ $m_2 = 0.00520$ $m_3 = 0.696$	0.9088	8.0	1.070
	Case 4			
$K'_{BO} = 0.00169$ $K'_{BC} = 0.0232$ $K'_{CO} = 0$ $K'_{CB} = 0.475$ $K'_{CD} = 0.0305$ $K'_{DO} = 0$ $K'_{DC} = 0.00741$	$A'_1 = 0.162$ $A'_2 = 0.909$ $A'_3 = -1.07$ $m_1 = 2.28 \times 10^{-4}$ $m_2 = 0.021$ $m_3 = 0.483$	0.863	9.8	0.7274 2.400[c]

[a] The results are averages of the values obtained from separate fits to data corresponding to six different doses. [b] Results for Case 3 were obtained only for data corresponding to the 0.300-mg./kg. dose. [c] Value of the sum of squares corrected to enable direct comparison to the value of 1.2227 obtained for the unconstrained fit to the pooled and averaged data.

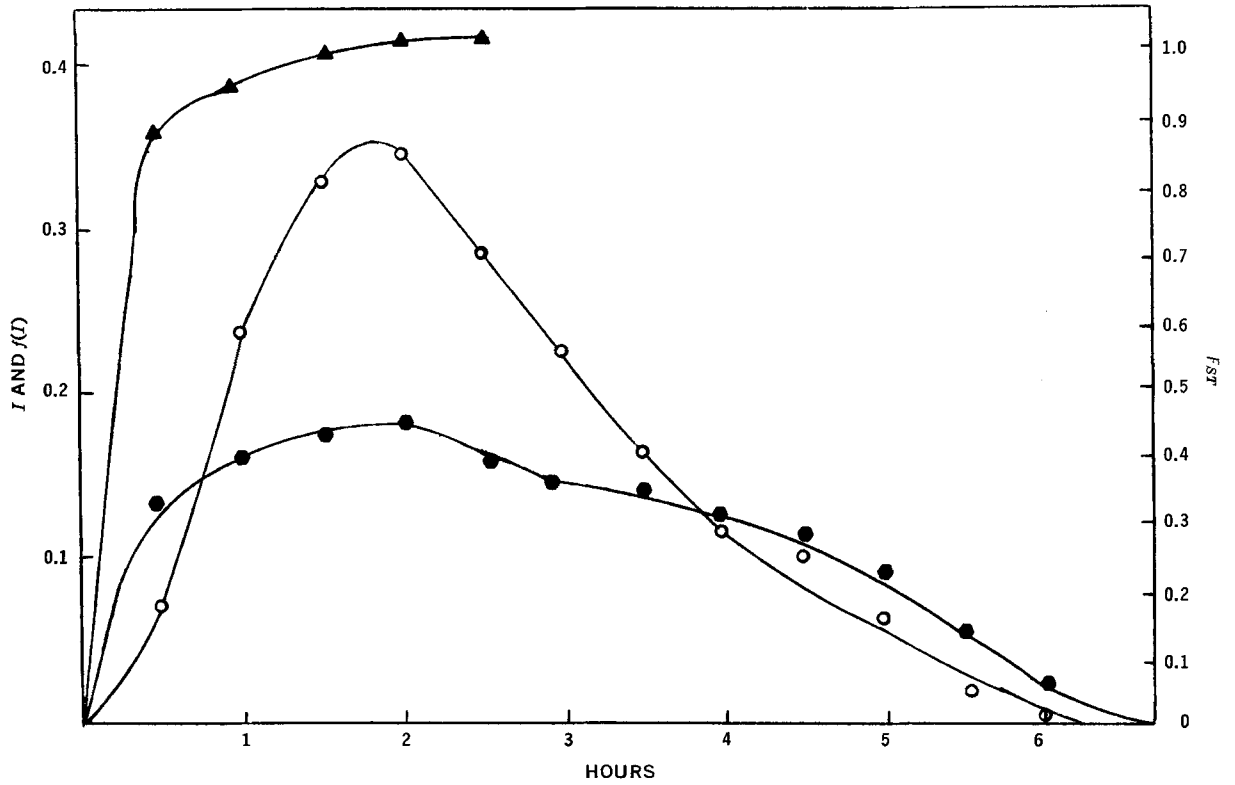


Figure 6—*The temporal variation of* I, *mydriatic response intensity* (—○—); *relative quantity of drug in the biophase,* f(I) (—●—); *and relative quantities of drug systemically absorbed,* F_{ST} (—▲—), *following oral dosing of rabbits with 35 mg./kg. tridihexethyl chloride. Each point is the average of a minimum of three determinations.*

acterize a biexponential model and six can be derived for a triexponential model with their corresponding three and five degrees of freedom, respectively (20).

Two-Compartment Model Fit—Eliminating Compartment D, shown in Scheme I, from consideration is tantamount to assuming that the biophase is indistinguishable from other tissue compartments. By setting $K_{BC} + K_{BO} = K^*_{Bo}$, all four transfer constants can be evaluated. This was accomplished by adding additional constraints on the values of the equation parameters such that the iterative systematized guessing computer program would compute the least-squares values of the transfer constants which are also nonnegative and nonimaginary; these values are included in the computer printouts. The values of the equation parameters obtained from the unconstrained least-squares fit to the data may be employed as the initial estimates for the constrained fit to the data. In the search for least-squares values of the equation parameters consistent with the constraints imposed on the transfer constants, each equation parameter is allowed to assume values in the limits between zero and nearly twice the value of its initial estimates. Exceeding these limits would serve no purpose, since the resulting theoretical curve would be grossly inconsistent with experimental results. When a fit to a model providing positive and real values for the transfer constants was not obtained within these limits, the model was discarded as a grossly inaccurate representation of the system's biokinetic behavior. This was found to be the case with 16 out of the 18 attempts in which data for six doses were separately fitted to each of three cases of two-compartment models. These included, in addition to the case where the evaluation of all four transfer constants was sought, two models in which K_{BO} and K_{CO} were set equal to zero, respectively. These results, shown in Table II, clearly indicate that a two-compartment model is inadequate in simulating the behavior of this system. Consequently, test fits to models with an additional two degrees of freedom were performed in an effort to obtain an improved fit to the data.

Three-Compartment Model Fit—A total of seven transfer constants are shown to characterize the three-compartment model (Schemes I and II). Again, employing the constraint that $K_{BC} + K_{BO} = K^*_{Bo}$ allows six of these constants to be calculated from the data. The remaining constant(s) can be assigned an arbitrary value which can appropriately be taken as zero.

Attempts were made to fit the data for each dose to five cases of the three-compartment model shown in Schemes I and II. These cases included setting: (1) $K_{BO} = K_{CO} = 0$; (2) $K_{BO} = K_{DO} = 0$; (3) $K_{DO} = 0$; (4) $K_{DO} = K_{CO} = 0$; (5) $K_{BO} = 0$; and (6) $K_{CO} = 0$. Each case assumes negligible or zero contributions for different paths of drug elimination from the body. All the cases assume the operation of a peripheral, pharmacologically inert drug depot compartment, D, communicating with the central, systemic compartment.

Cases 1 and 2 provided fits to the data derived from all six doses studied. Case 3 was found compatible with only one of the six doses. The averaged results are summarized in Table III. The relations between the transfer constants and equation parameters for the three cases, to which it was found that the data could be fitted, are given in Appendix IV along with appropriate expressions for the calculation of Q_{BS} and F_{ST}.

Generally, the selection of a compartment model with regard to its adequacy in simulating the experimentally observed biokinetic behavior of a system has been based only upon the value for the sums of squares. However, in the present study the agreement between (*a*) calculated and observed values of $t_{max.}$ and (*b*) calculated values of $\beta_2 t_{max.}/\beta t_{max.}$ with the observed value of $f(I_{max.})/f(I_{2max.})$ serves as additional criteria for the selection of a model. Inspection of Table III, however, reveals that except for the transfer constants, the results for Cases 1 and 2 are identical and clearly demonstrate the lack of uniqueness of fitted compartment models. Figure 4 also contains a plot of the equation for Cases 1 and 2, where it can be compared to the scattered experimental values and the curves corresponding to the unconstrained least-squares fit to the data. The systematic inconsistency of the fit to Cases 1 and 2 clearly indicates their inadequacy in simulating the behavior of the system. To obtain an improved fit, the requirement that $K_{BC} + K_{BO} = K^*_{Bo}$ was removed and the data again fitted to Cases 1, 2, and 4. Cases 1 and 2 could not be fitted to any of the data, while Case 4 provided fits for each of the doses. The averaged results are reported in Table III. The corresponding curve shown in Fig. 4 demonstrates a considerable improvement over the fits obtained to Cases 1 and 2. However, none of the constrained fits to the two- and three-compartment models which were obtained is exceptionally good. Presumably, further improvement in the simulation of the data could be obtained by

complicating the model with additional compartments. However, no further attempts of this nature were pursued in the present study.

If Models 1 and 2 had provided an acceptable fit to the data, it would not have been possible to choose between them. A need for additional criteria on which to base the selection of a model is clearly indicated. Since the purpose of the model is its application in the computation of Q_{BS} and F_{ST} for systemic routes of administration, a model could best be chosen on the basis of the accuracy with which it allows known, experimental values of these quantities to be computed. The experimental results necessary for the implementation of this criterion, however, were not obtained in the present study.

Oral Administration of Tridihexethyl Chloride—Figure 6 illustrates results obtained by orally dosing rabbits with 35 mg./kg. of tridihexethyl chloride contained in gelatin capsules. The method employed in its administration has been reported (21). The values of F_{ST} plotted in Fig. 10 were calculated using relations appropriate for Case 4. These expressions are contained in Appendix IV.

SUMMARY AND CONCLUSIONS

All behavior of the data predicted as necessary to the verity of using pharmacological response-intensity data to describe the biokinetics of tridihexethyl chloride was observed. A three-compartment model provided the best simulation of the temporal variation of the biophasic drug levels. In general, when a reversible, gradual response to a drug is measurable and the applicability of the pharmacological technique is suspected, further confidence in its use may be readily gained from a minimum of pharmacological data obtainable from intravenous dosing with the drug of interest.

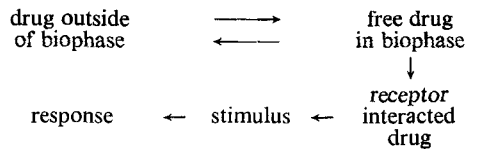
Unmodified, the presently described method may be expected to fail for cases where: (*a*) the biological response to the drug is not a continuous, single-valued function of the equilibrium drug level in the biophase, *e.g.*, it may exhibit hysteresis; (*b*) a continuously graded intensity of response is not detectable; (*c*) the effects of the drug are not immediately reversed by the removal of the drug from the biophase; and (*d*) the biokinetic behavior of the drug at some stage following its absorption into the central compartment or the biophase from its site of administration occurs by processes not describable by linear compartment modeling. Some of these conditions can be corrected for by suitable and sometimes simple modifications of the mathematical relations derived to describe the system behavior. In other cases, the innate behavior of the systems will preclude the use of pharmacological data for drug-absorption analysis. In every case, indirect or direct verification of the pharmacological method should be obtained prior to its application to new drugs or biological systems.

The sought therapeutic effect of some important classes of drugs, (*e.g.*, psychotherapeutic agents) cannot readily be quantitated or expressed as a continuous graded intensity of effect. However, many such drugs produce side effects, such as the hypothermic response to chlorpromazine, which can be continuously monitored and which may be appropriate for the implementation of the presently described pharmacological method of drug-absorption analysis. In this manner, the use of pharmacological data can provide an analytical tool which may allow the elucidation of the biokinetics and drug-absorption behavior of labile or highly potent drugs whose detection in corporeal fluids or tissues cannot be accomplished by direct chemical or radiological assay techniques. When the direct use of the observed pharmacological response intensity itself is inappropriate, it may occur that a rate of change—as expressed by numerically evaluated first or higher order derivatives of the observed response intensity with respect to time—or some other functional transformation of the pharmacological data may possess the behavior required for the application of the presently described method in an unmodified form. The use of transformed pharmacological response-intensity data in pharmacokinetic studies has been reported for anticoagulants (22, 23).

APPENDIX I

The rate processes responsible for the manifestation of a reversible pharmacological response may be diagrammed as in Scheme IA. The drug receptors are contained within the compartment termed the biophase.

The biokinetic treatment which is presently developed may be assumed to be strictly applicable when the rate-limiting process in the manifestation of the pharmacological response at any time is the drug's ability to gain access to its biological sites of action, *i.e.*, to penetrate into the biophase. The subsequent interaction with the receptor sites and the induction of the stimulus resulting in the observed response are sufficiently rapid that their extents are representative of reversible equilibrium with the levels of drug in the biophase at any time. As indicated by the results gained in the present study, this appears to be the case with tridihexethyl chloride. This is particularly indicated by the constancy of the $f(I)$ ratios read from the two different dose–effect curves shown in Fig. 3. With other drugs, if $f(I)$ ratios are clearly found not to be constant it may be suspected that the rate of flux of drug into and from the biophase from the systemic compartment is fast relative to the subsequent occurrence of the other processes necessary to the manifestation of the drug response. It might also be observed that due to the rapid passage of drug, the systemic and biophasic compartments are mathematically the same.



Scheme IA

When the described complications are met, it may become necessary to derive other mathematical models to relate intensities of response to biophasic drug levels which are based on a postulated mechanism of drug–receptor site interaction. As occurred with tridihexethyl chloride, this is not necessary if the rate-limiting step is merely the entrance and efflux of drug from the biophase. When it is necessary, however, a difficulty in deriving this relation becomes evident when it is considered that an equilibrium between the density of drug-affected receptor sites and the quantity of drug in the biophase cannot be expected. In fact, this precisely is the cause of the complication. However, some insight into the nature of the kinetic processes may again be gained from consideration of the intravenous dose–effect curve.

If the curve is constructed using $I_{max.}$ values, then considering I to be a monotonic increasing function of the equilibrium concentration (*i.e.*, concentration of drug in equilibrium with the receptor sites), the most propitious time to assume this equilibrium to obtain would be such that the rate of change of the concentration of drug in the biophase is minimal and therefore allows a maximum opportunity for the receptor sites to come into equilibrium with it. This time is obviously $t_{max.}$. The dose–effect curve using $I_{max.}$ values may then be assumed to characterize the equilibrium between drug-affected and free-receptor sites with free drug. This information may, in turn, be used to provide the insight necessary for the construction of a kinetic model to apply at other times besides $t_{max.}$ and provide the needed relationship between the observed intensity of response and quantity of drug in the biophase. Such a final relation is then valid irrespective of the path by which the drug gains entry to the biophase and can be used for drug-absorption analysis in lieu of the dose–effect curve which would, in this case, not be applicable for this purpose.

APPENDIX II

The resolution of the compartment system shown in Scheme I into that in Scheme II in practice can be accomplished by using a control eye. The verity of this approach can perhaps be further understood by considering for the moment that the quantity of the total drug absorbed systemically, Q_{TS}, is hypothetically in some way distinguishable from the quantity of the drug absorbed directly into the biophase[5], Q_{TB}. At any time the total quantity of drug absorbed from the site of administration, Q_T, is the sum of these quantities as given by Eq. 1A:

$$Q_T = Q_{TB} + Q_{TS} \qquad \text{(Eq. 1A)}$$

Mass balance dictates that Q_T at any time is the sum of the quantities of drug in the biophase, Q_B, occurring in the central compartment, Q_C, present in the depot compartment, Q_D, and which has been eliminated from the system, Q_E:

$$Q_T = Q_B + Q_C + Q_D + Q_E \qquad \text{(Eq. 2A)}$$

[5] This could be exemplified by the simultaneous administration of two hypothetical drugs differing only in the routes by which they are capable of gaining access to the biophase.

Since drug absorbed directly and systemically is being assumed to be distinguishable, similar relations exist for Q_{TB} and Q_{TS}; *i.e.*, at any time,

$$Q_{TB} = Q_{BB} + Q_{CB} + Q_{DB} + Q_{EB} \quad \text{(Eq. 3A)}$$

$$Q_{TS} = Q_{BS} + Q_{CS} + Q_{DS} + Q_{ES} \quad \text{(Eq. 4A)}$$

It is obvious that the sum of Eqs. 3A and 4A is equal to both Eqs. 1A and 2A and the quantity of drug in the biophase at any time is the sum of the quantities originating from systemic and direct absorption. Subtracting Eq. 4A from Eq. 2A produces a result equivalent to Eq. 3A, from which Eq. 5A can be obtained:

$$Q_{BB} = Q_B - Q_{BS} \quad \text{(Eq. 5A)}$$

Since the pharmacological response intensity, I, can be assumed to be a continuous function of the quantity of drug in the biophase over the range considered, the difference in $f(I)$ values observed for the experimental eye and that simultaneously observed for the control eye reflects the quantity of drug, Q_{BB}, in the biophase that arises by direct absorption. Therefore, Eq. 5A can be rewritten as Eq. 6A:

$$Q_{BB} = \beta[f(I)_{\text{exptl.}} - f(I)_{\text{control}}] \quad \text{(Eq. 6A)}$$

APPENDIX III

Equations 3*a* and 4 can readily be obtained by referring to Eq. 4A and to the compartment system in Scheme II. Equation 4A can be rewritten in differential form as Eq. 7A for the model reduced to two compartments by eliminating D; *i.e.*, K_{CD}, K_{DC}, and K_{DO} are set equal to zero.

$$dQ_{TS} = dQ_{BS} + dQ_{CS} + dQ_{ES} \quad \text{(Eq. 7A)}$$

Referring to System II in Scheme II, the following relations can be generated:

$$\frac{dQ_{BS}}{dt} = K_{CB}Q_{CS} - (K_{BC} + K_{BO})Q_{BS} \quad \text{(Eq. 8A)}$$

$$\frac{dQ_{ES}}{dt} = K_{CO}Q_{CS} + K_{BO}Q_{BS} \quad \text{(Eq. 9A)}$$

Equations 10A and 11A are obtained by solving for and expressing dQ_{CS} and dQ_{BS} in terms of Q_{BS}:

$$dQ_{CS} = \left[\frac{K_{BC} + K_{BO}}{K_{CB}}\left(\frac{dQ_{BS}}{dt}\right) + \frac{1}{K_{CB}}\left(\frac{d^2Q_{BS}}{dt^2}\right)\right]dt \quad \text{(Eq. 10A)}$$

$$dQ_{ES} = \left\{\frac{[K_{CO}(K_{BC} + K_{BO}) + K_{CB}K_{CO}]}{K_{CB}}Q_{BS} + \frac{K_{CO}}{K_{CB}}\frac{dQ_{BS}}{dt}\right\}dt \quad \text{(Eq. 11A)}$$

Substituting Eqs. 10A and 11A into Eq. 1 and integrating yield Eq. 12A:

$$Q_{TS} = \frac{K_{CO}(K_{BC} + K_{BO}) + K_{CB}K_{BO}}{K_{CB}}\int_0^t Q_{BS}\,dt + \left(\frac{K_{CB} + K_{BC} + K_{BO} + K_{CO}}{K_{CB}}\right)Q_{BS} + \frac{1}{K_{CB}}\frac{dQ_{BS}}{dt} \quad \text{(Eq. 12A)}$$

In the limit when $t = \infty$, Eq. 12A reduces to Eq. 13A:

$$Q_{TS\infty} = \frac{K_{CO}(K_{BC} + K_{BO}) + K_{CB}K_{BO}}{K_{CB}}\int_0^\infty Q_{BS}\,dt \quad \text{(Eq. 13A)}$$

In a similar manner, Eqs. 3*a*–3*e* can be derived for a three-compartment system.

APPENDIX IV

When the biophase is constituted, as in the present case with tridihexethyl chloride, by a compartment kinetically distinguishable from the central systemic compartment, the K_{iJ} values to be used in expressions for the cumulative amount of drug absorbed, *e.g.*, in equations such as 3*a*–3*e* and 12A, are not necessarily the values obtained from a computerized fit of a compartment model to the observed relationship of $f(I)/D$ to time. The computed values of (K'_{iJ})'s must be corrected for the fact that they are calculated from values of A'_i, where $A'_i = A_i/\beta$, rather than A_i values; see Eq. 9. No correction is necessary for single-compartment models or multiple-compartment models in which the biophase and central systemic compartments are identical; *i.e.*, $t_{\max.} = 0$ and, therefore, $\beta = 1$. Each K_{iJ} can be functionally related to the determined values of the (A'_i)'s, m_i's, and (K'_{iJ})'s and an unknown value of β. The value of β can be computed by considering that, for a rapid intravenous dose, Q_{TS} = dose at all times from $t = 0$ to $t = \infty$ and $f(I)$ at any time is given by the sum of exponentials providing the best fit for the rapid intravenous dosing data; *i.e.*, $f(I) = D\sum_{i=1}^{n} A'_i e^{-m_i t}$. The integral and n derivatives of $f(I)$ can obviously also be expressed in terms of sums of exponentials involving (A'_i)'s and m_i's. Substituting these theoretical expressions into an equation for Q_{TS} written in terms of (K'_{iJ})'s and β and equating Q_{TS} to the intravenous dose at all times leave only β unknown. Substituting an arbitrarily chosen value for the time ($t = 0$ or $t = \infty$ may be chosen to simplify the expression) allows a solution for β in terms of known values of (K'_{iJ})'s, (A'_i)'s, and m_i's. It may often be found that β drops out of equations for Q_{TS}, and its evaluation is then not necessary for the calculation of Q_{TS} and F_{ST}. In fact, in such a case the absolute value of β apparently cannot be computed.

Equations derived for the model parameters expressed in terms of the equation parameters—which were used in providing the constraints in the systematized iterative guessing computer programs—appropriate for the four cases of three-compartment model fits to the intravenous dosing data are given.

For all cases:

$$g = K'_{DC} + K'_{DO} = \frac{K'_{CB}m_1 + A_1(m_2 - m_1)(m_3 - m_1)}{K'_{CB}} \quad \text{(Eq. 14A)}$$

$$K'_{CB} = -m_1A'_1 - m_2A'_2 - m_3A'_3 \quad \text{(Eq. 15A)}$$

$$K^*_{BO} = K'_{BO} + K'_{BC} \quad \text{(Eq. 16A)}$$

The numerical value of K^*_{BO} is obtained experimentally from pharmacological data resulting from ophthalmic dosing.

Case 1:

$$K'_{BO} = 0 \quad \text{(Eq. 17A}a\text{)}$$

$$K'_{CO} = 0 \quad \text{(Eq. 17A}b\text{)}$$

$$K'_{BC} = K^*_{BO} \quad \text{(Eq. 17A}c\text{)}$$

$$K'_{CD} = m_1 + m_2 + m_3 - K'_{CB} - K'_{BC} - Q \quad \text{(Eq. 17A}d\text{)}$$

$$K'_{DO} = \frac{m_1m_2m_3}{K'_{BC}K'_{CD}} \quad \text{(Eq. 17A}e\text{)}$$

$$K'_{DC} = g - K'_{DO} \quad \text{(Eq. 17A}f\text{)}$$

$$K_{CB} = \beta K'_{CB} \quad \text{(Eq. 18A}a\text{)}$$

$$K_{BC} = K'_{BC} \quad \text{(Eq. 18A}b\text{)}$$

$$K_{BO} = K'_{BO} \quad \text{(Eq. 18A}c\text{)}$$

$$K_{CO} = K'_{CO} \quad \text{(Eq. 18A}d\text{)}$$

$$K_{CD} = K'_{CD} + (1 - \beta)K'_{CB} \quad \text{(Eq. 18A}e\text{)}$$

$$K_{DO} = K'_{CD}K'_{DO}\left[\frac{m_1m_2m_3}{-K'_{CD} + (1 - \beta)K'_{CB}}\right] \quad \text{(Eq. 18A}f\text{)}$$

$$K_{DC} = K'_{DC} + K'_{BO}\left[1 - \frac{K'_{CD}(m_1m_2m_3)}{K'_{CD} + (1 - \beta)K'_{CB}}\right] \quad \text{(Eq. 18A}g\text{)}$$

Case 2:

$$K'_{BO} = 0 \quad \text{(Eq. 19A}a\text{)}$$

$$K'_{DO} = 0 \quad \text{(Eq. 19A}b\text{)}$$

$$K'_{BC} = K^*_{BO} \quad \text{(Eq. 19A}c\text{)}$$

$$K_{DC} = g \quad \text{(Eq. 19A}d\text{)}$$

$$K'_{CO} = \frac{m_1 m_2 m_3}{K'_{DC} K'_{BC}} \quad \text{(Eq. 19A}e\text{)}$$

$$K'_{CD} = m_1 + m_2 + m_3 - K'_{CO} - K'_{CB} - K'_{DC} - K'_{BC} \quad \text{(Eq. 19A}f\text{)}$$

$$K_{BO} = K'_{BO} \quad \text{(Eq. 20A}a\text{)}$$

$$K_{DO} = K'_{DO} \quad \text{(Eq. 20A}b\text{)}$$

$$K_{BC} = K'_{BC} \quad \text{(Eq. 20A}c\text{)}$$

$$K_{CB} = \beta K'_{CB} \quad \text{(Eq. 20A}d\text{)}$$

$$K_{DC} = K'_{DC} \quad \text{(Eq. 20A}e\text{)}$$

$$K_{CO} = K'_{CO} \quad \text{(Eq. 20A}f\text{)}$$

$$K_{CD} = K'_{CD} + (1 - \beta) K'_{CB} \quad \text{(Eq. 20A}g\text{)}$$

Case 3:

$$K'_{DO} = 0 \quad \text{(Eq. 21A}a\text{)}$$

$$K'_{DC} = g \quad \text{(Eq. 21A}b\text{)}$$

$$\lambda = m_1 m_2 m_3 - K'_{CB} - K'_{DL} \quad \text{(Eq. 21A}c\text{)}$$

$$\mathrm{X} = m_1 m_2 + m_2 m_3 + m_1 m_3 - K'_{DC}(K^*_{BO} + K'_{CB}) - K^*_{BO}(\lambda - K^*_{BO}) \quad \text{(Eq. 21A}d\text{)}$$

$$\phi = \frac{m_1 m_2 m_3}{K'_{DC}} \quad \text{(Eq. 21A}e\text{)}$$

$$K'_{CO} = \frac{\mathrm{X} - \phi}{K'_{DC} - K^*_{BO}} \quad \text{(Eq. 21A}f\text{)}$$

$$K'_{CD} = \lambda - K^*_{BO} - K'_{CO} \quad \text{(Eq. 21A}g\text{)}$$

$$K'_{BO} = (\phi - K^*_{BO} K'_{CO})/K'_{CB} \quad \text{(Eq. 21A}h\text{)}$$

$$K'_{BC} = K^*_{BO} - K'_{BO} \quad \text{(Eq. 21A}i\text{)}$$

$$K_{DO} = K'_{DO} \quad \text{(Eq. 22A}a\text{)}$$

$$K_{DC} = K'_{DC} \quad \text{(Eq. 22A}b\text{)}$$

$$K_{CB} = \beta K'_{CB} \quad \text{(Eq. 22A}c\text{)}$$

$$K_{CO} = K'_{CO} + \frac{(K'_{DC} - K^*_{BO})(1 - \beta) K'_{CB}}{K'_{DC} - K^*_{BO}} \quad \text{(Eq. 22A}d\text{)}$$

$$K_{CD} = K'_{CD} + K'_{CB}(1 - \beta) + \frac{(K'_{DC} - K^*_{BO})(1 - \beta) K'_{CB}}{K'_{DC} - K^*_{BO}} \quad \text{(Eq. 22A}e\text{)}$$

$$K_{BO} = \frac{K'_{BO}}{\beta} - \left[\frac{K^*_{BO} K'_{CB}(K'_{DC} - K'_{BO})(1 - \beta)}{K'_{DC} - K^*_{BO}}{\beta K'_{CB}}\right] \quad \text{(Eq. 22A}f\text{)}$$

$$K_{BC} = K^*_{BO} - K_{BO} \quad \text{(Eq. 22A}g\text{)}$$

Case 4:

$$K_{BC} = + K_{BO} \neq K^*_{BO} \quad \text{(Eq. 23A}a\text{)}$$

$$K'_{CO} = 0 \quad \text{(Eq. 23A}b\text{)}$$

$$K'_{DO} = 0 \quad \text{(Eq. 23A}c\text{)}$$

$$K'_{DC} = g \quad \text{(Eq. 23A}d\text{)}$$

$$K'_{BO} = \frac{m_1 m_2 m_3}{K'_{DC} K'_{CB}} \quad \text{(Eq. 23A}e\text{)}$$

$$\mathrm{X} = m_1 m_2 + m_2 m_3 + m_1 m_3 - K'_{DC} K'_{BO} - K'_{BO} K'_{CB} - K'_{DC} K'_{CB} \quad \text{(Eq. 23A}f\text{)}$$

$$\Delta = (m_1 + m_2 + m_3 - K'_{DC} - K'_{BO}) K'_{DC} \quad \text{(Eq. 23A}g\text{)}$$

$$K'_{CD} = {}^1/_2[m_1 + m_2 + m_3 - K'_{CB} - 2K'_{DC} + \sqrt{(m_1 + m_2 + m_3 - K'_{CB} - 2K'_{DC})^2 - 4(\mathrm{X} - \Delta)}] \quad \text{(Eq. 23A}h\text{)}$$

$$K'_{BC} = m_1 + m_2 + m_3 - K'_{CD} - K'_{DC} - K'_{BO} - K'_{CB} \quad \text{(Eq. 23A}i\text{)}$$

$$K_{CO} = K'_{CO} \quad \text{(Eq. 24A}a\text{)}$$

$$K_{DO} = K'_{DO} \quad \text{(Eq. 24A}b\text{)}$$

$$K_{DC} = K'_{DC} \quad \text{(Eq. 24A}c\text{)}$$

$$K_{CB} = \beta K'_{CB} \quad \text{(Eq. 24A}d\text{)}$$

$$K_{BO} = \left(\frac{K'_{BO}}{\beta}\right) \quad \text{(Eq. 24A}e\text{)}$$

$$K_{CD} = {}^1/_2[m_1 + m_2 + m_3 - \beta K'_{CB} - 2K'_{DC} + \sqrt{(m_1 + m_2 + m_3 - \beta K'_{CB} - 2K'_{DC}) - 4\{[m_1 m_2 + m_2 m_3 + m_1 m_3 - K'_{DC} K'_{BO} - \beta K_{CB}(K_{BO} + K_{DC})] - \Delta\}}] \quad \text{(Eq. 24A}f\text{)}$$

$$K_{BC} = K'_{BC} + K'_{CD} - K_{CD} + K'_{BO}\left(1 - \frac{1}{\beta}\right) + K'_{CB}(1 - \beta) \quad \text{(Eq. 24A}g\text{)}$$

Calculating the cumulative amount of drug systemically absorbed, the general equation for Q_{TS} which includes all elimination paths, *i.e.*, K_{BO}, K_{CO}, and $K_{DO} \neq 0$, can be derived as:

$$Q_{TS} = \beta\left[R\frac{d^2 f(I)}{dt^2} + S\frac{df(I)}{dt} + Tf(I) + U\int_0^t f(I)dt\right] \quad \text{(Eq. 25A}a\text{)}$$

where:

$$K_{CB} K_{DC} R = 1 \quad \text{(Eq. 25A}b\text{)}$$

$$K_{CB} K_{DC} S = K_{BO} + K_{BC} + K_{DO} + K_{DC} + K_{CO} + K_{CB} + K_{CD} \quad \text{(Eq. 25A}c\text{)}$$

$$K_{CB} K_{DC} T = K_{DC} K_{CB} + K_{DC} K_{BC} + K_{DC} K_{BO} + K_{BC} K_{CO} + K_{BC} K_{CD} + K_{BO} K_{CO} + K_{BO} K_{CB} + K_{BO} K_{CD} + K_{DO} K_{BC} + K_{DO} K_{BO} + K_{DO} K_{CO} + K_{DO} K_{CB} + K_{DO} K_{CD} + K_{CO} K_{DC} \quad \text{(Eq. 25A}d\text{)}$$

$$K_{CB} K_{DC} U = K_{DC} K_{BC} K_{CO} + K_{DC} K_{BO} K_{CO} + K_{BC} K_{DO} K_{CO} + K_{BO} K_{DO} K_{CO} + K_{BC} K_{DO} K_{CD} + K_{BO} K_{DO} K_{CD} + K_D K_{BO} K_{CB} + K_{BO} K_{DO} K_{CB} \quad \text{(Eq. 25A}e\text{)}$$

These relations can also be written in terms of the equation parameters shown.

$$K_{CB} K_{DC} S = m_1 + m_2 + m_3 \quad \text{(Eq. 26A}a\text{)}$$

$$K_{CB} K_{DC} T = m_1 m_2 + m_2 m_3 + m_1 m_3 \quad \text{(Eq. 26A}b\text{)}$$

$$K_{CB} K_{DC} U = m_1 m_2 m_3 \quad \text{(Eq. 26A}c\text{)}$$

For a case in which any of the K_{iO}'s = 0, the equation for Q_{TS} is a degenerate case of the preceding. The relationships between the m_i's and the coefficients (R, S, T, and U) are not altered. This can be seen from inspection of the equation written in the following form:

$$\frac{Q_{TS}}{D} = \left[\frac{\beta}{K_{CB} K_{DC}}\right]\frac{d^2\left[\frac{f(I)}{D}\right]}{dt^2} + (m_1 + m_2 + m_3)\left[\frac{d\left[\frac{f(I)}{D}\right]}{dt}\right] + (m_1 m_2 + m_2 m_3 + m_1 m_3)\left[\frac{f(I)}{D}\right] + (m_1 m_2 m_3)\left\{\int_0^t\left[\frac{f(I)}{D}\right]dt\right\} \quad \text{(Eq. 27A)}$$

$$\frac{Q_{TS}}{D} = \left[\frac{\beta}{K_{CB} K_{DC}}\right]\vec{\theta} \quad \text{(Eq. 28A}a\text{)}$$

Three of the four cases for the three-compartment model have $K_{DO} = 0$. From these equations,

$$K_{DC} = m_1 + \left[\frac{A_1(m_2 - m_1)(m_3 - m_1)}{K_{CB}}\right] \quad \text{(Eq. 28A}b\text{)}$$

Since

$$K_{CB} = -m_1A_1 - m_2A_2 - m_3A_3 \quad \text{(Eq. 28Ac)}$$

$$= \beta K'_{CB} \quad \text{(Eq. 28Ad)}$$

for these cases,

$$K_{DC} = K'_{DC} \quad \text{(Eq. 28Ae)}$$

Inserting these equalities into Eq. 28A*a*,

$$\frac{Q_{TS}}{D} = \left[\frac{\beta}{\beta K'_{CB}K'_{DC}}\right]\theta \quad \text{(Eq. 29A}a\text{)}$$

$$= \left[\frac{1}{K'_{CB}K'_{DC}}\right]\theta \quad \text{(Eq. 29A}b\text{)}$$

Therefore, β does not enter as a factor into the equation for Q_{TS} when $K_{DO} = 0$. However, referring to Eqs. 18A*a*–18A*g* for Case 1, it is found that $K_{DC} \neq K'_{DC}$, as occurs for Cases 2, 3, and 4; a correction for β is, therefore, required. Appropriate substitutions using the equations for Case 1 into Eqs. 28A*a*–28A*e* and setting Eq. 28A*a* equal to unity at all times for a rapid intravenous dose allow solution for β. The solution given is appropriate for both the cases, $K_{BO} = 0$, and $K_{BO} = K_{CO} = 0$.

$$\beta = \left\{\frac{(K'_{CD} + K'_{CB})\,\theta\,(K^*_{BO} - Q) - K'_{CB}\,g(K^*_{BO} - g)\,(K'_{CD} + K'_{CB}) + K'_{CB}\,\zeta}{K'_{CB}\,\theta\,(K^*_{BO} - g)}\right\} \quad \text{(Eq. 30A}a\text{)}$$

$$\zeta = m_1m_2m_3 - g(m_1m_2 + m_2m_3 + m_1m_3 - gK^*_{BO} - K^*_{BO}K'_{CD} - K^*_{BO}K'_{CO}) \quad \text{(Eq. 30A}b\text{)}$$

The derivation of expressions for Q_{TS} is based on mass balance and requires the adoption of a compartment model. However, it is apparent from the mentioned considerations that the model parameters, K_{iJ}'s, can be replaced by equalities and Q_{TS} can be expressed entirely in terms of equation parameters, *i.e.*, the A_i's and m_i's. The values of the A_i's and m_i's corresponding to a model are generally distorted from their unconstrained least-squares values by the imposed constraint that requires the corresponding K_{iJ}'s to be positive and real in order to be consistent with physically realizable values. The unconstrained least-squares values of the A_i's and m_i's obviously provide the best representation of the experimental results.

Compartment models are not necessarily unique and realistic descriptions of the biokinetic behavior of a system. It is suggested that for the purposes of absorption analysis, it may be justifiable to abandon all pretenses of requiring compartment models to possess physical reality. On this basis an expression for Q_{TS} could be derived to correspond to a generalized compartment model and expressed entirely in terms of unconstrained least-squares values of A_i's and m_i's without concern to whether these values correspond to physically realizable values for the model parameters. Although the model may be completely fictitious for this reason, more realistic values for Q_{TS} might conceivably be obtained. The justification for this approach could only be confirmed experimentally through the comparison of known and computed values of Q_{TS}. The required experimental results were not obtained in the present study.

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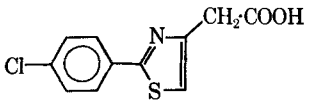
Pharmacokinetics of Fenclozic Acid in Animals and Man

D. S. PLATT

Abstract □ The pharmacokinetic properties of the anti-inflammatory agent, fenclozic acid, have been established in the dog, calf, sheep, and horse after intravenous administration and also in the rat, mouse, guinea pig, monkey, and man after oral dosage. At doses equivalent to those therapeutically effective in the rat, the pharmacokinetics of fenclozic acid are described in all species by the concept of the two-compartment open model. In all species, the compound is restricted mainly within the central compartment, the volume of which is less than 20% of the body weight. Due to serum protein binding, the vascular lymphatic system constitutes a large part of the central compartment volume. The biological half-life of fenclozic acid varies between 3 hr. in the monkey and 118 hr. in the horse; in the guinea pig, rat, dog, and man the half-life is in the range 26–31 hr. There is a direct relationship between the logarithm of the serum concentration and the activity in the adjuvant-induced arthritis test in rats. The pharmacokinetic information obtained has been used in the design of human clinical trials.

Keyphrases □ Fenclozic and ^{14}C-fenclozic acids—pharmacokinetic properties □ Pharmacokinetic parameters—fenclozic acid in animals, humans □ Arthritic subjects—fenclozic acid serum level relation, activity □ UV spectrophotometry—analysis □ TLC—separation, identification

The experimental syndrome of adjuvant-induced arthritis in rats has been used in these laboratories for several years for the detection and evaluation of compounds that may have use in the treatment of rheumatoid arthritis in man (1). Fenclozic acid (I.C.I. 54,450) (Structure I) is one of a series of substituted phenyl-



fenclozic acid [I.C.I. 54,450; 2-(*p*-chlorophenyl)thiazol-4-yl acetic acid]

I

thiazolyl acetic acids synthesized in these laboratories which possesses activity in this screening test (2). The pharmacological properties (3) and the metabolism (4) of this compound have been reported. The results of preliminary studies in man appeared recently (5).

In this paper, the pharmacokinetic properties of fenclozic acid in various animal species including man are discussed. The objectives of these serum concentration studies were: (*a*) to compare the properties of fenclozic acid in the different species; (*b*) to define the relationship between serum concentration and activity in the rat arthritis test; and (*c*) to use the information obtained in the design of human clinical trials.

EXPERIMENTAL

Dosing Procedures—Fenclozic acid was administered orally to mice, rats, guinea pigs, and rhesus monkeys by stomach tube as a ball-milled suspension in an inert dispersing fluid. In dogs, fenclozic acid was administered intravenously as either the sodium or *N*-methylglucamine salt dissolved in isotonic saline; for oral dosing, the drug was formulated into plain, uncoated tablets. For intravenous experiments in a calf, sheep, and horse, fenclozic acid was given as a solution of the *N*-methylglucamine salt in isotonic saline; the same solution was used in an oral dose experiment in the calf.

Fenclozic acid was formulated into plain, uncoated tablets or plain capsules for administration to man. In the radioactive experiment reported in this paper, 100 mg. ^{14}C-labeled fenclozic acid (specific activity 0.227 μc./mg.) was filled into a capsule for oral administration.

Analytical Procedure—Fenclozic acid was extracted from serum and assayed by the following method: To 1.0 ml. serum in a stoppered tube were added 1.0 ml. 1.5 *N* HCl and 5 ml. 2,2,4-trimethylpentane containing 5% v/v amyl alcohol. The mixture was shaken for 5–10 min. and centrifuged at low speed to separate the layers. The concentration of fenclozic acid was determined directly by measuring the absorbance of the organic layer at 300 nm. in a conventional spectrophotometer against a reference blank prepared by substituting water for serum in this procedure.

Fenclozic acid is stable in serum for several weeks at 4°. The overall recovery of drug added in known amounts to serum and extracted as described was 75% at initial concentrations in serum ranging from 1 to 500 mcg./ml. The recovery factor is the same for serum from all species, and replicate analyses gave a coefficient of variation not exceeding 5%. A standard solution of fenclozic acid in the organic solvent (1 mcg./ml.) gives an absorbance at 300 nm. of 0.073 in a 1-cm. cell; the response is linear in the range 1–30 mcg./ml.

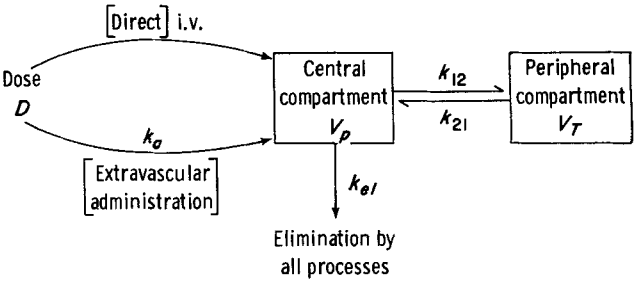
The identity of the extracted material was checked by subjecting aliquots of the organic extract to TLC on fluorescent silica gel plates, using an isopropanol–ammonia–water solvent (20:1:2). Under UV illumination, fenclozic acid and derivatives such as the decarboxylated product or phenyl-ring hydroxylation products quench the background fluorescence; only free fenclozic acid was detected in the organic serum extracts from all species.

Pharmacokinetic Analysis—After intravenous administration, the serum levels of fenclozic acid followed a biexponential decay according to Eq. 1:

$$Cp = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where Cp is the serum concentration at any time (t) after the dose, α and β are first-order rate constants of the serum level curve, and A and B are zero-time intercepts of the two components of the biexponential curve.

This biexponential decay is implicit to the two-compartment open-model shown in Scheme I. The serum level data of fenclozic acid were subjected, therefore, to a two-compartment pharmacokinetic analysis (6–8) to define both the rate constants of distribu-



Scheme I—The two-compartment open model; k_{12}, k_{21}, *and* k_{el} *are first-order rate constants;* V_p *and* V_T *are the volumes of the two compartments; and* k_a *is a first-order absorption rate constant, when drug is administered by extravascular route.*

Table I—Two-Compartment Analysis of Serum Concentration Data in the Dog, Calf, Sheep, and Horse after Single Intravenous Doses

Constant[b]	Units	Dog (n = 9)[a]	Calf (n = 1)	Sheep (n = 2)	Horse (n = 1)
Dose	mg./kg.	13.7 ± 2.0	10.0	10.0	10.0
A	mcg./ml.	42.9 ± 6.9	36.0	46.5	22.2
B	mcg./ml.	55.8 ± 9.0	100.0	87.0	55.1
Cp^0	mcg./ml.	98.7 ± 13.6	136.0	133.5	77.3
α	hr.$^{-1}$	0.619 ± 0.055	1.925	5.732	0.330
β	hr.$^{-1}$	0.0252 ± 0.002	0.0636	0.152	0.0059
k_{12}	hr.$^{-1}$	0.240 ± 0.022	0.471	1.818	0.091
k_{21}	hr.$^{-1}$	0.361 ± 0.044	1.432	3.832	0.237
k_{el}	hr.$^{-1}$	0.0432 ± 0.004	0.0855	0.235	0.0082
V_p	%[c]	13.9 ± 0.9	7.35	7.60	12.9
V_T	%[c]	9.2 ± 1.1	2.40	3.55	5.0
Vd_{ss}	%[c]	23.1 ± 1.8	9.75	11.15	17.9
$Vd_{ext.}$	%[c]	24.6 ± 2.1	10.00	11.50	18.1
Total area	mcg. hr./ml.	2290 ± 323	1590	600	9400
Half-lives					
$0.693/\beta$	hr.	28 ± 2.5	11	4.5	118
$0.693/k_{el}$	hr.	16 ± 1.2	8	3	84

[a] Values expressed as mean ± *SE*. [b] See *Experimental* section for definition of constants. [c] Volumes expressed as a percentage of total body weight.

tion and elimination and the volumes of distribution *in vivo*. The pharmacokinetic constants, A, B, C_p^0, α, β, k_{12}, k_{21}, k_{el}, V_p, V_T, Vd_{ss}, and $Vd_{ext.}$ have the same meaning as given by Riegelman *et al.* (6, 8). The material balance equations of Riegelman *et al.* (6) were used to derive the fractions of the dose in each compartment at various times after the intravenous dose.

The technique given by Loo and Riegelman (9) was used to calculate the extent of absorption of fenclozic acid into a two-compartment system after oral administration and, where possible, a first-order rate constant for absorption (k_a) was derived. In those cases where intravenously derived constants were not available, serum concentrations were fitted with the aid of a digital computer (GEIS time-shared system), programmed to solve the equations of the two-compartment system with a first-order rate of absorption into the central compartment (10). (Neither iterative digital procedures nor analog facilities were available; the data generated are, therefore, only approximate.)

It was shown (11, 12) that in certain circumstances the kinetics of the two-compartment system can be simplified to those of the one-compartment system when it is required to predict, using single-dose data, the blood levels after multiple oral or intravenous doses of a drug. In general, this simplified technique can be employed when the area (A/α), associated with distribution, is small relative to the total area under the curve. It is a necessary assump-

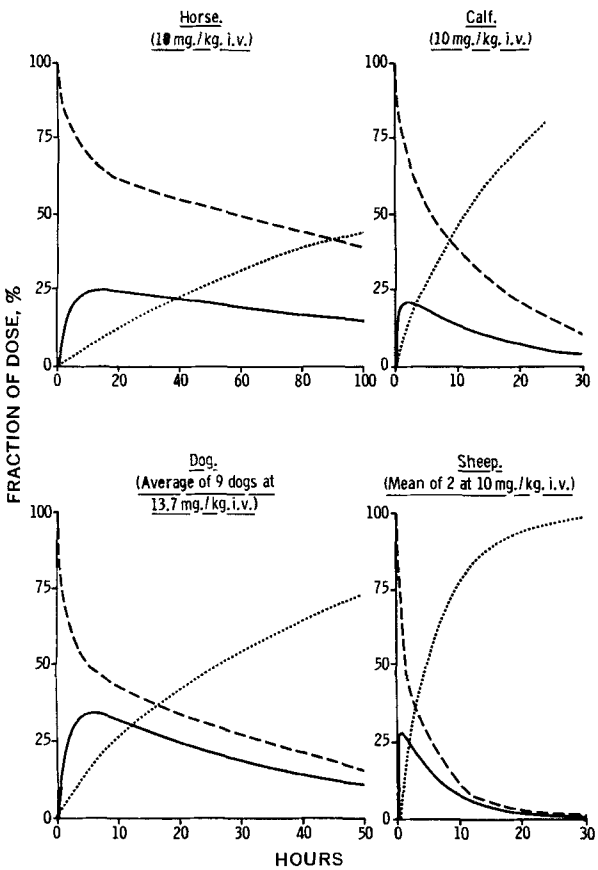


Figure 1—*Distribution and elimination of fenclozic acid in the dog, horse, calf, and sheep following intravenous administration. Curves are generated from the pharmacokinetic constants given in Table I. Key: ..., eliminated; ---, central compartment; and ——, peripheral compartment.*

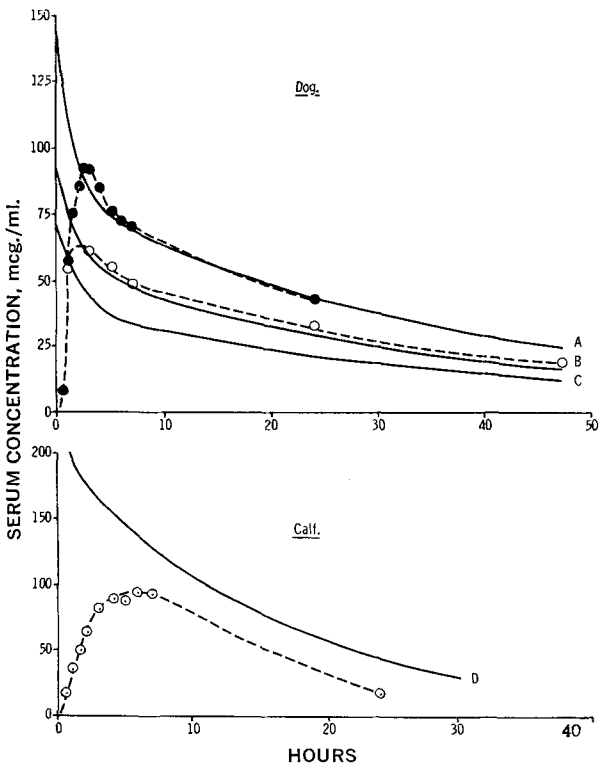


Figure 2—*Comparison of serum concentrations in dogs and a calf after oral or intravenous administration of fenclozic acid. Solid curves are generated from biexponential equations fitting intravenous data. Broken curves are observed concentrations after oral administration. Key: ●---●, dog, 20 mg./kg.* p.o.; *O---O, dog, 10 mg./kg.* p.o.; *and ⊙---⊙, calf, 20 mg./kg.* p.o. *Curve A = dog, 20 mg./kg. i.v. from equation* $Cp = 62.6e^{-0.619t} + 81.4e^{-0.0252t}$. *Curve B = dog, 13.7 mg./kg. i.v. from equation* $Cp = 42.9e^{-0.619t} + 55.8e^{-0.0252t}$. *Curve C = dog, 10 mg./kg. i.v. from equation* $Cp = 31.3e^{-0.619t} + 40.7e^{-0.0252t}$. *Curve D = calf, 20 mg./kg. i.v. from equation* $Cp = 72e^{-1.925t} + 200e^{-0.0636t}$.

Table II—Serum Concentrations of Fenclozic Acid after Single Oral Doses

Hours after Dose	Serum Concentration, mcg./ml.[a] Dog 10-mg./kg. Dose	Dog 20-mg./kg. Dose	Calf, 20-mg./kg. Dose	Rat, 10-mg./kg. Dose	Guinea Pig, 10-mg./kg. Dose	Rhesus Monkey, 10-mg./kg. Dose[b]	Mouse, 10-mg./kg. Dose[c]
0.5		8.4(2)	17.9				
1	54.0 ± 3.4(12)	57.1(2)	36.0	41.5(2)	51.4(2)	53.8 ± 4.3(6)	38.5
1.5		75.4(2)	49.6				
2		85.4(2)	64.0				
2.5		92.3(2)					
3	61.1 ± 1.4(12)	92.4(2)	81.1	53.0(2)	56.4 ± 6.4(4)	37.3 ± 1.9(6)	45.0
4		85.0(2)	89.3				
5	55.3 ± 1.9(12)	76.2(2)	87.5	61.8 ± 1.3(4)	55.7(2)	20.2 ± 2.7(6)	40.0
6		73.2(2)	93.7	56.6 ± 2.0(5)			
7	49.0 ± 1.8(12)	71.0(2)	93.0	66.5(2)	57.8 ± 4.6(4)	13.8 ± 1.2(6)	41.0
24	32.7 ± 1.4(12)	43.0(2)	17.6	37.0 ± 1.5(7)	44.9 ± 3.2(4)		11.5
30					34.4(2)		
48	19.2 ± 1.3(12)		0	20.8 ± 1.9(4)	17.0(2)		
72	14.4 ± 1.3(12)			10.0(1)	10.9(2)		
96				7.5(2)			

[a] When more than two results, mean expressed ±*SE*, with the number of animals in parentheses. [b] See text. [c] Each result from assay of pooled serum sample from five identically treated mice.

tion that each oral dose is completely absorbed. The pharmacokinetic properties of fenclozic acid are in this category. Since serum levels should achieve a steady-state level after multiple administration of a fixed dose at fixed time intervals, the average plateau serum levels after multiple doses to rats, guinea pigs, and dogs were calculated using Eq. 2 (13):

$$C_{av.} = \frac{1.44 \cdot D \cdot t_{1/2}}{Vd_{ext.} \cdot \Delta t} \qquad \text{(Eq. 2)}$$

where $C_{av.}$ is the average plateau serum level over the time interval between doses (Δt), D is dose, $t_{1/2}$ is the biological half-life (0.693/β), and $Vd_{ext.}$ is the biased estimate of the true volume of distribution (8).

When a compound is given by an extravascular route and the terminal, linear segment of the semilogarithmic plot of serum concentration is extrapolated back to zero time, the result obtained by dividing the intercept value into the dose is usually an underestimate of $Vd_{ext.}$ (=dose/V for an intravenous dose) and is a complex of several variables (14). If, however, evidence from a comparison with intravenous data indicates that absorption is both rapid and complete and if α is large relative to β, then the ratio of dose to extrapolated zero-time intercept will give a reasonable estimate of $Vd_{ext.}$. It will be shown later that this approximation can be used at low extravascular doses of fenclozic acid.

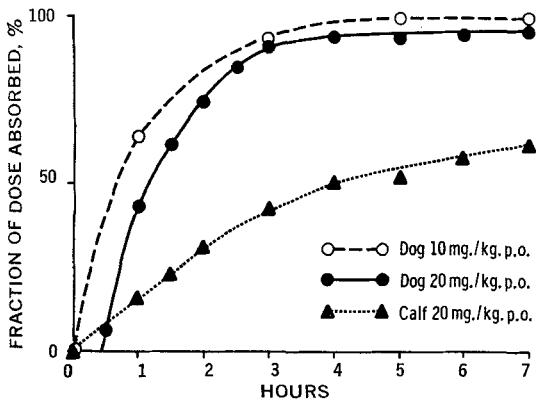


Figure 3—*Extent of absorption of fenclozic acid after oral administration to dogs and a calf. Constants used in the calculations were:* (a) *Dog (10 mg./kg.); total dose, 120 mg.;* k_{12}, *0.240;* k_{21}, *0.361;* k_{el}, *0.0432;* V_p, *1.235 l.** (b) *Dog (20 mg./kg.); total dose, 240 mg.;* k_{12}, *0.240;* k_{21}, *0.361;* k_{el}, *0.0432;* V_p, *1.67 l.** (c) *Calf (20 mg./kg.); total dose, 3320 mg.;* k_{12}, *0.471;* k_{21}, *1.432;* k_{el}, *0.0855;* V_p, *12.2 l. (The* k_{12}, k_{21}, *and* k_{el} *are in hr.*$^{-1}$ *units.)*

**The* V_p *value at the 10-mg./kg. dose level is that calculated from oral dose serum concentration observations (see text). The* V_p *value at the 20-mg./kg. dose level is that observed after intravenous administration (Table I).*

RESULTS

Intravenous Administration—Serum concentrations of fenclozic acid were measured in nine male dogs, one calf, two sheep, and a horse after single, rapid, intravenous injections. The dose ranged from 4 to 20 mg./kg. body weight in dogs; in the remaining species, the dose level was 10 mg./kg.

In every case, the serum levels exhibited a biexponential decay with time, consistent with the kinetics of the two-compartment open model discussed in the *Experimental* section. Biexponential equations to fit the data were derived graphically, using the technique of residuals. The rate constants of distribution and elimination and the volumes of distribution were calculated from these equations; the results are shown in Table I.

The results from the nine dogs were combined since there was a virtually linear relationship between dose and both zero-time concentration (Cp°) and the total area under the serum level–time curve in the dose range tested; a dose of 1 mg./kg. gave a mean Cp° value of 7.2 mcg./ml. and a total area of 167 mcg. hr./ml. The biological half-life (0.693/β), the half-life of elimination (0.693/k_{el}), and the steady-state volume of distribution (Vd_{ss}) were not affected by increasing the dose from 4 to 20 mg./kg. The biological half-life varied between 22 and 41 hr. in the individual dogs (average 28 hr.), whereas the half-life of elimination was in the range of 10–22 hr. (average 16 hr.). The steady-state Vd (Vd_{ss}) varied between 14.5 and 31.3% of body weight (average 23.2%); the biased estimate of total Vd ($Vd_{ext.}$) was on the average only 6% higher than the average Vd_{ss} result.

In the calf, sheep, and horse, as in the dog, the Vd_{ss} of fenclozic acid was low (9.75, 11.15, and 17.9% body weight, respectively); $Vd_{ext.}$ was only marginally higher than Vd_{ss} in all three species. There was an appreciable species variation in biological half-life (11, 4.5, and 118 hr., respectively).

Equilibrium dialysis studies showed that fenclozic acid is extensively bound to serum albumin in all species including man. The low values of Vd_{ss} (Table I) are consistent with a preferential retention of fenclozic acid within the central compartment, which is due, at least in part, to serum protein binding.

The amounts of fenclozic acid in the two body compartments and the amount eliminated from the body by all processes of metabolism and excretion at various times after the dose were calculated for all four species, using the constants given in Table I; the results are shown in Fig. 1. Despite time-scale differences, the pattern of distribution was similar in all four species, the central compartment predominating over the peripheral compartment at all times. The vascular lymphatic system constitutes a large part of the volume of the central compartment.

Oral Administration to Animals—Single oral doses of fenclozic acid were administered to dogs, a calf, mice, rats, guinea pigs, and rhesus monkeys. Twelve dogs (six males and six females) received

Table III—Serum Concentrations of Fenclozic Acid in Rats, Guinea Pigs, and Dogs after Multiple Oral Doses; Comparison of Observed and Calculated Values

Dose, mg./kg.	Serum Concentration (mcg./ml.) ±SE (n)[a]					
	Rat[b]		Guinea Pig[c]		Dog[d]	
	Observed	Calculated[e]	Observed	Calculated[e]	Observed	Calculated[e]
2.5	34 ± 5(3)	29	29 ± 2(3)	29	28 ± 3(4)	22
5	48 ± 1(3)	58	60 ± 3(3)	59		45
10	108 ± 4(10)	116	125 ± 10(3)	118	79 ± 7(8)	90
20	160 ± 14(3)	232	150 ± 4(3)	236	141 (2)	180
40	230 (2)	464	283 (1)	472	213 ± 24(3)	360
50	266 ± 11(15)	580			213 ± 16(6)	450
60					252 ± 34(4)	540
70					288 ± 20(7)	630
75	312 (2)	870				675
80	352 ± 9(4)	928	460 (2)	944	307 ± 19(7)	720
100	378 ± 23(3)	1160				

[a] (n) = number of individual animals tested. [b] Where $t_{1/2}$ = 29 hr., Δt = 24 hr., and $Vd_{ext.}$ = 15.2% body weight (body weight = 200 g.). [c] Where $t_{1/2}$ = 26 hr., Δt = 24 hr., and $Vd_{ext.}$ = 13.2% body weight (body weight = 400 g.). [d] Where $t_{1/2}$ = 30 hr., Δt = 24 hr., and $Vd_{ext.}$ = 20% body weight (body weight = 10 kg.). [e] Calculated using Eq. 2.

a single dose of 10 mg./kg., and two other dogs (one of each sex) had a dose of 20 mg./kg. No sex difference in serum concentrations of fenclozic acid was detected, and the mean levels are shown in Table II. Serum levels were measured in a calf (the same animal was used in the intravenous study) after a dose of 20 mg./kg. (Table II). Male rats, mice, and guinea pigs received oral doses of 10 mg./kg., with the results shown in Table II. Two female rhesus monkeys were examined on three separate occasions; in two experiments the animals received a dose of 5 mg./kg. and in the third experiment, 25 mg./kg. The serum concentrations in these monkeys were linearly related to dose; to simplify comparison with the other species, the observed levels were adjusted to a dose of 10 mg./kg. and the resultant concentrations combined to give the mean results shown in Table II.

Dogs—Experiments involving either intravenous or oral administration of fenclozic acid to dogs were carried out in different groups. Since a linear relationship between dose and serum level was observed in dogs after single intravenous doses of from 4 to 20 mg./kg., it was possible by manipulation of the mean zero-time intercepts *A* and *B* (Table I) to generate intravenous serum level–time curves for doses of 10 and 20 mg./kg. i.v. for comparison with the serum levels observed after equivalent oral doses. At a dose of 20 mg./kg., there was good agreement between generated intravenous data and observed oral data (Fig. 2); but at 10-mg./kg. doses, the oral results were in excess of, but not significantly different from, intravenous levels. In the latter instance, the oral data were virtually identical to the intravenous data for a mean dose of 13.7 mg./kg. Extrapolation of the terminal, linear segment of the semilogarithmic serum concentration plot (oral dose of 10 mg./kg.) to zero time gave an intercept of 55 mcg./ml., indicating a $Vd_{ext.}$ value not in excess of 18% body weight; the approximate mean biological half-life was 30 hr. (range 19–41 hr.). After intravenous administration, the mean $Vd_{ext.}$ was 24.6% body weight and the mean biological half-life was 28 hr. with a similar scatter to that observed after oral dosing. Thus, the oral dose serum levels gave an estimate of $Vd_{ext.}$ lower than that after intravenous doses.

The data in Fig. 2 indicated that absorption of the oral dose was complete in dogs; this was confirmed using the technique of Loo and Riegelman (9) as shown in Fig. 3. Absorption was essentially complete at 5 hr. after either dose level, although there was a delay of about 24 min. at the 20-mg./kg. dose; k_a values of 0.91 and 1.02 hr.$^{-1}$ were calculated, giving absorption half-lives of 0.76 and 0.68 hr. for the 10- and 20-mg./kg. doses, respectively. The calculations were checked by generating serum concentration curves with a digital computer (*Experimental* section), confirming that fenclozic acid was absorbed from dog gut by first-order kinetics into a two-compartment system.

Calf—From the intravenous constants given in Table I, and assuming linearity in the dose range 10–20 mg./kg., a serum level curve for a dose of 20 mg./kg. i.v. was calculated and compared with the oral data at the same dose (Fig. 2). Incomplete absorption from the gut was indicated. The overall extent of absorption, calculated by the technique of Loo and Riegelman (9), was about 70% (Fig. 3), with a first-order rate constant (k_a) of 0.295 hr.$^{-1}$ (absorption half-life, 2.4 hr.). The time required for maximal absorption was in excess of 7 hr., possibly due to the different anatomy of the ruminant alimentary tract.

Rats—The oral dose rat data given in Table II were fitted approximately by a digital computer programmed to solve the equation for first-order absorption into a two-compartment system (10). The following constants were generated: k_{12}, 0.154 hr.$^{-1}$; k_{21}, 0.437 hr.$^{-1}$; k_{el}, 0.033 hr.$^{-1}$; k_a, 0.5 hr.$^{-1}$ (half-life of absorption, 1.4 hr.); Vd_{ss}, 13.5% body weight; V_p, 11% body weight; α, 0.6 hr.$^{-1}$; β, 0.024 hr.$^{-1}$ (biological half-life, 29 hr.); and *B*, 66 mcg./ml. If, however, the terminal, linear segment of a simple semilogarithmic plot of the serum concentrations was extrapolated to zero time, an approximate intercept (*B*) value of 66 mcg./ml. was obtained, giving an approximate $Vd_{ext.}$ value of 15.2% body weight, with a biological half-life of 29 hr., *i.e.*, identical to the computer-generated values. In rats, therefore, reasonable estimates of *B*, β, and $Vd_{ext.}$ were obtained by the simple extrapolation technique. Absorption was complete at 7 hr. after the dose.

Guinea Pigs and Monkeys—Extrapolation of the guinea pig and monkey semilogarithmic serum concentration–time plots to zero time gave intercepts of 76 and 66 mcg./ml., indicating $Vd_{ext.}$ values of the order of 13.2 and 15.2% body weight, respectively. These approximations are in close agreement with the values observed in all other species at equivalent doses. The estimated biological

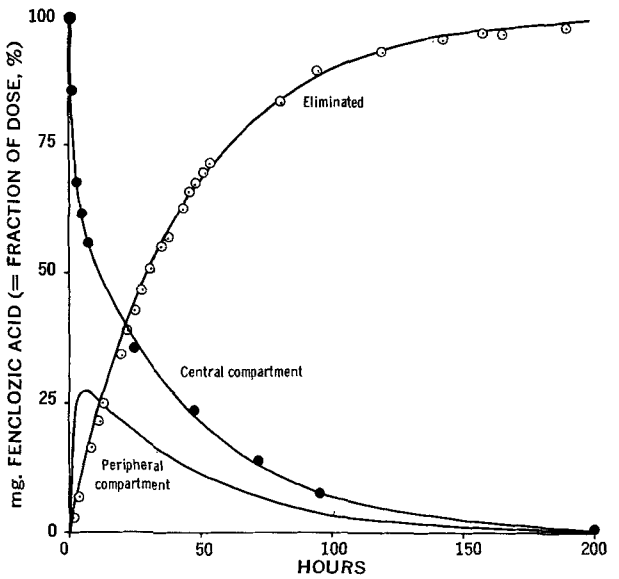


Figure 4—*Distribution and elimination of fenclozic acid in man after an oral dose of 1.43 mg./kg. Solid lines are data generated from the intravenous equation* $Cp = 6e^{-0.49t} + 11e^{-0.0224t}$, *since the observed absorption rate constant* (k_a = 7 hr.$^{-1}$) *was greatly in excess of* α *and* β. *Key:* ⊙, *observed total urinary excretion; and* ●, *observed serum concentration data expressed as a percentage of the zero-time concentration* (Cp° = 17 mcg./ml.).

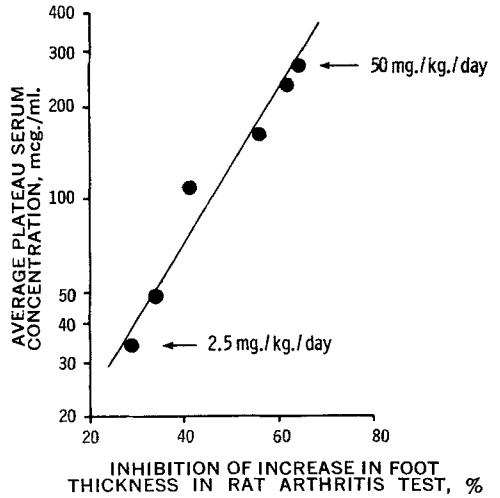


Figure 5—*Relationship of activity on Day 14 in the adjuvant-induced arthritis test in rats and serum concentration.*

half-lives were 26 and 3 hr. for guinea pigs and monkeys, respectively. By analogy with dog data, absorption of the oral dose was probably complete in both species, although the rate appeared to be faster in monkeys and somewhat slower in guinea pigs, the latter being very similar to rats.

Mice—At 10 mg./kg., the observed serum levels were lower than in dogs, rats, and guinea pigs. Accurate biological half-life estimation was not possible from the available data, but the results indicated a value not in excess of 11 hr. and a $Vd_{ext.}$ of not greater than 20% body weight. The shape of the serum level curve indicated a combination of slow absorption with a relatively rapid elimination, rather than incomplete absorption from the gut.

Oral Administration to Man—Serum concentrations of fenclozic acid were determined in human volunteers after single oral doses (in tablet or capsule form) in the range 1.5–6 mg./kg. The results of these studies were reported (5). Extrapolation of semilogarithmic serum level plots to zero time gave an average biological half-life of 25 hr., with an approximate $Vd_{ext.}$ value of 10.6% body weight; *i.e.*, the pharmacokinetic properties in man were similar to those in dogs, guinea pigs, and rats in respect to half-life and similar to all other species in respect to volume distribution.

A patient with rheumatoid arthritis weighing 70 kg. was given a single oral dose of 100 mg. ^{14}C-labeled fenclozic acid. Urine samples were collected for up to 190 hr. after the dose for determination of total radioactivity; 97% of the dosed radioactivity was recovered in the urine. Serum levels of fenclozic acid were determined up to 96 hr. The maximum serum level was observed at 1 hr. after the dose; thereafter the data had the appearance of a typical intravenous dosed serum level curve. A k_a value of about 7 hr.$^{-1}$ was required to allow a fit of the serum level data to be made, with $\alpha = 0.49$ hr.$^{-1}$ and $\beta = 0.0224$ hr.$^{-1}$; *i.e.*, k_a was at least 14-fold greater than α and 300-fold greater than β. [For the other human data (4), k_a values in excess of 2 hr.$^{-1}$ were required to give an approximate fit of the data.] The absorption phase, therefore, in this particular case can be effectively ignored and the data analyzed according to two-compartment intravenous kinetics (6–8).

The serum level data were fitted by the equation: $Cp = 6e^{-0.49t} + 11e^{-0.0224t}$, which on further analysis yielded the following constants: k_{12}, 0.154 hr.$^{-1}$; k_{21}, 0.325 hr.$^{-1}$; k_{el}, 0.0338 hr.$^{-1}$; Vd_{ss}, 12.4% body weight; and $Vd_{ext.}$, 13% body weight. The biological half-life ($0.693/\beta$) was 31 hr.

The amounts of fenclozic acid at various times after the dose in the central and peripheral compartments and also the amounts eliminated were calculated (6); the results are shown in Fig. 4. There was good agreement between observed urinary excretion data and elimination data calculated from the observed serum concentrations, confirming the validity of the two-compartment model in quantitating the fate of fenclozic acid in man and showing that the rate of elimination of human metabolites is dependent upon the rate of formation. The pattern of distribution and elimination in man, as shown in Fig. 4, was similar to that found in other species (Fig. 1).

The observed urinary excretion of total radioactivity confirmed that the oral dose of 100 mg. was completely absorbed, and a similar conclusion was reached following analysis of the serum level data using the technique of Loo and Riegelman (9).

Accumulation in Rat, Guinea Pig, and Dog Serum after Multiple Oral Doses—Serum concentrations of fenclozic acid were measured after 14 oral doses to rats, guinea pigs, and dogs; the results are shown in Table III. These results are compared with the average plateau concentrations, calculated using observed pharmacokinetic properties in Eq. 2. In all three species, there was good agreement between observed and calculated values in the dose range 2.5–10 mg./kg.; but at higher doses the observed values became progressively less than the calculated values. The reason for this discrepancy is not known but may be due in part to incomplete absorption. This factor may be of particular relevance at high doses of fenclozic acid, where ulceration of the gut is a common toxicological finding.

Relationship of Serum Concentrations in Rat to Activity in Adjuvant-Induced Arthritis Test—In the adjuvant-induced arthritis test in rats, compounds are given orally once daily for 14 days, starting on the day before injection of the adjuvant into the footpad. Activity is assessed by measuring the percentage inhibition of the increase in foot thickness of the injected foot on the 13th day following injection of the adjuvant (3).

Gibaldi (16) discussed the relationship between the magnitude of a pharmacological response and the amount of drug in the body and indicated the importance of establishing in pharmacodynamic studies the relationship between therapeutic response and body levels of drug, or some measure of the latter, such as blood concentrations. This relationship tends to be more meaningful than the dose–response relationship since the latter is influenced by changes in the mechanisms of absorption, distribution, and elimination of the drug in question.

The average observed plateau serum concentrations in rats after 14 days of dosing were compared with the observed activities in the rat arthritis test, expressed as percentage inhibitions of the increase in foot thickness (Fig. 5). There was a direct relationship between the logarithm of the serum concentration and therapeutic effectiveness, 50% inhibition occurring at a level of 120 mcg./ml. and 30% at a level of 40 mcg./ml.

DISCUSSION

Serum levels of fenclozic acid followed a biexponential decay in all species given an intravenous dose (dog, calf, sheep, and horse); a similar pattern of distribution and elimination was indicated in rats, guinea pigs, mice, monkeys, and man after oral administration. Application of the kinetic principles of the two-compartment open model (6–8) allowed further analysis of the data. In all species, the volume of the central compartment was small, being generally less than 20% of the animal body weight. At all times after the dose, the amount of fenclozic acid in this central compartment was in excess of that in the peripheral compartment; the vascular lymphatic system constitutes a large fraction of the central compartment since fenclozic acid is bound to serum albumin. The biological half-life varied over a wide range in the species tested, from 3 hr. in the rhesus monkey to 118 hr. in the horse; the dog, rat, guinea pig, and man exhibited similar half-lives (about 30 hr.), although there is considerable interspecies variation in the metabolism of fenclozic acid (4); glutamine conjugation is the major pathway in man (17).

First-order absorption kinetics were indicated in the dog, calf, rat, and man after oral administration either in tablet form, as a salt solution, or as a suspension. In each case the absorption rate constant (k_a) was considerably greater than the elimination rate constant (k_{el}). The most rapid absorption occurred in man (half-life 6–20 min.), followed by dog (about 40 min.), rat (about 80 min.), and calf (140 min.). The oral dose data in guinea pigs suggested a rate similar to that in rats; in monkeys, the rate was probably similar to that in man.

The relationship between distribution and elimination was such that serum levels of fenclozic acid after multiple doses could be adequately predicted using the simplified concept of the one-compartment model. There was good agreement between observed serum levels after multiple doses in rats, dogs, and guinea pigs and levels calculated from the single-dose data, but this was only true

at the lower doses tested; at higher doses, the observed levels were less than expected.

A direct relationship was observed between the logarithm of serum concentration and therapeutic effect in the adjuvant-induced arthritis test in rats. Significant activity in this test (30–50% inhibition) was associated with plateau serum levels in the range 40–120 mcg./ml., given by daily doses of about 5–10 mg./kg. Preliminary volunteer and clinical trials in man were designed on the basis of these observations in the belief that it is more rational to transpose from one animal species to another (in this case, rat to man) on the basis of comparative serum level data (18). Volunteer and patient studies established that doses of 100–200 mg. administered every 12 hr. gave rise to serum levels of fenclozic acid in the approximate range 50–100 mcg./ml. (5). Clinical effectiveness of fenclozic acid (100–200 mg. b.i.d.) was assessed in patients with rheumatoid arthritis in a double-blind crossover trial against aspirin (900 mg. q.i.d.), the results of which (19) lent support to this approach to clinical trial design. The results also allowed evaluation of the rat screening test in terms of human effectiveness.

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S. G. SCHULMAN

Abstract □ Dissociation constants of four isomeric aminoquinolines were determined spectrophotometrically and are compared with the same constants determined potentiometrically. The shifts in electronic absorption spectra of the compounds studied, upon protonation, are employed to assign the positions of protonation in the aminoquinolines. It was found that, in neutral and dilute acid solutions, a proton is added to the nitrogen atom in the heterocyclic rings in all compounds studied. Addition of a second proton, to the amino nitrogen, occurs only in strong acid solutions. 2-Aminoquinoline appears to exist in solution predominantly as an imino tautomer, while the other aminoquinolines studied exist predominantly in amino forms.

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The states of ionization of pharmacologically active substances and of biologically significant molecules are extremely important in determining the action of drugs. If these molecules have more than one group capable of dissociating or accepting protons in solutions, the sites of ionization may be equally important. Consequently, accurate knowledge of the sites of ionization corresponding to pKa values of drug molecules is vital to the study of chemical structure–biological activity relationships. Of several methods available for the determination of pKa values, potentiometry and spectrophotometry enjoy, by far, the greatest popularity. Potentiometry permits rapid and simple pKa determination but, in the case of polyacidic or polybasic molecules, does not permit simple evaluation of the site of dissociation or protonation unless the functional groups have widely differing characteristic pKa values in monofunctional molecules. In the latter case, the site of protonation can usually be inferred from the pH of the buffer regions of the titration curve. Unequivocal assignment of protonation sites, potentiometrically, can only be made by making derivatives of the molecule of interest in which all acidic or basic sites but one are blocked so that the ionizations can be evaluated one at a time. This is a time-consuming and frequently frustrating experience.

at the lower doses tested; at higher doses, the observed levels were less than expected.

A direct relationship was observed between the logarithm of serum concentration and therapeutic effect in the adjuvant-induced arthritis test in rats. Significant activity in this test (30–50% inhibition) was associated with plateau serum levels in the range 40–120 mcg./ml., given by daily doses of about 5–10 mg./kg. Preliminary volunteer and clinical trials in man were designed on the basis of these observations in the belief that it is more rational to transpose from one animal species to another (in this case, rat to man) on the basis of comparative serum level data (18). Volunteer and patient studies established that doses of 100–200 mg. administered every 12 hr. gave rise to serum levels of fenclozic acid in the approximate range 50–100 mcg./ml. (5). Clinical effectiveness of fenclozic acid (100–200 mg. b.i.d.) was assessed in patients with rheumatoid arthritis in a double-blind crossover trial against aspirin (900 mg. q.i.d.), the results of which (19) lent support to this approach to clinical trial design. The results also allowed evaluation of the rat screening test in terms of human effectiveness.

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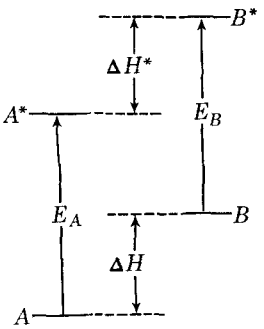
Conventional spectrophotometric pKa determination generally suffers from the same problems as potentiometry in assigning sites of protonation. This is due to the fact that, in spectrophotometry, changes in band intensities are used to evaluate base–acid ratios which, in conjunction with pH measurements, yield pKa values (1). Little attention, however, is paid to the shifts in the positions of the band maxima which occur upon protonation.

Shifts in the long wavelengths band maxima of acidic or basic molecules, upon protonation or dissociation, can be related to the difference between the dissociation constant of the molecule in the ground electronic state (pKa) and in the lowest excited singlet state (pKa*), the latter state being the one to which the transition represented by the long wavelength absorption band has occurred. The approximate relationship between excited state dissociation constants and spectral band shifts was developed by Förster (2) on the basis of a thermodynamic cycle and will be discussed briefly here (Scheme I).



Scheme I—The Förster cycle

There are two alternative pathways to reach the excited state of a base starting with the ground state of the conjugate acid. The first pathway is dissociation of the ground state acid (A) to the ground state conjugate base (B). This process is attended by an energy change, ΔH, the enthalpy of dissociation in the ground state. B then absorbs light energy E_B to arrive in the excited state B^*. The total energy absorbed in the process is thus $\Delta H + E_B$. Alternatively, A can first absorb light of energy E_A to arrive in its excited state A^*, followed by dissociation in the excited state with enthalpy change ΔH^*. The total energy absorbed in this process is $\Delta H^* + E_A$. Since this represents a closed thermodynamic cycle, Eq. 1 results:

$$\Delta H + E_B = \Delta H^* + E_A \qquad \text{(Eq. 1)}$$

Now, E_A and E_B are related to the wavelengths of absorption by $E_A = Nh(c/\lambda_A)$ and $E_B = Nh(c/\lambda_B)$, where N is the Avogadro number, h is Planck's constant, c is the speed of light, and λ_A and λ_B are, respectively, the wavelengths of absorption of A and B. If it is assumed that the vibrational structures of A, A^*, B, and B^* are similar, λ_A and λ_B may be taken at the maxima of the acid and conjugate-base long wavelength absorption bands, respectively. By substituting these relationships into Eq. 1 and rearranging,

$$\Delta H - \Delta H^* = Nhc\left(\frac{1}{\lambda_A} - \frac{1}{\lambda_B}\right) \qquad \text{(Eq. 2)}$$

From the second law of thermodynamics,

$$\Delta H = \Delta G + T\Delta S = 2.3\,RT\,\text{pKa} + T\Delta S \qquad \text{(Eq. 3)}$$

in which ΔG and ΔS are, respectively, the Gibbs free energy and entropy of dissociation in the ground state, and T is the absolute temperature, and

$$\Delta H^* = \Delta G^* + T\Delta S^* = 2.3\,RT\,\text{pKa}^* + T\Delta S^* \qquad \text{(Eq. 4)}$$

where ΔG^*, ΔS^*, and pKa* are, respectively, the Gibbs free energy, the entropy of dissociation, and the equilibrium constant for the corresponding dissociation in the excited state.

Substitution of Eqs. 3 and 4 into 2 yields:

$$\text{pKa} - \text{pKa}^* + \frac{T(\Delta S - \Delta S^*)}{2.3\,RT} = \frac{Nhc}{2.3\,RT}\left(\frac{1}{\lambda_A} - \frac{1}{\lambda_B}\right) \qquad \text{(Eq. 5)}$$

If it is now assumed that the entropies of protonation in ground and excited states are equal (a reasonable assumption, since the entropies of protonation in the respective electronic states are predominantly configurational and result in very similar energy differences),

$$\text{pKa} - \text{pKa}^* = \frac{0.625}{T}\left(\frac{1}{\lambda_A} - \frac{1}{\lambda_B}\right) \qquad \text{(Eq. 6)}$$

or, at 25°,

$$\text{pKa} - \text{pKa}^* = 2.10 \times 10^{-3}\left(\frac{1}{\lambda_A} - \frac{1}{\lambda_B}\right) \qquad \text{(Eq. 7)}$$

Equation 7 shows that by knowing the ground state pKa value for a particular dissociation equilibrium and the long wavelength absorption maxima of the acid and conjugate base, the lowest excited singlet state pKa* value for the corresponding equilibrium can be calculated. Since the process of electronic absorption is always accompanied by a change in electric dipole moment of the absorbing species, pKa* is almost always different from pKa. In general, it has been found that aromatic molecules containing —OH, —NH_2, or —SH groups become more acidic in the lowest excited singlet state (3); *i.e.*, pK* < pK. On the other hand, aromatic molecules containing —C(=O)—OH, —C(=O)—R, —(=)N (heteroaromatic nitrogen) groups become more basic in the excited state (pK* > pK). Now, if pKa* < pKa, then $1/\lambda_a > 1/\lambda_b$ and $\lambda_a < \lambda_b$. In other words, protonation of —OH, —NH_2, or —SH to form —OH_2^+, —NH_3^+, or —SH_2^+ always shifts the long wavelength absorption band to shorter wavelengths. Dissociation of the former groups to —O^-, —NH^-, or —S^- shifts the long wavelength absorption maxima to longer wavelengths (this is the reason for the yellow color of phenols in basic solutions while they are colorless in acidic solutions). Similarly, if pKa* > pKa, then $1/\lambda_a < 1/\lambda_b$ and $\lambda_a > \lambda_b$; so protonating —C(=O)—OH, —C(=O)—R, and —(=)N to form —C(=OH^+)—OH, —C(=OH^+)—R, and —(=)N^+—H shifts the long wavelength absorption band to longer wavelengths, while dissociation to yield —C(=O)—O^- shifts the long wavelength band to shorter wavelengths.

In molecules containing two functional groups, where both groups are of the same type (both with pKa* < pKa or both with pKa* > pKa), there has been insufficient study, to date, to determine the overall effect of one group upon the other. However, if one

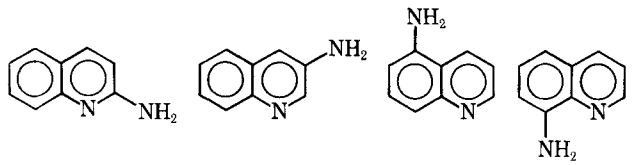
Table I—Long Wavelength Absorption Maxima of the Aminoquinolines

Aminoquinoline	$H_0 = -7.0$ λ, nm.	$H_0 = -7.0$ ε	pH = 1.93 λ, nm.	pH = 1.93 ε	pH = 12.00 λ, nm.	pH = 12.00 ε
2-Aminoquinoline	327	12,500	327	6760	328	5100
3-Aminoquinoline	317	10,600	372	4200	340	4000
5-Aminoquinoline	313	15,500	415	2680	333	3900
8-Aminoquinoline	312	13,900	382	2000	332	4100

Table II—pKa Values of the Aminoquinolines

Aminoquinoline	pKa_1	pKa_2
2-Aminoquinoline	—	7.27 ± 0.08
3-Aminoquinoline	−0.57 ± 0.12	4.96 ± 0.09
5-Aminoquinoline	+0.49 ± 0.08	5.63 ± 0.08
8-Aminoquinoline	−0.52 ± 0.09	3.92 ± 0.04

group has pKa* > pKa and the other has pKa* < pKa, it is almost always found that the values of pKa − pKa* for either group is greater, in absolute magnitude, than for either group alone in a monofunctional molecule. In the present study, the pKa values of four isomeric aminoquinolines, the 2-, 3-, 5-, and 8-isomers (the 8-isomer is a precursor of pamaquine), were determined spectrophotometrically. This is an excellent group of molecules with which to illustrate the foregoing discussion. These compounds each contain an aromatic amino group and a heterocyclic nitrogen atom. Both of these basic functions have similar pKa values in monofunctional molecules (*e.g.*, for pyridine pKa = 5.1 and for aniline pKa = 4.6), although, occurring in the same molecule, the electron-withdrawing effect of the heterocyclic nitrogen should tend to decrease pKa for the amino group while the electron-releasing effect of the amino group should tend to increase pKa for the ring nitrogen slightly (neglecting resonance effects due to positional relationships). As a solution containing any of the aminoquinolines is titrated with acid, it might be expected that the ring nitrogen would be protonated first but that some molecules would be singly protonated on the amino group before all of the ring nitrogens were protonated. Addition of a second proton to all of the molecules should occur at higher acidities, perhaps even in such strongly acidic solutions that potentiometric titration with a glass electrode would be impossible. The foregoing arguments are used here to test this hypothesis.



2-amino-quinoline | 3-amino-quinoline | 5-amino-quinoline | 8-amino-quinoline

EXPERIMENTAL

Spectrophotometric titrations were carried out on 1.00×10^{-4} *M* solutions of the aminoquinolines in buffered solutions with a Cary 15 recording spectrophotometer, using 1-cm. silica cells. Buffer solutions in the acidic region [pH 3 to Hammett acidity − 7.0 (4)] were made from analytical reagent grade perchloric acid[1] diluted with water and standardized against sodium carbonate. In the basic region (pH 10–13), buffer solutions were aqueous sodium hydroxide solutions prepared by diluting the carbonate-free sodium hydroxide with water, purging with and storing under nitrogen, and standardizing against potassium acid phthalate. Buffer solutions in the neutral region (pH 4.01–9.00) were prepared from Beckman standard buffers. 2-Aminoquinoline, 3-aminoquinoline, 5-aminoquinoline, and 8-aminoquinoline[2] were recrystallized from ethanol–water solutions.

Values for pKa were calculated from the relationship:

$$pKa = pH - \log \frac{\epsilon_A - \epsilon}{\epsilon - \epsilon_B} \qquad \text{(Eq. 8)}$$

where ϵ_A and ϵ_B are the molar absorptivities of acid and conjugate-base, respectively, at the wavelength chosen for measurement, and ε is the ratio of absorbance to concentration (*i.e.*, $1.00 \times 10^4 \times$ absorbance) at this wavelength.

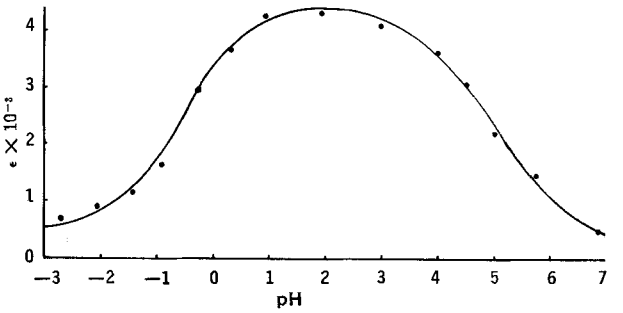


Figure 1—*Variation of the absorptivity (ε) of the 372-nm. absorption band of 3-aminoquinoline with pH. (The pH scale below pH 1.0 is represented by the Hammett acidity scale, H_0.)*

RESULTS AND DISCUSSION

The wavelength absorption maxima of the aminoquinolines in 11.7 *M* $HClO_4$ ($H_0 = -7.0$), 1.17×10^{-2} *M* $HClO_4$ (pH = 1.93), and 1.00×10^{-2} *M* NaOH (pH = 12.00) are listed in Table I. The variation of ε at 372 nm. with pH for 3-aminoquinoline is typical of the titration curves obtained in these studies and is shown in Fig. 1.

Since the absorptions in strong acid and basic solutions should correspond to the doubly protonated and neutral species, respectively, the absorptions in dilute acid solutions must correspond to

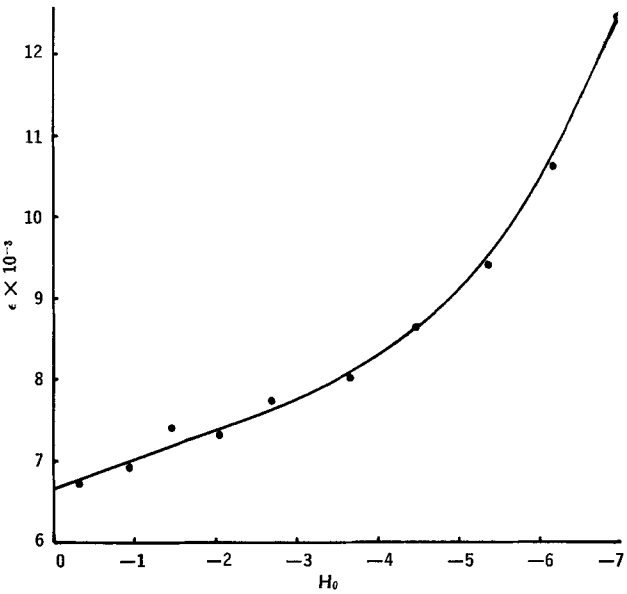


Figure 2—*Variation of the absorptivity (ε) of the 327-nm. absorption band of 2-aminoquinoline with Hammett acidity, H_0.*

[1] Mallinckrodt Chemical Works, Inc., St. Louis, Mo.

[2] K and K Chemicals, Inc., Plainview, N. Y.

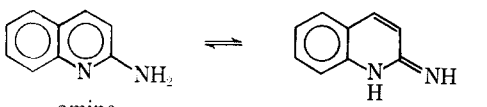
Table III—Values of ΔpKa (pKa − pK*) and pKa* Corresponding to the Spectral Data of Table I and the Titration Data of Table II in Conjunction with Eq. 7

Amino-quinoline	ΔpKa_1	pKa_1	pKa_1^*	ΔpKa_2	pKa_2	pKa_2^*
2-Amino-quinoline	—	—	—	+0.4	7.27	6.9
3-Amino-quinoline	+9.9	−0.57	−10.5	−5.3	4.96	10.3
5-Amino-quinoline	+16.6	+0.49	−16.1	−12.4	5.63	18.0
8-Amino-quinoline	+12.4	−0.52	−12.9	−8.2	3.92	12.1

the singly protonated species. Values for pKa for equilibria between the doubly protonated and singly protonated species (pKa_1) and between the singly protonated and neutral species (pKa_2) were calculated from Eq. 8, the data of Table I, and several solutions of the aminoquinolines at intermediate pH and H_0 values. These results are presented in Table II.

For 2-aminoquinoline, pKa_1 is not given in Table II because, in the most acidic solutions studied here ($H_0 = -7.0$), the titration curve for the formation of the doubly protonated species (or for the disappearance of the singly protonated species) was not completed. Furthermore, the absorbance of the doubly protonated species was increasing at such a rate that at $H_0 = -7.0$, the inflection point of the titration curve had not been passed (Fig. 2). Hence, it may be inferred that for 2-aminoquinoline, $pKa \ll -7.0$. Another striking feature of the 2-aminoquinoline spectra was the similarity at all pH values (Table I). This may be interpreted in terms of a small difference between ground and excited state pKa values, as will be discussed.

In a potentiometric study of the isomeric aminoquinoline, Albert and Goldacre (5) determined the pKa_2 values of the compounds studied here. Their values for pKa_2 and the values in this study are in excellent agreement. In that study it was assumed, but not proven, that protonation occurred on the ring nitrogen. The anomalously high value of pKa_2 for 2-aminoquinoline was attributed to a possible tautomerism between the amino form and the imino (or carbamidine) form (Scheme II). The greater basicity of the ring nitrogen



amino tautomer ⇌ amino (carbamidine) tautomer

Scheme II

in carbamidines relative to aromatic amines or ring nitrogens would account for the anomalously low acidity of the 2-amino isomer. Although no supportive evidence was offered by Albert and Goldacre (5) for this tautomerism, a similar tautomerism is known, from IR studies, to occur for 2-hydroxyquinoline with its 2-quinolone tautomer (6).

In Table III are tabulated the values of ΔpKa (pKa − pKa*) and pKa*, calculated with the help of Eq. 7 and the data of Table II.

The pKa* values of Table III indicate that with the exception of the 2-isomer, for which pKa_1^* cannot be determined in perchloric acid, the aminoquinolines studied here have $pKa_1^* < pKa_1$ and $pKa_2^* > pKa_2$. Since in this type of compound the ring nitrogen becomes more basic in the excited state while the amino nitrogen becomes less basic (or more acidic) in the excited state, pKa_2^* and pKa_2 must correspond to protonation of the ring nitrogen while pKa_1^* and pKa_1 must correspond to protonation of the amino group. Hence, the first protonation of the aminoquinolines, which shifts the absorption spectrum to longer wavelengths, involves protonation of the ring nitrogen only. The second protonation occurs some 5 log units to the acid side of the pH scale and shifts the absorption spectrum to shorter wavelengths. Therefore, pKa_1 must correspond to protonation of the singly protonated species (*i.e.*, at the amino site). Consequently, this experiment supports the speculations of Albert and Goldacre (5) as to the site of the first protonation. It is interesting that the gain in basicity in the excited state of the ring nitrogen is approximately the same as the loss in basicity of the amino group. This implies that the excitation process is accompanied by charge transfer from the amino group to the ring nitrogen.

The excited state pKa* values calculated in this experiment should not be taken literally as thermodynamic dissociation constants. Ground state equilibrium constants are determined not for isolated molecules but for molecules solvated by water in accordance with the distributions of electronic charge in the molecules. The energy of solvation contributes to the chemical potentials of each of the species involved in the dissociation equilibria and, therefore, to the experimentally determined pKa values. Since the process of excitation to the lowest excited singlet state alters the charge distributions in the absorbing molecules, the ground state equilibrium solvent cage is not at equilibrium with respect to the excited species. The absorption of light takes about 10^{-15} sec., while the time required for the solvent to relax to the equilibrium excited state solvent cage configuration is about 10^{-12} sec. Hence, in calculating pKa* values from shifts in absorption spectra, the calculation is performed for excited species in the ground state solvent cage, and the equilibrium energies of the excited species are not truly represented. Nevertheless, the results of absorption shift measurements do give reliable indications of charge distribution changes upon excitation and are perfectly adequate for the scope of the present study. If true pKa* values are required, they are better evaluated from shifts in fluorescence spectra with changing pH or from studies of the variation of fluorescence quantum yields with pH (7). Since the radiative lifetimes of fluorescent molecules are of the order of 10^{-8} sec., thermodynamic properties of excited molecules calculated from fluorescence data will reflect the energies of excited molecules in an equilibrium excited state solvent cage configuration.

The slight increase in the acidity of 2-aminoquinoline with respect to the first protonation, in the excited state, can be explained by two possible mechanisms. First, if protonation in the excited state occurs at the amino group instead of at the ring nitrogen, a shift to shorter wavelengths of the 2-aminoquinoline absorption spectrum is to be expected. However, the shift observed here is anomalously small compared with protonation of the amino groups of the other aminoquinolines. Alternatively, if the 2-aminoquinoline–2-iminoquinoline tautomerism proposed by Albert and Goldacre (5) does occur, the heterocyclic ring nitrogen has some of the character of a secondary amine with respect to the homocyclic ring. Consequently, in this case, protonation of the ring nitrogen might be expected to shift the absorption spectrum to shorter wavelengths (*i.e.*, $pKa_2 > pKa_2^*$) or, at most, only very slightly to longer wavelengths. If an equilibrium mixture of the amino and imino tautomers was present, two spectra, corresponding to the neutral species, should be observed. Protonation of this mixture should shift one spectrum to shorter wavelengths (the imino form) and the other to longer wavelengths (the amino form). Since this is not observed, this possibility is excluded. The best explanation seems to be that 2-aminoquinoline exists entirely in the imino form. The value of pKa_1 of 2-aminoquinoline, which is indeterminate in this experiment, is apparently much more acidic than pKa_1 for any of the other aminoquinolines. This anomaly cannot be explained at present.

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Michaelis–Menten Kinetics of Renal Tubular Secretion of D-(−)-*p*-Methyl Mandelic Acid and D-(−)-*p*-Ethyl Mandelic Acid in Rats

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Abstract □ Studies were carried out to determine the Michaelis–Menten kinetic parameters (V_m and K_m) of renal tubular secretion of *para*-alkylated mandelic acids, namely, D-(−)-*p*-methyl mandelic acid and D-(−)-*p*-ethyl mandelic acid, in rats. It is shown that the maximum initial secretion rates (V_m) of these compounds are similar, and they share a common "carrier" mechanism for their renal tubular secretion. Since the value of the intravenous dose (μmole/kg.) required to produce half the maximum secretion rate (K_m) for D-(−)-*p*-ethyl mandelic acid is found to be about one-half that for D-(−)-*p*-methyl mandelic acid, it is concluded that the affinity of the former for the "carrier" is approximately twice that of the latter. Evidence was obtained to indicate that the *para*-alkylated mandelic acids used in this study are secreted by the same mechanism responsible for the secretion of D-(−)-mandelic acid and its homologs such as tropic acid. Although the volume of distribution available in rats for these *para*-alkylated mandelic acids is demonstrated to be similar, it is shown to be strikingly smaller than that for D-(−)-mandelic acid and its homologs obtained by introducing methylene group(s) in the side chain of the mandelic acid molecule. The biological significance of the data obtained for the "model compounds" is discussed.

Keyphrases □ D-(−)-*p*-Methyl- and D-(−)-*p*-ethyl mandelic acids—renal tubular secretion □ Renal tubular secretion—*p*-alkylated mandelic acids □ Kinetic parameters, Michaelis-Menten—renal tubular secretion, *p*-alkylated mandelic acids □ Absolute configuration—methyl and ethyl mandelic acids □ GLC—analysis

Recent studies in rats from this laboratory (1) showed that the introduction of methylene group(s) in the side chain of a model compound, D-(−)-mandelic acid, decreases the biological half-lives of the compounds as compared to that of the parent compound. The various compounds studied were D-(−)-4-hydroxy-4-phenylbutanoic acid, D-(−)-benzyllactic acid, L-(+)- and D-(−)-tropic acid, and D-(+)- and L-(−)-phenyllactic acid. Furthermore, these compounds were shown to exhibit a mutual inhibitory effect on their renal tubular secretion and to share a common "carrier" mechanism for their secretion (1). Through the Michaelis–Menten kinetic treatment of the initial rates of renal tubular secretion data obtained for D-(−)-mandelic acid (hereafter referred to as mandelic acid) and DL-tropic acid (hereafter referred to as tropic acid), it was demonstrated that these compounds have similar maximum initial secretion rates and that the affinity of tropic acid for the "carrier" is approximately twice that of mandelic acid (2). In addition to being completely excreted unchanged in the urine of rats, since mandelic acid and its above mentioned homologs are neither metabolized nor bound to the plasma proteins, these compounds of low pKa values (3.7–4.7) were considered as the model compounds in the context of the objective of the studies (1, 2). The kinetic studies of urinary excretion of these model compounds were carried out to distinguish certain structural characteristics around the positively charged site of the carrier molecules believed to be present in the renal tubular membrane of rats for secretion of the anionic drugs.

The purposes of the work presented here were: (*a*) to study the kinetics of urinary excretion of the *para*-alkylated mandelic acids, namely, D-(−)-*p*-methyl mandelic acid and D-(−)-*p*-ethyl mandelic acid (hereafter referred to as methyl mandelic acid and ethyl mandelic acid), in rats, and (*b*) to determine if these compounds also share the same "carrier" for their renal tubular secretion as do mandelic acid and its above mentioned homologs. These objectives were accomplished by studying the Michaelis–Menten kinetics of renal tubular secretion of these compounds in rats.

EXPERIMENTAL

Materials—The following were used: D-(−)-mandelic acid[1], m.p. 132–133°, $[\alpha]_D^{25}$ − 149° [2]; D-(−)-*p*-methyl mandelic acid, m.p. 130–131°, $[\alpha]_D^{25}$ − 159°[2], obtained by resolving from the racemic acid synthesized by the method of Corsow *et al.* (3); D-(−)-ethyl mandelic acid, m.p. 119–120°, $[\alpha]_D^{25}$ − 120°[2], obtained by resolving from the racemic acid synthesized by the method of Klingenberg (4); and DL-tropic acid, m.p. 118–119°.

Apparatus—Mandelic acid and its homologs appearing in the urine of rats were quantitated by the gas chromatographic method previously described (1, 2), using 5% ethylene glycol succinate as well as 15% silicone rubber as the liquid phases. The specific rotation of the compounds was determined with the aid of a Perkin-Elmer 141 polarimeter, using a 1-ml. capacity polarimeter tube. The urine pH determinations were made on a Beckman model 76 Expandomatic pH-meter. The optical rotatory dispersion (ORD) curves of the compounds were obtained with the aid of a Cary model 60 recording spectropolarimeter.

Methodology—The procedure employed for preparing the rats for the study and for urine collection following the intravenous administration of the compounds was the same as previously described (1). About 30 Sprague-Dawley male rats, weighing between 185 and 235 g., were repeatedly used. None of the rats was used more than five times in the studies, and the rest period allowed between the successive use of a rat was about 2 weeks. The aqueous solutions of sodium salts of each compound used for intravenous administration were made isotonic, and the volume of intravenous injections was 2 ml. The urinary excretion kinetics of methyl mandelic acid were studied over the dosage range of 1.43–15.06 × 10^2 μmoles/kg.[3] (5–50 mg. per rat) to determine the dependency of the biological half-life of the compound on its intravenous dose. The urinary excretion kinetics of ethyl mandelic acid were studied over the intravenous dosage range of 1.27–3.94 × 10^2 μmoles/kg.[4] (5–15 mg. per rat).

To study the Michaelis–Menten kinetics of renal tubular secretion of the compounds in rats, their initial glomerular filtration rates (and, consequently, the initial secretion rates) were determined at the appropriate dosage levels in the presence of suitable competitive

[1] Aldrich Chemical Co., Milwaukee, Wis.

[2] For specific rotation determinations, the solutions of these compounds were prepared in 95% ethanol.

[3] Doses used were 143, 172, 320, 342, 391, 430, 512, 512, 523, 573, 573, 885, 903, 1420, and 1506 μmoles/kg.

[4] Doses used were 126, 149, 218, 222, 230, 288, 300, 377, and 391 μmoles/kg.

Table I—Conditions Employed for Gas Chromatographic Analysis and Retention Times Observed for the Compounds

	Oven Temperature[a]	He Flow Rate, ml./min.	Retention Time, min.
Methyl-*p*-methyl mandelate	180°	40	5.8
Methyl-*p*-ethyl mandelate	180°	40	7.2
Methyl-*p*-ethyl mandelate	160°	40	13.5[b]

[a] The injection port and detector temperatures were maintained at 210°. [b] As stated in the text, 15% silicone rubber was used as a liquid phase column to analyze methyl-*p*-ethyl mandelate in the presence of methyl tropate.

inhibitors of secretion, according to the method previously described (2). To determine the initial glomerular filtration rates of methyl mandelic acid at the dosage levels of 1.36–4.52 × 10^2 μmoles/kg. (5–15 mg. per rat) i.v., the intravenous dose of the sodium salt of the compound was injected into the rat 20 min. after the administration of a 5.46–13.36 × 10^3-μmoles/kg. (200–400 mg. per rat) i.p. dose of tropic acid as the sodium salt contained in 5 ml. of water at a pH of about 7.0. In a similar manner, the apparent initial glomerular filtration rates of ethyl mandelic acid at the dosage levels of 2.45–3.03 × 10^2 μmoles/kg. (8–10 mg. per rat) i.v. were determined in the presence of a 12.05 × 10^3-μmoles/kg. (400 mg. per rat) i.p. dose of methyl mandelate or 11.92–11.98 × 10^3-μmoles/kg. (400 mg. per rat) i.p. dose of tropic acid. The initial glomerular filtration rates of ethyl mandelic acid were also determined in the presence of simultaneously administered 25.74–26.02 × 10^2-μmoles/kg. (80 mg. per rat) i.v. doses of methyl mandelic acid.

The intravenous doses of sodium methyl mandelate and sodium ethyl mandelate employed in the studies designed to determine the maximum initial secretion rates (V_m), as well as their doses required to produce one-half of the maximum initial secretion rates

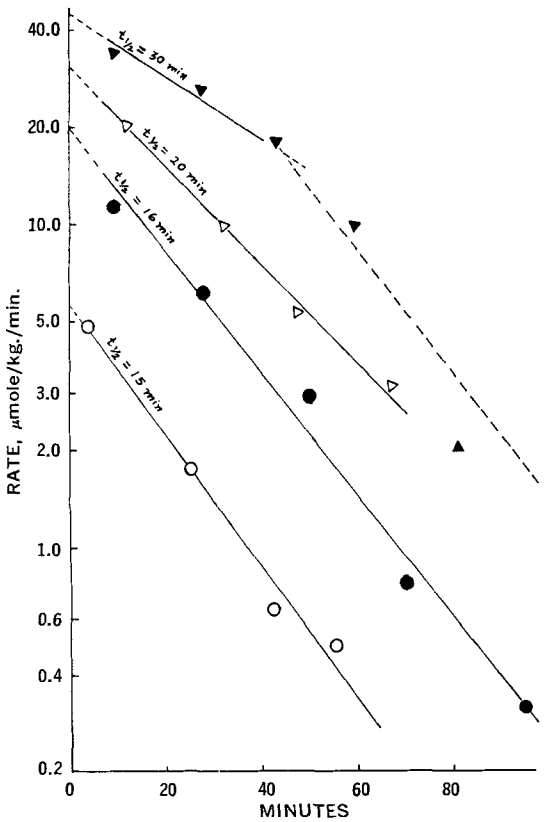

Figure 1—*Apparent first-order urinary excretion of* D-(−)-p-*methyl mandelic acid, showing the dependence of biological half-life* ($t_{1/2}$) *of the compound on the intravenous dose. Key:* ○, *143 μmoles/kg.;* ●, *512 μmoles/kg.;* △, *885 μmoles/kg.; and* ▲, *1506 μmoles/kg.*

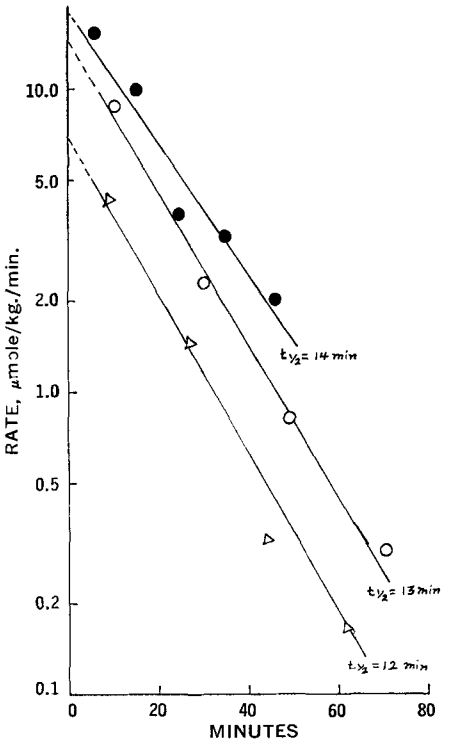

Figure 2—*Apparent first-order kinetics of urinary excretion of* D-(−)-p-*ethyl mandelic acid following its intravenous administration of 126-μmoles/kg.* (△), *288-μmoles/kg.* (○), *and 377-μmoles/kg.* (●) *doses to rats.*

(K_m), were in the range of 1.43–15.06 × 10^2 and 1.27–3.94 × 10^2 μmoles/kg., respectively.

To determine whether the inhibition of renal tubular secretion of methyl mandelic acid by tropic acid is competitive or noncompetitive, the initial secretion rates of methyl mandelic acid (dosage range 2.94–9.03 × 10^2 μmoles/kg.) were determined in the presence of 21.39–24.09 × 10^2-μmoles/kg. (80 mg. per rat) i.v. doses of tropic acid. In these studies both the substrate and inhibitor compounds, contained in the same intravenous solution, were administered as their sodium salts.

Gas Chromatographic Analysis of the Compounds—The urine samples were quantitatively analyzed for mandelic acid, methyl mandelic acid, and ethyl mandelic acid by a gas chromatographic method previously described (1). The liquid phase column used for the gas chromatographic analysis of these compounds was 5% ethylene glycol succinate. However, a 15% silicone rubber liquid phase column was used to obtain a distinct peak for ethyl mandelic acid in the presence of methyl mandelic acid. The conditions employed for the gas chromatographic analysis and retention times observed for methyl esters of the compounds are described in Table I.

Determination of Dissociation Constants and Apparent Partition Coefficients of the Compounds and Their Binding to Whole Rat Blood—These determinations were carried out by the methods previously described for mandelic acid and its several homologs (1).

Determination of Absolute Configuration of the Compounds—The ORD curves for methyl mandelic acid and ethyl mandelic acid were obtained and compared with that obtained for D-(−)-mandelic acid.

RESULTS AND DISCUSSION

Selection of the Compounds—Various properties considered ideal for use of the compounds in view of the objective of the studies were previously discussed (1). Since it was observed in preliminary studies that, of the administered dose of 10–20 mg. i.v. per rat, approximately 58% of the racemic methyl mandelic acid and 40% of the racemic ethyl mandelic acid are recovered unchanged in the urine of rats, the racemic compounds were resolved into their levorotatory optical isomers and their urinary recovery was studied in rats. The preliminary studies showed that 90–100% of the ad-

ministered doses (10–20 mg. i.v. per rat) of the levorotatory isomers of each of the two compounds was excreted unchanged in the urine of rats. Therefore, the other properties (dissociation constants, partition coefficients, and binding to plasma proteins) of the compounds were studied to determine their suitability for use in the present study.

The pKa value determined for levorotatory methyl mandelic acid is 3.4, and that determined for levorotatory ethyl mandelic acid is also 3.4 (lit. 3.5). The partition coefficient ($C_{organic}/C_{buffer}$) observed for methyl mandelic acid as well as ethyl mandelic acid between the pH 6.5 phosphate buffer solution (10 ml. solution containing 5 mg. of the acid) and chloroform or ether (10 ml.) was zero, as was found previously for mandelic acid, tropic acid, or phenyllactic acid (1). The results of the binding studies indicated that, at equilibrium, the concentration of methyl mandelic acid or ethyl mandelic acid in the solution (4 ml.) outside of the dialysis bag (containing 4 ml. of whole rat blood) was the same as in the solution (4 ml.) outside of the dialysis bag (containing 4 ml. of pH 7.4 phosphate buffer solution) of the control dialysis setup, indicating that neither of the acids is bound to the plasma proteins of rat. The amount of either acid used per dialysis setup was 1 mg. (1). As previously noted (1), the pH of rat urine was generally found to be in the range of 6.5–8, even after administration of a 400-mg. i.p. dose of tropic acid to the rats. Since these compounds possessed these ideal properties, the levorotatory isomers of *p*-methyl mandelic acid and *p*-ethyl mandelic acid were considered as the model compounds for use in the present study.

The absolute configurations of the levorotatory isomers of methyl mandelic acid and ethyl mandelic acid were determined by comparing the ORD curves of the compounds with that of D-(−)-mandelic acid (1). Since the plain ORD curves obtained for these compounds were similar to that of D-(−)-mandelic acid over the wavelengths of 600–200 nm., D absolute configuration was assigned to the levorotatory isomers of these compounds.

Apparent First-Order Urinary Excretion of the Compounds—As observed for mandelic acid and its several homologs (1), the urinary excretion of methyl mandelic acid and ethyl mandelic acid was found to occur by an apparent first-order process in rats. Since these compounds are entirely excreted in the urine in the unchanged form, the urinary excretion data were treated according to the following equation (1):

$$\log \Delta A_e/\Delta t = \log kA_0 - kt/2.303 \qquad \text{(Eq. 1)}$$

where $\Delta A_e/\Delta t$ is the rate of excretion of the compound at time t, A_0 is the amount of the compound in the body at zero time, and k is the apparent first-order rate constant for excretion of the compound. The rate constant, k, was obtained from the slope ($-k/2.303$) of the straight line, obtained upon plotting $\log \Delta A_e/\Delta t$ *versus* t. The straight line in each case was obtained by the method of least squares. The time t in these plots represented the midpoints of the urine-collection intervals. The biological half-lives of the compounds were calculated from $0.693/k$.

As shown in Fig. 1, it was observed that, while the biological half-life for methyl mandelic acid remained reasonably constant (16 ± 1.7 min.) over the dosage range of 1.43–9.03 × 10^2 μmoles/kg. i.v., it increased with the increase in the dose above this dosage level during the initial period of about 40 min. This may mainly be due to the increased saturation of the renal tubular secretory process with the increase in the dose of the compound. Although the two kinetic studies were performed at high doses (1420 and 1506 μmoles/kg.), unfortunately, it did not become possible to obtain from the rats the urine samples at shorter intervals (12 min. or so) to characterize the data curve after 50 min. and thereby be able to indicate that the half-life of the compound became about 16 min. Nevertheless, data in Fig. 1 tend to indicate that, after the initial period, the compound was excreted at a faster rate (shorter biological half-life), as can be expected. The apparent initial excretion rates of the compound at various dosage levels were determined from the values of the intercepts obtained by extrapolating the straight lines to zero time (1). The apparent first-order urinary excretion data obtained for ethyl mandelic acid over the dosage range of 1.27–3.94 × 10^2 μmoles/kg. are shown in Fig. 2. The biological half-life of the compound over this dosage range was found to be fairly constant (13.5 ± 1.6). As shown later, since it was demonstrated that methyl mandelic acid and tropic acid are able to inhibit the secretion of ethyl mandelic acid and that the data obtained at these dosage levels of ethyl mandelic acid were sufficient to determine the Michaelis–Menten kinetic parameters of its secretion, it was not necessary to carry out additional studies at higher doses of the compound to show the saturation effect on its renal tubular secretory process, as was done in the case of methyl mandelic acid (Fig. 1).

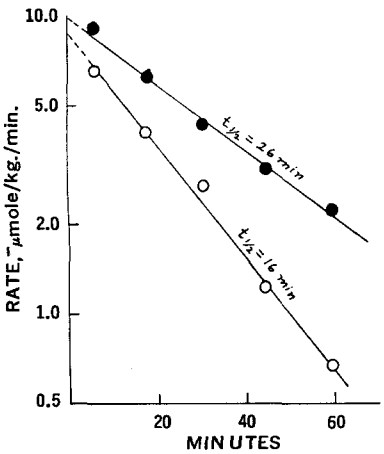

Figure 3—*Urinary excretion of* D-(—)-*mandelic acid* (●, *343 μmoles/kg.*) *and* D-(−)-*ethyl mandelic acid* (○, *219 μmoles/kg.*) *following their simultaneous intravenous administration to a rat.*

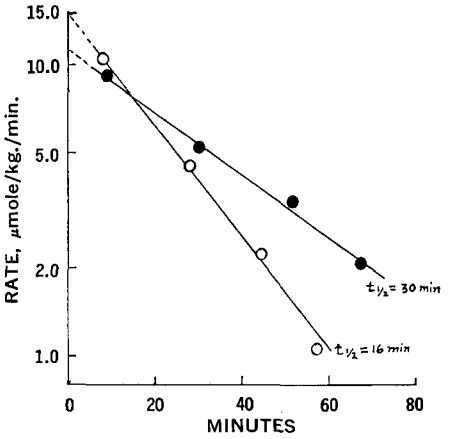

Figure 4—*Apparent first-order urinary excretion of* D-(−)-p-*methyl mandelic acid following intravenous administration of a 391-μmoles/kg. dose in the absence* (○) *and a 452-μmoles/kg. dose in the presence* (●) *of a 1.2* × 10^4-*μmoles/kg. i.p. dose of* DL-*tropic acid to rats.*

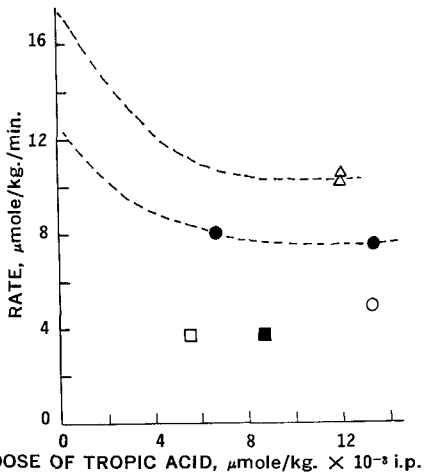

Figure 5—*Plots showing that the apparent limiting initial excretion rates* (i.e., *apparent initial glomerular filtration rates*) *of* D-(−)-p-*methyl mandelic acid* (*dosage range 136–452 μmoles/kg. i.v.*) *can be obtained in the presence of a 1.2* × 10^4-*μmoles/kg. i.p. dose of* DL-*tropic acid. Key:* □, *136 μmoles/kg.;* ■, *146 μmoles/kg.;* ○, *167 μmoles/kg.;* ●, *334 μmoles/kg.; and* △, *452 μmoles/kg.* (*The rates of the compound in the absence of tropic acid were obtained by extrapolating the data in Fig. 1.*)

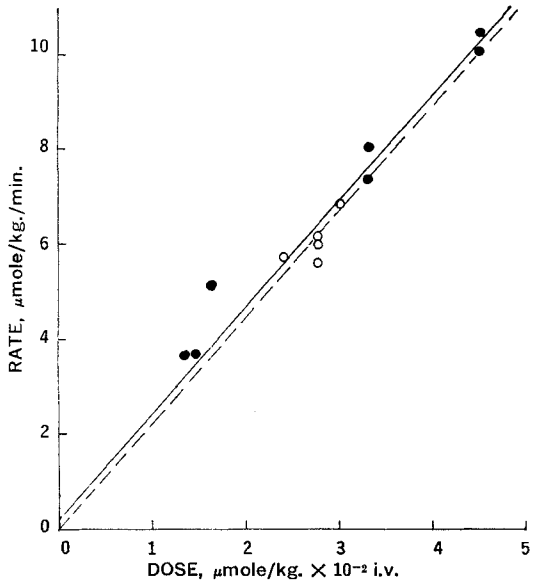

Figure 6—*Relationship between the apparent initial glomerular filtration rate and the intravenous dose for* D-(−)-p-*methyl mandelic acid* (●) *and* D-(−)-p-*ethyl mandelic acid* (○). (*The straight lines are obtained by the method of least squares.*)

The biological half-life of ethyl mandelic acid is found to be significantly shorter than that of methyl mandelic acid ($p < 0.01$).

The biological half-life observed for methyl mandelic acid or ethyl mandelic acid in this study is considerably shorter than that noted for mandelic acid (30 ± 4 min.) by Randinitis *et al.* (1) in most of their studies. However, it was noted that, while the volume of intravenous injection used in the present studies for the administration of various doses of the compounds to each rat was 2 ml., the volume of intravenous injection used by Randinitis *et al.* (1) for the administration of similar doses of mandelic acid to each rat varied from 0.2–0.7 ml. To make a valid comparison of the biological half-lives of these compounds, it was considered desirable to

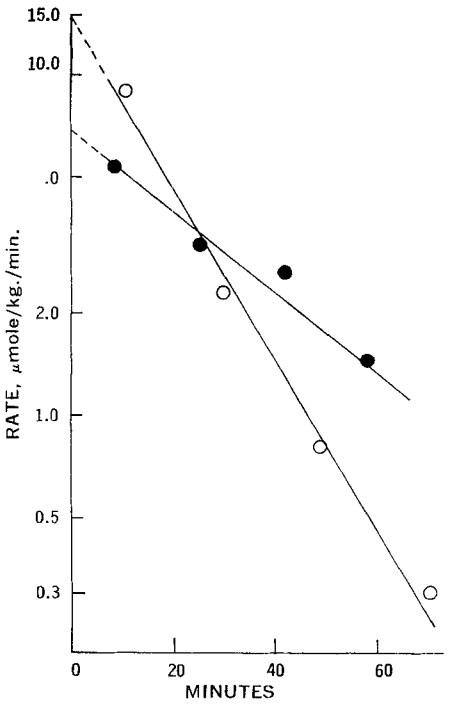

Figure 7—*Apparent first-order urinary excretion of* D-(−)-p-*ethyl mandelic acid following the intravenous administration of a 291-μmoles/kg. dose in the absence* (○) *and 303-μmoles/kg. dose in the presence* (●) *of a 2.6 × 10³-μmoles/kg. simultaneously administered intravenous dose of* D-(−)-p-*methyl mandelic acid to rats.*

Table II—Michaelis–Menten Kinetic Parameters of Renal Tubular Secretion for the Compounds in Rats

Compound	Apparent V_m, μmole/kg./min.	Apparent K_m, μmole/kg. × 10^{-2}
D-(−)-Methyl mandelic acid	21.6	10.2
D-(−)-*p*-Ethyl mandelic acid	18.5	4.9
D-(−)-Mandelic acid[a]	18.8	11.1
DL-Tropic acid[a]	20.8	5.2

[a] Values for these compounds are adapted from *Reference 2.*

determine an obvious influence of the injection volume on the biological half-lives of these compounds. In one study, the urinary excretion kinetics of mandelic acid (493 μmoles/kg.) were studied following its administration as the sodium salt contained in a 2-ml. i.v. injection volume; in the other study, the excretion kinetics of mandelic acid (343 μmoles/kg.) and ethyl mandelic acid (219 μmoles/kg.) were studied following their simultaneous administration as the sodium salts contained in a 2.5-ml. i.v. injection volume. The biological half-life determined for mandelic acid in the latter study was 26 min. (Fig. 3) and that determined in the former study was 32 min., both these values being within the range previously reported (1). The biological half-life of 16 min. observed for ethyl mandelic acid (Fig. 3) was equal to the uppermost limit value in the range noted in the present study. Therefore, no attempt was made to ascertain if mandelic acid had exerted a slight inhibitory effect on the renal tubular secretion of ethyl mandelic acid at these dosage levels. Since none of the homologs of mandelic acid previously studied (1) significantly influenced the biological half-life of mandelic acid at such low doses, it may be assumed that the half-life of mandelic acid was not influenced by ethyl mandelic acid in the study (Fig. 3). Thus, it seems that the injection volume (up to 2.5 ml.) has no apparent effect on the biological half-lives of the compounds.

As noted by Randinitis *et al.* (1) for mandelic acid and its homologs, in this study also the metabolism and the binding of methyl mandelic acid and ethyl mandelic acid to blood proteins of rats were negligible and their pKa values were considerably lower than the pH values of urine of rats. In view of these facts, the shorter biological half-lives observed for methyl mandelic acid and ethyl mandelic acid than that observed for mandelic acid may be attributed to the smaller volume of distribution available in rats for these compounds than that for mandelic acid and/or to the greater affinity of these compounds for the "carrier" for renal tubular secretion in rats than that of mandelic acid, if these compounds share a common "carrier" for their secretion. The influence of each factor is evaluated from the subsequent studies carried out to determine the initial glomerular filtration rates and the Michaelis–Menten kinetic parameters of renal tubular secretion of these compounds in rats.

Determination of Apparent Initial Glomerular Filtration Rates of the Compounds—In the studies designed to determine the initial glomerular filtration rates of methyl mandelic acid, the compound used to inhibit its secretion was tropic acid, which has a greater affinity for the renal tubular secretion "carrier" than that of mandelic acid (2). In the urinary excretion kinetic studies, the dose (5.46–13.36 × 10^3 μmoles/kg. i.p.) of tropic acid was administered 20 min. prior to the intravenous administration of methyl mandelic acid to allow substantial absorption of the inhibitor dose from the site of injection (1). As shown in Fig. 4, the biological half-life of methyl mandelic acid increased[5], and its initial excretion rate decreased, in the presence of tropic acid. Also, as depicted in Fig. 5, it is seen that the 10–13 × 10^3-μmoles/kg. i.p. dose of tropic acid produced a limiting initial excretion rate of methyl mandelic acid over its dosage range of 136–452 μmoles/kg. i.v. Since a linear relationship (Fig. 6) was observed between the intravenous dose of methyl mandelic acid and its limiting initial excretion rate at the corresponding dose, the limiting initial excretion rates noted for the compound were considered as its apparent initial glomerular filtration rates at the corresponding dosage levels (2).

[5] The range of biological half-life values observed was 22–38 min., depending on the dose of tropic acid used as the inhibitor.

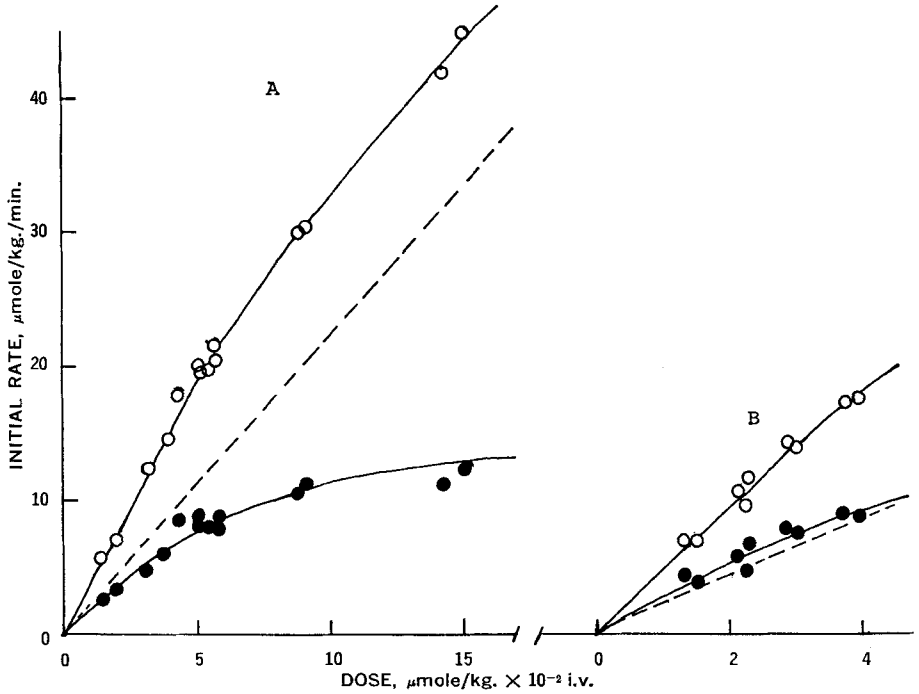

Figure 8—*Apparent initial excretion rates, ○; initial glomerular filtration rates, - - -; and initial secretion rates, ●; observed for* D-(−)-p-*methyl mandelic acid (A) and* D-(−)-p-*ethyl mandelic acid (B) following the intravenous administration of appropriate doses to rats.*

To determine the initial glomerular filtration rates of ethyl mandelic acid in rats, the compounds used as the inhibitors of its secretion were methyl mandelic acid and tropic acid. The purpose of using both these compounds as the inhibitors was to obtain additional evidence that all these compounds are secreted by a common "carrier" mechanism. Initially, two glomerular filtration-rate determination studies were carried out for the compound in the presence of 12.05×10^3-μmoles/kg. i.p. doses of methyl mandelic acid, which was administered to the rats 20 min. prior to the intravenous administration of the compound. But this dose of methyl mandelic acid caused bleeding in the urine of rats. Therefore, the additional studies were carried out using lower doses ($25.74–26.02 \times 10^2$ μmoles/kg. i.v.) of methyl mandelic acid as the inhibitor. In this event, both ethyl mandelic acid (dose of $2.45–3.03 \times 10^2$ μmoles/kg. i.v.) and methyl mandelic acid, contained in a 2-ml. intravenous injection volume as their sodium salts, were administered to the rats. When tropic acid was used as the inhibitor of secretion of methyl mandelic acid, a dose (12.0×10^3 μmoles/kg. i.p.) of the former was administered to the rats 20 min. prior to the administration of the intravenous dose of the latter. A typical kinetic study showing the inhibitory effect of methyl mandelic acid on the secretion of ethyl mandelic acid is shown in Fig. 7. When the apparent initial excretion rates obtained in these studies for ethyl mandelic acid were plotted against its corresponding intravenous doses, a linear relationship was observed (Fig. 6). This demonstrated that not only approximately 12×10^3-μmoles/kg. i.p. doses of methyl mandelic acid or tropic acid but also the simultaneously administered about 25×10^2-μmoles/kg. i.v. dose of methyl mandelic acid brought about an apparent complete inhibition of secretion of ethyl mandelic acid over its intravenous dosage range of $2.43–3.03 \times 10^2$ μmoles/kg.

An examination of the data in Fig. 6 indicated that, at the equimolar doses, the apparent glomerular filtration rates of methyl mandelic acid and ethyl mandelic acid are similar in rats. Therefore, it is concluded that the volume of distribution available for these compounds in rats is similar.

Determination of Michaelis–Menten Kinetic Parameters for Renal Tubular Secretion of the Compounds—To determine the V_m and K_m values for methyl mandelic acid and ethyl mandelic acid, the data regarding the apparent initial secretion rates (V) *versus* intravenous doses (D) were obtained by subtracting the apparent initial glomerular filtration rates from the apparent initial excretion rates at the corresponding dosage levels of each of these compounds (Fig. 8). The apparent initial glomerular filtration rates at the intravenous doses above those shown in Fig. 6 were obtained by extrapolation of the straight lines.

As previously shown (2), the plots of $1/V$ *versus* $1/D$ were constructed (Fig. 9) according to the Lineweaver–Burk equation:

$$1/V = K_m/V_m(D) + 1/V_m \qquad \text{(Eq. 2)}$$

The respective values of V_m for these compounds were calculated from the intercepts on the y-axis, and those of K_m were calculated from the slopes of the straight lines. The straight lines were obtained by the method of least squares. The values of V_m and K_m determined for the compounds are listed in Table II. The values of V_m for the two compounds are considered to be comparable by the reasoning offered for considering comparable the values of V_m determined for mandelic acid (18.8 μmoles/kg./min.) and tropic acid (20.8 μmoles/kg./min.) in the previous report (2). Therefore, it is concluded that methyl mandelic acid and ethyl mandelic acid are secreted by the same carrier mechanism present in the renal tubular membrane of rats. It is further concluded from comparing the values of K_m (Table II) for these compounds that the affinity of ethyl mandelic acid for the "carrier" molecules of renal tubular transport is approximately twice that of methyl mandelic acid.

Nature of Inhibition of Secretion of Methyl Mandelic Acid by Tropic Acid—Although methyl mandelic acid, ethyl mandelic acid, and tropic acid may be assumed to exhibit the mutual competitive inhibitory effects on their renal tubular secretion, studies were

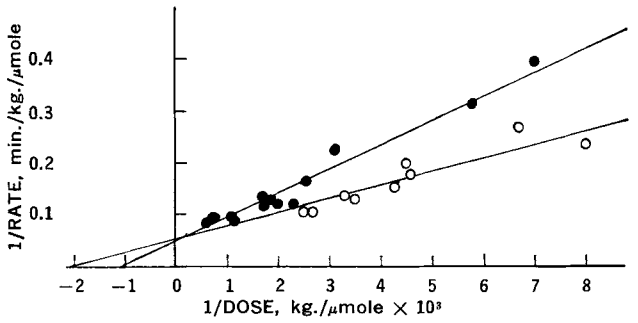

Figure 9—*Lineweaver–Burk plots of the reciprocal of the intravenous dose of* D-(−)-p-*methyl mandelic acid (●) and* D-(−)-p-*ethyl mandelic acid (○) against their reciprocal of the apparent initial secretion rates.*

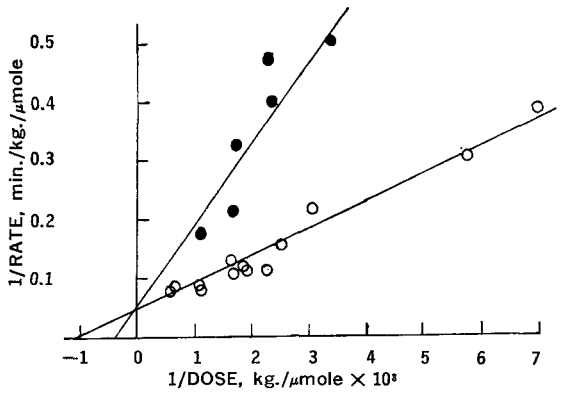

Figure 10—*Lineweaver–Burk plots indicating competitive inhibition of renal tubular secretion of* D-(−)-p-*methyl mandelic acid by* DL-*tropic acid (dose of 21–24 × 10² μmoles/kg. i.v.). Key:* ○, *inhibitor absent; and* ●, *inhibitor present.*

carried out to demonstrate that tropic acid competitively inhibits the secretion of methyl mandelic acid in rats. From the apparent initial excretion rates determined for methyl mandelic acid (2.94–9.03 × 10^2 μmoles/kg.) in the presence of simultaneously administered 21.4–24.1 × 10^2-μmoles/kg. i.v. doses of tropic acid, the apparent initial secretion rates were determined by subtracting from them the apparent glomerular filtration rates at the corresponding doses. The plot of $1/V$ *versus* $1/D$ obtained for the studies is shown in Fig. 10. It becomes evident from the apparent common *y*-intercept in this figure that tropic acid competitively inhibits the secretion of methyl mandelic acid.

Comparison of Michaelis–Menten Kinetic Parameters and Glomerular Filtration Rates of Mandelic Acid and Its Homologs—The values of V_m determined for methyl mandelic acid (21.6 μmoles/kg./min.) and ethyl mandelic acid (18.5 μmoles/kg./min.) in the present study are found to be remarkably similar to those reported (1) for mandelic acid (18.6 μmoles/kg./min.) and tropic acid (20.8 μmoles/kg./min.), thus indicating that the secretion of these compounds occurs in rats *via* the renal tubules by a common "carrier" mechanism. The comparison of the values of K_m for methyl mandelic acid (10.2 × 10^2 μmoles/kg.) and ethyl mandelic acid (4.9 × 10^2 μmoles/kg.) with those of mandelic acid (11.1 × 10^2 μmoles/kg.) and tropic acid (5.2 × 10^2 μmoles/kg.) suggests that the affinities of ethyl mandelic acid and tropic acid for the "carrier" molecules are similar, but these are approximately twice those of methyl mandelic acid and mandelic acid.

Although the affinity of ethyl mandelic acid for the "carrier" molecules is similar to that of tropic acid, the average biological half-life of ethyl mandelic acid (13 min.) observed in the present study is considerably shorter than that reported for tropic acid (22 min.). Also, the average biological half-life of methyl mandelic acid (16 min.) is considerably shorter than that reported for mandelic acid (30 min)., despite the fact that the affinities of these compounds for the "carrier" molecules are similar. These differences in the values of biological half-lives of the compounds can be explained by comparing their apparent glomerular filtration rates, as shown in Fig. 11. At the equimolar doses, the initial glomerular filtration rates obtained for *para*-alkylated mandelic acids (methyl mandelic acid and ethyl mandelic acid) are approximately twice those reported (1) for mandelic acid and its homologs obtained by introducing the methylene group(s) in the side chain of the mandelic acid molecule. This striking difference in the initial glomerular filtration rates of these compounds should be due to the difference in the volume of distribution available in rats for the compounds. Accordingly, the volume of distribution available for methyl mandelic acid and ethyl mandelic acid is almost half that available for mandelic acid and its homologs obtained by adding the methylene group(s) in the side chain of the mandelic acid molecule.

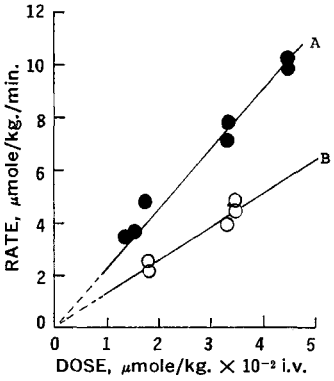

Figure 11—*Comparison of apparent initial glomerular filtration rates observed for* D-(−)-p-*methyl mandelic acid (Curve A) with that reported for* D-(−)-*mandelic acid (Curve B). (Curve A is obtained from Fig. 6 of this paper, and Curve B is adapted from Fig. 4 of* Reference 2.)

Significance of Results—The utilization of the model compounds, methyl mandelic acid and ethyl mandelic acid, in the present studies indicated that the substitution of an alkyl group containing at least two carbon atoms is necessary to increase the affinity of a resulting mandelic acid homolog for the "carrier" molecules of the renal tubular secretion system in rats. This also makes it possible to distinguish a further structural characteristic of the "carrier" molecules in addition to that mentioned in the previous studies (1). From the studies (1) of mandelic acid and its homologs [obtained by introducing methylene group(s) in the side chain of mandelic acid molecule], the possible structural characteristic distinguished was that the hydrophobic region around the cationic site of the carrier molecule probably does not extend uninterruptedly beyond the hydrophobic region represented by two carbon atoms (possibly two methylene groups). The results of the present study seem to support this suggestion, since the *para*-substitution of a methyl group in the mandelic acid molecule did not increase the affinity of the resulting molecule (methyl mandelic acid) for the "carrier," but the *para*-substitution of the ethyl group in the mandelic acid molecule increased the affinity of the resulting molecule (ethyl mandelic acid) for the "carrier." Furthermore, the present findings strongly suggest that the higher affinity of ethyl mandelic acid for the "carrier" molecules than that of mandelic acid or methyl mandelic acid is due to the additional interaction of its end methyl group with the hydrophobic portion that should be present next to the polar group of the "carrier" molecule implied to exist from the previous studies (1).

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Peel Oils of Different Types of *Citrus sinensis* L. and *C. aurantium* L. Growing in Egypt

M. S. KARAWYA*, S. I. BALBAA, and M. S. HIFNAWY

Abstract □ A comparative chromatographic study including column chromatography, TLC, and GLC was carried out on the peel essential oils (cold-pressed and steam-distilled) of different types and varieties of *Citrus sinensis* L. and *C. aurantium* L. growing in Egypt. A mineral oil capillary column was successfully employed in the GLC analysis of these oils. IR patterns of the oils were used in the differentiation between the cold-pressed and steam-distilled oils.

Keyphrases □ *Citrus sinensis, C. aurantium*—peel oil comparison □ Oils, *C. sinensis, C. aurantium*—partial component identification □ Chromatography, TLC, GLC, column—analysis □ IR spectrophotometry—identification

Since they fill an important position in the manufacture of perfumery, cosmetics, confectionery, and pharmaceutical formulations, the citrus essential oils have been the subject of numerous investigations. Mehlitz and Minas (1) used a combination of TLC and column chromatography to identify a number of oxygenated terpenes which were found in the steam-distilled oil of bitter orange peel. Karawya and Wahba (2) identified limonene, terpineol and/or linalool, geraniol and/or nerol, and methyl anthranilate in oil of sweet orange. The composition of certain citrus oils was studied by some workers (3–9) using GLC. In most of these investigations, the oils were separated into hydrocarbons and oxygenated fractions before examination with GLC. Stanley (10) employed TLC, GLC, and spectral methods for the analysis of hydrocarbons, esters, and alcohols in citrus oils. Many authors (11–14) employed IR analysis as a tool for the differentiation between synthetic and natural oils. Albaldejo (11) stated that the IR spectra of the common citrus oils lie between 2 and 16 μ.

The present work is a comparative chromatographic study using column chromatography, TLC, and GLC of the steam-distilled and cold-pressed peel oils of *Citrus sinensis* L. (sweet orange) types (Egyptian, navel, blood, and succari) and of *C. aurantium* L. (bitter orange) types (Egyptian and Spanish). IR analysis of cold-pressed and steam-distilled oils of Egyptian sweet orange was also undertaken.

EXPERIMENTAL

Materials—The cold-pressed and steam-distilled peel oils of the freshly collected mature fruits of four types of sweet orange (*C. sinensis* L.) (Egyptian, navel, succari, and blood) and two types of bitter orange (*C. aurantium* L.) (Egyptian and Spanish) growing in Egypt were used[1].

Procedures—*TLC*—The essential oils, as well as the pure constituents, were chromatographed side by side on silica gel G plates, using *n*-hexane–ethyl acetate (85:15). The chromatograms were inspected under UV light and sprayed with 1% w/v vanillin in sulfuric acid (Figs. 1 and 2). Results are shown in Table I.

Preparative TLC—The oils were chromatographed on silica gel G plates about 2 mm. thick. By this technique, most of the major constituents of the analyzed oils—*viz.*, linalool, terpineol, citral, geraniol and/or nerol, the terpene fraction, and esters were success-

Table I—Mean R_f Values of Separated Components of Cold-Pressed and Steam-Distilled Peel Oils[a]

Compound	Mean R_f	*C. sinensis* L. (Sweet Orange)								*C. aurantium* L. (Bitter Orange)			
		Egyptian		Navel		Succari		Blood		Egyptian		Spanish	
		C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.
Limonene	0.96	+	+	+	+	+	+	+	+	+	+	+	+
Linalyl acetate	0.75	+	±	+	±	+	±	+	±	+	±	+	±
Geranyl acetate	0.73												
Methyl anthranilate	0.67	+	−	+	−	+	−	+	−	+	−	+	−
Citronellal	0.68	+	+	+	+	+	+	+	+	+	−	+	−
Citral	0.51	+	±	+	±	+	±	+	±	+	±	+	±
Linalool	0.43	+	+	+	+	+	+	+	+	+	+	+	+
Terpineol	0.29	+	+	+	+	+	+	+	+	+	+	+	+
Farnesol	0.26												
Geraniol	0.24	+	+	+	+	+	+	+	+	+	+	+	+
Nerol	0.24												
Citronellol	0.25												
Unknown	0.55	+	−	+	−	+	−	+	−	+	−	+	−
Unknown	0.53	−	−	−	−	−	−	−	−	+	+	+	+
Unknown	0.46	−	+	−	+	−	+	−	+	−	−	−	−
Unknown	0.38	+	−	+	−	+	−	+	−	−	−	−	−
Unknown	0.15	−	+	−	+	−	+	−	+	−	+	−	+
Unknown	0.11	−	−	−	−	−	−	−	−	−	+	−	+
Unknown	0.08	+	+	+	+	+	+	+	+	+	−	+	−

[a] C.P. = cold pressed; S.D. = steam distilled; + = present; − = absent; and ± = located in traces.

[1] Species of oranges were identified by Dr. A. T. El Wakil, Division of Fruit Research, Horticulture Department, Ministry of Agriculture U.A.R.; voucher specimens have been deposited at this institution.

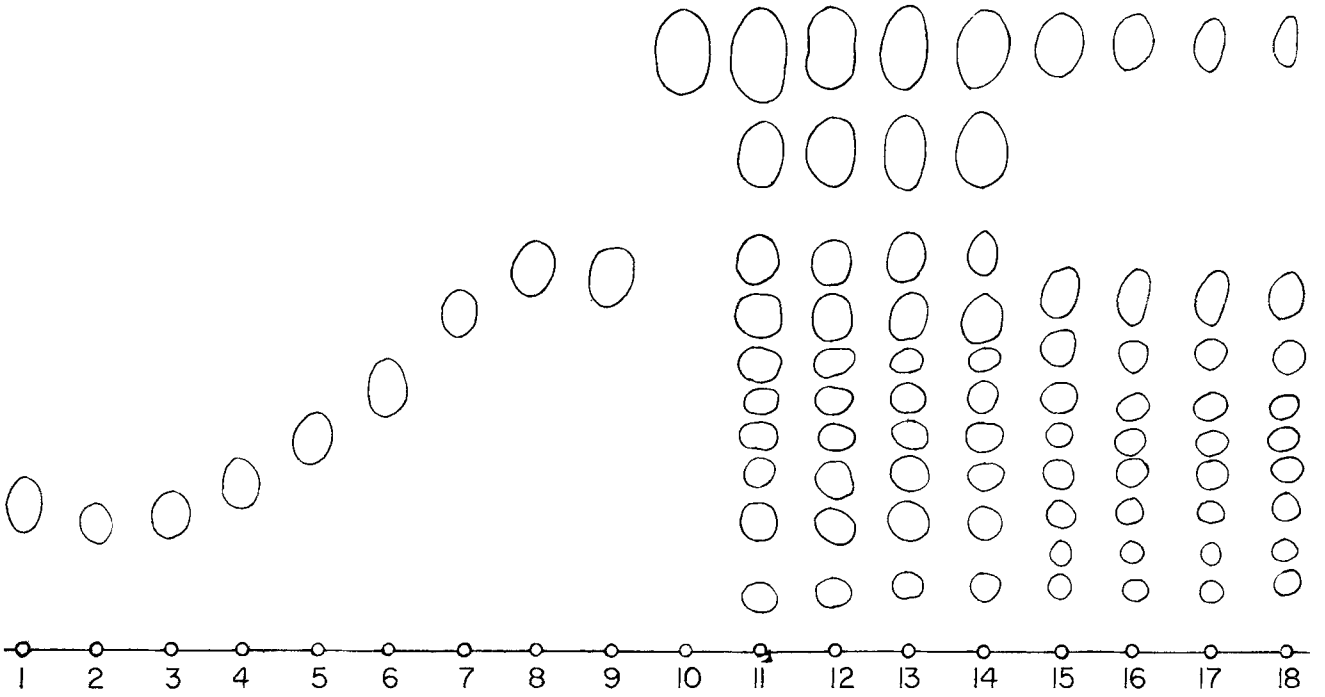

Figure 1—*TLC of the cold-pressed and steam-distilled peel oils of different types of* C. sinensis *L. The solvent used was* n*-hexane–ethyl acetate (85:15). Key: 1, citronellol; 2, nerol; 3, geraniol; 4, terpineol; 5, linalool; 6, citral; 7, citronellal; 8, geranyl acetate; 9, linalyl acetate; 10, limonene; 11, Egyptian orange oil (cold-pressed); 12, navel orange oil (cold-pressed); 13, blood orange oil (cold-pressed); 14, succari orange oil (cold-pressed); 15, Egyptian orange oil (steam-distilled); 16, navel orange oil (steam-distilled); 17, blood orange oil (steam-distilled); and 18, succari orange oil (steam-distilled).*

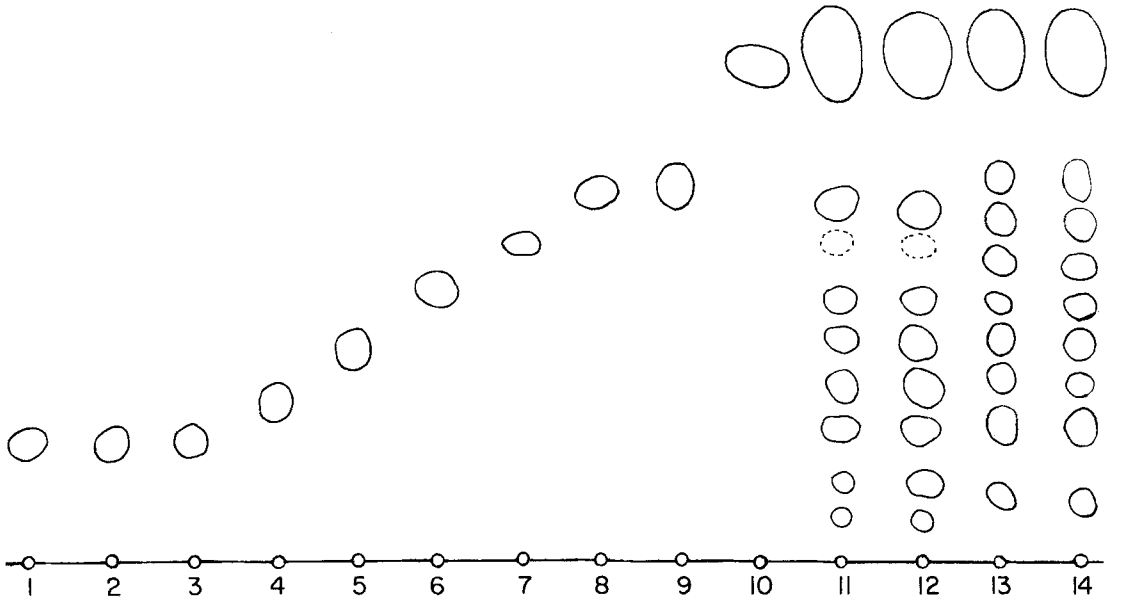

Figure 2—*TLC of the cold-pressed and steam-distilled peel oils of different types of* C. aurantium *L. The solvent used was* n*-hexane–ethyl acetate (85:15). Key: 1, citronellol; 2, nerol; 3, geraniol; 4, terpineol; 5, linalool; 6, citral; 7, citranellal; 8, geranyl acetate; 9, linalyl acetate; 10, limonene; 11, Egyptian bitter orange oil (steam-distilled); 12, Spanish bitter orange oil (steam-distilled); 13, Egyptian bitter orange oil (cold-pressed); and 14, Spanish bitter orange oil (cold-pressed).*

fully separated. These isolates could be used as reference materials for the GLC analyses of the investigated oils.

Column Chromatography—The oil of Egyptian bitter orange, containing most of the constituents of the investigated oils, was chosen for column chromatographic analysis. About 0.3 ml. of the oil was chromatographed on a 10-g. column of silica gel[2] (1-cm. diameter), using successively as eluents: petroleum ether (30–50°); petroleum ether containing 1, 2, and 5% ether; benzene; ether; and ethanol. Complete elution of any constituent was determined by applying a few drops of the effluent directly on silica gel G strips, evaporating the solvent, and spraying with the color reagent. The absence of any color indicated almost complete elution with the solvent. All fractions were then examined by TLC, using a series of microscope slides coated with silica gel G. The fractions were regrouped on the basis of identical R_f values, size, and color of spots, as well as their color under UV light. Results are shown in Table II.

GLC—GLC analyses were performed on an F&M model 500 equipped with flame-ionization detector apparatus (model 1609).

[2] British Drug House.

Table II—Column Chromatogram of the Cold-Pressed Peel Oil of *C. aurantium* L. (Egyptian Bitter Orange)

Number	Eluent	Fraction Number	Vol., ml.	R_f	Color with Spray Reagent	Authentic Sample
1	Petroleum ether	1 + 2	20	—	—	—
		3 to 11	140	0.96	Orange to green	Limonene
		12	10	—	—	—
2	(*a*) 1% Ether in petroleum ether	1 to 6	50	—	—	—
	(*b*) 2% Ether in petroleum ether	1 to 6	50	—	—	—
	(*c*) 5% Ether in petroleum ether	1	5	—	—	—
		2 to 4	60	1. 0.73	Purple to violet	Linalyl and geranyl acetate
				2. 0.40	Violet	Unknown
				3. 0.43	Orange to violet	Linalool
				4. 0.24	Orange to violet	Geraniol and/or nerol and/or citronellol
		5	5	5. —	—	—
3	Benzene	1	5	—	—	—
		2 to 4	30	0.72	Yellow	Unknown
		5 to 10	60	0.29	Orange to green	Terpineol
		11 to 13	15	—	—	—
4	Ether	1	10	—	—	—
		2 to 7	60	1. 0.51	Brown	Citral
				2. 0.08	Faint brown	Unknown
				3. 0.24	Fluorescent under UV	Unknown
				4. 0.19		Unknown
				5. 0.16		Unknown
				6. 0.12		Unknown
5	Ethanol	1	10	—	—	—
		2 to 4	50	Yellow band eluted and gave four unknown spots on TLC		

Mineral oil capillary column, 9144.0 cm. (300 ft.) in length, stainless steel, 0.025-cm. (0.01-in.) internal diameter, was used with the following operating conditions: injection port temperature, 275°; block temperature, 175°; column temperature, 90° (kept isothermal during the whole resolution); attenuation, 8; range, 10; chart speed, 30.48 cm. (12 in.)/hr.; carrier gas, nitrogen; inlet pressure, 7.2–7.5 kg.; and sample size, 0.5–0.8 μl.

The identification of the individual peaks of the chromatogram was achieved by comparing their relative retention distances with those of the different authentic samples under the same conditions. The relative retention distance of each peak was determined with respect to the peak corresponding to terpinene (No. 18), which occurred in all chromatograms of investigated samples and exhibited a sharp and well-defined peak.

The relative retention distances of the peaks in the sequence of their emergence, along with the weight percentages of the corresponding constituents, are listed in Tables III and IV. The numbers in the first column of the table refer to the numbers of the peaks in the order of their increasing relative retention distances.

Figures 3 and 4 show the gas chromatograms of the cold-pressed

Table III—Relative Percentage (w/w) of Terpene Hydrocarbons of Citrus Peel Oils[a]

Peak Number	Compound	d_{Rrel}	*C. sinensis* L. (Sweet Orange)								*C. aurantium* L. (Bitter Orange)			
			Egyptian		Navel		Succari		Blood		Egyptian		Spanish	
			C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.
1	Unknown	0.12	6.4	6.6	7.1	7.4	15.7	14.2	6.6	9.0	4.3	13.5	9.4	13.5
2	Unknown	0.18	2.8	1.6	4.00	4.0	6.9	5.9	4.1	8.2	3.4	7.1	5.1	7.2
2′	Unknown	0.24	—	—	—	—	—	0.9	—	—	—	—	—	—
3	Unknown	0.25	1.1	0.6	2.2	Traces	3.1	2.4	1.2	0.9	1.3	1.4	1.9	1.2
3′	Unknown	0.26	—	—	—	—	—	0.5	—	—	—	—	—	—
4	Unknown	0.32	—	0.4	Traces	—	1.0	1.4	0.4	0.5	—	1.6	—	1.5
5	α-Pinene	0.37	1.5	1.1	1.3	1.5	1.8	1.8	1.6	0.9	3.4	1.6	1.3	1.4
6	Sabinene	0.43	0.3	0.5	—	—	1.4	1.0	0.6	—	—	1.0	—	1.0
7	β-Pinene	0.47	5.3	1.2	5.1	2.6	3.2	2.2	3.9	2.8	8.8	4.0	3.8	3.8
8	Camphene	0.53	0.3	1.6	0.6	0.6	0.7	0.7	0.7	2.6	0.8	—	0.6	—
9	Unknown	0.55	—	—	—	—	—	—	—	0.2	—	—	—	—
10	Myrcene	0.61	0.5	1.1	2.7	1.1	1.7	Traces	2.6	1.3	0.7	2.5	0.9	2.3
12	Limonene	0.72	55.5	31.9	47.7	30.7	30.7	21.5	49.7	46.1	47.5	23.0	38.3	23.0
13	β-Phellandrene	0.83	—	—	—	—	—	0.6	Traces	2.1	1.7	2.8	Traces	1.6
14	Ocimene	0.85	0.5	2.5	Traces	2.5	Traces	0.4	2.4	5.8	1.1	—	2.6	—
15	*p*-Cymene	0.88	1.1	2.3	2.7	2.3	2.9	1.2	1.3	1.7	1.5	1.6	2.0	1.6
16	Unknown	0.90	0.3	1.7	1.2	1.7	1.3	2.4	0.5	Traces	1.1	2.3	1.0	2.2
17	Unknown	0.95	—	—	1.1	—	1.1	—	1.1	—	—	2.0	—	2.0
18	Terpinene	1.00	2.2	10.4	2.3	10.6	2.3	3.6	3.2	6.5	4.2	5.4	3.00	5.4
19	Unknown	1.04	2.8	5.3	2.7	5.3	5.8	3.2	2.7	0.9	0.1	1.8	4.2	1.8
19′	Unknown	1.06	—	—	—	—	—	1.0	—	—	—	—	—	—
20	Unknown	1.08	2.0	3.2	1.8	3.2	2.9	6.3	1.7	1.1	1.0	4.3	2.5	4.2
20′	Unknown	1.09	—	—	—	—	—	—	—	—	0.2	—	—	—
	Total Terpene Hydrocarbons		77.7	74.0	82.1	74.5	81.5	71.4	84.3	90.5	81.0	76.5	76.9	73.7

[a] d_{Rrel} = relative retention distance; C.P. = cold pressed; and S.D. = steam distilled.

Table IV—Relative Percentage (w/w) of Oxygenated Hydrocarbons of Citrus Peel Oils[a]

Peak Number	Compound	d_{Rrel}	*C. sinensis* L. (Sweet Orange)								*C. aurantium* L. (Bitter Orange)			
			Egyptian		Navel		Succari		Blood		Egyptian		Spanish	
			C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.	C.P.	S.D.
21	Unknown	1.15	2.0	0.5	0.6	0.5	0.5	Traces	0.6	0.7	0.6	—	0.6	—
22	Terpineol	1.18	0.6	0.7	1.0	0.7	1.0	1.4	1.0	1.1	1.1	1.6	1.4	1.6
23	Unknown	1.23	2.0	0.6	—	0.6	—	1.1	—	—	—	1.6	—	1.6
24	Citronellal	1.26	0.7	0.5	0.5	0.5	0.6	1.0	0.4	0.1	1.1	0.7	0.3	0.8
25	Unknown	1.3	—	—	2.5	Traces	1.1	Traces	—	—	0.1	0.6	0.5	0.6
26	Linalool	1.33	2.1	5.2	1.2	3.2	4.0	7.2	2.1	2.8	1.9	3.0	4.7	3.1
27	Linalyl acetate	1.37	2.2	1.4	2.5	1.5	2.6	2.1	2.0	1.2	1.9	1.0	1.5	1.0
27′	Unknown	1.43	—	—	—	—	—	—	—	—	1.3	—	—	—
28	α-Citral	1.51	1.1	1.1	1.1	0.9	1.0	1.0	1.0	0.1	0.5	1.0	0.9	1.0
29	Citronellol	1.64	1.0	—	—	—	—	—	0.7	—	0.4	—	—	—
29′	β-Citral	1.76	—	—	—	1.1	—	Traces	—	Traces	—	0.3	4.2	0.2
30	Nerol, and farnesol, and unknown	1.82	7.1	5.5	0.5	5.6	6.4	2.8	7.8	2.3	5.2	5.0	3.7	5.1
31	Geraniol	1.89	0.8	1.1	1.4	1.1	1.1	4.9	1.1	0.2	0.7	3.0	2.0	3.0
32	Geranyl acetate	2.05	1.0	6.5	2.1	5.5	0.4	2.2	0.7	0.2	0.7	3.7	1.4	3.7
32′	Unknown	2.14	—	4.4	—	4.4	—	—	—	—	—	—	—	—
33	Methyl anthranilate	2.4	0.5	0.1	0.4	0.1	0.4	0.4	Traces	Traces	Traces	1.0	0.3	1.0
34	Unknown	2.5	—	—	—	Traces	—	—	—	—	—	0.3	—	0.6
35	Unknown	2.6	—	—	—	—	—	—	—	—	—	0.3	—	0.3
	Total Oxygenated Hydrocarbons		21.1	27.6	13.7	25.7	19.0	24.10	16.3	8.7	15.5	23.1	21.5	23.6

[a] d_{Rrel} = relative retention distance; C.P. = cold pressed; and S.D. = steam distilled.

and steam-distilled peel oils of the Egyptian bitter orange, respectively.

IR Analysis—To study the potentialities of IR spectroscopy in the qualitative comparison between cold-pressed and steam-distilled peel oils of different oranges, those of Egyptian orange were studied as an example. The spectra of both the cold-pressed and steam-distilled peel oils of the Egyptian orange were made with a Unicam SP 200 spectrophotometer, using potassium bromide disks smeared lightly with the investigated oil. The charts are shown in Figs. 5 and 6.

RESULTS AND DISCUSSION

TLC chromatograms (Figs. 1 and 2) of the citrus peel oils investigated were well developed by *n*-hexane–ethyl acetate (85:15). Constituents corresponding to the major spots could be isolated by preparative TLC and analyzed by GLC. It was possible to chromatograph 5 ml. of the oil on 10 plates at one time. The terpenes were successfully separated from the oxygenated hydrocarbons by column chromatography.

In a previous communication (15), it was reported that mineral oil capillary columns were very efficient in the separation of the constituents of the volatile oils of some *Labiatae* plants. This process has been successfully applied to the citrus peel oils under investigation. The following constituents were determined qualitatively and quantitatively.

Hydrocarbons—The terpene hydrocarbons gave one spot on TLC and emerged together in the front zone from the column with petroleum ether. However, the GLC study of the terpene fraction, isolated by TLC and by silica gel column, showed the following. α-Pinene, β-pinene, limonene, and terpinene were identified in all the oils under investigation. The occurrence of the other constituents in the investigated oils is shown in Tables II and III.

Limonene normally accounted for the largest content of any constituent of citrus oils being present in higher concentration in the cold-pressed (30.7–55.5%) than in the steam-distilled peel oils (21.5–46.1). α-Pinene occurred in low concentrations in the investigated oils, ranging from 0.9 to 3.4%. β-Pinene, however, was more abundant than α-pinene; its concentration ranged from 1.12 to 8.8% in the examined oils. The cold-pressed oil of the Egyptian bitter orange showed the highest concentration of α-pinene and β-pinene, 3.4 and 8.8%, respectively.

Sabinene content reached up to 1.4% in cold-pressed oil of succari orange. Camphene occurred in low concentrations, ranging from 0.3 to 2.5% in the investigated oils. Myrcene and β-phellandrene were identified (up to 2.6 and 2.8%, respectively) in all the oils. Ocimene and *p*-cymene were present in low to moderate concentrations, up to 5.8 and 2.9%, respectively. Terpinene occurred in higher concentrations in the steam-distilled than in the cold-pressed oils, being 3.6–10.6% in the former oils and 2.2–4.2% in the latter oils.

Thirteen additional unknown peaks were located in the chromatograms of the investigated oils, as presented in Table III.

From the previous data, it is clear that the terpene hydrocarbons constitute the higher proportion of the total makeup of the investigated peel oils, being slightly higher in the cold-pressed oils (77.5–85.6%) than in the steam-distilled oils (72.0–75.6%). The hydrocarbon content of the cold-pressed oil of blood orange (85.6%)

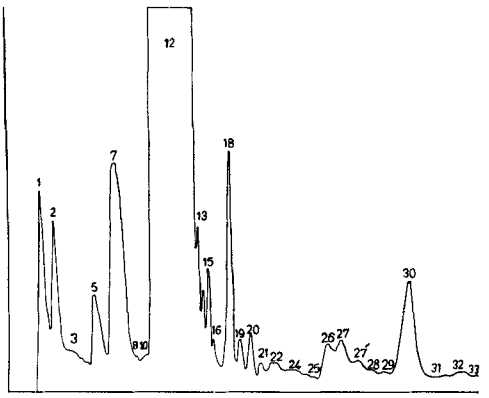

Figure 3—*GLC of the cold-pressed peel oil of Egyptian bitter orange.*

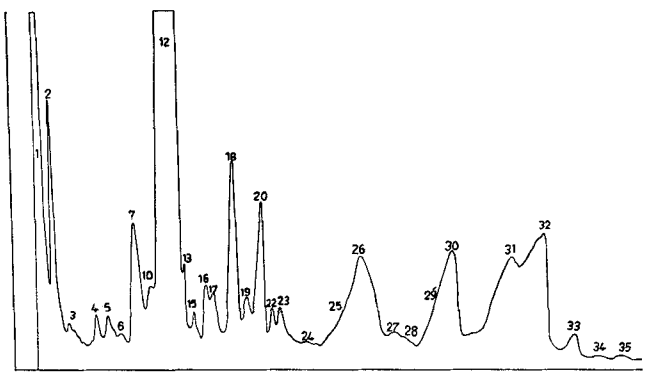

Figure 4—*GLC of the steam-distilled peel oil of Egyptian bitter orange.*

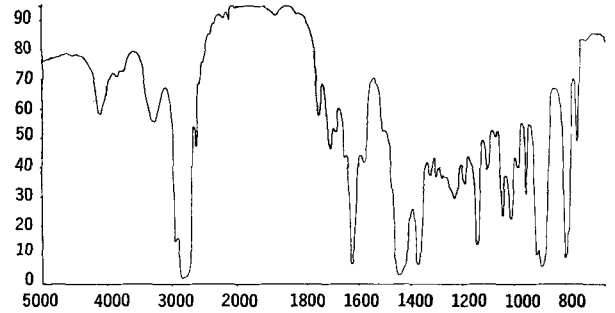

Figure 5—*IR spectrum of cold-pressed peel oil of Egyptian sweet orange.*

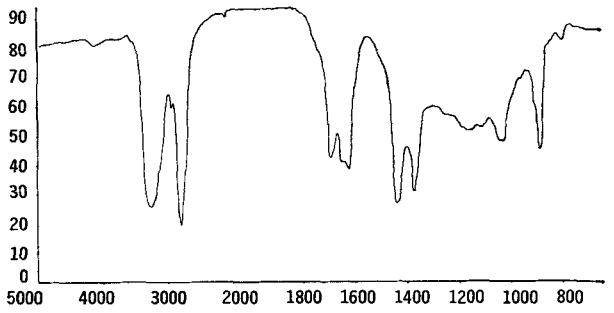

Figure 6—*IR spectrum of steam-distilled peel oil of Egyptian sweet orange.*

is exceptionally lower than that of the respective steam-distilled oil (90.4%).

Oxygenated Hydrocarbons—The oxygenated hydrocarbons were resolved into many spots by TLC, emerged into different zones by various eluents on a silica gel column, and gave numerous peaks by GLC. The following constituents were identified when compared with the respective authentic samples.

Linalool, terpineol, geraniol and/or nerol and/or citronellol and/or farnesol, citronellal, citral, linalyl acetate, and geranyl acetate were of common occurrence in the investigated oils. Methyl anthranilate, although detected in all the oils by GLC, could not be located in the TLC chromatograms of the steam-distilled oils (blue fluorescence under UV). This indicates that the steam-distilled oils may contain a substance that separates in the same zone as methyl anthranilate, quenching its fluorescence.

Linalool was located as one spot by TLC; it was eluted from the column together with other alcohols such as geraniol, nerol, citronellol, farnesol, and certain esters by 5% ether in petroleum ether. However, it gave a well-defined peak by GLC. Linalool content ranged from 2.8 to 7.2% in the steam-distilled oils and from 1.2 to 4.7% in the cold-pressed oils. Terpineol and geraniol were of low concentration in the investigated oils, ranging from 0.6 to 1.4% and from 0.2 to 4.9%, respectively. Terpineol gave one spot on TLC and was eluted from the column by benzene.

Citronellol was identified in the cold-pressed oils of Egyptian orange (1.0%), blood orange (0.7%), and Egyptian bitter orange (0.4%). Nerol and farnesol, together with other unidentified components, emerged as a broad peak, ranging from 0.5 to 7.8%. Citronellal gave almost the same R_f as esters by TLC, yet it could be identified in the investigated oils by GLC. It ranged from traces to 1.1%. Citral gave one spot on TLC and emerged from the column together with other unidentified components on ether elution. By GLC, authentic citral showed two peaks of different retention distances (1.5 and 1.8), which are expected to correspond to α- and β-citral, respectively. The first peak was identified in all the investigated oils, ranging from trace to 1.1%; the second peak appeared as a shoulder in steam-distilled oils of navel orange, succari orange, blood orange, and both kinds of bitter orange and in a comparatively high concentration (4.2%) in cold-pressed oil of Spanish bitter orange.

Linalyl acetate occurred in the cold-pressed oils in a comparatively moderate concentration, ranging from 1.5 to 2.6% and from 2.3 to 2.1% in the respective steam-distilled oils. Geranyl acetate was of a lower concentration than linalyl acetate. In the case of the steam-distilled oils of Egyptian and navel orange, the peak corresponding to geranyl acetate was flat due to the probable presence of other components of the same retention distance; its concentration was 6.5% in both oils. Methyl anthranilate concentration in the cold-pressed oils was usually higher than in the steam-distilled oils and ranged from a trace to 0.5%.

Seven unknown peaks were also located in the chromatograms of one or more of the investigated oils, as shown in Table IV. The total oxygenated constituents ranged from 14.4 to 19.2% in the cold-pressed oils and from 9.5 to 25.6% in the steam-distilled oils.

With respect to the IR spectroscopy, the spectra of the cold-pressed oil (Fig. 5) and steam-distilled oil (Fig. 6) of Egyptian orange peel show common bands and differ in others with respect to their position and comparative intensity. The broad band at about 3430 cm.$^{-1}$ measures the free alcohols, *e.g.*, citronellol, geraniol, linalool, and some other low boiling alcohols. This band is more intense in the steam-distilled than in the cold-pressed oil due to the probable hydrolysis of the esters during the distillation process resulting in a higher alcohol content. The unresolved bands at about 2960, 2930, and 2860 cm.$^{-1}$ are due to the C—H stretching frequencies of the CH_3 and CH_2 groups and are a measure of the aliphatic constituents in the oil. They are more pronounced in the cold-pressed than in the steam-distilled oil. Acetic esters appear clearly at about 1740 cm.$^{-1}$ in the cold-pressed oil and are almost absent in the steam-distilled oil. The small band at 1665 cm.$^{-1}$ is the C=C stretching frequency of the isopropylidene group, common to the constituents of both oils. There is a small band at about 1680 cm.$^{-1}$ which corresponds to citral and appears for both steam-distilled and cold-pressed oils. Citronellol and geraniol show absorptions at 1059 and 1003 cm.$^{-1}$, respectively, and are more distinct in the cold-pressed oil than in the respective steam-distilled oil. A common band at 890 cm.$^{-1}$, somewhat larger in the cold-pressed oil than in the steam-distilled oil, is due to the isopropenyl band of the sesquiterpenes.

On examining the IR patterns of the investigated cold-pressed and steam-distilled peel oils of Egyptian orange, it is evident that the cold-pressed oil spectra show numerous bands which are weak or completely absent in the spectra of the steam-distilled oil of the same species. Bands at about 845–920, 1300–1500, and 3900–4400 cm.$^{-1}$ are much stronger in the case of the cold-pressed oil. Bands at about 740–765, 800, and 940–970 cm.$^{-1}$, although strongly exhibited in the spectra of the cold-pressed oil, are nearly absent in those of the steam-distilled oil.

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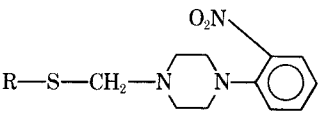
Keyphrases □ Hydrazones of 1-phenyl-, 1-benzyl-, and 1-benzhydryl-4-aminopiperazines—synthesis □ Antimicrobial activity—hydrazones □ Structure-activity relationships—hydrazones □ IR spectrophotometry—structure

Numerous piperazine derivatives have been reported in the literature to have antibacterial activity. For example, Nakanishi and Muro (1) noted that the unsymmetrically 1,4-disubstituted piperazines (I) were



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useful bactericides and fungicides. Pedrazzoli and Dall'-Asta (2) synthesized 1-[2-(2,4-dichlorobenzoyloxy)-2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine and a number of other related 1-phenethyl-4-methylpiperazine derivatives, and they reported that these compounds exhibited both trichomonicide and amebicide activities. Hydrazones containing the piperazine nucleus have recently been examined as potential antimicrobial agents. Kozhukharov and Kharizanova (3) reported that the diisonicotinyl hydrazone of 1,4-bis(benzoylethyl)piperazine showed tuberculostatic activity. Recent work by Prescott *et al.* (4) indicated that the bishydrazones obtained by reacting 1,4-diaminopiperazine with salicylaldehyde, 5-nitrosalicylaldehyde, and 3,4-dichlorobenzaldehyde showed a high *in vitro* antibacterial activity against *Staphylococcus aureus*.

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EXPERIMENTAL[1]

Chemical Synthesis—All melting points were determined using a Thomas-Hoover Unimelt apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer model 237B spectrophotometer and were in agreement with the assigned structures.

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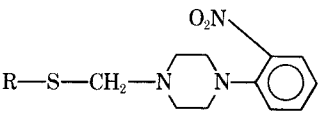

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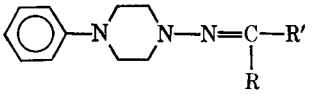
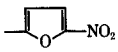
Table I—Hydrazones of 1-Phenyl-4-aminopiperazine



Compound Number	R	R′	Yield, %	M.p.	Recrystallization Solvent	Formula	Analysis, % Calcd.	Found
1	H	2-HOC_6H_4	74	139.5–141.5°	EtOH	$C_{17}H_{19}N_3O$	C, 72.57 H, 6.81 N, 14.93	C, 72.59 H, 7.02 N, 14.61
2	H	3-HOC_6H_4	34	195–197°	EtOH	$C_{17}H_{19}N_3O$	C, 72.57 H, 6.81 N, 14.93	C, 72.68 H, 6.87 N, 14.54
3	H	4-$(C_2H_5)_2NC_6H_4$	76	192.5–193.5°	EtOH–$CHCl_3$	$C_{21}H_{28}N_4$	C, 74.96 H, 8.39 N, 16.65	C, 75.15 H, 8.17 N, 17.02
4	H	5-NO_2-2-HOC_6H_3	98	223–225°	—[a]	$C_{17}H_{18}N_4O_3$	C, 62.57 H, 5.56 N, 17.17	C, 62.92 H, 5.78 N, 17.08
5	H	3,4-$Cl_2C_6H_3$	93	176–177.5°	Me_2CO	$C_{17}H_{17}Cl_2N_3$	C, 61.09 H, 5.13 N, 12.57	C, 60.76 H, 4.68 N, 12.98
6	H		40	152–154°	EtOH–Me_2CO	$C_{15}H_{16}N_4O_3$	C, 59.99 H, 5.37 N, 18.66	C, 60.35 H, 5.37 N, 18.62
7	H		98	163–164.5°	EtOH	$C_{16}H_{18}N_4$	C, 72.15 H, 6.81 N, 21.04	C, 72.19 H, 6.81 N, 20.82
8	H		72	116.5–118.5°	EtOH–H_2O	$C_{16}H_{18}N_4$	C, 72.15 H, 6.81 N, 21.04	C, 72.22 H, 6.86 N, 20.86
9	C_2H_5	4-$CH_3OC_6H_4$	18	140–142°	EtOH–H_2O	$C_{20}H_{25}N_3O$	C, 74.27 H, 7.79 N, 12.99	C, 74.09 H, 7.89 N, 12.95
10	CH_3	2-$HOC_6H_4CH{=}CH$	40	181–182°	EtOH–H_2O	$C_{20}H_{23}N_3O$	C, 74.74 H, 7.21 N, 13.07	C, 74.63 H, 6.55 N, 12.84
11	CH_3	4-$CH_3OC_6H_4CH{=}CH$	38	179–181°	EtOH	$C_{21}H_{25}N_3O$	C, 75.19 H, 7.51 N, 12.53	C, 75.35 H, 7.57 N, 12.68

[a] Washed with acetone.

mixture was refluxed for 1 hr. and cooled. The product was collected and recrystallized from a suitable solvent.

Solvents for recrystallization and physical data for the hydrazones are listed in Tables I–III.

Microbiological Evaluation—Representative members of six genera of bacteria and two genera of fungi were used in the screening of the new hydrazones for antimicrobial activity.[2] The six bacterial genera were represented by two Gram-positive bacteria, *S. aureus* and *Bacillus subtilis*, two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, one acid-fast bacterium, *Mycobacterium phlei*, and one anaerobic Gram-positive bacterium, *Clostridium perfringens*. The two fungi were *Saccharomyces cerevisiae* and *Candida albicans*. With the exception of *Cl. perfringens*, the bacteria were grown at 37° for 18 hr. and maintained thereafter at 4° on brain-heart infusion agar slopes[3]. *Cl. perfringens* was grown and maintained under similar conditions in cooked meat medium.[4] The fungi were similarly treated, but grown on slopes of Sabouraud's dextrose agar.[3] Subcultures were made from these stock cultures when necessary.

Preparation of Microorganisms for Evaluation—S. aureus, B. subtilis, E. coli, and *P. aeruginosa* were subcultured from the stock cultures to brain-heart infusion slopes and these were incubated at 37° for 18–20 hr. The bacterial growth was then washed from these slopes with brain-heart infusion broth (1 ml., Difco). This suspension of organisms was diluted 1:1000 with the same broth to provide a count of 1×10^6 to 1×10^7 viable organisms/milliliter.

Cl. perfringens was subcultured into cooked meat medium and incubated in a water bath for 18–20 hr. at 37°. This subculture (1 ml.) was then transferred to brain-heart infusion broth (10 ml.) containing sodium thioglycollate (0.1% w/v), which had been previously boiled to drive off dissolved oxygen from the medium. This broth culture was incubated in a water bath at 37° for 3 hr. and diluted 1:1000 in similar broth.

In the case of *M. phlei*, transfer was made from the stock culture to a sterile conical flask containing brain-heart infusion broth (20 ml.) and an aqueous solution of polysorbate 80[5] (10% w/v, 0.01 ml.). The flask was incubated at 37° for 3 days on a shaker. A dilution of 1:1000 was made at the end of the incubation period in similar broth.

The two cultures of fungi were prepared by inoculating Sabouraud's dextrose broth (20 ml.) with the organisms from the stock cultures. The subcultures after the incubation period of 18–20 hr. at 37° were diluted 1:1000 with the same broth.

Determination of Antimicrobial Activity—The test compounds in acetone were screened for antimicrobial activity at a concentration of 30 mcg./ml. Hydrazones that exhibited activity at this level were studied further to determine their minimum inhibitory concentrations (MIC) by an *in vitro* serial twofold dilution procedure. Broth cultures containing no compound were incorporated as positive controls. After each MIC determination, a loopful of solution from each dilution tube showing complete inhibition of bacterial growth was streaked out on brain-heart infusion agar plates, or on Sabouraud's dextrose agar plates in the case of *S. cerevisiae*, to determine whether the action of the compound was bactericidal or bacteriostatic in nature. The results are listed in Table IV.

RESULTS AND DISCUSSION

The hydrazones synthesized in this work were found to be insoluble in water and, in spite of the basic piperazine nucleus, they were only slightly soluble in 5 *N* HCl. A search of the literature (11, 12) revealed that ethanol and acetone had been used as solvents in screening potential antibacterial compounds. Since the hydrazone

[2] These microorganisms were obtained from the Department of Microbiology, Dalhousie University, Halifax, Nova Scotia, Canada.
[3] Baltimore Biological Laboratories, Baltimore, Md.
[4] Difco Laboratories, Detroit, Mich.
[5] Tween 80.

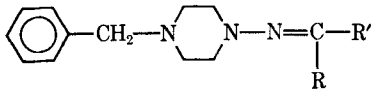


Table II—Hydrazones of 1-Benzyl-4-aminopiperazine

Compound Number	R	R′	Yield, %	M.p.	Recrystallization Solvent	Formula	Analysis, % Calcd.	Found
12	H	2-HOC_6H_4	77	135–136.5°	EtOH	$C_{18}H_{21}N_3O$	C, 73.19 H, 7.17 N, 14.23	C, 73.48 H, 7.09 N, 14.01
13	H	3-HOC_6H_4	80	179–181°	EtOH	$C_{18}H_{21}N_3O$	C, 73.19 H, 7.17 N, 14.23	C, 73.30 H, 6.89 N, 13.90
14	H	4-$(C_2H_5)_2NC_6H_4$	75	78.5–80°	EtOH–H_2O	$C_{22}H_{30}N_4$	C, 75.39 H, 8.63 N, 15.99	C, 75.67 H, 8.59 N, 15.88
15	H	5-NO_2,2-HOC_6H_3	67	159–160°	EtOH–Me_2CO	$C_{18}H_{20}N_4O_3$	C, 63.52 H, 5.92 N, 16.46	C, 63.54 H, 5.96 N, 15.70
16	H	3,4-$Cl_2C_6H_3$	75	83–85°	EtOH	$C_{18}H_{19}Cl_2N_3$	C, 62.08 H, 5.50 N, 12.07	C, 62.23 H, 5.52 N, 12.34
17	H		49	96.5–98°	EtOH	$C_{16}H_{18}N_4O_3$	C, 61.14 H, 5.77 N, 17.82	C, 61.12 H, 5.96 N, 17.59
18	H		63	95–98°	EtOH–H_2O	$C_{17}H_{20}N_4$	C, 72.83 H, 7.19 N, 19.98	C, 73.28 H, 7.14 N, 19.78
19	H		64	104.5–106°	EtOH–H_2O	$C_{17}H_{20}N_4$	C, 72.83 H, 7.19 N, 19.98	C, 72.85 H, 6.96 N, 19.85
20	C_2H_5	4-$CH_3OC_6H_4$	98	68–70°	Me_2CO	$C_{21}H_{27}N_3O$	C, 74.74 H, 8.07 N, 12.45	C, 74.79 H, 8.11 N, 12.30
21	CH_3	2-HO—C_6H_4CH=CH	51	145–147°	—[a]	$C_{21}H_{25}N_3O$	C, 75.19 H, 7.51 N, 12.53	C, 74.94 H, 7.36 N, 12.38
22	CH_3	4-$CH_3OC_6H_4$CH=CH	33	103.5–104.5°	EtOH–H_2O	$C_{22}H_{27}N_3O$	C, 75.61 H, 7.79 N, 12.02	C, 75.80 H, 7.89 N, 11.84

[a] Washed with petroleum ether.

compounds dissolved readily in acetone, all microbiological evaluations were carried out in this solvent. In a series of experiments designed to study the effect of acetone on the growth of *S. aureus*, *B. subtilis*, and *E. coli*, acetone did not have the ability to inhibit the growth of these bacteria below 12.5% v/v. The acetone content of all the test solutions was very much below this concentration. For this reason, it was assumed that any activity observed in the testings was due to the compound tested and not to the solvent itself.

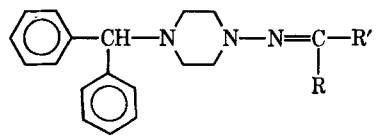


Table III—Hydrazones of 1-Benzhydryl-4-aminopiperazine

Compound Number	R	R′	Yield, %	M.p.	Recrystallization Solvent	Formula	Analysis, % Calcd.	Found
23	H	2-HOC_6H_4	50	165–166°	EtOH–Me_2CO	$C_{24}H_{25}N_3O$	C, 77.60 H, 6.78 N, 11.31	C, 77.48 H, 6.76 N, 11.41
24	H	4-$(C_2H_5)_2NC_6H_4$	42	123–124°	EtOH	$C_{28}H_{34}N_4$	C, 78.83 H, 8.03 N, 13.13	C, 79.11 H, 8.00 N, 13.26
25	H	5-NO_2,2-HOC_6H_3	64	193.5–195°	Me_2CO	$C_{24}H_{24}N_4O_3$	C, 69.21 H, 5.81 N, 13.45	C, 69.43 H, 5.81 N, 13.18
26	H	3,4-$Cl_2C_6H_3$	67	135.5–136.5°	EtOH–Me_2CO	$C_{24}H_{23}Cl_2N_3$	C, 67.93 H, 5.46 N, 9.90	C, 68.20 H, 5.43 N, 9.69
27	H		50	147–148°	EtOH	$C_{22}H_{22}N_4O_3$	C, 67.68 H, 5.68 N, 14.35	C, 67.42 H, 5.62 N, 14.29
28	H		73	122–124°	EtOH–H_2O	$C_{23}H_{24}N_4$	C, 77.50 H, 6.79 N, 15.72	C, 77.43 H, 6.77 N, 15.65
29	H		82	128.5–130.5°	EtOH–H_2O	$C_{23}H_{24}N_4$	C, 77.50 H, 6.77 N, 15.72	C, 77.35 H, 6.52 N, 15.63
30	CH_3	4-$CH_3OC_6H_4$CH=CH	52	175–177°	EtOH–Me_2CO	$C_{28}H_{31}N_3O$	C, 79.02 H, 7.34 N, 9.87	C, 78.91 H, 6.94 N, 9.88

Table IV—Approximate MIC (mcg./ml.) of Some Hydrazones

Compound Number	Compound	*S. aureus*[a]	*B. subtilis*[a]	*Cl. perfringens*[b]	*M. phlei*[c]	*S. cerevisiae*[a]
6	1-Phenyl-4-(5-nitro-2-furfurylidene-amino)piperazine	—[d]	1.56[g]	15[g]	—	—
15	1-Benzyl-4-(5-nitro-2-hydroxybenzyl-ideneamino)piperazine	25[e,f]	—	—	—	—
17	1-Benzyl-4-(5-nitro-2-furfurylidene-amino)piperazine	—	6.25[g,h]	—	—	—
22	1-Benzyl-4-[4-(4-methoxyphenyl)-3-butene-2-ylideneamino]piperazine	—	—	—	15[f]	—
27	1-Benzhydryl-4-(5-nitro-2-furfuryl-ideneamino)piperazine	—	0.78[g]	15[g,i]	—	—
28	1-Benzhydryl-4-isonicotinylidene-aminopiperazine	6.25[f]	6.25[f]	—	30[f,j]	15[k]
29	1-Benzhydryl-4-nicotinylideneamino-piperazine	—	—	—	30[f,j]	—

[a] Determined after 18–20 hr. of incubation at 37°. [b] Determined after 16–18 hr. of incubation at 37° under anaerobic conditions. [c] Determined after 48 hr. of incubation at 37°. [d] No inhibition of growth at 30 mcg./ml. [e] Partial inhibition at 12.5, 6.25, and 3.13 mcg./ml. [f] Bacteriostatic at MIC. [g] Bactericidal at MIC. [h] Partial inhibition at 3.13 and 1.56 mcg./ml. [i] Partial inhibition at 7.5, 3.8, and 1.9 mcg./ml. [j] Partial inhibition at 15 mcg./ml. [k] Fungistatic at MIC.

The new compounds were screened for the antimicrobial activity against six genera of bacteria and two genera of fungi, as listed in the *Experimental* section, at a concentration of 30 mcg./ml. It was believed that any significant activity should be detected at this level. Results of the screening indicated that *S. aureus* was susceptible to Compounds 15 and 28; *B. subtilis* to Compounds 6, 17, 27, and 28; *Cl. perfringens* to Compounds 6 and 27; and *M. phlei* to Compounds 22, 28, and 29. The Gram-negative *E. coli* and *P. aeruginosa* were not sensitive to any hydrazone. Only Compound 29 showed antifungal activity against *S. cerevisiae*. Not one hydrazone was active against *C. albicans*.

On the basis of the results of the microbiological assays, it was not possible to correlate clearly the structure of the hydrazones with the antimicrobial activity. However, a few observations were rather obvious.

1. The hydrazones of 1-phenyl-, 1-benzyl-, and 1-benzhydryl-4-aminopiperazines derived from ketones [for example, 4-methoxypropriophenone and 1-(2-hydroxyphenyl)-3-buten-2-one] and from substituted benzaldehydes (for example, 2-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 4-diethylaminobenzaldehyde, and 3,4-dichlorobenzaldehyde) did not show any *in vitro* antibacterial or antifungal activity. However, the hydrazones prepared from the heterocyclic aldehydes (for example, 5-nitro-2-furfural, isonicotinaldehyde, and nicotinaldehyde) were more promising as potential antimicrobial agents.

2. Of the four hydrazones that showed antibacterial activity against *B. subtilis*, three were 5-nitrofuran derivatives. Compound 27 was the most active, having a MIC value of 0.78 mcg./ml. Replacing the 5-nitro-2-furfuryl group in this compound with the 4-pyridyl group caused a decrease in activity, as indicated by the higher MIC value (6.25 mcg./ml.) for Compound 28. However, replacing the 5-nitro-2-furfuryl group with the 3-pyridyl group destroyed or greatly reduced the activity against *B. subtilis*.

3. When the aldehyde moiety in the hydrazone structures was identical, the 1-benzhydrylpiperazine derivatives appeared to give the best activity. For example, Compound 27 was more active than Compounds 6 and 15 against *B. subtilis*. Although both Compounds 6 and 27 had the same MIC value (15 mcg./ml.) against *Cl. perfringens*, Compound 27 caused partial inhibition of bacterial growth at a concentration of 1.9 mcg./ml. Again, Compound 28 was more active against *S. aureus* and *B. subtilis* than the corresponding Compounds 7 and 18.

4. The presence of a nitro group seemed to have a favorable effect on the antimicrobial activity of the compounds. Four of the seven active hydrazones contained a nitro group in the molecule. Perhaps the best illustration was that while Compound 12 did not show any activity against *S. aureus* at a concentration of 30 mcg./ml., Compound 15 did inhibit bacterial growth at a concentration as low as 3.13 mcg./ml.

5. Prescott *et al.* (4) reported that 1,4-bis(2-hydroxybenzylideneamino)piperazine, 1,4-bis(5-nitro-2-hydroxybenzylideneamino)-piperazine, and 1,4-bis(3,4-dichlorobenzylideneamino)piperazine could completely suppress the growth of *S. aureus* at a concentration of 4 mcg./ml. With the exception of Compound 15, the corresponding hydrazones of 1-phenyl-, 1-benzyl-, and 1-benzhydryl-4-aminopiperazines were not growth inhibitors of *S. aureus*. Even Compound 15 was about six times less active than 1,4-bis(5-nitro-2-hydroxybenzylideneamino)piperazine. On the basis of these observations, it would appear that in this type of compound the antibacterial activity against *S. aureus* was a function of the hydrazone moiety, although the piperazine nucleus might have had some favorable influence.

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Tumor-Localizing Agents X: Radioiodinated Hypoglycemic Agents

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Abstract □ This study was undertaken to find a pancreas-specific photoscanning agent. 1-(*p*-Iodobenzenesulfonyl)-3-*n*-propylurea-^{125}I was synthesized for this purpose, since it contains a gamma-emitting radionuclide and there seemed to be a reasonable possibility that a sulfonylurea such as this might concentrate in the pancreas to a greater extent than in other tissues and organs. Tissue-distribution studies with this compound showed that it has no particular predilection for the pancreas of dogs.

Keyphrases □ Tumor-localizing agents—radioiodinated hypoglycemic agents □ 1-(*p*-Iodobenzenesulfonyl)-3-*n*-propylurea-^{125}I—synthesis □ Tissue distribution—radioiodinated hypoglycemic agent □ TLC—separation, identification □ IR spectrophotometry—identification □ NMR spectroscopy—identification □ Scintillometry—analysis

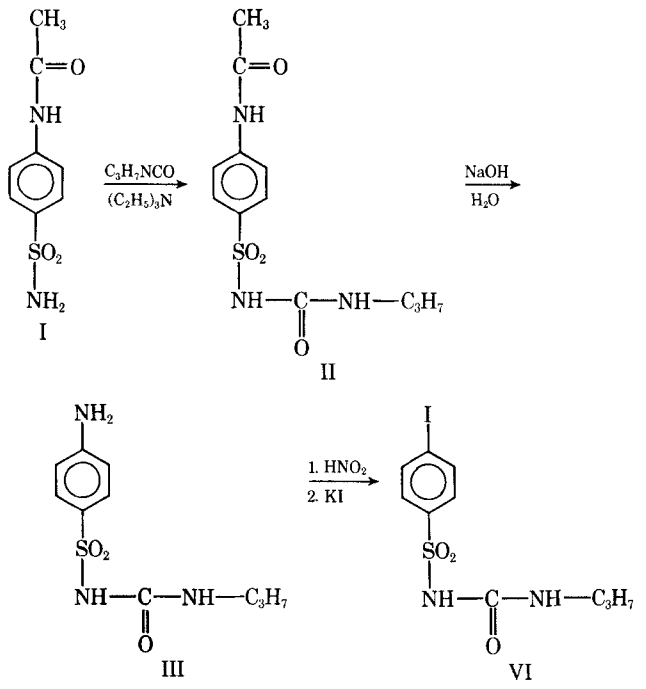
This research is part of a continuing program to develop new radioscanning agents for selected tumors. The present study was undertaken to find an agent that would selectively concentrate in the pancreas. Such a compound, when labeled with a gamma-emitting radionuclide, could be used in conjunction with external scintillation scanners and cameras for the diagnosis of pancreatic carcinoma. This type of carcinoma is particularly difficult to diagnose by existing methods (1).

The task of designing an agent to accomplish this purpose was somewhat difficult because very few substances are known to concentrate specifically in pancreatic tissue. A number of ^{14}C- and ^{35}S-labeled amino acids have been shown to concentrate in the pancreas following their intravenous administration (2–4). These substances do not, however, contain gamma-emitting radionuclides and cannot be used for external scanning purposes. Further studies along these lines, however, indicated that replacement of the sulfur of methionine with gamma-emitting ^{75}Se could be accomplished without destroying the amino acid's predilection for the pancreas (5). Subsequent clinical evaluations of 1-selenomethionine-^{75}Se resulted in mixed conclusions regarding its value as a pancreatic scanning agent (6, 7). More recently, it was found that certain radioiodinated phenylalanine analogs also display a high degree of specificity for concentrating in the pancreas of mice (8–10), but no selective localization could be achieved in dogs or monkeys (11). Consequently, studies with radioiodinated phenylalanine derivatives were discontinued.

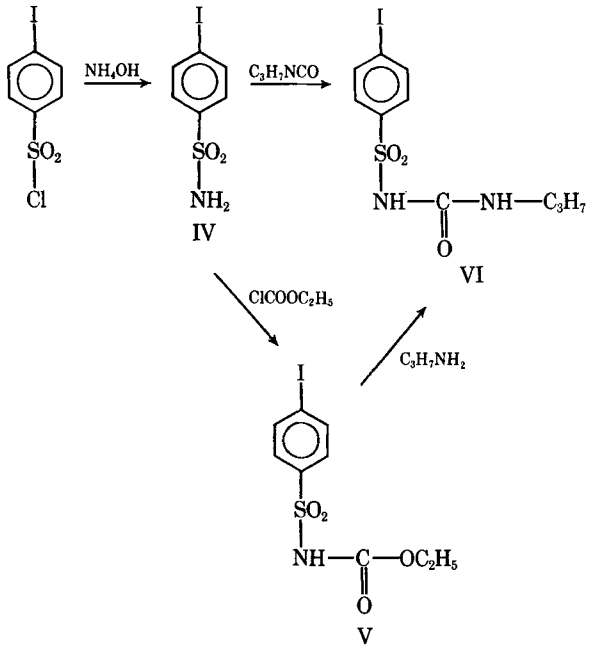
As a continuation of studies in this area, 1-(*p*-iodobenzenesulfonyl)-3-*n*-propylurea-^{125}I was selected as a compound that might prove useful as a pancreatic scanning agent. This compound is structurally similar to chlorpropamide [1-(*p*-chlorobenzenesulfonyl)-3-*n*-propylurea] and other sulfonylurea hypoglycemic agents. Although the sulfonylureas are known to have extrapancreatic effects, it is generally agreed that they exert their effect primarily by stimulating the release of insulin from the pancreatic beta cells (12). Accordingly, it seemed quite possible that these agents might concentrate in pancreatic tissue to a greater extent than in other tissues and organs. Relatively little research has been carried out on the tissue distribution of sulfonylureas. It has been reported that ^{35}S-carbutamide [1-(*p*-aminobenzenesulfonyl)-3-*n*-butylurea-^{35}S] concentrates to a greater extent in the liver than in other organs (13). However, some evidence indicates that carbutamide may differ from other sulfonylureas in this regard (14).

CHEMISTRY

1-(*p*-Iodobenzenesulfonyl)-3-*n*-propylurea (VI) was initially synthesized by a series of reactions which terminated in a diazotization step to introduce the iodine. This approach was taken because it was felt that the diazotization reaction would provide a convenient means of introducing the ^{125}I into the molecule. The starting material (I) was obtained by the chlorosulfonation of acetanilide (15), followed by reaction of the product with ammonia (16). Compound I was converted to II by reaction with *n*-propyl isocyanate. The *N*-acetyl group was removed by base-catalyzed hydrolysis. Iodine was introduced by diazotizing III and reacting the diazonium salt with potassium iodide to yield VI (Scheme I). The yield for the diazotization reaction was poor, thus making it seem impractical to introduce the ^{125}I in this manner. Accordingly, other methods for the synthesis of VI were explored. The most direct method involved treatment of *p*-iodobenzenesulfonyl chloride with ammonia to form the sul-



Scheme I



Scheme II

fonamide (IV), which was converted to VI by reaction with *n*-propyl isocyanate (Scheme II). An alternate method utilizes the formation of the intermediate sulfonylcarbamate (V) which was prepared from IV by refluxing with ethyl chloroformate. Conversion of V to VI proceeded smoothly by warming V with an excess of *n*-propylamine. The final products (VI) prepared by all three methods were identical.

Attempts to effect an isotope exchange on VI were unsuccessful; it underwent decomposition to *p*-iodobenzenesulfonamide when heated to 170° in any of a wide variety of solvents. Therefore, the isotope exchange was carried out on IV to produce *p*-iodobenzenesulfonamide-^{125}I, which was then converted to 1-(*p*-iodobenzenesulfonyl)-3-*n*-propylurea-^{125}I by reaction with *n*-propyl isocyanate.

TISSUE-DISTRIBUTION STUDIES

1-(*p*-Iodobenzenesulfonyl)-3-*n*-propylurea-^{125}I (100 μc.) was injected intravenously into four adult beagle dogs. The dogs were sacrificed at the end of 30 min. or 4, 24, and 72 hr., respectively. Other details pertaining to this phase of the study have been reported (17).

Data presented in Table I show that the desired uptake by the pancreas was not obtained. However, autoradiographic studies revealed that most of the radioactivity present in the pancreas was associated with the islet cells (17). Since the islet cells comprise only 1–4% of the total pancreatic weight (18), the concentration of drug in the islet cells could actually be quite high. Accordingly, this compound may have potential as a scanning agent for certain pancreatic islet cell tumors. Studies are currently in progress to assess its value for this purpose.

EXPERIMENTAL[1]

Melting points were taken on a Fisher-Johns melting-point apparatus and are corrected. IR spectra were taken on a Perkin-Elmer 337 spectrophotometer. The NMR spectra were obtained with a Varian A-60 spectrometer in $CDCl_3$ at a concentration of 10% with trimethylsilane as an internal reference. TLC was run with 2.54-cm. (1-in.) wide Eastman chromatograms, type K301R, with fluorescence indicator, and spots were detected with UV light. Chromatograms of radioiodinated compounds were scanned with an Atomic Associates RCS-363 radiochromatogram scanner. Sodium iodide-^{125}I in 0.05 *M* sodium bisulfite was purchased[2]; the specific activity was 0.17 mc./mg. of iodine. The specific activity of the final compound was determined with a Picker isotope calibrator.

[1] Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich.

[2] New England Nuclear Corp.

Table I—Tissue Distribution of 1-(*p*-Iodobenzenesulfonyl)-3-*n*-propylurea-^{125}I in the Dog[a]

Tissue	30 min.	4 hr.	24 hr.	72 hr.
Pancreas	0.008	0.009	0.019	0.017
Blood	0.079	0.044	0.056	0.060
Liver	0.018	0.026	0.031	0.021
Spleen	0.043	0.045	0.081	0.088
Kidney	0.024	0.027	0.051	0.031
Thyroid	0.006	0.006	0.016	0.171
Muscle	0.005	0.008	0.014	0.012
Fat	0.002	0.005	0.010	0.003

[a] Values are expressed as percent of dose per gram of tissue.

1-(*p*-Acetamidobenzenesulfonyl)-3-*n*-propylurea (II)—A solution of I (20 g., 0 1 mole) and triethylamine (3 ml.) in *n*-propylisocyanate (50 ml., 0.5 mole) was heated on a steam bath for 14 hr. and was then poured into 10% AcOH (250 ml.). The product precipitated as a yellow oil, which was dissolved in 10% NaOH (50 ml.). The pH of the solution was adjusted to 5 by the addition of HCl, and II precipitated as a solid. Recrystallization (EtOH–H_2O) afforded pure II (14 g., 50%), m.p. 197–198° [lit. (19) m.p. 198°]. The IR spectrum was as expected.

1-(*p*-Aminobenzenesulfonyl)-3-*n*-propylurea (III)—1-(*p*-Acetamidobenzenesulfonyl)-3-*n*-propylurea (II) (12 g., 0 04 mole) was dissolved in 15% NaOH (75 ml.), and the solution was refluxed for 3 hr. The product precipitated as a yellowish solid when the pH was adjusted to 5 with HCl. It was recrystallized (H_2O) to give 9 g. (88%) of III, m.p. 140–141° [lit. (20) m.p. 141°]. The IR spectrum was as expected.

***p*-Iodobenzenesulfonamide (IV)**—A solution of *p*-iodobenzenesulfonyl chloride (20 g., 0.065 mole) in NH_4OH (800 ml.) was refluxed with stirring for 3 hr. The hot reaction mixture was filtered, and the filtrate was placed in a refrigerator overnight. The product precipitated and was isolated by filtration. Recrystallization (EtOH–H_2O) produced pure IV (17 g., 91%), m.p. 190–191° [lit. (21) m.p. 189°]. The IR spectrum was as expected.

Ethyl *p*-Iodobenzenesulfonylcarbamate (V)—A mixture of IV (2.8 g., 0.01 mole) and anhydrous K_2CO_3 (3.6 g., 0.025 mole) in acetone (12 ml.) was stirred while ethyl chloroformate (1.25 ml., 0.013 mole) was added dropwise. The mixture was refluxed with stirring for 2 hr. A second portion of acetone (20 ml.) was added, and heating and stirring were continued for an additional 30 hr. The reaction was cooled to room temperature and filtered; the filtrate was acidified with 4.2 ml. of HCl, and an oil separated. The supernatant was decanted and the oil rubbed under water. The oil solidified on standing. Recrystallization (EtOAc–petroleum ether) produced pure V (1.54 g., 43%), m.p. 81–82°. The IR and NMR spectra were as expected.

Anal.—Calcd. for $C_9H_{10}INO_4S$: C, 30.43; H, 2.84; N, 3.94. Found: C, 30.60; H, 2.86; N, 3.83.

1-(*p*-Iodobenzenesulfonyl)-3-*n*-propylurea (VI)—*Procedure A*—*p*-Iodobenzenesulfonamide (IV) (300 mg., 0.001 mole), triethylamine (0.05 ml.), and *n*-propyl isocyanate (0.1 ml., 0.001 mole) were dissolved in anhydrous dimethylformamide (0.5 ml.) and sealed in a thick-walled test tube. The mixture was heated at 90° for 12 hr. The tube was opened and the reaction mixture was diluted with 10% AcOH (10 ml.). The product was collected by filtration and dissolved in 10% NaOH (10 ml.). The solution was filtered and the pH was adjusted to 5 with HCl. The resulting white solid was recrystallized (EtOH–H_2O) to furnish 230 mg. (60%) of VI, m.p. 155–156°. The IR and NMR spectra were as expected.

Anal.—Calcd. for $C_{10}H_{13}IN_2O_3S$: C, 32.62; H, 3.56. Found: C, 32.50, H, 3.57.

Procedure B—Ethyl *p*-iodobenzenesulfonylcarbamate (V) (400 mg., 0.001 mole) was dissolved in *n*-propylamine (0.4 ml., 0.05 mole) and shaken for 1.5 hr. The excess amine was removed under reduced pressure, and the residue was heated at 110° under reduced pressure (35 mm.) for 0.5 hr. An oil resulted. Recrystallization (EtOH–H_2O) furnished pure VI (195 mg., 47%), m.p. 155–156°. The IR spectrum was as expected.

Anal.—Calcd. for $C_{10}H_{13}IN_2O_3S$: C, 32.62; H, 3.56; I, 34.47; N, 7.61; Found: C, 32.62; H, 3.48; I, 34.77; N, 7.61.

Procedure C—1-(*p*-Aminobenzenesulfonyl)-3-*n*-propylurea (III) (7.7 g., 0.03 mole) was dissolved in HCl (15 ml.), and the solution was cooled to −5° with an ice–salt bath. A solution of $NaNO_2$ (2.4 g., 0.05 mole) in H_2O (10 ml.) was added dropwise at a rate that maintained the temperature of the reaction between 0 and 5°. Stirring was continued for an additional 15 min., and then the reaction mixture was filtered through glass wool into a solution of KI (18 g., 0.1 mole) in H_2O (60 ml.). A brown resin precipitated which was dissolved in 10% NaOH (100 ml.). The product precipitated as a yellowish solid when the pH of the solution was adjusted to 5 with HCl. Several recrystallizations (MeOH–H_2O) gave pure VI (1.5 g., 14%), m.p. 155–156°. A mixed melting point of this compound with the product from *Procedure A* showed no depression.

p-Iodobenzenesulfonamide-^{125}I—*p*-Iodobenzenesulfonamide (IV) (100 mg.) was treated with Na-^{125}I (3 mc.) in ethylene glycol (2 ml.) at 170° for 12 hr. as described previously (22). The radioiodinated product was recrystallized (EtOH–H_2O) to give a pure product (75 mg.), which was shown by TLC to be identical with the unlabeled material (IV): R_f = 0.91 in EtOAc–MeOH (4:1). A radiochromatogram of the strip showed that the radioactivity was present in the same spot.

1-(p-Iodobenzenesulfonyl)-3-n-propylurea-^{125}I—*p*-Iodobenzenesulfonamide-^{125}I (75 mg., 0.3 mmole), triethylamine (0.05 ml.), and *n*-propyl isocyanate (0.05 ml., 0.04 mmole) were dissolved in anhydrous dimethylformamide (0.5 ml.) and placed in a 10-ml. round-bottom flask equipped with a magnetic stirrer and a reflux condenser. The reaction was heated at 70° with stirring for 10 hr. and then diluted with H_2O (10 ml.). The product was collected by filtration and washed well with water. Recrystallization (EtOH–H_2O) gave a pure product (50 mg., 53%), which was identical with the unlabeled material (VI) on TLC in EtOAc–MeOH (4:1), R_f = 0.74. Radiochemical purity was established by TLC. The specific activity was 24 μc./mg., representing 80% exchange.

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Drug Permeation through Thin Model Membranes II: Permeation Characteristics of a Polymeric Model Biomembrane

KARL A. HERZOG* and JAMES SWARBRICK†

Abstract □ A novel synthetic model biomembrane consisting of a polymeric matrix containing several natural membrane constituents has been developed that exhibits permeation characteristics often attributed to natural biological membranes. When used to separate two aqueous phases buffered at physiological pH's, the model membrane constitutes an experimental system simulating gastrointestinal absorption. The transport of salicylic acid from a chamber at pH 2.0 (*A*) through the synthetic membrane and into a receiving chamber at pH 7.4 (*C*) follows apparent first-order kinetics. Support is given to a proposed kinetic model of $A \rightarrow C$ because of negligible membrane retention and minimal back-transport of the test drug. The rate of disappearance from *A* approximated the rate of appearance in *C*. The model system more closely simulates *in vivo* conditions than previously used three-phase liquid *in vitro* models. The pH-partition hypothesis is examined using this experimental system, the results indicating that the unionized, lipid-soluble species is the only form of the drug to permeate the synthetic biomembrane readily.

Keyphrases □ Biomembrane, polymeric—permeation characteristics □ Salicylic acid transport—model biomembrane □ pH-partition hypothesis–salicylic acid absorption—correlation □ UV spectrophotometry—analysis

There is continuing interest in model membrane transport phenomena. Thus, Lieb and Stein (1), working with nonelectrolyte transport across several polymeric films, related diffusion across these homogeneous networks to diffusion across biological barriers. Other investigators (2) impregnated several polymeric materials with various liquid lipoidal materials and used these membranes in a combination dissolution-partition apparatus. More recently, Bates *et al.* (3) used Millipore membrane filters impregnated with olive oil to study the effect of caffeine complexation on transport. Bimolecular (bilayer) lipid membranes have also been subjected to permeation studies employing organic solutes (4).

In a previous report (5), developmental aspects of a thin polymeric model biomembrane were presented. Salicylic acid was used as the test drug, and the apparent first-order disappearance rate constant from a pH 2.0 to a pH 7.4 aqueous environment, through the membrane, was used to assess the permeation capabilities of the membrane. A standard membrane consisting of 44% ethylcellulose, 44% total biological materials, and 12% mineral oil on a dry-weight basis was adopted. This composition afforded a compromise between membrane pliability, stability, and rate of drug transport. The absence of solvent flux through this membrane was also demonstrated, indicating the absence of pores in the polymer structure.

The present communication details some permeation characteristics of the membrane, namely: (*a*) the equivalence of the apparent first-order disappearance and appearance rate constants, k_d and k_a, respectively; (*b*) the concentration independence of the disappearance rate constant; (*c*) the extent of drug retention and the absence of dimerization within the membrane; and (*d*) the adherence to the pH-partition hypothesis of unionized drug absorption.

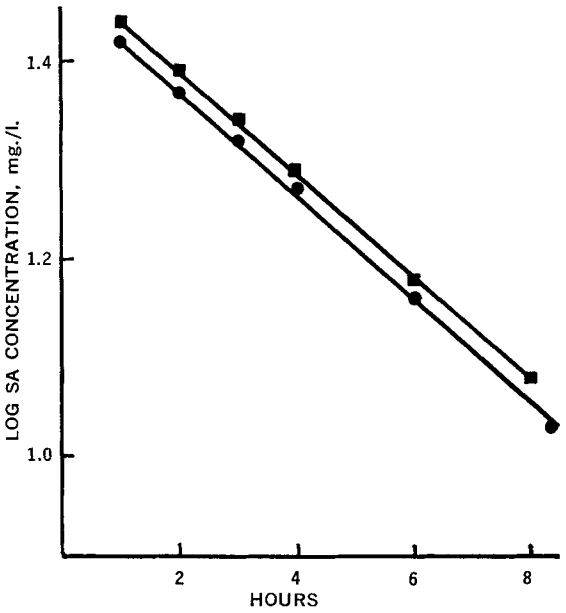

Figure 1—*Semilogarithmic plot of the disappearance and appearance of salicylic acid across the standard model biomembrane, pH 2.0–7.4. Key: ●, disappearance; and ■, appearance.*

EXPERIMENTAL

Materials—The materials used were reported previously (5) with the exceptions of reagent grade citric acid, sodium citrate, and sodium chloride, used as received.

General Experimental Protocol—The procedures for membrane formation, permeation studies using Plexiglas dialysis cells, and salicylic acid determination are as published (5) and remain unchanged, unless noted. All experiments were carried out at 37° using 0.1 *M* constant ionic strength buffers.

Partition Studies—A membrane (cross sectional area of 11.34 cm.^{2}) was placed into a flask containing 75 ml. of a solution of salicylic acid in a pH 2.0 buffer. After 3 hr. of agitation, which was sufficient for equilibrium concentrations to be obtained, the membrane was removed from the drug-containing buffer and the drug content of the buffer was determined spectrophotometrically. Four salicylic acid concentrations in pH 2.0 buffer (323, 422, 634, and 787 mg./l.) and one in pH 7.4 buffer (294 mg./l.) were employed. All determinations were made in triplicate.

Compliance with the pH-Partition Hypothesis—The buffer solutions used were 0.1 *N* HCl (pH 1.2), a hydrochloric acid buffer (pH 2.0), citric acid and sodium citrate (pH 3.0 and pH 4.0), and a phosphate buffer (pH 7.4). Salicylic acid (pKa = 3.0) determinations in pH 1.2, 3.0, and 4.0 buffers were made at 303, 301, and 298 nm., respectively, the wavelength of maximum absorbance. The duration of each experimental run was well past one half-life, with the exceptions of pH 4.0 and 7.4, which were followed for a period of 44 ($t_{42\%}$) and 68 hr., respectively. The apparent first-order disappearance-rate constant, k_d, was converted to the true first-order disappearance-rate constant, k_d', by correcting for the

Table I—Effect of Initial Salicylic Acid Concentration on the Disappearance-Rate Constant from pH 2.0 Compartment

Initial Concentration of Salicylic Acid, mg./l	Apparent Disappearance-Rate Constant, $k_d \times 10^3$ (hr.$^{-1}$)
30.8	117
300	123
772	122

relative amount of unionized drug species present in the system using the following equation:

$$k_d' = k_d/f \qquad \text{(Eq. 1)}$$

where f is the fraction of unionized salicylic acid.

RESULTS AND DISCUSSION

Equivalence of k_d and k_a—Figure 1 depicts the results of salicylic acid permeation through the model membrane within the *in vitro* transport system. The lower line represents the disappearance of salicylic acid from the pH 2.0 chamber, corrected for the equilibrium concentration of this chamber. The upper line denotes the appearance of salicylic acid in the pH 7.4 receiving chamber. This line is constructed by considering the equilibrium concentration in the receiving chamber and subtracting from it the observed concentrations in the chamber. Essentially all of the salicylic acid was transported, thereby allowing the equilibrium concentration in the receiving chamber to be taken as C_A^0 (Eq. 3) and the equilibrium concentration in the initial chamber to be considered as zero. Figure 1 is presented in this fashion to demonstrate the parallelism observed; that is, the equivalence of the apparent first-order rate constants, k_d and k_a, obtained from this plot. By using the method of least squares, these constants were derived from sampling periods after the attainment of steady-state conditions (which appears to be a rapid process) and were shown to be experimentally identical. From these plots, the amount of salicylic acid in the membrane under steady-state condition was computed to be 3.14% of the initial concentration.

Diffusion may be quantitated in terms of Fick's first law, which in turn may be modified when considering transport through a barrier quite thin in cross sectional dimension. Upon rearrangement and term collection, a simple first-order rate expression may be derived, the integrated form of which is characteristically plotted when first-order transport kinetics are suspected. These expressions appear as Eqs. 2 and 3, respectively, where: C_A^0 is the initial concentration in Compartment A, which represents the lumen of the gastrointestinal tract; C_A is the drug concentration within the same compartment at time equal to t; and k_d is as previously defined. Thus,

$$-\frac{dC_A}{dt} = k_d C_A \qquad \text{(Eq. 2}$$

$$\log C_A = \log C_A^0 - \frac{k_d t}{2.303} \qquad \text{(Eq. 3)}$$

From Eq. 3, a plot of log C_A *versus* t should yield a straight line, from the slope of which is obtained the value of k_d. Figure 1 is such a representation. The use of Fick's law as a basis for these passive diffusion equations requires that two assumptions be made: (*a*) that the barrier is thin and hence solute content is small, and (*b*) that a single rate constant expresses the material flux. Essentially, this describes a two-compartment transport model.

The first assumption is fulfilled by the negligible salicylic acid retention within the model membrane, also demonstrated by partition studies. Although k_d appears to be a hybrid rate constant reflecting a reversible exchange of unionized drug with Compartment A, the large partition coefficient in favor of the membrane phase indicates the exchange is essentially unidirectional. Regardless of the complexity of the process, k_d is experimentally equivalent to k_a, thereby satisfying the second assumption. Accordingly, Eq. 4, depicting overall unidirectional transport expressed by a single rate constant, best reflects the kinetics of this *in vitro* transport system:

$$A \xrightarrow{k_d \text{ (or } k_a)} C \qquad \text{(Eq. 4)}$$

Table II—Partition of Salicylic Acid between pH 2.0 Buffer and the Standard Model Biomembrane

Total Salicylic Acid in System, mg.[a]	Salicylic Acid in Membrane, mg.[b]	Salicylic Acid in Membrane, %	Partition Coefficient, k_p[c]
24.25	0.54	2.23	75.6
31.62	0.65	2.06	69.7
47.57	1.05	2.21	74.9
59.05	1.21	2.05	69.4
22.04[d]	Nil	Nil	—

[a] Volume of aqueous phase = 75 ml. [b] Volume of membrane = 22.6×10^{-3} ml. [c] k_p = mg./ml. in membrane/mg./ml. in aqueous phase. [d] Between pH 7.4 and membrane.

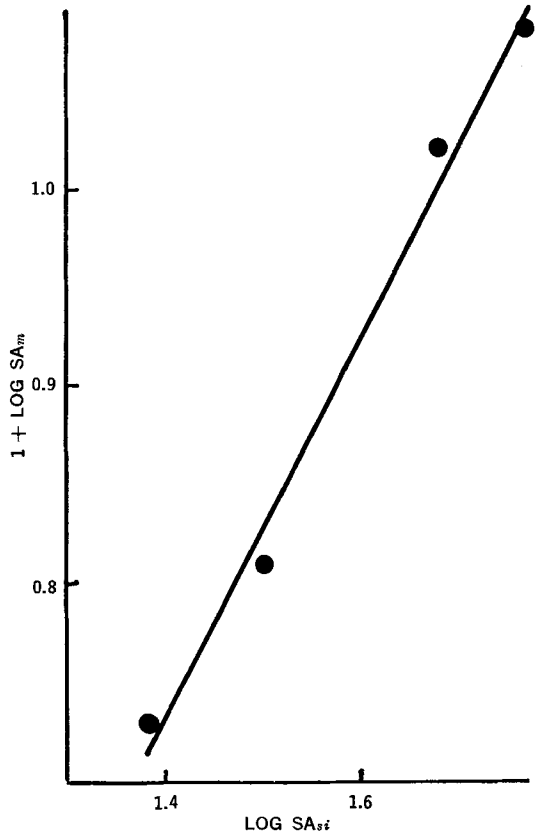

Figure 2—*Log–log plot of salicylic acid in the membrane* (SA_m) versus *salicylic acid initially present in the system* (SA_{si}).

Effect of Initial Concentration on k_d—A series of salicylic acid solutions in pH 2.0 buffer covering a 25-fold concentration range was employed to study the effect of initial concentration on the disappearance-rate constant across the model membrane. The results are contained in Table I; the right-hand column suggests that k_d is constant over the concentration range examined, *i.e.*, characteristic of first-order rate processes.

Partition Studies—Partition studies were undertaken to clarify the extent of solute association and the percent solute retention within the membrane. Initial salicylic acid concentrations were noted and again determined after 3 hr. in the buffer. As Table II indicates, both the amount of drug within the membrane and the percent of the total amount within the membrane were computed. The average percent retention of salicylic acid was 2.14% and is of the same magnitude as the value obtained from the permeation studies. The percent retention is also presented as an apparent partition coefficient, here defined as the ratio of drug/milliliter in the membrane to drug/milliliter in the aqueous environment. The results obtained from the pH 7.4 buffer demonstrate that the

Table III—Effect of pH on Disappearance-Rate Constant of Salicylic Acid Using the Standard Model Biomembrane

pH of Initial Compartment	Apparent Disappearance-Rate Constant, $k_d \times 10^3$ (hr.$^{-1}$)	Unionized Salicylic Acid, %	True Disappearance-Rate Constant, $k_d' \times 10^3$ (hr.$^{-1}$)[a]
1.2	139	98.4	141
2.0	123	90.9	135
3.0	69.5	50.0	139
4.0	12.4	9.1	136
7.4	Nil	0.0	Nil

[a] See Eq. 1.

ionized form of salicylic acid is unable to enter the model membrane.

Of interest, when considering the actual mechanism of permeation within the solid polymeric barrier, is the phenomenon of intermolecular associations of the penetrant molecules and their effects on the observed rate constants. Gonzales *et al.* (6), studying the diffusion and solubility of various compounds through polyethylene, observed that both benzyl alcohol and benzoic acid had the lowest diffusion of the test compounds. They attributed this observation to the potential of these compounds to exhibit intermolecular hydrogen bonding, thereby forming dimers and trimers whose diffusion within the polymer was thought to be restricted. From the partition data in Table II and Eq. 5, it is possible to demonstrate whether or not this phenomenon is operative in the standard model biomembrane. Thus,

$$\log SA_m = \log SA_{si}/n + \log MCR/n \qquad \text{(Eq. 5)}$$

where *MCR*, the membrane content ratio, is the ratio of drug in the membrane to the drug initially present in the system; SA_m and SA_{si} are the amounts of salicylic acid within the membrane and initially present in the system, respectively; and n is the degree of association of salicylic acid in the membrane. From Eq. 5 a log–log plot of SA_m *versus* SA_{si} should yield a straight line, the slope of which is $1/n$. If n assumes the value of unity, it may be assumed that dimer and trimer formation of salicylic acid is absent in this experimental system. Figure 2 illustrates such a plot, and the value of n was found to be 1.04. Hence, it is reasonable to assume the absence of solute interaction in the polymeric barrier.

Membrane Compliance with the pH-Partition Hypothesis—To establish whether or not the model membrane simulated biological membrane functionality with regard to passive drug absorption, an evaluation of the pH-partition hypothesis (7–11) was conducted (Table III). The apparent first-order disappearance-rate constants for the five pH values examined were converted to true first-order disappearance-rate constants by correcting for the amount of unionized drug present. The consistency of these data and the absence of any detectable transfer at pH 7.4 strongly suggest that only the unionized form of the drug crosses the barrier. Accordingly, these results are in good agreement with the predictions made by the pH-partition hypothesis relating the biological membrane to a nonpolar barrier which permits only nonpolar substances to cross readily.

SUMMARY

As a result of the low retentive qualities of the membrane and the equivalence of the rate constant for disappearance from Compartment A (k_d) and the rate constant for appearance in Compartment C (k_a), a two-compartment absorption model has been shown to fit the experimental system. The observed lack of concentration influence on k_d is consistent with first-order kinetics. A lack of molecular association within the membrane has been demonstrated, and the model membrane has been shown to adhere to the pH-partition hypothesis. Attempts to correlate *in vitro* drug permeation to *in vivo* drug absorption are currently in progress and will be the subject of future reports.

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Kinetics and Mechanisms of Lactonization of Coumarinic Acids and Hydrolysis of Coumarins I

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Abstract □ The lactonization of coumarinic acid and its anions and the hydrolysis of coumarin have been studied in the pH range of −0.3–11 in various buffer solutions between 10 and 25° by spectrophotometric methods. The significant influence of buffer concentration on the apparent first-order rate constant for lactonization was assigned to the acetic acid-catalyzed lactonization of the monoanion, HC^-. The dependency of the apparent first-order rate constants extrapolated to zero buffer concentrations on the hydrogen-ion concentration was derived. It was consistent with hydrogen-ion-catalyzed lactonization and apparent spontaneous lactonization of undissociated coumarinic acid, H_2C, to form coumarin, C, the spontaneous lactonization of the monoanion, HC^-, and the nonreactivity of the dianion, C^{-2}. An apparent discrepancy exists between the kinetic (3.0) and the spectral and potentiometric (4.0) pKa_1' which can only be rationalized by a more complex mechanism. This mechanism assumes the intramolecular formation of an ortho-acid, $H_2C‡$, which dehydrates spontaneously and by hydrogen-ion catalysis to give coumarin. The steady-state assumption for $H_2C‡$ permits the fitting of the log k–pH profiles consistent with the analytical pKa_1' values and is consistent with this mechanism. Coumarin is hydrolyzed by specific hydroxyl-ion-catalyzed solvolysis and has been characterized as a function of pH. The hydrolyses of 3-chlorocoumarin, 3-bromocoumarin, and 3- and 4-methylcoumarin were studied in 30% dioxane for the acid–lactone equilibrium. The halogen substituents showed pronounced accelerating effects consistent with the electronegative group acceleration of hydroxyl-ion attack on the carbonyl carbon, whereas the methyl substituents did not significantly modify the reactivity of coumarin.

Keyphrases □ Coumarinic acids—lactonization □ Coumarins—hydrolysis □ Kinetics, mechanisms—coumarinic acid lactonization, coumarin hydrolysis □ pKa′ values—coumarinic acid □ Buffer effects—coumarinate lactonization □ UV spectrophotometry—analysis

Although the hydrolyses of coumarins (5,6-benzo-α-pyrons) have been studied in alkaline solution (1, 2), the ring closure of the resultant coumarinic acids and their salts to the respective coumarins has not been investigated. The purpose of these studies on the kinetics of lactonization was to determine the relative reactivities of undissociated coumarinic acid and its monoanions and dianions. The rate–pH profile for the hydrolysis

Table I—Apparent First-Order Rate Constants, k, in sec.$^{-1}$ for the Lactonization of Coumarinic Acid

Medium[a]		10.0° pH[b]	10.0° 10^3k	17.5° pH[b]	17.5° 10^3k
10^3 [HCl]					
238		−0.46	57.0	−0.44	129
98.3		0.09	22.2	0.09	40
73.8		0.23	17.2	0.24	27.9
49.2		0.42	11.1	0.43	22.2
24.6		0.72	5.79	0.73	11.4
8.25		1.18	3.13	1.18	6.53
4.13		1.48	2.64	1.48	4.87
0.971		2.11	1.82	2.47	2.28
0.425		2.47	1.44		
[$H_2PO_4^-$]	[HPO_4^{-2}]				
0.065	0.011	6.29	0.0120	6.21	0.0275
0.0167	0.0277	7.23	0.00887	7.11	0.0202
0.00125	0.0329	8.38	0.00795	8.33	0.0193

Medium[a]		25.0° pH[b]	25.0° 10^3k	
10^3[HCl]				
207.5		−0.32	148	
89.7		0.15	64.0	
65.5		0.30	55.7	
45.3		0.47	36.3	
21.7		0.78	20.5	
9.5		1.12	13.9	
3.36		1.57	8.50	
1.04		2.08	5.48	
0.302		2.42	3.82	
[$HC_2H_3O_2$]	[$C_2H_3O_2^-$]		10^3k	
1.48		2.23	6.11	(6.02)[c]
0.183		2.79	3.80	(3.67)[c]
2.2	0.086	3.18	3.47	(2.42)[c]
1.25	0.1	3.50	1.99	(0.92)[c]
0.703	0.1	3.79	1.34	(0.495)[c]
0.269	0.086	4.10	0.640	(0.300)[c]
0.006	0.086	5.38	0.135	(0.115)[c]
[$H_2PO_4^-$]	[HPO_4^{-2}]		10^3k	
0.065	0.011	6.05	0.0995[d]	
0.0167	0.0277	7.07	0.0868	
0.00125	0.0329	8.26	0.0895[e]	
[NaOH]	[HCO_3^-]			
0.005	0.04	9.40	0.0689	
0.013	0.04	9.73	0.0691	
0.020	0.04	10.05	0.0828	
0.0215	0.04	10.11	0.0725	
0.030	0.04	10.50	0.162	
0.030	0.04	10.50	0.128	
0.0315	0.04	10.59	0.238	
0.033	0.04	10.70	0.281	

[a] NaCl was added to maintain the ionic strength at $\mu = 0.1$ when possible. [b] The pH values in HCl at concentrations greater than 0.1 M were calculated from pH = $-\log f$[HCl], where the activity coefficient, f, was obtained from the literature (4). All other values were measured by a pH meter. [c] These apparent first-order rate constants in the acetate buffer region were calculated after correcting for the catalytic effect of the acetic acid in accordance with $k_c = k - f_{HC^-} k'_{HAc}$ [HAc], where $f_{HC^-} = Ka_1'/([H^+] + Ka_1)$, $Ka_1' = 1.00 \times 10^{-4}$, and $k_{HAc} = 3.65 \times 10^{-3}$ l./mole-sec. at 25.0°. [d] At $^2/_3$ and $^1/_3$ of these buffer-ion concentrations, there were no differences among the apparent first-order rate constants. [e] When this phosphate buffer solution was also made 0.1 N in acetate ion, the rate constant was 8.88×10^{-5} sec.$^{-1}$.

Table II—Apparent First-Order Rate Constants, k, in sec.$^{-1}$ for the Hydrolysis of Coumarin

		$[HCO_3^-]^a$												
		0.040								0.000				
[NaOH]		0.006	0.019	0.024	0.026	0.027	0.029	0.031	0.032	0.035	0.0098	0.0375	0.0491	0.075	0.09
pH[b]		9.45	10.00	10.20	10.30	10.35	10.42	10.55	10.65	10.90	11.85	12.50	12.61	12.77	12.88
10^4k	25.0°	0.63	0.72	0.96	1.16	1.22	1.33	1.76	2.45	3.72	—	135	—	254	—
	17.5°	—	—	—	—	—	—	—	—	—	17.1	—	93.0	—	169
	10.0°	—	—	—	—	—	—	—	—	—	9.65	—	59.6	—	119

[a] NaCl added where possible to maintain ionic strength at $\mu = 0.1$. [b] The pH was experimentally determined in the bicarbonate–carbonate buffers but was calculated for the NaOH solutions from $pH = pK_w - \log f'[NaOH]$, where the activity coefficient f' was obtained from the literature (4).

of coumarin and the lactonization of the resultant coumarinates was determined to define the extent of the equilibrium between the open forms and the lactone as a function of pH and the catalytic species that are involved in such transformations. Abernethy (3) stated that the undissociated coumarinic acid has not been isolated nor have sufficiently sophisticated chemical methods been used to reveal directly its transient existence when coumarin is formed on addition of acids to sodium coumarinate. These studies will prove its existence, and the pKa′ values of coumarinic acid determined by kinetic, spectral, and potentiometric methods will be compared.

EXPERIMENTAL

Lactonization—Coumarin[1] was used as received. The reactions were investigated at various pH values between −0.3 and 11 in hydrochloric acid, acetate, phosphate, and carbonate buffers and sodium hydroxide solutions at 10.0, 17.5, and 25.0°. All solutions were made up with nitrogen-purged distilled water. The pH at the temperature of the kinetic experiments was read with a Radiometer pH meter and a Sargent glass electrode or was calculated and extrapolated from the known activities (4) in strong acid and alkaline solutions, *i.e.*, $pH = -\log f[HCl]$ or $pH = pK_w - \log f'[NaOH]$, where the pK_w and activity coefficients f and f' were obtainable at the desired temperatures (4). The ionic strength in all buffer solutions and in hydrochloric acid and sodium hydroxide solutions below 0.1 M was adjusted to $\mu = 0.1$ with sodium chloride.

Generally, 0.05 ml. of about 5×10^{-3} M coumarin, which had been completely solvolyzed in 0.01 N NaOH (pH about 11.9), was added to 3 ml. of the appropriate buffer solution to produce a final concentration in the range of 7.30–8.40 $\times 10^{-5}$ M. The compositions of the buffer solutions are listed in Table I. All solutions of sodium monocoumarinate and dicoumarinate were excluded from light to prevent possible *cis–trans* isomerization and photolytic degradation (1). A Cary model 15 recording spectrophotometer equipped with a repetitive scan accessory, automatic sample changer, and a thermostatically controlled cell compartment was used to follow the reactions. The change of absorbance in the appropriate buffer was automatically recorded either at one wavelength or by repetitive scan techniques. The thermally equilibrated solutions were mixed directly in the 1 × 1-cm. cells of the spectrophotometer. Water was used as the blank.

The plots of the logarithms of the differences in the final absorbance, A_∞, and the absorbance at any time, A, at 280 nm. against time gave straight lines; the rate constants (Table I) were determined from the slopes in accordance with the equation for an apparent first-order reaction:

$$\log |A_\infty - A| = \frac{-kt}{2.303} + \log |A_\infty - A_0| \qquad \text{(Eq. 1)}$$

Typical plots are given in Fig. 1. Kinetic runs exposed to continuous UV irradiation by repetitive scanning had the same final spectra and the same absorbances as those from which aliquots were taken. The fact that the same coumarin spectra resulted with the same absorbance as equimolar coumarin is adequate proof that the solutions were not photolytically degraded in the spectrophotometer during the reaction, that nonlactonizable *trans*-coumarinic acid was not formed on automatic spectrophotometric monitoring of lactonization under the conditions used.

A differential spectrophotometric method was used for kinetic runs above pH values of 9 where the overall absorbance changes were small, since coumarin and coumarinate ions achieved a final equilibrium mixture in the pH 9–10 region. Coumarin, which had been completely solvolyzed at pH 12, was added to the appropriate buffer in what was normally the reference cell. The cell that was normally the sample cell contained material that had been completely reacted to the lactone–anion equilibrium at the same temperature and concentration (8.4×10^{-4} M) and in the same buffer. The differences in the absorbances were thus magnified and were plotted in accordance with Eq. 1. The reason for the reversal of the sample and reference cells was that the finally reacted solutions had greater absorbance because the absorbance of coumarin was greater than that of the anions at the specified wavelength (Fig. 2).

The spectrum (Fig. 2) of the 7.30×10^{-5} M coumarin was recorded in 0.1 N HCl, and that of the equivalent concentration of coumarinate dianion was obtained after complete hydrolysis in 0.01 N NaOH (pH 11.9). The spectrum of the 7.30×10^{-5} M coumarinate monoanion was recorded on the Cary spectrophotometer immediately after mixing 0.05 ml. of 4.45×10^{-3} M coumarin which had been completely solvolyzed in 0.01 N NaOH and 3 ml. of pH 7.07 phosphate buffer solution at 25.0° (Table I). The spectrum of the 7.30×10^{-5} M undissociated coumarinic acid was obtained by mixing 0.05 ml. of the alkaline solution of solvolyzed coumarin and 3 ml. of 0.055 N HCl. The rapid change in absorbance was automatically recorded with time at each of 10-nm. intervals between 220–360 nm. The spectrum of coumarinic acid (Fig. 2) was obtained by graphical extrapolation of the recorded absorbance *versus* time plot to time zero for each wavelength.

Hydrolysis—The hydrolysis of coumarin was investigated in the pH region of 9–13. The compositions of the buffer solutions are listed in Table II. The decreasing absorbances of the 7.55×10^{-5} M solutions were monitored with time and plotted for $\lambda = 280$ nm. in accordance with Eq. 1. At pH values below 9.5, the previously

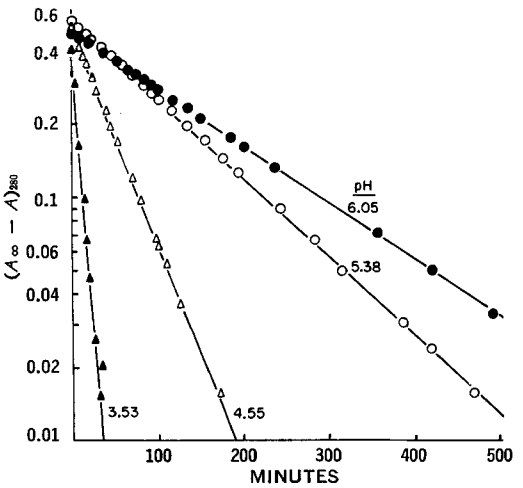


Figure 1—*Typical apparent first-order plots for the lactonization of coumarinic acid in acetate and phosphate buffers at 25° at various pH values ($\mu = 0.1$). The absorbance, A, is at 280 nm. and A_∞ is the absorbance at infinite time.*

[1] Aldrich Chemical Co., Cedar Knolls, N. J.

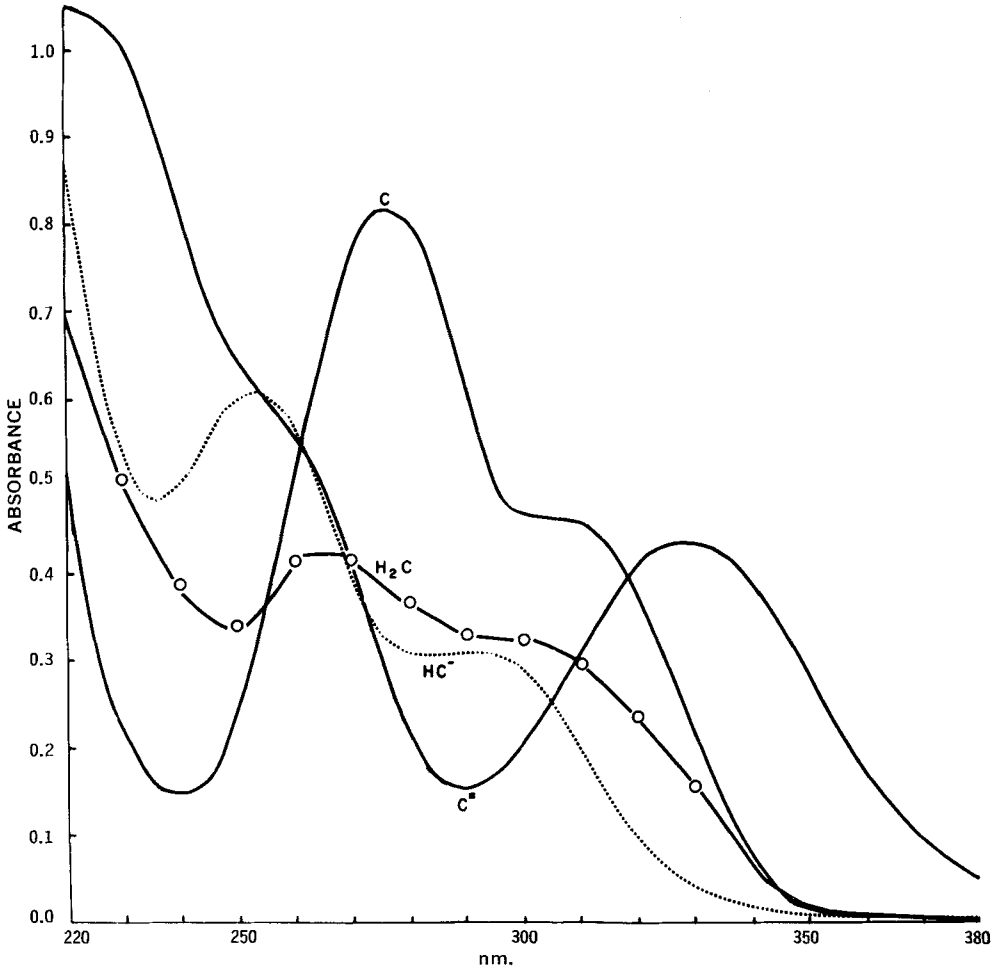


Figure 2—*UV spectra of 7.30 × 10⁻⁵ M undissociated coumarinic acid (based on extrapolated time zero values after introduction of sodium coumarinate in 0.055 N HCl), coumarinate monoanion, HC^- (in phosphate buffer of pH 7.2), coumarinate dianion, C^{-2} (in 0.1 N NaOH), and of coumarin, C (in 0.1 N HCl).*

described differential spectrophotometric method was used. However, the reacting coumarin solution was in the usual sample cell, and the reference cell contained the same concentration of coumarin which had been completely reacted in the same buffer, temperature, and pH value. Typical spectral changes of alkaline-treated (pH 10.35) coumarin are shown in Fig. 3 as a function of time.

The hydrolyses of 3- and 4-substituted coumarins were investigated spectrophotometrically in 30.0% dioxane–water mixtures (v/v) because of the low solubility of these compounds. The compositions of the buffer solutions are listed in Table III, as are the measured apparent pH values. The decreasing absorbances were recorded with time at 280, 290, 295, 280, and 273 nm. for coumarin

Table III—Apparent First-Order Rate Constants, k, in sec.$^{-1}$ for the Hydrolyses of 3- and 4-Substituted Coumarins at 25.0° in Dioxane (30%)–Water Mixtures

Medium[a]							
[NaOH]	[HCO_3^-]	pH[b]	Coumarin, 10^5k	3-Chloro-coumarin, 10^4k	3-Bromo-coumarin, 10^4k	3-Methyl-coumarin, 10^4k	4-Methyl-coumarin, 10^4k
0.0015	0.04	10.05	—	2.88	—	—	—
0.005	0.04	10.3	—	5.33	—	—	—
0.0095	0.04	10.52	—	10.05	1.98	—	—
0.011	0.04	10.6	5.23	—	—	—	—
0.015	0.04	10.78	5.62	14.5	—	—	—
0.0185	0.04	10.97	7.78	—	—	—	—
0.0195	0.04	11.02	—	—	7.16	—	—
0.0225	0.04	11.15	10.6	—	—	—	—
0.027	0.04	11.38	16.5	—	—	—	—
0.0285	0.04	11.45	—	—	—	2.27	—
0.030	0.04	11.53	—	—	—	2.35	—
0.031	0.04	11.6	22.1	—	—	—	—
0.032	0.04	11.65	—	—	—	2.72	—
0.0325	0.04	11.7	—	—	—	3.62	—
0.033	0.04	11.73	29.6	—	—	—	—
0.034	0.04	11.8	—	—	—	—	4.0
0.035	0.04	11.88	—	—	—	—	—
0.1		12.85	—	—	—	—	36.0

[a] NaCl was added when possible to maintain the ionic strength at $\mu = 0.1$. [b] The pH values were measured by a Radiometer pH meter and a Sargent combination electrode.

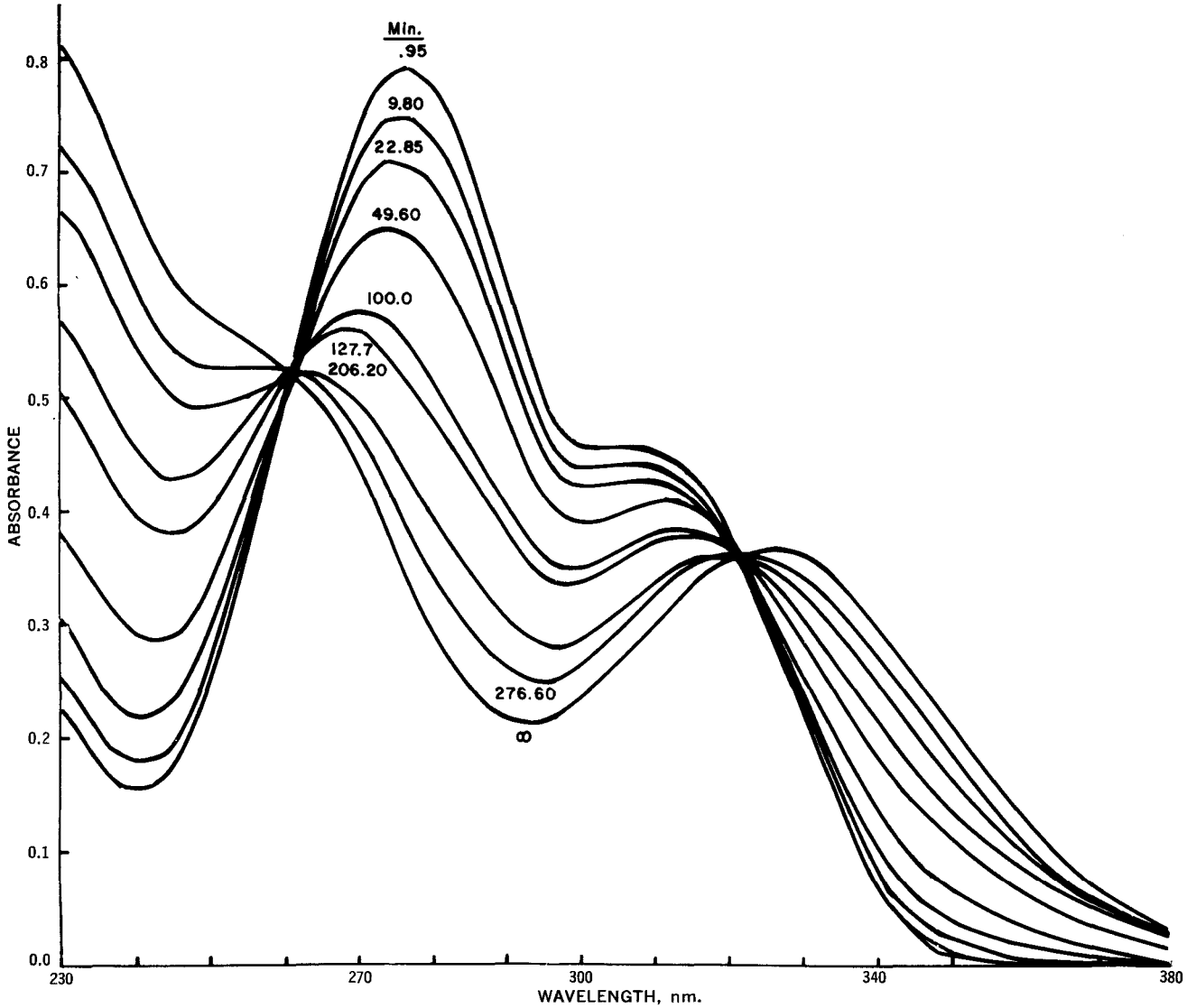


Figure 3—*Typical spectral changes for the hydrolysis of 7.55 × 10⁻⁵ M coumarin in carbonate buffer, pH 10.35, μ = 0.1, at 25°. The curves are labeled as to minutes after the start of the reaction.*

(for comparison), 3-chlorocoumarin[2], 3-bromocoumarin, 3-methylcoumarin[3], and 4-methylcoumarin[4], respectively. The plots of the logarithm of the differences of the absorbance at time zero, A_0, and the absorbance at any time, A, gave straight lines in accordance with Eq. 1. The coumarin compounds were obtained from the stated companies and were used as received. 3-Bromocoumarin was synthesized by the method of Ghiya and Marathey (5) and used after recrystallization in 0.001 *N* acetic acid (m.p. 109° uncorrected). 4-Methylcoumarin was prepared by the methods of Woodruff (6) and used after recrystallization in petroleum ether–benzene (1:1) mixture (m.p. 83–84°).

Spectrophotometric Determination of pKa′ Value of Coumarinic Acid—The UV spectra of mixtures of completely solvolyzed coumarin in 0.01 *N* NaOH with appropriate buffers (pH 1–7, Table I) at a final concentration of 7.30×10^{-5} *M* were recorded at 250, 280, and 320 nm. by the Cary recording spectrophotometer as a function of time immediately after mixing in the spectrophotometric cells. The apparent first-order rate plots for the lactonization in the different buffer solutions (Fig. 1) permitted extrapolation of the log $|A_\infty - A|$ values to an intercept value of log $|A_\infty - A_0|$, so that the absorbance values, A_0 at time zero, could be estimated for the various buffer solutions of Table I. Plots of these time zero absorbances assigned to equilibrium mixtures of coumarinic acid and its anion before lactonization are shown in Fig. 4. Similar plots of time zero absorbances assigned to equilibrium mixtures of coumarinate monoanion and dianion also are shown in Fig. 4.

Potentiometric Determination of pKa′ Values of Coumarinic Acid—A 0.5-ml. aliquot of a solution of 0.100 *M* coumarin, completely solvolyzed in 0.300 *N* NaOH, was diluted to 30.0 ml. with nitrogen-purged 0.1 *M* KCl solution. Different varying known amounts (0–100 μl.) of 0.5 *N* HCl were added to 3.00-ml. aliquots, and the pH of each aliquot was recorded immediately using a Radiometer pH meter and a Sargent combination electrode (S-30070-10). Similarly, as a blank, a 0.5-ml. aliquot of 0.100 *N* NaOH was diluted to 30.0 ml. with 0.1 *N* KCl solution, the microliters of 0.5 HCl added, and the pH read.

RESULTS AND DISCUSSION

Estimation of Apparent pKa′ Values—Apparent pKa values were estimated (7) at a specific wavelength by

$$\log \left| \frac{A_{HC^-} - A_0}{A_0 - A_{H_2C}} \right| = pKa' - pH \qquad \text{(Eq. 2)}$$

where the A_0 values are the time zero estimates of absorbances at a given pH value, A_{H_2C} is the asymptotic absorbance in increasingly acidic solutions and is assigned to the absorbance of 7.30×10^{-5} *M* undissociated coumarinic acid (Fig. 2 at pH 1.0), and A_{HC^-} is the asymptotic absorbance achieved in neutral solution and is assigned

[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] K & K Laboratories Inc., Plainview, N. J.
[4] Pfalz and Bauer, Flushing, N. Y.

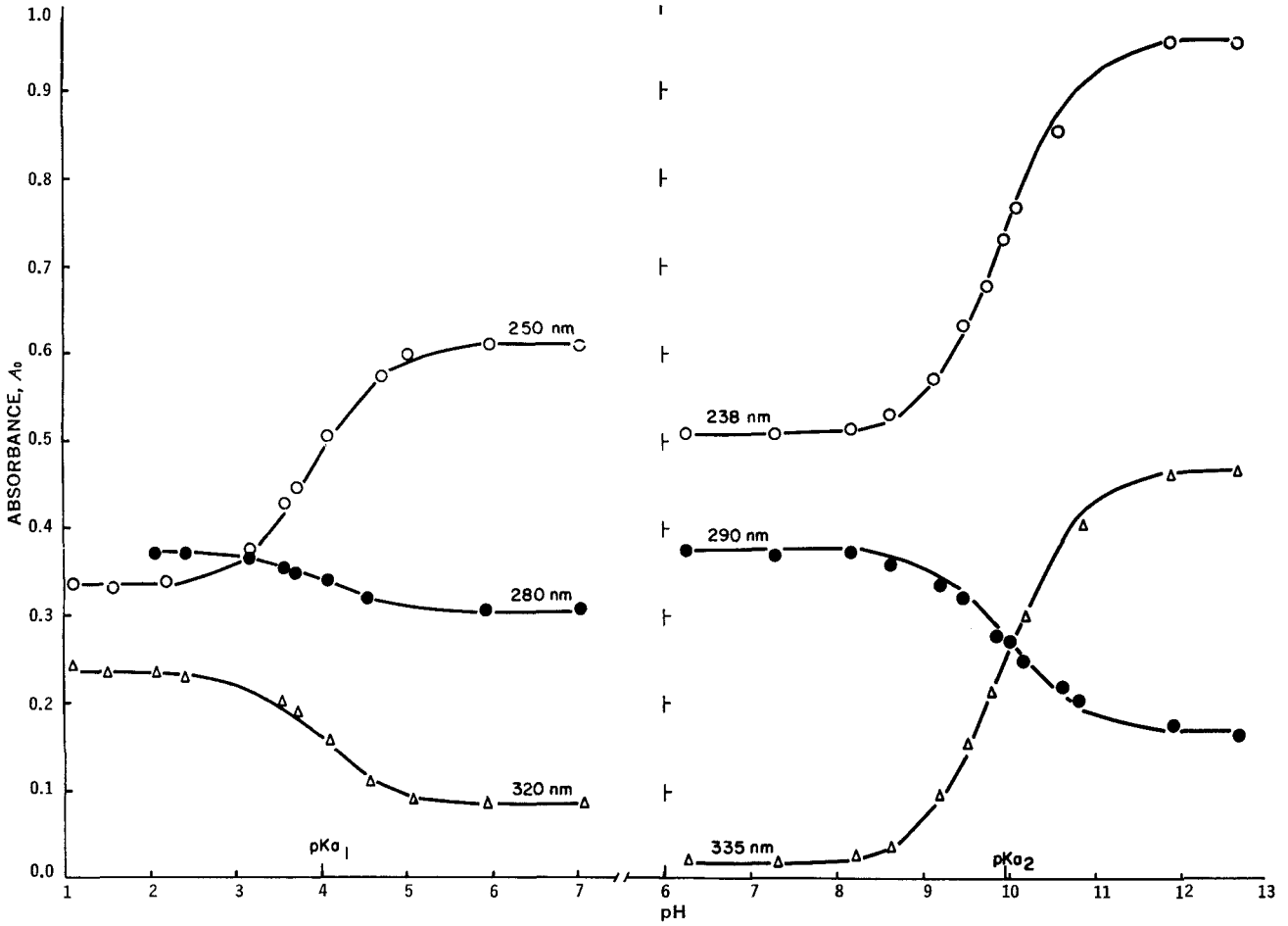


Figure 4—*Spectrophotometric titration curves for coumarinic acid at 25° at several wavelengths. The pKa_1' was made at 7.30×10^{-6} M and the pKa_2' was determined at 7.86×10^{-5} M. Absorbances were determined from extrapolated time zero values.*

to the absorbance of 7.30×10^{-5} *M* coumarinate monoanion. The obtained pKa_1' value was 4.00 at 25.0°. Similar studies were made at 238, 290, and 335 nm. after mixing solvolyzed coumarin and the appropriate buffers (Table I) in the pH range 6–13. The calculated pKa_2' from an expression similar to Eq. 2 for the dissociation of the phenolic monoanion to the coumarinate dianion was 9.95 at 25.0°. This was estimated as 10.7 by Mattoo (1). However, inspection of Mattoo's spectral Fig. 2 (1) does not show a clear isosbestic point. Since the monoanion lactonizes in this pH region, spectral reading should be made immediately or the spectrum of coumarin will interfere with the analyses. The spectral evidence was inconsistent with the previously (1) postulated dimers (Figs. 2 and 4) and yielded a pKa′ of 9.95 in the present studies.

The immediately read pH values of 3.0-ml. acidified aliquots of 0.00167 *M* coumarinic acid obtained from alkaline-solvolyzed coumarin when plotted against the microliters of 0.5 *N* HCl added provided the potentiometric titration curves of Fig. 5. The difference between the numbers of milliliters necessary to adjust these aliquots and a blank solution of the alkali used to a given pH are given as a difference curve in Fig. 5. The pH values of half-neutralization can be observed from this difference curve (8, 9) and used to estimate pKa′ as 4.00 and pKa_2' as 9.90. The asymptotic value in the acidic region was based upon the fact that 10 μl. of HCl had to be consumed for complete liberation of the coumarinic acid. Of course, the greatest error in pH measurement is in the region of pH 3.0, since the half-life for ring closure below pH 3.0 is less than 5.0 min.

Buffer Effects on Rates of Coumarinate Lactonization—The apparent first-order rate constants, *k*, for the lactonization of coumarinic acid (H_2C) and its anions (HC^- and C^{-2}) vary markedly with pH (Table I and Fig. 1). It was necessary to quantify any possible buffer effects on rate so that exact equations could be written for rate-constant dependence on pH. Significant buffer effects were observed in the acid region with acetate buffers (Table I and Fig. 6) but not with phosphate buffers (Table I, footnote *e*).

There was no significant difference in the rate of lactonization between a phosphate buffer solution (pH 8.26) of hydrolyzed coumarin (*i.e.*, as the monoanion) and when the same solution was 0.1 *M* in acetate ion, Ac^- (Table I, footnote *e*).

Thus, any catalytic effect of $k'_{Ac^-}[Ac^-][HC^-]$ is negligible, and the observed acetate buffer catalytic effects in the pH range 2.2–5.4 (Table I and Fig. 6) must be restricted to acetate-ion-, Ac^-, catalyzed lactonization of the coumarinic acid, H_2C (or its kinetically equivalent acetic acid-, HAc, catalyzed lactonization of the coumarinate monoanion, HC^-) and/or the nonkinetically equivalent acetic acid-, HAc, catalyzed lactonization of the undissociated coumarinic acid, H_2C.

The apparent first-order rate constant, *k*, for the lactonization of coumarinic acid, H_2C, and its anion HC^- can be constructed (10) at pH values below 9 as

$$k = \{k_{H^+}[H^+] + k_{HAc}[HAc] + k_{Ac^-}[Ac^-] + k_{H_2O}\} f_{H_2C} + \{k_{H^+}'[H^+] + k'_{HAc}[HAc] + k'_{Ac^-}[Ac^-] + k'_{H_2O}\} f_{HC^-} \quad \text{(Eq. 3)}$$

where

$$f_{H_2C} = \frac{[H_2C]}{[H_2C] + [HC^-]} = \frac{[H^+]}{[H^+] + K_{a_1}'} \quad \text{(Eq. 4)}$$

and

$$f_{HC^-} = \frac{[HC^-]}{[H_2C] + [HC^-]} = \frac{K_{a_1}'}{[H^+] + K_{a_1}'} \quad \text{(Eq. 5)}$$

are the fractions of total coumarinic acid, undissociated and anionic, respectively, and the k_i's are the indicated microscopic catalytic rate constants. These simple expressions for f_{H_2C} and f_{HC^-} can be used when K_{a_1}' and K_{a_2}' differ widely and when the dianion concentration, $[C^{-2}]$, is negligible (10) as it is in this case. The term $k'_{Ac^-}[Ac^-]$ has previously been shown to be negligible, and the

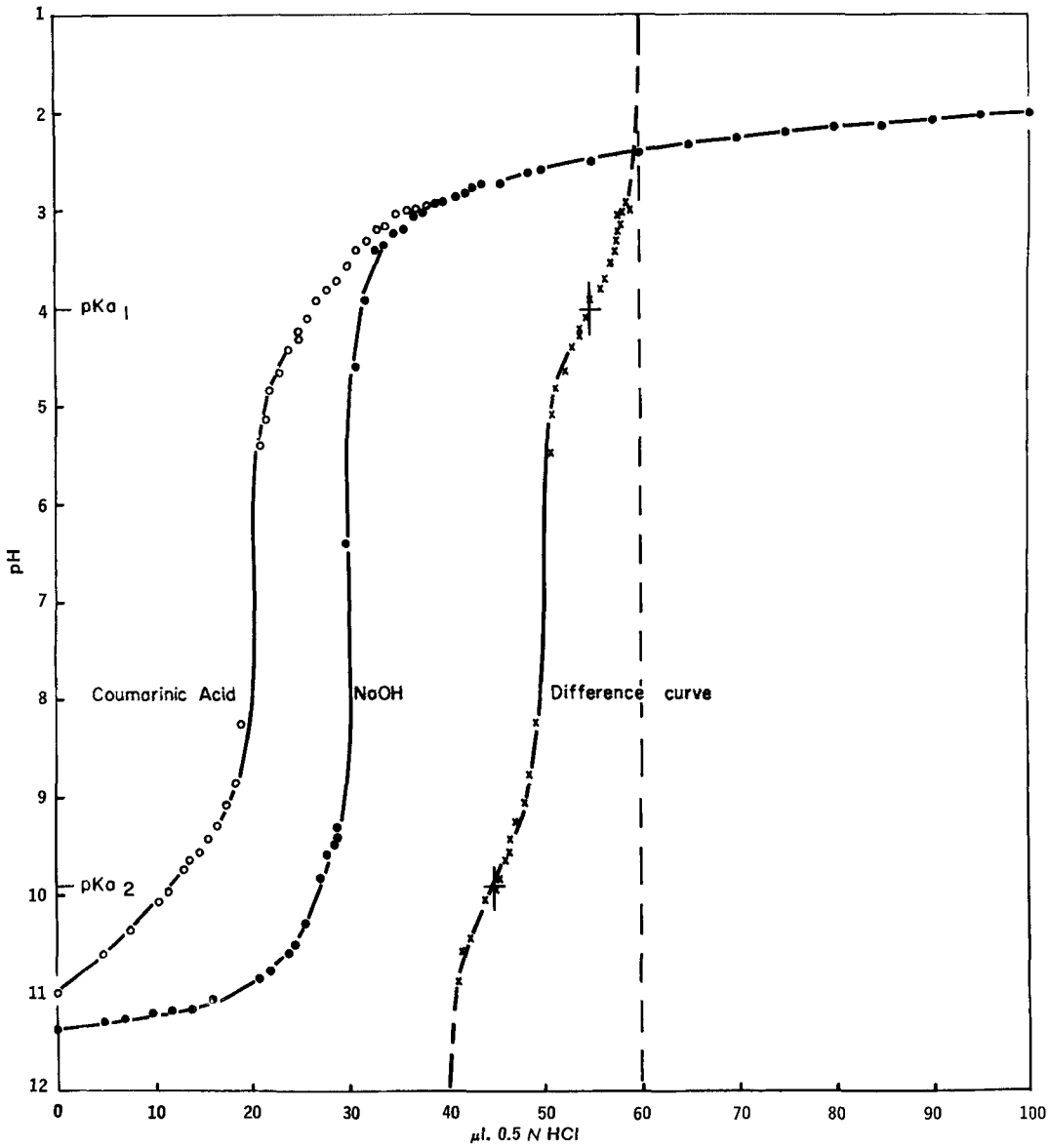


Figure 5—*Potentiometric titration curve for coumarinic acid at 25°. The titration curve of coumarinic acid is for 3 ml. of 1.67 × 10⁻³ M completely solvolyzed coumarin in 5.0 × 10⁻³ N NaOH, μ = 0.1. The blank titration curve is for 3 ml. 5.0 × 10⁻³ N NaOH, μ = 0.1.*

terms k_{Ac^-} [Ac⁻] f_{H_2C} and k'_{HAc}[HAc] f_{HC^-} are kinetically equivalent, as are $k_{H_2O}f_{H_2C}$ and $k_{H'}$ [H⁺] f_{HC^-}, so that Eq. 3 can be reduced to

$$k = \{k_{H^+}[H^+] + k_{HAc}[HAc] + k_{H_2O}\}f_{H_2C} + \{k'_{HAc}[HAc] + k'_{H_2O}\}f_{HC^-} \quad \text{(Eq. 6)}$$

At any constant [H⁺], this equation becomes

$$k = k_{HAc}[HAc]f_{H_2C} + k'_{HAc}[HAc]f_{HC^-} + (k_0 + k_0') \quad \text{(Eq. 7)}$$

where at constant pH, the constants

$$k_0 + k_0' = \{k_{H^+}[H^+] + k_{H_2O}\}f_{H_2C} + k'_{H_2O}f_{HC^-} \quad \text{(Eq. 8)}$$

where f_{H_2C} and f_{HC^-} of Eqs. 4 and 5 are also constants at constant [H⁺].

Since $K_{a_1}' = \{[H^+][HC^-]\}/[H_2C]$ and $K_{HAc} = \{[H^+][Ac^-]\}/[HAc]$, then

$$k'_{HAc}[HAc]f_{HC^-} = k'_{HAc}[HAc]\frac{[HC^-]}{[H_2C] + [HC^-]} = \frac{k'_{HAc}K_{a_1}'[HAc]}{[H^+]}\left\{\frac{[H_2C]}{[H_2C] + [HC^-]}\right\} = \frac{k'_{HAc}K_{a_1}'}{[H^+]}[HAc]f_{H_2C} \quad \text{(Eq. 9)}$$

If Eq. 7 is valid, substitution of Eq. 9 permits reduction of Eq. 7 to

$$k = (k_{HAc} + k'_{HAc}K_{a_1}/[H^+])f_{H_2C}[HAc] + (k_0 + k_0') \quad \text{(Eq. 10)}$$

Thus, a plot of the apparent first-order rate constant, k, *versus* f_{H_2C}[HAc] at any specific pH value should be a straight line of slope $(k_{HAc} + k'_{HAc}K_{a_1}'/[H^+])$ and intercept $(k_0 + k_0')$. The slope should decrease with increasing [H⁺] or decreasing pH values if acetic acid catalyzes the lactonization of the coumarinate monoanion. Similarly,

$$k_{HAc}[HAc]f_{H_2C} = k_{HAc}[HAc]\frac{[H_2C]}{[H_2C] + [HC^-]} = \frac{k_{HAc}[H^+][HC^-]}{K_{a_1}\{H_2C + HC^-\}} = \frac{k_{HAc}[HAc][H^+]}{K_{a_1}}f_{HC^-} \quad \text{(Eq. 11)}$$

If Eq. 7 is valid, substitution of Eq. 11 reduces Eq. 7 to

$$k = \left(k'_{HAc} + \frac{k_{HAc}[H^+]}{K_{a_1}}\right)f_{HC^-}[HAc] + (k_0 + k_0') \quad \text{(Eq. 12)}$$

Thus, a plot of the apparent first-order rate constant, k, *versus* f_{HC^-}[HAc] at any specific pH value should be a straight line of slope $(k'_{HAc} + k_{HAc}[H^+]/K_{a_1}')$ and intercept $(k_0 + k_0')$. The slope should increase with increasing [H⁺] or decreasing pH values if acetic acid catalyzes the lactonization of undissociated coumarinic acid.

The facts that slopes of k *versus* f_{H_2C}[HAc] do decrease with increasing pH in accordance with Eq. 10 and that slopes of k *versus* f_{HC^-} [HAc] do not significantly change with pH (Fig. 6) in accordance with the postulates of Eq. 12 prove that acetic acid does catalyze the lactonization of coumarinate monoanion but does not catalyze the lactonization of undissociated coumarinic acid.

The plots of Fig. 6 can be rationalized only on the presumption that the supposed catalytic rate constant, k_{HAc}, from acetic acid-

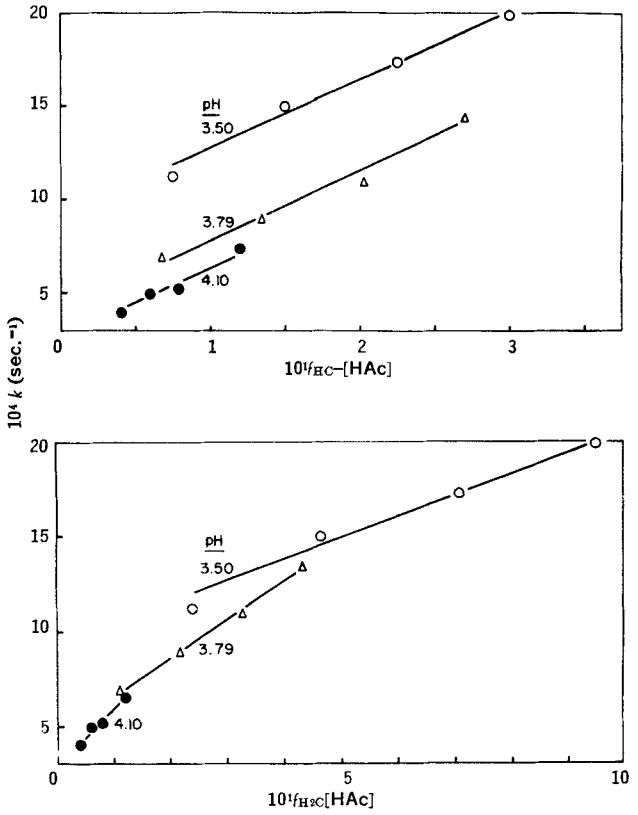


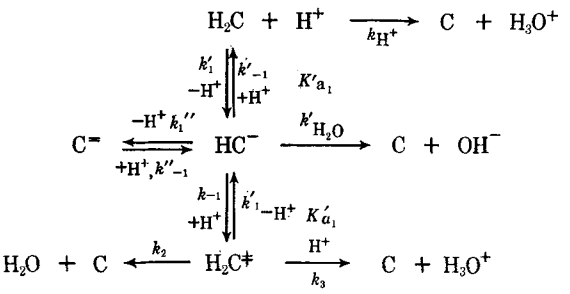
Figure 6—*Apparent first-order rate constants for the lactonization of coumarinic acid at 25°, μ = 0.1, as a function of the product of the concentration of acetic acid and the fraction as coumarinate monoanion (top) and the fraction of the undissociated coumarinic acid (bottom) at various pH values.*

catalyzed lactonization of undissociated coumarinic acid in Eqs. 7 and 9–12 is negligible. The values of $k_0 + k_0'$ can be obtained from the intercepts of the plots of Fig. 6 in accordance with Eqs. 10 and 12. The value of the catalytic rate constant, k'_{HAc}, for acetic acid-catalyzed lactonization of coumarinate monoanion, as determined from the parallel slopes of Fig. 6 in accordance with Eq. 12, is 3.65×10^{-3} l./mole-sec. at 25.0°. The observed apparent first-order rate constants at any pH can be corrected for this acetic acid catalysis by

$$k = k_{observed} - k'_{HAc} f_{HC^-}[HAc] \qquad \text{(Eq. 13)}$$

These are the k values for known $[H^+]$ and [HAc] values plotted in the log k–pH profiles of Fig. 7, where the apparent K_{a_1}' value of $10^{-4.00}$ was taken for the calculation of f_{HC^-} from Eq. 5.

The Log *k*–pH Profile for Lactonization of Coumarinic Acid and Its Anions—The apparent first-order rate constants plotted in the log k–pH profiles of Fig. 7 below the pH of 9.0, after correction for



Scheme I

general acid catalysis as by Eq. 13, may conform to the dependency of

$$k = \{k_{H^+}[H^+] + k_{H_2O}\} f_{H_2C} + \{k_{H^+}'[H^+] + k'_{H_2O}\} f_{HC^-} \qquad \text{(Eq. 14)}$$

Since $k_{H_2O} f_{H_2C}$ and $k_{H^+}'\,[H^+]\, f_{HC^-}$ are kinetically equivalent, Eq. 14 can be reduced to

$$k = \{k_{H^+}[H^+] + k_{H_2O}\} f_{H_2C} + k'_{H_2O} f_{HC^-} \qquad \text{(Eq. 15)}$$

The asymptotic slope of unity is approached with decreasing pH in Fig. 7 since Eq. 15 approaches $k = k_{H^+}[H^+]$ with increasing $[H^+]$. Thus the log k values at pH = 0 provide estimates of the k_{H^+} values (Table IV) for several temperatures.

The remainder of the log k–pH profile of Fig. 7 for lactonization was fitted by adjusting values of k_{H_2O} and k'_{H_2O} in Eq. 15 and assuming various values of K_{a_1}' for the calculation of the f_{H_2C} and f_{HC^-} values from Eqs. 4 and 5. To obtain a reasonable fit, it was necessary to assume a kinetic pK_{a_1}' of 3.00 for Eqs. 4 and 5 (Fig. 7). This differs significantly from the determined spectral (Fig. 4) and potentiometric (Fig. 5) pK_{a_1}' of 4.0. The derived parameters of Eqs. 13 and 15 are given in Table IV, as well as the Arrhenius and absolute rate thermodynamic factors obtained from the Arrhenius plots of Fig. 8.

Possible Explanation to Reconcile Apparent Kinetic, Spectral, and Potentiometric pK_{a_1}'s of Coumarinic Acid—A possible reconciliation of this discrepancy between the apparent kinetic pK_{a_1}' of 3.0 and the spectrophotometric and potentiometric titration pKa's of 4.0 may be based on an explanation similar to that offered by Eriksson (11) for similar discrepancies between kinetic and titrimetric pKa′ values observed in the alkaline solvolyses of substituted anilides and barbituric acids.

The simplest mechanistic scheme to explain the observations on lactonization of coumarinic acid, H_2C, to coumarin, C, can be given as Scheme I.

The total rate of loss of coumarinic acid to coumarin in all its forms, *i.e.*,

$$[H_2C]_T = [H_2C] + [HC^-] + [H_2C\ddagger] + [C^{-2}] \qquad \text{(Eq. 16)}$$

can be expressed as

$$-d[H_2C]_T/dt = k[H_2C]_T = -(d[H_2C]/dt + d[HC^-]/dt + d[H_2C\ddagger]/dt + d[C^{-2}]/dt) \qquad \text{(Eq. 17)}$$

Table IV—Microscopic Rate Constants[a] and Thermodynamic Parameters for the Lactonization of Coumarinic Acid and the Hydrolysis of Coumarin

Temperature	$10^2 k_{H^+}$	$10^3 k_{H_2O}$	k_{-1}	$10^{-2}P$	$10^2 Q$	$10^4 P/Q$[b]	$10^3 k_{HAc}$	$10^5 k'_{H_2O}$	k_{OH^-}
10.0°	2.2	1.9	19	5.4	5.8	0.93	—	0.80	0.14
17.5°	4.5	2.9	29	12.0	7.0	1.7	—	2.4	0.25
25.0°	10.0	5.0	50	8.8	8.0	1.1	1.2	9.2	0.45
ΔEa	17.8	10.9	10.9	—	—	—	—	22.8	13.2
log pZ[c]	12.0	9.2	5.7	—	—	—	—	12.5	9.9
$\Delta S\ddagger$ (e.u.)[d]	−5.5	−15.3	−34.5	—	—	—	—	−3.0	−18.0

[a] The apparent first-order rate constants, k, in sec.$^{-1}$ for the lactonization can be defined in terms of the various constants as: $k = k_{H^+}[H^+] f_{H_2C} + k_{H_2O} f'_{H_2O} + \{k'_{H_2O} + k'_{HAc}[HAc]\} f_{HC^-} = k_{H^+}[H^+] f_{H_2C} + (Q + P[H^+])/(1 + P[H^+]) k_{-1} Ka_1' f_{H_2C} + \{k'_{H_2O} + k'_{HAc}[HAc]\} f_{HC^-}$, where $(Q + P[H^+])/(1 + P[H^+]) = \{(k_2 + k_3[H^+])\}/(k_1 + k_2 + k_3[H^+])$, $Q = k_2/(k_1 + k_2)$, and $P = k_3/(k_1 + k_2)$. The f'_{H_2C} is an artificial expression of the fraction undissociated consistent with the log k–pH profile on the presumption of a kinetic pKa at 3 rather than at the true value of 4, $f_{H_2C} = [H^+]/([H^+] + Ka_1')$ and $f_{HC^-} = Ka_1'/([H^+] + Ka_1')$ in the pH region <7 and $f_{HC^-} = [H^+]/([H^+] + Ka_2')$ in the pH region >7, where $Ka_1' = 1.00 \times 10^{-4}$ and $Ka_2' = 1.48 \times 10^{-10}$ at 25.0°. All rate constants are in l./mole-sec. except k_{H_2O} and k'_{H_2O} which are in sec.$^{-1}$. [b] $P/Q = k_3/k_2$. [c] Ea in kcal./mole and log pZ are obtained from the slopes and intercepts of the Arrhenius plots of log k *versus* $1/T$ (Fig. 7), where T is the absolute temperature and $k = pZ\, e^{-\Delta Ea/RT}$. [d] Where $k = kT/h\, e^{-\Delta S\ddagger/R}\, e^{-\Delta H\ddagger/RT}$ and $\Delta H\ddagger = \Delta Ea - 0.6$ kcal.

where k is the apparent first-order rate constant and

$$-d[H_2C]/dt = (k_{H^+}[H^+] + k_1')[H_2C] - k_{-1}'[H^+][HC^-] \quad \text{(Eq. 18)}$$

$$-d[HC^-]/dt = (k_{-1}'[H^+] + k_1'' + k_{H_2O}' + k_{-1}[H^+])[HC^-] - k_1[H_2C\ddagger] - k_1'[H_2C] - k_{-1}''[H^+][C^{-2}] \quad \text{(Eq. 19)}$$

$$-d[C^{-2}]/dt = k_{-1}''[H^+][C^{-2}] - k_1''[HC^-] \quad \text{(Eq. 20)}$$

$$-d[H_2C\ddagger]/dt = (k_1 + k_2 + k_3[H^+])[H_2C\ddagger] - k_{-1}[H^+][HC^-] \quad \text{(Eq. 21)}$$

so that from Eqs. 17–21,

$$k = k_{H^+}[H^+][H_2C]/[H_2C]_T + k_{H_2O}'[HC^-]/[H_2C] + (k_2 + k_3[H^+])[H_2C\ddagger]/[H_2C]_T$$
$$= k_{H^+}[H^+]f_{H_2C} + k_{H_2O}'f_{HC^-} + (k_2 + k_3[H^+])f_{H_2C\ddagger} \quad \text{(Eq. 22)}$$

where f_{H_2C} and f_{HC^-} are defined by Eqs. 4 and 5, respectively, if $f_{H_2C\ddagger}$ is of negligible quantity. This latter postulate is consistent with the steady-state assumption that $d[H_2C\ddagger]/dt \sim 0$; when this condition is imposed on Eq. 21,

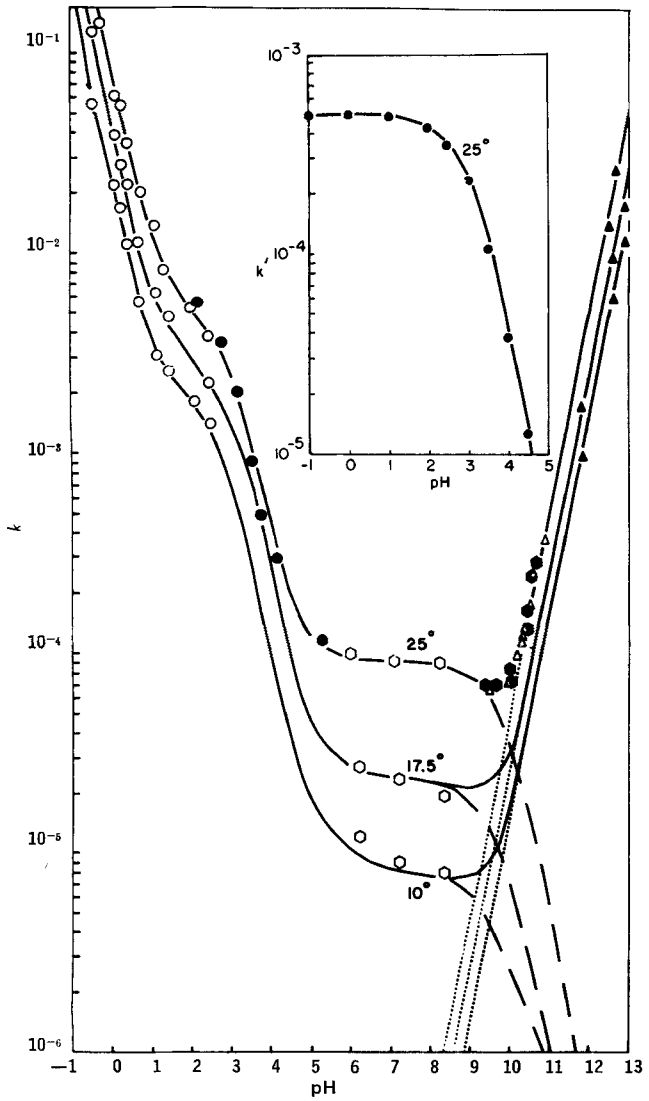


Figure 7—*Log* k–*pH profile for the lactonization of coumarinic acid and the hydrolysis of coumarin at 25.0, 17.5, and 10.0°. Key:* ○, *lactonization in HCl;* ●, *extrapolated from acetate buffer and acetic acid without general acid catalysis;* ○, *lactonization in phosphate;* ●, *lactonization in carbonate buffer;* △, *hydrolysis in carbonate buffer; and* ▲, *hydrolysis in NaOH. The inset is a plot of log* k′ versus *pH at 25.0° where* $k' = k - (k_{H^+}[H^+]f_{H_2C} + k'_{H_2O}f_{HC^-})$. *The drawn lines are calculated from the derived microscopic rate constants.*

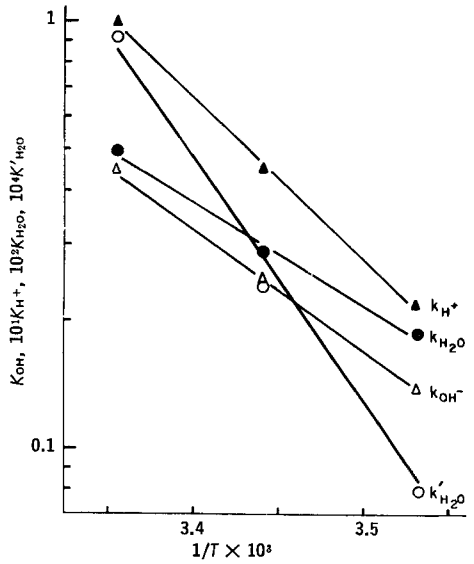


Figure 8—*Arrhenius plots of the microscopic rate constants for the lactonization of coumarinic acid and the hydrolysis of coumarin.*

$$[H_2C\ddagger] = [HC^-]k_{-1}H^+/(k_1 + k_2 + k_3[H^+]) \quad \text{(Eq. 23)}$$

so that Eq. 22 may be transformed into

$$k = k_{H^+}[H^+]f_{H_2C} + k_{H_2O}'f_{HC^-} + \frac{(k_2 + k_3[H^+])k_{-1}[H^+]}{k_1 + k_2 + k_3[H^+]}f_{HC^-} \quad \text{(Eq. 24)}$$

Since

$$[H^+]f_{HC^-} = K_{a_1}'f_{H_2C} \quad \text{(Eq. 25)}$$

the last term of Eq. 24 may be equated to

$$\frac{(k_2 + k_3[H^+])K_{a_1}'k_{-1}}{k_1 + k_2 + k_3[H^+]}f_{H_2C} \quad \text{(Eq. 26)}$$

which shows Eq. 24 to be equivalent to Eq. 15.

The value of k_{H^+} can be readily obtained from the intercept of the linear asymptote of the log k–pH plot with a slope of 1 at the pH values below 2 (Fig. 7). The value of k'_{H_2O} can be estimated from the plateau in the log k–pH profile reached between pH 6 and 8 where only the coumarinate monoanion, HC^-, exists and before the formation of a significant amount of nonlactonizable coumarinate dianion, C^{-2}, lowers the monoanion's apparent rate of lactonization. Thus, an apparent rate constant, k', can be calculated for the pH region between 2 and 6 and would be defined by consideration of Eqs. 24–26 as

$$k' = k - (k_{H^+}[H^+]f_{H_2C} + k_{H_2O}'f_{HC^-}) = \frac{(k_2 + k_3[H^+])k_{-1}K_{a_1}'}{(k_1 + k_2) + k_3[H^+]}f_{H_2C}$$
$$= \frac{Q + P[H^+]}{1 + P[H^+]}k_{-1}K_{a_1}'f_{H_2C} \quad \text{(Eq. 27)}$$

where

$$Q = k_2/(k_1 + k_2) \quad \text{(Eq. 28)}$$

and

$$P = k_3/(k_1 + k_2) \quad \text{(Eq. 29)}$$

Thus, at high $[H^+]$ concentrations or at pH values less than 2 where $f_{H_2C} \rightarrow 1$ and

$$(Q + P[H^+])/(1 + P[H^+]) = (k_2 + k_3[H^+])/(k_1 + k_2 + k_3[H^+]) \rightarrow 1 \quad \text{(Eq. 30)}$$

then,

$$\lim_{[H^+]\rightarrow\infty} k' = k_{-1}K_a' = k_{H_2O} \quad \text{(Eq. 31)}$$

and k_{-1} may be estimated (Table IV) from the known K_{a1}' and the apparent k_{H_2O} values, where the latter was obtained from the best fit of Eq. 15 and is 5×10^{-3} at 25°.

Thus, at low $[H^+]$ concentrations, possibly at pH values greater than 5, where $k_3[H^+] \ll k_2$

$$\lim_{[H^+]\to\infty} k' = \left(\frac{k_2}{k_1 + k_2}\right) k_{-1}K_{a1}'f_{H_2C} = Qk_{-1}K_{a1}'f_{H_2C} \to Qk_{-1}[H^+] \qquad \text{(Eq. 32)}$$

since from Eq. 4, $f_{H_2C} \to [H^+]/K_{a1}'$ at $[H^+] \ll K_{a_1}'$. Thus Q, as defined in Eq. 28, may be estimated (Table IV) since k_{-1} (Eq. 31), K_{a_1}', and $[H^+]$ are known.

At intermediate pH values, possibly at pH values between 2 and 5 where the more exact Eq. 27 holds, where k_{-1} (Eq. 31) and $Q = k_2/(k_1 + k_2)$ (Eq. 32) have been estimated (Table IV) and all other factors such as $[H^+]$, K_{a_1}', and f_{H_2C} (Eq. 4) are known, the value of P may be calculated as

$$P = \frac{k' - Qk_{-1}K_{a_1}'f_{H_2C}}{(k_{-1}K_a'f_{H_2C} - k')[H^+]} \qquad \text{(Eq. 33)}$$

and is given in Table IV.

The curves drawn through the points in Fig. 7 are based on the evaluated values of Q, P, k_{-1}, k_{H^+} and k'_{H_2O} given in Table IV.

Mechanism in Explanation of Kinetic Dependencies—The kinetic transformations of Scheme I are consistent with the fitting of the log k–pH profiles of Fig. 7 on the basis of the kinetic dependencies of Eqs. 22 and 24–27. These relations may have the mechanistic bases outlined in Scheme II which are consistent with the classical explanations of esterification (12).

The sequence I–IV, Scheme II,

$$H_2C + H^+ \rightleftarrows [H_3C^+] \rightleftarrows [H_3C\ddagger] \xrightarrow{-H_3O^+} C \qquad \text{(Eq. 34)}$$

is typical of hydrogen-ion attack on carbonyl to produce a protonated monoester of an orthoacid, $H_3C\ddagger$ (III), which dehydrates to coumarin, C (IV), and is consistent with a rate dependency of the apparent first-order rate constant on $k_{H^+}[H^+]f_{H_2C}$. The ionization equilibria of coumarinic acid, H_2C (I), provide a monoanion, HC^- (V), and a dianion, C^{-2} (IX), where the latter may be completely stabilized against intramolecular esterification by repulsion of its two negative charges. The coumarinic acid and/or its monoanion can form the anionic orthoacid, $HC\ddagger$ (VII), by intramolecular nucleophilic attack of phenate anion on carboxyl (VI → VII) after intramolecular general base attack of the monoanion (V → VI) or direct dissociation of the coumarinic acid (I ⇆ VI). Of course, the direct closure of the coumarinate monoanion (V → VIII) is a possibility. The anion orthoacid, $HC\ddagger$ (VII), may directly dissociate into hydroxyl ion and coumarin (VIII → IV). This is a relatively slow process and is consistent with the observed dependency of the apparent first-order rate constant on $k'_{H_2O}f_{HC^-}$ to form coumarin (Eq. 15). The anion orthoacid, $HC\ddagger$ (VII), may equilibrate with its conjugate monoesterified orthoacid, $H_2C\ddagger$ (VIII), which may spontaneously dehydrate to coumarin (VIII → IV) with first-order rate constant k_2 or be attacked by hydrogen ion with a second-order rate constant k_3 to form the more highly unstable protonated monoester of the orthoacid $H_3C\ddagger$ (III), which more readily forms coumarin (VIII → III → IV). These sequences are consistent with the complex dependency of the apparent first-order rate constant as represented by Eq. 27 on the premise of the steady-state approximation that $H_2C\ddagger$ (VIII) is highly unstable and in low concentration and that $k_3/k_2 = P/Q$ is in the ratio of 10,000:1.

Reactivity of Coumarin and Coumarinate Anion in Alkaline Solutions—If the coumarinate dianion C^{-2} (IX) is unreactive, due to the repulsion of the phenate and carboxylate anions, the apparent first-order rate constant for lactonization will plateau in the pH range 6–8 (Fig. 7) at a value of k'_{H_2O}, where all the coumarinic acid is in the monoanionic form, and will decrease in accordance with $k'_{H_2O}f_{HC^-}$ above pH values of 8 (dashed lines in Fig. 7). The plots of log k *versus* pH for the hydrolysis of coumarin to coumarinate dianion are linear above pH 10 in accordance with the expression

$$\frac{-dC}{dt} = \frac{d[H_2C]_T}{dt} = kC = k_{OH^-}[OH^-]C \qquad \text{(Eq. 35)}$$

where, on a logarithmic transformation,

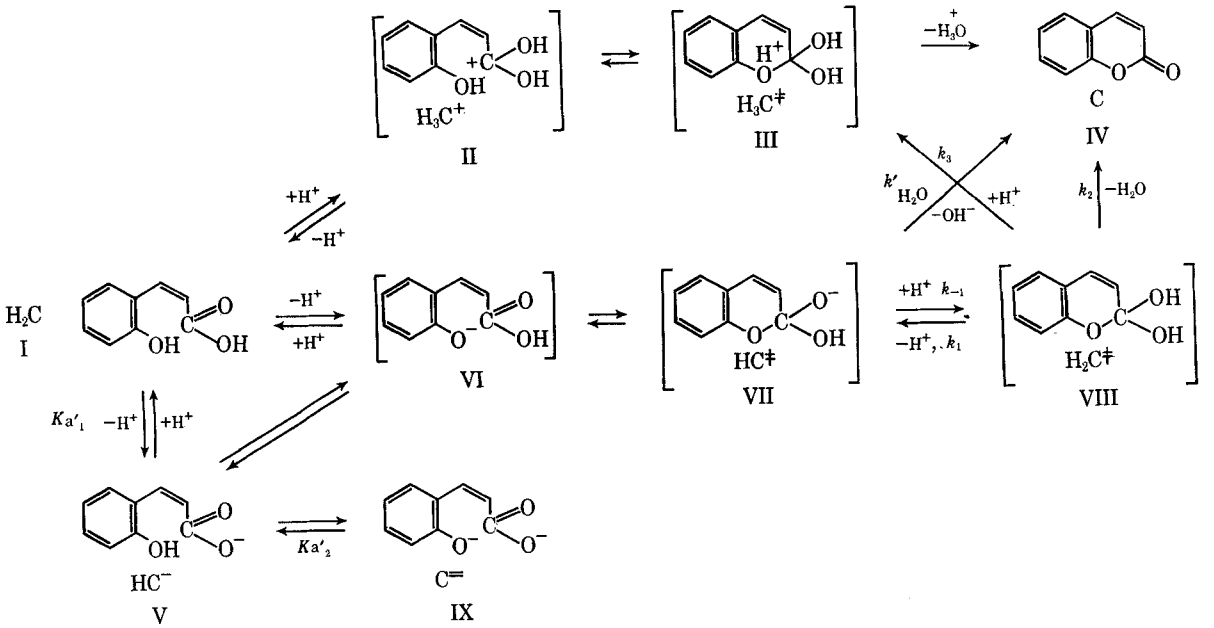
$$\log k = \log k_{OH^-} pK_w + \text{pH} \qquad \text{(Eq. 36)}$$

where the k_{OH} values (Table IV) are estimated from the intercepts. The sums of the observed apparent first-order rate constants for lactonization and hydrolysis are drawn in the pH region 8.5–11.5 and are consistent with the plotted overall apparent first-order rate constants obtained from appropriate plots of the spectral data in accordance with Eq. 1. This is consistent with a rate of achievement of a coumarinic acid monoanion and dianion–coumarin equilibrium, where the overall apparent first-order rate constant, k, to achieve this equilibrium in this pH region is actually

$$k = k'_{H_2O}f_{HC^-} + k_{OH}[OH^-] \qquad \text{(Eq. 37)}$$

since only nonreactive dianion, C^{-2}, and reactive monoanion, HC^-, are present in this pH region.

The equilibrium constant can be defined as a function of hydrogen- and hydroxyl-ion concentrations when Eqs. 17 and 35 are equated,



Scheme II

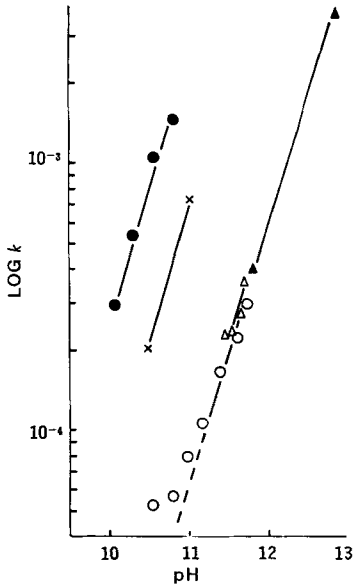


Figure 9—*Log* k–*pH relationship for the hydrolysis of 3-chlorocoumarin* (●), *3-bromocoumarin* (×), *3-methylcoumarin* (△), *coumarin* (○), *and 4-methylcoumarin* (▲) *in dioxane* (*30%, v/v*)–*water at 25°.*

Eqs. 18–26 and the fact that C^{-2} has been shown to be nonreactive are considered, and the resulting expression is rearranged to

$$K = \frac{[C]_{eq.}}{[H_2C]_{T_{eq.}}} = \frac{[C]_{eq.}}{[H_2C]_{eq.} + [HC^-]_{eq.} + [C^{-2}]_{eq.}} = \frac{k'_{H_2O}f_{HC^-} + \left(k_{H^+}[H^+] + \frac{(k_2 + k_3[H^+])k_{-1}K_{a1}'}{k_1 + k_2 + k_3[H^+]}\right) f_{H_2C}}{k_{OH^-}[OH^-]} \quad \text{(Eq. 38)}$$

where the subscript "eq." refers to the respective concentrations at equilibrium. However, coumarin and coumarinic acid ions only exist in solution together in the pH region 8.5–11.5 (Fig. 7), where f_{H_2C} is negligible, so that the equilibrium constant of Eq. 38 for lactonization simplifies to

$$K = k'_{H_2O}f_{HC^-}/k_{OH^-}[OH^-] \quad \text{(Eq. 39)}$$

Some typical values of this equilibrium constant at 25.0° and at various pH values are: 7.0 (pH), 2200 (*K*); 8.0, 210; 9.0, 18.3; 9.5, 4.8; 10.0, 0.96; 10.5, 0.142; and 11.0, 0.017.

Hydrolysis of 3- and 4-Substituted Coumarins in 30% (v/v) Dioxane–Water—Halogen substitution in the 3-position has a pronounced accelerating effect on hydrolysis (Table III). This can be rationalized by the electron-withdrawing action of these atoms, which is greater for the chloro than for the bromo compound. This property should and does promote the attack of the hydroxyl ions on the carbonyl carbon. Methyl substitutions for hydrogens in both the 3- and 4-positions do not significantly affect hydrolysis rates. This can be clearly seen from the bimolecular rate constants, k_{OH^-}, for the hydrolysis, determined from the intercepts of the unit slope plots of log *k versus* pH, plotted in accordance with Eq. 36 (Fig. 9). The k_{OH^-} (l./mole-sec.) values at 25.0° in 30.0% (v/v) dioxane–water were 2.5 for 3-chloro-, 0.7 for 3-bromo-, 0.07 for 3-methylcoumarin, 0.065 for coumarin, and 0.058 for 4-methylcoumarin. Further investigations of the reactivity of these compounds are planned in aqueous solutions.

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Multiple-Dosing Drug Kinetics: Nomographic Method of Estimating the Number of Doses Needed to Approach within a Fixed Percent of Asymptotic Minimum Drug Level

BERTON E. BALLARD and EFRAIM MENCZEL*

Abstract □ Under multiple-dosing conditions, where a drug disappears from a compartment in an apparent monoexponential manner after each dose administration, equations can be derived which permit the calculation of the number of doses needed to approach within a predetermined fixed percent of the asymptotic minimum drug level in the compartment. Nomograms are presented which illustrate how these equations can be used in dosing calculations.

Keyphrases □ Kinetics—multiple-dosing drugs □ Dosing calculations—asymptotic minimum drug level □ Nomographic method—multiple-dosing kinetics

In recent years a number of authors (1–9) have been concerned with the mathematics of designing optimal drug dosing regimens whereby initial and constant maintenance doses are administered to achieve compartment levels of the drug resulting in an oscillation between some maximum and minimum limiting values under steady-state conditions. This steady-state condition or plateau effect occurs when the input of drug to the compartment is equal to the output of drug from the same compartment in a given dosing time interval. Such drug dosing calculations are most useful when an empirical relationship has been found between the amount or concentration of drug in a given compartment and the intensity of a particular pharmacological response at a given time.

One problem with using the proposed drug dosing regimen calculations is that it is often tedious to estimate how many maintenance doses are required to reach the steady-state condition. From a mathematical point of view, with certain ratios of initial to maintenance doses and biological half-life to dosing time intervals, an infinite number of maintenance doses are required to achieve steady-state conditions. From a practical point of view, it is usually not necessary to reach steady-state conditions *exactly* in order to elicit the desired pharmacological response from the proposed drug dosing regimen. It is sufficient in a great many cases to approach the steady-state conditions within a small predetermined percent of the asymptotic values. How close the actual drug amounts or concentrations in the compartment should be to the theoretically calculated asymptotic values in order to elicit the desired pharmacologic response depends upon answers to at least three questions.

How sensitive is the pharmacological response to changes in drug amounts or concentrations in the compartment at particular times during the multiple-dosing regimen? How sensitive are the analytical techniques used in detecting changes in drug amounts or concentrations in the fluids present in the compartment studied? And how reproducible is the apparent biological half-life measured in the same compartment when the drug is administered at widely spaced times or under frequent dosing conditions?

The purpose of this article is to derive equations from which nomograms can be constructed to permit an estimate of the number of initial and maintenance doses needed to approach within a predetermined constant percent of the asymptotic minimum drug level in a given compartment. Since there is unlikely to be any general agreement as to the magnitude of this fixed percent, an arbitrary choice of ±1% of the asymptotic minimum value was selected for the purpose of illustration. Other fixed percentages of the asymptotic value, say ±5 and ±10%, would result in nomograms similar to the ones generated here and, therefore, will not be discussed.

THEORETICAL

Consider the case where the initial and maintenance doses of a drug are directly introduced into a given compartment from which the drug disappears in an apparent monoexponential manner by all processes. The number of doses necessary to approach within ±1% of the asymptotic minimum drug level can be estimated from Eq. 1, which was derived previously (10)

$$n \geqslant \left(\frac{t_{0.5}}{\tau}\right)\left(\frac{1}{\ln 2}\right) \ln Q \qquad \text{(Eq. 1)}$$

where n is the number of doses including the first one at zero time, $t_{0.5}$ is the apparent half-life for drug disappearance from the compartment, τ is the dosing interval in the same time units as for the half-life, and Q is defined by Eqs. 2–7. Let

$$B_i = B_m + B'' \qquad \text{(Eq. 2)}$$

where B_i is the initial dose, B_m is the constant maintenance dose, and B'' is the amount of drug in the compartment administered along with B_m as a part of the initial dose, and $B_i \geqslant B_m$.

Let

$$\beta = \frac{B''}{B^{\infty}_{\text{min.}}} = \frac{B''/V}{B^{\infty}_{\text{min.}}/V} \qquad \text{(Eq. 3)}$$

where β is the ratio of amounts or concentrations of drug, $B^{\infty}_{\text{min.}}$ is the asymptotic minimum amount of drug in the compartment one τ after a very large (or infinite) number of maintenance doses has been administered, and V is the constant for compartment volume (or apparent distribution volume for the compartment).

Let

$$\alpha_n = \frac{B^{(n)}_{\text{min.}}}{B^{\infty}_{\text{min.}}} = \frac{B^{(n)}_{\text{min.}}/V}{B^{\infty}_{\text{min.}}/V} \qquad \text{(Eq. 4)}$$

where α_n is the ratio of amounts or concentrations of drug compatible with integer values for n, $B^{(n)}_{\text{min.}}$ is the minimum amount of drug in the compartment one τ after the administration of the nth dose, and $B^{\infty}_{\text{min.}}$ and V have the same meaning as before. In this paper the value of $B^{(n)}_{\text{min.}}$ or $[B^{(n)}_{\text{min.}}/V]$ was arbitrarily set within ±1%

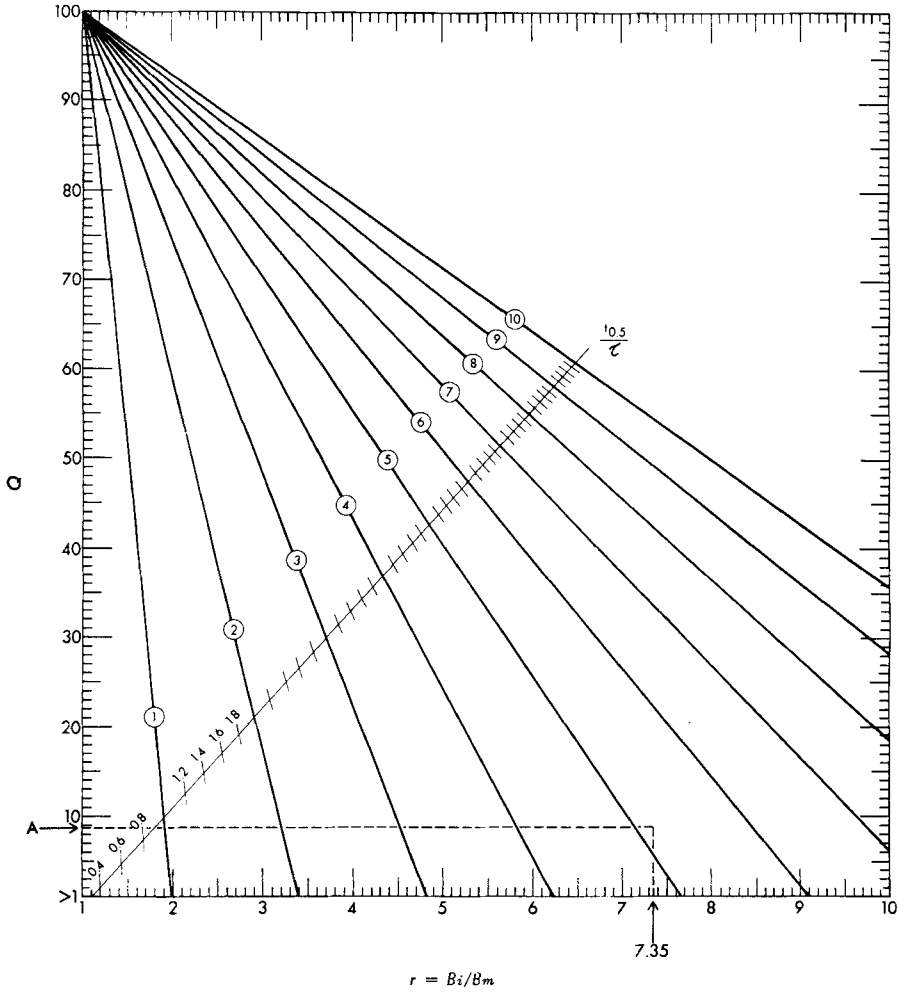

Figure 1—*Nomogram shows the relationship between* r *and* Q *for various* $t_{0.5}/\tau$ *values where* $\alpha = 0.99$. *Example for Animal A shows that* Q = 8.83 *when* r = 7.35 *and* $t_{0.5}/\tau = 5.18$.

of $B^{\infty}_{\text{min.}}$ or $(B^{\infty}_{\text{min.}}/V)$, so that it might not be possible to find values for α_n resulting in integer values for n. For α arbitrarily close to 1, say $|\alpha - 1| = 0.01$, it is desired to find the smallest n such that (10)

$$|\alpha_n - 1| = |e^{-n(\tau/t_{0.5})\ln 2}(\beta - 1)| \leqslant |\alpha - 1| \quad \text{(Eq. 5)}$$

Solving Eq. 5 for n yields

$$n \geqslant \left(\frac{t_{0.5}}{\tau}\right)\left(\frac{1}{\ln 2}\right)\ln\left(\frac{\beta - 1}{\alpha - 1}\right) \quad \text{(Eq. 6)}$$

Let

$$Q = \left(\frac{\beta - 1}{\alpha - 1}\right) = \left(\frac{1 - \beta}{1 - \alpha}\right) \quad \text{(Eq. 7)}$$

Substitution of Eq. 7 into Eq. 6 yields Eq. 1.

In this model, there is only one ratio, R, of the initial to the maintenance dose for a particular $t_{0.5}/\tau$ that results in constant values for $B^{\infty}_{\text{min.}}$ one τ after the first and all succeeding doses up to and including the nth dose. This ratio (11) is

$$R = \frac{B_i}{B_m} = \left[\frac{1}{1 - e^{-(\tau/t_{0.5})\ln 2}}\right] \quad \text{(Eq. 8)}$$

For purposes of convenience, let $X = (\tau/t_{0.5})\ln 2$.

There are many other possible values for the ratio B_i/B_m. Let

$$r = \frac{B_i}{B_m}, \qquad \text{where } r \neq R \quad \text{(Eq. 9)}$$

Substitution of Eq. 2 into Eq. 3 yields

$$\beta = \frac{B_i - B_m}{B^{\infty}_{\text{min.}}} = \frac{[(B_i - B_m)/V]}{(B^{\infty}_{\text{min.}}/V)} \quad \text{(Eq. 10)}$$

Substitution of Eq. 9 into Eq. 10 yields

$$\beta = \frac{(rB_m - B_m)}{B^{\infty}_{\text{min.}}} = \frac{(rB_m - B_m)/V}{B^{\infty}_{\text{min.}}/V} \quad \text{(Eq. 11)}$$

or

$$\beta = \frac{B_m(r - 1)}{B^{\infty}_{\text{min.}}} = \frac{B_m(r - 1)/V}{B^{\infty}_{\text{min.}}/V} \quad \text{(Eq. 12)}$$

It has been shown previously (11) that

$$B^{\infty}_{\text{min.}} = B_m\left(\frac{e^{-X}}{1 - e^{-X}}\right) \quad \text{(Eq. 13)}$$

Substitution of Eq. 8 into Eq. 13 yields

$$B^{\infty}_{\text{min.}} = B_m R e^{-X} \quad \text{(Eq. 14)}$$

Substitution of Eq. 12 into Eq. 7 yields

$$Q = \left(\frac{1}{1 - \alpha}\right)\left[1 - \frac{B_m(r - 1)}{B^{\infty}_{\text{min.}}}\right] \quad \text{(Eq. 15)}$$

Substitution of Eq. 14 into Eq. 15 yields

$$Q = \left(\frac{1}{1 - \alpha}\right)\left[1 - \frac{B_m(r - 1)}{B_m R e^{-X}}\right] \quad \text{(Eq. 16)}$$

and

$$Q = \left(\frac{1}{1 - \alpha}\right)\left[1 - \frac{(r - 1)}{Re^{-X}}\right] \quad \text{(Eq. 17)}$$

or

$$Q = \left(\frac{1}{1 - \alpha}\right)\left(1 - \frac{r}{Re^{-X}} + \frac{1}{Re^{-X}}\right) \quad \text{(Eq. 18)}$$

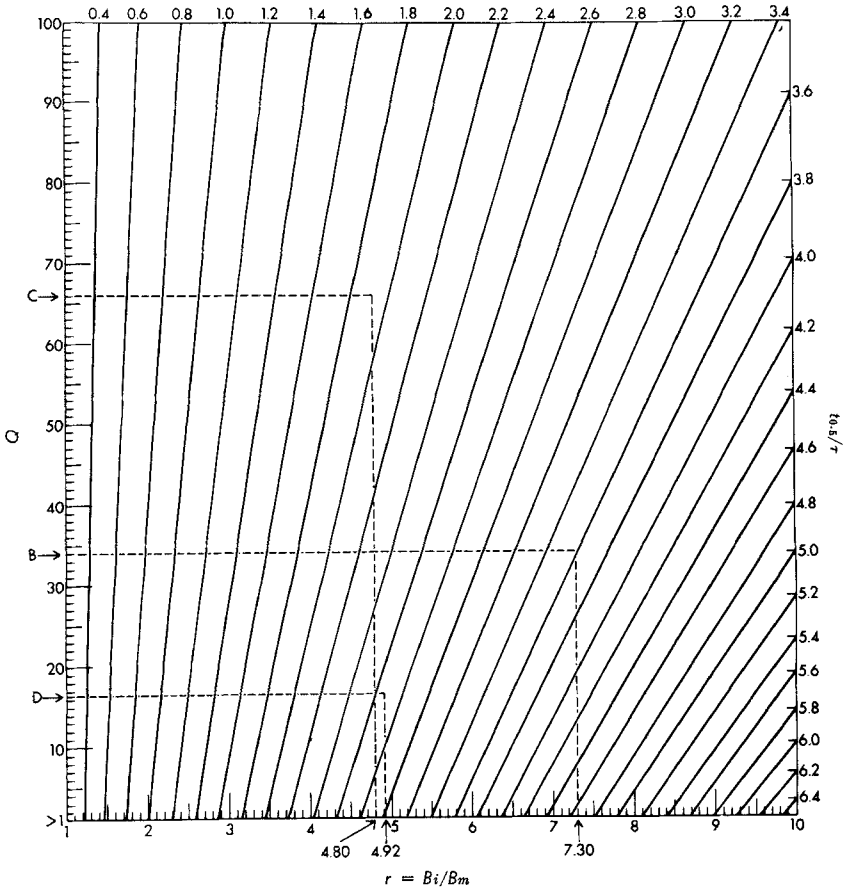

Figure 2—*Nomogram shows the relationship between* r *and* Q *for various* $t_{0.5}/\tau$ *values where* $\alpha = 1.01$. *Example for Animal B shows that* Q = 34.2 *when* r = 7.30 *and* $t_{0.5}/\tau = 3.60$. *Consult Table I for data on Animals C and D.*

Rearranging Eq. 18 gives

$$Q = \left(\frac{1}{1-\alpha}\right)\left(1 - \frac{re^{+X}}{R} + \frac{e^{+X}}{R}\right) \qquad \text{(Eq. 19)}$$

Substitution of Eq. 8 into the right-hand term of Eq. 19 gives

$$Q = \left(\frac{1}{1-\alpha}\right)\left[1 - \frac{re^{+X}}{R} + (e^{+X})(1 - e^{-X})\right] \qquad \text{(Eq. 20)}$$

and

$$Q = \left(\frac{1}{1-\alpha}\right)\left(e^{+X} - \frac{re^{+X}}{R}\right) \qquad \text{(Eq. 21)}$$

or

$$Q = e^{+X}\left(\frac{1 - \frac{r}{R}}{1-\alpha}\right) \qquad \text{(Eq. 22)}$$

Equation 22 can be rearranged into an equation for a straight line:

$$Q = \left[\frac{e^{+X}}{(1-\alpha)}\right] - \left[\frac{e^{+X}/R}{(1-\alpha)}\right] r \qquad \text{(Eq. 23)}$$

Thus, a plot of *r versus Q* would result in an intercept of

$$\left[\frac{e^{+(\tau/t_{0.5})\ln 2}}{1-\alpha}\right]$$

When *r* is 1, the value of *Q* will be +100 when α is 0.99, and it will be −100 when α is 1.01. The slope

$$\left[\frac{e^{+(\tau/t_{0.5})\ln 2/R}}{1-\alpha}\right]$$

will be negative when α is 0.99 and positive when α is 1.01.

The time, *t*, needed for amounts or concentrations to reach within ±1% of the corresponding asymptotic values one τ after the *n*th dose can be calculated from the relationship

$$t = n\tau \qquad \text{(Eq. 24)}$$

RESULTS AND DISCUSSION

Figures 1 and 2[1] show the plot of *r versus Q* where *r* ranges from 1 to 10. In Fig. 1 the value of $t_{0.5}/\tau$ varies from 0.4 to 10, and in Fig. 2 the value of $t_{0.5}/\tau$ varies from 0.4 to 6.4. The values for *Q* in both figures vary from >1 to 100. The reasons that *Q* must be greater than +1 at all times have been discussed elsewhere (10). Two examples, using literature data, will illustrate how Figs. 1–3 can be used to solve for the quantities *n* and *t*. The first example involves multiple subcutaneous drug administration and the second involves multiple oral drug administration.

Example 1: Multiple Subcutaneous Injections—Ballard and Menczel (10) administered dilute aqueous benzyl alcohol solutions directly onto the subcutaneous tissue of anesthetized rats and followed drug concentration changes as a function of time. The drug disappeared from the subcutaneous region compartment in an apparent monoexponential manner after each dose administration. A summary of the data obtained from Animals A through D (10, 11) is found in Table I. In Fig. 1 it can be seen for Animal A, when $r = 7.35$, $\alpha = 0.99$, and $t_{0.5}/\tau = 5.18$, that $Q \cong 8.83$. In Fig. 2 the same procedure used for Animal D is followed for Animals B and C to determine *Q* since they, too, have a value for $\alpha = 1.01$.

When using Figs. 1 and 2, it is not necessary in a particular case to know in advance whether $\alpha = 0.99$ or $\alpha = 1.01$. For example, in Fig. 1, only Animal A has the $t_{0.5}/\tau$ value needed to make an estimate of *Q* for its *r* value. Similarly, in Fig. 2, only Animals B, C, and D have the $t_{0.5}/\tau$ value needed to make an estimate of *Q* for their respective *r* values.

In Fig. 3 a plot of *n versus* $(t_{0.5}/\tau)$ is based upon Eq. 1. Once a given value for *Q* can be estimated from Fig. 1 or 2 using the appropriate values for *r* and $t_{0.5}/\tau$, it is possible to estimate *n* from

[1] Enlarged copies of Figs. 1, 2, and 3 are available upon request.

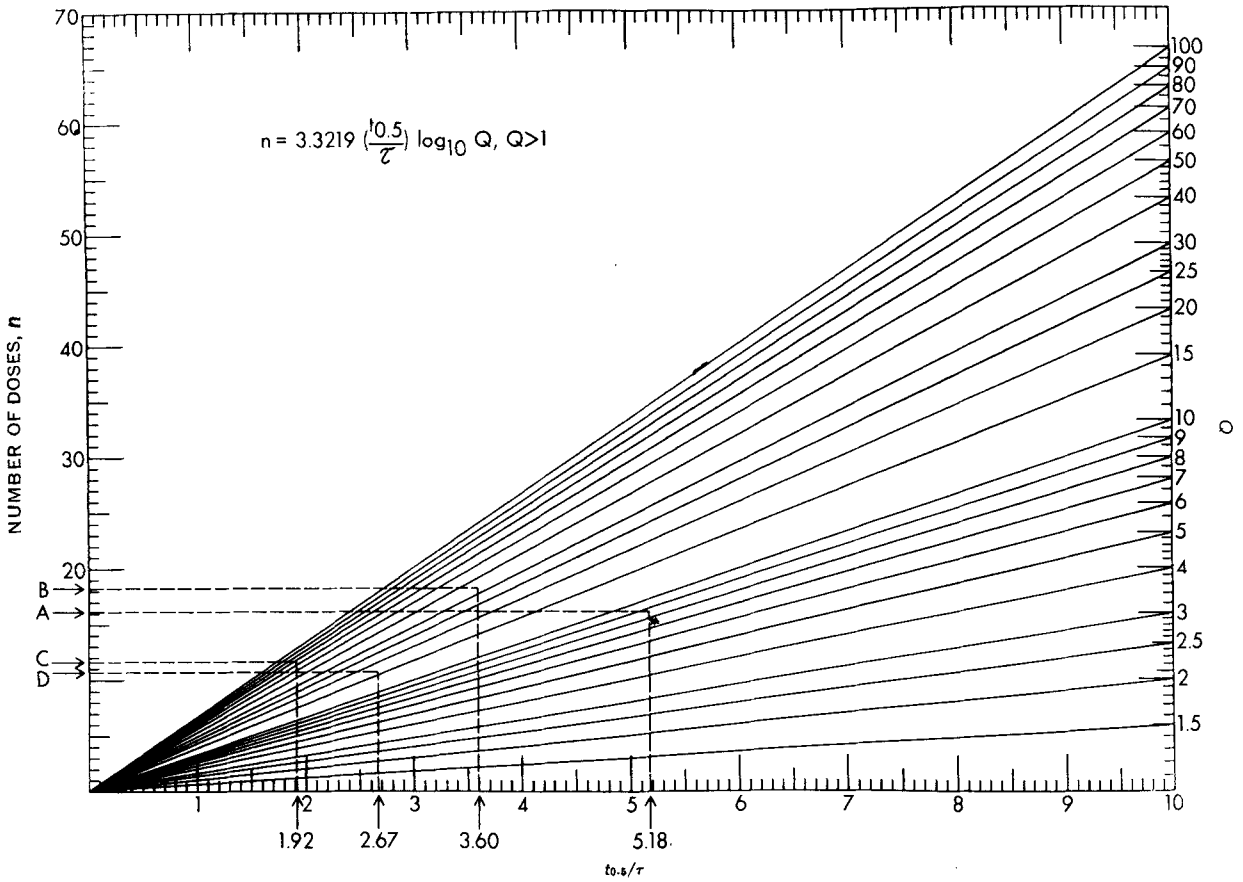

Figure 3—*Nomogram shows the relationship between* Q *and* n *for various values of* $t_{0.5}/\tau$. *Example for Animal A shows that when* Q = *8.83 and* $t_{0.5}/\tau$ = *5.18, then* n > *16. Consult Table I for data on Animals B, C, and D.*

Fig. 3 for the same values for Q and $t_{0.5}/\tau$. Obviously, it is also possible to proceed in the reverse direction; if a given value for n is desired, it should be possible to use Fig. 3 and the appropriate Fig. 1 or 2 to estimate the required B_i/B_m value for a fixed value[2] of $t_{0.5}/\tau$.

When apparent monoexponential loss of drug occurs from a compartment such as a subcutaneous absorption cell, the previous mathematical methods are correct. However, when the concentration *versus* time course of a drug in a given compartment is best described by some polyexponential function, the previous mathematical derivations must be used cautiously. Wagner *et al.* (8) discussed other phenomena which may invalidate the procedures discussed for multiple-dosing calculations.

Nevertheless, with carefully selected clinical data, it is possible, without introducing too much error, to use equations derived in this paper and the nomograms shown in Figs. 1–3 to estimate the number of doses and time needed to approach within ±1% of the asymptotic minimum drug level in the compartment. An example using these equations on data obtained from a sulfonamide having a short absorption half-life and long biological half-life in humans will illustrate the method of calculating n from a knowledge of the two ratios: $t_{0.5}/\tau$ and B_i/B_m.

Example 2: Multiple Oral Dosing—Krüger-Thiemer *et al.* (12) administered sulfamethoxypyrazine orally to three healthy test subjects using two different dosing regimens. In the first experiment, the subjects received an initial dose of 400 mg. of the drug followed by six[3] 100-mg. maintenance doses at a dosing interval of 24 hr. The plasma concentration of the drug was followed for 216 hr. In the second experiment, the same subjects received an initial dose of 2000 mg. followed by three 1500-mg. maintenance doses at a dosing interval of 168 hr. The plasma concentration of the drug was followed for 624 hr. in one subject and for 672 hr. in the other two subjects. The authors assumed that a single body compartment provided a sufficient description of drug distribution which took into account drug binding to plasma proteins. In this model, two first-order rate constants were considered to describe drug absorption from the gastrointestinal tract and drug elimination mainly by metabolism and renal excretion. Since the absorption half-life of the drug from the gastrointestinal tract was short (0.4–0.7 hr.) compared to the elimination half-life (50–69 hr.), only a small error is made if one assumes that the drug was being administered by a rapid intravenous injection instead of by oral administration. If it is valid to assume that the absorption step is nearly instantaneously rapid, then it should be possible to use the pharmacokinetic data summarized in the top half of Table II for the three subjects to estimate the number of doses, n, required to approach

Table I—Data and Derived Constants from Subcutaneous Multiple-Dosing Experiments with Benzyl Alcohol Required for Substitution into Figs. 1–3 to Estimate the Total Number of Doses Needed to Approach within ±1% of $B^{\infty}_{min.}$ (10, 11)

Constant	Animal A	Animal B	Animal C	Animal D
$t_{0.5}/\tau$[a]	5.18	3.60	1.92	2.67
α[b]	0.99	1.01	1.01	1.01
r[c]	7.35	7.30	4.80	4.92
R[d]	7.97	5.70	3.29	4.37
Q[e]	8.83	34.2	65.9	16.4
n[f]	>16	>18	>11	>10

[a] This ratio is defined in Eq. 1. [b] This term was arbitrarily set at 0.99 or 1.01 so that $B^{n}_{min.}$ would approach within ±1% of $B^{\infty}_{min.}$. [c] Defined by Eq. 9. [d] Defined by Eq. 8. [e] Defined by Eq. 7. [f] Defined by Eq. 1.

[2] The model 9100 Hewlett-Packard calculator (Palo Alto, Calif.) was programmed to solve the equations needed to construct Figs. 1–3 and to calculate the values contained in Tables I and II.

[3] In the work by Krüger-Thiemer *et al.* (12), Fig. 5 shows that six 100-mg. maintenance doses were administered to the three subjects. Tables VIII and XII were in error (13) because they indicated that only four 100-mg. maintenance doses were administered.

Table II—Data and Derived Constants from Oral Multiple-Dosing Experiments with Sulfamethoxypyrazine Required for Substitution into Figs. 1–3 to Estimate the Total Number of Doses and Time Needed to Approach within ±1% of $B^{\infty}_{min.}$ (12, 14)

Constant	Subject: Be.[i] Expt. 1	Be.[i] Expt. 2	Ste.[i] Expt. 1	Ste.[i] Expt. 2	Su.[i] Expt. 1	Su.[i] Expt. 2
$t_{0.5}$ (hr.)[a]	61.5	69.2	58.7	64.6	50.4	51.1
τ (hr.)[b]	24	168	24	168	24	168
$t_{0.5}/\tau$	2.56	0.41	2.45	0.39	2.10	0.30
B_i[c]	400 mg.	2000 mg.	400 mg.	2000 mg.	400 mg.	2000 mg.
B_m[d]	100 mg.	1500 mg.	100 mg.	1500 mg.	100 mg.	1500 mg.
r[e]	4	1.33	4	1.33	4	1.33
R[f]	4.22	1.23	4.05	1.20	3.56	1.11
Q[g]	6.81	45.87	1.71	68.68	17.32	191.89
n[h]	>7	>2	>1	>2	>8	>2
	(7.09)	(2.27)	(1.89)	(2.35)	(8.64)	(2.31)
t (days)[i]	7	14	1	14	8	14

Constant	Age Groups: Newborns	Infants	Children	Adults	Aged
$t_{0.5}$ (hr.)[a]	135.6	53.9	51.0	63.3	98.2
τ (hr.)[b]	24	24	24	24	24
$t_{0.5}/\tau$	5.65	2.25	2.13	2.64	4.09
B_i[c]	12 mg./kg.	8.2 mg./kg.	12 mg./kg.	750 mg.	600 mg.
B_m[d]	1.34 mg./kg.	1.9 mg./kg.	3 mg./kg.	150 mg.	100 mg.
r[e]	8.96	4.32	4.0	5.0	6.0
R[f]	8.66	3.77	3.59	4.33	6.42
Q[g]	3.83	19.89	15.70	20.23	7.70
n[h]	>10	>9	>8	>11	>12
	(10.96)	(9.69)	(8.44)	(11.44)	(12.05)
t (days)[i]	10	9	8	11	12

[a] Defined by Eq. 1. [b] Dosing time interval. [c] Initial dose. [d] Maintenance dose. [e] Defined by Eq. 9. [f] Defined by Eq. 8. [g] Defined by Eq. 7. [h] Defined by Eq. 1. [i] Defined by Eq. 24. [j] Abbreviations for the subjects used in *Reference 12.*

within ±1% of the asymptotic minimum drug level and the time, t, needed to reach this level for the two dosing regimens.

In Table II, it can be seen that under conditions defined in Experiment 1 for the three subjects, 1–8 days are required to approach within ±1% per cent of the asymptotic minimum drug level in the body expected one τ after a large (or infinite) number of maintenance doses has been administered. On the other hand, under conditions defined in Experiment 2 for the three subjects, the time needed to approach within ±1% of the asymptotic minimum drug level is 14 days. Thus, if it is therapeutically desirable to approach within ±1% of the asymptotic minimum drug level rapidly, the dosage regimen defined by the conditions of Experiment 1 would be preferred over that defined by the conditions of Experiment 2.

Sereni *et al.* (14) used the Krüger-Thiemer methods to study the pharmacokinetics of orally administered sulfamethoxypyrazine, using subjects of five different age groups. The subjects were newborns (2–3 days), infants (1–12 months), children (4–9 years), adults, and the aged (>70 years). The bottom half of Table II summarizes some of the data and other derived constants for the various age groups from the paper by Sereni *et al.* (14). It can be seen from Table II that from 8–12 days are needed to reach within ±1% of the asymptotic minimum drug level expected one τ after a large (or infinite) number of doses has been administered to these five different age groups.

In this example, the basic assumption is that the apparent biological half-life of the drug remains substantially the same after one or many doses has been administered. The data presented by Krüger-Thiemer *et al.* (12) seem to support this assumption for sulfamethoxypyrazine. However, with other drugs the apparent biological half-life may change, depending upon the number of maintenance doses administered. For example, when 500 mg. of tetracycline as the phosphate complex was administered orally to 10 test subjects every 12 hr., the apparent biological half-life increased from 6.3 hr. for the 1st day to 10.0 hr. for the 5th to 6th days (15). It is not yet clear why the apparent biological half-life of tetracycline as the phosphate complex administered orally to humans seems to change depending upon the number of doses administered. The equations derived in this paper cannot be used in cases where the apparent half-life of the drug in a given compartment varies during the dosing regimen.

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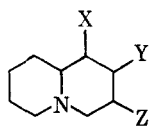
Esters of Bicyclic Aminoalcohols V: Duration of Corneal Anesthesia *versus* Enzymatic Hydrolytic Rate of Benzoates of 1-, 2-, and 3-Methyl-2-hydroxyquinolizidines

M. A. ZOGLIO*, T. O. SOINE†, and E. G. RIPPIE

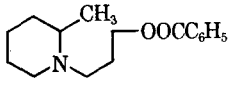
Abstract □ Benzoates of the 1-, 2-, and 3-methyl-2-hydroxyquinolizidines were prepared, and the relative *in vitro* rates of enzymatic hydrolysis of these esters together with quinolizidin-2-yl benzoate and piperocaine by pseudocholinesterase were determined. The duration of corneal anesthesia on rabbits was measured for these compounds. A comparison of the relative rate of enzymatic hydrolysis with duration of corneal anesthesia within the group of compounds and enzyme system studied was made and indicates no correlation between duration of corneal local anesthesia and rate of hydrolytic cleavage.

Keyphrases □ Aminoalcohol esters, bicyclic—synthesis □ Enzymatic hydrolysis—bicyclic aminoalcohol esters □ Corneal anesthesia—bicyclic aminoalcohol esters □ UV spectrophotometry—analysis, enzymatic hydrolysis □ IR spectrophotometry—identification □ TLC—separation □ NMR spectroscopy—structure □ GLC—identification

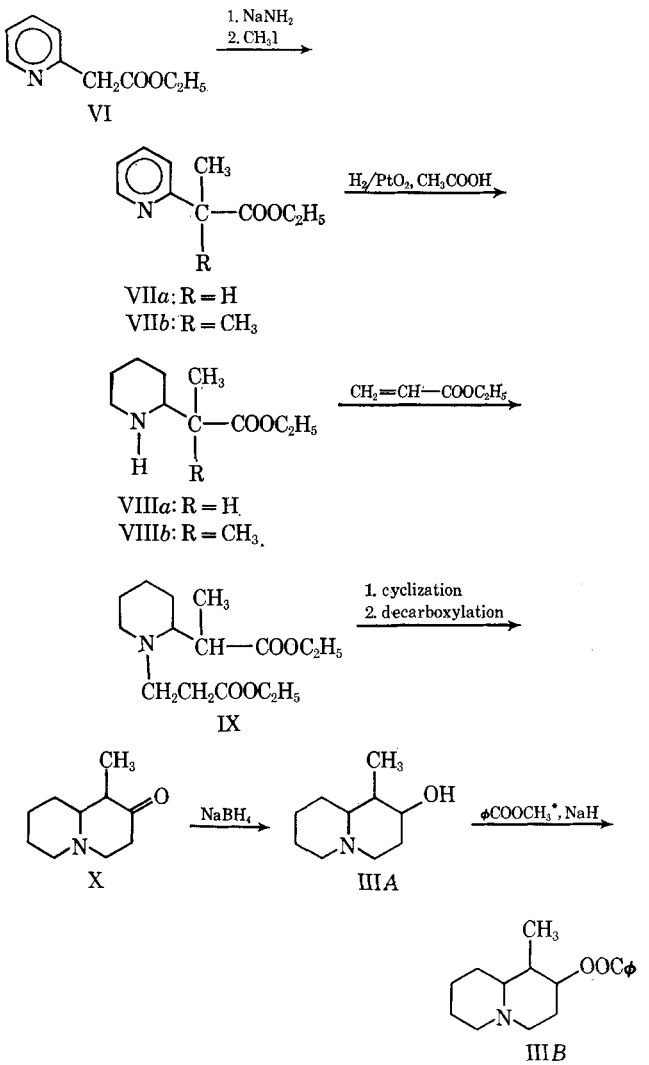
Metabolic inactivation of aminoester-type local anesthetics usually involves enzymatic cleavage at the ester linkage (1), presumably by nonspecific esterase activity. Prolongation of activity was achieved in a number of cases (2–4) by sterically inhibiting hydrolysis of the ester linkage. Kalow (5) correlated the duration of activity with the hydrolytic rate using human serum cholinesterase. In contrast to these observations, piperocaine hydrochloride was shown to be a longer acting local anesthetic than procaine (6), in spite of being hydrolyzed six times more rapidly by human serum cholinesterase (5). Most studies to date concern the effects of serum cholinesterases on local anesthetics; few have been conducted to determine whether duration of local anesthetic action in the cornea can be correlated with enzymatic hydrolytic rates. The literature is not very revealing as to the esterase activity of corneal tissue, but it can be presumed that any esterase activity would very likely be nonspecific. Thus, termination of corneal anesthesia could occur by enzymatic inactivation of an aminoester local anesthetic, by simple diffusion from the corneal tissue, or by protein binding, which then, in effect, becomes the rate-limiting factor in the diffusion process.



I*a* X = $OOCC_6H_5$, Y = Z = H
I*b* X = Z = H, Y = $OOCC_6H_5$
I*c* X = Y = H, Z = $OOCC_6H_5$

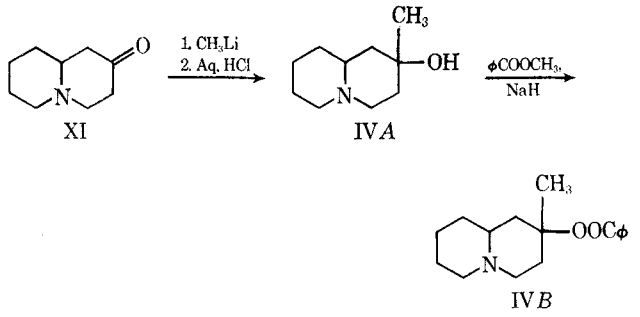


II



Scheme I—Synthesis of 1-Methyl-2-hydroxyquinolizidine Benzoate

The objective of the present work was to examine the relationship between *in vitro* enzymatic hydrolytic rate and corneal duration of action, using selected aminoesters with varying degrees of steric complexity around the hydrolytic site. The esters selected arose initially from a study (7) of the isomeric hydroxyquinolizidine benzoates (I*a*,*b*,*c*) in which the esters were compared with piperocaine (II), an open-chain analog. I*b* was found to be 1.72 times as active as II in the intradermal wheal test, whereas I*a* and I*c* were markedly less active. This suggested that I*b* and modifications of it might be useful for the indicated studies where the modification caused steric hindrance to es-

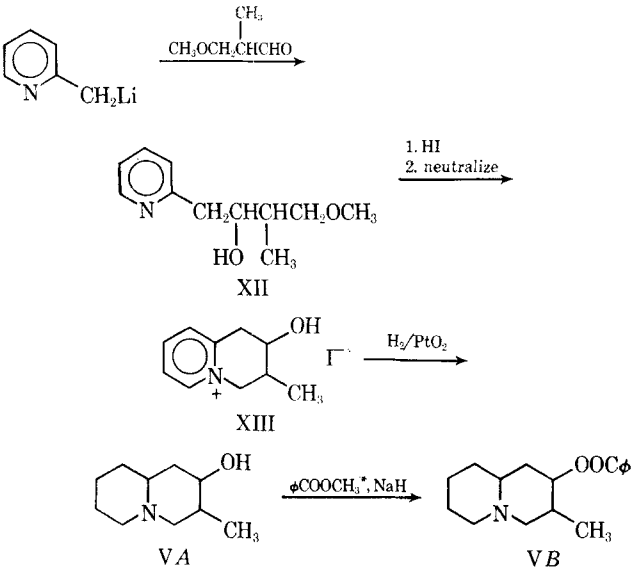


Scheme II—Synthesis of 2-Methyl-2-hydroxyquinolizidine Benzoate

terase hydrolysis through introduction of suitably placed methyl groups. Thus, the synthesis of the 1-, 2-, and 3-methyl-2-hydroxyquinolizidines (III*A*, IV*A*, V*A*) and the corresponding benzoates (III*B*, IV*B*, V*B*) was undertaken. Two racemates of IV*B* and V*B* were obtained, whereas III*B* provided only one. These racemates, together with I*b* and II, were the compounds used for the present study. No stereochemical designations were attempted, since it was obvious from the pharmacological results that stereochemistry was a minimal factor affecting activity and hydrolytic rate.

DISCUSSION

Synthesis—The synthesis of 1-methyl-2-hydroxyquinolizidine (III*A*) (Scheme I) was initiated by alkylating ethyl 2-(α-pyridyl) acetate (VI) to obtain ethyl 2-(α-pyridyl)-2-methylacetate (VII*a*) which, however, was contaminated with starting material and the corresponding dialkylated product [ethyl 2-(α-pyridyl)-2-dimethylacetate (VII*b*)] as determined by chromatographic procedures. Separation of the mixture by fractional distillation or chromatography could not be achieved. Therefore, the mixture was reduced catalytically to provide a mixture of the desired ethyl 2-(α-piperidyl)-2-methylacetate (VIII*a*) and the corresponding reduced forms of VI and the dialkylated contaminant (VIII*b*). A single product was not achieved, although fractional distillation provided a product without the reduced form of VI and with a much lower concentration of VIII*b*, as demonstrated by GLC. Further purification of VIII*a* could not be effected by the usual techniques and, therefore, it was subjected to Michael addition with ethyl acrylate in the impure form. Ethyl piperidyl-2α-propionate-1β-propionate (IX) was obtained in good yield, since VIII*b* apparently failed to react and was



Scheme III—Synthesis of 3-Methyl-2-hydroxyquinolizidine Benzoate

separated by fractional distillation. Dieckmann cyclization of IX followed by decarboxylation yielded the desired aminoketone (X), which was then reduced with sodium borohydride to yield III*A* after chromatographic purification. The NMR showed a doublet (J = 6 Hz.) in the C-methyl region and showed homogeneity under GLC and TLC examination.

The synthesis of 2-methyl-2-hydroxyquinolizidine (IV*A*) (Scheme II) was initiated by the preparation of 2-ketoquinolizidine (XI) by the method of Rhodes and Soine (8), which had been shown to be superior to that of Clemo *et al.* (9). Conversion to IV*A* was effected by the addition of methyl lithium according to McElvain and Berger (10) and Leonard *et al.* (11). The product had a broad melting point (60–74°) and was not separable into its isomeric forms by distillation or chromatographic procedures. However, when used as such for the preparation of the benzoate ester (IV*B*), it provided two isomeric racemic benzoates. Coincidentally, the melting points of the ester hydrochlorides were identical, but a mixed melting point showed marked depression; furthermore, the IR spectra differed significantly.

The synthesis of 3-methyl-2-hydroxyquinolizidine (V*A*) (Scheme III) could not be effected by the procedure used for III*A* and IV*A*. It was initiated by a modification of the Boekelheide and Gall (12) synthesis of 2-hydroxyquinolizidine in which β-methoxyisobutyraldehyde, prepared by the method of Elderfield *et al.* (13), was treated with α-picolyl lithium to yield 4-methoxy-1-(α-pyridyl)-3-methyl-2-butanol (XII). Hydriodic acid cleavage of XII yielded the cyclization product, 2-hydroxy-3-methyl-1,2-dihydro-3*H*,4*H*-quinolizinium iodide (XIII), which, upon catalytic reduction, yielded V*A* as a mixture of its isomers. Fractional distillation and chromatographic methods failed to provide a separation of the isomers. However, conversion to the benzoate form (V*B*) provided an easy separation of two isomeric racemic esters as the hydrochloride salts.

With respect to the conversion of the aminoalcohols to the benzoate esters, it was found, in keeping with the experiences of Feldkamp (14) and of Counsell and Soine (7), that the ester-interchange method was far superior to the usual Schotten–Baumann procedure.

Hydrolytic Rate Studies—An appropriate enzyme system was used in an attempt to approximate, by *in vitro* studies, the metabolic inactivation of a select group of topically active local anesthetics. Pseudocholinesterase was chosen for this study because of its possible role in synaptic transmission (15), its abundance in a variety of tissues in man and other animals (16), and its nonspecific nature. It is thought that the properties of this enzyme would allow a meaningful measure of the effects on enzymatic hydrolysis produced by the steric restrictions imposed when the alkyl ester chain of piperocaine (II) is fixed into the relatively rigid nucleus of quinolizidin-2-yl benzoate (I) and when additional restriction is produced by placing a methyl group in the 1-, 2-, and 3-positions of the quinolizidine nucleus. The type of kinetic data found in this study is predictable from classical theoretical considerations of enzyme catalysis (17) according to the Michaelis–Menten scheme. Both the Michaelis constant, K_m, and the maximum or zero-order rate were determined.

Although reaction velocities can be considered a function of K_m at low substrate concentrations, local anesthetics are injected in such high concentrations that the maximum or zero-order rate would seem to provide the best set of rates for comparison with duration of local anesthetic activity. The K_m values were determined as a measure of the effects produced by synthetic molecular manipulation on the ability of the enzyme and drug molecule to form a complex. K_m represents an upper limit to the value of the enzyme-substrate dissociation constant. The method used to measure hydrolysis was based on the UV absorption spectra of the local anesthetics and their hydrolytic products.

The results of the rate studies shown in Table I indicate that introduction of the quinolizidine nucleus reduces the rate of enzymatic hydrolysis considerably. A 300-fold reduction in rate is produced when the alkyl ester chain of piperocaine hydrochloride is fixed into the rigid nucleus of quinolizidin-2-yl benzoate hydrochloride. The Michaelis constants for the two compounds are of the same order of magnitude, suggesting that the reduction in rate is possibly due to a misalignment of the ester group in the complex with the enzymatic moieties functioning in hydrolysis rather than to a significantly reduced ability of the latter compound to form an enzyme–substrate complex.

Table I—Enzymatic Hydrolytic Data

Compound	Relative Hydrolytic Rates (Maximum or Zero-Order Velocity) with Piperocaine Arbitrarily Set at 1000.0	Michaelis Constants[a] (K_m) as Determined by Lineweaver–Burk Treatment of Data from Hydrolytic Curves
Piperocaine hydrochloride (II)	1000.0	5.0×10^{-6}
Quinolizidin-2-yl benzoate hydrochloride (I*b*)	2.83	7.6×10^{-6}
1-Methylquinolizidin-2-yl benzoate hydrochloride (III*B*)	0.56	9.5×10^{-5}
2-Methylquinolizidin-2-yl benzoate hydrochloride (IV*B*, isomer *a*)	0.11	—
2-Methylquinolizidin-2-yl benzoate hydrochloride (IV*B*, isomer *b*)	0.05	—
3-Methylquinolizidin-2-yl benzoate hydrochloride (V*B*, isomer *a*)	1.66	3.5×10^{-5}
3-Methylquinolizidin-2-yl benzoate hydrochloride (V*B*, isomer *b*)	1.54	3.8×10^{-5}

[a] Molar concentration units.

Introducing a methyl group into the 1- and 3-positions of the quinolizidine nucleus does, however, affect the attraction between enzyme and substrate, as is suggested by the higher K_m values for these compounds. The reduction in rate for these compounds as compared with piperocaine hydrochloride is most likely due to a combination of reduced complex formation and relative inaccessibility of the catalytic groups of the enzyme to the ester portion of the drug molecule.

The approximately 10,000-fold decrease in rate noted when comparing IV*B* (isomers *a* and *b*) with II is probably due to both of the previously mentioned factors. The extent of this produced by reduced attraction of the molecule to the enzyme surface cannot be estimated, since the K_m values for these compounds were not determined.

Differences in stereochemistry about the ester grouping (IV*B* isomers and V*B* isomers) did not affect the hydrolytic rate to a significant extent. The very slow reactions caused some difficulty in that the enzyme–substrate solutions became turbid before completion of the reaction. The rate data from the last portion of the reaction curves, which are important in K_m calculations, were erratic so that only the zero-order rates could be calculated for the IV*B* isomers in Table I.

Pharmacological Testing—The testing procedure employed parallels that of Patel and Soine (18) in most respects, with the only probable exception occurring in interpretation of the end-point of anesthesia. This may account for the fact that the duration ratio, as found for I*b* in this study, is approximately 20% below that found by the previous authors. It is assumed, however, that interpretation of the end-point in this study was consistent and that relative values for duration of activity are as accurate as this type of testing will permit. The testing results are tabulated in Table II.

The first and most striking observation is that, if the rates found using pseudocholinesterase are taken as representative of nonspecific esterase activity, no differences in duration of activity can be attributed to differences in rates of hydrolytic cleavage of the ester grouping.

The possibility remains, however, that an enzyme or group of enzymes with properties distinct from pseudocholinesterase, capable of cleaving all of the esters at nearly the same rate, could be present in the rabbit cornea and surrounding tissues. The literature contains little information pertaining to the isolation of esterases from this area.

At any rate, it would seem that the steric restrictions imposed in the quinolizidinyl esters of Table II should cause an enzyme some difficulty if proximity of one or more side chains of the enzyme to the carboxy portion of the ester is necessary for esterase activity. If metabolic inactivation of this type is not a dominant factor in termination of local anesthesia, other factors remain to be considered.

Table II—Local Anesthetic Activity

Compounds	Duration of Local Anesthesia, min.[a]	Duration Ratio[b]
Piperocaine hydrochloride (II)	15	—
Quinolizidin-2-yl benzoate hydrochloride (I*b*)	19	1.22 (±0.18)[c]
1-Methylquinolizidin-2-yl benzoate hydrochloride (III*B*)	21	1.38 (±0.27)
2-Methylquinolizidin-2-yl benzoate hydrochloride (IV*B*, isomer *a*)	24	1.60 (±0.43)
2-Methylquinolizidin-2-yl benzoate hydrochloride (IV*B*, isomer *b*)	19	1.24 (±0.43)
3-Methylquinolizidin-2-yl benzoate hydrochloride (V*B*, isomer *a*)	17	1.12 (±0.14)
3-Methylquinolizidin-2-yl benzoate hydrochloride (V*B*, isomer *b*)	25	1.64 (±0.51)

[a] Expressed as the mean duration of activity. [b] This ratio represents the mean ratio of activity. Each ratio used in calculating the mean is equal to the duration of activity of the compound divided by the duration of activity of piperocaine hydrochloride on the same rabbit. [c] 95% confidence limits.

Of these, the physicochemical factors involved in the removal or diffusion of drug from its site of action would appear to be potentially most influential. These factors, however, were not investigated in the current study.

EXPERIMENTAL

UV spectra were determined on a Bausch and Lomb 505 recording spectrophotometer, IR spectra on a Perkin-Elmer 237B grating IR spectrophotometer, and NMR spectra on a Varian Associates A-60 instrument in CCl_4 or $CDCl_3$ using tetramethylsilane as the internal standard. Melting points were determined in capillary tubes in a Thomas-Hoover melting-point apparatus and are uncorrected[1]. Fractional distillation was carried out on a Nester-Faust semimicro spinning band unit rated at 23 theoretical plates. GLC studies were done on a F&M model 500 instrument, equipped with a flame-ionization detector, using 0.63 × 60.9-cm. (0.25 × 24-in.) copper tubes. Preparatory scale gas chromatography (PGC) was attempted with 1.91 × 243.8-cm. (0.75 × 96-in.) copper columns using a thermal conductivity detector. Unless otherwise specified, columns were packed with 10% Carbowax on Chromosorb W. Silica gel for column chromatography (CC) refers to Baker Analyst 3405 activated at 120°, and silica gel for TLC refers to silica gel G[2] activated at the same temperature. The neutral form of alumina[2] for columns was used in all cases.

Ethyl 2-(α-Pyridyl)-2-methylacetate (VII*a*)—Sodium amide (14.4 g., 0.37 mole) in ether (200 ml.) was stirred and treated dropwise with ethyl 2-(α-pyridyl)acetate (VI) (60 g., 0.36 mole) without heating over a 1-hr. period. The mixture was then heated over a steam bath for 15 hr. followed by portionwise addition of methyl iodide (52.5 g., 0.37 mole) and an additional 5-hr. heating period. On cooling, the mixture was filtered, stripped of solvent, and fractionally distilled to yield 45 g. of a yellow liquid, b.p. 87° (1.25 mm.). GLC examination of the liquid indicated that it was a three-component mixture in the ratio of 10:4:1, with the lowest concentration being unreacted VI as shown by potentiation of its peak when added to the liquid. The peak of the intermediate concentration was shown to be ethyl 2-(α-pyridyl)-2,2-dimethylacetate (VII*b*) by again subjecting a small portion of the liquid product to the original alkylating conditions, since this peak in the GLC of the treated mixture was augmented. The peak in greatest abundance was, therefore, assumed to be VII*a* and this was confirmed by the NMR spectrum of the liquid which clearly showed the expected doublet for the methyl group. Further fractional distillation and attempts at PGC failed, although TLC confirmed the GLC results by showing three spots. These spots, developed with petroleum ether (30–60°)–acetone–ether (8:2:1), showed a similar pattern of behavior, although

[1] Microanalyses were determined by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis, Minn., or by the Schwartzkopf Microanalytical Laboratories, Woodside, N. Y.
[2] Supplied by Brinkmann Instruments, Inc., Great Neck, N. Y.

Table III—Analytical Data

Compound	Molecular Formula[a]	M.p. or B.p. (mm. Hg)	Analysis, % Calcd.	Found
III*A*	$C_{10}H_{19}NO \cdot HCl$	259.5–260°	C, 58.38	57.98
			H, 9.80	9.69
III*B*	$C_{17}H_{23}NO_2 \cdot HCl$	254–254.5°	C, 65.90	66.05
			H, 7.90	7.86
IV*A*	$C_{10}H_{19}NO \cdot 1/2H_2O$	74–79°	C, 67.41	67.79
			H, 11.23	11.23
IV*B* (isomer *a*)	$C_{17}H_{23}NO_2 \cdot HCl \cdot 1/2H_2O$	190–191°	C, 64.04	64.31
			H, 7.90	7.89
IV*B* (isomer *b*)	$C_{17}H_{23}NO_2 \cdot HCl \cdot 1/2H_2O$	190–191.5°	C, 64.04	64.48
			H, 7.90	7.86
V*A*	$C_{10}H_{19}NO \cdot HCl \cdot$	190–215°	C, 58.38	58.26
			H, 9.80	9.98
V*B* (isomer *a*)	$C_{17}H_{23}NO_2 \cdot HCl \cdot 1/4H_2O$	282–282.5°	C, 64.97	64.70
			H, 7.72	7.95
V*B* (isomer *b*)	$C_{17}H_{23}NO_2 \cdot HCl \cdot 1/4H_2O$	262–262.5°	C, 64.97	64.67
			H, 7.72	8.18
IX Picrate	$C_{11}H_{21}NO_2 \cdot C_6H_3N_3O_7$	129°	C, 47.65	47.55
			H, 5.65	5.66
XII	$C_{11}H_{17}NO_2$	96° (0.25)	C, 67.66	67.41
			H, 8.78	9.00
XIII	$C_{10}H_{14}NO \cdot I$	124–128°	C, 41.25	41.19
			H, 4.84	4.95

[a] Samples containing water of crystallization were dried in a desiccator following crystallization without the application of heat and then analyzed. Heating the samples in an abderhalden pistol at 110° resulted in loss of weight but, on exposure to the atmosphere, the samples rapidly regained the weight lost.

this system applied to a silica gel column failed to achieve separation. Since attempts at separation proved ineffective, the impure product was used as such for the following reaction.

Ethyl 2-(α-Piperidyl)-2-methylacetate (VIII*a*)—The above product (41 g.), dissolved in glacial acetic acid (100 ml.), was reduced with platinum oxide (1 g.) and hydrogen at 60 p.s.i. in a conventional Parr hydrogenation apparatus for 3 hr. The catalyst was removed by filtration, an additional 0.25 g. of platinum oxide was added, and the hydrogenation was resumed until hydrogen uptake ceased. Following removal of the catalyst by filtration and distillation of most of the acetic acid under reduced pressure, the residue was made alkaline with 15% (w/v) potassium hydroxide solution and extracted with ether (3 × 50 ml.). The dried (anhydrous Na_2SO_4) ethereal extracts were stripped of solvent and fractionally distilled to yield 32 g. of a colorless liquid, b.p. 74° (0.8 mm.). GLC examination of this liquid on a column containing 10% zinc stearate on Chromosorb W showed two peaks of differing magnitude (5:1), whose retention times did not correspond to that of ethyl 2-(α-piperidyl) acetate[3] since the latter showed a third peak when mixed with the product. Further fractional distillation and CC failed to show substantial separation; therefore, this product was used as such in the next step.

Ethyl Piperidyl-2α-propionate-1β-propionate (IX)—The above product (25 g.) was dissolved in absolute ethanol (200 ml.) to which was added freshly distilled ethyl acrylate (15 g., 0.15 mole), and the mixture was allowed to stand for 20 days at room temperature. Stripping off of the solvent and fractional distillation yielded two fractions, the first weighing 4 g., b.p. 75° (0.8 mm.), and the second weighing 33 g., b.p. 130° (0.8 mm.). The lower boiling fraction showed N—H stretching in the IR and corresponded in GLC retention time to the material in lowest concentration in the impure VIII*a* from the previous step. The higher boiling fraction showed no N—H stretching in the IR and was assumed to be the addition product (IX). Its homogeneity was demonstrated by a single GLC peak when a 10% silicone gum rubber on Chromosorb W packing was used. A picrate, m.p. 129°, was prepared in the usual manner.

1-Methyl-2-ketoquinolizidine (X)—Sodium hydride (6 g., 0.25 mole) in a 50.2% mineral oil dispersion was added to dry xylene (120 ml.) under a nitrogen atmosphere followed by 1 ml. of absolute ethanol, and the whole was raised to reflux temperature. To this, IX (27.1 g.) was slowly added over a 1-hr. period followed by 3 hr. of reflux. The reaction mixture was cooled in ice and treated with glacial acetic acid (16 g., 0.27 mole) followed by 50 ml. of water to dissolve the formed sodium acetate. The xylene layer was separated from the aqueous phase, and the latter was extracted with xylene (2 × 25 ml.), the extractions being added to the original xylene phase. The combined xylene extracts were then extracted with 6 *N* hydrochloric acid (3 × 80 ml.), and the combined acid extracts were heated under reflux for 8 hr. On cooling, the acid solution was neutralized with 20% (w/v) potassium hydroxide solution, saturated with potassium carbonate, and extracted with ether (4 × 25 ml.). The dried (anhydrous Na_2SO_4) ether extracts were stripped of solvent and fractionally distilled to yield 10.5 g. of product, b.p. 68.5° (0.6 mm.).

1-Methyl-2-hydroxyquinolizidine (III*A*)—Compound X (10 g.) was dissolved in water (25 ml.) and added to a cool solution of sodium borohydride (0.6 g.) in water (30 ml.); the whole was allowed to stand at room temperature for 5 hr. Ammonium hydroxide solution (10 ml.) was added, and the solution was extracted with hot benzene (4 × 25 ml.). The dried (anhydrous Na_2SO_4) benzene solution was stripped of solvent and distilled to yield 8.5 g. of product, b.p. 94° (0.6 mm.), which was free of C=O absorption in the IR. GLC and TLC (on alumina) examination showed a major component in approximately 85% concentration and two minor components. The solvent system for TLC was petroleum ether (30–60°)–acetone–ether (5:4:1) and was also the solvent system used to effect separation of 500 mg. of the product on an alumina column [100 g. alumina in a 1.91 × 45.7-cm. (0.75 × 18-in.) column]. GLC showed the column-chromatographed material to be devoid of the minor peaks, and its NMR clearly showed a doublet (J = 6 Hz.) in the *C*-methyl region (1.1 p.p.m.) which integrated for three protons. The hydrochloride salt, m.p. 259.5–260°, was prepared by adding an ethereal solution of HCl to a solution of the compound in ether and recrystallization from ethanol–ethyl acetate.

2-Methyl-2-hydroxyquinolizidine (IV*A*)—2-Ketoquinolizidine (9.4 g., 0.06 mole) was dissolved in dry ether (150 ml.) and treated by dropwise addition of methyl lithium (2.7 g., 0.12 mole) in a nitrogen atmosphere accompanied by stirring and reflux. Following the addition, the lithium complex was decomposed with a mixture of hydrochloric acid and water (11.5 ml. : 7 ml.), after which the ether layer was washed successively with water (10 ml.) and diluted hydrochloric acid (5 ml. acid:10 ml. water). The aqueous extracts were alkalinized with potassium carbonate and extracted with ether to obtain an ethereal solution which was dried (anhydrous Na_2SO_4) and stripped of solvent. Fractionation of the residue yielded a fraction, b.p. 123° (7 mm.), with a C=O free spectrum which solidified on standing and, following recrystallization from isopropyl ether and sublimation, gave 1.74 g. (18.5% theory) of IV*A*, m.p. 74–79°. Further attempts at fractionation and crystallization did not improve the product.

4-Methoxy-1-(α-pyridyl)-3-methyl-2-butanol (XII)—This compound was prepared smoothly according to the general method of

[3] Obtained from previous studies in these laboratories.

Walter (19), employing β-methoxyisobutyraldehyde prepared by the Elderfield *et al.* (13) method. The yield was 36.5% of theory, b.p. 96° (0.25 mm.).

2-Hydroxy-2-methyl-1,2-dihydro-3*H*,4*H*-quinolizinium Iodide (XIII)—Compound XII (10 g., 0.05 mole) was refluxed for 15 hr. with concentrated hydriodic acid (225 ml.) and then concentrated to 25 ml. under vacuum. The residue was placed in a separator with 25 ml. of chloroform; to this was added dropwise a saturated solution of potassium carbonate. The liberated oil dropped into the chloroform layer, and this layer was then withdrawn, cooled in a refrigerator overnight, and filtered. The yellow granular material (6 g., 42% of theory) was recrystallized twice from ethanol–ether, m.p. 124–128°.

3-Methyl-2-hydroxyquinolizidine Hydrochloride (V*A*)—Compound XIII (2 g., 0.007 mole), dissolved in ethanol (25 ml.) and combined with platinum oxide (0.15 g.), was subjected to atmospheric pressure hydrogenation until 3 equivalents of hydrogen was absorbed (3 hr.). The catalyst was removed by filtration, the residue was dissolved in water, and the solution was saturated with potassium carbonate. The alkaline solution was extracted thoroughly with ether; the ether extract was dried (CaH_2), filtered, and treated with ethereal hydrogen chloride. Recrystallization of the precipitated salt from ethyl acetate–ethanol yielded 0.6 g. (42% of theory) of white crystals, m.p. 190–215°.

1-Methylquinolizidin-2-yl Benzoate Hydrochloride (III*B*)—This ester was prepared from III*A* by the ester-interchange method of Counsell and Soine (7) and recrystallized from ethyl acetate–petroleum ether (30–60°) to give an 81% yield of white crystals, m.p. 254–254.5°. TLC examination of the ester prior to hydrochloride formation was carried out on alumina using petroleum ether (30–60°)–acetone–ether (5:4:1) and acetone–petroleum ether (30–60°) (1:9). No evidence for a second isomer was noted.

2-Methylquinolizidin-2-yl Benzoate Hydrochloride (IV*B*)—This ester was prepared from IV*A* by the ester-interchange method of Counsell and Soine (7). However, instead of converting the ester directly to the hydrochloride form, it was examined by TLC on alumina using the solvent systems employed for examination of III*B*. In the case of IV*B*, the TLC procedure indicated a mixture of two closely related materials. The acetone–petroleum ether (30–60°) (1:9) system seemed to be the most suitable and was applied to a 100-g. alumina column, 1.91 × 45.7-cm. (0.75 × 18-in.), for separation of the isomeric forms. The fastest moving material (isomer *a*), monitored by the use of Mayer's reagent, was collected, stripped of solvent, dissolved in ether, and treated with ethereal hydrogen chloride. The white crystals so obtained (10% of theory) were recrystallized from ethyl acetate–petroleum ether (65–70°), m.p. 190–191°. The slower moving fraction (isomer *b*), monitored in the same way and similarly converted to the hydrochloride form, was recrystallized from ethanol–ethyl acetate–petroleum ether (65–70°) and yielded 30% of theory of white crystals, m.p. 190–191.5°. A mixed melting point with the hydrochloride of the first fraction showed a 30° depression, and IR spectra of the two hydrochlorides showed significant differences.

3-Methylquinolizidin-2-yl Benzoate Hydrochloride (V*B*)—This ester was prepared from V*A* by the ester-interchange method of Counsell and Soine (7). Instead of converting the free base to the hydrochloride form directly, it was examined using a TLC system of 5% acetone in petroleum ether (30–60°) on alumina. This system was useful for examining both the aminoalcohol (V*A*) and its benzoate (V*B*). Separation of the isomeric esters was achieved on an alumina column as described for 2-methylquinolizidin-2-yl benzoate, and conversion to the hydrochloride form was similarly effected. The faster moving benzoate (isomer *a*), after conversion to the hydrochloride, was obtained in 14% yield, m.p. 282–282.5°, after recrystallization from isopropanol–isopropyl ether. The slower moving benzoate (isomer *b*) was obtained in 10% yield as the hydrochloride and, after recrystallization from isopropanol–petroleum ether (65–70°), gave m.p. 262–262.5°.

Kinetic Studies—One milliliter of a solution (at 37°) of pseudocholinesterase[4] in 0.05 *M* phosphate buffer (pH = 7.23 ± 0.02) was pipeted into a cell (1-cm. pathlength) containing 2 ml. of a solution (at 37°) of the ester to be studied. The cell was then stoppered, shaken to mix the solutions thoroughly, and placed into a jacketed cell holder (at 37 ± 0.02°) contained in a Bausch & Lomb model 505 recording spectrophotometer. The enzyme solution, ester solution, and pipets used to withdraw samples were kept in a constant-temperature bath at 37° before each run. The ester solution was pipeted into a cell, stoppered, and placed in the cell holder 10 min. before mixing. A blank was prepared in the same manner, substituting 2 ml. of the buffer solution for the ester solution. The ester concentrations were calculated to give an absorbance of about 0.9 when mixed with the enzyme solution.

With the instrument set at 240 nm., the decrease in absorption was followed as a function of time. The hydrolysis of II was rapid enough to allow a continuous recording to be made on the Bausch & Lomb 505. The hydrolysis of I*b* was followed on an external Honeywell recorder with a chart drive of 2.54 cm. (1 in.) per minute. The remaining esters were studied by taking single absorbance measurements at definite intervals of time. Each compound was studied at a minimum of two enzyme concentrations, with a minimum of two runs per specific enzyme concentration. All data from these runs were used in preparing absorbance *versus* time plots. The curves obtained were then analyzed to give the relative rates and Michaelis constants listed in Table I.

The observed zero-order rates are directly proportional to the enzyme concentration. To obtain a set of rates that would provide comparison on an equal basis, all rates were corrected to the enzyme concentration used in a standard run.

The standard run employed a 7.925 mg. % enzyme solution which hydrolyzed piperocaine hydrochloride at the rate of 4.80×10^{-6} moles/min. Besides providing a basis for comparison in this study, the standardization, which represents an average of three very close runs, allows other investigators to compare the potency of the particular lot of crystalline pseudocholinesterase they are using to that used in this study. This certainly would be essential if any comparison of rates to those shown in Table I were to be made. Hydrolysis of all the esters by the buffer solution alone at 37° was checked and found negligible for a period of 96 hr.

Pharmacological Testing—One-half milliliter of a 1% solution of the ester salt was introduced into the conjunctival sac from a blunt-tipped syringe and allowed to act upon the cornea for 1 min. before the measurements of duration of anesthesia were started. The compounds tested all induced anesthesia within the 1-min. period, as evidenced by the absence of the wink reflex. This was determined by attempting stimulation of the cornea with a blunt glass rod. The length of time required for the animal to regain the wink reflex was recorded. Six rabbits were used, as a minimum, to determine the duration of activity for each compound. To arrive at a duration ratio, each measurement was divided by the duration of anesthesia for piperocaine hydrochloride on the same rabbit. The mean of these ratios was determined, and the 95% confidence limits of the mean were calculated assuming a normal distribution for the mean and a χ-square distribution for the squares of the deviations (20). During the testing, no corneal irritation was observed and at no time were both eyes subjected to anesthesia simultaneously. One-percent solutions of the ester salts tested were prepared in distilled water immediately prior to use. Young adult white rabbits, male and female, were used for testing. The eyelids were carefully trimmed to remove the eyelashes. Each animal was allowed a 24-hr. rest period between tests.

[4] Carefully weighed crystalline human pseudocholinesterase from Cohn fraction IV-458 (Cutter Laboratories, Berkeley, Calif.) was dissolved in the buffer to yield solutions ranging in concentration from 7.925 mg. % for the hydrolysis of piperocaine hydrochloride to 80.0 mg. % for hydrolysis of the stereoisomers of 2-methylquinolizidin-2-yl benzoate hydrochloride. One milligram of this preparation contains the pseudocholinesterase activity of 5 ml. of normal human plasma.

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Anti-Inflammatory and Liver Sulfhydryl Content-Altering Effects of Certain Nonsteroids in the Rat

YI-CHI CHANG* and MARVIN H. MALONE†

Abstract □ Selected agents were evaluated orally in the male rat for their capacity to prevent carrageenin-induced pedal edema and to alter liver sulfhydryl levels. Indomethacin was the most potent anti-inflammatory agent, followed by tetrabenazine and chlorpromazine. Cryogenine and phenylbutazone were equieffective, with cyproheptadine, aspirin, and sparteine being least potent. Carrageenin-induced pedal edema alone did not lower liver sulfhydryl content. Aspirin elevated the sulfhydryl concentration both in the presence and absence of carrageenin-induced edema, while chlorpromazine, indomethacin, phenylbutazone, cryogenine, tetrabenazine, and sparteine were without significant effect at anti-inflammatory dosages. Cyproheptadine lowered liver sulfhydryl levels, but this was considered to be a nonspecific or toxic effect resulting from the high dosages employed.

Keyphrases □ Anti-inflammatory action—nonsteroids □ Sulfhydryl content alteration, liver—nonsteroids □ Carrageenin-induced pedal edema, rats—anti-inflammatory testing

The work of Marozzi and Malone (1) indicated that a correlation might exist between a compound's ability to protect against stress-induced hepatic sulfhydryl depletion and its potential in inhibiting carrageenin-induced acute pedal edema in the rat. The present study is concerned with a comparison of such activities, using both clinically useful antirheumatic agents (aspirin, phenylbutazone, and indomethacin) and compounds that are potent inhibitors of several models of inflammation in animals but which are not used clinically as anti-inflammatory agents (chlorpromazine and cyproheptadine). Cryogenine (vertine), an alkaloid shown to be equipotent to phenylbutazone in both carrageenin-induced acute inflammation and in adjuvant-induced (*Mycobacterium butyricum*) chronic inflammation (2), as well as sparteine and tetrabenazine, was also investigated. The latter two agents bear some structural resemblances to possible degradation products of cryogenine.

EXPERIMENTAL

Carrageenin-Induced Pedal Edema—Adult male rats of the Wistar strain[1] were allowed to equilibrate in air-conditioned quarters for at least 1 week after receipt of shipment. Animals were allowed free access to Purina laboratory chow and tap water at all times. Experimental procedures used were similar to those described by Van Arman *et al.* (3). On the day of the experiment, rats were removed from food at −1 hr. and dosed orally to avoid the parenteral "counterirritant" effect. The test drugs were administered either dissolved or suspended in 0.25% agar solution at a constant dosage volume of 10 ml./kg. Control animals received the agar vehicle alone. Plantar injections of 0.1 ml. of aged, 1% carrageenin were made into the rat hind paw at 0 hr. The paw volume of all animals was recorded plethysmographically immediately afterward and again at +3 hr. (peak edematous response) just prior to sacrifice and removal of liver samples. On the basis of preliminary testing, a predictable high and a predictable low effective oral anti-inflammatory dose were selected for each experimental compound[2].

Each experimental run consisted of 60 animals divided into six interrelated groups: Group I, agar control (10 ml./kg. of 0.25% aqueous agar orally at −1 hr.); Group II, carrageenin control (agar solution at −1 hr. followed by plantar injection of carrageenin at 0 hr.); Group III, low drug control (at −1 hr. the lower dosage of the test drug shown by preliminary experimentation to be effective orally in reducing carrageenin-induced acute inflammation); Group IV, high drug control (the higher dosage of the test drug); Group V, low drug experimental (the lower oral dose of the drug being evaluated at −1 hr., followed by plantar injection of carrageenin at 0 hr.); and Group VI, high drug experimental (the higher dosage of the test drug followed by carrageenin injection).

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[2] Sources of the test agents were: aspirin, Merck and Co., Inc., Rahway, N. J.; indomethacin and cyproheptadine HCl, Merck Sharp & Dohme Research Institute, West Point, Pa.; phenylbutazone, Geigy Pharmaceuticals, Ardsley, N. Y.; chlorpromazine HCl, Smith Kline & French Labs., Philadelphia, Pa.; tetrabenazine, Hoffmann–La Roche, Inc., Rahway, N. J.; sparteine SO_4, K and K Labs., Inc., Plainview, N. Y.; and cryogenine, Dr. A. E. Schwarting, Division of Pharmacognosy, University of Connecticut, Storrs, Conn. The cryogenine (mol. wt. = 435.53) used in this study is an alkaloid isolated from *Heimia salicifolia* Link and Otto, and not the trade name product Cryogénine (phenylsemicarbazide, mol. wt. = 151.2), a specialty of Lumière of Lyons, France, and distributed by Laboratoires Sarbach of Châtillon, France.

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Table I—Relative Anti-Inflammatory Potency against Carrageenin-Induced Acute Pedal Edema in the Rat

	Oral Dosage, mg./kg.	Mean Increase in Paw Volume, ml.	Inhibition, %	Relative Potency	Molar Potency
Control	—	1.32(0.95–1.63)[a]	—		
Aspirin	160.0	1.11(0.86–1.30)	16.0	1.0	1.0
Aspirin	400.0	0.83(0.49–1.26)	37.2		
Control	—	1.31(1.23–1.42)	—		
Cyproheptadine HCl	125.0	1.01(0.87–1.24)	23.2	1.33(0.71–2.51)[b]	2.41(1.28–4.53)[b]
Cyproheptadine HCl	312.5	0.88(0.54–1.19)	32.7		
Control	—	1.27(1.06–1.48)	—		
Indomethacin	5.0	1.00(0.62–1.18)	21.0	34.43(19.26–61.53)	67.95(38.02–121.45)
Indomethacin	12.5	0.83(0.67–0.99)	34.6		
Control	—	1.29(1.15–1.49)	—		
Phenylbutazone	50.0	1.14(0.91–1.33)	11.6	1.62(0.79–3.28)	2.76(1.36–5.59)
Phenylbutazone	125.0	1.02(0.83–1.20)	21.6		
Control	—	1.10(0.97–1.41)	—		
Chlorpromazine HCl	40.0	0.74(0.52–0.99)	32.7	7.90(3.35–18.64)	15.74(6.67–37.12)
Chlorpromazine HCl	100.0	0.53(0.10–0.92)	51.8		
Control	—	1.28(0.77–1.61)	—		
Cryogenine	100.0	1.04(0.73–1.35)	18.8	1.59(0.99–2.56)	3.93(2.44–6.34)
Cryogenine	250.0	0.86(0.61–1.40)	32.9		
Control	—	1.13(0.75–1.43)	—		
Tetrabenazine	15.0	0.87(0.73–1.11)	23.0	8.94(5.72–14.05)	17.64(11.24–27.71)
Tetrabenazine	37.5	0.72(0.53–0.81)	36.3		
Control	—	1.22(1.01–1.37)	—		
Sparteine SO_4	80.0	1.13(0.48–1.47)	7.4	0.81(0.45–1.45)	1.49(0.83–2.67)
Sparteine SO_4	200.0	0.99(0.92–1.75)	18.9		

[a] Figures in parentheses in this column represent the observed range of experimental values. [b] Figures in parentheses represent the 95% confidence limits of the potency.

Liver Sulfhydryl Determinations—The amperometric titration of hepatic sulfhydryl groups (expressed as milligrams percent glutathione), requiring a platinum rotating electrode in neutral buffered media, followed essentially the method of Benesch and Benesch (4). All rats were sacrificed by craniovertebral dislocation immediately after the +3-hr. paw volume measurements. Sacrifice was timed to occur between 1800 and 1900 hr. to avoid diurnal variation, unless otherwise specified. Portions (about 3 g.) of the central lobe of the liver were rapidly removed and frozen with a dry ice–acetone mixture in marked, capped, individual vials. All metal surfaces contacting the tissue were silicone coated. The livers were kept frozen with dry ice until the time for glutathione extraction, at which time 9.2 ml. of a 2.5% solution of sulfosalicylic acid was added to each gram (wet weight) of liver tissue and the combination was homogenized with a motor-driven glass homogenizer in an ice jacket. The homogenate was then centrifuged for 1.5 min. at maximum speed in a Safeguard centrifuge[3], and the supernatant was filtered through hardened filter paper (Whatman No. 50). Filtrates were collected in individual tubes, corked, and maintained in a frozen state until time of amperometric titration. This was done in a 150-ml. beaker containing the following: 4 ml. of 1.0 *M* tris-(hydroxymethyl)aminomethane (Fisher certified primary standard), 3.4 ml. of 1.0 *M* HNO_3, 0.3 ml. of 1.0 *M* KCl, 1.0 ml. of 9×10^{-4} *M* calcium disodium versenate (Riker), 1.0 ml. of liver filtrate, and water to make a total volume of 30 ml. (pH = 7.4).

All solutions concerned with the titration procedure were made with deionized, distilled water. Because of the instability of glutathione in alkaline solutions, this neutral, aqueous, and buffered solution was preferable to the alcoholic, ammoniacal system commonly used (5). The titrant (0.002 *M* $AgNO_3$) was made fresh daily from a 0.1 *M* stock solution. Titrant was delivered by means of a 2.0-ml. micrometer syringe (0.002 ml./division)[4]. The end-point was determined graphically by plotting microammeter deflections *versus* the volume of $AgNO_3$ added. The point of intersection of the two resulting straight lines was considered the end-point of the titration. The accuracy of the titration system was checked periodically throughout the day when known amounts of standard glutathione were recovered from identical aliquots of liver filtrates. The average recovery of standard glutathione over the range of 0.005–0.3 mg. was 98%. All data were analyzed according to the analysis of variance methods of Bliss (6).

[3] Clay-Adams, Inc.
[4] Roger Gilmont Instruments, Inc.

RESULTS AND DISCUSSION

That stress is a real phenomenon is accepted generally; however, the study of stress is difficult. The assessment of stress, aside from those noxious stimuli that cause actual cellular damage, often involves a certain degree of subjective and emotional interpretation which becomes increasingly complex and important as one ascends the evolutionary ladder. Within a species, the ability to withstand stress, like pain, varies. The ability of a noxious stimulus to act as a stressor also depends on both the intensity and duration of such a stimulus. Moreover, Hudak and Buckley (7) demonstrated a sex variation in that stress-induced hypertension in rats peaks after 22 weeks of exposure in the male and 28 weeks in the female. Usually, the more general the effect of a stressor on the body, the more efficient it is in eliciting the stress response.

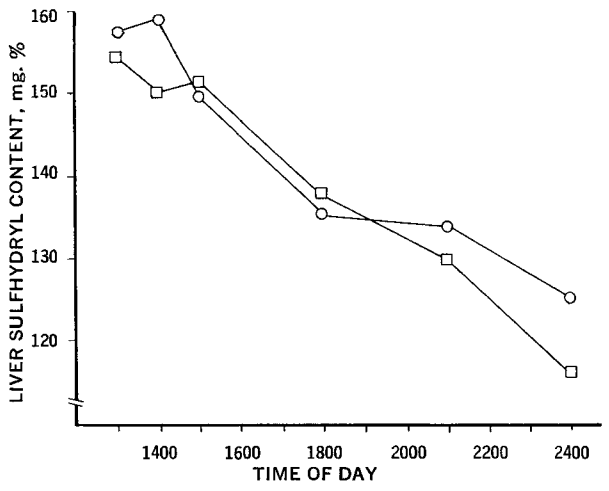

Figure 1—*Variation with time of male rat hepatic sulfhydryl concentration (calculated as glutathione) in the presence and absence of carrageenin-induced hind paw edema. Key:* O, *control; and* □, *carrageenin-treated. At each time period, levels in carrageenin-treated rats were not significantly different* ($p > 0.05$) *from the controls (7–10 animals/group). Corresponding levels at 1800 hr. and following were significantly lower* ($p < 0.05$) *than at 1300 hr. Pooled* SE *for controls = 6.3; pooled* SE *for carrageenin-treated = 6.4.*

Table II—Relative Drug Effects on Rat Liver Sulfhydryl Content at 1800 Hours

	Oral Dosage, mg./kg.	Mean Liver Glutathione[a] — No Carrageenin: mg. % (SE)	p	With Carrageenin: mg. % (SE)	p	Significance, p
Control	—	127.2(5.0)	—	125.9(4.6)	—	>0.20
Aspirin	160.0	144.7(4.7)	<0.01	137.1(5.6)	<0.01	>0.20
Aspirin	400.0	146.3(10.9)	<0.01	150.6(4.4)	<0.01	>0.20
Control	—	133.9(5.8)		127.8(2.6)	—	>0.20
Cyproheptadine HCl	125.0	109.4(4.3)	<0.01	126.6(4.2)	>0.20	<0.10
Cyproheptadine HCl	312.5	104.5(4.0)	<0.01	115.5(4.0)	<0.05	>0.05
Control	—	133.5(5.1)	—	141.0(4.6)	—	>0.20
Indomethacin	5.0	138.3(6.6)	>0.20	147.0(6.1)	>0.20	>0.10
Indomethacin	12.5	140.0(7.4)	>0.20	155.3(3.8)	>0.05	>0.05
Control	—	135.9(7.2)	—	125.7(7.4)	—	>0.20
Phenylbutazone	50.0	118.8(10.5)	>0.10	113.8(6.7)	>0.20	>0.20
Phenylbutazone	125.0	126.3(4.9)	>0.10	125.5(6.7)	>0.20	>0.20
Control	—	121.7(6.5)	—	121.1(7.9)	—	>0.20
Chlorpromazine HCl	40.0	131.5(5.5)	>0.20	118.4(3.0)	>0.05	<0.05
Chlorpromazine HCl	100.0	129.1(6.8)	>0.20	106.3(7.1)	>0.10	<0.05
Control	—	140.1(4.8)	—	128.4(4.0)	—	>0.05
Cryogenine	100.0	130.9(2.5)	>0.10	143.8(4.7)	<0.05	<0.05
Cryogenine	250.0	133.4(4.2)	>0.10	133.4(2.9)	>0.20	>0.20
Control	—	128.2(7.6)	—	129.6(9.7)	—	>0.20
Tetrabenazine	15.0	126.3(6.8)	>0.20	131.4(4.2)	>0.20	>0.05
Tetrabenazine	37.5	128.7(5.6)	>0.20	139.0(6.7)	>0.20	>0.20
Control	—	124.5(4.4)	—	137.6(5.8)	—	>0.05
Sparteine SO_4	80.0	124.5(6.2)	>0.20	133.5(6.7)	>0.20	>0.20
Sparteine SO_4	200.0	121.3(5.5)	>0.20	122.7(4.2)	<0.05	>0.20

[a] Liver glutathione is expressed as glutathione equivalents. Drugs were administered orally at −1 hr., carrageenin at 0 hr., and sacrifice with liver sampling just after the +3-hr. plethysmographic recordings.

Concomitant with physiological changes, stress-induced biochemical alterations such as increased lipolysis and glycogenolysis as well as adrenal ascorbic acid and cholesterol depletion take place. Bartlett and Register (8) showed that a reduction in liver sulfhydryl content occurs in response to cold or restraint. This has been shown to happen in various other tissues such as brain, kidney, and blood and in response to other types of stress such as heat, pain, hemorrhage, and various drugs (9–11). While the mechanism of stress-induced glutathione depletion is not well understood, it generally is thought to be the result either of activation of the pituitary–adrenocortical axis with the release of corticosteroids (during which process glutathione is rapidly oxidized and finally hydrolyzed to glycine and glutamylcysteine) or of stimulation of the sympathoadrenal system with the release of epinephrine and subsequent glycogenolysis (the reduction is thought to be the result of glutathione acting as a tightly bound prosthetic group of glyceraldehyde-3-phosphate dehydrogenase) (12).

Marozzi and Malone (1) indicated that a possible correlation exists between a compound's ability to protect against stress-induced (hind leg tourniquets) hepatic glutathione depletion and its potential in inhibiting carrageenin-induced acute pedal inflammation. However, in the present study, carrageenin-induced pedal edema in itself did not deplete liver sulfhydryl content (Fig. 1), possibly because of the localization of the swelling and the relative mildness of the phlogiston. While the lack of effect on liver sulfhydryl content was somewhat unexpected, it was still felt that measurement of this parameter might be useful in defining and separating antistress and anti-inflammatory activity during drug screening.

All of the oral anti-inflammatory potencies in Table I were calculated in terms of aspirin since it is still the most widely used nonsteroidal anti-inflammatory agent. Because the compounds used in this study differ widely in their molecular mass as well as in their structures, relative anti-inflammatory potency based on moles per kilogram was also calculated for each. The λ value (s/b) is generally regarded as the best single mathematical characteristic for a specific assay procedure. The mean λ value for the seven assays in Table I was 0.32 (SE = 0.018).

In support of the results depicted in Fig. 1, the glutathione levels of control animals receiving carrageenin (Table II) were never significantly different from the levels detected in those controls without the carrageenin challenge.

Histamine, serotonin, and bradykinin have been suggested as possible mediators of the inflammatory response. Cyproheptadine, a potent antagonist of these agents, has been shown to be active in several experimental models of inflammation but inactive in the carrageenin-induced acute rat hind paw edema at 3 mg./kg. orally (13) and at 0.5 mg./kg. s.c. (3). During the preliminary work of the present study, cyproheptadine was found to be inactive against such edema at 20 and 50 mg./kg. orally. The antiphlogistic activity observed in Table I with high dosages (125 and 312.5 mg./kg. orally) is probably the result of a secondary nonspecific effect. It is well known that diverse pharmacologic agents can inhibit this inflammatory response if sufficiently high dosages are employed. This toxic effect concept is supported by the significantly lowered hepatic glutathione levels reported in Table II.

Aspirin was unique among the drugs studied, since it caused a significant elevation of hepatic glutathione content both in the presence and absence of carrageenin-induced pedal edema. Like aspirin, indomethacin and phenylbutazone possess analgesic and antipyretic activity and are capable of uncoupling oxidative phosphorylation (14). More recently, Gerber *et al.* (15) showed that these agents are also capable of accelerating a disulfide interchange reaction between human serum sulfhydryl groups and 5,5′-dithiobis(2-nitrobenzoic acid). The relative ability of these agents to facilitate this reaction is of the same order as their anti-inflammatory potency determined by other laboratory tests. The exact mechanism by which indomethacin and phenylbutazone exert their anti-inflammatory activity is not known, although it is generally thought to be independent of pituitary involvement. Unlike aspirin, both of these clinically useful compounds had no detectable effect on rat liver glutathione content at effective oral anti-inflammatory dosages.

The Mexican plant, *Heimia salicifolia*, has been reputed to possess sudorific, antipyretic, diuretic, hemostatic, and vulnerary activities (16). One of its alkaloids, cryogenine, has been shown to possess hypothermic and selective CNS depressant activity (17), as well as anti-inflammatory activity against both acute carrageenin-induced pedal edema and chronic adjuvant-induced edema (2). No deaths were reported in rats treated with cryogenine orally at 100 mg./kg./day for 13 days; histopathologic examinations showed no lesions of the myocardium, liver, lung, kidney, or spleen (2). In a comprehensive recent study (18), chronic dosage of 100 mg./kg./day orally in rats for 21 days did not produce any significant histopathologic lesions. Since no alterations were noted in regard to thymus, adrenals, liver, and blood, these authors felt that cryogenine's anti-inflammatory activity was probably not mediated by the endogenous release of corticosteroids. Jiu (19) independently reported that extracts of *Heimia salicifolia* were effective in two additional models of inflammation (yeast-induced foot edema and cotton pellet granuloma). Trottier and Malone (20) showed that cryogenine can antagonize the effects of furtrethonium, histamine, and serotonin on

various isolated tissues, although with less potency and specificity than diphenhydramine and cyproheptadine. In the isolated guinea pig uterus, cryogenine evidenced some specificity for the histaminic receptor (20).

The present study confirms the previous findings on the anti-inflammatory efficacy of orally administered cryogenine and reports that cryogenine by itself was without effect on liver glutathione. While there was an apparent elevation of glutathione levels in animals receiving 100 mg./kg. of cryogenine plus carrageenin, no such effect was seen at the 250-mg./kg. dosage level, and the agar control (without carrageenin) had a statistically comparable high value. Therefore, the "elevation" can be dismissed as an artifact arising out of the experimental design. In a similar fashion, the statistically significant lowering of glutathione by 200 mg./kg. of sparteine in the presence of carrageenin also seems to be without real significance.

Tetrabenazine was consistently without effect on liver glutathione. Although tetrabenazine has been used as an edemogen in the rat (inducing local inflammation with a marked depletion of local histamine) (21), the present study is the first where tetrabenazine has been shown to possess oral antiphlogistic activity. Its potency was second to that of indomethacin in the series of agents studied.

Chlorpromazine, although not classified therapeutically as an anti-inflammatory agent, has been shown to be effective in suppressing yeast-, serotonin-, and formalin-induced edema and 45° thermic- and granuloma-edema, in addition to carrageenin-induced edema. The mechanism of chlorpromazine's inhibitory activity is not well understood. Results obtained in this study indicate that its activity appears not to be related to its antihistamine, antiserotonin, or antibradykinin action, since cyproheptadine is a more potent antagonist of these suspected mediators of inflammation and this agent showed a relatively low order of inhibitory activity. Although chlorpromazine is known to possess peripheral adrenergic blocking activity, the effects seen with tetrabenazine (negligible peripheral effect) suggest that such activity cannot account for the potent anti-inflammatory effect seen with chlorpromazine.

The present findings of antiphlogistic activity without significant changes in liver glutathione content would be consistent, therefore, with those of Brown *et al.* (22) which support the CNS as a possible site of action for some clinically useful anti-inflammatories. Bhattacharya and Marks (23) recently noted that a single large dose of 25 mg./kg. i.p. of chlorpromazine would cause a prompt rise in plasma corticosteroid levels 30 min. after drug treatment and that it would last for 48 hr. after injection. Hypothermia seemed not to be involved in this effect, since no significant differences were found between animals kept at room temperature and those maintained at an ambient temperature of 31°. Unlike those of tetrabenazine and cryogenine, the chlorpromazine dosages employed in the present study caused marked CNS depression. Such depression would be an important limiting factor for its use in a clinical situation. Therefore, the results of this study suggest that chemical modification of tetrabenazine (and possibly, cryogenine) could result in a new class of potent, nonsteroidal, anti-inflammatory drugs.

Within the limitations of the experimental design of this study (all glutathione determinations made at equivalently effective anti-inflammatory dosages), there appeared to be no correlation between anti-inflammatory capacity and effects on liver sulfhydryl content.

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Morphine-Induced Fetal Malformations II: Influence of Histamine and Diphenhydramine

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Abstract □ The results of this investigation indicate that teratogenic effects of high subcutaneous doses of morphine sulfate administered on Day 8 or 9 of gestation in CF-1 mice are not augmented by histamine dihydrochloride or decreased by diphenhydramine. On the contrary, histamine caused a decrease in the incidence of exencephaly and cryptorchid testes and diphenhydramine caused an enhancement of the teratogenic response when both of these agents were used in combination with morphine sulfate.

Keyphrases □ Morphine induced—fetal malformation □ Fetal malformation, morphine induced—histamine, diphenhydramine effects □ Teratogenic effects—morphine, high doses □ Histamine, diphenhydramine effects—morphine-induced teratogenesis

A previous investigation (1) demonstrated that high subcutaneous doses of morphine sulfate administered to nontolerant CF-1 mice on the 8th or 9th day of gestation are capable of inducing a variety of fetal malformations. The most prominent anomaly observed with drug challenge on Day 8 was exencephaly, while axial skeletal fusion was the most conspicuous malformation occurring with the drug insult on Day 9.

It can be assumed that an evaluation of the pharmacologic actions of morphine may provide some insight into its teratogenicity. Because a decrease in food consumption is not involved in the teratogenicity of morphine (1), the next most obvious pharmacologic action of the drug which should be investigated is its ability to release histamine.

Many investigations lead one to believe that histamine release may be involved in the production of congenital anomalies by morphine. Among these are those of Ciuchta and Gautieri (2) who, working with perfusion studies on full-term human placentas, showed that histamine consistently caused constriction of placental vessels. This action may result in a decrease in the oxygenation of fetal blood flowing through such vessels and, hence, contribute to fetal asphyxia which, therefore, could conceivably be responsible for the production of congenital malformations. Also, several investigators noted that mice, subjected to anoxia on Day 8 or 9, gave birth to a large number of fetuses with exencephaly and axial skeletal fusions (3, 4). Furthermore, X-radiation has been shown to cause exencephaly and axial skeletal fusions in mice (5), as well as an increase in the histamine content of adult females (6, 7). Consequently, the possibility exists that morphine may produce its teratogenic effects by histamine release and the ensuing placental anoxia.

Therefore, the purposes of the present study were: (*a*) to reconfirm the teratogenic effects of high doses of morphine in CF-1 mice, and (*b*) to ascertain whether histamine is involved in the production of congenital malformations by morphine. The latter may be determined by observing an enhancement of malformations with the prior administration of histamine, or a reduction of malformations with the prior administration of an antihistamine.

EXPERIMENTAL

In all experimental groups, CF-1 albino mice[1] were employed. The females were placed in groups of 25–30 in aggregate cages and were not mated until they weighed at least 25 g. Males were placed in individual metal cages[2] measuring 12.5 × 15 × 10 cm. with a wire mesh front and floor. Excreta pans were elevated to the cage floor to permit coprophagy. The mice were maintained on Purina laboratory chow and tap water *ad libitum*.

Sodium chloride (0.9%), morphine sulfate[3] (4.0%), diphenhydramine HCl[4] (1.0%), and histamine dihydrochloride[5] (1.0%) were prepared weekly in distilled water and kept under refrigeration until the time of administration. At this time, the prepared solutions were allowed to equilibrate to room temperature, and all injections were made using 1-ml. B-D tuberculin syringes equipped with 1.27-cm. (0.5-in.) 26-gauge needles.

To produce timed pregnancies, two female mice were placed in a fertile male's cage at 4:00 p.m. The following morning at 8:00 a.m. the females were removed and inspected for the appearance of a vaginal plug which represents the last part of the ejaculate. Females exhibiting this plug were considered gravid; the morning of its appearance was designated as Day 0 of gestation. These gravid mice were then weighed and placed in individual cages, similar to the males, and remained undisturbed until the morning of Day 7 when they were weighed a second time. Pregnancy was confirmed by a weight gain of 2 g. or more.

The pregnant animals were assigned at random to one of 31 experimental categories. Each category was treated on either Day 8 or 9 of gestation. The treatments included: two saline control groups receiving 0.3 ml. of normal saline solution; six morphine-treated groups receiving single injections of 200, 300, or 400 mg./kg. of morphine sulfate; four groups receiving 50 or 100 mg./kg. of the antihistamine diphenhydramine HCl; two groups receiving 50 mg./kg. of histamine dihydrochloride; six groups injected with 50 mg./kg. of diphenhydramine HCl followed in 15 min. by an injection of 200, 300, or 400 mg./kg. of morphine sulfate; four groups injected with histamine followed in 15 min. by an injection of either 200 or 300 mg./kg. of morphine sulfate; four groups injected with 0.3 ml. of saline followed in 15 min. by the injection of either 200 or 300 mg./kg. of morphine sulfate; and two groups injected with saline followed in 15 min. by a second injection of saline. One group represented the untreated controls to establish the norms of the mice.

The morphine injections, single saline injections, and the second injection of the saline–saline treatment were administered subcutaneously in the upper right abdomen. All other injections were given subcutaneously in the upper left abdomen.

Following the respective treatments, the mice were allowed to continue their gestation uninterrupted until Day 18 which is 1 day prior to the expected delivery. On this day, each pregnant mouse was sacrificed by cervical dislocation and its weight was recorded. The abdominal cavity was opened, exposing the uterine horns. The numbers of fetuses and resorption sites (metrial glands) were determined and recorded. The exposed fetuses were removed by blunt dissection, blotted dry, and weighed to the nearest hundredth of a

[1] Obtained from Carworth Farms, Inc., New City, N. Y.
[2] Norwich Wire Works.
[3] Morphine sulfate USP, Merck and Co., Rahway, N. J.
[4] Marketed as Benadryl by Parke-Davis and Co., Detroit, Mich.
[5] Histamine dihydrochloride, Nutritional Biochemicals Corp., Cleveland, Ohio.

Table I—Mean Values of Test Groups Receiving Single-Injection Treatments on Day 8

Treatment	Number of Litters	Maternal Wt.[a] S	Maternal Wt.[a] T	Fetuses Right Horn	Fetuses Left Horn	Resorptions Right Horn	Resorptions Left Horn	$\bar{X}$ Fetal Weight, g.	Number of Males	Number of Females
Control (untreated)	6	25.5	48.0	6.2	3.7[b]	0.17	0.33	1.07	4.0	5.6
Saline, 0.3 ml.	6	26.8	51.0	5.8	6.2	0.33	0.50	1.13	6.5	5.5
Morphine, 200 mg./kg.	6	27.0	44.3[b]	6.3	5.0	0.50	0	0.92[b]	5.8	5.2
Morphine, 300 mg./kg.	6	25.7	48.5	5.5	5.0	0.50	0.33	1.10	5.2	5.3
Morphine, 400 mg./kg.	6	28.0	47.8	5.7	4.8	0.50	0.67	1.03	7.5	3.0
Diphenhydramine, 50 mg./kg.	6	27.8	40.2[b]	5.5	5.3	0	0.17	0.85[b]	5.6	4.6
Diphenhydramine, 100 mg./kg.	5	25.8	43.6[b]	6.2	5.2	0	0.60	0.96	4.6	6.8
Histamine, 50 mg./kg.	6	27.5	48.3	5.2	4.8	1.0	1.20	1.15	5.7	4.3

[a] S = starting weight; T = terminal weight[b]. Statistically significant in comparison with saline control, $p < 0.05$.

Table II—Mean Values of Test Groups Receiving Single-Injection Treatments on Day 9

Treatment	Number of Litters	Maternal Wt.[a] S	Maternal Wt.[a] T	Fetuses Right Horn	Fetuses Left Horn	Resorptions Right Horn	Resorptions Left Horn	$\bar{X}$ Fetal Weight, g.	Number of Males	Number of Females
Saline, 0.3 ml.	6	25.2	49.7	6.2	4.0	0.50	0.33	1.20	5.7	4.5
Morphine, 200 mg./kg.	6	26.2	47.8	4.5	5.8	0.33	0.17	1.02[b]	6.0	4.3
Morphine, 300 mg./kg.	6	25.8	41.3[b]	5.3	3.3	0	1.50	1.15	4.3	4.3
Morphine, 400 mg./kg.	6	27.5[b]	44.5[b]	6.0	4.7	0.17	0.17	1.05	6.3	4.3
Diphenhydramine, 50 mg./kg.	6	26.0	39.8[b]	6.5	3.8	0.33	0.50	1.0	6.0	4.3
Diphenhydramine, 100 mg./kg.	4	26.0	46.3	6.0	5.8	0	0.50	0.95[b]	5.0	6.8
Histamine, 50 mg./kg.	5	22.2	44.2	4.0[b]	6.0	0.20	0.80	1.06	5.6	4.8

[a] S = starting weight; T = terminal weight. [b] Statistically significant in comparison with saline control, $p < 0.5$.

gram on a torsion balance. Each fetus was examined for external defects, and the sex was determined on the basis of their external genitalia. Fetal viability was determined by reflex movement of the limbs in response to mechanical stimulation of the fetus with a blunt probe after removal from the uterus. Every second fetus was processed for skeletal examination according to the method of Staples and Schnell (8). The remaining fetuses were fixed and decalcified in Bouin's fixative. After 2 weeks, the fixative was replaced by 70% ethyl alcohol in which the specimens were stored until freehand sections were made with a thin double-edged stainless razor blade according to the method of Wilson (9).

Statistical Methods and Analysis—The significance of observed variations among the experimental groups was determined by using standard statistical routines. These included the Student t test for continuous variables and the uncorrected χ-square test for binomial proportions (10). The t values were calculated on the IBM 360-60 computer. The probability, p, was determined for the t and χ^2 values by utilization of standard probability tables.

RESULTS

Maternal Effects of the Drugs—Maternal stimulation, followed by depression and a recurrent hyperactive period of recovery, was experienced as a result of all morphine treatments. With the higher doses of morphine, convulsive seizures accompanied the excitement stage and continued to interrupt the depression. Acute toxicity to morphine was found to be very pronounced when the mice were treated with 400 mg./kg. This dosage caused deaths to occur in 13 of 25 animals (52%) when given on Day 8 and in 11 of 23 (43%) when injected on Day 9.

Excitatory responses were also evoked upon the administration of diphenhydramine. When given alone, this antihistamine produced no convulsive activity at the dosage levels employed. However, when it was administered in combination with morphine, convulsive seizures appeared more frequent and severe than those observed with morphine alone. In no case did single injections of 50 mg./kg. diphenhydramine cause any maternal deaths. However, when it was administered 15 min. prior to the 400-mg./kg. dose of morphine, a definite increase in death rate was observed. When this combination treatment was performed on Day 8, four of five of the animals were killed; with this same combination on Day 9, all eight pregnant mice succumbed. As a result of this extremely high death rate, the decision was made to delete the 400-mg./kg. dose of morphine in combination with other test drugs. By employing the two lower doses of 200 and 300 mg./kg. in these combinations, enhancement or reduction in fetal effects as compared to those produced by morphine alone could still be observed and would satisfy the purpose of this investigation. In all cases, local tissue irritation occurred at the site of the diphen-

Table III—Mean Values of Test Groups Receiving Combination Treatments[a] on Day 8

Treatment	Number of Litters	Maternal Wt.[b] S	Maternal Wt.[b] T	Fetuses Right Horn	Fetuses Left Horn	Resorptions Right Horn	Resorptions Left Horn	$\bar{X}$ Fetal Weight, g.	Number of Males	Number of Females
Saline, 0.3 ml. Saline, 0.3 ml.	5	26.0	47.2[c]	5.8	6.2	0.40	0.80	1.14	7.0	4.2
Diphenhydramine, 50 mg./kg. Morphine, 200 mg./kg.	6	26.8	46.3	5.8	5.0	0	0.30	1.03	5.8	5.4
Diphenhydramine, 50 mg./kg. Morphine, 300 mg./kg.	6	26.0	42.8[d]	5.0	4.7	0.70	0.50	1.02	4.0	5.3
Histamine, 50 mg./kg. Morphine, 200 mg./kg.	5	25.8	46.4	5.2	4.4	0.20	0.20	1.08	5.4	4.6
Histamine, 50 mg./kg. Morphine, 300 mg./kg.	5	25.2	41.2[d]	4.6	4.0	0.40	0	1.04	4.0	4.2
Saline, 0.3 ml. Morphine, 200 mg./kg.	6	26.8	44.7	4.2[d]	5.7	0.20	0.20	1.03	4.4	5.0
Saline, 0.3 ml. Morphine, 300 mg./kg.	6	27.8[d]	48.7	4.5	5.0	0.30	0.30	1.05	5.3	5.2

[a] 15-min. interval between injections. [b] S = starting weight; T = terminal weight. [c] Statistically significant in comparison with saline control, $p < 0.05$. [d] Statistically significant in comparison with single injections of the respective dose of morphine, $p < 0.05$.

Table IV—Mean Values of Test Groups Receiving Combination Treatments[a] on Day 9

Treatment	Number of Litters	Maternal Wt.[b] S	Maternal Wt.[b] T	Fetuses Right Horn	Fetuses Left Horn	Resorptions Right Horn	Resorptions Left Horn	$\bar{X}$ Fetal Weight, g.	Number of Males	Number of Females
Saline, 0.3 ml. Saline, 0.3 ml.	5	25.4	45.0[c]	5.0	4.2	0.20	0.40	1.10	3.6	5.6
Diphenhydramine, 50 mg./kg. Morphine, 200 mg./kg.	6	25.0	41.0	3.8	3.8	1.70	2.20	1.12	3.3[d]	4.3
Diphenhydramine, 50 mg./kg. Morphine, 300 mg./kg.	6	25.8	46.3	4.8	4.8	1.20	0.80	1.03	5.2	4.5
Histamine, 50 mg./kg. Morphine, 200 mg./kg.	6	25.5	42.5	5.2	4.7	1.0	0.50	1.07	5.3	4.5
Histamine, 50 mg./kg. Morphine, 300 mg./kg.	5	25.4	46.0	5.2	6.2	0.40	0.80	1.08	5.6	4.8
Saline, 0.3 ml. Morphine, 200 mg./kg.	5	25.8	46.4	5.6	4.2	0.40	0.60	1.08	5.8	4.0
Saline, 0.3 ml. Morphine, 300 mg./kg.	6	26.2	42.0	3.7	4.5	1.0	1.0	0.98[d]	3.8	4.3

[a] 15-min. interval between injections. [b] S = starting weight; T = terminal weight. [c] Statistically significant in comparison with saline control, $p < 0.05$. [d] Statistically significant in comparison with single injections of the respective dose of morphine, $p < 0.05$.

hydramine injection; ulceration and necrosis at the injection site became evident a few days after the administration.

Histamine when given alone caused an overall depressive symptom in the animals. The administration of either histamine or saline 15 min. prior to morphine had no effect on the normal maternal symptoms caused by the narcotic.

Tables I–IV represent the cumulative mean values for each experimental group employed in this study. It appears from the results shown in Tables I and II that subcutaneous injections of morphine tend to cause a reduction in the mean terminal weights of the pregnant mice when compared to saline controls. This result was usually more apparent when the drug was administered on Day 9 of gestation. It can be seen from Tables II and III that in no instance did the administration of normal saline 15 min. before the injection of morphine cause any significant change in the effect of morphine alone on this parameter. It can also be seen from these tables that in almost every case the administration of diphenhydramine on either Day 8 or 9 resulted in a decrease in the mean terminal weight compared to saline, but the greater effect usually occurred at the lower dose. Diphenhydramine or histamine, when employed in the combination treatments with 300 mg./kg. morphine, both caused a reduction in the maternal mean terminal weight compared to single injections of morphine at this same dose.

Fetal Resorptions and Sex Ratios—In practically all cases, there were no differences in the fetal ratio (number of fetuses in the right horn *versus* number of fetuses in the left horn). In the few cases where there were significant differences, they only occurred on one side of this ratio which, therefore, would tend to lessen their significance. No significant differences were observed among the resorption ratios in any of the test groups. In every case, no change was observed in the male *versus* female ratio except when 50 mg./kg. diphenhydramine followed by 200 mg./kg. morphine was administered on Day 9. Here the number of males was less compared to 200 mg./kg. morphine given on the same day.

Mean Fetal Weight—A significant decrease in the mean fetal weight was observed with the administration of the lowest dose of morphine on both Days 8 and 9 of gestation. Diphenhydramine on Day 8 at the 50-mg./kg. dosage and on Day 9 at the 100-mg./kg. dosage caused a decrease in the fetal weight as compared to the saline controls. Although 300 mg./kg. morphine on Day 9 had no effect on the mean fetal weight, in combination with saline a significant decrease in this weight was observed.

Production of Anomalies—*Exencephaly*—The number of exencephalic fetuses and the number of litters containing defective young are tabulated in Tables V–VIII. From Table V it can be seen that in the groups treated with morphine alone, exencephaly was found in three of six litters after treatment with 300 mg./kg. on Day 8. Only one fetus with exencephaly was found in the group treated with 200 mg./kg. on Day 8, which has been shown to be insignificant. The administration of diphenhydramine alone produced no exencephalic fetuses. However, with the combination of 50 mg./kg. diphenhydramine and 200 mg./kg. morphine given on Day 8 (Table VII), five more exencephalic fetuses were found than with morphine given alone.

Although exencephaly was not observed in the groups receiving the combination of 50 mg./kg. histamine and 300 mg./kg. morphine on Day 8, this was a significant decrease in this effect compared to the three litters with exencephaly occurring with 300 mg./kg. morphine on the same day.

In all cases, the injection of saline 15 min. before the morphine administration compared to the injection of morphine alone produced no significant changes in the occurrence of the anomaly.

One case of exencephaly appeared in the following groups having morphine administered on Day 9: 200 mg./kg. morphine; 50 mg./kg. diphenhydramine in combination with 300 mg./kg. morphine; and saline in combination with 300 mg./kg. morphine. Two exencephalic fetuses were found in the group treated with 50 mg./kg. histamine in combination with 300 mg./kg. morphine. In no case was the appearance of exencephaly found to be significant as a result of any treatment given on Day 9.

In contrast to normal fetuses where the eyes are usually closed until 14 days after birth, all but one of the exencephalic fetuses observed had both eyes open. Sections made through these eyes disclosed the vitreous chamber and the lens as being stellate in shape

Table V—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 8

Treatment	Exencephaly Litters Absent	Exencephaly Litters Present	Exencephaly Normal Fetuses	Exencephaly Abnormal Fetuses	Cryptorchid Testes Litters Absent	Cryptorchid Testes Litters Present	Cryptorchid Testes Normal Fetuses	Cryptorchid Testes Abnormal Fetuses	Axial Skeletal Fusions Litters Absent	Axial Skeletal Fusions Litters Present	Axial Skeletal Fusions Normal Fetuses	Axial Skeletal Fusions Abnormal Fetuses
Control (untreated)	6	0	59	0	6	0	31	0	6	0	28	0
Saline, 0.3 ml.	6	0	72	0	6	0	38	0	6	0	34	0
Morphine, 200 mg./kg.	5	1	68	1	3	3[a]	37	3	6	0	29	0
Morphine, 300 mg./kg.	3	3[a]	60	3	4	2	29	3	6	0	31	0
Morphine, 400 mg./kg.	6	0	63	0	3	3[a]	29	3	5	1	29	2
Diphenhydramine, 50 mg./kg.	6	0	65	0	4	2	32	2	6	0	31	0
Diphenhydramine, 100 mg./kg.	5	0	57	0	2	3[a]	25	4[a]	5	0	28	0
Histamine, 50 mg./kg.	6	0	61	0	5	1	31	1	6	0	29	0
Saline, 0.3 ml. Saline, 0.3 ml.	5	0	56	0	4	1	23	1	5	0	32	0

[a] Significant $(\chi)^2 > 3.84$ = significantly different from saline control.

Table VI—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 9

Treatment	Exencephaly				Cryptorchid Testes				Axial Skeletal Fusions			
	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Saline, 0.3 ml.	6	0	61	0	6	0	31	0	6	0	30	0
Morphine, 200 mg./kg.	5	1	61	1	6	0	32	0	5	1	27	3
Morphine, 300 mg./kg.	6	0	52	0	4	2	25	2	3	3[a]	20	5[a]
Morphine, 400 mg./kg.	6	0	64	0	6	0	34	0	4	2	26	4[a]
Diphenhydramine, 50 mg./kg.	6	0	62	0	4	2	29	2	6	0	31	0
Diphenhydramine, 100 mg./kg.	4	0	47	0	3	1	22	1	3	1	22	2
Histamine, 50 mg./kg. Saline, 0.3 ml.	5	0	52	0	5	0	25	0	5	0	27	0
Saline, 0.3 ml.	5	0	46	0	4	1	20	1	5	0	25	0

[a] Significant $(\chi)^2 > 3.84$ = significantly different from saline control.

rather than the respective crescent and circular shapes seen in the normal state.

Other Soft-Tissue Defects—It can be seen from Tables V and VI that morphine caused a retardation of testicular descent (cryptorchid testes) and that this anomaly usually occurred more frequently when the drug was administered on Day 8 of gestation. This defect was found to be significant in the groups treated with 200 mg./kg. morphine on Day 8 and 400 mg./kg. morphine on Day 8 when compared to the saline controls. In no case did any drug combination significantly alter the effect of morphine on either day with respect to this defect. The group treated with 100 mg./kg. diphenhydramine on Day 8 showed a statistical significance in the appearance of this anomaly.

Other than exencephaly and cryptorchid testes, only a few soft-tissue malformations were found. One fetus demonstrating hydrocephalus was observed in the saline control group injected on Day 8. One fetus with hydronephrous was seen in each of the three groups treated with single injections of morphine on Day 8 and also in the group treated with 400 mg./kg. morphine on Day 9. This same anomaly was found in one fetus in each of the following groups: histamine, 50 mg./kg., on Day 8; diphenhydramine, 50 mg./kg., in combination with morphine, 200 mg./kg., on Day 8; and saline in combination with morphine, 200 mg./kg., on Day 9. An ectopic kidney was found in one fetus belonging to the group treated with 50 mg./kg. diphenhydramine on Day 8.

Rib and Vertebral Fusions—Table VI shows that 300 and 400 mg./kg. morphine, when given on Day 9, produce a significant occurrence of rib and vertebral fusions. With 200 mg./kg. morphine administered on Day 9, three fetuses occurred with either rib or vertebral fusions, the results being of borderline significance. However, in the combination of 50 mg./kg. diphenhydramine with 200 mg./kg. morphine given on this same day (Table VIII), the incidence of these fusions was found to be significant not only when compared with the saline controls but also when compared with results seen with 200 mg./kg. morphine given alone on Day 9.

In no case did the administration of saline 15 min. before the treatment with morphine significantly alter the effect of the single injections of morphine with respect to skeletal fusions.

DISCUSSION

The observations in this study concerning the CNS stimulation and convulsive seizures with higher doses of morphine have been routinely shown in experimental animals (1, 11, 12). Also, Gruhzit and Fiskin (13) reported that stimulation was observed with diphenhydramine in mice and rats irrespective of the mode of administration. Therefore, one would anticipate that a combination of diphenhydramine with morphine would produce more severe convulsive reactions than morphine alone, which was shown to be the case, due possibly to additive effects.

The local irritation observed at the site of diphenhydramine injection must be related to its cytotoxic action due to its local anesthetic properties (14), and this further reconfirms the observations reported by Gruhzit and Fiskin (13).

It can be concluded from the results that single injections of saline do not affect the maternal mean terminal weight when compared to untreated controls. This would indicate that when single injections of test drugs were found to cause a decrease in the maternal terminal weight, it was due to the drug injected and not the trauma of the injection itself.

In contrast to single injections, it was found that double injections of saline did significantly alter the maternal mean terminal weights on both days when compared to the single injection of saline, although this decrease did not differ greatly from that occurring with morphine alone. It was also shown that when the combination of saline and morphine was compared to morphine alone, no significant difference occurred. These results suggest that the trauma experienced by the double injection has some influence on the maternal mean terminal weight. However, a significant change in this parameter found with the combinations employed must be

Table VII—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 8 (Compared with Morphine, 200 or 300 mg./kg.)

Treatment	Exencephaly				Cryptorchid Testes				Axial Skeletal Fusions			
	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Saline, 0.3 ml. Morphine, 200 mg./kg.	6	0	59	0	5	1	30	1	6	0	28	0
Saline, 0.3 ml. Morphine, 300 mg./kg.	5	1	53	4	2	4	25	5	5	1	24	3
Diphenhydramine, 50 mg./kg. Morphine, 200 mg./kg.	3	3	59	6[a]	4	2	31	3	6	0	31	0
Diphenhydramine, 50 mg./kg. Morphine, 300 mg./kg.	4	2	55	3	3	3	27	3	5	1	27	1
Histamine, 50 mg./kg. Morphine, 200 mg./kg.		1	49	1	4	1	24	2	5	0	24	0
Histamine, 50 mg./kg. Morphine, 300 mg./kg.	5	0[a]	43	0	5	0	23	0	5	0	20	0

[a] Significant $(\chi)^2 > 3.84$ = significantly different from single morphine injection.

Table VIII—Occurrence of Exencephaly, Axial Skeletal Fusions, and Cryptorchid Testes with Treatment on Day 9 (Compared with Morphine, 200 or 300 mg./kg.)

Treatment	Exencephaly				Cryptorchid Testes				Axial Skeletal Fusions			
	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Saline, 0.3 ml. Morphine, 200 mg./kg.	5	0	49	0	4	1	25	1	5	0	23	0
Saline, 0.3 ml. Morphine, 300 mg./kg.	5	1	48	1	1	5	21	6	3	3	16	6
Diphenhydramine, 50 mg./kg. Morphine, 200 mg./kg.	6	0	46	0	4	2	22	2	1	5[a]	11	11[a]
Diphenhydramine, 50 mg./kg. Morphine, 300 mg./kg.	5	1	57	1	5	1	30	1	4	2	20	7
Histamine, 50 mg./kg. Morphine, 200 mg./kg.	6	0	59	0	6	0	30	0	4	2	26	3
Histamine, 50 mg./kg. Morphine, 300 mg./kg.	4	1	55	2	4	1	28	1	4	1	20	8

[a] Significant $(\chi)^2 > 3.84$ = significantly different from single morphine injection.

caused by the drug treatment because no change was observed with the combination of saline and morphine when compared to morphine alone.

A direct correlation between the degree of maternal mean terminal weight gain and the mean fetal weights was only noted in the two groups treated on Day 8 with 200 mg./kg. morphine and 50 mg./kg. diphenhydramine. It was also observed that in the groups treated on Day 9 with 200 mg./kg. morphine, 100 mg./kg. diphenhydramine, and saline with 300 mg./kg. morphine, the decreased mean fetal weights were not reflected in the maternal mean terminal weights. Furthermore, many groups showed significant decreases in the maternal mean terminal weights with no reflection on the mean fetal weights. Therefore, it must be concluded that the maternal weight gain during gestation cannot serve as an indicator of fetal size or development.

The fetal defects observed consequent to single injections of morphine sulfate reconfirm the results of those in a previous investigation (1), in which it was demonstrated that the drug administered in high doses on either Day 8 or 9 caused the pronounced fetal abnormalities, exencephaly and axial skeletal fusions. As in the previous investigation, it was also noted in the present study that the occurrence of exencephaly was more prominent when the drug was administered on Day 8, while the skeletal fusions were more pronounced with the treatment on Day 9.

Injection of normal saline 15 min. before the morphine did not modify the occurrence of exencephaly or axial skeletal fusions caused by the drug. This implies that if any drug, administered 15 min. before morphine, does generate a significant variation in the incidence of these two anomalies, this variation must be due to some action or interaction of both drugs and not to any trauma that might occur from the first injection.

The importance of cryptorchid testes found in the groups treated with morphine is not very apparent at this time. The retardation in testicular descent may be a result of the overall retardation of fetal growth, with the testes descending to their normal position at a later period of development. This possibility is supported by the observations that cryptorchid testes were more numerous in smaller fetuses and that they frequently occurred in the groups where other treatments produced smaller and lighter fetuses.

In the combination studies with morphine and diphenhydramine, only the 50-mg./kg. dose of the antihistamine was employed. The results of this investigation suggest that when such combinations are utilized, there is an enhancement in the ability of morphine to induce both exencephaly and axial skeletal fusions. This was demonstrated by the observation that when morphine was given alone at the 200-mg./kg. dose, there was no significant occurrence of either of the two anomalies. However, when this same dose was given in combination with diphenhydramine, the occurrence of both defects on Days 8 and 9, respectively, becomes significant not only when compared to saline but also when compared to the single injection of morphine.

Histamine, when administered alone, produced no significant soft-tissue anomalies and only skeletal defects that could be attributed to the retardation of fetal growth. Combinations of histamine and morphine did not enhance the incidence of exencephaly or axial skeletal fusions. On the contrary, the combination with morphine, 300 mg./kg., on Day 8 resulted in a significant decrease in the occurrence of exencephaly as well as a lower incidence of cryptorchid testes. Therefore, the results seem to indicate that morphine is not eliciting its teratogenic effects *via* histamine release. The fact that diphenhydramine is ineffective in preventing the anomalies further supports the conclusion that histamine release is not an etiologic factor in the production of morphine-induced congenital defects.

An interesting outcome of this investigation was that diphenhydramine potentiated the teratogenic action of morphine. Since it can be concluded from the findings that it was not the antihistaminic activity of diphenhydramine that enhanced the teratogenicity of morphine, it must be assumed that some other activity of diphenhydramine was responsible for the increased effect. Diphenhydramine, aside from being an antihistaminic, is a potent anticholinergic (15) and mild local anesthetic (14); therefore, it seems imperative that these and other properties of diphenhydramine should be investigated to determine whether they are involved in the enhancement of the teratogenic ability of morphine.

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(15) *Ibid.*, p. 636.

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Interaction of 3-Methylcholanthrene with Lecithin–Cholesterol Mixed Films

NORMAN D. WEINER*, IFTIKHAR CHAWDRY†, and ALVIN FELMEISTER†

Abstract □ The interaction of a carcinogenic hydrocarbon, 3-methylcholanthrene, with mixed films of various mole fractions of cholesterol and lecithin was investigated. The extent of interaction between 3-methylcholanthrene and cholesterol in the mixed films is greatly influenced by the competitive interaction between cholesterol and the phospholipid. At 50:50 cholesterol–lecithin molar ratios, where these lipids interact to the greatest extent, the interaction with 3-methylcholanthrene is weakest. These competitive interactions may account for the experimental observations that phospholipids retard while cholesterol accelerates the formation of tumors induced by polycyclic aromatic hydrocarbons.

Keyphrases □ 3-Methylcholanthrene interaction—lecithin–cholesterol mixed films □ Lecithin–cholesterol mixed films—competitive interaction □ Cholesterol–lecithin film competitive interaction effect—3-methylcholanthrene–film interaction □ Surface pressure—lecithin–cholesterol mixed film

Monomolecular films represent a relatively simple type of membrane model having a well-defined organized structure. They provide one of the most convenient and promising methods of studying molecules in a fixed orientation, as well as in a single layer where orientation can be changed by compression of the monomolecular film. As such, they constitute an important model system for the study of many natural phenomena that involve surfaces of an oriented array of molecules.

The production of cancer by pure hydrocarbons was discovered by researchers in England (1, 2). Since that time numerous substances have been demonstrated to have carcinogenic activity. These include polycyclic aromatic hydrocarbons, azo dyes, chemical agents of known and unknown composition, carcinogenic viruses, and physical agents (3).

Dickens and Weil-Malherbe (4, 5) observed that phospholipids retard, whereas cholesterol promotes, the formation and growth of tumors when injected simultaneously with the carcinogen, 3,4-benzpyrene.

Altman (6) also demonstrated that phospholipids retard the formation of tumors induced by a polycyclic hydrocarbon. He postulated that the protective activity of phospholipids must be due to their ability to form molecular associations with the polycyclic hydrocarbons, just as they do with cholesterol. Such associations promote the transportability of the carcinogen and thus prevent them from acting on the cell membrane. He felt that the question of why phospholipids only temporarily retard, and not permanently prevent, the formation of tumors must be seen in the scope of quantitative proportions in which phospholipids, on the one hand, and carcinogen plus cholesterol, on the other, occur in animal tissue.

Altman (7) suggested that carcinogenic substances induce cancer because they act as lipophilic sensitizers. They produce this effect either by occupying the available open spaces or by displacing the lipophilic sensitizers naturally occurring in the cell membrane. "Every change (even the slightest) in the structure of the membrane, imperatively leads to a change in its permeability and selectivity and consequently in the composition and properties of the whole cell." Therefore, a modification in the structure of the cell membrane can lead to the transformation of the original normal cell into another cell which accidently might be a cancer cell (6, 7).

Altman (6) felt that since phospholipids increase cell permeability while cholesterol decreases permeability, an excess of sterol (which condenses the cell membrane) promotes the fixation of carcinogenic molecules, while an excess of phospholipids (which expands the cell membrane) prevents such fixation. Absolute values of the ratio of phospholipids to cholesterol would then have a decisive influence on the incidence of cancer. Since values of phospholipids–cholesterol ratios are specific for each tissue, this situation offers an explanation of why one tissue is more accessible to tumor formation than another.

Finean (8) also explained the interactions of the polycyclic aromatic hydrocarbons on the basis of molecular association. Phospholipids and cholesterol both react with the carcinogenic compound; the association with the former promotes the motility of the carcinogen which is then prevented from sticking to the cell membrane.

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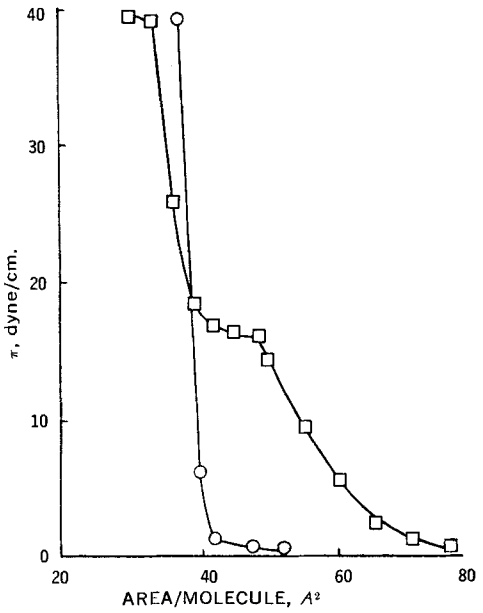

Figure 1—*Surface pressure–surface area (π–A) curves of cholesterol alone (○) and of cholesterol–3-methylcholanthrene (50:50 molar ratio) mixture (□).*

Clowes *et al.* (9, 10) studied the interactions of a variety of polycyclic hydrocarbons with cholesterol, β-dihydrocholesterol, and ergosterol spread as monomolecular films. Their work showed that the hydrocarbons fall into two classes with respect to the families of pressure–area curves obtained with the sterol films. Analysis of their data indicated that most of the hydrocarbons, in spite of their inability to spread on water, displayed sufficient reactivity toward sterol molecules to be held between the sterol ring system at an air–water interface.

The first class of interactions results in the formation of two-dimensional solutions from which the hydrocarbon can enter or leave at some particular surface pressure. The second class of interactions can be explained in terms of association complexes formed with the sterol molecules. It was suggested that this type of interaction may have significance in the transport of the hydrocarbon in the animal organism and in modifying biological structures.

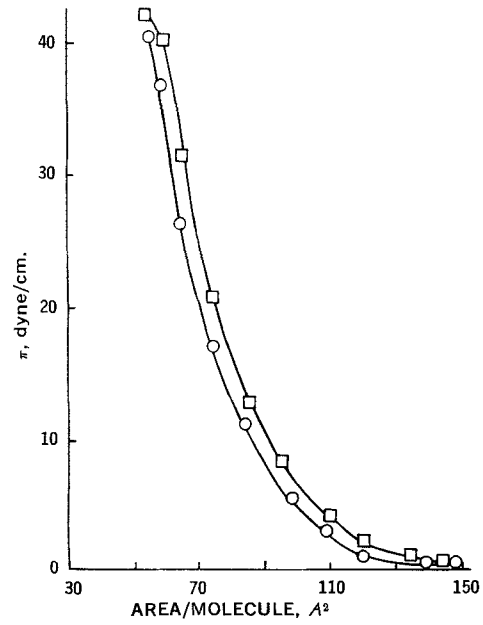

Figure 2—*Surface pressure–surface area (π–A) curves of egg lecithin alone (○) and of egg lecithin–3-methylcholanthrene (50:50 molar ratio) mixture (□).*

Snart (11) studied the monolayer behavior of various aromatic hydrocarbons with lecithin and cholesterol. He reported that hydrocarbons associate with cholesterol and lecithin in these films to a limited extent, after which additional hydrocarbon does not contribute to the film area but instead forms an excess solid phase. The association was treated in terms of solubility of the hydrocarbon in a two-dimensional solution and compared with data obtained from phase equilibria studies.

Phospholipids and cholesterol are major components of biological membranes composed of bimolecular leaflets or lipoprotein subunits (12). Mixed films of lecithins and cholesterol were first studied by de Bernard (13), who noted that cholesterol apparently was able to condense lecithin to a smaller cross-sectional area than it can exhibit alone. This condensation effect may be due to either a molecular interaction or a cavity effect (14).

The extent of the interactions of the carcinogenic hydrocarbons with cholesterol and lecithin would be modified by preexisting intermolecular interactions at the cell membrane between cholesterol and lecithin. The purpose of this investigation was to study the interaction of a carcinogenic hydrocarbon, 3-methylcholanthrene, with mixed films of various mole fractions of cholesterol and lecithin in order to gain some insight into the effect of membrane composition on the carcinogen–membrane interaction.

EXPERIMENTAL

The egg lecithin (mol. wt. 790)[1] and cholesterol (mol. wt. 386.7)[2] used were specified as chromatographically pure. Methylcholanthrene (mol. wt. 268.4)[3] and *n*-hexane, spectrograde[4], were also used.

Water used in this study was deionized by passing through a Bantam demineralizer and then distilled in an all-glass still. Glassware was cleaned in chromic acid solution and rinsed in hot-distilled water prior to use. All inorganic chemicals used were of reagent grade.

In the surface balance[5] used in these experiments, the Teflon-coated trough was removable to facilitate cleaning. The precision lead screw, which drives the reinforced Teflon barrier, allows for changes in surface area of the trough as small as 0.0125 cm². The barrier could be disengaged from the lead screw for rapid sweeping of the surface.

Surface pressure values were measured by the Wilhemy plate method (15). The platinum plate, roughened to ensure wetting, was suspended from a Torsion balance[6]. The balance can measure surface pressure changes of 0.1 dyne/cm.

The trough was filled with 0.9% sodium chloride solution, and the surface was swept several times with the barrier to clean it. Suction was then used to adjust the level of the subphase and to remove any remaining traces of dust or other insoluble contaminents.

The platinum plate was then lowered into position and allowed to remain beneath the surface while a known volume of mixtures of phospholipid–cholesterol–hydrocarbons dissolved in *n*-hexane

[1] Obtained from the Sylvania Chemical Co., Milburn, N. J.
[2] Obtained from Applied Science Laboratories, Inc., State College of Pennsylvania.
[3] Eastman Organic Chemicals, Rochester, N. Y.
[4] Obtained from Fisher Scientific Co., Fairlawn, N. J.
[5] Frater Instrument Co., Corona, N. Y.
[6] Supplied by Federal Pacific Electric Co.

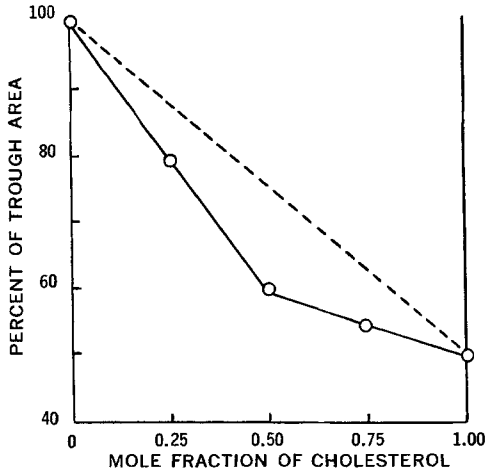

Figure 3—*Percent trough area of egg lecithin–cholesterol monolayers at a surface pressure of 10 dynes/cm. The broken line represents the additivity rule.*

was spread with the aid of an Agla micrometer syringe. This syringe can deliver accurately volumes as small as 0.001 ml. Generally, 0.07 ml. of the spreading solution was applied onto the subphase by allowing small drops to fall from the syringe held a few millimeters away from the subphase surface. At least 5 min. was allowed for the system to reach equilibrium before compression was initiated. The area available to the film molecules was then reduced in small increments, and surface pressure readings were taken 1 min. after each area change. Compression was continued until surface pressure no longer changed with area, indicating collapse of the film.

Each surface pressure–surface area (π–A) curve is the result of three separate experiments. The curves were always superimposable, with deviations of less than 0.2 dyne/cm. at all areas tested.

RESULTS AND DISCUSSION

Two-Component Systems—Figure 1 shows the surface pressure–surface area (π–A) plots of spread films of cholesterol alone and in combination with 3-methylcholanthrene. Alone, cholesterol does not exhibit any resistance to the barrier until each molecule occupies an area of about 40A^2. A further slight reduction in area then causes a sharp increase in pressure until the film collapses. These results are in agreement with those reported earlier by Clowes (16) and Demel and Joos (17).

Since 3-methylcholanthrene contains no hydrophilic groups, it has no tendency, when alone, to spread at the air–water interface. When 3-methylcholanthrene was spread alone, no surface pressure was observed upon compression at all areas tested.

When a 50:50 molar ratio mixed film of cholesterol and 3-methylcholanthrene was spread, the film area exceeded that of pure cholesterol at low surface pressures (Fig. 1), indicating that the hydrocarbon interacts strongly with cholesterol. At a surface pressure of about 17 dynes/cm., a further increase in pressure produced a gradual decrease in film area. This indicates removal of hydrocarbon molecules from an area-determining position to a non-area-determining position, *i.e.*, to an excess phase outside of, but in very close contact with, the film (16).

The fact that the area/molecule of the mixed film in the high pressure region is less than that of the pure cholesterol film at equal surface pressures is characteristic of mixed films in which the excess component, 3-methylcholanthrene, solubilizes the other (18).

Figure 2 shows the π–A curve of a spread film of egg lecithin. In the presence of 3-methylcholanthrene, the curves obtained for the mixed film were displaced only slightly. Similar results were reported by Clowes (16), who concluded that no discernible interaction between polycyclic hydrocarbons and lecithin could be found.

Three-Component Systems—Figure 3 shows the average area/molecule (percent trough area) *versus* the mole fraction of cholesterol for mixed films of cholesterol and egg lecithin at a surface pressure of 10 dynes/cm. While a condensation effect is observed at all mole fractions (the average area/molecule is always less than that predicted by the additivity rule), this effect is most striking at a

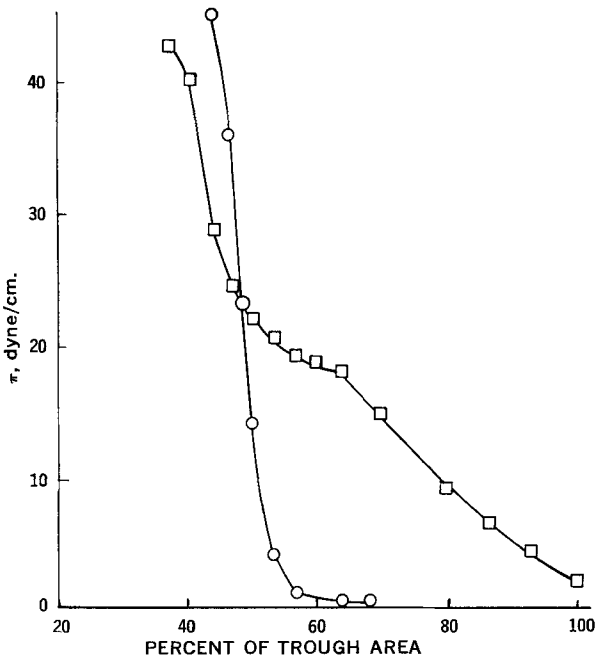

Figure 4—*Surface pressure–surface area* (π–A) *curves of cholesterol–egg lecithin (75:25 molar ratio) mixture alone* (○) *and in the presence of 3-methylcholanthrene* (□).

50:50 mole ratio. The results obtained are in agreement with those reported by other investigators (19, 20).

Figures 4–6 show the effect of 3-methylcholanthrene on the π–A curves of lecithin–cholesterol mixed films at various mole fractions. In each case the number of molecules of 3-methylcholanthrene added was equal to the total number of lipid molecules in the film. Since 3-methylcholanthrene interacts to a much greater extent with cholesterol as compared to egg lecithin, one would expect the degree of interaction of the carcinogen with the mixed films to be proportional to the mole fraction of cholesterol in the film. However, whereas the degree of interaction (as measured by comparing the increase on surface pressure at a given surface area) is greatest for the 75:25 cholesterol–lecithin mixed film, there is no discernible difference between the effect of 3-methylcholanthrene on the 50:50

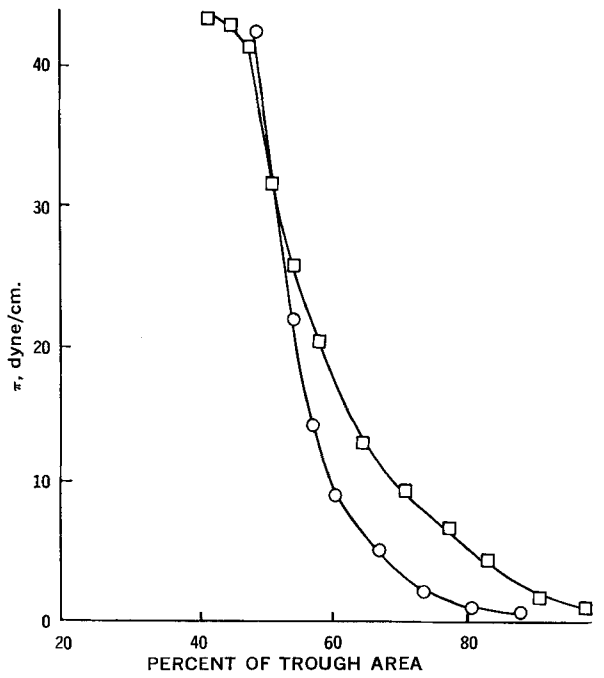

Figure 5—*Surface pressure–surface area* (π–A) *curves of cholesterol–egg lecithin (50:50 molar ratio) mixture alone* (○) *and in the presence of 3-methylcholanthrene* (□).

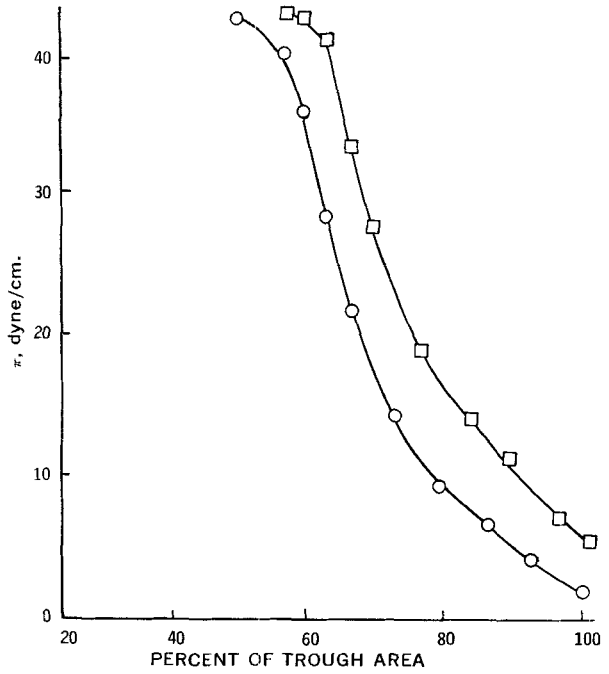

Figure 6—*Surface pressure–surface area (π–A) curves of cholesterol–egg lecithin (25:75 molar ratio) mixture alone (○) and in the presence of 3-methylcholanthrene (□).*

and 25:75 cholesterol–lecithin mixed films at low surface pressures, while the degree of interaction appears to be greater in the case of the 25:75 cholesterol–lecithin film at higher pressures.

It appears that the extent of interaction between 3-methylcholanthrene and cholesterol in the three-component mixed films is greatly influenced by the competitive interaction between cholesterol and the phospholipid. Therefore, at 50:50 cholesterol–lecithin molar ratios, where these lipids interact to the greatest extent, the interaction with 3-methylcholanthrene is weakest.

Altman (6, 7) demonstrated that phospholipids actually do retard the formation of tumors induced by a polycyclic hydrocarbon, when administered subcutaneously. He postulated that this effect could be explained on the basis of greater affinity of cholesterol to phospholipid than to 3-methylcholanthrene due to the presence of the OH group in the cholesterol molecule. This enables cholesterol to release 3-methylcholanthrene from its association with phospholipid, a process he expressed by the following equation:

phospholipid – 3-methylcholanthrene + cholesterol →
phospholipid – cholesterol + 3-methylcholanthrene (Eq. 1)

Based on this reaction, he postulated that the value of the ratio of phospholipid–cholesterol may have a decisive influence on the incidence of cancer.

Whereas the data support the postulation that the absolute value of the ratio of phospholipid to cholesterol influences the interaction with carcinogen, it appears that cholesterol, rather than the phospholipid, is the major interactor with 3-methylcholanthrene. This postulation is more direct than the one suggested by Altman (6, 7) to account for the experimental observation that phospholipids retard, while cholesterol accelerates, the formation of tumors induced by polycyclic aromatic hydrocarbons (4, 5).

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Dialysis of Ephedrine and Pentobarbital from Whole Human Saliva and Simulated Saliva

ROBERT E. DAVIS*, CHARLES W. HARTMAN†, and JULIAN H. FINCHER

Abstract □ It was shown *in vitro* that whole human saliva inhibits the passage of ephedrine through a dialysis membrane, whereas the passage of pentobarbital is unaffected. The binding of ephedrine in whole human saliva was found to be independent of pH. A simulated saliva was prepared which afforded the same results as whole human saliva.

Keyphrases □ Saliva, human, simulated—drug dialysis rates □ Dialysis rates—drug in simulated and human saliva □ Ephedrine, sodium pentobarbital—dialysis rates, saliva □ UV spectrophotometry—analysis

The sublingual and buccal routes for the administration of drugs have certain advantages over other routes (1). Drugs absorbed here are not exposed to gastrointestinal secretions which may cause decomposition. Moreover, they bypass the liver in the first circulatory pass (2) and are not exposed to microsomal enzymes before entering the general circulation, as is usually the case in gastrointestinal absorption. Other routes of administration may have these advantages but are generally less desirable than the sublingual and buccal routes for esthetic reasons.

A number of drugs are reported to readily enter the circulation by absorption from the oral cavity, but the physicochemical aspects of the process have not been extensively investigated. The present study, using a dynamic dialysis system, was undertaken to investigate the interactions that might occur between drug molecules and the constituents of salivary secretions. Such interactions, if they occur, might influence drug absorption through the oral mucosa. Ephedrine and pentobarbital were chosen for this research on the basis that pentobarbital reportedly is absorbed sublingually and bucally in therapeutic doses, whereas ephedrine is not (3).

EXPERIMENTAL

Materials—A 3% w/v aqueous solution of ephedrine and a 5% w/v aqueous solution of sodium pentobarbital[1] were used for the dialysis experiments.

Standard dialysis tubing[2] was used in the experimentation. This tubing had an average pore diameter of 4.8 nm. and was expected to retain materials having molecular weights greater than 12,000.

Dialysis Media—Three dialysis media were employed: a control, a simulated saliva, and whole human saliva.

The control medium was 0.165% w/v solution of sodium chloride, which possesses approximately the same osmotic characteristics as whole human saliva.

A formula for simulated saliva was not found in the literature. Even though the USP (4) contains formulas for simulated gastric and intestinal fluids, one major component of human gastrointestinal secretions, mucin, was omitted. The simulated saliva prepared for these studies was composed of substances found in appreciable quantities in whole human saliva. The quantities used were based on a resolution of the literature findings (5–26). A supplier for salivary mucin could not be found. However, salivary mucin consists of two principal types: those rich in *N*-acetyl galactosamine and neuraminic acids and those containing L-fucose (5). Gastric mucins also consist of the same general mucoproteins and mucopolysaccharides (27). Thus, gastric mucin was used in the place of salivary mucin. The formula for the simulated saliva is given in Table I. The physical properties of the simulated and the whole human saliva are compared in Table II.

Table I—Formula for Simulated Saliva

Material	meq./l.	Wt./l.
Mucin, gastric		1.0 g.
α-Amylase		2.0 g.
Sodium chloride	2.0	0.117 g.
Potassium chloride	20.0	0.149 g.
Sodium bicarbonate	25.0	2.100 g.
Distilled water		q.s. 1000 ml.

The whole human saliva used in the studies was collected from volunteers without external aid. For the large quantities needed, the subjects collected saliva for 4–5 hr. to minimize the nonuniformity among samples that is often encountered with forced flow. Loss of carbon dioxide and deterioration of the saliva were minimized by storage in tightly stoppered containers and by refrigeration.

The drugs were dialyzed into a 0.9% w/v solution of sodium chloride. This solution was selected to simulate the osmotic qualities of the blood and other body fluids.

Dialysis Procedure—For the dialysis of the drugs, a single dialysis tube was suspended by a string into 500 ml. of a 0.9% w/v solution of sodium chloride in an 8-fl. oz. conical graduate situated in a 2000-ml. beaker The temperature was maintained at 37° by pumping water to and from the beaker through a heat exchanger. Agitation and stirring were maintained within the conical graduate by a magnetic stirrer with a 1.27-cm. (0.5-in.) Teflon-coated stirring bar.

Each dialysis tube was sealed by tying one end, and 5.0 ml. of the dialysis medium was added to the tube. A volume of stock solution of sodium pentobarbital or ephedrine was added to effect a desired

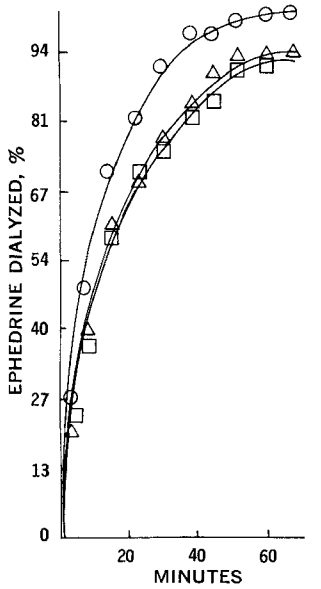

Figure 1—*Dialysis of ephedrine from the control* (○), *simulated saliva* (△), *and whole human saliva* (□) *media at a pH of 10.0.*

[1] Nembutal sodium, Abbott Laboratories, North Chicago, Ill.
[2] Oxford Laboratories, San Mateo, Calif.

Table II—Physical Comparison of Simulated Saliva to Whole Human Saliva

	Whole Human Saliva	Simulated Saliva
Color	Egg-shell white	Orange tint
Clarity	Opalescent	Clear
Specific gravity[a]	1.015	1.007
pH		
Initial	7.3	7.2
Final[b]	7.8–8.1	7.8–8.0

[a] Average values. [b] Rise of pH due to loss of carbon dioxide.

concentration. The volume of stock solution used was in each case insignificant in terms of the total volume in the bag. Ephedrine was tested in the control medium and both salivary media at three pH values, and pentobarbital was tested at one pH.

The pH range of the three media when the ephedrine was added without any buffers was 9.7–10.0. Sodium dihydrogen phosphate and citric acid, 10 g./l., were added to the media to give pH ranges of 7.3–7.4 and 4.5–4.8, respectively. The pH of the three media from which pentobarbital was dialyzed was 9.4.

Assay Procedures—Spectrophotometric assays for ephedrine and pentobarbital were carried out using a Beckman DU spectrophotometer[3], model 2400. Ephedrine was measured at 257 nm. after the method of Wheeler and Kaplan (28); pentobarbital was measured at 260 nm. as described by Conners (29).

RESULTS AND DISCUSSION

The effects of salivary constituents on the dialytic behavior of ephedrine at pH 10.0 are illustrated in Fig. 1. Data obtained at other pH values gave profiles that were qualitatively similar to Fig. 1. It is readily seen that ephedrine dialyzes more rapidly from the control medium than from the salivary media. Such difference might be explained by an interaction between the mucoproteins of salivary mucin and the ephedrine species present.

Interactions between mucin and amines were previously reported by Levine *et al.* (30). Also, organic molecular complex formation of the polymer type (31–33) involving mucin appears feasible, since the mucoproteins of mucin possess multiple carbonyl functions.

In the absence of interacting species in the dialysis medium, it would be expected that the dialytic rate of ephedrine would follow

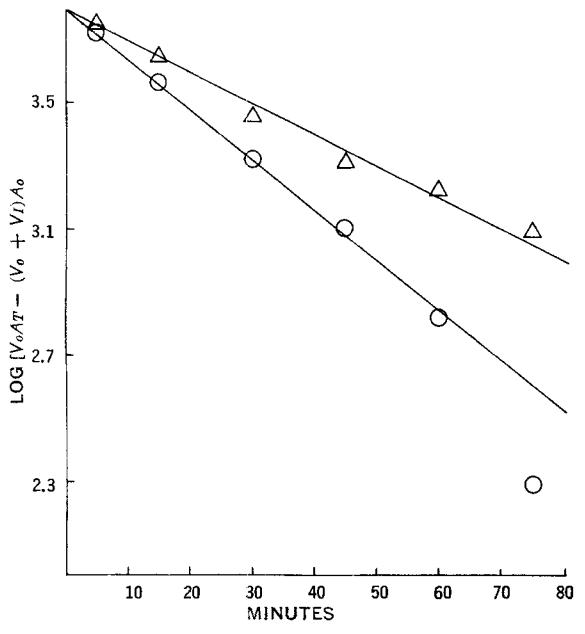

Figure 2—*Plot of the data of the dialysis of ephedrine at pH 10.0 using an expression of Fick's law. Key:* ○, *control medium; and* △, *whole human saliva medium.*

[3] Beckman Instruments, Inc., Fullerton, Calif.

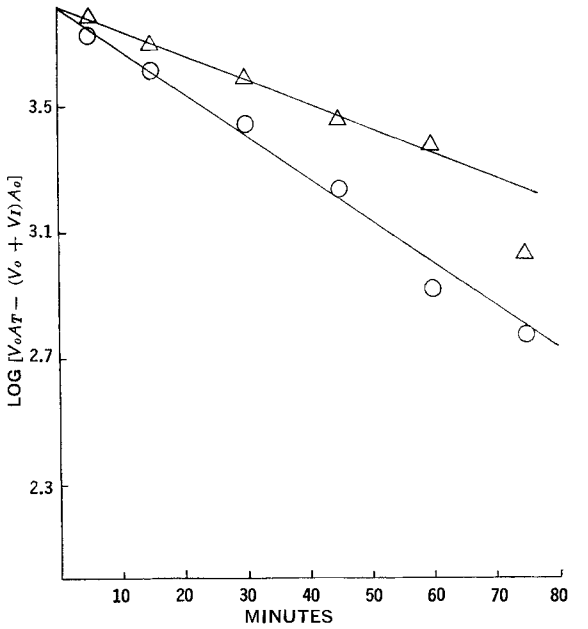

Figure 3—*Plot of the data of the dialysis of ephedrine at pH 4.5 using an expression of Fick's law. Key:* ○, *control medium; and* △, *whole human saliva medium.*

Fick's law (34). Where the pH's of the internal and external media are equal, Fick's law can be written as

$$\frac{dA_o}{dt} = k\left(\frac{A_I}{V_I} - \frac{A_o}{V_o}\right) \qquad \text{(Eq. 1)}$$

where:

A_o = amount of drug dialyzed into outside medium
A_I = amount of drug inside dialysis bag
V_o = volume of outside reservoir
V_I = volume inside dialysis bag
t = time
k = dialysis rate constant

A solution to this differential equation has the following form:

$$\log [V_oA_T - (V_o + V_I)A_o] = -\frac{V_o + V_I}{2.3V_oV_I}kt + \log (V_oA_T) \qquad \text{(Eq. 2)}$$

where $A_T = A_o + A_I$.

The data taken from the dialysis of ephedrine from the pH 10.0 control medium are plotted according to this equation as Curve B of Fig. 2. The linearity over 2–3 half-lives suggests that the model is appropriate.

Curve A of Fig. 2 shows the data collected on the dialysis from the whole human saliva medium plotted in the same manner. The drug dialyzed slower from the saliva, and this is taken to indicate a complexation of the ephedrine by saliva. The excellent linearity of this plot may be explained by assuming complex formation between

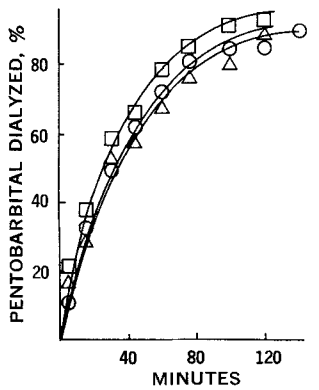

Figure 4—*Dialysis of pentobarbital from control* (○), *simulated saliva* (△), *and whole human saliva* (□) *media at a pH of 9.4.*

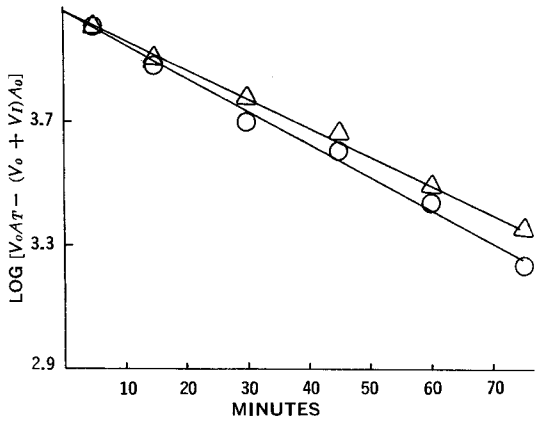

Figure 5—*Plot of the data of the dialysis of pentobarbital at pH 9.4 using an expression of Fick's law. Key: ○, control medium; and △, whole human saliva medium.*

ephedrine species and binding sites of the mucoproteins if the number of bound sites is negligible compared to the total available sites in this system. By assuming that the binding sites on the mucoproteins are comparable, the complexation of ephedrine by individual sites may be written:

$$E + S \overset{K}{=} C \qquad \text{(Eq. 3)}$$

where E represents free ephedrine species, S represents a binding site, C represents the complex, and K is the binding constant.

It can be shown that the concentration of unbound ephedrine in the dialysis bag can be expressed as

$$[E] = A_I/\{V_I(K[S] + 1)\} \qquad \text{(Eq. 4)}$$

If the bound ephedrine cannot be dialyzed, then Fick's law can be written:

$$\frac{dA_o}{dt} = k\left([E] - \frac{A_o}{V_o}\right) \qquad \text{(Eq. 5)}$$

Substituting Eq. 4 into Eq. 5 and utilizing the fact that $A_T = A_o + A_I$, this differential equation may be written:

$$\frac{dA_o}{dt} = k\left(\frac{V_oA_T - (V_o + V_I + K[S]V_I)A_o}{V_IV_o(K[S] + 1)}\right) \qquad \text{(Eq. 6)}$$

Under the conditions of the experimentation, V_I is much less than V_o. If $K[S]$ is of the order of unity, then $K[S]V_I$ is negligible compared to $(V_I + V_o)$, and the solution to the equation becomes

$$\log[V_oA_T - (V_o + V_I)A_o] = -\frac{V_I + V_o}{2.3V_IV_o(K[S] + 1)}kt + \log(V_oA_T) \qquad \text{(Eq. 7)}$$

That the slope of Curve A is less than the slope of Curve B is in keeping with the assumed model. From Eq. 2:

$$\text{slope of Curve B} = \frac{V_o + V_I}{2.3V_oV_I}k \qquad \text{(Eq. 8)}$$

while from Eq. 7:

$$\text{slope of Curve A} = \frac{V_o + V_I}{2.3V_oV_I(K[S] + 1)}k \qquad \text{(Eq. 9)}$$

The ratio of the slopes, under identical conditions of pH, allows estimation of the extent of binding:

$$\frac{\text{slope B}}{\text{slope A}} = K[S] + 1 \qquad \text{(Eq. 10)}$$

Since the number of binding sites on the mucoproteins has not been characterized in this research, the value of K cannot be known from these data. It can be shown, however, from the data collected at pH 10.0 that $K[S]$ has a value of 0.8. When the data collected at pH 4.5 were treated in the same manner, $K[S]$ was also found to be 0.8. It is concluded, therefore, that pH has no overall effect on the binding of ephedrine species by mucoproteins of saliva. The data collected do, however, show that pH has a marked effect on the rates of dialyses. For example, the slope of Curve A in Fig. 3 for the dialysis of ephedrine from a pH 4.5 buffered solution is 0.0134; the slope of Curve A in Fig. 2 for the dialysis of ephedrine from a pH 10.0 solution is 0.0180.

It is clear from the data at pH 4.5, where for practical purposes the ephedrine is 100% protonated, that the protonated form dialyzes at a significant rate. The rate of dialysis at pH 10.0, where 81% of the drug is unprotonated, is greater than that at pH 4.5, indicating that the free base passes the membrane at a significantly greater rate than the protonated form. The fact that the complex formation was pH independent does not appear to be consistent with the findings of Levine *et al.* (30), who postulated that the ammonium ion was necessary for complexation of quaternary amines by mucin.

A similar experiment was performed using pentobarbital. The data, as plotted in Fig. 4, show no significant differences in the rate of pentobarbital being dialyzed from the control, whole human saliva, or synthetic saliva media. When the data were plotted according to Eq. 2, the linearity obtained again demonstrated the appropriateness of the model (Fig. 5).

Studies of oral mucosal drug absorption, *in vitro*, should ideally be conducted using whole human saliva. Collection of adequate quantities of whole human saliva for experimentation in this area is difficult. To circumvent these difficulties, a synthetic saliva was prepared. The synthetic saliva afforded similar results in dialysis experiments.

These studies with ephedrine suggest that the constituents of salivary secretions could significantly affect the release of other amine drugs from drug systems. Since the passage of ephedrine through dialytic membranes was affected by saliva constituents, it is possible that the absorption of other amine drugs from the oral cavity would be influenced similarly. Additional experiments using other amine drugs are necessary to substantiate these possibilities. It is concluded, however, from these data that salivary secretion does slow the rate of passage of ephedrine through dialytic membranes, and that this decrease in rate is due to a binding of the drug to saliva constituents. This binding is pH independent.

A simulated saliva was prepared which afforded the same results as whole human saliva. It is suggested that such a solution would be an appropriate substitute for whole human saliva in future *in vitro* studies.

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Solubility of Sodium Salicylate in Mixed Solvent Systems

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Abstract □ The solubility of sodium salicylate was determined in aqueous mixtures of dioxane, acetone, 1-propanol, ethanol, and methanol. The solubility had fair parallelism to dielectric constants over a wide range of concentration, indicating adherence to the coulomb relationship. The solubility curves for sodium salicylate are examined from a suppressive dissociative point of view, insofar as ionization may be taking place without complete dissociation. Comparison of the solubility curves for an acid and its sodium salt is considered. The possibility that uni-univalent electrolytes in solutions below a dielectric constant of 30 are completely associated is shown for several systems in plots of various concentration notations. Adherence of the predicted linearity from the Born equation for these systems is discussed. However, the theoretical ionic radius is found to be about twice the expected value.

Keyphrases □ Salicylate, sodium, solubility—mixed solvent systems □ Alcohols, acetone, dioxane–water mixtures—sodium salicylate solubility □ Solubility, sodium salicylate—dielectric constant relationship □ Ion, radii—sodium, salicylate

The solubility of chemical compounds described as salts are normally thought of as having maximum solubility in water relative to other less polar solvents such as alcohols. The direction of decreasing solubility with decreasing polarity is generally based on the charge-separating ability of the solvent or the dielectric constant.

It is of interest to consider the solubility changes that occur as a function of the dielectric constant for a typical, useful pharmaceutical such as sodium salicylate. This pharmaceutical was also chosen because data are available (1) on salicylic acid in the same solvent systems and a comparison could be made for an acid and its sodium salt.

The Born equation (2), which deals with the relationship between solubility and the reciprocal of the dielectric constant, was also tested in this study.

The diminution of solubility of a salt with a concomitant decrease in dielectric constant obviously would be related to the degree of dissociation of the particular salt and might be related to the water content of these binary mixtures.

EXPERIMENTAL

Reagents—The reagents used were methanol[1], 1-propanol[1], 1-pentanol[2], 1-octanol[2], 1-butanol[3], 1-decanol[4], ethanol[5], and acetone[6]. Deionized water was used for all binary mixtures prepared. The sodium salicylate[7] was USP grade. Dioxane[8] (*p*-dioxane), stabilized, was also used in these studies.

The purity of the solvents was tested by the following procedure. The refractive indexes of each solvent were determined initially and throughout their use with a Bausch and Lomb Abbe-3 refractometer at 25°. The results are given in Table I.

The instrument was checked against 99 mole % free benzene (Fisher certified reagent), and the experimental value of 1.4983 was obtained at 25° *versus* the literature value of 1.498 at 25°.

[1] Fisher Certified, Fisher Scientific Co.
[2] Baker Analyzed, J. T. Baker Co., Phillipsburg, N. J.
[3] Eastman Kodak No. 50, Eastman Kodak, Rochester, N. Y.
[4] No. 5189, Matheson, Coleman & Bell, East Rutherford, N. J.
[5] U. S. Industrial Chemical Corp.
[6] Analytical Reagent, No. 2240, Mallinckrodt.
[7] USP, J. T. Baker Co., Phillipsburg, N. J.
[8] No. C 608258, Amend Drug and Chemical Co., Inc.

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† Deceased.

Solubility of Sodium Salicylate in Mixed Solvent Systems

ANTHONY N. PARUTA and JOHN W. MAUGER

Abstract □ The solubility of sodium salicylate was determined in aqueous mixtures of dioxane, acetone, 1-propanol, ethanol, and methanol. The solubility had fair parallelism to dielectric constants over a wide range of concentration, indicating adherence to the coulomb relationship. The solubility curves for sodium salicylate are examined from a suppressive dissociative point of view, insofar as ionization may be taking place without complete dissociation. Comparison of the solubility curves for an acid and its sodium salt is considered. The possibility that uni-univalent electrolytes in solutions below a dielectric constant of 30 are completely associated is shown for several systems in plots of various concentration notations. Adherence of the predicted linearity from the Born equation for these systems is discussed. However, the theoretical ionic radius is found to be about twice the expected value.

Keyphrases □ Salicylate, sodium, solubility—mixed solvent systems □ Alcohols, acetone, dioxane–water mixtures—sodium salicylate solubility □ Solubility, sodium salicylate—dielectric constant relationship □ Ion, radii—sodium, salicylate

The solubility of chemical compounds described as salts are normally thought of as having maximum solubility in water relative to other less polar solvents such as alcohols. The direction of decreasing solubility with decreasing polarity is generally based on the charge-separating ability of the solvent or the dielectric constant.

It is of interest to consider the solubility changes that occur as a function of the dielectric constant for a typical, useful pharmaceutical such as sodium salicylate. This pharmaceutical was also chosen because data are available (1) on salicylic acid in the same solvent systems and a comparison could be made for an acid and its sodium salt.

The Born equation (2), which deals with the relationship between solubility and the reciprocal of the dielectric constant, was also tested in this study.

The diminution of solubility of a salt with a concomitant decrease in dielectric constant obviously would be related to the degree of dissociation of the particular salt and might be related to the water content of these binary mixtures.

EXPERIMENTAL

Reagents—The reagents used were methanol[1], 1-propanol[1], 1-pentanol[2], 1-octanol[2], 1-butanol[3], 1-decanol[4], ethanol[5], and acetone[6]. Deionized water was used for all binary mixtures prepared. The sodium salicylate[7] was USP grade. Dioxane[8] (*p*-dioxane), stabilized, was also used in these studies.

The purity of the solvents was tested by the following procedure. The refractive indexes of each solvent were determined initially and throughout their use with a Bausch and Lomb Abbe-3 refractometer at 25°. The results are given in Table I.

The instrument was checked against 99 mole % free benzene (Fisher certified reagent), and the experimental value of 1.4983 was obtained at 25° *versus* the literature value of 1.498 at 25°.

[1] Fisher Certified, Fisher Scientific Co.
[2] Baker Analyzed, J. T. Baker Co., Phillipsburg, N. J.
[3] Eastman Kodak No. 50, Eastman Kodak, Rochester, N. Y.
[4] No. 5189, Matheson, Coleman & Bell, East Rutherford, N. J.
[5] U. S. Industrial Chemical Corp.
[6] Analytical Reagent, No. 2240, Mallinckrodt.
[7] USP, J. T. Baker Co., Phillipsburg, N. J.
[8] No. C 608258, Amend Drug and Chemical Co., Inc.

Table I—Refractive Index of Solvents Used

Solvent	Literature[a]	Determined	Percent Deviation
Methanol	1.326	1.3288	0.2
Ethanol	1.359	1.3640	0.4
1-Propanol	1.383	1.3840	0.1
1-Butanol	1.397	1.4025	0.4
1-Hexanol	1.416	1.4215	0.4
1-Octanol	1.427	1.4320	0.4
1-Decanol	1.4366[b]	1.4410	0.3
p-Dioxane	1.4224[b]	1.4024	0.1

[a] "Handbook of Chemistry and Physics," 48th ed., The Chemical Rubber Co., Cleveland, Ohio, 1967. [b] Values at 20° from *a*.

Table II—Dielectric Constant of Solvents Used

Solvent	Literature[a]	Determined	Percent Water
Methanol	32.63	32.7	0.2
Ethanol	24.30	24.4	0.4
1-Propanol	20.1	20.2	0.5
1-Butanal	17.1	17.2	0.6
1-Hexanol	13.3	13.4	0.8
1-Octanol	9.86[b]	9.9	0.4
1-Decanol	7.93[b]	8.0	0.8
p-Dioxane	2.21	2.2	0.4

[a] "Table of Dielectric Constants of Pure Liquids," U. S. Dept. of Commerce, NBS Circular 514. [b] Calculated from literature value at 20° and temperature coefficients to adjust value to 25°.

Further, a sample of each solvent was tested initially and throughout its use by measuring its dielectric constant value on a Sargent model V oscillometer. Deviations from literature values of the dielectric constant were considered to be due to the water content. The results are given in Table II.

Procedure—Solubilities were determined by either a spectrophotometric or gravimetric determination in a manner previously given (3). Spectrophotometric[9] determinations were done at 231 nm. Densities were determined by weighing the withdrawn samples previous to drying. In the case of the pure solvent scan, for the higher membered alcohols, the solubility was determined after 24-hr. equilibration in a 25° bath. Twenty-four hours was chosen since the equilibrium value of solubility occurred in about 10 hr. for the slowest rate in 1-decanol.

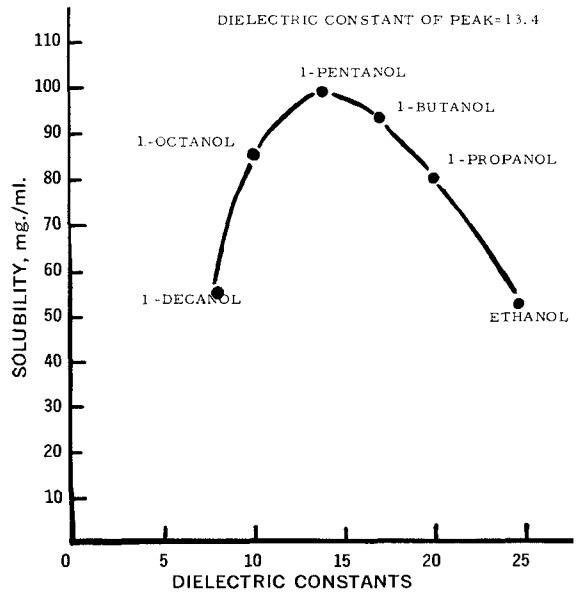

Figure 1—*Solubility of sodium salicylate in milligrams/milliliter at 25°* versus *the dielectric constants of pure alcohols.*

[9] Cary model 16 spectrophotometer.

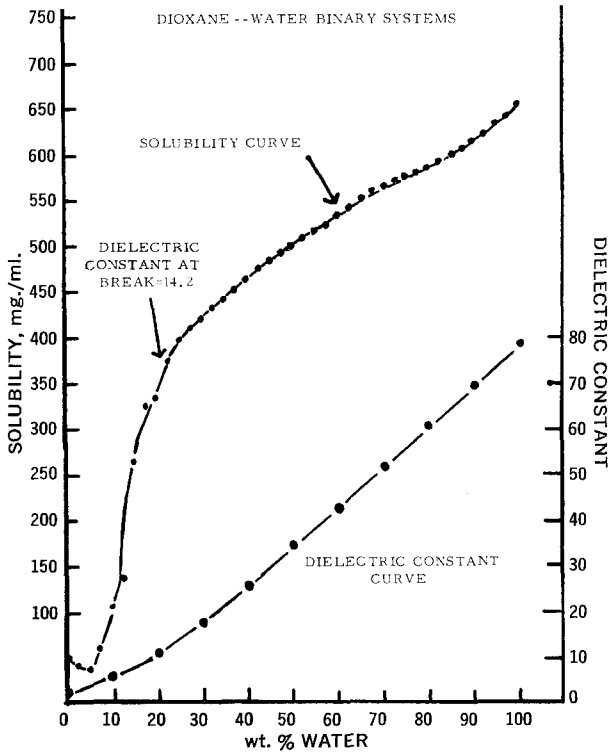

Figure 2—*Solubility of sodium salicylate in milligrams/milliliter* versus *the percent w/w water in dioxane–water mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

In the cases of alcohol–water, dioxane–water, and acetone–water systems, the solubility was tested at various concentrations, especially high nonpolar components, at 15-min. intervals for several

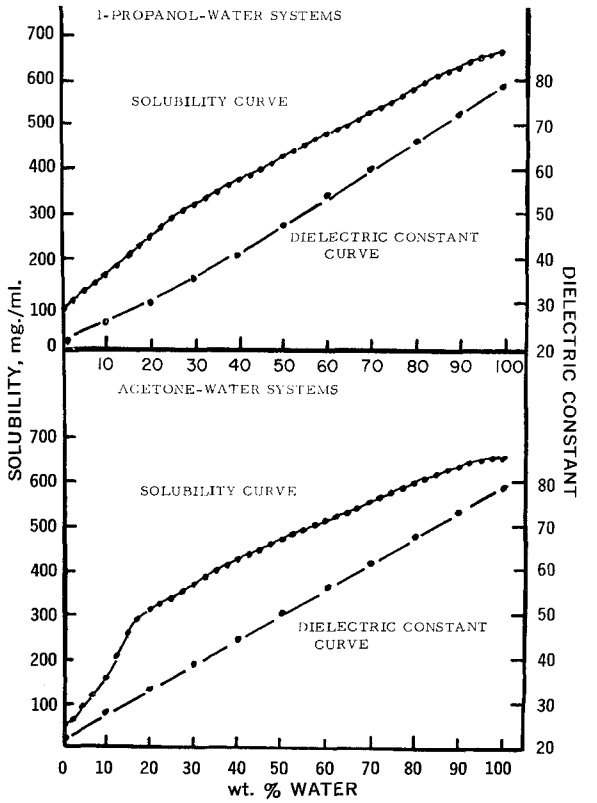

Figure 3—*Solubility of sodium salicylate in milligrams/milliliter at 25°* versus *the percent w/w water for the acetone–water and 1-propanol–water binary mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

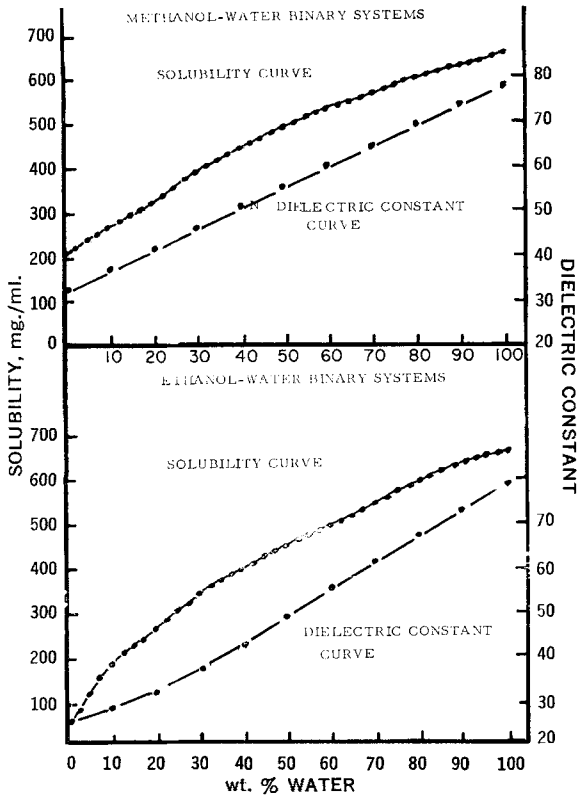

Figure 4—*Solubility of sodium salicylate in milligrams/milliliter at 25° versus the percent w/w water for the ethanol–water and methanol–water binary systems. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

hours. In no case did asymptotic solubility values occur after 2 hr. Since solutions of sodium salicylate are known to be light sensitive, the experimentally determined equilibrium solubility occurred in about 2 hr., and 4 hr. was chosen as the end-point. No discoloration of these solutions was noted during the procedure.

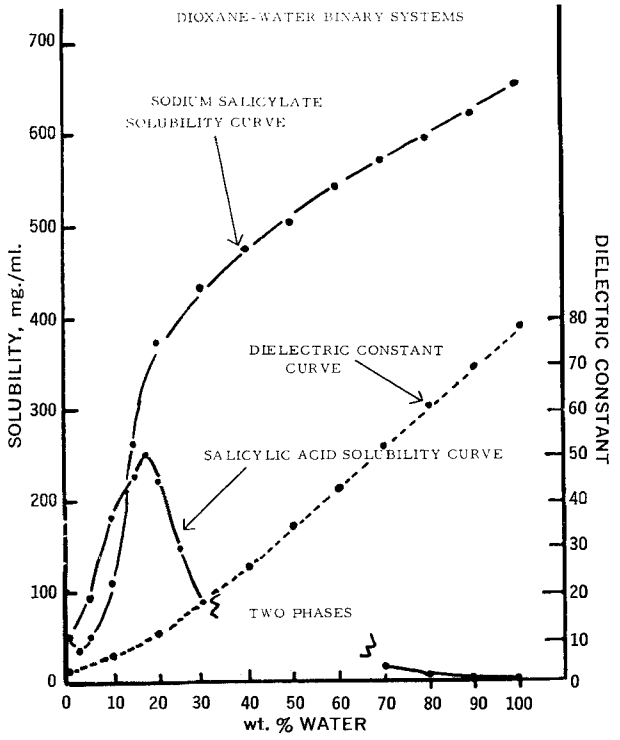

Figure 5—*Solubility of both salicylic acid and sodium salicylate in milligrams/milliliter at 25° for dioxane–water mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

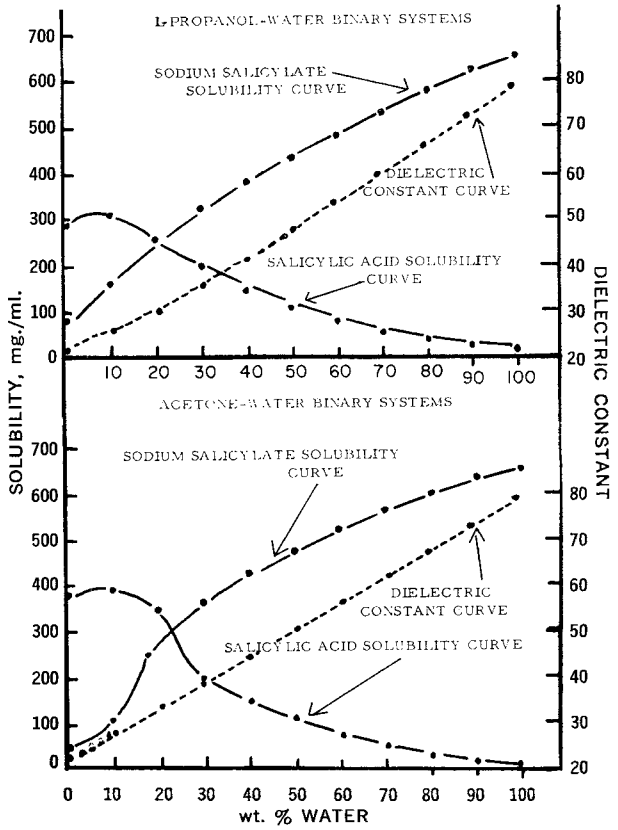

Figure 6—*Solubility of salicylic acid and sodium salicylate in milligrams/milliliter at 25° for acetone–water and 1-propanol–water mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

RESULTS AND DISCUSSION

The solubility of sodium salicylate was initially determined in a series of pure alcohols and is shown in Fig. 1. The solubility is plotted in milligrams/milliliter *versus* the dielectric constants of the pure solvents. Sodium salicylate dissolves to the greatest extent in 1-pentanol, producing a peak in this pure solvent scan. The dielectric requirement over this limited range of alcohols is equal to the dielectric constant of 1-pentanol or 13.9, which is strikingly similar to that observed for salicylic acid (1) which was found to have a value of about 15. This could indicate that at this relatively low value of the dielectric constant, sodium salicylate is essentially undissociated and acts as a neutral molecule or nonelectrolyte. It has been stated (4) that in media of dielectric constants below 60, divalent ions will be associated; and below a dielectric constant of 30, univalent ions will be undissociated. It may be assumed that a uni-univalent salt would then behave as a nonelectrolyte possessing semipolar character and inherent polarity.

The solubility of sodium salicylate was also determined in binary mixtures of water with dioxane, acetone, 1-propanol, ethanol, and methanol. The data obtained are plotted in Figs. 2–4 as the solubility in milligrams/millititer *versus* the weight percent of water. The dielectric constants of the binary mixtures are also shown in these figures, being drawn below the solubility curve. In the case of dioxane, a fair degree of parallelism is seen to exist between dielectric constants and solubility over the range of about 25% w/w water to pure water. The solubility curve deviates at about 25% w/w water, possessing a dielectric constant value of 14; again there is good agreement with the value of 15 for salicylic acid. In the case of the alcoholic systems, 1-propanol, ethanol, and methanol, a fair degree of parallelism is observed for the solubility curves. Only in the case of acetone–water mixtures does a deviation seem to exist at about a 20% w/w concentration of water. This occurs at a dielectric constant value of about 31, which supports the pre-

vious contention of complete association at a dielectric constant value of 30.

A comparison of the solubility curves observed for a weak acid and its sodium salt was of interest. Thus, the solubility data obtained are replotted in Figs. 5–7 as the solubility in milligrams/milliliter *versus* the weight percent of water. Included in these figures is the solubility of salicylic acid in the same solvent mixtures obtained previously (1).

In the case of the dioxane–water mixtures shown in Fig. 5, the solubility profile for sodium salicylate gives a sharp change of slope at approximately the same point as the peak observed for salicylic acid in the same mixture. Since the solubility profile for sodium salicylate is a repressive ionization curve, the "salt" acts as if it was completely associated at low dielectric constant values. The decrease in dielectric constants with increasing nonpolar solvent concentration causes the solubility to decrease, since the work required to separate the charges (ionization $\leftrightharpoons$ dissociation) becomes larger.

In the case of acetone–water mixtures, the solubility of the salt continuously decreases to the lowest value in pure acetone, whereas the salicylic acid shows a peak at a dielectric constant value of 25. However, after about 20% w/w water, there seems to be a fair degree of symmetry for these solubility curves about the line drawn through about 310 mg./ml.

Thus, the approximate solubility of salicylic acid and sodium salicylate is a fairly constant amount below and above this line at each concentration increment. The decrease in solubility of the sodium salt is due to the decrease in the charge-separating ability of the solvent mixture, whereas the increase in solubility of salicylic acid is due to the more favorable semipolar environment produced by the solvent blends.

With the three alcohols studied, although there is no line of symmetry between the solubility curves, the aforementioned statement still obtains. In the case of 1-propanol, a peak is observed for salicylic acid at a dielectric constant value of 22.

In the acetone, 1-propanol, and ethanol systems, the sodium salicylate intersects the salicylic acid solubility curve at the respective dielectric constants of 33, 30, and 34, whereas in methanol–water systems the value is 36. This supports the value of about 30 being the point where uni-univalent electrolytes become completely associated. Further, sodium salicylate becomes isodielectric with salicylic acid. That is, it possesses the same solubility as salicylic acid.

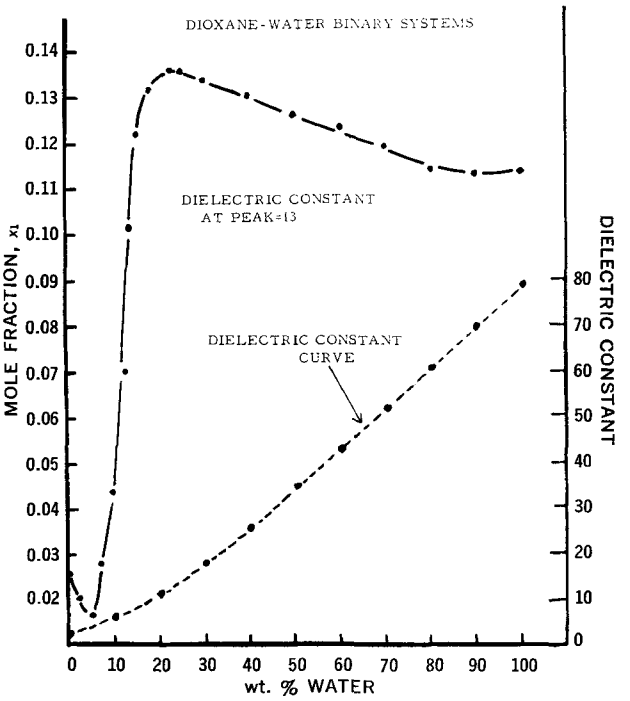

Figure 8—*Mole fraction solubility of sodium salicylate as a function of the percent w/w composition of dioxane–water mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

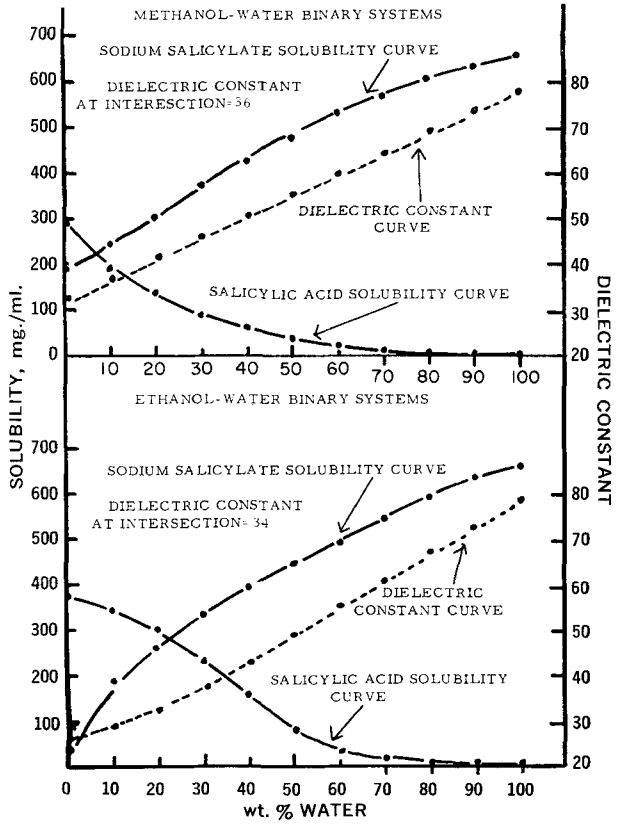

Figure 7—*Solubility of both salicylic acid and sodium salicylate in milligrams/milliliter at 25° for ethanol–water and methanol–water mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

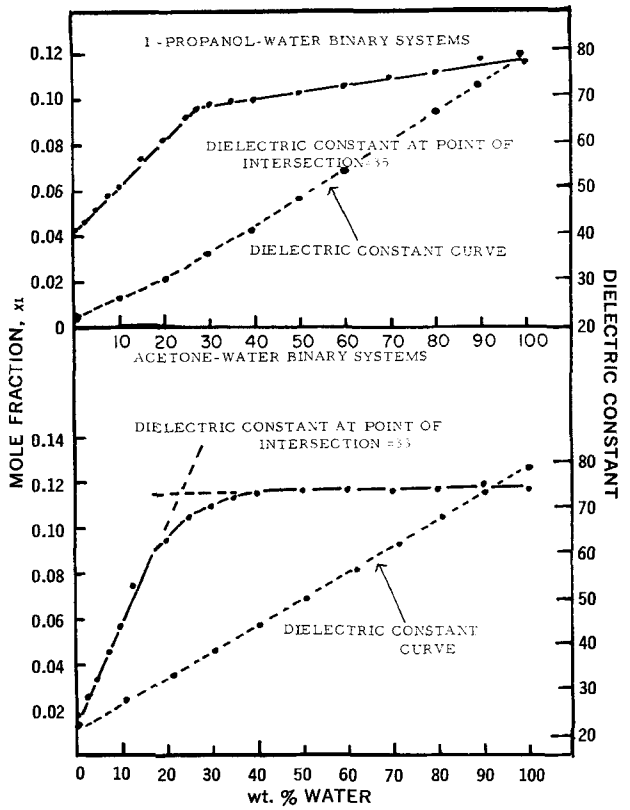

Figure 9—*Mole fraction solubility of sodium salicylate as a function of the percent w/w composition of acetone–water and 1-propanol–water binary mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

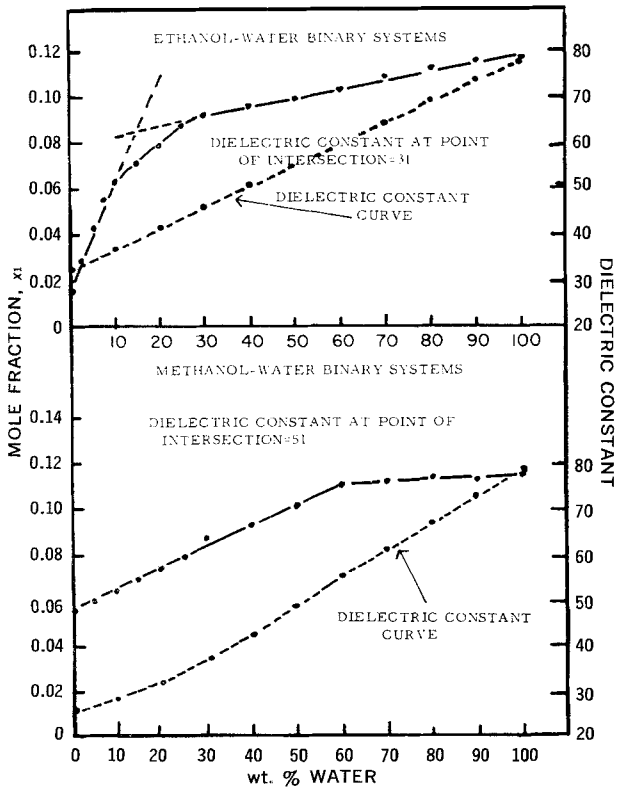

Figure 10—*Mole fraction solubility of sodium salicylate as a function of the percent w/w composition of ethanol–water and methanol–water binary mixtures. The dielectric constants of the binary solvent systems are also shown using the right-hand scale.*

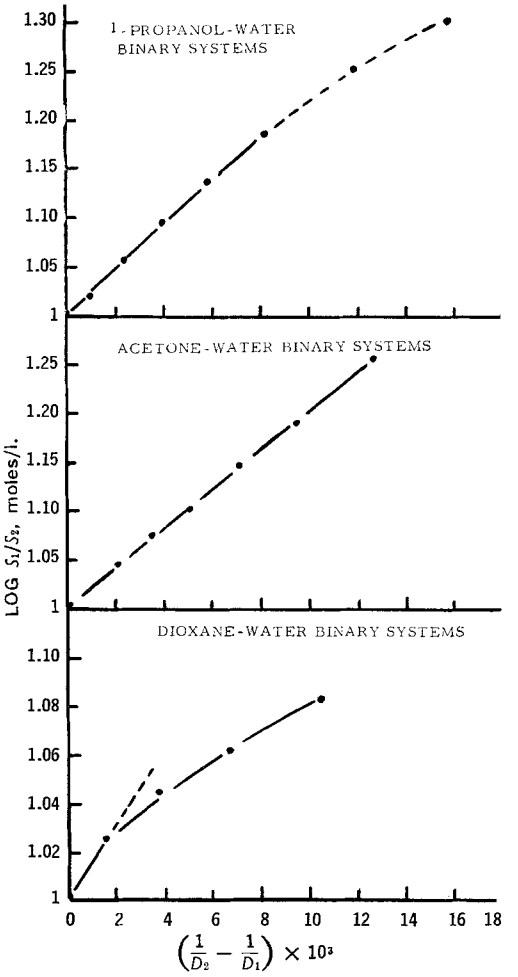

Figure 11—*Logarithm of the ratio of the molar solubility* (S_1/S_2) versus *the differences of the reciprocals of the dielectric constants for the systems shown.*

At the point of intersection, it might be assumed that the inherent polarities of salicylic acid and sodium salicylate are similar in magnitude. When the sodium salicylate values become less than salicylic acid, the sodium salicylate might act as a neutral molecule or nonelectrolyte possessing an inherent polarity below that of salicylic acid. This might be due to the presence of the sodium in the salt.

The solubility of sodium salicylate was also considered from a mole fraction point of view, and the results obtained were plotted in Figs. 8–10. In the case of dioxane, a peak is observed at a dielectric constant value of about 13. For acetone, 1-propanol, ethanol, and methanol systems, two essentially linear portions are evidenced which indicate "breaks" at dielectric constant values at 33, 35, 31, and 51, respectively. Further, the mole fraction solubility of sodium salicylate is essentially invarient over the range of 40% w/w water to pure water in acetone–water systems and from 60% w/w water to pure water in methanol–water systems.

Born (2) and Larson and Hunt (4) developed a theory which relates the solubility of a weakly ionized, relatively insoluble salt to the dielectric constant. This theory can be further broadened to relate the ratio of solubilities for a solute in two solvents to the differences of the reciprocals of the dielectric constant, as given in Eq. 1:

$$\log \frac{S_1}{S_2} = \frac{e^2}{(2.303)2rkT}\left(\frac{1}{D_2} - \frac{1}{D_1}\right) \qquad \text{(Eq. 1)}$$

where S_1 and S_2 are the molar solubilities at the respective dielectric constants D_1 and D_2. The charge on an electron e is taken in e.s.u., r is an effective ion radius, k is Boltzman's constant, and T is the absolute temperature. Obviously, a straight line having a slope of $0.4343e^2/2rkT$ should be observed when the log S_1/S_2 is plotted *versus* $(1/D_2 - 1/D_1)$. A theoretical slope can be calculated by assuming an average ion radius of 2.6 A in the following manner. By inspection, the slope has no units when r is used in centimeters:

$$\text{slope} = \frac{e^2}{(2.303)2rkt} \qquad \text{(Eq. 2)}$$

$$\text{slope} = \frac{(4.8 \times 10^{-10} \text{ stat coulomb})^2}{(4.606)\,(2.6 \times 10^{-8} \text{ cm.})(298 \text{ deg.})(1.38 \times 10^{-16} \text{ ergs/deg.}} \qquad \text{(Eq. 3)}$$

By converting stat coulomb to dyne-centimeters, squaring, and collecting constants:

$$\text{slope} = \frac{23 \times 10^{-20} \text{ dyne-cm.}^2}{4930 \times 10^{-24} \text{ cm.-ergs}} \qquad \text{(Eq. 4)}$$

Table III—Elements Generated from the IBM 360/50 Least-Squares Program and the Ranges of Concentration and Dielectric Constants for the Systems Given

System	Slope	*Y*-Intercept	Correlation Coefficient	Range	
				Concentration	Dielectric Constants
Acetone–water	19.6	0.004	0.9994	30–100% water	39.3–78.5
1-Propanol–water	21.5	0.002	0.9957	40–100% water	35.0–78.5
Ethanol–water	21.3	0.002	0.9982	30–100% water	37.5–78.5
Methanol–water	25.5	0.001	0.9992	30–100% water	46.4–78.5

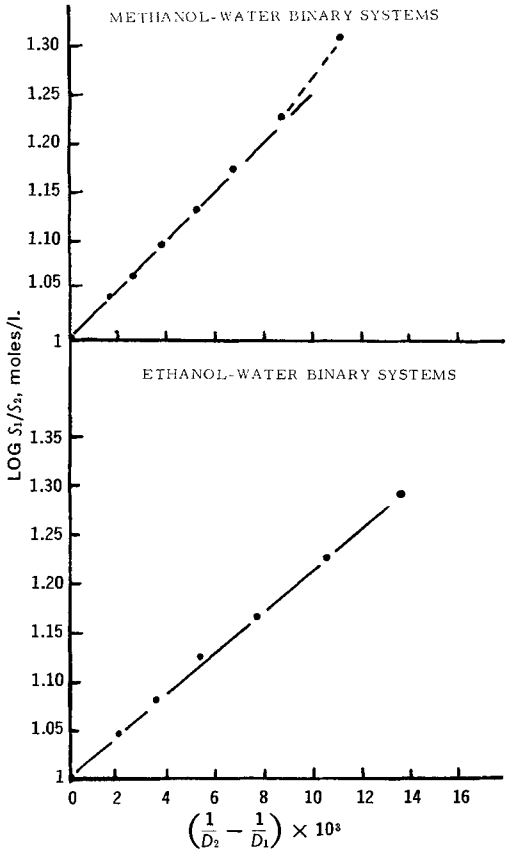

Figure 12—*Logarithm of the ratio of the molar solubility* (S_1/S_2) versus *the differences of the reciprocals of the dielectric constants for ethanol–water and methanol–water binary mixtures.*

Since an erg is a dyne-centimeter,

$$\text{slope} = \frac{23 \times 10^{-20}\ \text{dyne-cm.}^2}{0.493 \times 10^{-20}\ \text{dyne-cm.}^2} = 47.2 \qquad \text{(Eq. 5)}$$

Thus, the sodium salicylate systems were treated in this manner and the data were plotted as shown in Figs. 11 and 12. It is rather amazing that linearity is observed for the acetone, 1-propanol, ethanol, and methanol systems from about 30–40% w/w water up to pure water while dioxane–water systems show only limited linearity. Sodium salicylate possesses solubility in the range of about 1.0–4.1 *M* in these systems, and its adherence to the linearity in the Born equation is interesting.

The slopes of these lines were handled by a least-squares method programmed on an IBM 360/50 digital computer; the characteristics are shown in Table III. From the slopes generated from the experimental data, the average radius *r* is calculated to be about 5.6 Å, which is about two times too large for the average ion radius. The value of the average ion radius was calculated from the ionic size of sodium possessing a water number of 4 and the salicylate anion from molecular models with a monomolecular sheath of water molecules surrounding the polar functions. The hydrated sodium ion was calculated to be about 2.4 Å and the hydrated salicyl anion about 2.8 Å or an average value of 2.6 Å. The results are summarized in Table IV. With this value of *r* in the Born equation, the theoretical slopes are about two times too great, having a value of about 47.

Table IV—Average Ionic Radius from the Born Equation (Theoretical) and the Calculated Average Ionic Radius

System	*A*, Calculated Radius	*B*, Theoretical Radius	Ratio *A*/*B*
Acetone–water	6.2 Å	2.6 Å	2.4
1-Propanol–water	5.7 Å	2.6 Å	2.2
Ethanol–water	5.7 Å	2.6 Å	2.2
Methanol–water	4.8 Å	2.6 Å	1.9

Although the activity factor of Debye–Huckel can be included as an extension of the Born equation, that being the mean ionic activity coefficient, log $\gamma \pm$, the value of the coefficient of the ionic strength varies from about −0.51 in water to −2.98 in alcohols and −3.83 in acetone; no improvement was noted in the ionic size in the Born relationship in systems with high water concentration.

The actual effect of the Debye–Huckel correction is probably small and approximately cancels each other out in the solubility term of the Born equation. It has been stated (5, 6) that the "mean activity coefficient in a saturated solution is almost independent of the dielectric constant of the media."

While this situation may be expected for relatively insoluble compounds, there may be a rather substantial effect for highly soluble substances such as sodium salicylate.

At this point, the rather large deviation between calculated and theoretical ionic size cannot be resolved.

CONCLUSIONS

1. An alcohol solvent scan of the solubility of sodium salicylate indicates a dielectric requirement of 14, which is the maximum solubility at a particular dielectric constant value, remarkably similar to that observed for salicylic acid.
2. The solubility of sodium salicylate in mixed systems of varying dielectric constants shows a fair parallelism over a wide range of concentration of the nonpolar solvent added. This indicates general adherence to the coulomb relationship or the work required to separate a charge.
3. The solubility curves observed are considered to be repressive dissociation effects, and general correlation is found at a dielectric constant value of 30 where uni-univalent electrolytes may be completely associated. Mole fraction solubility plots, being essentially two linear curves, also support this postulation in several systems.

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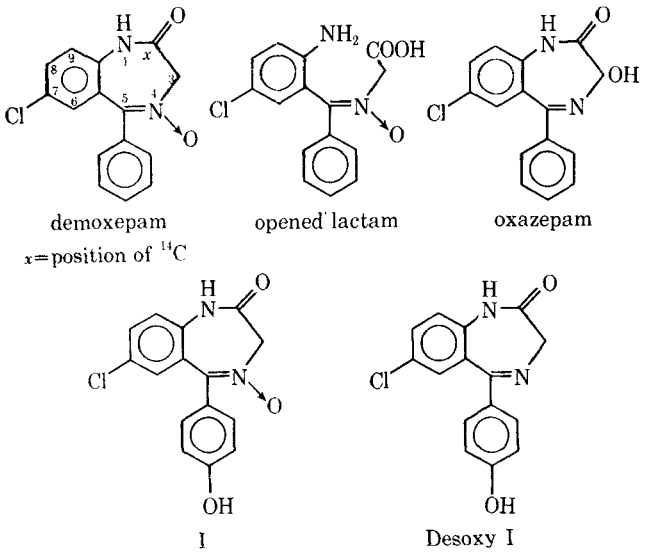
Metabolism of Demoxepam, a Chlordiazepoxide Metabolite, in the Dog

MORTON A. SCHWARTZ, EDWARD POSTMA, and STANLEY J. KOLIS

Abstract □ The pharmacokinetic evaluation of the disposition of intravenous and oral doses of demoxepam, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, in the dog revealed that: (*a*) the disposition was adequately described by a two-compartment open-system model; (*b*) oral demoxepam was apparently well absorbed; (*c*) the rate of elimination (β) varied between dogs with a half-life of 10–20 hr., but within an individual dog, it did not appear to be appreciably influenced by the route of administration; and (*d*) elimination proceeded primarily by biotransformation, with the excretion of intact drug limited to 10% in the urine and less than 2% in the feces. Pathways of biotransformation were studied with the aid of demoxepam-2-^{14}C. Two oxidative pathways led to the formation of two phenolic metabolites, the 5-(4-hydroxyphenyl) derivative (I) and the 9-hydroxy derivative (II) of demoxepam. Another pathway involved the removal of the *N*-oxide function resulting in the formation of *N*-desoxydemoxepam (III), oxazepam, and the *N*-desoxy phenols, Desoxy I and Desoxy II. Finally, the hydrolysis of demoxepam to *N*-(2-amino-5-chloro-α-phenylbenzylidene)glycine *N*-oxide ("opened lactam") was also demonstrated. The finding of *N*-desoxy metabolites in the feces and the corresponding *N*-oxides in the bile suggested that the site of reduction of the *N*-oxide function was the intestinal tract.

Keyphrases □ Chlordiazepoxide metabolite—demoxepam □ Demoxepam and demoxepam-2-^{14}C—metabolism, dog □ Biotransformation, metabolite formation, excretion—demoxepam □ Metabolites, demoxepam—separation, identification □ TLC—separation, identification, estimation □ Scintillometry—analysis □ NMR—structure □ Mass spectrometry—structure

Demoxepam (formerly designated Ro 5-2092 or "lactam"), 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, was first shown to be a plasma metabolite of chlordiazepoxide[1] in man and dog by Koechlin and D'Arconte (1). In further studies, Koechlin *et al.* (2) confirmed this finding and presented evidence that the urinary metabolites of ^{14}C-chlordiazepoxide in man included demoxepam, *N*-(2-amino-5-chloro-α-phenylbenzylidene)glycine *N*-oxide ("opened lactam") and an unidentified derivative of either demoxepam or opened lactam. While the urinary metabolites of ^{14}C-chlordiazepoxide in the dog were not subjected to detailed investigation, they appeared on paper chromatography to be the same as those in man.



In addition to opened lactam, three more chlordiazepoxide metabolites had to be considered as possible metabolites of demoxepam. One was oxazepam[2], which was reported by Kimmel and Walkenstein (3) to be excreted in very small amounts (1% of the dose in the urine and in the feces) by a dog given oral ^{14}C-chlordiazepoxide. The other two were phenols, I and Desoxy I[3], which were found (4) to be metabolites of chlordiazepoxide in the rat. The structures of these compounds and of the labeled demoxepam used in the present study are shown.

The current experiments were designed to gain pharmacokinetic information on the disposition of demoxepam in the dog in terms of absorption, distribution, and elimination. Unlabeled demoxepam was used for this purpose, and a small portion of this pharmacokinetic data was included in the study of chlordiazepoxide disposition in the dog (5). A second objective was to elucidate the biotransformation of demoxepam in the dog. For this purpose, demoxepam-2-^{14}C was employed.

EXPERIMENTAL

Experimental Design—Three apparently normal, nonfasted, male dogs weighing 8–11 kg. were administered intravenous and oral doses of unlabeled demoxepam. At least 1 month elapsed before a dog was redosed with the drug. Dogs 1 and 2 received oral and intravenous doses of 10 mg./kg., while Dog 3 received a 5-mg./kg. i.v. dose. Demoxepam was dissolved in propylene glycol for intravenous injection and was weighed into a gelatin capsule for oral administration. At zero time and at frequent intervals after drug administration, 10 ml. of heparinized blood was drawn and centrifuged, and the separated plasma was stored frozen until analyzed.

Dog 3 also received oral and i.v. 5-mg./kg. doses of ^{14}C-demoxepam (specific activity of 1.57 μc./mg.). This experiment is designated Dog 3-^{14}C to denote the administration of the labeled compound. In addition to frequent blood sampling, a complete collection of urine and feces was made until the excretion of ^{14}C was no longer measurable. Urine collection intervals were 0–2, 2–5, 5–12, 12–24, 24–30, and 30–48 hr. and daily thereafter, while feces were collected daily. Feces were homogenized in 50% ethanol prior to counting.

[1] Chlordiazepoxide hydrochloride, 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide hydrochloride, is the active ingredient in Librium; Hoffmann–La Roche Inc., Nutley, N. J.

[2] Oxazepam, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one, is the active ingredient in Serax; Wyeth Labs., Inc., Radnor, Pa.

[3] I is 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2*H*-1,4-benzodiazepin-2-one 4-oxide. Desoxy I differs from I only in that the 4-oxide (*N*-oxide) is absent.

An additional dog, Dog 4, was anesthetized with pentobarbital sodium[4] and used in a study of the biliary excretion of drug and metabolites. Prior to ^{14}C-demoxepam administration, the cystic duct was ligated and the bile duct was cannulated. Additional ligatures were placed at the pylorus and at the juncture of the small and large intestine. ^{14}C-Demoxepam (specific activity of 0.62 μc./mg.) in 0.1 *N* NaOH was injected intravenously at a dose of 5 mg./kg. The drug was injected within 3 min. after solution so as to prevent alkaline hydrolysis. Thirty-minute collections of bile were made throughout the 5-hr. experiment; at the end, urine was collected from the bladder.

Determination of Plasma Demoxepam—The fluorometric method of Koechlin and D'Arconte (1) was used to determine plasma levels of demoxepam after the administration of demoxepam or ^{14}C-demoxepam. The specificity of this assay was confirmed on finding that the phenolic derivative of demoxepam, Compound I, gave no fluorescence under the conditions of the analysis.

Labeled Demoxepam and Counting Techniques—Demoxepam-2-^{14}C was synthesized by Kaegi and Bader[5]. Its radiochemical purity was confirmed by TLC with Systems 1 and/or 2 (described later). On counting silica gel segments of the chromatoplate, 99% of the ^{14}C was found to have migrated as authentic demoxepam. Periodic checking of the radiochemical purity revealed that there was no decomposition with time.

All the counting was done in a liquid scintillation spectrometer[6], and the counts were converted to disintegrations per minute (d.p.m.) by the external standard-channels ratio procedure for quench correction. Aliquots of urine (0.2–1.0 ml.) and extracts of plasma, urine, bile, and feces were counted in Phosphor I (6). Aliquots of plasma (0.2–1.0 ml.), bile, homogenates of feces in 50% ethanol, aqueous homogenates of stomach and intestinal contents, and silica gel from chromatoplates were counted as suspensions in Phosphor II (6).

Extraction of Labeled Metabolites—The radioactivity excreted in the urine, feces, and bile was fractionated by extraction with ethyl acetate. The bile samples and the fecal homogenates were diluted 1:5 with water prior to extraction. All pH adjustments were accomplished by the addition of dilute acid or alkali. At each designated pH, two consecutive volumes of ethyl acetate, each equaling the aqueous volume, were shaken with the aqueous phases for approximately 10 min. each. The combined extract was counted and, if sufficient ^{14}C was present, was concentrated and examined by TLC.

Following the initial extraction of each sample at pH 7.0, the aqueous phase was incubated with a commercial preparation of β-glucuronidase and arylsulfatase[7], which was added to a final concentration of 1% (v/v). This incubation at pH 5.5 and 37° for 2–3 hr., which previously yielded maximum hydrolysis of conjugated benzodiazepine metabolites (6), was followed by serial extractions first at pH 7.0 and finally at pH 2.0.

TLC for Metabolite Separation, Identification, and Estimation—Silica gel containing a fluorescent indicator[8] was used for TLC with the following solvent systems: 1, chloroform–acetone–ethanol (80:5:5), 2, ethyl acetate–ethanol (90:10); 3, heptane–chloroform–ethanol–acetic acid (5:5:1:1); 4, heptane–chloroform–ethanol–concentrated ammonia (5:5:2:0.1); 4*a*, solvents of 4 in proportions of 5:5:4.5:0.1; 4*b*, solvents of 4 in proportions of 5:5:1:0.03; 5, isopropanol–concentrated ammonia (20:5); 6, heptane–chloroform–ethanol (5:5:2); 6*a*, solvents of 6 in proportions of 2:2:1; 7, chloroform–ethanol (97:3); 8, chloroform–methylene chloride–ethanol (75:25:5); and 9, heptane–ethyl acetate–ethanol (40:80:10).

The procedure utilizing TLC for metabolite identification and estimation was essentially the same as that used previously (6) in the investigation of the deconjugated urinary metabolites of ^{3}H-diazepam. In brief, identification consisted of demonstrating that, on two-dimensional TLC with two different solvent system pairs, a consistent amount of extracted ^{14}C migrated as a known compound added as an internal standard. Also obtainable from such consistent data was quantitation of labeled metabolite. The reference compounds were located by viewing the chromatoplate under shortwave UV light, and the silica gel containing each reference compound was counted as a suspension. The solvent system pairs were designated by the number of each system in the order used; for example, System 1–4 signifies that the plate was run in System 1 and then, after a turn of 90°, in System 4.

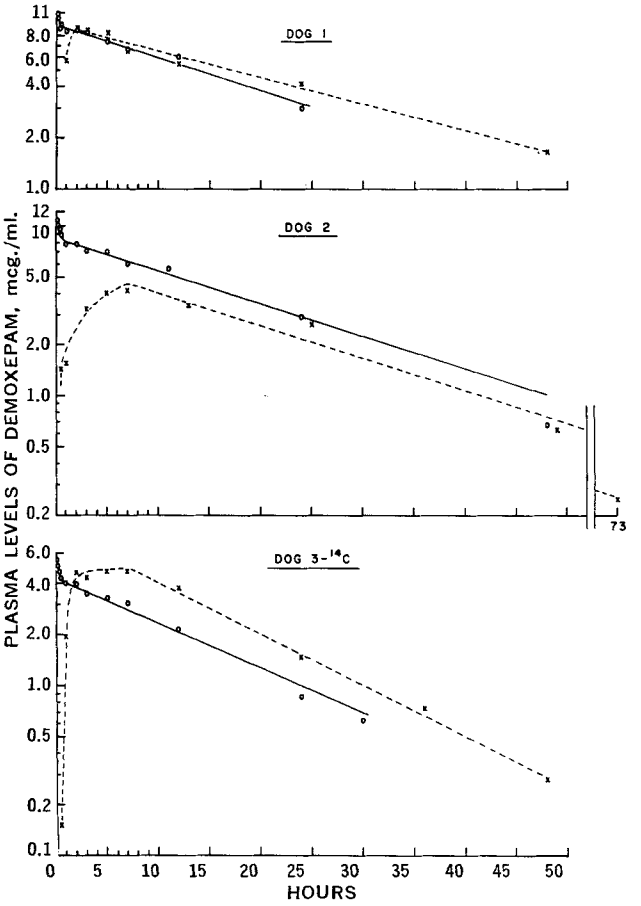


Figure 1—*Semilogarithmic plot of the plasma levels of demoxepam after oral and intravenous drug administration to three dogs. Dogs 1 and 2 received unlabeled doses of 10 mg./kg., and Dog 3-^{14}C received 5 mg./kg. of ^{14}C-demoxepam. Key: O—O, intravenous drug; and X- - -X, oral drug.*

Estimation of Urinary Opened Lactam—To facilitate the development of an assay, ^{14}C-opened lactam was synthesized from labeled demoxepam by an alkaline hydrolysis procedure similar to those previously described (7, 8). The crystallized product melted at 148–152° and was radiochemically pure by TLC.

Human control urine containing 20 mcg./ml. and 5 mcg./ml. of this ^{14}C-opened lactam was extracted with ethyl acetate at pH 7.0 and 2.0. While less than 5% of the ^{14}C was removed at pH 7.0, 93% was extracted at the acidic pH. To produce clearly defined spots on TLC, the extracted opened lactam was converted into its methyl ester (7). To accomplish this, the acidic extract was dried under nitrogen; the resulting oil, dissolved in 2 ml. of ethanol, was mixed with 2 ml. of ethereal diazomethane. After standing 15 min., the solution was evaporated to dryness and the residue dissolved in 0.2 ml. of ethanol. The methyl ester, separated by TLC in Systems 7 and 8, was located by spraying with the Bratton–Marshall reagents (9) and was scraped from the plate and counted. Recovery of opened lactam was approximately 50% by this procedure.

The assay of ^{14}C-opened lactam consisted of adding carrier opened lactam to the urine, which was extracted first at pH 7.0 and then at pH 2.0 with two equal volumes of ethyl acetate. The latter combined extract was concentrated, treated with diazomethane, and chromatographed. The ^{14}C migrating as opened lactam methyl ester was determined. A correction was made for incomplete recovery by the concomitant analysis of control urine to which ^{14}C-opened lactam was added at a concentration equaling that of carrier opened lactam added to the experimental urine.

[4] Nembutal.
[5] This synthesis by H. H. Kaegi and G. Bader has not as yet been published.
[6] Model 3380, Packard Instrument Co., Downers Grove, Ill.
[7] Glusulase, Endo Labs., Garden City, N. Y.
[8] SilicAR 7-GF-5, Mallinckrodt Chemical Works, St. Louis, Mo.

Table I—Pharmacokinetic Evaluation of Demoxepam Disposition in the Dog

Parameter	Parameter Value—Dog 1	Dog 2[a]	Dog 3-^{14}C	Dog 3
Dose (i.v. and oral), mg./kg.	10	10	5	5
Intravenous Demoxepam				
Dog wt., kg.	8.4	9.5	9.5	7.6
A, mcg./ml.	3.4	3.1	1.7	2.1
B, mcg./ml.	9.2	8.4	4.3	3.6
α, hr.$^{-1}$	8.35	3.4	4.6	4.2
α, half-life, hr.	0.083	0.20	0.15	0.17
β, hr.$^{-1}$	0.043	0.044	0.061	0.050
β, half-life, hr.	16	16	11	14
Rate constants of the two-compartment model				
k_{12}, hr.$^{-1}$	2.21	0.89	1.25	1.48
k_{21}, hr.$^{-1}$	6.12	2.48	3.34	2.69
k_{el}, hr.$^{-1}$	0.059	0.060	0.084	0.078
Compartment distribution of demoxepam				
V_p[b], % of body wt.	80	87	83	88
$F_c \times 100$[c]	73	73	73	64
Oral Demoxepam				
Dog wt., kg.	8.3	10.7	10.1	Not done
Peak plasma level, mcg./ml.	8.6	4.1	4.6	
Apparent elimination rate (β), hr.$^{-1}$	0.035	0.044	0.069	
Half-life of above, hr.	20	16	10	
Percent of dose absorbed[d]	99	70	134	

[a] The intravenous data of Dog 2 were previously reported (Dog 1 of *Reference 5*). [b] V_p in percent of body wt. = 100 × dose (mg./kg.)/A + B (mg./l.). [c] This is the percent of total body drug present in the central compartment during the elimination phase. Its calculation is described in the text. [d] Absorption was estimated from the areas under the plasma level curves; % absorption = 100 × oral dose area/i.v. dose area.

Further Metabolite Identification Procedures—The use of TLC for metabolite isolation and high-resolution mass spectrometry and/or NMR spectroscopy for identification was previously described (4, 10)[9].

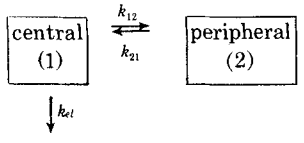
RESULTS

Pharmacokinetic Analysis of Demoxepam Disposition—The plasma levels of drug after intravenous administration exhibited a biexponential decline (Fig. 1). By means of an 'N-LIN' computer program (5, 11), all intravenous plasma level data were fitted to the biexponential equation

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where C_t is the plasma level at time t, A is the zero-time intercept and α the rate constant of the fast disposition rate, and B and β are the corresponding parameters of the slow disposition rate. This equation may also be derived from the two-compartment open system (12) in which k_{12} is the rate of transfer from the central to peripheral compartment, k_{21} is the reverse transfer rate, and k_{el} is the rate at which the drug is eliminated from the central compartment (Scheme I).

The values for the parameters of Eq. 1 were used in the calculation, described by Riegelman *et al.* (12), of the transfer rates and elimination rate of the model. All results obtained from pharmacokinetic analysis of the plasma level data are shown in Table I. The half-life of the fast disposition rate (α) ranged from 0.1–0.2 hr., indicating a very rapid transfer of a portion of the drug to the peripheral compartment. The elimination rate (β) was markedly slower, with a half-life ranging from 11–16 hr. This rapid distribution and slow elimination were also reflected by the difference in magnitude of k_{12} and k_{el}. The relatively large central compartment volume (V_p), which ranged from 80–88% of the body weight, indicated uptake of demoxepam by readily accessible tissues. Nagashima *et al.* (13) showed that the fraction of total body drug present in the central compartment (F_c) during the elimination phase remains constant and is equal to β/k_{el}. From this relationship, 64–73% of the body demoxepam was calculated to be present in the central compartment during the elimination phase (Table I). The peripheral compartment, therefore, was smaller in that it contained 36–27% of the drug. It should be noted that Dogs 3 and 3-^{14}C, which were essentially duplicate experiments in the same animal, yielded results that indicated fairly good reproducibility.



Scheme I

Table II—Plasma Levels of Demoxepam in Dog 3-^{14}C by *Fluorometric* and Radiometric Assay

Hours	Intravenous 5-mg./kg. Dose—Plasma Level by Radio. Assay[a], mcg. equiv./ml.	Intravenous 5-mg./kg. Dose—Ratio of Fluoro.[b] to Radio. Assay	Oral 5-mg./kg. Dose—Plasma Level by Radio. Assay[a], mcg. equiv./ml.	Oral 5-mg./kg. Dose—Ratio of Fluoro.[b] to Radio. Assay
0.083	5.92	0.92	No sample	
0.17	5.27	0.96	Nil	
0.33	4.90	0.94	No sample	
0.50	4.75	0.91	0.13	1.15
1	4.47	0.89	1.70	1.14
2	4.31	0.90	4.49	1.02
3	3.90	0.89	4.57	0.94
5	3.83	0.84	5.03	0.94
7	3.69	0.82	5.16	0.92
12	2.53	0.83	4.20	0.89
24	1.09	0.79	1.78	0.83
30	0.79	0.80	No sample	
36	No sample		0.80	0.91
48	0.11	—[c]	0.35	0.80
72	0.02	—[c]	0.06	—[c]

[a] The d.p.m./ml. of plasma were divided by the specific activity of administered ^{14}C-demoxepam to obtain mcg. equivalents of drug/ml. [b] The plasma levels obtained by fluorometric assay were shown in Fig. 1. [c] Fluorometric assay yielded no measurable levels.

Data obtained from the oral administration of demoxepam are also shown in Fig. 1 and Table I. Although Dogs 1 and 2 both received 10 mg./kg., the peak plasma level of demoxepam in Dog 1 was about twice that of Dog 2. This appears to be related to the faster rate of absorption of drug by Dog 1 (Fig. 1). In addition, a difference in the extent of absorption was also evident. Absorption, estimated from a comparison of the areas under the plasma level curves (*per os* area/intravenous area), was found to be virtually complete in Dog 1 and 70% in Dog 2 (Table I). In Dog 3-^{14}C, however, the absorption of labeled demoxepam was calculated to be 134%. A comparison of the areas under the plasma level curves obtained after intravenous administration of nonlabeled and labeled drug to this dog (Dog 3 and Dog 3-^{14}C) revealed that these areas were almost identical, and, therefore, reproducible. Apparently, the plasma levels found after oral dosing of Dog 3-^{14}C were markedly higher than would be expected for complete absorption. Although no explanation for this anomalous result can be given at the present time, other evidence (presented later) does indicate that absorption was complete. It is further seen from Table I that the rate of elimination, β, in each dog was virtually independent of the route of administration of demoxepam.

Fate of ^{14}C-Demoxepam—Table II gives the plasma levels of ^{14}C-demoxepam determined radiometrically in Dog 3-^{14}C and compares them with those shown in Fig. 1 which were determined fluorometrically. The ratio shown is a measure of how much plasma ^{14}C is accounted for as demoxepam. After both routes of administration, the ratios are close to 1.0 at the early times and show a tendency toward lower values with time. This finding indicates that essentially all the plasma ^{14}C was present as demoxepam

[9] The mass spectra, run on a Consolidated Electrodynamics Corp. 21-110 mass spectrometer, were interpreted by Dr. W. Benz. The NMR spectra, obtained with a Varian HA-100 spectrometer using acetone-d_6 as the solvent and internal tetramethylsilane as a reference, were interpreted by Mr. R. Pitcher.

Table III—Excretion of Radioactivity by Dog 3-^{14}C following 5-mg./kg. Oral and Intravenous Doses of ^{14}C-Demoxepam

Day	Urinary Excretion, % of Dose	Fecal Excretion, % of Dose	Total Excretion, % of Dose
	Oral Dose		
1	27.7	24.3	52.0
2	12.2	15.3	27.5
3	2.3	5.6	7.9
4	0.4	0.9	1.3
5	0.1	—[a]	0.1
	42.7	46.1	88.8
	Intravenous Dose		
1	27.5	26.6	54.1
2	10.3	16.3	26.6
3	0.9	2.2	3.1
4	0.1	0.2	0.3
5	Nil	Nil	Nil
	38.8	45.3	84.1

[a] Feces were not collected.

in the first few hours and that a slight amount of labeled metabolite(s) entered the plasma with time.

The total excretion of fecal radioactivity was greater than that of urinary radioactivity after both routes of administration (Table III). Moreover, the magnitude of the daily excretion of ^{14}C in urine and feces was not materially influenced by whether labeled drug was given orally or intravenously. This finding suggests that ^{14}C-demoxepam was completely absorbed (but does not explain the 134% absorption shown in Table I).

The nature of the urinary radioactivity was investigated by solvent extraction and TLC. As shown in Table IV, the percent of urinary ^{14}C extracted before and after glusulase treatment was not influenced significantly by the route of drug administration. Sizable amounts of ^{14}C were extracted at pH 7.0 (pre- and post-glusulase) with very little ^{14}C extracted at pH 2.0. After both routes of administration, roughly two-thirds of the directly extractable radioactivity was intact demoxepam and 12–13% was Metabolite I. Following deconjugation with glusulase, about one-half of the extracted metabolites was I, 6–7% was Desoxy I, and 3–4% was oxazepam. The validity of these estimates is supported by the good agreement obtained with both solvent system pairs.

Urinary opened lactam was also determined, even though the excretion of acidic metabolites was relatively slight. This metabolite accounted for 2.1% of the urinary ^{14}C excreted in 48 hr. after intravenous drug and 1.7% of that excreted following oral drug.

Table V—Urinary Excretion of Labeled Demoxepam and Metabolites by Dog 3-^{14}C Given Oral and Intravenous 5-mg./kg. Doses of ^{14}C-Demoxepam[a]

Compound Form[b]	Urinary Excretion in Percent of Dose: Demoxepam	I	Desoxy I	Oxazepam	Opened Lactam	Unknown
After intravenous drug:						
Nonconjugated	9.3	1.8	Nil	Nil		2.9
Conjugated	0.2	8.2	1.1	0.5		7.2
Acidic					0.6	1.9
Unextracted						4.2
After oral drug:						
Nonconjugated	9.6	1.7	Nil	Nil		2.7
Conjugated	0.3	7.7	0.8	0.6		5.7[c]
Acidic					0.8	2.2
Unextracted						7.9

[a] The excretion of drug and metabolites in the first 48 hr. is presented. The radioactivity excreted in this period is shown in Table III and represents the excretion of over 90% of the total amount of ^{14}C excreted in the urine after both intravenous and oral drug. [b] The nonconjugated compounds were those extractable directly at pH 7.0, the conjugated metabolites were those extracted at this pH after glusulase treatment, and the acidic metabolites were those extracted at pH 2. [c] About one-third of this radioactivity, or 2% of the dose, was subsequently identified (see text) as Metabolite II.

Table V summarizes the urinary excretion of demoxepam and metabolites. After both routes of administration, the following is evident: (*a*) 10% of the dose was excreted as intact drug (the 0.2–0.3% found in the conjugated fraction probably represented demoxepam that was not removed in the prior extraction); (*b*) Metabolite I was the major metabolite (9–10% of the dose) and was excreted predominantly as a conjugate; (*c*) very small amounts of conjugated Desoxy I, oxazepam, and opened lactam were excreted; and (*d*) approximately 17% of the dose was excreted as unknown metabolites. The lack of influence of the route of administration on the magnitude of excretion of drug and metabolites further supports the contention that demoxepam was completely absorbed by Dog 3-^{14}C.

That portion of the unknown urinary radioactivity excreted as conjugates was further investigated. Starting with 85 ml. of a pool of the 0–48-hr. urine excreted after oral ^{14}C-demoxepam, 37.5% of the radioactivity was extracted after glusulase treatment; 15% of this ^{14}C migrated between demoxepam (R_f 0.57) and Desoxy I

Table IV—Extractability of the Urinary Radioactivity and Estimation of the Extracted Metabolites by Two-Dimensional TLC

Extract[a]	Percent of Urinary ^{14}C Extracted	System	Composition of the Extracted Radioactivity[b]: Demoxepam, %	I, %	Desoxy I, %	Oxazepam, %	Unaccounted for, %
			After Intravenous Drug				
Preglusulase pH 7, EtAc	37.1	1–5	66.5	12.9	0.6	1.2	18.8
		1–6	66.0	12.5	1.5	0.7	19.3
			66.3	12.7	N.S.	N.S.	19.1
Postglusulase pH 7, EtAc	45.4	1–5	1.1	51.0	5.0	3.0	39.9
		1–6	0.8	44.9	8.1	2.5	43.7
			N.S.	48.0	6.6	2.8	41.8
pH 2, EtAc	6.2						
			After Oral Drug				
Preglusulase pH 7, EtAc	35.2	1–3	66.3	11.9	<0.6	<0.6	21.8
		1–4	70.5	12.0	0.7	<0.6	16.8
			68.4	12.0	N.S.	N.S.	19.3
Postglusulase pH 7, EtAc	37.9	1–3	1.9	52.5	5.5	4.3	35.8
		1–4	1.9	49.1	5.7	3.4	39.9
			N.S.	50.8	5.6	3.9	37.9
pH 2, EtAc	6.8						

[a] A pool of 0–48-hr. urine excreted by Dog 3-^{14}C after intravenous or oral ^{14}C-demoxepam was extracted serially as shown in the table. EtAc = ethyl acetate. [b] The percent of extracted ^{14}C migrating with the designated internal standard is shown for each solvent system, and the average value is shown below the line. N.S. signifies that less than 2% of the ^{14}C migrated as an internal standard and the results are not considered to be significant.

Table VI—Extractability of the Fecal Radioactivity[a] of a Dog Given ^{14}C-Demoxepam Orally and Estimation of the Directly Extractable Metabolites by Two-Dimensional TLC

Extract	Percent of Fecal ^{14}C Extracted	System	Composition of the Extracted Radioactivity[b] Demoxepam, %	I, %	Desoxy I, %	Oxazepam, %	Unaccounted for, %
Preglusulase							
pH 7, EtAc	44	1–4*b*	9.6	7.4	20.6	3.4	59.0
		1–3	10.6	7.3	20.4	3.2	58.5
			10.1	7.4	20.5	3.3	58.7
			(1.1)[c]	(0.8)[c]	(2.2)[c]	(0.4)[c]	(6.3)[c]
Postglusulase							
pH 7, EtAc	9.0						
pH 2, EtAc	3.0						
Aqueous remaining	40						

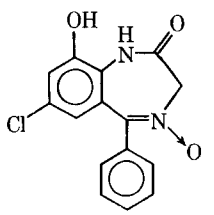
[a] An aliquot of the 1st day's feces representing the excretion of 24.3% of the dose was extracted. [b] The percent of extracted ^{14}C migrating with the designated internal standard is shown for each solvent system, and the average value is shown below the line. [c] Excretion in terms of the percent of dose administered is shown in parenthesis.

(R_f 0.36) with System 1, while 19% barely moved off the origin with System 4. From these results, it was estimated that this new metabolite comprised 6% of the urinary ^{14}C (37.5% × 17%) or roughly 2% of the dose. This is about one-third of the 5.7% of the unidentified conjugated radioactivity (Table V).

The new metabolite, purified by TLC in System 1 and rechromatographed in System 4, was analyzed by high-resolution mass spectrometry. Its empirical formula was found to differ from that of demoxepam ($C_{15}H_{11}ClN_2O_2$) by the addition of one oxygen atom. The location of this oxygen atom, however, could not be determined from the mass spectrum.

A definitive structure was obtained from a greater amount of this metabolite which was isolated in a second experiment. The extract, obtained after glusulase treatment of pooled urine which contained 6.9 × 10^7 d.p.m. (20 mg. equivalents of ^{14}C-demoxepam), was first evaporated to dryness. Then the residue was dissolved in ether–ethanol (10:1) which was extracted with an equal volume of 0.1 *N* NaOH. The alkaline phase, after adjustment to pH 7.0, was then extracted twice with ethyl acetate. This preliminary purification, based on the known ability of alkali to remove demoxepam from ether (1), was required to separate the metabolite from urinary constituents that would interfere in subsequent TLC. The final combined ethyl acetate extract, dried over anhydrous sodium sulfate, was concentrated and subsequently chromatographed with System 4*a*. The metabolite, eluted with ethanol followed by methanol, was rechromatographed with System 1. It was eluted this time with 0.1 *N* NaOH which was adjusted to pH 7.0 and extracted with ethyl acetate. The residue resulting from evaporation of the solvent was dissolved in chloroform and filtered through a cotton plug. The filtrate was again dried, and 0.65 mg. of purified metabolite was dissolved in acetone-d_6 for NMR spectroscopy.

A single scan showed the CH_2 at δ 4.71 and indicated that the oxygen was not added at the C-3 position of demoxepam. On time averaging for 144 scans, an *AB* pattern was obtained in the aromatic region (δ 6.52 and δ 7.08), with a typical *meta*-coupling of 2.5 Hz. This clearly showed that substitution had occurred at the C-9 position and that the metabolite was II, 7-chloro-1,3-dihydro-9-

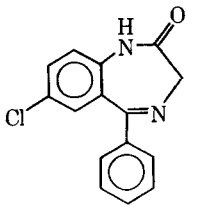


II

hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide. Confirmation of structure was obtained following the synthesis of II; the isolated and synthetic compounds were identical on examination by NMR spectroscopy and TLC.

The nature of the fecal radioactivity was also investigated by solvent extraction and TLC. It is seen in Table VI that, in contrast to the urinary ^{14}C (Table IV), relatively little fecal ^{14}C was excreted as conjugates. TLC of the major fraction revealed that 21% of the ^{14}C directly extractable at pH 7.0 was Desoxy I, 10% was intact demoxepam, 7.4% was I, and 3.3% was oxazepam (Table VI). A striking difference in the urinary and fecal excretion of I and Desoxy I was noted. Metabolite I predominated in the urine with little Desoxy I present (Table V), while in the feces Desoxy I was excreted to a much greater extent than was I. The very low extraction of ^{14}C at pH 2 (Table VI) indicated that no significant fecal excretion of opened lactam occurred.

Further investigation of the directly extractable radioactivity resulted in the identification of two more fecal metabolites. The high-resolution mass spectrum of one, purified by TLC with System 1 (R_f 0.67) followed by rechromatography with System 4*b* (R_f 0.40), clearly showed that the metabolite was III, 7-chloro-1,3-dihydro-5-

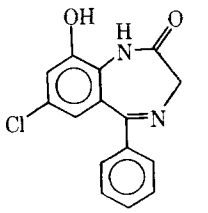


III

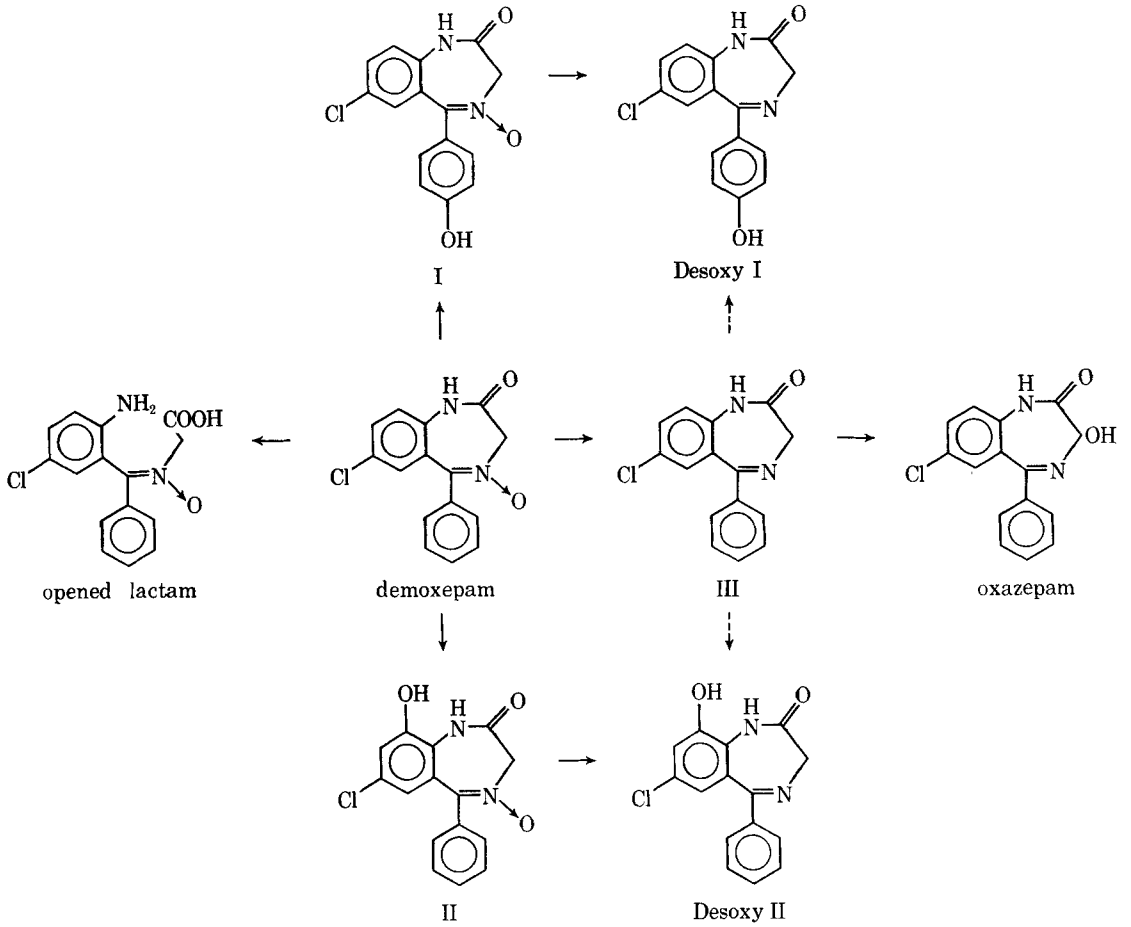
phenyl-2*H*-1,4-benzodiazepin-2-one, which is the *N*-desoxy derivative of demoxepam. Confirmation of structure and an estimate of the fecal excretion of III were obtained by two-dimensional TLC. Cochromatography of an aliquot of the directly extractable radioactivity with authentic III, a known compound (7, 8), revealed that a consistent amount of ^{14}C was recoverable as III. With System 3–4*b*, 17.9% of the radioactivity migrated as III; with System 1–4*b*, 16.0 and 18.6% migrated as III in two separate experiments. From the average distribution (18%), it was estimated that 1.9% of the dose was excreted in the feces on the 1st day as III.

The second metabolite was purified *via* System 1 (R_f 0.63), System 6*a* (R_f 0.65), and System 4*b* (R_f 0.29). The high-resolution mass spectrum showed that the metabolite no longer retained the *N*-oxide function and that it had the same empirical formula as Desoxy I ($C_{15}H_{11}ClN_2O_2$). However, TLC with System 4*b* clearly separated the metabolite and Desoxy I, thereby demonstrating that they were not identical. The subsequent isolation and identification of II as a urinary metabolite suggested that this fecal metabolite, which was an isomer of Desoxy I, might be 7-chloro-1,3-dihydro-9-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one (Desoxy II).

TLC of purified metabolite and authentic Desoxy II with System



Desoxy II



Scheme II

6 revealed that 83% of the ^{14}C migrated as the reference compound. This evidence, together with that from mass spectrometry, was considered sufficient to designate the metabolite as Desoxy II. It was estimated from the recovery of the metabolite on successive chromatographic purification that the amount of Desoxy II excreted in the feces was about one-half that of III, *i.e.*, roughly 1% of the dose. This identification of two new *N*-desoxy metabolites, together with the threefold greater excretion of Desoxy I than I (Table VI), indicated that the *N*-desoxy metabolites predominated in the feces.

The mode of secretion of labeled metabolites into the gastrointestinal (GI) tract and their identity were studied in the bile duct-cannulated Dog 4. Five hours after an intravenous dose, 3.9% of the dose was found in the urine, 4.3% in the bile, less than 0.1% in the stomach contents, and 0.3% in each (small and large) intestinal contents. Most of the radioactivity excreted in the feces after intravenous ^{14}C-demoxepam entered the GI tract *via* the bile.

Table VII shows that the major portion of the biliary ^{14}C could not be extracted with ethyl acetate. However, with respect to the ^{14}C extracted, the major components were demoxepam, conjugated I, and conjugated II. Conjugated oxazepam was also present but the other *N*-desoxy metabolites, Desoxy I, Desoxy II, and III, were not found in significant amounts. Since the latter three metabolites were definitely present in the feces, these results suggest that they were formed in the GI tract from the corresponding *N*-oxides secreted in the bile.

DISCUSSION

The pharmacokinetic data presented in this study support the previously reported (5) conclusion that a two-compartment open-system model adequately describes the disposition of demoxepam in the dog. In addition, evidence was also obtained that oral demoxepam was well absorbed. The rate of demoxepam elimination by an individual dog appeared to be independent of the route of drug administration. However, in different dogs, this rate was quite variable, with a half-life ranging from 10 to 20 hr. in these studies, and as high as 28 hr. in a previous dog (5).

^{14}C-Demoxepam was eliminated by both excretion and biotransformation. Ten percent of the labeled demoxepam administered either orally or intravenously was excreted as intact drug in the urine. Since 45% of the intravenously injected radioactivity was excreted in the feces, secretion of labeled products into the GI tract was evident. In the bile duct-cannulated dog, this radioactivity was shown to enter the GI tract mainly *via* the bile. Furthermore, about 10% of this biliary ^{14}C was found to be present as intact drug. This suggests that an enterohepatic circulation of demoxepam may be in operation in the dog. The absence of appreciable fecal excretion of demoxepam was demonstrated after oral ^{14}C-demoxepam administration. It is estimated that less than 2% of the dose was excreted as intact drug in the feces.

Since the total excretion of intact drug was less than 12% of the dose, demoxepam was eliminated primarily by biotransformation. The postulated metabolic pathways shown in Scheme II involve hydroxylation of each aromatic ring, the complete reduction of the *N*-oxide function, and the hydrolysis of the lactam bond. At least two pathways resulted in phenolic metabolites. *para*-Hydroxylation of the demoxepam 5-phenyl ring was apparently preferred over hydroxylation of the benzodiazepine aromatic ring since 10% of the oral ^{14}C-demoxepam was excreted as urinary I (free and conjugated) and only about 2% was excreted as urinary conjugated II.

The reduction of demoxepam to III in the GI tract was evident from the finding that intact drug, but not III, was detectable in the bile while a small amount of III (roughly 2% of the dose) was excreted in the feces. Although a reexamination of dog urine failed to detect III, the reduction of demoxepam in the tissues cannot be entirely excluded. III, a prominent metabolite of the tranquilizer diazepam[10] in dog and man (6), is not excreted in the urine to any

[10] Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, is the active ingredient in Valium; Hoffmann–La Roche Inc., Nutley, N. J.

Table VII—Extractability of the Biliary Radioactivity[a] and Estimation of Metabolites in a Dog Given a 5-mg./kg. i.v. Dose of ^{14}C-Demoxepam

Extract	Percent of Biliary ^{14}C Extracted	Composition of Extracted Radioactivity[b]								
		System	Demoxepam, %	I, %	Desoxy I, %	II, %	Desoxy II, %	III, %	Oxazepam, %	Unknown, %
Preglusulase										
pH 7, EtAc	10.4	1–4	81.5	2.2	N.M.	N.M.	N.M.	N.M.	N.M.	16.3
Postglusulase										
pH 7, EtAc	17.7	1–4	5.9	29.0	1.7	33.9	1.4	N.M.	4.9	23.2
		1–9	5.3	28.5	1.1	28.8	(1.4)[c]	N.M.	(5.0)[c]	29.9
			5.6	28.8	1.4	31.4	1.4	N.M.	5.0	26.6
pH 2, EtAc	5.1									
Remaining aqueous	64.5									

[a] A 1-ml. aliquot of each bile sample was combined to form a 0–5-hr. pool which was extracted. [b] Metabolites were estimated with the designated two-dimensional solvent system. N.M. signifies that measurable amounts of ^{14}C were not found as the reference compound. The limit of detection was 2% of the radioactivity extracted at pH 7 preglusulase and 1% of that extracted postglusulase. [c] In System 1–9, 6.4% of the extracted ^{14}C migrated as unresolved Desoxy II plus oxazepam. This total was readily separated into the individual values shown in parenthesis by using the amounts found for each on TLC with System 1–4.

appreciable extent by the dog (6, 14). On the other hand, oxazepam, which has been shown (15, 16) to be a metabolite of III, is a major urinary metabolite (conjugated) of diazepam in dog and man (6, 14). In the present study, less than 1% of oral or intravenous ^{14}C-demoxepam was excreted as urinary conjugated oxazepam, thus indicating that relatively little III was metabolized in the tissues to oxazepam.

The deconjugation and reduction of biliary conjugated I and II in the GI tract to yield fecal Desoxy I and Desoxy II were also evident from the difference in composition of biliary and fecal radioactivity. However, the endogenous hydroxylation of III to Desoxy I and Desoxy II cannot be entirely excluded. Therefore, these reactions are included as possible minor (broken arrow) pathways in Scheme II.

The hydrolytic pathway did not appear to be of major significance in the dog, because less than 1% of oral and intravenous labeled demoxepam was excreted as urinary opened lactam. In a previous study (2) of ^{14}C-chlordiazepoxide metabolism in two human subjects, urinary opened lactam accounted for 4 and 8% of the dose. This suggests that the hydrolytic pathway is of greater significance in man.

Evidence that chlordiazepoxide was metabolized to demoxepam by a pathway involving Metabolite DM (Ro 5-0883/1), the *N*-desmethyl derivative of chlordiazepoxide, as an intermediate was previously reported (17). Recently, a pharmacokinetic evaluation of chlordiazepoxide disposition in the dog revealed (5) that the drug was quantitatively biotransformed to Metabolite DM and that up to 50% of this metabolite was converted to demoxepam. These results, together with the conversion of demoxepam to oxazepam shown in the present study, provide a series of metabolic reactions which explain the slight conversion of chlordiazepoxide to oxazepam observed by Kimmel and Walkenstein (3).

Present studies of demoxepam disposition and biotransformation are concerned with determining whether information obtained in the dog is applicable to man.

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Dissolution of Poorly Water-Soluble Drugs II: Excipient Dilution and Force of Compression Effects on Tablets of a Quinazolinone Compound

JEFFREY O. HIRSCHORN and SAUL S. KORNBLUM*

Abstract □ Tablets of three different dilution ratios, comprised of the same excipients with a constant quantity of drug, demonstrated a significant variation of the drug's dissolution behavior. A linear relationship was obtained when plotting $t_{50}\%$ *versus* tablet weight. The relationship appears to have resulted from the fact that the apparent surface area of the ruptured tablet is related to the number of discrete particles formed from disintegration and deaggregation of the tablet. An increase of the compression force for the three tablets (200, 400, and 600 mg.) provided divergent results when comparing the $t_{50}\%$ of each tablet size, and the dissolution behavior appears to be a function of the dilution factor.

Keyphrases □ Dissolution, poorly water-soluble drug—parameters affecting □ Compression force effect—quinazolinone compound, tablet dissolution □ Excipient–drug ratio—tablet dissolution effect □ UV spectrophotometry—analysis

Research reports (1–3) have demonstrated the possible variation of *in vitro* dissolution of drugs when the composition or manufacturing techniques of a dosage form are altered.

Levy *et al.* (4) indicated the significance for both the size and age of granules on the dissolution rate of salicylic acid from compressed tablets. In another report (5), Levy and Gumtow showed where employment of a hydrophobic lubricant substantially interfered with the dissolution rate of salicylic acid tablets.

In another instance, Higuchi *et al.* (6) offered their observations concerning the force of compression as related to *in vitro* dissolution behavior of aspirin. They proposed that the increased dissolution from a greater compression force resulted due to an increase of particle surface area from granule fragmentation.

The authors (7) reported, in a previous paper, that significant variation of dissolution behavior can exist when comparing a drug powder and a compressed tablet of the same. The present communication deals with the effect on dissolution where a constant quantity of drug was diluted with the same excipients to three different tablet weights. The significance of altering the compression force with reference to *in vitro* dissolution was investigated for the tablet sizes considered in this research.

EXPERIMENTAL

Materials—A heterocyclic nitrogen base compound of quinazolinone structure was synthesized in accordance with specific standards of quality control[1]. The drug was twice passed through a laboratory hammer mill[2] equipped with a plate with 1-mm. perforations.

The drug was dissolved in an alcohol–chloroform solvent and subsequently spray dried in the manner outlined previously (7).

[1] Chemical Development Dept., Sandoz Pharmaceuticals, Hanover, N. J.

[2] Raymond Mill Combustion Engineering Co., Chicago, Ill.

Table I—Composition of Tablets Manufactured by Direct Compaction from Spray-Dried Quinazolinone Compound

Materials	Tablet Weight, mg. 200	400	600
Quinazolinone compound	50.0 mg.	50.0 mg.	50.0 mg.
Lactose USP, spray-dried	128.0 mg.	306.0 mg.	484.0 mg.
Microcrystalline cellulose[a]	20.0 mg.	40.0 mg.	60.0 mg.
Stearic acid powder USP	2.0 mg.	4.0 mg.	6.0 mg.

[a] Avicel, FMC Corp., Marcus Hook, Pa.

The spherical, amorphous drug particles of spray-dried drug were microscopically determined to fall into a diameter range from 1–15 μ, and the specific surface area was determined by the Brunauer, Emmett, and Teller technique to be 1.37 $m.^2/g.$

Direct Compaction Tablet Preparation Employing Spray-Dried Quinazolinone Compound—Compressed tablets were prepared by direct compaction in a hydraulic press equipped with a gauge[3] measuring forces from 0 to 5000 lb. and calibrated in 100-lb. increments. A special steel holder held the bottom punch and die in a fixed position during compression. The specific powder formulation was individually weighed and transferred into the die cavity; the top punch was then gently inserted into the die cavity, and the powder was compressed to a predetermined force. Reproducible dwell time was achieved for each tablet by applying the force to a specific load, and at this point the hydraulic valve was immediately released to reduce the force.

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

The compositions for tablets manufactured by direct compaction employing the spray-dried drug are given in Table I.

The amount of drug was maintained constant while the quantities of binder-disintegrant and lubricant were varied as related to tablet weight, so that their percentages were the same for each tablet size. Lactose was employed to adjust the dilution ratios to 1:4, 1:8, and 1:12 for drug to total tablet weight, respectively.

Direct Compaction Microscale Manufacturing Procedure—The ingredients listed in Table I were accurately weighed and passed through a No. 20-mesh U. S. Standard sieve. The materials were transferred to a glass mortar and gently blended without force so that the drug's particle structure would not be markedly altered. The premixed blend was placed into a 1-pint twin-shell blender[4] and tumbled for 5 min. The blended powders were chemically analyzed to assure content uniformity for the drug. A specific quantity of the blended powder was filled into the die cavity and compressed, using flat-face, beveled-edge punches of the appropriate size.

The tablet thickness for each size tablet was measured using a micrometer with vernier; each tablet employed in the dissolution studies had to adhere to a tolerance of 0.02 mm. This physical measurement assured compliance of each tablet to similar disintegration times for consecutive tablets of the same size. Samples of each tablet size (200, 400, and 600 mg.) were subjected to the USP disintegration test. The tablet formulations were designed to disintegrate rapidly. This eliminated a lag period in the dissolution experiments, thus providing uninhibited dissolution. A compression

[3] Model C, Fred S. Carver, Inc., Summit, N. J.

[4] Patterson-Kelley, Inc., East Stroudsburg, Pa.

Table II—Physical Description and Compression Forces Relating to Tablets Prepared from Spray-Dried Quinazolinone Compound

Tablet weight, mg.	200	400	600
Punch diameter, mm.	8	10	12
Punch surface area, in.^{2}	0.079	0.123	0.177
Force applied (lb.)/ pressure applied (p.s.i.)	950/12,000	1500/12,000	2150/12,000
Force applied (lb.)/ pressure applied (p.s.i.)	1400/18,000	2200/18,000	3200/18,000
Force applied (lb.)/ pressure applied (p.s.i.)	2200/24,000	2950/24,000	4250/24,000

force of 12,000 p.s.i. was initially chosen since it provided all tablets with a 20–30-sec. disintegration time. A significant variation in the dissolution rates for the three tablet weights evaluated was observed. The fast disintegration gave rise to the formation of discrete particles composed of drug–diluents which resulted from rupture of the tablet matrix.

The physical parameters that were determined as controls in the manufacture of the variety of tablets are listed in Table II.

Dissolution-Rate Analysis for Tablets—The unprotonated form of the quinazolinone compound in the pure form formed a saturated solution of 2.7×10^{-4} moles/l. in distilled water. A quantity of 50 mg. of the drug per tablet was utilized in this study, since it provided a concentration well below the aqueous saturation level of 1.21 mg./ml. at pH 1.2. A rotating-bottle technique was employed, since it was found suitable for attaining reasonably precise data for similar consecutive experiments. The apparatus was immersed in a constant-temperature bath, maintained at 37 ± 0.5°, and rotated at a constant speed of 10 r.p.m.

Each tablet was placed into a 120-ml. capacity, amber glass bottle containing 100 ml. of aqueous solvent which had been previously adjusted to a pH of 1.2 with hydrochloric acid. The solvent had been preheated to 37° prior to the addition of the sample. At the moment of sample addition, the time was recorded as $t = 0$. The container was tightly closed with a bakelite cap lined with a polyethylene sealer.

Apparent sink conditions were adhered to in this study since the solubility of the pure drug at 37° and pH 1.2 was determined to be 4.3×10^{-3} moles/l. The concentration of hydrogen ion was calculated to be 6.3×10^{-2} moles/l., which is about 35 times greater than the concentration of the compound (1.8×10^{-3} moles/l.).

Dissolution of the drug from the tablets was conducted for a 30-min. interval inasmuch as two half-lives were encompassed, which permitted valid comparison of the $t_{50\%}$ for each tablet size.

Table III provides all the dissolution data obtained for each tablet size and compression force. The amount dissolved at each time interval represents an average of four runs.

The analytical details for following dissolution involved the withdrawal of a 1-ml. portion at specified time intervals using a pipet with a filter tip. The aliquot was diluted to 50 ml. with aqueous pH 1.2 fluid. The absorption of the diluted solution was measured in a Cary recording spectrophotometer over the UV spectra, and the maximum absorbance at 232 nm. was used to calculate the drug concentration per 100 ml. of pH 1.2 aqueous solution. Beer's law was obeyed with this drug over a range of 0.001 mg./ml. to 0.005 mg./ml.

Table IV—Physical Specifications Determined for Each Tablet Size Prepared from Spray-Dried Quinazolinone Compound at Various Compression Forces

Compression Force, p.s.i.	Average Tablet Thickness, mm.[a]	Average Disintegration Time, sec.[b]
	200-mg. Tablets	
12,000	3.40 ± 0.02	20 ± 5
18,000	3.30 ± 0.02	45 ± 5
24,000	3.20 ± 0.02	85 ± 5
	400-mg. Tablets	
12,000	4.20 ± 0.02	30 ± 5
18,000	4.04 ± 0.02	45 ± 5
24,000	3.96 ± 0.02	65 ± 5
	600-mg. Tablets	
12,000	4.38 ± 0.02	30 ± 5
18,000	4.25 ± 0.02	55 ± 5
24,000	4.12 ± 0.02	105 ± 10

[a] Represents an average of all tablets employed in dissolution experiments. [b] Represents an average of six tablets performed by USP basket method with plastic disk.

A correction factor was calculated for each sequential sampling as a result of the small volume change that occurred since the test solution withdrawn was not replaced after each sampling. At least five, and not more than six, samples were taken for each run.

RESULTS AND DISCUSSION

A typical direct compression tablet formulation was chosen to study the effect on dissolution behavior, where the excipient content was increased in a relationship to a constant quantity (50 mg.) of drug. The three tablet weights investigated, 200, 400, and 600 mg., provided ratios of 1:4, 1:8, and 1:12 of drug to total tablet weight, respectively. The information in Table I indicates the composition and quantities of diluent, binder-disintegrant, and lubricant for the three tablet weights; the only variables introduced were the extent to which the drug particles were diluted and the surface area of the tablets. Upon disintegration, the tablets gave rise to the formation of discrete particles composed of drug–diluent, with a different content of drug to diluent for each discrete particle depending upon the tablet weights. These discrete particles, in turn, deaggregated to provide even smaller particles. The dissolution process then resulted from the solvent action of the gastric fluid on the discrete particles. Lactose, the main component comprising the tablets, was selected for its hydrophilic characteristic, which would lead one to conclude that the larger the tablet, the more probable that it would possess a more rapid dissolution rate for a specific compression force.

The differences existing between the tablet dilution ratio and force of compression at 12,000, 18,000, and 24,000 p.s.i. for the different tablet sizes are revealed in Figs. 1–3. The curves in these figures demonstrate that, at each compression force, the dissolution rate for the larger tablet is greater at the portion of the curve where the dissolution half-life is approached. The tablet's disintegration time and the thickness specifications for each tablet size that was compressed at the various forces are compiled in Table IV. For each tablet size, an increase in the compression force resulted in an in-

Table III—Dissolution Data at pH 1.2 for All Directly Compacted Tablets Prepared from Spray-Dried Quinazolinone Compound

Solution Time, min.	Amount Dissolved[a], mg./100 ml. 200 mg. at 12,000 p.s.i.	200 mg. at 18,000 p.s.i.	200 mg. at 24,000 p.s.i.	400 mg. at 12,000 p.s.i.	400 mg. at 18,000 p.s.i.	400 mg. at 24,000 p.s.i.	600 mg. at 12,000 p.s.i.	600 mg. at 18,000 p.s.i.	600 mg. at 24,000 p.s.i.
2	8.2	6.3	—	17.3	8.6	5.7	23.7	20.6	8.7
5	16.0	15.9	7.2	27.5	21.7	14.6	31.3	29.3	24.9
10	27.6	26.4	17.5	33.3	31.2	24.5	38.0	36.2	35.4
15	—	—	25.1	—	—	32.6	—	—	39.3
20	36.5	37.3	31.1	38.6	39.5	36.8	42.2	42.6	42.6
30	43.2	43.5	37.3	41.2	44.1	42.1	44.8	45.0	47.8

[a] Represents average of four runs for each tablet weight and compression force.

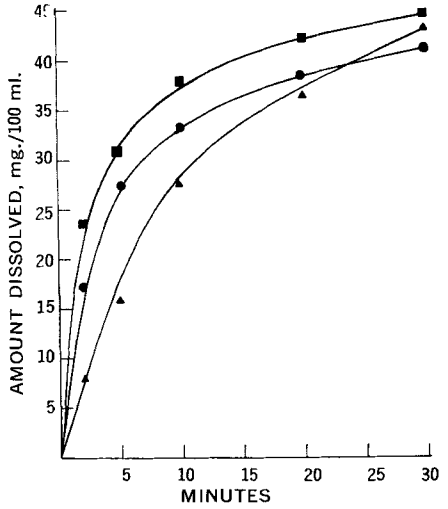

Figure 1—*Dissolution of quinazolinone compound in 100 ml. aqueous solution at pH 1.2 from compressed tablets at 12,000 p.s.i. Key: ▲, 200 mg.; ●, 400 mg.; and ■, 600 mg.*

creased disintegration time. The disintegration time for the three different tablet weights is about the same when compressed at 12,000 and 18,000 p.s.i., and, in all cases, it is under 55 sec. Those tablets compressed at 24,000 p.s.i. had a disintegration time that exceeded 1 min. but was less than 2 min.

The $t_{50\%}$ was determined from Figs. 1–3 for each tablet weight at various compression forces and may be observed in Table V. The half-life data for the 200-, 400-, and 600-mg. tablets indicate that the greater the pressure applied during compression, the longer the period for an equivalent amount of drug to be available in solution. The dissolution half-lives for the different tablet sizes supported the fact that the greater the drug's dilution, the more rapid was the dissolution rate. The apparent surface area, as a result of the number of discrete particles in contact with gastric fluid, appears to be the limiting factor. The greater the tablet weight, the less effect the force of compression exerts on the $t_{50\%}$. This phenomenon most likely relates to the significant increase that exists between the ratio of hydrophilic to hydrophobic materials as the tablet becomes greater in weight.

Figure 4 was prepared to determine the relationship of the half-life ($t_{50\%}$) to the specific tablet weight. When the pressure applied was maintained constant for the three different tablet sizes, a linearity existed for $t_{50\%}$ *versus* tablet weight. The smaller the tablet, the greater was the interference when varying the pressure applied in relation to the drug found in solution.

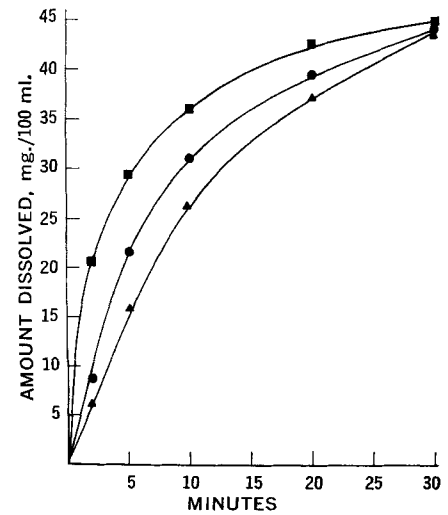

Figure 2—*Dissolution of quinazolinone compound in 100 ml. aqueous solution at pH 1.2 from compressed tablets at 18,000 p.s.i. Key: ▲, 200 mg.; ●, 400 mg.; and ■, 600 mg.*

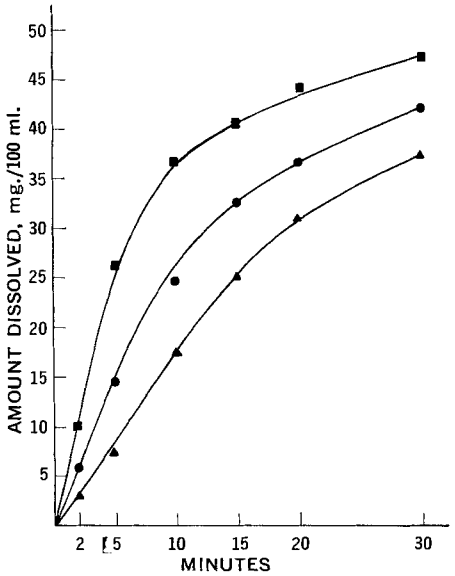

Figure 3—*Dissolution of quinazolinone compound in 100 ml. aqueous solution at pH 1.2 from compressed tablets at 24,000 p.s.i. Key: ▲, 200 mg.; ●, 400 mg.; and ■, 600 mg.*

The linear relationship for tablet weight *versus* dissolution rate at a specific compression force appears to indicate a physical similarity of the various tablet dilutions. This possibly points to a difference in number of the discrete particles as related to their apparent surface area which depends upon the tablet weight. Upon disintegration, the 600-mg. tablet provides relatively three times the number of discrete particles as the 200-mg. tablet. Each discrete particle for the 600-mg. tablet would possibly have one-third the amount of water-insoluble drug in combination with the hydrophilic tablet excipients as compared to the 200-mg. tablet. Therefore, the dissolution rate for various tablet sizes in this study could be dependent upon the aqueous fluid that was in contact with the greater number of discrete particles. A greater dilution of an equivalent amount of drug to be tableted proved to promote a corresponding enhancement of the dissolution rate.

SUMMARY AND CONCLUSIONS

The ratio of tablet diluent to drug has a pronounced effect on the dissolution behavior of this quinazolinone compound at pH 1.2. When a tablet disintegrates, it ruptures into discrete particles which are composed of the drug and diluents. The greater the quantity of diluent to drug in the directly compacted tablet, the larger is the surface area of the discrete particles in contact with alimentary fluid. This particular relationship makes the following assumption: the discrete particle-size distribution is similar for each tablet weight at the same compression force. The dissolution rate increase that occurred when increasing the excipient-to-drug ratio from 3:1 to 7:1 to 11:1 is directly related to the dilution factor.

The diluents selected for this study, with the exception of the lubricant, were hydrophilic, which provided an important capillary effect to the discrete particles in the simulated gastric fluid. Therefore, the greater the dilution of the hydrophobic drug with hydrophilic excipient, it would appear the more readily the solvent would be able to wet and dissolve the drug.

Table V—Half-Lives[a] (in Minutes) of Quinazolinone Compound in 100 ml. Aqueous Solution at pH 1.2 for Tablets of 200, 400, and 600 mg. at Various Compression Forces

Tablet Weight Half-Life Pressure Applied, p.s.i.	200 mg. $t_{50\%}$	400 mg. $t_{50\%}$	600 mg. $t_{50\%}$
12,000	6.8	4.2	2.5
18,000	9.4	6.4	3.4
24,000	14.8	9.4	4.8

[a] Each time interval was obtained from Figs. 1–3.

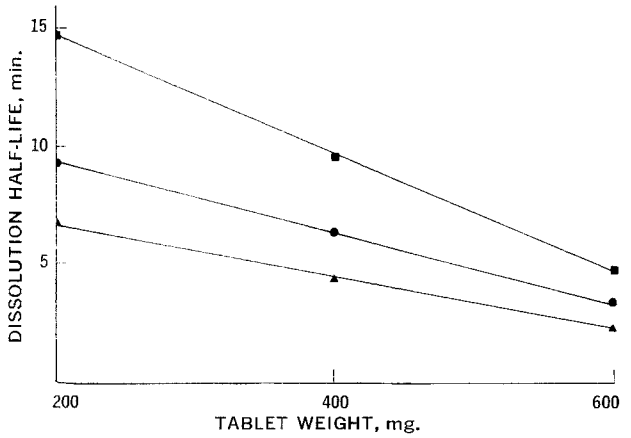

Figure 4—*Relationship of dissolution half-life* ($t_{50\%}$) versus *different tablet sizes compressed at the same pressure. Key:* ▲, $t_{50\%}$, *12,000 p.s.i.;* ●, $t_{50\%}$, *18,000 p.s.i.; and* ■, $t_{50\%}$, *24,000 p.s.i.*

When fabricating a tablet formulation, the research pharmacist many times will select the smallest tablet for a specific quantity of drug. This criterion is both economical and convenient from an oral administration viewpoint. In most cases, if the drug is poorly water soluble, micronization of the drug is utilized to maximize dissolution behavior. The data obtained in this study would recommend a larger tablet than is generally considered appropriate for a 50-mg. quantity of drug. The lesson taught with this poorly water-soluble drug indicates the necessity to consider the ratio of tablet excipient to drug in order to accomplish an optimum dissolution rate.

The force with which the tablet was compressed was also shown to affect dissolution behavior substantially. Figure 4 demonstrates that the larger the tablet size, the less effect compression force has on the dissolution rate.

The linear relationship which existed for tablet size *versus* $t_{50\%}$, when plotted for tablets compressed at 12,000, 18,000, and 24,000 p.s.i., was a good indication of the discrete particle-surface area's dependency on the dissolution characteristics. The discrete particle's total surface area possibly is related as a function of the ratio of diluent to drug when compressed at the same pressure.

The $t_{50\%}$ values reveal that a marked change in dissolution behavior occurs with the smaller tablet when the compression force is changed. This phenomenon is less pronounced as the dilution factor is increased. The dilution of the drug with excipient within each of the discrete particles from disintegration should provide for enhanced *in vivo* dissolution.

In conclusion, it is important to emphasize that when formulating a tablet of a poorly water-soluble drug, the following should be considered: optimum tablet size, hydrophilic nature of diluents, and optimum force of compression.

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DRUG STANDARDS

TLC Identification of Sulfonamides

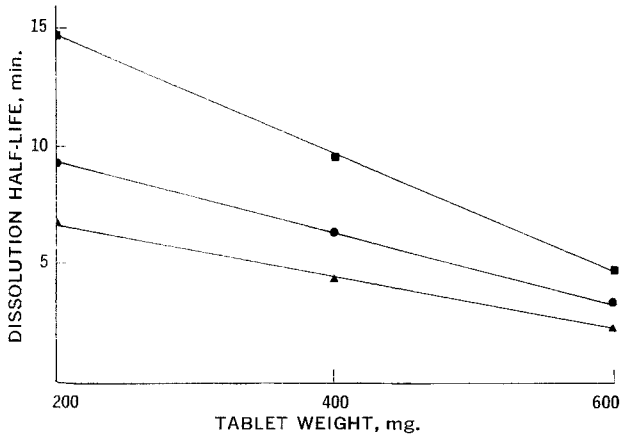
HANNAH R. KLEIN and WILLIAM J. MADER

Abstract □ An improved identification procedure for the official sulfonamides is presented. The method described uses TLC for separation; identification is accomplished on the plates using a specific detection reagent and suitable reference standards. By the use of three developing systems, the identity of any individual sulfonamide or the components of a mixture of sulfonamides may be established with certainty.

Keyphrases □ Sulfonamides, individual, mixed—identification procedure □ Chromatographic systems (TLC)—sulfonamide identification □ TLC—separation, identification

The USP and NF monograph procedures for identification of the official sulfonamides rely principally on the classical methods of organic chemistry for the recognition of a particular material. While used as identity tests, these procedures are extremely difficult and time consuming and often do not provide absolute proof in distinguishing individual substances of a class of compounds such as the sulfonamides.

The identity tests used in both compendia illustrate the difficulty in using these methods to identify and distinguish the sulfonamides. Roughly 80% of the methods used for official identity tests are based on visual observation of one of the following: heat decomposition, diazotization and coupling with β-naphthol, color or precipitate formed with cupric sulfate, color or fluorescence with resorcinol or phenol, ferric chloride, sodium bicarbonate solubility, or reduction. All of these procedures are subject to interferences, and none of them effectively distinguishes individual sulfonamides. The UV spectra of some compounds are used; and while they may be of considerable quantita-



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The identity tests used in both compendia illustrate the difficulty in using these methods to identify and distinguish the sulfonamides. Roughly 80% of the methods used for official identity tests are based on visual observation of one of the following: heat decomposition, diazotization and coupling with β-naphthol, color or precipitate formed with cupric sulfate, color or fluorescence with resorcinol or phenol, ferric chloride, sodium bicarbonate solubility, or reduction. All of these procedures are subject to interferences, and none of them effectively distinguishes individual sulfonamides. The UV spectra of some compounds are used; and while they may be of considerable quantita-

Table I—Systems Examined

No.	Thin Layer	Solvents	Reference
S-1	Silica gel G	Ethyl acetate–methanol–25% ammonium hydroxide (17:6:5)	12
S-2	Silica gel G	Petroleum ether–chloroform–*n*-butanol (1:1:1)	13
S-3	Silica gel	Chloroform–methanol (95:5)	14
S-4	Silica gel G	Chloroform–acetone–methanol–6 *N* ammonia (60:10:25:0.5)	5
S-5	Silica gel G	*n*-Propanol–0.05*N* hydrochloric acid (8:2)	5
S-6	Silica gel G	Butyl acetate–*n*-butanol–acetone–10% ammonia (3:3:4:1)	6
S-7	Silica gel G	Chloroform–*n*-butanol–acetone–85% formic acid (8:2:2:2)	6, 8
S-8	Silica gel G	Chloroform–methanol–*n*-butanol–2% ammonia (80:10:9:1)	7
S-9	Silica gel G	Chloroform–methanol (10:1)	8
S-10	Silica gel G	Chloroform–methanol–*n*-butanol–2% ammonia (8:10:10:1)	8
S-11	Alumina H	Ethyl acetate–water, buffered with ammonia (80:1)	9
S-12	Silica gel G	Ether–chloroform–*n*-butanol (1:1:1)	10
S-13	Silica gel G	Chloroform–methanol (9:1)	11
S-14	Alumina G	Ethyl acetate–methanol–25% ammonium hydroxide (17:3:3)	12
S-15	Silica gel G	Methyl isobutyl ketone–acetone–25% ammonium hydroxide (1:4:1)	12
S-16	Polyamide	Methyl isobutyl ketone–acetone–25% ammonium hydroxide (5:20:1)	12
S-17	Polyamide	Ethyl acetate–methanol–25% ammonium hydroxide (17:3:1)	12
S-18	Silica gel	Chloroform–methanol–acetic acid (94:5:1)	14
S-19	Silica gel	Chloroform–methanol–acetic acid (90:5:5)	14
S-20	Silica gel	Chloroform–*p*-dioxane (8:2)	14
S-21	Silica gel G	Chloroform–ethanol–heptane (1:1:1)	11
S-22	Alumina G	Methanol–chloroform (3:7)	15

tive value, they are of relatively little qualitative value since the spectra of all the sulfonamides are quite similar. In three instances the IR spectrum is used, and this will be discussed in the following paragraph. The only other official tests that could be used in identification are the melting points. Since many of the sulfonamides melt with decomposition and, consequently, over a wide range, this procedure must be used with caution.

Serious drawbacks in the IR characteristics of the sulfonamides prevent the use of IR spectra for identification. These drawbacks are: (*a*) the high incidence of polymorphism (1), giving rise to different spectra for different crystalline forms of the same compound; and (*b*) the low solubility of the sulfonamides in nonpolar solvents which prevents the use of solution spectra. Reproducible conversion between different forms of the sulfonamides is difficult to achieve and cannot be guaranteed by using similar solvent treatment on more than one form. The same can be said of recrystallization which, in addition, is undesirable since impurities may be excluded. Lyophilization of the sample, which has been reported to be successful using other compounds (2), was applied to separate forms of one of the sulfonamides but was unsuccessful.

It is readily apparent that there is currently no one procedure that will identify and differentiate all the official sulfonamides. Equally apparent is the desirability of replacing the numerous qualitative tests now employed by the USP and NF with a single procedure which could identify any of the official sulfonamides. In recent years, a number of reports (3, 4) dealt with TLC separation of various mixtures of sulfonamides. If a system could be found that would effectively separate all the sulfas, then a TLC separation coupled with a specific detection agent would provide an improved identification test.

In an attempt to find such a procedure, 22 separate TLC systems, which have been employed for some sulfonamides and on which data have been published, were examined. From these investigations, three separate solvent systems were found that will provide complete separation of the sulfonamides when used in conjunction. Complete separation cannot be achieved with less than three systems.

EXPERIMENTAL

Apparatus—Desaga-Brinkmann equipment for TLC was used throughout.

Adsorbents—The following were used: silica gel G and alumina H[1], polyamide[2], alumina and silica gel precoated glass plates, and aluminum-foil backed sheets[1].

Preparation of Layers—Glass plates, 20 × 20 cm., were coated with a 0.25-mm. thick layer of adsorbent as a slurry, air dried overnight, activated at 105° for 30 min., and allowed to cool to room temperature in a desiccator.

Solvent Systems—The 22 systems evaluated are listed in Table I. All solvents were reagent grade.

Samples—The sulfonamides tested were all either USP or NF grade. USP and NF reference standards were used where available. Solutions of each compound were prepared in acetone to contain 2 mg./ml. of the test substance.

Detection Reagents—The following were used:

1. *p*-Dimethylaminobenzaldehyde, 1% in 5% HCl.
2. Bratton–Marshall reagent: (*a*) 1 *N* HCl; (*b*) 5% sodium nitrite; (*c*) 100 mg. *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml. water. Spray with (*a*), then spray with (*b*), dry briefly at 100°, and then spray with (*c*).

Chromatographic Procedure—Chromatography chambers, lined with filter paper, were filled with the appropriate solvent mixture to a depth of about 1 cm. and allowed to equilibrate at least 1 hr. Thin-layer plates, prepared as previously described, were spotted with 2 μl. of each test solution at a point 1.5 cm. from the bottom of the plate and about 1 cm. apart. The spots were air dried, and the plate was placed in an equilibrated chamber and allowed to develop until the solvent front traveled 15 cm. above the point of application of the spots. The developed plates were air dried and sprayed with the detecting reagent.

RESULTS AND DISCUSSION

Evaluation of the solvent systems listed in Table I revealed that the best resolution could be achieved by using Solvents S-1, S-2, and S-3 in conjunction. None of the solvents, used individually, would separate all the compounds tested. The R_f values of the 16 official sulfonamides obtained with each of these three solvents are listed in Table II. The values represent an average of at least four determina-

[1] E. Merck AG, Darmstadt, West Germany.
[2] Woelm.

Table II—TLC R_f Values of Sulfonamides[a]

Compound	$R_f \times 100$ S-1	S-2	S-3
1. Acetylsulfisoxazole NF	56 ± 4	90 ± 2	54 ± 2
2. Phthalylsulfacetamide NF[b]	49 ± 2, and 29 ± 1	38 ± 2, and 3 ± 1	8 ± 2 and origin
3. Phthalylsulfathiazole NF	37 ± 4	62 ± 1	No migration
4. Succinylsulfathiazole USP	28 ± 1	5 ± 1	No migration
5. Sulfacetamide NF	44 ± 2	59 ± 3	9 ± 2
6. Sulfadiazine USP	42 ± 2	55 ± 3	24 ± 1
7. Sulfadimethoxine NF	59 ± 3	87 ± 2	36 ± 2
8. Sulfaethidole NF	57 ± 3	78 ± 6	17 ± 3
9. Sulfaguanidine NF	66 ± 1	18 ± 2	2 ± 1
10. Sulfamerazine USP	47 ± 2	67 ± 3	29 ± 2
11. Sulfamethazine USP	55 ± 2	68 ± 2	31 ± 2
12. Sulfamethizole NF	53 ± 5	71 ± 4	19 ± 1
13. Sulfamethoxazole NF	54 ± 3	84 ± 1	26 ± 2
14. Sulfanilamide USP	79 ± 1	42 ± 4	8 ± 2
15. Sulfapyridine USP	62 ± 2	54 ± 2	23 ± 2
16. Sulfisoxazole USP	56 ± 2	80 ± 2	14 ± 2

[a] Thin layer = silica gel G. Solvents = see Table I. Development distance = 15 cm. [b] Phthalylsulfacetamide produces two spots, apparently due to hydrolysis.

tions and are given with the mean deviation. The four official sodium salts of Compounds 1, 5, 6, and 10 are not separated from the free drug and can be identified as the corresponding sulfonamide. Since the R_f values of the salts are identical with those of the drugs, they are not listed separately. Examination of these R_f values shows that, by the use of appropriate reference standards, the identity of any individual sulfonamide, or the components of a mixture of sulfonamides, may be established with certainty.

Since the purpose of this method is both to separate and identify sulfonamides, the use of a reagent that gives a characteristic color reaction with sulfonamides is a valuable aid to identification in addition to R_f values. Visualization of the spots on the plate was studied with two specific reagents: (*a*) Bratton–Marshall, and (*b*) *p*-dimethylaminobenzaldehyde. Since the Bratton–Marshall reagent is useful only for N-4 unsubstituted sulfonamides and is the least sensitive of the two reagents, the *p*-dimethylaminobenzaldehyde reagent is preferred. This reagent produces bright-yellow spots on spraying with most of the compounds, but heating at 100° is necessary with some of the compounds before the spots are visible. Thin layers incorporating UV fluorescent indicators were not used, because they lack specificity and are not as sensitive as either of the spray reagents.

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Colorimetric Determination of Eriodictyol and Its Glycoside, Eriocitrin

MOHAMMED HASAN, EDGAR E. THEIMER, and VIJAY K. JAIN

Abstract □ A procedure has been devised to quantitate the flavanone, eriodictyol, and its glycoside, eriocitrin, by use of the color reagent, 2-aminoethyl diphenylborate. Also, some parameters in the use of this reagent in quantitative analysis have been studied. Application of the procedure to lemon bioflavonoid complex gave an average content of 2.5% eriocitrin.

Keyphrases □ Eriodictyol, eriocitrin—determination □ Lemon bioflavonoid complex—eriodictyol, eriocitrin determination □ 2-Aminoethyl diphenylborate—color reagent □ Colorimetric analysis—spectrophotometer

Several investigators (1–4) have shown interest in the biological and clinical effects of eriodictyol, a flavanone which occurs as the glycoside in *Citrus limon* (5–7).

In the course of an investigation of the biological effects of this flavonoid, it was found necessary to devise an analytical method for assaying the eriodictyol content of the materials being evaluated. Several methods for the quantitation of flavonoids are known. In general, they are either nonspecific (8–13) or involve time-consuming chromatographic separations (14–19). A control method avoiding these disadvantages was desired.

The following work describes assay methods for eriodictyol, both in the free aglycone form and in the form of eriodictyol glycoside, as it is present in the commercial product known as lemon bioflavonoid complex[1]. By TLC, the authors found this glycoside to coincide with the lemon glycoside described by Horowitz and Gentili (5) and characterized by them as eriodictyol 7-β-rutinoside (eriocitrin).

Neu (20) investigated the analytical use of diarylboric acids, including the photometric determination of flavones, and described the preparation of 2-aminoethyl diphenylborate. This reagent has come into use as a flavonoid spray on TLC and papergrams (21). Ogura *et al.* (8) presented UV spectra of the reaction products of aminoethyl diphenylborate with rutin and quercetin.

Except for eriodictyol and eriocitrin, available flavonoids which give colored aminoethyl diphenylborate reaction products have absorption peaks[2] ranging from 350 to 467 nm. Only eriodictyol and eriocitrin give red colors, with peaks at 505 and 520 nm., respectively (Table I) (Fig. 1). Of great interest was the observation that hesperidin and hesperetin, the most likely interferences in the samples, give negligible absorbance above 500 nm. Based on these observations, the following procedure was devised for eriodictyol in the absence of its glycoside. This procedure was then used as the basis of a procedure to assay for eriocitrin in lemon bioflavonoid complex.

Table I—Comparison of Absorbance Peaks of Some Flavonoids after Aminoethyl Diphenylborate Treatment under Standardized Conditions Using 0.1 mg. of the Compound

Flavonoids	$\lambda_{max.}$	A_{250}	A_{505}
Glycosides			
Flavanones			
Eriocitrin	520	0.33[a]	–
Hesperidin	420	0.00	–
Nonflavanones			
Quercitrin	453	0.03	–
Phlorizin	–	0.00	–
Aglycones			
Flavanones			
Eriodictyol	505	–	0.43
Hesperetin	417	–	0.00
Dihydroquercetin[b]	467	–	0.09
Nonflavanones			
Quercetin	467	–	0.22
Apigenin	350	–	0.00
Luteolin	445	–	0.08
Phloretin	–	–	0.00

[a] Calculated from lemon bioflavonoid complex reference standard results. [b] Dihydroquercetin may be classed as a 3-hydroxyflavanone or a dihydroflavonol; UV spectra of flavanones and dihydroflavonols are very similar.

When the methanol extractives from lemon bioflavonoid complex react with aminoethyl diphenylborate, the resultant solution is orange, as opposed to the red obtained from eriocitrin. The yellow component of this color is due to hesperidin and small amounts of other flavonoids, but it does not contribute significantly to the A_{520}, which can therefore be used as a measure of the eriocitrin content, relative to a standard lemon bioflavonoid complex run simultaneously. Because pure eriodictyol was available as a standard, a quantitative hydrolysis–extraction procedure was devised for assaying this (secondary) lemon bioflavonoid complex standard in terms of its eriodictyol (aglycone) equivalence, which may be recalculated to eriocitrin by multiplying by the ratio of the molecular weights. In this procedure, the use of hemicellulase as hydrolytic enzyme and ethyl acetate as extractant was adapted from work reported by Horowitz and Gentili (5). Acid hydrolysis was found to be too drastic.

EXPERIMENTAL

Eriodictyol Assay—The standard preparation is 0.1 mg. reference standard eriodictyol per milliliter in methanol. The sample preparation is 0.1 mg./ml. in methanol. The reagent is 2-aminoethyl diphenylborate[3]. Prepare a 2.5% solution in methanol. For the buffer, mix 1 volume 2 *M* aqueous acetic acid and 1 volume 2 *M* sodium acetate; adjust pH to 4.6.

[1] The lemon bioflavonoid complex used in this work was manufactured by Sunkist Growers, Inc., Corona, Calif., and by Test Laboratories Inc., Reseda, Calif.

[2] A Cary 15 spectrophotometer was used throughout this work.

[3] Diphenylboric acid–ethanolamine complex, Aldrich Chemical Co., Milwaukee, Wis.

Table II—Effect of Amount of Aminoethyl Diphenylborate Reagent on Color[a]

Aminoethyl Diphenylborate, mg.	$A_{max.}$ at 4 hr.	$A_{max.}$ at 95 hr.
25	0.284	0.629
50	0.430	0.879
75	0.522	0.979
100	0.601	1.090
125	0.645	1.128
150	0.676	1.188
175	0.729	1.218
200	0.733	1.198

[a] Buffer pH 4.6; 0.1 mg. eriodictyol.

Pipet 1 ml. sample and standard preparations into 10-ml. volumetric flasks. Add 1.00 ml. buffer, then add 4.00 ml. aminoethyl diphenylborate reagent, and bring to volume with methanol. Let stand exactly 4 hr. Measure A_{505} in 1-cm. cells against a reagent blank. Calculate purity from the absorbance ratio.

Assay of Lemon Bioflavonoid Complex for Its Eriocitrin Content–Method A—Mix 100 mg. lemon bioflavonoid complex, 200 mg. hemicellulase[4], and 10 ml. distilled water in a stoppered flask and store at 32° for about 20 hr. Using ethyl acetate, rinse quantitatively into a separator and extract with three 10-ml. portions of ethyl acetate. Transfer the combined organic extracts, with the aid of additional ethyl acetate, to a rotary evaporator flask, and evaporate to dryness *in vacuo*, below 50°. Rinse the residue with small portions of methanol into a 25-ml. volumetric flask, and add methanol to the mark. Pipet a 2-ml. aliquot, as sample preparation, into a 10-ml. volumetric flask, and proceed as directed under *Eriodictyol Assay*.

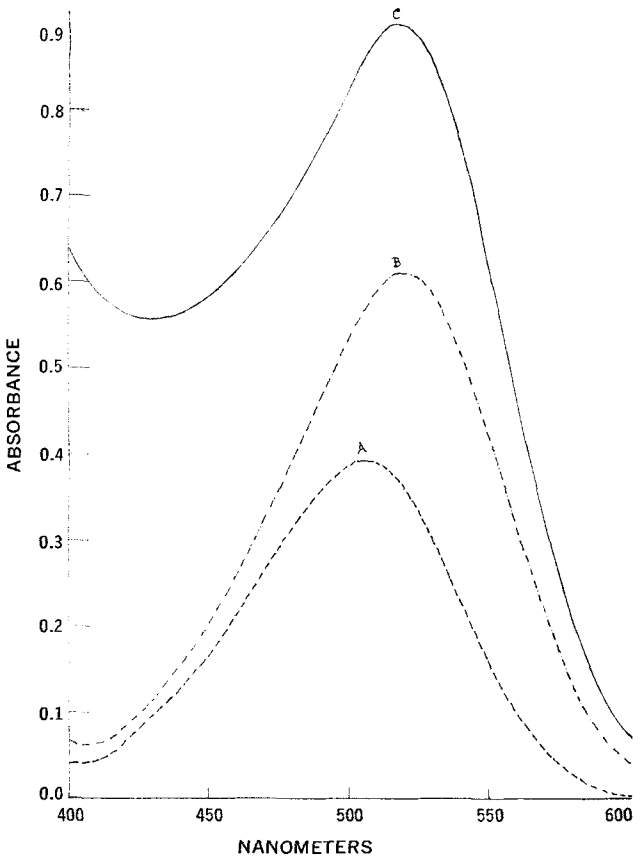

Figure 1—*Spectra of colored complex of eriodictyol, eriocitrin, and lemon bioflavonoid complex. Key: A, --- eriodictyol; B, -·-, eriocitrin; and C, ——, lemon bioflavonoid complex.*

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

Table III—Effect of Buffer pH on Color Development[a]

Buffer pH	Absorbance at $\lambda_{max.}$: 4 hr. A	4 hr. nm.	22 hr. A	At Max. A	At Max. nm.	At Max. Days	29 Days A	29 Days nm.
2.15	0.190	507	0.642	1.545	514	22	1.520	513
3.00	0.230	506	0.754	1.376	513	19	1.273	512
4.05	0.387	505	0.920	1.260	512	19	1.114	512
4.60	0.540	505	0.879	1.385	513	19	1.315	511
5.90	0.313	493	0.461					
6.72	0.251	488	0.358	Not determined				
8.50	0.191	485	0.276					

[a] 0.1 mg. eriodictyol, 100 mg. aminoethyl diphenylborate reagent, 1 ml. aqueous acetate buffer, and 9 ml. methanol.

Calculate the eriodictyol content from the absorbance ratio. Then, 2.07 × % eriodictyol × dilution factor = % eriocitrin in sample.

Alternative Assay of Lemon Bioflavonoid Complex–Method B—By using, as the reference standard, a lemon bioflavonoid complex that has been assayed by Method A, other lemon bioflavonoid complex samples can be assayed as follows.

Standard Preparation—Shake well 100 mg. reference standard lemon bioflavonoid complex with 100 ml. anhydrous methanol in a 100-ml. volumetric flask; then let stand overnight.

Sample Preparations—Similarly treat 100 mg. lemon bioflavonoid complex sample.

Aminoethyl Diphenylborate Reagent and Buffer—Proceed as described under *Eriodictyol Assay*.

Pipet 4 ml. clear supernatant solutions from standard and sample into 10-ml. volumetric flasks, and proceed as described under *Eriodictyol Assay*. Measure the absorbances at 520 nm. after exactly 4 hr. Calculate the eriocitrin content from the absorbance ratio and the assay value of the reference standard.

RESULTS AND DISCUSSION

Eriodictyol Assay—*Study of Conditions*—Absorbance values were found to vary somewhat between experiments. However, duplicates run at the same time always checked closely. Table II shows that even when aminoethyl diphenylborate concentration was increased to high values, an absorbance plateau could not be attained either at 4 or at 95 hr. Therefore, 100 mg. was chosen for the arbitrary standard conditions. The 4-hr. reaction time is also arbitrary. If more time is available, more intense color develops. When the pH 4.6 buffer is used, the reaction mixture pH is quite stable at 6.65 and is not influenced by addition of eriodictyol, lemon bioflavonoid complex, or aminoethyl diphenylborate. The pH values were measured by glass and calomel electrodes with a Beckman Zeromatic pH meter.

Table III shows that pH influences the rate of color development. The highest absorbance value for the 4-hr. measurement is attained when pH 4.6 buffer is used. There is a shift in wavelength of the absorbance peak when the pH or color development time is changed. No quantitative study was made of temperature dependence, but strong heating resulted in side reactions, while slight temperature variations had little effect. Artificial light had no noticeable effect. Absorbance is directly proportional to sample size up to at least 0.25 mg. eriodictyol.

Lemon Bioflavonoid Complex Assay–Method A—*Validity—Estimate of Interferences*—To estimate the contribution from other lemon bioflavonoid complex ingredients to the A_{505}, the methanolic solution obtained after hydrolysis of Sunkist lemon bioflavonoid complex was chromatographed on Whatman No. 1 paper by descending technique, using the solvent system of benzene–acetic acid–water (125:72:3) (22). The dried paper was edge-sprayed with 1% methanolic aminoethyl diphenylborate reagent; then the unsprayed part was cut into bands and separate color-forming ingredients were eluted with methanol. Several bands gave varying shades of yellow and there was the red eriodictyol band. By running the aminoethyl diphenylborate assay on these solutions in parallel, it was found that the total A_{505} due to noneriodictyol bands was about 5% of that from eriodictyol.

UV Spectrum—The isolation procedure specified in Method A gives a methanol solution whose UV curve is characteristic for

flavanones, such as hesperetin and eriodictyol ($\lambda_{max.}$ = 287 nm.), with hardly any distortion, which would be due to appreciable amounts of other flavonoids. That is, flavonoids which might interfere in the color test, *e.g.*, luteolin and quercetin (Table I), do not appear in the flavanone solution. Actually, at least 90% of the UV absorbance, in this case, was accountable to eriodictyol, with most of the balance probably due to hesperetin formed by partial hydrolysis and recovery from the hesperidin present in the lemon bioflavonoid complex. Dihydroquercetin was shown by TLC to be absent from the methanolic extract, and diosmin seems not to be present in lemon bioflavonoid complex in interfering quantity.

The UV spectrum of the aqueous layer remaining after ethyl acetate extraction of the enzymatic hydrolyzate showed the presence of different absorbers, probably other flavonoids. Examination by TLC and by the borohydride test (9) confirmed the absence of flavanones.

Reaction Course—Varying the enzymatic hydrolysis conditions by stirring magnetically for various times or by letting the reaction continue for 3 days did not affect the result.

Monitoring the reaction by TLC showed that, when insufficient time was allowed for complete hydrolysis, a product with a different R_f value, also giving a red color with aminoethyl diphenylborate spray, was formed as an intermediate. This may be eriodictyol glucoside. When this spot and the eriocitrin spot were no longer present, and only a product having the R_f value of eriodictyol gave a red product with aminoethyl diphenylborate spray, the hydrolysis was complete.

To determine if there was any loss of eriodictyol between hydrolysis and color measurement, two 100-mg. portions of reference standard lemon bioflavonoid complex were taken. To one of these, exactly 1 mg. of standard eriodictyol was added. Both were carried through the Method A procedure simultaneously. The difference between the resultant absorbances showed that very close to 100% of the added eriodictyol was recovered. Evidently the enzyme converts eriocitrin completely to eriodictyol and has no effect on the eriodictyol formed.

Lemon Bioflavonoid Complex Assay–Method B—*Study of Conditions—Extraction*—To justify the simple extraction procedure, varying proportions of methanol and lemon bioflavonoid complex were mixed. From each flask, an aliquot equivalent to the soluble portion from 4 mg. lemon bioflavonoid complex was carried through the aminoethyl diphenylborate reaction. Since no significant difference was observed, it may be concluded that solubility in methanol is not a color-limiting factor. In another experiment, a 2-week-old mixture of lemon bioflavonoid complex–methanol gave the same result.

Color Reaction—The reaction conditions for eriodictyol were adapted to the assay of eriocitrin in lemon bioflavonoid complex, and the procedure was standardized as described earlier. As in the case of eriodictyol, duplicate samples run simultaneously checked closely, even when reaction time was extended to 94 hr. Also, as with eriodictyol, the peak slowly shifts to higher wavelengths. Also, varying the amount of aminoethyl diphenylborate reagent had qualitatively the same effect on the $A_{max.}$ in the case of lemon bioflavonoid complex as with eriodictyol. Beer's law is obeyed up to at least 10 mg. lemon bioflavonoid complex.

Validity—To obtain an estimate of the contribution of interfering lemon bioflavonoid complex ingredients to the A_{520}, a concentrated methanolic extract of Sunkist lemon bioflavonoid complex was chromatographed on Whatman No. 1 paper by descending technique, using the solvent system of *n*-butanol–acetic acid–water (40:10:50). The separated ingredients were treated as in the case of the chromatographed hydrolyzed lemon bioflavonoid complex previously described. In addition to the eriocitrin band, several other bands giving colors with aminoethyl diphenylborate spray were observed. The total A_{520} due to noneriocitrin bands was not more than 5% of that from eriocitrin.

Roughly similar results were obtained when this experiment was repeated using Testlab lemon bioflavonoid complex.

APPLICATIONS

Assay of five different lots of lemon bioflavonoid complex from the two manufacturers gave eriocitrin contents of 2.1–3.0% when tested by Method A. When Method B was used, the absorbances gave the expected proportionality, with a maximum deviation of 10%.

A sample of orange bioflavonoid complex (Sunkist) was examined both by TLC and by the tests described previously. It failed to show the presence of any eriocitrin.

A lemon bioflavonoid complex sample tested after heating to 45° for 1 week gave the same assay result as the fresh material.

Preliminary experiments in the determination of eriodictyol in blood plasma by extraction and reaction with aminoethyl diphenylborate have been performed and have given promising results.

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Rapid Simultaneous Determination of Salicylic Acid and Aspirin by GC I: Analysis of Synthetic Aspirin–Salicylic Acid Mixtures and of Single-Component Aspirin Tablets

J. R. WATSON, P. CRESCUOLO, and FUMI MATSUI

Abstract □ A rapid, sensitive GC method for the simultaneous analysis of salicylic acid and aspirin in admixtures and in single-component acetylsalicylic acid (aspirin) tablets is presented. The procedure involves conversion of the two drugs to their methyl esters with diazomethane in tetrahydrofuran solution and subsequent isothermal elution of the components from a 5% OV-210 on Diatoport S glass column. Quantitation is effected by means of an electronic digital integrator with methyl *o*-methoxybenzoate as the internal standard. The average total drug recovery of 16 synthetic mixtures was 99.70%, with a standard deviation of ±0.58%, while the mean total recovery using the USP procedure was 99.40 ± 0.68%. When applied to the analysis of commercial single-component compressed and enteric coated aspirin tablets, the proposed technique gave results which were, in the majority of cases, in excellent agreement with the USP values for both aspirin and salicylic acid content. With a few formulations, the salicylic acid content was found to be slightly higher with the GC procedure than with the method described in the USP.

Keyphrases □ Salicylic, acetylsalicylic acids—simultaneous determination □ Aspirin tablets—analysis □ GC—analysis □ Methyl *o*-methoxybenzoate—internal standard, GC

In recent years, the drug field has witnessed the proliferation of methods for the analysis of salicylic acid and acetylsalicylic acid (aspirin) in pharmaceuticals and in various biological media. Surprisingly few, however, have been developed expressly for their simultaneous determination, and most require a separation step prior to quantitation of the individual components.

In the USP (1) and in the work of Levine and Weber (2, 3), the salicylic acid is trapped as a complex on a ferric chloride–urea–siliceous earth column prior to elution and subsequent spectrophotometric measurement. A second aliquot is then employed for the estimation of the aspirin. The BP (4) monograph for aspirin includes a limit colorimetric test for salicylic acid and an aqueous residual titration procedure for aspirin. Lin (5) differentiated the two drugs in synthetic mixtures by nonaqueous potentiometric titration, but this method would not appear to be sufficiently sensitive for the analysis of salicylic acid at the low levels normally encountered in aspirin raw material and commercial dosage forms. Lee *et al.* (6) reported a quantitative separation by Sephadex gel filtration and spectrophotofluorometric measurement of the column effluent but gave no data on the recoveries of the components. More recently, Shane and Miele (7) and Miles and Schenk (8) described rapid fluorometric methods for the determination of salicylic acid and aspirin in aspirin products. Several GC procedures have been proposed for the estimation of aspirin and/or salicylic acid as the free acids (9) or after derivatization to the trimethylsilyl (10) or methyl esters (11), but these methods either do not involve simultaneous measurement or otherwise present no supporting data for the quantitation of salicylic acid.

In this paper, a rapid, sensitive GC method for the simultaneous analysis of salicylic acid and aspirin in admixtures and in single-component aspirin tablets is presented. The drugs are converted to their methyl esters with diazomethane prepared in tetrahydrofuran solution and are eluted isothermally from a 5% OV-210 on Diatoport S system contained in a glass column. Peak areas are accurately measured with a printout digital electronic integrator, with methyl *o*-methoxybenzoate as the internal standard. The results are compared for precision and accuracy against those obtained with the USP method, and the merits of the proposed procedure are evaluated.

EXPERIMENTAL

Preparation of Diazomethane—*Materials*—The following were used: (*a*) tetrahydrofuran, B.D.H. reagent grade; (*b*) 2-(2-ethoxyethoxy)ethanol (Carbitol), Baker grade; and (*c*) *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Baker grade.

Procedure—The method selected for the preparation of diazomethane was essentially that of Deboer and Backer (12) but was scaled down 10-fold and employed tetrahydrofuran instead of diethyl ether. The apparatus was also modified to consist of a 50-ml. distilling flask with a side arm bent so as to fit through a cork stopper into a 50-ml. receiving conical flask. Through a second hole in the stopper was inserted a U-shaped outlet tube passing into and below the surface of tetrahydrofuran (4 ml.) contained in a second unstoppered conical flask. Both receiving flasks were cooled in an ice–salt mixture.

The solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (4.3 g.) in tetrahydrofuran (25 ml.) was added dropwise over 60–75 min. through a 60-ml. long-stemmed dropping funnel adjusted so that the tip was just above the surface of the magnetically stirred solution in the distilling flask [KOH, 1.2 g.; 2-(2-ethoxyethoxy)ethanol, 7 ml.; tetrahydrofuran, 2 ml.; and H_2O, 2 ml.] heated in a water bath at 80–90°. Distillation of diazomethane was carried out *in situ* with the recommended safety measures.

Preparation of Salicylic Acid–Internal Standard and Aspirin–Internal Standard Calibration Curves—*Materials*—The following were used: (*a*) salicylic acid, Anachemia, reagent grade; (*b*) aspirin; USP reference standard; and (*c*) methyl *o*-methoxybenzoate (internal standard), Eastman, highest purity.

Solutions—The following were used: (*a*) diazomethane in tetrahydrofuran, prepared as described previously; (*b*) salicylic acid in tetrahydrofuran, accurately weighed to contain about 5 mg./ml.; and (*c*) internal standard in tetrahydrofuran, accurately weighed to contain about 5 mg./ml.

Preparation of Mixtures, Methylation, and GC—For the salicylic acid–internal standard calibration curve, the exact volume of solution required to give 5.00 mg. of internal standard was dispensed into each of eight separate flasks (5-ml. volumetrics are convenient) from a 5-ml. microburet (graduated in 0.01 ml.) with a 24-gauge 1.9-cm (0.75-in.) hypodermic needle delivery tip. The exact volumes of salicylic acid solution calculated to give weight ratios of salicylic acid–internal standard of 0.30, 0.70, 0.90, 1.10,

1.30, 1.70, 2.10, and 2.30 were then dispensed into respective flasks.

For the aspirin–internal standard calibration curve, samples containing approximately 50, 80, 100, 125, 150, and 170 mg. of aspirin, respectively, were accurately weighed into six separate small stoppered flasks. The exact volume of solution equivalent to 5.00 mg. of internal standard was then dispensed into each flask.

Each individual drug–internal standard solution was then treated as follows. No more than 15 min. prior to being chromatographed, the solution was treated with diazomethane (added dropwise with shaking) to a permanent yellow color, and then immediately placed in a frost-free freezer at −15° along with all solutions to be subsequently methylated. Within 10 min. after preparation, 1 μl. of the esterified solution was injected by means of a Hamilton microsyringe into a GC[1] equipped with a flame-ionization detector unit and fitted with a 5% OV-210 on Diatoport S (80/100 mesh) coiled glass column, 1.5-m. × 0.32-cm. (5-ft. × 0.13-in.) o.d. preconditioned at 265° for 18 hr. Salicylic acid and aspirin were isothermally eluted as their methyl esters under the following conditions: column temperature, 110°; injection port temperature, 180°; and detector temperature, 205°. Gas flows were: nitrogen, 28 ml./min.; and hydrogen, 28 ml./min. The detector signal was supplied to a continuous balance 1-mv. recorder[2], with a chart speed of 0.63 cm./min. (0.25 in./min), connected to a fully automatic printout electronic digital integrator[3] with an effective input signal range of 0–100 mv., thus permitting a constant attenuation setting of 1 × 4.

The area ratios of the methyl esters to internal standard were plotted against the weight ratios of salicylic acid or aspirin to internal standard, and the respective slopes of the lines of best fit were determined.

Preparation of Synthetic Aspirin–Salicylic Acid Mixtures, Tablet Sampling, and Analysis by GC—*Materials and Solutions*—The following were used: (*a*) aspirin, Merck, USP grade (salicylic acid content predetermined to be 0.12% by the USP procedure); (*b*) salicylic acid in tetrahydrofuran, accurately weighed to contain about 0.5 mg./ml. and 4 mg./ml.; and (*c*) internal standard in tetrahydrofuran, accurately weighed to contain about 5 mg./ml.

Preparation of Synthetic Mixtures—Into a flask (10-ml. volumetric is convenient), 75–150 mg. of aspirin was weighed accurately. Then the exact volume of solution equivalent to 5.00 mg. of internal standard was dispensed through a 5-ml. microburet. The appropriate volume of salicylic acid solution was then added to give the desired percentage of salicylic acid based on the total aspirin–salicylic acid weight.

Tablet Sampling—Ten tablets were selected at random, weighed, and finely powdered on a Micro-Mill[4] apparatus. A quantity of the powder equivalent to about 100 mg. of aspirin was accurately weighed out into a 10-ml. stoppered flask, and the exact volume of solution equivalent to 5.00 mg. of internal standard was added through the microburet.

Analysis—Each simulated mixture and powdered tablet sample was treated in turn with diazomethane and chromatographed as described previously. The amount of salicylic acid and aspirin in the synthetic mixture and powdered tablet aliquot was computed from the relationship $y = mx$, using m values of 0.971 and 1.069 for the aspirin–internal standard and salicylic acid–internal standard calibration curves, respectively, and y as the experimental peak area ratio of methyl ester to internal standard.

Analysis of Synthetic Aspirin–Salicylic Acid Mixtures and of Single-Component Aspirin Tablets by the USP Method—The simulated mixtures were prepared as described under *Preparation of Synthetic Mixtures*, except that the addition of internal standard was omitted. The solvent was removed by evaporation at room temperature, and the residue analyzed for salicylic acid content as stated in the USP monograph for aspirin tablets, with quantity modifications being made where necessary. The amount of aspirin was determined by spectrophotometric measurements at 280 nm. of the appropriately diluted initial chloroform eluate (which is discarded in the USP procedure) from the ferric chloride–urea column. No interference from any UV-absorbing species originating from the urea, ferric chloride, or siliceous earth was encountered.

[1] Varian Aerograph series 200, model 204-B.
[2] Minneapolis-Honeywell, Electronik 15 strip-chart recorder.
[3] Kent, model Chromalog 2.
[4] Chemical Rubber Co., Cleveland, Ohio.

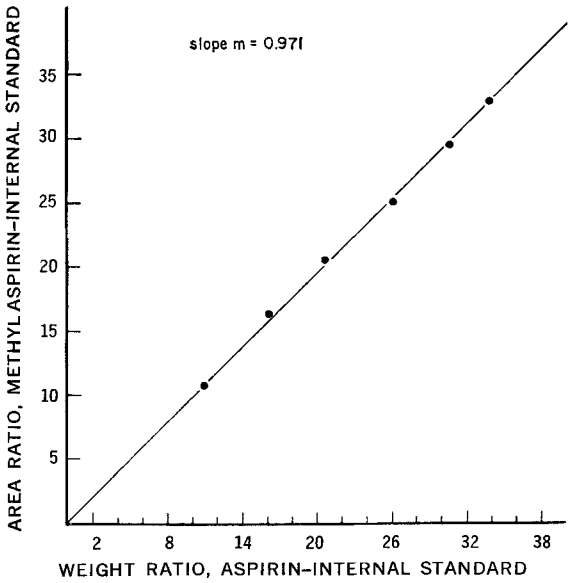

Figure 1—*Aspirin–internal standard calibration curve.*

The procedure followed for the single-component aspirin tablets was exactly as indicated in the monograph for aspirin tablets.

RESULTS

The area ratio *versus* weight ratio calibration curves for aspirin and salicylic acid are shown in Figs. 1 and 2, respectively. Both are straight lines passing through the origin ($y = mx$), and each point on the line represents the average of duplicate injections. With tetrahydrofuran as solvent, the precision between injections was excellent; the coefficient of variation computed from seven successive injections of a sample solution (kept at −15°) was ±0.35%. Detector response was linear for the weight ratio ranges investigated; salicylic acid was determined up to a ratio of 3.0:1 (not shown in the figure), and aspirin was determined up to a ratio of about 35:1. The mean slope for the aspirin line was 0.971, with a coefficient of variation of ±1.74%, while the values in the case of salicylic acid were 1.069 ± 1.12%. The calibration curves were checked about once a week over a 4-month period, but the drift from the lines was always less than their respective mean slope errors.

Because of the relatively large inaccuracies in weighing very small quantities of material (less than 10 mg.), a stock solution of salicylic acid in tetrahydrofuran was prepared and the calculated volume dispensed from a microburet into a flask containing an accurately weighed amount of aspirin. The theoretical percentages of salicylic

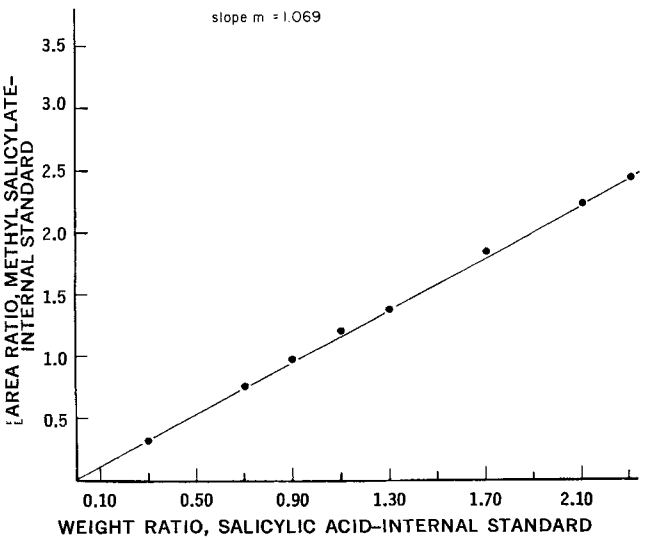

Figure 2—*Salicylic acid–internal standard calibration curve.*

Table I—Analysis of Aspirin–Salicylic Acid Synthetic Mixtures

Synthetic Mixture Number	Theoretical, %		Found—GC Method, %			Found—USP Method, %		
	Salicylic Acid	Aspirin	Salicylic Acid	Aspirin	Total Recovery	Salicylic Acid	Aspirin	Total Recovery
I	0.20	99.80	0.24	98.30	98.54	0.21	100.1	100.3
II	0.20	99.80	0.21	98.90	99.11	0.20	100.1	100.3
III	0.40	99.60	0.43	98.70	99.13	0.40	98.79	99.19
IV	0.40	99.60	0.40	98.81	99.21	0.39	99.35	99.74
V	0.60	99.40	0.57	100.0	100.6	0.56	98.57	99.13
VI	0.60	99.40	0.59	98.94	99.53	0.58	98.69	99.27
VII	0.80	99.20	0.75	98.97	99.72	0.76	98.71	99.47
VIII	0.80	99.20	0.77	98.75	99.52	0.77	98.92	99.69
IX	1.00	99.00	0.94	99.56	100.5	0.95	98.71	99.66
X	1.00	99.00	0.92	98.62	99.54	0.91	97.89	98.80
XI	3.00	97.00	2.99	96.51	99.50	2.89	95.86	98.75
XII	3.00	97.00	2.88	97.72	100.6	2.97	96.76	99.73
XIII	6.00	94.00	5.92	93.98	99.90	5.57	92.20	97.77
XIV	6.00	94.00	5.88	93.99	99.87	5.91	93.17	99.08
XV	10.00	90.00	10.14	89.66	99.80	9.83	89.25	99.08
XVI	10.00	90.00	10.21	89.96	100.2	9.78	90.71	100.5
					Mean 99.70% ±0.58%			Mean 99.40% ±0.68%

acid and aspirin in the synthetic mixtures were deduced on the basis of the total weight of drug. This type of sampling procedure was necessitated by the difficulty in achieving homogeneity of synthetic aspirin–salicylic acid mixtures made by addition of stock solution of both drugs followed by slow solvent evaporation; for all intents and purposes, samples analyzed by the GC and USP procedures were considered to be identical since they had been prepared in the same manner and from the same materials and solutions.

Inspection of Table I readily demonstrates the accuracy and precision obtainable with the GC procedure. The percentages of aspirin and salicylic acid found in the simulated mixtures were, without exception, very close to the theoretical values. The amount of drug found was calculated from the relationship $x = y/m$, where y is the experimental drug–internal standard area ratio, and m is the mean slope of the calibration curve. The average total drug recovery was 99.70%, with a standard deviation of ±0.58%. The mean total recovery with the USP procedure was 99.40 ± 0.68%.

In Table II, the results obtained when the GC technique was applied to the analysis of six brands of compressed and four brands of enteric coated single-component aspirin tablets are compared to those obtained with the USP procedure. In all cases, the agreement for aspirin percentage of label claim values was excellent. Product VII was the only sample found to be outside the USP limits for drug content and then only in a labeling sense, since it appears fairly evident from the data that it was formulated to contain 324 mg. aspirin per tablet. Nonaqueous titration of this sample against KOH in methanol with thymol blue as indicator gave an average percentage of label claim of 108.2%, further confirming the validity of the GC and USP results.

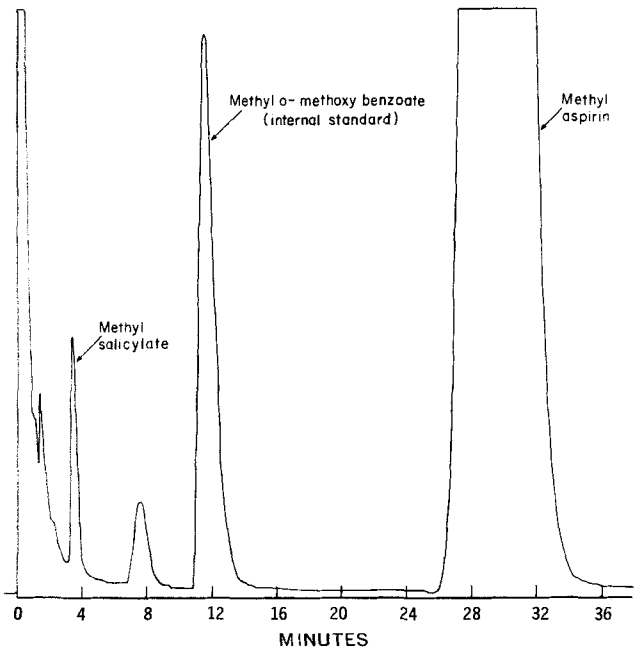

Figure 3—*GC of a methylated aspirin–salicylic acid mixture (salicylic acid ≃ 0.40%).*

The percentage of salicylic acid content determined by the two methods was found to be in line for most of the tablets examined; however, with Formulations I, VII, IX, and X, the amount of salicylic acid recovered was higher with the GC method than with the USP procedure. A problem that has been recognized as important in the determination of salicylic acid, particularly in buffered products, is the possibility of at least partial binding of the free acid by certain excipient materials to form chloroform-insoluble salts, resulting in underestimation of the true salicylic acid content. With this in mind, the USP assay for the mentioned samples was modified in turn to use different extraction media: (*a*) prior trituration of the powder with 1 ml. tetrahydrofuran, (*b*) the extraction medium of Guttman and Salomon (13) (citric acid and chloroform), and (*c*) that suggested by Shane and Miele (7) (HCl–citric acid and chloroform). None of these media, however, effected any increase in the salicylic acid recovery by the USP method, nor did injection of the methylated mixture at various time intervals give any change by the GC procedure.

DISCUSSION

Preparation of Diazomethane and Reaction with Aspirin and Salicylic Acid—Of the methods encountered in the literature for the preparation of diazomethane, the procedure described by Deboer and Backer (12) was considered to be the least hazardous from the point of view of toxicity of starting materials and of relative safety from explosion. All recommended precautionary measures were observed and the reaction quantities were scaled down 10-fold using tetrahydrofuran as the solvent. The yield of diazomethane thus obtained was conveniently sufficient for the methylation of several samples (usually about 10). The choice of solvent was dictated by several factors.

1. The much greater solubility of aspirin in tetrahydrofuran precluded the possibility of loss by fractional crystallization (thereby changing the aspirin–salicylic acid weight ratio) and thereby allowing a higher degree of precision than is possible with the more volatile diethyl ether.

2. The considerably smaller flame-ionization detector (FID) response to the former solvent gave better resolution and concomitant greater reliability of the methyl salicylate peak area integration.

3. A greater yield of diazomethane was obtained with tetrahydrofuran despite a generally longer reaction time.

The ease of hydrolysis of aspirin to salicylic acid when subjected to even trace amounts of hydroxyl ion necessitated distillation of

Table II—Analysis of Single-Component Aspirin Tablets

Product Number	Type	Label Dosage Level, mg./tablet	Found—GC Method, % Label Claim		Found—USP Method, % Label Claim	
			Salicylic Acid	Aspirin	Salicylic Acid	Aspirin
I	Compressed	453.6	0.11	100.8	0.05	98.40
			0.11	99.56	0.05	100.0
II	Compressed	324.0	0.14	100.7	0.13	100.1
			0.14	100.5	0.13	99.38
III	Compressed	324.0	0.07	99.91	0.05	100.0
			0.08	98.37	0.05	100.1
IV	Enteric coated	324.0	0.39	97.93	0.35	101.4
			0.42	98.76	0.38	100.4
V	Enteric coated	324.0	0.22	94.96	0.17	95.63
			0.22	96.71	0.18	95.95
VI	Compressed	324.0	0.12	98.42	0.08	100.6
			0.15	99.71	0.08	100.2
VII	Compressed	300.0	0.19	108.4	0.10	110.1
			0.21	107.4	0.11	110.1
VIII	Enteric coated	600.0	0.93	103.1	0.91	103.0
			0.90	101.5	0.96	102.9
IX	Enteric coated	324.0	4.59	92.37	3.07	93.97
			4.52	95.18	3.13	95.03
X	Compressed	81.0	0.20	101.2	0.12	102.6
			0.21	101.2	0.11	103.6

the product from the strongly alkaline reaction mixture. The yellow distillate was stable over a period of about a week when kept tightly stoppered under refrigeration at −15°.

In a study of phenolic aromatic acids, Seoane and Carnicer (14) concluded, on the basis of pKa considerations, that diazomethane should react completely with salicylic acid without forming any dimethylated compound, but they did not discuss the somewhat analogous case of aspirin in the presence of excess diazomethane. In the present work, when aspirin solutions were treated with diazomethane and stored at room temperature for 3 hr. or more, the conversion of aspirin to methylaspirin was attended by the slow formation of an artifact having retention time closely similar to that of methyl *o*-methoxybenzoate. Whatever the nature of this reaction, it was completely prevented by cooling the methylated sample solution in a frost-free freezer at −15° immediately after preparation and between successive injections of the same sample solution.

As an added measure to preclude artifact formation, thereby ensuring the correctness of the internal standard (methyl *o*-methoxybenzoate) peak area integration, addition of diazomethane to each sample solution was carried out no more than 15 min. prior to chromatography. The esterification was rapid and complete within 5 min. at −15°, and it was without vigorous effervescence when tetrahydrofuran was employed as the solvent. Preparation of the trimethylsilyl derivatives with the BSA[5] reagent was also attempted, but the results appeared to be less satisfactory. The observation of Rowland and Riegelman (10) on the appearance of two derivative peaks for salicylic acid was confirmed but was not investigated further.

GC of the Methyl Esters—GC of aspirin and salicylic acid as the free acids is often accompanied by considerable tailing of the peaks, due mainly to binding of the hydroxyl groups with active adsorption sites on the solid support. It is, therefore, usually necessary to convert these drugs to less polar derivatives (15) and to select a liquid stationary phase which will be relatively nonretentive in order to achieve optimum separation with a minimum of tailing. This is especially important if the peak areas are to be measured accurately. OV-210 is particularly suitable in this respect; under normal conditions of usage, a column of this type exhibits little or no bleedoff.

Figure 3 shows the clearcut separation of the peaks of interest at the low column temperature used in this work. The injection port temperature was kept at an optimum 180°; higher temperatures were found to induce some hydrolysis of the methylaspirin, giving spurious results. The extraneous peak at around 8 min. was due to the stabilizing agent, butylated hydroxyanisole, added to tetrahydrofuran to prevent excessive peroxide formation on storage. However, it was well resolved from the highly symmetrical methyl salicylate, internal standard, and methylaspirin peaks and did not affect the accuracy of the results. Operating isothermally, the total running time for a complete aspirin–salicylic acid analysis was 35 min., only 15 min. being required for the determination of salicylic acid.

Methyl *o*-methoxybenzoate fulfilled all of the criteria of a suitable internal standard; it was well resolved from and eluted close to the other peaks of interest and is of closely similar molecular structure to the compounds being measured. Indeed, area ratio *versus* weight ratio data interpolated from the calibration curves (Figs. 1 and 2) implied, as expected, an almost identical FID response to methyl salicylate, methylaspirin, and methyl *o*-methoxybenzoate. The low levels of salicylic acid usually found in aspirin raw material and commercial dosage forms will generally require a low attenuation setting and a methylaspirin solution of sufficiently high concentration (20–30 mg./ml.) in order to obtain a methyl salicylate peak of area amenable to accurate quantitation. As a result, the methylaspirin peak will invariably be off-scale so that its accurate measurement can be most conveniently achieved with an electronic digital integrator capable of accepting a wide range of input signals (up to 100 mv.). In practice, the signal level output from the detector on methylaspirin elution was in the range of 15–30 mv., which permitted a constant attenuation setting and a minimum of dial switching.

CONCLUSION

The present official procedures for the analysis of salicylic acid and aspirin are tedious and time consuming and require the expertise of a skilled analyst to obtain optimum accuracy and precision. They may be satisfactory for small numbers of individual samples but are not entirely feasible for large-scale investigational or quality control work which demands a relatively high data output. The GC method described here for the simultaneous analysis of aspirin and salicylic acid in synthetic mixtures is rapid, accurate, and precise and would appear to be more suitable for the types of surveys mentioned. A subsequent paper will deal with the analysis of multicomponent aspirin tablets by the proposed technique.

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R. A. SHARP* and A. M. KNEVEL

Abstract □ An analytical method was developed for the determination of barium in barium sulfate and diagnostic meals containing barium sulfate using atomic absorption spectroscopy. The procedure provides satisfactory levels of accuracy and precision and overcomes many disadvantages of currently used procedures such as lack of barium specificity, lengthy analysis time, and interference by diagnostic meal additives.

Keyphrases □ Barium—determination □ Diagnostic meals, barium sulfate—barium determination □ Atomic absorption spectroscopy—analysis

Barium sulfate has been the medium of choice for roentgenographic examination of the gastrointestinal tract for over half a century. In spite of its long use, an assay procedure was not included in the USP until publication of the 18th revision. Major drawbacks that contribute singly or collectively to the shortcomings of existing methods of barium sulfate analysis include lack of accuracy, lack of precision, excessive cost, and lengthy analysis time.

Several analytical techniques have been developed for barium analysis. These include: complexometric procedures using ethylenediaminetetraacetic acid (EDTA) as the titrant (1, 2), flame emission spectroscopy (3), X-ray (4), anodic arc excitation spectrography (5), gravimetry (6–8), and turbidimetry (9, 10). A gravimetric method based on fusion of the sample with sodium carbonate followed by precipitation of barium as barium chromate is the official assay procedure found in USP XVIII (11).

The introduction of atomic absorption spectroscopy (AAS) as a new analytical tool offers another approach for the assay of barium sulfate. The method is very sensitive and highly specific for barium ion (12). Roe *et al.* (13) reported a method for the estimation of low concentrations of sulfur and sulfate in biological materials by conversion of sulfate to barium sulfate and subsequent barium determination by AAS. The objective of this investigation is to evaluate atomic absorption spectroscopy as a method for barium content determination of barium sulfate and diagnostic meals containing barium sulfate.

EXPERIMENTAL

Equipment—A Perkin-Elmer model 290 atomic absorption spectrophotometer with a single slot, model 290-1169, burner head and a Perkin-Elmer Intensitron barium lamp were used for barium content determinations. A lamp current of 7 mamp. was used. The instrument slit width was set at 7 Å, and a meter damper setting of 3 was used. The instrument was adjusted to a coarse select element setting of approximately 709.7. Standard tanks of compressed air and acetylene were used with regulator settings of 40 and 8 psig., respectively. The instrument fuel flow was adjusted to approximately 14.1 and the air flow to approximately 14.8.

Reagents—The initial consideration in the development of an AAS method of analysis for barium sulfate was to find a solvent system in which barium sulfate would be readily soluble. This chemical is virtually insoluble in water and other solvents suitable for use in AAS (14, 15). Organic solvents, such as dimethyl sulfoxide and *N,N*-dimethylformamide, and aqueous solutions containing selected inorganic salts, such as zirconium nitrate and zirconium chloride, were evaluated experimentally and found to be unsuitable as solvent systems. In the course of the solubility study, ethylenediaminetetraacetic acid and its salts were also investigated. It was found that a 0.05 *M* disodium ethylenediaminetetraacetic acid (Na_2EDTA) solution USP XVIII, adjusted to pH 10 with 2 *N* sodium hydroxide solution had solubility properties suitable for the analytical procedure.

Because USP reference standard barium sulfate was not available for the preparation of a barium standard curve, barium sulfate USP was purified in the following manner. The material was dissolved in concentrated sulfuric acid, precipitated by dilution with distilled water, collected by vacuum filtration through a fritted-glass filter, dried, and finally ignited at 1000° for 4 hr. The process was repeated twice, and the purified barium sulfate was stored over silica gel. The barium chloride evaluated for standard curve samples was reagent grade containing 2 moles of water of crystallization. Lanthanum chloride was obtained from a commercial supply house and was of unknown grade. Barium sulfate USP and two commercial diagnostic meals containing barium sulfate were used in the evaluation study. The barium sulfate was obtained from a single lot and assayed following the USP XVIII gravimetric procedure with one exception. The barium sulfate and sodium carbonate mixture was fused using a muffle furnace instead of a blast burner. After the clear melt was obtained, it was then heated at 1000° for 30 min. The results of three determinations were 98.95, 99.92, and 100.0%. The two commercial sources of diagnostic meal were also obtained from single lots.

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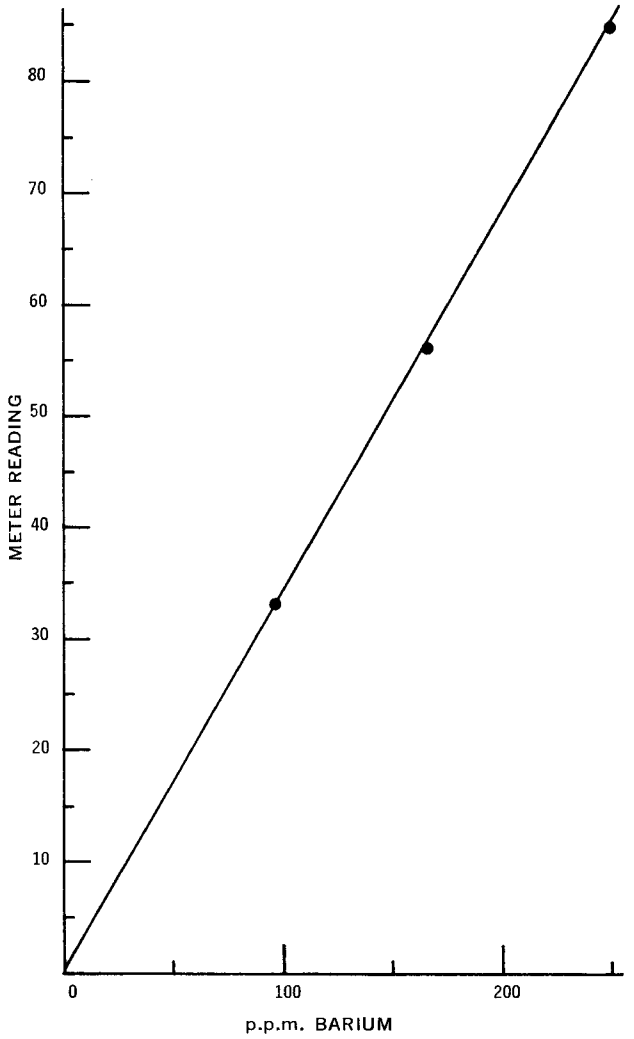

Figure 1—*Barium standard curve using barium sulfate in 0.05* M *Na_2 EDTA, pH 10.*

Selection of Standard—Water-soluble and water-insoluble barium salts were evaluated for construction of a standard barium curve. Aqueous solutions of barium chloride gave a nonlinear plot, and solutions of barium chloride in 0.05 *M* Na_2EDTA (pH 10) gave excessive scatter. Both plots were unsatisfactory for barium determination. As shown in Fig. 1, a standard barium curve, prepared by dissolving purified barium sulfate USP in 0.05 *M* Na_2EDTA (pH 10) solution, gave a linear plot. The reason for the unsuitability of barium chloride was not apparent but may have been caused by the presence of chloride ions.

The addition of lanthanum chloride was evaluated for the enhancement of barium absorption (13). This chemical did not significantly increase the barium response and therefore was not used in the final method.

Sample Preparation—Approximately 127 mg. of barium sulfate USP (150 p.p.m. barium) was mixed for 30 min. at 80° in 400 ml. of the solvent system (0.05 *M* Na_2EDTA, pH 10). The sample solution was cooled to room temperature and quantitatively transferred to a 500-ml. volumetric flask. The solvent system was added to volume, and the sample solution was thoroughly mixed. Diagnostic meal samples were prepared in a similar manner.

Assay Procedure—The atomic absorption spectrophotometer was allowed to warm up for approximately 1 hr. and adjusted for optimum response to a high concentration barium sulfate in the solvent system solution. Distilled water was used for zero adjustment. A standard barium curve was constructed from solutions of purified barium sulfate. Each sample solution containing barium sulfate was remixed by hand-shaking immediately prior to aspiration into the burner flame. Five individual instrument readings were made of each sample, and the average was used to calculate the barium content. Distilled water was aspirated between sample determinations to flush the system of residual sample. The burner head slot was cleaned by scraping with a razor blade after each five-sample determinations, and the nebulizer and pickup tube were cleaned after 10 sample determinations.

Ten individually weighed samples of barium sulfate USP and four individually weighed samples of each diagnostic meal were prepared and analyzed for barium content using AAS. To determine if meal components interfered with the assay method, studies were conducted using the meal formulations without barium sulfate under the same analytical conditions. Negligible absorbance (>0.02) was observed for both mixtures.

Table I—Results of Barium Sulfate USP[a] Assay

Theoretical p.p.m. Barium	Experimental[b] p.p.m. Barium	Percent Recovery
151.4	152	100.4
150.4	149	99.1
164.5	162	98.5
157.4	155	98.5
160.4	159	99.4
161.4	159	98.8
152.4	151	99.1
140.6	140	99.6
147.6	147	99.6
160.4	159	99.1
	Mean	99.2
	Relative *SD*	0.57

[a] 99.63% barium sulfate—average of three determinations. Assayed by USP XVIII modified method. [b] Calculated from average of five instrumental readings. Intrasample variations were all within ±1.0 p.p.m. barium.

RESULTS AND DISCUSSION

Results of the assay of barium sulfate USP are shown in Table I. The mean percent recovery for 10 samples indicates good accuracy. Each determination was well within the limits of purity set forth in USP XVIII. The precision of the method, as shown by the relative standard deviation and the intersample variations, was also very good.

Table II shows the results obtained for the assay of the two diagnostic meals. Since the exact amount of barium sulfate in these samples is unknown, neither the mean percent recovery nor the standard deviation values are as significant as those of the known barium sulfate USP samples. However, the data do show that diagnostic meals can be assayed satisfactorily by AAS.

CONCLUSIONS

A precise and accurate method of analysis was developed for barium sulfate using AAS. The procedure is particularly useful

Table II—Results of Diagnostic Meal Analysis for Barium Sulfate

Labeled	Experimental[a]	Percent Recovery
	Meal A, p.p.m. Barium	
152	153	100.6
156	160	102.5
153	153	100.0
146	148	102.0
	Mean	101.3
	Relative *SD*	1.16
	Meal B, p.p.m. Barium	
154	157	101.9
146	149	102.0
149	150	100.6
147	145	98.6
	Mean	100.8
	Relative *SD*	1.57

[a] Calculated from average of five instrumental readings. Intrasample variations were all within ±1.0 p.p.m. barium.

because it is applicable to the analysis of diagnostic meals containing barium sulfate.

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Rapid Assay of Hydrogen Peroxide Solution (USP XVIII) *via* UV Spectrophotometry

J. TIETJEN and A. MANCOTT

Abstract □ Assay of hydrogen peroxide solution (USP XVIII) was accomplished by direct UV spectrophotometric analysis. This determination can be done in less than 30 sec. with a calibrated spectrophotometer and is comparable in accuracy to the USP XVIII titrometric procedure.

Keyphrases □ Hydrogen peroxide solution—analysis □ UV spectrophotometry—analysis

The spectrum and absorbance of hydrogen peroxide solutions in the UV range have been investigated (1–6). Bessun and Spitz (6) reported that dilute hydrogen peroxide solutions (10 mcg./ml.) followed Beer's law when measured at wavelengths between 195 and 220 nm. The molar absorptivity of hydrogen peroxide solutions decreases rapidly at wavelengths above 275 nm., and no appreciable absorbance is recorded at wavelengths greater than 375 nm.

To perform a direct UV spectrophotometric assay on 3% hydrogen peroxide solutions (USP XVIII) without employing time-consuming and less accurate dilution techniques, a wavelength of 304 nm. was chosen, which is substantially higher than that of maximum absorption. The very low molar absorptivity at 304 nm. permits direct measurement of hydrogen peroxide solutions in the 2.0–4.5% range.

The UV spectrophotometric assay reported here can be done in a matter of seconds with a calibrated spectrophotometer. It requires no chemicals or reagents and is comparable in accuracy to the USP XVIII titrometric procedure (7).

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with a spectrophotometer[1]. Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—Hydrogen peroxide solution (30%)[2,3], 0.1 *N* potassium permanganate solution[4], and hydrogen peroxide solutions USP XVIII[5] were assayed. All other reagents used were of the highest commercial grade available.

Procedure—The spectrophotometer (slit width 5 Å, deuterium lamp) was adjusted to zero absorbance with distilled water. The wavelength was calibrated against a mercury emission spectrum and set at 304.0 nm. with the controls locked. Solutions of hydrogen peroxide were prepared by dilution of 30% hydrogen peroxide and standardized with 1.0 *N* potassium permanganate solution. Absorbances of the standard hydrogen peroxide solutions were measured. Samples of hydrogen peroxide solutions USP XVIII were assayed by measuring their absorbance at 304.0 nm. and comparing with the standards. The same samples were also assayed according to the USP XVIII titrometric procedure.

RESULTS AND DISCUSSION

Absorbance readings for standardized hydrogen peroxide solutions in the concentration range of 2.1–4.3 g./100 ml. were obtained (Table I). A graph of concentration *versus* absorbance was linear, with a slope of 4.386. The concentration of hydrogen peroxide in a given sample is found from Eq. 1:

$$C = 4.386A \qquad \text{(Eq. 1)}$$

where A = absorbance, and C = grams of H_2O_2 per 100 ml. of solution.

[1] Bausch & Lomb, model 505.
[2] Fisher certified reagent.
[3] B&A reagent, Allied Chemical.
[4] Fisher certified reagent.
[5] Parke-Davis & Co., Detroit, Mich.

because it is applicable to the analysis of diagnostic meals containing barium sulfate.

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Rapid Assay of Hydrogen Peroxide Solution (USP XVIII) *via* UV Spectrophotometry

J. TIETJEN and A. MANCOTT

Abstract □ Assay of hydrogen peroxide solution (USP XVIII) was accomplished by direct UV spectrophotometric analysis. This determination can be done in less than 30 sec. with a calibrated spectrophotometer and is comparable in accuracy to the USP XVIII titrometric procedure.

Keyphrases □ Hydrogen peroxide solution—analysis □ UV spectrophotometry—analysis

The spectrum and absorbance of hydrogen peroxide solutions in the UV range have been investigated (1–6). Bessun and Spitz (6) reported that dilute hydrogen peroxide solutions (10 mcg./ml.) followed Beer's law when measured at wavelengths between 195 and 220 nm. The molar absorptivity of hydrogen peroxide solutions decreases rapidly at wavelengths above 275 nm., and no appreciable absorbance is recorded at wavelengths greater than 375 nm.

To perform a direct UV spectrophotometric assay on 3% hydrogen peroxide solutions (USP XVIII) without employing time-consuming and less accurate dilution techniques, a wavelength of 304 nm. was chosen, which is substantially higher than that of maximum absorption. The very low molar absorptivity at 304 nm. permits direct measurement of hydrogen peroxide solutions in the 2.0–4.5% range.

The UV spectrophotometric assay reported here can be done in a matter of seconds with a calibrated spectrophotometer. It requires no chemicals or reagents and is comparable in accuracy to the USP XVIII titrometric procedure (7).

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with a spectrophotometer[1]. Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—Hydrogen peroxide solution (30%)[2,3], 0.1 *N* potassium permanganate solution[4], and hydrogen peroxide solutions USP XVIII[5] were assayed. All other reagents used were of the highest commercial grade available.

Procedure—The spectrophotometer (slit width 5 Å, deuterium lamp) was adjusted to zero absorbance with distilled water. The wavelength was calibrated against a mercury emission spectrum and set at 304.0 nm. with the controls locked. Solutions of hydrogen peroxide were prepared by dilution of 30% hydrogen peroxide and standardized with 1.0 *N* potassium permanganate solution. Absorbances of the standard hydrogen peroxide solutions were measured. Samples of hydrogen peroxide solutions USP XVIII were assayed by measuring their absorbance at 304.0 nm. and comparing with the standards. The same samples were also assayed according to the USP XVIII titrometric procedure.

RESULTS AND DISCUSSION

Absorbance readings for standardized hydrogen peroxide solutions in the concentration range of 2.1–4.3 g./100 ml. were obtained (Table I). A graph of concentration *versus* absorbance was linear, with a slope of 4.386. The concentration of hydrogen peroxide in a given sample is found from Eq. 1:

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Table I—Absorbance of Known Hydrogen Peroxide Solutions

Concentration of H_2O_2, g./100 ml.	Absorbance at 304.0 nm.
4.335	0.988
3.902	0.890
3.468	0.790
3.034	0.691
2.601	0.593
2.167	0.495

The USP XVIII standard for hydrogen peroxide solution (7)[6] is that 100 ml. of solution contains not less than 2.5 g. and not more than 3.5 g. of H_2O_2.

Determinations of seven random samples of hydrogen peroxide solutions were performed by both spectrophotometric and titrometric procedures. Agreement between both methods was within ±2 parts per 1000. The error introduced by working on a steep area of the absorption band is small when compared to the errors encountered using dilution techniques. Also, an appreciable saving in time, labor, and materials results by direct measurement of the hydrogen peroxide solution. An accurate calibration of the wavelength (either with a mercury emission spectrum or a solution of known hydrogen peroxide concentration) is essential for the reproducibility of this method[7]. By locking the controls of the spectrophotometer once it has been calibrated, many samples of hydrogen peroxide can then be assayed in a very brief period.

[6] Preservatives in hydrogen peroxide solution USP XVIII total not more than 0.05%. The stabilizer did not interfere with the spectrophotometric determination reported here.

[7] Calibration of the spectrophotometer using a sample of hydrogen peroxide whose concentration was found titrometrically requires more time but gives more accurate results.

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Fluorometric Determination of Amphetamines with 3-Carboxy-7-hydroxycoumarin

J. T. STEWART and D. M. LOTTI

Abstract □ A fluorometric method of analysis for amphetamines based on the interaction between aliphatic and/or cyclic amines and 3-carboxy-7-hydroxycoumarin to yield highly fluorescent coumarin–amine salts was investigated. The procedure was applied to the analysis of several amphetamines and amphetamine mixtures. Comparison of the coumarin method to other existing fluorometric methods for amines in the analysis of amphetamines was performed.

Keyphrases □ Amphetamines—analysis □ 3-Carboxy-7-hydroxycoumarin–cyclic amine reaction—fluorescence □ Fluorometry—analysis

The objective of this investigation was to evaluate a fluorometric method of analysis for amphetamines based on the interaction between aliphatic and/or cyclic amines and 3-carboxy-7-hydroxycoumarin to yield highly fluorescent coumarin–amine salts. The assay procedure applied to aliphatic and cyclic amines was reported previously from this laboratory (1). Use of the method permits determination of trace quantities of amines based upon fluorometric measurement of coumarin–amine salt, even in the presence of excess fluorescent coumarin reagent.

In this study, the method was applied to the analysis of several amphetamines and amphetamine mixtures. A study demonstrating the usefulness of the procedure to commercial dosage forms containing amphetamines was made. Comparison of this procedure to other existing fluorometric methods for amines was performed.

EXPERIMENTAL

Apparatus—Fluorescence spectra and measurements were made with a spectrophotofluorometer[1]. Clear, fused quartz cells (12.5 × 47 mm.) were used as sample cells.

Reagents and Chemicals—Powdered samples of dextroamphetamine sulfate[2], methamphetamine hydrochloride[3], benzphetamine hydrochloride[4], chlorphentermine hydrochloride[5], methylphenidate hydrochloride[6], phenmetrazine hydrochloride[7], and phendimetrazine tartrate[8] were used in the analytical procedure for preparation of standard solutions. 3-Carboxy-7-hydroxycoumarin was synthesized according to the procedure of Woods and Sapp (2). All other chemicals used were the highest grade of the commercially available materials.

Solutions of amphetamine salts (8×10^{-5} *M*) and 3-carboxy-7-hydroxycoumarin (6.8×10^{-4} *M*) were prepared by dissolving

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[5] Warner-Lambert Research Institute, Morris Plains, N. J.; research affiliate of Warner-Chilcott Laboratories.
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J. T. STEWART and D. M. LOTTI

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Keyphrases □ Amphetamines—analysis □ 3-Carboxy-7-hydroxycoumarin–cyclic amine reaction—fluorescence □ Fluorometry—analysis

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[7] Geigy Pharmaceuticals, Ardsley, N. Y.
[8] Ayerst Laboratories, Inc., New York, N. Y.

Table I—Analysis of Amphetamines

Amphetamine	Range[a], mcg./25 ml.
Amphetamine sulfate	7.4–118.4
Methamphetamine hydrochloride	3.7–59.2
Benzphetamine hydrochloride	5.5–88.0
Chlorphentermine hydrochloride	4.4–70.4
Methylphenidate hydrochloride	5.4–86.4
Phenmetrazine hydrochloride	4.3–68.8
Phendimetrazine tartrate	27.3–109.2

[a] Range observed for linear response extending through the origin.

weighed amounts of the amphetamine salts in water and 3-carboxy-7-hydroxycoumarin in ethanol.

Procedure—A quantity of amphetamine salt, indicated in Table I, dissolved in 1 ml. of water was placed in a 30-ml. separator followed by the addition of 3 drops of 0.1 *N* sodium hydroxide solution. The solution was extracted once with 5 ml. of diethyl ether, and the extract was washed with two 1-ml. portions of water. The ether extract was then transferred to a 25-ml. volumetric flask and evaporated to near dryness on a steam bath. Two milliliters of ethanol was added, followed by the addition of 1 ml. of an ethanolic solution of 3-carboxy-7-hydroxycoumarin. Samples, together with control solutions of reagent, were shaken at room temperature for 5 min. and diluted to volume with ethanol. The increases in fluorescence of reaction mixtures compared to their corresponding reagent blanks were measured using activation and emission wavelength settings of 420 and 450 nm., respectively.

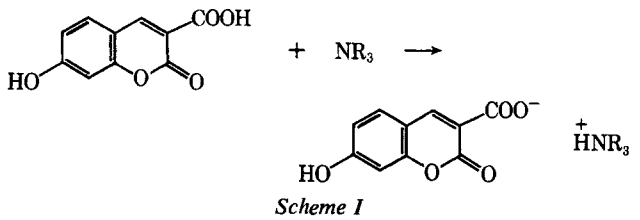
RESULTS AND DISCUSSION

In the analytical procedure, an amphetamine interacts with 3-carboxy-7-hydroxycoumarin to yield a highly fluorescent coumarin–amine salt (Scheme I). This interaction results in an increase in fluorescence and is accompanied by a change in the activation wavelength of the reaction mixture. The use of 420 and 450 nm. as the excitation and emission wavelengths, respectively, for the procedure can be correlated with known amphetamines run as analytical solutions and corresponds to pure synthesized coumarin–amine salts formed from various aliphatic and cyclic amines in the original method (1). The analysis is applicable to organic bases having pKa values of approximately 8 or greater. Reproducibility of the procedure can be shown by the standard deviation of the mean, which is 0.87%.

The method permits detection of fluorescence of the final product and correlation of fluorescence to concentration of drug even in the presence of a 2- to 32-fold excess of fluorescent coumarin reagent.

Analytical results for amphetamines successfully examined by this method are shown in Table I. The analysis of unknown concentrations of these drugs in this procedure requires direct comparison of the fluorescence of an unknown to that of a known concentration under the same conditions. The linear fluorescence-to-concentration range indicates the range over which samples could be compared.

Quantitative data from several systems shown in Table II reveal that use of this procedure permits the determination of amphetamines in the presence of various medicinals found in combination with them in commercial dosage forms. Earlier studies showed that primary, secondary, and/or tertiary aromatic amines, aromatic heterocycles, aromatic and aliphatic amides, and carbonyl-containing compounds also do not interfere with this method (1). However, due to the nature of the assay procedure, the presence of other extractable strongly basic organic compounds can significantly interfere with the fluorescent readings of the various amphetamines.



Scheme I

Table II—Analysis of Known Amphetamine Mixtures for Amphetamine

Mixture, Concentration of 3.2×10^{-6} *M*	Amphetamines—Found[a] $\times 10^{-6}$ *M*	Amphetamines—Percent of Theory
Amphetamine sulfate–amobarbital	3.15	98.4
Methamphetamine hydrochloride–nicotinic acid–thiamine mononitrate	3.16	98.8
Benzphetamine hydrochloride–aspirin–phenacetin	3.14	98.0
Chlorphentermine hydrochloride–chlorpromazine hydrochloride	3.20	100.0
Methylphenidate hydrochloride–meprobamate	3.15	98.4
Phendimetrazine tartrate–ascorbic acid–riboflavin	3.07	96.0

[a] Values represent average of four determinations.

Generally, it has been shown in this laboratory that inorganic acids and bases and organic acids interfere with the analytical determination unless the amphetamine can be successfully removed from them with the use of a suitable separation technique. In this study, solvent extraction of the free amphetamine bases with ether was sufficient to permit determination of these substances in mixtures containing various acidic and basic components (Table II).

No heating of the analytical solution is required to ensure a more complete reaction. Maximum fluorescence development and, hence, yield of product can be obtained upon shaking the solution at room temperature for 3–5 min. Repeated readings for a series of different samples indicated that the fluorescence of the solutions was stable for periods up to 1 day. Great care was taken to prevent overexposure of the samples to the xenon source of the fluorometer as well as to prevent contamination of solutions and cells used in the analysis.

The method of analysis of amphetamines by the 3-carboxy-7-hydroxycoumarin approach was performed on various dosage forms; comparative analyses were conducted using other known fluorometric procedures for aliphatic and/or cyclic amines (3, 4). The literature does not reveal that any of these existing fluorometric methods have been used for the analysis of amphetamines. The coumarin method possesses the advantages that: (*a*) only one re-

Table III—Comparative Determinations of Various Dosage Forms Containing Amphetamines by the 3-Carboxy-7-hydroxycoumarin and Other Known Fluorometric Methods

Amphetamine	Mean Percent of Labeled Amount—3-Carboxy-7-hydroxycoumarin Method	Acetylacetone Method[a]	Aconitic Anhydride Method[b]	Dansyl Chloride[d] Method[b]
Amphetamine sulfate[c]	97.7	95.7	—[d]	96.0
Methamphetamine hydrochloride[e]	100.0	—	—	95.3
Benzphetamine hydrochloride[f]	97.0	—	97.3	—
Methylphenidate hydrochloride[g]	100.0	—	—	90.0
Phenmetrazine hydrochloride[h]	96.3	—	—	93.3
Phendimetrazine tartrate[i]	96.1	—	—	—

[a] *Reference 3.* [b] *Reference 4.* [c] Benzedrine tablets, Smith Kline & French. [d] Blank denotes method not applicable to particular type of amine listed. [e] Desoxyn tablets, Abbott Laboratories. [f] Didrex tablets, The Upjohn Laboratories. [g] Ritalin tablets, Ciba Corp. [h] Preludin tablets, Geigy Pharmaceuticals. [i] Plegine tablets, Ayerst Laboratories.

agent is necessary for a wide variety of amines, and (*b*) concentration sensitivity is comparable and in most cases better than the other procedures listed (1).

Four determinations using the various methods were performed on each tablet. The mean percent of labeled amount for each drug is shown in Table III for all the methods.

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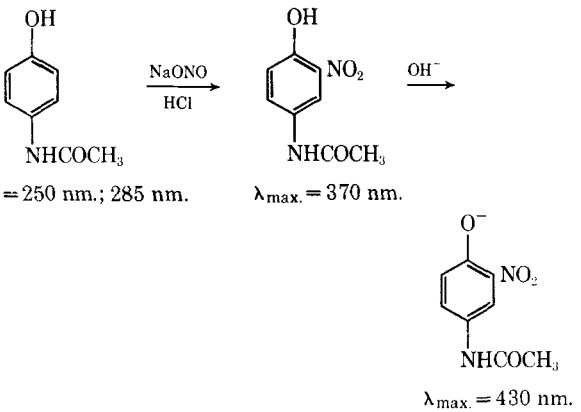
Selective Colorimetric Determination of Acetaminophen

LESTER CHAFETZ, ROBERT E. DALY, HERBERT SCHRIFTMAN, and JOSEPH J. LOMNER

Abstract □ A method for the quantitative determination of acetaminophen as 2-nitro-4-acetamidophenol, formed by the reaction of nitrous acid with the drug in aqueous solution, was compared with methods using this reaction reported by others. Phenacetin causes only negligible interference in the assay method; however, salicylamide forms a chromophore which interferes in the determination of acetaminophen. No other interferences were encountered. The procedure is stability indicating with respect to hydrolysis, and it appears to be adaptable to automated apparatus. Good recovery and precision data were obtained on application of the procedure to conventional and sustained-action tablet formulations declaring acetaminophen, phenacetin, phenylpropanolamine HCl, and phenyltoloxamine dihydrogen citrate.

Keyphrases □ Acetaminophen in dosage forms—analysis □ Nitrous acid–acetaminophen reaction—analysis method □ Colorimetric analysis—spectrophotometer

Most colorimetric methods (1–6) described for acetaminophen require preliminary hydrolysis of it to *p*-aminophenol. A significant contribution to methods for acetaminophen was provided by Le Perdriel *et al.* (7). They discovered that acetaminophen and nitrous acid react under mild conditions to form 2-nitro-4-acetamidophenol, which can be measured by its color in alkaline solution (Scheme I). They found no interference from structurally similar drugs such as phenacetin (acetaminophen *O*-ethyl ether) or acetanilide. Unlike the colorimetric method described by Brockelt (8), where the same chromophore is produced by nitration with nitric acid, there is no interference from *p*-aminophenol; thus the reaction of Le Perdriel *et al.* (7) is stability indicating for acetaminophen with respect to hydrolysis. Independently and later, Inamdar and Kaji (9) reported use of the chromophore formed by the reaction of nitrous acid with acetaminophen for dosage form assay; however, they measured the yellow color in acid solution instead of the orange-red color of the phenolate ion, and they attributed the chromophore to nitroso derivatives.



Scheme I

This report describes further investigations on the reaction of acetaminophen with nitrous acid and extends the observations of previous workers (7, 9). A modified procedure is proposed, and results obtained in the assay of some acetaminophen dosage forms are described.

EXPERIMENTAL

Equipment and Supplies—Acetaminophen NF, salicylamide NF, phenacetin USP, 6 *N* hydrochloric acid, 10% sodium nitrite, 15% sulfamic acid, and 10% sodium hydroxide were used. Spectra were determined in 1-cm. silica cells in a Cary model 14 recording spectrophotometer or in a Beckman DU fitted with the Gilford model 222 modification.

Proposed Method—*Standard Preparation*—Accurately weigh about 100 mg. of acetaminophen reference standard NF, dissolve it in water, and dilute to 100 ml. in a volumetric flask. Further dilute 10.0 ml. to 100 ml. with water to obtain a standard concentration of about 100 mcg./ml.

Assay Preparation—Weigh and finely powder not less than 20 tablets. Transfer a portion of the powder equivalent to about 100 mg. of acetaminophen to a glass-stoppered 250-ml. conical flask, add exactly 100.0 ml. of water, shake the mixture mechanically for 10 min., and filter. Dilute 10.0 ml. of the filtrate to 100 ml. with water in a volumetric flask.

Procedure—Transfer 10.0-ml. portions of the assay preparation and the standard preparation to 50-ml. volumetric flasks. Add successively to each flask 2.0 ml. of 6 *N* hydrochloric acid and 5.0 ml. of 10% sodium nitrite. Mix, let stand 15 min., and then destroy excess nitrous acid with 5.0 ml. of 15% sulfamic acid. After nitro-

agent is necessary for a wide variety of amines, and (*b*) concentration sensitivity is comparable and in most cases better than the other procedures listed (1).

Four determinations using the various methods were performed on each tablet. The mean percent of labeled amount for each drug is shown in Table III for all the methods.

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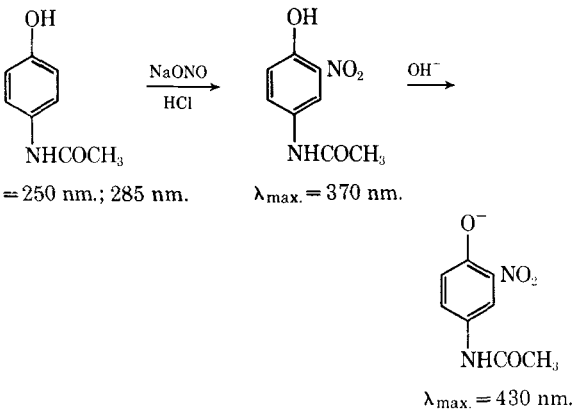
Selective Colorimetric Determination of Acetaminophen

LESTER CHAFETZ, ROBERT E. DALY, HERBERT SCHRIFTMAN, and JOSEPH J. LOMNER

Abstract □ A method for the quantitative determination of acetaminophen as 2-nitro-4-acetamidophenol, formed by the reaction of nitrous acid with the drug in aqueous solution, was compared with methods using this reaction reported by others. Phenacetin causes only negligible interference in the assay method; however, salicylamide forms a chromophore which interferes in the determination of acetaminophen. No other interferences were encountered. The procedure is stability indicating with respect to hydrolysis, and it appears to be adaptable to automated apparatus. Good recovery and precision data were obtained on application of the procedure to conventional and sustained-action tablet formulations declaring acetaminophen, phenacetin, phenylpropanolamine HCl, and phenyltoloxamine dihydrogen citrate.

Keyphrases □ Acetaminophen in dosage forms—analysis □ Nitrous acid–acetaminophen reaction—analysis method □ Colorimetric analysis—spectrophotometer

Most colorimetric methods (1–6) described for acetaminophen require preliminary hydrolysis of it to *p*-aminophenol. A significant contribution to methods for acetaminophen was provided by Le Perdriel *et al.* (7). They discovered that acetaminophen and nitrous acid react under mild conditions to form 2-nitro-4-acetamidophenol, which can be measured by its color in alkaline solution (Scheme I). They found no interference from structurally similar drugs such as phenacetin (acetaminophen *O*-ethyl ether) or acetanilide. Unlike the colorimetric method described by Brockelt (8), where the same chromophore is produced by nitration with nitric acid, there is no interference from *p*-aminophenol; thus the reaction of Le Perdriel *et al.* (7) is stability indicating for acetaminophen with respect to hydrolysis. Independently and later, Inamdar and Kaji (9) reported use of the chromophore formed by the reaction of nitrous acid with acetaminophen for dosage form assay; however, they measured the yellow color in acid solution instead of the orange-red color of the phenolate ion, and they attributed the chromophore to nitroso derivatives.



Scheme I

This report describes further investigations on the reaction of acetaminophen with nitrous acid and extends the observations of previous workers (7, 9). A modified procedure is proposed, and results obtained in the assay of some acetaminophen dosage forms are described.

EXPERIMENTAL

Equipment and Supplies—Acetaminophen NF, salicylamide NF, phenacetin USP, 6 *N* hydrochloric acid, 10% sodium nitrite, 15% sulfamic acid, and 10% sodium hydroxide were used. Spectra were determined in 1-cm. silica cells in a Cary model 14 recording spectrophotometer or in a Beckman DU fitted with the Gilford model 222 modification.

Proposed Method—*Standard Preparation*—Accurately weigh about 100 mg. of acetaminophen reference standard NF, dissolve it in water, and dilute to 100 ml. in a volumetric flask. Further dilute 10.0 ml. to 100 ml. with water to obtain a standard concentration of about 100 mcg./ml.

Assay Preparation—Weigh and finely powder not less than 20 tablets. Transfer a portion of the powder equivalent to about 100 mg. of acetaminophen to a glass-stoppered 250-ml. conical flask, add exactly 100.0 ml. of water, shake the mixture mechanically for 10 min., and filter. Dilute 10.0 ml. of the filtrate to 100 ml. with water in a volumetric flask.

Procedure—Transfer 10.0-ml. portions of the assay preparation and the standard preparation to 50-ml. volumetric flasks. Add successively to each flask 2.0 ml. of 6 *N* hydrochloric acid and 5.0 ml. of 10% sodium nitrite. Mix, let stand 15 min., and then destroy excess nitrous acid with 5.0 ml. of 15% sulfamic acid. After nitro-

Table I—Synopsis of Methods for Acetaminophen Colorimetry with Nitrous Acid

Method	Proposed	*Reference 7*	*Reference 9*
Acetaminophen, mcg./ml.	100	100	2000
Milliliters taken	10.0	1.0	1.0
Sample solvent	Water	Alcohol or water	1.0 ml. methanol, then 10 ml. water
HCl added	2.0 ml. 6 *N*	0.5 ml. 6 *N*	10 ml. 10%
10% NaONO added	5.0 ml.	1.0 ml.	1.0 ml.
Minutes of reaction	15	15	15
15% H_2NSO_3H added	5.0 ml.	Nil	Nil
Diluted with	15.0 ml. 10% NaOH	5 ml. 5% Na_2CO_3	5 ml. alcohol
Wait time (minutes)	To cool	Nil	1
Dilute to	50 ml. with water	–	100 ml. with water
Blank	Water	Reagent	Modified[a] reagent
Absorbance maximum	About 430 nm.	440–445 nm.	395 nm.

[a] See text under *Chemistry of the Reaction.*

gen evolution has ceased, add 15.0 ml. of 10% sodium hydroxide, cool under the tap, and dilute to volume with water. Concomitantly determine the absorbances of the solutions from the assay preparation and the standard preparation in 1-cm. cells in a suitable spectrophotometer at the wavelength of maximum absorbance at about 430 nm., using water as the blank. Calculate the quantity, in milligrams per tablet, of $C_8H_9NO_2$ by the formula $C(A_U/A_S)(TW/SW)$, where: C is the concentration, in micrograms per milliliter, in the standard preparation; A_U and A_S are the absorbances of the solutions from the assay preparation and the standard preparation, respectively; and TW and SW are the weights, in milligrams, of the average tablet and of the sample taken for assay, respectively.

Experiment on Phenacetin Interference—*Acetaminophen Stock Solution*—Dissolve an accurately weighed quantity of about 250 mg. of acetaminophen in water, and dilute the solution to 100 ml.

Acetaminophen Reference Solution—Transfer 10 ml. of alcohol to a 100-ml. volumetric flask. Add exactly 40.0 ml. of the acetaminophen stock solution, and dilute to volume with water. Further dilute 10.0 ml. with water to obtain a solution containing about 100 mcg./ml. of acetaminophen.

Acetaminophen–Phenacetin Test Solution—Dissolve an accurately weighed quantity of about 100 mg. of phenacetin in 10 ml. of alcohol in a 100-ml. volumetric flask. Add exactly 40 ml. of acetaminophen stock solution, and dilute the solution to volume with water. Further dilute 10.0 ml. to 100.0 ml. with water to obtain a solution containing about 100 mcg./ml. each of acetaminophen and phenacetin.

Procedure A—Transfer 10.0-ml. portions of the acetaminophen reference solution and the acetaminophen–phenacetin test solution to 50-ml. volumetric flasks. Add successively to each flask 2.0 ml. of 6 *N* hydrochloric acid and 5.0 ml. of 10% sodium nitrite. Mix, let stand 15 min., and then destroy excess nitrous acid with 5.0 ml. of 15% sulfamic acid. After nitrogen evolution has ceased, dilute to volume with water. Concomitantly determine the absorption spectra of the solutions in 1-cm. cells in a suitable recording spectrophotometer, using a 1-in-100 solution of alcohol in water as the blank, and compare the spectrograms.

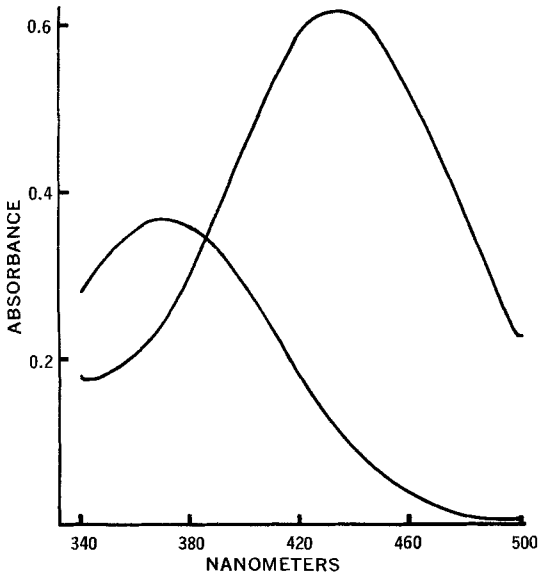


Figure 1—*Absorption spectra of acetaminophen chromophore in acid (low wavelength maximum) and in base.*

Procedure B—Follow Procedure A but change the directions following: "After nitrogen evolution has ceased,..." to "add 15.0 ml. of 10% sodium hydroxide, cool under the tap, and dilute to volume with water." Continue as directed under Procedure A with "Concomitantly determine...."

Experiment on Salicylamide Interference—*Acetaminophen Stock Solution and Reference Solution*—Use solutions described under *Experiment on Phenacetin Interference.*

Salicylamide Reference Solution—Dissolve about 300 mg. of salicylamide, accurately weighed, in 10 ml. of alcohol in a 100-ml. volumetric flask and dilute to volume with water. Further dilute 40.0 ml. to 100.0 ml. with water.

Acetaminophen–Salicylamide Test Solution—Dilute 40.0 ml. of acetaminophen stock solution to 100.0 ml. with water. Transfer 10.0-ml. portions of the dilution and of salicylamide reference solution to a 100.0-ml. volumetric flask; dilute the solution to volume with water to obtain a solution containing about 100 mcg./ml. of acetaminophen and about 120 mcg./ml. of salicylamide.

Procedure—Dilute 10.0 ml. of salicylamide reference solution to 100.0 ml. with water. Transfer 10.0-ml. portions of this dilution, of acetaminophen reference solution, and of acetaminophen–salicylamide test solution to separate 50-ml. volumetric flasks. Follow Procedure B under *Experiment on Phenacetin Interference* beginning with: "Add successively to each flask...."

Recovery Experiments—Add accurately weighed amounts of acetaminophen to portions of an analytically prepared blend of the remaining constituents of each of two commercial acetaminophen tablet formulations. Assay the samples by the method described under *Proposed Method.*

RESULTS AND DISCUSSION

Comparison of Methods—The similarities and differences among the proposed method and those described by Le Perdriel *et al.* (7) and Inamdar and Kaji (9) are evident in Table I.

Chemistry of the Reaction—Le Perdriel *et al.* (7) provided convincing evidence that 2-nitro-4-acetamidophenol is the reaction product—*viz.*, its spectra and that of its acid hydrolysis product are qualitatively and quantitatively superimposible on those of authentic 2-nitro-4-acetamidophenol and 2-nitro-4-aminophenol, respectively. Furthermore, TLC of a chloroform extract of the solution after color development provides a spot identical in R_f and reaction to that of the authentic compound. Moreover, nitration with nitrous acid is a well-known reaction of phenols (10). Rapoport *et al.* (11) determined the electronic absorption spectral constants for 2-nitro-4-acetamidophenol in 0.1 *N* sodium hydroxide and in 0.1 *N* hydrochloric acid, reporting maxima at 429 nm. (ϵ = 4170) in base and at 375 nm. (ϵ = 2250) in acid. Using the

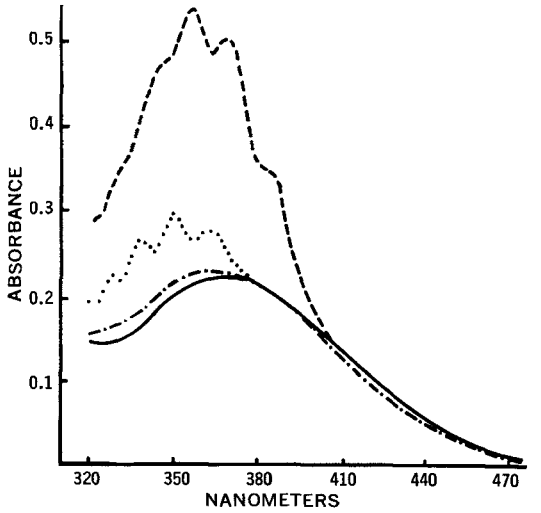


Figure 2—*Absorption spectra of acetaminophen chromophore prepared by the Inamdar and Kaji (9) method. Key: . . ., determined as in* Reference 9; - - -, *determined* versus *water;* —·—, *sulfamic acid added and water used as the blank; and* ——, *determined* versus *reagent blank.*

method proposed in this report, the acetaminophen reaction product shows maxima at about 432 nm. (ϵ = 3800) in base and at about 371 nm. (ϵ = 2010) in acid solution, *i.e.*, where no 10% sodium hydroxide is added after destruction of excess nitrous acid. Spectra of the chromophore are shown in Fig. 1. From the ratio of the absorptivities determined for the acetaminophen reaction product by the proposed method to those reported for the authentic compound (11), one may estimate that about 90% of the drug is converted to the chromophore.

Inamdar and Kaji (9) reported maxima at 375 and 395 nm., and they recommended use of the longer wavelength for determination of the acetaminophen reaction product. Their observations are at variance with the spectral data reported by Rapoport *et al.* (11). From the close similarity of the Le Perdriel *et al.* (7) and Inamdar and Kaji (9) methods evident in Table I, one would expect the chemistry to be identical. This was confirmed using the TLC system described by Le Perdriel *et al.* (7). Inamdar and Kaji (9) recommended a blank made by substituting water for the sample; *i.e.*, the sample solution contains 1 ml. of methanol, but the blank does not. They averred that addition of 95% alcohol destroys excess nitrous acid. In the present study, their method (9) was found to give spectra which showed nitrous acid peaks, and the wavelength designated by them for measurement lay on the slope of the curve (Fig. 2). Addition of sulfamic acid to destroy excess nitrous acid afforded a spectrum with a maximum at about 370 nm. (ϵ = 1570), indicating a reaction efficiency of about 70%. A similar curve was obtained when a true blank was used.

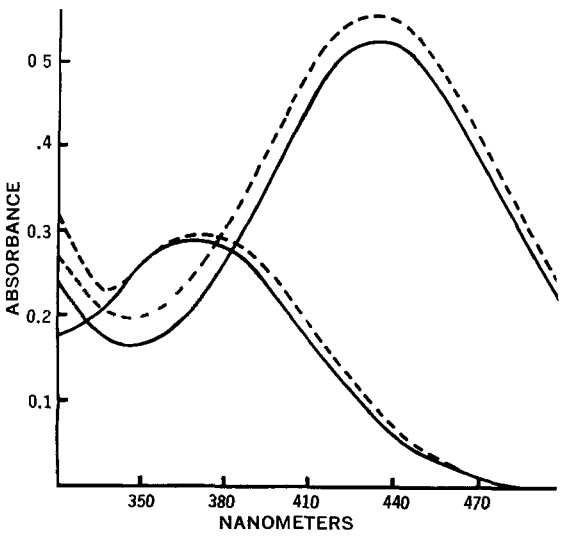


Figure 3—*Absorption spectra of acetaminophen chromophore formed in the presence of only acetaminophen* (——) *and in the presence of an equal concentration of phenacetin* (- - -).

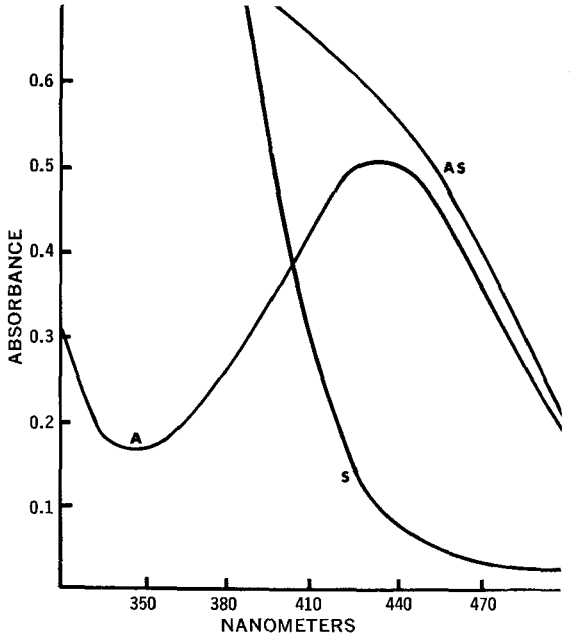


Figure 4—*A = absorption spectra of chromophore representing 20.5 mcg./ml. of acetaminophen. S = absorption spectra of chromophore representing 24.1 mcg./ml. of salicylamide. AS = absorption spectra from chromophore representing mixture of acetaminophen and salicylamide.*

Solvent Effect—Substituting methanol for ethanol in the Le Perdriel *et al.* (7) method lessened the absorptivity obtained by about 15%. The standard curve, using either ethanol or methanol, deviated from Beer's law at acetaminophen concentrations above about 35 mcg./ml. Using the wholly aqueous system of the proposed method, Beer's law conformance was obtained up to about 60 mcg./ml., the highest concentration tested, corresponding to an absorbance reading of about 1.47 units. An hypothesis that the difference in behavior of the aqueous solution from that of the alcohol solutions could be due to carbonyl compound impurities in the alcohols proved untenable: solutions of acetaminophen containing formaldehyde or acetone gave identical spectra with those prepared in water alone. The small percentage of alcohol in the solution determined served to displace the maximum to a slightly higher wavelength.

Selectivity of the Assay—Le Perdriel *et al.* (7) reported that aspirin, acetanilide, phenacetin, phenobarbital, tripelennamine, lidocaine, eucalyptol, glyceryl guaiacolate, sodium camphorsulfonate, *para*-hydroxybenzoate ester preservatives, and a number of tablet excipients did not interfere. The present authors have extended the list of drugs that do not interfere in the assay to include phenylpropanolamine and phenyltoloxamine.

Phenacetin Effect—Because nitration with nitrous acid is a reaction characteristic of phenols and phenolic ethers, the lack of

Table II—Recovery of Acetaminophen Added in Tablet Formulations

Formulation	mg. Acetaminophen Added per Average Tablet Weight	mg. Acetaminophen Found per Average Tablet Weight
Tablet A[a]	150.6	154.0, 151.8, 150.3 150.2, 148.7, 149.8
Tablet B[b]	300.0	299.3, 303.8, 304.9 296.2, 303.3, 299.4

[a] Marketed as Sinutab Tablets by Warner-Chilcott Laboratories, Division of Warner-Lambert Co. [b] Marketed as Sinubid Tablets by Warner-Chilcott Laboratories, Division of Warner-Lambert Co.

Table III—Assay of Acetaminophen in Commercial Tablet Formulations

Formulation	mg. Acetaminophen Claimed per Tablet	mg. Acetaminophen Found per Tablet	NF Assay
Tablet A	150.0	157.2,150.4,151.2 152.4,150.3,153.4	—
Tablet B	300.0	310.7,311.0,308.9 304.9,309.3,309.7	—
Acetaminophen tablets NF			
(Lot X)	325.0	324.2	319.0
(Lot Y)	325.0	320.8	319.1

interference of phenacetin, the *O*-ethyl ether of acetaminophen, would appear paradoxical. Recently, Hanegraaff and Chastagner (12) reported a study of the formation of nitro derivatives by acetaminophen, phenacetin, and a number of other derivatives of acetanilide using different nitrating agents—*viz.*, nitric acid, a mixture of sulfuric acid and sodium nitrite solution, a mixture of sodium nitrite and nitric acid, and a mixture of nitric acid and procaine. The last mixture did not effect nitration of any of the compounds, a fact they attributed to complete removal of nitrous acid by procaine. Using sodium nitrite and sulfuric acid, they produced a chromophore from acetaminophen with maximum absorbance at 375 nm. (ϵ = 630) and a chromophore from phenacetin with a maximum at 355 nm. (ϵ = 1880). They attributed the lack of interference by phenacetin in the Le Perdriel *et al.* (7) method to the fact that the alkaline pH used for measurement converts the acetaminophen chromophore to the phenolate ion, which absorbs at a higher wavelength than the phenacetin chromophore, which does not undergo a bathochromic shift in alkali. The conditions used by Hanegraaff and Chastagner (12), however, differ significantly from those used by Le Perdriel *et al.* (7) and in the method proposed here. The nitration mixture used by Hanegraaff and Chastagner (12) was 10 *N* with respect to sulfuric acid content, while it is about 0.7 *N* in hydrochloric acid content in the proposed method. A definitive test of the effect of phenacetin in the determination of acetaminophen, ignoring solubility considerations, was provided by comparing the colors formed by a mixture of equal concentrations of acetaminophen and phenacetin and by acetaminophen alone. Figure 3 shows that phenacetin interferes to a negligible extent in the acetaminophen determination by this method, even in acid solution. Moreover, the proposed method requires dissolution of the sample in water, in which phenacetin has less than 1 mg./ml. solubility. The excellent recovery values obtained for acetaminophen added to phenacetin-containing tablet formulations and the correspondence of assay values to label claim on commercial tablets provide further confirmation that phenacetin does not interfere in the method.

Salicylamide Interference—Trials of the proposed method on a proprietary analgesic formulation containing 194.4 mg. (3 gr.) of aspirin, 64.8 mg. (1 gr.) of caffeine, 97.2 mg. (1.5 gr.) of acetaminophen, and 129.6 mg. (2 gr.) of salicylamide per tablet provided an average assay value of 122.2 mg. acetaminophen per tablet. Figure 4 shows the effect of salicylamide in the reaction. At 430 nm., the salicylamide reaction product exhibits an absorptivity of about 0.9 l./g. cm., where the acetaminophen chromophore has an absorptivity of about 5.0 l./g. cm. Assuming that the tablets purchased contain the formula amounts of acetaminophen and salicylamide, the difference between the formula and determined values for acetaminophen can be accounted for by the salicylamide content. The structure of the salicylamide chromophore was not determined.

Recovery and Precision—The results of adding known amounts of acetaminophen to each of two analytically prepared commercial tablet formulations are shown in Table II.

One formulation tested (Tablet A) has a label declaration of 150 mg. each of acetaminophen and phenacetin, 22 mg. of phenyltoloxamine dihydrogen citrate (DHC), and 25 mg. of phenylpropanolamine HCl. The other (Tablet B) is a sustained-action tablet formula of the wax matrix type with a claim of 300 mg. each of acetaminophen and phenacetin, 66 mg. phenyltoloxamine DHC, and 100 mg. phenylpropanolamine HCl. The average recoveries were 100.1 ± 1.7% [relative standard deviation (*RSD*)] for the first and 100.4 ± 1.1% (*RSD*) for the sustained-action tablet.

Assay of Commercial Tablets—The proposed method was tested with the two marketed dosage forms described in the recovery experiments. Two different lots of acetaminophen tablets NF were assayed by the proposed method and the UV spectrometric method of NF XIII. The results are shown in Table III. The percent of declared amount and relative standard deviations obtained were 101.7 ± 1.57% for Tablet A, 103.3 ± 0.71% for Tablet B, and 99.7 and 98.4%, respectively, for the two lots of acetaminophen tablets NF.

SUMMARY AND CONCLUSIONS

A modified procedure for the colorimetric determination of acetaminophen was evaluated. The method is based on the observations of Le Perdriel *et al.* (7), who discovered that acetaminophen reacts with nitrous acid to form 2-nitro-4-acetamidophenol, which is orange-red in alkaline solution. A similar method proposed by Inamdar and Kaji (9), who measured the color in acid solution at its peak in the near UV, was found to have identical chemistry. The modified method employs a wholly aqueous system; the color thus obtained conforms to Beer's law, at least to an acetaminophen concentration of 64 mcg./ml. in the solution determined. Interference by phenacetin was found to be negligible; however, salicylamide forms a chromophore which interferes in the determination. Since the recommended method requires only the successive addition of reagents and dilution, it appears to be well suited to automated techniques.

Recovery of acetaminophen added in each of two different tablet formulations containing phenacetin, phenyltoloxamine, and phenylpropanolamine was close to theoretical. Relative standard deviations determined during recovery experiments and trials of the method on commercial products (6 trials each) ranged from ±0.71 to ±1.7%.

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Content Uniformity Test for Esterified Estrogen Tablets

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Abstract □ A rapid, automated, analytical procedure for the content uniformity of esterified estrogen tablets is described. The test is based on the measurement of sulfuric acid-induced fluorescence; it uses, as the standard, the batch of esterified estrogen powder used to manufacture the tablets. Data on content uniformity are given, and examples of calibration curves are shown.

Keyphrases □ Estrogen (esterified) tablets—content uniformity test □ Automated analysis—esterified estrogen tablets, content uniformity □ Diagram—automated analyzer, esterified estrogen tablets

The present emphasis on control of tablet content uniformity (1) prompted the search for a rapid and precise test for esterified estrogen tablets. No such procedure is available in the literature, and the assay available in the USP XVIII (2) is much too slow and tedious to be useful as a content uniformity test.

The acid-induced fluorescence of estrogens is a well-known phenomenon (3) and has been useful in automated analytical schemes (4–6). All such assays use a sample of the pure estrogen to generate a calibration curve, which is continuously updated with replicate standards and is used to determine the estrogen content of the samples. Because of the variable composition of esterified estrogens (2), it is not possible to obtain a single material that would be a valid standard for all batches of tablets. A solution to this problem is achieved by using as the standard a sample of the same lot of esterified estrogen powder that is used to prepare the batch of tablets. The esterified estrogen powder to be used was previously assayed by the USP XVIII procedure (2) and found acceptable.

This paper reports on the development of such a content uniformity test.

EXPERIMENTAL

Apparatus—A liquid sampler[1] and proportioning pump[1] are connected to a filter fluorometer[1] as shown in Fig. 1. The excitation filter is a Wrattan 47B[2] and the emission filter is a Wrattan 4[2]. Neutral density filters passing 1, 10, or 25% of the fluorescence are used, depending on the sample size. The water-jacketed mixing coil[3] is modified by the addition of an input line, 1 mm. in diameter, located three coils from the input end of the mixing coil. The fluorometer signal is recorded on a standard single or two-pen recorder[1]. A high-speed kitchen blender[4] equipped with semimicro blades and a stainless steel mixing vessel[5] of 360-ml. capacity with a friction-fit stainless steel cover is used to prepare samples and standards.

[1] Technicon Corp.
[2] Kodak Co.
[3] Technicon Corp., Part No. 114-0209-01.
[4] Waring Co., Winsted, Conn.
[5] Eberbach Co.

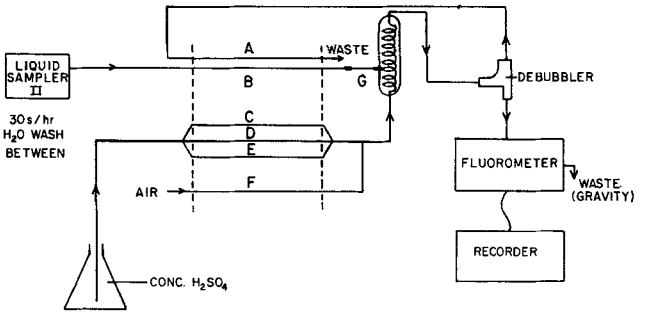

Figure 1—*Autoanalyzer flow diagram for content uniformity of esterified estrogen tablets. Composition and internal diameter (inches) of pump tubes are: A, acid flex, 0.056; B, clear standard, 0.065; C, D, and E, acid flex, 0.110; and F, clear standard, 0.040.*

Reagents—Concentrated sulfuric acid[6] and esterified estrogen powder from the same batch used to manufacture the tablets are used.

Standard Preparation—Esterified estrogen powder from the same lot used to make the tablets is used as the standard for the content uniformity test. Accurately weigh a quantity of esterified estrogen powder exactly equal to 10 times the target amount in a single tablet into the blender vessel, add a 100-ml. aliquot of water, cover, and blend until the contents are homogeneous. Decant and centrifuge the solution until clear. Dilute 6-, 8-, 10-, and 12-ml. aliquots of the clear solution to 100 ml. with water to give solutions corresponding to 60, 80, 100, and 120%, respectively, of the target weight of esterified estrogen powder per tablet.

Sample Preparation—Place a single tablet in the blender, add 100 ml. water, and blend until homogeneous. Filter a portion (about 20 ml.) of the homogenate through filter paper[7], collect in a screw-cap test tube, and cap.

Selection of Neutral Density Filters—Put a portion of the 100% standard solution into the liquid sampler and run it through the system. If the recorder peak height is off scale or less than half-scale deflection, then use the next larger or smaller neutral density filter, respectively. Repeat the procedure until the 100% standard response is approximately 50% of full-scale deflection.

Assay Procedure—The sampling probe of the liquid sampler is aligned to achieve satisfactory aspiration from the sample and wash cups. The fluorometer slit is set at 30× and the liquid sampler at 30 samples/hr. After about 1.0 hr. of conditioning of the flow system using 100% standards, the standards and samples are introduced. The set of four theory standards (60, 80, 100, and 120%) in quadruplicate is sampled initially, and the average fluorescence value is determined for each set. Five samples are then assayed, followed by the 100% standard in duplicate. If the mean of the 100% standards is different from the mean of the 100% standards of the calibration curve by less than 2%, the esterified estrogen powder content of the samples is read directly from the calibration curve. If the difference of the means is greater than 2%, a new calibration curve is drawn, passing through the new 100% standard point and parallel to the original calibration curve. The results of the five samples associated with the adjusted calibration curve are then determined. This procedure corrects for any slight baseline drift.

[6] Du Pont Yellow Label.
[7] Whatman No. 1.

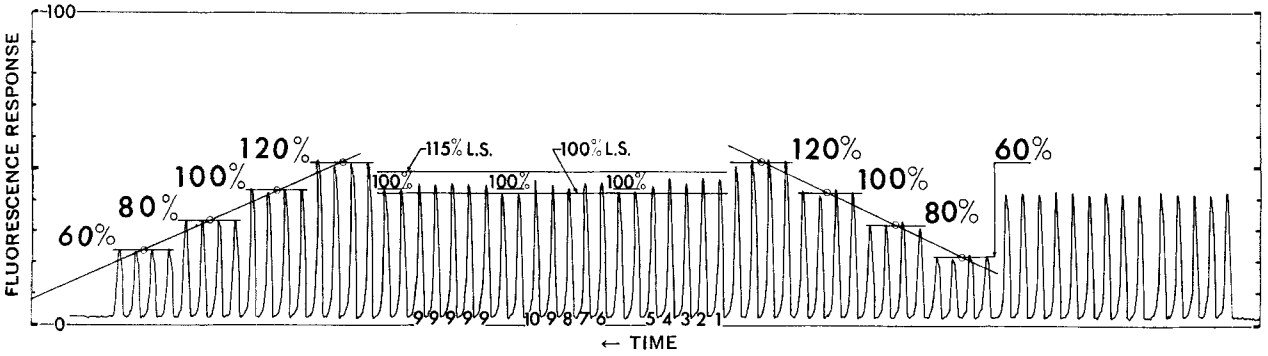

Figure 2—*Typical autoanalyzer curves showing linearity of the fluorescence response of esterified estrogen powder standards and the content uniformity of typical tablets. Peaks 1–10 are individual tablet results. Tablet 9 was run repeatedly to demonstrate precision. Target strength for all tablets is 107.5% L.S.*

RESULTS AND DISCUSSION

An example of a typical recorder output is shown in Fig. 2 and demonstrates the linearity of the calibration curve. Typical recorder peaks of production tablets and repeated runs of a single-tablet solution are also shown. The precision of the test is shown by the 95% confidence interval (106.6 ± 0.44%) calculated from the six replicate determinations of a single-tablet extract. The tablets contain a 7.5% overage which is reflected in the result. Typical results for a production batch of esterified estrogen tablets are given in Table I and in Fig. 2. All results are within the USP limits of 85–115% of label claim.

The validity of this test is based on the fact that the esterified estrogen powder used as the standard was previously assayed for estrone sulfate, equilin sulfate, and total estrogen sulfates by the USP XVIII procedure and found acceptable. Therefore, based on the content uniformity test results and the USP assay, it is possible to control adequately the absolute amounts of estrone and equilin sulfates present in a tablet, even though the fluorescence response may include substances in the esterified estrogen powder other than estrone and equilin sulfates. The variation of fluorescence response between lots of esterified estrogen powder has no effect on the validity of the uniformity test, because each batch of esterified estrogen powder is used as its own standard. A comparison of fluorescence responses of several batches of esterified estrogen powder is shown in Table II. The response of D is much higher than that of A, but the estrogen sulfate content, as measured by the USP XVIII assay, is within 2% of Sample A. This indicates that appreciable variation in the kind and/or quantity of unspecified material in esterified estrogen powder can occur. It was found that the fluorescence response of intentionally degraded tablets is significantly less than the response for undegraded tablets, but correlation of the results with the USP assay showed that the uniformity test overestimates the extent of degradation.

It should be possible to use this test to control the content uniformity of conjugated estrogen tablets (2) as well as esterified estrogen tablets, since the active principle differs only in quantity and not in type.

Table I—Typical Content Uniformity Results of a Production Batch of Esterified Estrogen Tablets

Tablet	Percent Target	Tablet	Percent Target	Tablet	Percent Target
1	101.4	11	97.2	21	97.7
2	97.7	12	100.9	22	94.9
3	101.4	13	93.0	23	96.7
4	99.5	14	93.0	24	99.7
5	97.7	15	94.9	25	93.0
6	99.5	16	101.4	26	94.9
7	99.5	17	101.4	27	97.7
8	101.4	18	94.4	28	97.7
9	96.7	19	102.8	29	98.8
10	96.7	20	102.3	30	95.3

Mean = 97.9% of target

Table II—Variation of Sulfuric Acid-Induced Fluorescence of Esterified Estrogen Powder USP

Lot	Percent Response[a]	Percent Estrogen Sulfate[b]
A	—	—
B	99.5	100.1
C	99.6	102.7
D	108.3	101.8

[a] Results are expressed as percent of Lot A. [b] The USP XVIII assay for total conjugated estrogens was used. The results are expressed as percent of Lot A.

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Metabolism of Morphine by Cats

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Abstract □ Cats excreted 19.2 ± 1.5% of free morphine and 48.4 ± 2.4% of conjugated morphine in urine during the first 48 hr. after subcutaneous injection of morphine. Fecal excretion during 2 days following drug administration ranged from 2.0 to 20% of the injected drug. The major morphine conjugate was isolated and characterized as morphine-3-ethereal sulfate by UV and IR spectra, morphine content, and elemental analysis. In addition to morphine-3-ethereal sulfate, morphine-3-glucuronide and normorphine were also identified as minor metabolites.

Keyphrases □ Morphine—metabolism, metabolites, cats □ Metabolites, urinary, fecal—morphine, cats □ IR spectrophotometry—analysis □ UV spectrophotometry—analysis

One of the morphine conjugates excreted by cats was isolated and characterized as morphine ethereal sulfate (1, 2). The present article describes the isolation and characterization of this and other morphine metabolites and gives data on the urinary and fecal excretion of free morphine and conjugated morphine up to 48 hr. following drug administration.

METHODS

Animal Experiments—Domestic male and female cats, weighing 1.4–3.0 kg., were injected subcutaneously with 20 mg./kg. (as the base) of *N*-$^{14}CH_3$-morphine HCl. Urine and feces were collected daily in a metabolism pan for 48 hr. (at least two samples collected). The pan was thoroughly washed after each collection, and the pooled urine and washings were frozen immediately.

Three cats were sacrificed 3 hr. after drug administration. The animals were given an intrathoracic injection of 50 mg./kg. of sodium pentobarbital followed by exsanguination *via* the carotid artery. Bile was obtained by aspiration.

Determination of Total Radioactivity, Free Morphine, and Conjugated Morphine—Free morphine, conjugated morphine, and total radioactivity in the urine were determined by the procedure described previously (3).

Isolation and Purification of the Major Morphine Conjugate from Cats—The urine sample (450 ml.) was loaded on 10 g. of acid-washed and activated charcoal[1]. The column was washed with 100 ml. of water. Morphine and its metabolites were either eluted with 350 ml. of 50% acetic acid in water or 400 ml. of ammoniacal (30% NH_3) methanol (1:1) from the column, and the eluate was evaporated to dryness at 50° under reduced pressure. The residue was triturated with methanol and crystallized from about 50 ml. of boiling water. After refrigeration overnight the colorless needle-shaped crystals were filtered, washed with methanol, and dried at 60° *in vacuo* for 8 hr. The yield of morphine conjugate from several experiments was 110 mg., 35% of injected morphine.

The methanolic supernatant was evaporated to dryness, and the residue was dissolved in a small amount of dilute HCl, adjusted to pH 10 with NH_4OH, saturated with NaCl, and extracted with ethylene chloride containing 30% of *n*-amyl alcohol. The extract was evaporated to dryness under reduced pressure. Chromatography of the residue, dissolved in methanol on Whatman 3 MM paper buffered with citrate–sodium phosphate solution at pH 6.8 (4) and developed with *n*-butanol and water (9:1), showed two spots having R_f values of 0.12 (normorphine) and 0.66 (morphine) after spraying with potassium iodoplatinate solution or 3% ninhydrin in methanol.

[1] 50–200 mesh, Fisher Scientific Co., Fair Lawn, N. J.

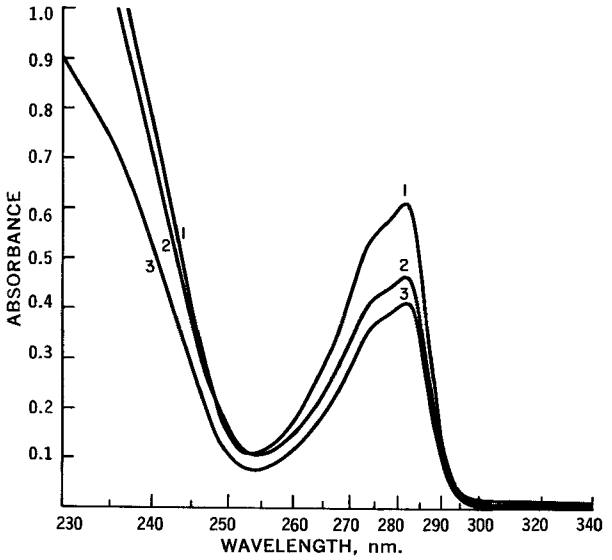

Figure 1—*UV spectrum of morphine conjugate isolated from urine of cats (morphine-3-ethereal sulfate). Key: 1, H_2O; 2, NaOH; and 3, HCl.*

Synthesis of Morphine Ethereal Sulfate—Morphine-3-ethereal sulfate was synthesized according to the procedure of Stolnikow (5).

RESULTS

Urinary, Fecal, and Biliary Excretion of Morphine by Cats—Urinary excretion of free morphine and conjugated morphine by nine cats after subcutaneous injection of morphine was 19.2 ± 1.5% and 48.4 ± 2.4%, respectively, of injected dose.

Fecal excretion of total morphine from six out of nine cats during 2 days following drug administration ranged from 2.0 to 20% of the injected dose, 2.1 ± 0.41% being free morphine and 8.3 ± 2.54% being conjugated morphine. One percent of the injected radioactivity was found in the 2–6 day feces of three animals which did not defecate within 48 hr.

Total drug excreted in the urine and feces was about 80% of the injected dose.

Recoveries of total morphine in the bile 3 hr. after subcutaneous injection of 20 mg./kg. of morphine from three cats were 22.7, 14.5, and 5.1% of the injected dose. The ratios of conjugated to free morphine were 5.7, 4.0, and 2.1.

Detection of Morphine Metabolites from Cats—The paper chromatograms of (*a*) the cat urine and (*b*) the eluate from the charcoal column on Whatman 3 MM paper, developed with *n*-butanol–acetic acid–water (4:1:2), showed two spots having R_f values of 0.42 (major metabolite, later identified as morphine-3-ethereal sulfate) and 0.70 (morphine). The mother liquor, after crystallization of the major morphine conjugate from the concentrated eluate, showed three spots having R_f values of 0.29 (morphine-3-glucuronide), 0.42, and

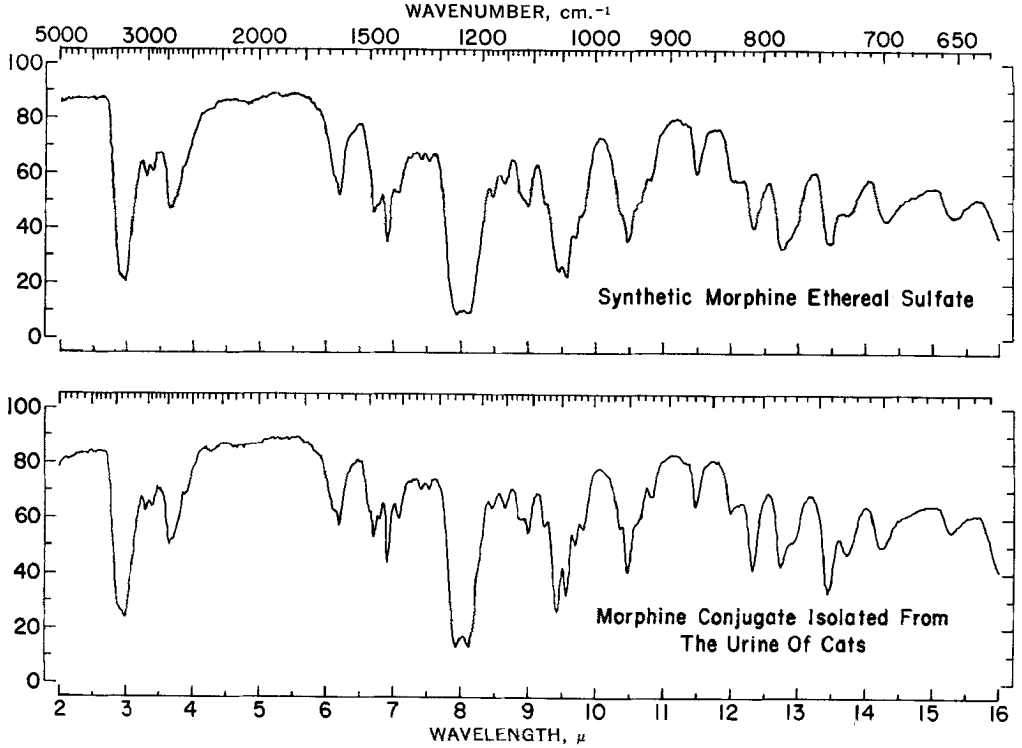

Figure 2—*IR spectra of morphine-3-ethereal sulfate.*

0.70. Normorphine was detected in the extract from the mother liquor after crystallization of morphine conjugate from the eluate, as described in the *Method* section.

Acid hydrolysis of the metabolite gave morphine, detected by the paper chromatography, indicating that the metabolite was a conugated morphine.

The results of incubation of 1-ml. aliquots of the urine or the isolated morphine conjugate solution (buffered with 3 ml. of 0.2 *M* sodium acetate at pH 5.0) with 15 mg. (60 units) of limpet phenol sulfatase[2], 0.1 ml. of glusulase[3], 0.2 ml. of ketodase[4], or 37.5 mg. (800 units) of β-glucuronidase[2] (buffered with 3 ml. of 0.2 *M* phosphate at pH 7.0) for 20 hr. in air showed that the morphine conjugate in the urine was hydrolyzed with glusulase but not with limpet phenol sulfatase and β-glucuronidase. The isolated morphine conjugate was completely hydrolyzed with glusulase and phenol sulfatase, but not by ketodase and β-glucuronidase. The results indicated that the isolated morphine conjugate contained a sulfate group.

Characterization of the Major Morphine Metabolite from Cats—The metabolite is insoluble in water, dilute acid solution, and organic solvents; it is sparingly soluble in hot water and soluble in alkaline solution. The metabolite does not melt nor discolor at 300° and does not show the typical morphine phenolic reaction after sequential treatment with alkaline ferricyanide and acid chloride solution.

The UV spectrum of the metabolite in NaOH solution did not show bathochromic shift (*a*, 285 nm. ≃6.0) (Fig. 1).

The IR spectrum of the metabolite as a pressed KBr pellet showed strong absorption bands at 1240–1280 cm.$^{-1}$, indicating that the metabolite contained a sulfate group. Presence of an absorption band at 2600–2860 cm.$^{-1}$ indicates the tertiary amine salt formation and suggests that the compound exists as a zwitterion. Absorption in the 3600-cm.$^{-1}$ region apparently is due to the secondary alcohol group (Fig. 2).

The morphine conjugate isolated from cats was identical with the synthetic morphine ethereal sulfate as shown by the IR and UV spectra, morphine content (76.8%) determination, phenolic test, and elemental analysis[5].

Anal.—Calcd. for $C_{17}H_{19}NO_6S$: C, 55.8; H, 5.24; N, 3.83; S, 8.78. Found: C, 55.46; H, 5.33; N, 3.84; S, 8.42.

[2] Sigma Chemical Co., St. Louis, Mo.
[3] Endo Lab., Garden City, N. Y.
[4] Warner-Chilcott Lab., Morris Plains, N. J.
[5] Huffman Lab., Wheatridge, Colo.

Data obtained in the present studies confirm the result of Fujimoto and Haarstad (2) that the major morphine metabolite in the cat is morphine-3-ethereal sulfate.

No attempt was made to isolate the minor morphine metabolites, *i.e.*, morphine-3-glucuronide (1–2% of injected dose) and normorphine detected by paper chromatography.

DISCUSSION

Urinary excretion of free morphine (19%) by cats obtained in the present studies is higher than the values (3–12% of injected dose) reported by Keeser *et al.* (6) and (6–10%) by Yoshikawa (7).

Five to twenty-three percent of the injected morphine was found in the bile of the gall bladder 3 hr. after injection. Since only 1% of the injected drug was found in the feces of three cats collected 48 hr. after injection, the data indicated that morphine and/or conjugated morphine excreted from the bile ducts was reabsorbed into the bloodstream.

In addition to morphine-3-ethereal sulfate, morphine-3-glucuronide and normorphine were detected as minor metabolites in the urine of cats injected with morphine. Detection of free normorphine as a metabolite of morphine confirms the report of Tampier and Penna-Herreros (8), who found normorphine in the cat liver homogenate 1 hr. after injection of morphine. Although morphine-3-glucuronide was detected in the mother liquor after crystallization of morphine ethereal sulfate, it was not detected in the urine with paper chromatography and the enzymatic hydrolysis method because of low concentration.

Welch *et al.* (9) reported that phenol sulfatase will completely hydrolyze phenol sulfates in 24 hr. in presence of cat urine if the urine concentration does not exceed 10%. The failure of phenol sulfatase to split off morphine ethereal sulfate in the present study may be due to a higher concentration of cat urine (25%) used for hydrolysis.

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Effect of Vagal Stimulation on Duodenal Serotonin in the Guinea Pig

FRANK J. HOHENLEITNER, MARTIN F. TANSY, and RICHARD H. GOLDER

Abstract □ The serotonin concentration of duodenal tissue was examined immediately after peripheral or central vagal stimulation, and an 18 or 36% reduction of serotonin, respectively, was found. This is the first demonstration of a decrease in gastrointestinal serotonin in response to autonomic nerve stimulation.

Keyphrases □ Serotonin, duodenal—effect of vagal stimulation, guinea pig □ Gastrointestinal serotonin, reduction—response to autonomic nerve stimulation □ Spectrophotofluorometry—analysis

Enterochromaffin cells (EC) are found throughout the gastrointestinal tract, but in the guinea pig their greatest concentration is in the duodenum, *juxta* the pyloric sphincter (1, 2). They are most frequently found deep in the crypts of Lieberkuhn and can be identified by staining the cell granules with silver salts after precipitation by formalin (3). These granules are believed to contain 5-hydroxytryptamine (5-HT, or serotonin), and a high degree of correlation has been reported between EC number and mucosal serotonin concentration in laboratory and domestic animals.

Stimulation of the vagi was shown to result in approximately a 75% decrease in EC counted in the proximal duodenum of the guinea pig (4). Reduction of cells counted merely reflected cellular degranulation, or at least a decrease in stainable substance within the cells. Since serotonin is most frequently identified with these cells, the purpose of this study was to determine if stimulation of the vagi is also associated with a change in tissue serotonin concentration.

EXPERIMENTAL

Animals and General Procedure—Seventy guinea pigs were secured and maintained as previously described (4). Anesthetized animals were divided into three groups. Group I animals (controls) were sacrificed in three subgroups: (*a*) immediately after achieving surgical anesthesia, (*b*) 30 min. later, or (*c*) 60 min. later. Group II animals had the vagi stimulated peripherally and were sacrificed 30 or 60 min. later. Group III animals were sacrificed at 30 or 60 min. after the initiation of central vagal stimulation. In all animals the duodenal tissue *juxta* the pyloric sphincter was removed for spectrophotofluorometric analysis of serotonin according to the method of Wise (5). One group of unanesthetized animals was sacrificed by decapitation. Of these, four received 10 mg./kg. reserpine intraperitoneally 24 hr. prior to decapitation. Results were analyzed for significance at the 0.05 level using a two-tailed Student *t* test.

Surgical and Recording Procedure—Under intraperitoneal urethan anesthesia (1.5 g./kg.), the vagi were exposed, divided, and stimulated high in the neck. At the cervical level the vagi were stimulated peripherally or centrally. Carotid arterial pressure was recorded continuously using a Statham pressure transducer. Scalar lead II of the electrocardiogram and tachograph was also recorded. All variables were charted on a polygraph (Grass model 7).

Electrical Stimulation—Bipolar electrodes were attached to the caudad or central portion of the vagi and surrounded with liquid petrolatum to isolate the stimulating current. The vagi were electrically stimulated with a Grass model S8 stimulator *via* an isolation transformer. The stimulator delivered a rectangular pulse (5 v., 30 Hz., 1 msec.), which was applied for a 5-min. duration followed by a rest interval of equal duration for a total of three times.

RESULTS

The results of this study are found in Fig. 1. The concentration of serotonin in unanesthetized animals was about 4.42 mcg./g. duodenal tissue, whereas after reserpine administration the concentration decreased to 0.43 mcg./g. or by approximately 90%.

Immediately after the guinea pigs were anesthetized, the duodenal tissue had a serotonin concentration of 3.09 mcg./g. In unstimulated controls the slight average increase to 3.29 mcg./g. after 30 min., as well as a marginal decrease to 2.63 mcg./g. at 60 min., was not significant. Stimulation of the peripheral vagi decreased serotonin concentration to 2.48 and 2.73 mcg./g. at 30 and 60 min., respectively. The 30-min. concentration was significantly different from both prestimulation and 30-min. controls, but at 60 min. this change was not significant. Central vagal stimulation decreased duodenal serotonin to 1.97 mcg./g. at 30 min. and 2.29 mcg./g. at 60 min. Both concentrations were significantly different with respect to prestimulation control, but only the 30-min. sample was statistically different from its respective control.

DISCUSSION

Pentilla (6) demonstrated a significant correlation between the number of EC cells and duodenal serotonin concentration in the mouse, rat, guinea pig, rabbit, sheep, pig, cow, and horse. The regression correlation coefficient of his study was determined on interspecies data, but the duodenal serotonin data from any one species generally did not appear to reflect this correlation. However, there was a modest correlation of EC and serotonin concentration in the rat duodenum during the first 2 months of life (6), and a strong correlation for developing chicken embryonic duodenum (7). Even more significantly, Pentilla (8) reported serotonin concentra-

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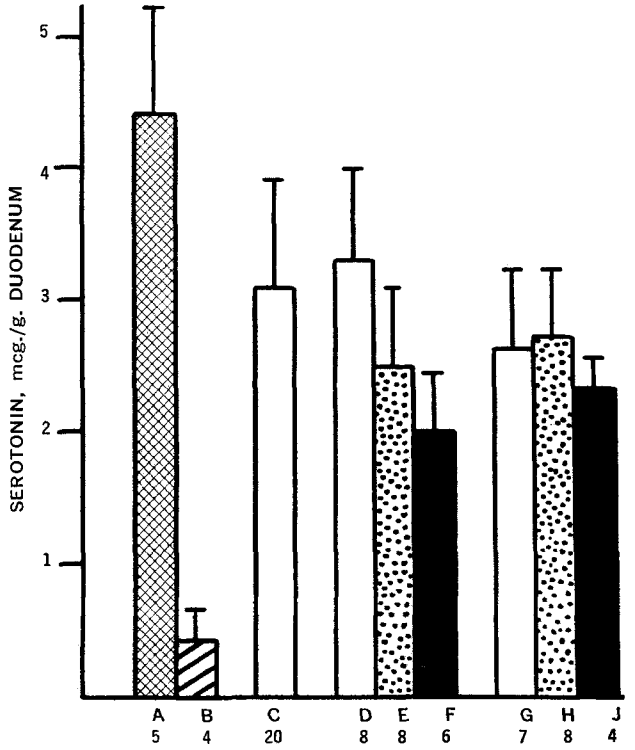

Figure 1—*Serotonin concentrations in guinea pig duodenum. Bars A and B: unanesthetized; Bar B: reserpinized; Bars C–J: anesthetized; Bar C: immediately after anesthetization; Bars D–F: 30 min. after stimulation; D: control; E: peripheral stimulated; F: central stimulated; Bars G–J: 60 min. after stimulation; G: control; H: peripheral stimulated; and J: central stimulated. Each bar represents the mean value plus standard deviation. The numbers under the bars represent the number of animals in each group.*

tions and cell counts throughout the alimentary tract in the guinea pig and the correlation was highly significant.

Linear correlation of the cell count with serotonin content must be restricted, however, to normal animals, since in reserpine-treated guinea pigs no such correlation exists (6). Reserpine administration reduces the staining response to silver salts (9), suggesting release of reactive substances from the EC cells. Pentilla observed about a 30–57% decrease in EC counted, whereas serotonin concomitantly decreased by approximately 90%. In the present study, reserpine produced a 90% decrease in serotonin concentration. In another report, a 90% reduction in cells counted was associated with reserpine administration (10).

The serotonin concentrations in guinea pig duodenum determined in this study are in good agreement with Erspamer (11), Nobili (12), and Hagmuller *et al.* (13). Pentilla (6, 8) reported much higher concentrations, which may have been due partly to the use of female guinea pigs in his study, since it is known that they contain a greater number of EC (14). Reserpine administration in the present study was used to demonstrate that the serotonin assay was capable of detecting greater changes in tissue serotonin.

Stimulation of the peripheral vagi produced approximately a 75% reduction in EC counted in the guinea pig duodenum (4), whereas similar stimulation resulted in only an 18% decrease in duodenal serotonin concentration. Central vagal stimulation produced a 46% decrease in cells counted (10) and a 36% decrease in tissue serotonin. A lack of correlation between cells counted and serotonin concentration should be expected. Cell counting is an all-or-none experience, *i.e.*, a cell is counted only if it contains sufficient stain to recognize the granules by which they are currently identified. Once a cell contains an unknown threshold concentration of stainable material, the addition of more stainable substrate would not alter the count. Conversely, cells may contain a considerable concentration of serotonin and yet, if subthreshold or in an unstainable form, may be insufficient to produce visual detection of granules. It should also be emphasized that tissue serotonin assay does not distinguish between serotonin granules within EC and released amines. Nonetheless, the observed reduction in granulation by peripheral vagal stimulation would at least be expected to be associated with a decrease in serotonin concentration, which is what the authors observed. Extension of the sampling time 30 min. beyond the end of stimulation to permit degradation or removal of released serotonin did not bring about any further decreases in serotonin.

Other investigators claimed demonstration of significant release of serotonin into the venous circulation of the vascularly perfused dog small intestine by administration of catechol amines (15) or by stimulating arterial sympathetic nerves. However, they failed to consider several factors. First, both parasympathetic and sympathetic nerves are grossly indistinguishable on the surface of the intestinal arteries; since no evidence was offered to support the statement that only sympathetic nerves were stimulated, it may be assumed that both types of fibers were involved in their study. Secondly, they reported changes in serotonin concentration in the effluent of the perfused intestine without indicating any flow data. Catechol amines and nerve stimulation would be expected to alter greatly the flow through the intestine; accordingly, without flow information, net changes in serotonin release are unknown. Therefore, the data they presented do not seem to demonstrate their conclusions unequivocally. On the other hand, this report is the first to demonstrate a reduction in intestinal tissue concentration of serotonin in response to autonomic nerve stimulation. The types of nerve fibers involved in this response are considered elsewhere (10).

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Effect of Cobalt Chloride and Sodium Cobaltinitrite on the Growth of Established Epithelial Tumors Induced by Methylcholanthrene

GERALD P. O'HARA*, DAVID E. MANN, Jr., and RONALD F. GAUTIERI

Abstract □ Previous investigations showed that when cobaltous chloride or sodium cobaltinitrite is administered in conjunction with the topical application of the carcinogen, methylcholanthrene, the tumorigenic response is significantly decreased. The experimental design of this study involved the administration of each cobalt compound, following completion of the minimal carcinogenic dose$_{50}$ (MCD$_{50}$) regimen of methylcholanthrene when tumorigenesis was firmly established, to ascertain whether these agents could retard the growth of formed tumors in addition to inhibiting those during the period of tumorigenesis. It was demonstrated that cobaltous chloride and sodium cobaltinitrite have no influence on the growth of established epithelial tumors induced by methylcholanthrene and that the antitumorigenic capacity of these compounds is restricted solely to the incipient stages of cancerization.

Keyphrases □ Tumors, epithelial, induced by methylcholanthrene—effect of cobalt chloride and sodium cobaltinitrite on growth □ Methylcholanthrene-induced tumors—effect of cobalt chloride and sodium cobaltinitrite on growth □ Cobalt chloride, sodium cobaltinitrite—effect on growth of methylcholanthrene-induced tumors

Since the observation by Kennaway and Heiger (1) during the early thirties that carcinogenesis could be initiated following repetitive stimulation by a single chemical entity, vindictive efforts to elucidate the nature of this exceedingly complex disease have been more multitudinous than fruitful. One presumptive concept pertaining to the origin of the cancerization process concerns the appearance of abnormal cells in response to stimuli that have caused delicate growth control mechanisms to go awry (2–5). Setala (6) recently called attention to the theory, postulated almost 100 years ago by Cohnheim, that suggests an embryologic accident as being the primary etiologic factor in initiating cancer in higher mammals. According to this supposition, during embryonic development, not all of the dividing cells become differentiated and mature but remain for some unknown reason undifferentiated and dormant. Later when challenged by certain chemical agents, carcinogens, they respond at the level of susceptibility they enjoyed during that much earlier time when embryonic arrest occurred. Certain similarities between teratogens, agents that act upon young cells to cause malformations in later life, and carcinogens, which induce cancer at all ages, become even more comparable when the Cohnheim hypothesis is considered. A gross dissimilarity cannot escape unnoticed, however, between the two types of stimuli. Teratogens generally interfere with cellular development to cause growth retardation (inhibition), while carcinogens inevitably precipitate uncontrolled growth (stimulation).

Because this laboratory has been involved in both teratogenic and carcinogenic studies in recent years, with a special interest in the inhibitory influence of certain cobalt compounds on the development of cortisone-induced cleft palate in mice (inhibition of an inhibition) and the prevention of methylcholanthrene-induced carcinogenesis (inhibition of a stimulation), it became the ultimate purpose of this investigation to determine whether or not these cobalt compounds could inhibit the growth of established tumors that had responded to methylcholanthrene administration in mice. If the administration of cobalt, after the tumors formed, failed to affect their developmental course, it might indicate that these cells have been basically changed to types that are no longer susceptible to the action of cobalt and indeed might suggest that methylcholanthrene transformed those latent immature embryonic cells into an adult type of proliferating cell.

Table I—Tumor Response of Individual Groups

Group	Effective Total Mice per Group	Tumorous Mice	Tumor Percent	Number of Males with Tumors	Number of Females with Tumors
A	57	28	49.1	15	13
B	60	30	50.0	17	13
C	58	29	50.0	16	13
D	58	34	58.6	17	17
Total	233	121	51.9	65	56

Table II—Mortality Rate of Individual Groups during Cobalt Therapy

Group	Number of Deaths	Deaths with Tumors	Deaths without Tumors
A	3	2	1
B	7	3	4
C	5	3	2
D	11	8	3
Total	26	16	10

EXPERIMENTAL

Two hundred and forty CF-1 mice were divided into four groups of 60, each containing 30 males and 30 females. Cages employed, diet, handling, shaving, and application procedure were discussed in a previous paper (7). All groups were initially subjected to the minimal carcinogenic dose$_{50}$ (MCD$_{50}$) of methylcholanthrene

Table III—Response of Individual Groups to Cobalt Therapy

Group	Number of New Tumors	Percent of New Tumors	Number of Regressions	Percent of Regressions	Percent Tumor Incidence at End of Therapy
A	11	20.4	4	7.4	61.1
B	11	21.2	5	9.6	63.5
C	11	21.2	3	5.8	65.4
D	5	11.1	8	17.8	51.1

Table IV—Comparison of Animals with Multiple Tumors to Total Number of Animals with Tumors before and after Cobalt Therapy

Group	Treatment Regimen Period, days 1–70	76–108	Status of Tumor Incidence on Day 72: Number with Tumors	Number with Multiple Tumors	Percent with Multiple Tumors	Status of Tumor Incidence on Day 111: Number with Tumors	Number with Multiple Tumors	Percent with Multiple Tumors
A	MCA[a]	No drug	28	9	32.1	33	23	69.7
B	MCA	$CoCl_2$ (25 mg./kg.)	30	9	30.0	33	21	63.6
C	MCA	$Na_3Co(No_2)_6$ (50 mg./kg.)	29	8	27.6	34	22	64.7
D	MCA	$Na_3Co(NO_2)_6$ (60 mg./kg.)	34	6	17.6	23	12	52.2

[a] Methylcholanthrene in the minimal carcinogenic dose$_{50}$(MCD$_{50}$).

(504 mcg.), applied through 21 biweekly applications of 0.02 ml. of a 0.12% solution of methylcholanthrene in acetone. Two days after the final administration of the carcinogen, all animals were examined closely, and those with growths measuring 1 mm. or greater (width *versus* height) were recorded. Table I shows the tumorigenic responses of each of the four groups at the end of the 10.4-week application period. On the 6th day after the cessation of methylcholanthrene applications, cobalt administration was commenced. Group A received no additional treatment and served as a control group; Group B received 25 mg./kg. of cobaltous chloride; Group C received 50 mg./kg. of sodium cobaltinitrite; Group D received 60 mg./kg. of sodium cobaltinitrite. All agents were administered intraperitoneally on Monday afternoons and Friday mornings for a period of 5 weeks (10 administrations). On the 3rd day after the final administration of the cobalt compounds, the animals were again closely examined for additional tumors and/or regressions.

RESULTS

Epilation was noted in the majority of animals approximately 10 days after the initial application of the carcinogen. This condition persisted for about 2 or 3 weeks and was characterized by an almost total loss of hair in the intrascapular region of most animals. A period of increased hair growth followed, which lasted for the remainder of the MCD$_{50}$ applications.

In Table I, the tumorigenic response of each group to the methylcholanthrene can be seen. The percent tumor incidence is in good agreement with all previously published data and serves, once again, to confirm the MCD$_{50}$ dose of methylcholanthrene in acetone (7) in this strain of mice. Table II shows the mortality in each group during cobalt therapy, the majority of deaths appearing in the group that received 60 mg./kg. of sodium cobaltinitrite (Group D). Most of these deaths can be attributed to the drug, because they occurred within a 2-hr. period following drug administration in which all of the animals exhibited marked hypoxia. In Table III the group receiving 60 mg./kg. of sodium cobaltinitrite (Group D) had twice as many regressions and less than half the amount of new tumor formations as compared to those of the control group. This finding, however, does not represent a significant change (*i.e.*, $\chi^2 < 3.84$). Table IV indicates the percentage of animals with tumors actually showing more than one recordable growth (multiple tumors) both before and after cobalt therapy. Again, there is no apparent difference between the control group and the treated groups, because the percent of animals with multiple tumors increased in all groups by approximately the same factor.

DISCUSSION

Cobaltous chloride and sodium cobaltinitrite have no significant effect on the fate of tumor development when these compounds are administered during the posttumorigenic period (after the application of the MCD$_{50}$ of methylcholanthrene in acetone for 10.4 weeks), although each compound is capable of decreasing the tumorigenic response when given concurrently with the MCD$_{50}$ of methylcholanthrene (8, 9). It appears that these cobalt compounds are only effective during the early stages of carcinogenesis, thus supporting the theory that cancer cells arise from embryologic cellular rests or cells that have failed to mature and differentiate (6). Therefore, during the early stages of carcinogenesis, before these cells have differentiated into a cancerous type, cobalt compounds are effective antitumor agents because the cells maintain their susceptibility to the inhibitory action of cobalt which is inherent in embryologic tissue. However, once the growths become firmly established and differentiated into cancerous types, they are resistant to the inhibitory action of cobalt because they are no longer, in effect, embryologic or undifferentiated cells.

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Determination of a Quaternary Ammonium Compound, Emepronium Bromide, in Human Urine by An Ion-Pair Extraction Method

STAFFAN EKSBORG, BENGT-ARNE PERSSON, JÖRGEN VESSMAN*, and BIRGITTA ENELL*

Abstract □ Emepronium bromide, an anticholinergic quaternary ammonium compound, was determined quantitatively in human urine by an ion-pair extraction method. This was constructed by the use of a theoretical approach, and favorable conditions were calculated from extraction constants and constants for side reactions. The principle of choosing selective extraction conditions is discussed on a theoretical basis; it has a general application in drug isolation from samples of such a complicated nature as biological objects. The present method includes extraction of the drug as perchlorate ion pair, purification of the extract, and determination with bromthymol blue. Using a concentration step in the method, a minimum concentration of 0.2 mcg./ml. urine could be determined with an absolute recovery of 98 ± 3%. Utilizing this method, the excretion of emepronium bromide in urine was followed after oral and rectal administration.

Keyphrases □ Emepronium bromide, determination in human urine—by ion-pair extraction method □ Ion-pair extraction method—determination of emepronium bromide in human urine □ Spectrophotometry—analysis

The isolation of quaternary ammonium ions from biological samples often can be made by an ion-pair extraction technique. Under suitable conditions, a very selective extraction method is obtained, which is of great importance when the object has such a complicated nature as the biological sample. Ion-pair extraction can also be used for transformation of the drug molecule to a form that makes the determination of low concentrations possible.

Investigation of the human urinary excretion of two quaternary antispasmodics by an ion-pair extraction method was made by Pfeffer *et al.* (1, 2). They used picric acid and tropaeolin 00 for the extraction and photometric determination. The recovery was low and varied greatly between subjects. This was assumed to be due to the presence of anions in urine with higher affinity than the anionic dyes for the quaternaries.

By the use of a theoretical approach in the method construction, more rapid and reliable results are obtained than by a purely empirical investigation technique. The calculation of favorable conditions can be based on extraction constants and constants for side reactions (3–7).

A demonstration of this principle is made in the present study, where an anticholinergic ammonium compound, emepronium bromide[1], is determined in human urine. The method is applied in a study of the excretion of the drug after oral and rectal administration.

Table I—Constants[a]

Anion	log E_{QX}	log E_{QHB}	log E_{Q_2B}	log $k_{diss.}$	pK'_{HB^-}
Perchlorate	3.58			−4.74	
BTB[b] (H_2B)		8.66	8.14		7.12

[a] Organic phase dichloromethane and aqueous phase phosphate or carbonate buffer solutions $\mu = 0.1$. [b] From *Reference 3*.

EXPERIMENTAL

Apparatus—The photometric measurements were performed with a Zeiss PMQ II Spektral-photometer in 10-mm. cells. The pH measurements were made with a Radiometer PHM 4 using glass and calomel electrodes.

Chemicals and Reagents—Dichloromethane of Fisher Certified ACS quality was equilibrated with water. Bromthymol blue (BTB) of analytical grade was purified according to Borg *et al.* (7). Tetrabutylammonium hydroxide, 0.1 *M*, in toluene–methanol was of BDH quality. All other chemicals were also of analytical grade. Phosphate and carbonate buffer solutions contained sodium as the only cation and had an ionic strength of 0.1. All aqueous reagents were equilibrated with dichloromethane.

Determination of Constants—The constants necessary for the construction of the method are given in Table I. The extraction constant of the emepronium ion pair with BTB, as well as the second acid dissociation constant of BTB, was determined by Schill (3).

The determination of the extraction constant of emepronium perchlorate was performed with samples containing equivalent amounts of emepronium and perchlorate ions, obtained by extracting an aqueous phase containing emepronium bromide and an excess of perchloric acid with dichloromethane. The organic phase was then equilibrated with an equal volume of 0.1 *M* NaH_2PO_4 for 1 hr.; after centrifugation the phases were separated. The emepronium content of the organic phase was determined by the BTB method (8) before and after equilibration. The concentrations of emepronium and perchlorate in the aqueous phase were then calculated from the difference between these measured concentrations. The found concentrations were used in the evaluation of the constants for the extraction and the dissociation of the ion pair as demonstrated earlier (9).

Analytical Method—A urine sample, 75.00 ml., is mixed with 10.00 ml. 2 *M* $HClO_4$ and 10.00 ml. dichloromethane in a 100-ml. glass-stoppered centrifuge tube. After shaking for 5 min. and centrifuging for 15 min., the organic phase is siphoned into a new centrifuge tube containing 10.00 ml. 0.1 *M* ClO_4^- in phosphate buffer pH 12. After shaking for 2 min. and centrifuging, the organic phase is siphoned into a centrifuge tube containing 5.00 ml. BTB (3×10^{-4} *M* in carbonate buffer pH 9.0). This mixture is shaken carefully for 5 min. to avoid emulsion, and is centrifuged for 20 min. The organic phase is siphoned and measured photometrically at 635 nm. after addition of 1 micro drop of 0.1 *M* tetrabutylammonium hydroxide into the cell. A blank from the same subject is prepared in the same way when no emepronium bromide is consumed. A theoretical recovery of 103% is obtained, depending on the solubility of dichloromethane in the urine sample (0.5 v/v%), for which compensation has to be done. Samples with a concentration down to 5×10^{-7} *M* (0.2 mcg./ml.) can be determined with the method.

Excretion Study—By the use of the described analytical method, urine blanks were determined for 12 volunteers employed in the excretion study. After administration of emepronium bromide, urine samples were collected every 2nd hr. and frozen for preservation.

[1] Cetiprin.

Table II—Total Urine Excretion of Emepronium Bromide 12 hr. after Administration

Formulation	Dose, mg.	Number of Subjects	Total Excretion mg.	δ	% of Dose
Tablets	200	12	1.11	±0.46	0.55
Suppository	100	11	2.96	±0.87	3.0

In a comparative study, the excretion was followed after an oral administration of 100 mg. at 7 a.m. and 100 mg. at 1 p.m. (therapeutic doses) and after a rectal single dose of 100 mg. at 7 a.m. In both these experiments, the subjects took the same doses the day before.

In another rectal experiment without premedication and with a lower dose, four of the test subjects were given 50 mg. of emepronium bromide in a suppository taken in the morning of the trial day. The excretion by urine was followed 12 hr. after administration.

RESULTS AND DISCUSSION

Calculation of Extraction Conditions—The drug concentration in the biological sample is often low, so it is essential to use a determination technique that gives the highest sensitivity possible. In the present method the BTB complex, with a molar absorptivity of 4.9×10^4, is used; it gives a minimum concentration of the complex of about 4×10^{-6} M (1.5 mcg./ml.) if reasonable precision in the photometric measurement is obtained. It is, however, possible to determine a lower concentration in the urine sample by the use of a large ratio (volume of aqueous phase)/(volume of organic phase) in the initial extraction step, which increases the concentration of the drug. In the present method the phase volume ratio is about 8, and the minimum urine concentration of the drug is then 5×10^{-7} M (0.2 mcg./ml.).

Suitable extraction conditions can be estimated from published data about the relation between the structure of the ion pair, the nature of the organic phase, and the extraction constant (4, 6, 10). In the actual case, a favorable system for the extraction of the quaternary ammonium compound from the urine sample was assumed to be found with perchlorate as the counterion in the ion pair and dichloromethane as the organic phase.

The calculation of extraction conditions was based on the extraction constant of emepronium perchlorate by the use of the following expression:

$$P_{QX} = 100/(1 + V_{aq.} \times V_{org.}^{-1} \times D_{QX}^{-1}) \qquad \text{(Eq. 1)}$$

where P_{QX} is the percentage extraction of the quaternary ammonium ion as ion pair with perchlorate; $V_{aq.}$ and $V_{org.}$ are the volumes of the aqueous and organic phases, respectively; and D_{QX} is the partition ratio defined by

$$D_{QX} = E^*_{QX} \times C_X \qquad \text{(Eq. 2)}$$

E^*_{QX} is the conditional extraction constant which can be calculated from the extraction constant, E_{QX}, and constants for side reactions. In this case, dissociation of the ion pair in the organic phase is the only side reaction, and E^*_{QX} can then be calculated from Eq. 3:

$$E^*_{QX} = E_{QX} + (E_{QX} \times k_{diss.})^{1/2} \times (C_Q \times C_X)^{-1/2} \qquad \text{(Eq. 3)}$$

As seen from this equation, E^*_{QX} will increase with decreasing values of $(C_Q \times C_X)$, which will favor the extraction in the low concentration range.

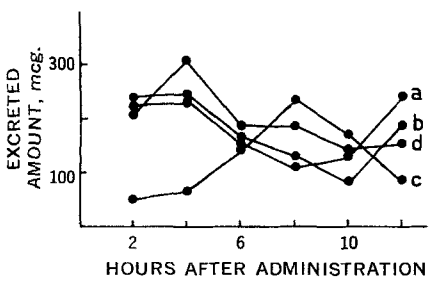

Figure 1—*Excretion of emepronium bromide in urine after oral administration of 2 × 100 mg. to four subjects (a, b, c, and d).*

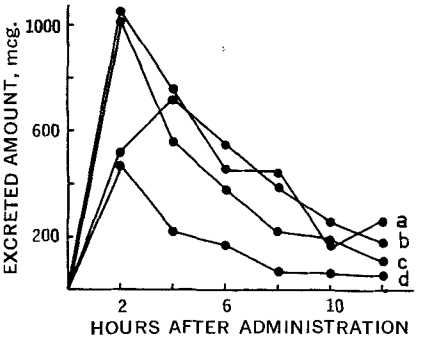

Figure 2—*Excretion of emepronium bromide in urine after rectal administration of 50 mg. to four subjects (a, b, c, and d).*

If a good selectivity is to be obtained by an extraction method, a system with unnecessarily high extraction capacity has to be avoided, since this will proportionally promote extraction of impurities. In general, an extraction degree of 99% is quite sufficient. An approximate calculation of extraction conditions by Eqs. 1 and 2 can, for the actual system, be based on the following values: $P_{QX} = 99$, $V_{aq.}/V_{org.} = 8$, and $E^*_{QX} = E_{QX} = 3.8 \times 10^3$ (Table I). These give a suitable perchlorate concentration of the aqueous phase, $C_X = 0.2$ M.

If a more exact calculation is wanted, E^*_{QX} has to be known. From Eq. 3, it follows that the value depends on the concentrations of the perchlorate and ammonium ion in the sample. The minimum sample concentration of Q^+ is according to the method 5×10^{-7} M, which gives a $C_Q = 5 \times 10^{-9}$ M after extraction. Calculations by Eq. 3 with this value of C_Q, $C_X = 0.2$ M, and $k_{diss.} = 10^{-4.74}$ (Table I) give $E^*_{QX} = 5.8 \times 10^3$, corresponding to a percentage extraction of 99.3.

The extraction conditions found by calculations were tested on aqueous solutions of emepronium bromide. The urine components urea, creatinine, and histidine, in normally occurring concentrations (11), were added to some samples. An emepronium recovery of 99 ± 2% was obtained in all cases after determination by the BTB method (8).

Isolation from Urine—When the perchlorate extraction was tested with urine samples free from emepronium bromide, in all cases the organic phase was found to contain components that gave response with BTB. This will give rise to a rather high blank and limit the sensitivity of the method.

It seemed, however, reasonable to assume that most of these coextracted substances were more polar than emepronium perchlorate with considerably smaller distribution ratios. If this was the case, the first extract from the urine could be purified by extraction with an aqueous phase of such perchlorate content that the emepronium would remain in the organic phase as an ion pair.

Calculations by Eqs. 1 and 2 proved that equal phase volumes and $C_{ClO_4} = 0.1$ M would prevent losses of emepronium. The aqueous phase was, in this case, alkalized (pH 12) to remove coextracted organic acids which might disturb the determination by the BTB method. At this pH, amines were extracted as bases. Tests showed that a single reextraction of this kind decreased the blank to one-fourth of its first value, while further extraction only gave small reductions.

The blank values varied considerably between tested subjects, with 0.15 as the highest and 0.04 as the lowest absorbance. Repeated tests showed small blank variations for each subject.

Photometric Determination—In the determination step the perchlorate ion in the organic phase was exchanged for a BTB anion. The main part of the perchlorate is present in the organic phase as an ion pair with emepronium but also to some extent with other urine components causing the blanks. The disturbance from components giving more polar BTB ion pairs than emepronium can be decreased by using a system without excessive extraction capacity. With a BTB reagent solution of a high pH, this is avoided and the influence of ion-pair forming amines will also diminish because of competitive base partition.

Emepronium can be extracted with both monovalent and divalent anions of BTB (3), but the extraction of the ion pair with the divalent anion is negligible at pH = 9.0. This can be shown by a logarithmic diagram using consecutive extraction constants according to Modin and Schill (12).

The BTB concentration necessary for a quantitative exchange of the perchlorate for BTB (HB^-) can be calculated by the use of α-

coefficients (3). The following equations will be valid:

$$D_{QHB} = E_{QHB} \times C_B \times \alpha_{HB}^{-1} \times \alpha_Q^{-1} \quad \text{(Eq. 4)}$$

α_Q is given by Eq. 5, where X^- means ClO_4^-:

$$\alpha_Q = ([Q^+] + [QX]_{org.} + [X^-]_{org.})/[Q^+] = 1 + E_{QX} \times C_X + (k_{diss.} \times E_{QX} \times C_X/C_Q)^{1/2} \quad \text{(Eq. 5)}$$

Eq. 5 is valid under the assumption that $[X^-]_{org.} = [Q^+]_{org.}$. If the constants in Table I, $C_X < 10^{-4.5}$, and $C_X/C_Q = 100$ are used, the calculation will give $\alpha_Q = 10^{0.55}$.

The calculation of α_{HB} at pH = 9.0 is easily made from Eq. 6:

$$\alpha_{HB} = C_B/[HB^-] = 10^{pH-pK'_{HB}} = 10^{1.88} \quad \text{(Eq. 6)}$$

When equal phase volumes are used, a percentage extraction of 99.0 is obtained if $D_{QHB} = 100$. If this value, E_{QHB} from Table I, and the found α_Q and α_{HB} are inserted in Eq. 4, a $C_B = 10^{-4.2}$ is obtained. This is the minimum concentration of BTB necessary for exchanging perchlorate for BTB in the emepronium ion pair. In the method, a slightly higher concentration is used to avoid losses due to small changes in the pH of the buffer.

The method was tested repeatedly on urine samples with added known amounts of emepronium bromide giving an initial concentration of the urine of 0.2 mcg./ml. and higher. Including the compensation for the solubility of dichloromethane in the urine, the absolute recovery of the method was 98 ± 3%, which is in good agreement with the theoretical recovery. For some single objects, somewhat lower precision in the determination was obtained due to incomplete phase separation.

Urinary Excretion—The described determination method was applied in some excretion studies; examples of excretion curves after oral administration of therapeutic doses are presented in Fig. 1. The comparative study of the total urinary excretion 12 hr. after oral and rectal administration is presented in Table II, where the mean values and the standard deviation between the subjects are given. A rather small part of the drug was excreted in the urine, the value for the rectal administration being higher than for the oral. In the rectal experiment, about half of the volunteers complained of side effects typical for anticholinergic drugs, *i.e.*, drynes in the mouth and accommodation disturbances.

In another rectal experiment, when the dose was lowered and no premedication was given, no side effects were noted. The four persons participating were identical with those in Fig. 1, and their excretion curves are given in Fig. 2. Maximal excretion in this case occurred within the first 2 hr. after administration.

Further studies of the absorption, metabolism, and excretion of emepronium bromide are in progress.

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Cactus Alkaloids X: Isolation of Hordenine and *N*-Methyltyramine from *Ariocarpus kotschoubeyanus*

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Abstract □ Hordenine HCl and *N*-methyltyramine HCl were crystallized from extracts of the cactus *Ariocarpus kotschoubeyanus* (Lemaire) Schumann. Other alkaloids, which have been found in closely related *Ariocarpus* species, were not detected in this species. Extraction of the isolated alkaloids by percolation gave higher yields than continuous extraction.

Keyphrases □ *Ariocarpus kotschoubeyanus*—isolation of hordenine, *N*-methyltyramine □ Hordenine—isolation from *Ariocarpus kotschoubeyanus* □ *N*-Methyltyramine—isolation from *Ariocarpus kotschoubeyanus* □ TLC—separation

Ariocarpus kotschoubeyanus (Lemaire) Schumann, like some other species of *Ariocarpus*, is commonly called pezuña de venado, pata de venado, chaute, or peyote in areas of northern and central Mexico where the cactus is indigenous (1–5). The common name of peyote and the purported poisonous nature of the plant (2, 4) aroused interest in this laboratory concerning its alkaloid chemistry. No previous phytochemical studies have been reported regarding *A. kotschoubeyanus;* however, *N*-methyl-3,4-dimethoxy-β-phenethylamine, hordenine, and *N*-methyltyramine have been identified and/or isolated from *A. fissuratus* (Engelmann) Schumann (6–8), *A. trigonus* (Weber) Schumann (9), and *A. retusus* Scheidweiler (10, 11), and *N*-methyl-4-methoxy-β-phenethylamine has been isolated from *A. retusus* (11).

In this investigation, alkaloids were initially extracted from defatted and basified plant material, using chloroform in large continuous extraction apparatuses; an anion-exchange resin was used to separate the purified alkaloids into phenolic and nonphenolic fractions (12). As evidenced by TLC, the phenolic fraction contained hordenine and *N*-methyltyramine, which were resolved by preparative TLC and crystallized as the hydro-

coefficients (3). The following equations will be valid:

$$D_{QHB} = E_{QHB} \times C_B \times \alpha_{HB}^{-1} \times \alpha_Q^{-1} \quad \text{(Eq. 4)}$$

α_Q is given by Eq. 5, where X^- means ClO_4^-:

$$\alpha_Q = ([Q^+] + [QX]_{org.} + [X^-]_{org.})/[Q^+] = 1 + E_{QX} \times C_X + (k_{diss.} \times E_{QX} \times C_X/C_Q)^{1/2} \quad \text{(Eq. 5)}$$

Eq. 5 is valid under the assumption that $[X^-]_{org.} = [Q^+]_{org.}$. If the constants in Table I, $C_X < 10^{-4.5}$, and $C_X/C_Q = 100$ are used, the calculation will give $\alpha_Q = 10^{0.55}$.

The calculation of α_{HB} at pH = 9.0 is easily made from Eq. 6:

$$\alpha_{HB} = C_B/[HB^-] = 10^{pH-pK'_{HB}} = 10^{1.88} \quad \text{(Eq. 6)}$$

When equal phase volumes are used, a percentage extraction of 99.0 is obtained if $D_{QHB} = 100$. If this value, E_{QHB} from Table I, and the found α_Q and α_{HB} are inserted in Eq. 4, a $C_B = 10^{-4.2}$ is obtained. This is the minimum concentration of BTB necessary for exchanging perchlorate for BTB in the emepronium ion pair. In the method, a slightly higher concentration is used to avoid losses due to small changes in the pH of the buffer.

The method was tested repeatedly on urine samples with added known amounts of emepronium bromide giving an initial concentration of the urine of 0.2 mcg./ml. and higher. Including the compensation for the solubility of dichloromethane in the urine, the absolute recovery of the method was 98 ± 3%, which is in good agreement with the theoretical recovery. For some single objects, somewhat lower precision in the determination was obtained due to incomplete phase separation.

Urinary Excretion—The described determination method was applied in some excretion studies; examples of excretion curves after oral administration of therapeutic doses are presented in Fig. 1. The comparative study of the total urinary excretion 12 hr. after oral and rectal administration is presented in Table II, where the mean values and the standard deviation between the subjects are given. A rather small part of the drug was excreted in the urine, the value for the rectal administration being higher than for the oral. In the rectal experiment, about half of the volunteers complained of side effects typical for anticholinergic drugs, *i.e.*, drynes in the mouth and accommodation disturbances.

In another rectal experiment, when the dose was lowered and no premedication was given, no side effects were noted. The four persons participating were identical with those in Fig. 1, and their excretion curves are given in Fig. 2. Maximal excretion in this case occurred within the first 2 hr. after administration.

Further studies of the absorption, metabolism, and excretion of emepronium bromide are in progress.

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Cactus Alkaloids X: Isolation of Hordenine and *N*-Methyltyramine from *Ariocarpus kotschoubeyanus*

J. M. NEAL, P. T. SATO, C. L. JOHNSON, and J. L. McLAUGHLIN

Abstract □ Hordenine HCl and *N*-methyltyramine HCl were crystallized from extracts of the cactus *Ariocarpus kotschoubeyanus* (Lemaire) Schumann. Other alkaloids, which have been found in closely related *Ariocarpus* species, were not detected in this species. Extraction of the isolated alkaloids by percolation gave higher yields than continuous extraction.

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In this investigation, alkaloids were initially extracted from defatted and basified plant material, using chloroform in large continuous extraction apparatuses; an anion-exchange resin was used to separate the purified alkaloids into phenolic and nonphenolic fractions (12). As evidenced by TLC, the phenolic fraction contained hordenine and *N*-methyltyramine, which were resolved by preparative TLC and crystallized as the hydro-

chlorides. Unlike the other *Ariocarpus* species investigated, the nonphenolic fraction was surprisingly devoid of any alkaloids in isolable quantity.

A second portion of plant material was extracted identically, but using cold percolation with chloroform rather than continuous extraction, to be assured that some thermolabile alkaloids had not been destroyed during the continuous extraction. Again, only minute amounts of nonphenolic alkaloids were detected; however, hordenine and *N*-methyltyramine were isolated in higher yields from the percolated extract. These alkaloids are weak sympathomimetics (13, 14) and might be responsible for some stimulatory effects upon ingestion of the plant.

EXPERIMENTAL

Plant Material—A total of 185 plants of *A. kotschoubeyanus* were purchased[1] and several are being maintained as greenhouse specimens[2]. The remaining plants were sliced, dried in a forced-air oven at 48° (78% moisture), and ground through a 3-mm. screen in a Wiley mill.

Extraction of Crude Alkaloids—Using large continuous extraction apparatuses, 1.5 kg. of the dried plant material was defatted, basified, and extracted with chloroform as previously described (12). A second portion of the plant material (1.2 kg.) was defatted in the large continuous extraction apparatuses, basified, and extracted with chloroform, using two large (10 × 50 cm.) percolators and adjusting the combined flow rate to about 1 l./hr. for 40 hr. The residues from the two chloroform extractions were processed identically, with one significant variation from a published procedure (10), to yield the crude alkaloids; the variation involved an additional chloroform extraction after adjusting the extracted aqueous solution at pH 9.5 to pH 10.5. The crude alkaloids were then resolved into phenolic and nonphenolic fractions with an anion-exchange column as previously described (12).

Preparative TLC—Each of the four resulting extracts was assayed by TLC, using previously described methods (11, 12). The nonphenolic extracts both contained scarcely detectable quantities of several unidentifiable alkaloids, while the phenolic fractions both contained appreciable quantities of compounds corresponding in R_f values and color reactions to hordenine and *N*-methyltyramine. These alkaloids were subsequently isolated from both phenolic fractions, using preparative TLC. The methods for the separation and preparation of the hydrochlorides were as previously described (11), with the exceptions that SGPF-254[3] was used as the adsorbant and the acid–base partitioning steps were omitted.

The continuous extraction method yielded 455 mg. (0.030%) of hordenine HCl (m.p. 181.5–183°)[4]; the percolation extract yielded 702 mg. (0.059%) of hordenine HCl which melted at the same temperature. The mixed melting points with authentic hordenine HCl[5] (m.p. 182–183°) were not depressed, and IR spectra[6] of the reference and natural compounds were essentially identical.

N-Methyltyramine HCl (55 mg., yield 0.004%), melting at 149–151°, was obtained from the continuous extraction method; 176 mg. (yield 0.015%) of this compound, melting at 148.5–150°, was obtained from the percolation extract. The two yields were combined and recrystallized twice from absolute ethanol–ethyl ether to afford 148 mg. of material melting at 151–152°. Synthetic *N*-methyltyramine HCl[7] also melted at 151–152°, as did a mixture of the synthetic and isolated material. IR spectra of the *N*-methyltyramine HCl from the two sources were indistinguishable.

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[1] Southwest Cactus Co., Alpine, TX 79830

[2] Identification confirmed by Dr. Edward F. Anderson, Department of Biology, Whitman College, Walla Walla, WA 99362

[3] Brinkmann Instruments, Inc.

[4] Fisher-Johns melting-point apparatus, uncorrected.

[5] Prepared from hordenine sulfate from Mann Research Labs.

[6] KBr pellets, Beckman IR-5A.

[7] Prepared from *N*-methyltyramine HBr supplied by Dr. A. Brossi, Hoffmann-La Roche, Inc.

Azulene Analogs of Pharmacologic Agents II: Amides

PETER H. DOUKAS* and TULLY J. SPEAKER

Abstract □ The paper describes the synthesis of nine azulene analogs of the benzenoid pharmacologic agent lidocaine, as part of a study of the pharmacodynamic effects of nonbenzenoid aromatic compounds. Two series of compounds were prepared: 1-azulyl amides and 1-amido-3-acetyl azulenes.

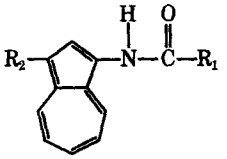
Keyphrases □ 1-Azulyl amides (lidocaine analogs)—synthesis □ 1-Amido-3-acetyl azulenes (lidocaine analogs)—synthesis □ Lidocaine analogs—synthesis, 1-azulyl amides, 1-amido-3-acetyl azulenes □ Column chromatography—separation

To study further (1) the pharmacological effects of replacing a benzenoid moiety by a nonbenzenoid, nonphysiologic azulyl structure, several azulyl amides (Types I and II) related to lidocaine (III) were synthesized as shown in Scheme I.

The 1-azulyl amides (Type I compounds) were prepared from 1-nitroazulene by reduction to 1-aminoazulene which, upon extraction from the reduction medium, was immediately reacted with the appropriate Ω-halo acid chloride in a Schotten–Baumann-type (2) synthesis to yield Ω-haloazulyl amides. It was essential that the aminoazulene be reacted immediately upon extraction, since even when kept cold, it underwent substantial decomposition. Once purified, the Ω-haloazulyl amides were reacted with the appropriate amines to yield the desired amino amides.

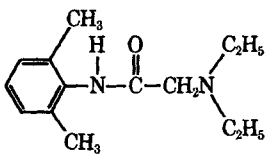
To introduce an electron-withdrawing substituent in the 3-position, various attempts to nitrate the azulyl amides were undertaken, all of which produced large amounts of amorphous decomposition products. Similar results were reported (3) for the nitration of the analogous *S*-acetyl thioazulene. It was subsequently decided to utilize the acetyl function in the 3-position as an electron-withdrawing function.

Attempts to prepare 1-amido-3-acetyl azulenes (Type II compounds) utilizing Friedel–Crafts conditions gave very poor yields of the desired intermediates due to difficulty in breaking the complex formed between the azulene and the Friedel–Crafts catalyst. The use of the Vilsmeier reaction was reported for the preparation of several azulyl ketones (4), and this approach gave satisfactory yields (about 40–50%) of appropriate intermediates. These amidoketones were very difficult to purify and required repeated chromatographic separation and recrystallization. Analytical data and NMR spectra indicated that the Vilsmeier product obtained

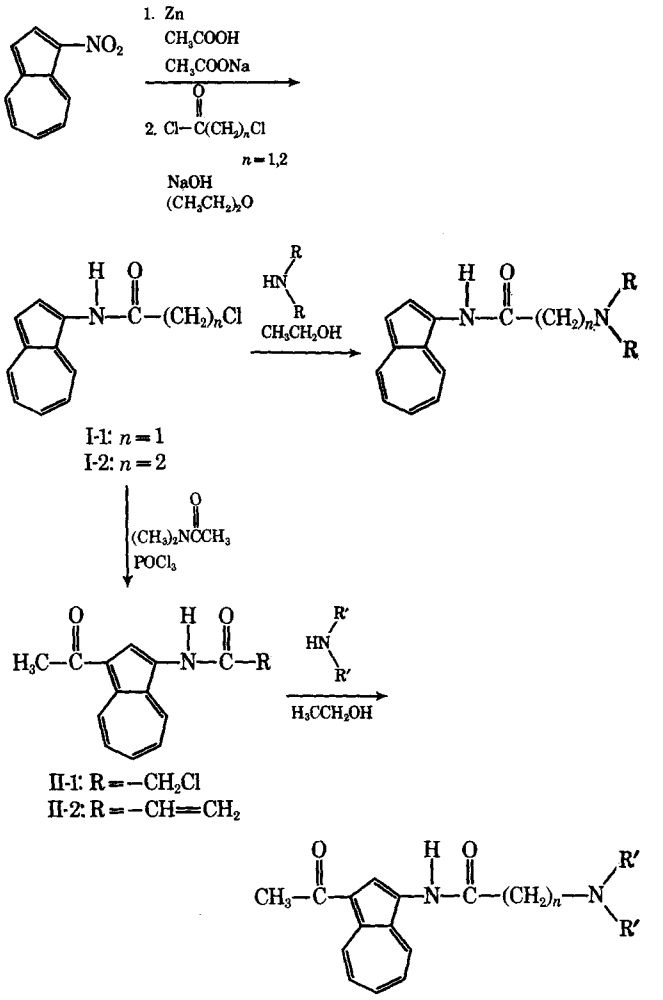


Type I: $R_2 = H$

Type II: $R_2 = CH_3C(=O)-$



III



Scheme I

from 1-(3-chloropropionamido)-azulene (I-2) had lost HCl to give 1-propenamido-3-acetylazulene (II-2).

The 1-propenamido-3-acetyl azulene was utilized in a Michael addition reaction to give the desired product. Acetylation of chloracetamido azulene (I-1) gave the expected 1-chloracetamido-3-acetylazulene (II-1). The biological properties of these compounds will be reported shortly.

EXPERIMENTAL[1]

Materials and Instrumentation—All melting points were taken on a Thomas-Hoover Uni-Melt apparatus and are uncorrected. UV and visible absorption spectra were obtained with a Cary model 15 recording spectrophotometer, utilizing ethanol as the solvent. IR spectra were obtained with a Perkin-Elmer Infracord model 127 recording spectrophotometer, utilizing potassium bromide disks. NMR spectra were obtained with Varian A-60 and Jeolco C-60H spectrometers, utilizing tetramethylsilane as the internal standard. The azulene used in this study was purchased[2],

[1] Analyses were performed by the Schwarzkopf Microanalytical Laboratories, Woodside, N. Y.

[2] Henley and Co., Inc., New York, N. Y.

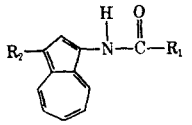


Table I—Azulylamides

Number	R_2=	R_1 =	M.p.	Formula	Percent Calcd.	Percent Found	Spectral Data: $IR_{max.}$ cm.$^{-1}$	Spectral Data: $UV_{max.}$/ Vis., nm.	Spectral Data: log e	Percent Yield	Recrystallization Solvent
I-1	H—	$-CH_2-Cl$	132.5–133.5°	$C_{12}H_{10}NOCl$	C, 65.58 H, 4.59 N, 6.37	C, 65.42 H, 4.60 N, 6.01	3200 1650	620 378 352 345 285 235	2.41 3.64 3.59 3.44sh 4.39 4.00	63.0	Chloroform–hexane
I-2	H—	$-CH_2-CH_2-Cl$[a]	112–113°	$C_{13}H_{12}NOCl$[a]	C, 66.81 H, 5.18 N, 5.99	C, 66.58 H, 4.80 N, 5.42	3200 1645	620 377 360 345 285 235	2.46 3.57 3.53 3.43sh 4.59 4.23	61.0	Chloroform–hexane
I-3	H—	$-CH_2-N(CH_2-CH_3)_2$	99[b]–100°	$C_{22}H_{23}N_5O_7$[b]	C, 56.28[b] H, 4.94 N, 14.92	C, 56.22[b] H, 4.98 N, 14.74	3200 1650	625 382 355 348 295 285	2.53 3.73 3.55 3.50sh 4.32sh 4.45	42.7	Ethanol
I-4	H—	$-CH_2-CH_2-N(CH_2-CH_3)_2 \cdot HCl$	187–189°	$C_{17}H_{22}N_2O$ HCl	C, 66.54 H, 7.55 N, 9.13	C, 66.52 H, 7.51 N, 8.90	3200 1650	625 385 368 355 295 288	2.60 3.73 3.71 3.60sh 4.46 4.54	68.6	Ethanol–ether
I-5	H—	$-CH_2-CH_2-N(CH_2-CH_2)_2O \cdot HCl$	243–245° dec.	$C_{17}H_{20}N_2O_2$ HCl	C, 63.64 H, 6.59 N, 8.73	C, 63.88 H, 6.86 N, 8.70	3210 1650	625 376 358 345 295 286	2.54 3.71 3.68 3.57sh 4.54 4.55	75.0	Ethanol–ether
II-1	$CH_3-C(=O)-$	$-CH_2-Cl$	180–183°	$C_{14}H_{12}NO_2Cl$	C, 64.25 H, 4.62 N, 5.35	C, 64.40 H, 4.58 N, 5.45	3200 1675 1645	570 395 300 245	2.68 3.81 4.43 4.23	64.2	Chloroform–benzene
II-2	$CH_3-C(=O)-$	$-CH=CH_2$[c]	187–189° (char 168°)	$C_{15}H_{13}NO_2$[c] H_2O	C, 70.02 H, 5.88 N, 5.44	C, 70.18 H, 6.00 N, 5.49	3150 1710 1650	575 402 305 240	2.69 3.82 4.50 4.07	49.0	Aqueous ethanol
II-3	$CH_3-C(=O)-$	$-CH_2-N(CH_2-CH_3)_2$	102–104°	$C_{18}H_{22}N_2O_2$	C, 72.45 H, 7.45 N, 9.39	C, 72.63 H, 7.31 N, 9.29	3250 1690	575 400 305 285 245	2.72 3.83 4.46 4.44 4.30	69.1	Ethyl acetate–*n*-heptane
II-4	$CH_3-C(=O)-$	$-CH_2-CH_2-N(CH_2-CH_3)_2$	93–95°	$C_{19}H_{24}N_2O_2$	C, 73.04 H, 7.74 N, 8.97	C, 72.99 H, 7.64 N, 9.06	3125 1675	575 400 305 245 245	2.71 3.88 4.58 4.58 4.45	70.0	Ethyl acetate–*n*-heptane

[a] NMR ($CDCl_3$): δ to (m, 7 protons), 3.7 (t, 2 protons), 2.7 (t, 2 protons). [b] Values are for the trinitrobenzene complex. [c] NMR (deuteroacetone): δ 7 to 10 (m, 6 protons), 6.5 (m, 2 protons), 5.75 (q, 1 proton), 2.65 (s, 3 protons).

and 1-nitroazulene was prepared after the method of Anderson *et al.* (5).

General Chromatographic Procedure—Chromatographic separations were done on Merck reagent aluminum oxide (alumina) No. 71707, utilizing the following procedure: concentrated solutions containing starting materials, reaction products, and decomposition products were adsorbed on a small amount of alumina (5–15 g.). The alumina adsorbate was air dried with frequent stirring. The resulting free-flowing adsorbate was packed above a 2 × 10-, 4 × 20-, or 4 × 40-cm. column of fresh alumina and eluted with solvents of increasing polarity. The following solvents and combinations thereof were used: petroleum ether, benzene, toluene, dichloromethane, chloroform, and ethanol.

General Method for Ω-Chloroacylamidoazulenes—Powdered zinc, 1.600 g. (0.024 g. atom), was added over a 0.5-hr. period to a mechanically stirred solution of 0.500 g. (0.0029 mole) of 1-nitroazulene and 0.700 g. (0.0086 mole) of sodium acetate in 30 ml. of glacial acetic acid in a 100-ml. beaker at room temperature. After complete addition of the zinc, during which the color of the solution slowly changed from red to violet, the reaction mixture was allowed to stir for an additional 20 min. The solution was diluted with 50 ml. of cold water, and the resulting mixture was decanted from the unreacted zinc. The mixture was exhaustively extracted with 30-ml. aliquots of peroxide-free ether; the combined green ether extracts were washed free of acid with 5% sodium hydroxide solution and then were washed with successive 50-ml. aliquots of water until the washings were substantially free of yellow color The ether solution was placed in a 500-ml. three-necked flask containing 50 ml. of 5% sodium hydroxide solution, and the whole mixture was placed in an ice bath. The biphasic mixture was stirred, and 0.0031 mole of the appropriate Ω-chloroacyl chloride in 10 ml. of ether was added dropwise. After complete addition, the mixture was allowed to stir for 20 min. (during which the color of the solution changed from green to blue). The phases were separated, and the ether layer was washed once with 50 ml. of 5% hydrochloric acid and twice with 50-ml. portions of water, dried over sodium

sulfate, and allowed to evaporate at room temperature. The resulting solids were recrystallized from chloroform–hexane to yield dark-green needles. Compounds I-1 and I-2 were prepared by this method. Percentage yields and physical and spectral properties of these compounds are presented in Table I.

General Method for 1-(Ω-Dialkylaminoacylamido)-azulenes—A solution of 0.50 mmole of the appropriate Ω-haloacylamidoazulene and 1.0 mmole of the appropriate secondary amine in 30 ml. of anhydrous ethanol was refluxed overnight, diluted to opacity with water, and extracted with chloroform. The chloroformic extract was extracted with dilute mineral acid; the resultant aqueous solution was basified and reextracted with chloroform. The organic phase was washed with water, dried over sodium sulfate, filtered, and allowed to evaporate at room temperature to yield a green oil. The oils prepared by this method were converted to their respective crystalline derivatives, I-3, I-4, and I-5, the physical and spectral properties of which are presented in Table I.

General Method for 1-(Acylamido)-3-acetylazulenes—A 10-fold molar excess (0.250 g.) of phosphorus oxychloride dissolved in 10 ml. of dimethylacetamide was added dropwise to a stirred solution of 0.22 mmole 1-chloracylamidoazulene in 15 ml. of dimethylacetamide. During the addition, the color of the solution gradually turned from blue to green. After completion of the addition, the stirred reaction mixture was heated in an 80° water bath for 1 hr., another 10-fold molar excess of $POCl_3$ was added, and the stirred mixture was kept in the 80° water bath for a 2nd hr. The flask was then removed from the water bath, and the stirred mixture was allowed to come slowly to room temperature. During this cooling, a large amount of crystalline white material separated from the green liquid. The mixture was chilled in an ice bath, 50–60 ml. of cold water was added, and the stirred resultant red solution was gradually made alkaline (pH 10) by the cautious addition of small portions of solid sodium carbonate.

The basic solution was exhaustively extracted with chloroform, and the combined green organic extracts were dried over sodium sulfate, filtered, concentrated, and chromatographed on alumina in accord with the general procedure previously described. Specifically, petroleum ether–dichloromethane (50:50) separated a blue band of starting material, leaving behind a slow moving green band which was extruded and packed atop a fresh column of alumina. Sequential development of the green band from the reaction mixture for II-1 with benzene and chloroform–benzene (30:70) separated a light-orange band and then II-1 as a dark-green band, leaving behind a faint yellowish-green band. Sequential development of the green band from the reaction mixture for II-2 with petroleum ether–dichloromethane (50:50) and chloroform eluted a faint-green band and then II-2 as a dark-green band. Evaporation of the eluting solvents gave solid or semisolid products which, after recrystallizing twice, gave crystals. The physical and spectral properties and yields of these compounds are given in Table I.

General Method for 1-(Ω-Diethylaminoacylamido)-3-acetylazulenes—A solution of 1.0 mmole of the appropriate 1-(Ω-haloacylamido)-3-acetylazulene and 2.0 mmoles of diethylamine in 15 ml. of anhydrous ethanol was refluxed overnight, diluted with 50 ml. of water, and exhaustively extracted with chloroform. The green organic phase was counterextracted with dilute hydrochloric acid, and the resultant red aqueous phase was made alkaline with dilute sodium hydroxide and chilled in an ice bath to give a green precipitate. The green precipitates were chromatogrammed on alumina with benzene to remove minor orange, yellow, and light-green bands and with chloroform to elute II-3 and II-4 as major dark-green zones. Spontaneous evaporation of the chloroform gave a green semisolid or oil, which solidified after standing for several days and which gave II-3 and II-4 as green crystals from ethyl acetate–*n*-heptane (50:50) when the solvent mixture was allowed to evaporate spontaneously. The physical and spectral properties and yields of these compounds are given in Table I.

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* Temple University Predoctoral Fellow, 1967–1969.

Effect of Vanadium on Metabolism of Glucose in the Rat

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Abstract □ A radiorespirometry experiment was conducted to determine the effects of the trace element vanadium on the metabolism of glucose-^{14}C in adult male rats by the collection of $^{14}CO_2$. Ammonium metavanadate solution was injected intraperitoneally, and labeled glucose was given *per os*. At the 0.05 significance level, an effect on metabolism was indicated by reduced levels of $^{14}CO_2$ after 1 and 3 hr. of collection but not at later times.

Keyphrases □ Vanadium—effect on glucose metabolism, rat □ Glucose metabolism—vanadium effect, rat □ Radiorespirometry—determination □ Liquid scintillation counting—analysis

Vanadium, a metallic trace element, is ubiquitous in the environment. Plants, animals, and man contain various amounts of vanadium, which has a predilection for biogenic fats and oils (1). In attempts to determine the biological functions of this element, several studies dealing primarily with the effect of vanadium on lipid metabolism were made. Bernheim and Bernheim (2), using a Warburg apparatus, demonstrated that vanadium increased the oxygen uptake of phospholipids in rat liver protein suspensions. The oxidation of other substrates including glucose, amino acids, and the lower fatty acids was not reported to be affected by vanadium.

Other investigators reported a decrease in rats in the hepatic synthesis of cholesterol (3), a decrease in the incorporation of ^{32}P into liver phospholipids (4), and a decrease in the activity of hepatic coenzyme A, coenzyme Q, and succinoxidase with vanadium present (5). The point of cholesterol inhibition has been suggested to be upon the enzyme squalene synthetase (6).

Since vanadium does affect lipid metabolism, it is reasonable to believe that the metal may also affect the metabolism of carbohydrate materials. Although the *in vitro* study of Bernheim and Bernheim (2) showed no effect of vanadium on glucose substrate, the response of the intact animal as measured with sensitive radiotracer techinques appeared to be worthy of investigation. This paper reports a study designed to determine the effects of vanadium on the metabolism of uniformly labeled glucose-^{14}C in intact rats by measuring amounts of expired $^{14}CO_2$.

EXPERIMENTAL

Radiochemical—The radiochemical purity of uniformly labeled D-glucose[1] (specific activity = 3.0 mc./mmole) was determined by paper chromatography and autoradiography. Fifteen micrograms of labeled glucose (0.25 μc.) and 10 mcg. of carrier glucose, each in a water solution, were spotted on three strips of Whatman No. 1 chromatography paper. Each strip was developed in one of three solvent systems: phenol–water (3:1 w/v), *n*-butanol–ethanol–water (52:30:18), and *n*-butanol–acetic acid–water (4:1:5). Glucose was located by lightly spraying with aniline phthalate solution[2] followed by heating 5–10 min. in an oven at 105°; brown spots formed. After development, an amount of the labeled glucose solution equal to 1% of that originally spotted was placed on each strip as a sensitivity spot. The strips were exposed to X-ray film[3] with an exposure time sufficient to allow 10^7 disintegrations from the sensitivity spots. No impurities at the 1% concentration level were revealed.

Metabolism Chambers—Four 15.24-cm. (6-in.), controlled-ventilation, glass metabolism chambers[4] were used to house the rats during CO_2 collection. A vacuum pump was used to draw CO_2-free room air through the chambers at the rate of 500 ml./min. Carbon dioxide was trapped in at least 75 ml. of a solution of 2-ethoxyethanol and 2-aminoethanol (2:1) contained in gas scrubbing towers. A secondary trap was included to ensure quantitative collection, although the efficiency of the primary trap was found to approach 100%. To prevent water quenching in the samples during counting, the chamber exhaust air was dehydrated with anhydrous calcium sulfate[5] prior to entering the primary CO_2 trapping solution.

Liquid Scintillation Counting—Activity determinations for ^{14}C were made with an internal liquid scintillation spectrometer[6] utilizing external standardization. Three-milliliter aliquots of the CO_2 trapping solution were added directly to 15 ml. of a liquid scintillator composed of 1 part toluene, 1 part 2-ethoxyethanol, and 0.8% 2,5-diphenyloxazole (8.0 g./l.). Duplicate aliquots were taken from each trap solution, and each sample was counted twice to minimize instrument error. The repeated counts on duplicate aliquots were averaged to obtain the sample activity. Primary trap samples were counted with a 1% counting error. Secondary trap samples with activities less than 1% of the primary trap activity were arbitrarily discarded. Sample counting efficiencies ranged from 70 to 73%.

Preliminary Studies—An investigation was made to determine the levels of ammonium metavanadate (NH_4VO_3) that could be safely administered to rats without causing sickness or death during the experimental period. Male rats[7] weighing approximately 200 g. were used. A single intraperitoneal dose of 11.0 mg. vanadium/kg. of body weight was found to be severely toxic, while a daily dose of 1.0 mg. vanadium/kg. was tolerated for 8 days with little observable effect. Daily doses of 2.0 and 3.0 mg. vanadium/kg. produced marked loss of weight after 7 and 5 days, respectively. As a result of these studies, single doses of 2.0 and 6.0 mg. vanadium/kg. and a daily dose of 2.0 mg. vanadium/kg. for 7 days were chosen for the respiration experiment.

Experimental Design and Procedures—The CO_2 respiration experiment was based on a completely randomized design utilizing 24 male rats weighing approximately 200 g. A control and three treatment groups, each containing six animals, were established. Four animals were tested each day for 6 consecutive days, with both treatment and day for testing assigned at random. All animals used in the respiration study were fasted for 24 hr. before dosing to reduce endogenous levels of glucose.

On the day for testing, each animal was given the appropriate amount of NH_4VO_3, dissolved in normal saline, by intraperitoneal injection followed immediately by 3.0 ml. of a 50% (w/v) glucose–water solution containing 4.01 μc./3 ml. *per os*. Control animals received labeled glucose, but saline solution was substituted for the NH_4VO_3 solution. Animals receiving repeated doses of vanadium were injected with the appropriate amount of NH_4VO_3 solution on each of 6 days prior to the respective day for testing.

Upon completion of dosing, the animals were immediately placed in the metabolism chambers where water, but no food, was pro-

[1] Amersham/Searle Corp., Des Plaines, Ill.
[2] Solution contained 0.93 g. of aniline, 1.66 g. of *o*-phthalic acid, and 100 ml. of water-saturated *n*-butanol.
[3] Kodak No-Screen Medical X-ray film, Eastman Kodak Co., Rochester, N. Y.
[4] Delmar Scientific Laboratories, Inc., Maywood, Ill.
[5] Drierite.
[6] Tri-Carb, Packard Instrument Co., Downers Grove, Ill.
[7] Sprague-Dawley descendants, Laboratory Supply Co., Indianapolis, Ind.

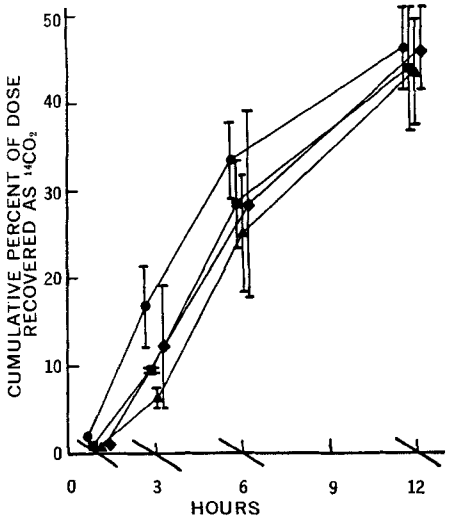

Figure 1—*Amount of $^{14}CO_2$ expired by rats receiving uniformly labeled glucose-^{14}C. Data points are staggered to include error bars which indicate 95% confidence intervals. Key: ●, control; ■, 2.0 mg. vanadium/kg.; ▲, 6.0 mg. vanadium/kg.; and ◆, 2.0 mg. vanadium/kg. daily for 7 days.*

vided. Carbon dioxide was collected for 12 hr., with the trapping solutions being changed at 1, 3, and 6 hr. At the end of the experimental period, the animals were sacrificed and discarded.

RESULTS AND CONCLUSIONS

The data from the CO_2 respiration study are shown in Fig. 1. At the end of each sampling interval, the control and the treatments were tested for significant differences at the 0.05 level by analysis of variance. Significant differences in the cumulative amounts of $^{14}CO_2$ collected were found at the 1- and 3-hr. collection intervals but not at the 6- and 12-hr. intervals. Further statistical comparison of the means by the Newman–Keul method (7), at the 0.05 level, indicated that at the 1-hr. interval each of the treatments produced significantly lower amounts of $^{14}CO_2$ than the control. At the 3-hr. interval, only the single-dose treatments were significant. Large biological variations appeared to obscure statistical significance at the 6- and 12-hr. intervals. Steadily decreasing amounts of labeled glucose in the body may also have reduced the effects of vanadium at the 12-hr. period.

Vanadium was thus found to affect glucose metabolism. The 6.0-mg. vanadium/kg. dose produced the greatest effect, but the 2.0-mg. dose also lowered $^{14}CO_2$ production significantly. Further studies are needed to determine the mode and site of action.

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Assignment of Configuration of Dehydrochlorination Products of Mitotane

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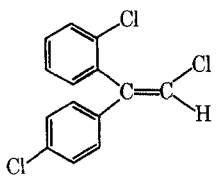
Abstract □ The drug of choice in the treatment of adrenal cortical carcinoma is mitotane (*o,p'*-DDD). The two geometric isomers obtained on dehydrochlorination of mitotane were separated for the first time and characterized. Analysis of the NMR spectra and the results of dipole moment measurements conclusively prove that the assignment of configuration of these compounds in the literature is incorrect. These compounds may be metabolites of mitotane.

Keyphrases □ Mitotane—separation, characterization, configuration assignment of dehydrochlorination products □ Configuration assignment—mitotane isomers, correction □ NMR spectroscopy—configuration, mitotane isomers

Approximately two people per million population are afflicted with cancer of the adrenal cortex (1). The drug of choice in the treatment of this condition is 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane [mitotane (*o,p'*-DDD)] a compound closely related in structure to the pesticide chlorophenothane (*p,p'*-DDT). The latter compound is readily dehydrochlorinated in the body to 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene (*p,p'*-DDE). Since the normal dose of mitotane is quite high, 10 g./day, it is possible that one of its metabolites, such as the dehydrochlorination products, could be responsible for the antitumor activity. Dehydrochlorination of mitotane leads to the formation of two geometric isomers: *cis*- and *trans*-1-chloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethylene (I and II), where the terms *cis* and *trans* refer to the relative orientations of the 1-chloro and 2-(*o*-chlorophenyl) groups. This report is concerned with the assignment of the configuration of these compounds using NMR and dipole moment measurements and conclusively shows that the previous structural assignments in the literature are incorrect (2, 3).



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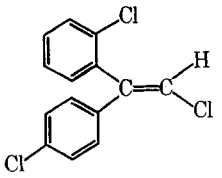


II

EXPERIMENTAL

Compounds I and II were prepared by dehydrochlorination of mitotane[1]. In a typical synthesis, 12.8 g. (0.04 mole) of mitotane was dissolved in 160 ml. of 1 *N* ethanolic NaOH, and the solution was refluxed for 1 hr. This was accompanied by the precipitation of NaCl. Water was added to the reaction mixture, and the resulting mixture was extracted with diethyl ether and dried with anhydrous calcium sulfate[2]. The solvent was removed under vacuum, the weight of the crude product being 95% of theoretical. The mixture was resolved into two components, with no indication of starting material by TLC on an alumina plate. Prior to chromatography, the plate was dipped in a solution of 1:3 DMF–diethyl ether, developed with 2,2,4-trimethylpentane, and sprayed with silver nitrate solution. The average R_f values for the isomers were 0.63 (I) and 0.77 (II), and they were present in the mixture in approximately equal concentrations.

The isomers were ultimately separated in gram quantities by column chromotography. In a typical separation, approximately 1 g. of the mixture of Isomers I and II dissolved in 50 ml. of petroleum ether was added to a 2 × 30-cm. column packed with 30 g. of neutral alumina. The column was eluted with petroleum ether, and 10 fractions of approximately 100 ml. each were collected. The separation of the components in the various fractions was followed by gas chromatography, employing an F and M model 700 unit equipped with a thermal conductivity detector using a 1.8 m. × 0.64-cm. (6 ft. × 0.25-in.) glass column packed with 3% diethylene glycol succinate on 80/100 mesh Gas Chrom Q at the following temperatures: injection port, 235°; oven, 190°; and detector, 230°. The flow rate of the helium carrier gas was 45 ml./min. The retention times under these conditions were approximately 9.5 (II) and 11 (I) min. Fractions rich in the individual isomers, I and II, were pooled and crystallized from hexane. Final crystallization from ethanol in the case of I and cyclohexane in the case of II resulted in melting points of 54–55° and 53.5–54°, respectively. Melting points are uncorrected and were determined on a Kofler micro hot stage.

Anal.—Calcd. for $C_{14}H_9Cl_3$; C, 59.30; H, 3.20. Found: (I) C, 59.26; H, 3.01. (II) C, 59.09, H, 3.34.

The NMR spectra were determined with a JNM-C-60H spectrometer. Spectra were taken in deuterochloroform in a concentration of about 100 mg./0.5 ml. with tetramethylsilane as the internal reference. The chemical shifts of the vinylic protons of I and II were 6.73 and 6.37 δ, respectively. The UV spectra were obtained on a Cary 14 spectrophotometer.

The dipole moments were obtained in dry benzene employing weight fractions of solute W_2 of approximately 0.005, 0.01, 0.015, and 0.02. The dielectric constants (e) were measured at 25° using a Dipolmeter DM 01 WTW, and refractive indexes (n) at the same temperature were determined with an Abbe refractometer. In each case, several readings were taken for the solution, the solvent, and the air in various sequences (4).

The orientation molar polarization of the solute P_2 was obtained by the Guggenheim method (5), plotting e and n^2 *versus* W_2 and evaluating the slopes of the graphs, α and ν, respectively. Plots were linear within experimental error; in each case, a straight regression line was obtained by the least-squares method. The value of P_2 was obtained from the equation

$$P_2 = 3\frac{M_2}{d_1}\frac{\alpha - \nu}{(e_1 + 2)^2} \qquad \text{(Eq. 1)}$$

where M_2 is the molecular weight of the solute, and d_1 and e_1 are the density and dielectric constant of the pure solvent, respectively. The dipole moment μ (in Debye) was calculated from P_2 (in milliliters/mole) by the equation

$$\mu = 0.01282\sqrt{P_2 T} \qquad \text{(Eq. 2)}$$

By using $d_1 = 0.87368$ and $e_1 = 2.2726$, the results in Table I were obtained.

RESULTS AND DISCUSSION

Configurational Assignment of I and II Using NMR—The compound 1-chloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethylene was first synthesized in 1962 (6). However, mention was not made of the possibility of two geometric isomers. More recently, Sharpless and Bradley (2) synthesized the mixture of I and II. Although they

[1] Aldrich Chemical Co. Inc., Milwaukee, Wis.
[2] Drierite.

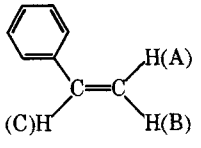
were unable to separate the isomers, they did obtain the NMR spectra which showed two sharp singlets in the vinylic region indicative of two nonequivalent vinylic protons. Their results showed that the aromatic portion of the spectrum was an unresolved multiplet and that the difference in chemical shift of the two vinylic signals was 0.34 p.p.m., which compares favorably with the difference of 0.36 p.p.m. in the current investigation. On the basis of the well-known fact that close proximity to other nuclei such as chlorine deshields protons (7, 8), they assigned the *trans*-configuration to the isomer in which the vinylic signal is the farther downfield. From an examination of a planar representation of the structure, this vinylic proton appears to be closer to the *o*-chloro group.

The same interpretation of the NMR results was given by Keith *et al.* (3). This interpretation of the NMR spectra, however, ignored possible differences in the chemical shift of the vinylic signals due to the magnetic anisotropy of the aromatic rings and differences in the preferred conformations of these rings in the two compounds. Indeed, when these factors are considered and in light of the dipole measurements, it is apparent that the assignment of structure made by these two groups is incorrect and that the isomer in which the vinylic signal is at the lowest field is actually the *cis*-isomer, I. This is the isomer in which, in the most favorable conformation, the ring *cis* to the vinylic hydrogen is in the same plane as the double bond, and the vinylic hydrogen is then in the region of maximum deshielding due to the magnetic anisotropy of the aromatic ring. An examination of molecular models indicates that there is more steric interference to a planar arrangement for the aromatic group *cis* to the vinylic proton in II than in I; thus the vinylic hydrogen in II is not deshielded as much and may actually be in the region of shielding. The difference in chemical shift for the signals of the vinylic protons in I and II of 0.36 p.p.m. is consistent with the downfield shift of 0.38 p.p.m. for the signal of the *cis*-β-proton in styrene as compared to ethylene (9).

The effect of the position of substitution on an aromatic ring and the resulting influence on the NMR of adjacent vinylic protons are shown by an examination of data obtained by Gurudata *et al.* on some aryl substituted styrenes (10). An examination of the chemical shift for the α-proton (Structure III, C) in the *o*-chloro-substituted derivative compared to the *p*-chloro compound shows that this proton is deshielded 0.45 p.p.m. in the former compound as compared to the latter. In compounds of this type, the preferred planar conformation is that in which the β-carbon is as far removed from the *ortho*-substituent as possible. The result is that the α-proton is in close proximity to the *ortho*-substituent, but not in such close proximity as to prevent a planar conformation and the α-proton is consequently deshielded. Thus, the *o*-chlorine is not adjacent to the *cis*-β-proton at all, but rather to the α-proton in these compounds.

It is instructive to consider the chemical shift of the A proton in the *ortho*- and *para*-chloro-substituted derivatives; there is very little difference in chemical shift for this proton in these two compounds with δ values of 5.59 and 5.67, respectively. This fact does not support the argument that proximity between the A proton and the chloro group is an important factor in the assignment of configuration in these compounds, and it shows that the aromatic ring has approximately the same degree of coplanarity with the vinylic group in the two compounds.

From an examination of molecular models of I and II, it is apparent that coplanarity of either ring with the vinyl group is exceedingly difficult in II. However, the *p*-chlorophenyl group in I has freedom of rotation and, therefore, can exist in the preferred planar orientation, resulting in deshielding of the *cis*-vinylic proton. Van der Linde *et al.* (11) obtained the NMR spectra of some 1,1-diarylalkenes. When one of the aryl groups carries an *ortho*-methyl substituent, the coplanarity of this ring is particularly disturbed and the absorption signal of the *cis*-hydrogen is shifted to higher field as compared to the compound in which both aryl groups are phenyl. The result is, however, that the other styrene system possessing only *ortho*-hydrogens can now more easily assume planarity, which then results in a downfield shift of 0.33 p.p.m. for the vinylic



III

Table I—Results of Dipole Moment Measurements for Isomers I and II

Parameter	I[a]	II[a]
α	1.541 ± 0.093	3.742 ± 0.089
ν	0.265 ± 0.019	0.259 ± 0.014
P_2 (milliliters/mole)	68.1 ± 5.1	185.8 ± 4.8
μ (Debye)	1.83 ± 0.07	3.02 ± 0.04

[a] Each figure is given with its probable error.

hydrogen *cis* to this phenyl group. The present situation is very similar; in I, coplanarity of the *p*-chlorophenyl group can occur, the result being the deshielding of the *cis*-vinylic proton. The UV data also support the idea of greater planarity in I (ϵ = 16,000, $\lambda_{max.}$ 258 nm.) than in II (ϵ = 14,900, $\lambda_{max.}$ 241 nm.).

The NMR signals for the aromatic portion of these molecules are also of interest but are difficult to interpret. The aromatic signals for I are a complex multiplet centered at about δ 7.32, whereas the analogous signal in II is essentially a singlet at 7.28. There may be greater deshielding of the *ortho*-hydrogens of the *p*-chlorophenyl ring by the vinylic group in I where a greater degree of coplanarity is possible, leading to a more complex signal. From inspection of molecular models, it appears that the most favorable conformation of the aromatic rings in II is that in which they are approximately perpendicular to the vinyl group. If this is true, the long-range effects of the aromatic rings on each other would be similar and the chemical shift of all aromatic protons signals would be nearly identical.

Configurational Assignment of I and II from Dipole Moment Measurements—Evaluation of the expected dipole moments of I and II by vector addition of bond moments (12) is easily done since only C—Cl bonds give a significant contribution, the moment of the H—$C_{vinylic}$ and H—$C_{aromatic}$ bonds being approximately zero. The value of the dipole moment of chlorobenzene (μ = 1.58) (13) was used for every C—Cl bond in I and II. Thus, for I, since the compensation of the C—Cl bonds is independent of rotation, the residual moment is that of the chlorobenzene molecule, *i.e.*, 1.58 D, which agrees well with the experimental value of 1.83 D. In the case of II, the vector addition of the Cl—$C_{vinylic}$ and *p*—Cl—C bonds always occurs in the vinylic plane, while the third vector (*o*—Cl—C bond) is out of this plane. In the limiting case of a 90° rotation, the vector addition in three-dimensional space gives a moment of 3.71 D. Even if the groups in II are planar, the calculated dipole moment would be 3.16 D. Thus, the calculated value for II varies between 3.16 and 3.71 D according to the amount of deviation from coplanarity, and these values are comparable to the experimental value of 3.02 D (Table I).

The experimental values obtained for I and II do not exactly match the calculated values because of certain approximations made in the calculations, such as the assumption of 120° angles and the additivity of bond moments, and neglect of any contribution from conjugation. Since the difference in the calculated dipole moments for the two isomers is large and the error introduced through the simplifying assumptions is small, there is no doubt concerning the assignment of configuration and the dipole moment measurements confirm the NMR results.

Compounds I and II are currently undergoing screening for antitumor activity at the Cancer Chemotherapy National Service Center.

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TLC Analysis of Mannich Bases Derived from Indolizines

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Abstract □ Seven Mannich bases derived from indolizines were studied using TLC procedures. Alumina, cellulose, and silica gel plates and a number of solvent systems were employed.

Keyphrases □ Mannich bases, from indolizines—TLC studies □ TLC—characterization, Mannich bases derived from indolizines □ Indolizine Mannich base derivatives—TLC requirements, characterization

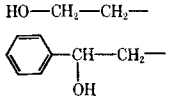
It was established in previous work that certain indolizines with dialkylaminomethyl side chains at the C-3 position exhibit pronounced CNS depression (1, 2). As part of a continuing exploration of indolizines with potential biological activity, a series of Mannich bases with *N*-substituted cyclohexylaminomethyl groupings were synthesized using 1,2-diphenylindolizine as the starting compound. The chemistry and biological activities of these compounds are reported elsewhere (3). In the present investigation, the characterization of these compounds is attempted using TLC procedures.

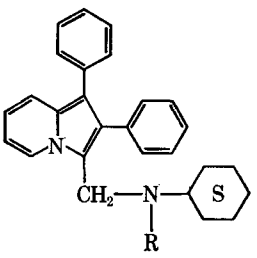
EXPERIMENTAL

Materials—3-Cyclohexylaminomethyl derivatives of 1,2-diphenylindolizine (Structure I) were used, where R was substituted as indicated in Table I. These compounds were prepared according to the methods described by Harrell *et al.* (3).

Developing Systems—Nineteen different solvent systems, indicated in Table II, were employed.

Table I—Compounds Used

Compound Number	R =	Melting Point
I		182–183°
II	$-C_2H_5$	123–124°
III	$-CH_3$	112–113°
IV	$(CH_3)_2CH-$	127–128°
V	$NC-CH_2-CH_2-$	152–153°
VI	$HO-CH_2-CH_2-$	138–139°
VII		137–138°



I

Detection—In most cases the spots were observed visually, giving a characteristic green color. The color was developed immediately after the plates were removed from the tank. A UV lamp[1] was also used. All compounds appeared to fluoresce under the longwave UV light.

Preparation of Plates—The plates (20 × 20 cm.) were coated with alumina[2], cellulose[3], or silica gel[4], 250 nm. thick, according to Stahl (4).

General Procedure—Solutions of the compounds were prepared by dissolving 10-mg. quantities into 3 ml. of acetone, chloroform, dioxane, or dimethylformamide (DMF). Samples of the solutions were applied 1.5 cm. from the bottom edges of the alumina, cellulose, or silica gel plates, and the ascending chromatograms were allowed to travel 10 cm. at room temperature. All experiments were repeated in a dark room at the same temperature (21°).

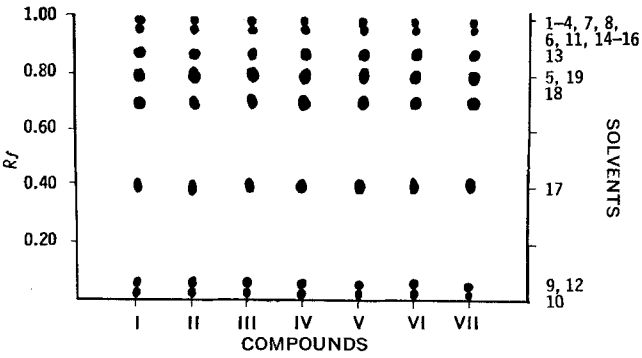


Figure 1—*Spot detection on silica gel plates. Left: R_f values. Right: solvent systems used (Table II). Compounds I–VII are those listed in Table I.*

[1] B. L. E. Spectroline, model C-3F.
[2] Aluminum Oxide G, Merck Co.
[3] MN-Cellulose Powder 300, Macherey, Nagel and Co.
[4] Silica gel G, Merck Co.

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TLC Analysis of Mannich Bases Derived from Indolizines

N. H. CHOULIS* and W. B. HARRELL†

Abstract □ Seven Mannich bases derived from indolizines were studied using TLC procedures. Alumina, cellulose, and silica gel plates and a number of solvent systems were employed.

Keyphrases □ Mannich bases, from indolizines—TLC studies □ TLC—characterization, Mannich bases derived from indolizines □ Indolizine Mannich base derivatives—TLC requirements, characterization

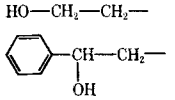
It was established in previous work that certain indolizines with dialkylaminomethyl side chains at the C-3 position exhibit pronounced CNS depression (1, 2). As part of a continuing exploration of indolizines with potential biological activity, a series of Mannich bases with *N*-substituted cyclohexylaminomethyl groupings were synthesized using 1,2-diphenylindolizine as the starting compound. The chemistry and biological activities of these compounds are reported elsewhere (3). In the present investigation, the characterization of these compounds is attempted using TLC procedures.

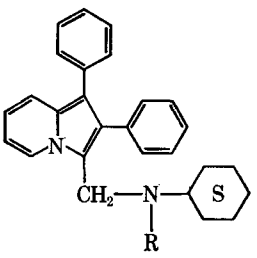
EXPERIMENTAL

Materials—3-Cyclohexylaminomethyl derivatives of 1,2-diphenylindolizine (Structure I) were used, where R was substituted as indicated in Table I. These compounds were prepared according to the methods described by Harrell *et al.* (3).

Developing Systems—Nineteen different solvent systems, indicated in Table II, were employed.

Table I—Compounds Used

Compound Number	R =	Melting Point
I		182–183°
II	$-C_2H_5$	123–124°
III	$-CH_3$	112–113°
IV	$(CH_3)_2CH-$	127–128°
V	$NC-CH_2-CH_2-$	152–153°
VI	$HO-CH_2-CH_2-$	138–139°
VII		137–138°



I

Detection—In most cases the spots were observed visually, giving a characteristic green color. The color was developed immediately after the plates were removed from the tank. A UV lamp[1] was also used. All compounds appeared to fluoresce under the longwave UV light.

Preparation of Plates—The plates (20 × 20 cm.) were coated with alumina[2], cellulose[3], or silica gel[4], 250 nm. thick, according to Stahl (4).

General Procedure—Solutions of the compounds were prepared by dissolving 10-mg. quantities into 3 ml. of acetone, chloroform, dioxane, or dimethylformamide (DMF). Samples of the solutions were applied 1.5 cm. from the bottom edges of the alumina, cellulose, or silica gel plates, and the ascending chromatograms were allowed to travel 10 cm. at room temperature. All experiments were repeated in a dark room at the same temperature (21°).

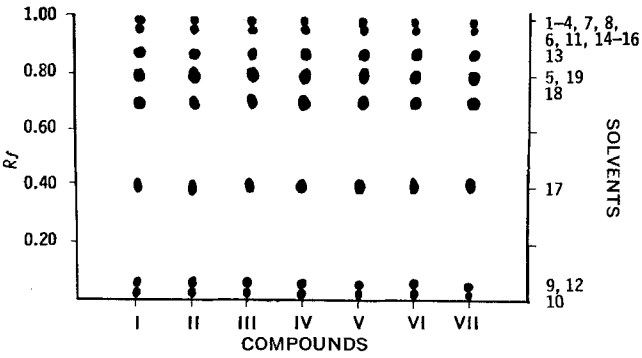


Figure 1—*Spot detection on silica gel plates. Left:* R_f *values. Right: solvent systems used (Table II). Compounds I–VII are those listed in Table I.*

[1] B. L. E. Spectroline, model C-3F.
[2] Aluminum Oxide G, Merck Co.
[3] MN-Cellulose Powder 300, Macherey, Nagel and Co.
[4] Silica gel G, Merck Co.

Table II—Solvent Systems Used

System Number	Composition
1	Chloroform–methanol (90:10)
2	Chloroform–methanol (90:10) (saturated with 10% ammonia)
3	Benzene–acetone (90:10)
4	Benzene–ethanol (90:10)
5	Acetone–chloroform–acetic acid–water (40:40:20:5)
6	Acetone–chloroform–acetic acid–water (30:30:30:10)
7	Chloroform–ethanol (99:1)
8	Acetone
9	*n*-Hexane
10	Water
11	Benzene
12	Cyclohexane
13	Methanol
14	Dichloromethane
15	*n*-Butanol saturated with water
16	*n*-Butanol–acetic acid–water (30:50:20)
17	*n*-Hexane–benzene (70:30)
18	*n*-Hexane–benzene (50:50)
19	Methanol–cyclohexane–acetone (60:40:20)

After development, the chromatoplates were removed, air dried, and visualized. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

The results (Table III and Fig. 1) indicate that although a number of solvent systems (Table II) were employed, using layers of alumina, cellulose, and silica gel, all seven compounds (I–VII, Table I) gave the same R_f value, although different for the various solvent systems and layers (Table III).

Two main possibilities should be examined for the explanation of these results. First, the steric effects of the introduced (at the position R of the ring) groups are not profound enough to overcome the effect of the bulky main component on the adsorption properties of the various compounds in the series.

Martin (5) stated that a dependence between chemical structure and chromatographic behavior exists (6, 7) and that R_f values are affected by the substitution on the ring. However, deviations from the rule have also been observed (8). Here, the contribution of the introduced group to the *Rm* value ($Rm = \log 1/R_f$) is probably dependent on the total molecule, on the position of this group in the molecule, and upon the rest of the molecule. Moreover, the adsorbents and solvents used may have suppressed possible R_f deviations due to chain substitutes. As a result, all compounds traveled the same distance, producing the same R_f.

Studies of the separation of the compounds between the liquid phase of the support system and that of the moving solvent (partition coefficient) did not produce any valid information. The use of cellulose layers (where partition is predominant) as well as silica gel and alumina layers left no doubt that differences in partition will not be conclusive, since all seven compounds produced the same R_f value whether on cellulose, alumina, or silica gel layers (although different for the various solvent systems and/or layers, Table III).

Moreover, to avoid any changes in the stationary phase, the plates were equilibrated with the vapor of the developing solvent mixture, and the chromatograms were run in a solvent-saturated tank.

Second, although stability studies indicated that all seven compounds remained unchanged under normal conditions (3), some chemical changes may have occurred. These changes probably occurred after the compounds were dissolved in the solvents or were applied on the plates, resulting in the formation of a common compound, which gave the same R_f value in any given solvent system or layer for all seven compounds.

Table III—$R_f \times 100$ Values of 1,2-Diphenylindolizine Derivatives[a]

Compounds	Solvents 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Silica Gel Plates																			
I	100	100	100	100	80	95	100	100	5	0	100	4	85	98	100	100	40	70	80
II	100	100	100	100	80	95	100	100	5	0	100	4	85	98	100	100	40	70	80
III	100	100	100	100	80	95	100	100	5	0	100	4	85	98	100	100	40	70	80
IV	100	100	100	100	80	95	100	100	5	0	100	4	85	98	100	100	40	70	80
V	100	100	100	100	80	95	100	100	5	0	100	4	85	98	100	100	40	70	80
VI	100	100	100	100	80	95	100	100	5	0	100	4	85	98	100	100	40	70	80
VII	100	100	100	100	80	95	100	100	5	0	100	4	85	98	100	100	40	70	80
Alumina Plates																			
I	98	—	100	—	—	95	—	—	—	0	—	—	—	—	—	86	60	—	—
II	98	—	100	—	—	95	—	—	—	0	—	—	—	—	—	86	60	—	—
III	98	—	100	—	—	95	—	—	—	0	—	—	—	—	—	86	60	—	—
IV	98	—	100	—	—	95	—	—	—	0	—	—	—	—	—	86	60	—	—
V	98	—	100	—	—	95	—	—	—	0	—	—	—	—	—	86	60	—	—
VI	98	—	100	—	—	95	—	—	—	0	—	—	—	—	—	86	60	—	—
VII	98	—	100	—	—	95	—	—	—	0	—	—	—	—	—	86	60	—	—
Cellulose Plates																			
I	99	—	100	—	—	99	—	—	—	0	—	—	—	—	—	—	100	—	—
II	99	—	100	—	—	99	—	—	—	0	—	—	—	—	—	—	100	—	—
III	99	—	100	—	—	99	—	—	—	0	—	—	—	—	—	—	100	—	—
IV	99	—	100	—	—	99	—	—	—	0	—	—	—	—	—	—	100	—	—
V	99	—	100	—	—	99	—	—	—	0	—	—	—	—	—	—	100	—	—
VI	99	—	100	—	—	99	—	—	—	0	—	—	—	—	—	—	100	—	—
VII	99	—	100	—	—	99	—	—	—	0	—	—	—	—	—	—	100	—	—

[a] Compounds I–VII are the ones depicted in Table I; Solvents 1–19 are the ones depicted in Table II.

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Influence of Pentobarbital on Some Cardiovascular Effects of Cryptenamine in Dogs

BERNARD KOROL

Abstract □ In a series of 32 conscious and anesthetized paired dogs, pentobarbital anesthesia significantly lowered basal systolic/diastolic arterial pressures, raised basal heart rate, and altered their responses to selected autonomimetic treatments. Pentobarbital anesthesia also significantly increased the effective hypotensive and/or bilateral carotid occlusion pressor response inhibiting dose of cryptenamine. Further examination of the data revealed that the conscious dog systolic depressor response to cryptenamine is the most sensitive and accurate bioassay criterion for the evaluation of cryptenamine potency.

Keyphrases □ Pentobarbital–cryptenamine—cardiovascular effects, dogs □ Cryptenamine–pentobarbital—cardiovascular effects, dogs □ Anesthesia, pentobarbital—influence on cryptenamine cardiovascular effects, dogs

In an earlier study (1) the carotid sinus pressor reflex bioassay procedure for the hypotensive principles of *Veratrum viride* was examined. The results obtained indicated that the date on which the experiment was performed significantly influenced the mean basal arterial pressure level and the magnitude of the pressor response to bilateral carotid occlusion, before as well as after drug treatment, but did not significantly alter the relationship between the drug dose and its blood pressure lowering response. It was speculated that seasonal influences are possibly the results of rhythmic changes in disposition and deposition of adipose tissue and other physiological changes, as reported by Fisher *et al.* (2) and Hilditch (3).

The assays presently employed (4, 5) to determine the ratio of emetic to effective hypotensive potency of *V. viride* samples obtains these measures separately in conscious (emetic assay) and anesthetized (hypotensive assay) dogs. Conceivably the reported (4) superior ratio of emetic to hypotensive dose effects of cryptenamine is due to the physiological state of the animal as used in each assay procedure. Ideally, both assays should be performed simultaneously in the same preparation so that a more meaningful ratio can be obtained.

It is well known that the physiological state of the experimental preparation can profoundly influence both the quality as well as the amplitude of a fixed treatment response. Schneider and Rinehart (6) reported that, in the anesthetized dog, the alkaloid ibogaine produced a marked hypotension, while the conscious dog was devoid of this effect. The alkaloid yohimbine was reported by Gershon and Lang (7) to produce a hypertensive response in the conscious dog while evoking a depressor response when the dog was anesthetized with pentobarbital.

A series of experiments was performed in chronically prepared dogs in the conscious and anesthetized states in an attempt to elucidate the influence of anesthesia on the nature and intensity of some cardiovascular effects of cryptenamine. This report is concerned with the findings obtained in this study.

Table I—Effects of Pentobarbital Anesthesia on the Systolic/Diastolic Arterial Pressures and Heart Rates and Its Influence on the Responses by Cryptenamine, Bilateral Carotid Occlusion (BCO), Tyramine (Tyr), Noradrenaline (Nad), and Isoproterenol (Isup) in Dogs

	Conscious			Anesthetized		
	Systolic, mm. Hg	Diastolic, mm. Hg	Heart Rate, beats/min.	Systolic, mm. Hg	Diastolic, mm. Hg	Heart Rate, beats/min.
Control:						
Basal	132.6[a]	60.7[a]	112.1[a]	110.6[a]	73.1[a]	164.8[a]
BCO	+32.5[a]	+30.6[a]	+3.8[a]	+25.9[a]	+25.6[a]	+16.8[a]
Tyr	+27.9	+18.5	−35.0	+36.5	+20.6	−40.8
Nad	+107.1[a]	+62.7[a]	−64.5	+155.0[a]	+92.9[a]	−56.2
Isup	−39.0	−39.0[a]	+136.7[a]	−41.6	−48.4[a]	+105.8[a]
After 1 mcg./kg. i.v. cryptenamine (total dose):						
Basal	−5.0[a, b]	−2.9	−6.9	−0.8[b]	−0.3	+0.3
BCO	+27.1	+27.4	+1.61[b]	+23.6	+24.4	+15.3[b]
After 3 mcg./kg. i.v. cryptenamine (total dose: 4 mcg./kg.):						
Basal	−22.1[a, b]	−18.4[a, b]	−20.0[a]	−6.3[b]	−7.3[b]	−16.0[a]
BCO	+17.7[a]	+20.2[a]	+0.3[b]	+16.2[a]	+16.7[a]	+11.8[b]
Tyr	+30.0	+23.0	−14.0[a]	+36.4	+24.1	−23.6[a]
Nad	+91.0[b]	+61.0	−19.9[a, b]	+147.7[b]	+98.6[b]	−47.7[b]
Isup	—	—	—	−31.4	−51.4	+115.0
After 10 mcg./kg. i.v. cryptenamine (total dose: 14 mcg./kg.):						
Basal	−48.8[a, b]	−44.8[a, b]	−49.8[a]	−23.7[a],	−27.4[a, b]	−42.4[a]
BCO	+4.8[a]	+5.0[a]	+2.5	+3.6[a]	+3.1[a]	0[a]
Tyr	+28.8[b]	+20[b]	−29.3[b]	+46.7[b]	+34.7[a, b]	−17.0[a, b]
Nad	+95.7[b]	+72.2[b]	−37.6[a]	+165.9[b]	+105.7[b]	−29.4[a]
Isup	−26.2[a]	−30.4[a, b]	+96.4[a]	−33.1[a]	−45.3[b]	+107.8

[a] $p = 0.05$ for differences between groups, conscious *versus* anesthetized. [b] $p = 0.05$ for differences between groups, control and after cryptenamine.

Table II—Variance, Difference in Variance, and Power of the Test Analysis on the Influence of Pentobarbital Anesthesia on Basal Systolic Level and the Systolic Depressor Response to Doses of 1, 3, and 10 mcg./kg. Cryptenamine

Treatment	Conscious		Anesthetized		F	p
	Variance	N	Variance	N		
Basal	156.6	8.09(80) 15.88(95)	479.52	8.09(80) 15.99(95)	14.79	$\ll 0.005$
1 mcg./kg.	82.5	21.1(80) 41.3(95)	22.5	11,000(80) 22,000(95)	6.66	<0.005
3 mcg./kg.	898.9	5.03(80)	158.3	187.6(80) 367.6(95)	15.38	$\ll 0.005$
10 mcg./kg.	2700	1.03(80) 2.02(95)	733.3	13.3(80) 26.2(95)	32.32	$\ll 0.005$

MATERIALS AND METHODS

Eight male and female beagle dogs were surgically prepared with bilaterally externalized carotid artery loops by a modification (8) of the Van Leersum method (9). From this preparation, it is quite convenient to measure systolic and diastolic arterial pressure directly, to induce a reflex pressor response to bilateral carotid occlusion, and to measure concomitantly other autonomic functions such as electrocardiogram (EKG), heart rate, and respiration while the animal is fully conscious and only slightly restrained.

Direct systolic and diastolic arterial pressure (lateral) was measured from a Medicon cannula (model AR-3218) inserted and fixed into an externalized carotid artery. The cannula was connected to a Statham pressure transducer (model P23AA), which supplied input into a Beckman strain gauge coupler (model 9872) of the recorder. The cannula was maintained patent by the slow retrograde infusion of saline through the pressure transducer, the rate of infusion not altering the arterial pressure measurements.

Heart rate was measured by the conversion of the R-R wave interval by a Beckman cardiotachometer coupler (model 9857) from a bipolar electrocardiograph input obtained through Beckman biopotential electrodes positioned and affixed to the skin over the area of the sternum. A homemade electrode paste composed of NaCl (0.07 mole/454 g.) was used with these electrodes to facilitate conduction.

The indirect rate and amplitude of respiration were measured through a pneumograph belt positioned around the thoracico-abdominal junction and connected to the pressure transducer connected, in turn, to the strain gauge coupler.

All recording was made on a Beckman Type R dynograph at a paper speed of 5 mm./sec.

To control for order effect, the eight animals were treated in a random order as follows:

Dog No.	Order Code
43	1, 2, 1, 2
44	2, 1, 2, 1
50	1, 2, 2, 1
51	2, 1, 1, 2
52	1, 1, 2, 2
53	2, 2, 1, 1
19	1, 2, 1, 2
1110	2, 1, 2, 1

with 1 or 2 indicating that the dog was to be used in the conscious or anesthetized state, respectively. Each dog was used in accordance with the schedule shown on a frequency of twice per week. After this schedule was completed ($N = 32$), a 1-week recovery period was allowed; then the procedure was repeated, but each dog was moved down one step in this schedule. In this manner, a total of 64 tests was performed, 32 each in conscious and anesthetized pairs. Thus, each dog served as its own control and received a series of eight tests, four in the conscious state and four while anesthetized.

All dogs received an indwelling venocatheter (PE-50) in the brachialcephalic or saphenous vein for all drug solution administration. When the dogs were to be anesthetized, sodium pentobarbital, 30–35 mg./kg., was administered through the indwelling venous catheter. Care was taken to position the pressure transducer at the appropriate position (heart-horizontal) for each dog, particularly when anesthesia was employed.

A 10–15-min. equilibration period was allowed following the preparation of the animal into its experimental condition. After the equilibration period, the animal received a 30-sec. (A) bilateral carotid occlusion (BCO); noradrenaline bitartrate (NAd), 2 mcg./kg.; tyramine hydrochloride (Tyr), 200 mcg./kg.; isoproterenol hydrochloride (Isup), 2 mcg./kg.; and a second 30-sec. BCO episode (B). Intravenous doses of 1, 3, 10, and 30 mcg./kg. of cryptenamine[1] were administered, with a 30-sec. BCO episode after each dose. The maximum dose of cryptenamine administered was determined by the degree of inhibition of the BCO-induced pressor response; *i.e.*, the dose was not increased when the BCO pressor response was maximally inhibited. At this time, the fixed-dose treatments with noradrenaline, tyramine, and isoproterenol were repeated.

The data were analyzed for statistical significance by one of two standard methods. When applicable, the matched-pair t test (10) was used. Otherwise, Student's t test was used (11).

RESULTS AND DISCUSSION

The data from this study on the influence of pentobarbital anesthesia in dogs on basal systolic/diastolic arterial pressures and heart rate and on their response to selected autonomimetic treatments, before and after graded doses on cryptenamine, are summarized in Table I.

It was reported (12) that the intravenous administration of barbiturate (thiopental) in man resulted only in a transient lowering of arterial pressure shortly after drug treatment. In the study reported here, the intravenous administration of sodium pentobarbital, 30–35 mg./kg., produced a statistically significant change of basal systolic/diastolic arterial pressure and an associated increase in heart rate. Moreover, pentobarbital anesthesia resulted in a significant lowering of the arterial pressure pressor response to a 30-sec. BCO episode while increasing the tachycardia produced by this treatment. The noradrenaline-induced pressor response, on the other hand, was significantly enhanced by the induced anesthesia without apparent change in heart rate. The tyramine pressor response was also greater during anesthesia, but the difference did not achieve statistical significance. The isoproterenol-induced diastolic depressor response was also significantly enhanced by the anesthetic, although the positive chronotropic response was considerably less after anesthesia.

These divergent results can be explained on the basis of pentobarbital-evoked partial inhibition of buffer mechanisms. Since the carotid occlusion pressor response is largely dependent upon a functional buffer mechanism, inhibition of this buffer mechanism would result in a diminished pressor response to carotid occlusion. Likewise, removal of this homeostatic control would also result in an increased pressor response to noradrenaline, which acts primarily at the effector site rather than through an established "pressor reflex."

It is also possible to explain the apparent influence of pentobarbital anesthesia on these responses by applying the Law of Initial Values (13). Prochnik *et al.* (14) reported that the carotid occlusion response is basal level dependent, with increasing response amplitude associated with increased basal level. The authors previously reported (15) that the amplitude of the pressor response to tyramine is inversely related to basal level, *i.e.*, increasing response amplitude with diminished basal level, while the pressor response to noradrenaline is independent of basal level. Therefore, it is

[1] Active component of Unitensen Aquenous, supplied by Dr. Arvid Zuber, Neisler Laboratories, Decatur, Ill.

conceivable that the observed difference in response amplitude, at least to tyramine, appearing in the conscious and anesthetized physiological states may have been reflections of the differences in basal level between the two preparations.

The intravenous dose of 1 mcg./kg. cryptenamine produced a significant hypotensive response in the conscious dog; the response was also significantly different from the almost complete lack of arterial pressure response this dose of cryptenamine produced in the pentobarbital-anesthetized matched pairs. After the higher doses of 3 and 10 mcg./kg. i.v. cryptenamine, the systolic/diastolic hypotensive responses of the conscious dogs were significant when compared with their respective pretreat levels. Only the hypotensive response produced by the 10 mcg./kg. cryptenamine differed significantly from the control in the anesthetized preparation. Most interesting was the finding that the arterial pressure hypotensive response of the conscious dog continued to demonstrate greater sensitivity than that exhibited by the anesthetized dog. The systolic/diastolic hypotension was statistically significantly greater in the conscious dog than in the anesthetized pair after both these higher doses of cryptenamine.

The pressor response to BCO was inhibited to the same degree in the conscious and anesthetized dog pairs following high dose treatment with cryptenamine. Although the inhibition of the BCO response significantly differed from their respective controls, there was insignificant difference between the conscious and anesthetized pairs. However, the apparent inhibition of the BCO response could have been secondary to the hypotension, which would profoundly diminish the amplitude of the pressor response (14).

After treatment with the 3- and 10-mcg./kg. doses of cryptenamine, the tyramine, noradrenaline, and isoproterenol response relationships between the conscious and anesthetized pairs remained consistent with their respective differences obtained during the control evaluation. These findings are somewhat inconsistent with the findings of Jandhyala and Buckley (16) who concluded that, based on a variety of organ system functions, cryptenamine appeared to potentiate the response of isoproterenol on β-adrenergic receptors. Findings in the present study do not indicate any consistent effect of treatment with cryptenamine on the systolic/diastolic and heart rate responses to the β-adrenergic stimulant isoproterenol in either the conscious or anesthetized dog.

The data were subsequently analyzed for difference in variance of the basal systolic level and the systolic depressor response to cryptenamine between the conscious and anesthetized matched pair dogs by the analysis of variance *F*-test (17). Calculation was also made from the available data for the minimum number (N) of animal tests for basal level systolic and systolic depressor response at the 80 or 95% Power of the Test (18). These two sets of calculated data are presented in Table II.

The variance of the conscious dog basal systolic level was significantly lower ($p \ll 0.005$) than that calculated for the anesthetized population. However, after receiving treatment with cryptenamine (1, 3, and 10 mcg./kg.), the dose-dependent increasing variance was significantly greater ($p < 0.005$) in the conscious dogs compared to the matched paired anesthetized preparation. It should be emphasized here that the cryptenamine mean systolic depressor responses were significantly more pronounced in the conscious dog, which may account for some of the apparent increased variance.

It is speculated that cryptenamine alters homeostatic mechanisms operating in the conscious state more profoundly than those operating in the anesthetized state, thus resulting in the increased variance. The reciprocal variance relationship obtained for the basal systolic levels can be explained as due to a partial inhibition of homeostatic mechanisms by the anesthetic, while in the conscious dog these mechanisms are maximally homeostatic.

The 80 and 90% Power of the Test analysis revealed that the minimum number (N) of matched paired tests necessary to demonstrate significant difference between conscious and anesthetized dog basal systolic levels was 8.09 and 15.88, respectively. The minimum N necessary to exhibit a significant systolic depressor response to cryptenamine, at the three doses employed, was considerably larger in the anesthetized dog than in the conscious dog.

It is concluded, therefore, from the Power of the Test analysis that the conscious dog systolic depressor response is the most sensitive, accurate, and efficient bioassay criterion for the evaluation of cryptenamine potency.

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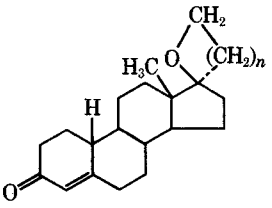
New Compounds: Steroidal Spiroethers

BALDEV SINGH and ROBERT G. CHRISTIANSEN

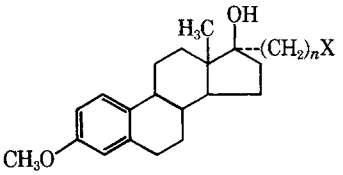
Abstract □ 3-Methoxyspiro[estra-1,3,5(10)-triene-17,2′-oxetane] (17β-O) (III*a*) and tetrahydro-3-methoxyspiro[estra-1,3,5(10)-triene-17,2′-pyran](17β-O) (III*b*) were synthesized from 3-methoxy-19-nor-17α-pregna-1,3,5(10),20-tetraen-17-ol (IV) and 17-(4-hydroxy-1-butynyl)-3-methoxyestra-1,3,5(10)-trien-17β-ol (V). Additionally, III*b* was transformed into tetrahydrospiro[estr-5(10)-ene-17,2′-pyran]-3-one (17β-O) (VII) *via* tetrahydro-3-methoxyspiro[estra-2,5(10)-diene-17,2′-pyran] (17β-O) (VI).

Keyphrases □ Steroidal spiroethers—synthesis □ Antiestrogenics—synthesis, steroidal spiroethers □ Spiroethers, steroidal—synthesis □ NMR spectroscopy—structure

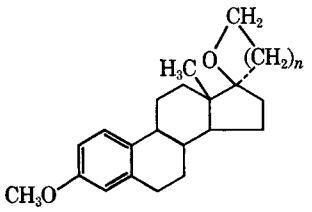
The disclosure by Brown (1) concerning the synthesis of the spiroether I*c* and the attempted synthesis of the spiroether I*a* prompted the authors to report the results obtained in their laboratory. Their interest in this area stemmed from the reported potent antiestrogenic activity of the spiroether I*b* (2). The intermediates, II*a* and II*d*, were prepared by different routes than those utilized by Brown (1). Hydroboration (3) of 3-methoxy-19-nor-17α-pregna-1,3,5(10),20-tetraen-17-ol (4) gave the diol II*a*, which was converted to the tosylate II*b* by means of *p*-toluenesulfonyl chloride in pyridine at 0–5° overnight. Brown (1) reported that at room temperature a mixture of II*b* and II*c* was isolated. The catalytic hydrogenation of 17-(4-hydroxy-1-butynyl)-3-methoxyestra-1,3,5(10)-trien-17β-ol (V) (5) afforded the diol II*d*. Treatment of II*d* with *p*-toluenesulfonyl chloride in pyridine at 0–5° gave the tosylate II*e* which could not be crystallized. The cyclizations of II*b* to III*a* and of II*e* to III*b* were effected with sodium hydride in refluxing tetrahydrofuran. The yields of III*a* and III*b* appear to be much better than those reported by Brown (1), who utilized potassium *tert*-butoxide in refluxing *tert*-butyl alcohol. Since the observations on the transformation of III*b* to I*c* and the attempted preparation of I*a* parallel those of Brown (1), they will not be duplicated here; however, VII [not reported by Brown (1)] was prepared by the oxalic acid hydrolysis of VI [not characterized by Brown (1)].



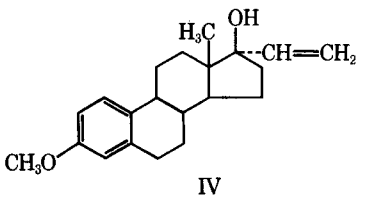
I*a*: $n = 1$
I*b*: $n = 2$
I*c*: $n = 3$



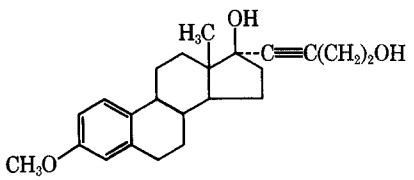
II*a*: $n = 2$; X = OH
II*b*: $n = 2$; X = O-tosyl
II*c*; $n = 2$; X = Cl
II*d*: $n = 4$; X = OH
II*e*: $n = 4$; X = O-tosyl



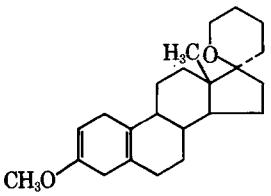
III*a*, $n = 1$
III*b*, $n = 3$



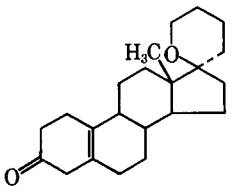
IV



V



VI



VII

EXPERIMENTAL[1]

3-Methoxy-19-nor-17α-pregna-1,3,5(10)-triene-17,21-diol (II*a*)—To an ice-cold solution of 62 g. of IV in 300 ml. of dry tetrahydrofuran was added 250 ml. of 1.0 *M* solution of disiamylborane in tetrahydrofuran. The resulting solution was allowed to stand at 0–10° for 72 hr. and was then stirred in an ice bath; 200 ml. of 3 *N* sodium hydroxide solution was added to it carefully, followed by the dropwise addition of 210 ml. of 30% hydrogen peroxide. Stirring was continued for another 2 hr., and then the unreacted hydrogen peroxide was destroyed by the addition of 40 ml. of a saturated solution of sodium bisulfite. The mixture was partitioned between chloroform and water. Evaporation of chloroform gave a white

[1] Melting points were determined in open capillaries in an oil bath and are uncorrected. Optical rotations were determined using 1% solutions of the compounds in chloroform; UV spectra were recorded on a Cary model 11 spectrophotometer in 95% ethanol solutions. IR spectra were determined with 0.5% KBr pellets on a Perkin-Elmer model 21 spectrophotometer, and the NMR spectra were recorded in $CDCl_3$ solutions using trimethylsilane as the internal standard on a Varian model A-60 spectrometer.

residue which was crystallized from acetonitrile to yield 40 g. of II*a*, m.p. 160–162° [lit. (1) m.p. 162–163°].

3-Methoxy-19-nor-17α-pregna-1,3,5(10)-triene-17,21-diol 21-*p*-Toluenesulfonate (II*b*)—A solution of 5.0 g. of II*a* and 6.0 g. of *p*-toluenesulfonylchloride in 30 ml. of dry pyridine was left at 0–5° overnight and then poured into ice-cold water. The product was extracted with ether. The ethereal extract was washed with dilute hydrochloric acid to remove pyridine. After it was dried ($MgSO_4$), the solution was concentrated to crystallize 5.8 g. of II*b*, m.p. 150–152° [lit. (1) m.p. 147–148°]; UV max. (95% EtOH) 224 nm. (21,000); $\lambda_{max.}^{KBr}$ 2.86, 8.43, and 8.53 μ; NMR signals at 390–480 (7 aromatic protons), 259 (t, —O_2SOCH_2—) and 145 Hz. (s, Ar—CH_3).

Anal.—Calcd. for $C_{28}H_{36}O_5S$: C, 69.40; H, 7.48; S, 6.61. Found: C, 69.62; H, 7.46; S, 6.58.

3-Methoxyspiro[estra-1,3,5(10)-triene-17,2′-oxetane] (17β-O) (III*a*)—A mixture of 6.0 g. of II*b*, 1.0 g. of sodium hydride, and 300 ml. of dry tetrahydrofuran was stirred under reflux for 120 hr. (the reaction was followed by TLC) and then allowed to cool to room temperature. The unreacted sodium hydride was destroyed by adding methanol (2 ml.), and the mixture was partitioned between ether and water. The ethereal extract was dried ($MgSO_4$) and then evaporated to give an oily residue, which was crystallized from *n*-pentane–ether to afford 3.5 g. of III*a*; m.p. 108–109°; NMR spectrum displayed peaks at 390–440 (aromatic protons), 223 (—OCH_3), 262 (m, —OCHz—), and 47 Hz. (18—CH_3).

Anal.—Calcd. for $C_{21}H_{28}O_2$: C, 80.72; H, 8.97. Found: C, 81.01; H, 9.09.

17-(4-Hydroxybutyl)-3-methoxyestra-1,3,5(10)-trien-17β-ol (II*d*)—V, 20.5 g., was hydrogenated over 1.2 g. of 10% palladium on charcoal catalyst in 300 ml. of dry tetrahydrofuran. The theoretical amount of hydrogen was taken up in 3.5 hr. Crystallization from ether gave 19.2 g. of II*d*, m.p. 124–126° [lit. (1) m.p. 127–128°].

Tetrahydro-3-methoxyspiro[estra-1,3,5(10)-triene-17,2′-pyran] (17β-O) (III*b*)—A solution of 3.8 g. of the diol II*d* and 4.1 g. of *p*-toluenesulfonyl chloride in 35 ml. of dry pyridine was allowed to stand in an ice bath for 6.5 hr. and then was partitioned between ether and water. The ethereal extract was washed with dilute hydrochloric acid three times and finally with water. After it was dried ($MgSO_4$), the solution was evaporated to dryness to give 4.94 g. of IV*e* as a semisolid material which failed to crystallize and was used as such in the next step (TLC examination indicated only one spot).

A slurry of 4.9 g. of the tosylate IV*e*, 300 mg. of sodium hydride, and 150 ml. of dry tetrahydrofuran was refluxed for 24 hr. After cooling to room temperature, the unreacted sodium hydride was destroyed by adding a few drops of methanol, and the mixture was partitioned between ether and water. The ethereal solution was dried ($MgSO_4$) and then evaporated to dryness. The residue was crystallized from *n*-pentane–ether to afford 3.1 g. of III*b*; m.p. 106–108°; NMR 390–440 (3 aromatic protons), 222 (OCH_3), 218 (m, —OCH_2—), and 51 Hz. (18—CH_3).

Anal.—Calcd. for $C_{28}H_{32}O_2$: C, 81.13; H, 9.44. Found: C, 81.03; H, 9.28.

Tetrahydro-3-methoxyspiro[estra-2,5(10)-diene-17,2′-pyran] (17β-O) (VI)—To a stirred solution of 16.7 g. of III*b* in 1600 ml. of dry ether and 1600 ml. of anhydrous liquid ammonia, held under reflux with a dry ice condenser, was added 20 g. of lithium wire over a period of 20 min. The resulting blue solution was further stirred for 30 min. At this stage, the reaction was quenched by the dropwise addition of 325 ml. of ethanol to give a colorless slurry. Ammonia was evaporated on a water bath, and the mixture was diluted with 800 ml. of water. The ethereal layer was decanted, dried ($MgSO_4$), and evaporated to dryness to give an oily residue which was crystallized from ether–*n*-pentane to afford 14.5 g. of VI, m.p. 140–142°; $\lambda_{max.}^{KBr}$ 5.90 and 6.0 μ; NMR 312 (m, C_2 proton), 212 (—OCH_2), 218 (m, OCH_2), and 51 Hz. (18—CH_3).

Anal.—Calcd. for $C_{23}H_{34}O_2$: C, 80.65; H, 10.01. Found: C, 80.80; H, 9.95.

Tetrahydrospiro[estr-5(10)-ene-17,2′-pyran]-3-one (17β-O) (VII)—A solution of 7.8 g. of VI, 13 ml. of water, 8.0 g. of oxalic acid dihydrate, 20 ml. of methanol, and 200 ml. of tetrahydrofuran was stirred at room temperature for 1 hr. and then diluted with 400 ml. of water. The product was extracted with ether. After drying ($MgSO_4$), the ethereal solution was evaporated to dryness to afford a white residue which was crystallized from ether to give 6.2 g. of VII, m.p. 164–166°; $\gamma_{max.}^{KBr}$ 5.81 μ; NMR spectrum displayed peaks at 200 (m, —OCH_2), 163 (s, 2 protons), 146 (s, 4 protons), and 51 Hz. (18—CH_3).

Anal.—Calcd. for $C_{22}H_{32}O_2$: C, 80.44; H, 9.83. Found: C, 80.80; H, 9.82.

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GLC Assay of Sodium Thyroxine

Keyphrases ☐ Thyroxine, sodium—analysis ☐ Diazomethane–thyroxine reaction—thyroxine analysis ☐ Estradiol benzoate—GLC internal standard ☐ GLC—analysis

Sir:

In the course of evaluating chemical assays of thyroid principles, it became clear that GLC held promise of reliable assays which would be simple, rapid, and highly specific. This communication reports our initial experience with a useful derivative of thyroxine prepared by reaction with diazomethane in a mixed solvent of methylene chloride and dimethyl sulfoxide under ambient conditions of temperature, pressure, and moisture.

Several groups reported other derivatives of thyroxine which allowed successful chromatography, and these are outlined here. Based partly on experience with several of those derivative sequences, we concluded that an alternative sequence was necessary. We wanted a one-step reaction in a simple vessel that would be relatively insensitive to other materials present, particularly moisture and salts. Because the USP and the NF list the sodium salts[1] of the optical isomers of thyroxine, the sequence had to be directly applicable to the sodium salt so that monograph purity and identity tests and assays could be developed. Methylation with diazomethane is commonplace for acids and has been used with phenols to a more limited extent.

The following sequence illustrates the methodology. Weigh 8 mg. of undried sodium *d*- or *l*-thyroxine into a narrow tube[2] and add 0.1 ml. dimethyl sulfoxide[3] containing aqueous HCl, 5% by volume. Solution is rapid. Add 1.0 ml. chromatographic grade methylene chloride containing 1.00 mg. estradiol benzoate NF reference standard as the assay internal standard. At this point, fine droplets may appear which consist of sodium chloride and any water present. In a hood, add 1.0 ml. diazomethane reagent[4], about 0.6 *M* in 2:1 ether–methylene chloride prepared by distillation of ethereal diazomethane into methylene chloride. Mix and allow to stand 30 min. using a polyethylene stopper. Uncover and evaporate the solution to a volume of about 0.2 ml. in a stream of N_2. The tube may be placed in warm water to speed the evaporation. Add 1 ml. methylene chloride and mix. A clear, colorless solution is obtained, containing a fine precipitate of salt. Inject 1.5 μl. or about 12 mcg. of derivative, on-column, in the following system: 0.6 m. × 4 mm. i.d. glass column, packed with 3% OV-1 on 80/100 silanized, acid-washed, flux-calcined diatomite[5], no-flow cured at 340° for 4 hr., and conditioned at 280°; helium carrier gas, 80 ml./min.; column temperature, 250°; and detector and injection port, 260°. The methylated T_4 derivatives elute in about 10 min., with a relative retention of 1.82 and 2.07, based on estradiol benzoate. The peak area of the methylated T_4 pair, the internal standard, and any other peaks are measured, and the ratio of areas of methylated T_4/estradiol benzoate is calculated. Peak height data are not acceptable.

Samples of sodium thyroxine, corrected for loss on drying, are compared quantitatively by this procedure, using a three-point calibration curve based on duplicate 6-, 8-, and 10-mg. samples of an arbitrary standard batch of either the salt or free acid. Moderate precision, 1.08%, was obtained without planimeter on multiple injection of a single 8-mg. sample. The column used to obtain the precision data gave a rectilinear calibration curve for the sample concentrations of 4, 6, 8, and 10 mg. Duplicates were within 1%.

For chromatographic purity, an internal standard is not added and suitable changes in electrometer attenuation are made to allow calculation of area percent of all peaks. Liothyronine (T_3) elutes as a pair of peaks at relative retention of 0.82 and 0.93; diiodothyronine occurs at 0.48; diiodotyrosine elutes too rapidly, 0.07, for accurate quantitation in this isothermal system, so that where this substance is important, temperature programming is necessary. The latter peak of T_3 predominates with longer reaction times and is the only peak after 5 hr. Slower methylation of the T_3 phenol compared to T_4 is consistent with the lesser acidity, *i.e.*, pKa 8.4 compared to pKa 6.6 (1). Tetraiodothyroacetic acid gives a peak at relative retention 1.02, but this offers no advantage as an internal standard, other than reaction control, over the steroid ester which is available in a controlled form.

Methylation of T_4 leads to a major peak, which is 93% of the total area, and one less mobile peak. The major peak exhibits no tailing and a symmetry of 0.93 due to the presence of another minor, but more mobile, methylated T_4 species. Initial NMR observations on the analogous diiodotyrosine suggest complete methylation of both phenolic and acid hydroxyls with possible methylation of the amine. Removal of excess diazomethane prior to injection is necessary to prevent side-product formation.

The column reported here, about 0.75 m., gave 1100 apparent plates for methylated T_4 and 1300 plates for estradiol benzoate with a 1.2 tailing factor. A column yielding 1000–1200 plates for estradiol benzoate, with a tailing factor less than 1.3, would be presumed adequate for this assay because the steroid is more chromatographically demanding than any of

[1] *Levo*-isomer as a thyroid hormone and *dextro* as an antihypercholesterolemic.

[2] Small centrifuge tubes or screw-cap vials. Silane treatment is not essential.

[3] Spectroquality, N_2 sparged.

[4] Diazald, Aldrich Chemical Co., prepared as directed except as noted.

[5] Gas-chrom Q and Supelcoport were used, both self-coated and supplier coated.

the iodoaminoacids. Samples as small as 1 mcg. of the methylated T_4 are symmetric, but the secondary peak is relatively larger. At higher sample sizes, greater than 20 mcg., severe front-edge leading develops, and this reflects the unusual solubility relationships of thyroxine. Linear calibration lines passing nearly through zero can be prepared by injection either of varying amounts of a single sample (1–30 mcg.) showing negligible, nonlinear adsorptive losses or by varying sample sizes of sodium thyroxine over a final sample concentration equivalent to 1–18 mg./ml. thyroxine. Using a 3% liquid coating, optimum chromatography requires injection of 5–20 mcg. of thyroxine. Smaller samples are best analyzed on 1% coatings, but caution is necessary. In our hands, a 1% OV-1 column showed distinct overload effects with samples in excess of 5 mcg. of thyroxine. This 1% loading allowed the detection of less than 50-ng. samples using flame ionization. Thermal instability causes significant losses, 24% at 340°, when very high injection port temperatures are used.

Flame-ionization detector (FID) response is notable. On a mass basis alone, the relative area response of methylated T_4 is only 15.7% that of estradiol benzoate. The response of the FID is commonly related (2) to the carbon content of the species, specifically those carbons not bonded to heteroatoms. The methylated T_4 response relative to the steroid when corrected for carbon content of the molecules is 47%. The interference of the heteroatoms, particularly the iodo groups, with the usual FID response is obvious.

Other reaction systems were investigated. Derivatization in plain methylene chloride led to a different peak pattern, with the minor, more mobile, methylated species becoming more abundant. Reaction of the phenol was slower, and initial solution of samples was less reliable. Although reaction in 40% dimethyl sulfoxide in methylene chloride led to the usual peak pattern, methylated T_4 is less soluble in dimethyl sulfoxide than in methylene chloride, so that calibration curves were not possible using concentrations above 4–5 mg./ml. in this solvent. The reverse holds for sodium thyroxine, which is directly soluble in dimethyl sulfoxide to at least 80 mg./ml. but which is only slowly soluble in methylene chloride. Solutions of the salt are yellow-brown. The methylated derivative, however, is soluble in methylene chloride and gives linear calibration curves up to at least 18 mg./ml. These solubility relations are the key to reliable quantitation of sodium thyroxine by GLC. A polar solvent, dimethyl sulfoxide, is necessary to dissolve the sample crystals; acid is necessary to convert the salt to the free acid which is soluble both in dimethyl sulfoxide and the much less polar 10% dimethyl sulfoxide–methylene chloride. Methylation leads to a derivative that is soluble in methylene chloride but which is much less soluble in polar solvents such as dimethyl sulfoxide and also in ether. Methylene chloride appears to be the solvent of choice for methylated thyroxine.

Dimethyl sulfoxide is desirable to dissolve the sodium salt, but the final concentration in the sample should be kept low for several reasons: diminished solubility of the derivative and the adverse effects of high-boiling solvents on reproducibility of elution data and analysis of rapidly eluted solutes. The effect of 40% dimethyl sulfoxide solvent on methylated T_4 was to make peak heights and retention times highly variable, so that the standard procedure, even with the more reproducible 10% dimethyl sulfoxide solvent, is to measure only peak areas. Formic or acetic acids in place of hydrochloric acid gave incomplete reaction. Direct reaction of the sodium salt was not rapid or quantitative, and a different peak pattern developed.

Alternative liquid phases were studied. OV-61 and OV-17 consist of 2:1 and 1:1, respectively, methyl–phenyl radicals in the polysiloxane. These gave much greater resolution but required much greater temperatures and carrier flow. Solvent elution was prolonged. Both were usable as 1.5% loadings, but the OV-61 was clearly less stable over long periods of use. All columns required occasional replacement of the first 10 cm. of packing, preferably after 30–50 injections. The OV-17 system may find application in biological studies or in a study of the reaction of diazomethane with iodoaminoacids. Detection by a hot-wire katharometer failed as sensitivity decreased with each injection, presumably due to chemical reaction with the halogenated species.

Two other approaches have led to successful GLC of thyroxine. Multiple-step syntheses of *N,O*-diacyl alkyl esters were reported by Stouffer *et al.* (3), Jaakonmaki and Stouffer (4), Hagen *et al.* (5), and Richards and Mason (6). None of these workers reported quantitative aspects. Variable solubility relations in the preparation of *N,O*-bisacetyl methyl esters and the need to control several steps diminished our interest in this approach.

Silylation methods have been applied, with the apparent advantage of one-step derivatization. GLC of iodoaminoacid trimethylsilyl derivatives was reported by several groups (7–9) using several silylation reagents. None of these workers considered solubility effects or the presence of polar materials or moisture on these reactions. We were unable to obtain reproducible silylation from concentrates by evaporation from solutions containing moisture and buffer salts. Also, the limited solubility of T_3 and T_4 made the direct use of silylating reagents uncertain. Sodium T_4 did not react readily due to failure to dissolve. Backer and Pileggi (10) made the very important observation that serum extracts using either cation- or anion-exchange resin workups did not yield samples suitable for silylation. Funakoshi and Cahnmann (11) made the additional observation that the sodium salts reacted only sluggishly. The structure of the trimethylsilane derivatives of iodoaminoacids is unresolved (8, 11). Observations made by the various workers on the problems of reaction of sodium salts and the presence of polar materials were confirmed in our hands and led us to search for an alternative to silylation. Work is planned to determine the applicability of the methylated derivative to biological extracts.

Osborn and Simpson (12) recently reported a sequential derivatization scheme for the identification of ^{125}I metabolites by TLC. They combined six solvents with four reactions: acetylation, hydrolysis, methyl

esterification, and methyl etherification. Methanolic diazomethane was used to prepare methyl ethers. The scheme allowed identification of a T_3 isomer in plaice, a flatfish (*Pleuronectes platessa*).

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N^{α},N^{α}-Dimethylhistamine, the Hypotensive Principle of the Sponge *Ianthella* sp.

Keyphrases ☐ *Ianthella* sp.—hypotensive constituent ☐ N^{α},N^{α}-Dimethylhistamine—isolation, identification ☐ Hypotensive activity—N^{α},N^{α}-dimethylhistamine

Sir:

The ubiquity of histamine in nature is well known; however, the occurrence of histamine derivatives in biological systems is less well known (1–8). We wish to report the isolation and identification of the potent hypotensive agent 4(or 5)-[2-(dimethylamino)ethyl]-imidazole from the sponge *Ianthella* sp.[1]. A previous report suggested the presence of this compound in the sponge *Geodia gigas*, but insufficient data were presented to afford unambiguous structure assignment (7, 8). Isolation of N^{α},N^{α}-dimethylhistamine from aqueous extracts of the sponge *Ianthella* sp. was effected by a combination of absorption and partition chromatography. Purification was followed by hypotensive activity in the anesthetized dog preparation.

Experimental[2]—Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. IR spectra were determined on a Beckman model IR-8 spectrometer. NMR spectra were determined on a Varian A-60 spectrometer, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. Mass spectra were determined on a Hitachi-RMU-6H mass spectrometer.

Isolation of 4(or 5)-[2-(Dimethylamino)ethyl]imidazole—A 100-g. sample of dried pulverized sponge *Ianthella* sp. was shaken with two 600-ml. portions of water for 2 hr. The two fractions were combined, and water was removed under reduced pressure at a temperature less than 50°. The residue was triturated with two 600-ml. portions of absolute ethanol. Combination of the fractions and removal of solvent gave 9 g. of residue, which had appreciable activity as a hypotensive agent.

Chromatography—The residue from initial extractions (9 g.) was dissolved in a minimum of absolute ethanol and placed on a 550-g. Florisil[3] column. The column was developed with increasing concentrations of ammonium hydroxide in absolute ethanol. Column effluence was monitored both by residue weight and hypotensive activity as determined in the anesthetized dog. Material eluted with 1 and 2% ammonium hydroxide in ethanol contained the desired activity. These fractions were combined, and the solvent was removed under reduced pressure to give 1.7 g. of material. TLC (acetonitrile–ammonium hydroxide) analysis of the residue indicated one principal component.

Further purification was effected by partition chromatography in the solvent system 2-butanol–ethyl acetate–triethylamine–water (600:400:10:990) using Celite 545[4] as the column support. Fractions (10 ml.) were collected automatically. Column fractions were monitored by weight and hypotensive activity. The fractions (homogeneous by TLC) were combined, and the solvent was removed under reduced pressure to give 685 mg. of desired material.

Characterization of 4(or 5)-[2-(Dimethylamino)ethyl]-imidazole—An analytical sample prepared by molecular distillation of the active component from column chromatography had an elemental analysis consistent with the formula $C_7H_{13}N_3$. The mass spectrum was characterized by a parent ion peak at *m/e* 139 with additional principal peaks at 124, 95, and 81 mass units. The NMR spectrum (D_2O) exhibited a sharp 6-proton absorption at δ2.19 (CH_3), a 4-proton absorption at δ2.65 (CH_2), and 2 broad singlets (1-proton each) at δ6.83 and δ7.62 (imidazole ring protons). A picrate salt, m.p. 223–226° [lit. (1) 223–226°], and the dihydrochloride salt, m.p. 183.5-184° [lit. (1) 182–184°], were prepared and characterized by elemental analysis. Comparison of the material from the sponge *Ianthella* sp. with a synthetically prepared sample of 4(or 5)-[2-(dimethylamino)-

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Unexpected Variable in the USP–NF Rotating Basket Dissolution Test

Keyphrases ☐ Dissolution test—rotating basket method ☐ Rotating basket dissolution test—vibration effect

Sir:

To increase our capability for dissolution-rate testing by the USP XVIII–NF XIII rotating basket method[1], we recently designed and constructed a mechanized apparatus capable of testing six dosage units simultaneously. To our surprise, this apparatus gave significantly longer dissolution times, especially at rotation speeds of 150 r.p.m. or less, than were obtained previously under the same conditions with a nonmechanized apparatus.

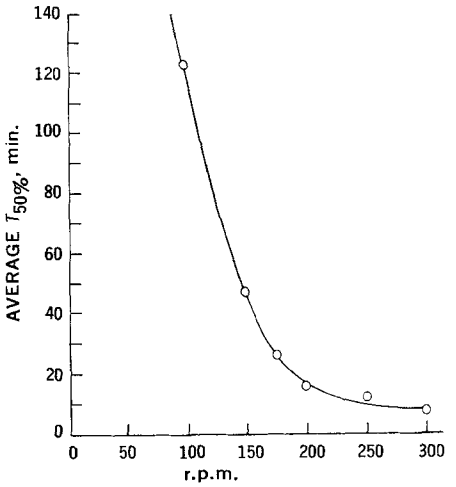

Figure 1—*Effect of rotation speed on dissolution rate of tolbutamide tablets USP, using the USP XVIII procedure with the mechanized apparatus.*

An investigation was undertaken to find the cause of this variation. Both pieces of apparatus conform to the requirements of the compendia (2, 3). By inspection, both permit smooth rotation of the basket assembly without perceptible wobble. The flask and stirrer assembly of the nonmechanized apparatus is mounted with a Fisher frame. The mechanized apparatus is of a unitized design; the six stirrers operate from a single-drive shaft; the flasks, held in place by a common manifold plate, rest on conical springs. The speeds of rotation for both pieces of equipment, as measured by a strobe light (or by counting revolutions at low speed), were well within the ±5% tolerance limits specified in the compendia. Both incorporate the same specified flask, which is sampled by a continuous flow system of the same pumping rate with similarly positioned inlet and outlet tubes of identical hold-up volumes.

Investigation finally revealed that a very subtle difference existed in the vibrational levels of the two pieces of equipment. Vibration was suspected when it was observed that the introduction of vibration by gentle tapping of the dissolution flask caused particles of a disintegrating tablet to spray from the basket; this was accompanied by an abrupt deflection in the dissolution profile toward higher percent dissolution. The vibrational levels associated with the two pieces of equip-

Table I—Effect of Vibration on Dissolution Times of a Single Lot of Tolbutamide Tablets USP by the USP Rotating Basket Method

Apparatus	Vibration[a] Displacement, mils	Vibration[a] Frequency, c.p.m.	Magnitude[b] of Vibration	Minutes for 50% Dissolution[c] 150 r.p.m.	300 r.p.m.
Nonmechanized unit	0.8	3600	Slightly rough	8.2	5.0
Nonmechanized unit, water pump off	0.2	3000	Very good	24	4.7
Mechanized unit	0.05	600	Extremely smooth	48	7.4

[a] Determined with Vibration Analyzer, model 600, International Research and Development Co., Columbus, Ohio. [b] From General Machinery "Vibration Severity Chart," International Research and Development Co., Columbus, Ohio. [c] Average of six tablets.

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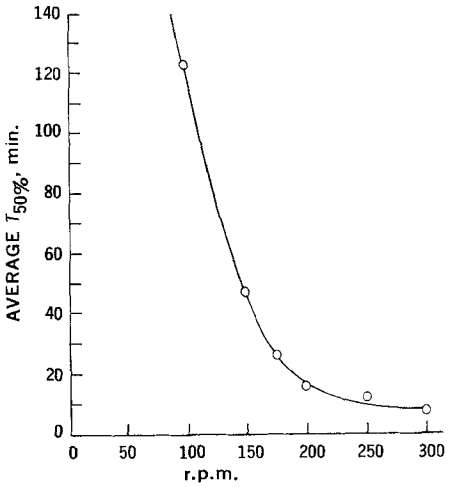

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ment were then tested with a vibration analyzer[2]. The analyzer probe was touched to various parts of the apparatus that contact the dissolution flask, and the vibration displacement and frequency values were recorded and averaged to give an estimate of the magnitude of vibration. These data are given in Table I, together with dissolution times obtained for a lot of tolbutamide tablets USP. Using the "Vibration Severity Chart" supplied by the manufacturer of the vibration analyzer, we rated the mechanized apparatus "extremely smooth" and the nonmechanized apparatus "slightly rough." Sources of vibration in the nonmechanized unit were traced to the benchtop, floor, and water circulator motor, with the latter being the principal source. When the circulator motor was turned off, the apparatus was rated "very good."

It is apparent from Table I that the effect of vibration on the dissolution rate of tolbutamide tablets USP is quite pronounced when the basket is rotated at 150 r.p.m., but it is almost insignificant when the basket is rotated at 300 r.p.m. In fact, for this lot of tablets the dissolution time obtained with the nonmechanized apparatus at 150 r.p.m. is about equivalent to that obtained with the mechanized apparatus at 300 r.p.m.

Some insight into the effect of vibration may be gained from a dissolution time–revolutions per minute profile. The profile shown in Fig. 1 was obtained, using the mechanized apparatus, for the same lot of tablets for which data are presented in Table I. Note that the slope is quite steep at 150 r.p.m. but quite flat at 300 r.p.m. Since additional vibration has the same effect on dissolution as additional rotation speed, it becomes immediately apparent why the effect of vibration is much more pronounced at 150 r.p.m. than at 300 r.p.m.

We have found that the shape of the dissolution time–revolutions per minute profile varies considerably from product to product, and to a lesser extent, from lot to lot of the same product. Therefore, control of vibration is more important for some products than for others. Undoubtedly, the nature of ingredients and the disintegration characteristics of the formulation are important factors.

It is not the purpose of this communication to cast doubt on the value of the USP and NF rotating basket dissolution test. In fact, we believe that this test holds much promise for the control of lot-to-lot dissolution-rate uniformity. However, if meaningful interlaboratory or even intralaboratory comparisons are to be made using this test, such variables as the one we have discussed must be identified and controlled.

When establishing dissolution specifications for products using the rotating basket method, the data presented here suggest two courses of action: either vibration must be carefully controlled within a specified limit as defined by an objective vibration test procedure, or its effects must be reduced by rotating the basket at sufficient speed.

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[2] Model 600 Vibration Analyzer, International Research and Development Co., Columbus, Ohio.

Preparation and Characterization of Lincomycin Cyclamate

Keyphrases ☐ Lincomycin cyclamate—synthesis, characterization ☐ Cyclamate interference—lincomycin therapy

Sir:

In recent studies, small quantities of the synthetic sweetening agents, sodium or calcium cyclamate[1], were shown to reduce the absorption of the antibiotic lincomycin hydrochloride to 25–30% of control values obtained in the absence of these agents (1). This activity was observed during carefully controlled clinical pharmacology studies, in which blood levels and/or urinary excretion were measured, in the development of a pediatric syrup of this antibiotic. It was found that depression in absorption of lincomycin occurs not only when the cyclamate is present with the antibiotic in the syrup ingested by both adults and children but also when the antibiotic is taken as the syrup and the cyclamate is ingested in the form of a diet drink[2], and mixing of the two fluids occurs in the human stomach. One molar equivalent of cyclamate essentially produced maximum depression of absorption of lincomycin hydrochloride, whereas no interference was found with the absorption of tetracycline hydrochloride.

In view of the obvious hazard to lincomycin therapy posed by the apparent interference of common cyclamates, we investigated the possibility of a metathetic reaction leading to precipitation of lincomycin cyclamate. Lincomycin cyclamate was expected to be easily precipitated from aqueous media by analogy with the known sparingly soluble hexadecylsulfamate and octadecylsulfamate salts of lincomycin (2) and from the fact that cyclamate salts have been prepared and characterized from several widely used classes of drugs such as antihistaminic, autonomic, myospasmolytic, central stimulant, neuroleptic, antitussive, antibiotic, and local anesthetics (3).

To our surprise, no precipitate formed when nonsaturated aqueous solutions of equivalent quantities of lincomycin hydrochloride were treated with either sodium or calcium cyclamate. Slightly impure linco-

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[2] Such as Diet-Rite Cola or Like.

ment were then tested with a vibration analyzer[2]. The analyzer probe was touched to various parts of the apparatus that contact the dissolution flask, and the vibration displacement and frequency values were recorded and averaged to give an estimate of the magnitude of vibration. These data are given in Table I, together with dissolution times obtained for a lot of tolbutamide tablets USP. Using the "Vibration Severity Chart" supplied by the manufacturer of the vibration analyzer, we rated the mechanized apparatus "extremely smooth" and the nonmechanized apparatus "slightly rough." Sources of vibration in the nonmechanized unit were traced to the benchtop, floor, and water circulator motor, with the latter being the principal source. When the circulator motor was turned off, the apparatus was rated "very good."

It is apparent from Table I that the effect of vibration on the dissolution rate of tolbutamide tablets USP is quite pronounced when the basket is rotated at 150 r.p.m., but it is almost insignificant when the basket is rotated at 300 r.p.m. In fact, for this lot of tablets the dissolution time obtained with the nonmechanized apparatus at 150 r.p.m. is about equivalent to that obtained with the mechanized apparatus at 300 r.p.m.

Some insight into the effect of vibration may be gained from a dissolution time–revolutions per minute profile. The profile shown in Fig. 1 was obtained, using the mechanized apparatus, for the same lot of tablets for which data are presented in Table I. Note that the slope is quite steep at 150 r.p.m. but quite flat at 300 r.p.m. Since additional vibration has the same effect on dissolution as additional rotation speed, it becomes immediately apparent why the effect of vibration is much more pronounced at 150 r.p.m. than at 300 r.p.m.

We have found that the shape of the dissolution time–revolutions per minute profile varies considerably from product to product, and to a lesser extent, from lot to lot of the same product. Therefore, control of vibration is more important for some products than for others. Undoubtedly, the nature of ingredients and the disintegration characteristics of the formulation are important factors.

It is not the purpose of this communication to cast doubt on the value of the USP and NF rotating basket dissolution test. In fact, we believe that this test holds much promise for the control of lot-to-lot dissolution-rate uniformity. However, if meaningful interlaboratory or even intralaboratory comparisons are to be made using this test, such variables as the one we have discussed must be identified and controlled.

When establishing dissolution specifications for products using the rotating basket method, the data presented here suggest two courses of action: either vibration must be carefully controlled within a specified limit as defined by an objective vibration test procedure, or its effects must be reduced by rotating the basket at sufficient speed.

(1) M. Pernarowski, W. Woo, and R. O. Searl, *J. Pharm. Sci.*, **57**, 1419(1968).

(2) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, pp. 934, 935.

(3) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, pp. 802, 803.

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[2] Model 600 Vibration Analyzer, International Research and Development Co., Columbus, Ohio.

Preparation and Characterization of Lincomycin Cyclamate

Keyphrases ☐ Lincomycin cyclamate—synthesis, characterization ☐ Cyclamate interference—lincomycin therapy

Sir:

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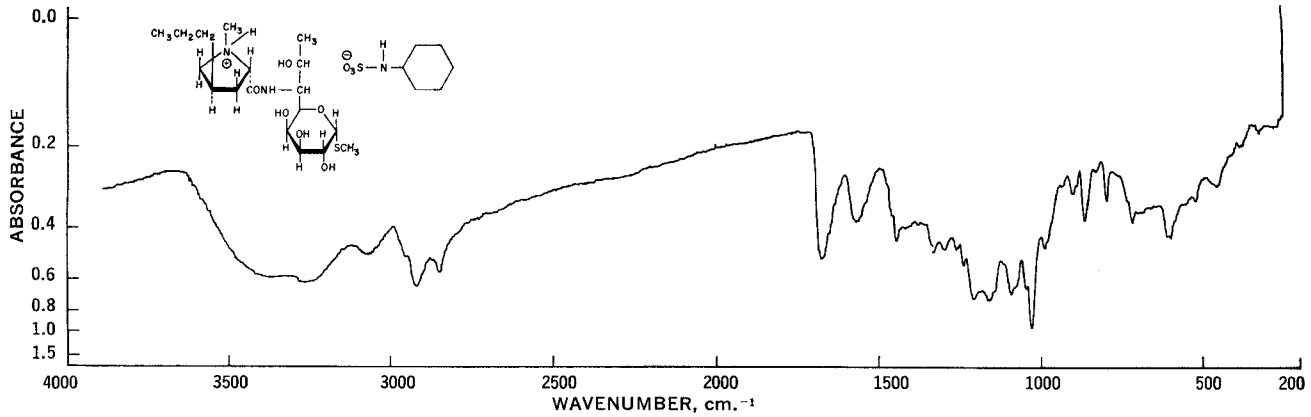

Figure 1—*Split mull IR spectrum (Perkin-Elmer 621 spectrometer) of lincomycin cyclamate dihydrate.*

mycin cyclamate, however, could be obtained as a waxy-white solid by slow, dropwise addition of a saturated aqueous solution of calcium cyclamate to a saturated aqueous solution of lincomycin hydrochloride. Solidification of the reaction mixture occurs suddenly, just short of complete addition of the equivalent quantity of calcium cyclamate. The precipitate is insoluble in excess saturated aqueous calcium cyclamate, but it is easily dissolved by water (solubility ~ 84 mg./ml.). It was possible to collect the lincomycin cyclamate at the stage of initial appearance of the waxy-white solid by treating the mixture with the minimal volume of absolute ethanol required to effect transfer. When, however, the preparation of lincomycin cyclamate was attempted from a concentrated solution of lincomycin HCl·H_2O in absolute ethanol (100 mg./1.5 ml.) by dropwise addition of a saturated aqueous solution of calcium cyclamate, no crystallization occurred on standing either at room temperature or at 0°, and no crystallization (neither lincomycin cyclamate nor calcium cyclamate) could be induced by further addition of absolute ethanol.

The lincomycin cyclamate prepared under these saturated aqueous conditions was characterized by mixed melting-point determinations and by comparison of IR spectra obtained from the same salt prepared under nonaqueous conditions. Lincomycin free base was generated from lincomycin HCl·H_2O (100 mg., 0.231 mmole) on treatment with excess aqueous am-

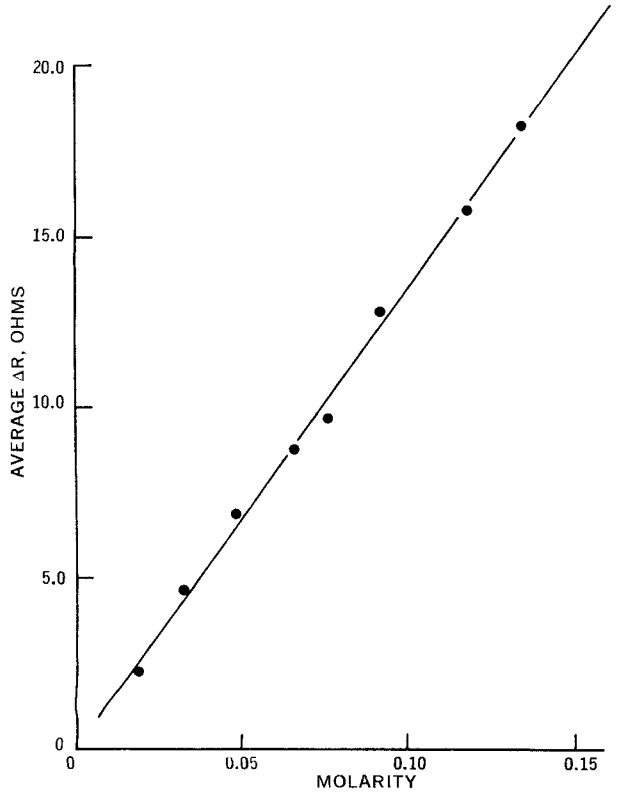

Figure 2—*Thermoelectric vapor phase osmometric measurements on individually prepared aqueous solutions of lincomycin cyclamate dihydrate at 37°.*

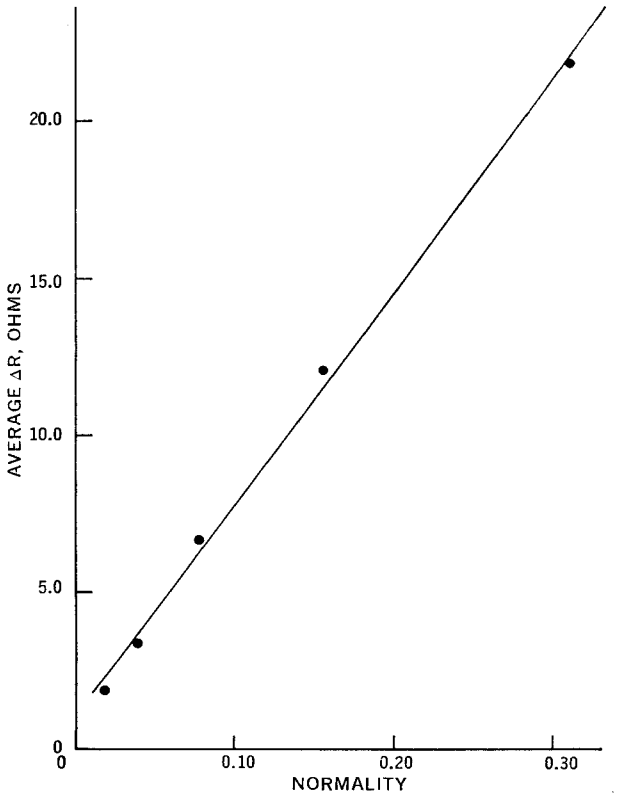

Figure 3—*Thermoelectric vapor phase osmometric dilution study of the effect of calcium cyclamate dihydrate on lincomycin cyclamate dihydrate at 37°. Dilution was followed from an aqueous solution 0.0163* M *(0.0490* N*) in calcium cyclamate dihydrate and 0.133* M *(0.265* N*) in lincomycin cyclamate dihydrate from an initial solution 0.314 total normality.*

monia and extracted with diethyl ether. After drying over anhydrous magnesium sulfate, the ethereal lincomycin solution was treated with a nearly saturated solution of cyclohexylsulfamic acid (42 mg., 0.231 mmole) in methyl ethyl ketone. Instant crystallization occurred during the addition. The crystals were collected, washed with diethyl ether, dried over P_2O_5, and found to melt at 149–151° (Kofler).

Anal.—Calcd. for $C_{18}H_{34}N_2O_6S \cdot C_6H_{13}NO_3S \cdot 2H_2O$: C, 46.36; H, 7.62; N, 6.76; S, 10.31. Found: C, 46.74; H, 8.14; N, 7.45; S, 10.49.

IR spectra (Fig. 1) of the lincomycin cyclamate dihydrate showed a broad band centered at 3400 cm.$^{-1}$ for ν_{O-H} from the water of hydration as well as from other OH groups within the lincomycin molecule itself and strong bands at 1165 and 1035 cm.$^{-1}$ for asymmetric and symmetric $\nu_{S=O}$ of the sulfonic acid moiety in the ionic form characterized by Detoni and Hadzi (4). These features are also consistent with $\nu_{O=H}$ at 3412 cm.$^{-1}$ and $\nu_{S=O}$ at 1168 and 1060 cm.$^{-1}$ found for calcium cyclamate dihydrate.

An attempt was made to prepare the anhydrous lincomycin cyclamate salt by subjecting the dihydrate to a pumping vacuum (0.1 mm. Hg) while heating the salt at 100° for 2–3 hr. No dehydration could be detected by comparison of IR spectral features. While these conditions have usually been satisfactory for dehydrating a wide range of salts (5), thermal gravimetric analysis (TGA) later showed no significant weight loss (2% up to 175°, probably due to surface moisture) by the material below 230°. An upper temperature limit is imposed by melting of the salt at 150°. We were also concerned about possible thermal degradation of the cyclamate salt, having observed a remarkably easy degradation of the hydrochloride salt (6) during evaporation of an aqueous ethanolic solution (pH 2) at 50° and 15 mm. Hg in a rotary evaporator. Differential thermal analysis (DTA) indicated the cyclamate salt to be much more stable than the hydrochloride and showed the following features: a small broad endothermic peak at 157° with no weight change (possibly a softening reaction) and large exothermic reactions accompanied by weight loss at 239, 323, and 506°. The sample was totally volatilized in air at 600°.

In view of these observations and our considerable difficulty in preparing lincomycin cyclamate, we conclude that the reported interference to absorption of lincomycin hydrochloride by common cyclamates does not involve a simple metathetic reaction. Since the next most likely cause of interference could arise from association of cyclamate anions with lincomycin cations in aqueous media, thermoelectric vapor phase osmometry (7, 8) was employed over the largest possible concentration range (Figs. 2 and 3) in an attempt to detect evidence of aggregation. In the absence of discontinuities in the curves of Figs. 2 and 3, it follows that no association of cyclamate with lincomycin ions occurs even in solutions slightly supersaturated with respect to lincomycin cyclamate (*e.g.*, 0.134 *M*, Fig. 2, and 0.133 *M*, Fig. 3). Other possibilities, therefore, apart from these simple physical phenomena must be considered to explain the apparent interference of common cyclamates to lincomycin therapy.

(1) J. G. Wagner, Abstracts, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, November 1968, p. 55.
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Comparative Systemic Availability of Acetaminophen when Administered Orally as Such and as Acetophenetidin

Keyphrases ☐ Acetaminophen systemic availability—acetaminophen, acetophenetidin administration ☐ Systemic availability, correlation—acetaminophen-precursor administration

Sir:

Phenolic drugs are metabolized to glucuronides and sulfates in man and animals (1). The systemic availability of some of these drugs upon oral administration may be decreased appreciably by biotransformation in the gastrointestinal mucosa and during the "first pass" through the liver (2–4). Acetaminophen (4′-hydroxyacetanilide) is a widely used analgesic and antipyretic agent which is eliminated mainly by glucuronide and sulfate formation (5). The related compound acetophenetidin (4′-ethoxyacetanilide) is largely converted in the body to acetaminophen (6). If acetaminophen is metabolized appreciably during absorption, its systemic availability should be relatively increased by administering its precursor, acetophenetidin, which has its phenolic group blocked and therefore protected from conjugation in the gut wall.

Prescott *et al.* (7) determined acetaminophen concentrations in the plasma upon oral administration of 1.8 g. acetaminophen or acetophenetidin to groups of normal subjects. They also determined the total urinary

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Table I—Systemic Availability of Acetaminophen when Administered Orally as Such and as Acetophenetidin[a]

Administered Drug	Number of Subjects	A, mcg./ml./hr. Uncorrected	A, mcg./ml./hr. Corrected for Difference in "Dose"[b]	$A \cdot \beta$, mcg./ml.	$A \cdot \beta$ Ratio, Acetaminophen to Acetophenetidin
Acetaminophen, Trial I	8	102	—	34.8	1.04
Acetophenetidin, Trial I	9	65.0	85.0	33.5	
Acetaminophen, Trial II	8	80.4	—	31.5	1.06
Acetophenetidin, Trial II	7	53.4	69.9	29.7	

[a] Based on mean data from *Reference 7*. [b] A, from acetophenetidin trials multiplied by 1.19 (ratio of molecular weights, acetophenetidin: acetaminophen) and by 1.1 (ratio of urinary recovery of acetaminophen and metabolites from acetaminophen and acetophenetidin).

excretion of acetaminophen and its conjugates. These data permit the determination of areas under the plasma concentration *versus* time curve, acetaminophen disposition-rate constant, and total amount of acetaminophen derived from acetophenetidin.

The amount of free acetaminophen entering the systemic circulation (D) can be calculated from the area under the plasma concentration curve (A), the apparent volume of distribution (V), and the disposition-rate constant for acetaminophen (β), according to the following relationship (8):

$$D = AV\beta \qquad \text{(Eq. 1)}^1$$

If the value of V is not known, one can determine D/V:

$$D/V = A\beta \qquad \text{(Eq. 2)}$$

Assuming that the average apparent volume of distribution did not differ appreciably in the two groups of subjects, the calculations indicate that the systemic availability of acetaminophen when administered as such is essentially the same as when administered in the form of its precursor acetophenetidin (Table I). This suggests that the extent of inactivation of acetaminophen during absorption from the gastrointestinal tract is minor under the experimental conditions.

[1] Equation 1 applies to all systems in which elimination is by apparent first-order kinetics from the central compartment [W. J. Westlake, *J. Pharm. Sci.*, **59**, 722(1970)].

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REVIEWS

Inflammation Biochemistry and Drug Interaction. Proceedings of an International Symposium, Como, Italy, October 11–13, 1968. Edited by A. BERTELLI and J. C. HOUCK. Excerpta Medica Foundation, Amsterdam (New York Office: New York Academy of Medicine Building, 2 East 103rd Street), The Netherlands, 1969. 359 pp. 17 × 24.5 cm. Price 9.8 Od Sterling/dfl. 81.00.

Within the last six years there were at least six international symposia which were devoted to the studies on inflammation and its related subjects. This present volume is the record of the second meeting sponsored by the International Inflammation Club and held within two years. It was devoted to the biochemical studies on inflammation and the mechanism of action of anti-inflammatory agents. The rapid progress, newer approaches, and the participation of other basic disciplines were some of the reasons accounting for the frequent meetings. The outcomes of these meetings were also very fruitful in promoting better world-wide communications between researchers and in unifying the basic interdisciplinary vocabularies.

There were forty-four papers by seventy-seven authors reported. Most of these papers were progress reports. The contents were divided into four sections and there were unavoidable overlaps.

It is very disappointing and regrettable that no discussion of the papers was included. Discussions in any symposium are always an important part of the meeting. There was one Foreword and one Summative Introduction to Inflammation but there was no conclusion or summary of the meeting.

The four sections are:

I. Proteolytic Enzymes in Inflammation—The role of proteases in inducing inflammation was discussed extensively in eight papers. The basic evidence is threefold: (*a*) inflammation is associated with activation of protease systems; (*b*) application of proteases can initiate the inflammatory process; and (*c*) many anti-inflammatory drugs were shown to have inhibitory effects on protease. There were a number of humoral and cellular proteases or protease systems involved in inflammation reported; however, none of them has ever been characterized.

II. Tissue Reaction and Collagenous Metabolism in Inflammation—This section includes seven papers. Anti-inflammatory agents have been shown to induce collagenolytic and proteolytic enzyme systems in rat skin and mouse and human fibroblasts, probably *via* the depression of an operon. The induced skin protease activated plasminogen, digested fibrin directly, and destroyed bradykinin, all of which could be important in anti-inflammation. Newer evidence for the possible role of lysosomal enzymes in the breakdown of a collagen and protein polysaccharide complex was presented.

III. Kinins and Other Factors in Phlogistic Reaction—This section includes thirteen papers. Further evidence of the involvement of kinins, mast cells, and platelet aggregation in inflammation was reported.

IV. Drug Interaction in Phlogistic Reaction—The antiproteolytic and prevention of protein denaturation were the two main mechanisms of action of anti-inflammatory agents discussed in this section, containing fourteen papers. No new idea or proposal has been suggested but a few new anti-inflammatory agents have been studied.

This book is a good library reference and it is also an excellent progress report on the biochemical study of inflammation.

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San Francisco, CA 94122

Lewis's Pharmacology. 4th Edition. By JAMES CROSSLAND. Williams and Wilkins Co., Baltimore, MD 21202, 1970. xviii + 1360 pp. 14.5 × 22 cm. Price $21.75.

The respected text of J. J. Lewis has been completely revised, rearranged, and rewritten. Outdated material has been deleted rigorously and replaced with much significant new material (299 pages more than the third edition). The page size of the previous editions has been retained and although the weight of paper used has been decreased (the print on the verso side of the page is quite readable), a very chunky book is the result.

Considering content and disregarding format, this revision is easily the most distinguished British text of pharmacology now available. It is interesting to note that this book may be ordered from continental distributors with a $9.00 saving in price (same sheets, different binding, different title page, plus a dust wrapper). The English publisher is E. & S. Livingstone.

It should be emphasized that the book is about pharmacology or perhaps more specifically about pharmacodynamics—it is not a text of materia medica or pharmacotherapeutics. Chemical structures are given in great profusion as well as 56 well-organized tables. A unique feature of the book is the actual illustration of pharmacologic procedures and apparatuses (classic techniques) along with typical records or tracings.

The text is organized into 56 specific chapters and divided into four major divisions: General Pharmacology (introduction, drug administration–mobilization, drug metabolism–storage–elimination, drug dependence, receptor theory, biostatistics, screening and testing methodology, experimental aspects of pharmacology); Humoral Substances and Mechanisms (autonomic agents, serotonin, histamine and antihistamines, polypeptides and acidic phospholipids); Systematic Pharmacology (local anesthetics, analgesics, general anesthetics, anticonvulsants, psychotropic agents, emetics and antiemetics, expectorants and antitussives, respiratory agents, drugs acting on blood and blood forming tissues, cardiovascular agents, diuretics and antidiuretics, gastrointestinal principles, agents affecting the liver, hormones, vitamins, metals, dermatologic agents); and Chemotherapy (including antineoplastic drugs). The section dealing with humoral pharmacology is especially effective.

Each chapter contains a selective bibliography divided into two parts: (*a*) Books, Monographs, and Reviews; and (*b*) Original Papers. The index is adequate. Occasionally the generic names are different from their U. S. counterparts, but this does not present a serious obstacle for the serious reader. This reviewer has used the past editions of this book as the required text for undergraduate pharmacy students, and the book earned general student acceptance. The present edition seems to be equally appreciated. The principles and concepts and drug functions that are stressed in the book are timeless in their validity while drug names come and go.

The text is reasoned closely and the words weighed judiciously to present an excellent international overview of what pharmacology is all about. This overview is unique and valuable, especially since certain American texts emphasize only current therapeutic novelties, others ignore fundamental principles, others follow clique theory, and still others ignore that pharmacology is once again an international science. This book should be in every library concerned with the teaching of the health sciences. While this volume cannot be regarded as a primary source book for the professional pharmacologist, it can function as an excellent orientation book for the specialist seeking perspective for himself within the total scope of pharmacology.

Reviewed by Marvin H. Malone
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REVIEWS

Inflammation Biochemistry and Drug Interaction. Proceedings of an International Symposium, Como, Italy, October 11–13, 1968. Edited by A. BERTELLI and J. C. HOUCK. Excerpta Medica Foundation, Amsterdam (New York Office: New York Academy of Medicine Building, 2 East 103rd Street), The Netherlands, 1969. 359 pp. 17 × 24.5 cm. Price 9.8 Od Sterling/dfl. 81.00.

Within the last six years there were at least six international symposia which were devoted to the studies on inflammation and its related subjects. This present volume is the record of the second meeting sponsored by the International Inflammation Club and held within two years. It was devoted to the biochemical studies on inflammation and the mechanism of action of anti-inflammatory agents. The rapid progress, newer approaches, and the participation of other basic disciplines were some of the reasons accounting for the frequent meetings. The outcomes of these meetings were also very fruitful in promoting better world-wide communications between researchers and in unifying the basic interdisciplinary vocabularies.

There were forty-four papers by seventy-seven authors reported. Most of these papers were progress reports. The contents were divided into four sections and there were unavoidable overlaps.

It is very disappointing and regrettable that no discussion of the papers was included. Discussions in any symposium are always an important part of the meeting. There was one Foreword and one Summative Introduction to Inflammation but there was no conclusion or summary of the meeting.

The four sections are:

I. Proteolytic Enzymes in Inflammation—The role of proteases in inducing inflammation was discussed extensively in eight papers. The basic evidence is threefold: (*a*) inflammation is associated with activation of protease systems; (*b*) application of proteases can initiate the inflammatory process; and (*c*) many anti-inflammatory drugs were shown to have inhibitory effects on protease. There were a number of humoral and cellular proteases or protease systems involved in inflammation reported; however, none of them has ever been characterized.

II. Tissue Reaction and Collagenous Metabolism in Inflammation—This section includes seven papers. Anti-inflammatory agents have been shown to induce collagenolytic and proteolytic enzyme systems in rat skin and mouse and human fibroblasts, probably *via* the depression of an operon. The induced skin protease activated plasminogen, digested fibrin directly, and destroyed bradykinin, all of which could be important in anti-inflammation. Newer evidence for the possible role of lysosomal enzymes in the breakdown of a collagen and protein polysaccharide complex was presented.

III. Kinins and Other Factors in Phlogistic Reaction—This section includes thirteen papers. Further evidence of the involvement of kinins, mast cells, and platelet aggregation in inflammation was reported.

IV. Drug Interaction in Phlogistic Reaction—The antiproteolytic and prevention of protein denaturation were the two main mechanisms of action of anti-inflammatory agents discussed in this section, containing fourteen papers. No new idea or proposal has been suggested but a few new anti-inflammatory agents have been studied.

This book is a good library reference and it is also an excellent progress report on the biochemical study of inflammation.

Reviewed by K. H. Lee
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Lewis's Pharmacology. 4th Edition. By JAMES CROSSLAND. Williams and Wilkins Co., Baltimore, MD 21202, 1970. xviii + 1360 pp. 14.5 × 22 cm. Price $21.75.

The respected text of J. J. Lewis has been completely revised, rearranged, and rewritten. Outdated material has been deleted rigorously and replaced with much significant new material (299 pages more than the third edition). The page size of the previous editions has been retained and although the weight of paper used has been decreased (the print on the verso side of the page is quite readable), a very chunky book is the result.

Considering content and disregarding format, this revision is easily the most distinguished British text of pharmacology now available. It is interesting to note that this book may be ordered from continental distributors with a $9.00 saving in price (same sheets, different binding, different title page, plus a dust wrapper). The English publisher is E. & S. Livingstone.

It should be emphasized that the book is about pharmacology or perhaps more specifically about pharmacodynamics—it is not a text of materia medica or pharmacotherapeutics. Chemical structures are given in great profusion as well as 56 well-organized tables. A unique feature of the book is the actual illustration of pharmacologic procedures and apparatuses (classic techniques) along with typical records or tracings.

The text is organized into 56 specific chapters and divided into four major divisions: General Pharmacology (introduction, drug administration–mobilization, drug metabolism–storage–elimination, drug dependence, receptor theory, biostatistics, screening and testing methodology, experimental aspects of pharmacology); Humoral Substances and Mechanisms (autonomic agents, serotonin, histamine and antihistamines, polypeptides and acidic phospholipids); Systematic Pharmacology (local anesthetics, analgesics, general anesthetics, anticonvulsants, psychotropic agents, emetics and antiemetics, expectorants and antitussives, respiratory agents, drugs acting on blood and blood forming tissues, cardiovascular agents, diuretics and antidiuretics, gastrointestinal principles, agents affecting the liver, hormones, vitamins, metals, dermatologic agents); and Chemotherapy (including antineoplastic drugs). The section dealing with humoral pharmacology is especially effective.

Each chapter contains a selective bibliography divided into two parts: (*a*) Books, Monographs, and Reviews; and (*b*) Original Papers. The index is adequate. Occasionally the generic names are different from their U. S. counterparts, but this does not present a serious obstacle for the serious reader. This reviewer has used the past editions of this book as the required text for undergraduate pharmacy students, and the book earned general student acceptance. The present edition seems to be equally appreciated. The principles and concepts and drug functions that are stressed in the book are timeless in their validity while drug names come and go.

The text is reasoned closely and the words weighed judiciously to present an excellent international overview of what pharmacology is all about. This overview is unique and valuable, especially since certain American texts emphasize only current therapeutic novelties, others ignore fundamental principles, others follow clique theory, and still others ignore that pharmacology is once again an international science. This book should be in every library concerned with the teaching of the health sciences. While this volume cannot be regarded as a primary source book for the professional pharmacologist, it can function as an excellent orientation book for the specialist seeking perspective for himself within the total scope of pharmacology.

Reviewed by Marvin H. Malone
Professor of Physiology-Pharmacology
University of the Pacific
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Essentials of Molecular Pharmacology. By ANDREJUS KOROLKOVAS. Wiley-Interscience, New York, NY 10016, 1970. xv + 339 pp. 15 × 23.5 cm. Price $16.50.

This book, which also could have been titled *Essentials of Medicinal Chemistry*, is an attempt to integrate various unifying principles, concepts, theories, and models concerning structure–activity relationships in order to present a coherent view of the interaction between ligands and biological systems.

The introductory chapter is philosophical in nature and includes a definition of molecular pharmacology. Chapter 2 (6 pp.) provides information on elementary thermodynamics, the Ferguson principle, and the classification of drugs into those that are structurally nonspecific and structurally specific. The influence of physicochemical properties on biological activities is discussed in Chapter 3 (23 pp.), and Chapter 4 (43 pp.) covers the role of specific moieties on biological activities. Included in Chapter 5 (32 pp.) are discussions on the principles of conformational analysis, stereoisomerism, and the role of drug geometry in drug–receptor interactions. Chapters 6 and 7 (61 pp.) treat the structure and nature of drug receptors and the forces involved in the combination of drugs with receptors. Under the title of *Topography of Receptors*, Chapter 8 (71 pp.) deals with the numerous attempts to determine the spatial requirements of different classes of receptors in excitable and nonexcitable tissue. Finally, theories and mechanisms of drug action are dealt with in Chapters 9 and 10 (52 pp.). These include theories based on occupation, rate, and receptor perturbation together with discussions on enzyme inhibition and metabolic antagonism.

The organization of this book is a welcome change from some of the more traditional approaches to this subject. However, because a large volume of material is covered, many topics are discussed in an extremely abbreviated fashion. In many cases, much reliance has been placed on space-consuming tables and diagrams without adequate discussion. As a result, discussions relevant to specific tables frequently are on different pages.

Although in-depth discussion generally is lacking, this should not be a serious obstacle to the student who wishes to pursue a particular topic further, since adequate, up-to-date lead-references are supplied at the end of each chapter. This book should be useful to the undergraduate and beginning graduate student in medicinal (pharmaceutical) chemistry as a supplement to more in-depth texts and literature studies.

Reviewed by Philip S. Portoghese
Department of Medicinal Chemistry
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University of Minnesota
Minneapolis, MN 55455 ■

NOTICES

Crystals and the Polarising Microscope. By N. H. HARTSHORNE and A. STUART. American Elsevier, New York, NY 10017, 1970. xi + 614 pp. 15.5 × 24 cm. Price $29.50.

Cavitation. By R. T. KNAPP, J. W. DAILY, and F. G. HAMMITT. McGraw-Hill, New York, NY 10036, 1970. xix + 578 pp. 15.5 × 23.5 cm. Price $25.00.

The Fate of Drugs in the Organism. Vol. I and Vol. II. Under the Chairmanship of J. HIRTZ. Masson et Cie Editeurs, Paris, France, 1970. (Volume I) xiii + 168 pp. and (Volume II) xiii + 338 pp. 18.5 × 24 cm. Price 187 fr. for 2-vol. set. (*French and English*)

Formulation and Function of Cosmetics. By J. STEPHAN JELLINEK. Wiley, New York, NY 10016, 1970. xix + 586 pp. 15.5 × 23 cm. Price $27.50.

Synthetic Peptides, Vol. I. By G. R. PETTIT. Van Nostrand Reinhold, New York, NY 10001, 1970. xii + 467 pp. 16 × 23.5 cm. Price $19.95.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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Journal of Pharmaceutical Sciences

APRIL 1971
VOLUME 60 NUMBER 4

REVIEW ARTICLE

β-Lactam Antibiotics: Their Physicochemical Properties and Biological Activities in Relation to Structure

J. P. HOU and J. W. POOLE

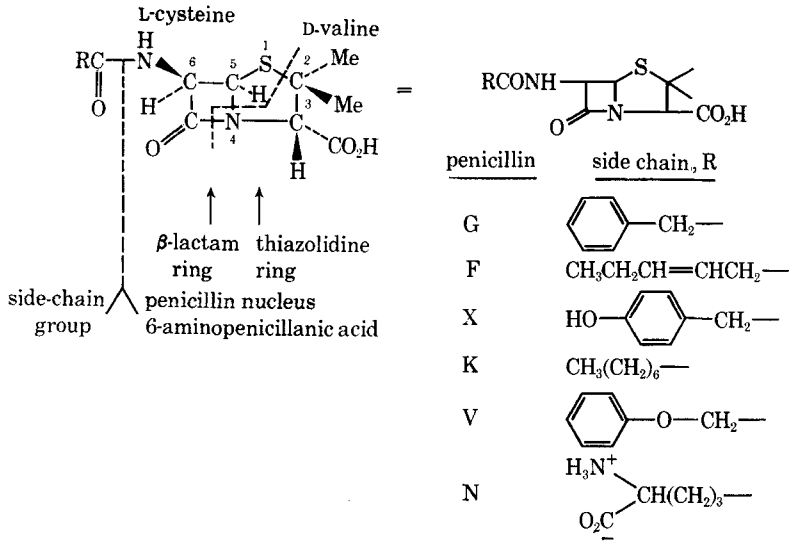
Keyphrases ☐ Antibiotics, β-lactams—review ☐ β-Lactam antibiotics—physical, chemical properties ☐ Biological activity–structure relationships—β-lactam antibiotics ☐ Penicillin, cephalosporins, inactivation—bacterial enzymes

CONTENTS

PHYSICAL AND CHEMICAL PROPERTIES 505
Degradation Reactions in Solution 505
Reactions of the 3-Carboxyl Group in Penicillins 506
Reactions of 3-Acetoxy and 4-Carboxyl Groups in Cephalosporins 507
Reactions of 6-Aminopenicillanic Acid 508
Rate of Degradation in Solution and Stabilization 508
Solubility Characteristics 510
Acid Dissociation Constants 510
Other Physicochemical Properties 511
BIOLOGICAL ACTIVITY IN RELATION TO STRUCTURE 512
Fundamental Structural Requirements 513
Effect of Side Chain on Activity 513
Intrinsic Antibacterial Activity–Structure Relationships 513
Absorption and Serum Concentrations 518
Factors Influencing Oral Absorption 520
INACTIVATION OF PENICILLINS AND CEPHALOSPORINS BY BACTERIAL ENZYMES 521
β-Lactamases: Their Distribution and Properties 521
β-Lactamase Resistance of Penicillins 522
β-Lactamase Resistance of Cephalosporins 523
Kinetic Studies on β-Lactamase Inactivation 523
Synergistic Activity and Increasing Usefulness of Antibiotics 526
CONCLUSION 527

The laboratory and clinical study of antibiotics, particularly the β-lactam-containing group (penicillins and cephalosporins), has been pursued vigorously during the past 30 years. The molecular modification of the naturally occurring penicillins and cephalosporins, either by fermentation or chemical synthesis, has been remarkably successful. The availability of the β-lactam nucleus has made it possible to prepare semisynthetic penicillins and cephalosporins with superior clinical effectiveness. There have been many excellent reviews of these advances, such as those by Abraham (1–4), Doyle and Nayler (5), Van Heyningen (6), Klein and Finland (7), Price *et al.* (8), Price (9), and Smith *et al.* (10). The present review attempts to cover the most significant recent progress made in this very broad field. Special attention has been paid to the physicochemical and biological properties and structure–activity relationships of the clinically important β-lactam drugs. Also discussed are some of the latest developments in the field of bacterial enzymes, particularly the β-lactamases, and their relationships to the β-lactam antibiotics.

Fleming's discovery of penicillin in 1929 (11) and the characterization of the compound by Chain *et al.* (12) and Abraham *et al.* (13) marked the beginning of a new era in chemotherapy. Extensive chemical and physicochemical studies, particularly with the aid of X-ray crystallography, provided an unequivocal demonstration of the fused β-lactam–thiazolidine



Basic structure of penicillin and the structures of naturally occurring penicillins G, F, X, K, and N and the biosynthetic penicillin V

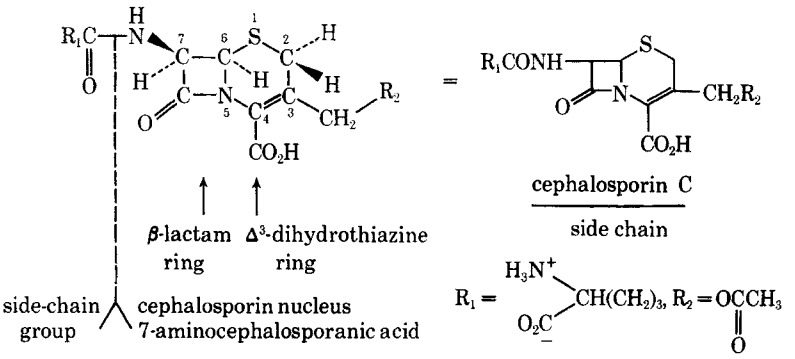
structure of penicillin (14). The penicillin molecule consists of a nucleus and a condensed side-chain group. The nucleus, known as 6-aminopenicillanic acid, is made of two amino acids, L-cysteine and D-valine, twisted together biogenetically into a cyclic dipeptide. The basic structure of the penicillin molecule and of natural and biosynthetic penicillins is shown above.

Since 1945, it has been realized that molds of the species of *Cephalosporium* produce antibiotics possessing some activity against Gram-positive and Gram-negative bacteria. These antibiotics were classified as cephalosporins N, C, and P (1, 2). Cephalosporin N was shown to be identical in structure to synnematin B, isolated by Gottshal *et al.* (15). Due to the identity of its nucleus, cephalosporin N should be called penicillin N (16). Cephalosporin P is known to be a group of tetracyclic compounds of steroid structure (17). Cephalosporin C resembles penicillin in possessing a β-lactam ring, but it is fused with a six-membered dihydrothiazine ring instead of a five-membered thiazolidine ring, and cephalosporin C also has a D-α-aminoadipoyl side chain (18). The full structure of cephalosporin C was elucidated by Abraham and Newton (19) and was confirmed by X-ray crystallographic analysis (20). The basic structure of cephalosporin and cephalosporin C is shown below.

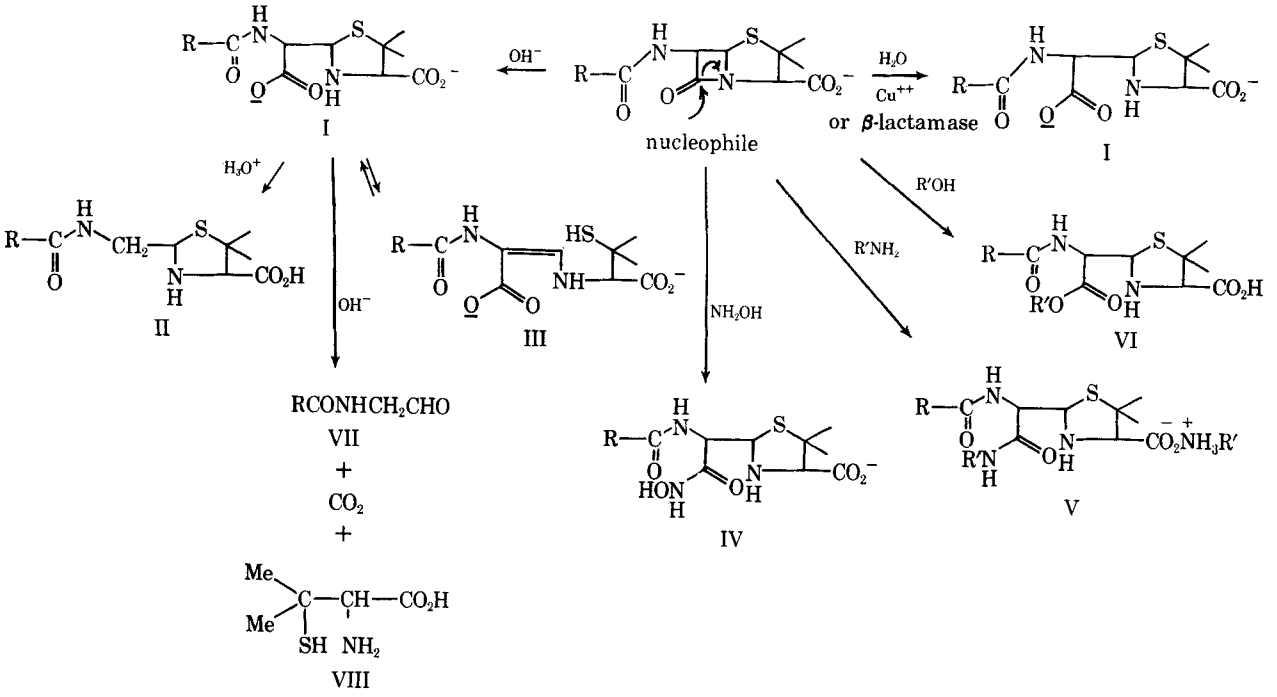
Early attempts to synthesize new penicillins were made to improve clinical usefulness of the antibiotic. Sheehan and his colleagues achieved the β-lactam synthesis (21, 22), which led to the formation of 5-phenylpenicillin (23, 24) and the sulfonyl analog of benzylpenicillin (25). This group also succeeded in the total synthesis of penicillin V (26, 27) and other penicillins (28). In 1959, Batchelor *et al.* (29) were successful in isolating 6-aminopenicillanic acid from penicillin G fermentation liquor. Several other research groups (30–39) independently reported that penicillins G and V could be hydrolyzed into 6-aminopenicillanic acid by amidase in good yield. Nonenzymic conversion of penicillin to 6-aminopenicillanic acid was also reported (40, 41).

Unlike natural penicillins, cephalosporin C and related analogs are relatively resistant to the enzyme penicillinase (42) and are acid stable. Attempts to prepare other cephalosporins by a fermentation process have been unsuccessful. Although the D-α-aminoadipoyl side chain of cephalosporin C can be removed by acid hydrolysis (43) to prepare 7-aminocephalosporanic acid, the yield is very poor. A vast improvement in the synthesis of 7-aminocephalosporanic acid was accomplished by Morin *et al.* (44). Woodward and his associates (45, 46) were successful in the total synthesis of cephalosporins.

The availability of 6-aminopenicillanic acid, 7-aminocephalosporanic acid, and the synthetic techniques has permitted the preparation of numerous new



Basic structure of cephalosporin and the structure of naturally occurring cephalosporin C



Scheme I

semisynthetic penicillins (47–71) and cephalosporins (72–80), varying in side-chain groups as well as derivatives, which were not previously available through fermentation[1]. Those of clinical importance have been listed in Tables I and II, respectively, with their names, side-chain structures, and special activities and properties.

PHYSICAL AND CHEMICAL PROPERTIES

Degradation Reactions in Solution—The bicyclic β-lactam–thiazolidine structure is more sensitive than the simple β-lactam structure to nucleophiles, electrophiles, oxidizing agents, and even water molecules (14, 81). The initial β-lactam cleavage has been shown to be responsible for many succeeding degradation reactions of penicillins in solution. It has been proposed that the nonplanarity of the fused penicillin nucleus may cause a great deal of suppression of the usual amide resonance as compared with that caused by the dipolar stabilized forms in the normal β-lactam structure (81).

Nucleophilic Attack Reactions—All penicillins are extremely susceptible to nucleophilic attack (Scheme I) by hydroxyl ions, primary and secondary amines, *etc.* The initially hydrolyzed product of penicillin is biologically inactive penicilloic acid (I). Penicilloic acid is stable in the form of salts or esters in neutral solutions, but on acidification it readily loses one molecule of carbon dioxide, giving the corresponding penilloic acid (II) (81, 82). Under drastic conditions, penicilloic acid may transfer to its tautomeric penamaldic acid (III) (83). Hydroxylamine reacts with penicillins quantitatively to form hydroxamic acid (IV). Similarly, penicillins interact readily with two molecules of amines, such as the alkylamine, to form corresponding alkylammonium penicilloic alkylamide (V). Primary alcohols (81), sugars, carbohydrates, and glycols at a neutral pH (84) react with penicillins to form monoesters of α-penicilloate (VI). Penicillin β-lactamases (3, 4) and metal ions, such as copper (85), are powerful catalysts to hydrolyze penicillins to penicilloic acids or a penicilloyl–copper-ion complex. The end hydrolytic degradation products of penicillins are penicilloaldehyde (VII), carbon dioxide, and a sulfur-containing amino acid, penicillamine (VIII) (81).

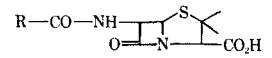
Cephalosporins are readily attacked similarly by nucleophilic reagents and cephalosporin β-lactamases (2, 6). The initial degradation product corresponding to penicilloic acid, however, is not stable and is readily fragmented in aqueous solution (86, 87).

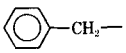
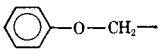
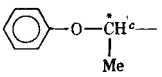
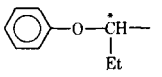
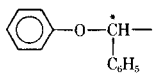
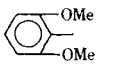
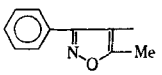
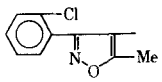
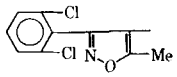
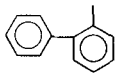
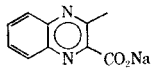
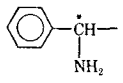
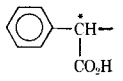
Electrophilic Attack Reactions—Penicillins are also sensitive to electrophilic attack both at the β-lactam nitrogen and the sulfur atom (Scheme II). In strong acid, penicillins are isomerized to penillic acid (IX) by a mechanism involving the transient oxazolone structure (X) (81). Benzylpenillic acid has a typical UV absorption band near 228 nm. (88). In weak acid or in neutral solution, penicillins inevitably undergo transformation to penicillenic acid (XI), which possesses a characteristic UV band near 320 nm. regardless of the type of penicillin used as the source (89, 90). Penicillenic acid is very unstable; it quickly isomerizes to either penicilloic or penillic acid, depending on the pH of the solution (90, 91). Metal ions are able to attack the sulfur atom of the thiazolidine ring, as is shown by the degradation of benzylpenicillin in neutral solution by $HgCl_2$ (90).

Evidently, the side-chain group of penicillins is directly involved in the penillic acid–penicillin transformation reactions. Substitution with a powerful electron-attracting group somewhere in the side chain, particularly in the α-position of benzylpenicillin, has been shown to stabilize the penicillin markedly in the acid solution (92, 93). Positive linear correlations

[1] Reports on the preparation of semisynthetic penicillins and cephalosporins are voluminous; only those for the preparation of compounds listed in Tables I, II, and XII and some of their derivatives are cited.

Table I—Name, Structure, Special Activity, and Properties of Several Clinically Useful Penicillins



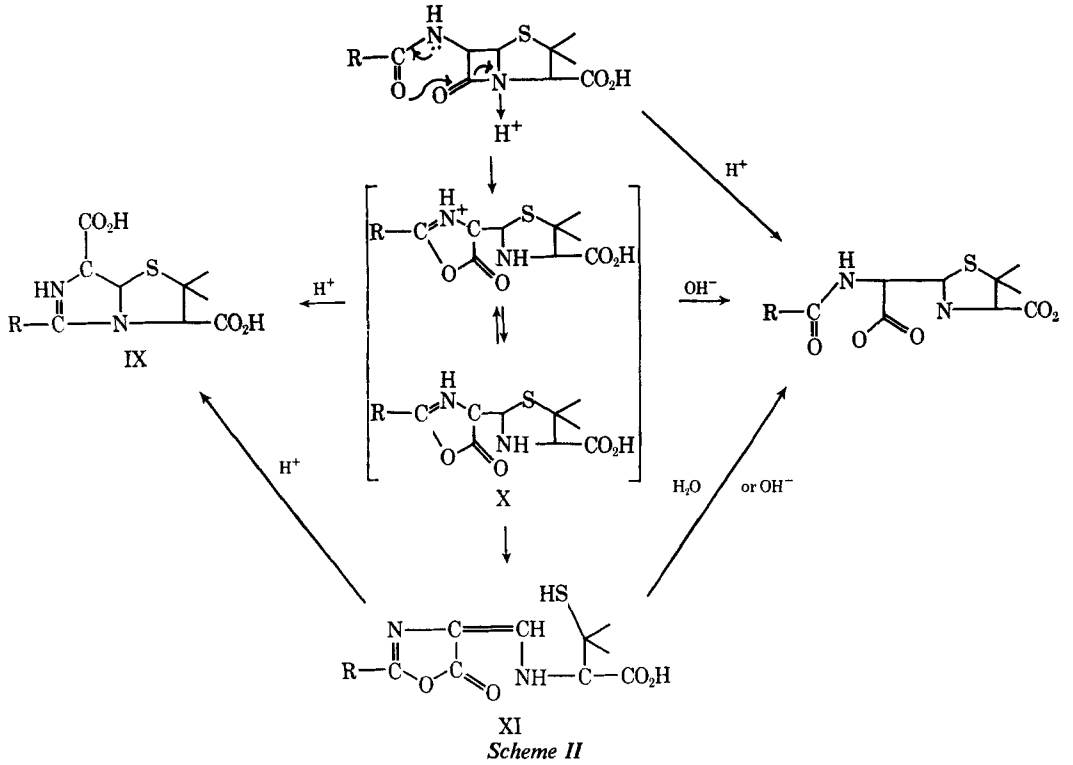
Nonproprietary Name	Chemical Name	Structure[a], R	Activity and Properties[b]
Penicillin G	Benzylpenicillin		G(+)-effective, very acid unstable, penicillin β-lactamase sensitive
Penicillin V	Phenoxymethylpenicillin		
Phenethicillin	Phenoxyethylpenicillin		G(+)-effective, acid stable, sensitive to penicillin β-lactamase, well absorbed
Propicillin	Phenoxypropylpenicillin		
Phenbenicillin	Phenoxybenzylpenicillin		
Methicillin	2,6-Dimethoxyphenylpenicillin		G(+)-effective, acid unstable, resistant to some penicillin β-lactamase
Oxacillin	3-Phenyl-5-methyl-4-isoxazolylpenicillin		
Cloxacillin	3-*o*-Chlorophenyl-5-methyl-4-isoxazolylpenicillin		
Dicloxacillin	3-*o*,*o*-Dichlorophenyl-5-methyl-4-isoxazolylpenicillin		G(+)-effective, acid stable, resistant to most β-lactamase, highly serum bound, orally absorbed
Nafcillin	2-Ethoxy-1-naphthylpenicillin		
Diphenicillin	2-Biphenylpenicillin		
Quinacillin	3-Carboxy-2-quinoxalinylpenicillin		
Ampicillin	D-α-(−)-Aminobenzylpenicillin		Broad spectrum, very acid stable, penicillin β-lactamase sensitive, well absorbed
Carbenicillin	α-Carboxybenzylpenicillin		Broad spectrum and *Ps. aeruginosa* effective, very acid unstable, orally ineffective

[a] Penicillins are available in soluble or sparingly soluble salts or in acidic or zwitterionic forms. [b] G(+) and G(−) represent Gram-positive and Gram-negative organisms; broad spectrum means effective against both Gram-positive and Gram-negative organisms; β-lactamases refer to the β-lactam-inactivating enzymes from both Gram-positive and Gram-negative bacteria; penicillin β-lactamase refers to the enzyme preferentially attacking susceptible penicillins (*References 3, 7, 10*). [c] Penicillins carrying asymmetric carbon atom in the side chain (shown by asterisk) are stereospecific.

of the acid stability of the corresponding penicillins with the acidity of the side-chain acids were obtained. For example, substitution of the α-hydrogen atom with the amino or halogen atom in benzylpenicillin (92), or into the benzene ring in methicillin (94) or in phenylpenicillin (95), has been shown to stabilize the substituted penicillins drastically.

Cephalosporins are relatively stable in acid and do not undergo the penicillin–penillic acid type of rearrangement even when they have the same side chain as the penicillins (3). This is probably due to the lower anionoid reactivity of the nitrogen in the dihydrothiazine ring (2, 3).

Reactions of the 3-Carboxyl Group in Penicillins—In general, the penicillin molecule is a fairly strong organic acid with a pKa value of about 2.6–2.7 in water (96, 97). The C-3 carboxyl group rapidly forms crystalline soluble (inorganic) and sparingly soluble (organic) salts or esters (98). Sodium, potassium, and calcium salts are the most useful soluble salts, while procaine (99), benzathine (100–103), benethamine (104), and dehydroabietylethylenediamine (hydrabamine) (105, 106) salts are the most commonly used organic salts. Similarly, various sparingly soluble penicillin esters (107, 108), amides (109–111), and other derivatives such as methyl alcohol (112), nitrile (113),



Scheme II

and thiopenicillanic acid (114) have also been prepared by substitution at C-3 in place of the carboxyl group.

Reactions of 3-Acetoxy and 4-Carboxyl Groups in Cephalosporins—The most reactive group in cephalosporins is the 3-acetoxy group. The chemistry of cephalosporin C, as elucidated by Abraham and Newton (19, 115), has greatly aided later workers in the area of the semisynthetic cephalosporins.

Scheme III illustrates the general reactions of the cephalosporins. Treatment of cephalosporin C with dilute acid gives not only a small amount of 7-aminocephalosporanic acid but also 7-aminocephalosporanic acid lactone (XII) and cephalosporin C lactone, known as cephalosporin C_e (XIII) (19, 43, 116). With the treatment of cephalosporin C with acetyl esterase from orange peel, or under mild acid hydrolysis, the acetoxy group is cleaved without lactonization and the corresponding 3-methyl alcohol (XIV) is obtained (116). This desacetylcephalosporin C is not readily acylated but, instead, is lactonized under acid and anhydrous conditions. In basic solution and by reacting with aromatic acid chloride, however, cyclization and esterification take place (XV) (73). Similarly, 3-*O*-carbamoyldesacetylcephalosporanic acid (XVI) is formed in a basic medium through a reaction with isocyanate (6).

Cephalosporin C absorbs hydrogen; if the hydrogenation is carried out in the presence of palladium on charcoal, the product will be desacetoxycephalosporin C (XVII) (19, 86, 116). Stedman *et al.* (117, 118)

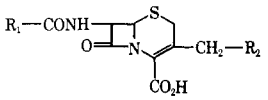
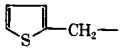
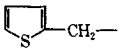
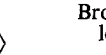
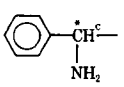
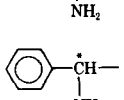
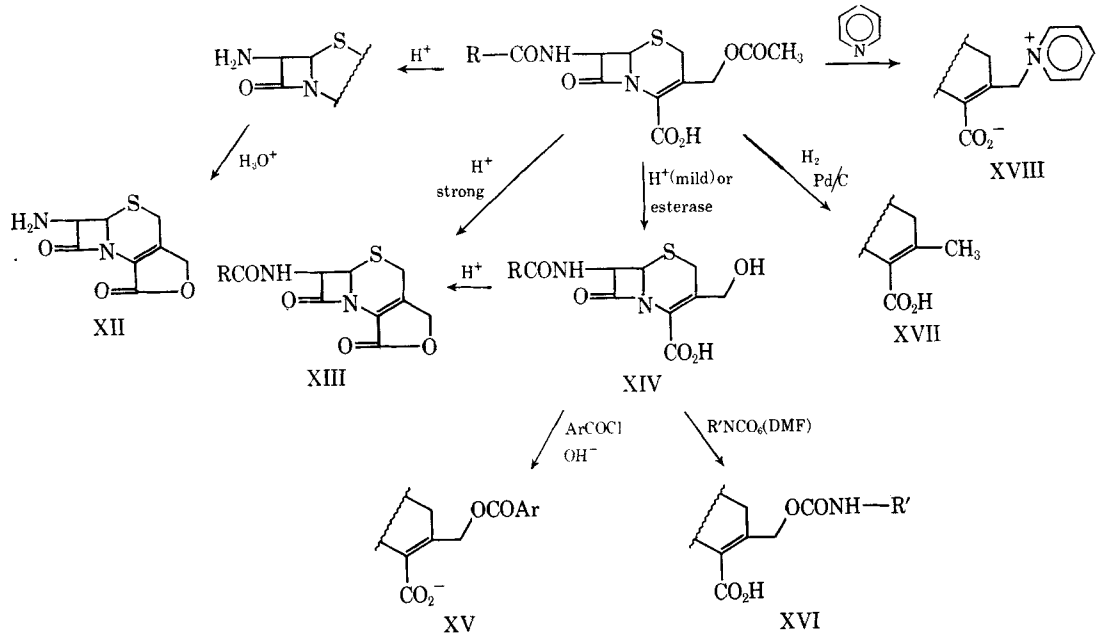


Table II—Name, Structure, Special Activity, and Properties of Several Clinically Useful Cephalosporins

Nonproprietary Name	Chemical Name	Structure[a] R_1	Structure[a] R_2	Activity and Properties[b]
Cephalothin	7-(Thiophene-2-acetamido)-cephalosporanic acid		$-OCOCH_3$	Broad spectrum, poorly absorbed
Cephaloridine	7-(2-Thienyl)acetamido-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine			Broad spectrum, poorly absorbed, less serum bound
Cephaloglycin	7-(D-α-Aminophenylacetamido)-cephalosporanic acid		$-OCOCH_3$	Broad spectrum, well absorbed
Cephalexin	7-(D-α-Amino-α-phenyl-acetamido)-3-methyl-3-cephem-4-carboxylic acid		$-H$	Broad spectrum, well absorbed

[a] Most of the cephalosporins are available in soluble salts or in zwitterions. [b] Broad spectrum means effective against both Gram-positive and Gram-negative organisms. Cephalosporins are usually penicillin β-lactamase resistant but very susceptible toward cephalosporin β-lactamases (*References 1–3, 6, 10*). [c] Cephalosporins carrying asymmetric carbon atom (shown by asterisk) are stereospecific.



Scheme III

showed that this reaction can be carried out at low pressure by using a large amount of palladium as the catalyst.

In neutral solution, cephalosporin C reacts with pyridine to form cephalosporin C_A (XVIII) and also becomes quaternized (119). It is now known that any agent of greater nucleophilicity than oxygen will displace the acetoxy group (6). Nucleophiles such as bivalent sulfur compounds, tertiary amines, azides, thiourea, thiocarbamates, xanthates, and pyridines have been used (74, 75, 119–121). The kinetics of the displacement reactions (122) have been reported. Cephalosporins such as cephalothin (123) and cephaloglycin (124) have been shown to have essentially the same chemical properties as cephalosporin C.

The 4-carboxyl group in cephalosporin, as in penicillin, can be modified to esters or amides. Many esters and amides of cephalothin (125) have been prepared, all of which have been shown to be much less active than the parent compound.

Reactions of 6-Aminopenicillanic Acid—Since 6-aminopenicillanic acid contains no side chain, it cannot undergo intramolecular rearrangement to form a penillic acid type of product. It is, therefore, relatively stable toward acids (29). However, 6-aminopenicillanic acid is readily attacked by carbon dioxide, to which penicillins are inert. The carbonation product of 6-aminopenicillanic acid is biologically inactive, having the structure of XIX (126, 127).

Grant *et al.* (128) showed that in neutral pH and at room temperature, a concentrated aqueous solution of 6-aminopenicillanic acid gave a high molecular weight polymer of poly-6-aminopenicillanic acid (XX). Such a product showed little antistaphylococcal activity.

Another interesting reaction of 6-aminopenicillanic acid is that of the 6-amino group with the hydroxyl group of a reducing sugar at room temperature and neutral pH, giving derivatives of *N*-glycosyl-6-aminopenicillanic acid (XXI). Though sodium salts of the glycosyl, maltoxyl, and lactosyl derivatives of 6-aminopenicillanic acid are stable toward both staphylococcal and bacillary β-lactamases, the antibacterial activity of these derivatives is much lower than that of 6-aminopenicillanic acid (129).

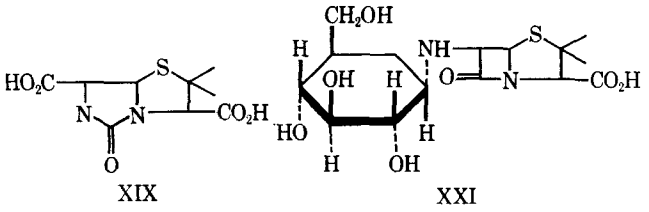
Rate of Degradation in Solution and Stabilization—*Kinetics*—The instability of penicillin in aqueous solution has been known since the time of its discovery. Abraham and Chain (130) were the first to report that the maximum stability range of barium penicillin in solution is between pH 5.5 and 7.5. Other workers (131–134) also investigated the decomposition of natural penicillins in aqueous media. They found that the destruction of penicillin in aqueous solution is a first-order irreversible reaction.

Brodersen (135–138) indicated that the rate of inactivation of penicillin G is largely dependent on hydrogen-ion concentration and temperature. The molecular and the ionized forms of penicillin interact at different rates with hydrogen ion. Within a pH range of 1–10 and at a total ionic strength of 0.5 at 30°, the rate of degradation of penicillin G follows pseudo-first-order kinetics and the pH of minimum degradation is at 6.5. The degradation reactions observe Arrhenius' law, and the apparent energies of activation vary with pH (17.57, 20.98, and 9.8 kcal./mole at a pH of 1.20, 4.54, and 9.57, respectively) but are independent of temperature (136–138).

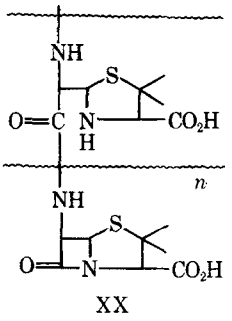
The rate constant for the overall degradation of penicillin G and the rate of formation of penicillenic acid were reported by Krejci (139).

Swintosky *et al.* (140, 141) demonstrated that the degradation of a procaine–penicillin G suspension is of zero order, since only the material in solution degrades, and that the decreased solubility could be utilized to stabilize the penicillins.

The degradation kinetics of phenethicillin in a pH range of 1–11.4, with a constant ionic strength of 0.5 and at 35°, was reported by Schwartz *et al.* (142). The apparent pH of minimum degradation occurred



XIX XXI



XX

at about 6.5. At a pH of 6.7, the energy of activation was 17.6 kcal./mole. The half-life of potassium phenethicillin at a pH of 1.5 and 35° is about 1 hr., in comparison with about 4 min. for penicillin G. In the acid region, the phenethicillin anion was shown to undergo degradation about 13 times faster than the molecular form.

Methicillin is very unstable in acid (143, 144), and its decomposition is roughly 2–3 times faster than that of phenethicillin. The reactions, in general, are very dependent on pH and have a much narrower stability zone near a pH of 6.5.

Finholt *et al.* (145) were the first to investigate the effect of buffer on the degradation of penicillin G. They found that the dihydrogen citrate ion, the monohydrogen phosphate ion, and the borate ion imposed significant catalytic effects on the penicillin ion, while acetic acid, the acetate ion, the monohydrogen citrate ion, the unprotonated citrate ion, the dihydrogen phosphate ion, and boric acid were noncatalytic or nearly so. No primary salt effect was observed at a pH of 4.5, but a significant salt effect was shown at 6.8 and 8.75. The log *k*–pH profile at zero buffer concentration gave a pH of minimum degradation at 6.65 at 60°. The penicillin G degradation due to nucleophilic attack is about 5000 times faster than in acid solution.

Stability studies on oxacillin and penicillin V in aqueous solution at several pH values and temperatures were reported by Kondrat'eva and Bruns (146). These penicillins are most stable at a pH of 6.0–7.0.

Saccani and Pansera (147) recently reported the apparent stabilities of ampicillin and hetacillin at 27° in several buffers (without controlling the ionic strength). The pH of minimum degradation occurred at 4.4 for both ampicillin and hetacillin.

Hou and Poole (148) investigated the kinetics of degradation of ampicillin in several buffer systems of pH 0.8–10, with a constant total ionic strength of 0.5 and at several temperatures. The observed rates were shown to follow pseudo-first-order kinetics and were significantly affected by general acid–base catalysis, particularly by the polycharged phosphate ions. At pH 4.94, no primary salt effect was observed in buffers of widely varied ionic strength. In 0.08 *N* HCl solution, however, the apparent rates were increased with the ionic strength but decreased with the dielectric constant of the solvent. The pH of minimum degradation (pH minimum) occurred at 4.85, which is the isoelectric point of ampicillin under the conditions used. In other words, the zwitterionic form of the ampicillin molecule is the most stable species toward both acids and bases. At zero buffer concentrations, however, the pH minimum shifts to a neutral value of 5.85, and degradation at this pH is mainly due to a spontaneous reaction. Inactivation of ampicillin in base is about 1400 times faster than in acid. The apparent energies of activation for the degradation of ampicillin are 16.4, 18.3, and 9.2 kcal./mole in buffers of pH 1.35, 4.93, and 9.78, respectively.

Kinetic Assay Procedures—The rate of degradation of a penicillin in solution can be determined by measuring the change in the amount of intact penicillin or, alternatively, by determining the amount of degradation product, either biologically or chemically (149, 150). Since the reaction mechanism of penicillins is complex, the quantitative determination of the initial degradation product (penicillenic or penicilloic acid) is most difficult. On the other hand, the microbiological, hydroxylamine (151), and iodometric (152–154) methods can rather accurately measure the residual intact penicillin and have been widely used.

Other methods, such as the polarimetric, spectrophotometric, manometric, acidimetric, or alkalinetitrimetric, are also available (150). These procedures have been chiefly used in studies on penicillinase.

Stabilization of Penicillins—The β-lactam antibiotics can be classified into the monobasic and the zwitterionic (ampicillin, cyclacillin, cephaloglycin, *etc.*). The optimum pH of stability for the former lies between 6 and 7; for the latter, it apparently coincides with the isoelectric point of the antibiotic. Accordingly, the pH of maximum stability of the zwitterionic antibiotics is at 4.85 for ampicillin (148, 155), 5.0 for cyclacillin (96), and 4.5 for cephaloglycin (72).

To date, no agent has been found to preserve or stabilize the β-lactam ring. However, numerous reports show that the degradation of penicillin in solution can be retarded in a particular type of buffer system or in the presence of certain chemical additives (156). Citric acid–citrate buffer has less catalyzing effect than phosphate buffer of similar buffer concentration (157–160). A combined citric–phosphate buffer is superior for the stabilization of ampicillin and hetacillin (147, 148).

The penicillins, cephalosporins, and their salts all have a strong tendency to form crystalline hydrates. For example, ampicillin (anhydrate) transforms to the long-needle crystalline trihydrate in a cold acidic solution (161). Grant and Alburn (162) reported that ampicillin monohydrate is less stable than its anhydrate analog when exposed to high humidity and elevated temperature. Similarly, ampicillin (anhydrate) has been shown to be more stable than ampicillin trihydrate in pharmaceutical preparations (163). On the other hand, sodium nafcillin monohydrate is more stable than its anhydrate in air (163). Normally, the anhydrous salt absorbs moisture relatively faster than the crystalline hydrate form. Accordingly, it was shown that sodium

Table III—Equilibrium Solubility (Milligrams per Milliliter) of β-Lactam Antibiotics and Their Salts in Several Solvents at Room Temperature[a]

Compound	Solvent												
	Water	Methanol	Ethyl Alcohol	Isopropanol	Acetone	*p*-Dioxane	Ethyl Ether	Chloroform	Carbon Tetrachloride	Ethyl Acetate	Isooctane	Pyridine	Carbon Disulfide
Sodium penicillin G	>20	>20	10	0.75	0.19	1.9	0.06	0.05	0.042	0.4	0.03	1.15	0.08
Potassium penicillin V	>20	>20	1.35	0.11	0.22	0.56	0.84	0.45	0.04	0.65	0	0.13	0.07
Penicillin V	0.9	>20	>20	>20	>20	12.6	11.75	>20	0.1	>20	0.04	>20	0.3
Sodium methicillin	>20	>20	>20	>6.54	0.26	0.52	0.02	0.61	0	0.05	0.22	>20	0.04
Sodium oxacillin	>20	>20	11.7	3.58	0.16	0.66	0.01	0.05	0	0.03	0	>20	0.02
Sodium cloxacillin	>20	>20	>20	9.2	2.72	4.22	0.08	1.82	0.01	0.6	0	>20	0.06
Sodium dicloxacillin	>20	>20	>20	9.5	13.44	4.57	0.09	0.54	0	1.4	0	6.38	0.02
Sodium nafcillin	>20	>20	>20	>20	20	20	0.41	>20	0	>20	0	>20	0.06
Procaine penicillin G	6.8	>20	>20	6.5	14.95	9.8	0.6	>20	0.12	3.35	0	>20	0.51
Benzathine penicillin G	0.32	16.9	15.4	3.65	3.0	2.4	0.4	2.1	0.57	1.2	0.09	>20	0.5
Benzathine penicillin V	0.32	>20	14.6	7.15	>20	>20	1.2	20	0.73	9.0	0.2	>20	0.55
Hydrabamine penicillin G	0.075	7.3	5.2	1.7	3.4	14.65	0.7	>20	0.5	1.65	0.5	>20	1.4
Ampicillin (anhydrous)	10.1	2.97	0.39	0.05	0.12	0.60	0.02	0.09	0.01	0.025	0	2.1	0.015
Ampicillin (trihydrate)	7.5	6.65	2.54	—	8.95	2.77	0.03	0.07	0.025	0.225	0.02	12.13	0.02
Cephaloridine	>20	2.91	1.95	0.21	0.18	0.46	0.01	0.24	0.05	0.04	0.05	0.74	0.02
Sodium cephalothin	>20	11.93	1.77	0.08	0.06	0.52		0.07	0.02	0.02	0.03	0.10	0.01

[a] Data from *References 165–167.*

oxacillin monohydrate and sodium methicillin monohydrate tended to absorb moisture much more slowly than the anhydrous sodium salts (164).

Solubility Characteristics—Nearly all sodium and potassium salts of penicillins and cephalosporins are: freely soluble or highly soluble (>20 mg./ml.) in water, 0.1 *N* NaOH, the lower alcohols, dimethylformamide, and formamide (some are also soluble in pyridine); sparingly soluble (1–20 mg./ml.) to slightly soluble (0.1–1 mg./ml.) in the higher alcohols, acetone, and ethyl acetate; and practically insoluble (<0.1 mg./ml.) in isooctane, cyclohexane, benzene, diethyl ether, petroleum ether, chloroform, carbon disulfide, and carbon tetrachloride. The amine salts, esters, and the free acid forms of the monobasic penicillins are generally more soluble in nonpolar than in polar solvents (165, 166).

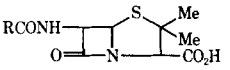
On the other hand, penicillin and cephalosporin zwitterions are freely soluble in formamide and dimethylformamide, slightly soluble in water or other polar solvents, and practically insoluble in nonpolar solvents such as ether or carbon tetrachloride (165–167). Since the zwitterionic penicillins and cephalosporins carry both ionic and nonpolar groupings, they behave like both ions and organic molecules; which is dominant depends on structural and environmental factors. Because of the dual functionality, significant effects of pH, salt, dielectric constant (96), and temperature (168) on the aqueous solubility of ampicillin have been observed.

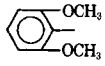
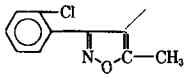
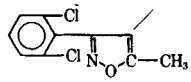
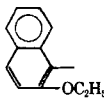
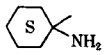
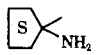
Penicillin salts were shown to be significantly more soluble in a nonpolar solvent in the presence of a small amount of water. Similarly, the water of crystallization in penicillin hydrates may help to increase the solubility in a nonpolar solvent. Ampicillin trihydrate is more soluble than its corresponding anhydrate in several organic solvents such as acetone, ethyl acetate, dioxane, and ether. Table III shows the solubilities of several penicillins, cephalosporins, and their salts in various commonly used solvents at room temperature.

Acid Dissociation Constants—Caution is required in determining the dissociation constants of penicillins and cephalosporins by conventional potentiometric titration methods. The difficulty arises from the fact that most of the monobasic penicillins precipitate when the pH drops or when a sufficient amount of penicillin-free acid is formed. This is particularly true for those penicillins with a bulky side-chain group. The problem is not usually encountered with amphoteric penicillins or cephalosporins.

The apparent pKa value of the carboxyl group of all penicillins ranges from 2.6–2.8 in water at 25° (88, 96, 97). The fairly strong acidity is believed to be due to the adjacent strong electronegative groups, namely the *S*-atom and β-lactam peptide linkage (88). The peptide and other groups in the side chain, because of the wide separation, probably do not contribute any inductive effect. Accordingly, the (nucleus) carboxyl group of all penicillins (except 6-aminopenicillanic acid) (97) has essentially the same acid dissociation constant (pKa) regardless of the side-chain group. On the other hand, penicillins carrying one or more ionizable groups in the side chain usually give rather varied pK

Table IV—Apparent Acid Dissociation Constants of Penicillins in Water



Penicillin	Side Chain, R	Temperature	pKa		References
			pK_1, COO^-	pK_2, NH_3^+	
Penicillin G	$C_6H_5CH_2$—	25°	2.76	—	88
		25°	2.72	—	97
		23°	2.71, 2.73	—	88
Penicillin X	*p*-$HOC_6H_5CH_2$—	23°	2.62	—	88
p-Heptylpenicillin	$C_7H_{13}C_6H_5$—	23°	2.66	—	88
Penicillin V	$C_6H_5OCH_2$—	25°	2.73, 2.74	—	97
Phenethicillin	$C_6H_5OCH(CH_3)$—	25°	2.72, 2.74	—	97
Propicillin	$C_6H_5OCH(C_2H_5)$	25°	2.72, 2.72	—	97
Methicillin		25°	2.76, 2.78	—	97
Cloxacillin		25°	2.73, 2.70	—	97
Dicloxacillin		25°	2.67	—	96
Nafcillin		25°	2.65	—	96
p-Aminobenzylpenicillin	*p*-NH_2-C_6H_5-CH_2—C_6H_5CH(—NH_2)—	—	2.6	4.6	169
Ampicillin		25°	2.66, 2.64	7.25, 7.24	96
			2.52, 2.53	7.24, 7.25	97
Cyclacillin (WY-4508)		25°	2.68	7.50	96
WY-7953		25°	2.62, 2.61	7.60, 7.62	96
6-Aminopenicillanic acid		25°	2.29, 2.30	4.90, 4.92	97

values as a result of the inductive effect of the adjacent groups (96). This is true, for instance, in the case of amphoteric penicillins such as penicillin N (16), ampicillin, cyclacillin, BL-P875, and 6-aminopenicillanic acid (97) but not *p*-aminobenzylpenicillin (169).

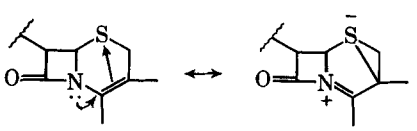
In the case of ampicillin (96), a change in the dielectric constant of the titration medium affects the pK_1 (carboxyl group) more than the pK_2 (amino group); a change in temperature exerts the opposite effect. The formation of ampicillin zwitterion from its uncharged species is an exothermic reaction, with a heat of formation of about 10 kcal./mole.

Tables IV and V list the acid dissociation constants of several penicillins and cephalosporins, respectively, in water and/or partially aqueous solvent at 25°.

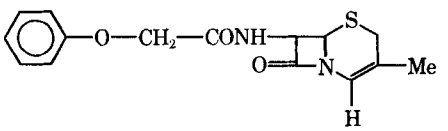
Because of the leveling effect, the acidic groups of penicillins and cephalosporins can be titrated in a nonaqueous or partially aqueous solvent. The pK value of penicillin G is increased by 2 pK units when the titration is carried out in 80% ethanol (88). Similarly, the pK_1 values of ampicillin and cyclacillin are increased by about 1.5 units, while the pK_2 values are decreased by 0.27 (ampicillin) and 0.46 (cyclacillin) in 50% *p*-dioxane (96). By varying the amount of the organic solvent, the pKa value in water for certain insoluble or sparingly soluble penicillins can be obtained by extrapolation. The pK values of nafcillin and dicloxacillin, thus obtained, were shown to be essentially identical to those of other penicillins determined in water (96).

Other Physicochemical Properties—*UV Absorption*—Pure 6-aminopenicillanic acid and cyclacillin, because they lack a chromophore group, have no absorption band in the UV region. Penicillin G, like other monoaromatic penicillins, has a weak end-absorption in water (88), with $\lambda_{max.}$ at 264 (ϵ, 173, l/m./cm.), 257.5 (ϵ, 256), 252 (ϵ, 255), and 257 nm. (ϵ, 285) (96). These bands are obviously typical absorption bands of the benzyl group. Sodium nafcillin, however, absorbs strongly in water at 332, 292, 280 (ϵ, 5680), and 227 nm. (ϵ, 6100). Clearly, these bands are from the conjugated naphthalene group.

Cephalosporins and their salts give a typical UV absorption band near 260 nm. in aqueous solution,



XXII



XXIII

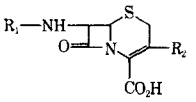
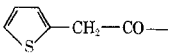
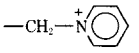
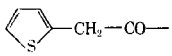


Table V—Acid Dissociation Constants of Cephalosporins at Room Temperature

Cephalosporin	R_1	R_2	pKa: Side Chain COO^-	pKa: Nucleus COO^-	pKa: Side Chain NH_3^+	References
Cephalosporin C	$H_3N^+—CH(CH_2)_3—CO—$ O_2C^-	$—CH_2OCOCH_3$	<2.6	3.1	9.8	2
Desacetyl-cephalosporin C	$H_3N^+—C(CH_2)_3—CO—$ O_2C^-	$—CH_2OH$	<2.5	3.0	9.7	116
D-Cephaloglycin	$C_6H_5—CH(NH_3^+)—CO—$	$—CH_2OCOCH_3$	—	4.4	7.1	78
L-Cephaloglycin	$C_6H_5—CH(NH_3^+)—CO—$	$—CH_2OCOCH_3$	—	4.6	7.1	78
	$C_6H_5—CH(NH_3^+)—CO—$	$—CH_2OH$	—	4.9	7.6	124
Cephaloridine			—	3.4	—	75
		$—CH_2SC(=S)—alkyl$	—	5.0	—	74
	H	—H	—	2.38	4.79	118
7-Aminocephalosporanic acid	H	$—CH_2OCOCH_3$	—	1.75	4.63	118

which is claimed to be due to the O=C—N—C=C— linkage (72), with possible participation of the nucleus sulfur atom (170), as shown in XXII. The 4-carboxyl group is not an essential part of the cephalosporin chromophore, since descarboxylated compounds (XXIII) also have a $\lambda_{max.}$ at 256 nm. (80). This particular band is dependent on the integrity of the β-lactam ring as well as on the C-3 and C-4 double-bond conjugation in the Δ^3-cephalosporin system (125). The $\lambda_{max.}$ at 260 nm. for cephalosporin C and at 267 nm. for desacetylcephalosporin C disappears upon alkaline hydrolysis (86). New semisynthetic cephalosporins such as cephalothin (72, 73), cephaloglycin (78), desacetylcephaloglycin (124), and desacetylcephalothin methyl ester (123), as well as other cephalosporin derivatives (79), all have a typical $\lambda_{max.}$ at or near 260 nm. Cephaloridine and many pyridine-substituted cephaloridines, however, show absorption at 230–240 and 250–260 nm., respectively (75, 76). The cephalosporin C band ($\lambda_{max.}$ 260 nm.) is shifted at 257 nm. upon lactonization in dilute acid (2, 124). As a result of formation of Δ^2-isomers of cephalosporin amides and esters, the original absorption at 260 nm. also disappears (125).

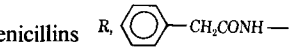
IR Absorption—Because of the fused β-lactam ring, both penicillins and cephalosporins exhibit a characteristic strong absorption band in the $\nu_{max.}$ 5.62–5.65-μ region (171, 172). This band disappears on hydrolysis by either base or β-lactamase, and a new band near 5.75 μ (the normal absorption band for penicilloyl ester or amide) forms. Similarly, bands at 5.69 μ (COOH), 3.00–3.05 μ (NH stretching), 6.0–6.1 μ (Amide I), and 6.5–6.7 μ (Amide II) of C=O stretching are also exhibited for all penicillins.

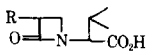
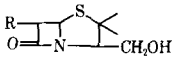
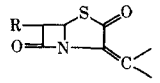
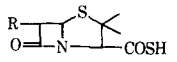
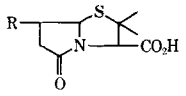
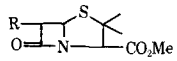
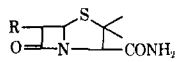
The IR absorption bands of cephalosporins and their esters, salts, and analogous derivatives have also been reported (72, 173); the new bands at 5.7 and 9.7 μ are due to the C-3 ester group (2, 72).

NMR Spectroscopy—NMR spectroscopy is another useful technique for characterization of the β-lactam drugs. Normally, the NMR spectra of the soluble salts of the penicillins and cephalosporins can be determined in D_2O. Penicillins usually exhibit a two-proton signal at τ 4.42 for the β-lactam hydrogen atoms, a sharp one-proton signal at τ 5.71 for the proton next to the carboxylate group, and sharp two- or three-proton signals at τ 8.38 and 8.47 for the gem-dimethyl group. In cephalosporins, the Δ^3-cephalosporin methylene protons adjacent to the sulfur exhibit doublets centered near τ 6.4 and 6.7; these signals disappear upon transformation to the Δ^2-isomer, and new single peaks appear near τ 3.6 and 5.0 (125). The centers of the single-proton quartet and the single-proton doublet, representing the β-lactam hydrogens at C-7 and C-6, are generally τ 0.8 unit apart in the Δ^3-esters but more than τ 0.4 unit apart in the Δ^2-isomers (125, 173). Upon acid hydrolysis of cephaloglycin, the acetyl group, which originally shows a singlet at τ 7.9, is lost and a new signal for acetate anion appears at τ 8.05 (124, 174).

BIOLOGICAL ACTIVITY IN RELATION TO STRUCTURE

In the last decade, attempts to develop new penicillins through molecular modification were extensive and successful. The main efforts were directed toward finding the so-called "ideal penicillin" or "all-purpose penicillin" (5, 7–9, 175). The ideal penicillin should

Table VI—Inactive or Less Active Penicillins 

Number	Compound	Structure	References
1	Desthiobenzylpenicillin		176
2	Benzylpenicillin alcohol		112
3	Anhydrobenzylpenicillin		177
4	Benzylthiopenicillin		114
5	Benzylhomopenicillin (γ-lactam)		178
6	Benzylpenicillin methyl ester		110
7	Benzylpenicillin amide		109

be stable toward acids and β-lactamases, well absorbed, less serum bound, and nonallergenic, and it should have a broad spectrum and high antibacterial activity. Because of the inherent acid stability and staphylococcal β-lactamase-resistant character of the cephalosporin nucleus, the semisynthetic cephalosporins have received considerable attention in the search for the ideal antibiotic. The antibacterial activities and some of the most important biological properties of the β-lactam antibiotics in relation to their chemical structure are discussed in this section.

Fundamental Structural Requirements—The antibacterial activity of the β-lactam antibiotics is no doubt related directly to the fused β-lactam–thiazolidine ring (penicillin nucleus) or fused β-lactam–dihydrothiazine ring (cephalosporin nucleus), since a breakage at any point leads to complete loss of activity, irrespective of the side chain (3, 8). Table VI shows the structures of a number of inactive and less active penicillins. Desthiobenzylpenicillin (Compound 1) is inactive (176); consequently, the sulfur atom is considered necessary. The free carboxyl group in both the thiazolidine ring and the dihydrothiazine ring is also necessary, since all types of derivatives of this carboxyl group of penicillins (3, 8, 9, 107–109, 111) and cephalothin (125) have been shown to be much less active or inactive. Accordingly, penicillin alcohol (112) (Compound 2), anhydrobenzylpenicillin (177) (Compound 3), benzylpenicillin methyl ester (110) (Compound 6), and benzylpenicillin amide (109) (Compound 7) have only slight antibacterial activity. A thioacid of benzylpenicillin (Compound 4) (114) exhibits about 90% of the antistaphylococcal activity of the active penicillin G. The γ-lactam-containing homopenicillin (Compound 5), unlike the β-lactam compound, is also biologically inactive (3, 178).

X-ray analysis has shown that, stereochemically, the β-lactam rings of penicillin and cephalosporin are essentially identical (21, 179) but the exocyclic carboxyl groups are different (180). Therefore, the observed fundamental differences in activity, as well as in stability, between penicillins and cephalosporins may be due to differences in the stereospecificity of the carboxyl groups as well as to the geometry of the fused ring systems (175).

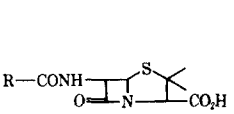
It was reported earlier that the penicillin β-lactam ring is energetic (81) and that the activity of the β-lactam antibiotics is related to the lability of their β-lactam ring structure (181). Correlations have been obtained between β-lactam ring stability and antibiotic activity and, similarly, between β-lactam stability and absorption in the IR spectrum (182). That is, with the β-lactam drugs, the more labile the β-lactam, the lower is the wavelength of absorption in the IR spectrum and, generally, the greater is the observed antibacterial activity. Conversely, the more resonant the β-lactam ring, the more stable is the ring toward base and, accordingly, the longer is the wavelength of absorption in the IR spectrum and, generally, the lower is the observed antibacterial activity (182).

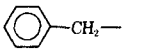
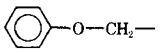
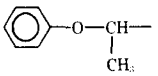
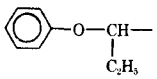
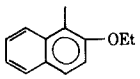
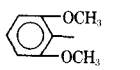
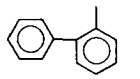
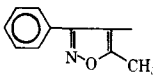
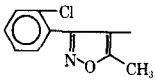
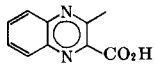
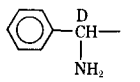
Effect of Side Chain on Activity—Although the penicillin or cephalosporin nucleus is essential for antibacterial activity, the potency of the various compounds is controlled to a great extent by the nature of the side chain (3, 8, 9). Since 6-chloro- and 6-bromopenicillanic acids are inactive (183), the amino groups of both 6-aminopenicillanic acid and 7-aminocephalosporanic acid are considered essential. Penicillins with benzylamino, phenylamino, and *N*-benzylphenylacetamido side chains (8) are also inactive compared with 6-aminopenicillanic acid. Penicillins with side chains having carboxamido, alkyl- and arylsulfonamido (8), and alkyl- and arylphosphinylacetamido groups (68) are much more active than 6-aminopenicillanic acid, but none is superior to penicillin G against staphylococci. The urido derivatives of 6-aminopenicillanic acid (69) have slightly lower antistaphylococcal activity than penicillin G but are better against penicillin-resistant strains. Penicillins with a phenylacetamido or thienylacetamido side-chain group appear to be the most active compounds. All compounds (both penicillins and cephalosporins) that have high antibacterial activity possess a continuous —C—CO—NH—C—CO—NH—C—COO— linkage, beginning in the side chain (—C—CO—NH—) and continuing along the β-lactam and the thiazolidine or dihydrothiazine nucleus.

The D-amino acid side chain of penicillin N and the phenylacetic acid side chain of penicillin G play a significant role in determining the biological capacities of these antibiotics (184). Penicillins with side chains having different physicochemical and spatial characteristics, such as methicillin (185) and the phenoxyalkylpenicillins (186), have properties significantly different from those of penicillin G.

Intrinsic Antibacterial Activity–Structure Relationships—*Gram-Positive Activity*—The first series of semisynthetic penicillins prepared for clinical use were the acid-stable α-phenoxyalkylpenicillins (186–190). α-Phenoxyalkylpenicillins are effective against infections caused by Gram-positive organisms. In this group of

Table VII—Minimal Inhibitory Concentrations (MIC) of Some Penicillins against Sensitive and Resistant Staphylococci



Number	Penicillin	R	MIC (mcg./ml.), Range		References
			Sensitive	Resistant	
1	Penicillin G		0.005–0.05 0.03–0.06	5–125 1–6	5 189
2	Penicillin V		0.03–0.06	0.4–3.0	189
3	Phenethicillin		0.06–0.125	0.4–3.0	189
4	Propicillin		0.06–0.125	0.25–0.6	189
5	Nafcillin		0.12–0.5	0.12–1.0	204
6	Methicillin		0.6–1.25 0.5–2.0	1.25–2.5 0.5–4.0	5 204
7	Biphenecillin		0.25– 0.12–1.0	0.5–5.0 0.25–1.0	5 204
8	Oxacillin		0.05–0.5	0.25–1.25	5
9	Cloxacillin		0.05–0.25 0.06–0.25	0.25–0.5 0.06–0.5	5 204
10	Quinacillin		0.25–2.0	0.5–4.0	204
11	Ampicillin		0.1–0.8	>100	7

compounds, as the α-substitution of the side chain becomes longer, the antistaphylococcal activity decreases, as well as the activity against other Gram-positive organisms (186, 188, 189). However, some derivatives (α-*tert*-isobutyl) show an increase in activity against β-lactamase-forming staphylococci (186, 188, 191). A series of analogous substitutions in the benzene ring of phenoxyethylpenicillin (phenethicillin) resulted in only a minor change in activity (188), and no direct relationship could be shown between antistaphylococcal activity and the electronic or steric effects obtained by the substitutions. However, one fact was apparent: the higher the molecular weight of the substituted side chain, the greater the serum binding and the lower the *in vivo* activity. Hansch and his coworkers (192, 193) demonstrated a correlation between the antistaphylococcal activity of 22 phenoxyalkylpenicillins and the negative lipophilic character of the side chain; the more hydrophilic the side chain, the greater was the antistaphylococcal activity of the penicillin. Similarly, among a series of substituted α-methoxypenicillins, 3,4 - dichloro - α - methoxybenzylpenicillin (194) was shown to be acid stable and to give good clinical results (195).

The majority of clinically used penicillins are 6-aminopenicillanic acid acyl derivatives of substituted acetic acid. For example, benzylpenicillin is a monosubstituted (phenyl), ampicillin is a disubstituted (phenyl and α-amino), and triphenylpenicillin is a trisubstituted (triphenyl) acetic acid derivative. Price *et al.* (8) showed that, in general, the disubstituted acetic acid penicillins have lower intrinsic antistaphylococcal activity than the monosubstituted derivatives, particularly if the substituents are bulky groups. The antistaphylococcal activity of the monosubstituted acetic acid derivatives was shown to be from 25 to 100% of that of penicillin G, with the majority in the range of 75–100%; the range for disubstituted derivatives was from 5 to 75%, with the majority in the 20–50% range; the trisubstituted derivatives were the least active, none being more than 10% as active as penicillin G.

The 6-aminopenicillanic acid derivatives of aromatic and heterocyclic acids, such as methicillin (196, 197), oxacillin (198, 199), cloxacillin (200), dicloxacillin (201), nafcillin (202), and quinacillin (203), also possess lower antistaphylococcal activity (about 20% of that of penicillin G), although they are all resistant to β-lactamase staphylococci (8, 9). In general (204), penicillin G is sufficiently active against non-β-lactamase-producing *Staphylococcus aureus* (Oxford), *Streptococcus pyogenes*, and *Listeria monocytogenes*, while methicillin, nafcillin, the isoxazolylpenicillins, quina-

cillin (203), and certain cephalosporins (204) are particularly active against the β-lactamase-producing staphylococci. As a result of both intrinsic resistance and enzymic inactivation (10), however, a few strains of *S. aureus* are resistant to methicillin, the isoxazolylpenicillins (205), and quinacillin (203).

Table VII shows that the penicillinase-resistant penicillins (Compounds 5–10) are about as active against resistant as against sensitive staphylococci [similar minimum inhibitory concentration (MIC) value], but penicillin G, the phenoxyalkylpenicillins, and ampicillin (Compounds 1, 2–4, and 11) are not.

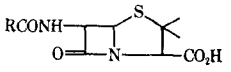
Gram-Negative Activity and Antibacterial Spectrum—Gram-negative activity here refers to activity against the Gram-negative bacteria. This activity was initially thought to be due to the presence of the free amino group in the penicillin or cephalosporin side chain (184, 206), this group being capable of attaining and maintaining close contact with susceptible sites on the Gram-negative bacteria (207). Penicillin N was the first penicillin used in the treatment of infections caused by Gram-negative bacteria, although it was considerably less active against the Gram-positive organisms than penicillin G (16, 184). *p*-Aminobenzylpenicillin (169) has a spectrum similar to that of penicillin N. The *ortho*- and *meta*-aminobenzylpenicillins (8, 9) have spectra similar to that of the *para*-isomer toward Gram-positive bacteria, but they are slightly less active against the Gram-negative organisms. Ampicillin (66, 206) has become one of the most effective broad spectrum antibiotics developed to date. It is effective against infections of the gastrointestinal, respiratory, and urinary tracts due to *Staphylococci, Streptococci, Haemophilus influenzae, Proteus mirabilis, Neisseria gonorrhoeae,* certain strains of *Escherichia coli,* and some species of *Salmonella, Shigella,* and *Moraxella.* The main disadvantage of ampicillin is that it is rather unstable toward penicillin-inactivating β-lactamases; its usefulness in infections caused by β-lactamase-forming *Staphylococcus, Proteus, Pseudomonas,* and *Aerobacter* is quite limited (208). Other amino group-containing penicillins, such as cyclacillin (WY-4508) (209) and BL-P875 (210), are also excellent broad spectrum penicillins but may be similarly sensitive to penicillinase.

The presence of a free amino group in the side chain is not the only requirement for Gram-negative activity; α-hydroxybenzylpenicillin (8, 9), azidobenzylpenicillin (211), pyridylmethyl derivatives (212) and isothiazolylmethyl derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid (213), and α-carboxybenzylpenicillin (carbenicillin) (214–216) are all broad spectrum β-lactam antibiotics, even though they do not contain such a free amino group. Table VIII lists the known penicillins with Gram-positive and Gram-negative activities. These compounds do have a side chain with sufficient polarity or hydrophilic property, and these characteristics are now thought to be essential for Gram-negative activity (71, 211–213, 217).

N-Acylation or condensation of penicillin N (3), *p*-aminobenzylpenicillin (169), ampicillin (218, 219), or cyclacillin (219) causes a drastic decrease in Gram-negative activity. The low activity of the phenoxyalkylpenicillins (220), methicillin, and the isoxazolylpenicillins (198–201, 221) against Gram-negative bacteria was shown not to be due to inactivation by β-lactamase (222). Nafcillin (223), quinacillin (224), diphenicillin (225), and penicillin dimer (226), none of which has the hydrophilic character, are also inactive against Gram-negative bacteria.

The mechanism responsible for the insusceptibility of the Gram-negative bacteria to penicillins is apparently more complex than that of the Gram-positive bacteria to penicillins. In general (10, 227), the resistance nature or insusceptibility of the coliform bacteria to penicillins may be chiefly due to three factors: (*a*) inherent resistance plus β-lactamase inactivation, as in the case of the resistance of β-lactamase-producing *Pseudomonas aeruginosa* (*pyocyanea*) and indole-positive *Proteus* and *Enterobacter* sp. to ampicillin (10); (*b*) inactivation due solely or primarily to β-lactamase, as in the case of the relative resistance of *Aerobacter aerogenes, Proteus vulgaris, Proteus morganii,* and *Proteus rettgeri* (10) to ampicillin, or the initial resistance of *S. aureus* to this drug (227); and (*c*) inherent resistance alone, as in the case of the *Salmonella* species and some strains of *Pr. mirabilis* and *Klebsiella,* which are sensitive to ampicillin and penicillin G

Table VIII—Broad Spectrum Penicillins



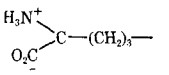
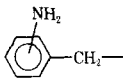
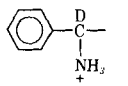
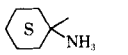
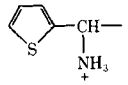
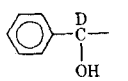
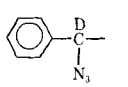
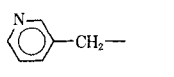
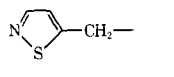
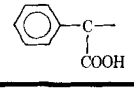
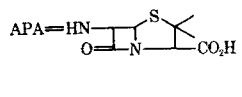
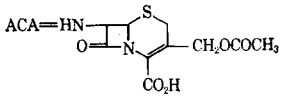
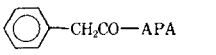
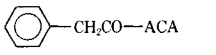
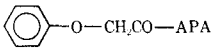
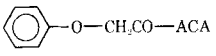
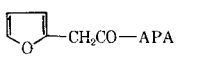
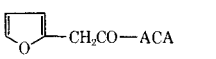
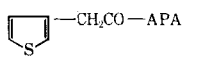
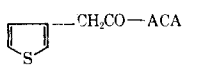
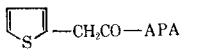
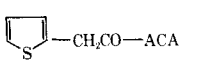
Number	Compound: Name	Compound: Side Chain R	References
1	Penicillin N		184
2	*p*-Aminobenzylpenicillin		169
3	*o*-Amino- or *m*-aminobenzylpenicillin		8, 9
4	Ampicillin		66, 206
5	Cyclacillin		209
6	BL-P875		210
7	α-Hydroxybenzylpenicillin		8, 9
8	Azidopenicillin		211
9	3-Pyridylmethylpenicillin		212
10	Isothiazolylmethylpenicillin		213
11	Carbenicillin		214–216

Table IX—Comparison of Minimal Inhibitory Concentrations (MIC) of Derivatives of 6-Aminopenicillanic Acid and 7-Aminocephalosporanic Acid in a Gradient Plate Test (mcg./ml.)[a]





Number	Structure of Compound	*Shigella* sp.	*Escherichia coli*	*Klebsiella* sp.	*Aerobacter* sp.	*Shigella sonnei*
1		32	42–48	13–109	42	44
2		33	24–37	2–27	15	27
3		112	110–144	138–>200	>200	105
4		107	101–132	7–>200	110	102
5		31	41–59	13–>200	84	40
6		15	10–36	7–11	8	17
7		15	25–33	10–105	38	26
8		31	17–33	2–20	7	8
9		28	38–46	10–122	56	169
10		11	8–15	6–8	5	11

[a] Data from *Reference 72.*

but which, even though producing little or no β-lactamase, are generally resistant to the phenoxyalkylpenicillins, methicillin, and the isoxazolylpenicillins.

Antibacterial Activity of Cephalosporin—Cephalosporin C is much less active than penicillin G against both Gram-positive and Gram-negative bacteria (2), probably because of its D-α-aminoadipoyl side chain since substitution of a phenylacetyl, 2-furylacetyl, 2- or 3-thienylacetyl, aryl- or alkylmercaptoacetyl, or other similar side-chain group results in a drastic increase in activity. In general, derivatives of 7-aminocephalosporanic acid are slightly less active than the corresponding derivatives of 6-aminopenicillanic acid against staphylococci but, as shown in Table IX, are several times more active against most Gram-negative bacteria (72, 204, 228).

Cephalothin is essentially similar to the furan-2-acetamido derivatives in antibacterial activity. Clinically, cephalothin is effective against a wide variety of organisms, particularly against the β-lactamase-forming *S. aureus* and some Gram-positive cocci, but is ineffective against *Pseudomonas*, the indole-positive *Proteus*, some strains of the *Klebsiella-Aerobacter* group, and the enterococci (229–232).

Cephaloridine (79) is moderately to highly effective against *A. aerogenes*, pyogenic cocci, most strains of *E. coli*, *Klebsiella*, *Pr. mirabilis*, *Salmonella* sp., and *H. influenzae* (233, 234) and is equally active against both sensitive and resistant staphylococci. Its inhibitory capacity for staphylococci is about 8–26 times greater than that of methicillin (234). Although many other desacetoxy derivatives of cephalosporins have been prepared, none is more active than cephaloridine, particularly against Gram-negative bacteria (74–76, 230). Any substitution into the pyridine ring in cephaloridine results in a decrease in activity (75).

The α-aminocephalosporins (78, 79) are cephalosporanic acid derivatives having a broad spectrum of activity, stability to gastric acid, and effective gastrointestinal absorption. Examples of such compounds are the α-aminophenylacetamido derivative (known as cephaloglycin) and α-aminothienylacetamidocephalosporanic acid. Among cephalothin, cephaloridine, and cephaloglycin, cephaloridine is the most effective of the three against sensitive Gram-positive organisms, its activity approaching that of penicillin (235); but cephaloglycin is the most effective against the Gram-negative bacteria (235–238).

In a series of α-substituted phenylacetamidocephalosporanic acids, the α-aminophenylacetamido and α-chlorophenylacetamido derivatives were found to be most active against *E. coli*, while the α-phenoxyphenylacetamido derivative was most active against β-lactamase-forming staphylococci (239). Another series of cephalosporins, prepared from acids such as the monocarboxylic, aromatic and heterocyclic diacetic, and sulfur-containing aliphatic dicarboxylic (240, 241), showed promising activity *in vitro* but negligible activity *in vivo*.

Cephalexin differs from cephaloglycin only by the lack of an acetoxy group attached to the dihydrothiazine ring. The excellent oral absorption and lack of serum binding of cephalexin compensate for its lower *in vitro* activity compared to cephaloglycin (242–244). Although cephalexin is less active against many organisms and is relatively slow acting compared to ceph-

aloridine, it is more active than cephaloridine against *N. gonorrhoeae* and more active than ampicillin toward penicillin-resistant staphylococci. Its overall activity is about 20% that of cephaloridine or ampicillin (243). It is rapidly inactivated by most strains of enterococci that are generally susceptible to ampicillin (244).

Both cephalexin and cephaloglycin (245) are moderately to highly effective against strains of group A hemolytic streptococci, viridans streptococci, pneumococci, gonococci, meningococci, and penicillin-sensitive *S. aureus*, but cephaloglycin is about 2–8 times more active than cephalexin toward these strains. On the other hand, both are moderately inactive against *H. influenzae*, enterococci, and most of the common Gram-negative bacilli. The activity of cephalexin is not significantly affected by the pH of the medium within the clinical range (pH 5.5–8.5). In contrast, the activity of cephaloglycin was shown to be quickly destroyed at the higher pH.

Studies on therapy of staphylococcal infections in monkeys (246, 247) showed that cephaloridine is the most active compared to cephaloglycin, cephalexin, and cephalothin.

Cephalexin (248), an orally absorbed cephalosporin antibiotic, is basically bactericidal in action. *Strep. pyogenes*, *Strep. viridans*, *Diplococcus pneumoniae*, *N. gonorrhoeae*, *Neisseria meningitidis*, *Corynebacterium diphtheriae*, *Clostridium tetani*, and *Clostridium perfringens* were consistently sensitive (MIC about <3 mcg./ml.) to this agent; most staphylococci (both penicillin-sensitive and penicillin-resistant strains), many of the Gram-negative enteric bacilli, including *Klebsiella*, *E. coli*, *Pr. mirabilis*, *Salmonella*, and *Shigella*, were moderately sensitive (MIC about 7.8 to 15.6 mcg./ml.). The strains of *H. influenzae*, isolates of *Enterobacter*, *Serratia*, *Enterococcus*, indole-positive *Proteus*, and *Pseudomonas* were usually resistant to cephalexin.

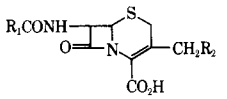
A cephalosporanic acid derivative, BL-P1322 [sodium 7-(pyrid-4-yl-thioacetamido)cephalosporanate], possesses antibacterial activity essentially the same as that of cephalothin (249). However, BL-P1322 was found to be significantly more effective against strains of *D. pneumoniae*, *Enterobacter* sp., and *Mycobacterium tuberculosis*.

Cefazolin (250, 251) is a new bactericidal cephalosporin active *in vitro* against most Gram-positive and Gram-negative bacteria except *Ps. aeruginosa;* it is also resistant to staphylococcal β-lactamase (250). The overall activity was shown to be parallel to that of cephaloridine; however, intramuscular administration of cefazolin resulted in blood levels twice as high as those observed with cephaloridine after similar administration (251).

A comparison of the antimicrobial activity of the various cephalosporanic acids and derivatives, the MIC's against both penicillin-sensitive and penicillin-resistant staphylococci and Gram-negative bacteria, are listed in Tables X and XI, respectively.

Activity against Strains of Pseudomonas *and* Proteus—*Ps. aeruginosa* (*pyocyanea*), as well as other strains of *Pseudomonas*, are inherently resistant to all forms of penicillins, whether or not assisted by inactivating β-lactamase (252). Carbenicillin was the first penicillin shown to be clinically efficacious against strains of *Proteus*, *Pseudomonas*, and *E. coli* (214, 215, 253–255). Carbenicillin has been used in patients with *Pseudomonas*-infected burns as well as in respiratory, urinary tract, and generalized *Pseudomonas* infections (255–257). It is relatively unstable toward staphylococcal β-lactamases. It is less active than ampicillin against *H. influenzae*, *E. coli*, *Salmonella* sp., *Shigella* sp., and *Pr. mirabilis*, as well as against staphylococci and streptococci (215, 253, 258–260), and it is ineffective against infections caused by *Klebsiella* sp. (215, 253) and *Serratia* sp. (258). The greatest disadvantage of carbenicillin is that it is extremely unstable toward acids and must be administered parenterally

Table X—Minimal Inhibitory Concentrations (MIC) of Some Cephalosporins against Sensitive and Resistant Strains of Staphylococci



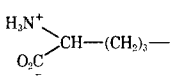
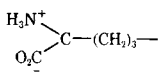
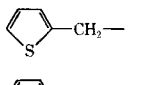
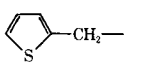
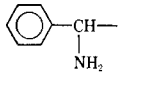
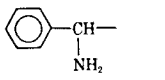
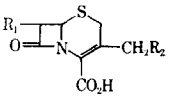
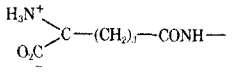
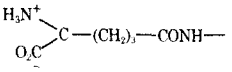
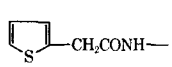
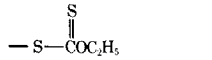
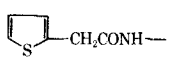
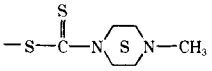
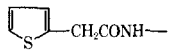
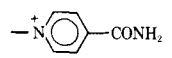
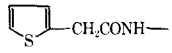
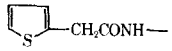
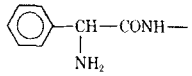
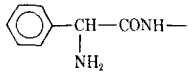
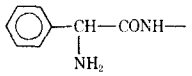
Number	Cephalosporin	Side-Chain Structure R_1	Side-Chain Structure R_2	MIC (mcg./ml.), Range Sensitive	MIC (mcg./ml.), Range Resistant	References
1	Cephalosporin C			60–125	60–125	5
2	Cephalosporin C_A			5–20	5–20	5
3	Cephalothin			0.3–0.6 0.25	0.3–0.6 0.25–0.5	5 204
4	Cephaloridine			0.12	0.12–0.25	204
5	Cephaloglycin			0.78–3.12	6.25–12.25	235
6	Cephalexin		—H	1.6–3.12	6.25–8.0	242 243

Table XI—Minimal Inhibitory Concentrations (MIC) of 7-Aminocephalosporanic Acid and Derivatives in a Gradient Plate Test



Number	Structure R_1	Structure R_2	*Shigella* sp.	*Escherichia coli*	*Klebsiella* sp.	*Aerobacter* sp.	*Shigella sonnei*	References
1			75	79	38	48	83	230
2			22	26	23	>50	23	230
3			78	83	6	67	—	230
4			2	3	2	4	—	230
5			6	2	3	2	2	75
6			5.6	2.4–2.8	4.2–4.5	3.8	3.8	75
7			11	8–15	6–8	5	11	72
8			9.1	12.6	9.7	8.2	—	124
9			11.6	16.3	14.4	16.4	—	124
10			2.1	3.6	3.6	3.9	—	124

(215). In addition, the emergence of resistant strains has occurred during therapy of *Pseudomonas* infections (259).

An α-sulfoamino derivative of ampicillin, BL-P1462 [6-(D-α-sulfoaminophenylacetamido)penicillanic acid and disodium salt] (261, 262), was shown to be active against a wide variety of Gram-negative bacilli, particularly *Ps. aeruginosa*. This drug was also shown to be slightly more active than carbenicillin against the same isolated strains of *Pseudomonas*, *Enterobacter*, and *Klebsiella* sp. However, it was not as active as carbenicillin against other Gram-negative bacilli, such as *E. coli* and *Proteus* sp. (262).

Table XII shows the antimicrobial spectrum and relative potency of several available penicillins and cephalosporins against both Gram-positive and Gram-negative organisms.

Effect of Diastereoisomers on Activity—Strikingly different antibacterial activity has been observed with the diastereoisomers of penicillins (187, 190, 211) and cephalosporins (78, 230). In a comparison of L-phenethicillin, D-phenethicillin, and DL-phenethicillin, McCarthy *et al.* (220) and English and McBride (263) pointed out that the L-epimer is the most active and the D-epimer the least active while the DL-form (phenethicillin) is intermediate in activity toward Gram-positive organisms.

Ampicillin [D-(−)-α-aminobenzylpenicillin], on the other hand, is about 2–6 times more active than its L-(+)-epimer and about 10 times more active than penicillin G against certain Gram-negative organisms (3). Similarly, the D-epimer of α-azidopenicillin is about 3–10 times more active than its L-epimer against both Gram-positive and Gram-negative organisms (211).

Spencer *et al.* (78) and Flynn (230) reported that L-cephaloglycin has about 5% of the antistaphylococcal activity (penicillin resistant) of its D-isomer; against Gram-negative organisms, D-cephaloglycin is at least 25 times more potent than its L-epimer.

Absorption and Serum Concentrations—Table XIII summarizes the peak serum concentrations of several penicillins and cephalosporins in human subjects.

The phenoxyalkylpenicillins are stable to gastric acid and well absorbed from the gastrointestinal tract (264, 267). The peak serum concentrations listed for these penicillins were reached at 1 hr. after oral administration in healthy volunteers, regardless of the dose. After oral administration of 250 mg. in solid dosage forms, the peak serum concentrations ranged as follows: penicillin V, 1–2 mcg./ml.; phenethicillin (189), 2–5.3 mcg./ml., or about twice that of penicillin V; and propicillin and phenoxyisopropylpenicillin, 2–7 mcg./ml., or about 2–3 times that of penicillin V (264–267). The L-isomer and the DL-isomer of the α-phenoxyethylpenicillins gave about twice the serum concentrations of the D-isomers (268). Similarly, the L-isomer of propicillin gave higher

penicillin levels than its D-isomer in dogs and rats (269). Food apparently interferes with the efficient absorption of these antibiotics, and the serum penicillin concentrations are decreased by about 30–60% when they are administered after a meal compared to those observed under fasting conditions (268, 270). There was no indication that a difference in the activity of human subjects (*i.e.*, as between ambulatory and bedridden patients) significantly influences the peak serum levels after oral administration of penicillin V (271). Water-soluble salts (potassium, sodium, and calcium) of penicillin generally give higher and earlier serum concentrations than the sparingly soluble salts (*i.e.*, benzathine penicillin) or the penicillin-free acid form (271, 272).

The peak serum levels of oxacillin, cloxacillin, and dicloxacillin (273–280) were also attained at 1 hr. after oral dosing in fasting volunteers. The serum concentrations of oxacillin were shown to be slightly lower than those attained after oral administration of phenethicillin or penicillin V (221, 275). After oral administration of a single dose of 1.0 g. of oxacillin, the peak serum concentrations were from 9 to 12 mcg./ml., which are well in excess of the MIC for resistant staphylococci (276, 278). The oral absorption characteristics of the three isoxazolylpenicillins were compared after a single dose of 500 mg. in fasting volunteers. The peak serum levels ranged from 5 to 7 mcg./ml. for oxacillin and from 7.5 to 14.4 mcg./ml. for cloxacillin, or about twice that of oxacillin (201, 277–279), and about 10–17 mcg./ml. for dicloxacillin (279, 280). The isoxazolylpenicillins are about 5–10 times more active than methicillin, while dicloxacillin is 2–3 times more active than cloxacillin and oxacillin against penicillinase-producing *S. aureus* (280). The observed therapeutic activity of the isoxazolylpenicillins seems to correlate well with their serum concentrations.

Methicillin (281, 282), nafcillin (282), diphenicillin (283, 284), and quinacillin (224) give relatively less serum levels. This may be due to either low stability or low aqueous solubility, or both of these factors may be operative for the higher molecular weight penicillins. Nafcillin was shown to be absorbed better than methicillin (281), but the absorption was quite irregular and significantly affected by food (202). However, the addition of buffer to nafcillin formulations results in an improvement in the oral absorption characteristics of this agent (202).

In dogs the peak serum level of ampicillin (7.2 mcg./ml.) is obtained 1 hr. after a single oral dose of 20 mg./kg., while those for phenethicillin and penicillin V (about 4.0 and 2.6 mcg./ml., respectively) are observed at only 30 min. after dosing. In dogs, as well as in humans, the ampicillin serum levels are more persistent than those of penicillin V and phenethicillin (285). After single oral doses of 250, 500, 750, and 1000 mg. of ampicillin in fasting volunteers, the mean peak serum concentrations were 1.9, 3.8, 5.1, and 6.8 mcg./ml., respectively (286). These peaks usually occurred at about 2 hr. after dosing in human subjects (compared to 1 hr. in dogs), and significant serum concentrations were maintained even at 6 hr. Doubling the dose virtually doubles the peak serum concentration. Probenecid delays the excretion of ampicillin (287). In rats, after either oral or intraduodenal administra-

Table XII—Antibacterial Spectra of Several β-Lactam Antibiotics[a]

Organism	Penicillin G	Penicillin V, Phenethicillin Propicillin, Phenbenicillin	Methicillin, Nafcillin, Ancillin, Quinacillin	Oxacillin, Cloxacillin, Dicloxacillin	Ampicillin, Hetacillin, Cyclacillin, BL-P875, Azidopenicillin	Carbenicillin, α-Sulfonamidobenzylpenicillin (BL-P1462)	Cephalothin, Cephaloridine, Cephaloglycin, Cephalexin, Cefazolin
S. aureus, β-lactamase producing	±	+	++	++	±	+	++
S. aureus, non-β-lactamase producing	+++	++	++	++	+++	++	++
Strep. pneumoniae	+++	++	++	++	+++	+	++
Strep. faecalis	++	++	++	+	++	+	+
Strep. pyogenes	+++	++	+++	++	+++	++	+++
Strep. viridans	+++	++	++	++	++	+	++
N. meningitidis	++	++	++	++	++	+	++
H. influenzae	++	+	+	+	++	++	+
Salmonella sp.	+	±	−	−	+	+	+
E. coli	±	−	−	−	+	+	++
Klebsiella, Aerobacter spp.	−	−	−	−	±	±	++
Pr. mirabilis, non-β-lactamase producing	−	−	−	−	+	++	+
Pr. mirabilis, β-lactamase producing	−	−	−	−	−	+	+
Pr. rettgeri, morganii, vulgaris	−	−	−	−	−	+	−
Ps. aeruginosa	−	−	−	−	−	+	−
References	7, 10, 187, 189, 206	7, 10, 189 222, 225	7, 10, 196, 202, 204, 223, 224, 225	7, 10, 204, 221, 222	7, 10, 204, 206, 209, 210, 211, 222, 292	10, 214, 215 253, 259, 260, 261	7, 10, 72, 124, 204, 235, 250, 251

[a] Minimum inhibitory concentration (median): +++, active at <0.1 mcg./ml.; ++, active at 0.1–10 mcg./ml.; +, active at > 10 mcg./ml.; ±, active at 250 mcg./ml; and −, no activity at >250 mcg./ml.

Table XIII—Representative Peak Serum Concentrations of Several β-Lactam Antibiotics in Human Subjects after a Single Oral Dose[a]

Antibiotics	Form	Dose, mg.	Dosage Form	Peak Serum Concn. (Range) mcg./ml.	Peak Time (hr.)	References
Penicillin V	Pot. salt	250	Unspecified	1.6	1	189
	Pot. salt	500	Unspecified	6.0	1	265
Phenethicillin	Pot. salt	250	Unspecified	3.3	1	189
	Pot. salt	500	Unspecified	8.8	1	265
Propicillin	Pot. salt	250	Unspecified	6.2	1	189
	Pot. salt	500	Unspecified	12.2	1	265
Phenbenicillin	Pot. salt	125	Capsules	4.9	0.5	267
	Pot. salt	250		7.5	1	267
Nafcillin	Sod. salt	1000	Tablets	6.4	1	202
			Capsules	6.6		
Oxacillin	Sod. salt	250	Capsules	1.9	1	277
	Sod. salt	500		6.0(5.0–7.0)	1	277,278
Cloxacillin	Sod. salt	250	Capsules	3.5	1	277
	Sod. salt	500		10.5(7.5–14.4)		277,278,279
Dicloxacillin	Sod. salt	250	Capsules	8.5	1	279
	Sod. salt	500		17.0		
Ampicillin, anhydrous	Acid	250	Suspension	2.2	1	291
			Capsules	2.0	2	
Ampicillin, trihydrate	Acid	250	Suspension	1.7	2	
			Capsules	1.9	2	
Hetacillin	Acid	250	Capsules	1.7	2	292
		500		2.5(2.3–2.8)		
Cephaloglycin	Acid	500	Unspecified	2.6	2	238
Cephalexin	Acid	250	Capsules	8.4	1	244
		500		15.0(11–18.4)	1	243,244
		1000		23.0	1	243

[a] In fasting human volunteers.

tion, ampicillin and penicillin V are both poorly absorbed. In dogs and humans, however, ampicillin is well absorbed and gives serum levels about twice those of penicillin V (288). Ampicillin is destroyed in the stomach by acid and in the intestine by the β-lactamases present there, but it remains intact in the bile (286, 288–290); thus, an absorbed–excreted–reabsorbed cycle of ampicillin in the body may be partly responsible for the long-lasting serum levels of this agent compared to those of the phenoxyalkylpenicillins. Because of the cycle, very little intact ampicillin remains in the middle and lower part of the gut, and none is present in the feces (290).

Ampicillin is available in two crystalline forms, the anhydrate and the trihydrate, having different properties. For instance, the anhydrous form has greater aqueous solubility and a higher apparent rate of dissolution than the hydrated substance (168). This difference is probably responsible for the fact that, after oral dosing to both dogs and humans, the anhydrous form gives higher and generally earlier peak serum levels and an overall more efficient absorption (greater area under the time–serum level curve) than the hydrated form (291).

Hetacillin, a condensation product of ampicillin and acetone which is less soluble than either form of ampicillin, gives serum levels about one-half of those obtained with ampicillin in volunteers (292, 293). After an oral dose of 500 mg. of hetacillin or ampicillin to fasting human subjects, the peak serum levels attained with hetacillin are only about 1–3 mcg./ml. 2 hr. after dosing, in comparison with 3–5 mcg./ml. for ampicillin (293, 294).

Cephalosporins N and C are rather acid stable but are very poorly absorbed from the gastrointestinal tract in both humans and animals. The poor absorption, however, does not completely depend on the α-aminoadipoyl side chain, since cephalothin and cephaloridine are also poorly absorbed (232–234). The absorption of cephaloridine can be improved if the antibiotic is given in combination with sodium lauryl sulfate (295). Cefazolin gives serum levels about twice as high as cephaloridine after intramuscular injection of a single 500-mg. dose (251). Many of the derivatives of 7-aminocephalosporanic acid, such as *N*-phenylacetyl, *N*-phenoxyacetyl, and phenylglycol, are absorbed much less readily than are the corresponding derivatives of 6-aminopenicillanic acid.

Cephaloglycin is well absorbed in comparison with cephaloridine or penicillin V (235). Two hours after oral administration of 250- and 500-mg. doses to fasting volunteers, the mean peak serum concentrations were about 1.3 and 2.3–2.6 mcg./ml., respectively (238, 296), which were slightly lower than those obtained from ampicillin with equal doses. Cephalexin has been shown to be absorbed more efficiently; in fasting volunteers in a crossover study, the peak serum concentrations were 8.4 and 18.8 mcg./ml. after oral administration of 250- and 500-mg. doses, respectively, and were reached 1 hr. postadministration (244). The serum concentration obtained from a single dose of 0.5 g. of cephalexin is virtually equal to that of cephaloridine by intramuscular injection of the same size dose (244). The rather high serum concentrations and the nearly complete recoveries of cephaloglycin and cephalexin indicate that the human is able to absorb these antibiotics substantially better than the rat and the dog (243).

Factors Influencing Oral Absorption—It is well known that the acid stability of the penicillins and cephalosporins is an important factor in their oral absorption. However, acid stability is obviously not the only property governing the oral absorption of the β-lactam antibiotics. β-Lactamase inactivation may

play an important role in the relatively low serum levels attained with some of these agents. Solubility and dissolution rate are other factors that may contribute to the faster and more efficient absorption of such drugs, as was shown in the case of anhydrous ampicillin in comparison with ampicillin trihydrate (291) and sodium and potassium ampicillin in comparison with ampicillin trihydrate (297).

Well-documented reports (202, 286, 298) have shown that stomach conditions, such as occur with fasting or nonfasting, may affect absorption. In general, the presence of food retards the rate and extent of penicillin absorption.

Similarly, formulation factors play a significant role in oral absorption. For example, dicloxacillin was shown to be more efficiently absorbed in suspension than in gelatin capsules (298). The physical and chemical properties of the drug (*i.e.*, crystalline state, stability, and metabolism in the body) conceivably are other key factors governing the blood serum levels.

A relationship certainly exists between protein binding and serum penicillin levels. For instance, the serum binding of the isoxazolylpenicillins is about 92–94% for oxacillin, 94–96% for cloxacillin, and 98–99% for dicloxacillin, and the observed serum penicillin levels of these drugs correlate with their respective magnitudes of serum binding (299). Measurement of serum penicillin levels alone, however, does not accurately reflect efficiency of oral absorption, since penicillins differ in tissue distribution, rate of removal from the blood, and rate of inactivation and excretion; each of these factors may be important in determining the serum levels (299). Dicloxacillin, for example, shows slower and lower renal excretion and is less well distributed in the pericellular space than oxacillin or cloxacillin (295, 299). Nafcillin and oxacillin, on the other hand, are equally well absorbed, the lower serum levels of nafcillin compared to those from oxacillin or cloxacillin being mainly due to the facts that nafcillin is better distributed in the tissues and is less serum bound than the isoxazolylpenicillins (299).

INACTIVATION OF PENICILLINS AND CEPHALOSPORINS BY BACTERIAL ENZYMES

The bacterial enzymes capable of attacking either the penicillins or the cephalosporins, or both, are the β-lactamases, the acylases (amidases), and the acetyl esterases (300, 301). β-Lactamases hydrolyze the β-lactam linkage, with production of the antibiotically inactive penicilloic or cephalosporoic acid. The acylases are enzymes which hydrolyze the *N*-acyl side chain (peptide bond) (300, 302). Acetyl esterases are of minor interest because they attack selectively the C-3 acetyl ester group in cephalosporins to desacetyl cephalosporins (300). β-Lactamases and acylases can both be produced by the same microbial strain, such as during incubation of penicillin G with strains of *E. coli*, *Streptococcus lavendulae*, or *Klebsiella aerogenes*, which usually produce more than one decomposition product (37, 300, 302, 303). In the cephalosporins, the opening of the β-lactam ring may be accompanied by the simultaneous expulsion of the C-3 ester or pyridine group due to strains of *Bacillus cereus* (β-lactamase II) or *Ps. aeruginosa* (304, 305). The distribution, specificity, and selective preparation of penicillin acylases (306–308) and the properties and sources of the known β-lactamases (309, 310) were reported. Comprehensive reviews on β-lactamase were recently published by Citri and Pollock (309), Pollock (301, 310), Abraham (311), and Rauenbusch (312).

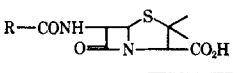
β-Lactamases: Their Distribution and Properties—Although β-lactamase was first discovered by Abraham and Chain in 1940 (311, 313), studies before 1960 were mostly confined to the β-lactamases of Gram-positive origin (314–319). The activity of enzymes from different sources and preparations was determined by various biological and chemical techniques, such as manometric, alkalititrimetric, hydroxylamine, optical rotatory, and iodometric (320). These studies were directed largely toward the production, purification, and elucidation of the enzymic properties of β-lactamase from *B. cereus* (316, 318, 319, 321), *Bacillus subtilis* (317, 322), *Bacillus licheniformis* (323), and *S. aureus* (324–328). Interest in the β-lactamases produced by the Gram-negative coliform bacteria arose only after the introduction of ampicillin (206) and other broad spectrum β-lactam antibiotics; bacteria such as *E. coli* (328–331), *K. aerogenes* (331, 332), *Ps. aeruginosa* (305, 333), *Proteus* sp. (331, 334–336), *Enterobacter cloacae* (337), and *E. coli* carrying the resistant (R) factor (338) gradually became resistant to the broad spectrum drugs. It is now known that β-lactamases are very widely distributed among bacteria of both Gram-positive and Gram-negative origin (324, 339), and the β-lactamase inactivation of penicillins and cephalosporins has been shown to be the major factor responsible for bacterial resistance to them (10, 300, 310).

Like other enzymes, β-lactamases are proteinoid macromolecules. Although the purified β-lactamases from Gram-positive organisms have a similar molecular weight (about 30,000) and an identical hydrolytic action toward penicillins, they differ greatly in other physicochemical properties, such as in electrophoretic mobility, adsorption and elution properties, optimal pH, isoelectric point, and overall amino acid composition (300, 301, 310, 312, 324, 340). β-Lactamases from *S. aureus* are rich in lysine; those from *B. licheniformis* are rich in aspartic acid (340, 341); and those from *B. cereus* are rich in alanine (310).

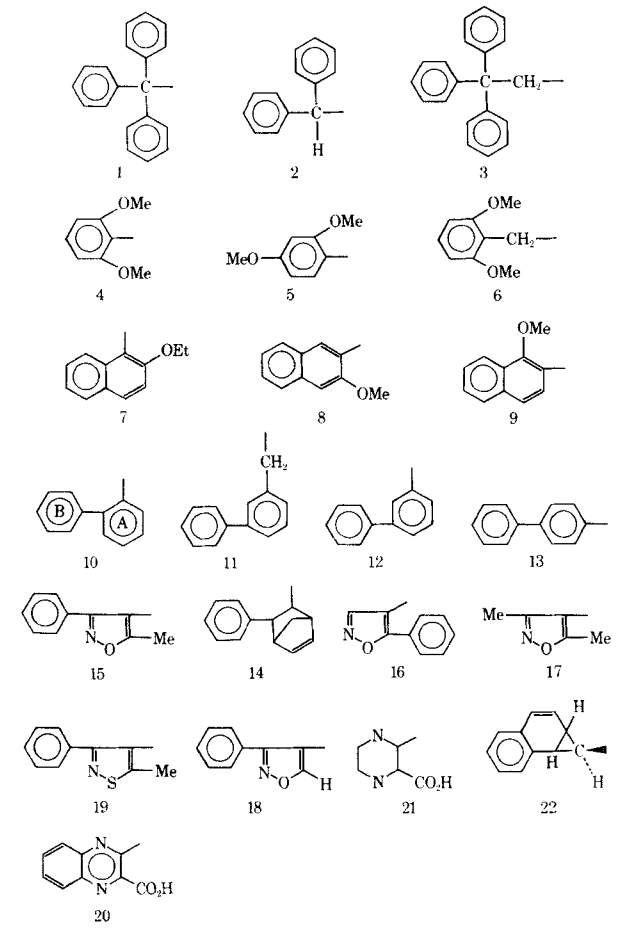
The β-lactamases from Gram-positive organisms are "extracellular" enzymes and are normally liberated in their growth media, while those from Gram-negative coliform bacteria are cell bound, or "endocellular," the enzymic activity appearing to take place inside the barrier of the cells. With the latter, liberation of the enzyme into the growth medium can be achieved only after disruption of the cell wall (300).

The β-lactamases of Gram-positive origin appear to be inducible (317); that is, they are formed in response to the presence of a substrate (such as methicillin), have greater enzymic activity, and have a higher energy of activation (about 7 kcal./mole or higher). The β-lactamases of Gram-negative bacteria cannot be induced; they are "constitutional," have a markedly varied differential permeability barrier, and have a rather low

Table XIV—Structural Comparison of Penicillins Resistant and Susceptible to β-Lactamase-Producing Staphylococci



Resistant Penicillin, R	Susceptible Penicillin, R



energy of activation (about 4–5 kcal./mole) (300, 342, 343).

In general, the β-lactamases from *S. aureus*, *B. cereus* (β-lactamase I), *B. licheniformis*, *Klebsiella* sp., *Pr. mirabilis*, and *Aerobacter cloacae* selectively attack the β-lactam of penicillins. These bacterial enzymes are often referred to as "penicillinases" or "penicillin β-lactamases" (310). On the other hand, bacterial enzymes from *B. cereus* (β-lactamase II), *E. coli*, *Enterobacter* sp., *Pr. vulgaris*, *Pr. rettgeri*, *Pr. morganii*, and *Ps. aeruginosa* are known to hydrolyze cephalosporins rapidly but to attack penicillins only of certain types (10, 300). These enzymes are often called "cephalosporinases" or "cephalosporin β-lactamases."

β-Lactamase Resistance of Penicillins—One of the most important organisms causing clinical resistance to penicillins is the β-lactamase-producing *S. aureus*. To overcome the effect of this enzyme, penicillins having appropriate *N*-acyl side chains have had to be used. Thus, in this case, the same feature of the side chain structure determines both the intrinsic activity and the stability toward β-lactamase (3). When the side chain of an α-phenoxyalkylpenicillin (186, 188, 191) is made longer, the activity against the penicillin-sensitive strains of *S. aureus* decreases but that against the resistant strains increases. Phenoxyisopropylpenicillins and phenoxyisobutylpenicillins show the highest stability toward β-lactamase-forming staphylococci. Such a sterically hindered group in the side chain may act by interfering with the enzyme attachment (8, 9).

Doyle *et al.* (92, 93) showed that marked antistaphylococcal β-lactamase activity is obtained when the penicillins are prepared from six-membered aromatic or heterocyclic carboxylic acids substituted in both *ortho*-positions with relatively bulky groups. Table XIV illustrates the structural requirements of the side-chain group of several types of penicillins for staphylococcal β-lactamase resistance. Triphenylmethylpenicillin (Compound 1) (344) was noted to have pronounced stability toward β-lactamase-forming staphylococci and *B. cereus*. Omission of one of the phenyl groups (Compound 2) or interpolation of a methyl group, as in β,β,-β-triphenylethylpenicillin (Compound 3), results in a β-lactamase-sensitive compound. 2,6-Dimethoxyphenylpenicillin, known as methicillin (Compound 4) (93), was the first of a series of penicillins proved to be effective and useful against staphylococcal infection, while 2,4-dimethoxyphenylpenicillin (Compound 5) and 2,6-dimethoxybenzylpenicillin (Compound 6) were found to be β-lactamase susceptible. Several methicillin analogs that possess a sterically hindered ring, such as 2-alkoxy-1-naphthylpenicillin (345), are stable toward β-lactamase. For example, 2-ethoxy-1-naphthylpenicillin (known as nafcillin) (Compound 7) and 2-methoxy-1-naphthylpenicillin (345, 346) are β-lactamase stable. Other similar forms, such as 2-methoxy-3-naphthylpenicillin (Compound 8) and 1-methoxy-2-naphthylpenicillin (Compound 9), are unstable toward the enzyme.

2-Biphenylpenicillin (Compound 10) has been shown to be 3–5 times more stable toward staphylococcal β-lactamase than methicillin. Substitution in either of the two aromatic rings results in a loss of activity (347). To exert maximal antistaphylococcal activity, the phenyl Ring A (proximal to the penicillin nucleus) must be directly attached to the side-chain peptide linkage, while Ring B (distal to the penicillin nucleus) must occupy the *ortho*-position of Ring A (Compound 10). Accordingly, the 2-biphenylmethylpenicillin, 3-biphenylpenicillin, and 4-biphenylpenicillin (Compounds 11, 12, and 13, respectively) are all unstable toward staphylococcal β-lactamase. If Ring A is changed to cyclohexyl or a bridged ring instead of an aromatic ring, the antistaphylococcal activity is lowered about 20–1000 times compared to that of 2-biphenylpenicillin (348); for example, *endo*-phenyl-*exo*-carboxamidopenicillin (Compound 14) and its analog are β-lactamase unstable. However, the penicillinase-resistant property will be retained if Ring A is replaced by a heterocyclic ring with sufficient size and aromaticity and provided there is a phenyl or an equivalent substituent adjacent to the side-chain peptide linkage. Ring B can be replaced by an aromatic heterocyclic system without losing activity. Also, replacement of the phenyl rings with α- or β-naphthyl groups results in no drastic loss of activity (349).

Some 3,5-disubstituted-4-isoxazolylpenicillins are also highly stable toward staphylococcal β-lactamase (93,

222). One example is 5-methyl-3-phenyl-4-isoxazolylpenicillin, known as oxacillin (Compound 15) (222), which was the first of several isoxazolylpenicillins used against Gram-positive penicillin-resistant organisms. Its 2-chlorophenyl and 2,6-dichlorophenyl analogs, known as cloxacillin and dicloxacillin, are particularly effective against β-lactamase-forming staphylococci. In contrast, 5-phenyl-4-isoxazolylpenicillin, 3,5-dimethyl-4-isoxazolylpenicillin, and 3-phenyl-4-isoxazolylpenicillin (Compounds 16, 17, and 18, respectively) are all unstable to staphylococcal β-lactamase (5, 8, 9).

Naito *et al.* (350) reported that some of the 3,5-disubstituted isothiazolylpenicillins have similar or improved activity compared to oxacillin. 5-Methyl-3-phenyl-4-isothiazolylpenicillin (Compound 19) and its 2,6-dichlorosubstituted compounds (not shown) have significantly greater stability toward staphylococcal β-lactamase than either oxacillin or dicloxacillin.

6-Aminopenicillanic acid derivatives prepared with vicinal dicarboxylic acids, such as quinoxacillin-2,3-dicarboxylic acid, known as quinacillin (Compound 20), are effective against penicillin-resistant staphylococci (203, 221). Other dicarboxylic acids, such as quinoline-2,3-dicarboxylic acid, have activity similar to quinacillin, but not the derivatives from pyrazin-2,3-dicarboxylic acid (Compound 21) or pyridine-2,3-dicarboxylic acids. Penicillins derived from cycloheptatriene carboxylic acid (351) and benznorcaradiene carboxylic acid (352) (Compound 22) show little β-lactamase resistance in comparison to methicillin or other β-lactamase-resistant penicillins.

β-Lactamase Resistance of Cephalosporins—Cephalosporin C and its derivatives, such as cephalothin and cephaloglycin (235), are effective against most β-lactamase-forming staphylococci and *B. cereus* and are moderately active against some coliform bacilli (3, 342, 353, 354). In addition, cephalosporin C is able to protect penicillin G from inactivation by these enzymes (3, 115). The resistance of cephalosporin C to staphylococcal penicillinases apparently is related more closely to the nucleus than to the nature of the side chain, since cephalosporin N (penicillin N) is very labile toward these β-lactamases (3, 115). Crompton *et al.* (42) showed that desacetylcephalosporin C and cephalosporin C itself are the most resistant toward staphylococcal β-lactamases, while the pyridine derivative (cephalosporin C_A) is less stable and the lactone derivative is the least stable. Apparently the nature of the side chain at C-3 in cephalosporin C as well as in other cephalosporins must also play an important role in staphylococcal β-lactamase stability.

Data obtained from the study of methicillin and its cephalosporin analogs against the β-lactamases from Gram-negative organisms indicate that the structure of the substitution in the 7-position of cephalosporin, like in penicillins, is the main determining factor in the resistance or inhibition of the β-lactamase (355). However, the structure of the group in the 3-position of cephalosporin modifies this character; it is suggested that a labile group in the C-3 position which can easily accept electrons from β-lactam conjugation gives the cephalosporin a higher affinity for the bacterial enzyme and thus increases its effectiveness as a competitive inhibitor to protect a susceptible penicillin from being inactivated.

Kinetic Studies on β-Lactamase Inactivation—The study of the bacterial β-lactamase inactivation of the β-lactam antibiotics from an enzymic kinetics approach provides valuable information concerning the magnitude of the interaction between enzyme and substrate, the possible mechanism of inactivation, and the inhibition of enzyme activity by substrate analog or competitive inhibitor (309, 356–360). The β-lactamases, like other biologically important enzymes, have been shown to be extremely specific and highly dependent on: (*a*) source and preparation, (*b*) experimental conditions, (*c*) accessibility of substrate, and (*d*) nature of the enzyme–substrate complex.

According to the conventional Michaelis–Menten expression (361), $V_{max.}$ is the maximum velocity in the presence of excess substrate, and K_m is the Michaelis constant, representing the dissociation constant of the enzyme–substrate complex. This constant, K_m, is inversely proportional to the affinity of the enzyme for the substrate (penicillin or cephalosporin). The enzyme (β-lactamase) and the substrate react to form an intermediate enzyme–substrate complex, which dissociates to the product and the original enzyme. The initial rate is usually measured over a wide range of substrate concentrations. From steady-state treatment, the $V_{max.}$ and K_m can normally be obtained by using Lineweaver–Burk (362) plots.

The efficiency of the β-lactamase can be expressed in terms of either the speed or the specificity of the reaction that they catalyze. The relative physiological efficiency of the activity of a β-lactamase can be indicated by the value of the product of its $V_{max.}$ and K_m with any substrate (323). The effectiveness of a competitive inhibitor in preventing the activity of a particular β-lactamase is determined from the ratio of the inhibitor to the substrate required for 50% inhibition of the initial rate of hydrolysis (42, 363).

β-Lactamase Kinetics—The kinetics of the penicillinase from *B. cereus* and *S. aureus* have been studied in great detail. In 1946, Henry and Housewright (364) found that the β-lactamase *B. cereus* 569 inactivates penicillins G and X at an approximately equal rate and penicillins F and K at a somewhat slower rate. The reactions were pseudo zero order in the presence of saturating concentrations of the substrate. The apparent energy of activation for the hydrolysis of penicillin G was about 7.5 kcal./mole.

Abraham and Newton (357) reported their manometrically obtained data on the *B. cereus* NRRL569 hydrolysis of penicillins V, G, and N; synnematin B; and cephalosporin C at pH 7 and 30°. Compared to penicillin G, penicillin V was hydrolyzed 1.25 times as fast; penicillin N and synnematin B were hydrolyzed 0.54 times as fast. Under identical conditions, cephalosporin C underwent no hydrolysis. The energies of activation were 5.3 and 5.7 kcal./mole for penicillins G and N, respectively, compared to 14.5 kcal./mole for the nonenzymic hydrolysis of penicillin G in dilute sodium hydroxide solution. Cephalosporin C behaved as a competitive inhibitor of the activity of β-lactamase from *B. cereus*.

The interaction of *B. cereus* 569/H with several penicillins and cephalosporins was explored by Citri and coworkers (365–370). The conformation of this extracellular enzyme can be reversibly modified in various ways. A conformational change in its active site was reflected, for instance, in an increased susceptibility to iodination, urea, and heat. The activity of the enzyme was found to depend on a definite and precise orientation of the active site, which was distorted by competitive inhibitors (358). The relative rates of hydrolysis of the penicillins and cephalosporins at pH 6 and 30° were: ampicillin, 120; phenoxyalkylpenicillins, 60–130; penicillin G, 100; 6-aminopenicillanic acid, 40; ancillin, 28; nafcillin, 17; oxacillin, 5; cloxacillin, 3; cephaloridine, 2; and 7-aminocephalosporanic acid = cephalosporin C = benzylcephalosporin = cephalothin, <0.1. All the competitive inhibitors exerted a powerful effect on the conformation of the active site of the enzyme (369), an effect described as "conformative response" (370).

Kuwabara and Abraham (371) studied the behavior of purified enzyme from *B. cereus* 569/H with regard to several penicillins and cephalosporins. They were able to separate the β-lactamase I (penicillinase) from the β-lactamase II (cephalosporinase) in a crude enzyme culture.

The kinetics of interaction of the β-lactamase from *B. subtilis* and penicillin G were investigated by Banfield (372) by an alkalimetric titration technique at varied pH, temperature, and ionic strength. From Lineweaver–Burk (362) plots, both the K_m and $V_{max.}$ values varied as the pH was increased from about 5.5 to 8.5. The heat of activation was approximately 5.7 kcal./mole at pH 7.14. Varying the ionic strength through the range 0.002–0.08 was found to have little effect on the reaction rate.

Novick (373) investigated the kinetics of several penicillins with both cell-bound and free staphylococcal β-lactamase. Measurements of the Michaelis constant revealed that the effectiveness of β-lactamase resistance is inversely proportional to the affinity of the β-lactam compounds for the enzyme. Methicillin, with a K_m of 2.8×10^{-2} *M*, is by far the most resistant. The K_m of other penicillins ranges from 3.8×10^{-6} *M* (penicillin V) to 17×10^{-6} *M* (propicillin), and these penicillins are unstable toward the enzyme. Usually, higher $V_{max.}$ and lower K_m values are obtained from the free than from the cell-bound form of penicillinase.

Gourevitch *et al.* (374) compared the $V_{max.}$ and K_m values of 11 penicillins with their *in vitro* MIC values against both penicillin-sensitive and penicillin-resistant staphylococci. In accordance with their data, they classified the penicillins into three groups. The nonpenicillinase-resistant compound had very high $V_{max.}$ and low (about $4–10 \times 10^{-5}$ *M*) K_m values and a large difference in MIC against the two types of staphylococci. Penicillin G and the phenoxyalkylpenicillins belong to this group. The moderately resistant penicillins have a K_m 30–80 times larger (about $2–3 \times 10^{-3}$ *M*) than that of the nonresistant penicillins, although the rates of reaction are about 20–50% lower and the MIC values for the two groups are fairly close. α-Phenoxyisobutylpenicillin and 2-(*N*-ethyl-*N*-phenylcarbamoyl)-phenylpenicillin belong to this group. The resistant penicillins are characterized by very low $V_{max.}$ and very high (about $2–5.4 \times 10^{-2}$ *M*) K_m values and a nearly identical MIC against sensitive and resistant staphylococci. Diphenicillin, methicillin, and the isoxazolylpenicillins belong to this group.

Knox and Smith (375) found that methicillin was the best inducer for staphylococcal penicillinase. Compared to the rate of hydrolysis of penicillin G, this enzyme hydrolyzed ampicillin about twice as fast; phenethicillin, penicillin V, and propicillin were hydrolyzed at about the same rate; 6-aminopenicillanic acid about 80 times slower; and methicillin about 120 times slower. The K_m obtained in cell-free staphylococcal β-lactamase is usually about 20 μ*M* for penicillin G and 50,000 μ*M* for methicillin, but only one-fifth of these values is obtained with either penicillin in the case of the cell-bound enzyme (376). DePue *et al.* (377) pointed out that the Michaelis constants of the penicillins are relatively little affected by interchange of the phenyl, benzyl, or aliphatic side chains. However, substitution of a side chain having an amino group (as in 6-aminopenicillanic acid, ampicillin, or *p*-aminobenzylpenicillin) or a nitro group caused a marked increase in K_m values or a lowering of the affinity for the enzyme. This drastic change in K_m values suggests that the electron-attracting nature of the side chain plays an important role in the formation of the enzyme–substrate complex.

Staphylococcal exopenicillinase was further investigated by Dyke (378) at pH 7.0 and 35°. The Michaelis constants for several penicillins were found to be in the order of: penicillin G (2.5 μ*M*) < cloxacillin (0.35 m*M*) < quinacillin (1.5 m*M*) < methicillin (24 m*M*). The $V_{max.}$, in comparison to that of penicillin G (1.0), was methicillin (1.54) > quinacillin (0.85) > cloxacillin (0.43). No change in K_m was observed for hydrolysis of quinacillin at a pH of 7.0, 5.9, or 4.8, but the maximum rate was about 10 times higher at pH 4.8 than at pH 7.0.

Studies on β-lactamase from Gram-negative bacteria opened another area of interesting enzymic kinetics. Jago *et al.* (333) reported that the enzyme *Ps. pyocyanea* NCYC-8023 hydrolyzes cephalosporin C faster than it does benzylpenicillin. Similarly, Sabath *et al.* (305) pointed out that the $V_{max.}$ of hydrolysis of cephalosporin C and several other cephalosporins by a crude extract of this enzyme was about 5 times as high as that of penicillin G. Methicillin, 6-aminopenicillanic acid, 7-aminocephalosporanic acid, and cloxacillin were all resistant to hydrolysis. Methicillin and cloxacillin were also powerful competitive inhibitors protecting cephalosporin C against inactivation.

Hamilton-Miller and coworkers reported broad studies (379, 380) on the β-lactamases from Gram-negative organisms. Toward the β-lactamases from several strains of *K. aerogenes*, penicillin G and ampicillin were more labile than cephaloridine and other cephalosporins, and cephaloridine was hydrolyzed at a relatively higher rate than cephaloram, cephalothin, or cephalosporin C. One strain of *A. cloacae* resembled *Klebsiella* in its activity toward cephalosporins. Strains of *E. coli* that do not contain an R-factor also had homogeneous specificity profiles; that is, all cephalo-

Table XV—Relative Rates of Hydrolysis of β-Lactam Antibiotics by Several β-Lactamases[a] (Maximum Rate, $V_{max.}$, of Hydrolysis of Penicillin G = 100)

	β-Lactamase									
	Penicillin β-Lactamase						Cephalosporin β-Lactamase			
Antibiotics	*S. aureus*	*B. cereus* β-Lactamase I	*Klebsiella* sp.	*Pr. mirabilis*	*Aerobacter cloacae*	*B. cereus* β-Lactamase II	*E. coli*	*Pr. rettgeri*	*Pr. morganii*	*Ps. aeruginosa*
Penicillin V	95–160	102–125	85–119	—	60–70	—	**50–82**	—	**38–44**	**76–79**
Phenethicillin	90–100	103	—	—	57–100	—	**<10**	—	—	**<1.0**
Propicillin	60–77	56	—	—	40–45	—	**<10**	—	—	—
Phenbenicillin	167	91	—	—	49–64	—	**<10**	—	—	—
6-APA	<0.1	40	31–34	4	14	—	**5–22**	—	—	**<1.0**
Ampicillin	120–200	100–120	182–287	9	100–120	64	**3**	—	—	**2–14**
Methicillin	**0.02–5.0**	**<0.1–3**	**<10**	**<0.1**	**<0.1**	89	**7**	—	—	**<1.0**
Oxacillin	**<1.0–7.0**	**<0.1–5**	**<10**	**<0.1**	**20–40**	89	**2–<10**	—	—	**<1.0**
Cloxacillin	**0.1–3.5**	**<0.1–3**	**<10**	**0.1**	**<10**	89	**1.0**	—	—	**<1.0**
Nafcillin	—	**17**	—	—	—	—	—	—	—	—
7-ACA	—	**<0.1**	—	—	—	—	—	—	—	<1.0
Cephalosporin C	**<0.1–1.2**	**<0.1**	**2–14**	**<2.0**	**4**	80	400–1250	6040	1010–1090	614
Cephaloram	**0.1**	**<0.1**	**10–20**	**<2.0**	**15**	—	360–560	1300	371–372	573
Cephalothin	**0.01**	**<0.1**	**4–15**	**<2.0**	**15**	89	350–515	2320	205–233	420–450
Cephaloridine	**0.2**	**<0.1–2**	**45**	**<2.0**	**64**	41	172–232	2280	300–350	700
Cephaloglycin	—	**<0.1**	—	—	—	50	—	—	—	—
References	3, 309, 312, 342, 374, 377, 378	3, 342, 357, 369, 371	3, 309, 379, 380	3, 309	342, 379, 380	3, 371	312, 342, 379, 380	379, 380	309, 379, 380	305, 309

[a] The maximum rate constant can be varied drastically in accordance with the temperature, pH, and buffer of the system used as well as the origin and preparation of the enzyme. The boldface numbers indicate the hydrolytic rates are very slow in comparison with that of penicillin G.

sporins tested were more labile than penicillin G toward these β-lactamases. *Pr. morganii* strains were found to hydrolyze cephalothin, cephaloridine, and cephaloram about 2–3 times faster than penicillin, while *Pr. rettgeri* hydrolyzed all cephalosporins about 13–60 times faster than penicillin G. The β-lactamases from the Gram-negative bacteria studied, *i.e.*, the *E. coli* and *Proteus* strain, preferentially attacked the cephalosporins, while those from *Klebsiella* sp. were found to inactivate the penicillins (380).

The maximum rates of inactivation of penicillins and cephalosporins by known β-lactamases of different origin are listed in Table XV. The wide variation in reported rates is due mainly to the great difference in origins and preparations of the enzymes and in assay techniques, as well as to the lack of uniformity of the experimental conditions. Nevertheless, the study of β-lactamase kinetics to date is far from complete. Particularly lacking is knowledge on the mechanism of β-lactamase inactivation of different penicillins and cephalosporins, the effect of pH on the affinity of a substrate for the enzyme, and the nature of the active site.

Inhibition of β-Lactamase—Methicillin and the isoxazolylpenicillins that are relatively resistant to hydrolysis by penicillinases from *B. cereus*, *S. aureus*, *B. licheniformis*, and *K. aerogenes* have been reported to be capable of inhibiting the inactivation of penicillin G by these enzymes (42, 340, 358, 359, 381, 382).

Hamilton-Miller and Smith (383) reported that neither methicillin, cephalosporin C, nor the isoxazolylpenicillins were found to exert any measurable inhibitory effect on the staphylococcal β-lactamase. However, methicillin did inhibit the hydrolysis of penicillin G, the phenoxyalkylpenicillins, phenbenicillin, and ampicillin by the penicillinase from *B. cereus* as well as that from *B. licheniformis*. Cephalosporin C had the same effect on *B. cereus* penicillinase. For a given amount of enzyme, the inhibition was proportional to the logarithm of the inhibitor concentration. The Gram-negative coliform enzymes are much more susceptible to inhibition than are those of Gram-positive origin (383). Compounds such as the isoxazolylpenicillins are very resistant to β-lactamases from *Pr. morganii* and *E. coli*. Against *K. aerogenes* β-lactamase, methicillin has a greater inhibitory effect than the isoxazolylpenicillins (383); against β-lactamase from *Ps. pyocyanea*, both methicillin and cloxacillin are powerful inhibitors (333, 384).

β-Lactamases from strains of *A. aerogenes*, *K. aerogenes*, *Pr. morganii*, *Pr. rettgeri*, *Pr. vulgaris*, and *Ps. aeruginosa* can readily inactivate all the cephalosporins (385). However, the inactivation, particularly that of cephaloridine, is inhibited in the presence of methicillin or cloxacillin. The magnitude of enzyme inhibition depends directly on the nature of the 7-acyl group. It was shown that the cephalosporin analog of methicillin is about 7 times more effective than methicillin itself in protecting cephaloridine against *A. aerogenes* (385).

The interaction of the competitive inhibitor with the enzyme (penicillin β-lactamase) leads to the formation of an inhibitor–enzyme complex. The bulky side-chain group or steric effect of these inhibitors causes repulsion, resulting in a conformational change in the enzyme, with a progressive loss of activity (309). Cephalosporins exert an inhibitory effect toward penicillin β-lactamase which may be due to the geometry

of their nucleus (7-aminocephalosporanic acid), which is considered to be unfavorable to attack by the penicillinases (3).

Reports have appeared in the literature (386–392) on the protection of penicillin G against penicillinase-catalyzed hydrolysis in the presence of certain kinds of compounds, including sodium sulfanilate, benzoic acid, imidazoles, 2-aminomethylthiazole, histamine, sodium benzoate, *p*-aminobenzoic acid, sulfanilic acid, salicylic acid, pyrogallol, quinine, chloroquine, quinacrine, proteolytic enzymes of trypsin and chymotrypsin, and some surface-active agents such as sodium tridecyl-2-sulfate and sodium diodecylbenzene sulfonate. Except at very high concentrations (about 1 m*M*), sodium or potassium chloride, nitrate, sulfate, phosphate, and acetate ions showed no measurable inhibitory effect on the activity of β-lactamases isolated from *E. coli* harboring transmissible R-factor, R_{GN238} and R_{GN14} (393). To date, no full investigations have been carried out on the magnitude and mechanism of inhibition of β-lactamases by these chemical agents and no attempt has apparently been made to compare their actions with those exerted by the resistant penicillins.

Synergistic Activity and Increasing Usefulness of Antibiotics—The combined use of antibiotics may sometimes result in a synergistic effect, as shown earlier in the case of cephalosporin C, which is a competitive inhibitor of *B. cereus* β-lactamase and protects penicillin G from being hydrolyzed by this enzyme (357). The penicillin G–cephalosporin C pair also acts synergistically against *S. aureus* (354).

Several studies have shown ampicillin to be synergistic with other antibiotics. In combination with methicillin, it had either an additive or a synergistic effect against a series of 17 methicillin-resistant staphylococcal strains (394). A mixture of ampicillin or penicillin G with methicillin or one of the isoxazolylpenicillins showed synergistic action against several Gram-negative bacteria, namely *E. coli*, *A. aerogenes*, *Proteus* sp., *Ps. aeruginosa*, and *K. pneumoniae*. For example, against *E. coli* R136, ampicillin alone was inhibitory only when the concentration reached 25 mcg./ml., and cloxacillin alone was without effect at a concentration of 10 mcg./ml.; together, however, 10 mcg./ml. of cloxacillin and only 2.5 mcg. of ampicillin were inhibitory (395). In the treatment of mice infected with *Shigella flexneri*, a combination of ampicillin and dicloxacillin showed significant synergism (396). This combination also showed significant synergism against strains of *E. coli* resistant to both of these penicillins (397).

Against *Pr. mirabilis*, the best antibiotic combination was found to be ampicillin and gentamicin; this pair was considerably more effective than gentamicin–penicillin G, ampicillin–kanamycin, or several others, all of which exhibited some synergistic action (398). Against *Pr. morganii*, ampicillin and cloxacillin were synergistic (399). *Ps. aeruginosa* was inhibited fairly well with a combination of ampicillin and oxacillin (400). In urinary tract infections where patients had a significant bacteriuria, a combination of ampicillin or penicillin G with nafcillin, methicillin, or one of the isoxazolylpenicillins produced a prolonged antibacterial activity against *Ps. aeruginosa*, *E. coli*, the *Enterobacter* group, and *Proteus* sp. (401, 402).

Other synergistic combinations include carbenicillin and gentamicin, which demonstrated a striking bactericidal effect against *Ps. aeruginosa*, whereas neither drug alone was effective in low dosage (403). Another such combination is penicillin G and dicloxacillin against *Sh. flexneri*. High levels of these antibiotics (200 and 250 mcg./ml., respectively) are required to inhibit this organism when the drugs are used alone, but in combination only 20 mcg./ml. of each demonstrated complete inhibition. For synergism against *Shigella* β-lactamase, it is essential that the inhibitor and substrate have a very different affinity for it (396).

The observed synergism through the combined use of antibiotics may be due to the following mechanisms (404): (*a*) a competitive inhibition of the inhibitor for the β-lactamases, involving either a tie-up of the active site or the rendering of a conformational change of the enzyme (366–370); (*b*) the effect of a favorable pH of the medium; and (*c*) a delayed excretion of the antibiotics; for example, probenecid has negligible antibacterial activity, but if given with a penicillin or cephalosporin, the serum levels of the antibiotic are notably increased and prolonged, thus indirectly resulting in a sustained antibacterial activity (405, 406).

Jawetz (407) objected to the combined use of antibiotics because: (*a*) adequate information is not available concerning the mechanism of synergy; (*b*) the use of a combination may encourage the emergence of drug-resistant organisms; and (*c*) adverse reactions may be lessened by using the individual drugs. Others have strongly favored synergistic combinations, arguing that the proper use of antibiotics in combination is not only scientifically sound but also often lifesaving (408). Several examples of the rational use of combinations have been reported. For instance, best results were obtained with a penicillin G–streptomycin combination in the treatment of bacteremia or subacute bacterial endocarditis (SBE) caused by either *Strep. viridans* or *Streptococcus faecalis*. The penicillin G–erythromycin pair was also very effective against strains of *S. aureus* highly resistant to both antibiotics. Cephalothin–streptomycin was shown to be effective for severe urinary tract infections and SBE due to *Strep. faecalis* (408, 409). A carbenicillin–gentamicin combination demonstrated synergistic activity against severe infections caused by *Ps. aeruginosa* (408).

To maximize the activity of the antibiotic combination, the pH of the medium should also be adjusted to the most favorable value (410). Ampicillin was shown to be about 10 times more active against *E. coli* and *Strep. faecalis* at a pH of 5.5 than at pH 8 (206). The MIC of streptomycin against *S. aureus* was about 100 times less at pH 5.2 than at pH 7.7 (411). Similarly, it was recently reported that penicillin G, ampicillin, cephalothin, cephaloridine, and novobiocin are considerably more active against strains of *Streptococcus* (*Enterococcus*) at pH 5.0 than in neutral or alkaline (pH 8.5) media (412). On the other hand, lincomycin, clindimycin, erythromycin, and gentamicin are moderately to markedly more active at pH 8.5 than in acidic (pH 5.0) media. This clearly indicates that

different molecular forms of an antibiotic (ionized, unionized, or zwitterion) may exert a drastically different antibacterial action (412, 413). Varying the pH may thus change the charge of the susceptible receptor on the bacterial cell, consequently altering its affinity for, or susceptibility to, the antimicrobial agent (413). Accordingly, for proper therapy of urinary tract infection, an adjunctive alkalinizing (410, 414) or acidizing medication may be required to maintain the urine at the optimal pH (412).

CONCLUSION

The semisynthetic β-lactam antibiotics have provided outstanding examples of how naturally occurring medicinal agents can be improved by molecular modification. Although the art of semisynthesis has overshadowed that of total chemical synthesis, the latter certainly has great potential, because it may lead to the exploration of the fundamental structure of other novel antibiotics having an even broader spectrum than the presently available penicillins and cephalosporins (3, 175). Another important goal for drug research is the development of new antibiotics that are effective against infectious diseases caused by protozoa and viruses as well as by bacteria.

The fundamental structural requirements of the β-lactam antibiotics include: (*a*) the fused β-lactam structure; (*b*) a free carboxyl group; and (*c*) one or more properly substituted amino side-chain groups (3–9). The expansion of the thiazolidine ring (as in cephalosporins) does not destroy activity as long as a free carboxyl group is retained in the same position (415). Although the role of this free carboxyl group in the overall bactericidal activity is not clear, it is known that the nucleus of penicillin and cephalosporin must contain a free carboxylate ion. The *N*-condensation side chain apparently is the key group governing the hydrophilic hydrophobic character as well as the overall spatial relationship within the two-ring system, which in turn affects not only the antibacterial activity but also the stability of the compounds.

In general, penicillins kill Gram-positive bacteria more effectively than they do Gram-negative bacteria (416–418). They destroy the dividing rather than the resting cell and induce a morphological alteration in the treated culture (415–417). The particular lability of Gram-positive organisms is due to the fact that their cell wall structure is relatively simple and devoid of lipids in comparison with that of the Gram-negative bacteria. Penicillins directly interfere with the glycopeptide cross-linking reaction and thus inhibit the synthesis of the bacterial cell wall. The relative insensitivity of *E. coli* to penicillin G and similar penicillins is probably due to failure of the penicillin to reach the sensitive site of the organism. In contrast, the greater bactericidal effect of ampicillin and other broad-spectrum analogs toward the Gram-negative bacteria is due to the greater ability of these drugs to penetrate the cell wall (416, 417). A mechanistic explanation has also been advanced, *i.e.*, that the antimicrobial activity of the energetic β-lactam ring is directly related to the inactivation or inhibition, *via* acylation, of the cell wall-synthesizing enzyme (transpeptidase) of the bacteria (418).

In studies on the kinetics and mechanisms of action of the β-lactamases, the aim is to gain first-hand information on the chemical nature of the catalysis. The search for more effective β-lactamase inhibitors will no doubt continue.

Although many of the criteria for the "ideal" or "all-purpose" penicillin and/or cephalosporin have been attained, a greater problem—that of allergenicity—remains unsolved. From immunological studies on the mechanism of allergenicity, it has been shown that the penicilloylated and cephalosporoylated protein impurity in the natural penicillins and cephalosporins may be largely responsible for the induction of hypersensitivity and anaphylactic reactions in patients treated with these drugs (419–424). Recently it was demonstrated that some dosage forms of penicillins G and V contain a small amount of highly antigenic penicilloylated protein (423) and that removal of this impurity reduces the incidence of allergic responses in sensitive patients (422, 424–426).

Besides the proteinaceous contaminants, the β-lactam antibiotics contain polymers that are formed in solution by internal reactions between the intact and degraded molecules (427, 428). The polymerization normally takes place as a chain reaction, initiated by the degradation product and influenced by pH, temperature, oxygenation, and other factors known to increase conversion to penicillenic acid (429).

When purified, either by dialysis, molecular sieving, or chromatography, penicillins have been tolerated without adverse reaction by patients whose hypersensitivity to the unpurified compounds was proven. Thus, to eliminate penicillin and cephalosporin allergenicity, the final product must be pure and free of contaminants. Obviously, the pharmaceutical formulator must remain aware of the possible degradations of the β-lactam antibiotics and must exercise due care in the design of new dosage forms to ensure that no adjuvant is added that could interact to produce allergenic by-products (430).

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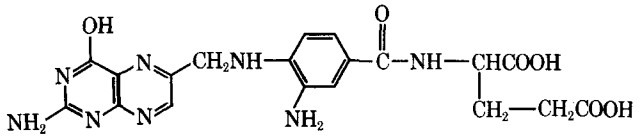
Synthesis of 3′-Aminopteroic Acid

J. N. WELLS and M. S. STRAHL

Abstract □ The synthesis of 3′-aminopteroic acid by three alternative routes is reported. The title compound was designed for possible folic acid antagonism but instead showed slight positive growth activity.

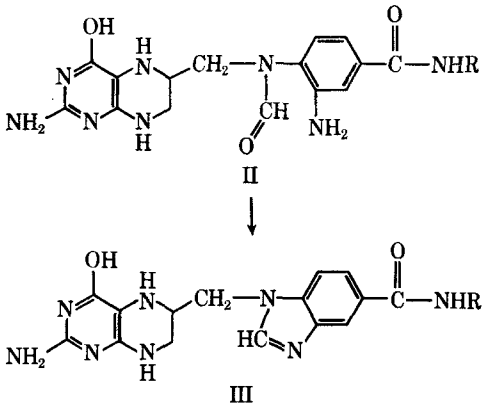
Keyphrases □ 3′-Aminopteroic acid—synthesis □ Folic acid activity—3′-aminopteroic acid effect □ Mass spectroscopy—identification, structure □ UV spectrophotometry—identification □ IR spectrophotometry—identification

The existence of ^{10}N-formyltetrahydrofolic acid as the source of the C_2 atom of the purine skeleton (1–3) suggested that 3′-aminofolic acid (I) could be a folic



I

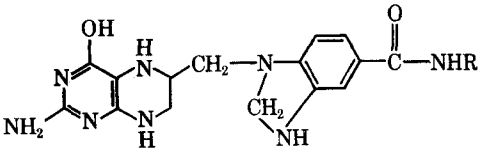
acid antagonist. 3′-Aminofolic acid could form ^{10}N-formyl-3′-aminotetrahydrofolic acid (II) through the same pathways that folic acid forms ^{10}N-formyltetrahydrofolic acid (Scheme I). If the formyl group, by



Scheme I

Schiff base formation with the 3′-amino function, could then be incorporated in the benzimidazole ring of III, the ^{10}N-carbon would be firmly bound and could not be readily transferred to an acceptor molecule. This would make III a likely competitive inhibitor of ^{10}N-formyltetrahydrofolic acid, since III would be unable to be utilized as a coenzyme and could block the active site of the transformylase enzyme (4) that mediates the C_1 transfer for purine synthesis. Similarly, III might also prevent ^{10}N-formyltetrahydrofolic acid from being transformed to $^{5}N,^{10}N$-anhydroformyltetrahydrofolic acid, the immediate source of the C_8 atom of the purine (1–3), by competing for the active site of the enzyme that effects that reaction.

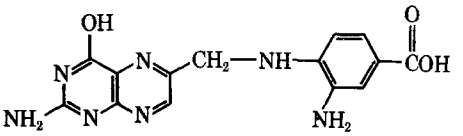
Another possibility for the production of an antimetabolite from II is that ^{10}N-hydroxymethyl-3′-aminotetrahydrofolic acid which might be formed through the serine-glycine interchange (5) may condense intramolecularly to form IV, since *N*-hydroxy-



IV

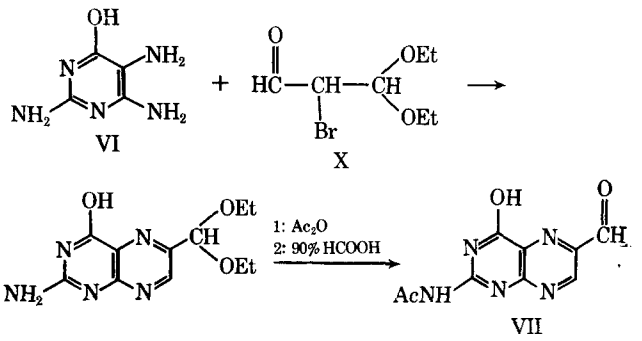
methylamines have a tendency to combine with another ligand (6). Compound IV could then be a competitive inhibitor of ^{10}N-hydroxymethyltetrahydrofolic acid by blocking the active site of cyclohydrolase (2). This would inhibit the formation of both $^{5}N,^{10}N$-methylenetetrahydrofolic acid used for thymine synthesis (7, 8) and $^{5}N,^{10}N$-anhydroformyltetrahydrofolic acid used to form the imidazole portion of the purine ring.

These speculations, plus the report that 3,4-diaminobenzoic acid has bacteriostatic activity antagonized by folic acid (9), prompted interest in the synthesis of 3′-aminopteroic acid (V).



V

2,4-Diamino-6-hydroxypyrimidine was nitrosated to give 2,4-diamino-5-nitroso-6-hydroxypyrimidine (10, 11). Reduction of this compound to 2,4,5-triamino-6-hydroxypyrimide (VI) (Scheme II) was effected in high



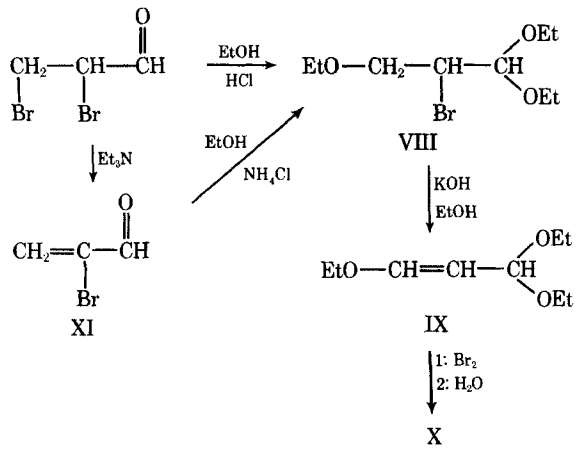
Scheme II

yield with either 44% ammonium sulfide (10) or by catalytic hydrogenation in aqueous sodium hydroxide (12). It was found in this study that appreciable 4-amino-2,6-dihydroxy-5-nitrosopyrimidine was formed by alkaline hydrolysis during the latter procedure. The slight modification of using 1 equivalent of base for solubilization minimized this hydrolysis.

The original synthesis of 2-bromo-3-ethoxypropanal diethyl acetal (VIII) (13), needed in the approach to the pteridine ring system, was described in a vague and ambiguous manner which is subject to many interpretations, all leading to low yields. Merely removing ethyl halide and water from the reaction mixture of 2,3-dibromopropanal in ethanol nearly doubles the published yield.

In the present study, the physical properties of 2-bromo-3-ethoxypropanal diethyl acetal (VIII) (b.p. 113–118°/14 mm., n_D^{25} 1.4440) did not correspond to the reported values [b.p. 103–104°/14 mm. (13), n_D^{25} 1.4593 (14)]. The confusion arising from this discrepancy and the importance of the intermediate prompted an independent synthesis of 2-bromo-3-ethoxypropanal diethyl acetal.

The reaction of 2,3-dibromopropanal (15) with triethylamine gave 2-bromopropenal (XI) (Scheme III).



Scheme III

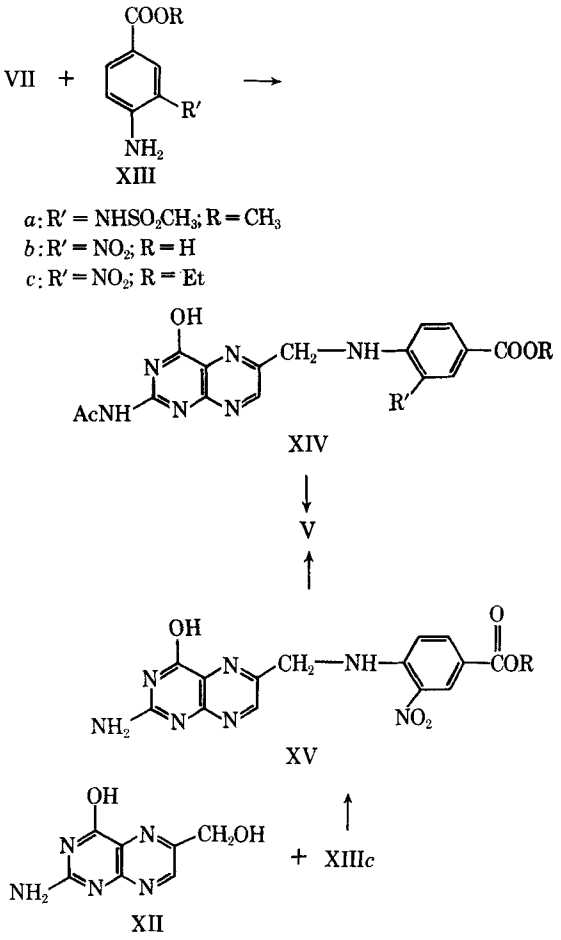
Reaction of XI with ethanol and ammonium chloride (16) gave 2-bromo-3-ethoxypropanal diethyl acetal (VIII), with physical properties and spectral characteristics identical to that obtained by the method of Fischer and Giebe (13).

Thermal degradation of X occurred so readily that even distillation at 45°/0.4 mm. [lit. (17) b.p. 65–70°/4 mm.] afforded pure material in yields not greater than 25%. In this study the yield of X was increased to an apparent 95%, based on pteridine formation, by using the material directly after removal of the solvents. Satisfactory results also were obtained by using the dibromo precursor of X directly.

2-Amino-4-hydroxy-6-diethoxymethylpteridine was prepared according to published methods (17) from X and VI. Acetylation followed by treatment with 90% formic acid gave 2-acetamido-4-hydroxy-6-formylpteridine (VII).

Three syntheses of 3′-aminopteroic acid were developed. Two of these involved the reaction of alternative precursors of 3,4-diaminobenzoic acid with VII. The third was the reaction of ethyl 4-amino-3-nitrobenzoate (18) with 2-amino-4-hydroxy-6-hydroxymethylpteridine (XII) (19).

Methyl 4-amino-3-*N*-mesylaminobenzoate (XIII*a*) (Scheme IV) was first prepared by catalytic hydrogena-



Scheme IV

tion of methyl 3-*N*-mesylamino-4-nitrobenzoate in methanol. Isolation of XIII*a* resulted in some decomposition since the compound is sensitive to oxygen. To minimize this decomposition, the reduction was thereafter run in ethylene glycol monoethyl ether and this solution was used directly in the reaction with VII.

Compounds VII and XIII*a* were heated to reflux in ethylene glycol monoethyl ether containing *p*-thiocresol. The product (XIV*a*) was demesylated with HBr in acetic acid (20) and then hydrolyzed in aqueous sodium hydroxide. The mass spectrum of the resulting 3′-aminopteroic acid (V), obtained by placing the sample directly in the electron beam, exhibited, among others, ions at *m/e* 309 (M-18) and *m/e* 294 (M-33).

4-Amino-3-nitrobenzoic acid (XIII*b*) and VII were refluxed in ethylene glycol monoethyl ether containing *p*-thiocresol to give XIV*b*. This was then hydrolyzed in base and catalytically reduced to form 3′-aminopteroic acid (V). The mass spectrum of this product was run at 400°, which caused some decomposition and decarboxylation. Fragments at *m/e* 324 (M-3), 280 (M-3-CO_2), 162 (2-amino-4-hydroxypteridine ion), and 44 (CO_2) were apparent and, except for CO_2, were of low

abundance. The mass spectra of known pteridines run at 300–350° showed that loss of H_2 is a facile process.

Ethyl 4-amino-3-nitrobenzoate (XIII*c*) and XII were refluxed in anhydrous formic acid (21). The material isolated from this reaction was hydrolyzed in base. TLC utilizing silica gel H adsorbant and *sec*-butyl alcohol–acetic acid–water (8:2:5) as the solvent system (19) showed the presence of precursor plus a new pteridine (XV) under long wavelength UV. No new pteridine could be detected, and only XII was recovered when XIII*c* was omitted from this reaction.

Catalytic hydrogenation of the alkaline solution of XV gave a compound identical to the 3′-aminopteroic acid prepared by the other methods described. The mass spectrum of this compound, obtained by placing the sample directly in the electron beam, exhibited ions at *m/e* 309 and 294. The conventional mass spectrum run at 400° exhibited a spectrum identical to the one already described for these conditions.

Compound V did not burn completely during its elemental analysis and, therefore, gave somewhat low carbon and hydrogen values with much nitrogenous residue, a characteristic of pteridines (22, 23).

3′-Aminopteroic acid (V) was tested for growth inhibition against *Lactobacillus casei* (ATCC 7469) in yeast extract veal infusion 0.1% glucose broth. Aliquots of a 64 mcg./ml. solution of V were added to the test cultures, and the medium was incubated at 35° for 20 hr. Comparatively massive amounts of V resulted in only slight positive growth activity.

EXPERIMENTAL[1]

2-Bromopropenal—To 56 g. (1.0 mole) of acrolein in 200 ml. of anhydrous ether at 0–5° was added, with stirring, 160 g. (1.0 mole) of bromine. Triethylamine (101 g.) was added dropwise to the well-stirred mixture while the temperature was maintained at 0°. The solution was filtered and the solid was washed with anhydrous ether. The washings were combined with the filtrate, and the solvent was removed *in vacuo* at room temperature. The residue was distilled to give 61 g. of compound, b.p. 80–84° (123 mm.), $n_D^{26.5}$ 1.5029. Thermal degradation limited the yield to 45%, although the theoretical amount of triethylamine hydrobromide was recovered. IR (neat) 3.20 μ(=CH_2), 3.51 μ(C$\underline{H}$O), 5.85 μ(C=O), 6.21 μ(C=C); NMR (CCl_4) δ 6.84 (d of d, 2, CH_2), 9.28 (s, 1, C$\underline{H}$O). This compound was used without further purification.

2-Bromo-1,1,3-triethoxypropane—*Method A*—To 61 g. (0.452 mole) of 2-bromopropenal were added 74 ml. of absolute ethanol and 3.0 g. of NH_4Cl. The reaction mixture was treated as described by Alberti and Sollazzo (16) to yield 36 g. (31%) of product, b.p. 113–118° (14 mm.), $n_D^{28.5}$ 1.4439.

Method B—A modified procedure of Fischer and Giebe (13) was followed. To a stirred solution of 366 g. (6.55 moles) acrolein in 500 ml. anhydrous ether was added dropwise 1062 g. (6.64 moles) of bromine. The temperature was maintained at 0°. The ether was removed *in vacuo* to leave 1143 g. of residue. This was added to 2800 ml. of anhydrous ethanol containing 16 g. of dry HCl. The reaction mixture was refluxed for several hours; then the liquid distilling between 45–50° was removed until no more ethyl halide (b.p. 37°) was evolved. Benzene (1000 ml.) was added to the reaction mixture, and the tertiary azeotrope of water, benzene, and ethanol was collected in a Dean-Stark trap. The solution was cooled to room temperature, and solid sodium bicarbonate was added with stirring until the solution was alkaline. The solution was filtered, and the solvents were removed *in vacuo*. The residue was distilled to yield forerun plus the desired compound, b.p. 113–118° (14 mm.), n_D^{21} 1.4440. The forerun was recycled through the same procedure to yield a combined weight of 1290 g. (76%) of product. The IR, NMR, and mass spectra of the products formed by Methods A and B were identical: NMR (CCl_4) δ 1.32 (m, 9, CH_3), 3.70 (m, 9, CH_2, αH, βH), 4.52 (C_1H).

1,3,3-Triethoxypropene-1—A modified procedure of Price and Moos (24) was followed. A stirred solution of 111 g. (0.437 mole) of 2-bromo-1,1,3-triethoxypropane and 70 g. of potassium hydroxide in 226 ml. of absolute ethanol was refluxed under nitrogen for 2 hr. Most of the ethanol was removed *in vacuo* at a maximum temperature of 45°. Then 100 ml. of water was added to dissolve the solids, and the top layer was collected. The aqueous layer was extracted with ether, and the organic layers were combined. The ether was removed *in vacuo*, and the residue was distilled to yield 65.9 g. (86%) of product distilling at 78–84° (9 mm.), n_D^{25} 1.4225 [lit. (24) b.p. 94–96° (20 mm.), n_D^{25} 1.4220]. IR showed absorbance at 6.01 μ(C=C), and the NMR was consistent with the structure (IX).

2-Bromo-3,3-diethoxypropanal—A modification of the procedure of Sletzinger *et al.* (17) was followed. To 1,3,3-triethoxypropene (30.6 g., 0.174 mole) dissolved in 100 ml. of dry ether was added 28 g. (0.174 mole) of bromine dropwise, with stirring at 0°. The ether was removed *in vacuo* at room temperature, and the residue was added dropwise to a stirred solution of 100 ml. water and 25 ml. acetone at 0°. The reaction mixture was stirred for 1 day at 0° and then extracted with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo* at room temperature. The residue was distilled to yield 8 g. (23%) of product, b.p. 46–47° (0.4 mm.), $n_D^{23.5}$ 1.4530. IR (neat) 5.77 μ(CHO); NMR (CCl_4) δ 1.28 (t, 6, CH_3), 3.82 (q, 4,CH_2), 4.25 (d of d, 1, αH), 4.96 (d, 1, βH), 9.68 (d, 1, C$\underline{H}$O).

Methyl 3-*N*-Mesylaminobenzoate—Methyl 3-nitrobenzoate (25) (46.8 g., 0.256 mole) was dissolved in 300 ml. of methanol and reduced in a Parr hydrogenator at an initial hydrogen pressure of 50 p.s.i., using 5 g. of Raney nickel as catalyst. After reduction was complete, the catalyst was removed by filtration and the methanol removed *in vacuo* at a temperature of 50°. The residue (methyl 3-aminobenzoate) was added to a solution of 27.1 g. triethylamine in 300 ml. of dry ether. To this was added, with stirring, a solution of 30.7 g. (0.268 mole) mesyl chloride in 50 ml. dry ether. The temperature was maintained at 0–5°. After the addition of mesyl chloride, the solution was stirred overnight at room temperature and then filtered; the solid was washed with dry ether. The white solid was stirred with 600 ml. of hot water and the mixture was filtered. The remaining solid was dried overnight at 60° (10 mm.) and then added to 500 ml. of refluxing methanol. The hot solution was filtered, and the remaining solid was washed with hot methanol. The combined methanol filtrates were concentrated to 250 ml. and chilled at 0° overnight. The precipitate was collected and recrystallized from methanol to yield 45.84 g. (78%) of product, m.p. 123°; IR (KBr) 5.92 μ(C=O), 7.55–7.90 μ and 8.75 μ(—SO_2—); mass spectrum *m/e* 229 (M), 198 (M-MeO), 150 (M-Ms), 79 (Ms). The M^+2 peak (about 4% of M) indicated one sulfur atom present in the molecule (26).

Anal.—Calcd. for $C_9H_{11}NO_4S$: C, 47.15; H, 4.84. Found: C, 46.64; H, 4.95.

Methyl 3-*N*-Mesylamino-4-nitrobenzoate—To a solution of 11.95 g. (52.2 mmoles) of methyl 3-*N*-mesylaminobenzoate in 100 ml. of acetic anhydride was added dropwise, with stirring, 8.0 ml. of 70% nitric acid. The reaction mixture was heated for 20 min. on a steam bath and then stirred overnight at room temperature. The solution was filtered, and the solid was recrystallized from methanol to yield 1.01 g. (7.0%) of yellow crystals, m.p. 168–169°. For analysis, the compound was sublimed at 130° (0.6 mm.), m.p. 168.5–169°; IR (KBr) 5.80 μ(C=O), 6.54 μ(—NO_2), 7.75–7.80 μ and 8.75 μ(—SO_2—); mass spectrum *m/e* 274 (M), 243 (M-MeO), 195 (M-Ms). The compound was hydrolyzed in 15% HCl (27) to yield 3-amino-4-nitrobenzoic acid (28), thus confirming the position of the nitro group.

[1] All melting and boiling points are reported uncorrected. Melting points were determined using a Büchi capillary melting-point apparatus. Manometers were calibrated with several compounds of confirmed boiling points to ensure correct pressure readings. A Perkin-Elmer model 21 IR spectrophotometer was used to obtain IR spectra. UV spectra were obtained by using a Bausch and Lomb model 505 recording spectrophotometer. NMR spectra were obtained using a Varian A-60-A spectrometer at sweep widths of 500 or 1000 Hz., with tetramethylsilane as internal standard. Solvents are specified. Mass spectra were obtained using a Hitachi RMU-6A mass spectrometer operating at 70 ev. unless otherwise indicated. Refractive indexes were obtained with a Bausch and Lomb Abbe refractometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN 37912, and Midwest Microlab, Inc., Indianapolis, IN 46226.

Anal.—Calcd. for $C_9H_{10}NO_6S$: C, 39.47; H, 3.68. Found: C, 39.24; H, 3.87.

Methyl 3-*N*-Mesylamino-4-aminobenzoate—Methyl 3-*N*-mesylamino-4-nitrobenzoate (500 mg., 1.89 mmoles) was dissolved in 50 ml. of methanol and catalytically hydrogenated at an initial pressure of 50 p.s.i. using 0.5 g. of Raney nickel. After the requisite amount of hydrogen had been taken up, the solution was filtered; the filtrate was evaporated *in vacuo* at room temperature to yield a white solid which darkened upon exposure to air. Mass spectrum *m/e* 244 (M), 213 (M-MeO), 165 (M-Ms). To minimize contact with atmospheric oxygen, this reduction was thereafter run in ethylene glycol monoethyl ether and the solution was used in the next step.

3′-Aminopteroic Acid—*Method A*—2-Acetamido-4-hydroxy-6-formylpteridine (17) (0.50 g., 2.14 mmoles), 3-nitro-4-aminobenzoic acid (0.50 g., 2.7 mmoles), and 1.87 g. of *p*-thiocresol were refluxed for 6 hr. in ethylene glycol in a nitrogen atmosphere. The product was isolated in the manner described by Sletzinger *et al.* (17) and then hydrolyzed in 100 ml. of 0.1 *N* NaOH (98°) for 1 hr. under nitrogen. This solution was catalytically hydrogenated for 2 days at a pressure of 60 p.s.i. using 5 g. of Raney nickel. After reduction was complete, the catalyst was removed by filtration; dilute HCl was added to the filtrate to precipitate the product. The solid was collected by centrifugation, washed with water and acetone, and dried at 100°/10 mm. to yield 37.3 mg. of product; m.p. > 300°. Satisfactory combustion analysis could not be attained due to the tendency of this compound to form a noncombustible residue. Some decarboxylation and decomposition occurred when the compound was heated to over 400°, necessary to obtain the mass spectrum due to the low volatility of V. Mass spectrum, *m/e* 324 (M-3), 280 (M-3)-CO_2, 162 and 44 (CO_2).

Method B—2-Amino-4-hydroxy-6-hydroxymethylpteridine (XII) (19) (0.193 g., 1 mmole) and ethyl 4-amino-3-nitrobenzoate (18) (0.420 g., 2 mmoles) were refluxed under nitrogen in a preformed solution of 20 g. 90% formic acid and 11.3 g. acetic anhydride for 15 hr. The reaction mixture was cooled, and the products were precipitated by addition of dry ether. The solid was dissolved in 50 ml. of 0.2 *N* NaOH and refluxed under nitrogen for 1 hr. TLC showed the presence of unreacted XII plus another pteridine. The alkaline solution containing the new pteridine was catalytically reduced for 10 hr. with hydrogen at 60 p.s.i. using 2 g. of Raney nickel. The solution was filtered, and the filtrate was brought to the pH of Congo red paper with dilute HCl. The residue was collected by filtration and dried at 130°/10 mm. for 8 hr. The mass spectrum of this residue, obtained by placing a sample directly in the electron beam, exhibited ions at *m/e* 309 (M-18) and 294 among others.

Method C—Methyl 3-*N*-mesylamino-4-nitrobenzoate (0.300 g., 1.23 mmoles) in 15 ml. of ethylene glycol monoethyl ether was hydrogenated overnight at a pressure of 50 p.s.i., using 5 g. of Raney nickel. The solution was filtered with suction, and 0.23 g. of 2-acetamido-4-hydroxy-6-formylpteridine (1 mmole) was added to the colorless filtrate. This reaction mixture was then refluxed for 12 hr. under nitrogen, the solution was cooled, and 3.5 g. of *p*-thiocresol was added. The solution was again refluxed for 4 hr. in a nitrogen atmosphere. The product was isolated after the method of Sletzinger *et al.* (17). This residue was allowed to stand in a solution of 0.5 g. of phenol in 3.0 ml. of 38% HBr in acetic acid for 2 days with intermittent shaking. The solution was filtered through glass wool into 50 ml. of water. Sodium hydroxide was added to make the solution just alkaline, and then 1.2 g. of sodium hydroxide was added and the solution was heated under nitrogen for 3 hr. on a steam bath. The solution was cooled and filtered, and the filtrate was acidified to the pH of Congo red paper. The precipitate was collected by filtration, washed with copious amounts of water, and then washed with acetone and ether. The solid was dried at 120°/10 mm. for 24 hr. to yield 22.4 mg. of compound whose mass spectrum exhibited ions at *m/e* 309 and 294 when a solid sample was placed directly in the electron beam. Again large amounts of uncombustible residue resulted upon combustion in an attempt to obtain an elemental analysis.

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Quantitative Interpretation of *In Vivo* Buccal Absorption of *n*-Alkanoic Acids by the Physical Model Approach

NORMAN F. H. HO and WILLIAM I. HIGUCHI

Abstract □ The human studies of Beckett and Moffat on the buccal absorption of *n*-alkanoic acids were quantitatively and mechanistically interpreted by the theoretical physical model approach. It is believed that a determination of the incremental partition coefficient for the methylene group in the relevant biophase has been achieved. Exceptionally good agreement of the absorption rate–buffer pH profiles between the experimental results and theory was found. The greater rates of absorption of the higher molecular weight acids in the homologous series at constant buffer pH of the drug solution are attributed entirely to the higher partition coefficients for these acids because their pKa values are essentially identical. However, the rightward shifts of the profiles of the homologous series relative to the dissociation curve are due not only to the increasing lipid solubility but also to the presence of an aqueous diffusion layer on the mucosal side of a biphasic aqueous–lipid barrier. A self-consistent, biophysically meaningful factor of 2.33 for the buccal lipoidal membrane–aqueous incremental partition constant for a methylene group was found. Significantly, this evaluative study probably represents the first example of the rigorous application of the physical model approach to the quantitative and mechanistic interpretation of the *in vivo* absorption of drugs.

Keyphrases □ Buccal absorption, *n*-alkanoic acids—quantitative interpretation □ *n*-Alkanoic acids, absorption—theoretical consideration □ Absorption rates, buccal—*n*-alkanoic acids

Recently, models based upon simultaneous chemical equilibria and diffusional mass transfer of acidic, basic, neutral, and amphoteric drugs through homogeneous and nonhomogeneous multicompartment barriers were studied and reported in detail (1, 2). This physical model approach to the understanding of drug transport was applied to various *in situ* drug absorption situations involving the intestinal, gastric, and rectal tracts with generally satisfactory results. It was also shown that the pH-partition theory is a limiting case of the more general approach presented.

In recent years, Beckett and his coinvestigators utilized the buccal absorption of drugs as an *in vivo* model for the study of drug transfer across physiological membranes (3–8). Employing a wide variety of organic drugs from acids to bases, they showed that the passive transfer of nonionic species across the lipid membrane of the buccal cavity was the primary transport mechanism.

In one particular paper, Beckett and Moffat (5) reported the influence of alkyl substitution on three groups of acids on buccal absorption, *i.e.*, those with the same pKa value and different solubilities, those with different pKa values and the same lipid solubility, and those with different pKa values and different lipid solubilities. The comparative absorption–pH profiles of the acids reflected the differences in their pKa values and/or their lipid–water partition characteristics. Indirectly, they attempted to correlate the buccal absorption data with partition coefficients of the acids in *n*-heptane–aqueous systems and with pharmacokinetic models (7, 8). Interestingly, the present authors noted that their physical models (1, 2) are explicitly related to the buccal absorption situation. Thus, the purpose of this paper is to examine quantitatively the absorption of the *n*-alkanoic acids with the physical model approach.

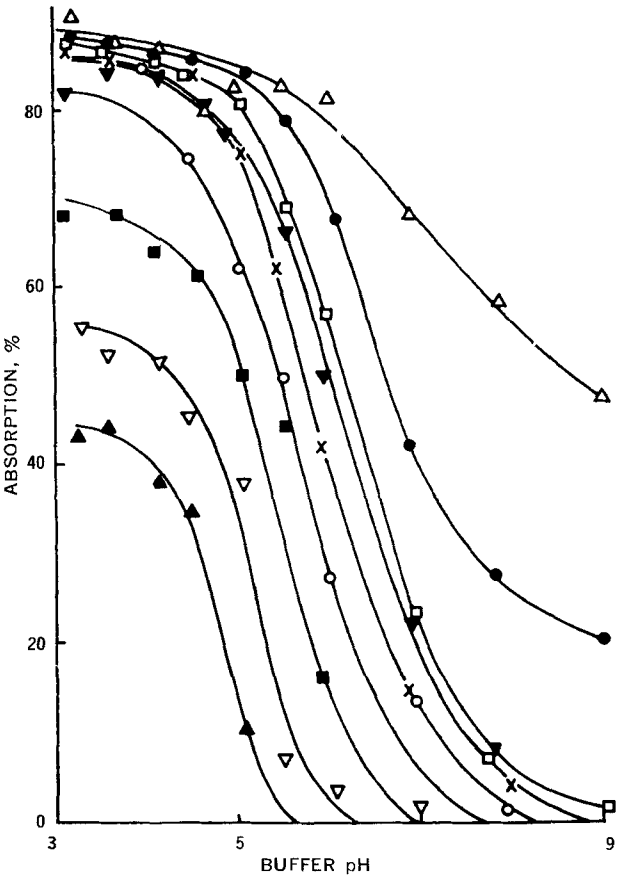

Figure 1—*Buccal absorption of* n-*alkanoic acid in man [redrawn from Beckett and Moffat (5)]. Key:* ▲, *butyric;* ▽, *valeric;* ■, *hexanoic;* ○, *heptanoic;* ×, *octanoic;* ▼, *nonanoic;* □, *decanoic;* ●, *undecanoic; and* △, *dodecanoic.*

THEORETICAL CONSIDERATIONS

The buccal absorption–pH curves of *n*-alkanoic acids ranging from butyric to dodecanoic acids (5) are shown in Fig. 1. The percent absorbed at any buffer pH is the fraction of drug absorbed after 5 min. of contact time in the buccal cavity. Beckett and Moffat (5) concluded that the measured absorption is a true passive transfer into the lipid membrane of the mouth. Since the pKa values for the acids are the same (pKa ~4.82–4.85 at 25°), it was also concluded that the differences in the relative absorption rates are related to the differences in the lipid–aqueous partition coefficients of the available nonionized species at any one buffer pH. Beckett and Moffat (5)

Table I—Buccal Absorption Data Treated in Accordance with the Physical Model[a]

n-Alkanoic Acid	Buffer pH	$f_{abs.}$ in 5 min.	Experimental K_u, sec.$^{-1}$ × 10^{-3}	$f(T)_{expt.}$	$T_{expt.}$
C_4	3	0.44	1.933	0.279	2.547
	4	0.41	1.759	0.254	2.563
	4.5	0.325	1.31	0.189	2.935
	5	0.15	0.542	0.078	4.789
	5.7	0.0	0.0	0.0	—
				Average	3.208
C_5	3	0.55	2.662	0.384	1.581
	4	0.52	2.447	0.353	1.599
	4.5	0.47	2.116	0.305	1.559
	5	0.35	1.436	0.207	1.556
	6	0.04	0.136	0.020	3.202
	6.4	0.0	0.0	0.0	—
				Average	1.9
C_6	3	0.70	4.013	0.579	0.7166
	4	0.67	3.696	0.533	0.7644
	4.5	0.625	3.269	0.472	0.7652
	5	0.525	2.481	0.358	0.7284
	6	0.16	0.581	0.084	0.7000
	7	0.0	0.0	0.0	—
				Average	0.735
C_7	3	0.82	5.720	0.825	0.2091
	4	0.79	5.202	0.751	0.2892
	4.5	0.74	4.490	0.648	0.3715
	5	0.665	3.645	0.526	0.3660
	6	0.30	1.189	0.172	0.3185
	7	0.05	0.171	0.025	0.2682
	7.8	0.0	0.0	0.0	—
				Average	0.3109
C_8	3	0.85	6.324	0.912	0.0946
	4	0.84	6.109	0.881	0.1178
	4.5	0.81	5.536	0.799	0.1723
	5	0.765	4.827	0.696	0.1774
	5.5	0.635	3.360	0.485	0.1891
	6	0.425	1.845	0.266	0.1765
	6.5	0.245	0.937	0.135	0.1354
	7	0.12	0.426	0.062	0.1037
	8.3	0.0	0.0	0.0	—
				Average	0.1459

[a] K_u, $f(T)_{expt.}$, and $T_{expt.}$ were calculated using Eqs. 3, 10, and 12, respectively.

stated that the absorption of the C_{11} and C_{12} acids are complicated by their increased lipid solubility of the ionized forms and surface-active properties.

One principal difficulty in relating the absorption data (Fig. 1) to a physical model is the lack of certain other kinetic information on which the model is based. However, as will be seen, reasonable assumptions can be made from the physical-chemical standpoint.

Initially the assumption is made that there are three homogeneous compartments, of which the first and third compartments are aqueous and separated by a lipid one[1]. The first compartment (mucosal side) consists of the bulk aqueous drug solution phase and a diffusion layer of thickness L_1. The second compartment is a lipid phase of thickness L_2, and the third compartment (serosal side) consists of the aqueous diffusion layer of thickness L_3 at pH 7.4. After the third compartment, there is assumed to be a perfect sink. The pH of each of the aqueous phases is assumed to be constant everywhere. This would be reasonable when the buffer capacity is high and no significant net transport of acids or bases is involved. Only nonionized drug species are assumed to transfer across the lipid membrane.

If the steady-state rate of buccal absorption may be approximated by a first-order process, then, as before (1, 2),

$$\ln \frac{Y_{w,1}}{Y_{w,1}(0)} = -K_u t \qquad \text{(Eq. 1)}$$

where $Y_{w,1}$ is the total aqueous concentration of alkanoic acid species on the mucosal side at any time t, and K_u is the rate constant.

[1] This corresponds to Model II-a in *Reference 2.*

In terms of the data given in Fig. 1, the fraction of drug absorbed[2] is

$$f_{abs.} = \frac{Y_{w,1}(0) - Y_{w,1}(t)}{Y_{w,1}(0)} \qquad \text{(Eq. 2)}$$

Consequently, with Eq. 1, the experimental rate constant at any buffer pH can be calculated by

$$K_u = -\frac{1}{t} \ln (1 - f_{abs.}) \qquad \text{(Eq. 3)}$$

From the theory (1, 2), the expression for the rate constant takes the general form

$$K_u = B_1 f(T) \qquad \text{(Eq. 4)}$$

where, in this three-compartment model,

$$K_u = B_1 \cdot \frac{1}{1 + (B_2/KX_1) + (B_3X_3/X_1)} \qquad \text{(Eq. 5)}$$

with

$$B_1 = \frac{AD_{w,1}}{VL_1} \qquad B_2 = \frac{L_2D_{w,1}}{L_1D_0R_2} \qquad B_3 = \frac{L_3D_{w,1}}{L_1D_{w,3}R_3} \qquad \text{(Eq. 6)}$$

Here, the $f(T)$ is a dimensionless constant with the limits $0 < f(T) \leq 1$; A and V are the geometrical surface area and volume of the drug solution, respectively; $D_{w,1}$ and $D_{w,3}$ are the diffusion coefficients in the aqueous diffusion layers in the first and third compartments, respectively; and D_0 is the lipid diffusion coefficient. The D_w of all ionized and nonionized species in a compartment are assumed equal. The lipid–aqueous partition coefficient[3] is K. R_2 and R_3 are the ratios of the true interfacial area to the geometrical area at the first and second compartments and at the second and third compartments, respectively.

The pH dependency on the absorption rate is given by the parameters X_1 and X_3, the fractions of nonionized species in the first and third aqueous compartments, which in the general case of alkanoic acids is

$$X = \frac{(H^+)}{(H^+) + K_a} \qquad \text{(Eq. 7)}$$

where (H^+) is the hydrogen-ion concentration and K_a is the dissociation constant.

The argument can now be presented that, particularly for the lower molecular weight alkanoic acids, B_3X_3/X_1 might easily be negligible compared to $1 + (B_2/KX_1)$ when the pH in the mucosal compartment is less than 7. First, L_3 is smaller than L_1, since L_1 can be easily the order of 50 μ and L_3 is probably the order of cell dimensions. Secondly, the two diffusion coefficients, $D_{w,1}$ and $D_{w,3}$, should be of the same order of magnitude. When the mucosal pH is less than around 6.0 or, at higher pH, if the absorption rate is small compared to the maximum rates (see Fig. 1), it follows that B_3X_3/X_1 should be negligible. This condition should apply to most of the data from butyric to octanoic acids. Thus, for the C_4 to C_8 acids, Eq. 5 for the absorption rate constant of acidic drugs reduces to a more simple expression[4] of an aqueous–lipid phase model:

$$K_u = B_1 \cdot \frac{1}{\left[1 + \frac{K_a}{(H^+)_1}\right]T + 1} \qquad (pH_1 \leq 7) \qquad \text{(Eq. 8)}$$

where

$$T = \frac{D_{w,1}L_2}{L_1KD_0} = \frac{P_{w,1}}{P_{0,2}R_2} \qquad \text{(Eq. 9)}$$

where T is the diffusion efficiency coefficient and is the ratio of the

[2] Unfortunately, the data (5) are single-point (in time) determinations. Therefore, a nonsteady-state (partitioning of binding) component is present in these data. It is believed, however, that the steady-state approximation is reasonable, as the mouth-rinse experiments indicated relatively little membrane partitioning of the solutes and binding.

[3] In *References 1* and *2*, the notation P was used for the partition coefficient.

[4] This corresponds to Model I in *Reference 2* or the model in *Reference 1.*

aqueous diffusion layer permeability ($P_{w,1}$) and lipoidal membrane permeability ($P_{0,2}$).

The two-phase model is probably a poor approximation of the three-phase model in the case of the buccal absorption of amines. The aqueous barrier on the serosal side may generally not be negligible in the case of amines (9, 10).

In Fig. 1 the percent absorption in 5 min. at pH $\leq$ 3 reaches approximately 87.5% maximum absorption with higher acids. By means of Eq. 3, one obtains $B_{1,expt.} = 6.931 \times 10^{-3}$ sec.$^{-1}$, where $B_{1,expt.}$ is the K_u value when $f(T) = 1$. Therefore, the experimental $f(T)$ for any alkanoic acid at any pH may be calculated by the relationship

$$f(T)_{expt.} = \frac{K_{u,expt.}}{B_{1,expt.}} \qquad \text{(Eq. 10)}$$

whereupon from the theory, *i.e.*, Eqs. 4 and 8, the experimental diffusion efficiency coefficient, T, is calculable. Thus,

$$f(T)_{expt.} = \frac{1}{\left[1 + \frac{K_a}{(H^+)_1}\right] T_{expt.} + 1} \qquad \text{(Eq. 11)}$$

or

$$T_{expt.} = \frac{1 - f(T)_{expt.}}{\left[1 + \frac{K_a}{(H^+)_1}\right] f(T)_{expt.}} \qquad \text{(Eq. 12)}$$

where $T_{expt.}$ is independent of buffer pH of the drug solution but is a characteristic property of the particular acid.

Since the pKa's of the acids are essentially equal, the theory predicts that the relative rates of absorption at constant pH among the alkanoic acids in the homologous series will be a reflection of their partition coefficients *via* T through Eq. 11. Referring to Eq. 9, one observes that T is inversely proportional to the partition coefficient. It follows then that T is small when K is large, and $f(T)$ approaches unity to give a maximum rate constant for absorption, B_1. Thus, $T_{expt.}$ has great significance in the evaluation of the data given in Fig. 1.

If it is reasonable to assume that the diffusion coefficients in the lipid membrane and in the aqueous phases change little with molecular weight, one may write

$$\frac{T_i}{T_{i+1}} = \frac{P_{0,2,i+1}}{P_{0,2,i}} = \frac{K_{i+1}}{K_i} \qquad \text{(Eq. 13)}$$

(i = number of C atoms in the *n*-alkanoic acid series) which relates the change in T or the permeability of the lipid membrane to the change in the partition coefficient. When a methylene group is added to the molecule, the increase in the partition coefficient within the homologous series may be expressed by

$$K_{i+1} = nK_i \qquad \text{(Eq. 14)}$$

where n is the incremental coefficient for a methylene group. Therefore, Eq. 13 becomes

$$\frac{T_i}{T_{i+1}} = n \qquad \text{(Eq. 15)}$$

Although n is not a true constant (11), it would be expected to show relatively little change from the C_4 to the C_8 acids in most organic solvents that do not allow appreciable dimerization.

RESULTS AND DISCUSSION

The results of the treatment of the absorption data (Fig. 1) with the aqueous diffusion layer–lipid membrane physical model are summarized in Table I. The rate constant K_u and the function $f(T)_{expt.}$ decrease with increasing buffer pH of the drug solution. It is significant that, as theoretically expected, the $T_{expt.}$ values of each alkanoic acid at various pH are essentially constant, with the exception of C_4 and C_5 acids at the high pH where the fraction of drug absorbed is fairly small. Since the scatter of $T_{expt.}$ for C_6 acid is small, the average $T_{expt.}$ (=0.735) was taken as the reference value for the subsequent analysis of the data and is, henceforth, referred to as T_6.

Table II—Comparison of $f(T)_{expt.}$ with Theoretical $f(T)$ Using Average $T_{expt.}$ Values and T Values with Incremental Partition Constants for a Methylene Group in the Homologous Series of *n*-Alkanoic Acids[a]

n-Alkanoic Acid	Buffer pH	$f(T)_{expt.}$	$f(T)$ with Average $T_{expt.}$	$f(T)$ with T_6 and $n = 3.15$	$f(T)$ with T_6 and $n = 2.3$
C_4	3	0.279	0.235	0.119	0.202
	4	0.254	0.214	0.107	0.183
	4.5	0.189	0.176	0.086	0.150
	5	0.078	0.112	0.053	0.095
	5.7	0.0	—	—	—
			($T = 3.208$)	($T = 7.293$)	($T = 3.888$)
C_5	3	0.384	0.342	0.299	0.368
	4	0.353	0.315	0.274	0.340
	4.5	0.305	0.265	0.228	0.288
	5	0.207	0.176	0.149	0.194
	6	0.020	0.033	0.027	0.037
	6.4	0.0	—	—	—
			($T = 1.9$)	($T = 2.315$)	($T = 1.691$)
C_6	3	0.579	0.573	0.573	0.573
	4	0.533	0.543	0.543	0.543
	4.5	0.472	0.482	0.482	0.482
	5	0.358	0.356	0.356	0.356
	6	0.084	0.080	0.080	0.080
	7	0.0	—	—	—
			($T_6 = 0.735$)	($T_6 = 0.735$)	($T_6 = 0.735$)
C_7	3	0.825	0.760	0.809	0.755
	4	0.751	0.737	0.789	0.732
	4.5	0.648	0.688	0.746	0.682
	5	0.526	0.566	0.636	0.560
	6	0.172	0.171	0.216	0.167
	7	0.025	0.022	0.028	0.021
	7.8	0.0	—	—	—
			($T = 0.311$)	($T = 0.233$)	($T = 0.320$)
C_8	3	0.912	0.871	0.930	0.876
	4	0.881	0.857	0.922	0.863
	4.5	0.799	0.824	0.902	0.831
	5	0.696	0.736	0.846	0.745
	5.5	0.485	0.550	0.706	0.561
	6	0.266	0.305	0.464	0.315
	6.5	0.135	0.127	0.223	0.132
	7	0.062	0.045	0.084	0.057
	8.3	0.0	—	—	—
			($T = 0.146$)	($T = 0.074$)	$T = 0.139$)

[a] With the exception of $f(T)_{expt.}$, all $f(T)$ values were calculated using Eq. 11 and the T values in parentheses. With the exception of the average $T_{expt.}$ and T_6, all T values were calculated using Eq. 15.

In Table II the $f(T)_{expt.}$ values are compared to the $f(T)$ values calculated both from the average $T_{expt.}$ values and from T values calculated with Eq. 15 employing T_6 as the reference. With regard to the latter, an incremental partition constant of 3.15 for the methylene group was used as a first approximation. This value was taken from the work of Iwasa *et al.* (12) and Hansch *et al.* (13) on the partitioning of esters, alcohols, ketones, and ethers between *n*-octanol and water at 25°. With this value for n, the $f(T)$ did not correlate well with the $f(T)_{expt.}$. Even a generous 5% correction of this constant for the temperature change from 25° to the physiological 37° based upon a ΔH of oil–water transfer of 500 cal. mole^{-1} (14) for a methylene group was insufficient to improve the correlation. As shown in Table III, incremental partition constants determined from the data in Table I yielded much smaller values, with a mean of 2.33. With $n = 2.3$ and T_6 as reference, the agreement between the theoretical $f(T)$ and $f(T)_{expt.}$ was very good.

From the data in Table II, profiles of the absorption rate function $f(T)$ *versus* buffer pH of the drug solution were constructed (Fig. 2). The exceptionally good agreement between the numerical values and shapes of the theoretical curves and the experimental results probably represents the first explicit application of the physical model approach for the quantitative and mechanistic interpretation of the *in vivo* absorption of drugs. Only the absorption–pH curve for butyric acid follows the dissociation curve characteristic of

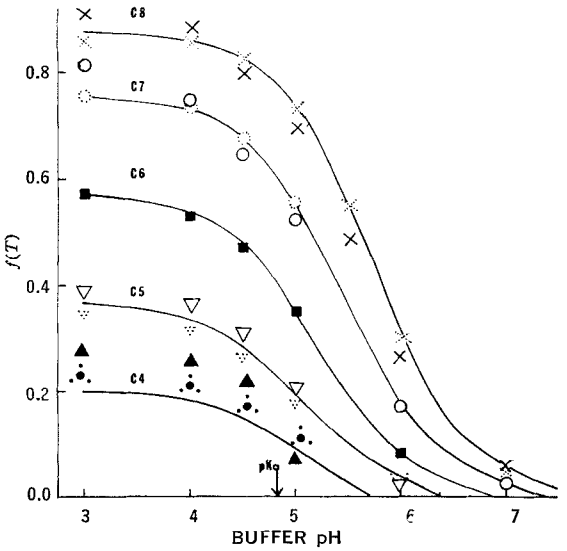

Figure 2—*Profiles of buccal absorption rate function* f(T) versus *buffer pH of the drug solution. Solid curves are* f(T) *based on* T_6 *with* n = *2.3.*

n-Alkanoic Acid	$f(T)_{expt.}$	f(T) *Based on Average* $T_{expt.}$
C_4	▲	△
C_5	▽	▽
C_6	■	□
C_7	○	○
C_8	×	×

these alkanoic acids. The increased rate in the absorption of the acids at constant buffer pH is attributable mainly to the partition coefficient characteristic of each acid. On the other hand, the shifts of the profiles of the homologous series to the right of the dissociation curve are due not only to the increasing lipid solubility but also to the presence of the aqueous diffusion layer on the mucosal side. These effects constitute a departure of real systems from the classical pH–partition theory for drug absorption.

In view of the preceding discussion, the absorption rate–pH profiles of the acids and also the extents of the pH shifts could be predicted *a priori* on the basis of the profile of one of the acids by employing Eqs. 11, 13, and 15. For example, when $f(T)_i = f(T)_{i+1}$ for i = number of carbon atoms,

$$\frac{1 + K_a/(H^+)_{1,i+1}}{1 + K_a/(H^+)_{1,i}} = \frac{T_i}{T_{i+1}} = \frac{P_{0,2,i+1}}{P_{0,2,i}} = n \qquad \text{(Eq. 16)}$$

In this case, if, for example, $K_a/(H^+)_{1,i+1}$ is large compared to unity, $(H^+)_{1,i}/(H^+)_{1,i+1} = n$. Thus, for the high pH data of the higher acids, the shifts are indeed observed to be rather constant (Fig. 2).

An interesting result of the theoretical analysis of the *in vivo* absorption data is the self-consistent factor, 2.33, for the buccal lipid membrane–aqueous incremental partition constant for the methylene group within a linear homologous series. This number is highly significant, since it is the first time to the authors' knowledge that a biophysically based and biologically meaningful factor has been deduced. Further investigations along these lines should lead to a mechanistic understanding of various structure–activity relationships based not upon simulated biological lipids but rather where the relevant membrane lipid in the dynamic real system is studied.

Table III—Buccal Lipid Membrane–Aqueous Incremental Partition Constant for the Methylene Group within the Homologous Series of *n*-Alkanoic Acids Derived from Buccal Absorption Experiments at 37°[a]

Buffer pH	Incremental Constant *n*			
	C_4–C_5	C_5–C_6	C_6–C_7	C_7–C_8
3	1.61	2.21	3.43	2.21
4	1.61	2.09	2.64	2.46
4.5	1.88	2.04	2.06	2.16
5	3.08	2.14	1.99	2.03
6	—	4.57	2.20	1.81
7	—	—	—	2.59
Average	2.05	2.61	2.47	2.21

[a] The incremental constant is calculated by $T_{expt.}$ values in Table I using Eq. 15.

Table IV—Estimates of the Buccal Membrane Permeability Coefficient of *n*-Alkanoic Acids from *In Vivo* Absorption Data

n-Alkanoic Acids	Average $T_{expt.}$	Membrane Permeability $P_{0,2}$, cm. sec.$^{-1}$, at 37°	
		Equation 9[a]	Equation 17[b]
C_4	3.208	5.20×10^{-4}	5.40×10^{-4}
C_5	1.90	8.77×10^{-4}	9.12×10^{-4}
C_6	0.735	2.27×10^{-3}	2.35×10^{-3}
C_7	0.3109	5.36×10^{-3}	5.57×10^{-3}
C_8	0.1459	11.43×10^{-3}	11.88×10^{-3}

[a] Calculations based on Eq. 9 using $D_{w,1} = 5 \times 10^{-6}$ cm.^{2} sec.$^{-1}$; $L_1 = 3 \times 10^{-3}$ cm. [b] Calculations based on Eq. 17 using $V = 25$ ml.; $A = 100$ cm.^{2}; $B_{1,expt.} = 6.931 \times 10^{-3}$ sec.$^{-1}$.

This number also implies that the lipoidal membrane of the buccal cavity is not strongly nonpolar. Consequently, *in vitro* partitioning studies with the hexane–water system or even the oleyl alcohol, octanol, or olive oil–water systems may not yield the expected information concerning the buccal situation. An incremental constant of 2.4–2.8 for the homologous series of *n*-alkyl acids, alcohols, and esters in the butanol–water system at 20°, as was shown by the studies of Collander (15, 16), appears to be appropriate. By considering a temperature correction to 37°, this *in vitro* system would probably provide good agreement with the present 2.33 factor. Collander and Barlund (17, 18) found incremental constants ranging from 1.7 to 2.5 per unshielded CH_2 group among chosen homologous pairs of an ether, alcohol, amide, and ester from permeation determinations using the plant cell *Chara ceratophylla.*

As described, the sequence of the quantitative analysis was based upon the apparent maximum absorption rate of 87.5% in 5 min., with the higher alkanoic acids in the series at pH < 3 in Fig. 1. This corresponds to $B_{1,expt.} = 6.93 \times 10^{-3}$ sec.$^{-1}$. To avoid the possible criticism that the 5-min. data for the higher acids are very close to 100% absorption and, therefore, unreliable for these calculations, an alternate method of data analysis was also used. Utilizing only the K_u *versus* buffer pH profile of hexanoic acid and calculating T_6 from the theory by

$$T_6 = \frac{K_{u,A} - K_{u,B}}{K_{u,B}[1 + K_a/(H^+)_B] - K_{u,A}[1 + K_a/(H^+)_A]}$$

$$[K_{u,A} \text{ at } (H^+)_A > K_{u,B} \text{ at } (H^+)_B] \qquad \text{(Eq. 17)}$$

the average $T_6 = 0.6564$ as compared to the average $T_6 = 0.7350$ in Table I, representing a difference of 10%. Using Eq. 8, one gets $B_{1,expt.} = 6.52 \times 10^{-3}$ sec.$^{-1}$, which is in good agreement with the $B_{1,expt.}$ determined before. Furthermore, the incremental constant found by the pH shifts of the absorption rate profiles by Eq. 16 is 2.22. Thus, the entire analysis is self-consistent.

In Table IV some estimates of the permeability coefficients for the buccal membrane are made. These were calculated with Eq. 9 using $D_{w,1} = 5 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ and $L_1 = 3 \times 10^{-3}$ cm. and also with

$$P_{0,2,i} = \frac{VB_{1,expt.}}{AT_{expt.}} \qquad \text{(Eq. 18)}$$

using $V = 25$ ml. according to Beckett and Moffat (5) and the area of the buccal cavity $A \sim 100$ cm.^{2}. It is significant that Eq. 18 permits the estimation of the $P_{0,2}$ value of an alkanoic acid from the experimental data alone and, in turn, with Eq. 13 predicts the $P_{0,2}$ values of other acids in the homologous series.

These $P_{0,2}$ values give rise to D_0 values of the order of 10^{-12} to 10^{-10} cm.^{2} sec.$^{-1}$ when $L_2 \simeq 100$ Å and reasonable values for K are employed. For example, for caproic acid, $K \simeq 50$ when the oil is oleyl alcohol or butanol; therefore, $D_0 = 4 \times 10^{-11}$ cm.^{2} sec.$^{-1}$.

Such small D_0 values are of the same order of magnitude as those values found when the rat gastrointestinal absorption data were treated (1, 2) and when data on drug transport across the hydrated *stratum corneum* were recently analyzed (19) by means of the physical model approach.

In summary, the physical model approach has been shown to be extremely successful in analyzing the buccal absorption data involving the alkanoic acids from C_4 to C_8. The mechanistic conclusions based upon the analysis appear to be very firm, because the agreement between the appropriate physical model and experiments was very good.

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Keyphrases □ Silicone elastomer—steroid release, *in vitro* □ Chlormadinone acetate release mechanism—silicone elastomer matrix □ Membranes, silicone elastomer—steroid transport rates □ Particle-size effect—steroid release from silicone matrix □ Colorimetric analysis—spectrophotometer

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By assuming that diffusion is the slow step in the overall release process, Eq. 1:

$$Q = \sqrt{DC_s(2A - C_s)t} \qquad \text{(Eq. 1)}$$

was derived by T. Higuchi (8) to describe drug release from ointment systems into a receptor sink when excess drug is suspended in that system. The Q is the amount released at time t/square centimeter of surface contact with the receptor sink; D is the diffusion constant of the drug molecule in the ointment or matrix system, A is the concentration of drug in the ointment given in amount/cubic centimeter, and C_s is the solubility of the drug in the ointment expressed in amount/cubic centimeter.

This paper is a report of the results of release experiments from a drug–matrix system analogous to the one described previously (8). The validity of Eq. 1 is tested.

Such small D_0 values are of the same order of magnitude as those values found when the rat gastrointestinal absorption data were treated (1, 2) and when data on drug transport across the hydrated *stratum corneum* were recently analyzed (19) by means of the physical model approach.

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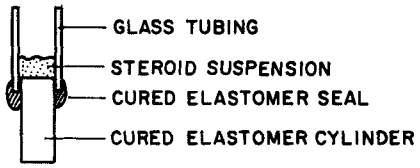

Figure 1—*Apparatus used for determination of solubility of chlormadinone acetate in silicone elastomer.*

The release dependencies on the time, concentration, and surface area parameters are examined.

THEORETICAL CONSIDERATIONS

The three rate processes necessary for drug depletion from the drug–matrix system are: (*a*) dissolution of the drug in the matrix; (*b*) diffusion of the solute to the matrix–receptor interface; and (*c*) transfer of the solute across the interface.

Equation 1 assumes that the diffusion process ultimately controls drug release. Two other possibilities exist. (*a*) Release may be controlled by the rate of drug dissolution into the matrix. In such a case, the surface area of the suspended drug particles affects the release. (*b*) The rate of solute transfer across the matrix–receptor interface may control the release. In such a situation, constant release rates are observed.

METHODS AND MATERIALS

Solubility in Silicone Elastomer—Cylinders of solid silicone elastomer[1], cured with stannous octoate[2], were prepared by drawing the fluid material into a 4-mm. (inner diameter) glass tube. The elastomer cylinders were removed from the tubes, sectioned into 2-cm. lengths, and partially inserted into a short length of empty glass tubing (Fig. 1). An aqueous suspension of tritium-labeled chlormadinone acetate[3] (6-chloro-17α-hydroxypregna-4,6-diene-3,20-dione acetate) was layered over the exposed elastomer surface in the tube. Several of these glass tubes were sealed in small glass vials and held at 37°. Thin slices of the protruding silicone elastomer cylinder were assayed periodically. The experiment continued until the assay values indicated that the concentration was constant. The thin sections of silicone elastomer (containing the tritium-labeled drug) were placed in 10 ml. of scintillation fluid. The fluid consisted of 4 g. of 2,5-diphenyloxazole[4] and 0.1 g. of 1,4-bis-[2-(4-methyl-5-phenyloxazoylyl)] benzene[4] in 1 l. of toluene. The samples were then counted on a liquid scintillation counter[5].

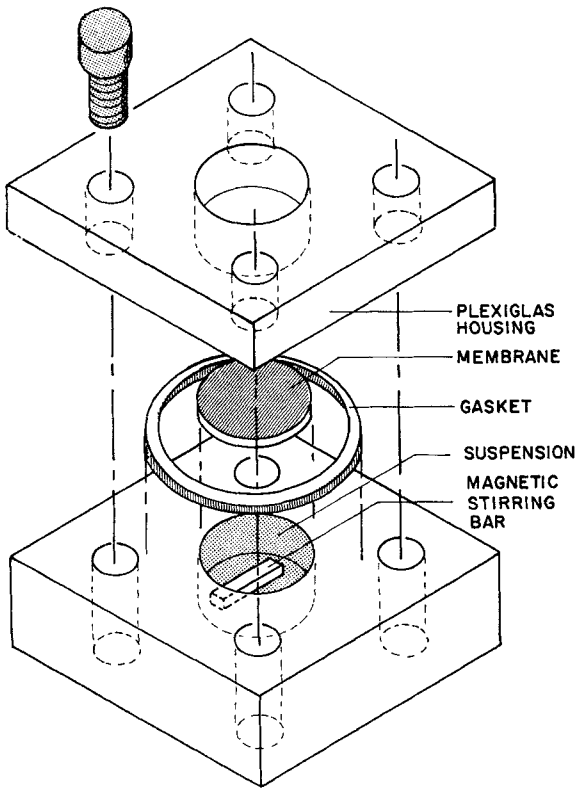

Figure 2—*Apparatus used for determination of transport rates of chlormadinone acetate across silicone elastomer membrane.*

Preparation of Matrixes—The drug–matrix release systems were prepared by levigating chlormadinone acetate into a silicone elastomer. The appropriate amount of stannous octoate was then levigated into the dispersion. The resulting fluid was then poured into tared glass petri dishes. The following systems were studied in triplicate:

1. Matrixes of 0.2, 2, 10, 20, and 40% chlormadinone acetate having the same depth and exposed surface area.
2. Matrixes of 2% chlormadinone acetate having the same depth but with 3.88, 7.15, and 59.7 cm.^{2} of exposed surface area.
3. Matrixes of 2% chlormadinone acetate with the same surface area but having depths of 0.635 and 1.91 cm.
4. Matrixes of 10% chlormadinone acetate with the same depth and exposed surface area as in System 1 but using macrocrystals. The macrocrystals were approximately 100–300 μ in length.

Release Experiments—The petri dishes were placed in beakers containing distilled water. The volumes and sampling times were such that the concentration of chlormadinone acetate never exceeded 10% of its solubility in water. The experiments were conducted in a water bath at 37°.

Six beakers were stirred simultaneously at 50 r.p.m. by a multiple stirring apparatus[6]. The 7.5 × 2.5-cm. stirring blades were lowered halfway into the receptor phase. Aliquots of the distilled water were extracted with chloroform, passed through anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved in 5 ml. of an acidified methanol solution of 1% 4-aminoantipyrine hydrochloride[7]. The solutions were placed in the dark for 20 min. to allow color development. Each sample was read at 393 nm. against a methanol blank on a UV spectrophotometer[8], and the drug concentration was calculated from a standard reading.

Membrane Transport Rates—Membranes of silicone elastomer were cast in thicknesses of 0.132, 0.226, and 0.48 cm. The membranes were placed in Plexiglas housings (Fig. 2) in contact with an aqueous suspension of tritium-labeled chlormadinone acetate. The apparatus was placed in a beaker of distilled water, and conditions as described under *Release Experiments* were maintained. The magnetic stirring bar in the suspension chamber was rotated by a submerged water-driven motor. The distilled water was sampled at appropriate times, and aliquots were assayed as described under *Solubility in Silicone Elastomer*.

RESULTS

Solubility—The solubility of chlormadinone acetate in the cured silicone elastomer was determined to be 82 mcg./ml. at 37°. The solubility of chlormadinone acetate in distilled water at 37° is approximately 1 mcg./ml. The low aqueous solubility necessitated the use of large receptor volumes to maintain sink conditions at all times.

Release from Matrixes—The release profiles of the 0.2, 2, 10, 20, and 40% matrixes are shown in Fig. 3. The release data in Fig. 3 were plotted *versus* the square root of time, as suggested by the diffusion-controlled release model discussed earlier (Fig. 4). If the release of chlormadinone acetate from the matrixes is *via* the diffusion-controlled mechanism, the plot in Fig. 4 would be linear in all of the early times (*i.e.*, $t < t$ for complete depletion).

[1] Silastic Medical Grade Elastomer No. 382, Dow Corning Corp., Midland, Mich.
[2] Catalyst M, Dow Corning Corp., Midland, Mich.

[3] The authors wish to express their appreciation to Dr. W. Hafferl and Mr. Andre Hary of Syntex Research, Palo Alto, Calif., for supplying the radioactive tracer.
[4] Arapahoe Chemicals, Division of Syntex Corp., Boulder, Colo.
[5] Model 720, Nuclear Chicago Corp., Des Plaines, Ill.
[6] Phipps and Bird, Inc., Richmond, Va.
[7] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.
[8] Beckman DU spectrophotometer, model 2407, Beckman Industries, Fullerton, Calif.

Table I—Release of Chlormadinone Acetate from 2% Silicone Elastomer Matrixes Having a Depth of 0.64 cm. (0.25 in.) into an Aqueous "Sink"

Hours	Release (Average of Three Experiments) mcg./cm.²	Variance	Cumulative Release, mcg./cm.²
8.0	46.6	(40.2–53.6)	47
14.5	52.3	(47.0–59.1)	99
30.5	65.6	(47.0–90.6)	165
38.5	45.4	(44.1–47.2)	210
54.5	60.6	(51.2–69.0)	270
70.5	62.9	(61.5–65.1)	333

Table II—Release of Chlormadinone Acetate from 2% Silicone Elastomer Matrixes Having a Depth of 1.9 cm. (0.75 in.) into an Aqueous "Sink"

Hours	Release (Average of Three Experiments) mcg./cm.²	Variance	Cumulative Release, mcg./cm.²
12	94.0	(86.5–101.0)	94
20	46.5	(41.8–50.8)	141
28	29.4	(26.3–33.9)	170
40	50.3	(44.6–57.0)	220
58	61.3	(49.3–79.1)	282
74.5	51.6	(45.2–60.5)	333

Release profiles were obtained with 2% matrixes having surface areas of 3.88, 7.15, and 59.5 cm.² exposed. As expected, the release was directly proportional to the area exposed.

Tables I and II present release data for the 2% matrixes having identical exposed surface areas but with thicknesses of 0.635 and 1.91 cm. Drug release is independent of the thickness of the matrix for the time period studied.

Of the total amount of drug present at the beginning of the experiments, approximately 2% was depleted. Attempts were made to determine concentration changes at various depths in the matrixes but they were unsuccessful. Apparently, the precision of the assay did not allow determination of minor changes in high concentrations.

The possibility that the dissolution rate of the drug in the matrix might affect the release prompted the experiment employing 10% chlormadinone acetate matrixes prepared with particles presenting vastly different surface areas. Figure 5 shows the release profiles for 10% chlormadinone acetate matrixes prepared with micro- (0.5–12 μ) and macro- (100–300 μ) particles. The particles were assumed to be spherical; by using average particle diameters, the surface area of the microparticles was estimated to be 60 times that of the macroparticles.

Membrane Transport—Drug transport across silicone elastomer membranes of various thicknesses was followed to a steady state. The lag time (1, 9, 10) was obtained from a plot of amount *versus* time. The diffusion coefficient was then calculated using Eq. 2:

$$L = \frac{l^2}{6D} \qquad \text{(Eq. 2)}$$

In this equation, L is the lag time in seconds, l is the membrane thickness in centimeters, and D is the diffusivity in square centimeters/second. The results listed in Table III represent the average of two experiments. The steady-state transport rates are roughly proportional to the inverse of the membrane thickness as predicted by Fick's first law. The numbers obtained for D are of the same magnitude as those calculated for the diffusion of radioiodide in petrolatum (6).

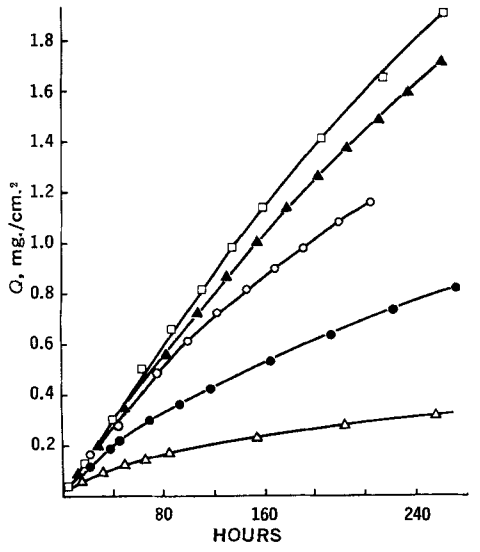

Figure 3—*Release of chlormadinone acetate (micronized) from several concentrations of silicone elastomer matrixes into an aqueous "sink." (Points indicate the average of three experiments.) Key: △, 0.2%; ●, 2%; ○, 10%; ▲, 20%, and □, 40%.*

DISCUSSION

Time Dependence—The release curves in Fig. 3 for 0.2 and 2% chlormadinone acetate appear to have the time dependency of a diffusion-controlled process. They are similar in shape to those reported by Patel *et al.* (11), for the release of radioiodide from an ointment solution, and later treated by W. Higuchi (6), who fitted the data to equations developed from diffusion theory. The release profiles for the 10, 20, and 40% matrixes, however, appear different enough that one might question whether the release mechanism is the same as that for the 0.2 and 2% matrixes. The release from the 40% matrixes was apparently linear with time for at least 140 hr. The release rates for the 10 and 20% matrixes were apparently constant also but for shorter times. Presumably, the 0.2 and 2% matrixes behave similarly, but assays would have to begin much earlier to detect the linear release period. Thus, there seems to be a maximum achievable release rate which is independent of total drug concentration.

A similar observation might have been made for the data reported by Whitworth and Becker (12) on the release of sulfacetamide and sulfathiazole from 5% ointments. Assuming that there was no receptor phase–solid drug contact, the initial part of the curves is difficult to rationalize within the diffusion-controlled model.

The experiments of Laug *et al.* (13) showed that, over a time interval of 0–24 hr., there was no increase in the amount of mercury absorbed when ointments ranging from 4–50% were applied to the skin of rats. Absorption from a 0.9% ointment was lower, however. Strakosch and Clark (14) reported similar results for the penetration of sulfonamides into the intact skin of guinea pigs. Data from the present study parallel that of the above-mentioned investigators (13, 14). Drug release over the 0–40-hr. time interval was the same

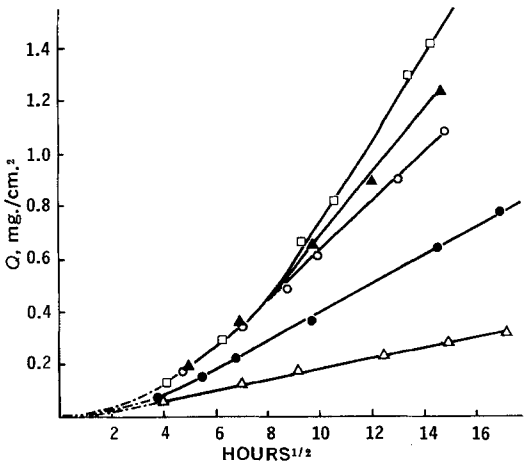

Figure 4—*Release of chlormadinone acetate (micronized) from several concentrations of silicone elastomer matrixes into an aqueous "sink" as a function of the square root of time. (Points indicate the average of three experiments.) Key: △, 0.2%; ●, 2%; ○, 10%; ▲, 20%; and □, 40%.*

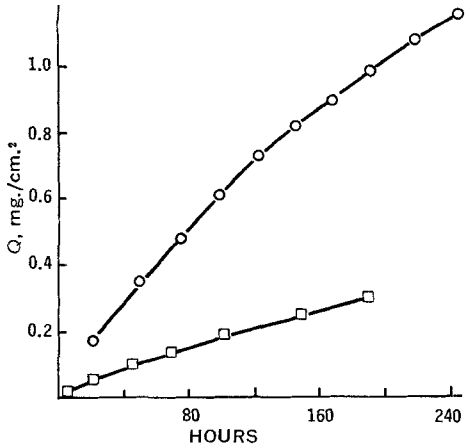

Figure 5—*Release of chlormadinone acetate from 10% silicone elastomer matrixes prepared with micronized and macrosize particles. (Points indicate the average of three experiments.) Key: □, macroparticles; and ○, microparticles.*

for 10, 20, and 40% matrixes but was lower for the 0.2 and the 2% matrixes.

Particle-Size Effect—The release profiles for 10% matrixes prepared with micro- and macrosize drug particles (Fig. 5) demonstrate the effect of surface area of undissolved drug. After 200 hr. the matrixes prepared with micronized chlormadinone acetate released approximately four times more than those prepared with macroparticles. This effect is probably due to a decreased dissolution rate because of the reduced total surface area of drug particles. The surface area effect is complicated because the matrix–drug system is not stirred and the net flux in that system is essentially unidirectional. Apparently, the surfaces actively involved in dissolution are some fraction of the total, and the depth (in the matrix) at which this process takes place increases with time.

The particle-size effect on release from ointments was demonstrated by a number of investigators, using various drugs. Barrett *et al.* (15) observed this effect in the *in vivo* release of fluocinolone acetonide from petrolatum ointments. Similar results were reported for salicylic acid (16) and for calomel (13, 17).

Equation 1 has failed to predict the release time dependence. This is graphically illustrated in Fig. 4 where definite curvature is seen in the early portions of the curves. Equation 1 further suggests that drug release should be proportional to the square root of the total concentration. The early portions of the curves in Fig. 3 display a release that is independent of concentration; these results are substantiated by the work of others (12–14). Finally, while it is true that Eq. 1 was developed for fine particles (8), this parameter is not reflected in the release equations, and changes in excess drug particle size do affect release.

The maximum release rate may be explained if the rate of desorption, from the matrix surface into the sink, controls the overall release process. Garrett and Chemburkar (4) considered such a case in their discussion of drug permeation through silicone membranes. They pointed out that if desorption controls the transport process, then the thickness of the membrane would have no effect on the steady-state transport rates. In the present experiments, the membrane thickness did affect those rates. The steady-state transport rate was roughly proportional to the inverse of the membrane thickness. The conclusion, therefore, is that the maximum release rate from the matrixes is not enforced by a limiting rate of desorption.

In membrane diffusion, steady state is defined as the condition where the rate of transport is constant because the concentrations in the first and last monolayers of the membrane are invariant with time. This definition assumes that the dimensions of the membrane do not change. If the concentration at the depot side monolayer is at a maximum for a given solute in a particular membrane, a maximum steady-state transport rate would be achieved. By applying this reasoning to the drug–matrix system, a maximum rate of release is not implausible since, regardless of the total drug concentration, C_s does not increase. The diffusing species are the solute molecules, not drug particles. What does change with concentrations (A), however, is the ability to maintain a concentration gradient across a diffusion barrier. The barrier here is the film of matrix material separating the embedded particles, nearest to the surface, from the aqueous receptor. All particles are assumed to be covered with silicone elastomer.

Table III—Steady-State Transport Rates and Calculated Diffusion Coefficients for Chlormadinone Acetate in Silicone Elastomer[a]

Membrane Thickness, cm.	Steady-State Transport Rates $\left(\frac{\text{mcg.}}{\text{cm.}^2/\text{hr.}}\right)$	L, hr.	D, cm.²/sec.
0.132	0.52	3	2.8×10^{-7}
0.226	0.34	8	3.0×10^{-7}
0.480	0.19	32	3.3×10^{-7}

[a] The transport rates are averages of two experiments.

This line of reasoning suggests that the chlormadinone acetate concentration was at a maximum at time zero in all the matrixes, all throughout the matrixes. Further, the initial rate of release was the same for all because the gradient initially established was the same. The more solid drug surface area exposed for dissolution, the longer the linear release period can be sustained. A decrease in the total concentration then shortens the linear release period but does not affect the initial release rate, provided excess solid drug is present initially.

There seems to be little doubt that the release model and the parameters affecting release are more complex than Eq. 1 indicates. The particle-size effect is inescapable. It is concluded that to describe adequately the release from these matrix systems, equations are needed that will reflect the effect of drug dissolution on the release.

SUMMARY

By employing distilled water as a receptor sink, release from several concentrations of chlormadinone acetate–silicone elastomer matrixes was studied. The 10% matrix was prepared with microparticles and macroparticles of chlormadinone acetate. Drug release from these systems was found to be a function of time, concentration, and surface area of the excess drug particles. Release from the 10% micronized matrixes was approximately four times that of the 10% macromatrixes, suggesting that the overall release process was controlled by the dissolution rate of the drug in the matrix. These results and their implications are pertinent to the analogous release system of an ointment containing excess suspended drug. The conclusion that drug dissolution rate affects release explains results in some early reports which previously could not be reconciled with the predictions of the accepted release equation. Equations are needed which will describe release and which will contain all the parameters that affect release.

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GLC Determination of Ethylene Chlorohydrin following Co-Sweep Extraction

JOSEPH WEINBERGER

Abstract □ A Co-Sweep extraction method was developed for removing and concentrating trace amounts of ethylene chlorohydrin from a variety of materials which were sterilized with ethylene oxide. Samples placed inside glass tubes were extracted in an apparatus with the aid of heat, solvents, and a continuous flow of carrier gas. The volatilized solvents were condensed in a cooling coil and swept into a collection tube. The entire extraction procedure was accomplished within 35 min. GLC was used to analyze the extract for ethylene chlorohydrin in the nanogram range. An ethylene chlorohydrin recovery study was performed in the microgram per gram range on different types of samples. The results indicate that the Co-Sweep technique is reliable, simple to operate, and potentially applicable to a wide variety of materials.

Keyphrases □ Ethylene chlorohydrin—determination □ Ethylene oxide-sterilized materials—ethylene chlorohydrin extraction, determination □ Extraction, Co-Sweep—ethylene chlorohydrin □ GLC—analysis

Ethylene oxide is now being used extensively for the sterilization of foods, pharmaceuticals, and manufactured goods. Numerous researchers have reported on various aspects of ethylene oxide sterilization. Until 1965, the only residues found from the use of this epoxide were ethylene oxide *per se*, ethylene glycol, and diethylene glycol (1–8). Wesley *et al.* (9) showed that chlorohydrins could be formed in foods fumigated with ethylene oxide or propylene oxide in the presence of inorganic chloride. Methods for analyzing ethylene oxide-sterilized foods, plastic, and rubber materials for residual ethylene chlorohydrin (2-chloroethanol) were reported by various researchers (10–17). Spitz and Weinberger (18) recently reported on a GLC method for the determination of ethylene oxide and the simultaneous determination of ethylene chlorohydrin and ethylene glycol in cellulose-type materials.

A rapid method was developed for the extraction of trace amounts of ethylene chlorohydrin from fabrics, cellulose-type materials, and a conglomerate of various materials sterilized by ethylene oxide. An aqueous extraction of ethylene chlorohydrin was achieved with a Co-Sweep extraction apparatus using heat, solvent, and a continuous flow of nitrogen gas. The entire extraction procedure was completed within 35 min. The aqueous extract obtained was quantitatively determined for ethylene chlorohydrin by GLC analysis.

EXPERIMENTAL

Apparatus—A Kontes Sweep Co-Distiller[1] (No. K-500750) was employed for the extraction and was equipped with a four-bay manifold flowmeter (K-628100-000), Teflon connection tubing (K-500500-307), and specially made glass side-arm sample tubes (13 mm. o.d. × 11 mm. i.d. × 33 cm. in length, with a 4-mm. Luer joint[2]). The open end of the tube was wrapped with two turns of Teflon ribbon tape [1.27-cm. (0.5-in.) width] and sealed with a 13-mm. o.d. silicone septum and a 0.97-cm. (0.38-in.) stainless steel hex-nut (Swagelock). The extracts were collected in 4-ml. graduated concentrator or collection tubes with a 19/22 ground-glass joint connected to glass extenders[3]. The end of the Teflon coil was held at the bottom of the collection tube by passing the tubing through a one-hole silicone septum (6 mm. o.d. × 8 mm. in length) inserted into a medicine dropper which was clamped in the bracket above the cooling bath. A cylinder of compressed nitrogen gas was equipped with a two-stage regulator and a 5A molecular sieve. A gas chromatograph[4], equipped with a dual-flame ionization detector and a stainless steel column [0.318 cm. (0.125 in.) × 2.44 m. (8 ft.)] packed with 10% polyethylene glycol[5] on 60–80-mesh acid-washed diatomaceous silica[6], was employed for all the experiments. The GLC operating temperatures were: column, 115°; injector, 135°; and detector, 200°. The GLC gases and flow rates were: helium, 30 ml./min. at 40 psig.; air, 300 ml./min. at 29 psig.; and hydrogen, 45 ml./min. at 27 psig.

Procedure—Prior to starting the extraction, the oven was adjusted to the horizontal position and preheated to 140°; the collection tubes and Teflon cooling coils were immersed in ice water baths, and the nitrogen carrier gas flow was adjusted in each of the four bays of the manifold flowmeter to approximately 75–80 ml./min. at 10 psig. A soap bubble meter was used to adjust the flow.

Four samples were extracted simultaneously in the Co-Sweep apparatus. The sample materials to be extracted were cut, weighed, and placed inside the glass sample tubes. A hex-nut and silicone rubber septum were used to seal each tube. Approximately 1 ml. of purified water was injected into the sample tubes with a hypodermic syringe and needle. The sample tubes were placed inside the preheated oven (140°) and connected to the Teflon cooling coil and then to the nitrogen carrier gas flow. The extraction time was started at this point. After 15 min. of sweeping carrier gas through the

[1] Kontes Glass Co., Vineland, N. J.
[2] Drawing No. 002172-29, Kontes Glass Co., Vineland, N. J.
[3] Kontes No. K-570050-0425 and K-570100, part 355.
[4] F & M model 5750.
[5] Carbowax 20M, Union Carbide, New York, N. Y.
[6] Chromosorb W, Johns-Manville, New York, N. Y.

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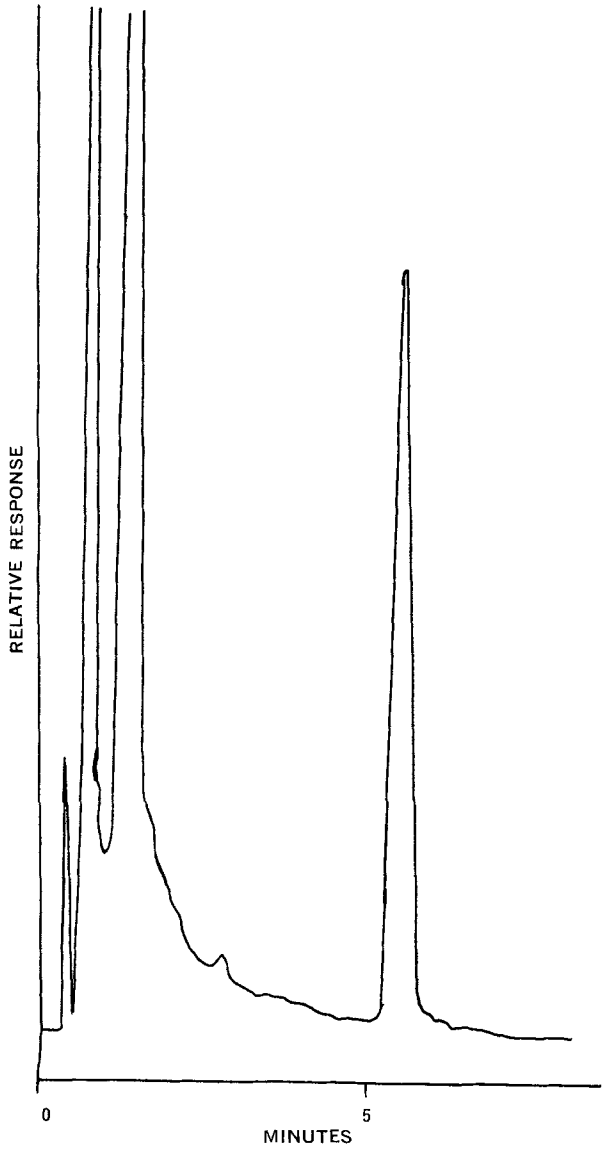

Figure 1—*Gas chromatogram of a standard aqueous solution of ethylene chlorohydrin, representing 40 ng. Retention time = 5.5 min. Injection volume = 1 μl. Attenuation range = 1×4.*

sample tubes and Teflon cooling coils, a 1-ml. injection of isooctane[7] was made into each sample tube through the septum using a 5-ml. hypodermic syringe and needle. Two additional 1-ml. injections of isooctane were performed at 5-min. intervals. After 30 min. of extraction, each cooling coil was disconnected from the sample tube and rinsed with 0.5 ml. of isooctane. The Teflon cooling coil was then connected again to the sample tube so that the carrier gas flow could force the rinse into the collection tube. The extraction was terminated after 35 min., and the collection tubes were removed from the water bath. The tubes were centrifuged at 1500 r.p.m. for 1 min. to remove any water droplets trapped in the isooctane layer. The volume of aqueous phase (bottom layer) in each tube was read to the nearest 0.01 ml. from the graduations on the tube. The Co-Sweep aqueous extracts were then quantitatively analyzed for ethylene chlorohydrin by GLC analysis on 1.0-μl. injections.

The elution time for ethylene chlorohydrin from the time of injection was approximately 5 min. Standard aqueous solutions of ethylene chlorohydrin[8] were prepared in the same concentration range as present in the samples. The peak height method was used to calculate the ethylene chlorohydrin content. Recovery studies were performed by carrying out the same extraction procedure on

[7] Matheson Coleman & Bell.
[8] Aldrich Chemical Co.

Table I—Recovery of Ethylene Chlorohydrin

Material[a]	Micrograms Added	Percent Recovery[b]
Plastic[c], cotton, and rayon	0	0
	1.5	101
	2.35	102
	4.60	93.5
Wood pulp nonwoven fabric	0	0
	11.5	94.7
	23.0	92.9
	57.8	91.7
Rayon nonwoven fabric	0	0
	48.0	91.7

[a] Weight of material ranged from 1.0 to 1.6 g. [b] Average value based on duplicate sample injections. [c] Polyvinyl chloride plasticized with dioctyl phthalate; sheet thickness = 0.1524 mm. (0.006 in.).

1-ml. aliquots of aqueous standard solutions of ethylene chlorohydrin which were pipeted onto unsterilized samples of material inside the sample tube. Ethylene oxide-sterilized counterparts of the sample materials were also subjected to the Co-Sweep technique and analyzed for ethylene chlorohydrin by GLC analysis.

RESULTS AND DISCUSSION

The recovery of ethylene chlorohydrin obtained with the Co-Sweep extraction method on various spiked materials is presented in Table I. The high recoveries obtained for ethylene chlorohydrin in the range of 1.5–57.8 mcg. seem to indicate that no appreciable adsorption took place on the spiked materials. Unspiked samples of materials were also extracted to eliminate the possibility of interfering peaks having the same retention time as ethylene chlorohydrin; for this reason, unsterilized counterparts of ethylene oxide-sterilized materials should also be extracted and analyzed for ethylene chlorohydrin as a precautionary measure. Table II shows the concentration of ethylene chlorohydrin found in ethylene oxide-sterilized counterparts of the sample materials used in Table I.

The rate of removal of ethylene chlorohydrin from an aqueous solution seems to be directly related to the nitrogen gas flow rate through the solution with time. A 13% loss of ethylene chlorohydrin occurred from a 50-mcg./ml. solution after bubbling nitrogen gas into the collection tube at a flow rate of 120 ml./min. for 1 hr. At a nitrogen flow rate of 50 ml./min., the loss of ethylene chlorohydrin was 3% in 1 hr. Therefore, to minimize the possible loss of ethylene chlorohydrin, a short extraction time of 35 min. and a low nitrogen flow rate of 75–80 ml./min. were chosen for the Co-Sweep extraction method.

Table III shows the recovery of ethylene chlorohydrin obtained from spiked rayon samples under different conditions of Co-Sweep extraction. Lower recoveries of ethylene chlorohydrin were obtained at extended extraction times and higher nitrogen flow rates (and, also, when helium was substituted for the carrier gas). Higher extraction temperatures above 140° caused additional volatiles to show up on the gas chromatogram of some samples which interfered with the analysis.

The isooctane injected into the sample tube does not affect the aqueous volume and was used to drive the condensed water droplets

Table II—Ethylene Chlorohydrin Found in Ethylene Oxide-Sterilized[a] Materials by Co-Sweep Extraction

Material[b]	Percent Weight/Weight Ethylene Chlorohydrin[d]
Plastic[c], cotton, and rayon	0.00017
Wood pulp nonwoven fabric	0.00046
Rayon nonwoven fabric	0.00003

[a] Samples of materials were previously sterilized in a commercial ethylene oxide sterilization cycle. [b] Weight of material analyzed ranged from 1.0 to 2.9 g. [c] Polyvinyl chloride plasticized with dioctyl phthalate; sheet thickness = 0.1524 mm. (0.006 in.). [d] Average value based on duplicate injections.

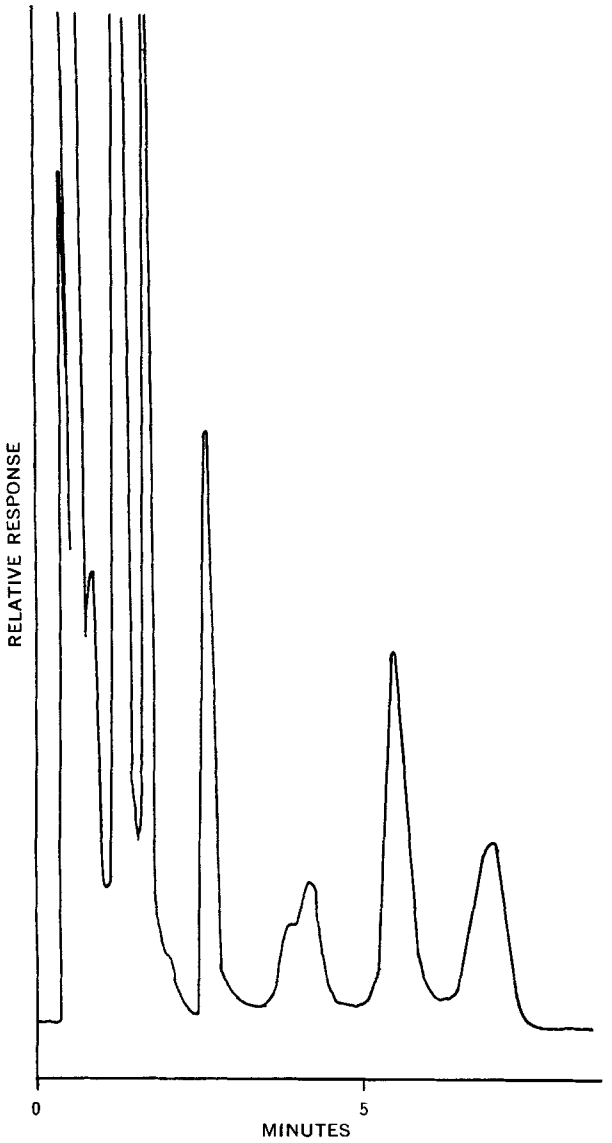

Figure 2—*Typical gas chromatogram obtained from a Co-Sweep extract of an ethylene oxide-sterilized material composed of plastic, cotton, and rayon. Retention time of ethylene chlorohydrin = 5.5 min. Injection volume = 1 μl. Attenuation range = 1×4.*

into the collection tube. The extracted ethylene chlorohydrin remains preferentially in the aqueous layer; this was determined by GLC analysis on both layers. A gas chromatogram of a standard aqueous solution of ethylene chlorohydrin, representing 40 ng., is shown in Fig. 1. A typical gas chromatogram obtained from a Co-Sweep extract of an ethylene oxide-sterilized material composed of plastic[9], cotton, and rayon is shown in Fig. 2. The complexity of the gas chromatogram in Fig. 2 as compared to the aqueous standard (Fig. 1) is due to additional components extracted from the plastic material.

The sensitivity of the GC instrument for ethylene chlorohydrin is approximately 0.2 ng./μl. injection. Therefore, the total weight of sample to be extracted by the Co-Sweep technique must contain more than 0.2 mcg. of ethylene chlorohydrin for a positive detection by GLC analysis (based on a 1-μl. injection from 1 ml. of aqueous extract). The amount of water used in the extraction can be reduced below 1 ml. for samples containing a high moisture content or, if desired, to lower the ethylene chlorohydrin detection level.

[9] Polyvinyl chloride sheet [thickness 0.1524 mm. (0.006 in.)] plasticized with dioctyl phthalate.

Table III—Recovery of Ethylene Chlorohydrin from Rayon Staple[a] under Varying Conditions of Co-Sweep Extraction[b]

Carrier Gas	Flow Rate, ml./min.	Minutes	Number of Injections, 1 ml. Water	Number of Injections, 1 ml. Isooctane	Percent Recovery[c], Range
Helium	120	60	1	5	78–83
Nitrogen	120	60	1	5	82–90
Nitrogen	120	45	1	5	82–84
Nitrogen	66	45	1	5	86–87
Nitrogen	120	35	1	3	85–87
Nitrogen	80	35	1	3	89–95

[a] Recovery based on spiking samples with 48 mcg. of ethylene chlorohydrin/g. [b] The oven temperature was 140°. [c] Based on duplicate injections from replicate sample extractions.

The Co-Sweep extraction method achieves a low level for determining ethylene chlorohydrin by increasing the sample weight relative to the aqueous extract volume. The method is potentially applicable to a wide range of materials and achieves a detection level for ethylene chlorohydrin at the micrograms per gram range in ethylene oxide-sterilized materials. In view of the foregoing results, the Co-Sweep extraction technique may be adaptable for the determination of various trace quantities of compounds or impurities in a wide variety of materials.

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Flow Rates of Solid Particulate Pharmaceuticals

F. Q. DANISH* and E. L. PARROTT

Abstract □ The flow rates of particles of sodium chloride and lactose through circular orifices were measured. Equations are presented relating the flow rate (Q), diameter of the particles (D_p), and diameter of the orifice (D_o). The addition of fine particles increased the flow rate to a maximum value; then a further increase in concentration of fines caused a decrease in flow rate. For 10% fines, a decrease in particle size caused an increase in flow rate to a maximum value, after which a further size reduction resulted in a slower flow rate.

Keyphrases □ Flow rates—diameter of particles, orifice □ Particulate solids—flow rates □ Fines—maximum flow rate □ Flowometer—granulation flow rates □ Repose angle—flow rates

The flow of particulate solids is an important factor in the production of pharmaceuticals. High-speed filling machines are used to deliver a given quantity of a powder or granulation into its package. The material must flow readily and consistently to ensure the desired fill. Solid dosage forms, such as capsules, tablets, and divided powders, require measured filling for the production of each unit. The uniformity of the final product requires a uniform flow rate of the powdered or granular formulation.

A review of the literature (1–5) reveals that little is known of the basic nature of the flow of particulate solids. Flowing particulate solids do not behave as fluids or solids. The free surface of a fluid in a gravitational field is horizontal, but the surface of a particulate solid can be inclined at slopes up to its repose angle. A particulate solid must expand locally to change its shape (6). It may show deformation similar to plastic deformation of solids (7).

Most investigations (8–12) of the flow of particulate solids have been conducted on monosized systems and with apparatus of much smaller or larger dimensions than those commonly encountered in tableting and filling operations. This investigation was initiated to demonstrate a relationship between flow rate, particle size, and diameter of orifice through which the flow occurs. The diameters of the circular orifices were the approximate size of the flow path found in tablet presses and filling machines. The particle sizes ranged from 0.03 to 0.154 cm., which included the sizes encountered in most pharmaceutical production. The particulate solids were composed of lactose and sodium chloride. A lactose granulation prepared with starch paste was selected because it is one of the most frequently used tablet bases. Sodium chloride granules were used to represent a particulate system that may be directly compressed.

Table I—Influence of Height of Fill in Flowometer on Flow Rate of 45/60-Mesh Size Fraction of Sodium Chloride through 0.635- and 0.925-cm. Circular Orifices

Initial Fill, cm.	0.925-cm. Orifice: Fill after Measurement, cm.	0.925-cm. Orifice: Flow Rate, g./sec.	0.635-cm. Orifice: Fill after Measurement, cm.	0.635-cm. Orifice: Flow Rate, g./sec.
6.0	3.0	14.7	4.9	5.0
9.0	6.0	14.9	7.9	5.0
12.0	9.3	15.5	10.9	5.0
15.0	12.3	14.5	14.0	4.9
18.0	15.3	14.5	17.0	4.9
21.0	18.1	14.3	20.0	4.8
24.0	21.2	14.1	23.0	4.9
27.0	24.1	14.0	26.0	4.9
30.0	27.2	14.0	29.0	4.9
33.0	30.2	14.0	32.0	4.9

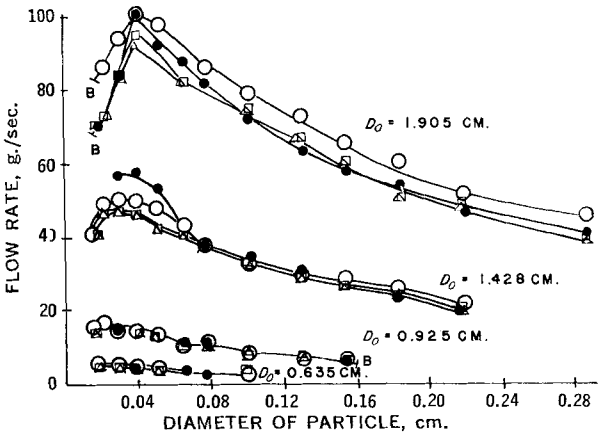

Figure 1—*Influence of relative humidity on the flow rate of various sizes of sodium chloride particles through several sizes of a circular orifice. Key: ○, 0; □, 30; △, 60; and ●, 80% relative humidity; and B, blocked.*

EXPERIMENTAL

Preparation of Monosized Particles—Sodium chloride[1] was milled through a No. 4 circular screen in a FitzMill with the blunt edges forward. The sodium chloride crystals were milled at slow, medium, and fast speeds to obtain an adequate quantity of various sizes required for the investigation. Five hundred grams of the milled sodium chloride was separated into size fractions by shaking for 5 min. in a Tyler sieve shaker containing appropriate U. S. standard sieves. The size fraction is expressed as a diameter which is the arithmetic mean of the openings of adjacent sieves. Size fractions larger than 0.030 cm. were treated with compressed air to remove any fine particles.

The lactose granulation containing 3% of starch was prepared by a wet granulation method. A 10% starch paste was added to the hydrous lactose USP in a Hobart mixer. After the starch paste and lactose were blended, the wet mass was passed manually through a 6-mesh screen. The wet granulation was collected on trays lined with paper and dried at 38° for 17 hr. The dried lactose granulation was then processed in the same manner as the sodium chloride.

Measurement of Flowability—The repose angle (θ) was measured by a fixed funnel and free-standing cone method (13). The reported value is the average of five determinations.

The flowometer consisted of a vertically mounted, cylindrical, stainless steel tube with an internal diameter (D_c) of 6.0 cm. and a height of 35.0 cm. A circular orifice of 1.905 cm. was accurately

[1] Sterling brine crystals, International Salt Co., Summit, Pa.

Table II—Flow Rate through a 1.905-cm. Circular Orifice and Repose Angle for Various Sizes of a Sodium Chloride and a Lactose Granulation

Mesh	Arithmetic Mean Diameter, cm.	Lactose		Sodium Chloride	
		Flow Rate, g./sec.	Repose Angle	Flow Rate, g./sec.	Repose Angle
12/14	0.1540	28.5	25.8°	—	—
14/16	0.1300	31.2	27.0°	66.6	33.2°
16/20	0.1015	34.4	28.0°	73.8	33.6°
20/25	0.0775	36.7	28.4°	82.9	32.1°
25/30	0.0650	38.6	28.8°	88.0	32.1°
30/40	0.0505	41.3	29.9°	93.8	32.8°
40/45	0.0385	42.0	30.9°	99.6	34.0°
45/60	0.0300	42.4	34.0°	101.0	35.2°
60/80	0.0213	38.8	33.9°	95.5	35.4°
80/100	0.0163	22.2	37.0°	91.3	36.6°
100/140	0.0127	21.7	37.7°	72.6	36.6°
140/200	0.0090	14.9	39.8°	Blocked	43.0°
200/325	0.0059	Blocked	42.1°	Blocked	50.6°

drilled in the center of the horizontal base plate. A spring shutter was fitted within guides on the base of the flowometer to allow rapid operation. Circular stainless steel plates, accurately drilled with orifice diameters (D_o) of 1.428, 0.925, and 0.635 cm., could be placed in the base of the flowometer to vary the orifice through which flow occurred.

A flat-bottom flowometer was selected so that the particles above the orifice would flow over each other rather than on a wall as in flow from a funnel-shaped vessel. Thus, any effect of the cone angle on flow rate was eliminated, and the flow rate more closely reflected the actual flow of the particles.

When particulate solids flow from a flat-bottom container, a dead or stationary region from which particles do not flow is at the periphery of the base of the container. This influences the flow rate and is known as the wall effect. A wall effect is encountered when the ratio of the diameter of the container to the diameter (D_p) of the particles is small. A number of relationships have been suggested to describe this effect (9, 14–16). The wall effect is not significant if the difference between the diameter of the container and the diameter of the orifice is greater than 30 times the diameter of the particles, *i.e.*, $(D_c - D_o) > 30\ D_p$. Literature (8, 12, 17) shows that the flow rate is independent of the diameter of the flowometer if $D_c/D_o > 2.5$.

The flowometer used in this study had a diameter of 6.0 cm., so with all orifices used the wall effect was insignificant for particles smaller than 0.20 cm. in diameter.

Since the weight of a stationary and dynamic column of a particulate solid is supported by the vertical walls and not by the base of a container, the height of the column should not affect the flow rate (15, 18). The flow rate is independent of the height or head of a column of particulate solid if the head is greater than twice the diameter of the container (9, 17, 19–21). When the head of a column falls below $2.5D_c$, the flow rate is increased. The flow rates at various heights using particles of sodium chloride with a diameter of 0.030 cm. are shown in Table I for two orifices. The data show that if the height of fill remaining exceeded approximately $2.5D_c$ or 18 cm., the influence of head on the flow rate was negligible. If the height of fill remaining was less than 18 cm., the flow rate was increased until the quantity of material was insufficient to flow. Therefore, throughout this study the height of fill remaining exceeded 18 cm. so that the influence of head on flow rate was negligible.

The flow rate was determined by collecting and weighing the material that flowed from the flowometer in a given time. A discharge time of 6 sec. was used with orifice diameters of 1.905 and 1.428 cm.; a discharge time of 12 sec. was used with orifice diameters of 0.925 and 0.635 cm. The flow rate (Q) is expressed in grams discharged per second. The reported flow rate is the average of five determinations. The variation of the flow rate did not exceed 5%.

Humidity—A high moisture content is known to reduce the flowability of some granular materials (22). To determine the influence of humidity on the flow of sodium chloride particles, they were exposed for 36 hr. to 0, 30, 60, and 80% relative humidity (23). The moisture content was determined with an Ohaus moisture balance; it did not exceed 0.4%. As shown in Fig. 1, at low and moderate humidities the effect of humidity on the flow rate of particles of sodium chloride was not significant. At 80% relative humidity, the particles tended to cake, but they flowed after shaking.

The moisture content of the lactose granulation after exposure to 60% relative humidity did not exceed 0.4%. It has been reported that lactose below 65% relative humidity contains negligible moisture (24) and remains free flowing (25).

Because the flow of sodium chloride and lactose particles was not affected by the humidities in the laboratory, no further effort was made to control humidity. All measurements were made at ambient humidity and temperature.

RESULTS AND DISCUSSION

Repose Angle and Flow Rate—Angular properties have been used to classify powders and granules; however, a relationship between an angular property and flow rate has not been expressed (22, 26–28). The flow rate and repose angle of various size fractions of lactose and sodium chloride are given in Table II and are plotted in Figs. 2 and 3. The results support the conclusion of Gold *et al.* (29) that there is no correlation between actual flow properties and repose angle.

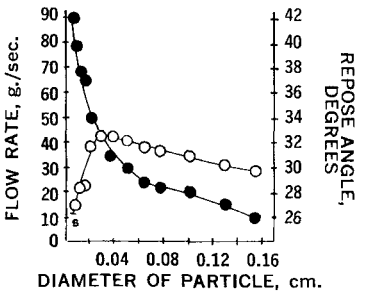

Figure 2—*Flow rate through a 1.905-cm. circular orifice and repose angle for various sizes of lactose granulation. Key: ○, flow rate; ●, repose angle; and B, blocked.*

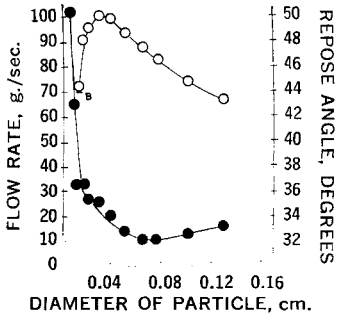

Figure 3—*Flow rate through a 1.905-cm. circular orifice and repose angle for various sizes of sodium chloride particles. Key: ○, flow rate; ●, repose angle; and B, blocked.*

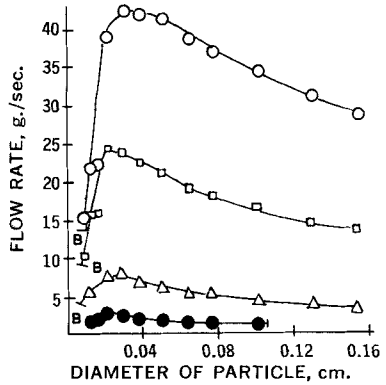

Figure 4—*Flow rate of various sizes of lactose granulation through circular orifices with diameters: ○, 1.905; □, 1.428; △, 0.9250; ●, 0.6530 cm.; and B, blocked.*

Since active flow is an important factor in pharmaceutical manufacturing, it seems valid to characterize particulate solids in a dynamic term. Because the repose angle expresses static interparticulate friction, it was decided to study the flow of particulate solids by means of a flowometer, which measures a dynamic flow rate that closely simulates the actual production conditions.

The most important single factor controlling flow rate is the diameter of the orifice (8, 30). As shown in Figs. 1 and 4, the flow rate is increased as the diameter of the orifice is increased. The flow rate is proportional to a power of the diameter of the orifice. The power may be empirically evaluated by a plot of log Q against log D_o (15, 31, 32). The linear relationship of log Q to log D_o for lactose and sodium chloride is shown in Figs. 5 and 6.

Figures 1 and 4 show that for a given orifice the flow rate is increased to a maximum rate as the particle size is decreased and that a further reduction of particle size results in a slower flow rate. For both materials the maximum flow rate through various orifices occurred when the particles were 0.03 cm. in diameter.

Relation of Q, D_p, and D_o—Brown and Richards (33), in their examination of the empty annulus adjacent to the edge of an orifice, developed an equation for the purpose of finding the orifice giving rise to zero flow. The equation is:

$$(4Q/\pi\rho\sqrt{g})^{0.4} = mD_o + C \qquad \text{(Eq. 1}$$

where Q is the flow rate in grams per second, ρ is the density of the material, g is the acceleration of gravity, and m and C are constants. Figures 7 and 8 are plots of the equation using experimental values for monosized particles of lactose and sodium chloride. The constants m and C may be evaluated from the slope and intercept with the ordinate axis, respectively, of this linear plot. Attempts (15, 34–36) have been made to relate bulk density to flow rates; however, it is doubtful if any arbitrarily selected bulk density can be correlated with flow because a particulate solid expands with a decrease in density when it begins to flow (12, 37). Thus, rather than select some bulk density, which is not representative of the density of the flowing particles, the density of the material was used (19).

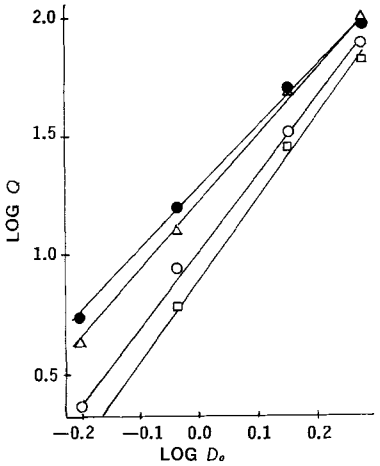

Figure 6—*Log–log relationship of diameter of circular orifice to the flow rate of sodium chloride granules of various size. Key: □, 0.1540; ○, 0.1015; △, 0.0505; and ●, 0.0300 cm.*

Upon rearrangement of Eq. 1 and substitution of $k = -C/m$,

$$D_o = 1/m(4Q/\pi\rho\sqrt{g})^{0.4} + k \qquad \text{(Eq. 2)}$$

The general use of the equation of Brown and Richards (33) is supported by Jones and Pilpel (38) and this study. The relationship applies in the region where a decrease in particle size causes an increase in flow rate. When the particle diameter is less than 0.025 cm., other forces, which were discussed by Jones and Pilpel (39), influence the flow rate. In pharmaceutical operations involving flow of particulate solids, the sizes of the particles are usually larger than 0.059 cm. For these reasons the present investigation was restricted to particles with a diameter larger than 0.025 cm.

The intercept with the ordinate axis and the slope of each straight line in Fig. 8 were determined. A plot of the reciprocal ($1/m$) of the slope of each particle size against D_p is linear and is expressed by the relationship

$$1/m = 0.75D_p + 1.36 \qquad \text{(Eq. 3)}$$

A plot of log k against log D_p is linear and can be used to evaluate the constants for the relationship.

$$k = 1.202D_p^{0.768} \qquad \text{(Eq. 4)}$$

Upon the substitution of $1/m$ from Eq. 3 and k from Eq. 4 into Eq. 2,

$$D_o = (1.36 + 0.75D_p)(4Q/\pi\rho\sqrt{g})^{0.4} + 1.202D_p^{0.768} \qquad \text{(Eq. 5)}$$

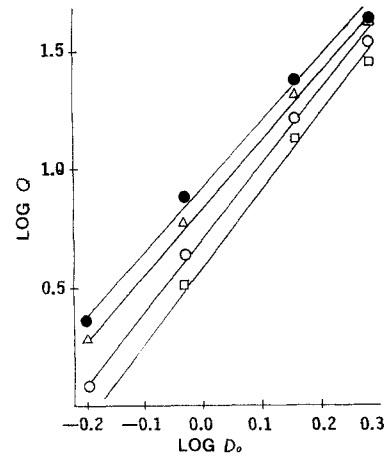

Figure 5—*Log–log relationship of diameter of circular orifice to the flow rate of lactose granulation of various particle size. Key: □, 0.1540; ○, 0.1015; △, 0.0505; and ●, 0.0300 cm.*

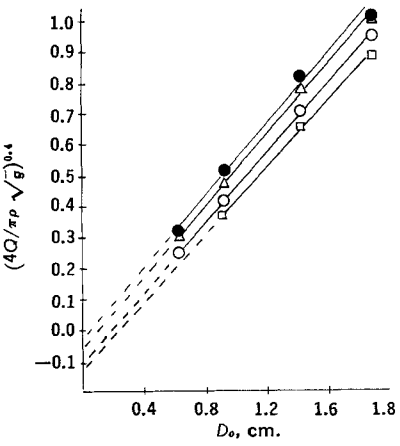

Figure 7—*Influence on relationship of $(4Q/\pi\rho\sqrt{g})^{0.4}$ to diameter of orifice of lactose granulation of various particle size. Key: □, 0.1540; ○, 0.1015; △, 0.0505; and ●, 0.0300 cm.*

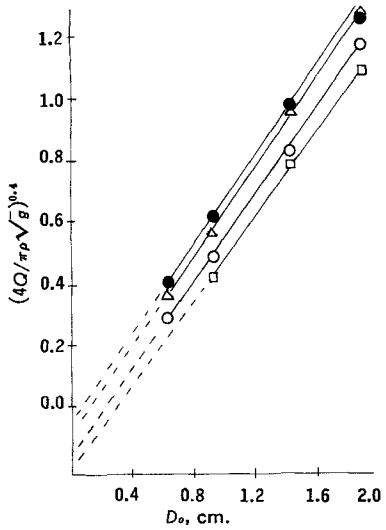

Figure 8—*Influence on relationship of* $(4Q/\pi\rho\sqrt{g})^{0.4}$ *to diameter of orifice of sodium chloride granules of various particle size. Key:* □, *0.1540;* ○, *0.1015;* △, *0.0505; and* ●, *0.0300 cm.*

After rearrangement, the flow rate of particles of sodium chloride is related to the diameter of the orifice and the diameter of the particles by the equation

$$Q = [(D_o - 1.202D_p^{0.768})/(1.36 + 0.75D_p)]^{2.5}(\pi\rho\sqrt{g}/4) \quad \text{(Eq. 6)}$$

Adjacent to the periphery of the orifice, there is an empty space or annulus with a width ($k/2$) that is independent of the size of the orifice (19, 33, 35, 40). Thus, the effective diameter of a circular orifice is ($D_o - k$). If Eq. 2 is written

$$D_o - k = 1/m(4Q/\pi\rho\sqrt{g})^{0.4} \quad \text{(Eq. 7)}$$

it is readily seen that the flow rate is a function of the effective diameter of the orifice. When $(4Q/\pi\rho\sqrt{g})^{0.4}$ is plotted against the effective diameter, a straight line passing through the origin is produced (Fig. 9). The relation of this function of flow rate to effective diameter of the orifice is constant regardless of particle size. Therefore, the statement that flow rate is a function of particle size requires qualification. The flow rate in the actual region of movement of particles is independent of particle size. Flow is affected by particle size only to the extent that particle size changes the value of k.

It may be more practical to use the measured diameter (D_o) than the effective orifice diameter, which requires the evaluation of k. Since k is a function of D_p, Eq. 2 may be written

$$D_o = 1/m(4Q/\pi\rho\sqrt{g})^{1/n} \quad \text{(Eq. 8)}$$

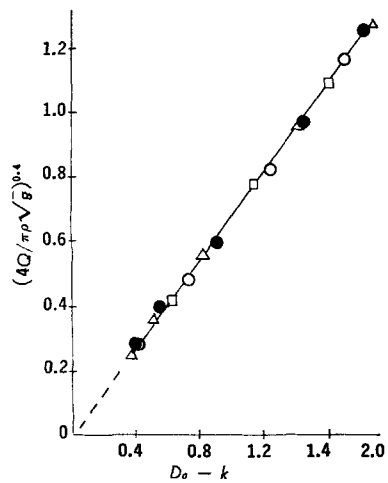

Figure 9—*Relationship of* $(4Q/\pi\rho\sqrt{g})^{0.4}$ *to effective diameter of the orifice for various particle size. Key:* □, *0.1540;* ○, *0.1015;* △, *0.0505; and* ●, *0.0300 cm.*

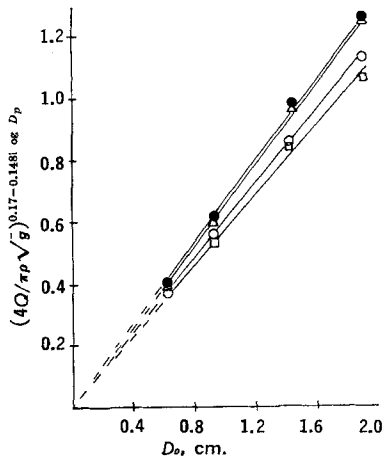

Figure 10—*Influence on relationship of* $(4Q/\pi\rho\sqrt{g})^{0.17 - 0.148 \log D_p}$ *to diameter of orifice for sodium chloride granules of various particle size. Key:* □, *0.1540;* ○, *0.1015;* △, *0.0505; and* ●, *0.0300 cm.*

where $1/m$ and $1/n$ are functions of particle size. If the reciprocals of the slopes of the plot of log Q against log D_o in Fig. 5 are plotted against log D_p, there is a linear relationship, which may be expressed

$$1/n = 0.17 - 0.148 \log D_p \quad \text{(Eq. 9)}$$

Substitution of Eq. 9 into Eq. 8 gives

$$D_o = 1/m(4Q/\pi\rho\sqrt{g})^{0.17 - 0.148 \log D_p} \quad \text{(Eq. 10)}$$

When the experimental data are used in Fig. 10 to plot $(4Q/\pi\rho\sqrt{g})^{0.17 - 0.148 \log D_p}$ against D_o, a straight line, which passes through the origin, is produced. The slopes of these lines are used to relate Q, D_o, and D_p. For each particle size, the reciprocal of the slope was plotted against D_p to evaluate the constants for the relationship

$$1/m = 1.41 + 2.5D_p \quad \text{(Eq. 11)}$$

Upon substitution of $1/m$ from Eq. 11 into Eq. 10 and rearrangement,

$$Q = [D_o/(1.41 + 2.5D_p)]^{1/(0.17 - 0.148 \log D_p)}(\pi\rho\sqrt{g}/4) \quad \text{(Eq. 12)}$$

which is equivalent to Eq. 6 for particles of sodium chloride.

The constants for the lactose granulation were evaluated in a similar manner; however, to conserve space only Eqs. 13 and 14 and Figs. 7 and 11 are presented:

$$Q = [(D_o - 1.202D_p^{0.781})/(1.72 + 0.757D_p)]^{2.5}(\pi\rho\sqrt{g}/4) \quad \text{(Eq. 13)}$$

$$Q = [D_o/(1.677 + 2.11D_p)]^{1/(0.243 - 0.08 \log D_p)} \times (\pi\rho\sqrt{g}/4) \quad \text{(Eq. 14)}$$

The comparison of the experimental and calculated flow rates in

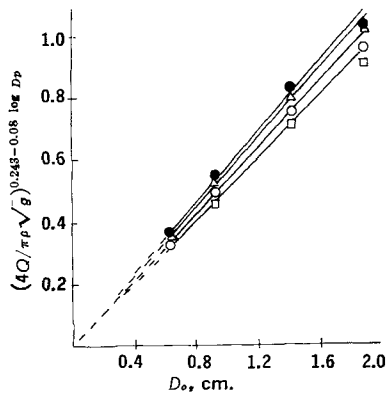

Figure 11—*Influence on relationship of* $(4Q/\pi\rho\sqrt{g})^{0.243 - 0.08 \log D_p}$ *to diameter of orifice for lactose granulation of various size. Key:* □, *0.1540;* ○ *0.1015;* △, *0.0505; and* ●, *0.0300 cm.*

Table III—Experimental Rate (Q) and Calculated Rate ($Q_{calcd.}$) of Flow through a 1.428-cm. Circular Orifice for Various Monosized Particles

Mesh	Size, cm.	Q, g./sec.	$Q_{calcd.}$, g./sec.	Error, %	$Q_{calcd.}$, g./sec.	Error, %
Lactose		Experimental	Equation 13		Equation 14	
12/14	0.1540	13.5	12.1	−9.5	13.0	−2.4
14/16	0.1300	14.5	13.3	−7.8	14.4	−0.3
16/20	0.1015	16.4	15.0	−9.3	16.3	−0.3
20/25	0.0755	17.9	16.8	−6.1	18.3	2.4
25/30	0.0650	18.9	17.6	−7.1	19.2	1.5
30/40	0.0505	20.7	18.7	−9.3	20.6	−0.6
40/45	0.0385	22.3	19.8	−11.2	21.8	−2.5
45/60	0.0300	23.9	20.6	−13.7	22.7	−5.0
Sodium Chloride		Experimental	Equation 6		Equation 12	
8/10	0.2190	21.2	21.1	−0.1	16.3	−22.6
10/12	0.1840	25.3	24.6	−2.1	20.2	−20.2
12/14	0.1540	28.6	28.0	−1.9	24.2	−15.4
14/16	0.1300	30.0	31.1	3.6	27.8	−7.0
16/20	0.1015	32.7	35.4	8.3	32.9	0.4
20/25	0.0755	38.3	39.5	3.3	37.9	−0.8
25/30	0.0650	42.4	41.9	−1.0	40.3	−4.9
30/40	0.0505	47.9	45.0	−6.2	43.5	−9.2
40/45	0.0385	49.8	47.7	−4.1	46.2	−7.1
45/60	0.0300	49.8	49.8	0	48.2	−3.1

Table III demonstrates the validity of Eqs. 6 and 12 for monosized particles of sodium chloride and of Eqs. 13 and 14 for monosized particles of lactose granulation. Similar comparisons for other orifices show that, in general, the value of the calculated and experimental flow rates are within a 10% variation.

Because real particulate solids are composed of various sizes of particles, it would be useful to have an equation that could be applied to calculate the flow rates of polysized particulate solids. Following the pattern of Jones and Pilpel (38), binary and ternary mixtures were prepared, and the flow rates were measured. The particle diameter ($D_{pav.}$) of the mixture was represented by the geometric mean diameter, *e.g.*, for a binary mixture

$$D_{pav.} = \sqrt{D_{p_1}^{m_1/0.5} \times D_{p_2}^{m_2/0.5}} \quad \text{(Eq. 15)}$$

where m_1 and m_2 are the weight fractions of particles of diameter D_{p1} and D_{p2}, respectively.

In applying Eqs. 13 and 14 for monosized particles to the calculation of flow rates of mixtures of lactose, it was found that the best correlation between experimental and calculated flow rate was obtained if the right side of the equation was multiplied by an empirical factor of 1.14. Thus, for mixtures of the lactose granulation,

$$Q = 1.14\,[(D_o - 1.202D_{pav.}^{0.781})/(1.72 + 0.757D_{pav.})]^{2.5} \times (\pi\rho\sqrt{g}/4) \quad \text{(Eq. 16)}$$

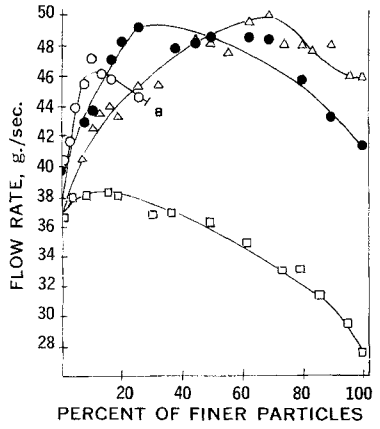

Figure 12—*Influence of percent of fine particles of sodium chloride on the flow rate through a 1.428-cm. circular orifice of 16/20-mesh size fraction of sodium chloride. Key to diameter of fine particle: ○, 0.0090; ●, 0.0163; △, 0.0385; □, 0.1540 cm; and B, blocked.*

and

$$Q = 1.14\,[D_o/(1.677 + 2.11D_{pav.})]^{1/(0.243 - 0.08 \log D_p)} \times (\pi\rho\sqrt{g}/4) \quad \text{(Eq. 17)}$$

The calculated flow rates using Eqs. 16 and 17 for binary and ternary mixtures of lactose granulation are compared to the experimental flow rates in Tables IV and V.

Similarly, for sodium chloride particles, the flow rate of the mixtures may be expressed by multiplying the right side of Eqs. 6 and 12 by the factor of 1.14 so that for mixtures:

$$Q = 1.14\,[(D_o - 1.202D_{pav.}^{0.768})/(1.36 + 0.75\,D_{pav.})]^{2.5} \times (\pi\rho\sqrt{g}/4) \quad \text{(Eq. 18)}$$

$$Q = 1.14\,[D_o/(1.41 + 2.5D_{pav.})]^{1/(0.17 - 0.148 \log D_{pav.})} \times (\pi\rho\sqrt{g}/4) \quad \text{(Eq. 19)}$$

In general, for both materials the experimental and calculated flow rates of binary and ternary mixtures using these equations are within a 10% variation.

The bulk density of a polysized particulate bed is greater than that of a monosized system because the smaller particles tend to fill the void between the larger particles. Similarly, in a flowing polysized particulate system the smaller particles fit within the void between the larger particles. Consequently, there is a greater mass of material per unit volume of discharge and there is a greater flow rate. Thus, the factor 1.14 may be considered as a correction for the increased bulk density of the polysized system during actual flow.

Fines—The influence of fine particles on the flow rate of larger particles was demonstrated by the addition of finer, monosized

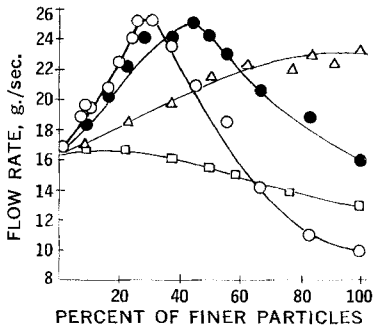

Figure 13—*Influence of percent of fine particles of lactose granulation on the flow rate through a 1.428-cm. circular orifice of 16/20-mesh size fraction of lactose granulation. Key to diameter of fine particle: ○, 0.0090; ●, 0.0163; △, 0.0385; and □, 0.1540 cm.*

Table IV—Experimental Rate (Q) and Calculated Rate ($Q_{calcd.}$) of Flow through a 1.428-cm. Circular Orifice for Binary Mixtures of Monosized Particles of Lactose Using Eqs. 16 and 17

D_{p1}, cm.	Percent	D_{p2}, cm.	Percent	Q, g./sec.	Equation 16: $Q_{calcd.}$, g./sec.	Equation 16: Error, %	Equation 17: $Q_{calcd.}$, g./sec.	Equation 17: Error, %
0.1015	75	0.1540	25	16.5	16.4	−0.6	17.7	7.8
	50		50	15.3	15.5	1.6	16.8	10.1
	25		75	13.9	14.7	5.8	15.9	14.4
0.1015	75	0.0385	25	18.5	18.5	1.8	20.5	11.1
	50		50	21.4	20.3	−5.1	22.2	3.8
	25		75	22.4	21.5	−3.6	23.6	5.7
0.1015	75	0.0163	25	22.4	20.1	−9.9	22.0	−1.5
	50		50	24.1	22.4	−6.9	24.0	2.1
	25		75	19.2	24.0	25.3	26.5	38.0

Table V—Experimental Rate (Q) and Calculated Rate ($Q_{calcd.}$) of Flow through a 1.428-cm. Circular Orifice for Ternary Mixtures of Monosized Particles of Lactose Using Eqs. 16 and 17

Percent of D_p, cm.: 0.1015	Percent of D_p, cm.: 0.0650	Percent of D_p, cm.: 0.0127	Q, g./sec.	Equation 16: $Q_{calcd.}$, g./sec.	Equation 16: Error, %	Equation 17: $Q_{calcd.}$, %	Equation 17: Error, %
25	50	25	25.0	21.7	−13.0	23.8	−4.5
33.3	33.3	33.3	24.4	22.0	−9.6	24.2	−0.7
50	25	25	23.2	21.2	−8.6	23.2	−0.5
25	25	50	24.1	23.4	−2.9	25.7	6.7

particles to particles with a diameter of 0.1015 cm. After blending for 5 min. in a V-blender, the flow rate was measured. As the concentration of the finer particles was increased and more of the void between the larger particles was filled, the flow rate was increased to a maximum value; then as a greater concentration was added, the flow rate was decreased.

As shown in Fig. 12, the smallest size of the finer particles required the lowest concentration of the finer particles to obtain the maximum flow rate. For example, maximum flow rates of particles with a diameter of 0.1050 cm. were obtained upon the addition of 9.9, 25.9, and 69% of fine particles with diameters of 0.0090, 0.0163, and 0.0385 cm., respectively. Although a maximum flow rate was obtained at a lower concentration of the finest particles, the value of the maximum flow rate may be less than the maximum flow rate obtained upon the addition of a greater concentration of a larger-sized fine particle. For example, the addition of 9.9% of fine particles with a diameter of 0.0090 cm. to particles with a diameter of 0.1050 cm. produced a maximum flow rate of 47.1 g./sec., while the addition of 25.9% of fine particles with a diameter of 0.0163 cm. produced a maximum flow rate of 49.3 g./sec.

As shown in Fig. 13, for the lactose granulation having a diameter of 0.1050 cm., maximum flow rates were 25.2, 25.2, and 22.3 g./sec. upon the addition of 25.9% of particles having a diameter of 0.0090 cm., 44.4% of particles having a diameter of 0.0163 cm., and 62.5% of particles having a diameter of 0.0385 cm., respectively.

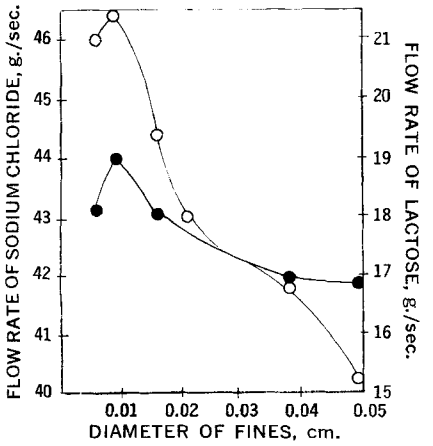

Figure 14—*Influence of size of fines on the flow rate through a 1.428-cm. circular orifice of 16/20-mesh size fraction when the percent of fines is maintained at 10%. Key: ○, sodium chloride; and ●, lactose granules.*

The influence of the size of fine particles on the flow rate of particles of sodium chloride and lactose with a diameter of 0.1050 cm. by the addition of 10% fines is shown in Fig. 14. As the diameter of the finer particles was decreased to 0.009 cm., the flow rate was increased because the weight per unit volume of flow was greater. When the diameter is less than 0.009 cm. and the void space is mostly filled by the smaller particles, it appears that the number of points of contact is so large that interparticular friction becomes great enough to slow the flow rate.

SUMMARY

1. A flowometer was used to study the flow of a sodium chloride and a lactose granulation through a circular, horizontal orifice.

2. Equations are presented relating the flow rate, diameter of the particles, and diameter of the orifice through which flow of monosized particles occurs.

3. An empirical factor is introduced into these equations so that they may be applied to binary and ternary blends of various monosized particles.

4. The addition of fine particles to a monosized particulate solid increased the flow rate to a maximum value; then as a greater concentration of fines was added, the flow rate was decreased.

5. For a given concentration of fines, the flow rate was increased to a maximum rate as the diameter of the fines was decreased to approximately 90 μ; then further reduction in diameter of the fines resulted in slower flow rates.

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Synthesis, Antileukemic Activity, and Stability of 3-(Substituted-Triazeno)pyrazole-4-carboxylic Acid Esters and 3-(Substituted-Triazeno)pyrazole-4-carboxamides

Y. FULMER SHEALY and C. ALLEN O'DELL

Abstract □ Representative 3-triazeno derivatives of the ethyl and methyl esters of pyrazole-4-carboxylic acid and of pyrazole-4-carboxamide were synthesized from the corresponding 3-diazopyrazoles. It was shown that certain triazenopyrazoles in solution are decomposed by light, and transformations of 3-(3-methyl-1-triazeno)pyrazole-4-carboxamide and 3-[3,3-bis(2-chloroethyl)-1-triazeno]pyrazole-4-carboxamide in solution in the dark to 3-aminopyrazole-4-carboxamide and to a *v*-triazolinium salt, respectively, were observed. All of these transformations are analogous to reactions of triazenoimidazoles observed previously. Triazenopyrazoles having both the ester and the amide groups increased the average survival time in the standard mouse L-1210 leukemia assay. In some of these tests, increases in lifespan of 60–120% were observed.

Keyphrases □ Triazenopyrazoles—synthesis, decomposition by light □ Leukemia L-1210 inhibition—triazenopyrazoles □ Antileukemic activity—triazeno derivatives of pyrazole esters, amides □ Diazopyrazole esters—antimicrobial activity

Various triazenoimidazolecarboxamides (*Ia*) (1–5) and triazenoimidazolecarboxylates (*Ib*) (6, 7) have been synthesized, and a number of these have displayed antineoplastic activity (2, 3, 5–10). Several triazeno-*v*-triazoles (*e.g.*, II) were prepared as ring analogs of the imidazoles, and activity was also found among derivatives of this ring system (11). It seemed logical, therefore, to extend these studies to related heterocycles. The synthesis and antileukemic activity of 3-(substituted-triazeno)pyrazole-4-carboxylic acid esters (V–VIII) and 3-(substituted-triazeno)pyrazole-4-carboxamides (X–XIV) are described in the present report. The bis(2-fluoroethyl)triazenopyrazoles (IX and XV) comprised part of a recent communication (12); after most of this work had been completed, one of the other derivatives, the dimethyltriazeno derivative (X) of the amide series, was reported by Noell and Cheng (13). The dimethyltriazeno derivative of antipyrine, a pyrazolone, was prepared by Stolz (14) in 1908.

The triazenopyrazoles (V–XV) were prepared by the methods utilized for the synthesis of triazenoimidazoles (I), namely, by isolating a diazopyrazole and treating it in nonaqueous media with the appropriate amine or by diazotizing the aminopyrazole and then adding the amine to the aqueous solution without isolating the intermediate diazo derivative. Both methods afforded the simple dialkyltriazenopyrazolecarboxylate esters (V–VIII). The first method was used in syntheses, or attempted syntheses, of unstable triazenes, such as bis(2-haloethyl)triazeno derivatives and the monomethyltriazene (XIII); it is preferable for the preparation of triazenopyrazolecarboxamides because of the competing intramolecular cyclization reaction to 3,7-

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Synthesis, Antileukemic Activity, and Stability of 3-(Substituted-Triazeno)pyrazole-4-carboxylic Acid Esters and 3-(Substituted-Triazeno)pyrazole-4-carboxamides

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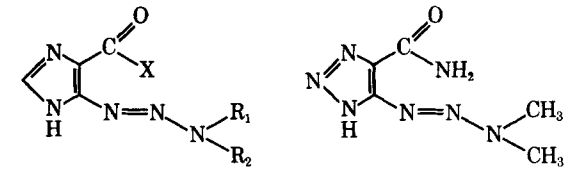
Abstract □ Representative 3-triazeno derivatives of the ethyl and methyl esters of pyrazole-4-carboxylic acid and of pyrazole-4-carboxamide were synthesized from the corresponding 3-diazopyrazoles. It was shown that certain triazenopyrazoles in solution are decomposed by light, and transformations of 3-(3-methyl-1-triazeno)pyrazole-4-carboxamide and 3-[3,3-bis(2-chloroethyl)-1-triazeno]pyrazole-4-carboxamide in solution in the dark to 3-aminopyrazole-4-carboxamide and to a *v*-triazolinium salt, respectively, were observed. All of these transformations are analogous to reactions of triazenoimidazoles observed previously. Triazenopyrazoles having both the ester and the amide groups increased the average survival time in the standard mouse L-1210 leukemia assay. In some of these tests, increases in lifespan of 60–120% were observed.

Keyphrases □ Triazenopyrazoles—synthesis, decomposition by light □ Leukemia L-1210 inhibition—triazenopyrazoles □ Antileukemic activity—triazeno derivatives of pyrazole esters, amides □ Diazopyrazole esters—antimicrobial activity

Various triazenoimidazolecarboxamides (*Ia*) (1–5) and triazenoimidazolecarboxylates (*Ib*) (6, 7) have been synthesized, and a number of these have displayed antineoplastic activity (2, 3, 5–10). Several triazeno-*v*-triazoles (*e.g.*, II) were prepared as ring analogs of the imidazoles, and activity was also found among derivatives of this ring system (11). It seemed logical, therefore, to extend these studies to related heterocycles. The synthesis and antileukemic activity of 3-(substituted-triazeno)pyrazole-4-carboxylic acid esters (V–VIII) and 3-(substituted-triazeno)pyrazole-4-carboxamides (X–XIV) are described in the present report. The bis(2-fluoroethyl)triazenopyrazoles (IX and XV) comprised part of a recent communication (12); after most of this work had been completed, one of the other derivatives, the dimethyltriazeno derivative (X) of the amide series, was reported by Noell and Cheng (13). The dimethyltriazeno derivative of antipyrine, a pyrazolone, was prepared by Stolz (14) in 1908.

The triazenopyrazoles (V–XV) were prepared by the methods utilized for the synthesis of triazenoimidazoles (I), namely, by isolating a diazopyrazole and treating it in nonaqueous media with the appropriate amine or by diazotizing the aminopyrazole and then adding the amine to the aqueous solution without isolating the intermediate diazo derivative. Both methods afforded the simple dialkyltriazenopyrazolecarboxylate esters (V–VIII). The first method was used in syntheses, or attempted syntheses, of unstable triazenes, such as bis(2-haloethyl)triazeno derivatives and the monomethyltriazene (XIII); it is preferable for the preparation of triazenopyrazolecarboxamides because of the competing intramolecular cyclization reaction to 3,7-

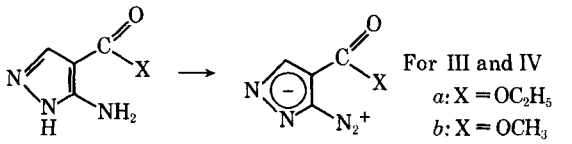


I

a: X = NH_2

b: X = OR

II



III IV

For III and IV

a: X = OC_2H_5

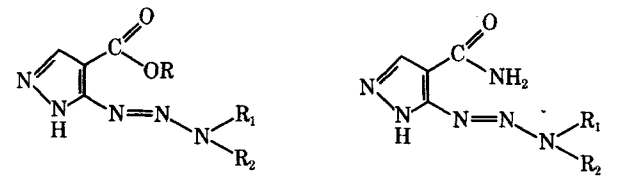
b: X = OCH_3

c: X = NH_2

Scheme I

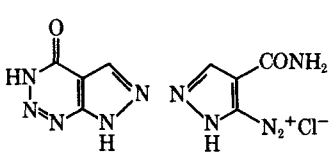
dihydro-4*H*-pyrazolo[3,4-*d*]-*v*-triazin-4-one (XVI) (15). Although 3-(3-butyl-3-methyl-1-triazeno)pyrazole-4-carboxamide (XI) was obtained without isolating 3-diazopyrazole-4-carboxamide (IV*c*), the dimethyltriazeno derivative (X) was more readily obtained pure (86% yield) by using the anhydrous medium (ethyl acetate) employed in some of the triazenoimidazole syntheses (5). This method was also used in the previously reported synthesis (13). The two bis(2-fluoroethyl)triazeno derivatives (IX and XV) were isolated and purified without causing serious difficulties (12), but 3-[3,3-bis(2-chloroethyl)-1-triazeno]pyrazole-4-carboxamide (XIV), like the analogous imidazole (4, 10), is less stable and more difficult to isolate. A specimen was eventually obtained that, according to NMR assay, contained only about 3% of the *v*-triazolinium salt formed by intramolecular cyclization. (See *References 4* and *16* for characterization of the analogous imidazole.)

Both ethyl and methyl 3-diazopyrazole-4-carboxylates (IV*a* and IV*b*) were isolated as pure crystalline compounds from small-scale diazotization reactions either by adding the aminopyrazoles (III*a* and III*b*) to aqueous sodium nitrite or by employing the reverse mode of addition (Scheme I). A diazotization on a modestly increased scale performed by adding the nitrite solution to III*b* yielded principally the bis(pyrazolyl)triazene (XVIII). This compound was then deliberately prepared by using an excess of III*b*. Employment of III*c* free base and only one equivalent of hydrochloric acid resulted in the precipitation of the diazo derivative (IV*c*). However, the procedure (13) of diazotizing the aminopyrazole hemisulfate, without added acid, is preferable because preparation of the free base is not required. When excess hydrochloric acid was used in diazotizing III*c* sulfate or free base, attempts to isolate the diazo amide (IV*c*) or to form a triazene *in situ* usually gave mixtures of the desired product and the pyrazolo-*v*-triazinone (XVI). As already intimated, the isolation of pure XI was an exception. A possible explanation for the adverse effect of excess acid on the isolation of IV*c* is that the latter compound is protonated to the diazonium chloride (XVII), which should be soluble and should cyclize readily to XVI.



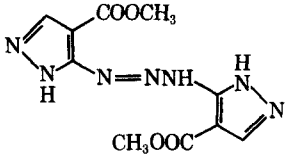
R	R_1	R_2
V: C_2H_5	CH_3	CH_3
VI: CH_3	CH_3	CH_3
VII: C_2H_5	CH_3	*n*-C_4H_9
VIII: CH_3	CH_3	*n*-C_4H_9
IX: C_2H_5	$-CH_2CH_2F$	$-CH_2CH_2F$

	R_1	R_2
X:	CH_3	CH_3
XI:	CH_3	*n*-C_4H_9
XII:	CH_3	$-CH_2CH_2OH$
XIII:	CH_3	H
XIV:	$-CH_2CH_2Cl$	$-CH_2CH_2Cl$
XV:	$-CH_2CH_2F$	$-CH_2CH_2F$



XVI

XVII



XVIII

The monomethyltriazeno derivative (XIII), like the analogous imidazole (2), decomposed spontaneously in the dark in aqueous methanol to 3-aminopyrazole-4-carboxamide (III*c*).

This study included observations on the stability of 3-(3,3-dimethyl-1-triazeno)pyrazole-4-carboxamide (X) and of the methyl ester (VI) in comparison with the analogous triazenoimidazoles. These observations differ considerably from previous statements relating to the stability of X; it was reported (13) that X is unchanged during 3 days of "exposure to daylight" in aqueous buffer at pH 7, and the implied stability to light was contrasted with the behavior of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, which is known to be dissociated in solution under certain conditions of light exposure and to remain unchanged during several days under other conditions (1, 15, 17). For this study, solutions of X and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide contained in Pyrex volumetric flasks placed side-by-side were exposed simultaneously[1], and changes were observed by determining the UV spectra of aliquots withdrawn from the two solutions. 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide and its pyrazole analog (X) were exposed in this way to light as follows: (*a*) in 50% aqueous ethanol in direct sunlight (filtered through window glass); (*b*) in 50% aqueous ethanol at a distance of 10 cm. from a UV light source that emits radiation principally at 365 nm.; and (*c*) in a Krebs–Ringer phosphate buffer (18) at a distance of 15 cm. from the same UV source. Two generalizations summarize the results: (*a*) in all three experiments, both the triazenopyrazole (X) and the triazenoimidazole [5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide] decomposed, as evidenced by the decrease or disappearance of their long wavelength absorption

[1] Comparisons of the relative stabilities of light-sensitive compounds (or of the rates of change of a given compound in different solutions) are valid only if the conditions of exposure are identical. The wavelength and intensity of sunlight reaching exposed compounds are determined by a number of variables such as latitude, time of the day, time of the year, transmission properties of the glass through which the light passes, and the clarity of the atmosphere. When two solutions are placed side-by-side, these conditions are equalized, but they may not be reproducible.

Table I—Triazenopyrazoles and Diazopyrazoles *versus* Leukemia L-1210[a]

Compound	Dose[b], mg./kg., and Schedule[c]	Mortality, Deaths/Total	Average Weight Change, g., T/C	Average Survival Time	
				T/C, Days	T/C Ratio[d], %
V	400 D2	0/6	−1.2/+2.6	10.3/8.0	128
	200 D2	0/6	−0.6/+2.6	10.0/8.0	125
	600	0/6	−4.6/+2.4	12.0/9.3	129t
	400 D2	0/6	−1.3/+2.4	13.2/9.3	141
	267 DR	0/6	−0.7/+2.4	10.5/9.3	112
	178	0/6	+0.8/+2.4	10.3/9.3	110
	200	2/6	−3.5/+1.8	12.3/9.1	135
	120 q.d. 1–9	0/6	−1.7/+1.8	12.2/9.1	134
	72 DR	0/6	−1.0/+1.8	11.5/9.1	126
	42	0/6	−0.9/+1.8	10.3/9.1	113
VI	400 D2	0/4	−3.0/+2.4	12.0/9.8	122t
	200 DR	0/4	−1.9/+2.4	11.8/9.8	120t
	100	0/4	−0.5/+2.4	10.5/9.8	107
	400 q.d. 1–9	2/6	−5.2/+1.1	7.0/9.0	77t
	200 q.d. 1–9	0/12	−3.3/+1.4	14.6/8.9	164[e]
	300	1/6	−5.3/+1.1	7.2/8.1	88
	200 q.d. 1–9	0/6	−3.7/+1.1	10.5/8.1	129
	133 DR	0/6	−2.8/+1.1	13.3/8.1	164
	88	0/6	−2.1/+1.1	14.2/8.1	175
	88	0/5	−1.5/+0.9	13.6/8.3	163
	58 q.d. 1–9	0/6	−1.8/+0.9	13.0/8.3	156
	38 DR	0/6	−1.3/+0.9	11.3/8.3	136
	25	0/6	−0.5/+0.9	10.0/8.3	120
VII	400 D1	0/6	−0.5/+1.9	10.3/9.0	114
	400 q.d. 1–9	0/13	−1.5/+1.1	13.8/8.5	162[e]
	600	3/6			t
	400 q.d. 1–9	0/6	−2.9/+1.6	11.5/8.9	129
	266 DR	0/6	−2.1/+1.6	15.0/8.9	168
	177	0/6	−1.3/+1.6	12.3/8.9	138
VIII	400 D1	0/6	+0.3/+1.9	10.0/9.0	111
	400 q.d. 1–9	0/6	−3.6/+0.7	14.0/8.7	160
	600	1/6	−2.6/+1.5	8.0/9.0	88
	400 q.d. 1–7	0/6	−2.2/+1.5	12.3/9.0	136
	268 DR	0/6	−1.8/+1.5	13.5/9.0	150
	176	0/6	−1.4/+1.5	11.8/9.0	131
X[f]	400 D1	0/6	−2.0/+2.5	12.0/8.5	141t
	300	0/6	−2.0/+1.0	12.0/8.3	144
	150 D1	0/6	−1.4/+1.0	10.2/8.3	122
	75 DR	0/6	−0.6/+1.0	8.3/8.3	100
XI	400 D1	0/6	−3.6/−0.3	9.2/8.6	106
	200 D1	1/6	−1.5/+1.7	10.6/9.2	115
	400 q.d. 1–9	7/7			t
	100 q.d. 1–9	0/12	−2.5/+1.8	12.3/8.5	145[e]
	150	0/6	−3.7/+1.4	9.5/8.3	114
	100 q.d. 1–9	0/6	−2.9/+1.4	11.7/8.3	140
	66 DR	0/6	−1.6/+1.4	15.3/8.3	184
	44	0/6	−1.0/+1.4	13.7/8.3	165
XII	400 D1	0/6	+2.1/+2.6	9.7/8.8	110
	400 q.d. 1–9	0/12	−1.2/+1.4	14.9/8.9	167[e]
	600	0/6	−1.2/+3.0	16.7/7.5	222
	400 q.d. 1–9	0/6	−1.5/+3.0	15.5/7.5	205
	268 DR	0/6	−0.2/+3.0	12.7/7.5	169
	176	0/6	+1.2/+3.0	11.2/7.5	149
	176 q.d. 1–9	0/6	−0.6/+1.6	13.2/8.3	159
	118 DR	0/6	−0.5/+1.6	11.2/8.3	134
	80	0/6	−0.2/+1.6	10.8/8.3	130
XIV[g]	300	3/6	−0.3/+1.2	11.7/9.3	125t
	150 D1	0/5	+0.7/+1.2	10.4/9.3	111
	75 DR	0/6	+1.7/+1.2	9.7/9.3	104
	150	0/6	+0.6/+1.7	12.3/8.3	148
	100 q.d. 1–9[h]	0/6	+0.6/+1.7	12.0/8.3	144
	67 DR	0/6	+0.5/+1.7	10.2/8.3	122
IV*a*	40 D1	6/6			t
	20 D1	0/6	−1.1/+2.0	9.2/9.0	102
	10 D1	0/6	+0.8/+2.0	8.7/9.0	96
	20 q.d. 1–9	6/6			t
	10 q.d. 1–9	0/6	−1.9/+1.9	7.5/8.8	85t
	5 q.d. 1–9	0/6	−0.6/+0.7	9.0/8.4	107

Table I—*Continued*

Compound	Dose[b], mg./kg., and Schedule[c]	Mortality, Deaths/Total	Average Weight Change, g., T/C	Average Survival Time— T/C, Days	T/C Ratio[d], %
IV*b*	40 D1	6/6			t
	20 D1	0/6	−0.8/+2.0	8.8/9.0	97
	20 q.d. 1–9	6/6			t
	10 q.d. 1–9	1/6	−1.9/+1.9	7.2/8.8	81t
	5 q.d. 1–9	0/6	−1.1/+0.7	9.2/8.4	109
XVIII	400 D1	0/6	+0.9/+2.8	7.5/8.7	86
	400 q.d. 1–9	0/6	−2.4/+1.0	8.0/9.2	86

[a] Solutions or suspensions of the compounds (VI, IV*a*, and IV*b* in physiological saline, V in physiological saline containing 0.4% carboxymethylcellulose, and the remaining compounds in physiological saline containing 0.15% polysorbate 80) were protected from light and were injected immediately after they were prepared. Mice were implanted intraperitoneally on Day 0 with 10^5 L-1210 cells. Treatment schedules designated D1 (Day 1) or D2 (Day 2) mean that a single dose was injected intraperitoneally on the 1st day (about 24 hr.) or on the 2nd day (about 48 hr.), respectively, after implantation; q.d. 1–9 means that daily injections at the specified dose were initiated on Day 1 and continued through Day 9 or until the death of the animal. Average weight change = average weight change of host animals. Mortality and weight changes of host animals were determined 4 days after the first (for daily treatment) or only (for Day 1 or Day 2) injection. T = treated mice, C = control (untreated leukemic) mice. [b] Some of the higher (toxic) and lower (inactive) doses are not included. [c] DR = dose response. These doses were administered on the designated schedule on the same day in the same controlled experiment. [d] t = toxic. A rating of toxic may be based upon mortality (>2/6), a value of T/C of 85% or less, or a weight-change difference exceeding −4 g. after Day 1 or Day 2 treatment. The latter criterion is not applied to results of daily administration. [e] Values in the last three columns are approximate averages of two tests. [f] *cf.*, *Reference 13* for additional data. [g] NMR assays showed that the specimen used for daily treatment contained about 25% of the isomeric *v*-triazolinium salt at the beginning of the tests; the specimen used for the D1 tests was similarly shown to contain about 3–4% of the *v*-triazolinium salt. [h] The dose of 150 mg./kg./day was administered q.d. 1–8.

maxima; and (*b*) the triazenoimidazole disappeared more rapidly than did its pyrazole analog.

Solutions of the pyrazole methyl ester (VI) and the analogous imidazole methyl ester (*Ib*, R = R_1 = R_2 = CH_3) (6) in 50% aqueous ethanol were exposed simultaneously in the same way to 365-nm. radiation at a distance of 10 cm. Again, both compounds decomposed, and the imidazole derivative decomposed significantly faster than the pyrazole derivative. The following two comparisons also appear to be justified, but there is less assurance that the experimental conditions for the photochemical change were equalized: (*a*) although the two methyl esters were not exposed simultaneously with their amide analogs, the last-mentioned experiment was comparable to Experiment *b* with the two amides, and the results indicate that the esters change more slowly in 365-nm. light than do their amide analogs; and (*b*) exposure of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide and its *v*-triazole analog similarly, but not simultaneously, in 50% methanol to 365-nm. radiation resulted, in agreement with an earlier suggestion (11), in slower disappearance of the triazeno-*v*-triazole. Consistent with, but not directly comparable to, the foregoing observations of light sensitivity of triazenopyrazoles was an earlier observation of slow decomposition of XI in absolute methanol under ambient lighting conditions. Although the triazenopyrazoles may not be rapidly degraded under conditions of normal use, the observations described here show that they should be handled as light-sensitive compounds until their stability to prevailing lighting conditions is determined.

BIOLOGICAL ACTIVITY

Results of tests of Compounds V–VIII, X–XIV, and XVIII *versus* murine lymphoid leukemia L-1210 are summarized in Table I. The data show that triazeno derivatives of both the ester and amide series prolong the survival time of treated animals. Increases in survival time of 60–122% (T/C = 160–222%) were recorded in some tests of the esters VI–VIII and of the amides XI–XII administered in multiple daily doses (q.d. 1–9), and the dose–response data for these derivatives are internally consistent. Compounds V, X, and XIV also significantly increased the lifespan of treated mice. The 3-(2-hydroxyethyl)-3-methyl-1-triazeno derivative (XII) resembles three 5-[3-(2-hydroxyethyl)-3-methyl-1-triazeno]imidazoles (5, 7) in inhibiting L-1210 at doses higher than tolerated doses of similar dialkyltriazeno derivatives. The three 3,3-[bis(2-haloethyl)-1-triazeno]pyrazoles (IX, XIV, and XV) were less effective in these initial screening tests than were the corresponding imidazole derivatives (Table I) (10, 12). The fact that the activity of XIV is less than that of the analogous imidazole (NSC-82196) may be the result of a faster rate of transformation to the isomeric *v*-triazolinium salt, but there are no data to support this supposition. The bulk material used in the single-dose tests was at least comparable in quality to specimens of NSC-82196 used in similar tests; the specimen used for chronic therapy contained about 25% of the *v*-triazolinium salt.

In tests *in vitro* of Compounds V–XV and XVIII in the paper-disk-agar-plate method, there was no observable inhibition of several Gram-negative and Gram-positive bacteria or of several species of fungi, except for evidence of slight inhibition of *Streptococcus faecalis* (ATCC 8043) by Compounds V, VI, and IX at 100 mcg./disk. The absence of antimicrobial activity contrasts with the antimicrobial activity of triazenoimidazoles, particularly with the broad-spectrum activity of the esters (6, 7, 19). The diazopyrazole esters (V and VI), but not the amide (X), displayed antibacterial and antifungal activity *in vitro*.

EXPERIMENTAL

During all operations employed in the preparation, isolation, or purification of the triazenopyrazoles, they were routinely protected from light. Isolated specimens were routinely stored in a refrigerator, except for bis(2-haloethyl)triazeno derivatives which were stored in a freezer at −10 to −15°. UV and IR spectra were recorded with a Cary spectrophotometer (model 14) and with Perkin-Elmer spectrophotometers (models 521 or 621), respectively. The IR bands listed were taken from spectra of samples in pressed KBr disks, unless stated otherwise, and are in $cm.^{-1}$; s = strong, m = medium, w = weak, b = broad, and sh = shoulder. Solutions used to determine UV data were prepared by diluting a 5-ml. aliquot of an ethanol solution of the compound to 50 ml. with 0.1 *N* HCl, phosphate buffer (pH 7), or 0.1 *N* NaOH; absorption maxima are in nanometers.

Melting or decomposition temperatures were determined in a capillary tube with a Mel-Temp or a Mettler FP-1 apparatus unless otherwise stated. TLC was performed by applying 20- and 40-mcg. portions of a compound in ethanol (IV*a*, IV*b*, VI–VIII, and X–XII) or methanol (IX, XIV, XV, and XVIII) to silica gel plates. The developing solvents are given in the experimental procedures. The methods of detection of spots on all chromatograms were both UV absorption (254 and 365 nm.) and UV light (254 nm.) after spraying with an optical whitening agent[2]. In addition, some chromatograms were also examined after exposure to iodine vapor.

[2] Ultraphor WT, BASF Colors and Chemicals, Inc., Charlotte, N. C.

Ethyl 3-Diazopyrazole-4-carboxylate (IV*a*)—*Procedure A*—To a solution of 10 mmoles (1.55 g.) of ethyl 3-aminopyrazole-4-carboxylate (III*a*) in 25 ml. of 1 *N* HCl at 0° was added, during 5 min., a solution of 11 mmoles (760 mg.) of sodium nitrite in 5 ml. of water. The resulting turbid solution was stirred at 0° for 0.5 hr. and filtered, the filtrate was extracted with three 50-ml. portions of chloroform, and the chloroform layers were combined. The total chloroform extract was washed with 50 ml. of saturated NaCl solution, dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The residual yellow syrup crystallized: yield, 849 mg. (51%); m.p. 67–68° (Kofler Heizbank); TLC, 1 spot (9:1 $CHCl_3$–CH_3OH); IR bands at 2290w, 2110w, 2210s (N_2^+), 1700s (ester CO), 1530ms, and 768 ms.

Anal.—Calcd. for $C_6H_6N_4O_2$: C, 43.38; H, 3.64; N, 33.72. Found: C, 43.29; H, 3.54; N, 33.67.

Procedure B—This procedure was the same as Procedure A except that: (*a*) the solution of III*a* was added to the solution of sodium nitrite at such a rate that the temperature did not exceed 3°, and (*b*) the reaction mixture was stirred for 1 hr. prior to the chloroform extraction. The yield of IV*a*, identical with a specimen obtained by Procedure A, was 44%.

Methyl 3-Diazopyrazole-4-carboxylate (IV*b*)—This compound was prepared from 10 mmoles of III*b* by a procedure that otherwise duplicated Procedure A for the preparation of IV*a*: yield, 21%; m.p. 72–73°; TLC, 1 spot (9:1 $CHCl_3$–CH_3OH); IR bands at 2285w, 2120w, 2215s (N_2^+), 1710s (ester CO), 1535ms, 1525sh, and 760ms.

Anal.—Calcd. for $C_5H_4N_4O_2$: C, 39.48; H, 2.65; N, 36.84. Found: C, 39.24; H, 2.69; N, 36.71.

A similar diazotization of 8 g. of III*b*, performed for the purpose of preparing VIII without isolating IV*b*, gave chiefly the bis(pyrazolyl)triazene (XVIII) (see the procedures for VIII). Addition of the solution of III*b* (8 g. in 100 ml. of 1 *N* HCl) to the nitrite solution (4.31 g. in 50 ml. of water) during 40 min. at 0–3°, followed by stirring for 0.5 hr. and isolation as in Procedure A for IV*a*, gave IV*b* in 41% yield (m.p. 72–73°). Recrystallization of a specimen by diluting an ethyl acetate solution (previously treated with activated carbon and concentrated *in vacuo*) with cyclohexane gave pale-yellow crystals which were apparently in a different crystal form: m.p. 91–92°; TLC, 1 spot (98:2 $CHCl_3$–CH_3OH); IR spectrum, identical with that of the specimen obtained by the first procedure except for some differences in the 1300–850 $cm.^{-1}$ region.

Anal.—Calcd. for $C_5H_4N_4O_2$: C, 39.48; H, 2.65; N, 36.84. Found: C, 39.39; H, 2.60; N, 36.82.

3-Diazopyrazole-4-carboxamide (IV*c*)—This compound was prepared by Procedure A for IV*a* with the following exceptions: (*a*) equimolar amounts of III*c* (free base) and 1 *N* HCl were used, and (*b*) the product was separated by filtration after 2.5 hr. of stirring at 0°. The yellow solid (42% yield) exploded on a Kofler Heizbank apparatus at about 180–185°. The IR spectrum was identical with that of a specimen prepared by the procedure subsequently reported by Cheng *et al.* (15): IR bands at 2215sb (N_2^+), 1680s, 1625s, 1535s, 775mw, 750mw, and 722s.

Ethyl 3-(3,3-Dimethyl-1-triazeno)pyrazole-4-carboxylate (V)—Procedure A for the preparation of IV*a* was duplicated with the following exceptions: (*a*) the reaction time was 1 hr.; (*b*) isolation of IV*a* by chloroform extraction was omitted; and (*c*) excess nitrosating agents were decomposed with sulfamic acid. The cold aqueous solution of IV*a* was diluted with 15 ml. of 25% aqueous dimethylamine, and the resulting solution was stirred at room temperature for 2.5 hr. Extraction of the reaction mixture with chloroform as described in Procedure A for IV*a* afforded a pale-yellow crystalline solid (1.9 g., 90%). Dissolution of the crude triazene in 20 ml. of hot ethyl acetate, treatment of the solution with activated carbon, dilution of the hot filtrate with 20 ml. of cyclohexane, and cooling the resulting solution afforded yellow platelets: wt., 1.4 g. (71%); m.p. 113.5–115.5°; IR bands at 1710s and 1685s (ester CO), 1570m, 1495s, and 775m. $UV_{max.}$ ($\epsilon \times 10^{-3}$): 226 (9.7) and 312 (12.1) in 0.1 *N* HCl; 230 (11.0) and 313 (11.2) at pH 7; 244 (14.2) and 314 (8.8) in 0.1 *N* NaOH.

Anal.—Calcd. for $C_8H_{13}N_5O_2$: C, 45.49; H, 6.20; N, 33.16. Found: C, 45.64; H, 6.19; N, 32.89.

Methyl 3-(3,3-Dimethyl-1-triazeno)pyrazole-4-carboxylate (VI)—*Procedure A*—By a procedure similar to that described for the preparation of V, Compound VI was prepared from 2 g. of III*b*. A small quantity of an unidentified solid was filtered from the reaction mixture before the extraction with chloroform. Recrystallization of crude VI from ethyl acetate–cyclohexane (1:3) in the manner described for V gave white crystals: yield, 62%; m.p. 103°; TLC, 1 spot (95:5 $CHCl_3$–CH_3OH); IR bands at 1720s (ester CO), 1568ms, 1485ms, and 772m. $UV_{max.}$ ($\epsilon \times 10^{-3}$): 226 (9.5) and 312 (12.1) in 0.1 *N* HCl; 230 (10.9) and 313 (11.3) at pH 7; 244 (13.7) and 315 (8.95) in 0.1 *N* NaOH.

Anal.—Calcd. for $C_7H_{11}N_5O_2$: C, 42.63; H, 5.62; N, 35.52. Found: C, 42.41; H, 5.64; N, 35.65.

Procedure B—Anhydrous dimethylamine was bubbled into 35 ml. of ethyl acetate at 0–5° until the volume had increased to about 40 ml., and 1.8 g. of IV*b* was added in small portions during 1 hr. at 0°. The mixture was stirred for an additional hour at 0°, treated with activated carbon, and concentrated *in vacuo* to a yellow solid which was triturated with a mixture (9:1) of cyclohexane–ethyl acetate. Recrystallization of the residual solid from ethyl acetate–cyclohexane gave 1.22 g. of VI (m.p. 103–104°) identical with the specimen obtained from Procedure A.

Ethyl 3-(3-Butyl-3-methyl-1-triazeno)pyrazole-4-carboxylate (VII)—To a solution of 1.96 g. of sodium nitrite in 10 ml. of water at 0° was added dropwise a cold solution of 4.0 g. of III*a* in 50 ml. of 1 *N* HCl at a rate that kept the temperature at 0–5°. The yellow solution was stirred at 0° for 1 hr., excess nitrosating agents were destroyed by the addition of sulfamic acid, the mixture was filtered, the filtrate was diluted with 10 ml. of *n*-butylmethylamine, and the resulting reaction mixture was stirred in the dark at room temperature for 3 hr. The chloroform solution obtained by extracting the reaction mixture with three 70-ml. portions of chloroform was washed with 70 ml. of saturated aqueous NaCl, dried with $MgSO_4$, treated with activated carbon, filtered through diatomaceous earth[3], and evaporated *in vacuo*. The residual yellow syrup (6.5 g.) was dissolved in purified cyclohexane and a small quantity of acetone (to effect dissolution) and applied to a column (60 g.) of magnesia-silica gel[4] prewashed with cyclohexane. The column was eluted with cyclohexane and then with acetone, and the fractions shown by TLC to contain pure VII were combined and concentrated *in vacuo*. The residue was a very viscous, faintly yellow syrup which was kept *in vacuo* for a prolonged period to remove the last traces of solvents: yield, 5.7 g. (88%); TLC, 1 spot (95:5 or 99:1 $CHCl_3$–CH_3OH); IR bands (film) at 1715sh and 1690s (ester CO), 1565ms, 1490sh, and 775ms. $UV_{max.}$ ($\epsilon \times 10^{-3}$): 313 (12.3) in 0.1 *N* HCl; 231 (10.6) and 313 (11.4) at pH 7; 245 (14.4) and 313 (8.8) in 0.1 *N* NaOH.

Anal.—Calcd. for $C_{11}H_{19}N_5O_2$: C, 52.16; H, 7.56; N, 27.65. Found: C, 52.33; H, 7.67; N, 27.77.

Methyl 3-(3-Butyl-3-methyl-1-triazeno)pyrazole-4-carboxylate (VIII)—This compound was obtained from III*b* (6.3 g.) by a procedure similar to that described for the preparation of VII. The yellow syrup obtained from the chloroform extract was precipitated from an ether solution by the addition of cyclohexane. This process was repeated, the purified syrup was then treated in ether with activated carbon, and the ether filtrate was concentrated *in vacuo* to a viscous, colorless syrup which was kept *in vacuo* for 16 hr. at room temperature and for 1 hr. at 56°: yield, 59%; TLC, 1 spot (99:1 $CHCl_3$–CH_3OH); IR bands (film) at 1715sh and 1695s (ester CO), 1565ms, 1480sh, and 780m. $UV_{max.}$ ($\epsilon \times 10^{-3}$): 313 (12.2) in 0.1 *N* HCl; 230 (10.6) and 314 (11.7) at pH 7; 244 (14.1) and 314 (9.1) in 0.1 *N* NaOH. When VIII was stored at low temperature, it crystallized to a low melting solid.

Anal.—Calcd. for $C_{10}H_{17}N_5O_2$: C, 50.19; H, 7.16; N, 29.27. Found: C, 50.03; H, 7.28; N, 29.29.

A pure specimen of VIII was initially obtained in 27% yield from 2 g. of III*b* by a similar procedure, except that the diazotization was performed by adding the nitrite solution immediately to III*b*. However, on a somewhat larger scale (8 g. of III*b* in 100 ml. of 1 *N* HCl and 4.32 g. of sodium nitrite in 20 ml. of water), this procedure gave chiefly XVIII (5.3 g.), which was removed by filtration after excess nitrite was decomposed. The filtrate afforded only a slight yield of VIII.

Dimethyl 3,3′-Diazoaminodipyrazole-4-carboxylate (XVIII) (*cf.*, the preceding paragraph)—To a solution of 1.411 g. (10 mmoles) of III*b* in 25 ml. of 1 *N* HCl at 0° was added, over a 5-min. period, a solution of 390 mg. (5.65 mmoles) of sodium nitrite in 5 ml. of water. A heavy yellow precipitate was present in the solution (after 1.5 hr.), but the reaction mixture gave a positive Bratton–Marshall test. An additional 706 mg. (5 mmoles) of III*b* in 10 ml. of water was

[3] Celite, Johns-Manville.
[4] Florisil, The Floridin Co.

added. The mixture was stirred at room temperature for 18 hr., at which time a second solution of 706 mg. of III*b* in 10 ml. of water was added and stirring was continued for 2 hr. The yellow precipitate was collected by filtration, washed thoroughly with water, and dried *in vacuo* at 56°: yield, 1.50 g.; m.p. 153–155° dec. The analytical sample was obtained by recrystallizing 500 mg. of the crude product from 140 ml. of boiling ethanol: recovery of pale-yellow crystals, 350 mg.; m.p. 162–163° dec.; TLC, 1 spot (5:1 $CHCl_3$–CH_3OH or 86:14 butanol–water); IR bands at 1715s and 1690s (ester CO), 1615w, 1580s, 1570sh, 1550ms, 1520m, 1492s, and 778s. $UV_{max.}$ ($\epsilon \times 10^{-3}$): 232 (13.1) and 347 (15.3) at pH 7.

Anal.—Calcd. for $C_{10}H_{11}N_7O_4$: C, 40.95; H, 3.78; N, 33.44. Found: C, 40.75; H, 3.90; N, 33.44.

3-(3,3-Dimethyl-1-triazeno)pyrazole-4-carboxamide (X)—This compound was prepared from IV*c* by a procedure similar to Procedure B for VI, except that the additional stirring time was 2.5 hr. and the precipitated X was isolated by filtration (86% yield) and could be recrystallized from ethyl acetate containing about 2% ethanol: m.p. 188–190° dec. (inserted at 170°, heating rate 3°/min.); TLC, 1 spot (5:1 $CHCl_3$–CH_3OH); IR bands at 1640s, 1595ms, 1500m, 1480m, and 778w. UV extinction coefficients were somewhat higher and the decomposition temperature was somewhat lower than those reported (13). $UV_{max.}$ ($\epsilon \times 10^{-3}$): 317 (14.5) in 0.1 *N* HCl; 230 (11.2) and 318 (13.0) at pH 7; 243 (12.1) and 325 (11.2) in 0.1 *N* NaOH.

Anal.—Calcd. for $C_6H_{10}N_6O$: C, 53.52; H, 5.53; N, 46.13. Found: C, 53.71; H, 5.59; N, 45.95.

3-(3-Butyl-3-methyl-1-triazeno)pyrazole-4-carboxamide (XI)—A solution of IV*c* was prepared by adding slowly a solution of 301 mg. of sodium nitrite in 5 ml. of water to a suspension of 500 mg. of III*c* hemisulfate in 8.3 ml. of 1 *N* HCl at 0°, stirring the mixture for 15 min., destroying excess nitrosating agents with sulfamic acid, and filtering to remove a yellow precipitate. The filtrate was diluted with 3 ml. of *n*-butylmethylamine, the reaction mixture was stirred at room temperature for 1 hr., and the pH of the solution was adjusted to 6.6 with aqueous HCl. Addition of 50 ml. of chloroform caused the separation of platelets which were collected by filtration after the mixture had been chilled: yield, 550 mg. (62%); m.p. 142–145° dec. Recrystallization of 450 mg. of the crude product from ethyl acetate–cyclohexane (3:5) in the manner described for V gave white crystals: recovery, 325 mg.; m.p. 148–150° dec.; TLC, 1 spot (9:1 $CHCl_3$–CH_3OH); IR bands at 1645s, 1595s, 1505ms, 810mb, and 790sh. $UV_{max.}$ ($\epsilon \times 10^{-3}$): 319 (16.2) in 0.1 *N* HCl; 232 (11.3) and 319 (14.3) at pH 7; 244 (12.3) and 325 (12.6) in 0.1 *N* NaOH. The extinction coefficient (14.7) at 320 nm. of a methanol solution of XI left unprotected from light decreased by about 10% during 48 hr.

Anal.—Calcd. for $C_9H_{16}N_6O$: C, 48.20; H, 7.18; N, 37.48. Found: C, 48.25; H, 6.90; N, 37.20.

3-[3-(2-Hydroxyethyl)-3-methyl-1-triazeno]pyrazole-4-carboxamide (XII)—To a solution of 2.06 g. (27.5 mmoles) of freshly distilled 2-(methylamino)ethanol in 25 ml. of anhydrous ethanol at 0° was added, during 0.5 hr., 1.25 g. (9.1 mmoles) of IV*c*; the resulting mixture was stirred at 0° for 0.5 hr. The yellow precipitate was collected by filtration, washed with ethanol, and dried *in vacuo* at 56°: yield, 1.50 g. (78%); m.p. 172–174° dec. Recrystallization of a specimen from ethanol, with the aid of activated carbon, gave white crystals: m.p. 173–175° dec.; TLC, 1 spot (3:1 $CHCl_3$–CH_3OH); IR bands at 1650s, 1615s, 1510ms, 1480ms, and 780m. $UV_{max.}$ ($\epsilon \times 10^{-3}$): 225 (10.1) and 318 (15.2) in 0.1 *N* HCl; 230 (11.4) and 318 (13.6) at pH 7; 243 (12.6) and 325 (12.1) in 0.1 *N* NaOH.

Anal.—Calcd. for $C_7H_{12}N_6O_2$: C, 39.62; H, 5.71; N, 39.61. Found: C, 39.72; H, 5.37; N, 39.25.

3-(3-Methyl-1-triazeno)pyrazole-4-carboxamide (XIII)—The monomethyltriazeno derivative was prepared from 500 mg. of IV*c* and anhydrous methylamine in methanol by a procedure similar to that described for XII, except that IV*c* was added during 5 min.: yield of yellow precipitate, 267 mg. (44%); m.p. 105–115° dec.; IR bands at 1665s, 1610s, 1520mb, and 780mw. Analytical data were satisfactory for a hemihydrate of XIII but not for anhydrous material. It is likely that the material was not entirely pure, but further purification was not attempted because of the instability of XIII.

Anal.—Calcd. for $C_5H_8N_6O \cdot {}^1/_2H_2O$: C, 33.89; H, 5.12; N, 47.44. Found: C, 33.83; H, 5.05; N, 47.15.

A mixture of 150 mg. of XIII and 12 ml. of water–methanol (5:1) became homogeneous within 4.5 hr.; TLC after 5 hr. indicated that decomposition to III*c* was complete. Concentration of the solution *in vacuo* and trituration of the residual syrup with ether gave a yellow solid which was collected by filtration, washed with ether, and dried *in vacuo* at 56°: yield, 88 mg. (79%); m.p. 175–182°. Comparison of IR, UV, and TLC (5:3:2 butanol–H_2O–acetic acid) data from this material with those of an authentic sample of III*c* (m.p. 184–186°) showed the two samples to be identical.

3-[3,3-Bis(2-chloroethyl)-1-triazeno]pyrazole-4-carboxamide (XIV)—A solution of bis(2-chloroethyl)amine free base was prepared by the previously described (4) procedure, except that ethyl acetate was substituted for dichloromethane. The solution obtained from 8.85 g. of the hydrochloride and two 40-ml. portions of ethyl acetate was diluted with 8 ml. of anhydrous methanol; then 1.01 g. of IV*c* was added in portions, with stirring, during 30 min. The reaction mixture was stirred for 45 min., diluted with 50 ml. of cyclohexane, and filtered to remove a water-soluble precipitate (156 mg.). The filtrate was concentrated *in vacuo* to a volume of about 15 ml. and then diluted with 150 ml. of cyclohexane. A gummy solid precipitated; it was separated by decanting the supernatant liquid and was stirred for 15 min. with 50 ml. of a 1:1 mixture of ethyl acetate–cyclohexane. A granular solid was separated by filtration, washed with the same solvent mixture, and dried *in vacuo* at room temperature: yield, 1.30 g. (64%); m.p. 178–182° dec. An NMR analysis indicated that about 53% of this solid was the *v*-triazolinium salt formed from XIV. Therefore, the solid (1.2 g.) was stirred in 15 ml. of water at room temperature for 5 min., separated by filtration, washed with water, and dried *in vacuo* at 56° for 15 min. and at room temperature for 3.5 hr.: wt., 480 mg.; m.p. 110° with resolidification and remelting at 185–188° dec.; IR bands at 3375ms, 1645s, 1588ms, 1560sh, 1505mb, 780m, and 742mw. During TLC (9:1 $CHCl_3$–CH_3OH), some of the *v*-triazolinium salt was formed and appeared as a weak spot at the origin and as a streak to the fast moving major spot (XIV).

Anal.—Calcd. for $C_8H_{12}Cl_2N_6O$: C, 34.42; H, 4.33; N, 30.11; Cl, 25.40. Found: C, 34.58; H, 4.37; N, 29.93; Cl, 25.24.

An NMR analysis indicated that the quantity of the isomeric *v*-triazolinium salt present in this material was about 3–4% or less. This specimen was used in the D1 tests summarized in Table I. Although there was evidence of deterioration of some earlier specimens of XIV stored at low temperatures, the amount of the *v*-triazolinium salt in this specimen remained essentially unchanged during 4 months at −10°.

Stability Observations[5]—Observations of the relative stability of triazeno derivatives in solution were made by preparing the solutions in the dark in Pyrex volumetric flasks, withdrawing aliquot portions at convenient time intervals, diluting the aliquots with phosphate buffer (pH 7), and recording the UV spectra at pH 7. When the stabilities of the two compounds were to be compared, the solutions were placed side-by-side (with one exception) to equalize the conditions. The decrease in absorbance of the long wavelength maximum was considered to correspond to the disappearance of the triazeno derivative. The concomitant formation of a bicyclic *v*-triazinone (XVI, 2-azahypoxanthine, or 2,8-diazahypoxanthine) could be observed if substantial dissociation of an amide derivative occurred, but it was not established that the *v*-triazinone was the sole product. The difference between the wavelengths of the absorption maxima at pH 7 of 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (330 nm.) and 2-azahypoxanthine (286 nm., *Reference 1*) is large, and the absorbance of the latter compound is essentially zero at 330 nm. This difference is less for the pyrazole analog (X) (317 nm.) and the pyrazolo-*v*-triazinone (XVI) (doublet at 275 and 286 nm.), and absorption by XVI extends beyond the long wavelength maximum of X. There is more over-

[5] These experiments were performed to determine whether triazenopyrazoles are light sensitive and to compare compounds under like conditions. The data recorded here are presented in support of conclusions summarized in the *Discussion;* they should not be regarded as controlled rate determinations. In these experiments, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide decomposed in sunlight and in light in the 360–370-nm. region more rapidly than it had in earlier experiments (5) monitored by TLC, but exposure conditions differed. In the earlier experiments, the concentration was greater (25 mg./5 ml.), exposure to sunlight (in 50% methanol) occurred at different times of the year, and exposure of the 50% ethanol solution to light in the 365-nm. region was at a distance of 15 cm. (6 in.).

lapping of absorption in mixtures of X and XVI than in mixtures of 2-azahypoxanthine and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide and less accuracy in estimating substantial decreases in the molar absorbance of X than of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide. There is essentially no absorption by 2,8-diazahypoxanthine ($UV_{max.}$ 278 nm.) at the long wavelength maximum (315 nm.) of the *v*-triazole analog (II). For all solutions placed in radiation in the 365-nm. region[6], the two solutions being compared were kept in a small dark room at room temperature (about 26–28°). Exposure to light caused increases of a few degrees in the temperature of the solutions[5]. The following experiments were performed according to the procedures and under the conditions described above.

Experiment 1—Solutions of X (about 4 mg.) and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (about 6 mg.) in 50% aqueous ethanol contained in 25-ml. Pyrex volumetric flasks were placed side-by-side and exposed simultaneously to bright, direct sunlight passing through window glass. The temperature of the solutions reached 40° after 2 hr. The observed changes in absorbance (*A*) of X at 317 nm. were as follows (hr., percent change): 0.5, −7; 1, −9; 2, −31; and 3.5, −46. The disappearance of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide was virtually complete within 0.5 hr. (change of $A \geq 98\%$) and complete within 1 hr. The final spectra [after 1 hr. for 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide and after further exposure of X the following day] indicated that XVI and 2-azahypoxanthine were the predominant products formed from X and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, respectively.

Experiment 2—Solutions of X (about 5 mg.) and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (about 5 mg.) in 50% aqueous ethanol contained in 25-ml. Pyrex volumetric flasks were suspended side-by-side at a distance of 10 cm. (distance from the surface of the lamp filter to the wall of the flask) from a UV source[6] with maximum light emission at 365–366 nm. The changes in *A* of X at 317 nm. were as follows (hr., percent change): 0.5, −6; 1, −41; 2.5, −58; and 3.5, −71. The changes in 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide were the same as those described for Experiment 1.

Experiment 3—Solutions of X (14.6 mg.) and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (9.9 mg.) in 50 ml. of a phosphate buffer of the Krebs–Ringer type were exposed to 365-nm. light as described in Experiment 2, except that the distance was 15 cm. The changes in *A* of X at 317 nm. were as follows (hr., percent change): 1, +3; 4, −5; 7, −8; 24, −31; 48, −62; 72, −88. The changes in *A* of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide at 330 nm. were as follows (hr., percent change): 1, −85; and 4, −99. The decreased rates of change in the Krebs–Ringer solution relative to those in 50% ethanol are probably due in part to the increased distance from the light source.

Experiment 4—Solutions of II (6.8 mg./50 ml.) and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (12.5 mg./50 ml.) in 50% methanol were exposed to UV light as described in Experiment 2, except that the distance was 15 cm. and the two solutions were not irradiated simultaneously. The changes in *A* of II at 315 nm. were as follows (hr., percent change): 1, −25; 4, −93; and 6, −97. The disappearance of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide was almost complete within 1 hr. and complete within 2.5 hr. (A weak shoulder remained in the 330-nm. region after 1 hr., decrease in *A* about 96–97%.)

[6] The UV light source was a General Electric Bonus Line mercury reflector flood lamp, No. H100PFL 38-4, 100 watts (Hanovia Type 16106 power supply), fitted with a red-purple glass filter, Corning No. 5874, to remove most of the visible light. According to the manufacturer's data, the spectral radiant characteristics of the lamp and the transmission properties of the filter are such that about 80% of the radiation emitted by the lamp-filter combination should be between 360 and 370 nm. Since the mercury-emission spectrum has lines at 365–366 nm., the light emitted by the lamp-filter combination is assumed to be principally at 365–366 nm. No attempt was made to evaluate the influence of aging of the lamp and filter on the spectral properties of the emitted light.

Experiment 5—Solutions of VI (10 mg.) and methyl 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxylate (Ib, R = R_1 = R_2 = CH_3) (10 mg.) in 50 ml. of 50% aqueous ethanol were exposed to 365-nm. light as described in Experiment 2. The changes in *A* of VI at 313 nm. were as follows (hr., percent change): 1, −10; 3, −17; 7, −20; 25, −45; 50, −90; and 75, −100. The changes in *A* of the imidazole analog at 330 nm. were as follows (hr., percent change): 1, −8; 3, −28; 7, −64; 25, −99; and 50, −100. The final spectra showed maxima at 221 and 235 nm., respectively.

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Acetaminophen III: Dissolution Studies of Commercial Tablets of Acetaminophen and Comparison with *In Vivo* Absorption Parameters

G. L. MATTOK, I. J. McGILVERAY, and C. A. MAINVILLE

Abstract □ Dissolution studies of eight different lots of acetaminophen tablets were investigated by means of three simple dissolution procedures. None of the methods provided complete correlation with physiological availability of acetaminophen when estimated from blood or urine profiles. Two lots of tablets, which were several years old, released the drug more slowly *in vitro* than more recently purchased lots of the same brands. All of the recently manufactured products required less than 15 min. for 50% dissolution; this time appears to be a reasonable limit for tablet formulations of acetaminophen.

Keyphrases □ Acetaminophen tablets—dissolution □ Absorption–dissolution parameters, comparison—acetaminophen □ Aging effects—acetaminophen tablet dissolution □ UV spectrophotometry—analysis

The design of an apparatus, suitable for the measurement of dissolution rates of solid dosage forms, is relatively straightforward. Hersey (1) commented recently that: "The number of dissolution rate methods described in the literature is almost equal to the number of workers in the field." However, there is a paucity of data comparing dissolution data from the various dissolution tests with quantitative *in vivo* availability results.

Levy (2) emphasized the need for *in vitro* tests that reflect faithfully dissolution rate-limited absorption of drugs from pharmaceutical dosage forms. A number of studies showed that dissolution characteristics of selected dosage forms of certain drugs (3–9), obtained using specific dissolution conditions, parallel blood or urine levels of the drug. However, no dissolution apparatus has been evaluated which would predict physiological availabilities of a variety of formulations of different drugs. [Many examples are cited in a recent bibliography by Wagner (10).]

In this study, eight lots of tablets, representing six brands of the moderately soluble drug acetaminophen, were examined with three simple dissolution tests. The dissolution times from the three tests with all the tablets were compared with physiological availability parameters obtained in 10 subjects in a separate study (11).

EXPERIMENTAL

Materials—Eight lots of tablets[1] (K_1, K_2, K_3, N, P, Q, R, and S) were used. The tablets, which had been evaluated in physiological availability studies, contained 300–500 mg. of acetaminophen according to label strength (11). Four newer lots (Q_2, Q_3, R_2, and R_3) and one other product (U) containing sorbitol were also studied.

Methods—Literature methods with some modifications were used. The resin kettle method (RK) differed from the original description (12) in that 900 ml. of dissolution fluid was used and the stirrer was centered in the jar so that the bottom of the shaft was 6.3 cm. from the bottom of the jar. The stationary basket method (SB) was similar to that described by Cook *et al.* (13), but for the present work the basket was constructed with a 30-mesh wire screen. The USP XVIII/NF XIII Method I or rotating basket method (14) was used with a stirring rate of 100 r.p.m. and 900 ml. dissolution fluid. Disintegration times were measured by an official method (15) using six tablets in simulated gastric and intestinal fluids.

Dissolution Fluids—Simulated gastric (0.1 *N* HCl) and intestinal (0.05 *M* phosphate buffer, pH 7.4) fluids were used.

Sample Assay—The same sampling procedure was used for the three methods. A 1-ml. Luer fitting tuberculin syringe, graduated in 0.01-ml. units, was attached to a medium-porosity glass filter by means of a female Luer joint (16). This device eliminated most particulate matter from the transfer of the sample to volumetric flasks. The sample (1.0 ml.) was diluted to 25 ml. with the dissolution fluid; its absorbance was measured at 243 nm. with a Beckman DU-2 spectrophotometer. The calibration curve for acetaminophen reference standard in gastric buffer is linear up to 25 mg./l.

Calculations—The label strength of each tablet was accepted. The concentration of each sample, corrected for the volume change caused by sample removal, was calculated from the calibration

[1] Letter indicates brand, number indicates different lots.

Table I—Dissolution Times of Acetaminophen Tablets in Gastric Buffer Using USP Method

Brand	Range[a], min.	t_{50}, min.	$\pm SD$	CV	T_{50}, min.
K_1	2.5–3.5	3.0	0.45	14.9	3.0
K_2	3–5	3.9	0.74	18.7	4.0
K_3	2–3.5	2.8	0.52	19.0	2.5
N	1.5–2.5	2.0	0.32	15.8	2.0
P	1.5–2.5	1.3	0.60	45.3	1.0
Q_1	16–22.6	20.2	3.47	17.2	20.0
R_1	14–29	20.2	6.71	33.2	18.5
S	1.0	1.0	0.00	0.0	1.0
Q_2	7.0, 6.5[b]	—	—	—	—
Q_3	5.0, 4.5[b]	—	—	—	—
R_2	4.0, 3.5[b]	—	—	—	—
R_3	1.5, 1.5[b]	—	—	—	—
U[c]	3.0, 3.0[b]	—	—	—	—

[a] Six-tablet test. [b] Two-tablet test. [c] Contained sorbitol.

Table II—Dissolution Times of Acetaminophen Tablets in Gastric Buffer Using RK Method

Brand	Range[a], min.	t_{50}, min.	$\pm SD$	CV	T_{50}, min.
K_1	3.5–13	5.3	2.81	53.6	4.5
K_2	2–11	4.9	2.80	57.7	3.5
K_3	3–12.5	7.7	3.82	49.9	6.0
N	2–3	2.6	0.39	15.2	3.0
P	0.5–1	0.95	0.16	16.6	1.0
Q_1	23–37	30.3	4.6	15.2	30.0
R_1	23.5–39	31.5	4.7	14.8	30.5
S	1.0	1.0	0.00	0.0	1.0
Q_2	5.5, 4.0[b]	—	—	—	—
Q_3	14.5, 12.0[b]	—	—	—	—
R_2	3.0, 3.0[b]	—	—	—	—
R_3	2.0, 2.0[b]	—	—	—	—
U[c]	4.5, 4.0[b]	—	—	—	—

[a] Ten-tablet test. [b] Two-tablet test. [c] Contained sorbitol.

Table III—Individual Dissolution Times (t_{50}) for Acetaminophen Tablets

Brand	t_{50}, min., in: Gastric Buffer SB Method	Intestinal Buffer SB Method	Intestinal Buffer RK Method
K_1	5.5, 5.0	6.0, 3.0	7.5, 4.0
K_2	5.0, 5.5	7.5, 14.0	8.5, 14.5
K_3	7.0, 6.0	9.5, 16.0	8.0, 25.0
N	7.0, 5.5	4.5, 3.0	2.0, 2.0
P	5.0, 4.0	2.0, 1.0	1.0, 1.0
Q_1	36.0, 35.0	105, 106	110, 126
R_1	32.0, 21.0	24.0, 27.0	15.0, 28.0
S	5.0, 5.0	1.0, 3.0	1.0, 0.5
Q_2	21.5, 18.5	76 , 69	
Q_3	13.5, 13.5	8.0, 14.0	
R_2	5.0, 5.0	6.5, 7.5	
R_3	5.0, 4.0	8 , 4.5	
U[a]	5.0, 6.5	6.0, 4.0	

[a] Contained sorbitol.

curve. The t_{50} values (or times for 50% of the labeled drug content to pass into solution) were obtained from a semilog plot of the percent undissolved drug against time. Mean t_{50} values, *i.e.*, $\bar{t}_{50}$, based on measurements with 10 tablets (RK method) or six tablets (USP/NF method), standard deviations, and coefficients of variation were then calculated. A plot of the average percent undissolved for 10 (RK method) or six (USP/NF method) tablets against sampling time provides a "mean dissolution rate curve" from which T_{50} was estimated. Dissolution measurements with the SB method were carried out on two tablets.

Physiological Availability—The protocol and regimen for the administration of the eight lots of acetaminophen tablets to 10 volunteers were described (11, 17). Blood samples collected at 20 and 40 min. and 1, 1.5, 2, 4, and 6 hr. after ingestion provided data for the estimation of the area under the free drug blood level curve (corrected to the same dose) for each tablet. Similarly, urine was collected at 1, 1.5, 2, 2.5, 3, 4, 5, 8, 11, 14, and 24 hr.; the mean availability of each dosage form was obtained from the total urinary excretion.

RESULTS

Dissolution—Dissolution characteristics of the original eight lots of acetaminophen tablets and five more recent lots are summarized in Tables I, II, and III. A typical set of curves used for calculating T_{50} values is shown in Fig. 1. Dissolution profiles for Q_1 with the RK method are compared in intestinal and gastric buffers in Fig. 2.

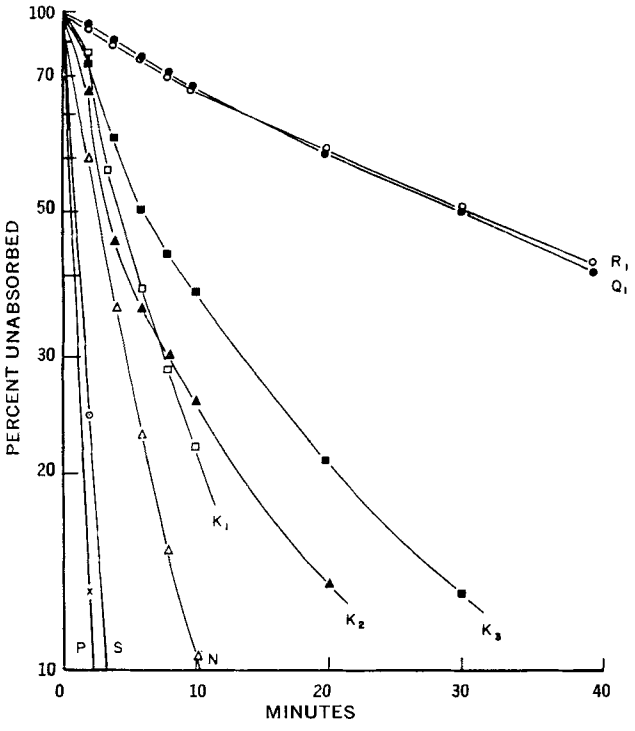

Figure 1—*Acetaminophen tablets. Curves for derivation of* T_{50} *in gastric buffer using RK method.*

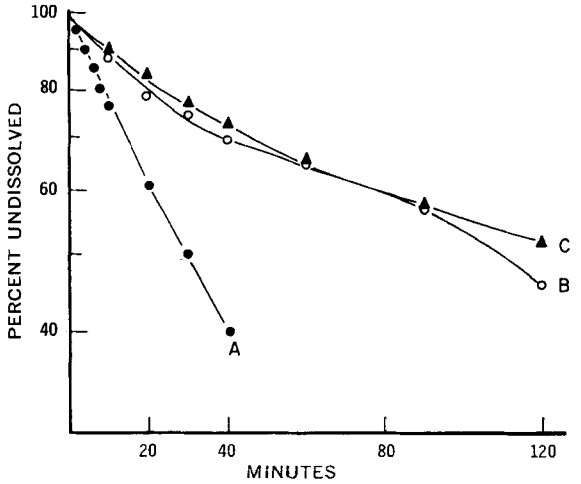

Figure 2—*Dissolution rate profiles with RK method for acetaminophen formulation* Q_1. *Key: A, average values* (±SD) *for 10 tablets in gastric buffer; B and C, individual tablets in intestinal buffer.*

Disintegration—Disintegration times of six tablets from lots K_1, Q_1, Q_2, Q_3, and R_1 in simulated gastric and intestinal buffers are shown in Table IV.

Physiological Availability—The results of a physiological availability study of acetaminophen dosage forms were fully described previously (11) and are summarized in Table V.

DISCUSSION

Comparison of Brands—Most of the acetaminophen tablets examined had $\bar{t}_{50}$ and T_{50} values in gastric buffer of less than 10 min. with the RK and USP/NF methods (Tables I and II). This limit was also indicated by single-tablet t_{50} values obtained with the SB method where two tablets of each lot number were tested (Table III). In the original group of eight lot numbers, brands Q_1 and R_1 were outstanding. These products had dissolution half-lives ($\bar{t}_{50}$ or T_{50}) more than twice as long as the other formulations in all the tests used. Since the formulations of these products were not known, only speculations can be made concerning the longer dissolution times. These differences in dissolution properties (Tables I–III) cannot be attributed to disintegration characteristics since disintegration times (Table IV) of Q_1 and R_1 in gastric buffer were comparable to those of K. However, it was known that these two samples had been on the shelf for about 3 years and they could thus have been "aged" compared to the others.

To establish whether more recently prepared lots of the same br nds would give altered dissolution characteristics, two tablet screening tests were done on two other lots of brands Q and R where the sequence of ages was $Q_1 > Q_2 > Q_3$ and $R_1 > R_2$ and R_3. The results are presented at the foot of Tables I, II, and III. Although there was apparently some interlot variation, the newer lots were closer to the other brands in dissolution behavior; in the Q series,

Table IV—Disintegration Times of Acetaminophen Tablets

Brand	Dissolution Medium	Range[a], min.	Mean[a], min.
K_1	Gastric	3–10	6
Q_1	Gastric	3–4	3
Q_2	Gastric	1–2	1
Q_3	Gastric	1.0	1
R_1	Gastric	1.5–2	2
K_1	Intestinal	3.5–4	4
Q_1	Intestinal	29–36	33
Q_2	Intestinal	28–38	31
Q_3	Intestinal	4–9	6
R_1	Intestinal	0.5–1	1

[a] Six tablets.

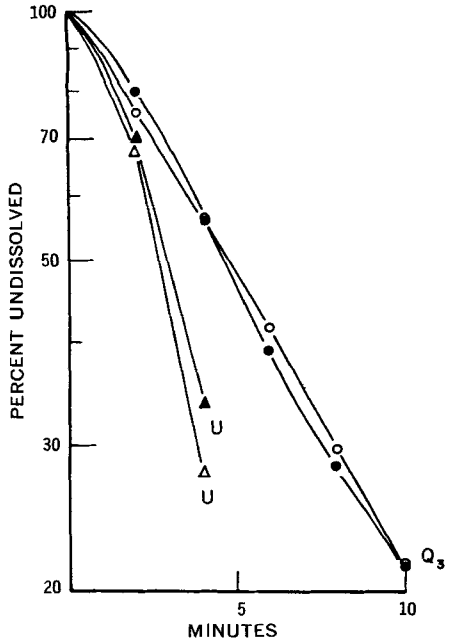

Figure 3—*Acetaminophen formulations Q_3 and U (with sorbitol). Individual curves for two tablets in gastric buffer using USP XVIII/NF XIII method.*

dissolution time increased with the age of the product. These findings suggest that either the formulations were changed to shorten the dissolution times or that the dissolution times of these formulations had increased with age.

A similar gradation in dissolution time in intestinal buffer was also observed (Table III). The dissolution half-life (t_{50}) of Q_1 increased three- to fourfold, and the mean disintegration time increased from 3 to 33 min. when the tests were done in intestinal buffer. Similar increases in t_{50} values were found for Q_2 and Q_3, t_{50} increasing with the age of the product, *i.e.*, $Q_1 > Q_2 > Q_3$. Furthermore, whereas dissolution of Q_1 in gastric buffer in the RK apparatus followed a regular exponential curve, first-order dissolution did not occur in intestinal fluid (Fig. 2). This was probably due to the poor disintegration properties of Q_1 in intestinal fluid which may

Table V—Physiological Availabilities from Blood Levels of Free Drug (PAB) and Urinary Excretion (PAU) of Acetaminophen Dosage Forms

Formulation	PAB, %	PAU, %
K_1	92.4	88.9
K_2	85.1	89.8
K_3	78.4	85.3
N	87.3	90.7
P	102.7	97.7
Q_1	91.8	89.3
R_1	98.9	92.9
S	97.1	88.1
T (control)	100.0	100.0

have resulted from aging. It was not due to a change in the intrinsic dissolution characteristics of acetaminophen at this concentration, since all the other formulations could be expected to be affected in a similar manner by the change of medium.

Tablets from three lot numbers of formulations K showed modest increases in t_{50} when the dissolution medium was changed from gastric to intestinal buffer in the SB method (Table III). However, the mean disintegration times for K_1 in intestinal fluid (4 min.) was somewhat less than in gastric buffer (6 min.). The increased t_{50} values in intestinal fluid must, therefore, be attributed to formulation rather than aging.

Formulation U, a commercial acetaminophen tablet, has a base containing sorbitol which is reported to improve the absorption of the drug (18). The dissolution half-lives of these tablets with any of the three methods were unremarkable compared to the normal group. These results are in contrast to the findings of Walters (19) who reported differences in $t_{0.9}$ (or $t_{90\%}$)—the time for solution of 90% of the drug—when two commercial formulations of acetaminophen were compared with another containing sorbitol. Since the dissolution apparatus used by Walters (19) was different from any of the three used in this study, any comparison with his results must be made with caution. Walters (19) found that the sorbitol-containing preparation gave t_{50} values of 3–4 min. However, dissolution times for U were close to those for all other brands examined except the "aged" formulations Q_1 and R_1 (Table I). Furthermore, the dissolution rate curve (Fig. 3) is similar to that of many other brands.

Comparison of Dissolution Methods—On the basis of the results of the present investigation, a comparison can be made of the RK and USP/NF dissolution methods with gastric fluid as the dissolu-

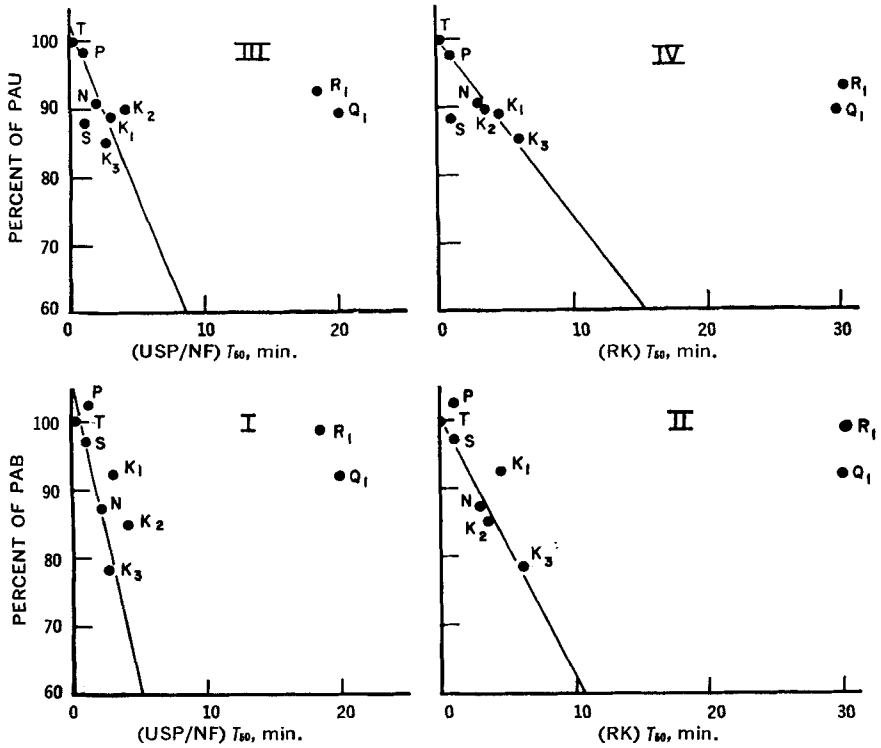

Figure 4—*Acetaminophen dosage forms. Plot of physiological availabilities (PA) from blood levels (I and II) and total urinary excretion (III and IV) against dissolution half-lives (T_{50}) in gastric buffer.*

tion medium. Rankings of the various formulations according to their $\bar{t}_{50}$ and T_{50} values are similar for both methods. Brands S and P release acetaminophen very rapidly, with T_{50} less than 1 min., while Q_1 and R_1 are the slowest to dissolve. These slower dissolving brands reverse their order of ranking (based on T_{50} values) in the two methods. This is attributed to intertablet variation, since the spread of individual t_{50} values is quite wide.

The most serious difference in the order of ranking and magnitude of values occurs with K_3, where $\bar{t}_{50}$ moves from 2.8 to 7.7 min. and T_{50} from 2.5 to 6.0 min. in the USP/NF and RK methods, respectively. The SB method shows a longer individual t_{50} value (6–7 min.) for K_3 compared to the USP/NF method. Individual tablet results for all lots of K indicate that the RK method is more sensitive to tablet variation than the USP/NF method. This is reflected in the coefficients of variation which are in the range 15–19 for the USP/NF method (Table I) and 50–58 for the RK method (Table II).

Individual tablet variation was also evident from the $\bar{t}_{50}$ values obtained with the SB and RK methods using intestinal buffer. The intertablet variations appear to be real, rather than artefacts of the RK system, since some other formulations tested showed similar coefficients of variation in both USP/NF and RK methods. In only two cases, P and R, was the USP/NF method more sensitive, based on coefficients of variation, than the RK method (Tables I and II). However, in the case of P the difference is exaggerated since t_{50} is low and the rate of dissolution is too rapid to derive a well-defined dissolution curve.

The USP/NF method gave shorter dissolution half-lives than the RK method for all brands except P and S which dissolved extremely rapidly, and discrimination was beyond the sensitivity of these intermittent sampling methods. This was to be expected (20) in view of the increased agitation in the USP/NF system. In addition to the increased stirring rate (100 *versus* 60 r.p.m.) used in the USP/NF method, the rotating basket of the USP/NF apparatus has a slight abrading effect on a tablet surface whereas the RK method depends only on liquid turbulence. Both USP/NF and RK methods appear to give an adequate assessment of acetaminophen tablet formulations for the purpose of a control laboratory. With brand K, where more than one lot was sampled, the RK method gave larger interlot and intralot variations and for this formulation it was the more discriminating method. The two-tablet tests done with Q_2 and Q_3 indicate that this conclusion may also be valid for formulation Q.

Comparison of Physiological Availability with Dissolution Data—Several authors have presented dissolution and absorption or physiological availability data on a few formulations of the same drug without attempting a direct correlation, *e.g.*, phenylbutazone (5), tolbutamide (8), and sulfisoxazole (7). Any correlation of availability–dissolution properties assumes that dissolution is the rate-limiting step in the absorption process. Most correlations which have been attempted were done with very few dosage forms and, even then, the correlation did not embrace all the dosage forms. Middleton *et al.* (21) were among the first to attempt to establish such a relationship. They plotted percent availability (from urinary excretion data) against disintegration and dissolution times (T_{50}). These results were used to demonstrate the increased predictive value of dissolution characteristics over disintegration in assessing the physiological availabilities of formulations. When this graphical method was applied to the acetaminophen dosage forms and the control solution (T), which was considered to have T_{50} of zero, most points were grouped close together except for the two older dosage forms, Q_1 and R_1, which had longer dissolution times (Fig. 4). With this type of curve, since there is no significant difference in availabilities (11), the availabilities of the different dosage forms should tend to the same value; therefore, the dissolution tests were more sensitive to changes in formulation than the biological tests. Any effect that the slower dissolution times of tablets Q_1 and R_1 may have had on the total absorption of acetaminophen must be hidden in the other variables of the trial.

A more sophisticated correlation of dissolution and absorption of drugs was introduced by Levy and his coworkers (22, 23). The rate of absorption of each dosage form was calculated and compared directly with the dissolution rate. The assumption was made that the kinetic model fitting the drug absorption process would usually also apply to the *in vivo* dissolution process when the absorption rate is dissolution rate-limited. It was further assumed that *in vitro* dissolution parallels *in vivo* dissolution. Thus, if percent dissolved *in vitro* is multiplied by a factor equal to the ratio of the first-order rate constants for *in vivo* absorption and *in vitro* dissolution and plotted against percent absorbed *in vivo* at the same time, a straight line of slope unity is obtained (23). With the present results, subject variations in the *in vivo* data prevented calculation of meaningful absorption rates by the Wagner–Nelson method (24) for each dosage form. Studies of these variations in acetaminophen metabolism are currently underway. For the same reason, the correlation of Cressman *et al.* (9) was inapplicable.

The dissolution rates of the acetaminophen tablets in this study provided T_{50} values varying from 1 to 30 min. with the RK method and from 1 to 20 min. with the USP/NF method. These differences in dissolution rates were not reflected in the physiologic availability. The more recently produced lots of tablets gave dissolution T_{50} values with either the USP/NF or RK method of under 15 min., and this would be a reasonable maximum value to propose for tablet formulations of this drug. (For the USP/NF method, a 10-min. maximum would appear to suffice.) The effect of tablet aging on tablet dissolution rate deserves further study.

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Solubility of Amino Acids in Pure Solvent Systems

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Abstract □ The effects of various solvents and pH on the solubilities of glycine, L-alanine, L-valine, L-phenylalanine, and DL-aminooctanoic acid were studied in a series of pure aqueous and alcoholic solutions. The aqueous solubility was found to be inversely proportional to the size of the nonpolar portion of the molecule. A low nonaqueous solubility seemed to be due to a dominance of the amino acid by the charged α-amino carboxylic acid portion of the molecule. In aqueous and alcoholic solutions, an isoelectric band of minimum solubility was formed. A distinct increase in solubility proportional to the addition of acid or base was seen as the pH exceeded the limits of the isoelectric band. In the alcoholic solvent systems studied, the addition of either acid or base produced a greater divergence from the isoelectric pH than would be seen in a pure aqueous system

Keyphrases □ Amino acid solubility—pure solvent systems □ Alcoholic, aqueous systems—amino acid solubility □ Isoelectric pH—amino acid minimum solubility □ Acid, base addition effects—amino acid solubility, pure solvents

The importance of amino acids in biological systems is due to the unique properties of this class of chemical compounds. These substances, possessing both acidic and basic properties within the same molecule, are the building blocks leading to complex peptides and proteins which are the basic materials of life.

In 1933, Edsall and Blanchard (1) examined the dissolution and dissociation of several amino acids in aqueous systems. Other studies (2, 3) tabulated the solubility of several amino acids in water and ethanol and presented some theory as to their behavior.

At this time, much research has been done (4, 5) and is being conducted on the selective uptake of several amino acids by various body organs. The emphasis has been placed on qualifying the activity of the *in vivo* receptors. With this in mind, it was felt that a solubility study of a series of amino acids in a series of related solvents at pH above, at, and below the isoelectric point would be useful in predicting solubility patterns which might be related to the nature and properties of these amino acids and their behavior *in vivo*.

EXPERIMENTAL

Equipment—A rotating apparatus[1] capable of holding multiple samples was immersed in a water bath kept at constant temperature by a controlled temperature circulating pump[2]. The pH and apparent pH of nonaqueous systems (pH′) were measured using a combination electrode.

Systems Employed—The amino acids used in this study were glycine, L-alanine, L-valine, L-phenylalanine, and DL-aminooctanoic acid. The solvents used were water (which had been distilled and deionized), anhydrous menthanol, absolute ethanol, *n*-propanol, isopropanol, and tertiary butanol. The label purity of the manufacturer was accepted, and no pretreatment of the chemicals was felt necessary. The water was deionized using a mixed column bed to minimize the possibility of complex formation. Hydrochloric acid and sodium hydroxide were used to adjust pH. All work was performed at a temperature of 25 ± 0.2°.

[1] Menold Rotating Apparatus, Lester, Pa.
[2] Porta-temp., Precision Scientific Co., Chicago, Ill.

Table I—Molar Solubility of Amino Acids in Pure Solvents

Solvent Systems	Amino Acids[a] Glycine	L-Alanine	L-Valine	L-Phenylalanine
H_2O[b]	2.90	1.63	0.474	0.172
Methanol	0.009	0.012	0.013	0.022
Ethanol[b]	0.004	0.002	0.003	0.002
n-Propanol	0.011	0.002	0.003	0.002
Isopropanol	0.004	0.005	0.003	0.001
Tertiary butanol	0.004	0.001	0.007	0.001

[a] Each value is the average of at least three determinations. [b] Solubility in agreement with original data published by Cohn (7).

Procedure—The solubilities of the amino acids in pure solvents were all determined by the same method. At least four samples containing equal volumes of solvent were prepared, an excess amount of the selected amino acid was added, and the sample bottles were rotated in the constant-temperature bath for 24 hr. or until equilibrium was attained. Either 1- or 5-ml. samples were withdrawn from the sample bottles, using a pipet with a pledget of glass wool on the end as a filtering medium. The size of the sample to be withdrawn was determined by preliminary experimentation to minimize error during gravimetric analysis. The sample was immediately transferred to a tared vial, weighed to determine density, placed in an oven, and dried to constant weight. All drying was done at a temperature of 95° or less to avoid decomposition of the amino acids (6). Immediately after the sampling process, the pH or pH′ of each solution was taken.

In the nonaqueous solvents, the relationship between total amino acid solubility and pH′ was the main concern. In the pure aqueous solvent system, the experimental interest included salt formation as well as the difference in total solubility as a function of pH.

After initial sample withdrawal and pH′ check of the nonaqueous systems, incremental amounts of acid were added to half of the samples in succeeding 24-hr. periods, the pH′ was taken, and a gravimetric analysis was made on the samples. Base was added to the remaining samples and a similar analysis was performed. For the aqueous systems, the same analysis was performed as well as an additional procedure designed to quantitate the amount of salt present at a given pH. One hundred milliliters of water, a specified

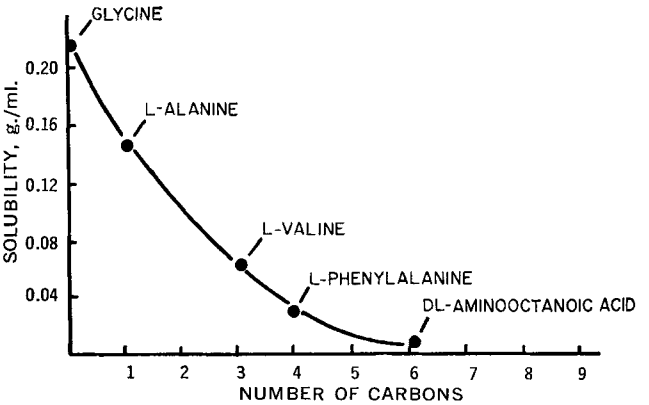

Figure 1—*Solubility of amino acids as a function of the number of carbons attached to the α-amino group.*

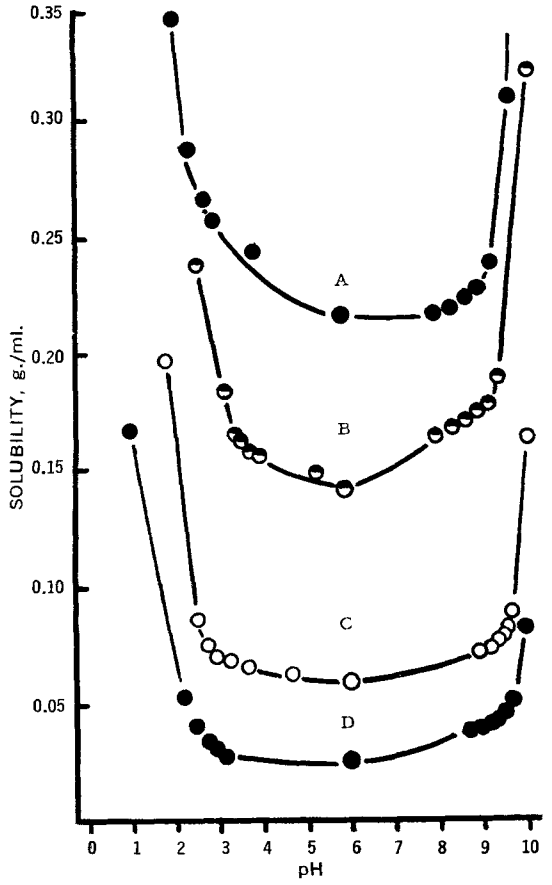

Figure 2—*Change in aqueous solubility of the amino acids as a function of pH. Key: Curve A, glycine; Curve B,* L-*alanine; Curve C,* L-*valine; and Curve D,* L-*phenylalanine.*

amount of HCl or NaOH, and an excess of amino acid were placed in a conical flask, capped, and mixed with a magnetic stirrer until equilibrium was reached. After 24 hr., at least fifteen 5-ml. samples were withdrawn from the flask and examined. To those samples previously mixed with HCl, five accurately measured incremental amounts of NaOH solution were added in sets of three each, so that the pH increased to just below the isoelectric point. Precipitation occurred and the samples were allowed to equilibrate for 24 hr. The amount of precipitate, the amount of combined amino acids remaining in the solutions, and the pH of the solutions were determined using the previously described procedure. A similar process was followed for the samples mixed with NaOH, except that HCl was added in incremental amounts to a pH just above the isoelectric point.

RESULTS AND DISCUSSION

The aqueous solubility of the series of amino acids studied seems to decrease with increasing length of the nonpolar portion of the molecule. If the α-amino carboxylic acid group of glycine is considered to be the primary polar unit of the amino acids, then an increase in the nonpolar portion of the amino acid molecules should produce a solubility decrease (7). The total solubility was due to the combined solubilities of the polar and nonpolar portions of the molecule. In Fig. 1, this expected solubility is shown by a plot of experimental data which shows a nonlinear decrease when the number of carbon atoms attached to the α-amino carboxylic group is increased. The assumption that the solubility of an aromatic ring is approximately equivalent to three CH_2 groups (2) accounts for the inclusion of L-phenylalanine at the fourth carbon, between L-valine and DL-aminooctanoic acid rather than at the seventh carbon, and further substantiates the adoption of glycine as a basic solubility unit.

Table II—Effect of Adding Acid on Total Aqueous Solubility of Amino Acids[a]

Amino Acid	*Y*-Intercept	Slope	Correlation Coefficient
Glycine	2.92	0.6583	0.9990
L-Alanine	1.67	0.2738	0.9988
L-Valine	0.514	0.4360	0.9948
L-Phenylalanine	0.208	0.2094	0.8818

[a] Moles/liter HCl as a function of amino acid solubility (moles/liter).

Table III—Effect of Adding Base on Total Aqueous Solubility of Amino Acids[a]

Amino Acid	*Y*-Intercept	Slope	Correlation Coefficient
Glycine	2.90	0.3986	0.9936
L-Alanine	1.75	0.4715	0.9978
L-Valine	0.458	0.5402	0.9968
L-Phenylalanine	0.167	0.5069	0.9910

[a] Moles/liter NaOH as a function of amino acid solubility (moles/liter).

As expected from Cohn's (7) work in ethanolic solvents systems, all alcoholic solvents decreased the solubility of the amino acids to a point considerably below that of water alone. Table I illustrates the magnitude of the reduction in solubility when solvents other than water were used. When the decrease in solubility from aqueous to nonaqueous media was compared (Table I), it was found that the percentage reduction in amino acid solubility was inversely related to the length of the nonpolar portion of the molecule. However, the molar solubilities in the alcoholic solvents were all of the same magnitude, which seemed to indicate that, in alcohols, the α-amino carboxylic acid group dominates the solubility characteristics of those amino acids studied.

The addition of acid or base to an amino acid in aqueous solution at the isoelectric point produced a change in the species present. The addition of HCl to a neutral solution of an amino acid produced cations in proportion to the first dissociation constant (K_1). Conversely, the addition of NaOH to a neutral aqueous solution of amino acid produced anionic species as a function of the K_2 (1).

$$K_1 = \frac{(H^+)\,(^+H_3NRCOO—)}{(^+H_3NRCOOH)} \qquad \text{(Eq. 1)}$$

$$K_2 = \frac{(H^+)\,(H_2NRCOO—)}{(^+H_3NRCOO—)} \qquad \text{(Eq. 2)}$$

The isoelectric point of those amino acids studied was defined (2) as the pH value at which the net charge of the dipolar molecule is equal to zero and pH = $^1/_2(pK_1 + pK_2)$. However, if the solubility is plotted as a function of the pH, as illustrated in Fig. 2, the presence of an invariant band of similar solubility over a range of pH

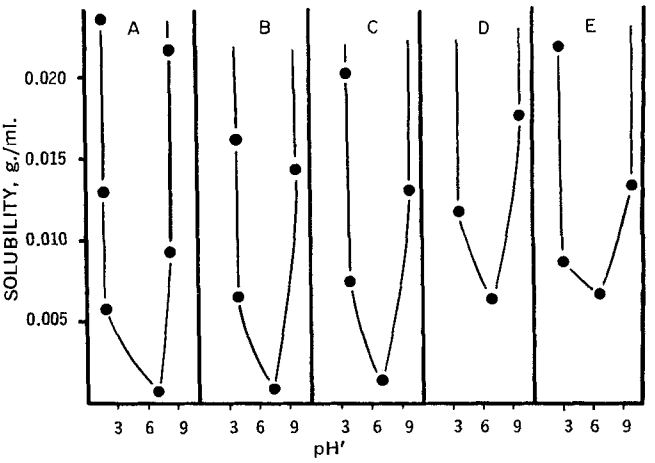

Figure 3—*Solubility of amino acids in pure methanol as a function of pH'. Key: Column A, glycine; Column B,* L-*alanine; Column C,* L-*valine; Column D,* L-*phenylalanine; and Column E,* DL-*aminooctanoic acid.*

Table IV—Effect of Acid and Base Addition on pH and Solubility of Glycine in Methanol and Water[a]

Water			Methanol		
pH	Total Solubility, g./ml.	Acid or Base Added, g./ml.	pH	Total Solubility, g./ml.	Acid or Base Added, g./ml.
2.7	0.3287	0.1417			
3.0	0.3036	0.0631			
3.8	0.2328	0.0080	2.20	0.0260	0.0224
4.4	0.2236	0.0041	3.30	0.0126	0.0074
5.3	0.2182	0.0002	3.25	0.0054	0.0037
6.2	0.2176	0.0000	7.05	0.0009	0.0000
6.8	0.2180	0.0004	9.65	0.0093	0.1053
8.1	0.2212	0.0073	9.75	0.0217	0.0107
8.3	0.2254	0.0142	9.9	0.0458	0.0213
9.0	0.2725	0.0622			
9.3	0.2957	0.1103			

[a] Each value is the average of at least three determinations.

on either side of the calculated isoelectic point is seen for each amino acid. This invariance is followed by increases in solubility as the pH is increased or decreased on either side of the band. The presence of an isoelectric band rather than a single isoelectric point was described by Cohn and Edsall (2). They postulated that for a simple amino acid, whose K_1 and K_2 are sufficiently separated, an isoelectric band is produced, throughout which the concentration of cations and anions is very low and the dipolar species dominates. This behavior can be further explained if Eqs. 1 and 2 are rearranged in terms of the Henderson–Hasselbach buffer equation:

$$\text{pH} = pK_1 + \log (C_A/C_S) \quad \text{(Eq. 3)}$$

$$\text{pH} = pK_2 + \log (C_S/C_A) \quad \text{(Eq. 4)}$$

where C_A is the solubility attributed to the dipolar ionic form of the amino acid, and C_S is the solubility due to the anion or cation that forms an associated salt with the added acid or base. It can be seen that when the pH is equal to the pK, the ratio of the C_S to C_A or C_A to C_S will be one, and the maximum buffer effect will be present. As expected, total solubility shows an increase upon addition of acid or base without significant change in pH.

It would seem that the total solubility of an amino acid is the sum of the solubility due to the zwitterion plus that due to the anion or cation. To examine this hypothesis quantitatively, the solubility range of each amino acid was separated into two parts: that influenced by acid addition and that influenced by base addition. A computer program was prepared to calculate, using a least-squares analysis, the first-degree equation and the correlation coefficients for two variables. The moles of acid or base added was designated as the independent variable, and the total molar solubility of the amino acid was designated as the dependent variable. In all calculations of total solubility, the assumption was made that the amino acid combined in a mole-to-mole ratio with the acid or base.

Tables II and III show the calculated first-degree equation parameters when X moles of either HCl or NaOH was added. The Y-intercepts indicate the initial solubility of the amino acid, and these closely approximate the experimentally observed values. In the general first-degree equation, $Y = mX + b$, the slope should be one if total solubility was equal to the solubility due to the salt form plus the initial solubility of the zwitterionic species. However, the calculated slopes show a deviation from the expected values. Since the intercepts closely approximate their intended values, these differences are probably due to a combination of acid and/or base with amino acid in other than the expected 1:1 ratio. It would seem that the half HCl salts mentioned in the literature (3) are not formed but that the reciprocal is true because more moles of acid are required per mole of salt formed. Since there is a rather wide deviation of slope from one amino acid to another, it would also seem that the ratio of the association salt formed depends on the individual characteristics of each amino acid, including the affinity of each amino acid for protons and the possible solute–solute interactions. This can be seen in Figs. 2 and 3 by examining the reciprocal behavior of the slopes upon addition of acid and base. This behavior may be due in part to the ability of the amino acid to accept or release hydrogen ions.

It can be seen from Fig. 3 that the addition of acid or base to a neutral solution of the alcoholic solvents produced a solubility profile similar to that seen in water. This figure illustrates the relative solubility of the amino acids in neutral solutions of methanol and the subsequent increase in solubility as the pH′ was varied. This illustration is representative of the solubility changes observed with variation of pH′ in each of the semipolar solvents tested. Note, for example, the increase in the pH′ at minimum solubility in neutral solution as compared to that seen in Fig. 2 for aqueous solvents. This change in pH′ was expected since methanol has a lower autoprotolysis constant than water. In ethanol, *n*-propanol, isopropanol, and tertiary butanol, the shift in the pH′ at minimum solubility was also attributable to difference in autoprotolysis.

Table IV illustrates the difference in solubility and pH′ as a function of acid or base added for glycine in water and methanol. In all semipolar solvents studied, the addition of either acid or base produced a greater divergence from the isolectric pH than would be seen in a similar aqueous system. It would seem that this change in behavior is mainly due to a lower buffer capacity produced in these less solubilizing solvents.

In conclusion, for the amino acids studied in aqueous solvent systems, a band of minimum solubility was found at pH values above and below the isoelectric point and a distinct increase in total solubility was seen as the pH exceeded the limits of the isoelectric band. Further, the increase in total solubility of the amino acids was linearly related to the moles of acid or base added. In the alcoholic solvents, a similar solubility–pH profile was found; but due to the lower solubility of the amino acids in these solvents, the addition of acid or base produced a greater divergence from the isoelectric pH than was seen in aqueous systems. The isoelectric region in the alcoholic systems seemed to be shifted as a function of the autoprotolysis constant of the specific solvent.

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Toxicity of Ethylene Chlorohydrin I: Acute Toxicity Studies

W. H. LAWRENCE, J. E. TURNER, and J. AUTIAN

Abstract □ The LD_{50} of ethylene chlorohydrin was evaluated in mice, rats, rabbits, and guinea pigs, and the range of values was found to fall between 64 and 98 mg./kg. The mean time of death from vapors of the compound was calculated to be 13.3 min. when mice were used as the test animals. Dermal tests in rabbits produced no significant irritant response even when applied undiluted; but when the compound was injected intradermally or instilled into the eye, it was extremely irritating. Ethylene chlorohydrin was found to be cytotoxic in cell culture and to have an inhibitory effect upon cell growth in cultures. Since plastic devices are being ethylene oxide-sterilized and since ethylene chlorohydrin may form as a reaction product, it is necessary to be aware of the toxic potentials of this compound.

Keyphrases □ Ethylene chlorohydrin—acute toxicity studies □ Dermal, intradermal, ocular toxicity—ethylene chlorohydrin □ Inhalation toxicity—ethylene chlorohydrin □ Cell culture growth inhibition—ethylene chlorohydrin

One useful method of sterilizing plastic and elastomeric medical devices is by the use of ethylene oxide alone or in combination with an inert gas. This method of sterilization is now common practice in the medical and paramedical industries which produce nearly an unlimited number of disposable devices for the hospital. Most hospital pharmacies and central supply departments also have installed ethylene oxide units as an adjunct to the autoclave. Small portable units are now appearing in doctors' and dentists' offices.

The presence of residual amounts of ethylene oxide in a polymeric device can present potential toxicologic problems if the ethylene oxide is released to tissue. In recent years, it also has been found that foods and various types of plastic items might contain reaction products of ethylene oxide, namely, ethylene glycol and ethylene chlorohydrin. The latter is formed in the presence of chloride ions, in food or a polymeric material. In 1965, Wesley *et al.* (1) published their results of the production of ethylene chlorohydrin in foods fumigated with ethylene oxide. Two years later, Cunliffe and Wesley (2) brought to the attention of the medical world that ethylene chlorohydrin could result from the sterilization of polyvinyl chloride medical tubings with ethylene oxide. Since then a great deal of interest has developed concerning the potential toxic response from materials sterilized with ethylene oxide.

Ethylene chlorohydrin, also known as 2-chloroethanol and β-chloroethyl alcohol, is a colorless liquid at room temperature, with an odor resembling a mixture of ethyl alcohol and ether[1]. It has a molecular weight of 80.52, a specific gravity of 1.213 (at 20°), and a boiling point of 128.8°. It is miscible in all proportions with water, ethyl alcohol, and ether.

Ethylene chlorohydrin has a number of industrial uses such as solvents for certain types of polymers and lacquers and as a starting material for synthesis of a host of organic compounds. It has been investigated also in agricultural sciences for hastening the early sprouting of dormant potatoes and treating seeds for the inhibition of biological activity (3, 4).

One of the first well-substantiated toxicity observations in humans due to ethylene chlorohydrin was reported by Koelsch in 1927 (5). Of nine cases of chlorohydrin toxicity, two were fatal. Koelsch also conducted animal experiments on the compound. Since then, others (6–9) have presented evidence on the toxicity of ethylene chlorohydrin. Blecket and Strube (10) reported on percutaneous toxicity in humans exposed to chlorohydrin in industrial settings. More recently, Guess (11) presented data on its toxicity to animal tissues and cells in culture.

The formation of ethylene chlorohydrin in polymeric materials due to ethylene oxide sterilization and the possible toxic effects of this compound when released to biological systems prompted this toxicity study of the compound in the hopes of confirming and supplementing existing toxicity data. Since ethylene chlorohydrin is used in the food industry or may be produced in foods containing chlorides when the food is treated with ethylene oxide, the same toxicity information may have further applicability outside the medical and paramedical fields. The results of acute toxicity experiments provide the basis for this report. A subsequent report will describe the results of subacute toxicity studies.

EXPERIMENTAL

Materials—A detailed analysis for purity was not performed on the ethylene chlorohydrin[2] used in these experiments, but gas chromatography revealed the liquid was essentially 99% ethylene chlorohydrin. The liquid was used without further purification throughout the experiments.

LD_{50} Determinations—Experiments were conducted on male mice (Swiss-Webster), male rats (Sprague-Dawley), male and female rabbits (New Zealand albino), and female guinea pigs (Hartley strain). Graded doses of chlorohydrin were administered to groups of four or five animals per dose level, and the animals were observed 1 week for mortalities[3]. The effect of two different routes of administration on the LD_{50} of chlorohydrin was studied in three species of the animals used. The LD_{50} values were then calculated according to Cornfield–Mantel's modification (12) of Karber's method and/or the method of Weil (13).

Dermal administration of chlorohydrin in rabbits was performed by adding the required dose of the compound to a single Webril patch of sufficient size to absorb the agent. The patch was then placed in contact with a nonabraded area of the rabbit's skin (previously clipped of hair) and secured with a polyethylene overwrap. Twenty-four hours later, the bandage was removed, and the rabbit

[1] Ethylene chlorohydrin and chlorohydrin will be used interchangeably throughout this paper.

[2] Obtained from Matheson, Coleman and Bell, East Rutherford, N. J.

[3] Administered as milliliters per kilogram and then converted to milligrams per kilogram.

was observed an additional 6 days for signs of toxicity or mortality. The LD_{50} was calculated on the basis of mortalities that occurred during the 7-day period.

Inhalation Toxicity—Groups of mice were exposed to air–chlorohydrin vapors for periods ranging from 10 to 15 min. The animals that survived the exposure were removed and observed for 7 days for deaths.

The inhalation chamber consisted of an 8.75-l. all-glass desiccator. Filtered air, at a rate of 1 l./min., was bubbled through ethylene chlorohydrin in a glass flask; the resulting air–chlorohydrin mixture was passed into the chamber. Five mice were exposed in the chamber at one time, and the procedure was repeated to give a total of 10 mice for each exposure duration.

It was estimated, by the method of Silver (14), that the concentration of chlorohydrin vapor in the chamber would reach 80% equilibrium with the incoming air vapor in approximately 14.1 min.

Dermal Irritation—Dermal irritation of ethylene chlorohydrin was tested in albino, New Zealand rabbits using a modification of the Draize method (15) and that of Wolven and Levenstein (16). A volume of 0.2 ml. of the test liquid was placed on a 0.5-in. square Webril cotton patch which was then applied to the shaved backs of New Zealand rabbits. The Webril patch was covered by a 1.27- × 2.54-cm. (0.5 × 1-in.) 12-ply cotton gauze, which in turn was covered with a polyethylene sheet of approximately the same dimensions and secured with cellophane tape. Four to six test sites were employed per rabbit, as well as a positive (8% w/v sodium lauryl sulfate in water) and a negative (normal saline) control site. All sites were covered with an unfolded piece of gauze and secured with adhesive tape encircling the torso of the animals. To impede further the volatilization of the test material and to protect the test and control sites, the torso was covered with a film of polyethylene and secured with cellophane tape. The rabbits were placed in individual cages for 24 hr., after which the bandages were removed and the sites of application examined.

Initial trials with the method described indicated that rapid absorption of the compound and the total dose of chlorohydrin employed led to death of the animals within 24 hr.[4]. For this reason the dose was reduced and fewer sites were used in each animal to permit the animal to survive for at least 24–48 hr.

Intradermal Irritation—A volume of 0.2 ml. of the test liquid, as such or diluted with normal saline solution, was injected intradermally on the dorsal surfaces (previously freed of hair) of a group of female albino rabbits (New Zealand strain). Dilutions of chlorohydrin were prepared of 10, 20, 40, and 80% (v/v). Further dilutions were made at increments from 0.625 to 5%. Each animal was injected with two of the test solutions at each of four different sites. Two positive control sites (20% ethyl alcohol) and two negative control sites (normal saline solution) were also included in each rabbit. Fifteen minutes after the last injection, trypan blue solution (1% w/v) was injected into the marginal ear vein at a dose of 1 ml./kg. At 0.5, 1, 5, 10, 15, 45, and 60 min., the sites of injections were examined and scored for irritancy as compared to the control sites. The scoring system used was as follows:

0 = No color (no irritation)—equivalent to saline control
± = Doubtful response
1 = Light-blue coloration (slightly irritating)
2 = Distinct blue coloration throughout (moderately irritating)
3 = Ischemic central area surrounded by deep-blue halo—equivalent to the ethyl alcohol control (markedly irritating)

The highest irritation response regardless of time was used in calculating the average irritant score of each solution but, in most instances, the maximal intensity was observed at the 60-min. period.

Eye Irritation—Solutions of chlorohydrin in normal saline were prepared in concentrations similar to those used for the "intradermal test." A volume of 0.1 ml. of the test solution was instilled into the superior temporal quadrant of the rabbit's right eye, while the left eye served as an untreated control. At least two rabbits were used for each test solution. Each of the rabbit's eyes was examined every 30 min. for 3 hr. following instillation of the test sample, and the response was recorded using the following scoring system:

0 = No irritation—comparable to untreated control eye
± = Doubtful irritation
1 = Definite conjunctiva and palpebral irritation with significant edema
2 = Either iritis or palpebral irritation with significant edema
3 = Eye swollen shut, with or without purulent discharge, and generally accompanied by iritis and corneal damage

The average response for each solution was calculated based upon the highest response observed.

Tissue Culture—*Agar Overlay Method*—Ethylene chlorohydrin, as such, and dilutions in distilled water were tested on monolayers of mouse fibroblasts (L-cells) according to the method of Guess *et al.* (17). A volume of 0.2 ml. of the test solution was pipeted onto a paper disk which was previously placed firmly on the agar[5]. Each Petri dish was kept in an incubator for 24 hr.; it was then removed and the cells under and immediate to the paper disk were examined for a cytotoxic response as indicated by loss of a vital strain (neutral red) which has been added to the cells. The test solutions were prepared in concentrations similar to those used in the "eye test."

Inhibitory Dose, 50% (ID_{50})—A method similar to that of Rosenbluth *et al.* (18) was employed to evaluate the growth-inhibiting effect of chlorohydrin using mouse fibroblast L-cells, strain 929. The medium used for maintenance of stock cultures consisted of modified Basal Medium Eagle (BME)[6] supplemented with 5% newborn calf serum, 1% L-glutamine, and 50 mcg./ml. of streptomycin sulfate. Stock cultures were subcultured every 72 hr.; cells for inoculum were harvested with the aid of 0.05% trypsin in phosphate-buffered saline, the trypsin was removed from the monolayer by aspiration, and the trypsinized cells were resuspended in BME supplemented with 10% calf serum and 2% L-glutamine.

A 2-ml. semiautomatic Cornwall syringe was used to deliver a 1.0-ml. aliquot of cell suspension (2×10^5 cells) into each culture tube. Various concentrations of chlorohydrin, dissolved in BME (no serum or glutamine), were prepared immediately prior to use. These were selected to provide adequate data between 20 and 80% inhibition. One milliliter of the appropriate toxicant–BME solution was added to each tube containing 1 ml. of the cell suspension.

Table I—LD_{50} Values of Ethylene Chlorohydrin

Species	Sex	Route of Administration	Acute Toxicity: LD_{50}, mg./kg.	Acute Toxicity: 95% Confidence Limits
Mice	Male	Intraperitoneal	98.3	71.9–134.6
	Male	Oral	81.4	66.4– 99.7
Rats	Male	Intraperitoneal	64.0	53.7– 76.9
	Male	Oral	71.3	57.8– 88.6
Rabbits	Either sex	Intraperitoneal	84.6	63.7–112.5
	Either sex	Dermal	67.8	41.2–111.7
Guinea pigs	Female	Intraperitoneal	85.8	—[a]

[a] 95% confidence could not be calculated.

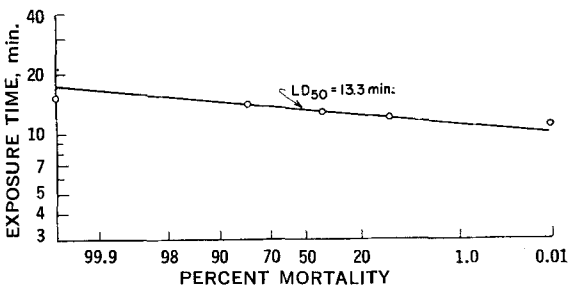

Figure 1—*Inhalation toxicity of ethylene chlorohydrin to mice plotted as exposure time* versus *percent mortality.*

[4] This test was performed before the "dermal toxicity test" leading to the LD_{50} value as described in the previous section.

[5] In this procedure, a thin layer of agar is placed over the surface of the monolayer of cells.

[6] BME was prepared according to Eagle (19, 20), except that magnesium was added as magnesium chloride in a concentration of 177 mg./l.

Controls received identical treatment except for the absence of chlorohydrin.

Both treated and control culture tubes were incubated for 72 hr., and the protein content of each tube was determined by the colorimetric method of Oyama and Eagle (21). Percent growth was calculated using the following expression:

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

where A is the mean absorbance for cultures receiving a specific treatment, A_0 is the mean absorbance for the untreated control cultures, and B is the initial (zero time) mean absorbance of untreated control cultures. Mean absorbance values were obtained from 5 replicate tubes for the treated cultures and 10 replicate tubes for zero-time and 72-hr. untreated control cultures.

RESULTS AND DISCUSSION

Determination of LD_{50}—The LD_{50} values for chlorohydrin in the various species are summarized in Table I and, as indicated, fall into a relatively narrow range. Goldblatt (8) reported an LD_{50} of 72 mg./kg. in rats by the oral route and 56 mg./kg. by the intraperitoneal route, and Johnson (22) found an oral LD_{50} in rats of 77 mg./kg., all of which are comparable to the values reported here.

The routes of administration employed in this study appear to have little influence on the LD_{50} of chlorohydrin (Table I). Dermal absorption of chlorohydrin in the rabbit is rapid and complete as indicated by its lethal activity. Absorption of chlorohydrin through the skin was reported by Goldblatt (8) in mice, by Ambrose (9) and Guess (11) in rabbits, and by Blecket and Strube (10) in man.

Inhalation Toxicity—When the data were analyzed graphically by plotting mortality against duration of exposure (as Bliss probits *versus* log time) (23), a linear relationship was obtained from which it was possible to evaluate the time required to kill 50% of the animals (Fig. 1). This lethal time dose (LT_{50}) was found to be 13.3 min.

As conducted in this study, inhalation toxicity experiments did not permit calculation of an LD_{50} but did permit an approximation of the lethality of the compound based upon the duration of inhalation required to kill 50% (LT_{50}) of the mice. However, the actual concentration of ethylene chlorohydrin in the inhalation chamber was not constant. It increased from zero when the animals were initially placed into the chamber to 80% equilibrium with the air–chlorohydrin mixture after 14 min. The extremely short period of time for death to occur confirms the high degree of toxicity of the compound when inhaled. Ambrose (9) reported concentrations of 7.5 p.p.m. for 1 hr. as being lethal to rats. The threshold limit value (TLV)[7] for chlorohydrin is given as 5 p.p.m.

Intradermal Irritation and Eye Tests—Chlorohydrin, undiluted, produced an intradermal irritation score of 3 (marked irritation), while dilutions equivalent to 1 and 5% (v/v) solutions gave an average score of ± (doubtful) and 1 (slight irritation), respectively.

In the eye test, a value of 3 (highest irritancy) was recorded for the undiluted chlorohydrin; dilutions of chlorohydrin from 20 to 80% also produced a 3 response. On further dilution, in the range of 2.5–10%, an average score of 2 resulted while a 1.25% solution gave a response of 1. Dilutions containing less than 1% chlorohydrin were nonirritating.

Guess (11) reported the responses of the rabbit to ethylene chlorohydrin when it was placed in contact with skin, injected intradermally or intramuscularly, placed on penile tissue, and instilled into the eye. He noted that undiluted ethylene chlorohydrin did not produce any significant irritant response when placed on the surface of the skin. When undiluted ethylene chlorohydrin was injected intradermally or intramuscularly, a very pronounced irritation developed at the sites of the injections. Irritation of the eye was produced by instillation of the undiluted compound. Similar results were observed when the compound was placed on penile mucosa. Irritation and tissue damage were progressively less pronounced as the solutions became more dilute. When the compound was sufficiently diluted, it produced no tissue damage or irritation. In the study by Guess (11), ethyl alcohol was employed as a standard irritant and was compared with the irritant response produced by chlorohydrin. The author noted that the tissue responses to chlorohydrin was much greater than to comparable concentrations of alcohol.

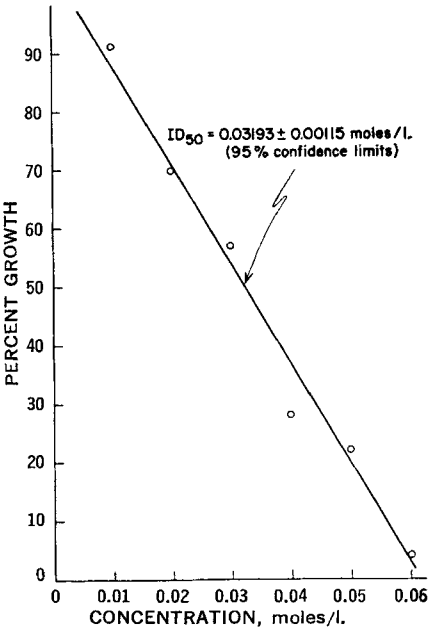

Figure 2—*Growth of L-cells in media containing various concentrations of ethylene chlorohydrin.*

In the current study, no significant irritant response was evident when the compound was applied to the skin of rabbits for 24 or 48 hr. This finding confirms the results of Guess (11). The lack of an irritant response is thought to be due to the rapid absorption of the compound into the systemic circulation, probably at a rate equal to or greater than penetration of chlorohydrin through the epidermis. This rapid passage of the chemical through the skin prevents accumulation of the agent to a critical concentration in dermal tissue. However, when the compound is injected directly into the tissues, a sufficient local concentration is achieved to produce a definite damaging effect. Dilution of the compound to a 5 or 1% solution reduced and abolished the intradermal response. Marked eye irritation with possible corneal damage was noted in this study when the undiluted compound was instilled into the eye. The response persisted even when the concentration of the agent was progressively diluted to 20%. Dilutions below 1.25% failed to produce eye irritation, and intermediate concentrations produced graded degrees of irritation. Guess (11) reported that chlorohydrin failed to cause eye irritation at a 10% concentration.

Tissue Culture—In this test where monolayers of cells were cultured in Petri dishes and covered with a thin layer of agar, the response from a test solution was recorded as an "all or none" response. Chlorohydrin in concentrations of 10–100% was found to be cytotoxic. Dilution to 5% or less abolished the cytotoxic response, A similar test by Guess (11) on chlorohydrin was comparable, except he reported a "noncytotoxic" response at 2% and less.

Cell growth inhibition tests permitted the data to be plotted as percent growth *versus* concentration of chlorohydrin (Fig. 2). By the method of least squares, it was possible to calculate the ID_{50} (inhibitory dose necessary for a 50% reduction in cell growth). The ID_{50} was found to be 0.03193 ± 0.00115 mole/l. ($p = 0.05$) or approximately 2570 mg./l. For comparison, the ID_{50} of ethyl alcohol was found to be 0.155 mole/l. Although it is not valid to compare LD_{50} values in animals with LD_{50} values in cell culture for a number of apparent reasons, it is interesting to note that the toxicity of chlorohydrin in cell culture is approximately 5 times that of ethyl alcohol but approximately 100 times as lethal as alcohol in rats.

According to Johnson (24), the toxic action of ethylene chlorohydrin in rats is due to metabolism of the compound to chloroacetaldehyde in target organs. Cells in tissue culture may not con-

[7] Threshold Limit Values, American Conference of Government Industrial Hygienists, Cincinnati, Ohio (1970). The Royal Institute of Chemistry (1960) placed the maximum allowable concentration (MAC) at 5 p.p.m., Goldblatt and Chiesman (1944) suggested it be 2 p.p.m., while the Merck Index (1960) suggests it should probably be below 1 p.p.m.

vert the chlorohydrin into the more toxic metabolic product, chloroacetaldehyde, or may simply be less sensitive to its effects and therefore can tolerate relatively higher concentrations of ethylene chlorohydrin.

Toxic Liability to Chlorohydrin—The results of the acute toxicity experiments reported here contribute additional evidence that ethylene chlorohydrin can be dangerous to life when inhaled or when the liquid comes in contact with skin. Small amounts of ethylene chlorohydrin falling on the skin may lead to systemic toxicity without showing significant signs of local irritation. If the dermal LD_{50} for rabbits (Table I) could be extrapolated to man, a volume slightly more than a teaspoonful could be lethal to the average (70 kg.) man if it contacts the skin and is not washed off immediately.

Plastic Devices—It is now well established that in the presence of chlorides, ethylene oxide sterilization of plastic medical devices can generate, as one of the reaction products, the highly toxic ethylene chlorohydrin. A potential local and systemic toxic hazard might thus be created if sufficient chlorohydrin or ethylene oxide is present in the device and is released to tissue or biological fluids (25). Proper degassing procedures for a plastic device after ethylene oxide sterilization can generally remove all of the residual ethylene oxide present in the device but this may not be the case for ethylene chlorohydrin (if present). To ensure safety to the patient, all ethylene oxide-sterilized devices should be biologically tested for possible toxic reaction products prior to their release for patient use. In most instances, manufacturers of plastic medical and dental devices are performing toxicity tests on their devices. Unfortunately, in many hospitals this type of testing program is not being conducted when "inhospital" ethylene oxide sterilization is performed on "reusable devices."

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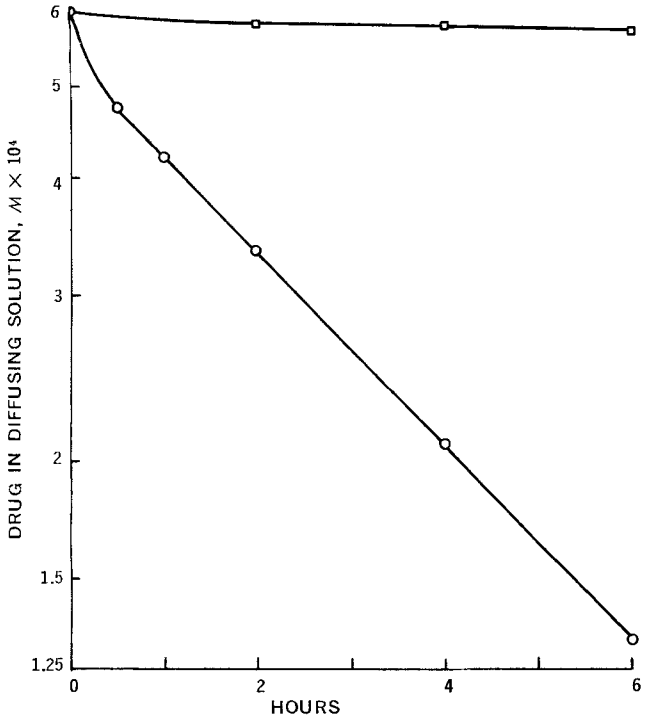

Figure 1—*Disappearance of chlorpromazine from the diffusing solution (in pH 6 citric acid buffer) through a 0.6-mil nylon membrane (□) and 5-mil dimethyl polysiloxane membrane (○) to the desorbing solution (5 × 10⁻³ N HCl) at 37°.*

permeation of drugs through such membranes. To approach this problem, it seems desirable to assess the physicochemical factors that would control this physiological process in the absence of biochemical factors.

This report is concerned with studies of the influences of a wide variety of substances such as surfactants, endogenous substances, dietary substances, adsorbents, and tablet excipients on the permeation of a drug through a model membrane. The choice of the diffusion technique for this purpose was based upon the belief that the absorption of drugs is a rate process and can best be studied kinetically. Silicone rubber membranes, which were chosen for this study, have been successfully used (1, 2). Chlorpromazine was selected as a model drug since it represents not only phenothiazine-type tranquilizers but also a good number of drugs with an aromatic group and an aliphatic amino side chain. Although chlorpromazine has been widely used for psychiatric treatment, only a few physicochemical studies on the interactive nature of this drug have been made. These include interfacial properties (3, 4), effects of adsorbents on gastrointestinal absorption (5–9), protein binding (10–12), and complex formation (13).

The objectives of the present work were to follow the time course of drug accumulation in the desorbing solution[1] and to discuss the effects of various interactions taking place in the diffusing solution[1] in the presence of various substances. Under these conditions, the influence of substances present in the diffusing solution on the permeative behavior of the drug through the synthetic membrane should mimic to a certain extent the effect of these substances on the absorption of the drug from gastrointestinal tracts. Some physiological effects of these substances, however, cannot be accounted for by this physicochemical model. It has been reported (14–16) that the submicellar concentrations of surfactants modify the permeability of biological membranes. Synthetic membranes, however, do not appear to have this property (17). In addition, the effect due to metabolic changes of the drug in the membrane has to be taken into account with live membranes.

[1] In this paper, a solution containing chlorpromazine at time zero is referred to as a diffusing solution, and a solution on the other side of the membrane is referred to as a desorbing solution.

EXPERIMENTAL

Materials—Chlorpromazine hydrochloride[2] was used without further purification. The following substances, which might interact with the drug and so affect its permeation, were examined: polysorbate 80[3], sodium lauryl sulfate[4], gastric mucin[5], sodium glycocholate[6], sodium taurocholate[4], caffeine[7], skim milk[8], saccharin sodium[9], riboflavin phosphate sodium[10], kaolin[6], activated charcoal[11], a sulfonic acid-type cation-exchange resin[12], lactose[6], gelatin[4], and talc[6]. Most of these materials were used as received. Buffers used in studies on the effect of pH were citric acid[7] buffer over the pH range 4.1–6.0 and 3,3-dimethylglutaric acid[13] buffer over the pH range 6.4–7.4 (18). The cation-exchange resin was washed several times with pH 6 citric acid buffer until no change in pH was observed.

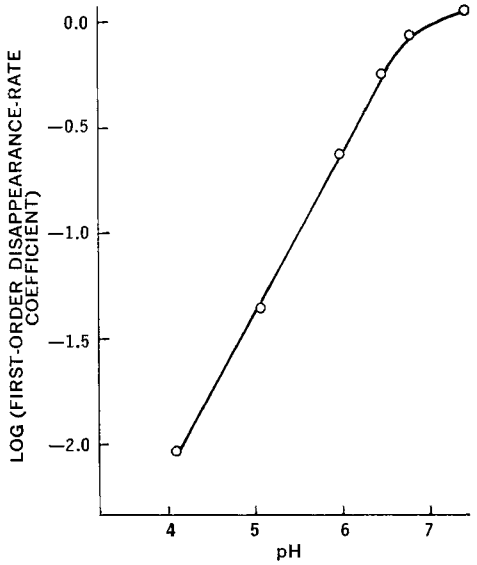

Figure 2—*pH profile of first-order disappearance-rate coefficient of chlorpromazine from the diffusing solution at 37°. Chlorpromazine concentration was 0.6 mM except at pH 7.4 where a 0.3 mM solution was used. Buffers were 0.05 M citric acid buffer over the pH range 4.1–6.0 and 0.05 M 3,3-dimethylglutaric acid buffer over the pH range 6.4–7.4. The desorbing solution was 5 × 10⁻³ N with respect to HCl.*

[2] Poulenc, Montreal, Canada.
[3] Tween 80, Atlas Chemical Industries, Wilmington, Del.
[4] Fisher Scientific Co., Chemical Manufacturing Div., Fair Lawn, N. J.
[5] Nutritional Biochemical Corp., Cleveland, Ohio.
[6] Merck & Co., Montreal, Canada, and Rahway, N. J.
[7] British Drug Houses, Toronto, Canada.
[8] Borden Co., Toronto, Canada.
[9] Abbott Laboratories, North Chicago, Ill.
[10] Sigma Chemical Co., St. Louis, Mo.
[11] Darco G-60, Anachemia Chemicals, Montreal, Canada.
[12] Dowex 50W-X12, 50–100 mesh, Dow Chemical Co., Midland, Mich.
[13] Eastman Organic Chemicals, Rochester, N. Y.

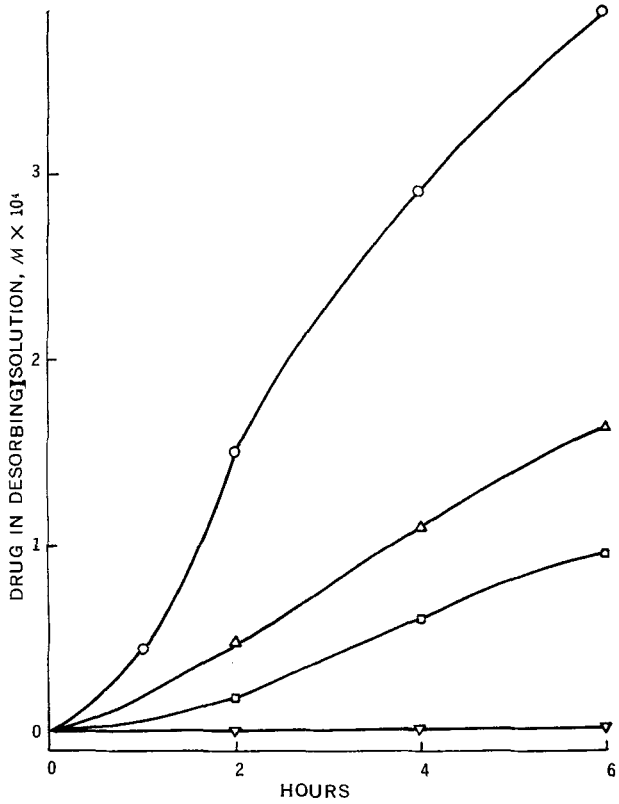

Figure 3—*Effect of surfactants on the permeation of chlorpromazine at 37°. Key: ○, no additive; △, 1% polysorbate 80; □, 0.6 mM sodium lauryl sulfate; and ▽, 2 mM and 6 mM sodium lauryl sulfate. Initial concentration of chlorpromazine was 0.6 mM in 0.05 M pH 6 citric acid buffer, and the desorbing solution was 5 × 10⁻³ N with respect to HCl. A point at 1 hr. in the control experiment was included to show sigmoidal increase in drug concentration in the desorbing solution.*

Diffusion Cell—A diffusion cell, similar to that of polymethy methacrylate described by Patel and Foss (19), was employed with the following modifications: (*a*) stainless steel was used instead of polymethyl methacrylate to avoid possible adsorption of the drug to the cell material (20); and (*b*) the height of the cell was increased by 2 cm. so that the openings on the top were still above the water level when the main part of the cell was immersed in a constant-temperature shaker water bath[14]. The overall dimensions were 10.0 × 7.7 × 3.8 cm.; each compartment occupied about 12 ml.; and the area available for diffusion was 9.4 cm.^{2}. The diffusion membrane employed was medical grade dimethyl polysiloxane sheeting[15] in a labeled thickness of 5 mil. The nylon membrane[16] used for preliminary work was 0.6 mil in thickness.

Procedure—The two cavities of the assembled cell with the membrane were washed several times with water and methanol and then both compartments were filled with methanol overnight to facilitate the leaching of the drug absorbed within the membrane. They were washed thoroughly with water and filled with water until the cell was used. The water was completely removed prior to use, and the cell was placed in the shaker water bath maintained at 37° so that nine-tenths of the cell was immersed in water. A 10-ml. portion of a chlorpromazine solution (1.2 m*M*) in pH 6 citric acid buffer was mixed with an equal volume of an additive solution of appropriate concentration (see the figures). The mixture was placed in the water bath to equilibrate at 37°. A hydrochloric acid solution (5×10^{-3} *N*) was similarly warmed to the same temperature.

After equilibration, a 10-ml. portion of the hydrochloric acid solution was placed in one compartment of the cell to ionize the permeated drug so that the concentration of permeable species would be maintained at a zero value; thus, "sink" conditions apply. The same volume of the drug solution was placed in the other. Shaking was started immediately, and sampling (0.5 ml.) from the diffusing and desorbing solutions was made at 2, 4, and 6 hr. of incubation. When the effect of hydrogen-ion concentration was examined, sampling (0.2 ml.) was made at appropriate time intervals, depending on the rate of permeation of the drug at each pH. The shaking rate was maintained at 95 ± 2 oscillations/min. with the length of the stroke 2.4 cm. The concentrations of the drug were determined spectrophotometrically at 255 nm. (or 308 nm. when additives interfered with the assay at 255 nm.) after appropriate dilution. Although the decrease in volume due to sampling reached 10% of the original volume, no volume correction was made because the main interest in this study was centered on the relative effects of interactive species rather than the calculation of a permeability constant in the presence of each interactive species. Because of the photochemical instability of the drug, appropriate precautions were taken during the experiments.

RESULTS AND DISCUSSION

Choice of Membrane—Although a nylon film has been reported to be permeable to chlorpromazine (*21*), preliminary diffusion studies with a nylon membrane in a labeled thickness of 0.6 mil indicated rather slow permeation of the drug through this membrane in the pH range below 7.4 (Fig. 1). A dimethyl polysiloxane membrane in a labeled thickness of 5 mil, on the other hand, was found to be about 60 times more permeable to the drug than the nylon membrane. This observation as well as the lipidlike nature of the silicone rubber led to the use of the dimethyl polysiloxane membrane for the present investigation of the permeative behavior of chlorpromazine and the effect of additives on the permeation of the drug. The permeation of a steroid through dimethyl polysiloxane was also noted by Kincl *et al.* (22) to be about 100 times faster than through a nylon membrane of comparable thickness.

Permeation Characteristics of Chlorpromazine—A plot of log chlorpromazine concentrations of the diffusing solution against time is shown in Fig. 1. It can be seen from the figure that the depletion of the drug from the diffusing solution followed first-order elimination except in the initial period of permeation. The

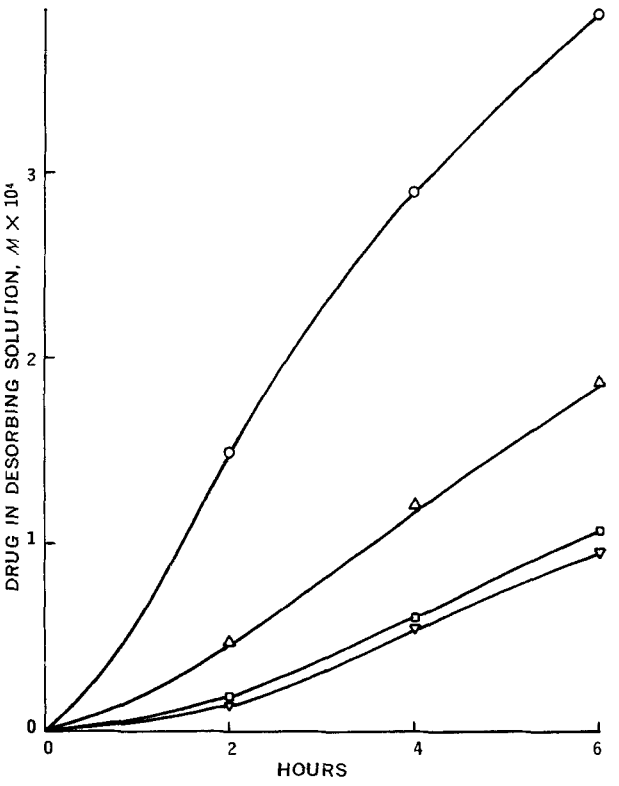

Figure 4—*Effect of endogenous substances on the permeation of chlorpromazine at 37°. Key: ○, no additive; △, 1% gastric mucin; □, 10 mM sodium glycocholate; and ▽, 10 mM sodium taurocholate.*

[14] Model MSB-1122A-1 constant-temperature shaker bath, Blue M. Electric Co., Blue Island, Ill.

[15] Silastic, Medical Products Div., Dow Corning Corp., Midland, Mich.

[16] Capran 77C, Allied Chemical Canada, Belleville, Ontario, Canada.

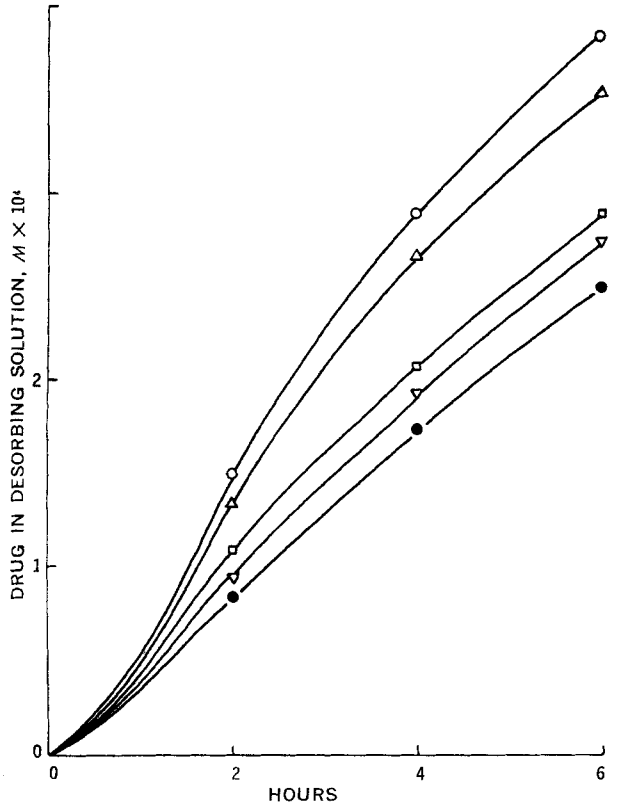

Figure 5—*Effect of dietary substances on the permeation of chlorpromazine at 37°. Key: ○, no additive; △, 10 mM saccharin sodium; □, 2.5% skim milk; ▽, 20 mM caffeine; and ●, 10 mM riboflavin phosphate sodium.*

initial sudden decrease in drug concentrations was attributed by some workers (21, 23) to the initial build-up of the drug in the membrane.

The change in first-order disappearance-rate coefficient[17] of the drug from the diffusing solution through the membrane to the desorbing solution over the physiological pH range is presented in Fig. 2. An essentially linear relationship was obtained between log rate coefficient and pH over pH 4.1–6.4. Beyond pH 6.8, there was deviation from the linear relationship. Among factors that control the permeation rate of the drug through the membrane, the partition coefficient is considered to influence the rate-limiting step in the pH range 4.1–6.4. Chlorpromazine with a pKa of 9.3 (24, 25) has an extremely high oil–water partition coefficient when the aqueous phase is alkaline (26). In this pH range, as the pH of the diffusing solution is increased, the proportion of unprotonated molecule increases and partition would then be favored. This factor is likely to be responsible for the observed linear relationship in the pH range 4.1–6.4. At higher pH values, however, the partition coefficient may no longer influence the rate-limiting step; instead, diffusion through the bulk solution–membrane interfacial diffusion layer (27) or through the membrane may limit the permeation rate.

Effect of Additives on Permeation of Chlorpromazine—The analyses of desorbing solutions were used mainly in this study because the additives frequently interfered with the assay procedure in the diffusing solutions. The lack of concentration–time data for diffusing solutions, however, made simple calculations of permeation coefficients of the drug impossible because the concentrations of both diffusing and desorbing solutions are required for calculation of the rate coefficients for nonsteady-state permeations such as those of the present study. The effects of additives are, therefore, discussed on the basis of the graphical representation of the amount of drug permeated into the desorbing solution at time t. A pH of 6 was chosen for these experiments due to the convenient rate of permeation of the drug at this pH.

[17] A term "rate coefficient" is used in this paper instead of "apparent rate constant."

Surfactants—The effects of a nonionic surfactant, polysorbate 80, and an anionic surfactant, sodium lauryl sulfate, on the permeation of the drug are shown in Fig. 3. Both surfactants exhibited a rate-retarding effect. The effect of polysorbate 80 is most likely due to a micellar effect (14). The drug present in micelles would be expected to be less or unavailable for permeation. This surfactant, with a very small critical micelle concentration (CMC) of 0.0013% (28), is considered to be in micelle form in the concentration employed.

The effect of sodium lauryl sulfate is, on the other hand, likely to stem from both insoluble complex formation and micellar effect[18]. Sodium lauryl sulfate seems to form an insoluble complex with chlorpromazine below its CMC and soluble micellar aggregates above it. Solutions of chlorpromazine in 6 m*M* sodium lauryl sulfate (above CMC) were clear, but the drug was found to be almost unavailable for permeation. This could indicate that most of the drug was present in micelles and unavailable for permeation. The drug solutions in 0.6 and 2 m*M* sodium lauryl sulfate (below CMC) were cloudy, and the degree of turbidity depended upon the concentration of the surfactant. Furthermore, when a turbid mixture containing a small amount of the surfactant was warmed, it gave a clear solution. From these observations of concentration and temperature effects of the phenomenon, it would be safe to assume that the turbidity was indicative of the formation of a poorly soluble complex between chlorpromazine and the surfactant. The reasonably fast rate of permeation observed from the 0.6 m*M* sodium lauryl sulfate solution indicates that some portion of the drug was still uncomplexed and available for permeation. The percentages of complexed drug increased with increasing concentration of the lauryl sulfate. Practically none was available for permeation at the lauryl sulfate concentration of 2 m*M*, which is

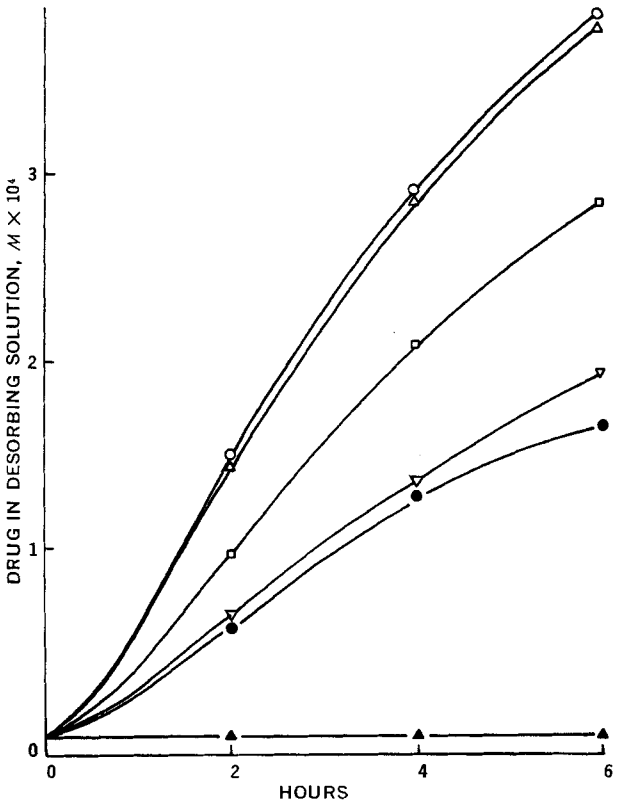

Figure 6—*Effect of adsorbents and tablet excipients on the permeation of chlorpromazine at 37°. Key: ○, no additive and 2% lactose; △, 1% gelatin; □, 1% talc; ▽, 1% kaolin; ●, 1% cation-exchange resin; and ▲, 1% activated charcoal.*

[18] Although the CMC of sodium lauryl sulfate is reported to be 8 m*M* (0.23%) from conductivity measurement (29, 30), it appears to vary considerably in the presence of solubilizates. The CMC determined from solubility measurements of steroids is reported to be 2 m*M* (31). In the presence of 0.6 m*M* chlorpromazine, the CMC was estimated to be around 4 m*M* at 25° (17). The CMC estimated from the solubility diagram of prednisolone at 30° (32) is also around 4 m*M*.

still below its CMC[18]. The formation of a poorly soluble complex between chlorpromazine cation and lauryl sulfate anion may be similar to the formation of procaine penicillin between procaine cation and penicillin G anion.

Endogenous Substances—The rate-retarding effect of gastric mucin, sodium glycocholate, and sodium taurocholate is presented in Fig. 4. The effect of the mucin may be attributed to at least one of the following factors: protein binding and the increased viscosity of the drug solution. Increased viscosity was reported to retard the absorption rate of salicylic acid from the stomach of the rat (33). Bile salts, on the other hand, form micelles (34) and make the drug less available for permeation. Insoluble complex formation was also noted below the CMC's of the bile salts. At concentrations of the bile salts employed in this study, however, the effect may be attributed mainly to micelle formation. The CMC's of sodium taurocholate and sodium glycocholate are reported to be around 8 m*M* and 10 m*M*, respectively, at 37° (34). Increase in membrane permeability in the presence of bile salts in submicellar concentrations as observed in the everted rat intestine (35, 36) and in goldfish (37) appears to be absent in the silicone rubber membrane.

Dietary Substances—Results with caffeine, riboflavin, saccharin, and skim milk are shown in Fig. 5. All these substances reduced the permeation rate of the drug. The effect of caffeine and riboflavin may be attributed to complex formation, since they are known to be very good complexing agents (38, 39). These complexes, even though they are soluble, are reported to be less or not permeable (40). The observed permeation of the drug may thus be considered mainly due to the free (uncomplexed) drug. Riboflavin phosphate, although it is an anion, did not form an insoluble complex with chlorpromazine cation. The effect of saccharin is small but appears to be real since the data were reproducible. This effect may also be due to soluble complex formation, because the alternative of insoluble complex formation between saccharin anion and chlorpromazine cation was not observed. The effect of skim milk can be ascribed to protein binding, since the protein binding of phenothiazine tranquilizers by bovine serum albumin is well documented (10–12).

Adsorbents—Among adsorbents investigated, activated charcoal was found to be extremely effective in retarding the permeation rate of chlorpromazine (Fig. 6). The adsorption of phenothiazine derivatives by charcoal was reported (5, 8) to be more extensive than by kaolin. Adsorption to these solid materials may be presumed to be the reason why the drug permeated to a much less extent that it did in their absence. Chlorpromazine, which is mostly protonated at pH 6, would be adsorbed by the cation-exchange resin. This would cause the conversion of unprotonated species to a protonated form in order to maintain the equilibrium at this pH. Decrease in permeable unprotonated species can then be considered to render the drug less available for permeation in the presence of the cation-exchange resin than in its absence.

Tablet Excipients—Lactose, gelatin, and talc were selected from many possible excipients of tablets. Lactose did not appreciably modify the permeation of chlorpromazine (Fig. 6). It is reasonable to expect that other sugars such as sucrose and glucose also do not interact with the drug significantly. Gelatin also failed to modify markedly the permeation rate of the drug. This may be attributed to weak interactive properties of gelatin which lacks tryptophan and contains only small amounts of other aromatic amino acids (41). Aromatic amino acids may be considered to be a strong binding group within protein molecules if hydrophobic bonding plays a dominant role in protein binding (42), although the exact mechanism of protein binding has not been established. The rate-retarding effect of talc, on the other hand, may be explained by adsorption of the drug to the solid surface (5, 8).

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Quantum Chemical Approach to Structure–Activity Relationships of Tetracycline Antibiotics

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Abstract □ Bacteriostatic activities for tetracyclines, determined *in vitro* by microbial growth kinetics, should be related to the perturbation energy, ΔE_i, which characterizes the interaction between these antibiotics and their receptor substance. Quantum perturbation molecular orbital theory, combined with multiple-regression techniques, are applied in gaining estimates of the relative variations in ΔE_i for a family of tetracyclines which act in an identical way on a receptor region of unknown structure. The results of this theoretical approach are in good agreement with a number of independent observations relating to the bacteriostatic effects of tetracyclines and are consistent with the hypothesis that bacterial growth inhibition by tetracyclines is a consequence of an inhibition in protein synthesis due to the association of the antibiotic with a ribosomal site. The derived relationships between the bacteriostatic activities and the electronic structure of the tetracycline molecules implicate the oxygens at positions 10, 11, and 12 of the tetracycline nucleus as being intimately involved in leading to the bacteriostatic effects. This relationship also leads to satisfactory estimates for the inhibition rate constants of other tetracyclines, provided that specific effects such as intramolecular hydrogen bonding are taken into account. The effect of substituents on the electronic properties of the C_6 and phenoldiketone tetracycline regions is to produce a gradation in the inhibitory properties of this family of antibiotics.

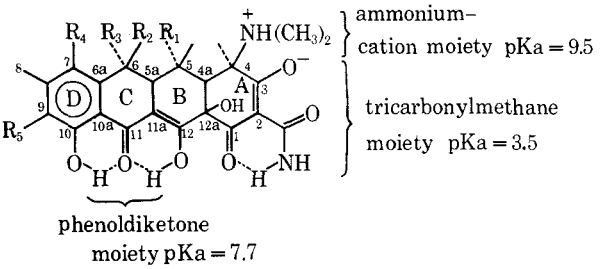
Keyphrases □ Tetracycline antibiotics—structure–activity relationships □ Quantum chemical approach—structure–activity relationships, tetracyclines □ Bacterial protein growth inhibition hypothesis—tetracyclines □ Molecular orbital perturbation theory—tetracycline activity □ Ribosomes, aminoacyl site—tetracycline binding

In both the past and the present, most clinically useful drugs have been discovered empirically or through synthesis of congeners of preexisting successful types. It was estimated (1) that only about one out of every 3000–5000 compounds synthesized is found to be a useful therapeutic agent. For this reason, even an imprecise or qualitative knowledge of molecular structure–activity relationships and mode of action of each family of compounds can be very useful in developing a more rational and direct approach to the synthesis of new drugs.

Since the isolation in 1947 (2) of the first known member of the tetracycline family, the clinical importance of these compounds as therapeutic and prophylactic agents against a wide range of infections has stimulated efforts (3–6) to define their mode of action as inhibitors of bacterial reproduction. Because of the advances to date in unraveling the mode of action of tetracyclines, this class of drugs is particularly well adapted for such investigations. This paper uses quantum chemical methods in an attempt to advance the knowledge of the relationship between electronic structure and the action of tetracyclines at the receptor site. An analysis of the possibilities and shortcomings of applying principles of quantum chemistry to the study of biological properties of drugs can be found in *Reference 7*.

EXPERIMENTAL DATA AND MODELS

Tetracyclines are derivatives of an octahydronaphthacene hydrocarbon system composed of four rings (Structure I). For useful



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clinical activity, previous work has shown that they clearly depend more upon the presence of their phenoldiketone, tricarbonylmethane, and ammonium-cation moieties than upon the presence of substituents at carbons 5 to 9. Modifications of the tricarbonylmethane structure, which is not readily reversible by simple aqueous hydrolysis, lead to drastic reductions in biological activity.

The oxygen functions at positions 10, 11, and 12 appear to be essential for biological activity, since alteration of this chromophoric group leads to compounds that are nearly or totally inactive. These functions are also among the most likely sites for metal chelation. The binding of tetracyclines to several macromolecules in the presence of metal ions was reported (8–10). The ability of the tetracyclines to chelate divalent cations is probably involved in their biological action. Alterations of the dimethylamino group at carbon 4 strongly reduce antibacterial activity. Substituent changes at carbons 5 to 9 produce derivatives with quantitatively different activities, affording a series of derivatives that offers a good possibility for studies of structure–activity relationships (11). The relative significance of these structures toward the mechanism of the biological action of these compounds has not yet been precisely determined and needs to be clarified in order to relate fully the structural characteristics to the activities.

For tetracyclines, the close similarity among the chemical reactions, their similar antibacterial spectra, the fact that the bacteriostatic effects of the most active compounds occur at similar concentrations, and the common occurrence of cross-resistance have led to the general assumption (4) that the mechanisms by which they inhibit the growth of microbial cells are similar if not identical. Although other effects may play a part in tetracycline bacteriostasis, many investigators presently feel that a general inhibition of protein biosynthesis appears to be the primary mechanism by which the tetracyclines exert their antibiotic activity. Tetracycline probably exerts its inhibitory effects on protein synthesis by binding to the ribosomes, thus interfering with the formation of the necessary complex between ribosome, messenger-RNA, and aminoacyl-*t*-RNA. This idea, widely accepted, is the basis for most of the recent research in this area (9, 12–15).

Quantitative biological activities of a large number of compounds, obtained under identical conditions in a precise manner, are required to establish structure–activity relationships. Ideally, these activities should be directly related to the mechanism of action of the compounds and be free of extraneous competing equilibria. Most of the available activities for tetracycline antibiotics have been summarized by Barrett (16), Boothe (17), and Plakunov (18). Many of these activities have been determined under conditions such that the results parallel clinical activity. This type of activity may be different than activity at the receptor site.

In a recent work (6, 15), activities suitable for the study of structure–activity relationships were established for a series of com-

Table I—Experimental Inhibitory Rate Constants

Number, i	Compound, T_i	k_i^I
1	7-NO_2-6-Demethyl-6-deoxytetracycline	748 ± 55
2	7-Cl-6-Demethyltetracycline	517 ± 34
3	7-Cl-Tetracycline	401 ± 15.9
4	Tetracycline	271 ± 11.3
5	5-Oxytetracycline	257 ± 24.5
6	7-NH_2-6-Demethyl-6-deoxytetracycline	181 ± 7.82
7	9-NH_2-6-Demethyl-6-deoxytetracycline	145 ± 8.79
8	6-Demethyl-6-deoxytetracycline	94.4 ± 3.06
9	7-Br-6-Demethyl-6-deoxytetracycline	51.8 ± 3.14
10	9-NO_2-6-Demethyl-6-deoxytetracycline	44.3 ± 1.51
11	9-$N(CH_3)_2$-6-Demethyldeoxytetracycline	23.6 ± 1.52
12	5*a*(6)-Anhydrotetracycline	15.5 ± 1.23
13	12*a*-Deoxytetracycline	2.54
14	7-Cl-5*a*(11*a*)-Dehydrotetracycline	0.34
15	4-Dedimethylaminotetracycline	24.1 ± 1.19
16	4-CH_3I-Tetracycline	2.18 ± 0.22
17	2-CN-Tetracycline	0.00
18	7-Cl-Isotetracycline	0.00
19	7-Cl-6-Demethyl-6-deoxytetracycline	(84.52)[a]
20	7-Dimethylamino-6-demethyldeoxytetracycline	(145.49)[a]

[a] Calculated based on Eq. 17.

pounds, including both clinically active and "inactive" tetracycline antibiotics. These activities were obtained by three types of kinetic measurements on exponentially growing *Escherichia coli* W inhibited by the antibiotics. Inhibition of cell division, protein synthesis, and nucleic acid synthesis was simultaneously studied so that activities might be more directly related to the mode of action. Generation rate constants were determined in the presence of low, graded concentrations of tetracyclines. The effect of the action of each antibiotic, T_i, is to cause a reduction in the generation rate constants:

$$[N_i]_i = [N_0]e^{k_i t} \qquad k^0 > k_i > 0 \qquad \text{(Eq. 1)}$$

where $[N_i]$ and $[N_0]$ are concentrations of cells, proteins, or nucleic acids in the culture; k_i is the corresponding generation rate constant in the presence of antibiotic T_i; and k^0 is the generation rate constant in the absence of antibiotic. The constants k^0 were found to be experimentally identical. The rate constants k_i were obtained by application of regression analysis to Eq. 1. Rate constants k_i were found to be related to antibiotic concentration $[T_i]$ by the equation:

$$k_i = k^0 - k_i^I [T_i] \qquad \text{if } k_i > 0 \qquad \text{(Eq. 2)}$$

where k_i^I are the inhibitory rate constants. Within experimental accuracy, the inhibitory rate constants for cell division, for protein synthesis, and for nucleic acid synthesis are practically identical for each antibiotic. The constants k_i^I are accurate measures of the potency of the antibiotics under the test conditions and should be useful in structure–activity relationship studies. Values of k_i^I for the compounds studied are given in Table I.

From the experimental results on the kinetics of inhibition, kinetic models for the interference of tetracycline antibiotics with protein synthesis in the individual cell can be constructed (6, 19). These models are presented in terms of reactions involved in protein synthesis in a bacterial culture. The models consider that tetracyclines bind to the aminoacyl site of ribosomes and thereby prevent the binding of aminoacyl-*t*-RNA. They also assume that there is a reversible passage of tetracyclines from outside to inside the cells. Tetracyclines inside the cell bind to ribosome-RNA (RIB) and form a complex (RIB–T_i) which is not capable of synthesizing protein. This may be written as follows:

$$[T_i'] \overset{K_i^p}{\rightleftharpoons} [T_i] \qquad \text{(Eq. 3)}$$

$$[T_i] + [\text{RIB}] \overset{K_i^c}{\rightleftharpoons} [\text{RIB}\text{–}T_i] \qquad \text{(Eq. 4)}$$

The stoichiometry of this equation is that suggested by Day (8) and Maxwell (3). As a starting point, the simplest model involving the least number of reactions is postulated. Considering the limiting cases of this model, a simple expression for the inhibitory constants can be written (19):

$$k_i^I = k^0 K_i^p K_i^c \qquad \text{(Eq. 5)}$$

This approach is, of course, an oversimplification and is used here because of this simplicity. Several variations of this model have been discussed (6). Equation 5 shows the principal factors responsible for the variation of k_i^I when passing from one molecule to another in a series of tetracycline antibiotics. It affords a basis for the theoretical study of structure–activity relationships.

THEORETICAL APPROACH

The present theoretical work is based on several considerations and assumptions supported by the experimental results:

1. The unusual structural groupings in the tetracyclines produce three macroscopic acidity constants in aqueous solution which, it is generally agreed (20–22), are approximately equal to 3.5, 7.7, and 9.5. The functional groups responsible for the thermodynamic pKa values are located in the tricarbonyl–methane system, the phenoldiketone moiety, and the ammonium cation, respectively. On this basis, and from studies on the effect of broth pH changes on tetracycline-inhibited cultures (15, 23), the authors adopted the dipolar ion molecular model in Structure I.

2. It is assumed that all the investigated compounds act in the same way by binding either directly or through metal chelates to the site on the ribosome which normally binds the transfer-RNA molecules (4, 6, 12, 15).

3. If the locus of action of an antibiotic lies within the microbial cell, the ability to permeate the cells, represented in Eq. 5 by the factor K_i^p, is an important factor in antibiotic activity; it is difficult to distinguish between structural changes that alter the ability of the tetracyclines to react at the receptor site and changes that alter the tetracycline's ability to permeate the cell. Removal or specific alteration of the functional groups responsible for the macroscopic acidity constants significantly alters the acidic characteristics of the molecules. This alteration could lead to important modifications of the ability to permeate the cells (24, 25). To reduce, as much as possible, the influence of the K_i^p factor, and as a first step to gain insight on action of the tetracycline antibiotics at the molecular level, only those members of the tetracycline family (normal tetracyclines) that have intact 4-dimethylamino, tricarbonylmethane, and phenoldiketone structures (Compounds 1–14, Table I) were included in this study. It is then assumed (15, 24, 25) that, for the compounds of the series considered here, the factor essentially responsible for the variation in k_i^I values is the variation of the equilibrium constant, K_i^c, due to differences in electronic molecular structures.

In accord with these assumptions, Eq. 5 can be written, for normal tetracyclines, in the following approximative form:

$$\log k_i^I = \text{constant} + \log K_i^c \qquad \text{(Eq. 6)}$$

This relation can be utilized to estimate the k_i^I values, for molecules belonging to this series, from K_i^c calculations. From Eq. 4 and according to the principles of statistical thermodynamics, the follow-

Table II—Empirical Parameters for π-Structure Calculations (39, 40)

Atom, p	δ_p	Bond, p—q	η_{pq}
C	0.00	C—C	1.00
O (carbonyl)	0.70	C—O (carbonyl)	2.00
O (hydroxyl)	2.00	C—O (hydroxyl)	0.90
O (nitro)	1.00	C—N	0.80
N (amino)	1.80	C—Br	0.30
N (nitro)	2.00	C—Cl	0.40
Br	1.60	N—O	0.70
Cl	2.10		

ing set of equations can be written:

$$K_i^c = \frac{f_{RT_i}}{f_R f_{T_i}} e^{-\Delta\epsilon/kT} \quad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 7)}$$

where $\Delta\epsilon_i = \epsilon_{RT_i} - (\epsilon_R + \epsilon_{T_i})$. The quantity $\Delta\epsilon_i$ is the difference in ground-state energies between the complex RIB–T_i and the reactants RIB and T_i; the f terms are partition functions; and k and T stand, respectively, for Boltzman's constant and the absolute temperature. The set of equations in Eq. 7 can be written in the more convenient form:

$$\log K_i^c = \log \frac{f_{RT_i}}{f_R f_{T_i}} + \text{constant} \cdot \Delta\epsilon_i \qquad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 8)}$$

To separate the solvation energy contribution, ΔE_i(solv.), the term $\Delta\epsilon_i$ can be approximated by the sum (26):

$$\Delta\epsilon_i = \Delta E_i + \Delta E_i(\text{solv.}) \qquad \text{(Eq. 9)}$$

where the quantity ΔE_i(solv.) is given by the equation:

$$\Delta E_i(\text{solv.}) = E_{RT_i}(\text{solv.}) - \{E_R(\text{solv.}) + E_{T_i}(\text{solv.})\} \qquad \text{(Eq. 10)}$$

Since the equilibrium reactions were carried out under the same experimental conditions for all the compounds, and because of the close structural similarity between the selected tetracyclines, the usual simplifications can be utilized; *i.e.*, the terms $\log (f_{RT_i}/f_R f_{T_i})$ and ΔE_i (solv.) do not significantly vary with respect to the variation of ΔE_i. The assumption that the partition function terms are constant appears to be generally valid (27), provided that no specific effects (such as steric hinderance) are involved. The assumption of constant solvation energy terms is supported (26) by the fact that each of the two compound series, RT_i and T_i, are composed of molecules of the same size, having similar structure and a similar charge distribution in their zwitterionic moieties. The set of Eq. 8 can then be written in the following form:

$$\log K_i^c = a + b\,\Delta E_i \qquad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 11)}$$

where a and b are assumed to be practically constant. Substituting Eq. 11 in Eq. 6, one has the set of equations

$$\log k_i^I = \text{constant}_1 + \text{constant}_2\,\Delta E_i \qquad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 12)}$$

Then, within the limitations of the theory and the assumed simplifications, k_i^I activities can be related to differences in the molecular electronic ground-state energies, ΔE_i. To study the ΔE_i variation in the present case, by using the familiar molecular orbital theory (28), the perturbation energy of the two systems, T_i and RIB, is evaluated when they weakly interact in the complex RIB–T_i. As a basis for a general theory of organic chemistry, Dewar (29) developed a method wherein, instead of calculating the coefficients of the molecular orbitals of a mesomeric system AB by the usual variational procedure, he showed that they can be obtained to a sufficiently good approximation by regarding formation of the bonds linking A to B as a perturbation and applying perturbation theory.

The formation of charge-transfer complexes and the reactivity of conjugated molecules, for a definite type of reaction with a definite reagent, were similarly discussed, as a function of the magnitude of the perturbation energy, by Fukui *et al.* (30, 31) and Morokuma *et al.* (32). From an approximate expression of the perturbation energy, the delocalizability indexes of reactivity were derived by these authors. More recently, Klopman and Hudson (33–35) described a more general perturbation treatment of chemical reactivity, in which allowance is made for ionic interaction and the theory is not restricted to π-conjugated molecules.

If T_i and RIB approach such that an atom t of T_i and an atom r of RIB interact, being weakly linked in the RIB–T_i complex, this interaction produces a change in energy ΔE_i, the magnitude of which depends on the reactants, the atoms t and r, and their distance d_{tr}. If, as indicated, one is only concerned with electronic effects, then perturbation theory as applied by Klopman and Hudson (33–35) to weakly interacting compounds gives, for p pairs of t and r bonding atoms, the following set of expressions[1]:

$$\Delta E_i = \Sigma_p \left\{ Q_{i_{t_p}} Q_{r_p}(tt|rr) + \Sigma_{ki}\Sigma_m \frac{2c_{ki}^2{}_{t_p}}{\epsilon_{ki} - \epsilon_m} c_{m r_p}^2 \beta_{t_p r_p}^2 - \Sigma_{1i}\Sigma_n \frac{2c_{1i}^2{}_{t_p}}{\epsilon_{1i} - \epsilon_n} c_{n r_p}^2 \beta_{t_p r_p}^2 \right\} \qquad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 13)}$$

where Σ_p holds for the p pairs, t_p–r_p, of interacting atoms in the complex; $Q_{i_{t_p}}$ and Q_{r_p} are the net charges of atoms t_p and r_p, respectively; $(tt|rr)_p$ is the coulombic electron-repulsion energy: $(tt|rr)_p = \int \chi_{t_p}{}^2\,(1)\,(1/d_{12})\,\chi_{r_p}{}^2\,(2)\,dz$; the c_{us}'s are the atomic orbital coefficients in the molecular orbital development: $\phi_u = \Sigma_s c_{us}\chi_s$; ϵ_{ki} and ϵ_{1i}, respectively, stand for the energies of the occupied and unoccupied molecular orbitals of T_i; ϵ_m and ϵ_n have the same significance for RIB; and β_{tr} represents the resonance integral associated with the t_p–r_p bond.

By combining Eqs. 12 and 13, one can write the following set of equations:

$$\log k_i^I = c + d\Sigma_p \left\{ Q_{i_{t_p}} Q_{r_p}(tt|rr)_p + \Sigma_{ki}\Sigma_m \frac{2c_{ki}^2{}_{t_p}}{\epsilon_{ki} - \epsilon_m} c_{m r_p}^2 \beta_{t_p r_p}^2 - \Sigma_{1i}\Sigma_n \frac{2c_{1i}^2{}_{t_p}}{\epsilon_{1i} - \epsilon_n} c_{n r_p}^2 \beta_{t_p r_p}^2 \right\} \qquad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 14)}$$

c and d being constants.

The complex RIB–T_i probably does not involve (19) covalent bonding, and it is expected to be charge controlled. That is to say, the differences $\epsilon_{ki} - \epsilon_m$ and $\epsilon_{1i} - \epsilon_n$ for all pairs of orbitals will be large, with very little transfer of charge. The small differences between the various ϵ_m and between the various ϵ_n values can then be neglected (35). Thus, equations in Eq. 14 can be written in the following simplified form:

$$\log k_i^I = c + d\Sigma_p \left\{ Q_{i_{t_p}} Q_{r_p}(tt|rr)_p + \left(\Sigma_{ki} \frac{2c_{ki}^2{}_{t_p}}{\epsilon_{ki} - \bar{\epsilon}_m}\right)(\Sigma_m c_{m r_p}^2) \times \beta_{t_p r_p}^2 + \left(\Sigma_{1i} \frac{2c_{1i}^2{}_{t_p}}{\epsilon_{1i} - \bar{\epsilon}_n}\right)(\Sigma_n c_{n r_p}^2)\beta_{t_p r_p}^2 \right\} \qquad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 15)}$$

where $\bar{\epsilon}_m$ and $\bar{\epsilon}_n$ are average values. The terms $\Sigma_{ki}[2c_{ki}^2{}_{t_p}/(\epsilon_{ki} - \bar{\epsilon}_m)]$ and $\Sigma_{1i}[2c_{1i}^2{}_{t_p}/(\epsilon_{1i} - \bar{\epsilon}_n)]$ are very closely related to Fukui's *et al.* (30, 31) electrophilic ($E_{i_{t_p}}$) and nucleophilic ($N_{i_{t_p}}$) delocalizabilities, $E_{i_{t_p}} \approx \Sigma_{ki}(2c_{ki}^2{}_{t_p}/\lambda_{ki}\beta_{cc})$ and $N_{i_{t_p}} = \Sigma_{1i}(2c_{1i}^2{}_{t_p}/\lambda_{1i}\beta_{cc})$, where λ_k is the energy of the kth molecular orbital in units of β_{cc} with reference to α_c, α_c and β_{cc} being the Coulomb and the resonance integrals of benzene. This circumstance, as well as the fact that the quantities $Q_{r_p}(tt|rr)_p$, $(\Sigma_m c_{m r_p}^2)\beta_{t_p r_p}^2/\beta_{cc}$, and $(\Sigma_n c_{n r_p}^2)\beta_{t_p r_p}^2/\beta_{cc}$ are essentially constant for all the equations of the set, transforms the equations in Eq. 15 into the following set of simultaneous equations:

$$\log k_i^I = c + \Sigma_p\{f_p Q_{i_{t_p}} + g_p E_{i_{t_p}} + h_p N_{i_{t_p}}\} \qquad i = 1, 2, \ldots, 14 \qquad \text{(Eq. 16)}$$

where c, f_p, g_p, and h_p are constants. This expression is formally similar to expression 14 in *Reference 36*.

In principle, this system of simultaneous equations holds for the atoms p of the tetracyclines directly perturbed by bonding in the complex with ribosomes. Combined with the usual multiple-regression techniques, these equations can be usefully applied to estimate the relative variation of ΔE_i for the family of tetracyclines which act in an identical way on the RIB of unknown structure. They can also be used to determine which tetracycline atoms, p, are directly concerned in complex formation.

[1] It is assumed in this work that there is no degeneracy between molecular orbital energies. See following discussion.

Table III—Empirical Parameters for Electronic Localized Structure Calculations (3, 9, 41, 43, 44)

$\delta°$ Values

H	C(Ternary)	C(Quaternary)	N(Ternary)	N(Quaternary)
0.00	0.12	0.07	0.24	0.31

O(Hydroxyl)	O(Carbonyl)	$O^{-1/2}$(Nitro)	Cl	Br	O . . .	H—O
0.40	0.28	0.33	0.35	0.30	+0.04	−0.04

ϵ_{AB} Values

C—C	C—H	C—N	C—O	C=O	C—Cl	C—Br	N—H	O—H	$C—O^{-1/2}$	$C—N^+R_3$	$N^+—R_3$	O . . . H
1.00	1.00	1.00	0.95	0.70	0.65	0.58	0.45	0.45	0.80	1.33	0.60	0.07

$\gamma_{A(B)}$ Values

	A					
B	H	C	N	O	Cl	Br
H	—	0.3	0.3	0.3	—	—
C	0.4	0.1	0.1	0.1	0.4	0.5
N	0.4	0.1	—	0.1	—	—
O	0.4	0.1	0.1	—	—	—
Cl	—	0.2	—	—	—	—
Br	—	0.23	—	—	—	—

Table IV—Calculated Atomic Charge Indexes, $Q_p(\sigma + \pi)$, for Atoms 6, O_{10}, O_{11}, and O_{12}

Compound, T_i	Q_6	Q_{O10}	Q_{O11}	Q_{O12}
1	−0.0610	−0.2866	−0.2719	−0.2955
2	+0.0951	−0.3191	−0.2759	−0.2955
3	+0.1415	−0.3191	−0.2759	−0.2955
4	+0.1393	−0.3183	−0.2760	−0.2955
5	+0.1264	−0.3183	−0.2759	−0.2955
6	−0.0610	−0.3221	−0.2758	−0.2955
7	−0.0610	−0.3213	−0.2760	−0.2955
8	−0.0610	−0.3183	−0.2760	−0.2955
9	−0.0590	−0.3188	−0.2759	−0.2955
10	−0.0614	−0.2869	−0.2997	−0.2955
10′	−0.0614	−0.2869	−0.2760	−0.2955
11	−0.0614	−0.3381	−0.2751	−0.2955
11′	−0.0614	−0.3213	−0.2760	−0.2955
12	−0.0716	−0.3144	−0.3515	−0.1596
13	+0.1393	−0.3184	−0.2646	−0.3213
14	+0.1524	−0.3189	−0.2061	−0.2773
19	−0.0590	−0.3189	−0.2760	−0.2955
20	−0.0614	−0.3221	−0.2760	−0.2955

CALCULATIONS

The general molecular structure (I) assumed for tetracyclines is based on the experimental data of Donohue *et al.* (37). The two unsaturated phenoldiketone and tricarbonylmethane moieties can be considered as being practically planar. The σ–π separability has been assumed for these two local molecular regions. The two local π-electronic structures were calculated by using the conventional Hückel method as modified by Wheland and Pauling (38). The empirical parameters utilized (39, 40) are given in Table II. Since the number of valence atomic orbitals in the electronic localized structure of tetracyclines is exceedingly large, the localized electronic properties were evaluated by use of Del Re's method (41) as modified by Berthod and Pullman (39, 42). The latter modification was used to take into account the σ–π interaction and hydrogen-bond contributions. The empirical parameters utilized (39, 41, 43, 44) are reproduced in Table III. The calculated atomic net charge indexes Q_{it_p} $(\sigma + \pi)$, for atoms C_6, O_{10}, O_{11}, and O_{12}, and their π-delocalizability indexes, E_{it_p} and N_{it_p}, are shown, respectively, in Tables IV and V[2]. The sigma part of the delocalizability indexes was neglected.

Equation 16, which relates the inhibitory activity of the tetracyclines to their electronic structure, was analyzed by multiple-regression models (45) involving the inhibitory rate constants $k_i{}^I$ and the terms Q_{it_p}, E_{it_p}, and N_{it_p}. Ideally, all possible combinations of the variables should be examined, but this rapidly becomes impracticable. The difficulty can be resolved by an appreciation of the technical background of the problem. A few general rules exist for the handling of problems involving many variables. It is a common practice to begin by fitting a regression on the variable that is thought, on informed opinion, most likely to be related to the dependent variable and then to introduce other variables in turn, retaining those that significantly increase the regression sum of squares. Following this procedure, the authors considered a great number of possible combinations of variables, as many as were judged necessary to avoid the possibility that a significant contribution might be overlooked. A total of 356 regressions was investigated. Some of the regression equations tested are given in Table VI[3].

RESULTS AND DISCUSSION

The multiple-regression equation, relating the tetracycline inhibitory index, $k_i{}^I$, to the perturbation energies, ΔE_i, that was found to best satisfy statistically the simultaneous system of Eqs. 16 is

$$\log k^I = 18.3975 + 56.1733Q_{O10} + 16.9155E_{O10} + 48.7956Q_{O11} - 1.1063E_{O11} + 71.3254Q_{O12} + 18.3655E_{O12} + 3.3880Q_{C_6} \quad \text{(Eq. 17)}$$

Equation 17 has: (*a*) a multiple-correlation coefficient, $R = 0.993$, which is highly significant [the 0.1% point for 12 degrees of freedom being 0.78 (45)]; (*b*) an estimated standard deviation, SD, of 0.1627; and (*c*) a standard error of the mean, SEM, of 0.0434.

The *t*-test for the significance of the coefficients is shown in Table VII. The analysis of variance for the multiple regression is shown in Table VIII. This type of analysis gives the value $F_6{}^7 = 61.068$ for the significance of the regression equation. This value is highly significant ($p < 0.001$). Table IX compares experimental and predicted values for $\log k_i{}^I$. Figure 1 gives a graphic representation of experimental *versus* predicted $\log k_i{}^I$ values. The result is very satisfactory when one considers the number of approximations used.

Within the accuracy of the approximations used for the determination of ΔE_i, the results obtained in this study are consistent both with the existence of a direct dependence between the variation in activity and the equilibrium constant $K_i{}^c$ and, within the assumed mode of action, the existence of a direct inhibition of protein synthesis by ribosome–tetracycline interaction. These results are consistent, more generally, with any mode of action of the general type described in the model, Eqs. 3–5. Equation 17 indicates that the variation in activity in normal tetracyclines is directly related with the electronic characteristics of the phenoldiketone and C_6 molecular regions as a function of the effects of substituents. The introduction in the regression of any variable other than those appearing in

[2] The charge and delocalizability indexes for atoms other than C_6, O_{10}, O_{11}, and O_{12} will be sent to the interested reader on request.

[3] The analysis of all tested regressions will be sent on request.

Table V—Calculated π-Electronic Delocalizability Indexes, E_p and N_p, for Atoms 6, O_{10}, O_{11} and O_{12}

Compound, T_i	E_6	N_6	$E_{O_{10}}$	$N_{O_{10}}$	$E_{O_{11}}$	$N_{O_{11}}$	$E_{O_{12}}$	$N_{O_{12}}$
1	—	—	+0.9946	+0.0547	+0.8185	−0.5831	+1.0451	−0.1101
2	—	—	+1.0592	−0.0680	+0.8277	−0.5949	+1.0453	−0.1103
3	—	—	+1.0592	−0.0680	+0.8277	−0.5949	+1.0453	−0.1103
4	—	—	+1.0532	−0.0694	+0.8273	−0.5948	+1.0452	−0.1103
5	—	—	+1.0532	−0.0694	+0.8273	−0.5948	+1.0452	−0.1103
6	—	—	+1.0832	−0.0634	+0.8294	−0.5952	+1.0453	−0.1104
7	—	—	+1.0863	−0.0665	+0.8273	−0.5932	+1.0452	−0.1103
8	—	—	+1.0532	−0.0694	+0.8273	−0.5948	+1.0452	−0.1103
9	—	—	+1.0577	−0.0684	+0.9276	−0.5948	+1.0453	−0.1103
10	—	—	+1.0043	−0.0499	+0.8270	−0.5916	+1.0452	−0.1103
10′	—	—	+1.0043	−0.0449	+0.8270	−0.5916	+1.0452	−0.1103
11	—	—	+1.0863	−0.0665	+0.8273	−0.5932	+1.0452	−0.1103
11′	—	—	+1.0863	−0.0665	+0.8273	−0.5932	+1.0452	−0.1103
12	+1.2601	−0.8270	+1.0776	−0.0852	+1.0655	−0.0958	+0.6633	−0.5550
13	—	—	+1.0570	−0.0679	+1.4467	−0.4570	+1.0326	−0.0606
14	—	—	+1.0588	−0.0687	+0.7274	−0.6773	+0.6131	−0.6131
19	—	—	+1.0592	−0.0680	+0.8277	−0.5949	+1.0452	−0.1103
20	—	—	+1.0832	−0.0634	+0.8294	−0.5952	+1.0453	−0.1104

Eq. 17 reduces the significance of the regression equation as measured by F- and t-tests.

Consider the two regression equations:

$$\log k^I = 7.23 + 52.41Q_{O_{10}} + 19.54E_{O_{10}} + 27.55Q_{O_{11}} - 1.80E_{O_{11}} + 47.27Q_{O_{12}} + 14.02E_{O_{12}} + 3.37Q_6 - 0.79Q_9 + 19.65Q_{10a} \quad \text{(Eq. 18)}$$

and

$$\log k^I = -333.75 + 184.59Q_{6a} + 65.52E_{6a} - 4.38Q_7 - 180.11E_7 - 1491.56Q_{10a} + 178.73E_{10a} + 657.51Q_{11} + 206.62E_{11} + 124.77Q_{11a} - 9.11E_{11a} \quad \text{(Eq. 19)}$$

Regression 18, which differs from Eq. 17 only by including Q_9 and Q_{10a}, has a multiple-correlation coefficient $R = 0.994$; but it has a significance given by $F_4^9 = 37.53$ ($p < 0.005$), which is less significant than the F-test for Eq. 17. The t-tests for the significance of the coefficients in Eq. 18 are shown in Table X; this test shows that the newly introduced variables, Q_9 and Q_{10a}, are not significant. Regression 19 has a multiple-correlation coefficient $R = 0.981$ but this equation is not acceptable statistically, $F_3^{10} = 7.683$ ($p < 0.1$). The t-test for significance of coefficients is shown in Table XI. Both the F-test and the t-test show the nonsignificance of this regression. Similar conclusions are obtained from the analysis of the other tested regressions (see Table VI).

It is important to realize that it is the dependence of the variation of the inhibitory ability, Δk_i^I, on the variation of the perturbation energy, $\Delta\Delta E_i$, as a whole, that has a real physical meaning. The quantity ΔE_i is made up of a combination of several more or less arbitrarily defined "reactivity indexes," charges, and so on, of which the nature depends on the particular method used in evaluating ΔE_i. In dealing with chemical reactivity problems, it is necessary to analyze the whole variation of ΔE_i. The simultaneous analysis of the different reactivity indexes making up ΔE_i indicates the type of "controlling effect" in each type of reaction. As pointed out by Hudson and Klopman (33), it is intrinsically incorrect to relate reactivity to a particular reactivity index. In the case of this series of tetracycline compounds, all component terms of ΔE_i that do not significantly contribute to the $\Delta\Delta E_i$ variation do not explicitly appear in Eq. 17. The ones that appear, Q_6, $Q_{O_{10}}$, $Q_{O_{11}}$, $Q_{O_{12}}$, $E_{O_{10}}$, $E_{O_{11}}$, and $E_{O_{12}}$, are not significant considered in isolation. In fact, each one frequently does not vary, or varies little, when passing from one molecule to another in the series (Tables IV and V).

Table VI—Some Regression Equations Tested

1. $\log k^I = 22.8809 + 34.0946Q_{O_{10}} + 6.7370E_{O_{10}}$ (1.3) (0.5) $+ 51.6236Q_{O_{11}} - 0.5822E_{O_{11}}$ (2.6) (−0.7) $+ 69.6836Q_{O_{12}} + 17.5739E_{O_{12}}$ (3.3) (4.3)
   $R = 0.9534$, $F_7^6 = 11.673$ ($p < 0.005$)
2. $\log k^I = 18.3975 + 56.1733Q_{O_{10}} + 16.9155E_{O_{10}}$ (4.9) (3.1) $+ 48.7956Q_{O_{11}} - 1.1063E_{O_{11}}$ (5.9) (−3.1) $+ 71.3254Q_{O_{12}} + 12.3655E_{O_{12}}$ (8.1) (10.7) $+ 3.3880Q_6$ (5.8)
   $R = 0.9930$, $F_6^7 = 61.068$ ($p < 0.001$)
3. $\log k^I = 11.1498 + 0.0818Q_9 + 12.5640E_9$ (0.0) (0.5) $+ 45.3364Q_{O_{10}} + 2.8236E_{O_{10}}$ (1.6) (0.1) $- 19.4518Q_{O_{11}} - 3.6663E_{O_{11}}$ (−0.2) (−0.9) $- 24.0750Q_{O_{12}} + 3.1213E_{O_{12}}$ (−0.2) (0.2)
   $R = 0.9668$, $F_5^8 = 8.9546$ ($p < 0.025$)
4. $\log k^I = 31.1534 + 1.2606Q_7 + 7.4356E_7$ (1.8) (5.3) $+ 52.2854Q_{O_{10}} + 1.7786E_{O_{10}}$ (1.6) (0.1) $+ 74.6820Q_{O_{11}} - 0.6185E_{O_{11}}$ (2.4) (−0.8) $+ 78.4148Q_{O_{12}} + 22.1390E_{O_{12}}$ (3.1) (3.6)
   $R = 0.9678$, $F_5^8 = 9.2443$ ($p < 0.025$)

Table VII—t-Test for Significance of Coefficients in the Multiple-Regression Eq. 17

Variable	t-Value	p
Q_0	4.895	<0.005
$E_{O_{10}}$	3.077	>0.01
$Q_{O_{11}}$	5.89	<0.005
$E_{O_{11}}$	−3.087	>0.01
$Q_{O_{12}}$	8.095	<0.005
$E_{O_{12}}$	10.681	<0.005
Q_6	5.779	<0.005

Table VIII—Analysis of Variance for the Multiple Regression (17)[a]

Source	Sum of Squares	Degrees of Freedom	Mean Squares
Due to regression	11.3170	7	1.6167
About regression	0.1588	6	0.0265

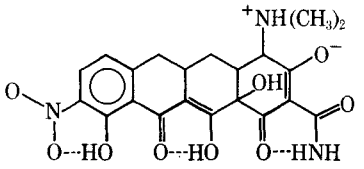
[a] $F_6^7 = 61.068$, and $p < 0.001$.

Table IX—Actual and Predicted Values for the Logarithm of the Inhibitory Rate Constants

Compound, i	log K_i^I (exp.)	log k_i^I (calcd.)	Residuals
1	2.874	2.860	+0.014
2	2.714	2.454	+0.260
3	2.604	2.611	−0.007
4	2.434	2.541	−0.107
5	2.410	2.502	−0.092
6	2.259	2.165	+0.094
7	2.161	2.252	−0.091
8	1.975	1.861	+0.114
9	1.714	1.923	−0.209
10	1.647	1.641	+0.006
11	1.374	1.352	+0.022
12	1.190	1.190	0.000
13	0.399	0.399	0.000
14	−0.469	−0.470	−0.001

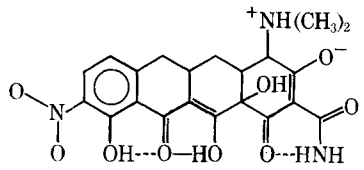
Regression 17 underlines the electrostatic or charge-controlled character of tetracycline action. The introduction of the N_{t_p} variables associated with atoms O_{10}, O_{11}, and O_{12} gives nonsignificant t-values and important lowering of the F-values for the significance of the multiple correlation (Table VI). The fact that the E_{t_p} delocalizabilities are significant indicates that the π-electronic donor character of the phenoldiketone structures are also important. This fact is consistent with the possibility that ribosome–tetracycline interaction occurs through metal chelates.

Inasmuch as Eq. 17 gives an indication of the relationship between inhibitory activity and the molecular structure for the normal tetracycline series, it should be possible to predict the influence of specific factors, such as steric hindrance, on the inhibitory index k_i^I. The phenolic hydroxyl groups in the tetracycline molecules play an essential role in the interaction process. As a consequence, when the 10-hydroxyl group is hindered by hydrogen bonding with an *ortho*-nitro substituent, the activity is greatly reduced. This is the case (46) for the 9-NO_2-6-demethyl-6-deoxytetracycline (Compound 10 in this series). In the calculations, the authors considered this type of interaction of the hydroxyl group by using the molecular model shown here for Compound 10.



Compound 10

It is easy to account for the quantitative importance of this hindering factor by considering a compound with the ideal structure (Compound 10′), where the hydroxyl group-10, as in the other molecules in the series, is not *ortho*-inhibited (Compound 10′). Equation 17 gives a rate inhibitory constant of $k_{10'}{}^I = 635.25$ for Compound 10′. As one would expect, this value for the activity is very near to that of the 7-NO_2-compound, $k_1{}^I = 748.78$. The experimental $k_{10}{}^I$ is 44.33, an activity which is well correlated with the calculated value for Compound 10 of 43.72. A similar case was observed with 9-dimethylamino-6-demethyl-6-deoxytetracycline, Compound 11. When $k_{11}{}^I$ is calculated neglecting the hydrogen bonding between the methylamino substituent and the hydroxyl group-10, the predicted value for activity is $k_{11}{}'^I = 178.81$. This value lies between the activities for the 7-NH_2 and the 9-NH_2 derivatives and it is significantly different from the experimental activity value $k_{11}{}^I = 23.67$. The calculated value for the 10-hydroxyl-hindered compound is 23.66.



Compound 10′

Table X—t-Test for Significance of Coefficients in the Multiple Regression (18)

Variable	t-Value	p
$Q_{O_{10}}$	3.788	≤ 0.01
$E_{O_{10}}$	2.825	≤ 0.025
$Q_{O_{11}}$	1.037	>0.1
$E_{O_{11}}$	1.982	<0.1
$Q_{O_{12}}$	1.584	≤ 0.1
$E_{O_{12}}$	2.573	<0.05
Q_6	4.423	<0.01
Q_9	0.641	>0.1
Q_{10a}	0.784	>0.1

Table XI—t-Test for Significance of Coefficients in the Multiple Regression (19)

Variable	t-Value	p
Q_{6a}	2.294	<0.1
E_{6a}	2.176	<0.1
Q_7	−0.624	>0.1
E_7	−2.357	$=0.05$
Q_{10a}	−2.220	<0.1
E_{10a}	1.665	≤ 0.1
Q_{11}	2.457	<0.05
E_{11}	2.377	≤ 0.05
Q_{11a}	2.146	<0.1
E_{11a}	−2.365	$=0.05$

Insofar as Eq. 17 is correct, it is possible to use it for the predictions of the activity of untested normal tetracycline derivatives. For instance, in the experimental work of Miller *et al.* (15), the rate activity constant for 7-chloro- and 7-dimethylamino-6-demethyl-6-deoxytetracyclines (Compounds 19 and 20 in the tables) were not determined. The predicted values, from Eq. 17, are, respectively, $k_{19}{}^I = 84.52$ and $k_{20}{}^I = 145.49$. The predicted activity $k_{20}{}^I$ is of the same order (*cf.*, Table I) as those for the 7-NH_2- and 9-NH_2-6-deoxytetracycline derivatives. Also, the predicted activity $k_{19}{}^I$ is of the same order as those for the 6-demethyl-6-deoxytetracycline and 7-Br-6-demethyl-6-deoxytetracycline derivatives. The latter result is in good agreement with the experimental results of Hlavka *et al.* (47) on the *in vitro* activities for these three compounds.

CONCLUSION

In spite of the large number of approximations used, the results of this theoretical approach seem to explain reasonably well a great quantity of experimental data on the inhibitory properties of normal tetracyclines by relating them to the electronic structure of the molecules.

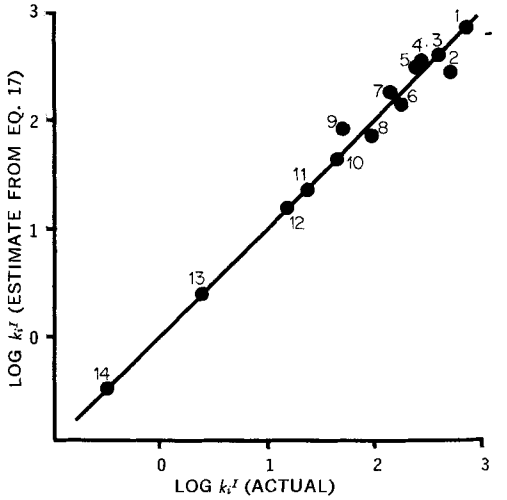


Figure 1—*Graphic representation of actual* versus *estimated log* $k_1{}^I$ *values.* $F_6{}^7 = 61.07$ ($p < 0.001$).

The theoretical results are consistent with the hypothesis of a direct inhibition of *E. coli* caused by complexation of tetracyclines with a biological structure, for example, inhibition of protein synthesis by complexation with a ribosome.

The tetracycline action seems to involve a direct interaction of C_6 and the phenoldiketone region with the proposed ribosome receptor site. The process seems to be charge controlled. The interaction of phenoldiketone oxygen functions with the ribosome may be achieved through metal chelates, since this interaction is a function of the donor character of the oxygen atoms.

The effect of substituents on the electronic properties of the C_6 and phenoldiketone regions is to produce a graduation of the inhibitory properties of normal tetracyclines.

Equation 17 permits a satisfactory estimation of rate inhibitory constants k_i^I for normal tetracyclines, provided that specific effects such as steric hindrance are taken into account. Furthermore, it allows the estimation of activities of tetracyclines not yet tested *in vitro* or clinically for inhibitory activities.

This paper serves as an example of the use of quantum chemical techniques in elucidating biological action and provides an indication of the changes one can make in a drug molecule to alter biological action. These methods could also be used to suggest molecular changes needed for greater stability, enhanced penetration through membranes, and general solubility and partitioning characteristics.

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Stereospecific Hydrogenations V: Hydrogenation Rates Using Palladium-on-Poly-S-leucine and Palladium-on-Poly-S-valine

ROBERT L. BEAMER and WILLIAM D. BROWN

Abstract □ The effect of polyamino acid secondary structure (helical or nonhelical) on the rates of hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid catalyzed by palladium-on-poly-*S*-leucine and by palladium-on-poly-*S*-valine was investigated. For each substrate, the hydrogenations proceeded at a faster rate with palladium-on-poly-*S*-leucine (helical catalyst) than with palladium-on-poly-*S*-valine (nonhelical catalyst). That α-methylcinnamic acid and α-acetamidocinnamic acid occupy entirely different sites on the catalyst surface was shown by product and cross-inhibition studies. The observation that dihydro-α-methylcinnamic acid increases the rate of hydrogenation of α-acetamidocinnamic acid on palladium-on-poly-*S*-valine indicates a possible allosteric mechanism analogous to that observed with enzyme catalysis.

Keyphrases □ Palladium-on-poly-*S*-leucine—α-acetamidocinnamic acid, α-methylcinnamic acid hydrogenation rates □ α-Acetamidocinnamic acid, α-methylcinnamic acid hydrogenation rates—palladium-on-poly-*S*-valine □ Helical conformation—polyamino acid □ Catalyst surface-active sites—inhibition studies

Asymmetry in polypeptides and proteins can arise both from the asymmetric carbon atoms of the individual amino acid residues (primary structure) and from helical folding of the polymeric chains (secondary structure) (1). Stereospecific hydrogenations using palladium-on-poly-*S*-leucine, palladium-on-poly-*S*-valine, palladium-on-poly-γ-benzyl-*S*-glutamate, and palladium-on-poly-β-benzyl-*S*-aspartate were described in earlier papers of this series (2–4). α-Methylcinnamic acid and α-acetamidocinnamic acid, both of which form asymmetric carbon atoms upon hydrogenation, were used as substrates in the previous investigations. The absolute configurations of the reduced substrates have been determined (5).

Hydrogenations using palladium-on-poly-*S*-leucine, which possesses a right-handed helical structure (6), *versus* those using palladium-on-poly-*S*-valine, which is nonhelical (7), indicated that the chirality of the helical conformation had a greater influence on the enantioselectivity of the hydrogenations than the dissymmetry arising from the amino acid residues (4).

Hydrogenation of α-methylcinnamic acid using palladium-on-poly-*S*-amino acids, which possess a right-handed helical structure (*viz.*, poly-*S*-leucine and poly-γ-benzyl-*S*-glutamate), produced R(−)-dihydro-α-methylcinnamic acid; hydrogenation using these same catalysts produced *S*(−)-phenylalanine (after hydrolysis of the hydrogenation product) when α-acetamidocinnamic acid was the substrate (2–4). Conversely, *S*(+)-dihydro-α-methylcinnamic acid and R(+)-phenylalanine were formed from this substrate when either a left-handed helical polyamino acid (*viz.*, poly-β-benzyl-*S*-aspartate) or a nonhelical polyamino acid (*viz.*, poly-*S*-valine) was used as the carrier for the palladium (3, 4).

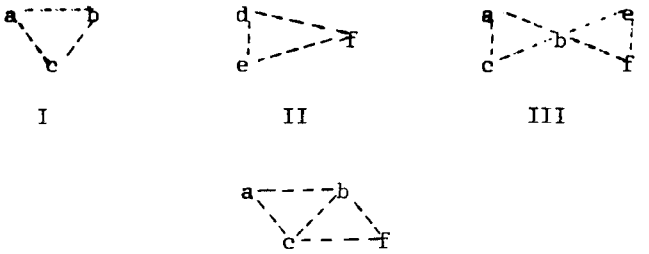


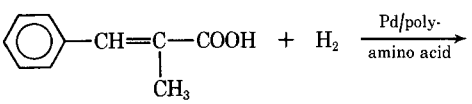
Figure 1—*Hypothetical active sites on a catalyst surface.*

The formation of products of opposite configuration when α-methylcinnamic acid and α-acetamidocinnamic acid were hydrogenated in the presence of the same catalyst led to the conclusion that these two substrates were occupying—at least in part—different sites on the catalyst surface.

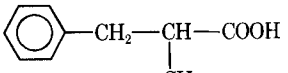
The investigation described in this paper was undertaken to determine the influence of helical or nonhelical conformation (secondary structure) upon the hydrogenation rates of α-methylcinnamic acid and α-acetamidocinnamic acid. The helical or nonhelical influence on hydrogenation rates can be assessed by measuring the rate of hydrogen uptake by the respective substrate under identical conditions when catalyzed by palladium-on-poly-*S*-leucine (a helical catalyst) as compared with that catalyzed by palladium-on-poly-*S*-valine (a nonhelical catalyst).

Another objective of this investigation was to ascertain the degree to which α-methylcinnamic acid or α-acetamidocinnamic acid might occupy the same or different sites on the catalytic surface. Figure 1 illustrates substrate specific centers which could exist on a stereospecific catalytic surface.

Sites I and II each represent separate substrate-specific sites. The two substrate-specific sites present in III have one common point, while IV illustrates two sites having two points in common. Although the configurational evidence provided in the previous investigations of this series (2–4) showed that the



α-methylcinnamic acid



dihydro-α-methylcinnamic acid

Scheme I

α-methylcinnamic acid and α-acetamidocinnamic acid must occupy different sites on the catalytic surfaces, these sites may or may not have points in common.

The hydrogenation of α-methylcinnamic acid is represented by Scheme I. Scheme II illustrates the hydrogenation of α-acetamidocinnamic acid to *N*-acetylphenylalanine followed by hydrolysis to phenylalanine.

Using a minimal catalyst (8, 9) and conducting the hydrogenation of α-methylcinnamic acid (Scheme I) in the presence of varying concentrations of the product, dihydro-α-methylcinnamic acid, the concentration of dihydro-α-methylcinnamic acid that produces product inhibition can be determined. In a similar manner, the concentration of *N*-acetylphenylalanine that inhibits the hydrogenation of α-acetamidocinnamic acid also can be determined.

By hydrogenating α-methylcinnamic acid in the presence of *N*-acetylphenylalanine and by hydrogenating α-acetamidocinnamic acid in the presence of dihydro-α-methylcinnamic acid (cross-inhibition studies), a determination of the degree to which the two substrates occupy the same catalytic site can be made. If dihydro-α-methylcinnamic acid inhibits the hydrogenation of α-acetamidocinnamic acid or if *N*-acetylphenylalanine inhibits the hydrogenation of α-methylcinnamic acid, the two substrates must be occupying the same site. On the other hand, if no cross-inhibition is seen in either hydrogenation, one must conclude that the two substrates are not occupying the same catalytic site. The concentrations of the inhibitors should correspond closely to the concentration of product that produced inhibition.

EXPERIMENTAL

Reagents—The following were used: α-methylcinnamic acid[1], α-acetamidocinnamic acid dihydrate[1], *N*-carbobenzyloxy-*S*-valine[2], leucine[3], and carbobenzyloxy chloride[2].

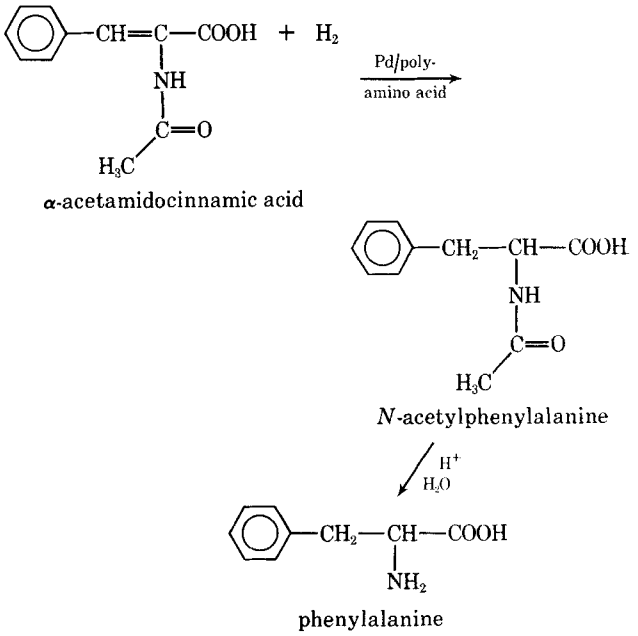
Absolute Alcohol—This reagent was prepared by taking commercial absolute alcohol and refluxing over calcium oxide for at least 24 hr. and then distilling. The product distilled at 78.5°.

Poly-*S*-leucine—This compound was prepared by the procedure of Beamer *et al.* (2), starting with *S*-leucine and carbobenzyloxy chloride.

Poly-*S*-valine—This compound was prepared by the method described by Beamer *et al.* (3), starting with *N*-carbobenzyloxy-*S*-valine.

Dihydro-α-methylcinnamic Acid—This compound was prepared by using 8.10 g. (0.05 mole) of α-methylcinnamic acid in 100 ml. of absolute alcohol and 1.0 g. of palladium-on-charcoal [100 mg. $PdCl_2$/g. charcoal prepared in the presence of sodium acetate (10)]. The hydrogenation was carried out in a Parr low-pressure hydrogenator at an initial pressure of 4.2 kg./cm.^{2}. The time required for complete hydrogenation was 1 hr. Following hydrogenation, the catalyst was removed by filtration and the ethanol was evaporated under reduced pressure. The residue was distilled under reduced pressure. The boiling point of the product was 114° at 0.25 Torr. [lit. (11) 160°/13 Torr.]. A neutralization equivalent of the product agreed with that calculated for dihydro-α-methylcinnamic acid. An IR spectrum of the product was identical with one reported for dihydro-α-methylcinnamic acid (12).

***N*-Acetylphenylalanine**—This compound was prepared by the hydrogenation of α-acetamidocinnamic acid dihydrate, 4.8 g. (0.023 mole), using 1.0 g. palladium-on-charcoal [100 mg. $PdCl_2$/g. charcoal prepared in the presence of sodium acetate (10)]. Following hydrogenation, the ethanol was evaporated under reduced pressure and the white crystalline solid product recrystallized from absolute alcohol. The melting point of the product was 171–172° [lit. (13) 172°].



Scheme II

Preparation of Catalysts—The catalysts were prepared by depositing palladium (from a solution of palladous chloride, 2.5%) upon the respective polyamino acids according to Method II of Beamer *et al.* (2). The concentrations employed were 200, 100, 125, and 150 mg. of palladous chloride/g. of carrier[4]. In each study, the polyamino acid used to make the catalyst and the catalyst used came from a single batch [*i.e.*, they were identical catalysts (8)].

Hydrogenation Studies—These studies were carried out in a Thomas microhydrogenator[5] modified by immersing the reaction flask in a 1000-ml. beaker into which water from a constant-temperature bath was circulated. All hydrogenations were conducted at a temperature of 36.0°.

The hydrogenation procedure of Ogg and Cooper (14) was used for these studies. In all studies, 30.0 mg. of catalyst was employed along with 4 ml. of absolute alcohol; the amount of substrate used was 1×10^{-4} mole (16.2 mg. of α-methylcinnamic acid and 24.1 mg. of α-acetamidocinnamic acid dihydrate). The amount of product necessary to produce product inhibition was determined to be 8 times the concentration of the substrate. An initial concentration of 8×10^{-4} mole of product or inhibitor/4 ml. of absolute alcohol was used in both product and cross-inhibition studies (131.4 mg. of dihydro-α-methylcinnamic acid and 165.8 mg. of *N*-acetylphenylalanine). During the hydrogenations, the volume of hydrogen consumed was recorded at set intervals. The results of the studies were processed using an IBM 7040 computer and a least-squares FORTRAN IV program[6]. This program converted all data into volumes of hydrogen at standard temperature and pressure. The program then converted the volumes into millimoles of hydrogen. It also gave values for the *y*-intercept and slope for each set of data. The calculated slope was an average slope, because only the first portion of the curve was linear. The various runs for each study were averaged, and the standard deviation between runs was determined.

RESULTS

The results of the hydrogenation studies are given in Tables I, II, and III and in Figs. 2–7. The catalyst concentration employed is indicated with each table.

[1] Aldrich Chemical Co.
[2] Nutritional Biochemicals.
[3] Mann Biochemical Corp.
[4] Represent minimal catalyst concentrations (8, 9).
[5] H. R. Thomas Co.
[6] The authors will make the FORTRAN IV computor program available to any interested investigator.

Table I—Hydrogenations of α-Methylcinnamic Acid and α-Acetamidocinnamic Acid Using Palladium-on-Poly-*S*-leucine and Palladium-on-Poly-*S*-valine as Catalysts

Minutes	α-Methylcinnamic Acid		α-Acetamidocinnamic Acid	
	Poly-*S*-leucine[a], 0.00176 *SD*, mmole H_2	Poly-*S*-valine[a], 0.00132 *SD*, mmole H_2	Poly-*S*-leucine[a], 0.00135 *SD*, mmole H_2	Poly-*S*-valine[a], 0.00187 *SD*, mmole H_2
0.00	0.0000	0.0000	0.0000	0.0000
	0.008719	0.001191	0.001698	
1.00	0.03309	0.002252	0.005434	0.000682
	0.05596	0.004497	0.011380	
2.00	0.07220	0.006975	0.017326	0.001535
	0.08152	0.009448	0.024799	
3.00	0.08694	0.011357	0.030909	0.002216
	0.08856	0.013718	0.037193	
4.00	0.09060	0.016194	0.043562	0.003326
	0.09127	0.018896	0.049334	
5.00	0.09511	0.021497	0.054689	0.004519
	0.09545	0.023498	0.059781	
6.00	0.09613	0.025750	0.064534	0.005543
		0.028112	0.069628	
7.00		0.030361	0.073452	0.006481
		0.032266	0.077355	
8.00		0.034064	0.081007	0.007504
		0.036055	0.084318	
9.00		0.038343	0.087458	0.008698
		0.039905	0.089835	
10.00		0.041476	0.091876	0.009722
		0.043390	0.093144	
11.00		0.045304	0.094845	0.010829
		0.047555	0.096373	
12.00		0.049132	0.097216	0.011682
		0.050819	0.096913	
13.00		0.052506	0.098272	0.012621
		0.054083	0.098986	
14.00		0.055206	0.100140	0.013388
		0.056555		
15.00		0.058132		0.014327
16.00		0.061175		
17.00		0.063764		
18.00		0.067127		
19.00		0.069378		
20.00		0.071856		0.018761
21.00		0.073881		
22.00		0.075684		
23.00		0.077488		
24.00		0.079175		
25.00		0.080641		0.023632
26.00		0.081991		
27.00		0.083717		
28.00		0.084909		
29.00		0.085759		
30.00		0.086610		0.027716
35.00		0.090178		0.032370
40.00		0.093265		0.036800
45.00		0.095468		0.041457
50.00		0.097213		0.045659
55.00		0.098199		0.049976
60.00		0.098344		0.053610
65.00				0.056336
70.00				0.058948
75.00				0.061325
80.00				0.073073
85.00				0.075969
90.00				0.078354
95.00				0.086578
100.00				0.089305
105.00				0.092032

[a] 200 mg. $PdCl_2$/g. polyamino acid.

Table II—Inhibited and Uninhibited Hydrogenations of α-Methylcinnamic Acid and α-Acetamidocinnamic Acid Using Palladium-on-Poly-*S*-leucine as Catalyst

Minutes	Uninhibited, 0.000000 *SD*, mmole H_2	Inhibitor	
		N-Acetylphenylalanine, 0.000095 *SD*, mmole H_2	Dihydro-α-methylcinnamic Acid, 0.00056 *SD*, mmole H_2
	α-Methylcinnamic Acid[a]		
0.00	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000
1.00	0.000677	0.000678	0.000506
	0.000846	0.001356	0.000506
2.00	0.001354	0.002034	0.001012
	0.002032	0.002375	0.001350
3.00	0.002711	0.002820	0.002026
	0.003387	0.003560	0.002026
4.00	0.004064	0.003899	0.002364
	0.004742	0.004409	0.002701
5.00	0.005419	0.005086	0.003376
6.00	0.006096	0.006106	0.004051
7.00	0.007452	0.006784	0.004558
8.00	0.008468	0.007802	0.005064
9.00	0.009314	0.008649	0.005739
10.00	0.009823	0.009836	0.006246
11.00	0.011347	0.010851	0.006920
12.00	0.012024	0.011866	0.007427
13.00	0.012720	0.012981	0.007933
14.00	0.013549	0.014236	0.008440
15.00	0.014226	0.015083	0.009115
20.00	0.017952	0.019319	0.012154
25.00	0.020324	0.021687	0.014348
30.00	0.024049	0.025074	0.016204
35.00	0.027635	0.028128	0.017892
40.00	0.030993	0.030155	0.019412
45.00	0.034042	0.032864	0.020930
50.00	0.034890	0.034898	0.002450
55.00	0.039429	0.037273	0.023800
60.00	0.040139	0.039307	0.025150
	α-Acetamidocinnamic Acid[b]		
	0.000003 *SD*	0.000120 *SD*	0.000054 *SD*
0.00	0.0000	0.0000	0.000000
1.00	0.000681	0.000000	0.000677
2.00	0.001022	0.000678	0.001354
3.00	0.001362	0.000678	0.002032
4.00	0.002044	0.001356	0.002710
5.00	0.002725	0.001356	0.003386
6.00	0.003406	0.002034	0.004074
7.00	0.004088	0.002034	0.004741
8.00	0.004769	0.002712	0.005418
9.00	0.005450	0.002712	0.006096
10.00	0.006132	0.003390	0.006774
11.00			
12.00			
13.00			
14.00			
15.00	0.008516	0.005084	0.009482
20.00	0.011242	0.006779	0.012192
25.00	0.015329	0.008135	0.015239
30.00	0.018056	0.008982	0.017949
35.00	0.020099	0.009830	0.019980
40.00	0.023164	0.010846	0.021674
45.00	0.025548	0.011863	0.023706
50.00	0.027592	0.012710	0.026754
55.00	0.029636	0.013728	0.027770
60.00	0.031680	0.014744	0.029802

[a] 100 mg. $PdCl_2$/g. [b] 125 mg. $PdCl_2$/g.

Table III—Hydrogenations of α-Methylcinnamic Acid and α-Acetamidocinnamic Acid using Palladium-on-Poly-*S*-valine as Catalyst in the Presence of Dihydro-α-methylcinnamic Acid and *N*-Acetylphenylalanine

Minutes	Uninhibited, 0.000018 *SD*, mmole H_2	Inhibitor: Dihydro-α-methylcinnamic Acid, 0.000007 *SD*, mmole H_2	Inhibitor: *N*-Acetylphenylalanine, 0.000009 *SD*, mmole H_2
	α-Methylcinnamic Acid[a]		
0.00	0.000000	0.000000	0.000000
1.00	0.000844	0.000000	0.000849
2.00	0.001689	0.000676	0.001529
3.00	0.002365	0.000676	0.002380
4.00	0.003042	0.001014	0.003295
5.00	0.003717	0.001352	0.004080
6.00	0.004392	0.001690	0.004760
7.00	0.005068	0.002198	0.005440
8.00	0.005744	0.002536	0.006120
9.00	0.006425	0.002874	0.006800
10.00	0.007096	0.003044	0.007480
15.00	0.010474	0.004903	0.010880
20.00	0.014022	0.006763	0.014110
25.00	0.017063	0.008623	0.017340
30.00	0.019766	0.010314	0.020569
35.00	0.022132	0.011836	0.023800
40.00	0.024158	0.013357	0.026860
45.00	0.026186	0.014879	0.029921
50.00	0.028213	0.016062	0.032810
55.00	0.030240	0.017416	0.035530
60.00	0.032098	0.018768	0.038081
	α-Acetamidocinnamic Acid[b]		
		0.000078 *SD*	0.000047 *SD*
0.00		0.000000	0.000000
1.00		0.001015	0.000507
2.00		0.002031	0.000845
3.00		0.003046	0.001182
4.00		0.004062	0.002196
5.00		0.005077	0.003210
6.00		0.006262	0.003886
7.00		0.007277	0.004730
8.00		0.008292	0.005068
9.00		0.009308	0.005575
10.00		0.010324	0.006082
15.00		0.015569	0.009292
20.00		0.020816	0.014192
25.00		0.026062	0.017908
30.00		0.031139	0.021118
35.00		0.036216	0.023484
40.00		0.041463	0.026862
45.00		0.046370	0.030240
50.00		0.051448	0.032606
55.00		0.055848	0.034632
60.00		0.060587	0.036998

[a] 150 mg. $PdCl_2$/g. [b] 200 mg. $PdCl_2$/g.

Table I and Figs. 2 and 3 give the results of hydrogenating α-methylcinnamic acid and α-acetamidocinnamic acid using palladium-on-poly-*S*-leucine and palladium-on-poly-*S*-valine as catalysts.

The results of the product inhibition and the cross-inhibition experiments using palladium-on-poly-*S*-leucine are given in Table II and Figs. 4 and 5. Table III and Figs. 6 and 7 give the results of the product inhibition studies using the palladium-on-poly-*S*-valine catalyst.

DISCUSSION

From the results obtained from the hydrogenation experiments involving poly-*S*-leucine and poly-*S*-valine as carriers for palladium, one must conclude that the helical character of the poly-*S*-leucine favorably influences the hydrogenation rate. Table I and Figs. 2 and 3 show that both α-methylcinnamic acid and α-acetamidocinnamic acid were reduced faster with palladium-on-poly-*S*-leucine than with palladium-on-poly-*S*-valine. Previous studies (4)

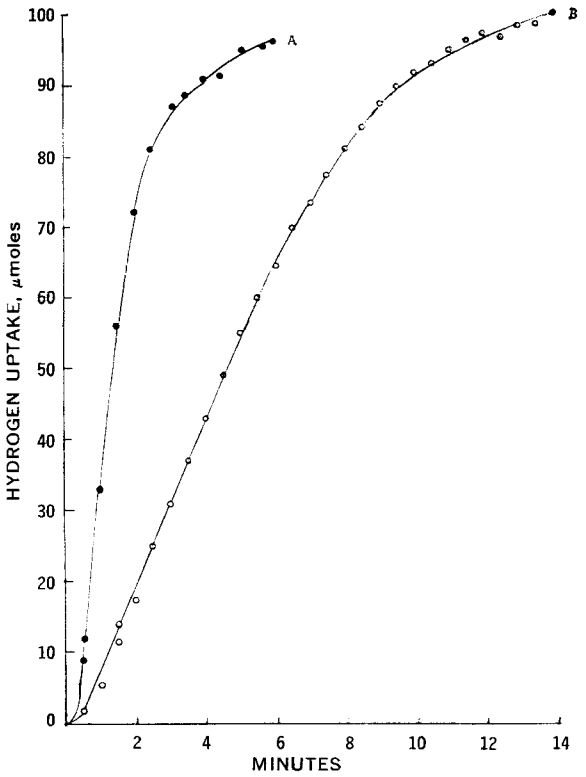
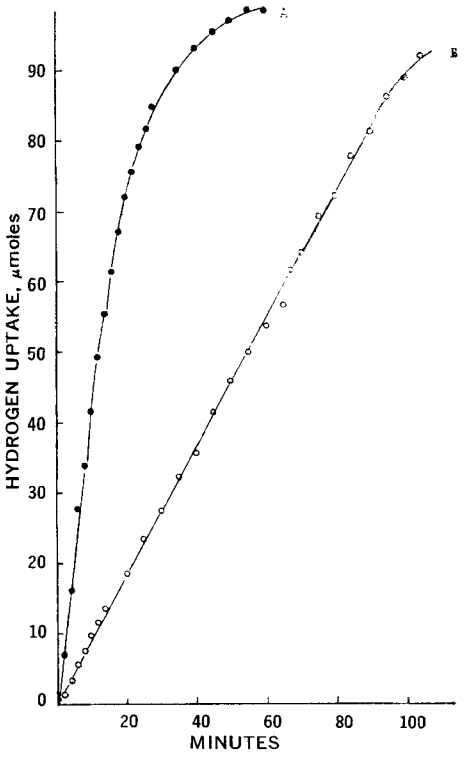


Figure 2—*Hydrogenations of α-methylcinnamic acid and α-acetamidocinnamic acid by palladium-on-poly-S-leucine. Key: A, ●, α-methylcinnamic acid; and B, ○, α-acetamidocinnamic acid.*



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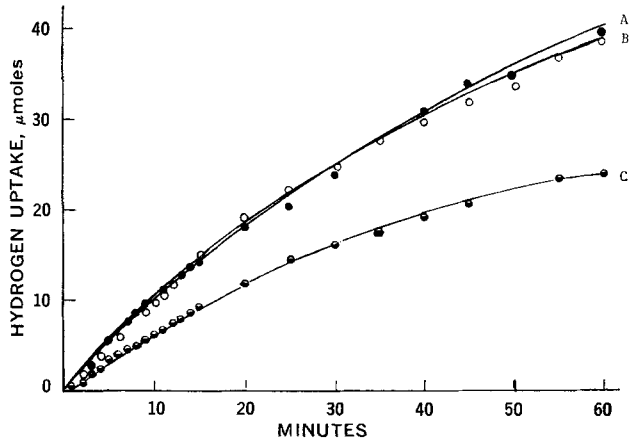

Figure 4—*Hydrogenations of α-methylcinnamic acid by palladium-on-poly-S-leucine in the presence of* N-*acetylphenylalanine and dihydro-α-methylcinnamic acid. Key: A, ●, α-methylcinnamic acid; B, ○, α-methylcinnamic acid with* N-*acetylphenylalanine; and C, ◒, α-methylcinnamic acid with dihydro-α-methylcinnamic acid.*

using these same catalysts and substrates showed a consistently higher optical yield with the poly-*S*-leucine catalyst than with the poly-*S*-valine catalyst. Apparently, not only does the α-helix provide increased stereoselection, but it also provides a closer fit between the catalyst and substrate. With both catalysts, α-methylcinnamic acid was hydrogenated at a faster rate than α-acetamidocinnamic acid. The faster rate could result from a greater affinity of α-methylcinnamic acid for the catalyst surface, from the greater steric hindrance provided by the more bulky acetamido group as compared to the smaller methyl group, or from a combination of both of these effects.

The hydrogenation of 1×10^{-4} mole of α-methylcinnamic acid using palladium-on-poly-*S*-leucine was inhibited by the addition of 8×10^{-4} mole of the product of the hydrogenation, dihydro-α-methylcinnamic acid, to the reaction mixture. A similar inhibition was seen with the hydrogenations of α-methylcinnamic acid using palladium-on-poly-*S*-valine. However, these same reactions were not inhibited by the presence of 8×10^{-4} mole of *N*-acetylphenylalanine, the product of the reduction of α-acetamidocinnamic acid.

The hydrogen uptake by 1×10^{-4} mole of α-acetamidocinnamic acid catalyzed by 30 mg. of palladium-on-poly-*S*-leucine was product inhibited by the presence of 8×10^{-4} mole of *N*-acetylphenylalanine, but 8×10^{-4} mole of dihydro-α-methylcinnamic acid in the reaction mixture did not inhibit the reaction. The same results were obtained when α-methylcinnamic acid was hydrogenated in the presence of the palladium-on-poly-*S*-valine catalyst. Dihydro-α-methylcinnamic acid (8×10^{-4} mole/4 ml. of solvent) increased the rate of the palladium-on-poly-*S*-valine-catalyzed hydrogenation of α-acetamidocinnamic acid, while *N*-acetylphenylalanine in the same concentration slowed the hydrogenation rate.

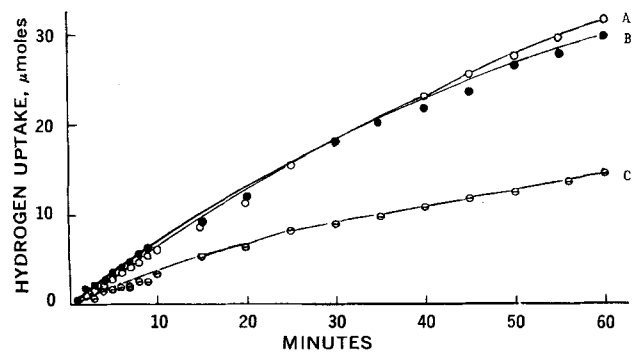

Figure 5—*Hydrogenations of α-acetamidocinnamic acid by palladium-on-poly-S-leucine in the presence of* N-*acetylphenylalanine and dihydro-α-methylcinnamic acid. Key: A, ○, α-acetamidocinnamic acid; B, ●, α-acetamidocinnamic acid with dihydro-α-methylcinnamic acid; and C, ◒, α-acetamidocinnamic acid with* N-*acetylphenylalanine.*

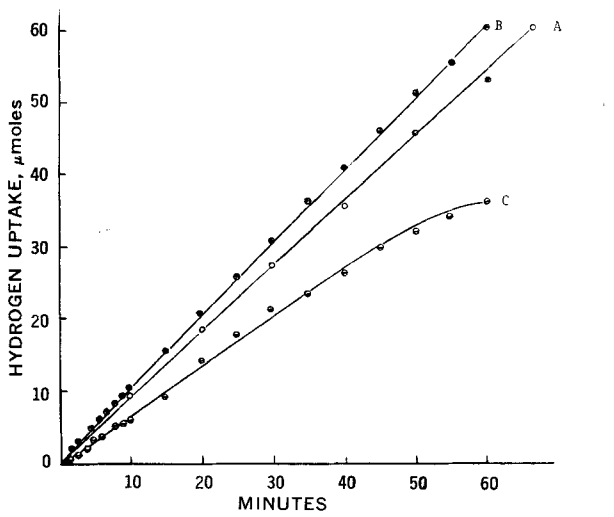

Figure 6—*Hydrogenations of α-acetamidocinnamic acid by palladium-on-poly-S-valine in the presence of* N-*acetylphenylalanine and dihydro-α-methylcinnamic acid. Key: A, ○, α-acetamidocinnamic acid with no inhibitor; B, ●, α-acetamidocinnamic acid with dihydro-α-methylcinnamic acid; and C, ⊖, α-acetamidocinnamic acid with* N-*acetylphenylalanine.*

The increase in hydrogenation rate observed with α-acetamidocinnamic acid when catalyzed by palladium-on-poly-*S*-valine in the presence of dihydro-α-methylcinnamic acid is reminiscent of allosteric facilitation of some enzyme-catalyzed reactions (15).

The results of the inhibition experiments lead to the conclusion that the two substrates occupy two entirely different reaction sites (*i.e.*, I and II of Fig. 1). This not only confirms the conclusion arising from the configurational studies (2–4), but extends the conclusions of these studies by eliminating partial interaction between sites (*i.e.*, as seen in III and IV of Fig. 1).

SUMMARY AND CONCLUSIONS

The polyamino acid secondary structures of α-methylcinnamic acid and α-acetamidocinnamic acid were found to affect hydrogenation rates in reactions catalyzed by palladium-on-poly-*S*-

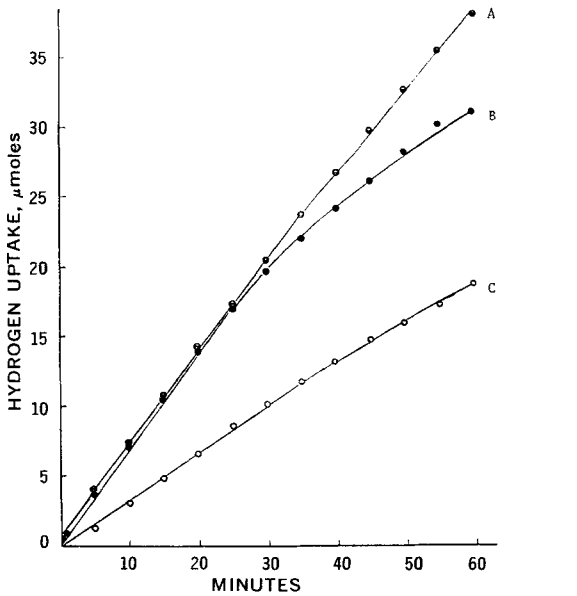

Figure 7—*Hydrogenations of α-methylcinnamic acid by palladium-on-poly-S-valine in the presence of* N-*acetylphenylalanine and dihydro-α-methylcinnamic acid. Key: A, ⊖, α-methylcinnamic acid with* N-*acetylphenylalanine; B, ●, α-methylcinnamic acid with no inhibitor; and C, ○, α-methylcinnamic acid with dihydro-α-methylcinnamic acid.*

leucine *versus* those catalyzed by palladium-on-poly-*S*-valine. The catalyst possessing the α-helical conformation (palladium-on-poly-*S*-leucine) gave a faster rate of hydrogenation than the nonhelical catalyst (palladium-on-poly-*S*-valine).

The hydrogenation rates for α-methylcinnamic acid were faster than those for α-acetamidocinnamic acid with both of the catalysts.

Inhibition studies show that the two substrates, α-methylcinnamic acid and α-acetamidocinnamic acid, occupy entirely different sites on the catalyst surface in both the palladium-on-poly-*S*-leucine- and the palladium-on-poly-*S*-valine-catalyzed reactions.

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DRUG STANDARDS

Use of Ceric Sulfate and Cupric Perchlorate for Titrimetric Analyses of Phenothiazine Derivatives

L. G. CHATTEN, R. A. LOCOCK, and R. D. KRAUSE

Abstract □ A method was developed in which phenothiazine derivatives were titrated visually to a colorless end-point with ceric sulfate. Quantitative recoveries were obtained only for chlorpromazine, acetylpromazine, trifluoperazine, and triflupromazine. The method was also applied to pharmaceutical dosage forms of these drugs. UV photometric detection of the end-point was found to be applicable only to thiethylperazine and thioridazine. Attempts to develop a quantitative procedure for phenothiazines by photometric titration with cupric perchlorate in acetonitrile were unsuccessful.

Keyphrases □ Phenothiazine derivatives and dosage forms—analysis □ Ceric sulfate—phenothiazine titration □ Titration, visual—phenothiazine derivatives, analysis □ UV photometric titration—phenothiazine derivatives, analysis

In 1940, Michaelis *et al.* (1) showed, by potentiometric titration, that the oxidation of phenothiazine (I) and several of its derivatives proceeds through two successive and distinct one-electron steps. Scheme I illustrates the sequence in the oxidation of an *N*-substituted phenothiazine. A highly colored free radical or semiquinone (II) results from the first oxidation step. The loss of another electron from this intermediate gives a phenazathionium ion (III). The phenazathionium ion from the second step can then react with water to produce a sulfonium base (IV), which will lose a proton to yield the phenothiazine sulfoxide (V).

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The purposes of the present investigation were: (*a*) to develop a simple assay for phenothiazine drugs

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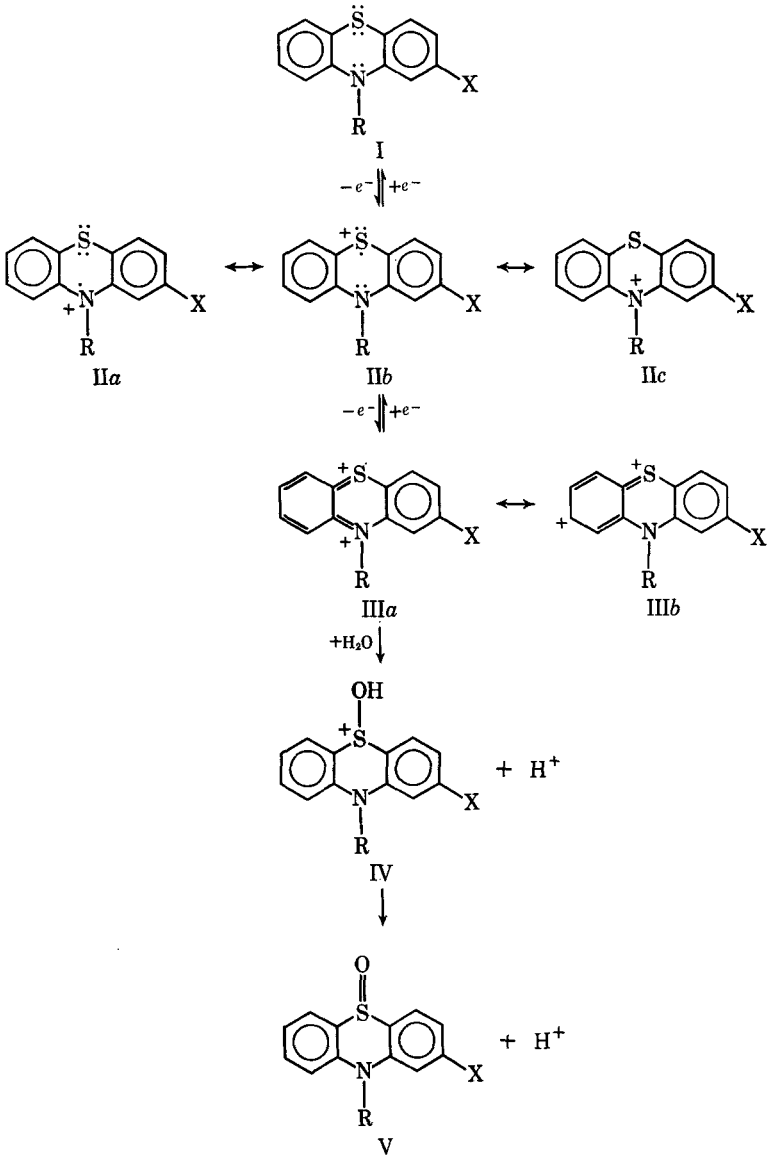

Scheme I—Oxidation pathway of an N-substituted phenothiazine derivative

using aqueous ceric sulfate or cupric perchlorate in acetonitrile as the titrant, and (*b*) to apply the procedure to various dosage forms on the Canadian market.

EXPERIMENTAL

Apparatus—The following were used: low actinic volumetric flasks, 5-ml. microburet equipped with a platinum tip, a Beckman model DB spectrophotometer equipped with a Beckman 96160 flow cell, and a titration vessel originally described by Rehm *et al.* (19).

Reagents—The following were used: glacial acetic acid; 0.1 *M* perchloric acid in glacial acetic acid (standardized against primary standard potassium acid phthalate); 6% mercuric acetate in glacial acetic acid; 0.01 *M* disodium ethylenediaminetetraacetic acid; copper metal[1]; 0.1% α-pyridyl-β-azonaphthol in 95% ethanol; acetonitrile; 0.001 and 0.005 *M* cupric perchlorate in acetonitrile; sulfuric acid; ceric ammonium nitrate; arsenic trioxide (primary standard); 0.025 *M ortho*-phenanthroline–ferrous sulfate complex; 1 in 400 solution of osmium tetroxide in 0.05 *M* sulfuric acid; chloroform; and methanol.

Phenothiazine Derivatives and Dosage Forms Investigated—The following phenothiazine drugs were used in this investigation: acetylpromazine maleate, aminopromazine fumarate, chlorpromazine and chlorpromazine hydrochloride, fluphenazine dihydrochloride, levomepromazine hydrochloride, mepazine acetate hydrate, methdilazine, perphenazine, pipamazine, prochlorperazine and prochlorperazine dimaleate, promazine and promazine hydrochloride, promethazine, pyrathiazine, thiethylperazine dimaleate, thiopropazate and thiopropazate dihydrochloride, thioridazine and thioridazine hydrochloride, trifluoperazine and trifluoperazine dihydrochloride, triflupromazine hydrochloride, and trimeprazine tartrate.

The purity of these compounds was established by titration of 0.1 meq. of the phenothiazine derivative dissolved in glacial acetic acid with 0.1 *M* acetous perchloric acid, using crystal violet as the indicator. Mercuric acetate in glacial acetic acid was added for halide salts. All compounds had a purity of 98% or greater, with the exception of pipamazine (97.85%) and pyrathiazine (93.79%).

Dosage forms were analyzed for the following: chlorpromazine[2], fluphenazine[3], triflupromazine[4], and trifluoperazine[5].

[1] British Drug House, Analar.

[2] Chlorpromanyl "10" tablets and Chlorpromanyl injection (Paul Maney Laboratories); Largactil tablets, Largactil Spansule capsules, Largactil injection, Largactil suppositories, and Largactil liquid (Poulenc Ltd.)

[3] Moditen tablets, E. R. Squibb and Sons Ltd.

[4] Vesprin injection, E. R. Squibb and Sons Ltd.

[5] Stelazine tablets and Stelazine injection, Smith Kline & French.

Table I—Variation of Recovery with 2-Position Substituent in Titration of Phenothiazine Derivatives wih Ceric Sulfate

Drug	Average Percent Recovery[a]	2-Position Substituent
Acetylpromazine maleate	101.7	—$COCH_3$
Trifluprom azine hydrochloride	101.9	—CF_3
Trifluoperazine dihydrochloride	103.9 (4 *M*)	—CF_3
Fluphenazine dihydrochloride	106.0 (2 *M*)	—CF_3
Pipamazine	104.4	—Cl
Chlorpromazine	104.1 (2 *M*)	—Cl
Mepazine acetate hydrate	109.6	—H
Levomepromazine hydrochloride	113.3	—OCH_3
Thioridazine	120.4	—SCH_3
Thiethylperazine dimaleate	163.0	—SCH_2CH_3

[a] Solvent used was 0.2 *M* H_2SO_4 unless otherwise indicated.

Preparation and Standardization of 0.05 *M* Ceric Sulfate—The procedure followed was as outlined in the USP (20) for 0.1 *M* ceric sulfate. Appropriate adjustments were made in quantities of ceric ammonium nitrate and sulfuric acid.

Visual Titration of Phenothiazine Derivatives with Ceric Sulfate—About 10 mg. of the phenothiazine was accurately weighed into a 10-ml. beaker and dissolved in 5 ml. of dilute sulfuric acid. The titrant was 0.05 *M* ceric sulfate. The solution was titrated with the aid of a magnetic stirrer and a titration lamp. The end-point was taken at complete decolorization of the solution.

Visual Titration of Pharmaceutical Dosage Forms with Ceric Sulfate—*Tablets*—Twenty tablets were weighed and powdered. A sample of powder approximately equivalent to 10 mg. of the active ingredient was accurately weighed into a 10-ml. beaker, dissolved in 5 ml. of 1 *M* sulfuric acid, and titrated as already described.

Injections and Solutions—An aliquot of the liquid preparation containing about 10 mg. of the active ingredient was accurately pipeted into a 10-ml. beaker. After dilution to 5 ml. with 1 *M* sulfuric acid, titration proceeded as for tablets.

Suppositories—Ten suppositories were weighed and reduced to fine particles. An amount of material approximately equivalent to 10 mg. of the active ingredient was accurately weighed into a 10-ml. beaker and dissolved in 3 ml. of chloroform. Three milliliters of methanol was added, and the titration was completed as for the tablets.

Photometric Titration of Phenothiazine Derivatives with Ceric Sulfate—An aliquot containing about 0.25 mg. of the drug in 1 *M* sulfuric acid was transferred to the photometric titration vessel and diluted to 25 ml. with 1 *M* sulfuric acid. The titrant, 0.005 *M* ceric sulfate, was added in 0.05-ml. increments from a 5-ml. microburet; the absorbance at 270 nm. (275 nm. for thiethylperazine or thioridazine) was read 30 sec. after the addition of each increment. The absorbance was plotted against the volume of titrant added, and the end-point was taken at the change of slope of the graph.

Standardization of Cupric Perchlorate Titrant in Acetonitrile—A 0.01 *M* solution of the disodium salt of ethylenediaminetetraacetic acid in water was standardized against pure copper metal. The indicator employed was a 0.1% α-pyridyl-β-azonaphthol solution in ethanol, which changed at the end-point from violet to yellow.

The standardized ethylenediaminetetraacetic acid solution was then used to standardize 0.005 and 0.001 *M* solutions of cupric perchlorate in acetonitrile. A 5-ml. aliquot of the acetonitrile solution was treated in the same manner.

Photometric Titration of Phenothiazine Derivatives with Cupric Perchlorate—An aliquot containing about 4 mg. of the drug in acetonitrile was transferred to the photometric titration vessel and diluted to 25 ml. with acetonitrile. One milliliter of 0.005 *M* cupric perchlorate in acetonitrile was added to the solution, and the visible spectrum was scanned. The wavelength of maximum absorbance was used for the photometric titration. The titration vessel and flow-through cell were then drained and rinsed with acetonitrile; another aliquot of drug was added and diluted. The titrant was added from a 5-ml. microburet in 0.4-ml. increments, and the absorbance was read 30 sec. after the addition of each increment. The absorbance was plotted against the volume of titrant added, and the end-point was taken at the change of slope of the graph.

RESULTS AND DISCUSSION

Titrations with Ceric Sulfate—The use of ceric ion in aqueous solutions as a titrant for the analysis of phenothiazines has been extensively investigated (4–13). Since ceric ion is a very strong oxidizing agent (E° = 1.44 v. in 1 *M* H_2SO_4), phenothiazines are oxidized to the corresponding sulfoxides; thus, the equivalence point in the titrations corresponds to the addition of 2 moles of ceric ion per mole of phenothiazine.

The titrations were carried out in aqueous solutions of sulfuric acid where the free radical intermediate is stabilized. During the titrations, the color of the solution, due to the presence of the free radical species, increased to a maximum after 1 equivalent of titrant was added; then the color decreased in intensity and disappeared entirely after 2 equivalents were added. It appears, therefore, that a simple procedure could be devised in which this self-indicating property of phenothiazine derivatives might be utilized for their analysis. Accordingly, a series of titrations were carried out in which a pure phenothiazine derivative in dilute sulfuric acid was titrated to a colorless end-point with a standard solution of ceric sulfate. Each derivative was titrated in several different concentrations of sulfuric acid, but a consistent correlation could not be found between the results and the concentration of acid in the solvent.

The recoveries obtained were invariably greater than 100%. A correlation was seen between the extent of the error and the electron-withdrawing power of the substituent in the 2-position of the phenothiazine nucleus. The best results were obtained when this substituent was strongly electron withdrawing. Typical results are listed in Table I.

From these results, it appears that ceric sulfate is such a strong oxidizing agent that the phenothiazine derivatives are oxidized past the sulfoxide stage during the titration. When the end-point, chosen by the disappearance of the color due to the free radical species, is reached, some titrant already has been consumed in converting the sulfoxide to a higher oxidation state, probably the corresponding 3-hydroxyphenothiazine or 3-phenothiazone derivative. This subsequent reaction would be expected to proceed to a greater extent in compounds that are more easily oxidized. Kabasakalian and McGlotten (21), in a polarographic study of phenothiazines, found that oxidation was more difficult when electron-withdrawing groups were substituted in the 2-position of the phenothiazine nucleus.

Analysis of Pharmaceutical Dosage Forms—The visual titration method with ceric sulfate was also applied to certain pharmaceutical

Table II—Results of Visual Titration of Phenothiazine Dosage Forms with Ceric Sulfate

Dosage Form	Percent Recovered
Chlorpromazine hydrochloride, 11.2 mg./tablet	101.8
Chlorpromazine hydrochloride, 55.8 mg./tablet	99.1
Chlorpromazine hydrochloride, 30.0 mg./tablet	109.0
Chlorpromazine, 25.0 mg./suppository	109.0
Chlorpromazine hydrochloride injection, 27.9 mg./ml.	99.1
Chlorpromazine hydrochloride injection, 5.58 mg./ml.	96.7
Chlorpromazine hydrochloride liquid, 27.9 mg./5 ml.	127.6
Fluphenazine hydrochloride, 1 mg./tablet	108.1
Trifluoperazine dihydrochloride, 10 mg./tablet	101.6
Trifluoperazine dihydrochloride injection, 1 mg./ml.	110.4
Trifluoperazine hydrochloride injection, 20 mg./ml.	100.5

Table III—Results of Photometric Titration of Phenothiazine Derivatives with Cupric Perchlorate

Drug	R[a] at End-Point: Without Perchloric Acid, First End-Point	R[a] at End-Point: Without Perchloric Acid, Second End-Point	R[a] at End-Point: With Perchloric Acid
Chlorpromazine	1.23	2.82	1.49
Levomepromazine	0.85	2.54	1.49
Prochlorperazine	0.72	2.76	1.57
Promethazine	0.86	2.04	1.34
Thioridazine	0.92	2.64	1.58
Trifluoperazine	1.14	2.73	1.46
Methdilazine	1.16	2.65	1.47

[a] R = (moles cupric perchlorate/mole drug).

dosage forms of those phenothiazine derivatives for which the best results were obtained. Results of these titrations are shown in Table II.

Compressed tablets were analyzed simply by stirring a quantity of the powdered tablet material with dilute sulfuric acid for several minutes and then titrating to the disappearance of color with ceric sulfate. Although insoluble tablet excipients remained suspended throughout the titration, there was no difficulty in determining the decolorization end-point. Good results were obtained for chlorpromazine and trifluoperazine tablets. The recovery obtained for fluphenazine tablets was high, but only slightly higher than the recovery obtained when the pure drug was analyzed by this method.

The chlorpromazine hydrochloride injections contained a preservative which was more easily oxidized by ceric sulfate than was chlorpromazine. Thus, initial addition of titrant did not result in the production of a red coloration, but a color did appear after all the preservative was oxidized. Readings were taken at the first appearance of color and at complete decolorization of the solution. The difference was the amount of titrant consumed by the drug. Recoveries obtained for several dosage forms were higher than expected, probably because of the presence of other oxidizable substances in the product.

UV Photometric Titrations with Ceric Sulfate—UV absorption spectra of dilute sulfuric acid solutions of phenothiazine derivatives substituted with —H, —Cl, —OCH_3, or —CF_3 in the 2-position show a strong peak at 250–255 nm. and a weaker, broad peak centered around 300 nm. Derivatives substituted with —SCH_3 or —SCH_2CH_3 in the 2-position also showed a weak, broad peak at 300 nm., but the position of the stronger peak was shifted to 260 nm. Addition of 2 equivalents of ceric sulfate to a solution of chlorpromazine converted the chlorpromazine spectrum to that of chlorpromazine sulfoxide, which exhibited absorption maxima at 240, 273, and 299 nm. Similarly, the addition of 2 equivalents of ceric sulfate to solutions of all phenothiazine derivatives with —H, —Cl, —OCH_3, or —CF_3 substituents in the 2-position resulted in spectra with strong absorption in the 240-nm. region and absorption peaks between 269 and 273 nm. The position of this latter peak was shifted to 275 nm. when the 2-substituent was a thioalkyl group.

Since the sulfoxide and the free radical species derived from thioalkyl substituted phenothiazines absorb at different wavelengths in the UV regions, the absorption due to the sulfoxide species produced during the titration can be measured without interference from the free radical intermediate. Photometric titrations of these compounds at a wavelength of 275 nm. resulted in a curve in which the absorbance increased linearly with the addition of ceric sulfate until 2 equivalents of titrant were added and then remained constant with the addition of further increments of titrant. Recoveries of thiethylperazine and thioridazine obtained by this method were 98.3 and 98.1%.

Photometric titrations with ceric sulfate were also attempted for phenothiazine derivatives with substituents other than thioalkyl. With these compounds, the absorption of the free radical interfered with that of the sulfoxide at 270 nm. Thus, a peak was produced in the photometric titration curve after the addition of 1 equivalent of titrant, corresponding to the maximum intensity of the free radical absorption. The absorption at 270 nm. decreased with the addition of the 2nd equivalent of titrant as the free radical was being converted to the sulfoxide. After 2 equivalents of ceric sulfate were added, the absorbance, now due only to the sulfoxide, remained constant with the addition of further increments of titrant. Recoveries obtained for the latter class of compounds ranged from 84 to 125%.

Titrations with Cupric Perchlorate—Although the use of nonaqueous solvents as media for acid–base reactions in analytical chemistry is now well established, little work has been done to investigate the use of these solvents in oxidation–reduction reactions. A large proportion of the work reported in this area involved the utility of glacial acetic acid as the solvent. Some interest has been focused on acetonitrile as a solvent because it is highly resistant to oxidation or reduction. Its moderately high dielectric constant promotes ionization of solutes and, therefore, more rapid electron transfer between species. Kratochvil *et al.* (22) found that cupric perchlorate is a powerful oxidizing agent in acetonitrile. Consequently, the titration of the free bases of several phenothiazine derivatives in acetonitrile with a standard solution of cupric perchlorate was attempted. The end-point was determined photometrically by following the absorbance at the wavelength of maximum absorption of the colored free radical intermediate produced upon partial oxidation of a phenothiazine derivative, which occurs at 510 nm. in phenothiazines not substituted in the 2-position, at 500 nm. if the 2-substituent is a —CF_3 group, at 525 nm. if —Cl, at 575 nm. if —OCH_3, and at 645 nm. in thioridazine where the substituent is —SCH_3.

Photometric titrations were attempted both with and without the prior addition of perchloric acid. If acid was not added to the solution before titration, initial addition of cupric perchlorate did not result in the production of any color. The free radical is stabilized by the presence of hydrogen ions; apparently when none is added to the solution, the free radical intermediate does not exist. Under these conditions, the oxidation does not stop at the free radical stage but continues to the formation of the phenazathionium ion. However, after the addition of 0.7–1.2 moles of cupric perchlorate per mole of phenothiazine, a stable color was produced in the solution, resulting in a linear increase in absorbance with the addition of more titrant, and an apparent photometric end-point. Stabilization of the free radical form is thought to result from accumulation of hydrogen ions produced in the reaction of phenazathionium ion and traces of water present in the solvent.

Addition of perchloric acid to the solution before titration changes the shape of the titration curve. In this instance, the first addition of titrant results in the formation of a color since the free radical is being stabilized by the acid added. As more titrant is added, a linear increase in absorbance is seen. The end-point is selected as that place where the slope of the graph changes from highly positive to zero or slightly negative. The ratio of moles of cupric perchlorate added at the end-point to moles of phenothiazine present in solution is about 1.5:1 for most of the phenothiazines titrated (Table III).

That this ratio is not integral is probably a result of more than one reaction proceeding at the same time. Initially, addition of titrant produces the free radical species which is stabilized by the presence of acid in the solution. If this was the only reaction, the photometric curve would increase to a maximum after 1 equivalent of cupric perchlorate was added and then decrease at the same rate as more titrant was added, since the additional titrant would oxidize the free radical to the colorless phenazathionium ion. However, it appears that these reactions occur simultaneously; by the time the absorbance due to the free radical species reaches a maximum, some of the free radical has reacted further.

If this hypothesis is correct, an increase in acid concentration would tend to decrease the rate of the second reaction relative to that of the first reaction because of the increased stability of the free radical formed by the first reaction. Thus, the ratio of moles of cupric perchlorate to moles of drug found at the end-point should decrease to a limit of unity with increasing acid concentration. The effect of the concentration of perchloric acid on the molar ratio obtained in the titration of methdilazine was investigated and the results do support the theory proposed. From a value of 1.47 at a perchloric acid concentration of 0.005 *M*, the molar ratio decreased to a value of 1.42 at an acid concentration of 0.1 *M*, 1.27 at an acid concentration of 1.0 *M*, and 1.18 at an acid concentration of 2.0 *M*. As the acid concentration was increased, the photometric titration curve became increasingly rounded in the end-point region. If the acid concentration in the solvent was raised to more than 2 *M*, this curvature made it impossible to determine an end-point in the titration.

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Determination of Zinc in Pharmaceutical Products by Neutron Activation Analysis

MICHEL MARGOSIS and JOSEPH P. F. LAMBERT*

Abstract □ Nondestructive neutron activation analysis was applied as a rapid, efficient, and specific method for the determination of zinc at either low or major concentration levels in various pharmaceutical products in bulk or dosage forms. This technique yielded results that compare well with more conventional methods and offers decided advantages over them.

Keyphrases □ Zinc in pharmaceuticals—determination □ Impurity interference—zinc determination □ Neutron activation analysis—zinc

Official methods for the analysis of zinc involve ashing followed by gravimetry (1) or complexometric (2, 3) or alkalimetric titration (1). Zinc in insulin is determined by a dithizone colorimetric procedure subsequent to extraction (2); in zinc bacitracin, it is determined by complexometric titration with edetic acid (3). However, there is no official procedure for determining zinc in various zinc bacitracin dosage forms such as ointments. The advent of atomic absorption spectroscopy has increased the efficiency and specificity of the determination of zinc, but in many laboratories this technique has been limited to simple dilute aqueous systems.

Table I—Nuclear Properties of Zinc for Activation with Thermal Neutrons

Stable Nuclide	Abundance, %	Cross Section, Barns	Radionuclide Formed	Half-life ($t_{1/2}$)	Energy of Principal γ-Ray, Mev.
^{64}Zn	48.89	0.46	^{65}Zn	245 days	1.115
^{66}Zn	27.81				
^{67}Zn	4.11				
^{68}Zn	18.56	1.0	^{69}Zn	52 min.	None
^{68}Zn	18.56	0.1	^{69m}Zn	13.8 hr.	0.439
^{70}Zn	0.62	0.10	^{71}Zn	2.3 min.	0.12
^{70}Zn	0.62	0.01	^{71m}Zn	4.1 hr.	

The application of neutron activation analysis (NAA) to zinc in pharmaceutical products offers increased utility, specificity, and accuracy (4). Advantages of NAA accrue from the facts that the technique: (*a*) is normally unaffected by the complex organic matrixes composing drug systems; (*b*) requires no physicochemical separation procedure; and (*c*) is both qualitative and quantitative in a presumptive assay method at either microconcentration or macroconcentration levels. This study shows the application of NAA for the deter-

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Table II—Determination of Zinc in Various Pharmaceutical Compounds

Sample	Percent Zn Found: ^{69m}Zn[a]	^{65}Zn	AA[b]	EDTA[c]	Theoretical (or Limits), %	Reference Number
Zinc bacitracin (Batch 1)	3.10	5.87	5.46	5.81	<10	(3)
Zinc bacitracin (Batch 2)	4.44	5.00	5.12	5.54	<10	(3)
Zinc bacitracin (Batch 3)	3.38	6.39	5.89	6.13	<10	(3)
Zn oxide	80.3	83.7	76.3	—	80.34	(2)
Zn stearate	12.1	12.6	11.1	—	12.5–14.0	(2)
Zn undecylenate	14.5	15.2	15.0	—	14.83–15.44	(1)
Zn phenolsulfonate	14.8	12.0	11.7	—	11.70–12.29	(1)
Zn metal (wire)	100.2	—	—	—	100.0	

[a] Unreliable results attributed to high analyzer dead-time. [b] Atomic absorption method. [c] Edetic acid titration method.

Table III—Determination of Zinc in Various Pharmaceutical Dosage Forms

Sample	^{69m}Zn Found, mg./g.	^{65}Zn Found, mg./g.	Zn Expected[a], mg./g.
Zn bacitracin ointment 1[b]	—	0.453	0.4–0.5
Zn bacitracin ointment 2	—	0.427	0.4–0.5
Zn bacitracin ointment 3	—	0.408	0.4–0.5
Zn bacitracin ointment 4	—	0.514	0.4–0.5
Zn bacitracin ointment 5	—	0.461	0.4–0.5
Zn bacitracin ointment 6	—	0.439	0.4–0.5
Neomycin tablet (Zn stearate)	0.732/tablet	0.767/tablet	0.78/tablet
Zn undecylenate ointment 1[c]	30.7	31.0	30.1
Zn undecylenate ointment 2[c]	30.7	29.9	30.1

[a] The expected zinc in the zinc bacitracin ointment is estimated on the basis of 500 units/g. ointment, about 50 units of activity/mg., and about 5–6% zinc in the zinc bacitracin used in making the ointment. [b] The six bacitracin ointments are from different lots, types, and/or manufacturers. [c] Duplicate analysis from a different weighed sample from the same tube.

mination of zinc in such diverse products as antibiotics and insulin and in such salts as the stearate, undecylenate, and phenolsulfonate, which are used as a lubricant, antifungal agent, and astringent, respectively.

EXPERIMENTAL

Irradiation Containers—Snap-cap polyethylene vials of approximately 1- or 5-ml. capacity[1] were used for all samples.

Standards—A stock solution of zinc standard was prepared by dissolving 100.0 mg. of reagent grade metal in about 10 ml. of dilute nitric acid and diluting to 100 ml. with water. Aliquots of 1.00 or 2.00 ml. were transferred to the polyvials for comparison with samples containing milligram quantities of zinc. A secondary dilution of the stock standard solution containing 50.00 mcg./ml. was prepared for comparison with samples of lower zinc content.

Samples—Several milligrams of zinc salts and about 1 g. of ointment were accurately weighed into the polyvials. Tablets were individually placed in polyvials; 1.00-ml. aliquots of insulin samples were transferred to the 5-ml. polyvials and evaporated overnight at 60° on a block hot-plate with an airflow through the hood.

Apparatus—Irradiations were performed in the high flux exposure tube of a 1-Mw. nuclear research reactor (swimming pool type)[2], which provides a thermal neutron flux of approximately 10^{13} n./cm.^{2}-sec. Gamma-ray spectra were obtained with a 1024-channel pulse height analyzer[3] (PHA), using a 7.62 × 7.62-cm. (3 × 3-in.) NaI(Tl) scintillation detector[4] housed in a 6-ton steel shield, and a 7.62 × 7.62-cm. (3 × 3-in.) NaI well-type detector. The detector, which was covered with a thick plastic β-absorber, had a resolution of 8% at 662 kev. The PHA had a gain setting of 10 kev. per channel, and four independent counts could be stored prior to readout. The readout equipment consisted of a teletype printer and an X-Y recorder[5].

Procedure—Three 5-ml. or four 1-ml. polyvials were packaged together with a standard into an irradiation bucket and lowered into the exposure tube. Samples containing milligram amounts of zinc were irradiated for 3 min., whereas the other samples (antibiotic ointments and insulin) were irradiated for 20 min. After a group irradiation, the samples were allowed to cool several hours or overnight and were placed in larger polyethylene containers. ^{69m}Zn-activity was then measured by counting from 4 to 20 min. at a maximal distance of 5 cm. from the detector, depending upon the total activity of the samples, so as to obtain a PHA dead-time lower than 30%. After several more days of cooling, ^{65}Zn-activity of samples and standards was measured again by counting in the well-type detector. In all measurements, the Covell method (5) for photopeak analysis was used to quantitate the zinc activity.

[1] Olympic Plastics, Los Angeles, Calif.
[2] At the Naval Research Laboratory, Washington, D. C.
[3] Northern Scientific model 610.
[4] Harshaw 12SW12-W 3/H.
[5] Moseley model 7590.

RESULTS AND DISCUSSION

Elemental zinc is a mixture of five stable nuclides which react with thermal neutrons to produce radionuclides (Table I), two of which are useful in this application (6). The ^{69m}Zn-nuclide, with a 14-hr. half-life and a major photopeak at 0.44 Mev., is produced in substantial quantity, as shown in Fig. 1, allowing a shorter analysis

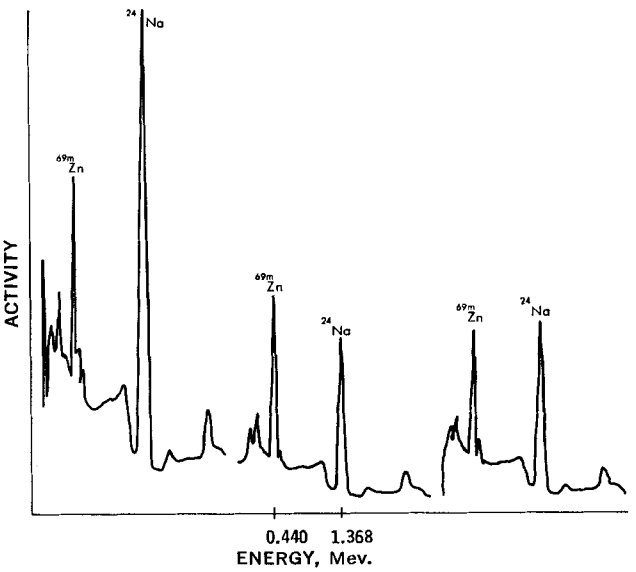

Figure 1—*Spectra of ^{69m}Zn from three different zinc bacitracin ointments.*

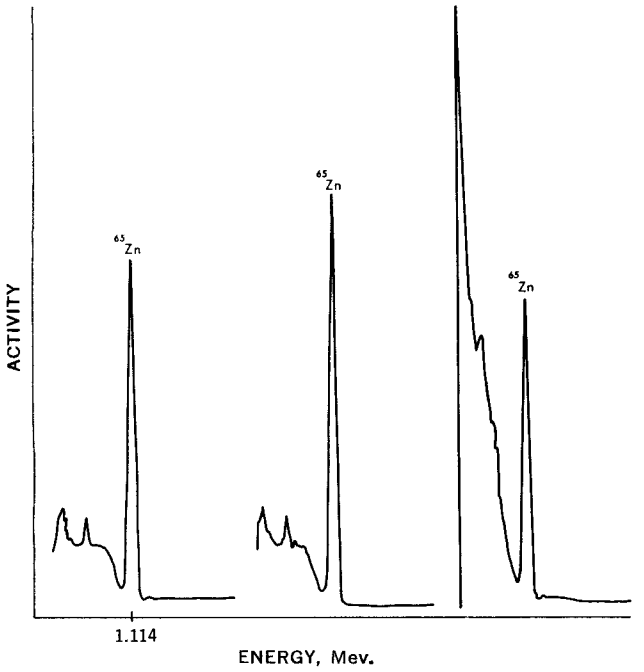

Figure 2—*Spectra of 245-day half-life ^{65}Zn from zinc bacitracin bulk, standard zinc sulfate solution, and a zinc bacitracin ointment.*

time while retaining the nondestructive feature of the technique. However, within the time of irradiation, impurities are also activated, with the combined effect of increasing both analyzer dead-time and background interference. Some of this interference is reduced by allowing a cooling/wait period of several hours before counting. This permits shorter-lived foreign nuclides to dissipate and correspondingly reduces the analyzer dead-time to a more practical level. Yet, it appears that in samples of lower concentration of zinc (*i.e.*, less than 0.5 mg./g. in ointments), the background is still large enough to lower the results generally by 10–25%, rendering them less reliable as evidenced by a lack of reproducibility and also by comparison to the ^{65}Zn-analysis. Although the Covell (5) method for spectral analysis is used, accuracy and precision still depend upon the purity of the photopeaks and the number of channels selected for integration, particularly with a sodium iodide detector.

To achieve maximal accuracy and precision, the ^{69m}Zn-nuclide is allowed to decay for several days to an insignificant activity level so that the 245-day half-life ^{65}Zn-nuclide can be measured at 1.12 Mev. (Fig. 2). By that time, the PHA dead-time is low enough for use of the well-type detector, which possesses superior geometry, and the background interference has been so reduced as to optimize conditions for measurement by the Covell method.

Results of analysis of several zinc-containing drugs by NAA are listed in Table II and are compared to results obtained by atomic absorption spectroscopy and by complexometric titration with edetic acid. The poor results obtained with ^{69m}Zn in the case of these compounds, particularly the zinc bacitracins, are mostly attributed to high-analyzer dead-time as well as some Compton contribution from ^{24}Na.

Various antibiotic ointments from different commercial sources, together with a neomycin tablet and one zinc undecylenate ointment, were analyzed for zinc (Table III). As previously mentioned, more reliable results are obtained by exploiting the longer-lived ^{65}Zn-radionuclide. Table IV lists results obtained from the analysis of various insulin samples and compares them to those obtained by

Table IV—Determination of Zinc in Insulin by NAA (Found, mcg./ml.)

Sample	Type[a]	NAA (^{65}Zn)	Atomic Absorption	Dithizone Colorimetric Method Lab I	Lab II
1	IZS-40	86.3	91	87	86
2	GZI-40	130	123	123	134
3	GZI-40	132	130	137	133
4	PZI-80	184	—	179	174
5	IZS-80	175	191	186	190
6	ISO-40	10.9	—	10	9
7	ISO-80	17.9	20	18	17
8	IZS-40	88.7	93	84	88

[a] IZS = insulin zinc suspension, GZI = globin zinc insulin, PZI = protamine zinc insulin suspension, and ISO = isophane insulin suspension.

atomic absorption spectroscopy and the dithizone colorimetric method. In this analysis, it was found necessary to evaporate the initial aliquot to dryness prior to irradiation, because the heat generated by the reactor caused loss of sample. There is good correlation among the results of all three methods.

Since the counting time is relatively short compared to the half-life of the nuclides, a decay factor correction is not necessary and has been omitted. Elemental zinc is then quantitated by direct comparison of activity to concentration; other variables are compensated for by irradiating samples and standards at the same time.

Additive interferences from accompanying nuclear reactions (7) with epithermal and fast neutrons producing ^{65}Zn or ^{69m}Zn from copper, nickel, gallium, or germanium are negligible, chiefly because the quantity of these elements, if present at all in these products, is very limited; the probability of such reactions is extremely small because of the relative abundance and cross section of these reactions.

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GLC Determination of Methamphetamine Hydrochloride in Methyl Acrylate–Methyl Methacrylate Sustained-Release Tablets

LAWRENCE T. SENNELLO

Abstract □ A rapid and precise GLC method was developed for the detection and determination of methamphetamine hydrochloride in methyl acrylate–methyl methacrylate copolymer sustained-release tablets. After dissolution of whole tablets in chloroform, *in situ* conversion of the methamphetamine hydrochloride to its free base with potassium hydroxide, and addition of *n*-tridecane internal standard, an aliquot of the resulting solution can be injected into a gas chromatograph for analysis. An OV-101 chromatographic column was used.

Keyphrases □ Methamphetamine HCl sustained-release dosage form—analysis □ Methyl acrylate–methyl methacrylate matrix—methamphetamine HCl dosage form □ GLC—analysis □ *n*-Tridecane—GLC analysis standard

A large number of pharmaceutical preparations has become available in recent years in the form of sustained-release tablets and capsules. Such products have the obvious advantage of maintaining drug blood concentrations at desired levels for prolonged periods. One method of preparing sustained-release tablets entails the use of a methyl acrylate–methyl methacrylate copolymer matrix (1, 2). In this system, the drug is intimately mixed with the plastic, compressed to a tablet, and treated to give the desired prolonged drug-release rate (3, 4). The resulting tablets are extremely hard and insoluble in most solvents.

In the development of assay procedures for the active components of such tablets, the extreme hardness frequently precludes manual grinding. Early experiments in this laboratory included attempts to leach the active components from tablets with water. The prolonged leaching times required for the tablet make it time consuming for recovery of drug for total assay. Maximum leaching efficiencies are about 98%.

Methods have been reported for the GLC resolution of amphetamine and methamphetamine after conversion of the former to 1-benzylethylisothiocyanate (5), for determination of methamphetamine in blood and/or urine (6–9), and for determination of that same drug in pharmaceutical preparations after extraction of the amine base into diethyl ether (10, 11).

A very convenient procedure was developed in the author's laboratory for the GLC determination of methamphetamine hydrochloride in methyl acrylate–methyl methacrylate tablets. The proposed method does not require extraction of the drug, but it involves dissolving entire tablets in chloroform, *in situ* conversion of the drug to its free base with methanolic potassium hydroxide, and injection of an aliquot of the reaction mixture into a gas chromatograph.

EXPERIMENTAL

Operational Parameters—The instrument used for this work was a Varian Aerograph model 1800, equipped with hydrogen-flame detectors. The column was a stainless steel coil, 1.52 m. (5 ft.) long and 0.15 cm. (0.062 in.) i.d., packed with 5% OV-101 on 80/100-mesh Gas Chrom Q. The temperatures were: column, 100°; injection port, 150° with a Pyrex insert; and detector, 150°. The flow rates were: carrier gas, nitrogen, 12–15 ml./min.; detector gas, hydrogen, 30 ml./min.; and air, 300 ml./min. All injections were made with a microsyringe, using an injection volume of approximately 1.5 μl. The instrument was operated at a range of 10^{-11} amp./mv. and 64× attenuation. The recorder used was a 0–1-mv. Varian Aerograph model 20. Peak areas were measured with a Varian Aerograph model 471 digital integrator. A sample chromatogram is shown in Fig. 1.

Standard Preparation—Accurately weigh about 70 mg. of *n*-tridecane[1] and 60 mg. of methamphetamine hydrochloride into a 50-ml. volumetric flask. Add about 20 ml. of chloroform, and swirl to dissolve. Add 5 ml. of 0.1 *N* methanolic potassium hydroxide, dilute to volume with chloroform, and mix.

Sample Preparation—Accurately weigh about 70 mg. of *n*-tridecane into a 50-ml. volumetric flask. Add whole methamphetamine hydrochloride tablets equivalent to about 60 mg. of drug. Add about 20 ml. of chloroform, and agitate on a wrist-action shaker until all the tablets are dissolved. Add 5 ml. of 0.1 *N* methanolic potassium hydroxide, dilute to volume with chloroform, and mix.

Sample Analysis—Aliquots of standard and sample (1.5 μl.) are alternately injected into the instrument under the aforementioned conditions. After elution of the *n*-tridecane peak, the instrument is ready for another injection. A total elution time of about 9–10 min. can be expected.

Calculations—*Relative Response Factor for Methamphetamine Hydrochloride*—

$$K = \frac{(\text{mg. of } n\text{-tridecane in standard})(\text{area of methamphetamine peak for standard})}{(\text{mg. of methamphetamine·HCl in standard})(\text{area of } n\text{-tridecane peak for standard})} \quad \text{(Eq. 1)}$$

$$\text{mg. methamphetamine hydrochloride per tablet} = \frac{(\text{mg. of } n\text{-tridecane in sample})(\text{area of methamphetamine peak for sample})}{(K)(\text{area of } n\text{-tridecane peak for sample})(\text{number of tablets used for sample})} \quad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Chloroform was the only solvent tried in which the methyl acrylate–methyl methacrylate copolymer was sufficiently soluble to permit dissolution of the tablets. The appreciable solubility of both methamphetamine and methamphetamine hydrochloride in chloroform, combined with the relatively low response it induces in a hydrogen-flame detector, made this the solvent of choice.

In the author's laboratory, it was found that a complete analysis could be completed within about 1 hr. of receipt of a sample. This combination of speed and simplicity makes the method most attractive.

Although a small amount of the dissolved plastic matrix was injected into the chromatograph with the sample, no interference was noted in any of the chromatograms due to pyrolysis of the polymer. The simple composition of this type of tablet greatly

[1] K & K Laboratories, Inc., Plainview, N. Y.

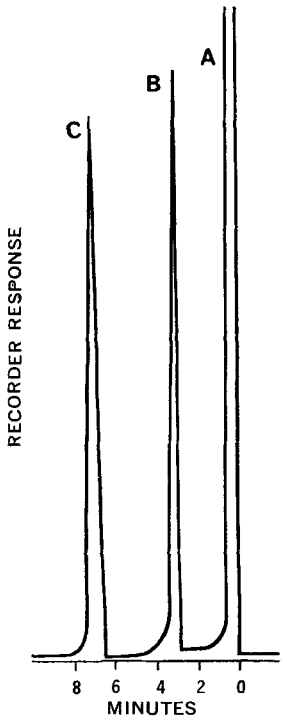

Figure 1—*Gas chromatogram showing relative retention times of methamphetamine and* n-*tridecane. Key: A, solvent; B, methamphetamine; and C,* n-*tridecane.*

simplifies analysis. It was found that unless the methamphetamine hydrochloride is converted to its free base, chromatographic results are spurious and unpredictable.

Both recovery of a known amount of methamphetamine hydrochloride and precision were excellent. Addition of measured amounts of drug to a placebo, with subsequent analysis, yielded 100.3% recovery. Relative standard deviations of numerous analyses were about ±1%.

Investigations are being conducted on the application of this approach to the analysis of other drugs in similar matrixes.

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Determination of Lactate in Parenterals Containing Reducing Sugars

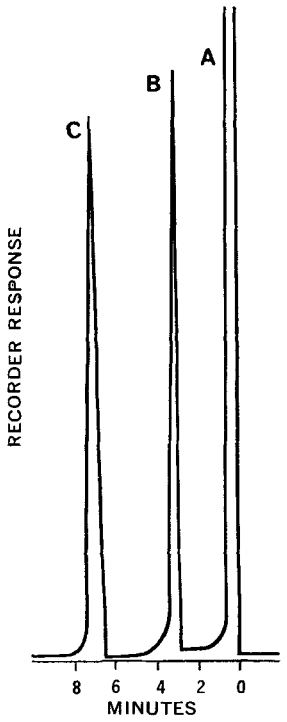
CLAUDE E. TOLBERT* and CHARLES T. KENNER†

Abstract □ Reducing sugars in parenterals containing sodium lactate interfere with the determination of the lactate. An acid–diatomaceous earth column separation of sodium lactate (as lactic acid) is proposed which uses ether as the eluting solvent. After esterification, the lactate is converted to a hydroxamic acid which reacts with iron(III) to form a reddish-purple complex which absorbs at 515 nm. The standard deviation of the method is 1.07% of the average amount declared as being present. The method is rapid and can be used for parenterals containing 20% or less of reducing sugar. Parenterals containing partially hydrolyzed protein give high results.

Keyphrases □ Lactate determination—parenterals containing reducing sugars □ Hydroxamic acid formation—lactate analysis □ Column chromatography—separation □ Colorimetric analysis—spectrophotometer

The charring and titration method for the determination of sodium lactate given in the USP (1) is satisfactory for preparations listed in that compendium. The official method, however, does not work satisfactorily for the analysis of sodium lactate in parenterals and other nonofficial products that contain reducing sugars, phosphates, stabilizers, and other substances which produce titratable bases or color interferences when charred.

Hillig (2) suggested a continuous ether extraction of lactic acid from an acidified dairy product sample followed by a colorimetric assay. Boisson (3) used a similar extraction procedure for the removal of lactic acid from solutions that also contain dextrose, except the sample solution was saturated with ammonium sulfate prior to extraction. Dalrymple (4) analyzed nonofficial parenterals for sodium lactate by using the Hillig extraction procedure followed by titration of the extracted lactic acid with sodium hydroxide solution. Staruszkiewicz (5) proposed a GLC method for lactic and succinic acids in eggs, in which the acids were extracted into ether by a continuous extraction procedure, esterified, and extracted into chloroform before injection. These methods are time consuming and



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suffer from interference by stabilizers, such as sulfites, and by carryover of the inorganic acid.

This paper reports the development of an acid–diatomaceous earth column separation of sodium lactate (as lactic acid) from reducing sugars and other interfering substances, using ether as the eluant. After esterification, the lactate is converted to a hydroxamic acid which reacts with iron(III) to form a reddish-purple complex which is measured photometrically at a wavelength of 515 nm. The color development is a modification of the Goddu *et al.* (6) procedure for organic esters.

EXPERIMENTAL

Apparatus—A chromatographic tube, Pyrex, 2.20 × 25 cm., constricted at one end to 0.4 × 5.0 cm., was used. An aluminum tamping tool was used to fit the chromatographic column. All photometric measurements were made on a Cary model 15 or model 11 spectrophotometer.

Reagents and Solutions—All reagents were ACS, USP, or NF grade. The following were used:

1. Diatomaceous earth[1], acid-washed grade.
2. Ethyl ether, neutral—Agitate 400 ml. of ethyl ether in a separator with 25.0 ml. of 1.0 *N* NaOH and separate. Wash ether with 25-ml. portions of water by agitation and separation until the wash water is neutral to litmus.
3. Sodium hydroxide (12.5% w/v methanolic)—Reflux 12.5 g. of NaOH pellets with 100 ml. of methanol for several minutes and cool.
4. Sodium hydroxide (0.1% w/v methanolic)—Add 1 ml. of 12.5% methanolic NaOH to 124 ml. of methanol.
5. Hydrochloric acid (5% v/v methanolic)—Add 5 ml. of HCl (37% w/w) to 95 ml. of methanol.
6. Hydroxylamine hydrochloride (12.5% w/v methanolic)—Dissolve 12.5 g. of $NH_2OH \cdot HCl$ in 100 ml. of methanol. Adjust, using phenolphthalein, so that 5.0 ml. requires 2.5 ± 0.2 ml. of 12.5% methanolic NaOH.
7. Ferric perchlorate stock solution—Add 10.0 ml. of perchloric acid (70% w/w) to 0.800 g. of standard iron wire in a 50-ml. beaker. Cover beaker with a watch glass and place on a steam bath until the iron dissolves, approximately 5 min. (CAUTION: the iron dissolves with almost explosive violence if the solution is heated too rapidly.) Cool to room temperature and dilute to 100 ml. with absolute ethanol.
8. Ferric perchlorate analytical solution—Dilute 10.0 ml. of stock ferric perchlorate and 3.0 ml. of perchloric acid (70% w/w) to 250 ml. with absolute ethanol. Discard after 3 days.
9. Calcium lactate standard solution (0.5 mg./ml.)—Dry hydrous calcium lactate to constant weight at 120°. Dissolve 100.0 mg. of anhydrous standard in 10 ml. of water and dilute to 200 ml. with alcohol USP. This standard is equivalent to 0.413 mg./ml. lactic acid.

PROPOSED METHOD

Preparation of Chromatographic Column—Place a loose pad of glass wool in the bottom of a chromatographic column; add approximately 30 g. of anhydrous, granular sodium sulfate; and cover with a loose pad of glass wool. Thoroughly mix 0.5 ml. of ammonium sulfate (50% w/v) with 1.0 g. of diatomaceous earth and transfer to the column. Pack evenly with the tamping rod. Prepare the sample and transfer to the column as directed under *Preparation of Sample*.

Preparation of Sample—Dilute an appropriate aliquot of sample with alcohol USP to give a sodium lactate concentration of between 0.25 and 0.45 mg./ml. Pipet a 10.00-ml. aliquot into a 100-ml. beaker and evaporate to dryness on a steam bath. Cool residue to room temperature and dissolve in 2 ml. of 1 *N* HCl. Mix thoroughly with 3.0 g. of diatomaceous earth and transfer to the column in two portions, packing each evenly. Dry wash the beaker with 0.5 g. of diatomaceous earth and transfer to column. Cover with a loose pad of glass wool.

[1] Celite 545, Johns-Manville Corp., New York, N. Y.

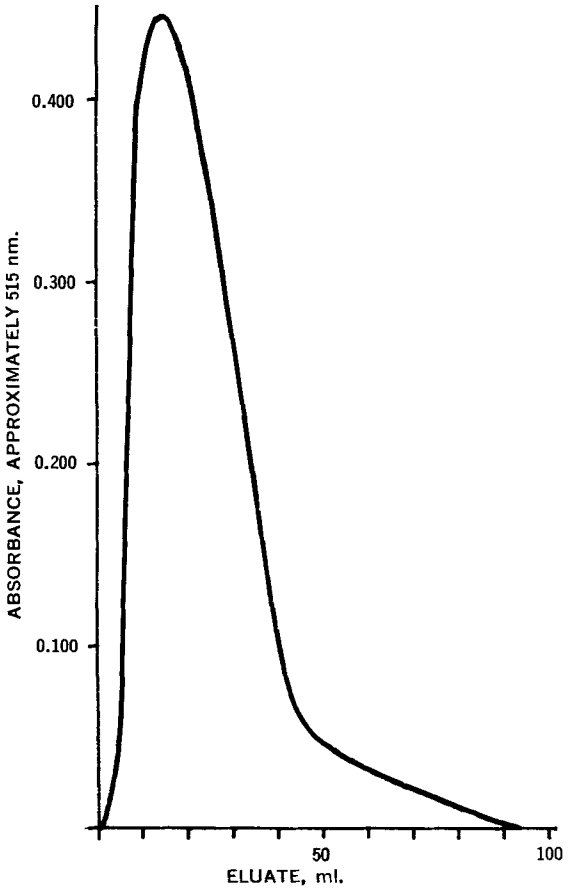

Figure 1—*Typical lactic acid elution curve.*

Preparation of Standards—Pipet 0-, 2.0-, 4.0-, 6.0-, and 8.0-ml. aliquots of the standard calcium lactate into separate 125-ml. glass-stoppered conical flasks. These aliquots are equivalent to 0 (blank), 0.83, 1.65, 2.48, and 3.30 mg. of lactic acid, respectively. To each, add 1.0 ml. of 0.1% methanolic NaOH, and evaporate to dryness on the steam bath. Continue as directed under *Color Development* beginning with "add 1.0 ml. of 5% methanolic HCl. . .." In certain of the recovery studies, the standards were treated as samples and adsorbed on diatomaceous earth for column separation. For this

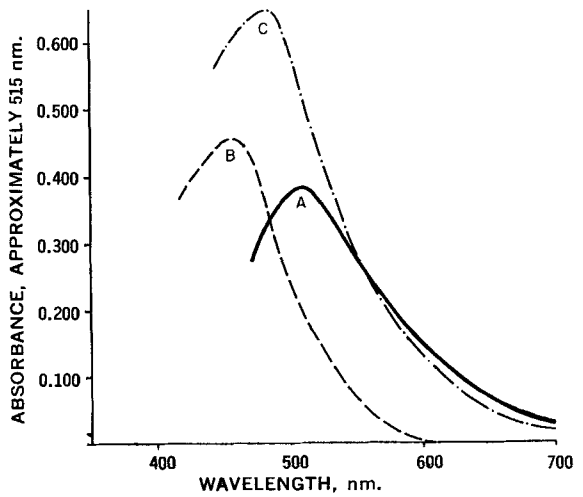

Figure 2—*Dextrose interference in absorbance measurements. Key: A, sodium lactate, 1.5 mg.; B, dextrose, 100 mg.; and C, sodium lactate, 1.5 mg. plus 100 mg. of dextrose.*

Table I—Sodium Lactate in Commercial Preparations

Product	Sodium Lactate Declared, mg./100 ml.	Found mg./100 ml.	Found Percent of Declared
5% Dextrose with electrolyte No. 75[a]	220	230.6 228.2	104.8 103.7
5% Invert sugar with electrolyte No. 2[a]	280	307.0 308.6	109.6 110.2
Sodium lactate injection USP	187	182.5 180.8	97.8 96.9
5% Dextrose with electrolyte No. 48[a]	260	273.7 267.1	105.3 102.7
10% Dextrose[b] with electrolyte No. 48	260	250.4	96.3
20% Dextrose[b] with electrolyte No. 48	260	257.1	98.9

[a] These electrolyte solutions contain various amounts of $NaC_3H_5O_3$ NaCl, KCl, $MgCl_2$, K_2HPO_4, NaH_2PO_4, and $NaHSO_3$. [b] Prepared from the commercial sample containing 5% dextrose in electrolyte No. 48 by addition of dextrose.

procedure, pipet the aliquots into 100-ml. beakers and continue as directed under *Preparation of Sample*, beginning with "evaporate to dryness on a steam bath. . .."

The color is stable for at least 1 hr. and follows Beer's law in the range of 0.5–4.0 mg. of lactic acid per 50 ml. Even though a previously prepared calibration curve can be used satisfactorily in many cases, a series of standards should be run with each set of samples to guard against unexpected variations in temperature affecting the results.

Chromatographic Separation—Use a 125-ml. glass-stoppered conical flask as the column eluate receiver. Rinse the sample beaker with two 10-ml. portions of neutral ether and add to the column. Follow with neutral ether as needed to maintain a flow rate of approximately 6 ml./min. After 100–110 ml. of the ether eluate has been collected, rinse the tip of the tube with neutral ether and remove the flask. Add 2.0 ml. of 0.1% methanolic NaOH to the flask and evaporate to dryness on a steam bath.

Color Development—(Keep the flasks stoppered during the color development procedure except for addition of reagents.) To each individual flask, add 1.0 ml. of 5% methanolic HCl and swirl to hasten solution. After the sample has dissolved, add 1.5 ml. of 12.5% methanolic hydroxylamine reagent, swirl, and allow to stand for 20 min. Add 1.5 ml. of 12.5% methanolic NaOH, mix, and allow to stand for 10 min. Add 25.0 ml. of the ferric perchlorate analytical solution, and agitate thoroughly to ensure complete solution of any precipitated ferric hydroxide. Allow to stand 10 min. Filter the solution quantitatively through dry Whatman 41H paper into a 50-ml. volumetric flask, using alcohol USP to rinse the flask and paper. Dilute to volume with alcohol USP. Measure the absorbance at 515 nm. against the blank. If a recording spectrophotometer is used, record the visible spectrum from 490 to 700 nm.

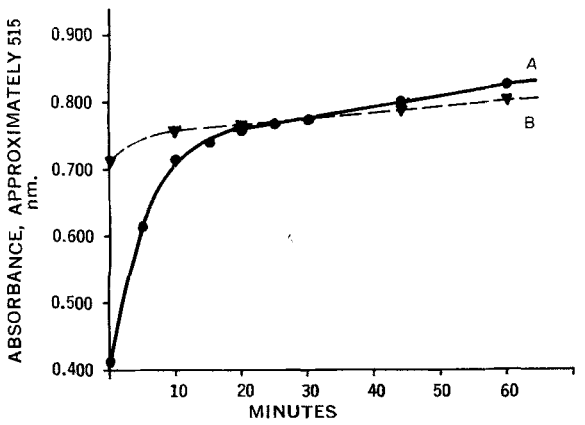

Figure 3—*Effect of time in alkaline and acidic media on net absorbance of 2.1 mg. of sodium lactate. Key: A, acidic solution at room temperature (25°); and B, alkaline solution at room temperature (25°).*

METHOD DEVELOPMENT

Chromatographic Separation—In the proposed procedure, the sodium lactate in the sample is converted to lactic acid by dissolving in 1 *N* HCl before adsorption on diatomaceous earth for transfer to the column. As a consequence, the chromatographic separation involves the selective removal of lactic acid from the column by partition between the ether eluant and the hydrochloric acid solution adsorbed on the diatomaceous earth. Elution studies indicate that hydrochloric acid concentrations up to 6 *N* apparently have no effect upon the elution pattern, but concentrations between 3 and 6 *N* often cause colored blanks, probably due to the leaching of iron from the diatomaceous earth even though the acid-washed grade is used. The proposed method utilizes 1 *N* HCl, which does not cause colored blanks and which gives a satisfactory elution pattern. With this concentration, 99.4% of a 6.6-mg. sample of lactic acid is removed from the column in the first 50 ml. of ether eluant; therefore the 100–110 ml. specified in the method ensures complete removal of even larger lactic acid samples than are recommended. A typical elution pattern for 3.90 mg. of lactic acid is shown in Fig. 1.

Interference Studies—Diethyldithiocarbamate, which is often used as a preservative for ether, interferes with the color development step. It reacts with the color reagent to form a magenta-colored compound which absorbs in the same region as the compound produced with lactic acid (7). The ether is washed with sodium hydroxide to remove the diethyldithiocarbamate and then with water to remove the excess base.

Early results indicated the loss of lactic acid during ether evaporation. Two milliliters of 0.1% methanolic NaOH is sufficient to convert the lactic acid to sodium lactate and does not interfere with the esterification step. Larger volumes tend to cause erratic results in the absorbance developed, probably due to partial neutralization of the methanolic HCl used in the esterification step.

Water interferes with both the esterification and color development by causing a decrease in absorbance of approximately 3%/0.05 ml. of water. Consequently, it is essential that the eluate be evaporated to complete dryness, even though the sodium sulfate trap used in the column is effective in the removal of water.

Dextrose and other reducing sugars interfere with the color development step and must be eliminated by the chromatographic separation. Figure 2 shows the amount of interference in the color development step caused by 100 mg. of dextrose on the absorbance of a 1.5-mg. sample of sodium lactate. In many parenterals the ratio of sugar to sodium lactate is approximately 20 to 1, so the column must be capable of removing at least 100 mg. of dextrose for the sample sizes recommended in the proposed procedure.

Early studies indicated that some dextrose was eluted from the column with the ether eluate when the column was prepared using the acidic sample layer only. A 50% ammonium sulfate solution adsorbed on diatomaceous earth was instituted as a trap layer below the sample layer, and it improved the results significantly. However, replicate studies indicated occasional high values which could not be explained satisfactorily except that the ether was evidently removing small amounts of water and dextrose from the aqueous traps. Incorporation of a layer of anhydrous sodium sulfate below the ammonium sulfate trap effectively removes any water and dextrose. Certain organic acids, such as acetic, succinic, citric, tartaric, and ascorbic acids, are partially or completely eluted from the column with the lactic acid and, consequently, interfere with the procedure by producing iron(III) hydroxamate chelates which absorb in the same region. Since none of the known parenterals containing sodium lactate includes other organic salts in the formulation, these acids need not be considered unless their presence is stated in the declared formulation of the parenteral.

One sample which contained partially hydrolyzed casein in addition to dextrose, sodium lactate, and other electrolytes gave suspiciously high results when run by the recommended procedure. The interference was believed to be due to amino acids or other decomposition products which were being eluted with the lactic acid during the chromatographic step. Efforts to eliminate or offset the interference were unsuccessful, even though the interference could be reduced by precipitation of the unhydrolyzed casein before evaporation of the diluted original sample. An average value

of 130% of the declared amount was obtained by the recommended procedure. This value was reduced to 115% of the declared amount by precipitation of the unhydrolyzed casein before analysis. Thus, samples that contain hydrolyzed protein cannot be run satisfactorily by the recommended procedure.

Esterification and Hydroxamic Acid Formation—The reaction in the acid medium is assumed to be mainly esterification and that in the basic medium mainly hydroxamate formation (8). Most esterification procedures for methylation of lactic acid call for a dehydrating agent or heat during the esterification (9). Various dehydrating agents including gaseous hydrogen chloride, concentrated sulfuric acid, and anhydrous calcium chloride (10) were investigated. The reaction was also carried out in methylene chloride and ethylene chloride to eliminate the necessity of a dehydrating agent. Satisfactory yields of methyl lactate were obtained with each procedure. However, the small amount of lactic acid which must be esterified in the proposed procedure suggested that the reaction might be quantitative in the presence of the large excess of hydroxylamine without the presence of a dehydrating agent or anhydrous solvent, since the reaction of the hydroxylamine with the ester should help drive the esterification reaction to completion. Time studies on the esterification and hydroxamate formation reactions were instituted.

The amount of time allowed in the acid medium before addition of the methanolic base was varied while the amount of time between addition of the methanolic base and the iron color-producing reagent was kept constant. The amount of time allowed between the addition of the methanolic base and the iron color-producing reagent was then varied while the time in the acid medium was kept constant. The results are shown in Fig. 3. Both reactions increase rapidly at first and then reach a plateau of slow increase after a given length of time. The optimum times were found to be 20 min. in the acid medium and 10 min. in the basic medium.

The actual absorbing species measured is the iron(III) chelate of the hydroxamic acid formed by the reaction of methyl lactate and hydroxylamine (6). The reaction is quantitative at room temperature and should not be heated since the absorbance of the chelate is affected greatly by temperature. After the color is developed, raising the temperature to approximately 60° causes rapid loss in absorbance and a change in the absorption maximum. If the solution is then cooled to room temperature, the original color returns but the absorbance is permanently decreased by varying amounts, depending upon the length of time at the elevated temperature. At room temperature, the color is stable for approximately 60 min. but then fades slowly. After 3 hr. the absorbance is reduced to approximately 94%.

The presence of even small amounts of water affect the stability of the color developed. For example, the use of 95% ethanol USP instead of absolute ethanol in the preparation of the iron(III) perchlorate solutions causes the fade rate to increase to approximately 14%/hr.

RESULTS AND DISCUSSION

The results of the analyses of representative commercially available parenterals which contain sodium lactate and reducing sugars are shown in Table I. The standard deviation, as calculated from the difference in duplicates (11), is 2.62 mg./100 ml., 1.07% of the average amount declared as present. The results are satisfactory in the presence of as much as 20% dextrose, as shown in the last sample in the table.

The standard deviation for six replicate determinations on a sample containing 5% dextrose was 0.054 mg., which represented 1.90% of the average amount found. Recovery of 4.10 mg. of standard by the procedure averaged 4.09 mg., with a standard deviation calculated from the range (12) of 0.053 mg. or 1.29%.

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Effect of Vehicle Dielectric Properties on Rectal Absorption of Acetaminophen

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Abstract □ The rectal absorption of acetaminophen from an aqueous suspension, a propylene glycol suspension, and a theobroma oil suppository was measured by urinary excretion. The results are discussed in terms of their relationship to the dielectric constants of the vehicles.

Keyphrases □ Acetaminophen—rectal absorption from aqueous suspension, propylene glycol suspension, theobroma oil suppository, relationship to dielectric constants □ Rectal absorption, acetaminophen—effect of vehicle dielectric constant □ Dielectric constant—role in rectal absorption of acetaminophen in different vehicles

Kakemi *et al.* (1) demonstrated the influence of water-soluble suppository bases on the rectal absorption of sulfonamides in specially prepared rats. Their technique was one of recirculation *in situ* and was designed to elucidate the mechanism of rectal absorption and to evaluate the influence of pharmaceutical materials upon it. These investigators observed that water-miscible compounds such as propylene glycol decreased the absorption of poorly water-soluble sulfonamides through a decrease in lipid–vehicle partition. This decrease in lipid–vehicle partition was related to the dielectric constant of the water-miscible vehicle, because the larger the dielectric constant (or the nearer the dielectric constant to the dielectric constant of water) the smaller the reduction in rectal absorption. In other words, sulfonamides were poorly absorbed from low dielectric media because they were more soluble in that media than in the rectal mucosa.

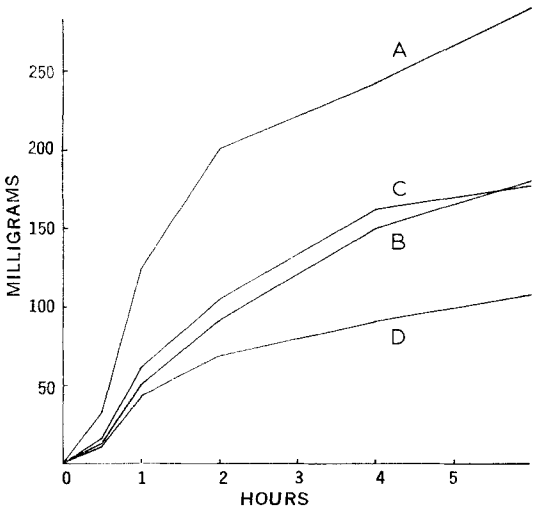

Figure 1—*Urinary excretion means of acetaminophen according to vehicle and route of administration. Key: A, water, oral; B, MCC–CMC gel, rectal; C, theobroma oil, rectal; and D, propylene glycol, rectal.*

Kakemi *et al.* (1) were not able to evaluate the effect of a nonwater-miscible vehicle. Such a vehicle would have a very low dielectric constant—a dielectric constant low enough to be out of the dielectric range required for sulfonamide solubility.

This investigation is an extension of their work; vehicles of all three dielectric ranges—too high, too low, and optimum for solubility—are studied. In addition, this investigation represents an application of their conclusions to a practical formulation–absorption problem in humans.

For human studies, acetaminophen was selected in place of a sulfonamide. Acetaminophen has dielectric-related solubilities similar to those of sulfonamides (2); it is absorbed rectally (3, 4), it can be measured in the urine (3, 5, 6), and it is not known to be sensitizing like the sulfonamides. In addition, detailed pharmacokinetic studies have been reported (5, 6).

Water, with a dielectric constant of 80.37 at 20° (7), had the highest dielectric constant used in this investigation. Propylene glycol represented the optimum range of dielectrics, since it has a dielectric constant of 32.0 at 20° (8). Theobroma oil represented the lower range of dielectrics. The dielectric constant of liquid theobroma oil could be expected to be from 2 to 6, as judged from reported values for vegetable acids and monopalmitin (9).

For comparison, an orally administered solution was included in the study.

EXPERIMENTAL

Materials—Acetaminophen NF micropowder[1] was used in all preparations. Excipients were of USP quality.

Preparation of Dosage Forms—A suspension of acetaminophen, 500 mg./5 ml., buffered to pH 6.0 was prepared in a 4.0% microcrystalline cellulose–carboxymethylcellulose[2] (MCC–CMC) gel by a previously described method (10).

A suspension of acetaminophen in propylene glycol, 500 mg./2 ml., was prepared without additives.

Theobroma oil suppositories of acetaminophen, 500 mg./2 g., were prepared by the fusion process.

The orally administered solution was simply 500 mg. of acetaminophen dissolved in a liter of water.

Urinary Excretion Studies—All subjects were healthy males, ranging from 24 to 39 years of age and from 62 to 95 kg. in weight.

[1] S. B. Penick and Co., New York, N. Y.
[2] Avicel RC, American Viscose Division, FMC Corp., Marcus Hook, Pa.

Table I—Urinary Excretion Means of Acetaminophen ±95% Confidence Limit (mg.)

Vehicle and Route	Number of Subjects	Hours 0.5	1	2	4	6
Water, oral	10	33.3 ±16.7	123 ±36.7	200 ±58.3	242 ±68.3	290 ±75.0
MCC–CMC gel, rectal	10	13.3 ±8.2	44.9 ±28.3	91.5 ±43.3	149 ±58.2	180 ±58.2
Theobroma oil, rectal	10	15.8 ±26.8	60.0 ±41.6	104 ±41.8	162 ±58.2	177 ±29.4
Propylene glycol, rectal	5	11.5 ±8.3	43.3 ±30.0	68.2 ±51.6	90.0 ±63.3	107 ±66.7

The same 10 subjects were used throughout the program. However, only five of these subjects were available for the propylene glycol suspension experiments. These five ranged from 25 to 39 years in age and from 62 to 88 kg. in weight.

The following protocol was utilized. Breakfast was light without coffee or tea. The bowel and bladder were evacuated before inserting the medication. Each subject drank 1 l. of room temperature water after inserting the medication. Urine samples were collected at 0.5, 1, 2, 4, and 6 hr. Abstinence from liquids and drugs during the test was required.

The aqueous and propylene glycol suspensions were administered with the aid of a short piece of polyethylene tubing attached to a glass syringe.

Urine Analysis—Urine analysis for total acetaminophen was by the method of Brodie and Axelrod (11).

Solubility Studies—Ancillary data on the solubility of acetaminophen in the vehicles at 37° were obtained. Each vehicle and an excess of the drug were placed in 75-ml. cylindrical bottles and rotated at 43 r.p.m. for 24 hr. on an apparatus designed for testing sustained-release products. This apparatus is described in the NF (12). For the aqueous and propylene glycol systems, filtered samples were quickly pipeted with warmed pipets, diluted with acidified methanol, and assayed spectrophotometrically at 249 nm. (13).

In the case of theobroma oil, after rotation, the bottle was set in an upright position for 24 hr. in the water bath. The undissolved drug settled to the bottom of the bottle, and a clear zone appeared near the surface of the liquid. After the theobroma oil solidified at room temperature, 500 mg. was scraped from the surface. This material was extracted with warmed acidified methanol. The acidified methanol was then cooled, filtered, and assayed spectrophotometrically at 249 nm.

RESULTS AND DISCUSSION

The urinary recovery means and their 95% confidence limits are listed in Table I. The means themselves are illustrated in Fig. 1.

Table II—*t*-Test Values for Urinary Excretion

X_1, Vehicle and Route	Hours	X_2, Vehicle and Route: Aqueous, Rectal	Theobroma Oil, Rectal	Propylene Glycol, Rectal
Water, oral	0.5	2.0	1.2	1.8
	1	3.1[a]	2.1	2.6[a]
	2	2.9[a]	2.8[a]	2.8[a]
	4	5.3[a]	4.7[a]	5.7[a]
	6	5.6[a]	5.7[a]	7.6[a]
MCC–CMC gel, rectal	0.5		−0.12	0.24
	1		−0.45	0.74
	2		−0.11	0.63
	4		−0.10	3.2[a]
	6		0.17	3.3[a]
Theobroma oil, rectal	0.5			0.18
	1			0.48
	2			0.74
	4			2.8[a]
	6			3.0[a]

[a] Significant difference at 95% level.

The *t*-test values are listed in Table II, and the solubilities are listed in Table III.

As one might expect, in none of the experiments was the total dose excreted within the 6 hr. Even in the case of the oral solution, only 58% of the dose was excreted. With the rectal dosage forms, the percent recovered was much less. Since it was the intent of the investigation to evaluate only the relative bioavailability from the rectal dosage forms and since it was impossible to police both the bowel and bladder habits of the participants for a longer period, a 6-hr. study was considered sufficient.

The excretion rate of acetaminophen from the rectal dosage forms declined during the last sampling period. Since the excretion kinetics of acetaminophen would be the same in all cases, it is difficult to see how the relative bioavailability rankings of the drug from the rectal dosage forms could change at some later time.

From the data, no significant differences in the 0.5-hr. recoveries are observed. After that, the orally administered solution is significantly better than all of the rectal dosage forms. At 4 and 6 hr., the aqueous and theobroma oil vehicles ranked significantly better than the propylene glycol suspension. The differences between the two better formulations and the propylene glycol formulation—whether significant or not—increased with each increasing sampling period. The aqueous and theobroma oil formulations were equivalent at all time periods. By comparing the excretion after 6 hr., the amount excreted from the better formulations was 1.7 times greater than from the propylene glycol formulation.

The solubility of acetaminophen in the various vehicles is in agreement with the predictions of Paruta and Irani (2). At 37°, propylene glycol was 6.6 times and 72 times more effective as a solvent than the aqueous vehicle and theobroma oil, respectively.

The aqueous vehicle contained electrolytes to buffer the system to pH 6.0. These electrolytes would increase the dielectric properties of the system, and their buffer capacity would maintain acetaminophen in its least soluble form (14).

Although rheological evaluations indicated an interaction between acetaminophen and MCC–CMC (10), the solubility studies showed that such an interaction is not particularly significant in regards to *in vitro* availability. The exact role this interaction plays in *in vivo* availability is difficult to assess.

These investigations demonstrated that if acetaminophen is dispersed in a vehicle that has a dielectric constant that meets the dielectric requirement for high solubility, then its rectal absorption is apt to be relatively low. And, if acetaminophen is dispersed in vehicles that have dielectric constants that are too high or too low for high solubility, then its rectal absorption is apt to be relatively high. This work with acetaminophen is a verification and extension of the work of Kakemi *et al.* (1) that was done with sulfonamides in specially prepared rats. This work is also an application of their theories to a practical formulation–absorption problem in humans. However, one must be cautious in making generalizations. Several factors including the alteration of the rectal mucosa, the absorption of the vehicles *per se*, the effects of complexation or binding, and

Table III—Solubility of Acetaminophen in Vehicles at 37°

Vehicle	Solubility
Propylene glycol	156 mg./ml.
MCC–CMC gel	23.6 mg./ml.
pH 6.0 buffer	23.8 mg./ml.
Theobroma oil	2.16 mg./g.

the variation of dosage form mass were not evaluated in this investigation.

SUMMARY

The theories of Kakemi *et al.* (1) that relate the dielectric constants of water-miscible vehicles to rectal absorption have been extended to include a water-immiscible vehicle and have been applied to a practical formulation-absorption problem in humans.

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*Graduate Fellow of the American Foundation for Pharmaceutical Education.

Extrinsic Cotton Effects of Bishydroxycoumarin when Bound to Bovine Serum Albumin

J. H. PERRIN and P. IDSVOOG

Abstract □ The binding of bishydroxycoumarin to bovine serum albumin at pH 7.4 was investigated by circular dichroism and differential absorption. No major conformational changes were observed on binding, but optical activity was induced at wavelengths above 290 nm. and investigated for various drug-to-protein ratios. Several possible explanations for the experimental observations are made.

Keyphrases □ Bishydroxycoumarin—extrinsic Cotton effects when bound to bovine serum albumin □ Albumin, bovine serum—bound bishydroxycoumarin, extrinsic Cotton effects □ Circular dichroism—analysis □ Differential absorption—analysis

Bishydroxycoumarin[1] was found to be over 99% bound to human plasma proteins, even when the concentration in whole blood was 1000 mg./l. (1). Paper electrophoresis studies suggest that the drug is entirely bound to the albumin fraction (2). In this report the binding of bishydroxycoumarin to bovine serum albumin in 0.1 *M* pH 7.4 phosphate buffers is investigated. Other highly protein bound drugs which have shown extrinsic Cotton effects include the *N*-arylanthranilates (3) and the pyrazolone analgesics (4, 5).

MATERIAL AND METHODS

Bishydroxycoumarin USP was used as supplied[2]. The bovine serum albumin was crystallized and lyophilized[3]. All buffer materials were reagent grade. The circular dichroism (CD) spectra were obtained at 25° in 5-, 10-, or 20-mm. cells, using the 6002 attachment to a Cary 60 spectropolarimeter[4]. The dynode voltage was never allowed to exceed 0.35, and the signal-to-noise

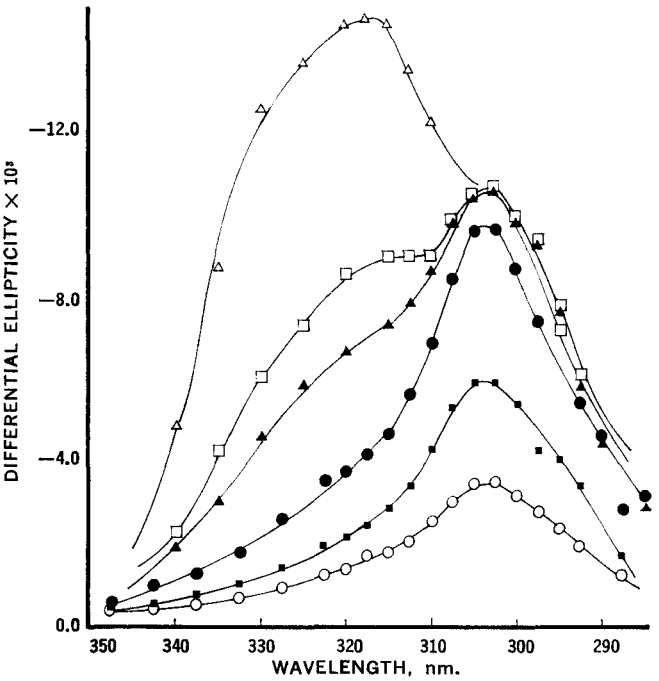

Figure 1—*Extrinsic CD curves for bishydroxycoumarin binding to bovine serum albumin measured in 0.1* M *phosphate buffer, pH 7.4, corrected to 1-cm. pathlength. D/P molar ratios are:* △, *10.26;* □, *6.16;* ▲, *5.13;* ●, *1.44;* ■, *0.82; and* ○, *0.4.*

[1] Dicumarol.
[2] Abbott Laboratories, North Chicago, Ill.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Cary Instruments, Monrovia, Calif.

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MATERIAL AND METHODS

Bishydroxycoumarin USP was used as supplied[2]. The bovine serum albumin was crystallized and lyophilized[3]. All buffer materials were reagent grade. The circular dichroism (CD) spectra were obtained at 25° in 5-, 10-, or 20-mm. cells, using the 6002 attachment to a Cary 60 spectropolarimeter[4]. The dynode voltage was never allowed to exceed 0.35, and the signal-to-noise

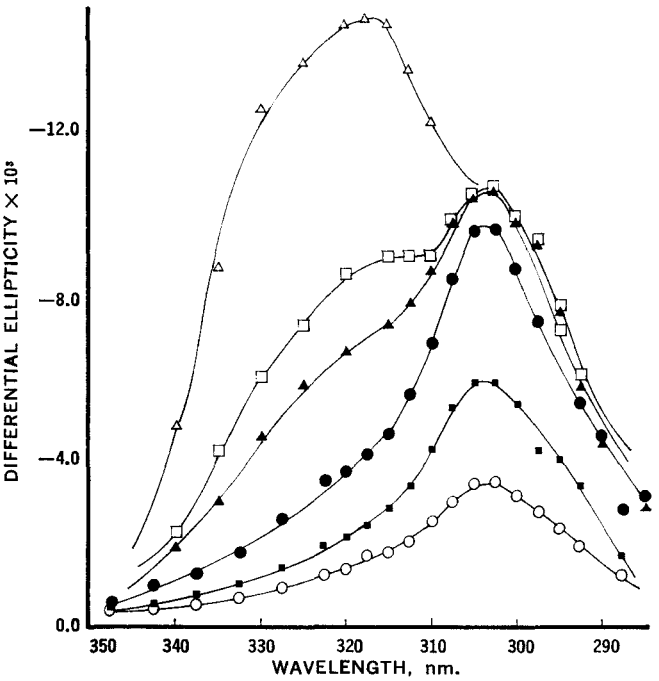

Figure 1—*Extrinsic CD curves for bishydroxycoumarin binding to bovine serum albumin measured in 0.1* M *phosphate buffer, pH 7.4, corrected to 1-cm. pathlength. D/P molar ratios are:* △, *10.26;* □, *6.16;* ▲, *5.13;* ●, *1.44;* ■, *0.82; and* ○, *0.4.*

[1] Dicumarol.
[2] Abbott Laboratories, North Chicago, Ill.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Cary Instruments, Monrovia, Calif.

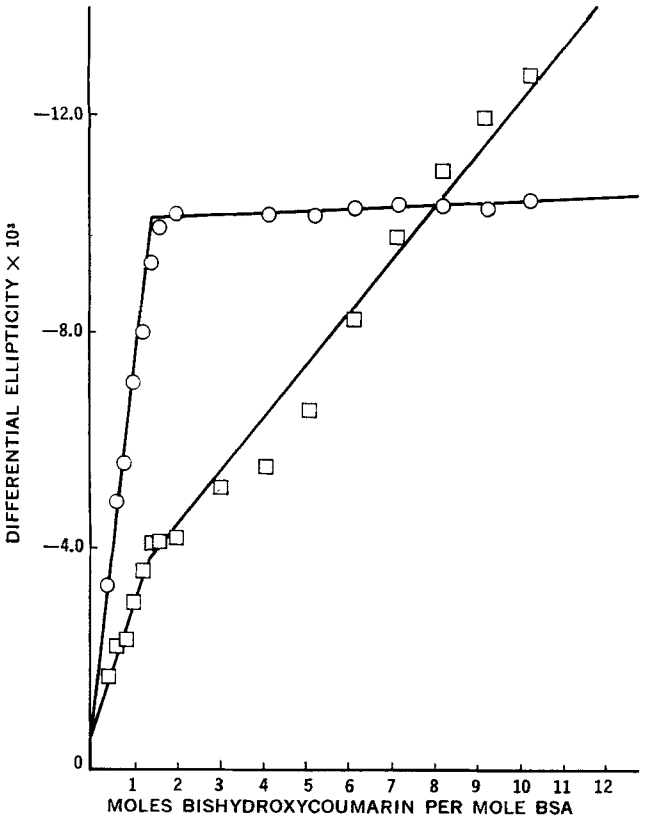

Figure 2—*Plot of observed ellipticity against D/P ratio for bishydroxycoumarin binding to bovine serum albumin. Key:* ○, *wavelength of 303 nm.; and* □, *wavelength of 318 nm. corrected to 1-cm. pathlength.*

ratio was always greater than 40 for the optically clear solutions. CD data are presented as differential ellipticity, which is taken as the observed ellipticity minus the ellipticity due to the constant concentration of serum albumin at the same wavelength. The differential UV spectra were obtained by determining the spectra of the drug alone, the protein alone, and then the mixture, using the buffer as the reference in a Cary 14 spectrophotometer.

A 1.45×10^{-5} *M* bovine serum albumin concentration (mol. wt. 69,000) and drug concentrations up to 1.49×10^{-4} *M* were used in these investigations.

RESULTS AND DISCUSSION

The extrinsic Cotton effects produced above 290 nm. are shown in Fig. 1; any small contribution of the protein was subtracted from these observed ellipticities. Below 290 nm., the ellipticity of the protein becomes dominant and the absorbance of the solutions rises so that good quantitation is impossible and, as such, is not reported. Investigations in shorter pathlength cells and on diluted solutions, down to 200 nm., suggest that there is little or no departure from the α-helical structure associated with bovine serum albumin at pH 7.4.

Various drug-to-protein (D/P) ratios were used, and the shapes as well as the magnitudes of the induced CD curves were dependent upon this ratio (Fig. 1). At low D/P ratios, there is a peak at 303 nm. and a slight shoulder at 318 nm. However, at higher D/P ratios, this shoulder develops into a dominant peak. Figure 2 shows the plot of observed ellipticity against D/P ratio at fixed wavelengths of 303 and 318 nm. Both plots show a break at a D/P ratio of approximately 1.5; there is a leveling off of induced ellipticity at 303 nm. above this D/P ratio, but there is a continued increase in ellipticity at 318 nm. with all solutions investigated.

The explanations of these differences and the break at the D/P ratio of 1.5 are complicated by the lack of information of the pKa's of bishydroxycoumarin. Bishydroxycoumarin is a dibasic acid whose pKa's are extremely difficult to measure because of poor aqueous solubility and instability in extremes of pH. However, values of pKa_1 have been reported as 5.7 (6) and 6.5 (7); the simi-

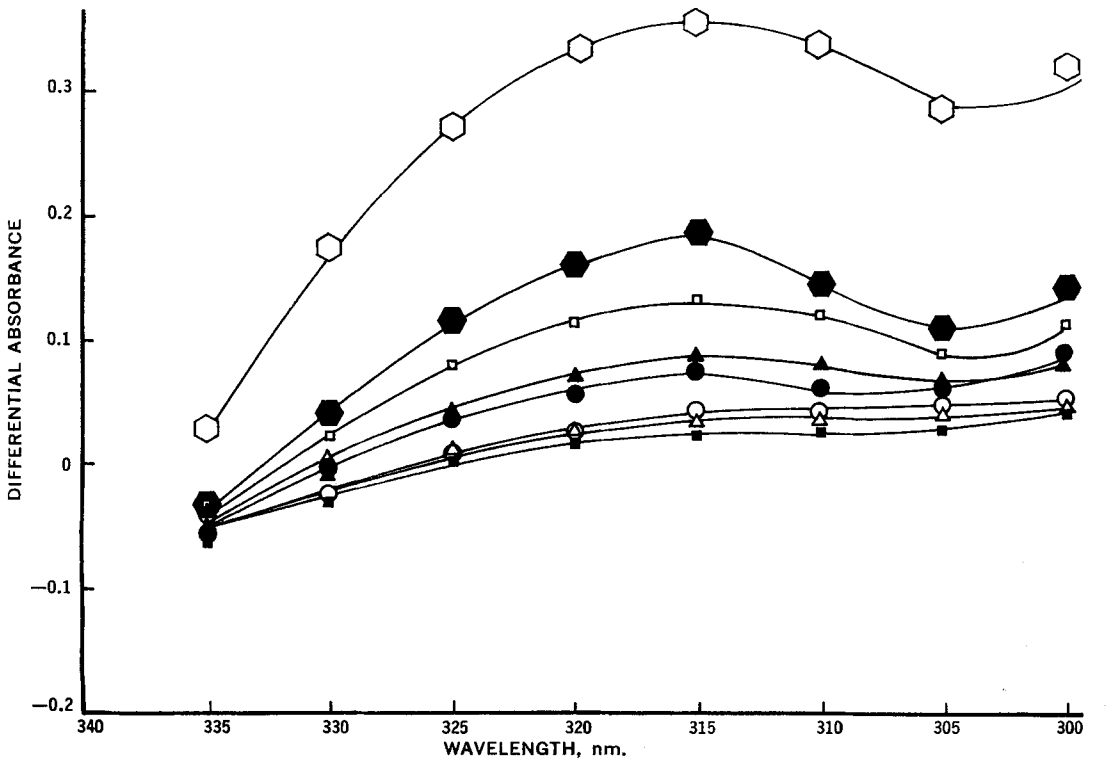

Figure 3—*Differential UV plot for binding of bishydroxycoumarin to bovine serum albumin. Differential absorbance = absorbance of drug alone + absorbance of protein alone − absorbance of mixture of drug and protein in 0.1* M *phosphate buffer, pH 7.4, in 1-cm. cells. Key:* ○, *5.13;* ●, *4.10;* □, *3.08;* ▲, *2.46;* ●, *2.05;* ○, *1.44;* △, *1.03; and* ■, *0.82.*

larity of the second acid grouping to the first suggests that its pKa is similar to that of the first. It seems probable, therefore, that all three species are present to some extent in the pH 7.4 buffer of these investigations.

The UV curve of bishydroxycoumarin at pH 7.4 shows a peak at 303 nm., a shoulder above 310 nm., and a binding to bovine serum albumin. There are a slight peak shift and a diminished absorbance, as shown by the differential UV plot of Fig. 3. This plot shows a large decrease in absorbance on binding for D/P ratios greater than 1.5 at wavelengths near 315 nm. The lack of pKa knowledge and the possibility of tautomerism make the interpretation of the effect of pH on UV data impossible at this time, and the CD peaks cannot be associated with any specific species of bishydroxycoumarin. It is possible that the observations reported here are due to the different binding of the various ionized or unionized forms of bishydroxycoumarin.

Another possible explanation for the CD data is that there is a primary binding of a single molecule of bishydroxycoumarin followed by association of more drug molecules at the binding site, as has been suggested for the binding of dyes to polypeptides (9, 10). However, no new peaks nor wavelength shifts are observed at high D/P ratios, apparently obviating the possibility of self-association involving drug chromophores.

Perhaps a more probable explanation for the break in observed ellipticity against D/P ratio plot and the difference in behavior at the two wavelengths is that there is a primary binding site and a series of other less specific binding sites on the bovine serum albumin molecule, with binding to both types of sites occurring to some extent simultaneously. The less specific nature of this secondary binding may be supported by the optical activity being induced only at the higher wavelengths. It is possible that the secondary binding sites become available only after the primary binding, although there is no major conformational change on drug binding.

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Pseudomonas aeruginosa Contamination of Liquid Antacids: A Survey

ELIZABETH P. ROBINSON

Abstract □ In a recent survey, 279 retail packages of liquid antacid containing magnesium hydroxide as an active ingredient from 11 manufacturers, 21 raw materials, and six in-process samples were examined for *Pseudomonas aeruginosa*. Eighty-five of the finished bottles (30.5%) and two in-process samples (33%) were positive. The aerobic plate count ranged from <100 to 9,300,000 organisms/g. Nineteen of the total samples (6.8%), including one water sample used as a raw material, were contaminated with coliforms or *Alcaligenes* sp. These samples had an aerobic plate count from <100 to 500,000 organisms/g.

Keyphrases □ *Pseudomonas aeruginosa* contamination—liquid antacids, survey □ Antacids, liquid—contamination by *Pseudomonas aeruginosa*, survey □ Contamination, *Pseudomonas aeruginosa*—in liquid antacids

Contamination of nonsterile pharmaceuticals by pathogenic yeasts, molds, and bacteria is rapidly becoming a matter of worldwide concern (1–7). This laboratory recently encountered bacterial contamination in liquid antacids manufactured by pharmaceutical firms in northeastern United States. Previous experience with contamination of liquid antacids had revealed the presence of *Pseudomonas aeruginosa*. In a recent survey of antacid products of a similar type, with samples obtained both during manufacturing and from the finished product, the author encountered a high level of bacterial contamination, with *P. aeruginosa* being the predominating organism. Magnesium hydroxide was the common active ingredient in all products examined.

Table I—Tests to Differentiate *Pseudomonas aeruginosa* from *Alcaligenes* sp.

Test	*P. aeruginosa*	*Alcaligenes* sp.
Glucose utilization	Oxidation	Nonutilized
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Growth at 42°	+	+
Motility	+	+
Fluorescence	+	−
Pigment	Pyocyanine Pyorubrin	−
Flagella	1 Polar	Peritricious

EXPERIMENTAL

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Growth at 42°	+	+
Motility	+	+
Fluorescence	+	−
Pigment	Pyocyanine Pyorubrin	−
Flagella	1 Polar	Peritricious

EXPERIMENTAL

In this survey, 279 retail packages of liquid antacid representing 11 manufacturers and 34 different labels, 21 raw materials, and six in-process samples were examined.

A sample portion of 10 ml. or 10 g. was thoroughly mixed with

Table II—Results of a Survey for Bacterial Contamination of Liquid Antacids, In-Process Samples, and Raw Materials

Type of Sample	Number Tested	*P. aeruginosa*	*Alcaligenes* sp.	Coliforms
Raw materials	21	0	1	0
In-process	6	2	0	0
Finished bottles	279	85	16	2

A summary of contaminated samples from the three manufacturers is presented in Table III. From the wide aerobic plate-count range, it can be readily seen that *Pseudomonas* contamination is extremely nonuniform, even within a given batch. By enrichment procedures, it is possible to recover small numbers of *Pseudomonas* from contaminated products.

Samples of magnesium hydroxide and other major raw materials used in manufacturing the contaminated batches of antacid were not available for testing. The raw materials examined included six samples of magnesium hydroxide used in the manufacture of non-

Table III—Summary of Contaminated Samples

Manufacturer	Aerobic Plate Count	Total Samples Tested	*P. aeruginosa* Number Positive	*P. aeruginosa* Percent	Other Gram-Negatives Number Positive	Other Gram-Negatives Percent
A	<100–9,300,000	132	65	49.2	16	12.1
B	36,000–165,000	16	5	31.3	0	0
C	100,000–361,000	17	15	88.2	2	11.8

90 ml. of sterile distilled water containing 1% of a surfactant[1], and 10-fold serial dilutions to 1:100,000 were prepared in sterile distilled water. One milliliter of each dilution and 1 ml. of the undiluted product were seeded into both 10 ml. of tryptic soy broth (Difco) and 10 ml. of Koser citrate medium (BBL). After 48 hr. incubation at 35°, each dilution was streaked on plates of desoxycholate citrate agar (Difco) and incubated for 24 hr. Typical colonies were transferred to triple sugar iron agar slants. All cultures giving alkaline slant and butt reactions, with or without hydrogen sulfide production, were transferred to biochemical media for identification.

The cytochrome oxidase test of Ewing and Johnson (8), glucose utilization using OF Medium (BBL) (8), growth at 42° (9), gelatin liquefication (10), arginine dihydrolase production (11), fluorescence under longwave UV light on *Pseudomonas* agar F (Difco) containing 1% glycerol, and production of pyocyanine or pyorubrin water-soluble pigments on Tech agar (BBL) (11) were performed. Difco nitrate broth containing 0.5% bacto-agar was employed for the nitrate reduction test. The 0.5% agar gave tentative motility results, which were substantiated by wet mount preparations and Bailey's flagella stain (10). Cultures giving positive reactions according to the scheme of Sutter (11) were considered to be *P. aeruginosa* (Table I).

Aerobic plate counts were performed as described elsewhere (12), using tryptic soy agar (Difco) as the plating medium.

RESULTS AND DISCUSSION

Eighty-five of the finished product samples (30.5%), all from three manufacturers but representing 24 private labels, and two in-process samples, were positive for *P. aeruginosa* (Table II). The aerobic plate-count range for all contaminated samples was from <100 to 9,300,000 organisms/g. Samples contaminated with other Gram-negative organisms had a plate-count range from <100 to 500,000 organisms/g. All samples from which no Gram-negative organisms were isolated had plate counts of <100 organisms/g.

The original cultures isolated were subjected to more extensive biochemical testing. The tests used proved to be the most valuable in identification along with the consistent presence of pyocyanine and/or pyorubrin pigment in all cultures identified as *P. aeruginosa*. These cultures were also examined by electron microscopy to determine the position of the flagella. Unidentified cultures classed as *Alcaligenes* sp. were late cytochrome oxidase positive (greater than 2 min.), did not utilize glucose, and had peritricious flagella. These were isolated from one sample of water used as a raw material and from 16 finished product samples. Coliforms fermented lactose on desoxycholate citrate agar or gave an acid reaction on triple sugar iron and were isolated from five finished product samples.

[1] Tergitol Anionic 7, Union Carbide.

contaminated samples (aerobic plate counts of <100 organisms/g.).

The effect that *P. aeruginosa* has on the composition, and possibly on the therapeutic activity, of antacid preparations still needs to be studied. No changes in color or odor were noted in the *Pseudomonas*-contaminated samples tested. However, two batch samples contaminated with coliforms had a strong ammonia-like odor rather than the characteristic odor of peppermint, suggesting that some product decomposition had occurred.

The high level of contamination with a potential pathogen in commercial antacids from a few manufacturers indicates that a need exists for control of all raw materials (including the manufacture of extracted natural materials such as magnesium hydroxide from sea water), manufacturing processes of nonsterile drugs, and finished product material.

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pH-Dependent Hydrolysis of 4,4′-Diformamidodiphenylsulfone

L. C. GARG

Abstract □ The hydrolysis of the antimalarial agent, 4,4′-diformamidodiphenylsulfone (I), was studied at five hydrogen-ion concentrations and four different temperatures. Under all conditions, 4,4′-diformamidodiphenylsulfone was first hydrolyzed to 4-amino-4′-formamidodiphenylsulfone (II) and then to 4,4′-diaminodiphenylsulfone (III) according to the reaction: $I \xrightarrow{k_1} II \xrightarrow{k_2} III$. The k_1 rate constants were greater than k_2. Both k_1 and k_2 followed apparent first-order kinetics with respect to 4,4′-diformamidodiphenylsulfone and 4-amino-4′-formamidodiphenylsulfone concentrations, respectively, and were dependent on pH in the order of $k_{pH\,2} >>> k_{pH\,8} \simeq k_{pH\,10} > k_{pH\,4} \simeq k_{pH\,6}$. Thus, at the stomach pH, extremely rapid nonenzymatic hydrolysis is to be expected. It is, however, limited by the solubility of the drug which is extremely low. 4,4′-Diformamidodiphenylsulfone was found to be most stable at pH ~6.

Keyphrases □ 4,4′-Diformamidodiphenylsulfone—pH-dependent hydrolysis □ Hydrolysis, pH dependent—4,4′-diformamidodiphenylsulfone □ TLC—reaction monitoring

4,4′-Diformamidodiphenylsulfone has a suppressive action against *Plasmodium berghei* infections in mice (1) and shows a modest repository activity (2, 3). It has an antimalarial activity similar to, but more prolonged than, 4,4′-diaminodiphenylsulfone against chloroquine-resistant strains of *Plasmodium falciparum* infections in man (4). The observed reversal of antimalarial action of 4,4′-diaminodiphenylsulfone against *P. falciparum* infections in man (5) and of 4,4′-diformamidodiphenylsulfone against *P. berghei* infections in mice (6) by *p*-aminobenzoic acid suggests that 4,4′-diformamidodiphenylsulfone acts as an antimalarial by hydrolysis to the 4,4′-diaminodiphenylsulfone or 4-amino-4′-formamidodiphenylsulfone. Therefore, a knowledge of the hydrolysis of 4,4′-diformamidodiphenylsulfone in solutions of various pH's at various temperatures seemed vital before reaching conclusions about the mechanism of its antimalarial activity. This report presents the rates of hydrolysis of 4,4′-diformamidodiphenylsulfone at several pH's and temperatures.

METHODS

Because of its low solubility in water (~10 mcg./ml.), a stock solution was prepared by dissolving 4,4′-diformamidodiphenylsulfone in dimethyl sulfoxide to make a concentration of 5 mg./ml. One- or two-milliliter portions of this solution were added to 2 or 3 ml. of buffer at pH 1.7, 4.05, 5.95, 7.85, and 9.65 (buffer compositions given in Table I), which had been kept at the relevant temperature. The ionic strength of all the buffers was kept constant by adding KCl. The final concentration of 4,4′-diformamidodiphenylsulfone was 2500 or 1250 mcg./ml. A few experiments were done starting with 4-amino-4′-formamidodiphenylsulfone in concentrations of 2500 mcg./ml. In all cases, the reactions were followed either to completion or through one half-life.

At various time intervals, 0.1-ml. aliquots were withdrawn and analyzed for free arylamine by the method of Bratton and Marshall (7) as modified in this laboratory. The method permitted the determination of 4,4′-diaminodiphenylsulfone or 4-amino-4′-formamidodiphenylsulfone in presence of 4,4′-diformamidodiphenylsulfone without any hydrolysis of 4,4′-diformamidodiphenylsulfone during analytical procedures. The method involved addition of 4.5 ml. of trichloroacetic acid–sodium citrate buffer[1] to a final 0.5-ml. volume of sample plus water.

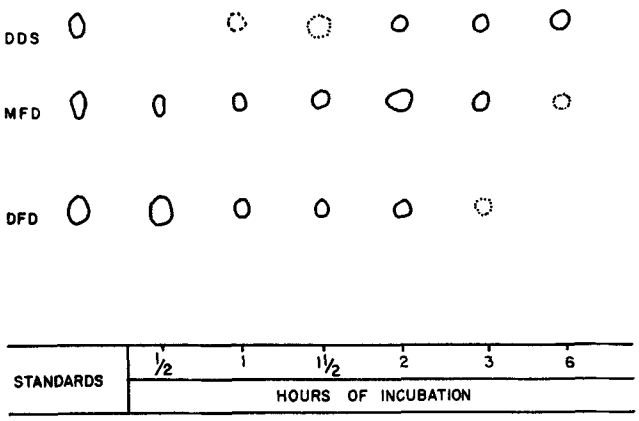

Figure 1—*TLC of 4,4′-diformamidodiphenylsulfone (DFD) (after hydrolysis in buffer of pH 1.7 at 37°) on silica gel, developed twice in ethyl acetate and seen under shortwave UV light. DDS = 4,4′-diaminodiphenylsulfone; MFD = 4-amino-4′-formamidodiphenylsulfone.*

Duplicate 2-ml. aliquots were then diazotized and coupled by adding 0.3 ml. each of 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (coupling reagent) in the order mentioned, mixing, and waiting for 1 min. after addition of each reagent. The reaction mixtures were then allowed to stand at room temperature for 10 min. One milliliter of 100% ethanol was added, and the absorbance was measured at 540 nm., using 1-cm. lightpath. The method was sensitive enough to detect 1.5 mcg. of 4,4′-diaminodiphenylsulfone per sample. Total arylamines were determined after boiling for 45 min. 0.1-ml. aliquots of the incubation mixtures in 0.2 ml. of 5 *N* HCl, 1 ml. of 15% trichloroacetic acid, and 3.7 ml. water. This procedure hydrolyzed all the 4,4′-diformamidodiphenylsulfone and 4-amino-4′-formamidodiphenylsulfone to 4,4′-diaminodiphenylsulfone. Duplicate 2-ml. aliquots of the hydrolyzed samples were diazotized and coupled, and the A_{540} was determined as previously described. The detailed discussion of the methods as applied to the mixtures of 4,4′-diformamidodiphenylsulfone 4-amino-4′-formamidodiphenylsulfone, and 4,4′-diaminodiphenylsulfone in aqueous solutions, urine, or plasma will be published (8, 9).

The hydrolysis of 4,4′-diformamidodiphenylsulfone was monitored by TLC using silica gel-coated glass plates (F254). After spotting the samples, the plates were run twice to the same mark in ethyl acetate with intermittent drying. This gave a better separation of hydrolytic products. The resulting separation of hydrolytic products was observed under shortwave UV light. The dried plates were then sprayed with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde, HCl) which produced yellow spots, reacting with free amine of 4,4′-diaminodiphenylsulfone and 4-amino-4′-formamidodiphenylsulfone. A light-yellow color was also seen at 4,4′-diformamidodiphenylsulfone spots, probably due to deformylation of tiny amounts of 4,4′-diformamidodiphenylsulfone releasing free amine with the Ehrlich reagent spray. Standard solutions of 4,4′-diaminodiphenylsulfone, 4-amino-4′-formamidodiphenylsulfone, and 4,4′-diformamidodiphenylsulfone were similarly treated along with test samples for the purpose of identification of hydrolytic products of 4,4′-diformamidodiphenylsulfone.

[1] Trichloroacetic acid, 245 g., and sodium citrate, 163 g., dissolved in distilled water and the volume adjusted to 500 ml.; 40 ml. of 1% polysorbate 80 (Tween 80) added and pH adjusted, if necessary, to 2.35 with 60% trichloroacetic acid.

Table I—Conditions, Observed[a] First-Order Rate Constants, and Energy of Activation for the Hydrolysis of 4,4′-Diformamidodiphenylsulfone[b] in Various Buffers at Different Temperatures

Buffer Composition[c]	Buffer pH	$10^5 k$ in min.$^{-1}$ at Temperatures 25° k_1	25° k_2	37° k_1	37° k_2	60° k_2	70° k_2	80° k_2	ΔH_a for k_2, kcal. mole^{-1}
25 ml. of 0.2 *M* KCl 6.5 ml. 0.2 *M* HCl	1.7	920	460	—	1160 1200[e]	6000	12,000	25,000	14.186
10 ml. of 0.5 *M* CH_3COONa 10 ml. of 2 *M* CH_3COOH	4.05	—	1.3[d]	6.2	3	13	24	40	12.886
25 ml. of 0.1 *M* KH_2PO_4 2.8 ml. of 0.1 *M* NaOH	5.95	—	1.1[d]	4.7	2.4	10	17	27	12.039
25 ml. of 0.1 *M* KH_2PO_4 23 ml. of 0.1 *M* NaOH	7.85	—	9.9[d]	37	18 16[f]	69	116	190	11.669
25 ml. of 0.05 *M* $NaHCO_3$ 5.3 ml. of 0.1 *M* NaOH	9.65	—	5.1[d]	23	11	42	73	120	12.035

[a] Unless otherwise stated. [b] 2.5 mg./ml. in 50% dimethyl sulfoxide unless otherwise stated. [c] Ionic strength μ adjusted to 1.4 with KCl. [d] Calculated from ΔH_a values. [e] Hydrolysis was started with 4-amino-4′-formamidodiphenylsulfone. [f] Using 1250 mcg./ml. of 4,4′-diformamidodiphenylsulfone in 25% dimethyl sulfoxide.

RESULTS AND DISCUSSION

TLC analysis of the reaction mixture at various time intervals showed that 4,4′-diformamidodiphenylsulfone (I) is initially deformylated to 4-amino-4′-formamidodiphenylsulfone (II), which is then deformylated to 4,4′-diaminodiphenylsulfone (III) according to the reaction: $\text{I} \xrightarrow{k_1} \text{II} \xrightarrow{k_2} \text{III}$.

A typical TLC tracing is shown in Fig. 1. R_f values of 4,4′-diformamidodiphenylsulfone, 4-amino-4′-formamidodiphenylsulfone, and 4,4′-diaminodiphenylsulfone were found to be 0.29, 0.53, and 0.7, respectively, under these conditions.

The logarithm of the difference in A_{540} for free amine at time t (A_t) and at time of complete hydrolysis (A_∞) was plotted against time From the equation:

$$\log (A_\infty - A_t) = \frac{-k \cdot t}{2.303} + \log A_\infty \qquad \text{(Eq. 1)}$$

k_1 was calculated taking the data of the time period before appearance of any 4,4′-diaminodiphenylsulfone (as seen by TLC). Similarly, k_2 was calculated using the data of the time period after disappearance of 4,4′-diformamidodiphenylsulfone from the incubation mixture (as seen by TLC). A_∞ for k_2 was the A_{540} due to the complete hydrolysis of the reaction mixture starting with 4,4′-diformamidodiphenylsulfone. A_∞ for k_1 was taken as half of A_∞ for k_2, because only one end group is removed during the initial reaction period. In some cases, the rate constants k_2 were also obtained in reaction mixtures starting with 4-amino-4′-formamidodiphenylsulfone.

The conditions and the first-order rate constants, k_1 and k_2, in buffers of various pH's at various temperatures are given in Table I. The rate constants k_1 were found to be two times k_2 (Table I). The overall rate constant, k', for conversion of 4,4′-diformamidodiphenylsulfone $\xrightarrow{k'}$ 4,4′-diaminodiphenylsulfone could not be determined accurately due to the limitation of the analytical method. By using this method, 4-amino-4′-formamidodiphenylsulfone concentration could be determined in the presence of 4,4′-diformamidodiphenylsulfone. Similarly, 4,4′-diaminodiphenylsul-

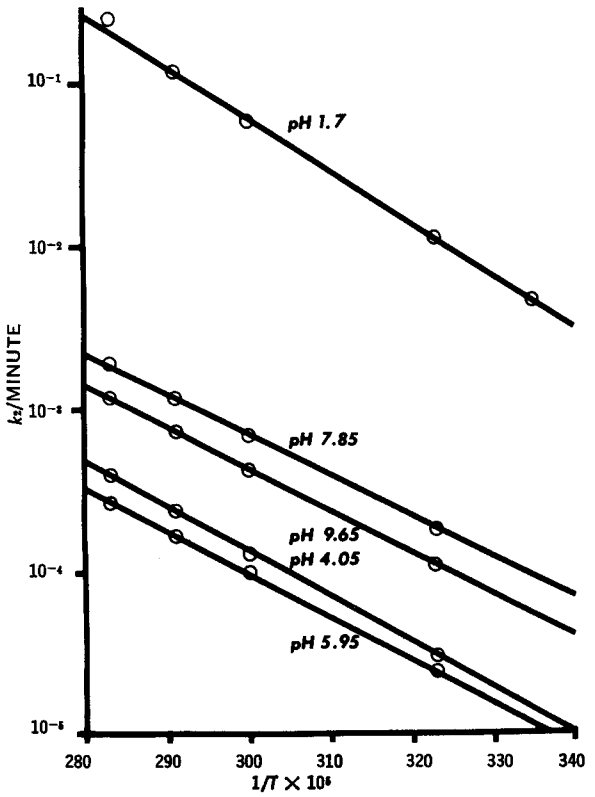

Figure 2—*Arrhenius plots;* k_2 *for 4,4′-diformamidodiphenylsulfone → 4,4′-diaminodiphenylsulfone as a function of pH.*

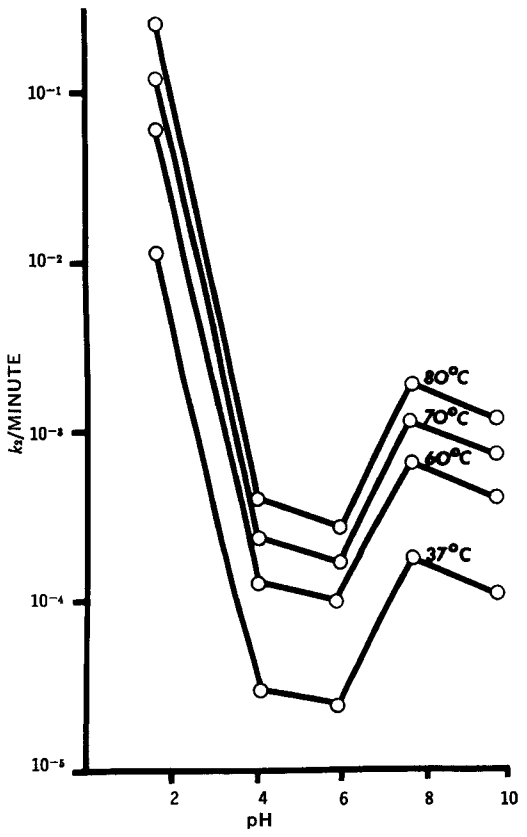

Figure 3—*Log* k_2*-pH profiles for the hydrolysis of 4,4′-diformamidodiphenylsulfone at various temperatures.*

fone concentration could be determined in the presence of 4-amino-4′-formamidodiphenylsulfone or 4,4′-diformamidodiphenylsulfone. However, the concentrations of 4,4′-diaminodiphenylsulfone, 4-amino-4′-formamidodiphenylsulfone, and 4,4′-diformamidodiphenylsulfone cannot be determined in a mixture of all three. As $k_1 > k_2$, then k' would be practically the same as the slower rate constant k_2.

The logarithmic version of the Arrhenius expression is:

$$\log k_2 = \log P - \frac{\Delta H_a}{2.303R} \cdot \frac{1}{T} \qquad \text{(Eq. 2)}$$

where ΔH_a is the energy of activation in cal. mole^{-1}, R is 1.987 cal. deg.$^{-1}$ mole^{-1}, T is the absolute temperature, and log P is the intercept of the plot of log k_2 against the reciprocal of the absolute temperature. The observed k_2 rate constants are given in Table I. The Arrhenius plots for the k_2 rate constants at various pH's are shown in Fig. 2. The ΔH_a values obtained from the slopes of Arrhenius plots for k_2 rate constants are listed in Table I. The k_2 rate constants at 25° at all pH's except pH 1.7 were calculated from the Arrhenius expression and are also included in Table I.

The log k_2–pH profiles for the hydrolysis of 4,4′-diformamidodiphenylsulfone at several temperatures are given in Fig. 3. At low pH (1.7), which is comparable to stomach pH, 4,4′-diformamidodiphenylsulfone is very rapidly hydrolyzed. At pH 4–6 the hydrolysis of 4,4′-diformamidodiphenylsulfone is much slower than at pH 1.7 (about 1/500). The hydrolysis is, however, enhanced at pH 7.85 and is about 5 times faster than at pH 4–6. For unknown reasons, at pH 9.65 the hydrolysis appeared to slightly decrease again. The results indicate that the hydrolysis of 4,4′-diformamidodiphenylsulfone is affected by pH and is first order with respect to 4,4′-diformamidodiphenylsulfone concentration. It may also be first order with respect to H^+-ion concentrations. 4,4′-Diformamidodiphenylsulfone is most stable at pH $\sim$ 6. Another arylamine substituted compound, *N*-acetyl-*p*-aminophenol, shows a similar log *k*–pH profile with a most stable pH $\sim$ 6 (10).

The present results agree with the *in vitro* and *in vivo* results reported by Gleason and Vogh (11) on the enzymic hydrolysis of 4,4′-diformamidodiphenylsulfone in mouse plasma. In their experiments, analysis of plasma showed a 4,4′-diformamidodiphenylsulfone undergoing a stepwise hydrolysis to 4,4′-diaminodiphenylsulfone through 4-amino-4′-formamidodiphenylsulfone as an intermediate.

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Drug Biotransformation Interactions in Man V: Acetaminophen and Salicylic Acid

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Previous studies in this series revealed mutual inhibitory effects in the biotransformation of acetaminophen and salicylamide to their respective glucuronides and sulfates (1), and of salicylic acid and salicylamide to their glucuronides (2). There was also some indication of an inhibitory effect of salicylate on the formation of salicylamide sulfate (2). To elucidate further the biotransformation interactions of these widely used analgesics and antipyretics, the effect of salicylate on the pharmacokinetics of acetaminophen was studied in normal adults. The results of this investigation will help to identify the major determinant in the previously described interactions, since each of the three interacting drugs has now been studied in the presence of the two others.

In addition to these mechanistic considerations, there has been some concern about a trend among pediatricians to prescribe full doses of aspirin and acetaminophen every 4 hr. so that the patient receives one or the other drug every 2 hr. (3). Acetaminophen is eliminated mainly by the formation of its glucuronide and sulfate (1). Salicylic acid is also subject to glucuronidation, and it inhibits the formation of at least some types of sulfates (2, 4, 5). Concern about the possible consequences of the

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Table I—Verification of Assay Method for Acetaminophen and Its Metabolites and for Salicylic Acid and Its Metabolites in Urine Samples Containing Both Groups of Substances

Metabolite[a]	Sample	Concentrations, as mg. % APAP or SA Equivalent			
		A: Urine after Salicylate Administration	B: Urine after Acetaminophen Administration	Equal Parts, A and B[b]: Assay	Equal Parts, A and B[b]: Theoretical
SA	1	1.6	0.0	0.8	0.8
	2	1.0	0.0	0.5	0.5
	3	1.3	0.0	0.7	0.7
SAG	1	16.6	0.0	8.3	8.3
	2	14.1	0.0	7.2	7.1
	3	18.6	0.0	9.3	9.3
SU	1	40.7	0.0	20.7	20.4
	2	42.5	0.0	20.5	21.3
	3	45.9	0.0	22.0	23.0
APAP	4	0.0	4.2	2.2	2.1
	5	0.0	2.0	1.1	1.0
	6	0.0	4.3	2.2	2.2
APAPG	4	0.0	17.5	8.8	8.8
	5	0.0	32.2	16.0	16.1
	6	0.0	68.7	35.1	34.4
APAPS	4	0.0	17.8	8.7	8.9
	5	0.0	22.1	11.3	11.1
	6	0.0	38.0	18.1	19.0

[a] Abbreviations: SA, salicylic acid; SAG, salicylic acid glucuronide; SU, salicyluric acid; APAP, acetaminophen; APAPG, acetaminophen glucuronide; and APAPS, acetaminophen sulfate. [b] Equal volumes of A and B were mixed in the following manner: 1 + 4, 2 + 5, 3 + 6.

concomitant use of full doses of acetaminophen and salicylate was therefore justified in principle and made a formal pharmacokinetic investigation desirable.

EXPERIMENTAL

Five healthy male volunteers, 27–33 years old, participated in this study. They received oral doses of 1 g. acetaminophen in one test, 1 g. acetaminophen and salicylic acid (1 g. 2 hr. before and 0.5 g. 4 hr. after acetaminophen) in the second test, and a total of 1.5 g. salicylic acid (1 g. initially and 0.5 g. 6 hr. later) in the third test. The drugs were given in aqueous solution (salicylic acid as sodium salicylate) in the morning on an empty stomach. Food was withheld for at least 2 hr. The tests were carried out in random order at approximately 1-week intervals. Urine was collected by three subjects every 0.5 hr. for 4 hr., every hour for 8–10 hr. thereafter, and then at longer intervals for a total of 24 hr. (acetaminophen alone) or 48 hr. (tests with salicylate). Two additional subjects collected urine at longer and irregular intervals. Their data are, therefore, not shown in the figures.

Acetaminophen and its metabolites in the urine were determined by the method described by Levy and Yamada (1) for the determination of acetaminophen and its metabolites in the presence of salicylamide, with the following modifications: (*a*) isoamyl acetate (analytical grade, Fisher) rather than ether was used for the extractions, since some manufacturers' ether, even if purified by repeated washing, interfered in the assay; (*b*) the borate buffer was saturated with sodium chloride and was prepared by adding hydrochloric acid to 0.16 *M* sodium tetraborate to apparent pH 8.0; and (*c*) all enzymatic hydrolyses were carried out for 16 hr. When separating the glusulase incubation mixture and isoamyl acetate by centrifuging, a gel phase formed at the aqueous–organic interphase. This gel was mixed gently into the aqueous phase with a stirring rod, and the system was then recentrifuged, resulting in a clean separation of the phases.

Total acetaminophen (free and conjugated drug) by acid hydrolysis was determined by adding 1 ml. concentrated hydrochloric acid and 2 ml. water to 3 ml. urine and heating this solution for 2.5 hr. at 100°. After cooling, water was added to yield a total volume of 10 ml. Five grams potassium phosphate (dibasic) was added to 5 ml. of the solution, and this was extracted with 10 ml. ethylene dichloride. Five milliliters of the organic phase was extracted with 5 ml. 2 *N* hydrochloric acid. To 1 ml. of the aqueous phase was added 10 ml. of phenol–hypobromite mixture (6), and the absorbance of this solution was determined at 625 nm. after 40 min. of color development.

Salicylic acid and its metabolites were determined as described by Levy and Procknal (2). Assay of acetaminophen and its metabo-

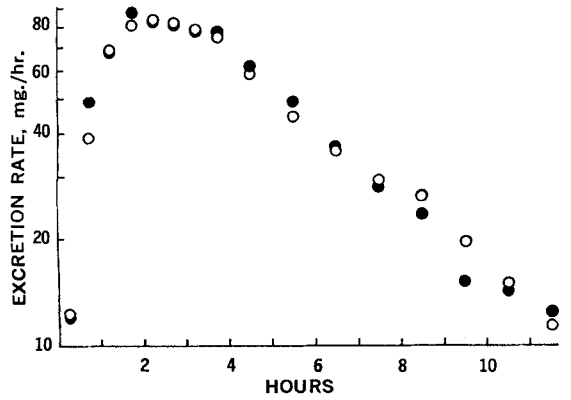

Figure 1—*Excretion rate of acetaminophen glucuronide (in terms of acetaminophen) as a function of time after oral administration of 1 g. acetaminophen alone (●) and with salicylate (○). Average of Subjects A, B, and C.*

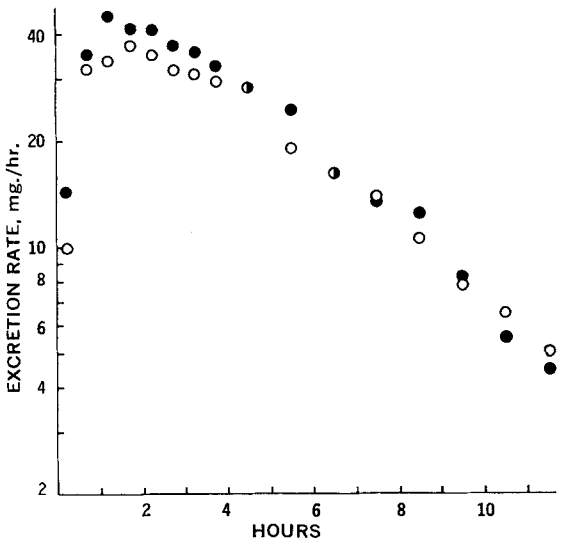

Figure 2—*Excretion rate of acetaminophen sulfate (in terms of acetaminophen) as a function of time after oral administration of 1 g. acetaminophen alone (●) and with salicylate (○). Average of Subjects A, B, and C.*

Table II—Effect of Salicylate on Biotransformation and Biologic Half-Life of Acetaminophen in Man[a]

Subject	Amount (and Percent)[b] Excreted, mg. APAP Equivalent										Half-Life, $t_{1/2}\beta$, hr.	
	Free APAP		APAPG		APAPS		Total (Glusulase)		Total (Acid)			
	Control	With SA	Control	With SA	Control	With SA	Control	With SA	Control	With SA	Control	With SA
A	18 (2.0)	29 (3.4)	685 (77.0)	681 (80.3)	187 (21.0)	138 (16.3)	890	848	878	849	2.2	2.2
B	33 (4.0)	50 (6.0)	497 (60.0)	518 (62.3)	299 (36.0)	263 (31.7)	829	831	862	878	2.8	2.8
C	15 (1.8)	23 (2.7)	484 (58.0)	464 (55.0)	335 (40.2)	357 (42.3)	834	844	803	846	2.5	2.5
D	26 (3.0)	24 (2.8)	590 (72.6)	640 (74.5)	199 (24.4)	195 (22.7)	813	859	815	827	1.6	2.2
E	32 (4.0)	25 (3.1)	593 (73.4)	583 (73.0)	183 (22.6)	194 (23.9)	808	802	799	827	1.8	1.8
Mean	25 (3.0)	30 (3.6)	570 (68.2)	577 (69.0)	241 (28.8)	229 (27.4)	835	837	831	845	2.2	2.3

[a] Abbreviations as in Table I. [b] Percent data are based on total recovery by glusulase hydrolysis and are listed in parentheses.

lites in urine samples obtained after acetaminophen administration, assay of salicylate and its metabolites in urine samples obtained after salicylate administration, and reassay of these substances in combined acetaminophen and salicylate urines showed that there was no interference by any of the substances in the analysis of the others (Table I).

RESULTS AND DISCUSSION

The time course of acetaminophen glucuronide excretion after acetaminophen administration was not affected by salicylate (Fig. 1). There was also no apparent effect on the time course of acetaminophen sulfate excretion (Fig. 2). Despite the very limited excretion of free acetaminophen (about 3% of the dose), semilogarithmic plots of free acetaminophen excretion rates *versus* time yielded a terminal linear (β-phase) segment which reflects the elimination of this drug from the body (Fig. 3). The average half-life of the β-phase was not affected by salicylate (Fig. 3 and Table II). Rapid absorption of acetaminophen by some subjects in some experiments also revealed an initial distributive phase as described previously (1).

Salicylate had no apparent effect on the terminal composition of urinary excretion products of acetaminophen and on the total recovery of this drug and its metabolites. Total acetaminophen was determined by enzymic (glusulase) as well as acid hydrolysis; the average results obtained by these methods differed by less than 1%. The three subjects represented in Fig. 3 excreted more free acetaminophen with salicylate than in the control experiments, but this was not the case with the other two subjects (Table II).

The dosage regimen of salicylate was designed to maintain relatively constant body levels of this drug over 12 hr. Since about half of a rapidly absorbed 1-g. dose of salicylate is eliminated by normal adults in 6 hr. (7), 1 g. was given initially and 0.5 g. was given 6 hr. later. Acetaminophen did not inhibit the formation and excretion of salicylurate and salicylic glucuronides (Fig. 4).

This investigation revealed no apparent interaction in the biotransformation of acetaminophen and salicylate. This does not rule out the possibility of interactions at higher doses or at elevated body levels attained after repeated dosings of these drugs. However, the ability of acetaminophen and salicylate to inhibit glucuronide and sulfate formation is clearly much less than that of equivalent doses of salicylamide, as is evident upon a review of the previous investigations in this series (1, 2). The pronounced competitive

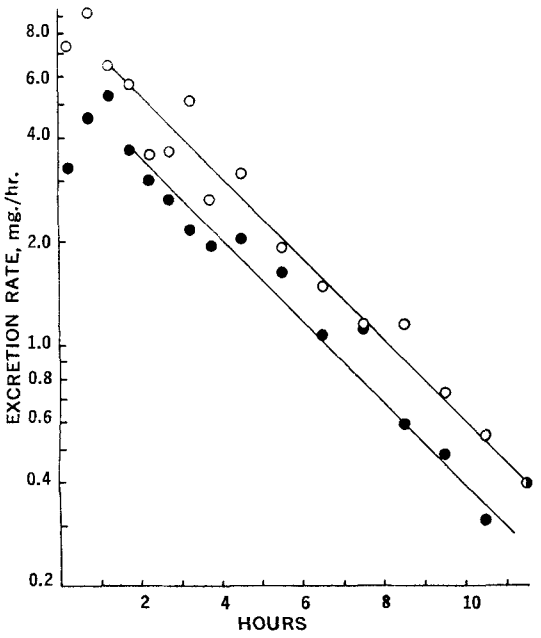

Figure 3—*Excretion rate of free acetaminophen as a function of time after oral administration of 1 g. acetaminophen alone* (●) *and with salicylate* (○). *Average of Subjects A, B, and C.*

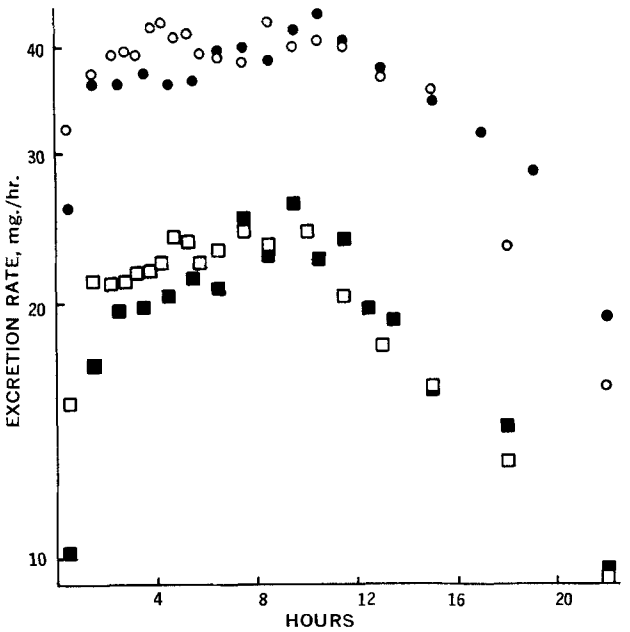

Figure 4—*Excretion rate of salicyluric acid (circles) and salicylic glucuronides (squares) after oral administration of 1 g. salicylic acid at zero time and 0.5 g. at 4 hr. All data are expressed in terms of salicylic acid. Closed symbols, control experiment; open symbols, with 1 g. acetaminophen given at 2 hr. Average of Subjects A, B, and C.*

inhibitory effect of salicylamide on glucuronide and sulfate formation is most likely due to its very rapid metabolism. The nonlinear nature of salicylamide elimination prevents comparisons of formation-rate constants, but an admittedly approximate estimation based on available data (1, 8, 9) indicates that the formation of salicylamide glucuronide and sulfate proceeds about 10 times more rapidly than the formation of acetaminophen glucuronide and sulfate at body levels of 1 g. of these drugs. It is now evident that of the several combinations of acetaminophen, salicylate, and salicylamide studied in this laboratory, salicylamide is the major determinant in the biotransformation interactions encountered (1, 2).

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Keyphrases □ MAO activity—human liver, normal, cirrhotic, noncirrhotic abnormal, comparison □ Enzyme activity—human liver, normal cirrhotic, noncirrhotic abnormal, comparison □ Liver, human, normal, cirrhotic, noncirrhotic abnormal—determination, comparison of MAO activity

It is difficult to classify diseases of the liver since pathological conditions affecting the liver may arise from cell degenerative process, circulatory disturbance, necrosis, and tumor formation. Liver diseases can be characterized, however, by careful histological study of the tissue. Cirrhosis of the liver is best characterized by distorted reconstruction of lobules throughout the entire liver or in a considerable part of it. One widely believed theory concerning cirrhosis states that the degeneration of the liver cells results from an accumulation of toxic amines in the organ to a level that exceeds the deamination ability of the metabolic processes (1). The accumulation of toxic amines may be due to the lack of an adequate MAO activity. The purpose of this investigation was to study the activity of this enzyme system in normal, cirrhotic, and noncirrhotic but abnormal human livers.

EXPERIMENTAL

Human liver specimens were obtained from autopsy as well as by biopsy from hospital patients[1]. Liver specimens were stored in the freezer as soon as they were obtained. The specimens preserved in cold showed reproducible results (2). The isolation technique of liver mitochondria was similar to that reported by Hogeboom *et al.* (3).

In this procedure, carried out in a cold atmosphere, an approximately 0.7-g. portion of fresh or frozen liver was chopped into small pieces with a dissecting scissor onto a watch glass and transferred to a tared glass homogenizing tube; the exact weight of the sample was recorded. Homogenization of this chopped tissue was carried out in 5 ml. of 0.88 *M* sucrose solution for 5 min. The homogenate was quantitatively transferred to a 50-ml. polyethylene tube. The homogenizing tube and pestle were thoroughly rinsed with three or four portions of 0.88 *M* sucrose solution, and the rinses were added to the polyethylene tube containing liver homogenate. The final total volume of homogenate, representing approximately 20 ml., was then centrifuged (cold room 0–5°) at $600\times g$ for 10 min., and the supernatant fraction containing mitochondria and microsomes was transferred by means of a pipet into a 50-ml. polyethylene tube. The supernatant containing mitochondria was centrifuged at $24{,}000\times g$ for 20 min. to sediment the mitochondria, and the supernatant portion containing microsomes was discarded.

The solid mass of mitochondria was resuspended in 20 ml. of 0.88 *M* sucrose solution and centrifuged at $24{,}000\times g$ for an additional 20 min. This supernatant fraction was discarded. The packed mass of mitochondria was quantitatively transferred into a homogenizing tube, and the suspension of mitochondria in phosphate buffer pH 6.9 (0.5 *M*) was facilitated by careful homogenization. The mitochondrial solution was then transferred into a 10-ml. volumetric flask, the homogenizing tube was carefully rinsed with phosphate buffer, and the volume was made to the mark with

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Table I—Normal Human Livers

Number	Subject	Sex[a]	Age	Liver[b] Specimen	mg. N_2/g. Liver	mg. Mitochondrial N_2/g. Liver	MAO Activity Expressed as: MAO Units[c]	MAO Units/Liver Nitrogen	MAO Units/Mitochondrial Nitrogen
1	R.D.	M	28 days	a	28.10	3.21	444	15.80	138.3
2	S.Mc.	F	2½ yr.	a	29.30	3.81	1381	47.15	362.5
3	D.C.	F	4 yr.	a	30.05	4.16	2170	35.34	255.2
4	S.V.	M	5½ yr.	a	39.75	5.16	2787	70.11	540.1
5	L.J.	F	8 yr.	a	28.46	2.91	972	34.15	340.2
6	J.W.	F	15 yr.	a	34.40	—	2649	77.00	—
7	L.H.	M	18 yr.	b	32.70	4.00	2715	83.02	678.2
8	C.H.	M	19 yr.	a	32.70	5.13	3150	96.21	614.0
9	B.S.	F	26 yr.	a	—	5.59	2880	—	515.2
10	T.B.	M	31 yr.	a	30.98	4.27	2640	85.21	618.3
11	A.T.	M	41 yr.	a	27.08	4.50	2520	93.05	560.0
12	M.G.	F	46 yr.	a	32.65	5.23	2580	80.59	493.9
13	M.B.	M	47 yr.	a	26.13	4.15	1968	74.80	478.2
14	M.K.	F	47 yr.	b	—	3.86	1892	—	490.2
15	C.J.	M	48 yr.	a	32.90	4.16	1924	59.40	462.5
16	O.M.	M	49 yr.	a	27.07	4.16	1087	40.15	261.3
17	R.D.	M	49 yr.	a	26.17	4.87	1358	51.89	280.0
18	W.P.	M	57 yr.	b	27.90	—	1852	66.37	—
19	S.R.	F	59 yr.	b	34.45	4.16	2805	81.42	674.3
20	R.S.	M	60 yr.	a	32.75	4.92	1845	52.33	375.0
21	C.W.	M	63 yr.	a	32.55	3.45	1380	42.39	400.0
22	S.M.	M	64 yr.	a	31.25	5.28	2327	74.43	440.5
23	W.F.	M	64 yr.	a	34.40	—	1800	52.32	—
24	B.H.	F	65 yr.	a	29.50	4.32	1897	64.37	439.6
25	C.B.	M	65 yr.	a	27.65	4.18	1253	45.31	299.8
26	E.W.	M	66 yr.	a	33.65	3.88	1713	50.90	441.5
27	A.Mc.	F	68 yr.	a	31.56	3.90	1704	53.99	436.9
28	L.B.	M	69 yr.	b	—	—	2565	—	—
29	W.T.	M	70 yr.	a	—	—	2527	—	—
30	C.T.	M	76 yr.	a	29.15	3.91	2037	69.87	520.9
31	C.D.	M	78 yr.	b	—	5.27	4493	—	852.6
32	E.Mc.	M	84 yr.	b	35.40	5.12	3070	86.72	599.6
33	M.T.	F	86 yr.	a	26.18	—	834	31.85	—
34	P.E.	M	94 yr.	a	27.15	4.17	1665	60.83	399.3
				Mean	30.78	4.37	2085	61.21	463.1
				SD	± 3.28	±0.70	± 785	±19.92	±144.0

[a] M = male, F = female. [b] a = autopsy, b = biopsy. [c] One unit is amount of MAO causing the disappearance of 1 mcg. tyramine/g. tissue/hr.

additional buffer solution. The enzyme activity of a 1.0-ml. aliquot of a mitochondrial solution was determined by the method of Sjoerdsma *et al.* (4), a method based on the colorimetric determination of substrate (tyramine) disappearance.

MAO activity was expressed on the basis of liver nitrogen as well as mitochondrial nitrogen. Nitrogen determination of liver was carried out by the semimicro Kjeldahl procedure using 100.0 mg. of sample. Nitrogen determination of mitochondrial solution was performed by the semimicro Kjeldahl procedure using 3.0 ml. of the aliquot solution. The results of the analysis are shown in Tables I–III. Statistical evaluation was by the Student *t* test.

RESULTS AND DISCUSSION

The data for 49 liver specimens are shown in Tables I–III, representing analysis of normal livers, cirrhotic livers, and noncirrhotic abnormal livers, respectively. It is difficult to state that a given specimen of biological material is normal. However, by common agreement, the histological appearance of biological material is used to establish normal, cirrhotic, and noncirrhotic abnormal categories. Livers showing hepatitis, fatty degeneration, and malignant changes were included in samples classified as noncirrhotic abnormal liver. Neither sex, age, nor mode of obtaining specimens (autopsy or biopsy) seemed to affect the results, so these parameters were ignored in evaluating the data.

Nitrogen content of normal, cirrhotic, and noncirrhotic abnormal human livers did not differ, and the values agree with the literature value of 27.3 mg./g. liver (5). However, data for the N_2 content of mitochondria of human liver have not been published. Various values of N_2 content of mitochondria of rat liver have been reported, depending upon the sedimentation technique. Liver mito-

Table II—Cirrhotic Human Livers

Number	Subject	Sex[a]	Age, yr.	Liver[b] Specimen	mg. N_2/g. Liver	mg. Mitochondrial N_2/g. Liver	MAO Activity Expressed as: MAO Units[c]	MAO Units/Liver Nitrogen	MAO Units/Mitochondrial Nitrogen
1	D.B.	F	9	b	—	—	135	—	—
2	D.V.	M	14	a	29.65	3.11	510	17.20	164.0
3	R.B.	F	26	b	29.44	—	536	18.20	—
4	E.O.	M	54	b	—	—	105	—	—
5	T.W.	M	59	a	22.85	—	203	10.72	—
6	E.H.	F	62	a	33.35	4.72	436	13.07	92.4
7	C.B.	M	62	a	28.09	1.98	934	33.26	477.2
				Mean	28.68	3.27	408	18.49	244.5
				SD	± 3.39	±0.35	±270	± 7.87	±160.0
							$p<0.001$	$p<0.001$	$p<0.05$

[a] M = male, F = female. [b] a = autopsy, b = biopsy. [c] One unit is amount of MAO causing the disappearance of 1 mcg. tyramine/g. tissue/hr.

Table III—Noncirrhotic Abnormal Human Livers

Number	Subject	Sex[a]	Age, yr.	Liver[b] Specimen	mg. N_2/ g. Liver	mg. Mitochondrial N_2/ g. Liver	MAO Activity Expressed as— MAO Units[c]	MAO Units/ Liver Nitrogen	MAO Units/ Mitochondrial Nitrogen
1	M.M.	F	11	a	29.56	4.56	1200	40.59	263.1
2	P.P.	F	31	a	21.19	3.40	1260	59.46	370.6
3	C.W.	M	32	a	31.70	4.79	2055	64.82	429.0
4	F.Z.	M	44	a	25.58	5.27	1449	56.64	274.9
5	J.D.	M	63	a	32.86	4.08	1875	57.06	495.5
6	L.C.	M	64	a	26.00	2.93	1155	44.42	394.2
7	G.W.	M	65	a	32.30	1.17	None[d]	None[d]	None[d]
8	P.W.	M	77	a	29.45	4.65	1320	44.82	283.9
				Mean	28.59	3.85	1473	52.54	358.9
				SD ±	3.77	±1.24	±326.3	± 8.48	± 81.6
							$p<0.10$	$p<0.30$	$p<0.10$

[a] M = male, F = female. [b] a = autopsy, b = biopsy. [c] One unit is amount of MAO causing the disappearance of 1 mcg. tyramine/g. tissue/hr. [d] Excluded in calculating the mean value.

chondria sedimented four times have been shown to have an N_2 value of 4.8 mg./g. liver (3), which also agrees well with the value found in this study. Unfortunately, the authors have values for mitochondrial N_2 in only three cirrhotic livers and cannot determine if the values differ from normal.

MAO activity of cirrhotic human liver is 80% less than that of normal liver if based on liver weight. That of noncirrhotic abnormal liver is 35% less than the normal liver when expressed similarly. These values further agree when MAO activity is expressed on the basis of liver N_2 as well as mitochondrial N_2.

Various enzyme activities such as esterase (7, 8) and oxidase (9) have been reported to be reduced in damaged hepatic tissue. In contrast, alkaline phosphatase activity has been shown higher in all types of hepatic injury (10). This study's data add one more example of a decrease in the liver enzyme activity in cirrhotic and noncirrhotic abnormal human livers. The deoxyribonucleic acid content of the same cirrhotic human liver has been reported to be 80% higher than the normal liver (11). The fall of hepatic MAO activity and rise in deoxyribonucleic acid content constitute a sensitive index of cirrhosis of human liver. Rats fed with pantothenic acid-deficient diet have also shown low MAO activity and high deoxyribonucleic acid content. However, those rats do not develop cirrhosis (12). Therefore, cirrhosis cannot be implicated as a cause of decreased MAO activity.

This study indicates 80 and 35% less MAO activity in cirrhotic liver and noncirrhotic abnormal liver, respectively, as compared to the normal liver. Nitrogen content of whole liver and mitochondria remain essentially constant in normal and pathological conditions.

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Hypocholesterolemic Agents I: 3-Methyl-4-phenylbutenoic Acids

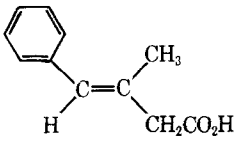
SHARON G. BOOTS, MARVIN R. BOOTS, and KENNETH E. GUYER

Abstract □ Three isomers of 3-methyl-4-phenylbutenoic acid were examined as potential inhibitors of yeast β-hydroxy-β-methylglutaryl-CoA reductase. All were shown to be inactive when assayed over a concentration range of 8–17 m*M*.

Keyphrases □ 3-Methyl-4-phenylbutenoic acid isomers—tested as β-hydroxy-β-methylglutaryl-CoA reductase inhibitors □ β-Hydroxy-β-methylglutaryl-CoA reductase inhibitors—3-methyl-4-phenylbutenoic acid isomers tested

A recent report by Porcellati *et al.* (1) provided evidence for the inhibition of cholesterol biosynthesis (*in vitro*) by β-benzal butyric acid (BBA) at a site located between acetate and mevalonate. Inhibition of acetyl-CoA:ligase (E.C.6.2.1.1) and acetyl-CoA acetyl transferase (E.C.2.3.1) reactions was reported (1) to be absent except at high concentrations.

In an effort to locate the specific site of inhibition, the effects of BBA and its double-bond isomers (I and II) on yeast β-hydroxy-β-methylglutaryl-CoA reductase (HMG-CoA reductase, E.C.1.1.1.34) were investigated.



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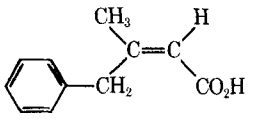

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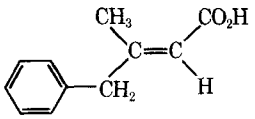


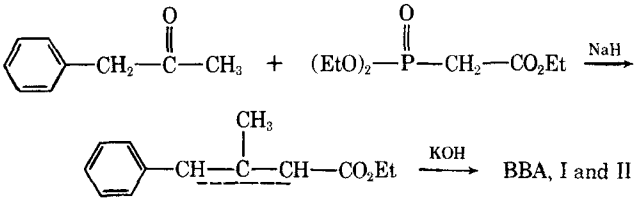
II

The synthetic scheme for the butenoic acids is presented in Scheme I.

Treatment of phenylacetone with triethyl phosphonacetate afforded a mixture of the desired α,β- and β,γ-unsaturated esters. Saponification of the mixture and chromatography on a silicic acid column yielded the desired pure acids.

EXPERIMENTAL[1]

Chemical Synthesis—*Preparation of BBA and* cis-(*I*)- *and* trans-(*II*)-*3-Methyl-4-phenyl-2-butenoic Acids*—The procedure of Wadsworth and Emmons (2) was used. To a cooled suspension of 2.4 g. of 50% sodium hydride (0.05 mole) in 100 ml. of dimethoxyethane (distilled from lithium aluminum hydride) was added dropwise with stirring 11.2 g. (0.05 mole) of triethyl phosphonoacetate. The temperature was maintained below 30° during the addition, and the mixture was stirred an additional 45 min. after the addition was complete. To this mixture was added dropwise 6.7 g. (0.05 mole) of phenyl-2-propanone, maintaining the temperature below 30°. The reaction mixture was stirred for 30 min. at 25°, and then 20 ml. of water was added. The mixture was extracted with 300 ml. of ether, washed with water, washed with a saturated sodium chloride solution, and then dried over anhydrous sodium sulfate.



Scheme I

The solvent was removed under reduced pressure to give a colorless liquid, which was distilled, b.p. 118° (1 mm.), to afford 5.3 g. of a mixture of esters which was not characterized but was saponified directly.

To this mixture of esters were added 3.0 g. of 85% potassium hydroxide pellets, 30 ml. of methanol, and 15 ml. of water. The mixture was heated under reflux for 4 hr.; then the methanol was removed under reduced pressure. The mixture was extracted with ether. The basic aqueous phase was acidified with 5% hydrochloric acid solution and then was extracted with ethyl acetate. The organic phase was washed with water, washed with a saturated sodium chloride solution, and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give 2.5 g. of a colorless oil (0.014 mole), 28%.

The colorless oil was chromatographed on 60 g. silicic acid (Table I). Fractions 4 and 5 were combined and rechromatographed on 40 g. of silicic acid (Table II). Fractions 8 and 9 were combined and recrystallized twice from petroleum ether, b.p. 60–75°, to give 130 mg. of BBA as white plates, m.p. 111–112°, lit. (3) m.p. 112–113°.

Fractions 7 and 8 from the second column were combined and recrystallized from petroleum ether to give 51 mg. of I as white prisms, m.p. 66–69°. Table III lists the NMR data.

Anal.—Calcd. for $C_{11}H_{12}O_2$: C, 75.0; H, 6.8. Found: C, 75.2; H, 6.8.

Fractions 10–12 were combined and recrystallized from petroleum ether to give 100 mg. of II as white prisms, m.p. 73–76°. Table III lists the NMR data.

Anal.—Calcd. for $C_{11}H_{12}O_2$: C, 75.0; H, 6.8. Found: C, 74.8; H, 7.0.

Preparation of β-Hydroxy-β-methylglutaric Anhydride[2]—To an ice-cold solution of 2.88 g. (17.8 mmoles) of β-hydroxy-β-methylglutaric acid[3] in 40 ml. of dry acetone was added dropwise with stirring 3.6 g. (17.4 mmoles) of dicyclohexylcarbodiimide[4] in 20 ml. of dry acetone. Immediately, a white precipitate began to form. The mixture was stirred at room temperature for 17 hr. Dry ether (50 ml.) was added, and the mixture was filtered. The solvent was removed under reduced pressure to afford a white solid, which was slurried with ether–petroleum ether. The mixture was cooled and then filtered to give 2.54 g. (17.2 mmoles), 97%, of white prisms, m.p. 100–102° [lit. (5) m.p. 102–103°].

Preparation of HMG-CoA—The method of Louw *et al.* (4) was used, except that the bubbling of nitrogen through the system was found to be unnecessary.

[1] Melting points, determined with a Thomas-Hoover capillary melting-point apparatus, are corrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

[2] The authors experienced the same difficulty as Louw *et al.* (4) in the preparation of β-hydroxy-β-methylglutaric anhydride. Therefore, an alternate method was developed, using dicyclohexylcarbodiimide, which afforded the anhydride in high yield.

[3] Mann Research Lab.

[4] Aldrich Chemical Co.

Table I—Chromatographic Separation of BBA[a]

Fraction	Weight, mg.	Description
1. 100% P.E.	—	
2. 100% P.E.	—	
3. 5% E–P.E.	—	
4. 5% E–P.E.	600	Colorless liquid
5. 5% E–P.E.	500	Colorless liquid
6. 5% E–P.E.	350	Colorless liquid
7. 5% E–P.E.	130	Colorless liquid
8. 50% E–P.E.	400	White solid
9. 100% E	150	White solid
Total	2.130 g.	

[a] Fractions are 100 ml. of petroleum ether (P.E.), b.p. 60–75°; diethyl ether (E); or mixtures as indicated (v/v).

Assay of Substrate—HMG-CoA was assayed by using Ellman's method (6) to determine reduced Coenzyme A before and after saponification (0.05 *N* KOH for 10 min. at 30°). This is similar to the method recently reported by Louw *et al.* (4).

Enzyme Preparation—The enzyme, HMG-CoA reductase, was prepared from locally obtained Fleischman's bakers yeast by the procedure of Durr and Rudney (7). This procedure consists of freezing crumbled yeast in liquid nitrogen, extraction with K_2HPO_4 solution, precipitation at pH 4.7, dissolving the precipitate in 0.15 *M* K_2HPO_4 containing 1.0 m*M* ethylenediamine tetraacetate, titration to pH 7 with 1 *N* KOH, centrifugation at 105,000×*g* for 1 hr. (or 37,000×*g* for 170 min. at 5°), and filtration of the supernatant through glass wool. The enzyme solution was transferred to capped serum bottles and stored at −10° until it was used.

Enzyme Inhibition—Enzyme activity was determined at 30° in a Gilford recording spectrophotometer by measuring the decrease of optical density at 340 nm. according to a modification of the procedure of Durr and Rudney (7). To each cell were added 1.6 ml. of 0.15 *M* NaH_2PO_4 buffer (pH 7.0) containing 1 m*M* ethylenediamine tetraacetate, 0.2 ml. ethylene glycol monoethyl ether, 0.5 ml. aqueous cysteine solution containing 6.8 mg. cysteine, 0.05 ml. reduced triphosphopyridine nucleotide (TPNH) solution containing 0.36 μm. TPNH, and 0.1 ml. enzyme solution (about 14 mg. protein/ml. in buffer). The optical density was allowed to stabilize after stirring prior to the addition of 0.05 ml. HMG-CoA solution containing 44 nmoles of DL-HMG-CoA and stirring again. (Inhibition was studied by dissolving the inhibitor in ethylene glycol monoethyl ether.) Albumin was deleted from the procedure of Durr and Rudney (7) since its addition did not seem to improve the assay.

RESULTS

BBA has been assigned a *cis*-configuration with regard to the phenyl and methyl groups. This assignment was been made on the basis of steric considerations.

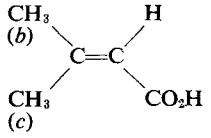
Structural assignment of the acids, I and II, was made on the basis of NMR spectroscopy. A tabulation of the NMR data is found in Table III.

Table II—Chromatographic Separation of Compounds I and II[a]

Fraction	Weight, mg.	Description
1. 100% P.E.	—	
2. 100% P.E.	—	
3. 1% E–P.E.	—	
4. 1% E–P.E.	—	
5. 1% E–P.E.	—	
6. 2% E–P.E.	—	
7. 2% E–P.E.	37	White solid
8. 2% E–P.E.	100	White solid
9. 2% E–P.E.	91	Colorless liquid
10. 2% E–P.E.	106	White solid
11. 2% E–P.E.	60	White solid
12. 2% E–P.E.	46	White solid
13. 100% E	544	Yellow oil
Total	984	

[a] Fractions are 100 ml. of petroleum ether (P.E.), b.p. 60–75°; diethyl ether (E); or mixtures as indicated (v/v).

Table III—Chemical Shift Data[a]

Compound	Vinyl	Methylene	Methyl
BBA	6.44	3.24	1.97
I	5.82	4.00	1.77
II	5.73	3.42	2.10
	5.72	—	1.93[b] 2.18[c]

[a] The chemical shifts were determined in deuterochloroform using a Varian A-60 NMR spectrophotometer. The values are reported in p.p.m. relative to tetramethylsilane as an internal standard. All peaks for BBA, I, and II were observed as singlets using a sweep width of 1000 Hz.

The stereochemistry of I and II has been assigned after comparing the chemical shifts of the methyl protons of the acids, I and II, with the methyl protons of 3-methyl-2-butenoic acid.

The methyl protons of I appear at 1.77 p.p.m., which is shifted upfield from the expected position of approximately 1.93 p.p.m. observed when the methyl group is *cis* to the hydrogen atom as in 3-methyl-2-butenoic acid. This diamagnetic shift can be readily explained by the steric crowding of the carboxyl and benzyl groups. To minimize this steric interaction between the two groups, the molecule must assume a conformation that allows shielding of the methyl group by the aromatic ring. Upon examination of Dreiding stereomodels, this conformation would appear to be a favorable one.

The stereochemical assignment of II is also based on the chemical shift of the methyl group. The methyl protons of II appear at 2.10 p.p.m., which is in close agreement with the position of 2.18 p.p.m. observed when the methyl group is *trans* to the hydrogen atom as in 3-methyl-2-butenoic acid.

No inhibition of HMG-CoA reductase activity was noted either for concentrations up to and including 22 μmoles/cell (500 times substrate concentration) of Compounds I and II or for 44 μmoles/cell (1000 times substrate concentration) of BBA.

DISCUSSION

Although it was reported that in rat liver BBA strongly inhibits the *in vitro* incorporation of acetate but not mevalonate into cholesterol (1), the exact step inhibited is not known. Acetyl-CoA: ligase from pigeon liver is 58% inhibited by 10 m*M* BBA, but acetyl-CoA acetyl transferase from pigeon liver shows no inhibition in the presence of 20 m*M* BBA. The finding that even acetyl-CoA:ligase was not inhibited at concentrations of BBA below 1 m*M*, which markedly inhibited incorporation of acetate into sterol, suggested to Porcellati *et al.* (1) that another enzyme between acetate and mevalonate was also inhibited by BBA. The present investigation clearly shows no inhibition of HMG-CoA reductase at concentrations up to 17 m*M* BBA. Even though the degree of similarity between HMG-CoA reductase from yeast and that from mammalian sources is not known, other known inhibitors of mammalian cholesterol biosynthesis also have been observed to be potent inhibitors of yeast HMG-CoA reductase (8).

Having investigated the *in vitro* effect of BBA on yeast HMG-CoA reductase, the authors intend to investigate the effect of mammalian HMG-CoA reductase in a similar manner. The present results suggest, however, that BBA inhibits yet another step between acetate and mevalonate. Alternatively, it could increase the activity of other enzymes that utilize intermediates of the acetate ⟶ mevalonate pathway and thus make these intermediates unavailable. For example, an increase in activity of certain enzymes of fatty acid biosynthesis would be expected to result in less substrate available for mevalonic acid biosynthesis.

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(8) K. E. Guyer, M. R. Boots, and S. G. Boots, unpublished data.

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Technical assistance by Mrs. Catherine Faley is gratefully acknowledged.

Sterically Hindered Esters of Vitamin A III: Biological Availability of Vitamin A from Sterically Hindered Esters

A. J. FORLANO

Abstract □ Previous studies demonstrated that sterically hindered esters of vitamin A, such as the α,α-dimethylpalmitate and α-methyl-α-ethylcaproate, had some superior chemical properties compared to the palmitate. The biological availability of vitamin A from these esters, compared with the palmitate and cod liver oil as controls, was determined in rats. Maximum availability of vitamin A was obtained with the palmitate followed by cod liver oil. Availability from the α,α-dimethylpalmitate was 71% that of the palmitate, while the α-methyl-α-ethylcaproate had a poor availability pattern.

Keyphrases □ Vitamin A—biological availability from sterically hindered esters □ Biological availability—vitamin A from sterically hindered esters □ Biological assay—comparison of vitamin A esters

A previous study by Forlano and Harris (1) suggested that maximal stability of vitamin A esters could be obtained by the introduction of electropositive groups in the α-position of the esters. Based on this concept, sterically hindered esters of vitamin A were prepared and their chemical stability pattern was determined. Some of the more promising compounds in this series were the vitamin A pivalate, α,α-dimethylvalerate, triethylacetate, α-methyl-α-ethylcaproate, and α,α-dimethylpalmitate. These were subjected to chemical evaluation tests such as oxidation, base-catalyzed hydrolysis, acid-catalyzed elimination, and acid-catalyzed isomerization (2, 3). It was then considered desirable to determine the biological availability of the esters that showed the best overall chemical stability when compared with the palmitate. Vitamin A α-methyl-α-ethylcaproate (2) and the α,α-dimethylpalmitate (3) were chosen, with cod liver oil and vitamin A palmitate functioning as standards. Two parameters were considered in the biological tests: (*a*) the amount of biological activity by a growth assay (4), and (*b*) the death rate in the various groups of rats, which was a further indication of the animals' ability to utilize the vitamin A ester (5).

EXPERIMENTAL

Chemical and spectrophotometric assays for vitamin A preparations may not give a true indication of the biological availability of vitamin A derivatives, especially new vitamin A derivatives that have unknown biological availability patterns. For this reason, a biological assay is very valuable in the final determination of potency.

The procedure used was a modification of the USP bioassay (5). It was based on the fact that the growth rate of a rat varies directly with the consumption of vitamin A (4). Vitamin A esters that are completely available will have higher growth rates than equimolar doses of esters that are not completely absorbed. Animals will die from avitaminosis A when fed derivatives that are poorly absorbed or not absorbed at all (5).

Table I—Average Weight (in Grams ±1 Standard Deviation) of Rats Fed Different Forms of Vitamin A

Days	Control Group	Cod Liver Oil Group	Vitamin A Palmitate Group	Vitamin A α-Methyl-α-ethylcaproate Group	Vitamin A α,α-Dimethylpalmitate Group
0	61	63	60	63	62
7	85	90	85	85	90
13	108	110	108	108	108
23	138	153	156	136	144
25	146 ± 13.5	165 ± 12.4	167 ± 9.2	141 ± 11.2	152 ± 9.2
30	144 ± 18.0	189 ± 14.3	187 ± 12.2	138 ± 18.1	167 ± 8.9
35	146 ± 14.2[a]	208 ± 15.4	210 ± 12.6	155 ± 16.9[b]	179 ± 12.8
38	156[c,d]	212 ± 8.9	216 ± 12.8	164 ± 14.0[e]	186 ± 13.8
41	157[d]	220 ± 10.2	232 ± 13.7	166 ± 17.0[e]	196 ± 11.6
45	144[d]	227 ± 10.8	242 ± 14.5	158 ± 24.3[e]	201 ± 12.9
52	128[d]	247 ± 11.5	264 ± 14.3	165 ± 26.4[a]	211 ± 15.8
55	—[f]	250 ± 11.1	271 ± 15.3	160 ± 27.8[g]	212 ± 18.8

[a] Represents 50% mortality. [b] Represents 30% mortality. [c] Represents 80% mortality. [d] No standard deviation was run in these groups because only one rat remained. [e] Represents 40% mortality. [f] Represents 100% mortality. [g] Represents 60% mortality.

(5) H. Hilz, J. Knappe, E. Ringelmann, and F. Lynen, *Biochem. Z.*, **329**, 476(1958).
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EXPERIMENTAL

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[a] Represents 50% mortality. [b] Represents 30% mortality. [c] Represents 80% mortality. [d] No standard deviation was run in these groups because only one rat remained. [e] Represents 40% mortality. [f] Represents 100% mortality. [g] Represents 60% mortality.

Table II—Average Weight Gains of Rats in Grams (Column A) and the Percent Increase as Compared to Vitamin A Palmitate Standard (Column B)

Days	Control		Cod Liver Oil		Vitamin A Palmitate		Vitamin A α-Methyl-α-ethylcaproate		Vitamin A α,α-Dimethylpalmitate	
	A	B	A	B	A	B	A	B	A	B
0	—	—	—	—	—	—	—	—	—	—
7	23.4	—	27.5	—	24.9	100	22.5	—	28.0	—
13	47.0	97.5	47.5	98.5	48.2	100	45.0	93.4	45.7	94.8
25	84.0	79.0	101.3	95.3	106.3	100	78.2	73.6	90.0	84.7
35	84.8[a]	50.0	144.8	96.8	149.6	100	85.0[b]	56.8	116.5	77.9
45	83.0[c]	45.7	164.1	90.4	181.6	100	95.0[d]	52.3	137.9	75.0
55	—[e]	—	186.3	88.8	209.9	100	97.0[f]	46.2	149.6	71.3

[a] Represents 50% mortality. [b] Represents 30% mortality. [c] Represents 80% mortality. [d] Represents 40% mortality. [e] Represents 100% mortality. [f] Represents 60% mortality.

Fifty young male rats, weighing between 60 and 65 g. each, were randomly divided into five groups. All groups received a vitamin A-free diet[1] and water *ad libitum*. In addition, each group, except a control which received plain cottonseed oil, was given 0.1 ml. of a cottonseed oil solution of the vitamin A source containing the molar equivalent of 78 USP units once a week. The freshly prepared dose was administered with an oral syringe placed directly down the rat's throat.

The five groups were designated as follows:

1. Control group (cottonseed oil alone).
2. Cod liver oil group (cod liver oil USP diluted with cottonseed oil).
3. Vitamin A palmitate group [23 mg. of vitamin A palmitate (Pfizer) dissolved in 50 ml. of cottonseed oil].
4. Vitamin A α-methyl-α-ethylcaproate group [18 mg. of this ester (2) dissolved in 50 ml. of cottonseed oil].
5. Vitamin A α,α-dimethylpalmitate group [25 mg. of this ester (3) dissolved in 50 ml. of cottonseed oil].

The rats were weighed at regular intervals, using a conventional triple-beam balance. The average weights for the different groups are presented in Table I, and the average weight gains are presented in Table II.

RESULTS AND DISCUSSION

The control group required 55 days for complete mortality; however, at 35 and 38 days, there was 50 and 80% mortality, respectively. There were definite signs of vitamin A deficiency, such as loss of weight and xerophthalmia, at 30 days. These figures represent a typical vitamin A depletion pattern for rats in this weight group (4).

During this same 55-day period, the vitamin A palmitate group had the greatest rate of weight gain when compared to the other groups receiving equivalent amounts of vitamin A. For this reason, the palmitate group was considered the standard to which the other groups were compared. The average weight gain for the cod liver oil group was 89% that of the vitamin A palmitate group, while for the α-methyl-α-ethylcaproate group it was 46% in the rats that survived (60% death rate in this group), and for the α,α-dimethylpalmitate group it was 71% with no deaths.

The 40% surviving rats in the α-methyl-α-ethylcaproate group demonstrated an appreciable degree of variability in their weight-gain pattern as indicated by the large standard deviation. These data indicate that the rats that died could not effectively utilize this ester to an appreciable extent. The surviving rats also experienced considerable difficulty in their utilization of this compound, as suggested by their poor and erratic growth-rate pattern. None of the survivors had a weight-gain pattern comparable to the vitamin A palmitate group. One could speculate that the steric hindrance in the α-position of this ester may have caused substantial resistance to the action of esterases required to hydrolyze these compounds.

The availability pattern in the cod liver oil group was comparable to that of the vitamin A palmitate group, indicating that cod liver oil esters are reasonably well utilized.

There were no deaths in the vitamin A α,α-dimethylpalmitate group, even though the esters had steric hindrance in the α-position. The average weight gain of 71% after 55 days showed that while this ester was not as efficiently utilized as the palmitate, there was sufficient utilization to maintain life and growth.

Only the control and vitamin A α-methyl-α-ethylcaproate groups showed the physical signs of avitaminosis A. The rats in these two groups, in which death occurred, showed the following symptoms of avitaminosis A: (*a*) loss of weight; (*b*) conjunctivitus; (*c*) puffing of fur; (*d*) odd gait while walking, indicating improper bone formation; and (*e*) death.

If the biological availability patterns from the two sterically hindered esters are compared with those of nonsterically hindered esters, one can see that steric hindrance in the α-position of the ester exerts an inhibition of biological utilization. It is possible that steric hindrance in the α-position could affect the rate of enzymatic hydrolysis of these esters, with a subsequent decrease in utilization.

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

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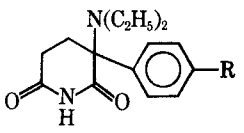
3-Aminopiperidones V: 2-Ethoxy and 2-Carbethoxy Derivatives of 2-*p*-Methoxyphenylglutarimide

B. H. MOON* and C. F. MARTIN

Abstract □ The syntheses of two new modifications of the model compound, 2-(*N*,*N*-diethylamino)-2-phenylglutarimide (I), bearing, in both instances, the methoxy substituent on the *para*-position of the phenyl group and, respectively, the ethoxy and the carbethoxy groups in place of the substituted amino function on carbon 2 are described. The preparation of these compounds is an extension of work designed to provide a basis for studies of structure *versus* potential anticonvulsant activity relationships among the 3-aminopiperidones.

Keyphrases □ 3-Aminopiperidones—2-ethoxy, 2-carbethoxy derivatives of 2-*p*-methoxyphenylglutarimide synthesized for structure–activity studies □ 2-*p*-Methoxyphenylglutarimide—synthesis of 2-ethoxy, 2-carbethoxy derivatives □ NMR—structure □ IR—structure

Pharmacological screening of the model compound, 2-(*N*,*N*-diethylamino)-2-phenylglutarimide (I) (1, 2), demonstrated that it possessed potential anticonvulsant activity but that it was detoxified rapidly in the biological system. Based upon pharmacological evaluations of similar compounds (3, 4), it was reasoned that of several possibilities, *para*-hydroxylation of the phenyl group, with subsequent glucuronide conjugation of I in the biological system, could account, at least in part, for its short duration of action. Therefore, routes to the synthesis of modifications I*a* and I*b* were developed, wherein the *para*-position of the phenyl group was occupied in one case by a chlorine atom and in the other by the methyl group (5). These compounds were designed to block the position noted from metabolic attack and also to provide a basis for studies of the effect on activity of substituents that would reasonably be expected to have opposite electronic influences on the phenyl group and, in turn, on the molecular stability, drug receptor interactions, and biotransportation of the compounds.



I: R = H
I*a*: R = Cl
I*b*: R = CH_3

DISCUSSION

During the present study, which has dealt with routes to the synthesis of other *para*-phenyl-substituted derivatives of the model compound (I), it was found (Scheme I) that in an attempted conversion of α-(*N*,*N*-diethylamino)-*p*-methoxyphenylacetonitrile (II) (6) to ethyl α-(*N*,*N*-diethylamino)-*p*-methoxyphenylacetate (III) by alcoholysis in the presence of sulfuric acid, the major product of the reaction was α-ethoxy-*p*-methoxyphenylacetonitrile (IV). Because the diethylaminomethoxyphenylacetonitrile and, in turn, the ethoxy derivative are both obtained in good yield by this route, it was determined that the synthesis of the corresponding ethoxy glutarimide would be worthy of investigation. This was accomplished by α-carbon alkylation of IV with methylacrylate in the presence of base to produce methyl 4-cyano-4-ethoxy-4-*p*-methoxyphenylbutyrate (VI), followed by cyclization of the cyano-ester in an acid system to yield 2-ethoxy-2-*p*-methoxyphenylglutarimide (VII).

As this work developed, it was proposed that for comparative purposes a corresponding carbethoxy derivative of the glutarimide system would be helpful to delineate further the relationships of structure to activity in the series. It was reasoned that relationships among molecules bearing the ester group, the ether group, and the tertiary amino group would provide bases for determining the importance of variations in polarity in this part of the glutarimide structure, along with information on the significance of the amino group, *per se*, in conferring pharmacological activity on the molecule. Synthesis of the desired carbethoxy derivative was accomplished, as shown in Scheme II, by the initial condensation of *p*-methoxyphenylacetonitrile (VIII) with diethylcarbonate in the presence of sodium to yield α-carbethoxy-*p*-methoxyphenylacetonitrile (IX) (7). This, in turn, was converted to methyl 4-carbethoxy-4-cyano-4-*p*-methoxyphenylbutyrate (X) by α-carbon alkylation with methylacrylate in the presence of base, followed by cyclization in acid to yield 2-carbethoxy-2-*p*-methoxyphenylglutarimide (XI).

EXPERIMENTAL[1]

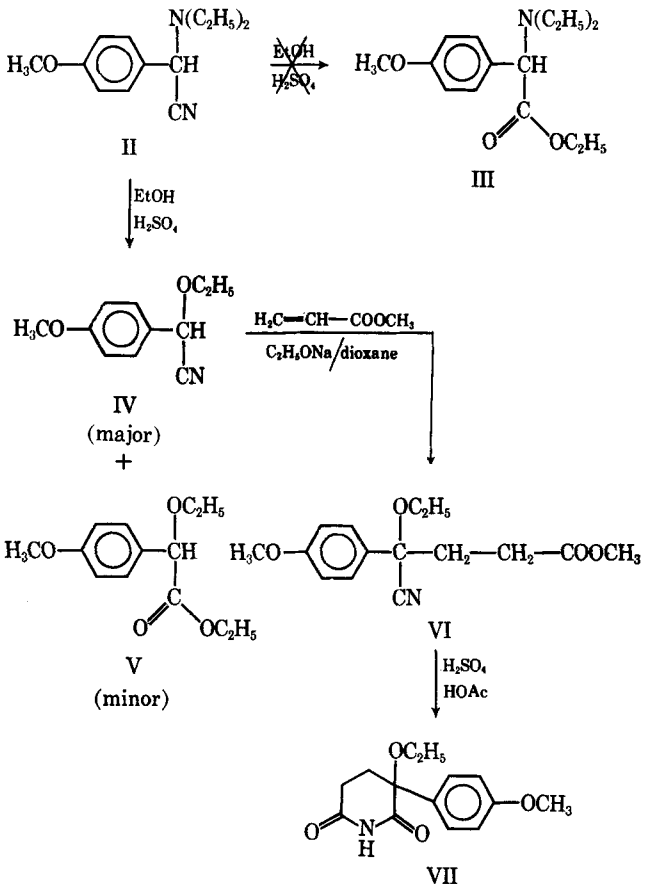
General—Melting points were determined on the Fisher-Johns melting-point block and in open capillary tubes using the Thomas-Hoover apparatus; they are uncorrected. IR spectra were recorded with Beckman IR-5 and IR-8 spectrophotometers. NMR spectra were determined on Varian A-60 and T-60 spectrometers using tetramethylsilane as the internal standard.

α-Ethoxy-*p*-methoxyphenylacetonitrile (IV)—To a stirred solution of ethanol (142 g.) and concentrated sulfuric acid (110 g.) was added α-(*N*,*N*-diethylamino)-*p*-methoxyphenylacetonitrile (II, 110 g.) at such a rate as to maintain gentle refluxing. The mixture was refluxed for an additional 10 hr., cooled, and poured into 300 ml. of ice water. The upper organic layer was separated and washed successively with Na_2CO_3 (10%) and water. After drying, the product was distilled to yield 57 g. (60%) of a 5:1 mixture of IV and V as a pale-yellow liquid, b.p. 110–115° (0.2 mm.). Separation of the mixture was accomplished chromatographically using acid-washed alumina and magnesium silicate gel[2] (60–200 mesh) (1:1) in a 25.4 × 2.54-cm. (10 × 1-in.) column with benzene–petroleum ether (10:1); b.p. (IV) 104° (0.2 mm.); IR (10% $CHCl_3$): 2250 (CN); 1120, 1145 (OC_2H_5 ethoxy); NMR (20% CCl_4): singlet 5.1 p.p.m. (methine proton); singlet 3.8 p.p.m. (methyl protons of *p*-methoxy group); quartet 3.6 p.p.m. (methylene protons of ethoxy group); triplet 1.2 p.p.m. (methyl protons of ethoxy group) with phenyl protons centered at 7.1 p.p.m.

Anal.—Calcd. for $C_{11}H_{13}NO_2$: C, 69.11; H, 6.80; N, 7.33. Found: C, 68.90; H, 6.82; N, 7.19.

Ethyl α-Ethoxy-*p*-methoxyphenylacetate (V)—This compound, b.p. 115° (0.2 mm.), obtained as a minor product from the reaction that yielded IV, was identified by the presence of the characteristic ester carbonyl stretching frequency in the IR (1730) and the absence of the nitrile peak (2250). NMR showed overlapping triplets at 1.2 p.p.m. (methyl protons of both the ester ethyl and the ethoxy groups), a quartet at 4.2 p.p.m. (methylene protons of ester ethyl), a quartet at 3.6 p.p.m. (methylene protons of ethoxy group), a

[1] Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.
[2] Florisil.



Scheme I

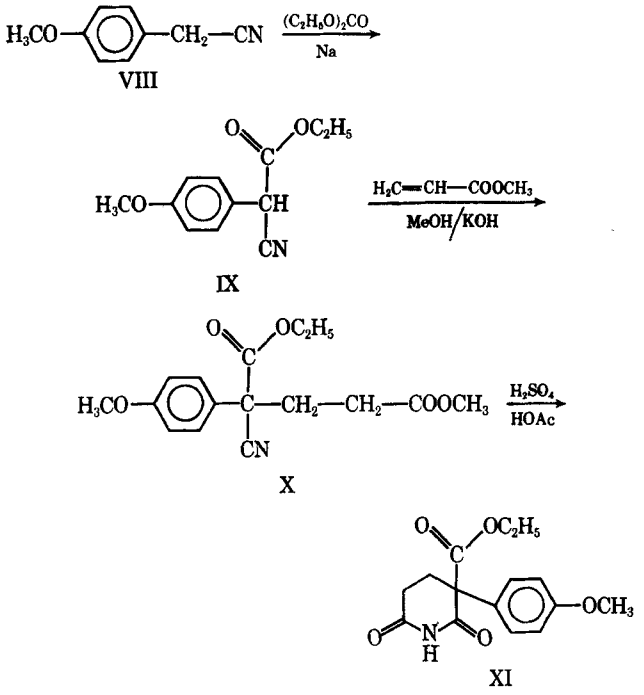
singlet at 4.8 p.p.m. (methine proton), and a singlet at 3.8 p.p.m. (methyl protons of *p*-methoxy group).

Anal.—Calcd. for $C_{13}H_{18}O_4$: C, 65.54; H, 7.56. Found: C, 65.57; H, 7.68.

Methyl 4-Cyano-4-ethoxy-4-*p*-methoxyphenylbutyrate (VI)—To a stirred solution of IV (19.1 g., 0.1 mole) and methylacrylate (10.4 g., 0.12 mole) in 70 ml. of dioxane at room temperature was added, dropwise, a solution of sodium (1 g.) in 20 ml. of ethanol. With this addition, the temperature of the reaction mixture gradually increased until it reached 50°, at which point it was maintained by cooling until all of the sodium ethoxide solution had been added. At the end of this period, the mixture was allowed to cool slowly to room temperature and was filtered; the filtrate was diluted with 70 ml. of water. After extraction with ether, the combined extracts were washed with water, dried, and flash evaporated; the residue was distilled to yield 15 g. (54%) of VI as a pale-yellow liquid, b.p. 132–135° (0.01 mm.); IR (10% $CHCl_3$): 1725 (C=O ester); 2250 (CN) was "quenched" (8); NMR (20% CCl_4): singlet 3.6 p.p.m. (methyl protons of ester methyl); singlet 3.8 p.p.m. (methyl protons of *p*-methoxy group); triplet 1.2 p.p.m. (methyl protons of ethoxy group); multiplets 2.4 p.p.m. (methylene protons of adjacent carbons in carbomethoxyethyl group).

Anal.—Calcd. for $C_{15}H_{19}NO_4$: C, 64.96; H, 6.91; N, 5.05. Found: C, 64.98; H, 7.23; N ,4.74.

2-Ethoxy-2-*p*-methoxyphenylglutarimide (VII)—To a stirred solution of concentrated sulfuric acid (65 ml.) in glacial acetic acid (130 ml.), VI (11.1 g., 0.04 mole) was added in small portions. The mixture was heated to 110° and maintained at this temperature for 30 min. After cooling, the mixture was poured into ice water, rendered alkaline with NH_4OH (28%) (cooling), and extracted with chloroform. The combined extracts were washed twice with water and dried. After flash evaporation of the solvent, the oily residue solidified to yield 0.5 g. (5%) of VII, m.p. 197°, after recrystallization from chloroform–ethanol; IR (KBr pellet): 3500 (NH imide); 1690 (C=O imide). NMR (20% CCl_4): singlet 3.8 p.p.m. (methyl protons of *p*-methoxy group); triplet 1.2 p.p.m. (methyl protons of ethoxy group); quartet 3.6 p.p.m. (methylene protons of ethoxy group); multiplets 2.4 p.p.m. (methylene protons of adjacent unsubstituted carbons in glutarimide ring system).



Scheme II

Anal.—Calcd. for $C_{14}H_{17}NO_4$: C, 63.86; H, 6.50; N, 5.32. Found: C, 63.38; H, 6.02; N, 5.24.

Methyl 4-Carbethoxy-4-cyano-4-*p*-methoxyphenylbutyrate (X)—To a solution of α-carbethoxy-*p*-methoxyphenylacetonitrile (IX, 21.9 g., 0.1 mole) in 40 ml. of tertiary butanol (warmed to 40°) was added 12.3 ml. of 30% methanolic KOH dropwise with stirring. This was followed immediately by the addition of methylacrylate (8.6 g., 0.1 mole) at a rate that permitted maintenance of reaction temperature between 35 and 40°. The mixture was stirred for an additional 3 hr., diluted with water (100 ml.), and neutralized (cooling) with HCl (10%). After extraction with ether, the extracts were washed with water, dried, and flash evaporated; the residue was distilled to yield 18 g. (59%) of X, b.p. 169–170° (0.1 mm.); IR (10% $CHCl_3$): 1750 (C=O ester); 2250 (CN); NMR (20% CCl_4): singlet 3.6 p.p.m. (methyl protons of ester methyl); singlet 3.8 p.p.m. (methyl protons of *p*-methoxy group); triplet 1.2 p.p.m. (methyl protons of ester ethyl); quartet 4.2 p.p.m. (methylene protons of ester ethyl); multiplets 2.4 p.p.m. (methylene protons of adjacent carbons in carbomethoxyethyl group).

Anal.—Calcd. for $C_{16}H_{19}NO_5$: C, 62.95; H, 6.23. Found: C, 62.79; H, 6.34.

2-Carbethoxy-2-*p*-methoxyphenylglutarimide (XI)—A mixture of methyl 4-carbethoxy-4-cyano-4-*p*-methoxyphenylbutyrate (X, 8.0 g., 0.03 mole) in 26 ml. of a 1:1 solution of glacial acetic and concentrated sulfuric acids was heated on the steam bath for 30 min. The reaction mixture was poured into an equal volume of ice water, neutralized with 6 *N* NaOH, and extracted with chloroform. The combined extracts were washed several times with water and dried. After evaporation of the solvent from the dried extract, the product solidified upon rubbing with a chloroform–hexane mixture to yield 3.5 g. (40%) of XI, m.p. 115–117°, after recrystallization from chloroform–hexane; IR (KBr pellet): 3200, 3400 (NH imide); 1725 (C=O ester) fused with 1710 (C=O imide); NMR (20% CCl_4): singlet 3.8 p.p.m. (methyl protons of *p*-methoxy group); triplet 1.2 p.p.m. (methyl protons of ester ethyl); quartet 4.2 p.p.m. (methylene protons of ester ethyl); multiplets 2.4 p.p.m. (methylene protons of adjacent unsubstituted carbons in glutarimide ring system).

Anal.—Calcd. for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.84; N, 4.81. Found: C, 61.84; H, 5.77; N, 4.85.

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N-Phenylacetylsulfamide: Sulfone Analog of Anticonvulsant Acylureas

CECIL C. CHAPPELOW, Jr.*, and WILLIAM J. ROST†

Abstract □ *N*-Phenylacetylsulfamide was synthesized under three different conditions. If ethyl acetate was used as a solvent, a higher yield of the monoacylated product was obtained than if pyridine was used as the solvent. If the lithium salt of sulfamide was used, a maximum yield of the diacylated product and a minimum yield of the monoacylated product were obtained. The *N*-phenylacetylsulfamide was less than half as active as trimethadione when tested as an anticonvulsant in the cat.

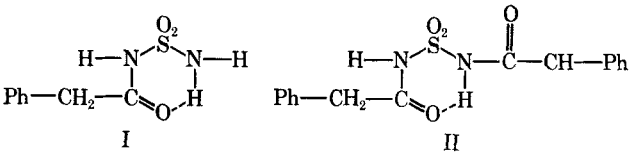
Keyphrases □ *N*-Phenylacetylsulfamide—synthesis, three methods □ Acylureas—synthesis of sulfone analog □ Anticonvulsant acylureas—synthesis of the sulfone analog *N*-phenylacetylsulfamide

The acylureas such as phenacemide[1], Ph—CH_2—CO—NH—CO—NH_2, represent a class of anticonvulsant drugs. Hence, it was desired to prepare the sulfone analog, Ph—CH_2—CO—NH—SO_2—NH_2, for comparison. A search of the literature revealed that only a few simple acyl derivatives of sulfamide have been prepared and characterized (1).

To prepare the desired compound, phenylacetyl chloride and sulfamide were condensed. Even though an equimolar ratio of the two chemicals was employed, an appreciable amount of *N,N'*-bis(phenylacetyl)sulfamide was obtained. If ethyl acetate was used as the solvent, a 40% yield of the monoacylated product and 15% yield of the diacylated product were obtained. If the reaction was run in pyridine, a 30% yield of the monosubstituted product and an 8% yield of the disubstituted product were obtained.

In an attempt to increase the yield of the monoacylated product and/or decrease the yield of the diacylated product, the reaction was run with the lithium salt of sulfamide. Unexpectedly, this reaction gave only a 10% yield of the monoacylated and a 42% yield of the diacylated products. The high yield of the diacylated product was probably the result of a transmetallation reaction between the *N*-lithiosulfamide and the monoacylated products.

Inspection of the NMR spectra of *N*-phenylacetylsulfamide and *N,N'*-bis(phenylacetyl)sulfamide revealed an unusually large chemical shift for one of the nitrogen-bonded protons of these compounds. These shifts are indicative of intramolecular hydrogen bonding (2). Based on these data, it is proposed that in solution the monosubstituted and disubstituted compounds exist as the following intramolecular hydrogen-bonded structures:



I II

These structures are compatible with most of the general rules governing the formation of intramolecular hydrogen-bonded rings set forth by Wheland (3). Also, the formation of an intramolecular hydrogen bond structure for *N*-phenylacetylsulfamide would explain the high incidence of disubstitution, especially in the reaction where *N*-lithiosulfamide was employed as the reactant.

An exchange reaction of *N*-phenylacetylsulfamide and deuterium oxide was conducted according to the procedures of Ouchi and Moeller (4). This was readily driven to completion by employing an excess of deuterium oxide. NMR analysis of the reaction product confirmed the structure assignment and revealed the location of all nitrogen-bound proton signals.

N-Phenylacetylsulfamide was studied for activity as a potential petit mal anticonvulsant by recording its protective ability, as compared to the standard anticonvulsant, trimethadione, against pentylenetetrazol-in-

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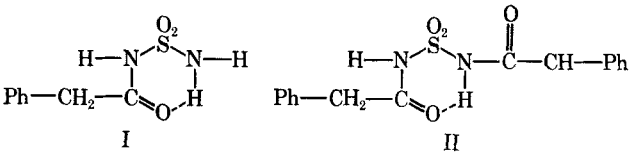

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duced electrographic spiking in each of eight CNS sites in the cat. The areas studied were the central lateral nucleus of the thalamus, anterior dorsal nucleus of the thalamus, dorso-lateral nucleus of the thalamus, mesencephalic reticular formation, central hippocampus, head of the caudate nucleus, anterior amygdala, and the fronto-occipito cortex. Recordings were made on a Grass model 7 polygraph using model 7P5 EEG preamplifiers. Recording electrodes were made of nichrome wire and stereotaxically implanted into each recording site. Recordings were made following administration of convulsant doses of pentylenetetrazol to cats protected and unprotected by trimethadione and *N*-phenylacetylsulfamide.

Findings indicate that *N*-phenylacetylsulfamide was less than 0.5 times as effective as the standard, trimethadione, on a molar basis as an anticonvulsant tested against convulsant doses of pentylenetetrazol. The compound was also found to lack significant anticonvulsant activity against the excitant effects of pentylenetetrazol at considerably higher dose levels. At these higher dose levels, the compound exhibited strong emetic properties.

EXPERIMENTAL[2]

The melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. The IR spectra of the compounds were determined on a Perkin-Elmer model 137 Infracord recording spectrophotometer. The spectra were run as mineral oil mulls. A Varian Associates model A-60 NMR spectrometer was used to determine the proton magnetic resonance spectra. Tetramethylsilane was used as a standard reference, and hexadeuterodimethylsulfoxide was used as the solvent.

Synthesis of Phenylacetylsulfamide and *N,N'*-Bis(phenylacetyl)-sulfamide Using Pyridine—To a rapidly stirred solution of sulfamide (9.6 g., 0.1 mole) in 100 ml. of pyridine, phenylacetyl chloride (19.0 g., 0.11 mole) was added dropwise over 30 min. The addition was accompanied by a slight temperature rise. After addition was complete, the reaction mixture was stirred for 6 hr. while the temperature was maintained at 60°. Then the reaction mixture was poured onto cracked ice and stirred until the ice melted. The resultant two-phase mixture was extracted twice with 100-ml. portions of chloroform. The extracts were combined, washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue (23.0 g.), an orange-colored semicrystalline solid, was dissolved in absolute alcohol, and the two products were obtained by repeated concentration and cooling in the following order:

N,N'-Bis(phenylacetyl)sulfamide—Crude product (2.5 g.), m.p. 166–168° dec., which after one recrystallization from absolute alcohol gave 2.0 g. (7% yield) of final product, a white powder m.p. 170–171° dec. The IR spectrum of the product has an intense band at 3500 cm.$^{-1}$ (—NH—), a sharp intense band at 1710 cm.$^{-1}$ (C=O), a broad band centered at 1160 cm.$^{-1}$ (SO_2), and a characteristic series of three bands at 760, 710, and 690 cm.$^{-1}$ (aromatic). The NMR spectrum has a sharp singlet at 3.55δ (—CH_2—), another sharp singlet at 7.17δ (—C_6H_5), and a broad singlet centered at 12.25δ (—NH— — —O=C).

Anal.—Calcd. for $C_{16}H_{16}N_2O_4S$: C, 57.82; H, 4.85; N, 8.43; S, 9.65. Found: C, 57.64; H, 4.84; N, 8.25; S, 9.56.

N-Phenylacetylsulfamide—Crude product (8.0 g.), m.p. 153° dec., which after one extraction with hot benzene and two recrystallizations from absolute alcohol, gave 7.0 g. (30% yield) of final product, a white powder, m.p. 164–165°. The IR spectrum had a sharp band at 3800 cm.$^{-1}$ and a moderately broad band at 3500 cm.$^{-1}$ (—NH— and —NH_2), an intense band at 1690 cm.$^{-1}$ (C=O), a broad intense band centered at 1135 cm.$^{-1}$ (SO_2), and a characteristic series at 865, 810, 765, and 695 cm.$^{-1}$ (aromatic). The NMR spectrum has a sharp singlet at 3.48δ (—CH_2—), a second sharp singlet at 7.15δ (—C_6H_5 and —NH_2), and a broad singlet centered at 11.43δ (—NH— — —O=C).

Anal.—Calcd. for $C_8H_{10}N_2O_3S$: C, 44.85; H, 4.70; N, 13.08; S, 14.97. Found: C, 44.79; H, 4.93; N, 12.71; S, 14.71.

***N*-Phenylacetylsulfamide Using Ethyl Acetate**—The previously described reaction was repeated using the same amount of reactants, except that 200 ml. of ethyl acetate was employed as the solvent according to the procedure of Anderson and Degering (5). The by-product HCl distilled out of the reaction mixture, and the products were isolated by concentrating and cooling the reaction mixture. A 40% yield of *N*-phenylacetylsulfamide and a 15% yield of *N,N'*-bis(phenylacetyl)sulfamide were obtained.

***N*-Phenylacetylsulfamide *via* *N*-Lithiosulfamide**—To a stirred solution of sulfamide (9.60 g., 0.1 mole) in 300 ml. of tetrahydrofuran, a 15% solution of *n*-butyllithium in hexane (6.4 g., 0.1 mole) was added dropwise. After refluxing the resultant slurry of *N*-lithiosulfamide for 30 min., phenylacetyl chloride (15.5 g., 0.1 mole) was added dropwise with stirring. The reaction mixture was refluxed for 30 min. The resultant clear solution was evaporated to dryness, and the residue was extracted with boiling ethyl acetate. The extracts were washed with water, dried over anhydrous sodium sulfate, and concentrated until a precipitate began to separate. A crude product was obtained by the addition of toluene and subsequent cooling. Recrystallization from absolute ethanol yielded 7.0 g. (41.9%) of *N,N'*-bis(phenylacetyl)sulfamide, m.p. 171–172°, and 2.2 g. (10.0%) of *N*-phenylacetylsulfamide, m.p. 155–158°.

These products had IR absorption and NMR spectra identical to the previously reported product.

[2] Elemental analyses were performed by the Analytic Laboratory of the Midwest Research Institute, Kansas City, Mo.

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Solubility of Alkyl Benzoates III: Hydrogen Bonding and Solubility—A Theoretical Approach

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Abstract □ The theoretical aspects of the solubility features of a series of alkyl *p*-hydroxybenzoates (parabens), singly or as a combination (benzylparaben with each of the alkyl parabens in a homologous series ranging from methyl to *n*-butyl), were evaluated. The involvement of hydrogen bonding was considered. Both IR and NMR spectrometry were employed for analyses. The interrelation of molecular symmetry, solubility, and entropy was discussed.

Keyphrases □ Parabens, solubility—theoretical aspects □ Alkyl *p*-hydroxybenzoates—solubility, hydrogen bonding, theoretical aspects □ Solubility, alkyl *p*-hydroxybenzoates—theoretical aspects □ IR spectrophotometry—analysis □ NMR spectroscopy—analysis

The experimental results of previous communications (1, 2) on the solubility features of esters of *p*-hydroxybenzoic acid (parabens) revealed that the dielectric constant is a necessary, but not the only, property of a solvent accounting for its solubility. This became particularly obvious when the solubility of a homologous series of alkyl parabens (methyl, ethyl, *n*-propyl, and *n*-butyl) together with benzylparaben and methyl *p*-methoxybenzoate were determined separately and compared with the solubility values obtained when a combination of two of these parabens was used (each alkyl paraben was combined with benzylparaben and then dissolved). In the latter case, the solubility of both parabens increased considerably. Using 60% polyethylene glycol in water (PEG–H_2O) as a solvent in these experiments, the solubility data for benzylparaben in various concentrations of alkyl parabens were compared with the dielectric constant values of various concentrations of alkyl parabens. The results (2) were indicative of the involvement of a contributory factor, other than the dielectric constant of the solvent or solution, to solubility.

The mutual solubilizing potential observed as a result of combining two parabens was envisaged to be due to the participation of hydrogen bonding. Accordingly, several experiments were designed to determine the validity and the theoretical aspects of the proposed postulate. The theoretical consideration possesses the merit of applicability in a number of solubility problems confronting pharmaceutical scientists.

EXPERIMENTAL

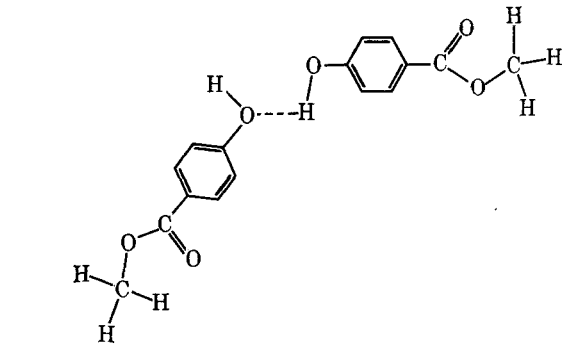
The solvents and solutions were prepared according to the previously reported method (1). The solutions were prepared in 60% PEG–H_2O, except for IR experiments where a spectral quality chloroform was employed. The IR spectra were determined using a Beckman IR-8 spectrophotometer. The NMR spectra were determined using a Varian Associate A-60 spectrophotometer.

The chloroform solutions for the determination of C=O stretching frequency were injected by means of a hypodermic syringe into liquid cells, pathlengths 0.25 and 0.50. A polystyrene film was used as a standard to make the necessary corrections for the positions of the bands.

RESULTS AND DISCUSSION

The results obtained previously (1) on the solubility features of a mixture of two parabens were indicative of the existence of a mutual solubilizing phenomenon among these esters. The pairs of parabens subjected to this evaluation were benzylparaben with methyl, ethyl, *n*-propyl, *n*-butyl paraben, or methyl *p*-methoxybenzoate.

The theoretical aspects of the solubility enhancement of combined parabens are expressed by considering the fact that a decrease in the dielectric constant of the solvent as a result of dissolving any one of the parabens should induce the solubility of its closely related homolog. The reported data (2) on the dielectric constant



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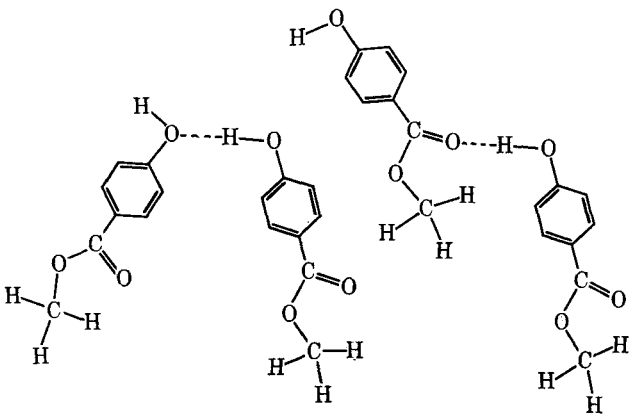

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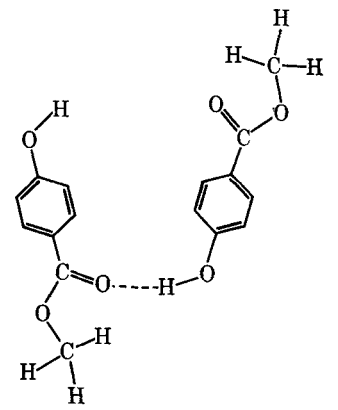

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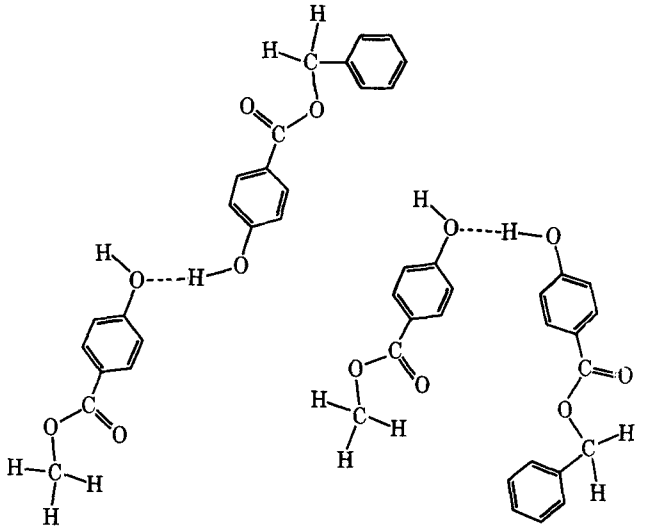

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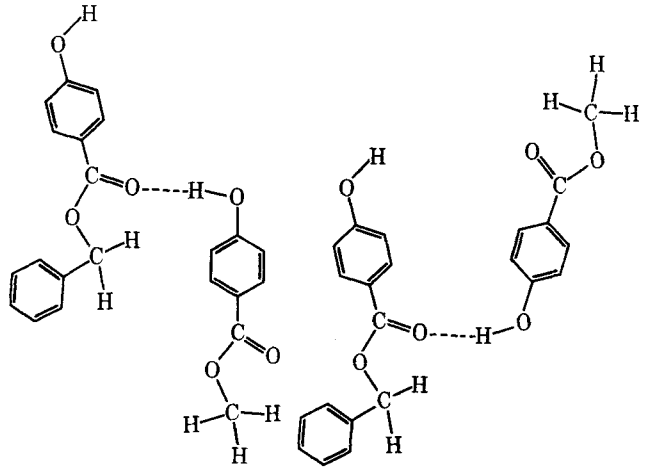

VII VIII

values of various concentrations of paraben solutions and the potential of each of the solutions to effect the solubility of benzyl paraben revealed that the dielectric constant was not a major factor in the enhancement of solubility. Both 0.1 *M* of methyl *p*-methoxybenzoate and 0.1 *M* of ethylparaben, prepared separately in 60% PEG–H_2O, possess nearly identical dielectric constants, 43.20 and 43.23, respectively. Their solubilizing potentials on benzylparaben are, however, quite different, 0.4412 *M* of benzylparaben in the former solution and 0.3753 *M* in the latter.

In the solution containing two different esters, the formation of dimers from two nonidentical molecules is more probable than from identical molecules. This argument is based on the fact that dimers I and II, as shown by the structural formulas, both possess elements of symmetry, whereas V and VI do not. The symmetry number, σ, which represents the number of equivalent orientations a molecule can assume as a result of rotation around an axis, enters into entropies of molecules. The contribution of entropy due to symmetry is $-R \ln \sigma$. Dimers I and II (transoid and cisoid, assuming both dimers are flat on the paper) thus possess an entropy value 2.76 cal./deg. mole lower than their counterparts V and VI.

In the case of hydrogen bonding between the C=O group of the ester and the phenolic OH, formation of dimeric species such as III and VII is not probable due to the proximity of the alkyl group and phenyl hydrogens. This can be visualized easily by observing the molecular models of these compounds. In the cisoid configurations (IV and VIII), the dimer VIII, because of possessing an entropy value 1.38 cal./deg. mole more than IV, will be the predominant dimeric species of such type. Thus, a solution containing two different parabens will consist of a high ratio of dimers comprised of two nonidentical moieties to dimers with identical moieties.

The hydrogen bonding between the C=O, HO, or the HO, HO does not necessarily require that both esters exist in the same plane. The coplanarity, however, is favored by considering the existence of orbital overlap in hydrogen bonding and the fact that overlap is most effective with orbitals having axes on a common plane. Nevertheless, assuming that some dimers are formed from monomers on two different planes, there seems to be a greater steric hindrance for dimers constructed *via* the C=O, OH than *via* the two phenolic OH's. Even if this relatively high probability of formation of the latter type of dimer is disregarded and only dimers having their monomers on a common plane are envisioned, dimers of types V and VI ought to predominate over VIII. This is because the entropy value of V and VI is 1.36 cal./deg. mole larger than the entropy value of VIII, a reasoning based on the analogy with two diastereoisomers, such as *cis*-1,3- and *trans*-1,3-dimethylcyclohexane, of which the *trans*, due to existing in *dl*-forms, contains an entropy value of $R \ln 2$ larger than *cis* (3). Another factor which may not favor the predominant existence of VIII is the requirement of a high energy of activation for the formation of this relatively rigid molecule.

The monomeric species of the esters that are in equilibrium with dimers will therefore originate to a larger extent from V and VI. An abundance of monomeric species or dimers of types V and VI in the solution of a mixture of two parabens *versus* the solution of a single paraben was shown by determining separately the IR spectra of 0.1 and 0.05 *M* methylparaben and benzylparaben and a 0.1 *M* solution of a mixture of these two parabens. The carbonyl stretching frequencies of 0.1 and 0.05 *M* of the solution of a single paraben (either methylparaben or benzylparaben) were at 1690 and 1703 $cm.^{-1}$, respectively. The band for 0.1 *M* of the mixture of the two was at 1698 $cm.^{-1}$. The presence of dimer V or VI may not be solely responsible for this shift. As the reported data, based on NMR spectrometry, indicate (1), in comparison with the individual solubility of benzylparaben and methylparaben, dissolving a mixture of these two increases solubility from 0.3 to 0.6 and 1.1 to 1.3 *M*, respectively. Such difference in solubility enhancement, therefore, cannot be attributed only to the abundance of V and VI but rather to the presence of more monomeric species.

The effect of dilution on the OH stretching frequency of the paraben solutions could not be investigated easily because of the limitation of the instrument. The regions between 3400 and 3600 $cm.^{-1}$ is quite condensed, and a variation of about 20 or 30 $cm.^{-1}$ due to twofold dilution cannot be detected readily. The NMR spectrometry for the OH resonance peak position as a function of dilution was explored. The OH proton peak positions for the solutions of single parabens and the mixture of parabens were found to be: 0.1 *M* methylparaben, δ 6.12; 0.1 *M* benzylparaben, δ 6.08; and 0.1 *M* of the mixture of the two, δ 6.10 (exactly at midpoint). In spite of these NMR data, the postulate relating solubility enhancement to a high proportion of monomers V and VI still holds correct. The occurrence of hydrogen bonds between relatively acidic phenolic protons and relatively electronegative Cl of chloroform prevents observing a shift in the position of OH, by using either IR or NMR, whereas the C=O stretching frequency is not affected by such bonding.

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Some Considerations as to the Determination and Significance of Biologic Half-Life

MILO GIBALDI and HOWARD WEINTRAUB*

Abstract □ The biologic half-life of a drug as determined from the terminal exponential phase of appropriate semilogarithmic plots is shown to dictate the steady-state levels of drug upon repetitive dosing, regardless of how low the plasma concentrations are or how little drug remains in the body, after a single dose, before the terminal exponential phase occurs. Accordingly, premature termination of pharmacokinetic studies may yield erroneous underestimates of half-life and, in turn, poor predictions of steady-state levels. Since the plasma levels of a drug after administration of a single dose may be so low upon attainment of the terminal exponential phase that they are not detected by presently available analytical methods, the true biologic half-life of certain drugs may be indeterminable from single-dose studies. The possible lack of attainment of the terminal exponential phase is also of concern in repetitive dosing pharmacokinetic studies where the half-life is determined after each dose. Under these conditions, not only may the true half-life be underestimated but the artifactual, apparent half-life may increase with increasing dose, erroneously suggesting self-inhibition of biotransformation processes or capacity-limited elimination.

Keyphrases □ Biologic half-life—theoretical considerations of determination, significance □ Computer-simulated data—determination, significance of biologic half-life □ Half-life, drug biologic—theoretical considerations for correct interpretation

Biologic half-life is probably the most critical pharmacokinetic parameter of a drug, since it markedly influences the duration of drug action as well as the degree of accumulation of the drug in the body upon repetitive dosing. Knowledge of the half-life of a drug can be extremely useful in a predictive sense, particularly with respect to the design of rational dosing regimens. However, since access to compartments in the biologic system and frequency of sampling are usually limited, experimental design is a critical consideration in pharmacokinetic studies, and even the determination of biologic half-life of a drug may pose considerable problems. Wagner (1) discussed several possible errors in the plotting and interpretation of semilogarithmic plots of blood level and urinary excretion data that may lead to gross inaccuracies in the estimation of half-life. In addition, the lack of a specific assay for a drug in the plasma or urine and the reliance upon levels of total radioactivity may yield a biased estimate of half-life (2, 3).

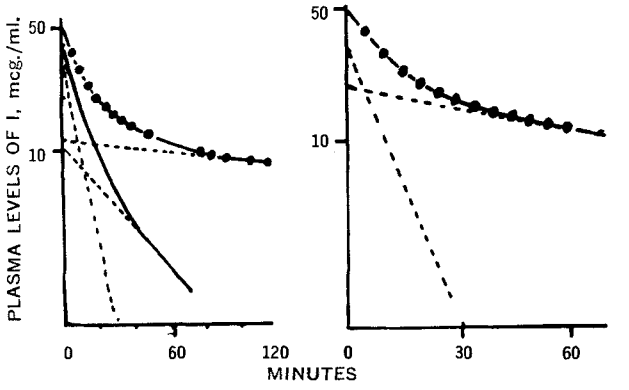
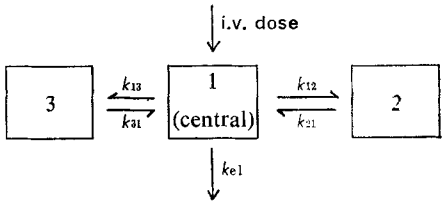


Figure 1—*Plasma levels of I in the dog after a single injection (20 mg./kg. i.v.) during a 1-hr. and 2-hr. experiment. Based on Figs. 1 and 2 in* Reference 4.



Scheme I—*Three-compartment open model proposed to describe the time course of I in the dog. According to Loo* et al. *(4),* $k_{12} = 2.28\ hr.^{-1}$, $k_{21} = 3.60\ hr.^{-1}$, $k_{13} = 1.50\ hr.^{-1}$, $k_{31} = 0.84\ hr.^{-1}$, *and* $k_{el} = 0.72\ hr.^{-1}$.

Another problem, which has received little attention to date, is the application of inappropriate mathematical models to blood level or urinary excretion data because of the limited duration of the sampling period. Pharmacokinetic studies may be terminated too early for several reasons. An arbitrary experimental protocol may call for blood sampling over a fixed period without considering the pharmacokinetic properties of the drug. Moreover, analytical methods may not be sufficiently sensitive, so that low but persistent levels of drug are not detected.

Under these conditions, significant underestimates of half-life may be obtained if two-compartment kinetics are erroneously treated as single-compartment kinetics or if a three-compartment model is interpreted in terms of the two-compartment model. An example of the latter situation is shown in Fig. 1. According to Loo *et al.* (4), the plasma levels of 5-(dimethyltriazeno)-imidazole-4-carboxamide (I), a cancer chemotherapeutic agent, over a 2-hr. period after intravenous administration of 20 mg./kg. in a dog can be described in terms of a three-compartment open model. Plasma concentrations (C_p) as a function of time (in minutes) are given by $C_p = 30.5 \exp(-0.117t) + 10.3 \exp(-0.028t) + 11.4 \exp(-0.003t)$, yielding a half-life of 231 min. for the terminal exponential phase (β-phase). However, if the determination of plasma level is halted 1 hr. after administration, a markedly different and incorrect pharmacokinetic profile emerges. In this case, plasma concentrations as a function of time (in minutes) are given by $C_p = 32.7 \exp(-0.111t) + 19.2 \exp(-0.008t)$, yielding a half-life of 87 min. for the apparent terminal exponential phase, which represents a threefold error.

The present report concerns the consequences of this type of error with respect to the prediction of drug accumulation and steady-state blood levels upon multi-

Table I—Nonlinear Least-Squares Regression Analysis of Simulated Central Compartment Levels of I after Several Doses of 100 mg. at Hourly Intervals According to the Equation: Levels = $A \exp(-\alpha)t + B \exp(-\beta)t$

Dose	Sum of Squared Deviations	A[a], mg.	α[a], hr.$^{-1}$	B[a], mg.	β[a], hr.$^{-1}$	$(t_{1/2})\beta$, hr.
1	0.024	63.91 (0.25)	6.418 (0.071)	34.92 (0.33)	0.479 (0.011)	1.45
2	0.035	65.42 (0.30)	6.273 (0.082)	54.95 (0.39)	0.387 (0.008)	1.79
4	0.041	65.94 (0.31)	6.217 (0.086)	82.71 (0.40)	0.314 (0.005)	2.21
8	0.043	66.09 (0.32)	6.200 (0.086)	115.00 (0.40)	0.274 (0.004)	2.53
12	0.044	66.12 (0.32)	6.194 (0.087)	130.58 (0.40)	0.262 (0.003)	2.65
16	0.044	66.14 (0.32)	6.193 (0.087)	138.10 (0.40)	0.257 (0.003)	2.70

[a] Parenthetic values beneath each parameter estimate denote the standard error of the estimate.

ple dosing as well as with respect to the determination of apparent half-lives during repetitive dosing.

METHOD

The pharmacokinetics of distribution and elimination of I in the dog were assumed to follow the three-compartment open model (Scheme I) suggested by Loo *et al.* (4). Simulated "body" and compartment levels as a function of time after initial and repetitive intravenous administration of I were obtained by using the appropriate differential equations and rate constants as input data for the "MIMED" digital computer analog-simulation program (5). These simulated levels were given equal weight and were used as input data for the digital computer program of Marquardt (6) to obtain nonlinear least-squares regression fits to the data.

RESULTS

Steady-State Blood Levels in Multicompartment Models—According to Wagner *et al.* (7), the average blood (serum) or plasma concentration of drug ($\bar{C}$) at steady state after multiple dosing is given by:

$$\bar{C} = F \cdot D/V \cdot K \cdot \tau \qquad \text{(Eq. 1)}$$

where D is the dose given at the beginning of each dose interval, F is the fraction of each dose absorbed, τ is the length (in units of time) of the dosage interval, and V is the apparent volume of distribution of the drug. According to Wagner *et al.* (7), K is the "first-order rate constant for overall loss of drug from the blood." It is also implicit in the work of a number of authors that the total area under the plasma concentration *versus* time curve (AUC) after a single dose is given by:

$$\text{AUC} = \int_0^\infty C(t)dt = F \cdot D/V \cdot K \qquad \text{(Eq. 2)}$$

Hence,

$$\bar{C} = \int_0^\infty C(t)dt/\tau \qquad \text{(Eq. 3)}$$

While it is clear that Eqs. 1 and 3 can be readily applied to a one-compartment model, some questions are raised as to how to apply these very useful relationships to multicompartment models. The major question relates to the meaning of K and V in the multicompartment model. A resolution of the problem is apparent based on the relationships developed by Gibaldi *et al.* (8). These workers showed that in a two-compartment model:

$$\int_0^\infty C(t)dt = F \cdot D/V \cdot \beta \qquad \text{(Eq. 4}$$

where V is an apparent volume of distribution[1], and β is 2.303 times the slope of the terminal exponential phase of the semilogarithmic plot of plasma concentration or amount of drug in the body *versus* time. It is implicit in Eqs. 22*a* and 23*a* of *Reference 8* that Eq. 4 is valid for any multicompartment model. The required assumptions are that elimination occurs from the central compartment and that the term β is related to the terminal exponential phase of the semilogarithmic plot of plasma or body level of drug *versus* time, regardless of the number of exponential phases that precedes the terminal phase. This relationship was also derived somewhat differently and independently by Westlake (9). Therefore,

$$\bar{C} = (F \cdot D)/V \cdot \beta \cdot \tau = (F \cdot D)\, t_{1/2}/0.693V \cdot \tau \qquad \text{(Eq. 5)}$$

where V is equivalent to $(V_d)_{\text{area}}$ or $(V_d)_\beta$ (8).

According to Eq. 5, it is clear that in the multicompartment situation, the plasma levels of drug or the amount of drug in the body, in the steady-state after multiple dosing, are related to the half-life of the drug as determined from the terminal exponential phase of the appropriate semilogarithmic plots. This half-life dictates the steady-state conditions, regardless of how low the plasma concentrations are or how little drug remains in the body after a single dose, before the terminal exponential phase occurs.

Hence, the type of error in the estimation of half-life discussed by Loo *et al.* (4) can have a profound effect on the prediction of steady-state levels. This is readily apparent if the erroneous β obtained from the plasma levels of I during the 1-hr. period after intravenous administration is used to predict steady-state levels of I upon repetitive dosing of 100 mg. every hour. Use of this incorrect estimate yields a prediction of $(\bar{C} \cdot V)$ equal to 208 mg. This is considerably lower than a $(\bar{C} \cdot V)$ value of 555 mg. predicted by using the β-value obtained from the plasma levels of I during the 2-hr. period after intravenous administration.

Since plasma levels of a drug after administration of a single dose may be so low upon attainment of the true β-phase that they are not detected by presently available analytical methods, it follows that the true biologic half-life of a drug, *i.e.*, $0.693/\beta$, may be indeterminable from single-dose pharmacokinetic studies. Hence, the authors suggest that the half-life of certain drugs be estimated from steady-state studies using Eq. 5.

Determination of Half-Life during Repetitive Dosing—An apparent advantage in repetitive dosing pharmacokinetic studies is that the half-life may be determined after each dose. Presumably, drug-related enzyme induction or inhibition, or capacity-limited biotransformation, may be revealed by monitoring the half-life as a function of the number of doses or the degree of drug accumulation. This approach, however, can artifactually produce apparent changes in half-life which have no relationship to changes in drug elimination. Obviously, during the course of a rational repetitive dosing study, the subsequent dose is administered long before the blood

[1] As discussed in *Reference 8*, V is actually a proportionality constant, with units of volume, which relates the plasma concentration of drug to the amount of drug in the body, once pseudodistribution equilibrium is attained.

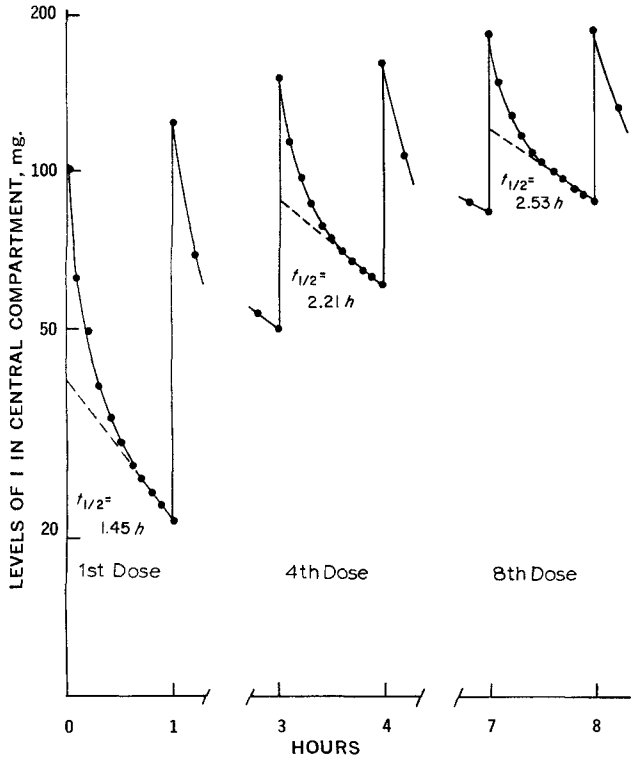


Figure 2—*Simulation of central compartment levels of I in the dog upon repetitive dosing of 100 mg. at hourly intervals employing the three-compartment open model shown in Scheme I. Interpretation of the curves according to a two-compartment model suggests erroneously that the half-life increases upon multiple dosing.*

levels of drug from the previous dose have run their time course and diminished to zero. Hence, determination of half-life during repetitive dosing may be based on blood levels that do not represent the terminal exponential (β) phase.

This artifact in itself can introduce serious error, but the repetitive dosing situation is still more complicated. During the course of dosing, the pharmacokinetically distinguishable body compartments that are in equilibrium with the central (plasma-containing) compartment contain more and more drug at the end of each dosing interval, and the levels asymptotically approach steady-state conditions. Hence, with each subsequent dose, a smaller and smaller fraction of the total amount of drug present must leave the central compartment and enter a peripheral compartment to achieve distribution equilibrium which signals the initiation of monoexponential loss of drug from the body (*i.e.*, the β-phase). This fraction becomes constant once steady state is achieved. Therefore, more and more of the true pharmacokinetic profile of a drug may be revealed during a given dosing interval as steady state is approached.

A possible consequence of this interesting distribution phenomenon is that a two-compartment model may be needed to describe plasma levels of the drug after the *n*th dose, while a one-compartment model is all that is *apparently* needed to describe plasma levels of the drug after the first dose. On the other hand, the net fraction of the drug distributed from the central compartment to a peripheral compartment may be so small at steady state that the existence of the compartment may not be apparent, whereas its existence is quite apparent after a single dose. However, given the limited data, particularly in human studies, a more likely consequence of this distribution phenomenon is that the apparent half-life of the drug will be assumed to increase upon repetitive dosing or upon elevating the body levels of drug.

To exemplify this situation, computer simulations were obtained of levels of I in the dog upon repetitive dosing. Figure 2 shows the central compartment levels of drug after intravenous administration of the first, fourth, and eighth doses. The time course of these curves are identical to the time course of plasma concentration curves. The simulated data after each dose were then subjected to nonlinear least-squares regression analysis. In agreement with Loo *et al.* (4), the data in each case were well described in terms of the two-compartment model, despite the fact that they were generated according to a three-compartment model. The appropriate pharmacokinetic and statistical parameters resulting from the curve fitting after each dose are summarized in Table I. It is evident from inspection of these data as well as from Fig. 2 that if the drug levels in the central compartment after each dose are interpreted in terms of the two-compartment model, then the apparent half-life increases upon repetitive dosing. Although application of the two-compartment model to these data is in error, such application is certainly reasonable from a statistical point of view (Table I).

Interestingly, Doluisio and Dittert (10) reported that the apparent half-life of several tetracyclines increased upon repetitive dosing. These workers found that the half-lives of tetracycline, demethylchlortetracycline, methacycline, and doxycycline increased from 6.3, 11.0, 7.0, and 8.3 hr., respectively, after a single dose to 10.8, 13.6, 14.3, and 16.6 hr., respectively, during multiple (4-day) administration of therapeutic doses every 12 hr. Van Rossum (11) recently suggested that these data should be interpreted in terms of a two-compartment model rather than the one-compartment model used by Doluisio and Dittert (10). Hence, although cursory examination of the data might suggest self-inhibition or capacity-limited elimination of tetracycline, it is more likely that the apparent changes in half-life upon repetitive dosing are due to the distribution phenomenon and mathematical artifacts already discussed.

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GLC Determination of New Antiarrhythmic Drug

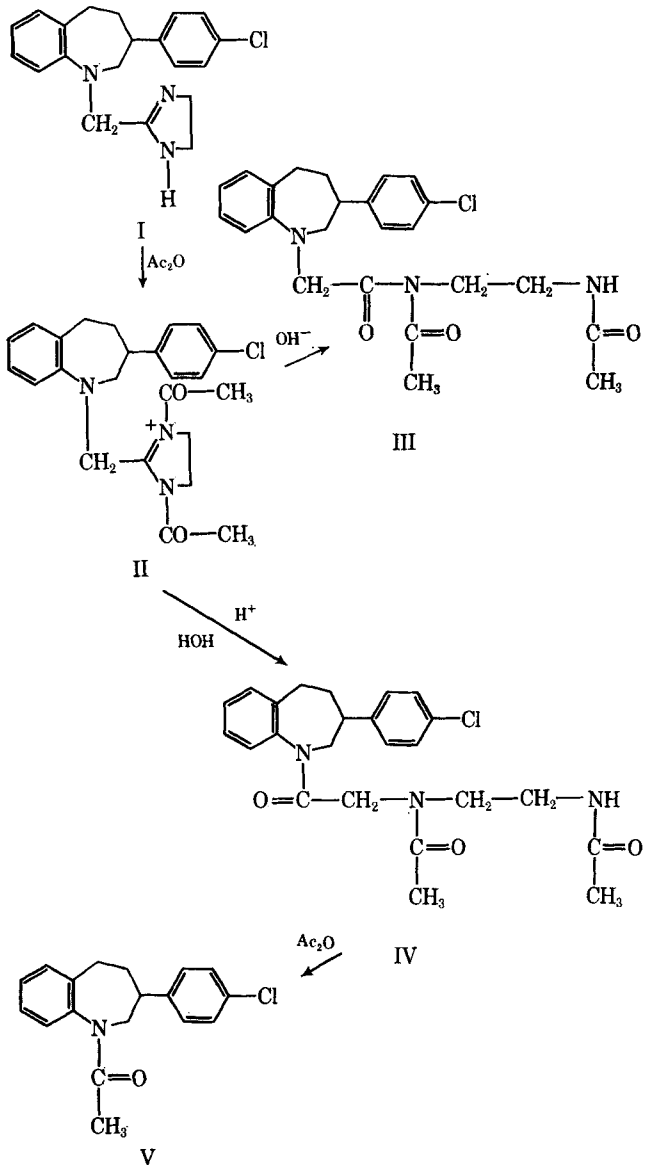
R. LUDERS*, P. O'BRIEN, and F. TISHLER†

Abstract □ A GLC method for the determination of a new antiarrhythmic compound is described. In an attempt to define the compound being chromatographed, isolation and identification techniques were carried out on the derivative and a mechanism for the unusual reaction is proposed.

Keyphrases □ 3-(*p*-Chlorophenyl)-2,3,4,5-tetrahydro-1-[(2-imidazolin-2-yl)-methyl]-1-benzazepine—GLC method of microgram quantification □ Derivative formation, antiarrhythmic drug—isolation, identification, mechanism □ GLC—determination, antiarrhythmic drug

The clinical introduction of a new antiarrhythmic compound, 3-(*p*-chlorophenyl)-2,3,4,5-tetrahydro-1-[(2-imidazolin-2-yl)-methyl]-1-benzazepine (Compound I, Scheme I) has made it necessary to develop methodology capable of detecting microgram quantities of the compound for pharmacokinetic studies.

Preliminary work indicated that Compound I could not be suitably chromatographed as the parent compound. Since Tishler *et al.* (1) showed previously that similar compounds could be acetylated at elevated conditions, this approach was undertaken and found to yield, at around 140°, a derivative suitable for quantitation work at the micro level. In an attempt to define the derivative, the reaction product was isolated and characterized by physical and chemical means, thereby leading to the proposed mechanism in Scheme I.



Scheme I—Proposed mechanism for the derivatization of 3-(p-chlorophenyl)-2,3,4,5-tetrahydro-1-[(2-imidazolin-2-yl)-methyl]-1-benzazepine

EXPERIMENTAL

Apparatus—GLC determinations were carried out on an instrument (Barber Colman model 5000) equipped with glass columns [2.44 m. (8 ft.) × 4 mm. i.d.], containing 1% polyethylene glycol (Carbowax) 20 M on 100–120 mesh Gas Chrom S, and a flame-ionization detector. The operating parameters were: nitrogen flow rate, 40 ml./min.; temperature of column, 235°; temperature of detector, 250°; temperature of flash heater, 250°; and electrometer sensitivity, 3×10^{-11} amp. full-scale.

Procedure—Five milliliters of the standard solution containing 0.5 mcg. of the HCl salt and 5 ml. of the sample solution are made alkaline with 0.4 ml. of 6 *N* sodium hydroxide. The samples are extracted twice with 5-ml. portions of a mixed solvent containing 10% methylene chloride, spectroquality, in ether. The appropriately combined extracts are evaporated to dryness with the aid of a stream of nitrogen in a 10-ml. centrifuge tube. The residues are reacted with 0.1 ml. of a 1:1 mixture of acetic anhydride–pyridine in a capped centrifuge tube for 20 min. in a 135° oil bath. The samples are again evaporated to dryness under a stream of nitrogen.

The residue is diluted with 10 μl. of dimethylformamide, and a 2-μl. sample is chromatographed. The concentration of the sample solution is obtained by graphically comparing its area (height and width at height/2) to the area of the standard solution. The response of the standard to the flame-ionization detector is linear over the microgram range needed.

For identification purposes, milligram quantities of the derivative were prepared as already described. The residue obtained was shown by TLC to be essentially one spot and therefore was used for characterization purposes without further purification.

RESULTS AND DISCUSSION

Based on earlier data generated by Tishler *et al.* (1), one would normally anticipate that either Compound III or IV would be the compound being chromatographed. However, UV, IR, NMR, and mass spectra data clearly indicated that the derivative was singly acetylated and that the acetyl group was connected to the nitrogen of the seven-membered ring.

To validate completely the proposed structure, Compound V was synthetically prepared by an alternate route. The various physical parameters, including gas chromatography retention time, obtained on the synthesized compound were identical to those of the residue isolated in the acetylation reaction, thereby confirming the proposed structure, V.

Although several pathways for the formation of Compound V can be proposed, the one shown in Scheme I is favored, based on data previously generated by Tishler *et al.* (1) on related morphanthridine compounds. The amide interchange (Compound IV to Compound V) was also reported by Rodionov and Zvorykina (2)

in their work on acylation reactions (Scheme II):

$$R{-}CH_2{-}CO{-}NH{-}Ph \xrightarrow[Ac_2O]{reflux} Ac{-}NH{-}Ph$$

Scheme II

The application of the gas chromatographic procedure was applied to plasma samples. A more detailed discussion (data substantiating the selection of the various parameters described under *Procedure*) of this application for pharmacokinetic studies will be presented at a later date.

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pH Dependence of Fluorescence of Riboflavin and Related Isoalloxazine Derivatives

S. G. SCHULMAN

Abstract □ pKa values were determined for several flavins by absorptiometry and by fluorimetry. The poor agreement between absorptiometrically and fluorimetrically determined pKa values is attributed to the fluorimetrically measured quantities being due to equilibria in the fluorescent state (lowest excited singlet state). The excited-state pKa's and the ground-state pKa's do not correspond to protonation at the same sites in the isoalloxazine ring. The significance of excited-state equilibria for fluorimetric analysis is considered.

Keyphrases □ Fluorescence, pH dependence—riboflavin, related isoalloxazine derivatives □ Riboflavin, related isoalloxazine derivatives—fluorescence pH dependence □ pH dependence—fluorescence of riboflavin, related isoalloxazine derivatives

Fluorimetric assay is probably the most widely employed method for the determination of riboflavin (1). The intense green fluorescence of riboflavin (2) and that of its photodecomposition product lumiflavin (3–5) have been used in quantitative analysis.

Riboflavin fluorescence is constant in intensity between pH 3 and 8 but is quenched at pH <3 and >8. As a result, it is necessary to maintain pH 3–8 in solutions upon which riboflavin assay is performed. Early studies of the fluorescence intensity of riboflavin as a function of pH yielded two pKa values of 2 and 10 (6). However, absorptiometric determination of the pKa's in the same acidity regions gave values of -0.2 and 9.8 (7). Subsequent measurements of the second pKa of riboflavin by electrochemical methods yielded values of 9.9 (8) and 10.0 (9). In light of the good agreement between the fluorimetrically, absorptiometrically, and electrometrically determined values of the second pKa, it seems reasonable to conclude that they all correspond to the same dissociation. However, the substantial difference between the value of the first pKa as determined by fluorimetric titration and that determined by absorptiometry suggests that the two pKa values do not correspond to identical protolytic processes.

The most obvious explanation for the latter dilemma is that the absorptiometric measurements yield a conventional pKa value corresponding to a protolytic dissociation in the ground state of the molecule, while the fluorescence measurements yield a pKa corresponding to a protolytic reaction of riboflavin in the electronically excited state from which fluorescence originates: the lowest excited singlet state (10). Hereafter, pKa values corresponding to electronically excited states will be referred to as pKa*. Prototropism in the lowest excited singlet state derives from the fact that rates of protonation and dissociation are often fast enough to compete with fluorescence for deactivation of the excited state of a fluorescent molecule. Consequently, the fluorescence intensity may reflect the relative concentrations of acid and conjugate base in the electronically excited state rather than the corresponding quantities in the ground state (as determined by absorptiometry or electrometry). Because the electronic distribution is generally different in an electronically excited molecule from that in the ground state of the same molecule, the binding energy of a proton and hence the pKa is different from that in the ground-state molecule. Obviously, it is as important to know pKa* values in fluorimetric analysis as it is to know pKa values in absorption spectrophotometric analysis in order to establish the pH range of optimum analytical utility.

To investigate more closely the differences between the pH dependencies of the fluorescence spectra and

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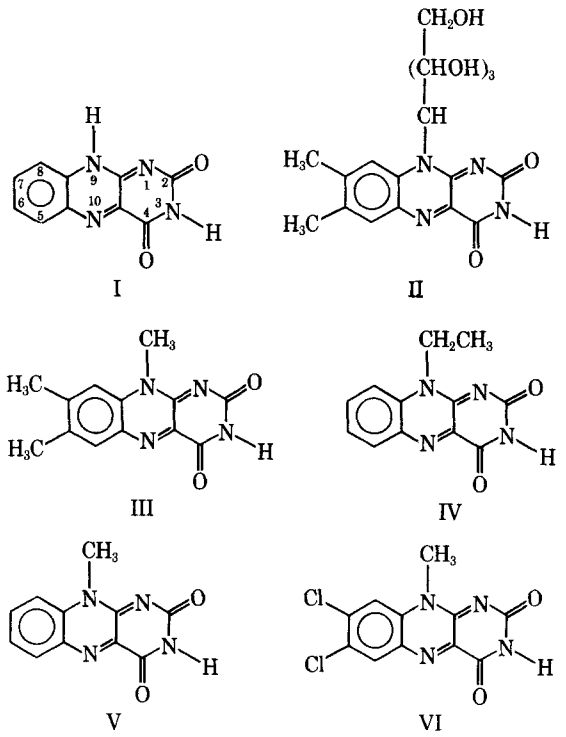
To investigate more closely the differences between the pH dependencies of the fluorescence spectra and

Table I—Features of the Absorption and Fluorescence Spectra of Riboflavin and Some Related Isoalloxazine Derivatives

	Long Wavelength Absorption Maxima				Fluorescence Maxima, pH 7.00, λ, nm.
	4.65 M $HClO_4$ (H_o = 2.02)		pH 7.00		
	λ, nm.	ε	λ, nm.	ε	
Riboflavin	395	1.18×10^4	447	1.19×10^4	517
Lumiflavin	368	3.43×10^4	433	3.22×10^4	509
9-Ethylisoalloxazine	370	3.36×10^4	433	3.33×10^4	507
9-Methylisoalloxazine	368	3.35×10^4	435	3.33×10^4	507
6,7-Dichloro-9-methyl-isoalloxazine		—		—	514

absorption spectra of riboflavin and some related isoalloxazine derivatives (flavins), the present study was undertaken. The structures of isoalloxazine (I), riboflavin (II), lumiflavin (III), 9-ethylisoalloxazine (IV), 9-methylisoalloxazine (V), and 6,7-dichloro-9-methylisoalloxazine (VI) are relevant to this study.



EXPERIMENTAL

Analytical reagent grade riboflavin was purchased[1] and was further purified by several recrystallizations from distilled deionized water. Pure samples of lumiflavin, 9-ethylisoalloxazine, 9-methylisoalloxazine, and 6,7-dichloro-9-methylisoalloxazine were used without further purification (11).[2] Absorption and fluorescence spectra were taken on 4.00×10^{-5} M solutions of all compounds studied in 1-cm. square quartz cells. Wherever possible, the same solutions were employed in luminesence and absorption studies. All solutions were deoxygenated by purging with dry nitrogen for 10 min. prior to recording of spectra. Mallinckrodt reagent grade perchloric acid and Beckman standard buffers were used to make solutions of known pH and Hammett acidity (12).

Fluorescence spectra were taken on a Farrand Mark I spectrofluorometer and recorded on a Heath model EU-20B strip chart recorder. The emission and excitation monochromators of the spectrofluorometer were calibrated against the mercury lines from a Pen-Ray low pressure mercury lamp. Emission and excitation spectra were not corrected for the wavelength dependencies of monochromator and phototube responses. However, this correction is not critical for the present work.

Absorption spectra were taken on a Beckman DB-G spectrophotometer. The pKa values were calculated from the pH (or Hammett acidity) dependences of the long wavelength absorption bands of the conjugate acid species according to

$$\text{pKa} = \text{pH (or } H_o) - \log \frac{\epsilon_A - \epsilon}{\epsilon - \epsilon_B} \qquad \text{(Eq. 1)}$$

where ϵ_A and ϵ_B are the ratios of absorbance to concentration at the acidic and basic limits, respectively, of the inflection region; ϵ represents the value of absorbance/concentration for any point in the inflection region of the titration; and H_o is the Hammett acidity. The pKa* values were estimated graphically from the value of pH at 50% fluorescence quenching in the plot of relative fluorescence intensity *versus* pH.

RESULTS AND DISCUSSION

The principal features of the absorption and fluorescence spectra of the flavins are listed in Table I. The absence of absorption spectral data for 6,7-dichloro-9-methylisoalloxazine in Table I is a result of the extremely low solubility of that compound in water. Consequently, no pKa was determined for it. However, due to the greater sensitivity of fluorescence compared with absorption, a value of pKa* was estimable even at the low concentration level of the 6,7-dichloro derivative at saturation in water. The absorptiometric titration curves are shown in Fig. 1, while the fluorimetric titration curves are presented in Fig. 2. The pKa and pKa* values estimated from the data of these curves are given in Table II.

The excellent agreement between all of the pKa values of Table II indicates that protonation occurs at the same site in the ground state for all of the compounds studied here. Similarly, the closeness of the pKa* values indicates that in the lowest excited singlet states of the flavins, the same site in the isoalloxazine ring is being protonated for all compounds studied. The slightly lower pKa* value of the 6,7-dichloro compound probably is due to the inductive effects of the chlorine atoms.

That the fluorescences of the flavins arise from electronically excited states having the same structures as the corresponding ground states in neutral solution is strongly suggested by the mirror-image relationships (13) extant between the fluorescence bands and the long wavelength absorption bands of the corresponding flavins.

Clearly, in all cases, the value of pKa* relative to pKa indicates that the protonated form of the fluorescent species is a significantly weaker acid in the excited state than in the ground state. The problem of the discrepancy between the fluorimetrically and absorptio-

Table II—pKa and pKa* Values of the Flavins[a]

	pKa	pKa*
Riboflavin	−0.1	1.7
Lumiflavin	0.0	1.7
9-Ethylisoalloxazine	0.0	1.7
9-Methylisoalloxazine	0.1	1.7
6,7-Dichloro-9-methyl-isoalloxazine	—	1.3

[a] Estimated from the data represented in Figs. 1 and 2, respectively.

[1] Mallinckrodt Chemical Works, Inc., St. Louis, Mo.
[2] Obtained from Dr. Gordon Tollin of the University of Arizona.

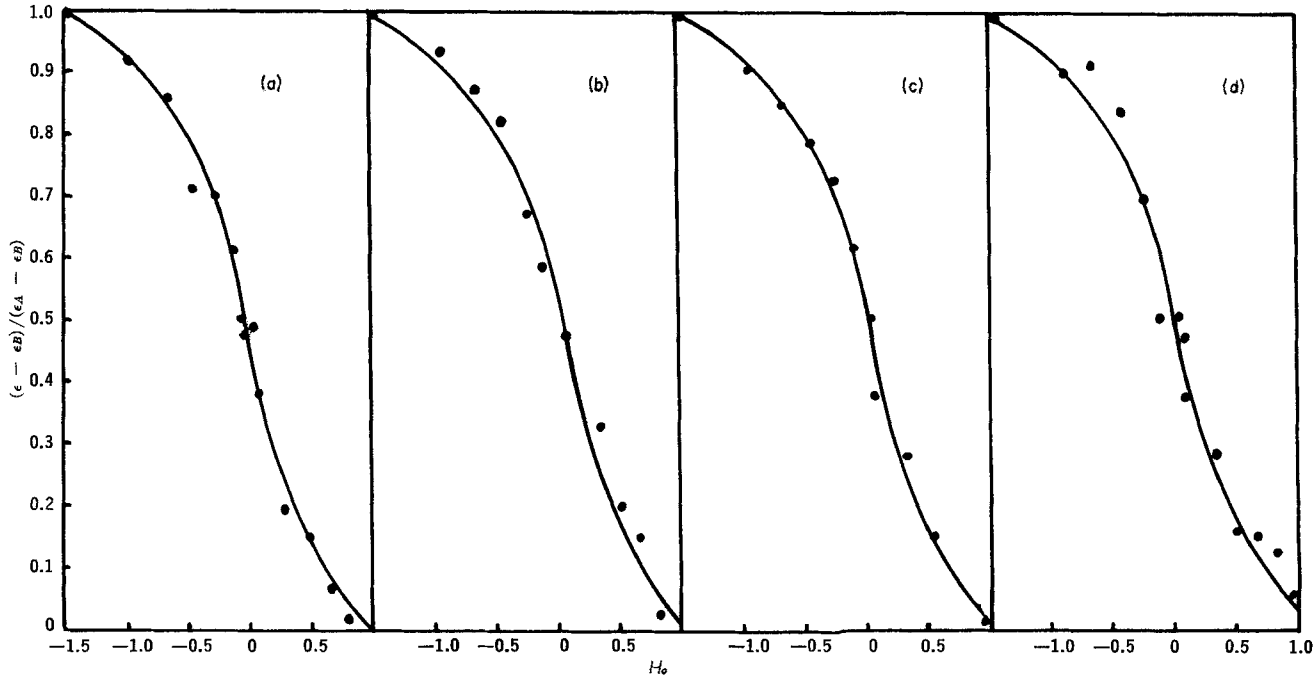


Figure 1—*Absorptiometric titrations of the flavins;* $(\epsilon - \epsilon_B)/(\epsilon_A - \epsilon_B)$ *is the fraction of flavin in the protonated form. Key: (a), riboflavin; (b), lumiflavin; (c), 9-ethylisoalloxazine; and (d), 9-methylisoalloxazine.*

metrically determined values of pKa in acid solution was considered by Weber (14). He concluded, from the shortening of the mean lifetime of the excited state as the riboflavin fluorescence was quenched by hydrogen ion, that the quenching occurred in the excited state (dynamic quenching). This is in agreement with the basic premise here. If quenching of the riboflavin fluorescence occurred as a result of a ground state acid–base reaction (static quenching), it would not be accompanied by an alteration of excited-state properties (lifetime). However, it is implicit in Weber's treatment that the excited-state protonated form and the ground-state protonated form of riboflavin are identical. This is not necessarily the case. In a polyfunctional molecule like a flavin, the redistribution of electronic charge accompanying electronic excitation often results in the site of protonation in the excited state being different from that in the ground state. For example, the quinolinols exist as neutral phenols in the ground state and are protonated at the ring nitrogen in acidic solutions. However, in the lowest excited singlet state, the quinolinols are zwitterions in neutral solution and are protonated at the phenolic oxygen in strongly acidic media (15). A rough estimate of the pKa* value of the excited-state equilibrium corresponding to the species involved in the absorptiometric titration (pKa*′) is to be had from the shift in the long wavelength absorption maxima, upon protonation, according to a thermodynamic treatment by Forster (16). In this treatment, pKa*′ is calculated from

$$\text{pKa}^{*\prime} = \text{pKa} - 2.10 \times 10^{-3}(\bar{\nu}_a - \bar{\nu}_b) \qquad \text{(Eq. 2)}$$

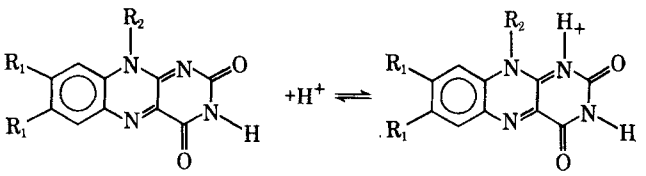
where pKa is the ground-state equilibrium involving the identical conjugate acid–base pair to which pKa*′ corresponds, and $\bar{\nu}_a$ and $\bar{\nu}_b$ are the wavenumbers of the long wavelength maxima in the absorption spectra of acid and conjugate base, respectively. It must be borne in mind that pKa*′ values calculated from absorption spectral shifts frequently are different from those determined by fluorimetric titration, by as much as 3 pH units. This is a consequence of the differences between the vibrational and solvent structures of ground- and excited-state species (15). However, if these calculations are carefully interpreted, they may yield useful information. Application of Eq. 2 to the absorbance data of Table I yields the results presented in Table III.

Table III—pKa*′ Values and Absorption Maxima of Conjugate Acid ($\bar{\nu}_a$) and Conjugate Base ($\bar{\nu}_b$) Species Derived from the Flavins

	$\bar{\nu}_a$ (cm.$^{-1}$)	$\bar{\nu}_b$ (cm.$^{-1}$)	pKa*′
Riboflavin	2.53×10^4	2.24×10^4	−6.2
Lumiflavin	2.72×10^4	2.31×10^4	−8.5
9-Ethylisoalloxazine	2.70×10^4	2.30×10^4	−8.2
9-Methylisoalloxazine	2.72×10^4	2.30×10^4	−8.9

The pKa*′ values of Table III are substantially different from the pKa* values of Table II, even if the approximate nature of the calculation is taken into account. Consequently, it must be concluded that the excited-state prototropic equilibrium responsible for the quenching of the flavin fluorescence does not correspond to the ground-state equilibrium studied absorptiometrically. Since the conjugate base species are probably the same in both equilibria, the excited- and ground-state equilibria appear to differ in the conjugate acid species; *i.e.*, the site of protonation of the isoalloxazine ring is different in the excited state than in the ground state.

Self-consistent field molecular orbital calculations on isoalloxazine (17) have indicated that, in the ground state, the nitrogen atom in the 1-position has the highest π-electron charge density and should therefore be the most basic site on the isoalloxazine ring (18) (Scheme I). Some experimental work supports this conclusion (19). The molecular orbital calculations also predict that, in the lowest excited singlet state, the nitrogen atom in the 1-position becomes considerably less basic, in agreement with the values of pKa*′ calculated here from absorption spectra, and that the nitrogen atom in the 10-position becomes the most basic (Scheme II). In light of the supporting evidence, it is concluded that the ground-state equilibria of the flavins studied by absorptiometric titration are of the type shown in Scheme I, whereas the equilibria



Scheme I

studied in the lowest excited singlet state by fluorimetric titration are of the type shown in Scheme II.

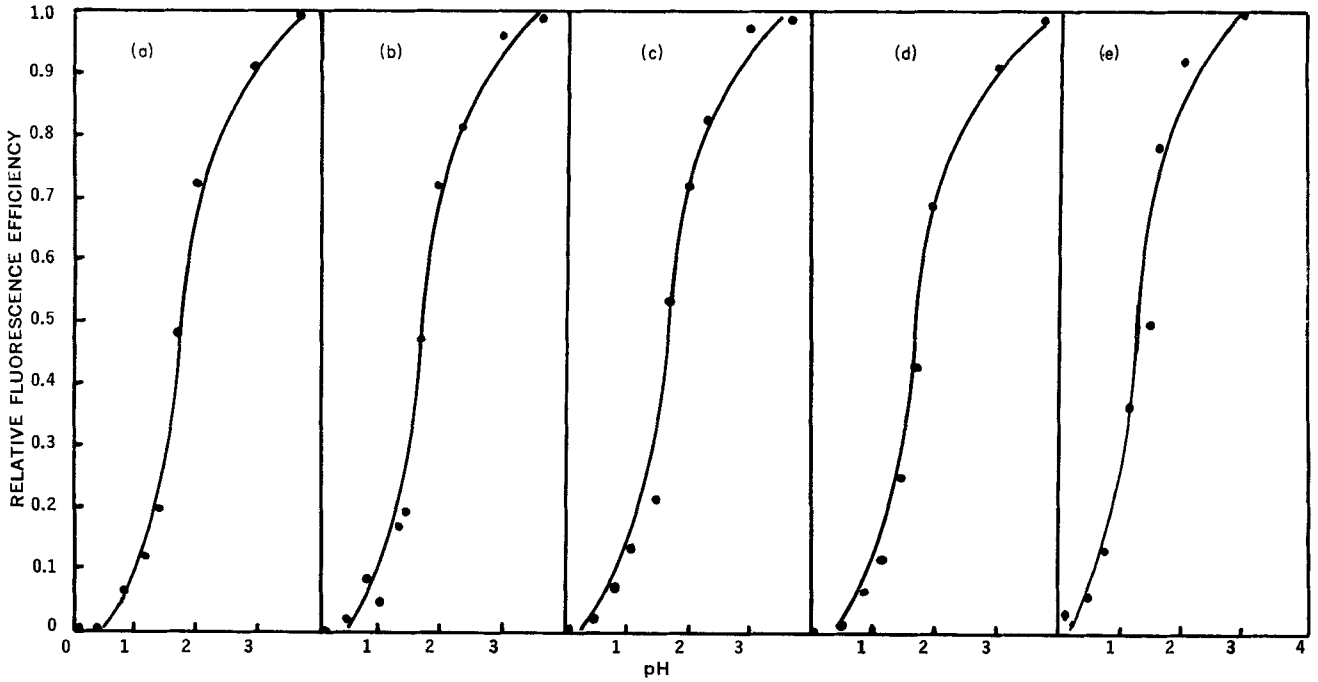
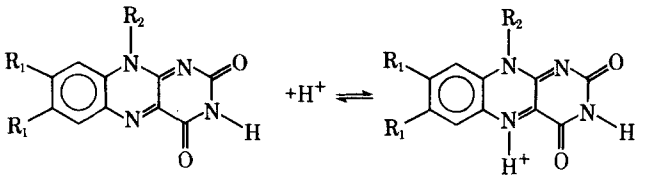


Figure 2—*Fluorimetric titrations of the flavins. Key: (a), riboflavin; (b), lumiflavin; (c), 9-ethylisoalloxazine; (d), 9-methylisoalloxazine; and (e), 6,7-dichloro-9-methylisoalloxazine.*



Scheme II

The excellent agreement between the pKa values of the flavins in the basic region (pKa ~ 10), whether determined by absorptiometry, electrometric measurement, or fluorimetry, is no doubt due to the fact that all three methods are determining the same ground-state pKa (probably of the abstraction of the amidic proton on the nitrogen in the 3-position by OH^-). The failure of fluorimetric titration in weakly basic solution to represent the excited-state dissociation is likely due to the diffusion-limited nature of the hydrogen-ion abstraction by OH^-. Consequently, in weakly basic solution where OH^- concentration is low, the reaction is not fast enough to compete with fluorescence for deactivation of the excited state. Therefore, the concentration of the absorbing species has prime importance in determining the intensity of fluorescence, so that the fluorescence intensity *versus* pH characteristics of the titration are much the same as a plot of absorbance *versus* pH. In support of this conclusion, Weber (14) found the mean lifetime of the excited state of the fluorescent species to vary only slightly as the fluorescence is quenched by OH^-, implying that the quenching in this case is static rather than dynamic.

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cis- and *trans*-2-(3,4,5-Trimethoxyphenyl)cyclohexylamines: *N*-Methyl and *N,N*-Dimethyl Derivatives

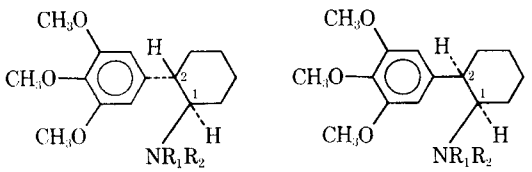
BETTY R. LOWRY and ALAIN C. HUITRIC

Abstract □ The *N*-methyl and *N,N*-dimethyl derivatives of *cis*- and *trans*-2-(3,4,5-trimethoxyphenyl)cyclohexylamines were prepared for evaluation of psychotropic activity. Synthesis by catalytic reductive methylation and characterization by NMR spectroscopy are reported. Magnetic nonequivalence of the methyl groups was observed in the *trans-N,N*-dimethyl derivative under acidic conditions where inversion of the nitrogen atom is suppressed.

Keyphrases □ 2-(3,4,5-Trimethoxyphenyl)cyclohexylamines, *cis* and *trans*—*N*-methyl and *N,N*-dimethyl derivatives, synthesis as potential psychotropic agents □ TLC—analysis □ NMR—structure

N-Methyl and *N,N*-dimethyl derivatives of *cis*- and *trans*-2-(3,4,5-trimethoxyphenyl)cyclohexylamines were prepared from known (1) *cis*- and *trans*-2-(3,4,5-trimethoxyphenyl)cyclohexylamines (II and I) by catalytic reductive alkylation with formaldehyde (2)[1]. The rate of the reductive alkylation is sufficiently faster on the primary than the secondary amines to afford predominance of the secondary amines III and IV when about 1 equivalent of formaldehyde is used. The use of excess formaldehyde produced the tertiary amines V and VI in good yields. A greater degree of monomethylation selectivity was obtained with the *cis*-amine II than with the *trans*-isomer I. This indicates a greater difference in the energy of activation for the reductive methylation of the primary *versus* secondary amine in the *cis*-isomer than in the *trans*-isomer, presumably because of steric factors.

An alternative two-step synthesis of *trans* secondary amine III by lithium aluminum hydride reduction of the formamide gave a lower overall yield than the reductive alkylation. The tertiary amine VI was also prepared in comparable yields by the less convenient sequence involving preparation of the methiodide from II, followed by dequaternization with sodium thiophenoxide in butanone (3). The Clark–Eschweiler preparation of V and VI utilizing formaldehyde and formic acid was not applicable in this system because of the highly activated aromatic ring which favors Pictet–Spengler cyclization. Cyclic derivatives of I and II will be reported elsewhere. The direct preparation of tertiary amines from 2-(3,4,5-trimethoxyphenyl)nitrocyclohexanes by catalytic reductive alkylation with formaldehyde, as reported for aromatic nitro compounds (2), was unsuccessful with these alicyclic compounds. Only a trace amount of V was obtained from the *trans*-nitro compound by this method.



I: $R_1 = R_2 = H$
III: $R_1 = H$, $R_2 = CH_3$
V: $R_1 = R_2 = CH_3$

II: $R_1 = R_2 = H$
IV: $R_1 = H$, $R_2 = CH_3$
VI: $R_1 = R_2 = CH_3$

Table I—60-MHz. NMR Spectra of 2-(3,4,5-Trimethoxyphenyl)-cyclohexylamine Hydrochlorides in 88% HCOOH [Chemical Shift, τ (p.p.m.)]

Compound	ArH	OCH_3 (3,5)	OCH_3 (4)	H-1	H-2	N-CH_3[a]
I	3.28	6.10	6.16	~6.28	~7.2	–
II	3.30	6.10	6.14	~6.1	~6.8	–
III	3.22	6.06	6.12	~6.45	~7.16	7.19 t
IV	3.25	6.06	6.11	~6.2	~6.7	7.21 t
V	3.23	6.09	6.15	~6.26	~7.15	7.01 d 7.13 d
VI	3.15	6.07	6.12	~6.30	~6.30	7.08 d

[a] t and d stand for triplet and doublet, respectively.

The extent of methylation was readily determined by NMR through integration of the methylamino protons. The multiplicity of the signals of the *N*-methyl protons in highly acidic media provided additional NMR differentiation between monomethylamino and dimethylamino compounds. Under highly acidic conditions, the rate of N—H proton exchange is greatly decreased, and coupling can be seen between the ammonium and *N*-methyl protons, giving distinctive triplets or doublets for the signals of the *N*-methyl groups for the secondary and tertiary amines, respectively. The entire series was, therefore, characterized by NMR as the hydrochloride salts in 88% formic acid.

The spectral data are summarized in Table I, including the data of the nonmethylated precursors I and II for reference. The N—CH_3 signals of the secondary amines III and IV accounted for three protons and occurred as triplets with J = 5.2–5.5 Hz. Products of exhaustive reductive alkylations were characterized as the dimethylamine derivatives by the N—CH_3 signals accounting for six protons and their occurrence as doublets with peak separations of 5.2 Hz. The two methyl groups had identical chemical shifts in the *cis*-isomer VI but were nonequivalent in the *trans*-isomer V, in which two sets of doublets occurred (τ 7.01 and 7.13). The nonequivalence results from a time-average unequal population of rotamers about the C_1—N bond causing the two methyl groups to be affected differently by the long-range shielding effects of the magnetic anisotropy of the aromatic ring. Suppression of the inversion of the nitrogen atom[2]

[1] The compounds were submitted to Eli Lilly and Co. for pharmacological evaluation, January 1969.

[2] This suppression of inversion in the acidic medium is indicated from the observed coupling between the ammonium and N–CH_3 protons which requires a slow N–H proton exchange on the NMR time scale.

(on the NMR time scale) is required for the observed nonequivalence. As expected, the nonequivalence is not seen in the spectra of the free amine in deuteriochloroform or pyridine. The chemical shifts of the two 3,5-methoxy groups were equivalent in all cases but different from the group at position 4. The two aromatic hydrogens were equivalent in all cases. The signals of H-1 were broad unresolved multiplets, sometimes partially overlapped with the signals of the methoxy groups, and their chemical shifts could not always be assigned with certainty. Coupling of H-1 with the N—H protons contributes to the broadening of these signals. Likewise, accurate assignment of chemical shifts to signals of H-2 was not possible in all cases because of partial overlapping by other signals.

The NMR spectra of the tertiary amines V and VI were also examined in deuteriochloroform and pyridine solution. In deuteriochloroform, V gave a singlet at τ 3.56 for two aromatic protons, a singlet at τ 6.15 for all methoxyl protons, a singlet at τ 7.81 for methylamino protons, and a broad signal centered at τ 7.40 representing overlapping H-1 and H-2 signals. Similarly, VI exhibited a singlet at τ 3.28 for the two aromatic protons, a singlet at τ 6.17 for the nine methoxyl protons, and a singlet at τ 7.88 for the six *N*-methyl protons. The signal of H-2 was partially overlapped by signals of cyclohexane protons on unsubstituted positions. The signal of H-1 gave an unresolved multiplet of half-width of about 11 Hz. at τ 6.88. The width of the signal of this equatorial H-1 was slightly wider than for the corresponding *cis* primary amine II measured in tetrachloroethylene (1). This suggests that in deuteriochloroform the *cis*-*N*,*N*-dimethylamino compound, VI, may not be entirely in a single chair conformation. In Compound II, the high predominance of a single chair conformation was verified from the signal of H-2 (1). The same analysis is not possible for VI because of partial overlapping of the signal of H-2. In the *cis*-isomers, deviation from preponderance of a single chair conformation with the aromatic group in an equatorial orientation is not unexpected because the bulkiness of the substituted amino group is increased. This deviation will be greater for the protonated form where inversion of the nitrogen atom is suppressed and where nitrogen has the sp^3 hybridization. In the present series the deviation should be greatest for Compound VI in strongly acidic medium. The broad signal of H-1 for VI in formic acid is consistent with this interpretation. The signal of H-2 is also partially overlapped in formic acid.

EXPERIMENTAL[3]

The NMR spectra were recorded either on a Varian A-60 or Varian T-60 spectrometer at an operating temperature of about 37°. The melting points were determined on a Kofler micro hot stage unless otherwise indicated.

[3] Elemental analyses were done by A. Bernhardt, Mulheim, Germany, or Huffman Laboratories, Wheatridge, Colo.

The *cis*- and *trans*-2-(3,4,5-trimethoxyphenyl)cyclohexylamines, I and II, were prepared by the method reported earlier (1). The free base II, previously described as a liquid, was obtained as a crystalline solid, m.p. 45–47°, upon crystallization from hexane.

General Methylation Procedure—A solution of 1–4 mmoles of primary amine I or II in 50–100 ml. 95% ethanol was treated with aqueous 37% formaldehyde solution and then hydrogenated on a Parr apparatus at an initial pressure of 30 p.s.i. in the presence of 10% palladium on charcoal until hydrogen uptake ceased. For monomethylation, 1.1–1.2 equivalents of formaldehyde was employed, while a large excess (about 60 mmoles) of the aqueous solution was used for dimethylation. About 5–10% of the catalyst was used based on weight of the amine. The catalytic reductive methylation of I was rapid, but was much slower with II, presumably due to slow formation of the imine intermediate. Following removal of catalyst by filtration, the solution was acidified with acetic acid and evaporated. The free base was regenerated with KOH and extracted with benzene, and the resulting solution was dried (anhydrous Na_2SO_4) and evaporated under reduced pressure to give the crude amine.

Hydrochloride salts were prepared by saturation of a benzene–petroleum ether solution of the amine with HCl gas and isolated by evaporation and crystallization of the residue from an appropriate solvent.

***N*-Methyl-*trans*-2-(3,4,5-trimethoxyphenyl)cyclohexylamine (III)**—TLC of crude III indicated contamination with small amount of I and V. Separation was effected by chromatography on silica gel with acetone and acetone–methanol, whereupon III eluted last. A benzene–petroleum ether solution of this amine was filtered and converted to the hydrochloride in the usual manner. Crystallization yielded 52% of III hydrochloride, m.p. 190–192.5° dec., from isopropanol–ethyl acetate, and m.p. 202–202.5° dec. from isopropanol–hexane.

Anal.—Calcd. for $C_{16}H_{26}ClNO_3$: C, 60.84; H, 8.30; N, 4.44. Found: C, 60.76; H, 8.07; N, 4.47.

***N*-Methyl-*cis*-2-(3,4,5-trimethoxyphenyl)cyclohexylamine (IV)**—TLC of crude IV indicated little contamination. The product was readily isolated as the free base in 83.5% yield by crystallization from hexane and had m.p. 65.5–67.5° (Fisher-Johns). IV hydrochloride had m.p. 241–241.5° dec. upon crystallization from ethanol.

Anal.—Calcd. for $C_{16}H_{26}ClNO_3$: C, 60.84; H, 8.30; N, 4.44. Found: C, 60.53; H, 8.05; N, 4.45.

***N*,*N*-Dimethyl-*trans*-2-(3,4,5-trimethoxyphenyl)cyclohexylamine (V)**—Isolation of the product afforded 86.1% as the hydrochloride, m.p. 247–248° dec., upon crystallization from isopropanol.

Anal.—Calcd. for $C_{17}H_{28}ClNO_3$: C, 61.90; H, 8.56; N, 4.25. Found: C, 61.89; H, 8.54; N, 4.27.

The free base V was crystallized from hexane, giving colorless plates, m.p. 75–76.5°.

***N*,*N*-Dimethyl-*cis*-2-(3,4,5-trimethoxyphenyl)cyclohexylamine (VI)**—Isolation afforded 76.7% of VI hydrochloride, m.p. 210–210.5° dec., on crystallization from isopropanol–ethyl acetate.

Anal.—Calcd. for $C_{17}H_{28}ClNO_3$: C, 61.90; H, 8.56; N, 4.25. Found: C, 62.06; H, 8.53; N, 4.29.

Free amine VI crystallized from hexane, m.p. 75.5–77.0°.

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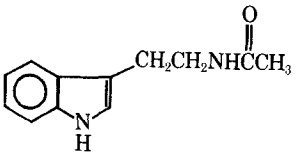
Hydroxyindole-*O*-methyltransferase V: Effects of Substituents on Hydrolysis of *N*-Acyltryptamines in Rats

BENG T. HO, G. EDWARD FRITCHIE, MICHAEL B. NOEL, and WILLIAM M. McISAAC

Abstract □ The effects of substituents on the hydrolysis of the amide linkage were studied in rats with five tritium-labeled, substituted *N*-benzoyltryptamines and *N*-phenylacetyltryptamines. In the blood, amides of phenylacetic acid were hydrolyzed at a faster rate than those of benzoic acid. Methylation of the amide nitrogen facilitated hydrolysis rather than retarding it. These five inhibitors of hydroxyindole-*O*-methyltransferase accumulated in pineal glands 15 min. after intravenous injection. Substitution of the chlorine atom on 3- and 4-positions of the phenyl ring decreased the transport into the pineal glands but retarded the metabolism in the organ. Of the compounds studied, *N*-phenylacetyltryptamine demonstrated the longest duration in the rat pineal gland.

Keyphrases □ Hydroxyindole-*O*-methyltransferase—substituted *N*-acyltryptamines, effect on hydrolysis, rats □ *N*-Acyltryptamines, substituted—effect on hydroxyindole-*O*-methyltransferase hydrolysis, rats □ TLC—separation

During studies of hydroxyindole-*O*-methyltransferase, a series of *N*-acyltryptamines was synthesized and tested as inhibitors of the enzyme (1–3). Replacement of the methyl group of *N*-acetyltryptamine (I) by a phenyl



I

or phenylmethylene group gave Compounds II and V which were four and eight times, respectively, better inhibitors than I (1). The activity was increased further by chlorine substitution on the phenyl ring, bromine substitution on the C-5 position of the indole nucleus, or both. The 3,4-dichlorophenyl compound (III) and the 5-bromo-3,4-dichloro compound (IV) were 70 and 280 times, respectively, more active than I (2). Methylation of the amide nitrogen, however, did not cause any change in the activity of V.

To explore the relative stability of these inhibitors *in vivo*, tritium-labeled compounds were synthesized, and the effects of substituents on the hydrolysis of amide linkages were studied in rats.

EXPERIMENTAL

Tritiation of 5-bromotryptamine was performed by a tritium–hydrogen exchange method (4). The 5-bromotryptamine-^{3}H was then acylated as previously described (1, 2). The specific activities (microcuries per milligram) obtained are as follows: II, 166; III, 120; IV, 80; V, 174; and VI, 123. 5-Bromoindole-3-acetic acid was purchased[1] and 5-bromotryptamine (m.p. 77–80°, ether) was prepared as previously described (2). 5-Bromotryptophol was synthesized by the following procedure.

To a stirred suspension of 150 mg. (4 mmoles) of $LiAlH_4$ in 150 ml. of anhydrous ether was added 508 mg. (2 mmoles) of 5-bromoindole-3-acetic acid in 30 ml. of ether. The mixture was refluxed for 3 hr., decomposed with 10% NaOH, and then filtered. The solid on the filter was extracted with hot ether (four 150-ml. portions). The combined ether filtrate and extracts were dried (Na_2SO_4), treated with charcoal, filtered, and evaporated *in vacuo*. The crude product crystallized from $CHCl_3$ upon cooling to give 400 mg. (83%) of yellow-pink solid, m.p. 84–87°. Subsequent recrystallizations from chloroform gave an analytical sample, m.p. 87–88°. $\nu_{max.}$ (KBr): 3400 cm.$^{-1}$ (OH), 60 MHz. NMR (CD_3—$COCD_3$): 2.28 (singlet, H—4); 2.80 (multiplet, H—2, H—5, H—6); 6.20 (triplet, J = 7.0 Hz., CH_2—OH); 7.05 J (triplet, J = 7.0 Hz., CH_2).

Anal.—Calcd. for $C_{10}H_{10}BrNO$: C, 50.02; H, 4.20; N, 5.83. Found: C, 50.06; H, 4.23; N, 5.59.

Male Sprague-Dawley rats, weighing 200–250 g., were injected intravenously with 25 mmoles/kg. of each tritiated compound in 0.1 ml. of propylene glycol. The animals were sacrificed by decapitation at the designated time intervals. Three rats were used for each time interval, except for Compound IV which had four rats per time interval. A 0.1-ml. aliquot of blood was diluted with 3 ml. of methanol and 15 ml. of liquifluor containing phenyl-oxazolyl-phenyl-oxazolyl-phenyl, 2,5-diphenyloxazole, and toluene, and it was assayed for tritium in a Nuclear Chicago Mark I liquid scintillation spectrometer. All values were corrected for 100% efficiency (channel ratio) and recovery. The brains and pineal glands were dissected and homogenized separately in 4 and 200 parts of water, respectively. Aliquots (0.1 ml.) of the homogenates were assayed for tritium.

The blood and tissue homogenates were then extracted with acetone until over 95% of the radioactivity was taken up (2 × 10 ml.). The acetone was evaporated to a small volume, spotted on silica gel TLC plates, and developed in ethyl acetate. From the chromatograms, silica in bands (0.5 cm. wide) was scraped into counting vials, digested with the methanol and liquifluor mixture, and then assayed for tritium. On each plate, the unchanged compound was identified by its R_f value and isotope dilution. Percent unchanged was calculated from: disintegrations per minute of unchanged compound/total disintegrations per minute from TLC plate × 100.

DISCUSSION

Fifteen minutes following the intravenous injections of the five compounds (II–VI), high radioactivity appeared in all pineal glands, indicating the accumulation of the hydroxyindole-*O*-methyltransferase inhibitors at the site of action (Table I). Substitution of the chlorine atom on 3- and 4-positions of the phenyl ring of II decreased the transport of the compound (III) into the brain and pineal gland. This was likely due to a lower level of III in blood as compared to II. The low uptake of III in the brain was improved considerably by placing a bromine atom on the 5-position of the indole nucleus to give IV.

In the blood, amides of phenylacetic acid (V and VI) were hydrolyzed at a faster rate than those of benzoic acid (II–IV), as seen in the presence of lesser amounts of unchanged compounds (Table I). Although the methylation of the amide nitrogen of V resulted in a higher level of VI in the blood, it facilitated hydrolysis rather than retarding it. Concurrently, the unchanged compounds with phenylmethylene linkage (V and VI) were present in lower percentages in the brain than those with a phenyl group (II–IV).

Of the five compounds studied, V demonstrated the longest duration in rat pineal gland. The concentration of unchanged molecules remained highest at all time intervals. No unchanged V was detected in the brain after 2 hr.

The effects of substitution on the rate of metabolism were observed. The metabolism of III and IV appeared to be slow in the brain and pineal gland as a result of introducing 3,4-dichlorine sub-

[1] Aldrich Chemical Co.

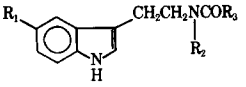
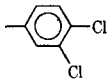
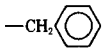
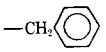


Table I—Distribution of Total and Unchanged Compounds in Rat Tissues[a]

Number	Compound R_1	Compound R_2	Compound R_3	Tissue	Micromoles per Gram Tissue or per Milliliter Blood[b] 15 min. Total	15 min. Unchanged[c]	1 hr. Total	1 hr. Unchanged[c]	2 hr. Total	2 hr. Unchanged[c]	4 hr. Total	4 hr. Unchanged[c]
II	H	H		Brain	30.3	20.3	6.6	4.6	4.7	3.9	7.7	2.6
				Pineal	100.4	74.3	30.5	9.8	21.2	6.6	1.1	0
				Blood	33.6	24.5	14.8	1.8	5.2	0.6	12.2	0.2
III	H	H		Brain	8.5	7.1	2.1	1.8	2.4	1.2	1.6	1.3
				Pineal	59.2	48.5	25.3	16.4	16.6	10.1	0.6	0.4
				Blood	5.6	4.5	1.4	1.2	1.8	1.7	1.3	1.1
IV	Br	H		Brain	30.1	26.2	14.7	12.8	7.5	4.7	3.6	2.0
				Pineal	60.8	31.0	33.6	14.4	16.2	3.9	10.2	1.6
				Blood	11.2	9.9	10.0	3.5	10.3	2.4	5.3	0.9
V	H	H		Brain	29.5	13.0	13.2	11.6	7.4	7.1	8.2	8.1
				Pineal	101.4	75.0	5.5	1.5	6.6	2.9	5.3	1.9
				Blood	11.2	4.4	8.2	3.8	8.3	1.5	5.4	0.3
VI	H	CH_3		Brain	33.4	12.0	17.5	4.0	13.9	0	9.4	0
				Pineal	147.2	83.9	56.2	17.4	62.3	16.2	64.3	4.5
				Blood	21.1	3.8	12.6	3.2	6.6	1.3	10.0	0.2

[a] Each compound (25 mmoles/kg.) was administered intravenously. [b] Each value represents the mean of three rats (four in IV); each determination was done in duplicate. [c] Silica gel TLC in ethyl acetate. R_f values were II, 0.85; III, 0.91; IV, 0.85; V, 0.85; and VI, 0.75.

Table II—Distribution of Metabolites of 5-Bromo-3,4-dichlorobenzoyltryptamine in Rat Tissue

Tissue	Time	Unchanged Compound %	Unchanged Compound μmole[e]	5-BrT[a, d] %	5-BrT[a, d] μmole[e]	5-BrIAA[b, d] %	5-BrIAA[b, d] μmole[e]	5-BrTOL plus Unknown[c, d] %	5-BrTOL plus Unknown[c, d] μmole[e]
Brain	15 min.	87	26.0	3	0.9	8	2.4	2	0.6
	1 hr.	87	12.8	3	0.4	9	1.3	1	0.2
	2 hr.	63	4.7	7	0.5	11	0.8	19	1.4
	4 hr.	55	2.0	17	0.6	16	0.6	12	0.4
Pineal	15 min.	51	31.0	11	6.7	15	9.1	13	7.9
	1 hr.	43	14.4	16	5.4	18	6.0	23	7.7
	2 hr.	24	3.9	22	3.6	28	4.5	26	4.2
	4 hr.	16	1.6	24	2.4	22	2.2	48	4.9
Blood	15 min.	88	9.9	2	0.2	1	0.1	9	1.0
	1 hr.	35	3.5	16	1.6	19	1.9	28	2.8
	2 hr.	23	2.4	24	2.5	21	2.2	22	2.3
	4 hr.	17	0.9	32	1.7	28	1.5	23	1.2

[a] 5-BrT = 5-bromotryptamine. [b] 5-BrIAA = 5-bromoindole-3-acetic acid. [c] 5-BrTOL = 5-bromotryptophol. [d] Silica gel TLC in ethyl acetate. R_f values were 5-BrIAA, 0.58; 5-BrTOL, 0.26; and 5-BrT, 0.15. [e] Each value represents the mean of four rats; each determination was done in duplicate.

stitution to II. Placement of the methyl group on V resulted in a decrease in the metabolism of VI in the pineal but not in the brain.

Since IV was the best *in vitro* hydroxyindole-*O*-methyltransferase inhibitor, its metabolism in rats was studied in a more detailed fashion. Hydrolysis of the amide linkage *in vivo* gave 5-bromotryptamine, which was then subjected to the attack of monoamine oxidase to form 5-bromoindole-3-acetaldehyde. The aldehyde was either oxidized by aldehyde dehydrogenase to 5-bromoindole-3-acetic acid or reduced by alcohol dehydrogenase to 5-bromotryptophol. In rat brain, the metabolism of IV was slower than in the pineal or blood (Table II). In most of the later time intervals, the 5-bromotryptamine, 5-bromoindole-3-acetic acid, and possibly the 5-bromotryptophol were present in approximately the same amounts in the three tissues studied.

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Hydroxyindole-*O*-methyltransferase VI: Inhibitory Activities of Substituted Benzoyltryptamines and Benzenesulfonyltryptamines

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Abstract □ A number of benzoyltryptamines and benzenesulfonyltryptamines substituted on the phenyl ring with OH, NH_2, or $NHCOCH_3$ were synthesized, and their inhibitory activities on hydroxyindole-*O*-methyltransferase were evaluated. The results showed that the enzyme has tolerance for the $NHCOCH_3$ group on either the *meta*- or *ortho*-position.

Keyphrases □ Hydroxyindole-*O*-methyltransferase—tryptamines, benzoyl, benzenesulfonyl substituted, synthesis, inhibitory activities □ Tryptamines, benzoyl, benzenesulfonyl substituted, synthesis, inhibitory activities—hydroxyindole-*O*-methyltransferase □ Inhibitory activities—substituted tryptamines

A number of substituted *N*-benzoyltryptamines and benzenesulfonyltryptamines have been found to be good inhibitors of hydroxyindole-*O*-methyltransferase (1, 2). Chlorine substitution on the benzene ring of *N*-benzoyltryptamine, in general, increases inhibition of the enzyme (2). In the present work, some *N*-acetamidobenzoyltryptamines were synthesized to test tolerance of the enzyme for the $NHCOCH_3$ group at the *ortho*-, *meta*-, or *para*-position.

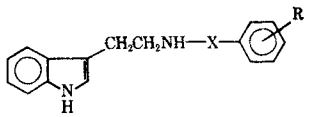
At the pH (7.8) at which the *in vitro* enzyme assay was performed, anilino groups of II, III, and IV exist mostly in the protonated form (Table I). From the observation that II and I were inhibitors of equal activity and III was only 1.2-fold less active than I (Table I), it seems that the *para*- and *meta*-NH_3^+ groups did not participate in enzyme-inhibitor attachment, nor did they exert any effect on binding of the phenyl ring to the enzyme. The phenolic OH group of VIII, however, was probably bound to the enzyme *via* hydrogen bonding and/or charge-transfer complex of the type HO:→*A*–*E*. This argument is based on the finding that the NH_3^+ of IV, which cannot be a donor, caused a 2.5-fold decrease in activity relative to I.

Acetylation of anilino groups of III and IV improved inhibitory activities 4.6 and 3.5 times, respectively, (see VI and VII), whereas the same substitution on II resulted in a 1.4-fold decrease in activity which, if significant, may suggest a limited bulk tolerance of the enzyme for the $NHCOCH_3$ group at the *para*-position. The increased activity from the acetyl group of VI and VII could be attributed to the presence of a carbonyl group whose oxygen atom is capable of serving as the donor in a hydrogen-bonding and/or charge-transfer interaction between the inhibitor and the enzyme. Attempts to prepare *N*-(*O*-acetylsalicyloyl)tryptamine were unsuccessful due to the instability of the acetoxy group; hydrolysis always occurred during workup as followed by GLC.

It was difficult to rationalize the twofold loss of inhibition when a *p*-amino group was substituted on the benzene ring of IX to give X, particularly since no difference in activity was observed between I and IX and between I and II. The *p*-$NHCOCH_3$ group gave further reduction of inhibition (see XI).

Results from the present study indicate that the enzyme has tolerance for the $NHCOCH_3$ group on either the *meta*- or *ortho*-position of the benzene ring of *N*-benzoyltryptamine. Thus, inhibitors bearing a group at one of these two positions, which is capable of forming a covalent bond with a nucleophilic group on the enzyme (3), might possibly be potential candidates for active-site-directed irreversible inhibition of hydroxyindole-*O*-methyltransferase. An irreversible inhibitor is needed to prolong the inhibition of hydroxyindole-*O*-methyltransferase *in vivo*, and an active-site-directed inhibitor would be advantageous for specificity. Syntheses of these compounds are currently being pursued in these laboratories.

Table I—Inhibition of Hydroxyindole-*O*-methyltransferase by



Compound	X	R	I_{50}[a], m*M*
I	CO	H	0.37[b]
II	CO	*p*-NH_2	0.32
III	CO	*m*-NH_2	0.46
IV	CO	*o*-NH_2	0.92
V	CO	*p*-$NHCOCH_3$	0.45
VI	CO	*m*-$NHCOCH_3$	0.10
VII	CO	*o*-$NHCOCH_3$	0.26
VIII	CO	*o*-OH	0.23
IX	SO_2	H	0.37[b]
X	SO_2	*p*-NH_2	0.75
XI	SO_2	*p*-$NHCOCH_3$	—[c]

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from *Reference 1*. [c] Due to limited solubility of the compound in the medium, the highest concentration tested was 1×10^{-3} *M*. A 15% inhibition was observed at this concentration.

EXPERIMENTAL[1]

***N*-*p*-Nitrobenzoyltryptamine**—To a solution of 4.7 g. (29 mmoles) of tryptamine and 3.5 g. (35 mmoles) of triethylamine in 50 ml. of chloroform was added dropwise a solution of 5.4 g. (30 mmoles) of *p*-nitrobenzoyl chloride. Precipitation took place almost immediately. After stirring the mixture at ambient temperature for 2 hr., 50 ml. of water was added. The solid was filtered and washed successively with 10% HCl (2 × 20 ml.), 2 *N* NaOH (2 × 20 ml.), and water (2 × 20 ml.); yield 8.5 g. (94.5%); m.p. 197–198°. Recrystallization from methoxyethanol–water gave 6.7 g. (74%), m.p. 200–201°.

Anal.—Calcd. for $C_{17}H_{15}N_3O_3$: C, 66.01; H, 4.89; N, 13.59. Found: C, 65.90; H, 4.75; N, 13.56.

[1] Melting points are corrected and were taken on a Mel-Temp apparatus.

N-m-**Nitrobenzoyltryptamine**—In a similar manner as described in the preparation of *N-p*-nitrobenzoyltryptamine, tryptamine was reacted with *m*-nitrobenzoyl chloride. The mixture was stirred overnight at ambient temperature, during which period precipitation occurred. The product weighed 8.4 g. (93.5%), m.p. 141.5–142°. After one recrystallization from methoxyethanol–water, the melting point was not changed.

Anal.—Calcd. for $C_{17}H_{15}N_3O_5$: C, 66.01; H, 4.89; N, 13.59. Found: C, 65.92; H, 4.69; N, 13.70.

N-o-**Nitrobenzoyltryptamine**—To a cooled solution of 16.7 g. (0.1 mole) of *o*-nitrobenzoic acid in 150 ml. of chloroform was added 10 ml. (0.13 mole) of thionyl chloride, and the mixture was stirred at 60° for 2 hr. After evaporation of the solvent and excess thionyl chloride, the oily residue was dissolved in 50 ml. of chloroform. It was added dropwise to a mixture prepared by neutralizing 20 g. (0.1 mole) of tryptamine hydrochloride in 100 ml. of water with 100 ml. 2 *N* NaOH and adding 75 ml. of chloroform to dissolve the liberated amine. Stirring the reaction mixture at ambient temperature for 1 hr. brought about the formation of an oily solid, which was filtered and washed with chloroform. The wet solid was dissolved in chloroform, and the solution was dried (Na_2SO_4), treated with charcoal, and filtered through diatomaceous earth[2]; upon chilling, it gave 20.8 g. (67%) of a bright-yellow solid, m.p. 110–112°. From the chloroform layer of the original reaction mixture and the mother liquor, an additional 20% of product was obtained (total yield 87%). Subsequent recrystallizations from chloroform gave an analytical sample as lustrous yellow platelets, m.p. 111–113°.

Anal.—Calcd. for $C_{17}H_{15}N_3O_5$: C, 66.01; H, 4.89; N, 13.59. Found: C, 65.89; H, 4.89; N, 13.62.

N-p-**Nitrobenzenesulfonyltryptamine**—A solution of 5 g. (25 mmoles) of tryptamine hydrochloride in 25 ml. of water was treated with charcoal and then filtered. The solution was shaken with 50 ml. of chloroform and 25 ml. of 2 *N* NaOH. *p*-Nitrobenzenesulfonyl chloride was added, and the shaking was continued for 5 min. A thick yellow oil, which separated from the mixture, solidified upon chilling. Recrystallization from methanol gave 7.2 g. (83.5%), m.p. 135–139°. Subsequent recrystallizations from chloroform gave an analytical sample as flocculent white needles, m.p. 138–139.5°.

Anal.—Calcd. for $C_{16}H_{15}N_3O_4S$: C, 55.64; H, 4.38; N, 12.17. Found: C, 55.49; H, 4.23; N, 12.07.

N-p-**Aminobenzoyltryptamine (II)**—A solution of 6 g. (19.4 mmoles) of *N-p*-nitrobenzoyltryptamine in 200 ml. of ethanol was shaken with hydrogen in the presence of 0.5 g. of 5% palladium on carbon catalyst until the consumption of hydrogen ceased (about 30 min.). The filtered solution was evaporated *in vacuo*, leaving 5.2 g. of product, m.p. 162–165°. Two recrystallizations from aqueous ethanol gave 3.4 g. (63%) of analytical sample, m.p. 167–169°.

Anal.—Calcd. for $C_{17}H_{17}N_3O$: C, 73.09; H, 6.13; N, 15.04. Found: C, 72.85; H, 6.14; N, 14.95.

N-m-**Aminobenzoyltryptamine (III)**—Hydrogenation of *N-m*-nitrobenzoyltryptamine was carried out in a similar manner as in the preparation of *N-p*-aminobenzoyltryptamine. The crude product (m.p. 153–155°) in 10% HCl was extracted with chloroform; the chloroform extracts were discarded, and the aqueous layer was made basic (pH 10) with 10% NaOH to precipitate the free amine. After recrystallization from aqueous ethanol, the yield of product (m.p. 156–157°) was 50%.

Anal.—Calcd. for $C_{17}H_{17}N_3O$: C, 73.09; H, 6.13; N, 15.04. Found: C, 72.95; H, 6.13; N, 14.89.

N-o-**Aminobenzoyltryptamine (IV)**—The amine, m.p. 160–161.5° (methanol), was obtained in 93% yield by catalytic reduction of *N-o*-nitrobenzoyltryptamine.

Anal.—Calcd. for $C_{17}H_{17}N_3O$: C, 73.09; H, 6.13; N, 15.04. Found: C, 73.18; H, 6.07; N, 15.08.

N-p-**Aminobenzenesulfonyltryptamine (X)**—Catalytic reduction of *N-o*-nitrobenzenesulfonyltryptamine gave 90% of the amine, m.p. 144–146° (aqueous acetone).

Anal.—Calcd. for $C_{16}H_{17}N_3O_2S$: C, 60.93; H, 5.43; N, 13.22. Found: C, 61.13; H, 5.44; N, 13.25.

N-p-**Acetamidobenzoyltryptamine (V)**—A solution of 1.4 g. (5 mmoles) of *N-p*-aminobenzoyltryptamine and 15 g. of acetic anhydride was stirred at ambient temperature for 2 hr., during which a pink precipitate deposited. The product was filtered and washed with water; yield 1.4 g. (87%); m.p. 204–205.5°. For recrystallization, the solid was dissolved in toluene with a small amount of ethanol and heptane added to turbidity. The melting point of the product remained unchanged after the recrystallization.

Anal.—Calcd. for $C_{19}H_{19}N_3O_2$: C, 71.01; H, 5.96; N, 13.08. Found: C, 71.24; H, 6.04; N, 12.91.

N-m-**Acetamidobenzoyltryptamine (VI)**—Acetylation of *N-m*-aminobenzoyltryptamine gave 88% of crude product, m.p. 158–160°. Recrystallization from the same solvent system as described for V yielded 67% of analytically pure sample, m.p. 160.5–161.5°.

Anal.—Calcd. for $C_{19}H_{19}N_3O_2$: C, 71.01; H, 5.96; N, 13.08. Found: C, 70.99; H, 5.70; N, 12.86.

N-o-**Acetamidobenzoyltryptamine (VII)**—To a chilled solution of 2.35 g. (8.5 mmoles) of *N-o*-aminobenzoyltryptamine in 50 ml. of chloroform, containing 2 ml. of pyridine, was added 4 ml. (42.4 mmoles) of acetic anhydride. The mixture was stirred overnight at ambient temperature. Chloroform was evaporated *in vacuo*, and the residual oil solidified upon trituration with ice water. Recrystallization of the crude product from ethyl acetate–ether gave 2.58 g. (96%) of white solid, m.p. 148–150°. Subsequent recrystallizations from ethyl acetate gave an analytical sample, m.p. 152–154°, as white needles.

Anal.—Calcd. for $C_{19}H_{19}N_3O_2$: C, 71.01; H, 5.96; N, 13.08. Found: C, 71.04; H, 6.09; N, 13.15.

N-p-**Acetamidobenzenesulfonyltryptamine (XI)**—A solution of 0.83 g. (2.65 mmoles) of *N-p*-aminobenzenesulfonyltryptamine in 10 ml. of acetic anhydride containing 2 ml. pyridine was stirred overnight at ambient temperature and then poured with vigorous stirring into ice water. The yellow solid was collected on a filter; yield 0.86 g. (92%); m.p. 124–129°. Recrystallizations from aqueous methanol and then chloroform gave 0.70 g. (75%) of analytically pure sample, m.p. 136–136.5°.

Anal.—Calcd. for $C_{18}H_{19}N_3O_3S$: C, 60.49; H, 5.36; N, 11.76. Found: C, 60.62; H, 5.41; N, 11.65.

N-**Salicyloyltryptamine (VIII)**—To a stirred solution of 2 g. (10 mmoles) of tryptamine in 15 ml. of benzene was added 0.8 g. (5 mmoles) of salicyloyl chloride, prepared according to the literature procedure (4). The mixture was stirred overnight at ambient temperature, and the precipitate was collected on a filter and washed successively with hot 10% HCl (two 100-ml portions) and hot water (three 100-ml. portions). Recrystallization of the crude product from benzene–petroleum ether gave 0.8 g. (57%), m.p. 143.5–144.5°.

Anal.—Calcd. for $C_{17}H_{16}N_2O_2$: C, 72.84; H, 5.75; N, 9.99. Found: C, 72.61; H, 5.81; N, 9.71.

Assay—Hydroxyindole-*O*-methyltransferase was isolated from beef pineal gland and purified according to the method of Axelrod and Weissbach (5). Incubation was carried out with *N*-acetylserotonin and *S*-adenosyl-L-methionine-methyl-^{14}C, as previously described (6). The stock solutions of all the inhibitors were prepared in dimethyl sulfoxide. Previous findings showed that the same magnitude of inhibitory activity was obtained regardless of the use of the organic solvent (6).

[2] Celite, Johns-Manville.

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Comparative Neutralization of Lung- and Mucosal-Derived Heparin by Protamine Sulfate Using *In Vitro* and *In Vivo* Methods

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Abstract □ The objective of this investigation was to compare the number of heparin units neutralized by protamine sulfate as a function of heparin source, potency, and manufacturing process. Two experiments were conducted. In the first, results obtained by the *in vivo* response in rabbits were shown to agree with results obtained by the USP *in vitro* assay. The second compared the neutralization of several samples of sodium heparin by the USP *in vitro* procedure according to the parameters outlined. Results reported establish significant neutralization differences.

Keyphrases □ Heparin, lung, mucosal derived—comparison of neutralization by protamine sulfate, *in vivo*, *in vitro* □ Protamine sulfate, neutralization of heparin—comparison, lung, mucosal derived, *in vivo*, *in vitro* □ Potency, heparin—correlation with neutralization values

In recent years, some clinicians have expressed concern over the apparent variability in patients of the neutralization of sodium heparin by protamine sulfate. Walton *et al.* (1) reported that the number of heparin units neutralized by protamine sulfate varies with the source and specific activity of the sodium heparin.

Results similar to those reported by Walton *et al.* (1) were observed in evaluating heparin samples derived respectively from lung and mucosa, currently the primary market sources. Sodium heparin derived from different source materials was neutralized by protamine sulfate according to the USP *in vitro* assay procedure. Protamine sulfate did not neutralize all sodium heparin units to the same degree.

A two-phased study[1] was conducted to establish the significance of these observations: (*a*) to ascertain whether the differences observed in the *in vitro* assay paralleled the *in vivo* response; and (*b*) to compare the ability of protamine sulfate to neutralize heparin samples derived from different source materials, different potencies, or different manufacturing processes. These factors were not always known by previous investigators.

[1] Assays associated with the first-phase study were performed by Eli Lilly and Co. Assays for the second-phase study were performed by Cohelfred Laboratories, Inc., The Upjohn Co., and Eli Lilly and Co.

Table I—*In Vitro* Assay Data[a]

Heparin Source	Potency, USP Heparin Units/mg. Number of Assays	Potency, USP Heparin Units/mg. Mean ± *SE*	Heparin Units Neutralized/mg. Protamine Sulfate Number of Assays	Heparin Units Neutralized/mg. Protamine Sulfate Mean ± *SE*	Relative Neutralization, %
Beef lung	27	135.6 ± 1.8	7	84.6 ± 0.9	100
Swine mucosa	6	159.7 ± 3.9	4	114.6 ± 1.6	135

[a] All standard errors are for $P_{0.05}$.

Table II—*In Vivo* Assay Data[a]

	Protamine Sulfate, mg./kg.	Mean Clotting Time, min.	Number of Rabbits
Beef lung heparin, units/kg.			
100	0.174	22.32	10
100	0.292	16.45	10
100	0.554	11.23	20
Swine mucosal heparin, units/kg.			
100	0.174	20.78	10
100	0.292	12.80	10
100	0.554	6.64	20
Controls	—	4.26	10

[a] A comparison of the two curves, calculated as per Finney (4), established that protamine sulfate neutralized 30% more heparin units derived from swine mucosa than from beef lung.

FIRST-PHASE STUDY

Two samples of sodium heparin, one of lung origin and one of mucosal origin, were assigned potencies by the USP assay procedure. They were then evaluated by the USP *in vitro* protamine sulfate neutralization assay.

Methods—The sodium heparin potencies were assigned by the official procedure (USP XVII, p. 611). The *in vitro* heparin neutralizations were determined by the official procedure (USP XVII, p. 539).

The *in vivo* heparin neutralization method is a modification of a procedure described by Gross (2). Rabbits were injected intravenously with 100 units/kg. of sodium heparin. Two and one-half minutes later, they were injected with sufficient protamine sulfate to produce a dose–response curve as measured by clotting time. Clotting time was determined 7.5 min. after the administration of protamine sulfate by using the capillary tube method described by Peterson and Mills (3).

Results and Discussion—A summary of the *in vitro* assay data is found in Table I; a summary of the *in vivo* assay data is found in Table II. The *in vitro* assays established that 1 mg. of protamine sulfate neutralized 84.6 heparin USP units of lung origin. (This approximates the 85.8 units expected by the USP *in vitro* neutralization assay as specified under *Heparin preparation*.) One milligram of protamine sulfate neutralized 114.6 heparin USP units of mucosal origin (35% more than for lung heparin). The *in vivo* study demonstrated a 30% difference for the two materials. These results indicate that neutralization values, obtained by the USP *in vitro* assay, are indicative of the *in vivo* response in rabbits.

Table III—Data from Second-Phase Protamine Sulfate–Sodium Heparin Neutralization Study

Sodium Heparin Lot No.	Laboratory	Sulfur, %	Potency, USP Heparin Units/mg. "As Is" Number of Assays	Range	Mean	Heparin Units Neutralized/mg. Protamine Sulfate Number of Assays	Range	Mean	Relative Neutralization, %[a]
			Source—Beef, Lung						
C	A	—	3	114–117	116	2	86	86	100
C	B	—	2	116	116	4	84–94	88	103
C	C	12.24	4	115–117	115	2	86	86	100
	Average				116			87	101
B	A	—	2	129–133	131	3	92	92	107
B	B	—	5	131–137	133	6	87–97	93	108
B	C	13.15	4	131–137	134	2	87	87	101
	Average				133			91	106
A	A	—	2	150–152	151	2	101–104	103	120
A	B	—	2	157	157	5	101–107	103	120
A	C	13.70	4	157–163	160	4	86–91	89	104
	Average				156			98	115
			Source—Beef, Mucosa						
11148-27.5	A	—	4	98	98	2	77	77	90
11148-27.5	B	—	3	102–106	105	3	76–79	77	90
11148-27.5	C	11.91	4	101–104	103	5	78–81	79	92
	Average				102[b]			78	91
11148-27	A	—	3	126–128	127	2	95	95	110
11148-27	B	—	3	130–135	133	4	96–102	98	114
11148-27	C	11.42	4	128–130	129	3	91–94	93	108
	Average				130			95	111
			Source—Swine, Mucosa						
LP09138L	A	—	4	128–132	130	2	94	94	110
LP09138L	B	—	3	132–138	136	3	94–104	98	114
LP09138L	C	11.60	4	134–139	136	2	104	104	121
	Average				134			99	115
LP09138	A	—	4	153–155	154	2	109–112	111	129
LP09138	B	—	5	151–158	154	3	110–117	114	133
LP09138	C	11.65	4	156–160	158	2	107–111	109	127
	Average				155			111	130
LP01299	A	—	8	166–174	169	3	121–127	123	143
LP01299	B	—	3	167–175	170	2	128	128	149
LP01299	C	13.68	4	168–173	170	2	114–117	116	135
	Average				170			122	143
			Source—Swine, Mucosa[c]						
11018-26C	A	—	3	143	143	2	104–107	105	122
11018-26C	B	—	3	144–148	146	5	102–105	105	122
11018-26C	C	12.73	4	141–146	144	2	108–112	110	128
	Average				144			107	124
11208-28	A	—	4	167–171	169	1	116	116	135
11208-28	B	—	3	167–171	170	3	112–115	114	133
11208-28	C	12.45	4	169–174	171	2	111	111	129
	Average				170			114	132
X-10018	A	—	3	177	177	2	125–128	127	148
X-10018	B	—	3	167–177	172	6	114–124	120	140
X-10018	C	12.10	4	177–194	184	2	114–117	116	135
	Average				178			121	141

[a] Relative percent neutralization was determined by dividing number of heparin units neutralized by 85.8 units. [b] Sample 11148-27.5 does not meet the USP potency requirement. [c] Inorganic sulfate used in manufacture.

SECOND-PHASE STUDY

Sodium heparin samples known to be from different sources, of different potencies, and from different manufacturing processes were procured. These were first assigned heparin unit potencies by the USP procedure, and they were then neutralized by the USP protamine sulfate assay. The sulfur content of all materials was also determined[2].

Methods—The sodium heparin potencies and the *in vitro* neutralization assays were performed as previously indicated. After cation removal, sulfur determinations were made by the Schöniger method.

Results and Discussion—A summary of data from the second-phase study is found in Tables III and IV. Among the several so-

Table IV—Summary of Results

Heparin Source	Potency, Units/mg.	Heparin Units Neutralized/mg. Protamine Sulfate	Relative Neutralization, %
Beef lung	116–156	87–98	101–115
Beef mucosa	102–130	78–95	91–111
Swine mucosa	134–178	99–122	115–143

[2] The beef lung heparin was supplied by The Upjohn Co.; the heparin of mucosal origin (swine and beef) was supplied by Cohelfred Laboratories, Inc.; and the protamine sulfate was supplied by Eli Lilly and Co.

dium heparin samples examined that met the USP potency requirement, 1 mg. of protamine sulfate neutralized from 87 to 122 sodium heparin USP units, a maximum variation of 42%.

Within each type of sodium heparin examined, an apparent correlation was observed between heparin units neutralized by 1 mg. protamine sulfate and heparin potency. No correlation was observed between sulfur content and the neutralization values.

SUMMARY

Although all types of sodium heparin materials were not included, and no attempt was made to quantitate the parameters examined, namely tissue, species, potency, and process, this study established that there is a significant difference in the neutralization of different types of sodium heparin by protamine sulfate.

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Hexahydrocoenzyme Q_4 in Pseudohypertrophic Muscular Dystrophy

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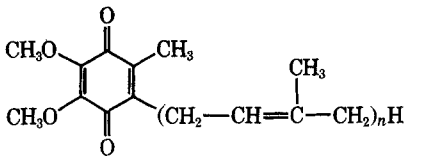
Abstract □ Hexahydrocoenzyme Q_4, 250 mg. per day for 8 months followed by 1000 mg. per day for 4 months, did not improve muscle strength or alter serum and urine creatine and creatinine, serum creatine phosphokinase, or aldolase, or a battery of other clinical and laboratory indexes including oral glucose tolerance and associated insulin, growth hormone, and inorganic phosphorus levels in 19 boys with pseudohypertrophic muscular dystrophy of the Duchenne type. The failure to observe beneficial or other changes during the ingestion of hexahydrocoenzyme Q_4 might have been because of: (*a*) the intrinsic inactivity of the substance in Duchenne dystrophy, and (*b*) the low dose level, particularly if this dystrophy should be one of the vitamin-dependent diseases of genetic nature which involves vitamins of both the water- and oil-soluble category. In any case, the effective dosage of hexahydrocoenzyme Q_4 for the genetic muscular dystrophy of mice in a previously reported study was approximately 10–50 times that used in this clinical study. The dosage for the mice was "massive" in terms of their body content of coenzyme Q. Hence, the studies herein reported do not exclude the possibility that higher homologs of the coenzyme Q group, *i.e.*, Q_5–Q_{10}, might have beneficial effects in human muscular dystrophy. In such trials, coenzyme Q_{10} would certainly be the most important, since it is present in human tissues.

Keyphrases □ Muscular dystrophy, pseudohypertrophic—treatment with hexahydrocoenzyme Q_4, evaluation □ Hexahydrocoenzyme Q_4—evaluation of use in pseudohypertrophic muscular dystrophy □ Coenzyme Q_4 homologs—evaluated in muscular dystrophy treatment

Coenzyme Q_{10}, a relatively new vitamin (1), is widely distributed in mammalian species. Certain rodent tissue such as that of mice and rats may contain mostly coenzyme Q_9 but also some Q_{10}. The normal members of the coenzyme Q group, represented by I, differ in the number, *n*, of the isoprenoid units in the side chain. From the viewpoint of mammalian metabolism, coenzymes Q_9 and Q_{10} may be regarded in the category of the oil-soluble group of vitamins such as vitamin A and vitamin D.



I

n = 1–10

n = 10 for human tissue

n = $-CH_2CH{=}C(CH_3){-}CH_2(CH_2{-}CH_2{-}CH(CH_3){-}CH_2)_3H$ for hexahydrocoenzyme Q_4

Coenzyme Q_{10} is naturally present in the human body. It was found in every organ and tissue analyzed (2) and, presumably, is in every cell of the human body that has mitochondria. Coenzyme Q_{10} is a component of the bioenergetic reactions of respiration and coupled oxidative phosphorylation which reside in the inner mitochondrial membrane. The presence of coenzyme Q_{10} in these electron-transfer processes is indispensable, and the molecule has the general structural specificities of a vitamin. It is evident that increasing deficiencies of coenzyme Q_{10} would be increasingly deleterious to health and be reflected by some nature of disease, depending upon the distribution of the deficiency in the body.

Human muscle tissue and heart tissue obtained at autopsy from three individuals showed 20–30 mcg. CoQ_{10}/gram of wet weight (gww) tissue and 50–80 mcg. CoQ_{10}, respectively (2).

dium heparin samples examined that met the USP potency requirement, 1 mg. of protamine sulfate neutralized from 87 to 122 sodium heparin USP units, a maximum variation of 42%.

Within each type of sodium heparin examined, an apparent correlation was observed between heparin units neutralized by 1 mg. protamine sulfate and heparin potency. No correlation was observed between sulfur content and the neutralization values.

SUMMARY

Although all types of sodium heparin materials were not included, and no attempt was made to quantitate the parameters examined, namely tissue, species, potency, and process, this study established that there is a significant difference in the neutralization of different types of sodium heparin by protamine sulfate.

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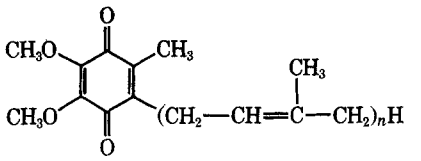
Hexahydrocoenzyme Q_4 in Pseudohypertrophic Muscular Dystrophy

T. S. DANOWSKI*, K. FOLKERS†§, H. A. WISSINGER*, T. C. HOHMANN*, J. A. GERNETH*, and J. W. VESTER‡

Abstract □ Hexahydrocoenzyme Q_4, 250 mg. per day for 8 months followed by 1000 mg. per day for 4 months, did not improve muscle strength or alter serum and urine creatine and creatinine, serum creatine phosphokinase, or aldolase, or a battery of other clinical and laboratory indexes including oral glucose tolerance and associated insulin, growth hormone, and inorganic phosphorus levels in 19 boys with pseudohypertrophic muscular dystrophy of the Duchenne type. The failure to observe beneficial or other changes during the ingestion of hexahydrocoenzyme Q_4 might have been because of: (*a*) the intrinsic inactivity of the substance in Duchenne dystrophy, and (*b*) the low dose level, particularly if this dystrophy should be one of the vitamin-dependent diseases of genetic nature which involves vitamins of both the water- and oil-soluble category. In any case, the effective dosage of hexahydrocoenzyme Q_4 for the genetic muscular dystrophy of mice in a previously reported study was approximately 10–50 times that used in this clinical study. The dosage for the mice was "massive" in terms of their body content of coenzyme Q. Hence, the studies herein reported do not exclude the possibility that higher homologs of the coenzyme Q group, *i.e.*, Q_5–Q_{10}, might have beneficial effects in human muscular dystrophy. In such trials, coenzyme Q_{10} would certainly be the most important, since it is present in human tissues.

Keyphrases □ Muscular dystrophy, pseudohypertrophic—treatment with hexahydrocoenzyme Q_4, evaluation □ Hexahydrocoenzyme Q_4—evaluation of use in pseudohypertrophic muscular dystrophy □ Coenzyme Q_4 homologs—evaluated in muscular dystrophy treatment

Coenzyme Q_{10}, a relatively new vitamin (1), is widely distributed in mammalian species. Certain rodent tissue such as that of mice and rats may contain mostly coenzyme Q_9 but also some Q_{10}. The normal members of the coenzyme Q group, represented by I, differ in the number, *n*, of the isoprenoid units in the side chain. From the viewpoint of mammalian metabolism, coenzymes Q_9 and Q_{10} may be regarded in the category of the oil-soluble group of vitamins such as vitamin A and vitamin D.



I

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Human muscle tissue and heart tissue obtained at autopsy from three individuals showed 20–30 mcg. CoQ_{10}/gram of wet weight (gww) tissue and 50–80 mcg. CoQ_{10}, respectively (2).

The response of mice with a hereditary muscular dystrophy to therapy with hexahydrocoenzyme Q_4 was first reported by Farley *et al.* (3), who observed that a dosage of 100 mg./kg. i.p. 7 days per week resulted in physical improvement but no increase of survival. Subsequently, Scholler *et al.* (4) increased the dosage to 250 mg./kg. 7 days a week by the oral route. At this higher level, there was a physical improvement in the dystrophic mice and the number of days from onset of therapy to death was four times that of the corresponding period for the control mice.

Zuckerman *et al.* (5) confirmed the response of these dystrophic mice to therapy with hexahydrocoenzyme Q_4 and found that the therapy increased by eightfold the "hanging-time" of the mice.

Analysis of the pooled livers, hearts, and kidneys of severely dystrophic mice showed a level of 112 mcg. of CoQ/gww. The corresponding level for the three pooled organs of nondystrophic littermates as controls showed 209 mcg. of CoQ/gww according to Nilsson *et al.* (6). Not only was there about a 50% deficiency of CoQ in these organs of the severely dystrophic mice, but the rate of biosynthesis of CoQ for severely diseased animals was about 50% lower than that of potentially dystrophic animals at a weaning age.

The muscular dystrophy (gene symbol dy), which occurred as a spontaneous autosomal mutation in a strain of mice 129 Re, causes muscular weakness, atrophy, and a reduced lifespan. These dystrophic mice show clinical, histological, and physiological similarities to myotonic dystrophy of man, Erb's dystrophy, and, except for the difference in inheritance, the Duchenne dystrophy (7). While there are several similarities between the genetic dystrophy of the mouse and the Duchenne type of muscular dystrophy in man, some metabolic differences also have been reported (8). Nevertheless, a trial of hexohydracoenzyme Q_4 in human muscular dystrophy was undertaken.

MATERIALS AND METHODS

Nineteen boys, 5–17 years of age and in the early, intermediate, or late stages of pseudohypertrophic (*i.e.*, Duchenne type) muscular dystrophy, received synthetic hexahydrocoenzyme Q_4 *per os* for 1 year. The initial dosage of 250 mg./day was continued for 8 months and then increased to 1000 mg./day for an additional 4 months.

Prior to and during therapy, the observations cited in the *Results* section were made at intervals of from 1 to 12 months.

RESULTS

The medication did not produce any untoward effects. All but one of the children increased in body weight during the year of therapy. The gains ranged from 3 to 14 lb. with an average of 7.5 lb. Blood pressure, pulse rates, and electrocardiographic tracings remained within pretherapy limits.

Motor Performance—None of the patients reported an increase in muscle strength and motor performance, nor was any improvement observed by their families. Six of the families felt that their children lost ground during the year of observation. The performance of 110 muscle groups against resistance and gravity was evaluated in each patient by a physical therapist prior to and at the end of the treatment period without evidence of improvement. Serial recordings of 105 successive contractions of either one or both hands in 105 sec. on an ergometer (9) confirmed the lack of an increase in strength.

Measurements of the time necessary for the patient to rise from the floor to a standing position did not indicate improvement in strength. The same conclusion was reached with respect to maximal extension of the lower leg while sitting on the edge of the examining table. When present, contractures at elbows, hips, and knees did not diminish during CoQ_4 therapy.

Serum Enzymes and Solutes—Serum creatine phosphokinase levels remained elevated in the high range characteristic of Duchenne muscular dystrophy (10, 11). The mean pretherapy value, based on three or more measurements in each child, was 473 ± 212 I.U. The 12th-month levels, comparable to those recorded after 3, 6, and 9 months of therapy, averaged out to 431 ± 212 I.U. The difference is not statistically significant by the *t*-test (12). Serum aldolase (13), when elevated, did not decrease, and alkaline and acid phosphatase (14) levels also remained within the pretherapy range.

Serum creatine and creatinine (15) did not change significantly during the 12 months of treatment and observation. Other serum solutes, comprising CO_2, Cl, Na, K, total protein, albumin, globulin, calcium, inorganic phosphorus (16, 17), uric acid (18), cholesterol (19), triglycerides (20), and nonesterified fatty acids (21), fluctuated within the normal range.

Urine Data—Urine creatine and creatinine excretion (15) and the renal clearance of the two measured during the 12th month of therapy were found to be within the pretreatment range. Similarly, the 24-hr. excretion of 17-ketosteroids (22), Porter–Silber chromogens (23), 11-desoxycortisol metabolites (24), and urinary pressors (aortic strip assay) (25) was of the same order of magnitude in the control and treatment periods.

Glucose Tolerance and Serum Insulin—Oral glucose tolerance, judging by the Glucose Tolerance Sum (26), based on the 0-, 0.5-, 1-, and 2-hr. blood glucose levels following 1.75 g. of glucose/kg. of body weight, remained below 500. As established in evaluations in this laboratory (26), each of these glucose tolerance tests would be classified as normal by the World Health Organization, the U. S. Public Health Service, the British Diabetic Association, and Fajans and Conn. Also, the serum immunoassayable insulin levels (27) recorded in the glucose tolerance test performed in the 12th month of treatment were about the same as those observed in the pretherapy period; the difference between the mean values was not statistically significant.

Serum growth hormone (27) and inorganic phosphorus (17) levels recorded in these glucose tolerance tests were not affected by the 1 year of treatment.

Other Indexes—The relative blood cell volume, the hemoglobin level, and the sedimentation rate did not change during treatment. This was also true of hepatic indexes including serum bilirubin, cephalin flocculation, and thymol turbidity and the thyroidal indexes [serum PBI (28), uptake of exogenous T_3 by blood cells *in vitro* (29), and the thyroidal ^{131}I uptake at 24 hr.].

DISCUSSION

In part, this trial of hexahydrocoenzyme Q_4 in human pseudohypertrophic muscular dystrophy was undertaken to follow up the demonstration that this compound improved hind-limb strength in genetically dystrophic mice and prolonged their survival (4). Coenzyme Q is functional at two enzyme sites in the mitochondrial electron-transfer processes, one in succin-oxidase and the other in DPNH-oxidase.

As a working hypothesis, Farley *et al.* (3) and Gale *et al.* (2) suggested that the muscular dystrophy occurring as a genetic trait in mice or in humans might result from a deficiency of coenzyme Q which might result primarily from a genetic defect in its biosynthesis. The deficiency might be augmented by absence of dietary factors required for the biosynthesis and peroxidation of coenzyme Q and its isoprenoid precursors. A deficiency of coenzyme Q would lead to an impairment of electron transfer in the enzyme systems of succin-oxidase and DPNH-oxidase and, in turn, there could be a deficiency in the biosynthesis of adenosine triphosphate through the coupled oxidative phosphorylation which would result in the low levels of adenosine triphosphate that characterize dystrophic muscle (30).

These trials with hexahydrocoenzyme Q_4, undertaken as a possible test of this hypothesis, did not result in improved muscle strength and motor performance in this group of patients with pseudohypertrophic muscular dystrophy of the Duchenne type. Apparent improvement in hand strength during the early months of treatment was not continued and probably reflected increased skill

in manipulating the rubber bulb. Theoretically, the medication could have retarded a loss of muscle strength without evident improvement, but the control observations of the natural history of this disorder (31) exclude this possibility. Moreover, in these studies the ingestion of hexahydrocoenzyme Q_4 at a dosage of 250–1000 mg./day during a 12-month interval was not associated with any decrease in serum creatine phosphokinase nor with statistically significant changes in serum or urine creatine and creatinine; other blood and serum solutes; urinary steroids; hematologic, hepatic, or thyroidal indexes; or oral glucose tolerance.

When the dystrophic mice were given hexahydrocoenzyme Q_4 at a dosage of 10 mg./kg. p.o. 5 days per week, the improvement in physical performance was marginal; but at 100 mg./kg. i.p. 7 days per week, improvement of physical status was clearly evident (3). At 250 mg./kg. p.o. 7 days per week, not only was physical performance improved, but survival was significantly increased (4). In comparison, these 19 boys, 5–17 years of age and in early, intermediate, or late stages of Duchenne dystrophy, received approximately 5 mg./kg. of hexahydrocoenzyme Q_4 for 8 months and then approximately 20 mg./kg. for an additional 4 months based on an average body weight of 50 kg. Based on a direct extrapolation of the most effective dosage in the dystrophic mice, these boys received only about one-fiftieth of the extrapolated dosage for 8 months and only about one-tenth of the extrapolated dosage for the remaining 4 months. On the other hand, the dosage administered to the boys can seem high when one considers that the total body content of coenzyme Q_{10} appears to be in the range of 0.5–1.5 g. (2). Assuming a deficiency of coenzyme Q_{10} in the body of a boy with Duchenne dystrophy, his total body content of coenzyme Q_{10} could be expected to be not much less than perhaps 75% of 0.5–1.5 g. While the actual clinical dosage might seem adequate to restore such a deficiency of coenzyme Q_{10}, this reasoning may be inappropriate on the basis of a comparison of the effective dosage for the dystrophic mouse with this animal's total body content of coenzyme Q. The effective dosage for the mouse is far in excess of the total body content of coenzyme Q. The dosage for the mice and the clinical dosage may correlate with a report by Rosenberg (32), who summarized the background on "inherited" vitamin-dependency states which have been defined as genetic disturbances that lead to specific biochemical abnormalities which involve catalysis by a vitamin, but respond only to "massive" amounts of the vitamin. "Massive" amounts may be from 10- up to 1000-fold the dose level of the same vitamin which corrects a primary deficiency state due to dietary inadequacy. Reaction specificity, genetic etiology, and quantitative responsiveness are understood to characterize a genetic vitamin-dependency state. A primary vitamin-deficiency state is generally acquired through dietary inadequacy and is generally corrected by very low dose levels of the vitamin. On such a genetic basis, the effective dosage of hexahydrocoenzyme Q_4 for the dystrophic mice might be explained, and the dosage range of 250–1000 mg./kg. for these boys may have been low. However, the absence of benefit from the clinical dosage may be due to the intrinsic inactivity of hexahydrocoenzyme Q_4 in the Duchenne type of dystrophy.

Despite the absence of any beneficial or any other effects of hexahydrocoenzyme Q_4 in these studies, the possibility still exists that higher homologs might stay the course of muscular dystrophy. It recently became known that these mice with a hereditary dystrophy also respond to treatment with coenzyme Q_7 (33); companion studies (34) revealed a deficiency of coenzyme Q in the succinate dehydrogenase–coenzyme Q reductase of the hearts and hindleg muscles of the dystrophic mice and that this deficiency increases with increasing severity of the dystrophy. Studies with the higher homologs of coenzyme Q are certainly in order. It is the highest homolog, *i.e.*, coenzyme Q_{10}, that is biosynthesized in human tissues and that ultimately will be the coenzyme Q of choice for final appraisal. These studies were initiated with hexahydrocoenzyme Q_4 because other homologs were not available in quantities sufficient for clinical trial.

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§ Number CXXX in the Coenzyme Q series.

Relationship between Antioxidant and Antihemolytic Activities of Vitamin E Derivatives *In Vitro*

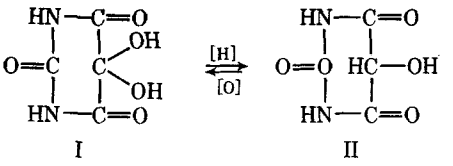
W. A. SKINNER*, H. L. JOHNSON, M. ELLIS, and R. M. PARKHURST

Abstract □ Various derivatives of vitamin E and other antioxidants were evaluated *in vitro* for their antihemolytic effects on the blood from vitamin E-deficient rats. These results were compared with the activities of some of these compounds as antioxidants in protecting β-carotene from air oxidation in corn oil. The lack of a good correlation between these effects would indicate that different mechanisms are involved in their antihemolytic and antioxidant effects.

Keyphrases □ Vitamin E derivatives—antioxidant, antihemolytic activities, relationship □ Antihemolytic effects, vitamin E derivatives—correlated with antioxidant activities □ Antioxidants, vitamin E derivatives—*in vitro* evaluation of antihemolytic activities, correlation

During an investigation of the relation of dietary factors to the development of diabetes in the alloxan-treated rat, Rose and György (1) discovered that vitamin E-deficient rats experienced marked hemolysis after administration of the alloxan. Further studies led to the finding that, *in vitro*, alloxan (I) was not active in hemolyzing red blood cells but that its reduction product, dialuric acid (II), was quite active (Scheme I).

Friedman *et al.* (2) studied the dialuric acid-induced hemolysis as a bioassay method for vitamin E, pointing out some precautions necessary for increased accuracy of the determination. The relative *in vivo* potency of α- and γ-tocopherol was also studied. The statement was made that α-tocopherol was active by addition



I ⇌ II

Scheme I

to the blood of deficient rats *in vitro* but that the biological specificity was lost in this test because the several tocopherols exerted the same quantitative effects.

Rose and György (3) found that the activity of various tocopherols evaluated *in vivo* for prevention of this blood hemolysis varied, with α-tocopherol being the most active.

Deficiency of vitamin E in premature infants results in increased susceptibility of their erythrocytes to hemolysis *in vitro* by dilute hydrogen peroxide solutions (4). Thus, vitamin E deficiency in various animal species results in susceptibility of the blood to hemolysis *in vitro* in the presence of either H_2O_2 or dialuric acid.

An interesting review of the biological effects of the tocopherols and of various antioxidants summarized the relative effectiveness of the tocopherols in the *in vitro* hemolysis test using dialuric acid (5). The results derived from studies by Rose and György (3) and Bunyan *et al.* (6) indicated that α-tocopherol was most effective while diisooctylhydroquinone was of com-

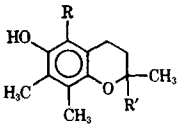


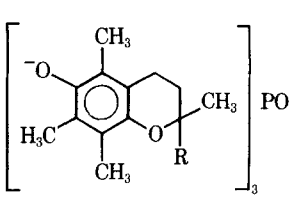
Table I—Antioxidant Activity and Antihemolytic Activity of Vitamin E Analogs *In Vitro*

R	R′	Antioxidant Activity (Half-Life), hr.[a]	Antihemolytic Activity (Median Effective Dose), mcg.[a]
H	$CH_3(C_{16}H_{33})$	394 (>570)	0.5–1 (1–5)
CH_3	$CH_3(C_{16}H_{33})$	375 (570)	0.5 (0.2–0.3)
CH_2OH	$CH_3(C_{16}H_{33})$	244	5–10 (1–5)
$CH_2OCH_2C_6H_5$	$CH_3(C_{16}H_{33})$	179 (263)	100 (25–50)
$CH_2OC_2H_5$	$CH_3(C_{16}H_{33})$	171 (225)	1–5 (10–25)
CH_2OCH_3	$CH_3(C_{16}H_{33})$	169 (221)	5–10 (10)
$CH_2OCOC_6H_5$	CH_3	66	>100
CHO	CH_3	62	5–10
Orthoquinone	CH_3	41	5
	$CH_3(C_{16}H_{33})$	39 (58)	50 (>100)
	$CH_3(C_{16}H_{33})$	38 (37)	50–100 (50–100)
$CH_2N(CH_3)_2$	$CH_3(C_{16}H_{33})$	35 (24)	10 (2.5–5)
$CH_2SC(NH)NH_2 \cdot HCl$	CH_3	27	1–5
$CH_2SC(NH)NH_2 \cdot HCl$ (acetate)	CH_3	—	10–25
$CH_2SC_2H_5$ (acetate)	$CH_3(C_{16}H_{33})$	—	10 (>100)
CH_2Cl (acetate)	$CH_3(C_{16}H_{33})$	< 12	>100 (>100)
Control		12	—

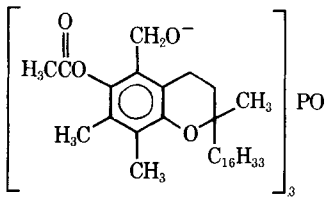
[a] Values in parentheses are for the corresponding compounds in which R′ is the normal tocopherol side chain ($C_{16}H_{33}$) in place of CH_3.

Table II—Antihemolytic Activities of Vitamin E Analogs *In Vitro*

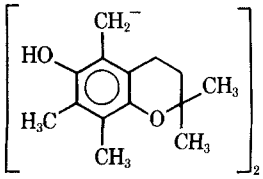
Compound	Antihemolytic Activity (Median Effective Dose), mcg.
6-Hydroxy-2,2,5,7-tetramethylchroman	>100
2,2,6,7-Tetramethylchroman-5,8-quinone	50
1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline	1–5
Compound 1	>100
Compound 2	100
Compound 3	>100
2,2,5,7,8-Pentamethylchroman-6-phosphate	0.5–1
Tocopherolquinone model	>100
2,2,7,8-Tetramethyl-5-hydroxymethyl-6-phosphate	50
Compound 4	0.5–1
Compound 5	1–5
Methylene blue	5–10
Butylated hydroxytoluene	1–2.5
Butylated hydroxyanisole	2.5–5
α-Tocopherol nicotinate	>100
4,4′-Methylenebis(2,6-di-*tert*-butylphenol)	50
4,4-Thiobis(6-*tert*-butyl-*o*-cresol)	1
1,3,5-Trimethyl-2,4,6,tris[3,5-di-*tert*-butyl-4-hydroxybenzyl]benzene	>100
N,*N*′-Diphenyl-*p*-phenylenediamine	0.5–1
Pyrogallol	1
Ascorbic acid	>100



(1) R = $C_{16}H_{33}$
(2) R = CH_3



(3)



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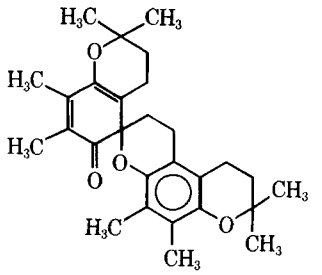

(5)

parable potency and *N*,*N*′-diphenyl-*p*-phenylenediamine was quite affective but less potent. Moore and Sharman (7) reported that when *N*,*N*′-diphenyl-*p*-phenylenediamine, methylene blue, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline[1], or sodium selenide was administered to vitamin E-deficient rats, only *N*,*N*′-diphenyl-*p*-phenylenediamine offered significant protection against blood hemolysis induced by dialuric acid. Similar tests conducted by Bunyan *et al.* (6) failed to show correlations in protective efficacy in tests conducted *in vivo versus* those conducted *in vitro*.

More recently, Gloor *et al.* (8) reported that α-tocopheramine (6-amino-5,7,8-trimethyltocol) was as active as α-tocopherol in the blood hemolysis test, whereas γ-tocopherol had only 10% of the activity of α-tocopherol. *N*-Methyl-γ-tocopheramine(6-*N*-methylamino-7,8-dimethyltocol) was 13 times more active than γ-tocopherol in this test.

Because it was felt that the biological activity of vitamin E could be related to its antioxidant effect, a study was initiated to determine the ability of certain antioxidant compounds to afford protection against the dialuric acid-induced hemolysis of the blood from vitamin E-deficient rats. This activity was compared with antioxidant activities of these compounds as measured *in vitro*. Most of the compounds were derivatives of α-tocopherol or of 6-hydroxy-2,2,5,7,8-pentamethylchroman.

EXPERIMENTAL

An adaptation of the methods of Bunyan *et al.* (6) and Friedman *et al.* (2) was used for the hemolysis studies. Test compounds were preincubated with saline suspensions of washed red cells from vitamin E-deficient rats (37°, 1 hr.). These treated red cells were resuspended in saline–phosphate buffer (pH 7.4) and incubated with dialuric acid (15 min. at 37° and then 45 min. at room temperature). After centrifugation and dilution of the supernatant, the optical density of the latter was determined at 415 nm. This value was compared with values obtained for samples not exposed to dialuric acid and for samples completely hemolyzed with distilled water (2). The red cells used exhibited 80–100% hemolysis by dialuric acid in the absence of protecting agents. Compounds were evaluated at 100-, 50-, 25-, 10-, 5-, 1.0-, 0.5-, 0.25-, and 0.1-mcg. levels.

Synthetic and antioxidant data were previously reported by Skinner and Parkhurst (9), who determined the protection of β-carotene in corn oil against air oxidation.

RESULTS AND DISCUSSION

A comparison of the *in vitro* antioxidant activities with the *in vitro* antihemolytic activities of these vitamin E analogs (Table I) indicates some overall correlation between the two activities, but notable exceptions are present. For example, the thiourea derivative protects β-carotene only 27 hr. (half-life) but is quite active in protecting blood cells from hemolysis. The same is true of the 5-dimethylaminomethyl derivatives. The orthoquinone and the 5-aldehyde are also better antihemolytic agents than the antioxidant results indicate. The 5-hydroxymethyl and 5-methoxymethyl derivatives are quite active in both tests.

It is obvious from a study of Table II that all antioxidants are not equally effective in preventing the dialuric acid-induced hemolysis of red blood cells of vitamin E-deficient rats. None of the compounds evaluated was as effective as α-tocopherol, but some of the model chroman derivatives were quite active; *e.g.*, 2,2,5,7,8-pentamethylchroman-6-phosphate; Compound 4; Compound 5; 2,2,5,7,8-pentamethyl-6-hydroxychroman; 2,2,7,8-tetramethyl-6-hydroxychroman; 5-ethoxymethyl-2,2,7,8-tetramethyl-6-hydroxychroman; and 5-thioureiodomethyl-2,2,7,8-tetramethyl-6-hydroxychroman hydrochloride. The most active of the nontocopherol antioxidants were pyrogallol, *N*,*N*′-diphenyl-*p*-phenylenediamine, butylated hydroxytoluene, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, and 4,4′-thiobis(6-*tert*-butyl-*ortho*-cresol). Ascorbic acid, which is an excellent antioxidant in certain systems, was inactive in preventing hemolysis.

It would be of interest to compare the most active of these derivatives for their *in vivo* effectiveness against dialuric acid-induced hemloysis of red blood cells of vitamin E-deficient rats.

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(5) v. G. Brubacher and H. Weiser, *Deut. Ges. Ernaehr., Wissenschaftl. Veroeffenslich.*, **16**, 50(1967).
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COMMUNICATIONS

Direct Gas Chromatographic Analysis of Long-Chain Alcohols and Alkyltrimethylammonium Bromides

Keyphrases ☐ Alkyltrimethylammonium bromides—analysis ☐ Alcohols, long chain—analysis ☐ TLC—analysis ☐ GLC—analysis

Sir:

Quaternary ammonium compounds have numerous industrial applications [for example, see Schwartz *et al.* (1)]. In pharmacy, alkyltrimethylammonium halides are important both for their antibacterial action, which may be linked to their micellar properties (2–4), and when used in combination with long-chain fatty alcohols to form mixed emulsifiers (5–8). We are studying the self-bodying action of mixed emulsifiers, including those of the alkyltrimethylammonium bromide–long-chain alcohol type (5, 6).

Commercial alkyltrimethylammonium bromides, including cetrimide BP, and alcohols, such as cetostearyl alcohol BP, are mixtures of homologs, and it is desirable to have a simple, direct method of analysis suitable for all materials.

Link and Morrissette (9) described the analysis of long-chain alcohols by GLC, using a nonpolar substrate on a solid support treated with alkali. However, there is some controversy over the analysis of quaternary compounds in alkaline conditions. Metcalfe (10) used a similar column to analyze long-chain quaternary compounds; under the conditions described, the corresponding tertiary amines were produced. Analysis of these provided a measure of the total homolog composition of the original mixture. No peak had a retention time that corresponded to any of the postulated higher olefins which would result from a Hofmann degradation.

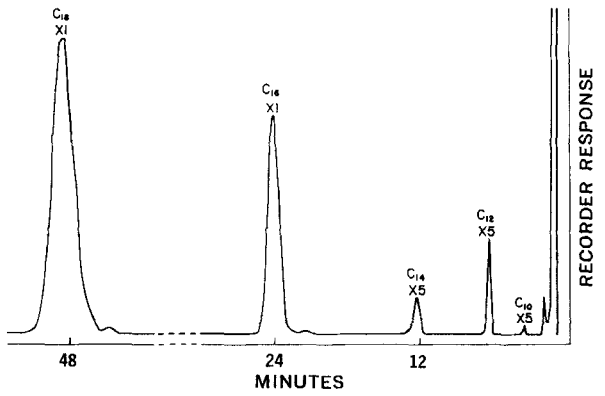

Figure 1—*Chromatogram of cetostearyl alcohol BP.*

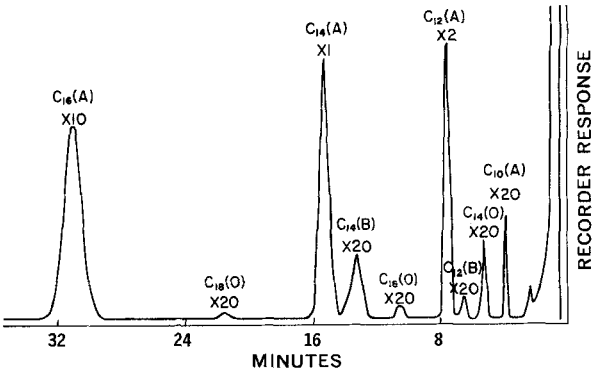

Figure 2—*Chromatogram of cetrimide BP. Key: (A) = tertiary amine; (O) = olefin; and (B) = alkyl bromide. For certain peaks, detector sensitivity increased by the factor shown.*

Laycock and Mulley (11) were unable to reproduce Metcalfe's (10) experiments, and thus they modified the column and analyzed quaternary ammonium compounds in the form of the hydroxides. Under their conditions, each compound decomposed quantitatively into a mixture of tertiary amine and olefin.

Neither paper reported checks on the original quaternary compounds for tertiary amine content which, if present, would make the analyses inaccurate.

We have investigated the homolog composition of a series of alcohols and alkyltrimethylammonium bromides using a similar column to that described by Metcalfe (10). A Perkin-Elmer F.11 chromatograph with flame-ionization detector was used. The column was stainless steel, 2 m. long, 0.3175 cm. (0.125 in.) o.d., packed with acid-washed Chromosorb W (60–80 mesh) coated with 10% w/w potassium hydroxide. The flow rate of the carrier gas, nitrogen, was 25 ml./min. The injection and column temperature for 4% w/v solutions of the alcohols dissolved in *n*-heptane were 225–230

(4) H. H. Gordon and J. P. DeMetry, *Proc. Soc. Exp. Biol. Med.*, **79**, 446(1952).
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COMMUNICATIONS

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Keyphrases ☐ Alkyltrimethylammonium bromides—analysis ☐ Alcohols, long chain—analysis ☐ TLC—analysis ☐ GLC—analysis

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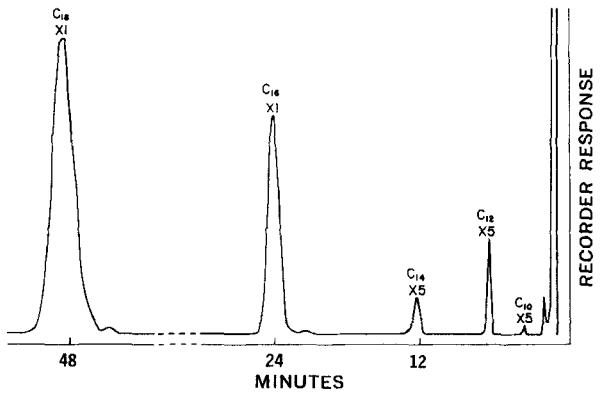

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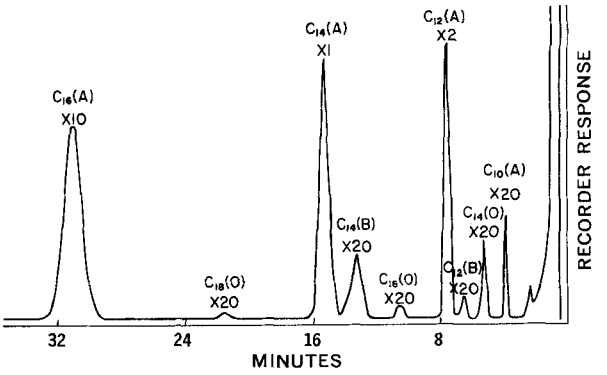

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Table I—Analysis of Long-Chain Alcohols by GLC[a]

Constituents Present	Commercial Long-Chain Alcohols					Retention Time, min.
	Cetostearyl Alcohol BP	Dodecanol	Tetradecanol	Hexadecanol	Octadecanol	
Decanol	(0.2)	(0.4)	—	—	—	3.4
Dodecanol	1.3	99.2	0.5	0.5	0.5	6.3
Tetradecanol	0.5	(0.2)	99.0	0.7	1.4	12.3
Impurity	(0.4)	—	(0.2)	(0.3)	—	—
Hexadecanol	24.7	(0.4)	(0.3)	98.0	0.6	24.3
Impurity	(0.4)	—	(0.1)	0.6	0.5	—
Octadecanol	72.8	—	—	—	97.5	49.3

[a] The figures in parentheses are estimated values.

Table II—Analysis of Alkyltrimethylammonium Bromides by GLC[a]

Constituents Present	Commercial Alkyltrimethylammonium Bromides						Retention Time[b], min.
	Cetrimide BP (I.C.I.)	Cetrimide BP (Compass)	Dodecyltrimethylammonium Bromide	Tetradecyltrimethylammonium Bromide	Hexadecyltrimethylammonium Bromide	Octadecyltrimethylammonium Bromide	
Decyltrimethylammonium bromide	(0.4)	0.5	0.6	—	—	—	3.9
1-Bromododecane	(0.1)	0.3	1.3	—	—	—	7.2
Dodecyltrimethylammonium bromide	19.9	21.9	94.6	2.0	1.5	1.5	7.6, 2.7
1-Bromotetradecane	0.7	1.6	—	0.9	—	—	14.4
Tetradecyltrimethylammonium bromide	68.5	68.4	2.3	92.5	1.9	1.9	15.6, 5.5
1-Bromohexadecane	(0.2)	(0.2)	—	(0.1)	1.5	—	29.6
Hexadecyltrimethylammonium bromide	9.9	8.0	0.5	4.0	91.5	2.4	30.6, 11.3
1-Bromooctadecane	—	—	—	—	—	1.6	55
Octadecyltrimethylammonium bromide	—	—	—	—	3.0	91.2	58.9, 22
Eicosyltrimethylammonium bromide	—	—	—	—	(0.7)	1.5	110

[a] The figures in parentheses are estimated values. [b] Retention times for quaternaries are those of tertiary amine and olefin, respectively.

and 240°, respectively. For the alkyltrimethylammonium bromides dissolved in isopropyl alcohol (4% w/v), they were 265–270 and 232°, respectively. Sample sizes varied from 0.5–2 μl., and during runs the detector sensitivity was varied to estimate small traces of impurity. The peak areas were measured by electronic integration on a Digital Integrator type IE 165A[1]. Narrow sharp peaks were obtained for both types of compound (Figs. 1 and 2).

The response of the flame-ionization detector is essentially proportional to the carbon content of the compound being analyzed. An approximate correction, C, for the flame-ionization response has been suggested (12, 13). This converts the response per gram of material to response per gram of carbon:

$$C = \frac{\text{molecular weight of material}}{\text{number of carbon atoms} \times 12} \qquad \text{(Eq. 1)}$$

Each peak area was multiplied by this correction factor, and the percentage of each component was determined by the internal normalization method; that is, each corrected peak area was expressed as a percentage of the total corrected peak area. The results for cetostearyl alcohol[2] and other long-chain alcohols[3] are shown in Table I. These results are the mean of at least two analyses, and in general repetitive runs differed by less than 1%. There were trace unidentified impurities before the main C_{16} and C_{18} peaks.

The main peaks for all the alkyltrimethylammonium bromides[4] had the retention times of corresponding tertiary amines, although traces of corresponding olefins were detected (less than 2% total in every analysis). Thus, the main reaction was the decomposition of quaternary ammonium compound to tertiary amine and alkyl halide, as reported by Metcalfe (10). However, the alternative method of decomposition in alkaline conditions, the Hofmann degradation to tertiary amine and olefin did occur, but to a lesser extent. The peak areas were corrected for carbon content, and impurities before the main peaks were identified as corresponding alkyl bromides, using standards of the authentic materials[5].

[1] Gas Chromatograph Ltd., Maidenhead, England.

[2] Cetostearyl alcohol BP, Loveridge Ltd., Southampton, England.

[3] Dodecanol, puriss. grade, and tetradecanol, purum grade, Fluka, Buchs S. G., Switzerland. Hexadecanol and octadecanol, nominal purities 99%, B.D.H. Chemicals Ltd., Poole, England.

[4] Cetrimide BP, a commercial mixture of alkyltrimethylammonium bromides, I.C.I., Macclesfield, Cheshire, England, and Compass Chemicals Ltd., London, England. The alkyltrimethylammonium bromides: dodecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, and octadecyltrimethylammonium bromide, Schuchardt München, Munich 8, Germany; nominal purities: dodecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, octadecyltrimethylammonium bromide, 95%; hexadecyltrimethylammonium bromide, 98–99%.

[5] 1-Bromododecane, B.D.H. Chemicals Ltd., Poole, England; 1-bromotetradecane, Ralf N. Emanuel Ltd., Alperton, England; and 1-bromohexadecane, Hopkins and Williams Ltd., Chadwell Heath, England.

The alkyltrimethylammonium bromides were checked for tertiary amine content using TLC. The 20 × 20-cm. glass plates were coated with a 0.25-mm. thickness of aluminum oxide G, using a Desega spreader, and were air dried. For spotting, pure tertiary amines[6] were diluted with ethanol, and the alkyltrimethylammonium bromides were dissolved in water. The dilutions were calculated so that the weight of tertiary amine deposited from a calibrated capillary tube was approximately 0.1% of the weight of quaternary surfactant deposited. The developing solvent was a mixture of acetone and water (100:5), and Dragendorff's reagent was used to color the quaternaries and the tertiary amines orange. The more polar quaternaries were strongly adsorbed onto the substrate and thus moved only a short distance from the point of application. It was estimated that there was less than 0.2% tertiary amine in each quaternary compound examined. These quantities would not significantly alter the analyses based on GLC.

The results for the alkyltrimethylammonium bromides are shown in Table II; they are the mean of at least two analyses, and repetitive runs in general differed by less than 1%.

These results have been used to confirm the validity of Shinoda's equation (14) for predicting the CMC of mixtures of quaternary ammonium surfactants containing up to seven components (15). They have also been used to investigate the mechanism of the self-bodying action of mixed emulsifiers of the type alkyltrimethylammonium bromides–cetostearyl alcohol (5, 6).

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[6] Dimethylaminododecane, dimethylaminotetradecane, and dimethylaminooctadecane, Fluka, Buchs S. G., Switzerland.

Octahedral Hybridization of Hydrogenic Orbitals in Molecular Orbital and Ligand Field Calculations

Keyphrases ☐ Hydrogenic orbitals—octahedral hybridization ☐ Molecular orbital, ligand field calculations—octahedral hybridization of hydrogenic orbitals

Sir:

Various quantum chemical methods have been applied to the correlation of theoretical indexes of drug and biochemical activity with experimentally determined quantities (1). Among these are molecular orbital theory and ligand field theory. The applications of these treatments to any molecular system containing hexacoordinate atoms (*e.g.*, transition metal ions in metalloenzymes) necessarily entails knowledge of a set of six d^2sp^3 hybrid orbitals, composed of linear combinations of the hydrogenic s, p, and d orbitals, to describe the bonding situation and to provide the appropriate orbitals for the calculation of π-electron energies, charge densities, and other indexes of molecular reactivity (2). The construction of d^2sp^3 hybrid orbitals was originally discussed by Pauling (3). The set of d^2sp^3 hybrids given by Pauling is:

$$\psi_1 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{2}}p_z + \frac{1}{\sqrt{3}}d_z \qquad \text{(Eq. 1)}$$

$$\psi_2 = \frac{1}{\sqrt{6}}s - \frac{1}{\sqrt{2}}p_z + \frac{1}{\sqrt{3}}d_z \qquad \text{(Eq. 2)}$$

$$\psi_3 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z + \frac{1}{2}d_x + \frac{1}{\sqrt{2}}p_x \qquad \text{(Eq. 3)}$$

$$\psi_4 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z + \frac{1}{2}d_x - \frac{1}{\sqrt{2}}p_x \qquad \text{(Eq. 4)}$$

$$\psi_5 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z - \frac{1}{2}d_x + \frac{1}{\sqrt{2}}p_y \qquad \text{(Eq. 5)}$$

$$\psi_6 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z - \frac{1}{2}d_x - \frac{1}{\sqrt{2}}p_y \qquad \text{(Eq. 6)}$$

where ψ_1–ψ_6 are the d^2sp^3 hybrids and s, p_x, p_y, p_z, and d_z are hydrogenic orbitals. Necessary and sufficient conditions for the set ψ_1–ψ_6 to form a complete set of d^2sp^3 orbitals are that the orbitals are equivalent (*i.e.*, they each contain probability density of s, p, and d character in the ratio of 1:3:2), they are normalized (*i.e.*, $\int \psi_i \psi_i dT = 1$, where dT is the volume element in space), and they are orthogonal to one another (*i.e.*, $\int \psi_i \psi_{j \neq i} dT = 0$). Upon examination of the atomic orbital coefficients, it can be seen that the hybrid orbitals fulfill the required conditions of equivalence and normalization but do not form an orthogonal set. For example, the integral of the product of ψ_1 and ψ_4 is not zero but rather $1/3$.

The alkyltrimethylammonium bromides were checked for tertiary amine content using TLC. The 20 × 20-cm. glass plates were coated with a 0.25-mm. thickness of aluminum oxide G, using a Desega spreader, and were air dried. For spotting, pure tertiary amines[6] were diluted with ethanol, and the alkyltrimethylammonium bromides were dissolved in water. The dilutions were calculated so that the weight of tertiary amine deposited from a calibrated capillary tube was approximately 0.1% of the weight of quaternary surfactant deposited. The developing solvent was a mixture of acetone and water (100:5), and Dragendorff's reagent was used to color the quaternaries and the tertiary amines orange. The more polar quaternaries were strongly adsorbed onto the substrate and thus moved only a short distance from the point of application. It was estimated that there was less than 0.2% tertiary amine in each quaternary compound examined. These quantities would not significantly alter the analyses based on GLC.

The results for the alkyltrimethylammonium bromides are shown in Table II; they are the mean of at least two analyses, and repetitive runs in general differed by less than 1%.

These results have been used to confirm the validity of Shinoda's equation (14) for predicting the CMC of mixtures of quaternary ammonium surfactants containing up to seven components (15). They have also been used to investigate the mechanism of the self-bodying action of mixed emulsifiers of the type alkyltrimethylammonium bromides–cetostearyl alcohol (5, 6).

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[6] Dimethylaminododecane, dimethylaminotetradecane, and dimethylaminooctadecane, Fluka, Buchs S. G., Switzerland.

Octahedral Hybridization of Hydrogenic Orbitals in Molecular Orbital and Ligand Field Calculations

Keyphrases ☐ Hydrogenic orbitals—octahedral hybridization ☐ Molecular orbital, ligand field calculations—octahedral hybridization of hydrogenic orbitals

Sir:

Various quantum chemical methods have been applied to the correlation of theoretical indexes of drug and biochemical activity with experimentally determined quantities (1). Among these are molecular orbital theory and ligand field theory. The applications of these treatments to any molecular system containing hexacoordinate atoms (*e.g.*, transition metal ions in metalloenzymes) necessarily entails knowledge of a set of six d^2sp^3 hybrid orbitals, composed of linear combinations of the hydrogenic s, p, and d orbitals, to describe the bonding situation and to provide the appropriate orbitals for the calculation of π-electron energies, charge densities, and other indexes of molecular reactivity (2). The construction of d^2sp^3 hybrid orbitals was originally discussed by Pauling (3). The set of d^2sp^3 hybrids given by Pauling is:

$$\psi_1 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{2}}p_z + \frac{1}{\sqrt{3}}d_z \qquad \text{(Eq. 1)}$$

$$\psi_2 = \frac{1}{\sqrt{6}}s - \frac{1}{\sqrt{2}}p_z + \frac{1}{\sqrt{3}}d_z \qquad \text{(Eq. 2)}$$

$$\psi_3 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z + \frac{1}{2}d_x + \frac{1}{\sqrt{2}}p_x \qquad \text{(Eq. 3)}$$

$$\psi_4 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z + \frac{1}{2}d_x - \frac{1}{\sqrt{2}}p_x \qquad \text{(Eq. 4)}$$

$$\psi_5 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z - \frac{1}{2}d_x + \frac{1}{\sqrt{2}}p_y \qquad \text{(Eq. 5)}$$

$$\psi_6 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{12}}d_z - \frac{1}{2}d_x - \frac{1}{\sqrt{2}}p_y \qquad \text{(Eq. 6)}$$

where ψ_1–ψ_6 are the d^2sp^3 hybrids and s, p_x, p_y, p_z, and d_z are hydrogenic orbitals. Necessary and sufficient conditions for the set ψ_1–ψ_6 to form a complete set of d^2sp^3 orbitals are that the orbitals are equivalent (*i.e.*, they each contain probability density of s, p, and d character in the ratio of 1:3:2), they are normalized (*i.e.*, $\int \psi_i \psi_i dT = 1$, where dT is the volume element in space), and they are orthogonal to one another (*i.e.*, $\int \psi_i \psi_{j \neq i} dT = 0$). Upon examination of the atomic orbital coefficients, it can be seen that the hybrid orbitals fulfill the required conditions of equivalence and normalization but do not form an orthogonal set. For example, the integral of the product of ψ_1 and ψ_4 is not zero but rather $1/3$.

These errors have been carried over into standard texts on chemical bonding (2, 4). It is, therefore, in order to call attention to this matter; the use of the nonorthogonal set of d^2sp^3 hybrids in molecular orbital and ligand field calculations will result in errors in off-diagonal terms of matrixes used to calculate values of physical variables. A correct set of equivalent, normalized orthogonal d^2sp^3 hybrids is:

$$\psi_1 = \frac{1}{\sqrt{6}}s + \frac{1}{\sqrt{2}}p_z + \frac{1}{\sqrt{3}}d_z \quad \text{(Eq. 7)}$$

$$\psi_2 = \frac{1}{\sqrt{6}}s - \frac{1}{\sqrt{2}}p_z + \frac{1}{\sqrt{3}}d_z \quad \text{(Eq. 8)}$$

$$\psi_3 = \frac{1}{\sqrt{6}}s - \frac{1}{\sqrt{12}}d_z + \frac{1}{2}d_x + \frac{1}{\sqrt{2}}p_x \quad \text{(Eq. 9)}$$

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$$\psi_5 = \frac{1}{\sqrt{6}}s - \frac{1}{\sqrt{12}}d_z - \frac{1}{2}d_x + \frac{1}{\sqrt{2}}p_y \quad \text{(Eq. 11)}$$

$$\psi_6 = \frac{1}{\sqrt{6}}s - \frac{1}{\sqrt{12}}d_z - \frac{1}{2}d_x - \frac{1}{\sqrt{2}}p_y \quad \text{(Eq. 12)}$$

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Hydrophilic–Lipophilic Balance and Distribution Coefficients of Nonionic Surfactants

Keyphrases ☐ Hydrophilic–lipophilic balance, nonionic surfactants—distribution coefficient correlation ☐ Distribution coefficient correlation—hydrophilic–lipophilic balance, nonionic surfactants ☐ Nonionic surfactants—hydrophilic–lipophilic balance and distribution coefficients

Sir:

The hydrophilic–lipophilic balance (HLB) of nonionic surfactants has been correlated with many of their physical properties such as dielectric constants, heats of hydration, critical micelle concentrations, cloud points, phase inversion temperatures of emulsions, spreading coefficients of oil on water or of water on oil, and interfacial tensions at oil–water interfaces. In general, good correlations were found between the HLB and these properties within a given homologous series of surfactants. However, as soon as the chemical characteristics of the hydrophilic or lipophilic moieties were changed, *e.g.*, by going from a polyoxyethylated surfactant to a sorbitan derivative or from a *n*-alkanol base to an alkylphenol base, different correlations were obtained. So far, the search for a universal correlation between the HLB and another property of the surfactant, which could be determined more readily than the HLB, has not been successful.

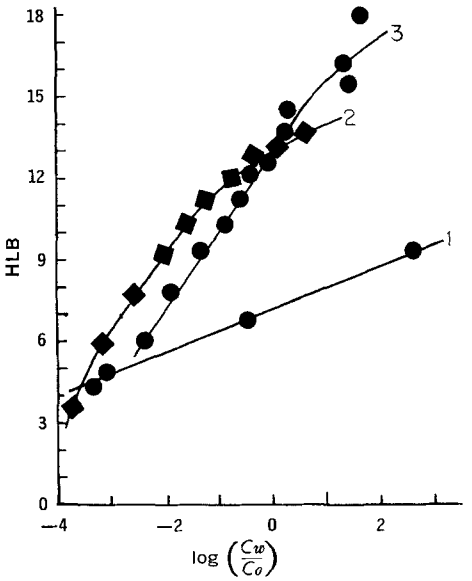

Figure 1—*Relationship between correct HLB values and the logarithms of the water–oil distribution coefficients. Key: Curve 1, sorbitan monoesters; Curve 2, homogeneous polyoxyethylated octylphenols; and Curve 3, normally distributed polyoxyethylated octylphenols.*

Intuitively, the distribution coefficient of the surfactants between water and oil, C_w/C_o, would seem to be well suited for correlation with the balance between their hydrophilic and lipophilic tendencies. Davies and Rideal (1) combined the kinetic treatment of emulsion coalescence with the free energy change of transferring a surfactant molecule from water to oil and arrived at the following correlation:

$$\text{HLB} = 7 + 0.829 \log (C_w/C_o) \quad \text{(Eq. 1)}$$

Table I—Comparison between Experimental HLB Values and Values Derived from Distribution Coefficients

Surfactant	Experimental HLB[a]	$\left(\frac{C_w}{C_o}\right)^b$	HLB from Eq. 1
Sorbitan monooleate[c]	4.3	3.70×10^{-4}	4.2
Sorbitan monostearate[d]	4.7	6.19×10^{-4}	4.3
Sorbitan monopalmitate[e]	6.7	0.314	6.6
Polyoxyethylene sorbitol oleate[f]	9.2	367	9.1
	HLB from Eq. 2a	$\left(\frac{C_w}{C_o}\right)^g$	**HLB from Eq. 1**
Octylphenol(EO)$_1$	3.5	1.84×10^{-4}	3.9
Octylphenol(EO)$_3$	7.8	3.13×10^{-3}	4.9
Octylphenol(EO)$_6$	11.2	5.92×10^{-2}	6.0
Octylphenol(EO)$_{10}$	13.6	3.85	7.5

[a] From *Reference 2*. [b] From *Reference 1*. [c] Span 80. [d] Span 60. [e] Span 40. [f] Atlox 1087. [g] From *Reference 3*.

These errors have been carried over into standard texts on chemical bonding (2, 4). It is, therefore, in order to call attention to this matter; the use of the nonorthogonal set of d^2sp^3 hybrids in molecular orbital and ligand field calculations will result in errors in off-diagonal terms of matrixes used to calculate values of physical variables. A correct set of equivalent, normalized orthogonal d^2sp^3 hybrids is:

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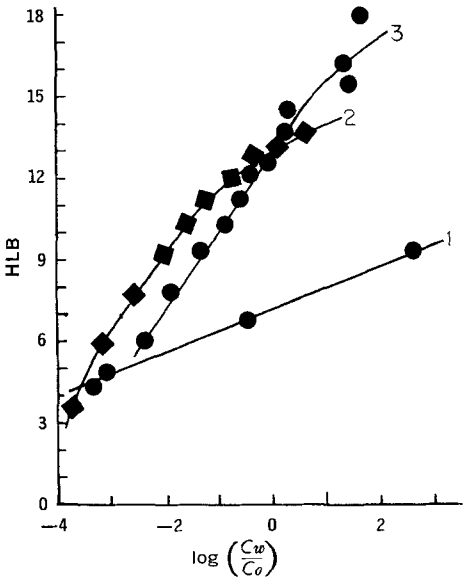

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It is shown in this communication that for a different category of nonionic surfactants, namely, polyoxyethylated octylphenols, $C_8H_{17}C_6H_4O(C_2H_4O)_pH$, Eq. 1 does not apply. These are homogeneous or single-species surfactants; each compound has a single value of p (3). Because experimental HLB determinations of these surfactants have not been made, their HLB values were calculated by

$$\text{HLB} = \frac{E}{5} \qquad \text{(Eq. 2)}$$

where E is the percentage of polyoxyethylene in the surfactant molecule. For polyoxyethylated octylphenols, Eq. 2 can be rewritten as

$$\text{HLB} = \frac{4405p}{5(206.32 + 44.05p)} \qquad \text{(Eq. 2a)}$$

where 206.32 is the molecular weight of octylphenol. Equation 2 was derived by Griffin from experimental measurements and is reported to be applicable to all polyoxyethylated nonionic surfactants (2); this is the basis of the HLB system for rating these surfactants. The HLB values calculated according to Eq. 2 are those listed in a recent compilation (2) and are considered to be correct.

These HLB values are compared in Table I with the HLB values calculated from the distribution coefficients (3) according to Eq. 1 for several surfactants of the homologous series. The correct HLB values (experimental or calculated by Eq. 2) for the entire series, as well as for the sorbitan-based surfactants, are plotted in Fig. 1 against the logarithms of the distribution coefficients. The curve for the polyoxyethylated octylphenols diverges considerably from the straight line of the sorbitan-based surfactants. A universal correlation between the distribution coefficients and the HLB, applicable to all nonionic surfactants, requires that the two curves coincide. An alternative, although less likely, explanation for the divergence is that Eq. 2 is not applicable to all nonionic surfactants; specifically, that it breaks down for homogeneous surfactants.

The distribution coefficients of normally distributed polyoxyethylated octylphenols (3, 4), where each surfactant has a range of p values, are also plotted against the HLB in Fig. 1. A broadened molecular weight distribution of the nonionic surfactants affects the relationship between HLB and distribution coefficient far less than a change in their chemical characteristics.

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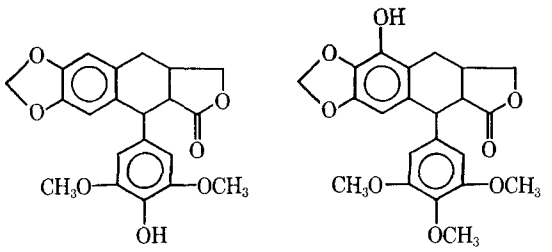
Isolation and Characterization of Cytotoxic Principles from *Hyptis verticillata* Jacq.

Keyphrases ☐ *Hyptis verticillata*—cytotoxic principles ☐ β-Peltatin, 4′-demethyldesoxypodophyllotoxin—isolation, identification, *H. verticillata* ☐ Antimitotic activity—*H. verticillata* extracts ☐ IR spectrophotometry—identification ☐ NMR spectroscopy—identification ☐ Mass spectroscopy—identification

Sir:

A recent observation (1) that aqueous extracts of *Hyptis verticillata* Jacq. (Labiatae) inhibited development of sea urchin eggs led to a systematic study of extractable substances from this source. As a result of this study, two antimitotic agents, 4′-demethyldesoxypodophyllotoxin (I) and β-peltatin (II) were isolated and identified by comparison with authentic materials.

The ubiquity of lignans related to the 2,3-dimethylphenylnaphthalenes (2–10) is well documented, and the breadth of occurrence of cytotoxic substances related to podophyllotoxin is continually expanding (11–12). Isolation of these materials from *H. verticillata* represents, to our knowledge, the first report of their occurrence in the Labiatae family and attests to their widespread occurrence.



I II

Experimental[1]—Melting points were determined on a Hoover-Thomas capillary melting-point apparatus. IR spectra were determined in chloroform solution on a Beckman IR-8. NMR spectra were determined on a Varian A-60 spectrometer. Mass spectra were determined on a Hitachi RMU-6H mass spectrometer. Antimitotic activity of the fractions and materials obtained as a consequence of purification was determined[2] using Earle's L929 mouse fibroblast cell line as the test system.

Antimitotic Assay—Earle's L929 mouse fibroblast cells (1.5×10^5 cells/tube) were incubated 10 hr. at 36° in culture tubes, each tube containing 1 ml. of Eagle's minimum essential medium supplemented with 10% calf serum. To pairs of tubes containing the incubated cells was added 0.1 ml. of the test solution containing the suspected antimitotic agent. The cells were incubated an additional 18–24 hr. Generally, three to four dilutions varying by factors of 10 were

[1] Microanalyses were performed by Mr. Malcolm Stone of the A. H. Robins Co.

[2] By Dr. R. W. Tankersley, A. H. Robins Co.

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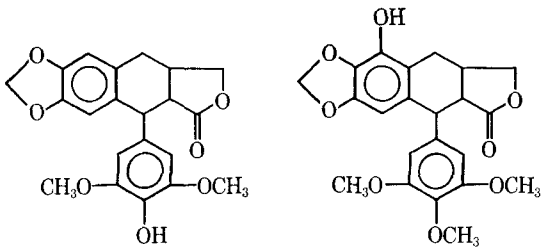

I II

Experimental[1]—Melting points were determined on a Hoover-Thomas capillary melting-point apparatus. IR spectra were determined in chloroform solution on a Beckman IR-8. NMR spectra were determined on a Varian A-60 spectrometer. Mass spectra were determined on a Hitachi RMU-6H mass spectrometer. Antimitotic activity of the fractions and materials obtained as a consequence of purification was determined[2] using Earle's L929 mouse fibroblast cell line as the test system.

Antimitotic Assay—Earle's L929 mouse fibroblast cells (1.5×10^5 cells/tube) were incubated 10 hr. at 36° in culture tubes, each tube containing 1 ml. of Eagle's minimum essential medium supplemented with 10% calf serum. To pairs of tubes containing the incubated cells was added 0.1 ml. of the test solution containing the suspected antimitotic agent. The cells were incubated an additional 18–24 hr. Generally, three to four dilutions varying by factors of 10 were

[1] Microanalyses were performed by Mr. Malcolm Stone of the A. H. Robins Co.

[2] By Dr. R. W. Tankersley, A. H. Robins Co.

prepared for each compound. Colchicine (1:10,000–1:10,000,000 w/v) was run concomitantly with unknown compounds as standards.

At the termination of incubation, cells were assayed microscopically for the morphology of cells in the metaphase block. Metaphase arrest was characterized by round, chunky cells and was easily distinguished from the diamond shape of normal cells. The end-point was taken as the minimum concentration that produced over 50% of cells blocked in the metaphase.

Extraction and Preliminary Fraction of H. verticillata *Jacq.*—Coarsely milled leaves and stems of *H. verticillata* Jacq.[3] (100 g.) were extracted at room temperature with four 500-ml. portions of 95% ethanol. The extracts were combined, and solvent was removed under reduced pressure. The dark residue (4.2 g.) was partitioned between chloroform–water. The aqueous fraction had marginal antimitotic activity and was discarded. The chloroform-soluble material was repartitioned between heptane and 70% aqueous methanol. Negligible antimitotic activity was found in the residue from the heptane layer. Removal of solvent from the aqueous methanol layer gave 1.4 g. of a light-green residue containing greater than 95% of the antimitotic activity of the original extract.

Isolation of 4′-Demethyldesoxypodophyllotoxin and β-Peltatin—The residue from the aqueous methanol–heptane partitioning experiment was dissolved in 5 ml. of chloroform and placed on a magnesia-silica gel[4] column (50 g.). The column was eluted with increasing concentrations of methanol in chloroform. The desired materials, as determined by antimitotic activity, were present in the 2 and 5% methanol in chloroform fractions. Combination of these fractions (homogeneous by TLC) and removal of the solvent gave 166 mg. of active material.

The residue from absorption chromatography was dissolved in a minimum of benzene and placed on a partition column (40 g. of diatomaceous earth[5]; 50% v/w formamide as the stationary phase). The column was developed with benzene saturated with formamide. Fractions (3 ml.) were collected automatically on a Gilson linear fraction collector. Bioassay indicated that active materials were present in fractions 5–20 and 58–90.

Fractions 5–20 were pooled and solvent was removed under reduced pressure. The residue was crystallized from ethyl acetate–heptane (49 mg., 0.05%), m.p. 251–253° [lit. (13) m.p. 247°]. Comparison with an authentic sample established identity of the material as 4′-demethyldesoxypodophyllotoxin.

Fractions 58–90 were pooled and evaporated to dryness. The residue was crystallized from ethyl acetate–heptane (12 mg., 0.012%), m.p. 234.5–236°. Identity of the material as β-peltatin was established by comparison of the IR, mass, and NMR spectra with an authentic sample. Melting points were identical and mixture melting point was undepressed.

[3] A voucher sample of *H. verticillata* Jacq. is maintained at the Department of Botany, University of the West Indies, Kingston, Jamaica.

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Synthesis and Antibacterial Activity of 5-Nitro-2-furaldehyde Phenylhydrazones

Keyphrases ☐ 5-Nitro-2-furaldehyde phenylhydrazones—synthesis ☐ Antibacterial activity—5-nitro-2-furaldehyde phenylhydrazones

Sir:

The antibacterial activity of 5-nitro-2-furaldehyde derivatives has been known for some time (1–3). Although many of them have been synthesized, their phenylhydrazones have not been studied.

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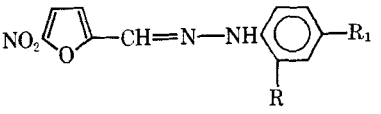

Table I—5-Nitro-2-furaldehyde Phenylhydrazones[a]

Number[b]	R	R_1	Yield, %	M.p.[c]	Formula	Anal., %[d] Calcd.	Found
1	H	F	91	171–173°	$C_{11}H_8FN_3O_3$	C, 52.96 H, 3.23 N, 16.86	C, 53.18 H, 3.31 N, 16.59
2	H	Cl	70	194–196°	$C_{11}H_8ClN_3O_3$	C, 49.73 H, 3.03 N, 15.81	C, 50.00 H, 3.25 N, 16.00
3[e]	H	Br	75	185–186°	$C_{11}H_8BrN_3O_3$	C, 42.59 H, 2.58 N, 13.55	C, 42.46 H, 2.28 N, 13.46
4	H	I	92	215–217°	$C_{11}H_8IN_3O_3$	C, 36.99 H, 2.25 N, 11.76	C, 36.98 H, 1.96 N, 11.80
5	H	CH_3	70	165–167°	$C_{12}H_{11}N_3O_3$	C, 58.77 H, 4.52 N, 17.13	C, 58.50 H, 4.30 N, 16.94
6	H	OCH_3	92	188–190°	$C_{12}H_{11}N_3O_4$	C, 55.17 H, 4.24 N, 16.08	C, 54.95 H, 4.42 N, 15.89
7	H	COOH	90	240° dec.	$C_{12}H_9N_3O_5$	C, 52.37 H, 3.30 N, 15.27	C, 52.60 H, 3.51 N, 15.10
8	F	H	75	165–167°	$C_{11}H_8FN_3O_3$	C, 52.96 H, 3.23 N, 16.86	C, 53.21 H, 3.50 N, 17.02
9	Cl	H	75	168–169°	$C_{11}H_8ClN_3O_3$	C, 49.73 H, 3.03 N, 15.81	C, 49.60 H, 3.42 N, 15.90
10	Br	H	95	148–150°	$C_{11}H_8BrN_3O_3$	C, 42.59 H, 2.58 N, 13.55	C, 42.85 H, 3.02 N, 13.55
11	I	H	90	146–148°	$C_{11}H_8IN_3O_3$	C, 36.99 H, 2.25 N, 11.76	C, 37.24 H, 2.24 N, 11.75
12	CH_3	H	93	168–169°	$C_{12}H_{11}N_3O_3$	C, 58.77 H, 4.52 N, 17.13	C, 58.80 H, 4.29 N, 16.83
13	OCH_3	H	70	148–150°	$C_{12}H_{11}N_3O_4$	C, 55.17 H, 4.24 N, 16.08	C, 54.95 H, 4.53 N, 15.80
14	COOH	H	95	250° dec.	$C_{12}H_9N_3O_5$	C, 52.37 H, 3.30 N, 15.27	C, 52.60 H, 3.54 N, 15.38

[a] The UV, IR, and NMR spectra are in agreement with the proposed structures. [b] All compounds in this table were recrystallized from ethanol or ethanol–water. [c] Melting points are uncorrected. [d] Elemental analyses were performed by Mrs. M. Casanello. [e] Lit. (8) m.p. 201°.

II). Compounds 5–7 and 9–14 were active against *Bacillus subtilis* at concentrations of about 200–400 mcg./ml. The minimum inhibitory concentrations (M.I.C.) were determined by the cup method; the M.I.C. values for medium containing 10% human serum (5) were unchanged, except for the genera Salmonella which showed increased values (1200 mcg./ml.). Compound 1 exhibits a very low acute toxicity level compared with some clinically useful nitrofurans (1), although comparisons are rather difficult since the toxicity of nitrofurans is dependent on the crystal size (6). No deaths occurred when doses of 1000 mg./kg. were given to white mice, either orally or intraperitoneally.

Table II—Activity of Compound 1

Test Organism	M.I.C., mcg./ml.
Escherichia coli ATCC 9637	180
Pseudomonas aeruginosa ACM 69	240
Proteus vulgaris ATCC 13315	200
Salmonella typhimurium AMC ETS 9	400
S. paratyphi B (I. Malbran)	400
S. paratyphi C (I. Malbran)	400
Bacillus subtilis ATCC 6633	180
Shigella flexneri (I. Malbran) serotype 10	240

Stability tests performed with Compound 1, as described by Garrett and Carper (7), showed no appreciable decomposition, even during 30 days at 60°, when protected from light.

(1) K. Miura and H. K. Reckendorf, in "Progress in Medicinal Chemistry," vol. 5, G. P. Ellis and G. B. West, Eds., Butterworths, London, England, 1967.

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Cycloheptaamylose Inclusion Complexes of Barbiturates: Correlation between Proton Magnetic Resonance and Solubility Studies

Keyphrases □ Cycloheptaamylose inclusion complexes—barbiturates □ Proton magnetic resonance—analysis

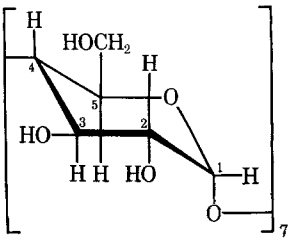
Sir:

In the past few years, Lach and his coworkers (1–5) reported extensively on the complexation of cycloamyloses (cyclodextrins) with various medicinally useful molecules. These studies, based upon the solubility method of Higuchi and Lach (6), are indicative of stereospecific interactions between drug and cycloamylose molecules; however, they do not provide *direct* evidence for inclusion of the solute within the cycloamylose cavity. Elucidation of the mechanism of such interactions, therefore, remains largely speculative.

We recently described a proton magnetic resonance (PMR) method for examining the mode of interaction of cycloheptaamylose with a variety of aromatic substrates (7). Our method is based upon the rationale that when an aromatic moiety is included within the cavity of the doughnutlike cycloheptaamylose molecule (I)[1], protons located within the cycloheptaamylose cavity (H-3, H-5, and, possibly, H-6) undergo appreciable shielding due to the anisotropy of the aromatic moiety, whereas protons located at the exterior of the torus (H-1, H-2, and H-4) are relatively unaffected. We have now extended our studies to complexation of some pharmacologically active barbituric acid derivatives (II). In the present communication, we demonstrate a correlation between results obtained by the conventional solubility method and by our PMR method.

Formation constants (K_f), determined by the solubility method, and the substrate-induced chemical shift changes ($\Delta\delta$) for a variety of cycloheptaamylose–barbiturate complexes are listed in Table I. It is evident from the results shown in this table that a parallel trend exists between K_f and $\Delta\delta$; *i.e.*, K_f and $\Delta\delta$ for H-5 are of the order: barbital < amobarbital < pentobarbital < phenobarbital. From a comparison of the relative magnitudes of $\Delta\delta$ for H-5 and H-3, it appears that association takes place by approach of the barbiturate from the primary hydroxyl side of cycloheptaamylose. For phenobarbital, $\Delta\delta$ for H-5 is the largest, which indicates that the anisotropic moiety (the phenyl side chain) of this barbiturate penetrates the cycloheptaamylose cavity. This penetration is apparently rather shallow when compared with that previously observed for simple aromatic substrates (7), since $\Delta\delta$ for H-3 is minimal. For the other barbiturates also, $\Delta\delta$'s for H-5 are significant and positive. This observation would be consistent with the well-recognized upfield shifts due to hydrophobic interactions. Such interactions could result from inclusion of the nonpolar, aliphatic side chains of the barbiturates within the cavity.

The fact that barbital, amobarbital, and pentobarbital, with their respective ethyl, isopentyl, and *n*-pentyl side chains, show a corresponding order in the $\Delta\delta$ of H-5 and in the formation constants supports the suggested mode of interaction. The formation constant for phenobarbital is the highest, an apparent consequence of the snug fit of the phenyl ring relative to the aliphatic side chains of the other barbiturates. Hydrogen bonding between the heterocyclic barbiturate nucleus and the primary hydroxyl groups of cycloheptaamylose also could be partly responsible for the interaction. Such a possibility would not be precluded by the suggested hydrophobic interaction.



cycloheptaamylose

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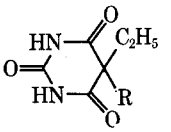

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barbital	$-CH_2-CH_3$
amobarbital	$-CH_2-CH_2-CH(CH_3)_2$
pentobarbital	$-CH(CH_3)-CH_2-CH_2-CH_3$
phenobarbital	$-C_6H_5$

barbiturates

II

[1] The constituent glucose units of cycloamyloses are known to have the Cl chair conformation (8–10). Space-filling molecular models (Corey–Pauling–Koltun) show that the interior of the cycloheptaamylose cavity has, from the primary hydroxyl side, successive layers of H-6, H-5, the ring oxygens, and H-3.

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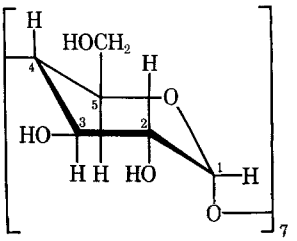

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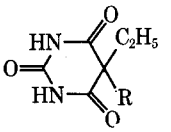

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[1] The constituent glucose units of cycloamyloses are known to have the Cl chair conformation (8–10). Space-filling molecular models (Corey–Pauling–Koltun) show that the interior of the cycloheptaamylose cavity has, from the primary hydroxyl side, successive layers of H-6, H-5, the ring oxygens, and H-3.

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Two new chapters have been added entitled, "Therapeutic Incompatibility" and "The Coordinator of Health Services."

Throughout the text references are cited in the body of the chapters and many additional references have been added. Tables and figures representing recent study and research data have been included.

Chapter I dealing with "The Prescription" retains the basic information necessary for processing a physician's medication order. The family prescription record form has been added. Chapter II entitled, "Biopharmaceutical Considerations in Dosage Form Design," presents the topics fundamental to an introductory treatment of physical, chemical, and biological factors that affect therapeutic efficacy of active ingredients. The material presented requires considerable elaboration by an instructor.

The presentations on dosage forms include the basic background information descriptive of the classes, such as aerosols, powders, capsules, tablets, suppositories, and solutions. Composition, design, compounding methods, and prescriptions serve to illustrate dispensing needs. There are some obsolete prescription examples, such as the salol and camphor combination. The physical–chemical principles applicable to design and evaluation are presented in terms of such concepts as rheology, bioavailability, chemical and mathematical principles, and others where involved.

Recognition of the significance of drug reactions and interactions has led to a chapter on "Therapeutic Incompatibilities." Mechanisms of drug interactions are presented and typical examples are cited. Many tables are included as well as 203 references.

The inclusion of chapters on "Prescription Accessories," "Hospital Pharmacy," and "Radiopharmaceuticals" expands the usefulness of this text for several areas of the pharmacy curriculum. In other words, the book does not lend itself as a textbook for any one particular pharmacy course. Study assignments in this book could be valuable for courses in pharmacy technology, product development, dispensing pharmacy, biopharmaceutics, and clinical or professional practice.

The trends in pharmacy curriculum design are reflected in the selection of subject matter of all fifteen chapters which deal with the health-care needs of patients.

Reviewed by M. L. Neuroth
Virginia Commonwealth University
Richmond, VA 23219 ■

Principles of Aerosol Technology. By PAUL SANDERS. Reinhold, 450 West 33rd St., New York, NY 10001, 1970. x + 418 pp. 15.5 × 24 cm. Price $17.50.

After the publication of a number of books and chapters on aerosols, most of which follow a now familiar pattern, Mr. Sanders has managed to write a book on aerosols with a new approach. This does not mean that everything in the book is new but the emphasis definitely is, that emphasis being on the fundamental principles that govern the characteristics and behavior of pressurized aerosol systems involving mainly fluorochlorohydrocarbon propellants. A serious shortcoming of the book is the lack of significant material on the flammable hydrocarbon or compressed gas systems.

This failure is easily understood in that the book is a modified and expanded version of lectures presented by members of The Freon Products Division of the E. I. du Pont Company to groups interested in various areas of aerosol technology. Subjects include spray characteristics, vapor pressure, solubility, viscosity, density, stability, and flammability. The physical–chemical principles are presented in a clear, step-by-step manner which should be comprehensible to any person involved in formulation or production but will, no doubt, be criticized by some as being too elementary. However, it is a significant advance in most areas over present books in the field.

The section in the book on emulsions, foams, and suspensions is particularly outstanding and truly unique to the aerosol literature. It is "must reading" for all those engaged in cosmetic and pharmaceutical aerosol formulation. The exclusion of nonfluorochlorohydrocarbon propellants systems is less damaging to this section than it is to others.

The chapter on miscellaneous aerosol systems appearing in the last section of the book is the only textbook treatment available on such subjects as co-dispensing, bag-in-can, and aspirator systems. Mr. Sanders has also chosen to include three chapters by other authors; the one on food aerosols is more or less academic due to emphasis on the fluorochlorohydrocarbon propellants. The others on sampling of aerosol systems and toxicity are excellent, the latter being a good starting point for those interested in the present aerosol toxicity controversy.

The book should be included in the library of researchers and teachers and is highly recommended reading for all students interested in aerosol systems.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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MAY 1971
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REVIEW ARTICLE

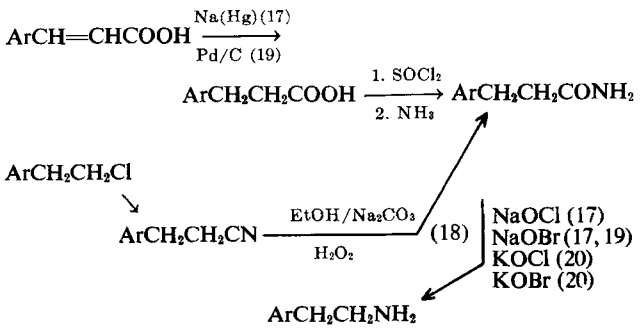
Psychotomimetic Phenethylamines

KARL A. NIEFORTH

Keyphrases ☐ Psychotomimetic phenethylamines—review ☐ Phenethylamines, psychotomimetics—biological, chemical properties ☐ Biosynthesis, synthesis—psychotomimetic phenethylamines ☐ Structure–activity relationships—psychotomimetic phenethylamines ☐ Biological activity, effects—phenethylamines

Psychotomimetic activity has been observed following ingestion, inhalation, or injection of materials representing numerous chemical classes, many of which are rather complex (*e.g.*, lysergic acid diethylamide, yohimbine, and tetrahydrocannabinol). Synthetic difficulties have, therefore, precluded extensive studies into the various chemical and physical properties that might influence mind-altering capabilities. Substituted phenethylamines are relatively easy to synthesize; with minimal time and effort, several compounds may be obtained with a variety of ring modifications which may then be related to biological potency.

This review, which is supplemental to an excellent manuscript by Patel (1), covers four basic areas: synthesis and biosynthesis, identification and assay, biological implications, and structure–activity relationships. The period covered is from 1966 to early 1970. It will familiarize the reader with past accomplishments in this area and also indicate the direction future research will follow. During the next few years, research should decide the conformational requirements for phenethylamine psychotomimetics and perhaps yield more information concerning their mechanism of activity.

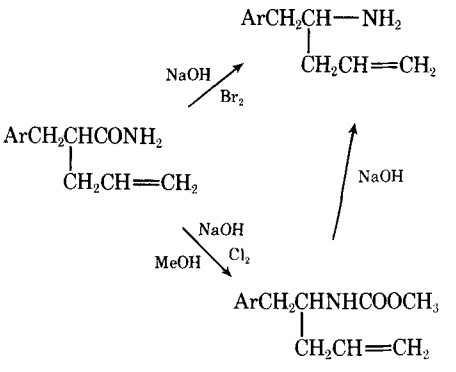


Scheme I

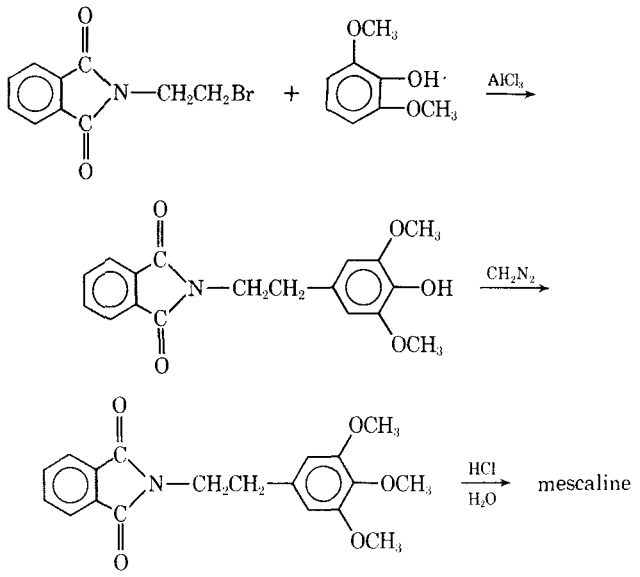
SYNTHETIC ROUTES

A variety of procedures are available to the medicinal chemist desirous of preparing possible psychotomimetic methoxylphenethylamines. The nature of the selected synthetic route is limited by the sensitivity of substituent groups and the imagination of the scientist.

Greatest synthetic utility is made of the reaction between appropriately substituted aldehydes or olefins



Scheme II



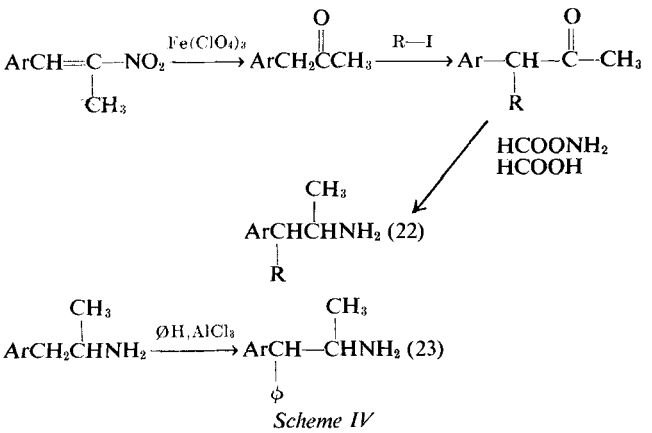
Scheme III

and nitroalkanes followed by reduction of the resultant nitrostyrenes (2–11). Excellent yields are obtained from either the primary nitro compound: $ArCHO + CH_3NO_2 \rightarrow ArCH{=}CH{-}NO_2$, or the secondary compound: $ArCHO + C_2H_5NO_2 \rightarrow Ar{-}CH{=}C(-CH_3){-}NO_2$ (4, 11), or $ArCH{=}CH{-}CH_3 + C(NO_2)_4 \rightarrow Ar{-}CH{=}C(-CH_3){-}NO_2$ (3, 11).

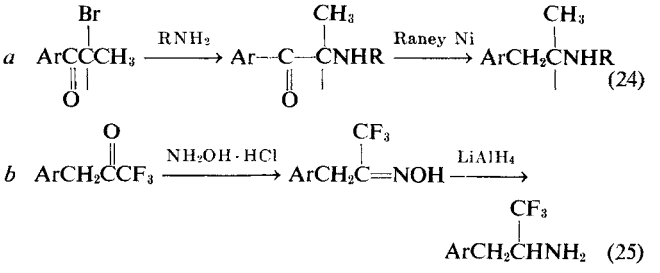
Reduction of the nitrostyrene to the corresponding phenethylamine is usually accomplished with $LiAlH_4$ [$LiAlH_4$-^3H (5)] in ether or tetrahydrofuran (2–5, 7–9, 11). The same reduction has been conducted using amalgamated zinc and hydrochloric acid (10) or palladium (6).

A longer synthetic pathway involves the conversion of a substituted benzyl alcohol to the chloride, then to the cyanide, and finally to the amine (12–16): $ArCH_2OH \rightarrow ArCH_2Cl \rightarrow ArCH_2CN \rightarrow ArCH_2CH_2NH_2$. Earlier references to this procedure (1) indicated that $LiAlH_4$ is the agent of choice to reduce the nitrile. More recent publications showed the utility of a variety of reducing conditions, including Al/Ni (15) and Ni/Cr_2O_3 (13, 14). This more lengthy route appears to offer few advantages over the nitrostyrene pathway and would be selected only where suitably substituted aldehydes are not available.

Some investigators made use of the Hofmann reaction to prepare suitably substituted phenethylamines (17–20)



Scheme IV



Scheme V

(Scheme I). This procedure was used for the conversion of naturally occurring materials such as coumarin (17) and piperonal (20) to biologically active derivatives of mescaline. Careful control of the reaction conditions (Scheme II) permits the preparation of interesting compounds (19).

Other recognizable reactions have been utilized in the synthesis of the title compounds with varying success. Rabusic and Gregor (21) (Scheme III) conducted an interesting sequence of reactions, with the overall yield of 30% competing quite favorably with previous procedures.

Beta-substituted compounds may be synthesized by the routes shown in Scheme IV.

Several mescaline analogs have been prepared by reduction of a suitable precursor, as in the examples shown in Scheme V.

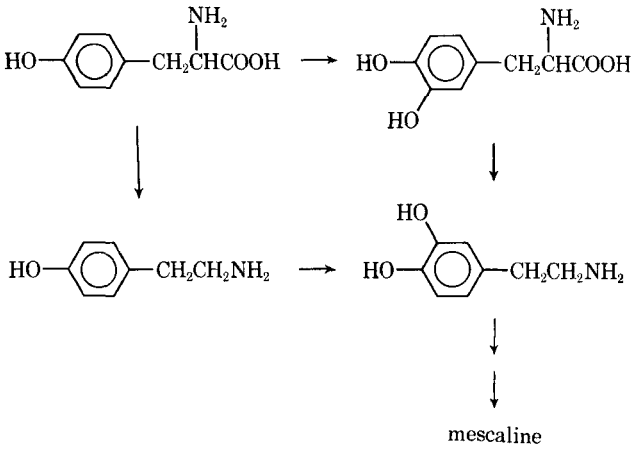
BIOSYNTHESIS

Mescaline occurs in several cacti and has been utilized as a sacrament in religious ritual. It has been isolated from the cacti *Lophophora williamsii*, *Trichocereus pachanoi* Br. and R., *Trichocereus bridgessi* (SD) Br. and R., and *Trichocereus macrogenus* (26).

Through the use of radiolabeled precursors, certain assumptions have been made concerning the biogenesis of mescaline. Agurell *et al.* (27) reported the presence of two possible routes to mescaline because *L. williamsii* was capable of incorporating both ^{14}C-labeled tyramine and DL-3,4-dihydroxyphenylalanine into mescaline. Since both are possible products of tyrosine, the route shown in Scheme VI was indicated.

An excellent chromatographic technique (28) was designed to separate the possible precursors. Later work by the same investigators (29) indicated that phenethylamine, tyramine, dopamine, and 3,4,5-trihydroxyphenethylamine were progenitors of mescaline, although phenethylamine was only slightly incorporated. Neither 4-methoxyphenethylamine nor 3,4-dimethoxyphenethylamine was an efficient precursor. This finding is not in complete agreement with a later publication (30) which reported efficient incorporation of 3,4,5-trihydroxyphenethylamine. Agurell (26) published a proposed biosynthesis (Scheme VII) of mescaline which is common to *L. williamsii* and *T. pachanoi*.

Compounds 5, 6, and 7 have not been tested as precursors, and Compound 6 has not been isolated



Scheme VI

from the mescaline-producing cacti. Compound 7*a* occurs in *Trichocereus werdermannianus* and *T. pachanoi*, and Compound 7*b* occurs in peyote. 3,4-Dimethoxyphenethylamine is described as a by-product arising from methylation of Compound 5.

Paul *et al.* (30), working exclusively with *L. williamsii*, reported a rather high (3.75%) incorporation of 3,4-dimethoxyphenethylamine, which strongly supported their hypothesis that it was a direct precursor resulting in a different biosynthesis (Scheme VIII).

Subsequent publications by Paul's group (31, 32) supported Agurell's pathway based on later findings of poor incorporation of 3,4-dimethoxy-5-hydroxyphenethylamine into mescaline isolated from *L. williamsii*. Their separation procedure did not exclude contamination of the mescaline by the labeled 3,4-dimethoxyphenethylamine precursor.

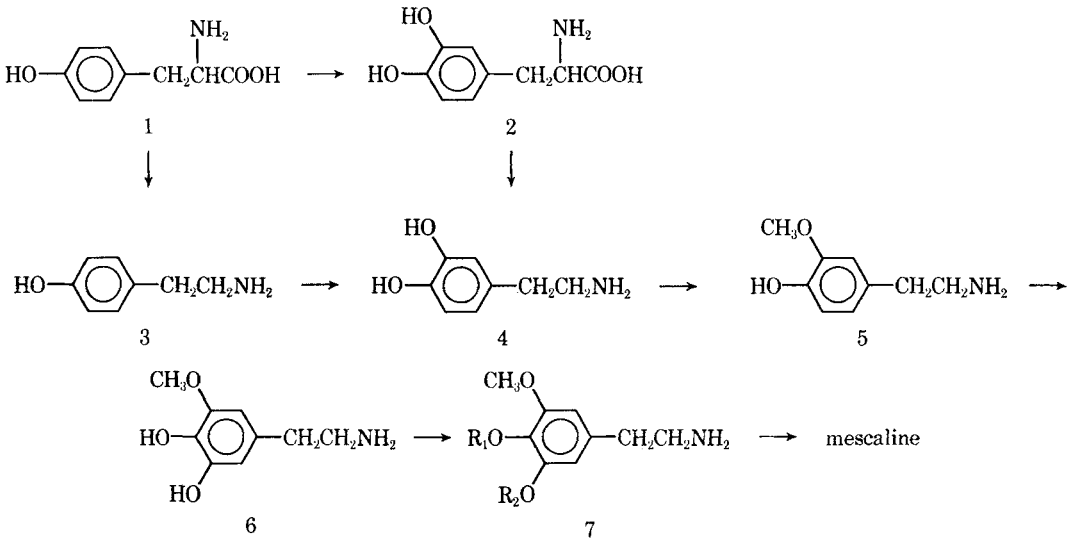
IDENTIFICATION AND ASSAY

Major interest in this area has centered around two topics, schizophrenia and drug abuse. Rather widespread acceptance of the methadone rehabilitation program for heroin addiction has necessitated a great number of urine analyses. Such programs require periodic unannounced urine analysis for the presence of opium derivatives, other depressants, stimulants, and hallucinogens. Most of the procedures are based on TLC techniques (33–38), although paper chromatography (36, 39), GC (28, 40, 41), IR spectrophotometry (40, 42), mass spectrometry (43), and ion exchange (44) have been utilized.

Fike (33), working in a coroner's office, determined R_f values for 140 drugs, including many of the presently abused agents. The determinations were conducted in five different systems, using a 1% solution of iodine in methanol as a general locating agent. Specific color sprays were used, although total separation and identification of all agents were not attempted. General correlations were made between the structure of the drug and the R_f values in the five systems. Fike observed that steric hindrance adjacent to groups responsible for binding to silica, basicity of the compounds, and presence or absence of a pyridyl ring had the greatest influence on the R_f.

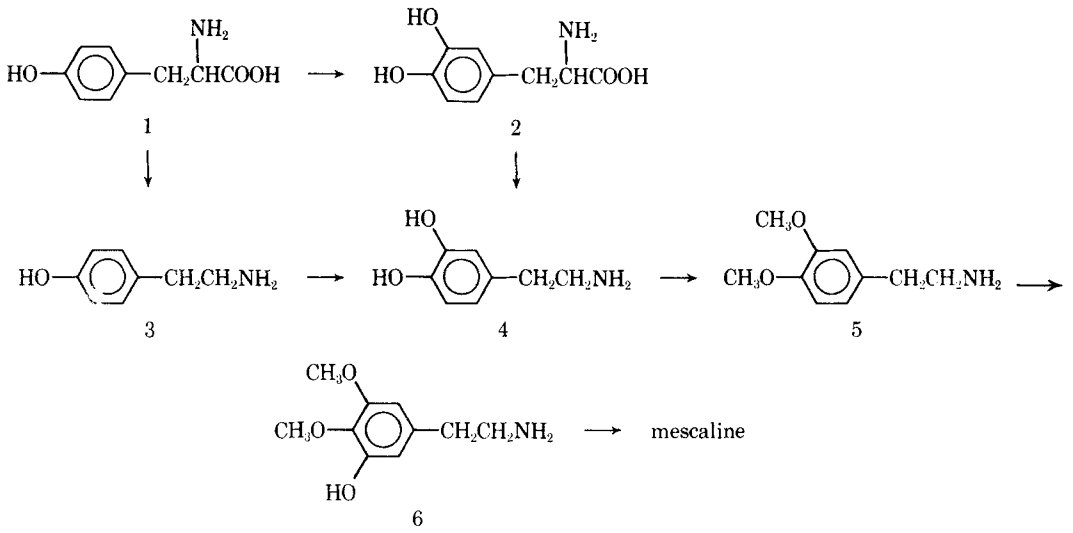
One- and two-dimensional separations of 1-dimethylaminonaphthalene-5-sulfonyl derivatives of several phenethylamines were reported (35). Seventy-one compounds of possible biological interest were evaluated in various systems. The amide derivatives were also separated by the use of thin-layer electrophoresis.

Closely related compounds of pharmaceutical (34) and forensic (34, 38) interest were separated and identified using TLC. Silica gel was used as the absorbing layer in the majority of the reports. Clarke (36) published a separation and identification procedure for LSD, methylsergide, psilocin, psilocybin, bufotenin, 6- and 7-hydroxytryptamine, 5-methoxydimethyltryptamine, and mescaline which uses both ascending paper chromatography and TLC. Particular interest in 4-methyl-2,5-dimethoxyphenyl-2-aminopropane stimulated the publication of a separation and identification procedure for



7*a*: $R_1 = H$, $R_2 = CH_3$
7*b*: $R_1 = CH_3$, $R_2 = H$

Scheme VII



Scheme VIII

the title compound in the presence of amphetamine, methamphetamine, mescaline, bufotenine, and dimethyltryptamine. Compound identification was accomplished by spraying with 10% sodium acetate and 1% 2,6-dibromo-*p*-benzoquinone-4-chlorimine in ethanol and exposing to iodine vapor. They also reported GC separation of the same compounds.

Separation of those compounds closely related to mescaline was effected (37) in several solvent systems. Those compounds that serve as biogenic precursors are separated on the basis of the presence or absence of phenolic groups. Successful separation (28) of these compounds was prerequisite to the publication of the probable mescaline biosynthetic pathway.

GC separation (28, 36, 40) has been particularly useful in combination with mass spectra for identification of the title compounds. Bistrimethylsilylacetamide was found to be quite useful in the separation of 3,4-dimethoxyphenethylamine and tryptamine, both of interest in Parkinson's disease (40). Further discussion of the implications of these materials in the urine appears later in this paper.

Several procedures have been recommended for application in urine-monitoring programs with limited success (45 and references therein). These procedures must be specific, rapid, sensitive, uncomplicated, and inexpensive due to the large number of samples that must be analyzed in a large methadone program. A very successful procedure, developed by Dole *et al.* (46), used ion-exchange paper to absorb the drugs followed by elution with a series of buffer solvent systems. This procedure is reported (44) to satisfy the listed requirements for the identification of narcotic analgesics, barbiturates, amphetamines, and several psychoactive drugs.

Several assay procedures were described in Patel's review (1) and were used by this reviewer. Optimum results in this laboratory were achieved with the procedure described by Woods *et al.* (47).

Bell and Somerville (39) described a fluorescence method for identification and quantitative assay of biologically interesting amines. The materials were spotted on paper and dried. The papers were sprayed with 5% glycine, adjusted to pH 3.0 with HCl, and suspended in a Kilner jar containing moistened paraformaldehyde. After heating at 80° for 3 hr., the papers were viewed under UV light at 360 nm. The fluorescent compounds were eluted with 0.1 *N* HCl and measured in a spectrofluorometer.

Mescaline may be determined spectrophotometrically in unadulterated systems (48). Concentrations of approximately 20 mg./100 ml. may be assayed directly by scanning from 330 to 248 nm. after previously blanking with water at 330 nm. The maximum absorbance, which occurs at approximately 268 nm., is compared to a standard solution of the sulfate or hydrochloride.

The procedures were utilized and modified by several scientists (49–63) studying the significance of 3,4-dimethoxyphenethylamine, which is proposed to be the end-product of aberrant metabolism of the catecholamines. The presence of this compound, the "pink spot" in schizophrenic urine, has been discarded as an artifact by some authors (49, 50, 55, 60–62) and isolated conclusively by others (52, 54, 63) (Table I).

Factors such as patient's diet, time of urine collection, volume of collection, and drug history appear to be critical in this research. A negative nitrogen balance during night hours results in very low concentrations in morning urine. Plant-free diets have given rise to negative findings. Perry and his coworkers (61, 63) carefully screened chronic schizophrenics' urine for 3,4-dimethoxyphenethylamine and bufotenine without success. The patients had been taken off drugs 6 weeks before assay and fed a diet free of plants and cheese for 2 weeks. Urine was collected during 48 hr. and subjected to rigorous assay. Their failure to locate the spots may have been the result of the special diet or drug restriction, or it may possibly have been due to the use of chronic rather than acute schizophrenics. They concluded that no convincing evidence has yet been presented to implicate either ring-methoxylated phenethylamines or *N*-methylated indoleamines in the etiology of the schizophrenias.

The presence of 3,4-dimethoxyphenethylamine in the urine of schizophrenics was apparently confirmed by Creveling and Daly (56), as the presence of 3,4-dimethoxyphenylacetic acid has been by Kuehl *et al.* (64).

They utilized chromatography and mass spectrometry to confirm identification. No further implications concerning the presence of the material were made. The "pink spot" was shown to consist of a mixture of at least seven different compounds. Pind and Faurbye (53) published a critique of the various analytical procedures, including a possibility of confusion between 3,4-dimethoxyphenethylamine and a metabolite of chlorpromazine. Two excellent reviews are available (52, 57) for further reading in this area.

BIOLOGICAL IMPLICATIONS

For a drug to possess hallucinogenic properties, it must, of course, be capable of penetrating the blood–brain barrier. Of all the psychotomimetic methoxylated phenethylamines, mescaline appears to be one of the least successful. Denber and Teller (65) showed that <0.002 of an intravenously injected dose of mescaline was taken up by the CNS of young white rats. A maximum of 0.14% of the dose in the brain was attained in 30 min. (66). Although the mitochondrial fraction bound a small amount of drug, the largest amount appeared in the nerve-ending fraction. The mitochondrial fraction increased with time in mescaline content at the expense of nerve endings, probably reflecting drug metabolism. The authors suggested that the large number of empty vesicles in the myelin supernatant would indicate that the pharmacologic effect of mescaline may be clarified by studying the mechanism of catecholamine release (67).

Korr *et al.* (68) determined the half-life of mescaline in mice to be approximately 55 min. One hour after injection, the highest levels of the drug were to be found in the cortex and the brain stem. Six hours after injection, the radioactive material was found in the hippocampus. Interestingly, the increase in motility of the animals paralleled the increasing concentration in the *cornu ammonis*.

The close structural relationship of amphetamine and its hallucinogenic methoxylated derivatives suggests a subtle difference in the distribution of these compounds. Amphetamine, although an active stimulant, is not usually considered as hallucinogenic. In a recent study (69), amphetamine, methamphetamine, 4-methoxyamphetamine, 3,4-dimethoxyamphetamine, 2,4,5-trimethoxyamphetamine, and 2,5-dimethoxy-4-methylamphetamine were evaluated as to their sites of action on the basis of EEG alerting. The effective mean doses for EEG alerting were 4.0 ± 0.74 mg./kg., 2.8 ± 0.44 mg./kg., 2.6 ± 0.30 mg./kg., 6.0 ± 0.30 mg./kg., 3.1 ± 0.31 mg./kg., and 0.3 ± 0.15 mg./kg., respectively. Those compounds without methoxy groups induced typical EEG alerting in the midbrain level, whereas the methoxy compounds brought about alerting in an area caudal to the midbrain and cephalad to the first cervical spinal segment. 3,4-Dimethoxyamphetamine and 3,4,5-trimethoxyamphetamine were capable of EEG alerting at both the midbrain and medullary regions. The potencies of these drugs for evoking EEG alerting approximated to some degree their psychotomimetic potencies in man. The mechanism of activity of these drugs at the synaptic level is not known, but different sites for EEG alerting would indicate different mechanisms for this response. Fujimori and Himwich (69) suggested that the psychotomimetic methoxy derivatives of amphetamine may act by inhibiting the synchronizing mechanism in the lower brain stem, releasing from its restraint the midbrain-activating system. Bridger and Mandel (70) also suggested different sites of activity for mescaline, 3,4-dimethoxyphenethylamine, and amphetamine based on studies in intact animals.

Table I—Identification of 3,4-Dimethoxyphenethylamine in Urine

Group	Source	Present	Absent	Inconclusive	Total
A	Normal volunteers	1	249	—	250
	Mentally normal inpatients	—	120	—	120
B	Schizophrenics	44	12	11	67
	Paranoid schizophrenics	2	15	—	17
C	Nonschizophrenics	—	16	1	17
	Schizophrenics	20	30	19	69
	Paranoid schizophrenics	2	54	6	62
	Schizophreniform syndromes	5	58	25	88
	Nonschizophrenics	1	68	8	77

Synaptic activity of mescaline was further indicated by findings of Denber and Teller (71). Subcellular levels in the cortical region were attained in 30 min. in the myelin microsomal supernatant and in 45 min. in the nerve endings. 2,5-Dimethoxy-4-methylamphetamine was further studied by Idanpaan-Heikkila *et al.* (72). High levels of radioactivity were found in the hippocampus, amygdaloid nucleus, medial and lateral genticulate bodies, and the putamen. Closer to the midline, the drug appeared to accumulate in the thalamic nuclei, caudate nucleus, and hypothalamus. In the frontal region of the brain, the drug was found in the cerebellum, fastigial nucleus, and olfactory nuclear area. As discussed later, the drug appeared to concentrate in those areas that would be suggested by its symptomatology.

Peripheral distribution of mescaline was thoroughly discussed by Patel (1). The proposed implications of methoxy derivatives of phenethylamines encouraged studies of the binding of these agents (72–74). VanVunakis *et al.* (75) utilized mescaline and other compounds as haptens and elicited the production of antibodies toward the 3,4,5-trimethoxyphenyl, 3,4-dimethoxyphenyl, and 4-methyl-2,5-dimethoxyphenyl groups. This immunological approach offers a new tool for the detection of these compounds in biological fluids, a method of determining cross-tolerance, and possibly an antidote for the pharmacological effects of these compounds. Rundire and Sehon (76) were able to inhibit the subcutaneous effects of serotonin in mice by using a specific antiserum.

Oh *et al.* (73) studied the binding of mescaline and other biologically active amines to plasma protein fractions. Considerable binding of 3,4-dimethoxyphenethylamine was realized with Cohn fraction III, with negligible binding by other fractions. Mescaline and the other amines were not bound to any extent by the various plasma fractions. No significant differences

between normal and schizophrenic plasma binding were noted.

Proteolipid protein (77) bound mescaline and related compounds irreversibly. This binding was much stronger than the complexes formed between mescaline, 3,4-dimethoxyphenethylamine, or serotonin and casein, ovalbumin, serum albumin, or a serum protein mixture.

The close structural relationships of psychotomimetic phenethylamines and the catecholamines would suggest some impact of these compounds on adrenergic receptors. Evidence (78) was presented to demonstrate the activity of mescaline at several α-adrenergic sites as an agonist. It is capable of antagonizing the effects of α-stimulatory drugs and, in the presence of specific β-blocking agents, can inhibit the effects of epinephrine. The β-stimulatory action of mescaline appeared to be very slight, and it did not stimulate cholinergic receptors.

Structurally unrelated hallucinogens including LSD, mesaline, psilocin, and 2,5-dimethoxy-4-methylamphetamine have been observed to facilitate the flexor reflex and evoke the stepping reflex in the chronic spinal dog. Similar responses have been reported for methoxamine, amphetamine, and mescaline. The facilitory action of the former compounds was felt to be an agonistic action (79) because chlorpromazine, which does not affect the flexor reflex, does antagonize the facilitory actions of D-lysergic acid diethylamide, mescaline, psilocin, 2,5-dimethoxy-4-methylamphetamine, and the serotonin antagonist, methysergide. The agonistic actions of the hallucinogens were distinguished from the anorexics in the following manner: (*a*) phenoxybenzamine antagonized the effects of amphetamine and methoxamine but not the hallucinogens; and (*b*) cyproheptadine antagonized the actions of the hallucinogens and facilitated the effects of the anorexics. The similarity of the actions to tryptamine responses suggested that LSD-like hallucinogens owe at least a part of their activity in the spinal chord to agonistic activity at the tryptamine receptor.

Early hypotheses by Gaddum (80), concerning the mechanism of action of LSD, mentioned possible interaction with serotonin, increasing concentration and decreasing turnover. The site of this activity was proposed to be at the serotonin neurons of the raphe nuclei (81) because minute doses of LSD produced a reversible cessation of the firings of single neurons in these midbrain nuclei. Aghajanian *et al.* (82) studied the interaction of the raphe neurons with 2-bromolysergic acid diethylamide, *N,N*-dimethyltryptamine, mescaline, 2,5-dimethoxy-4-methylamphetamine, scopolamine, atropine, and phencyclidine. The latter three drugs produce psychoses of a different type than LSD (83, 84). Inhibitory responses were noted with LSD, *N,N*-dimethyltryptamine, and 2-bromolysergic acid diethylamide, although the action of 2-bromolysergic acid diethylamide was incomplete. An inhibitory effect isolated in a subgroup of raphe cells was observed after mescaline and 2,5-dimethoxy-4-methylamphetamine. No effect on raphe units was seen after scopolamine, atropine, and phencyclidine.

The subtle difference in the activity of LSD and *N,N*-dimethyltryptamine as compared to mescaline and 2,5-dimethoxy-4-methylamphetamine has been reflected in their effect on serotonin metabolism. 5-Hydroxyindoleacetic acid levels are moderately lowered by LSD and *N,N*-dimethyltryptamine in a wide range of doses (85). The relative raphe inhibitory potency was roughly comparable to behavioral potency in rats.

2-Bromolysergic acid diethylamide is much less potent than LSD as a psychotomimetic, although structurally it is quite similar. A postulate for this observation has been proposed by the fact that serotonin creatinine sulfate (2×10^{-6} *M*) markedly decreased in size the postsynaptic potential induced *in vitro* in guinea pig superior colliculus slices in response to optic tract stimulation. Total block of this response was achieved by LSD (10^{-6} *M*), psilocybin (10^{-5} *M*), and mescaline (10^{-4} *M*). It was not blocked by 10^{-5} *M* 2-bromolysergic acid or morphine (86).

The total significance of the overlapping activities of the various psychotomimetics is not entirely clear at this time. Cross-tolerance exists among these compounds, and changes in metabolism resulting in conservation of serotonin (87, 88) are common to both the indolealkylamines and the phenethylamines. Mescaline as well as LSD produced slight increases in 17-ketogenic steroid excretion (89) which, although often associated with stress, was poorly correlated with other clinical or physiological signs of stress. Both drugs also produced very similar effects on eye movements, as reported by Hebbard and Fischer (90).

Recognized disruption of learned behavior upon mescaline administration and the present molecular theory of learning associated with ribonucleic acid molecules suggested that this disruption may be related to mescaline-induced instability of ribonucleoprotein particles in the brain (91). Mescaline did not, however, affect enzymic activities of isolated normal brain cortex ribosomes so that observed ribosomal changes may simply reflect generalized cellular disorganization. Demethylation of mescaline in brain cortex slices is associated with appreciable methylation of ribonucleic acid species (92). It is not unlikely that the reported instability is related to partially blocked hydrogen bonding sites in ribonucleic acid by methylation on mescaline treatment, as indicated by hyperchromicity effects.

The biphasic activity of mescaline as observed in conditioned avoidance responses suggested activity of a metabolite (93). Conditioned avoidances are first inhibited and then enhanced. Pretreatment of animals with iproniazid enhanced the activity, supporting the hypothesis that activity is due to the parent compound rather than to a metabolite (93). Tolerance and cross-tolerance studies (94) supported the concept of biphasic activity by demonstrating that successive doses of mescaline induced tolerance to the vegetative phase and increased the excitatory phase. In a like manner, tolerance developed to the predominantly inhibitory effect of 3,4-dimethoxyphenethylamine and to the excitatory effect of *N,N*-dimethylmescaline. Cross-tolerance existed among all three compounds.

Presentday abuse of several methoxyphenethylamines has necessitated the development of dependable bioassay tests for identification (95). Researchers (95 and references cited therein) have developed test procedures that consist of several tests to identify psychotomimetic drugs and test procedures that may be used to evaluate relative potencies among compounds in the same class. Corne and Pickering (95) reported a possible correlation between drug-induced hallucinations in man and a characteristic head twitch in mice. Excellent relationships between effective doses in mice and hallucinogen doses in man were found for at least 15 compounds. The head twitch did not occur upon administration of compounds closely related to the hallucinogens but not hallucinogenic. Similar relationships have not been established between teratogenic effects (96, 97) in animals and man.

2,5-Dimethoxy-4-methylamphetamine has been the most popular mescaline derivative within the drug culture since its first appearance in 1967 (98). Preliminary pharmacology indicated it to be 25–50 times more potent than mescaline and substantially different than amphetamine (99, 100). Brain concentrations of 2,5-dimethoxy-4-methylamphetamine may be related to several of its actions (75): the hissing and violent rage of cats by stimulation or destruction of certain areas of the hypothalamus; the modification of information from the eye, ear, and skin by alteration of hippocampus activity; *etc.* High brain concentrations of 2,5-dimethoxy-4-methylamphetamine were found in those areas that could account for the induced behavioral effects. Chlorpromazine is not always successful in decreasing the effects of 2,5-dimethoxy-4-methylamphetamine (101). It appears doubtful that chlorpromazine is a specific antidote but its sedative effects may ameliorate some aspects of the reaction (102). Halasz *et al.* (101) suggested that the interaction between 2,5-dimethoxy-4-methylamphetamine and chlorpromazine is dose related and that it is possible to attain a tranquilizer dose which can aggravate rather than protect the patient from the hallucinogen.

3,4-Dimethoxyphenethylamine has been rather thoroughly studied (103–112) because of its possible implications in mental disorders. In high doses (200 mg./kg.), it produces catatonia or hypokinetic rigidity in rats (104). *N*-Acetyl-3,4-dimethoxyphenethylamine is more potent than 3,4-dimethoxyphenethylamine in rodents (107). In both dogs and cats, behavioral responses to the drugs would indicate hallucinogenic activity. In rats, blood pressure was initially elevated (pressor response) and subsequently lowered (β-block) (104). Antihistaminic activity of 3,4-dimethoxyphenethylamine was present in dogs but absent in guinea pigs and rabbits (105). Tolerance (94) and an absence of tolerance (106) have both been reported for this agent. 3,4-Dimethoxyphenethylamine–schizophrenic plasma (108) complexes caused amphetaminelike response in mice unlike normal plasma–3,4-dimethoxyphenethylamine complexes. Masur *et al.* (109) were unable to verify these findings.

Clinical trials of 3,4-dimethoxyphenethylamine in doses up to 800 mg. (103) revealed no psychotomimetic effects similar to those experienced with mescaline, in contrast to those experiments conducted with lower animals. This finding may be the result of rather complete metabolism of 3,4-dimethoxyphenethylamine to the corresponding acid, in contrast to the significant amounts of mescaline excreted as the free amine.

3,4-Dimethoxyphenethylamine in the rat is rapidly concentrated (within 5 min. of injection) in the kidney, liver, spleen, and heart, with relatively small amounts entering the brain (110). Within the brain, highest concentrations were found in the cerebellum and slightly less in the hemispheres, midbrain, and pons medulla. Over 90% of the drug was metabolized within 60 min., with a majority being biotransformed to 3,4-dimethoxyphenylacetic acid by monoamine oxidase. It did not appear to accumulate in sympathetic nerve endings. Clark *et al.* (111) studied the rate of oxidative deaminations of a series of ring-methoxylated phenethylamines. "Mescaline oxidase" is inhibited by semicarbazide whereas "tryptamine oxidase," both from the rabbit liver, is not. Deamination of 3,4-dimethoxyphenethylamine was found to be only partially inhibited by semicarbazide, indicating alternate routes for its metabolism. Mescaline deamination is completely blocked by semicarbazide (111, 112).

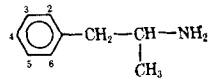
Metabolism of 3,4-dimethoxyphenethylamine was reported (58) to result in the formation of 3,4-dimethoxyphenylacetic acid, 2(3′,4′-dimethoxyphenyl)ethanol, *N*-acetyl-3,4-dimethoxyphenethylamine, *N*-acetyl-3-methoxy-4-hydroxyphenethylamine, and 2(3′-methoxy-4′-hydroxyphenyl)ethanol. Relative percentage isolations of each of the compounds were, respectively, 77, 0.1, 0.1, 6.2, and 0.1 with 15.5% of the material excreted as the free amine. This agrees with the observation of Sargent *et al.* (113) that demethylation at the C^4-methoxy was 15 times as rapid as at C^3-methoxy.

STRUCTURE–ACTIVITY RELATIONSHIPS

Ring Substituents—Smythies *et al.* (114, 115) studied the relationship between the hallucinogenic potency of phenethylamines and the methoxy substituent. Monosubstituted compounds (*o*, *m*, *p*) were inactive in doses up to 25 mg./kg. All disubstituted compounds (2,3; 2,4; 2,5; 2,6; 3,4; and 3,5) were inactive in the Sidman avoidance response technique. Previous reports (104–107) indicated rodent activity of the 3,4-isomer (3,4-dimethoxyphenethylamine). Only mescaline among the trisubstituted derivatives (2,3,4; 2,3,5; 2,3,6; 2,4,5; 2,4,6; and 3,4,5) was found to be active. Of the tetrasubstituted derivatives (2,3,4,5; 2,3,5,6; and 2,4,5,6), only the 2,3,4,5 was active (approximately 2 mescaline units). The pentasubstituted compound was the most active (7 mescaline units) of all compounds synthesized. Shulgin (116), in a recent review, reported 2,4,5-trimethoxyphenethylamine to be equal in potency to mescaline and 4-methoxyphenethylamine to be only slightly less potent than mescaline.

3,4-Methylenedioxyphenethylamine was less than 0.2 times as potent as mescaline, 3-methoxy-4,5-methylenedioxyphenethylamine about equipotent to mescaline, and 2-methoxy-3,4-methylenedioxyphenethylamine somewhat less than 5 times as potent as mescaline.

Table II—Structure–Activity Relationships



Compound Number	Substitution Pattern 2	3	4	5	6	Activity, Mescaline Units
1	H	H	OCH_3	H	H	5
2	OCH_3	OCH_3	H	H	H	—
3	OCH_3	H	OCH_3	H	H	5
4	OCH_3	H	H	OCH_3	H	8
5	OCH_3	H	H	H	OCH_3	—
6	H	OCH_3	OCH_3	H	H	1
7	H	OCH_3	H	OCH_3	H	—
8	H	OCH_3	OCH_3	OCH_3	H	2.2
9	OCH_3	H	OCH_3	OCH_3	H	17
10	OCH_3	OCH_3	OCH_3	H	H	2
11	OCH_3	OCH_3	H	OCH_3	H	4
12	OCH_3	OCH_3	H	H	OCH_3	13
13	OCH_3	H	OCH_3	H	OCH_3	10
14	O —CH_2—	O	H	H	H	—
15	H	O —CH_2—	O	H	H	3
16	H	OCH_3	O —CH_2—	O	H	2.7
17	OCH_3	H	O —CH_2—	O	H	12
18	OCH_3	O —CH_2—	O	H	H	10
19	O —CH_2—	O	OCH_3	H	H	3
20	O —CH_2—	O	H	OCH_3	H	—
21	O —CH_2—	O	H	H	OCH_3	—
22	OCH_3	O —CH_2—	O	OCH_3	H	12
23	OCH_3	OCH_3	O —CH_2—	O	H	5
24	H	OCH_3	O —$(CH_2)_2$—	O	H	1
25	OCH_3	OCH_3	OCH_3	OCH_3	H	6
26	OCH_3	OCH_3	OCH_3	H	OCH_3	—
27	OCH_3	OCH_3	H	OCH_3	OCH_3	—
28	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	—
29	OC_2H_5	H	OCH_3	OCH_3	H	7
30	OCH_3	H	OC_2H_5	OCH_3	H	15
31	OCH_3	H	OCH_3	OC_2H_5	H	7
32	OC_2H_5	H	OC_2H_5	OCH_3	H	—
33	OC_2H_5	H	OCH_3	OC_2H_5	H	—
34	OCH_3	H	OC_2H_5	OC_2H_5	H	—
35	OC_2H_5	H	OC_2H_5	OC_2H_5	H	—
36	OCH_3	H	CH_3	OCH_3	H	80

Larger doses of the related compounds (117) have been used to induce a hypokinetic rigid syndrome in cats which was, in turn, related to the structure. This response was caused by only the 4-methoxy, 3,4-dimethoxy, 3,4,5-trimethoxy, and 3-hydroxy-4-methoxy compounds. The latter compound was active only after iproniazid treatment, presumably prolonging the half-life of the compound permitting liver *O*-methyltransferase to synthesize 3,4-dimethoxyphenethylamine. 4-Methoxy substitution appeared to be prerequisite for activity. Ring hydroxylation apparently inhibited transfer across the blood–brain barrier. The duration of hypokinetic rigid syndrome was related to the number of methoxy groups adjacent to the 4-methoxy groups.

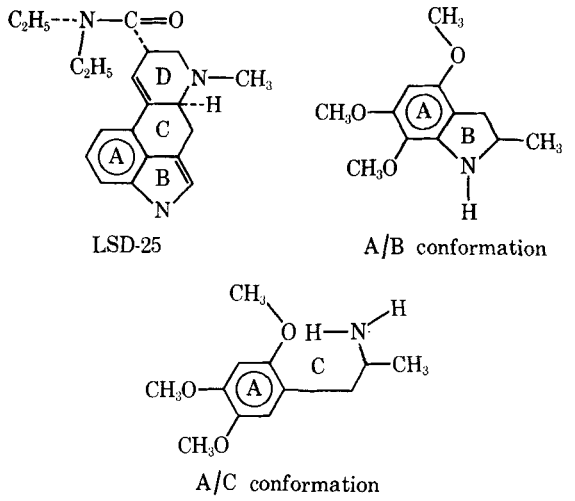
α-Methyl Derivatives—Table II summarizes the pertinent data concerned with the methoxy derivatives of phenylisopropylamine (116).

Compounds 2, 5, 7, 14, 20, 21, 26, 27, and 28 were not synthesized at the time of this report. Since that time, Compounds 2 and 7 have been synthesized (4). Compounds 32–35 have been synthesized (2) but not totally evaluated pharmacologically. Certain generalizations concerning the structure–activity relationships can be made with the information in Table II. The inclusion of an α-methyl group increases the activity of this series.

Optimum activity is found in those compounds bearing a *para*-methoxy group. The addition of an *ortho*-methoxy group usually causes an increase in activity which may be substantial. *Meta*-methoxylation with few exceptions causes a decrease in activity. Conversion of two adjacent methoxy groups to a methylenedioxy group generally brings about an increase in activity. *Para*-methylmercapto substitution is not reported (118) as inducing hallucinations.

Psychotomimetic effects have been claimed in related compounds which do not possess methoxy groups. *Ortho*-substituted *N*-methylphenyl-2-aminopropanes demonstrated remarkably high potencies expressed as approximate mescaline units (119). The authors prepared *ortho*-amino (6 mescaline units), nitro (28 mescaline units), iodo (8 mescaline units), chloro (27 mescaline units), and bromo (20 mescaline units). Similar substitutions in the *para*-position resulted in very potent psychostimulants without psychotomimetic activity. The effects of these compounds were discernible up to 48 hr. longer than LSD.

Disruption of visual discrimination with squirrel monkeys (120) yielded results very similar to those reported earlier (116). Compound 26 was approximately 5 times as potent as Compound 9, which was the most potent trimethoxy derivative. 2,5-Dimethoxy-4-ethylphenylisopropylamine was reported to be about twice as potent as Compound 26, which would give it a mescaline value of approximately 150 (121). Uyeno (122) evaluated a series of hallucinogenic compounds on the basis of their ability to alter escape



Scheme IX—Comparison of LSD to Mescaline Derivatives

tendencies of rats as measured by their swimming time through an escape tube. Compound 9 was about twice as potent as Compound 8, and both were more potent than Compound 10.

Fairchild *et al.* (123) evaluated several hallucinogen compounds by comparing their ability to produce patterns of distortion in frequency distribution of spontaneous brain electrical activity in the cat. The application of multivariate statistical techniques to the results of broad-band frequency analysis of spontaneous brain electrical activity showed Compounds 10, 15, and 16 to be more potent than Compound 6 which, in turn, was slightly more potent than mescaline.

Ho *et al.* (4) evaluated an expanded series of compounds as to their ability to disrupt mouse behavior as determined by a swim maze test. 4-Methyl-5-methoxyphenyl-2-aminopropane was as active as 2,5-dimethoxy-4-methylamphetamine and of longer duration, whereas 4-methyl-2-methoxyphenyl-2-aminopropane was inactive. 2,5-Dimethoxyphenyl-2-aminopropane was also inactive, indicating the necessity of the 4-methyl substituent. Several other new compounds were reported but not related in potency to known hallucinogens.

A limited number of novel compounds (124–127) were reported which bear resemblance to mescaline. A series of methoxyphenethylamines was synthesized (124) containing an α-trifluoromethyl group. These compounds were all less potent than corresponding methyl analogs, and only the 3,4,5-methoxy derivatives gave positive results in the head twitch assay (0.11 mescaline unit).

Walters and Cooper (125) prepared *trans*-2-(3,4,5-trimethoxyphenyl)cyclopropylamine in anticipation of prolonged mescalinelike effects. The title compounds exhibited identical changes in mice as mescaline, although its acute effects were briefer than either mescaline or tranylcypromine. It definitely had psychotomimetic effects in rodents which cannot be directly extrapolated to humans at this time.

The large number of synthesized methoxyphenethylamine derivatives has made possible certain theories concerning criteria for activity (128–130). Similar modes of activity among LSD, psilocybin, and mescaline suggest that common structural requirements may exist between the chemically unrelated series. Since LSD is the most potent compound and is additionally a rigid structure, it should make a suitable model for the hallucinogens. Only the D-lysergic acid conformation is naturally occurring and only D-LSD is psychotomimetic in man. Snyder and Richelson (128) postulated that a compound's ability to form the A and B or A and C rings of lysergic acid (Scheme IX) is critical insofar as psychotomimetic activity is concerned.

Table III—Tendency to Form A/B or A/C Ring Conformations

Compound	Tendency to Form—Ring B	Tendency to Form—Ring C	Potency, Mescaline Units
2,4,6-Trimethoxy	0	4	10
2,4,5-Trimethoxy	0.7	2	17
6-Methoxy-3,4-methylenedioxy	0.7	2	21
2-Methoxy-3,4-methylenedioxy	0.6	2	18
2,3,6-Trimethoxy	0	2	<10
3,4,5-Trimethoxy	2	—	2.2
3-Methoxy-4,5-methylenedioxy	2	—	2.7
2,3,5-Trimethoxy	1	0	<7
2,3,4-Trimethoxy	0.6	0	<2

Analogs of mescaline might possibly form either the A/B or A/C ring system of lysergic acid. Mescaline itself would be able to form the A/B ring system by intramolecular hydrogen bonding between the amine side chain and either *ortho* position. Negative π-charge calculations (129, 130) revealed that mescaline possesses the greatest charge at positions 2 and 6 and would, therefore, be most likely to form the B ring of LSD. 2-Methoxy-3,4-methylenedioxyphenethylamine may owe its greater potency to a tendency toward A/C ring conformations. 2,3,4-Trimethoxyphenethylamine is not as potent and would not share a similar tendency toward A/C ring conformations, because the 3-methoxy group would inhibit the required free rotation of the 2-methoxy group.

Methoxyphenyl-2-aminopropanes are more potent than their desmethyl counterparts (116). Major consideration in the formation of the B ring would be paid to the negative π-charge at positions 2 and 6 and the availability of these positions for hydrogen bonding. Those compounds with methoxy groups on position 2 and without substitution at position 3 would also have a tendency to form the A/C conformation. Snyder and Richelson (128) compared the combined tendencies to approximate LSD to potency for a series of methoxyphenyl-2-aminopropanes (Table III).

Snyder and Merrill (129, 130) made molecular orbital calculations for a variety of hallucinogenic drugs and their nonhallucinogenic counterparts and discovered several relationships between the electronic configurations and activity. Unfortunately, no methylenedioxy compounds were included in this study.

The energy of the highest filled molecular orbital was calculated for a series of mono-, di-, and trisubstituted phenethylamines. Progressive methoxylation resulted in an increase in highest filled molecular orbital energy. Mescaline had the highest filled molecular orbital in the series, with 2,3,4-methoxyphenethylamine a poor

Table IV—Relationship of Hallucinogenic Potency in Different Classes of Drugs to the Energy of Their Highest Filled Molecular Orbital

Drug	Energy of Highest Filled Molecular Orbital, B Units	Potency, Mescaline Units
LSD	0.2180	3700
Psilocin	0.4603	31
6-Hydroxydiethyltryptamine	0.4700	25
2,4,5-Trimethoxyamphetamine	0.4810	17
3,4,5-Trimethoxyamphetamine	0.5357	2.2

second. Confirmation of this possible relationship will depend on similar calculations for the other isomers. Activity was also related to superdelocalizability, which is a function of the highest filled molecular orbital energy related to each atom.

A series of trimethoxyphenyl-2-aminopropanes were subjected to the same calculations. The energy of the highest filled molecular orbital was greatest for 2,4,5-trimethoxy (17 mescaline units), intermediate for 3,4,5-trimethoxy (2.2 mescaline units) and lowest for 2,3,4-trimethoxy (<2 mescaline units). Activity did not correlate well with frontier electron density, π-charge, free valence, superdelocalizability, or lowest empty molecular orbital energy.

Energy calculations showed excellent relationships between highest filled molecular orbital energy and activity in a series of unrelated compounds (Table IV).

In spite of a rather crude calculation for LSD, the high potency is reflected by an energetic highest filled molecular orbital. The suggestive correlations between highest filled molecular orbital and psychotomimetic activity implies that these materials may act as electron donors. Of course, other compounds such as chlorpromazine are powerful electron donors and do not possess hallucinogenic activity. However, a combination of steric and electronic considerations would provide the possibility of designing hallucinogenic compounds of greater potency than are now in existence.

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In Vivo and *In Vitro* Availability of Commercial Warfarin Tablets

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Abstract □ Rate of absorption and bioavailability of warfarin following oral administration of four different commercial warfarin tablets was assessed by measurement of plasma concentrations of warfarin followed by pharmacokinetic and statistical analysis of the resulting data. In Study No. 1, results obtained with five 5-mg. tablets were compared with those obtained with one 25-mg. tablet, all tablets being made by the same manufacturer. The rate of absorption was about twice as fast from the five 5-mg. tablets as from the one 25-mg. tablet. Relative absorption from the one 25-mg. tablet was only about 80%, compared with 100% from the five 5-mg. tablets. In Study No. 2, three 5-mg. tablets, prepared by three different manufacturers, were compared in a crossover study in 12 normal subjects at a two-tablet (10-mg.) dose. The rate constant for absorption varied over about a fourfold range, but all three tablets were equally available and the entire dose of warfarin was absorbed, apparently, after each treatment. Results obtained in man correlated excellently with results of *in vitro* rate of dissolution tests but not with disintegration times of the tablets. The results will aid in delineating the critical area for *in vitro* dissolution rates of warfarin tablets prepared similarly to the commercial products and in quality control procedures.

Keyphrases □ Warfarin tablets—*in vitro*, *in vivo* availability □ Bioavailability—warfarin tablets □ Absorption-rate constants—warfarin tablets □ Pharmacokinetics—warfarin absorption □ Tablets, warfarin—plasma concentration comparison □ Dissolution, absorption-rate correlation—warfarin tablets

The importance of performing both clinical studies and *in vitro* rate of dissolution tests on different manufacturers' formulations of the same drug was recently stated in a "White Paper on the Therapeutic Equivalence of Chemically Equivalent Drugs," prepared by a subcommittee of the Policy Advisory Committee, Drug Efficacy Study of the National Research Board, National Academy of Sciences–National Research Council (1). Minimum variability of absorption rates in man and complete physiological availability are desired characteristics for all drugs, but these properties are especially important for an anticoagulant such as warfarin.

Previous investigations were made of the *in vitro* dissolution and *in vivo* absorption characteristics of different oral dosage forms of both warfarin (acid) and its sodium salt (2, 3). These studies strongly suggested that absorption of warfarin in man is dissolution-rate controlled. However, both these studies were performed using 100-mg. doses of warfarin, and the commercial formulations used were not identified. It appeared desirable to compare plasma concentrations of warfarin in man following oral administration of several commercial tablets at dose levels in the normal therapeutic range. Warfarin presents a particularly interesting example in this area since plasma concentrations of warfarin have already been indirectly correlated with efficacy as measured by depression of "prothrombin complex activity" using a pharmacokinetic approach (4).

This report compares plasma concentrations of warfarin (acid) measured in two different crossover studies. In the first study, six subjects ingested one 25-mg. tablet or five 5-mg. tablets of sodium warfarin; both tablets were made by the same manufacturer. Plasma samples were analyzed independently by two different methods of analysis. In the second study, 12 subjects ingested 10-mg. label doses of sodium or potassium warfarin administered as two tablets of three different manufacturers' kinds of warfarin. *In vitro* rate of dissolution tests were performed on five individual tablets of each lot of tablets tested clinically. Three lots of tablets containing 5 mg. of sodium warfarin and three lots of tablets containing 25 mg. of sodium warfarin were prepared; some of these tablets were shown to release the warfarin *in vitro* much faster than any commercial tablet studied.

EXPERIMENTAL

Screening of Subjects—Subjects screened to become members of each of the two panels received a drug history and physical examination which included measurement of blood pressure, pulse, and respiration. Hematology measurements included hemoglobin, hematocrit, mean corpuscular hemoglobin concentration, white cell count, and differential. Blood chemistry measurements included serum creatinine, bilirubin (both free and total), serum glutamic oxalacetic transaminase, and alkaline phosphatase. Each prospective panel member also had a urinalysis, chest X-ray, and electrocardiogram. In the second study, prothrombin time was measured during the screening a few days before the study began and 96 hr. after the last dose of warfarin was administered.

In the first study, 10 subjects were screened and seven were utilized. In the second study, it was necessary to screen 20 subjects to obtain a satisfactory panel of 12 "normal" subjects. Only those subjects who had all parameters in the normal range were selected.

Subjects Selected—*Study No. 1*—Subject 6, who received Treatment B (one 25-mg.) in Phase I was replaced by Subject 6*a* who received Treatment A (five 5-mg.) in Phase II. The other five subjects received both treatments as scheduled. Subjects 1 and 4 were females, and the remainder were males. Group I consisted of Subjects 1, 2, and 3 in both Phases I and II; Group II consisted of Subjects 4, 5, and 6 in Phase I and Subjects 4, 5, and 6*a* in Phase II. The group number, average age and range of ages, and average body weight and range of body weights were as follows: I, 25 (21–30) years, 68 (54.5–90.5) kg.; II (Phase I), 27 (23–31) years, 70.5 (59.0–77.5) kg.; and II (Phase II), 28 (23–31) years, 71.5 (59.0–77.5) kg.

Study No. 2—Group I consisted of Subjects 1 through 4, Group II consisted of Subjects 5 through 8, and Group III consisted of Subjects 9 through 12. Subjects 2 and 12 were females, and the

Table I—Dosage Schedules Employed in the Two Clinical Studies

Group	Subjects	Study No. 1 Phase I	Study No. 1 Phase II
I	1, 2, 3	A (five 5-mg.)	B (one 25-mg.)
II	4, 5, 6	B (one 25-mg.)	
	4, 5, 6[a]		A (five 5-mg.)

Group	Subjects	Study No. 2 Phase I	Study No. 2 Phase II	Study No. 2 Phase III
I	1 to 4	A (two 5-mg.)	C (two 5-mg.)	D (two 5-mg.)
II	5 to 8	C (two 5-mg.)	D (two 5-mg.)	A (two 5-mg.)
III	9 to 12	D (two 5-mg.)	A (two 5-mg.)	C (two 5-mg.)

remainder were males. The statistics (as already explained) were as follows: I, 24 (23–32) years, 72.0 (59.0–90.0) kg.; II, 24 (21–31) years, 78.0 (68.0–92.0) kg.; and III, 24 (21–27) years, 74.5 (60.5–94.0) kg. Four subjects were common to both studies; their subject numbers in Study No. 2 followed by their subject numbers in Study No. 1 in brackets were as follows: 2 (4), 3 (3), 6 (5), and 12 (1).

Informed consent forms were signed by all subjects who participated in these studies.

Protocols—All subjects received no barbiturates or other known enzyme-inducing agents for a period of 30 days and no medication other than the prescribed medication for study for a period of 7 days preceding initiation of the studies and during the studies. Subjects were fasted overnight and for 4 hr. postadministration of warfarin. On the mornings medication was administered, each subject drank 240 ml. (8 oz.) of water within the 1st hr. after arising and 240 ml. of water when the tablets were ingested at zero time for each subject (between 8 and 9 a.m., accurately determined in each case). Tablets were swallowed whole.

During Study No. 1, each subject was administered two tablets of menadiol diphosphate (tetrasodium salt)[1], 5 mg., the day before administration of warfarin, 4 hr. following the dose of warfarin, and 3 days following the dose of warfarin. In Study No. 2, no medication other than warfarin was administered. All subjects were ambulatory during the daytime and performed their normal jobs in the hospital.

In Study No. 1, 20 ml. of whole blood, and in Study No. 2, 15 ml. of whole blood, was taken from a forearm vein at zero time and at 1, 4, 8, 12, 24, 48, 72, and 96 hr. postdosing. The blood was drawn into one or two vacutainer tubes containing ethylenediaminetetraacetic acid as anticoagulant. Each blood sample was centrifuged as rapidly as possible after collection, and the plasma was quick-frozen and kept in the frozen state (about −20°) until just prior to assay. All samples were double labeled with paste labels and tags bearing subject numbers, phase, collection time in hours, and date. In both studies, treatments were separated by a period of 3 weeks (504 hr.) which is equivalent to about 10 or more average half-lives of warfarin in plasma.

Treatments and Schedules—Four tablets, designated A, B, C, and D, were studied[2]. In Study No. 1, treatments were A (five 5-mg.), meaning five 5-mg. tablets of type A, and B (one 25-mg.), meaning one 25-mg. tablet of type B, both of which were made by the same manufacturer. In Study No. 2, treatments were A (two 5-mg.), meaning two tablets of type A; C (two 5-mg.), meaning two tablets of type C; and D (two 5-mg.), meaning two tablets of type D. Dosage schedules are given in Table I.

Assay of Plasma Samples—In Study No. 1, 4-ml. aliquots of each plasma sample were independently analyzed by a modification of the simple extraction–UV spectrophotometric method of O'Reilly *et al.* (5) (Method E) and by a TLC method similar to that of Lewis and Ilnicki (6) but employing a UV spectrophotometric rather than a fluorescence end-point (Method T). The details of these methods were described in a previous publication (7). Menadiol diphosphate (tetrasodium salt) was shown not to interfere in either method.

In Study No. 2, a second modification of the simple extraction–UV spectrophotometric method of O'Reilly *et al.* (5) was employed. In this method, 40 ml. of 1,2-ethylene dichloride, 7 ml. of plasma, 3 ml. of distilled water, and 2 ml. of 2.6 *N* hydrochloric acid were shaken for 10 min. in a Kahn shaker in a 100-ml. bottle fitted with a polyethylene-lined screw cap. The 1,2-ethylene dichloride layer was separated and washed with 10 ml. of 0.1 *M* phosphate buffer, pH 7.2. A 30-ml. aliquot of the washed 1,2-ethylene dichloride extract was extracted with 5 ml. of 2.5 *N* sodium hydroxide solution.

The absorbances of the alkaline aqueous extract at 308 and 360 nm. were read in a 7.5-cm. pathlength cell in a Gilford single-beam UV spectrophotometer. The instrument was zeroed for each extract at each wavelength by adjusting the slit width with water in the same cell as used to measure the absorbance of the sample extracts. The plasma concentrations were calculated from the formula:

$$\text{plasma concentration of warfarin (acid) in mcg./ml.} = \frac{(As_{308} - As_{360}) - (Ab_{308} - Ab_{360})}{0.320} \quad \text{(Eq. 1)}$$

where *As* is the absorbance of the extract derived from plasma obtained after dosing with warfarin, and *Ab* is the absorbance of the extract derived from the same subject's zero hour (predose) plasma.

As a quality control procedure, a spiked blood bank plasma sample (spiked at 2 mcg. warfarin/ml.) and a blank blood bank plasma sample were carried through the assay procedure with each daily batch. A daily batch constituted all the plasma samples of one particular subject on one phase of the crossover.

The factor 0.320 in Eq. 1 is based on extensive assay standardization, described in the previous report (7), employing reference standard warfarin (acid) USP.

Assay of Tablets—Each lot of tablets was assayed by the official USP XVIII method, using a group of 20 tablets from each bottle used in the clinical studies. Instead of assaying only one aliquot of powder derived from the 20 tablets, as prescribed by the USP, three different aliquots of powder were taken to obtain an indication of assay error. In addition, five individual tablets from each lot were assayed by a modification of the USP XVIII method. In the modified method, the single tablet was dissolved in 0.1 *N* sodium hydroxide by agitation; then the solution was shaken for 15 min. The volume of sodium hydroxide solution to be acidified and back-extracted into chloroform was adjusted to 15 ml. for the 5-mg. tablets and 3 ml. for the 25-mg. tablet. Appropriate calculations made from the observed absorbances enabled the total warfarin in individual tablets to be assigned.

Solubility of Warfarin (Acid) in 0.1 *N* Hydrochloric Acid—Six determinations of the solubility of warfarin in 0.1 *N* hydrochloric acid were made at both room temperature (about 25°) and at 37°. For each temperature, two bottles contained 5 mg., two bottles contained 25 mg., and two bottles contained 50 mg. of sodium warfarin. One hundred milliliters of 0.1 *N* hydrochloric acid was added to each bottle, and the bottles were equilibrated at the appropriate temperatures in a shaking bath overnight. Similar net absorbances ($A_{308} - A_{360}$) were obtained from each bottle at a given temperature.

***In Vitro* Rate of Dissolution Studies**—The pH of the fluids and exposure times were the same as those used by O'Reilly *et al.* (2). Single tablets were tested individually from each lot. Each tablet was exposed initially to 900 ml. of 0.1 *N* hydrochloric acid for 30 min. After 30 min, 100 ml. of a strong phosphate buffer was added to raise instantaneously to and maintain the pH at 7.5 ± 0.1 for the rest of the dissolution test. The dissolution assembly was similar to that described by Poole (8), being a three-necked, round-bottom, 1-l. flask. The stirring shaft was 25.4 cm. (10 in.) long; the stirring paddle was half-moon shaped, being 3.08 cm. (2 in.) wide and 1.91 cm. (0.75 in.) deep at the widest part of the arc. Both shaft and paddle were made of stainless steel. The shaft and paddle were centered in the middle hole of the flask and positioned so that the bottom of the paddle was exactly 2.5 cm. from the bottom of the flask. The shaft was rotated at 50 r.p.m. by means of a GT21 laboratory mixer and motor controller[3].

[1] Synkayvite, Roche.

[2] Tablet A was Coumadin, sodium, 5 mg. (Endo Laboratories, Inc.), Lot No. BA500A; Tablet B was Coumadin, sodium, 25 mg. (Endo Laboratories, Inc.), Lot No. 7H0212; Tablet C was Athrombin-K, 5 mg. (Purdue-Frederick), Lot No. K687355; and Tablet D was Panwarfarin (sodium), 5 mg. (Abbott Laboratories), Lot No. 830-0755-21.

[3] Gerald K. Keller Co.

Table II—Plasma Warfarin Concentrations[a] (Micrograms/Milliliter) Measured in Study No. 1 following 25-mg. Doses of Warfarin Sodium

Subject	Treatment	Analytical Method	Body Weight, kg.	Hours 1	4	8	12	24	48	72	96
1	A (five 5-mg.)	E[b]	59.0	2.84	4.08	3.19	3.25	2.69	1.47	0.81	0.48
		T[c]		2.85	4.54	2.80	3.22	2.25	1.67	0.93	0.62
	B (one 25-mg.)	E		2.93	3.86	3.17	3.03	2.31	1.60	1.04	0.53
		T		2.96	3.97	3.24	3.20	2.10	1.60	0.93	0.62
2	A (five 5-mg.)	E	54.4	2.19	3.36	3.21	2.66	1.90	1.16	0.61	0.34
		T		2.35	2.71	2.66	2.69	2.44	1.26	0.63	0.59
	B (one 25-mg.)	E		0.14	0.86	2.34	2.29	1.80	1.01	0.60	0.33
		T		0.14	0.76	2.23	2.32	1.77	0.96	0.52	0.34
3	A (five 5-mg.)	E	90.3	2.44	2.43	1.89	1.86	1.70	1.22	0.84	0.70
		T		2.71	2.54	2.00	1.85	1.47	0.96	0.58	0.34
	B (one 25-mg.)	E		1.70	2.01	1.86	1.83	1.49	0.97	0.72	0.53
		T		1.35	1.89	1.68	1.61	1.35	0.92[d]	0.67	0.41
4	A (five 5-mg.)	E	59.0	1.38	3.73	2.91	2.96	2.15	1.28	0.83	0.48
		T		1.63	3.42	2.95	3.11	2.11	1.24	0.61	0.53
	B (one 25-mg.)	E		0.48	1.89	2.22	2.44	1.92	1.02	0.54	0.16
		T		0.70	2.21	2.40	2.78	1.98	1.36	0.83	0.44
5	A (five 5-mg.)	E	77.1	2.57	2.54	2.62	2.18	1.37	0.99	0.58	0.34[e]
		T		2.86	2.64	2.62	2.26	1.57	1.13	0.56	0.51
	B (one 25-mg.)	E		0.79	1.35	1.57	1.86	1.42	1.22	0.91	0.68
		T		0.46	1.20	1.58	1.77	1.25	1.05	0.70	0.47
6*a*	A (five 5-mg.)	E	77.1	2.62[f]	2.89	2.42	2.30	1.93	1.74	1.02	0.82
		T		2.50	3.02	2.38	2.44	1.81	1.10	0.95	0.62
6	B (one 25-mg.)	E	74.8	2.01	2.30	2.08	2.10	1.39	0.83	0.53	0.42
		T		1.89	2.32	1.76	1.76	1.08	0.86	0.49	0.32

[a] All zero-hour plasmas were zero. Plasma hemolyzed; could not be assayed. [b] E = modified O'Reilly extraction method. [c] T = TLC method. [d] Value estimated from semilogarithmic plot of 24-, 72-, and 96-hr. plasma concentrations. (Plasma hemolyzed; could not be assayed.) [e] Value estimated from semilogarithmic plot of 48- and 72-hr. plasma concentrations. (Plasma hemolyzed; could not be assayed.) [f] Value estimated from 0-, 4-, 8-, and 12-hr. plasma concentrations by nonlinear interpolation.

The dissolution fluids were maintained at 37 ± 1° by means of a constant-temperature bath. Fluid was continuously circulated through a small syringe barrel fitted with glass wool and small glass beads (one end of the barrel being immersed in the dissolution medium) into 0.29 cm. (0.125 in.) i.d. silicone tubing, through a Sigmamotor pump, then through a silica flowcell in a Gilford UV spectrophotometer, and back into the three-necked dissolution flask again. A V-shaped glass tube filled partly with glass beads was placed in the silicone tubing line to act as a debubbling device. For the 5-mg. tablets, a 10-mm. lightpath was used; for the 25-mg. tablets, a 1-mm. pathlength flowcell was used. The rate of flow of fluid in the system was of the order of 70 ml./min.

The absorbance of the filtered dissolution medium was read manually at 308 nm., the wavelength of maximum absorbance of warfarin, at very frequent intervals. When greater than 85% of the warfarin had dissolved, the stirring speed was increased to 277 r.p.m. until a constant absorbance value was obtained. Reference standard warfarin (acid) USP was used for calibration purposes. Values obtained with this standard were then used to calculate the equivalent amount of sodium or potassium warfarin dissolved at different times from the tablets. The amount of sodium or potassium warfarin corresponding to the constant absorbance value was used as the denominator to calculate the percent of warfarin sodium or potassium dissolved at each sampling time.

Disintegration Times—Disintegration times were determined using at least 12 tablets of each lot. The USP XVII disintegration apparatus, without plastic disks, and water at 37° were employed.

Interpretation of Results—Symbolism employed is as follows:

C = plasma concentration of warfarin (acid) at time t after administration (micrograms/milliliter).

D = dose of warfarin (acid) based on the official assay of 20 tablets (milligrams).

F = fraction of the dose of warfarin that was absorbed (dimensionless).

Peak = observed peak plasma concentration of warfarin (micrograms/milliliter).

W = body weight of an individual subject (kilograms).

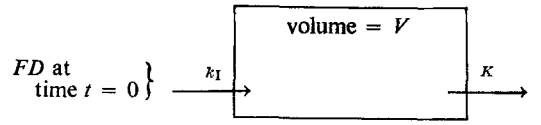
A = estimated area under the plasma concentration curve from time zero to infinite time. This is sometimes symbolized by area $0 \rightarrow \infty$ and was estimated by the usual method (4). The dimensions are (micrograms/milliliter) × hours.

$T_{1/2}$ = half-life calculated from the terminal plasma concentrations by the method of least squares when ln C was plotted *versus* t. Only those points that appeared to be randomly distributed about a straight line were utilized. Usually the earliest time value used was 24 or 48 hr. The line fitting the points has the equation: $\ln C = \ln C^0 - (0.693/T_{1/2})t$. $T_{1/2}$ is expressed in hours. In Table VIII, it is shown that $0.693/\beta$, where β was obtained by two-compartment analysis, is essentially the same as $T_{1/2}$ estimated above; this is not necessarily true generally but is true with these warfarin data.

Normalized area = $0.693A/[(T_{1/2})(D/W)]$, which is the same as the equation of Wagner (9).

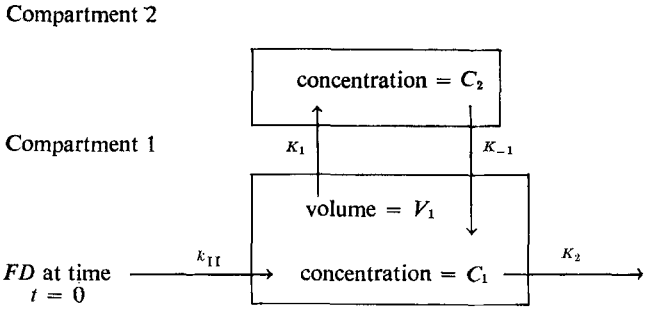
V_d = apparent volume of distribution of warfarin when the two-compartment open model is viewed as a one-compartment open model. This has formerly (10) been termed $V_{d\ \text{area}}$. It may be shown that $V_d = V_1\alpha/K_{-1}$, where V_1, α, and K_{-1} are defined below. V_d is expressed in liters. V_d/W is the V_d expressed as a fraction of body weight.

The one-compartment open model with first-order absorption may be written as shown in Scheme I:



Scheme I

The two-compartment open model with first-order absorption may be written as shown in Scheme II:



Scheme II

where V = apparent volume of distribution obtained by one-compartment analysis (Scheme I); K = apparent elimination-rate constant obtained by one-compartment analysis (Scheme I); k = the first-order rate constant for absorption of warfarin (hours^{-1}); k_I refers to the value obtained by one-compartment analysis (Scheme I); and k_{II} refers to the value obtained by two-compartment analysis (Scheme II). K_1 and K_{-1} are first-order distribution-rate constants (hours^{-1}) (Scheme II); K_2 = first-order rate constant for elimination of warfarin by all processes (hours^{-1}) (Scheme II); V_1 = volume of the inner compartment of which plasma is considered representative (liters) (Scheme II); and C_1 = concentration of warfarin (acid) in the plasma and inner compartment at time t (micrograms/milliliter) (Scheme II).

$$\alpha = {}^1/_2[(K_1 + K_{-1} + K_2) + \sqrt{(K_1 + K_{-1} + K_2)^2 - 4K_{-1}K_2}] \text{ (hours}^{-1}\text{)} \quad \text{(Eq. 2)}$$

$$\beta = {}^1/_2[(K_1 + K_{-1} + K_2) - \sqrt{(K_1 + K_{-1} + K_2)^2 - 4K_{-1}K_2}] \text{ (hours}^{-1}\text{)} \quad \text{(Eq. 3)}$$

For Scheme II, it may be shown that:

$$C_1 = \frac{kFD}{V_1}\left[\left\{\frac{K_{-1} - \alpha}{(k - \alpha)(\beta - \alpha)}\right\} e^{-\alpha t} + \left\{\frac{K_{-1} - \beta}{(k - \beta)(\alpha - \beta)}\right\} e^{-\beta t} + \left\{\frac{K_{-1} - k}{(\alpha - k)(\beta - k)}\right\} e^{-kt}\right] \quad \text{(Eq. 4)}$$

It may be shown that following oral administration of warfarin:

$$FD = V_1K_2A = V_d\beta A = V_d\left(\frac{0.693A}{T_{1/2}}\right) \quad \text{(Eq. 5)}$$

If both sides of Eq. 5 are divided by W, then rearrangement yields

$$\frac{0.693A}{(T_{1/2})(D/W)} = \frac{F}{(V_d/W)} \quad \text{(Eq. 6)}$$

where D/W is the milligrams/kilogram dose, and V_d/W is the apparent volume of distribution of warfarin expressed as a fraction of body weight. Equation 6 provides a pharmacokinetic interpretation of the "normalized area."

Statistical treatment of data included the following. Individual subject values of C at each sampling time, peak, area 0–24 hr., area 0–96 hr., A, normalized A value, time of occurrence of peak plasma concentration, and $T_{1/2}$ were analyzed by analysis of variance for crossover design using the data collected in each study separately. The significance levels shown in the tables opposite treatment averages are those obtained by an F-test using the among or between treatment mean square and the residual mean square in each analysis of variance. Four subjects were common to both studies; hence, there were five $T_{1/2}$ values estimated for each of these four subjects directly from their observed terminal plasma concentrations. Correlation coefficients were obtained for each subject when these $T_{1/2}$ values were correlated with the dose number, the dosing day number, and the dose of warfarin administered. All possible correlation coefficients were calculated for the correlations of peak, A, and the normalized areas with D/W, D, $1/W$, and peak; when N = 56, the values obtained with Treatments A (five 5-mg.), A (two 5-mg.), C (two 5-mg.), and D (two 5-mg.) were included, plus those from literature (11) obtained after a much higher dose; when N = 18, only those values obtained with Treatments A (five 5-mg.) and A (two 5-mg.) were included.

Pharmacokinetic analysis of the data included: (*a*) calculation of the normalized areas and interpretation by means of Eq. 6, and (*b*) fitting of some individual subject and average plasma concentrations observed following each treatment to the equations appropriate to Schemes I and II[4]. In each fitting, the plasma concentrations and corresponding time values, initial estimates of the parameters obtained graphically, the appropriate equations[4], and the program NONLIN were appropriately fed to an IBM 360/67 digital computer. The output of the computer was the least-squares estimates of the parameters, their standard deviations, and confidence intervals.

The rate of availability of warfarin (*i.e.*, rate of absorption) is indicated by the k_{II} values or by the half-absorption times [(0.693)(60)/k_{II}] in minutes. To relate the data obtained in both clinical studies with *in vitro* data, two approaches were used. The first approach made no assumption about efficiency of absorption and utilized Eq. 7:

$$\frac{\text{milligrams of warfarin (acid) absorbed in 1 hr.}}{V_d \text{ expressed as a fraction of body weight}} = \left(\frac{F}{V_d/W}\right)(D)[1 - e^{-k_{II}}] \quad \text{(Eq. 7)}$$

The second approach assumed that F has a value of unity (*i.e.*, entire dose absorbed) following Treatments A (five 5-mg.), A (two 5-mg.), C (two 5-mg.), and D (two 5-mg.); following Treatment B (one 25-mg.), F was assigned a value of 0.806[5]. Equation 8 was then utilized to obtain the *in vivo* parameter:

$$\text{milligrams of warfarin absorbed in 1 hr.} = FD[1 - e^{-k_{II}}] \quad \text{(Eq. 8)}$$

Bioavailability of warfarin (*i.e.*, relative absorption efficiency) was assessed within each study by two methods. First, normalized areas were compared by analyses of variance for crossover design. Equation 6 indicates this is equivalent to a comparison of $F/(V_d/W)$ values. If one assumes that the average V_d/W remained constant for the same panel given two or three treatments, then the difference between or among normalized areas theoretically yields the differences between or among F values. Also if the difference between two average normalized areas is significant, then the ratio of the normalized areas theoretically is equivalent to the ratio of F values following the two treatments. Secondly, V_1/F values, estimated by two-compartment analysis, were compared, taking cognizance of their 95% confidence intervals. If the assumption is made that the average V_1 remains constant for each treatment for a given panel of subjects, then the comparison of these values reflects significance or nonsignificance of differences in F values following two or more treatments.

[4] For Scheme I, the equation was $C = C^0\,(k_I/k_I - K)[e^{-Kt} - e^{-K_I t}]$. For Scheme II, Eqs. 2, 3, and 4 were used.

[5] $F_B = (D_A/D_B)\cdot(\beta_B/\beta_A)\cdot(A_B/A_A) = (22.7/23.9)\ (0.0187/0.0187)\ (140.9/165.9) = 0.806$, where the subscripts refer to Tablets A and B, and the β and A values were those estimated by nonlinear fitting to the two-compartment open model with first-order absorption.

Table III—Plasma Warfarin Concentrations[a] (Micrograms/Milliliter) Measured in Study No. 2 following 10-mg. Doses of Either Warfarin Sodium or Warfarin Potassium as Two 5-mg. Tablets

Subject	Tablets	Body Weight, kg.	Hours 1	4	8	12	24	48	72	96
1	A	72.6	1.08	0.81	0.78	0.79	0.63	0.37	0.30[b]	0.25
	C		0.86	1.54	1.02	1.09	0.96	0.77	0.58	0.46[c]
	D		0.08	1.04	0.81	0.83	0.60	0.46	0.35	0.25
2	A	59.0	1.33	1.26	0.98	0.93	0.68	0.44	0.36	0.23
	C		1.68	1.03	1.04	1.03	0.79	0.63	0.41	0.31
	D		0.34	1.68	1.13	1.03	0.87	0.56	0.40	0.26
3	A	89.4	1.02	1.00	0.66	0.66	0.61	0.46	0.33	0.27
	C		1.56	1.40	1.29	1.24	0.69	0.35	0.26	0.15[d]
	D		0.46	0.93	0.76	0.76	0.64	0.47	0.34	0.21
4	A	65.8	0.91	1.12	0.98	0.85	0.72	0.54	0.36	0.28
	C		0.47	0.87	1.01	0.93	0.77	0.53	0.45[e]	0.31
	D		0.46	1.17	1.02	0.93	0.73	0.56	0.39	0.29
5	A	73.5	0.74	0.91	0.81	0.72	0.54	0.46	0.31	0.23
	C		0.98	0.99	0.78	0.66	0.53	0.35	0.24	0.08
	D		0.70	0.93	0.82	0.86	0.66	0.49	0.40	0.27
6	A	77.1	1.06	0.95	0.76	0.89	0.60	0.36	0.25	0.20
	C		0.28	0.89	0.80	0.63	0.47	0.37	0.23	0.19
	D		0.30	1.10	0.93	0.90	0.74	0.55	0.40	0.31
7	A	91.6	0.56	1.00	0.85	0.79	0.63	0.43	0.33	0.22
	C		0.84	0.83	0.64	0.64	0.46	0.31	0.26	0.19
	D		0.24	0.89	0.82	0.74	0.62	0.45	0.33	0.24
8	A	68.0	1.52	1.17	0.89	0.78	0.69	0.45	0.28	0.13
	C		0.60	0.95	0.73	0.80	0.57	0.36	0.26	0.18
	D		0.88	0.98	0.93	0.87	0.66	0.51	0.33	0.22
9	A	68.0	0.95	1.30	0.93	0.79	0.69	0.55	0.37	0.27
	C		1.15	1.32	1.00	0.91	0.73	0.60	0.41	0.31
	D		1.14	1.14	0.93	0.81	0.59	0.43	0.37	0.23
10	A	74.8	1.16	0.86	0.86	0 77	0.57	0.36	0.30	0.20
	C		0.63	1.13	0.91	0.81	0.68	0.49	0.40	0.29
	D		0.62	0.89	0.87	0.69	0.59	0.43	0.36	0.26
11	A	93.9	0.95	0.87	0.75	0.74	0.56	0.36	0.28	0.18
	C		0.74	0.82	0.65	0.57	0.48	0.32	0.19	0.11
	D		0.41	1.00	0.76	0.74	0.55	0.41	0.30	0.25
12	A	60.3	2.01	1.68	1.58	1.15	0.83	0.67	0.31	0.21
	C		0.56	1.74	1.18	1.27	0.80	0.68	0.58	0.41
	D		1.29	1.45	1.61	1.08	0.97	0.61	0.49	0.35

[a] All zero-hour plasmas were zero. [b,c,d,e] Samples were hemolyzed and could not be assayed. [b] Value estimated from semilogarithmic plot of 48- and 96-hr. plasma concentrations. [c] Value estimated from semilogarithmic plot of 12–72-hr. plasma concentrations. [d] Value estimated from semilogarithmic plot of 24–72-hr. plasma concentrations. [e] Value estimated from semilogarithmic plot of 48–96-hr. plasma concentrations.

RESULTS

Plasma Concentration—The plasma concentrations of warfarin measured in Study No. 1 are shown in Table II, while those measured in Study No. 2 are shown in Table III. With respect to the data shown in Table II, it was shown previously (7) that a plot of the *E versus T* assay values gave a plot with a least-squares line forced through the origin having a slope of 1.00. It was concluded (7) that both methods of assay measured only unchanged warfarin following single doses of drug at the dose levels studied. Hence, in Study No. 2, only the extraction (*E*) assays were performed. The quality control chart for the assays performed for Study No. 2 is shown as Fig. 1. This gives the net absorbance/concentration ratio, observed each day assays were performed, for spiked blood bank plasma. The mean value of 0.315 agrees very closely with the factor 0.320 used in calculating the plasma concentration. The latter value was based on extensive calibration data.

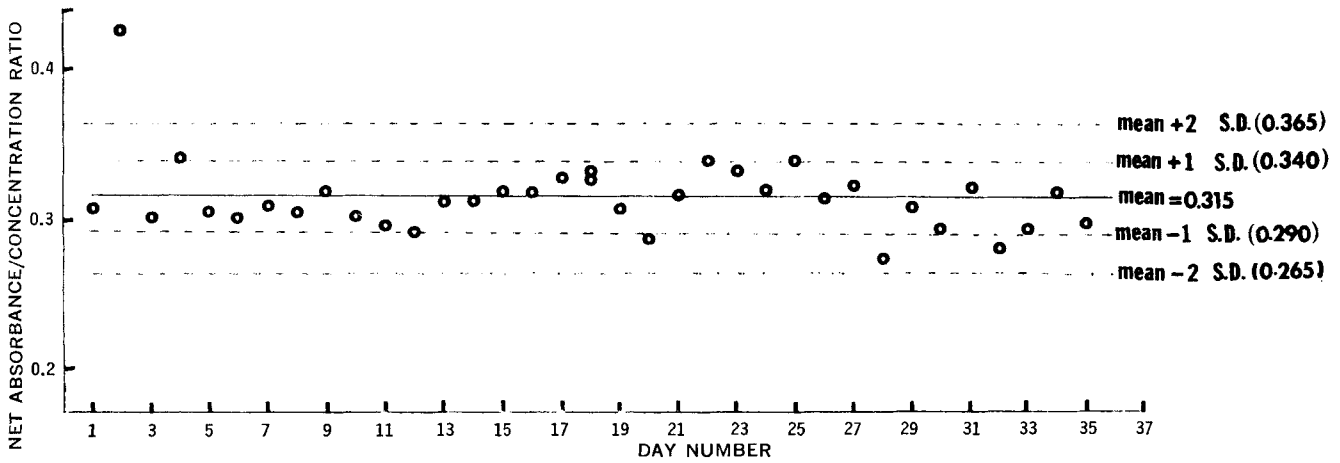


Figure 1—*Quality control chart for warfarin Study No. 2 based on spiked blood bank plasma and blood bank plasma carried through the assay along with subjects' plasmas each day assays were performed.*

Table IV—Summary of Averages for Study No. 1

Plasma Concentration, mcg./ml.	Analytical Method	Treatment: A (five 5-mg.) Average	A (five 5-mg.) CV, %[a]	B (one 25-mg.) Average	B (one 25-mg.) CV, %	ANOVA Results	Average B/ Average A × 100
At 1 hr.	*E*[b]	2.34	22.1	1.34	78.8	Sig. ($0.05 > p > 0.025$)	57
	T[c]	2.48	18.7	1.25	83.9	Sig. ($0.05 > p > 0.025$)	50
At 4 hr.	*E*	3.17	20.9	2.05	50.2	Borderline ($p \simeq 0.05$)	65
	T	3.14	24.0	2.06	54.1	Sig. ($0.025 > p > 0.01$)	66
At 8 hr.	*E*	2.71	18.7	2.21	24.7	Sig. ($0.05 > p > 0.025$)	81
	T	2.57	13.1	2.15	29.1	Borderline ($p = 0.54$)	84
At 12 hr.	*E*	2.53	20.5	2.26	19.8	Sig. ($0.025 > p > 0.01$)	89
	T	2.59	20.1	2.24	28.7	Sig. ($0.01 > p > 0.05$)	86
At 24 hr.	*E*	1.96	22.7	1.72	20.9	N.S. ($0.10 > p > 0.05$)	88
	T	1.94	19.9	1.59	26.4	Sig. ($0.05 > p > 0.025$)	82
At 48 hr.	*E*	1.31	20.0	1.11	24.5	N.S.	85
	T	1.23	19.8	1.12	25.9	N.S.	91
At 72 hr.	*E*	0.78	20.9	0.72	29.1	N.S.	92
	T	0.71	25.3	0.69	24.8	N.S.	97
At 96 hr.	*E*	0.53	37.0	0.44	41.1	N.S.	83
	T	0.53	19.8	0.43	24.9	N.S.	81
Peak plasma concentration, mcg./ml.	*E*	3.19	20.3	2.47	29.0	Sig. ($0.025 > p > 0.01$)	77
	T	3.21	21.9	2.51	31.9	Sig. ($0.005 > p > 0.001$)	78
Time of occurrence of peak plasma concentration, hr.	*E*	6.3	35.7	7.3	53.7	Borderline ($p \simeq 0.07$)	—
	T	3.0	38.4	8.0	54.8	Borderline ($p \simeq 0.07$)	—
Area 0–24 hr. mcg./ml. × hr.	*E*	58.6	17.8	47.1	25.5	Sig. ($0.025 > p > 0.01$)	80
	T	58.7	15.7	45.8	30.7	Sig. ($0.025 > p > 0.01$)	78
Area 0–96 hr. mcg./ml. × hr.	*E*	138.6	15.5	117.0	21.5	N.S.	84
	T	134.9	16.4	113.6	26.1	N.S.	84
A (estimated area 0–∞) mcg./ml. × hr.	*E*	165.4	17.5	144.9	22.3	N.S.	88
	T	157.7	16.9	135.8	24.6	N.S.	86
$T_{1/2}$, hr.	*E*	35.4	23.6	39.4	33.4	N.S.	—
	T	35.0	26.2	35.4	15.9	N.S.	—
$\frac{0.693A}{(T)_{1/2} \times (D/W)}$	*E*	9.90	11.5	7.52	16.6	Sig. ($0.01 > p > 0.001$)[d]	76
	T	9.61	9.62	7.58	18.0	Sig. ($0.025 > p > 0.01$)	79

[a] *CV* (%) = standard deviation/average × 100. [b] *E* = modified O'Reilly extraction method. [c] *T* = TLC method. [d] Result obtained by both paired *t*-test and ordinary *t*-test as well as ANOVA.

Table IV summarizes treatment averages for Study No. 1. Absorption of warfarin was slower and less efficient following Treatment B (one 25-mg.) than following Treatment A (five 5-mg.). This is of particular interest since both tablets were made by the same manufacturer. There were significant differences between treatment averages for plasma concentrations measured at 1, 4, 8, and 12 hr.; peak plasma concentrations; areas 0–24 hr., and normalized areas. For each measurement or parameter, the average for Treatment B (one 25-mg.) expressed as a percentage of the average for Treatment A (five 5-mg.) is shown in the last column of Table IV. The poorer tablet, B, gave higher coefficients of variation, in general, than the better tablet, A. This is particularly noticeable for plasma concentrations measured during the absorption phase and for the normalized areas reflecting relative absorption.

Table V summarizes treatment averages for Study No. 2. There were significant differences among treatment averages for plasma concentrations at 1 hr. and for time of occurrence of peak plasma concentrations. There were no significant differences among treatment averages for plasma concentrations measured at 4, 8, 12, 24, 48, 72, and 96 hr.; area 0–24 hr.; area 0–96 hr., *A*; normalized area; and $T_{1/2}$. The order for average plasma concentrations measured at 1 hr. was Tablet A > Tablet C > Tablet D; the order for time of occurrence of peak plasma concentrations was Tablet A < Tablet C < Tablet D.

Estimates of Bioavailability—Table VI summarizes average values of the normlized areas for all subjects in each study and for the four subjects common to both studies and the results of statistical analyses. The four subjects common to both studies gave average normalized area values very similar to the corresponding averages given by all subjects. There were no significant differences among normalized area treatment averages for all 12 subjects in Study No. 2, indicating equal bioavailability for Tablets A, C, and D following the two-tablet dose of each. By both assay methods, the differences between normalized area treatment averages for Tablets A and B were highly significant at the 25-mg. dose level in Study No. 1. The ratio of the average normalized area for Treatment B (one 25-mg.) to the average normalized area for Treatment A (five 5-mg.) was 0.84 for the *E* assays and 0.79 for the *T* assays. These values are theoretically equivalent to F_B/F_A, where the subscripts refer to the tablets. The same ratio obtained by two-compartment analysis of average plasma concentration data was 0.806 (see *Footnote 5*). Hence, absorption of warfarin following Tablet B was about 80% of absorption of warfarin following Tablet A at the 25-mg. dose level.

The results of the nonlinear least-squares fitting of average plasma concentrations following each of the five treatments is shown in Table VII (one-compartment analysis) and Table VIII (two-compartment analysis). The measures of fit, r^2 and Corr., indicate better fits to the two-compartment model than to the one-compartment model. Also, there were distinct trends in areas of poor fit in the case of the one-compartment analysis. The 95% confidence intervals of V_1/F were 6.64–7.32, 5.53–7.45, and 5.12–7.57 l. for Treatments A (two 5-mg.), C (two 5-mg.), and D (two 5-mg.), respectively. These confidence intervals overlap. This result indicates equal bioavailability of warfarin from Tablets A, C, and D.

Estimates of Absorption Rate—The k_{11} values in Table VIII are the estimated first-order rate constants for absorption of warfarin following the various treatments. The larger this number, the more rapid was the absorption. The order of absorption rates were: Tablet A > Tablet B in Study No. 1, and Tablet A > Tablet C > Tablet D in Study No. 2. The half-absorption times are summarized in Table XIV.

Variability of $T_{1/2}$—There were marked intersubject and intrasubject variations in $T_{1/2}$ of warfarin. The *average* $T_{1/2}$ of 35.4 and 39.4 hr. following Treatments A (five 5-mg.) and B (one 25-mg.), respectively, in Study No. 1 were not significantly different. Similarly, the *average* $T_{1/2}$ of 54.7, 53.1, and 53.8 hr. following Treatments A (two 5-mg.), C (two 5-mg.), and D (two 5-mg.), respectively, in Study No. 2 were not significantly different. However, the same subject administered warfarin at different times exhibited different $T_{1/2}$ values. The standard deviations caleulated from the mean squares for among subjects, among time periods, and among

Table V—Summary of Averages for Study No. 2

Plasma Concentration, mcg./ml.	Treatment: A (two 5-mg.) Average	A (two 5-mg.) *CV*, %[a]	C (two 5-mg.) Average	C (two 5-mg.) *CV*, %	D (two 5-mg.) Average	D (two 5-mg.) *CV*, %	ANOVA Results
At 1 hr.	1.11	34.1	0.86	49.1	0.58	63.7	Sig. ($0.01 > p > 0.005$)[b]
At 4 hr.	1.08	23.0	1.13	27.1	1.10	21.9	N.S. ($p > 0.25$)
At 8 hr.	0.90	25.9	0.92	22.3	0.95	24.7	N.S. ($p > 0.25$)
At 12 hr.	0.82	15.4	0.88	27.1	0.85	13.9	N.S. ($p > 0.25$)
At 24 hr.	0.65	12.6	0.66	24.1	0.68	18.2	N.S. ($p > 0.25$)
At 48 hr.	0.45	20.8	0.48	33.1	0.49	12.9	N.S. ($p > 0.25$)
At 72 hr.	0.31	11.6	0.36	38.0	0.37	13.3	N.S. ($0.25 > p > 0.10$)
At 96 hr.	0.22	19.3	0.25	47.3	0.26	15.1	N.S. ($p > 0.25$)
Peak plasma concentration, mcg./ml.	1.20	25.6	1.21	28.5	1.11	23.8	N.S. ($p > 0.25$)
Time of occurrence of peak plasma concentration, hr.	2.3	64.4	3.6	53.6	4.1	37.0	Sig. ($0.05 > p > 0.01$)
Area 0–24 hr. mcg./ml. × hr.	20.0	18.6	20.4	23.6	19.7	18.4	N.S. ($p > 0.25$)
Area 0–96 hr. mcg./ml. × hr.	48.9	14.3	51.4	27.5	51.9	15.2	N.S. ($p > 0.25$)
A (estimated area 0–∞) mcg./ml. × hr.	67.1	12.0	72.7	36.6	72.7	15.4	N.S. ($p > 0.25$)
$T_{1/2}$, hr.	54.7	24.3	53.1	26.4	53.8	17.9	N.S. ($p > 0.25$)
$\frac{0.693A}{(T_{1/2}) \times (D/W)}$	7.15	18.5	7.82	23.8	7.46	17.4	N.S. ($p > 0.25$)

[a] CV (%) = standard deviation/average × 100. [b] Multiple-range tests indicated that the average for Treatment D was significantly different from the averages for Treatments A and C but that the averages for Treatments C and D were not significantly different.

treatments were 14.1, 14.9, and 2.7 hr., respectively, using mean squares from the analysis of variance of $T_{1/2}$ in Study No. 2. This indicates that intrasubject variation of $T_{1/2}$ was of the same order of magnitude as intersubject variation of $T_{1/2}$.

This variation in $T_{1/2}$ *cannot* be explained by continued absorption of warfarin when the $T_{1/2}$ was estimated. The average $T_{1/2}$'s for treatments *within each study* are essentially equal, yet the absorption rates vary widely from treatment to treatment within each study. The average $T_{1/2}$'s after the 25-mg. label doses are *lower* than the average $T_{1/2}$'s after the 10-mg. doses. The 25-mg. doses were absorbed over longer time periods, in general, than the 10-mg. doses. If absorption was continuing when $T_{1/2}$ was estimated, one would expect the average $T_{1/2}$ to be *higher*, not lower, after 25-mg. doses than after 10-mg. doses. The $T_{1/2}$ was usually estimated from plasma concentrations observed in either the 48–96- or 24–96-hr. range; due to the rapid absorption of warfarin, absorption is complete long before 24–48 hr.

Table IX lists the $T_{1/2}$ values estimated for the four subjects common to both studies. In general, these data suggest random variation of $T_{1/2}$ for each subject from one time of administration to the next. Such variation is probably caused by changing activity of microsomal metabolizing enzymes (which change K_2) and changes in the distribution-rate constants (K_{-1} and K_1) from one administration to the next. This follows since the $T_{1/2}$'s estimated are essentially identical to $0.693/\beta$ and β is a function of all three of these rate constants, as indicated by Eq. 3.

Correlation coefficients were calculated from the data for each subject in Table IX as follows: the correlation of $T_{1/2}$ with dose number, the correlation of $T_{1/2}$ with dosing day number, and the correlation of $T_{1/2}$ with dose. All but one of the correlation coefficients were not significant ($p > 0.05$); most of the r values were not as large as the critical r for the $p = 0.10$ level. Subject B gave a significant correlation for $T_{1/2}$ with dose ($r = 0.926$; $0.05 > p > 0.02$). Overall, however, there is little statistical evidence of a significant trend linking change in $T_{1/2}$ of warfarin with either time or dose in the range studied.

Correlations of Parameters—Table X summarizes the correlation coefficients and significance levels for all possible correlations of peak, A, and $(0.693A)/T_{1/2}$ with D/W, D, $1/W$, and peak. The correlations of $(0.693A)/T_{1/2}$ with $1/W$ support a correction for body weight such as is made in Table VI. It may also be seen that $(0.693A)/T_{1/2}$ correlates better with D/W (*i.e.*, milligrams/kilogram

Table VI—Comparison of Average Normalized Areas

Study	Treatment	Number of Subjects	Average Dose of Warfarin, mg.	Assay	$0.693A/(T_{1/2})(D/W)$: All Subjects Average	All Subjects *SD*	Four Subjects Common to Studies No. 1 and 2 Average	Four Subjects Common to Studies No. 1 and 2 *SD*
No. 2, this report	A (two 5-mg.)	12	9.07	*E*[a]	7.15	1.32	6.93	1.56
	C (two 5-mg.)	12	8.93	*E*	7.82	1.86	8.29	2.64
	D (two 5-mg.)	12	9.40	*E*	7.46	1.30	8.61	1.55
					Av. 7.48		Av. 7.94	
No. 1, this report	A (five 5-mg.)	6	22.7	*E*	9.90	1.14	9.92	1.20
				T[b]	9.61	0.93	9.77	0.89
	B (one 25-mg.)	6	23.9	*E*	7.52	1.25	7.88	1.40
				T	7.58	1.36	7.46	1.49
O'Reilly *et al.*	1.5 mg. sodium warfarin/kg.	14	93.3[c]	*E*	8.71	1.70		
Weighted average	*E* assays only, without B (one 25-mg.)				8.04			

[a] E = simple extraction assay. [b] T = TLC assay. [c] The 1.5 mg. sodium warfarin/kg. body weight (average dose of 100 mg. sodium warfarin) is equivalent to 1.4 mg. warfarin acid/kg. body weight (average dose of 93.3 mg. warfarin acid). [d] No significant difference among averages ($p > 0.25$). [e] Significant difference among averages ($p < 0.001$). [f] Significant difference between averages ($0.01 > p > 0.001$). [g] Significant difference between averages ($0.025 > p > 0.01$). [h] Significant difference between averages ($0.02 > p > 0.01$). [i] Significant difference between averages ($0.05 > p > 0.02$).

Table VII—Parameters Estimated by Nonlinear Least-Squares Fitting of Average Plasma Concentrations (Extraction Assay), Their Standard Deviations, and Two Measures of Closeness of Fit. Fitting Was to the One-Compartment Open Model with First-Order Absorption

Estimated Parameter	Treatment				
	Study No. 1		Study No. 2		
	A (five 5-mg.)	B (one 25-mg.)	A (two 5-mg.)	C (two 5-mg.)	D (two 5-mg.)
k_1, hr.$^{-1}$	1.34 (0.124)	0.695 (0.0994)	2.55 (0.494)	1.45 (0.193)	0.680 (0.135)
K, hr.$^{-1}$	0.0198 (0.00108)	0.0174 (0.00161)	0.0168 (0.00257)	0.0168 (0.00168)	0.0163 (0.00233)
C^0, mcg./ml.[a]	3.23[b] (0.0696)	2.51[b] (0.0941)	1.07 (0.06161)	1.09 (0.0448)	1.10 (0.0644)
Measures of fit					
r^2	0.999	0.996	0.994	0.996	0.994
Corr.	0.997	0.993	0.981	0.984	0.976

[a] $C^0 = F \cdot D/V_d$. [b] $F_B = D_A C^0_B / D_B C_A = (22.7)(2.51)/(23.9)(3.23) = 0.74$, where the subscripts refer to Tablets A and B. Calculation assumes Vd constant for both treatments and $F_A = 1$.

dose) than does A (area $0 \rightarrow \infty$). This supports the calculation of normalized areas to estimate bioavailability.

Regressions (not reported) also indicate excellent agreement between data of this study and that of O'Reilly *et al.* (11). For example, using the regression equations for peak on D/W and $(0.693A)/T_{1/2}$ on D/W, derived from the 18 points resulting from Treatments A (five 5-mg.) and A (two 5-mg.), one can estimate expected values of peak and $(0.693A)/T_{1/2}$ after a 1.5-mg./kg. dose of sodium warfarin by extrapolation; these estimated values agreed very closely with the average peak and $(0.693A)/T_{1/2}$ values calculated from the data of O'Reilly *et al.* (11). This also suggests that warfarin kinetics are linear and *not* dose dependent in man in the range of 10–100-mg. doses of sodium warfarin. A future paper (12) also supports this and the applicability of the two-compartment open model to warfarin administered intravenously.

Tablet Potencies—Tablet potencies obtained by official assay of tablets are given in Table XI. These drugs are labeled in terms of their sodium or potassium salt contents. All tablets were within acceptable limits. Since the plasma concentrations were expressed in terms of warfarin (acid), the dose values used in the various calculations were based on 4.54, 23.9, 4.47, and 4.70 mg. of warfarin (acid) per tablet for Tablets A, B, C, and D, respectively. Use of such values for individual subjects would be valid if there were not large tablet-to-tablet variations. Here, five tablets of each lot were individually assayed. The results, also shown in Table XI, indicate that tablet-to-tablet variability in warfarin content was quite small in the samples studied. Hence, use of the average assay value to estimate dosage for the individual subject appears to be justified in this case.

Solubility of Warfarin in 0.1 *N* Hydrochloric Acid—At room temperature, the solubility measured was 4.35 (*SD* 0.25) mcg. warfarin (acid)/ml. At 37°, the solubility measured was 7.36 (*SD* 0.49) mcg. warfarin (acid)/ml. Hence, during exposure to the 900 ml. of 0.1 *N* hydrochloric acid in the dissolution-rate studies, a maximum of 6.62 mg. (*SD* 0.41) of warfarin (acid) can be in solution. Since one 5-mg. tablet of sodium warfarin contains 4.665 mg. of warfarin (acid), the solution would be 70% saturated with warfarin if the tablet completely dissolved during the 30-min. exposure time

Table VIII—Parameters Estimated by Nonlinear Least-Squares Fitting of Average Plasma Concentrations (Extraction Assay), Their Standard Deviations, and Two Measures of Closeness of Fit and Several Other Parameters Calculated from Them. Fitting Was to Two-Compartment Open Model with First-Order Absorption

Estimated Parameter	Treatment				
	Study No. 1		Study No. 2		
	A (five 5-mg.)	B (one 25-mg.)[a]	A (two 5-mg.)	C (two 5-mg.)	D (two 5-mg.)
k_{11}, hr.$^{-1}$	0.836 (0.0741)[b]	0.450	2.19 (0.129)	1.06 (0.102)	0.531 (0.0545)
K_1, hr.$^{-1}$	0.132 (0.0201)	0.132	0.0412 (0.0028)	0.0554 (0.0093)	0.0673 (0.0115)
K_{-1}, hr.$^{-1}$	0.315 (0.0206)	0.315	0.116 (0.0030)	0.140 (0.0102)	0.116 (0.0075)
K_2, hr.$^{-1}$	0.0270 (0.0016)	0.0270	0.0206 (0.0004)	0.0195 (0.0010)	0.0201 (0.0012)
V_1/F, liters	5.065 (0.300)	6.283	6.98 (0.108)	6.49 (0.301)	6.35 (0.386)
Measures of fit					
r^2[c]	1.000	0.992	1.000	1.000	1.000
Corr.[d]	1.000	0.983	1.000	0.998	0.998
Other parameters					
α, hr.$^{-1}$	0.455	0.455	0.164	0.201	0.191
β, hr.$^{-1}$	0.0187	0.0187	0.0144	0.0136	0.0122
$T_{1/2} = \frac{0.693}{\beta}$, hr.	37.1 [38.0][e]	37.1 [36.8]	48.1 [45.9]	50.9 [52.0]	56.8 [52.5]
$\frac{V_d}{F} = \frac{V_1}{F}\frac{\alpha}{K_{-1}}$, liters	7.29	9.08	9.84	9.35	10.48
$\frac{F}{V_d/W}$	9.52 [9.90][f]	7.61 [7.52]	7.57 [7.15]	7.97 [7.82]	7.11 [7.46]

[a] Plasma concentrations fitted holding K_1, K_{-1}, and K_2 constant and same values as Treatment A (five 5-mg.). [b] Numbers in parentheses are standard deviations of estimated parameters. [c] $r^2 = (\Sigma_{obs.}{}^2 - \Sigma_{dev.}{}^2)/\Sigma_{obs.}{}^2$. [d] Corr. is correlation coefficient for regression of predicted and observed plasma concentrations. [e] Numbers in square brackets are half-lives calculated directly from *average* terminal plasma concentrations and are included for comparison purposes. These numbers are not the same as those in Tables IV and V, which are average half-lives of individual subjects. [f] Numbers in square brackets are treatment averages calculated from individual subject's data directly, using the formula $0.693A/(T_{1/2})(D/W)$.

Table IX—Warfarin Half-Lives Estimated from Terminal Plasma Concentrations for Four Subjects Common to Both Clinical Studies

Subject	Subject Number in Study No. 1	Subject Number in Study No. 2	Half-Life, hr. Study No. 1 Phase I	Study No. 1 Phase II	Study No. 2 Phase I	Study No. 2 Phase II	Study No. 2 Phase III	Average
B	4	2	26.2[b]	33.7[a]	51.3[c]	49.9[d]	42.0[e]	40.0
Cr	3	3	47.2[a]	55.0[b]	59.9[c]	34.1[d]	34.5	46.1
Ch	5	6	56.9[b]	31.1[a]	52.1[d]	56.8[e]	74.5[c]	54.3
R	1	12	28.8[a]	34.3[b]	59.9[e]	42.7[c]	76.8[d]	48.5
Dose number			1	2	3	4	5	
Dosing day number			1	22	64	85	106	

[a] Treatment A (five 5-mg.). [b] Treatment B (one 25-mg.). [c] Treatment A (two 5-mg.). [d] Treatment C (two 5-mg.). [e] Treatment D (two 5-mg.).

Table X—Correlation Coefficients (r) and Significance Levels (p) for Pairs of Parameters

		N	Ordinate: Peak r	Peak p	A r	A p	$0.693A/T_{1/2}$ r	$0.693A/T_{1/2}$ p
Abscissa	D/W	56[a]	0.971	<0.001	0.932	<0.001	0.972	<0.001
		18[b]	0.970	<0.001	0.891	<0.001	0.975	<0.001
	D	56	0.969	<0.001	0.959	<0.001	0.924	<0.001
		18	0.913	<0.001	0.942	<0.001	0.940	<0.001
	$1/W$	56	0.251	$0.10 > p > 0.05$	0.227	$0.10 > p > 0.05$	0.338	$0.02 > p > 0.01$
		18	0.526	$0.02 > p > 0.01$	0.277	>0.10	0.451	$0.05 > p > 0.02$
	Peak	56	—	—	0.945	<0.001	0.959	<0.001
		18	—	—	0.910	<0.001	0.981	<0.001

[a] Includes the six subjects in this study given A (five 5-mg.); the 12 subjects in this study each given A (two 5-mg.), C (two 5-mg.), and D (two 5-mg.); plus 14 subjects given 1.5 mg./kg. (average dose 100 mg.) of sodium warfarin equivalent to 1.4 mg./kg. (average dose 93.3 mg.) of warfarin acid reported by O'Reilly *et al.* (11). [b] Includes the six subjects in this study given A (five 5-mg.) and the 12 subjects given A (two 5-mg.).

in the acid solution.

***In Vitro* Rate of Dissolution Studies and Disintegration Times**—Figure 2 is a plot of the dissolution data for Tablets A and B used in Study No. 1. The points correspond to the average percent dissolved of five tablets of each lot. The vertical bars mark off one standard deviation on either side of the average. Almost negligible amounts of warfarin dissolved from these tablets during the 30-min. exposure to 0.1 N hydrochloric acid. The average times required to dissolve 25, 50, and 75% of the warfarin from these two tablets are summarized in Table XII. The differences between the averages were highly significant in each case. Tablet A dissolved more rapidly than Tablet B by all criteria. Since readings were made at very frequent time intervals, one may also estimate the rate of dissolution from these data. A plot (not shown) of rate of dissolution of Tablet A *versus* time has a sharp peak just after 30 min. and then falls off rapidly. However, a similar plot (not shown) for Tablet B is low and flat over most of the observation period.

Figure 3 is a plot of the dissolution data for Tablets A, C, and D used in Study No. 2. The points to the far left of both Figs. 2 and 3 are the same since Tablet A was used in both studies. Again, almost negligible amounts of warfarin dissolved from all three tablets during the 30-min. exposure to 0.1 N hydrochloric acid. The average times required to dissolve 25, 50, and 75% of the warfarin from these three tablets are summarized in Table XII. The differences among the tablet averages are highly significant ($p < 0.001$) in each case as determined by analysis of variance. There were no significant differences among the trial averages in each case ($p > 0.10$). The order of rates of dissolution was Tablet A > Tablet C > Tablet D.

Table XI—Tablet Potencies as Measured by Official Assay Procedure

Aliquot of Powder from 20 Tablets	Potency (mg./Tablet)[a] Tablet A Salt[c]	Tablet A Acid[d]	Tablet B Salt[c]	Tablet B Acid[d]	Tablet C Salt[e]	Tablet C Acid[d]	Tablet D Salt[c]	Tablet D Acid[d]
1	4.84	4.51	25.9	24.2	5.02	4.47	4.94	4.61
2	4.93	4.60	25.4	23.7	4.99	4.44	5.10	4.76
3	4.82	4.50	25.5	23.8	5.05	4.50	5.08	4.74
Average	4.86	4.54[b]	25.6	23.9[b]	5.02	4.47[b]	5.04	4.70[b]
SD	0.059	0.055	0.265	0.265	0.030	0.030	0.087	0.081
Percent of label	97.2	—	102.4	—	100.4	—	100.8	—
Tablet No.								
1	4.75	4.43	24.3	22.7	4.91	4.37	4.79	4.47
2	4.81	4.49	24.4	22.8	5.00	4.45	4.91	4.58
3	4.74	4.42	24.5	22.9	4.93	4.39	4.89	4.56
4	4.89	4.56	24.7	23.0	4.97	4.42	5.07	4.73
5	4.83	4.51	24.7	23.0	4.92	4.38	4.83	4.51
Average	4.80	4.48	24.5	22.9	4.95	4.40	4.90	4.57
SD	0.061	0.058	0.179	0.130	0.038	0.033	0.107	0.099
Percent of label	96.0	—	98.0	—	99.0	—	98.0	—

[a] Ratios of molecular weights are: warfarin acid/sodium warfarin = 0.933 and warfarin acid/potassium warfarin = 0.890. [b] These averages were used to estimate milligrams/kilogram dosage, D/W, following oral administration of the various tablets. [c] As sodium warfarin. [d] As warfarin (acid). [e] As potassium warfarin.

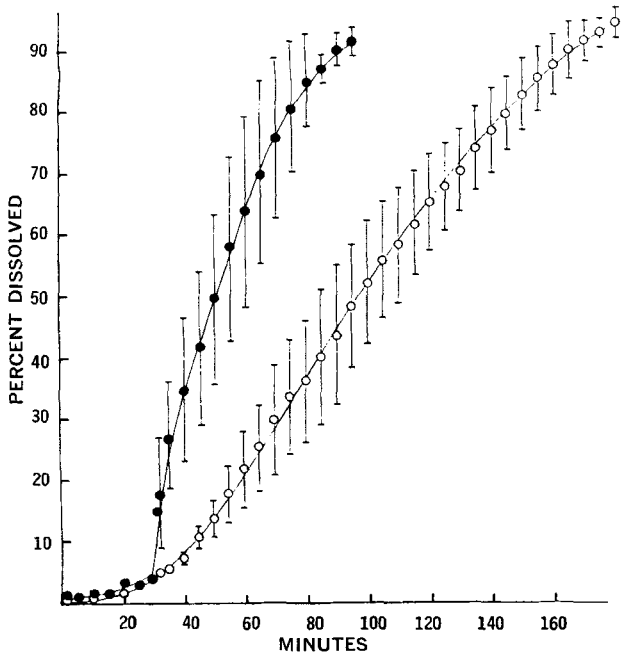


Figure 2—*Dissolution results obtained with Tablets A and B used in Study No. 1. Key: ●, Tablet A; and ○, Tablet B. Points are averages for five tablets. Bars mark off 1* SD *on either side of the average.*

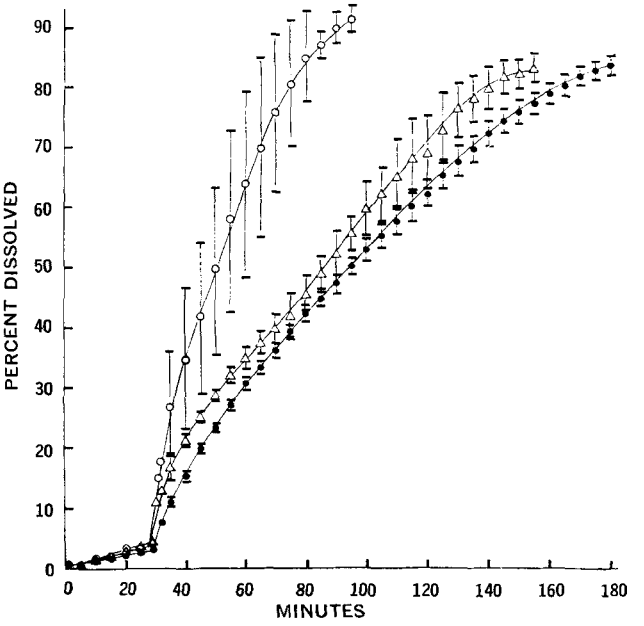


Figure 3—*Dissolution results obtained with Tablets A, C, and D used in Study No. 2. Key: ○, Tablet A; △, Tablet C; and ●, Tablet D. Points are averages for five tablets. Bars mark off 1* SD *on either side of the average.*

The disintegration times determined in water in the USP apparatus without plastic disks and the times to dissolve 50% of the warfarin ($t_{50\%}$) in the dissolution test are compared in Table XIII for the four tablets studied. Although the disintegration test distinguished between Tablets A and B, it failed to distinguish between Tablets A, C, and D. This is more evidence of the advisability of dissolution-rate criteria rather than disintegration time for quality control purposes.

The authors prepared 5-mg. sodium warfarin tablets containing 20% Veegum-F, spray-dried dextrose, and calcium stearate by direct compaction on a Stokes single-punch tablet press. These tablets released warfarin 16 times more rapidly *in vitro* than the best commercial 5-mg. tablets on the basis of $t_{50\%}$ values. These tablets also disintegrated in 0.1 *N* hydrochloric acid under static conditions in about 1 min., whereas all three commercial 5-mg. tablets remained intact in such an acid environment under similar conditions for at least 30 min. However, a 25-mg. tablet of similar composition released its warfarin only 1.3 times more rapidly than Tablet B. Further investigations are being made on an improved 25-mg. tablet; results will be published separately.

Correlation of *In Vivo* with *In Vitro* Results—The results obtained in man may be correlated in many ways with the results obtained *in vitro*. One may use the observed data directly. For example, Fig. 4 is a plot of the average warfarin plasma concentration at 1 hr. postadministration against the average amount of warfarin (acid) dissolved *in vitro* in 1 hr. To obtain the abscissa values for this plot, the average amount of warfarin (acid) dissolved per tablet *in vitro* in 1 hr. was multiplied by the number of tablets administered in the clinical study (*i.e.*, either 1, 2, or 5).

Pharmacokinetically, it is more desirable to correlate indirectly the *in vivo* with *in vitro* results. Three approaches were taken:

1. Table XIV lists the half-absorption times in man (calculated from the k_{II} values in Table VIII) and the times required to dissolve 50% of the warfarin in the dissolution test. There appears to be a dose dependency in the results achieved by this method. At the 25-mg. dose level (Study No. 1), the *in vitro* $t_{50\%}$ values almost exactly predict the half-absorption times in man. At the 10-mg. dose level (Study No. 2), there is a perfect rank order correlation but, in all cases, the half-absorption times are less than the *in vitro* $t_{50\%}$ values.

2. Figure 5 is a plot of the milligrams of warfarin (acid) absorbed in 1 hr. divided by V_d/W against the average amount of warfarin (acid) dissolved *in vitro* in 1 hr. The ordinate values for this plot were calculated using Eq. 7, the $F(V_d/W)$ values and k_{II} values listed in Table VIII, and the doses listed in Table VI. The abscissa values were obtained as already indicated. This plot makes no assumptions about efficiency of absorption (*i.e.*, the absolute amount of warfarin absorbed) from any tablet.

3. Figure 6 is a plot of estimated milligrams of warfarin (acid) absorbed in man in 1 hr. against the average amount of warfarin (acid) dissolved *in vitro* in 1 hr. The ordinate values for this plot were calculated using Eq. 8, the k_{II} values listed in Table VIII, the doses listed in Table VI, and F values of unity for Tablets A, C, and D and 0.806 for Tablet B (see *Footnote 5*). The first four points appear to be randomly distributed about a straight line; the least-squares line free to pass through any intercept has the equation: *in vivo* = 0.527 + 1.18 (*in vitro*), (r = 0.953; p = 0.05). The line forced through the ori-

Table XII—Summary of Average Times Required to Dissolve 25, 50, and 75% of the Warfarin in Tablets A, B, C, and D

Percent Dissolved	Tablet				Results of Statistical Analyses	
	A	B	C	D	A *versus* B	Among A, C, and D
25[a]	35.9	65.8	45.1	51.8	$p < 0.001$	$p < 0.001$
50[b]	51.3	96.6	86.4	94.6	$p > 0.001$	$p < 0.001$
75[c]	68.1	135.8	125.9	147.4	$p > 0.001$	$p < 0.001$

[a] Trial averages for Tablets A, C, and D were 45.1, 43.1, 45.0, 45.9, and 42.3. [b] Trial averages for Tablets A, C, and D were 81.0, 75.6, 80.2, and 70.8. [c] Trial averages for Tablets A, C, and D were 121.8, 107.5, 115.0, 114.9, and 109.8. There were no significant differences among any of the trial averages above in each set ($p > 0.10$).

Table XIII—Comparison of Disintegration Times with Time for 50% of Warfarin to Dissolve *In Vitro*

Tablet	Disintegration Time[a], min.		$t_{50\%}$[b], min.	
	Average	*SD*	Average	*SD*
A	11.0	1.18	51.3	9.39
B	20.5	4.05	96.6	13.0
C	10.7	1.15	86.8	5.66
D	11.2	0.51	94.6	2.64

[a] Determined in water in the USP apparatus without plastic disks at 37°. [b] Determined by method described in *Experimental* section of this report.

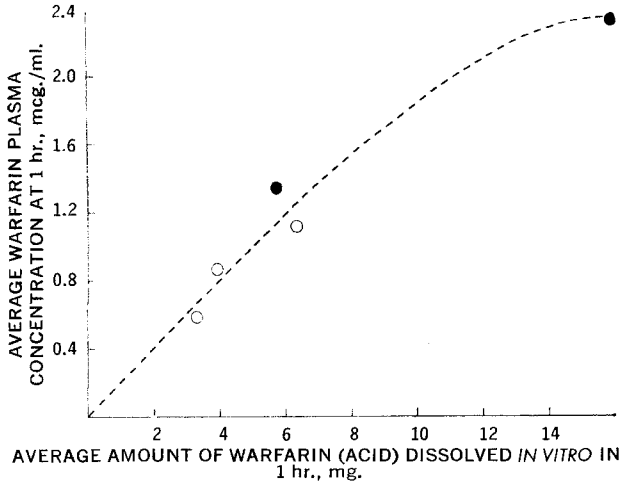


Figure 4—*Correlation of* in vivo *with* in vitro *results. Plot of average plasma concentration of warfarin at 1 hr. against average amount of warfarin dissolved* in vitro *in 1 hr. Key:* ●, *25-mg. dose;* ○, *10-mg. dose; points from left to right refer to Treatments D (two 5-mg.), C (two 5-mg.), B (one 25-mg.), A (two 5-mg.), and A (five 5-mg.).*

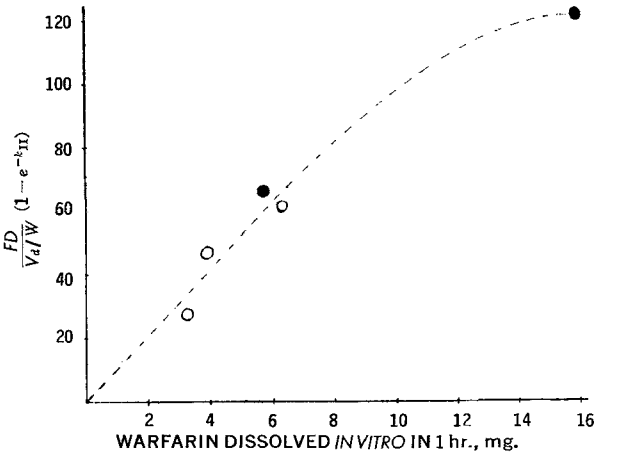


Figure 5—*Correlation of* in vivo *with* in vitro *results. Plot of* FD/(V_d/W)($1 - e^{-k_{II}}$) *against milligrams of warfarin (acid) dissolved* in vitro *in 1 hr. The ordinate is equivalent to the estimated milligrams of warfarin (acid) absorbed in 1 hr. divided by* V_d *expressed as a fraction of body weight. Key: points from left to right refer to Treatments D (two 5-mg.), C (two 5-mg.), B (one 25-mg.), A (two 5-mg.), and A (five 5-mg.).*

gin has the equation: *in vivo* = 1.27 (*in vitro*), and this is the line drawn through the points on the plot.

DISCUSSION

Normalized areas, calculated in the manner indicated in the *Experimental* section, were used for the following reasons: (*a*) In fitting individual subject sets of plasma concentrations to Eqs. 2, 3, and 4, convergence was not always obtained. Hence, the parameters could not be estimated; this also occurred with average plasma concentrations for Treatment B (one 25-mg.), which explains *Footnote* [a] of Table VIII. (*b*) Values of A estimated by the usual method (9) agreed very closely with values obtained by integration of the triexponential equation obtained by two-compartment fitting. (*c*) Values of $T_{1/2}$ estimated directly from terminal plasma concentrations agreed very closely with corresponding values of $0.693/\beta$, where β was obtained by exponential fitting to Eqs. 2, 3, and 4. Fitting of individual subject plasma concentrations, where feasible, gave similar parameters for a given treatment to those estimated from the average plasma concentrations. Parameters listed in Tables VII and VIII were obtained by assigning equal weights to the plasma concentrations in a given set. Reciprocal weighting gave similar results in these cases. Since it was shown formerly (7) that the error involved in a measured warfarin plasma concentration is independent of the magnitude of the plasma concentration in the range of concentrations reported here, it appeared to be more valid *in these cases* to use equal weights.

Some readers may question the emphasis on average peak plasma concentrations in Tables IV, V, and X. The senior author has noticed high correlations of peak with D/W and high correlations of peak with $0.693A/T_{1/2}$ in similar studies with other drugs. High correlations between these pairs of parameters were also observed in these studies (Table X) and appeared worthy of reporting. Pharmacokinetically, the peak plasma concentration is a complicated function. However, these high correlations suggest that the widespread use of peak plasma concentration by the medical profession may, in many cases, be supported by sound statistical evidence. Moreover, in Tables IV and V, the average peak plasma concentration of individual subjects is often considerably higher than the peak of the average plasma concentration curve; too frequently the latter is used rather than the former and leads to a distortion not warranted by the data.

The results indicate that absorption of warfarin in man is dissolution-rate controlled, as suggested from previous investigations (2, 3), and that such control extends down into the therapeutic dose range. Some of the commercial tablets studied have dissolution rates *in vitro*, and presumably *in vivo*, that are just in the critical range. The warfarin in Tablet B was not completely available for absorption in man, while another tablet, A, made by the same manufacturer, allowed complete bioavailability of its contained warfarin.

Tablets A, C, and D provided equal bioavailability of the warfarin in Study No. 2, but the rate constants for absorption of warfarin from these tablets at the 10-mg. dose level varied over a fourfold range. The data suggest that the *in vivo* dissolution rate of warfarin from Tablet D is just inside the critical range; if the rate was much slower, the warfarin contained in such a tablet may not be completely available to man.

It may be theorized that one would expect absorption from five 5-mg. tablets to be faster than from one 25-mg. tablet on the basis of difference in surface area of the intact tablets. This concept is most probably erroneous. First, this difference in surface area of intact tablets is almost negligible when related to the available surface when the tablets disintegrate. When a tablet disintegrates, the increase in surface area is of the order of 1000–10,000 times the surface area of the intact tablet. Second, the opposite results with capsules have already been reported in the literature. Riegelman (13) showed that average plasma levels of griseofulvin in 16 subjects were *higher* after two 250-mg. capsules than after four 125-mg. capsules. It is realized that tablets are different from capsules, but these results are worthy of repetition. Third, there is no *a priori* reason to expect that the rate of dissolution of drug from a higher potency tablet will always be less than that from a lower potency tablet. This can readily be perceived if one considers that the higher dose could be punched in a tablet with lactose and a good disintegrant while the lower dose of drug could be directly compressed without adjuvants or, even worse, encased in cement. The way the tablet is made is the important thing, and both good high and low potency tablets of almost any drug may be prepared.

The results reported here do not imply that other commercial warfarin tablets, made by the same or different manufacturers, will provide satisfactory results in man. For example, a 10-mg. tablet, made by the same manufacturer that made Tablet C, was found to have an average *in vitro* $t_{50\%}$ of 144 min.; this tablet was the slowest dissolving of six different commercial tablets tested *in vitro*. If the *in vivo–in vitro* correlations established in these studies are valid when extrapolated to lower *in vitro* rates of dissolution, one would expect absorption in man following oral administration of this 10-mg. tablet to be quite slow relative to that for two A tablets and perhaps to show a lower bioavailability than even Tablet B.

Plasma concentrations of individual subjects following intravenous administration of sodium warfarin have also been shown to be fit by the two-compartment open model (12), thus supporting the preference for this model for plasma concentrations observed after oral administration. The analysis of the intravenous data gave an average value of 0.114 for V_d/W, which has a reciprocal, $1/(V_d/W)$, of 8.77. The average value of $V_{d\ \text{extrap.}}/W$, reported by O'Reilly *et al.* (3), was 0.125, which has a reciprocal of 8.00. It is shown in the *Appendix* that, for warfarin, $V_{d\ \text{area}}$ and $V_{d\ \text{extrap.}}$ are essentially identical. When these values are substituted into Eq. 6, using either the average normalized areas of Table VI or β and A values of Table VIII, F values for Treatment A (five 5-mg.) range from 1.13–1.27. The fact that F values greater than unity are obtained by this method is incongruous

Table XIV—Half-Absorption Times of Warfarin Estimated from Average Plasma Concentrations Compared with Times Required to Dissolve 50% of Warfarin *In Vitro*

Study	Treatment	Time Required to Dissolve 50% of Warfarin *In Vitro*, min.	Half-Absorption Time in Man, min.
No. 1	A (five 5-mg.)	51.3	49.4
	B (one 25-mg.)	96.6	92.4
No. 2	A (two 5-mg.)	51.3	19.0
	C (two 5-mg.)	86.8	39.2
	D (two 5-mg.)	94.6	78.3

but does suggest strongly that the absorption of warfarin following Tablet A was essentially complete (*i.e.*, 100% absorption). Since it is unlikely that efficiency of absorption of warfarin after two A tablets in Study No. 2 would be less than after five A tablets in Study No. 1, absorption of warfarin following Tablet A in Study 2 was probably essentially complete. Since the average normalized area ratios for Treatments A (two 5-mg.), C (two 5-mg.), and D (two 5-mg.) were not significantly different in Study No. 2, absorption of warfarin following Tablets C and D was probably essentially complete in Study No. 2. However, the discrepancy between the average normalized areas for Tablet A in Studies No. 1 and 2 (Table VI) is difficult to explain. For each of the four subjects common to both studies given Treatments A (five 5-mg.) and A (two 5-mg.), the ratios $A_2/A_1 \cdot D_1/D_2$ and $(T_{1/2})_2/(T_{1/2})_1$, where the subscripts refer to the study number, were calculated. The average dose corrected area ratio was 1.10 (range 0.942–1.32), and the average half-life ratio was 1.77 (range 1.27–1.91). These results in relation to Eq. 6 indicate that for these subjects either F was lower in Study No. 2 than in Study No. 1, or that V_d/W was higher in Study No. 2 than in Study No. 1, or that both factors were operative. Since the data for these four subjects gave similar average normalized areas to the average normalized areas obtained for the entire panel, this indicates a similar hypothesis is valid for the entire panels also.

APPENDIX: VOLUMES OF DISTRIBUTION

In terms of the parameters of the two-compartment open model, various volumes of distribution were described by Riegelman *et al.* (10). From the equations given by these authors, the following equations were derived:

$$V_{d\ \text{area}} = V_1\left(\frac{\alpha}{K_{-1}}\right) \qquad \text{(Eq. 1A)}$$

$$V_{d\ \text{extrap.}} = V_1\left(\frac{\alpha - \beta}{K_{-1} - \beta}\right) \qquad \text{(Eq. 2A)}$$

$$V_{d\ \text{ss}} = V_1\left(\frac{\alpha + \beta - K_2}{K_{-1}}\right) \qquad \text{(Eq. 3A)}$$

Since for warfarin, β is much smaller than either α or K_{-1}, for warfarin the $V_{d\ \text{area}}$ is approximately the same value as $V_{d\ \text{extrap.}}$. Since the calculations in Table VI relate to $V_{d\ \text{area}}$ and the estimates of volume of distribution of warfarin made by O'Reilly *et al.* (3) following intravenous injection are really estimates of $V_{d\ \text{extrap.}}$, these are directly comparable in the case of warfarin but not necessarily for another drug. This appendix was included to validate the comparison made under *Estimates of Bioavailability* in the text.

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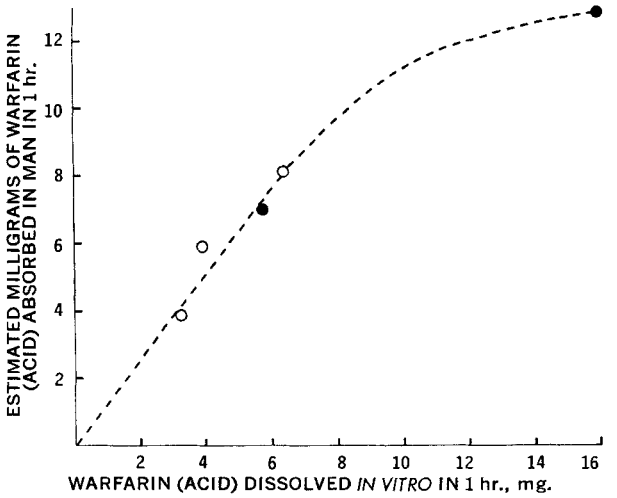

Figure 6—*Correlation of* in vivo *with* in vitro *results. Plot of estimated amount of warfarin absorbed in man in 1 hr. against amount of warfarin dissolved* in vitro *in 1 hr. Key:* ○, *10-mg. label dose; and* ●, *25-mg. label dose. Points from left to right refer to Treatments D (two 5-mg.), C (two 5-mg.), B (one 25-mg.), A (two 5-mg.), and A (five 5-mg.).*

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Pharmacokinetics of Phosphonomycin in Man I: Intravenous Administration

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Abstract □ The disposition of phosphonomycin following intravenous administration is adequately described by a two-compartment open model. Pharmacokinetic parameters derived from serum concentration data correctly predict the urinary excretion profile for the same individual. Lack of self-consistency between serum and urine profiles at any time is in every case accompanied by apparent deviations in renal clearance rate. The adequacy of the model is further illustrated by the good agreement between serum and urinary excretion profiles predicted with pharmacokinetic parameters derived for individual subjects and those actually observed on repeated administration to the same subjects.

Keyphrases □ Phosphonomycin—pharmacokinetics □ Pharmacokinetics—phosphonomycin, intravenous administration □ Renal, serum clearance, human—phosphonomycin □ Distribution-rate constants, volumes, human—phosphonomycin

Phosphonomycin[1], (−)(1R,2S)-1,2-epoxypropylphosphonic acid, is a newly discovered antibiotic produced by strains of *Streptomyces*. Its isolation, characterization, synthesis, and biological properties have been the subjects of recent publications (1–7). Early pharmacologic observations following the oral and intravenous administration in man also have been reported (8–10).

The present communication is concerned only with the pharmacokinetic analysis of serum concentration and urinary excretion data from single and repeated intravenous administrations in apparently healthy, male volunteers. In particular, evidence is developed in support of the two-compartment open model in representing data for individual subjects who received single doses of 250 or 500 mg. of phosphonomycin as the disodium salt. Pharmacokinetic parameters derived for a given individual are shown to give reasonable predictions for the serum and urinary excretion profiles on the same individual on repetitive dosing. Apparent departures from the model, manifest as inconsistencies between serum and urine data, are in every case accompanied by deviations in measurements of serum renal clearance in the same direction. Throughout this discussion, emphasis is placed on self-consistency of the proposed model for individual subjects. Hopefully, the analysis will also help to demonstrate some aspects of pharmacokinetic modeling which heretofore have only been asserted.

EXPERIMENTAL

Pharmacokinetic analysis was performed on results of exploratory studies originally designed to determine the fate of phosphonomycin in man. The experimental design was not originally intended to include pharmacokinetic modeling, but the available data appeared to be sufficient to permit analysis. The data to be discussed were obtained in four separate studies involving apparently healthy, male volunteers. Some subjects participated in more than one study; in these instances, the same numeral identifications are retained throughout so as to facilitate comparison. In studies involving single intravenous injections, the dose of phosphonomycin as the lyophilized powder of the disodium salt was dissolved in 30 ml. of 5% dextrose and was injected into an antecubital vein in 10 min. or less. The procedure for the renal clearance study was already reported (8). Serum and urine specimens were bioassayed by a conventional cup-plate method, employing *Proteus vulgaris* (ATCC 21100) as the test organism.

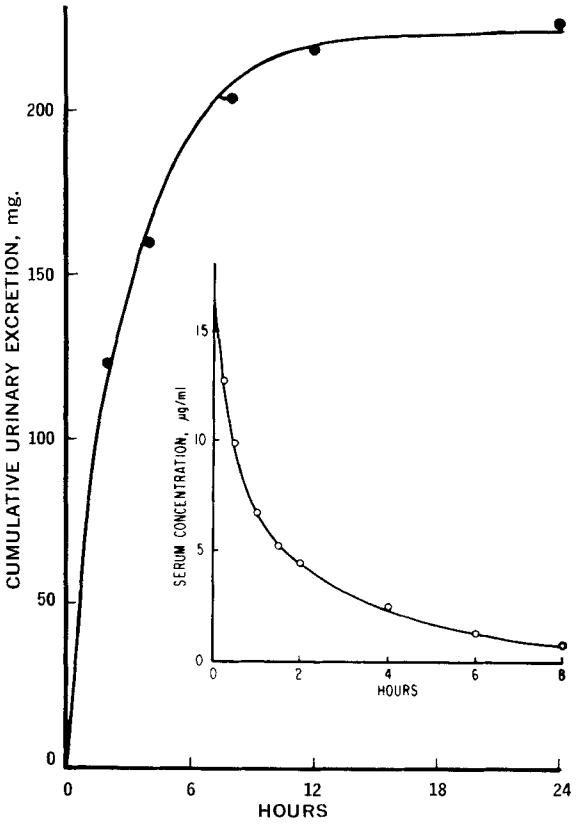


Figure 1—*Typical serum and urinary excretion profiles. Key: Observed serum* (○) *and urine* (●)*; predicted* (——). *Subject 7.*

Study 1—Eight subjects received 250 mg. of phosphonomycin as single injections. Blood samples were taken at 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, and 8 hrs. Fractional urine samples were collected at intervals of 0–2, 2–4, 4–8, 8–12, ad 12–24 hr.

Study 2—Six subjects received 500 mg. of phosphonomycin as single injections. Blood samples were taken at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 hr. Urine samples were collected fractionally at intervals of 0–2, 2–4, 4–8, 8–12, 12–24, and 24–36 hr.

Study 3—Six subjects received 500-mg. doses of phosphonomycin repetitively at 6-hr. intervals as single injections. A total of eight doses was given. Three of the subjects (6, 10, and 13) had participated in either Study 1 or 2. The other three (15, 16, and 17) had participated in another study (not reported here) in which two 500-mg. doses were given 4 hr. apart as single injections; hence, their pharmacokinetic parameters were also known. Blood samples were taken at 0, 0.25, 1, 2, 6, 6.25, 12, 12.25, 18, 18.25, 24, 24.25, 30, 30.25, 36, 36.25, 37, 38, 40, 42, 42.25, and 48 hr. Fractional urine collections were made at 6-hr. intervals for a total of 72 hr.

[1] Phosphonomycin has recently been redesignated as fosfomycin by the United States Adopted Names Council.

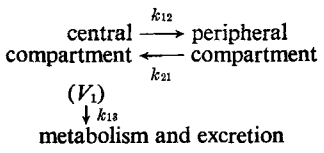
Table I—Estimated Rate Constants and Volumes of Distribution for Phosphonomycin in Man

Subject	Dose, mg.	Rate Constants, hr.$^{-1}$ k_{12}	k_{21}	k_{13}	Volume of Distribution, V_1
1	250	1.16	1.64	0.46	14.8
2	250	2.47	1.74	1.07	7.7
3	250	0.84	1.07	0.53	12.4
4	250	0.68	1.01	0.52	16.0
5	250	0.88	1.83	0.51	17.5
6	250	0.32	1.19	0.46	16.6
7	250	0.85	1.25	0.57	14.4
8	250	0.81	1.03	0.68	11.1
9	500	0.36	0.99	0.45	14.6
10	500	0.70	0.84	0.61	10.1
11	500	1.24	1.26	0.69	10.5
12	500	0.93	0.87	0.72	11.0
13	500	1.92	1.34	0.91	7.4
14	500	0.32	0.64	0.46	16.5
Averages					
(1–8)	250	1.00	1.34	0.60	13.8
(9–14)	500	0.91	0.99	0.64	11.7
(1–14)	—	0.96	1.19	0.62	12.9

Study 4—Renal clearances were determined by a conventional method. Six subjects received single-loading doses of 250 mg. as rapid injections; constant infusions began at the 2nd hour and terminated at the 4th hour. The rates of infusion were individually calculated with the intent of maintaining constant serum levels of 4–5 mcg./ml. Serum samples were taken at 0, 0.25, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, and 10 hr.; urine samples were collected fractionally at 0–2, 2–3, 3–4, 4–6, 6–8, 8–12, and 12–24 hr.

TWO-COMPARTMENT OPEN MODEL

Sufficient blood sample points are available to permit an attempt at compartmental analysis. The data appear to be adequately described by the two-compartment open model, which is schematically represented in Scheme I, where k_{12}, k_{21}, and k_{13} are first-order rate constants for the designated processes; and V_1 is the apparent volume of distribution for the central compartment, of which blood serum is considered to be a part. The appropriate differential equations for this model and their solutions are well known (11, 12). Only the final expressions describing the time course of events for the central and urine compartments are repeated here to facilitate subsequent discussion:



Scheme I

$$C_1(t) = \frac{D}{V_1}\left[\frac{(k_{21} - \alpha)e^{-\alpha t}}{(\beta - \alpha)} + \frac{(k_{21} - \beta)e^{-\beta t}}{(\alpha - \beta)}\right] \quad \text{(Eq. 1)}$$

and

$$X_3(t) = k_{13}fD\left[\frac{(k_{21} - \alpha)(1 - e^{-\alpha t})}{\alpha(\beta - \alpha)} + \frac{(k_{21} - \beta)(1 - e^{-\beta t})}{\beta(\alpha - \beta)}\right] \quad \text{(Eq. 2)}$$

where α and β are the hybrid disposition constants such that $\alpha\beta = k_{21}k_{13}$ and $\alpha + \beta = k_{12} + k_{21} + k_{13}$; D is the dose injected; $C_1(t)$ is the concentration of antimicrobial activity in the central compartment (serum concentration) at time t; $X_3(t)$ is the cumulative amount of activity in the urine up to time t; and f is the fraction of the dose that is ultimately excreted in the urine.

Pharmacokinetic parameters depicted in the model for individual subjects were extracted from serum concentration data by the method of residuals and calculated with the aid of Eq. 1. These are shown in Table I. Urinary excretion data from the same subjects provided an internal verification on the consistency of the proposed model. Accordingly, individual urinary excretion profiles were gen-

Table II—Cumulative Urinary Excretion of Phosphonomycin following Single Injections, Observed *versus* Predicted[a]

Subject	Dose, mg.	Source	Hours: 2	4	8	12	24	36
			Milligrams Excreted					
1	250	Predicted	83.7	125.7	166.6	181.7	190.0	—
		Observed	110.2	136.7	168.5	183.3	190.5	—
2	250	Predicted	151.0	204.3	240.3	248.2	250.4	—
		Observed	151.8	199.3	235.6	245.2	256.5	—
3	250	Predicted	127.7	182.5	234.0	252.2	261.8	—
		Observed	144.0	188.2	238.1	253.1	262.5	—
4	250	Predicted	129.2	182.2	227.6	241.8	248.2	—
		Observed	134.6	184.4	223.9	235.8	248.5	—
5	250	Predicted	135.1	196.3	245.5	259.2	264.3	
		Observed	169.6	203.8	250.0	261.0	264.5	—
6	250	Predicted	136.2	193.5	237.4	248.9	252.7	—
		Observed	160.2	212.3	232.7	247.1	252.9	—
7	250	Predicted	117.7	166.5	207.7	220.2	224.3	—
		Observed	122.7	159.8	204.1	218.6	226.0	—
8	250	Predicted	131.8	178.2	215.9	226.5	230.5	—
		Observed	116.4	168.2	206.0	225.2	231.0	—
9	500	Predicted	246.8	353.3	441.4	468.0	478.7	479.5
		Observed	111.2	323.9	432.9	460.5	484.2	484.3
10	500	Predicted	200.1	274.1	341.0	364.0	374.7	375.4
		Observed	157.3	261.3	323.1	345.7	356.9	369.8
11	500	Predicted	245.6	343.2	425.5	450.0	460.9	461.4
		Observed	105.2	289.0	406.0	433.5	460.0	460.0
12	500	Predicted	331.6	446.6	548.9	581.8	597.4	598.0
		Observed	224.8	489.4	565.2	581.8	598.5	598.6
13	500	Predicted	246.4	336.7	410.1	430.5	437.5	438.0
		Observed	251.3	307.4	421.5	425.8	442.4	442.4
14	500	Predicted	206.7	290.8	365.6	392.5	407.8	408.2
		Observed	85.6	311.7	366.8	395.1	401.2	402.3

[a] With the aid of Eq. 2 using parameters from Table I.

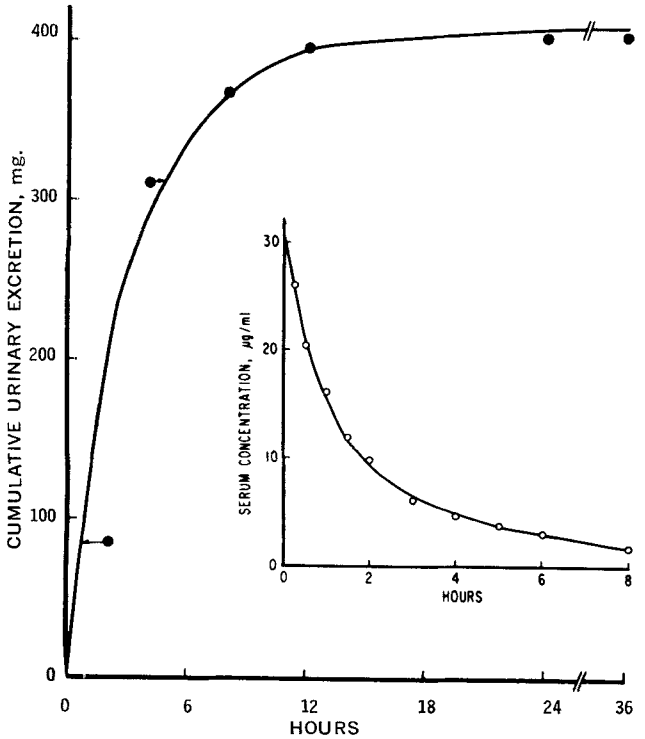


Figure 2—*Typical serum and urinary excretion profiles. Key: observed serum* (○) *and urine* (●)*; predicted* (——). *Subject 14.*

erated with Eq. 2, using parameters given in Table I. A comparison between calculated and experimentally observed urinary excretion profiles is shown in Table II. Typical serum concentration and urinary excretion profiles are also shown in Figs. 1 and 2. There is good agreement between the observed and calculated profiles except during the early time periods in the urinary excretion profiles of some individuals.

Renal and Serum Clearance—One of the seldom discussed prerequisites in the application of Eq. 2 is that renal clearance is presumed to be constant (11, 13). The single-dose data permit the evaluation of serum clearance and also of renal clearance in several ways. These are discussed in turn because their interrelationship provides some insight into the experimental situation and helps to strengthen the proposed model for phosphonomycin.

Renal clearance is usually defined as follows:

$$\dot{V}cl,r \equiv \frac{C_3 \cdot \dot{V}\mu}{C_{1,m}} = \frac{X_3(t_2) - X_3(t_1)}{(t_2 - t_1)C_{1,m}} \qquad \text{(Eq. 3)}$$

where $\dot{V}cl,r$ is the renal clearance, C_3 is the urine concentration, $\dot{V}\mu$ is the rate of urine formation, $X_3(t)$ is the cumulative amount excreted in the urine at time t, and $C_{1,m}$ is the serum concentration taken at the midpoint of the urine collection interval between t_1 and t_2. Values of $\dot{V}cl,r$ were calculated by Eq. 3 for each subject over time intervals for which the data were amenable to this treatment. They are shown in Table III. It is quite apparent that renal clearance calculated in this manner is not constant either incrementally or cumulatively.

Table III—Renal Clearance of Phosphonomycin, Milliliters per Hour, Estimated in Accordance with Eq. 3

$$\dot{V}cl,r = \frac{X_3(t_2) - X_3(t_1)}{C_{1,m}(t_2 - t_1)}$$

Subject	Dose, mg.	Urine Collection Intervals, hr. 0–2	2–4	4–8	0–4	0–8	0–12
1	250	7,344	—	3,975	5,792	8,775	7,637
2	250	10,259	—	8,820	10,831	13,328	20,437
3	250	10,919	—	6,905	7,844	10,262	11,162
4	250	9,750	—	7,601	10,975	12,169	15,113
5	250	12,852	—	8,702	9,611	13,588	16,733
6	250	10,539	—	3,913	10,406	12,118	15,842
7	250	9,158	—	8,533	9,077	10,206	14,009
8	250	8,086	—	7,875	9,347	11,198	15,640
9	500	2,940	12,972	9,400	6,921	8,590	13,234
10	500	4,184	6,755	4,681	5,834	6,732	8,732
11	500	3,507	12,416	10,446	6,752	9,759	12,900
12	500	6,811	23,211	7,584	14,919	13,853	19,395
13	500	8,786	6,936	9,834	7,182	9,579	12,235
14	500	2,750	18,842	9,497	8,027	10,047	11,437

Table IV—Renal Clearance of Phosphonomycin, Milliliters per Hour, Estimated in Accordance with Eq. 5

$$\dot{V}cl,r = \frac{X_3(t_2) - X_3(t_1)}{\int_{t_1}^{t_2} C_1(t)dt}$$

Subject	Dose, mg.	Urine Collection Intervals, hr. 0–2	2–4	4–11	11–12	12–24
1	250	6,779	3,246	4,000	5,051	4,484
2	250	8,270	7,392	8,000	11,115	41,500
3	250	7,736	5,525	6,634	5,639	6,801
4	250	8,602	7,774	7,199	6,779	16,149
5	250	11,789	5,247	8,828	7,600	6,340
6	250	9,165	7,076	3,621	9,848	11,755
7	250	7,762	5,636	8,009	8,635	10,717
8	250	6,158	7,778	7,000	12,617	9,981
9	500	2,854	12,641	7,856	6,525	13,877
10	500	3,626	9,815	5,879	4,616	5,691
11	500	2,847	12,534	9,049	7,267	17,333
12	500	6,396	21,635	6,990	4,774	9,982
13	500	5,971	3,582	9,025	1,254	12,545
14	500	2,613	16,552	4,526	6,437	2,468

A second method for the evaluation of renal clearance takes cognizance of the fact that $C_{1,m}$ in Eq. 3 should reflect the mean serum concentration for the urine collection period (14). The serum concentration at the midpoint of the interval would reflect the mean only if $C_1(t)$ varies linearly between t_1 and t_2. Such is not the case when the data appear to fit the proposed model. However, the mean serum concentration $\bar{C}_1$ can be estimated from its first noncentral moment (Law of the Mean)[2], in that

$$\bar{C}_1 = \frac{\int_{t_1}^{t_2} C_1(t)dt}{t_2 - t_1} \qquad \text{(Eq. 4)}$$

where the numerator is the area under the serum concentration *versus* time curve between t_1 and t_2. Substituting $\bar{C}_1$ as shown in Eq. 4 for $C_{1,m}$ in Eq. 3,

$$\dot{V}cl,r = \frac{X_3(t_2) - X_3(t_1)}{\int_{t_1}^{t_2} C_1(t)dt} \qquad \text{(Eq. 5)}$$

Equation 5 is in the same form as that which Wagner (15, 16) asserted to be the preferred method in calculating $\dot{V}cl,r$.

Areas under the curve over the appropriate time intervals were obtained analytically, using the integrated form of Eq. 1 and parameters from Table I. Values of $\dot{V}cl,r$ calculated in accordance with Eq. 5 are shown in Tables IV and V. Within any given subject, there is considerable variation in renal clearance from one incremental urine collection to the next (Table IV). However, when cumulative urinary excretion is considered as in Table V, this variability is dramatically reduced, and a pattern of relatively constant renal clearance values emerges as larger increments of urine collections are considered. This smoothing effect appears to be consistent with the nature of the experiment in that the body may require some time to adjust to the continuously changing serum concentrations, particularly during the early time periods when these changes are most rapid. Also, it is quite conceivable that a subject may not always be able to void completely and on time.

In comparing Tables II and V, it is evident that there is good agreement between calculated and observed urinary excretion pro-

[2] In relation to the present discussion, the required assumptions for $C_1(t)$ to be continuous for $0 \leq t \leq \infty$, differentiable for $0 < t < \infty$, and absolutely convergent appear reasonable.

$$\dot{V}cl,r = \frac{X_3(t_2) - X_3(t_1)}{\int_{t_1}^{t_2} C_1(t)dt}$$

Table V—Renal Clearance of Phosphonomycin, Milliliters per Hour, Estimated in Accordance with Eq. 5

Subject	Dose, mg.	Urine Collection Intervals, hr. 0–2	0–4	0–8	0–12	0–24	0–36	Mean, $\bar{\dot{V}}cl,r$
1	250	6,779[a]	5,597	5,205	5,192	5,161	—	5,289
2	250	8,270	8,043	8,036	8,134	8,430	—	8,182
3	250	7,736	7,072	7,415	6,879	6,876	—	7,196
4	250	8,602	8,362	8,139	8,058	8,270	—	8,286
5	250	11,789[a]	9,754	9,568	9,465	9,404	—	9,548
6	250	9,165[a]	8,546[a]	7,636	7,738	7,798	—	7,724
7	250	7,762	7,142	7,314	7,388	7,465	—	7,414
8	250	6,158	6,580	6,653	6,932	6,985	—	6,662
9	500	2,854[a]	5,807	6,215	6,233	6,406	—	6,165
10	500	3,626[a]	4,396	4,619	4,619	4,621	4,788	4,669
11	500	2,847[a]	5,598[a]	6,342	6,394	6,635	—	6,457
12	500	6,396[a]	13,095[a]	9,707	9,429	9,444	—	9,527
13	500	5,971	5,323	5,988	5,766	5,886	—	5,787
14	500	2,613[a]	6,617	6,191	6,208	6,069	—	6,271

[a] Not included in the mean.

files for times when renal clearances are constant. Furthermore, nonagreements between observed and calculated profiles are in every case accompanied by renal clearance values that deviate in the same direction. This appears to be strong evidence in support of the proposed model; when the criterion of constant clearance is satisfied, the model adequately describes the time course of events in two distinct, measurable body compartments. In any event, the apparent nonadherence to the model can be explained and, hence, does not constitute sufficient reason for rejecting the model.

While constancy in renal clearance provides a necessary criterion for modeling, a valid model must in turn provide correct estimates of pharmacokinetic parameters, particularly a tangible one like renal clearance. Therefore, if the results shown in Table V are valid, then the mean values, $\bar{\dot{V}}cl,r$, given in the last column should be reasonable estimates of renal clearance for each subject. That such indeed appears to be the case may be inferred from a comparison with the results of renal clearance estimated by the method of constant infusion (Study 4). Table VI summarizes the results of Study 4 and those obtained by the application of Eq. 5 (as shown in Table V). Inasmuch as two different sets of subjects are involved, the comparison is made on the basis of an average individual having a body surface area of 1.73 m.^{2}.

In the case of the proposed model, pharmacokinetic theory requires that

$$\text{serum clearance} \equiv \dot{V}cl,s = k_{13}V_1 \qquad \text{(Eq. 6)}$$

and

$$\text{renal clearance} \equiv \dot{V}cl,r = fk_{13}V_1 \qquad \text{(Eq. 7)}$$

Hence,

$$\frac{\dot{V}cl,r}{Vcl,s} = f \qquad \text{(Eq. 8)}$$

Table VII shows the estimates of renal clearance by Eq. 7 and of serum clearance by Eq. 6. In addition, a comparison is made between observed values of f and those obtained from the ratios of $\bar{\dot{V}}cl,r$ (Table V) to $\dot{V}cl,s$ (Eq. 6). It is seen that the calculated values of f correctly predict the observed. This may appear to be an exercise in triviality because such equality is required by the two-compartment open model. Precisely because it is required and because renal clearance values in the numerator agree with those independently determined by constant infusion, the equality in f constitutes additional support of the proposed model. In essence, this is another way of showing that the derived function for $C_1(t)$ based on the proposed model correctly predicts the incremental change in urinary excretion.

Dose–Area Relationship—If the pharmacokinetics of phosphonomycin are dose independent, one should expect that the total areas under the serum concentration curve, $\int_0^\infty C_1(t)dt$, are linearly related to the dose adjusted for differences in body weight and that the intercept of the regression line does not differ from the origin. Although only two doses were tested (at least three doses would be required to test for linearity), statistical analysis showed that the intercept of the weight adjusted regression line did not differ significantly from zero. Thus, the data do not suggest any evidence of dose dependence in the metabolism and/or the distribution of phosphonomycin at the dosage levels employed in the study.

Multiple-Dose Kinetics—One of the more important aspects of modeling is that it provides a means to predict the fate of the drug in the body and helps to devise dosage regimens to achieve and maintain the desired body levels. Hence, the ability to predict the time course of events upon repeated administration is still another test for the adequacy of the proposed model. Two types of comparison are considered. First, because every subject in Study 3 previously participated in another study from which pharmacokinetic parameters can be estimated, there is the opportunity to assess the reliability of parameters for individual subjects. Second, since dosage regimens are seldom devised for individual patients, almost never in whom the pharmacokinetic parameters are already known,

Table VI—Renal Clearance of Phosphonomycin: A Comparison of Estimates from Pharmacokinetic Analysis and Those Independently Obtained by Constant Infusion

Subject	Body Weight, kg.	Renal Clearance, ml./min./1.73 m.^{2}
1	61.4	115[a]
2	68.2	129[a]
3	61.4	111[a]
4	77.3	120[a]
5	88.6	112[a]
6	95.5	99[a]
7	90.9	109[a]
8	68.6	118[a]
9	66.4	105[a]
10	62.7	102[a]
11	66.4	105[a]
12	72.3	120[a]
13	60.0	115[a]
14	79.1	108[a]
Average (pharmacokinetic analysis)		112.8
18	86.8	84[b]
19	67.7	134[b]
20	87.7	129[b]
21	78.2	131[b]
22	66.4	106[b]
23	69.1	97[b]
Average (constant infusion)		113.5

[a] Same as $\bar{\dot{V}}cl,r$ in Table V but corrected for body surface area according to the equation: surface area = 11.0 × (body weight)$^{0.66}$ (*Reference 17*). [b] Estimated in accordance with the method described under Study 4; values taken from *Reference 8*.

Table VII—Renal and Serum Clearance of Phosphonomycin, Milliliters per Hour, and Estimation of the Fraction of Dose Excreted in the Urine

Subject	Serum Clearance[a], ml./hr.	Renal Clearance[b], ml./hr.	Fraction of Dose —Excreted in the Urine— Calculated[c]	Observed
1	6808	5188	0.777	0.762
2	8239	8453	0.993	1.026
3	6572	6900	1.095	1.050
4	8320	8270	0.996	0.994
5	8925	9443	1.070	1.058
6	7636	7728	1.012	1.012
7	8208	7420	0.903	0.904
8	7548	6974	0.883	0.924
9	6570	6366	0.938	0.969
10	6161	4559	0.758	0.740
11	7245	6665	0.891	0.920
12	7920	9480	1.203	1.197
13	6734	5960	0.859	0.885
14	7590	6102	0.826	0.804

[a] Estimated by Eq. 6. [b] Estimated by Eq. 7. [c] The ratio of $\bar{V}cl,r$ (from Table V) to $\dot{V}cl,s$ (from the second column of this table).

it is useful to determine whether the average pharmacokinetic parameters derived from a few volunteers can adequately predict the time course of events in some healthy individual.

The predicted time course of events in the central and the urine compartments for each subject were computer simulated using the appropriate sets of pharmacokinetic parameters from Table I. The calculated values are compared with those actually observed in Tables VIII (serum) and IX (urine). A typical set of serum and urine profiles is also given in Fig. 3. The results for the average subject in Tables VIII and IX constitute an attempt to compare the arithmetic average of the observed data and the simulation values based on the average parameters from Table I. The greatest deviations between calculated and observed serum levels are seen in regions where changes are most rapid, such as the 15-min. point following each dose. As with single injections, differences between observed and predicted urinary excretion tend to disappear as larger collection periods are considered. In view of the variability inherent in the bioassay, the cumulative nature of the urinary excretion profiles and minor differences in the rates of injection from dose to dose, including that from which the parameters are derived, the agreement obtained individually and on the average appears quite satisfactory.

DISCUSSION

Plasma or serum concentration data are generally preferred for the purpose of pharmacokinetic modeling. Among other considerations, this compartment is closest to the site of absorption and is readily accessible, provided the drug or metabolite(s) can be quantitatively detected therein. This is especially true in the case of intravenous administration. The urine is the body compartment that is perhaps even more conveniently accessible from the experimental viewpoint. However, urine is at least one more compartment removed from the source of input and is therefore somewhat less sensitive to perturbations. Rapid changes in drug distribution and metabolism require frequent sampling which, in the case of urine, calls for highly cooperative subjects whose ability to void on command and to retain bladder contents until a given hour to ensure complete sampling without catheterization may be less than ideal. Even among the most cooperative, there exists an irreducible minimum in the time between samples, and the subject must be able to void completely each time. Either by choice or by necessity, however, more elegant pharmacokinetic analyses have been performed on urinary excretion profiles since the pioneering studies of Nelson and his coworkers (13, 18–20).

When both serum and urine data are available, as they are in this case, there is the opportunity for immediate verification as to the adequacy of any proposed pharmacokinetic model. The additional experimental work involved appears to be disproportionately meager compared with the information so gained. Subsequent discussions presume that the evidence set forth in previous sections is sufficient to establish the two-compartment open model as adequate for the disposition of phosphonomycin following intravenous administration.

Discrepancies between predicted and observed urinary excretion, when they occur, are mostly confined to the first one or two urine collection periods. Possible causes of such nonagreement are numerous; some may be physiologic, others experimental. Variation in urine pH can have profound effects on urinary excretion (21, 22), but it is most unlikely in the case of phosphonomycin with pKa's of 1.5 and 6.4. Under physiologic conditions, phosphonomycin exists as the monovalent and the divalent anions in the urine. Inasmuch as drug concentrations are measured in terms of antimicrobial activity, it is conceivable that products of metabolism may be more or

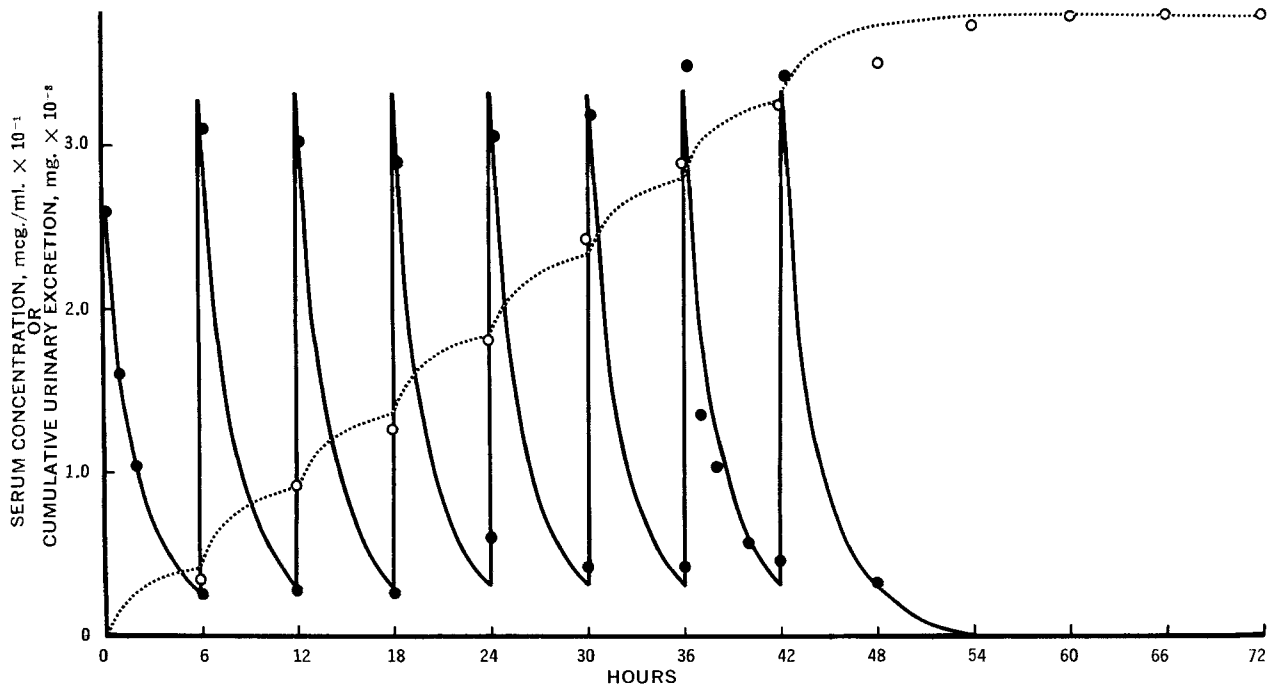


Figure 3—*Typical serum and urinary profiles of phosphonomycin on repeated intravenous injections of 500 mg. every 6 hr. Key: observed serum* (●) *and urine* (○)*; predicted serum* (——) *and urine* (- - -). *Subject 6.*

Table VIII—Observed and Predicted Serum Levels following Repetitive Administration of Phosphonomycin, 500 mg., Every 6 Hours

Subject	Source	Time from the First Dose, hr.																		
		0.25	1	2	6	6.25	12	12.25	18	18.25	24	24.25	30	30.25	36	36.25	37	38	40	42	42.25	48
		Serum Concentration, mcg./ml.																				
6	Simulated[a]	25.0	16.1	10.4	2.5	27.6	2.8	27.6	2.9	27.7	2.9	27.7	2.9	27.7	2.9	27.7	18.1	11.8	5.8	2.9	27.7	2.9
	Observed	26.0	13.7	9.0	7.0	31.0	3.2	30.3	2.7	29.0	6.0	30.6	4.3	31.9	4.3	34.8	13.6	10.4	5.7	4.6	34.2	3.3
10	Simulated[a]	36.4	18.2	10.9	3.4	39.5	4.1	40.2	4.2	40.3	4.2	40.3	4.2	40.3	4.2	40.3	21.4	13.3	7.3	4.2	40.3	4.2
	Observed	31.0	15.0	8.2	2.6	37.2	3.1	34.4	2.6	35.4	4.4	32.5	4.0	43.7	3.6	44.5	20.7	12.9	6.8	4.1	38.6	2.8
13	Simulated[a]	36.4	15.3	10.4	2.9	39.1	3.4	39.5	3.4	39.6	3.4	39.6	3.4	39.6	3.4	39.6	17.8	12.2	6.5	3.4	39.6	3.4
	Observed	30.6	15.0	10.6	3.1	34.1	3.4	35.7	4.6	34.4	4.1	34.1	5.0	34.1	5.0	42.0	24.9	15.0	7.6	4.9	37.0	2.9
15	Simulated[b]	38.2	17.5	11.8	3.8	41.8	4.6	42.4	4.7	42.6	4.7	42.6	4.7	42.6	4.7	42.6	21.1	14.6	8.3	4.7	42.6	4.7
	Observed	31.6	17.6	10.4	2.9	37.9	2.5	38.1	3.5	32.6	5.0	35.3	3.8	42.0	3.6	34.2	20.0	12.4	5.7	3.8	40.4	2.2
16	Simulated[c]	28.0	18.0	11.3	2.9	30.7	3.4	31.1	3.1	31.2	3.4	31.2	3.4	31.2	3.4	31.2	20.6	13.2	6.5	3.4	31.2	3.4
	Observed	23.6	10.2	6.8	1.8	27.8	1.9	25.8	1.6	30.9	3.6	33.5	2.7	29.5	2.7	29.5	13.6	8.4	5.2	2.7	30.0	2.1
17	Simulated[d]	26.3	15.8	9.7	2.6	28.7	3.1	29.1	3.1	29.2	3.1	29.2	3.1	29.2	3.1	29.2	18.1	11.4	5.8	3.1	29.2	3.1
	Observed	25.0	13.3	8.8	2.5	30.5	2.9	29.3	3.2	33.0	5.3	36.6	4.3	34.5	3.9	30.8	18.1	12.9	6.2	4.0	28.6	3.8
Average	Simulated[e]	27.1	13.7	8.9	2.7	29.5	3.1	30.0	3.2	30.0	3.2	30.0	3.2	30.0	3.2	30.0	16.1	10.7	5.8	3.2	30.0	3.2
	Simulated[f]	29.8	14.3	8.8	2.7	32.3	3.1	32.7	3.2	32.8	3.2	32.8	3.2	32.8	3.2	32.8	16.7	10.6	5.8	3.2	32.8	3.2
	Observed	28.0	14.1	9.0	3.3	33.1	2.8	32.2	2.9	32.8	4.8	33.8	3.9	35.9	3.9	36.0	18.5	12.0	6.2	4.0	34.8	2.9

[a] Parameters from Table I. [b] $k_{12} = 1.39$; $k_{21} = 1.20$; $k_{13} = 0.70$; $V_1 = 8.21$. [c] $k_{12} = 0.57$; $k_{21} = 1.14$; $k_{13} = 0.52$; $V_1 = 12.69$. [d] $k_{12} = 0.36$; $k_{21} = 0.84$; $K_{13} = 0.50$; $V_1 = 14.18$. [e] Average parameters for all subjects in Table I. [f] Average parameters for all subjects receiving 500 mg.

less active than the parent compound. However, there is no evidence that phosphonomycin is metabolized either in man or in animals. The possibility that there is variable and incomplete distribution in the body fluids during the early time periods is at least in qualitative agreement with the data. Since urinary recoveries depended on the cooperation of the subjects to void voluntarily on schedule as well as completely emptying their bladders, some error may have been introduced in the collections.

That these factors may be major causes of the discrepancies can be supported in several ways. Renal clearance values for each incremental urine collection period are not constant (Table IV). Constant and apparently correct values of renal clearance can be achieved by considering larger increments in a cumulative fashion. Since each cumulative increment under consideration also contains contributions from all preceding ones, correct estimates of $\dot{V}cl,r$ are possible only if the total amount excreted up to a given time is also estimated correctly. Hence, if cumulative amounts are correct but apparent deviations exist prior to that time, such deviations must be assigned to errors in timekeeping. These arguments suggest that discrepancies between observed and calculated urinary profiles should be considered as horizontal deviations rather than vertical ones (arrows in Figs. 1 and 2).

It would have been physiologically more realistic to take cognizance of the fact that some finite lag time should be incorporated into the urinary excretion profile[3]. The magnitude of this lag is estimated to be in the order of 2 or 3 min. and is usually experimentally determined. Inasmuch as the first urine sample point is at 120 min., the present data do not lend themselves to an estimation of the lag parameter with any degree of certainty. It should be noted, however, that changes attendant to a 2- or 3-min. displacement of the urinary excretion profiles would be minor in relation to the overall experimental error and, hence, should not materially influence the interpretations attempted herein.

The application of Eq. 5 requires further elaboration. The Law of the Mean guarantees that the value of C_1 used to estimate $\dot{V}cl,r$ is that of the mean serum concentration over the urine collection interval under consideration; thus, Eq. 5 is conceptually more acceptable than Eq. 3 in estimating renal clearance. In fact, Eq. 3 can be thought of as a special case of Eq. 5 in which $C_1(t)$ is linear between t_1 and t_2; hence, there is the need to achieve constant serum levels in the conventional methods of renal clearance determinations. Experimental errors notwithstanding, Eq. 5 leads to a correct estimate of $\dot{V}cl,r$ only when the denominator term can be properly estimated. $\int_{t_1}^{t_2} C_1(t)dt$ is the area under the serum concentration curve between t_1 and t_2; in practice, it may be estimated in a variety of ways, analytically if $C_1(t)$ is known, by a planimeter, by weighing, by the trapezoidal rule or Simpson's rules, *etc.* With respect to data presently under discussion, any of these methods of area estimation will lead to the same pattern for values of $\dot{V}cl,r$ when calculated by Eq. 5. However, not the same $\dot{V}cl,r$ values are achieved, and, therefore, they are not equally satisfactory as estimates of renal clearance. Hence, the applicability of Eq. 5 is largely dependent on the accuracy with which $\int_{t_1}^{t_2} C_1(t)dt$ can be estimated. The user must exercise judgement as to the adequacy of the method selected to estimate the area. It must be noted also that Eq 5. retains its present meaning only if the criteria of continuity and differentiability for $C_1(t)$ are satisfied. Otherwise, there is no assurance that the serum concentration implicit in Eq. 5 corresponds to that of the mean over the urine collection period. Consequently, when zero-order absorption, saturable process(es), or repetitive dosing is involved or suspected, there would be region(s) of $C(t)$ over which Eq. 5 should be applied only with due caution.

As in the case of spectinomycin (23), the foregoing seems to suggest that where it is safe and experimentally feasible, renal clearance can be estimated from a single rapid intravenous injection. In theory at least, this appears to be ideal in cases where an adequate model can be deduced from the data. Whenever self-consistency in the model between plasma (serum) and urine compartments can be demonstrated, a reasonable estimate of renal clearance would emerge. This is apparently the case with phosphonomycin, but the general applicability remains to be demonstrated, particularly with examples representing more complex mechanisms of excretion (24–28). There are obvious advantages

[3] The authors are indebted to Dr. J. G. Wagner and Dr. G. Levy for helpful discussions on this point.

Table IX—Observed and Predicted Urinary Excretion Profiles following Repetitive Administration of Phosphonomycin, 500 mg., Every 6 Hours

Subject	Source	Time from the First Dose, hr. 6	12	18	24	30	36	42	48	54	60	66	72
		Cumulative Urinary Excretion, mg.											
6	Simulated[a]	431	901	1375	1850	2324	2800	3274	3750	3804	3811	3812	3812
	Observed	362	942	1284	1834	2442	2896	3257	3505	3736	3784	3798	3801
10	Simulated[a]	386	823	1270	1719	2167	2616	3065	3514	3582	3582	3589	3490
	Observed	388	813	1317	1767	2170	2544	3011	3467	3561	3570	3586	3590
13	Simulated[a]	301	651	1008	1367	1725	2084	2442	2801	2846	2852	2853	2854
	Observed	183	400	802	1284	1832	2298	2326	2774	2850	2865	2866	2868
15	Simulated[b]	403	874	1358	1846	2333	2820	3308	3795	3872	3887	3890	3890
	Observed	214	647	1224	1767	2120	2676	3350	3824	3880	3895	3901	3901
16	Simulated[c]	401	838	1282	1727	2172	2617	3062	3507	3565	3574	3576	3576
	Observed	421	889	1274	1869	2211	2521	2978	3503	3539	3552	3555	3561
17	Simulated[d]	352	737	1128	1520	1912	2305	2698	3090	3143	3152	3153	3154
	Observed	120	532	918	1377	1824	2116	2554	3044	3112	3130	3136	3140
Average	Simulated[e]	379	804	1237	1671	2106	2540	2975	3409	3471	3481	3483	3483
	Simulated[f]	376	800	1233	1668	2102	2537	2971	3406	3468	3478	3481	3481
	Observed	281	704	1137	1650	2100	2508	2913	3353	3447	3468	3474	3477

[a–f] Footnotes same as those in Table VII.

to such a procedure, because it would eliminate the need to maintain constant serum levels and the potential sources of experimental error attendant thereto. It would undoubtedly be more attractive to the patient because he should be able to remain ambulatory except for sampling times. The elimination half-life and the extent of biotransformation are seen to be determining factors in considering the attractiveness of this alternative procedure. A drug that is extensively metabolized or slowly excreted in the urine is obviously not a good candidate.

Finally, words like valid, correct, adequate, proper, *etc.* have been used throughout. They are to be interpreted only in relation to this report which is based on data presently available. The authors recognize that modeling is only an approximation to the actual physical situation; refinements are always possible and are to be sought as new data are developed.

SUMMARY AND CONCLUSIONS

The two-compartment open model adequately describes the time course of events of phosphonomycin in the central and urine compartments following intravenous administration in man. Apparent inconsistencies in the proposed model are in every case accompanied by deviations from constant renal clearance. Consideration of likely causes strongly suggests that such deviations are attributed to the experimental design which relied on voluntary voiding of urine samples and the inability of the subjects to cooperate completely in performance as to completeness and time of voiding.

The adequacy of the model is further demonstrated in several ways. First, the model is shown to be adequate at two dose levels (250 and 500 mg.). Second, areas under the serum concentration curve are linearly related to dose, with an intercept not different from zero. Third, pharmacokinetic parameters derived for individual subjects satisfactorily predict the time course of events in the central and urine compartments of the same subjects following repeated administration. Average parameters also satisfactorily predict average levels. Fourth, evaluations of self-consistency of the proposed model between serum and urine lead to estimates of renal clearance in agreement with those obtained by constant infusion. Thus, a firm pharmacokinetic base has been established for the disposition of phosphonomycin in man. This is necessary to the evaluation and the understanding of the kinetics of absorption, which will be reported in a future communication.

In the process of evaluating the data, the possibility also has been suggested for the use of single intravenous injections as a potential alternate to conventional methods for the determination of renal clearance. This may be viewed as a useful by-product of pharmacokinetic analysis; it is apparently also practical in the case of phosphonomycin. The applicability of the integral method (Eq. 5) for calculating renal clearances was illustrated and discussed. Important limitations in its use are: (*a*) the conformance of the serum concentration function with the Law of the Mean requirements for continuity and differentiability, and (*b*) the accuracy in the area under the serum curve estimation.

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Stereochemical Investigations of Local Anesthetic Action

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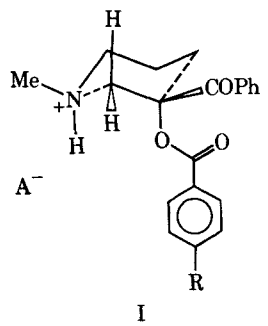
Abstract □ The toxicities and local anesthetic activities in a variety of assay systems are reported for a series of 1-alkyl-3-benzoyl-3-acyloxypiperidines. In solution, the procaine analog, XI, is conformationally more homogeneous than procaine about the 4-atom aminoethoxy unit and is 8 times more active and 16 times more toxic than procaine on a molar basis. This suggests that the preferred *gauche*-conformation of XI has positive biological significance for local anesthetic receptors on one hand and for CNS and/or cardiac receptors on the other hand, although this stereochemical feature discriminates in favor of CNS and/or cardiac receptors.

Keyphrases □ Anesthetics, local—conformation–activity relationship □ Stereochemical aspects—local anesthetic activity □ 1-Alkyl-3-benzoyl-3-acyloxypiperidines—synthesis □ Pharmacological screening—1-alkyl-3-benzoyl-3-acyloxypiperidines □ IR spectrophotometry—structure □ Polarimetry—conformation

The antipodal and diastereoisomeric potency and toxicity ratios of amino ester local anesthetics vary with the pharmacological tests used in their evaluation, but they never exceed 8 : 1 (1–5). Recent studies (6–9) on the synthesis and local anesthetic activity of asymmetric 1-methyl-3-benzoyl-3-chloropiperidine and 1-methyl-3-benzoyl-3-acyloxypiperidines (I) prompted a more rigorous pharmacological evaluation and comparison of this prototype with the chemically analogous but conformationally less restricted drug, procaine (II), in order to explore the potential relevance of conformational



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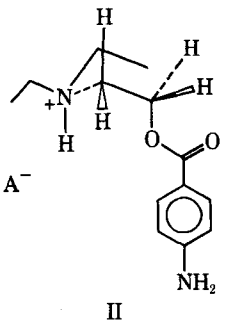

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restraints (*cf.*, 10, 11) about the 4-atom aminoethoxy unit to potency and/or selectivity. While this investigation is restricted to ethanolamine esters, it is of interest that conformational effects have been studied in the propanolamine series (12).

EXPERIMENTAL[1]

Chemistry

All melting points were obtained in a Hershberg-type (13) silicone (550-Dow)-filled melting-point apparatus equipped with Anschutz full-immersion thermometers and are uncorrected. The samples were placed in the silicone bath 10° below the reported melting points and heated at a rate of 1–2°/min.

Specific rotations were determined with a Zeiss 0.01° polarimeter in a modified (14) 2-dm., 2-ml. syringe-filling polarimeter tube.

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Petroleum ether refers to the fraction boiling from 30–60°. Solutions of free amines in apolar solvents were clarified with activated charcoal[2] and dried simultaneously with anhydrous sodium sulfate; they were then filtered through sintered glass. Solvents were evaporated in a water bath under reduced pressure. All base washings were performed with saturated aqueous $NaHCO_3$. All acid washings were performed with 3 *N* HCl. Unless otherwise stated, the HCl salts were prepared in Et_2O using HCl gas, dried, and recrystallized.

(+)-1-Methyl-3-benzoyl-3-benzoyloxypiperidine (III) Hydrochloride—To 13.0 g. (59.3 mmoles) of IV, (+)-1-methyl-3-benzoyl-3-hydroxypiperidine, m.p. 72.5–73.0°, $[\alpha]_D^{25}$ (absolute EtOH) +11.4 ± 0.3° (c 5.00) [lit. (10, 11) 72.5–73.0°, +11.4°] in 100 ml. of pyridine was added 18 g. (80 mmoles) of benzoic anhydride. The solution was allowed to reflux for 24 hr. After evaporation of the solvent, the residue was mixed with 100 ml. of 3 *N* HCl. The mixture was washed with $HCCl_3$, made basic with Na_2CO_3, and extracted with $HCCl_3$. The extract was dried, filtered, and evaporated to give an oil which, on clarification in and crystallization from petroleum ether, afforded 9.28 g. (28.7 mmoles, 48%) of III: m.p. 65–66°; $[\alpha]_D^{28}$ (absolute EtOH) +61.4 ± 0.4° (c 9.00); IR 1705 $cm.^{-1}$ (C=O, aromatic ester), 1676 $cm.^{-1}$ (C=O, aromatic ketone), no OH band in the 3500-$cm.^{-1}$ region.

Anal.—Calcd. for $C_{20}H_{21}NO_3$: C, 74.3; H, 6.55; N, 4.33. Found: C, 74.0; H, 6.62; N, 4.14.

The HCl salt was crystallized from 2-PrOH[3]: m.p. 234–235°.

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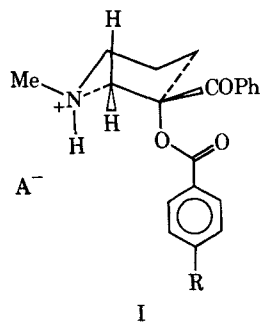

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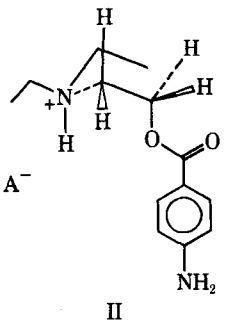

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[3] 2-PrOH = isopropyl alcohol.

Anal.—Calcd. for $C_{20}H_{22}ClNO_3$: C, 66.8; H, 6.16; Cl, 9.85; N, 3.89. Found: C, 66.8; H, 6.16; Cl, 9.71; N, 3.92.

(±)-1-Methyl-3-benzoyl-3-benzoyloxypiperidine (V) Hydrochloride—To 4.35 g. (18.7 mmoles) of VI, (±)-2-methoxy-2-phenyl-5-methyl-1-ox-5-azaspiro[2.5]octane, b.p. 115–119°/2 mm. [lit. (7, 11, 16) b.p. 70–71°/0.08 mm., *cf.*, 8, b.p. 117–119°/2 mm.], consisting of a mixture of both diastereoisomers (16–19) in 50 ml. of dry Et_2O, was added 5 g. (41 mmoles) of benzoic acid in 50 ml. of dry Et_2O. After 24 hr. at room temperature, the solvent was evaporated and replaced with $HCCl_3$. The mixture was washed with base and then with H_2O, dried, filtered, and evaporated. The residue was clarified in and crystallized from petroleum ether to give 3.9 g. (12.2 mmoles, 65%) of V: m.p. 114–115° [lit. (8) m.p. 114–115°]; IR 1708, 1675 cm.$^{-1}$. The HCl salt, m.p. 201–202° [lit. (8) m.p. 201–202°], was crystallized from 2-PrOH.

(±)-1-Methyl-3-benzoyl-3-(*o*-chlorobenzoyloxy)piperidine (VII) Hydrochloride—Treatment of 6.5 g. (41.5 mmoles) of *o*-Cl-benzoic acid in 300 ml. of Et_2O and 4.35 g. of VI, as in the preparation of V, afforded VII from Et_2O: m.p. 120–122°; IR 1732, 1686 cm.$^{-1}$; and subsequently 6.0 g. (15.3 mmoles, 82%) of the HCl salt from 2-PrOH: m.p. 221–223°: IR 1732, 1679 cm.$^{-1}$.

Anal.—Calcd. for $C_{20}H_{21}Cl_2NO_3$: C, 60.9; H, 5.37; Cl, 17.98; N, 3.55. Found: C, 60.7; H, 5.09; Cl, 17.71; N, 3.39.

(±)-1-Methyl-3-benzoyl-3-(*o*-bromobenzoyloxy)piperidine (VIII) Hydrochloride—Treatment of 8.5 g. (42.3 mmoles) of *o*-Br-benzoic acid and 4.35 g. of VI, as in the preparation of V, afforded VIII from Et_2O; m.p. 149–151°; IR 1743, 1679 cm.$^{-1}$; and subsequently 7.0 g. (15.9 mmoles, 85%) of the HCl salt from 2-PrOH: m.p. 216–218°; IR 1736, 1683 cm.$^{-1}$.

Anal.—Calcd. for $C_{20}H_{21}BrClNO_3$: C, 54.8; H, 4.83; Br, 18.21; Cl, 8.08; N, 3.19. Found: C, 55.1; H, 5.05; Br, 18.00; Cl, 8.00; N, 3.22.

(±)-1-Methyl-3-benzoyl-3-(*p*-hydroxybenzoyloxy)piperidine (IX) Hydrochloride—Treatment of 13.8 g. (0.1 mole) of *p*-OH-benzoic acid in 500 ml. of boiling Et_2O and 7.0 g. (30 mmoles) of VI, as in the preparation of V, afforded IX which was dissolved in 250 ml. of Me_2CO and treated with 5 ml. of 12 *N* HCl. The salt crystallized on cooling and was recrystallized from EtOH to give 8.1 g. (21 mmoles, 70%) of IX·HCl·$^1/_2H_2O$; m.p. 226–227° dec.

Anal.—Calcd. for $C_{20}H_{22}ClNO_4 \cdot {}^1/_2H_2O$: C, 62.4; H, 6.02; Cl, 9.21; N, 3.64. Found: C, 62.8; H, 6.09; Cl, 9.30; N, 3.71.

(±)-1-Methyl-3-benzoyl-3-(*p*-methoxybenzoyloxy)piperidine (X) Hydrochloride—Treatment of 15.2 g. (0.1 mole) of *p*-OCH_3-benzoic acid in 100 ml. of boiling tetrahydrofuran and 7.0 g. of VI, as in the preparation of V, afforded 8.9 g. (25.2 mmoles, 84%) of X. The HCl salt·H_2O was crystallized from EtOH–Et_2O, m.p. 196–198°.

Anal.—Calcd. for $C_{21}H_{24}ClNO_4 \cdot H_2O$: C, 61.8; H, 6.43; Cl, 8.70; N, 3.43. Found: C, 61.8; H, 6.20; Cl, 9.72; N, 3.46.

(±)-1-Methyl-3-benzoyl-(*p*-aminobenzoyloxy)piperidine (XI) Hydrochloride—Treatment of 6.0 g. (44 mmoles) of *p*-NH_2-benzoic acid in 75 ml. of boiling tetrahydrofuran and 4.35 g. of VI, as in the preparation of V, afforded 5.8 g. (17.2 mmoles, 92%) of XI from *n*-hexane–Et_2O, m.p. 145–147°.

Anal.—Calcd. for $C_{20}H_{22}N_2O_3$: C, 71.0; H, 6.55; N, 8.28. Found: C, 71.3; H, 6.35; N, 7.88.

The HCl salt was prepared by adding 17.2 meq. of standardized 2-PrOH–HCl to 5.8 g. of XI in 2-PrOH–tetrahydrofuran (60:40), evaporating the solvent, and recrystallizing the residue from 2-PrOH–Et_2O: m.p. 170–172°; IR 3310, 1705 cm.$^{-1}$.

Anal.—Calcd. for $C_{20}H_{23}ClN_2O_3$: C, 64.1; H, 7.47; Cl, 9.46; N, 7.47. Found: C, 64.0; H, 7.40; Cl, 9.21; N, 7.34.

(±)-1-Methyl-3-benzoyl-3-(*o*-chloro-*p*-aminobenzoyloxy)piperidine (XII) Hydrochloride—Treatment of 7.7 g. (45 mmoles) of *o*-Cl-*p*-NH_2-benzoic acid and 4.35 g. of VI, as in the preparation of XI·HCl, afforded 5.9 g. (15.9 mmoles, 85%) of XII and, subsequently, the HCl salt from 2-PrOH–Et_2O: m.p. 203–205°; IR 3340, 1708, 1673 cm.$^{-1}$.

Anal.—Calcd. for $C_{20}H_{22}Cl_2N_2O_3$: C, 58.7; H, 5.42; Cl, 17.32; N, 6.84. Found: C, 58.3; H, 5.72; Cl, 17.00; N, 7.04.

(±)-1-*n*-Propyl-3-benzoyl-3-hydroxypiperidine (XIII) Hydrochloride—Treatment of 3-benzoyl-3-hydroxypiperidine (XIV) obtained from 7.0 g. (18.5 mmoles) of the *p*-toluenesulfonate salt, m.p. 192–193° [lit. (9) m.p. 192–193°], with 2.75 g. of *n*-PrBr in 75 ml. of DMF at 60° for 48 hr., followed by evaporation of DMF, afforded a residue which was dissolved in H_2O. The solution was made basic with Na_2CO_3 and extracted with petroleum ether. The extract was dried, clarified, filtered, and evaporated to give 2.9 g. (11.7 mmoles, 63%) of XIII: IR 3440, 1678 cm.$^{-1}$. The HCl salt was crystallized from 2-PrOH: m.p. 226–228° dec.; IR 1672 cm.$^{-1}$.

Anal.—Calcd. for $C_{15}H_{22}ClNO_2$: C, 63.5; H, 7.81; Cl, 12.49; N, 4.94. Found: C, 63.5; H, 7.80; Cl, 12.52; N, 4.68.

(±)-1-*n*-Propyl-3-benzoyl-3-benzoyloxypiperidine (XV) Hydrochloride—Benzoylation of 2.47 g. (10 mmoles) of XIII, as in the preparation of III·HCl, afforded 2.72 g. (7.0 mmoles, 70%) of XV·HCl from 2-PrOH–Et_2O: m.p. 181–182°; IR 1719, 1680 cm.$^{-1}$; no OH band in 3500 region.

Anal.—Calcd. for $C_{22}H_{26}ClNO_3$: C, 68.1; H, 6.76; Cl, 9.14; N, 3.61. Found: C, 68.0; H, 6.49; Cl, 9.17; N, 3.88.

(±)-1-*iso*-Propyl-3-benzoyl-3-benzoyloxypiperidine (XVI) Hydrochloride—Treatment of 7.0 g. of XIV·*p*-tosylate with 3.14 g. of 2-PrI, as in the preparation of XIII, gave 2.15 g. (8.7 mmoles, 47%) of 1-*iso*-propyl-3-benzoyl-3-hydroxypiperidine (XVII) which was benzoylated as in the preparation of III·HCl to afford 2.86 g. (8.2 mmoles, 94%) of XVI, IR 1735, 1670 cm.$^{-1}$, and, subsequently, 2.53 g. (6.52 mmoles, 75%) of XVI·HCl from 2-PrOH: m.p. 196–197°; IR 1718, 1673 cm.$^{-1}$.

Anal.—Calcd. for $C_{22}H_{26}ClNO_3$: C, 68.1; H, 6.76; Cl, 9.14; N, 3.61. Found: C, 68.2; H, 6.68; Cl, 9.45; N, 3.63.

(±)-1-Methyl-3-benzoyloxy-3-(α-hydroxybenzyl)piperidine (XVIII)—To V, obtained from 1.64 g. (4.55 mmoles) of V·HCl, were added 10 ml. of 90% EtOH and 0.046 g. of $NaBH_4$. After 24 hr., the solvent was evaporated and the residue was boiled with Et_2O. The Et_2O was washed with H_2O, dried, clarified, filtered, and evaporated to give 1.13 g. (3.5 mmoles, 77%) of product (XVIII and V); IR 3460, 1712, 1678 cm.$^{-1}$. The mixture was dissolved in petroleum ether and, on cooling, afforded crystals which were recrystallized from petroleum ether to give 685 mg. (2.1 mmoles, 46%) of XVIII: m.p. 126–127°; IR 3460, 1712 cm.$^{-1}$. The stereochemistry of this apparently pure diastereoisomer is unknown.

Anal.—Calcd. for $C_{20}H_{23}NO_3$: C, 73.8; H, 7.12; N, 4.30. Found: C, 73.6; H, 7.15; N, 4.57.

(±)-1-*iso*-Propyl-3-benzoyloxy-3-(α-hydroxybenzyl)piperidine (XIX) Hydrochloride—Treatment of 0.3 g. of $NaBH_4$ in 100 ml. of 90% EtOH and XVI, obtained from 1.9 g. (4.85 mmoles) of XVI·HCl, as in the preparation of XVIII, afforded 1.2 g. of XIX: IR 3450, 1708 cm.$^{-1}$, slight absorption in 1680 region. The HCl salt, 1.17 g. (3.0 mmoles, 62%), was crystallized from 2-PrOH–Et_2O, m.p. 225–227°. The stereochemistry of this apparently pure diastereoisomer is unknown.

Anal.—Calcd. for $C_{22}H_{28}ClNO_3$: C, 67.8; H, 7.24; Cl, 9.09; N, 3.59. Found: C, 67.5; H, 7.00; Cl, 9.39; N, 3.51.

Biology

Animals and Experimental Conditions—Male albino mice, guinea pigs, and rabbits were used. These were obtained from a commercial breeder and were housed and fed under standard conditions for 1–2 weeks prior to use. Solutions of all test compounds were prepared immediately prior to use with physiological saline or with distilled water for injection.

ED_{50}—Four standard methods were used for the evaluation of local anesthetic activity.

1. Corneal anesthesia in rabbits: the procedure of Luduena and Hoppe (20) was followed, using four rabbits for each concentration of drug.

2. Intracutaneous wheal in guinea pigs: the technique of Bulbring and Wajda (21) was employed, with six tests on three guinea pigs being performed simultaneously.

3. Sciatic nerve block in guinea pigs: the method of Shackell (22) was used with four guinea pigs for each concentration of drug.

4. Mouse tail: the procedure of Bianchi (23) was employed with 10 mice (average weight 30–35 g.) for each concentration. A minimum of five concentrations was used to determine the dose–response curves.

$T_{1/2}$ Values—Using the method of Bianchi (23), a sufficient number of mice were injected with compounds at doses corresponding to the previously determined ED_{50} values. At least 10 responding mice were selected after 15 min. and retested for local anesthesia every 5 min. thereafter until all test animals recovered. The percentage of mice anesthetized was plotted *versus* time, and the line was fitted by means of regression analysis. The time at which 50% of the test animals remained anesthetized was obtained by extrapolation from the graph.

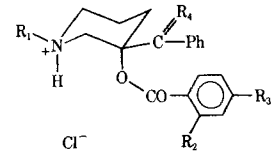

Table I—Local Anesthetic Potency and LD_{50} Values of Some Substituted Piperidines and Control Compounds Procaine, Lidocaine, and Cocaine

Number	Compound R_1	R_2	R_3	R_4	LD_{50}[a], Mouse, Subcutaneous Injection, mg./kg.	ED_{50}[a,b], Mouse Tail, mg./kg.	ED_{50}[a], Guinea Pig Wheal, mg./kg.	ED_{50}[a], Sciatic Nerve, mg./kg.	ED_{50}[d], Rabbit Cornea, %	*TI*[e]	$T_{1/2}$[e–h] at ED_{50}, min.	Sat.[e,h,i] Value, mg./kg.	TI_s[e,i]
III	Me	H	H	O	180 (160–230)	9 (7–14)	—	—	—	20	27 (20–45)	—	—
V	Me	H	H	O	176 (140–220)	9 (6–15)	8 (5–16)	60 (45–80)	0.5	20	26 (20–50)	69	2.5
VII	Me	Cl	H	O	190 (170–215)	26 (19–38)	6 (3–10)	35 (12–95)	1.0	7	30 (20–70)	—	—
VIII	Me	Br	H	O	165 (145–190)	38 (27–51)	8 (4–15)	35 (12–95)	1.5	4	29 (20–80)	—	—
IX	Me	H	HO	O	130 (110–140)	18 (15–23)	10 (6–19)	75 (38–100)	1.0	7	—	—	—
X	Me	H	MeO	O	165 (140–195)	9 (6–15)	10 (4–15)	35 (18–62)	0.8	18	—	—	—
XI	Me	H	H_2N	O	35 (28–44)	3 (2–4)	—	—	—	12	29 (20–65)	—	—
XII	Me	Cl	H_2N	O	30 (25–35)	6 (5–7)	4 (2–8)	4[c] (2–8)	0.13	5	26 (20–60)	22	1.4
XV	*n*-Pr	H	H	O	265 (215–285)	34 (27–39)	3 (2–6)	20 (4–120)	0.5	8	28 (20–80)	—	—
XVI	*iso*-Pr	H	H	O	32 (25–36)	10 (5–17)	—	—	—	3	28 (20–80)	—	—
XIX	*iso*-Pr	H	H	H,OH	300 (280–325)	16 (13–20)	18 (9–36)	—	1.8	19	—	—	—
Procaine·HCl					410 (360–465)	18 (12–20)	25 (11–50)	75 —	—	23	20 (20–45)	1500	0.27
Lidocaine·HCl					200 (170–225)	9 (5–14)	26 —	80 —	2.1	22	22 (20–50)	58	3.4
(−)-Cocaine·HCl					68 (55–86)	3 (1.5–4.5)	9 —	14 —	1.0	23	22 (20–55)	66	1.0

[a] Values in parentheses are 95% confidence limits. [b] At highest concentrations used, III–XIX caused severe tissue damage. [c] Convulsions noted in test animals. [d] At highest concentrations used, III–XIX caused local irritation in most instances. [e] Based on the mouse tail test. [f] Time at which one-half of the test animals were still anesthetized. [g] Values in parentheses represent the times at which anesthesia disappeared in the first and last test animals. [h] See the text for the details of determination. [i] The concentration that theoretically would afford local anesthesia of infinite duration. [j] Therapeutic index calculated on the basis of saturation values.

Saturation Values—The method used to determine $T_{1/2}$ at ED_{50} was also applied to previously determined ED_{30} and ED_{70} dose levels. The reciprocal of $T_{1/2}$ at each dose level was plotted against the log of the respective ED_{30}, ED_{50}, and ED_{70} values. The line was extrapolated to infinite time to obtain a value termed the "saturation value," *i.e.*, the amount of drug that theoretically would yield an infinitely long period of local anesthesia.

Nerve Action Potentials—Circumesophageal connections of crayfish, including the median giant axon, were dissected out in lengths of 2 cm. and mounted in a Lucite nerve chamber containing appropriate bathing solution (24). Nerve impulses were induced by electric shocks applied near one end of the axon by means of a pair of platinum electrodes and were recorded with another pair near the other end. The distance between the two sets of electrodes was 13 mm. The diameter of the platinum wire was 100 μ, and the distance between the electrodes in each pair was 1.5 mm. A vaseline sheath was put between each pair of electrodes. Medial giant axons, having diameters ranging from 120–150 μ, were used. The stimulus was amplified *via* a Tektronix 3A3 at 5-v. intensity, with a frequency of 1/sec. and a duration of 0.2 msec. The action potential was recorded on a Tektronix 561 oscilloscope through a Tektronix 3A3 d.c. amplifier. Experiments were conducted at 22°. The photographs from the oscilloscope were taken with a Grass polygraph camera. The negatives obtained were passed through a projector, and conduction time and amplitudes were measured from the projection. The action potential as a control was recorded, and solutions of the drugs were added in increasing concentration. The effects were recorded at regular intervals.

Blood Pressure—Female New Zealand rabbits, weighing 3–5 kg., were anesthetized by injecting 35–40 mg. /kg. of pentobarbital into the marginal ear vein. Following anesthesia, a cannula was inserted into the trachea, and the animals were administered artificial respiration by pump. The femoral vein was cannulated for drug injection. Arterial pressure was measured from the carotid artery by a Statham P-23A transducer connected to a Gilson polygraph.

Electrocardiograms—Lead II records were obtained on the Gilson polygraph using needle electrodes.

LD_{50}—These values were determined with mice (average weight 25–30 g.), using a minimum of 10 mice at three dose levels. The animals were injected subcutaneously in the nape of the neck. After 24 hr., the number of dead animals was used to calculate the LD_{50} using the method of Litchfield and Wilcoxon (25).

RESULTS

No statistically significant differences in potency, toxicity, or duration of effect are evident between V and the dextrorotatory antipode (III) cited in Table I. With the exception of the *p*-H_2N moiety, the LD_{50} values are relatively insensitive to substituents on the aromatic acyloxy unit. Thus, III–X are about 2–3 times more toxic than procaine, about as toxic as lidocaine, and 2–3 times less toxic than cocaine, while XI (*p*-H_2N) and XII (*o*-Cl-*p*-H_2N) are about 13, 6, and 2 times more toxic than the controls, procaine, lidocaine, and cocaine, respectively. On the other hand, the *p*-H_2N group affects LD_{50} more than ED_{50}. The former is 2 times more active than the latter, 6 times more active than procaine (8 times more active on a molar basis), 3 times more active than lidocaine, and equally as active as cocaine.

While the ED_{50} values for the mouse tail assay are sensitive to other aromatic substituents, the ED_{50} values are less sensitive in the other three assays employed. Comparison of the ED_{50} values for one assay with those of the other assays and/or comparison of the derived therapeutic indexes reveal a lack of rank-order coherence. Based upon therapeutic indexes calculated from mouse tail ED_{50}

Table II—Effect of Compounds on Amplitude and Conduction Velocity of the Stimulated Crayfish Nerve Fiber

Drug·HCl	Concentration, m*M*	Change in Amplitude[a], %	Change in Conduction Velocity[a], %	Minutes[b]	Recovery Time, min.
Procaine	10.00	−63	−27	13	5
	50.00	−100	−100	1	8
Lidocaine	10.00	−6	−7	4	1
	50.00	−100	−100	5	2
V	0.50	−6	−4	7.5	2
	1.00	−100	−100	8	2
XII	2.00	−77	−50	20	12
	10.00	−100	−100	4	—[c]

[a] Maximum change from initial values. [b] Time required to produce maximum percent change from initial values. [c] Irreversible block, *i.e.*, no return after washout.

values, there is little essential difference in therapeutic potential between the controls and III, V, X, and XIX, while V (rabbit cornea) and XV (guinea pig wheal, guinea pig sciatic nerve, and rabbit cornea) are inordinately more efficacious than the controls in the tests cited. On the basis of therapeutic indexes calculated with saturation values, V ranks only slightly below lidocaine but 2.5 and 10 times higher than cocaine and procaine, respectively.

As reflected by $T_{1/2}$ values, all compounds so evaluated produce longer lasting effects (26–30 min.) than the controls (20–22 min). At the highest doses used in the initial mouse tail assay, severe tissue damage is produced by the test compounds but not by the controls. The test compounds but not the controls produce slight injection of the vessels of the palpebral conjunctiva and sclera at the highest concentrations used in the rabbit cornea evaluation. However, all eyes appeared normal after 24 hr. No signs of irritation or tissue damage were observed in any assay at the ED_{50} dose. The slopes of dose–response curves are substantially the same for test compounds and controls.

Electrophysiological studies (Table II) show that V produces maximal reversible responses on conduction velocity and amplitude in stimulated crayfish median giant axon preparations at concentrations 50-fold below those required for procaine and lidocaine. While XII and procaine produce similar, reversible, submaximal responses at concentrations of 2 and 10 m*M*, respectively, at 10 m*M* the maximal effects on amplitude and conduction velocity afforded by XII are irreversible, suggesting profound cellular damage or chemical derangement.

In contrast to procaine, which produces no significant vasodepressor response in rabbits at a dose of 2.0 mg./kg., Compound XII, at 1.0 mg./kg., moderately decreases (35%) mean arterial blood pressure for about 4 min. (*cf.*, Table III). The vasodepressor effect is associated with a slight bradycardia and significant alteration in the character of the ECG as reflected by changes in P, QRS, and T waves. At 2 mg./kg., XII caused immediate death in 2 of 3 animals; the remaining rabbit displayed marked lowering of blood pressure (60–70%) associated with extreme bradycardia and marked changes in P, QRS, and T waves.

DISCUSSION AND CONCLUSIONS

Until the recent report of Stubbins *et al.* (26) established the existence of a local anesthetic receptor, support for such a hypothesis, based upon the small differences in diastereoisomeric and antipodal potency and toxicity ratios of amino ester local anesthetics, was tenuous, since small differences in biological effects could result from stereoselective metabolism, adsorption, excretion, transport, *etc.*, rather than from stereoselective receptor binding. The mono-β-chloroethyl analog of lidocaine acts as an alkylating agent, producing lasting local anesthetic action which cannot be washed out. When administered with lidocaine to protect competitively the reaction sites, normal local anesthetic response and washout are observed (26).

Since phospholipids contain the requisite nucleophile, a phosphodiesterate anion, as well as ester dipoles, hydroxyl groups, and nonpolar chains in the esteratic segment, which may interact with both the nonpolar and ester moieties of local anesthetic amino ester cations (27), and since phospholipids have been implicated in local anesthetic response (27–29), it is attractive to think of these, alone or in association with protein, as the "receptor." The facts that phospholipids are optically asymmetric (30) and that the asymmetric esteratic segments are chemically, structurally, and conformationally heterogeneous and relatively remote from the anionic center suggest a possible explanation for the characteristically poor stereochemical discrimination. The combination of phospholipid anion with local anesthetic amino ester cations, reenforced by dipole, hydrogen, van der Waals, and hydrophobic bands, can reasonably be expected to release bound inorganic cations and, in altering phospholipid conformation, effectively bury the anions. This suggests that electrochemical stimulation also effects changes in the membrane conformation of phospholipids and/or lipoprotein, resulting not in the release of normally bound Na cation but in its rapid structural transfer into and across the membrane.

In deciding to investigate the 1-alkyl-3-benzoyl-3-acyloxypiperidine prototype, it was reasoned that the several biological responses of local anesthetics were mediated in chemically different environments by receptors differing in their conformational and/or chemical characteristics. Thus, a change in the conformational population of a drug brought about by steric and/or electrostatic restraints in a molecule more rigid than contemporary local anesthetic amino esters was expected to affect potency and, at the same time, affect selectivity by taking advantage of the conformational adaptabilities of prototype and receptor and/or the topographical and environmental differences of the receptors or drug-bound receptors. Test system dependent variations in antipodal and diastereoisomeric potency ratios, like the observed variations in the rank-order potency of these piperidines from one assay system to the next, reflect subtle differences in permeability, clearance, metabolism, and/or receptor structure, even in the various test tissues. This prototype (I), in providing a higher population of the 4-atom aminoethoxy *gauche*-conformation than is possible with analogs such as procaine, presented a potential opportunity to observe the biological effects of conformational rigidity and homogeneity in local anesthetics.

The data reveal that this prototype produces local anesthesia by characteristic, reversible changes in the nerve membrane. The slopes of the dose–response curves of the test compounds and controls suggest a profound similarity in their mode of action (*27*). In addition, the prototype exhibits the classical side effects, myocardial depression and stimulation of the cerebral cortex as evidenced by convulsions (31, 32), although it does not exhibit antipodal partition of local anesthetic activity (*cf.*, III and V, Table I) in the mouse tail assay. Generally, the effects on potency and toxicity of substituents in the piperidine series do not differ markedly from those observed in the procaine series. What small differences do exist may be attributable to the effect of the benzoyl moiety on the stereochemistry, distribution, and fate of the piperidines.

The empirical formula used as a potential measure of the energy of binding to a receptor is virtually identical for both procaine ($C_{13}H_{21}ClN_2O_2$) and desbenzoyl-XI ($C_{13}H_{19}ClN_2O_2$). The minor role played by the benzoyl unit in determining the local anesthetic potency or energy of binding to the local anesthetic receptor may be reflected in the small difference in potency between XVI and XIX. The 6-carbon diethylaminoethyl unit in procaine and the *N*-methylpiperidine unit in these prototypes apparently subserve the same function in spite of their slight structural difference, since higher alkylation of the piperidine system does not inordinately affect local anesthetic potency (*cf.*, V, XV, and XVI). The generally higher $T_{1/2}$ values for the piperidine analogs compared to the controls may

Table III—Effect of Compound XII on Mean Arterial Blood Pressure of the Rabbit

Dose, mg./kg.	Mean Pressure, mm. Hg Before	Mean Pressure, mm. Hg After	Average Change, mm. Hg	Duration, min.	Heart Rate, beats/min.
0.25	115	115	0	—	215
0.50	120	115	−5	—	215
1.00	105	75	−35	4.0	185
2.00[a]	105	35	−70	8.3	95

[a] Two of three test animals died at this dose level.

be rationalized on the basis of the thermodynamically preferred axial orientation of the smaller of the 3-substituents, the ester group (33), and/or on the basis of the fact that the piperidine analogs are esters of stereochemically more hindered tertiary carbinols. Both structural features tend to depress hydrolysis rates and thus maintain higher concentrations of drug in the biophase. Alternately or additionally, higher $T_{1/2}$ values may reflect greater mechanical affinity for lipid or ionic entities or lower diffusion rates in or about the biophase.

The fact that XI is 8 times more potent than procaine on a molar basis in the mouse tail assay suggests that the *gauche*-conformation, with respect to the 4-atom aminoethoxy moiety in these drugs, has some positive biological significance. The high potency of V and XII relative to procaine in electrophysiological studies on isolated median giant axon of crayfish (Table II) reinforces this argument. Significantly, Sax *et al.* (34) came to the same conclusion. Using di-*p*-nitrophenylphosphoric acid as a model of a phospholipid, they subjected the procaine salt to X-ray crystallographic analysis and found the cation to exist in the *gauche*-conformation about the 4-atom aminoethoxy group. While the solid with its conformational homogeneity may be aphysiological, the established conformations and interactions of the ions may well be biologically relevant. The ester is in the thermodynamically more stable (33) *anti*-conformation with the unshared electron pair and the negative end of the ether oxygen dipole of the ester directed toward the cationic center, while the carbonyl oxygen is flanked by the 2-hydrogens of the carbonyl carbon (*cf.*, II). Structures I and II detail the striking similarity in chemical and stereochemical superimposability patterns of these anesthesiophores.

If the benzoyl group has little or no effect on toxicity also, the 16-fold difference in molar potency between procaine and XI suggests that the *gauche*-conformation has greater biological significance for CNS and cardiac receptors than for peripheral local anesthetic receptors. In effect, the *gauche*-conformation appears to contribute significantly to local anesthetic, cardiac, and CNS activity and acts as a stereochemical determinant, discriminating between local anesthetic receptor on the one hand and in favor of CNS and/or cardiac receptors on the other hand. Amide isosterism in the piperidine series and conformational restriction of the benzoate moiety with respect to the 4-atom aminoethoxy unit in this and in new prototypes are features now under investigation (35).

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Kinetics of Flocculation and/or Coalescence of Dilute Oil-in-Water Emulsions

D. F. BERNSTEIN*, W. I. HIGUCHI, and N. F. H. HO

Abstract □ The present study was part of a long-range attempt to describe quantitatively the coalescence or flocculation behavior of emulsions. Several baseline experiments of emulsion coalescence kinetics were conducted with stress placed on the elimination of possible artifact effects. Dibutyl phthalate, dioctyl phthalate, ditolyl, and "Unity Oil" were chosen as the oil phases in this investigation. Dilute emulsions of the oils in varying concentrations of sodium lauryl sulfate solutions were prepared ultrasonically, and the changes in the particle number and size distribution with time were studied in electrolyte solutions. The data obtained were treated by plotting the reciprocal of the cumulative number of particles greater than a specified size *versus* time. A theoretical analysis involving the consideration of finite emulsion droplet-size distributions showed that this method of data treatment is valid for the experimental systems in this study. For rapid reaction systems, the slopes of these Smoluchowski-type plots were directly related to the Smoluchowski rate constant. Rapid reaction conditions were found when the surfactant concentration was sufficiently low or when the electrolyte concentration was sufficiently high. The possible influences of wall effects, oil volatility, oil solubility, creaming (or sedimentation), shear, and mixing effects upon the rate constants were investigated. In the cases of ditolyl and the phthalates, it was found that these influences were negligible or could be eliminated by modifying the techniques involved in the procedure. In studies over a 10-fold range of oil concentration, 100-fold range of surfactant concentration, and a 6-fold range of electrolyte concentration, a maximum rate constant for coalescence of 4.0×10^{-12} cm.^{3}/sec. ($\pm 5\%$) was observed. Interestingly, this maximum rate constant is about 70% of the theoretical Smoluchowski rate constant.

Keyphrases □ Emulsions, oil/water—flocculation, coalescence kinetics □ Flocculation, coalescence behavior—diluted o/w emulsions □ Kinetics—o/w emulsion flocculation, coalescence □ Particle-size analysis—emulsions □ Aging effect, emulsions—particle number, particle-size distribution

The kinetics of coagulation (flocculation or coalescence) is one of the more important problems in the theory of disperse systems. The theory, first proposed by Smoluchowski (1), modified by Fuchs (2), and extended by Verwey, Overbeek, Derjaguin, and Landau (2), has never been completely developed analytically due to the complexities of the mathematical models and to the difficulty in handling the nonlinear integro-differential equation necessary for the analytical treatment.

Difficulties in designing suitable experimental verification of these theories have also been encountered. Little quantitative work has been reported in the literature, and the semiquantitative studies have involved little more than checks of the Schultze–Hardy rule or the effect of surfactant on the particle-size distribution of disperse systems (3, 4). Most work has been performed on solid sols or latex suspensions, since they are relatively monodisperse; few studies have involved emulsions.

Van den Tempel's (5) investigation of the stability of emulsions to the action of electrolyte correlated the rule of Schultze and Hardy, according to which the concentration of a cation necessary to coagulate a negatively charged sol is determined by the charge on the cation.

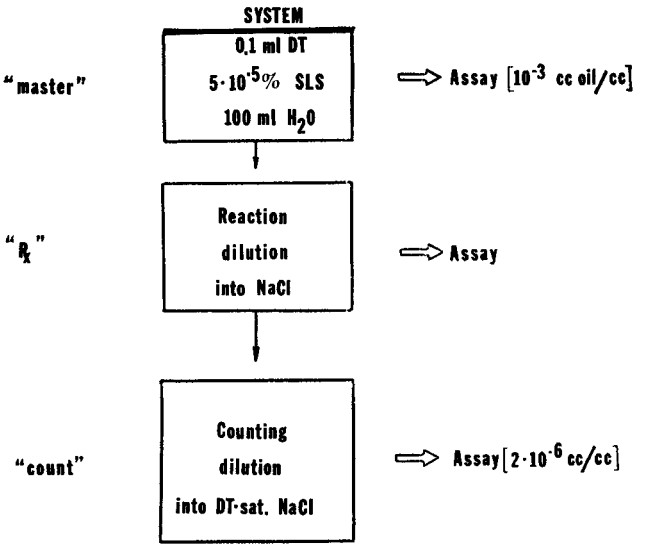

Figure 1—*Schematic representation of dilution steps.*

His system was not monodisperse, and the particles were counted microscopically.

The Coulter counter has been extensively used to characterize colloidal systems. Wachtel and LaMer (6) used the Coulter counter to size o/w emulsions, while Higuchi *et al.* (7, 8) first investigated parameters of emulsion aggregation by differentiating the relative amounts of aggregates. These studies were followed by Lemberger and Mourad (9) and Swift and Friedlander (10), the latter concentrating on the application of similarity or self-preserving functions to the Smoluchowski fundamental equation for aggregation. Rowe (4) and Mima and Kitamori (11) demonstrated the utility of the change in particle-size distribution as a parameter of emulsion stability. Mima and Kitamori (11) found that flocculation of oil droplets stabilized with acacia was dependent on the concentration of the oil and salt and on the size distribution of the primary (original) particles.

Matthews and Rhodes (12, 13) and Higuchi *et al.* (14) demonstrated the use of the Coulter counter in investigating the coagulation of monodisperse latexes. Matthews and Rhodes (12, 13) found coagulation times (or half-lives) to be about twice what the Smoluchowski theory predicted. Thus, the rate constants were also half of the Smoluchowski maximum of 5.6×10^{-12} cm.^{3}/sec. Johnson *et al.* (15), using polyvinyltoluene latex, found rate constants of 4.0×10^{-12} cm.^{3}/sec. for systems at 17°. Ho and Higuchi (16) found a 4% increase in the rate of aggregation of denatured ovalbumin due to the heterogeneity of their system. Their value of 4.148 for the self-preserving A_1 heterogeneity factor compared favorably to Smoluchowski's value of 4.0 for a monodisperse system.

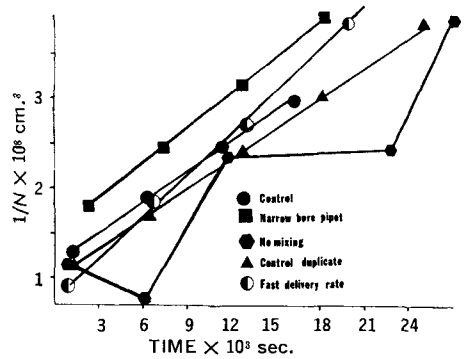

Figure 2—*Effect of artifacts in the dilution steps on the* 1/N versus *time plot.*

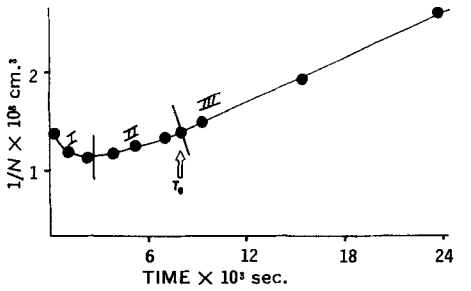

Figure 3—1/N versus *time plot indicating the growth of small particles into the Coulter counter range.*

THEORY

Smoluchowski developed a basic theory for aggregation of spherical particles in a colloidal suspension (1, 2, 17). His fundamental equation for the flocculation of a system controlled by Brownian diffusion is

$$\frac{dN_\infty}{dt} = -KN_\infty^2 \qquad \text{(Eq. 1)}$$

where N_∞ = the number of particles of all sizes, K = bimolecular rate constant, t = time, and

$$K = \frac{4\pi DR}{W} \qquad \text{(Eq. 2)}$$

and the Stokes–Einstein translational diffusion coefficient

$$D = \frac{kT}{6\pi\mu a} \qquad \text{(Eq. 3)}$$

where R = distance between the centers of the two particles, W = stability factor (= 1.0 for irreversible collision, >1.0 when a potential barrier exists), k = Boltzmann constant, T = Kelvin temperature, μ = viscosity, and a = radius of the particle.

At collision, $R = 2a$ (the particles are assumed to be spherical and conjoin into spheres). Thus the theoretical maximum (W = 1.0) rate constant at 30° in water is

$$K = \frac{4kT}{3\mu W} = 5.62 \times 10^{-12} \text{ cm.}^3\text{/sec.} \qquad \text{(Eq. 4)}$$

Integration of Eq. 1, where N_o is the number of original particles of all sizes,

$$\int_{N_o}^{N} \frac{dN}{N^2} = -K\int_0^t dt \qquad \text{(Eq. 5)}$$

yields Eq. 6:

$$\frac{1}{N} - \frac{1}{N_o} = Kt \qquad \text{(Eq. 6)}$$

Thus, plots of reciprocal numbers of particles *versus* time are linear with slope K and intercept $1/N_o$.

The half-life, τ, is

$$\tau = \left(\frac{4kT}{3\mu}\right)^{-1} \cdot \frac{1}{N_o} \qquad \text{(Eq. 7)}$$

Using Eqs. 4 and 6,

$$\frac{1}{N} - \frac{1}{N_o} = \frac{4kT}{3\mu}t \qquad \text{(Eq. 8)}$$

and Eq. 7:

$$N_o = \frac{3\mu}{4kT\tau} \qquad \text{(Eq. 9)}$$

and multiplying Eq. 8 by N_o, the relationship

$$\frac{N_o}{N} - 1 = \frac{4kTN_o}{3\mu}t \qquad \text{(Eq. 10)}$$

is transformed to

$$\frac{N_o}{N} = 1 + \frac{t}{\tau} \qquad \text{(Eq. 11)}$$

Data in this form are said to be normalized. The normalized graph of N_o/N *versus* time/τ allows all systems to be plotted on a common graph. The maximum theoretical system has both slope and intercept of 1.0.

EXPERIMENTAL

Materials—The oil, ditolyl[1], was redistilled under reduced pressure and had a measured density of 0.993 g./ml. at 20°, a n_D^{20} of 1.5940, and a UV maximum at 250 nm. Another oil phase, that of mixtures of dioctyl phthalate[2] and dibutyl phthalate[3], was investigated once the preliminary studies were completed.

The sodium lauryl sulfate surfactant[4] was repurified by recrystallization from ethanol and ethanol–water mixtures. Double-distilled water was used exclusively. Sodium chloride electrolytes were either 5, 0.9, or 0.8% sodium chloride USP solutions or were prepared by dilution of these. Magnesium sulfate was anhydrous analytical reagent grade material. All experiments were performed at room temperature (16–26°).

Preparation of Master Emulsion—The master emulsion was prepared by ultrasonification with the Biosonik II[5], equipped with a 20 kHz. piezoelectric probe. Oil, 0.1 ml., was added to 99.9 ml. of a 5×10^{-5}% solution of sodium lauryl sulfate in water and mechanically shaken prior to irradiation to disperse the larger globules. Emulsification is produced more rapidly and with far more efficiency when the oil and aqueous phase are mechanically agitated so that a gross mixture is first produced. The solution was then subjected to 20 kHz. for 15 sec., followed by 10 kHz. for 10 additional sec. Emulsions so produced were relatively monodisperse with particles in the 0.6–0.8-μ range (diameter).

The reaction dilution was made into a solution containing sufficient quantities of sodium lauryl sulfate and NaCl to give the desired concentration of surfactant and electrolyte. The counting dilution—a further 100-fold dilution to provide a particle concentration suitable for the Coulter counter—was made into saline solution presaturated with the oil under investigation.

Particle-Size Analysis—Particle size was determined using the Coulter counter, model A[6], equipped with a 50-μ aperture tube. A 400-channel RIDL analyzer was used for the distribution determinations. Calibration of the tube was achieved with polyvinyltoluene latexes[7] of 1.857- or 2.051-μ diameter. The manometer tube apparatus was completely shielded by copper screening to eliminate electronic noise.

[1] Supplied by Aldrich Chemical Co. as 3,3'-dimethylbiphenyl, puriss.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Union Carbide Chemical Co., New York, N. Y.
[4] Maprofix 563, Onyx Chemical Co., Wilmington, Del. (95% C-12).
[5] Bronwill Scientific, Rochester, N. Y.
[6] Coulter Industrial Sales, Hialeah, Fla.
[7] Polyvinyltoluene, Dow Chemical Co., Midland, Mich.

Table I—Ditolyl Initial Rate Constants for Several Salt and Surfactant Concentrations

Percent Sodium Lauryl Sulfate in Reaction	Dilution	Rate Constant $K \times 10^{-12}$ cm.³/sec. 0.9% NaCl	4% NaCl	1% $MgSO_4$
0.6×10^{-6}	1/4	4.169		
0.4×10^{-5}	1/12	3.707		
0.5×10^{-5}	1/10	3.899		
0.6×10^{-5}	1/8	3.787		
1.0×10^{-5}	1/5	3.848		
1.0×10^{-5}	1/5	3.935		
1.0×10^{-5}	1/5	4.077		
1.2×10^{-5}	1/4	4.262		
1.2×10^{-5}	1/4	4.287		
2.5×10^{-5}	1/2	4.318		
9.0×10^{-5}	1/5	4.016		
1.0×10^{-4}	1/5	2.261		
2.5×10^{-4}	1/5	1.315		
1.0×10^{-3}	1/5	0.933	4.187	3.525
1.2×10^{-3}	1/5	0.169	4.154	3.640
1.2×10^{-3}	1/5		4.285	3.740

Table II—Initial Rate Constants as a Function of Oil, Salt, Surfactant, and Temperature

Reaction Vessel Composition: Oil	Sodium Lauryl Sulfate, %	NaCl, %	T	$K \times 10^{-12}$ cm.³/sec. At T	At 25°[a]
Ditolyl	1.0×10^{-5}	0.9	23°	4.077	4.297
	1.2×10^{-5}	0.9	16°	3.395	4.352
	9.0×10^{-5}	0.9	23°	4.016	4.233
	1.0×10^{-4}	0.9	23°	2.261[b]	2.383
	2.5×10^{-4}	0.9	23°	1.315[b]	1.386
	1.0×10^{-3}	0.9	23°	0.933[b]	0.983
	1.2×10^{-3}	0.9	23°	0.169[b]	0.178
Dioctyl phthalate–dibutyl phthalate	1.2×10^{-5}	0.9	18°	3.341	4.042
	1.2×10^{-5}	0.9	16°	3.321	4.257
	1.2×10^{-5}	0.9	16°	2.931	3.757
	9.0×10^{-5}	0.8	27°	2.224	2.112
	3.9×10^{-4}	0.8	27°	0.610	0.579
	8.1×10^{-4}	0.8	16°	0.212	0.272
	3.8×10^{-3}	0.8	27°	0.126	0.120
	8.0×10^{-3}	0.8	16°	0.056	0.072
Hexadecane	1.2×10^{-5}	0.8	16°	2.845	3.647
	1.2×10^{-5}	0.9	16°	3.457	4.431
Unity Oil	1.2×10^{-5}	0.8	17°	2.873	3.576

[a] Temperature and viscosity corrections applied to obtain K at 25°. [b] 4% NaCl gave $K = 4.219 (\pm 1.5\%) \times 10^{-12}$ cm.³/sec.

Apparatus—All glassware used in these experiments was washed thoroughly and rinsed twice in tap water and once in distilled water before use. Pipets were subjected to four wash–rinse cycles before use.

Preliminary Studies—The first system was a slight modification of van den Tempel's (5) mixture of 57 volumes of chlorobenzene and 43 volumes of paraffin oil, resulting in a density of 1.006 g./ml. By using a ratio of 56 volumes of chlorobenzene and 44 volumes of mineral oil, a mixture was obtained having a density of 1.0027 g./ml. However, the chlorobenzene had some solubility in the aqueous phase and was partially volatilized by the ultrasonic irradiation. Thus, upon loss of the aromatic component from the system, the oil phase had a density of less than unity and quickly creamed. A search of the literature to find a nonpolar, water-insoluble, high boiling point liquid having a density close to unity indicated that ditolyl (3,3′-dimethylbiphenyl) would be suitable.

Early experimental results with ditolyl demonstrated a rapid loss of particles from the counter measuring system[8]. To determine the reason for this phenomenon, a quantitative assay for ditolyl was introduced. This involved extraction of the oil from the emulsion with a small amount of ether, followed by three cyclohexane extractions. It was demonstrated with both control solutions and emulsions that this technique gave quantitative recovery of oil (98.5–101.5%).

The possibility of the following artifacts contributing to the rapid disappearance of particles was examined: (*a*) unimolecular wall reaction between the oil and vessel wall; (*b*) volatility of the oil in dilute emulsions; (*c*) creaming of the oil phase; and (*d*) solubility of the oil in electrolyte solution.

Assays of aliquots drawn from the bulk emulsion as well as an assay of the residual volume of emulsion were quantitative. Thus the oil was not undergoing a wall reaction; if it were, the bulk assay would have been less than 100% recovery and the remainder (including the wall washing liquor) greater than 100%.

Assay experiments were performed with some tightly sealed beakers and some that were left open to the atmosphere. All assays were quantitative, indicating that (*b*) was not influencing the disappearance of the oil. Assays were conducted on a reaction system over a period of time to test the effect of creaming. These assays were quantitative over 7–8 hr. (about 3.5–4 τ in the system being investigated), but decreased in percent recovery after that time. However, since the counting procedure is of the order of a minute, the immediate loss of particles could not be explained in this way.

To investigate the solubility of ditolyl in saline solution, a saturated solution was first assayed (both by direct assay and by extraction assay) and the solubility was determined to be 1.5×10^{-6} ml. oil/ml. saline. Since the concentration of ditolyl in the dilute counting stage was 2×10^{-6} ml. oil/ml. saline, it was evident that some 75% of the oil was solubilized. Emulsions diluted into saline rapidly decreased (about 40%) in counts over 1 min., while an identical dilution into saline presaturated with the oil showed no decrease in counts over 10 min. All subsequent experiments were conducted using such a presaturated vehicle for the counting dilution. This solubility phenomenon was shown not to be important in the "reaction" dilution, since the emulsion is 100 times as concentrated in oil.

Dilution Technique—The system investigated most closely was a "master" emulsion composed of 0.1 ml. ditolyl, 5×10^{-5}% sodium lauryl sulfate, and water to make 100 ml. This master emulsion was diluted into the "reaction" vessel and then diluted into the "counting" vessel such that the total dilution was 500-fold. By diluting the master from 2 to 20 times (denoted $1/2$–$1/20$ dilution), a 10-fold oil concentration range was investigated. With the $1/2$ dilution, the counting dilution was $1/250$; mixing the 1-ml. aliquot with this volume of saline changed the particle-size distribution. Using a $1/20$ reaction dilution (and $1/25$ counting dilution) that had a half-life of 10 hr., the creaming effect occurred after 7.5 hr, and quantitative data could not be obtained from so short an experiment. Thus, a $1/5$ dilution was chosen as optimum. This system is represented schematically in Fig. 1.

The data obtained (time and the number of particles greater than a specified size) were treated by the typical Smoluchowski rate plot of $1/N$ *versus* time. While linear curves resulted, a sufficient amount of scatter generated a study of the actual pipeting and dilution steps involved in the procedure.

The technique of the dilution steps is as follows: the reaction dilution was effected by using a 5-ml. wide bore, blowout-type serological pipet (Kimax No. 37034B). Two such 5-ml. aliquots were taken from the master and diluted into 40 ml. of a solution containing the desired amounts of surfactant and electrolyte. The counting dilution was performed by quantitative transfer of 1 ml. of the reaction emulsion into 99 ml. of the presaturated saline. The saline was stirred before the transfer to ensure adequate mixing of the aliquot with the saline without unnecessary shear. The aliquot was introduced just below the surface of the solution and allowed to drain into the counting vessel at a rate of 1 ml. in 10–11 sec. No further mixing was performed. The particles were then immediately counted.

Using this technique, the number of particles in four[9] successive determinations did not differ from the average of the four by more than 3%. Using a 1-ml. "to deliver" pipet (small bore) for the transfer operation, a decrease in the number of counts (as compared to the described procedure) was attributed to shear-induced coalescence of the particles passing the narrow bore. Although the deviations from the average count were 4–7%, rate constants were similar to those obtained from the described procedure.

[8] About three orders of magnitude larger than Smoluchowski coalescence.

[9] Due to electronic polarity effects, an even number of readings must be taken.

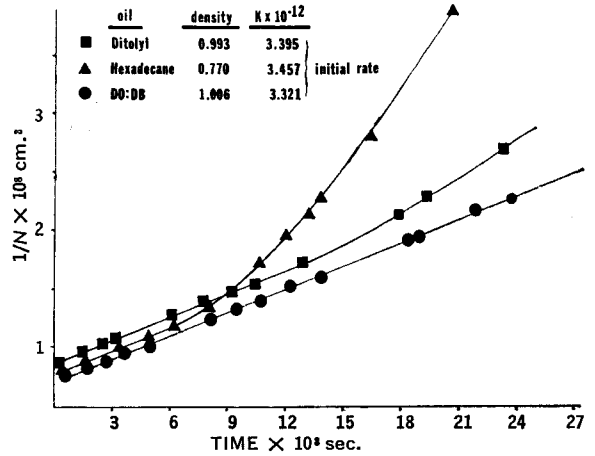

Figure 4—*1*/N versus *time plot of several oils of differing densities.*

When prestirring was not performed, a large amount of scatter was noticed in the 1/*N versus* time plot. When the aliquot was allowed to flow out of the wide-bore pipet simply by gravity (1 ml. in 1 sec.), the data obtained yielded a shear-accelerated rate constant. The effect of these artifacts on the treatment of data is shown in Fig. 2. The technique first presented was used in all experiments because of its accuracy and reproducibility.

This double-dilution technique was chosen over the *in situ* system (*e.g.*, one 500-fold dilution from master to counting vessel) so as not to sacrifice volumetric accuracy. To ensure that the two-stage dilution did not change the coalescence pattern at each step, one 500-fold dilution was made simultaneously with the same dilution in two steps. The particle counts in each system were within 7–10% of each other. This variation was also noted in the 500-fold dilution of dialyzed latex particles, and the difference in both cases is attributed to problems associated with mixing a 1-ml. aliquot into the large volume (500 ml.) of saline.

In addition, the second dilution in the two-step procedure was made into saline and into saline–surfactant solutions to check the possibility of desorption at this step. Absolute counts and rate constants determined from each method differed by less than 1%.

Aging of Emulsion—After the master emulsion was ultrasonified, it was allowed to stand undisturbed in its concentrated form for 5 min ; the reaction dilution was then made. Counting determinations at intervals of approximately 0.1 τ demonstrated an initial increase in the number of particles followed by the decrease one normally expects from coalescence (Fig. 3). In Region I of Fig. 3 (early times after dilution), the increase in the number of particles is due to the great number of small particles (small in the sense that they were below the minimum size seen by the Coulter counter) "growing" into the range of the instrument. Region II represents that in which the coalescence of the larger particles is becoming more important. Finally, in Region III, most of the small particles have coalesced to become large enough to be counted and thus represents the situation in which most of the mass of oil is accounted for by the Coulter counter.

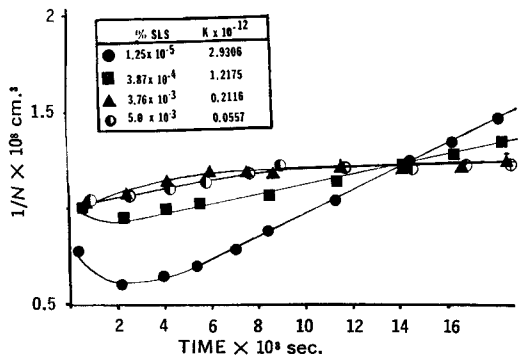

Figure 5—*Effect of surfactant concentration on the* 1/N versus *time plot.*

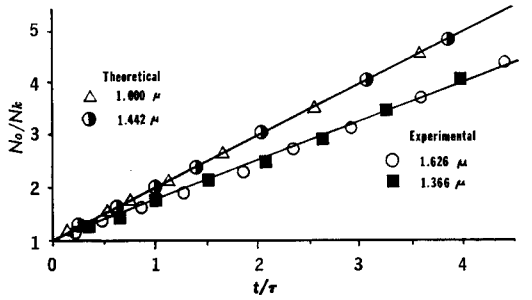

Figure 6—*Comparison of theoretical and experimental data on a normalized plot.*

Coulter Counter Thresholds—Adjustable threshold settings (voltage screenout) in the electronic counting circuit permit a count to be determined for the total number of particles larger than a given size. Experiments run at threshold settings of 3, 4, and 5 (corresponding to all particles larger than 1.366-, 1.509-, and 1.626-μ diameter, respectively) gave similar rate constants. Since linearity was observed in Region III of the Smoluchowski plot, it was accepted that the majority of particles were being counted in this region and that the small effect due to coalescence of small particles into the Coulter counter range could be neglected. Theoretical analyses involving computer studies demonstrated that this assumption was valid.

RESULTS AND DISCUSSION

Over 40 systems utilizing ditolyl were investigated, each being reproduced at least twice with rate constants within 5% of each other for similar systems. These data are summarized in Table I. In studies over 100-fold surfactant, 6-fold sodium salt, 4-fold magnesium salt, and 10-fold oil concentration ranges, a maximum rate constant of 4.0×10^{-12} cm.³/sec. (±5%) was observed. The rate constants were determined by least-squares regression analyses of the linear portion of the 1/*N versus* time plot (Region III, Fig. 3). The values reported are initial values only and do not reflect the deviations due to creaming. When the reaction vessel was a $^1/_5$ dilution, graphical deviations (increasing upward slope) were noted after 4 τ (about 35,000 sec.). These graphical deviations were accompanied by a corresponding decrease of the percent oil recovered from the quantitative UV extraction assay. Even swamping the system at this point with surfactant (the stabilized emulsion gave a nearly horizontal slope from that point on) did not inhibit the eventual slight upward trend of the 1/*N versus* time plot.

Another series of investigations was directed toward a 2:1 (v/v) mixture of dioctyl and dibutyl phthalates. The resulting density of 1.006 g./ml. was matched to within 0.5% of that of the 0.8% NaCl in the reaction vessel. The creaming effect (detected graphically or by assay) was absent for 7.5–8 τ (about 70,000 sec.)[10]. These experiments were performed at 17 ± 1°, and the decrease in temperature (resulting in an increase in the viscosity) was believed to be responsible for the decrease in the rate constants to values between 2.8 and 3.3 $\times 10^{-12}$ cm.³/sec. These determinations are summarized in Table II.

Figure 4 shows the similarity of initial rate constants independent of the oil employed, even when hexadecane (with a 23% density difference) systems were investigated. The effect of the density of the oil and, consequently, its importance are noted by the upward curvature of the graphs depicting the coalescence behavior of oils whose density differs from that of the saline vehicle employed.

Figure 5 is a typical surfactant study utilizing the dioctyl phthalate–dibutyl phthalate system. Interestingly, the higher surfactant concentrations permitted some coalescence to occur before a stable system (the horizontal portion of the graph) was reached. Particle-size distribution photographs showed almost no change over this stability period. This "limited coalescence" phenomenon is being pursued further.

[10] Even after 8 τ, the loss of particles from the system due to creaming is primarily due to the increased r^2 term and not the density difference in Stoke's equation.

Table III—Computer Input Data

Dielectric constant	80	Time	$20\ \tau$
Hamaker constant	10^{-13} erg	Number of classes	100
Surface potential	6 mv.	Initial	
		singlets	5%
Kappa ($cm.^{-1}$)	$1.26 \times 10^{+6}$	doublets	10%
Viscosity	1 cp	triplets	50%
kT	4.14×10^{-14}	4-mers	25%
Singlet radius	$0.5\ \mu$	5-mers	10%

COMPUTER STUDIES

The IBM 360/67 computer, utilizing a program (18) involving solutions to the set of nonlinear ordinary differential equations, was used to generate a set of theoretical data. These input data are given in Table III.

To check the validity of determining K from the $1/N$ *versus* time plot along with the arbitrary choice of threshold (minimum particle size seen), a normalized plot (Fig. 6) of the computer-generated data was made.

When all the particles were counted, the slope was 1.031 (corresponding to a rate constant of 5.794×10^{-12} $cm.^3/sec.$). A similar graph, excluding the 5% singlet contribution, gave a slope of 1.017 (rate constant: 5.715), while the slope of a graph omitting both singlets and doublets was 1.009 (rate constant: 5.671). Similar results were found experimentally; these data are summarized in Table IV.

The computer-generated rate constants were greater than the Smoluchowski maximum (5.62) due to the heterogeneity of the system. The validity of using the $1/N$ *versus* time plot to determine K was thus established. Also noteworthy was the independence of the rate constant on the minimum size cutoff point.

Studies now in progress include rigorous particle-size distribution determinations, salt–surfactant interplay, electrophoretic mobility studies, and investigations into the region of limited coalescence. By using the analytical tools at hand, experiment and theory can then be compared in a wide range of systems.

Table IV—Rate Constant as a Function of Omission of Small Particles

Threshold	Corresponding[a] Particle Diameter, μ	$K \times 10^{-12}$ $cm.^3/sec.$	Percent of Maximum Theoretical Rate
	Experimental		
3	1.366	4.252	75.65
4	1.509	4.169	74.18
5	1.626	4.077	72.54
	Computer Simulation (W = 1.0)		
N_k	1.000	5.794	103.1
N_k *less* singlets	1.260	5.715	101.7
N_k *less* singlets and doublets	1.442	5.670	100.9

[a] Particles having dimensions less than this are not counted.

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Placental Transfer of 2,3,5-Triiodobenzoic Acid in the Rat

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Abstract □ The placental transfer of 2,3,5-triiodobenzoic acid and/or metabolites was studied using ^{14}C-labeled 2,3,5-triiodobenzoic acid. Placental transfer of 2,3,5-triiodobenzoic acid and/or metabolites occurred in both cold-stressed and control rats. The maternal blood of cold-stressed and control rats contained significantly higher concentrations of 2,3,5-triiodobenzoic acid and/or metabolites than the blood of respective fetal rats. However, cold stress did not significantly alter either placental transfer or the level of 2,3,5-triiodobenzoic acid and/or metabolites in the maternal and fetal tissue. Cold stress had no apparent effect upon the plasma level of either 2,3,5-triiodobenzoic acid, 2,5-diiodobenzoic acid, or 3,5-diiodobenzoic acid in maternal or fetal animals. While nonpregnant rats acclimated to cold stress, pregnant rats did not, indicating cold stress and pregnancy may act synergistically.

Keyphrases □ 2,3,5-Triiodobenzoic acid-^{14}C—placental transfer □ Placental transfer, 2,3,5-triiodobenzoic acid-^{14}C—rats □ Fetal, maternal levels—2,3,5-triiodobenzoic acid-^{14}C, metabolites □ Cold-stress effect—urinary excretion, 2,3,5-triiodobenzoic acid-^{14}C □ Scintillometry—analysis

The presence of 2,3,5-triiodobenzoic acid in the environment has created concern regarding its toxicity. Results of various studies (1–3) show that the compound is tolerated in the adult rat, guinea pig, and man. Increased fetal malformations in mice and rats by environmental contaminants (4) strongly suggest that the degree of placental transfer is another parameter for the determination of the potential toxicity of an environmental contaminant.

The present study was conducted to determine the placental transfer, metabolism, and urinary excretion of 2,3,5-triiodobenzoic acid in rats. Cold-stressed as well as normal rats were studied since stress has been shown to alter the metabolism (5), the excretion (6), and the action (7) of various compounds. The influence of stress on placental transfer seems pertinent since many environmental stimuli have been found to cause a stressed condition in man and animals.

EXPERIMENTAL

Radiochemical—Carboxyl-^{14}C-labeled 2,3,5-triiodobenzoic acid (2,3,5-triiodobenzoic acid*) was available from the work reported by Spitznagle *et al.* (8) and was purified by thick-layer chromatography using the procedure of Jarboe *et al.* (9). Chemical purity was determined by spotting separately both 2,3,5-triiodobenzoic acid* and reference standard 2,3,5-triiodobenzoic acid[1] on thin-layer plates of purified silica gel[2]. Separate plates were developed in one of two solvent systems: petroleum ether–propionic acid (10:1 v/v) or propionic acid–methanol–benzene (1:2:10 v/v). UV light and a 0.1% bromcresol green solution were used to detect spots. No chemical impurities were found.

Radiochemical purity was determined by spotting 2,3,5-triiodobenzoic acid* on thin-layer plates of purified silica gel[3] and developing the plates in the solvent systems, already described. Radioactive spots were located with X-ray film[4]. The spot containing 2,3,5-triiodobenzoic acid* was scraped into a counting vial, and the radioactivity was determined. The radioactivity of the remaining sorbent on the plate was determined similarly. Of the total radioactivity recovered, 97.9 ± 0.3% was present as 2,3,5-triiodobenzoic acid*.

Radioactivity Measurements—Tissue samples (30–75 mg.) were placed in tared counting vials and weighed. Then 1 ml. of a tissue-digesting solution[5] was added to each vial, and the vials were heated for 2 hr. at 60° with occasional agitation. One-half milliliter of 30% hydrogen peroxide was added to decolorize the samples. The samples were heated at 60° for an additional hour and cooled. Glacial acetic acid, 0.5 ml., was added to each sample to reduce protein phosphorescence.

Urine samples were prepared by adding 50–100-μl. aliquots of urine to the counting vials.

Fifteen milliliters of scintillator[6] was added to each urine or tissue sample. The samples were counted in a liquid scintillation spectrometer[7] using the internal standard method (11) to determine the absolute disintegration rates. The maximum counting error was 1.2%.

Animal Care and Mating—Sprague-Dawley[8] female rats, weighing 200–250 g., were housed in individual screen-bottom hanging cages and allowed free access to food[9] and water. The animals were maintained in a lighted environment from 6 a.m. to 8 p.m. and were handled for 5–10 min. daily during pregnancy to reduce stress (12). They were mated during the period from October to April using the procedure described by Mork *et al.* (13). They were assigned to treatment groups by using a table of random numbers.

Placental Transfer of 2,3,5-Triiodobenzoic Acid*—Forty pregnant rats, weighing 290–350 g., were injected intraperitoneally with 15 μc. (1.77 mg.) of 2,3,5-triiodobenzoic acid* per 300 g. of body weight on Day 19 of gestation. Four days prior to injection, 20 pregnant rats were placed in individual metabolism cages in an environmental chamber[10] maintained at 4° and illuminated for 14 hr. daily. The remaining animals were placed in individual metabolism cages at room temperature with the same light conditions.

Five animals from each treatment group were sacrificed 2 hr. after injection. Additional groups of five animals from each treatment group were sacrificed at 4, 6, and 8 hr. after injection. Urine was collected at 2, 4, 6, and 8 hr. after injection.

Maternal animals were sacrificed with a small guillotine and exsanguinated. The blood was collected in a heparinized beaker. Samples were removed for measurement of radioactivity of whole blood. The remainder was transferred to a heparinized 15-ml. test tube and centrifuged. The plasma was decanted and stored frozen for metabolite studies. The maternal adrenals were removed, trimmed to remove connective tissue, weighed, frozen in liquid nitrogen, and stored frozen until assayed for ascorbic acid by the procedure of Maickel (14). Samples of maternal liver were obtained.

The fetuses were removed from the uterus of each maternal animal. Samples of whole fetal blood were collected in heparinized capillary tubes[11], centrifuged, and stored frozen for metabolite studies. Samples of fetal whole blood, fetal liver, and entire fetus were also obtained for each maternal animal. To obtain a homogeneous sample of the entire fetus, two fetuses per maternal rat were frozen in liquid nitrogen, placed between sheets of plastic, and pulverized. A small precooled spatula was used to mix the fragments thoroughly. Different fetuses were used for the different tissue samples.

[1] International Minerals and Chemical Corp., Skokie, Ill.
[2] A *mixture of Adsorbosil-1* and Adsorbosil P-1 (2:1), Applied Science Laboratories, State College, Pa.
[3] Adsorbosil-1.
[4] No-Screen X-Ray Film, Eastman Kodak Co., Rochester, N. Y.
[5] A methanolic solution of hyamine hydroxide prepared according to Bruno and Christian (10) from hyamine 10-X crystals, Rohm & Haas, Philadelphia, Pa.
[6] Contained 1 part *p*-xylene, 3 parts *p*-dioxane, 3 parts 2-ethoxyethanol, 8% naphthalene, and 1.0% 2,5-diphenyloxazole.
[7] Tri-Carb, Packard Instrument Co., Inc., Downers Grove, Ill.
[8] Sprague-Dawley, Inc., Madison, Wis.
[9] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.
[10] Model 683, Hotpack Corp., Philadelphia, Pa.
[11] The method of Mork *et al.* (13) was used.

Table I—Concentration of 2,3,5-Triiodobenzoic Acid[a] in Various Rat Tissues

Treatment[b]	Hours[c] 2	4	6	8
		Maternal Blood		
Cold-stressed	21.76 ± 3.90	15.90 ± 3.40	21.05 ± 4.08	15.93 ± 5.64
Control	13.44 ± 6.06	15.52 ± 5.18	20.23 ± 3.31	18.73 ± 2.65
		Fetal Blood		
Cold-stressed	3.80 ± 1.91	4.40 ± 0.98	5.01 ± 0.96	8.03 ± 4.04
Control	3.42 ± 0.51	3.81 ± 1.32	6.49 ± 1.07	5.61 ± 0.42
		Maternal Liver		
Cold-stressed	6.71 ± 2.09	5.31 ± 0.99	6.69 ± 1.26	5.14 ± 1.55
Control	5.66 ± 1.36	5.45 ± 1.06	6.15 ± 1.30	5.82 ± 0.48
		Fetal Liver		
Cold-stressed	2.28 ± 0.39	3.12 ± 0.56	4.26 ± 0.59	4.75 ± 2.19
Control	2.44 ± 0.90	2.92 ± 0.89	4.85 ± 1.34	4.31 ± 0.56
		Fetal Tissue[d]		
Cold-stressed	1.57 ± 0.52	2.50 ± 0.67	3.76 ± 0.98	4.08 ± 1.50
Control	1.29 ± 0.23	2.58 ± 0.59	3.89 ± 0.64	4.74 ± 0.67

[a] Expressed as micrograms of 2,3,5-triiodobenzoic acid and/or metabolites per gram of tissue ± standard deviation. [b] Five maternal animals per treatment group per time interval. [c] Time elapsed between injection and sacrifice. [d] Homogeneous sample of entire fetus.

Plasma Metabolites—Maternal and fetal blood plasma was analyzed to determine the relative amounts of 2,3,5-triiodobenzoic acid and its metabolites that transferred to the fetus. The plasma was extracted with ethanol, which was found in preliminary studies to remove greater than 99% of the 2,3,5-triiodobenzoic acid and its metabolites. Fifteen-microliter aliquots of the ethanol extracts were spotted on TLC plates of purified silica gel[2]. Reference standard 2,3,5-triiodobenzoic acid and the two suspected metabolites, 2,5-diiodobenzoic acid and 3,5-diiodobenzoic acid, were spotted on each plate. The plates were developed in petroleum ether–propionic acid (10:1 v/v). Each spot was located with UV light and was then scraped quantitatively into counting vials containing liquid scintillator. The vials were counted and the results were expressed as the percent of total radioactivity recovered from each sample spotted on the thin-layer plate.

Effect of Cold Stress on Urinary Excretion of 2,3,5-Triiodobenzoic Acid—Twelve nonpregnant female rats were injected intraperitoneally with 5.06 μc. (0.52 mg.) of 2,3,5-triiodobenzoic acid*. Six were placed in metabolism cages and were cold stressed as already described for 4 days previous to the administration of 2,3,5-triiodobenzoic acid* and during the time of urine collection. The other six were kept in metabolism cages at room temperature. Urine from each animal was collected at 3-hr. intervals over a 24-hr. period. Aliquots were prepared for counting and the ^{14}C was determined. The results were expressed as the percent of the ^{14}C dose excreted. After 24 hr., the animals were sacrificed and the adrenals were removed, weighed, frozen, and stored for ascorbic acid determination.

Statistical Analysis—Analysis of variance (15) was run to test for significant differences, at the 0.05 confidence level, in the amount of 2,3,5-triiodobenzoic acid in each tissue sample. Variables tested included treatment, time, and interaction between treatment and time. If no interaction between treatment and time was found, stress and control data were pooled at each time interval and com-

Table II—Relative Amount 2,3,5-Triiodobenzoic Acid and Metabolites in Blood Plasma

Hours[a]	Percent of Total Radioactivity[b] Origin	2,3,5-Triiodobenzoic Acid	2,5-Diiodobenzoic Acid	3,5-Diiodobenzoic Acid	Solvent Front
			Stress Maternal[c]		
2	8.7 ± 4.1	73.8 ± 5.3	12.1 ± 1.3	2.0 ± 0.6	1.2 ± 0.4
4	7.8 ± 3.9	71.8 ± 2.6	16.0 ± 4.2	2.6 ± 1.2	1.5 ± 1.4
6	10.6 ± 4.7	67.7 ± 8.6	21.0 ± 4.4	2.3 ± 0.7	0.5 ± 0.6
8	5.7 ± 0.1	64.6 ± 9.5	26.6 ± 9.3	2.4 ± 1.1	0.5 ± 0.5
			Stress Fetal		
2	7.4 ± 4.7	62.5 ± 10.8	18.0 ± 1.6	6.9 ± 3.5	5.2 ± 3.4
4	5.6 ± 2.5	65.5 ± 2.4	18.6 ± 3.7	7.0 ± 2.9	3.3 ± 1.5
6	5.2 ± 2.6	65.7 ± 7.1	22.3 ± 5.0	4.2 ± 3.5	2.4 ± 3.1
8	8.4 ± 3.9	57.1 ± 9.9	22.1 ± 5.6	6.6 ± 3.1	5.7 ± 3.7
			Control Maternal[d]		
2	15.5 ± 7.4	68.2 ± 8.7	14.4 ± 2.9	1.5 ± 0.4	0.4 ± 0.2
4	15.5 ± 7.5	63.9 ± 2.9	18.2 ± 7.0	1.9 ± 0.6	0.5 ± 0.4
6	11.3 ± 8.0	67.0 ± 8.0	17.6 ± 1.6	2.3 ± 1.2	1.4 ± 1.2
8	9.6 ± 2.6	63.4 ± 9.9	21.9 ± 11.4	1.7 ± 0.1	0.9 ± 0.9
			Control Fetal		
2	4.1 ± 2.9	61.5 ± 13.7	20.1 ± 8.6	7.6 ± 3.4	6.1 ± 4.8
4	10.4 ± 4.1	53.1 ± 8.4	23.7 ± 6.5	7.3 ± 3.6	5.5 ± 2.1
6	3.8 ± 1.9	56.5 ± 16.0	18.0 ± 4.2	7.8 ± 5.2	13.8 ± 10.7
8	8.6 ± 5.8	60.7 ± 8.2	20.9 ± 7.5	4.4 ± 2.3	5.4 ± 4.2

[a] Time elapsed between injection and sacrifice. [b] Percent of total radioactivity recovered from a sample spotted on a thin-layer plate expressed as mean value ± standard deviation. [c] Four maternal animals per time period. [d] Five maternal animals 2 hr. postinjection, six maternal animals 4 hr. postinjection, and four maternal animals each at 6 and 8 hr. postinjection.

Table III—Fetal/Maternal Blood Plasma Ratios of 2,3,5-Triiodobenzoic Acid[a]

Treatment	Hours[b] 2	4	6	8
Cold-stressed	0.85 ± 1.15	0.91 ± 0.04	0.98 ± 0.12	0.87 ± 0.15
Control	0.94 ± 0.14	0.84 ± 0.16	0.84 ± 0.20	0.97 ± 0.20

[a] Ratios calculated from data in Table II. [b] Time elapsed between injection and sacrifice.

pared to pooled data at other time intervals. The Student *t* test (16) was used to determine significant differences at the 0.05 confidence level for urine samples as well as for 2,3,5-triiodobenzoic acid and its metabolites in blood plasma.

RESULTS AND DISCUSSION

Placental Transfer of 2,3,5-Triiodobenzoic Acid—The ^{14}C activity detected in tissue was expressed as 2,3,5-triiodobenzoic acid even though metabolites may have been present. Data in Table I show that placental transfer of 2,3,5-triiodobenzoic acid occurred at each time interval studied. The 2,3,5-triiodobenzoic acid levels in the tissue of cold-stressed maternal and fetal rats in relation to 2,3,5-triiodobenzoic acid in respective control tissue showed no significant differences. Since no interaction between treatment and time was found, stress and control data were pooled at each time interval and compared statistically. Although occasional statistically significant differences were observed, the pooled data did not consistently indicate an influence of time on placental transfer or the level of 2,3,5-triiodobenzoic acid in maternal or fetal tissue.

The maternal blood of cold-stressed and control rats contained significantly higher concentrations of 2,3,5-triiodobenzoic acid than did the blood of respective fetal rats at each time interval. However, cold stress did not significantly affect the levels of 2,3,5-triiodobenzoic acid in either the maternal blood or the fetal blood; it would thus appear that exposure to cold did not affect the placental transfer of 2,3,5-triiodobenzoic acid.

Plasma Metabolites—Actual 2,3,5-triiodobenzoic acid, exclusive of metabolites, was determined in this phase of the investigation. The relative amount of 2,3,5-triiodobenzoic acid in the maternal blood plasma of cold-stressed and control rats (Table II) did not vary significantly over the 2–8-hr. postinjection period. Except for the 4-hr. postinjection interval, the blood plasma level of 2,3,5-triiodobenzoic acid in maternal cold-stressed rats was not significantly different from that present in respective control animals. No significant differences between cold-stressed and control rats were found at any time interval for fetal blood plasma levels of 2,3,5-triidobenzoic acid. No significant differences in ratios (Table III) of 2,3,5-triiodobenzoic acid in fetal blood plasma to maternal blood plasma due to treatment or time within a single treatment were found. Apparently a constant percentage of the 2,3,5-triiodobenzoic acid in maternal blood plasma was present in fetal blood plasma at each time interval. The data suggest that exposure to cold did not affect the placental transfer of 2,3,5-triiodobenzoic acid.

Comparison of the concentration of 2,5-diiodobenzoic acid in the fetal blood plasma with 2,5-diiodobenzoic acid in respective maternal blood plasma showed no significant difference in the 2,5-diiodobenzoic acid level for stressed or control animals, with the exception of the cold-stressed group at the 2-hr. interval. The concentration of 3,5-diiodobenzoic acid was significantly higher in fetal blood plasma than in respective maternal blood plasma for both stressed and control rats at 2 and 4 hr. postinjection and at 8 hr. postinjection for the control animals. The data suggest a selective transport mechanism for 3,5-diiodobenzoic acid which was not affected by cold stress. The results of this phase of the investigation indicate that exposure to cold did not affect the placental transfer of either 2,3,5-triiodobenzoic acid or its metabolites.

Effect of Cold Stress on Urinary Excretion of 2,3,5-Triiodobenzoic Acid—Nonpregnant cold-stressed rats excreted a significantly higher

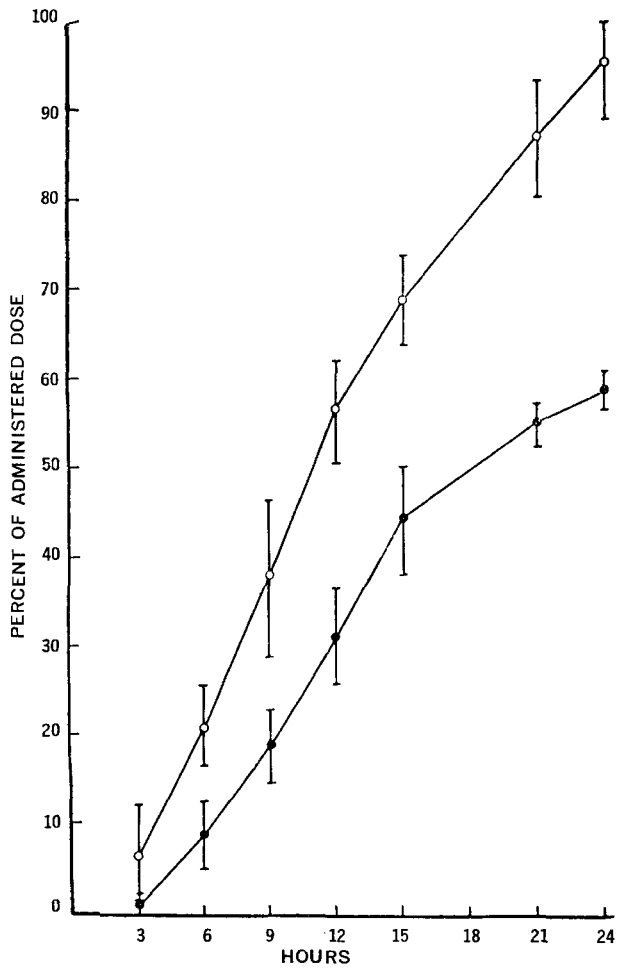

Figure 1—*Urinary excretion of 2,3,5-triiodobenzoic acid and/or metabolites by nonpregnant rats. Key: ○, cold-stressed; and ●, control.*

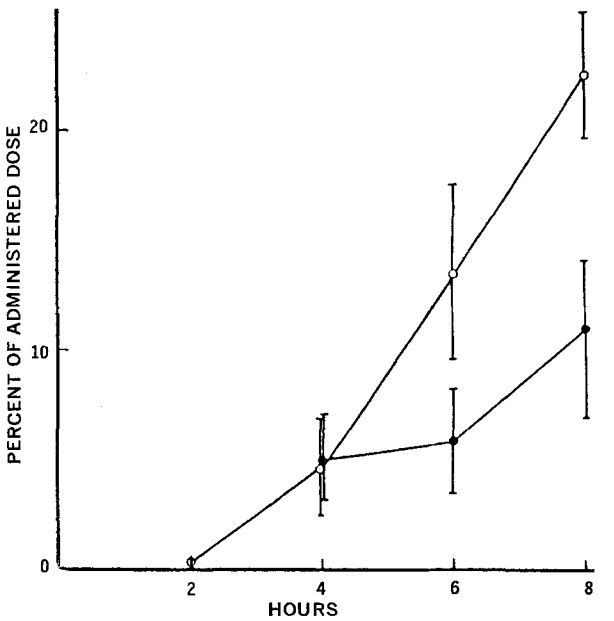

Figure 2—*Urinary excretion of 2,3,5-triiodobenzoic acid and/or metabolites by pregnant rats. Key: ○, cold-stressed; and ●, control.*

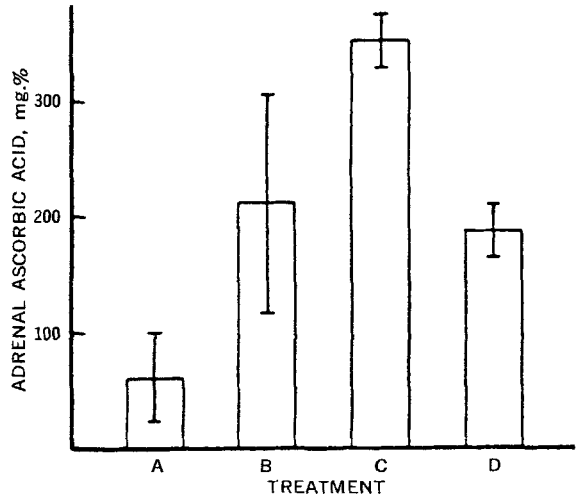

Figure 3—*Adrenal ascorbic acid. Key: A, cold-stressed pregnant; B, control pregnant; C, cold-stressed nonpregnant; and D, control nonpregnant.*

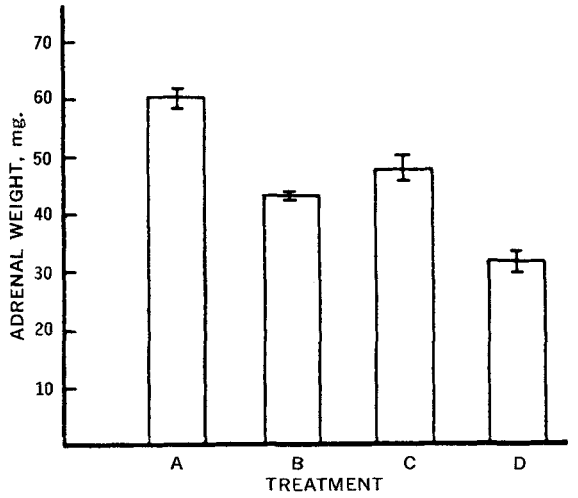

Figure 4—*Adrenal weight. Key: A, cold-stressed pregnant; B, control pregnant; C, cold-stressed nonpregnant; and D, control nonpregnant.*

percentage of injected 2,3,5-triiodobenzoic acid as 2,3,5-triiodobenzoic acid and/or metabolites than the nonpregnant controls for each time interval studied with the exception of the 3-hr. postinjection period (Fig. 1). Pregnant cold-stressed rats excreted a significantly higher percentage of injected 2,3,5-triiodobenzoic acid as 2,3,5-triiodobenzoic acid and/or metabolites than pregnant control rats at 6 and 8 hr. postinjection (Fig. 2). The nonpregnant cold-stressed rats excreted a higher percentage of administered 2,3,5-triiodobenzoic acid than the pregnant cold-stressed animals.

Adrenal Ascorbic Acid—The adrenal ascorbic acid level in the nonpregnant cold-stressed rats was significantly higher than in the nonpregnant controls (Fig. 3). The high levels of ascorbic acid indicate that the animals had acclimated to the stress (17). The pregnant cold-stressed rats had significantly lower levels of adrenal ascorbic acid than the pregnant and nonpregnant controls, which indicates that the pregnant cold-stressed rats did not acclimate to the cold. Pregnant control and nonpregnant control groups had similar levels of adrenal ascorbic acid, indicating no apparent stress from pregnancy on Day 19 of gestation. The data suggest that nonpregnant rats can acclimate to cold stress within 4 days while pregnant rats cannot. These data imply that cold stress and pregnancy act synergistically.

Cold stress has been shown to cause an increase in the size of the adrenal glands of rats (18). In the present study (Fig. 4), the pregnant cold-stressed rats had adrenal glands that were significantly larger than those of the other animals. The cold-stressed pregnant and cold-stressed nonpregnant rats had significantly larger adrenal glands than their respective controls; comparison between the two control groups revealed no significant difference.

SUMMARY

The results of the present investigation show that the placental transfer of 2,3,5-triiodobenzoic acid and two of its metabolites, 2,5-diiodobenzoic acid and 3,5-diiodobenzoic acid, occurred in cold-stressed and control rats on Day 19 of gestation.

Exposure to cold stress for a period of 4 days apparently did not influence the levels of 2,3,5-triiodobenzoic acid and/or its metabolites in the maternal and fetal tissue. The maternal blood of cold-stressed and control rats contained significantly higher concentrations of 2,3,5-triiodobenzoic acid and/or metabolites than the blood of respective fetal rats at each time interval studied. However, cold stress did not significantly affect the levels of 2,3,5-triiodobenzoic acid and/or metabolites in either the maternal blood or the fetal blood; it would thus appear that exposure to cold did not affect placental transfer. Cold stress had no apparent effect upon the plasma level of either 2,3,5-triiodobenzoic acid, 2,5-diiodobenzoic acid, or 3,5-diiodobenzoic acid in maternal or fetal animals. However, the data suggest a selective transport mechanism for 3,5-diiodobenzoic acid which was not affected by cold stress.

Pregnant cold-stressed rats excreted a significantly higher percentage of administered 2,3,5-triiodobenzoic acid as 2,3,5-triiodobenzoic acid and/or metabolites than the pregnant control rats. The same relationship was observed with nonpregnant cold-stressed animals when compared to nonpregnant control rats. Although an increased excretion of 2,3,5-triiodobenzoic acid and/or metabolites was observed in the cold-stressed pregnant rats in relation to pregnant controls, the increased loss of compound had no obvious effect on 2,3,5-triiodobenzoic acid and/or metabolite levels in cold-stressed rats.

The data obtained from the determination of adrenal ascorbic acid levels indicate that rats on Day 19 of gestation do not exhibit an apparent condition of stress from pregnancy. However, the additional insult to the pregnant animal from cold apparently resulted in a stressed condition. These data imply that cold stress and pregnancy act synergistically, suggesting that stress must be considered in the determination of the potential toxicity of an environmental contaminant as well as a medicinal agent.

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Crystal Structure of a 1:1 Aminopyrine–Barbital Complex

SETSUO KIRYU

Abstract □ The crystal structure of a 1:1 complex of aminopyrine and barbital was determined from three-dimensional X-ray diffraction data. The unit cell dimensions are $a = 11.915$, $b = 26.922$, $c = 7.199$ Å, and $\beta = 97° 17'$. The space group is $P2_1/c$; $Z = 4$. The intensity data were collected photographically, and the structure was solved directly by the symbolic addition method. Molecules in the crystal are arranged in the series of aminopyrine–barbital–barbital–aminopyrine linked by hydrogen bondings. The ethyl groups and phenyl groups are in contact with the same groups of other molecules, with normal van der Waals' interactions.

Keyphrases □ Aminopyrine–barbital complex—crystal structure □ Crystal structure—1:1 aminopyrine–barbital complex □ Molecular structure—aminopyrine, barbital □ X-ray diffractometry—analysis

Since aminopyrine diethylbarbiturate appeared as a potent analgetic about half a century ago, numerous physicochemical as well as pharmacological investigations of this well-known system have been reported.

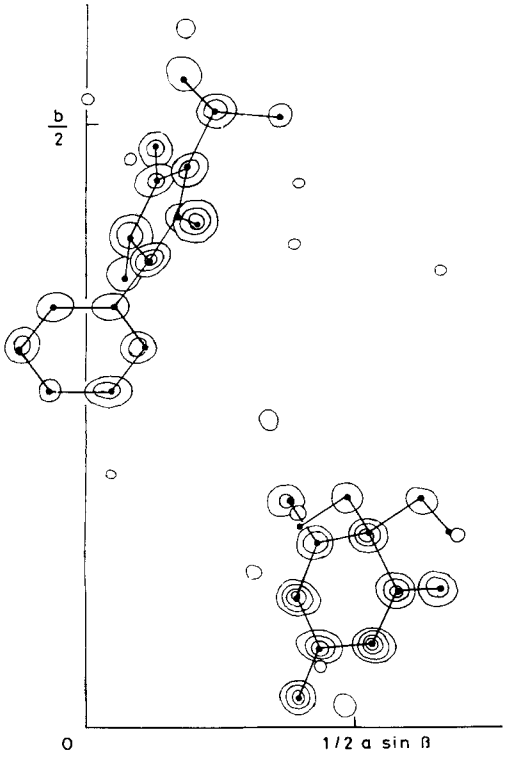

Figure 1—*Sections from a three-dimensional E-map projected along the c-axis. The contours are at equal intervals on an arbitrary scale.*

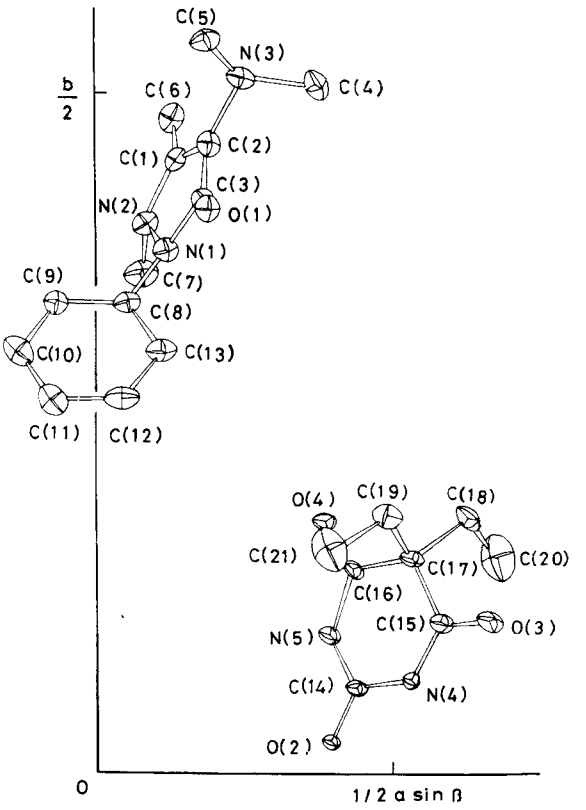

Figure 2—*Ellipsoidal representations of atomic thermal parameters and the atomic numbering scheme.*

Today a variety of information on this compound, such as its thermal or spectroscopic properties, is available; but the only study on its crystal structure was reported by Hertel in 1931 (1), and it does not offer sufficient information. Therefore, structure analysis by the X-ray diffraction method was undertaken to determine the exact crystal structure of this compound.

Table I—Initial Phase Assignments

h	*k*	*l*	|*Fo*|	|*E*|	Sign
5	10	5	35.2	4.34	+
−2	1	1	148.5	2.59	−
8	2	5	23.0	3.75	+
1	22	0	40.2	3.56	a
8	11	2	35.2	3.53	b
6	1	5	28.5	3.27	c

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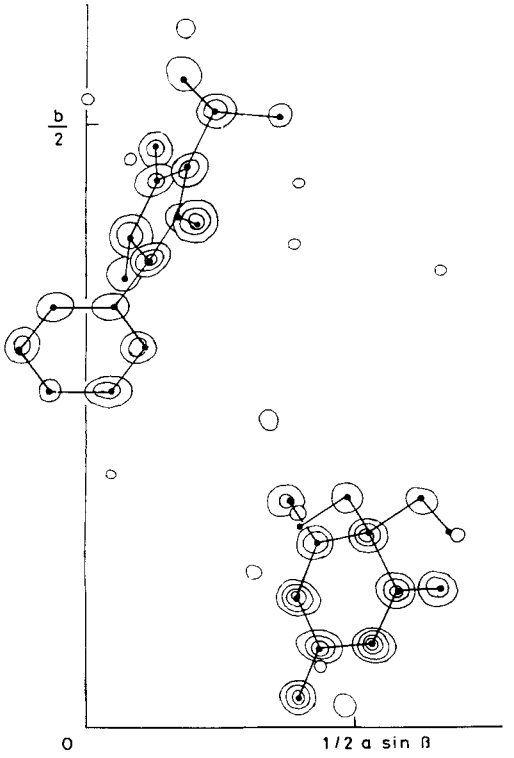

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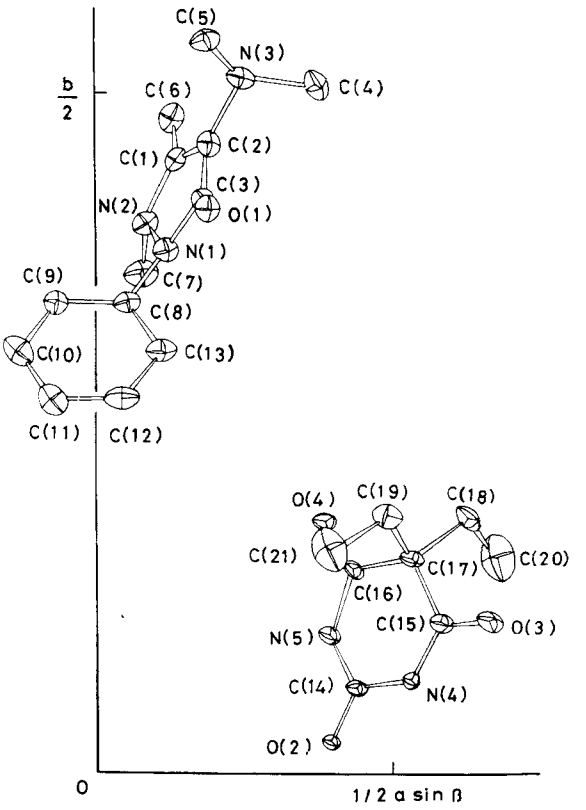

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8	11	2	35.2	3.53	b
6	1	5	28.5	3.27	c

Table II—Positional Parameters and Their Estimated Standard Deviations (Fractional Coordinate × 10^4)

	x	y	z
	Aminopyrine		
C (1)	1322 (4)	4522 (2)	0884 (7)
C (2)	1871 (4)	4638 (2)	2586 (7)
C (3)	1764 (4)	4227 (2)	3806 (7)
C (4)	3599 (6)	5055 (3)	3699 (11)
C (5)	1809 (7)	5374 (3)	4482 (11)
C (6)	1257 (5)	4818 (3)	−0893 (7)
C (7)	0726 (7)	3708 (3)	−0639 (8)
C (8)	0524 (5)	3495 (2)	3466 (7)
C (9)	−0653 (5)	3491 (2)	3058 (8)
C (10)	−1292 (7)	3136 (3)	3848 (9)
C (11)	−0727 (7)	2778 (3)	5079 (10)
C (12)	0434 (7)	2794 (3)	5478 (9)
C (13)	1092 (6)	3148 (2)	4668 (8)
N (1)	1169 (4)	3865 (2)	2721 (5)
N (2)	0819 (4)	4066 (2)	0934 (6)
N (3)	2385 (4)	5104 (2)	3077 (6)
O (1)	2070 (3)	4171 (1)	5520 (5)
	Barbital		
C (14)	4360 (4)	0651 (2)	3912 (7)
C (15)	5788 (5)	1146 (2)	5774 (8)
C (16)	4294 (5)	1539 (2)	3415 (8)
C (17)	5280 (5)	1619 (2)	4925 (8)
C (18)	6226 (7)	1912 (3)	4122 (11)
C (19)	4864 (7)	1931 (3)	6501 (11)
C (20)	6713 (11)	1617 (6)	2561 (16)
C (21)	3922 (10)	1675 (6)	7405 (16)
N (4)	5293 (4)	0705 (2)	5236 (6)
N (5)	3915 (4)	1069 (1)	3052 (6)
O (2)	3956 (3)	0243 (1)	3522 (5)
O (3)	6613 (5)	1152 (2)	6968 (7)
O (4)	3851 (5)	1886 (2)	2537 (8)

Table IV—Least-Squares Planes

1. Equations of Planes[a]

Plane[b]	A	B	C	D
1	0.87750	−0.38927	−0.38909	−3.6277
2	−0.17236	0.61039	0.78873	7.6042
3	−0.70524	0.08008	0.78816	−1.3226

2. Displacements from the Plane (Å × 10^3)

Plane	Displacement
1	C(1) 25, C(2) 0, C(3) −24, N(1) 38, N(2) −39; atoms not forming the plane C(6) 145, C(7) 680, C(8) −457, N(3) −88, O(1) −125
2	C(8) −1, C(9) 4, C(10) 0, C(11) −6, C(12) 9, C(13) −5
3	C(14) 17, C(15) −19, C(16) −17, C(17) 28, N(4) −3, N(5) −6; atoms not forming the plane O(2) 48, O(3) −34, O(4) −69

[a] The equations are in the form $AX + BY + CZ = D$, referred to the crystallographic axes, with X, Y, and Z in Å units. [b] Plane number: 1, pyrazolone ring of aminopyrine; 2, benzene ring of aminopyrine; and 3, pyrimidine ring of barbital.

EXPERIMENTAL

Single crystals were prepared by slow evaporation of an ether solution, containing 0.7 g. of aminopyrine and 1.0 g. of barbital, in a refrigerator, whereupon colorless thin needles extending along the *c*-axis were obtained. The lattice constants of the monoclinic unit cell were determined from Weissenberg and precission photographs with Cu$K\alpha$ radiation (λ = 1.5418 Å); a = 11.915 ± 0.007, b = 26.922 ± 0.005, c = 7.199 ± 0.011 Å, and β = 97° 17′ ±12′.

The cell contains four aminopyrine ($C_{13}H_{17}N_3O$) and four barbital ($C_8H_{12}N_2O_3$) molecules; the observed density is 1.26 g.·cm.$^{-3}$, calculated 1.20 g.·cm.$^{-3}$. Systematic absences of $0k0$ for k odd and $h0l$ for l odd indicate the space group to be $P2_1/c$. Linear absorption coefficient for Cu$K\alpha$ radiation is μ = 8.1 cm.$^{-1}$. The total number of electrons per unit cell is $F(000)$ = 888.

The dimensions of the specimen used for collecting the intensity data were 0.3 × 0.2 × 2.0 mm. Equi-inclination Weissenberg photographs, $hk0$-$hk6$ and $h0l$-$h2l$, were taken with Cu$K\alpha$ radiation, using the multiple-film technique. The intensities were estimated with a microphotometer and also visually by comparison with

Table III—Thermal Parameters and Their Estimated Standard Deviations (× 10^4)[a]

	B11	B22	B33	B12	B13	B23
			Aminopyrine			
C(1)	73 (4)	16 (1)	122 (11)	14 (3)	32 (10)	7 (4)
C(2)	78 (4)	15 (1)	102 (10)	0 (3)	−1 (9)	0 (4)
C(3)	72 (4)	14 (1)	110 (10)	4 (3)	−17 (9)	−3 (4)
C(4)	92 (6)	28 (2)	350 (21)	−30 (5)	50 (17)	28 (9)
C(5)	136 (8)	22 (1)	340 (21)	−2 (5)	94 (19)	−69 (8)
C(6)	104 (6)	25 (1)	96 (12)	20 (4)	20 (11)	28 (5)
C(7)	161 (8)	20 (1)	114 (13)	3 (5)	0 (14)	−34 (6)
C(8)	95 (5)	14 (1)	105 (11)	5 (3)	23 (10)	−5 (4)
C(9)	92 (5)	20 (1)	179 (13)	1 (4)	18 (12)	−10 (6)
C(10)	133 (7)	25 (1)	186 (15)	−15 (5)	74 (16)	2 (7)
C(11)	126 (7)	26 (1)	229 (16)	−19 (5)	70 (16)	−10 (7)
C(12)	185 (9)	16 (1)	180 (15)	4 (5)	64 (17)	20 (6)
C(13)	126 (6)	13 (1)	149 (12)	3 (4)	−3 (13)	3 (5)
N(1)	95 (4)	16 (1)	84 (8)	5 (3)	−18 (8)	2 (3)
N(2)	95 (4)	18 (1)	85 (9)	6 (3)	−9 (8)	−6 (4)
N(3)	111 (5)	16 (1)	139 (10)	−7 (3)	15 (10)	1 (4)
O(1)	95 (3)	17 (1)	103 (7)	1 (2)	−53 (7)	6 (3)
			Barbital			
C(14)	72 (4)	9 (1)	148 (11)	−1 (3)	−9 (9)	3 (4)
C(15)	98 (5)	11 (1)	224 (13)	−8 (3)	−47 (12)	6 (5)
C(16)	98 (5)	9 (1)	226 (14)	0 (3)	−26 (12)	10 (5)
C(17)	92 (5)	9 (1)	196 (13)	−13 (3)	−29 (11)	−2 (4)
C(18)	130 (8)	22 (1)	369 (21)	−45 (5)	0 (19)	57 (8)
C(19)	136 (8)	22 (1)	329 (20)	−2 (5)	10 (19)	−89 (8)
C(20)	195 (14)	65 (4)	445 (32)	−30 (12)	331 (35)	1 (19)
C(21)	179 (12)	57 (3)	432 (30)	−46 (10)	257 (30)	−145 (16)
N(4)	77 (4)	11 (1)	221 (11)	−4 (2)	−69 (9)	13 (4)
N(5)	89 (4)	10 (1)	161 (10)	1 (2)	−59 (9)	6 (3)
O(2)	78 (3)	9 (1)	235 (9)	−9 (2)	−69 (8)	2 (3)
O(3)	150 (5)	17 (1)	372 (14)	−23 (3)	−283 (14)	21 (5)
O(4)	160 (6)	10 (1)	426 (15)	−2 (3)	−237 (15)	42 (5)

[a] Thermal parameters are given according to the expression: $T = \exp[-(h^2B11 + k^2B22 + l^2B33 + hkB12 + hlB13 + klB23)]$.

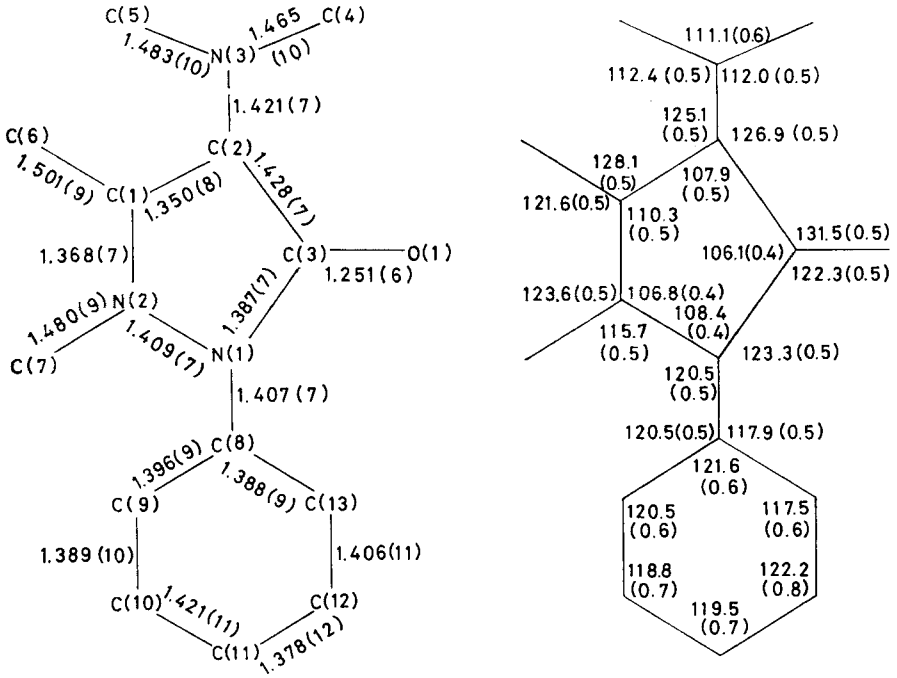

Figure 3—*Bond lengths (Å) and bond angles with their estimated standard deviations for aminopyrine.*

a calibrated scale. The number of unique reflections examined was 3669, among which were 1176 reflections of zero intensity. Corrections were applied for Lorentz and polarization factors but not for absorption.

STRUCTURE DETERMINATION

The structure was solved directly by the symbolic addition procedure for centrosymmetric crystals (2). Three reflections used to specify the origin and three additional reflections, each phase of which was designated by a symbol, are listed in Table I.

In the course of the phase determination, it became apparent that $a = b = +$ and $a = -c$. Phases were finally determined for 394 reflections of 477 with $|E| \geqq 1.5$, and 382 of them were found to be correct after refinement. The positions of the atoms were readily found in the E-map computed with these 394 reflections (Fig. 1). Six cycles of block diagonal least-squares refinement applied to the positional parameters and the isotropic temperature and overall scale factors reduced the R-index from 0.50 to 0.27. Further refinement was carried out, introducing anisotropic temperature factors; after eight cycles of refinement the R-value was reduced to 0.177 (0.108 for observed reflections). The weighting scheme was $\sqrt{w} = 0.2$ for $Fo = 0$, $\sqrt{w} = 1.0$ for $0 < Fo < 11$, and $\sqrt{w} = 11/Fo$ for $Fo \geqq 11$, and the function minimized was $\Sigma w(Fo - KFc)^2$.

The three-dimensional difference Fourier map, as well as the values of the positional and thermal parameters, showed no inconsistency with the structure obtained from the initial E-map, but few peaks of hydrogen atoms were clearly distinguishable in the difference Fourier map. Therefore, no further refinement, including the hydrogen atoms, was carried out. Maximum shifts of the positional and thermal parameters during the final cycle did not exceed a third of their standard deviations. The final positional and thermal parameters with their standard deviations are listed in Tables II and III, and the ellipsoidal representation of the thermal parameters is shown in Fig. 2.

The atomic scattering factors used for carbon, nitrogen, and oxygen were those of Berghuis *et al.* (3). Computations were carried out on the FACOM 230-60 of the Computer Center of Kyushu University with the programs UNICS (4), except for the computations of Fourier synthesis which were computed with a program written by the author.

DESCRIPTION OF THE STRUCTURE

The interatomic distances and the bond angles calculated on the basis of the parameters obtained are shown in Figs. 3 and 4.

Molecular Structure of Aminopyrine—In the five-membered pyrazolone ring, all the bond lengths lie between the corresponding

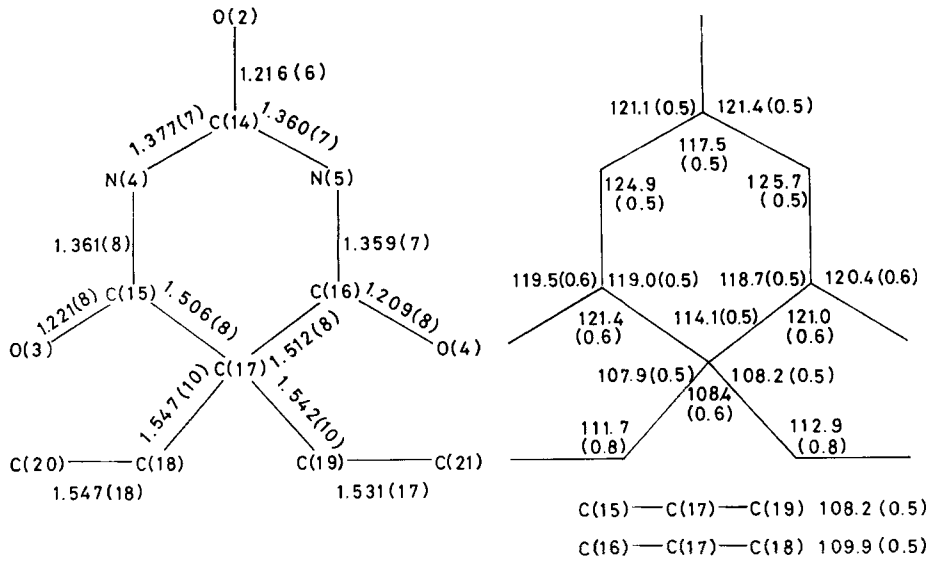

Figure 4—*Bond lengths (Å) and bond angles with their estimated standard deviations for barbital.*

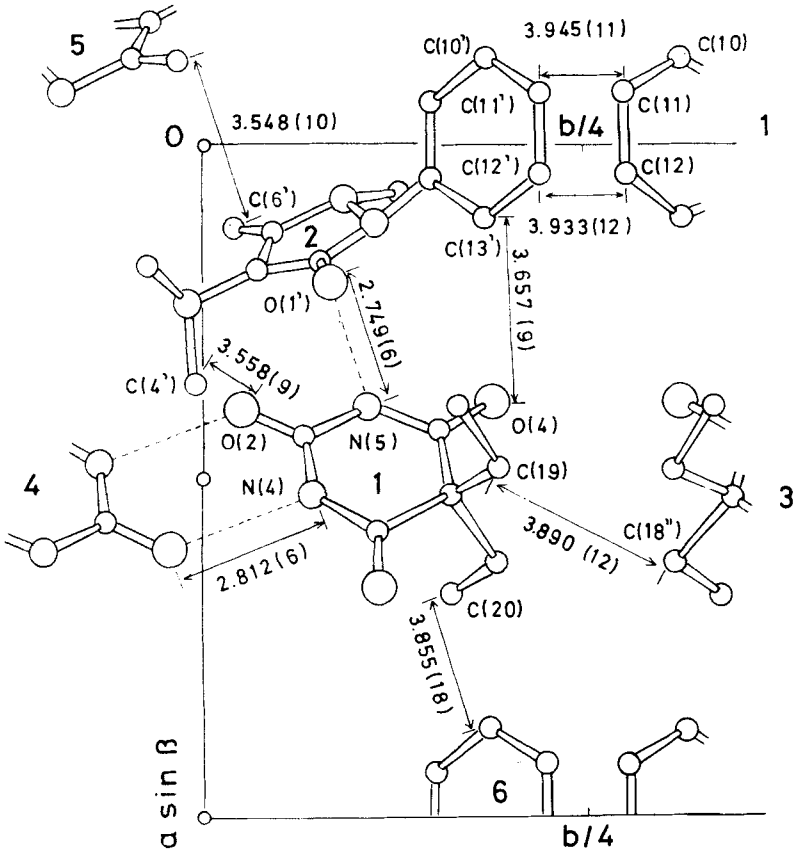

Figure 5—*Intermolecular atomic distances (Å) with their estimated standard deviations. The hydrogen bondings are shown by broken lines. The numbers on the molecules show following symmetry operations: (1)* x, y, z; *(2)* x, $^1/_2-$y, $-^1/_2+$ z; *(3)* x, $^1/_2-$y, $^1/_2+$ z; *(4) 1*−x, −y, *1*−z; *(5)*−x, $-^1/_2+$ y, $-^1/_2-$z; *and (6) 1* + x, $^1/_2-$y, $-^1/_2+$ z.

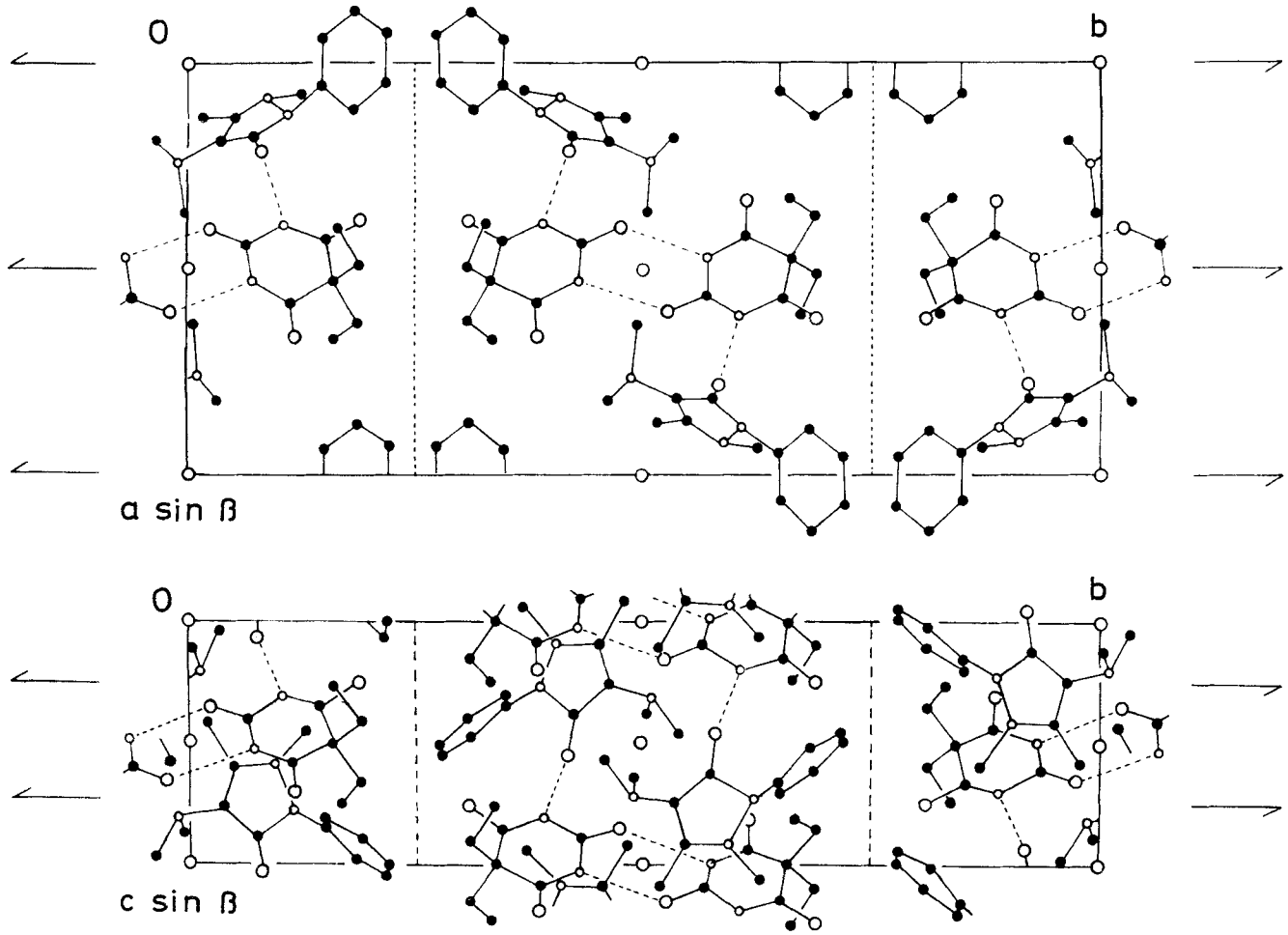

Figure 6—*The molecular arrangement viewed along the* c- *and* a-*axes. The hydrogen bondings are shown by broken lines.*

single- and double-bond values. The C(1)—N(2) bond length of 1.350 Å, however, shows an isolated double-bond character and the N(1)—N(2) distance 1.409 Å approximates a single-bond length.

Significant out-of-plane distortions occur in the ring (Table IV). The coordinations around the nitrogen atoms are not planar but pyramidal, and the substituents on N(1) and N(2), the phenyl and methyl groups, deviate from one another in a *trans*-configuration from the least-squares plane of the ring. The phenyl ring is completely planar (Table IV), and the C—C bond lengths (1.378–1.421 Å) and the bond angles (117.5–122.2°) are those of the typical benzenoid structure. The ring is inclined by 39° with respect to the plane of the pyrazolone ring. The dimethylamino group on C(2) is also of a pyramid configuration; the C(2)—N(3) bond length of 1.421 Å is a little shorter than that of a pure single bond at 1.47 Å (5) and is perhaps affected by the conjugated system of the pyrazolone ring.

Molecular Structure of Barbital—The values of bond lengths and angles are in reasonable agreement with the corresponding values for the same molecule in the barbital crystal (Form I) reported by Craven *et al.* (6). The C—N bond lengths of 1.359–1.377 Å in the pyrimidine ring show about 20–30% of double-bond characters, and there are no significant differences among the three C—O pure double-bond lengths. The C—C bond lengths of 1.506 and 1.512 Å in the pyrimidine ring approximate that of the single bond, while the steric requirement of the ring formation leads to a slight broadening of the angle at the tetrahedrally bonded atom C(17) from the tetrahedral angle of 109.4–114.1°. In the ethyl groups, the C—C—C angles and the C—C bond lengths agree quite well with those of normal paraffin. For an isolated barbital molecule, the ring atoms should be coplanar and the hydrocarbon chain of the ethyl groups should be normal to the ring plane. Some significant deviations of the ring atoms from the plane, however, were observed in this crystal (Table IV). The pyrimidine ring is slightly folded along the C(15)—C(16) line at an angle of about 4°. Similar distortions were also found in the pyrimidine rings in barbital I (6) and in other dialkylbarbiturate crystals (7, 8), in which the foldings of the planes were observed along the N(4)—C(16) diagonals.

Crystal Structure—The intermolecular atomic distances are shown in Fig. 5, and the arrangement of the molecules in the crystals is shown in Fig. 6. The short distances between the carbonyl and the imino groups, O(1)—N(5) 2.749 Å and O(2)—N(4) 2.812 Å, suggest hydrogen bondings. The interaction between two barbital molecules with hydrogen bonds is quite similar to that in barbital crystal I, in which the molecules are arranged in infinite planar ribbons with two kinds of hydrogen bonding (6). There are no infinite chains of molecules linked by hydrogen bonds in this compound; only two aminopyrine and two barbital molecules are linked in the series of aminopyrine–barbital–barbital–aminopyrine. The hydrocarbon groups, *i.e.*, the ethyl and phenyl groups, are in contact with one another in normal van der Waals' interactions. No other unusually close contacts or overlappings of molecules, which are found in some charge-transfer complexes, were observed.

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NED D. HEINDEL*, WILLIAM P. FIVES*, THOMAS F. LEMKE*, and RICHARD A. CARRANO†

Abstract □ A series of anthranilamides of diverse structure were prepared by the nucleophilic ring opening of isatoic anhydrides. Sixteen analogs, 10 of them previously unknown in the literature, were submitted to general pharmacologic screening. Several of the compounds displayed CNS depression, anti-inflammatory–analgesic, and antitremor activities.

Keyphrases □ Anthranilamides—synthesis from isatoic anhydrides □ CNS depressant activity—anthranilamides □ Anti-inflammatory, analgesic potential—anthranilamides □ Antitremor activity—anthranilamides

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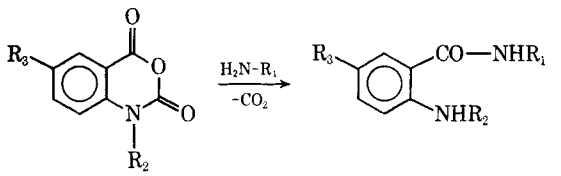
Synthetically, these compounds are obtained by nucleophilic ring opening of substituted isatoic anhydrides with amines (Scheme I). Although procedures



Scheme I

were suggested for these transformations (7, 8), an improved modification has been developed which simplifies the experimental method and enhances the yield by the use of *N*,*N*-dimethylformamide as a solvent when

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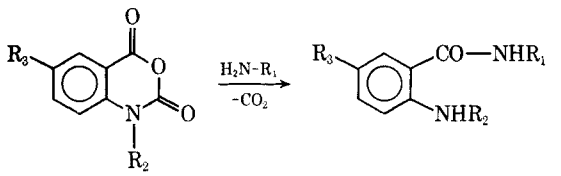

Scheme I

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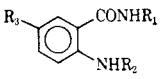
Table I—Anthranilamides



Compound	R_1	R_2	R_3	Method	Yield, %	M.p. (b.p./mm. Hg)	Formula	Anal., % Calcd.	Found
I	H	H	H	—[a]		107.5–108°			
II	H	C_6H_5	H	C	81	129.0–130.5°[b]			
III	H	H	I	C	61	203–204°	$C_7H_7IN_2O$	C, 32.08 H, 2.69 N, 10.69	C, 32.09 H, 2.72 N, 10.46
IV	CH_3	H	I	C	63	163.5–164.5°	$C_8H_9IN_2O$	C, 34.81 H, 3.29 N, 10.15	C, 35.08 H, 3.31 N, 10.08
V	CH_3	H	Cl	C	75	131–132°	$C_8H_9ClN_2O$	C, 52.04 H, 4.91 N, 15.17	C, 52.33 H, 5.01 N, 14.90
VI	H	H	NO_2	C	45	234–236°[c]			
VII	C_6H_5	CH_3	Cl	A	95	179–180°	$C_{14}H_{13}ClN_2O$	C, 64.49 H, 5.02 N, 10.75	C, 64.21 H, 5.09 N, 10.66
VIII	H	H	Cl	C	58	170–172°[d]			
IX	C_6H_5	H	Cl	A	65	154–155°	$C_{13}H_{11}ClN_2O$	C, 63.29 H, 4.49 N, 11.36	C, 63.15 H, 4.21 N, 11.56
X	2—ClC_6H_4—	CH_3	Cl	A	83	139–140°	$C_{14}H_{12}Cl_2N_2O$	C, 56.96 H, 4.10 N, 9.49	C, 57.25 H, 4.35 N, 9.55
XI	$Et_2N(CH_2)_3CH(CH_3)$—	CH_3	Cl	A	81	(194–196°/2.2)	$C_{17}H_{28}ClN_3O$	C, 62.66 H, 8.66 N, 12.89	C, 62.68 H, 8.61 N, 12.87
				B	73	(194–196°/2.2)			
XII	$Et_2N(CH_2)_3$—	H	H	A	80	(159–161°/0.1)	$C_{14}N_{23}N_3O$	C, 67.43 H, 9.30 N, 16.85	C, 67.49 H, 9.42 N, 16.90
				B	67	(159–161°/0.1)			
XIII	CH_3	CH_3	Cl	C	74	104–105°[e]	$C_9H_{11}ClN_2O$	C, 54.41 H, 5.58 N, 14.10	C, 54.68 H, 5.34 N, 13.96
XIV	H	CH_3	H	C	67	161–163°[f]			
XV	$Et_2N(CH_2)_3CH(CH_3)$—	H	H	A	69	(170–171°/0.18)	$C_{16}H_{27}N_3O$	C, 69.27 H, 9.81 N, 15.15	C, 69.13 H, 9.60 N, 15.07
XVI	H	CH_3	Cl	C	78	153–154°	$C_8H_9ClN_2O$	C, 52.05 H, 4.91 N, 15.17	C, 52.19 H, 4.90 N, 15.22

[a] Commercial material from Maumee Chemical Co., purified by double vacuum sublimation, lit. m.p., 107–108°, K. Butler and M. W. Partridge, *J. Chem. Soc.*, **1959**, 2396. [b] Lit. m.p. 127.5–128.5°, M. Goodman, N. Arbiter, and G. Powell, *J. Amer. Chem. Soc.*, **55**, 4294(1933). [c] Lit. m.p. 230°, "Dictionary of Organic Compounds," Oxford University Press, New York, N. Y., 1965, p. 176. [d] Lit. m.p. 172°, "Dictionary of Organic Compounds," Oxford University Press, New York, N. Y., 1965, p. 101. [e] Lit. (10) m.p. 95–97°. [f] Lit. m.p. 159–160°, H. Weddige, *J. Prakt. Chem.*, **36**, 150(1887).

high boiling amines are employed as nucleophiles. The reported methods for ring opening with ammonia (9) or with methylamine (10) were used without modification. The anthranilamides were refined to analytical purity and submitted to general pharmacologic screening. Table I lists the compounds studied.

EXPERIMENTAL

Preparation of Anthranilamides—Commercial anthranilamide (Compound I) was doubly sublimed in vacuum before biological evaluation. Other anthranilamides (Compounds II–XVI) were synthesized. Several of the required isatoic anhydrides (isatoic, *N*-methylisatoic, 5-chloro-*N*-methylisatoic, 5-chloroisatoic, and 5-nitroisatoic anhydride) were obtained as samples[1]. *N*-Phenylisatoic anhydride was prepared by a literature method (11), and 5-iodoisatoic anhydride was synthesized from 5-iodoanthranilic acid by the phosgenation technique reported for anthranilic acid (12). The 5-iodoisatoic anhydride melted with decomposition at 270–273° and was employed directly without further purification.

Method A—A solution of 20 mmoles of the required anhydride, 25–30 mmoles of the amine, and 300 ml. of anhydrous *N,N*-dimethylformamide was stirred at reflux for 4 hr. The solvent was removed *in vacuo*. If no crystallization ensued on chilling the oily mass, the product was vacuum distilled. The results of this technique with aniline, *o*-chloroaniline, 5-diethylamino-2-pentylamine ("Noval diamine"), and 3-diethylaminopropylamine are reported in Table I.

Method B—To 20 mmoles of the solid dry isatoic anhydride was added 25 mmoles of the liquid amine (5-diethylamino-2-pentylamine or 3-diethylaminopropylamine). The addition was carried out in small portions, with the resulting paste being stirred to miminize foaming (CO_2). After completion of the addition, the solution was stirred and heated at 80–90° until the evolution of gas ceased (usually 1–2 hr.). The heavy oil was cooled to room temperature, diluted with 400 ml. of chilled diethyl ether, and filtered to remove traces of insoluble solids. The filtrate was washed with 50 ml. of cold water, dried ($MgSO_4$), and concentrated to a yellow oil which was fractionated *in vacuo*.

Method C—A suspension of 25 mmoles of the anhydride and 500 ml. of 0.9 *M* aqueous amine was stirred for 12 hr. and filtered; the solid was washed with 100 ml. of a 0.9 *M* solution of the same amine. The dried solid was recrystallized from EtOH to analytical purity.

General Rat Screening—All compounds were tested and the results were analyzed according to the methods described by Malone and Carrano (13). Nonfasted albino rats (180–210 g.) were used. Injections were made intraperitoneally.

General CNS Testing—A log dose *versus* response curve was generated, using the inability of trained animals to walk a rotating wooden rod (28 mm. diameter, at 20 r.p.m.) for 1 min. as the response criterion. Six 18–22-g. nonfasted albino mice were used per group, and dosing was done intraperitoneally. The animals were tested at 0, 15, 30, 60, 90, 120, 150, and 180 min. postinjection. The time postinjection for the peak effect was determined, and the quantal response at that dose was used to plot the curve. A graphical ED_{50} was estimated. This dose and peak effect time was then used for

[1] Maumee Chemical Co., Toledo, Ohio.

Table II—*In Vivo* Rat Screening Data for Anthranilamides

Compound	Maximum Sublethal Dose, mg./kg. i.p.	ANS Score	CNSD Score	Significant Symptomatology	Reference Drug Match
I	>1000	0.95	12.50	Decreased motor activity, slight ataxia, slight lacrimation, piloerection, decreased rectal temperature, decreased rotorod ability, decreased body tone	None outstanding
II	>1000	2.75	5.86	Slightly decreased motor activity, ataxia, piloerection, decreased body weight, decreased body tone	None outstanding
III	316	3.92	21.30	Decreased motor activity, ataxia, loss righting reflex, screen grip loss, enophthalmos, piloerection, decreased rectal temperature, decreased rotorod ability, decreased body tone	Chlordiazepoxide
IV	562	3.17	19.06	Decreased motor activity, ataxia, loss righting reflex, screen grip loss, enophthalmos, lacrimation, piloerection, decreased rectal temperature, decreased rotorod ability, decreased body tone	Phenobarbital
V	316	3.60	21.30	Decreased motor activity, ataxia, loss righting reflex, screen grip loss, enophthalmos, ptosis, slight mydriasis, piloerection, decreased skin and connective tissue elasticity, decreased rectal temperature, decreased rotorod ability, decreased body tone	Chlorpromazine, chlordiazepoxide
VI	>1000	2.86	1.62	Slight mydriasis, decreased skin and connective tissue elasticity, decreased body weight, slightly decreased body tone	None outstanding
VII	>1000	2.54	10.11	Decreased motor activity, decreased respiratory rate, enophthalmos, piloerection, decreased body weight, decreased body tone, fearfulness	None outstanding
VIII	56	2.54	8.41	Decreased motor activity, ataxia, loss righting reflex, screen grip loss, slight enophthalmos, slightly decreased rectal temperature, decreased rotorod ability, decreased body tone	Phenobarbital
IX	>1000	4.34	3.94	Mydriasis, diarrhea, decreased body weight	Indomethacin
X	>1000	2.54	7.33	Decreased motor activity, decreased respiratory rate decreased body weight, decreased body tone	None outstanding
XI	177	1.06	3.01	Decreased motor activity, slight enophthalmos, decreased body weight, decreased rotorod ability, decreased body tone	None outstanding
XII	56	3.17	2.85	Decreased motor activity, ataxia, decreased rotorod ability, decreased body tone, enophthalmos, decreased body weight	None outstanding
XIII	>1000	2.96	3.24	None	None outstanding
XIV	316	6.14	15.43	Decreased motor activity, ataxia, loss righting reflex, miosis, lacrimation, salivation, decreased skin and connective tissue elasticity, decreased rectal temperature, decreased rotorod ability, decreased body tone	None outstanding
XV	>1000	2.61	0.00	None	None outstanding
XVI	177	5.29	27.55	Decreased motor activity, ataxia, loss righting reflex, decreased body tone, decreased rotorod ability, screen grip loss, increased respiratory rate, enophthalmos, lacrimation, decreased rectal temperature	Diphenylhydantoin
Phenobarbital sodium	100	7.32	28.32	Decreased motor activity, ataxia, loss righting reflex, loss corneal reflex, screen grip loss, enophthalmos, ptosis, mydriasis, lacrimation, decreased skin and connective tissue elasticity, decreased rotorod ability, decreased body tone	Reference compound
Diphenylhydantoin sodium	316	6.46	25.74	Decreased motor activity, ataxia, loss righting reflex, screen grip loss, exophthalmos, mydriasis, piloerection, decreased skin and connective tissue elasticity, decreased rectal temperature, decreased rotorod ability, decreased body tone	Reference compound
Indomethacin	316	2.01	3.86	Slightly decreased motor activity, mydriasis, mucosal blanching, decreased body tone, diarrhea, piloerection, decreased body weight	Reference compound
Chlorpromazine hydrochloride	100	13.40	33.28	Decreased motor activity, loss righting reflex, ataxia, analgesia, loss corneal reflex, loss pinna reflex, screen grip loss, clonic convulsions, enophthalmos, ptosis, mydriasis, lacrimation, piloerection, diarrhea, decreased skin and connective tissue elasticity, decreased rectal temperature, decreased rotorod ability, decreased body tone	Reference compound
Chlordiazepoxide hydrochloride	100	6.45	35.65	Decreased motor activity, loss righting reflex, ataxia, screen grip loss, enophthalmos, miosis, lacrimation, decreased skin and connective tissue elasticity, decreased rectal temperature, decreased rotorod ability, decreased body tone	Reference compound

Table III–Results with Anthranilamides in Selected Screening Tests

Compound	Rotorod Test: Est. ED_{50}, mg./kg. i.p.	Rotorod Test: Peak Effect Time, min.	Percent Protection at the Rotorod ED_{50}: Antistrychnine Test	Antimetrazol Test	Antielectroshock Test	Antioxotremorine Test	Acetylcholine Writhing Test: Dose, mg./kg. i.p.	Acetylcholine Writhing Test: Percent Protection
I	>200	30	0	0	17	89	300	0
III	68	30	0	0	0	36	300	67
IV	172	30	0	0	0	70	300	17
V	113	30	0	0	0	83	30	0
							300	67
VII	>200	30	0	0	0	0	300	0
VIII	62.5	30	0	0	0	76	30	0
							300	33
IX	>200	30	0	0	0	6	300	17
X	>200	30	0	0	0	0	300	17
XII	180		0	0	0	72	300	50
XIII	>200	15	16	16	0	59	50	17
							100	17
							500	83
XIV	103	15	0	0	16	82	30	0
							300	83
XV	>200	30	83	0	50	3	30	17
							300	67
XVI	130	15	0	0	0	82	500	67
Indomethacin	>200	30	0	0	0	21	1	33
							3	50
							7	83
							10	100
Phenobarbital sodium	68	90	33	100	100	60	—	—
Diphenylhydantoin sodium	48	120	0	0	66	29	—	—
Chlordiazepoxide hydrochloride	40	30	0	83	66	58	—	—
Chlorpromazine hydrochloride	2	90	0	0	0	27	1	17
							5	67
							10	100

subsequent work in the antistrychnine, antimetrazol, antielectroshock, and antioxotremorine tests. In these tests, the experimental drug was injected intraperitoneally into groups of six 18–22-g. nonfasted albino mice; after the time to peak effect, strychnine sulfate (1.1 mg./kg. s.c.), pentylenetetrazole (metrazol) (70 mg./kg. s.c.), oxotremorine (350 mcg./kg. s.c.), or a 50-amp., 0.2-sec. shock was administered to each animal. The animals were observed for 30 min. for tonic extensor seizures in the antistrychnine test and for clonic seizures in the antimetrazol test. In the antielectroshock test, the animals were observed for hind-limb tonic extensor seizures. In the antioxotremorine test, tremors were subjectively rated per animal on a scale of 0–3 and the total response for the entire group was calculated and compared to that of a control group.

In each test a group of animals receiving 0.9% saline, 5 ml./kg., was run simultaneously with the experimental groups. In the acetylcholine writhing test, mice were injected orally with the experimental agent and, after an elapse of the peak effect time, challenged with a 7.0-mg./kg. i.p. injection of acetylcholine bromide. The number of animals in the experimental group that elicited a writhing response within 2 min. post the challenge was compared to that of a control group receiving 0.9% saline.

Acute Anti-Inflammatory–Analgesic Testing—Nonfasted albino rats in the weight range of 160–180 g., equally divided between sexes, were used. The experimental drugs were administered orally. A modified Randall–Selitto (14) method was used; immediately following administration of the drug, 0.1 ml. of a 1% carrageenin suspension[2] was injected into the subplantar area of the right hind paw and the control foot volume was recorded by volume displacement. The control analgesic response was then estimated by placing the injected paw between two grooved disks which were slowly forced together *via* air pressure. The amount of pressure required to cause the animal to vocalize or attempt to bite the disks was recorded. The analgesic response was again estimated at 1 and 2 hr. postinjection, and foot volume was measured at 4 hr. postinjection. A control group that received 0.9% saline in place of the experimental drug was tested simultaneously for comparison.

[2] Marine Colloids, Inc.

BIOLOGICAL ACTIVITY AND DISCUSSION

Table II summarizes some important aspects of the results of general rat screening. Several reference agents are included for comparison. The maximum sublethal dose (MSLD) is the highest dose administered at which no death occurred. No doses were made over 1.0 g./kg.; therefore, in cases where there was no death at this dosage, the MSLD is assumed to be greater than 1.0 g./kg. Also presented in the table are scores that represent the extent of general autonomic nervous system (ANS) and general CNS depressant (CNSD) activity. The absolute values of these scores between ANS and CNSD are not comparable. The significant symptomatology and results of computer-matching operations against a library of 27 reference drugs are also included.

Compounds I, III, IV, V, VII, VIII, X, XIV, and XVI produced significant CNS depression. Some of the symptomatology exhibited by these drugs would be that expected of a general CNS depressant, tranquilizer, muscle relaxant, or anticonvulsant. However, Compounds III, IV, V, VIII, IX, and XVI displayed a particular similarity to specific reference drugs, as indicated in Table II. The majority of these reference agents are CNS depressants: chlorpromazine (tranquilizer), diphenylhydantoin (anticonvulsant), chlordiazepoxide (muscle relaxant/mild tranquilizer), and phenobarbital (sedative/hypnotic). Based on these similarities, several of the drugs were screened for their CNSD potential in the tests listed (Table III).

These results indicate that, in general, throughout the series there was significant activity in the acetylcholine writhing test, indicating potential anti-inflammatory–analgesic activity. Also, there seems to be a potential throughout the series for antitremor activity since several of the compounds (VIII, XII, XIII, XIV, XVI) had good activity in the antioxotremorine test.

Table IV—Anti-Inflammatory–Analgesic Activity of Anthranilamides

Compound	Dose, mg./kg. p.o.	Mean Percent Inhibition of Carrageenin Edema	Mean Percent Increase in Foot Pressure Pain Threshold (hr. postinjection)
II	50	0	8 (1,2)
	500	16	8 (1,2)
IX	50	16	12 (2)
	500	30	29 (2)
XII	30	0	17 (2)
	300	0	33 (2)
XIII	50	0	3 (1)
	500	13	48 (2)[a]
XIV	50	4	45 (2)
	500	12	120 (2)[b]
XVI	50	4	26 (2)
	500	9	204 (2)[b]
Indomethacin	3	39	10 (2)
	10	57	30 (1,2)
	30	51	18 (1,2)
Aspirin	100	18	16 (1)
	300	43	40 (1)
	1000	74	25 (1,2)

[a] The animals tested were slightly depressed. [b] The animals tested were moderately–markedly depressed.

Table IV summarizes the results of the acute anti-inflammatory–analgesic test for compounds selected because of their behavior in previous tests. Compound IX matched well with the reference drug, indomethacin, in the general rat screen. Compounds XII and XIII exhibited low toxicity in the general rat screen. Compounds XII, XIII, XIV, and XVI had good activity in the acetylcholine writhing test. Compound II was chosen for structure–activity relationship purposes, because it is similar in structure to known anti-inflammatory agents. None of the compounds tested exhibited outstanding anti-inflammatory activity. Compounds IX and XIV produced slight to moderate effects at 500 mg./kg. Compounds XIII, XIV, and XVI produced significant increases in pressure threshold in the analgesic portion of the test, but the animals were notably depressed.

Compounds IX, XIV, and XVI were tested intraperitoneally at 2, 10, and 50 mg./kg. for cardiovascular activity in an anesthetized cat because of the relatively high autonomic scores in the rat screen. The physical characteristics of these drugs made it impractical to test them intravenously. For all three drugs, there were no alteration in blood pressure, heart rate, or respiration and no significant effects on the responses of acetylcholine, norepinephrine, histamine, dimethylphenylpiperazinium, and sympathetic or vagal stimulation.

Compound XVI, which showed the most CNS depression in the rat screen, was found in other testing to protect significantly against amphetamine toxicity in aggregated mice at 100 mg./kg., both subcutaneously and orally. Major tranquilizer activity was indicated.

Compounds VIII and XII are the most toxic of the series, because death occurred at 56 mg./kg. in the rat screen. In general, as expected, addition of a halogen tends to increase activity and toxicity. Nitro in place of halogen seems to decrease activity and toxicity.

It seems probable that CNSD potential is related to *in vivo* binding at the *o*-amino function. Those compounds that reflected diminished basicity at this nitrogen (*i.e.*, II and VI) and those that reflected diminished steric accessibility at this site (*i.e.*, XI, XII, and XV) gave the lowest CNSD scores in the general rat screen.

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Pharmacokinetic and Metabolic Fate of Potassium Canrenoate (SC-14266) in Man

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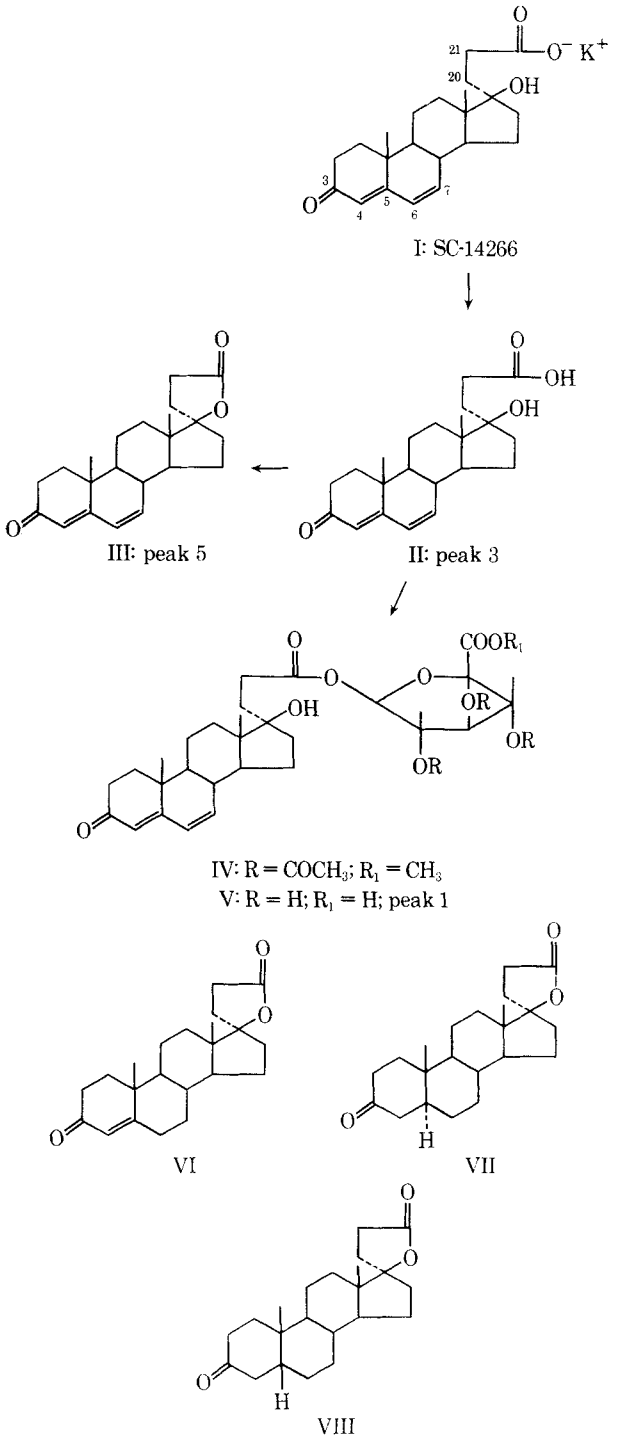
Abstract □ The metabolism of intravenously administered radioactive potassium canrenoate (SC-14266-^{3}H) was studied in three men. Within 5 days, 47.45 ± 4.17% of the administered radioactivity was recovered in urine and 14.36 ± 0.12% in the feces. In the urine, canrenone was identified as the major metabolite in the free fraction, and the major water-soluble metabolite was identified as the glucuronic acid ester conjugate of the hydroxy acid analog of SC-14266. The elimination of the total radioactivity in the plasma occurred in three phases, their half-lives being 0.073, 0.85, and 43.31 hr., respectively. The disappearance of the total label during the first and second phases was predominantly due to the excretion of the hydroxy acid derivative and canrenone. The third phase was a function of the excretion of the conjugate. Three hours after administration of the drug, the plasma levels of the hydroxy acid and canrenone were similar and declined, with a half-life of 8.25 hr.

Keyphrases □ Canrenoate-^{3}H, potassium (SC-14266)—pharmacokinetic, metabolic fate, human □ Pharmacokinetic parameters—potassium canrenoate-^{3}H in human □ Metabolites, potassium canrenoate-^{3}H—identification □ TLC—analysis □ Scintillometry—analysis □ GLC—analysis □ Spectrophotometry, IR, UV—analysis

SC-14266 [potassium canrenoate, potassium 3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl) propionate, I][1] is a water-soluble steroidal diuretic (1). It is structurally related to spironolactone [3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone][2], a useful aldosterone antagonist. The metabolism of spironolactone in humans has been studied by a number of investigators (2–5), but so far only one metabolite, canrenone [aldadiene, 3-(3-oxo-17β-hydroxy-4,6-androstadiene-17α-yl) propionic acid γ-lactone, III], has been fully characterized. Tentative characterization of the metabolites of SC-14266 was attempted by Wagner *et al.* (6), who reported the presence of seven metabolites in human urine. All of the work reported in the literature on the metabolism of spironolactone and its congeners in humans has been carried out using the unlabeled compound. With the availability of specifically tritiated SC-14266 (7), the present investigation was undertaken to identify and quantitate the major metabolites in the plasma and urine of human subjects and to study their kinetics of distribution and elimination after intravenous administration of the drug.

MATERIALS AND METHODS

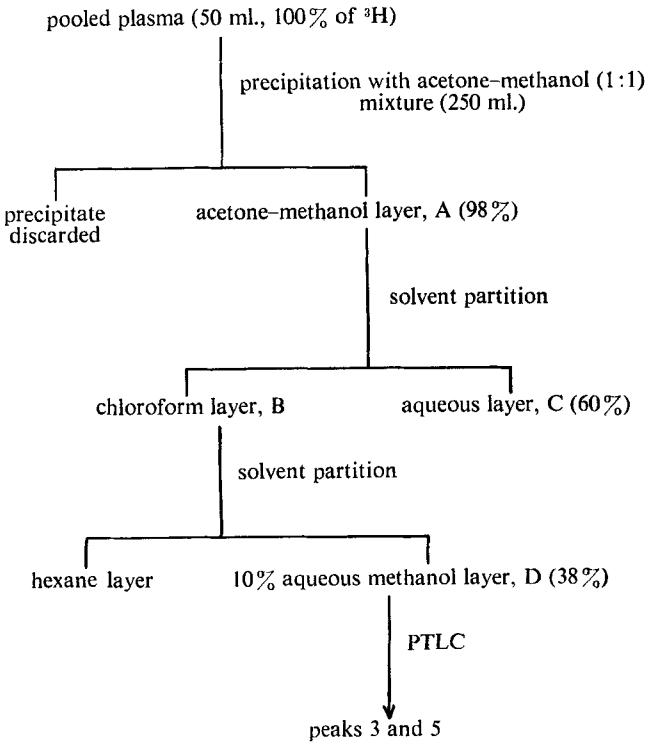
Labeled SC-14266 (I)—The studies on the metabolism of SC-14266 (Scheme I) were carried out using the ^{3}H-labeled compound, with a specific activity of 647 μc./mg.; the tritium label in I was present in the side chain of the steroid on C-20. The compound migrated as a single spot, and its radiochemical purity was 92% as determined by a TLC system.



Scheme I

Treatment of Subjects—Three adult male subjects were each given single intravenous injections of SC-14266 (200 mg. containing 182 μc. of ^{3}H). The sterile dry powder was dissolved in 10 ml. of sterile saline immediately before intravenous injection. An aliquot was

[1] Soldactone, G. D. Searle and Co.
[2] Aldactone, G. D. Searle and Co.



Scheme II—Isolation scheme for separation of metabolites from pooled plasma of Subject A

taken for radioactivity measurement to determine the actual amount of radioactivity injected. Blood samples were withdrawn at various intervals into heparinized vacutainers. Urine and fecal samples were taken for 5 days. Plasma samples and excreta were stored at −20° until analyzed.

Measurement of Radioactivity—All samples were counted in a Nuclear-Chicago Mark I liquid scintillation spectrometer. For counting aqueous solutions (plasma and urine) and extracts containing polar compounds, Bray's scintillation solution (8) was used; for samples scraped from thin-layer chromatograms, Snyder's scintillation mixture (9) was employed. For counting radioactivity in the feces, samples (10 g.) were freeze-dried and the dry powder was exhaustively extracted with methanol in a continuous extraction apparatus. The methanol extract was diluted to 100 ml. and 100 μl. counted in Bray's solution. Chemical quenching was corrected by a channels-ratio method, and color quenching was done by the addition of an internal standard (toluene-³H). Tritiated water in plasma and urine was determined by the method of Werbin *et al.* (10).

TLC—Analytical TLC was carried out on silica gel HF_{254}[3] coated (250-μ thickness) glass plates using the saturated chamber technique. The solvent system was composed of methanol–glacial acetic acid–chloroform (5:1:94). Compounds were visualized under UV and by spraying with either a 10% ethanolic solution of phosphomolybdic acid or a 5% solution of ceric sulfate in 10% (v/v) aqueous sulfuric acid. The sprayed plates were heated at 110° for 10 min. Preparatory TLC (PTLC) was run on 20 × 20-cm. glass plates coated with 1 mm. of silica gel HF_{254}.

For detecting radioactive compounds on a thin-layer plate, 3-mm. bands of silica gel were scraped from the plate, using a Snyder-Kimble zonal scraper[4] (11); the radioactivity in each zone was determined by liquid scintillation spectrometry. The radioactivity distribution pattern of the chromatogram was then plotted by a computer-plotter which was programmed to give the total radioactivity eluted from the plate, the number of radioactive peaks, their R_f values, and the percentage of radioactivity in each peak.

Separation of Metabolites from Plasma and Urine—Metabolites from plasma and urine were isolated according to the schemes outlined in Schemes II and III.

[3] E. Merck, Darmstadt, West Germany.
[4] Analabs Inc.

GLC—GLC studies were made using a Varian Aerograph model 2100 instrument having a flame-ionization detector with a 1.5-m. (5-ft.) long, 2-mm. i.d., silanized glass U-tube packed with 1.3% OV-17[5] on 80–100 mesh Chromosorb W-H.P.[6]. Operating conditions were as follows: column temperature, 250°; detector and injection port temperature, 275°; carrier gas helium flow rate, 27 ml./min.; hydrogen flow rate, 30.4 ml./min.; and air flow rate, 300 ml./min. Peak areas and retention times were obtained using a Varian Aerograph model 481 electronic digital integrator.

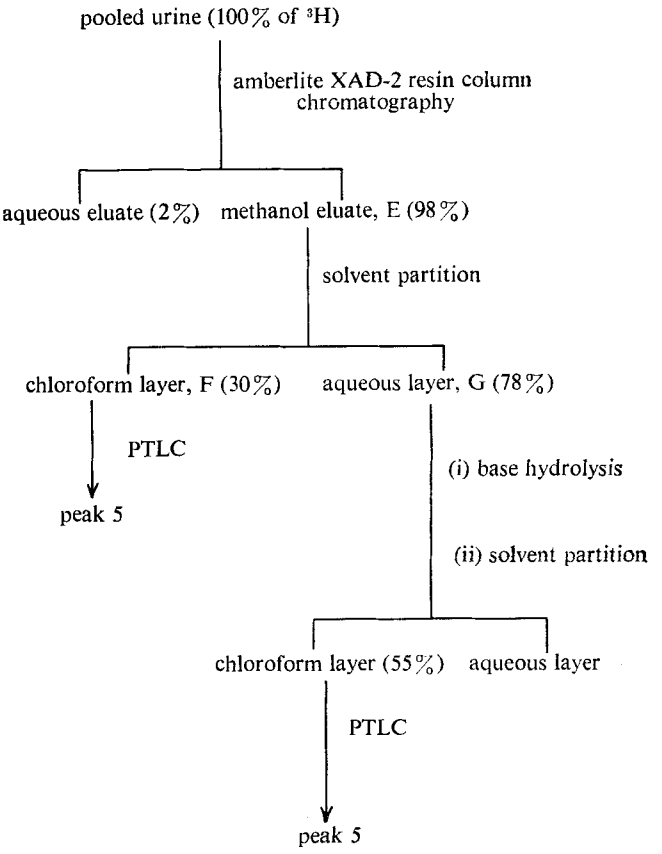
The smallest quantity of reference Compounds III, VI, VII, and VIII that could be accurately measured using these conditions was 0.05 mcg. To test the recoveries of canrenone (III) and hydroxy acid (II) from plasma and urine, known amounts of these compounds were added to the zero-hour urine and plasma samples, and the isolation procedures indicated in Schemes II and III were repeated. The recoveries of II and III from plasma ranged between 75 and 80%, while the recovery of III from urine was between 90 and 95%.

Spectroscopic Analysis—IR spectra were measured in chloroform using an ultramicro cell in a Beckman IR-12 instrument. UV absorption spectra were determined in methanol in a Beckman DK-2A ratio recording spectrometer.

Hydrolysis of Water-Soluble Conjugated Fraction—For base hydrolysis, potassium hydroxide (1.5 g.) was dissolved in an aliquot of Extract G (50 ml., 2 × 10^5 d.p.m.), which was left at room temperature for 12 hr. The extract was acidified (pH 3) with dilute hydrochloric acid, and the free steroids were extracted with chloroform. This procedure was repeated on another aliquot of Extract G without the addition of base.

Acidic hydrolysis was done by refluxing an aliquot of Extract G (4 × 10^6 d.p.m.) in 2 *N* hydrochloric acid (20 ml.) for 1 hr. The released aglycone was extracted with chloroform.

For enzyme hydrolysis, aliquots of conjugated Extract C or G (6 × 10^5 d.p.m.) in acetate buffer at pH 5.1 (25 ml.) were incubated at 37° for 48 hr. with 1 ml. (5000 Fishman units) of Ketodase[7]; the



Scheme III—Isolation scheme for separation of metabolites from pooled urine

[5] Applied Science.
[6] Johns-Manville.
[7] Warner-Chilcott.

Table I—Excretion of Radioactivity (Percent Administered ^3H) in the Urine and Feces of Men after Intravenous Administration of SC-14266-^3H

	Subjects					
	A		B		C	
Day	Urine	Feces	Urine	Feces	Urine	Feces
1	44.49	0.05	34.50	0.80	34.93	0.24
2	7.35	0.60	5.17	2.01	4.74	5.82
3	2.15	3.77	2.90	9.54	1.47	6.24
4	0.81	9.20	1.21	0.01	0.58	1.05
5	0.86	0.98	0.83	2.82	0.37	0.96
Total	55.66	14.60	44.61	14.18	42.09	14.31

major free steroid liberated was extracted with chloroform and purified by PTLC.

Preparation of Methyl Ester Acetate of the Conjugated Metabolite (Peak 1)—An aliquot of urinary Extract G (2×10^6 d.p.m.) in methanol (10 ml.) was treated with an excess of an ethereal solution of diazomethane at 0°. After 30 min., the solvent and the excess of diazomethane were removed in a stream of nitrogen, and the residue was dissolved in 20 ml. of pyridine–acetic anhydride (1:3) mixture and left at room temperature for 12 hr. The reagents were removed in a stream of nitrogen; the residue, dissolved in benzene (2 ml.), was passed through a 7.5-g. column (12×1 cm.) of silica gel packed in benzene. The column was first eluted with benzene (40 ml.) followed by chloroform (40 ml.) and then 5% methanol in chloroform (40 ml.). The last eluate, which contained 71% of the radioactivity applied to the column, was further purified twice by PTLC (solvent system of 2.5% methanol in chloroform). The UV-absorbing radioactive zone (R_f 0.4–0.6) was eluted with a methanol–methylene chloride (20:80) mixture, and the residue was subjected to spectral examination.

Calculation of Pharmacokinetic Parameters—The nonlinear plasma radioactivity curve obtained after an intravenous administration of SC-14266-^3H was analyzed by both biexponential and triexponential decay functions. The mathematical expressions for these relationships are given in Eqs. 1 and 2:

$$\text{biexponential decay:} \quad C_p = h_1 e^{-\alpha t} + h_2 e^{-\beta t} \qquad \text{(Eq. 1)}$$

$$\text{triexponential decay:} \quad C_p = h_1 e^{-\alpha t} + h_2 e^{-\beta t} + h_3 e^{-\gamma t} \qquad \text{(Eq. 2)}$$

where C_p = plasma radioactivity at time t; h_1, h_2, and h_3 = extrapolated zero-time intercepts of three phases; and α, β, and γ = hybrid rate constants corresponding to these three phases. The best values of constants h_1, h_2, h_3, α, β, and γ were obtained by the iterative least-squares method with a digital computer. This method gave values of the constants that were independent of personal error in drawing the semilogarithmic plots of plasma radioactivity concentration *versus* time.

The volume of the central compartment (V_p) in the case of the triexponential decay curve was evaluated by use of Eq. 3 given by Loo *et al.* (12):

$$V_p = \frac{\text{dose}}{h_1 + h_2 + h_3} \qquad \text{(Eq. 3)}$$

The plasma clearance was evaluated by use of Eq. 4:

$$\text{plasma clearance} = \frac{\text{dose}}{\text{plasma area}} \qquad \text{(Eq. 4)}$$

where area under blood level curve is from time = 0 to time = ∞.

RESULTS AND DISCUSSION

Distillation of water from plasma and urine samples showed that less than 1% of the ^3H in these samples was present as tritiated water. This finding suggests that the label on the steroid was not appreciably displaced during metabolic transformations. Since SC-14266 was labeled in the side chain, only those compounds that retained the side chain were detected.

The urinary and fecal excretions of the total radioactivity at different time intervals are listed in Table I. Within 5 days, 47.45 ± 4.17% of the administered radioactivity was excreted in the urine and 14.36 ± 0.12% in the feces; 37.97 ± 3.26% of the urinary radioactivity was excreted in the first 24 hr. The time course of the excretion of the radioactivity in the feces was very erratic. No attempt was made to investigate the fecal metabolic composition. Complete recovery of the label was not achieved, probably because of the short period for fecal collection and the relatively long half-life of the conjugated metabolite.

Plasma Metabolic Composition—The TLC distribution pattern of the total plasma metabolites 1 hr. after an intravenous administration of the drug to Subject A is shown in Fig. 1A. The R_f values of peak 5 (16.5%, R_f 0.76) and peak 3 (42.2%, R_f 0.29) were identical to canrenone (III) and the hydroxy acid (II), respectively. Since the parent drug (I) is a potassium salt of II, the conversion of I to II would be expected to take place instantaneously. Thus, the biotransformation and pharmacokinetic profile of the administered drug I would be that of the acid II. A large percentage of the radioactivity (31.6%) was associated with the polar metabolite (peak 1) which

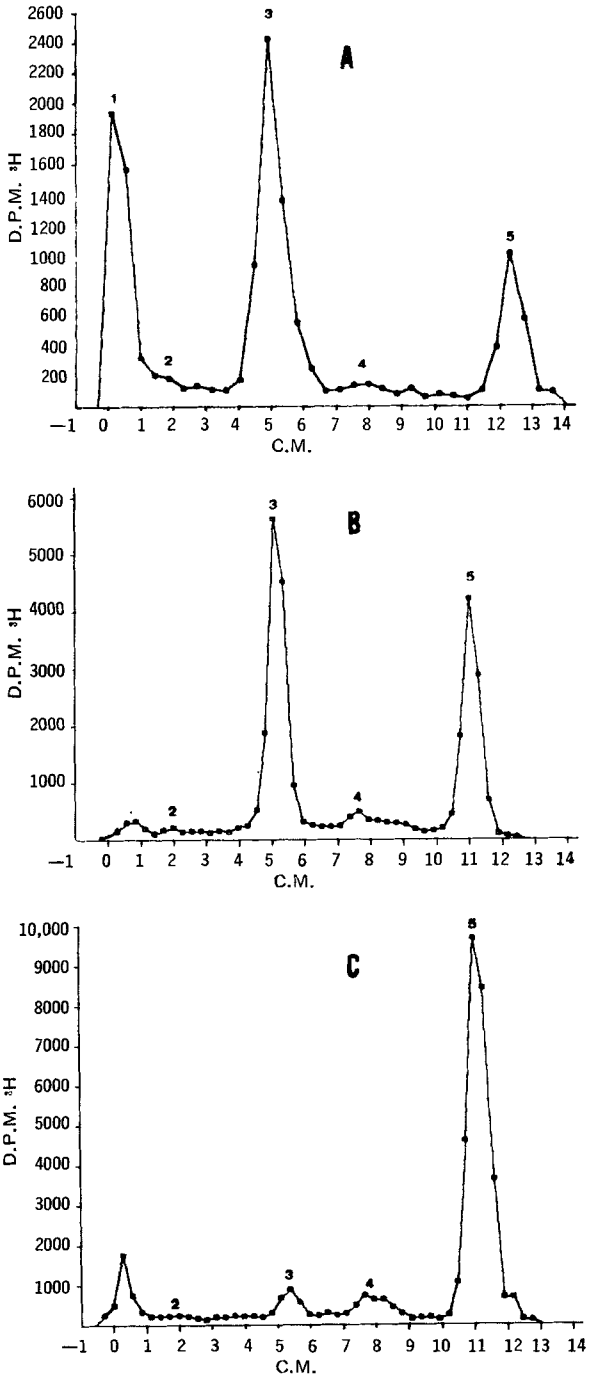


Figure 1—*Radiochromatograms of plasma metabolites. For identities of peaks, see text. Key: A, Extract A, free plus conjugated metabolites; B, Extract D, free metabolites; and C, free metabolites after enzymatic hydrolysis of Extract C.*

remained at the origin. This polar compound was also isolated from urine, and its structure is discussed later.

To characterize the plasma metabolites further, all plasma samples of Subject A were pooled and the total radioactivity was fractionated between the free fraction (Extract B) and the conjugated fraction (Extract C). To remove the plasma lipids, chloroform Extract B was fractionated between hexane and 10% aqueous methanol. The latter fraction contained 38% of the plasma radioactivity, while 60% was present as water-soluble metabolites in Extract C. The metabolic composition of the free fraction (Extract D) is given in Fig. 1B, which shows that 28.2% of the radioactivity was present in peak 5 whose mobility coincided with canrenone. This chromatographic system, however, did not resolve canrenone

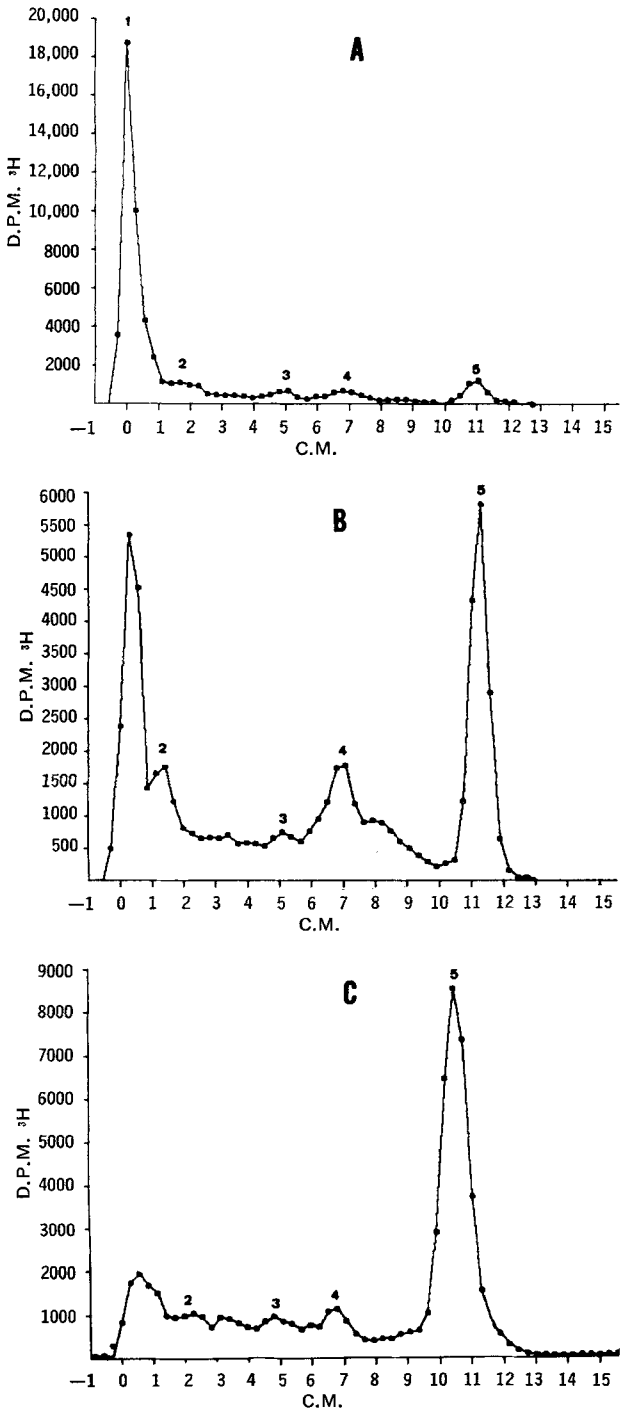


Figure 2—*Radiochromatograms of urinary metabolites. For identities of peaks, see text. Key: A, Extract E, free plus conjugated metabolites; B, Extract F, free metabolites; and C, free metabolites after enzymatic hydrolysis of Extract G.*

Table II—Total Radioactivity in Plasma after Intravenous Administration of SC-14266-³H in Subjects A, B, and C

Time	Percent ³H per Liter Plasma — Subject A	B	C	Mean	*SEM*, ±
1 min.	12.8	24.1	20.66	19.19	3.34
3 min.	10.9	18.9	13.41	14.40	2.36
5 min.	9.81	19.5	11.76	13.69	2.96
10 min.	7.10	10.9	9.73	9.24	1.12
15 min.	5.51	9.28	6.95	7.25	1.10
30 min.	4.02	6.67	4.90	5.20	0.78
45 min.	2.84	6.71	3.85	4.47	1.16
1 hr.	2.27	5.63	3.77	3.89	0.97
1.5 hr.	2.02	4.31	2.46	2.93	0.70
2.0 hr.	1.67	3.74	2.16	2.52	0.62
2.5 hr.	1.47	2.88	2.07	2.14	0.41
3.5 hr.	1.24	1.94	1.58	1.59	0.20
4.0 hr.	1.13	1.86	—	1.50	0.37
6.0 hr.	1.28	1.20	1.79	1.42	0.18
8.0 hr.	1.04	1.35	1.40	1.26	0.11
10.5 hr.	0.93	1.10	1.42	1.15	0.14
11.8 hr.	1.06	0.95	1.44	1.15	0.15
18.0 hr.	0.81	0.83	1.29	0.98	0.16
24.0 hr.	0.99	0.81	—	0.90	0.13

from its dihydro and tetrahydro derivatives VI, VII, and VIII. These compounds may be regarded as potential metabolites. An excellent separation of canrenone and its reduced derivatives was obtained by GLC, using an OV-17 column. The retention times of Compounds III, VI, VII, and VIII were 24.8, 22.7, 17.5, and 14.6 min., respectively. GLC analysis indicated that peak 5 was canrenone, and there was no evidence of Compound VI, VII, or VIII. On mild acid treatment of peak 3, a change in its polarity from R_f 0.31 to 0.70 was observed. The less polar product was identified as canrenone by GLC. This change in the polarity can be explained as lactonization of the hydroxy acid (II) to the γ-lactone (III).

The water-soluble conjugates in the plasma were present in Extract C. After enzymatic hydrolysis, 80% of the radioactivity was extractable by chloroform. The thin-layer radiochromatogram of the free fraction thus released is shown in Fig. 1C. The major aglycone (peak 5, 75%) was identified as canrenone (III) by TLC and GLC analyses.

Urinary Metabolic Composition—The Amberlite XAD-2 resin[8] (13) chromatography of the pooled urine removed large amounts of the nonradioactive materials in the aqueous eluate. The methanol eluate (Extract E) contained 98% of the radioactivity applied to the column. The metabolic composition of this extract, which represented the total urinary radioactive metabolites, is shown in Fig. 2A. The major urinary metabolite was a polar compound (peak 1, 70.8%) which remained at the origin of the TLC plate. The mobilities of peak 5 (6.7%, R_f 0.68) and peak 3 (4.8%, R_f 0.31) were similar to canrenone (III) and hydroxy acid (II). Peak 4 (6.1%, R_f 0.41) and peak 2 (9.6%, R_f 0.09) were due to unknown metabolites.

On further fractionation of Extract E, 30% of the radioactivity was extracted with chloroform (Extract F) while the remainder was present as water-soluble material (Extract G). The metabolic composition of the chloroform-soluble Extract F is shown in Fig. 2B. In this radiochromatogram, 27.6% ofthe radioactivity was associated with canrenone (peak 5, III), 23% with unknown metabolite peak 4 (R_f 0.45), and 12% with polar peak 2; 25% remained at the origin of the plate.

Structure Elucidation of the Conjugated Metabolite (Peak 1)—In both plasma and urine, the major metabolite was the water-soluble conjugate. To investigate the nature of the conjugated functionality, the urinary conjugate (Extract G) was subjected to various hydrolytic procedures. Chloroform extraction of the conjugated fraction at pH 3 gave 30% of the radioactivity in the organic phase. After acid hydrolysis, 83% of the radioactivity was extracted by chloroform. TLC analysis of the aglycone released after acid hydrolysis indicated the presence of a highly fluorescent compound with an R_f value of 0.64. This compound showed a maximum UV absorption at 355 nm. No further attempt was made to characterize this material. Riegelman *et al.* (14) found that spironolactones possessing a 4,6-dien-3-one grouping gave highly fluorescent com-

[8] Rohm & Haas, Philadelphia, Pa.

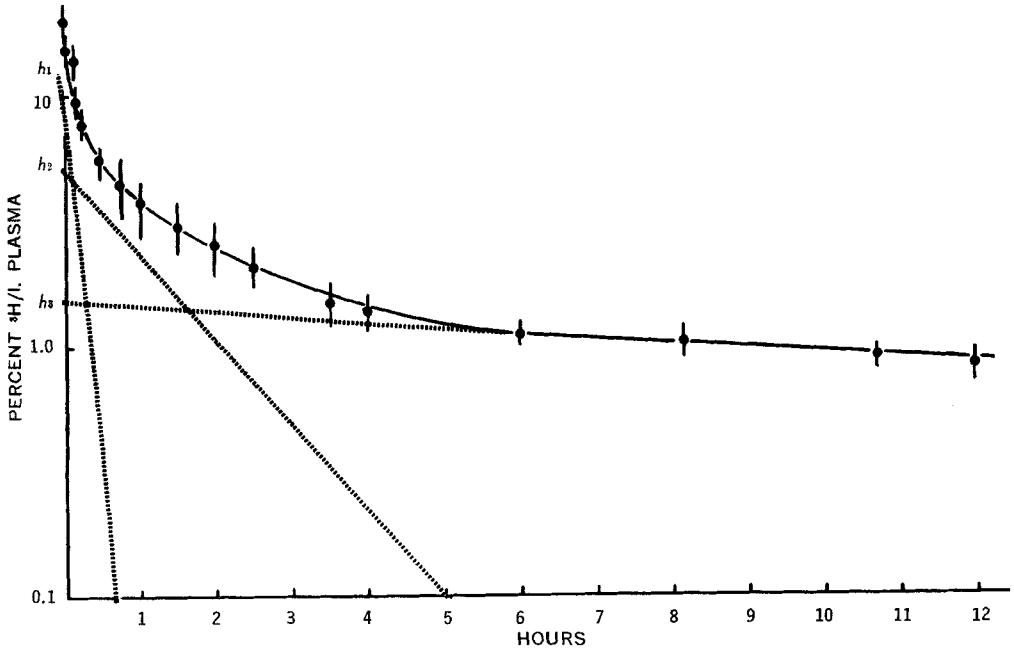


Figure 3—*Semilogarithmic plot of the mean plasma total radioactivity after intravenous administration of SC-14266-³H to Subjects A, B, and C. Vertical bars represent standard errors of the mean.*

pounds on treatment with an acid. The formation of the fluorescent compound with acid is also the basis for the estimation of canrenone in biological fluids (15). After mild base hydrolysis, 70% of the radioactivity was extracted by chloroform, giving an aglycone with TLC and GLC behavior identical to canrenone. Release of the free steroid on mild base hydrolysis suggested the presence of an ester linkage in the conjugate. After enzymatic hydrolysis, 60% of the radioactivity was extracted by chloroform; the major aglycone thus released (55%, peak 5, Fig. 2C) was identified as canrenone by TLC and GLC analyses.

To provide spectral evidences for the structure of the conjugate, the urinary water-soluble Extract G was methylated with diazomethane and the product acetylated with acetic anhydride–pyridine mixture at room temperature.. The acetylated methyl ester thus obtained was purified by silica gel column chromatography followed by PTLC. The IR absorption spectrum of this derivative gave strong absorption bands at 1745, 1245, and 1040 cm.$^{-1}$, which were characteristic of four ester groups of the sugar moiety (16). The absorption bands at 1670 and 1620 cm.$^{-1}$ and UV maximum at 282 nm. were indicative of the steroidal 4,6-dien-3-one chromophore. The IR spectrum also showed a weak broad absorption at 3480 cm.$^{-1}$, suggesting the presence of an unacetylated hydroxyl group. These spectroscopic data were compatible with Structure IV for the acetylated methyl ester derivative of the conjugate; thus, Structure V was proposed for the parent conjugate (peak 1).

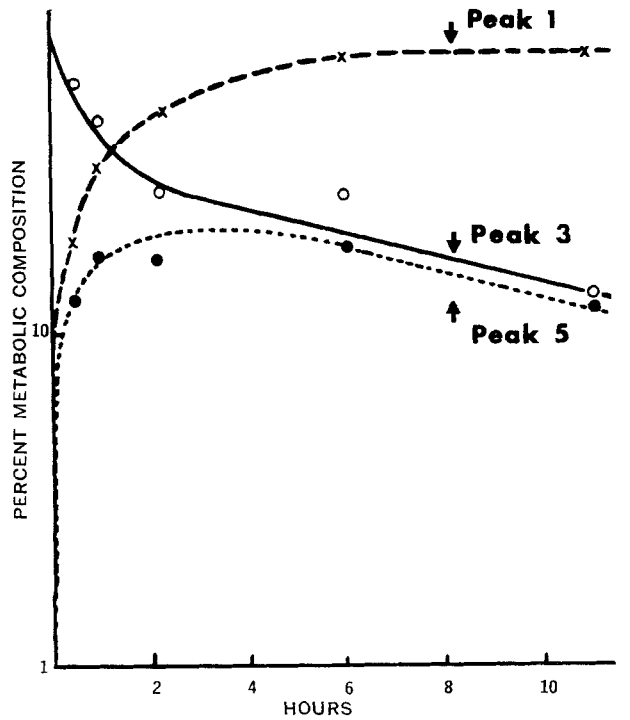


Figure 4—*Time course composition of peaks 1, 3, and 5 in plasma of Subject B. For identities of peaks, see text.*

Pharmacokinetic Analysis of Plasma Radioactivity—The plasma radioactivity (total) expressed as percent ³H per liter of plasma in Subjects A, B, and C is given in Table II. Figure 3 illustrates the semilogarithmic plot of the mean plasma levels. The curve in this figure is clearly nonlinear; by the method of residual analysis, it could be resolved into three distinct phases. The best values for the slope of each phase and their corresponding zero-ordinate concentration intercepts were derived by digital computer and are given in Table III. Immediately after injection of SC-14266-³H, there was a rapid phase of disappearance of the total label, with a half-life of only 0.073 hr. After 1 hr., a second phase, with a half-life of 0.849 hr., was observed; from 6 hr. onward, the total label was eliminated very slowly with a half-life of 43.31 hr.

To study the kinetics of elimination of individual plasma metabolites, the nature of compounds present in the plasma samples of Subject B were analyzed by TLC. The radioactivity in the plasma was attributed to three major compounds. These compounds were identified as already described as canrenone (peak 5), its hydroxy acid analog (peak 3), and the glucuronic acid ester conjugate of the hydroxy acid (peak 1). The time course composition of these plasma metabolites in Subject B is illustrated in Fig. 4. The percentage composition in the plasma due to the hydroxy acid (peak 3) decreased rapidly from 0 to 3 hr., while the percentage composition due to canrenone (peak 5) increased rapidly during this period. After 3 hr., the percentage composition of both hydroxy acid and canrenone remained about the same and decreased with a half-life of 8 hr. These results suggest that after 3 hr., an equilibrium between the hydroxy acid and its corresponding γ-lactone was established. The percentage composition of the conjugate (peak 1) increased very sharply; after 6 hr., 60% of the plasma radioactivity was associated with this compound.

For evaluation of pharmacokinetic parameters of the hydroxy acid (peak 3), the radioactivity in each plasma sample of Subject C was separated by TLC. The semilogarithmic plot of peaks 1, 3, and 5 is given in Fig. 5. The results were essentially similar to those obtained with Subject B. The plasma radioactivities due to the hydroxy acid and to canrenone were similar after 3 hr., and their levels declined with a half-life of 8.25 hr. The plasma level due to the conjugate (peak 1) decreased sharply initially, showed a gradual rise between 1 and 4 hr., and then declined very slowly with a half-life of 40

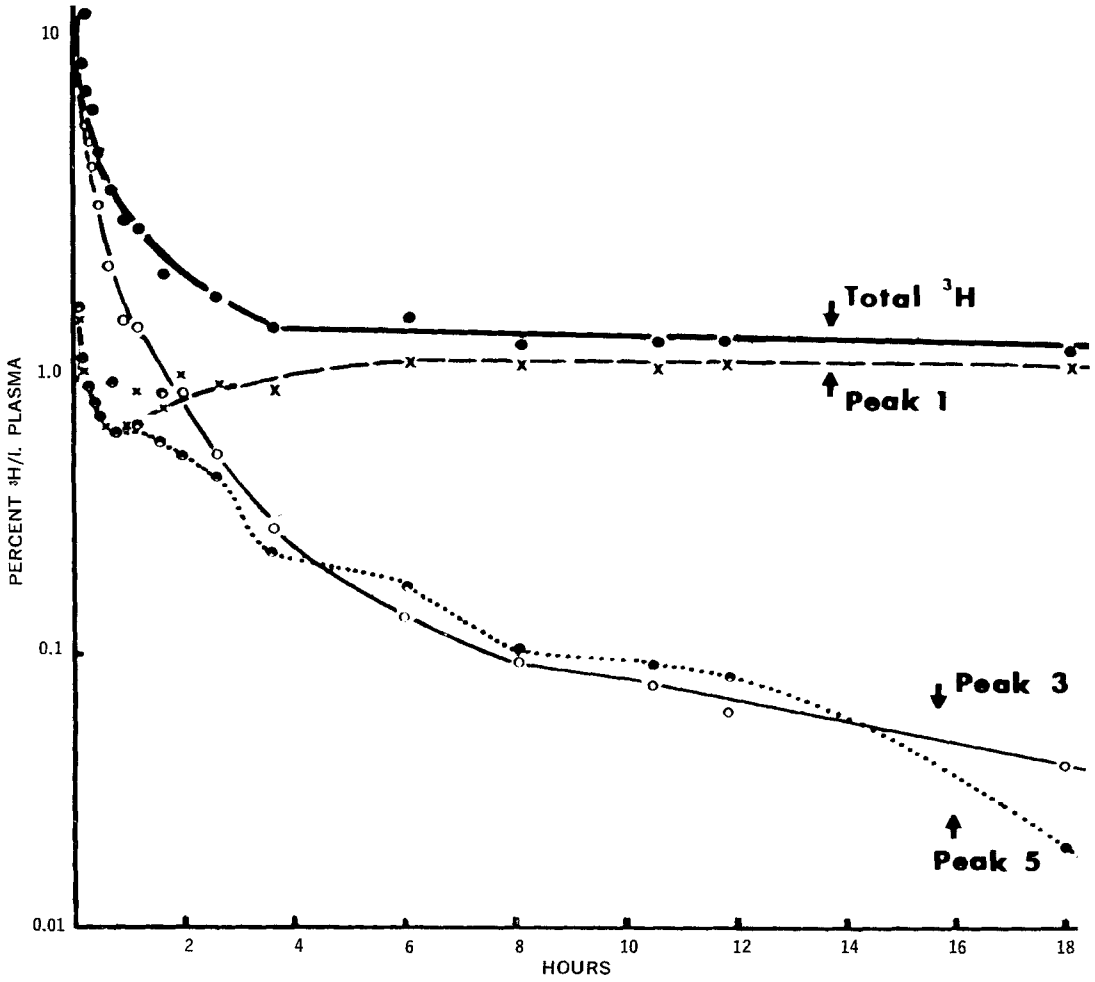


Figure 5—*Semilogarithmic plot of peaks 1, 3, and 5 in plasma of Subject C. For identities of peaks, see text.*

Table III—Kinetic Parameters[a] Calculated from Plasma Radioactivity Data Obtained after Intravenous Injection of SC-14266-³H

Subject	Radioactivity	h_1, % ³H/l.	α, hr.$^{-1}$	$t_{1/2}$, hr.	h_2, % ³H/l.	β, hr.$^{-1}$	$t_{1/2}$, hr.	h_3, % ³H/l.	γ, hr.$^{-1}$	$t_{1/2}$, hr.	Area, % ³H·hr./l.	Clearance, l./day
Mean of A, B, and C	Total ³H	13.86	9.461	0.073	5.661	0.816	0.849	1.390	0.016	43.31	95.28	25.19
C	Total ³H	10.26	5.903	0.117	4.224	0.931	0.744	1.727	0.018	38.5	102.22	23.48
C	Peak 3 ³H	8.028	4.721	0.147	2.422	0.712	0.973	0.193	0.084	8.25	9.98	240.48

[a] See text for definition of symbols.

Table IV—Analysis of the Radioactivity[a] in the Urine after Intravenous Administration of SC-14266-³H to Subjects A, B, and C

	Total ³H					"Free Fraction" ³H					Percent Free Fraction				
	Subject				*SEM*,	Subject				*SEM*,	Subject				*SEM*,
Hours	A	B	C	Mean	±	A	B	C	Mean	±	A	B	C	Mean	±
0–1	7.71	4.36	6.35	6.14	0.97	4.28	2.53	3.54	3.45	0.51	55.5	58.0	55.7	56.4	0.80
1–2	5.82	5.01	2.86	4.56	0.88	1.94	1.92	0.97	1.61	0.32	33.3	38.3	33.9	35.17	1.58
2–3	5.04	4.94	5.59	5.19	0.20	1.42	1.61	1.46	1.50	0.06	28.2	32.6	26.1	28.97	1.92
3–4	4.03	2.46	3.18	3.22	0.45	1.14	0.63	0.96	0.91	0.15	28.3	25.6	30.2	28.03	5.33
4–8	8.90	7.23	6.02	7.38	0.83	2.45	1.76	1.86	2.02	0.22	27.5	24.3	30.9	27.57	1.91
8–12	4.18	3.61	4.34	4.04	0.22	1.11	0.87	1.25	1.08	0.11	26.5	24.1	28.8	26.47	1.36
12–24	8.81	6.89	6.57	7.42	0.70	2.18	1.53	1.93	1.88	0.19	24.7	22.2	29.4	25.43	2.11
24–36	6.39	3.87	3.23	4.50	0.96	1.60	0.99	0.99	1.19	0.20	25.0	25.6	30.6	27.07	1.78
36–48	0.96	1.30	1.51	1.26	0.16	0.25	0.32	0.48	0.35	0.07	26.0	24.6	31.8	27.47	2.20
48–72	2.15	2.90	1.47	2.17	0.41	0.61	0.71	0.54	0.62	0.05	28.4	24.5	36.7	29.87	3.60
72–96	0.81	1.21	0.58	0.87	0.18	0.25	0.34	0.19	0.26	0.04	30.9	28.1	32.7	30.57	1.34
96–120	0.86	0.83	0.37	0.69	0.16	0.23	0.24	0.14	0.20	0.03	26.7	28.9	37.8	31.13	3.39
Total	55.66	44.61	42.07	47.45	4.17	17.46	13.45	14.31	15.07	2.11					

[a] Expressed as percent administered ³H.

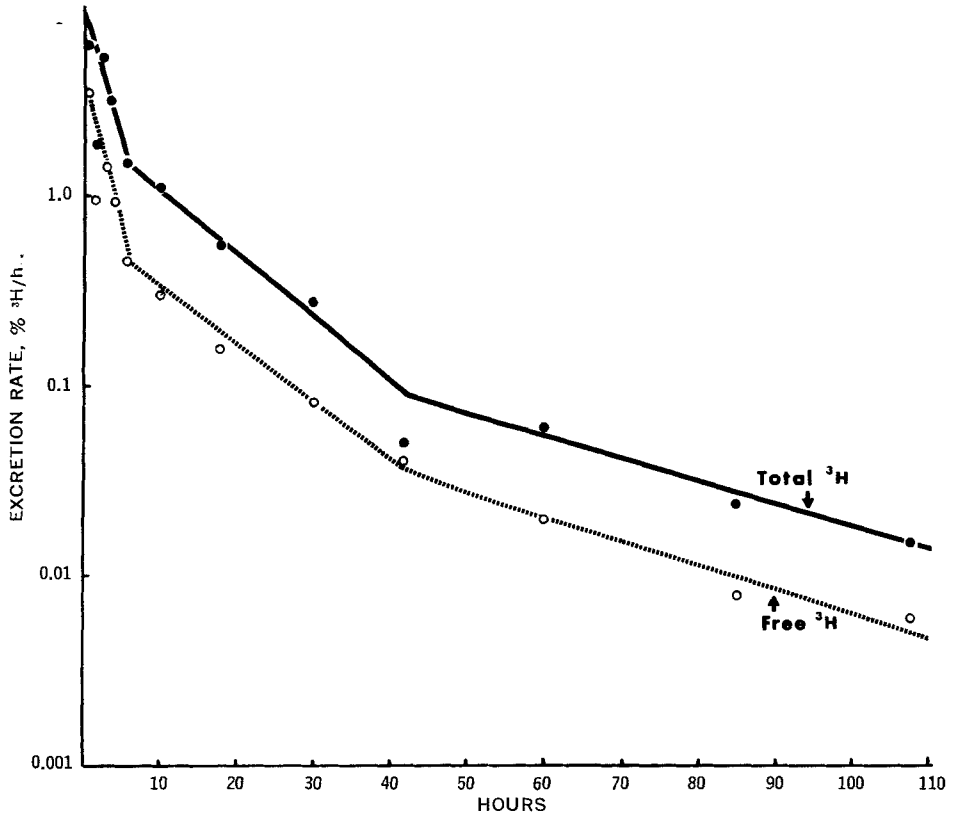


Figure 6—*Urinary excretion rate of total label and urinary free fraction in Subject C.*

hr. The rapid initial fall in the plasma level of the conjugate seen in Fig. 5 is difficult to rationalize. One explanation for this may be that the rapid decline was an artifact and not due to the conjugate. This view is supported by the facts that the injected SC-14266-^{3}H had 8% of a polar impurity and the radioactivity due to the polar compound 1 min. after intravenous administration was 9.2% of the total plasma radioactivity.

For evaluating the best model for the plasma disappearance of the hydroxy acid (peak 3), its plasma levels were tested for the best fit using both biexponential and triexponential functions. In the former case, the best values of the parameters describing the curve were

$$C_p = 8.243e^{-4.566t} + 2.367e^{-0.562t} \quad \text{(Eq. 5)}$$

For the triexponential equation, the best values of the parameters were

$$C_p = 8.028e^{-4.721t} + 2.422e^{-0.711t} + 0.193e^{-0.084t} \quad \text{(Eq. 6)}$$

Comparison of the theoretical curves obtained by use of these equations with experimental points showed that the best fit was obtained with the triexponential decay function. It is interesting to compare the half-lives of the three phases of elimination in Subject C due to total radioactivity and that due to the hydroxy acid. The half-lives and their corresponding zero time intercepts of the total label for the first and second phases were similar to that of the hydroxy acid, suggesting that, in these two phases, the elimination of the total label was predominantly a measure of the elimination of the hydroxy acid. However, the half-life of the third phase of the total plasma tritium was very long compared to that of the hydroxy acid, indicating that, in this phase, the elimination of the total label was predominantly a measure of the disappearance of the conjugated metabolite.

The volume of the central compartment (V_p) of the hydroxy acid in Subject C was evaluated as 9.4 l. and the plasma clearance of the total label in this subject was 23.4 l./day (Table III). This clearance value was one-tenth the value of the clearance of hydroxy acid.

Pharmacokinetic Analysis of Urinary Radioactivity—The total label excreted in the urine after intravenous administration of SC-14266-^{3}H to Subjects A, B, and C is given in Table IV. Within 5 days, 47.45 ± 4.17% of the administered radioactivity was recovered in the urine. The metabolic composition in the urine has already been described. Chloroform extraction of the pooled urine sample gave 30% of the urinary radioactivity as the free fraction in which canrenone (peak 5) was identified as the major metabolite. Seventy percent of the urinary radioactivity was present as the water-soluble metabolite which was identified as the glucuronic acid ester conjugate (peak 1) of the hydroxy acid (peak 3).

To study the kinetics of the urinary excretion of these metabolites, each urine sample of Subjects A, B, and C was extracted with chloroform to separate free and conjugated metabolites. Table IV shows the values of the free fraction present in the urine collected at different intervals. Except for the first urinary collection (0–1 hr.), the percentage of the free fraction excreted in the urine remained essentially constant (24–37%) in all three subjects. This is in contrast to the ratios of free and conjugated metabolites in the plasma, where in both Subjects B and C the percentage of the conjugate increased sharply after administration of the drug; after 24 hr., greater than 90% of the plasma radioactivity was associated with the conjugate. These differences in the ratio of the conjugate between plasma and urine suggest that some hydrolysis of the conjugate must have taken place after disappearance from the plasma and prior to excretion in the urine.

The urinary excretion rate (17) of both total label and urinary free fraction for Subject C is illustrated in Fig. 6. In both cases, three distinct phases of elimination were seen. For the free fraction, the half-lives of the three phases were 1.7, 10, and 29 hr.

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Keyphrases □ Ampicillin, sodium and trihydrate—pharmacokinetics □ Dicloxacillin, sodium—pharmacokinetics □ Absorption rates, half-lives, intramuscular injection—penicillins □ Biological availability—penicillins

The absorption rates of various dosage forms can be quantitated, at least comparatively, without knowledge of the absolute amount of drug absorbed (1); but this amount must be known to estimate availability reliably. If biological availability is defined in terms of the amount of drug that appears in the blood, the only method of drug administration certain to provide 100% of the dose to the blood is intravenous injection. Loo and Riegelman (2) developed a method for calculating the rate and extent of absorption of a drug from an extravascular dosage form when intravenous data are available. Often it is not practical during early stages of drug product development to administer a new drug intravenously to humans, and comparisons must be made between the dosage form candidate and a "readily available" extravascular dosage form. However, the use of extravascular reference dosage forms has important limitations which must be recognized by the investigator. It is not unusual to find that some drugs are not completely absorbed even from oral solutions. On the other hand, it is generally considered safe to assume that an intramuscular injection is completely absorbed. Intramuscular injections of penicillins do not appear to be completely absorbed; this paper deals with the hazards of interpreting penicillin serum data pharmacokinetically, especially with regard to biological availability, on the basis of intramuscular data.

The intramuscular route of administration is traditional for the penicillins, and many methods have been used to prolong their release from intramuscular injection sites. For example, suspensions of relatively insoluble penicillin salts have been used for this purpose. Ampicillin trihydrate is a relatively water-insoluble, chemically stable powder, and its potential use in formulating a sustained-release intramuscular dosage form of ampicillin was investigated. A dosage form that produces slow absorption of a drug which is rapidly eliminated presents an interesting pharmacokinetic case. If the kinetic model is assumed to be:

$$\text{dosage form} \xrightarrow{k_a} \text{blood} \xrightarrow{k_e} \text{eliminated drug}$$

Scheme I

and both processes are first order, it can be shown (3) that the rate constant corresponding to the descending curve of a semilogarithmic plot of blood level *versus* time is not necessarily k_e. In fact, such a procedure yields the smaller of the two rate constants whether it be k_a or k_e. In this paper, penicillin serum level data will be presented which demonstrate this point, and methods for handling such data will be discussed.

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The purposes of these studies were: (*a*) to compare the rates of release from intramuscular injection sites

Table I—Intramuscular Ampicillin and Dicloxacillin (20 Subjects)

Dose and Dosage Form	Mean Serum Levels (±*SD*), mcg./ml.							
	0	15 min.	30 min.	1 hr.	2 hr.	3 hr.	4 hr.	6 hr.	8 hr.
Ampicillin trihydrate suspension									
250 mg.	0	0.48 ±0.27	1.09 ±0.49	1.68 ±0.63	1.84 ±0.59	—	1.10 ±0.34	0.54 ±0.24	0.24 ±0.17
500 mg.	0	0.52 ±0.25	1.12 ±0.51	2.03 ±0.95	2.75 ±0.86	—	2.55 ±0.66	1.57 ±0.38	0.95 ±0.43
1000 mg.	0	1.30 ±2.0	2.27 ±2.0	3.67 ±2.1	4.84 ±2.6	—	4.71 ±1.6	3.12 ±1.1	2.28 ±0.78
Ampicillin sodium solution									
500 mg.	0	3.37 ±1.6	5.40 ±2.0	6.79 ±1.6	5.07 ±0.74	—	1.50 ±0.42	0.45 ±0.14	0.14 ±0.06
Dicloxacillin sodium solution with lidocaine									
250 mg.	0	—	4.67 ±1.0	5.07 ±0.79	4.61 ±0.65	3.12 ±0.46	2.18 ±0.39	1.14 ±0.34	—

of ampicillin trihydrate suspensions with that of ampicillin sodium solution, and (*b*) to determine the percent of the doses of ampicillin absorbed from the trihydrate suspensions and from the solution of the sodium salt administered intramuscularly. For comparison purposes, the rate of release and percent of the dose of sodium dicloxacillin, a highly protein-bound penicillin derivative absorbed following intramuscular injection, were also studied.

EXPERIMENTAL

Materials—The sodium ampicillin solution used in this study was commercially available[1] and reconstituted according to directions on the package insert. All other drug products were experimental formulations[2].

Clinical Protocol—The subjects were healthy adult volunteers ranging in age from 21 to 63 years and weighing from 125 to 215 lb. The majority were males; no females of childbearing age were included. To assure a relatively steady metabolic state, the subjects were fasted from 8 p.m. on the day preceding the administration of the drugs until after the 4-hr. postdrug blood specimen was drawn. The studies were usually begun at about 8 a.m. Following the 4-hr. postdrug specimen, the subjects were allowed food and water *ad libitum*. Venous blood specimens were collected at appropriate time intervals following drug administration.

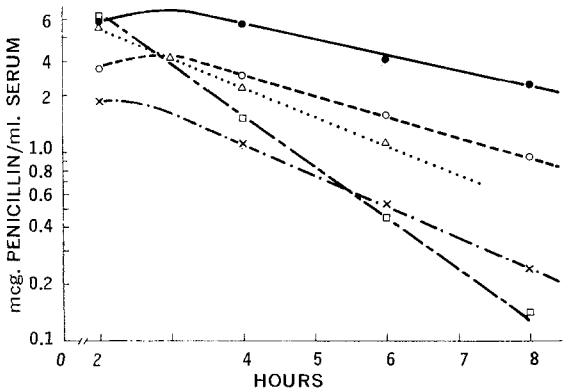


Figure 1—*Semilogarithmic plot of 20-subject average serum levels of penicillin activity following intramuscular administration of 1000 mg. (●), 500 mg. (○), and 250 mg. (×) of ampicillin trihydrate suspension; 500 mg. (□) of ampicillin sodium solution; and 250 mg. (△) of dicloxacillin sodium solution with lidocaine.*

Analytical Procedure—The serum was separated from the blood specimens by centrifugation and was frozen and shipped to be assayed[3] for penicillin activity by the standard cup-plate method (4).

RESULTS AND DISCUSSION

Twenty-subject average serum levels (± standard deviations) of ampicillin and dicloxacillin activity following intramuscular injection of various dosage forms of the two drugs are shown in Table I. Figure 1 shows semilogarithmic plots of the data; half-lives corresponding to the straight-line descending portions of the plots are summarized in Table II. With the exception of the ampicillin sodium solution, these half-lives are all significantly longer than 1 hr.

It is often assumed that the half-life obtained from the descending curve of a plot such as that shown in Fig. 1 corresponds to the half-life for elimination of the drug, *i.e.*, the biological half-life; and the rising portion of the curve is assumed to be associated with the absorption as well as the elimination process. Based upon these assumptions, Wagner and Nelson (1) developed a method for calculating the half-life of the absorption process. Their method involves calculation of the amount of drug unabsorbed; for drugs apparently absorbed by a first-order process, the absorption half-life is determined from a semilogarithmic plot of this quantity against time.

The apparent absorption half-lives for the five intramuscular penicillin dosage forms, as calculated by the method of Wagner and Nelson (1) using the half-lives from the descending curves shown in Fig. 1, are shown in the third column of Table II. These half-lives are all 1 hr. or less; with the exception of the ampicillin sodium solution, they correspond fairly closely with the elimination half-lives of the drugs following intravenous administration shown in the second column of Table II (5). These results suggest that the "absorption" half-lives calculated by the Wagner–Nelson (1) method are, in fact, the biological half-lives for elimination of the drugs, and the half-lives for the descending curves in Fig. 1 may correspond to the absorption half-lives of the dosage forms. The one exception is the ampicillin sodium solution for which the descending curve agrees with the intravenous elimination half-life, and the Wagner–Nelson "absorption" half-life is significantly shorter than those of the other preparations.

These apparent anomalies are the result of the mathematics of the kinetic model assumed for the *in vivo* behavior of these drugs and dosage forms.

If the model is:

$$\text{muscle} \xrightarrow{k_a} \text{blood} \xrightarrow{k_e} \text{eliminated drug}$$

Scheme II

[1] Polycillin-N for Injection, Bristol Laboratories, Syracuse, N. Y.
[2] Prepared by Bristol Laboratories, Syracuse, N. Y.
[3] At the Microbiology Control Department of Bristol Laboratories, Syracuse, N. Y.

Table II—Absorption and Elimination Half-Lives for Ampicillin and Dicloxacillin following Intramuscular Injection (20 Subjects)

Dose and Dosage Form	Elimination Half-Lives, hr. From Fig. 1[a]	Elimination Half-Lives, hr. From i.v. Data[b]	Absorption Half-Lives, hr. W–N Calc.[c]	Absorption Half-Lives, hr. W–N Calc.[d]	Absorption Half-Lives, hr. L–R Calc.[e]	Absorption-Rate Constant, hr.$^{-1}$, L–R Calc.
Ampicillin trihydrate suspension						
250 mg.	1.8	1.0	0.7	1.4	1.55	0.45
500 mg.	2.8	1.0	0.9	1.9	2.3	0.30
1000 mg.	3.6	1.0	1.0	2.9	3.3	0.21
Ampicillin sodium solution						
500 mg.	1.1	1.0	0.5	0.6	0.78	0.89
Dicloxacillin sodium solution with lidocaine						
250 mg.	2.0	0.88	0.8	2.0	1.3	0.53

[a] Half-lives determined from the slopes of the descending portions of the plots in Fig. 1. [b] Elimination half-lives from intravenous data (5). [c] Wagner–Nelson calculation (1) using elimination half-lives from Fig. 1. [d] Wagner–Nelson calculation (1) using elimination half-lives from intravenous data (5). [e] Loo–Riegelman calculation (2) using k_{12}, k_{21}, and k_{el} from intravenous data (5).

where k_a and k_e are the rate constants controlling first-order processes, then the blood levels of drug may be described in terms of the following biexponential equation:

$$\text{blood level} = \text{constant}\ (e^{-k_a t} - e^{-k_e t}) \qquad \text{(Eq. 1)}$$

For such a case, the slope of the descending curve of a semilogarithmic plot of blood levels against time yields the half-life of the *slower* of the two processes (3).

With many drugs, elimination is slower than absorption; the slope of the descending curve yields the biological half-life for elimination. The penicillins, however, are rapidly eliminated drugs having biological half-lives of about 1 hr. If the absorption of the penicillins from intramuscular injection sites is relatively slow, *i.e.*, if the half-lives are significantly longer than 1 hr., the slopes of the descending curves will be a function of the absorption half-lives. The results in Table II suggest that this is the case for all the penicillin dosage forms listed except the ampicillin sodium solution which has an apparent absorption half-life of 0.5 hr.

The Wagner–Nelson (1) method of calculation is useful with drugs for which no intravenous data are available to estimate elimination half-lives and for which it can be assumed that absorption is faster than elimination. In these cases, the calculation may be carried out using an elimination half-life estimated from the descending blood level curve following extravascular drug administration. But with drugs such as the penicillins, when elimination is faster than absorption, the half-life estimated from the descending curve will be the absorption half-life, and the half-life estimated from the Wagner–Nelson calculation will be the elimination half-life. On the other hand, if the elimination half-life of such drugs can be estimated from intravenous data, the Wagner–Nelson calculation should yield a reliable estimate of the absorption half-life even if elimination is faster than absorption. A somewhat similar approach was employed by Gibaldi and Schwartz (6) to calculate the rate of oral absorption in dogs of penamecillin, a prodrug of penicillin G. In this case, the biological half-life of penicillin G after oral administration to dogs was utilized in calculating the oral absorption rate of penamecillin by the Wagner–Nelson method.

Since intravenous elimination half-lives for ampicillin and dicloxacillin are available (5), they were employed in a repetition of the Wagner–Nelson calculation. The resulting absorption half-lives, shown in the fourth column of Table II, are in agreement with the half-lives corresponding to the descending curves shown in Fig. 1. The only exception is the ampicillin sodium solution for which the intravenous half-life is essentially the same as the half-life for the descending curve in Fig. 1; the absorption half-lives for the two Wagner–Nelson calculations are, therefore, equivalent. These results show with actual data how the slope of the descending portion of a semilogarithmic plot of drug blood levels following extravascular administration can be misleading with regard to the half-lives of the absorption and elimination processes. Wagner and Metzler (7) discussed the difficulties in estimating elimination half-lives in cases where absorption and elimination are occurring simultaneously. In such cases, the estimated elimination half-life may be significantly higher than the "best" value. In the present case, however, where absorption is slower than elimination, the estimates will be completely erroneous unless intravenous elimination half-lives are used.

The Wagner–Nelson calculations of absorption and elimination half-lives for the penicillin intramuscular dosage forms are based on the assumption that the body behaves as a one-compartment open model with respect to penicillin. But, a two-compartment body model was shown to be more consistent with intravenous penicillin data; the parameters of the two-compartment models for ampicillin and dicloxacillin were established (5). Furthermore, Loo and Riegelman (2) developed a method for calculating absorption half-lives for such drugs when the parameters of the two-compartment model are known. The fifth column of Table II shows the results of the Loo–Riegelman calculation of absorption half-lives for the intramuscular penicillin dosage forms. These results are probably the best estimates of the absorption half-lives, and they are in good agreement with the half-lives corresponding to the descending curves of Fig. 1, the only exception being the ampicillin sodium solution. Although there are some discrepancies among the absorption half-lives calculated by the two methods, both the Loo–Riegelman and the Wagner–Nelson calculations rank the dosage forms in the same order. Thus, either calculation could be used in ranking intramuscular dosage forms of the penicillins with regard to their absorption rates so long as the correct elimination-rate constants are used in the Wagner–Nelson calculation.

The results in Table II are of interest from a formulation point of view. The absorption half-life for the 500-mg. dose of ampicillin trihydrate suspension is about three times longer than that for a 500-mg. dose of ampicillin sodium solution, suggesting that slow dissolution of the particles in the trihydrate suspension can significantly prolong release of ampicillin from the intramuscular site. The differences in the absorption half-lives for the various doses of ampicillin trihydrate suspension are more difficult to rationalize, but the fact that absorption rate decreases as dose increases suggests that the surface area in contact with the injected dose may be a limiting factor when dose is varied.

The slow absorption rate of dicloxacillin solution compared with that of ampicillin solution suggests that some factor other than solubility or dissolution rate reduces the rate of diffusion of dicloxacillin from the intramuscular injection site. Similarities in the molecular weights and structures of ampicillin and dicloxacillin suggest that their diffusion coefficients in water would probably be similar. However, dicloxacillin is reported to be about 95% bound, whereas ampicillin is reported to be only about 20% bound, to plasma proteins (8); it is conceivable that binding to proteinaceous muscle tissue or to the proteins in interstitial fluids significantly slows the diffusion of dicloxacillin from the injection site. The absorption of dicloxacillin solutions was about as slow as that of ampicillin trihydrate suspensions.

In addition to absorption rates, which often are governed by rate of drug release, dosage forms are also evaluated in terms of the total amount of drug they release in the body, *i.e.*, their biological availabilities. Biological availability may be expressed in terms of the area under the plasma level–time curve following administration of the test dosage form compared with the area following administration of a "standard" dosage form (assuming the plasma clearance is constant). Usually, the standard dosage form is a solu-

Table III—Absorption Efficiencies and Biological Availabilities of Ampicillin and Dicloxacillin Intramuscular Dosage Forms (20 Subjects)

Dose and Dosage Form	Area under Serum Curve, hr. × mcg./ml.	Absorption Efficiency[a]		Biological Availability[c], $Area_{i.m.}/Area_{i.m.soln.}$
		$Area_{i.m.}/Area_{i.v.}$	L–R Calc.[b]	
Ampicillin sodium solution				
250 mg. i.v.	13[d]	100%	100%	—
500 mg. i.m.	20	77%[e]	78%	100%
Ampicillin trihydrate suspension				
250 mg. i.m.	8.2	63%	64%	82%
500 mg. i.m.	17	65%[e]	66%	85%
1000 mg. i.m.	37	71%[f]	71%	92%
Dicloxacillin sodium solution				
250 mg. i.v.	28[d]	100%	100%	—
Dicloxacillin sodium solution with lidocaine				
250 mg. i.m.	21	75%	76%	100%

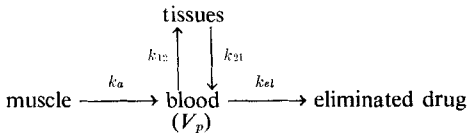
[a] Based on the intravenous dosage form as a reference standard representing 100% absorption. [b] $A_{max.}/dose \times 100$ (2). [c] Based on the intramuscular solution dosage form as a reference standard representing 100% bioavailability; *i.e.*, maximum absorption from the intramuscular site. [d] See *Reference 5*. [e] Based on 2× the area for the 250-mg. i.v. dose. [f] Based on 4× the area for the 250-mg. i.v. dose.

tion administered *via* the same route as the test dosage form, and it is assigned an availability of 100%. According to this conception of availability, only drug that is absorbed, *i.e.*, that reaches the blood, is "available." Often for practical reasons with new drugs intended for oral administration in solid dosage forms, the standard dosage form will be an oral solution; occasionally, an intramuscular injection will be used as a standard for oral availability. The latter approach is inconsistent with the above definition of biological availability and may even be misleading, because extravascular parenteral administration is not always equivalent to 100% absorption efficiency.

This point is illustrated by the data in Table III in which areas under serum level curves for intramuscular ampicillin and dicloxacillin dosage forms are compared with the areas for equivalent doses administered intravenously. Absorption efficiencies can also be computed by dividing the $A_{max.}$ values from the Loo–Riegelman calculations (2) by the doses administered, and these values are included in Table III for comparison purposes. The results show that, compared to the intravenous standards, a 250-mg. intramuscular dose of dicloxacillin sodium solution is only about 75–76% absorbed, as previously reported (8); a 500-mg. intramuscular dose of ampicillin sodium solution is only about 77–78% absorbed. Many factors may be responsible for an apparent absorption of less than 100% following an intramuscular injection of a solution. In the case of the penicillins, some of the drug may have been decomposed chemically or enzymatically at the injection site. Or, a fraction of the dose may have been released from the injection site so slowly that it produced undetectable blood and urine levels of active drug. It is less likely that unabsorbed drug remained permanently bound to the muscle tissue, although this is also possible. Intramuscular ampicillin trihydrate suspensions are apparently less well absorbed than intramuscular ampicillin sodium solution, the suspensions having biological availabilities of 82–92% based on the intramuscular solution standard.

These results illustrate that the intramuscular route of administration can be misleading as a standard for evaluating the availability of an oral dosage form. In fact, an early report (9) suggested that dicloxacillin was 100% absorbed orally because urinary output was identical to that found after intramuscular administration. Although this is not true, it can be said that dicloxacillin is as available orally as it is intramuscularly. The rates of absorption *via* the two routes are markedly different (10).

To test the agreement between the experimental data and the biological availabilities and absorption rate constants calculated by the Loo–Riegelman method, an analog computer was programmed with the following model:



Scheme III

where "muscle" represents the amount of drug remaining at the intramuscular injection site; "blood" represents the amount of drug in the "central compartment" (11) (the blood level at any time can be calculated by dividing this amount by the volume of the central compartment, V_p); "tissues" represents the amount of drug in the body but outside the central compartment; "eliminated drug" represents the amount of drug that has disappeared from the body by metabolic and excretory pathways; and k_a, k_{12}, k_{21}, and k_{el} are the rate constants controlling transfer among the "compartments."

The following rate constants and volumes were used for ampicillin and dicloxacillin (5):

	Ampicillin	Dicloxacillin
k_{12}	1.7 $hr.^{-1}$	2.0 $hr.^{-1}$
k_{21}	1.6 $hr.^{-1}$	2.0 $hr.^{-1}$
k_{el}	2.0 $hr.^{-1}$	2.1 $hr.^{-1}$
V_p	9.5 l.	4.4 l.

For each dosage form, the absorption-rate constant, k_a, was taken from the last column of Table II; the dose administered was the $A_{max.}$ value from the Loo–Riegelman calculation (2). (Actually, $A_{max.}/V_p$ was put into the computer so that it generated blood concentrations directly.) In each case, a slight lag time was also introduced.

Figure 2 shows the results of a typical analog simulation. The solid line was generated by the computer (with a lag time of 0.2 hr.), and the solid points are 20-subject average serum concentrations of ampicillin activity following intramuscular administration of 500 mg. of ampicillin sodium solution. A similar figure was

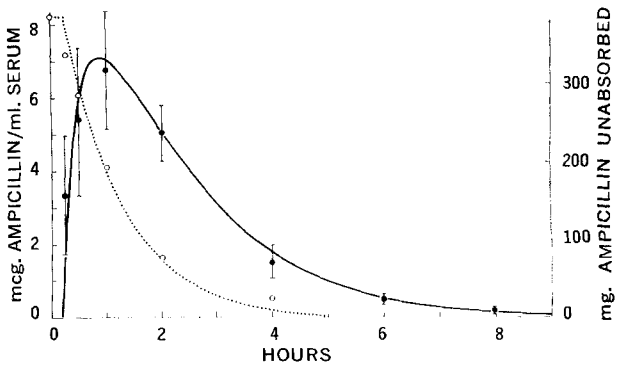


Figure 2—*A comparison between an analog computer-generated curve (solid line) using Scheme III and 20-subject average serum data points (●) (with standard deviations) following intramuscular administration of 500 mg. of ampicillin sodium solution. The dotted line is the analog computer-generated amount of ampicillin remaining unabsorbed and the open circles (○) are for this amount calculated by the Loo–Riegelman method (2).*

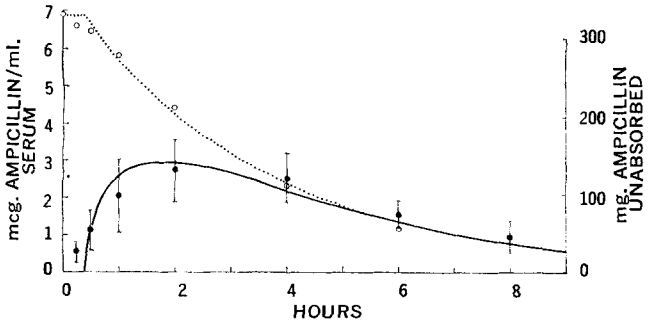


Figure 3—*A plot similar to that shown in Fig. 2 for 500 mg. of ampicillin trihydrate suspension administered intramuscularly.*

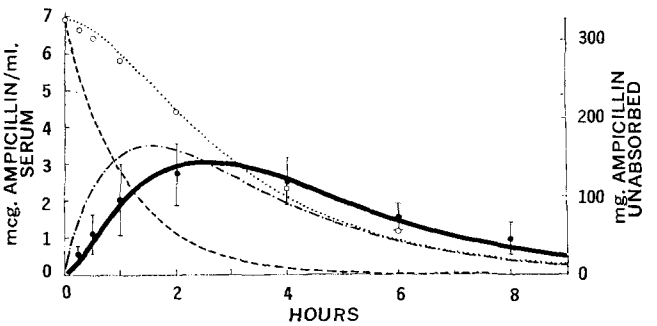


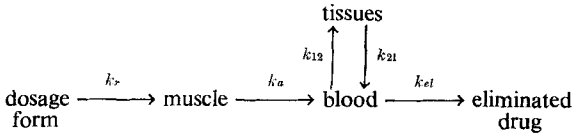
Figure 4—*A plot similar to that shown in Fig. 2 for 500 mg. of ampicillin trihydrate suspension administered intramuscularly. In this case, the mathematical model given by Scheme IV was used. The value of* $k_r = 0.45$ *hr.*$^{-1}$. *The lines (generated by the computer) are as follows:* ——, *serum level;* . . ., *amount of ampicillin remaining unabsorbed;* - - -, *amount of ampicillin remaining in the dosage form; and* - · - · - · -, *amount of ampicillin solution in the muscle.*

published previously (10) for intramuscular administration of 250 mg. of dicloxacillin sodium solution with lidocaine. These results suggest that previously published intravenous data for ampicillin and dicloxacillin (5) are consistent with the present intramuscular data for these drugs.

The dotted line in Fig. 2 is the analog computer estimate of the amount of drug remaining in the "muscle" compartment, and the open circles are the "amount remaining unabsorbed" as calculated by the Loo–Riegelman method (2). The agreement here suggests that the Loo–Riegelman calculation and the analog model are mutually consistent.

Figure 3 shows a comparison of the experimental blood levels (solid points) following intramuscular injection of 500 mg. of ampicillin trihydrate suspension and the theoretical blood curve (solid line) generated by the computer programmed with the model given by Scheme III using the absorption-rate constant given in Table II and a lag time of 0.35 hr. The dotted line is the analog computer estimate of the amount of drug remaining in the "muscle" compartment, and the open circles are the "amount remaining unabsorbed" as calculated by the Loo–Riegelman method (2). Although these lines agree fairly well with the experimental points, the overall shapes of the curves at early times are less than satisfactory. Many other absorption- and elimination-rate constants as well as many $A_{max.}/V_p$ values were tried with the program, but no combination fit the early blood points better than the combination of constants derived from the Loo–Riegelman calculations. In every case, the theoretical blood curve rose too rapidly at early times.

Absorption from a suspension depends upon both dissolution of the solid particles and subsequent absorption of the drug in solution. Therefore, the following modified model was tested:



Scheme IV

where "dosage form" represents the amount of ampicillin remaining in solid particles, "muscle" represents the amount of ampicillin solution remaining in the muscle, k_r is the first-order rate constant controlling dissolution of the solid drug, and k_a is the first-order rate constant controlling absorption of ampicillin in solution. This model is similar to a model used by Turco *et al.* (12) to describe plasma levels of sulfamethazine following oral administration of a suspension of the drug to humans. The absorption-rate constant, k_a, was set at 0.89 hr.$^{-1}$, the same rate constant that fits the 500-mg. ampicillin sodium solution data (Table II and Fig. 3); k_r was varied to give the best fit to the data points.

Figure 4 shows that, when k_r is set at 0.45 hr.$^{-1}$, the theoretical blood curve (bold solid line) agrees very well with the experimental blood concentrations (solid points) at all times without introducing a lag time. In addition, the theoretical "amount remaining unabsorbed" curve (dotted line) agrees with the amounts calculated by the Loo–Riegelman method (open circles). The latter curve is the sum of the amounts of drug in the "dosage form" and "muscle" compartments, and the curves for these compartments are also shown in Fig. 4 (dashed and dot-dashed lines, respectively).

It should be emphasized that, although Scheme IV fits the data very well, it was not the only model tested. Overall dissolution and absorption of suspensions may approximate a zero-order process, and many models were tested which involved pure zero-order and mixed zero- and first-order absorption mechanisms. None of the models tested agreed with the data points as well as the consecutive first-order model (Scheme IV), but this must not be construed as proof or disproof that the mechanism depicted by Scheme IV is or is not the true operating mechanism. These results prove only that Scheme III is probably an oversimplification for ampicillin trihydrate suspensions administered intramuscularly.

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Interaction of Bishydroxycoumarin with Polyvinylpyrrolidone

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Abstract □ Bishydroxycoumarin forms a highly soluble complex with polyvinylpyrrolidone in aqueous solution. A 1.0% solution of polyvinylpyrrolidone at pH 7.4 increased the solubility of bishydroxycoumarin, the increase being approximately 21.5 times that observed for the drug in the absence of the macromolecule. Equilibrium dialysis, viscometric, and spectrophotometric methods were used to study binding mechanisms. A polyvinylpyrrolidone molecule with a molecular weight of 40,000 provides approximately 50 identical sites for the bishydroxycoumarin molecule. This implies that one binding site consists of seven or eight monomer units of the polymer. The intrinsic association constant is in the order of 3×10^3 l./mole at 20° and decreases with an increase in temperature. Thermodynamic data indicate that hydrophobic bonds form at low temperature. UV spectral changes of the drug in the presence of the macromolecule suggest that the α,β-unsaturated lactone structure of bishydroxycoumarin is involved in the complexation. Intrinsic viscosity data indicate that coiling occurs during the binding process.

Keyphrases □ Bishydroxycoumarin, polyvinylpyrrolidone—interaction □ Complex formation—bishydroxycoumarin, polyvinylpyrrolidone □ Dialysis, equilibrium—bishydroxycoumarin, polyvinylpyrrolidone complex □ Viscometry—bishydroxycoumarin, polyvinylpyrrolidone complex □ UV spectrophotometry—analysis

Polyvinylpyrrolidone has several properties of pharmaceutical interest, including its ability to complex with drugs, dyes, toxins, and other substances (1). The results reported herein indicate that a highly soluble complex is formed when the polymer and bishydroxycoumarin are combined at a pH of 7.4. Studies were carried out to evaluate the mechanism of this interaction.

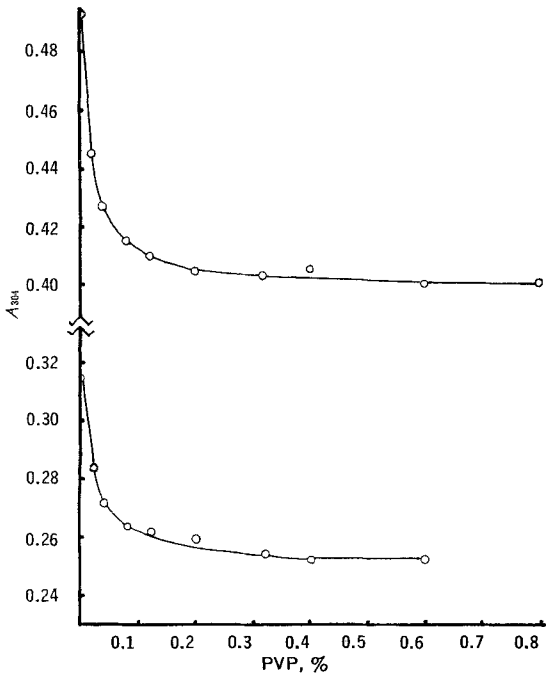

Figure 1—*Effect of polyvinylpyrrolidone on the* A_{304} *value for bishydroxycoumarin (upper curve: 26.5* $\times$ 10^{-6} *mole/l. bishydroxycoumarin; lower curve: 16.9* $\times$ 10^{-6} *mole/l.).*

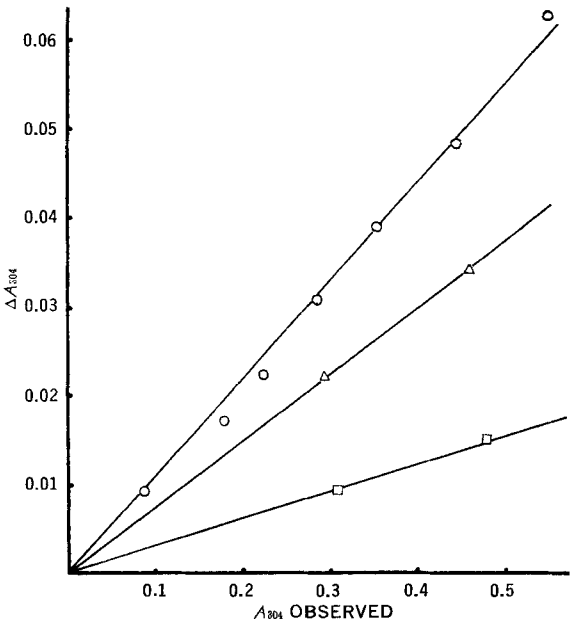

Figure 2—*Calibration curves for the analysis of bishydroxycoumarin in the presence of polyvinylpyrrolidone. Polyvinylpyrrolidone concentrations: 0.004%, □; 0.01%, △; and 0.02%, ○.*

EXPERIMENTAL

Materials—Specifications for bishydroxycoumarin, tris(hydroxymethyl)aminoethane, and dialyzer tubing were given in a previous paper (2). Polyvinylpyrrolidone[1] was extracted with anhydrous ether in a continuous-extraction apparatus for 24 hr. to remove any subfractions or impurities and then dried in a vacuum oven at 25–40°. The average water content, based on loss of weight at 110–115° for 24 hr., was 3.8%. A buffer system of pH 7.4 and ionic strength 0.15 [0.18 mole/l. tris(hydroxymethyl)aminoethane and 0.15 *N* HCl] was used in this investigation.

Spectrophotometric Analysis—Bishydroxycoumarin absorbs a maximum of radiant energy at 304 nm. The A_{304} value decreases as polyvinylpyrrolidone concentration increases. A series of solutions containing a constant quantity of bishydroxycoumarin and varying quantities of polyvinylpyrrolidone in tris(hydroxymethyl)aminoethane buffer was prepared, and A_{304} values were measured[2]. Tris(hydroxymethyl)aminoethane buffer was used as a reference solution. Results for two concentrations of bishydroxycoumarin in the presence of varying quantities of polyvinylpyrrolidone are shown in Fig. 1.

Calibration curves for the analysis of bishydroxycoumarin in the presence of polyvinylpyrrolidone are shown in Fig. 2. Three series of solutions containing varying amounts of bishydroxycoumarin and 0.004, 0.01, and 0.02% polyvinylpyrrolidone in tris(hydroxymethyl)aminoethane buffer were prepared. Polyvinylpyrrolidone solutions containing no bishydroxycoumarin were used as reference solutions. The A_{304} values were measured. A second set of solutions

[1] Plasdon C, General Aniline Corp., New York, N. Y.; average molecular weight, 40,000.

[2] A Beckman DU-2 spectrophotometer equipped with a thermospacer and maintained at 20° was used to measure A_s values.

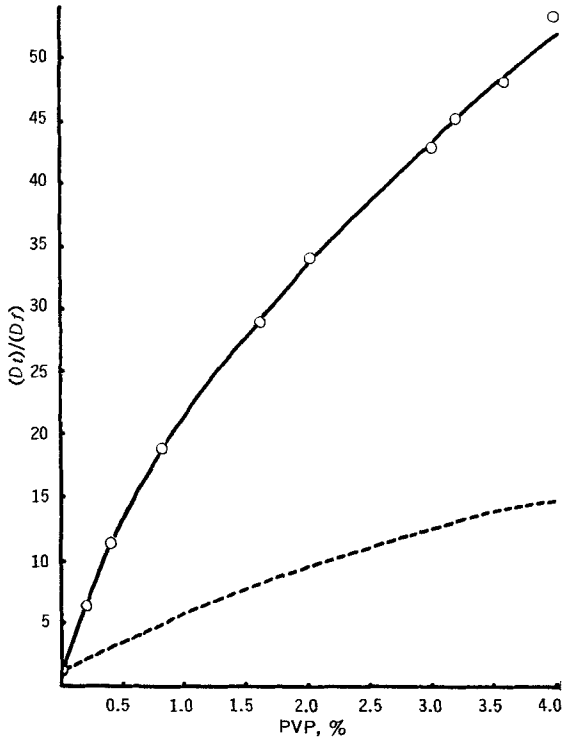

Figure 3—*Effect of polyvinylpyrrolidone on the apparent solubility of bishydroxycoumarin at 20°. Dotted line (see* Reference 2) *shows the effect of human serum albumin.*

containing identical quantities of bishydroxycoumarin but no polyvinylpyrrolidone was prepared. Tris(hydroxymethyl)aminoethane buffer was used as the reference solution. The A_{304} values were measured. For each pair of solutions containing identical quantities of bishydroxycoumarin, the former A_{304} value was subtracted from the latter A_{304} value. This yielded the ΔA_{304} value, which was then plotted against the A_{304} value for solutions containing polyvinylpyrrolidone. Results are shown in Fig. 2. For example, if a bishydroxycoumarin solution in 0.02% polyvinylpyrrolidone had an A_{304} value of 0.400, the corrected value of 0.443 was used to calculate bishydroxycoumarin concentrations.

Solubility Analysis and Equilibrium Dialysis Studies—Solubility and dialysis procedures were described in a previous paper (2). Dialysis experiments were carried out at 10, 20, 30, and 40° using 0.1 and 0.4% polyvinylpyrrolidone. A correction factor of 4% of the total bishydroxycoumarin was used to correct for losses due to membrane binding (2). It was reported (3) that some polyvinylpyrrolidone passes through the membrane even if the average molecular weight is over 40,000. A colorimetric method of analysis for polyvinylpyrrolidone (4) showed that approximately 0.25% of the total amount of polyvinylpyrrolidone present passed through the membrane. No correction, therefore, was made for polyvinylpyrrolidone losses through the membrane.

Viscometric Analysis—Using capillary viscometers[3], the intrinsic viscosity of polyvinylpyrrolidone in tris(hydroxymethyl)aminoethane buffer was determined in the presence and absence of bishydroxycoumarin at 10, 20, 30, and 40°. Temperatures were controlled to ± 0.05°. Polyvinylpyrrolidone concentrations ranged from 0.5 to 4.0%. The bishydroxycoumarin concentration was 1.85×10^{-4} mole/l. Procedures for the determination of intrinsic viscosity are given in the literature (6). The relative viscosities of bishydroxycoumarin solutions and tris(hydroxymethyl)aminoethane buffer were considered to be unity in the calculation of relative viscosity. Densities were measured using a calibrated Westphal balance[4] maintained at a constant temperature.

[3] Cannon Fenske routine viscometer (size 50), Cannon Instrument Co., State College, Pa. The instrument was calibrated with freshly distilled water. The kinetic energy correction term (5) was neglected.

[4] E. Machlett & Son Scientific Apparatus, New York, N. Y. The reproducibility was ±0.0001 density unit.

RESULTS AND DISCUSSION

Spectrophotometric Analysis—Polyvinylpyrrolidone alters the spectral characteristics of bishydroxycoumarin. A similar phenomenon was observed for the human serum albumin–bishydroxycoumarin interaction (2). It was suggested in that paper (2) that the α,β-unsaturated lactone structure present in bishydroxycoumarin was involved in the interaction. Figure 1 shows that the A_{304} value does not decrease beyond a certain polyvinylpyrrolidone concentration. This implies that, beyond that point, the bishydroxycoumarin molecules are in the bound form. On the basis of these data and those obtained from bishydroxycoumarin solutions containing no polyvinylpyrrolidone, the ε values for free (1.87×10^4) and bound (1.5×10^4) bishydroxycoumarin can be calculated. The concentration of free drug (D_f) and the r value can be estimated from the ε values for free and bound drug.

Solubility Analysis—The effect of polyvinylpyrrolidone on the apparent solubility of bishydroxycoumarin is illustrated in Fig. 3 which, for comparison, includes the effect of human serum albumin (2). The solubility of bishydroxycoumarin in tris(hydroxymethyl)-aminoethane buffer of pH 7.4 is 1.90×10^{-4} mole/l. The solubility curve is linear to about 0.4% polyvinylpyrrolidone.

The molar ratio of total drug to free drug, $(D_t)/(D_f)$, at a given macromolecule concentration has been widely used as a measure of binding. Literature values for approximately 40 compounds in 1.0% polyvinylpyrrolidone show that the ratio rarely exceeds 1.2. Bishydroxycoumarin has the unusually high value of 21.5. The molar ratio of bound drug to total macromolecule, $(D_b)/(M_t) = r$, is also frequently used as a measure of binding. The r value, calculated from the initial slope of Fig. 3, is 20.

Equilibrium Dialysis—Results for the dialysis experiments are shown in a Langmuir-type plot (Fig. 4). The binding curve is expressed by Eq. 1, which is derived from the law of mass action (7, 8):

$$r = \frac{nk(D_f)}{1 + k(D_f)} \qquad \text{(Eq. 1)}$$

where n is the maximum number of independent binding sites on the polymer, and k is the intrinsic association constant. The Langmuir-type plot (Fig. 4) shows that saturation of the binding sites was not achieved by the dialysis method. However, an upper limit to the binding curve was obtained from the solubility data, *i.e.*, $r = 20 = n$.

Equation 1 can be rearranged in a number of ways for convenient plotting to yield values of k and n by extrapolation, *e.g.*:

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nk(D_f)} \qquad \text{(Eq. 2)}$$

i.e., a plot of $1/r$ *versus* $1/(D_f)$ is a straight line with a slope of $1/nk$ and an intercept of $1/n$. The curves pass through approximately the same intercept, the reciprocal of which (*i.e.*, 50) was taken as the maximum number of binding sites on the polymer. Intrinsic association constants were calculated from the slopes at different temperatures and are listed in Table I.

Thermodynamic Analysis and Mechanism of Interaction—A van't Hoff-type plot is shown in Fig. 6. The standard enthalpy change, $\Delta H°$, at a given temperature was estimated from the tangent to the curve. Other thermodynamic functions were calculated in the usual way, including the "unitary" entropy change, ΔSu (9), and are reported in Table I. The unitary function depends only on factors that involve the interaction of bishydroxycoumarin molecules and the binding sites with the solvent and with each other. Contributions due to randomness of mixing with the solvent are excluded.

Table I—Thermodynamic Data for Interaction of Bishydroxycoumarin with Polyvinylpyrrolidone

Temperature	$k \times 10^{-3}$, l./mole	$\Delta H°$, kcal./mole	$\Delta G°$, kcal./mole	$\Delta S°$, e.u.	ΔSu, e.u.
10°	3.64	−2.74	−4.61	+6.63	+14.61
20°	3.05	−2.96	−4.67	+5.86	+13.84
30°	2.48	−4.69	−4.71	+0.05	+8.03
40°	1.72	−8.57	−4.64	−12.56	−4.58

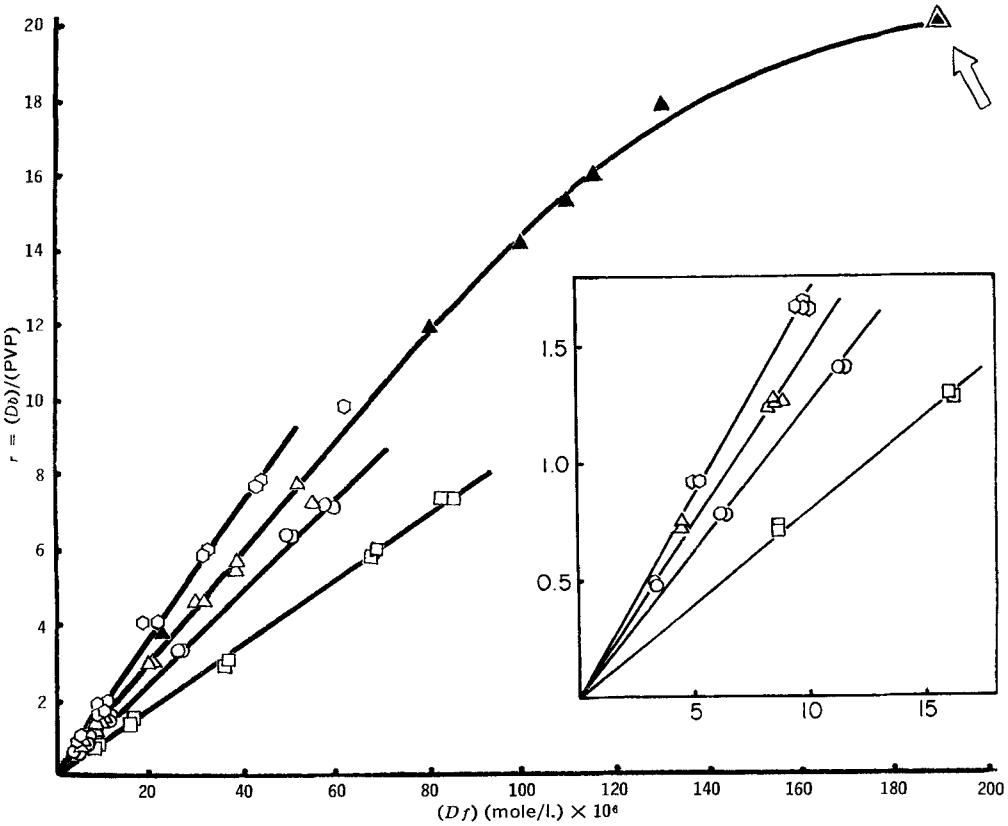

Figure 4—*Langmuir-type plot for the polyvinylpyrrolidone–bishydroxycoumarin interaction at 10°, ○; 20°, △; 30°, ○; and 40°, □. Polyvinylpyrrolidone concentrations are 0.4% (open symbols) and 0.1% (closed symbols). Solubility data are indicated by an arrow.*

The thermodynamic parameters obtained were interpreted on the basis of the "iceberg" concept of water structure (10). The number of hydrogen bonds surrounding the polyvinylpyrrolidone–bishydroxycoumarin complex is less than the number around the two unbound entities. Hence, it might be expected that complexation should be accompanied by a positive enthalpy change. Table I, however, shows that binding is exothermic from 10 to 40°. In studies in the interaction of polyvinylpyrrolidone with a number of substances, Molyneux and Frank (11) calculated that the net enthalpy change associated with: (*a*) the heat needed to overcome specific interaction between water and the polymer and water and the small molecule (*i.e.*, "dehydration"); (*b*) exothermic binding between the "dehydrated" entities to form a complex; and (*c*) exothermic interaction between the complex and neighboring water (*i.e.*, "rehydration") is essentially a constant value of −5 kcal./mole (ΔHb in *Reference 11*). If this value is subtracted from $\Delta H°$ in Table I, the net enthalpy changes associated with the melting of the icebergs around the two separate entities and the reformation of hydrogen bonds in icebergs around the complex become +2.3, +2.0, +0.3, and −3.6 kcal./mole at 10, 20, 30, and 40°, respectively. The decrease in enthalpy with increasing temperature may be explained by the decrease in "icelikeness" around hydrocarbons with increasing temperatures (Table IX in *Reference 12*).

Positive entropy changes on the interaction of polyvinylpyrrolidone with a variety of small molecules or ions have been attributed to the formation of hydrophobic bonds (11, 13, 14). It is postulated that the polyvinylpyrrolidone bishydroxycoumarin complex is accompanied either by a less ordered iceberg or by an

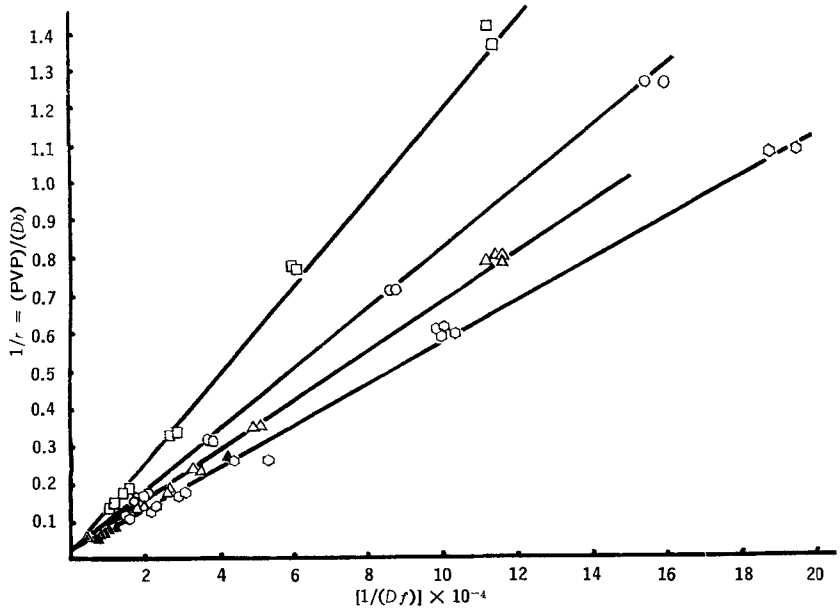

Figure 5—*Double reciprocal plot for the polyvinylpyrrolidone–bishydroxycoumarin interaction (symbols as in Fig. 4).*

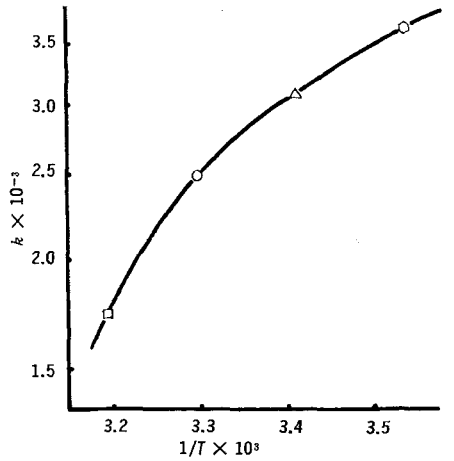

Figure 6—*Van't Hoff plot (log* k versus *1/*T*) for the polyvinylpyrrolidone–bishydroxycoumarin interaction.*

iceberg containing a smaller number of water molecules than the icebergs of the two separate entites. The release of water molecules from the ordered structure should produce a proportional gain in positive entropy (increase in randomness). Thus, the positive entropy values observed between 10 and 30° (Table I) is attributed to the formation of hydrophobic bonds. The contribution of the entropy term to free energy is 41 and 37% at 10 and 20°, respectively. At higher temperatures the binding process is increasingly exothermic; the role of hydrophobic bonding, which is essentially endothermic (9, 15), becomes less important.

At pH 7.4, bishydroxycoumarin exists mainly as the anion (2). However, polyvinylpyrrolidone has no ionizable groups (16) and it is unlikely that electrostatic interactions will be involved in complex formation. The dependence of binding strength on temperature also rules out this possibility (17). However, Frank *et al.*

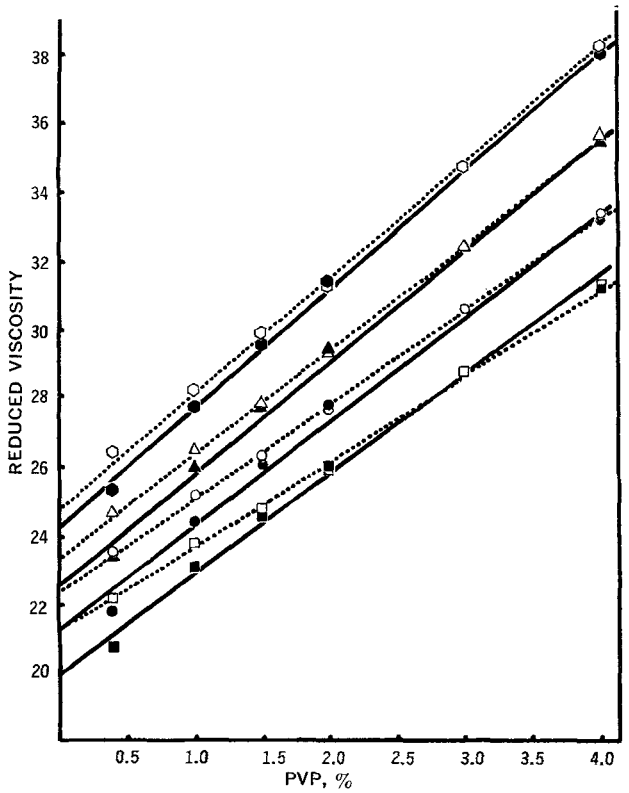

Figure 7—*Reduced viscosity of polyvinylpyrrolidone as a function of polyvinylpyrrolidone concentration at various temperatures (symbols as in Fig. 4). Solid and dotted lines represent the viscosities in the presence and absence of bishydroxycoumarin (185 × 10⁻⁶ mole/l.), respectively.*

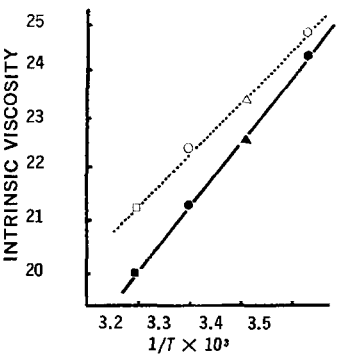

Figure 8—*Effect of temperature on the intrinsic viscosity of polyvinylpyrrolidone in the presence of bishydroxycoumarin (symbols as in Fig. 4).*

(18) reported that the lactam bond in the pyrrolidone ring is likely to undergo ion-dipole interaction with an anion, and van der Waals' forces will stabilize the complex.

A polyvinylpyrrolidone molecule with a molecular weight of 40,000 has approximately 360 monomer units. The binding data shown in Fig. 5 indicate that the average number of binding sites on a polyvinylpyrrolidone molecule is approximately 50, irrespective of temperature; *i.e.*, on the average, 7.2 repeating units provide a binding site for one bishydroxycoumarin molecule. Molecular models show that a bishydroxycoumarin molecule fits on approximately eight pyrrolidone rings. The polyvinylpyrrolidone model shows that the pyrrolidone rings appear to provide a channel-type cavity in both sides of the paraffin backbone. The possibility of another bishydroxycoumarin anion binding on the other side of the backbone is unlikely because of repulsive forces between the bound anion and an oncoming anion.

Viscometric Analysis—The relationship between changes in intrinsic viscosity and configurational changes in macromolecules is discussed in *References 19* and *20*. Viscometry has been widely used for complexation studies of polyvinylpyrrolidone with a variety of substances (18, 21–23).

In Fig. 7, the reduced viscosity is plotted *versus* polyvinylpyrrolidone concentration. The correlation coefficient between these two variables is in the range of 0.980–1.048. The intercept (*i.e.*, intrinsic viscosity) was determined by means of the method of least squares. No attempts were made to interpret changes in the Huggins constant (24) associated with the interaction since they are, in general, too irregular to be correlated with any definite molecular effect (22).

In the absence of bishydroxycoumarin, the intrinsic viscosity of polyvinylpyrrolidone decreases with increase in temperature. This finding was explained in terms of a progressive coiling of the polymer with temperature (25). A monoionized bishydroxycoumarin molecule is expected to have few rotational degrees of freedom around the methylene bridge because of the one remaining intramolecular hydrogen bond (Scheme 1b in *Reference 2*). A model of the complex indicates that a better fit is achieved by a slight bending or folding of the polymer toward the bishydroxycoumarin anion. This postulate is supported by the decrease in intrinsic viscosity on binding which suggests coiling of the polymer chain.

The variation of intrinsic viscosity with temperature is shown in an Arrhenius-type plot in Fig. 8. From the slope, a term analogous to activation energy was estimated to be 0.92 and 1.13 kcal./mole in the presence and absence of bishydroxycoumarin, respectively. The difference in slopes indicates a reduction in the extent of coiling on complexation as the temperature is decreased. Theoretically, at approximately −5°, no configurational changes will occur.

In contrast to these results, Molyneux and Frank (22) found an increase in intrinsic viscosity of polyvinylpyrrolidone in the presence of many organic anions. This was explained in terms of expansion of the polymer due to coulombic repulsion between the bound anions. However, in tris(hydroxymethyl)aminoethane buffer of ionic strength 0.15, repulsion between bound anions is reduced by the "screening" effect of the free counterions (RNH_3^+ form) and coiling of the polymer becomes possible.

Changes in intrinsic viscosity are not, in general, a linear function of the concentration of the cosolute (21). Due to the low solubility of bishydroxycoumarin, viscosity determinations were impractical, except at a concentration near the solubility limit. Hence, the changes measured serve only to ascertain the existence of an effect at that concentration and the general nature of this effect.

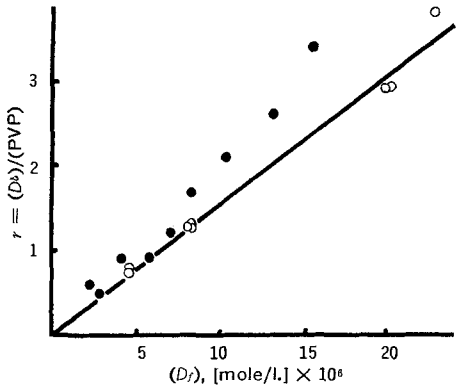

Figure 9—*Comparison of binding data obtained from spectrophotometric analysis* (●) *with data obtained from equilibrium dialysis* (○) *for the polyvinylpyrrolidone–bishydroxycoumarin interaction at 20°.*

Summary and Comparison of Methods Used to Evaluate Binding—Four methods were used to evaluate the binding mechanism of bishydroxycoumarin and polyvinylpyrrolidone: spectrophotometric, equilibrium dialysis, solubility, and viscometric. The first three methods are based on the analysis of changes in the properties of bishydroxycoumarin due to complex formation. Viscometry measures changes in rheological properties occurring in polyvinylpyrrolidone.

Figure 9 compares spectrophotometric data with the results obtained from dialysis experiments. At low (D_f), data from both methods agree. The agreement may be due to the fact that polyvinylpyrrolidone has only one type of binding site for bishydroxycoumarin. However, the precision of the spectrophotometric method falls off rapidly at higher (D_f).

The spectrophotometric method is important since information on the binding process can be obtained with accuracy at very low (D_f) and it is unnecessary to separate free and bound drug as in the dialysis technique. Binding data obtained in this way must be complemented by other methods because drug concentrations are limited by Beer's law.

Solubility analysis has been used widely to study molecular interaction in solution (26). If there is a sharp break in the solubility curve, the overall step stability constant, K_{ov}, can be estimated[5]. However, polyvinylpyrrolidone has a high aqueous solubility and formed a soluble complex with bishydroxycoumarin. The solubility curve was nonlinear and failed to show a break. The unusually high solubility of the complex deserves further investigation. Coprecipitates of bishydroxycoumarin and polyvinylpyrrolidone would be expected to produce a significant increase in dissolution rate.

The value of n obtained by the solubility method was approximately 20, compared with a value of 50 obtained from dialysis experiments. The molecular model of the complex supports the latter value. It is suggested that all binding sites are not accessible to bishydroxycoumarin molecules and that uptake of bishydroxycoumarin anion interferes with the interaction at other sites.

Viscometry does not provide quantitative information; in the present study, its applicability was limited by the low solubility of bishydroxycoumarin at pH 7.4. However, a configurational change in polyvinylpyrrolidone was detected from measurements made at one bishydroxycoumarin concentration. Coiling of the polymer, as a result of binding, is a possible explanation of the inaccessibility of all binding sites to bishydroxycoumarin molecules.

[5] The value of K_{ov} is defined by $(MD_n)/(M_f)(D_f)^n$ for the reaction $nD + M = MD_n$. The relationship of this constant with the intrinsic association constant, k, is (27):

$$K_{ov} = k^n \left[n \left(\frac{n-1}{2}\right)\left(\frac{n-2}{3}\right)\left(\frac{n-3}{4}\right) \cdots \left(\frac{1}{n}\right)\right]$$

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Biological Disposition of Pentylenetetrazol-10-^{14}C in Rats and Humans

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Abstract □ Female white rats were dosed orally with pentylenetetrazol-10-^{14}C. Total radioactivity in different tissues was determined at five times after dosing. The kidney and liver showed slightly higher levels of radioactivity than did the blood and other tissues analyzed. During the first 24 hr., approximately 73% of the administered ^{14}C was recovered in the urine; less than 1% was recovered in the feces. TLC of urine followed by autoradiography showed the presence of pentylenetetrazol-10-^{14}C and at least five radioactive metabolites. Acetone extracts of the blood were chromatographed and were found to contain pentylenetetrazol-10-^{14}C and four of the five labeled metabolites detected in the urine. TLC of urine collected from humans dosed orally with pentylenetetrazol-10-^{14}C showed the presence of pentylenetetrazol-10-^{14}C and at least four radioactive metabolites. The metabolites in human urine occurred at the same R_f positions as the labeled metabolites of rat urine when cochromatographed, indicating possible similar metabolism by rats and humans.

Keyphrases □ Pentylenetetrazol-10-^{14}C—biological disposition □ Tissue distribution—pentylenetetrazol-10-^{14}C □ Metabolism, pentylenetetrazol-10-^{14}C—rats, humans □ Autoradiograms, TLC—analysis □ Scintillometry—analysis

Subconvulsive oral doses of pentylenetetrazol have been used in the treatment of geriatric patients with symptoms of mental confusion, depression, or arteriosclerosis psychosis. The pharmacological activity and metabolism of pentylenetetrazol have been widely studied. However, the literature contains conflicting reports concerning the biotransformation of the compound and the chemical forms in which it is eliminated from the body. Some authors reported detection of no pentylenetetrazol, or only very low amounts, in the urine of experimental animals dosed with pentylenetetrazol (1–5). Esplin and Woodbury (4) reported that only one labeled compound, chromatographically identical to pentylenetetrazol, was detected in the urine of rats dosed intravenously with ^{14}C-labeled pentylenetetrazol. Stiver (5) reported the presence of pentylenetetrazol and three labeled metabolites in the urine of rats dosed intravenously with pentylenetetrazol-10-^{14}C. The purpose of the work reported here was to study the biological disposition of subconvulsive oral doses of pentylenetetrazol in rats and humans. The study was accomplished by radiotracer techniques, using oral doses of pentylenetetrazol-10-^{14}C.

EXPERIMENTAL

Materials and Methods—Pentylenetetrazol-10-^{14}C, synthesized and purified by the method of Stiver *et al.* (6), was used throughout this study. The radiochemical purity was greater than 99%, as shown by TLC, autoradiography, and liquid scintillation counting techniques. All other chemicals or reagents used were analytical grade. For the animal studies, pentylenetetrazol-10-^{14}C was dissolved in sterile water. Each milliliter of this solution contained 30.4 μc. of ^{14}C and 2.84 mg. of pentylenetetrazol. The pentylenetetrazol-10-^{14}C dosages for humans were reported by Ebert *et al.* (7). Urine collected from that study was used to study urinary metabolites.

Fifteen Sprague-Dawley[1] female rats (152–177 g.) were randomly divided into five groups and fasted for 20 hr. Each was then orally dosed with 12.1 μc./1.13 mg. of pentylenetetrazol-10-^{14}C, an amount well below the convulsant dose (8). The animals were placed individually in metal metabolism cages. Fasting was continued but water was allowed *ad libitum* for the remainder of the study. Urine and feces from each of the three animals in the five groups were collected until the groups were sacrificed at 1, 4, 8, 12, or 24 hr. by decapitation. Samples of blood, portions of abdominal fat, intestinal feces, and muscle from the right rear leg were removed from the carcass as well as the entire heart, brain, lung, spleen, liver, and kidney. Tissues were washed in normal saline solution, and all samples except feces were weighed and frozen until analyzed. Blood was pretreated with sodium citrate. Feces were stored over Drierite, weighed, and pulverized before assay.

Radioanalysis—The radioactivity in all samples was measured at 4° in low potassium vials with a liquid scintillation spectrometer[2] equipped with bialkali phototubes. The scintillator used for all samples consisted of 1 part toluene, 1 part 2-ethoxyethanol, and 0.4% 2,5-diphenyloxazole. All samples were then fortified with toluene-^{14}C internal standard and recounted to determine the counting efficiency.

Aliquots of urine in duplicate and radioactive zones on chromatograms were counted in vials containing 15 ml. of the scintillator solution. Samples, duplicate when possible, of tissue and blood (50–80-mg. aliquots) and dried and crushed feces (3.6–90.8-mg. aliquots) were digested for 6–8 hr. at 70° in tightly capped vials. The digesting agent was 0.2 ml. of a mixture of equal amounts of 70% perchloric acid and 2-ethoxyethanol and 0.4 ml. of 30% hydrogen peroxide. When solubilization was complete, the samples were cooled and counted in 50 ml. of scintillator.

TLC and Autoradiography—Metabolites in the blood and urine of rats and the urine of humans were separated chromatographically with methyl ethyl ketone as the solvent. Aliquots of urine, treated urine, or treated plasma were cochromatographed with authentic pentylenetetrazol-10-^{14}C on desiccator-stored, precoated, 250-μ silica gel G plates with fluorescent indicator[3]. Each plate was developed three times in the same solvent system (13 cm. per development). Previous work by Stiver (5) showed that other solvent systems for TLC of pentylenetetrazol did not give as complete a separation as this system.

Autoradiograms were made from the developed chromatograms by first spraying the plates twice[4]. The plates were then exposed to medical X-ray film, in a freezer at −10°, for 7–28 days to allow the detection of spots representing 1% or less of the total ^{14}C applied. The radioactive zones thus detected were further quantitated by liquid scintillation counting of the absorbent scraped from the plates.

To determine whether any conjugates of pentylenetetrazol were present in rat urine, hydrolysis treatments similar to those reported by Ice *et al.* (9) were employed prior to chromatography. Samples of urine were subjected to steam heat, sodium hydroxide, sodium hydroxide plus steam heat, hydrochloric acid, and hydrochloric acid plus steam heat.

Because of the very low levels of activity in human urine samples, the samples were filtered through Whatman No. 1 filter paper to remove precipitated salts; 1-ml. aliquots of the filtrates were lyophilized at 0.2–0.3 mm. Hg, using dry ice and 2-methoxyethanol as a

[1] Sprague-Dawley, Madison, Wis.
[2] Packard Instrument Co., Inc., Downers Grove, Ill.
[3] Brinkmann Instruments, Inc., Des Plaines, Ill.
[4] With a thin coat of Omnispray Intensifier for Autoradiography, New England Nuclear, Boston, Mass.

Table I—Tissue Distribution of ^{14}C at Various Times following Oral Administration of Pentylenetetrazol-10-^{14}C to Rats

Tissue	Hours after Administration: 1	4	8	12	24
Blood	0.72 ± 0.04[a]	0.53 ± 0.01	0.16 ± 0.03	0.14 ± 0.04	0.04 ± 0.00
Brain	0.63 ± 0.01	0.47 ± 0.04	0.18 ± 0.03	0.14 ± 0.04	0.03 ± 0.00
Fat	0.31 ± 0.03	0.21 ± 0.05	0.10 ± 0.07	0.05 ± 0.03	0.01 ± 0.01
Heart	0.75 ± 0.06	0.53 ± 0.03	0.18 ± 0.04	0.15 ± 0.05	0.04 ± 0.00
Kidney	0.99 ± 0.03	0.79 ± 0.01	0.26 ± 0.05	0.20 ± 0.05	0.05 ± 0.00
Leg muscle	0.61 ± 0.05	0.50 ± 0.03	0.19 ± 0.04	0.14 ± 0.04	0.03 ± 0.00
Liver	0.91 ± 0.03	0.69 ± 0.05	0.25 ± 0.05	0.19 ± 0.05	0.04 ± 0.00
Lung	0.74 ± 0.04	0.55 ± 0.03	0.19 ± 0.04	0.15 ± 0.04	0.04 ± 0.00
Spleen	0.71 ± 0.01	0.53 ± 0.01	0.16 ± 0.04	0.14 ± 0.04	0.03 ± 0.00

[a] The mean of three animals ± *SD* is given and expressed as the percent of the administered 12.1-μc. dose per gram of tissue.

coolant until the resulting samples contained about 2% moisture. The lyophilized urine residues were reconstituted in 0.2 ml. of 70% ethanol, and the mixtures were chromatographed.

To study metabolites in rat plasma, 3-ml. aliquots of whole blood were centrifuged and 1-ml. aliquots of the centrifugates were then lyophilized. Each lyophilized sample was mixed with 0.2 ml. of water in the lyophilization ampul, and then about 0.2 ml. of acetone was added with thorough mixing. The acetone–water supernatant was chromatographed and analyzed for radioactivity.

RESULTS AND DISCUSSION

Tissue Distribution Study—Table I shows that the concentration of pentylenetetrazol-10-^{14}C and/or its labeled metabolites was about equal in the blood, brain, heart, leg muscle, lung, and spleen at the different times studied. The level of ^{14}C detected in the liver was always slightly higher than that detected in the mentioned tissues, and the level of ^{14}C present in the kidney was always slightly higher than that detected in the liver. Fat showed the lowest level of ^{14}C of all tissues assayed. The concentration of ^{14}C in all tissues studied decreased steadily with time.

Table II—Cumulative Urinary and Fecal Excretion of ^{14}C by Rats Dosed Orally with Pentylenetetrazol-10-^{14}C

Hours	Number of Animals	Cumulative ^{14}C: Excreted in Urine, %	Excreted in Feces, %[a]
1	3	2.5 ± 3.5	0.14 ± 0.00[b]
2	12	8.3 ± 8.4	—[c]
4	12	14.2 ± 8.3	0.16 ± 0.06[d]
6	9	32.1 ± 16.1	—[c]
8	9	48.2 ± 14.1	0.21 ± 0.30
10	6	56.1 ± 14.1	—[c]
12	6	63.0 ± 17.0	0.35 ± 0.39
18	3	63.5 ± 9.8	—[c]
24	3	73.4 ± 5.6	0.79 ± 0.22

[a] Feces collected from metabolism cages and feces obtained from the rats' intestines were assayed. Values represent the mean ± *SD* of three animals, except where otherwise noted. [b] Value for one animal only. [c] No animals were sacrificed at this time. [d] Value for two animals only.

Table III—Relative Occurrence of Labeled Compounds in Rat Urine after Oral Administration of Pentylenetetrazol-10-^{14}C

R_f[a]	Time of Urine Sample Collection, hr.: 0–2	2–4	4–6	6–8	8–10	10–12	12–18	18–24
0.00	16.7[b]	18.9	15.8	16.0	17.1	17.2	15.0	16.6
0.15	30.7	18.3	14.2	10.6	7.6	7.6	5.2	8.9
0.62	28.2	37.0	39.4	38.2	35.2	33.7	30.0	26.2
0.73	1.8	4.3	1.9	3.0	3.0	2.7	3.5	3.1
0.78	21.3	22.9	28.0	31.9	36.8	38.5	44.0	43.0

[a] Labeled compounds are specified according to their typical R_f on the thin-layer plates. The compound at R_f 0.78 was identified as pentylenetetrazol on the basis of cochromatography with known pentylenetetrazol-10-^{14}C. The metabolites were not identified. [b] The relative occurrence of each compound is expressed as the average percent of the total ^{14}C in all urine samples collected at each time interval. Values reported through 12 hr. are the average of the same four rats; values beyond 12 hr. are the average of two of these rats. For each time interval, values used for the average did not differ by more than 5%.

The results of the study were in general agreement with those reported previously (3, 4) for the tissue distribution of intravenously administered pentylenetetrazol. The notable exception is that Stiver (5) reported that concentrations of pentylenetetrazol-10-^{14}C and/or its labeled metabolites were about twice as high in kidney tissue as in liver tissue after intravenous administration of pentylenetetrazol-10-^{14}C to male rats.

At the end of 24 hr., approximately 73% of the administered ^{14}C had been excreted in the urine and less than 1% had been excreted in the feces (Table II). These results are in general agreement with those reported by other investigators (4, 5) who studied the excretion of intravenously administered ^{14}C-labeled pentylenetetrazol. Esplin and Woodbury (4) reported a 24-hr. urinary recovery of 63% of the administered ^{14}C and a 48-hr. fecal recovery of 2–5% of the administered ^{14}C. Stiver (5) reported excretion of 66% of the administered ^{14}C in the urine during a 24-hr. study.

Rat Urine Metabolite Study—A compound tentatively identified as pentylenetetrazol-10-^{14}C and at least five labeled metabolites were detected in all rat urine samples studied (Table III). The four most prominent metabolites detected in all rat urine samples were seen at the origin and at R_f 0.15, 0.62, and 0.73. Known pentylenetetrazol-10-^{14}C and the compound in the urine tentatively identified as pentylenetetrazol on the basis of cochromatography in one solvent system were both visible on the autoradiograms at R_f 0.78. Three additional minor labeled metabolites were visible on some autoradiograms; these could not be separated for radioassay from neighboring spots because of tailing. None of the labeled metabolites was identified. The number of labeled metabolites detected was greater than that reported by previous investigators (4, 5).

The relative occurrence of pentylenetetrazol in the urine increased steadily from 21% in the 0–2-hr. sample to 43% in the 18–24-hr. sample. The reason for this increase is not clear. The fluctuations in relative occurrence of the various labeled compounds in the urine with time were similar in general to those reported by Stiver (5) who analyzed urine collected from male rats dosed intravenously with pentylenetetrazol-10-^{14}C.

Table IV—Relative Occurrence of Pentylenetetrazol-10-^{14}C and Its Labeled Metabolites in Rat Urine Subjected to Various Hydrolysis Treatments

R_f[a]	Treatment: None	Heat	HCl	HCl plus Heat	NaOH	NaOH plus Heat
0.00	15.3[b]	15.5	2.3	2.0	25.4	26.5
0.08	2.8	2.6	32.2	31.3	0.0[c]	0.0[c]
0.15	17.6	17.4	0.0[c]	0.0[c]	10.7	9.5
0.37	1.3	1.4	0.0[c]	0.0[c]	0.9	1.1
0.62	36.7	37.2	38.1	39.1	37.3	37.2
0.73	2.3	2.1	2.4	2.2	2.3	2.3
0.78	24.0	23.9	25.0	25.5	23.4	23.3

[a] Labeled compounds are specified according to their typical R_f on the thin-layer plates. The compound at R_f 0.78 was identified as pentylenetetrazol on the basis of cochromatography with known pentylenetetrazol-10-^{14}C. The metabolites were not identified. [b] The relative occurrence of each compound is expressed as the percent of the total ^{14}C in each sample analyzed. Rat urine collected from one rat 2–6 hr. after oral administration of pentylenetetrazol-10-^{14}C was used. [c] No darkening of the autoradiogram was observed at this R_f, indicating that a negligible amount of ^{14}C was present.

Table V—Relative Occurrence of Labeled Compounds in Rat Plasma after Oral Administration of Pentylenetetrazol-10-^{14}C

Sacrifice Time, hr.	R_f	Labeled Compounds, %[a]
1	0.00[b]	1.5 ± 0.53
	0.08	1.7 ± 0.76
	0.62	11.1 ± 1.36
	0.73	2.2 ± 0.79
	0.78	83.4 ± 2.14
8	0.00	3.4 ± 1.36
	0.08	1.9 ± 2.37
	0.62	15.6 ± 0.59
	0.73	4.0 ± 1.25
	0.78	75.1 ± 2.28

[a] The relative occurrence of each compound is expressed as the percent of the total ^{14}C in each sample and is given as the mean of three samples ± *SD*. [b] Labeled compounds are specified according to their typical R_f on the thin-layer plates. The compound at R_f 0.78 was identified as pentylenetetrazol on the basis of cochromatography with known pentylenetetrazol-10-^{14}C. The metabolites were not identified.

The relative occurrence of pentylenetetrazol-10-^{14}C and its labeled metabolites in urine subjected to various hydrolysis treatments is given in Table IV. No new labeled compounds were detected in any of the treated urine samples. If any conjugates of pentylenetetrazol were originally present in the urine, they were not hydrolyzed under the conditions of the treatment employed. However, the data given in Table IV suggest that the metabolites seen at R_f 0.00, 0.15, and 0.37 in untreated urine are acid-hydrolyzable conjugates of the metabolite at R_f 0.08, and that the compounds at R_f 0.08 and 0.15 are base-hydrolyzable conjugates of the metabolite(s) remaining at the origin. Because the NaOH treatment only partially hydrolyzed the material at R_f 0.15, probably at least two labeled metabolites have an R_f of 0.15.

Rat Plasma Metabolite Study—TLC of acetone-extracted blood plasma samples showed the presence of pentylenetetrazol-10-^{14}C at R_f 0.78 and at least four radioactive metabolites: at R_f positions 0.00, 0.08, 0.62, and 0.73. On some autoradiograms, a fifth minor labeled metabolite, which appeared as a very faint spot at R_f 0.50, was detected. None of the labeled metabolites was identified. The five labeled metabolites detected in the plasma extracts occurred at the same R_f's as five of the labeled metabolites detected in the urine sample chromatographed on the same plates.

Table V shows that the relative occurrence of the various metabolites was greater in the plasma extracts of animals sacrificed at 8 hr. than in those rats sacrificed at 1 hr. The major metabolite, at R_f 0.62, increased from 11% at 1 hr. to 16% at 8 hr. Pentylenetetrazol represented 83% of the total ^{14}C in the 1-hr. plasma extract but only 75% of the ^{14}C in the 8-hr. plasma extract.

Stiver (5) detected only pentylenetetrazol-10-^{14}C and no labeled metabolites in the plasma of male rats dosed intravenously with pentylenetetrazol-10-^{14}C. However, the detection technique reported by Stiver (5) was less sensitive than the one reported here.

Human Urine Metabolite Study—TLC of urine collected from one human during the period extending from 24 to 36 hr. after the first of three 100-mg. doses of pentylenetetrazol-10-^{14}C, which contained 22.8% of the administered radioactivity, was analyzed. Chromatographs showed the presence of a compound tentatively identified as pentylenetetrazol at R_f 0.78 and at least three unidentified labeled metabolites at R_f positions 0.00, 0.25, and 0.62. Pentylenetetrazol, tentatively identified on the basis of cochromatography with known pentylenetetrazol-10-^{14}C, accounted for about 24% of the ^{14}C in the sample.

TLC of urine collected from four humans during the period extending from 6 to 9 hr. after a single 100-mg. oral dose of pentylenetetrazol-10-^{14}C showed the presence of pentylenetetrazol at R_f 0.78 and at least four labeled metabolites at R_f 0.00, 0.25, 0.62, and 0.73. The labeled metabolites were not identified. Three of the labeled metabolites detected in human urine occurred at the same R_f's as three metabolites detected in rat urine at R_f 0.00, 0.62, and 0.73.

Table VI—Relative Occurrence of Labeled Compounds in the Urine of Four Human Subjects 6–9 Hr. after Oral Administration of Pentylenetetrazol-10-^{14}C

R_f	Labeled Compounds, %[a]
0.00[b]	22.6 ± 3.0
0.25	6.4 ± 3.1
0.62	55.5 ± 4.8
0.73	5.7 ± 2.0
0.78	9.9 ± 3.3

[a] The relative occurrence of each compound is expressed as the percent of the total ^{14}C in each urine sample. [b] Labeled compounds are specified in terms of their typical R_f on the thin-layer plate. Pentylenetetrazol was identified on the basis of cochromatography with known pentylenetetrazol-10-^{14}C. The labeled metabolites were not identified.

The relative occurrence of pentylenetetrazol-10-^{14}C and its labeled metabolites in the four human urine samples analyzed is given in Table VI. Pentylenetetrazol accounted for an average of 9.9% of the ^{14}C in the samples, and the major metabolite at R_f 0.62 accounted for 55%. The proportion of pentylenetetrazol in the 6–9-hr. human urine (9.9%) was well below that detected in rat urine at any time during a 24-hr. study—*viz.*, 21–44%.

The metabolism of orally administered pentylenetetrazol-10-^{14}C by rats and humans was similar in that both species excreted unchanged pentylenetetrazol and at least three labeled metabolites which were chromatographically similar. Pentylenetetrazol and the three similar metabolites detected in the urine of both species accounted for 94% of the ^{14}C in human urine collected during the 6–9-hr. period and for 89% of the ^{14}C present in rat urine collected during the 6–8-hr. period after dosing. The metabolism of pentylenetetrazol-10-^{14}C by the two species differed in that the human urine contained one labeled metabolite not detected in rat urine, and some rat urine samples contained four minor labeled metabolites which were not detected in human urine.

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Fluorometric Analysis of Ampicillin in Biological Fluids

WILLIAM J. JUSKO

Abstract □ Ampicillin forms a strongly fluorescent yellow product in acid solution during hydrolysis at elevated temperature. The quantity and rate of formation of the fluorescent material are enhanced appreciably by formaldehyde, and the fluorescent product exhibits extraction properties of an organic acid. In alkaline solution, the fluorescent compound has uncorrected excitation and emission maxima at 346 and 422 nm., respectively. An assay procedure based on these observations permits detection of less than 0.05 mcg./ml. of ampicillin and/or α-aminobenzylpenicilloic acid. By combining fluorometric and microbiological methods, measurement can be made of ampicillin degradation products as well as the unchanged antibiotic in serum and urine.

Keyphrases □ Ampicillin analysis—biological fluids □ Biological fluids—ampicillin and degradation product, analysis □ pH effect—ampicillin product fluorescence □ Hydrolysis products—ampicillin □ TLC—separation □ Microbiological—fluorometric analysis—ampicillin □ Fluorometry—analysis

Several methods for the quantitative measurement of ampicillin (α-aminobenzylpenicillin) in aqueous solution have been described. These utilize techniques such as UV spectrophotometry (1), iodine absorption (2), and hydroxamate color formation (3). Recently, Smith *et al.* (4) described a spectrophotometric assay method based on the hydrolysis of ampicillin to α-aminobenzylpenicillenic acid which exhibits absorption at 322 nm. Because of its relative insensitivity, this procedure is unsuitable for measurement of ampicillin at low concentrations in biological fluids. To date, procedures based on microbiological assay (5, 6) have been the only nonisotopic methods suitable for detection of ampicillin, as well as many other penicillins, at the low concentrations encountered in serum and urine following therapeutic doses of the drug.

During the investigation of the spectrophotometric method of Smith *et al.* (4), it was found that a strongly fluorescent product could also be formed from ampicillin. This observation led to the development of a highly sensitive fluorometric method for quantitative measurement of ampicillin and at least one of its degradation products in serum and urine.

EXPERIMENTAL

Apparatus—An Aminco-Bowman spectrophotofluorometer was employed for measurement of fluorescence. A Beckman Expandomatic SS-2 pH meter and a Beckman DB spectrophotometer were used for pH and UV absorption determinations, respectively. Electrophoresis was performed in agar gel using a Laurell-type electrophoresis apparatus[1].

Materials—Analytical grade sodium ampicillin[2] (potency: 865 mcg./mg.) was used as the reference material for all studies. Stock solutions containing 100 mcg./ml. of ampicillin (corrected for potency) were either prepared fresh or were stored frozen and used within 5 days. Potassium hetacillin[2], potassium benzylpenicillin[3], and cephalexin[4] were analytical grade materials provided by the manufacturers. Methicillin, oxacillin, cephalothin, and ampicillin powder for injection were used by dilution of the contents of proprietary vials of parenteral material.

Buffers—Measurements carried out at specific pH values involved use of the following buffer systems: at pH < 2, HCl–KCl (7); at pH 2–8, citric acid–disodium phosphate (2); at pH 8–11, disodium phosphate–NaOH (7); and at pH > 11, KCl–NaOH (7). All pH values were obtained at 20° at the time of use of the buffered solutions.

Effect of pH on Ampicillin Product Fluorescence—A solution of the fluorescent product was obtained by heating 100 mcg. of ampicillin in 40 ml. of pH 3.4 citrate–phosphate buffer for 2 hr. at 75°. One-milliliter aliquots of this stock solution were mixed with 15 ml. of each of 15 buffers which covered the pH range of 0–14. The fluorescence intensity and final pH of each solution were then measured.

Effect of pH on Formation of Product—One milliliter containing 100 mcg. ampicillin was added to 39 ml. of various buffers (pH range 1–10) which were preheated to 75°. At 30-min. intervals, 1-ml. aliquots were withdrawn from each of the 11 solutions and added to 2 ml. of 2 *N* NaOH. Fluorescence readings were obtained until increases in intensity were no longer apparent. The study was also repeated at a higher temperature in the buffer system that was found to yield maximum fluorescence (pH 2). Solutions containing 0.25 *M* citrate buffer and 5 mcg./ml. of either ampicillin or hetacillin were heated at 90° in the presence and absence of 0.8% formaldehyde. Aliquots of these mixtures were removed for fluorescence measurements at various times up to 5 hr.

Solvent Extraction—Spectroscopic grade organic solvents were used to determine whether ampicillin or the fluorescent product could be extracted from aqueous solution. The solvents tested were: acetone–chloroform (1:1), chloroform, ethylene dichloride, hexane, heptane, and carbon tetrachloride. Known concentrations of ampicillin or its fluorescent product were placed in 5 ml. solution buffered to pH 1, 2, 3.4, or 7 and shaken with an equal volume of organic solvent. After agitation for 1 hr., fluorometric measurement of the concentration of drug or product remaining in the aqueous phase was made.

Other Assay Methods—Ampicillin in aqueous solutions at a concentration of about 500 mcg./ml. was quantitated by UV spectrophotometry (1). In particular, the contents of four vials of ampicillin for injection were diluted with water and the absorbance measured at a wavelength of 258 nm.

A microbiological assay was employed along with the fluorometric method to measure ampicillin in aqueous solutions and in biological samples at concentrations less than 5 mcg./ml. The agar-well diffusion assay described by Bennett *et al.* (5) was used, employing *Bacillus subtilis*[5] as the microorganism.

Biological Samples—Serum and urine specimens were obtained after intravenous administration of 570 mg. ampicillin to a normal adult male subject (85 kg. weight). In addition, urine samples were collected for 36 hr. after oral administration of 500 mg. ampicillin to the same subject. All samples were refrigerated and analyzed within 3 days.

Electrophoresis—Serum samples, which were obtained after intravenous administration of ampicillin were subjected to agar gel electrophoresis. Aliquots of 0.01 ml. of serum were applied to the agar gel which was buffered with tris–maleate at pH 5.6 (8) Electrophoresis of these, as well as ampicillin standards, was carried out at approximately 240 v. and 60 ma. for 45 min. The agar gel was then overlayed with 1.5% nutrient agar[5] containing *B. subtilis* spores. After incubation at 35° for 16–20 hr., the bioautogram was examined for zones of bacterial inhibition.

[1] Metaloglass Inc., Boston, Mass.
[2] Bristol Laboratories, Syracuse, N. Y.
[3] Chas. Pfizer & Co., Brooklyn, N. Y.
[4] Eli Lilly and Co., Indianapolis, Ind.
[5] Difco Laboratories, Detroit, Mich.

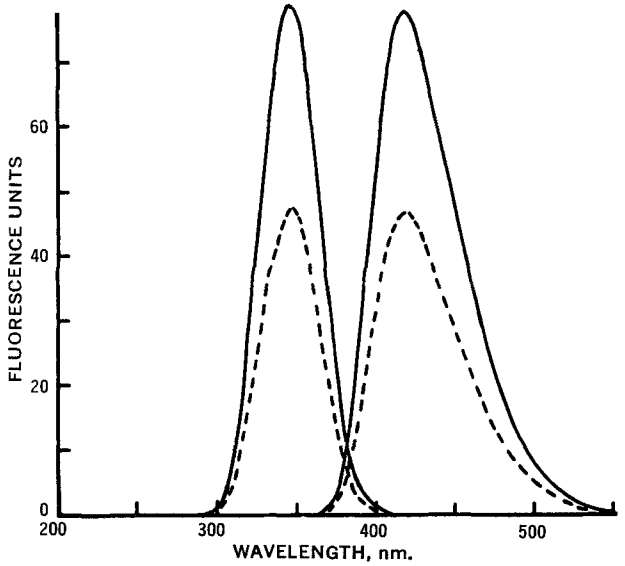


Figure 1—*Spectrophotofluorometric excitation (346 nm.) and emission (422 nm.) spectra for the fluorescent product of ampicillin reference material* (——) *and from urine* (- - -).

Chromatography—A composite sample of urine collected after oral administration of ampicillin was diluted, buffered to pH 3.4, and heated at 75° for 2 hr. Extracts of the fluorescent product in 4 ml. of acetone–chloroform or 4 ml. of chloroform were then used to initiate 16-tube countercurrent distribution (CCD) studies. The aqueous phase consisted of 6 ml. of pH 3.4 citrate–phosphate buffer. At the completion of 15 transfers, 1 ml. of 10 *N* NaOH was added to each tube. Following equilibration, an aliquot of the aqueous phase was fluorometrically analyzed. The method of Williamson and Craig (9) was then used to generate calculated distribution profiles for the two CCD systems.

Similar acetone–chloroform extracts of fluorescent product from ampicillin in urine and from the reference material were subjected to TLC on Eastman silica gel sheets. The solvent systems used included: A, chloroform–acetone (1:1), B, 1-butanol–methanol (4:6), and C, ethylene dichloride–dioxane–water (2:1:1).

Ampicillin Conjugates—Test for the presence of glucuronide or sulfate metabolites of ampicillin was carried out on composite urine samples obtained after oral dosage of the drug. Two milliliters of urine was mixed with 1 ml. of 0.4 *M* pH 5.2 acetate buffer and 1 ml. of either glusulase[6], containing 18,000 units of glucuronidase and 5000 units of sulfatase, or Ketodase[7], containing 5000 units of β-glucuronidase. Samples with and without enzyme were incubated at 35° for 20 hr. and then assayed microbiologically.

Effect of Penicillinase—A degradation product of ampicillin, α-aminobenzylpenicilloic acid, was formed in aqueous solution by hydrolysis of ampicillin with penicillinase[8]. A solution containing 20 mcg./ml. ampicillin and 80 kinetic units/ml. of the enzyme in *M*/15 pH 7.4 Sorensen's buffer was incubated at 37° for 1 hr. At various intervals, 1 ml. of solution was removed and added to 3 ml. of 10% trichloroacetic acid maintained at 0°. One portion of this sample was analyzed by the fluorometric method while another portion was neutralized and assayed microbiologically.

RESULTS

Formation and Properties of the Ampicillin Product—When ampicillin is subjected to heating in acid solution, a strongly fluorescent yellow product is formed. The absorption and emission spectra of this product are shown in Fig. 1. The fluorescence profiles for products formed from analytical grade ampicillin and from ampicillin excreted in urine are identical. All fluorescence measurements in this study were obtained at the excitation maximum at 346 nm. and the emission maximum at 422 nm. (uncorrected).

[6] Endo Laboratories, Garden City, N. Y.
[7] Warner-Chilcott, Morris Plains, N. J.
[8] Bioquest Laboratories, Cockeysville, Md.

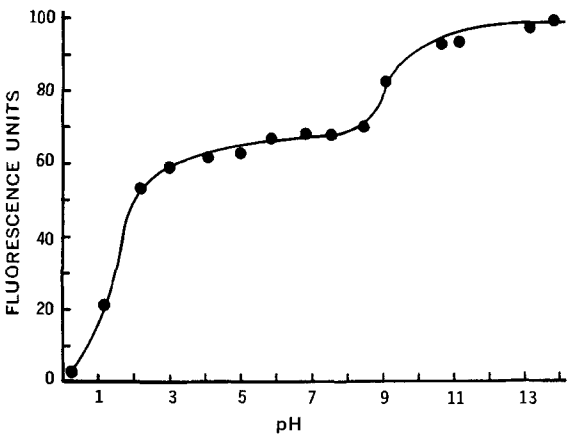


Figure 2—*Relative fluorescence intensity of equal concentrations of the ampicillin product in solutions of various pH values.*

Measurements of the fluorescence intensity of equal concentrations of the ampicillin product in solutions at various pH values yielded the results shown in Fig. 2. The fluorescence of the ampicillin product is greatest at pH values greater than 12. All measurements of ampicillin product fluorescence were, therefore, usually obtained in 1 *N* NaOH solution. The pH–fluorescence profile is an indication that the ampicillin product has at least two ionization constants, namely between pKa 1 and 2 and between pKa 9 and 10.

The effect of pH on the relative amount of fluorescent product formed from ampicillin after heating for 4 hr. at 75° is shown in Fig. 3. An appreciable quantity of product is generated in acid medium, with the maximum amount formed over the pH range of 2–3. Practically no fluorescent material is produced in solutions at pH values greater than 7. The rate of formation of the fluorescent product at 90° in pH 2 buffer in the presence and absence of formaldehyde is shown in Fig. 4. Formaldehyde exerts a catalytic effect on the reaction, causing both an increased rate of formation of the product and an increased total amount of the material. A similar experiment with hetacillin, which is known to hydrolyze rapidly in aqueous solution to ampicillin (10), produced essentially identical results.

The extractibility of the fluorescent product from acid media was tested with several organic solvents. The compound is readily extracted into acetone–chloroform (1:1), chloroform, and ethylene dichloride but is practially insoluble in hexane, heptane, and carbon tetrachloride. From either acetone–chloroform or chloroform, the product can be subsequently partitioned into 1 *N* NaOH. Ampicillin itself, on the other hand, could not be extracted from acidic solution into any of the solvents tested.

At ampicillin concentrations less than 50 mcg./ml., only a single fluorescent product appears to be formed. This was apparent from TLC of acetone–chloroform extracts of the reaction mixture. In the solvent systems used, single fluorescent spots were found with R_f values of 0.80 (A), 0.74 (B), and 0.62 (C). However, at appreciably higher ampicillin concentrations, TLC revealed multiple fluorescent products with R_f values less than that of the primary product These may have been the result of some polymerization since the

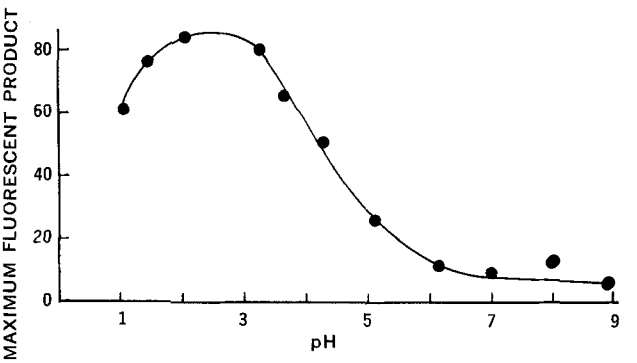


Figure 3—*Effect of pH on the maximum amount of fluorescent product formed from 2.5 mcg./ml ampicillin at 75°.*

1. *Nonprotein solution:* mix 1 ml. of solution with 1 ml. of *M*/2 pH 2 citrate buffer.

1*a*. *Protein solution:* mix 0.5 ml. of sample with 2 ml. of 10% trichloroacetic acid. Centrifuge to obtain 2.0 ml. of clear supernatant.

2. Add 0.5 ml. of 7% formaldehyde in 0.4 *M* pH 2 citrate buffer.
3. Heat solutions in water bath at 90° for 2 hr. and then cool to room temperature.
4. *Direct:* add 1 ml. of 2 *N* NaOH to each solution.

4*a*. *Extraction:* add 7 ml. of acetone–chloroform (1:1). Shake 30 min.

4*b*. Shake 5 ml. of organic phase with 2 ml. of 1 *N* NaOH for 10 min.

5. Measure fluorescence of alkaline solutions at 346-nm. excitation and 422-nm. emission wavelengths.
6. Reference standards of ampicillin should be simultaneously included.

Scheme I—Procedure for fluorometric assay of ampicillin

fluorescence profile showed an additional absorption peak at 285 nm. (1).

Proposed Method—The procedures evolved for the fluorometric analysis of ampicillin in biological and nonbiological fluids are outlined in Scheme I. The method is based on the observations outlined and is useful for ampicillin concentrations as high as 20 mcg./ml. and as low as 0.01 mcg./ml. When very low concentrations of ampicillin are being measured, reference standards should include a drug-free blank from each individual studied. This ensures against patient variation in the fluorometric blank characteristics of samples containing proteins.

Dosage Form Analysis—Microbiological, spectrophotometric, and fluorometric methods of analysis were used to determine the ampicillin content of four proprietary vials of injectible sodium ampicillin. The results of these determinations are shown in Table I. A chi-square analysis of the data ($\chi^2 = 1.26$ on 6 *df*) showed that all techniques gave comparable results.

Other Antibiotics—The assay procedure was tested, using several penicillin or cephalosporin derivatives as shown in Table II. As expected (10), hetacillin forms, on an equimolar basis, as much of the fluorescent product as does ampicillin. Methicillin, oxacillin, and cephalothin formed no detectable fluorescence. Of particular interest is that benzylpenicillin, which lacks the α-amino group of ampicillin, forms no fluorescent product. Cephalexin, however, yielded a small amount of the fluorescent material. This cephalosporin derivative has the α-aminobenzyl group as does ampicillin, but the β-lactam ring is more resistant to acid hydrolysis. These data show that the aminobenzyl group and a cleaved β-lactam ring are necessary for the formation of the fluorescent product.

Biological Sample Analysis—Known concentrations of ampicillin in aqueous solution and added to pooled human plasma were subjected to the fluorometric assay procedure without the organic extraction steps. Over the range of 0.2–10 mcg./ml., there was a linear relationship between ampicillin concentration and fluorometer response, as shown in Fig. 5. The relative recovery of the antibiotic from the protein solutions compared to the aqueous solutions was 99.6 ± 5.2%. Thus, ampicillin can be quantitatively recovered from plasma using trichloroacetic acid as the protein precipitant.

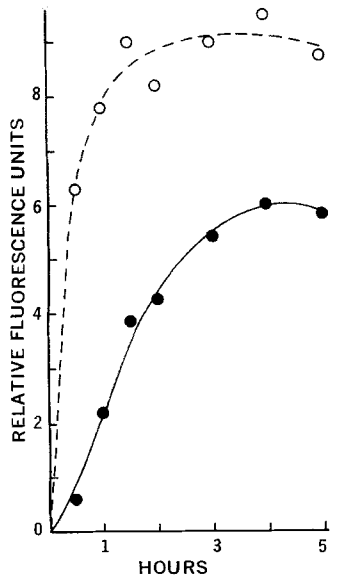


Figure 4—*Rate of formation of the fluorescent product from 2.5 mcg./ml. ampicillin at 90° in the presence* (○) *and absence* (●) *of 0.8% formaldehyde.*

Table I—Comparison of Three Assay Methods: Recovery of Ampicillin from Commercial Vials of Injectable Sodium Ampicillin

Product	Labeled Contents, mg.	Percent of Label Claim[a] Microbiological	UV	Fluorometric
1	1000	112	114	118
2	500	122	118	127
3	500	125	107	114
4	500	110	107	116

[a] Chi-square test: 1.26 on 6 *df*.

Serum samples, obtained after intravenous administration of 570 mg. ampicillin to a normal male subject, were analyzed for ampicillin by the fluorometric and microbiological methods. The two methods yielded some appreciable differences (Fig. 6), particularly in samples removed during the later part of the collection period. Such differences are characteristic of the presence of a metabolite or degradation product in the samples. The specificity of the microbiological assay was checked by agar gel electrophoresis and bioautography. Unchanged ampicillin was found to be the only substance in plasma that inhibited the growth of the test organism.

Urine samples were also collected after ampicillin administration, and the fluorometric and microbiological assays were applied. Differences in results obtained by the two methods were similar to those noted in serum. Enzymatic treatment of composite urine samples with β-glucuronidase and/or sulfatase yielded no change in the quantity of microbiologically active ampicillin. Thus, the metabolite of ampicillin does not appear to be a glucuronide or sulfate conjugate.

The fluorescent product obtained from ampicillin excreted in urine was subjected to two CCD systems. The aqueous/organic distribution coefficient of the ampicillin product was found to be 0.18 in the acetone–chloroform system and 0.43 in the chloroform system. To calculate the expected CCD profiles for a single solute, these values were used with the mathematical relationships described by Williamson and Craig (9). The calculated data and the experimental measurements are shown in Fig. 7 for the two CCD systems. The relatively good agreement between the calculated lines and the data points suggests strongly that a single fluorescent product is formed from ampicillin and its metabolites in urine when present in low concentrations. This observation was further confirmed by TLC of an acetone–chloroform extract of the fluorescent product from ampicillin excreted in urine and ampicillin reference material.

Products of Hydrolysis—Two possible degradation products of ampicillin are its penicillenic acid and its penicilloic acid derivatives. The present assay method utilizes hydrolytic conditions similar to those employed by Smith *et al.* (4), who were able to form and measure *in vitro* the penicillenic acid product of ampicillin. At the low ampicillin concentrations encountered in the biological samples collected in the present study, it was not possible to detect penicillenic acid absorbance at 322 nm. before or after acid hydrolysis.

Table II—Relative Amount of Fluorescent Material Formed from Various Penicillin or Cephalosporin Derivatives

Antibiotic	Concentration, mcg./ml.	Percent of Ampicillin Fluorescence
Ampicillin	1,3,5	100
Hetacillin	1,3,5	102 ± 7
Benzylpenicillin	5	0
Methicillin	~5	0
Oxacillin	~5	0
Cephalexin	1,3,5	8.9 ± 0.4
Cephalothin	~5	0

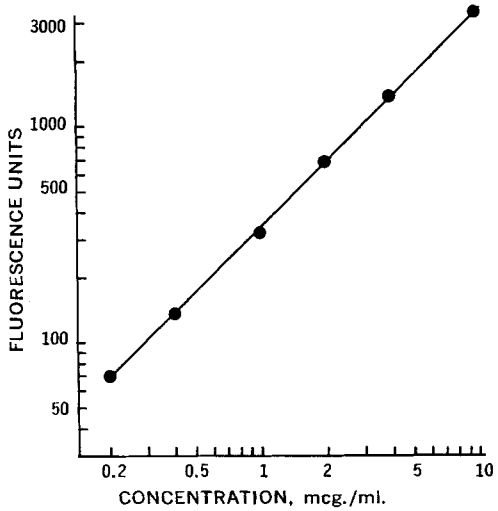


Figure 5—*Fluorometric standard curve obtained for ampicillin added to pooled human plasma.*

The penicilloic acid product of ampicillin is readily formed in the presence of the enzyme penicillinase. The ability of the fluorometric procedure to measure this product is demonstrated by the results of the study shown in Fig. 8. Microbiologically active ampicillin rapidly disappears in the presence of the enzyme, while fluorometrically the total apparent concentration remains constant. These data show that equimolar concentrations of both ampicillin and α-aminobenzylpenicilloic acid yield identical amounts of the fluorescent product. This indicates that either ampicillin degrades to the acid during the formation of the fluorescent product or that both compounds can be directly transformed into the same intermediate.

An attempt to separate the small quantity of degradation product from ampicillin in urine using chromatographic techniques was not successful. This was primarily due to the similar physicochemical properties of these compounds. Direct identification of the apparent metabolites of ampicillin in serum and urine was, therefore, not possible at this time.

DISCUSSION

The fluorometric assay for ampicillin described in this report is an indirect but highly sensitive method for the measurement of the antibiotic in solution. Although the precise chemical identity of the fluorescent product was not determined, various physicochemical properties of it, as well as of ampicillin, have been elucidated. Such information provides interesting insights into the chemistry of these penicillin derivatives.

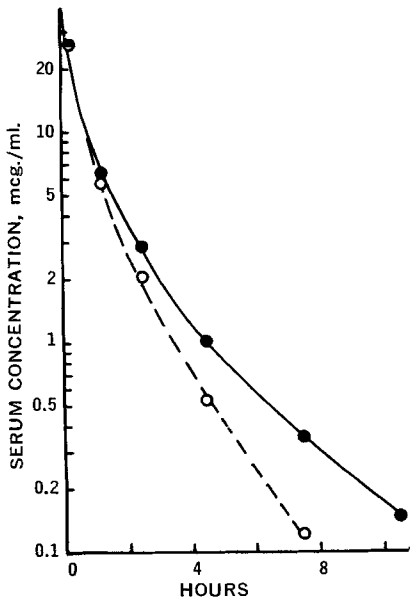


Figure 6—*Serum concentrations of apparent ampicillin as a function of time after intravenous administration of 570 mg. to a male subject. Concentrations were obtained with the fluorometric* (●) *and microbiological* (○) *methods.*

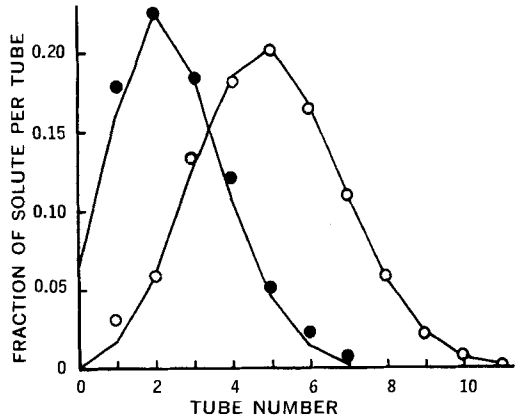


Figure 7—*CCD profile for the fluorescent product of ampicillin in urine using acetone-chloroform* (●) *and chloroform* (○) *solvents and pH 3.4 buffer. Lines represent theoretical calculations (9).*

Hou and Poole (11) were the first to note that aging of ampicillin mixtures includes the formation of a substance with absorption at 340–355 nm. This material was probably identical to the fluorescent product found in the present investigation. Based on UV absorption spectra, kinetic data, and other information, these investigators (11) as well as others (12) proposed various pathways of degradation of ampicillin. These mechanisms primarily involved cleavage of the β-lactam portion of the molecule, and the benzylamino group was assumed to remain intact. The results of the present study (Table II) have shown that both portions of the ampicillin molecule are required in the formation of the fluorescent product. Addition of formaldehyde, a type of formol titration (13), enhances the reaction by reducing the basicity of the amino group. A chemical pathway for formation of a fluorescent material which takes these factors into account is shown in Scheme II. The formation of a 3,6-substituted diketopiperazine (III) from either ampicillin (I) or α-aminobenzylpenicilloic acid (II) is consistent with the ability of amino acid esters to condense to form diketopiperazines (15). The imino protons of product III could subsequently dissociate in solution above pH 10 to produce a more fluorescent species, which would account for the pH–fluorescence profile of Fig. 2. It is possible for the remaining portion (R) of the piperazine molecule (III) to undergo a variety of other chemical reactions (11, 12) that are characteristic of penicillins rather than amino acids. Further physicochemical analysis of the fluorescent product will be required for direct confirmation of the suggested pathway.

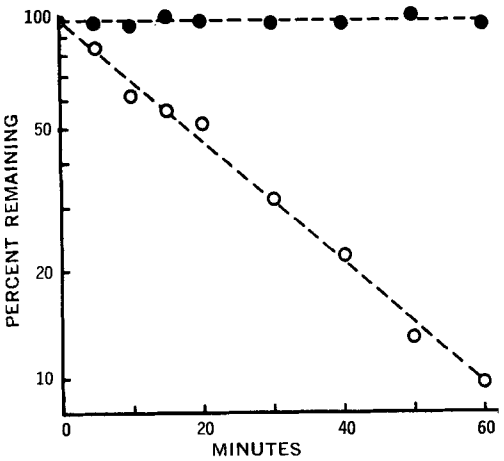
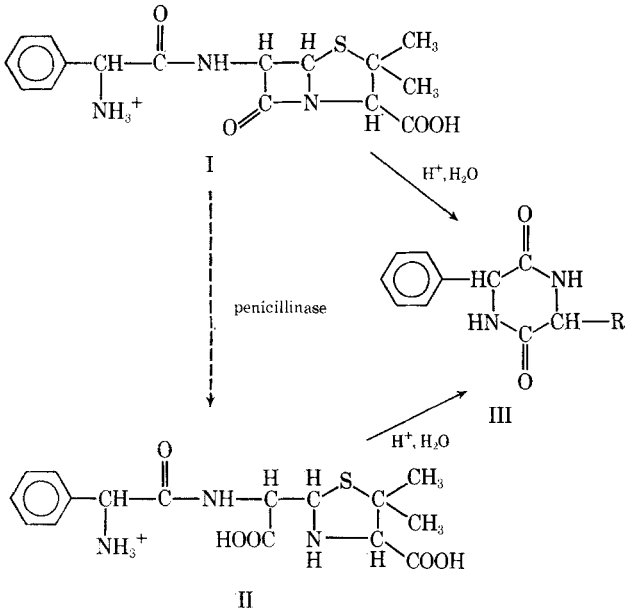


Figure 8—*Effect of penicillinase on ampicillin stability as a function of time as determined by the fluorometric* (●) *and microbiological* (○) *assays.*



Scheme II—Proposed pathway for formation of a 3,6-disubstituted diketopiperazine (III) from ampicillin (I) and/or α-aminobenzylpenicilloic acid (II)

The results of the experiment with penicillinase showed that α-aminobenzylpenicilloic acid and ampicillin can be detected equally well by the fluorometric assay. The major *in vivo* degradation products of other penicillins such as penicillin G and phenoxymethyl and phenoxyethyl penicillin have been shown to be their respective penicilloic acid derivatives (14). Since all penicillins share a labile β-lactam portion of the molecule, it is very likely that the detected degradation product of ampicillin in serum and urine of man is its penicilloic acid derivative.

A search of the literature did not reveal a direct method for analysis of ampicillin in biological fluids. The feasibility of a direct assay is limited by the poor solubility of the amphoteric compound in organic solvents. In addition, the isolation of small amounts of the unchanged antibiotic is hampered by its relative instability in solution. For these reasons, measurements of apparent ampicillin in serum and other biological samples are usually done by microbiological methods which are easily performed. However, such assays are often used with the assumptions that the microorganism is sensitive only to the unchanged drug and that metabolites of the antibiotic do not act as competitive inhibitors of the bacteriocidal properties of ampicillin. It was found in the present study that the procedure (5) utilizing *B. subtilis* is specific for ampicillin in the presence of its degradation products. The microbiological method is, therefore, useful in conjunction with the fluorometric method as a means of differentiating between ampicillin and its *in vivo* metabolites or degradation products. The detection and quantitation of the latter compounds are of importance since the allergenic properties of the penicillins have been ascribed to their degradation products (12).

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Nature of Bonding in Montmorillonite Adsorbates I: Surface Adsorption

J. THURØ CARSTENSEN* and KENNETH S. E. SU

Abstract □ It has been shown that organic solutes that do not have groups which are cation exchangeable adsorb on the surface of neutralized montmorillonite and do not (unlike several solvents such as alkanols and water) penetrate to the interior of the crystal. The number of available sites on the surface is of the order 10^{18}–10^{19} sites per m.[2] of surface area. The number of interior sites available is about 600 times as high. The fact that surface adsorption is the process involved explains in part the fact that such adsorbates are physiologically available when used in pharmaceutical preparations.

Keyphrases □ Montmorillonite adsorbates—bonding nature □ Bonding nature—montmorillonite adsorbates □ Benzoic acid adsorption—montmorillonite □ Moisture isotherm—montmorillonite □ Particle-size determination—montmorillonite

MacEwan (1) showed by X-ray studies that some clays, including montmorillonite, have a micalike structure, being built up in layers; the interlaminar distance is large, and layers are held together by second-order forces. The interlaminar distance in montmorillonite can vary from 10 to 20 Å or even more, depending on its water content. Bradley *et al.* (2) showed that these water molecules form layers about 3 Å thick, whereas the thickness of the aluminum silicate framework is 9.6 Å (3). The distance in dehydrated montmorillonite can be more than 9.6 Å, due to the presence of various cations; according to Hendricks (4), these cations place themselves in the same fashion as do the water molecules. Mering (5) showed that the structure of montmorillonite (Fig. 1) is one of a repeating unit of $[Al_{2-x}Mg_x, Si_{4-y}Al_y, O_{10}(OH)_2]$ where $(x + y)$ represents the negative charge; this is compensated for by cations which position themselves on the crystal surface and, hence, are exchangable. In "neutralized" species[1], this cation is H^+.

MacEwan (1) pointed out that water can be replaced by organic molecules. He showed that the number of layers of organic solvent molecules present between silicate layers is zero for heptane; one for heptanol, ethylenediamine, and 1-propanol; two for 1,2-propanediol, ethanol, ethylene glycol, methanol, and acetone; and three for acetonitrile. This type of work has been extended and verified in many subsequent publications (6–10). Studies of this type have most frequently been conducted by exposing the montmorillonite to the organic vapor and measuring the change in crystal spacing.

MacEwan and Talib-Uddin (11), in the case of α-zinc hydroxide, showed that the sodium salt of 1-hydroxy-2,4-dinitro-7-naphthalenesulfonic acid "adsorbed" by placing itself in the interlaminar positions. Wai and Banker (12) showed that, for many organic

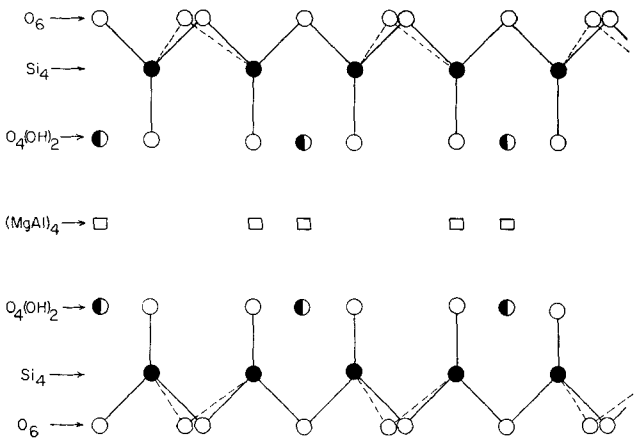

Figure 1—*Structure of montmorillonite according to Mering (5).*

compounds, adsorption on montmorillonite, which is not neutralized, is one of ion exchange. Zentner (13) showed that "neutralized" montmorillonite may be employed to make adsorbates which serve the purpose of taste-masking of bitter compounds. These adsorbates are physiologically available (14); it would, therefore, appear that they could not be too strongly adsorbed (*e.g.*, could not be chemisorbed).

In the case of protonized substituted amines (and other protonized compounds), the adsorbates on neutralized montmorillonite are presumably of an ion-exchange nature. In the case of uncharged species, however, this cannot be the process. From the description given, the adsorbates could be either a surface adsorption or they could be brought about by an adsorption of the type described for α-zinc hydroxide, where the organic molecule actually penetrates the crystal.

An investigation of the nature of the bonding involved is presently being conducted by the authors. This preliminary publication directs itself to the question whether, in the case of neutralized montmorillonite, solutes adsorb on the surface only or whether adsorption in the interlaminar space is also taking place.

EXPERIMENTAL AND RESULTS

Mering (5) pointed out that treatment of montmorillonite with mineral acids may give rise to structural changes of the surface. Micronized neutralized montmorillonite[2] was, therefore, used as received from the supplier; the entire study refers to one single lot[2], except where noted. Drying by heat also may cause structural alterations (5), so the montmorillonite was dried by exposure (on a vacuum rack with high vacuum capability) to less than 0.5-μ pressure. After exposure for 7 days, the samples had equilibrium pressures of less than 1 μ Hg. Nevertheless, the samples contained 5.6% structural water, as determined by Karl Fischer titrations.

[1] Veegum Neutral, marketed by R. T. Vanderbilt Co., Inc., New York, NY 10017

[2] Micronized Veegum Neutral, Type S-6814, Lot FX 329, marketed by R. T. Vanderbilt Co., Inc., New York, NY 10017

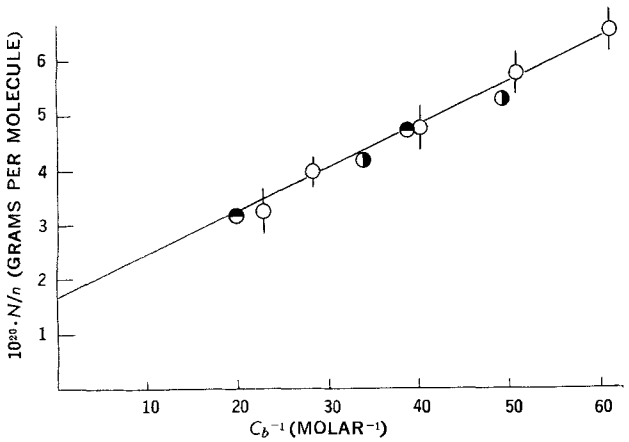

Figure 2—*Benzoic acid isotherm from anhydrous isopropanol. Ordinate represents grams of montmorillonite per molecule of benzoic acid, and abscissa is reciprocal molarity of benzoic acid. Key:* ○, *represents the procedure described in the text;* ◓, *represents pre-extracted montmorillonite (see text); and* ◑, *represents a log of montmorillonite different from the lot used in the main portion of the study. The equation for the line is:* $1/n = 1.67 \cdot 10^{-20} + [8.31 \cdot 10^{-22}/C_b]$.

Benzoic acid isotherms were determined by dissolving a weighed amount of benzoic acid in the appropriate solvent. An aliquot was titrated with 0.02 *N* NaOH; another aliquot was exposed to a weighed amount of dried montmorillonite in a sealed conical flask and shaken mechanically at 25° for 48 hr. The sample was then centrifuged, and an aliquot of the supernatant was titrated; the amount adsorbed was determined by difference. A blank sample, containing no benzoic acid, was prepared in a similar fashion and showed zero titer.

Reciprocal plotting of the data yields straight lines, as exemplified in Fig. 2. Since anhydrous solvents (*e.g.*, methanol) have been reported to (and were found to) extract and replace the structural water, the solvent after equlibration contains small amounts of water. One to six grams of montmorillonite was used per 100 ml. of solvent, so the moisture content of the solvent, in reality, would be 60–360 mg./100 ml. To ascertain that this small amount did not affect the results, two runs were made with montmorillonite pre-extracted with solvent. Results from these fall in line with the remaining results (Fig. 2); hence, the effect of the small amount of water is within experimental error. Two runs were made with a different lot of montmorillonite, also giving consistent results (Fig. 2).

Moisture isotherms were performed by exposing 5 g. of montmorillonite to 100 ml. of water–isopropanol mixtures for 3 weeks in sedimentation tubes of 4.2-cm. i.d., of the type described elsewhere (16). The water content of the solvent was determined before and after equilibration by means of Karl Fischer titrations; a plot of moisture content of the montmorillonite as a function of water content of solvent is shown in Fig. 3.

The cross-sectional area of benzoic acid was estimated by molecular model to be 7 Å^2 from a lengthwise direction and 14 Å^2 from the side.

Microscopic examination of the lot of montmorillonite used revealed the majority of particles to be of a 1-2-μ dimension. A few (less than 0.05% by number) were 5–10 μ. A 2-μ cube, employing a density figure obtained by displacement in hexane, results in a calculated surface area of 1 m.^{2}/g. B.E.T. nitrogen isotherms give higher results but may involve not only external, but also internal, laminar surfaces. It is, furthermore, to be expected that the experimental figure is larger than geometrically expected, since the latter is predicated on smooth surfaces and on monodispersity.

Table I—Benzoic Acid Isotherms in Various Solvents

Solvent	Intercept	Sites per Gram
Isopropanol	$1.67 \cdot 10^{-20}$	$6.0 \cdot 10^{19}$
Water–isopropanol (50% w/w)	$2.07 \cdot 10^{-20}$	$4.6 \cdot 10^{19}$
Ethanol	$2.51 \cdot 10^{-20}$	$4.0 \cdot 10^{19}$
Butanol	$2.34 \cdot 10^{-20}$	$4.3 \cdot 10^{19}$
Methanol	$2.01 \cdot 10^{-20}$	$5.0 \cdot 10^{19}$
Average		$4.8 \cdot 10^{19}$
Standard deviation		$0.8 \cdot 10^{19}$

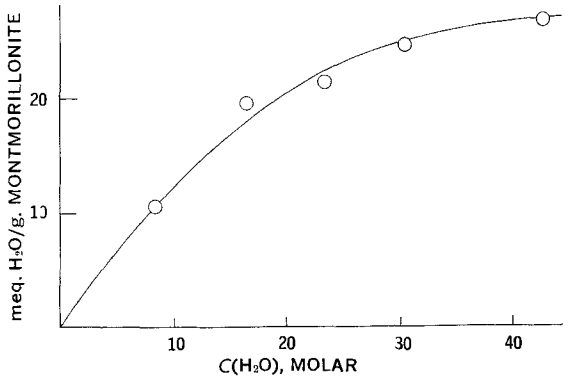

Figure 3—*Moisture isotherm of montmorillonite from aqueous solutions of isopropanol.*

DISCUSSION

Isotherms of benzoic acid and of water on neutral montmorillonite were determined since they give information relative to the number of adsorption sites. If the adsorption of benzoic acid is a competition between solvent (*o*) and benzoic acid (*b*), then the surface fraction covered (θ) is given in terms of the concentrations in solution (*C*) by (15):

$$\theta_b = \frac{K_b C_b}{1 + K_b C_b + K_o C_o} \qquad \text{(Eq. 1)}$$

If $K_o C_o$ is negligible compared to unity, then (taking reciprocals) this equation becomes:

$$\frac{1}{\theta_b} = 1 + \frac{1}{K_b C_b} \qquad \text{(Eq. 2)}$$

If 1 g. of montmorillonite contains *N* sites in total, and the number of sites occupied per gram is *n* (*i.e.*, there are *n* molecules adsorbed per gram), then $\theta_b = n/N$ is the fractional coverage. If (as is conventionally assumed) the number of sites *N* equals the saturation value of *n* for high values of C_b, then $N = n_\infty$ and $\theta_b = n/n_\infty$ is a dimensionless quantity. Equation 2 may be written:

$$\frac{N}{n} = 1 + \frac{1}{K_b C_b} \qquad \text{(Eq. 3)}$$

Since this equation is dimensionless, K_b is in reciprocal concentration units; molarity is used here so this unit is M^{-1}. Equation 3 may be written:

$$\frac{1}{n} = \frac{1}{N} + \frac{1}{K_b N} \cdot \frac{1}{C_b} \qquad \text{(Eq. 4)}$$

so that a plot of the reciprocal of the number of molecules adsorbed per gram of montmorillonite *versus* reciprocal concentration yields an intercept of $1/N$ and an intercept/slope ratio of K_b. These considerations assume that $K_o C_o$ is negligible compared to unity. If $K_o C_o$ is not negligible, then Eq. 4 becomes:

$$\frac{1}{n} = \frac{1}{N} + \frac{[K_o C_o + 1]}{K_b N} \cdot \frac{1}{C_b} \qquad \text{(Eq. 5)}$$

For the benzoic acid isotherms, the concentration of benzoic acid is low, so C_o is practically invariant; reciprocal plotting, therefore, still yields a straight line (Fig. 2) and the intercept still reflects the reciprocal of the number of sites (Eq. 5). However, the intercept-to-slope ratio only yields the value for K_b if the value of $K_o C_o$ is negligible compared to unity.

Figure 2 shows that in the case of isopropanol, the intercept is $1.67 \cdot 10^{-20}$; hence, $N = 6.0 \cdot 10^{19}$. Intercept and *N*-values for other

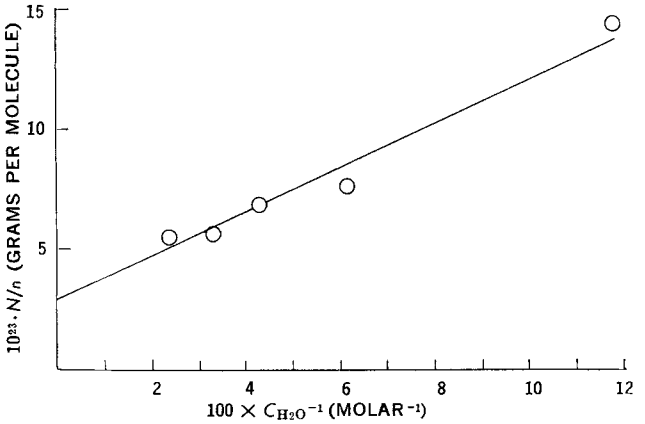

Figure 4—*Langmuir plot of data from Fig. 3. The equation of the line is:* $1/n = 2.86 \cdot 10^{-23} + [9.4 \cdot 10^{-22}/C_{H_2O}]$.

solvents are listed in Table I, and it is seen that these do not differ greatly from solvent to solvent; $N = 4.8 \cdot 10^{19}$ is the average value. From Fig. 2, it is seen (from intercept-to-slope ratio) that, provided K_oC_o is negligible compared to unity, $K_b = 20\ M^{-1}$.

The adherence to a Langmuir plot should preclude multiple-layer adsorption, and estimates of surface area should be possible from saturation data. The benzoic acid molecule has a cross-sectional area of about 10 Å^2, so the surface of the neutral montmorillonite in contact with solution may be estimated at 5 m.^{2}/g., *i.e.*, there are about 10^{19} sites per m.^{2} of surface.

As mentioned in the introduction, solvent (and water) molecules migrate into the crystal space in the montmorillonite. It is, *a priori*, possible that the solute molecules behave likewise (11). If so, then the total number of interstitial and surface sites should be available for adsorption, and the number of sites obtained from a water isotherm should equal that from a benzoic acid isotherm. For this reason, the amount of water removed by montmorillonite from hydroisopropanolic solutions was determined. In this case, the concentration of water in Eq. 5 is represented by C_b, and C_o is concentration of isopropanol. Since C_b in this case is not small and is varied over a large range, C_o is not invariant. If, however, K_oC_o is negligible (compared to unity), Eq. 4 should be obeyed.

A plot of the data is shown in Fig. 3, and plotting according to Eq. 4 is shown in Fig. 4. The linearity of the data in Fig. 4 is good, and the number of sites calculated is $N = 3.5 \cdot 10^{22}$. This is about 600 times larger than the number of sites available for benzoic acid adsorption, and it may be concluded that the adsorption of the latter is strictly a surface phenomenon on the neutralized montmorillonite.

It is important to note that the number of sites available to benzoic acid adsorption does not change appreciably with water content, as seen from Table I; the number of sites calculated from the benzoic acid isotherm in 50% isopropanol equals those obtained in anhydrous isopropanol and other solvents.

Mering (5) showed that three to four monolayers of water are introduced per silicate layer in the intercalation process. He showed that the thickness of these is 3 Å each; the thickness of the silicate layer, as mentioned, is 9.6 Å. If four molecular layers of water exist between each layer of silicate and if there are n layers, the interior sites should amount to approximately $4 \cdot n \cdot A \cdot \gamma$, and the platelet surface sites should number $2 \cdot A \cdot \gamma$, where γ is a proportionality constant related to area, and A is the site density on the platelet. The ratio of internal to surface sites (assuming A to be constant) should then be $2 \cdot n = 600$, *i.e.*, n should be approximately 300. The average thickness of the montmorillonite particles in contact with hydrous solvent should then be $300 \cdot [9.6 + 12] = 6500$ Å $= 0.7\ \mu$, which is not in conflict with the microscopically found dimensions. In this description the adsorption energies of the different layers cannot differ greatly, since the adsorption isotherm, otherwise, would not be of the Langmuir type.

Regarding the assumption that $K_oC_o \ll 1$, it is seen from Fig. 4 that reciprocal plotting yields a straight line; for linearity to prevail in the water isotherm, it is necessary that $K_oC_o \ll 1$ (Eq. 5). The value of $K_b = 20\ M^{-1}$, therefore, represents a realistic figure. In the benzoic acid isotherms, C_b is in the range $0.02 \cdot 0.05\ M$ so $K_bC_b = 0.4$–1; since K_bC_b, therefore, is of the order of unity and K_oC_o is much smaller than unity, it can also be stated with acceptable credibility that $K_bC_b \gg K_oC_o$.

SUMMARY

Benzoic acid adsorbs from alkanolic solutions on the surface of neutralized montmorillonite by Langmuir isotherms.

The number of surface sites estimated from the benzoic acid isotherms is much less than that of water (which adsorbs by intercalation), showing that the benzoic acid does not penetrate the crystal.

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Linear Free Energy Models for Ester Solvolysis with a Critical Examination of the Alcohol and Phenol Dissociation Model

RICHARD J. WASHKUHN*, VITHAL K. PATEL, and JOSEPH R. ROBINSON†

Abstract □ Rate constants for the alkaline hydrolysis of *para*-substituted benzoate esters, with a variety of aliphatic and aromatic leaving groups, were determined under constant reaction conditions. It was found that acyl variation encompassing both aliphatic and aromatic leaving groups could be correlated using the Hammett equation, since the interaction mechanisms involved in both rate processes are apparently proportional to those involved in the equilibrium process. Application of either the Hammett equation or phenol dissociation model to aromatic substituents in the alkyl portion of the ester resulted in nonlinearity. The incomplete success observed in the comparison of the rate processes to these models was not due to a change in mechanism or in rate-determining step, as was shown by solvent isotope, temperature, and catalytic studies, but was apparently due to differences in resonance and solvation interactions in rate and equilibrium processes. Aromatic substituent effects in the alkyl portion of the ester could be quantitated by using a linear combination of the Hammett and phenol dissociation model analogous to the Yukawa–Tsuno equation. In addition, the Swain and Lupton approach quantitated the effect of the alkyl substituent on the rate of reaction. Application of the alcohol dissociation model for aliphatic substituents in the alkyl portion of the ester was not totally effective in correlating rates of reaction with pKa of the leaving group because curvature was noted, when substituents with low pKa were employed, in the plot of rate constant *versus* pKa. In this case the nonlinearity was ascribed to mesomeric stabilization of the anion in the equilibrium process. Besides the nonlinearity in the free energy plots of rate against phenol or alcohol dissociation, there was a dispersion of approximately 5 pKa units between the lines for aliphatic and aromatic leaving groups; this was attributed to the resonance differences observed in the pKa's of alcohols and phenols.

Keyphrases □ Benzoate esters, *p*-substituted—alkaline hydrolysis □ Hydrolysis rates—*p*-substituted benzoate esters □ Deuterium isotope solvent effects—alkaline hydrolysis rates, esters □ Activation parameters—alkaline hydrolysis, benzoate esters □ Alcohol, phenol dissociation models, Hammett relationship—structure–reactivity relationship, benzoate esters □ UV spectrophotometry—ester hydrolysis monitoring

The quantitative prediction of rates and equilibria of organic reactions has long plagued physical chemists due to the complexity of organic molecules. Numerous treatments utilizing the extrathermodynamic approach (1) have been partially successful in analysis of structural and medium effects on reactivity. This approach usually involves a comparison of the free energies in a specific rate or equilibrium process with that of a suitable model or set of models.

The choice of the appropriate model for linear free energy relationships has been mainly dependent upon the specific reaction considered. In the case of ester hydrolysis, substituent effects in the acyl portion of the molecule have been successfully correlated with reactivity using either the Hammett or Taft relationship, depending upon the type of ester involved in the series. Substituent variations in the alkyl portion of the ester molecule have been investigated in the past by several groups; attempts to correlate structure with reactivity using either the Hammett or Taft relationship have not been successful (2–7). A variety of approaches (6, 8, 9) have been used to develop linear relationships between structure and reactivity for substituents in the alkyl portion, most of which deal with the free energy of ionization of the conjugate acid of the leaving group.

Kirsch and Jencks (8) and Bruice *et al.* (9) demonstrated that the rates of solvolysis of some acetate esters could be correlated with the pKa of the conjugate acid of the leaving groups; however, the two studies disagree as to whether a single correlation, encompassing both aromatic and aliphatic leaving groups, or a dual correlation, one for each type, is necessary. Robinson and Matheson (6) showed that dissociation of aliphatic alcohols is an excellent model for ester hydrolysis involving aliphatic substituents in the alkyl portion of the ester molecule. Additional results were reported concerning the effectiveness of the alcohol dissociation model in correlating structure with reactivity for a variety of *para*-substituted benzoates with aliphatic leaving groups (10).

Recently, a number of investigators (3, 4, 7, 11) reported the observation of nonlinear correlations of the free energy of activation with the free energy of ionization of the conjugate acid of the leaving group or nucleophile. Ryan and Humffray (3, 4) observed that a linear combination of σ and σ^- constants was required to give linearity in the plot of rate of alkaline hydrolysis for aryl benzoates and aryl acetates. Kirsch *et al.* (5) also observed nonlinearity in Hammett plots for the alkaline hydrolysis of aryl benzoates and suggested that a special value of σ for the *p*-NO_2 substituent be used to attain linearity. Milton *et al.* (7) also observed nonlinearity in the $\Delta G‡$–ΔG^0 plot for the basic methanolysis of aryl acetates and carbonates; they suggested the nonlinearity may be due to differences in resonance interactions in the two processes or changes in the structure of the transition state as the reactant structure is altered. Nonlinear $\Delta G‡$–ΔG^0 relations have also been observed for carbonyl displacement reactions involving variations in nucleophile and have been explained by either a change in rate-determining step or a change in transition state configuration (12).

The present study examines some linear free energy relationships that have been used in ester solvolysis, with a thorough exploration of the use of alcohol and phenol dissociation as a model process for ester solvolysis. The intents are to evaluate the extent to which both dissociation processes would serve as effective models and to offer explanations for the curvature in such structure–reactivity free energy correlations.

EXPERIMENTAL

Reagents—Fisher certified acetonitrile was used in all reaction mixtures and stock solutions without further purification after it was established that it had no effect on the rate of alkaline hydrolysis and that it was spectrally pure (from 230 to 400 nm.). All other chemicals were of analytical or reagent grade. The reaction mixture for the solvent deuterium isotope studies was prepared with 99.8% D_2O[1] and NaOD (minimum 99% purity)[2]. Water was doubly distilled from acid permanganate in an all-glass distillation apparatus.

All esters were prepared according to procedures outlined by Shriner *et al.* (13) with slight modification. The acyl chloride was reacted with the appropriate alcohol by heating under reflux for 30 min. The reaction mixture was then dissolved in chloroform, extracted with 5% sodium carbonate followed by water, and finally dried with magnesium sulfate. After removal of chloroform under vacuum, the remaining ester was purified by repeated recrystallization from mixtures of water and the alcohol representing the alkyl portion of the ester or, in the case of liquids, by vacuum distillation. In the case of esters derived from phenols, the acid chloride was added either directly to the phenol, with subsequent heating above the melting point of the phenol for about 30 min., or to an ethyl ether solution of pyridine followed by addition of an ethereal solution of the phenol and refluxed for about 1 hr. Purification of these esters involved the same procedures as described for the esters of alcohols, with the modification that repeated recrystallization was carried out from aqueous methanol solutions. The identification and purity of the esters were determined by melting-point or boiling-point comparison with literature values and by spectral properties.

Apparatus—The pH measurements and adjustments were made using a Corning model 12 pH meter with an expanded scale and a Beckman type E3 wide-range glass electrode. The pH meter and electrode system was standardized against phosphate buffer, as described by Bates (14). To ensure accuracy of pH measurements, particularly at elevated temperatures, adjustment of pH of the reaction mixture as well as standardization of the pH meter was carried out at the reaction temperature, using a water-jacketed beaker at the appropriate temperature. Water bath temperatures for the pH measurements and also the kinetic measurements were maintained to ± 0.1° with Sargent Thermonitor electronic relays. Progress of the reaction was followed using either a Cary model 11, 14, or 15 recording spectrophotometer equipped with thermostated cell compartments.

Procedures—*Solvent System*—Fifty milliliters of acetonitrile was added to an equal volume of 0.02 *M* dibasic phosphate buffer, and the resulting solution was brought to the appropriate pH with a few drops of concentrated NaOH solution. It was determined that phosphate buffer did not catalyze the reaction. The acetonitrile–phosphate buffer solution gave a volume somewhat less than 100 ml. and was used as such for all kinetic determinations. For the solvent deuterium isotope studies, the phosphate buffer was eliminated since the pD was sufficiently high to act as a buffer by itself; consequently, pseudo-first-order conditions were maintained since the pD change was negligible during the course of the reaction.

The reaction media for the solvent study were composed of various combinations of acetonitrile and 0.02 *M* phosphate buffer (acetonitrile–phosphate buffer, 5:95, 30:70, and 50:50). These solutions were adjusted to the appropriate pH as described previously. The reaction medium for the imidazole study was prepared by dissolving an appropriate amount of imidazole in a 50% v/v solution of acetonitrile–water such that the final concentration of imidazole would be 0.05 or 0.1 *M*. The resulting solution was adjusted to the appropriate pH with a few drops of saturated NaOH solution.

Continuous Spectrophotometric Procedure—For rapid reactions, the following procedure was used: after temperature equilibration of the buffer system in a 1- or 2-cm. photometer cell, 50 μl. of a stock solution of ester in acetonitrile was introduced and mixed thoroughly within the cell. A 1×10^{-2} *M* stock solution was used for esters derived from alcohols, while a variety of concentrations varying from 6×10^{-3} to 5×10^{-2} *M* in acetonitrile was used as a stock solution for esters derived from phenols. The reaction progress was monitored by following the disappearance of ester at an appropriate wavelength: benzoates at 244 nm., toluates at 250 nm., *p*-chlorobenzoates at 254 nm., *p*-fluorobenzoates at 244 nm., and *p*-cyanobenzoates at 250 nm., and by the appearance of the acid anion at 290 nm. in the *p*-nitrobenzoate case for all esters derived from alcohols. For the esters of phenols, the appearance of the phenolate anion was monitored at 300 nm. for all cases except for the *p*-nitrophenolate and 2,4-dinitrophenolate anions which were monitored at 400 nm.

Discontinuous Spectrophotometric Procedure—For esters with inconveniently slow rates of reaction, the following procedure was used: 100 ml. of buffer solution was prepared and adjusted to the appropriate pH. Two milliliters of a 1×10^{-2} *M* ester stock solution was added to the buffer solution after temperature equilibration, and the resulting mixture was placed in 5-ml. volumetric flasks which were then placed in a water bath at the appropriate temperature. At periodic time intervals, two flasks were removed from the bath and quenched in an ice–brine solution, and their absorbances were determined at an appropriate wavelength. The average absorbance of the two solutions was used, and the data were treated using either a log $(A_t - A_\infty)$ *versus* time plot or the method described by Guggenheim (15).

Data Treatment—All experiments were carried out with a large excess of hydroxide ion; thus, pseudo-first-order kinetics were observed. Pseudo-first-order rate constants were converted to second-order rate constants using hydroxide-ion activity as calculated from the measured pH and the appropriate pKw (16).

For the solvent deuterium isotope studies, second-order rate constants were determined using Eq. 1 as described by Bates (14):

$$\text{pD} = \text{pH meter reading} + 0.40 \qquad \text{(Eq. 1)}$$

with subsequent conversion to deuteroxide-ion activity using a value of 14.955 for the ionization constant of deuterium oxide at 25° (16).

Kinetics studies using imidazole were done at two concentrations of imidazole (0.05 and 0.1 *M*). Rate constants due to imidazole catalysis (k_{IM}) were calculated from the pseudo-first-order rate constants using Eq. 2, after establishing that catalysis was first order with respect to imidazole over the pH range used in this study:

$$k_{obs.} = k_{OH}(OH) + k_{IM}(IM) \qquad \text{(Eq. 2)}$$

The activation parameters, $\Delta G‡$, $\Delta H‡$, and $\Delta S‡$, were calculated at 25° using Arrhenius plots for the determination of *Ea* and the following equations:

$$\Delta H‡ = Ea - RT \qquad \text{(Eq. 3)}$$

$$\Delta G‡ = 2.303RT\left(\log\frac{kT}{h} - \log k_r\right) \qquad \text{(Eq. 4)}$$

$$\Delta S‡ = \frac{\Delta H‡ - \Delta G‡}{T} \qquad \text{(Eq. 5)}$$

RESULTS

Although the literature abounds with rate data on solvolysis of esters, there are insufficient data, under constant conditions (temperature, solvent composition, buffer composition, *etc.*) to carry out the analysis described in the introduction. Thus, second-order alkaline hydrolytic rate constants were determined for a variety of acyl- and alkyl-substituted benzoate esters under constant conditions, and they are presented in Table I together with standard deviations and number of determinations. Rate constants for some of these esters were presented in an earlier publication (10) but, for the sake of completeness, they are reproduced here. At least two rate determinations were made for each ester, and these were carried out at more than one pH value to ensure that the reaction was first order with respect to hydroxide ion. The pH range employed for all esters was 11–13.4 since the buffer capacity of the solvent system, for the concentration of ester employed in this study, was satisfactory over this range.

Solvent deuterium isotope effects on the rate of alkaline hydrolysis of some benzoate esters were determined, and the results are

[1] Stohler Isotope Chemicals.
[2] Brinkmann Instruments, Inc.

Table I—Second-Order Rate Constants for Alkaline Hydrolysis of Some Benzoate Esters[a]

Ester Series	$k \times 10^4$, M^{-1} sec.$^{-1}$	SD^b	Number of Determinations
p-Toluates			
Methyl	2.65	0.002	4
Ethyl	0.879	0.001	2
n-Propyl	0.671	0.049	3
Isopropyl	0.168	0.015	2
n-Butyl	0.568	0.044	2
Isobutyl	0.449	0.013	2
Phenyl	15.1	0.4	3
p-Chlorophenyl	33.7	0.5	5
p-Nitrophenyl	232	3	5
p-Fluorophenyl	26.2	0.1	4
p-Cyanophenyl	158	3	3
p-Tolyl	10.5	0.5	4
Benzoates			
Methyl	6.08	0.08	2
Ethyl	1.98	0.04	2
n-Propyl	1.67	0.08	3
Isopropyl	0.319	0.07	2
n-Butyl	1.41	0.02	2
Isobutyl	1.18	0.13	2
Phenyl	33.6	1.4	4
p-Chlorophenyl	77.9	3.9	4
p-Nitrophenyl	536	18	5
p-Fluorophenyl	56.5	1.2	4
p-Cyanophenyl	414	4	3
p-Tolyl	22.5	1.2	4
m-Cyanophenyl	226	9	3
Monochloroethyl	12.4	0.1	2
Dichloroethyl	31.9	0.3	4
Trichloroethyl	57.8	0.7	6
2,4-Dinitrophenyl	3580	37	4
p-Fluorobenzoates			
Methyl	12.1	0.2	3
Ethyl	4.05	0.08	3
n-Propyl	2.65	0.09	3
Isopropyl	0.623	0.04	3
n-Butyl	2.44	0.06	3
Isobutyl	1.90	0.24	3
Phenyl	67.3	1.2	5
p-Chlorophenyl	115	2	3
p-Nitrophenyl	772	15	4
p-Fluorophenyl	89.3	6.8	4
p-Cyanophenyl	552	13	4
p-Tolyl	35.6	0.8	4
p-Chlorobenzoates			
Methyl	19.1	0.1	3
Ethyl	6.51	0.39	3
n-Propyl	5.11	0.65	2
Isopropyl	1.21	0.02	2
n-Butyl	3.49	0.29	4
Isobutyl	3.36	0.08	2
Phenyl	103	2	4
p-Chlorophenyl	235	3	3
p-Nitrophenyl	1460	15	3
p-Fluorophenyl	191	3	4
p-Cyanophenyl	1070	9	4
p-Tolyl	71.9	1.6	4
2,4-Dinitrophenyl	7430	44	4
p-Cyanobenzoates			
Methyl	195	2	4
Ethyl	79.7	1.4	5
n-Propyl	52.6	0.3	3
Isopropyl	12.6	0.2	5
n-Butyl	41.9	1.2	3
Isobutyl	39.4	1.5	4
Phenyl	736	14	4
p-Chlorophenyl	1660	26	5
p-Nitrophenyl	12,700	150	5
p-Fluorophenyl	1310	28	4
p-Cyanophenyl	7630	110	5
p-Tolyl	518	10	4
2,4-Dinitrophenyl	100,600	200	4
p-Nitrobenzoates			
Methyl	276	7	6
Ethyl	98.8	3.9	6
n-Propyl	76.0	1.8	5
Isopropyl	19.6	1.2	4
n-Butyl	63.4	0.8	3
Isobutyl	60.0	2.0	4
Phenyl	1140	21	3
p-Chlorophenyl	2570	11	4
p-Nitrophenyl	20,900	290	4
p-Fluorophenyl	2000	36	3
p-Cyanophenyl	10,800	250	4
p-Tolyl	752	29	4
m-Cyanophenyl	8450	200	6
Monochloroethyl	504	50	10
Dichloroethyl	1816	22	5
Trichloroethyl	3220	22	8

[a] In a 50% (v/v) acetonitrile–0.02 *M* phosphate buffer at 25°. [b] Standard deviation.

shown in Table II. A suitable correction for the pKa of deuterium oxide was employed in the calculation of the rate constants, as described in the *Experimental* section. Also included in Table II are the second-order rate constants for imidazole catalysis of the same benzoate esters. The imidazole studies were carried out at more than one concentration of imidazole and at different pH's to ensure that the catalytic constant for imidazole was first order with respect to imidazole. For both the solvent deuterium isotope studies and the imidazole studies, a minimum of three determinations was carried out for each ester; the standard deviations for these rate constants are in the range of 2–4% of the rate constants.

The effect of solvent composition on the rate of alkaline hydrolysis of some benzoate esters was investigated, and the results are presented in Table III. Due to solubility problems associated with the esters, it was not possible to carry out the reactions in a fully aqueous system. A minimum of three determinations was employed for each ester at each solvent composition. The standard deviations for the rate constants are in the range of 2–4% of the rate constants.

Temperature effects on the rate of alkaline hydrolysis of some benzoate esters were also investigated, and the results are shown in Table IV. Determination of the appropriate pH at the various temperatures, as well as calculation of the activation parameters, was discussed in the *Experimental* section. At least three determinations were made for each ester at each temperature. Standard deviations for these rate constants are in the range 3–5% of the rate constants. The activation parameters calculated from the temperature studies are presented in Table V.

DISCUSSION

Acyl Variation—The success of the Hammett relationship in correlation of structure with reactivity for acyl variations in benzoates has been widely known and accepted since its conception (17, 18). Hence, successful correlations for *para*-substituted ben-

Table II—Solvent Deuterium Isotope Effects and Imidazole Catalytic Effects Relative to Alkaline Hydrolysis of Some Benzoate Esters

Benzoate Esters	k_{OH}/k_{OD}	k_{OH}/k_{IM}	$k_{OH} \times 10^4$, M^{-1} sec.$^{-1}$
2,4-Dinitrophenyl	0.650	2.01	3580
p-Nitrophenyl	0.745	1.37	536
m-Cyanophenyl	0.820	2.14	227
p-Tolyl	0.875	1.86	22.5
Methyl	0.360	2.02	6.08
Ethyl	0.408		1.98
n-Propyl	0.441		1.67

Table III—Effect of Solvent Composition upon the Second-Order Rate Constants for Alkaline Hydrolysis of Some Benzoate Esters

Benzoate Esters	$k_{OH} \times 10^4$ (M^{-1} sec.$^{-1}$) 50%[a]	30%[a]	5%[a]
2,4-Dinitrophenyl	3,580	15,900	150,000
p-Nitrophenyl	536	2,740	28,200
m-Cyanophenyl	227	1,220	21,500
p-Tolyl	22.5	144.0	3,200
Ethyl	1.98	21.4	267

[a] Percent v/v acetonitrile in 0.02 *M* phosphate buffer.

zoates with alcoholic and phenolic leaving groups are expected. Treatment of the data in the present study using this procedure gives excellent results, as shown by the slopes, standard deviations of points from the line, and correlation coefficients for all of the benzoates (Table VI). The fact that the rates of hydrolysis may be correlated successfully suggests that all factors operative in the rate process are the same as, or at least parallel to, those involved in the equilibrium process.

Comparison of the ρ-values for the two types of leaving groups, aliphatic and aromatic, reveals that the sensitivity of alkaline hydrolysis to polar effects is relatively independent of the type of leaving group, since the ρ-values are approximately the same (2.2 for aliphatic leaving groups as compared to 2.1 for aromatic leaving groups). This slight difference in the ρ-values may conceivably be a result of inductive or resonance differences involved in the two types of leaving groups rather than a steric effect; in the case of aliphatic leaving groups, the ρ-values are insensitive to a wide variety of steric variations when considering the series of methyl through isopropyl leaving groups.

Treatment of the present data for variations in the acyl component of the ester molecule, using the Swain and Lupton approach (19), is demonstrated in Fig. 1. The average percent resonance (%R) contribution for each type of leaving group was determined from the slopes and intercepts in this figure. The %R for the alkaline hydrolysis of *para*-substituted benzoates with aromatic leaving groups was calculated to be 51; while for *para*-substituted benzoates with aliphatic leaving groups, the %R was 50. Since the %R involved in the benzoic acid ionization in water is 53, based on the Hammett substituent constants (19), one might expect that substituent effects in the acyl component of the benzoate esters would be successfully correlated with σ and this is indeed the case.

Alkyl Variation—Neither Hammett's substituent constant, σ, nor σ^- could successfully correlate rates of alkaline hydrolysis when variations were made in the alkyl portion of aryl benzoates, suggesting some factor or factors may not be taken into account when the comparison is made. A variety of approaches have been used to linearize Hammett plots when curvature in the line exists. Several authors (5, 11) suggested that the σ-values for strong electron-withdrawing substituents be adjusted to fit the line defined on the basis of other weaker electron-withdrawing substituents. This forced linearization of Hammett plots, however, might lead to erroneous mechanistic interpretation of the reaction at hand.

Another approach involves a linear combination of model processes, benzoic acid dissociation and phenol dissociation, as proposed by Ryan and Humffray (3, 4), who suggested that the Hammett

Table IV—Effect of Temperature on the Second-Order Rate Constants for Alkaline Hydrolysis of Some Benzoate Esters

Benzoate Esters	$k_{OH} \times 10^4$ (M^{-1} sec.$^{-1}$) 20°	25°	30°	35°
Methyl	4.40	6.08	11.0	15.0
Ethyl	1.35	1.98	3.41	4.20
n-Propyl	1.18	1.67	2.43	3.88
p-Tolyl	12.2	22.5	23.7	31.7
Phenyl	17.8	33.6	39.1	50.7
p-Fluorophenyl	32.5	56.5	64.8	84.9
p-Chlorophenyl	40.7	77.9	86.2	113
m-Cyanophenyl	132	227	252	326
p-Cyanophenyl	218	412	425	546
p-Nitrophenyl	302	533	592	845
2,4-Dinitrophenyl	2930	3580	4770	5040

Table V—Activation Parameters for Alkaline Hydrolysis of Some Benzoate Esters as a Function of the Alkyl Component

Substituent	$\Delta G^{\ddagger}_{25°}$, kcal./mole	$\Delta H^{\ddagger}_{25°}$, kcal./mole	$\Delta S^{\ddagger}_{25°}$, cal./mole-deg.
Methyl	21.8	14.7	−23.8
Ethyl	22.5	13.5	−30.2
n-Propyl	22.6	14.1	−28.5
p-Tolyl	21.1	10.5	−35.6
Phenyl	20.8	12.2	−28.9
p-Fluorophenyl	20.5	11.5	−30.2
p-Chlorophenyl	20.3	12.1	−27.5
m-Cyanophenyl	19.7	10.1	−32.2
p-Cyanophenyl	19.3	10.8	−28.5
p-Nitrophenyl	19.2	11.5	−25.8
2,4-Dinitrophenyl	18.1	6.5	−38.9

substituent constant (σ) is associated with nucleophilic attack of the hydroxide ion at the carbonyl carbon of the ester while the phenol dissociation constant (σ^-) is most closely associated with decomposition of the tetrahedral intermediate. Treatment of the present data using this procedure is shown in Fig. 2. Table VII indicates the slopes, standard deviations, and correlation coefficients for the ester data using this approach. The magnitude of the proportionality constant, r, required for the linear combination of σ and σ^- has been used to indicate the rate-determining step. The value of r necessary to linearize the Hammett plot for the data in this study is 0.3, which suggests that attack of hydroxide ion is rate limiting in the reaction sequence, which coincides with the observation of Ryan and Humffray (3, 4).

Comparisons of the slopes in the two plots, as seen in Table VIII, for acyl and alkyl variation show that the acyl component has a greater sensitivity to polar effects than does alkyl variation. Kirsch *et al.* (5) and Tommila and Hinshelwood (20) also observed that a greater sensitivity exists in the acyl portion than in the alkyl portion. If the rate-determining step for all of these reactions involves only the addition of hydroxide ion to the ester carbonyl carbon, this greater sensitivity can be understood in terms of the reacting bond being one atom closer to the substituent.

Treatment of the data in this study for alkyl variations, using the Swain and Lupton approach, is exemplified in Fig. 3 and Table IX. The correlation coefficients for each series of leaving groups are quite good; that linear correlations using this concept could be obtained suggests that at least two interaction mechanisms are involved as in the case for acyl variation. However, some factor(s)

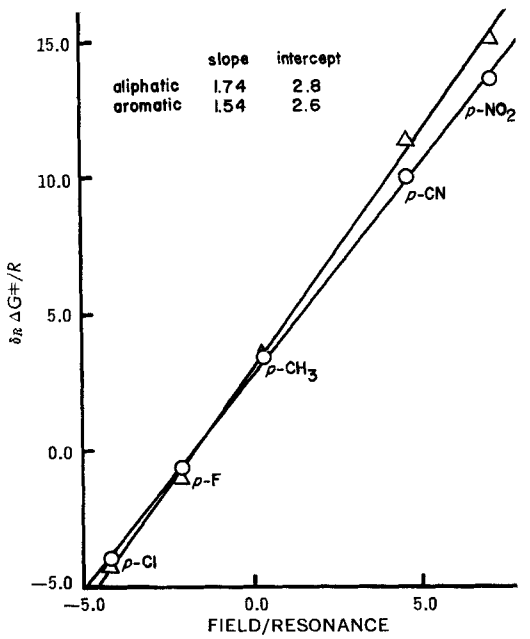

Figure 1—*Graphical representation of Swain and Lupton's treatment for acyl variations on the alkaline hydrolysis of benzoate esters with aromatic and aliphatic leaving groups. Key: ○, aromatic leaving groups; and △, aliphatic leaving groups.*

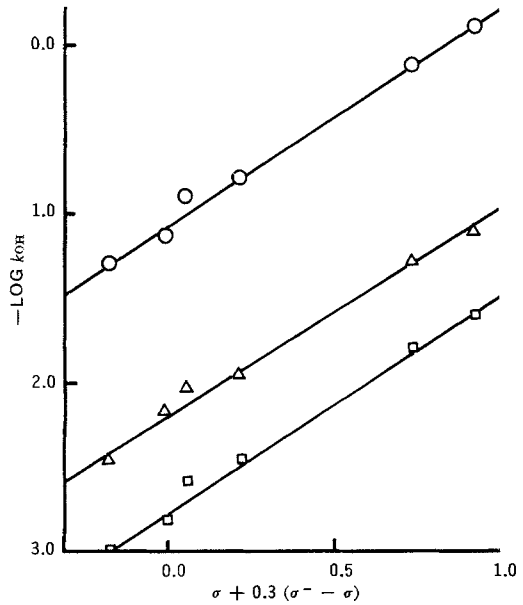

Figure 2—*Plot of the second-order rate constants for alkaline hydrolysis of some benzoate esters with variations in the aromatic leaving group* versus *a modified form of the Yukawa and Tsuno equation. Key:* ○, p-*cyanobenzoates;* △, p-*fluorobenzoates; and* □, p-*methylbenzoates.*

is not contributing equally to the overall interaction mechanism involved in the rate process in comparison to benzoic acid (σ) or phenol (σ^-) dissociation. No correlation is apparent between the $\%R$ involved in the reaction series and the acyl substituent; however, a striking difference in $\%R$ is observed when comparing the $\%R$ involved in the rate process, 43%, with that of the two equilibrium processes (53% for benzoic acid dissociation and 56% for phenol dissociation). There is a 10–15% difference in resonance contributions in the rate process as compared with either dissociation model, suggesting that this difference may account for the nonlinearity observed in the Hammett plot or log k_{OH} *versus* σ^- plot. Milton *et al.* (7) observed an average of 46% resonance for the methoxide-catalyzed methanolysis of aryl acetates and carbonates; they also observed nonlinearity for both series when the rate data were plotted against σ or σ^-.

Successful correlations of alkyl variations in structure and reactivity for *para*-substituted benzoate esters derived from alcohols were reported previously (10), but over a limited range of substituents involving small polar effects. Expansion of the series of substituent variation to encompass more polar groups, such as the halogenated ethyl substituents, did not result in the continued linearity of the correlation, as evidenced by the lines on the right-hand side of the pKa − log k_{OH} plot shown in Fig. 4. This nonlinearity might arise from the mesomeric effects associated with stabilization of the anion of the alcohol in the dissociation process.

Table VI—Correlation of Relative Rates of Alkaline Hydrolysis (log k/k_0) with Hammett Substituent Constants (σ) for Some Benzoate Esters

Ester Series[a]	ρ[b]	SD[c]	R[d]
Methyl benzoates	2.138	0.079	0.996
Ethyl benzoates	2.213	0.093	0.995
n-Propyl benzoates	2.184	0.049	0.999
Isopropyl benzoates	2.233	0.061	0.998
n-Butyl benzoates	2.158	0.077	0.996
Isobutyl benzoates	2.243	0.055	0.998
p-Tolyl benzoates	1.910	0.093	0.992
Phenyl benzoates	1.973	0.079	0.995
p-Fluorophenyl benzoates	2.020	0.056	0.998
p-Chlorophenyl benzoates	1.990	0.091	0.998
p-Cyanophenyl benzoates	2.095	0.117	0.998
p-Nitrophenyl benzoates	2.095	0.028	0.999

[a] Each series was composed of the following acyl substituents: *p*-methyl, hydrogen, *p*-fluoro, *p*-chloro, *p*-cyano, and *p*-nitro. [b] ρ is the least-squares fitted slope of a log (k/k_0) *versus* σ plot. [c] Standard deviation of the points from the line. [d] Correlation coefficient for the line.

Table VII—Correlation of Relative Rates of Alkaline Hydrolysis (log k/k_0) with Substituent Constants for Some Aryl Benzoate Esters Using the Modified Yukawa and Tsuno Equation

Ester Series[a]	ρ[b]	SD[c]	R[d]
p-Toluates	1.409	0.095	0.992
Benzoates	1.495	0.104	0.998
p-Fluorobenzoates	1.400	0.128	0.982
p-Chlorobenzoates	1.340	0.139	0.986
p-Cyanobenzoates	1.383	0.100	0.989
p-Nitrobenzoates	1.445	0.094	0.993

[a] Each series was composed of the following phenolic leaving groups: *p*-methyl, hydrogen, *p*-fluoro, *p*-chloro, *p*-cyano, and *p*-nitro. [b] ρ is the least-squares fitted slope of a log (k/k_0) *versus* $\sigma + 0.3\ (\sigma^- - \sigma)$ plot. [c] Standard deviation of the points from the line. [d] Correlation coefficient for the line.

As can be seen from Fig. 4, there are two distinct families of curves, depending on the types of leaving group; both types of leaving groups show definite curvature in the free energy plots. Kirsch and Jencks (8) also observed two separate correlations as a function of leaving group, but they did not observe the curvature since a limited number of substituents were used in the correlation. Two questions are apparent: (*a*) Why is there curvature in the free energy plot? (*b*) Why are there two families of curves, one for aromatic leaving groups and one for aliphatic leaving groups, and what accounts for the separation between these curves?

Nonlinearity in the $\Delta G\ddagger$–ΔG^0 Plot—The nonlinearity observed in the $\Delta G\ddagger$–ΔG^0 plot may result from nonlinearity in either the rate process or the comparison of the rate process to the equilibrium process. When considering the rate process, three possible explanations may be postulated involving: (*a*) a change in rate-determining step, (*b*) a change in mechanism, and (*c*) a change in the transition state structure as a result of substituent variations. When comparison of the rate process to the equilibrium process is considered, nonlinearity may arise from: (*a*) the differences in the interaction mechanisms involved in the two processes, or (*b*) a solvent effect since the rate process was carried out in 50% acetonitrile–water and the dissociation process in water.

If a change in rate-determining step or mechanism is the explanation for the nonlinearity, then the transition state for nucleophilic attack of hydroxide ion at the carbonyl carbon of the ester must be stabilized by the substituent variation to a degree such that the transition state associated with decomposition of the tetrahedral intermediate would have a greater energy of activation and thus be rate determining in the reaction mechanism. Bender and Thomas (21) showed that the ratio of hydrolysis to exchange ($k_H/k_{Ex.}$) varied with the electron-withdrawing power of the acyl substituent in a series of *para*-substituted methyl benzoates, but in all cases $k_H/k_{Ex.}$ was greater than unity, thus implying that the rate-determining step in all instances was the formation of the tetrahedral intermediate.

It has been suggested that a change in mechanism occurs as the leaving group is varied in the case of imidazole-catalyzed hydrolysis of esters (22, 23). General base catalysis occurs for esters with poor leaving groups, while nucleophilic catalysis occurs for esters with good leaving groups, as deduced from the order associated with the imidazole concentration in the rate expression. Solvent deuterium isotope effects and imidazole catalysis studies are important tools

Table VIII—Effect of Substituents in the Acyl Component Relative to the Alkyl Component

Substituent	ρ Acyl[a]/ρ Alkyl[b]
p-Nitro	1.469
p-Cyano	1.391
p-Chloro	1.485
p-Fluoro	1.442
Hydrogen	1.319
p-Methyl	1.355

[a] ρ Acyl is the value of the least-squares fitted slope of log (k/k_0) *versus* σ plot for acyl variations. [b] ρ Alkyl is the value of the least-squares fitted slope of log (k/k_0) *versus* $\sigma + 0.3\ (\sigma^- - \sigma)$ plot for alkyl variations.

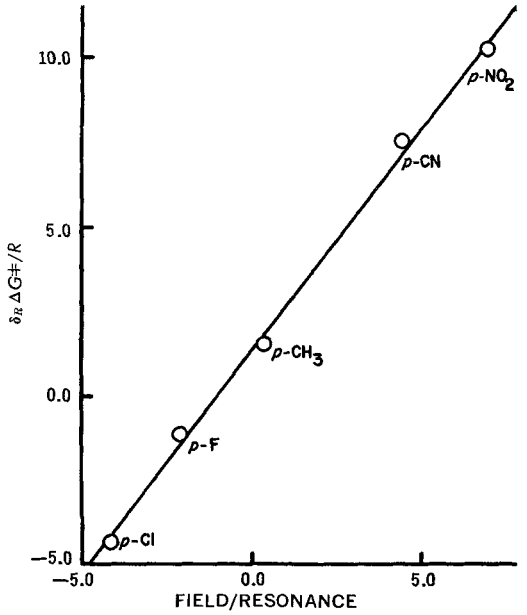

Figure 3—*Graphical representation of Swain and Lupton's treatment for variations in aromatic leaving group on the alkaline hydrolysis of benzoate esters.*

in suggesting the mechanism involved in ester hydrolysis, and both were investigated in the present study. The results of these studies are tabulated in Table II as a function of leaving group. As can be seen from the table, the rates of deuteroxide-catalyzed hydrolysis are greater than that of hydroxide ion; this inverse isotope effect suggests that the reaction involves specific base catalysis rather than general base catalysis. Similarly, the imidazole catalysis study implies that the mechanism involved in all cases of ester variations is nucleophilic attack rather than general base catalysis, since the ratio of rate constants associated with each ester is quite small. Milton *et al.* (24) observed similar solvent deuterium isotope effects in their study of basic methanolysis of aryl acetates and carbonates and suggested that a change in rate-determining step was not in

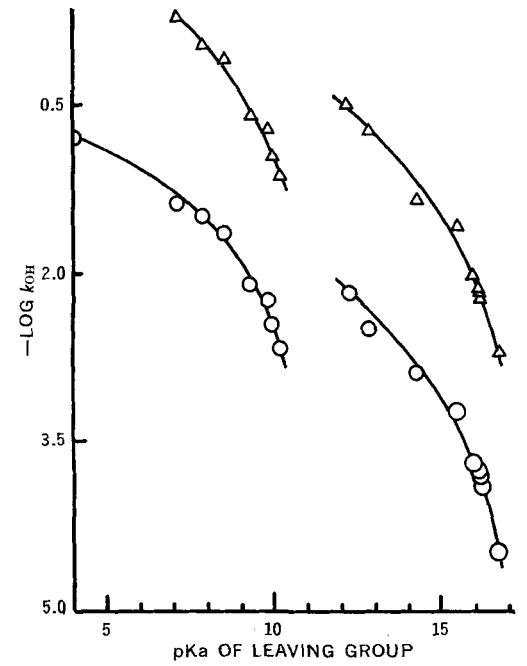

Figure 4—*Plot of the second-order rate constants for alkaline hydrolysis of benzoates and* p-*nitrobenzoates with variations in the leaving groups* versus *the pKa of the corresponding leaving group. Key:* △, p-*nitrobenzoates; and* ○, *benzoates.*

Table IX—Correlation of Free Energy of Activation for Alkaline Hydrolysis of Some Benzoate Esters with the Field (F) and Resonance (R) Constants of Swain and Lupton

Ester Series[a]	Slope[b]	Intercept	R[c]	%R[d]
p-Toluates	1.226	1.403	0.990	42
Benzoates	1.244	1.591	0.987	44
p-Fluorobenzoates	1.037	1.713	0.981	50
p-Chlorobenzoates	1.291	1.040	0.994	34
p-Cyanobenzoates	1.223	1.345	0.987	41
p-Nitrobenzoates	1.264	1.458	0.988	42

[a] Each series is composed of the following phenolic leaving groups: -methyl, *p*-fluoro, *p*-chloro, *p*-cyano, and *p*-nitro. [b] Value of the least-squares fitted slope of $\delta_R \Delta G\ddagger/R$ *versus* F/R plot, where $\delta_R \Delta G\ddagger$ is the effect of substituent R on the free energy of activation. [c] Correlation coefficient for the line. [d] Percent resonance involved in the reaction series as calculated by the method of Swain and Lupton.

order for the explanation of the nonlinearity observed in the $\Delta G\ddagger$–ΔG^0 plot.

Exner (25, 26) suggested that if a linear correlation of the free energies of activation at two temperatures can be obtained for a reaction, then the reaction is occurring *via* the same mechanism at the two temperatures throughout the series of substituent variations. Temperature effects on the rates of alkaline hydrolysis reveal that a linear correlation may be obtained; this is demonstrated by Fig. 5 for benzoate esters with both aromatic and aliphatic leaving groups. The slope and correlation coefficient for this plot are 0.933 and 0.999, respectively, providing additional evidence that the nonlinearity in the free energy plots is not due to a change in mechanism or rate-determining step. Also, the %R as calculated from the Swain and Lupton approach is not affected to any appreciable extent by the temperature.

Another possible explanation for nonlinearity might involve changes in the structure of the transition state as the substituent is varied. Fersht and Jencks (27) recently investigated the reactions of nucleophilic reagents with acylating agents of extreme reactivity and unreactivity, and they suggested that the symmetry of the transition state involved in the reaction may account for the nonlinearity observed in the free energy plot. This, however, cannot be applied to the case of ester hydrolysis since a relatively stable tetrahedral intermediate is involved in the mechanism, and asymmetry in the transition state would be reflected in a change in rate-determining step. The effect of the leaving group on the solvation cage around the carbonyl carbon in the ester molecule as the hydroxide ion approaches it may conceivably be a more appropriate explanation for the nonlinearity. Since the dispersal of the developing negative charge in the transition state is affected by the electron-withdrawing power of the leaving group, the effects of substituents

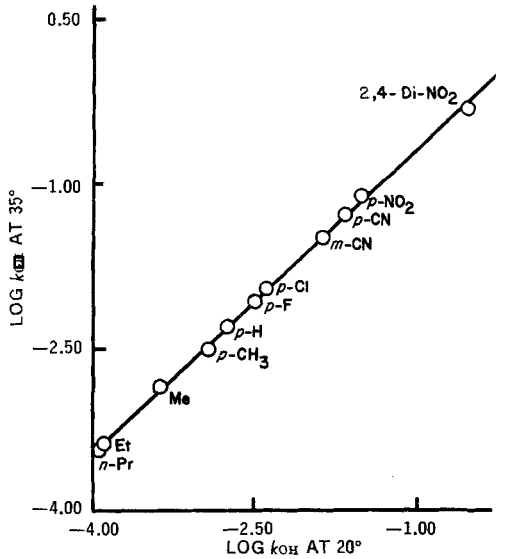

Figure 5—*Linear dependence of the substituent effects on the second-order rate constants for alkaline hydrolysis of benzoate esters at 20° and 35°, encompassing both aromatic and aliphatic leaving groups.*

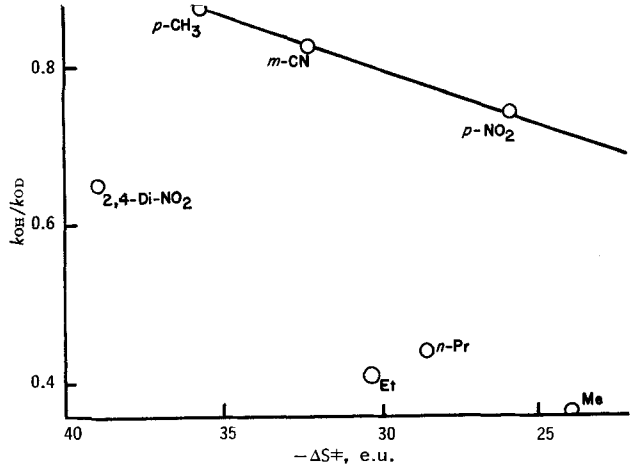

Figure 6—*Plot of substituent effects on the solvent deuterium isotope effects* versus *entropies of activation for the alkaline hydrolysis of benzoate esters with variations in leaving group.*

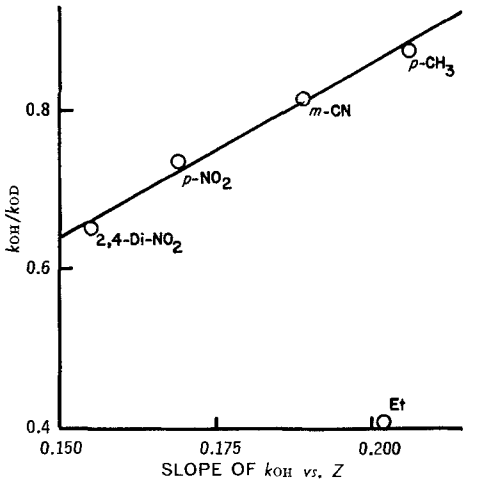

Figure 8—*Plot of the linear dependence of the solvent deuterium isotope effect on the slope of the Kosower plot for alkaline hydrolysis of benzoate esters with variations in leaving group.*

on the entropy of activation and the magnitude of the inverse solvent deuterium isotope effect would be related since both are dependent on the hydrogen bonding of the solvent in the transition state. Figure 6 indicates that this is indeed the case for leaving groups with similar steric requirements at the carbonyl carbon. The deviations from the line may be attributed to either variation in steric parameters or resonance.

Solvent effects on the second-order rate constants were correlated using the Kosower Z values for solvent polarity, and linear correlations were obtained as shown in Table X. Generally, the rate of reaction increased as the dielectric constant of the medium increased, suggesting that the activated complex is more polar than the ground state of the ester and the values of the slopes of this plot are dependent on the electron-donating or withdrawing power of the leaving group. These sensitivities to medium changes parallel the entropies of activation and the inverse solvent deuterium isotope effects as a function of leaving group involved in the ester. Figure 7 demonstrates the linearity of the entropies of activation to the slope from the log k_{OH} *versus* Z plot for *para*-substituted aromatic leaving groups, with the deviations from the line reflecting the steric differences involved in the ethyl and 2,4-dinitrophenyl leaving group. Figure 8, on the other hand, illustrates the linear dependence of the inverse solvent deuterium isotope effects on the slope from the log k_{OH} *versus* Z plot for all the aromatic leaving groups and the displacement from the line for the aliphatic leaving group.

Comparison of the rate process to the equilibrium process may involve either differences in the interaction mechanisms involved in the two processes or a solvent phenomenon, since the rate process was carried out in a semiaqueous system and compared to the equilibrium process in a completely aqueous system. Considering the interaction mechanisms involved in the two processes first, resonance effects in the phenol dissociation process are definitely known to exist and result from stabilization of the anion. Calculations show that there is 56%R in the dissociation of the phenols (19) as compared to 43% for the alkaline hydrolysis of esters with variations in the aromatic leaving group. In the case of alcohol dissociation, no resonance is capable of stabilizing the anion, since there are no π-electrons present in the saturated alcohol. However, for halogenated ethanols, the mesomeric effects of the halogen may result in stabilization of the anion. Hence the nonlinearity in the $\Delta G‡$–ΔG^0 plot may be a result of the differences in resonance involved in the two processes. Furthermore, the enthalpies of ionization of phenols in water are approximately one-half of the enthalpies of activation involved in the alkaline hydrolysis of the esters with corresponding leaving groups, while the entropies involved in both processes are very similar.

No distinct relationship was observed in the $\Delta H‡$ *versus* $\Delta S‡$ plot, as shown in Fig. 9, as a function of leaving group for the alkaline hydrolysis of these esters; however, if the enthalpies and entropies of activation are a result of external and internal contributions as suggested by Hepler for ionization of phenols (28), a line may be drawn in the plot with a slope corresponding to a value of the isokinetic temperature of 340°K. The deviations from this

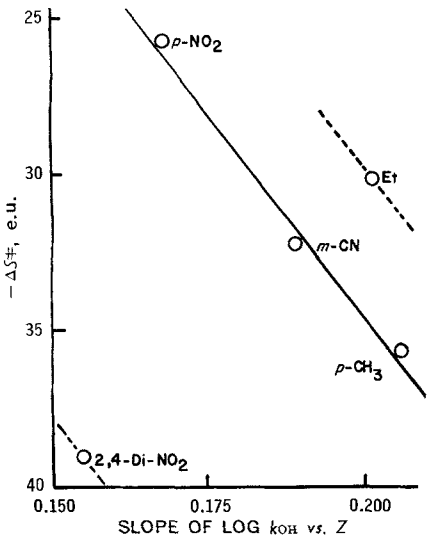

Figure 7—*Plot of the entropies of activation* versus *the slope of the Kosower plot for alkaline hydrolysis of benzoates with variations in leaving group.*

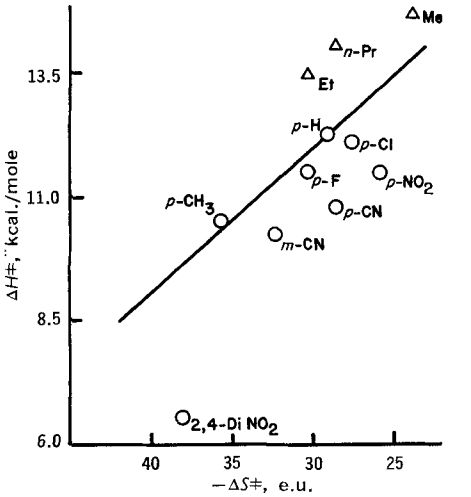

Figure 9—*Enthalpies of activation* versus *entropies of activation for the alkaline hydrolysis of benzoate esters with variations in the leaving group. The line drawn on the plot has a value of 340°K. for the slope. Key:* ○, *aromatic leaving groups; and* △, *aliphatic leaving groups.*

Table X—Correlation of Solvent Effect on the Alkaline Hydrolysis of Some Benzoate Esters with Kosower's Z Values

Benzoate Ester	Slope[a]	Intercept[b]	R[c]
2,4-Dinitrophenyl	0.155	1.32	0.999
p-Nitrophenyl	0.168	0.68	0.999
m-Cyanophenyl	0.189	0.47	0.996
p-Tolyl	0.206	0.34	0.997
Ethyl	0.202	−1.30	0.999

[a] Slope of the least-squares fitted line of log k_{OH} *versus* Z plot. [b] Intercept value of log k_{OH} corresponding to 100% water. [c] Correlation coefficient for the line.

line would then reflect the internal contributions to the enthalpies of activation exhibited by the substituted aromatic leaving groups, and a linear plot of these substituent effects on the internal contributions to enthalpy of activation *versus* Hammett's substituent constants is obtained (Fig. 10). The substituent effects on the free energies of ionization of phenols is defined by:

$$\delta_R \Delta G^0 = \delta_R \Delta H_i^0 + (280° - T)\delta_R \Delta S^0 \qquad \text{(Eq. 6)}$$

while the free energies of activation for the alkaline hydrolysis of esters with the corresponding leaving groups is defined by:

$$\delta_R \Delta G‡ = \delta_R \Delta H_i‡ + (340° - T)\delta_R \Delta S‡ \qquad \text{(Eq. 7)}$$

suggesting that differences in the solvation may be responsible for the nonlinearity since the substituent effect on ΔG^0 in the ionization of phenols is primarily dependent on the internal contributions to enthalpy, with minor solvation contributions as reflected by the temperature differential.

A solvent effect may also explain the nonlinearity in the $\Delta G‡$–ΔG^0 plot if nonlinearity exists in the plot of the pKa of the leaving groups in water as compared to the pKa of the leaving group in the buffer system. Extrapolation of the rate constants to 100% water for each leaving group was performed, and the logarithms of these extrapolated values were plotted *versus* the pKa of the conjugate acid of the leaving group (Fig. 11). Included in Fig. 11 are the logarithms of the rate constants at a variety of other water concentrations; nonlinearity has not been eliminated, suggesting that a solvent effect is not the cause for the nonlinearity.

SEPARATION OF AROMATIC AND ALIPHATIC LEAVING GROUP CURVES IN THE $\Delta G‡$–ΔG^0 PLOT

Kirsch and Jencks (8) observed two distinct correlations as a function of the type of leaving group in the alkaline hydrolysis of acetate esters and suggested that the phenolic esters were less reactive than alcoholic esters of comparable pKa because of steric hindrance of the bulky benzene ring. Bruice *et al.* (9), however, observed a single correlation for the alkaline hydrolysis of acetates as a function of the pKa of the leaving group. According to Jencks, this could be explained by the fact that their results were obtained under a variety of conditions and could not be compared directly. If a steric effect is operative in determining this separation between the two types of leaving groups, the entropies of activation involved in each type should reflect this.

As can be seen in Table V, no apparent trend or regularity in $\Delta S‡$ for either the phenolic or alcoholic esters is evident. However, the $\Delta H‡$'s for the alcoholic esters apparently are several kilocalories greater than those of the phenolic esters, suggesting that resonance effects play a greater role in determining the rate than steric effects; resonance effects would be reflected in $\Delta H‡$ whereas steric effects would primarily be reflected in $\Delta S‡$. Furthermore, if comparisons of the pKa's of the alcohols and phenols with that of the corresponding acids are made, as in Fig. 12, one observes two distinct correla-

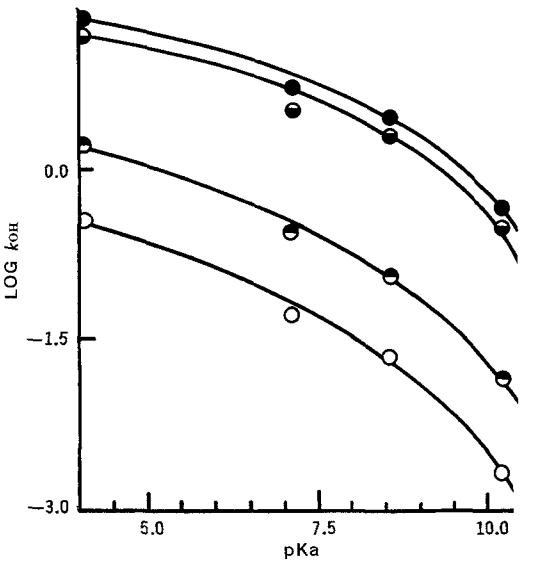

Figure 11—*Effect of solvent composition on the second-order rate constants for alkaline hydrolysis of benzoate esters with aromatic leaving groups* versus *the pKa of the corresponding phenol. Key:* ●, *100% water;* ◒, *5% (v/v) acetonitrile in water;* ◓, *30% (v/v) acetonitrile in water; and* ○, *50% (v/v) acetonitrile in water.*

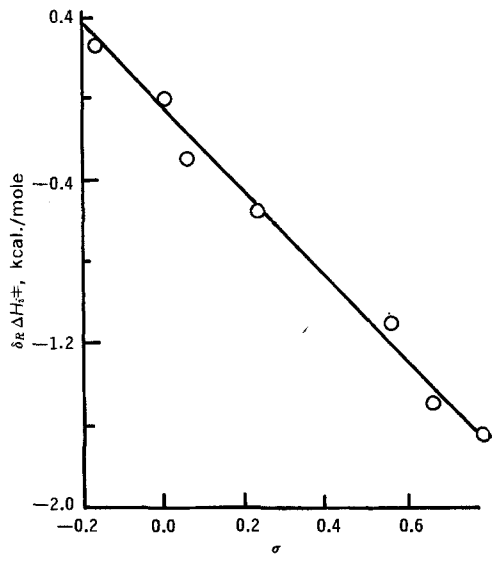

Figure 10—*Hammett plot of substituent effects in the internal contributions to enthalpy of activation for the alkaline hydrolysis of benzoates with aromatic leaving groups.*

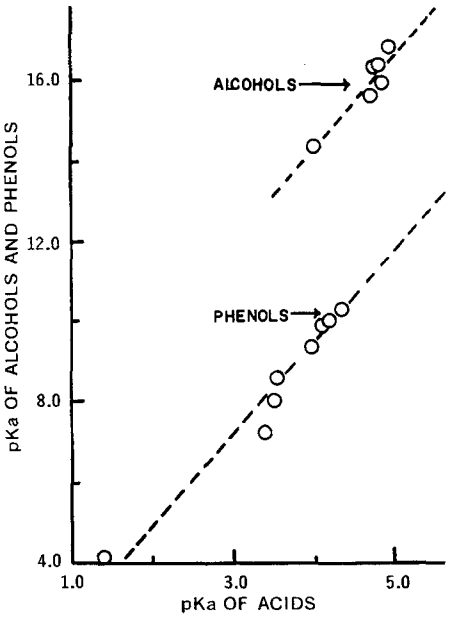

Figure 12—*Plot of the pKa of alcohols and phenols (ROH)* versus *the pKa of the corresponding acids (RCOOH).*

tions separated by approximately 5 pKa units, which coincides with the 5 pKa unit displacement between the rates of hydrolysis of esters with alcoholic and phenolic leaving groups in the $\Delta G‡$–ΔG^0 plot. Thus, the separation observed in the $\Delta G‡$–ΔG^0 plot may be a result of the differences involved in comparing the two equilibrium processes to the rate process.

CONCLUSIONS

Correlation of substituent effects with reactivity for acyl-substituted benzoate esters, composed of both aliphatic and aromatic leaving groups, is successfully accomplished using the Hammett equation. This successful correlation is apparently due to equivalent contributing factors involved in both the rate and the equilibrium processes. One of these factors is the resonance interaction, which was found to be essentially equal in the equilibrium process (dissociation of *para*-substituted benzoic acids) and in the rate process (alkaline hydrolysis of acyl-substituted benzoate esters).

Neither the Hammett equation nor the alcohol and phenol dissociation model is entirely effective in correlating structure with reactivity for variation in the alkyl portion of the ester molecule. However, the alcohol dissociation model is effective over a larger range of substituents than either the Hammett relationship or the phenol dissociation model.

Plotting the rate constant for alkaline hydrolysis of some benzoates *versus* the dissociation constant of the conjugate acid of the leaving group yields two distinct families of curves, one for aliphatic and one for aromatic leaving groups, and each ester series shows distinct curvature. The curvature in the $\Delta G‡$–ΔG^0 plot is not due to a change in mechanism or rate-determining step in the rate process, based on the evidence presented here, but rather is due to multiple interaction mechanisms which are apparently not involved to the same extent when comparing the two processes. One of these interaction mechanisms, resonance, may account for the nonlinearity since the resonance contributions are approximately 10–15% less in the alkaline hydrolysis of aryl benzoates than are involved in the dissociation of benzoic acids or phenols.

Another interaction mechanism that must be considered to account for the nonlinearity is the solvation differences that exist between the rate and equilibrium processes. Substituent effects on $\Delta H‡$ and $\Delta S‡$ can be divided into internal and external contributions; it has been shown here that only the internal contributions to $\Delta H‡$ can be successfully correlated with Hammett's polar substituent constant, σ, suggesting that the nonlinearity in $\Delta G‡$–ΔG^0 plots might be a result of the external contributions arising from solvation of the transition state. However, when the entropies involved in the two processes are considered, evidence suggests that the nonlinearity involved may not be a result of solvation differences since the $\Delta S‡$ for the rate process are very similar to the ΔS^0 for the equilibrium process. Hence, the resonance interaction mechanism is favored as an explanation of the nonlinearity observed in the $\Delta G‡$–ΔG^0 plot.

The two families of curves observed by plotting the rate constant for alkaline hydrolysis against the pKa of the leaving group may best be explained on the basis of resonance involved in the dissociation process. In phenol dissociation, there is resonance stabilization of the anion; in alcohol dissociation, no such stabilization is possible. Thus, the 5 pKa unit difference between the two families of curves, one for aliphatic and one for aromatic, is the same as is observed when plotting the pKa of phenols and alcohols against the pKa of the corresponding aliphatic and aromatic acids.

In summary, the alcohol and phenol dissociation model does not linearly correlate with rate of ester solvolysis when large ranges of substituent leaving groups are employed. Moreover, the incomplete correlation can be used to provide mechanistic insight into alkaline hydrolysis of esters. The alcohol dissociation model is effective over a larger range of substituents than phenol dissociation, conceivably because of the general lack of resonance interaction mechanism involved in alcohol dissociation as compared to phenol dissociation.

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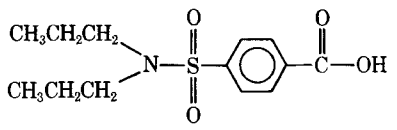
Combined GC and High Resolution Mass Spectrometric Determination of Probenecid

KHALID SABIH, CURTIS D. KLAASSEN, and KHAWLA SABIH

Abstract □ A sensitive GC method for measurement of probenecid in biological fluids is described. This method involves the conversion of probenecid to its methyl ester by treatment with dimethylsulfate under basic conditions. Analysis was performed on Gas Chrom Q coated with DC-200. The major metabolic product of probenecid, the glucuronide conjugate of the unchanged drug, was identified by combined GC and high resolution mass spectrometry following enzymatic hydrolysis.

Keyphrases □ Probenecid determination—biological fluids □ Biological fluids—probenecid determination □ Dimethylsulfate–probenecid reaction—probenecid analysis □ GLC–mass spectroscopy, combined—analysis

Probenecid [(di-*n*-propylsulfamyl)benzoic acid] (I), a sulfonamide derivative, is used as a uricosuric agent in the treatment of gout. It has also been useful in the laboratory for studying the hepatic and renal organic acid secretory mechanisms that excrete drugs and other chemicals. Several spectrophotometric methods have been described (1–4) to determine the levels of the drug in biological fluids. However, most of these methods are neither sensitive nor specific and the "blank" is often quite high.



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Guarino and Schanker (5), in their study of the biliary excretion of probenecid, pointed out that the drug undergoes metabolic changes and that these metabolites are excreted as the glucuronide.

While this work was in progress, Guarino *et al.* (6) identified the metabolites of probenecid by mass spectrometry. They reported that probenecid was excreted into the bile of rats unchanged to the extent of about 60% and that the rest was excreted as the glucuronide conjugate of the side-chain oxidized products of probenecid and also the dealkylated product. However, the present work indicates that these metabolites are quantitatively minor in relation to the simple probenecid glucuronide.

This report describes a very sensitive, quantitative combined GC–mass spectrometric method for the determination of probenecid in biological fluids.

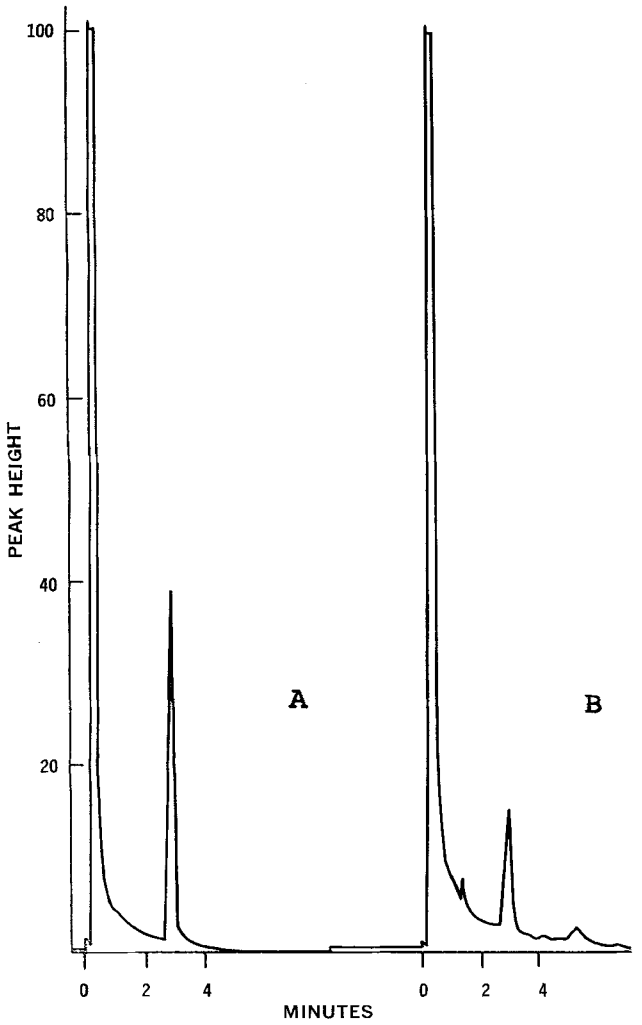

Figure 1—*Gas chromatogram of: (A) pure probenecid, and (B) probenecid extracted from bile treated with β-glucuronidase. Curve A represents 5-μl. injection of 40 mcg. probenecid in 100 μl. chloroform.*

EXPERIMENTAL

Reagents—Probenecid[1] and dimethylsulfate[2] were used. Chloroform and heptane were analytical redistilled reagents. Acetate buffer, 0.2 *N*, pH 5.6, was made by mixing 4.8 ml. of AcOH solution (0.2 *N*; 11.55 ml. in 1000 ml. H_2O) and 45.2 ml. of sodium acetate solution (0.2 *N*; 16.4 g. in 1000 ml. H_2O) and diluting the mixture with water to 100 ml. Methanolic potassium carbonate solution was made by mixing 1 ml. of 5% aqueous solution of K_2CO_3 and 9 ml. of MeOH.

Preparation of Probenecid Methyl Ester—A solution of 0.5 g. of probenecid in 20 ml. methanolic K_2CO_3 was placed in a flask fitted with a magnetic stirrer. Ten milliliters of Me_2SO_4 was added, and the reaction was allowed to proceed for 15 min. at 70°. MeOH was then removed under reduced pressure, and the residue was treated with 20 ml. acetate buffer, pH 5.6. The reaction mixture was then extracted with 50 ml. $CHCl_3$. The chloroform extract was dried (Na_2SO_4), and solvent was removed under reduced pressure. TLC of the residue, using freshly prepared silica gel G plates, gave only one uniform spot, R_f 0.83 with chloroform and 0.17 with benzene. The residue was purified on a silica column. The fraction

[1] Supplied by Merck Institute for Therapeutic Research, West Point, Pa.

[2] Matheson Coleman and Bell, Norwood, Ohio.

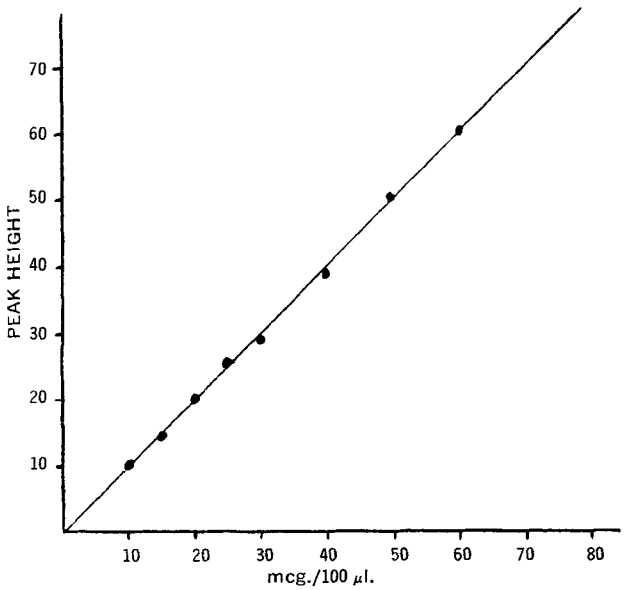

Figure 2—*Relationship between peak height and amount injected. Five microliters injected in each case.*

eluted with $CHCl_3$ was collected and gave a white solid (m.p. 50–51°) upon removal of solvent. The IR spectrum of the product showed no absorption in the hydroxyl region.

Anal.—Calcd. for $C_{14}H_{21}NO_4S$: C, 56.18; H, 7.02; N, 4.68. Found: C, 56.24; H, 7.13; N, 4.56.

The methyl ester of probenecid was also prepared using HF–CH_3OH reagent, but the yield was not quantitative as in the case of the Me_2SO_4–K_2CO_3 method.

GC—An F&M model 5755B gas chromatograph, equipped with a flame-ionization detector, was used for chromatography. The column was 1.4 m., 0.32 cm. (4.5 ft., 0.125 in.) stainless steel tubing packed with Gas Chrom Q, 80–100 mesh, coated with 5% DC-200. The operating temperatures used for analysis of probenecid were: column, 190°; injection port, 310°; and detector, 320°. Helium was used as the carrier gas at a flow rate of 40 ml./min.

Standards—The sodium salt of probenecid was added to water, plasma, urine, or bile samples in varying amounts (2.5–60 mcg./ml.). The resulting mixtures were acidified with 1 ml. 1 *N* HCl and then extracted by shaking with 5 ml. of chloroform for 5 min. The chloroform extracts were taken to dryness under a stream of air, and each residue was treated with 1 ml. of MeOH–K_2CO_3 and 0.1 ml. of Me_2SO_4. The reaction was allowed to proceed for 5 min. in a water bath (70°). Methanol was then removed under a stream of air at the temperature of the water bath, and 1 ml. of pH 5.6 buffer was added to each residue. Extraction of probenecid methyl ester was accomplished by shaking each standard with 10 ml. of chloroform or heptane. The organic layer was separated and dried under a stream of air. GC analysis was carried out by adding 100 μl. of chloroform to each residue and injecting 1–5 μl. onto the column under the conditions cited previously.

Biological System—Simonsen Sprague-Dawley rats (300–350 g.) were anesthetized with pentobarbital sodium (45 mg./kg.). The femoral vein and artery were cannulated with polyethylene tubing (PE-50) for administering the probenecid and collecting blood samples, respectively. The bile duct was cannulated with PE-10 tubing for collection of bile samples.

Probenecid was administered intravenously at a dose of 40 mg./kg. Bile samples were collected from the cannulated bile duct at hourly intervals. Blood samples were obtained from the cannulated femoral artery at 10 min., 1, 2, 3, and 4 hr. after administration of the probenecid; 0.2 ml. plasma or bile was used for the analysis. Heparin was used as the anticoagulant. Urine samples were collected from another group of rats at 6-hr. intervals for 48 hr.

Each sample was acidified with 1 ml. 1 *N* HCl and extracted with 5 ml. $CHCl_3$. The free probenecid partitioned into the $CHCl_3$, and the conjugated metabolite remained in the aqueous phase. The layers were separated, and the aqueous layer from each sample was adjusted to pH 5.0 (1 *N* NaOH and checked with pH meter) and incubated with 2 ml. β-glucuronidase solution (1 mg./ml. in acetate buffer, pH 5.0) overnight. The mixture was acidified and extracted with 5 ml. chloroform. The chloroform extracts (before and after treatment with the enzyme) were taken to dryness separately under a stream of air. Each residue was then treated with 1 ml. MeOH–K_2CO_3 solution followed by 0.1 ml. Me_2SO_4, and the reaction was allowed to proceed for 5 min. in a water bath at 70°. Methanol was then removed under a stream of air, and 1 ml. of pH 5.6 buffer was added followed by 5 ml. $CHCl_3$. Extraction was aided by shaking for 2 min. The chloroform layer was taken to dryness and redissolved in 100 μl. of chloroform. Analysis was carried out by injecting 1–5 μl. of the resulting solutions onto the column under the conditions cited previously.

Mass Spectrometry—The medium resolution mass spectra of probenecid methyl ester and the methylated drug obtained from biological samples were determined on a Finnigan quadrupole mass spectrometer. All samples were introduced to the ionization chamber through a Varian Aerograph model 1740 gas chromatograph. The column used was 3.05 m., 0.32 cm. (10 ft., 0.125 in.) stainless steel tubing packed with Gas Chrom Q coated with 3% OV-1. The oven temperature was programmed from 180 to 250° at 6°/min. Helium was used as the carrier gas, with a flow rate of 30 ml./min.

The high resolution mass spectrum of probenecid methyl ester was obtained using an A.E.I. MS-9 double-focusing mass spectrometer. The MS-9 was coupled to a digital data system[3]; computer processing of the digitalized data was carried out on Battelle's CDC-6400 computer. Samples were introduced by direct probe operating at 120°. The ionizing voltage was 70 ev., the source temperature was 200°, the multiplier setting was 5.0, and the accelerating voltage was −8 kv.

Table I—Recovery of Probenecid from Plasma

Amount Added, mcg./ml.	Percent Recovered
10	98
20	95
40	100
60	93
100	96
200	94

Table II—Plasma Disappearance and Biliary Excretion of Probenecid[a]

10 min.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
Plasma Disappearance of Probenecid, mcg./ml.					
172	109	43.1	3.1	0	0
181	61	6.2	1.2	0	0
206	119	12.5	0.6	0	0
Free Probenecid in Bile, mcg./ml.					
	469	250	156	43.7	6.2
	375	281	148	85.9	12.5
	625	516	148	93.7	7.8
Conjugated Probenecid in Bile, mcg./ml.					
	364	463	55	58.6	11.2
	199	165	311	26.5	12.5
	366	62	60	16.5	12.6
Biliary Flow, μl./min./kg.					
	80	67	59	45	32
	83	71	52	43	38
	68	60	44	31	13

[a] Probenecid given intravenously (40 mg./kg.).

RESULTS AND DISCUSSION

Methylation—The methyl derivative of probenecid was obtained in quantitative yield using Me_2SO_4 as the methylating agent under basic conditions. The methyl derivative of probenecid, however, underwent some hydrolysis on standing for a long time. This was shown by high resolution mass spectrometry.

[3] Designed and built at Battelle.

GC—The gas chromatograms of pure probenecid methyl ester and that obtained from biological material are shown in Figs. 1A and B, respectively. Under the conditions cited in the method, the methyl ester of probenecid had a retention time of 3.0 min. Chromatograms of control samples did not contain any peaks with the retention time of probenecid. Addition of increasing amounts of probenecid to samples of known concentrations of the drug produced an increase in the peak height having the retention time of the drug.

The relationship between peak height and the quantity of the drug injected was linear at optimum instrument sensitivity, as shown in Fig. 2. Duplicate runs were reproducible; for example, with a 50 mcg./ml. solution, the standard error was ±0.23.

The GC method is applicable to biological fluids such as plasma, bile, and urine. The extraction procedure for probenecid from these fluids was adequate and gave 93–100% recovery of added drug (Table I).

Table II demonstrates the application of this GC method for determining the plasma disappearance and biliary excretion of probenecid in rats. Three hours after a 40-mg./kg. dosage of probenecid, essentially all the probenecid had disappeared from the plasma. No conjugated probenecid was detectable in the plasma at any time interval.

The excretion of probenecid into the bile was also measured. Both free and conjugated probenecid were detected in the bile, in contrast to only free probenecid being detected in the plasma. Probenecid is detected in the bile for 5 hr. after the administration of 40 mg./kg. of probenecid, whereas no detectable amounts were found in the plasma after the 3rd hr. This suggests that the probenecid is stored in the liver, as are many compounds that are excreted into the bile. The results indicate that the amount of the free probenecid extracted in the bile represents 55–60% of the total probenecid present in the bile.

Identification of Probenecid Glucuronide—Tillson (1) reported in 1954 that probenecid is excreted both as the unchanged free acid and as its simple acyl glucuronide. However, Guarino and Schanker (5) later reported that the glucuronide is that of metabolically changed probenecid. In a more recent study, Guarino *et al.* (6) demonstrated that probenecid undergoes side-chain W and W-1 oxidation, followed by glucuronide formation and excretion of these glucuronides into the bile. *N*-Dealkylated probenecid was also detected in the bile, but no simple acyl glucuronide was found.

In the present study, free probenecid was removed from the bile of rats administered probenecid by acidifying the bile with 1 *N* HCl and extracting it into chloroform. To make sure that all of the unconjugated probenecid was removed by this extraction, the aqueous layer was adjusted to pH 5.0, allowed to stand overnight, and then reextracted with chloroform after acidification with HCl. GC of this second extract showed no peak corresponding to probenecid. This evidence demonstrated that the drug extracted from bile samples after treatment with β-glucuronidase was due to the presence of the glucuronide conjugate and not due to the presence of unextracted residues of probenecid.

Table III—High Resolution Mass Spectral Data of Probenecid and Its Methyl Ester

m/e	Relative Intensity	*m/e*	Relative Intensity
14	1.7	83	2.6
17	2.6	85	2.3
18	16.0	91	3.2
27	4.6	95	1.3
28	16.8	97	3.9
29	3.6	103	5.0
30	2.4	104	3.9
36	2.6	105	2.0
39	2.4	109	1.2
40	3.4	111	1.5
41	9.5	120	1.5
42	6.6	121	31.7
43	16.5	122	2.9
44	4.2	135	37.2
45	2.5	136	3.5
50	1.7	149	1.0
51	1.4	185	34.2
55	4.7	186	2.5
56	9.9	187	1.8
58	2.0	197	7.3
60	1.2	199	26.2
64	1.1	200	3.4
65	6.3	201	1.9
67	1.3	214	8.8
69	3.2	228	3.8
70	8.3	256	100.0
71	3.8	257	13.5
72	2.2	258	6.0
73	1.4	268	3.3
75	2.9	270	79.2
76	5.4	271	10.2
77	3.6	272	5.0
81	2.2	285	3.5
82	1.2	299	3.5

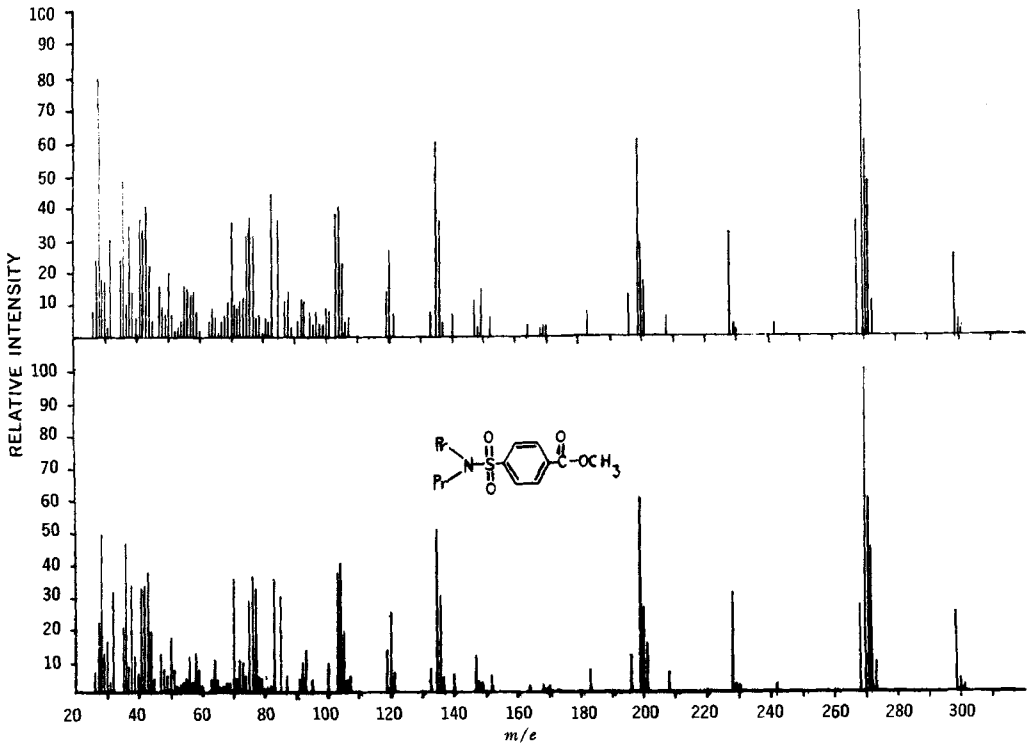

Figure 3—*Mass spectra of probenecid methyl ester and of probenecid metabolite.*

After the bile was treated with β-glucuronidase, additional peaks were observed on the gas chromatogram (Fig. 1). The major peak has the same retention time after enzymatic cleavage as does the parent probenecid (Fig. 1).

Further evidence was obtained that this peak was probenecid by the combined GC–mass spectrometry described previously. To establish firmly the masses of the prominent ions in the mass spectrum of the probenecid methyl ester, a high resolution mass spectrum was obtained (Table III) on the standard sample introduced into the mass spectrometer in the direct probe. The mass spectra of probenecid and that obtained by enzymatic hydrolysis of the glucuronide conjugate were determined by combined GC–mass spectrometry, and both spectra were identical (Fig. 3). The computer printout of probenecid spectrum was identical to that reported by Guarino *et al.* (6). Thus, no attempt will be made to identify fractions or fragmentation pattern.

Therefore, from this study, it would appear that the major metabolic product of probenecid in the rat is the simple acyl glucuronide. The authors do not feel that this is the only metabolite of probenecid, but it is the major metabolite because it is the only easily detectable peak observed by GLC when the probenecid peak remains on scale. However, when a larger quantity of metabolites is placed on the column so that the probenecid peak goes off scale, additional peaks are observed. By examining the data reported by Guarino *et al.* (6), it would appear that their results are similar but that they inadvertently disregarded their major metabolite, the metabolite that was in such great quantity that it went off scale and whose retention time was identical to the simple acyl glucuronide of probenecid. Therefore, it would appear that the major metabolic product of probenecid found in rat bile is the simple acyl glucuronide of probenecid.

CONCLUSION

The GC method described is accurate, sensitive, and specific for the determination of probenecid. The method is applicable to biological fluids such as blood, bile, and urine, using only very small amounts of these fluids.

The major metabolic product of probenecid was identified to be the simple acyl glucuronide conjugate. Conclusive evidence was obtained from the retention time and mass spectrometry following enzymatic hydrolysis. The mass spectra are simple and can be described mainly in terms of bond fission.

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DRUG STANDARDS

Determination of Chloride in Aluminum Hydroxide Gels by Use of a Chloride-Selective Electrode

R. M. SPEIGHTS*, J. D. BROOKS, and A. J. BARNARD, Jr.

Abstract ☐ The determination of the chloride content of aluminum hydroxide wet gels and dried powders is important for the control of both in-process materials and finished products. The application of the chloride-selective electrode was studied, and the response was found to be Nernstian in slurries containing 0.010–4.0% Cl and 1.00–2.50% in Al_2O_3 over the pH range 5.7–7.8. The modest equipment costs and operator skill required and the rapidity commend the method.

Keyphrases ☐ Chloride determination—aluminum hydroxide gels ☐ Chloride-selective electrode—chloride determination in aluminum hydroxide gels ☐ pH, aluminum hydroxide content, effects—chloride-selective electrode response

Aluminum hydroxide, as a wet gel or dried powder, is a major antacid and is manufactured by the controlled neutralization of an acidic solution of an aluminum salt, commonly aluminum chloride. The product is freed of chloride by persistent washing. Consequently, the determination of residual chloride is important both for in-process control and as a specification for the finished pharmaceutical grade product.

Chloride is conventionally determined in the products by a Volhard titration or by a turbidity comparison. In the Volhard titration the product is dissolved in nitric acid, a known volume of a standard silver nitrate solution is added, and the silver chloride precipitate is rendered inactive by addition and shaking with nitrobenzene. The excess of silver ion in the supernatant is titrated with a standard thiocyanate solution after addition of iron (III) nitrate as the indicator.

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Ion-selective electrodes offer exciting possibilities for applied analysis, especially where many samples of a

single material must be examined rapidly and routinely. The properties, feasibility, and applicability of various ion-selective electrodes were reported and reviewed (1–4).

The sensing element of the "solid-state" chloride-selective electrode consists of a solid silver chloride crystal in contact with an internal filling solution, containing silver ion at a fixed concentration (5). The potential developed between the internal reference electrode and the internal filling solution is due only to the changes in the silver-ion activity, which in turn is influenced by the chloride-ion activity in the sample solution.

Therefore, at a given temperature the potential developed can be written in the following equation:

$$E = E_b - \frac{2.3RT}{F} \log a_{Cl^-} \qquad \text{(Eq. 1)}$$

When the concentration of chloride ion, C_{Cl^-}, is substituted for its activity, Eq. 1 reduces to

$$E = E_b - k \log C_{Cl^-} \qquad \text{(Eq. 2)}$$

The value of k, given by $2.3RT/F$, is 59.16 mv.; however, for a practical ion-selective electrode the obtained value may depart slightly from the theoretical one.

When the Nernstian response predicted by Eq. 2 is secured, the plot on semilogarithmic paper of the voltage of the system (linear) against the chloride content in percent (log) should give a straight line. The slope of the resulting line is the change in cell voltage for a unit change in chloride content.

Some practical applications of the chloride-selective electrode reported so far include the determination of chloride in ground water (6), sweat and urine (7), cheese (8), biological fluids (9), drugs (10), and alkali metal chlorides (11).

The present work was undertaken to establish whether the chloride-selective electrode could be applied to the rapid establishment of the chloride content of aluminum hydroxide process samples as well as final products. A detailed study was needed since anion penetration accompanies or follows the formation of oleated compounds, such as hydrous metal oxides (12), and thus the electrode response might not correspond completely to the chloride content. Parameters requiring study included chloride content, assay of the gel, and pH. Small variations in ambient temperature were found not to have an effect in this study; consequently, temperature is not a variable of practical concern. Additionally, it was established that the leak rate of the saturated calomel electrode makes only negligible contribution to the sample chloride concentration over the course of a practical analysis.

It is important to know how effective the discrimination of the electrode for the anion of interest is in the presence of other anions. The available chloride-selective electrode[1] is reported (3) to be 15 times more sensitive to chloride ion than to bromide ion and 360 times more sensitive to chloride than to iodide. Of cations, silver (I) is the only common interference. For a 0.1 M halide solution, Nernstian response is reported (5) for the entire pH range 0–14.

[1] Orion Research Inc.

Table I—Chloride Response of the Electrode

Cl, %	1.5% Al_2O_3, pH 7.43, mv.	1.5% Al_2O_3, pH 7.80, mv.	1.5% Al_2O_3, pH 5.70, mv.	2.43% Al_2O_3, pH 7.43, mv.	1.00% Al_2O_3, pH 7.43, mv.
0.0101	135.5	134.0	135.5	135.0	130.0
0.0201	119.0	118.0	120.0	115.7	117.0
0.0302	109.5	105.8	109.0	106.0	105.8
0.0402	102.0	97.6	101.7	97.3	97.5
0.0502	96.2	92.8	94.0	92.0	92.4
0.0603	92.0	88.4	89.5	86.5	88.0
0.0803	85.0	81.0	83.0	80.0	80.8
0.1002	79.6	76.0	77.5	74.8	75.7
0.1992	63.6	60.4	61.0	59.0	59.9
0.2971	54.0	51.0	51.7	50.0	50.5
0.3937	47.7	44.5	45.0	44.0	44.5
0.9509	28.3	25.0	25.0	24.4	25.0
1.8000	13.5	10.0	10.0	10.0	10.5
2.3429	7.0	4.0	4.0	3.3	4.0
2.8857	1.5	−0.5	−0.0	−1.0	−0.3
3.4286	−2.3	−4.4	−4.0	−5.0	−4.0
3.9714	−6.3	−8.0	−7.5	−8.0	−8.0

MATERIALS

Reagents—A "chloride-free" stock slurry was prepared by techniques analogous to those used in the large-scale production of aluminum hydroxide gel; however, aluminum nitrate was used rather than aluminum chloride in order to reduce the level of chloride to a negligible amount. This slurry was assayed by an ethylenediaminetetraacetic acid titration procedure, paralleling that of the USP (13). The solution in nitric acid is buffered to pH 3.5 by addition of saturated ammonium acetate solution. A volume of 100 ml. of methanol is added along with a known volume of a standardized ethylenediaminetetraacetic acid solution; the excess of ethylenediaminetetraacetic acid is back-titrated with 0.1 M zinc sulfate. The indicator is dithizone, conveniently added as a methanolic solution (1 mg./ml.), which is stable if stored in a refrigerator (14, 15). This assay procedure gives precise and accurate results in the hands of an experienced worker. The average of a number of titration results indicated the assay of this stock slurry to correspond to 2.43% as Al_2O_3. The pH of this slurry was established to be 7.40 with an expanded-scale pH meter. The chloride content was investigated using a 25-g. portion of the slurry and a conventional Volhard titration and was found to be less than 0.0001%. The slurry was, therefore, more than satisfactory for the study since the chloride content of aluminum hydroxide process samples and final products is in practice substantially higher and commonly above 0.01%.

A 1.5% Al_2O_3 slurry was prepared as required by mixing 61.7 g. of the 2.43% Al_2O_3 chloride-free slurry with sufficient distilled water

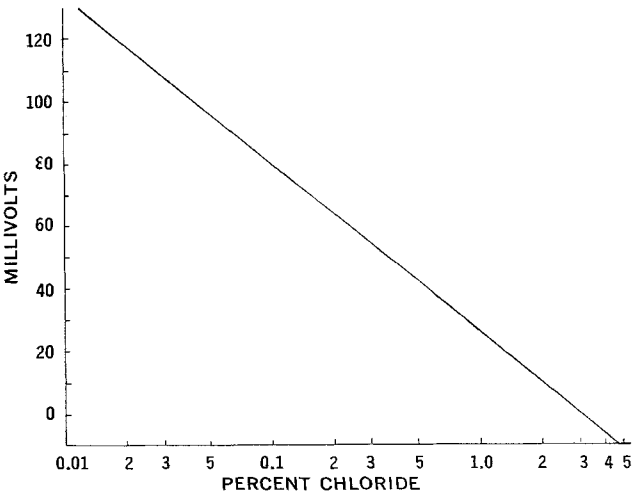

Figure 1—*Response of electrode to chloride content.*

Table II—Electrode Response as Function of pH for Two Chloride Levels

0.050% Cl		1.80% Cl	
pH	mv.	pH	mv.
7.95	+90.2	7.7	+10.0
5.2	+95.0	5.3	+10.3
5.6	+93.8	5.6	+ 9.8
6.0	+93.0	6.0	+ 9.8
6.4	+92.7	6.35	+ 9.8
6.9	+92.7	6.8	+10.0
7.3	+92.7		
7.7	+92.5	7.7	+10.0

Table III—Comparison of Chloride Content (Percent) of Aluminum Hydroxide Dried Powder by Three Methods

Sample Number	Turbidimetric	Volhard	Chloride Electrode	
			1st day	2nd day
1	0.30	0.31	0.328;0.328	—
2	0.40	0.40	0.325;0.334	0.370;0.387
3	0.20	0.28	0.231;0.219	0.241;0.232
4	0.30	0.33	0.247;0.247	—
5	0.50	0.40	0.472;0.472	—
6	0.40	0.36	0.393;0.383	—
7	0.60	0.59	0.571;0.571	0.625;0.602

(chloride free) to give a final weight of 100.0 g. The mixture was thoroughly mixed using magnetic stirring. A 1.00% Al_2O_3 slurry was prepared as needed in the same way, mixing 41.2 g. of the 2.43% Al_2O_3 slurry with water to a weight of 100.0 g.

A chloride standard solution was prepared by dissolving 27.39 g. of reagent grade sodium chloride (shown by Volhard titration to have an assay of 99.9% NaCl) in chloride-free water and diluting with water to 100 ml. using a Class A volumetric flask. The solution corresponds to 0.166 g. of chloride/ml.

Apparatus—An Orion chloride-ion electrode, model 94-17, was used in conjunction with an Orion single-junction reference electrode, model 90-01, with the manufacturer's filling solution (Orion 90-00-01). A Corning research pH meter, model 12, with an expanded scale was used in the measurement of the voltages developed.

The chloride-selective electrode was prepared for use by applying a small amount of silicone oil (Orion 94-00-03) *via* an impregnated piece of absorbent tissue paper to the electrode body and the sensing element. This treatment causes the electrode to shed water and thereby reduces the rinsing of the electrode necessary to obviate contamination by the previous sample (5).

The reference electrode was readied for use by adding a small amount of the filling solution through the filling hole, retracting the sleeve, and then allowing the solution to wet the inner cone. Finally, the filling solution was added until the volume level reached within 0.63 cm. (0.25 in.) of the filling hole. This operation was performed daily before use of the electrode. It was reported (5) that with the careful maintenance described, the rate of contamination of the sample with chloride ion due to electrode seepage is about 0.2 mmole/min. An operator must make the voltage measurement in the shortest time possible with samples especially low in chloride.

EXPERIMENTAL

Chloride Response of Electrode—The chloride response of the chloride-selective electrode was evaluated with the chloride-free 1.50% Al_2O_3 slurry (pH 7.43). A magnetic stirring bar was added to the 100.0 g. of the slurry in a 250-ml. beaker, taking care to avoid loss of material through splashing. Magnetic stirring was commenced at such a rate that full suspension of the slurry was secured. The chloride standard solution was then added in small increments *via* a 10-ml. Class A buret with 0.02-ml. divisions. Care was taken to assure that each increment reached the slurry by touching the tip of the buret to the beaker wall and allowing the resulting drop to mix with the suspension. After thorough mixing *via* magnetic stirring and standing for 1 min., the stable reading on the expanded millivolt scale was recorded. The results of this experiment are shown in Table I in the second column, and the plot of voltage against the percent of chloride is shown in Fig. 1.

Effect of pH on Electrode Response—The effect of pH on the response of the chloride-selective electrode was then studied at constant aluminum hydroxide concentration. Three experiments were developed, including the one already mentioned (pH 7.43). The same chloride-addition schedule was followed with the 1.5% Al_2O_3 gel, but the pH was adjusted by adding either 10% (w/w) sodium hydroxide solution or reagent grade nitric acid to pH 7.80 and 5.70, respectively. The results are tabulated in Table I; if plotted in the style of Fig. 1, the experimental points would essentially define a single line only.

Experiments were conducted to establish any interaction of pH and chloride concentration over the chloride contents that might be encountered with process samples, namely, 0.05 and 1.80% chloride. Slurries of these contents were obtained by addition of 0.30 and 12.00 ml. of the chloride standard solution to 100.0-g. amounts of the 1.50% chloride-free slurry, respectively. The pH was then adjusted to various values by the addition of sodium hydroxide and nitric acid. The results are summarized in Table II.

Effect of Al_2O_3 Assay on Electrode Response—The effect of the aluminum hydroxide content of the slurry on the response of the chloride-selective electrode was then considered. This effect was investigated by recording the electrode response following the addition of chloride incrementally to slurries of various assay values. First, to a 100.0-g. portion of the chloride-free 2.43% Al_2O_3 stock slurry, the chloride standard solution was added in small increments and the cell voltage was read after each addition. A chloride-free slurry of 1.00% Al_2O_3 content was treated similarly. The results for these two slurries are summarized in Table I; if plotted in the style of Fig. 1, the experimental points would essentially define a single line only.

Procedure for Wet Gels—The recommended procedure for the determination of chloride ion in aluminum hydroxide wet gel is as follows: assay the gel according to the USP procedure. Based on this assay value, weigh an appropriate portion into a 250-ml. beaker, add water to 100.0 g., and stir magnetically, thereby obtaining 100 g. of a 2% Al_2O_3 slurry.

C_1 designates the percent concentration of chloride in this slurry. Insert the electrodes, read the expanded millivolt scale, and record the reading (E_1). From a 10-ml. buret, add 0.30 ml. of the chloride (0.050%) standard (0.166 g./ml.) and designate the increase ΔC. Record the reading of the millivolt scale (E_2) after the addition of chloride. Calculate the change in scale reading $E_1 - E_2$ and designate as ΔE. Calculate the percent chloride in the slurry by the formula:

$$C_1 = \frac{\Delta C}{10^{\Delta E/k} - 1} = \frac{\Delta C}{\left(\text{antilog}\,\frac{\Delta E}{k}\right) - 1} \qquad \text{(Eq. 3)}$$

The value of k, established by computer processing of all of the data presented in Table I, was found to be 53.96 mv. with a standard deviation of the mean of ±1.05 mv. However, for the purpose of calculation of C_1 by Eq. 3, the value of k was taken to be 54.0 mv.

Calculate the chloride content of the original wet gel sample by the formula:

$$\%\ \text{Cl} = \frac{C_1 \times 10{,}000}{\text{sample wt. in grams} \times \%\ \text{assay}} \qquad \text{(Eq. 4)}$$

In practical analysis, the use of Eq. 3 can be replaced by a semilog plot of C_1 *versus* E, since ΔC and k are constants (*e.g.*, see Fig. 1).

Procedure for Dried Powders—The recommended procedure for the determination of chloride in aluminum hydroxide dried powders is as follows: assay the powder according to the USP procedure. Weigh in a tared 250-ml. beaker about 4 g. of the powder (to the nearest milligram). Add water to make the final weight of slurry 100.0 g. and stir magnetically. Continue according to the second and subsequent paragraphs of the *Procedure for Wet Gels.*

Calculate the chloride content of the dried powder by Eq. 4.

RESULTS AND DISCUSSION

The results obtained with aluminum hydroxide wet gels are presented in Tables I and II and Fig. 1. From these data, Nernstian

Table IV—Comparison of Requirements for Three Chloride Methods

	Turbidimetric	Modified Volhard	Chloride Electrode
Equipment and solutions	Glassware	Glassware	Glassware, pH meter with expanded scale, and electrodes
	1 buret	2 burets	1 buret
	1 standardized solution of HCl and 1 stock solution of silver nitrate	2 standardized solutions of silver nitrate and ammonium thiocyanate plus 1 iron stock solution	1 standard sodium chloride solution
	Triple-beam balance	Analytical balance	Analytical balance
Special precautions	Protection from sunlight	Addition of nitrobenzene and shaking to "protect" precipitate	None
Operator skill required	Extreme (visual acuity and ability to differentiate turbidity)	Moderate (normal visual acuity)	Modest
Rapidity of determination	Fast	Slow	Moderate
Precision and accuracy	Low	High	Moderate

response was demonstrated for chloride contents in the range 0.010–4.0% for aluminum hydroxide slurries 1.00–2.50% in Al_2O_3 and with the pH in the range 5.7–7.8.

It was also of interest to examine the effect of various salts on the chloride electrode potential and thereby establish if any common interferences might be encountered with practical samples. To the 100 g. of the standard slurry of 0.30% chloride content, 1 g. of each of the following salts, as the reagent grade products, was consecutively added: $NaNO_3$, $Mg(NO_3)\cdot 6H_2O$, KNO_3, and $Ca(NO_3)_2\cdot 4H_2O$. The increases in the cell voltage after the additions amounted to 2.7, 2.0, 0.0, and 1.6 mv., respectively. The total increase in electrode response for the four salts was only 6.3 mv. This voltage increase corresponds to a decrease in the measured chloride content of 0.01% and can largely be explained in terms of the decrease in the weight concentration of the slurry by the salt additions. These findings suggest that the use of the chloride-selective electrode can probably be extended to aluminum hydroxide formulations.

Furthermore, it was found that the voltage measurements could be secured while the magnetic stirrer was operating without any disturbance in the meter response.

It was also of interest to demonstrate the validity of the method with aluminum hydroxide dried powders, since they also represent major pharmaceutical products. Seven dried gels meeting USP specifications for chloride were determined according to the *Procedure for Dried Powders*, except that a second standard addition of 0.3 ml. of the chloride standard solution was made and the calculations of the results were based on the first addition and the sum of the two. Additionally, the chloride content of these dried powders was determined by the USP turbidimetric procedure and by the modified Volhard titration. The results obtained by the three methods, tabulated in Table III, show excellent agreement. The repeatability of the chloride-sensitive electrode procedure, established for three of the samples, was satisfactory.

It was instructive to compare the requirements of the three methods in terms of equipment, precaution, operator skill, rapidity, *etc.*, when applied to aluminum hydroxide or similar products. This qualitative comparison is delineated in Table IV.

The value for the Nernst factor, 54.0 mv., secured for the chloride electrode used in this study was confirmed for a second similar electrode purchased from the same manufacturer at a later date. Consequently, the use of that value k in calculations based on Eq. 3 is further supported.

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Effect of Concentration and Size of Lubricant on Flow Rate of Granules

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Abstract □ The flow rates of lactose granulations containing 50% of phenobarbital, sulfadiazine, and sodium salicylate were compared to the flow rate of the basic lactose granulation. Hydrogenated castor oil, glyceryl monostearate, polyethylene glycol 4000, and stearic acid were investigated as lubricants by means of a flowometer. For each lubricant, there was an optimum concentration, not exceeding 1%, which produced a maximum flow rate of the sodium chloride and the lactose granulation. For a constant concentration of lubricant, the flow rate was increased to a maximum rate as the size of the lubricant was reduced to 0.0213 cm. A further reduction in size of the lubricant slowed the flow rate.

Keyphrases □ Granule flow rates—lubricant size, concentration effects □ Lubricant size, concentration effects—granule flow rates □ Flow rates, granules—optimum lubricant size, concentration □ Fines concentration in granules—flow effect

The flow of particulate solids is involved in many pharmaceutical operations such as blending, encapsulation, tableting, and the packaging of powders and granules. The flow properties of a formulation may be improved by operational procedures such as granulation or spray drying. If modification of the particles is not feasible, the flow properties may be improved by the addition of a lubricant.

The flow of particles *per se* is the primary consideration in the formulation of particulate solids. Any friction or adhesion between the material and the equipment is of secondary concern, although it cannot be ignored in tableting. Münzel and Kägi (1) classified lubricants used in the preparation of compressed tablets as: (*a*) true lubricants (die eigentlichen Gleitmittel), which improve the flow properties of the granules and do not deform significantly under compressional pressures; (*b*) antiadhesive agents (die Antiadhäsionsmittel), which prevent the adhesion of the tablet to the punches and die and easily deform under pressure; and (*c*) substances and mixtures that are concomitantly lubricants and antiadhesive agents. Inherent in this classification is the recognition that lubricants, *e.g.*, calcium stearate and magnesium stearate, often perform more than a single function and are not so precisely classified in reality. Thus, in this report on the flowability of granules, a lubricant is defined as any other substance added in small amounts to improve the flow of a particular solid. Selection of a lubricant is often on a trial-and-error basis because no single method has been widely accepted as suitable for the evaluation of lubricants (1–5). The flowometer offers a simple and objective method to evaluate the effectiveness of lubricants under dynamic conditions (6, 7).

Table I—Flow Rate of a 16/20-Mesh Size Fraction of a Lactose Granulation and of a Granulation Containing Equal Quantities of Drug and Lactose

Orifice Diameter, cm.	Flow Rate, g./sec. Lactose Granulation	Flow Rate, g./sec. Lactose and Drug (1:1) Granulation	Percent Change
	Phenobarbital		
1.428	16.4	15.8	−3.6
0.925	4.3	4.0	−7.2
	Sulfadiazine		
1.428	16.4	15.5	−5.3
0.925	4.3	4.0	−7.2
	Sodium Salicylate		
1.428	16.4	18.0	9.8
0.925	4.5	4.6	9.1

Lubricants are generally recommended to be used in concentrations up to 5% and as very fine powder (8–11). This investigation was conducted to determine the effect of size and concentration of the lubricant on the flow rate of sodium chloride and lactose granulations. The effect on flow rate of the incorporation of several drugs into the lactose granulation was examined.

EXPERIMENTAL

Preparation of Particles—The method of preparation and separation of size fractions of sodium chloride and lactose particles has been described (7). Lactose granulations containing 50% of a drug were prepared by a wet granulation method. All materials were USP grade. The drugs were phenobarbital, sulfadiazine, and sodium salicylate. Equal weights of lactose and the drug were mixed in a V-blender for 1 hr. The mixture was transferred to a Hobart mixer, and portions of a 10% starch paste were added until a total of 3% starch had been added. The water solubility of sodium salicylate necessitated the use of a 20% starch paste. The wet mass was passed manually through a 6-mesh screen. The wet granulation was collected on trays lined with paper and dried in an oven at 38° for 17 hr. The dried granulation was milled through a No. 4 circular screen in a FitzMill, with the blunt edges forward while operating at low speed. The 16/20-mesh size fraction was separated by a Tyler sieve shaker using U. S. standard sieves. The granulation was treated with compressed air to remove fine particles.

The lubricants were melted on a water bath and then allowed to solidify at room temperature. The lubricants were milled in a FitzMill with the aid of dry ice. To prepare large particles, a No. 10 screen was used with the mill operating at slow speed and the blunt edge forward. To prepare small particles, a No. 40 screen was used with the mill operating at fast speed. A Tyler sieve shaker was used to collect the 16/20- and 30/40-mesh size fractions. The 60/80-, 100/140-, and 140/230-mesh size fractions were separated by using a 10-g. sample in an Allen-Bradley sonic sifter for 3 min. at an amplitude of 10.

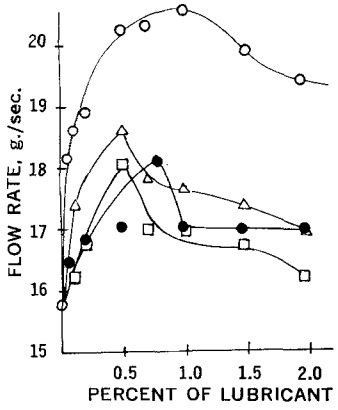

Figure 1—*Influence of concentration of 100/140-mesh size fraction of lubricants on flow rate through a 1.428-cm. circular orifice of a 16/20-mesh size fraction of lactose granulation. Key: ○, stearic acid: ●, hydrogenated castor oil; □, polyethylene glycol 4000; and △, glyceryl monostearate.*

The materials studied as potential lubricants were purified glyceryl monostearate[1], hydrogenated castor oil[2], polyethylene glycol 4000[3], and stearic acid USP. The lubricants were added in increasing proportions to 500 g. of the 16/20-mesh size fraction of sodium chloride and lactose granulations and mixed for 5 min. in a V-blender with a 2-qt. shell.

Measurement of Flow Rate—Flow rates through orifice diameters of 0.925 and 1.428 cm. were determined with a flowometer described in a previous report (7). The flow rate, expressed in grams per second, represents the average of five measurements.

RESULTS AND DISCUSSION

Influence of Drug on Flow Rate—Granulations containing equal quantities of lactose and a drug were used to determine the effect of a drug on the flow rate. The density of lactose is 1.59 g./ml. Since flow rate is a function of the density of the material (12), drugs were selected that had approximately the same density as lactose. Phenobarbital, sulfadiazine, and sodium salicylate were chosen because their densities are 1.30, 1.50, and 1.50 g./ml., respectively.

In the powdered form, the drugs flowed poorly. It was somewhat surprising that the flow rates of the granulations containing a drug did not change more than 10% from that of the lactose granulation. As shown in Table I, with sodium salicylate the flow rate was increased; and with sulfadiazine and phenobarbital the flow rate was decreased. It appears that these drugs do not greatly alter the flow rate of a basic lactose granulation. The granulation operation *per se* is important in the preparation of flowing particulate solids.

Influence of Concentration and Size of Lubricant—The influence of the concentration of a lubricant of a given size on the flow rate was determined by the addition of a 100/140-mesh size fraction of the lubricant to a 16/20-mesh size fraction of sodium chloride and lactose granulations. The effect on the flow rate of the lactose granulation upon the addition of various percentages of glyceryl monostearate, hydrogenated castor oil, polyethylene glycol 4000, and stearic acid is shown in Fig. 1. A maximum flow rate was obtained at 1% stearic acid, 0.7% hydrogenated castor oil, 0.5% polyethylene glycol 4000, and 0.5% glyceryl monostearate. A similar effect was demonstrated upon the addition of lubricants to sodium chloride particles. As shown in Fig. 2, a maximum flow rate was obtained at 0.1% hydrogenated castor oil, 0.05% polyethylene glycol 4000, and 0.05% glyceryl monostearate.

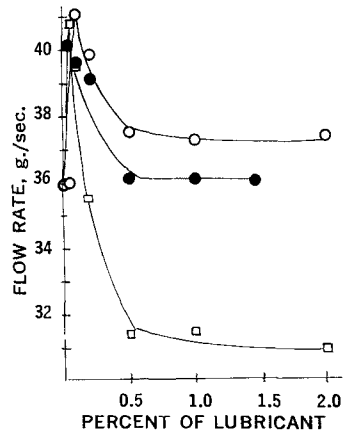

Figure 2—*Influence of concentration of 100/140-mesh size fraction of lubricants on flow rate through a 1.428-cm. circular orifice of a 16/20-mesh size fraction of sodium chloride granules. Key: ○, hydrogenated castor oil; ●, polyethylene glycol 4000; and □, glyceryl monostearate.*

[1] Fisher Scientific Co., Fair Lawn, N. J.
[2] The Baker Castor Oil Co., Bayonne, N. J.
[3] Union Carbide Corp., New York, N. Y.

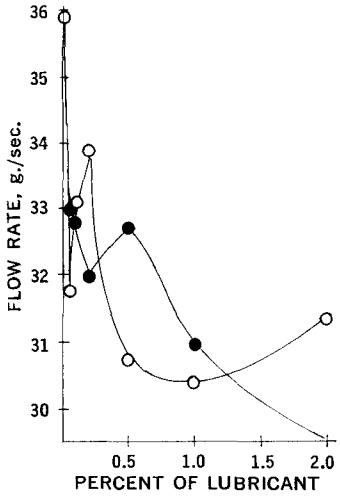

Figure 3—*Influence of concentration of 100/140-mesh size fraction of stearic acid on flow rate through a 1.428-cm. circular orifice of sodium chloride granules. Key: ○, 100/140-mesh size fraction; and ●, 30/40-mesh size fraction of sodium chloride.*

Münzel and Kägi (1) reported that the addition of 5 and 10% stearic acid and polyethylene glycol 6000 impaired the flow rate of sodium chloride and increased the flow rate of other substances. They did not investigate the influence of the lubricant at concentrations less than 1%. The present report demonstrates that the flow rate is increased by a small quantity of lubricant and that the maximum flow rate is usually attained with less than 1% lubricant.

The variation of behavior upon the addition of stearic acid to the sodium chloride particles is shown in Fig. 3. The addition of 0.05% stearic acid decreased the flow rate, and the addition of a greater concentration of stearic acid increased the flow to a maximum rate at 0.2% stearic acid. The variation was verified by adding stearic acid to a 30/40-mesh size fraction of sodium chloride particles. Again the flow rate was decreased as the concentration of the stearic acid was increased to 0.2%; as the concentration was increased to 0.5% stearic acid, the maximum flow rate was attained. An electrostatic effect was observed on the stearic acid particles and was assumed to be responsible for this variation (13).

The action of lubricants has been studied from the viewpoint of concentration, but apparently no research has been reported concerning the influence of size of the lubricant on flowability. It is usually assumed that particles of a lubricant have a diameter less than 0.015 cm., *i.e.*, 100 mesh (8).

In this investigation of the effect of size of a lubricant on flow rate, a constant concentration of lubricant was selected for which a 100/140-mesh size fraction of lubricant had produced a maximum

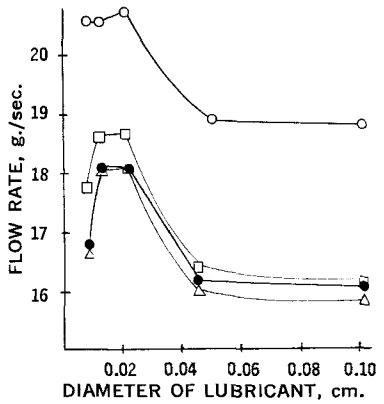

Figure 4—*Influence of various sizes of lubricant maintained at a constant concentration on the flow rate through a 1.428-cm. circular orifice of a 16/20-mesh size fraction of lactose granulation. Key: ○, 1% stearic acid; ●, 0.7% hydrogenated castor oil; △, 0.05% polyethylene glycol 4000; and □, 0.05% glyceryl monostearate.*

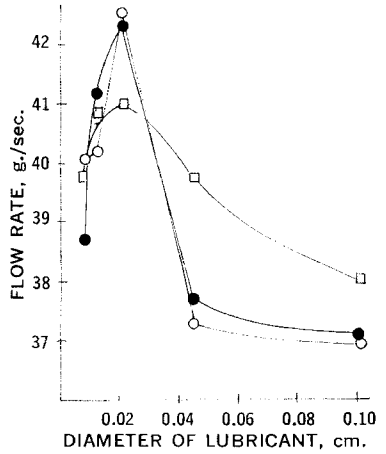

Figure 5—*Influence of various sizes of lubricant maintained at a constant concentration on the flow rate through a 1.428-cm. circular orifice of a 16/20-mesh size fraction of sodium chloride granules. Key: ●, 0.1% hydrogenated castor oil; ○, 0.05% polyethylene glycol 4000; and □, 0.05% glyceryl monostearate.*

flow rate. For example, 1% stearic acid had produced a maximum flow rate of the lactose granulation. Therefore, with the concentration of stearic acid maintained at 1%, various size fractions of stearic acid were added to a 16/20-mesh size fraction of lactose granulation, and the flow rates were measured. As shown in Fig. 4, the maximum flow rate occurred with stearic acid particles having a diameter of 0.0213 cm., *i.e.*, 60/80-mesh size fraction. Similarly, with 0.7% hydrogenated castor oil, the maximum flow rate occurred with the particles of lubricant having a diameter of 0.0213 cm. With 0.5% polyethylene glycol and 0.5% glyceryl monostearate, the maximum flow rate occurred when the lubricant had a diameter of 0.0213 cm.

As shown in Fig. 5, the maximum flow rates for the 16/20-mesh size fraction of sodium chloride particles were observed with the lubricant possessing an average diameter of 0.0213 cm. The concentrations of lubricants employed in these experiments were 0.1% hydrogenated castor oil, 0.05% polyethylene glycol 4000, and 0.05% glyceryl monostearate.

With both sodium chloride and lactose granulation, the maximum flow rate occurred when the lubricants had a diameter of 0.0213 cm. This finding suggests that there is an optimum diameter for a lubricant and that a smaller size will reduce flowability. Thus, when a lubricant is used in a formulation, there is an optimum concentration and size that should be used to obtain maximum flowability.

For a given material, the addition of fines increases the flow of a granulation to a maximum flow rate (14). As the concentration of the fines is increased and the void is progressively filled to a greater degree, the flow reaches a maximum rate. With a further increase in concentration of fines, the contact surface between the particles and the concomitant increase in interparticular friction becomes so great that the movement of the particles is less and the rate is slowed.

The smaller size of fines of the granule required a lower percent of fines to attain a maximum flow rate (7). When the concentration of fines was maintained at 10%, the flow rate was increased to a maximum value for sodium chloride and lactose granulations as the diameter of the fines was reduced to 0.009 cm., *i.e.*, 140/200-mesh size fraction. A further reduction of size of the fines produced a slower flow rate, presumably due to the larger specific surface and greater interparticular friction.

With the four lubricants used with the sodium chloride and lactose granulations, the maximum flow was attained if the diameter of the lubricant was 0.0213 cm. The difference in size of particles of lubricant and the size of fines needed to attain a maximum flow rate suggests that the mechanisms by which lubricants and fines increase flowability are different. It has been proposed for powders that lubricants act by mechanically separating the coarser particles, with a reduction in van der Waals' force (4, 15, 16). It appears that a lubricant increases flow rate of a granulation by a reduction of interparticular friction within the flowing stream of the particulate solid.

SUMMARY

1. The flow rate of a lactose granulation was not markedly affected by the addition of 50% phenobarbital, sulfadiazine, and sodium salicylate.

2. The effect of varying the concentration of lubricants—hydrogenated castor oil, glyceryl monostearate, polyethylene glycol 4000, and stearic acid—on the flow rate of sodium chloride and lactose granulations was investigated with a flowometer.

3. For the four lubricants investigated, a 1% or less concentration produced a maximum flow rate of sodium chloride and lactose granulations.

4. For a constant concentration of the four lubricants studied, a maximum flow rate was obtained when the diameter of the lubricant was 0.0213 cm.

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Automated IR Technique for Determination of Methylphenidate Hydrochloride in Tablet Formulation

TIBOR URBÁNYI and MARIA C. H. LIN

Abstract □ A new automated method has been developed for the determination of methylphenidate hydrochloride in pharmaceutical formulations using IR absorption. The weakly reactive ester possesses an isolated absorption band in the IR region. The method is specific for the ester carbonyl band and it is more reliable than any other method found in the literature. Since the determination is based on an extraction of the intact ester into a chloroform solution, the proposed method shows stability and will strengthen the application as an analytical method in the pharmaceutical industry.

Keyphrases □ Methylphenidate HCl tablets—automated analysis □ Automated procedure—methylphenidate HCl determination in tablets □ Stability-indicating procedure—methylphenidate HCl tablets □ IR spectrophotometry—automated analysis procedure

Spectrophotometric methods such as UV, visible, or fluorometric techniques are commonly available for the determination of active ingredients in unit dosage pharmaceutical preparations. The unique sensitivity of these methods in unquestionable. However, with the gain in sensitivity, there is a corresponding loss in specificity. The IR absorption has a unique specificity as a photometric method, but until now it has been neglected because of its lack of sensitivity for the quantitative determination of pharmaceutical preparations. Its application is becoming more significant with the introduction of commercially available high-resolution instruments.

The usefulness of IR absorption as an advantageous quantitative technique for the determination of an ester in pharmaceutical preparations is generally recognized. Preparations containing ester groups undergo chemical reactions fairly easily and may cause pharmacological deactivation of the active drug. Methods suitable for the determination of the decomposition product as well as for the content uniformity per unit dosage, with good precision, were needed. The IR technique was successfully used for the determination of the intact ester in one tablet formulation when the other methods failed to work.

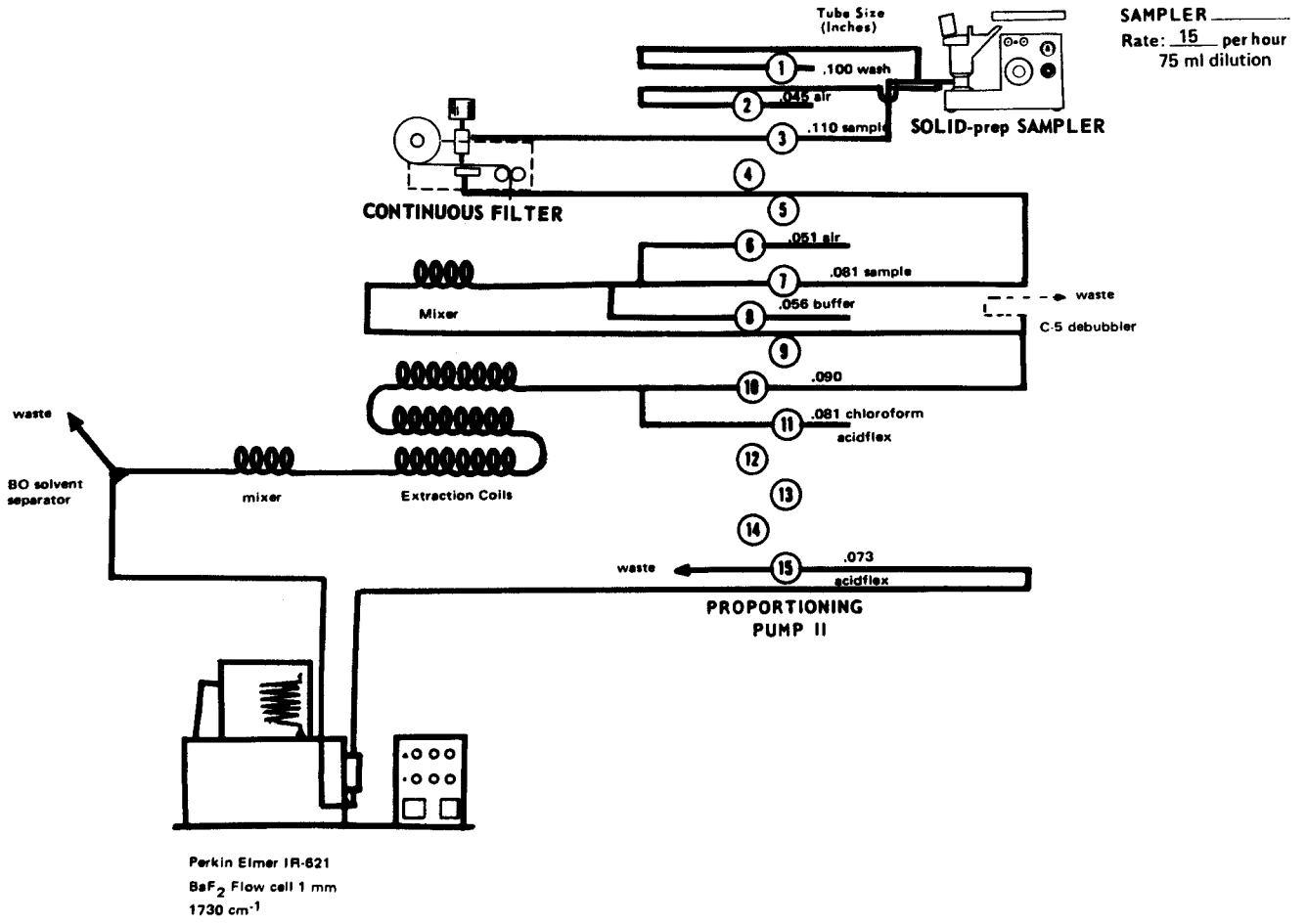

Figure 1—*Schematic diagram indicating the keys of the flow diagram, the automated equipment used for the analytical procedure, and the special tubing where necessary.*

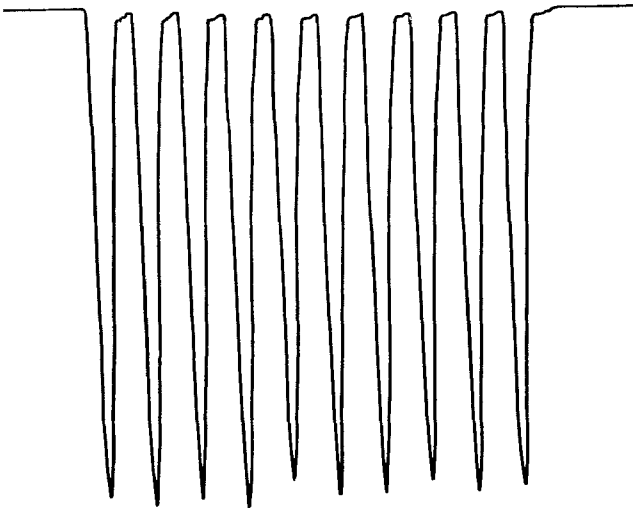

Figure 2—*Typical recording of consecutive standards.*

Several manual methods have been reported for the determination of compounds containing an ester as a functional group. Compounds that do not contain aromatic amino or hydroxy groups can be directly detected with 4-aminophenazone after complete saponification (1). This method is specific for 4-hydroxybenzoic acid rather than an ester. The specificity of the chromatographic determination of esters was also reported (2). The esters were separated on thick silica gel layers and determined colorimetrically using different reagents. This manual method is not suitable for automation because of technical difficulties.

IR spectra are often used for the identification of esters in the region of 1050–1350 cm.$^{-1}$ (3). This region is characteristic of the acyloxy moiety. Saturated aliphatic esters have characteristic absorption in the far IR region (350–50 cm.$^{-1}$) (4). The band frequency correlations are classified by the alcohol components for which these spectra provide particularly useful identifications. Although these bands are useful for qualitative identification, the band intensities are not strong enough for quantitative determination.

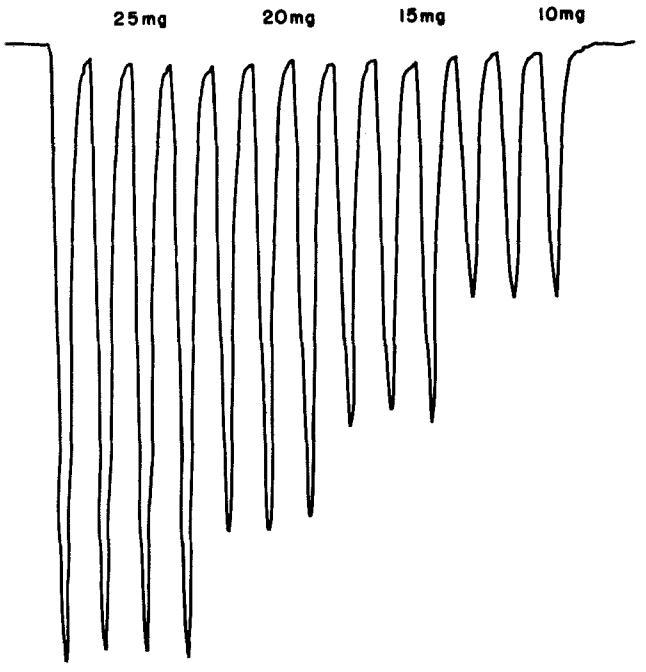

Figure 3—*Beer–Lambert curves for absorbance* versus *concentration.*

Table I—Repeatability of Standard Values by Automated Analysis

Number	Absorbance[a]
1	0.0226
2	0.0230
3	0.0229
4	0.0233
5	0.0221
6	0.0229
7	0.0228
8	0.0221
9	0.0228
10	0.0225

[a] Absorbance: low = 0.0221, average = 0.0227, high = 0.0233, and SD = 1.65%.

A very specific qualitative colorimetric test consists of converting the ester to hydroxamic acid, which forms a color complex with ferric ion (5). This qualitative test was lately converted into a quantitative method (6), but the color development is pH dependent and the precision is dependent on reagent purity.

Among the large number of substances subjected to automated analysis, the esters are perhaps the analytes that need more meaningful consideration in the technology for their determination. The IR technique undoubtedly will strengthen the overall analysis in the pharmaceutical field; perhaps it will soon become a more popular, common, analytical technique in automated analysis.

EXPERIMENTAL

Apparatus—A standard Technicon automatic AutoAnalyzer system consisting of the following modules was used: SOLIDprep sampler II, programmed at 15 samples/hr.; proportioning pump II; continuous filter, speed at 3.5; and Perkin-Elmer IR 621 equipped with 1-mm. Beckman barium fluoride liquid cells, steady wavelength at 1730 cm.$^{-1}$, with a chart speed of 10 min./in. and with 10× ordinate scale expansion.

Reagents and Solutions—*Solvents*—The following were used: chloroform, reagent grade; distilled water; and bicarbonate buffer (dissolve 25.2 g. sodium bicarbonate and 31.8 g. of sodium carbonate in 1000 ml. of distilled water).

Standard Preparation—Dissolve 426.4 mg. of methylphenidate hydrochloride in 100 ml. of distilled water.

Automated Procedure—The schematic diagram of Fig. 1 indicates the keys of the flow diagram, the automated equipment used for the analytical procedure, and the special tubing where necessary. In performing the complete automated analysis, tablets were placed individually into the SOLIDprep sampler turntable cups and the system was activated. The tablets were ground to a fine powder in the blender and dissolved in 75 ml. of water for a period of 1.75 min. (45% of the complete 4-min. cycle); the sampling time was 30% and the rinsing time 25% of the complete cycle. A continuous filter was adapted to filter off the undesirable, insoluble tablet excipients from the solution before mixing with solvents. The clear filtrate was segmented with air and combined with a stream of buffer solution. The sample stream and buffer passed through a single mixing coil to obtain complete mixing. Then the debubbled solution was pumped through three horizontal double-extraction coils to permit sufficient extraction. The chloroform–buffer mixture was passed again through a mixing coil before reaching the solvent separator. The chloroform solution then was sucked through the liquid cell for spectral measurement against the chloroform in the reference cell.

Five-milliliter aliquots of standards were introduced at the proper intervals during the measurements, and the absorption of the carbonyl band of the tablets' active ingredient and standards were re-

Table II—Comparison of Results Obtained by Automated and Official Methods for Methylphenidate Tablets

Automated Method[a]						Official Method[b]	
Number	Absorbance	Milligrams/ Tablet	Number	Absorbance	Milligrams/ Tablet	Number	Milligrams/ Tablet
1	0.0213	19.7	11	0.0209	19.4	1	20.5
2	0.0209	19.4	12	0.0215	19.9	2	19.7
3	0.0204	18.9	13	0.0215	19.9	4	20.2
4	0.0218	20.2	14	0.0204	18.9	4	19.6
5	0.0210	19.4	15	0.0205	18.9	5	19.7
6	0.0219	20.3	16	0.0204	18.9	6	20.2
7	0.0214	19.8	17	0.0215	19.9	7	20.0
8	0.0220	20.4	18	0.0205	18.9	8	20.7
9	0.0210	19.4	19	0.0202	18.7	9	20.5
10	0.0224	20.7	20	0.0222	20.6	10	20.6

[a] Low = 18.7 mg./tablet, average = 19.6 mg./tablet, high = 20.7 mg./tablet, and SD = 3.2%. [b] Low = 19.6 mg./tablet, average = 20.2 mg./tablet, high = 20.7 mg./tablet, and SD = 1.4%.

corded on the IR instrument recorder. Using the common baseline technique, the content per dosage form was calculated.

DISCUSSION

Automation as a routine chemical analysis has been used for 20 years in the field of analytical chemistry. Although automation has been used mostly for clinical testing, the technique has reached widespread application in industry, especially in pharmaceutical chemical analysis. Since content uniformity testing has become official in the USP and NF, automation achieved popularity as one of the most efficient and economical methods of analysis in process control. Because of the great demand in clinical and biological testing, the automated instruments available on the market were prepared especially for the handling of liquids rather than for solids for these laboratories. Difficulties were encountered in automating pharmaceutical preparations, namely in the disintegration and dissolution of tablets in suitable solvents using the SOLIDprep unit[1].

This paper describes a fully automated method; the sampling and dissolution processes do not require manual operations. Until now, very few such methods were proposed in the literature. Fortunately, in this study no difficulties were encountered in disintegration or dissolution of methylphenidate tablets, and the best synergism of disintegration and dissolution was found in distilled water.

In the initial phase of preparing the manifold, experiments were performed to determine the optimum buffer composition to obtain the most effective extraction of the active ingredient through the extraction coils. Various buffer solutions, such as borate, citrate, and phosphate with well-defined pH values of 9.5–10.0, were tested. While these buffer solutions followed the Beer–Lambert law for manual measurement, a continuous background change was observed. The cause of this change is unknown. It may be due to decomposition or from interaction between the methylphenidate and buffer solution. More realistic and reproducible extraction with a steady background was obtained when carbonate buffer solution was used to convert the acid salt to an undecomposed, chloroform-soluble free ester. Although the stability of the methylphenidate in this buffer solution is affected by time, significant decomposition was found only after 5–10 min. of standing in buffer solution. Since the time that elapsed in contact of sample solution with the buffer prior to extraction was less than 1 min., the possible decomposition of the sample solution was eliminated.

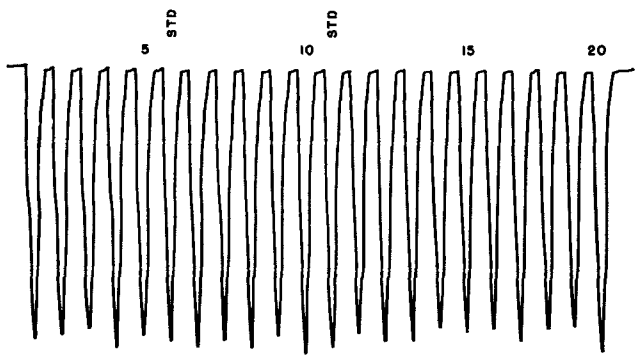

Figure 4—*Curves of regular production tablets.*

[1] Technicon Corp.

In the second phase of this study, the effectiveness of the chloroform extraction was investigated. Mechanical shaking of the sample solution with chloroform gave satisfactory extraction; however, technical difficulties appeared in the performance. Passing the chloroform–buffer solution through an ultrasonic bath resulted in a cloudy solution without clear separation. Using the Technicon extraction coil, a clear solution was obtained; however, the efficiency of one coil was inadequate. Three extraction coils in series gave about 80% recovery of the active ingredient, which was sufficient for spectrometric measurement.

To obtain reliable absorbance values, the instrument was set on 10× expansion scale and deviation of ±0.0006 absorbance was recorded on 10 replicate standard solutions. Figure 2 shows the consecutive standard curves using the IR absorption technique. Evaluating the recorded curves, one can see a stable baseline with fairly good reproducibility and clean backwash. The variation of the standard concentrations is demonstrated in Fig. 3. The plotted curve, prepared from the average peak readings *versus* its concentration, resulted in a straight line which passed through the origin. Table I summarizes the individual absorbance values on the standards with the standard deviation. After reliable indications on standards were obtained, regular production tablets containing 20 mg./tablet were subjected to complete automated testing. The results of this test can be seen in Fig. 4. Table II incorporates the comparative results obtained by official and automated techniques. From this table, it is evident that the automated method is reliable and useful for the determination of unit dosage in individual tablets. Although the results in Table II were obtained on 20-mg. tablets, the proposed method is applicable for 10-mg. tablets and, with a slight modification of the instrument, for 5-mg. tablets as well.

The instrumental system for this study was extremely stable; however, the acidflex tubes conducting the chloroform solutions lose their elasticity after a certain time, which may cause a significant change in the assay. This problem can be minimized by replacing the acidflex tubing periodically.

CONCLUSION

The goal of this study was to develop a quantitative method suitable for content uniformity testing that can be automated and is stability indicating.

The IR method was successfully applied for the determination of the intact ester carbonyl after a simple extraction procedure. The extraction was necessary to separate the ester from interfering materials that have absorption in the same region. The commonly used methods, *i.e.*, colorimetric and titrimetic, cannot be applied because of interferences.

This paper presents a preliminary work on a new technique for detection in automated analysis. The dependence of buffer solutions upon the nature of extraction and the intensity of measurable band with the other parameters is discussed. As a new application in automation, the advantages of the technique are demonstrated.

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E. N. HIESTAND, J. M. BANE, Jr., and E. P. STRZELINSKI

Abstract □ An impact–rebound method of estimating the hardness (*i.e.*, the pressure necessary to produce permanent deformation) of a compact of powdered solid is evaluated. A steel sphere arranged as a pendulum acts as the indenter. Since energy consumed during impact is used in doing pressure–volume work, this energy divided by the volume of the indentation provides an estimate of the mean deformation pressure. Two methods of estimating the dent volumes are compared. The simpler method uses equations adopted from metallurgy. These estimate the volume from the energy consumed during impact and the chordal radius of the dent. The alternate method estimates the volume from the displacement of a grid of lines projected onto the dented surface at a small angle of incidence. The latter method gives slightly smaller volumes than the former. When plotted in various ways, the data obtained yield slopes and/or intercepts consistent with the adopted metallurgical equations. Therefore, these equations are considered to provide: (*a*) an acceptable description of the indentation process and (*b*) the more satisfactory method of estimating the hardness of compacts. Plots of log pressure *versus* relative density appear to be linear. Extrapolations of these plots to a relative density of unity provide estimates of the properties of a single polycrystalline mass of the powdered material.

Keyphrases □ Powder compacts, compressed—impact test □ Compressed powder compacts—impact–rebound hardness test □ Indentation volume measurement—compressed powder compacts □ Diagram—pendulum impact device

In previous communications (1–3), the senior author indicated that the mechanical properties of solids are important fundamental properties which affect the flow and bonding properties of solids. Shlanta and Milosovich (4) studied some of the time-dependent effects, and Shlanta (5) reviewed the importance of plastic deformation in tableting. Huffine (6) also considered these properties. Because the anelastic properties of crystalline solids are dependent not only on the strength of the intermolecular interactions but also on the degree of imperfection of the molecular arrangement, the plastic yield pressure is not an intrinsic property of the solid state of a chemical compound in a given polymorphic form. The procedure followed in crystallizing the compound and the subsequent working of the solid may influence the plastic yield value.

Hardness may be defined as the resistance of a solid to permanent deformation (7). Indentation methods are standardized and are common practice for assessing the hardness of metals. Previous reports in the pharmaceutical literature (8–10) of the use of indentation methods have not established whether the equations used with metals may be applied to compacts of organic materials. It is the purpose of this article to explore this aspect for the dynamic, *i.e.*, the impact–rebound, method of hardness testing. The theoretical equations adopted from the metallurgical field are described later in this communication.

APPARATUS

The pendulum arrangement for controlling the pathway of a sphere is used in this apparatus. This arrangement simplifies the control and measurement of the initial and rebound height. Figure 1 shows the apparatus diagrammatically. Figure 2 is a photograph of the apparatus (an air shield was removed to expose the apparatus).

Two dies are used in these studies: one is of square cross section and the other is circular. Both have a cross-sectional area of 2.25 in.^{2}. Eight to twenty grams of powdered solid is used in the die. The compact is pressed with a hydraulic press[1]. The die is held between the punches only by the friction with the powder, thereby permitting both punches to move. To increase the probability of a series of tablets having identical properties, the pressure is raised to a selected value and maintained for a definite time. Figure 3 shows the two dies with one punch and the compact in place.

EXPERIMENTAL PROCEDURE

After removal from the press, the compact is pushed to within a couple of millimeters of the surface of the die. The die, F, containing the compact, B, and longer punch, G, are clamped into the apparatus by means of clamps, J. The back-up block, H, is moved up to the punch and its bolts are tightened only moderately. Bolt I is then used to push against the support post, N, until enough force is developed to push the back-up block, the punch, and the compact until the compact is moved flush with the die surface. The bolts on the back-up block are then tightened. Thus, only one face of the solid is left unsupported.

The sphere, A, is raised against the pointed pole of the magnetic hold, E. Both a.c. and d.c. currents are used initially to cause the sphere to vibrate against E and seek an equilibrium orientation. Before release, the a.c. signal is removed and the sphere comes to rest held by the d.c. field only. The sphere is released by opening the d.c.

[1] Model 341-20, Loomis Engineering and Manufacturing Co., Caldwell, N. J.

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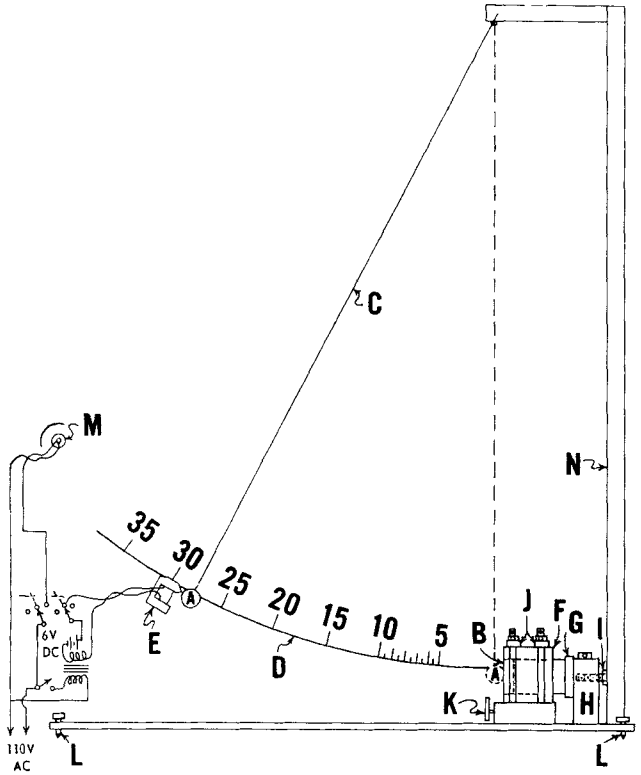

Figure 1—*Schematic of arrangement of pendulum impact device. Key: A, steel sphere; B, powder compact; C, wire support of pendulum; D, scale of angle from vertical; E, magnetic hold-release; F, die; G, punch; H, back-up block; I, back-up bolt; J, die clamp; K, stop (winch driven); L, leveling screws; M, light; and N, support post.*

circuit. At the same time a light, M, turns on and a camera shutter is opened to make a time exposure of the rebound of the sphere.

An electrically driven winch also starts when the sphere is released. With proper timing, it moves a metal stop between the compact and the sphere after the rebound occurs. This prevents a second impact from occurring.

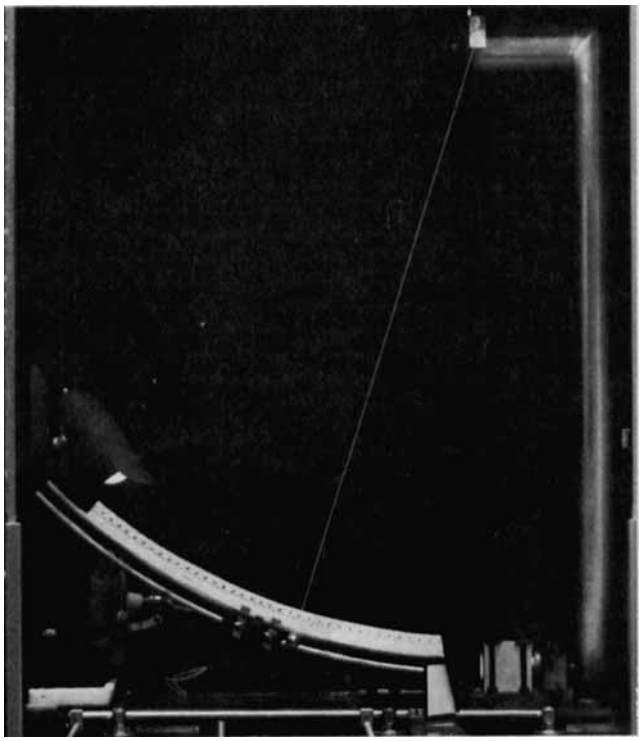

Figure 2—*Photograph of pendulum impact device.*

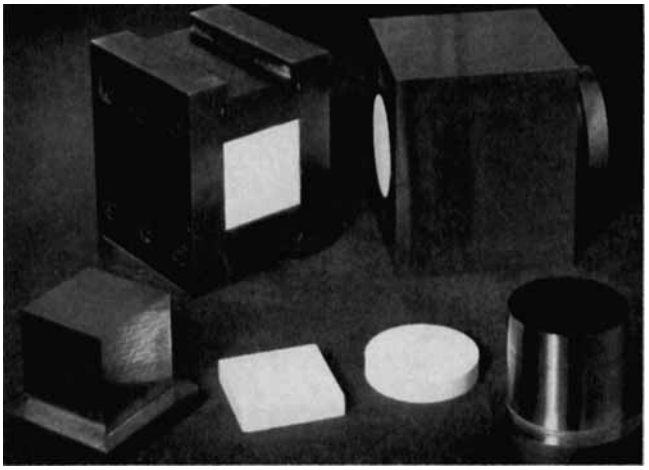

Figure 3—*Two dies and punches with compact in place as used in the impact test. A separate compact after removal from the die is shown also.*

If desired, additional hits may be made on the same spot. An "off-center" hit will be evident by an "off-side" rebound. However, the magnetic hold and release arrangement nearly always puts the sphere on target.

Measurement of Indentation—The volume of the indentation may be obtained by the optical procedure outlined in a subsequent section. This procedure permits an evaluation of the volume without removing the die and compact. This feature is essential if successive hits are to be made on the same compact.

Theoretical equations are derived for calculating the volume from the initial and rebound heights of the sphere and the chordal diameter of the dent. This diameter may be obtained either by direct microscopic measurement or by measurement from an enlarged photograph. With some materials, shadowing lightly with black paint increases the contrast at the edges sufficiently to make the diameter measurement easier and more precise. Spraying at a low angle of incidence produces the shadow. A mean value of five determinations and a statistical evaluation of errors are useful in these measurements.

Estimation of Mean Deformation Pressure—The assumption of a constant mean deformation pressure[2], P, is mathematically equivalent to the calculation of the average mean deformation pressure because

$$P = \frac{\int_0^{V_p} P_z \, \delta V}{\int_0^{V_p} \delta V} = \frac{E_p}{V_p} \qquad \text{(Eq. 1)}$$

where P_z is the mean pressure for penetration z, V is the variable volume, V_p is the permanent dent volume, and E_p is the work of deformation. If all the energy lost by the sphere is assignable to E_p, then $E_p = E_i - E_e$, where E_i and E_e are the respective initial and rebound potential energies of the sphere. Therefore, $P = (E_i - E_e)/V_p$. This simple equation is all that is needed to estimate P if the volume is measured directly. Since there is no real advantage to the awkward terminology, average mean deformation pressure, only mean pressure will be used hereafter.

Equation 10 permits P to be estimated by measuring the chordal diameter, $2a$, of the dent instead of its volume. A similar equation, 12, estimates the volume of the dent. These calculations introduce additional assumptions. Before they are used, it must be demonstrated that they yield good estimates. Since both Eqs. 10 and 12 contain the same assumptions in their derivation, it was considered sufficient to test only one of them. An independent measurement

[2] The pressure is not a constant over the sphere–compact interface because of friction and stress gradients in the compact.

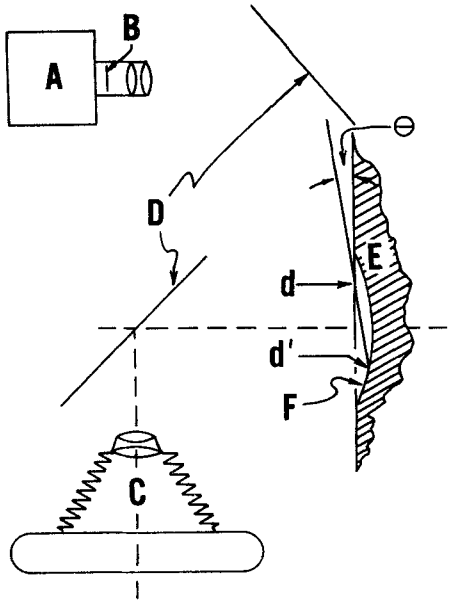

Figure 4—*Schematic of arrangement for determining dent volume. Key: A, slide projector; B, transparency containing grid of lines; C, camera; D, first surface mirrors; E, compact; F, surface of indentation; θ, angle of incidence of projected image; d, location of an image of vertical lines on undented surface; and d', location of image of the same vertical line on dented portion of surface.*

of the volume appeared to be easier than a direct measurement of pressure.

Measurement of Dent Volume—The surface characteristics and the fragile nature of some compacts restrict the suitable methods for measuring dent volume. The following optical method was selected because it did not disturb the compact. In fact, the data are obtained without removal of the compact from the apparatus.

An image of a grid of vertical lines was projected horizontally at a small angle of incidence, θ, onto the tablet surface. The lines of the grid were spaced to produce equally spaced vertical lines on the tablet surface. At the indentation, the vertical lines became curved, the horizontal displacement being proportional to the depth of the indentation. Figure 4 shows schematically the arrangement. By photographing the tablet, the image of the grid could be recorded. Figure 5 shows a typical photograph of a grid image on a dented compact. To estimate the displacement accurately, the photograph

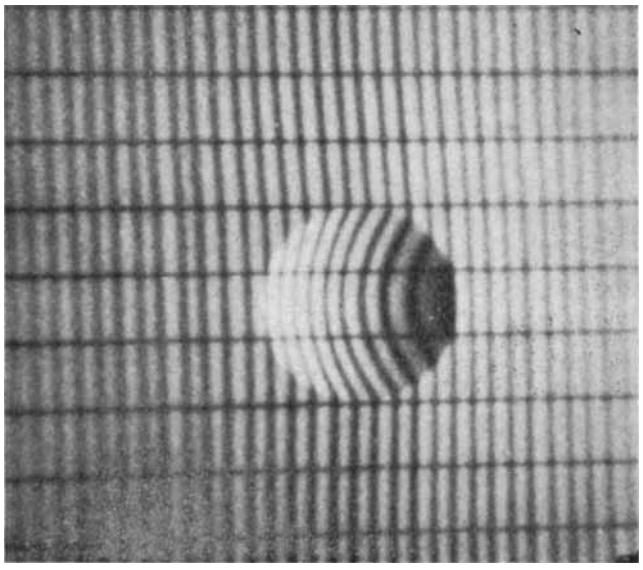

Figure 5—*Magnified photograph of grid image on compact surface. Horizontal line spacings on the compact surface are determined with a cathetometer. The photographic and projection magnification factor is determined by change in spacing of these lines.*

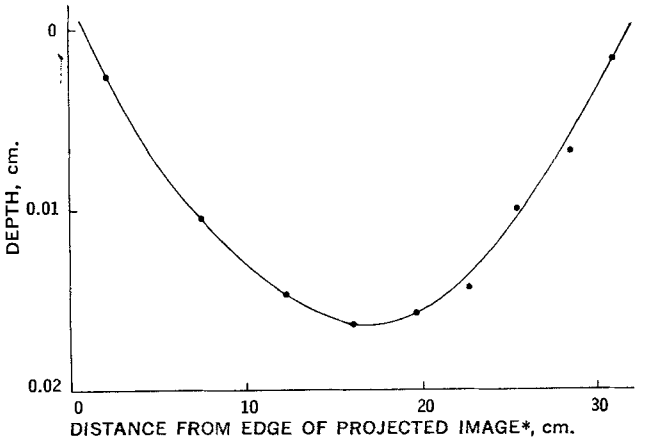

Figure 6—*Plot of data scaled from an enlarged image of the type shown in Fig. 5. * This scale is magnified 66.6 times the true dent size.*

was made with transparency film and projected onto a large sheet of plain white paper.

The depths determined from the photograph were plotted against the distance from an arbitrary point near one edge of the dent. A smooth curve was drawn through the points and the center, and maximum depth was obtained from these "smoothed data." To simplify the volume calculation, the data were replotted with transposed axes. The log of the distance above the maximum depth, d, was plotted against the log of the chordal radius, α_d. These data approximated a straight line on the log–log plot. Therefore, the cross-sectional surface of the dent is assumed to be described by the equation[3]

$$d = k\alpha_d^n \qquad \text{(Eq. 2)}$$

where k and n are constants which may be determined from the log–log plot. Figures 6 and 7 show typical examples of the two plots used to determine k and n. Using Eq. 2, a simple equation for the volume is obtained by assuming circular symmetry—*viz.*,

$$V = \int \pi\alpha_d^2\,\delta d = \int_0^a \pi n k \alpha_d^{n+1}\,\delta\alpha_d$$

$$= \frac{n\pi k}{n+2}\,a^{n+2} \qquad \text{(Eq. 3)}$$

where a is the maximum value of α_d. The value of a used in Eq. 3 is the same value used later in Eq. 12; n and k were obtained by an iteration procedure with a computer to yield a least-squares fit to the data. In Fig. 7, a line of slope 2 is shown to permit a comparison of the data to a spherical shape.

A Simple Theoretical Model—Tabor's (7) equations for the impact–rebound process are based on a simple model. To the authors' knowledge, this theory has not been discussed previously in the pharmaceutical literature. Therefore, a brief treatment of the concepts should be useful for clarifying the assumptions made and the deviations of experimental values from the calculated ones.

The rebound process is a result of elastic deformation only. It is equal to the reverse process of pushing the sphere back into the dent to produce the same contact area, assumed to be the contact area that has a chordal radius of a. Hertz' equation for the elastic deformation of a sphere and semiinfinite body of homogeneous, isotropic solids is used. Three elasticity relationships are useful—*viz.*,

$$z = \alpha^2\left(\frac{1}{r_1} - \frac{1}{r_2}\right) \qquad \text{(Eq. 4)}$$

$$F_\alpha \propto z^{3/2} \qquad \text{(Eq. 5)}$$

and

$$\frac{1}{r_1} - \frac{1}{r_2} = \frac{3}{4}\frac{F}{a^3}f(E) \qquad \text{(Eq. 6)}$$

[3] For a spherical indentation of radius r, when $d^2 \ll \alpha^2$, $k \approx 1/2r$, and $n = 2$. Note that $n = 2$ also is a parabola and as long as $d^2 \ll \alpha^2$, the volumes of the two configurations may be considered equal.

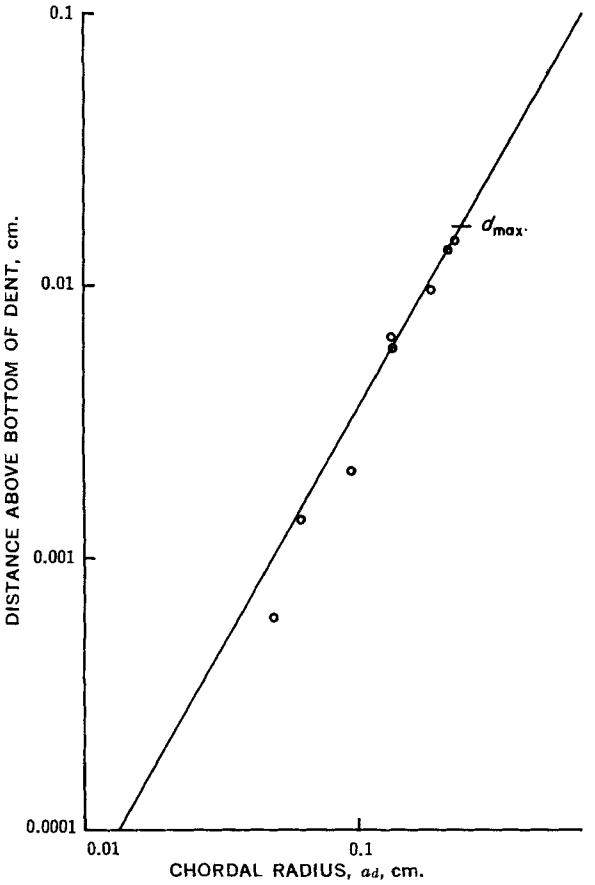

Figure 7—*The same data are used here as in Fig. 6 but the origin has been transposed to the bottom of the dent. The* k *and* n *for Eq. 2 are obtained from a computer analysis which gives the line of this plot;* $d_{max.}$ *is the maximum value of* d *based on Fig. 6.*

where z is the approach distance (elastic penetration) of the sphere into the dent, F_α is the force at penetration z, α is the chordal radius of the contact area at penetration z, r_1 and r_2 are the radii of curvature of the two surfaces before elastic deformation, and

$$f(E) = \frac{1 - \sigma_1^2}{E_1} + \frac{1 - \sigma_2^2}{E_2} \qquad \text{(Eq. 7)}$$

where σ is Poisson's ratio and E is the modulus of elasticity. Subscripts 1 and 2 refer to the spherical indenter and to the permanently dented surface, respectively. Equations 4 and 5 may be used to show that the energy of elastic deformation, E_e, required to push the sphere into the dent to produce contact over a circle of radius a is given by

$$E_e = \int F_\alpha \, \delta z = {}^2/_5 P \pi a^4 \left(\frac{1}{r_1} - \frac{1}{r_2}\right) \qquad \text{(Eq. 8)}$$

where the force at maximum penetration, F, is equal to $P\pi a^2$. Combining Eqs. 6 and 8 gives

$$E_e = {}^3/_{10} P^2 \pi^2 a^5 f(E) \qquad \text{(Eq. 9)}$$

Note that Eq. 9 depends only on the elastic deformation, as does the rebound process. Since $f(E)$ is not known, a complete check of Eq. 9 is not readily made. However, if P (here P is the pressure at maximum penetration) is a constant for different values of a, a plot of log E_e/mg *versus* log a should have a slope of 3. Figure 8 shows such a plot. For most of the examples, the correlation is good. Therefore, it is assumed that Eqs. 8 and 9 describe the rebound process satisfactorily and that P is essentially a constant.

Using Eq. 3, one may show that when the permanent dent is a spherical dent with the depth much less than a, it will have a volume, V_p, equal to $\pi a^4/4r_2$. This may be used to eliminate r_2 in Eq. 8. If it is also assumed that the P in Eq. 1 is equal to the P in Eq. 8, one may combine these equations and solve for P to obtain

$$P = \frac{4r_1}{\pi a^4}(E_p + {}^5/_8 E_e) \qquad \text{(Eq. 10)}$$

or

$$P = \frac{4mgr_1}{\pi a^4}(h_i - {}^3/_8 h_r) \qquad \text{(Eq. 11)}$$

where m is the mass of the sphere, g is the gravitational acceleration constant, h_i is the initial height of the sphere, and h_r is the rebound height. Equation 11 assumes no losses of energy other than the pressure volume work of the permanent deformation. Since $PV_p = mg(h_i - h_r)$, one may divide this by Eq. 11 to obtain V_p:

$$V_p = \frac{\pi a^4}{4r_1}\left(\frac{h_i - h_r}{h_i - {}^3/_8 h_r}\right) \qquad \text{(Eq. 12)}$$

Figure 9 is a plot of the volumes calculated using Eq. 12 *versus* the "photo" volumes, *i.e.*, the volumes estimated from photographs such as Fig. 5. Equation 12 yields slightly larger values than the photo volumes. Obviously, both values may be in error. Errors in the photo volumes are expected to have random distribution, but undetected systematic errors are possible. These could lead to an underestimate of the dent volumes. However, Eq. 12 estimates may be expected to be slightly larger than the true volumes because frictional, accoustical, and hysteresis losses have been neglected. Also the mean deformation pressure may not be a constant during the impact because of the dynamic nature of the process. The last assumes P is larger when the sphere is moving at a larger velocity and goes to a minimum as the sphere velocity goes to zero[4]. This too would result in an overestimate of the volume by Eq. 12. However,

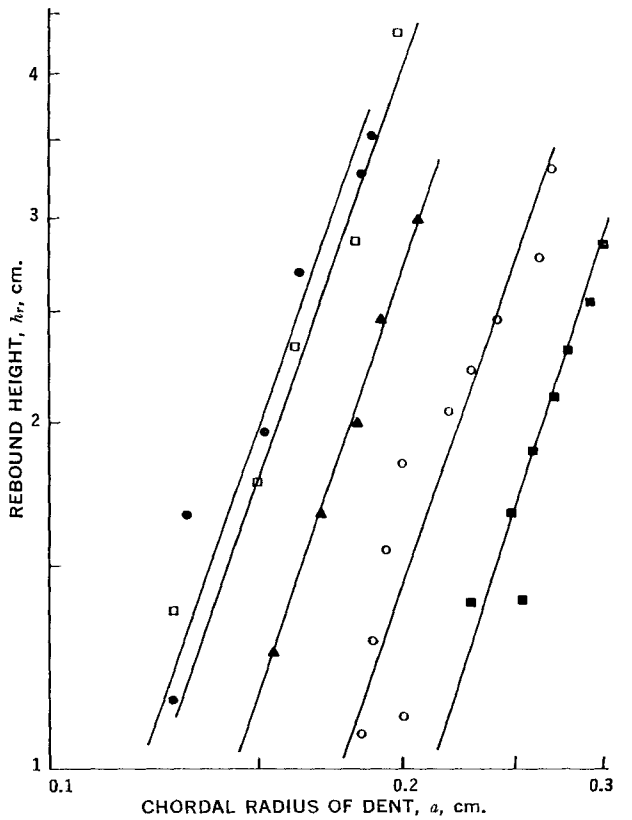

Figure 8—*Comparison of Eq. 9 with the experimental data. Solid lines have theoretical slope 3. Key:* ●, *spray-dried lactose, Lot B,* $\rho_r = 0.749$; □, *sitosterol,* $\rho_r = 0.868$; ▲, *sucrose,* $\rho_r = 0.818$; ○, *spray-dried lactose, Lot A,* $\rho_r = 0.690$; *and* ■, *a tablet formulation,* $\rho_r = 0.574$.

[4] Even so, P in Eq. 9 may be constant because it is concerned only with P at zero velocity of the sphere.

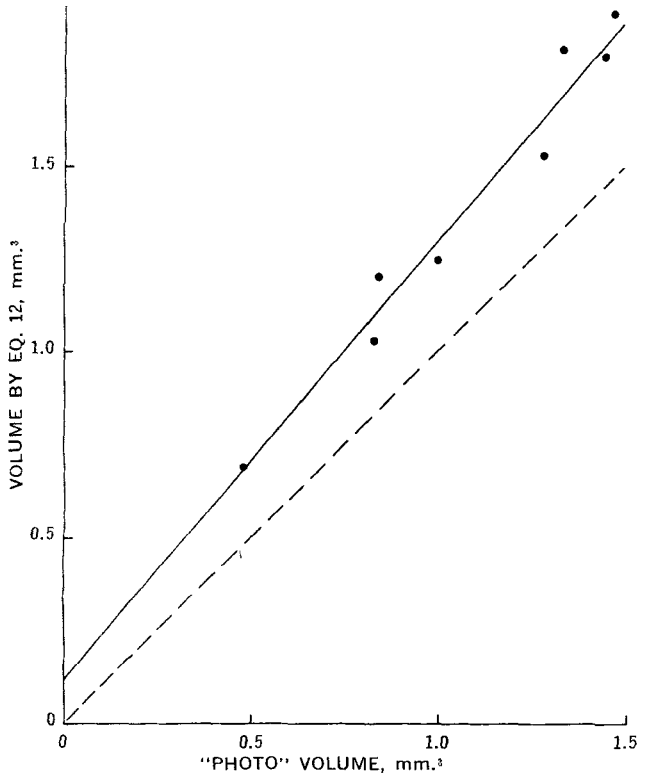

Figure 9—*Comparison of the values obtained by two methods of estimating the volumes of the permanent indentation. Values based on Eq. 12 are somewhat larger than those based on the photographs.*

consolidation beneath the dent during penetration could cause P to increase. This effect should be detected by a more rapid increase in a than for a slope of 3 in Fig. 8. Only one portion of one of the plots shows significant deviation in this direction. Therefore, this effect appears generally to be insignificant.

Based on these studies, the authors conclude that probably Eq. 12 overestimates V_p by a small amount and that the companion Eq. 11 therefore would underestimate the mean pressure of plastic deformation. However, these small deviations from the true values are not expected to be significant impediments to the application of this technique of hardness testing.

Figure 10 shows a plot of log P *versus* ρ_r, where ρ_r is the relative density of the compact, *i.e.*, the bulk density divided by the absolute density of the crystalline materials. Extrapolations to $\rho_r = 1$ may be questionable but are considered useful estimates of the value of P for the crystalline solid.

Additional Experimental Results—The area over which molecular forces may act after two solids have been pressed together depends on the effectiveness of the relieved elastic stresses in producing separation of the surfaces. For the geometric model considered here, this "contact area" is a function of the ratio of r_2/r_1. If, in deriving Eq. 10, r_2 had not been eliminated, one could have obtained

$$r_2/r_1 = 1 + \frac{5}{8}\frac{E_e}{E_p} \qquad \text{(Eq. 13)}$$

Rearranging Eq. 6 and replacing a^3 with $(F/p\pi)^{3/2}$, one notes that

$$\frac{r_1}{r_2} = 1 - \frac{3}{4}\frac{r_1 P^{3/2}\pi^{3/2}}{F^{1/2}} f(E) \qquad \text{(Eq. 14)}$$

Obviously, E_e/E_p is not a constant independent of the applied force F since r_1/r_2 is a function of F. Combining the above with Eq. 13 yields

$$\frac{E_p}{E_e} = \frac{5}{6}\frac{F^{1/2}}{\pi^{3/2} r_1 P^{3/2} f(E)} - \frac{5}{8} \qquad \text{(Eq. 15)}$$

Since $F^{1/2} = (P\pi)^{1/2}a$ when P is constant, this relationship may be tested in a manner analogous to the test of Eq. 9. A plot of E_p/E_e *versus* a is shown in Fig. 11. The lines in Fig. 11 were drawn through the $-5/8$ intercept. Equation 15 contains both the plastic and elastic parts of the impact process. Since this test is independent of the photo method and any associated errors in it, the results shown in Fig. 11 strongly reinforce the argument that the model and the equations provide good estimates of the yield pressure.

The following extension of these concepts to the interaction between particles was reported previously (11). If one considers a sphere of radius r_1 at rest in a spherical dent of radius r_2, the separation, S, of the two spherical surfaces at a chordal radius, α, from the point of tangency is given for $\alpha \ll r_1$ by

$$S = \frac{\alpha^2}{2}\left(\frac{1}{r_1} - \frac{1}{r_2}\right) \qquad \text{(Eq. 16)}$$

Using Eq. 13 to eliminate r_2 and rearranging give

$$\alpha^2 = 2r_1 S\left(1 + \frac{8E_p}{5E_e}\right) \qquad \text{(Eq. 17)}$$

If it is assumed that the attraction resulting from intermolecular forces is effective over an area $A_c = \pi a_c^2$ at which point $\alpha = a_c$ and S equals S_m, the maximum separation effective in bonding, then one obtains

$$A_c = \pi a_c^2 = 2\pi r_1 S_m\left(1 + \frac{8E_p}{5E_e}\right) \qquad \text{(Eq. 18)}$$

Combining this with Eq. 15 yields, for a single contact point,

$$A_c = \frac{8S_m F^{1/2}}{3\pi^{1/2}P^{3/2}f(E)} \qquad \text{(Eq. 19)}$$

This equation predicts that A_c should increase as the $^1/_2$-power of the force applied and decrease as the $^3/_2$-power of the plastic yield value of the yielding solid.

If one extends this to the application of a total force, F_t, on a given cross-sectional area of a powder bed of monodispersed particles, and if one assumes that only one of the particles deforms so that after elastic rebound one of each pair of contacting particles retains a radius of r_1, it is obvious that the total effective contact area will be

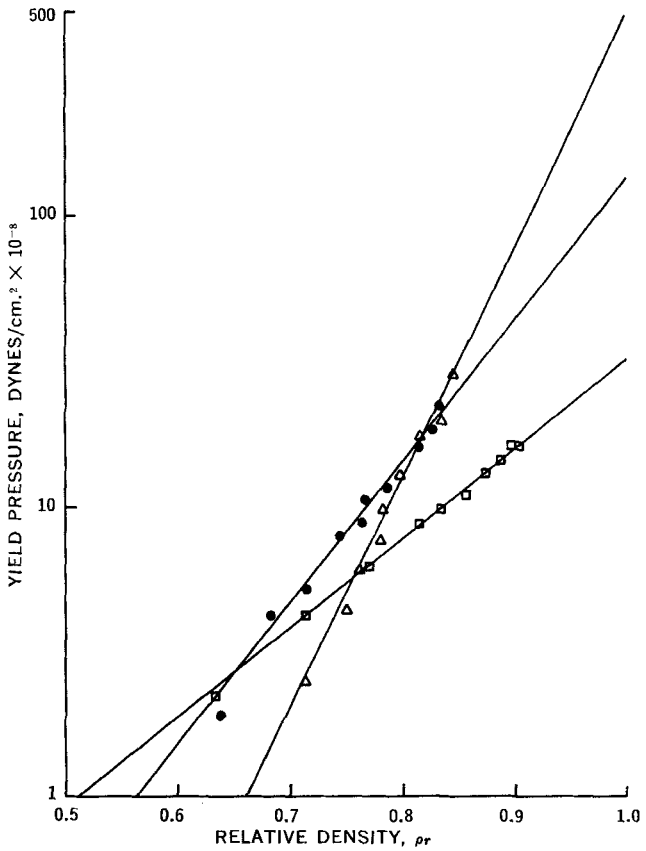

Figure 10—*Mean yield pressure appears to follow a logarithmic relationship with the bulk density of the compact. Key:* ●, *spray-dried lactose, Lot A;* △, *sucrose; and* □, *tablet formulation.*

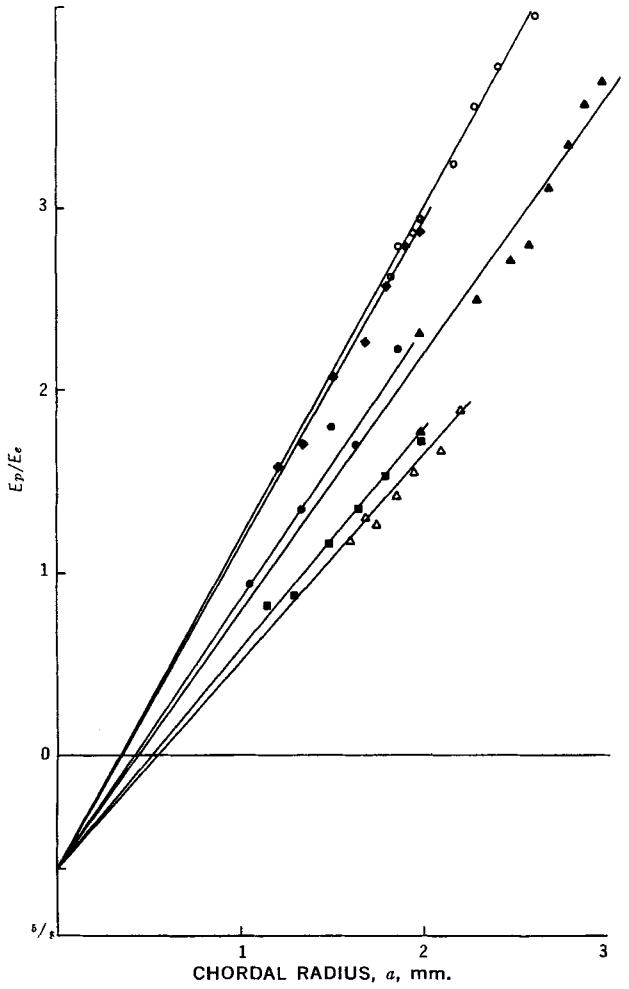

Figure 11—*Comparison of the experimental data with Eq. 15. The lines are drawn through the theoretical value of the intercept,* $-5/8$. *Key:* ○, *spray-dried lactose, Lot A,* $\rho_r = 0.690$; ●, *spray-dried lactose, Lot B,* $\rho_r = 0.749$; ◆, *sucrose,* $\rho_r = 0.818$; ▲, *tablet formulation,* $\rho_r = 0.574$; △, *tablet formulation,* $\rho_r = 0.752$; *and* ■, *sitosterol,* $\rho_r = 0.868$.

N_cA_c, where N_c is the number of contacts. If the packing remains the same, $N_c \propto 1/r_1^2$ and $F \propto r_1^2$. Therefore, $F_t = N_cA_c \propto 1/r_1$. Qualitatively, this conforms with experience, *i.e.*, smaller particles form more cohesive powder beds. According to Krupp (12), with small particles, attractive forces may deform contacting surfaces without externally applied loads. This effect must be added to the relationship that considers only the role of mechanical properties.

DISCUSSION

The relationship between the mean pressure of deformation and the yield stress is not simple, especially in a heterogeneous, porous compact. Friction and other factors influence this relationship. In the absence of friction, the mean pressure is equal to approximately three times the yield stress of the solid (7). Since the exact relationship is unknown in the real case, it is probably better to avoid any statement on the yield stress. The mean pressure required to produce the indentation may be used as an indication of the hardness, *i.e.*, the resistance to permanent deformation.

Obviously, pressures as large as those shown in Fig. 10 can only be developed by the rapid deceleration of the sphere. Tabor (7) derived Eq. 20 to estimate the time, t, after contact required to bring the velocity of the sphere to zero:

$$t = \frac{\pi}{2}\sqrt{\frac{m}{2\pi P r_1}} \qquad \text{(Eq. 20)}$$

Using this equation, values in the range of 10^{-4} sec. are obtained. The time of contact is not dependent on the velocity of the sphere unless P is a function of velocity. P has been assumed constant in all the equations adopted from Tabor's book (7). The time of impact is much less than the time of compression in a rotary tablet machine.

Since the impact–rebound technique permits the separation of the elastic from the anelastic displacement and energy, it may be feasible to characterize the elastic properties also. This aspect is being explored in the authors' laboratory.

The assumptions made in the use of this technique may cause one to question the validity of the method. Certainly, not all energy losses are due to plastic deformation. However, some workers (13) reported coefficients of restitution greater than 0.98 when hard materials were used with small impact velocities to avoid permanent deformation. Therefore, it is concluded that the basic concepts are essentially correct.

CONCLUSION

The use of an impact arrangement permits the energy and volume of the permanent deformation to be determined. The average deformation pressure is calculated from the same data. Equations used for metallurgical specimens have been shown to have applicability in the characterization of compacts of organic solids.

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Automated System for Analytical Microbiology I: Theory and Design Considerations

N. R. KUZEL and F. W. KAVANAGH

Abstract □ Quantitative microbiological assays use either of two responses, growth inhibition or growth promotion. Antibiotics and germicides are assayed by growth inhibition, and vitamins and amino acids by growth promotion. Previous automation efforts in these types of assays are critically reviewed. Design considerations are presented for a new automated system which meets all essential requirements of microbiological turbidimetric analysis. Optimum requirements are discussed separately for dilution, incubation, and measurement. The automated system duplicates all essential characteristics of well-known manual procedures. Dilution and measurement are performed separately and simultaneously, and incubation is made independent of both functions so that incubation time can be optimized for each analysis. This arrangement furnishes the flexibility, versatility, and precision so necessary in present-day automated analytical microbiology.

Keyphrases □ Microbiological assay, automated—theory □ Design consideration—automated microbiological assay □ Automated analysis, microbiological—theory, design

The increasing use of turbidimetric microbiological analysis has emphasized the need for a precise, versatile, automated system. Such a system should meet the needs of a large variety of users and the stringent requirements of a living biological system. The theory in this field is very complex and sometimes not well understood. Effective utilization of these theoretical considerations in the design of a practical and versatile automated system has required simplification of theory and its reduction to essentials. The present paper summarizes this theory, relates this work to other efforts in the field, and presents the design considerations that led to the construction of an automated system. In the accompanying paper (1), this system is described in detail, and a laboratory evaluation is presented of the systems used for analysis of certain antibiotics and vitamins.

BASIC THEORY

Quantitative photometric microbiological assays take advantage of two responses, growth inhibition or growth promotion. Growth-inhibiting substances and microbes interact to decrease the mass of the latter. The two parameters of bacterial growth affected by antibacterial agents are growth rate and lag time. Most antibiotics which have been studied in sufficient detail to discover the dynamics of their action reduce growth rate. A few substances increase lag time. A cell with a lag time of 5 hr. or more is dead for analytical purposes. Each population of organisms apparently makes one of two responses to inhibitors of growth. Either the entire population makes the same response to the same degree or it does not. The first type of response is characteristic of antibiotics that reduce growth rate. The second response is elicited by such substances as cetyl trimethylammonium bromide, which seem to affect only a portion of the population (2).

Requirements for a growth-inhibition assay are: (*a*) a substance capable of inhibiting growth rate or increasing lag time, (*b*) a test organism sufficiently susceptible to the test substance, and (*c*) a liquid medium nutritionally sufficient so as not be be altered significantly by substances accompanying the test substance in the assay solution or by metabolic products of the growing test organism.

Growth-promoting substances and microbes interact to increase the mass of the latter. The test substance must be required by the microbe, and the microbe must not be able to synthesize the test substance, at least at the rate needed. The substances most often determined by growth-promotion methods are the B vitamins and amino acids. Other organic compounds and certain minerals may also be measured.

Requirements for a growth-promotion assay are: (*a*) a substance essential for growth of the test organism, (*b*) the test organism, and (*c*) a liquid medium deficient only in the substance being measured. The preferred assays are those for which the deficiency is absolute, although assays for substances that affect only growth rate can be done. Kinetics of vitamin assays are simple and well known; the organism grows in the presence of the test substance and does not grow in its absence. Time of incubation and composition of the medium are such that the amount of the test substance in the assay tube is the only limiting factor. Under these conditions, the total cell mass is proportional to the amount of test substance in the tube.

If all the above-mentioned criteria are met for either growth-inhibition or growth-promotion assays, the one additional requirement is that the test substance be the only variable introduced into the assay. This means that all dilutions are performed precisely, that no variable environmental factors are introduced, and that the resultant growth can be measured with sufficient precision so as not to degrade the final result. In conventional manual performance of these assays, each of these factors can be directly influenced by the analyst; each sample or standard is handled individually and uniquely by the analyst. Not only do all the operations require considerable time if performed precisely, but the only assurance that any one sample is directly related to its standard is based on the confidence resulting from the analyst's previous performance. Thus, the results of the analysis are extremely operator dependent. The means usually used to overcome these deficiencies are a high degree of replication of the assay and the use of rather elaborate statistics to evaluate the results. These required replications of procedures cause a high cost of analysis and an undesired degree of error in estimations of sample concentration.

It is to avoid this high cost and/or concomitant error that automation of this assay has been attempted. Automated analysis, when performed properly, should assure that the dilution and measurement have been performed in a reproducible manner. Environmental changes must still be monitored by an appropriate number of standards run in the same manner as the samples. The greater the time difference between processing of sample and standard, the greater is the opportunity for some change to occur. Ideally, to avoid environmental influence, the sample and its reference standard should be analyzed at exactly the same time and under exactly the same conditions, or all conditions should be held so constant that differences in time have no influence on the end result. The extent to which various automated systems approach this ideal governs their usefulness as suitable assay systems.

DISCUSSION

Manual Analysis—Practical quantitative photometric microbiological assays were introduced by McMahan (3) in 1944 as an improvement over both the serial dilution tube assay and the diffusion assay. Very little was published on additional methods or theory until the publication of Treffers (4) in 1956 on the antibiotic dose–response line. Kavanagh (5, 6) continued the development begun by Treffers of the dose–response line and, in addition, discussed in detail the theory and present practice of photometric assaying.

Continuous-Flow Automation—Continuous-flow methods for automation of microbiological turbidimetric analysis have been reported in the literature for many years. A review of these early methods was published by Gerke and Ferrari (7). A more recent review was published by Kuzel *et al.* in 1969 (8). Gerke *et al.* (9) first described the use of automated continuous-flow bioassays for turbidimetric analysis. Haney *et al.* (10) introduced some improvements on this method. Further details on this method were presented by Gerke *et al.* (11). Shaw and Duncombe (12) used a biostat to provide continuously grown inoculum. Continuous dilution rather than intermittent sampling for turbidimetric analysis was introduced by Platt *et al.* (13), based on earlier work by Pagano *et al.* (14). A variation of the continuous-flow technique was described by Berg *et al.* (15). These authors prepared their dilutions by continuous-flow methods into discrete vessels and then incubated and read out the dilutions as discrete samples.

Very recently, two papers on continuous-flow turbidimetric analysis were published by Jones and Palmer (16) and Grimshaw and Jones (17). These authors state that three technicians, using two continuous-flow systems, can run 16 samples per day at a precision of ±3%. This experience appears to be indicative of the problem with application of continuous-flow methods to microbiological assays. These methods generally depend on precise and reproducible long-term control of all environmental conditions, including a continuous, dynamic supply of test organisms. Deviations from absolute control are seen as drift and are compensated for by very frequent analysis of standards and randomization of replications. The necessity of precise, long-term, environmental control also requires a relatively long period of equilibration of the system before useful data can be obtained. Incubation times are restricted by the back-pressures built up in the incubation coils and by aggregation of viable organisms which collect in various recesses of the system. Since incubation times are restricted to 30–60 min., these methods [with one exception, as described by Davis *et al.* (18)] cannot be used for analysis of vitamins. For these and other reasons, continuous-flow turbidimetric methods—despite years of experience—have had only scattered instances of routine applications.

Discrete Analysis Automation—An alternate approach to automation of turbidimetric microbiological assays has been by batch or discrete analysis methods. These, in some instances, have been somewhat more successful. McMahan (19) reported on an elaborate system for such analysis. The system automatically dilutes samples and standards simultaneously into a moving rack, adds the appropriate nutrient broth, controls the movement of the diluted tubes through a water bath, and then automatically transfers the contents of the tubes into a colorimeter for readout. All steps are controlled by punched cards, which are decoded at each work station. The analysis rate was reported to be about 50 samples/hr., each sample consisting of eight observations, four on the sample and four on a concurrent standard.

Gualandi and Morisi (20) described an automated discrete analyzer for both chemical and microbiological analyses. Samples were diluted, incubated, and read out automatically in a temperature-controlled air incubator. Capacity was limited to 18 samples per run. Each run required about 3.5–4 hr. for antibiotics and about 5.5–6.5 hr. for vitamins, so sample capacity varied from 18 to a maximum of 36 samples per working day. Simoncini *et al.* (21) reported on the use of this equipment in their laboratory.

Burns and Hansen (22) described a totally automated system for turbidimetric analysis. Their system accepted unfiltered fermentation broths, filtered them, diluted the filtrate, added inoculum, incubated the samples in an air incubator, measured the turbidity, and calculated the results by means of an on-line computer. In a later publication, Burns *et al.* (23) described a modified system which was faster (120 observations an hour) and used a batch dialysis scheme for effecting sample purification. Davis *et al.* (18) reported on the use of a commercial apparatus designed for chemical analysis, for turbidimetric microbiological analysis of folate activity. Millbank *et al.* (24) further elaborated on the use of this equipment.

Most of these systems involve straight-line or full automation by means of a series of integrated, timed, discrete steps. Such systems cannot be used for long incubation times such as are required for analysis of vitamins, unless the unit is loaded with samples, partially processed, shut off for a number of hours, and then restarted for the remainder of the processing. This ties up the entire apparatus for the duration of incubation and also limits the number of samples that can be processed to the capacity of the incubator associated with the apparatus.

DESIGN CONSIDERATIONS

General—Since manual turbidimetric analysis has been used by so many workers in the field and since the theory behind the use of this methodology is complex or sometimes unknown, it appears desirable to take advantage of these years of experience by staying with the same technology. This would dictate the use of the same test organisms and the same nutrient broth. It would also require measurement of the same microbiological response according to the same assay design. This parallelism of methodology should make the task of conversion from manual to automated methods rapid and simple. Official methods could then be accommodated with no significant changes, and acceptance of the automated results by regulatory agencies should be expedited.

The automated system should have a large sample-handling capacity because of the large number of assays normally performed in most microbiological laboratories. An analysis rate of at least one sample per minute would be desirable. The equipment should be convenient to use and easy to clean after use. There should be essentially no interaction between adjacent samples so that an unexpected high concentration of one sample would not affect succeeding samples. This elimination of interaction would also allow more than one kind of sample to be run without intermediate cleanup; that is, more than one antibiotic could be run in a group of samples, provided the test organism and nutrient broth are the same for the group of samples. Changeover from one test organism or nutrient broth to another should be easily performed since several changes need be made each day in many laboratories.

The system should be able to operate unattended for sufficiently long times so that an operator could perform other productive tasks such as preparing samples, setting up the next test, and cleaning the laboratory area. The analyzer should be small enough to fit in a conventional laboratory facility and should not require an unusual laboratory environment. Obviously, it should be mechanically simple and reliable for routine use.

A system that could perform analyses on both vitamins and antibiotics on the same day has some additional requirements. Vitamin assays normally require a least 16 hr. for incubation, while antibiotic assays require 3–5 hr. If samples can be diluted in a discrete module, incubated independently in one or more incubators, and then read out in a third module, dovetailed operations can be considered. The diluter module can dilute antibiotics in the morning and vitamins in the afternoon. The incubator can be used for antibiotics during the earlier part of the working day and for vitamins during the remainder of the day and overnight. The reader can be used for the readout of the previously incubated vitamin tests in the morning and for the antibiotic tests in the afternoon.

A careful study of the logistics of manual analysis revealed that two steps, the dilution step and the readout step, were the most difficult, time consuming, and inaccurate; the incubation step was not difficult or time consuming. Conversely, the incubation phase had been, in past automation attempts, the most difficult and costly to automate and had placed the greatest restrictions on the versatility of previous systems. Separation of the incubation process has other advantages. It gives the analyst freedom to select optimum incubation times and temperatures independent of the rest of the system. It allows the rest of the system to operate at the full speed of each module without consideration of incubation requirements. The incubation step can be divided among several incubators to provide different temperatures. These incubators can be stacked to conserve space. Temperature control of smaller units is easier to accomplish than is control of much larger units. Diluted tubes in appropriate carriers can be placed manually into and removed from the incubators, obviating the need for mechanical devices dynamically controlling the transport of tubes through the incubator.

All the above-mentioned requirements dictated the need for separate dilution, incubation, and readout modules in a system designed to duplicate essentially the basic steps required for conventional manual analysis. Consideration now had to be given to the optimum design for each of these modules.

Diluter Module—Many requirements were placed on this module. It had to make dilutions rapidly. If dilutions could be made so rapidly that no appreciable change in the concentration of the assay

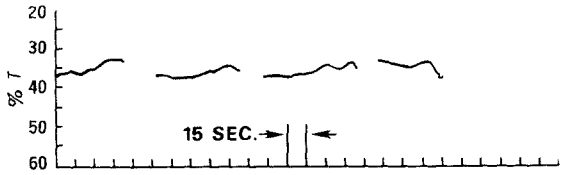

Figure 1—*Representative curves obtained by repetitive scans of a static suspension of* Lactobacillus leichmannii.

organism would occur during dilution, fewer standards would have to be run. (A change in concentration of assay organisms between samples and their corresponding standards causes a drift of test results.) Previous workers attempted to control short-term drift by keeping the inoculated nutrient medium chilled. Keeping the nutrient broth uniformly chilled has certain operational disadvantages. If the dilution time for any set of samples and their standard could be reduced to 10 min. or less, chilling should not be necessary for most assay organisms.

Samples should be routinely evaluated at more than one concentration so that parallelism of the slopes of response can be compared for the sample and its standard. This multiple dilution should be performed automatically for each sample. A module that automatically performed four dilutions might be a satisfactory arrangement. At least two different dilutions would be needed, and these could be performed in duplicate. The same equipment should also be easily convertible to produce up to four different dilutions, if necessary.

The dilution step should be performed with sufficient precision to eliminate this as a source of error in the test. The required precision was estimated to be better than $\pm 0.5\%$. The diluter must be constructed of materials that will withstand the corrosive action of the various salts contained in nutrient broths. All lines that contact the assay organisms should have a smooth lumen and no recesses where the organisms could be trapped. This would prevent a cluster of organisms from being accumulated and then subsequently released in a localized high concentration. Rapid sterilization of the equipment should be possible without requiring disassembly of the diluter. Hot water or normal sterilizing fluids must be accommodated. The diluter should also be able to dilute samples in solvents such as methanol, ethanol, and acetone. The diluter mechanism should be rugged, reliable, and easy to maintain. It should be easy to calibrate and retain its calibration for long periods of time.

Incubation Module—A water bath is required that provides a very uniform temperature, precisely controlled, throughout the entire bath. This is best accomplished with a precision temperature controller and very rapid and unobstructed water flow. The bath should be easily accessible and capable of being stacked to conserve space. It should be constructed of stainless steel and easy to clean. It was believed that the incubation process had been a long neglected variable in turbidimetric analysis; precise, uniform temperature control of the contents of the assay tubes would minimize one more important variable.

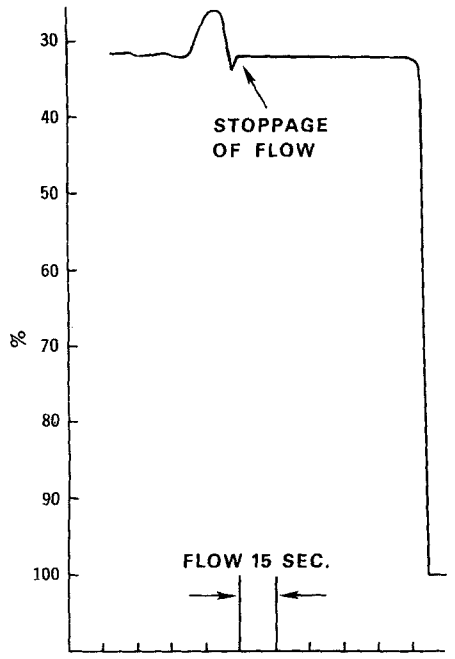

Figure 2—*Recording of the photometric response obtained on a fast-flowing stream of* Lactobacillus leichmannii.

Reader Module—The major design features of this module should include ruggedness, reliability, and maximum convenience. No appreciable interaction between the readings of adjacent samples should occur. The readings should be performed as rapidly as possible to be consistent with the speed of the diluter and meet the sample load requirements. It should perform the measurement of turbidity with such precision that it does not become a significant source of error in the assay. This would require the readout of fast-flowing streams. Previous experience with fast-flowing streams indicated their utility in solving the problems of flow birefringence.

Flow birefringence is a phenomenon exhibited by elongated particles, such as rod-shaped organisms, in which the particles align themselves with slow-moving currents in a container of liquid. When this container is a flowcell and the motion is imparted by the filling of the cell, many minutes are required before currents cease to exist. Additional impetus can be given to the currents if any appreciable heat is absorbed by the contents of the cell from the incident beam of the spectrophotometer. Since the slowly changing alignment of the elongated particles presents a variable cross section of particles to the beam, a variable reading is obtained. With certain flowcells and with flow rates of about 1 ml./sec. or more, these effects are not evident and stable readings are obtained even with long rod-shaped organisms such as *Lactobacillus leichmannii*. Figure 1 shows the effects of flow birefringence normally encountered in photometric analysis. Each recording is a new filling of the flowcell. There is a highly variable response at each filling with very little predictability of the exact result. Figure 2 shows the same suspension in a flowing stream; it is evident that a stable reading can be obtained in only a few seconds and the reading remains stable until the flow is stopped.

When flow is stopped, serious deviation from steady state is again observed.

CONCLUSION

The design considerations discussed in this paper were evaluated in terms of all previously reported approaches to automation of turbidimetric microbiological analysis. All previously reported systems did not meet the design goals in one or more major requirements. This led the authors to design and evaluate a prototype of a new automated system. This was constructed, and several improvements were subsequently made on later models of this system. Information on the latest system are presented in the accompanying paper (1), and a discussion is given on how closely the design goals stated here have been achieved.

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N. R. KUZEL and F. W. KAVANAGH

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Keyphrases □ Microbiological assay—automated □ Automated system—photometric microbiological analysis □ Antibiotics, vitamins—automated analysis procedures □ Turbidimetric analysis, microbiological—automated procedures

In a previous paper, basic theoretical and practical considerations for the design of an automated system for turbidimetric microbiological analysis were discussed (1). This paper reports on the construction of a system meeting these design requirements. Applications of this system to the analysis of several antibiotics and vitamins are reported, and some typical results are presented.

EXPERIMENTAL

Diluter Module—Figure 1 shows a photograph of the diluter module. Both this and the reader module employ a specially designed tube transport mechanism. Empty test tubes are placed in carriers shown in Fig. 2. Each carrier holds an 8 × 10 tube array. These carriers are stainless steel and have a oneway handle so that they can be inserted into the transport mechanism in only one orientation. Two of these carriers can be loaded into the tube transport mechanism at one time. The carriers are moved by a rake mechanism which advances the carriers as required under either manual or automatic control.

Associated with the diluter module is a control panel, shown at the upper right of the module. This control panel contains all the controls necessary for operation of the module. Mounted on the left upper portion of the module is an automatic sampler and turntable which can hold up to 40 tubes for automatic sampling. The struc-

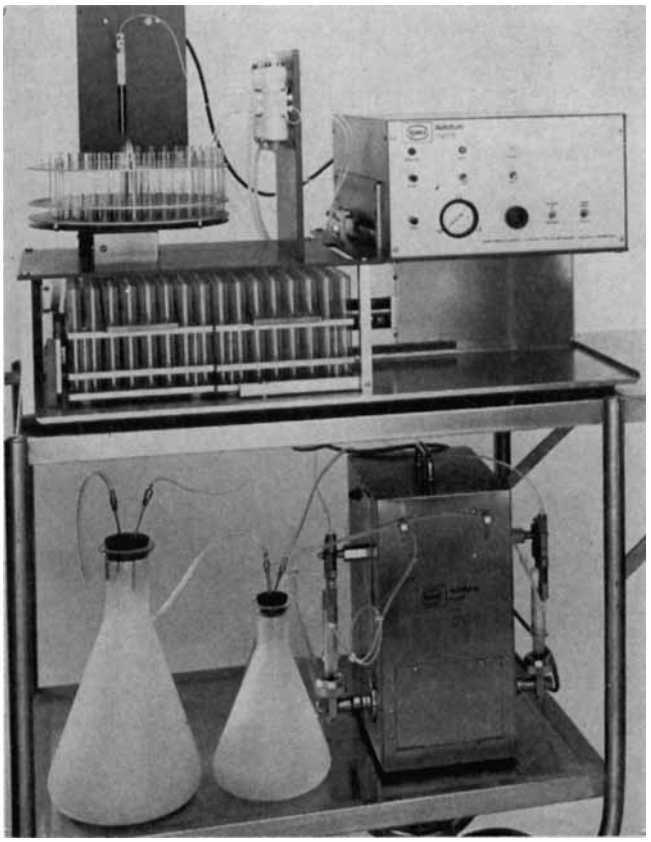

Figure 1—*Diluter module.*

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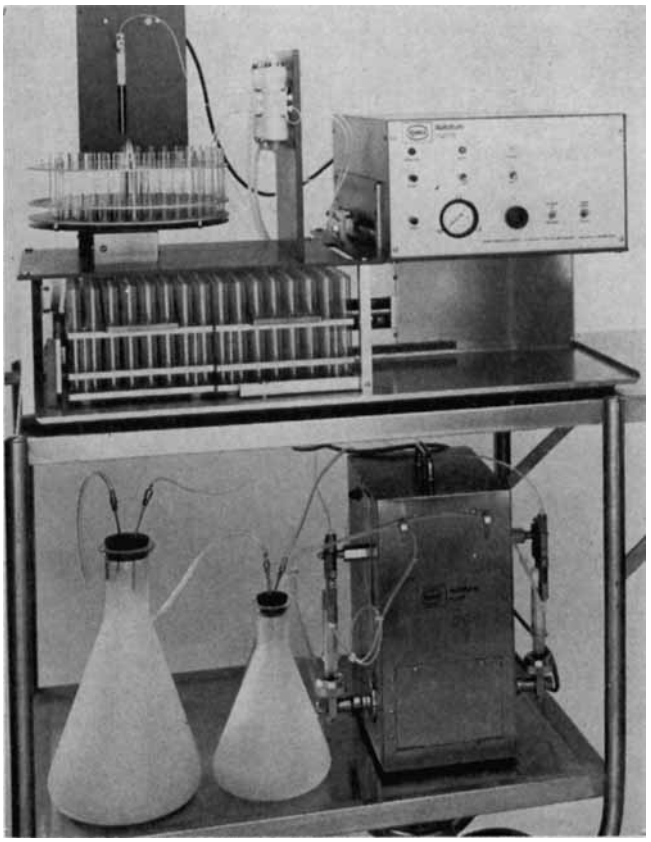

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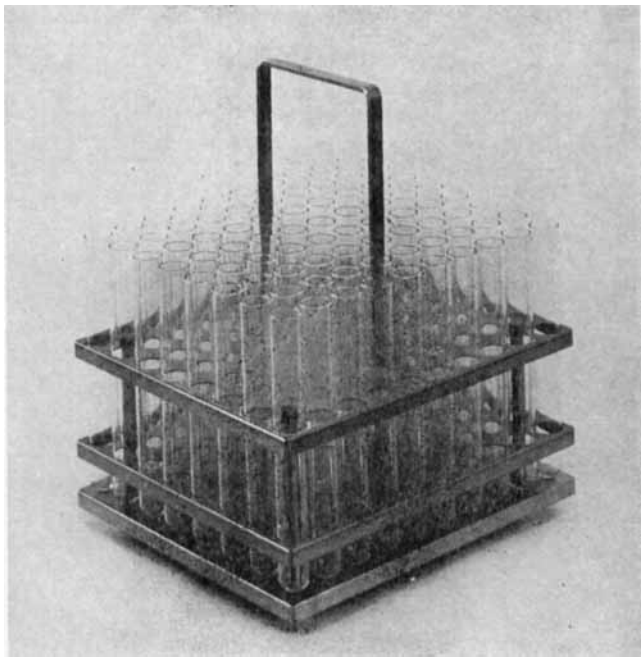

Figure 2—*Tube carrier.*

ture between the sampler and the control section is the valve system for metering the samples from the turntable. Below the diluter module is a delivery device for providing the necessary amount of diluent for each sample.

In practice, the operator places a container of nutrient broth, which may or may not be inoculated with test organism, in front of the diluent unit and drops the Teflon lines leading to the delivery unit into the nutrient broth. A wash trough (Fig. 3) is placed in one carrier, and the carrier is placed into the diluter unit. The diluter is automatically purged, and the washings are collected in the waste trough. Meanwhile, the operator places a turntable loaded with sample tubes on the sampler. One or two carriers of empty tubes are then placed into the diluter. The diluter starts processing the samples on the turntable as soon as the empty tubes are in position, and the operator presses the "start" switch.

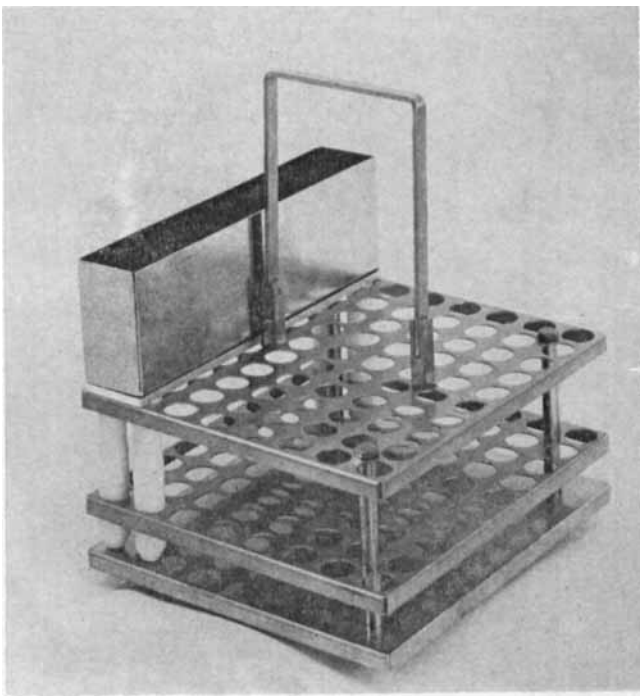

Figure 3—*Wash trough inserted in carrier.*

From this point until the last row of assay tubes in the carrier is filled, the diluter operates without attention, and the operator is free to do other tasks. Four dilutions are made from each sample in the turntable, and each dilution is performed in 5 sec. Thus, a complete load of 40 sample tubes, providing 160 dilutions, requires less than 14 min. of dilution time. This time interval is so short that, under most conditions, no significant growth of inoculum or interaction between organism and drug substance occurs during this period.

The entire load resides in two carriers or assay units. Since these two units are handled separately in subsequent steps, they should be considered as individuals; for maximum precision, each carrier must contain its own standard curve. Thus, each assay unit (carrier) requires less than 7 min. for dilution; since each unit contains its own standard curve, many such units can be processed from one batch of inoculated broth. The carriers are manually transferred to a specially designed water bath for incubation.

Cleanup of the diluter is also very easy and rapid. The rake is reversed, and another carrier with two waste troughs is inserted. The lines in the nutrient broth container are lifted out of the container, and then the lines are transferred to a container of hot water. The diluter is again started and run for less than 2 min. For longer sterilization, a "purge" switch can be used to cycle the diluent delivery unit continuously for as long as desired, or one or more extra wash troughs can be inserted initially. The hot water container is then removed and substituted by one of cool water or another of nutrient broth and the diluter is again turned on for a short time, depending on how many wash troughs are inserted. The diluter is now either cleaned or primed for the next samples, depending on whether the cooling liquid was water or inoculated nutrient medium. In either case, about 5 min. is required for cleanup or changeover from one nutrient broth to another. Some workers prefer to replace the hot water sterilization with another agent such as dilute formalin[1].

The dilution is performed in a unique manner. Two 8-port diluting valves are used, and both are connected by a T arrangement to a sample probe and a common waste receptacle. Each valve has two Teflon sample loops attached to it. In these laboratories, each valve is fitted with a 0.1- and a 0.15-ml. loop. These loops are easily removed and can be replaced with loops of other volumes in a matter of seconds. The two loops operate 180° out of phase with each other, so that while one is being filled, the other is being emptied of its contents. The loops are filled by vacuum and are emptied by discharging a predetermined quantity of nutrient broth through them.

Dilution is performed by a repetitive series of sequential events. A sample probe fitted with a small diameter line is lowered into a sample tube on the sampler turntable. Vacuum is applied through sample loop 1 to the probe, flushing out the previous contents of the sample loop and filling it with the new sample. The vacuum is then shut off, and the sample loop remains filled with the new sample. The 8-port valve is now moved pneumatically to its alternate position to connect the sample loop between the delivery line and the diluent pump. A metered amount of nutrient broth is then passed through the sample loop, washing out its contents into an empty test tube in the carrier located below the delivery line. While this action is occurring, the second sample loop is being filled with another portion of the same sample in the same manner as just described. The diluent valve is now returned to its original position, and the sample in loop 2 is washed into another test tube by a second portion of nutrient broth delivered in the same manner as before. During this time, sample loop 1 is filled with the contents of the next sample in the turntable.

Since two diluent valves operate in synchrony, four dilutions are automatically made from each sample. The extent of dilution is determined by the internal volume of each of the four loops attached to the diluent valves and the volume of broth delivered. These alternate actions are performed until all tubes in the carriers are filled. Since, in effect, the metering valves operate 180° out of phase with the sampler, only two dilutions of the last sample in the tray are delivered as diluted samples. To obtain four dilutions from each sample and to get complete samples in each assay rack, positions 20 and 40—or the last of any intermediate series in the sampler tray—should be buffer or tubes of sample dilution fluid. In practice, this turns out to be an advantage, since the first and

[1] This is possible since all materials in contact with the nutrient broth are Teflon, Kel-F, or stainless steel.

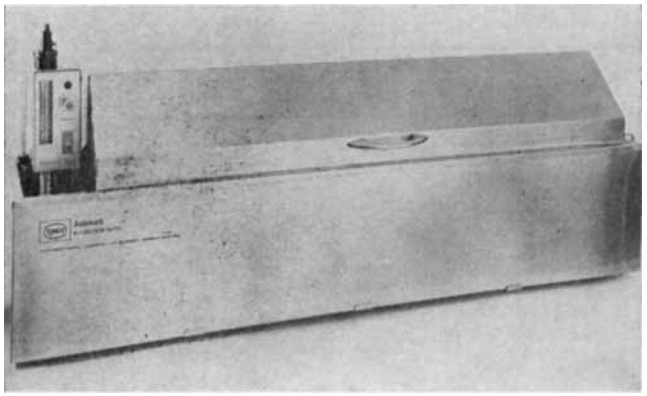

Figure 4—*Incubation bath.*

last two positions in each carrier contain no drug substance and any drift that might occur can thus be easily monitored.

The amount of sample removed from the sample tube is determined by the length of the withdrawal cycle, the diameter of the tubing, and the vacuum. Changes in the amount of sample withdrawn can be made by changing the vacuum by means of the vacuum control valve located on the control panel. This is especially useful if nonaqueous solutions, such as methanol or acetone, are to be sampled.

The dilution system used is a closed tubing system. Regardless of loop size, the volume of diluted sample remains the same because the contents of the sample loops are replaced with an equal volume of diluent during each cycle. For this reason, loop sizes can be changed without affecting the final volume of diluted sample; only the dilution ratio is changed. All the critical interconnecting tubing was carefully selected to be of minimum volume so that sample interaction would be essentially eliminated. The metering valves are of the zero dead-volume type and do not contribute any interaction. The only tubing that could be responsible for interaction between samples is either thoroughly washed with the next sample or remains filled with the diluent common to all samples. When the system is used properly, the only carryover from one sample to another is the minute amount on the outside of the sampler probe. This can be illustrated by use of concentrated dye solutions followed by water samples. Dye solutions up to 10 times the usual concentrations can be used before any indication of interaction is seen.

All solutions in this system flow in one direction, and there are no dead sample cavities or cross-connections. No part of the sample probe or tubing connected to it ever contacts the solutions containing the test organism. This arrangement of connections and flow pattern makes cleaning easy. Flushing the sample probe with a tube or two of sterile water while the medium lines are being sterilized by pumping hot water through them, as if performing a dilution, gives adequate cleaning. Depending upon the sequence of test organisms, media, and antibiotics, sterilization may not be necessary.

Incubation Module—After dilution, the assay carriers are moved manually to a specially designed water bath (Fig. 4). This bath holds four carriers, *i.e.*, 320 tubes equivalent to 76 samples and standards. The bath provides rapid water circulation and precise temperature control. The importance of proper incubation becomes evident when operational errors normally found in manual methods are eliminated. For example, some antibiotic assays can be influenced by a temperature nonuniformity of 0.05°. Most antibiotic assays are not that sensitive, but temperatures should be controlled so that the maximum difference between any two tubes in a test would be less than 0.1°.

After the optimum period of incubation for each test, the assay carriers are removed from the incubation bath and further growth of the test organism is stopped. A convenient method of temporarily stopping growth in antibiotic assays involves immersion of the carrier into an 80° water bath for 1–2 min., followed by subsequent cooling to about room temperature. With vitamin assays, cooling in a chilled water bath is adequate. Some assays may require shaking of the tubes before measuring turbidity. For those assays, a device is being designed to shake an entire carrier of tubes at one time.

Reader Module—The readout is accomplished automatically. Figure 5 shows the reader module. The basic tube transport mechanism is the same as for the diluter. A spectrophotometer is placed on the upper left portion of the transport mechanism. Its output is monitored by a digital voltmeter located on the control panel. The output of the spectrophotometer is adjusted so that the output voltage is 1.000 v. representing 100% T (transmittance). A reading such as 0.648 v. then represents 64.8% T. A probe assembly is substituted for the delivery-line-carriage assembly of the diluter module.

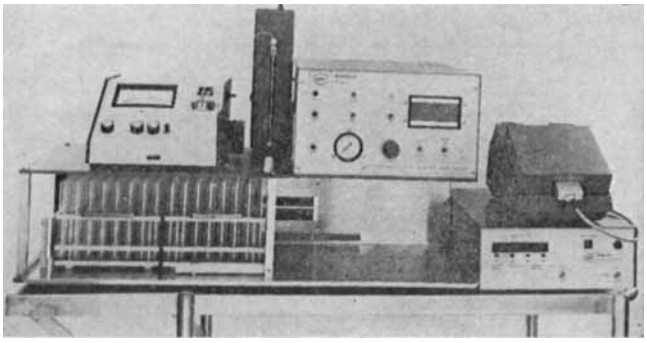

Figure 5—*Reader module.*

After the carrier of incubated tubes is placed in the reader and the "start" button pushed, the probe assembly advances to the position of the first tube, and the probe lowers into the tube directly below it. A solenoid valve in the vacuum line opens, and the suspension of bacteria in the tube is drawn rapidly through the flowcell of the spectrophotometer. The spectrophotometer continuously monitors the flowing stream. After about 6 sec. of flow, the reading on the voltmeter is automatically stored. The voltmeter reading can be read manually or the value can be recorded automatically by one of several means.

After the reading of the spectrophotometer is made, the flow is stopped, the probe is raised from the tube, and the probe assembly advances to the next position. If other tubes are to be read, the same cycle is repeated for each tube until the test is completed or until the "stop" switch is depressed. The inside tube of each row in the carrier is sensed; if any of these positions are vacant, the rake advances the carrier to the next occupied position or clears the carrier.

Each reading cycle takes 10 sec., and each carrier is read in about 13.5 min. With automated data recording, an operator can leave

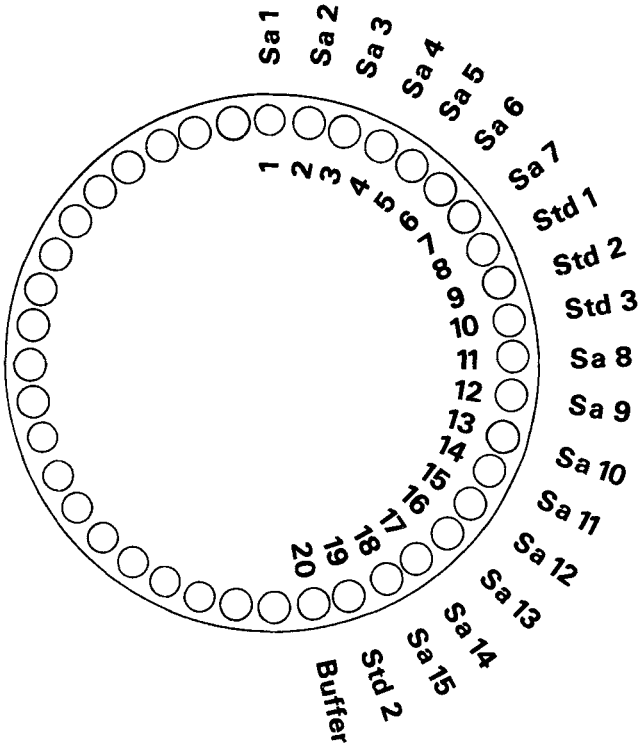

Figure 6—*Sampler turntable arrangement.*

Table I—Dilution Pattern

Tube	Row 8	7	6	5	4	3	2	1	Start
1	Sa 15	Sa 12	Sa 10	Std 3	Std 1	Sa 5	Sa 3	Buffer	From previous run
2	Sa 15	Sa 12	Sa 10	Std 3	Std 1	Sa 5	Sa 3	Buffer	
3	Sa 15	Sa 13	Sa 10	Sa 8	Std 1	Sa 6	Sa 3	Sa 1	Loop 1
4	Sa 15	Sa 13	Sa 10	Sa 8	Std 1	Sa 6	Sa 3	Sa 1	Loop 2
5	Std 2	Sa 13	Sa 11	Sa 8	Std 2	Sa 6	Sa 4	Sa 1	Loop 3
6	Std 2	Sa 13	Sa 11	Sa 8	Std 2	Sa 6	Sa 4	Sa 1	Loop 4
7	Std 2	Sa 14	Sa 11	Sa 9	Std 2	Sa 7	Sa 4	Sa 2	
8	Std 2	Sa 14	Sa 11	Sa 9	Std 2	Sa 7	Sa 4	Sa 2	
9	Buffer	Sa 14	Sa 12	Sa 9	Std 3	Sa 7	Sa 5	Sa 2	
10	Buffer	Sa 14	Sa 12	Sa 9	Std 3	Sa 7	Sa 5	Sa 2	

delivery line movement ↓

assay rack movement →

the reader unattended for periods up to about 27 min. It should be emphasized that the reader monitors a fast-flowing stream. The amount of sample withdrawn is regulated by the vacuum control valve on the control panel.

Automated Readout—All readers used in these laboratories have some form of automated data acquisition attached to them. The simplest system uses a digital converter to accept the BCD (Binary Coded Decimal) output from the digital voltmeter and to drive a paper tape printer. Printed on the tape is a test code, three digits of automatic sequence data, and four digits of data representing percent T to the nearest 0.1% T.

Several systems also contain a coupler for an IBM 526 summary card punch to punch simultaneously cards for off-line computer calculation of the data. One system drives a paper tape punch rather than a card punch. Most of these systems will eventually be interfaced directly to an on-line computer to provide computed data as soon as each carrier has been read. The interface is being designed and should be operational in the near future.

ANALYSIS PROCEDURE

The assay design is different for antibiotics and vitamins, and each will be treated separately. In each, the sample is automatically assayed at two concentrations because more than one concentration is required to detect aberrant samples. For example, in the assay of antibiotics, if the two potencies obtained from the two concentrations of sample differ from the mean by more than 5%, a problem should be suspected. If the two potencies differ from the mean by 10% or more, standard and sample differ in some significant manner, and the problem should be investigated. The capability to detect such problems was provided by the use of two sizes of measuring loops in each system.

Antibiotic Assay—A statistical study of this system showed that the potency of a sample was independent of the dilution at which it was assayed. Therefore, any dilution that provides a concentration of active material falling between the two ends of the standard curve is suitable. No special effort need be taken to dilute the sample exactly to an estimated predetermined concentration. The most accurate method of sample preparation should be used, even though the resulting concentration is estimated to be a number such as 5.67 mcg./ml. Standards and samples should be diluted by means of volumetric flasks and transfer pipets or by an *identical* schedule using automatic diluters. The diluting medium must be the same for samples and standards if highest accuracy is to be obtained.

Table II—Reproducibility Using Dye Solutions, Readings in Percent T

	0.1-ml. Loops		0.15-ml. Loops	
	Loop 1	Loop 2	Loop 3	Loop 4
	99.9	100.0	100.0	99.9
	47.9	47.9	33.2	33.2
	47.8	47.9	33.2	33.3
	47.8	47.8	33.2	33.3
	99.8	99.9	100.0	99.9
	99.9	99.9	99.9	100.0
	47.9	48.0	33.3	33.2
	47.8	47.9	33.2	33.3
	47.8	47.8	33.2	33.3
	47.8	48.0	33.2	33.3
Average	47.83	47.90	33.21	33.27
Range	0.1	0.2	0.1	0.1
CV, percent	0.102	0.170	0.113	0.146
Absorbance	0.321	0.320	0.479	0.478
Average absorbance of series of manual dilutions	0.319		0.479	
Ratio of 0.15/0.1-ml. loops 0.4785/0.3205 = 1.49				

A wide range of total growth (zero tube or the tubes without antibiotic) will give satisfactory assays. The range depends upon characteristics of the test bacterium, composition of medium, temperature of incubation, and time of incubation.

The standard curve should be selected so that the smallest concentration gives not more than 90% of the growth obtained in the zero tube while the high end of the curve gives not less than 20% of the growth of the zero tube. The range may be from 2- to 10-fold, depending upon the assay system. As an example, the range for the erythromycin standard curve can be 2, 4, 6, and 8 mcg./ml. in the sample tubes, resulting in concentrations of 0.02–0.12 mcg./ml. in the assay tubes. For samples whose content is likely to be unpredictable, this long curve is used and the sample is diluted to an estimated concentration of about 5 mcg./ml. If the product potency can be accurately estimated, a standard curve of 3, 4, and 5 mcg./ml. is used and the sample is diluted to an estimated 4 mcg./ml. concentration. Spacing of the concentrations of standards is on a linear scale, not a logarithmic one as for a diffusion assay.

Also needed is a tube of the solution used for diluting the samples, usually a buffer, water, or nonaqueous solvent. The samples and standards are placed in the sample turntable in the desired order. One possible arrangement is shown in Fig. 6 where the contents of one-half of a sample turntable are shown.

Standards can also be placed at the beginning or at the end of each test or can be randomly distributed throughout the array. The single duplicate standard is used as a check sample. It can be replaced with a secondary standard of known concentration. The last position must be a buffer or zero tube. The pattern shown in Fig. 6 would produce a diluted array as shown in Table I.

A single-strength nutrient medium is prepared and is inoculated with test organism just before dilution. Because of the speed of dilu-

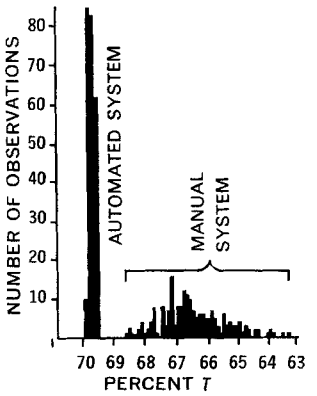

Figure 7—*Reproducibility of readings obtained by reading individual tubes of a pooled suspension of* Lactobacillus leichmannii.

Table III—Penicillin G Assay—Automated System

Known Potency of Sample, units/ml.	Day 1	Day 2	Day 3	Day 4	Day 5	Average	Range
1.50	1.49	1.50	1.50	1.49		1.50	1.49–1.50
2.00	2.03	2.03	2.04	2.01	2.00	2.02	2.00–2.04
2.50		2.49	2.52	2.55	2.54	2.52	2.49–2.55
		2.50	2.53	2.53	2.51		
3.00	2.97	3.04	2.99	2.97	3.05	3.00	2.97–3.05
3.33		3.33	3.34	3.37	3.43	3.37	3.33–3.43
4.00	4.04	4.05	4.00	3.99	4.04	4.03	3.99–4.05

tion, nutrient medium at room temperature is usually satisfactory if dilutions are performed as soon after inoculation as possible. In certain cases, the medium might have to be chilled or left at room temperature for some time after inoculating to obtain optimum results. Stirring of the medium is not always necessary and, in fact, may be harmful to the accuracy of the assay if significant heating of the medium is produced by the stirring device.

After incubation, the suspensions of killed organisms are measured on the reader module. When paired dilutions are used, the pairs can be observed for a quick check on the quality of the data. In general, if manual calculations are used, the results from each pair are averaged and separate standard curves are plotted for the 0.1- and the 0.15-ml. loops. Unknowns are referenced to the appropriate curves, and two assay values are obtained for each sample in the sample turntable. These two values, if they match the slope criterion, are averaged for the reported answer.

In most antibiotic assays, reduction in growth rate of the test organism by the test drug causes the test response. Therefore, the bacterial population at any time is proportional to the initial population (inoculum). An error in the inoculum causes an equal error in bacterial concentration at the termination of incubation. The volume of broth delivered to the assay tubes and, consequently, the concentration of inoculum differ by 0.5%, with the inoculum in the assay tubes receiving 0.15 ml. of sample being the smaller. This seemingly small difference in inoculum can cause an appreciable difference between the two dose–response lines. The difference in quantity of inoculum prevents the responses from the two sizes of samples from being portions of one dose–response line. The two sets of assay tubes are, in effect, different assays and must be treated as such; otherwise, accuracy of the system will be degraded. The influence of inoculum size is greater in certain assays than in others.

The millivolt output of the spectrophotometer should be plotted against concentration of antibiotic in the sample tube, using an inverse semilogarithmic graph (2) when manual interpolation is to be done. If a computer is used, millivolts are transformed to absorbance and log *A versus C* is plotted. This equation may also be used in manual computations, although it is less convenient than the inverse semilogarithmic graph. A point-to-point curve is constructed, and the results are interpolated from the curve. If a computer is used for calculation, it is preferable to calculate each loop against itself, resulting in four standard curves and four responses for each sample. The necessity for precise calibration of the loops and exact adjustment of the diluent unit is eliminated when each loop is compared only to itself. Rounding-off errors in the averaging of two measurements are also avoided.

Table V—Vitamin B_{12} Assay

Sample	Theory	Automated Assay	Manual
Secondary standard			
Day 1	107.1	107 mg./ml.	107
2		106 mg.	112
3		108 mg.	111
4		106 mg.	120
5		106 mg.	
6		107 mg.	
7		108 mg.	
8		108 mg.	
Lot A	42.5	45.0 mg./ml.	43.9
		41.8 mg.	50.2
Lot B	2.15	2.00 mg./ml.	2.22
		2.03 mg.	2.31
Lot C	28.0	28.0 mg./ml.	43.7
		28.4 mg.	29.8
		28.2 mg.	19.6
		28.0 mg.	27.7

Vitamin Assay—Total growth (concentration of microorganisms, not percent *T*) in a vitamin assay is proportional to the amount of vitamin put into the tube up to a limit set by the composition of medium and subsequent pH. There is a linear range with a span adequate for practical assays. The upper limit of the standard curve should be set at some point below the upper end of the straight-line portion of the curve. If points at 0 and at one-half of the high level standard are included for both standard and sample, the slope ratio method of calculating potency may be used as in common zero-five-point assays (3). Two separate assays would be obtained, just as in the antibiotic assay, because each size loop is treated separately. In this design, the standard would be at concentrations of 0, 1, and 2 and the samples at a concentration ratio of 1:2. Each sample would be assayed at two sample levels. This design is precise but may be more precise than is needed. This ideal response of concentration of bacteria is the one to use when investigating assay procedures and the quality of dose–response lines.

A more practical approach may be to use a multipoint standard curve and a single concentration of sample. A representation is then selected that gives a curve as nearly straight as possible. Such a curve may be obtained by first transforming millivolts into absorbance and plotting *A versus C*. The two different levels of sample, automatically produced, are different enough to detect abnormal samples.

Any desired design can be used, but the last tube must be a sample blank. Two tests can be loaded into the diluter at once. Single-strength medium without inoculum is used. After the tubes are diluted, they are capped and sterilized by autoclaving. The tubes are cooled prior to inoculation. Inoculation must be carried out very carefully since many vitamin tests are now run, at least partially, as growth-rate assays. The use of one drop of a suspension of bacteria per tube does not provide a sufficiently reproducible inoculum for each tube in the assay. A more satisfactory method is to dilute the inoculum by a factor of 10 and inoculate each tube with 0.5 ml. of this suspension. A precise repetitive pipeting device should be used to add a constant volume of inoculum to each tube.

Table IV—Precision Study on Erythromycin Using the Automated System

Assay Date	Sample Set	Test A, mcg./ml.				Test B, mcg./ml.			
		Std. 3	2.5	3.75	5.0	Std. 3	2.5	3.75	5.0
Day 1	1	3.02	2.25	3.35	4.42	2.94	2.21	3.33	4.38
	2	2.99	2.22	3.33	4.50	3.06	2.25	3.40	4.52
Day 2	3	2.99	2.20	3.41	4.46	3.01	2.26	3.42	4.46
	4	2.92	2.19	3.34	4.44	2.90	2.22	3.35	4.36
Day 3	5	2.99	2.26	3.40	4.45	2.99	2.25	3.32	4.51
	6	2.94	2.26	3.32	4.39	2.95	2.24	3.32	4.40
Day 4	7	3.01	2.24	3.34	4.39	2.92	2.25	3.38	4.42
	8	2.98	2.23	3.26	4.37	3.00	2.26	3.32	4.46
Day 5	9	2.96	2.22	3.35	4.44	2.99	2.25	3.32	4.47
	10	2.98	2.14	3.24	4.38	3.03	2.29	3.32	4.46
Average		2.98	2.22	3.33	4.42	2.98	2.25	3.35	4.44

Table VI—Nicotinic Acid

Sample		3-Day Acidimetric	1-Day Turbidimetric	Theory
Secondary standard		116.0	99.6	100
		95.2	100	
		99.5	98.8	
		97.3	101.6	
	$\bar{x}$	101.9	100.0	
Sample A		27.5	29.4	28
		30.3	27.7	
	$\bar{x}$	28.9	28.5	
Sample B		37.2[a]	27.9	28
		24.4	29.6	
	$\bar{x}$	24.4	28.8	
Sample C		26.1	29.1	28
		36.2[a]	28.1	
	$\bar{x}$	26.1	28.6	
Sample D		17.4	19.6	20
		14.2	20.1	
	$\bar{x}$	15.8	19.8	

[a] Invalid assays.

Incubation of the inoculated tubes is carried out in the normal manner. Termination of growth can be accomplished by chilling in a cold water bath before reading. Each tube must be shaken to resuspend the organisms before measuring turbidity with the reader module.

RESULTS

Dilution Error—Considerable effort was expended in designing a dilution system that would be rapid and precise and have a minimum of sample interactions. The system was evaluated using dye solutions to test these design goals. Table II shows the total error involved in the dilution and readout process using dye solutions. FD&C Blue No. 1 was used as the dye, and its percent *T* was read at 620 nm. The dye samples were interspersed with water, and all the readings are shown in Table II. Good precision is obtained and no significant interaction is observed between dye samples and water.

The loops can be cut to match exactly, if desired, and their ratios can also be exact, if necessary. Such matching requires three or four successive calibrations, with the loop length being trimmed each time. If a computer is used for calculating the results on each individual loop, only one calibration is necessary since the loops do not have to be closely matched. Loops have maintained their calibration for over 3.5 years. This is to be expected since the loops do not contact any moving parts and have walls heavy enough to prevent any change in internal volume. Since they are of small diameter [*i.e.*, 0.08 cm. (0.030 in.) i.d.] and are flushed with 10 ml. of liquid in about 5 sec., the high flow rate of liquid in every cycle thoroughly flushes the loops and prevents any accumulation of debris which could affect internal volume. The loops, being made of Teflon, provide a smooth inner surface which is not subject to coating by foreign materials. Microorganisms have no opportunity to grow on the walls of the tubing.

Table VII—Table of Coefficients of Variation[a], Production Control Department

	Manual	Initial Automated	Recent Automated
Erythromycin standard check	13.1	5.76	1.88
Erythromycin secondary standard	12.4	4.66	1.58
Tylosin standard check	12.7	4.40	1.37
Tylosin secondary standard	8.71	6.07	2.00
Vitamin B_{12} secondary standard	4.63	1.50	1.41
Calcium pantothenate standard	16.0[b]	4.50	3.20
Calcium pantothenate secondary standard	16.0[b]	6.00	3.42
Cephalexin	9.34[c]		1.79
Neomycin	19.9[c]		1.76

[a] Coefficient of variation is defined to mean 1 *SD* (calculated on single assays) divided by the average obtained on these assays. [b] This value is typical but obtained by another method. [c] These results represent plate assays.

Fast-Flow Readout—Figure 7 illustrates the practical application of the use of fast flow systems. A pooled suspension of *Lactobacillus leichmannii* was prepared and subdivided into 400 tubes. Two hundred tubes were read on the existing semiautomated system. This system consisted of a Lumetron with a conventional flow-through cell, a Leeds and Northrup type H potentiometer, a timer, a shaft position encoder, and a translator to drive an IBM 526 card punch. The operator emptied the suspension into the flowcell and, after a 13-sec. automatically timed interval, the result was punched on an IBM card. The results are seen on the left of Fig. 7 and range from 63.4 to 68.8% *T*, with one reading at 74.5% *T*. This variation is not due to drift since the results varied in a random manner. The readings of the other 200 tubes were made on the automated fast flow system and are shown at the right of Fig. 7. These readings range from 69.6 to 70.0% *T*. This same comparison has been conducted in these laboratories several times with essentially the same results.

Analysis of Antibiotics—Two illustrations of the use of the equipment for antibiotics will be presented. A large number of other examples could be given, but these results are typical of those obtained with many other antibiotics.

The data presented in Table III are a summary of 5 days' results on a series of dilutions of a standard penicillin solution. The first column is the known value and, reading across, the values obtained on each of the 5 days. Each value represents the result calculated from four individual diluted tubes or one sample in the sampler turntable. Each of these results is obtainable in 20 sec. of dilution time and 40 sec. of readout time. Standards were run at 1, 2, 4, and 6 units/ml., and the ratio of the number of sample tubes to standard tubes was 5 to 1. It can be seen from the range that all the results except one were accurate to ±2% of the true value, and the averages show no bias.

A more comprehensive precision study is shown in Table IV. This study was made on erythromycin base. The only components common to all the assays were: the stock standard solution, the operator, the slant of the test organism, the lot of assay broth, and the assay system. Ten weighings were made of slightly more than 100 mg. of the sample of erythromycin base into 100-ml. volumetric flasks. The base was dissolved in acetone, and the solution was diluted to give 1.000 mg. of solids/ml. Two freshly prepared dilutions were assayed each day in two tests. Each solution and its standard were diluted to assay level using volumetric pipets and flasks. Different flasks and pipets were used for the different tests. Two inocula were prepared. Two lots of media were prepared from the same lot of B.B.L.[2] Antibiotic Assay Broth No. 3. Both sets of samples and standards were assayed in each of the two daily assays. The standards were diluted to 2, 3, 4, and 5 mcg./ml. The samples were diluted to 2.5, 3.75, and 5 mcg. of solids/ml. Dilutions to assay levels were made in pH 8 phosphate buffer. Thus, each assay was linked to the other by no more events than if it had been performed on a different day. Each assay contained two independently prepared standards and samples.

The data were analyzed as a 2 × 5 × 3 factorial experiment with one covariate. The factors were days, dilutions, and "tests," with the standard measurement being the covariate. The dependent variable was observed in micrograms per milliliter. However, in addition to the analysis carried out on this variable, the observations were expressed in percent recovery and analyzed. This was necessary in order to see if the percent recovery was affected by dilution. For each "treatment," two replications were observed. There was no evidence to indicate a statistical difference in either days or dilutions or the interactions: "test" by day, "test" by dilution, day by dilution, and "test" by day by dilution.

From the results of this study, it appears that there is 95%

[2] Baltimore Biological Laboratories.

certainty that approximately 95% of the individual assays will lie within ±2.7% of the recovery percentage. This figure is derived from the calculated standard deviation and takes into consideration that S is only an estimate; S has been multiplied by a factor found in standard tolerance factor tables for normal distribution to obtain the quoted confidence interval (4). Also, it appears with 95% certainty that the true potency is within ±1.35% of the mean of five assays when the response variate is in terms of percent recovery. The statements are predicated on the assumption that there is a relative sample standard deviation of 1.05%. Of couse, 1.05% is an estimate (calculated from these data) and is itself subject to variation.

Analysis of Vitamins—Again, two of many possible examples will be given to illustrate the use of the automated system for vitamin analyses. Results on vitamin B_{12} are shown in Table V. This table shows the results of automated analysis *versus* manual analysis run on some of the same days. The results on a secondary standard run on 8 days have a range of ±1% of the average value, and the average value is the same as the average obtained on multiple chemical assays. Each of the automated assay values represents the average of the results of two tubes in the sampler turntable, one a high sample and the other a low sample. Manual results show a much greater variation.

The results obtained on nicotinic acid are shown in Table VI. Here, a 3-day acidimetric assay was replaced by a 1-day turbidimetric assay. Even though the assay time was reduced by 2 days and involved much less hand labor, the results by the automated method were far superior to those obtained by the manual method. In the manual method, an unusually large number of invalid assays was customarily obtained, but the automated method rarely produced an invalid assay using the same criteria of invalidity.

DISCUSSION

The system just described is a fourth-generation system, with all previous systems still being used. All the systems employ the same basic principles, but each successive generation improves convenience, is faster, and eliminates former minor maintenance problems. If all systems are included, cumulative experience with the basic concept totals over 11 years. In this period, it has been evident that the equipment can provide precise analytical results if the method is properly designed and if operators are well trained and observant.

In any automated system, the operator is still a big factor in realization of the maximum potential of the system. Table VII illustrates this point very clearly. This is a table of coefficients of variation obtained on known standards and secondary standards which are routinely run to check for validity of the assay. The first column represents the results of the last manual assays before conversion to the automated equipment. The second column represents the results of the automated assay after about 3 months' experience with the equipment. The last column shows the results of a more recent set of 25 consecutive values obtained on the automated equipment. Even with relatively little experience, the automated method had a markedly lower coefficient of variation than the manual method. The really significant comparison, however, is between the last two columns which compare the more recent work with prior automated results obtained over a year earlier. This clearly demonstrates the improvement possible with the automated equipment, minor optimization of the equipment, and the use of well-trained operators. It also illustrates the precision obtainable in a routine production control operation. During this time, there were no major changes in the equipment, no changes in methods or operators, and no changes in calculation of results. It should be emphasized that these calculations represent all the results obtained over each period of the study for all the methods, and strict validity criteria would undoubtedly reduce the coefficient of variation stated.

A further item of interest is that there are no early automated results for cephalexin and neomycin since these were not run by this method until recently, yet the present precision is consistent with other tests. This would indicate that good precision is not a matter of the operator learning how to run a specific test over a long period, but that training in the general technique carries over into new uses of the equipment.

The results shown in Table VII are only representative of the many assays to which the equipment has been applied; the selection was made only to present a wide spectrum of uses. The turbidimetric assay has been used as a replacement for plate assays in cases in which the unique properties of agar diffusion are not required. Higher precision and significant cost savings are realized when automated turbidimetric methods replace manual turbidimetric and diffusion methods.

In these laboratories, this equipment has been well received by the operators, their supervisors, and management. The operator is still very much involved in the assay. The significant difference is that the operator has been relieved from the routine, boring operations, yet he has an opportunity to interact with the assay and take pride in the results of the assay. Supervision has been satisfied since very few assays are lost because of failure to meet validity criteria. In addition, each operator has become much more productive and is processing 2–4 times as many samples as previously possible. Management can observe the much higher level of precision obtainable and has a much higher degree of confidence in the results produced by the laboratory. Fewer assays need be run on each sample, and yet the confidence interval can be substantially reduced. Running fewer assays generally can result in faster turn-around time and better service can be offered. Space requirements using the automated equipment are much smaller than that for the previous manual assay, and total sample capacity is still very high. If greater capacity is required in any one location, it can be obtained by extending the effective workday to beyond 8 hr. to take better advantage of the equipment.

SUMMARY

In summary, a highly versatile system for turbidimetric microbiological analysis has been described. It is a relatively simple, rugged, and reliable system with few moving parts. It is capable of analyzing a wide variety of antibiotics, vitamins, and other substances that can produce a microbiologial response. The equipment is compact so that it can be installed in a number of areas where needed, thus eliminating the necessity of a large central analytical service area and its attendant problems of sample transportation and delay. Starting up the equipment and its subsequent cleanup are easily and effectively accomplished because of the small interconnecting lines and ease of operation. The operation of the system is such that a well-trained laboratory technician can operate it with minimum support, much like the situation found in conventional automated chemical analyzers. Experience with the system shows it can provide rapid and reproducible results at a lower cost per assay than conventional manual methods. As a result of this experience, this equipment is now being used in several installations and is furnishing analytical information that assures management of a higher level of quality assurance than previously obtainable by manual methods.

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Instrumented Pilot Plant Lyophilizer: Its Versatility in Developing and Programming Production Cycles

PATRICK P. DeLUCA

Abstract □ A detailed evaluation of a pilot plant lyophilizer provided valuable information for the development and programming of drying cycles for production equipment. Temperature–time and pressure–time curves from the initial freeze-drying runs and a knowledge of the conductivity behavior of three products were found to be almost essential in the development of optimum lyophilization cycles.

Keyphrases □ Lyophilizer, instrumented—pilot plant □ Production cycle development—instrumented pilot plant lyophilizer □ Instrumentation—pilot plant lyophilizer □ Temperature–resistivity measurement, pilot plant lyophilizer—application to production equipment

In previous investigations (1–7), the various parameters believed to influence significantly the freezing and drying processes during the lyophilization of pharmaceuticals were elucidated. From these studies, it was also concluded that certain instrumentation and controls on a conventional freeze-dryer were essential equipment for optimum lyophilization. The purposes of this report are to describe the necessary instrumentation and controls and to demonstrate how the information obtained from both preliminary and pilot studies can be utilized to program cycles for a production scale operation. Most of the instrumentation is of the standard type and can be easily incorporated into most existing freeze-dryers.

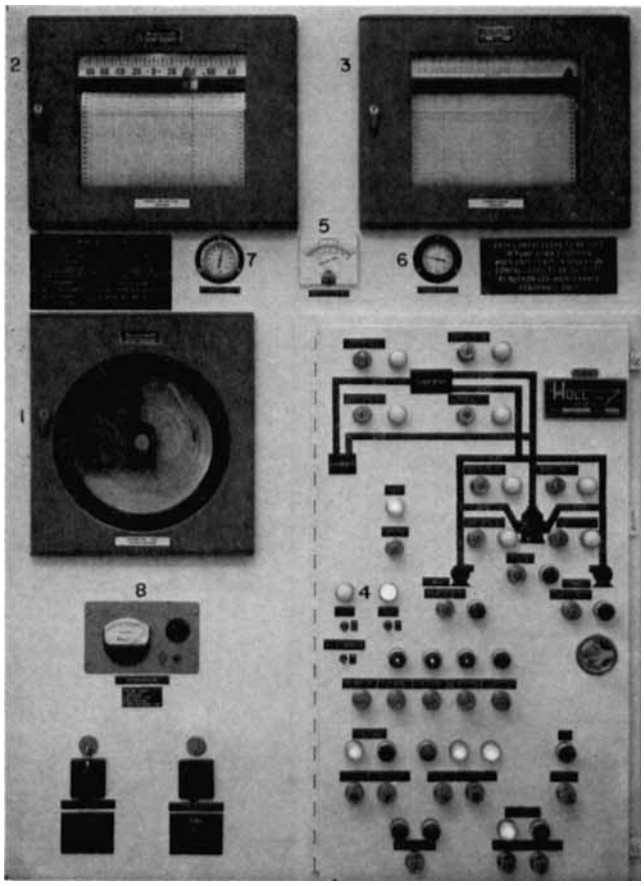

Figure 1—*Instrumentation and control panel. Key: 1, program controller; 2, temperature recorder; 3, vacuum recorder; 4, alarm system controls; 5, pyrometer; 6, vacuum and pressure gauge; 7, air pressure gauge; and 8, thermocouple vacuum gauge.*

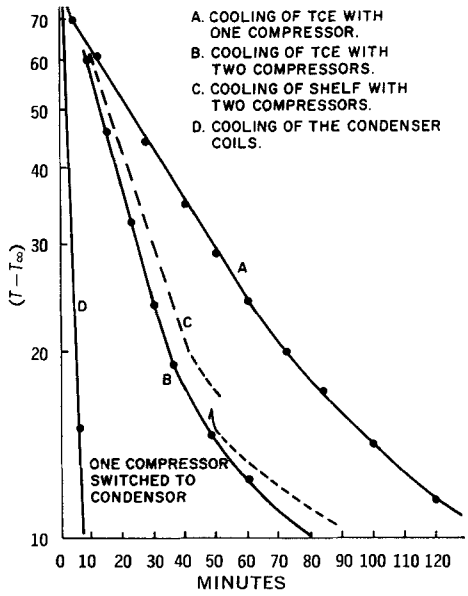

Figure 2—*Capability of the refrigeration system. The temperature controller was set at −50°.*

Essential in the development of a lyophilization cycle is a knowledge of the capabilities of the various systems comprising a freeze-drying unit. Once the capabilities of the various systems are evaluated, it is possible to use a more systematic approach to conducting preliminary studies and designing a cycle. Although the information resulting from such evaluation usually cannot be utilized directly with another lyophilizer in another plant, the methods employed to determine the necessary information will be described and the significance of the values obtained will be discussed. To make this presentation more meaningful, a brief description of the equipment will be presented.

DESCRIPTION AND EVALUATION OF EQUIPMENT

The unit[1] consists basically of a freeze-drying chamber, an external condenser, heating and chilling systems for the chamber shelves, a condenser refrigeration system, a vacuum pumping system, and a control and instrumentation panel.

Freeze-Drying Chamber—The chamber is provided with a suitable wall flange so that it could be sealed into the wall of a sterile

[1] A Hull Corp. model 653-F30 freeze-dryer.

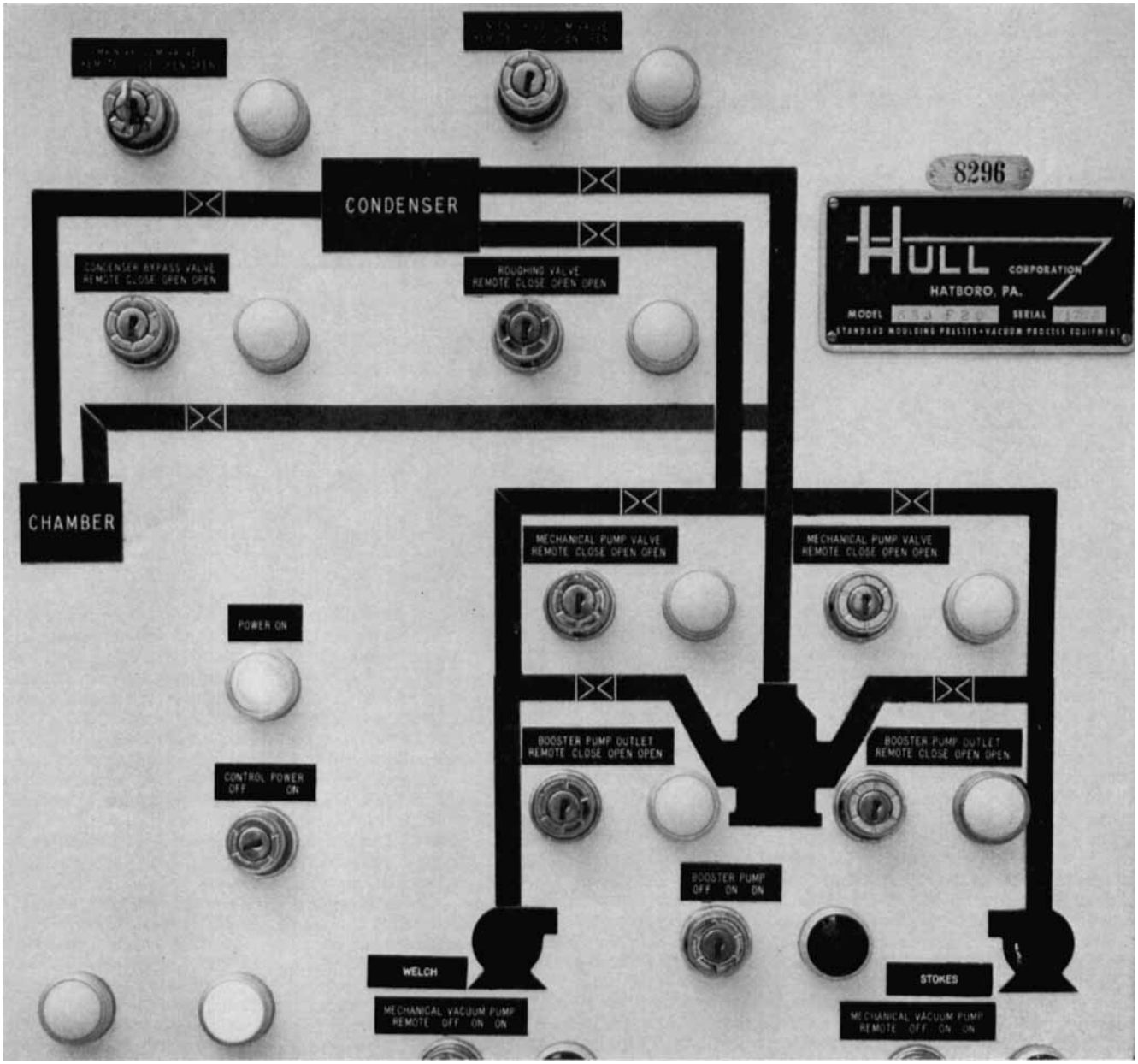

Figure 3—*Graphic representation of the vacuum system.*

room. During a run, handwheels equipped with Teflon washers seal the door of the chamber and two Thermopane-type windows permit visual observation into the chamber. A manual break valve, equipped with a stainless steel filter holder on the chamber door, allows for connection of an inert gas service in order to pressurize the chamber upon termination of the drying cycle.

The interior of the drying chamber is equipped with eight thermocouple probes for monitoring the temperature of selected samples throughout the chamber. A plug-in jack-panel arrangement permits easy connection of thermocouples which can be designed for specific purposes. Four 0.61 × 0.91-m. (2 × 3-ft.) product shelves provide a total product shelf area of 2.22 m.^{2}. The shelves are coated with a black phenolic resin for high emissivity, and stainless steel bumper strips along the front edge of each shelf preclude chipping of the coating during loading and unloading. For production purposes, stainless steel shelves are highly recommended.

External Condenser—The condenser is of a special design and, for maximum flow, is closely coupled to the drying chamber by a 25.4-cm. (10-in.) vacuum line. A 25.4-cm. (10-in.), remotely actuated, high vacuum valve isolates the drying chamber from the external condenser. The large condenser surface area in the form of plate coils, widely spaced, and the special internal baffling of the condenser contribute to a high rate of vapor removal and a good distribution of ice on the condensing surface. The unit was designed to handle at least 30 l. of moisture in the form of ice before defrosting is required.

Instrumentation and Controls—Figure 1 shows the instrumentation and control panel for the unit; a similar arrangement, either entirely or in part, can be adapted to almost any lyophilizer. This unit is equipped with a dual-control panel; one panel is located in the sterile drying room adjacent to the recording instrumentation and the door of the dryer, and the other is in the nonsterile machinery area. Key-type switches with local or remote positions permit operation of the equipment either from the sterile area or from the machinery area. To be operative from the machinery area, the key switches in the sterile room must be set to remote position.

The instrumentation includes a 24-point strip chart temperature recorder, calibrated for −85 to +85°, to record and monitor temperatures throughout the system such as product temperatures within the chamber, shelf temperature, recirculated trichloroethylene temperature, and condenser temperature. A strip chart vacuum recorder is provided, having a range of 1–1000 μ for recording the pressure within the drying chamber. This instrument is equipped with alarm switches set at 200 and 300 μ. Should the pressure within the drying chamber rise to 200 μ, a pilot light goes on and a buzzer alarm sounds. If the pressure within the chamber rises above 300

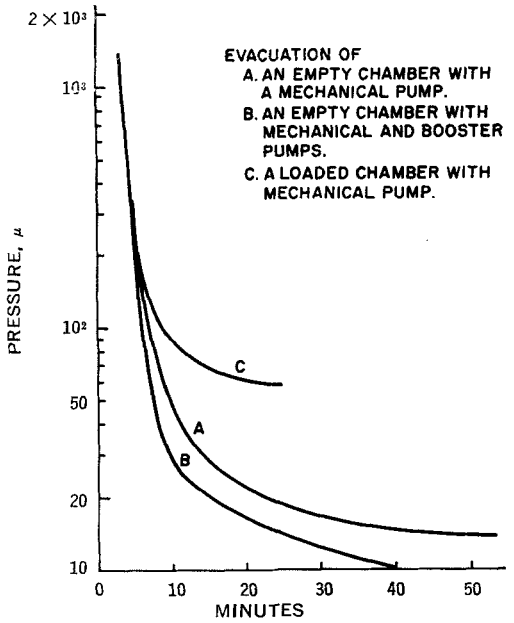

Figure 4—*Capability of the vacuum system.*

μ, a second pilot light goes on, an alarm bell sounds, the valve between the external condenser and the vacuum chamber closes, heat to the trichloroethylene is cut off (if the unit is on the heat cycle), the vacuum pump stops, and the shelf refrigeration starts. The pressure at which the alarms are set depends to some extent on the size of the chamber. Slightly higher settings can be used in larger production units of similar capability.

The panel also has a dial readout pyrometer to indicate the temperature of the defrost water leaving the external condenser; a combination dial-type vacuum pressure gauge to indicate whether the chamber is under vacuum or being pressurized during sterilization; a dial-type air pressure gauge for the pneumatically operated vacuum valves; and, lastly, a 5-station indicating-type thermocouple gauge. This gauge will permit reading the vacuum within the drying chamber, in the external condenser, at the inlet to the oil booster pump, and at the inlet to each mechanical high vacuum pump.

A contact recording program controller programs the drying cycle by controlling the temperature of the trichloroethylene recirculated through the drying chamber. The instrument, calibrated from −85 to +85°, moves a set point index through a time *versus* conditions pattern. The controller consists of a motor-driven plastic cam cut to the desired process pattern; a cam follower connected by cable to a standard control index, in this case, the temperature of the trichloroethylene entering the chamber; and a circular chart

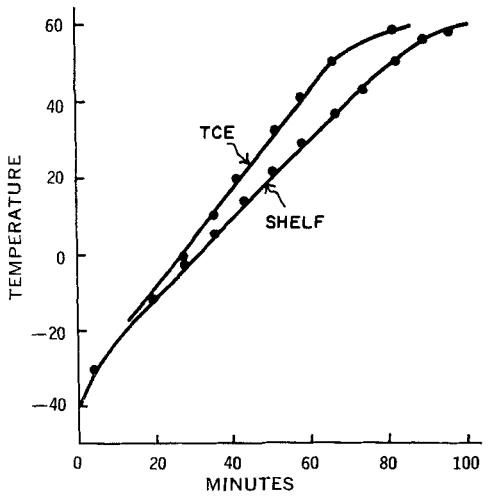

Figure 5—*Heating curves for the circulating trichloroethylene fluid and the surface of a shelf. The temperature controller was set at 60°.*

Table I—Rates of Cooling and Heating of Circulating Fluid and Shelves and Rate of Cooling of the Condenser Coils

	Cooling Rate, min.$^{-1}$	Heating Rate, deg./min.
Circulating fluid	0.0177[a]	1.35
Shelf 1	0.0165[a]	1.09
Shelf 2	0.0171[a]	1.12
Shelf 3	0.0169[a]	1.07
Shelf 4	0.0162[a]	1.05
Condenser coils	0.121[b]	—
Circulating fluid	0.00824[c]	1.35

[a] Both compressor units of trichloroethylene to shelf refrigeration. [b] One compressor on condenser. [c] One compressor on shelf refrigeration.

recorder which records the temperature of the trichloroethylene entering the chamber shelves.

Key-type stepping switches are used to control the chamber sterilization cycle and the external condenser defrost cycle. These switches, of course, preclude the accidental introduction of steam or water to either the drying chamber or the condenser during a drying run and make the sterilization and defrosting processes essentially foolproof. For sterilization, the steam enters *via* a remote-operated steam inlet valve on the chamber, and sterilizing conditions can be allowed to exist as long as desirable. A condensate remote-operated valve opens and permits the steam trap to discharge condensate from the chamber to the drain to maintain a level of 15 psig. in the chamber.

Defrosting of the condenser is accomplished by admitting 3 psig. steam *via* a remote-operated inlet valve on the condenser. The steam, of course, begins to melt the ice on the plates, and a valve to a steam condensate drain opens, permitting discharge of condensate. The defrosting may be observed through two sight glasses located at the rear of the condenser. When the ice is essentially melted, and before the discharge drain temperature rises too high, the steam inlet is closed and the water inlet valve is opened by the key stepping switch. Water fills the condenser and overflows *via* a drain at the top. When all the ice is melted and the condenser plate temperature is reduced, the key switch knob is turned to another position which closes the water inlet valve and opens a main drain valve, permitting the condenser to drain.

Refrigeration, Heating, and Vacuum Systems—The shelves in the drying chamber are chilled and heated by circulating trichloroethylene. The unit is equipped with two integral two-stage Freon 22, 10-hp. compressors. One of these maintains the temperature of the circulating trichloroethylene at any level from −40 to 70° for any length of time during the drying cycle without removing refrigeration from the external condenser. The second compressor normally serves the external condenser during the drying cycle. However, a provision exists for diverting the refrigeration from the condenser to the trichloroethylene heat exchanger to assist in the initial chilldown of the shelves. Therefore, the trichloroethylene can be initially chilled by means of the shelf compressor or by both shelf compressor and external condenser compressor, where each compressor is expanding Freon to the trichloroethylene heat exchanger, thus permitting rapid cooling of the trichloroethylene and freezing of the product. The initial chilldown of trichloroethylene and freezing of the product might be started while the external condenser is defrosting. To protect the refrigeration and heating systems from damage, an electrical interlock is provided to prevent heating or chilling of the trichloroethylene unless the pump that recirculates the trichloroethylene is operating.

The cooling capability of the refrigeration system was determined by measuring the cooling rates of the condenser coils and of the circulating fluid using either a single compressor or two compressors on the trichloroethylene. In Fig. 2, the cooling rates for the circulating fluid, condenser coils, and a shelf are illustrated. The log $(T - T_\infty)$ was plotted against time to account for the capacity of the compressor, which was rated at −50°, T_∞ being equal to −50°. Such a plot produces a straight line down to −32°, about 18° above the rated capacity of the compressor. Starting from ambient conditions, it requires over 120 min. for the trichloroethylene to reach −40° using one compressor and approximately 80 min. when both are employed.

Since the condenser coils are cooled by direct expansion of Freon, the cooling, as demonstrated by Curve C, was quite rapid.

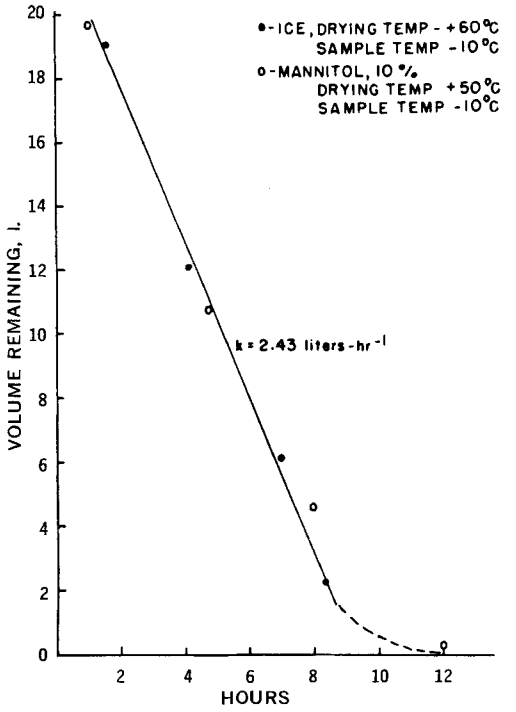

Figure 6—*A plot of the quantity of water remaining as a function of time. The rate of sublimation is represented as* k.

Within 6 min., the temperature of the condenser fell to −35°; within 15 min., it reached −50 to −55°. The temperature was lowered further with the application of vacuum.

The broken segment of Curve B resulted when the condenser was switched from shelf to condenser refrigeration. Using this procedure, the condenser coils can be cooled to below −50° within 15 min., as shown by D. Curve C represents the cooling of one of the shelves, and the heat transfer through the shelves is demonstrated by the difference between B and C.

Figure 3 shows the graphic representation of the vacuum system on the control panel. The vacuum system consists of a two-stage compound-type air-ballasted high vacuum pump, a single-stage high vacuum pump as a spare or standby, and an oil booster pump with associated piping and remote actuated valves. Thus, it is possible to evacuate the chamber, the condenser, or both together by either the single-stage or two-stage mechanical pump or by making use of the oil booster pump in conjunction with a mechanical pump to obtain a low chamber pressure. Low pressures during the drying cycles are often essential in the lyophilization of biologicals.

The capability of the vacuum system is illustrated in Fig. 4 where curves are plotted for the evacuation of an empty chamber using only the mechanical pump (Curve A) or using both the mechanical pump and the booster pump (Curve B), and for the evacuation of a chamber with a full load using only the mechanical pump (Curve C). By pulling on the condenser directly, the mechanical pump has the same efficiency in lowering the pressure to approximately 400 μ of Hg as it does when it is pulling through the booster pump. Below 400 μ, the rate of evacuation was greater when both the

Table II—Defrosting of Condenser Coils

Minutes	Condenser Temperature	Remarks
0	−60°	Steam injected into the condenser
8	3°	
26	42°	
32	Above 80°	Observation of condenser showed coils completely free of ice Water injected into condenser
38	20°	Water drain opened
44	21°	Water drain closed; compressor turned on to refrigerate coils
50	−35°	
62	−54°	

Table III—Sterilization of Chamber

Minutes	Chamber Temperature	Chamber Pressure, psig.	Remarks
0	40°		Steam injected
10	105°	3	
16	115°	10	
20	117°	12	
35	121°	15	
50	121°	15	Steam discontinued Drain opened
55	90°		Two compressors on shelf refrigeration
60	65°		Chamber opened and loaded

mechanical and booster pumps were employed simultaneously. In this manner, pressures as low as 5–10 μ can be attained; with the mechanical pump alone, pressures of approximately 13–15 μ are attainable. As illustrated by Curve C, with a charge of about 20 l. of frozen water at −30° in the chamber, the mechanical pump was capable of lowering the pressure to below 100 μ in less than 10 min.

During drying, the recirculating trichloroethylene is maintained at the desired temperature throughout the cycle in the range from −40 to 70°, as programmed by the cam controller. This controller will call for either refrigeration from the trichloroethylene compressor or the addition of heat from an electric immersion heater.

The capability of the heating system is demonstrated by the curves in Fig. 5. The circulating fluid was heated at the rate of 1.35° per minute over a range of −30 to 50°. Heating of the shelves was at a rate of approximately 1.1°/min.; within 80 min., a shelf temperature of 50° was attained. Although the increase of temperature with time deviates slightly from linearity, linear rate constants were calculated for the heating of the shelves, and these are summarized with the cooling rates in Table I.

Once the capabilities of the various systems are established, it is possible to utilize this information for the development of a cycle. During the freezing stage, if a rapid chilldown is desired, both compressors can be employed for cooling the circulating trichloroethylene fluid to the shelves. Once the desired temperature is reached, the condenser compressor can be switched to the coils in the freeze-trap. As illustrated by the broken line segment of Curve B in Fig. 2, a single compressor on the trichloroethylene is capable of maintaining this temperature or, if desired, it can be used to effect additional cooling. In the meantime, the condenser coils can be cooled to below −50° in approximately 15 min.

For the drying stage, the capabilities of the heating and vacuum system are considered. Generally, it is common practice to evacuate a chamber to a low pressure before applying heat to the shelves. But if it is possible to reduce the pressure in the chamber to below 100 μ in less than 10 min., as illustrated in Fig. 4, it is apparent, from a consideration of the curves in Fig. 5, that heat can be applied simultaneously with the vacuum. After 10 min. of heating, the trichloroethylene temperature is increased by approximately 15°, and there should be no risk of overheating before a sufficiently low

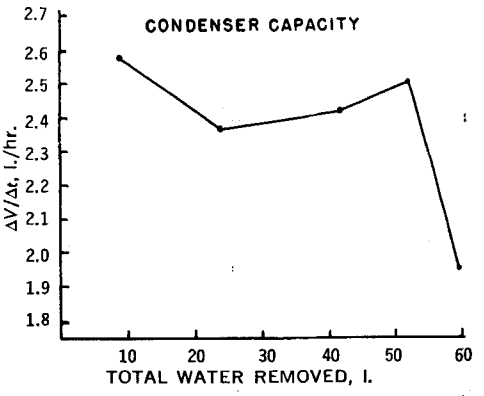

Figure 7—*A plot of the rate of sublimation as a function of total ice sublimed.*

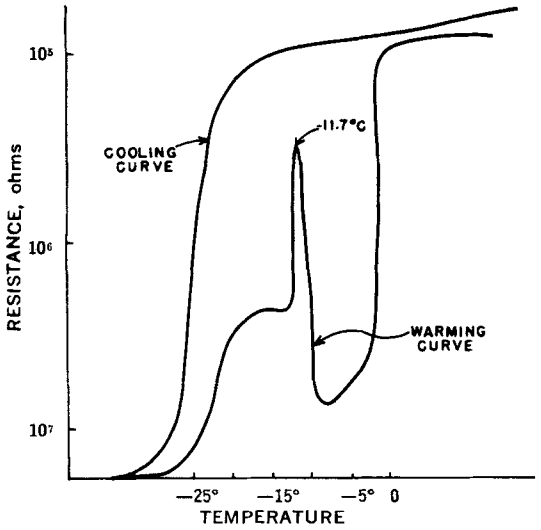

Figure 8—*Cooling and warming curves for a 0.3 molal methylphenidate HCl solution (0–10⁸-ohm decade).*

pressure is attained in the chamber. In fact, if a product necessitates cooling to a low temperature because it exhibits considerable supercooling, it might be advisable to initiate the heating phase 5–10 min. before applying vacuum.

Vaporization Rate and Condenser Capacity—The vaporization rate from pure ice and frozen solutions was determined by measuring the removal of water from crystallizing dishes during a drying cycle. The chamber was loaded with 20 l. of water or 10% mannitol solution by introducing 300-ml. quantities into the dishes and then interrupting the drying cycle at certain times during the period of maximum drying to measure the water remaining. A pressure–time curve, similar to that in Fig. 9, was used to determine the time periods for interruption of the cycle. For each subsequent time period, the water that was vaporized was replaced in the dishes. A plot of the quantity of water remaining as a function of time is illustrated in Fig. 6. The vaporization rate is almost linear during the primary drying stage; the maximum rate that can be expected during the drying cycle, using the indicated conditions, is approximately 2.43 l./hr. The open circles represent points obtained during the drying of a frozen 10% solution of mannitol; the rate of vaporization is similar to that for pure ice. This is due to the fact that for pure ice, although it has a low coefficient of thermal conductivity, the vapor flow is not obstructed by a front of dry material, while for the frozen mannitol solution, which has a higher coefficient of thermal conductivity, the obstruction to vapor flow continually increases as drying proceeds.

The capacity of the condenser is illustrated in Fig. 7, where the amount of water vaporized over specific intervals of time is plotted against the total amount of water sublimed. The capacity of the

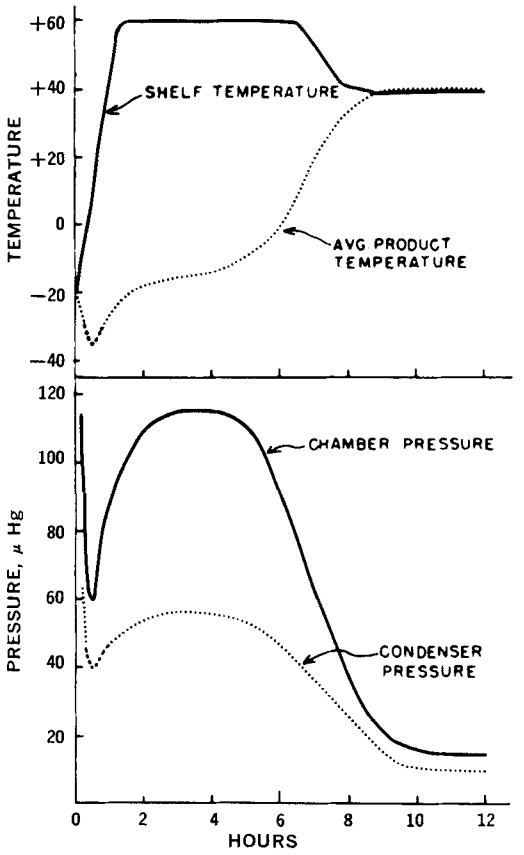

Figure 9—*Temperature–time and pressure–time curves characteristic of the drying cycle for methylphenidate HCl.*

condenser is in excess of 60 l., and an efficient rate of vaporization is maintained up to approximately 52 l.

Defrosting of Condenser Coils—With approximately 60 l. of water frozen on the condenser coils, the experiment to determine the condenser capacity was terminated and the coils were defrosted. Table II lists the details of the defrosting cycle. From Table II, it can be seen that it is possible to defrost 60 kg. of ice from the coils and have the condenser ready for another run within 1 hr.

Sterilization of Chamber—During sterilization of the chamber, spore strips of *Bacillus stearothermophilus* were located on each shelf and below the bottom shelf. Sterilization consisted of injecting steam into the chamber and allowing a gauge pressure of from 12 to 15 p.s.i. to be maintained in the chamber for a 30-min. period. Culturing of the spore strips on trypticase media showed no discernible growth after incubation for 1 week. The sterilization schedule is illustrated in Table III.

Table IV—Details of Lyophilization Cycle for Methylphenidate HCl

Hours	Shelf Temperature	Average Product Temperature	Pressure Differential (Chamber–Condenser), μ	Remarks
0	20°	25°	—	Both compressors on trichloroethylene shelf refrigeration
1	−20°	−17°	—	Condenser compressor switched to freeze trap
1.5	−20°	−20°	—	Vacuum applied; heat applied at a rate of 1°/min.
1.75	−5°	−33°	40	
3	60°	−22°	90	
5	60°	−15°	95	
6	60°	−11°	90	
7	60°	−5°	85	
8.5	60°	11°	25	Heat reduced to 40° at rate 0.25°/min.
10.5	40°	41°	13	
20	40°	40°	8	Run terminated

Table V—Details of Lyophilization Cycle for an Antibiotic Preparation

Hours	Shelf Temperature	Average Product Temperature	Pressure Differential (Chamber–Condenser), μ	Remarks
0	23°	27°	—	Both compressors used to cool samples to −20°
1	−20°	−20°	—	One compressor switched to condenser
1.25	−20°	−20°	—	Vacuum and heat applied; temperature increased at 1°/min. to 40°
2.5	40°	−22°	105	
6	40°	−12°	90	
8	40°	−8°	50	
12.25	40°	20°	20	Temperature increased at a rate 0.5°/min. to 60°
14.5	58°	42°	8	
16.75	60°	55°	5	Temperature decreased to 40°
21	40°	41°	5	Run terminated

DEVELOPMENT AND PROGRAMMING OF CYCLES

Prior to the development of a lyophilization cycle, of course, is the development of a suitable formulation. The optimum formula will permit the overall cycle to be carried out in the least amount of time, while providing a stable and efficacious product which contains a low moisture content, undergoes rapid reconstitution, and possesses the desired appearance. The potency of many pharmaceutical agents is of such a magnitude that relatively small amounts are required for the lyophilized injectable dosage form. Therefore, the need for a suitable filler or bulking agent is often indicated. Unfortunately, the list of materials to select from for this purpose is rather limited; even more important, selection of an agent is usually on an arbitrary basis.

For this paper, it will suffice to establish the importance of selecting the proper additives and to proceed with describing the development and programming of a cycle for an existing formula. For this purpose, three examples were selected, each representing or containing a relatively highly soluble medicament. Although this selection does not do justice in representing the many types of products being processed as lyophilized dosage forms, it demonstrates the approach for designing a cycle.

The first example is a drug intended for administration at a concentration of 10 mg./ml. Although the solution to be freeze dried contained 34 mg./ml. of active ingredient, a filler was required to add stability to the finished cake. A quantity of mannitol equal to that of the active substance was added. The eutectic temperature of the solution was determined by temperature–resistivity measurements (6), and Fig. 8 shows the conductivity behavior of this product during cooling and warming. In the liquid state, the resistivity of the solution is very low; but when solidification occurs, the resistivity increases sharply. The lowest temperature at which liquid can exist is the eutectic temperature. Since it is difficult to determine eutectic temperature during freezing because of supercooling, the warming curve is used. For methylphenidate HCl, the eutectic temperature was found to be −11.7°. During the freezing stage, the degree of supercooling should be considered when deciding to what temperature the samples are to be lowered to ensure complete solidification. Qualitatively, this information can be obtained from the cooling curve. For methylphenidate HCl, complete solidification occurred at −20°.

With a knowledge of the eutectic temperature and the supercooling characteristics of the product, a preliminary run consisting of one full tray of vials was then conducted. Temperature and pressure curves for the drying cycle are illustrated in Fig. 9. With the circulating fluid temperature set at 60°, all the probed samples passed through 0° within 6.5 hr. The heat was lowered gradually to 40° and allowed to remain at this temperature until the run was terminated. From the temperature and pressure curves, it can be seen that maximum drying took place between 1 and 6 hr. The max-

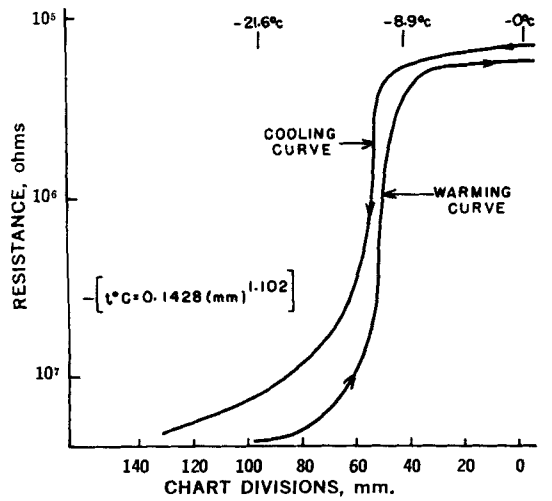

Figure 10—*Conductivity behavior during the freezing of 30% antibiotic solution and thawing of the frozen mass.*

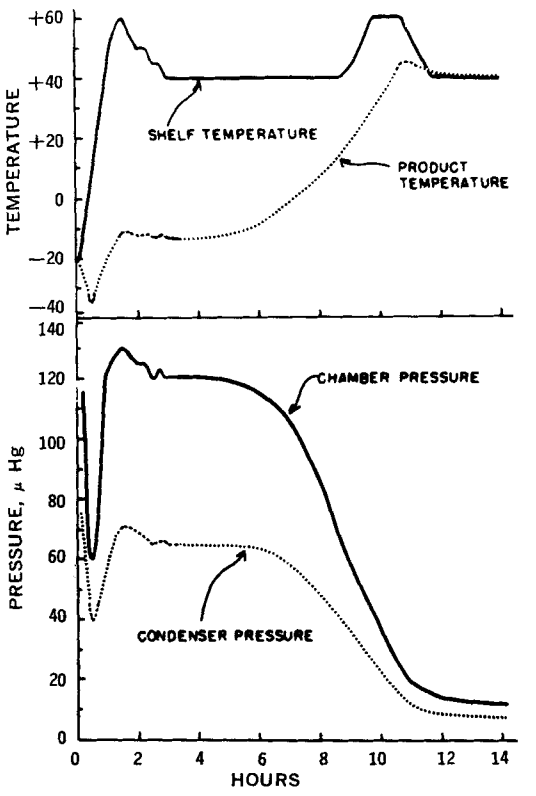

Figure 11—*Temperature–time and pressure–time curves characteristic of the drying cycle for an antibiotic preparation.*

Table VI—Details of Lyophilization Cycle for Pralidoxime Chloride

Hours	Shelf Temperature	Average Product Temperature	Pressure Differential (Chamber–Condenser), μ	Remarks
0	26°	26°	—	Both compressors on shelf refrigeration
1.5	−40°	−11°	—	
2.5	−40°	−35°	—	Compressor switched to condenser refrigeration; cam temperature set at −35°
2.75	−35°	−35°	—	Vacuum and heat applied with temperature set at 14°
6	14°	−22°	30	
20	14°	−20°	30	
40	14°	−20°	30	
52	14°	2°	20	Temperature increased at rate of 2°/hr. up to 24°
58	24°	15°	10	
64	60°	55°	8	Temperature lowered to 40°
66	40°	43°	5	Run terminated

imum vapor pressure difference between chamber and condenser occurred between 2 and 5 hr., with the chamber pressure reaching a minimum value of 15 μ after 10 hr. The leveling off of the product temperature below the eutectic point during the primary drying phase was an indication that the heat applied was not excessive.

With a knowledge of the drying characteristics of the product obtained from the temperature and pressure curves, a full chamber run of about 3500 vials (16 trays holding a total volume of 10.36 l. of solution) was made using a programmed drying cycle; the details are summarized in Table IV. Both compressors were put on shelf refrigeration to freeze the samples quickly. When the temperature of the lowest sample reached −20°, a specially cut plastic cam was attached to the programmer and the condenser compressor was switched to the freeze-trap. Within 30 min., all the samples were equilibrated at −20° and the condenser was at −55°. The plastic cam was designed to allow for a 1° rise per minute from −20 to 60° at the onset of the drying cycle.

Although the product temperature was at 40° after 10 hr., the product was allowed to remain in the chamber for an additional 10 hr. before terminating the run because the production operation is on a single shift basis. Moisture determination on 16 samples (one vial from each tray) gave results varying from 0.15 to 0.65%.

The cycle for the first production-size batch can then be programmed in the production lyophilizer by using the plastic cam designed and tested in research and development on the pilot plant lyophilizer. Slight modifications may be required at times to optimize the cycle further for the production-size batches but this is to be expected when scaling up an operation (8).

The second example is an antibiotic preparation, intended for freeze-drying as a 30% solution. Because of the high solid content, there is no need for a bulking agent. From the temperature–resistivity curves presented in Fig. 10, the eutectic point was found to occur at −8.9° and only negligible supercooling was encountered, with complete solidification at approximately −10°.

The temperature and pressure curves obtained from a preliminary run are illustrated in Fig. 11. The curves demonstrate the necessity of maintaining a temperature of 40° to eliminate the risk of melting the samples. With the drying temperature set initially at 60°, the product temperature increased steadily to −11°. Since it is desirable to hold the drying temperature at least 4–5° below the eutectic point, the trichloroethylene temperature was lowered, stepwise, until the product temperature was leveled at −13°. After the major drying had taken place and the product temperature was above 10°, the shelf temperature was increased to 60°.

On the basis of the temperature and pressure curves from the preliminary run, a drying cycle was designed for a full chamber load of 2400 units, each containing 4.5 ml. of solution. The details of this run are included in Table V. Moisture determination varied from 0.4 to 0.85%.

The third example is pralidoxime chloride, which has a eutectic temperature of −10.8° and exhibits a considerable degree of supercooling. From the temperature–resistivity curves presented in Fig. 12, complete solidification was not effected until a temperature below −30° was reached. Because of the high dosage, 5.0 g./20-ml. vial, a supporting material was not needed.

The preliminary run indicated that the drying temperature must be kept low due to the formation of a resistant film on the surface of the cake during the early stages of drying. The film obstructs

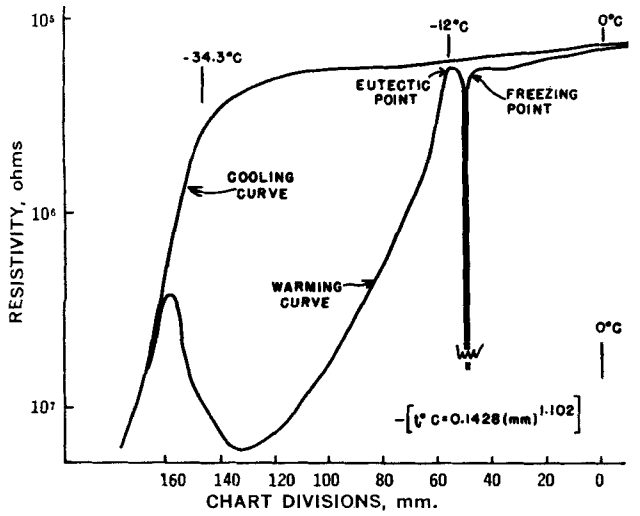

Figure 12—*Cooling and warming curves for a 2.9 M solution (50% w/v) of pralidoxime chloride.*

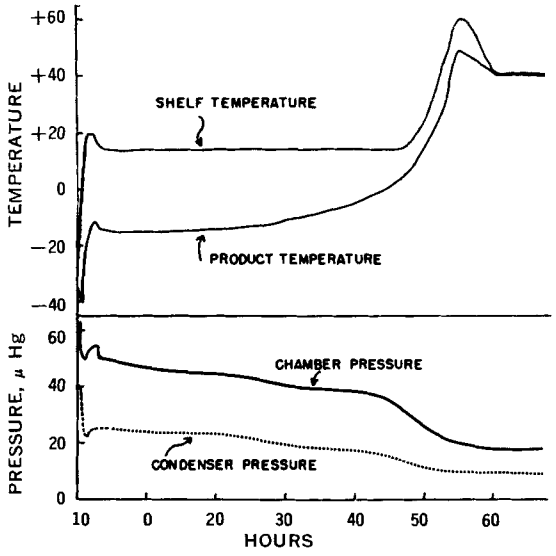

Figure 13—*Temperature–time and pressure–time curves characteristic of the drying cycle for pralidoxime chloride.*

vapor flow; as the drying front descends, this obstruction to vapor flow increases. If too much heat is applied, the sample will liquify. Figure 13 illustrates that, for this product, it was necessary to maintain a low drying temperature throughout the primary drying phase.

With the drying temperature set at 20°, the product temperature rose to within half a degree of the eutectic point. The drying temperature was lowered to 14°, and the product temperature dropped to −15°. The temperature of the product remained essentially constant for over 20 hr. before it began to rise gradually. However, it took over 45 hr. before the product passed through zero. The drying temperature was increased gradually to 60° and subsequently lowered to 40° when the product temperature passed through 40°. From the curves in Fig. 13, it was decided that the initial drying temperature should be no higher than 16°.

A full chamber load consisting of 1500 units, each containing 15 ml. of solution, was frozen to −35°, and the cycle proceeded as summarized in Table VI. With this product, it was necessary to run the cycle beyond 60 hr. to obtain a 100% acceptable yield. Moisture determination on the dried samples ranged from less than 0.1 to 0.25%.

An example for a poorly soluble drug has been omitted because eutectic temperatures for such medicinals are usually slightly below 0°, and the degree of supercooling is generally negligible. In such cases, the design of a cycle is dependent on the properties of the additive or bulking agent employed and not on the properties of the drug. A knowledge of the thermal conductivity of the frozen mass would be another refinement in the design of the freeze-drying cycles.

SUMMARY

A pilot plant lyophilizer was designed to permit versatility for the research and development of lyophilization cycles. The unit contains the necessary instrumentation to control temperatures accurately and to monitor temperature and pressure.

The equipment and instrumentation were described, and a procedure employed for evaluating the various systems comprising the unit was outlined. An explanation and examples were given to illustrate how the information obtained could be utilized to the greatest advantage in conducting preliminary studies and programming cycles.

Through the utilization of temperature–resistivity measurements obtained during preliminary screening and temperature–time and pressure–time curves from the initial freeze-drying runs on the pilot plant unit, a procedure was developed for programming drying cycles for production equipment.

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NOTES

Spectrophotometric Determination of Anthramycin

VLADIMIR STEFANOVICH* and M. Q. CEPRINI†

Abstract □ A quantitative UV spectrophotometric method for the determination of a new antitumor antibiotic, anthramycin, is described. The method is shown to be applicable for the determination of anthramycin in fermentation beers of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143) and in various preparations containing this antibiotic.

Keyphrases □ Anthramycin—spectrophotometric determination □ *Streptomyces refuineus* var. *thermotolerans*—spectrophotometric determination of anthramycin in fermentation beers □ Fermentation beers—spectrophotometric determination of antibiotic, anthramycin

Anthramycin (I*a*), an active antitumor antibiotic, produced by *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143), was recently isolated in the form of its crystalline methyl ether (I*b*) and as anhydroanthramycin (I*c*) (1, 2).

Anthramycin possesses a characteristic UV spectrum essentially identical with that of anthramycin methyl ether when measured in methanol (Fig. 1). Studies showed that the UV spectrum of anthramycin methyl ether conforms to Beer's law at concentrations of 2.0–10.0 mcg./ml. These data were used to establish an assay procedure for the quantitative determination of anthramycin based on the evaluation of UV absorption measurements.

Anthramycin can also be determined by *in vitro* biological assay, *e.g.*, *versus Bacillus* sp. TA (NRRL-B-3167).

EXPERIMENTAL AND RESULTS

Determination of Standard Curve—Anthramycin methyl ether (10.0 mg.) was dissolved in methanol and brought to 100 ml. in a volumetric flask. This stock solution was then used to prepare 10 different concentrations of anthramycin methyl ether in the range of 2–10 mcg./ml. Absorbances were read in the Beckman DU spectrophotometer at 335 nm. Figure 2 shows UV absorption curves of anthramycin and its methyl ether in methanol.

Ethanol and *n*-butanol may also be used in place of methanol.

vapor flow; as the drying front descends, this obstruction to vapor flow increases. If too much heat is applied, the sample will liquify. Figure 13 illustrates that, for this product, it was necessary to maintain a low drying temperature throughout the primary drying phase.

With the drying temperature set at 20°, the product temperature rose to within half a degree of the eutectic point. The drying temperature was lowered to 14°, and the product temperature dropped to −15°. The temperature of the product remained essentially constant for over 20 hr. before it began to rise gradually. However, it took over 45 hr. before the product passed through zero. The drying temperature was increased gradually to 60° and subsequently lowered to 40° when the product temperature passed through 40°. From the curves in Fig. 13, it was decided that the initial drying temperature should be no higher than 16°.

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NOTES

Spectrophotometric Determination of Anthramycin

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Abstract □ A quantitative UV spectrophotometric method for the determination of a new antitumor antibiotic, anthramycin, is described. The method is shown to be applicable for the determination of anthramycin in fermentation beers of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143) and in various preparations containing this antibiotic.

Keyphrases □ Anthramycin—spectrophotometric determination □ *Streptomyces refuineus* var. *thermotolerans*—spectrophotometric determination of anthramycin in fermentation beers □ Fermentation beers—spectrophotometric determination of antibiotic, anthramycin

Anthramycin (I*a*), an active antitumor antibiotic, produced by *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143), was recently isolated in the form of its crystalline methyl ether (I*b*) and as anhydroanthramycin (I*c*) (1, 2).

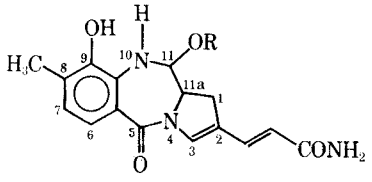
Anthramycin possesses a characteristic UV spectrum essentially identical with that of anthramycin methyl ether when measured in methanol (Fig. 1). Studies showed that the UV spectrum of anthramycin methyl ether conforms to Beer's law at concentrations of 2.0–10.0 mcg./ml. These data were used to establish an assay procedure for the quantitative determination of anthramycin based on the evaluation of UV absorption measurements.

Anthramycin can also be determined by *in vitro* biological assay, *e.g.*, *versus Bacillus* sp. TA (NRRL-B-3167).

EXPERIMENTAL AND RESULTS

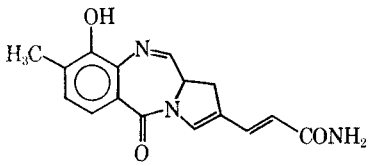
Determination of Standard Curve—Anthramycin methyl ether (10.0 mg.) was dissolved in methanol and brought to 100 ml. in a volumetric flask. This stock solution was then used to prepare 10 different concentrations of anthramycin methyl ether in the range of 2–10 mcg./ml. Absorbances were read in the Beckman DU spectrophotometer at 335 nm. Figure 2 shows UV absorption curves of anthramycin and its methyl ether in methanol.

Ethanol and *n*-butanol may also be used in place of methanol.



Ia: R = H, anthramycin

Ib: R = CH_3, anthramycin methyl ether



Ic: anhydroanthramycin

Method Precision and Recovery Experiments—With five standard solutions, prepared on consecutive days, absorbance values expressed as absorption of a 1% solution of anthramycin methyl ether with a cell length of 1 cm. (*a*) were 112.5 ± 0.38 (mean ± *SE*).

To establish the precision of the spectrophotometric method, a known quantity of anthramycin methyl ether was dissolved in water saturated with *n*-butanol (using 0.2 ml. of methanol as a primary solvent) and then extracted twice with equal volumes of *n*-butanol saturated with water. These combined *n*-butanol extracts were then diluted with methanol; the absorbance was read at 300, 335, and 370 nm. in a Beckman DU spectrophotometer. The concentration of anthramycin methyl ether in the combined *n*-butanol extracts were calculated using the absorbance value at 335 nm. and also by the modification of the baseline method (3) [$A = B - (C + D)/2$, where A = calculated absorbance, B = absorbance at 335 nm., C = absorbance at 300 nm., and D = absorbance at 370 nm.]. Both methods gave identical results when pure, crystalline anthramycin methyl ether was utilized. The starting material was 4.0 mg. of crystalline anthramycin methyl ether, and 3.89 ± 0.02 (mean value of six recovery experiments ±*SE*) was recovered (97.2%).

These results were essentially confirmed by repeating the recovery experiment with anthramycin methyl ether (20 mg.) labeled with ^{14}C in the methoxy group (1057 d.p.m./mg.) where, after two extractions with *n*-butanol saturated with water, 95.8% of the radioactivity (20,250 d.p.m.) was found in the *n*-butanol extract. All measurements of radioactivity were made in a Packard Tri-carb scintillation spectrophotometer at 20° with the following phosphor solution: PPO, 4 g./l.; and POPOP, 100 mg./l. toluene.

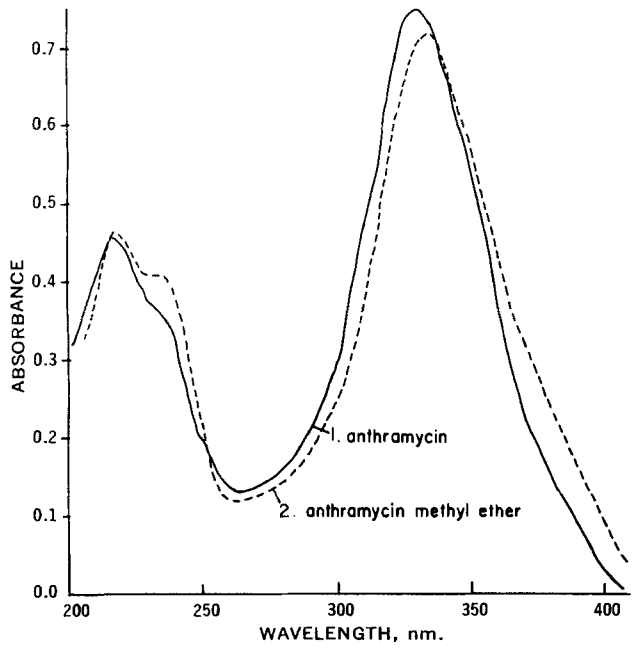


Figure 1—*UV spectra of anthramycin and anthramycin methyl ether at 0 hr. (in methanol). Key: 1, anthramycin, $\lambda_{max.}$ 333 nm., • = 37,000; and 2, anthramycin methyl ether, $\lambda_{max.}$ 334/5 nm., ϵ = 36,800.*

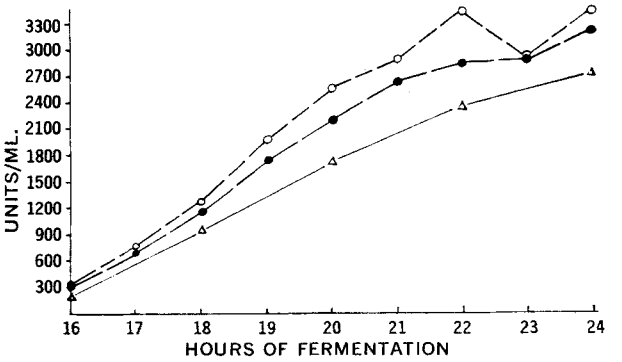


Figure 2—*Determination of anthramycin in fermentation beer. Key: ●, spectrophotometric assay of beer filtrate; ○, microbiological assay of beer filtrate; and △, microbiological assay of whole beer.*

In a separate experiment to determine the distribution coefficient (K) of anthramycin methyl ether in a *n*-butanol–water system containing 1% of methanol, 85.5% of the anthramycin methyl ether was extracted by *n*-butanol saturated with water. Total recovery of the antibiotic was 99.0%, and the distribution coefficient K = 6.3. On the basis of the K-value, two successive extractions with equal volumes of *n*-butanol saturated with water should give a total extraction of anthramycin methyl ether of 97.9%.

Determination of Anthramycin in Fermentation Beers—The determination of anthramycin in fermentation beers of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143) was conducted essentially as described previously.

A fermentation beer of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3134) was sampled every hour during fermentation. The cells from these samples were separated by centrifugation, and the aqueous supernatant was used for the spectrophotometric and microbiological analyses.

For UV spectrophotometric analysis, the following procedure was used. To 4.2 ml. of each supernatant (fermentation beer filtrate with pH adjusted between 6 and 7, if necessary) in a 15-ml. centrifuge tube, 0.8 ml. of *n*-butanol was added to saturate the aqueous phase with *n*-butanol. Each sample was then extracted twice with 5.0 ml. of *n*-butanol saturated with water; the phases were separated after each extraction by centrifugation and the butanol extracts were combined. The butanol extracts were transferred to 100-ml. volumetric flasks and diluted to the mark with methanol. Flasks were heated for 3 min. at 60° (to transform anthramycin into anthramycin methyl ether), and the UV absorbance was read at 300, 335, and 370 nm. in a Beckman DU spectrophotometer.

In all experiments, a corresponding blank (*n*-butanol saturated with water) and an anthramycin methyl ether standard control were run simultaneously. The amount of the anthramycin expressed as anthramycin methyl ether in micrograms per milliliter of fermentation beer filtrate was calculated as follows: anthramycin methyl ether (mcg./ml. of beer filtrate) = $(100 \times a)/(b \times c \times d)$, where a represents the baseline value of the sample, b = baseline value for anthramycin methyl ether at a concentration of 1 mcg./ml. = 0.067, c = recovery of anthramycin methyl ether = 0.97, and d = volume of fermentation beer filtrate in milliliters; in this case = 4.2.

To determine the validity of the technique applied to the fermentation beer filtrate, a known quantity of anthramycin methyl ether standard (0.5 mg. in 50 ml. of methanol) was added to the fermentation beer filtrate. Determination of anthramycin was then accomplished by the previously described procedure; it was found from the six determinations that the average recovery was 97.7% with extremes ±1.8%.

The method described was applied to several fermentation runs of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143), and results of the determination of anthramycin in two particular runs (one with a high concentration of antibiotic and the other with a much lower concentration) are presented in Figs. 2 and 3, respectively. In the same figures, along with the spectrophotometric determinations, are presented results of the biological assays. A plate-cup

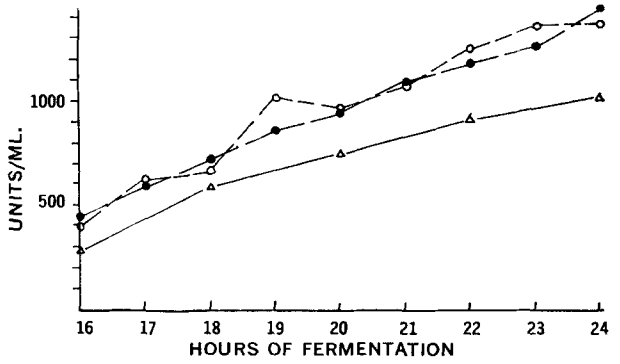


Figure 3—*Determination of anthramycim in fermentation beer. Key: ●, spectrophotometric assay of beer filtrate; ○, microbiological assay of beer filtrate; and △, microbiological assay of whole beer.*

assay against *Bacillus* sp. TA (NRRL B-3167) was simultaneously run with the spectrophotometric assay on aliquots of the fermentation beers and fermentation beer filtrates. One milligram of pure crystalline antibiotic anthramycin methyl ether was arbitrarily assigned 16,000 units of activity *versus Bacillus* sp. TA (NRRL B-3167).

It is evident from Figs. 2 and 3 that both determinations, spectrophotometric and microbiological, agree very well and that the quantity of anthramycin in fermentation beer filtrates can be directly, accurately, and rapidly determined by the presented UV spectrophotometric method.

Some discrepancy between the results of the spectrophotometric and microbiological determinations at different hours of fermentation can be explained by the fluctuation in microbiological assay rather than by inaccuracies of the spectrophotometric method.

The identical procedure can be applied for the determination of anthramycin methyl ether as a bulk drug and in pharmaceutical preparations. However, in these cases, the samples should first be suspended in a very small amount of methanol to which is added a volume of *n*-butanol (saturated with water) adequate to bring the concentration of methanol to 1%, followed by the same volume of water (saturated with *n*-butanol). After shaking, both phases should be separated, extraction with *n*-butanol (saturated with water) repeated, the butanol extracts combined, and then treated as described under *Determination of Anthramycin in Fermentation Beers.*

DISCUSSION

The quantitative determination of the antibiotic anthramycin as its methyl ether (I*b*) in pharmaceutical preparations appears to be a straightforward procedure; however, application of the same method for the determination of this antibiotic in the fermentation beer requires additional comment.

Anthramycin methyl ether is not present in the fermentation beer as such. It was established previously that the methoxy group at Position 11 is derived from methanol during crystallization of the crude antibiotic from this solvent (1). When the crude antibiotic was crystallized from acetone, anhydroanthramycin (I*c*) was obtained. On crystallization of this substance from methanol–water, anthramycin methyl ether was fomed. Anthramycin, which most likely exists as such in fermentation beers, was obtained only synthetically in a pure state by addition of water to anhydroanthramycin. Anthramycin, when dissolved in methanol (Fig. 1), is transformed into anthramycin methyl ether; the rate is slow at room temperature with incomplete reaction even after 24 hr. However, anthramycin is transformed quantitatively into anthramycin methyl ether by crystallization from hot methanol–water.

Differences in wavelength of maximum absorption ($\lambda_{max.}$) and molar absorptivity (ϵ) between anthramycin and anthramycin methyl ether are of such a low order that, regardless of the structure in which the antibiotic exists during quantitative determination, there is no appreciable effect on the accuracy of the method. The validity of the method is further confirmed by concordance of UV spectrophotometric determination with microbiological assay.

The concentration of the antibiotic is lower in the fermentation beer than in the beer filtrate. This can be explained by the fact that the whole fermentation beer contains cells that are inactive and comprise 11% of the total volume, consequently yielding a lower antibiotic content in the whole beer than in the corresponding beer filtrate.

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Effect of Viscosity on Thiamine and Riboflavin Absorption in Man

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Abstract □ Thiamine and riboflavin are absorbed from the upper small intestine by specialized transport processes. It was thought that oral administration of these vitamins in viscous solution would affect their absorption, since a previous investigation in rats showed that viscous solutions decrease the absorption of drugs from the stomach and retard gastrointestinal transit. Contrary to expectations, oral administration of thiamine and riboflavin in 50 ml. of highly viscous methylcellulose solution (3400 cps. at a shear rate of 5.1 sec.$^{-1}$) did not affect significantly the rate and extent of absorption of the two vitamins in healthy adult volunteers.

Keyphrases □ Thiamine, viscosity—effect on absorption, man □ Riboflavin, viscosity—effect on absorption, man □ Viscosity—effect on riboflavin, thiamine absorption, in man

Previous studies in rats showed that the absorption of drugs and the gastrointestinal transit of a drug solution are decreased when the viscosity of the solution is increased by methylcellulose (1). Seager (2) found that the gastrointestinal absorption of nitrofurantoin in man is decreased significantly when the drug is administered as a *suspension* in 5% methylcellulose (viscosity grade not specified) rather than in water. Apparently, no formal study of the effect of viscosity on the gastrointestinal absorption of drugs in *solution* has been carried out in man. Therefore, the absorption of two water-soluble vitamins, thiamine and riboflavin, was studied upon oral administration in aqueous solution and in highly viscous methylcellulose solution.

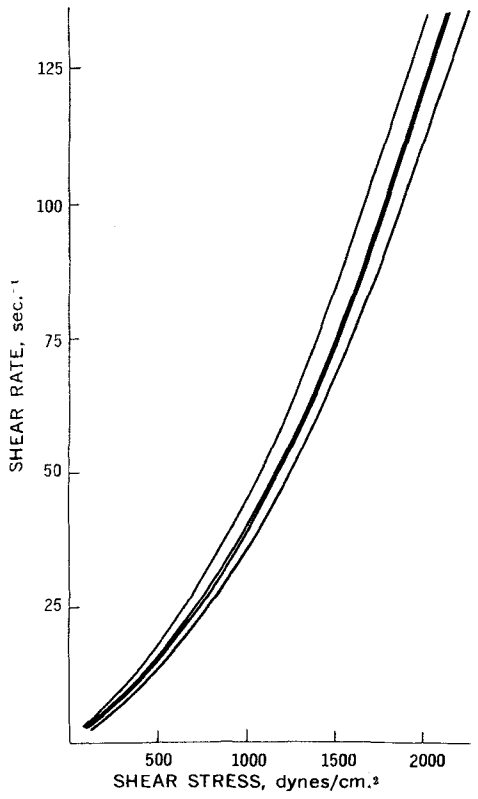

Figure 1—*Rheograms of the high viscosity (methylcellulose) vitamin solution used in the study. Each rheogram, obtained at 37°, represents one of the solutions prepared separately for each subject.*

Table I—Effect of Viscosity on Gastrointestinal Absorption of Thiamine and Riboflavin in Man

Subject	Thiamine		Riboflavin	
	Aqueous	Methylcellulose	Aqueous	Methylcellulose
	Percent of Dose Recovered in Urine			
SF	7.57	5.76	26.9	21.0
WJ	5.02	5.04	26.5	16.5
VS	9.62	6.92	20.8	19.8
WH	3.47	4.31	12.2	12.7
Mean	6.42	5.51	21.6	17.5

Thiamine and riboflavin are absorbed by specialized, saturable transport processes which are located in the proximal region of the small intestine (3–8). Oral dosage forms that remain at specialized absorption sites in the small intestine for longer periods of time than do ordinary aqueous solutions or tablets should increase the absorption of large, otherwise only partly absorbed, doses of the two vitamins. The administration of large doses of thiamine and riboflavin in highly viscous solution should, therefore, result in a decreased initial absorption rate but increased extent of absorption if the highly viscous solutions are emptied from the stomach and moved along the intestinal tract more slowly than ordinary aqueous solutions of these vitamins.

EXPERIMENTAL

Four young men, 24–28 years old, weighing 62–85 kg., served as test subjects. They received 41 mg. riboflavin-5′-phosphate·$2H_2O$ (equivalent of 30 mg. riboflavin) and 26.9 mg. thiamine hydrochloride (molar equivalent of 30 mg. riboflavin) in the morning on an empty stomach. The vitamins were dissolved in 50 ml. water or methylcellulose solution. The solutions were flavored with 1% citric acid and 0.06% sodium saccharin. Two of the subjects took the aqueous solution first and the methylcellulose solution several weeks later. The other two subjects took the solutions in the reverse order. The bottle containing the vitamin solution was rinsed with 30 ml. water which was also taken. The subjects had their normal lunch and dinner, but they were instructed not to take any vitamin preparation and drugs for at least 1 week preceding and during the study.

The subjects emptied their bladders immediately before taking the vitamin solution. Urine was then collected every 30 min. for 4 hr., every hour for the next 4 hr., every 2 hr. until bedtime, and at desired intervals thereafter for a total of 36 hr. Excretion was completed within that period of time. About 3 ml. glacial acetic acid was added to each 100 ml. urine immediately after collection, and the urine sample was placed in a refrigerator until assayed. All samples were protected from light. The subjects drank 50–100 ml. water after each

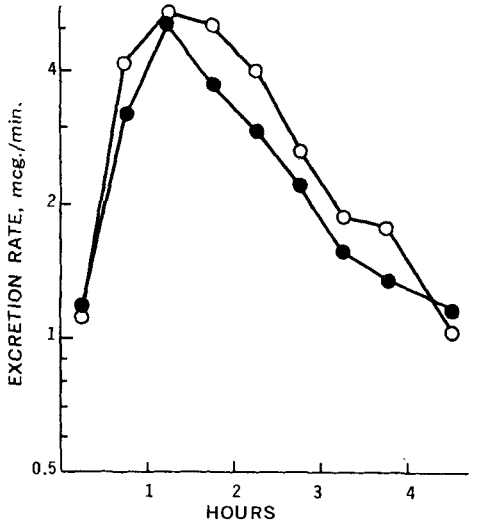

Figure 2—*Urinary excretion rate of thiamine as a function of time after oral administration of thiamine and riboflavin in water* (○) *and methylcellulose solution* (●). (*Average of four subjects.*)

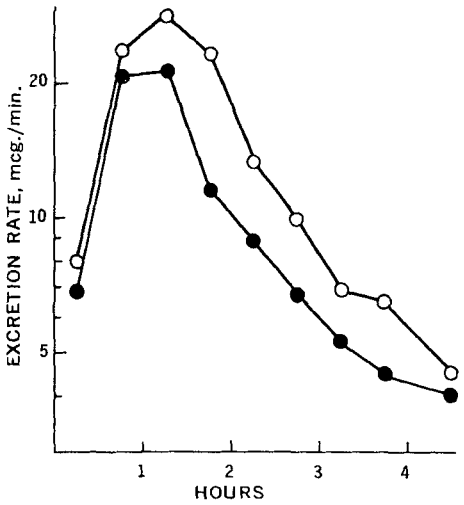

Figure 3—*Urinary excretion rate of riboflavin as a function of time after oral administration of riboflavin and thiamine in water* (○) *and methylcellulose solution* (●). (*Average of four subjects.*)

voiding to maintain an adequate urine output. Twelve-hour "blank" collections of urine were also carried out on each subject.

Riboflavin in the urine was determined fluorometrically by a modification of the USP XVI procedure (6). Thiamine was determined by the thiochrome method according to Mickelsen *et al.* (9), with minor modifications. Permutit was activated according to the procedure of the Association of Vitamin Chemists (10). Fluorescence was measured with the Turner fluorometer, model 111, with primary filter 7-60 and secondary filters 2A and 47B. Neither assay interfered to any measurable extent with the other, as determined by analysis of solutions containing known concentrations of riboflavin and thiamine. There was no evidence of degradation of the vitamins in acidified urine samples stored in the refrigerator for 2 weeks. All assays were performed within that time. Aliquots of the vitamin solutions were assayed together with the urine sample, and percent absorption was calculated relative to the vitamin content of the solutions as determined by the assay.

The methylcellulose solutions were prepared by dispersing methylcellulose[1] in hot water with a Waring Blendor. The vitamins and flavoring agents were dissolved separately in small volumes of water and added to the methylcellulose solution. The final concentration of methylcellulose in the solution was 2.5% w/w. The preparation was stored in a refrigerator. Rheograms were obtained after 3–4 days, and the solution was administered within 3–7 days after preparation. Fresh solutions were prepared for each experiment (*i.e.*, four solutions for the four test subjects).

Rheograms were obtained at 37° with the Epprecht Rheomat 15 viscometer, using measuring system C. The solutions were sheared initially at a rate of 5.14 sec.$^{-1}$ for 3 min. and then left undisturbed for 5 min. Shear stress determinations were made every minute at 15 shear rates, from the lowest to the highest rate and back to the lowest shear rate.

Five milliliters of vitamin solution containing methylcellulose was placed in Visking dialysis tubing and dialyzed at room temperature for 70 hr. against 50 ml. of vitamin solution without methylcellulose. Both solutions were assayed at 17 and 70 hr. for riboflavin and thiamine.

RESULTS AND DISCUSSION

The vitamin solutions with methylcellulose were so viscous that they had to be taken by spoon. They had the consistency of honey; rheograms of the solution are shown in Fig. 1. Since the solutions were pseudoplastic, their viscosity was shear rate dependent and averaged 3410, 2680, and 1560 cps. at shear rates of 5.14, 30.4, and 137 sec.$^{-1}$, respectively. Equilibrium dialysis of the vitamin–methylcellulose solution against vitamin solution without methylcellulose yielded thiamine and riboflavin concentration ratios of unity, showing that the macromolecule did not bind or complex with either vitamin. There was no significant difference in the total amount of thiamine and riboflavin excreted in the urine after oral administration of these vitamins in water and methylcellulose solution, respectively (Table I). There was also no significant difference in the initial excretion rates of the two vitamins (Figs. 2 and 3). The somewhat lower average excretion rate of riboflavin after the 1st hr. following administration of the viscous solution was primarily due to one of the four subjects (Subject WJ).

Similar results were obtained previously in this laboratory in an unpublished study in which 100-ml. solutions containing 10 mg. riboflavin or 10 mg. riboflavin and 1.5% methylcellulose were administered to four subjects in five experiments (11). The viscosity of the methylcellulose solution at low shear rates was about 370 cps., approximately one-tenth that of the more concentrated solution used in the present study. The average urinary recovery of riboflavin from the aqueous solution was 23.9% of the dose; that from the methylcellulose solution was 29.7%. This difference was not statistically significant.

The results of this study are not consistent with previous observations in animals (1) and with the results obtained by Seager in man with nitrofurantoin *suspensions* (2). It is possible that Seager's results were due to slower *in vivo* dissolution of the nitrofurantoin particles coated with methylcellulose rather than to slower gastric emptying of the suspension. Food retards the absorption of thiamine and increases the extent of absorption of riboflavin in man (12). Pathological conditions which decrease gastrointestinal motility increase the absorption of large doses of riboflavin (13).

The lack of effect of a relatively large volume (50 ml.) of highly viscous solution in the present study suggests that gastric emptying and intestinal transit were not affected significantly under the experimental conditions. Clearly, there is no justification for the assumption that a pronounced change in viscosity will invariably affect the gastrointestinal absorption of drugs from a solution.

[1] Methocel 4000, 60 HG, Dow Chemical Co., Midland, Mich.

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Degradation Products of Chloramphenicol

I. K. SHIH

Abstract □ Incubated aqueous solutions of chloramphenicol at various pH's (1–14) yielded detectable amounts of *p*-nitrobenzaldehyde (an oxidation product) and arylamine (a reduction product). Identical degradation products were also found in certain dosage forms (creams and capsules), although they were not found in ophthalmic ointment.

Keyphrases □ Chloramphenicol—degradation products determined □ *p*-Nitrobenzaldehyde—determined as degradation product in chloramphenicol □ Arylamine—determined as degradation product in chloramphenicol

The stability of chloramphenicol is known to be dependent in part upon two degradation pathways: amide hydrolysis at pH 2–7 and carbon–chlorine cleavage about pH 7 (1, 2). Amide hydrolysis leads to the formation of *p*-nitrophenyl-2-amino-1,3-propanediol. This compound was detected in dosage forms by Sahli *et al.* (3), Kassem *et al.* (4), Rousselet and Paris (5), and James and Leach (6). Other degradation products have not been reported in dosage forms of chloramphenicol below pH 7.0.

As suggested by Higuchi and Bias (2), the reactivity of chloramphenicol could lead to other degradation reactions. Initial studies in these laboratories revealed that aged chloramphenicol preparations contained arylamine and *p*-nitrobenzaldehyde. The present study was designed to illustrate the identification of these degradation products and to measure the concentrations of *p*-nitrobenzaldehyde in various chloramphenicol preparations.

EXPERIMENTAL

Materials and Apparatus—Reference standard chloramphenicol was used[1]. All other chemicals and solvents were of reagent grade. Silica gel sheets (chromatogram sheets, Eastman No. 6061) were used for TLC. The chromatographic solvent systems are shown in Table I. Spectrophotometric analyses were performed with the Bausch & Lomb 505 (visible) and the Beckman IR-8 (IR).

Spontaneous Oxidation and Reduction of Chloramphenicol in Aqueous Solution—Fourteen aqueous solutions (0.2%) of chloramphenicol were prepared in low actinic volumetric flasks ranging from pH 1 to 14, respectively. After standing at room temperature for 24 days or longer, 50 ml. of each sample solution was extracted with approximately 25 ml. ether. Evaporation of the ether extract yielded a residue. Tests were then performed for the presence of oxidation and reduction products by using the same methods as subsequently described for chloramphenicol dosage forms.

Identification of *p*-Nitrobenzaldehyde and Its Occurrence in Dosage Forms—The contents of 10 capsules, equivalent to 2.5 g. of chloramphenicol, were stirred with 400 ml. water for 0.5 hr. The suspension was filtered and the filtrate distilled. Approximately 300 ml. of distillate was collected and extracted with 50 ml. ether. Evaporation of the ether extract yielded a small amount of residue possessing the characteristic odor of *p*-nitrobenzaldehyde. The residue, Product A, was dissolved in 0.5 ml. methanol; 10–30 μl. was spotted on a thin-layer plate along with 5 μl. (containing 2 mcg.) of the reference *p*-nitrobenzaldehyde. After development, the TLC plates were sprayed with 0.5% alcoholic phenylhydrazine solution. A similar procedure was followed for chloramphenicol cream and ophthalmic ointment, except that a 20-g. sample was taken and a small amount of antifoam was introduced in the distillation step.

An alternate procedure was used as follows: the contents of five capsules were extracted with approximately 50 ml. ether and filtered. To the filtrate, a few drops of 0.5% alcoholic phenylhydrazine solution were added and the solution was evaporated. The orange-yellow phenylhydrazine derivative was extracted with 0.5 ml. of solvent mixture (two parts CCl_4 and one part $CHCl_3$). Then 10–30 μl. of this solution (Solution B) was spotted on a thin-layer plate along with 5 μl. (containing 2 mcg.) of a reference sample of *p*-nitrobenzaldehyde phenylhydrazone.

IR Identification of *p*-Nitrobenzaldehyde as Its Phenylhydrazone Derivative—The phenylhydrazone derivative was obtained as described previously. The orange-yellow spots of phenylhydrazone were cut out and extracted with methanol. Evaporation of methanol yielded a small amount of dark-orange residue, which was triturated with a small amount of potassium bromide. The IR spectrum of the sample in KBr pellets was compared to that of an authentic sample of *p*-nitrobenzaldehyde phenylhydrazone.

Estimation of *p*-Nitrobenzaldehyde in Chloramphenicol Dosage Forms—From the contents of 20 capsules, the powder equivalent to 1.0 g. of chloramphenicol, accurately weighed, was transferred to a 10-ml. volumetric flask and made to volume with ethanol. A filtered 5-ml. aliquot was mixed with 2 ml. of 0.5% alcoholic phenylhydrazine solution. After standing for 30 min., the absorbances of both the sample and standard solutions were measured against a reagent blank at 440 nm. A similar procedure was followed for chloramphenicol cream, except that a 5-g. sample was completely dissolved in an appropriate amount of ethanol and the cream base was separated by freezing. This ethanol extract was then treated as for capsules. The results are reported in Table II.

Identification of *p*-Nitrophenyl-2-amino-1,3-propanediol—The contents of four capsules were mixed in 40 ml. methanol and filtered. The filtrate was evaporated and the residue extracted with 2–3 ml. water. Then 30–60 μl. of the sample preparation (Solution C) was spotted along with 5 μl. (containing 10 mcg.) of reference *p*-nitrophenyl-2-amino-1,3-propanediol on a thin-layer plate. After development, the plate was sprayed with 0.2% alcoholic ninhydrin solution and heated in an oven for about 5 min. at 60°. For creams, a 5-g. sample was melted on a water bath, and 20 ml. of 95% ethanol was added. Most of the cream base separated on freezing, and the mixture was filtered through a cotton pad. The filtrate was evaporated to dryness, and the residue was treated as for capsules. A

[1] Obtained from Parke, Davis and Co.

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Degradation Products of Chloramphenicol

I. K. SHIH

Abstract □ Incubated aqueous solutions of chloramphenicol at various pH's (1–14) yielded detectable amounts of *p*-nitrobenzaldehyde (an oxidation product) and arylamine (a reduction product). Identical degradation products were also found in certain dosage forms (creams and capsules), although they were not found in ophthalmic ointment.

Keyphrases □ Chloramphenicol—degradation products determined □ *p*-Nitrobenzaldehyde—determined as degradation product in chloramphenicol □ Arylamine—determined as degradation product in chloramphenicol

The stability of chloramphenicol is known to be dependent in part upon two degradation pathways: amide hydrolysis at pH 2–7 and carbon–chlorine cleavage about pH 7 (1, 2). Amide hydrolysis leads to the formation of *p*-nitrophenyl-2-amino-1,3-propanediol. This compound was detected in dosage forms by Sahli *et al.* (3), Kassem *et al.* (4), Rousselet and Paris (5), and James and Leach (6). Other degradation products have not been reported in dosage forms of chloramphenicol below pH 7.0.

As suggested by Higuchi and Bias (2), the reactivity of chloramphenicol could lead to other degradation reactions. Initial studies in these laboratories revealed that aged chloramphenicol preparations contained arylamine and *p*-nitrobenzaldehyde. The present study was designed to illustrate the identification of these degradation products and to measure the concentrations of *p*-nitrobenzaldehyde in various chloramphenicol preparations.

EXPERIMENTAL

Materials and Apparatus—Reference standard chloramphenicol was used[1]. All other chemicals and solvents were of reagent grade. Silica gel sheets (chromatogram sheets, Eastman No. 6061) were used for TLC. The chromatographic solvent systems are shown in Table I. Spectrophotometric analyses were performed with the Bausch & Lomb 505 (visible) and the Beckman IR-8 (IR).

Spontaneous Oxidation and Reduction of Chloramphenicol in Aqueous Solution—Fourteen aqueous solutions (0.2%) of chloramphenicol were prepared in low actinic volumetric flasks ranging from pH 1 to 14, respectively. After standing at room temperature for 24 days or longer, 50 ml. of each sample solution was extracted with approximately 25 ml. ether. Evaporation of the ether extract yielded a residue. Tests were then performed for the presence of oxidation and reduction products by using the same methods as subsequently described for chloramphenicol dosage forms.

Identification of *p*-Nitrobenzaldehyde and Its Occurrence in Dosage Forms—The contents of 10 capsules, equivalent to 2.5 g. of chloramphenicol, were stirred with 400 ml. water for 0.5 hr. The suspension was filtered and the filtrate distilled. Approximately 300 ml. of distillate was collected and extracted with 50 ml. ether. Evaporation of the ether extract yielded a small amount of residue possessing the characteristic odor of *p*-nitrobenzaldehyde. The residue, Product A, was dissolved in 0.5 ml. methanol; 10–30 μl. was spotted on a thin-layer plate along with 5 μl. (containing 2 mcg.) of the reference *p*-nitrobenzaldehyde. After development, the TLC plates were sprayed with 0.5% alcoholic phenylhydrazine solution. A similar procedure was followed for chloramphenicol cream and ophthalmic ointment, except that a 20-g. sample was taken and a small amount of antifoam was introduced in the distillation step.

An alternate procedure was used as follows: the contents of five capsules were extracted with approximately 50 ml. ether and filtered. To the filtrate, a few drops of 0.5% alcoholic phenylhydrazine solution were added and the solution was evaporated. The orange-yellow phenylhydrazine derivative was extracted with 0.5 ml. of solvent mixture (two parts CCl_4 and one part $CHCl_3$). Then 10–30 μl. of this solution (Solution B) was spotted on a thin-layer plate along with 5 μl. (containing 2 mcg.) of a reference sample of *p*-nitrobenzaldehyde phenylhydrazone.

IR Identification of *p*-Nitrobenzaldehyde as Its Phenylhydrazone Derivative—The phenylhydrazone derivative was obtained as described previously. The orange-yellow spots of phenylhydrazone were cut out and extracted with methanol. Evaporation of methanol yielded a small amount of dark-orange residue, which was triturated with a small amount of potassium bromide. The IR spectrum of the sample in KBr pellets was compared to that of an authentic sample of *p*-nitrobenzaldehyde phenylhydrazone.

Estimation of *p*-Nitrobenzaldehyde in Chloramphenicol Dosage Forms—From the contents of 20 capsules, the powder equivalent to 1.0 g. of chloramphenicol, accurately weighed, was transferred to a 10-ml. volumetric flask and made to volume with ethanol. A filtered 5-ml. aliquot was mixed with 2 ml. of 0.5% alcoholic phenylhydrazine solution. After standing for 30 min., the absorbances of both the sample and standard solutions were measured against a reagent blank at 440 nm. A similar procedure was followed for chloramphenicol cream, except that a 5-g. sample was completely dissolved in an appropriate amount of ethanol and the cream base was separated by freezing. This ethanol extract was then treated as for capsules. The results are reported in Table II.

Identification of *p*-Nitrophenyl-2-amino-1,3-propanediol—The contents of four capsules were mixed in 40 ml. methanol and filtered. The filtrate was evaporated and the residue extracted with 2–3 ml. water. Then 30–60 μl. of the sample preparation (Solution C) was spotted along with 5 μl. (containing 10 mcg.) of reference *p*-nitrophenyl-2-amino-1,3-propanediol on a thin-layer plate. After development, the plate was sprayed with 0.2% alcoholic ninhydrin solution and heated in an oven for about 5 min. at 60°. For creams, a 5-g. sample was melted on a water bath, and 20 ml. of 95% ethanol was added. Most of the cream base separated on freezing, and the mixture was filtered through a cotton pad. The filtrate was evaporated to dryness, and the residue was treated as for capsules. A

[1] Obtained from Parke, Davis and Co.

Table I—R_f Values for Reference Materials and Degradation Products[a]

Compound	$CHCl_3$–CCl_4 (1:1)	$CHCl_3$–CCl_4 (2:1)	$CHCl_3$–CCl_4 (1:2)	CH_3OH–$CHCl_3$ (2:1)	CH_3OH–$CHCl_3$ (1:1)
p-Nitrobenzaldehyde reference	—	—	0.61	—	—
Degradation Product A	—	—	0.61	—	—
p-Nitrobenzaldehyde phenylhydrazone reference	0.56	0.69	0.17	—	—
Degradation Product B as phenylhydrazone derivative	0.56	0.69	0.17	—	—
p-Nitrophenyl-2-amino-1,3-propanediol reference	—	—	—	0.58	—
Degradation Product C	—	—	—	0.58	—
Arylamine reference	—	—	—	—	0.75
Degradation Product D	—	—	—	—	0.75

[a] No degradation products were found in expired ophthalmic ointments.

similar procedure was used for an ophthalmic ointment. The R_f values are reported in Table I.

Identification of Arylamine in Chloramphenicol Dosage Forms—Contents of five capsules were extracted with approximately 20 ml. water. The aqueous extract was shaken with about 40 ml. ether. Evaporation of the ether extract yielded a residue, to which was added 0.2 ml. water (Solution D). Then 40–60 μl. was spotted on a thin-layer plate along with 5 μl. of reference arylamine (containing about 1 mcg.), which was synthesized by zinc dust reduction of chloramphenicol in distilled water. After development, the thin-layer plates were sprayed consecutively with 0.1% sodium nitrite, 0.2% HCl, 0.4% ammonium sulfamate, and 0.2% *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution. A similar procedure was used for an ophthalmic ointment. As for the chloramphenicol cream, 3–5-g. samples were mixed slowly with about 30 ml. water to avoid emulsification. The solution was siphoned through cotton. Then 10–15 ml. of the clear solution was collected and extracted with about 20 ml. ether. Evaporation of ether extract yielded a residue, which was treated in the same manner as with capsules.

DISCUSSION

The experimental results (Table I) show clearly that both *p*-nitrobenzaldehyde and arylamine were present in certain chloramphenicol dosage forms which had been stored under normal conditions. *p*-Nitrophenyl-2-amino-1,3-propanediol was also found in this study, as had been previously reported. Expired water-free preparations such as ophthalmic ointment did not show the presence of any degradation product. This was in agreement with the results of James and Leach (6).

Aqueous solutions of chloramphenicol, ranging from pH 1 to 14, after standing at room temperature over a period of 24 days or longer yielded detectable amounts of *p*-nitrobenzaldehyde (an oxidation product) and arylamine (a reduction product), while freshly prepared solutions failed to show the presence of these products. Experimental findings thus give strong evidence to the effect of water on this new degradation pathway of chloramphenicol, which may well account for the presence of these two specific degradation products as found in certain water- or moisture-containing chloramphenicol dosage forms, *e.g.*, creams and capsules.

Table II—Quantitative Estimation of *p*-Nitrobenzaldehyde in Chloramphenicol Preparations

Sample	Manufacturers	*p*-Nitrobenzaldehyde, %[a]
Capsule	A	0.040
Capsule[b]	B	0.024
Capsule[b]	C	0.016
Cream[c]	C	0.090
Cream[d]	C	0.572

[a] Percentage of *p*-nitrobenzaldehyde was calculated on the basis of label claim of chloramphenicol. [b] Capsules of B and C were expired samples. [c] Label claim for chloramphenicol cream was 1%. [d] Cream was expired sample of 1 year.

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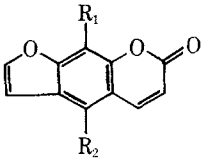
Natural Coumarins XII: Umbelliprenin, a Constituent of *Ammi majus* L. Fruits

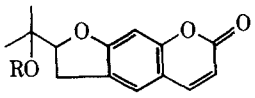
E. A. ABU-MUSTAFA, F. K. A. EL-BAY, and M. B. E. FAYEZ

Abstract □ The hitherto known free coumarins of *Ammi majus* L. fruits (xanthotoxin, imperatorin, bergapten, isopimpinellin, and isoimperatorin) are all psoralene derivatives. Umbelliprenin represents the first nonfuranoid coumarin to be found in this source, and its isolation is reported in this article.

Keyphrases □ Umbelliprenin, a nonfuranoid coumarin—isolation from *Ammi majus* L. fruits □ Coumarins, natural—isolation of umbelliprenin from *Ammi majus* L. fruits □ TLC—separation, identification

The well-established pharmaceutical utility of *Ammi majus* L. fruits, essentially as a source for photodynamically active agents used in the treatment of leucodermia (vitiligo) (1), made it one of the most important economic drug plants of Egypt. Five furocoumarin products—xanthotoxin (I) (2), imperatorin (II) (3), bergapten (III) (3), isopimpinellin (IV) (4), and isoimperatorin (V) (5)—and one dihydrofurocoumarin—marmesin (VI) (6), occurring naturally as the glucoside





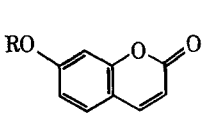
VI: R = H; VII: R = glucose

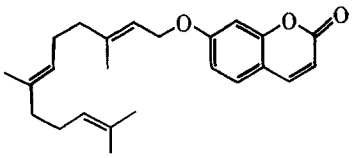
I: R_1 = OCH_3, R_2 = H
II: R_1 = $OCH_2CH{=}C(CH_3)_2$, R_2 = H
III: R_1 = H, R_2 = OCH_3
IV: R_1 = R_2 = OCH_3
V: R_1 = H, R_2 = $OCH_2CH{=}C(CH_3)_2$

marmesinin (VII) (7)—have so far been isolated from this umbelliferous plant which may be regarded as the richest known source for coumarins.

From TLC evidence, the presence of at least one additional product in the free (nonglycosidic) coumarin fraction of *A. majus* L. fruits has been suspected for some time. The isolation, from preparative chromatoplates, of yet another constituent of the drug is now reported. The product (m.p. 60–61°, optically inactive) was obtained in extremely small amounts and was shown to be a nonhydroxylic coumarin. The UV spectrum (showing one principal peak at 325 nm.) precluded the presence of a furanoid moiety (a feature in all the hitherto known coumarins of *A. majus* L.) since no perceptible absorption was observed in the 240–280-nm. region (7, 8).

The nature of the isolated product was revealed by mild treatment with mineral acid, which readily afforded a phenol identified as umbelliferone (VIII) and subsequently methylated to give herniarin (IX). By direct comparison, it was established that the compound was actually umbelliprenin [the *trans,trans*-farnesyl ether (9) of umbelliferone] (X), a rare product which had previ-





VIII: R = H; IX: R = CH_3 X

ously been isolated from *Angelica archangelica* (10). The mass spectrum[1] (Fig. 1) of umbelliprenin showed that the molecular ion (*m/e* 366) suffers an initial loss of 56 mass units, which may be attributed to expulsion of C_4H_8 giving ion formulated as *a* (measured *m/e* 310.1521, calculated for $C_{20}H_{22}O_3$ *m/e* 310.1565) (Scheme I) rather than to expulsion of two carbon monoxide particles (calculated for $C_{22}H_{30}O$ *m/e* 310.2294). This type of expulsion obviously occurs from the sesquiterpenoid side chain and is also observed to take place from other fragment ions. Elision of the entire farnesyl side chain, with transfer of a hydrogen atom favorably accommodated in a six-membered cyclic arrangement, leads to umbelliferone ion (*m/e* 162) and fragment *b* (*m/e* 204), which subsequently loses 43 mass units to give an ion at *m/e* 161. The more intense peaks in the spectrum result by fragmentations in the farnesyl group. These include ions *c*, *d*, and *e* showing at *m/e* 69, 137, and 205, respectively; the last two undergo further degradation by loss of C_4H_8 to give ions at *m/e* 81 and 149, respectively. The genesis of the ions resulting by loss of C_4H_8 may be difficult to envisage, although rearrangement may well be involved; *f* and *g* are not unlikely for ions *m/e* 81 and 149, respectively. The assigned structures are all substantiated by high resolution measurement of the respective peaks.

Umbelliprenin is the first nonfuranoid coumarin to be found in *A. majus* L. Its presence among the major furocoumarins may have biogenetic significance. It indicates that ether isoprenylation at C-7 (presumably with farnesyl pyrophosphate) may occur, though to a limited degree, prior to the more extensive and biogenetically favored carbon alkylation at C-6 which is considered, by the very reasonable postulations of Aneja *et al.* (11), to lead to the α-(hydroxyisopropyl)-dihydro-

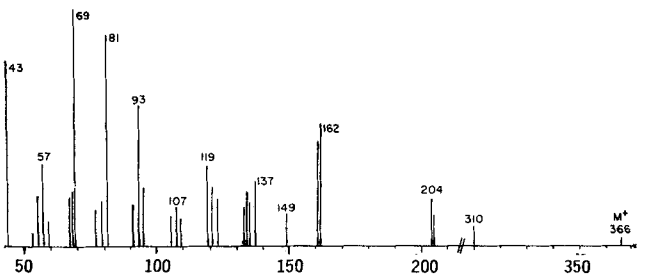
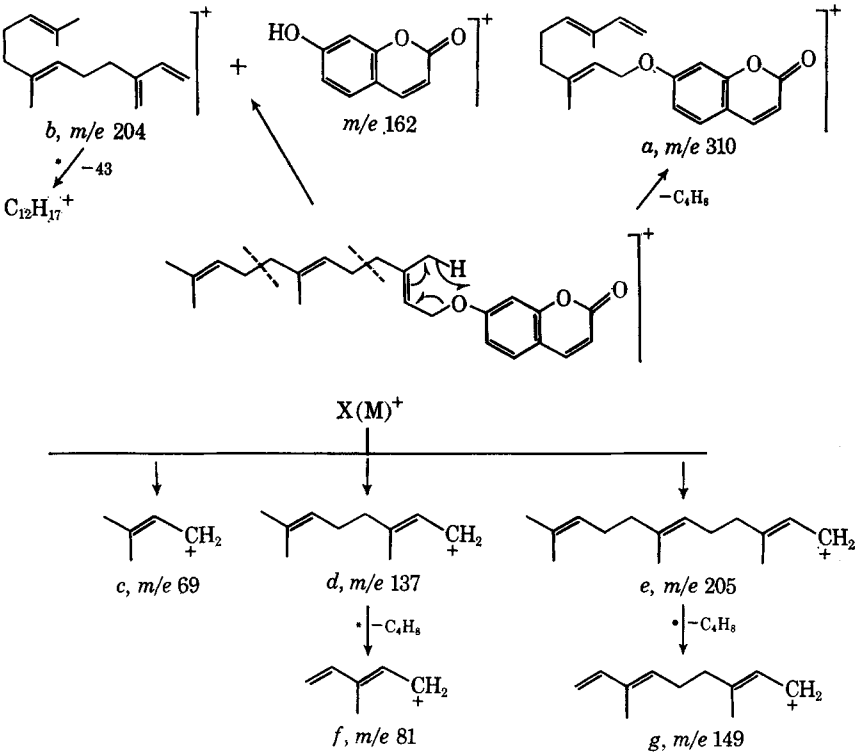


Figure 1—*Mass spectrum of umbelliprenin (X).*

[1] In Scheme I, the transitions supported by the observation of appropriate metastable ions are distinguished by an asterisk.



Scheme I

furan residue (as in marmesin, VI) and ultimately to the unsubstituted furan system (as in the psoralene derivatives, I–V). In fact, it appears particularly attractive to envision all oxygen and carbon isoprenylations as events that occur early in the biogenetic pathway of *A. majus* L. coumarins, quite possibly before the complete elaboration of the α-pyrone system[2].

EXPERIMENTAL

Isolation of Umbelliprenin from *A. majus* L.—The processing of 3.5 kg. of *A. majus* L. fruits for free coumarins, as previously described (5), afforded a crude mixture (18 g.) in which isopimpinellin, xanthotoxin, bergapten, imperatorin, and isoimperatorin were detected (R_f 0.36, 0.39, 0.42, 0.49, and 0.62, respectively) on silica gel G chromatoplates developed with benzene–ethyl acetate (9:1). In addition, a minor product showed up at R_f 0.63, giving brilliant violet fluorescence under UV light and orange, blue, and light-pink colors with the iodine–potassium iodide, 10% sodium hydroxide, and 50% stannous chloride spray reagents, respectively. It was isolated by repeated preparative layer chromatography, and the material collected was crystallized from ethanol–water to give a small amount (8 mg.) of yellowish needles, m.p. 60–61°. The new product was soluble in ethanolic alkali to give a yellowish solution (fluorescent in UV light) from which it was precipitated by acidification. The compound gave negative tests for methoxyl and phenol groups. The UV spectrum exhibited a principal absorption at 325 nm. ($\log \epsilon = 4.15$). Direct comparison (TLC, mixed melting point, and IR spectra) of the isolated material with authentic umbelliprenin revealed its identity.

Boiling the natural product for 2 hr. in 10% ethanolic hydrochloric acid solution, followed by the usual work-up, gave a residue (positive phenol reaction) from which umbelliferone (melting point and mixed m.p. 218–220°) was isolated. The latter was treated with methyl iodide in acetone solution containing sodium carbonate under reflux for 3 hr., and the product was shown to be herniarin. The identities of these two reaction products were established by direct comparison with authentic materials on the chromatoplates, using several solvent systems and spray reagents.

[2] In a recent report, Paikert and Floss (12) proposed that isoprenylation may take place at an early stage in the pathway of furocoumarin biosynthesis in *Pimpinella magna*.

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Effects of Aspirin on Gastric Mucosae of Albino Rat and Mongolian Gerbil (*Meriones unguiculatus*)

CLARENCE H. MORRIS*, JOHN E. CHRISTIAN, and WARREN G. HANSEN

Abstract □ When albino rats were orally administered aspirin in buffer at pH 4.60 at a dose level of 50.0 mg./kg. body weight, all of the animals developed gastric erosions and bleeding within 2 hr. Under the same conditions and at the same dosage, Mongolian gerbils failed to develop these lesions. At higher dose levels of 100 and 200 mg./kg., some gerbils developed mild to moderate gastric lesions with some bleeding.

Keyphrases □ Aspirin—effect on gastric mucosae, albino rat, Mongolian gerbil □ Gastric mucosae—effect of aspirin, albino rat, Mongolian gerbil □ Aspirin—inducing development of gastric erosions and bleeding, albino rat, Mongolian gerbil

It has been known for more than 50 years (1) that the oral administration of aspirin and closely related compounds results in erosions of the gastric mucosa and bleeding from the site of the lesion. These erosions were observed in human subjects (1, 2), dogs (3), cats (4), guinea pigs (5), and albino rats (6). Major biological differences between species with respect to this pathological response to aspirin have not been described.

In recent years, the Mongolian gerbil (*Meriones unguiculatus*) has become increasingly popular as a laboratory animal because of its docility, curiosity, and tractability (7). Therefore, during present studies concerned with mechanisms of aspirin-induced gastric lesions, it became of interest to compare the responses of the gastric mucosae of the Mongolian gerbil and the albino rat to aspirin.

MATERIALS AND METHODS

Twenty six male albino rats[1], weighing about 250 g. (range 200–300 g.), were divided into two groups. The experimental group, consisting of 18 animals, received individual doses of 50 mg. aspirin/kg. body weight; the control group of eight animals received the dose medium only.

Male gerbils[2], weighing about 58 g. (range 50–65 g.), were divided into three experimental groups and a control group. One group of 19 animals received individual doses of 50 mg. aspirin/kg. body weight, the second group of 7 received 100 mg. aspirin/kg. body weight, and the third group of 11 received 200 mg. aspirin/kg. body weight. A control group of 20 gerbils received the dose medium only.

All animals were fasted for at least 15 hr. prior to administration of the aspirin or dose medium. During the fast, they were allowed water *ad libitum*. Water was withdrawn at the time of administration of the drug or dose medium.

The chemical purity of the aspirin was established according to the procedure outlined in the USP (8). Eighty-five milligrams of pure aspirin was dissolved in 5.95 ml. of 0.15 *M* citrate buffer, pH 5.60. The pH of this mixture after solution of the aspirin was 4.60. A similar buffer solution for control animals was adjusted to pH 4.60 with the aid of the glass electrode and careful addition of 1 *N* HCl. Enough aspirin solution was administered to each experimental animal to give a dose of 50, 100, or 200 mg./kg. body weight. Corresponding volumes of buffer solution were administered to control animals. These solutions were administered orally to the animals by means of a No. 16 curved steel oral catheter which was dipped in mineral oil for lubrication.

Exactly 2 hr. after administration of the dose solution, each animal was killed by etherization. The stomach was removed, opened along the line of lesser curvature, stretched, and pinned on a large rubber stopper. The mucosal surface was washed with 0.9% saline to remove any mucous, ingested feces, or other debris. Each stomach was then examined grossly and graded on an eight-point scale developed by Morris *et al.* (9) with respect to the number and size of lesions present. (A rating of 1 corresponds to a normal stomach; a rating of 8 corresponds to the most severe condition, representing more than 10 lesions, one or more of which is larger than 1 mm. in diameter. Ratings 2 through 7 lie between the two extremes in severity.)

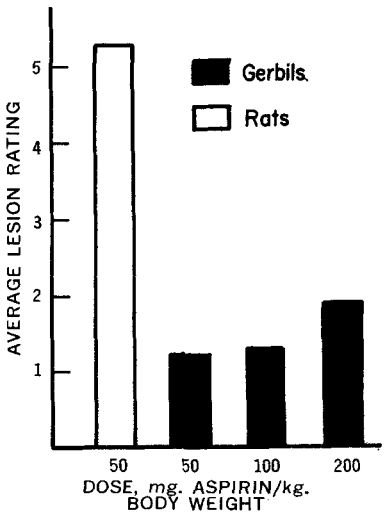

Figure 1—*Average lesion ratings of rats (that received individual doses of 50 mg. aspirin/kg. body weight) compared with average lesion ratings of gerbils (that received individual doses of 50, 100, or 200 mg. aspirin/kg. body weight).*

RESULTS AND DISCUSSION

The results are presented in Fig. 1, which shows the average lesion rating of the rats and gerbils plotted *versus* dose level. All of the rats that received aspirin developed lesions in the secretory epithelium of the gastric mucosa.

By contrast, 16 of the 19 gerbils in the first experimental group, which received 50 mg./kg. body weight, could not be distinguished from their controls. The gastric mucosae of these animals appeared normal in every respect on careful gross examination. In three of the gerbils that received this dose of aspirin, mild erythema was seen in the secretory epithelium, but no lesions of any kind could be found.

Of the second experimental group of gerbils, which received individual doses of 100 mg. aspirin/kg. body weight, one stomach was graded 3 (less than five lesions, all of which are less than 1 mm. in diameter) on the rating scale. All others of this group were entirely normal and indistinguishable from the controls. Of the 11 gerbils in the third experimental group, which received 200 mg. aspirin/kg. body weight, one was graded 4 (less than five

[1] Holtzman Rat Co., Madison, Wis.
[2] Chick Line, Vineland, N. J.

lesions of which one or more are larger than 5 mm. in diameter) and four were graded 3 on the rating scale. The remaining six stomachs were normal.

These observations indicate that the Mongolian gerbil is less susceptible to the induction of gastric lesions by aspirin than the Holtzman albino rat. The biological basis for the observed difference in resistance is not revealed by the present study. It is, however, clear that a dose level of aspirin causing gastric mucosal erosion in all of the male albino rats that received it did not cause such erosion in those male Mongolian gerbils to which it was administered. Higher dose levels do result in lesion formation in the gerbil, with a direct relationship between incidence and dose. However, even at a dose level fourfold higher than that causing lesions in all rats that received it, only 5 of 11 gerbils exhibited lesions, the severity of which was slight to moderate.

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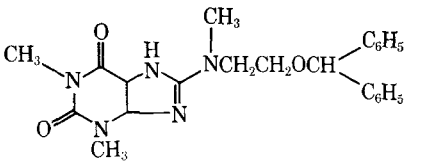
Isolation and Identification of 8-(2-Diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine from Dimenhydrinate

R. S. SANTORO and R. J. WARREN

Abstract □ Differences in solubility and in rate of solubility from previously tested chemical lots were observed in a dimenhydrinate chemical evaluated by the tests and specifications in the official monograph. An impurity was isolated from the lot in question and was identified as 8-(2-diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine based on spectral and elemental data.

Keyphrases □ 8-(2-Diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine—isolation, identification from dimenhydrinate □ Dimenhydrinate—isolation, identification of 8-(2-diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine □ Impurity of dimenhydrinate—isolation, identification of 8-(2-diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine

In a routine analysis of dimenhydrinate chemical according to the tests and specifications in the USP XVII monograph, it was observed that one lot did not completely dissolve in alcohol. Also, its solubility in water, which is the first step in the diphenhydramine assay, was less than complete. In both instances, a hazy solution resulted. However, except for these slight discrepancies, the chemical met the requirements detailed in the monograph. Previous lots dissolved rapidly in the solvents mentioned, while this lot had a poor rate of solubility.



I

Differential thermal analysis revealed one major endotherm at 105° which was identical to previous lots tested. The X-ray diffraction pattern was also the same as previous lots, ruling out polymorphism as a possible explanation for the solubility difference. The thin-layer chromatogram had spots corresponding to the diphenhydramine and 8-chlorotheophylline moieties and a

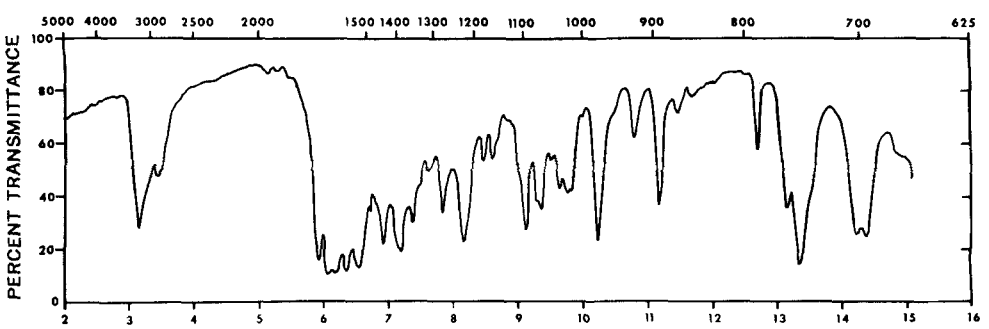


Figure 1—*IR spectrum o unknown.*

Stability of Morphine in Aqueous Solution IV: Isolation of Morphine and Sodium Bisulfite Interaction Product

S. Y. YEH* and JOHN L. LACH

Abstract □ The interaction product of morphine and sodium bisulfite was isolated and characterized as 8-sulfonic acid-dihydromorphinone based on UV and IR spectra and elemental analysis.

Keyphrases □ Morphine–sodium bisulfite interaction product—isolation of 8-sulfonic acid-dihydromorphinone □ 8-Sulfonic acid-dihydromorphinone—identification, isolation as morphine–sodium bisulfite interaction product □ IR—structure, identification □ UV—structure, identification

Previous studies on the stability of morphine in aqueous solution showed that morphine undergoes interaction with sodium bisulfite (1). This communication describes the nature and isolation of the interaction product.

EXPERIMENTAL

Materials—The following were used: morphine base USP, m.p. 255°; morphine sulfate USP; dihydromorphine HCl; and sodium bisulfite A.R.

Solubility Analysis—An excess quantity of morphine base was placed in one set of 125-ml. glass-stoppered bottles containing 25 ml. of distilled water with varying amounts of sodium bisulfite. The bottles were placed in a mechanical shaker in a constant-temperature bath at 30° and equilibrated for 20 hr. Aliquots were removed and assayed spectrophotometrically at wavelength 286 nm. for their morphine content.

In the analysis for the stoichiometric ratio of morphine and sodium bisulfite in the interaction product, 0.4 g. of morphine base was placed in ground-glass-stoppered bottles containing 25 ml. of distilled water and 0.15 g. of sodium bisulfite. The bottles were filled with N_2 and equilibrated at 30° for 4 hr. Aliquot samples were removed and assayed for morphine content spectrophotometrically at wavelength 286 nm. and for free bisulfite content by titration with iodine in acid solution.

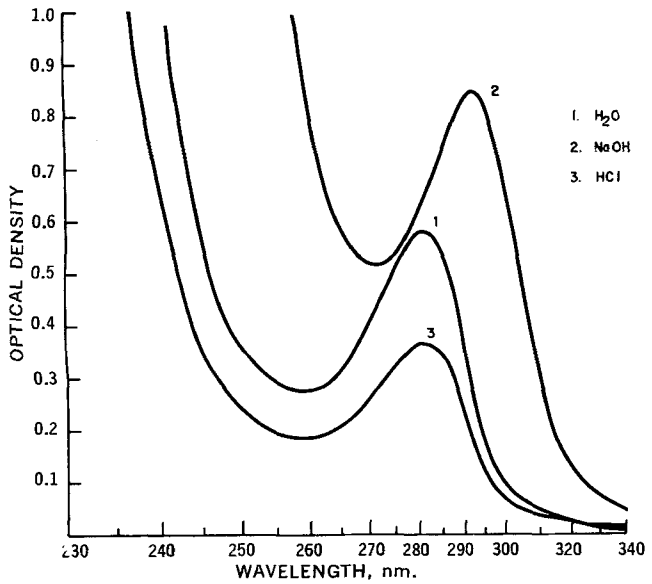


Figure 1—*UV spectrum of 8-sulfonic acid-dihydromorphinone.*

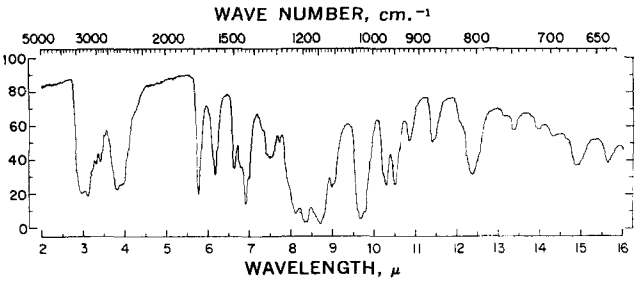


Figure 2—*IR spectrum of 8-sulfonic acid-dihydromorphinone.*

Isolation of Morphine–Sodium Bisulfite Interaction Product—Morphine sulfate (2.1 g.) with tracer amounts of ^{14}C-morphine (as indicator for chromatographic separation) and 7 g. of sodium bisulfite were dissolved in 700 ml. of distilled water, sealed in 20-ml. samples, and heated at 105° for 4 days, the time for the reaction to reach maximum (1). Two procedures were used to isolate the morphine–sodium bisulfite interaction product.

Procedure A—The solution (250 ml.) was chromatographed on an activated charcoal (50–200 mesh)[1] column (1 × 10 cm.). The column was washed with 75 ml. of water and eluted with 50% of acetic acid in water. The effluent was collected in 10-ml. fractions for 250 ml. Morphine and morphine–sodium bisulfite interaction product were completely adsorbed on the column and eluted from the column as indicated by counting 0.5-ml. aliquots of the effluent with 10 ml. of Bray's solution (2). The effluent was concentrated under reduced pressure to near dryness; the residue was suspended in 10 ml. of water, filtered, washed with methanol, recrystallized from boiling water, and dried in vacuum to yield 70 mg. (12% of morphine added) of white prisms, m.p. > 300°.

Anal.—Calcd. for $C_{17}H_{19}NO_6S$: C, 55.72; H, 5.24; N, 3.82; S, 8.73. Found: C, 55.28, 55.71; H, 5.10, 5.17; N, 3.66, 3.82; S, 8.72.

The UV spectrum of this compound in water and HCl medium showed a minimum at 258 nm. and a maximum at 280 nm.; in NaOH solution, the minimum was at 270 nm. and maximum at 292 nm. (Fig. 1). The IR spectrum showed strong absorption bands at 2600 cm.$^{-1}$ (R_3N^+), 1725 cm.$^{-1}$ (C=O), 1000–1200 cm.$^{-1}$ (RSO_3H), and 800 cm.$^{-1}$ (C—S—) (Fig. 2).

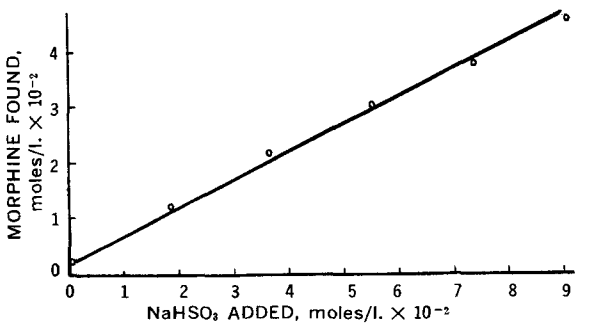


Figure 3—*Effects of sodium bisulfite on the solubility of morphine alkaloid in aqueous solution.*

[1] Fisher Scientific Co.

Table I—Stoichiometric Determination of Morphine Sodium Bisulfite Interaction in Water at 30°

Bottle Number	Free $NaHSO_3$ Found, moles/l. × 10^{-2}	Interacted $NaHSO_3$[a], moles/l. × 10^{-2}	Morphine Found[b], moles/l. × 10^{-2}	Ratio, Morphine / $NaHSO_3$
1	4.5387 (average)	—	—	—
2	3.345	1.194	1.265	1.06
	3.383	1.155		1.09
3	3.154	1.385	1.425	1.03
	3.365	1.174		1.21
4	3.358	1.481	1.458	1.00
	3.153	1.386		1.07

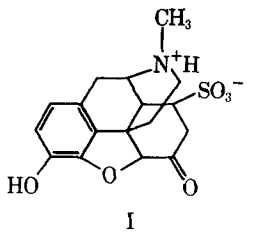
[a] The sodium bisulfite undergoing interaction was determined by subtracting the blank containing only sodium bisulfite from the bisulfite present in the other bottles. [b] The morphine concentrations were corrected for the free morphine in aqueous solution (0.1201 × 10^{-2} mole/l.).

Procedure B—Isolation with Amberlite XAD-2 Resins—The morphine–sodium bisulfite solution (120 ml.) was chromatographed on Amberlite XAD-2 resins[2] in a column 2.2 × 41 cm. (0.87 × 16 in.) previously washed with acetone, methanol, and water. The column was washed with 100 ml. of water and eluted with 160 ml. of methanol. The eluate collected from 60–160 ml. was concentrated to near dryness under a stream of N_2. The crystals were washed with methanol and dried (70 mg., 24% of morphine added). The crystals thus obtained from several batches were recrystallized from boiling water and dried in vacuum at 60°. The results of elemental analysis and the IR and UV spectra of these crystals were identical to those isolated from the charcoal column.

RESULTS AND DISCUSSION

The results obtained from solubility studies indicate that the amount of morphine dissolved in the solution increases with increased sodium bisulfite concentration (Fig. 3). The ratio of morphine to sodium bisulfite in the solution is 1 : 2. One part of the sodium bisulfite, presumably salt formation with morphine, is titratable with iodine solution. The other part of sodium bisulfite formed a stable interaction product with morphine and is untitratable with iodine solution. The stoichiometric ratio of morphine to sodium bisulfite in the interaction product was found to be 1 : 1 (Table I).

The isolated interaction product of morphine–sodium bisulfite is insoluble in water, in dilute HCl, and in common organic solvents. The compound contains no sodium ion as detected with flame analysis. The UV and IR spectra reveal that the compound contains a phenol, a carbonyl, and a sulfonic acid group. The tertiary amine is protonized, suggesting that the compound exists as an inner salt or zwitterion. The UV and IR spectra of this compound are quite different from that of morphine-3-ethereal sulfate (3).



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Based on the elemental analyses and UV and IR spectra, the structure of this compound was assigned as 8-sulfonic acid-dihydromorphinone (I).

Critchfield and Johnson (4) indicated that sodium bisulfite reacts with alpha-beta unsaturated compounds having a strong electron-attracting group on the alpha-position to saturate the double bond and to form a stable sulfonic acid. Since morphine has a similar structure, *i.e.*, with a hydroxyl group at the 6-position and an unsaturated double bond at the 7–8-position, it is probable that the (—SO_3) attaches on the 8-position. When the double bond of morphine was saturated, *e.g.*, dihydromorphine, no such reaction was observed under similar condition[3] whereas codeine does undergo this type of interaction[4]. It can be assumed that similar chemicals and pharmaceuticals with alpha-beta unsaturated compounds having a strong electron-attracting group on the alpha-position will react with bisulfite if the concentration of bisulfite ions is high enough. Some pharmaceutical preparations such as epinephrine (6) and steroids (7) containing bisulfite undergo such a bisulfite interaction.

The interaction of morphine and sodium bisulfite takes place very rapidly. Five minutes after addition of sodium bisulfite (1%) to the morphine solution at room temperature, 20% of the morphine (3 mg./ml.) underwent this type of reaction (1). The interaction of morphine and sodium bisulfite probably also takes place in commercial preparations, although the amount of the bisulfite preservative (0.5%) is usually small. Analgesic and toxicity aspects of this interaction product have not yet been studied.

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[2] Rohm & Haas Co., Philadelphia, Pa.

[3] The column partition chromatography used for determination of free morphine (5) had to be modified for determination of unreacted dihydromorphine. The modification was made as follows: 5 g. of diatomaceous earth buffered with 5 ml. of 1.0 *M* of K_2HPO_4, pH 9.2, as supporter. Three milliliters of dihydromorphine HCl (0.3%) with or without 1% of sodium bisulfite, 1.4 ml. of 5% of K_3PO_4 solution, and 0.7 ml. of 1 *M* of K_2HPO_4 solution were mixed thoroughly with 5 g. of diatomaceous earth. The mixture was made slush with chloroform and packed in the column on the supporter. The column was eluted with chloroform. The first 25-ml. fraction of the effluent was discarded, and the optical density of the effluent collected from the 25–125-ml. fraction was read at wavelength 286 nm.

[4] Codeine was determined as described for morphine (5), except that chloroform instead of chloroform containing 15% of *n*-butyl alcohol was used as eluent and the effluent was collected from 0–100 ml.

Effect of Dissolution Media on Disintegration and Dissolution of Hydrochlorothiazide Tablets

A. S. ALAM and E. L. PARROTT

Abstract □ The disintegration and dissolution times of hydrochlorothiazide tablets granulated with acacia, polyvinylpyrrolidone, or starch were measured in distilled water, 0.1 *N* hydrochloric acid, simulated intestinal fluid, and borate buffer at pH 10. The influence of 0.03% polysorbate 80 in the dissolution media on the disintegration and dissolution times is reported. No correlation is demonstrated between the disintegration and dissolution times.

Keyphrases □ Hydrochlorothiazide tablets, disintegration, dissolution—effect of dissolution media □ Dissolution media, hydrochlorothiazide tablets—effect on disintegration, dissolution □ Disintegration, dissolution times, hydrochlorothiazide tablets—effect of dissolution media

In the past decade, many rate studies on the release and dissolution of drugs were conducted (1–6). Various dissolution media were used.

In 1949, the Contact Committee[1] recognized that normally the stomach is acid in reaction, but the committee recommended distilled water as the test medium for the disintegration test in view of the variability of quantity and concentration of acid in both normal and sick persons. This recommendation was based on early observations that, in many instances, water and dilute acid gave closely comparable results (7). For the first time, simulated gastric fluid was specified in USP XV as the test medium for tablets containing ingredients that reacted more readily in acid solution than in water. This raised the question if gastric fluid should be used for all tablets since all orally administered tablets are subjected to the gastric juice. The USP XVIII disintegration test uses water; however, the unresolved question of which medium to use has been shifted to the dissolution test. At present, the USP monograph specifies the dissolution medium for each tablet that has a dissolution specification. The purpose of this investigation was to study the effect of several dissolution media on the disintegration and dissolution of three formulations of a hydrochlorothiazide tablet.

The incorporation of surface-active agents in tablets was reported to improve disintegration (8). The inclusion of a surface-active agent in the dissolution medium was reported to hasten the dissolution of powdered drugs (9–11) and drugs in tableted form (12–18).

Surface-active agents may enhance disintegration by a reduction of interfacial tension which promotes wetting and penetration of the liquid into the tablet. Surface-active agents may enhance dissolution processes of a poorly soluble drug in a tablet by a reduction of interfacial tension and micelle formation. This study was also undertaken to determine the influence of polysorbate 80 in several dissolution media used in tablet disintegration and dissolution tests.

EXPERIMENTAL

Preparation of Tablets—Each tablet contained 50 mg. of hydrochlorothiazide in a lactose base (19). All tablets were prepared by a wet granulation method using acacia, polyvinylpyrrolidone[2], or starch as a binding agent.

Disintegration—The USP XVIII tablet disintegration procedure and apparatus were used to determine the disintegration time.

Solubility and Dissolution—An excess of hydrochlorothiazide and the appropriate dissolution medium were rotated by a submersion rotor in a constant-temperature bath at 37 ± 0.1° for 24 hr. A portion was withdrawn from each bottle with a pipet fitted with a cotton filter. After a 200-fold dilution with the appropriate dissolution medium, the absorbance was measured spectrophotometrically using a blank solution of the appropriate dissolution medium. The wavelengths of maximum absorption in the various dissolution media were determined with a DK-2 spectrophotometer. In all dissolution media, a linear relationship was obtained between the absorbance and concentration of hydrochlorothiazide.

Dissolution Methods I and II are described in the NF XIII. For Method I, the rate of rotation was 60 r.p.m. The dissolution media are given in Table I. The intestinal fluid used was simulated intestinal fluid USP from which the pancreatin was omitted. The borate buffer was prepared by mixing 250 ml. of a 0.2 *M* boric acid and potassium chloride solution with 220 ml. of a 0.2 *M* sodium hydroxide solution and then adjusting to pH 10 by the addition of 28.0 ml. of the sodium hydroxide solution.

Since the surface tension of human gastric fluid ranges from 35 to 50 dynes/cm. (9), polysorbate 80 was added to the dissolution media so that their surface tensions would be within this range. The surface tension of each medium, given in Table I, was measured at 37° with a Fisher Du Nouy tensiometer.

The dissolution profile was obtained by plotting the percent of hydrochlorothiazide dissolved at 37° against time (19). From the dissolution profile of each tablet, the time required for 50 and 66.7% of the hydrochlorothiazide to dissolve was read and designated as $t_{1/2}$ and $t_{2/3}$, respectively. The value reported for the dissolution time represents the average of three tablets.

RESULTS AND DISCUSSION

In determining the disintegration time by the USP method, it was noticed that occasionally a single tablet would require considerably longer to disintegrate than the other five tablets. Thus, it was thought that the average of the individual disintegration time of six tablets might be more representative of disintegration. As shown in Tables II and III, the average disintegration time for tablets granulated with acacia and starch was from one-half to two-thirds of the USP disintegration time. As shown in Table IV for tablets granulated with polyvinylpyrrolidone, the average disintegration time was approximately 15% faster than the USP disintegration time. Although the average disintegration time was less than the USP disintegration time, there was a rank correlation between the two disintegration times in all dissolution media.

[1] Combined Pharmaceutical Contact Committee (members appointed from American Drug Manufacturers' Association and American Pharmaceutical Manufacturers' Association).

[2] Plasdone K 29-32, registered trademark of GAF Corp., New York, N. Y., for pharmaceutical grade polyvinylpyrrolidone.

Table I—Wavelength of Maximum Absorption and Solubility at 37° of Hydrochlorothiazide in Various Dissolution Media and the Surface Tension at 37° of the Dissolution Media

Dissolution Medium	$\lambda_{max.}$, nm.	Solubility, g./l.	Surface Tension, dynes/cm.
Distilled water	270.5	0.830	71.0
Distilled water with polysorbate 80[a]		1.152	38.4
0.1 *N* Hydrochloric acid	271.5	0.823	70.9
0.1 *N* Hydrochloric acid with polysorbate 80[a]		1.070	39.2
Intestinal fluid[b]	270.5	0.884	71.7
Intestinal fluid with polysorbate 80[a]		0.998	38.4
Borate buffer, pH 10	273.0	4.070	71.2
Borate buffer with polysorbate 80[a]		4.820	39.1

[a] 0.03% polysorbate 80. [b] Simulated intestinal fluid USP without pancreatin.

Table II—Disintegration and Dissolution of Hydrochlorothiazide Tablets Granulated with Acacia in Various Dissolution Media at 37°

Dissolution Medium	Disintegration Time, min.		Dissolution Time, min.			
		Average of	Method I[a]		Method II[b]	
	USP	Six Tablets	$t_{1/2}$	$t_{2/3}$	$t_{1/2}$	$t_{2/3}$
Distilled water	12.4	8.3	32.8	45.6	14.4	25.7
Distilled water with polysorbate 80[c]	12.0	7.7	24.9	35.2	17.9	33.6
0.1 *N* Hydrochloric acid	22.0	11.9	20.8	30.5	14.1	26.4
0.1 *N* Hydrochloric acid with polysorbate 80[c]	15.2	9.9	15.8	25.3	11.3	24.4
Intestinal fluid[d]	21.3	13.9	29.0	40.4	16.8	28.3
Intestinal fluid with polysorbate 80[c]	19.2	14.2	27.0	37.6	14.5	24.8
Borate buffer, pH 10	13.8	8.4	16.7	24.4	10.4	14.0
Borate buffer with polysorbate 80[c]	14.1	9.4	17.3	25.6	7.7	11.0

[a] USP method using 60 r.p.m. [b] NF Method II. [c] 0.03% polysorbate 80. [d] Simulated intestinal fluid USP without pancreatin.

There was no marked difference in disintegration time in the various media. The 0.1 *N* hydrochloric acid produced the greatest variation. For tablets granulated with acacia and starch, the hydrochloric acid produced a longer disintegration time than the other media. For tablets granulated with polyvinylpyrrolidone, the hydrochloric acid produced a shorter disintegration time.

As shown in Table I, the addition of 0.03% polysorbate 80 to the dissolution media lowered the surface tension. With a reduction of surface tension, it is conceivable that the tablet would be wetted and more easily penetrated by the dissolving medium, with a more rapid disintegration. As shown in Table III, this effect was observed only in the disintegration in borate buffer of the tablet granulated with starch. In all other observations, within the variance of the disintegration test, the addition of polysorbate 80 to the dissolution medium did not significantly alter disintegration time.

As shown in Tables II–IV, the dissolution times within a given method were comparable with distilled water, 0.1 *N* hydrochloric acid, and simulated intestinal fluid. On the basis of the Noyes and Whitney equation, this would be anticipated because the agitation and volume of the liquid are constant and, as shown in Table I, the solubility of hydrochlorothiazide for practical purposes is the same. In the borate buffer, the solubility of hydrochlorothiazide is approximately 4 times as great as in the other media, and a faster dissolution would be expected (12, 20). This is experimentally verified by a shorter $t_{1/2}$ and $t_{2/3}$ for all formulations in the borate buffer.

As shown in Table I, the addition of 0.03% polysorbate 80 increased the total solubility of hydrochlorothiazide in all dissolution media. The critical micelle concentration (CMC) of polysorbate 80 is 0.01 and 0.03%, as determined by the surface tension and the

Table III—Disintegration and Dissolution of Hydrochlorothiazide Tablets Granulated with Starch Paste in Various Dissolution Media at 37°

Dissolution Medium	Disintegration Time, min.		Dissolution Time, min.			
		Average of	Method I[a]		Method II[b]	
	USP	Six Tablets	$t_{1/2}$	$t_{2/3}$	$t_{1/2}$	$t_{2/3}$
Distilled water	3.3	2.0	27.4	43.9	24.8	45.6
Distilled water with polysorbate 80[c]	3.3	2.3	23.2	40.0	22.5	44.0
0.1 *N* Hydrochloric acid	5.8	2.8	19.3	35.4	14.8	31.6
0.1 *N* Hydrochloric acid with polysorbate 80[c]	2.9	2.0	21.9	44.6	18.1	37.0
Intestinal fluid[d]	3.3	2.1	25.0	40.0	18.6	40.5
Intestinal fluid with polysorbate 80[c]	4.0	2.6	26.3	47.6	18.1	40.1
Borate buffer, pH 10	3.7	2.5	6.6	10.2	4.4	7.0
Borate buffer with polysorbate 80[c]	2.0	1.8	7.0	10.4	4.8	7.0

[a] USP method using 60 r.p.m. [b] NF Method II. [c] 0.03% polysorbate 80. [d] Simulated intestinal fluid USP without pancreatin.

Table IV—Disintegration and Dissolution of Hydrochlorothiazide Tablets Granulated with Polyvinylpyrrolidone in Various Dissolution Media at 37°

Dissolution Medium	Disintegration Time, min. USP	Disintegration Time, min. Average of Six Tablets	Dissolution Time, min. Method I[a] $t_{1/2}$	Dissolution Time, min. Method I[a] $t_{2/3}$	Dissolution Time, min. Method II[b] $t_{1/2}$	Dissolution Time, min. Method II[b] $t_{2/3}$
Distilled water	11.9	10.5	27.3	39.1	17.7	32.0
Distilled water with polysorbate 80[c]	13.1	10.9	28.5	41.2	16.8	27.7
0.1 *N* Hydrochloric acid	9.5	7.5	29.4	43.5	12.1	20.7
0.1 *N* Hydrochloric acid with polysorbate 80[c]	11.3	9.9	24.6	37.0	12.6	24.3
Intestinal fluid[d]	13.6	11.9	21.1	33.4	15.3	29.0
Intestinal fluid with polysorbate 80[c]	12.9	11.4	23.7	35.4	15.0	25.3
Borate buffer, pH 10	13.6	11.3	17.7	26.1	8.8	13.1
Borate buffer with polysorbate 80[c]	14.3	11.8	16.7	24.4	8.9	12.9

[a] USP method using 60 r.p.m. [b] NF Method II. [c] 0.03% polysorbate 80. [d] Simulated intestinal fluid USP without pancreatin.

solubility method, respectively (21). Thus, there is sufficient polysorbate 80 to solubilize some hydrochlorothiazide.

Inspection of Table II indicates a slight increase in dissolution of tablets granulated with acacia upon the addition of polysorbate 80 to the dissolution medium. As shown in Tables III and IV with tablets granulated with polyvinylpyrrolidone and starch, the addition of polysorbate 80 did not change the dissolution time. In this limited investigation, the addition of a wetting agent did not significantly affect the dissolution of hydrochlorothiazide from the three tablet formulations. This seems to indicate that the inclusion of a wetting agent in a routine dissolution test is questionable until further studies are made.

Schroeter *et al.* (22), in a study of the dissolution and disintegration times of 76 lots of tablets, demonstrated that there is an extreme specificity in the presence or absence of a relationship between the disintegration and dissolution times. Undoubtedly, there are occasions when the relationship between dissolution time and disintegration time is significant (23); however, in general it appears that one should not expect a correlation between disintegration and dissolution (24–27).

With the three formulations studied, there is no correlation between disintegration and dissolution. For example, in comparing tablets granulated with starch to those granulated with polyvinylpyrrolidone, it is seen that the disintegration of the tablet containing starch is approximately 3 times as fast as the tablet prepared with polyvinylpyrrolidone; yet the dissolution times are of the same magnitude. Conversely, the disintegration in borate buffer of tablets granulated with acacia and polyvinylpyrrolidone is approximately the same as in other media, but the dissolution is faster in the borate buffer than in any other medium studied.

Unquestionably, the tablet granulated with starch disintegrates most rapidly in distilled water, 0.1 *N* hydrochloric acid, and simulated intestinal fluid, and in these media to which 0.03% polysorbate 80 has been added; however, there is no difference in the dissolution time from the three formulations. In the borate buffer, both the disintegration and dissolution are more rapid for the tablet granulated with starch than in other media. Since the addition of polysorbate 80 to the borate buffer does not alter the dissolution time, polysorbate 80 appears in this instance to function as a wetting agent to hasten disintegration.

SUMMARY

1. The disintegration times of hydrochlorothiazide tablets granulated with acacia, polyvinylpyrrolidone, or starch were measured in distilled water, 0.1 *N* hydrochloric acid, simulated intestinal fluid, and a borate buffer at pH 10.

2. The addition of 0.03% polysorbate 80 to the dissolution media did not alter the disintegration time of the tablets, except for a more rapid disintegration in the borate buffer of the tablet granulated with starch.

3. The dissolution times of the three tablet formulations were the same in distilled water, 0.1 *N* hydrochloric acid, and simulated intestinal fluid, and they were faster in the borate buffer.

4. The addition of 0.03% polysorbate 80 to the dissolution media did not significantly alter the dissolution times of the tablets.

5. There was no correlation between the disintegration and dissolution times.

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Controlled Potential Coulometric Analysis of Amine-Oxides of Pharmaceutical Interest

RICHARD W. JANSSEN* and CLARENCE A. DISCHER

Abstract □ Controlled potential coulometry was shown to be a valuable technique for the quantitative determination of certain amine-oxides. From 98 to 100% of chlordiazepoxide and the *N*-oxides of atropine, brucine, 3-bromopyridine, scopolamine, and strychnine was recovered.

Keyphrases □ Amine-oxides—quantitative analysis by controlled potential coulometry □ Electrolyses, controlled potential—quantitative analysis of amine-oxides □ Coulometry, controlled potential—quantitative analysis of amine-oxides

The polarographic reduction of a considerable number of amine-oxides has been reported, including chlordiazepoxide (1, 2), diazepam (3), adenine-*N*-oxide (4, 5), phenazine-*N*-oxide (6), and amine-oxides having a pyridine or quinoline nucleus (7–9). Foffani and Fornasari (10) reported the appearance of a single wave during the reduction of pyridine-*N*-oxide, this being produced by the cationic form. Later these workers (11) reported that the amine-oxide is reduced more readily when conjugation of its bond with the ring is lessened. Emerson and Rees (9) confirmed that diminished conjunction results in a lowered reduction potential.

Although these polarographic studies indicate the feasibility of controlled potential reduction of *N*-oxides, quantitative studies have not been reported.

Table I—Percentage Recovery of Atropine-*N*-oxide by Controlled Potential Coulometric Analysis under Varying Conditions

Sample, mg.	pH	Potential, v.	Recovery, %
19.3[a]	0.40	−0.9	99.0
19.3	0.40	−0.9	96.4
19.3	0.40	−0.9	97.7
38.6	0.40	−0.9	97.0
38.6	0.40	−0.9	97.0
19.3	0.88	−0.9	99.4
19.3	0.88	−0.9	95.8
19.3	0.88	−0.9	99.6
38.6	0.88	−0.9	96.5
38.6	0.88	−0.9	99.9
38.6	0.88	−0.9	93.5
38.6	0.88	−0.9	99.3
19.3	1.57	−1.0	100.7
19.3	1.57	−1.0	97.9
19.3	1.57	−1.0	99.3
38.6	1.57	−1.0	100.0
19.3	2.71	−0.9	93.7
19.3	2.71	−0.9	91.9
19.3	2.71	−1.0	95.7
19.3	2.71	−1.0	95.8
19.3	2.71	−1.0	95.8
38.6	2.71	−1.0	100.0
38.6	2.71	−1.0	97.0
19.3	4.63	−1.0	96.9
19.3	4.63	−1.0	94.7
19.3	4.63	−1.0	96.1

[a] Equivalent to 5.00×10^{-5} *M* atropine-*N*-oxide.

Table II—Percentage Recovery of Amine-Oxides by Controlled Potential Coulometric Analysis

Compound	Potential, v.	pH	Recovery, %[b]
Atropine-*N*-oxide	−1.00	1.57	99.3 ± 1.4
Brucine-*N*-oxide	−1.05	4.63	100.0 ± 2.5
3-Bromopyridine-*N*-oxide	−0.80	0.88	98.5 ± 0.4
Chlordiazepoxide[a]	−0.30	0.40	99.5 ± 0.3
Scopolamine-*N*-oxide	−0.75	4.63	98.0 ± 3.0
Strychnine-*N*-oxide	−0.90	0.88	98.0 ± 0.7

[a] Supplied by Hoffmann-La Roche, Inc. [b] Sample concentration 5×10^{-5} *M*.

EXPERIMENTAL

Equipment—Controlled potential electrolyses were performed using an electronic controlled potential coulometric titrator, model Q-2005, ORNL (12). A Non-Linear Systems digital voltmeter, model 484 A, displayed the readout voltages. Reductions were accomplished in a 125-ml. cell having a 30-ml. mercury pool as the working electrode (cathode). The surface area of the pool was 16 cm.^{2} at rest. Compartments for the saturated calomel reference electrode and the platinum wire nonworking electrode (anode) were isolated from the cathode chamber by glass frits and potassium nitrate-agar plugs. Nitrogen was bubbled through the solution during runs. A four-bladed impeller driven by a Sargent synchronous rotator served to agitate the surface of the mercury pool.

Solvents—Solvents utilized in the study were sulfuric acid–potassium sulfate systems of pH 0.40, 0.88, 1.57, and 2.71, and an acetic acid–sodium acetate system of pH 4.63. A solute concentration of 5×10^{-5} *M* was selected for the work.

Assay Procedure—The solvent was degassed with nitrogen, and the titrator was switched on until a steady current resulted. The titrator was then switched off, and a weighed sample was added to the cell and dissolved. The titrator and the timer were switched on simultaneously and allowed to run until there was no further decrease in the current. The readout voltage and time were recorded, and the readout due to the reduction of polarized water molecules was determined from the background. This was subtracted from the total readout; the equivalents of sample were then calculated by use of a previously determined instrumental calibration factor.

RESULTS AND DISCUSSION

The most effective reduction potential for the quantitative recovery of a given compound was a function of the pH, as demonstrated by the experimental results for atropine-*N*-oxide (Table I). Mean values, based on these data, demonstrate this reduction potential–pH relationship even more clearly. With a reduction potential of −0.9 v. (*versus* saturated calomel electrode) at pH's 0.40, 0.88, and 2.71, the percent recoveries are 97.7 ± 1.3, 98.3 ± 1.7, and 93.3 ± 1.4, respectively. With a reduction potential of −1.0 and pH's 1.57, 2.71, and 4.63, recoveries are 99.3 ± 1.4, 95.8 ± 0.05, and 95.9 ± 0.90%, respectively.

Results, under optimum conditions, for those *N*-oxides successfully determined are shown in Table II. Pyridine-*N*-oxide, 2,6-lutidine-*N*-oxide, and quinoline-*N*-oxide gave meaningless recoveries since their amine-oxide reduction waves overlapped the catalytic hydrogen wave.

Samples of less than 5×10^{-5} mole were not used because weighing errors adversely affected the accuracy of the coulometric results. Utilization of samples significantly greater than 5×10^{-5} mole led to unacceptably long coulometric reduction periods.

Vigorous agitation of the mercury pool surface during coulometry was critical. Decreased current efficiency resulted if the vigorous agitation was not maintained.

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Chemistry and Pharmacological Evaluation of 1-Phenyl-2-propanols and 1-Phenyl-2-propanones

CHARLES F. BARFKNECHT, ROBERT V. SMITH, DAVID E. NICHOLS, JAMES L. LESENEY, JOHN P. LONG, and JAMES A. ENGELBRECHT

Abstract □ Various ring-methoxylated 1-phenyl-2-propanols and 1-phenyl-2-propanones were synthesized and pharmacologically evaluated. Most compounds had depressantlike activity. The ketones were readily reduced by rabbit liver microsomes. No reductase activity was found in rat and mouse liver preparations. Partition coefficients were determined, and a linear correlation between LRA_{50}'s (loss of righting ability in 50% of the mice) and partition coefficients was observed for six of the compounds investigated.

Keyphrases □ 1-Phenyl-2-propanols, 1-phenyl-2-propanones, derivatives—synthesis, pharmacological evaluation as psychotomimetic agents □ Partition coefficients—1-phenyl-2-propanols, 1-phenyl-2-propanones, derivatives □ Structure–activity relationships—1-phenyl-2-propanols, 1-phenyl-2-propanones, derivatives □ TLC—identification □ GLC—analysis

In the study of psychotomimetic agents, the identity of active chemical species has been the subject of controversy. Harley-Mason *et al.* (1) suggested that the hallucinogenic action of mescaline was caused by a metabolite. Goldstein *et al.* (2) isolated 3,4,5-trimethoxyphenylethanol (I) as a product of mescaline metabolism. When I was injected into rabbits, a mescalinelike effect was observed. They proposed that the corresponding aldehyde, 3,4,5-trimethoxyphenylacetaldehyde (II), was the "active" intermediate.

Since methoxylated amphetamines are psychotomimetics related to, and generally more potent than, mescaline (3), it was decided that the oxygen analogs of methoxylated amphetamines, namely 1-phenyl-2-propanols (III) and 1-phenyl-2-propanones (IV), be synthesized and their pharmacology examined. Recent studies on the metabolism of amphetamine revealed that significant amounts of IV*a* are excreted by rabbits, while small amounts are excreted by man (4).

Initial studies were conducted on 1-(3,4-dimethoxyphenyl)-2-propanol (III*c*), which was found to have an immediate, but short-lived, depressantlike effect on a conditioned avoidance response in the rat (5). Encouraged by this action, a systematic study of the series was begun. The effects of ring methoxylation on potency, metabolism, and partition coefficients are reported here.

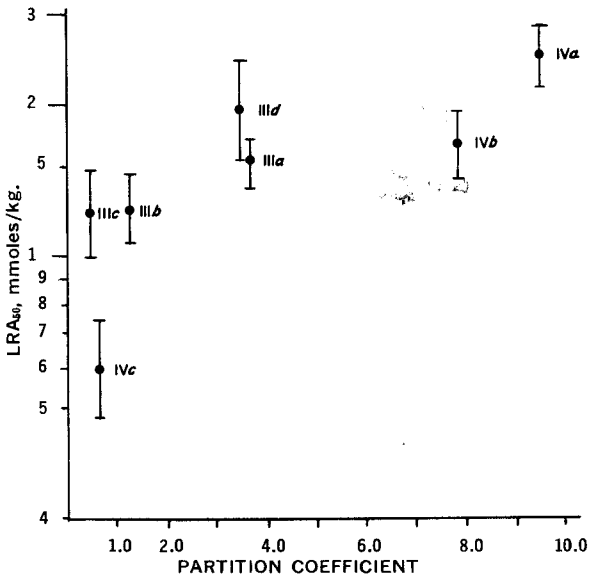

Figure 1—*Plot of* LRA_{50}*'s* versus *partition coefficients. For* n = 6 *(excluding IVc), the correlation coefficient = 0.84.*

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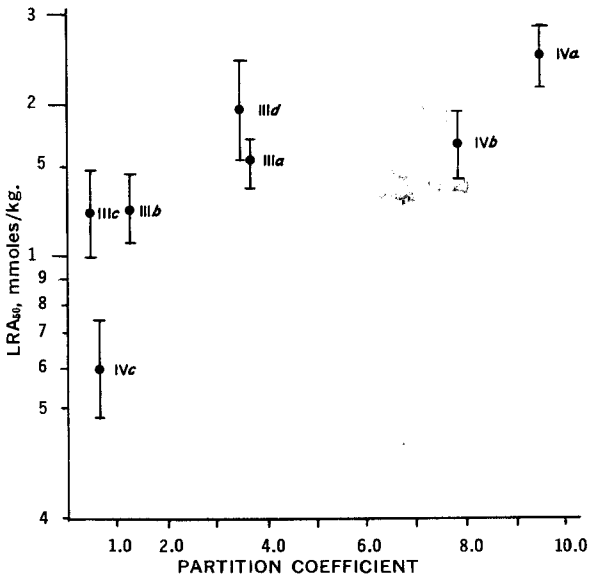
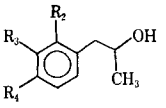
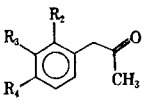


Figure 1—*Plot of* LRA_{50}*'s* versus *partition coefficients. For* n = 6 *(excluding IVc), the correlation coefficient = 0.84.*



III



IV

Table I—Pharmacological Data on 1-Phenyl-2-propanols and 1-Phenyl-2-propanones

Compound	R_2	R_3	R_4	LD_{50}, mg./kg.	LRA_{50}, mg./kg.	LRA_{50}, mmoles/kg.	Salivation	Onset, min.	Ataxia	Hyperactivity
III*a*	H	H	H	520 (423–640)[a]	215 (182–233)	1.58	No	<3	Yes, then sedation	No
III*b*	OCH_3	OCH_3	H	580 (518–650)	240 (205–281)	1.22	Perfuse, 100–300 None >300	<3	Yes	No
III*c*	H	OCH_3	OCH_3	650 (551–767)	240 (198–290)	1.22	No	<3	Yes	No
III*d*	H	H	OCH_3	820 (738–920)	320 (254–367)	1.93	No	<3	Yes	No
IV*a*	H	H	H	540 (486–599)	330 (289–376)	2.46	No	<3	Yes, then sedation	No
IV*b*[b]	OCH_3	OCH_3	H	740 (649–844)	320 (274–374)	1.65	Slight, 100–200 Perfuse, 300–400	<3	No, immediate sedation	No
IV*c*[b]	H	OCH_3	OCH_3	670 (520–724)	115 (93–143)	0.59	No	<3	Yes	No
IV*d*[b]	H	H	OCH_3	560 (483–649)	—[c]	—	No	<3	Tremors	Yes

[a] 95% fiducial limits. [b] Prepared according to the method of R. V. Heinzelman, in "Organic Syntheses," coll. vol. IV, Wiley, New York, N. Y., 1963, p. 573. [c] Effective only at toxic doses.

RESULTS AND DISCUSSION

1-Phenyl-2-propanols and 1-phenyl-2-propanones were synthesized according to literature methods. New compounds (III*b* and III*d*) were prepared by analogous routes. The results of the pharmacological testing are summarized in Table I. Most compounds had depressantlike activity. The data indicate that the 3,4-dimethoxyketone (IV*c*) is the most potent compound. Animals dosed with the LRA_{50}'s (loss of righting ability in 50% of the mice) exhibited varying refractivity to noxious stimuli (footpad and tail clamps). Deaths were apparently due to respiratory depression. Compound IV*d* demonstrated activity different from the remainder of the series and was the only compound showing hyperactivity associated with ataxia.

Compounds IV*a–c* were found to be readily reduced by the 10,000-g supernatant of rabbit liver (Table II); reversal of this reaction was not observed at pH 7.4. When similarly incubated with mouse and rat liver preparations, reductase or oxidase activity was not detected. Analogous results with substituted acetophenones were reported by Culp and McMahon (6).

The partition studies were patterned after those of Hansch *et al.* (7) and Quintana (8). The partition coefficients determined were of the relative order of magnitude expected for the compounds used (8). Interestingly, a plot of LRA_{50}'s (mmoles/kg.) *versus* partition coefficients (Fig. 1) was linear, with a correlation coefficient of 0.84 for III*a–d* and IV*a–b*.

Table II—Physical Properties of 1-Phenyl-2-propanols and 1-Phenyl-2-propanones and *In Vitro* Reduction of Ketones

Compound	Retention[a,b] Time, min.	R_f	$\lambda_{max.}$ (ϵ)[c]	Partition Coefficients	Rate of Reduction[d], μmoles/g. liver/hr.
III*a*	8.8	0.47	259 (188)	3.67	—
III*b*	4.8	0.45	272 (1230)	1.27	—
III*c*	8.0	0.35	277 (2740)	0.26	—
III*d*	—	—	278 (1810)	3.48	—
IV*a*	4.4	0.61	260 (232)	9.45	14.2
IV*b*	3.6	0.63	275 (1370)	7.85	14.8
IV*c*	5.6	0.52	281 (3410)	0.67	15.7
IV*d*	—	—	279 (1820)	6.73	—

[a] Column temperature at 115° for III*a* and IV*a*; 180° for III*b–c* and IV*b–c*; retention times relative to solvent front. [b] III*a–c* chromatographed as their acetates. [c] Maxima used in partition studies. [d] With rabbit liver, 10,000-g supernatant only.

The experiments performed were not capable of discerning whether the effects observed were central and/or peripheral in origin. However, the apparent depressant activity of these compounds is interesting in light of the work of Moffett *et al.* (9) with the phenone congeners of III and IV. They reported that 3′,4′,5′-trimethoxyacetophenone had some tranquilizer effect in schizophrenic patients, while 3,4-dihydroxyphenylcyclopentylketone received clinical study as a tranquilizer. Recently, the sleep-inducing properties of tryptophol and its 5-hydroxy and 5-methoxy analogs were reported by Feldstein *et al.* (10). They suggested that their compounds might be involved in sleep regulation. From the data reported here, it would appear that the depressant action of arylalkyl alcohols and the corresponding ketones may be a more general property of this class of compounds than previously implied.

Further studies on the structure–activity relationships and the mechanism of action of these systems are in progress.

EXPERIMENTAL

Materials—With the exception of III*b* and III*d*, all compounds were previously reported in the literature. Samples were prepared by the literature methods and had physical properties that agreed with the published values.

1-(2,3-Dimethoxyphenyl)-2-propanol (*III*b)—Compound III*b* was synthesized in essentially quantitative yield by sodium borohydride reduction of IV*b* and purification by distillation, b.p. 97°/0.25 mm.

Anal.—Calcd. for $C_{11}H_{16}O_3$: C, 67.35; H, 8.16. Found: C, 67.19; H, 8.26.

1-(p-Methoxyphenyl)-2-propanol (*III*d)—Compound III*d* was synthesized in essentially quantitative yield by a route analogous to that used for III*b*, b.p. 95°/0.1 mm.

Anal.—Calcd. for $C_{10}H_{14}O_2$: C, 72.12; H, 8.44. Found: C, 72.11; H, 8.37.

Sodium NADP[1], dipotassium glucose-6-phosphate[1], and glucose-6-phosphate dehydrogenase[1] were used as purchased. New Zealand rabbit livers[2] and livers from mature male albino mice and Holtzman rats were used for the preparation of 10,000-g supernatant in 0.25 *M* sucrose (1 ml. ≡ 0.5 g. liver) after the method of Lu and Coon (11). All solvents and reagents were analytical reagent grade.

Methods—*Liver Incubations*—In the standard procedure, 5 μmoles of phenylpropanone or phenylpropanol in 0.5 ml. of 0.2 *M* Tris–HCl buffer, pH 7.4, was mixed with 2.5 ml. of 10,000-g liver supernatant, 1 μmole NADP, 5 μmoles glucose-6-phosphate, and 2

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Pel-Freez, Rogers, Ark.

units glucose-6-phosphate dehydrogenase. Control mixtures were prepared in the same way but with liver preparation which had been boiled for 1 min. A blank mixture was also prepared in like manner but without substrate. The liver mixtures were incubated at 37° for 1 hr.; each mixture was quenched by boiling. The incubated mixtures were extracted with 3 × 1 ml. of ethyl acetate; after drying over anhydrous Na_2SO_4, the ethyl acetate extracts were reduced to dryness and then treated as indicated under GLC. For TLC, *n*-heptane extracts of the ethyl acetate residues were used.

TLC—Plates of 250-μ silica gel GF_{254} were used throughout with the following solvent system: benzene–ethyl acetate, 1:1 v/v; development was 10 cm. Detection was *via* fluorescence quenching in 254-nm. radiation. Attempts to locate phenolic metabolites were with diazotized sulfanilic acid of Axelrod and Pulliam (12).

GLC—A Hewlett-Packard 5750B gas chromatograph, equipped with 3% OV-17 on Gas Chrom Q (100/120 mesh) column (6 mm. i.d. × 180 cm.), was used with 60 ml./min. carrier gas flow and the column temperatures listed in Table II. Phenylpropanols were chromatographed as their acetates following reaction of 10–20 mg. of them in 0.05 ml. of pyridine–acetic anhydride (1:1) for 1 hr. at room temperature.

Partition Studies—UV spectra were recorded with a Beckman DK-2 spectrophotometer. Phenylpropanone derivative, 1–5 mg., dissolved in 25 ml. of *n*-heptane was shaken vigorously for 1.5 hr. with 25 ml. of citrate phosphate buffer (pH 7.0) at 25.0 ± 0.1°. The heptane layer was dried over anhydrous Na_2SO_4, diluted to 50 ml. in a volumetric flask with *n*-heptane, and compared to standard solutions at the maxima indicated in Table I with a Gilford model 240 spectrophotometer.

Pharmacological Evaluation—All compounds were administered as suspensions in less than 1% polysorbate 80. Graded doses were administered intraperitoneally to Swiss-Webster male mice weighing 25–30 g. Groups of 10 mice at each dose level were observed, and the gross effects were recorded. Loss of righting ability was considered positive in animals that could not right themselves for a period of at least 30 sec. The loss of righting ability dose (LRA_{50}) and the lethal dose (LD_{50}) were calculated by the method of Litchfield and Wilcoxon (13).

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R. J. WARREN, L. J. RAVIN, P. P. BEGOSH, E. G. SHAMI, and J. E. ZAREMBO

Abstract □ NMR spectroscopy was used to study the enolization of 1-*p*-chlorophenyl-1-hydroxy-2-propanone. At 55°, chloroform solutions of this material gave an equilibrium mixture consisting of the starting material, the enol form, and a third material. This third component was isolated and identified by spectral data as 3,6-bis-(*p* - chlorophenyl) - 1,4 - dimethyl - 2,5,7 - trioxabicyclo - [2,2,1]-heptane. The equilibrium mixture was found to contain 70% of the starting material and 30% of the two reaction products.

Keyphrases □ 1-*p*-Chlorophenyl-1-hydroxy-2-propanone—enolization followed by NMR, characterization of equilibrium mixture □ 3,6-Bis(*p*-chlorophenyl)-1,4-dimethyl-2,5,7-trioxabicyclo-[2,2,1]-heptane—isolation, identification from enolization of 1-*p*-chlorophenyl-1-hydroxy-2-propanone □ NMR spectroscopy—enolization of 1-*p*-chlorophenyl-1-hydroxy-2-propanone

In studies on 1-*p*-chlorophenyl-1-hydroxy-2-propanone, it became necessary to determine if enolization occurred and, if so, to what extent, under what set of conditions, and with what products, if any, being formed. NMR was the analytical method of choice because it was capable of following both the disappearance of the starting material and the appearance of the reaction products as they formed. NMR has been shown to be a valuable analytical tool in the study of reactions and reaction mechanisms (1, 2).

EXPERIMENTAL

All NMR spectra were recorded on a Jeolco C60H spectrometer equipped with a variable-temperature probe. Deuterated chloroform was used as the solvent. Chemical shifts were measured relative to tetramethylsilane.

IR spectra were recorded between 4000–625 $cm.^{-1}$ on a Perkin-Elmer model 21 spectrometer with a sodium chloride prism.

NMR Spectra—A sample of Compound I was dissolved in deuterated chloroform and transferred to an NMR sample tube. The tube was flushed with nitrogen and sealed, and an NMR spectrum was immediately run on the sample. After this spectrum was obtained, the sealed sample tube was placed in a constant-temperature bath set at 55°. The sample was withdrawn at selected intervals, and the NMR spectrum was recorded and integrated.

Isolation of Compound III—The solution containing Compounds I, II, and III was streaked onto 0.5-mm. thick silica gel GF plates. The plates, 20 × 20 cm. (8 × 8 in.), were developed in benzene

units glucose-6-phosphate dehydrogenase. Control mixtures were prepared in the same way but with liver preparation which had been boiled for 1 min. A blank mixture was also prepared in like manner but without substrate. The liver mixtures were incubated at 37° for 1 hr.; each mixture was quenched by boiling. The incubated mixtures were extracted with 3 × 1 ml. of ethyl acetate; after drying over anhydrous Na_2SO_4, the ethyl acetate extracts were reduced to dryness and then treated as indicated under GLC. For TLC, *n*-heptane extracts of the ethyl acetate residues were used.

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NMR Studies on Enolization of 1-*p*-Chlorophenyl-1-hydroxy-2-propanone

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Abstract □ NMR spectroscopy was used to study the enolization of 1-*p*-chlorophenyl-1-hydroxy-2-propanone. At 55°, chloroform solutions of this material gave an equilibrium mixture consisting of the starting material, the enol form, and a third material. This third component was isolated and identified by spectral data as 3,6-bis-(*p* - chlorophenyl) - 1,4 - dimethyl - 2,5,7 - trioxabicyclo - [2,2,1]-heptane. The equilibrium mixture was found to contain 70% of the starting material and 30% of the two reaction products.

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EXPERIMENTAL

All NMR spectra were recorded on a Jeolco C60H spectrometer equipped with a variable-temperature probe. Deuterated chloroform was used as the solvent. Chemical shifts were measured relative to tetramethylsilane.

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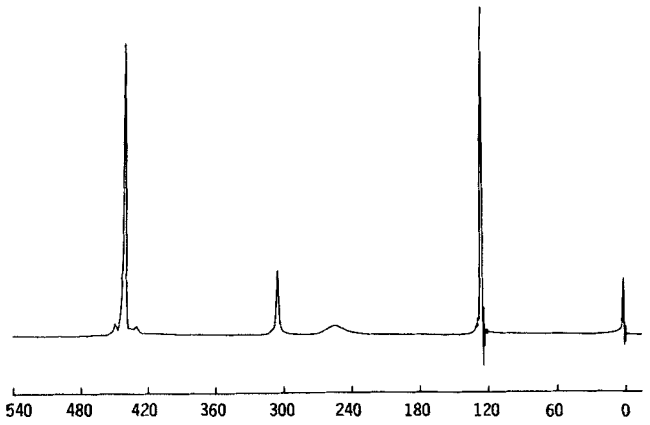


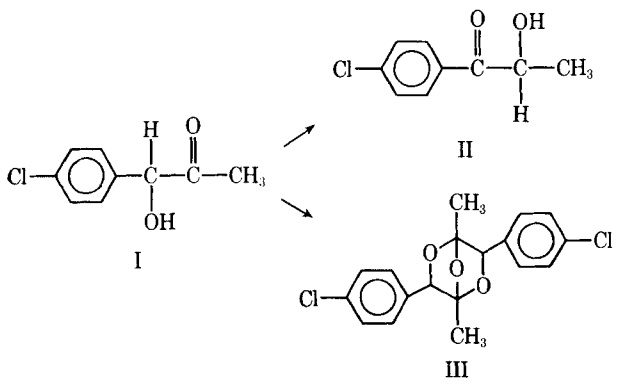
Figure 1—*NMR spectrum of starting material.*

with no equilibration. After the solvent front advanced 10 cm., the plates were dried in a hood and observed under 254-nm. UV. The zones where the various components were located were outlined with a scribe. The silica in the individual zones of interest was scraped from the plate, loaded in a 10-ml. hypodermic syringe fitted with a disk and Whatman No. 3 filter paper, and eluted with ether. The ether solution was evaporated to dryness, and the residue was taken up in 0.5 ml. of deuterated chloroform. An NMR spectrum was obtained on this solution of Compound III.

The IR spectrum was obtained by evaporating the solution used for NMR on a sodium chloride plate.

RESULTS AND DISCUSSION

The enolization of 1-*p*-chlorophenyl-1-hydroxy-2-propanone in chloroform solution at room temperature has been found to proceed very slowly. The process is accelerated with increasing temperature; at 55°, the reaction reaches an equilibrium state after approximately 168 hr. The equilibrium mixture was found to be as shown in Scheme I.



Scheme I

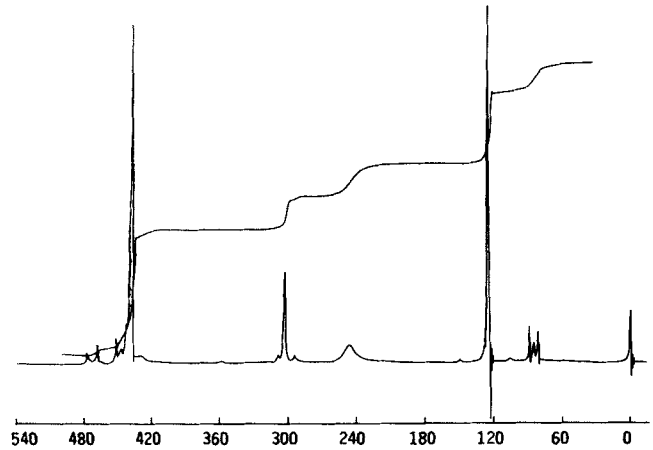


Figure 2—*NMR spectrum after 114 hr. at 55°.*

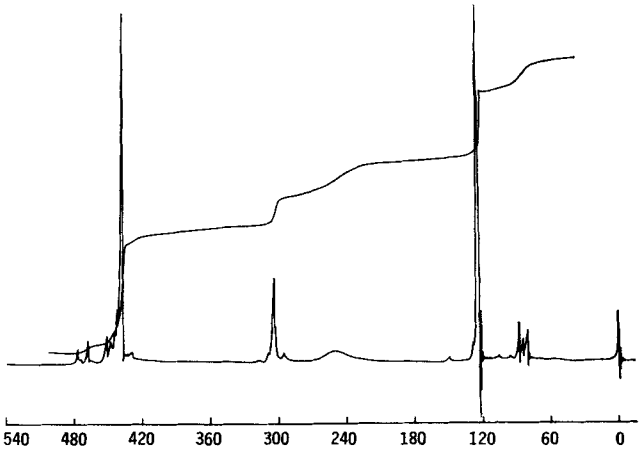


Figure 3—*NMR spectrum of equilibrium mixture.*

NMR Spectra—The NMR spectrum of Compound I is shown in Fig. 1. The NMR absorption peaks may be assigned as follows: methyl group appears as a singlet at 124 Hz., hydroxyl as a broad singlet at 255 Hz., methine as a singlet at 304.5 Hz., and aromatic protons at 439.5 Hz. Figure 2 illustrates a spectrum taken after 114 hr. at 55°. In addition to the peaks present in the original starting material (Fig. 1), additional peaks are evident in the spectrum in Fig. 2. NMR signals at 81, 84.5, and 88.5 Hz. represent the CH_3 groups in products II and III. The weak peaks in the area of 304.5 Hz. represent a quartet due to the CH group in II. The quartet is partially overlapped or masked by the CH in the starting material. A doublet at 450 Hz. and another at 472.5 Hz. arise from the aromatic protons in II. The additional chemical shift downfield is attributable to the increased proximity of the carbonyl group in the enol form. Figure 3 is the spectrum of the mixture at equilibrium. This spectrum, taken after 238 hr. at 55°, shows the increased buildup of the area represented by II and III. After 168 hr., there is no significant increase in intensity of the NMR absorption in these areas.

Identification of Compound III—The spectrum of Compound III (Fig. 4) reveals a singlet absorption peak at 89 Hz. assignable to the isolated methyl groups in the structure proposed for III. Another singlet at 294 Hz. is due to the CH groups, and the remaining signal at 438 Hz. is from the aromatic protons.

The IR spectrum of Compound III (Fig. 5) shows the absence of OH— and C=O functions and the presence of strong ether linkages (8.70, 9.25, and 10.0 μ.) and a *para*-substituted aromatic system (11.9–12.5 μ.).

The mass spectrum gave a molecular weight of 350 and supported the proposed structure.

All of the spectral data are compatible with the structure proposed for Compound III.

Calculation—The amount of remaining starting material and of products II and III at equilibrium may be calculated from the integrated peak intensity of their NMR signals. As mentioned, the signals from the various CH_3 groups in I, II, and III all fall in the area of 70–204 Hz. If one takes the total integrated area for this range to represent 100% of the CH_3 group present, then the amount

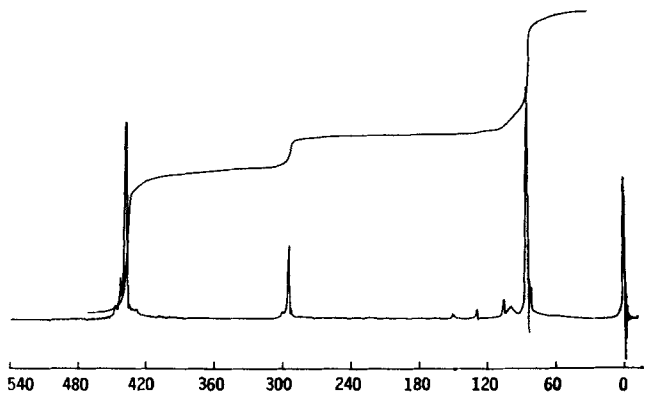


Figure 4—*NMR spectrum of Compound III.*

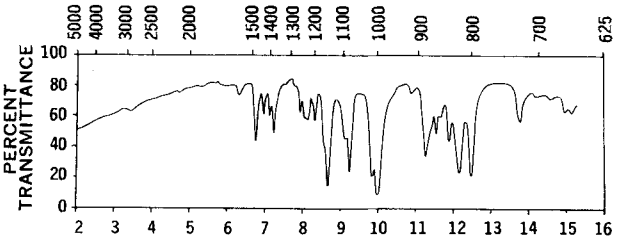
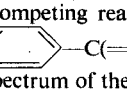


Figure 5—*IR spectrum of Compound III.*

of starting material present at any time may be calculated as:

$$\% \text{ Compound I} = \frac{\text{integral I}}{\text{integral I} + \text{II} + \text{III}} \times 100 \quad \text{(Eq. 1)}$$

Calculations on this basis were made for the spectra run at various time intervals. The data are shown in Table I.

Interferences—If oxygen is not excluded from the system in this experiment, a competing reaction involving the formation of the diketone Cl—  —C(=O)—C(=O)—CH_3 will result. Figure 6 is the NMR spectrum of the diketone. It is obvious from the chemical shift of the methyl group in the diketone spectrum that this material is easily detected in the reaction mixture if it is present.

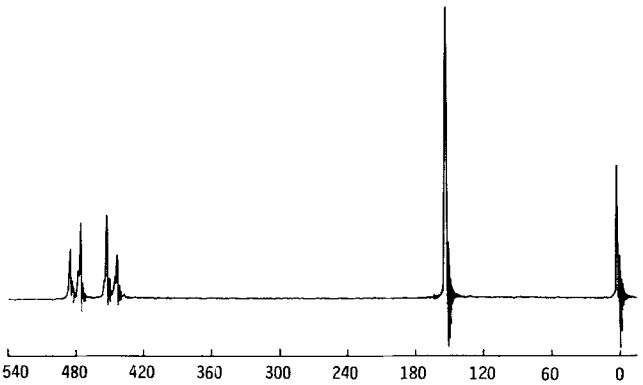


Figure 6—*NMR spectrum of diketone.*

Table I—Percent I, II, and III at Various Time Intervals

Hours	Percent I	Percent II and III
0	100	—
66	83.3	16.7
72	83.3	16.7
91	84.4	15.6
96	82.6	17.4
114	77.3	22.7
120	75.7	24.3
138	75.5	24.5
140	76.0	24.0
144.5	73.1	26.9
166	70.7	29.3
168	70.4	29.6
238	69.9	30.1

CONCLUSION

NMR has been used successfully to monitor the enolization of 1-*p*-chlorophenyl-1-hydroxy-2-propanone. In this study, an unexpected product (III) was formed. This material was isolated and identified by IR, NMR, and mass spectral data.

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New Compounds: 2,4-Dinitrobenzenesulfenamides

M. J. KORNET, T. C. HO, and L. ISENBERG

Abstract □ A compilation of 2,4-dinitrobenzenesulfenamide derivatives of 72 amines is given. Fifty-one of the derivatives are described for the first time.

Keyphrases □ 2,4-Dinitrobenzenesulfenamide derivatives—compilation and description of 72 amines □ Amines, 2,4-dinitrobenzenesulfenamide derivatives—description, compilation

In the course of other studies with 2,4-dinitrobenzenesulfenyl chloride (I), the authors found that this reagent is excellent for the derivatization of amines. This observation was made earlier by Billman *et al.* (1) who characterized 14 amines by converting them into the corresponding 2,4-dinitrobenzenesulfenamides (Scheme I):

$$2,4\text{-}(O_2N)_2C_6H_3SCl + 2R_2NH \rightarrow 2,4\text{-}(O_2N)_2C_6H_3SNR_2 + R_2NH \cdot HCl$$

Scheme I

Subsequently, Kharasch (2) showed that I can also be used for the preparation of derivatives of many other functional groups. Tables containing the physical properties of such derivatives have since been published (3).

More recently, I was advocated for the characterization of pharmaceutically important organic compounds such as barbituric acid, phenylbutazone, and saccharin (4). In addition, sulfenamides are valuable precursors for a number of pharmaceutically useful sulfonamides (5). In the biochemical area, Fontana *et al.* (6) and Scoffone *et al.* (7) showed that I may be used to determine both the cysteine and tryptophan content in polypep-

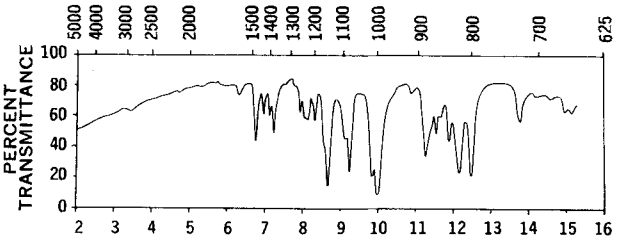
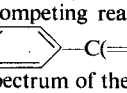


Figure 5—*IR spectrum of Compound III.*

of starting material present at any time may be calculated as:

$$\% \text{ Compound I} = \frac{\text{integral I}}{\text{integral I} + \text{II} + \text{III}} \times 100 \quad \text{(Eq. 1)}$$

Calculations on this basis were made for the spectra run at various time intervals. The data are shown in Table I.

Interferences—If oxygen is not excluded from the system in this experiment, a competing reaction involving the formation of the diketone Cl—  —C(=O)—C(=O)—CH_3 will result. Figure 6 is the NMR spectrum of the diketone. It is obvious from the chemical shift of the methyl group in the diketone spectrum that this material is easily detected in the reaction mixture if it is present.

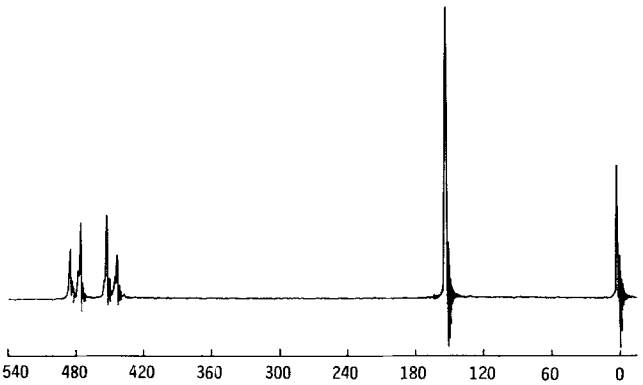


Figure 6—*NMR spectrum of diketone.*

Table I—Percent I, II, and III at Various Time Intervals

Hours	Percent I	Percent II and III
0	100	—
66	83.3	16.7
72	83.3	16.7
91	84.4	15.6
96	82.6	17.4
114	77.3	22.7
120	75.7	24.3
138	75.5	24.5
140	76.0	24.0
144.5	73.1	26.9
166	70.7	29.3
168	70.4	29.6
238	69.9	30.1

CONCLUSION

NMR has been used successfully to monitor the enolization of 1-*p*-chlorophenyl-1-hydroxy-2-propanone. In this study, an unexpected product (III) was formed. This material was isolated and identified by IR, NMR, and mass spectral data.

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New Compounds: 2,4-Dinitrobenzenesulfenamides

M. J. KORNET, T. C. HO, and L. ISENBERG

Abstract □ A compilation of 2,4-dinitrobenzenesulfenamide derivatives of 72 amines is given. Fifty-one of the derivatives are described for the first time.

Keyphrases □ 2,4-Dinitrobenzenesulfenamide derivatives—compilation and description of 72 amines □ Amines, 2,4-dinitrobenzenesulfenamide derivatives—description, compilation

In the course of other studies with 2,4-dinitrobenzenesulfenyl chloride (I), the authors found that this reagent is excellent for the derivatization of amines. This observation was made earlier by Billman *et al.* (1) who characterized 14 amines by converting them into the corresponding 2,4-dinitrobenzenesulfenamides (Scheme I):

$$2,4\text{-}(O_2N)_2C_6H_3SCl + 2R_2NH \rightarrow 2,4\text{-}(O_2N)_2C_6H_3SNR_2 + R_2NH \cdot HCl$$

Scheme I

Subsequently, Kharasch (2) showed that I can also be used for the preparation of derivatives of many other functional groups. Tables containing the physical properties of such derivatives have since been published (3).

More recently, I was advocated for the characterization of pharmaceutically important organic compounds such as barbituric acid, phenylbutazone, and saccharin (4). In addition, sulfenamides are valuable precursors for a number of pharmaceutically useful sulfonamides (5). In the biochemical area, Fontana *et al.* (6) and Scoffone *et al.* (7) showed that I may be used to determine both the cysteine and tryptophan content in polypep-

Table I—2,4-Dinitrobenzenesulfenamide Derivatives of Amines

Amine	Melting Point	Formula	Percent Nitrogen—Calcd.	Percent Nitrogen—Found
4-Acetylaniline	207–208.5°	$C_{14}H_{11}N_3O_5S$	12.61	12.50
2-Aminoaniline	166°[a]	$C_{12}H_{10}N_4O_4S$	18.30	18.56
2-Amino-4-methylpyridine	210.5–212.5°	$C_{12}H_{10}N_4O_4S$	18.30	18.38
Ammonia	119.5–120.5°[b]			
Aniline	142.5–143°[b]			
Benzylamine	86–87°	$C_{13}H_{11}N_3O_4S$	13.77	13.82
N-Benzylaniline	134.5–135°[b]			
Benzylethylamine	74–75°	$C_{15}H_{15}N_3O_4S$	12.61	12.57
Benzylmethylamine	99–101°	$C_{14}H_{13}N_3O_4S$	13.16	13.12
Benzyl *n*-propylamine	84–85°	$C_{16}H_{17}N_3O_4S$	12.10	12.02
2-Bromoaniline	146–147°	$C_{12}H_8BrN_3O_4S$	11.35	11.49
3-Bromoaniline	160–160.5°	$C_{12}H_8BrN_3O_4S$	11.35	11.41
4-Bromoaniline	180.5–181°[b]			
n-Butylamine	88.5–89°[b]			
tert-Butylamine	89.5–90.5°	$C_{10}H_{13}N_3O_4S$	15.49	15.70
2-Chloroaniline	125–126°	$C_{12}H_8ClN_3O_4S$	12.90	12.94
3-Chloroaniline	148.5–149.5°	$C_{12}H_8ClN_3O_4S$	12.90	12.94
4-Chloroaniline	164–164.5°[b]			
5-Chloro-2-methylaniline	204–206°	$C_{13}H_{10}ClN_3O_4S$	12.37	12.48
Cyclohexylamine	109.5–110°[b]			
N-Cyclohexylaniline	114–116°	$C_{18}H_{19}N_3O_4S$	11.25	11.23
Cyclohexylethylamine	96–96.5°	$C_{14}H_{19}N_3O_4S$	12.92	13.02
Cyclohexylmethylamine	95.5–96°[b]			
Dibenzylamine	106–107°	$C_{20}H_{17}N_3O_4S$	10.63	10.64
2,5-Dichloroaniline	156–157°	$C_{12}H_7Cl_2N_3O_4S$	11.67	11.62
Dicyclohexylamine	128.5–130.5°	$C_{18}H_{25}N_3O_4S$	11.07	10.91
Diethylamine	99–100°[b]			
N,*N*-Diethylaniline	146–147°[b,c]			
Diisobutylamine	108.5–109.5°	$C_{14}H_{21}N_3O_4S$	12.84	12.84
Diisopropylamine	98–99°	$C_{12}H_{17}N_3O_4S$	14.04	13.85
N,*N*-Dimethylaniline	175–176°[b,c]			
2,4-Dimethylaniline	160.5–161.5°	$C_{14}H_{13}N_3O_4S$	13.16	13.20
2,5-Dimethylaniline	172.5–174.5°	$C_{14}H_{13}N_3O_4S$	13.16	13.35
2,6-Dimethylaniline	202–203°	$C_{14}H_{13}N_3O_4S$	13.16	13.05
Diphenylamine	150–151.5°	$C_{18}H_{13}N_3O_4S$	11.44	11.47
Di-*n*-propylamine	95.5–97°	$C_{12}H_{17}N_3O_4S$	14.04	13.94
4-Ethoxylaniline	138–139°	$C_{14}H_{13}N_3O_5S$	12.53	12.64
4-Ethoxycarbonylaniline	153.5–155.5°	$C_{15}H_{13}N_3O_6S$	11.57	11.50
Ethylamine	66–66.5°[b]			
2-Fluoroaniline	148–149.5°	$C_{12}H_8FN_3O_4S$	13.59	13.68
4-Fluoroaniline	139.5–141°	$C_{12}H_8FN_3O_4S$	13.59	13.59
Furfurylamine	115–115.5°	$C_{11}H_9N_3O_5S$	14.23	14.07
Hexamethylenimine	126–128°	$C_{12}H_{15}N_3O_4S$	14.14	14.13
2-Hydroxyaniline	192.5–193.5°	$C_{12}H_9N_3O_5S$	13.68	13.67
4-Hydroxyaniline	155–156°[b]			
2-Iodoaniline	153.5–154°	$C_{12}H_9IN_3O_4S$	10.07	10.33
2-Methoxyaniline	155–156°	$C_{13}H_{11}N_3O_5S$	13.08	13.29
3-Methoxyaniline	145–146°	$C_{13}H_{11}N_3O_5S$	13.08	13.05
4-Methoxyaniline	158–159°[b]			
2-Methoxycarbonylaniline	161.5–162°	$C_{14}H_{11}N_3O_6S$	12.03	12.32
4-Methoxycarbonylaniline	188–190°	$C_{14}H_{11}N_3O_6S$	12.03	12.08
4-Methoxy-2-nitroaniline	192–193°[a]	$C_{13}H_{10}N_4O_7S$	15.30	15.38
Methylamine	99–99.5°[b]			
2-Methylaniline	155–156°[b]			
3-Methylaniline	142.5–143.5°	$C_{13}H_{11}N_3O_4S$	13.77	13.80
4-Methylaniline	161–161.5°[b]			
N-Methyl-4-nitroaniline	160–161°	$C_{13}H_{10}N_4O_6S$	16.00	15.83
3-Methylpiperidine	95–96°	$C_{12}H_{15}N_3O_4S$	14.14	14.24
4-Methylpiperidine	59–60°	$C_{12}H_{15}N_3O_4S$	14.14	14.20
Morpholine	164.5–165°[b]			
1-Naphthylamine	188.5–189°[b]			
2-Naphthylamine	167–168°[b]			
2-Nitroaniline	204.5–206°	$C_{12}H_8N_4O_6S$	16.66	16.75
3-Nitroaniline	172.5–173°	$C_{12}H_8N_4O_6S$	16.66	16.74
4-Nitroaniline	181–182.5°[a]	$C_{12}H_8N_4O_6S$	16.66	16.82
1-Phenylethylamine	99–100°	$C_{14}H_{13}N_3O_4S$	13.16	13.29
Piperidine	151.5–152.5°	$C_{11}H_{13}N_3O_4S$	14.84	15.03
1,3-Propanediamine[d]	105–107°	$C_{15}H_{14}N_6O_8S_2$	17.86	17.54
n-Propylamine	94–94.5°[b]			
Pyrrolidine	147–149°	$C_{10}H_{11}N_3O_4S$	15.61	15.35
1,2,3,4-Tetrahydroisoquinoline	157.5–159.5°	$C_{15}H_{13}N_3O_4S$	12.68	12.59
2,4,6-Trimethylaniline	170–171°	$C_{15}H_{15}N_3O_4S$	12.61	12.55

[a] Decomposition point. [b] Data are taken from *Reference 3*. [c] *Para*-sulfide, not amide. [d] Bis compound.

tides and proteins.

In this paper, the physical properties of 51 new 2,4-dinitrobenzenesulfenamides are described. A total of 72 amines have now been derivatized, and the present compilation should be of value in the qualitative identification of these compounds.

EXPERIMENTAL[1]

Melting points were determined with the Fisher-Johns melting-point apparatus and are corrected.

The procedure for 2,4-dinitrobenzenesulfenamide formation was identical to the one reported (3) except that methylene chloride was used as the reaction solvent. The results are summarized in Table I.

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[1] Microanalyses were performed by Dr. Kurt Eder, Geneva, Switzerland. 2,4-Dinitrobenzenesulfenyl chloride was obtained from Matheson Scientific.

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COMMUNICATIONS

Distribution of Ampicillin Administered Orally in Three Different Forms in Rabbit

Keyphrases ☐ Ampicillin distribution—rabbit ☐ Distribution, rabbit—anhydrous, trihydrate, and metampicillins

Sir:

Many physicochemical factors, like particle size (1), salt form (2), ester form (3), or crystal form (4), can affect the dissolution rate and the pharmacokinetics of antibiotics. Particularly, different plasma levels of antibiotic after administration of anhydrous or trihydrate ampicillin (5) and different biliary levels of antibiotic after administration of ampicillin or metampicillin have been described (6). We have reported the distribution of ampicillin in the rabbit after oral administration of three forms of 6-[D(−)-α-aminophenylacetamido]-penicillanic acid: anhydrous ampicillin (AA), trihydrate ampicillin (TA), and metampicillin (MA) (condensation product of ampicillin with formaldehyde).

Sixty male New Zealand rabbits, 2.8 ± 0.3 kg., were fed on a pellet diet with water *ad libitum* and kept at 21 ± 1° and relative humidity 50 ± 4%. The fasting animals were treated orally in random order with 50 mg./kg. of the ampicillins, the amounts of which were expressed as 6-[D(−)-α-aminophenylacetamido]penicillanic acid, and with a mean particle diameter of 14 ± 7 μ. At random order, the animals were killed by bleeding 0.5, 1, 2, and 4 hr. after antibiotic administration; all specimens of blood were taken with a sterile syringe. The kidney, liver, stomach, duodenum, lung, brain, heart, spleen, and muscle were aseptically homogenized for 5 min. with a 0.2 *M* buffer phosphate solution, pH 7; the homogenates were centrifuged for 10 min. at 3000 r.p.m., and assays were performed on the supernatants. The assays of ampicillin (in terms of micrograms per milliliter or micrograms per gram) were carried out on the same day the test was made.

Ampicillin levels were assayed by the cup-plate method, with *Bacillus subtilis* FB27 as the test organism. Samples of plasma, supernatants, and antibiotic standards were diluted when necessary in phosphate buffer, pH 7; several tests were also made with ampicillin-free plasma and supernatants, and no antibacterial activity was detected. The standard solutions were made with plasma or supernatants from untreated animals.

No statistical differences were evident (Table 1) in the distribution of the ampicillin administered as anhydrous ampicillin, trihydrate ampicillin, or condensation product with formaldehyde. Sutherland and Robinson (7) also found that the activities demonstrated by the condensation products of ampicillin with acetone or formaldehyde corresponded closely with the rates to which these compounds hydrolyzed to ampicillin.

(1) G. Harvey and J. O. Alexander, *Lancet*, **1**, 327(1962).

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Table I—Distribution of Ampicillin (Micrograms per Milliliter or Micrograms per Gram) after Oral Administration in the Rabbit of Anhydrous Ampicillin, Trihydrate Ampicillin, and Metampicillin at 50 mg./kg. as 6-[D(−)-α-Aminophenylacetamido]penicillanic Acid

Hours after Antibiotics Administration	Antibiotics[a]	Plasma	Organs							
			Kidney	Stomach	Duodenum	Liver	Lung	Brain	Muscle	Spleen	Heart
0.5	AA	1.31 ± 0.27	9.32 ± 2.52	20.09 ± 2.37	68.21 ± 9.01	0.42 ± 0.10	0.74 ± 0.17	0.22 ± 0.03	0.65 ± 0.11	0.64 ± 0.09	0.88 ± 0.17
	TA	1.06 ± 0.16	7.57 ± 1.52	19.97 ± 2.72	58.20 ± 6.51	0.58 ± 0.09	0.73 ± 0.06	0.39 ± 0.04	0.46 ± 0.03	0.72 ± 0.12	0.92 ± 0.12
	MA	1.20 ± 0.22	8.12 ± 1.13	25.46 ± 3.78	57.42 ± 5.56	0.34 ± 0.08	0.88 ± 0.10	0.29 ± 0.07	0.59 ± 0.11	0.66 ± 0.10	0.96 ± 0.07
1	AA	2.25 ± 0.29	15.89 ± 2.32	8.69 ± 1.91	18.54 ± 2.02	0.44 ± 0.09	1.94 ± 0.37	0.47 ± 0.11	0.94 ± 0.09	1.26 ± 0.23	1.52 ± 0.20
	TA	2.36 ± 0.34	19.91 ± 3.64	10.39 ± 1.24	22.29 ± 2.41	0.51 ± 0.09	1.86 ± 0.44	0.44 ± 0.04	1.00 ± 0.18	1.79 ± 0.11	1.88 ± 0.31
	MA	2.19 ± 0.38	17.27 ± 2.06	9.70 ± 1.66	21.24 ± 2.62	0.55 ± 0.04	1.67 ± 0.23	0.57 ± 0.12	1.00 ± 0.21	1.33 ± 0.25	1.63 ± 0.29
2	AA	1.16 ± 0.21	10.77 ± 1.62	1.59 ± 0.15	6.82 ± 1.05	0.40 ± 0.12	0.59 ± 0.11	0.26 ± 0.05	0.58 ± 0.06	0.55 ± 0.06	0.59 ± 0.08
	TA	0.95 ± 0.26	10.88 ± 1.33	1.49 ± 0.34	5.30 ± 0.57	0.40 ± 0.11	0.67 ± 0.16	0.32 ± 0.08	0.79 ± 0.25	0.64 ± 0.11	0.53 ± 0.11
	MA	1.11 ± 0.17	8.18 ± 1.10	1.87 ± 0.28	6.29 ± 0.50	0.48 ± 0.09	0.84 ± 0.13	0.22 ± 0.06	0.65 ± 0.08	0.61 ± 0.08	0.68 ± 0.07
4	AA	0.45 ± 0.06	3.66 ± 0.47	0.56 ± 0.10	1.97 ± 0.30	0.06 ± 0.03	0.32 ± 0.07	0.13 ± 0.03	0.42 ± 0.08	0.24 ± 0.07	0.30 ± 0.05
	TA	0.54 ± 0.11	3.80 ± 0.78	0.50 ± 0.07	1.67 ± 0.13	0.12 ± 0.06	0.39 ± 0.13	0.13 ± 0.03	0.40 ± 0.11	0.20 ± 0.03	0.29 ± 0.04
	MA	0.38 ± 0.04	3.28 ± 0.67	0.68 ± 0.12	2.45 ± 0.34	0.11 ± 0.04	0.38 ± 0.07	0.16 ± 0.04	0.47 ± 0.08	0.31 ± 0.06	0.37 ± 0.05

[a] AA = anhydrous ampicillin; TA = trihydrate ampicillin; and MA = metampicillin. $n = 5$.

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Translocation of Ligand Conformational Free Energy in Receptor Activation: A Possible Functional Role of Conformational Isomerism in Drug Action

Keyphrases □ Ligand conformational free energy—receptor activation □ Conformation isomerism relationship—biological activity

Sir:

Numerous attempts have been made to define the pharmacophoric conformations of physiologically active molecules (1). Some have postulated that the preferred conformation is the pharmacophoric species (2, 3), while others have concluded that, at least in one case, an energetically unfavorable conformation is bound to the receptor (4–6). On the other hand, Gill (7) implied that more than one conformation may be involved, and Shefter (8) recently pointed out the inadequacies of associating a unique conformation of cholinergic ligands with biological activity. None of these speculations has ascribed a functional role to conformational equilibria in the ligand–receptor interaction.

We wish to propose a model in which conformational free energy of the ligand is utilized to perform work in assisting conformational reorganization of the receptor assembly. The concept described is based on the widely accepted hypothesis (9–12) that a specific conformational change of the receptor assembly gives rise to a biological effect.

The model envisages ligand–receptor association and events leading to depolarization and recovery as a cyclic process (Scheme I). The agonist exists as an equilibrium mixture, $A \rightleftarrows B$, of a preferred (B) and a higher energy (A) conformer. It is assumed that A combines with resting receptor component R faster then does B and that $k_1 \gg k_{-1}$. The receptor component is considered to be a regulatory site (11) which is coupled to one or more macromolecular membrane subunits (M). The activated receptor component (R^*) is capable of triggering a conformational reorganization of M to M', thus giving rise to depolarization.

The salient features associated with various phases of the cycle are as follows:

1. The *higher energy* conformer (A) has greater affinity for R when compared to B; the difference in

Table I—Distribution of Ampicillin (Micrograms per Milliliter or Micrograms per Gram) after Oral Administration in the Rabbit of Anhydrous Ampicillin, Trihydrate Ampicillin, and Metampicillin at 50 mg./kg. as 6-[D(−)-α-Aminophenylacetamido]penicillanic Acid

Hours after Antibiotics Administration	Antibiotics[a]	Plasma	Organs							
			Kidney	Stomach	Duodenum	Liver	Lung	Brain	Muscle	Spleen	Heart
0.5	AA	1.31 ± 0.27	9.32 ± 2.52	20.09 ± 2.37	68.21 ± 9.01	0.42 ± 0.10	0.74 ± 0.17	0.22 ± 0.03	0.65 ± 0.11	0.64 ± 0.09	0.88 ± 0.17
	TA	1.06 ± 0.16	7.57 ± 1.52	19.97 ± 2.72	58.20 ± 6.51	0.58 ± 0.09	0.73 ± 0.06	0.39 ± 0.04	0.46 ± 0.03	0.72 ± 0.12	0.92 ± 0.12
	MA	1.20 ± 0.22	8.12 ± 1.13	25.46 ± 3.78	57.42 ± 5.56	0.34 ± 0.08	0.88 ± 0.10	0.29 ± 0.07	0.59 ± 0.11	0.66 ± 0.10	0.96 ± 0.07
1	AA	2.25 ± 0.29	15.89 ± 2.32	8.69 ± 1.91	18.54 ± 2.02	0.44 ± 0.09	1.94 ± 0.37	0.47 ± 0.11	0.94 ± 0.09	1.26 ± 0.23	1.52 ± 0.20
	TA	2.36 ± 0.34	19.91 ± 3.64	10.39 ± 1.24	22.29 ± 2.41	0.51 ± 0.09	1.86 ± 0.44	0.44 ± 0.04	1.00 ± 0.18	1.79 ± 0.11	1.88 ± 0.31
	MA	2.19 ± 0.38	17.27 ± 2.06	9.70 ± 1.66	21.24 ± 2.62	0.55 ± 0.04	1.67 ± 0.23	0.57 ± 0.12	1.00 ± 0.21	1.33 ± 0.25	1.63 ± 0.29
2	AA	1.16 ± 0.21	10.77 ± 1.62	1.59 ± 0.15	6.82 ± 1.05	0.40 ± 0.12	0.59 ± 0.11	0.26 ± 0.05	0.58 ± 0.06	0.55 ± 0.06	0.59 ± 0.08
	TA	0.95 ± 0.26	10.88 ± 1.33	1.49 ± 0.34	5.30 ± 0.57	0.40 ± 0.11	0.67 ± 0.16	0.32 ± 0.08	0.79 ± 0.25	0.64 ± 0.11	0.53 ± 0.11
	MA	1.11 ± 0.17	8.18 ± 1.10	1.87 ± 0.28	6.29 ± 0.50	0.48 ± 0.09	0.84 ± 0.13	0.22 ± 0.06	0.65 ± 0.08	0.61 ± 0.08	0.68 ± 0.07
4	AA	0.45 ± 0.06	3.66 ± 0.47	0.56 ± 0.10	1.97 ± 0.30	0.06 ± 0.03	0.32 ± 0.07	0.13 ± 0.03	0.42 ± 0.08	0.24 ± 0.07	0.30 ± 0.05
	TA	0.54 ± 0.11	3.80 ± 0.78	0.50 ± 0.07	1.67 ± 0.13	0.12 ± 0.06	0.39 ± 0.13	0.13 ± 0.03	0.40 ± 0.11	0.20 ± 0.03	0.29 ± 0.04
	MA	0.38 ± 0.04	3.28 ± 0.67	0.68 ± 0.12	2.45 ± 0.34	0.11 ± 0.04	0.38 ± 0.07	0.16 ± 0.04	0.47 ± 0.08	0.31 ± 0.06	0.37 ± 0.05

[a] AA = anhydrous ampicillin; TA = trihydrate ampicillin; and MA = metampicillin. $n = 5$.

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Translocation of Ligand Conformational Free Energy in Receptor Activation: A Possible Functional Role of Conformational Isomerism in Drug Action

Keyphrases □ Ligand conformational free energy—receptor activation □ Conformation isomerism relationship—biological activity

Sir:

Numerous attempts have been made to define the pharmacophoric conformations of physiologically active molecules (1). Some have postulated that the preferred conformation is the pharmacophoric species (2, 3), while others have concluded that, at least in one case, an energetically unfavorable conformation is bound to the receptor (4–6). On the other hand, Gill (7) implied that more than one conformation may be involved, and Shefter (8) recently pointed out the inadequacies of associating a unique conformation of cholinergic ligands with biological activity. None of these speculations has ascribed a functional role to conformational equilibria in the ligand–receptor interaction.

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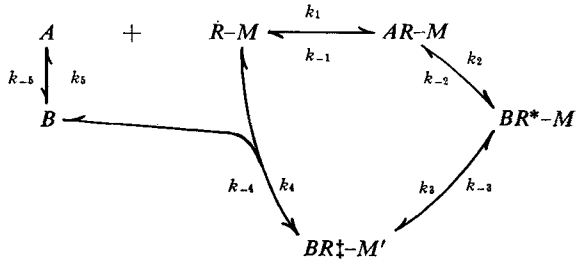

Scheme I

affinity resides primarily with association rate constant k_1.

2. Complex *AR–M* undergoes a conformational reorganization, resulting in the transfer of conformational free energy from the ligand to the receptor component. This process involves the decay of high energy conformer *A* to the lower energy conformer *B*, with a concomitant elevation of the receptor component to activated state *R**. It is conceivable that a *BRM* complex may also lead to an activated receptor component (*BR*–M*), although it would occur with greater difficulty because a higher activation energy would have to be surmounted for this process. The difference in activation energy would be equivalent to ΔG for the conformational equilibrium, $A \rightleftarrows B$.

3. Activated receptor assembly *BR*–M* undergoes conformational reorganization to *BR‡–M′*. The energy of *R** is considered to be dissipated in the triggering process. This is accompanied by a conformational change of the activated receptor component to *R‡*, which is in an energy state similar to that of *R*. Depolarization occurs in the *M′* state.

4. The drug–receptor assembly complex (*BR‡–M′*) dissociates to complete the cycle. This may involve several intermediates but, for the sake of simplicity, the model depicts this as a single step. The dissociation of *B* ($k_4 \gg k_{-4}$) is facilitated by the conformational state of the receptor component (*R‡*).

5. The low energy conformer, *B*, which is released from the complex, reestablishes equilibrium with *A* by the extraction of environmental thermal energy.

If this model is interpreted in terms of the rate theory of drug action (4, 6), then the effect of conformational isomerism on biological activity would depend on which step is rate limiting in the cycle. According to this concept, the affinity of the ligand for the receptor will reflect the ratio of k_1 and one of the rate-limiting kinetic constants (k_2, k_3, k_4, or k_5). A complete kinetic description of the model will be presented in a future publication.

The model, therefore, envisages the interaction of neurotransmitter agents with receptors as providing the necessary chemical information to allow this cycle to proceed in a clockwise fashion. The transformation of random thermal energy into a specific energy source (*i.e.*, conversion of the stimulant to a higher energy conformer) is viewed as a means of lowering the activation energy for receptor activation. While the model most probably is not applicable in all cases of conformational isomerism in drug action, it may be relevant to the naturally occurring neurotransmitters, particularly if evolutionary factors are of prime importance in shaping molecules possessing maximum efficiency as chemical messengers.

The model does not rule out the possibility that totally rigid ligands can act as stimulants, although it suggests that such molecules should be less active than the naturally occurring agonist. Unfortunately, there are no reported examples of totally rigid analogs of neurotransmitters. Elaboration and testing of totally rigid ligands might be an approach to testing this model.

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Molecular Orbital Studies in Chemical Pharmacology. Edited by LEMONT B. KIER. Springer-Verlag, New York, NY 10010, 1970. v + 290 pp. 18 × 26 cm. Price $9.40.

This volume is a collection of eleven contributions presented at a Battelle Seattle Research Center symposium dealing with applications of the molecular orbital approaches of quantum chemistry to studies in chemical pharmacology.

In an introductory lecture, B. Pullman outlines the history of the calculations on the electronic structure of the purine and pyrimidine bases making up the nucleic acids. The impact of the development of techniques of calculation on the nature and significance of the results is discussed by the analysis of the results for four representative problems concerning the properties of these bases: electronic charge distribution, molecular ionization potential, hydrogen-bonding and stacking intermolecular interactions, and purine tautomerism as a possible mechanism for mutations.

In the next lecture, J. R. Hoyland presents a valuable critical analysis of the nature, merits, and demerits of the most common "all valence-electrons" molecular orbital approaches. The "extended" and "iterative extended" Hückel methods, the non-empirical approaches (NEMO methods), the semi-empirical approaches in which atomic orbital differential overlap is neglected (CNDO, INDO, and MINDO methods), are discussed in detail. Deeply analyzed is the probable correctness which can be expected from each of these methods when applied to problems on molecular geometries, relative conformational energies, charge distribution, ionization potentials, hydrogen-bond strengths, heats of dimer formation, and reaction rates.

Three of the subsequent contributions are concerned with problems involving the calculations of the energy of molecular systems. R. Rein and his coworkers discuss the feasibility of a direct physical interaction between an amino acid and a nucleotide base as a primitive transcription mechanism in the recognition process required by the genetic code. This is done by calculating the total interaction energies between glycine and the nucleotide bases guanine, cytosine, adenine, and uracil. Rein's group, in a second contribution, considers the mechanism of thymine photodimerization to gain insight into the possible dimerization modes in DNA. Calculations are made for all the interaction energies as two ground state thymine molecules approach to within bonding distances, and these energies are compared to the interaction energies obtained when one of the thymine molecules is in an excited state. L. B. Kier and J. M. George present a study of the energetically preferred conformations of nine amino acid residues. This is done with the hope that the proper assemblage of the conformationally preferred residues may result in a structure which has some significance in relation to polypeptide conformation. Bradykinin structure is specifically approached in this way.

The other six contributions are more closely related to specific pharmacological problems. They are entitled:

- The Correlation of Electronic Structures of Indole Derivatives With Their Biological Activities, by J. P. Green and S. Kang.
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Apart from some conformational considerations, these contributions make use of the molecular orbital approaches to calculate a variety of quantum chemical electronic indexes. These are subsequently applied to gain correlations between drug activities (measured at different levels of biological complexity) and the calculated electronic index, used individually or in equations which may be subjected to multiple regression analyses. In several cases the electronic indexes are combined with free-energy related physicochemical parameters. The utilization of these empirically derived regression equations to gain insight into pharmacological problems (structure–activity relationships, rational drug design, submolecular mechanism of drug action and drug–receptor interaction) are discussed. Since, if it is not an established consequence of physical laws, it is incorrect to imply causality from a correlation, however high the correlation may be, discussions are also begun regarding the possible physical meaning of such correlations.

This book, dedicated to the first symposium dealing with the subject of quantum pharmacology, provides a good appreciation of the present state of knowledge in this difficult field, which is in an early, pioneering stage of development. A useful bibliography is provided for those theoreticians and experimentalists interested in doing more detailed studies in this emerging discipline.

Reviewed by Dr. Federico Peradejordi
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This book bears the subheading "A useful supplementation for the European Pharmacopoeiae." In the preface, the author refers to the actual task of pharmacognosy, *i.e.*, the identification and evaluation of drugs and their corresponding mixtures and preparations. For many drugs of the pharmacopoeia, the macroscopic and microscopic examination was the only possibility of analytical evaluation. It was only a small number of drugs which got an additional determination of value. The evaluation of chromatography, especially of thin-layer chromatography, enabled an analytical statement by separation and subsequent identification of such mixtures. This statement has reached an importance just as great as the classic microscopy. For this reason, the chromatography of drugs was already integrated into modern pharmacopeias, for example, into the European Pharmacopoeia.

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lesions of which one or more are larger than 5 mm. in diameter) and four were graded 3 on the rating scale. The remaining six stomachs were normal.

These observations indicate that the Mongolian gerbil is less susceptible to the induction of gastric lesions by aspirin than the Holtzman albino rat. The biological basis for the observed difference in resistance is not revealed by the present study. It is, however, clear that a dose level of aspirin causing gastric mucosal erosion in all of the male albino rats that received it did not cause such erosion in those male Mongolian gerbils to which it was administered. Higher dose levels do result in lesion formation in the gerbil, with a direct relationship between incidence and dose. However, even at a dose level fourfold higher than that causing lesions in all rats that received it, only 5 of 11 gerbils exhibited lesions, the severity of which was slight to moderate.

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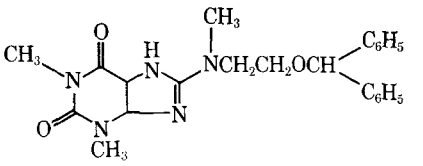
Isolation and Identification of 8-(2-Diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine from Dimenhydrinate

R. S. SANTORO and R. J. WARREN

Abstract □ Differences in solubility and in rate of solubility from previously tested chemical lots were observed in a dimenhydrinate chemical evaluated by the tests and specifications in the official monograph. An impurity was isolated from the lot in question and was identified as 8-(2-diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine based on spectral and elemental data.

Keyphrases □ 8-(2-Diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine—isolation, identification from dimenhydrinate □ Dimenhydrinate—isolation, identification of 8-(2-diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine □ Impurity of dimenhydrinate—isolation, identification of 8-(2-diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine

In a routine analysis of dimenhydrinate chemical according to the tests and specifications in the USP XVII monograph, it was observed that one lot did not completely dissolve in alcohol. Also, its solubility in water, which is the first step in the diphenhydramine assay, was less than complete. In both instances, a hazy solution resulted. However, except for these slight discrepancies, the chemical met the requirements detailed in the monograph. Previous lots dissolved rapidly in the solvents mentioned, while this lot had a poor rate of solubility.



I

Differential thermal analysis revealed one major endotherm at 105° which was identical to previous lots tested. The X-ray diffraction pattern was also the same as previous lots, ruling out polymorphism as a possible explanation for the solubility difference. The thin-layer chromatogram had spots corresponding to the diphenhydramine and 8-chlorotheophylline moieties and a

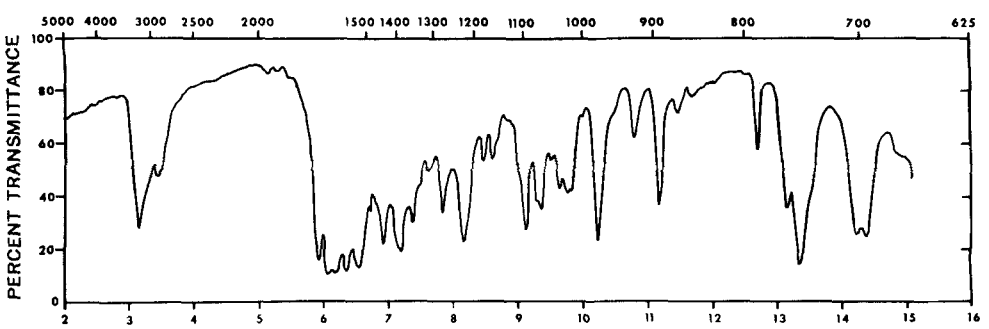


Figure 1—*IR spectrum o unknown.*

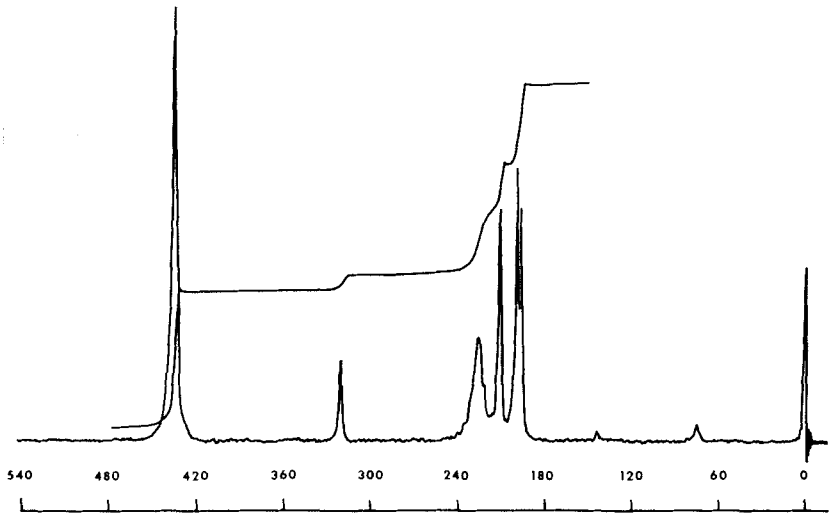


Figure 2—*NMR spectrum of unknown.*

third unknown constituent. This paper describes the isolation of the unknown constituent from dimenhydrinate chemical and its identification as 8-(2-diphenylmethoxy - *N* - methylethylamine) - 1,3 - dimethylxanthine (*I*).

RESULTS AND DISCUSSION

Isolation of the Unknown—Five grams of chemical was weighed into a 250-ml. separator and shaken with 75 ml. of water. The solution was made basic with 5 ml. of 0.1 *N* sodium hydroxide and extracted with 4 × 75-ml. portions of chloroform. The chloroform extracts were passed through a cotton pledget and combined, and the solvent was evaporated with nitrogen gas. The oily residue was transferred to a 12-ml. centrifuge tube with the aid of chloroform, and the chloroform was evaporated with nitrogen gas. The residue was mixed with 5 ml. of ethanol; the tube was capped and allowed to sit for 24 hr. The white precipitate was compacted by centrifugation, the supernatant was decanted, and the residue was washed several times with ethanol. Residual ethanol was evaporated with nitrogen gas. The residue, a white crystalline powder, weighed 35 mg., which represented 0.7% of the starting material.

Anal.—Calcd. for $C_{23}H_{25}N_5O_3$: C, 65.86; H, 6.01; N, 16.70. Found: C, 65.68; H, 6.01; N, 16.57.

TLC—One hundred micrograms was spotted from a chloroform solution of the unknown containing 10 mg./ml. onto a silica gel GF plate activated for 1 hr. at 100°. The plate was equilibrated in a tank for 15 min. before development in the presence of the developing solution. The developing solution was a mixture of chloroform–methanol–formic acid mixed in the proportions of 90 : 10 : 3, respectively. Authenticated dimenhydrinate was run under the same conditions.

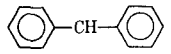
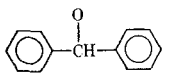
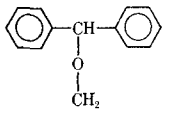
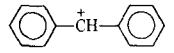
The developed chromatograms were made visible with UV light (254 nm.), iodine vapors, and iodoplatinate reagent. The following spots, along with the R_f values, were visible under UV light and in the presence of iodine vapors: diphenhydramine, origin; 8-chlorotheophylline, 0.36; and unknown, 0.50. With the use of iodoplatinate reagent, diphenhydramine at the origin and the unknown at 0.50 were visible. The chromatogram of the isolated material contained a single spot at R_f 0.50. The starting material contained three spots: diphenhydramine, origin; 8-chlorotheophylline, 0.36; and unknown, 0.50.

Spectroscopy—The IR spectrum of the unknown (Fig. 1) showed the presence of the theophylline moiety as indicated by absorption peaks at 3.15 (NH), 5.90, and 6.05 (C=O) μ. The presence of the amine side chain was shown by absorptions at 8.15 (CH_2—O—CH) and 13.25, 13.35, 14.20, and 14.39 μ. (monosubstituted phenyl groups).

The UV spectrum had a $\lambda_{max.}$ at 300 nm. which is compatible with the structure proposed.

The NMR spectrum (Fig. 2) revealed aromatic protons at 7.3 p.p.m., CH adjacent to aromatics at 5.35 p.p.m., and two methylene and two methyl groups at 3.0–4.0 p.p.m. Integration of the spectrum showed that the protons were presented in a ratio of 10 : 1 : 13, which is consistent with the proposed structure.

The mass spectrum gave a molecular weight of 419. The spectrum supports the proposed structure, and the major peaks may be interpreted as follows:

m/e Value	Fragment Ion Structure
419	molecular ion peak
252	M— 
236	M— 
222	M— 
167	

Conclusion—An impurity isolated from dimenhydrinate chemical was insoluble in water and ethanol. The spectral and elemental data on the unknown are compatible with the structure 8-(2-diphenylmethoxy-*N*-methylethylamine)-1,3-dimethylxanthine.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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Journal of Pharmaceutical Sciences

JUNE 1971
VOLUME 60 NUMBER 6

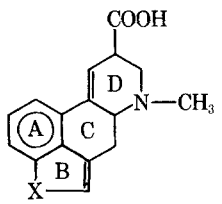
REVIEW ARTICLE

Structural Analogs of Lysergic Acid

E. CAMPAIGNE and D. R. KNAPP

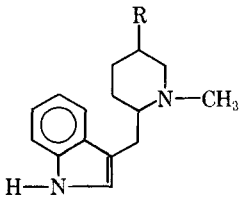
Keyphrases ☐ Lysergic acid structural analogs—literature review ☐ Structure–activity relationships—lysergic acid analogs ☐ Pharmacological activity—lysergic acid analogs

In the course of work on the preparation of the sulfur isosters (*Ia*) of lysergic acid (*Ib*), a literature search was made for other analogs of lysergic acid to see what types of molecular modification had already been applied to this structure. The following review of the published studies on analogs of lysergic acid is the result of this search. Most of these analogs consist of partial structures of lysergic acid or derivatives thereof, in which one or more of the rings have been opened or dissected away to discover the smallest fragment of the polycyclic system that might retain biological activity. The analogs are discussed in structural categories rather than by biological activity groups.

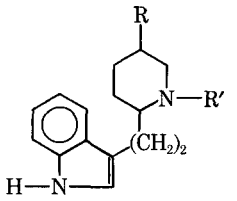


Ia: X = S
Ib: X = NH

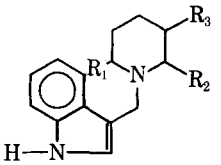
The analogs that probably bear the closest structural resemblance to the parent lysergic acid are indole derivatives of types II and III, in which only the C ring has been opened. The unsubstituted (R = H) and the ethyl-substituted (R = C_2H_5) derivatives of II showed high oxytocic activity *in vitro* on guinea pig uterus but



II



III

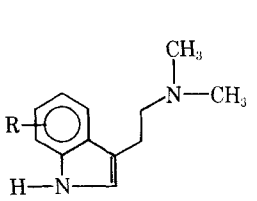


IV

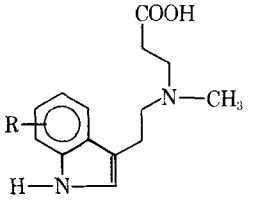
were not active *in vivo* (1, 2). Carboalkoxy substitution of II (R = COOR′) did not increase the activity (2). Castle and Whittle (3, 4) increased the distance between the indole and piperidine ring systems by one methylene group in a series of derivatives of type III. They prepared derivatives of III with both alkyl and carboxamido substituents for R but abandoned the series when the compounds failed to show any interesting activity in mouse behavior, oxytocic, and antimicrobial testing (4). Compounds of type IV (R_1, R_2 = H or CH_3, R_3 = H), although structurally less similar to lysergic acid, showed higher *in vivo* and *in vitro* oxytocic activity than those of type II (5, 6). When the indole system was replaced by naphthalene, the oxytocic dose caused blood pressure disturbances (6). Surprisingly, 3′-carboalkoxy substituents (IV, R_1, R_2 = H, R_3 = COOR) greatly reduced the oxytocic activity (6). When the amide group present in ergonovine was attached at the 3′-position [IV, R_1, R_2 = H, R_3 = CONHCH(CH_3)-

CH_2OH], the compound showed uterine inhibitory action (6).

Analogs of types V and VI (R = 4—Cl, 4—NO_2, 6—NO_2, 4—NH_2, 6—NH_2), in which both the C and D rings are open, have been prepared, but the testing results have not been reported (7).

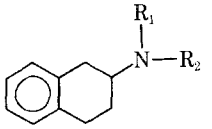


V

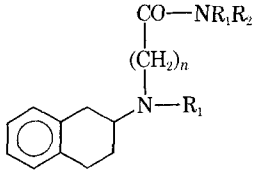


VI

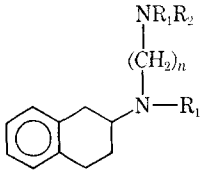
Dissection of the A and C rings from lysergic acid while keeping the amino group gives 1,2,3,4-tetrahydro-2-naphthylamine (VII, R_1, R_2 = H). Many derivatives



VII



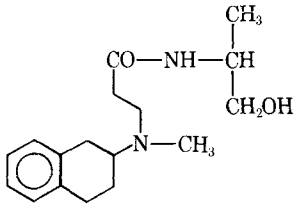
VIII



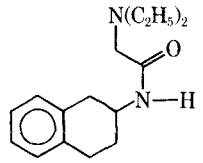
IX

of the basic structure of VII have been prepared as analogs of lysergic acid. The unsubstituted amine (VII, R_1, R_2 = H) exhibits both sympathomimetic and sympatholytic activity (8). Both the unsubstituted amine and the monomethyl derivative (VII, R_1 = H, R_2 = CH_3) showed pyretic and pressor activity in dogs (9). The pressor effect was not prevented by CNS destruction, indicating that it was not centrally mediated. The hyperthermic effect was, however, a central one (9). Ethyl substitution (VII, R_1 = H, R_2 = C_2H_5) or dimethyl substitution (VII, R_1, R_2 = CH_3) caused reduction in pressor activity, and the diethyl derivative (VII, R_1, R_2 = C_2H_5) showed hypotensive action (9).

Marini-Bettolo, Chiavarelli, Bovet, and their coworkers (10–20) prepared and tested over 200 derivatives of VII. The amides of type VIII and the diamines of type IX both showed sympatholytic activity, but only the amides were oxytocic (17). One of these amides, the ergonovine analog X, bore striking resemblance to the parent ergonovine in that it was a potent oxytocic with little vasomotor action (13). The tetrahydronaphthylamine derivative (XI) has been used clinically as an oxytocic (21). In this connection, it is surprising that the corresponding diethylaminoacetyl amide of tryptamine is less active (1/150 ergometrine) than 2-diethylaminoacetamidothiophene (1/100 ergometrine) (22). From their study of derivatives of VII, Marini-Bettolo *et al.* (10) concluded that the 1,2,3,4-tetrahydro-2-naphthylamine element in the structure of ergot al-



X

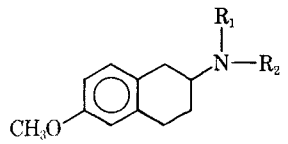


XI

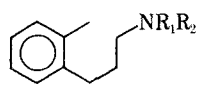
kaloids is essential for sympatholytic activity rather than the indole moiety.

Cymerman-Craig *et al.* (23, 24) also investigated derivatives of VII. As in the earlier work, the parent amine (VII, R_1, R_2 = H) and the lower alkyl derivatives showed pressor activity while the higher alkyl derivatives were hypotensive (25, 26). All of the derivatives showed effects upon body temperature and respiration. One of these (VII, R_1 = CH_3, R_2 = $CH_2CH_2CH_3$) was particularly interesting because it showed marked anti-5-hydroxytryptamine activity as well as optimum adrenolytic action and minimum respiratory depression. It was also hypothermic in rabbits, the opposite of the effect of the unsubstituted amine.

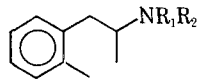
Kraushaar (27) studied a series of 6-methoxy-substituted derivatives (XII) and found them to have oxytocic activity. The parent amine (XII, R_1, R_2 = H) showed the strongest oxytocic action with the least side effects.



XII



XIII

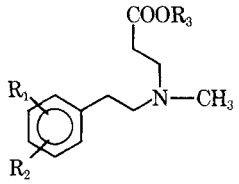


XIV

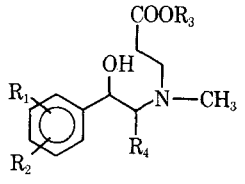
Cymerman-Craig *et al.* (28) prepared related compounds of types XIII and XIV, in which the saturated (C) ring was opened and where R_1 and R_2 were H or small alkyl groups. Work was suspended in this series when the derivatives were not found to be active.

Derivatives similar to XIII and XIV, in which the amino nitrogen bears more complex substituents, have, however, shown activity. Baltzly and his coworkers (29, 30) found that esters of types XV and XVI exhibited oxytocic action when the rings contained methoxy substituents (R_1, R_2 = OCH_3). The compounds without ring substituents were inactive, and the disubstituted derivatives were approximately 10 times as potent as the monosubstituted ones (29). The disubstituted derivatives had only 5–10% of the oxytocic activity of ergonovine. In the series containing hydroxyl substituents and chain branching (XVI), the addition of the hydroxyl appeared to increase the oxytocic action (30).

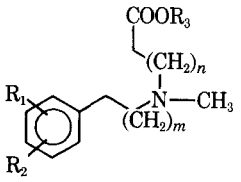
The effects of varying the distance between the nitrogen and the ring and that between the nitrogen and the carbonyl were studied in compounds of type XVII (31). Compounds of type XVII, where m = 1 and n = 0,



XV



XVI



XVII

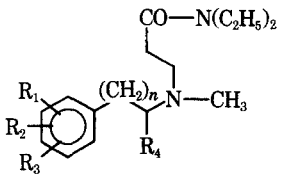
were inactive, indicating that at least two carbons must separate the amine and ester functions. Compounds with more than two carbons between the nitrogen and the carbonyl were also active, however. Lack of appropriate compounds limited the study of the distance between the nitrogen and the ring, but benzyl derivatives (XVII, $m = 0$) were inactive. In all cases, the ester substituent (R_3) did not appear to be critical.

Compounds of general structure XVIII were evaluated as cholinesterase inhibitors (32). Compounds with none, one, two, and three methoxy ring substituents (R_1, R_2, R_3), where R_4 = H or CH_3 and $n = 0$ or 1, were tested; although all were found to have some activity, none was as active as LSD.

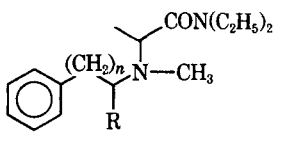
Compounds of type XIX as well as simple derivatives of type XX were also tested for cholinesterase inhibition (33). In the series of type XIX, the distance between the nitrogen and the ring appeared to be important since XIX (R = H, $n = 1$) was 10 times as active as XIX (R = H, $n = 0$). Although the simple derivatives of type XX were active, both series showed activity on the order of one one-thousandth that of LSD.

Plieninger (34) tested compounds of types XXI, XXII, and XXIII. All of these showed oxytocic activity, but the series with the double bond in the nitrogen-bearing ring (XXIII) had markedly higher potency.

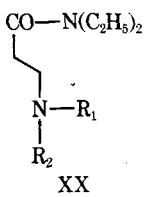
Compounds of general structure XXIV, which can be viewed as lysergic acid analogs, in which the indole system has been replaced by pyridine and where rings C and D are open, were found to have no anti-5-hydroxytryptamine activity (35). Two of these did, however, show a slight but significant 5-hydroxytryptamine potentiating action (XXIV, R_1 = H, R_2 = OEt, NEt_2).



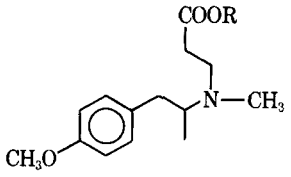
XVIII



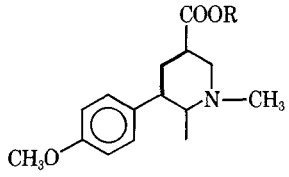
XIX



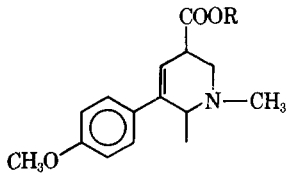
XX



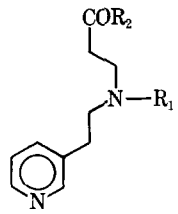
XXI



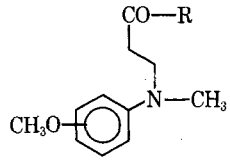
XXII



XXIII



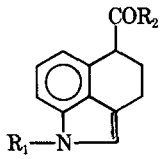
XXIV



XXV

In all of the series considered up to this point, the amino nitrogen was separated from the aromatic ring by a carbon chain. A series of compounds (XXV) has been tested in which the nitrogen is attached directly to the aromatic ring (36). The ethyl ester, amide, and diethylamide of XXV, with both *meta*- and *para*-methoxy substituents, were tested for anti-5-hydroxytryptamine activity. All except the amides in the *para*-series were found to be active but were weak compared to LSD. In addition, the ester in the *meta*-series showed anticholinergic action.

A series of compounds (XXVI) which contain the A, B, and C rings of lysergic acid was prepared, presumably for pharmacological evaluation, but testing results were not reported (37). The compounds were derived from intermediates of a new synthesis of lysergic acid recently reported (38).

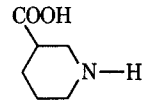


R_1 = H, CH_3CO
R_2 = OH, OCH_3, $N(C_2H_5)_2$

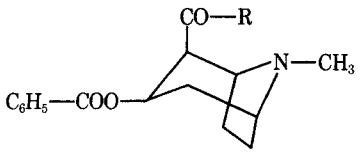
XXVI

The D ring of lysergic acid can be viewed as a piperidine-3-carboxylic acid (XXVII) derivative. Many derivatives of XXVII have been prepared and evaluated for biological activity, and these could be considered analogs of lysergic acid. Among the first such compounds to be tested were derivatives of the alkaloid cocaine (XXVIII, R = OCH_3). The diethylamide (XXVIII, R = NEt_2) was compared with cocaine and found to be a longer acting local anesthetic but was four times as toxic to mice as cocaine (39). The diethylamide specifically inhibited the effects of 5-hydroxytryptamine on rat uterus, whereas cocaine inhibited both 5-hydroxytryptamine and oxytocin in the same system.

Lasslo *et al.* (40, 41) prepared a series of piperidinecarboxylic acid derivatives of types XXIX, XXX, XXXI,

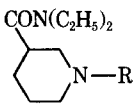


XXVII

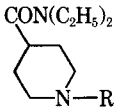


XXVIII

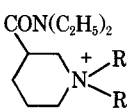
and XXXII, including some of types XXIX and XXX with ring unsaturation. The activities, if any, of these compounds were not reported, however.



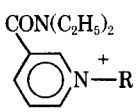
XXIX



XXX



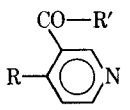
XXXI



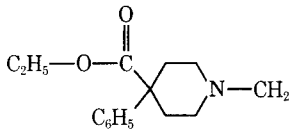
XXXII

Quintana and Schrader (42) extended this series with some additional derivatives like XXIX and XXX. These included some with different amide substituents and some dimeric compounds, but again no testing results were reported.

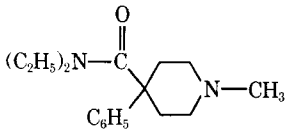
Lehrfeld (43) prepared a series of 6-substituted nicotinic acid derivatives (XXXIII) as analogs of lysergic acid, but no additional information has been published on these compounds.



XXXIII



XXXIV



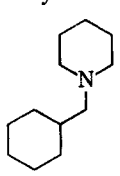
XXXV

The occurrence of psychic side reactions with meperidine (XXXIV) led Lasslo and Waller (44) to prepare the diethylamide derivative XXXV. In preliminary testing, both XXXIV and XXXV were found to have CNS effects.

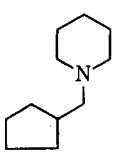
The work on analogs of lysergic acid has led to smaller and smaller fragments that show biological activity. Such simple derivatives as the *N*-substituted piperidines XXXVI, XXXVII, and XXXVIII show oxytocic activity of the order of one-tenth that of methylergonovine (45). Other simple compounds that show oxytocic activity are the tetrasubstituted glycinamides (XXXIX). An extensive series of these were tested; those with ethyl, propyl, and butyl substituents were found to be the most active (46).

Some completely open-chain analogs of lysergic acid (XL, R = OEt, NEt_2) were reported, but no testing results were given (47).

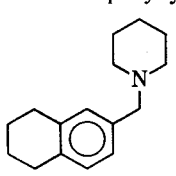
Comparatively lesser attention has been directed toward synthesis of the more complex and polycyclic



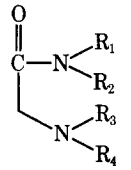
XXXVI



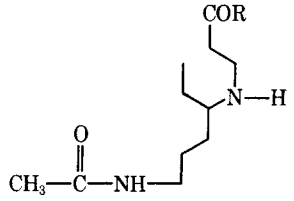
XXXVII



XXXVIII



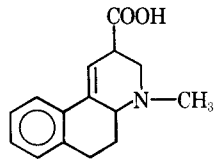
XXXIX



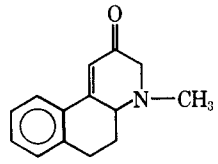
XL

analogs of lysergic acid, but recent developments and some as yet uncompleted efforts in this area deserve mention. An interesting analog of lysergic acid would be 2-carboxy-4-methyl-1,2,3,4,4*a*,5,6-hexahydrobenzo-[*f*]quinoline (XLI), lacking only the pyrrole nucleus; naturally, this molecule has attracted the attention of several groups of workers.

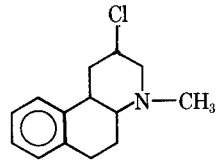
The crucial hexahydrobenzo[*f*]quinoline (XLII) necessary for the synthesis of XLI was assembled independently by Leemann and Fabbri (48) and Cymerman-Craig *et al.* (49), using a reaction scheme reminiscent of the original synthesis of lysergic acid by Kornfeld *et al.* (50). The ketone, XLII, has been reduced and converted



XLI



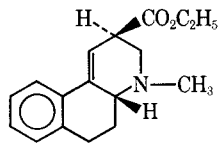
XLII



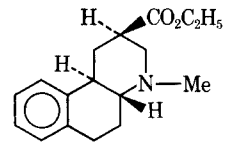
XLIII

to halide XLIII, but all attempts to displace the halogen in order to attach the carboxyl have been unsuccessful[1]. However, the desired acid XLI was obtained by Horii *et al.* (51), utilizing an entirely different synthetic approach, and was found to possess marked oxytocic activity. These workers claimed XLI and its derivatives as uterine-contracting agents in several patents (52).

These workers have prepared all stereoisomers of XLI and its 10,10*a*-dihydro derivative (53) and also their diethyl, *n*-butyl, and 2-hydroxyisopropylamide derivatives (54). The most potent oxytocics that have



XLIV



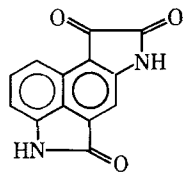
XLV

appeared from this work are XLIV and XLV, which show one-sixteenth and one-fifteenth the activity of ergometrine (51).

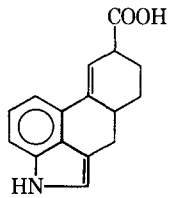
Godshall (55) attempted to synthesize compounds of a steroid nature incorporating features of the lysergic

[1] J. Cymerman-Craig, personal communication, September 1968.

acid structure. It is hoped that this "marriage" of the lysergic acid structure and the steroids might yield compounds that cross the blood–brain barrier more easily than do the normal steroids. Thus far, XLVI is the only related compound reported, and no testing results in this area are yet available[2].



XLVI



XLVII

The authors' efforts toward the sulfur analog (I*a*) of lysergic acid have progressed to the three-ring stage (56), but attempts to add the D ring have not yet been successful. The synthesis of nor-7-deazalysergic acid (XLVII) was recently described, along with the 10,10*a*-dihydro derivative (57), but again no biological activity studies on these compounds or derivatives are yet available.

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RESEARCH ARTICLES

Investigation of Semisolid Lipophilic Preparations by Small Strain and Continuous Shear Viscometry and Their Application to Texture Profile

B. W. BARRY and A. J. GRACE

Abstract □ The effects of grade variation of white soft paraffin on the rheological properties of paraffin ointment BP, simple ointment BP, and some nonpharmacopoeial paraffinic and emulsion preparations were investigated by continuous shear and creep viscometry. The creep curves were analyzed to determine discrete and continuous spectra of retardation times and transformed mathematically to determine dynamic parameters such as storage and loss compliances. The elastic properties of the white soft paraffins influenced the properties of preparations made with these paraffins. There was no general correlation between viscosity data for the preparations, possibly due to some specific interaction such as that described for hard paraffin and liquid paraffin. Dilution of white soft paraffin with liquid paraffin shortened the retardation times but did not significantly alter the type of retardation spectrum involved (bimodal), while emulsification radically altered the retardation mechanisms. The utility of the types of viscometry and various analyses of the creep curves is discussed in terms of correlation with sensory data for texture profile evaluation. It is concluded that small strain experiments are important in texture profile evaluation.

Keyphrases □ Ointments—paraffin effect on rheological properties □ Paraffin, soft—rheological effect, ointments □ Rheology—ointments, emulsions, soft paraffin □ Viscometry—continuous shear, creep, oscillatory □ Viscoelasticity—ointments, soft paraffin, emulsions

Manufacturers of therapeutic and cosmetic semisolid preparations must be able to reproduce the consistency of their products and to ensure that new products are of a consistency acceptable to the consumer. By correlating sensory data with rheological data, a texture profile may be determined, an important concept which has been developed in recent years in the food industry. Many of the parameters, such as viscosity, elasticity, and ductility, which contribute to a subjective assessment of consistency may be measured rheologically. Sherman (1) recently discussed the textural profile and evaluation of pharmaceutical products for external application, and he reviewed the published data correlating instrumental and sensory evaluations of textural properties.

Many rheological techniques are available for evaluation of pharmaceutical materials. Continuous shear viscometry has been a popular approach (2–4); in particular, the Ferranti–Shirley cone and plate viscometer with an automatic flow curve recorder unit and X-Y plotter has been used (5–9). However, the information derived from continuous shear experiments is of limited use in characterizing complex rheological properties such as viscoelasticity, because the high rates of shear involved often cause breakdown of the viscoelastic structure of the sample. To examine a viscoelastic material in its ground state, approximating to zero shear conditions, it is usual to employ small strain experiments such as creep (10) and oscillatory (11) methods.

It was shown that the rheological properties of white soft paraffin BP may vary considerably from grade to grade (10), and a brief discussion of the effect of such variation on the industrial use of the materials was given. The properties of a sample depend on the source of the crude petroleum, the type and degree of refining, blending processes, and the mechanical and thermal history of the material (12–14). Such variation in the properties of white soft paraffin is reflected in the properties of formulations containing significant amounts of this material. The purposes of the present work are: (*a*) to investigate the effects of grade variation of white soft paraffin on the rheological properties of selected lipophilic formulations, and (*b*) to indicate various rheological techniques and methods of experimental data treatment that are available and may be of use in texture profile studies of pharmaceutical preparations.

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Abstract □ The effects of grade variation of white soft paraffin on the rheological properties of paraffin ointment BP, simple ointment BP, and some nonpharmacopoeial paraffinic and emulsion preparations were investigated by continuous shear and creep viscometry. The creep curves were analyzed to determine discrete and continuous spectra of retardation times and transformed mathematically to determine dynamic parameters such as storage and loss compliances. The elastic properties of the white soft paraffins influenced the properties of preparations made with these paraffins. There was no general correlation between viscosity data for the preparations, possibly due to some specific interaction such as that described for hard paraffin and liquid paraffin. Dilution of white soft paraffin with liquid paraffin shortened the retardation times but did not significantly alter the type of retardation spectrum involved (bimodal), while emulsification radically altered the retardation mechanisms. The utility of the types of viscometry and various analyses of the creep curves is discussed in terms of correlation with sensory data for texture profile evaluation. It is concluded that small strain experiments are important in texture profile evaluation.

Keyphrases □ Ointments—paraffin effect on rheological properties □ Paraffin, soft—rheological effect, ointments □ Rheology—ointments, emulsions, soft paraffin □ Viscometry—continuous shear, creep, oscillatory □ Viscoelasticity—ointments, soft paraffin, emulsions

Manufacturers of therapeutic and cosmetic semisolid preparations must be able to reproduce the consistency of their products and to ensure that new products are of a consistency acceptable to the consumer. By correlating sensory data with rheological data, a texture profile may be determined, an important concept which has been developed in recent years in the food industry. Many of the parameters, such as viscosity, elasticity, and ductility, which contribute to a subjective assessment of consistency may be measured rheologically. Sherman (1) recently discussed the textural profile and evaluation of pharmaceutical products for external application, and he reviewed the published data correlating instrumental and sensory evaluations of textural properties.

Many rheological techniques are available for evaluation of pharmaceutical materials. Continuous shear viscometry has been a popular approach (2–4); in particular, the Ferranti–Shirley cone and plate viscometer with an automatic flow curve recorder unit and X-Y plotter has been used (5–9). However, the information derived from continuous shear experiments is of limited use in characterizing complex rheological properties such as viscoelasticity, because the high rates of shear involved often cause breakdown of the viscoelastic structure of the sample. To examine a viscoelastic material in its ground state, approximating to zero shear conditions, it is usual to employ small strain experiments such as creep (10) and oscillatory (11) methods.

It was shown that the rheological properties of white soft paraffin BP may vary considerably from grade to grade (10), and a brief discussion of the effect of such variation on the industrial use of the materials was given. The properties of a sample depend on the source of the crude petroleum, the type and degree of refining, blending processes, and the mechanical and thermal history of the material (12–14). Such variation in the properties of white soft paraffin is reflected in the properties of formulations containing significant amounts of this material. The purposes of the present work are: (*a*) to investigate the effects of grade variation of white soft paraffin on the rheological properties of selected lipophilic formulations, and (*b*) to indicate various rheological techniques and methods of experimental data treatment that are available and may be of use in texture profile studies of pharmaceutical preparations.

Table I—Composition of Ointment and Emulsion Bases

System	Constituents, %: Liquid Paraffin	Hard Paraffin	Wool Fat	Beeswax	Cetostearyl Alcohol	Water	Sorbitan Monooleate[a]	White Soft Paraffin[b]
White soft paraffin	—	—	—	—	—	—	—	100.0
Simple ointment BP	—	5.0	5.0	—	5.0	—	—	85.0
Emulsion of simple ointment	—	2.5	2.5	—	2.5	49.75	0.25	42.5
Paraffin ointment BP	—	3.0	—	2.0	5.0	—	—	90.0
Dilutions of soft paraffin with liquid paraffin:								
A	20.0	—	—	—	—	—	—	80.0
B	40.0	—	—	—	—	—	—	60.0
C	60.0	—	—	—	—	—	—	40.0
D	80.0	—	—	—	—	—	—	20.0
E	90.0	—	—	—	—	—	—	10.0
Emulsions:								
A	—	—	—	—	—	50.0	2.0	48.0
B	9.6	—	—	—	—	50.0	2.0	38.4
C	19.2	—	—	—	—	50.0	2.0	28.8
D	28.8	—	—	—	—	50.0	2.0	19.2
E	38.4	—	—	—	—	50.0	2.0	9.6
F	43.2	—	—	—	—	50.0	2.0	4.8

[a] Span 80. [b] The preparations made with the different samples of white soft paraffin are distinguished in the text by the sample number in parentheses.

EXPERIMENTAL

Materials—The materials used were of BP standard, except the surface-active agent[1] which was a commercial sample. Three grades of white soft paraffin were used, designated I, II, and III[2]. The compositions of the various preparations used are given in Table I, and in the text they are identified by name with the white soft paraffin sample number in parentheses.

Procedure—*Continuous Shear*—A Ferranti–Shirley cone and plate viscometer, with an automatic flow curve recorder unit and X-Y plotter, was used for continuous shear studies. The viscometer was used in two modes: (*a*) rates of shear varied from 0.0 to 1754.0 sec.$^{-1}$, and (*b*) rates of shear varied from 0.0 to 175.4 sec.$^{-1}$, using a 600-sec. sweep time in each case. The performance of the instrument was described previously (15). The test samples were prepared as described later and stored at 25 ± 1.2° for 24 hr. before use.

Creep Testing—Creep experiments were performed using a modification of a Westwind P.C.B. III pneumatic bearing and reaction turbine (10). The ointment bases were loaded into the viscometer at 60°; the emulsions, except emulsions of simple ointment, were mixed at 60°, using a Silverson mixer, and loaded into the viscometer while still warm. The simple ointment emulsions were made as described, but when cool they were milled using a triple-roller mill. Unless treated this way, the emulsions were unstable when large concentrations of water were incorporated, as reported for emulsions of hydrophilic petrolatum by Kostenbauder and Martin (3). The laboratory scale mill (Erwerka Apparatebau GMBH) had calculated maximum shear rates in the roller gaps of 6300 and 10,300 sec.$^{-1}$. All samples were left in the creep viscometer overnight at 25 ± 0.1° for stresses to relax, for temperature equilibration, and for crystallization to occur. Since crystallization of soft paraffins has been reported to be time dependent (16), the samples should be considered as 1 day old.

Approximate linearity checks were performed on each sample, and a creep curve was obtained in the linear region. All the curves were analyzed by the method of Warburton and Barry (17) to obtain discrete spectra. The accuracy of each analysis was checked by a computer program, which regenerated the curve when the analyzed parameters were used as input data. The analyses were accepted if the reconstituted data were within 2% of the experimental curve. Selected curves were also analyzed to obtain continuous retardation spectra. Dynamic, or oscillatory, data were derived from the creep curves using a computer program which employed Schwarzl's approximations for the transformation of transient into dynamic data (18).

[1] Span 80, Atlas Chemical Industries.
[2] Samples I and II are grades 783/L and 892/2/148 produced by Dalton & Co. Ltd., England. Sample III was produced by Plantar Industries.

RESULTS AND DISCUSSION

Continuous Shear—The continuous shear investigation of some preparations was inconclusive due to ejection of the test sample from the measuring gap of the viscometer. This phenomenon is concerned with the elastic recovery and/or fracture of the test sample during shear (8, 9, 19) and may also occur in concentric cylinder viscometers. The viscometer design of Kostenbauder and Martin (3) is evidence that they were aware of this problem.

White soft paraffin (I) and all the preparations containing hard waxes were ejected, to some extent, from the cone-plate gap of the viscometer, and apparent viscosity data could not be derived. The yield values obtained were higher for preparations of the soft paraffins and hard waxes than for the soft paraffins themselves. The yield stresses (Table II) are probably dependent upon the nature of the crystalline matrix (8), which was reinforced by the addition of the hard waxes. However, the validity of the yield stress data for these materials is questionable, because it is not known whether ejection of the test sample begins to occur during or after the formation of the spur point in the flow curve.

Continuous shear studies on the emulsions and paraffinic dilutions of soft paraffin (III) (Table II) indicated that the flow curves were anticlockwise hysteresis loops which sometimes contained spur points. The hysteresis may have been due to thixotropy or to irreversible shear breakdown; both the extent of hysteresis and the magnitude of the spur points decreased with a decrease in the percentage of white soft paraffin.

Figure 1 shows that the apparent viscosity at 1754.0 sec.$^{-1}$ of the emulsions and liquid paraffin dilutions of white soft paraffin (III) decreased as the percentage of soft paraffin decreased, but that the apparent relative viscosity of the emulsions increased [apparent relative viscosity is apparent viscosity of emulsion/apparent viscosity of liquid paraffin dilution, at any given concentration of white soft paraffin (III)]. Plotting the apparent viscosity data on semilogarithmic axes yielded linear graphs, indicating that the variation in apparent viscosity of the emulsions and paraffinic dilutions with the percentage of white soft paraffin (III) could be represented by an Arrhenius-type equation (20, 21). The data derived at 175.4 sec.$^{-1}$ were similar.

The inverse relationship between apparent relative viscosity and concentration of white soft paraffin (III) in the continuous phase of the emulsions indicated that as the percentage of white soft paraffin (III) was decreased, the contribution of the emulsified water to the apparent viscosity of the emulsion increased, especially when less than 20% white soft paraffin was present.

Creep: Discrete Spectra—The rheological behavior of a viscoelastic material that shears under a constant stress in the linear region, that is, where the stress–strain ratio is a function of time alone and not stress magnitude, may be expressed by the equation:

Table II—Continuous Shear Data

Preparation	0.0–1754.0 sec.$^{-1}$ Apparent Viscosity, poise	Yield Stress, dynes cm.$^{-2}$	0.0–175.4 sec.$^{-1}$ Apparent Viscosity, poise	Yield Stress, dynes cm.$^{-2}$
White soft paraffin				
I	*[a]	3,550	12.1+	2,600
II	1.2+	2,300	6.5	2,450
III	10.1+	7,700	33.4	5,100
Paraffin ointment				
I	*	8,150	*	6,100
II	*	5,150	1.3+	7,200
III	13.7+	15,500	8.8+	5,550
Simple ointment				
I	*	9,500	*	5,200
II	*	4,600	*	2,850
III	13.3+	22,300	31.6+	5,500
Simple ointment emulsion				
I	21.3+	22,500	62.4+	10,300
II	*	8,800	*	6,750
III	*	20,300	*	9,600
Emulsion				
IA	13.1	5,500	26.8	3,450
IIA	4.3	5,050	14.4	2,750
IIIA	17.4	6,750	37.9	4,600
IIIB	11.9	2,650	23.9	1,850
IIIC	9.4	1,300	16.5	850
IIID	8.1	—	14.2	450
IIIE	6.0	—	10.6	—
IIIF	5.8	—	7.9	—
Dilution of white soft paraffin III with liquid paraffin				
IIIB	5.9	4,200	14.2	1,650
IIIC	4.2	2,900	10.0	700
IIID	3.1	481	6.6	—
IIIE	2.2	—	3.3	—
IIIF	1.6	—	1.8	—

[a] Indicates total sample ejection, + indicates some sample ejection, data may be invalid. Rates of shear varied from (*a*) 0.0–1754.0 sec.$^{-1}$, and (*b*) 0.0–175.4 sec.$^{-1}$ in 600 sec., apparent viscosity determined at apex of flow curve.

$$J(t) = J_0 + \sum_{i=1}^{n} J_i(1 - e^{-t/\tau i}) + t/\eta_0 \qquad \text{(Eq. 1)}$$

where $J(t)$ is the total compliance (strain–stress ratio) at time t, J_0 is an initial elastic compliance, J_i is a time-dependent elastic compliance, τ_i is a retardation time, and η_0 is the residual Newtonian viscosity. Using the method of Warburton and Barry (17), a creep curve may be analyzed to determine the values of the parameters J_0, J_i, τ_i, and η_0. All the data derived by discrete spectrum analysis are given in Tables III and IV.

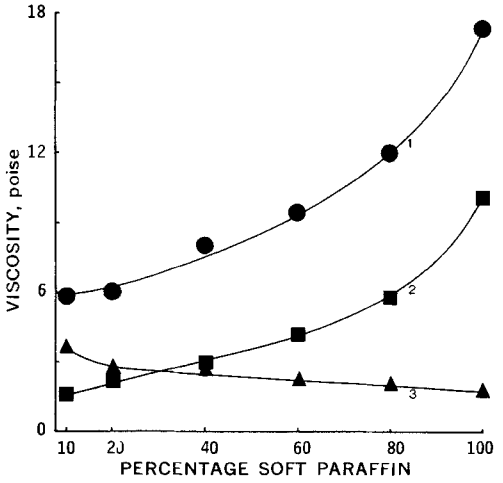

Figure 1—*Effect of concentration of white soft paraffin (III) on the apparent viscosity, at 1754.0 sec.$^{-1}$, of: (1) emulsions, (2) liquid paraffin dilutions of the soft paraffin, and (3) the apparent relative viscosity of the emulsions [apparent relative viscosity is apparent viscosity of emulsion/apparent viscosity of paraffinic dilution at any given concentration of white soft paraffin (III)].*

The three soft paraffins used represent the median and upper and lower extremes of a range of soft paraffins recently investigated (10). The total elastic compliance, $[J(t) - t/\eta_0]_{t\to\infty}$, of Sample III was more than three times that of Sample I, and their residual viscosities differed by more than a factor of 10. The ratio of the total elastic compliance (derived by summation of individual elastic compliances) of white soft paraffins (I, II, and III) is given in Table V, with the corresponding ratios for simple and paraffin ointments and simple ointment emulsion. In each case the preparation of soft paraffin (I) was the least compliant, and its compliance was considered as unity in the calculation of the ratio. The ratio between the total elastic compliances of soft paraffins (I and II) was approximately maintained for each of their preparations, but there was greater variation between the ratios of preparations of white soft paraffins (I and III). The conclusion derived is that the elastic properties of white soft paraffin are of importance in formulation, because they may affect the elastic properties of the final product.

The effect of grade variation of the soft paraffins was not so apparent when the residual viscosities of the preparations of the soft paraffins were compared (Table III). The paraffin ointments maintained the same order of magnitude of residual viscosity as the soft paraffins, but the difference between the values was reduced. With the simple ointments and their emulsions, the order

Table III—Discrete Spectra Data for Ointment Bases and White Soft Paraffins

Parameter	White Soft Paraffin I	White Soft Paraffin II	White Soft Paraffin III	Paraffin Ointment I	Paraffin Ointment II	Paraffin Ointment III	Simple Ointment I	Simple Ointment II	Simple Ointment III	Simple Ointment Emulsion I	Simple Ointment Emulsion II	Simple Ointment Emulsion III
J_0	35.0	44.0	76.0	32.0	46.0	47.0	40.0	46.0	43.0	41.0	56.0	50.0
J_1	31.0	52.0	98.0	19.0	29.0	22.0	7.8	12.0	10.0	96.0	180.0	520.0
J_2	10.0	19.0	45.0	2.7	7.9	9.4	—	7.0	6.0	23.0	54.0	59.0
J_3	6.2	14.0	39.0	4.0	7.7	8.6	—	5.8	15.0	48.0	43.0	80.0
J_4	5.5	2.4	46.0	6.2	12.0	11.0	—	6.1	—	—	43.0	56.0
Total elastic compliance, JT[a]	87.7	131.4	304	63.9	102.6	98.0	47.8	76.9	73.9	208	376	765
τ_1	1424	2059	1360	1282	655	1175	20	546	424	743	890	1442
τ_2	238	425	236	328	96	248	—	82	28	120	150	374
τ_3	38	25	45	60	19	7	—	7	6	18	35	80
τ_4	5	3	8	5	3	1	—	1	—	—	9	9
η_1	45.2	39.6	13.9	65.7	22.7	53.4	2.5	44.4	44.2	7.74	4.81	2.77
η_2	23.8	22.2	5.20	120.0	12.2	26.4	—	11.6	5.13	5.22	2.78	6.34
η_3	6.13	1.86	1.16	15.1	2.44	7.55	—	1.15	0.43	0.36	0.82	1.00
η_4	0.83	1.30	0.18	0.85	0.27	0.70	—	0.16	—	—	0.21	0.16
η_0	278.0	35.0	24.6	286.0	137.0	107.0	24.1	148.0	9.84	8.07	4.58	7.37

Units: J_0, J_1, J_2, J_3, J_4 — cm.^{2} dyne^{-1} × 10^{-8}; τ_1, τ_2, τ_3, τ_4 — seconds; η_1, η_2, η_3, η_4, η_0 — poise × 10^8.

[a] $JT = [J(t) - t/\eta_0]_{t\to\infty}$

Table IV—Discrete Spectra Data for Emulsions and Liquid Paraffin Dilutions of White Soft Paraffin (III)

Parameter		Emulsion IA	Emulsion IIA	Soft Paraffin III	Emulsion IIIA	Dilution IIIB	Emulsion IIIB	Dilution IIIC	Emulsion IIIC	Dilution IIID	Emulsion IIID	Emulsion IIIE	Emulsion IIIF
J_0	cm.^{2} dyne^{-1} × 10^{-6}	0.71	0.49	0.76	1.7	1.4	3.8	2.4	8.4	17.0	30.0	180.0	1600.0
J_1		0.78	0.41	0.98	4.5	0.6	8.0	5.5	18.0	13.0	14.0	66.0	910.0
J_2		0.36	0.22	0.45	1.5	1.1	2.9	2.4	66.0	4.5	12.0	33.0	540.0
J_3		0.31	0.18	0.39	1.4	1.0	1.2	1.2	51.0	19.0	11.0	16.0	780.0
J_4		0.24	—	0.46	0.9	—	2.0	—	2.4	—	—	—	—
τ_1	seconds	1369	1360	1360	1638	2440	1638	661	1470	222	354	180	305
τ_2		190	147	236	229	212	200	43	116	69	28	13	53
τ_3		26	5	45	31	19	62	1	21	12	5	4	7
τ_4		7	—	8	4	—	7	—	3	—	—	—	—
η_1	poise × 10^6	1750.0	3320.0	1390.0	364.0	4190.0	204.0	120.0	80.4	17.1	25.3	2.7	0.3
η_2		527.0	208.0	520.0	148.0	198.0	69.5	17.8	18.7	15.2	2.3	0.4	0.1
η_3		84.0	26.2	116.0	22.2	19.5	51.3	1.2	18.2	0.6	0.4	0.2	0.01
η_4		31.3	—	18.0	4.3	—	3.7	—	1.4	—	—	—	—
η_0		1760.0	2870.0	2460.0	617.0	1690.0	482.0	239.0	109.0	177.0	54.4	30.9	2.0

of magnitude of the residual viscosities did not correspond with that of the soft paraffins. Slippage effects did not occur significantly during the experiments, as confirmed by the creep recovery curves; the reasons for the lack of correlation between the data are not known, but some specific interaction between the components of the preparations may affect the viscosity. Such a phenomenon may be observed when a mixture of liquid paraffin and 5% hard paraffin is heated to 60° and cooled and the Newtonian viscosity of the mixture is measured. The addition of 5% hard paraffin causes a significant decrease in the viscosity of liquid paraffin (1.6–1.4 poise). This has been confirmed using a Haake Rotovisko viscometer and is, thus, not an instrumental effect due to cone and plate geometry. The decrease in the viscosity is thought to be due to entrapment or cocrystallization of the higher paraffin fraction of liquid paraffin with the crystals of hard paraffin. The removal from solution of the higher paraffins reduces the viscosity of the liquid paraffin more than it is increased by the suspended crystals of hard paraffin; therefore, there is an overall decrease in viscosity.

The three-dimensional crystal matrix which exists in soft paraffins and similar materials (22) was reinforced by the addition of crystalline waxes such as hard paraffin and beeswax, as shown by the decrease in the total elastic compliances of the paraffin and simple ointments in relation to the respective white soft paraffin. The reverse occurred with the simple ointment emulsions, because the milling process disrupted the crystalline matrix of the continuous phase.

Creep: Continuous Retardation Spectra—Analysis of creep curves into discrete viscoelastic spectra leads to mathematical data which may not always be easy to understand or compare, although the data may be such that they can be plotted to determine additional parameters. For example, the residual viscosity may be employed in Arrhenius-type curves to obtain the activation energy for viscous flow (8, 9). Analysis of creep curves to obtain continuous retardation spectra is simpler and yields graphical results which are often easier to use, because the spectra represent the viscoelastic behavior of materials in a more general way than other functions such as $J(t)$. The retardation spectrum, L, is defined by the continuous analog of Eq. 1:

$$J(t) = J_0 + \int_{-\infty}^{\infty} L(1 - e^{-t/\tau}) d \ln \tau + t/\eta_0 \quad \text{(Eq. 2)}$$

and has the nature of a distribution function with the dimensions of a compliance (23). Maxima in the curves indicate a concentration of retardation processes (time-dependent elastic strain mechanisms at molecular level) measured by their contribution to compliance. Several approximation techniques are available to determine retardation spectra (24, 25); the one used here is due to Schwarzl and Staverman (26–28) and was developed from a method proposed by Alfrey (29). The value of the distribution function at any given time may be derived using Eqs. 3 and 4:

$$L(t) \sim \frac{d}{d \ln t} [J(t) - t/\eta_0] \quad \text{(Eq. 3)}$$

$$L(t) \sim \frac{d}{d \ln t} \left[J(t) - \frac{dJ(t)}{d \ln t} \right] \Bigg|_{t=2\tau} \quad \text{(Eq. 4)}$$

The values of $J(t)$ and t/η_0 may be determined directly from an experimental curve or obtained from the discrete spectrum data by using the reconstitution program. Equations 3 and 4 were used by Sherman in the study of water-in-oil emulsions (30) and food products (31, 32). They may be used to investigate aging and temperature effects and to compare different formulations of a preparation or material.

Figure 2 indicates that while the retardation spectra of white soft paraffins (I and II) determined by Eq. 3 were broad and smooth, that of white soft paraffin (III) had two maxima. Paraffin ointment BP (III) in the same figure appears to have a very broad and flat distribution; however, expansion of the ordinate showed that it was bimodal, although the maxima were depressed. Emulsion (IIIA) had a single maximum, and the retardation processes involved appeared not to correspond with those of white soft paraffin (III). The continuous retardation spectra of dilutions of soft paraffin (III) with liquid paraffin were all bimodal, although the relative magnitude of the maxima varied and the spectra became narrower, with the peaks shifting to shorter times as the dilution increased (Fig. 3). Thus, emulsification of white soft paraffin (III) radically altered its retardation mechanisms, but dilution with liquid paraffin, although affecting the time scale of the spectrum, did not change the bimodal distribution of the retardation processes.

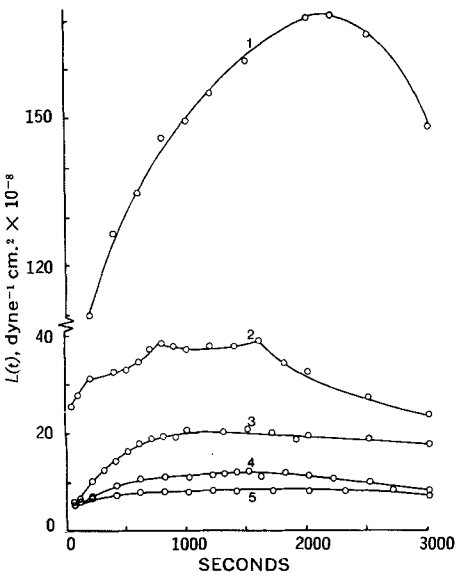

Figure 2—*Continuous retardation spectra. Key: 1, emulsion (IIIA); 2,3,4, white soft paraffins (III), (II), and (I), respectively; and 5, paraffin ointment BP (III).*

Creep: Transformation to Dynamic Parameters—Dynamic, or oscillatory, small strain experiments consist of imposing a sinusoidal

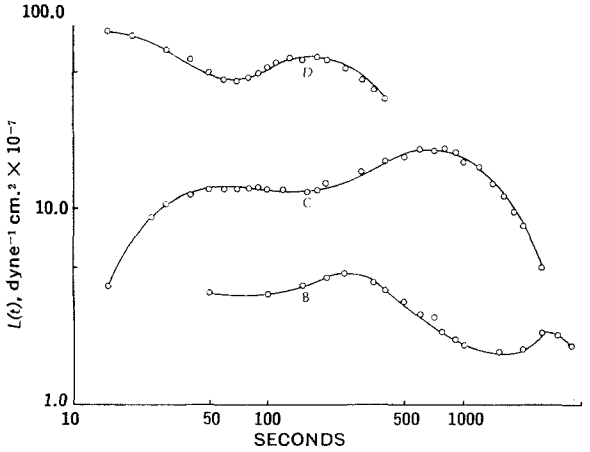

Figure 3—*Continuous retardation spectra of liquid paraffin dilutions (IIIB–IIID, Table I) of white soft paraffin (III).*

stress or strain on a test material and measuring the resultant strain or stress, respectively. By using small amplitudes of oscillation, the structure of the test material changes far less than when it is sheared continuously, even at low rates of shear. Takano (33) compared the effects of oscillatory and continuous shear viscometry. For studying viscoelastic materials which are linear only at low stress, a dynamic system of testing has the advantages that: (*a*) at low amplitudes the information obtained approximates closely to the zero shear condition, and (*b*) properties of the test material may be determined over a wide range of frequency of oscillation. The complex compliance determined in dynamic experiments may be resolved into parameters descriptive of the elastic and viscous characteristics of the material, the storage and loss compliances, respectively. A suitable instrument for oscillatory testing of pharmaceutical semisolids is the Weissenberg rheogoniometer with transfer function analyzer (11). This sophisticated instrument is expensive and would be available only in specialized laboratories. Alternative methods for obtaining dynamic information are either to convert a continuous shear viscometer to oscillatory mode (34) or to transform creep data into dynamic data.

This transformation is feasible if the test material is linear in its viscoelastic behavior, but the procedure is mathematically difficult (23, 35). Many approximation methods have been proposed for the interconversion of dynamic and transient viscoelastic data (23, 35, 36). The approximation due to Schwarzl (18) for calculation of dynamic compliances from creep data involves using values of $J(t)$ at various times in creep; for example, Eq. 5 is the most accurate for the determination of the storage compliance, $J'(\omega)$:

$$J'(\omega) \sim J(t) + 0.0007[J(32t) - J(16t)] - 0.0185[J(16t) - J(8t)] + 0.197[J(8t) - J(4t)] - 0.778[J(4t) - J(2t)] - 0.181[J(t) - J(t/2)] - 0.049[J(t/4) - J(t/8)] \quad \text{(Eq. 5)}$$

where $\omega = 1/t$. The loss compliance is calculated using a similar type of equation. The values of $J(t)$ required may be measured directly from an experimental creep curve. However, a computer program has been written to convert transient data derived by discrete spectrum analysis into dynamic data for linear viscoelastic materials. The advantage of such a method, as opposed to reading $J(t)$ directly, is that it facilitates the transformation over a wider range of frequency, especially at higher frequencies which correspond to creep times too short to be measured directly from the experimental curve.

A brief preliminary investigation was carried out to determine the accuracy of the approximation technique and this application of it. Experimental dynamic data were obtained using a Weissenberg rheogoniometer with transfer function analyzer. Creep curves were obtained for samples loaded into the viscometer while cold to simulate the loading procedure used with the rheogoniometer. The curves were analyzed graphically by the method of Warburton and Barry (17), the analyses were checked by computer, and the data were transformed by computer. Theoretical and experimental data for emulsion (IIIA) are plotted in Fig. 4. The agreement between the experimental storage compliances and those determined from the creep curve was satisfactory, the maximum difference between the values being 3.5×10^{-6} cm.^{2} dyne^{-1} at approximately 0.1 rad. sec.$^{-1}$. The agreement between the experimental and theoretical loss compliances was also satisfactory, except at higher frequencies. While the two loss compliance curves are similar, they diverge at frequencies of oscillation greater than 0.1 rad. sec.$^{-1}$, although the difference between their values is only 1.1×10^{-6} cm.^{2} dyne^{-1} at 0.5 rad. sec.$^{-1}$. This frequency corresponds qualitatively with a creep test of 2-sec. duration, and calculation of the loss compliance involves $J(t)$ at times less than 0.01 sec. As the short time values of $J(t)$ are important in the calculation of the loss compliance, the errors involved may be large and are unavoidable. It is probable that 0.1 rad. sec.$^{-1}$ represents the maximum frequency at which this method of data treatment should be used to calculate the loss compliance from any given creep curve.

The preparations used to indicate the application of this method of data transformation were the emulsions (IIIA–IIIF) of white soft paraffin (III). The curves for the storage compliances of emulsions (IIIE and IIIF) (Fig. 5) attain a maximum at frequencies below approximately 0.002 rad. sec.$^{-1}$, indicating that the emulsions attained the equivalent of transient steady-state flow. With reference to the model representation of viscoelastic behavior (37), the equilibrium attained by the storage compliance indicates maximum extension of the Voigt units and Hookean spring. Emulsions (IIIA–IIIC) attained a similar state at frequencies a power of 10 lower. The slopes of the curves before the plateau region was reached were

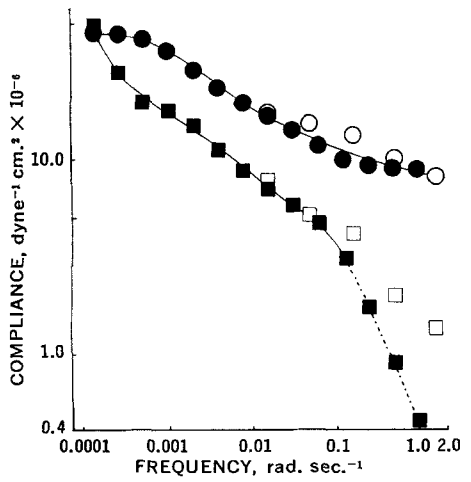

Figure 4—*Dynamic compliances for emulsion (IIIA). Key:* ○, □, *experimental storage and loss compliances, respectively, derived using a rheogoniometer; and* ●, ■, *theoretical storage and loss compliances, respectively, derived by transformation of creep data.*

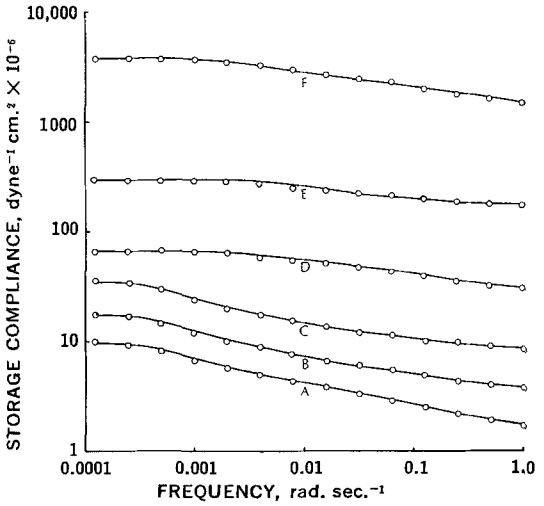

Figure 5—*Variation of storage compliance with frequency of oscillation for emulsions of white soft paraffin (III). See Table I for details of emulsion formulations.*

similar, showing that the elastic structures within the materials were similar. The loss compliances (Fig. 6) decreased as the frequency increased, and the slopes of the curves were similar at low frequency. The increase in the loss compliance slopes at frequencies greater than approximately 0.1 rad. sec.$^{-1}$ was found to occur experimentally using the rheogoniometer, but the error involved may be large.

Texture Profile Evaluation—The relationship between instrumental and sensory data has been studied for many years (38). Stevens (39) defined two types of sensory stimuli, prothetic and metathetic. The former may be represented by a double logarithmic equation and are quantitative (how much? how viscous? *etc.*). The latter are qualitative stimuli (of what kind?) and may be represented with simple logarithmic equations. Katz (40) found that psychological properties such as body and elasticity do not correspond directly to the physical parameters of viscosity and elasticity but are the result of a complicated cooperation of different senses. Thus, the sensory property called elasticity does not correspond to physical elasticity but is more accurately expressed by the viscosity/elasticity ratio. Therefore, in a sensory evaluation of rheological properties, there is a complex interrelationship between stress, strain, and time as the consumer cannot always differentiate the properties that are measured instrumentally, and it may be necessary to use power equations involving all three parameters (41, 42). Examples of textural studies on food and cosmetic products are those of Szczesniak (43), Matz (44), and Fryklöf (45).

Continuous shear viscometry would appear, initially, to be a suitable method for rheological investigation in texture profile studies, because the conditions, such as shear rate, involved in a subjective assessment of consistency may be simulated on a continuous shear viscometer. However, certain materials are not amenable to such a procedure, due to, for example, ejection of sample in a cone-plate viscometer or fracture and slippage in a couette viscometer. With suitable materials, information may be derived concerning the variation of apparent viscosity with rate of shear, temperature, and age of sample, and also the types of flow curve of the materials. Yield stresses may also be determined; while their cause is not always known, they are due, in part at least, to some property of the material (9). Davis (8) suggested that in some materials they are concerned with crystalline structures which are susceptible to mechanical strain. The parameters determined by continuous shear viscometry are only of use in textural studies if they can be correlated with sensory data. Difficulties arise in attempts to measure shear forces and rates of shear in a sensory evaluation, since certain parameters, such as hardness, may be assessed rapidly, while others, such as ductility, require a longer time. It is also necessary to know exactly what the consumer is assessing, a psychological problem, because there is overlap in property identification.

Small strain experiments to measure viscoelastic properties of test materials are useful in many ways. While the underlying mechanisms of the ultimate properties of a material (properties during structural breakdown) are not fully understood, they involve to some degree the same molecular mechanisms that are reflected in simpler viscoelastic properties (23). This correlation may be applied to the spreading of viscoelastic emulsions, ointments, gels, and creams, as spreading causes rupture of the viscoelastic structure within the material. Small strain experiments may also give an indication of the ductility (fiber or strand length) of a material (10), a parameter that is usually assessed subjectively, although a physical test has been proposed (46). The difficulty, as in continuous shear studies, is to correlate experimental and sensory data. The discrete spectrum analysis of creep curves yields detailed data by representing the time-dependent elastic properties of the test material by the algebraic sum of a set of hypothetical series compliances which may, or may not, have a direct physical significance in the system studied (23, 47).

The data may be useful in comparative studies, especially when they can be plotted against a controlled variable (48). For example, by extrapolation it may be shown that plots of creep parameters such as J_0 and η_0 of the emulsions and paraffinic dilutions of white soft paraffin (III) against soft paraffin concentration intercept at 20% or more of white soft paraffin. By correlating this with the apparent relative viscosity data derived in continuous shear, it is reasonable to propose that for concentrations of white soft paraffin (III) less than 20% of the continuous phase of the emulsions, the contribution of the emulsified water dominates the rheological properties of the emulsions, and the effect of white soft paraffin is minimal. However, correlation between small strain and continuous shear data is often difficult to achieve because most complex materials become non-Newtonian at higher rates of shear. For example, in creep, emulsion (IIIA) has a lower residual Newtonian viscosity than white soft paraffin (III), while in continuous shear the paraffin has a lower apparent viscosity than the emulsion. The utility of discrete spectra data in texture profile evaluation is difficult to assess. It is possible that the compliance (the inverse of the elastic modulus) or residual Newtonian viscosity may be correlated with some sensory information in a complex manner, as shown by Katz (40).

Table V—Comparison of Ratio (I:II:III) of Total Elastic Compliance of Preparations of Soft Paraffins (I, II, and III)

Preparation	I	II	III
White soft paraffin	1.0	1.5	3.5
Paraffin ointment	1.0	1.6	1.5
Simple ointment	1.0	1.6	1.5
Simple ointment emulsion	1.0	1.8	3.7

A retardation time is the product of a compliance and its associated viscosity:

$$\tau = J\eta \qquad \text{(Eq. 6)}$$

and discrete spectra analyses produce retardation times which may not have a basic physical significance. Therefore, when the sensory situation reported by Katz (40) is known to exist, it may be more useful to analyze creep curves to obtain continuous retardation spectra. For example, while discrete spectra analyses indicated that soft paraffins (I, II, and III) were dissimilar as regards compliance and residual viscosity, the retardation data did not show any large difference between the materials. However, the continuous spectra indicated that soft paraffin (III) possessed retardation mechanisms different to those of samples (I) and (II). Similarly, although discrete spectra analyses indicated that emulsion (IIIA) was weaker than soft paraffin (III), they did not show that emulsification altered the type of retardation mechanisms. That emulsification does alter the gel structure of white soft paraffin was recently shown by other workers (49), and the results derived here are thus not due to errors in the methods of analysis applied. Such differences in the retardation mechanisms of viscoelastic materials, which are not easily detected using discrete analyses, may possibly be correlated with sensory data when a consumer's subjective property identification concerns both viscosity and elasticity.

The maximum rates of shear involved in a sensory evaluation depend inversely on the general consistency of the samples being assessed. Furthermore, different sensory parameters are determined at different rates of shear. For example, the sensory evaluation of *consistency* of water-in-oil and oil-in-water semisolid emulsions was correlated with initial shear stress (a parameter derived using a vis-

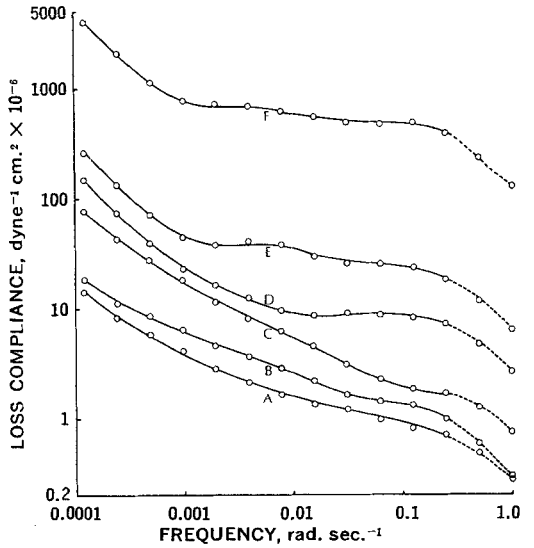

Figure 6—*Variation of loss compliance with frequency of oscillation for emulsions of white soft paraffin (III). See Table I for details of emulsion formulations.*

cometer with discontinuous shear rate variation, and equivalent to a yield stress at a low rate of shear), while *spreadability* was correlated with apparent viscosity at 400 sec.$^{-1}$ (50, 51). Barry and Grace (52) found a relationship between the sensory evaluation of consistency of work softened and unworked white soft paraffin and the yield stresses determined in continuous shear. Apparent viscosity data at rates of shear below 30 sec.$^{-1}$, but after the formation of a spur point in the flow curves, were not correlated with the sensory data. However, a linear relationship was found using discrete viscoelastic parameters determined in creep at very low rates of shear. It is thus apparent that: (*a*) small strain data may be correlated with sensory data, and (*b*) for a *full* texture profile evaluation, experimental data should be derived at various rates of shear. Dynamic small strain experiments may thus be of use in textural studies as measurements may be made over a wide range of frequency (rate of shear) in conditions that approximate to the rheological ground state. Transformation of creep data into oscillatory data is less accurate at high frequencies, especially for the loss compliance. However, by taking creep measurements at various temperatures and employing the method of reduced variables (23), it may be possible to extend the range of accurate transformation of data to higher frequencies.

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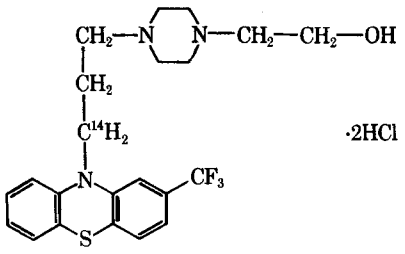
Biological Disposition and Metabolic Fate of Fluphenazine-^{14}C in the Dog and Rhesus Monkey

JACQUES DREYFUSS, JOHN J. ROSS, Jr., and ERIC C. SCHREIBER

Abstract □ Fluphenazine-^{14}C, 4-[1-^{14}C-3-[2-(trifluoromethyl)phenothiazin-10-yl]propyl]-1-piperazineethanol dihydrochloride, was administered orally to dogs at 10 and 40 mg./kg. and to monkeys at 10 mg./kg. After an oral dose of 10 mg./kg., dogs excreted 2–4% and 75–89% of the dose in the urine and feces, respectively. Monkeys that received 10 mg./kg. orally excreted 12–19% and 56–69% of the dose in the urine and feces, respectively. After an oral dose of 40 mg./kg., dogs excreted 5–18% and 85–110% of the dose in the urine and feces, respectively. After 10 mg./kg. i.v., a dog excreted 1.2, 62.0, and 0.03% of the dose in the urine, bile, and expired air, respectively, in 7 hr. After an oral dose of 40 mg./kg., dogs had the highest concentrations of radioactivity in the liver; lungs; combined retina, choroid, and sclera; and kidneys. Localization of radioactivity to gross areas of the brain was not demonstrable. In addition to fluphenazine sulfoxide, several metabolites were present in the urine of dogs and monkeys. In the feces, in addition to fluphenazine-^{14}C, one major and two minor metabolites were found in both species. As the oral dose of fluphenazine-^{14}C administered to dogs was increased from 10 to 40 mg./kg., the amount of unchanged fluphenazine-^{14}C increased relative to the major metabolite. The major metabolite was unconjugated in the feces but was present as the glucuronide conjugate in the bile.

Keyphrases □ Fluphenazine-^{14}C dihydrochloride—metabolic fate, dog, monkey □ Biliary, urinary, fecal excretion—fluphenazine-^{14}C and metabolites □ Metabolites, fluphenazine-^{14}C—isolation, identification □ Scintillometry, liquid—analysis

Fluphenazine[1] is a phenothiazine derivative bearing a trifluoromethyl substituent and a piperazine-containing side chain (Structure I). The compound was labeled with



I

Structure of fluphenazine-^{14}C

^{14}C in the 1-propyl position and administered to dogs, rhesus monkeys, and baboons in an attempt to determine the kinds of metabolites formed. It was hoped to synthesize subsequently some of these metabolites and examine their pharmacological activities, thus adding to the understanding of the structure–activity relationships within this class of potent drugs. The results obtained with rhesus monkeys and baboons did not differ appreciably. Therefore, for the most part, only the results found with dogs and rhesus monkeys, the subhuman primate species more commonly employed in experimental studies, are presented here.

[1] Prolixin.

Table I—Chromatographic Systems Employed to Separate Fluphenazine-^{14}C and Its Metabolites

System Number	Solvent System[a]	R_f Fluphenazine	R_f Fluphenazine Sulfoxide	R_f Metabolite C
1	Chloroform–95% ethanol (9:1)	0.37	0.11	—
2	Chloroform–95% ethanol–ammonia (80:20:1)	0.91	0.83	—
3	Benzene–ammonia–dioxane (10:10:80)	0.70	0.42	0.62
4	Benzene–ammonia–dioxane (60:5:35)	0.41	0.06	0.05
5	Chloroform–95% ethanol–ammonia (10:85:2.5)	0.82	0.58	0.60
6	Chloroform–100% ethanol–ammonia (80:10:1)	0.69	0.47	0.18
7	Chloroform–95% ethanol–ammonia (20:75:1)	0.82	0.37	—

[a] Activated thin-layer plates coated with silica gel PF (Brinkmann) were used.

METHODS AND MATERIALS

Purity and Specific Activity of Fluphenazine-^{14}C—Fluphenazine-^{14}C dihydrochloride was synthesized[2] and had a specific activity of 5.5 μc./mg. and a radiochemical purity of 98.4%, as determined by chromatography in Solvent Systems 3 and 4 (Table I).

Animals—Rhesus monkeys[3] (2.0 kg.) were maintained on a diet of Purina Monkey Chow. Primates and pure-bred beagles (7.8–14.9 kg.) were housed in cages that allowed for the separate collection of urine and feces. All animals received about 50 μc. of fluphenazine-^{14}C. Samples of blood were collected at frequent intervals from the jugular veins of the dogs or from the femoral veins of the primates. Samples of urine or feces were collected from all animals every 24 hr. for 14 days. All samples were stored in the frozen state while awaiting analysis.

Animal Surgery—A pure-bred male beagle was anesthetized with pentobarbital (30 mg./kg. i.v.). The radial vein of the dog was infused with a buffered solution of mannitol and pentobarbital at the rate of about 3 ml./min. during the entire 7-hr. experiment. The solution contained: mannitol, 100 g.; KH_2PO_4, 0.2 g.; K_2HPO_4, 0.9 g.; pentobarbital, 25.5 mg./kg. of body weight; and sufficient water to make a final volume of 2 l. An endotracheal tube was inserted in the trachea and a catheter in the urinary bladder. After a midline incision was made, the entrance to the gall bladder from the bile duct was clamped, and the common bile duct was cannulated with polyethylene tubing (No. 100) near its entrance into the duodenum.

Measurement of $^{14}CO_2$ Evolution—To remove respiratory water from the air expired by the dog through the endotracheal tube, it was led through a trap kept cold with dry ice. The air stream was then bubbled through a trap containing 250 ml. of a mixture of 2-methoxyethanol and 2-aminoethanol (3:1). The system was connected to a vacuum line with an appropriate bleeder valve so that the dogs were able to obtain enough air through a twoway valve located just past the endotracheal tube and still force the expired air through the CO_2-trap. The contents of the CO_2-trap were renewed every 105

[2] By Mr. A. Restivo.
[3] Obtained from Primate Imports, Inc.

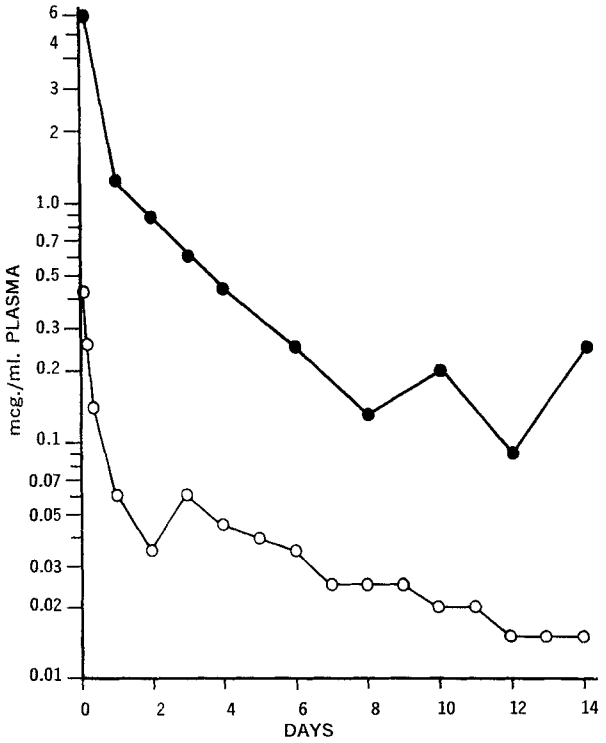


Figure 1—*Levels of fluphenazine-^{14}C equivalents in the plasma of dogs after 10 or 40 mg./kg. p.o. Key: ●, 40 mg./kg.; and ○, 10 mg./kg.*

min. The trapping solution was analyzed for the presence of radioactivity by placing 0.5 ml. into 1.0 ml. of solubilizer[4], followed by 15 ml. of Bray's scintillation fluid (1).

Analysis of Blood and Plasma—A 0.2-ml. sample of heparinized blood was digested in 0.5 ml. of 1.0 *N* NaOH by being heated overnight at 80°. The sample was then bleached with 30% hydrogen peroxide, neutralized with 0.2 ml. of 2-ethylhexanoic acid, and counted in 15 ml. of Bray's scintillation fluid.

A 0.4-ml. sample of plasma was dissolved in 2 ml. of solubilizer[4] and counted in 15 ml. of toluene scintillation fluid. This scintillation fluid contained, per liter of toluene, 5 g. of 2,5-diphenyloxazole and 300 mg. of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

Analysis of Urine and Bile—Samples of urine and bile were counted directly in 15 ml. of Bray's scintillation fluid.

Analysis of Feces—Fecal samples were homogenized in 2–3 volumes of methanol. A 1-g. sample of the homogenate was transferred to a vial, and 5 ml. of 0.5 *N* sodium hydroxide was added. The sample was digested for 16 hr. at 80°. One milliliter of the digested material was transferred to another vial, bleached with 30% hydrogen peroxide, neutralized with 0.2 ml. of 2-ethylhexanoic acid, and counted in 15 ml. of Bray's scintillation fluid. Certain samples of feces that contained the major portion of the dose were also assayed by the combustion method of Kelly *et al.* (2), as described by Ebert and Hess (3).

Extraction Procedure for Feces—A sample of feces was homogenized with 2–3 volumes of methanol and then centrifuged; the supernatant fluid was recovered and saved. Chloroform–methanol (1:1), equal in volume to the supernatant fluid recovered, was added to the fecal residue, and the mixture was shaken for 30 min. and centrifuged; the supernatant fluid was saved. The supernatants were combined and spotted directly for chromatography or were first concentrated to a suitable volume at 40–50° under reduced pressure. This extraction procedure removed an average of 71% of the radioactivity present in the feces.

Analysis of Tissues—The brain was grossly dissected into the cortex and subcortex, cerebellum, brain stem, and dorsal and hypothalamus (4). All samples of brain and other tissues taken at autopsy were homogenized with 2–3 volumes of methanol. About

[4] NCS, Amersham/Searle.

Table II—Summary of the Excretion of Fluphenazine-^{14}C and/or Its Metabolites by Dogs and Rhesus Monkeys

Animal	Dose, mg./kg.	Sex	Percent of Dose		
			Urine	Feces	Total
Dog 143[a]	10	M	1.86	88.99	90.85
Dog 144[a]	10	F	3.88	75.09	78.97
Monkey 231[a]	10	M	19.35	56.43	75.78
Monkey 232[a]	10	F	12.38	68.54	80.92
Dog Q7-5[a]	40	M	10.15	84.69	94.84
Dog Q8-137[a]	40	F	5.11	89.19	94.29
Dog 131[b]	40	M	5.77	109.86	115.63
Dog 107[b]	40	F	18.04	95.97	114.01

[a] Acute drug administration. [b] Nonradioactive fluphenazine was administered orally at 40 mg./kg./day prior to and following the administration of fluphenazine-^{14}C.

0.2 g. of homogenate was solubilized in 2 ml. of solubilizer[4] and counted in 15 ml. of toluene scintillation fluid.

Instrumentation—All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3375. Counting efficiency was determined by the use of external standardization. Chromatograms were examined for the presence of radioactivity with an Actigraph III scanner[5].

Enzymatic Hydrolysis—Samples of bile (0.5 ml.) were mixed with 0.4 ml. of 0.1 *M* acetate buffer, pH 4.5, and incubated at 37° with either 0.2 ml. of β-glucuronidase[6] or about 1 mg. of aryl sulfatase (Sigma, type III) for 18 or 3 hr., respectively.

Chromatography—With the exception of feces, all samples were spotted on thin-layer plates without prior extraction. Several solvent systems were employed to separate fluphenazine-^{14}C from its metabolites. These different solvent systems and the R_f values of the reference compounds are listed in Table I. Solvent Systems 3 and 4 were routinely employed to detect the presence of any unchanged fluphenazine-^{14}C. In Solvent System 4, metabolites were retained at the origin. Solvent Systems 5 and 6 were routinely employed to separate the polar metabolites. The other solvent systems were employed occasionally, as described under *Results*.

RESULTS

Plasma Levels—Figure 1 depicts the levels of fluphenazine-^{14}C equivalents present in the plasma of dogs that received 10 or 40 mg./kg. p.o. The kinetics of the elimination of radioactivity during the 24 hr. following drug administration (data not shown) indicate the occurrence of two first-order rate processes, with half-lives of 3–5 and 12–13 hr., respectively. Thereafter, the plasma levels decline with a half-life of 5–6 days in the dogs that received 10

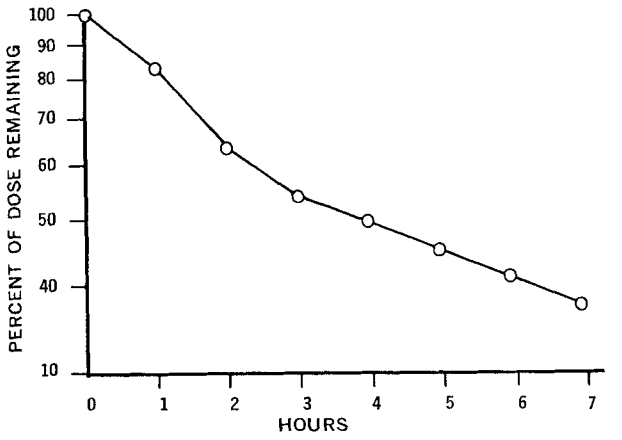


Figure 2—*Excretion of fluphenazine-^{14}C and/or its metabolites in the urine, bile, and expired air. A dog with an externalized bile duct received 10 mg./kg. i.v. of drug.*

[5] Nuclear Chicago.
[6] Ketodase, Warner Chilcott.

Table III—Excretion of Fluphenazine-^{14}C and/or Its Metabolites in the Urine, Bile, and Expired Air of Dog Q8-262[a]

Hours	Percent of Dose		
	Urine	Bile	$^{14}CO_2$
1.0	0.29	16.86	—
1.75	—	—	0.007
2.0	0.22	19.27	—
3.0	0.18	9.52	—
3.5	—	—	0.006
4.0	0.14	4.47	—
5.0	0.13	4.40	—
5.25	—	—	0.006
6.0	0.11	3.83	—
7.0	0.11	3.64	0.006
	1.18	61.99	0.025

[a] A female beagle received 10 mg./kg. i.v. of fluphenazine-^{14}C.

mg./kg. of drug; a good linear fit of the data was obtained (Fig. 1). Monkeys exhibited elimination kinetics similar in their magnitude to those found for the dogs, except that the phase having the shortest half-life in the dogs (3–5 hr.) was not present for the monkeys. In general, the complexity of the elimination kinetics points to the formation of a broad spectrum of metabolites.

The levels of radioactivity found in the plasma of the dogs that received 40 mg./kg. were much higher than those in dogs given 10 mg./kg. of fluphenazine-^{14}C, even when the fourfold difference in the level of drug administered is taken into account. These data suggest that dogs given the higher dose of drug saturated those sites that bind fluphenazine-^{14}C, its metabolites, or both more completely than did the dogs given the lower dose. In dogs that received 40 mg./kg., a larger proportion of the drug was available to the circulation, thus accounting for the higher plasma levels.

Excretion—Table II summarizes the elimination of radioactivity in the urine and feces of dogs and rhesus monkeys. Since the administered drug may tranquilize the animals during the early part of the 14-day test, the intake of food and water may be greatly reduced, resulting in a reduced and delayed excretion of feces. Thus, the data are useful for determining the routes of elimination of the radioactivity but are difficult to interpret from a kinetic standpoint. The primary route for the excretion of fluphenazine-^{14}C, its metabolites, or both was *via* the feces for both species (10 mg./kg.). Compared with the monkeys, the dogs excreted a greater percentage of the dose in the feces. Monkeys, as opposed to dogs, are unable to tolerate oral doses of 40 mg./kg. of fluphenazine-^{14}C; thus, data are available only for dogs given this dose level. Dogs Q7-5 and Q8-137 received single oral doses of 40 mg./kg. of fluphenazine-^{14}C. Dogs 131 and 107 received oral doses of 40 mg./kg. per day of nonradioactive fluphenazine before and after a single oral dose of 40 mg./kg. of fluphenazine-^{14}C. In all four animals, the major portion of the radioactivity was again excreted in the feces and a smaller portion in the urine. However, the amount excreted in the urine by dogs given 40 mg./kg. was several times higher than that excreted by dogs given 10 mg./kg. In each case, the major portion of the radioactivity was

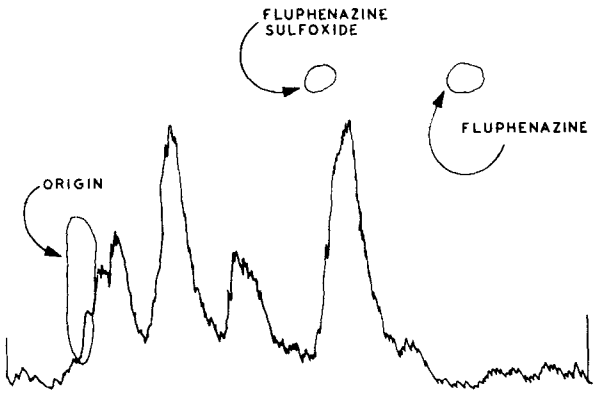


Figure 3—*Chromatogram of urine from the dog in Solvent System 3. The dog received 40 mg./kg. p.o. of fluphenazine-^{14}C.*

Table IV—Levels of Fluphenazine-^{14}C and/or Its Metabolites in Selected Tissues of Dogs

Tissue	Dog 107[a] (mcg./g.)	Dog 113[b] (mcg./g.)
Cerebellum	27.1	22.7
Brain cortex and subcortex	33.2	28.4
Dorsal and hypothalamus	33.6	25.1
Brain stem	31.8	27.9
Combined retina, choroid, and sclera	95.7	61.5
Aqueous and vitreous humors	0.31	0.19
Lens	0.32	0.23
Cornea	4.5	4.5
Heart	14.8	34.3
Kidneys	41.9	47.2
Liver	136.8	109.4
Lungs	61.3	134.3
Omental fat	8.1	7.0
Skeletal muscle	9.8	11.7
Skin with hair	17.0	12.1
Blood, mcg./ml.	1.5	1.4
Plasma, mcg./ml.	2.4	2.0

[a] A female beagle received 40 mg./kg. of fluphenazine-^{14}C by gavage. [b] A male beagle received 40 mg./kg. of fluphenazine-^{14}C by gavage.

excreted during the first 4 days, depending upon the physiological state of the particular animal. Thereafter, small amounts of radioactivity continued to be excreted in the urine and the feces for the duration of the test.

Biliary Excretion—A female beagle with an externalized bile duct received fluphenazine-^{14}C as an intravenous infusion lasting 5 min. The excretion of radioactivity by this dog in the urine and bile and as respiratory $^{14}CO_2$ is shown in Table III. The excretion of fluphenazine-^{14}C, its metabolites, or both takes place primarily in the bile; only 1.2% of the dose was eliminated in the urine. Small

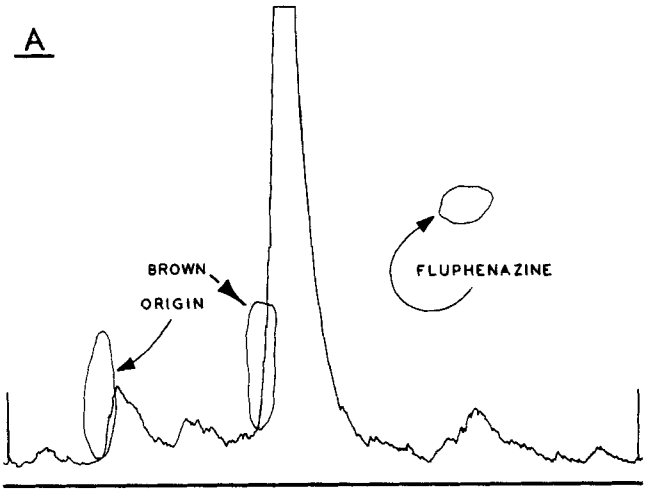


Figure 4—*Chromatograms of extracts of feces from dogs in Solvent System 6. Dogs received: (A) 10 mg./kg. p.o. of fluphenazine-^{14}C, or (B) 40 mg./kg. p.o. of fluphenazine-^{14}C.*

Table V—Summary of the Metabolism of Fluphenazine-^{14}C in the Urine of Dogs and Rhesus Monkeys

Animal	Time of Sample, hr.	Percent of Dose: Fluphenazine Sulfoxide	Percent of Dose: Fluphenazine-^{14}C
Dog 144[a]	0–4	0.95	1.14
Dog 144[a]	24–48	0.68	0.16
Monkey 231[a]	0–4	3.96	0.00
Monkey 232[a]	0–4	1.89	0.00
Dog Q7-5[b]	0–8	2.68	0.92
Dog Q8-137[b]	0–8	—	—
Dog 131[c]	8–24	1.85	0.13
Dog 107[c]	0–8	6.59	0.38

[a] Acute drug administration (10 mg./kg. p.o.). [b] Acute drug administration (40 mg./kg. p.o.). [c] Nonradioactive fluphenazine was administered orally at 40 mg./kg./day prior to and following a single dose of fluphenazine-^{14}C (40 mg./kg. p.o.).

amounts of radioactivity were eliminated in the expired air as $^{14}CO_2$. During the 7 hr. of the test, a total of 63% of the dose was excreted. The excretion of fluphenazine-^{14}C, its metabolites, or both is plotted in Fig. 2. The results show clearly that the radioactivity is eliminated according to a biphasic pattern.

Tissue Distribution—Two dogs, one male and one female, used in a chronic toxicity test, received *nonradioactive* fluphenazine dihydrochloride orally at a level of 40 mg./kg. per day for 52 weeks. Both dogs received a single dose of 40 mg./kg. of fluphenazine-^{14}C by gavage. The animals were sacrificed 19 hr. later, and various tissues were removed and analyzed for the presence of fluphenazine-^{14}C, its metabolites, or both (Table IV). Radioactivity was present in the four areas of the brain examined in approximately equal concentrations. Other organs with concentrations of radioactivity equal to or greater than those in the brain were the kidneys, liver, lungs, and the combined retinal, choroid, and scleral layers of the eye. The concentrations of radioactivity present in the brains of Dogs 107 and 113 were 18–21 times higher than those found in the blood at the times the animals were sacrificed. The dog with the externalized bile duct, which had received 10 mg./kg. i.v. of fluphenazine-^{14}C, had 20 times more radioactivity in the brain at autopsy than had been present in the blood at the time of sacrifice (7 hr. after drug administration). Thus, it is apparent that fluphenazine-^{14}C, its metabolites, or both are highly localized in the various tissues of the body including the target organ, the brain.

Biotransformation—The amounts of unchanged fluphenazine-^{14}C and fluphenazine sulfoxide present in the urine of dogs and monkeys are summarized in Table V. In the urine of the two species, about 1% of the dose or less is excreted as unchanged fluphenazine-^{14}C. Fluphenazine sulfoxide appears to be present in the urine to the extent of 4% of the dose or less. Fluphenazine sulfoxide was identified in the urine of Dog 144 by chromatography in Solvent Systems 3 and 4, with authentic fluphenazine sulfoxide as a reference. Each chromatogram yielded three radioactive peaks, one of which, in each case, corresponded in its R_f value to that of fluphenazine sulfoxide.

Table VI—Summary of the Metabolism of Fluphenazine-^{14}C in the Feces of Dogs and Rhesus Monkeys

Animal	Time of Sample, hr.	Percent of Dose: A	B	C	Fluphenazine-^{14}C
Dog 143[a]	72–96	2.04	8.45	72.5	2.84
Dog 144[a]	48–72	2.55	9.54	53.8	5.32
Dog 144[a]	72–96	1.43	5.03	57.9	4.06
Monkey 231[a]	24–48	3.49	5.70	30.8	9.70
Monkey 232[a]	0–24	3.42	6.51	39.4	13.4
Dog Q7-5[b]	24–48	2.94	3.44	20.2	17.6
Dog Q8-137[b]	24–48	13.0	8.29	20.6	3.12
Dog 131[c]	24–48	0.98	6.76	18.5	35.5
Dog 107[c]	24–48	0.58	7.12	29.0	26.6

[a] Acute drug administration (10 mg./kg. p.o.). [b] Acute drug administration (40 mg./kg. p.o.). [c] Nonradioactive fluphenazine was administered orally at 40 mg./kg./day prior to and following a single dose of fluphenazine-^{14}C (40 mg./kg. p.o.).

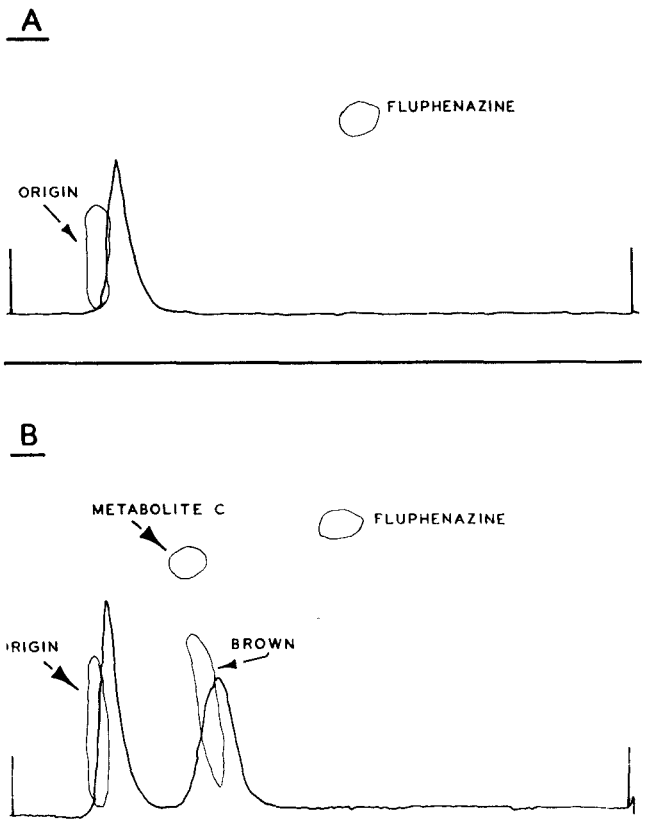


Figure 5—*Chromatograms of bile (0–1 hr.) from the dog (A) before and (B) after treatment with β-glucuronidase. Solvent System 6 was employed. The dog received 10 mg./kg. i.v. of fluphenazine-^{14}C.*

The amounts of fluphenazine and its sulfoxide present in the urine of dogs do not vary significantly as the dose administered is raised from 10 to 40 mg./kg. Figure 3 illustrates a chromatogram of urine from Dog 131 collected during the interval of 0–8 hr. A prominent peak corresponds in R_f value to that of fluphenazine sulfoxide; three, possibly four, other radioactive components are also observed.

Table VI summarizes the disposition of fluphenazine-^{14}C in the feces of dogs and monkeys. The distribution of metabolites in the feces of baboons was essentially similar to that for the rhesus monkey. Fluphenazine-^{14}C was identified in the feces of a baboon by chromatography in Solvent Systems 1, 2, and 3. All three chromatograms showed a minor peak that corresponded, in each case, with the R_f value of authentic fluphenazine. In the feces of animals that received oral doses of 10 mg./kg. of fluphenazine-^{14}C, the unaltered drug was excreted in the amounts of 3–5% of the dose by the dogs and 10–13% of the dose by the monkeys. One major and two minor components are present for each animal species. The results for the dog appear to represent the simplest case, since most of the radioactivity is present in a single peak, as shown in Fig. 4A. This large peak corresponds neither to fluphenazine nor to fluphenazine sulfoxide. In fact, no radioactivity present in the fecal extracts corresponds to fluphenazine sulfoxide. Since the rhesus monkeys and baboons showed a sizable component remaining at the origin of the chromatograms, an attempt was made to separate further the possible components of this peak. The peak that remains at the origin in Solvent System 6 separated into two peaks which moved with R_f values of 0.08 and 0.32 in Solvent System 5; these metabolites are designated in Table VI as A and B, respectively. The major peak shown in the chromatogram of a fecal extract from the dog (Fig. 4A) is designated as Metabolite C, which characteristically forms a brownish zone if the thin-layer plate is allowed to stand overnight. The presence of Metabolites A, B, and C could be detected in the fecal extracts of all three species. The most notable difference in distribution of these components was that Metabolite C was more abundant in the dog than in the primates.

Evidence was obtained that, at least in the baboon, urinary metabolites were distinctly different from those present in the feces. In a chromatogram of a urine sample, essentially all of the radio-

activity moves with an R_f value of 0.1. In a chromatogram of a fecal extract, the major portion of the radioactivity moves with an R_f value of 0.68. Since both of these chromatograms were developed in Solvent System 7, the results indicate that most of the radioactivity present in the urine is not of the same nature as that found in the feces.

As the dose of fluphenazine-^{14}C administered to dogs is raised from 10 to 40 mg./kg., a shift is observed in the relative amounts of unchanged fluphenazine-^{14}C and Metabolite C (Fig. 4B and Table VI). The amount of unchanged fluphenazine-^{14}C generally ranged from 18 to 36% of the dose. The fecal extracts from Dog Q8-137 contained only 3% unchanged fluphenazine-^{14}C, but this dog excreted a correspondingly higher amount of Metabolite A than did the other three animals. Although the fecal samples analyzed were not all excreted during the same interval, in each case they represented the first samples that contained appreciable amounts or the major portion of the dose. With the exception of Dog Q8-137, the other three animals again showed that Metabolite A was the component least present in the extracts of feces. Metabolite B was the next most prominent, and Metabolite C was the most abundant biotransformation product. It is obvious that as the dose of fluphenazine-^{14}C is raised from 10 to 40 mg./kg., the amount of Metabolite C decreases and, correspondingly, the amount of fluphenazine-^{14}C increases. No obvious differences are seen between the two groups of dogs that received 40 mg./kg. of fluphenazine-^{14}C with or without pretreatment with *nonradioactive* fluphenazine.

To determine whether the metabolites present in the feces of dogs had undergone any alteration in the intestines, a sample of bile collected during the 1st hr. after an intravenous dose of 10 mg./kg. of fluphenazine-^{14}C was examined. Chromatography in Solvent System 6 showed that none of the radioactivity present corresponded to unchanged fluphenazine-^{14}C; all of the radioactivity remained at the origin (Fig. 5A). This result is in sharp contrast to the chromatogram of feces shown in Fig. 4A, where most of the radioactivity moved as a single peak with an R_f value of about 0.4. Thus, the radioactive material present in the bile of dogs is not the same as the material extracted from their feces.

To determine whether the bile contained glucuronide conjugates that could have been hydrolyzed in the intestine, a sample of bile was hydrolyzed with a β-glucuronidase preparation. Chromatography now yielded two peaks in both Solvent Systems 5 and 6 instead of the single peaks found with the unhydrolyzed samples of bile (Solvent System 6 is shown in Fig. 5B). This peak, which only appears after hydrolysis, corresponds well to the R_f value of Metabolite C. One can conclude that, at least in the dog, the major metabolite of fluphenazine-^{14}C is excreted in the bile as the glucuronide conjugate. This conjugate is subsequently hydrolyzed in the gastrointestinal tract.

DISCUSSION

There appear to be no appreciable differences between the modes of excretion of fluphenazine-^{14}C in dogs that received 10 or 40 mg./kg. orally, whether as a single dose or chronically; however, at the higher dose, more radioactivity in the feces is unchanged fluphenazine-^{14}C. Also, at the higher dose, the levels of fluphenazine-^{14}C, its metabolites, or both present in the blood and plasma were much higher than would be expected from the fourfold difference in dose level, suggesting that saturable tissue-binding sites are present.

The localization of phenothiazine derivatives and their metabolites in body tissues has been well documented (5). In fact, the pharmacological effects of this class of drugs appear to be closely related to their distribution in certain tissues, like the brain. In the present studies, the authors were unable to demonstrate that fluphenazine-^{14}C or its metabolites localized preferentially in any of the gross anatomic areas of the brain.

Metabolite C is excreted in the bile of the dog as the glucuronide conjugate but appears in the feces as the phenolic compound. The hydrolysis of glucuronide conjugates by intestinal microorganisms has been well documented (6). Sulfoxidation, a metabolic pathway for the conversion of chlorpromazine and related compounds (7), also occurs with fluphenazine in rats (8), in man (9), and, as shown in the present study, in dogs and monkeys, although the excretion of fluphenazine sulfoxide in the urine accounts for only a small percentage of the dose.

As discussed in a companion article (10), Metabolite C was identified as 7-hydroxyfluphenazine.

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Identification of 7-Hydroxyfluphenazine as Major Metabolite of Fluphenazine-^{14}C in the Dog

JACQUES DREYFUSS and ALLEN I. COHEN

Abstract □ The major metabolite of fluphenazine-^{14}C {4-[1-^{14}C-3-[2-(trifluoromethyl)phenothiazin-10-yl]propyl]-1-piperazineethanol dihydrochloride} in dogs and rhesus monkeys is 7-hydroxyfluphenazine. This metabolite was isolated from the feces of dogs by a combination of solvent extraction, column chromatography, and preparative TLC. Mass spectral data and 220-MHz. NMR spectroscopy indicated that the metabolite was hydroxylated on either the C-7 or C-8 position of the phenothiazine ring system. Definitive identification of the metabolite as 7-hydroxyfluphenazine was provided by a comparison of the spectral and chromatographic properties of the metabolite with authentic samples of 7- and 8-hydroxyfluphenazine.

Keyphrases □ Fluphenazine-^{14}C metabolite—identification □ 7-Hydroxyfluphenazine—fluphenazine metabolite □ Fecal excretion—7-hydroxyfluphenazine □ Chromatography, column, thin-layer—isolation □ Mass spectroscopy—identification □ NMR spectroscopy—identification

A companion publication (1) reported the metabolic disposition of fluphenazine-^{14}C in the dog and rhesus monkey. That study pointed out that a major metabolite, designated "Metabolite C," was excreted in the feces of both species but was present in up to 73% of the dose in the feces of dogs. In addition, Metabolite C was present as the glucuronide conjugate in the bile of dogs but was found unconjugated in the feces.

This paper describes the isolation of Metabolite C from the feces of dogs and its unequivocal identification, with the aid of 220-MHz. NMR spectroscopy, as 7-hydroxyfluphenazine.

MATERIALS AND METHODS

Isolation of Metabolite C from Dog Feces—Dogs 143 and 144 (see *Reference 1*, Table II) were given 10 mg./kg. of fluphenazine-^{14}C orally. An average of 72% of the dose was excreted in the feces on the 4th day after dosing. These fecal samples were homogenized in 2–3 volumes of methanol; the homogenates were pooled and centrifuged, and the supernatant fluid (212 ml.) was recovered and saved. The remaining fecal residue was extracted with 212 ml. of chloroform–methanol (1:1) by shaking for 30 min. followed by centrifugation. The supernatant fluid was recovered and saved. The remaining residue was extracted a second time with 212 ml. of chloroform–methanol (1:1). The extracts were combined and evaporated at 30–40° under reduced pressure to about 100 ml. This procedure extracted 94% of the radioactivity present in the pooled fecal homogenate.

The concentrated extract was acidified to a pH of 1–2 with 6 *N* HCl. Enough alumina, grade 1 (Woelm), which had been previously washed with chloroform–methanol (5:1), was added to the concentrated extract to form a thick paste, which was dried overnight *in vacuo* at 37°. The dried sample was placed on top of a column of dry alumina, grade III (Woelm), 10 cm. high by 5 cm. wide, prepared, and eluted with chloroform–methanol (5:1) according to the method of Loev and Snader (2). Six fractions of 50 ml. each were collected. Fractions 2–4 were evaporated to about 8 ml., spotted on activated silica gel PF chromatograms (Brinkmann), 1 mm. thick, and developed in chloroform–100% ethanol–ammonia (80:10:1). The silica gel containing a UV-positive zone, migrating with an R_f of 0.25, was removed from the plate, packed into a small column, and eluted with chloroform–methanol (5:1). The eluate from the column was pooled and evaporated to about 3 ml.

To remove a number of fluorescent impurities, the sample was spotted on preparative thin-layer chromatograms coated with silica gel PF, 1 mm. thick, and developed in benzene–ammonia–dioxane (10:10:80). The silica gel containing a UV-positive zone, moving with an R_f of 0.4, was removed, packed into a short column, and eluted with chloroform–methanol (5:1). The concentrated eluate contained about 18 mg. of final product as the free base. This isolated metabolite, designated Metabolite C, was chromatographed in chloroform–100% ethanol–ammonia (80:10:1) and had a radiochemical purity of 93%. The presence of a radioactive component, representing 6% of the radioactivity applied to the plate, was also observed.

To determine whether the procedure described for the isolation of Metabolite C from the feces resulted in any degradation of the compound, authentic fluphenazine-^{14}C dihydrochloride or 7-hydroxyfluphenazine dihydrochloride was added to samples of fecal homogenates, obtained from dogs before dosing. The samples were extracted and then chromatographed, first on the alumina column and, subsequently, on the two silica gel chromatograms as described previously. This procedure for fluphenazine-^{14}C yielded a single peak, representing 98.5% of the radioactivity applied on the second silica gel plate and corresponded to the R_f value of authentic fluphenazine. Similarly, for 7-hydroxyfluphenazine, only a single UV-positive spot was found that corresponded to the R_f value of authentic 7-hydroxyfluphenazine in the two solvent systems used to develop the silica gel-coated plates. These results indicate that neither fluphenazine-^{14}C nor 7-hydroxyfluphenazine was degraded by the procedure used to isolate Metabolite C.

Identification of Metabolite C—NMR spectra were obtained on either a Varian Associates model A60 or HR220 spectrometer. Mass spectra were obtained on an Associated Electrical Industries

Table I—High-Resolution Mass Spectral Data for Metabolite C and Fluphenazine

Metabolite C		Fluphenazine	
Observed Mass	Formula[a]	Observed Mass	Formula[b]
453.1663	$C_{22}H_{26}N_3O_2SF_3$	437.1753	$C_{22}H_{26}N_3OSF_3$
435.1570	$C_{22}H_{24}N_3OSF_3$	419.1637	$C_{22}H_{24}N_3SF_3$
422.1492	$C_{21}H_{23}N_3OSF_3$	406.1550	$C_{21}H_{23}N_3SF_3$
409.1424	$C_{20}H_{22}N_3OSF_3$	393.1481	$C_{20}H_{22}N_3SF_3$
366.1025	$C_{18}H_{17}N_2OSF_3$	350.1053	$C_{18}H_{17}N_2SF_3$
352.0842	$C_{17}H_{15}N_2OSF_3$	336.0909	$C_{17}H_{15}N_2SF_3$
323.0587	$C_{16}H_{12}NOSF_3$	307.0632	$C_{16}H_{12}NSF_3$
322.0512	$C_{16}H_{11}NOSF_3$	306.0562	$C_{16}H_{11}NSF_3$
309.0443	$C_{15}H_{10}NOSF_3$	293.0486	$C_{15}H_{10}NSF_3$
296.0359	$C_{14}H_9NOSF_3$	280.0407	$C_{14}H_9NSF_3$
283.0268	$C_{13}H_8NOSF_3$	267.0332	$C_{13}H_8NSF_3$
282.0208	$C_{13}H_7NOSF_3$	266.0256	$C_{13}H_7NSF_3$
264.0640	$C_{14}H_9NOF_3$	248.0686	$C_{14}H_9NF_3$
171.1492	$C_9H_{19}N_2O$	171.1497	$C_9H_{19}N_2O$
157.1332	$C_8H_{17}N_2O$	157.1335	$C_8H_{17}N_2O$
143.1179	$C_7H_{15}N_2O$	143.1184	$C_7H_{15}N_2O$
125.1075	$C_7H_{13}N_2$	125.1070	$C_7H_{13}N_2$
113.1077	$C_6H_{13}N_2$	113.1084	$C_6H_{13}N_2$
100.0762	$C_5H_{10}NO$	100.0760	$C_5H_{10}NO$
98.0960	$C_6H_{12}N$	98.0958	$C_6H_{12}N$
98.0844	$C_5H_{10}N_2$	98.0843	$C_5H_{10}N_2$
84[c]	$C_5H_{10}N$	84.0810	$C_5H_{10}N$
70[c]	C_4H_8N	70.0653	C_4H_8N

[a] Calculated masses agreed within 2.3 millimass units of found masses, except for the M^+, which deviated by 3.6 millimass units. [b] Calculated masses agreed within 1.6 millimass units of found values. [c] Exact mass not measured.

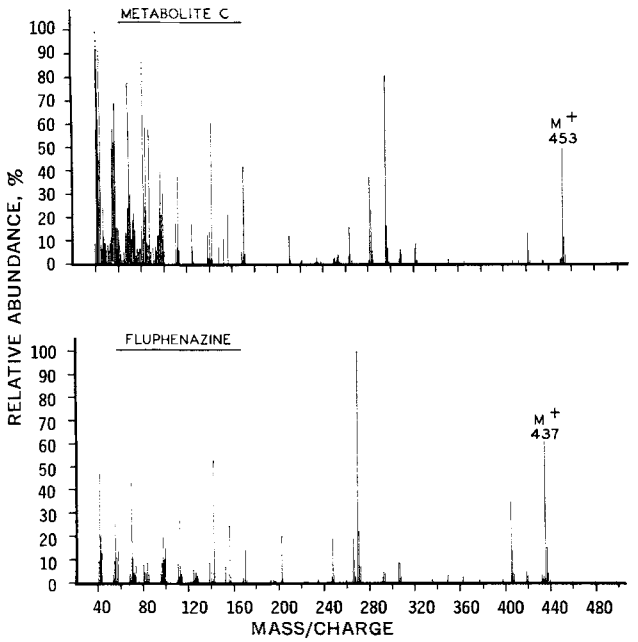

Figure 1—*Mass spectra of Metabolite C and fluphenazine.*

model MS902 spectrometer. Elemental compositions for selected mass spectral fragments were obtained either by peak-matching or by computer analysis of data recorded from the MS902 spectrometer.

RESULTS

The low-resolution mass spectra for Metabolite C and authentic fluphenazine dihydrochloride are shown in Fig. 1. The molecular ion for fluphenazine is found at *m/e* 437, which agrees with the calculated molecular weight for the free base. The molecular ion for Metabolite C is found at *m/e* 453, 16 a.m.u. higher than that of the parent molecule. In addition, prominent ions in Metabolite C are shifted 16 a.m.u. higher than the corresponding fragments observed for fluphenazine. A more detailed analysis for these fragments is provided by the data shown in Table I, which were obtained under conditions of high resolution.

By the reasoning of the mass spectrometric shift technique introduced by Biemann (3), a series of fragments occurs which is derived either from the side chain or from the tricyclic ring system. Those fragment ions arising exclusively from the alicyclic portion of the molecule, ions having *m/e* up to 171 a.m.u., were identical in both Metabolite C and fluphenazine. The empirical formulas obtained from the observed masses for Metabolite C and fluphenazine confirm that the additional 16 a.m.u. represents the introduction of an oxygen atom into the parent molecule. A series of fragments is observed having lower masses than the parent ions, which, for Metabolite C, contain an additional oxygen which is not present for the fragment ions of fluphenazine. Since the additional oxygen is not present on the piperazine-containing side chain, it must be located on the tricyclic ring system. The mass spectrum obtained with an authentic sample of fluphenazine sulfoxide indicated that Metabolite C was not the sulfoxide derivative.

Duffield *et al.* (4) reported that mass spectrometry is able to distinguish between oxygenation at C-3 *versus* C-6, C-7, and C-8 in chlorpromazine and 2-chlorophenothiazine. These authors also pointed out that the introduction of a side chain like dimethylaminopropyl to the phenothiazine ring system dramatically alters the mass spectral fragmentation process when compared with those compounds not having a side chain. In this study, the authors were unable to reason in an analogous manner from the fragmentation pattern reported for the oxygenated isomers of chlorpromazine (4), since fluphenazine contains a piperazine-containing side chain which results in a fragmentation pattern different from that observed when the phenothiazine ring system contains a dimethylaminopropyl side chain (5). Since they did not have a derivative of fluphenazine oxygenated at the C-3 position as a reference compound, they were not able to take an approach similar to that described for other substituted phenothiazines (4). Thus, localization of the additional oxygen atom of Metabolite C to either of the aromatic rings of the phenothiazine ring system was not possible on the basis of mass spectral data.

NMR spectroscopy was employed to obtain additional information regarding the location of the additional oxygen atom in Metabolite C. Examination of the 60-MHz. spectra for authentic fluphenazine base (not shown) and Metabolite C (Fig. 2) indicated that differences in the spectra had occurred only in the region of the aromatic proton resonances. This portion of the spectrum for Metabolite C contained six protons, whereas that for authentic fluphenazine contained seven protons, thus establishing Metabolite C as a phenolic compound. Since the three protons that resonate between 2.8 and 3.0τ correspond to the C-1, C-3, and C-4 protons of fluphenazine, it was possible to say that the hydroxyl group was not substituted on the ring bearing the trifluoromethyl group.

Craig and his coworkers (6, 7) reported that increased resolution was obtained for hydroxyl derivatives of chlorpromazine at 220 MHz., thus permitting positive structural assignments of these phenothiazine metabolites. The 220-MHz. spectrum for Metabolite C is shown in Fig. 3. The three protons resonating between 2.7 and 2.9τ were assigned to the ring containing the trifluoromethyl group, because the C-1 proton is coupled only to a *meta*-proton, while the C-4 proton is coupled only to an *ortho*-proton; the C-3 proton is coupled to both the C-1 and C-4 protons.

The remaining peaks in the spectrum, occurring from 3.1 to 3.4τ, are present as two sets of doublets and a quartet. The assignment of these three resonances is based on the fact that the low-field doublet at 3.13τ is coupled only to the intermediate-field quartet at 3.35τ which, in turn, is coupled to the *meta*-proton resonating at 3.40τ. The magnitude of the coupling constants between *ortho*-hydrogens is 7–9.2 Hz.; between *meta*-hydrogens, it is 1.1–3.1 Hz.; and between *para*-hydrogens, it is 0.0–0.7 Hz. (8). The coupling constants between the aromatic protons of Metabolite C, shown in Fig. 3, fall in the range given for *meta*- and *ortho*-coupled protons. These relationships among the protons only permit substitution of the hydroxyl group at C-7 or C-8.

To establish the structure of Metabolite C unequivocally, the IR and NMR spectra of Metabolite C were compared with those of authentic 7- and 8-hydroxyfluphenazine. Examination of the NMR spectra indicated that the aromatic proton resonances for 7- and 8-hydroxyfluphenazine were distinctly different at 60 MHz., whereas the spectra for 7-hydroxyfluphenazine and Metabolite C were superimposable at both 60 *and* 220 MHz. The IR spectra of 7-hydroxyfluphenazine and Metabolite C were also identical in all major features, except that more of the fine structure was exhibited by the authentic compound than by the isolated metabolite, a finding attributed to the greater purity and the crystalline properties of the former compound.

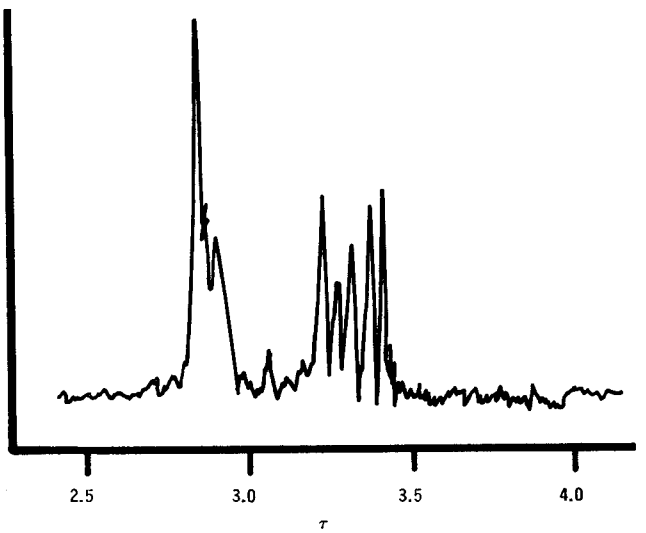

Figure 2—*NMR spectrum (60 MHz.) for the aromatic proton resonances of Metabolite C. The sample was dissolved in perdeuteriomethanol, with tetramethylsilane as a reference.*

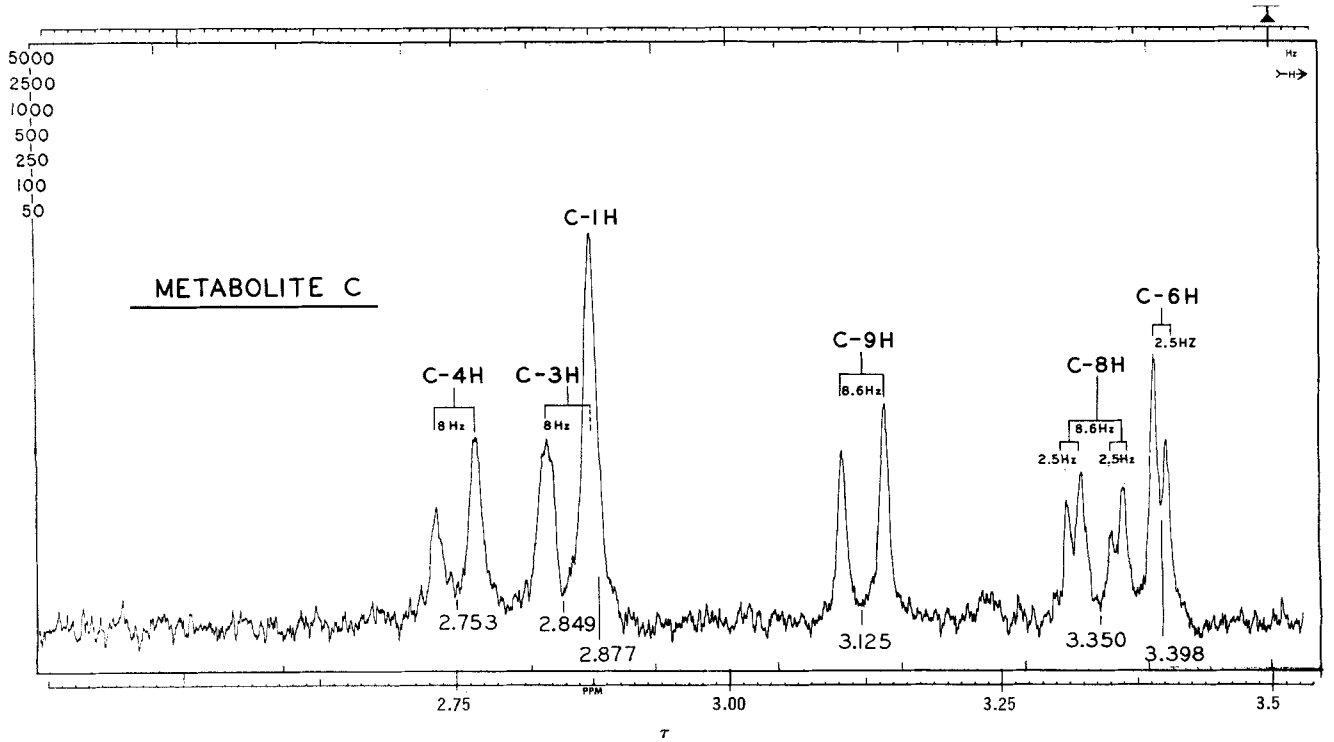

Figure 3—*NMR spectrum (220 MHz.) for the aromatic proton resonances of Metabolite C. The sample was dissolved in perdeuteriomethanol, with tetramethylsilane as a reference. The proton assignments are discussed in the text.*

TLC also confirmed the identity of Metabolite C as 7-hydroxyfluphenazine, since these compounds had identical R_f values in Solvent Systems 3, 4, and 6 (*Reference 1*, Table I). Both compounds exhibited the same reddish-brown color when the plates were allowed to stand in the laboratory for several hours. On the other hand, 8-hydroxyfluphenazine had a consistently lower R_f value than did Metabolite C in Solvent Systems 4 and 6 and, on standing, exhibited a yellow color.

DISCUSSION

The biotransformation of fluphenazine appears to resemble that previously reported for chlorpromazine (9–11). Both compounds are converted to the sulfoxide, a urinary metabolite, and to hydroxylated derivatives. In chlorpromazine, hydroxylation occurs in the dog and man primarily at C-7 and, to a lesser extent, at C-3. In the present study with fluphenazine (1), not all urinary and fecal metabolites were identified; however, if hydroxylation at C-3 does occur in the dog or rhesus monkey, it is a minor pathway. The metabolism of chlorpromazine and other substituted phenothiazines has also been studied in liver preparations *in vitro*. Under these conditions, hydroxylation of chlorpromazine at C-7 also occurred more readily than at C-3 in rat and human microsomal enzyme systems (12). Similarly, hydroxylation of various phenothiazine derivatives, including fluphenazine, with widely differing ring and side-chain substituents, occurred in rat liver preparations (13). Since fluphenazine contains a trifluoromethyl group on the phenothiazine ring system, instead of a chlorine atom as in chlorpromazine, it appears that this difference does not markedly influence the occurrence of hydroxylation at C-7. The same statement, by analogy, also applies to the lack of any marked influence of the nature of the side chain on ring hydroxylation in chlorpromazine and fluphenazine.

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Authentic samples of 7- and 8-hydroxyfluphenazine were supplied by Dr. F. Sowinski, Department of Organic Chemistry, Squibb Institute for Medical Research; the synthesis and spectra of these compounds will be published. Miss Barbara Keeler interpreted the IR spectra, and Mr. Leroy Johnson of Varian Associates determined the 220-MHz. NMR spectra.

Excretion and Biotransformation of the Enanthate Ester of Fluphenazine-^{14}C by the Dog

JACQUES DREYFUSS, JOHN J. ROSS, Jr., and ERIC C. SCHREIBER

Abstract □ Fluphenazine-^{14}C enanthate {4-[1-^{14}C-3-[2-(trifluoromethyl)phenothiazin-10-yl]propyl]-1-piperazineethanol ester with heptanoic acid} in a sesame oil formulation was administered intramuscularly to dogs. Only traces of radioactivity were found in the circulation. Excretion was predominantly in the feces; 2–3% of the dose was excreted in the urine. Radioactivity was still present at the sites of injection 21 days after the dogs had received the dose. Unformulated fluphenazine-^{14}C enanthate was excreted according to a biphasic, exponential decay curve after intravenous administration to a dog with an externalized bile duct. The half-life of the slower portion of this decay curve was considerably *faster* than that observed in dogs that had received formulated fluphenazine-^{14}C enanthate intravenously. Thus, the rate-limiting step in the biological disposition of formulated fluphenazine-^{14}C enanthate after intramuscular administration is probably the release from an oily depot rather than excretion. Unformulated fluphenazine-^{14}C enanthate, administered intramuscularly to a dog with an externalized bile duct, also represents a slow-release situation. Therefore, the sesame oil employed in the formulation serves as a convenient vehicle for the administration of the drug, although its presence is not an absolute requirement for slow release. Fluphenazine-^{14}C enanthate is hydrolyzed to fluphenazine-^{14}C by plasma esterases. Radioactivity present in the bile of a dog that had received fluphenazine-^{14}C enanthate intravenously was identical with the glucuronide conjugate of 7-hydroxyfluphenazine. Thus, fluphenazine-^{14}C enanthate appears to be metabolized by the dog according to the metabolic pathway established for fluphenazine-^{14}C.

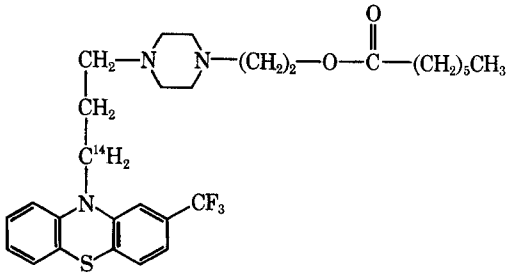
Keyphrases □ Fluphenazine-^{14}C enanthate—metabolism, excretion □ Biliary, urinary, fecal excretion—fluphenazine-^{14}C enanthate □ Tissue levels—fluphenazine-^{14}C, metabolites □ Metabolite—7-hydroxyfluphenazine glucuronide □ TLC—analysis □ Scintillometry—analysis

Fluphenazine enanthate[1] is a long-acting ester of fluphenazine. This ester (Structure I) is administered intramuscularly in a sesame oil-based formulation which permits a convenient mode of treatment for outpatients and obviates uncertainties attendant on administration of drugs to psychically disturbed individuals.

The present study with dogs was designed to answer these questions: Is the metabolism of the ester of fluphenazine similar to that of the unesterified compound, the metabolism of which was discussed in a companion publication (1)? Is the slow-release property of the formulated preparation a consequence of the physical characteristics of the drug molecule, the release characteristics of a drug molecule present in an oily depot, or both?

METHODS AND MATERIALS

Fluphenazine-^{14}C enanthate (Structure I) was synthesized[2] and had a specific activity of 2.47 μc./mg. and a radiochemical purity of 96.8%. Fluphenazine-^{14}C enanthate was injected into the thigh muscle of pure-bred beagles at a dose of 1 mg./kg. The standard formulation contained 25 mg./ml. of fluphenazine-^{14}C enanthate



I

Structure of fluphenazine-^{14}C enanthate

(free base), 1.5% benzyl alcohol, and sesame oil. In some experiments, the ester was administered intravenously as the dihydrochloride salt to facilitate dissolution of the compound in mixtures of water and ethanol. In other experiments, when unformulated ester was administered intramuscularly, the free base was dissolved in absolute ethanol. Methods pertaining to the collection and analysis of excreta, blood and plasma, and tissues, as well as surgical procedures, have been described (1).

Chromatography—Three solvent systems were employed to develop activated thin-layer plates of silica gel PF (Brinkmann): (*a*) 1, benzene–ammonia–dioxane (60:5:35); (*b*) 2, chloroform–95% ethanol–ammonia (10:85:2.5); and (*c*) 3, chloroform–100% ethanol–ammonia (80:10:1). The R_f values of fluphenazine-^{14}C enanthate and some of its potential metabolites are indicated on the individual chromatograms.

Hydrolysis by Plasma Esterases—Blood was obtained from several dogs of each sex and pooled. The plasma was recovered, and one portion was placed in a boiling water bath for 30 min.; the sample was centrifuged to recover the supernatant fluid. Fluphenazine-^{14}C enanthate was dissolved in 95% ethanol, and sufficient 0.1 *N* HCl was added to convert the compound to the dihydrochloride salt. The solution thus prepared contained 88 mcg. of drug per milliliter of plasma. Both undenatured and denatured plasma samples were incubated at 37° for 20 hr.; then the undenatured plasma sample was denatured with an equal volume of methanol and centrifuged. The supernatants from each tube were chromatographed in Solvent System 3.

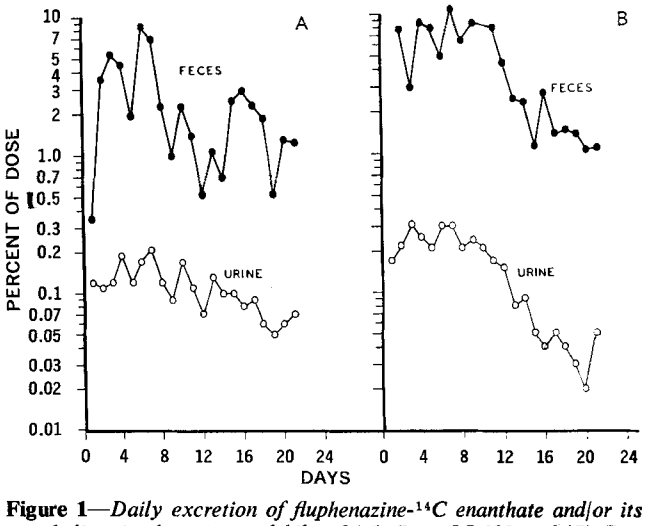


Figure 1—*Daily excretion of fluphenazine-^{14}C enanthate and/or its metabolites in the urine and bile of (A) Dog Q7-132 and (B) Dog Q7-222. The dogs received 1 mg./kg. i.m. of formulated fluphenazine-^{14}C enanthate.*

[1] Prolixin Enanthate, E. R. Squibb & Sons, Inc., New York, N. Y.
[2] By Mr. F. Dondzila.

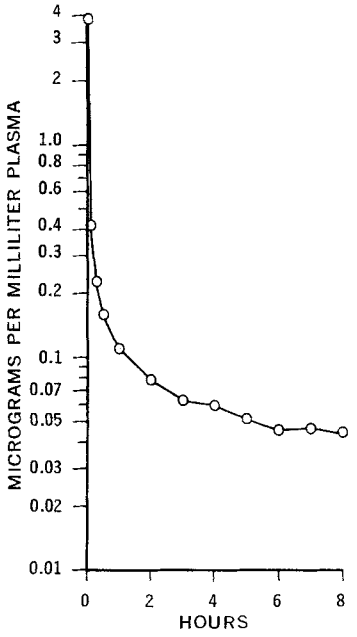


Figure 2—*Levels of fluphenazine-^{14}C enanthate equivalents in the plasma of Dog Q7-147. The dog received 1 mg./kg. i.v. of fluphenazine-^{14}C enanthate dihydrochloride in water made 28% in ethanol.*

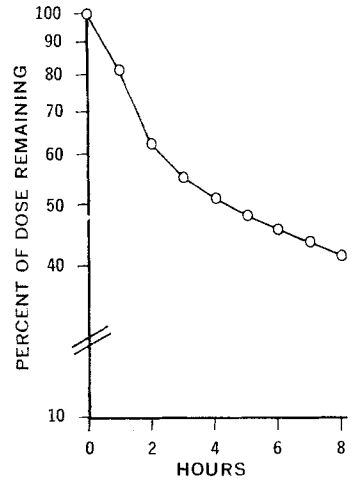


Figure 3—*Excretion of fluphenazine-^{14}C and/or its metabolites in the urine and bile of Dog Q7-147. The dog received 1 mg./kg. i.v. of fluphenazine-^{14}C enanthate dihydrochloride in water made 28% in ethanol.*

Enzymatic and Alkaline Hydrolysis of Bile—Bile (0.5 ml.) from Dog Q7-147 was placed into each of two tubes. One tube was made strongly alkaline with a small drop of 50% NaOH. To the other tube was added 0.4 ml. of 0.1 *M* acetate buffer, pH 4.5, and 0.2 ml. of β-glucuronidase (Ketodase). Alkaline hydrolysis was carried out at 90–100° for 1.5 hr. Enzymatic hydrolysis was carried out at 35–40° for 20 hr. The pH of both samples was adjusted to about 7 by the addition of acid or base. These samples were then chromatographed in Solvent Systems 2 and 3.

The reverse treatments were also conducted. The tube that had previously been made strongly alkaline had added to it 0.4 ml. of acetate buffer, pH 4.5, followed by 0.2 ml. of β-glucuronidase. The tube that had previously received β-glucuronidase was made strongly alkaline with NaOH. The conditions for incubation were the same as described previously. After incubation, the pH of the samples was again adjusted to about 7 and they were chromatographed in Solvent Systems 2 and 3.

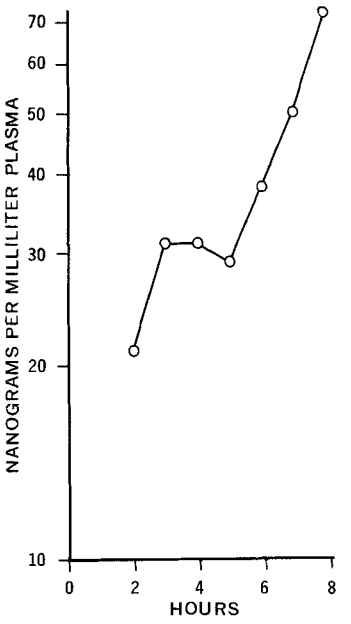


Figure 4—*Levels of fluphenazine-^{14}C enanthate equivalents in the plasma of Dog Q7-111. The dog received 1 mg./kg. i.m. of fluphenazine-^{14}C enanthate in absolute ethanol.*

RESULTS AND DISCUSSION

Disposition of Formulated Drug Intramuscularly—Figure 1 shows the values obtained for the daily excretion of fluphenazine-^{14}C enanthate, its metabolites, or both in the urine and feces of Dogs Q7-132 and Q7-222 after intramuscular injection of 1 mg./kg. of the standard formulation. During the 21 days, about 55–87% of the dose was excreted, chiefly in the feces; only 2–3% of the radioactivity was excreted in the urine. The rate of excretion was highest from the 4th through the 10th days. The half-lives for the excretion of radioactivity by these two animals (estimated graphically) ranged from 4 to 7 days in the urine and about 4 days in the feces. The total amounts of radioactivity excreted by Dogs Q7-132 and Q7-222, as well as the portions of the dose still present at the injection site, are summarized in Table I. A total of 74–99% of the dose was accounted for during the 21 days of the experiment. The very low levels of radioactivity found in the plasma of Dogs Q7-132 and Q7-222 ranged from 3 to 11 ng./ml. during the entire test. Two to three days after drug administration, levels of radioactivity in the plasma were found to be about one-half of the maximal levels.

Disposition of Unformulated Drug Intravenously—A dog with an externalized bile duct received a 1-mg./kg. i.v. dose of unformulated fluphenazine-^{14}C enanthate dihydrochloride. The levels of radioactivity in the plasma of this dog (Q7-147) are shown in Fig. 2. During the first several minutes following drug administration, the levels of radioactivity in the plasma declined precipitously. Elimination of radioactivity from the circulation then proceeded ever more slowly, with a half-life of about 9 hr. during the interval of 3–8 hr. During the 8-hr. experiment, only 1.40% of the dose was excreted in the urine; 56.95% was excreted in the bile. The excretion of fluphenazine-^{14}C enanthate, its metabolites, or both in the urine and bile of Dog Q7-147 is shown in Fig. 3. The decay curve fits a biphasic, exponential pattern. The portion of the curve for the first 3 hr. has a half-life of about 0.5 hr. (by the "method of residuals"), while the portion of the curve for the

Table I—Excretion of Fluphenazine-^{14}C Enanthate and/or Its Metabolites by Dogs

Dog[a]	Sex	Route	Formulated	Duration of Experiment	Percent of Dose			
					Urine	Feces or (Bile)	Injection Site	Total
Q7-132	M	i.m.	Yes	21 days	2.34	52.56	19.55	74.45
Q7-222	F	i.m.	Yes	21 days	3.19	83.88	12.12	99.19
Q7-111	M	i.m.	No	8 hr.	0.06	(0.49)	87.82	88.37
Q7-147	M	i.v.	No	8 hr.	1.40	(56.95)	—	58.35

[a] Dogs received 1 mg./kg.

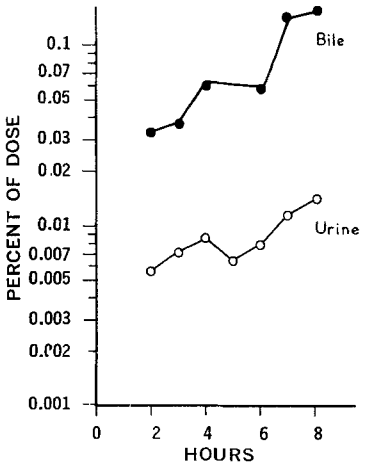


Figure 5—*Excretion of fluphenazine-^{14}C enanthate and/or its metabolites in the urine and bile of Dog Q7-111. The dog received 1 mg./kg. i.m. of fluphenazine-^{14}C enanthate in absolute ethanol.*

3rd to 8th hr. has a half-life of 12–13 hr. Even the longer of these half-lives (12–13 hr.) is much shorter than the value of 4–7 days cited earlier for the dogs that received formulated fluphenazine-^{14}C enanthate intramuscularly. Thus, when the formulated drug is administered intramuscularly, the rate-limiting step for the elimination of radioactivity from the body is not a function of the rate of excretion but probably of the rate of drug release from the site of injection.

Disposition of Unformulated Drug Intramuscularly—Unformulated fluphenazine-^{14}C enanthate was administered intramuscularly to a dog with an externalized bile duct. During the 8-hr. experiment, the levels of fluphenazine-^{14}C enanthate equivalents in the plasma of this dog (Q7-111) rose consistently (Fig. 4). The excretion of fluphenazine-^{14}C enanthate, its metabolites, or both in the urine and bile of Dog Q7-111 is shown in Fig. 5. Only about 0.55% of the dose was excreted (Table I), most of it, again, in the bile. The major portion of the dose (88%) remained at the injection site of Dog Q7-111. Thus, fluphenazine-^{14}C enanthate, which is itself an oily compound, is slowly released from the site of intramuscular injection after the unformulated drug has been administered.

Distribution—Dogs Q7-132 and Q7-222 were sacrificed at the end of 21 days. Only trace amounts of radioactivity were present in the brain, kidneys, fat, skin, and left thigh muscle (Table II). Of the tissues examined, the livers had the highest concentrations of radioactivity.

Dog Q7-111 was sacrificed after 8 hr. The lungs and liver contained the highest concentrations of radioactivity. The three areas of the brain examined showed approximately equal levels of radioactivity.

Dog Q7-147 was also sacrificed after 8 hr. All the tissues examined contained measurable amounts of radioactivity (Table

Table II—Residual Fluphenazine-^{14}C Enanthate and/or Its Metabolites in Selected Tissues of Dogs

Tissue	—After 21 Days— Dog Q7-132[a] (mcg./g.)	Dog Q7-222[a] (mcg./g.)	—After 8 Hr.— Dog Q7-111[b] (mcg./g.)	Dog Q7-147[c] (mcg./g.)
Lungs	—	—	0.20	2.73
Liver	0.35	0.25	0.20	3.69
Kidneys	0.05	0.06	0.05	0.69
Omental fat	0.03	0.03	0.03	0.24
Skin	0.03	0.01	0.02	0.15
Heart	—	—	0.06	0.42
Brain	0.04	0.03	—	—
Dorsal and hypothalamus	—	—	0.13	0.75
Brain cortex and subcortex	—	—	0.11	0.43
Brain stem	—	—	0.13	0.40
Cerebellum	—	—	—	0.52
Right thigh muscle (contains injection site)	4.78	3.61	21.77	—
Left thigh muscle	0.02	0.03	0.05	0.63

[a] Dogs Q7-132 and Q7-222 received 1 mg./kg. of formulated fluphenazine-^{14}C enanthate intramuscularly. [b] Dog Q7-111 received 1 mg./kg. of fluphenazine-^{14}C enanthate in absolute ethanol intramuscularly. [c] Dog Q7-147 received 1 mg./kg. of fluphenazine-^{14}C enanthate dihydrochloride in water made 28% with respect to ethanol, intravenously.

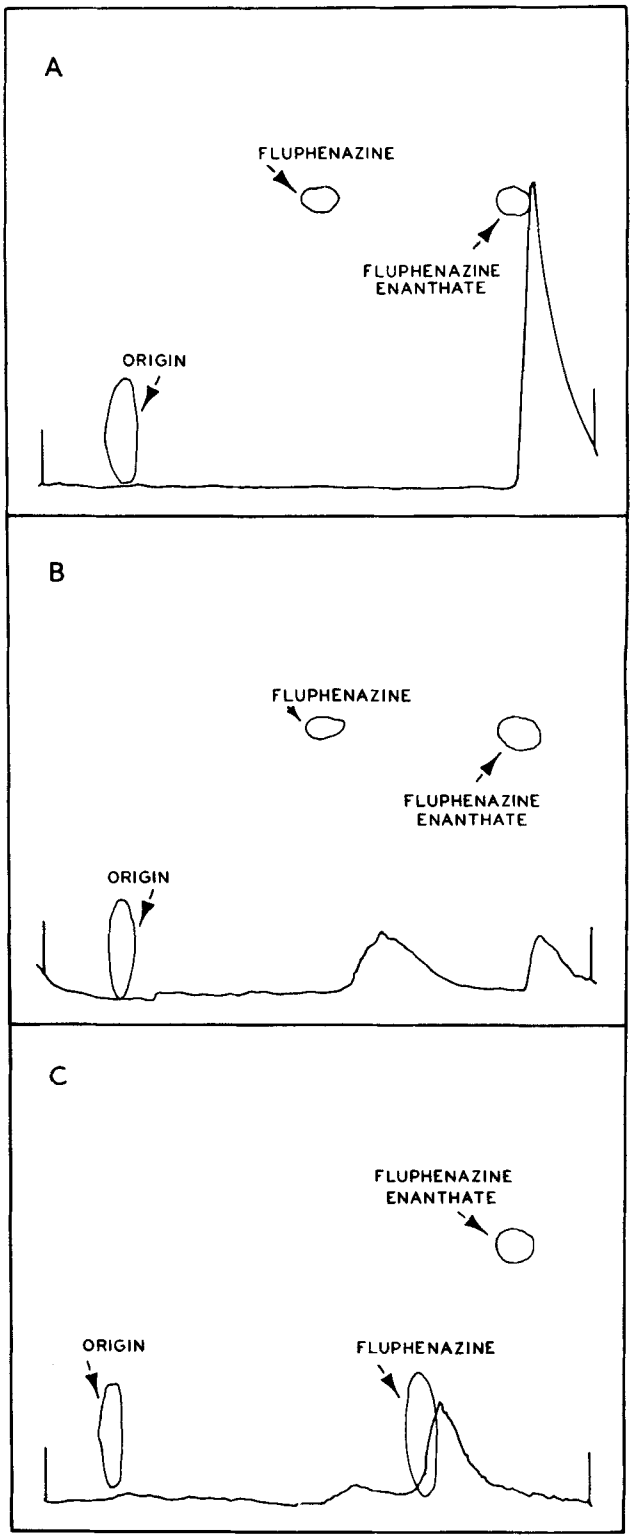


Figure 6—*Chromatograms of fluphenazine-^{14}C enanthate incubated with dog plasma: (A) denatured plasma, (B) intact plasma, and (C) intact plasma after alkaline hydrolysis. Solvent System 3 was employed.*

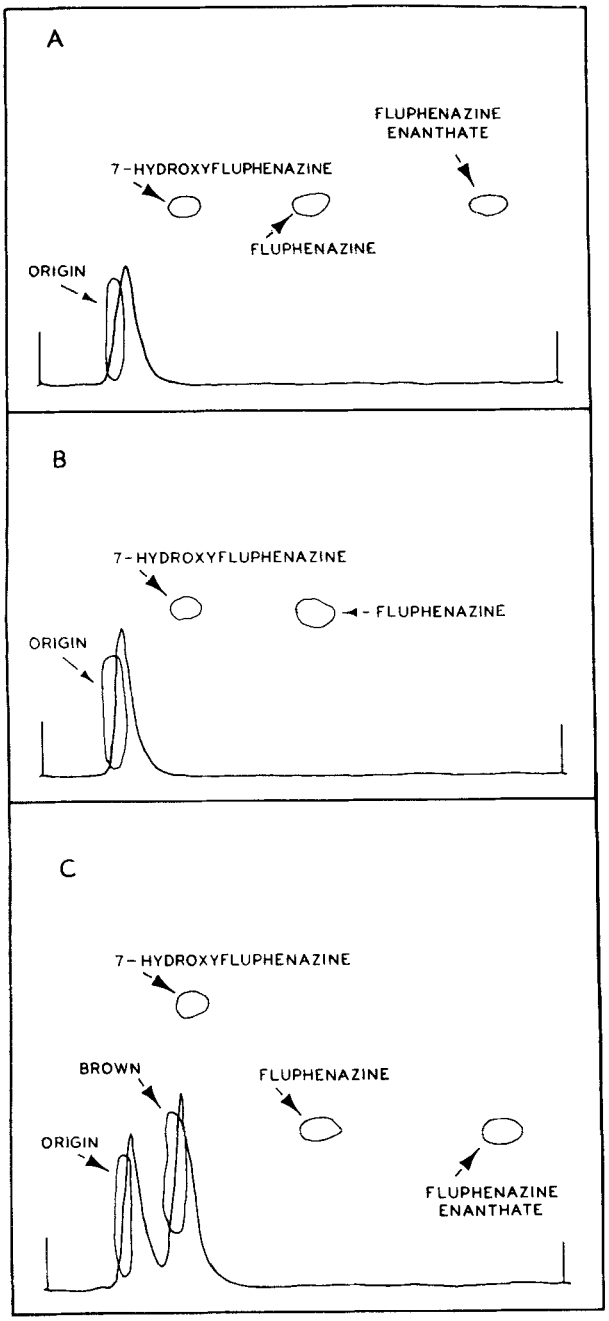


Figure 7—*Chromatograms of bile (0–1 hr.) developed in Solvent System 3: (A) bile from Dog Q7-147 was subjected to alkaline hydrolysis; (B) bile from Dog Q8-262 was chromatographed without any prior treatment; and (C) bile from Dog Q7-147 was subjected to hydrolysis with β-glucuronidase.*

II). No striking localization was noted in the areas of the brain examined. The lungs and liver, again, contained the greatest concentrations of radioactivity; the liver contained 8% of the dose at the time of sacrifice. The approximately 12% of the dose present in the various tissues of Dog Q7-147, plus the amounts excreted, accounted for about 70% of the dose.

Biotransformation—The biological disposition of fluphenazine-^{14}C dihydrochloride in dogs and subhuman primates was presented in a companion paper (1). There, evidence was presented to support the notion that the major metabolite of fluphenazine-^{14}C was unconjugated in the feces of dogs and was present as the glucuronide conjugate in the bile. To determine whether fluphenazine-^{14}C enanthate could be metabolized *via* the pathway previously established for fluphenazine-^{14}C, fluphenazine-^{14}C enanthate was incubated with either intact or denatured dog plasma. The samples were then chromatographed to determine the amount of fluphenazine-^{14}C enanthate remaining. Incubation of fluphenazine-^{14}C enanthate with denatured dog plasma led to the complete recovery of a peak corresponding in R_f value to fluphenazine enanthate (Fig. 6A). Incubation of fluphenazine-^{14}C enanthate with intact dog plasma led to two peaks on the chromatogram (Fig. 6B), one corresponding in R_f value to that of fluphenazine enanthate, the other to that of fluphenazine.

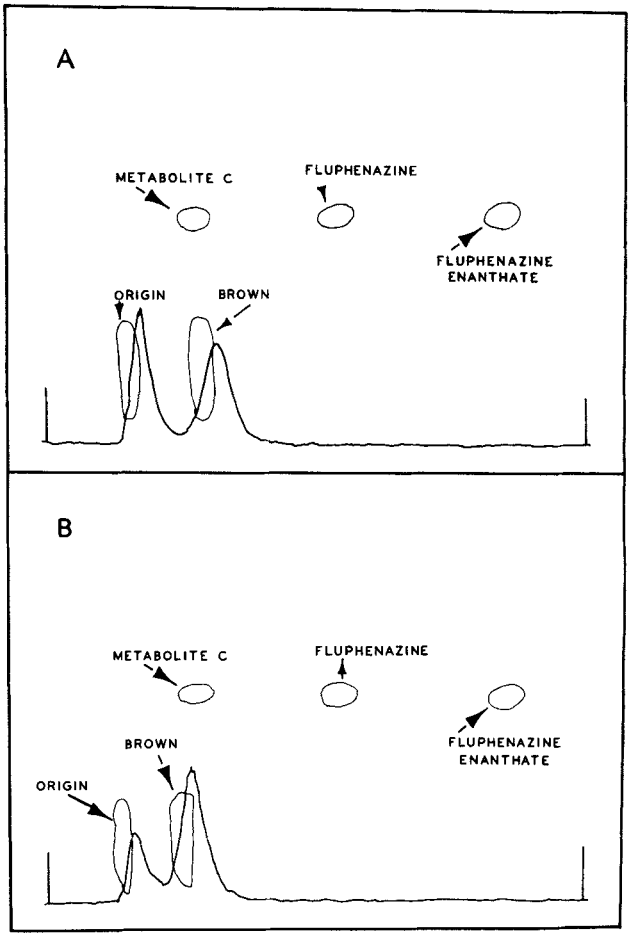


Figure 8—*Chromatograms of bile from Dog Q7-147 (0–1 hr.) developed in Solvent System 3: (A) bile was subjected to hydrolysis with β-glucuronidase followed by alkaline hydrolysis; and (B) bile was subjected to alkaline hydrolysis followed by hydrolysis with β-glucuronidase.*

To provide better evidence that the peak corresponding in R_f value to that of fluphenazine in Fig. 6B was, in fact, attributable to fluphenazine, the incubated sample of dog plasma was made alkaline, hydrolyzing the ester bond. The sample was then chromatographed again in Solvent System 3, as shown in Fig. 6C. Most of the radioactivity now appeared under a single peak that corresponds to the R_f value of fluphenazine; another minor, broad peak, with a slightly slower R_f value, probably represents products resulting from the alkaline hydrolysis of fluphenazine-^{14}C enanthate at elevated temperatures. Thus, the authors concluded that dog plasma contains esterases capable of hydrolyzing fluphenazine-^{14}C enanthate to fluphenazine-^{14}C. These results are comparable to those reported by Ebert and Hess (2). In their study of the metabolism of fluphenazine-^{14}C enanthate in the rat, fluphenazine-^{14}C was recovered after the direct injection of fluphenazine-^{14}C enanthate dissolved in sesame oil into the brains of rats.

Bile collected from Dog Q7-111 between the 7th and 8th hr. and from Dog Q7-147 during the 1st hr. and the 7th to 8th hr. was chromatographed in Solvent Systems 1 and 3. No unchanged fluphenazine-^{14}C enanthate was observed in any of these bile samples in either solvent system. Thus, no fluphenazine-^{14}C enanthate was recoverable after the administration of unformulated fluphenazine-^{14}C enanthate to dogs, whether intramuscularly or intravenously.

To determine whether conjugates were present in the bile, samples obtained from Dog Q7-147, which had received fluphenazine-^{14}C enanthate intravenously, were analyzed. Bile from this animal was treated with alkali in order to remove the heptanoic acid group. A chromatogram of this sample (Fig. 7A) shows that all of the radioactivity remained at the origin. Figure 7B shows the same result for a sample of bile from Dog Q8-262, which had received fluphenazine-^{14}C (10 mg./kg. i.v.) (1). Similar results are also obtained with these two samples of bile if the chromatograms are developed in Solvent System 2. Figure 7C reveals that a second peak, which corresponds in R_f value to Metabolite C of fluphenazine-^{14}C, is formed after bile from Dog Q7-147 has been treated with β-glucuronidase. Metabolite C was shown in a companion paper (3) to be identical with 7-hydroxyfluphenazine.

Confirmatory evidence that Metabolite C can be derived from the bile of a dog that had received fluphenazine-^{14}C enanthate was obtained by the following series of experiments. Bile obtained from Dog Q7-147 was given one of two sequential treatments. Either the bile was treated first with β-glucuronidase, followed by alkaline hydrolysis, or the bile was subjected first to alkaline hydrolysis and then treated with β-glucuronidase. In both cases, the samples were chromatographed after the second treatment in Solvent Systems 1, 2, and 3. The results obtained with Solvent System 3 are shown in Fig. 8. Regardless of the sequence of treatments, the same results are obtained in each solvent system; two peaks are observed, one of which corresponds to a brownish zone on the chromatogram and also to the R_f value of Metabolite C. These results are explainable only by the conversion of fluphenazine-^{14}C enanthate to the glucuronide of 7-hydroxyfluphenazine. Although the formation of the glucuronide of 7-hydroxyfluphenazine enanthate is a theoretical possibility for the metabolism of this compound, no 7-hydroxyfluphenazine enanthate was available as a chromatographic reference standard. Such a material would be expected to have an R_f value in Solvent System 3 intermediate between that of fluphenazine and fluphenazine enanthate. In fact, none of the radioactivity seen in Fig. 7C, for example, was observed to move with such an R_f value.

In conclusion, fluphenazine-^{14}C enanthate was shown to be hydrolyzed to fluphenazine-^{14}C by plasma esterases of the dog *in vitro*. Radioactivity present in the bile of a dog that had received fluphenazine-^{14}C enanthate was excreted as the glucuronide conjugate of 7-hydroxyfluphenazine. From the present studies, the authors are unable to determine whether fluphenazine-^{14}C enanthate is first hydroxylated and then has the ester bond cleaved, or whether the ester bond is first cleaved and the compound is then metabolized in a manner essentially like that previously described for fluphenazine-^{14}C (1).

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Abstract □ A sensitive spectrofluorometric assay of sotalol·HCl (*d,l*-4-(2-isopropylamino-1-hydroxyethyl)methanesulfonanilide hydrochloride; MJ 1999), a potent and specific β-adrenergic blocking agent, was developed. The compound fluoresces in alkali at 250/350 nm. and in acid at 235/309 nm. The maximum extractability was found at a pH of 9, where the substance is primarily the zwitterion with potentiometric pKa′ values of 8.30 and 9.80, where the former has been spectrophotometrically assigned to the dissociation of the conjugated sulfanilino group. A mixture of *n*-amyl alcohol and chloroform (1 : 3, v/v) at a volume ratio of organic solvent and pH 9 buffer solution of 8 : 1 extracted 85% of the compound, which was completely reextracted into 5.2 *N* HCl. The fluorescence of the monocharged molecule was measured at 235/309 nm. in the acid solution. The sensitivity of the assay was 0.1 mcg./ml. in plasma and 2.5 mcg./ml. in urine. Statistical analyses of assays of sotalol-spiked plasmas, urines, and acidic extraction blanks showed no dose × day interactions and no significant variations of the slopes of calibration curves within 3–4 days of assay; no significant assay differences when the drug was stored under refrigeration in water, acidic extraction blanks, whole blood, plasma, serum, and urine for 2–6 days; and no significant effects from blood and urine obtained from different dogs. The recovery of the drug was 69.4% from plasma and 81% from urine. The sensitivity of 0.1 mcg./ml. in plasma and 2.5 mcg./ml. in urine and the reproducibility of 2% at a midrange concentration of 0.5 mcg./ml. in plasma and of 2.2% at a midrange concentration of 10 mcg./ml. in urine allow the assay to be applicable for monitoring blood levels and urinary excretion of the drug when administered in therapeutic amounts.

Keyphrases □ Sotalol determination—blood, urine □ Partition coefficients—sotalol·HCl □ pKa determination—sotalol·HCl □ Variability parameters—sotalol·HCl fluorometric analysis □ Fluorometry—analysis, biological fluids

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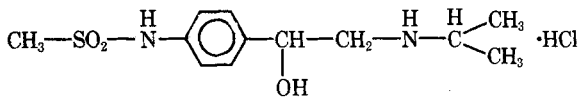

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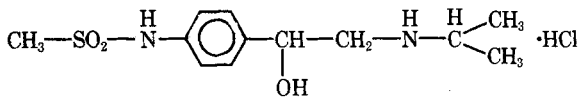

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mum dose regimens demands nonradioactive analytical methods. This paper describes the chemistry, extraction, and spectrofluorometric assay for sotalol·HCl in blood and urine.

EXPERIMENTAL

Equipment—A Farrand MK-1 spectrofluorometer was used in the analysis of sotalol·HCl[1]. The pKa′ values were determined with a Radiometer recording titrator, type TTT 1c/SBR 2c, equipped with a combination electrode, miniature (Sargent); a recording UV spectrophotometer (Cary model 15) was also used. All solutions used in the analytical procedure were prepared with all-glass-distilled water. The glassware was cleaned with Hemosol, rinsed repeatedly with tap water and deionized water, and soaked in 1:3 concentrated HNO_3–HCl mixture. After a final rinse with deionized water and distilled water, the glassware was dried and ready for use.

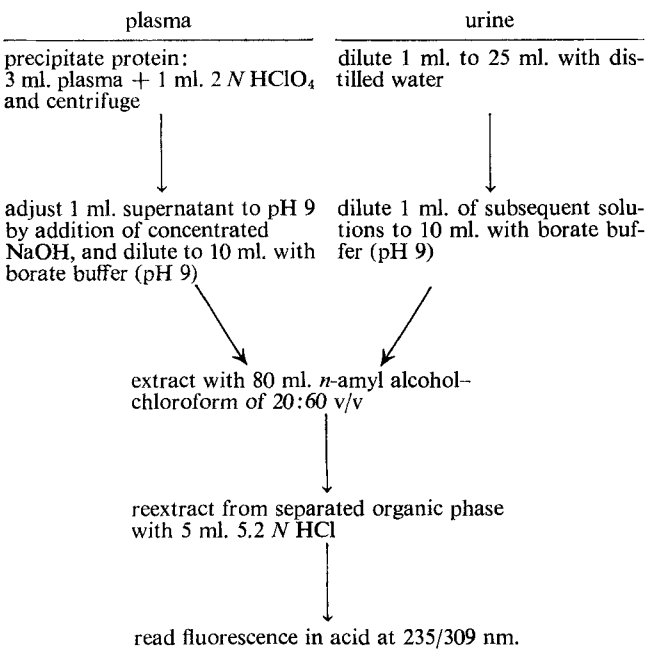
Reagents—The following were used: 5.2 *N* HCl (1:1 dilution of HCl reagent ACS), 2 *N* $HClO_4$ (analytical reagent), Na_2SO_4 (analytical reagent), and 0.5 *M* borate buffer pH 9 (5). All organic solvents used were of analytical reagent grade. The sotalol·HCl was used as the powder[2].

pKa′ Determinations—Sotalol·HCl (0.0054 *M*) was dissolved in 0.0050 *N* HCl, and the ionic strength was adjusted to 0.20 with added KCl. The solution was titrated with 0.3000 *N* NaOH. An equal volume of 0.0050 *N* HCl was also titrated with 0.3000 *N* NaOH in a blank titration.

The spectra of 6.7×10^{-5} *M* sotalol·HCl were recorded at various pH values in the range 5.5–11.0. Phosphate buffers (0.05 *M*) were used within the pH range of 5.5–8.2, borate buffers (0.05 *M*) within 8.4–9.6, and carbonate buffers (0.05 *M*) within 10.0–11.0 (5). All buffers were adjusted to a constant ionic strength of 0.20 with KCl.

Partition Coefficients—Sotalol·HCl (1.07×10^{-4} *M*) was analyzed spectrophotometrically in buffers of various pH values before and after extraction with buffer-saturated chloroform. Other organic solvents which were screened to obtain the solvent system chosen for maximum extraction from aqueous buffer solutions were cyclohexane, ethyl ether, ethyl acetate, *o*-dichlorobenzene, ethylene dichloride, *n*-amyl alcohol, and various mixtures of chloroform with ethyl acetate and *n*-amyl alcohol.

Analytical Procedure (Scheme I): Plasma Analysis—Heparinized dog blood was centrifuged at 3000 r.p.m. for 15 min. to separate the red blood cells from plasma. A stock plasma solution of 1 mcg./ml. sotalol·HCl was prepared by adding a concentrated aqueous solution of sotalol·HCl to this plasma. This stock plasma solution was diluted with plasma to prepare solutions that were 0.1, 0.3, and 0.6 mcg./ml. of sotalol·HCl. One milliliter of 2 *N* $HClO_4$ was added to 3 ml. of these plasma solutions to precipitate the protein. The samples were centrifuged for 5 min. at 3000 r.p.m. One milliliter of the clear supernatant was adjusted to pH 9 with concentrated NaOH and diluted to 10 ml. with 0.5 *M* borate buffer. The solutions were again centrifuged to remove traces of precipitate and were then transferred into 125-ml. separators containing 80 ml. of a H_2O–saturated *n*-amyl alcohol–chloroform mixture (20:60, v/v). The samples were shaken for 30 min. on an automatic shaker. After 30 min. of separation, the solvent was drained into conical flasks containing 3.5 g. anhydrous Na_2SO_4 to remove traces of water in the solvent. The aqueous phase was discarded. The dried solution was then decanted into separators containing 5 ml. 5.2 *N* HCl and shaken for 30 min. After 30 min. of separation, the solvent was drained and discarded. The aqueous phase was centrifuged for 2 min. to remove residual solvent. The samples were then analyzed on the spectrofluorometer at 309 nm. with excitation at 235 nm., using a slit width of 10 nm. for excitation and 20 nm. for fluorescence measurement at a meter sensitivity setting of 0.03.



Scheme I—Analysis of Sotalol·HCl in Plasma and Urine

[1] Mead Johnson & Co.
[2] Obtained from Mead Johnson & Co.

Table I—Results[a] of Repetitive Duplicate Assays of Drug Spiked in Blanks and Extracted on Different Days from Different Waters, Plasmas, and Urines Spiked on Different Days

Dose, mcg./ml.	Day 1		Day 2		Day 3		Day 4	
	1	2	1	2	1	2	1	2
Drug Added to Acidic Aqueous Extraction Blanks								
0	0.30	0.30	0.28	0.30	0.28	0.40	0.29	0.29
0.01	0.50	0.44	0.48	0.42	0.49	0.46	0.42	0.45
0.03	0.68	0.74	0.70	0.75	0.80	0.80	0.80	0.80
0.06	1.00	1.00	1.00	0.97	1.04	1.04	1.10	1.12
0.10	1.75	2.18	1.67	2.14	1.72	2.04	2.09	2.10
Drug Extracted from Water								
0	0.28	—	0.28	—	0.20	—		
0.1	0.38	0.45	0.38	0.38	0.38	0.34		
0.3	0.59	0.64	0.60	0.64	0.60	0.69		
0.6	0.98	0.98	0.99	1.02	0.99	0.94		
1.0	1.52	—	1.57	1.48	1.28	1.40		
Drug Extracted from Plasma								
0	0.24	—	0.27	0.24	0.23	0.24		
0.1	0.34	0.35	0.33	0.30	0.34	0.32		
0.3	0.50	0.55	0.50	0.51	0.54	0.54		
0.6	0.83	0.90	0.87	0.91	0.85	0.90		
1.0	1.40	1.34	1.36	1.30	1.29	1.30		
Drug Extracted from Urine								
0	0.32	—	0.34	0.34	0.33	0.35		
2.5	0.48	0.47	0.45	0.47	0.45	0.45		
5.0	0.62	0.63	0.56	0.56	0.54	0.54		
10.0	0.86	0.84	0.80	0.82	0.84	0.82		
15.0	1.17	1.10	1.14	1.18	1.15	1.12		
20.0	1.39	1.30	1.36	1.35	1.28	1.36		

[a] Results are given in the arbitrary fluorescence units of the Farrand MK-1 spectrofluorometer at a meter sensitivity setting of 0.03 and measured at 235/309 nm.

Urine Analysis—Dog urine was obtained by catheterization and centrifuged to spin down the sediment. A urine stock solution of 25 mcg./ml. sotalol·HCl was prepared by adding concentrated solutions of sotalol·HCl to this urine. This stock solution of urine was diluted with urine to prepare solutions that were 2.5, 5, 10, 15, and 20 mcg./ml. of sotalol·HCl. Aliquots (1.00 ml.) of these solutions were first diluted up to 25 ml. with distilled H_2O. Aliquots (1.00 ml.) of these subsequent solutions were then diluted up to 10 ml. with 0.5 *M* borate buffer of pH 9. The buffer capacity was sufficient to maintain this pH. The subsequent centrifugation, extraction into *n*-amyl alcohol–chloroform, drying with anhydrous Na_2SO_4, decanting and extraction into concentrated HCl, and spectrophotofluorometric analysis were carried out in the same manner as specified for plasma.

Sources of Variability in Analysis: Instrument Variability—Prior to starting a series of readings, the spectrofluorometer was adjusted to maximum sensitivity and the reading of a quinine bisulfate standard solution (10 mcg./ml. in 0.1 *N* H_2SO_4) was adjusted to a constant meter reading of 2.0 at range 0.03 at 235/309 nm., the

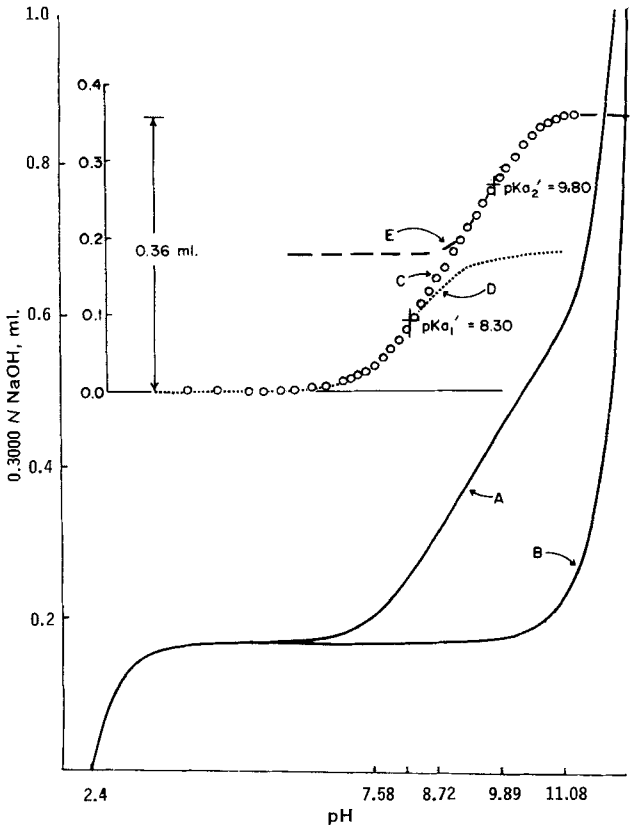


Figure 1—*Potentiometric titrations of 10 ml. 5.4 × 10^{-3} M sotalol·HCl, μ = 0.2 (Curve A), and 5 × 10^{-3} N HCl, μ = 0.2, as a blank (Curve B). The open circles (Curve C) mark the differential titration curve, i.e., the difference between the milliliters of 0.3000 N NaOH necessary to reach a given pH for sotalol (A) and blank (B). The dashed lines are the constructed titration curves (D and E) for the two acid equivalents of sotalol. The midpoints between two asymptotes serve to estimate the pKa_1' and pKa_2' values.*

wavelengths used for the analysis of sotalol·HCl. This reading was checked before and after each sample measurement.

Variability Due to Extraction Procedure—Drug was added to water (0.1, 0.3, 0.6, and 1.0 mcg./ml.), and duplicate extractions were carried out for each concentration on 3 subsequent days. The samples were stored at 4° and assayed on the 4th day; the yield of extraction was determined from direct analysis of aqueous acidic solutions of 0.01, 0.03, 0.06, and 0.1 mcg./ml., which encompass the range of concentrations after the dilution processes of the extraction procedure. These spiked aqueous acidic solutions were prepared from solutions run through the extraction procedures starting with water without drug. The data are given in Table I.

Variability Due to Differences among Animals—Blood and urine were obtained from three dogs, each on a separate day. Duplicate extractions were made from each plasma containing 0.1, 0.3, 0.6, and 1.0 mcg./ml. and each urine containing 2.5, 5, 10, 15, and 20 mcg./ml. A different plasma and urine were used on each of the 3 subsequent days. The resultant extracts in 5.2 *N* HCl were stored at 4° and assayed on the 4th day. The data are given in Table I.

Effect of Storage at 4° in Whole Blood, Plasma, and Serum—Sotalol·HCl was added to heparinized whole blood, plasma, and serum (0, 0.1, 1.0, and 10.0 mcg./ml.) and stored at 4°. Whole blood was centrifuged on 2 subsequent days, and the plasma was run through extraction and analyzed. Plasma and serum, containing the drug in the same concentrations, were extracted and assayed on 2 subsequent days and on the 6th day after adding the drug.

Effect of Storage at 4° in Water and in Urine—Drug was added to water (0, 0.3, and 0.6 mcg./ml.) and urine (0, 2, and 6 mcg./ml.). The samples were stored at 4° while carrying out extractions on 3 subsequent days. The samples were assayed on the 4th day.

Statistical Analysis—The calibration curves for sotalol·HCl were statistically evaluated by stepwise regression analysis, using a digital computer program (6), and by analysis of variance (7).

RESULTS AND DISCUSSION

Estimation of Dissociation Constants: Potentiometric pKa' Values—There are two dissociable acidic groups in sotalol·HCl (I): the neutral sulfonated aniline and the protonated amino group in the isopropylamino side chain. The differential potentiometric titration method of Parke and Davis (8, 9) was applied. The difference curve was constructed (open circles in Fig. 1) from the subtraction of the milliliters of standard alkali consumed in the blank titration from that consumed by sotalol·HCl to obtain the same pH value. The distance (0.36 ml.) between the two asymptotic values of the difference curve (inset, Fig. 1) represents the base in milliliters used to neutralize both of the hydrogen ions of sotalol·HCl since 0.18 ml. of 0.3000 *N* NaOH was necessary for one acid equivalent of the titrated 10 ml. of 5.4 × 10^{-3} *M* sotalol·HCl. The constructed titration curves (dashed lines in Fig. 1) for each acid equivalent were used to estimate pKa' values at the pH values of half-neutralization for each equivalent. The pKa_1' value of 8.30 and the pKa_2' value of 9.80 were so estimated.

Spectrophotometric pKa' Determination—Sotalol·HCl has two UV absorption peaks; the $\lambda_{max.}$ 227 nm. is favored below a pH of 8.5, and the $\lambda_{max.}$ 248 is favored above this pH value (Fig. 2). Two isosbestic points were observed, at 234 nm. and approximately at 215 nm. The absorbances at the maximum wavelengths were plotted as a function of pH (Fig. 3). The pKa_1' of 8.35 was calculated from the appropriate plot (Fig. 4) of the Henderson–Hasselbalch equation:

$$\text{pKa} - \text{pH} = \log \frac{|A - A_{(^-MJ)}|}{|A_{(HMJH^+)} - A|} \qquad \text{(Eq. 1)}$$

where A is the absorbance value at the pH of each buffer solution, $A_{(^-MJ)}$ is the asymptotic absorbance value obtained in alkaline solution, and $A_{(HMJH^+)}$ is the asymptotic value in acidic solution. This value can only be related to the spectrally affected dissociation of the anilino hydrogen in sotalol·HCl (I). The pKa_2' of 9.80 can now be assigned to the protonated amine group in the side chain.

The presence of four species of sotalol·HCl in solution can be based on these assignments: a positively charged ($HMJH^+$), a neg-

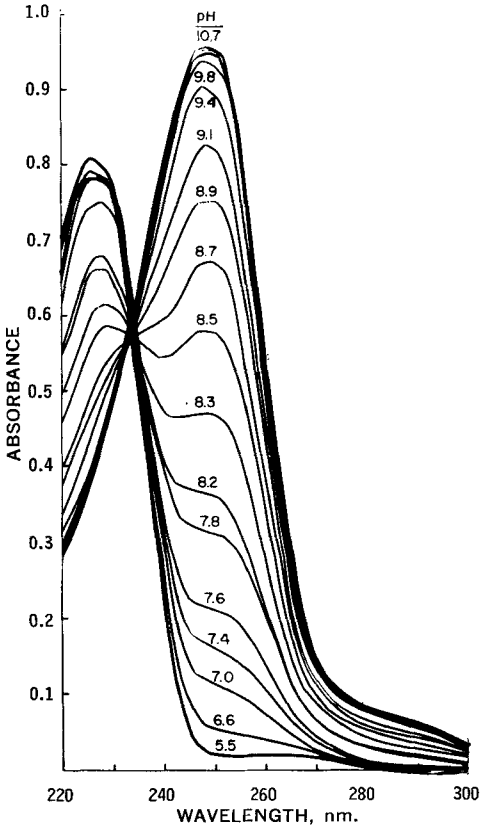


Figure 2—*UV absorption spectra of 6.7 × 10^{-5} M sotalol in buffers of μ = 0.2 as a function of pH.*

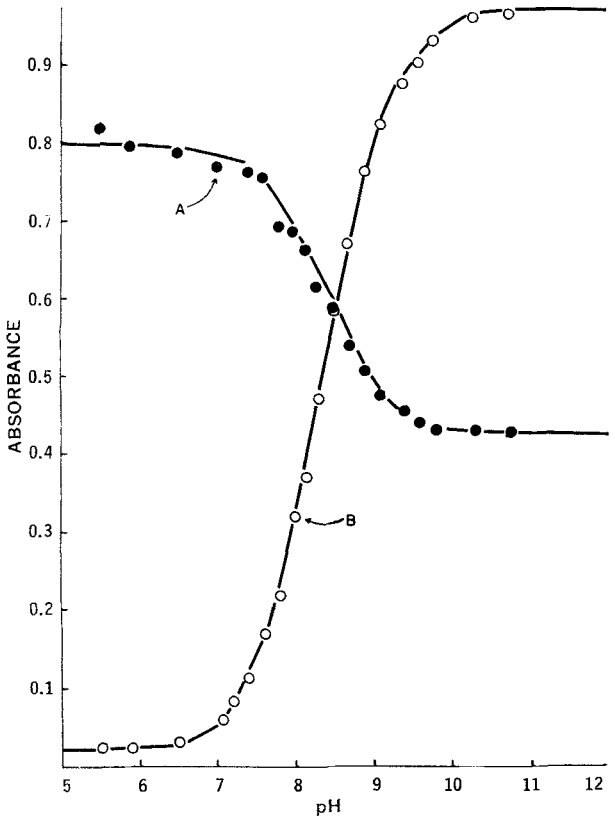


Figure 3—*Absorbance, A, of 6.7 × 10⁻⁵ M sotalol at λ_{max}. 227 nm. (Curve A) and λ_{max}. 248 nm. (Curve B). The lines drawn through these points were calculated on the basis of*

$$pKa' - pH = \log \frac{[A - A_{(^-MJ)}]}{[A_{(HMJH^+)} - A]}$$

for a pKa′ = 8.35, where $A_{(^-MJ)}$ is the absorbance reached in the most alkaline solution, and $A_{(HMJH^+)}$ is the absorbance reached in the most acidic solution.

atively charged ($^-$MJ), a neutral form (HMJ), and a zwitterion ($^-$MJH$^+$) (Scheme II). At high acidities, only the protonated species (HMJH$^+$) should exist; in strong alkaline solution, only the negatively charged form ($^-$MJ) should exist. All four species should be present at the intermediate pH values, and the dissociation con-

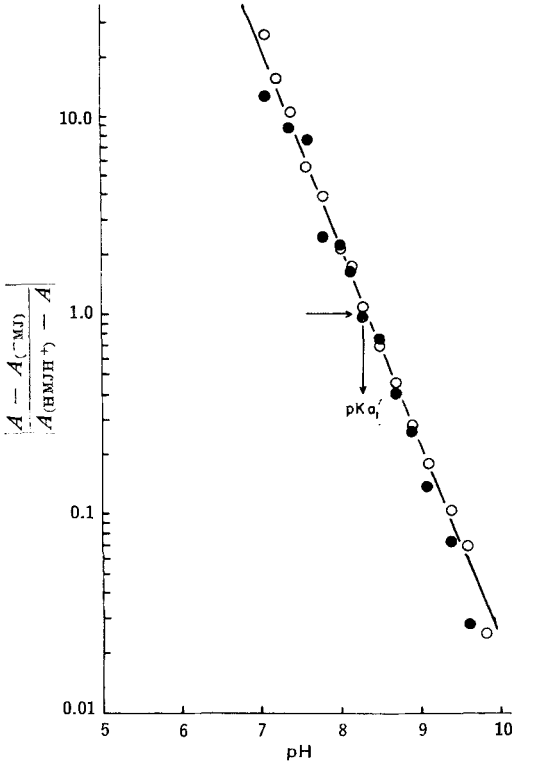


Figure 4—*Semilogarithmic plot for 6.7 × 10⁻⁵ M sotalol, μ = 0.2, in accordance with*

$$pKa' - pH = \log \frac{[A - A_{(^-MJ)}]}{[A_{(HMJH^+)} - A]}$$

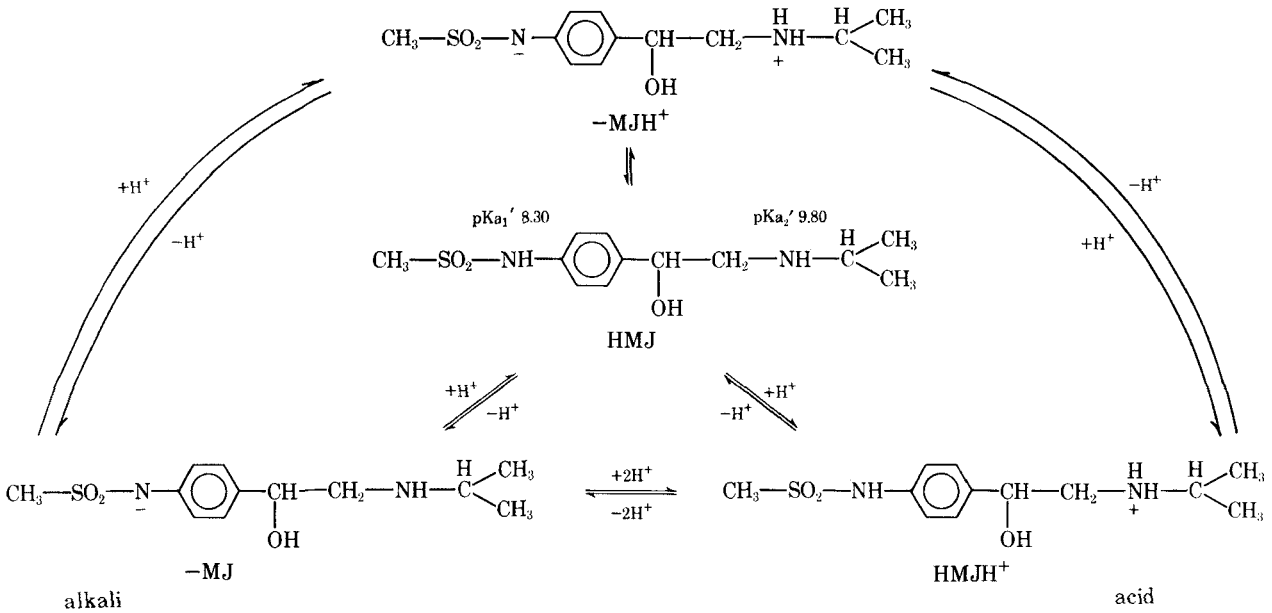
Key: ●, from values obtained at λ_{max}. 227 nm.; and ○, from values obtained at λ_{max}. 248 nm.

stants could be written as

$$K_{a_1}' = 10^{-8.30} = \frac{([HMJ] + [^-MJH^+])\,[H^+]}{[HMJH^+]} \qquad \text{(Eq. 2)}$$

and

$$K_{a_2}' = 10^{-9.80} = \frac{[^-MJ]\,[H^+]}{([HMJH^+] + [^-MJH^+])} \qquad \text{(Eq. 3)}$$



Scheme II—Possible Equilibria among Ionic Species Derived from Sotalol·HCl

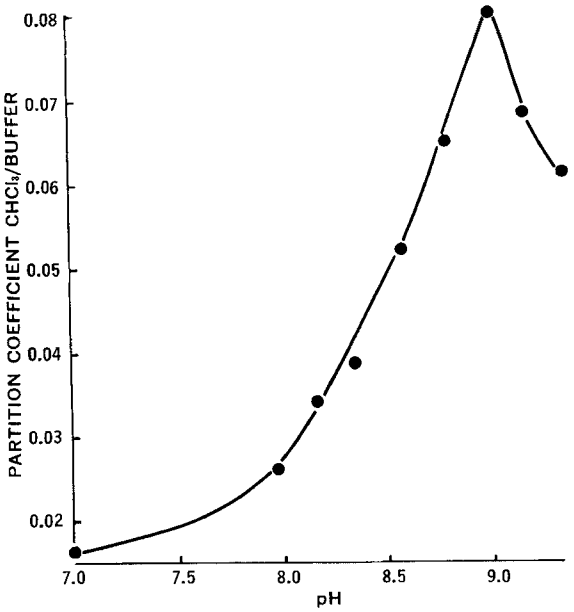


Figure 5—*Plot of apparent partition coefficient between chloroform–buffer for 1.07 × 10⁻⁴ M sotalol in buffer as a function of the pH of the aqueous phase.*

The relative amounts of the neutral species (HMJ) and zwitterion ($^-MJH^+$) present could be estimated from the shape of the plot of UV absorbance *versus* pH (Fig. 3), since it can be assumed that $^-MJH^+$ and ^-MJ have the same spectrum. If considerable amounts of neutral species were formed with increasing pH, the semilogarithmic plot (Fig. 4) of Eq. 1 would not be linear and of unit slope since the asymptotic absorbance only would be reached at much higher pH values, *i.e.*, > 10.8. The reason for this is that significant amounts of the uncharged form, HMJ, would exist through the process that gave the second pKa_2'. Since this is not so, it may be concluded that only very small amounts of the neutral species exist in solution.

Partition Coefficient—Sotalol·HCl must be extracted from body fluids to be assayed. It was anticipated that maximal extraction could be effected into an organic solvent at maximum concentration of the neutral form in equilibrium with the zwitterion, halfway between the two pKa's, *i.e.*, about pH 9. Sotalol·HCl, dissolved in

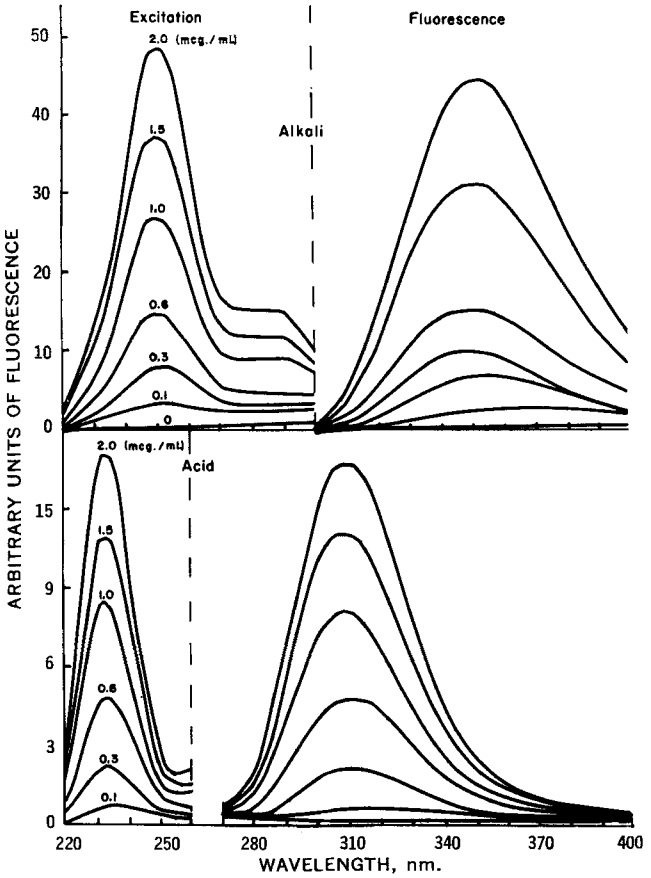


Figure 6—*Excitation and fluorescence spectra of various concentrations of sotalol·HCl labeled in micrograms/milliliter in both acid (5.2 N HCl) and alkali (1.0 N NaOH). The excitation peak was 235 nm. in acid and 250 nm. in alkali; the fluorescence peak was 309 nm. in acid and 350 nm. in alkali.*

Table II—Analyses of Variances of Table I Data

Source	Degrees of Freedom	Mean Square	Components of Variance[a]	*F*-Value	Significance at 5% Level
		Direct Analysis of Drug Added to Blanks			
Dose	4	3.4277		263.7	
Days	3	0.0102	$\sigma_D^2 = \sigma^2 + 10\,\sigma_1^2$	0.8	N.S.
Dose × days	12	0.0052		0.4	N.S.
Duplicates	20	0.0135	σ^2		
Total	39				
		Drug Extracted from Water and Analyzed			
Dose	4	1.1317		566.0	
Days	2	0.0095	$\sigma_D^2 = \sigma^2 + 7.14\,\sigma_1^2$	4.7	S.
Dose × days	8	0.0034		1.7	N.S.
Duplicates	11	0.0020	σ^2		
Total	25				
		Drug Extracted from Plasma and Analyzed			
Dose	4	1.1240		1605.7	
Days	2	0.0005	$\sigma_D^2 = \sigma^2 + 9.37\,\sigma_1^2$	0.6	N.S.
Dose × days	8	0.0009		1.1	N.S.
Duplicates	14	0.0008	σ^2		
Total	28				
		Drug Extracted from Urine and Analyzed			
Dose	5	0.8992		1284.6	
Days	2	0.0014	$\sigma_D^2 = \sigma^2 + 11.37\,\sigma_1^2$	2.0	N.S.
Dose × days	10	0.0009		1.2	N.S.
Duplicates	17	0.0007	σ^2		
Total	34				

[a] σ^2 = Mean square of error within duplicate determinations. σ_D^2 = Mean square of error within duplicate determinations plus variance component due to differences among days.

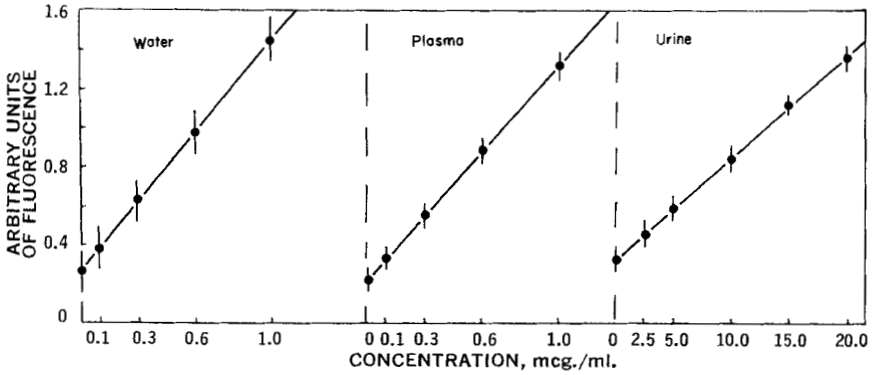


Figure 7—*Calibration curves for sotalol·HCl extracted from spiked water, plasma, and urine. The vertical lines indicate the 95% confidence intervals (twice the standard deviation) for the mean values at various concentrations obtained from duplicate extractions made from replicate solutions, each prepared and run through the analytical procedure on 3 successive days.*

buffers of various pH, was extracted with chloroform. A definite pH dependency of the partition coefficient was observed (Fig. 5), with a maximum partition coefficient of $C_{CHCl_3}/C_{buffer} = 0.08$ at pH 9. This was indicative of the presence of some neutral molecules (HMJ) in equilibrium with the zwitterion ($^-MJH^+$) which, although in low concentration, possessed a sufficiently high coefficient to permit partition into the organic solvent. The mixture of *n*-amyl alcohol–chloroform (1:3, v/v) was the most efficient, with a partition coefficient of 0.68. At a volume ratio of aqueous buffer phase and solvent phase of 1:8, about 85% of the compound was extracted into the solvent mixture. This amount was quantitatively reextractable into acid or alkali where practically all of the molecules were charged.

Spectrofluorometric Assay—After oral administration of a therapeutic dose of 1–2 mg./kg., plasma levels of sotalol·HCl may be expected to be 1 mcg./ml. and below. Since the limit of detection by means of UV spectrophotometry was about 2 mcg./ml., a more sensitive assay method for the drug had to be found. When the excitation and emission spectra of the compound on a spectrofluorometer were scanned, peaks were found in acid medium at 235/309 nm. and in alkali at 250/350 nm. (Fig. 6). The fluorescence was about three times higher in alkali than in acid. The lower limit of detection in both acid and alkali was 0.01 mcg./ml. Since the background reading for extracts from blood and urine in acid was lower and less variable than in alkali, the method of choice was to reextract the compound into 5.2 *N* HCl and to read the fluorescence in that medium.

Calibration Curves for Sotalol—Calibration curves for sotalol·HCl added to and extracted from water, plasma, and urine are given in Fig. 7. Each point represents the mean of three duplicate determinations carried out on each of 3 subsequent days. Each solution was separately prepared on each day, using plasma and urine from different animals. The curves were fitted by the method of least squares, and the 95% confidence intervals ($\pm 2\sigma$) of each point are indicated by the vertical lines in Fig. 7. The slopes of the combined response–dose data are 15.89 (spectrofluorometric response units per microgram/milliliter at 235/309 nm. using a meter sensitivity setting of 0.03) for direct analysis of drug added to the extraction blanks, 1.188 for the procedure when the drug was extracted from water, 1.102 when the drug was extracted from plasma (10-fold dilution compared to direct analysis), and 0.0515 for the procedure when the drug was extracted from urine (250-fold dilution compared to direct analysis). The data on which the calibration curves were based are listed in Table I.

After correction of the slopes for the dilutions made during the analytical procedure, the average recovery of the drug could be determined from the ratio of the corrected slopes to the slope of 15.89 obtained from the drug-spiked extraction blanks. The mean recovery values on extraction and analysis obtained in this manner were 74.8% from water, 69.4% from plasma, and 81.0% from urine. Of course, additional extractions would increase the effectiveness of the separation procedures.

Analysis of Variance—The analysis of variance tables for the four studies (Table I, Fig. 7) are given in Table II and are for concentrations of drug analyzed in extraction blanks and concentrations of drug extracted from water, plasma, and urine and subsequently analyzed. None of the dose × day-interactions was significant. Therefore, within any one study (either drug added to extraction blanks and analyzed or drug extracted from water, plasma, or urine and analyzed), the slopes of the lines on different days (Fig. 7) are the same. The slopes were previously summarized.

A significant difference due to days was found only in the extraction of the drug from water and subsequent analysis. In all other instances, the variation about regression was not significant among days and thus was not significant among the different plasmas and urines obtained from different dogs since plasmas and urines from different dogs were used on the different days. Since there were no significant differences among days for the assay of drug stored in refrigerated acidic extraction blanks for 4 days, it can be concluded that extracted samples may be safely stored for this length of time.

If the regression equation is taken as

$$y = a + bx \qquad \text{(Eq. 4)}$$

the variance of a predicted drug concentration x from a given response can be approximated by (10):

$$\text{variation } (\hat{x}) = \frac{\sigma^2}{b^2\sum(x-\bar{x})^2}\left[\frac{\sum x^2}{n} + \frac{2(y-a)\bar{x}^2}{b} + \left(\frac{y-a}{b}\right)^2\right] \qquad \text{(Eq. 5)}$$

Table III—Sotalol·HCl[a] Spiked in Whole Blood, Plasma, and Serum and Stored for Several Days and Analyzed

Dose, mcg./ml.	Whole Blood Days 1	Whole Blood Days 2	Plasma Days 1	Plasma Days 2	Plasma Days 6	Serum Days 1	Serum Days 2	Serum Days 6
0.1	0.11	0.11	0.11	0.11	0.10	0.16	0.14	0.16
1.0	0.44	0.50	0.56	0.58	0.53	0.54	0.58	0.55
10.0	3.95	4.35	4.00	3.75	4.10	4.35	4.50	4.10

[a] Results are given in the arbitrary fluorescence units of the Farrand MK-1 spectrofluorometer at a meter sensitivity of 0.03 and measured at wavelength 242/309 nm.

Table IV—Analysis of Variance of Table III Data

Source	Degrees of Freedom	Mean Square	*F*-Value	Significance at 5% Level
Dose	2	37.8169	2260.42	
Media	2	0.0424	2.53	N.S.
Dose × media	4	0.0301	1.80	N.S.
Error among days	15	0.0167		

Table V—Storage Effects on Sotalol·HCl[a] Spiked in Water and Urine and Analyzed

Dose, mcg./ml.	Water Days 1	Water Days 2	Water Days 3	Dose, mcg./ml.	Urine[a] Days 1	Urine[a] Days 2	Urine[a] Days 3
0.0	0.38	0.32	0.35	0.0	0.67	0.60	0.67
0.3	0.87	0.73	—	2.0	2.65	2.75	2.77
0.6	1.13	1.18	1.14	6.0	8.10	8.50	8.65

[a] Results are given in the arbitrary fluorescence units of the Farrand MK-1 spectrofluorometer at a meter sensitivity of 0.03 and measured at wavelength 235/309 nm. The urines were diluted 10-fold during the analytical procedure.

Table VI—Analysis of Variance of Table V Data

Source	Degrees of Freedom	Mean Square	*F*-Value	Significance at 5% Level
Drug Extracted from Urine				
Dose	2	48.5496		
Days	2	0.0384	1.59	N.S.
Dose × days	4	0.0242		
Drug Extracted from Water				
Dose	2	0.5269		
Days	2	0.0757	1.05	N.S.
Dose × days	4	0.0721		

where a = intercept, b = slope, σ^2 = mean square of error, x = dose, and $\bar{x}$ = mean dose.

The percent error in terms of concentrations for a given response is obtained by

$$\text{percent error} = \frac{\sqrt{\text{variation } (\hat{x})} \cdot 10}{x} \qquad \text{(Eq. 6)}$$

Therefore, for a drug concentration of 0.5 mcg./ml. in plasma, the error would be estimated as 1.99%; and for a drug concentration of 10 mcg./ml. in urine, it would be 2.23%.

Effect of Storage at 4° in Whole Blood, Plasma, and Serum—Sotalol·HCl was extracted and analyzed from the plasma prepared from spiked whole blood on 2 subsequent days. The drug was extracted and analyzed from spiked plasma and serum on 2 subsequent days and on the 6th day after spiking.

The data obtained are listed in Table III. The analysis of variance is given in Table IV.

The results indicate that there is no contribution of the different media to the error among days, *i.e.*, there is no difference in the spectrofluorometric response whether the medium spiked was whole blood, plasma, or serum.

Effect of Storage at 4° in Water and Urine—Sotalol·HCl was extracted from spiked water and urine on 3 subsequent days and analyzed. The data are listed in Table V.

Since there were no duplicate determinations, the dose × day-interaction term was used as the error term in the analysis of variance (Table VI). No difference was found among days when the drug was stored in urine or water.

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Keyphrases □ Hydroxamic acid derivative—vasomotor, α-block negating action □ β-Hydroxylamine cinnamonyl hydroxamic acid—hypotensive, α-block negating action □ α,β-Receptors, blockade effect—β-hydroxylamine cinnamonyl hydroxamic acid action □ Antihistamine effect—β-hydroxylamine cinnamonyl hydroxamic acid action

Hydroxamic acids may be described as *N*-hydroxylated amides occurring naturally in plants and microbes. Neilands (1) described the outstanding biochemical features of hydroxamic acids and reviewed their biological actions. Because phenylethyl hydroxamic acids (Ar—C—C—CO—NH—OH), analogous to vasoactive phenylethylamines, have never been examined for vasomotor activity, a number of hydroxamic acids with two- and three-carbon structures juxtapositioned between a phenyl ring and the hydroxamic acid moiety

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Table I—Cardiac and Vasodepressor Responses to Incremental Intravenous Bolus Doses of β-Hydroxylamine Cinnamonyl Hydroxamic Acid

β-Hydroxylamine cinnamonyl hydroxamic acid bolus dose, mg./kg.	0.3 $N = 9$	0.6 $N = 10$	0.9 $N = 8$	1.2 $N = 10$	1.5 $N = 11$	1.8 $N = 7$	2.1 $N = 6$
Systolic pressure reduction (mean ± *SE*), mm. Hg	23.7 ± 1.4	39.7 ± 2.2	57.1 ± 2.0	64.5 ± 1.5	76.3 ± 1.8	92.4 ± 2.4	96.5 ± 2.5
Diastolic pressure reduction (mean ± *SE*), mm. Hg	17.5 ± 1.2	27.6 ± 1.2	40.8 ± 1.6	45.6 ± 1.6	48.5 ± 1.3	53.3 ± 2.1	57.5 ± 1.3
Heart rate[a], beats/min.							
Control	138 ± 1.5	131 ± 1.5					
Experimental	141 N.S.	161 p 0.05					

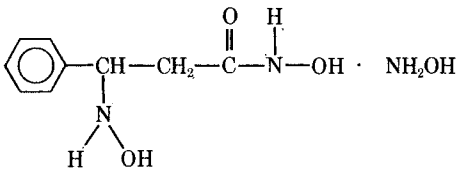
[a] Heart rate was recorded as the mean of the means of 9 dogs at the 0.3-mg./kg. dose level, and of 10 dogs at the 0.6-mg./kg. dose level. Control heart rate was recorded for 1-min. periods over a 10-min. interval prior to the bolus injection of β-hydroxylamine cinnamonyl hydroxamic acid. Experimental heart rate was measured at peak response after bolus injection and statistically evaluated by comparison with the control mean.

(Ar—$C_{2,3}$—CO—NH—OH) were synthesized and studied pharmacologically in this laboratory to examine structure–activity relationships (2).

β-Hydroxylamine cinnamonyl hydroxamic acid (Ar—CNOH—C—CO—NHOH) was observed to produce a rapidly reversible, yet well-controlled, dose-related hypotension in anesthetized dogs (3). This compound was of particular interest because of its special analogy with numerous vasoactive amines, its vasodepressor action, and its ability to negate an α-adrenergic block. These interesting pharmacodynamic actions of β-hydroxylamine cinnamonyl hydroxamic acid may be related to the unique electron density of the terminal nitrogen in the hydroxamate radical.

EXPERIMENTAL

Synthesis of β-Hydroxylamine Cinnamonyl Hydroxamic Acid—β-Hydroxylamine cinnamonyl hydroxamic acid was synthesized and prepared by the general method of Cooley *et al.* (4). The white powdery precipitate produced by the addition of ethylcinnamate to an alkaline methanolic hydroxylamine solution was purified in crystalline form from a 1:1 mixture of heptane–ethanol. Elemental, radical, and IR analyses established the molecular structure (I) of β-hydroxylamine cinnamonyl hydroxamic acid[1].



I

All physical characteristics of β-hydroxylamine cinnamonyl hydroxamic acid agreed well with those initially described by Possner (5). A positive $FeCl_3$ test for the presence of hydroxamic acid moiety and periodic IR analysis showed β-hydroxylamine cinnamonyl hydroxamic acid to be stable for approximately 30 months. β-Hydroxylamine cinnamonyl hydroxamic acid was soluble to the extent of 2.4 g. in 100 ml. water and was prepared for parenteral administration in isotonic saline.

Procedures—Mongrel dogs of both sexes, weighing 13–18 kg., were anesthetized with pentobarbital sodium (30 mg./kg. i.v.). A femoral artery was cannulated, and the systemic blood pressure was recorded with a Statham pressure transducer and Grass model 5 polygraph. Quantitative evaluation of blood pressure was achieved by internal calibration of the polygraph and checked against direct manometric readings. Respiration was recorded with a conventional pneumograph.

All drugs were administered intravenously through a polyethylene catheter inserted into the saphenous vein of a contralateral limb. Continuous infusions of β-hydroxylamine cinnamonyl hydroxamic acid at precise flow rates were made with an Aminco peristaltic pump and Manostat flowmeter. β-Hydroxylamine cinnamonyl hydroxamic acid was prepared for intravenous administration in concentrations expressed as the acid in isotonic saline; this preparation was neutral in pH.

Since the vasodepressor response to a bolus intravenous injection of β-hydroxylamine cinnamonyl hydroxamic acid was prompt and rapidly reversible, all experiments—except dose–response studies—were designed to elucidate the mechanism of action of β-hydroxylamine cinnamonyl hydroxamic acid under the condition of a standard, sustained, intravenous infusion (0.6 mg./kg./min.).

RESULTS

Cardiovascular Responses to β-Hydroxylamine Cinnamonyl Hydroxamic Acid—In 61 experiments with 20 dogs, the cardiovascular responses to progressively increasing incremental doses of β-hydroxylamine cinnamonyl hydroxamic acid were studied. An intravenous bolus injection of 0.3 mg./kg. β-hydroxylamine cinnamonyl hydroxamic acid produced a decrease in mean systolic blood pressure of about 24 mm. Hg at peak response, and no significant reflex tachycardia was observed. However, a 0.3-mg. increment increase in bolus dose (0.6 mg./kg.) produced a 40-mm. Hg decrease in mean systolic pressure and a significant compensatory reflex tachycardia (Table I).

Over a bolus dose range of 0.6–1.8 mg./kg., an essentially linear reduction in mean systolic and diastolic blood pressure was observed; with each 0.3-mg. increment in dose up to a total of 1.8 mg./kg., mean systolic blood pressure was reduced about 14 mm. Hg (Table I and Fig. 1).

The vasodepressor response to a bolus injection of β-hydroxylamine cinnamonyl hydroxamic acid at all dose levels was prompt and short in duration; and in no experiment did the duration of response exceed 1 min. A slightly increased respiratory rate (4–6/min.) was usually noted when systolic blood pressure was reduced sufficiently (40 mm. Hg) to recruit reflex tachycardia.

Repeated intravenous bolus injections of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg.) at 5- and 2-min. intervals over a period of 26 min. showed no evidence of tachyphylaxis or anaphylaxis; *i.e.*, the vasodepressor response neither decreased nor increased (Fig. 2).

In 30 dogs, a continuous intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid at the rate of 0.3 mg./kg./min. produced a sustained decrease in average blood pressure of 14 ± 2.3 mm. Hg. Doubling the dose (0.6 mg./kg./min.) decreased average blood pressure 33 ± 6.2 mm. Hg. Sustained infusions of β-hydroxylamine cinnamonyl hydroxamic acid at the latter dose level for periods up to 2 hr. showed no cumulative effects. Qualitative analysis for methemoglobin (6) in blood samples taken at 10-min. intervals over a 2-hr. period of infusion of β-hydroxylamine cin-

[1] Confirmational elemental and structural analyses were carried out by Alfred Bernhardt, Max Planck Institute, West Germany.

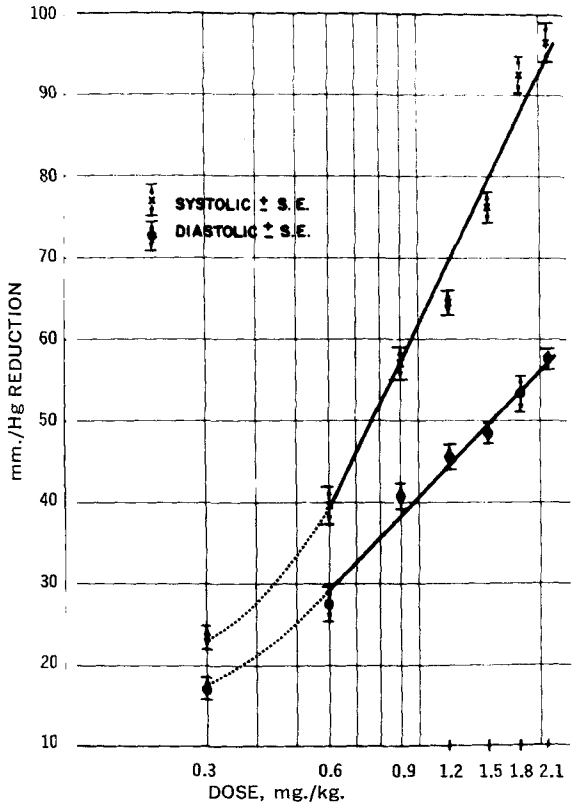


Figure 1—*Log dose–response plot of systolic and diastolic pressure reductions produced by progressively increasing incremental bolus doses of β-hydroxylamine cinnamonyl hydroxamic acid. Mean ± SD of response at each dose level was determined from 6–11 separate responses in an equal number of dogs. A least-squares fit applied to the dose range of 0.6–2.1 mg./kg. was employed to show that the response to the minimal effective dose was greater than that to any subsequent incremental increase.*

namonyl hydroxamic acid at several dose levels (0.6, 1.2, and 1.8 mg./kg./min.) was negative. Regardless of the dose–response relationship, blood pressure always returned promptly to preinfusion control levels on discontinuing β-hydroxylamine cinnamonyl hydroxamic acid. This is illustrated by a typical experiment in Fig. 3.

In 11 dogs rendered hypotensive (−30 ± 2.5 mm. Hg) by means of the standard infusion of β-hydroxylamine cinnamonyl hydroxamic acid, epinephrine HCl injected intravenously (1.5 mcg./kg.) produced a normal vasopressor response in all subjects. This response clearly suggested that α-adrenergic receptors were unaffected by the β-hydroxylamine cinnamonyl hydroxamic acid-induced vasodilatation.

Response to β-Hydroxylamine Cinnamonyl Hydroxamic Acid in the Presence of an Antihistamine—To determine if the vasodepressor response to β-hydroxylamine cinnamonyl hydroxamic acid was due in part to the release of endogenous histamine, β-hydroxylamine cinnamonyl hydroxamic acid was administered 30 min. before and after producing histamine block in seven dogs. An intravenous test dose of histamine diphosphate (0.01 mg./kg.) was injected 15 min. before and after the intravenous administration of diphenhydramine HCl (2.0 mg./kg.) in order to examine the efficacy of the histamine block. After establishing a satisfactory but varying degree of histamine block in each subject (Fig. 4), the intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg./min.) produced a vasodepressor response in all subjects that was identical in pattern and degree to the preblock control response (Table II).

Response to β-Hydroxylamine Cinnamonyl Hydroxamic Acid in the Presence of Atropine—To determine if β-hydroxylamine cinnamonyl hydroxamic acid produced elaboration of mediator acetylcholine, six dogs were treated with atropine sulfate (0.5 mg./kg. i.v.) 30 min. after a standard control infusion of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg./min.). An intravenous test dose of acetylcholine Cl (5.0 mcg./kg.) was administered 10 min. before and 40 min. after the atropine block. The marked vaso-

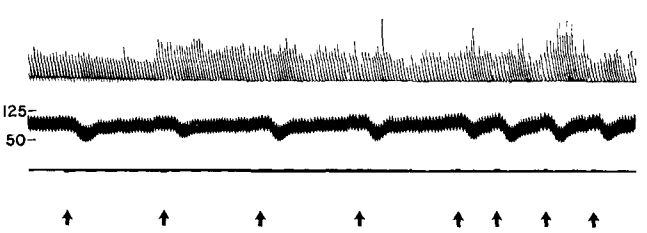


Figure 2—*Montage (eight frames) of a typical experiment showing complete absence of tachyphylaxis and anaphylaxis to repeated intravenous bolus injections of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg.) in the anesthetized dog. β-Hydroxylamine cinnamonyl hydroxamic acid was injected four times at 5-min. intervals and four times at 2-min. intervals as indicated. Regardless of the interval, each injection of β-hydroxylamine cinnamonyl hydroxamic acid produced a consistent 30-mm. reduction in average blood pressure. Respiratory rate (11/min.) and duration of response (50 sec.) to β-hydroxylamine cinnamonyl hydroxamic acid were unaffected by the repeated close-order injections.*

depressor and respiratory responses to injected acetylcholine were adequately blocked by atropine in all experiments (Fig. 5), and the postatropine vasodepressor response to the standard infusion of β-hydroxylamine cinnamonyl hydroxamic acid was unaltered (Table II). Because atropine adequately blocked the unusually dramatic vasodepressor response to the second challenging test dose of acetylcholine, it was clear that the lesser, yet characteristic, degree of vasodepression produced by the standard β-hydroxylamine cinnamonyl hydroxamic acid infusion was not mediated by acetylcholine.

Response to β-Hydroxylamine Cinnamonyl Hydroxamic Acid in the Presence of Physostigmine—To determine if the vasodepressor action of β-hydroxylamine cinnamonyl hydroxamic acid was potentiated or prolonged by acetylcholinesterase inhibition, the standard infusion of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg./min.) was administered before and after the intravenous administration of physostigmine salicylate (0.1 mg./kg.). In four of seven dogs, this acetylcholinesterase blocking dose of physostigmine produced measurable skeletal muscle fasciculations, with moderate increases in average blood pressure (Table II). Both the muscle fasciculation and elevated blood pressure persisted until β-hydroxylamine cinnamonyl hydroxamic acid was infused; however, the pattern and degree of vasodepressor response to β-hydroxylamine cinnamonyl hydroxamic acid were unaltered (Table II). The somatic neuroeffector response to physostigmine was regarded

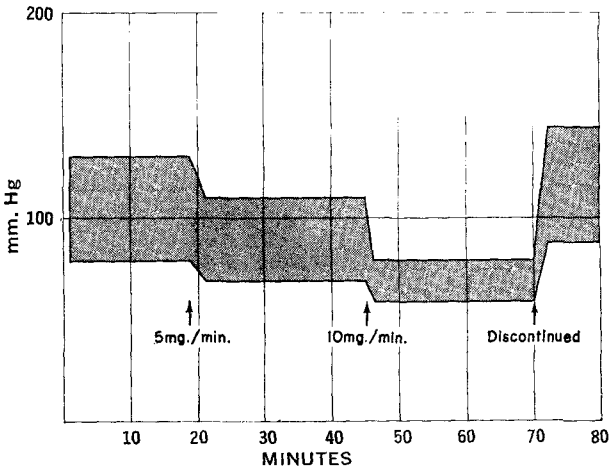


Figure 3—*Typical pattern of vasodepressor response to a continuous intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid at two dose levels. Note particularly the stability of the staircase reduction in systolic blood pressure at each dose level and the rapid recovery—with slight overshoot—of systolic-diastolic and pulse pressures following abrupt cessation of the β-hydroxylamine cinnamonyl hydroxamic acid infusion. Infusion rates at 5 and 10 mg./min. equal 0.3 and 0.6 mg./kg./min., respectively.*

Table II—Effect of Pretreatment with a Number of Blocking Agents on the Vasodepressor Action of β-Hydroxylamine Cinnamonyl Hydroxamic Acid in the Anesthetized Dog

1 Blocking Agent[a], Intravenous Dose	2 Average Control, Blood Pressure ± *SE*, mm. Hg	3 Response to Agent, Blood Pressure ± *SE*	4 Control Response to β-Hydroxylamine Cinnamonyl Hydroxamic Acid, Blood Pressure ± *SE*	5 Response to β-Hydroxylamine Cinnamonyl Hydroxamic Acid after Agent	6 Significance, Column 4 *versus* 5
Diphenhydramine, 2.0 mg./kg. (N = 7)	112 ± 7.7	−40 ± 6.8	−35 ± 2.1	−34 ± 2.4	N.S.
Atropine, 0.5 mg./kg. (N = 6)	111 ± 7.6	−1 ± 1.6	−27 ± 2.7	−26 ± 2.4	N.S.
Physostigmine, 0.1 mg./kg. (N = 7)	108 ± 7.5	+16 ± 7.1	−26 ± 2.4	−30 ± 2.5	N.S.
Dichloroisoproterenol, 10 mg./kg. (N = 5)	106 ± 5.5	−43 ± 2.1	−30 ± 1.7	−29 ± 1.6	N.S.
Chlorpromazine, 1.5 mg./kg. (N = 5)	110 ± 8.5	−5 ± 2.4	−33 ± 4.3	−32 ± 3.0	N.S.
Phenoxybenzamine, 12 mg./kg. (N = 6)	106 ± 7.7	−25 ± 1.3	−30 ± 1.5	−29 ± 3.0	N.S.

[a] A minimum of 30 min. was permitted to elapse between the administration of the blocking agent and the start of the standard infusion of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg./min.). The average control blood pressure in Column 2 was computed by adding the sums of the systolic and diastolic pressures and dividing by 2. The immediate response to the blocking agent (Column 3) was tabulated as the deviation from the average blood pressure in Column 2. The values in Column 4 represent the response to a standard β-hydroxylamine cinnamonyl hydroxamic acid infusion before the administration of the blocking agent. The significance in Column 6 was determined by the Student t test in a self-paired analysis.

as evidence of the significant acetylcholinesterase blocking action of physostigmine.

Atropine block (0.5 mg./kg.) of the muscarinic actions of elevated acetylcholine titers induced by physostigmine inhibition of acetylcholinesterase also failed to modify the characteristic vasodepressor response to β-hydroxylamine cinnamonyl hydroxamic acid. These experiments were identical in protocol and pattern of response to β-hydroxylamine cinnamonyl hydroxamic acid to those illustrated in Fig. 5.

Response to β-Hydroxylamine Cinnamonyl Hydroxamic Acid in the Presence of β-Adrenergic Blockade—To determine if β-hydroxylamine cinnamonyl hydroxamic acid produced its vasodepressor action through direct stimulation of β-adrenergic receptors, five dogs were treated with dichloroisoproterenol (10 mg./kg. i.v.) after a control standard infusion of β-hydroxylamine cinnamonyl hydroxamic acid. A challenging dose of isoproterenol (0.3 mg./kg. i.v.) was administered 30 min. after dichloroisoproterenol to appraise the adequacy of the β-adrenergic block in all subjects; the standard intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid produced its characteristic vasodepressor response (Table II).

Actions of β-Hydroxylamine Cinnamonyl Hydroxamic Acid in the Presence of α-Adrenergic Blockade—In six dogs pretreated with phenoxybenzamine HCl (12.0 mg./kg.) and five dogs pretreated with chlorpromazine HCl (1.5 mg./kg.), following epinephrine (1.5 mcg./kg.) verification of α-receptor blockade, the standard infusion of β-hydroxylamine cinnamonyl hydroxamic acid produced a vasodepressor response that was similar in all respects to the pretreated control response in 11 subjects (Table II).

Because β-hydroxylamine cinnamonyl hydroxamic acid failed to show β-adrenergic stimulating activity, it became of interest to determine if the epinephrine reversal produced by α-adrenergic blockade would be exaggerated or otherwise attenuated by β-hydroxylamine cinnamonyl hydroxamic acid. In the 11 α-block experiments previously described, wherein epinephrine reversal was observed in all subjects, a second bolus dose of epinephrine (1.5 mcg./kg.) administered during the infusion of β-hydroxylamine cinnamonyl hydroxamic acid now produced a clear vasopressor response (Fig. 6). On discontinuing the infusion of β-hydroxylamine cinnamonyl hydroxamic acid and again repeating the epinephrine, the classical epinephrine reversal was once again observed.

The β-hydroxylamine cinnamonyl hydroxamic acid-induced restoration of the epinephrine vasopressor response in the α-blocked subject (Arrow 2, Fig. 6) was in marked contradistinction to the epinephrine reversal observed before and after the infusion of β-hydroxylamine cinnamonyl hydroxamic acid (Arrows 1 and 3, Fig. 6). This response, produced by β-hydroxylamine cinnamonyl hydroxamic acid, could not be attributed to a temporary chronotropic action of epinephrine since a reflex bradycardia was observed in the majority of experiments.

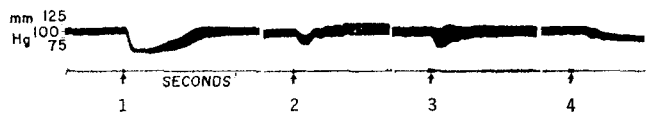


Figure 4—*Influence of histamine block on the vasodepressor actions of β-hydroxylamine cinnamonyl hydroxamic acid in the anesthetized dog. Histamine diphosphate (0.01 mg./kg.) was injected before and after (Arrows 1 and 3) the intravenous administration of a blocking dose (2.0 mg./kg.) of diphenhydramine HCl (Arrow 2). The initial histamine injection reduced blood pressure from 115/85 to 35/20 mm. Hg, while a second challenging dose of histamine, delivered 15 min. after diphenhydramine, reduced blood pressure from 120/85 to 110/40 mm. Hg. The confirmed and satisfactory degree of histamine block failed to modify the characteristic pattern and degree of vasomotor depression produced by a standard intravenous infusion (Arrow 4) of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg./min.). Evaluation of the systolic–diastolic and pulse pressure morphologies exemplified by histamine, diphenhydramine, and β-hydroxylamine cinnamonyl hydroxamic acid showed that endogenous histamine liberation is inconsequential to the consistent and stable blood pressure reduction produced by β-hydroxylamine cinnamonyl hydroxamic acid.*

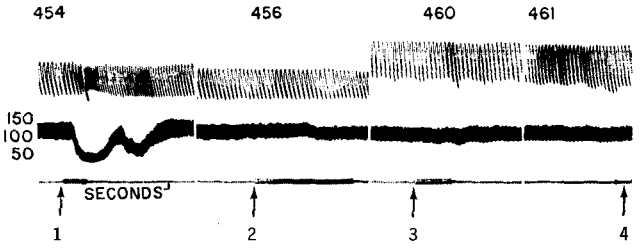


Figure 5—*Influence of cholinergic block on the vasodepressor actions of β-hydroxylamine cinnamonyl hydroxamic acid in the anesthetized dog. The vasomotor and respiratory responses to an intravenous test dose of acetylcholine Cl (5.0 mcg./kg.) were determined before (Arrow 1) and after (Arrow 3) the administration of 0.5 mg./kg. atropine sulfate (Arrow 2) in order to ascertain the adequacy of the atropine block. The atropine block, confirmed by the absence of significant respiratory and vasomotor responses to a challenging dose of acetylcholine (Arrow 3), did not alter the characteristic pattern of vasomotor responses to a standard infusion (0.6 mg./kg./min.) of β-hydroxylamine cinnamonyl hydroxamic acid (Arrow 4). It is apparent in this typical experiment that the vasodepressor action of β-hydroxylamine cinnamonyl hydroxamic acid is not dependent upon cholinergic mediators. (Respiration, upper tracing.)*

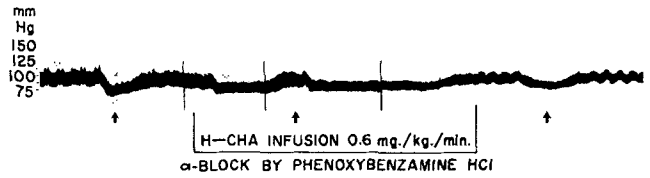


Figure 6—*Montage of a typical experiment demonstrating negation of α-adrenergic blockage by β-hydroxylamine cinnamonyl hydroxamic acid. In the phenoxybenzamine-pretreated dog (12.0 mg./kg.), a bolus injection of epinephrine (1.5 mcg./kg.) produced marked vasodepression (Arrow 1) and confirmed the adequacy of the α-block. Repeating the epinephrine during the infusion of β-hydroxylamine cinnamonyl hydroxamic acid now produced a definite vasopressor response (Arrow 2). Five minutes after discontinuing the infusion of β-hydroxylamine cinnamonyl hydroxamic acid, a third injecton of epinephrine (Arrow 3) showed reestablishment of the phenoxybenzamine-induced α-blockade. The infusion of β-hydroxylamine cinnamonyl hydroxamic acid clearly lifted the confirmed α-block; however, it did not permanently displace phenoxybenzamine nor prevent rapid reestablishment of the α-block.*

In control experiments utilizing direct-acting vasodilator agents (nitroglycerin, sodium nitrite, and papaverine) and hemorrhage (12% of calculated blood volume) to reduce systemic blood pressure about 30 mm. Hg, an intravenous bolus injection of epinephrine during the induced hypotension of the α-blocked dog failed to produce a vasopressor response. Only in the hemorrhaged hypotensive subject did epinephrine produce a small chronotropic response which was inadequate to modify significantly the epinephrine reversal produced by phenoxybenzamine α-blockade.

DISCUSSION

The most significant observations in this study relate to the ability of β-hydroxylamine cinnamonyl hydroxamic acid to exert a consistent hypotensive action on sustained intravenous infusion and to restore the pressor action of epinephrine in an established α-adrenergic blockade.

The vasodepressor responses to bolus injections of β-hydroxylamine cinnamonyl hydroxamic acid over a wide dose range were of short duration, lasting 50 sec. on the average, and no tachyphylaxis or anaphylaxis was observed following repeated close-order intravenous administration (Fig. 2). Prolonged intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid, producing 30-mm. Hg reductions of average systemic blood pressure for periods up to 4 hr., produced no apparent cumulative effects.

The ultrashort duration of action of β-hydroxylamine cinnamonyl hydroxamic acid is apparently related to its unique molecular structure. β-Aminopropionohydroxamic acids, synthesized and studied recently by Coutts *et al.* (7), produced hypotension in the anesthetized cat lasting from 5 to 120 min. following a single intravenous bolus injection. Substituting cyclic tertiary amines on the β-carbon of propionohydroxamic acids—in contrast to arylhydroxylamine substitutions—probably accounts for the minimal fivefold increase in duration of action.

The lowering of systemic blood pressure by β-hydroxylamine cinnamonyl hydroxamic acid in the anesthetized dog was not modified significantly by any of the mediator blocking agents studied. The pharmacodynamic action of β-hydroxylamine cinnamonyl hydroxamic acid, therefore, appears to be direct on vascular smooth muscle.

Pretreatment of dogs with large doses of diphenhydramine (2.0 mg./kg.) and atropine sulfate (0.5 mg./kg.) consistently failed to produce any alteration in the blood pressure responses to intravenous bolus injections or sustained infusions of β-hydroxylamine cinnamonyl hydroxamic acid. It was clear in all experiments that β-hydroxylamine cinnamonyl hydroxamic acid was not acting directly on histaminergic or cholinergic (muscarinic) receptors (Figs. 4 and 5).

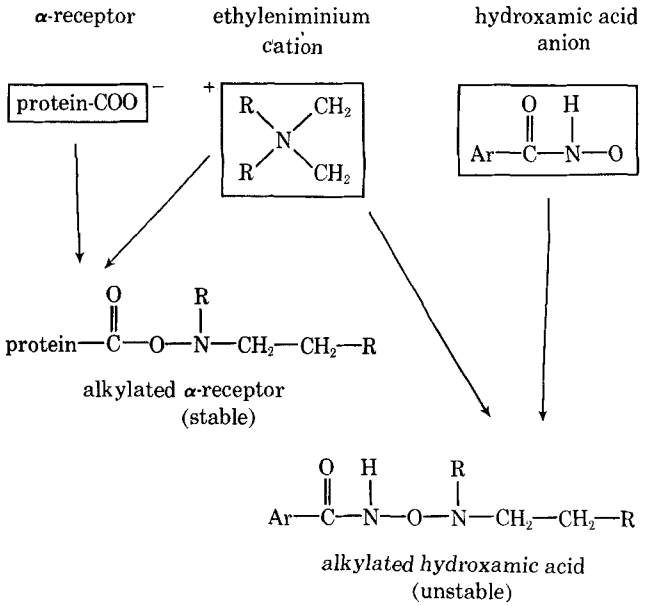
The infusion of β-hydroxylamine cinnamonyl hydroxamic acid in the physostigmine-pretreated dog produced no greater vasodepressor response than that observed in control experiments. Skeletal muscle fasciculations observed in four of seven experiments following intravenous administration of physostigmine was promptly and completely abolished by the standard intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid (0.6 mg./kg./min.). This observation strongly suggested a direct action of β-hydroxylamine cinnamonyl hydroxamic acid on skeletal muscle contractile mechanisms and provided further evidence that the action of β-hydroxylamine cinnamonyl hydroxamic acid is independent of cholinergic mediation. This antinicotinic action of β-hydroxylamine cinnamonyl hydroxamic acid is being investigated further.

The use of a β-receptor blocking agent (dichloroisoproterenol) demonstrated that β-hydroxylamine cinnamonyl hydroxamic acid did not produce its vasodepressor action through stimulation of β-receptor mechanisms. A bolus injection or a sustained infusion of β-hydroxylamine cinnamonyl hydroxamic acid in the β-blocked animal lowered systemic blood pressure to the same degree as in untreated controls (Table II).

α-Receptor blocking agents (phenoxybenzamine and chlorpromazine) in no way modified the vasodepressor response to β-hydroxylamine cinnamonyl hydroxamic acid. On the other hand, an infusion of β-hydroxylamine cinnamonyl hydroxamic acid was clearly able to restore—for the duration of the infusion—a confirmed α-blockade produced by either blocking agent; *i.e.*, during an infusion of β-hydroxylamine cinnamonyl hydroxamic acid, an intravenous injection of epinephrine that previously produced marked vasodilatation now produced a pronounced vasoconstriction accompanied by reflex bradycardia (Fig. 6). It is interesting indeed that the restoration of the epinephrine vasopressor response in the α-blocked dog produced by β-hydroxylamine cinnamonyl hydroxamic acid in no way interfered with normal cardiovascular buffer reflexes.

The apparent α-block lift produced by β-hydroxylamine cinnamonyl hydroxamic acid was both peculiar and unique inasmuch as the several types of α-block lift previously reported (8, 9) are seemingly of a different nature. For example, permanent α-block lift may be produced by trypsin enzymatic hydrolysis of haloalkylamine-alkylated α-receptors, and temporary lift may be produced by large doses of ephedrine or methoxamine. β-Hydroxylamine cinnamonyl hydroxamic acid-induced α-block lift occurred only for the duration of a standard intravenous infusion, and the α-receptor block produced by the haloalkylamines employed (phenoxybenzamine and chlorpromazine) was reestablished immediately after discontinuing the infusion (Fig. 6).

It now appears well established that β-haloalkylamines ionize to form ethyleniminium cations which alkylate and covalently bond to the carboxyl sites of α-adrenergic receptor enzyme profiles (10). In Scheme I, it is postulated that hydroxylamine cinnamonyl hydroxamic acid, a highly polar aralkyl molecule with anionic properties, would have a strong physicochemical affinity for the ethyleniminium cation. Acting as an anion, hydroxylamine cinnamonyl hydroxamic acid may combine with the labile cationic fraction



Scheme I

(ethyleniminium) of the β-haloalkylamine and prevent subsequent alkylation of "reserve adrenergic receptors" (11) brought forth through the vasodilator action of hydroxylamine cinnamonyl hydroxamic acid. In this way, hydroxylamine cinnamonyl hydroxamic acid would both restore the epinephrine vasopressor response and protect spare α-receptors from alkylation.

The β-hydroxylamine cinnamonyl hydroxamic acid–ethyleniminium bonding (Scheme I) is clearly unstable since it can be maintained only by a continuous infusion of β-hydroxylamine cinnamonyl hydroxamic acid. The dual actions of β-hydroxylamine cinnamonyl hydroxamic acid—rendering spare receptors accessible and bonding ethyleniminium—only mimic an α-block lift inasmuch as the alkylated α-receptors are not inactivated. This was evident by the observation that epinephrine reversal was immediately reinstituted on discontinuing the infusion of β-hydroxylamine cinnamonyl hydroxamic acid.

The maintenance of vascular smooth muscle tone is related to controlled neural and adrenal baroreceptor-triggered adrenomedullary release of catecholamines (12). Recent evidence indicates that norepinephrine may even be synthesized locally within the arterial wall (13), and the interaction of epinephrine and/or norepinephrine and adenosine triphosphate provides necessary energy for smooth muscle contraction and maintenance of vascular tone (14).

Adrenomedullary release of catecholamines has been shown to be adenosine triphosphate dependent, and it can be blocked with *N*-ethylmalimide which interferes with adenosine triphosphotase (15). The direct inhibitory action of β-hydroxylamine cinnamonyl hydroxamic acid at the vascular smooth muscle level is perhaps like that of *N*-ethylmalimide at the adrenomedullary level; *i.e.*, β-hydroxylamine cinnamonyl hydroxamic acid most likely interferes with energy-dependent processes within the arterial smooth muscle wall. The vasodilatation induced by β-hydroxylamine cinnamonyl hydroxamic acid may also be due in part to the formation of organometallic complexes (β-hydroxylamine cinnamonyl hydroxamic acid–Ca or β-hydroxylamine cinnamonyl hydroxamic acid–Mg) with calcium or magnesium ions that would interfere with adenosine triphosphate-dependent energy-generating mechanisms (15).

The metabolic fate of β-hydroxylamine cinnamonyl hydroxamic acid is unknown at this time. Utilizing the ferric chloride ($FeCl_3$) method for determining the hydroxamic acid moiety in β-hydroxylamine cinnamonyl hydroxamic acid, all attempts to detect β-hydroxylamine cinnamonyl hydroxamic acid in serum, whole blood, and ureteral urine met with only limited success, and then only after a 4-hr. period of administering the standard intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid. In a 20.0-kg. dog, this constituted a total dose of 2.88 g. The rapid inactivation of β-hydroxylamine cinnamonyl hydroxamic acid in body fluids is in complete accord with its ultrashort duration of action.

Chemical analyses for methemoglobin in blood samples taken at 30-min. intervals over a 4-hr. period of constant infusion (0.6 mg./kg./min.) of β-hydroxylamine cinnamonyl hydroxamic acid showed only a trace toward the end of the infusion period.

Preliminary studies of the toxicity of β-hydroxylamine cinnamonyl hydroxamic acid indicate that the intraperitoneal LD_{50} in the rat is of the order of 350 mg./kg. Lethal doses of β-hydroxylamine cinnamonyl hydroxamic acid, administered intraperitoneally, produced CNS depression, hypoxemia, and marked cyanosis, followed by clonic convulsions and death.

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Metal Complexes of Thiouracils II: Solubility Analyses and Spectrophotometric Investigations

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Abstract □ Complexes of Cu^{+2}, Cd^{+2}, and Pb^{+2} with 2-thiouracil, 6-*n*-propyl-2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, and 5,6-dimethyl-2-thiouracil have lower absorptivities than the parent thiouracils. On the premise that all metal-ion complexes of thiouracils have similar absorptivities, cupric ion is a much more highly complexing metal (4–10×) than cadmium and lead. The lack of spectral aberration on the addition of Fe^{+3} and Fe^{+2} to solutions of these thiouracils shows that no significant complexation occurs with these metal ions. None of the studied metals forms significantly spectrophotometrically observable complexes with 2-ethylmercapto-4-hydroxypyrimidine and 6-methyl-*N,N'*-diethyl-2-thiouracil. This fact implies that only those tautomerizable thiouracils that can form ionizable sulfhydryl groups are capable of metal complexation. Solubility analysis methods were devised for the determination of stability constants and solubilities of 1:1, 1:2, and mixed ligand complexes. However, thiouracil solubility was not significantly different in the absence and presence of metal ions. Also, metal-ion concentrations were not significant in saturated thiouracil solutions and demonstrated that the solubilities of the precipitating complexes were less than the accuracies of the spectrophotometric thiouracil and polarographic metal analyses. A previous literature proposal that a cuprous-2-thiouracil disulfide complex is the major result in mixtures of cupric ion and 2-thiouracil was denied by the demonstration that thiouracil is not oxidized by cupric ion in acidic solutions, that 2-thiouracil disulfide readily disproportionates in acid to the sulfinic acid and 2-thiouracil, and that the rate of such disproportionation is independent of cupric-ion concentration.

Keyphrases □ Thiouracil–metal complexes—solubility analyses, spectrophotometric investigation □ Complex formation—thiouracils–cupric, cadmium, ferric ions □ Solubility analyses—thiouracil–metal complexes □ Potentiometric titration—stability constant analysis, complexes □ UV spectrophotometry—stability constant analysis, complexes

It was shown (1) that the divalent metals (M^{+2}): Pb^{+2}, Cd^{+2}, Ni^{+2}, and Zn^{+2}, complex with the 5- and/or 6-alkyl-substituted thiouracil monanions, U^- (II), as shown in Scheme I. Significant concentrations of MU^+ (III) and MU_2 (IV) complexes, with bonding of metal ion to the sulfur anion, permitted estimation of the respective K_1 and K_2 constants by potentiometric titrations in homogeneous solutions. Precipitated complexes MU_2 and M_2U_2 (VII) were isolated and characterized for the more stable Pb^{+2} and Cd^{+2} complexes, where M_2U_2 is most probably the cyclic dimer, bis-(thiouracil–metal), formed by mutual head-tail bonding of the positively charged divalent metal ion complexed with the sulfide anion and the anionic oxygen of the dissociated 4-hydroxyl of the enolized pyrimidine ring.

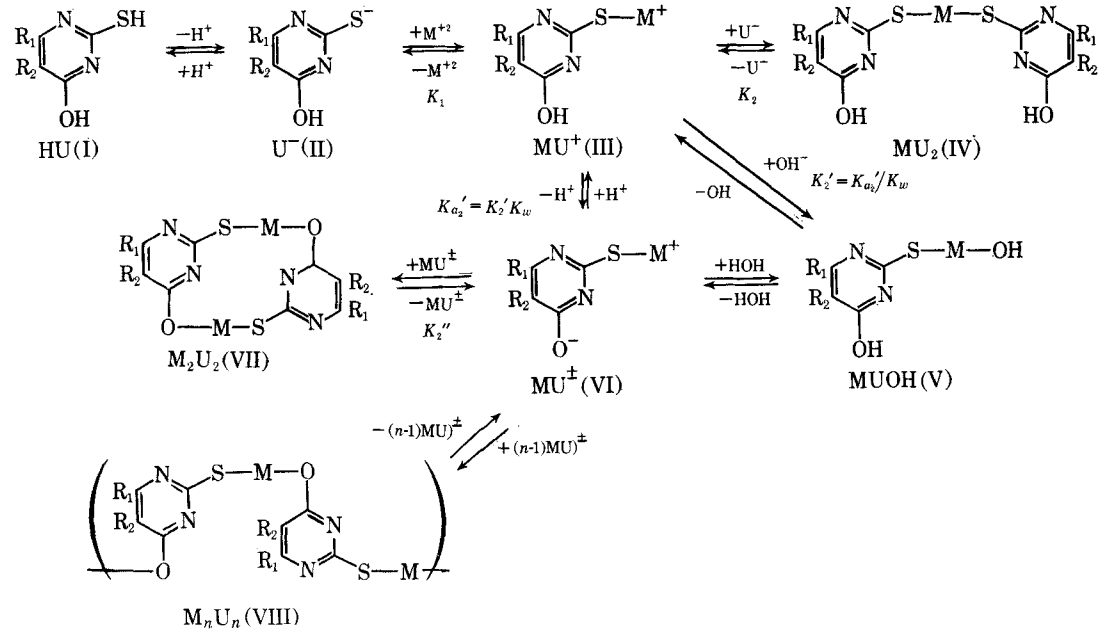
These subsequent studies were made to utilize spectrophotometric techniques to provide evidence for complex formation of these metal ions with the variously substituted thiouracils. A major purpose was to investigate the stability of the cupric-ion complexes by such methods since their high insolubility made potentiometric titration techniques impractical. Potentiometric titration techniques for the calculation of stability constants are limited in that the complexes formed must stay in solution and, in general, must be constituted with homogeneous or unmixed ligands. Another purpose of these studies was to evaluate the ability of solubility analyses under controlled conditions to identify the presence and stoichiometry of formed complexes and to calculate the solubilities and stability constants of such possible complexes as $MU^{\pm}$, MUOH, M_2U_2, and M_nU_n (Scheme I) which could not be determined potentiometrically (1).

Moreover, it had been proposed that the result of interaction of thiouracil and cupric copper was oxidation to the 2-thiouracil disulfide to form a stable complex with the simultaneously formed cuprous ion (2). This does not seem reasonable since 2-thiouracil disulfide is known to disproportionate readily to thio-

Table I—Absorbance Decrease from Additive Absorbances at $\lambda_{max.}$[a] of Thiouracil–Metal-Ion Mixtures

Compound	Absorbance at $\lambda_{max.}$[a] of 1.00×10^{-4} *M* TU	Metal, M^{+n}							
		Cu^{+2}		Cd^{+2}		Pb^{+2}		Fe^{+3}	Fe^{+2}
		10^4 $[M^{+n}]$							
		1.00	7.00	1.00	7.00	1.00	7.00	1.00[b]	1.00[c]
2-Thiouracil	1.328	0.208	0.341	0.051	0.086	0.050	0.120	−0.020	0.015
6-*n*-Propyl-2-thiouracil[d]	1.520	0.460	0.464	0.012	0.150	0.028	0.069	−0.012	−0.013
6-Methyl-2-thiouracil	1.512	0.512	0.567	0.011	0.085	0.025	0.065	0.006	−0.019
5-Methyl-2-thiouracil[e]	1.535	0.478	0.463	0.039	0.123	0.048	0.085	0.024	0.008
5,6-Dimethyl-2-thiouracil[d]	1.763	0.293	0.438	0.030	0.035	0.027	0.059	0.002	−0.003
2-Ethylmercapto-4-hydroxypyrimidine	0.550	−0.011	−0.020	−0.005	−0.013	0.003	0.006	0.024	0.000
6-Methyl-*N,N'*-diethyl-2-thiouracil	1.320	−0.001	−0.012	−0.001	−0.021	0.026	0.033	—	—

[a] $\lambda_{max.}$ for 2-ethylmercapto-4-hydroxypyrimidine was 284 nm., whereas the values for the other compounds lay between 272 and 278 nm. [b] A significant absorbance of about 0.210 was due to 1.00×10^{-4} *M* Fe^{+3}; in the noniron cases, the metals gave no absorbances at the $\lambda_{max.}$. This absorbance made reasonable differences unobtainable at 7.00×10^{-4} *M* Fe^{+3}. [c] The ready oxidation of ferrous ion to ferric ion by air at high concentrations gave anomalous interferences at 7.00×10^{-4} *M* Fe^{+2}. [d] Precipitates formed when these compounds were mixed with cupric ion so that the values under Cu^{+2} of absorbance decrease from additive absorbances for these compounds may not be considered as exact for complex formation in homogeneous solutions. No obvious precipitate was observed on mixing of the other thiouracils and Cu^{+2}. If precipitation had occurred in these cases, a relatively significant and wavelength-independent absorbance would have appeared at values above 310 nm. This was not observed (Fig. 1). [e] When the solvent was 0.001 *M* $HClO_4$ rather than water, there were negligible decreases in absorbance at the $\lambda_{max.}$ for mixtures of 10^{-4} *M* 5-methyl-2-thiouracil and 1.00×10^{-4} *M* and 7.00×10^{-4} *M* Cu^{+2} where the apparent absorbance decreases were 0.001 and 0.007, respectively.



Scheme I

uracil and the corresponding sulfinic acid (3, 4), and cuprous ion is known to readily disproportionate to cupric ion and metallic copper in solution (5). Spectral monitoring of these phenomena could give insight into the validity of this proposal.

EXPERIMENTAL[1]

Materials—The purification and characterization of the various thiouracils studied: 2-thiouracil, 6-*n*-propyl-2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, 5,6-dimethyl-2-thiouracil, 2-ethylmercapto-4-hydroxypyrimidine, and 6-methyl-*N*,*N*′-diethyl-2-thiouracil, and the metals used were reported previously (1).

Spectra of Thiouracil–Metal-Ion Mixtures—The UV spectra of aqueous solutions, 1.00×10^{-4} *M* in the thiouracils and 0.00, 1.00, or 7.00×10^{-4} *M* in cupric, cadmium, lead, ferric, or ferrous nitrates, were recorded on the Cary model 15 dual-beam UV recording spectrophotometer against a water blank (Table I). The general procedure was to dilute stock solutions appropriately so as to record the spectra of the metal nitrate and thiouracil alone and then in combination (Figs. 1–3) at the stated molarities. Since the only metal ion studied with a large UV absorption was ferric ion (Fig. 3), spectrophotometric studies of solutions of mixtures, 1.0×10^{-4} *M* in thiouracil and 7.0×10^{-4} *M* in ferric or ferrous ion, were impractical. In the ferrous-ion case, this was due to the ready oxidation of such a large concentration to the absorbing and interfering ferric ion.

Slight precipitates were observed when cupric ion was mixed with 6-*n*-propyl-2-thiouracil and 5,6-dimethyl-2-thiouracil, so the resultant absorbance values of such mixtures (Table I) are probably below those that would have resulted from complexation in homogeneous solution. No precipitate was observed when cupric ion was added to the solutions of 2-thiouracil, 6-methyl-2-thiouracil, 5-methyl-2-thiouracil, 2-ethylmercapto-4-hydroxypyrimidine, or 6-methyl-*N*,*N*′-diethyl-2-thiouracil when the concentrations were as stated. If precipitation had occurred in these cases, it would be anticipated that absorbance due to turbidity would be independent of wavelength and would have been observed at wavelengths greater than 310 nm. This was not observed (Fig. 1).

The UV spectra of cupric-2-thiouracil mixtures were recorded at five hydrogen-ion concentrations that ranged from 1.0 to 1×10^{-4} *M* in $HClO_4$. The cupric-ion concentration was 2×10^{-4} *M* and the 2-thiouracil concentration was 10^{-4} *M* in each mixture. The spectra were read against blank solutions with appropriate $HClO_4$ concentrations. Spectra of mixtures that were 9×10^{-5} *M* in 2-thiouracil, 0.05 *M* in $HClO_4$, and 9 or 0.43×10^{-4} *M* in Cu^{+2} were also obtained.

Method of Continuous Variations (6) Applied to 2-Thiouracil and Cupric Ions—Varying aliquots of 1.00×10^{-4} *M* 2-thiouracil solu-

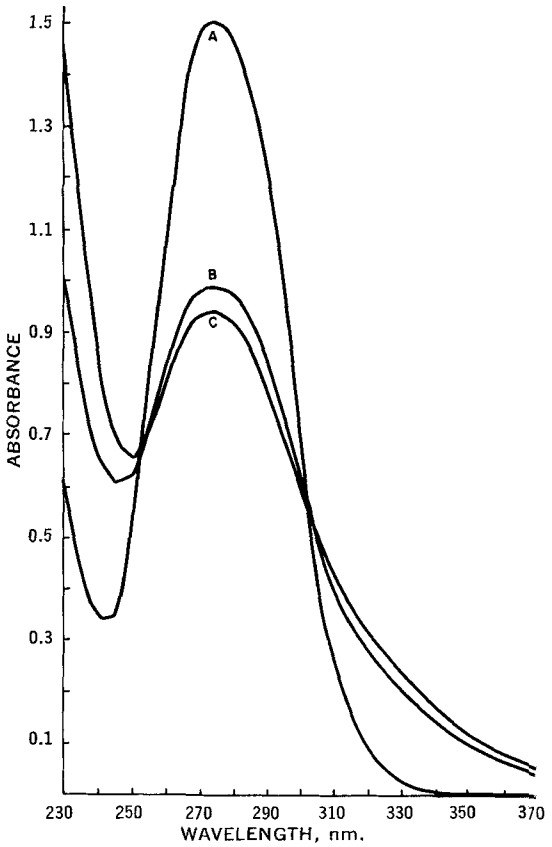


Figure 1—*UV spectra of mixtures of cupric nitrate and 6-methyl-2-thiouracil in water at 25.0°, where 1.00×10^{-4} M Cu^{+2} has no absorbance. Key: Curve A, 1.00×10^{-4} M 6-methyl-2-thiouracil; Curve B, 1.00×10^{-4} M $Cu(NO_3)_2$ and 1.00×10^{-4} M 6-methyl-2-thiouracil; and Curve C, 7.00×10^{-4} M $Cu(NO_3)_2$ and 1.00×10^{-4} M 6-methyl-2-thiouracil.*

[1] The elemental analyses and molecular weight and weight loss measurements in these investigations were performed by Huffman Laboratories, Inc., Wheatridge, Colo.

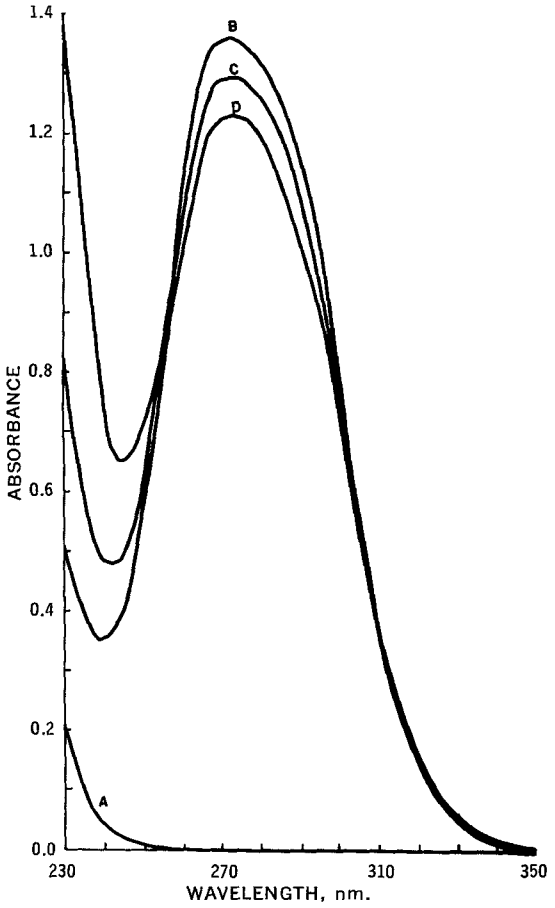


Figure 2—*UV spectra of mixtures of lead nitrate and 2-thiouracil in water at 25.0°. Key: Curve A, 1.00 × 10⁻⁴ M Pb⁺²; Curve B, 1.00 × 10⁻⁴ M 2-thiouracil; Curve C, 1.00 × 10⁻⁴ M 2-thiouracil and 1.00 × 10⁻⁴ M Pb⁺²; and Curve D, 1.00 × 10⁻⁴ M 2-thiouracil and 7.00 × 10⁻⁴ M Pb⁺².*

tion were added to aliquots of 1.00×10^{-4} *M* cupric nitrate solution so that the total number of moles of 2-thiouracil plus cupric ion was kept constant at 1.00×10^{-4}. The UV spectrum of each solution was recorded against a water blank.

Comparison of Total Thiouracil Solubilities at Various pH Values in the Absence and Presence of Cadmium Ion—Samples (200 mg.) of 2-thiouracil and 6-*n*-propyl-2-thiouracil were placed in volumetric flasks, and 40 ml. of solutions (ranging from 10^{-3} to 0.1 *M*) of varying concentrations of perchloric acid and sodium hydroxide was added. The final pH values ranged up to 6.5. The solutions with excess thiouracil were equilibrated at 25.0° in a controlled-temperature shaker bath, and the excess thiouracil was allowed to settle. Aliquots (5 ml.) of $Cd(NO_3)_2$ solutions were added slowly to some of the flasks; those that showed a precipitate on addition of the cadmium ions were discarded. The volume of each flask was made up to 50.0 ml. with the same aqueous solutions and reequilibrated at 25.0° in the shaker bath. The resultant molarity in Cd^{+2} was 0.001 *M*, although in some cases a 0.005 *M* Cd^{+2} solution was possible at lower pH values.

Filter sticks (Sargent No. S-30417) were used to remove samples from the equilibrated solutions. The first filtrate was discarded; 2.0-ml. aliquots of the filtered equilibrated solution were taken, appropriately diluted (50- or 100-fold) with 0.1 *M* $HClO_4$, and read on the Beckman DU spectrophotometer at $\lambda_{max.} = 272$ nm. against a 0.1 *M* $HClO_4$ blank solution to calculate the thiouracil in solution. Repetitive samples were taken with time to assure complete equilibration. The pH values of the equilibrated solutions were read in the presence of precipitate in a 10-ml. beaker.

Determination of Metal Content at Various pH Values in Saturated Thiouracil Solutions—Solutions (50 ml.), saturated with 2-thiouracil, 6-*n*-propyl-2-thiouracil, and 6-methyl-2-thiouracil, were prepared as described in the previous section; the final solutions were varied in $Cd(NO_3)_2$ from 1 to 15×10^{-3} *M*. Included in the 50-ml. volume were 1, 5, or 10 ml. of 0.01 *M* NaOH solutions. These solutions were equilibrated in a 25° shaker bath. At intervals, 5 ml. of the equilibrated solution was filtered through fritted filter sticks. Aliquots of these filtrates were diluted up to volume (50 ml.) with 1 *M* HNO_3, and 0.125 ml. 0.2% of a surface-active agent[2] solution was added to suppress the polarographic maxima. The solutions were purged with nitrogen and polarographically analyzed for cadmium ion, using a calibration curve made from similarly prepared solutions of known cadmium nitrate concentrations (6).

The $E_{1/2}$ in 1 *M* HNO_3 for Cd^{+2} is −0.59 v. against the saturated calomel electrode. A Sargent model XV polarograph was used. The H-type electrolysis cell was immersed in a constant-temperature bath at 25.0 ± 0.1°.

Preparation and Purification of Disodium Salt of 2-Thiouracil Disulfide Monohydrate, RSSR (X)—This material was prepared according to the procedure of Miller *et al.* (3). 2-Thiouracil (2.56 g.) was dissolved in 41 ml. of 1.0 *N* NaOH, and the solution was cooled in an acetone–dry ice bath until nearly frozen. Sodium iodide (4.57 g.) and iodine (2.54 g.) were dissolved in 20 ml. of water and also cooled in acetone–dry ice. The iodine–iodide solution was added slowly, with stirring, to the alkaline 2-thiouracil solution in an 800-ml. beaker. About 500 ml. of acetone was added, and the slow-forming white precipitate was collected. The precipitate was recrystallized from water–acetone twice and dried at 70°. Equivalent weight 156.6; calculated for $C_8H_4N_4Na_2O_2S_2 \cdot H_2O$, 158.1. UV spec-

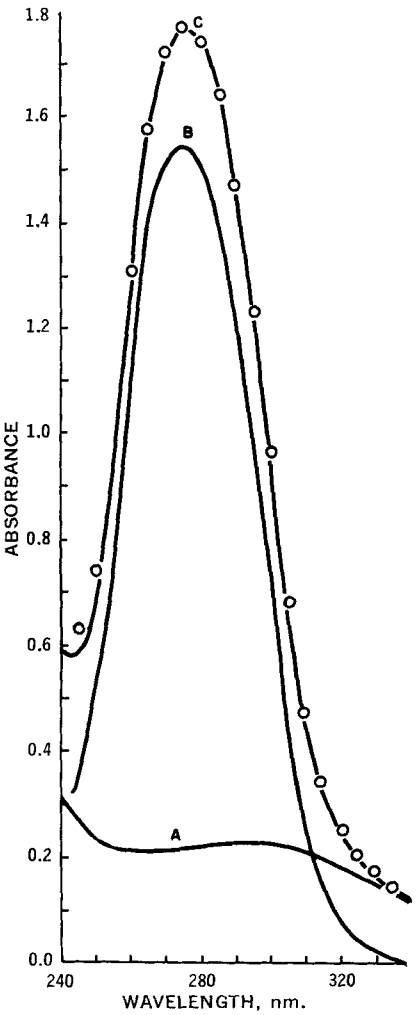


Figure 3—*UV spectra of mixtures of ferric nitrate and 6-methyl-2-thiouracil in water at 25.0°. Key: Curve A, 1.00 × 10⁻⁴ M Fe⁺³; Curve B, 1.00 × 10⁻⁴ M 6-methyl-2-thiouracil; and Curve C, 1.00 × 10⁻⁴ M 6-methyl-2-thiouracil and 1.00 × 10⁻⁴ M Fe⁺³. The plotted points are the summation of the absorbances of A and B.*

[2] Triton.

trum (3) (H_2O), $\lambda_{max.}$ 270 (ϵ 18,520), $\lambda_{max.}$ 211 (ϵ 25,485); (pH 9.56 ammonia buffer), $\lambda_{max.}$ 273 (ϵ 12,469), $\lambda_{max.}$ 213 (ϵ 31,537). IR spectrum, $\bar{\nu}$ in $cm.^{-1}$ (mineral oil mull): 3250 (OH), 1570, 1530, 1350, 1170, 1000, 828, 720. Molecular weight (ebullioscopic in water) 96; calculated for $C_8H_4N_4Na_2O_2S_2 \cdot H_2O$, 105.

Anal.—Calcd. for $C_8H_4N_4Na_2O_2S_2 \cdot H_2O$: C, 30.38; H, 1.91; N, 17.71; Na, 14.54; S, 20.27. Found: C, 29.87; H, 2.73; N, 14.38; Na, 15.15; S, 20.67.

Synthesis and Purification of Disodium Salt of 2-Sulfinyl-4-hydroxypyrimidine Trihydrate, RSO_2H (XI)—2-Thiouracil (2.56 g.) was dissolved in 26 ml. of 1.0 *N* NaOH, and 2.1 ml. of 34% hydrogen peroxide was slowly added with stirring to the acetone–dry ice chilled solution. Acetone was added to the point of precipitation, and the reaction mix was maintained cold overnight. The reaction mix was filtered and the precipitate washed with acetone; the washings were added to the filtrate, and the filtrate was put back into the refrigerator overnight. A white precipitate came down, which was collected, recrystallized twice from acetone–water, and dried at 50° in a vacuum oven. Equivalent weight 257.3; calculated for $C_4H_2N_2Na_2O_3S \cdot 3H_2O$, 256.1. UV spectrum (0.05 *M* $HClO_4$), $\lambda_{max.}$ 257 (ϵ 4924), $\lambda_{max.}$ 213 (ϵ 5885), $\lambda_{max.}$ 270 (ϵ 3510); (0.1 *M* $HClO_4$), $\lambda_{max.}$ 270 (ϵ 3631).

Anal.—Calcd. for $C_4H_2N_2Na_2O_3S$: C, 23.8; H, 1.00; N, 13.8; Na, 22.8; S, 15.87; weight loss for trihydrate, 21.15. Found: C, 23.96; H, 3.16; N, 12.38; Na, 21.03; S, 16.27; weight loss, 21.53.

For both Compounds X and XI, the consistencies of the C, S, and Na analyses of single samples were considered as sufficient evidence for the anticipated identities of the compounds since H and N analyses are notoriously difficult for the salts of such polyelemental compounds.

Degradation of 2-Thiouracil Disulfide in Absence and Presence of Cupric Ion—A solution (1.999×10^{-4} *M*) of the disodium salt of 2-thiouracil disulfide monohydrate was prepared by dissolving 6.322 mg. in 100 ml. of water. Aliquots (2.0 ml.) of the disulfide stock solution were added to: (*a*) 3.0 ml. H_2O or (*b*) 2.0 ml. H_2O and 1.0 ml. of 4.00×10^{-4} *M* cupric nitrate. Each solution was prepared when needed; then 5.00 ml. of 0.1 *M* $HClO_4$ was added very rapidly from a 5.00-ml. syringe (Hamilton) and mixed. The change in absorbance was recorded continuously at 270 nm. The UV absorption was measured using 1-cm. cells and a 0.05 *M* $HClO_4$ blank. A water-jacketed (25.0°) Beckman DU spectrophotometer, fitted with an automatic sample changer (Gilford) and connected to a strip-chart recorder (Sargent SRL), was used to record the absorbance as a function of time. The absorbances of all three solutions were recorded up to 400 min., well beyond the time when no further change occurred.

RESULTS AND DISCUSSION

Spectral Studies—The data of Table I summarize the spectral information for the solutions of mixtures of various thiouracils and metal ions obtained from the UV spectra shown in Figs. 1–3.

The premise of additivity of the absorbances attributable to the concentrations of thiouracil and metal ion should hold when there is no interaction. Only ferric ion of the studied metal ions had significant absorption at the $\lambda_{max.}$ values of the thiouracils. Since these particular studies (Figs. 1–3 and Table I) used a relatively high concentration of thiouracil (10^{-4} *M*) with high absorbances at the $\lambda_{max.}$ (about 1.5), differences between the anticipated and observed absorbances would have to exceed 0.02 to permit conclusions of significant interaction between the metal ion and the thiouracil.

On these premises, it is apparent (Table I) that Cu^{+2} (Fig. 1), Cd^{+2}, and Pb^{+2} (Fig. 2) show significant complex formation with almost all the studied thiouracils. The exceptions are the two compounds that cannot form an ionizable sulfhydryl group. These nontautomerizable compounds are 2-ethylmercapto-4-hydroxypyrimidine and 6-methyl-*N*,*N*′-diethyl-2-thiouracil. Absorbances of mixtures of the studied metals and these compounds were the same as the absorbance contributions of the compounds alone at all wavelengths.

There are no significant interactions of Fe^{+3} or Fe^{+2} with any of the listed thiouracils (Table I). This is clearly shown in the typical case of Fe^{+3} and 6-methyl-2-thiouracil (Fig. 3), where the plotted summed absorbances, points on Curve C, of the separate solutions of Fe^{+3} (Curve A) and 6-methyl-2-thiouracil (Curve B) are coincident with the recorded absorbances (Curve C) at all wavelengths of a mixture of the same concentrations.

The interaction of copper ions with the sulfhydryl-containing thiouracils, as monitored by the absorbance decrease from expected additivity, is obviously of a higher order of magnitude than such interactions with Cd^{+2} or Pb^{+2}, as can be seen in Table I and on comparison of Figs. 1 and 2 for equimolar Cu^{+2} and Pb^{+2}. The complexes in solution probably are similar in nature and have absorptivities that are not highly dependent on the nature of the complexed metal ion. It was shown previously (1) that the primary metal complexes of thiouracils formed in aqueous solution are predominantly MU^+ (III) and MU_2 (IV) (Scheme I) in the resultant homogeneous, acid solution.

The decrease in absorbance of thiouracil solutions, when the nonabsorbing metal ions are introduced, implies that Structures III and/or IV have lower absorptivities than the undissociated thiouracil I. It also follows on these premises that the greater the absorbance decrease for a given metal ion at equivalent concentrations and conditions, the greater the stability of the formed complex with thiouracil of that metal ion. Thus, the stabilities of the III and IV complexes of the thiouracils with Cu^{+2} are very much greater according to Table I than those for Cd^{+2} and Pb^{+2}. When the solvent was aqueous $HClO_4$ (0.001–1.0 *M*) rather than water alone, no significant decrease in absorbance from that expected from the thiouracil alone was observed for Cu^{+2}–5-methyl-2-thiouracil mixtures. This indicates that the equilibria of Scheme I are readily reversed by the addition of hydrogen ion and that noninteracting M^{+2} and thiouracil are in acidic solutions with minimal complexes such as III and IV.

The range of 5–10% loss of absorbance at $\lambda_{max.}$ values of about 272 nm. for the mixtures containing a sevenfold excess of Cd^{+2} and Pb^{+2} over thiouracil (10^{-4} *M*) is insufficient to permit studies of complex formation for these combinations by spectral means.

Method of Continuous Variations Applied to Cu^{+2} and 2-Thiouracil Mixtures—The spectra of Cu^{+2}–thiouracil mixtures indicate the formation of complexes by a decrease in the thiouracil absorption at the 272-nm. maximum (Fig. 1) and an increase in absorbance in the region of 345 nm. (Fig. 1). At both of these wavelengths, Cu^{+2} does not absorb.

The spectrophotometric method of continuous variations (7) can determine the apparent stoichiometry of a strong, major complex formed in solution. This method depends on the determination of the spectral absorbance of mixtures of equimolar solutions of the two complexing species. The mixtures are made in varying ratios so that the total number of moles of both species is kept constant, although the mole fraction of each species continuously varies.

The absorbances of such mixtures were obtained at 272 nm. (Curve B) and 345 nm. (Curve D) and are plotted against the mole fraction of each species in Fig. 4. The straight line (Curve A) represents the expected absorbances for all ratios if there are no interactions between the two species. Thus, the difference (Curve C) between Curves A and B represents the spectral aberration at 272 nm. attributable to complex formation. Curve D for the absorbance of the varying ratios is a similar representation *per se* since the reacting species do not absorb at 345 nm.

The method of continuous variations states that such difference plots (Curves C and D, Fig. 4) show maxima at mole fractions representative of the stoichiometry (with continuously varying mixtures of equimolar solutions) or of the stability constant (with continuously varying mixtures of nonequimolar solutions) of a single strong complex (7).

Unfortunately, the difference plots (Curves C and D, Fig. 4) are relatively small and the data have a relatively large variability; no decision can be made as to stoichiometry. This may be attributed to several factors: (*a*) the absorptivities of the metal complexes such as III and IV in Scheme I are only slightly less than that of the thiouracils; (*b*) under the conditions of study, the amount of complex formed is small; and (*c*) the conditions for a sharp maximum are destroyed when more complexes than one exist significantly in solution. Although all three of these reasons are valid, item *b* may be the most important. Complex formation is hindered in acidic solutions since addition of acid destroys the complex and reverses the equilibria of Scheme I; the "natural" pH values of these aqueous mixtures of Fig. 4 are acidic. When alkali was added to increase the concentration of the complexing thiouracil anion, II, ready precipitation of the complex occurred (1) and thus destroyed the utility of the spectral method to deduce the stoichiometry and stability constants of Cu^{+2} complexes of thiouracils in solution.

Limitations of Potentiometric Titration Methods in Stability

Constant Analysis of Thiouracil–Metal Complexes—The use of potentiometric titration data to determine the stoichiometries and stability constants of complexes demands the net substitution by one of the complexing species for a proton (or protons) on the other. It also demands the maintenance of homogeneous solutions during the simultaneous determination of pH (for the calculation of $\gamma_{\pm}[H^+]$ and/or $\gamma_{\pm}'[OH^-]$) and the amount of titer (alkaline or acid) necessary to obtain that pH for various mixtures of the complexing species, such as the doubly charged metal ion, M^{+2}, and thiouracil, HU.

In the present case, this information is sufficient to calculate the stability constants for the formation of MU^+ and MU_2 complexes (Scheme I). However, if $MU^{\pm}$ and/or MUOH complexes are formed and maintained in solution, the data from potentiometric titrations are inadequate for this purpose. As previously developed in detail (1), the following expressions define the various necessary dissociation and stability constants:

$$K_a' = \frac{\gamma_{\pm}[H^+][U^-]}{[HU]} \quad \text{(Eq. 1)}$$

where K_a' is the dissociation constant at the sulfhydryl group of the thiouracil, HU, to the thiouracil anion, U^-; and $\gamma_{\pm}$ is the activity coefficient for hydrogen ion:

$$K_1 = \frac{[MU^+]}{[M^{+2}][U^-]} \quad \text{(Eq. 2)}$$

where K_1 is the association constant for the formation of the simple complex, MU^+, due to the formation of a covalent bond between the divalent metal ion and the negatively charged sulfur of the thiouracil anion, U^-:

$$K_2 = \frac{[MU_2]}{[MU^+][U^-]} \quad \text{(Eq. 3)}$$

where K_2 is the association constant or step stability constant for the formation of the MU_2 complex due to the formation of the second covalent bond between the metal ion and the negatively charged sulfur of the second thiouracil anion so that the overall stability constant is:

$$\beta_2 = K_1K_2 = \frac{[MU_2]}{[M^{+2}][U^-]^2} \quad \text{(Eq. 4)}$$

The dissociation of the available proton from the hydroxyl in the 4-position of the MU^+ complex to form the $MU^{\pm}$ zwitterion may be defined as:

$$K_{a_2}' = \frac{[MU^{\pm}][H^+]\gamma_{\pm}}{[MU^+]} = K_2'K_w \quad \text{(Eq. 5)}$$

The association or step stability constant of a hydroxyl ion with the MU^+ complex to form a mixed ligand complex MUOH may be defined as:

$$K_2' = \frac{[MUOH]}{[MU^+][OH^-]\gamma_{\pm}'} = K_{a_2}'/K_w \quad \text{(Eq. 6)}$$

where K_w is the autoprotolytic constant for water, and $\gamma_{\pm}'$ is the activity coefficient for the hydroxyl-ion concentration. The overall stability constant for the formation of MUOH or $MU^{\pm}$ can be defined by:

$$\beta_{11} = K_1K_2'K_w = \frac{[MUOH][H^+]\gamma_{\pm}}{[M^{+2}][U^-]} = K_1K_{a_2}' = \frac{[MU^{\pm}][H^+]\gamma_{\pm}}{[M^{+2}][U^-]} \quad \text{(Eq. 7)}$$

where, in this particular case, the formation of a mixed ligand complex, MUOH, V cannot be distinguished from the formation of a zwitterion, $MU^{\pm}$, in terms of equilibrium constants.

It was shown previously (1) that the equation which relates the degree of formation, $\bar{n}$, *i.e.*, the average number of ligands bound to a metal ion, for the multiple equilibria in homogeneous solution of

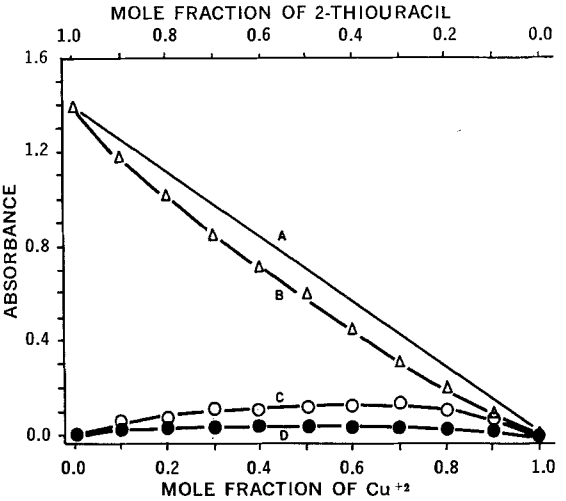


Figure 4—*Absorbances of continuously varied ratios of volumes of 10^{-4} M equimolar solutions of $Cu(NO_3)_2$ and 2-thiouracil so that the mole fraction of the solution, which is 10^{-4} M in both species, continuously varies. Curve A represents the absorbances at 272 nm. of 2-thiouracil if they are not affected by Cu^{+2}. Curve B represents the observed absorbances at 272 nm. of the mixtures of the specified molar ratios. Curve C represents the difference between Curves A and B at those molar ratios. Curve D represents the observed absorbances of the mixtures at 345 nm. where 2-thiouracil alone does not absorb.*

Scheme I to the respective dissociation and stability constants of Eqs. 1–7 is:

$$\frac{\bar{n}}{(1-\bar{n})[U^-]} = K_1 + \frac{\beta_{11}}{[H^+]\gamma_{\pm}} + \frac{(\bar{n}-2)}{(\bar{n}-1)}\beta_2[U^-] \quad \text{(Eq. 8)}$$

where $\bar{n}$ may be calculated from:

$$\bar{n} = \frac{[HU]_0 - \{[U^-] + [HU]\}}{[M^{+2}]_0} = \frac{[HU]_0 - [U^-](1 + [H^+]\gamma_{\pm}/K_a')}{[M^{+2}]_0} \quad \text{(Eq. 9)}$$

where $[HU]_0$ and $[M^{+2}]_0$ are the prepared concentrations of thiouracil and metal ion and are the sums of the respective concentrations in all forms and complexes and must refer to the amounts maintained in homogeneous solution, $[H^+]\gamma_{\pm}$ is calculable from the measured pH of the solutions, K_a' is the dissociation constant of thiouracil (Eq. 1), and the concentration of thiouracil anion may be calculated from:

$$[U^-] = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_{\pm}/K_a' - \beta_{11}[M^{+2}]/[H^+]\gamma_{\pm}} = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_{\pm}/K_a' - [MU^{\pm}]/[U^-]} = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_{\pm}/K_a' - [MUOH]/[U^-]} = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_{\pm}/K_a'} + \frac{[MU^{\pm}]}{[H^+]\gamma_{\pm}/K_a'} \quad \text{(Eq. 10)}$$

where [NaOH] is calculated on the basis that none of the added alkali to achieve the measured $\gamma_{\pm}[H^+] = 10^{-pH}$ had been consumed.

However, $[U^-]$ in Eq. 10 cannot be calculated for the multiple equilibria of Scheme I in homogeneous solution unless $\beta_{11}[M^{+2}]$ is also known, where $[M^{+2}]$ is the concentration of uncomplexed or free metal ion at any measured pH. This value of $[M^{+2}]$ cannot be obtained solely by the potentiometric titration of homogeneous solutions; its determination demands some selective analytical procedure, such as a specific metal/metal-ion electrode. The only exception would be when the potentiometric titrations could be carried out in a homogeneous solution in the presence of a large excess of metal ion and $[M^{+2}]$ could be taken as approximately equal to $[M^{+2}]_0$, where the amount of free metal ion lost to form the complex is insignificant with respect to the total metal ion originally

added. Such a case would demand small stability constants and soluble complexes. The latter is not so with these studied metal complexes of thiouracils.

When β_{11}, K_2', or $K_{a_2}' = 0$, *i.e.*, when there is no significant amount of MUOH or $MU^{\pm}$ formed or in solution (Eqs. 5–7), only then will $[U^-]$ be calculable from potentiometric titrations of homogeneous solutions since Eq. 10 then reduces to:

$$[U^-] = \frac{[HU]_0 - [NaOH]}{[H^+]\gamma_{\pm}/K_a'} \quad \text{(Eq. 11)}$$

and Eq. 8 reduces to:

$$\frac{\bar{n}}{(1 - \bar{n})[U^-]} = K_1 + \frac{(\bar{n} - 2)}{(\bar{n} - 1)}\beta_2[U^-] \quad \text{(Eq. 12)}$$

where $\bar{n}$ is calculable from Eq. 9 since $[U^-]$ is now calculable from Eq. 11. Thus, $\beta_2 = K_1K_2$ and K_1 can be obtained from the slope and intercept, respectively, of the plot of $\{\bar{n}/(1 - \bar{n})[U^-]\}$ *versus* $[U^-]$, all values obtainable from the potentiometric titrations of homogeneous solutions containing only MU^+ or MU_2 complexes (Eqs. 2–4).

At high acidities, Eqs. 11 and 12 can be used as valid approximations to obtain β_2 and K_1 since:

$$\beta_{11}[M^{+2}]/[H^+]\gamma_{\pm} = [MU^{\pm}]/[U^-] = [MUOH]/[U^-] \ll [H^+]\gamma_{\pm}/K_a' \quad \text{(Eq. 13)}$$

and Eq. 10 reduces to Eq. 11 at low pH values.

An alternate perspective is that $MU^{\pm}$ (or MUOH) formation is a result of MU^+ dissociation (or its association with $[OH^-]$, Eq. 6) and would be restricted by a high pKa_2 (or $pK_b = pK_2'$). The $[MU^{\pm}]$ should be relatively negligible at 2 pH units below pKa_2', and [MUOH] should be relatively negligible at 2 pH units below its $pK_b = -pK_2'$ (Eq. 6), where $K_b = 1/K_2'$ is its basic dissociation constant to yield hydroxyl ions and the MU^+ complex.

Possible Circumvention of Titration Method Limitations by Total Thiouracil Solubility Determination in the Presence of Complexing Metal Ions—One way to determine $[U^-]$ in the presence of an $MU^{\pm}$ (and/or MUOH) complex when Eqs. 8–10 must hold is to maintain the solutions saturated with thiouracil at various $[M^{+2}]_0$ and pH values. The concentration of undissociated thiouracil, [HU], is then fixed at its intrinsic solubility, S_i. Thus, $[U^-]$ is calculable from Eq. 1 at all known hydrogen-ion activities and is restricted to:

$$[U^-] = K_a'S_i/[H^+]\gamma_{\pm} \quad \text{(Eq. 14)}$$

The total solubility, S_0, of thiouracil as the undissociated acid and its anion in the absence of complexing metal ion on consideration of Eqs. 1 and 3 may be expressed by:

$$S_0 = [HU] + [U^-] = S_i(1 + K_a'/[H^+]\gamma_{\pm}) \quad \text{(Eq. 15)}$$

and Eq. 9, for all complexes in the equilibria of Scheme I, becomes:

$$\bar{n} = \frac{S_T - S_0}{[M^{+2}]_0} \quad \text{(Eq. 16)}$$

where $S_T = [HU]_0$, the total solubility of thiouracil as acid, anion, and all possible complexes in the presence of an initial concentration, $[M^{+2}]_0$, of metal ion and in a solution saturated with thiouracil acid, *i.e.*, $[HU] = S_i$.

Substitution of rearrangements of Eq. 14 into Eq. 8 with subsequent rearrangements can yield:

$$\frac{\bar{n}}{(1 - \bar{n})[U^-]^2} - \frac{K_1}{[U^-]} = \frac{\beta_{11}}{K_a'S_i} + \frac{(\bar{n} - 2)}{(\bar{n} - 1)}\beta_2 \quad \text{(Eq. 17)}$$

or

$$\frac{\bar{n}\gamma_{\pm}^2[H^+]^2}{(1 - \bar{n})K_a'S_i} - K_1\gamma_{\pm}[H^+] = \beta_{11} + \frac{(\bar{n} - 2)}{(\bar{n} - 1)}\beta_2K_a'S_i \quad \text{(Eq. 18)}$$

so that plots of left-hand sides of these expressions against $(\bar{n} - 2)/(\bar{n} - 1)$ will give intercept values of $\beta_{11}/K_a'S_i$ and β_{11}, respectively, and slope values of β_2 and $\beta_2K_a'S_i$, respectively. In saturated solutions of undissociated thiouracil, where $[HU] = S_i$, all values of $\bar{n}$ (Eq. 16), $[U^-]$ (Eq. 14), and $\gamma_{\pm}[H^+] = 10^{-pH}$ are calculable; K_a' and S_i are obtainable by the respective titration and solubility studies of HU, and K_1 may be well estimated from appropriate plots (Eq. 13) from the potentiometric titrations of homogeneous solutions in the more acidic regions. Thus, β_2 and β_{11} are obtainable from the slopes and intercepts of Eqs. 17 and 18 to permit the calculation of K_{a_2}' and K_2' (Eq. 7).

The concentrations of $MU^{\pm}$, its equivalent mixed ligand complex, MUOH, or the sum of both concentrations are also calculable if the amount of NaOH necessary to give a measured pH value where the total solubility, $S_T = [HU]_0$, of complexing thiouracil is measured and the intrinsic solubility, S_i, at that pH is known. This follows from the substitution of Eq. 14 into the rearranged Eq. 10, where:

$$[HU]_0 - [NaOH] = [H^+][U^-]\gamma_{\pm}/K_a' - [MU^{\pm}] = S_i - [MU^{\pm}] = S_i - [MUOH] \quad \text{(Eq. 19)}$$

and thus:

$$[MUOH] \text{ and/or } [MU^{\pm}] = S_i + S_T + [NaOH] \quad \text{(Eq. 20)}$$

where the alkali added is calculated in terms of [NaOH] in that solution to give $\gamma_{\pm}[H^+] = 10^{-pH}$.

Theoretical and Practical Limitations of Thiouracil Solubility Analysis in Stability Constant Determination of Thiouracil–Metal Complexes—The previous discussion and outlined procedures permit the determination of all stability constants—*viz.*, K_1, K_2, β_{11}, K_2', and/or K_{a_2}' for significant concentrations of the pertinent complexes in the multiple equilibria of Scheme I, provided that (*a*) the solutions of mixtures of thiouracil and metal ion are saturated with undissociated thiouracil at all equilibrated and measured pH values, and (*b*) no metal is precipitated as a thiouracil or mixed ligand complex or as its hydroxide at any equilibrated and measured pH value. If the latter is not so, the known total concentration of metal ion in the solutions, $[M^{+2}]_0$, cannot be used to estimate the sums of the concentrations of all forms (ions and complexes) containing metals. Thus, $\bar{n}$ would be unobtainable from Eq. 16.

A practical consideration of Eq. 16 is that the total experimentally determined solubility of thiouracil, S_T, as the sum of the concentrations of thiouracil in all forms as acid, ions, and complexes must significantly exceed the solubility, S_0, of thiouracil as the acid and anion at any given pH.

It can be readily shown from Eqs. 14 and 15 that:

$$\log \frac{S_0 - S_i}{S_i} = \log \frac{A - A_{H^+}}{A_{H^+}} = pH - pKa' \quad \text{(Eq. 21)}$$

where A is the spectrophotometric absorbance of an aliquot of the thermostatically filtered solution of a saturated thiouracil solution at a given pH which has been diluted 1:1 and made 0.1 N in $HClO_4$. The A_{H^+} is the absorbance of an aliquot of a saturated solution of thiouracil in 0.1 N $HClO_4$ diluted 1:1 and read in 0.1 N $HClO_4$. A typical plot of the logarithmic function of absorbances against pH is given in Fig. 5 in accordance with Eq. 21 for 2-thiouracil at 25.0°. This plot has the expected positive slope of unity, and the pKa′ is 7.52 as estimated from the intercept. Similar plots were obtained for the other thiouracils studied; the data were summarized in Table II of *Reference 1*.

When cadmium ion was added to these saturated solutions of thiouracils at various pH values, the filtrate diluted, and the absorbances measured in the manner described, no significant difference in absorbance was observed for a given pH value over those saturated solutions studied without cadmium ion. These solubility studies on 2-thiouracil and 6-*n*-propyl-2-thiouracil with cadmium ion had to be restricted to pH values less than 6 since the complex precipitation that occurred above that value (1) had to be avoided. Since the total solubility, S_T, in the presence of cadmium ion did not exceed the total solubility, S_0, in its absence for any measurable pH value, significant values of $\bar{n}$ could not be calculated from Eq. 16; this total thiouracil solubility method was not applicable in this case.

This is not unexpected, as can be shown by a specific set of calculations for 6-*n*-propyl-2-thiouracil and cadmium ion at 25.0° based on the data given in Table II of *Reference 1*, where $S_i = 7.07 \times 10^{-3}$ M/l. and $K_a' = 1.74 \times 10^{-8}$ M/l. The total solubility of all 6-*n*-propyl-2-thiouracil forms in solution with metal ions can

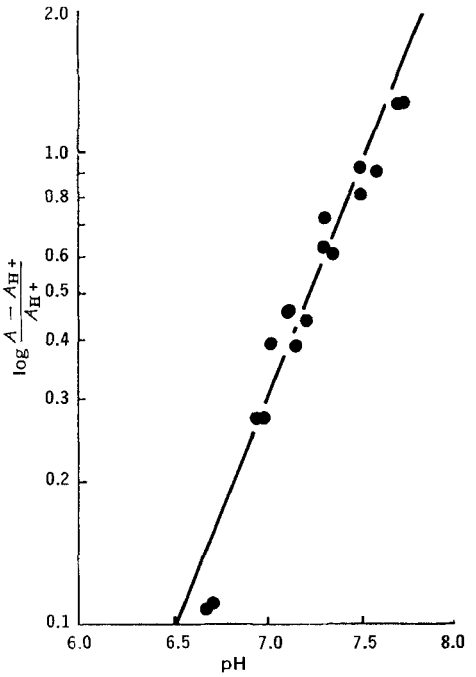


Figure 5—*Plot of log* $(A - A_{H^+})/A_{H^+}$ *against pH for aqueous, saturated 2-thiouracil solutions with and without added metal ions in solution at 25.0° according to log* $(A - A_{H^+})/A_{H^+} = pH - pKa'$, *where* A *is the absorbance of a saturated solution in the buffer region and* A_{H^+} *is the absorbance due to the intrinsic solubility as measured in 0.100* M $HClO_4$. *The slope is 1.00 and pKa' is 7.52.*

be defined as:

$$S_T = S_0 + [MU^+] + 2[MU_2] + [MU^{\pm}] + [MUOH] \quad \text{(Eq. 22)}$$

where S_0 is obtainable from Eq. 15.

If $[MU^{\pm}]$ and [MUOH] are considered to be exceedingly small and it is assumed that less than 0.1 of the total metal-ion concentration is complexed, so that $[M^{+2}] \sim [M^{+2}]_0$, then $\beta_2 = K_1K_2 = 4.49 \times 10^{-8}$ $(l./M)^2$, where $K_1 = 1.45 \times 10^{-4}$ $l./M$ and $K_2 = 3.09 \times 10^{-4}$ $l./M$ for Cd^{+2}–6-*n*-propyl-2-thiouracil complexes. Thus, $[MU^+]$ and $[MU_2]$ can be calculated from Eqs. 2 and 4, respectively, where $[U^-]$ in thiouracil-saturated solutions is known from Eq. 14 and the measured $\gamma_{\pm}[H^+] = 10^{-pH}$. The calculations of S_T (Eq. 22) for several cases gives the following results: 10^3S_T, 10^3 $[Cd^{+2}]$, and pH: 7.07, 0.00, and 4.00; 7.08, 1.00, and 4.00; 7.17, 5.00, and 4.00; 7.08, 0.00, and 5.00; and 7.30, 1.00, and 5.00. It must be concluded that the difference between S_T and S_0 up to pH 5 is not sufficiently significant for utilization of this method. It would be feasible to calculate significant differences, $S_T - S_0$, at higher alkalinities where the concentrations of complexes should be higher if their precipitation did not occur. However, complex precipitation was observed with the mixture of 2×10^{-3} *M* 6-*n*-propyl-2-thiouracil and 2×10^{-3} *M* Cd^{+2} in the region of pH 5 (1). At higher pH values, cadmium ion was observed to contaminate the excess insoluble 6-*n*-propyl-2-thiouracil, which implied that the cadmium complexes had precipitated and these methods were contraindicated.

Possible Circumvention of Titration Method Limitations by Analysis of Total Metal, M_S, in Solution—The concentration, M_S, of metal in solution prior to the precipitation of any metal–thiouracil complex is the same as the calculated total metal concentration (1), $[M^{+2}]_0$, so that a plot of M_S against $[M^{+2}]_0$ would be linear and of unit slope for any given pH value (Curves a–b, a–b′, and a–b″ of Fig. 6) until the solubility of any one complex given in the following expression (see Scheme I) is exceeded:

$$M_S = [M^{+2}] + [MU^+] + [MU^{\pm}] + [MU_2] \quad \text{(Eq. 23)}$$

For the sake of convenience in this development, $[MU^{\pm}]$ (VI) is considered as the sum of the concentrations of this complex and the mixed ligand complex, MUOH (V), which was shown in Eq. 7 to be stoichiometrically equivalent. Appropriate substitutions of rearranged Eq. 2 for $[MU^+]$, Eq. 4 for $[MU_2]$, and Eq. 7 for $[MU^{\pm}]$ in Eq. 23 result in:

$$M_S = [M^{+2}]\left\{1 + K_1[U^-] + \frac{\beta_{11}[U^-]}{[H^+]\gamma_{\pm}} + \beta_2[U^-]^2\right\} \quad \text{(Eq. 24)}$$

where $[U^-]$ of Eq. 11 may be calculated only if $[M^{+2}]$ can be obtained from direct analysis in solution.

$[MU^{\pm}]$ may be considered negligible at low pH values since the pKa_2' for the dissociation of MU^+ must be approached for β_{11} of Eq. 7 to become significant. If the conditions are such that MU_2 precipitates at these low pH values, then from Eq. 4,

$$[M^{+2}] = S_{MU_2}/\beta_2[U^-]^2 \quad \text{(Eq. 25)}$$

where S_{MU_2} is the solubility of MU_2. It follows that when the precipitation of MU_2 is just noted (at points b, b′, and b″ for various decreasing $[H^+]$ in Fig. 6), Eq. 24 becomes:

$$M_S = [M^{+2}]\{1 + K_1[U^-] + S_{MU_2}\} = S_{MU_2}\{(1/\beta_2)(1/[U^-]^2) + (K_1/\beta_2)(1/[U^-]) + 1\} \quad \text{(Eq. 26)}$$

where $[U^-]$ is calculable from Eq. 12 using the experimental values of $[HU]_0$, [NaOH], and $[H^+]$ when the $[MU^{\pm}]$ of Eq. 11 is negligible. This Eq. 26 is of the polynomial form:

$$M_S = a(1/[U^-])^2 + b(1/[U^-]) + c \quad \text{(Eq. 27)}$$

where $a = S_{MU_2}/K_1K_2 = S_{MU_2}/\beta_2$, $b = K_1S_{MU_2}/\beta_2 = S_{MU_2}/K_2$, and $c = S_{MU_2}$. Thus, a series of analyses of concentrations of metal ions in solution, M_S, at various known hydrogen-ion concentrations to permit calculations of $[U^-]$ from Eq. 12 when negligible $MU^{\pm}$ exists (see Fig. 6) can permit fitting of the constants of Eq. 27 and determination of the solubility of MU_2.

The data obtained on potentiometric titrations of metal–thiouracil mixtures (1) permitted observation of pH values at which the

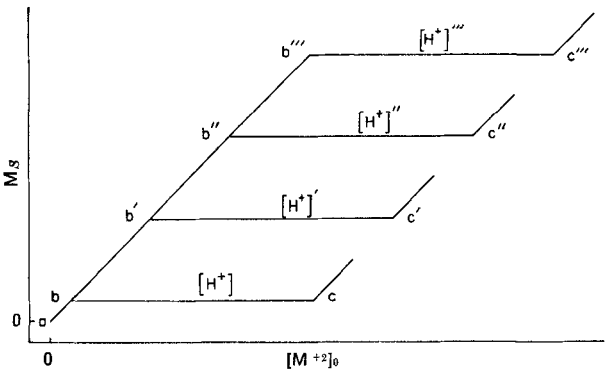


Figure 6—*Anticipated plots of concentrations of complexing metal,* M_S, *in solutions equilibrated at various hydrogen-ion concentrations* versus *the concentration of metal ion* $[M^{+2}]_0$ *that should have been present in these solutions if no complexation or interaction with thiouracil had occurred. The ordinate value of* M_S *at precipitation (b, b′, b″, or b‴) should decrease with decreased hydrogen-ion concentration,* i.e., $[H^+]''' > [H^+]'' > [H^+]' > [H^+]$. *Each plateau represents a constant pH condition for a given amount of added excess thiouracil where a higher concentration of metal would be needed to form and precipitate complexes at the lower pH values since the concentration of the complexing thiouracil anion is lessened. The abscissa lengths, bc, b′c′,* etc., *represent the concentration of metal ion consumed in the precipitation of the least soluble complex. The ordinate distance, b,b′,* etc., *either approaches the solubility of* MU_2 *if this is the preferable precipitated complex (Eq. 33) or approaches a value proportional to, but greater than, the solubility of* $MU^{\pm}$ *(or a* M_nU_n *complex) if this is the preferable precipitated complex (Eq. 36). The fact that, at the lowest and highest pH values studied, no significant values of b could be observed for such solubility studies on mixtures of* $Cd(NO_3)_2$ *with several thiouracils implies that the solubilities of such complexes are exceedingly small and more sensitive measurements of total metal in solution are needed than were available. The plot subsequent to points c, c′,* etc., *is not necessarily linear or of unit slope. For example, if* MU_2 *is precipitated first, on exhaustion of excess thiouracil it may redissolve and form* M_nU_n *complexes with the increased added metal.*

possible precipitation of complexes was initiated. If these complexes were MU_2, and if there was no significant supersaturation of MU_2, the calculated $[M^{+2}]_0$ should be just equal to M_S at this pH value. Since the $[U^-]$ values can be calculated (Eq. 11) from the known $[HU]_0 - [NaOH]$ and $[H^+]$ values, the necessary data for use of Eqs. 26 and 27 should be available if the complex whose solubility was first exceeded was MU_2.

Unfortunately, prior studies (1) indicated that the precipitating complex on potentiometric titration normally had a 1:1 stoichiometry and most probably was the cyclic dimer, M_2U_2, bis-(thiouracil–metal), obtained from the head-tail dimerization of the zwitterion, $MU^{\pm}$.

The known total metal concentrations, as prepared in the previously cited potentiometric titrations, were plotted against $1/[U^-]$, where $[U^-]$ was calculated from Eq. 11 for the $[H^+]$ at the pH of initial precipitation, in accordance with the assumptions underlying Eqs. 25–27. The resultant plots for Cd–2-thiouracil and Pb–2-thiouracil mixtures were linear and not parabolic as would be predicted by Eq. 27. There was no statistically significant intercept different than zero. The slopes were 7.3×10^{-8} and 4.57×10^{-8} M^2 for the respective mixtures of Cd–2-thiouracil, where $\log K_1 = 4.02$ and $\log K_2 = 3.49$ (1), and of Pb–2-thiouracil, where $\log K_1 = 4.74$ and $\log K_2 = 3.44$ (1). If these slope values are assigned to $b = S_{MU_2}/K_2$ of Eq. 27, then $S_{MU_2} = 2.26 \times 10^{-4}$ M for $Cd(TU)_2$ and $S_{MU_2} = 1.26 \times 10^{-4}$ M for $Pb(TU)_2$. These values are not inconsistent, within error, with the apparent zero intercepts. However, if one inserts these values for $S_{MU_2} = [MU_2]$ into Eq. 4 and uses the above ascertained values of K_1 and K_2, where $[U^-]$ is calculated from Eq. 11, an utterly impossible concentration of free metal ion, $[M^{+2}]$, is estimated. For example, in the case of the Cd–2-thiouracil mixture at a total metal concentration of $M_S = 2 \times 10^{-3}$ M,

$$[M^{+2}] = S_{MU_2}/K_1K_2[U^-]^2 = \frac{2.26 \times 10^{-4}}{(1.05 \times 10^4)(3.10 \times 10^3)}\left(\frac{1}{3.64 \times 10^{-5}}\right)^2 = 3.4 \times 10^{-3}\ M \quad \text{(Eq. 28)}$$

Since it is impossible that the free or uncomplexed metal-ion concentration, $[M^{+2}]$, can exceed the total metal concentration, M_S, the underlying assumptions of Eqs. 11 and 25–27 must be invalid. This is also consistent with the fact that MU_2 normally is not the stoichiometric precipitate in the potentiometric titration studies (1).

If $MU^{\pm}$ of Eq. 23 precipitates as M_2U_2 (1) before the concentration of MU_2 exceeds its solubility, Eq. 7 dictates that:

$$[M^{+2}] = S_{MU^{\pm}}[H^+]\gamma_{\pm}/\beta_{11}[U^-] \quad \text{(Eq. 29)}$$

where $S_{MU^{\pm}}$ is the solubility of $MU^{\pm}$.

It follows that when the precipitation of $MU^{\pm}$ is just noted (at points b, b′, and b″ for various decreasing $[H^+]$ as in Fig. 6), Eqs. 23 and 24 become the more complicated expressions:

$$M_S = [M^{+2}]\{1 + K_1[U^-] + \beta_2[U^-]^2\} + S_{MU^{\pm}} = S_{MU^{\pm}}\left\{\frac{[H^+]\gamma_{\pm}}{\beta_{11}[U^-]} + \frac{K_1[H^+]\gamma_{\pm}}{\beta_{11}} + \frac{\beta_2[U^-][H^+]\gamma_{\pm}}{\beta_{11}} + 1\right\} = \frac{S_{MU^{\pm}}}{K_{a_2}'}\left\{\frac{[H^+]\gamma_{\pm}}{[U^-]}\left(\frac{1}{K_1}\right) + [H^+]\gamma_{\pm} + K_2K_a'[HU] + K_{a_2}'\right\} \quad \text{(Eq. 30)}$$

where $[U^-]$ must be obtained from Eq. 10.

Possible Circumvention of Titration Method Limitations by Analyses of Total Metal, M_S, in Saturated Thiouracil Solutions—If the solution is saturated with thiouracil, application of Eq. 14, which considers S_i as the constant solubility of undissociated thiouracil, transforms Eq. 24 to:

$$M_S = [M^{+2}]\{1 + K_1K_a'S_i(1/[H^+]\gamma_{\pm}) + (\beta_{11}K_a'S_i + \beta_2(K_a')^2S_i^2)(1/[H^+]\gamma_{\pm})^2\} \quad \text{(Eq. 31)}$$

If MU_2 precipitates first and $[MU^{\pm}]$ is negligible, then from Eqs. 4 and 14,

$$[M^{+2}] = S_{MU_2}[H^+]^2\gamma_{\pm}^2/[\beta_2(K_a')^2S_i^2] \quad \text{(Eq. 32)}$$

where S_{MU_2} is the solubility of MU_2, and it follows that Eq. 26 becomes:

$$M_S = S_{MU_2}\{[H^+]^2\gamma_{\pm}^2/[\beta_2(K_a')^2S_i^2] + \gamma^{\pm}[H^+]/[\beta_2K_a'S_i] + 1\} \quad \text{(Eq. 33)}$$

Thus, a plot of the analyzed total metal concentration, M_S, in saturated thiouracil solution against the hydrogen-ion activity, $\gamma_{\pm}[H^+]$, should fit the expression:

$$M_S = a'\gamma_{\pm}^2[H^+]^2 + b'\gamma_{\pm}[H^+] + c' \quad \text{(Eq. 34)}$$

where $a' = S_{MU_2}/[\beta_2(K_a')^2S_i^2]$, $b' = S_{MU_2}/[\beta_2K_a'S_i]$, and $c' = S_{MU_2}$, when MU_2 is the first complex to exceed its solubility.

If $MU^{\pm}$ precipitates as the dimer M_2U_2 (1) in saturated thiouracil solutions before MU_2 exceeds its solubility, then it can be shown from Eqs. 7 and 14 that:

$$[M^{+2}] = S_{MU^{\pm}}[H^+]\gamma_{\pm}/[U^-]\beta_{11} = S_{MU^{\pm}}[H^+]^2\gamma_{\pm}^2/[\beta_{11}K_a'S_i] \quad \text{(Eq. 35)}$$

where $S_{MU^{\pm}}$ is the solubility of $MU^{\pm}$, and it follows that Eq. 30 becomes:

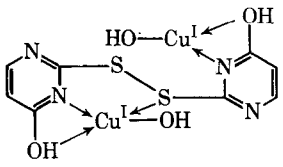
$$M_S = S_{MU^{\pm}}\{[H^+]^2\gamma_{\pm}^2/[\beta_{11}K_a'S_i] + K_1[H^+]\gamma_{\pm}/\beta_{11} + \beta_2K_a'S_i/\beta_{11} + 1\} \quad \text{(Eq. 36)}$$

Thus, a plot of the total metal concentration, M_S, in saturated thiouracil solution against the hydrogen-ion activity, $\gamma_{\pm}[H^+]$, should also fit the polynomial Eq. 34, but where the constants have a different significance, *i.e.*, $a' = S_{MU^{\pm}}/[\beta_{11}K_{a_2}'S_i]$, $b' = K_1S_{MU^{\pm}}/\beta_{11} = S_{MU^{\pm}}/K_{a_2}'$, and $c' = 1 + \beta_2K_a'S_i/\beta_{11} = 1 + K_2K_a'S_i/K_{a_2}'$, when $MU^{\pm}$ is the first complex to exceed its solubility.

The data obtained by analysis of metal concentrations, M_S, in saturated solutions of thiouracil at maintained pH values should give similar curves to those drawn in Fig. 6. The plateau values of M_S at known hydrogen-ion concentrations should permit estimation of the a', b', and c' values of Eq. 34, whether the MU_2 or the $MU^{\pm}$ complex precipitates first. No other measurements are necessary on the saturated thiouracil systems.

The metal concentration, M_S, assayed in the filtrate from the equilibrated saturated thiouracil solutions did not give the ideal plots of Fig. 6 when plotted against the concentrations of metal ion added. There was no significant increase in filtrate metal concentration with added metal until all the excess thiouracil was consumed in complex formation (point c, Fig. 5). Even then, the M_S values were widely variant and did not follow the ideal linearity for $[M^{+2}]_0$ values beyond points c, c′, and c″. This implies that: (*a*) the solubility of the precipitated complex (M_S values of idealized plateaus b–c, b′–c′, and b″–c″ of Fig. 6) was too small to be evaluated properly by this method within the error of the polarographic analysis of metals in the filtrate, and/or (*b*) metal-ion or metal–thiouracil complex is adsorbed from the solution onto the precipitated complex or onto the excess thiouracil under the conditions of the studies. Also, further proof of the difficulty of equilibration was the inability to obtain a constant pH after adding alkali over months of equilibration in the constant-temperature baths. Thus, although these equations and approaches are theoretically sound, they were not able to be put into practice in these studies.

Evaluation of Proposed Complex of Cuprous Ion and 2-Thiouracil Disulfide as a Result of Cupric Ion and 2-Thiouracil Interaction—Weiss and Venner (2) isolated precipitates after a day's presumed reaction of thiouracil with cupric ion in 1.0 *N* NaOH. They assigned the possible structure of a disulfide complex of cuprous ion, IX, on the basis of the elemental analysis of this precipitate.

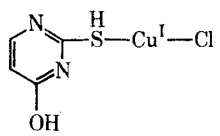


IX

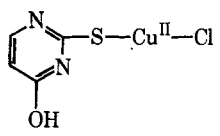
Since it has been demonstrated by the present authors that alkaline thiouracil solutions are susceptible to air oxidation, even in the absence of cupric ion, the possibility exists that the isolated complex contained cupric rather than the suggested cuprous ion.

A solution of thiouracil disulfide in water, reacted with cuprous

chloride in concentrated hydrochloric acid, produced a yellow product that Weiss and Venner (2), on the basis of nitrogen, copper, and chloride analysis, proposed to be IX except for the substitution of chlorine for hydroxyl on the copper. However, the extreme instability of thiouracil disulfide under such conditions (3, 4) casts doubt on this assertion. It is possible that the product may actually be X or even XI, if the great ease of disproportionation of cuprous to cupric ion (5) is considered.



X

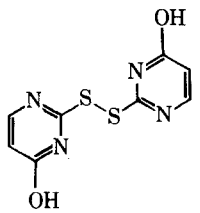


XI

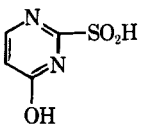
These proposals (2) imply that the complex, IX, results from the reaction of cupric ion and thiouracil as a consequence of the redox reaction:

$$2RSH + 2Cu^{+2} \rightarrow RSSR + 2Cu^{+} \rightarrow IX \quad \text{(Eq. 37)}$$

where RSH is the thiouracil, I, and RSSR is the 2-thiouracil disulfide, XII. Such statements could argue against possible assignments



RSSR, XII



RSO_2H, XIII

of the Structure III, IV, or V (Scheme I) for the copper complexes.

It is known (3, 4, 8) that the hydrolysis of the disulfide, RSSR, proceeds by disproportionation in acidic solutions to the thiouracil, RSH, and the sulfinic acid, RSO_2H (XIII) where for n moles of 2-thiouracil disulfide,

$$n\text{RSSR} + n\text{H}_2\text{O} \rightarrow (3n/2)\text{RSH} + (n/2)\text{RSO}_2\text{H} \quad \text{(Eq. 38)}$$

If n moles of cupric ion do react with the generated $3n/2$ moles of thiouracil, then,

$$(3n/2)\text{RSH} + n\text{Cu}^{+2} \rightarrow (n/2)\text{RSH} + (n/2)\text{RSSR} + n\text{Cu}^{+} \quad \text{(Eq. 39)}$$

and it follows that the resultant formed $n/2$ moles of RSSR should again disproportionate as:

$$(n/2)\text{RSSR} + (n/2)\text{H}_2\text{O} \rightarrow (3n/4)\text{RSH} + (n/4)\text{RSO}_2\text{H} \quad \text{(Eq. 40)}$$

When the sum of Eqs. 38–40 is simplified, the overall reaction of thiouracil disulfide with equimolar cupric ion, if cupric ion oxidized the thiouracil, should be:

$$n\text{RSSR} + (3n/2)\text{H}_2\text{O} + n\text{Cu}^{+2} \rightarrow (3n/4)\text{RSO}_2\text{H} + (5n/4)\text{RSH} + n\text{Cu}^{+} \quad \text{(Eq. 41)}$$

The overall reaction of 1 mole of the disulfide with 2 moles of cupric ion, if cupric ion oxidizes the thiouracil, should be:

$$n\text{RSSR} + 2n\text{H}_2\text{O} + 2n\text{Cu}^{+2} \rightarrow n\text{RSH} + 2n\text{Cu}^{+} + n\text{RSO}_2\text{H} \quad \text{(Eq. 42)}$$

The final absorbances at a given wavelength of solutions containing such 1:1 or 1:2 molar ratios of disulfide and cupric ion can be predicted from the obtained molar absorptivities for 2-thiouracil, RSH ($\epsilon_{270} = 13{,}680$), its sulfinic acid derivative, RSO_2H ($\epsilon_{270} = 3510$), and the known original concentration of the 2-thiouracil disulfide, RSSR (3.99×10^{-5} M). The predicted final absorbances of such solutions when cupric ion and thiouracil do not undergo the redox reactions of Eqs. 39, 41, and 42 should be the same as when the disproportionation of the disulfide occurs (Eqs. 38 and 40) in the absence of cupric ion, *i.e.*, 0.89. The predicted final absorbance should be 0.79 for equimolar cupric ion and 0.69 for twice equimolar cupric ion with thiouracil disulfide if the respective redox Eqs. 41 and 42 are applicable.

The observed absorbance when equimolar and twice equimolar solutions of cupric ion were equilibrated in 0.05 M $HClO_4$ with the 4.0×10^{-5} M 2-thiouracil disulfide was 0.83, which was the same absorbance observed for the disulfide alone equilibrated in 0.05 M $HClO_4$. The difference between the predicted absorbance for the disproportionation of the disulfide and the obtained final absorbances for all the solutions may be attributed to the partial oxidation of 2-thiouracil to its sulfonic acid derivative, *i.e.*, RSO_3H (8).

Thus, it can be concluded that thiouracil is *not* oxidized by cupric ion in acid solution and that the most probable copper complexes of thiouracils are with cupric ion and similar to those already postulated (1) for Pb^{+2}, Cd^{+2}, Zn^{+2}, and Ni^{+2} (Scheme I). Also, it was shown previously (1) and by the spectral evidence presented here that an apparent necessary prerequisite for metal complexation at a sulfur in these structures is a dissociable sulfhydryl group.

The apparent half-lives (6 min. at 25.0°) for the disproportionation of 4.0×10^{-5} M 2-thiouracil disulfide in 0.05 M $HClO_4$ (Eq. 38) were monitored by following absorbance changes with time at 270 nm. and were independent of the cupric-ion concentrations of 0.0, 4.0, and 8.0×10^{-5} M. This provides no evidence for cupric-ion complexation with the 2-thiouracil disulfide.

Additional evidence for the lack of cupric-ion oxidation of 2-thiouracil in 0.1 M $HClO_4$ was obtained when the absorbances, A, of 10^{-4} M thiouracil solutions containing cupric ion [0.0 M (A = 1.233), 2.15×10^{-4} M (A = 1.250), and 4.5×10^{-3} M (A = 1.305)] were determined. An absorbance of 0.995 would have been predicted if cupric ion had been the cause of sulfinic acid formation, *i.e.*, if Eqs. 39 and 40 had been valid.

The evidence denies the proposal of complexes similar to IX resulting from cupric-ion interaction with thiouracils. Thiouracil disulfide formation is most probably an artifact of sulfhydryl oxidation by atmospheric oxygen in alkaline solutions which may be cupric ion catalyzed. However, it is highly improbable that the resultant cuprous ion has sufficient stability to form a stable complex with any possible ligand in this system since chloride ion was excluded and, certainly, neither the cupric nor cuprous ion should be able to complex with the thiouracil disulfide. Metal ions should not significantly bind to the oxygen of the 4-hydroxyl groups in the thiouracil disulfide, since no complexation with metal ions was observed (1) with a compound whose dissociable proton was removed by alkylation, *i.e.*, 2-ethylmercapto-4-hydroxypyrimidine.

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Relative Toxicity of Three Homologous Series of Nonionic Surfactants in the Planarian

WITOLD SASKI*, MARIELLA MANNELLI, MARCO F. SAETTONE, and FRANCESCO BOTTARI

Abstract □ An investigation on the toxicity of three homologous series of nonionic surfactants was carried out using planarian *Dugesia lugubris* (O. Schmidt). The surfactants used in this study were polyoxyethylene stearic acid esters, polyoxyethylene sorbitan fatty acid partial esters, and polyoxyethylene alkyl ethers. The effect of surfactant concentration (C) on time of death (T) of planarian can be described by a regression equation, where $1/T$ is linearly related to C. Analysis of variance of $1/T$ showed that each regression was highly significant and that deviations from linearity were not significant. The behavior of the polyoxyethylene stearic acid esters and polysorbates was in good agreement with the Levy–Gucinski theory, which describes the effect of toxic agents on goldfish. No agreement with the same theory was shown by surfactants of the polyoxyethylene alkyl ethers, whose regression lines, in the C range 0.05–1.0%, failed to intersect the origin. Statistical differences in relative toxicity between the members of each series were assessed. In the polyoxyethylene stearic acid esters, a linear relationship between the length of the polyoxyethylene chain and lethal time was detected. Relative toxicity in the polysorbates decreases in the order of the 20, 40, 60, and 80 esters, thereby depending on the nature of the esterifying acid (lauric, palmitic, stearic, and oleic, respectively). No clear-cut pattern was found with the polyoxyethylene alkyl ethers; differences in relative toxicity between the ethers of the series were not statistically significant. The present study is indicative of the utility of the planarian in biopharmaceutical research.

Keyphrases □ Surfactants, nonionic—relative toxicity in planarian □ Toxicity, nonionic surfactants—polyoxyethylene chain-length effect □ Biologic membrane permeation—surfactants □ Surface tension, pH effects—surfactant absorption, toxicity

The part surface-active agents play in the passage of drugs across biological membranes continues to be of great interest in biopharmaceutical research (1–4). The use of surfactants to prepare micellar solutions of pharmaceutical materials may, in some cases, result in an increased absorption and biological activity, although cases are known in which solubilization affects drug absorption adversely or not at all (5). Effects on the absorbing membrane, interaction with the drug, and modification of the physical properties of the dosage form have been considered among the possible mechanisms by which surfactants may modify drug absorption (6).

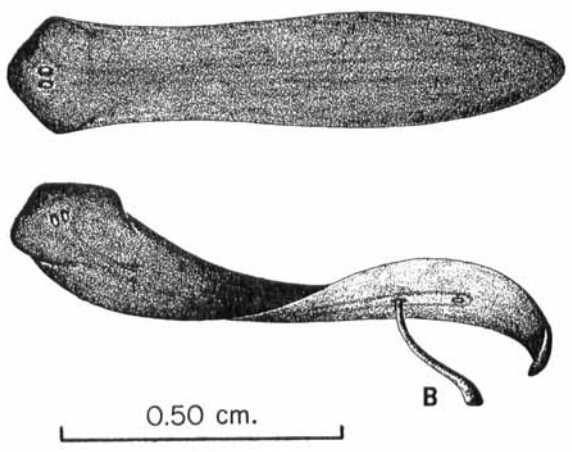

Figure 1—*Planarian* Dugesia lugubris. *Key: A, head; and B, pharynx.*

The effect of surface-active agents on biological membranes has been investigated using different approaches. Unicellular organisms such as bacteria, used in earlier studies, were later found unsuitable owing to the presence of enzymes and other vital cell constituents located in the cell membrane and, therefore, accessible to surface-active agents in the medium. Experimental systems using small animals or isolated intestines were recently employed (3, 7). The use of goldfish (*Carassius auratus*) for studies of factors influencing biological membrane permeation has been extensively illustrated in a recent series of papers (8–10). This animal has been used to investigate the influence of polysorbate 80 (1, 12) and of bile salts (4, 11) on pentobarbital and on 4-aminoantipyrine absorption.

The present authors set out to develop an experimental system involving an aquatic animal other than fish whereby studies on the kinetics of the transfer of drugs, both in the presence and in the absence of added surfactant, could be carried out satisfactorily. A platyhelminth, the planarian, was selected. The planarian, widely used in genetic, physiologic, and behavioral research (13), apparently has never been employed in pharmacology[1].

In the present paper, the effect on planarian of three homologous series of nonionic surfactants, which might be used as drug solubilizers, was investigated. The purposes of this study were: (*a*) to learn whether these agents exert any toxic action of their own, and (*b*) to assess on a statistical basis any functional relationship existing between toxic activity (*i.e.*, lethal time of planarian) and surfactant concentration. Indeed, nonionic surfactants may possess a pharmacologic activity of their own, due to their chemical structure or physical characteristics and independent of their surface-tension depressing activity (14). An investigation in this direction was considered to be an essential prerequisite to further studies dealing with membrane permeation by solubilized drugs.

[1] A possible objection to the use of planarian might be its low position in the biological scale. However, other low life forms have been successfully employed in pharmacologic research. Worms of the genera *Lumbricus* and *Tubifex*, for example, have been found useful in studies concerning evaluation of local anesthetic activity and standardization of chrysarobin, respectively; *cf.*, B. P. Block, D. J. Potts, and R. S. H. Finney, *J. Pharm. Pharmacol., Suppl.*, **16**, 85T(1964); and P. Boymond and P. Amacker, *Pharm. Acta Helv.*, **39**, 223(1964).

Table I—Effect of Surfactant Concentration on Time of Death of Planarian

Surface-Active Agent	Concentration, % w/v: 0.05	0.10	0.30	0.50	0.75	1.00
	Average Time of Death, hr.[a] (*CV*%)[b]					
Polyoxyethylene 20 stearate	19.60(12.0)	8.60(12.1)	3.32(13.4)	2.04(15.2)	1.34(3.8)	1.04(4.9)
Polyoxyethylene 30 stearate	48.43(11.8)	31.08(20.2)	10.14(6.5)	6.11(13.5)	4.18(20.3)	3.27(22.1)
Polyoxyl 40 stearate	—	40.75(48.1)	16.32(38.8)	8.90(28.9)	5.48(14.6)	3.75(12.1)
Polyoxyethylene 50 stearate	—	52.67(26.3)	17.94(13.7)	10.86(4.8)	7.65(15.3)	5.64(18.4)
Polyoxyethylene 100 stearate	—	—	51.45(14.7)	37.97(20.8)	24.81(14.2)	21.55(13.5)
Polysorbate 20	3.34(13.6)	1.86(36.0)	0.62(4.9)	0.37(2.9)	0.27(4.4)	0.20(7.3)
Polysorbate 40	—	8.96(27.5)	2.06(24.3)	0.98(27.8)	0.68(25.0)	0.47(16.7)
Polysorbate 60	27.68(22.8)	—	4.62(18.7)	2.74(18.2)	1.54(16.3)	1.21(17.3)
Polysorbate 80	9.85[c](4.5)	3.60[d](9.0)	3.16[e](15.2)	2.30[f](17.9)	1.82[g](7.8)	1.37[h](17.8)
Polyoxyethylene 23 lauryl ether	6.20(10.9)	5.32(5.7)	3.88(6.3)	2.74(10.3)	2.02(21.0)	1.74(6.4)
Polyoxyethylene 20 cetyl ether	1.13(24.8)	1.03(11.6)	0.72(11.4)	0.57(21.3)	0.47(17.5)	0.37(11.5)
Polyoxyethylene 20 stearyl ether	1.43(12.8)	1.16(12.4)	0.72(16.6)	0.58(22.9)	0.51(29.0)	0.43(31.4)
Polyoxyethylene 20 oleyl ether	1.54(6.3)	1.08(11.9)	0.82(15.8)	0.63(13.7)	0.48(10.9)	0.37(16.0)

[a] Average of 12 animals. [b] *CV*% = standard deviation/average × 100. Concentration, % w/v: [c] 1.00. [d] 2.00. [e] 2.50. [f] 3.00. [g] 4.00. [h] 5.00.

EXPERIMENTAL

The specimens of diploid type of *Dugesia lugubris* (O. Schmidt) (Fig. 1) were collected from an artificial pond[2]. The animals averaged 1.0 ± 0.2 cm. in length and 9.0 ± 1.0 mg. in weight.

Water and Food—All animals were kept in tap water brought into the laboratory directly from the mains *via* iron plumbing. It should be remembered that copper or brass tubing may cause cupric poisoning (15). Distilled water was used for the solutions in the experiments. Preliminary tests showed that planarians could survive for a long time in distilled water and that no significant differences in lethal times could be detected when the surfactant solutions were made with pond, tap, or distilled water. Animals were fed on beef liver cut into small pieces once a week, the water being changed and waste products removed the following day. All experiments were carried out on animals fasted for at least 10 days.

Nonionic Surface-Active Agents—The following compounds were used—*viz.*, a homologous series of polyoxyethylene stearic acid esters: polyoxyethylene 20 stearate, polyoxyethylene 30 stearate, polyoxyl 40 stearate, polyoxyethylene 50 stearate, and polyoxyethylene 100 stearate[3]; a homologous series of polyoxyethylene sorbitan fatty acid partial esters: polysorbate 20, polysorbate 40, polysorbate 60, and polysorbate 80 USP[3]; and a homologous series of polyoxyethylene alkyl ethers: polyoxyethylene 23 lauryl ether, polyoxyethylene 20 cetyl ether, polyoxyethylene 20 stearyl ether, and polyoxyethylene 20 oleyl ether[3]. All compounds were commercially available materials, and no attempt at further purification was made.

Determination of pH of Immersion Fluids—The pH of the solutions was determined by means of a Photovolt 111 pH meter.

Determination of Surface Tension of Immersion Fluids—The ring detachment method, employing the du Nöuy tensiometer[4], was used. Three successive measurements were taken for each of the concentrations; after application of the usual corrections (17), the average was calculated.

Determination of Time of Death of Planarian—Three animals were placed in a beaker containing 20 ml. of a solution of the surfactant of varying concentrations: 0.05, 0.10, 0.30, 0.50, 0.75, and 1.0% w/v in distilled water at 20 ± 1°. From the moment of immersion, the planarians were observed with the help of a magnifying glass, and the time elapsed between immersion of the animals into the solutions and death was recorded. To eliminate bias in time-of-death determination, each beaker was marked with a code number by an individual not involved in the study, the code being broken only after completion of all determinations. When immersed, the planarians contract and curl at regular intervals and then distend again and swell. Their motility gradually decreases. The planarians eventually remain distended and become covered with a whitish substance. The pharynx is extruded. This precedes the moment of death (*T*) which corresponds to the state of perfect immobility and is followed, after 2–3 min., by a complete disintegration of the planarian which confirms the accuracy of the time of death recorded[5].

RESULTS AND DISCUSSION

The Levy–Gucinski theory (8), developed to describe the absorption of drugs by goldfish, states: $1/T = DA/L \cdot C$, where T is time of death, L is the lethal dose, D is the apparent absorption-rate constant, A is the surface area of the absorbing membrane, and C is the drug concentration in the aqueous medium. Provided a number of assumptions are fulfilled, a plot of reciprocal time of death *versus* concentration of drug in the medium should give a straight line intersecting the origin and having a slope equal to DA/L. Levy and coworkers (8, 9) also demonstrated that the CT value (product of drug concentration and time of death), used frequently as an index of relative toxicity of drugs, is a function not only of the inherent toxicity but also of the rate of absorption of a compound.

The effect on planarian *Dugesia lugubris* of the 13 nonionic surfactants in aqueous solution of concentration ranging from 0.05 to 1.0% (1.0–5.0% for polysorbate 80) are presented in Table I. Four separate experiments were carried out with each of the agents, using three animals for each solution concentration in any one of the experiments performed. The time of death of planarian was found to vary inversely with the concentration of surfactant in solution. The regression equations calculated for each compound are shown in Table II. To test the statistical significance of the $1/T$ *versus* C slopes and the linearity of the regressions, the data obtained with individual animals were submitted to an ANOVA test, with the aid of an Olivetti Programma 101 computer. The ANOVA showed that all regressions were highly significant ($p \ll 0.0005$) and that deviations from linearity were not significant. Only the regression line for polysorbate 20 showed some deviation from linearity, since the F value for nonlinear terms was 3.106, which is significant at the 5% level.

The following statistical tests were also performed. Within each series, the significance of difference between slopes was calculated using a test analogous to the t-test between two sample means. The results of this analysis are reported in Table III.

Using the Student t test at each concentration, significance of difference between reciprocals of death times at each surfactant

[2] In the botanical garden of the Department of Botany, the University of Pisa. For a comprehensive treatise on anatomy and physiology of planarian, *cf. Reference 16.*

[3] Trademarked as Myrj 49, 51, 52, 53, and 59; Tween 20, 40, 60, and 80; and Brij 35, 58, 78, and 98, respectively, by Atlas Chemical Industries, Wilmington, Del.

[4] Cenco Co., Chicago, Ill.

[5] When planarians are immersed in solutions containing pharmacologically active compounds of different types (barbiturates, chlorphenesin, *etc.*), the death pattern is essentially the same, but disintegration occurs 1–2 hr. after pharynx extrusion. Disintegration invariably follows death of a planarian. The rapid disintegration in the presence of surfactants is probably due to the wetting properties of these compounds.

Table II—Regression Equations Describing the Effect of Surfactant Concentration (C) on Time of Death of Planarian (T)

Surface-Active Agent	Regression Equation, $1/T = bC + a$
Polyoxyethylene 20 stearate	$1/T = 0.9540C + 0.0179$
Polyoxyethylene 30 stearate	$1/T = 0.3183C + 0.0045$
Polyoxyl 40 stearate	$1/T = 0.2659C - 0.0061$
Polyoxyethylene 50 stearate	$1/T = 0.1786C + 0.0029$
Polyoxyethylene 100 stearate	$1/T = 0.0406C + 0.0078$
Polysorbate 20	$1/T = 4.7932C + 0.1443$
Polysorbate 40	$1/T = 2.3039C - 0.1289$
Polysorbate 60	$1/T = 0.8771C - 0.0271$
Polysorbate 80	$1/T = 0.1571C - 0.0522$
Polyoxyethylene 23 lauryl ether	$1/T = 0.4234C + 0.1406$
Polyoxyethylene 20 cetyl ether	$1/T = 1.8780C + 0.8350$
Polyoxyethylene 20 stearyl ether	$1/T = 1.8548C + 0.7374$
Polyoxyethylene 20 oleyl ether	$1/T = 2.0700C + 0.6191$

concentration was determined by comparing the mean death times of any two members of a series until all the members of all the concentrations were tested. The results are listed in Table IV.

Polyoxyethylene Stearate Series—A plot of reciprocal time of death of planarian *versus* concentration of the five members of the homologous series of polyoxyethylene stearate series is presented in Fig. 2. A linear relationship is observed in each case and, as predicted by the Levy–Gucinski theory, the regression lines extrapolate to origin. The intercepts are not significantly different from zero at the 95% probability level. Differences between slopes of the polyoxyethylene stearic acid ester series are all significant or highly significant. Differences between reciprocals of death times at the lower concentrations of polyoxyl 40 stearate *versus* polyoxyethylene 50 stearate are not significant. The differences at the concentrations of 0.1 and 1.0% of polyoxyethylene 30 stearate *versus* polyoxyl 40 stearate are also not significant. Reciprocals of time for all remaining members of the series as compared with each other are significantly different at each concentration.

One further observation is apparent. The slopes of the lines for the individual members of the series decline as the ethylene oxide mole ratio R (*i.e.*, the number of moles of ethylene oxide added per mole of stearic acid) increases, the sequence being polyoxyethylene 20 stearate, polyoxyethylene 30 stearate, polyoxyl 40 stearate, polyoxyethylene 50 stearate, and polyoxyethylene 100 stearate.

This is further evidenced by a plot of the R values for polyoxyethylene stearate solutions of identical concentration *versus* time of death of planarian (Fig. 3). The points lie in straight lines, with intercept at $R \sim 19$. It must be remembered that, in a $1/T$ *versus* C plot, the slope is related to absorption rate and toxicity. In this case, a separate evaluation of each effect was not possible; hence, the decrease in slope is only indicative of a decrease in relative toxicity of the surface-active agents.

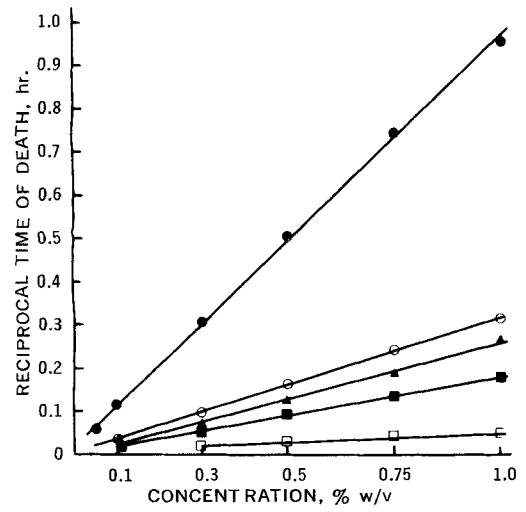

Figure 2—*Reciprocal time of death of planarian as a function of surfactant concentration at 20°. Key: ●, polyoxyethylene 20 stearate; ○, polyoxyethylene 30 stearate; ▲, polyoxyl 40 stearate; ■, polyoxyethylene 50 stearate; and □, polyoxyethylene 100 stearate.*

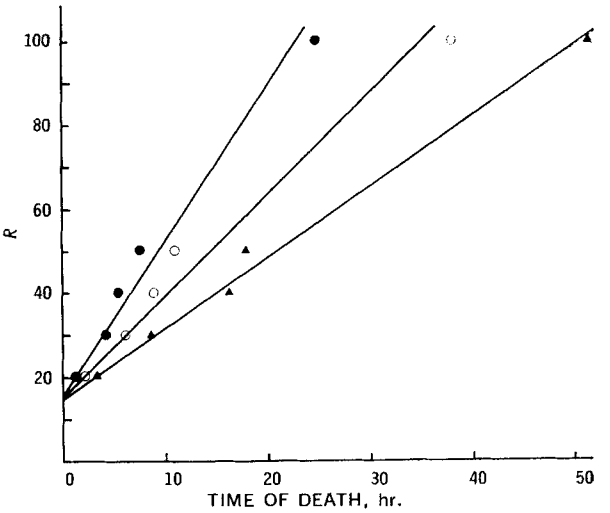

Figure 3—*Time of death of planarian as a function of ethylene oxide mole ratio* (R) *of polyoxyethylene stearates. Concentration of solutions, key: ▲, 0.30%; ○, 0.50%; and ●, 0.75%.*

The linear relationship existing between ethylene oxide mole ratio of surfactants and time of death of planarian appears rather interesting and deserves further investigation with other surfactants having a fixed hydrophobic moiety and different R values.

Since the polyoxyethylene moiety is an important segment of the polyoxyethylene stearates, the acute toxicity of polyethylene glycols is pertinent. Their acute oral toxicity (LD_{50} on a gram per kilogram basis) in the rat was reported to be decreasing with increasing molecular weight (18). Reduced absorption of polyethylene glycols from the gastrointestinal tract as the molecular weight was increased was also reported (18). The present findings are consistent with this report.

Products of surfactant concentration and time of death of planarian, *i.e.*, CT values based on actual experimental data, are presented in Table V. The CT values are relatively constant for each compound of the polyoxyethylene stearate series over the wide range of concentrations.

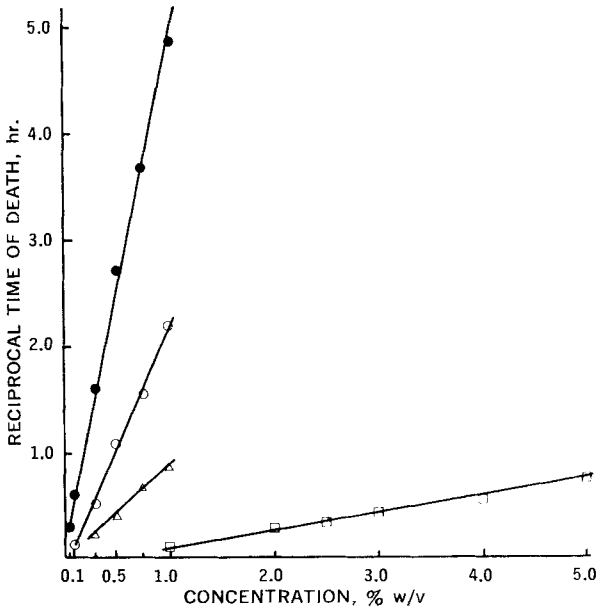

Figure 4—*Reciprocal time of death of planarian as a function of surfactant concentration at 20°. Key: ●, polysorbate 20; ○, polysorbate 40; △, polysorbate 60; and □, polysorbate 80.*

Table III—Significance of Difference between Slopes

Slopes	$F^{a,b}$	Student's t	Degrees of Freedom	Significance[b]
Polyoxyethylene 20 stearate *versus* polyoxyethylene 30 stearate	S	45.4	87	S
Polyoxyethylene 30 stearate *versus* polyoxyl 40 stearate	S	4.04	75	S
Polyoxyl 40 stearate *versus* polyoxyethylene 50 stearate	S	7.08	63	S
Polyoxyethylene 50 stearate *versus* polyoxyethylene 100 stearate	n.s.	34.1	104	S
Polysorbate 80 *versus* polysorbate 40	S	21.5	114	S
Polysorbate 80 *versus* polysorbate 60	S	37.8	85	S
Polysorbate 40 *versus* polysorbate 60	S	20.4	89	S
Polysorbate 40 *versus* polysorbate 80	S	34.8	59	S
Polyoxyethylene 23 lauryl ether *versus* polyoxyethylene 20 cetyl ether	S	28.2	74	S
Polyoxyethylene 23 lauryl ether *versus* polyoxyethylene 20 stearyl ether	S	10.5	70	S
Polyoxyethylene 23 lauryl ether *versus* polyoxyethylene 20 oleyl ether	S	19.8	70	S
Polyoxyethylene 20 cetyl ether *versus* polyoxyethylene 20 stearyl ether	S	0.167	98	n.s. ($p > 0.7$)
Polyoxyethylene 20 cetyl ether *versus* polyoxyethylene 20 oleyl ether	S	1.98	111	n.s. ($p > 0.05$)
Polyoxyethylene 20 stearyl ether *versus* polyoxyethylene 20 oleyl ether	S	1.36	116	n.s. ($p > 0.1$)

[a] F = variance ratio, s_1^2/s_2^2. [b] S = significant, $p < 0.001$. n.s. = not significant. Differences between slopes not indicated in the table were highly significant.

Polysorbate Series—Figure 4 is a plot of reciprocal time of death of planarian *versus* concentration of surfactants of the polysorbate series. All regression lines are highly significant; all four extrapolate virtually to origin, and each slope (*i.e.*, the action exerted on planarian by each surfactant) differs significantly from the others.

The toxicity of one of the surface-active agents, polysorbate 80, was so low that it was necessary to employ much higher concentrations (within 1.0–5.0% range) to obtain the end-point.

In this series, it can be seen that, the hydrophilic moiety of the surfactants being equal, relative toxicity depends largely on the nature of the hydrophobic portion, *i.e.*, the esterifying acid. The decreasing order of toxicity on planarian was polysorbate 20,

Table IV—Significance of Difference between Reciprocals of Death Times at Each Surfactant Concentration

	Concentration, %	Student's t	Significance
Polyoxyethylene Stearic Acid Ester Series			
Polyoxyl 40 stearate *versus* polyoxyethylene 50 stearate	0.1	2.26	n.s. ($0.05 > p > 0.02$)
	0.3	1.62	n.s. ($p > 0.1$)
	0.5	2.58	n.s. ($0.02 > p > 0.01$)
	0.75	4.91	S ($p < 0.001$)
	1.0	6.99	S ($p < 0.001$)
Polyoxyethylene 30 stearate *versus* polyoxyl 40 stearate	0.1	0.38	n.s. ($p > 0.1$)
	0.3	5.25	S ($p < 0.001$)
	0.5	3.93	S ($p < 0.001$)
	0.75	3.63	S ($0.001 < p < 0.002$)
	1.0	2.28	n.s. ($0.05 > p > 0.02$)
Polyoxyethylene 50 stearate *versus* polyoxyethylene 100 stearate; polyoxyethylene 30 stearate *versus* polyoxyethylene 50 stearate; polyoxyethylene 30 stearate *versus* polyoxyethylene 100 stearate; polyoxyethylene 20 stearate *versus* all others: all reciprocals of times are significantly different at each concentration			
Polysorbate Series			
Polysorbate 20 *versus* polysorbate 40; polysorbate 20 *versus* polysorbate 60; polysorbate 40 *versus* polysorbate 60: all reciprocals of times are significantly different at each concentration.			
Polyoxyethylene Alkyl Ether Series			
Polyoxyethylene 20 stearyl ether *versus* polyoxyethylene 20 oleyl ether	0.05	1.88	n.s. ($0.1 > p > 0.05$)
	0.1	1.27	n.s. ($p > 0.1$)
	0.3	1.90	n.s. ($0.1 > p > 0.05$)
	0.5	1.27	n.s. ($p > 0.1$)
	0.75	0.13	n.s. ($p > 0.1$)
	1.0	1.05	n.s. ($p > 0.1$)
Polyoxyethylene 20 cetyl ether *versus* polyoxyethylene 20 oleyl ether	0.05	3.00	S ($p < 0.01$)
	0.1	1.1	n.s. ($p > 0.1$)
	0.3	1.73	n.s. ($0.1 > p > 0.05$)
	0.3	1.46	n.s. ($p > 0.1$)
	0.75	0.66	n.s. ($p > 0.1$)
	1.0	0.11	n.s. ($p > 0.1$)
Polyoxyethylene 20 cetyl ether *versus* polyoxyethylene 20 stearyl ether	0.05	2.39	n.s. ($0.05 > p > 0.02$)
	0.1	2.32	n.s. ($0.05 > p > 0.02$)
	0.3	0.46	n.s. ($p > 0.1$)
	0.5	0.07	n.s. ($p > 0.1$)
	0.75	0.49	n.s. ($p > 0.1$)
	1.0	1.02	n.s. ($p > 0.1$)
Polyoxyethylene 23 lauryl ether *versus* all others: all reciprocals of times are significantly different at each concentration.			

Table V—Experimentally Determined *CT* Values in Grams per Deciliter × Hours for the Homologous Series of Polyoxyethylene Stearates

Surface-Active Agent	Concentration, % w/v 0.30	0.50	0.75	1.0	Average
Polyoxyethylene 20 stearate	0.99	1.02	1.00	1.04	1.0
Polyoxyethylene 30 stearate	3.04	3.05	3.13	3.27	3.1
Polyoxyl 40 stearate	4.89	4.45	4.11	3.75	4.3
Polyoxyethylene 50 stearate	5.38	5.43	5.74	5.64	5.5
Polyoxyethylene 100 stearate	15.40	18.98	18.60	21.55	18.6

Table VI—Effect of Low Concentrations of Polyoxyethylene Alkyl Ethers on Time of Death of Planarian

Surface-Active Agent	Concentration, % w/v 0.01	0.02	0.03	0.04
	Average Time of Death, hr.[a]			
Polyoxyethylene 23 lauryl ether	19.30(10.2)	11.68(77.7)	10.30(8.1)	6.75(10.0)
Polyoxyethylene 20 cetyl ether	3.08(19.5)	2.08(14.9)	1.35(11.6)	1.18(14.0)
Polyoxyethylene 20 stearyl ether	3.75(33.4)	2.21(12.2)	1.57(20.2)	1.35(18.2)
Polyoxyethylene 20 oleyl ether	5.15(9.1)	2.73(12.7)	1.80(10.0)	1.60(12.5)

[a] Average of 12 animals; *CV*% in parentheses.

polysorbate 40, polysorbate 60, and polysorbate 80, respectively. The acute oral toxicity (LD_{50} on a gram per kilogram basis) in the fasted rat was reported to be 34.7, 34.2, 33.8, and 38.0, respectively (18). The differences in relative toxicity between the individual members of the polysorbate series in the planarian are by far more pronounced. However, both in higher animals and in planarian, esterification of polyoxyethylene 20 sorbitan with oleic acid results in a significantly lower order of toxicity.

Polyoxyethylene Alkyl Ether Series—The results obtained for this series in the same concentration range (0.05–1.0%) as the other surfactants were somewhat different in that they did not seem to fit adequately the Levy–Gucinski theory. Indeed, although a linear relationship between reciprocal time of death of planarian and surfactant concentration was observed, all four lines extrapolated to the ordinate at a point (average intercept ± *SD*, 0.583 ± 0.31) which was significantly different from zero. The results are plotted in Fig. 5; one surfactant (polyoxyethylene 20 stearyl ether) is omitted for the greater clarity of the graph. As may be seen in Table III, the slopes of polyoxyethylene 20 cetyl ether, polyoxyethylene 20 stearyl ether, and polyoxyethylene 20 oleyl ether are significantly different from the slope of polyoxyethylene 23 lauryl ether but do not significantly differ from each other. In other words, the effect on planarian of the three former agents is practically identical, although they correspond to nominally different structures.

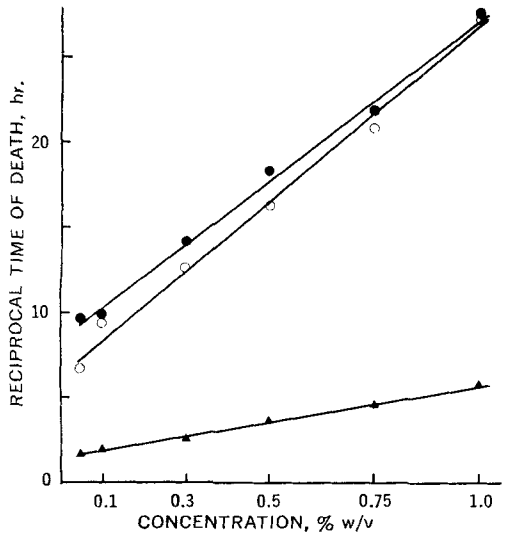

Figure 5—*Reciprocal time of death of planarian as a function of surfactant concentration at 20°. Key: ▲, polyoxyethylene 23 lauryl ether; ●, polyoxyethylene 20 cetyl ether; and ○, polyoxyethylene 20 oleyl ether.*

To gather additional data, the compounds were tested at concentrations ranging from 0.01 to 0.04%. The results are reported in Table VI and plotted in Fig. 6. It can be seen that the points lie on a curve ideally connecting the origin with the straight lines obtained in the experiments at higher concentrations of the surfactants.

These data indicate that polyoxyethylene 23 lauryl ether, the agent possessing the highest degree of hydrophilicity, is the least toxic of the series. This is in agreement with the acute toxicity data in terms of LD_{50} in grams per kilogram in fasted male rat (18).

Compounds of polyoxyethylene stearate and polysorbate series show a linear relationship between $1/T$ and C, and all lines have the origin as common intercept. This implies *absorption* of a toxic agent, whose concentration increases linearly with increasing overall surfactant concentration[6].

The concentration of these surfactants were well above their respective critical micelle concentrations (CMC's)[7]; therefore, the solutions contained micellar aggregates in equilibrium with surfactant monomers. Ultrafiltration studies of nonionic surfactant solutions have demonstrated that, above the CMC, monomers coexist with micelles, and the concentration of both species increases linearly with increasing overall surfactant concentration (19). Therefore, either species may be responsible for the toxic action on the planarian, although penetration of large micellar aggregates through a biological membrane seems less likely.

The behavior of the compounds of the polyoxyethylene alkyl ether series seems to indicate a much higher activity of the toxic species at low surfactant concentrations. A gradual decrease in activity occurs in the concentration range 0.01–0.05%; then a linear relationship between activity and overall surfactant concentration is observed. The effect results in a downward inflection of the $1/T$ *versus* C curve; hence, the linear portion of the curve extrapolates to the ordinate and not to the origin. Perhaps this phenomenon could be ascribed to the presence of toxic impurities undergoing gradual entrapment in surfactant micelles, *i.e.*, decreasing in activity, above the CMC. Indeed, GC analysis of commercial samples of polyoxyethylene alkyl ether surfactants has shown the presence of

[6] As an alternative hypothesis, it might be speculated that surface-active agents alter membrane permeability, with a loss of some essential planarian component, such as electrolyte.

[7] CMC's for Tween 20, 40, 60, and 80 are 0.0060, 0.0029, 0.0027, and 0.0013 g./dl., respectively; for Myrj 51, 52, 53, and 59, 0.0081, 0.011, 0.014, and 0.016 g./dl., respectively (*cf.*, P. Becher, in "Nonionic Surfactants," M. J. Schick, Ed., Marcel Dekker, New York, N. Y., 1967, p. 478). The CMC of Myrj 49, not reported in the literature, was found to be 0.015 g./dl. by the surface-tension method.

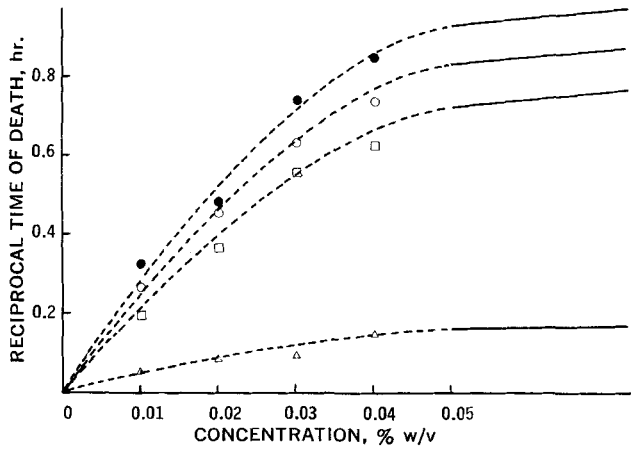

Figure 6—*Reciprocal time of death of planarian as a function of surfactant concentration at 20°. Key: △, polyoxyethylene 23 lauryl ether; ●, polyoxyethylene 20 cetyl ether; ○, polyoxyethylene 20 stearyl ether; and □, polyoxyethylene 20 oleyl ether.*

substantial amounts of alcohols and polyethylene glycols (20). From further work, now in progress, additional data may be obtained which might help clarify this question. The CMC's of the four surfactants, not reported in the literature, were determined by the surface-tension method. They were 0.012, 0.012, 0.010, and 0.018 g./dl. for polyoxyethylene 23 lauryl ether, polyoxyethylene 20 cetyl ether, polyoxyethylene 20 stearyl ether, and polyoxyethylene 20 oleyl ether.

Surface tension of the immersion solutions used throughout this study seems not to have influenced the results. The concentrations of the surface-active agents (except for compounds of the polyoxyethylene alkyl ether series at the 0.01% concentration) were above, or well above, their CMC's. Correspondingly, the variation in surface tension over the wide ranges in concentration was very slight (*cf.*, Table VII). The same can be said for pH of the solutions. Planarians have been reported to be rather indifferent to the pH of their environment, enduring without evident effect wide ranges in this factor (15). To verify this point, the survival time of planarians in solutions of pH ranging from 2.2 to 9.0 was recorded (Table VIII). All animals survived more than 7 days in the pH range 4–7. The pH of surfactant solutions at 1.0% concentration ranged from 4.5 to 7.0 (*cf.*, Table VII).

Table VII—Surface Tension and pH of Surfactant Solutions at 20°

Surface-Active Agent	Concentration, % w/v	Surface Tension, dynes/cm.	pH
Polyoxyethylene 20 stearate	0.05	41.4	
	1.0	38.0	6.3
Polyoxyethylene 30 stearate	0.05	43.0	
	1.0	38.3	5.1
Polyoxyl 40 stearate	0.05	42.7	
	1.0	40.0	4.9
Polyoxyethylene 50 stearate	0.05	44.6	
	1.0	43.4	4.5
Polyoxyethylene 100 stearate	0.05	48.1	
	1.0	47.2	5.0
Polysorbate 20	0.05	34.0	
	1.0	33.3	6.8
Polysorbate 40	0.05	38.8	
	1.0	37.0	6.6
Polysorbate 60	0.05	42.3	
	1.0	38.6	6.7
Polysorbate 80	1.0	40.8	6.7
	5.0	35.8	6.5
Polyoxyethylene 23 lauryl ether	0.05	40.7	
	1.0	40.0	6.5
Polyoxyethylene 20 cetyl ether	0.05	38.9	
	1.0	38.7	6.9
Polyoxyethylene 20 stearyl ether	0.05	40.6	
	1.0	40.3	7.0
Polyoxyethylene 20 oleyl ether	0.05	38.5	
	1.0	38.0	6.1

Table VIII—Survival Time of Planarian in Solutions of Different pH at 20°

pH of Solution[a]	Survival Time[b]
2.2	4.0 min.
3.0	1.0 hr.
4.3–7.5	7 days
8.0	30 hr.
9.0	3.0 hr.

[a] Solutions in the pH range 2.2–5.0 were prepared from 0.01 *N* HCl and 0.01 *M* dibasic sodium citrate; in the pH range 6.0–9.0 from 0.05 *M* tris(hydroxymethyl)aminomethane (Tris) and 0.05 *N* hydrochloric acid. [b] Average of six animals.

In conclusion, the data presented in this preliminary paper point out the utility of the planarian as an experimental animal. No direct evidence is presented concerning the nature of the toxic species (with the possible exception of the polyoxyethylene alkyl ethers at lower concentrations), although it is speculated that the agents in their monomeric state might be responsible for the toxic action.

Data on relative toxicity of surfactants obtained in this study are in satisfactory agreement with data in the literature obtained with higher animals. Planarians proved to be very sensitive to variations in type, structure, and concentration of nonionic surfactants and might prove a useful tool for investigations on these compounds. Further studies on the utility of the planarian in this and other areas of biopharmaceutical research are in progress.

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Toxogonin: Oral Administration to Man

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Abstract □ Toxogonin, administered orally in an aqueous solution to healthy male volunteers, produced dose-related blood levels of oxime. These levels were lower than those found with similar doses of 2-pyridinium aldoxime methochloride. A dose-independent symptom complex of facial warmth and/or numbness and a cool menthol sensation in the throat was similar to, but of less intensity than, that previously seen when toxogonin was given intramuscularly. If toxogonin shows further therapeutic promise, additional investigations into methods to facilitate its gastrointestinal absorption are indicated.

Keyphrases □ Toxogonin, oral administration—dose-related blood, plasma levels, oxime □ Absorption, toxogonin—oral administration □ Dose-independent symptoms—toxogonin □ Urinary excretion—toxogonin

Toxogonin [*N,N'*-oxydimethyl(pyridinium-4-aldoxime) dichloride] has been suggested as a replacement for pyridine-2-aldoxime methochloride as the oxime of choice in the therapy of anticholinesterase poisoning (1, 2). *In vitro* studies indicated that toxogonin is 15–30 times more effective than pralidoxime in reactivating cholinesterase inhibited by certain anticholinesterases (1, 2); animal studies showed it to be more effective (3–5), although not quite as much as suggested by *in vitro* studies. In cases of poisoning in man, it was more effective than (6), or at least as effective (7) as, pyridine-2-aldoxime methochloride.

By intramuscular administration to men, the dose of toxogonin necessary to produce plasma levels above 5 mcg./ml. is about one-third the dose of pyridine-2-aldoxime methochloride needed for similar levels (8), although the significance of this is not known since a therapeutic blood level has not been determined. Another point in its favor is that toxogonin produced hypertension in unpoisoned men (8), which is a possible benefit in a severely poisoned individual whose circulation is failing.

Although oxime should be given to a severely poisoned individual by intravenous injection, oral therapy might be useful in some circumstances. One instance might be in poisoning by a substance that is rather inactive *in vitro* but is metabolized (or "toxified") in the body to a toxic anticholinesterase; *e.g.*, parathion to paroxon. In this case, it is desirable to maintain a constant level of oxime over a period of time, which would require repeated injections if parenteral administration was used. A recent report describes the effectiveness of pyridine-2-aldoxime methochloride administered in this manner to poisoned rats (9).

Oral oximes also might be given prophylactically (10, 11) to men with a high risk of exposure to anticholinesterases, such as crop dusters. Finally, oral administration of oximes might be considered for a poisoned individual whose signs and symptoms are very mild, not requiring urgent treatment.

To assess how well toxogonin is absorbed when administered orally to men as determined by blood levels of oxime and to investigate the side effects caused by toxogonin administered orally were the purposes of this study.

SUBJECTS, METHODS, AND MATERIALS

The subjects were U. S. Army enlisted men between the ages of 19 and 31 years (mean 21.6) who volunteered for and were accepted into this study after a physical examination, chest X-ray, electrocardiogram, and laboratory tests[1] revealed no abnormalities. They weighed from 66.8 to 98.4 kg. (mean 78.1). They were told they were to receive a new and possibly useful antidote for anticholinesterase ("nerve agent") poisoning and that they might experience some symptoms (left unspecified). Their only reward for participating was a day off from duty.

Toxogonin was purchased[2]. Spectroanalysis showed it to be over 98% pure; the intraperitoneal LD_{50} [by the method of Bliss (12)] in 15–20-g. female mice was 109.6 (95% CL: 102.5–117.2) mg./kg.

Three or four subjects participated each test day. On each morning of the test, a fresh 25% (w/v) solution of toxogonin in distilled water was prepared and administered orally within 1 hr. of preparation. The doses selected were 1, 3, 5, 7, and 9 g.; the volumes of toxogonin solution were correspondingly 4 to 36 ml. The lowest dose and the highest dose were given to two subjects each; the other three doses were given to three subjects each.

Subjects were admitted to the test ward on the evening before the test. They were allowed no solid food from midnight until 3 hr. after they had taken the drug, which was about 8 a.m. A high fluid intake was urged from the time they awoke (about 6:30 a.m.) until the conclusion of the test.

In the hour before the drug was given, three measurements of blood pressure and heart rate were made with the subject supine. These measurements were repeated hourly for 6 hr. after drug administration. Also during this period, blood was drawn for control laboratory studies (hematocrit, hemoglobin, total and differential white blood cell counts, blood urea nitrogen, alkaline phosphatase, and serum glutamic oxaloacetic transaminase) which were repeated 1 and 7 days later. Each subject voided before receiving the drug; this urine specimen was used as a control for oxime analysis and urinalysis.

In three subjects (all 7-g. dose), leukocyte chromosomes were studied before the subjects received toxogonin and 4 hr. and 7 days afterwards. The preparations were made by the method of Moorhead *et al.* (13).

Blood was obtained at 0.5, 1, 2, 3, 4, 5, and 8 hr. after drug administration; the whole blood and plasma were analyzed for toxogonin content by methods previously described (14). The oxime content of the red cells was also measured and will be the subject of a separate report. Urine was collected and analyzed for oxime content (14) at hourly intervals for the first 10 hr.; thereafter, for 24 hr., all urine was collected and analyzed, but no time schedule for collection was imposed. Most of the urine specimens were refrigerated overnight and analyzed. A preliminary study, performed by adding a known amount of toxogonin to urine and analyzing aliquots daily, had shown that there was less than 5% loss of toxogonin over a week.

[1] Hematocrit, hemoglobin, total and differential white blood cell count, urinalysis, blood urea nitrogen, alkaline phosphatase, serum glutamic oxaloacetic transaminase, bilirubin, total protein and albumin, and red blood cell and plasma cholinesterase.

[2] E. Merck, Darmstadt, Germany.

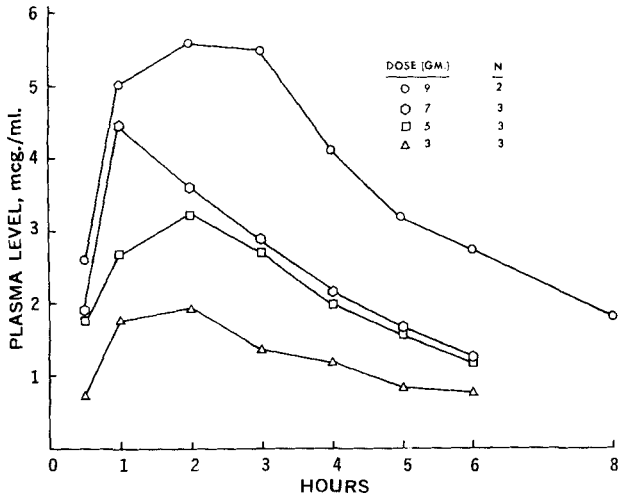

Figure 1—*Toxogonin: mean plasma oxime levels after oral administration.*

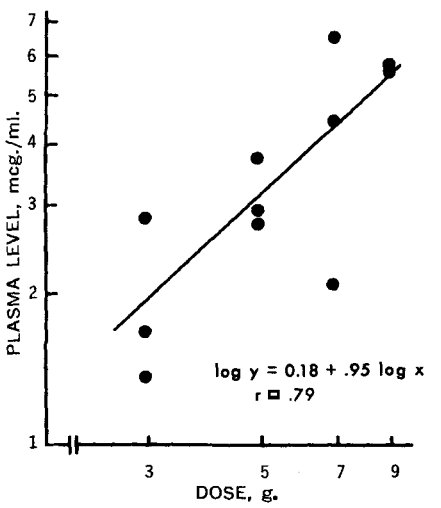

Figure 2—*Toxogonin* (per os)*: dose* versus *maximal plasma level.*

The subjects were allowed to be out of bed except for 10 min. preceding measurement of heart rate and blood pressure.

Because of the low amount of oxime excreted in the urine (see *Results*), it was thought that perhaps excretion was delayed. Three additional subjects were given toxogonin (two received 7 g. and one received 3 g.) under the same test conditions, except that blood was drawn at 2 and 3 hr. after drug only to ensure that toxogonin was present in about the same concentration as it was in the others; all urine was collected for oxime analysis for 48 hr. (These blood and urine values were not included in the tabulated results.)

RESULTS

Blood Levels of Oxime—The oxime plasma concentrations of the two subjects who received 1 g. of toxogonin reached maximum values of about 1 mcg./ml., but the lower values varied considerably. Since these levels are at the limits of the analytical method, these data were not tabulated.

Figure 1 shows the time courses of the mean plasma values for oxime for the other dose groups (through a laboratory error, the 8-hr. plasma value for three subjects, one at a dose of 5 g. and two at 7-g. doses, was discarded). The whole blood oxime concentrations were about 60–70% of the plasma values and are not shown. Maximal values were reached in 1 or 2 hr.; for all but the 7-g. dose group, a plateau level was maintained for the next 1–2 hr., undoubtedly because of continued absorption.

A plot of the dose *versus* the maximal measured plasma concentration for each subject is shown in Fig. 2.

In a previously reported study (15), conducted with similar subjects and test conditions, commercially available tablets of pyridine-2-aldoxime methochloride produced higher plasma levels than those found for toxogonin. Figure 3 shows these comparisons. The plasma concentration of toxogonin is roughly 50–60% of that produced by an equiweight dose of pyridine-2-aldoxime methochloride.

Urinary Excretion—Overall, 2.2% of the dose given was excreted in the urine during the 24-hr. collection period; percentage excretion by dose group was 1.4 for 3 g. (range 0.3–3.1), 2.4 for 5 g. (range 2.3–2.4), 2.2 for 7 g. (range 1.3–3.7), and 3.2 (range 3–3.4) for 9 g. This would not have increased over the next 24 hr.; the percentages excreted by the three subjects whose urine was collected for 48 hr. were 2.1 (7-g. dose), 3.5 (7-g. dose), and 0.7 (3-g. dose).

Half-Lives of Oxime—*Plasma*—By assuming that a drug is eliminated by first-order kinetics, the half-life for it in plasma ($t_p{}^1/_2$) and its rate constant of elimination (k_p) can be calculated from the slope of a line drawn through the terminal points when the plasma concentration is plotted on a logarithmic scale against time on a linear scale. For nine of the subjects, the coefficient of correlation of this line through the last five to six values was about 0.99; for one subject, the coefficient of correlation was 0.96; and for one subject (7-g. dose), the coefficient of correlation of such a line was poor and his data were not used in half-life calculations.

The mean plasma half-life of toxogonin for 10 subjects was 2.64 hr. (for 3 g., 2.41 hr.; for 5 g., 2.78 hr.; for 7 g., 2.29 hr.; and for 9 g., 3.10 hr.). The mean k_p value for the 10 subjects was 0.27 hr.$^{-1}$ (by dose group: 0.29 for 3 g., 0.24 for 5 g., 0.31 for 7 g., and 0.23 for 9 g.). This half-time is not significantly ($p > 0.1$ by t-test) different from the half-life of pyridine-2-aldoxime methochloride (mean 2.66 hr.) found in earlier studies.

Urine—The rate constant (k_u) and biological half life ($t_u{}^1/_2$) for urinary excretion can be obtained from the slope of the regression line of the plot of the amount to be excreted (total amount excreted over the entire collection period minus the amount excreted by time, t) as a percentage of the total amount excreted (on a logarithmic scale) against time, t (16).

The plots for the means of each dose group are shown in Fig. 4. The $t_u{}^1/_2$ values were 4.4 hr. for 3 g., 5.0 hr. for 5 g., 5.2 hr. for 7 g., and 4.6 hr. for 9 g., with a mean for all doses of 4.8 hr. Corresponding k_u values were 0.16 hr.$^{-1}$, 0.14 hr.$^{-1}$, 0.13 hr.$^{-1}$, and 0.15 hr.$^{-1}$.

This time is significantly ($p < 0.01$ by t-test) longer than the corresponding values for pyridine-2-aldoxime methochloride (mean of 2.44 hr.).

Other Laboratory Findings—There were no alterations in the values obtained in any of the routine laboratory tests at 1 or 7 days.

Physiological Effects—There were no significant changes in the heart rate or blood pressure of any subject.

Chromosome Studies—There was not an increased number of gaps or breaks, nor was there an increase in the mitotic index in the chromosomes of the three subjects (all 7-g. dose) in whom chromosomes were studied.

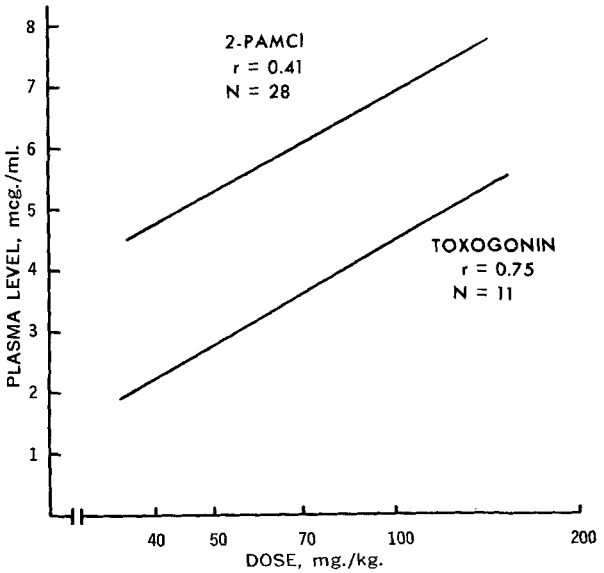

Figure 3—*Toxogonin and pyridine-2-aldoxime methochloride: comparison of plasma levels after oral administration.*

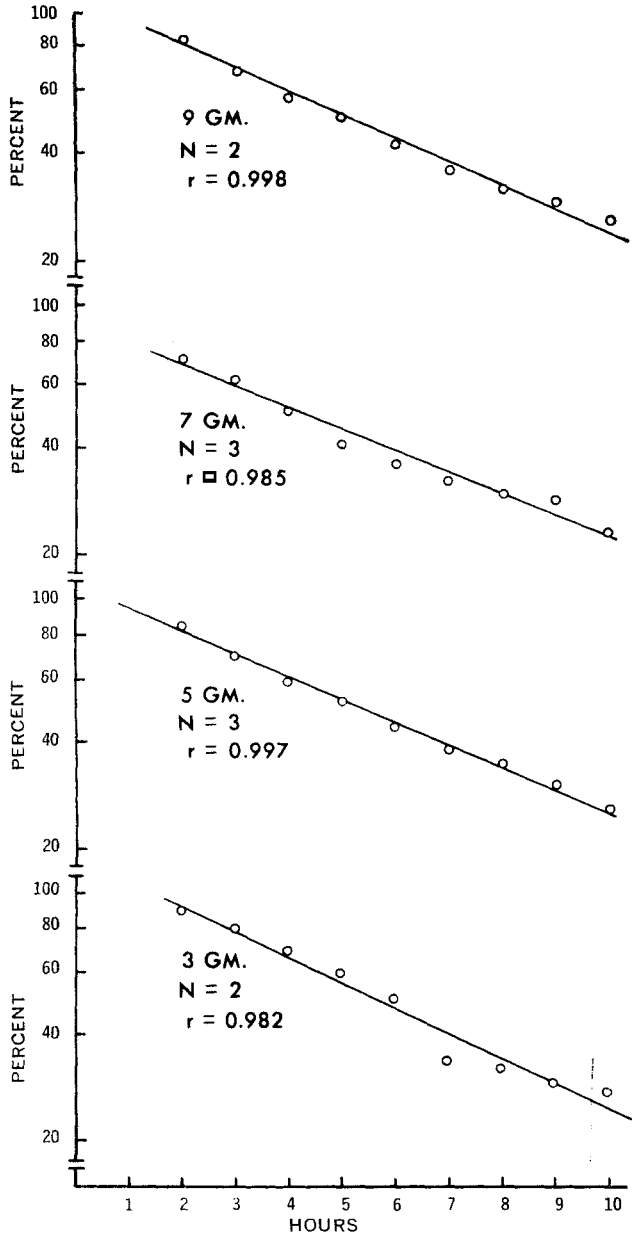

Figure 4—*Toxogonin: percent not excreted* versus *time.*

Side Effects—Most of the subjects described the taste of the solution as "bitter" or "salty" but tolerable.

The symptoms were similar to those reported after intramuscular toxogonin (*1, 8*), but their intensity was less. Two subjects receiving 3 g. noted numbness of their lips; one reported a cool sensation in his mouth, and one noted a dry mouth. At 5 g., one reported a cool sensation in his mouth, and one noted that his mouth felt dry. Of the five subjects receiving 7 g., two reported a tightness in their cheeks, four said their faces felt numb, and two spontaneously described a menthol taste and coolness in their throats. One subject at a dose of 7 g. became nauseated and vomited about 1.5 hr. after receiving the oxime. (This subject's oxime blood levels were among the highest in this dose group and were included in all analyses.) One subject receiving 9 g. said his face felt warm and numb. Several in each dose group complained of feeling drowsy or of their eyes feeling heavy.

DISCUSSION

Erdman and Okonek (17) reported that no measurable blood levels of oxime were found in three human subjects after the oral administration of 30 mg./kg. of toxogonin in a tablet also containing 24 mg./kg. of disodium ethylenediaminetetraacetic acid as an adjuvant. At a dose of 1 g. (about 12 mg./kg. in two subjects, each weighing 82 kg.) in the present study, maximum plasma levels of about 1 mcg./ml. were found, which is close to the limit of the analytical method. At higher doses, proportionally higher blood levels were found.

Orally administered toxogonin gives relatively low blood levels of oxime when compared to oral pyridine-2-aldoxime methochloride. Although, in general, an aqueous solution of a drug should be absorbed more quickly and completely than a drug compressed into a tablet which must undergo disintegration in the gastrointestinal tract before absorption, it is possible that toxogonin and perhaps other oximes are degraded by gastric acidity or other factors against which the tablet or capsule protects the active ingredient. Erdman and Okonek (17) demonstrated in rats that this may be true for toxogonin because the drug was more completely absorbed after instillation into the duodenum than from instillation into the stomach. This suggests that if it is in an appropriate pharmaceutical preparation, one resistant to gastric action, the gastrointestinal absorption of toxogonin might be greater.

The small amount of oxime excreted in the urine is perhaps a reflection of the poor absorption of toxogonin or could be due to the metabolism of the compound to a substance not detectable by this method. Since in a similar study on toxogonin administered by the intramuscular route, the authors found 82% of the compound in the urine (8), we tend to discard the latter suggestion, although the metabolism of a compound may be different when it is administered by different routes.

If it can be assumed that the amount of drug excreted in the urine is a direct reflection of the amount of drug absorbed, there is a disparity between plasma levels and excretion of toxogonin compared to 2-pyridinium aldoxime methochloride. For similar doses (milligrams per kilogram), the plasma levels of toxogonin are more than 50% of those obtained with 2-pyridinium aldoxime methochloride, but the percentage of the dose of toxogonin found in the urine is about one-fifth of that found after 2-pyridinium aldoxime methochloride (15), suggesting that less of the dose is absorbed. One possibility, as noted, is that toxogonin is metabolized to a compound not identified in the urine. Another possibility is that the volume of distribution (the hypothetical volume of body fluid in which the substance is present in the same concentration as it is in the plasma) is much smaller for toxogonin than for 2-pyridinium aldoxime methochloride, in which case higher blood levels would be produced by smaller amounts of drug. This latter possibility can only be verified by further work.

The exact therapeutic effectiveness that might be expected from these plasma concentrations is unknown, although evidence suggests that plasma levels of 2–5 mcg./ml. would be adequate. Sundwall (18), in *in vivo* studies, suggested that 4 mcg./ml. of the methanesulfonate salt of pralidoxime is adequate to reverse certain effects (*e.g.*, neuromuscular block) produced by an anticholinesterase. In *in vitro* studies, Erdman *et al.* (1) noted that 3 mcg./ml. of pralidoxime (exact salt not specified) was required to reactivate cholinesterase inhibited by paraoxon (a value close to that found by Sundwall *in vitro*), but only 0.1–0.2 mcg./ml. of toxogonin was needed to produce the same amount of reactivation under the same conditions. These latter investigators (1) also noted that cholinesterase depression produced in the blood of dogs and cats by 20 times the lethal dose of parathion was quickly regenerated by 1–3 mg./kg. of toxogonin, a dose which in man produces a plasma level of around 2–5 mcg./ml.

If, as suggested by Erdman and coworkers (1, 2), toxogonin is a better antidote than 2-pyridinium aldoxime methochloride for anticholinesterase poisoning, further investigations on mechanisms to facilitate intestinal absorption are indicated if oral administration is desirable.

SUMMARY AND CONCLUSIONS

Toxogonin administered orally in an aqueous solution to healthy male volunteers produced dose-related blood levels of oxime. These levels were lower than those found with similar doses of 2-pyridinium aldoxime methochloride. A dose-independent symptom complex of facial warmth and/or numbness and a cool menthol sensation in the throat was similar to but of less intensity than that previously seen when toxogonin was given intramuscularly. If toxogonin shows further therapeutic promise, additional investigations into methods to facilitate its gastrointestinal absorption are indicated.

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Kinetics of Rheological Properties of Acacia Solutions

DENNIS D. WARNER and OSCAR E. ARAUJO

Abstract □ The kinetics of the rheological properties of acacia solutions prepared from two different lots of USP grade acacia were investigated with respect to preservative, temperature, and pH. A rotating viscometer was used to measure the viscosity of samples stored at 40, 50, 60, and 70° for up to 6 weeks. Solutions were prepared using benzoic acid as a preservative. Control solutions containing no preservative were also studied. An analog computer was used to analyze the viscosity and time. An Arrhenius-type relationship was established for the apparent first-order rate constants.

Keyphrases □ Acacia solutions—rheological properties □ Rheological properties, acacia solutions—kinetics □ Temperature, aging effects—acacia solutions, viscosity □ Preservative effects, acacia solutions—rheological properties

It was reported that solutions of acacia undergo a change in viscosity with time (1–4). Taft and Malm (1) found that bacterial growth lowered the viscosity of acacia solutions, while Osborne and Lee (2) reported that unpreserved acacia solutions exhibited a greater decrease in viscosity with aging than did acacia solutions preserved with 0.2% benzoic acid. Joslin and Sperandio (3) studied the effect of temperature and method of preparation on acacia solutions. They reported that the temperature of the water used to prepare the solutions affected the viscosity of the acacia solutions. They also reported that storage temperature affected the change in viscosity.

More recently, Araujo (4) studied the effects of certain preservatives on the aging characteristics of acacia solutions for a period of 1 year; he reported that acacia solutions, in a range of 10–25% by weight, behaved as Newtonian systems. He observed that the viscosity of all preserved and unpreserved acacia solutions decreased with aging. After 6 weeks at room temperature, the reduction in viscosity of the acacia solutions appeared to follow a zero-order process.

The literature contains very few kinetic studies on change of viscosity of aqueous solutions of gums. Levy and Schwarz (5) characterized the viscosity reduction of tragacanth solutions as a zero-order process, while Tobolsky (6) reported that the degradation of most linear polymers followed a first-order process.

Acacia in solution can be hydrolyzed by using sulfuric acid (7) or by heating the solution for a period of time (8, 9).

One report indicated the possibility of a relationship between viscosity and molecular weight of acacia (10). An earlier investigation indicated that acacia with a molecular weight of 280,000 may be split into fragments with molecular weights of less than 10,000 after autohydrolysis for 1 day (11). This combined information seemed to imply that there might be a change in viscosity upon hydrolysis which may be related to the change in molecular weight. Therefore, it seemed reasonable that the rate of change in viscosity might be related to the rate of hydrolysis of acacia.

If bacterial growth affects the viscosity of acacia solutions, the rate of viscosity change might also be affected by the rate of bacterial growth. Araujo (4) speculated that the rate of bacterial decomposition of acacia solutions stored at room temperature appeared to decrease after a period of 6 weeks.

Since the greatest decrease in viscosity of acacia solutions appeared to occur in the first 6 weeks (3, 4), the kinetics of the rheological properties of acacia solutions over this initial time period warranted investigation,

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Table I—Mean and Range of Nine Viscosity Determinations of Acacia Solutions

Lot	Weeks	0.2% Benzoic Acid at 40°	0.2% Benzoic Acid at 50°	0.2% Benzoic Acid at 60°	0.2% Benzoic Acid at 70°	No Preservative at 40°	No Preservative at 50°	No Preservative at 60°	No Preservative at 70°
		Mean and Range of η, cps.							
1	0	74.5 72.5–76.7	74.6 73.3–75.8	71.0 70.0–72.7	66.3 65.2–67.5	74.8 72.6–77.5	72.4 70.7–74.1	70.8 69.0–72.3	69.0 66.9–71.0
	1	72.1 70.4–73.6	67.9 66.4–69.3	60.9 59.4–61.9	51.7 51.0–52.3	68.7 67.5–70.1	68.1 66.8–69.3	59.4 58.2–60.5	51.1 50.1–52.3
	2	65.7 64.9–66.6	59.6 57.1–61.6	52.2 50.5–53.4	47.2 46.2–47.9	65.7 64.3–67.5	61.9 61.0–62.7	53.1 51.8–54.0	48.9 47.5–50.3
	3	61.0 60.1–62.1	55.8 54.5–56.8	47.3 45.3–48.2	41.9 40.9–43.4	60.8 59.7–62.1	56.4 54.0–57.5	47.3 45.7–48.5	44.1 42.5–45.5
	4	61.4[a] 60.4–62.3	53.4[a] 52.6–54.0	45.6[a] 45.0–46.0	35.8 32.7–38.6	54.6 51.4–56.6	52.1 49.2–53.8	43.7 40.5–45.3	39.7 38.3–41.4
	5	58.2[a] 57.4–58.8	49.7 49.2–50.1	46.0 44.1–47.7	34.9 33.3–36.2	54.5[a] 54.0–54.7	49.9 49.4–50.5	44.2 43.0–45.7	38.5 36.4–39.9
	6	55.0 54.3–55.5	49.4 48.6–50.1	41.5 40.7–42.3	31.8[a] 30.5–33.3	50.1 49.2–50.8	48.7 47.9–49.9	43.2 42.3–43.9	36.1 34.3–37.9
2	0	55.2 54.2–57.1	54.9 53.6–56.2	53.1 51.8–54.2	51.5 49.2–52.9	55.6 54.3–57.1	54.7 53.6–56.2	53.8 52.9–54.9	53.6 53.0–54.5
	1	52.9 52.4–53.6	51.4 51.0–52.1	47.8 47.5–48.1	42.7 41.8–44.4	48.1 47.5–49.2	50.8 50.1–52.1	49.3 48.8–49.8	43.4 43.1–43.7
	2	49.1 46.6–50.4	46.9 44.9–48.2	43.0 42.5–43.6	35.6 34.4–36.2	43.9 42.9–44.9	48.5 47.7–49.1	45.3 44.2–46.2	37.5 35.7–38.9
	3	49.2 47.9–50.3	45.0 43.6–45.7	38.5 34.8–39.5	30.7 27.4–33.4	38.9 37.9–40.1	44.4 43.1–45.2	39.7 37.0–41.4	34.5 30.5–36.6
	4	46.9[a] 46.2–47.5	39.5 34.4–43.6	32.8 26.6–37.3	25.8 21.8–28.3	33.0 30.1–35.6	40.5 38.3–43.3	36.8 32.7–39.6	31.3 28.6–33.5
	5	44.5 43.7–45.3	41.4 40.9–41.8	36.4 35.6–37.5	30.3[a] 28.9–31.9	35.0[a] 34.6–35.3	41.0 40.3–41.6	38.0 37.4–38.8	31.3[a] 30.5–31.8
	6	44.5 42.7–45.7	40.2 39.2–40.9	34.0[a] 33.5–35.0	25.8[a] 25.4–26.1	31.9[a] 31.4–32.2	41.6 40.8–42.0	36.1 35.4–36.4	28.9[a] 28.5–29.0

[a] Six determinations of viscosity were made.

with the following objectives:

1. To determine the relationship between the change in viscosity of acacia solutions and storage time.
2. To determine the effect of preservative on the rate of change in viscosity of acacia solutions.
3. To determine the effect of storage temperature on the rate of change in viscosity of acacia solutions.
4. To determine the effect of lot variation on the parameters studied.

EXPERIMENTAL

Preparation of Acacia Solutions—All acacia solutions were prepared in the same manner. Distilled water (2400 ml.) containing the appropriate amount of preservative was heated until dissolution was effected. The preservative solution was cooled at 60° and placed in a blender[1]. Eight hundred grams of acacia USP was added, and the mixture was blended at low speed for 1 min. in order to prepare a 25% by weight solution. The acacia solutions were allowed to stand for 1 day to permit complete hydration of the acacia. The solutions were then placed in 50-ml. serum vials. The vials were sealed, using rubber stoppers and aluminum closures.

All acacia used came from two lots which met the USP requirements for acacia powder; they are designated as Lots 1[2] and 2[3].

Preservative Used—Two sets of acacia solutions with respect to preservative were prepared in this study. One contained no preservative, and the other contained 0.2% by weight of benzoic acid USP.

Temperature and Time Studies—The samples were divided and placed in four constant-temperature water baths set at 40, 50, 60, and 70°. These temperatures were maintained within ±0.5°. At given times, the samples were taken from the water baths, cooled, and measured rheologically; the pH was determined also.

Rheological Measurements—All rheological measurements were

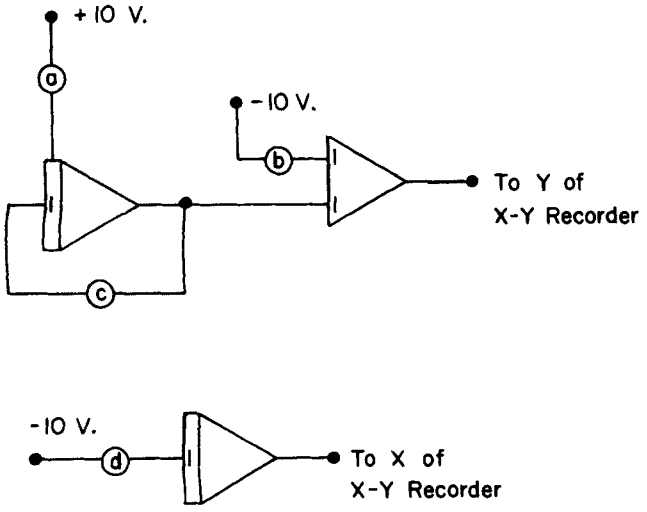

Figure 1—*Analog computer program for fitting viscosity* versus *time data.*

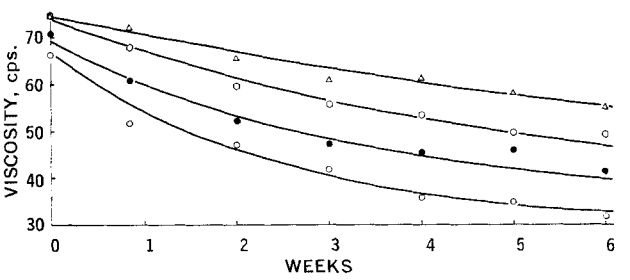

Figure 2—*Viscosity stability at different temperatures of acacia solutions from Lot 1 containing benzoic acid. Key:* △, *40°;* ○, *50°;* ●, *60°; and* ○, *70°.*

[1] Waring Blendor, model CB5.
[2] Fisher Scientific Co.
[3] Stein Hall and Co.

Table II—Values of k and η_∞ Calculated from Computer Data

Lot	Preservative	Temperature	k, weeks^{-1}	η_∞, cps.
1	Benzoic acid	40°	0.104	32.8
		50°	0.184	33.1
		60°	0.296	33.6
		70°	0.418	29.7
	None	40°	0.146	33.5
		50°	0.170	33.5
		60°	0.308	35.0
		70°	0.436	34.1
2	Benzoic acid	40°	0.084	25.2
		50°	0.126	24.6
		60°	0.226	25.9
		70°	0.430	24.7
	None	40°	0.258	25.9
		50°	0.138	26.5
		60°	0.192	26.7
		70°	0.426	26.6

made with a rotational viscometer[4] equipped with a dual measuring head which allows a greater range of viscosity measurements.

The temperature of the sample was maintained at 25 ± 0.2° using a constant-temperature circulator[5]. All samples were measured using rotary bob MV I and beaker MV supplied with the viscometer; either six or nine determinations were made for each sample, the number depending on the limitations of the viscometer.

Viscosity, η in cps., was calculated from Eq. 1:

$$\eta = K \times U \times S \qquad \text{(Eq. 1)}$$

where K is an instrumental constant dependent on the rotary bob and the measuring heat setting, U is the instrument setting which controls the revolutions per minute of the rotary bob, and S is the normal scale reading of the instrument. The mean and the range of the six or nine determinations of viscosity were used for analog computer analysis and are given in Table I.

pH Measurements—All pH measurements were made on a research pH meter[6] immediately after the viscosity determination.

Data Analysis—An analog computer[7] equipped with an X-Y recorder[8] was used to analyze the rheological data. An output of 1 v. on the ordinate was equivalent to 10 cps. of actual data, and 1 v. on the abscissa was equivalent to 1 sec. of computer time which was equivalent to 1 week of actual data.

RESULTS AND DISCUSSION

Time Studies—In this study, as seen in Table I, the viscosity of acacia solutions was determined at intervals up to 6 weeks for all samples. In all cases, the viscosity appeared to decrease with increasing time. However, in some samples, the viscosity decreased to a lesser extent than in others.

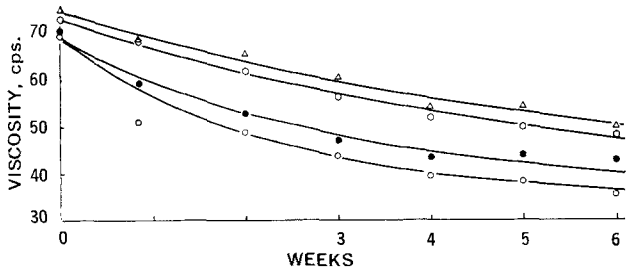

Figure 3—*Viscosity stability at different temperatures of acacia solutions from Lot 1 containing no preservative. Key:* △, *40°;* ○, *50°;* ●, *60°; and* ○, *70°.*

[4] Haake Rotovisko, instrument 67-369, Polyscience Corp., Evanston, Ill.
[5] Haake constant-temperature circulator, model Fe.
[6] Corning model 12-B.
[7] Pace TR-48, Electronic Associates, Inc.
[8] Mosely Autograt model 2D-2 X-Y, Hewlett-Packard, Inc.

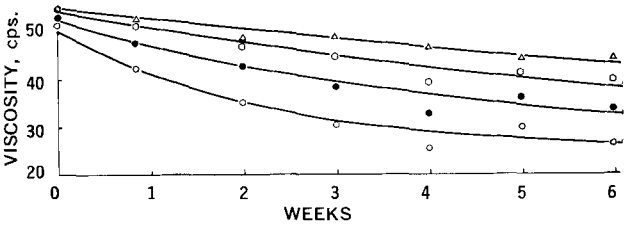

Figure 4—*Viscosity stability at different temperatures of acacia solutions from Lot 2 containing benzoic acid. Key:* △, *40°;* ○, *50°;* ●, *60°; and* ○, *70°.*

In Lot 1, the greatest decrease in viscosity was exhibited by the sample containing benzoic acid stored at 70°. It decreased a total of 34.5 cps. over 6 weeks, while the sample containing benzoic acid stored at 40° exhibited the smallest decrease, 19.5 cps.

In Lot 2, the sample containing the preservative stored at 70° also exhibited the largest decrease in viscosity, 25.7 cps. over 6 weeks, while the sample containing preservative at 40° again had the smallest decrease, 10.7 cps., over the same time period.

However, the overall change in viscosity does not provide information as to the path the change takes, *i.e.*, whether the change is slow and then fast or vice versa. By sampling at given times and using appropriate methods, the rate of the decrease may be measured.

If the viscosity decrease is due to the decrease in molecular weight of acacia, then it would appear that after complete hydrolysis of acacia, the viscosity measured would be the viscosity of a solution of the end-products of the hydrolysis. This would then mean that the viscosity would eventually become constant.

An analog computer was programmed for the model:

$$(\eta - \eta_\infty) = (\eta_0 - \eta_\infty)e^{-kt} \qquad \text{(Eq. 2)}$$

where η_0 is the initial viscosity, η_∞ is the viscosity at infinite time, k is the specific rate constant, and t is time in weeks. The program used to simulate Eq. 2 is shown in Fig. 1. The variable potentiometers, a, b, and c, correspond, respectively, to $\eta_0 - \eta_\infty$, η_∞, and k. Potentiometer d remained constant and corresponds to a constant change in time.

The rheological data were plotted on graph paper, and the graph paper was placed on the X-Y recorder. By adjusting potentiometers a, b, and c, a curve was drawn to fit the viscosity data. The potentiometer readings were recorded and used to calculate η_0, η_∞, and k. The calculated values of η_∞ and k are given in Table II. The computer-drawn curves for each set of viscosity *versus* time data are shown in Figs. 2–5 and appear to fit the experimental data reasonably well.

The pH of the acacia solutions remained fairly constant, with two exceptions which will be noted later.

The specific rate constants are relatively small, indicating a slow change in viscosity. Since it appears that a first-order relationship exists between viscosity and time, future studies might determine a definite relationship between the rate of hydrolysis of acacia and the rate of viscosity change.

Lot Effects—The most significant difference between the two lots is in the initial viscosities. The average initial viscosity of Lot 1 was 17.5 cps. greater than the average initial viscosity of Lot 2. This difference could possibly be accounted for by differences in the average molecular weight of the acacia due to variation in the natural source of the gum. The variation in molecular weight may be attributed to the composition of the acacia molecule itself. Studies have indicated that the acacia molecule contains D-ga-

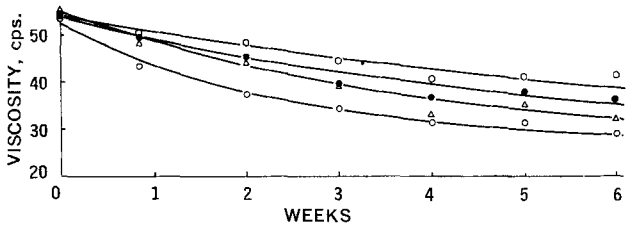

Figure 5—*Viscosity stability at different temperatures of acacia solutions from Lot 2 containing no preservative. Key:* △, *40°;* ○, *50°;* ●, *60°; and* ○, *70°.*

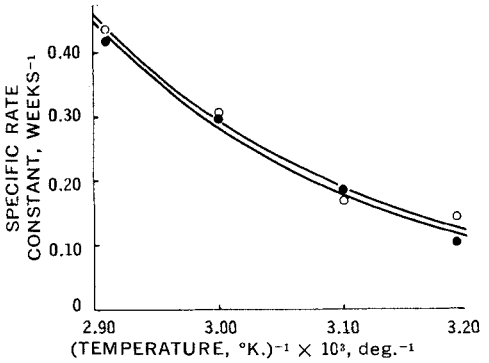

Figure 6—*Arrhenius-type plots for specific rate constants of acacia solutions from Lot 1. Key: ●, 0.2% benzoic acid; and ○, no preservative.*

lactose, L-arabinose, L-rhamnose, and D-glucuronic acid in varying proportions, depending upon the source of the gum (12–15).

Lot 1 also had a greater η_∞ than Lot 2, with the average difference being 7.4 cps., which might be explained by the varying proportions of major constituents of the acacia molecule.

Effect of Preservative—When the pH of the acacia solutions was measured, it was noted that the solutions with benzoic acid had an initial pH of 0.15–0.20 pH units lower than the corresponding solutions prepared without benzoic acid. This difference of pH might be attributed to the acidic nature of the preservative.

An unusual result, possibly due to preservative effect, involved the unexpectedly high value of the rate constant for Lot 2 at 40° with no benzoic acid. Since bacterial growth might be favored at 40°, it was thought that the unusually large rate constant could have been caused by bacterial hydrolysis. The pH of this group decreased from 4.14 to 3.67 over the 6-week period. Araujo (4) observed a sharp pH decrease for the first 6 weeks of his study at room temperature, which he attributed to bacterial attack.

However, if bacterial attack caused the inconsistency in the rate constant, then it appears that there would have been a disagreement in the rate constant for Lot 1 at 40° with no benzoic acid. This discrepancy is not evident by comparing the rate constants in Table II. However, a similar decrease from 4.11 to 3.69 pH units was also observed for this group. Therefore, no definite conclusion can be drawn to account for the inconsistency of the rate constant for Lot 2

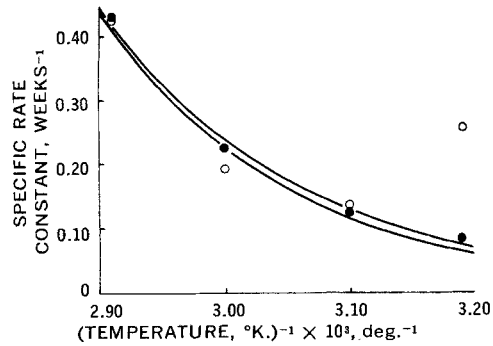

Figure 7—*Arrhenius-type plots for specific rate constants of acacia solutions from Lot 2. Key: ●, 0.2% benzoic acid; and ○, no preservative.*

Table III—Values of E_a Calculated from Computer Data

Lot	Preservative	E_a, kcal./mole
1	Benzoic acid	9.4
	None	8.7
2	Benzoic acid	12.4
	None	13.2

at 40° with no benzoic acid. However, an analysis of variance of the rate constants shows no significant effect of preservative on the rate of change in viscosity of the acacia solutions.

Effect of Temperature—The effect of temperature on the specific rate constants is highly significant. Therefore, an analysis of the effect was made using an Arrhenius-type equation:

$$k = Ae^{-E_a/RT} \qquad \text{(Eq. 3)}$$

where T is the absolute temperature, R is the gas constant, E_a is the energy of activation, and A is a preexponential factor. The rate constant–temperature data were analyzed by the analog computer. The computer plots are shown in Figs. 6 and 7.

The values for E_a were calculated for the rate constant change and are given in Table III. At the present stage of rheological scientific development, it is difficult to interpret the significance of this Arrhenius-type plot. Thus, E_a may or may not be related to the energy of activation for the hydrolysis of acacia. However, the fact that an Arrhenius-type relationship seemed to exist cannot be overlooked. It seems plausible to assume that the first-order rate for viscosity change may have a definite relationship to the rate of hydrolysis of acacia.

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Salicylate Absorption from Rectal Suppositories

E. L. PARROTT

Abstract □ The absorption in humans of aspirin and sodium salicylate incorporated in six suppository bases was studied by means of urinary salicylate elimination. It was found that the rectal dose of aspirin and sodium salicylate was equivalent to the oral dose. Rectal absorption was altered by pharmaceutical factors, which affected the release of the drug from the suppository. Aspirin was rapidly released from suppositories of polyethylene glycol and polyoxyethylene (4) sorbitan monostearate with glyceryl monostearate; aspirin was poorly released from a suppository of sorbitan monopalmitate. Sodium salicylate was rapidly released from a suppository of theobroma oil. Data for the release from theobroma oil suppositories containing cholesterol and glyceryl monostearate were inconclusive.

Keyphrases □ Salicylate absorption—rectal suppositories □ Aspirin, sodium salicylate absorption, suppositories—comparison □ Rectal suppositories—salicylate availability □ Suppository bases—rectal absorption

Suppositories are indicated for systemic action in patients who are in a coma or who cannot tolerate oral medication due to emesis or pathological conditions of the upper gastrointestinal tract. A rectal suppository is a convenient dosage form for the administration of medication to infants and mentally disturbed patients. Although the use of rectal suppositories is recognized for the administration of analgesics, antispasmodics, sedatives, and tranquilizers, there are conflicting opinions on the amount of drug that should be given rectally as compared to the oral dose. Generally, the suggested rectal dose ranges from one-half to twice the oral dose. By assuming that the physiological factors were constant, the proper rectal dose would be dependent on the physicochemical properties (*e.g.*, partition coefficient, physical state, and solubility) of the drug and on the properties (*e.g.*, melting point, solubility, and surface activity) of the suppository (1).

Atropine, chloral hydrate, methylene blue, morphine, and sodium salicylate were reported to be absorbed rectally more quickly than with oral administration (2). The dosage requirement of sulfanilamide is said to be the same for an oral tablet and a glycerinated gelatin suppository (3). Formulation may be the limiting factor in rectal absorption because the drug can be absorbed only after it is released from the suppository. On the basis of plasma levels in man 2 hr. after administration, it was reported that the absorption of aspirin from a polyethylene glycol suppository and from a glycerinated gelatin suppository was 93 and 53%, respectively, of the plasma level obtained from oral administration (4). Thus, pharmaceutical factors may influence the bioavailability and dose of a drug administered in a suppository.

This study was undertaken to compare, in terms of dose, the rectal and oral absorption of aspirin and sodium salicylate and to demonstrate the effect of several suppository bases on absorption. Since both drugs are analgesic, the clinical choice between aspirin and sodium salicylate might be based on their solubilities in the rectal fluid and the suppository base.

Table I—Yield Value and Dissolution Profile of Aspirin and Sodium Salicylate from Various Suppository Bases

Suppository Formula, g.		Yield Value, kg.	Minutes	Percent of Sodium Salicylate Dissolved 25°	Percent of Sodium Salicylate Dissolved 37°	Percent of Aspirin Dissolved 25°	Percent of Aspirin Dissolved 37°
PEG[a] 1540	0.51	2.2	15	66.9	88.3	38.3	90.2
PEG 6000	0.69		30	81.3	97.2	58.0	97.4
PEG 400	0.3		45	93.9	97.9	78.3	102.0
			60	100	100	92.0	103.5
Theobroma oil[b]	2.0	2.2	15	2.9	92.2	0.8	2.0
			30	4.3	98.2	2.6	5.6
			45	5.9	100	5.4	13.5
			60	6.8	100	11.7	16.9
Theobroma oil	1.27	1.9	15	6.3	94.0		
Glyceryl monostearate[c]	0.15		30	8.3	96.0		
Distilled water	0.08		45	9.4	100		
			60	10.7	100		
Theobroma oil	1.40	2.1	15	6.4	86.5	0.8	82.0
Cholesterol[d]	0.03		30	7.0	87.0	2.6	91.0
Distilled water	0.07		45	8.5	85.4	5.4	93.9
			60	8.8	85.4	11.7	98.3
SMP[e]	1.50	8.8	15	6.8	8.8	0.8	1.6
			30	8.8	10.7	1.0	2.9
			45	10.8	14.6	1.2	3.7
			60	13.6	20.5	1.8	4.2
PSMS[f]	1.35	0.4	15	5.9	49.2	1.1	3.4
Glyceryl monolaurate[g]	0.15		30	9.8	62.0	1.1	34.9
			45	13.6	63.0	1.1	48.8
			60	16.8	67.0	2.3	53.8

[a] Polyethylene glycol marketed as Carbowax, Union Carbide Chemicals. [b] Hershey's. [c] Purified, Fisher Scientific Co. [d] USP, Merck. [e] Sorbitan monopalmitate marketed as Span 40, Atlas Chemical Industries, Inc. [f] Polyoxyethylene (4) sorbitan monostearate marketed as Tween 61, Atlas Chemical Industries, Inc. [g] Marketed as Aldo MLD, Glyco Chemicals, Inc.

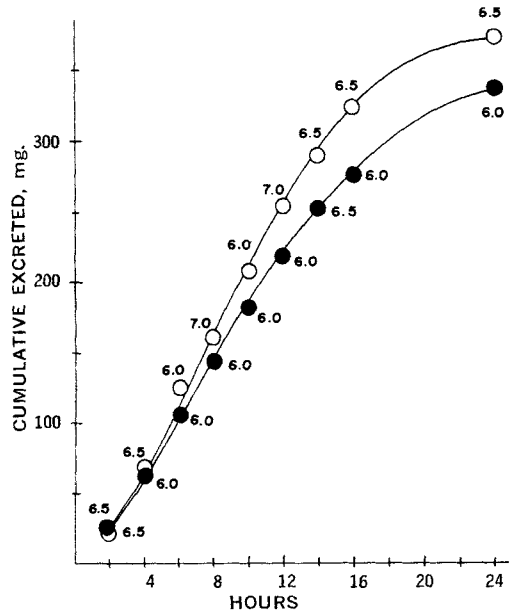

Figure 1—*Cumulative urinary salicylate excretion from Subject P. S. after the administration of 600 mg. of aspirin orally and rectally in a polyethylene glycol suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

EXPERIMENTAL

Protocol—Ten healthy male and two healthy female human subjects, from 24 to 37 years of age and weighing from 52 to 84 kg., were used in this study. Six hundred milligrams of aspirin powder or 534 mg. of sodium salicylate powder in a gelatin capsule was administered orally upon arising. Subjects were ambulatory and permitted to ingest food and fluid as desired. Urine samples were collected at 2-hr. intervals. Volumes were measured, and aliquots were retained for analysis. The pH of the urine was determined at the time of collection of each sample. No attempt was made to control the urinary pH.

After 1 week, a suppository containing the same dose of drug was administered rectally, and urine samples were collected according to the protocol. Each subject served as his control in the comparison of the urinary excretion of the drug from oral and rectal administration.

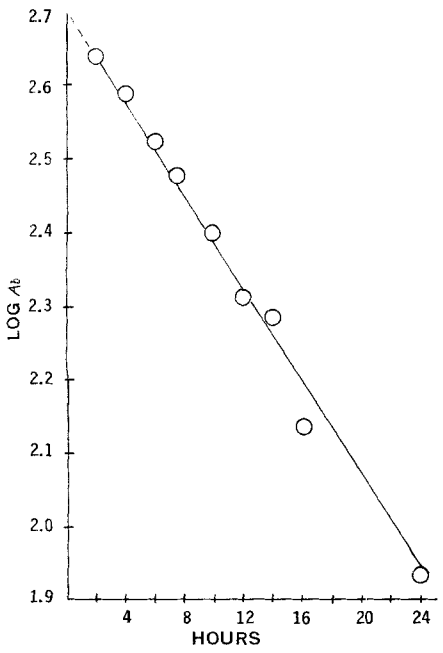

Figure 2—*Plot for Subject P. S. of logarithm of unexcreted salicylate against time used to evaluate* k. k = −2.303, *slope* = 0.073, *hr.*$^{-1}$.

Table II—Quantity of Salicylate, Volume, and pH of Urine Excreted at 2-hr. Intervals following Oral and Rectal Administration of 600 mg. of Aspirin

Hour	pH	Urine, ml.	Salicylate Excreted, mg.	Cumulative Excreted, mg.	Milligrams in Body
			Orally		
0	6.5	—	—	—	480
2	6.5	75	22.9	22.9	437.1
4	6.5	4	48.0	70.9	389.1
6	6.0	85	55.2	126.1	333.9
8	7.0	85	35.1	161.2	298.8
10	6.0	95	46.8	208.0	252.0
12	7.0	90	46.2	254.2	205.8
14	6.5	73	36.5	290.7	169.3
16	6.5	87	33.9	324.6	135.4
24	6.5	280	49.0	373.6	86.4
			Rectally		
0	—	—	—	—	
2	6.5	45.0	27.0	27.0	
4	6.0	55	37.1	64.1	
6	6.0	70	42.0	106.1	
8	6.0	60	39.0	145.1	
10	6.0	50	37.5	182.6	
12	6.0	45	36.6	219.2	
14	6.5	65	33.7	252.9	
16	6.0	55	25.9	278.8	
24	6.0	215	59.1	337.9	

Analytical Method—The analysis of the urine samples was carried out by the method of Trinder (5). Analysis was carried out during the day following the collection of urine. For the measurement of dissolution from dosage forms containing aspirin, an aliquot of the dissolution medium was subjected to alkaline hydrolysis. This solution was then acidified, appropriately diluted with 0.1 *N* hydrochloric acid, and assayed spectrophotometrically in terms of salicylic acid (6).

Preparation of Dosage Forms—The six formulations for the suppositories are given in Table I. The suppositories were prepared by

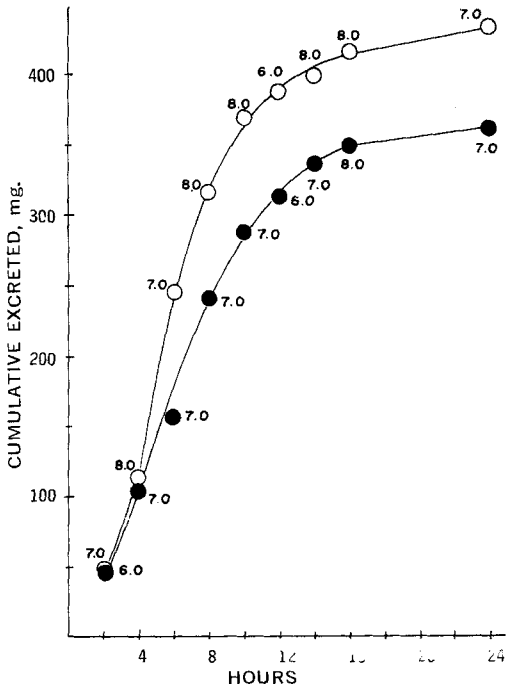

Figure 3—*Cumulative urinary salicylate excretion from Subject N. V. after the administration of 600 mg. of aspirin orally and rectally in a polyoxyethylene (4) sorbitan monostearate with glyceryl monolaurate suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

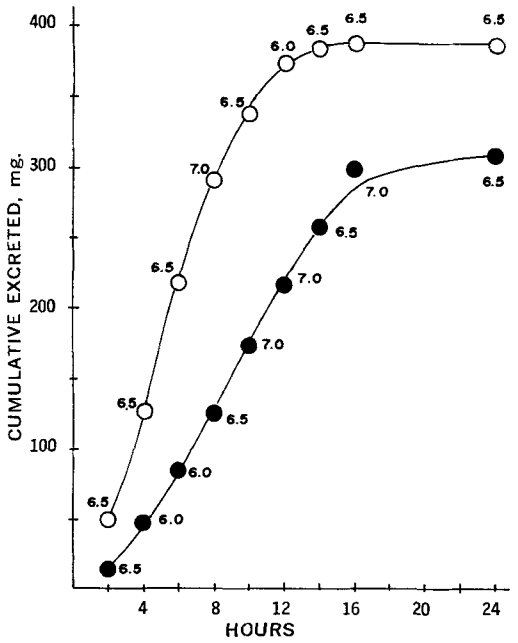

Figure 4—*Cumulative urinary salicylate excretion from Subject J. M. after the administration of 600 mg. of aspirin orally and rectally in a theobroma oil suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

the fusion method (7) and were utilized in 1 or 2 days after their preparation.

To determine the yield value, the suppository was first sliced in half, and the tapered end was discarded. The remaining uniform cylinder of the suppository was held in a vertical position by a small ring, and weights were added to a cardboard platform placed on the upper end. The yield value was considered to be the weight that caused deformation of the suppository at room temperature.

The percent of drug dissolved from the suppository was determined by use of the USP disintegration apparatus. A suppository was placed in the apparatus and held within a single glass cylinder

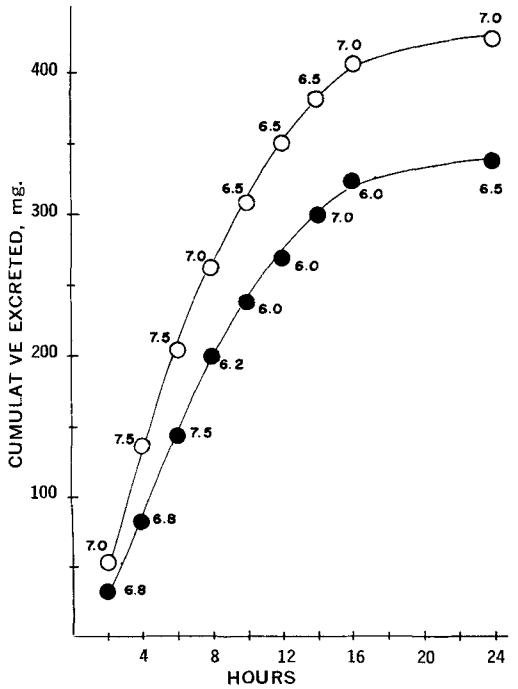

Figure 5—*Cumulative urinary salicylate excretion from Subject W. H. after the administration of 600 mg. of aspirin orally and rectally in a theobroma oil with cholesterol suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

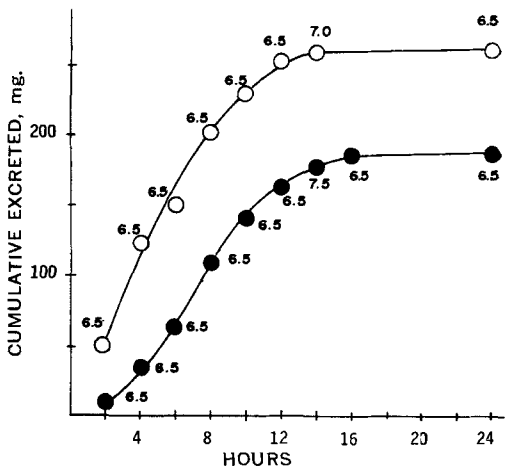

Figure 6—*Cumulative urinary salicylate excretion from Subject J. S. after the administration of 600 mg. of aspirin orally and rectally in a theobroma oil with glyceryl monostearate suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

by means of plastic tubing bent in the upper end of the cylinder so that it prevented the escape of the suppository. With the disintegration apparatus in operation in 1 l. of distilled water, samples were removed by a pipet fitted with a filter at 15, 30, 45, and 60 min. The samples were analyzed, and the percent dissolved at each time interval was calculated. Dissolution profiles were determined at 25 and 37°. The results are shown in Table I.

In preparing suppositories and capsules, a single batch of aspirin powder USP[1] and sodium salicylate powder USP[1] were used. Salicylate equivalents of each drug, *i.e.*, 600 mg. aspirin and 534 mg. sodium salicylate, were manually placed in a No. 0 hard gelatin capsule. The release of the drug from the capsule was determined in the USP disintegration apparatus, using distilled water and 0.1 *N* hydrochloric acid at 37°. The drugs were dissolved within 30 min.

RESULTS AND DISCUSSION

Typical experimental data for one subject are given in Table II. The pH of the urine did not fluctuate greatly, and this fluctuation

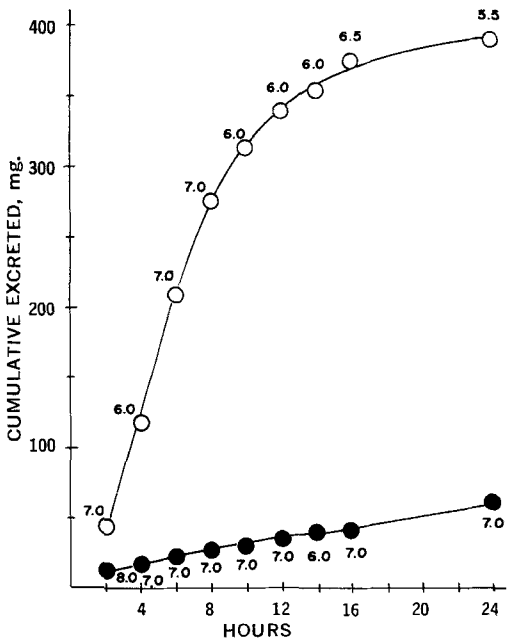

Figure 7—*Cumulative urinary salicylate excretion from Subject A. A. after the administration of 600 mg. of aspirin orally and rectally in a sorbitan monopalmitate suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

[1] Merck & Co., Inc.

Table III—Rate Constant for Salicylate Urinary Elimination and Comparison of f, the Fraction of 600-mg. Dose of Aspirin Recovered in Urine, at 4 and 24 hr. after Oral Ingestion of a Capsule and Rectal Administration in Various Suppository Bases

Subject	k, hr.$^{-1}$	For 24 hr. f_{oral}	For 24 hr. f_{rectal}	For 24 hr. $\frac{f_{rectal}}{f_{oral}}$	For 4 hr. $\frac{f_{rectal}}{f_{oral}}$	Suppository Base[a]
P.S.	0.073	0.81	0.73	0.90	0.90	Polyethylene glycol
N.V.	0.160	0.94	0.79	0.84	0.92	Polyoxyethylene (4) sorbitan monostearate with glyceryl monolaurate
J.M.	0.150	0.84	0.67	0.80	0.38	Theobroma oil
W.H.	0.120	0.92	0.74	0.80	0.60	Theobroma oil with cholesterol
J.S.	0.067	0.56	0.41	0.73	0.30	Theobroma oil with glyceryl monostearate
A.A	0.110	0.84	0.14	0.17	0.12	Sorbitan monopalmitate
Average	0.11	0.82				
Range	0.067–0.15	0.56–0.94				

[a] See Table I for complete formula.

Table IV—Rate Constant for Salicylate Urinary Elimination and Comparison of f, the Fraction of 534-mg. Dose of Sodium Salicylate Recovered in the Urine, at 4 and 24 hr. after Oral Ingestion of a Capsule and Rectal Administration in Various Suppository Bases

Subject	k, hr.$^{-1}$	For 24 hr. f_{oral}	For 24 hr. f_{rectal}	For 24 hr. $\frac{f_{rectal}}{f_{oral}}$	For 4 hr. $\frac{f_{rectal}}{f_{oral}}$	Suppository Base[a]
H.L.	0.110	0.81	0.74	0.91	0.64	Polyethylene glycol
I.H.	0.045	0.57	0.51	0.79	0.89	Theobroma oil
H.P.	0.093	0.81	0.71	0.87	0.50	Sorbitan monopalmitate
R.B.	0.095	0.79	0.63	0.80	0.68	Theobroma oil with glyceryl monostearate
C.R.	0.127	0.96	0.72	0.75	0.96	Theobroma oil with cholesterol
A.H.	0.064	0.68	0.31	0.45	0.62	Polyoxyethylene (4) sorbitan monostearate with glyceryl monolaurate
Average	0.09	0.78				
Range	0.045–0.127	0.68–0.96				

[a] See Table I for complete formula.

would not appreciably affect salicylate elimination because the dose is small (8). The cumulative amount of salicylate eliminated in the urine is shown as a function of time in Table II and Fig. 1 for the oral and rectal administration of 600 mg. of aspirin. In view of the known rapid and complete gastrointestinal absorption of aspirin, the total excretable drug was assumed to be equal to the administered dose (9). The amounts (A_b) of salicylate remaining in the body after each urine collection time are then readily calculated. If the logarithm of the unexcreted drug is plotted against time, the rate constant (k) for urinary elimination may be determined (10–12). From the plot in Fig. 2 of the data in Table II, the k value for this subject was 0.073 hr.$^{-1}$.

Similar experimental data were determined for the other subjects and are represented graphically in Figs. 3–7. The k value was determined graphically for each subject; the average k value was 0.11 hr.$^{-1}$, with a range from 0.067 to 0.15 hr.$^{-1}$ as shown in Table III. The fraction (f_{oral}) of the orally administered dose of aspirin re-

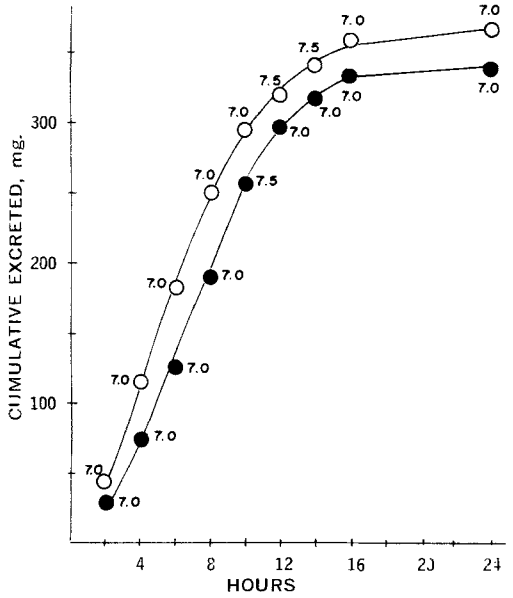

Figure 8—*Cumulative urinary salicylate excretion from Subject H. L. after the administration of 534 mg. of sodium salicylate orally and rectally in a polyethylene glycol suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

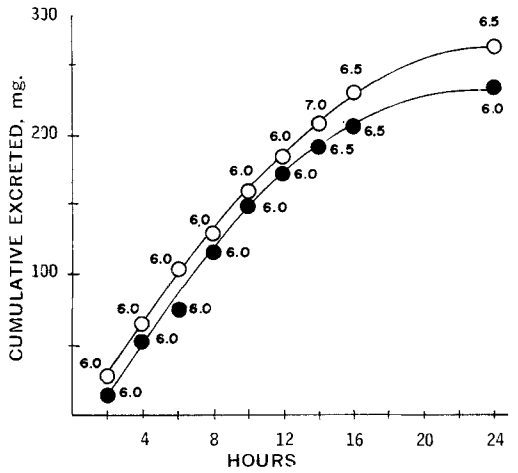

Figure 9—*Cumulative urinary salicylate excretion from Subject I. H. after the administration of 534 mg. of sodium salicylate orally and rectally in a theobroma oil suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

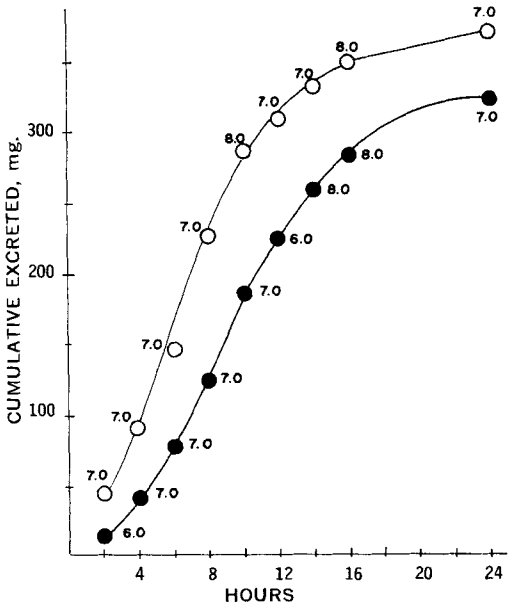

Figure 10—*Cumulative urinary salicylate excretion from Subject H. P. after the administration of 534 mg. of sodium salicylate orally and rectally in a sorbitan monopalmitate suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

covered in the urine in 24 hr. was calculated for each subject; it ranged from 0.56 to 0.94 with an average of 0.82.

By using the same protocol for the administration of an equivalent dose of sodium salicylate, the cumulative amounts of salicylate eliminated in the urine were experimentally measured as a function of time for each subject, as shown in Figs. 8–13. The k and f_{oral} values were calculated as previously described. The average k value was 0.09 hr.$^{-1}$, with a range from 0.045 to 0.127 hr.$^{-1}$ as listed in Table IV. The fraction of the orally administered dose of sodium salicylate recovered in the urine in 24 hr. ranged from 0.68 to 0.96 with an average of 0.78.

Thus, the excretion and absorption of equivalent doses of aspirin and sodium salicylate are kinetically the same because the f_{oral} and k values for both drugs are the same.

The assumption that the mucosa of the entire gastrointestinal tract possesses the same absorption characteristics for the passively

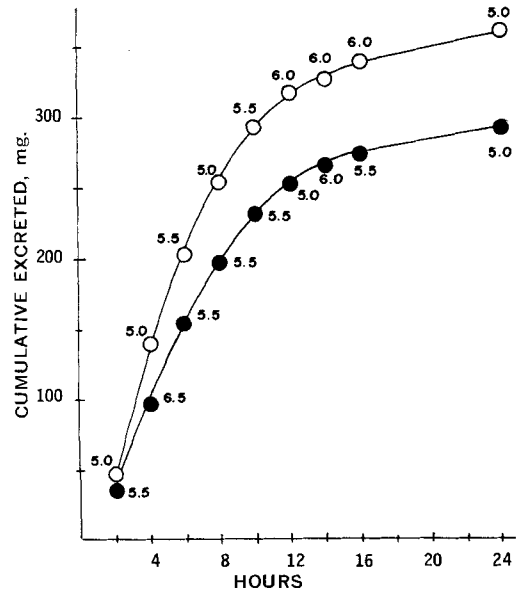

Figure 11—*Cumulative urinary salicylate excretion from Subject R. B. after the administration of 534 mg. of sodium salicylate orally and rectally in a theobroma oil with glyceryl monostearate suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

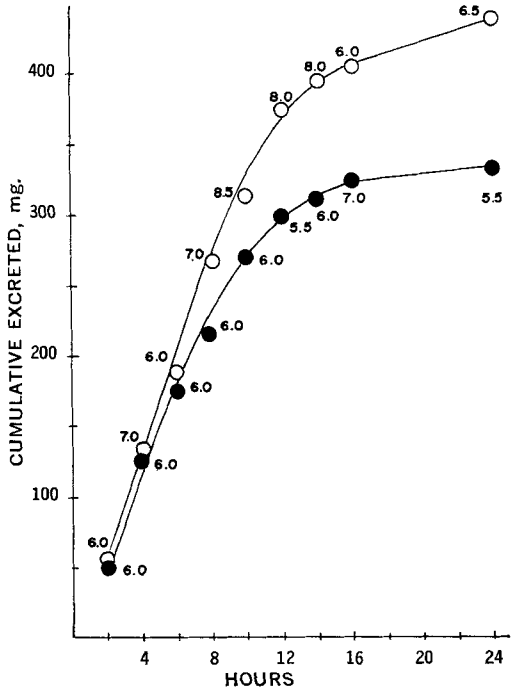

Figure 12—*Cumulative urinary salicylate excretion from Subject C. R. after the administration of 534 mg. of sodium salicylate orally and rectally in a theobroma oil with cholesterol suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

absorbed aspirin and sodium salicylate seems valid, since the f_{rectal}/f_{oral} for aspirin was approximately 0.9 from suppository bases of polyethylene glycol and polyoxyethylene (4) sorbitan monostearate with glyceryl monolaurate at 4- and 24-hr. intervals, as shown in Figs. 1 and 3, and the f_{rectal}/f_{oral} for sodium salicylate was approximately 0.9 for suppository base of theobroma oil, as shown in Fig. 9. This assumption is also supported by the report that the plasma levels of aspirin administered in a rectal suppository were comparable to the plasma levels of aspirin administered orally (4).

Effect of Suppository Bases—The formulation of a suppository may affect absorption by controlling the release of the drug. Sorbitan monopalmitate is insoluble in water and melts at approximately 45°. As shown in Table II, only 4% of the aspirin was dissolved in 30 min., during which pieces of the sorbitan monopalmitate suppository were cast off gradually. Knowing these *in vitro*

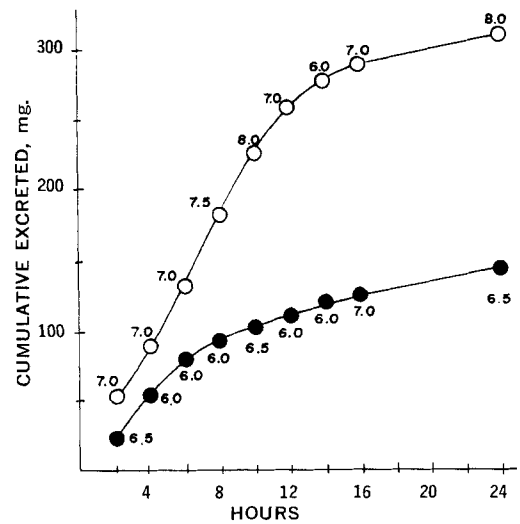

Figure 13—*Cumulative urinary salicylate excretion from Subject A. H. after the administration of 534 mg. of sodium salicylate orally and rectally in a polyoxyethylene (4) sorbitan monostearate with glyceryl monolaurate suppository. Values at each point are the urinary pH. Key: ○, capsule; and ●, suppository.*

characteristics, one would anticipate a poor release of the drug. *In vivo*, the suppository remained intact for 30 min. and was difficult to retain. As shown in Fig. 7, the release of aspirin from a suppository of sorbitan monopalmitate was very poor at both the 4- and 24-hr. intervals.

As demonstrated in Table I, the *in vitro* testing of suppositories should be done at body temperature. Obviously, temperature is most important with bases such as theobroma oil and polyoxyethylene (4) sorbitan monostearate, which melt at body temperature. This is strikingly demonstrated with sodium salicylate in theobroma oil from which only 7% was released in 1 hr. at 25° but from which 98% was released in 15 min. at 37°. As suggested by this *in vitro* release, the *in vivo* release was rapid because at 4 hr. the f_{rectal}/f_{oral} for sodium salicylate in theobroma oil was 0.79 as shown in Fig. 9.

For suppository bases containing theobroma oil, the f_{rectal}/f_{oral} for aspirin was approximately 0.8 at 24 hr.; however, at a 4-hr. interval the ratio was greatly reduced, as seen in Table III. Thus, to obtain the desired effect within 4 hr., the rectal dose of aspirin in theobroma oil should be 3–4 times as great as the oral dose. The addition of glyceryl monostearate and cholesterol to theobroma oil suppositories did not appear to have any significant effect, as shown in Figs. 5, 6, 11, and 12; however, the results were inconclusive.

For sodium salicylate in suppository bases of polyethylene glycol, sorbitan monopalmitate, and theobroma oil at 24 hr., the f_{rectal}/f_{oral} was approximately 0.9. At 4 hr., a larger f_{rectal}/f_{oral} value was obtained with sodium salicylate in theobroma oil than in polyethylene glycol and sorbitan monopalmitate suppositories. Since the diffusion of the drug is slow in the solid sorbitan monopalmitate suppository and time is required for the rectal fluid to dissolve the polyethylene glycol suppository, the effect is due to the rapid melting and increased contact between the melted theobroma oil and the aqueous rectal fluid, which rapidly partitions the water-soluble sodium salicylate from the oleaginous layer.

Effect of Drug—In addition to selecting a suitable suppository base for the administration of a particular drug, occasionally the pharmacist may be in a position to select the form of the drug best suited for rapid absorption. As a general practice, rapid and more complete rectal absorption is obtained by the choice of a water-soluble form of the parent drug (13).

Since rapid release is generally desired with systemic treatment, the comparison of f_{rectal}/f_{oral} at 4 hr. is more meaningful than the 24-hr. interval. According to the data in Tables III and IV, the f_{rectal}/f_{oral} at 4 hr. for sodium salicylate and aspirin in theobroma oil was 0.89 and 0.38, respectively. This absorption is presumably a consequence of the partition coefficient, which favors the partitioning of the water-soluble sodium salicylate from the liquid oleaginous phase but does not favor the release of the water-insoluble aspirin. Thus, if rapid release from an oleaginous suppository, which melts at body temperature, is desired, the water-soluble form of the drug should be used (13).

Similarly, at 4 hr. the f_{rectal}/f_{oral} for sodium salicylate and aspirin in sorbitan monopalmitate was 0.5 and 0.12, respectively. Although sorbitan monopalmitate does not melt and the diffusion of drugs would be anticipated to be slower than in the liquefied theobroma oil, the more water-soluble sodium salicylate was partitioned from the sorbitan monopalmitate more readily than the less water-soluble aspirin.

The absorption of aspirin and sodium salicylate was approximately the same from the polyethylene glycol suppository. Apparently, the limiting step in the absorption of both drugs was the dissolution of the polyethylene glycol suppository in the rectal fluid. Since the rate of dissolution of the polyethylene glycol suppository is the same, the release of aspirin and sodium salicylate was the same, as shown in Figs. 1 and 8.

In the suppository composed of polyoxyethylene (4) sorbitan monostearate and glyceryl monolaurate, absorption of aspirin was greater than sodium salicylate, as shown in Figs. 3 and 13. This is comparable to the report of Lowenthal and Borzelleca (14) in which salicylic acid was absorbed better than sodium salicylate from polyoxyethylene (4) sorbitan monostearate suppositories administered to dogs. The surface activity of the polyoxyethylene (4) sorbitan monostearate may peptize the mucus and make additional absorption surface available for the unionized aspirin (15).

SUMMARY

1. The rectal dose of aspirin and sodium salicylate is equivalent to the oral dose.

2. Rectal absorption may be altered by pharmaceutical factors which affect the release of the drug from the suppository base.

3. Polyethylene glycol and polyoxyethylene (4) sorbitan monostearate with glyceryl monolaurate are good suppository bases for the rapid release of aspirin.

4. Theobroma oil is a good suppository base for the rapid release of sodium salicylate.

5. In general, the water-soluble sodium salicylate is more readily released from a suppository base than the water-insoluble aspirin.

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Opium Alkaloids IX: Detection of Coreximine in *Papaver somniferum* L. Based on Its Biosynthesis from Reticuline

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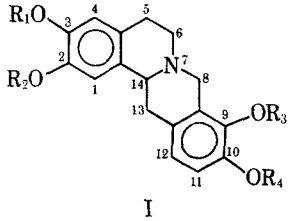
Abstract □ Protoberberines are biosynthesized in plants from reticuline in such a way that the *N*-methyl group of reticuline becomes the methylene group in the 8-position. It was demonstrated in this study that coreximine, a tetrahydro-ψ-berberine, is also derived from reticuline. (±)-Reticuline-(3-^{14}C) administered to intact opium poppies was incorporated into coreximine to an extent of 0.174%, and controlled degradation showed that the radioactivity was located at the C-6 position. Consequently, it could be concluded that the opium poppy is capable of converting reticuline to coreximine, and that coreximine, like scoulerine and isocorypalmine, is a normal member of the opium alkaloids. In the same way, it was shown that canadine, tetrahydropalmatine, stylopine, and berberine were not present in the plants in detectable amounts.

Keyphrases □ *Papaver somniferum*—coreximine detection □ Coreximine production from (±)-reticuline-(3-^{14}C)—*P. somniferum* □ IR spectrophotometry—identification □ NMR spectroscopy—identification □ GLC—identification □ Scintillometry—analysis

In 1962, Pfeifer and Teige (1) isolated a new opium alkaloid which they named somniferine (or base X), later shown to be identical with isocorypalmine (2) (I: $R_1 = R_3 = R_4 = CH_3$, $R_2 = H$). Scoulerine (I: $R_1 = R_4 = CH_3$, $R_2 = R_3 = H$) was isolated from opium in 1965 (3) and has proved to be an important intermediate on the biosynthetic pathways leading to protopines, phthalide-isoquinolines, and benzophenanthridines (4, 5). In a patented process for chromatographic separation, Ose *et al.* (6) described the isolation of appreciable quantities of berberine (II: $R_1 + R_2 = CH_2$, $R_3 = R_4 = CH_3$) from opium poppies. This was disputed by Hakim *et al.* (7) who obtained negative tests for berberine in several varieties of opium poppy with paper chromatography and paper electrophoresis.

The protoberberines occur in a number of plant families. The most common substitution pattern is 2,3,9,10. More rarely are additional substituents found



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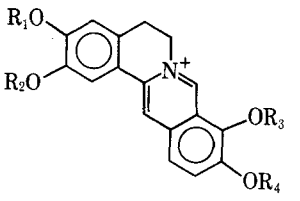
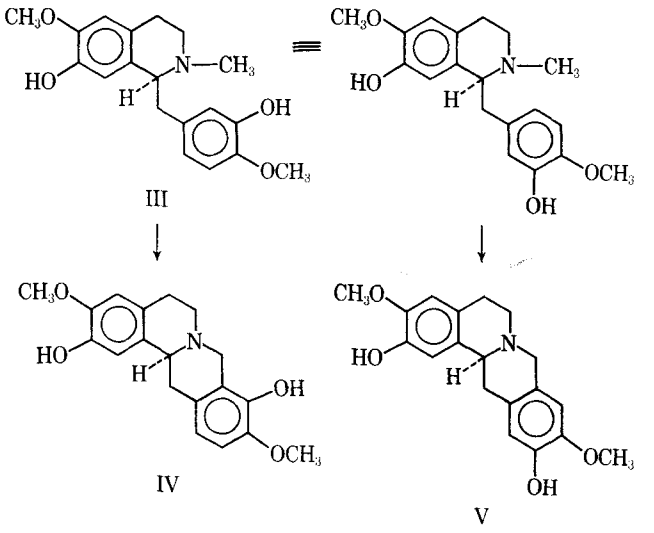


II

in positions 1, 5, or 13. Alkaloids having the same carbon skeleton but substituted in positions 2, 3, 10, and 11 are often referred to as tetrahydro-ψ-berberines (8).

The biosynthetic relationship of protoberberines to the benzylisoquinolines was recognized by Robinson (9) and demonstrated experimentally by Barton *et al.* (10), Battersby *et al.* (11), and Spenser and Gear (12). Based on feeding experiments performed with a great number of precursors and with different plants, it may be concluded that 2,3,9,10-substituted protoberberines (IV) are biosynthesized from (+)-reticuline (III) as illustrated in Scheme I.

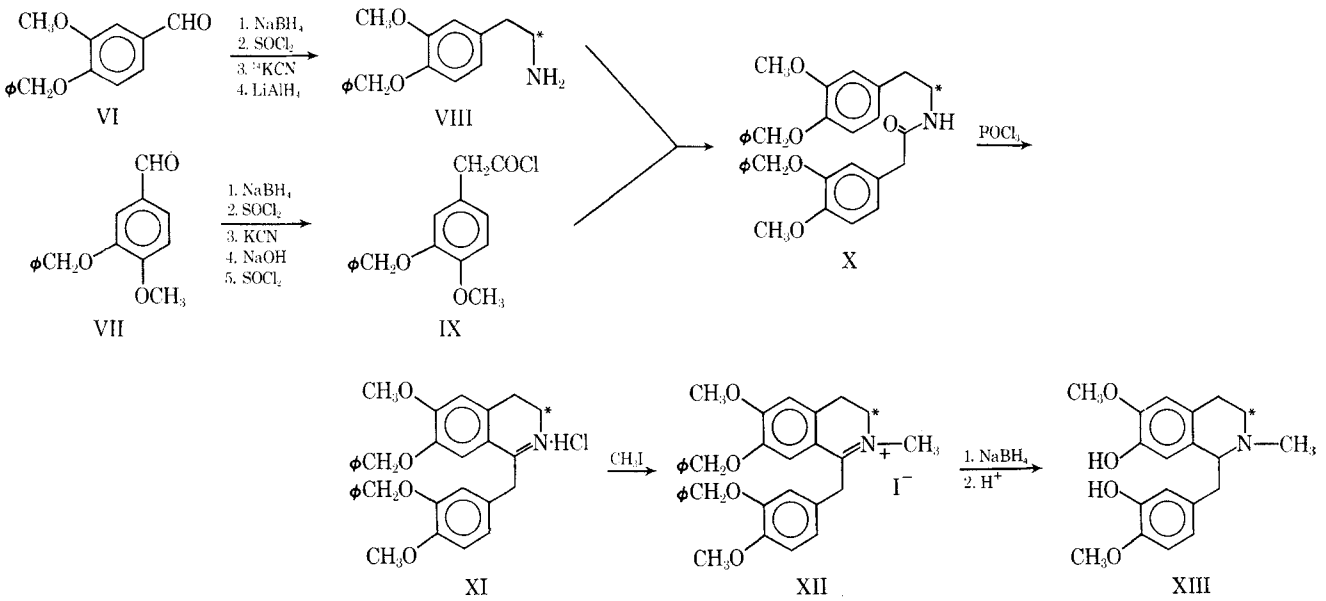


Scheme I—Proposed biosynthesis of protoberberines

The carbon atom in position 8, "berberine bridge," is derived from the *N*-methyl group of reticuline. The protoberberine (I and IV) is then oxidized spontaneously or enzymatically to the corresponding dehydro form (II). Several possible mechanisms have been proposed for the formation of the "berberine bridge" (10, 11).

From a mechanistic point of view, two products may be formed during the cyclization of reticuline. Coupling at the *ortho*-position to the hydroxyl group gives a tetrahydroberberine (IV), whereas coupling at the *para*-position gives a tetrahydro-ψ-berberine (V). In the living plant, this cyclization is probably under enzymatic control, and the product or products would depend on the presence of the required enzymes and on their specificity. Natural coreximine (V) is levorotatory and has the same absolute configuration as (−)-scoulerine (IV) and (+)-reticuline (III) (13, 14). It was, therefore, of interest to study the possibility that coreximine might be produced in the opium poppy since the potential precursor, (+)-reticuline, is known to be present (15–17). It was also decided to determine the presence or absence of related alkaloids, particularly in view of the conflicting observations regarding berberine.

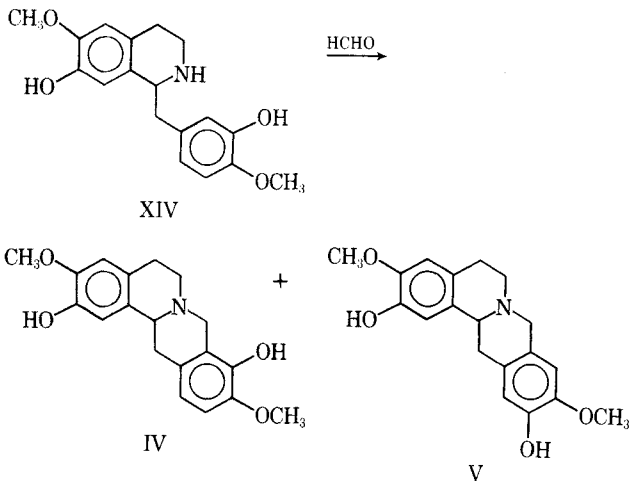
If these alkaloids exist in the opium poppy, the quantities would be too small to permit their actual isolation and characterization from a limited amount of plant material. The determination was, therefore, based on the hypothesis that they are biosynthesized in the plant by way of reticuline. Radioactively labeled (±)-reticuline was administered to opium poppies. Two weeks later the



Scheme II—Synthesis of (±)-reticuline-(3-^{14}C)

plants were harvested and extracted; the alkaloids, whose presence was to be determined, were added as "cold" carriers during the extraction (reverse isotope dilution). The alkaloids were then isolated and purified. Incorporation of the radioactive label in a position consistent with the biosynthetic pathways strongly suggested that the alkaloid is normally present in the plant. (±)-Reticuline-(3-^{14}C) and (±)-reticuline-(*N*-$^{14}CH_3$) were used as precursors. (±)-Reticuline-(3-^{14}C) was synthesized by the sequence of reactions illustrated in Scheme II (18).

(±)-Reticuline-(*N*-$^{14}CH_3$) was synthesized as described by Barton *et al.* (19). (±)-Coreximine for carrier dilution was prepared by condensation of (±)-norreticuline with formaldehyde (20) (Scheme III). This



Scheme III—Synthesis of (±)-scoulerine (IV) and (±)-coreximine (V)

gave a mixture of (±)-scoulerine and (±)-coreximine, which was readily separated by column chromatography on neutral aluminum oxide. (±)-Canadine was prepared by reduction of berberine sulfate with sodium borohydride (21). (±)-Stylopine was obtained by sodium borohydride reduction of coptisine which was isolated from coptis root.

RESULTS AND DISCUSSION

The conditions of the radioactive feedings and the results are given in Table I. The incorporation of radioactivity was calculated on the basis of the amount of "cold" carrier added.

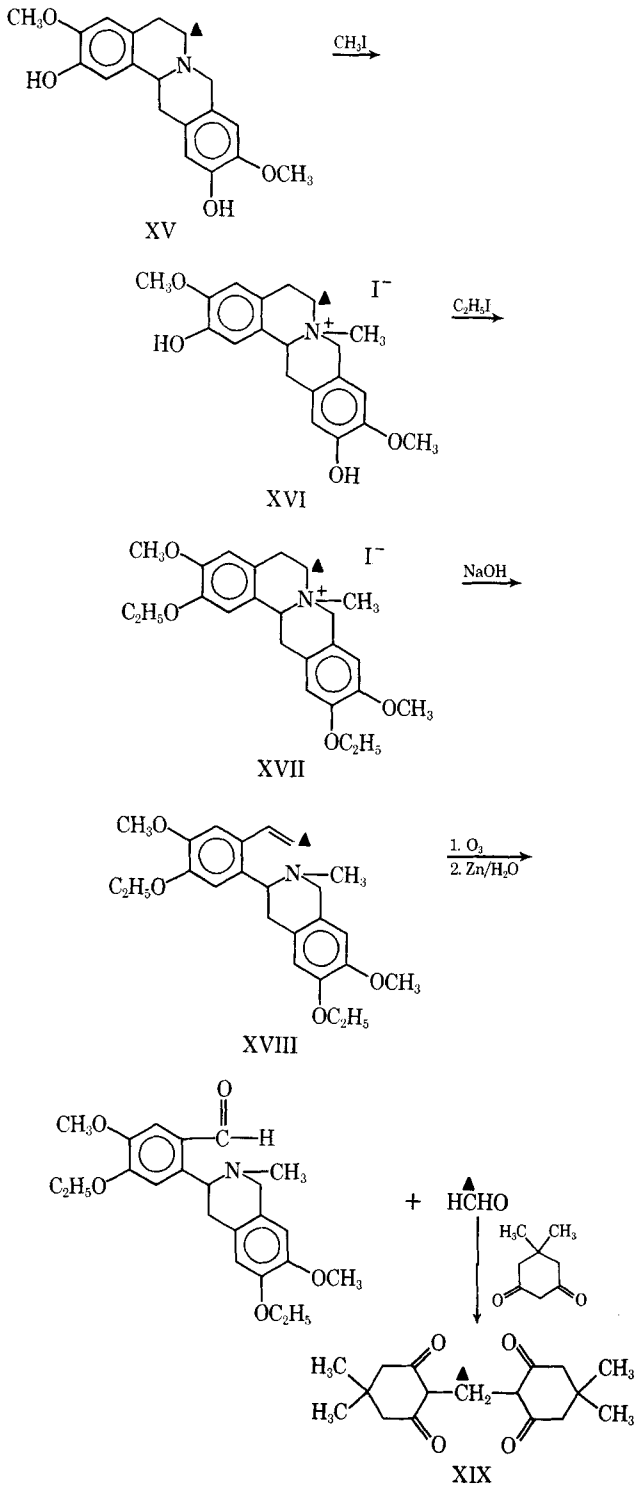
In view of the facts that (+)-reticuline is the proper precursor of the alkaloids studied, and that this substance is utilized by the plant for biosynthesis of a number of alkaloids, at least one of which occurs in large amounts (narcotine), an incorporation of 0.174% into coreximine is quite significant. It would not serve any useful purpose in this case to administer optically active reticuline, since this alkaloid is known to undergo rapid racemization in the plant (22). Radioactive coreximine was degraded to isolate the carbon atom in the 6-position, as illustrated in Scheme IV (23). The results showed that at least 90% of the radioactivity resided in this position. The slightly low specific activity of the dimedone derivative is a result of dilution by nonradioactive formaldehyde arising from the *N*-methyl group during the degradation process. A similar case was reported by Battersby *et al.* (24). Based on these results, one may conclude that the alkaloid coreximine is present in the opium poppy and that its biosynthesis proceeds along the same pathway as that reported for the protoberberines.

The incorporations of reticuline into canadine, tetrahydropalmatine, berberine, and stylopine were so small as to be negligible. Therefore, if these alkaloids were actually present in the varieties of opium poppies studied, the concentrations were too low to be detected. It is well known, however, that the alkaloid composition of *Papaver somniferum* varies greatly from one variety to another and that certain alkaloids may be virtually absent from some varieties while present in others.

EXPERIMENTAL

Equipment, Reagents, and Techniques—All melting points were determined with the Thomas-Hoover capillary melting-point apparatus and were uncorrected. The IR spectra were taken in potassium bromide with a Perkin-Elmer IR spectrometer, model 137. The NMR spectra were determined in deuterochloroform with the Varian Associates high-resolution spectrometer A 60-A, using tetramethylsilane as the internal standard. GLC analyses were carried out with a F&M biomedical gas chromatograph, model 400. Ozonolysis was performed on a Towers ozone apparatus, GE 150.

The radioactivity was determined by liquid scintillation counting with a Packard (model 3003) TriCarb liquid scintillation spectrometer. Two types of scintillation fluid were used: nonpolar (toluene type) and polar (dioxane type), depending on the solubility of the



Scheme IV—Controlled degradation of (±)-coreximine

substance. The sample, 0.5–2 mg., was weighed accurately on a Cahn gram electrobalance and dissolved in 0.1 ml. of methanol. Ten milliliters of scintillation fluid was added. The counting efficiency was calibrated with an internal standard of toluene-^{14}C. The efficiencies ranged from 80 to 84% for the nonpolar type and from 77 to 79% for the polar fluid. Berberine sulfate has an intense, yellow color and fluorescence, making a direct determination of its radioactivity difficult by scintillation counting. It was, therefore, reduced to (±)-canadine. About 0.1 mg. of sodium borohydride was added to the solution of berberine sulfate in methanol before addition of the scintillation fluid.

Cultivation of Plants and Administration of Labeled Precursors—The plants, *P. somniferum* L., Noordster and Indra varieties, were grown in flowerpots in a greenhouse, and the radioactive precursors were administered at the end of the flowering season as soon as the petals dropped. An aqueous solution of reticuline sulfate, containing the equivalent of about 3 mg./ml. of reticuline base, was injected into the top of the seed capsule as described by Battersby *et al.* (25). Each capsule received 0.3 ml. of the solution. The plants were allowed to grow normally for 1–2 weeks before they were harvested and stored in a freezer until they were required for extraction.

Four batches of plants were used. The first batch, consisting of 18 plants (Noordster variety), was fed a total of 19.9 mg. (80.31 μc.) of (±)-reticuline-(3-^{14}C); (±)-coreximine (210 mg.) and (±)-canadine (200 mg.) were used as "cold carriers." The second batch, 14 plants (Indra variety), was fed 36.6 mg. (144.2 μc.) of (±)-reticuline-(*N*-$^{14}CH_3$); tetrahydropalmatine (250 mg.) was used as carrier. The third batch, 17 plants (Indra variety), was fed 34.1 mg. (134.3 μc.) of (±)-reticuline-(*N*-$^{14}CH_3$), while berberine sulfate (250 mg.) was used for reverse isotope dilution. The fourth batch, consisting of 17 plants (Indra variety), was fed a total of 34.5 mg. (135.9 μc.) of (±)-reticuline-(*N*-$^{14}CH_3$); (±)-stylopine (250 mg.) was used as carrier.

Synthesis of Labeled Precursors—Several practice runs were always made with nonradioactive materials prior to the synthesis of the labeled compounds. The intermediates were characterized by TLC, NMR, IR spectroscopy, and melting points. The final products were compared with natural reticuline isolated from opium.

(±)-*Reticuline-(3-^{14}C)*—The synthesis did not differ in essential detail from that reported by Battersby *et al.* (18). The starting materials were isovanillin and vanillin. Benzylation with benzyl chloride under mildly basic condition yielded the corresponding *O*-benzyl ethers (VI and VII). The aldehyde groups of the *O*-benzyl ethers were reduced with sodium borohydride to the alcohols which, in turn, were converted to the chlorides with thionyl chloride. The 3-methoxy-4-benzyloxybenzyl chloride was reacted with potassium [^{14}C]-cyanide to give 3-methoxy-4-benzyloxyphenyl-[1-^{14}C]-acetonitrile. The nitrile was reduced with lithium aluminum hydride to the 3-methoxy-4-benzyloxyphen[1-^{14}C]ethylamine (VIII). The 3-benzyloxy-4-methoxybenzyl chloride was reacted with potassium cyanide to give 3-benzyloxy-4-methoxyphenyl acetonitrile. Hydrolysis with potassium hydroxide produced 3-benzyloxy-4-methoxyphenylacetic acid, which was converted to the acid chloride (IX) with thionyl chloride. Reaction of 3-methoxy-4-benzyloxyphen-[1-^{14}C]ethylamine (VIII) with 3-benzyloxy-4-methoxyphenylacetyl chloride (IX) under Schotten-Baumann conditions yielded *N*-(3-methoxy-4-benzyloxyphen[1-^{14}C]ethyl-3-benzyloxy-4-methoxyphenylacetamide (X).

1. 1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydro[3-^{14}C]isoquinoline hydrochloride (XI)—*N*-(3-Methoxy-4-benzyloxyphen[1-^{14}C]ethyl-3-benzyloxy-4-methoxyphenylacetamide (68 mg.) was dissolved in 5 ml. of dry toluene, and 0.5 ml. of freshly distilled phosphorus oxychloride was added. The solution was heated in an oil bath at 95–105° in a current of nitrogen, and the solution was concentrated in a rotary vacuum evaporator. The resulting yellow residue was dissolved in 2 ml. of 95% ethanol, and hydrogen chloride gas was bubbled through the solution for 2 min. The reaction mixture, after cooling, gave 52 mg. of yellow prisms, m.p. 202–203° [lit. (18) 201–203°].

2. 1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydro[3-^{14}C]isoquinoline methiodide (XII)—The dihydroisoquinoline hydrochloride (XI) (52 mg.), obtained in the preceding reaction, was suspended in 1.5 ml. of anhydrous methanol. A solution of 2 mg. of metallic sodium in 0.5 ml. of anhydrous methanol and 0.5 ml. of iodomethane were added, and the mixture was refluxed for 3 hr. under a stream of nitrogen. After 12 hr. in a refrigerator, the crystals were collected, giving 55 mg. of the methiodide, m.p. 195–196° [lit. (19) 197–198°].

3. (±)-Reticuline-(3-^{14}C) (XIII)—The crystals of 1-(3-benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydro[3-^{14}C]isoquinoline methiodide (XII) (55 mg.) were suspended in 2 ml. of methanol. The suspension was cooled in an ice bath, stirred with a magnetic stirrer, and 100 mg. of sodium borohydride was added in small portions. The reaction mixture was stirred for 14 hr. at room temperature. The solution was then evaporated to dryness, and 20 ml. of water and 1 ml. of 2 *N* sodium hydroxide were added. The mixture was extracted with chloroform (3 × 70 ml.) and the combined chloroform extracts were washed with water and evaporated to dryness to give *O,O*-dibenzylreticuline-(3-^{14}C), which was debenzylated without further purification.

Table I—Radioactive Feeding Experiments with *Papaver somniferum* L.

Precursor	Microcuries	Number of Plants	Year	Variety	Carrier Alkaloid	Incorporation, %	D g-radation, Activity in Isolated Fragment, %
(±)-Reticuline-(3-^{14}C)	80.31	18	1968	Noordster	(±)-Coreximine	0.174	90
(±)-Reticuline-(3-^{14}C)	80.31	18	1968	Noordster	(±)-Canadine	0.0011	—
(±)-Reticuline-(*N*-$^{14}CH_3$)	144.2	14	1969	Indra	(+)-Tetrahydropalmatine	0.00085	—
(±)-Reticuline-(*N*-$^{14}CH_3$)	134.3	17	1969	Indra	Berberine sulfate	0.00059	—
(±)-Reticuline-(*N*-$^{14}CH_3$)	135.9	17	1969	Indra	(±)-Stylopine	0.00058	—

The residue of *O,O*-dibenzylreticuline-(3-^{14}C) was dissolved in a mixture of 0.8 ml. of ethanol and 0.8 ml. of concentrated hydrochloric acid. The solution was heated in an oil bath at 130° for 1 hr., cooled, and evaporated to dryness. The residue was dissolved in 0.5 ml. of ethanol, and 10 ml. of ether was added to cause precipitation. The precipitate was collected, dissolved in a minimum amount of water, and washed twice with ether. The aqueous solution was basified with ammonium hydroxide to pH 8–9 and extracted with chloroform (3 × 10 ml.). The combined chloroform extracts were evaporated to dryness to give a residue of 30 mg. It was dissolved in ether, and a minimum amount of petroleum ether (b.p. 30–60°) was added to cause precipitation. This was repeated several times. TLC of the precipitate on silica gel with chloroform–methanol (9:1) and with ethanol–benzene (2:8) showed a single compound identical with natural reticuline isolated from opium. GLC analyses of the free base and the trimethylsilyl derivative, on columns containing *OV-1* and *OV-225* as stationary phases, gave only one peak having the same retention times as natural reticuline. The IR and NMR spectra of (±)-reticuline from the inactive runs were identical with those obtained with authentic reticuline. The (±)-reticuline-(3-^{14}C) had a specific radioactivity of 4.036 μc./mg. or 1.32 mc./mmole.

Synthesis of (±)-*Reticuline*-(N-$^{14}CH_3$)—This synthesis was essentially the same as described by Barton *et al.* (19). 3-Methoxy-4-benzyloxyphenethylamine was obtained by reaction of *O*-benzylvanillin with nitromethane, followed by reduction with lithium aluminum hydride.

1. 1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydroisoquinoline methiodide-(*N*-$^{14}CH_3$)—1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydroisoquinoline hydrochloride (490 mg.) was suspended in 25 ml. of ethyl acetate, and 25 ml. of a saturated solution of sodium bicarbonate was added. Nitrogen was bubbled through the solution until the solid was dissolved. The aqueous layer was extracted with ethyl acetate, and the combined ethyl acetate extracts were washed with water and evaporated to dryness. The residue was dissolved in 6 ml. of dry benzene and transferred to a reaction vessel 1 cm. in diameter and 10 cm. long. It was connected to a vacuum manifold system through which [^{14}C]-iodomethane (2 mc./141 mg.) was distilled into the benzene solution. The reaction vessel was sealed off and left at room temperature with occasional shaking. Yellow crystals appeared. After 4 days, the vessel was frozen in liquid nitrogen, and the seal was broken in a dry chamber. An excess of nonradioactive iodomethane was added, and the vessel was sealed again. After 2 days, the crystals were collected, m.p. 195–196° [lit. (19) 197–198°].

2. (±)-Reticuline-(*N*-$^{14}CH_3$)—The foregoing (*N*-methyl-^{14}C)-methiodide was reduced and debenzylated, and (±)-reticuline-(*N*-$^{14}CH_3$) was purified as described for the preparation of (±)-reticuline-(3-^{14}C); yield, 234 mg. of pure (±)-reticuline-(*N*-$^{14}CH_3$). It had a specific activity of 3.94 μc./mg. or 1.29 mc./mmole.

Alkaloids for Carrier Dilution—Tetrahydropalmatine and berberine sulfate were purchased[1].

Synthesis of Tetrahydroberberine[(±)-*canadine*] (*21*)—Berberine sulfate (500 mg.) was dissolved in 10 ml. of methanol, 500 mg. of sodium borohydride was added gradually, and the mixture was refluxed for 10 min. After addition of 50 ml. of water, the solution was extracted with chloroform (3 × 30 ml.), and the combined extracts were evaporated to dryness to give a residue of 400 mg. It was crystallized from 95% ethanol, m.p. 175–176° [lit. (21) 177–178°].

Synthesis of (±)-*Coreximine* (*20*)—

1. 1,2,3,4-Tetrahydro-7-hydroxy-1-(3-hydroxy-4-methoxybenzyl)-6-methoxyisoquinoline hydrochloride [(±)-norreticuline hydrochloride] (XIV)—1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydroisoquinoline hydrochloride (XI) (1.1 g.), prepared as described previously, was dissolved in 70 ml. of ethanol. The solution was shaken with hydrogen and 500 mg. of palladium (10%) on charcoal at room temperature and 1 atm. pressure. The uptake of hydrogen was complete in 3 hr. Removal of the catalyst and evaporation of the solvent left a residue which solidified on addition of water to give (±)-norreticuline hydrochloride monohydrate, 0.715 g., m.p. 165–166° [lit. (18) 165°]. The IR spectrum showed bands at 3250, 1500, 1450, 1300, 1270, 1240, 1120, 1030, and 860 cm.$^{-1}$.

2. 3,10-Dimethoxy-5,6,13,14-tetrahydro-8*H*-dibenzo(*a,g*)quinolizine-2,11-diol[(±)-coreximine] (20)—(±)-Norreticuline hydrochloride monohydrate (640 mg.) was dissolved in 4 ml. of ethanol. The solution was basified with concentrated ammonium hydroxide solution, and 100 ml. of water was added. It was then extracted with 3 × 40 ml. of chloroform. The combined chloroform extracts were evaporated to dryness to give a residue of 501 mg. The residue was dissolved in 24 ml. of methanol, and 5.5 ml. of 37% formaldehyde solution was added. The solution was refluxed for 1 hr. and the solvent evaporated, leaving a residue which consisted of a mixture of (±)-scoulerine and (±)-coreximine, as evidenced by TLC on aluminum oxide with chloroform–methanol (99:1). Authentic alkaloids were used for comparison of R_f values.

The residue was dissolved in a minimum amount of chloroform, transferred to a column of neutral aluminum oxide[2], and eluted with chloroform. The fractions containing coreximine were combined and evaporated to dryness. The residue (300 mg.) was crystallized from methanol, m.p. 237–240° [lit. (20) 238–239°].

(±)-*Stylopine*—Seventy grams of coptis root (botanical origin unknown) from Taiwan was ground, 100 ml. of 10% sulfuric acid was added, and the mixture was heated on a steam bath with frequent swirling for 20 min. After cooling to room temperature, the solution was made strongly alkaline with 80 ml. of 50% (w/v) sodium hydroxide solution and extracted with chloroform (4 × 200 ml.). The combined chloroform extracts were evaporated to dryness; the residue was dissolved in 50 ml. of methanol and reduced with sodium borohydride. After addition of 50 ml. of water, the solution was extracted with chloroform (3 × 100 ml.). Evaporation of the solvent gave a gum which was purified by column chromatography using neutral aluminum oxide and benzene. The fractions containing stylopine were combined and evaporated to dryness. The residue was crystallized from a mixture of chloroform and methanol to give 300 mg. of stylopine, m.p. 214–215° [lit. (26) 217°]. The mass spectrum[3] showed a fragmentation pattern characteristic of a protoberberine (27), with a molecular ion at *m/e* 323 and major fragments at *m/e* 148 and 174. Accurate mass determination of the molecular ion gave a mass of 323.114551 (calculated for $C_{19}H_{17}NO_4$, 323.115749).

Extraction, Separation, and Purification of Alkaloids—The plants were extracted by maceration with methanol in a high-speed

[1] Pierce Chemical Co.

[2] Woelm, activity IV.

[3] Associated Electronics Industries, model MS 902 high-resolution mass spectrometer.

blender[4], and the alkaloid carriers were added. The suspension was poured into a glass percolator and percolated with methanol until the extract gave negative tests for alkaloids. The extract was concentrated to a final volume of 1 l. in a rotary vacuum evaporator at 38° and shaken with about 150 ml. of ethyl acetate. The ethyl acetate layer was washed with 3 × 50 ml. of 0.5 *N* hydrochloric acid, and the washings were combined with the original aqueous solution (total alkaloids).

Canadine and Coreximine—The aqueous, acidic solution of total alkaloids from the plants to which (±)-canadine and (±)-coreximine had been added as cold carriers was extracted with several portions of chloroform. Evaporation of the chloroform gave a residue of weakly basic alkaloids. It was dissolved in chloroform (6 ml.) and transferred to a column of silica gel[5]; the alkaloids were eluted with a mixture of benzene and methanol (98:2). The elution of the alkaloids was monitored by micro-TLC. The fractions containing (±)-canadine were combined, evaporated to dryness, and rechromatographed on neutral alumina (50 g.) with benzene–chloroform (1:1). (±)-Canadine obtained in this way was crystallized from ethanol to constant radioactivity (10 d.p.m./mg.).

The aqueous solution remaining after extraction of the weakly basic alkaloids was basified to pH 8–9 with sodium bicarbonate and extracted with chloroform. The combined chloroform extracts were evaporated to dryness, and the residue was subjected to preparative TLC on silica gel with chloroform–methanol (9:1). The band corresponding to coreximine was extracted with methanol, and the extract was purified by chromatography on a column of neutral alumina (30 g.). Elution with chloroform gave coreximine, which was crystallized seven times from methanol to constant radioactivity (1480 d.p.m./mg.).

Tetrahydropalmatine—The weakly basic alkaloids, obtained as described from the batch of plants to which tetrahydropalmatine had been added as cold carrier, were chromatographed on a column of silica gel (60 g.). The elution was started with benzene, continued with a mixture of equal parts of ether and chloroform, and, finally, completed with chloroform–methanol (97:3). The fractions shown by micro-TLC to contain tetrahydropalmatine were combined and evaporated to dryness. The residue was crystallized three times to give constant radioactivity (11 d.p.m./mg.).

Stylopine—The aqueous, acidic solution of total alkaloid from the plants to which (±)-stylopine had been added as cold carrier was basified with ammonium hydroxide to pH 8–9 and extracted with chloroform (4 × 200 ml.). The chloroform extracts were evaporated to dryness, and the residue was chromatographed on a column of silica gel (70 g.) with chloroform. The fractions containing (±)-stylopine were combined, evaporated to dryness, and rechromatographed on neutral alumina (40 g.) with benzene. (±)-Stylopine obtained in this way was crystallized from a mixture of chloroform and methanol to constant radioactivity (7 d.p.m./mg.).

Berberine—The aqueous, acidic solution of total alkaloids obtained from the plants to which berberine had been added as cold carrier was extracted as described previously to remove the weakly basic alkaloids. The aqueous layer was then basified with ammonia and extracted several times with a mixture of chloroform–isopropyl alcohol (3:1). The extracts were combined and evaporated to dryness, and the residue was dissolved in 0.5 *N* hydrochloric acid. The solution was made strongly basic with sodium hydroxide and extracted repeatedly with chloroform. The combined chloroform extracts were washed with water and evaporated to dryness, yielding a residue of nonphenolic alkaloids. TLC showed it to contain berberine. It was chromatographed on a column of silica gel (65 g.), first with chloroform and then with chloroform–methanol (98:2). The fractions containing berberine were combined and concentrated to about 100 ml. Two milliliters of 10% sulfuric acid was added, and the solution was concentrated to a small volume (about 5 ml.) when yellow crystals of berberine bisulfate started to form. After 12 hr. in a refrigerator, the crystals were collected and recrystallized twice from ethanol to constant radioactivity (7 d.p.m./mg.).

Degradation of Radioactive Coreximine—Several practice runs were made with inactive material prior to the degradation of radioactive coreximine.

1. 2,11-Dihydroxy-3,10-dimethoxy-7-methyl-5,6,13,14-tetrahydro-8*H*-dibenzo(*a*,*g*)quinolizinium iodide [(±)-phellodendrine iodide] (XVI)—Coreximine (55 mg.) was dissolved in 5 ml. of methanol, and 3 ml. of iodomethane was added. The mixture was refluxed in a water bath. After 2 hr., the solution was evaporated to dryness. Addition of 1 ml. of warm methanol gave yellow crystals of (±)-phellodendrine iodide, 56 mg., m.p. > 300° [lit. (23) > 300°].

2. 2,11-Diethoxy-3,10-dimethoxy-7-methyl-5,6,13,14-tetrahydro-8*H*-dibenzo(*a*,*g*)quinolizinium iodide [(±)-*O*,*O*-diethylphellodendrine iodide] (XVII)—The crystals (±)-phellodendrine iodide (XVI) were dissolved in 4 ml. of 1 *N* alcoholic potassium hydroxide solution, and 4 ml. of iodoethane was added. The solution was refluxed in a water bath for 2 hr. Another 2 ml. of 1 *N* alcoholic potassium hydroxide and 2 ml. of iodoethane were added, and the reflux was continued for 2 hr. The resulting solution was evaporated to dryness to give a yellowish oily residue of *O*,*O*-diethylphellodendrine iodide (58 mg.).

3. *O*,*O*-Diethylphellodendrine methine (XVIII)—The residue (58 mg.) of *O*,*O*-diethylphellodendrine iodide was dissolved in 3 ml. of ethanol. Four grams of potassium hydroxide in 10 ml. of water was added. The solution was refluxed in an oil bath at 110–120° for 3 hr. and extracted with chloroform (5 × 20 ml.). The combined chloroform extracts were concentrated to dryness to give a residue of *O*,*O*-diethylphellodendrine methine (34 mg.), m.p. 116–117° [lit. (23) 116–117°]. The IR spectrum of the methine was identical with that reported by Tomita and Kunitomo (23).

4. Ozonolysis—The residue of *O*,*O*-diethylphellodendrine methine (XVIII) was dissolved in 5 ml. of ethyl acetate and cooled in a dry ice–chloroform bath; ozonized oxygen was passed through the solution. Finally, oxygen was passed through the resulting blue solution to expel excess ozone. The solution became colorless and was evaporated to dryness to give an oily residue. Zinc dust (0.2 g.), 20 ml. of water, and 10 mg. of silver nitrate were added. The mixture was refluxed in an oil bath for 30 min. Half of the water was then distilled at atmospheric pressure into a solution of 0.15 g. dimedone in 10 ml. of water and 4 ml. of ethanol. Water was added to the distilling flask and the distillation continued. The formaldehyde-dimedone, which separated over a period of 15 hr., was collected to give 16 mg. of crystals, m.p. 190–192°. It was recrystallized three times from 50% aqueous ethanol to a constant radioactivity of 1423 d.p.m./mg.

[4] Waring Products Co.
[5] Woelm, activity II.

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Determination of Time Course of *In Vivo* Pharmacological Effects from *In Vitro* Drug-Release Testing

VICTOR F. SMOLEN

Abstract □ A mathematical approach is described which permits the computation of the temporal variation of pharmacological response intensity from the results of *in vitro* drug-release testing that linearly correlate or can otherwise be functionally related to *in vivo* drug availability. The development of optimized drug-release tests is suggested. Through the application of the described approach, such tests would permit the predictive estimation from *in vitro* data of the dependency of the pharmacological behavior of appropriate drugs on formulation factors. The dependency of the time course of pharmacological effects of a mydriatic drug on the drug-release characteristics of several types of dosage formulations is graphically shown.

Keyphrases □ Pharmacological response intensity correlation—*in vivo* drug availability □ Bioavailability time course determination—pharmacological data □ Drug-release tests, *in vitro*—optimization □ Absorption analysis, drug—theoretical considerations

It is well known that formulation factors can markedly influence the therapeutic activity and toxicity of pharmaceutical products. The advent of ever increasingly more effective and potent drugs emphasizes the imperative of concomitantly developing pharmaceutical dosage forms for optimal effectiveness, safety, and reliability. It is seldom practical to perform the exhaustive *in vivo* human testing of drug formulations that may be necessary to obtain a formulation possessing optimal drug-release characteristics and, therefore, the pharmacological response behavior that may be precisely desired. It is presently proposed that human testing of drug formulations for this purpose could be minimized if, instead of directly testing the many formulations themselves, *in vivo* studies are performed to establish "optimized *in vitro* drug-release tests" capable of predicting the *in vivo* bioavailability behavior of the drug as a function of formulation factors. Presently employed *in vitro* drug-release tests are generally inadequate for this purpose.

Morrison and Campbell (1), in their 1965 review article, concluded that: "It is apparent that *in-vitro* disintegration tests, as presently used, have certain inherent faults, and eventually must be modified or replaced by more critical tests of physiological availability." Aside from discrediting disintegration tests and replacing them by dissolution tests, apparently only limited progress has been reported since this time with regard to the modification and further development of *in vitro* tests that can reflect *in vivo* drug behavior.

It is often stated that the problem is quite complicated because a correlation of *in vivo* to *in vitro* release that is found with a particular test for a particular drug in a particular dosage form may not exist if another drug is substituted or the formulation altered. Few attempts have been reported to determine the limits to which this may be the case. The *in vitro* to *in vivo* drug availability correlations that have been found have always been after the fact. Quantitative correlations for a spectrum of dosage forms were well exemplified by the work of Cressman *et al.* (2) for one drug. The correlations, however, were of a single-point nature, *e.g.*, time for 50% of the drug to be released from the dosage form was correlated with the 50% release time *in vivo*.

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Determination of Time Course of *In Vivo* Pharmacological Effects from *In Vitro* Drug-Release Testing

VICTOR F. SMOLEN

Abstract □ A mathematical approach is described which permits the computation of the temporal variation of pharmacological response intensity from the results of *in vitro* drug-release testing that linearly correlate or can otherwise be functionally related to *in vivo* drug availability. The development of optimized drug-release tests is suggested. Through the application of the described approach, such tests would permit the predictive estimation from *in vitro* data of the dependency of the pharmacological behavior of appropriate drugs on formulation factors. The dependency of the time course of pharmacological effects of a mydriatic drug on the drug-release characteristics of several types of dosage formulations is graphically shown.

Keyphrases □ Pharmacological response intensity correlation—*in vivo* drug availability □ Bioavailability time course determination—pharmacological data □ Drug-release tests, *in vitro*—optimization □ Absorption analysis, drug—theoretical considerations

It is well known that formulation factors can markedly influence the therapeutic activity and toxicity of pharmaceutical products. The advent of ever increasingly more effective and potent drugs emphasizes the imperative of concomitantly developing pharmaceutical dosage forms for optimal effectiveness, safety, and reliability. It is seldom practical to perform the exhaustive *in vivo* human testing of drug formulations that may be necessary to obtain a formulation possessing optimal drug-release characteristics and, therefore, the pharmacological response behavior that may be precisely desired. It is presently proposed that human testing of drug formulations for this purpose could be minimized if, instead of directly testing the many formulations themselves, *in vivo* studies are performed to establish "optimized *in vitro* drug-release tests" capable of predicting the *in vivo* bioavailability behavior of the drug as a function of formulation factors. Presently employed *in vitro* drug-release tests are generally inadequate for this purpose.

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as rate of agitation, geometry of the apparatus, composition of the release media, solubility volume of a sink, and permeability of membranous sources.

The author suggested (3) that the methods of operation research (3, 6) can be applied to optimize these conditions and, therefore, design an *in vitro* release test to function optimally in reflecting *in vivo* drug availability for a spectrum of drug formulations possessing varying drug-release characteristics. The experimentation could be designed so that the predicted *in vivo* behavior of the drug computed from *in vitro* data would be within predetermined statistical confidence intervals. Briefly, the author's suggested approach may be further understood from considering that if an *in vitro* drug-release test perfectly simulates *in vivo* drug availability from several different types of dosage forms, all points on a plot of quantities of drug absorbed *in vivo versus* quantities released at corresponding times *in vitro* would ideally fall upon a line having a slope of unity and an intercept equal to zero. The linear correlation coefficient would also be computed as unity. The deviations of the slope and intercept of least-squares regression lines and linear correlation coefficients from these ideal values may be utilized as criteria on which to gauge the adequacy of an *in vitro* drug-release test at any stage of its development. A systematic repetition of the test conducted under varying test conditions will provide the data necessary for the expression of these criteria as empirical functions of the test conditions. These functions may then be utilized to compute the set of test conditions that are optimal in minimizing the deviations and, therefore, in reflecting the *in vivo* drug-availability behavior of the dosage forms.

In anticipation of the implementation of this approach resulting in the development of *in vitro* drug-release tests possessing a predictive capability with regard to the influence of formulation factors on drug bioavailability, it is the purpose of this communication to describe a means by which such tests could also be utilized with appropriate drugs to predict quantitatively the time course of their pharmacological activity. When the method to be described is applicable, pharmacological response characteristics such as onset and duration of action, maximum intensity of effect, time of maximum effect, and rates of dissipation of the response intensity could be estimated from *in vitro* drug-release testing. Obviously, such results may be expected, within the limits of biological variation, to apply specifically only to the subject(s) donating the *in vivo* data which the *in vitro* release test has been designed to simulate optimally.

THEORETICAL

Pharmacological Method of Drug-Absorption Analysis—A mathematical approach was developed by the author which provides the necessary link between the quantity of drug cumulatively absorbed (A_t) *in vivo* from a site of administration at any time and the observed intensity of the pharmacological response at the corresponding time following dosing. The method permits the determination of the time course of drug bioavailability to be determined entirely from pharmacological data. The method is applicable to any route of administration and any type of dosage form. It has been successfully applied to drugs administered by parenteral, oral, and ophthalmic routes. The confirmation of the applicability of the method in its simplest form, as presented here, can be directly accomplished from pharmacological data alone. The details of this procedure are presented elsewhere (7, 8).

A reverse application of the pharmacological method of drug-absorption analysis permits the computation of the time course of variation of the pharmacological response intensity from the temporal variation of A_t. Therefore, if A_t values for a spectrum of dosage formulations are linearly correlated—in the manner reported by Gibaldi and Weintraub (5)—or functionally related—in the manner reported by Levy and Hollister (4)—to analogous values, R_t, obtained from the results of *in vitro* drug-release testing, the time course of pharmacological activity can be computed from *in vitro* data.

Assuming the biokinetics of the drug can be represented by a linear compartment model, the necessary mathematical relationships can be formulated as shown here. The quantity of drug in the biophase, Q_B, following a rapid intravenous dose, D, is given at any time by Eq. 1, where A_i and m_i are equation parameters and n denotes the number of body compartments. The biophase is defined as a hypothetical body compartment containing the sites of drug action:

$$Q_B = D \sum_{i=1}^{m} A_i e^{-m_i t} \qquad \text{(Eq. 1)}$$

An intravenous dose–effect curve may be constructed by plotting the intensity of pharmacological response, I, observed at an arbitrarily chosen time, t_r, as a function of dose. The functional relationship, $D = f(I_{t_r})$, between the dose and the intensity of response is graphically provided by the resulting curve. At a time t_r, Eq. 1 may be rewritten as Eq. 2, where β_{t_r} is constant:

$$Q_{Bt_r} = f(I_{t_r}) \sum_{i=1}^{m} A_i e^{-m_i t_r} = f(I_{t_r})\beta_{t_r} \qquad \text{(Eq. 2)}$$

Assuming Eq. 2 to be valid at times other than t_r, it can be rewritten as Eq. 3 to provide a general relationship between the observed intensity of response and the quantity of drug in the biophase:

$$Q_B = f(I)\beta_{t_r} \qquad \text{(Eq. 3)}$$

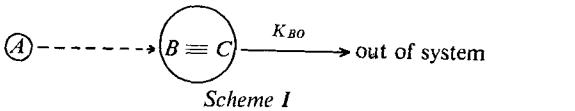
Equation 3 permits values of $f(I)$, which are directly proportional or equal to the quantity of drug in the biophase, to be obtained for any observed value of I directly from the corresponding value of the abscissa of the dose–effect curve irrespective of the time of observation, dose, and mode of administration. This is the case, provided, as generally assumed (9–11), that the pharmacological effect is an instantaneous, equilibrium, nonhysteretic result of the quantity of drug in the biophase at any time. Tests for the validity of this assumption as well as for the adequacy of a linear compartment model were described previously (7, 8).

Complete details of the pharmacological method of drug absorption analysis are presented in other reports. Briefly, however, the application of the pharmacological method routinely involves:

1. Experimental determination of the time course of pharmacological response intensity for several rapidly administered intravenous doses.
2. Construction of an intravenous dose–effect curve.
3. Transformation of observed I values, *via* the dose–effect curve, into $f(I)$ values normalized for dose.
4. Selection of a linear compartment model to simulate the biokinetic behavior of the system. The choice of the model, in addition to other criteria, is based upon the results of least-squares fits of sums of exponentials to the $f(I)/D$ *versus* time data.
5. Confirmation of the applicability of the method and the adequacy of the compartment model.

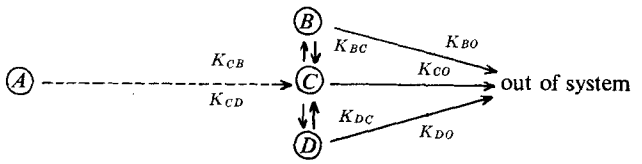
Upon the determination of a compartment model, suitable expressions for A_t as a function of $Q_B = \beta_{t_r} f(I)$ can be derived, which allow the cumulative amounts of drug systemically absorbed to be computed from the temporal variation of I observed following dosing by any route of administration. Similar results are also calculable for cases of, for example, percutaneous or transcorneal drug absorption directly into the biophase following topical application of the drug to the skin or eye, respectively, where it produces a local effect.

Equations 4 and 5 exemplify equations appropriate for the calculation of A_t for drugs whose biokinetic behavior can be described by single- and three-compartment models. The models are diagrammed in Schemes I and II, where C represents the central, plasma pool, compartment; B represents the biophase; D represents a pharmacologically inert depot compartment; the transfer constants are symbolized by the K_{ij}'s; and the site of administration is denoted by A. The calculation of A_t is independent of any assumptions concerning the nature of the kinetics and mechanisms of absorption. The single-compartment model treats compartments B and C as being kinetically indistinguishable and behaving as a single compartment, *i.e.*, $B \equiv C$.



Scheme I

$$A_t = \beta_{t_r}\left[f(I) + K_{BO}\int_0^t f(I)\,dt \right] \qquad \text{(Eq. 4)}$$



Scheme II

$$A_t = \beta_{t_r}\left[R\frac{d^2f(I)}{dt^2} + S\frac{df(I)}{dt} + Tf(I) + U\int_0^t f(I)\,dt \right] \qquad \text{(Eq. 5)}$$

where:

$$K_{CB}K_{DC}R = 1$$

$$\begin{aligned} K_{CB}K_{DC}S &= K_{BO} + K_{BC} + K_{DO} + K_{DC} + K_{CO} + K_{CB} + K_{CD} \\ &= m_1 + m_2 + m_3 \end{aligned}$$

$$\begin{aligned} K_{CB}K_{DC}T &= K_{DC}K_{CB} + K_{DC}K_{BC} + K_{DC}K_{BO} + K_{BC}K_{CO} \\ &\quad + K_{BC}K_{CD} + K_{BO}K_{CO} + K_{BO}K_{CB} + K_{BO}K_{CD} \\ &\quad + K_{DO}K_{BC} + K_{DO}K_{BO} + K_{DO}K_{CO} + K_{DO}K_{CB} \\ &\quad + K_{DO}K_{CD} + K_{CO}K_{DC} \\ &= m_1m_2 + m_2m_3 + m_1m_3 \end{aligned}$$

$$\begin{aligned} K_{CB}K_{DC}U &= K_{DC}K_{BC}K_{CO} + K_{DC}K_{BO}K_{CO} + K_{BC}K_{DO}K_{CO} \\ &\quad + K_{BO}K_{DO}K_{CO} + K_{BC}K_{DO}K_{DC} + K_{BO}K_{DO}K_{CO} \\ &\quad + K_DK_{BO}K_{CB} + K_{BO}K_{DO}K_{CB} \\ &= m_1m_2m_3 \end{aligned}$$

It is apparent that Eq. 4 is very similar to the expression made familiar by Wagner and Nelson (12). However, its applicability is extended to the utilization of pharmacological data in the calculation of A_t.

Values of A_t are obtained from $f(I)$ values through the numerical solution of Eqs. 3 and 4. For the purpose of relating *in vivo* pharmacological behavior to results of *in vitro* drug-release tests, it is necessary to solve such equations to yield $f(I)$, or Q_B, in terms of A_t. The solution of Eqs. 4 and 5 for Q_B can be accomplished readily through the use of Laplace transforms to yield Eqs. 6 and 7. Values of Q_B are directly proportional (Eq. 3) or equal (when t_r can be chosen as zero) to values of $f(I)$:

$$Q_B = A_t - K_{BO}e^{-K_{BO}t}\int_0^t A_te^{K_{BO}t}\,dt \qquad \text{(Eq. 6)}$$

$$\begin{aligned} Q_B = \frac{K_{CB}K_{DC}}{(m_1 - m_2)(m_2 - m_3)(m_3 - m_1)}\Bigg[& m_1(m_2 - m_3)e^{-m_1t} \times \int_0^t A_te^{m_1t}\,dt + m_2(m_3 - m_1)e^{-m_2t}\int_0^t A_te^{m_2t}\,dt + \\ & m_3(m_1 - m_2)e^{-m_3t}\int_0^t A_te^{m_3t}\,dt \Bigg] \end{aligned} \qquad \text{(Eq. 7)}$$

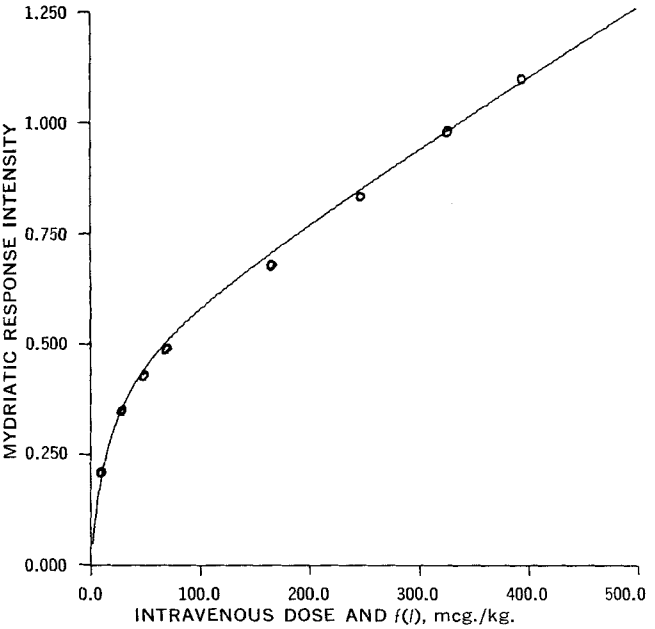


Figure 1—*Intravenous dose–effect curve for tropicamide illustrating the relationship of the mydriatic response intensity,* I, *to the dose and the quantity of drug,* $Q_B = f(I)$, *in the biophase.*

Assuming A_t can be directly or otherwise related to values of R_t, Eqs. 6 and 7 permit the calculation of $f(I)$ from the results of *in vitro* drug-release testing. The $f(I)$ values can be transformed graphically, *via* the dose–effect curve, or analytically to corresponding values of I. In this manner, the time course of pharmacological response intensity *in vivo* can be constructed from a succession of R_t values. The temporal variation of drug blood levels can be computed in a similar manner. For a single-compartment model, blood levels may be computed using Eq. 6 in its present form since the biophase and central compartments are identical and are equal to Q_B. For the three-compartment example, the level of drug in compartment C can be obtained from Eq. 8. The values of $f(I)$ for use in Eq. 8 are computed from A_t values using Eq. 7:

$$Q_C = \frac{\beta_{t_r}}{K_{CB}}\left[\frac{df(I)}{dt} + (K_{BO} + K_{BC})f(I) \right] \qquad \text{(Eq. 8)}$$

METHODS AND MATERIALS

The intravenous dose–effect curve, shown in Fig. 1, was determined using rabbits. Each point represents the average of a minimum of four replications on different animals. The method em-

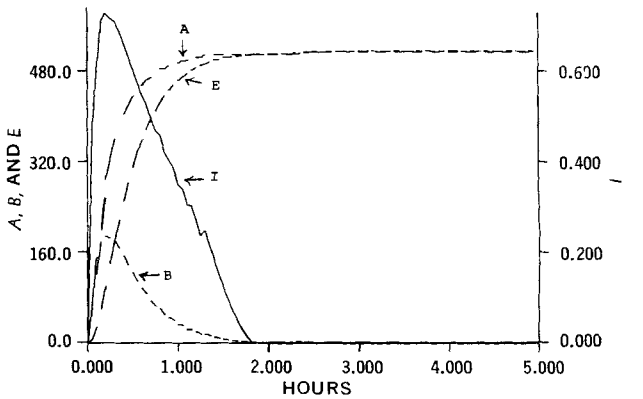


Figure 2—*Computerized plot of the time course of variation of the cumulative amount of drug excreted* (E), *the amount in the biophase* (B), *and the intensity of pharmacological response* (I), *computed from the cumulative amounts of drug absorbed* (A) *following dosing with a hypothetical fast-release dosage form.*

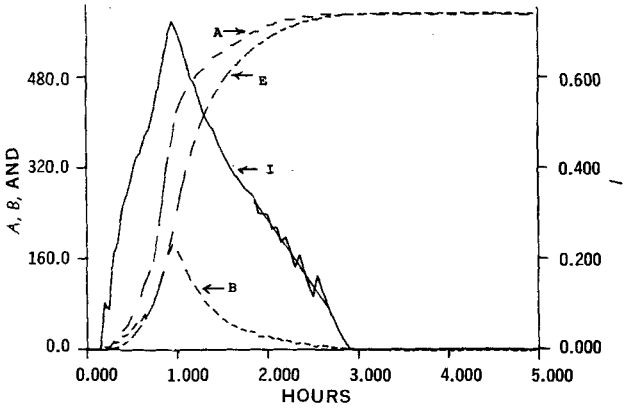


Figure 3—*Computerized plot of the time course of variation of the cumulative amount of drug excreted* (E), *the amount in the biophase* (B), *and the intensity of pharmacological response* (I), *computed from the cumulative amounts of drug absorbed* (A) *following dosing with a hypothetical slow-release dosage form.*

ployed for the measurement of pupillary diameters is presented elsewhere (8).

FORTRAN IV computer programs were prepared, and all calculations were performed with the aid of a CDC 6500 digital computer.

Figures 1–6 were constructed by a model 563 Calcomp Digital Incremental Plotter[1]. The jagged appearance of some curves is characteristic of the plotter.

RESULTS AND DISCUSSION

The dose–effect relationship for the mydriatic drug tropicamide was chosen to exemplify the theoretical considerations presented here. The confirmation of the applicability of the pharmacological method of drug absorption analysis to tropicamide was reported previously (8). The biokinetic behavior of this drug also has been determined to be well approximated by a single-compartment model in which the biophase and central systemic compartments are kinetically indistinguishable.

Figure 1 presents a plot of experimentally observed maximum mydriatic response intensities following the intravenous administration of the corresponding dose plotted on the abscissa. As discussed, the quantity of drug in the biophase, which in this case is identical to the central systemic compartment, can be obtained from observed mydriatic response intensities through the use of the dose–effect curve in a manner of a calibration curve. Alternative to the graphical estimation of the quantity of drug in the biophase, the curve in Fig. 1 may be described analytically by Eq. 9. The parameters, α = 1.0, K = 17.19 mcg./kg., R = 0.470 mcg./kg., and $P = 1.57 \times 10^{-3}$ kg./mcg., have biophysical significance as an intrinsic activity, drug–receptor association affinity constant, and density of receptor sites, respectively. The constant P has some connotation both as a distribution coefficient and an intrinsic activity. The manner in which these constants were evaluated was reported previously (8). The constant, β_{t_r}, has a value of unity, which is consistent in this case with the kinetic indistinguishability of the biophase and the central systemic compartment:

$$Q_B = \frac{\beta_{t_r}(I - \alpha R - KP) + [\beta(\alpha R + KP - I) + 4\beta^2 KPI]^{1/2}}{2\beta_{t_r}{}^2 p} \qquad \text{(Eq. 9)}$$

Transformation of observed values of I into corresponding values of Q_B can be accomplished directly through the use of the dose–effect curve or the application of an empirical equation fitted to the curve. No assumptions are necessary regarding the mechanism of the drug–receptor interaction. It is also not required to assign any physical significance to the parameters that would appear in an equation resulting from an empirical fit to the dose–effect curve.

The curves in Figs. 2–6 graphically exemplify the relationships between A_t, Q_B, I, and the quantity of drug, E, eliminated from the one-compartment system appropriate to describing the behavior of tropicamide. The curves were constructed using hypothetical values of A_t such as would be expected to be obtained from the results of optimized *in vitro* drug-release testing of dosage forms possessing the various drug-release properties indicated in the figures. The plotted A_t values were used for the computation of the remaining curves shown in each figure. Equations 6 and 9 were applied to

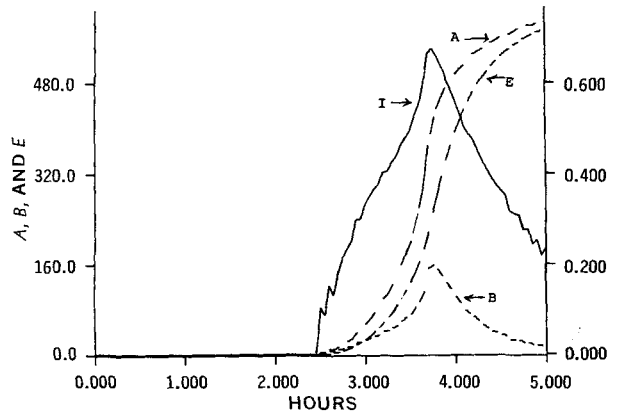


Figure 4—*Computerized plot of the time course of variation of the cumulative amount of drug excreted* (E), *the amount in the biophase* (B), *and the intensity of pharmacological response* (I), *computed from the cumulative amounts of drug absorbed* (A) *following dosing with a hypothetical enteric-coated oral dosage form.*

[1] California Computer Products, Inc., Anaheim, Calif.

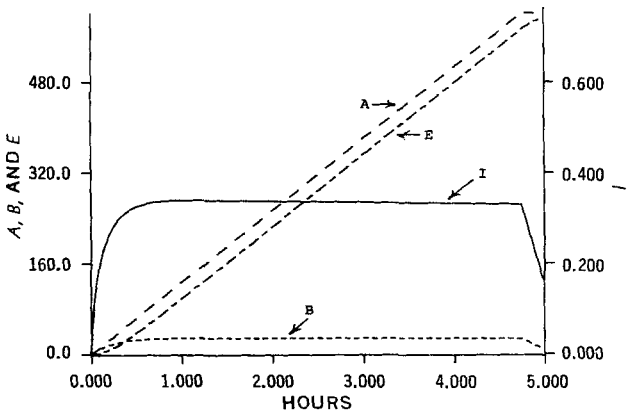


Figure 5—*Computerized plot of the time course of variation of the cumulative amount of drug excreted* (E), *the amount in the biophase* (B), *and the intensity of pharmacological response* (I), *computed from the cumulative amounts of drug absorbed* (A) *following dosing with a hypothetical zero-order sustained-release dosage form.*

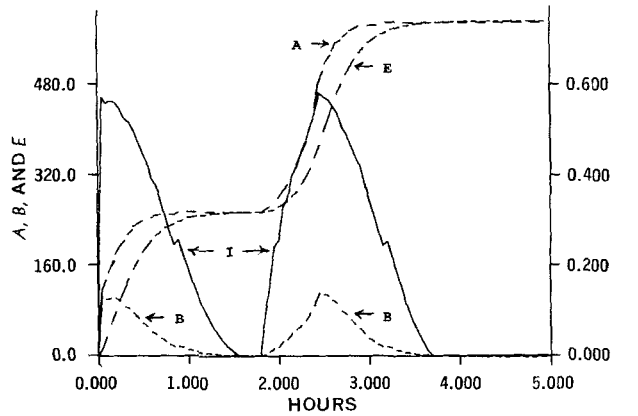


Figure 6—*Computerized plot of the time course of variation of the cumulative amount of drug excreted* (E), *the amount in the biophase* (B), *and the intensity of pharmacological response* (I), *computed from the cumulative amounts of drug absorbed* (A) *following dosing with a hypothetical repeat-action oral dosage form.*

compute Q_B and I, respectively. Values of E were simply obtained as the differences between computed values of Q_B and their corresponding values of A_t.

SUMMARY AND CONCLUSIONS

An approach is suggested for the development of "optimized *in vitro* drug-release tests" having a maximum capability of predicting *in vivo* drug bioavailability from dosage forms as a function of formulation factors. Such tests, appropriately applied with caution, could minimize the extent of human testing required to develop drug products with optimal *in vivo* drug-release characteristics. It is further demonstrated that with appropriate drugs for which a continuously graded pharmacological response intensity is observable, the results of optimized drug-release testing can be utilized to compute the time course of pharmacological activity.

The development of such a capability represents the ultimate in *in vitro* drug-release testing that can be sought. However, because of practical considerations such as the large magnitudes of intersubject and intrasubject variation commonly observed with *in vivo* data and the large amount of experimentation that may be necessary to develop optimized tests providing *in vivo* correlations within acceptable statistical limits, *in vitro* drug-release tests may remain constrained in their applicability. An intrinsic limitation is their applicability only to those cases where the systemic availability of the drug is rate limited by its release from the dosage form (3). Obviously, the development and subsequent application of *in vitro* drug-release tests should be undertaken with an awareness of such limitations. However, the inclusion of an appropriate membrane (13) as a permselective barrier in the *in vitro* drug-release testing apparatus could ultimately allow this latter serious limitation to be surmounted.

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Problems Associated with Analysis of Pharmacokinetic Models

WILFRED J. WESTLAKE

Abstract □ When a pharmacokinetic model is fitted to blood levels of a drug, the estimates of the pharmacokinetic parameters are likely to be subject to considerable error. These errors are probably unimportant as long as the model is used only to predict blood levels. However, when the parameters are used to predict other features of the system (*e.g.*, tissue drug levels), considerable errors in prediction may result. An example based on simulated data is devised to illustrate this possibility. It is further suggested that if the pharmacokinetic parameters are derived for the purpose of predicting the blood levels for different regimens or formulations of the drug—such as multiple-dose regimens or sustained-release capsules—this end can be met more expeditiously by using purely empirical techniques.

Keyphrases □ Pharmacokinetic models—analysis problems □ Blood levels, drugs—parameter predictions □ Lithium carbonate pharmacokinetics—two-compartment model □ Tissue drug levels—prediction errors

Over the past few years, it has become fashionable to conduct studies in which blood levels of a drug are determined at various times after administration; the resulting data then are fitted to a pharmacokinetic model. An example is the two-compartment model with oral administration, in which the usual assumptions of first-order kinetics lead to the expression:

$$C(t) = \frac{k_a D}{V_c}\left[\frac{(\alpha - k_{21})}{(\alpha - \beta)(k_a - \alpha)}e^{-\alpha t} + \frac{(k_{21} - \beta)}{(\alpha - \beta)(k_a - \beta)}e^{-\beta t} - \frac{(k_a - k_{21})}{(k_a - \alpha)(k_a - \beta)}e^{-k_a t}\right] \quad \text{(Eq. 1)}$$

for the drug concentration in the central (plasma) compartment. The constants in this expression are the rate constants k_a (gut to plasma compartment), k_{12} and k_{21} (plasma compartment to tissue compartment and vice versa), k_e (elimination from plasma compartment), and D/V_c (amount of drug absorbed divided by the volume of distribution of the plasma compartment). The parameters α and β are defined by the relations $\alpha\beta = k_e k_{21}$ and $\alpha + \beta = k_e + k_{12} + k_{21}$. The usual problem is to find the values for these five constants which best fit Eq. 1 to a set of empirical data. This is an exercise in nonlinear least squares, usually carried out with a computer program which utilizes iterative schemes for finding the best fit.

compute Q_B and I, respectively. Values of E were simply obtained as the differences between computed values of Q_B and their corresponding values of A_t.

SUMMARY AND CONCLUSIONS

An approach is suggested for the development of "optimized *in vitro* drug-release tests" having a maximum capability of predicting *in vivo* drug bioavailability from dosage forms as a function of formulation factors. Such tests, appropriately applied with caution, could minimize the extent of human testing required to develop drug products with optimal *in vivo* drug-release characteristics. It is further demonstrated that with appropriate drugs for which a continuously graded pharmacological response intensity is observable, the results of optimized drug-release testing can be utilized to compute the time course of pharmacological activity.

The development of such a capability represents the ultimate in *in vitro* drug-release testing that can be sought. However, because of practical considerations such as the large magnitudes of intersubject and intrasubject variation commonly observed with *in vivo* data and the large amount of experimentation that may be necessary to develop optimized tests providing *in vivo* correlations within acceptable statistical limits, *in vitro* drug-release tests may remain constrained in their applicability. An intrinsic limitation is their applicability only to those cases where the systemic availability of the drug is rate limited by its release from the dosage form (3). Obviously, the development and subsequent application of *in vitro* drug-release tests should be undertaken with an awareness of such limitations. However, the inclusion of an appropriate membrane (13) as a permselective barrier in the *in vitro* drug-release testing apparatus could ultimately allow this latter serious limitation to be surmounted.

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Abstract □ When a pharmacokinetic model is fitted to blood levels of a drug, the estimates of the pharmacokinetic parameters are likely to be subject to considerable error. These errors are probably unimportant as long as the model is used only to predict blood levels. However, when the parameters are used to predict other features of the system (*e.g.*, tissue drug levels), considerable errors in prediction may result. An example based on simulated data is devised to illustrate this possibility. It is further suggested that if the pharmacokinetic parameters are derived for the purpose of predicting the blood levels for different regimens or formulations of the drug—such as multiple-dose regimens or sustained-release capsules—this end can be met more expeditiously by using purely empirical techniques.

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Over the past few years, it has become fashionable to conduct studies in which blood levels of a drug are determined at various times after administration; the resulting data then are fitted to a pharmacokinetic model. An example is the two-compartment model with oral administration, in which the usual assumptions of first-order kinetics lead to the expression:

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It is well known, however, that the estimates of the parameters obtained are often extremely unreliable, especially if the values of any pair of the constants k_a, α, and β in the three exponential terms are at all close together (1). The purposes of this paper are to sound a warning note in this connection and to illustrate the problem by a simulation of actual blood level data. The unreliability of the estimates is related to the fact that, in many practical cases, wide variations in the estimates of the parameters give rise to only minute changes in the goodness of fit as measured by the residual sum of squares in the nonlinear estimation. In other words, there is a wide range of values for the constants k_a, α, β, k_{21}, and D/V_c for which essentially equally good fits of the model to the data are available. This is characterized in statistical terms by the fact that the confidence intervals associated with the estimates of the parameters are very wide. For example, if the least-squares estimate of k_a is found to be 3.85 hr.$^{-1}$, the 95% confidence interval might well be of the order of 0.90–6.80 hr.$^{-1}$. Extremely wide confidence intervals are likely to be the rule rather than the exception, except where the data accurately conform to the model under consideration and are obtained to an extremely high degree of precision.

Clearly, with the wide confidence intervals typically encountered, one cannot place great faith in the estimates of rate constants *per se*—at least not without a considerable amount of replication of the experiment. It can then be asked: To what practical purposes will the estimates of the rate constants be put? One answer is that they can be used to predict drug concentrations in the blood (or in other parts of the system) arising from given dosage regimens or new experimental formulations. This paper discusses this prediction problem and makes two main points:

1. While estimates of pharmacokinetic parameters from blood drug levels may be subject to considerable error, they usually allow reasonably accurate predictions to be made as long as these predictions are confined to blood levels. However, when one wishes to make such predictions (*e.g.*, for multiple-dose regimens or sustained-release formulations), this can be achieved more expeditiously by employing elementary superposition (or overlaying) techniques on the experimental blood level values themselves.

2. If the pharmacokinetic parameters are used to predict drug concentrations in some part of the system other than the blood compartment, considerable errors in prediction are possible. Examples are constructed to demonstrate this possibility.

As an example of prediction of blood levels of a drug, suppose that an experiment with a certain formulation of a drug gives hourly values $x_1 \ldots x_{12}$ of the blood drug levels from 0 to 12 hr. and that thereafter a few additional values are obtained (*e.g.*, at 18 and 24 hr.). If, during the first 12 hr. the samples are not obtained exactly on an hourly schedule, any missing hourly values are simply interpolated. To utilize simple superposition techniques, the hourly blood levels from 12 hr. on must be known; that is, x_i (for all integer values of $i > 12$) are needed. In practice, this is often not difficult to accomplish since the final exponential decay of the blood levels can frequently be modeled very easily. The values of x_t ($i > 12$) are then given by $Be^{-\beta(i-12)}$, where B and β are constants obtained by fitting the final exponential decay of the blood level curve. The values $x_1 \ldots x_{12}$ and the function $Be^{-\beta(i-12)}$ now become the input for a computer program which generates the blood levels from a given dosage regimen. For example, if the same formulation of the drug is given at 12-hr. intervals starting at 0 hr., then by superposition the predicted blood level at 27 hr. after the start of dosing (3 hr. after the third dose) is: $x_3 + Be^{-\beta(15-12)} + Be^{-\beta(27-12)}$, where the contribution x_3 is due to the dose at 24 hr., the second term is due to the dose at 12 hr., and the third term is due to the initial dose.

Steady-state blood levels, after infinite dosing, are easily obtained in a similar manner. For example, the predicted steady-state blood level at 3 hr. after a dose is taken is simply:

$$x_3 + Be^{-\beta(15-12)} + Be^{-\beta(27-12)} + Be^{-\beta(39-12)} \ldots = x_3 + Be^{-3\beta}/(1 - e^{-12\beta}) \quad \text{(Eq. 2)}$$

Most examples of predictions of blood levels from specified dosage regimens or experimental sustained-release formulations can be handled in this way. The only principles involved are those of superposition and

Table I—Simulated and Predicted Blood Level Data

Hours	Exact Blood Levels Generated by Two-Compartment Model	Exact Blood Levels with Added Noise	Blood Levels Predicted by Least-Squares Fit of Pharmacokinetic Parameters	Percentage Error in Predictions (Relative to Exact Levels)
0.25	0.18040	0.17489	0.17720	−1.8
0.5	0.25175	0.25877	0.25101	−0.3
0.75	0.26869	0.26085	0.27001	0.5
1.0	0.26015	0.26474	0.26200	0.7
1.5	0.21919	0.22221	0.21951	0.1
2	0.17869	0.17596	0.17770	−0.6
3	0.12672	0.12859	0.12788	0.9
6	0.08240	0.08374	0.09182	11.4
9	0.06991	0.08632	0.07846	12.2
12	0.06074	0.07290	0.06737	10.9
15	0.05287	0.05871	0.05784	9.4
18	0.04603	0.04384	0.04967	7.9
21	0.04007	0.04399	0.04265	6.4
24	0.03489	0.03410	0.03662	5.0

Table II—Least-Squares Estimates of Drug Concentration Parameters[a]

	Actual Values of Parameters	Least-Squares Estimates of Parameters	Approximate 95% Confidence Intervals	
k_a	2.389	1.774	−0.54	4.09
α	0.914	1.387	−0.61	3.38
β	0.0462	0.0508	0.036	0.065
k_{21}	0.250	0.333	0.211	0.455
D/V_c	0.441	0.570	−0.13	1.27
Residual sum of squares	0.00066	0.00037		

[a] Rate constants are in hr.$^{-1}$; D/V_c is in meq./l.

of scaling the blood levels by the appropriate factor whenever the dose differs from that on which the experimental data were obtained. This empirical "overlaying" principle depends only on the linearity of the transfers from one compartment to another, an assumption that is freely made in most pharmacokinetic analysis. Despite its simplicity, therefore, the technique is really a more sophisticated method than compartmental analysis for handling multiple-dose regimens and sustained-release formulations, since it is valid under less restrictive conditions than are required for any given compartmental analysis.

The second of the two points made earlier concerned the prediction of drug levels in parts of the system other than the blood compartment. In the two-compartment model, the obvious factor of interest that cannot be obtained without pharmacokinetic analysis is the concentration of drug in the tissue compartment. Gibaldi (2) made an interesting comparison of rapid intravenous injection and intravenous infusion by examining the differences in tissue drug levels that they generate. The warning referred to earlier is that the estimates of the pharmacokinetic parameters, although giving a good fit to the data, may be subject to considerable error. This error generally will not be of any consequence as long as the parameters are used only to predict future blood levels over the same time span for which data were obtained. However, if the parameters are used to predict any characteristic of the system other than blood levels, the errors in the parameter estimates may result in appreciable errors in these predictions. The following example was devised to demonstrate this possibility.

Table III—Exact and Predicted Tissue Drug Levels

Hours	Exact Tissue Drug Levels	Predicted Tissue Drug Levels	Percentage Error of Predicted over Exact
0.25	0.01358	0.02169	59.7
0.50	0.04198	0.06727	60.3
0.75	0.07398	0.11842	60.1
1.0	0.10435	0.16625	59.3
1.5	0.15321	0.24002	56.7
2	0.18552	0.28433	53.3
3	0.21562	0.31619	46.6
6	0.21074	0.28881	37.0
9	0.18496	0.24831	34.3
12	0.16112	0.21322	32.3
15	0.14027	0.18308	30.5
18	0.12212	0.15720	28.7
21	0.10631	0.13498	27.0
24	0.09255	0.11590	25.2

EXPERIMENTS ON SIMULATED DATA

From experiments with lithium carbonate administered as a solution in human volunteers, the pharmacokinetic parameters for a two-compartment model were fitted. Typical estimates in one volunteer (who was administered 300 mg. of lithium carbonate in solution) were $k_a = 2.389$ hr.$^{-1}$, $\alpha = 0.914$ hr.$^{-1}$, $\beta = 0.0462$ hr.$^{-1}$, $k_{21} = 0.250$ hr.$^{-1}$, and $D/V_c = 0.441$ meq./l. These parameters then were used to generate artificial data at various times based on the expression for drug concentration in the plasma compartment. These data are given in Column 1 of Table I. If one attempted to fit these data to the two-compartment model, a perfect fit would result, with the residual sum of squares essentially equal to zero and the parameter estimates identical to those used to generate the data. A low level of random noise was added to the blood levels in the first column, using random normal deviates with $\sigma = 0.01$. The second column shows the resulting set of data. These concentrations correspond to the sort of data one might obtain in an actual experiment if the system behaved as a perfect two-compartment model but with a small random error being introduced in the assay procedure for determining blood drug levels. The error σ is assumed to be the same for each data point. In practice, the error σ could be proportional to the actual drug concentration. This case is not treated here, although similar examples could easily be constructed for it.

The data in the second column were then fitted by least squares to the two-compartment model. This is the problem of estimating the five parameters (k_a, α, β, k_{21}, and D/V_c) in Eq. 1. The nonlinear least-squares program used is an adaptation of one written at the University of Wisconsin Computing Center and is based on the algorithm of Marquardt (3). Convergence was assumed complete with the iteration for which the relative change in the error sum of squares was less than 10^{-4}. The least-squares fit for the parameters is given in Table II, together with the actual values of the parameters. The 95% confidence intervals given are the simple (univariate) confidence intervals for each parameter individually.

Thus, the parameter values as estimated are appreciably different from the true values (note particularly a 26% error in k_a and a 50% error in α). However, the considerable width of the approximate 95% confidence intervals given in the last column should immediately warn one not to place too much faith in the actual values of the estimates. This simulation was performed with several sets of random noise. In a number of cases, the quite small amount of noise added was sufficient to disguise the two-compartment nature of the model, and the best least-squares fit reduced the model to the one-compartment case in which k_a and α were essentially equal. The example given here is typical of one of the "better" examples in which a reasonable distinction between k_a and α was possible.

It was suggested earlier that, even though the least-squares estimates of the pharmacokinetic parameters might be considerably in error, they will probably enable reasonably accurate predictions of blood levels to be made. That such, in fact, is the case can be seen by referring back to Columns 3 and 4 of Table I: the predicted blood levels and their percentage error over the correct levels, respectively. The error is, in general, less than 1% over the first 3 hr., when the levels of drug in the blood are appreciable. Thereafter, the proportional error becomes higher, although this is in large part due to the fact that the blood levels are becoming rapidly smaller and errors tend to be *proportionately* higher. Contrast this situation with the prediction of drug levels in the tissue compartment given by the equation:

$$T(t) = \frac{k_a k_{12} D}{V_T} \times \left[\frac{e^{-kat}}{(k_a - \alpha)(k_a - \beta)} - \frac{e^{-\alpha t}}{(\alpha - \beta)(k_a - \alpha)} + \frac{e^{-\beta t}}{(k_a - \beta)(\alpha - \beta)}\right] \quad \text{(Eq. 3)}$$

Table III shows the exact concentrations together with the predicted concentrations and the percentage error of predicted over exact. For convenience, the volume of distribution, V_T, for the tissue compartment has been assumed to be equal to that for the

Table IV—Exact and Predicted Blood and Tissue Drug Levels

Hours	Exact Blood Drug Levels	Predicted Blood Drug Levels	Percentage Error in Predictions (Relative to Exact)	Exact Tissue Drug Levels	Predicted Tissue Drug Levels	Percentage Error in Predictions (Relative to Exact)
0.25	0.13476	0.13202	−2.0	0.00449	0.00484	7.8
0.5	0.19500	0.19307	−1.0	0.01438	0.01555	8.1
0.75	0.21680	0.21596	−0.4	0.02632	0.02848	8.2
1.0	0.21919	0.21887	−0.1	0.03860	0.04171	8.1
1.5	0.20104	0.20012	−0.5	0.06136	0.06578	7.2
2	0.17570	0.17339	−1.3	0.08030	0.08505	5.9
3	0.13221	0.12830	−3.0	0.10725	0.11031	2.9
6	0.06899	0.06830	−1.0	0.13670	0.12909	−5.6
9	0.04900	0.05069	3.4	0.13612	0.12042	−11.5
12	0.04111	0.04266	3.8	0.12800	0.10748	−16.0
15	0.03671	0.03715	1.2	0.11847	0.09497	−19.8
18	0.03346	0.03264	−2.5	0.10914	0.08371	−23.3
21	0.03068	0.02873	−6.4	0.10040	0.07375	−26.5
24	0.02819	0.02530	−10.3	0.09233	0.06496	−29.6

central compartment, V_c. This has no significance, however, since the present concern is only with proportional errors.

The predicted parameters consistently overestimate the tissue drug levels, starting with a nearly 60% proportional error and decreasing to 25% by 24 hr. At the peak tissue levels (say from 2 to 9 hr.), the error ranges from about 50 to 35%. The consistency of the overestimation would lead to a substantial overestimation of the total amount of drug flowing into the tissue compartment.

A similar simulation is now presented based on the parameters: $k_a = 2.529\ \text{hr.}^{-1}$, $\alpha = 0.432\ \text{hr.}^{-1}$, $\beta = 0.028\ \text{hr.}^{-1}$, $k_{21} = 0.101\ \text{hr.}^{-1}$, and $D/V_c = 0.302$ meq./l. Again, the constants were suggested by experiments with lithium carbonate solution. After addition of random noise ($\sigma = 0.01$) to the blood drug levels, the least-squares estimates of the parameters were: $k_a = 2.327\ \text{hr.}^{-1}$, $\alpha = 0.5198\ \text{hr.}^{-1}$, $\beta = 0.0423\ \text{hr.}^{-1}$, $k_{21} = 0.1457\ \text{hr.}^{-1}$, and $D/V_c = 0.3166$. In contrast with the earlier simulation, k_a, α, and D/V_c are estimated reasonably accurately but there are larger errors in β and k_{21}. Table IV gives the exact blood and tissue drug levels and the predicted blood and tissue drug levels based on the least-squares estimators. In this case, the estimated parameters give predictions for the blood levels that are all within 4% of the true blood levels right up to the last two time intervals (21 and 24 hr.). By contrast, the predicted tissue drug levels show considerably greater errors. They are neither so consistently nor so grossly in error as the predictions of the previous simulation, but, nevertheless, they still give a distorted picture of the true tissue drug levels. Reasonably accurate predictions (say within 5%) are achieved only somewhere between 2 and 6 hr. Before 3 hr., the predictions overestimate drug tissue levels; after 6 hr., there is an ever-increasing underestimation.

In conclusion, two points should be made. First, these two examples of the considerable disparity between exact tissue drug levels and predicted levels based on least-squares estimates of the pharmacokinetic parameters are in no sense unusual. Many other similar examples have been constructed. Second, similar experiments with simulated data have confirmed the assertion made earlier that predictions of blood drug levels for sustained-release capsules or multiple-dose regimens can be made more accurately by using simple superposition techniques than by using estimations of pharmacokinetic parameters.

DISCUSSION

Certain problems involved in the fitting of pharmacokinetic models to blood level data are sometimes glossed over in published studies. The problems are statistical and are inherent in the attempt to fit data to functions consisting of sums of exponential terms. Confidence intervals for least-squares estimates of parameters are frequently wide, and there may be substantial errors in the estimates. A simple example was constructed to show the erroneous conclusions that can be drawn from these least-squares estimates when they are used to make predictions *other* than blood levels. It is further noted that when blood level prediction is sought, as for example in designing drug formulations, empirical methods should give predictions that are as good or better than those based on the parameter estimates derived from compartment models.

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Influence of Complex Formation on Membrane Transport

V. S. VAIDHYANATHAN

Abstract □ Theoretical aspects of the influence of complex formation on transport across a diffusional barrier of three species, participating in an association–dissociation reaction, are presented. On the basis of solution of a nonlinear differential equation derived from equations of continuity, valid for stationary states, when diffusion coefficients are constants and coupling between fluxes of different species is ignored, it is shown that the usual assumption that the complex formation reaction is at equilibrium at all locations in the system is not valid, except when the concentration of one of the reactant species is maintained equal on both sides of the membrane. In the general case, methods of calculation of reaction-rate profiles and concentration profiles to any desired order of approximation in the inhomogeneous diffusional barrier region from experimentally measurable quantities are included. The method of computing the association-rate and dissociation-rate constants from suitable transport measurements is presented.

Keyphrases □ Kinetics—complex formation membrane transport, theoretical □ Membrane transport—complex formation effect, theoretical □ Complex formation—effect on membrane transport, kinetic theory □ Association-, dissociation-rate constants—computation methods □ Reaction, concentration-rate profiles, inhomogeneous barrier region—calculation

In a series of papers on the effect of complex formation on drug absorption, Reuning and Levy (1–3) presented experimental information and analysis of a model describing the overall transfer of a drug across a diffusional barrier in the presence of a complexing agent. Their experimental setup is that specified concentrations of a mixture of α (caffeine) and β (salicylamide) are placed in a conical flask of relatively large volume and well stirred. A small amount of solution containing β is placed in a nylon bag and suspended in the large volume mixture. Samples of solution in the nylon bag are withdrawn at regular time intervals and analyzed for total concentrations of α and β in the nylon bag.

The basic assumption in these experiments is that α and β, when present together in solution, form a complex γ represented by the reaction:

$$\alpha + \beta \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \gamma \qquad \text{(Eq. 1)}$$

where k_1 and k_2 are the rate constants for the association–dissociation reaction. The equilibrium constant, $K = (k_1/k_2)$, is determined by various methods to be 30–50 l./mole. It is assumed that the reaction represented by Eq. 1 is at equilibrium at all locations in the system and that the species transport across the diffusional barrier at a rate proportional to their respective concentration gradients. Experimentally, it is determined that species α permeates the diffusional barrier much more slowly than species β.

A series of experiments with varying initial concentrations of β in the nylon bag and specified concentration of α and β in the outside solution was performed; the time variation of concentrations of β in the nylon bag was determined. The fraction of total amount of β present in complexed form was computed and utilized in the calculation of the stability constant for complex formation.

The principal objectives of this paper are to: (*a*) analyze the significance of experimental information obtained by the above-mentioned set of experiments, (*b*) examine the validity of the basic assumptions invoked in the interpretation of experimental data, and (*c*) present certain theoretical aspects of the influence of chemical reactions on the flux of species across a diffusional barrier which also participate in a reaction of the type presented in Eq. 1. If the reaction presented in Eq. 1 is not at equilibrium, then, by necessity, the reaction-rate profile $J_R(x)$.

$$J_R(x) = k_1 C_\alpha(x) C_\beta(x) - k_2 C_\gamma(x) \qquad \text{(Eq. 2)}$$

will be a function of position variable x in the inhomogeneous diffusional barrier under conditions of stationary states and nonsteady states. In Eq. 2, $C_\sigma(x)$ is the local concentration of species σ at a specified location x in the system. The position variable x is defined along a direction normal to the plane of the diffusional barrier. Both $J_R(x)$ and $C_\sigma(x)$ are independent of time under conditions of steady state and vary with time under nonsteady-state conditions.

Later in this paper, expressions for the reaction-rate profile for nonsteady states and steady states are presented. In the section, *Nonstationary-State Conditions*, the general aspects of the problem as dictated by equations of continuity are discussed. In the section, *Stationary State (Reaction at Equilibrium)*, considerations valid for steady states when the reaction is at equilibrium at all locations of the diffusional barrier are presented. In that section, it is shown that unless the concentration of one of the species, α or β, is maintained the same in solutions on both sides of the diffusional barrier, the reaction-rate profile will be nonvanishing in the inhomogeneous region. The inhomogeneous character of the diffusional barrier is responsible for the maintenance of a stationary-state reaction-rate profile. In a later section, an expression is presented for the reaction-rate profile valid in the inhomogeneous region when diffusion coefficients are regarded as constants and when coupling between fluxes of different species may be ignored. One may compute the reaction-rate profile and the effect of chemical reaction on flux of a specified species across a diffusional barrier.

Reuning and Levy (1–3) considered the possible occurrence of dimerization of α and the possibility of a 2:1 complex formation, in addition to the reaction of the type presented in Eq. 1, occurring in the system. For the sake of brevity, it is assumed that only the reaction represented by Eq. 1 occurs in the system presented here.

NONSTATIONARY-STATE CONDITIONS

It is both interesting and illuminating to begin with the conditions valid for nonstationary state. Experiments of the type mentioned previously yield measurement of (dC_σ^{II}/dt), where C_σ^{II} is the concentration of species σ in the nylon bag and t is the time variable. In the following, time derivatives are denoted with a super dot and quantities corresponding to stationary states are denoted with an asterisk to distinguish them from corresponding quantities of nonsteady-state conditions. When reaction of the type represented in Eq. 1 takes place in the system, one has the equations of continuity for the three species:

$$\dot{C}_\alpha = -\nabla\cdot J_\alpha(x) - J_R(x) \qquad \text{(Eq. 3}a\text{)}$$

$$\dot{C}_\beta = -\nabla\cdot J_\beta(x) - J_R \qquad \text{(Eq. 3}b\text{)}$$

$$\dot{C}_\gamma = -\nabla\cdot J_\gamma + J_R \qquad \text{(Eq. 3}c\text{)}$$

where J_σ is the matter flux (in moles per unit area per unit time) of species σ. So long as one considers only one-dimensional transport, *i.e.*, transport along an axis normal to the plane of the membrane, one may replace $\nabla\cdot J_\sigma$ by $(dJ_\sigma/dx) = J_\sigma'$. The first derivative with respect to position variable is denoted by a single prime. The second derivative with respect to x is denoted by a double prime.

Within the spirit of irreversible thermodynamics and existence of nonvanishing interactions between molecules across space, the flux of a molecule of a specified species is correlated with the position and fluxes of all other molecules present in the system (4–9). If one now assumes that the influence of fluxes of other species on the flux of a specified species may be ignored and that the flux of a specified species is proportional to its concentration gradient as a first approximation, in the spirit of Fick's equation, one may write:

$$J_\sigma = -D_\sigma C_\sigma' \qquad \text{(Eq. 4)}$$

where D_σ is the diffusion coefficient of species σ in the system. In principle, D_σ is dependent on concentration profiles of all species present in the inhomogeneous system, given by complicated expressions of molecular theory (10). Thus, the diffusion coefficient is a function of the position variable. If one adopts, as a second approximation, the assumption that diffusion coefficients may be regarded as constants, one may rewrite the equations of continuity as:

$$\dot{C}_\alpha = -D_\alpha C_\alpha'' - J_R \qquad \text{(Eq. 5}a\text{)}$$

$$\dot{C}_\beta = -D_\beta C_\beta'' - J_R \qquad \text{(Eq. 5}b\text{)}$$

$$\dot{C}_\gamma = -D_\gamma C_\gamma'' + J_R \qquad \text{(Eq. 5}c\text{)}$$

Under steady-state conditions, all explicit time derivatives must vanish. Thus one recovers a basic equation valid for stationary states:

$$-J_\alpha' = -J_\beta' = J_\gamma' = J_R \qquad \text{(Eq. 6)}$$

Equation 6, along with the assumed validity of Eq. 4, formed the basis for the derivation of a (nonlinear) differential equation for the reaction-rate profile by Blumenthal and Katchalsky (11)—*viz.*,

$$J_R^*(x) = \lambda^{-2}J_R^* + 2k_1C_\alpha'C_\beta' \qquad \text{(Eq. 7)}$$

$$\lambda^{-2} = k_1\{(\bar{C}_\alpha/D_\beta) + (\bar{C}_\beta/D_\alpha)\} + (k_2/D_\gamma) \qquad \text{(Eq. 8)}$$

In Eq. 8, $\bar{C}_\sigma$ refers to the stoichiometric concentration of species σ, when the reaction of Eq. 1 is at equilibrium and the J_R of Eq. 2 vanishes identically. The λ is defined as the relaxation length for the reaction in the system and is constant independent of position variable x due to the assumed constancy of diffusion coefficients. Equation 7 is a nonlinear differential equation for the reaction-rate profile in the inhomogeneous diffusional barrier under conditions of steady state. Blumenthal and Katchalsky (11) obtained the solution of Eq. 7, neglecting the nonlinear term $2k_1C_\alpha'C_\beta'$. A critical examination of this approximation is presented elsewhere (12).

Under conditions of steady states, one has from Eqs. 5*a–c* that:

$$C_\beta'' = (D_\alpha/D_\beta)C_\alpha'' \qquad \text{(Eq. 9}a\text{)}$$

$$C_\gamma'' = -(D_\alpha/D_\gamma)C_\alpha'' \qquad \text{(Eq. 9}b\text{)}$$

Under nonstationary-state conditions, one has:

$$C_\beta'' = (D_\alpha/D_\beta)C_\alpha'' + g_1 \qquad \text{(Eq. 10}a\text{)}$$

$$C_\gamma'' = -(D_\alpha/D_\gamma)C_\alpha'' + g_2 \qquad \text{(Eq. 10}b\text{)}$$

$$g_1 = (1/D_\beta)(\dot{C}_\alpha - \dot{C}_\beta) \qquad \text{(Eq. 10}c\text{)}$$

$$g_2 = -(1/D_\gamma)(\dot{C}_\alpha + \dot{C}_\gamma) \qquad \text{(Eq. 10}d\text{)}$$

The third basic assumption is now made—that both g_1 and g_2 are independent of the position variable. The implication of this assumption is that the departure of the state of the system from stationary states is small. If this assumption is valid, it is possible to express:

$$C_\beta(x) = (D_\alpha/D_\beta)C_\alpha(x) + (g_1/2)x^2 + r_1x + r_2 \qquad \text{(Eq. 11}a\text{)}$$

$$C_\gamma(x) = -(D_\alpha/D_\gamma)C_\alpha(x) + (g_2/2)x^2 + r_3x + r_4 \qquad \text{(Eq. 11}b\text{)}$$

Substitution of these equations into Eq. 2 enables one to express the reaction-rate profiles in a inhomogeneous diffusional barrier under conditions of nonstationary states as:

$$J_R(x) = \sum_{i=0} R_ix^i \qquad \text{(Eq. 12}a\text{)}$$

$$R_0 = J_R(0) = k_1C_\alpha(0)C_\beta(0) - k_2C_\gamma(0) \qquad \text{(Eq. 12}b\text{)}$$

$$R_1 = D_\alpha\eta^2a_1 + [k_1C_\alpha(0)p - k_2q] \qquad \text{(Eq. 12}c\text{)}$$

$$R_2 = D_\alpha\eta^2a_2 + k_1(D_\alpha/D_\beta)a_1^2 + k_1pa_1 + k_1C_\alpha(0)g_1/2 - k_2g_2/2 \qquad \text{(Eq. 12}d\text{)}$$

$$R_k = D_\alpha\eta^2a_k + k_1(D_\alpha/D_\beta)\sum_{\substack{i,j=1\\ i+j=k}}^{k-1} a_ia_j + k_1pa_{k-1} + (k_1g_1/2)a_{k-2} \qquad \text{(Eq. 12}e\text{)}$$

where:

$$r_1 = p = (1/D_\beta)(q_\beta - q_\alpha) \qquad \text{(Eq. 13}a\text{)}$$

$$r_2 = C_\beta(0) - (D_\alpha/D_\beta)C_\alpha(0) \qquad \text{(Eq. 13}b\text{)}$$

$$r_3 = q = (1/D_\gamma)(q_\alpha + q_\gamma) \qquad \text{(Eq. 13}c\text{)}$$

$$r_4 = C_\gamma(0) + (D_\alpha/D_\gamma)C_\alpha(0) \qquad \text{(Eq. 13}d\text{)}$$

$$\eta^2 = (k_2/D_\gamma) + (k_1/D_\alpha D_\beta)\{C_\alpha(0)D_\alpha + C_\beta(0)D_\beta\} \qquad \text{(Eq. 13}e\text{)}$$

$$a_i \equiv (1/i!)\{d^iC_\alpha(x)/dx^i\}|_{x=0} \qquad \text{(Eq. 13}f\text{)}$$

In these equations, $C_\alpha(0)$ is the concentration of species α at location $x = 0$, where inhomogeneity begins in the diffusional barrier. The constant parameter η^2 shares some similarity with λ^{-2} of Eq. 8 but is different from it; η becomes identical with λ^{-1} if the reaction of Eq. 1 is at equilibrium at location $x = 0$.

In a later section, the reaction-rate profile, $J_R^*(x)$, at steady state is evaluated as the solution of certain nonlinear differential equations and expressed as:

$$J_R^*(x) = \sum_{i=0} S_ix^i \qquad \text{(Eq. 14)}$$

Comparison of the expressions for constant coefficients S_i's and R_i's indicates that:

$$R_0 = S_0 \qquad R_1 = S_1 \qquad \text{(Eq. 15}a\text{)}$$

$$R_2 - S_2 = {}^1/_2\{k_1C_\alpha(0)g_1 - k_2g_2\} \qquad \text{(Eq. 15}b\text{)}$$

$$R_k - S_k = (k_1g_1/2)\,a_{k-2} \qquad k \geqslant 3 \qquad \text{(Eq. 15}c\text{)}$$

In other words, the departure of the state of the system from stationary states affects only coefficients of order greater than (x^2). So long as the reaction rate is either constant or a linear function of the position variable in the inhomogeneous diffusional barrier, the influence of chemical reactions on fluxes across a membrane approximates to the stationary-state calculations.

STATIONARY STATE (REACTION AT EQUILIBRIUM)

In this section, a nonlinear differential equation is derived for a function, $G(x)$, related to concentration profiles of the three species participating in the reaction; it is valid when diffusion coefficients are constants and coupling between fluxes may be ignored. On the basis of the assumption that the reaction of Eq. 1 is at equilibrium in the inhomogeneous diffusional barrier, the solution of the differential equation is analyzed, yielding relations between the observed flux of a specified species and the stability constant of the complex. The reaction can be at equilibrium in the inhomogeneous medium if and only if the concentration of one of the reacting species is maintained constant throughout the system. In other cases, the reaction rate of Eq. 2 cannot be nonvanishing inside the diffusional barrier under steady-state conditions subject to the validity of the assumptions contained in the derivation of the nonlinear differential equation for $G(x)$.

When one assumes that matter fluxes are independent of each other and proportional to their respective concentration gradients, as presented in Eq. 4, one has from Eq. 6:

$$-J_\alpha = I(x) + q_\alpha \quad \text{(Eq. 16a)}$$

$$-J_\beta = I(x) + q_\beta \quad \text{(Eq. 16b)}$$

$$J_\gamma = I(x) + q_\gamma \quad \text{(Eq. 16c)}$$

$$I(x) \equiv \int J_R^*(x)dx \quad \text{(Eq. 16d)}$$

$$J_\alpha^*(x) - J_\beta^*(x) = (q_\beta - q_\alpha) = \text{a constant} \quad \text{(Eq. 16e)}$$

$$J_\alpha^*(x) + J_\gamma^*(x) = (q_\gamma - q_\alpha) = \text{another constant} \quad \text{(Eq. 16f)}$$

If the species do not participate in the reaction to form a complex then the measured flux, J_σ, in the absence of a chemical reaction will equal $-q_\sigma$. When one has a mixture of chemically noninteracting species transporting across a diffusional barrier, the fluxes of each species are constant under stationary states. When three components of a mixture participate in the reaction of Eq. 1, then the fluxes of these three species are no longer independent of the position variable. However, linear combinations of the fluxes of two species, as presented in Eqs. 16*a–f*, remain constant independent of the position variable.

Equations 16*a–f*, along with Eq. 4 and the assumption of constant diffusion coefficients, yield the following relations for the concentration profiles of the three species:

$$C_\alpha(x) = (G/D_\alpha) + (q_\alpha/D_\alpha)x + K_\alpha \quad \text{(Eq. 17a)}$$

$$C_\beta(x) = (G/D_\beta) + (q_\beta/D_\beta)x + K_\beta \quad \text{(Eq. 17b)}$$

$$C_\gamma(x) = -(G/D_\gamma) - (q_\gamma/D_\gamma)x + K_\gamma \quad \text{(Eq. 17c)}$$

$$G \equiv G(x) = \int I(x)dx \quad \text{(Eq. 17d)}$$

where K_α, K_β, and K_γ are integration constants. It is evident that K_σ will equal $C_\sigma(0)$, if the constant term of any of the function $G(x)$ vanishes. Substituting expressions 17*a–d* into Eq. 2, one obtains the following nonlinear differential equation for $G(x)$:

$$G'' = \mu G^2 + \eta^2 G + \sigma G x + A x^2 + B x + J_R(0) \quad \text{(Eq. 18)}$$

where:

$$G'' = J_R^*(x)$$

$$J_R(0) = k_1 K_\alpha K_\beta - k_2 K_\gamma$$

$$\mu = (k_1/D_\alpha D_\beta)$$

$$\eta^2 = (k_2/D_\gamma) + \mu\{K_\alpha D_\alpha + K_\beta D_\beta\}$$

$$\sigma = \mu(q_\alpha + q_\beta)$$

$$A = \mu q_\alpha q_\beta$$

$$B = (k_2 q_\gamma/D_\gamma) + \mu\{K_\alpha D_\alpha q_\beta + K_\beta D_\beta q_\alpha\}$$

Equation 18 is valid for inhomogeneous regions provided the diffusion coefficients are constant and fluxes are given by Eq. 4. The coefficients μ, η^2, σ, A, B, and $J_R(0)$ are constants independent of the position variable x.

If the assumption that the reaction is at equilibrium inside every location of the diffusional barrier is valid, then it follows that:

$$G'' = J_R^*(x) = 0 \quad \text{(Eq. 19)}$$

Equation 19 demands that the function G, satisfying Eq. 18, can at best be a linear function of x, represented by:

$$G(x) = a + bx \quad \text{(Eq. 20)}$$

where a and b are constants. Substituting Eq. 20 into Eq. 18, one obtains

$$x^2 P + xQ + R = 0 \quad \text{(Eq. 21)}$$

$$P = \mu b^2 + \sigma b + A \quad \text{(Eq. 22a)}$$

$$Q = \eta^2 b + 2\mu a b + \sigma a + B \quad \text{(Eq. 22b)}$$

$$R = \mu a^2 + \eta^2 a + J_R(0) \quad \text{(Eq. 22c)}$$

Equation 21 is a polynomial in x, equal to zero. Recalling that in order for a polynomial of varying powers of x to equal zero, the coefficients of every power of x must identically vanish, one has $P = Q = R = 0$. One obtains from Eqs. 22*a* and 22*c* that:

$$a = -(\eta^2/2\mu) \pm (\eta^2/2\mu)[1 - \{4J_R(0)\mu/\eta^4\}]^{1/2} \quad \text{(Eq. 23a)}$$

$$b = -(\sigma/2\mu) \pm (\sigma/2\mu)[1 - \{4\mu A/\sigma^2\}]^{1/2} \quad \text{(Eq. 23b)}$$

Since the reaction is at equilibrium everywhere, one has

$$a = 0 \quad \text{or} \quad a = -(\eta^2/\mu) \quad \text{(Eq. 24a)}$$

$$b = -q_\alpha \quad \text{or} \quad b = -q_\beta \quad \text{(Eq. 24b)}$$

Substituting these results in Eq. 22*b*, which should also vanish, one obtains:

$$B = \eta^2 q \quad \text{when } b = -q_\alpha \text{ and } a = 0, \text{ or } -\eta^2/\mu \quad \text{(Eq. 25a)}$$

$$B = \eta^2 q_\alpha \quad \text{when } b = -q_\beta \text{ and } a = 0, \text{ or } -\eta^2/\mu \quad \text{(Eq. 25b)}$$

The two expressions for B and $\eta^2 q_\alpha$ obtained from Eq. 18 are equal if either:

$$q_\alpha = q_\beta = q_\gamma \quad \text{(Eq. 26)}$$

or:

$$q_\alpha = \{k_1 D_\gamma q_\beta + k_2 D_\beta q_\gamma\}\{k_2 D_\beta + k_1 K_\alpha D_\gamma\}^{-1} \quad \text{(Eq. 27)}$$

Similarly, the two expressions B and $\eta^2 q_\beta$ become identical if either Eq. 26 is satisfied or:

$$q_\beta = \{k_2 D_\alpha q_\gamma + k_1 K_\beta D_\gamma q_\alpha\}\{k_2 D_\alpha + k_1 K_\beta D_\gamma\}^{-1} \quad \text{(Eq. 28)}$$

The validity of Eq. 26 along with Eqs. 17*a–d* and 4 demands that the gradients of all three species vanish across the diffusional barrier. In other words, Eq. 26 describes the equilibrium state of the whole system. When one chooses the value $b = -q_\beta$, one has:

$$C_\beta' = 0 \quad \text{(Eq. 29a)}$$

$$C_\alpha' = (q_\alpha - q_\beta)/D_\alpha \quad \text{(Eq. 29b)}$$

The rigorous validity of Eq. 24*b*, when the reaction is at equilibrium in the diffusional barrier, and constant diffusion coefficients require that the concentration gradient of either α or β should vanish. Conversely, if the gradients of the reacting species α and β do not vanish across the diffusional barrier, then Eq. 20 cannot be a solution of Eq. 18 and, therefore, the reaction cannot be at equilibrium in the inhomogeneous diffusional barrier. In addition, the reaction rate cannot even be constant in the inhomogeneous diffusional barrier if either of the gradients of the reacting species does not vanish. Inspection of Eq. 18 suggests that under no circumstance can the function G be a quadratic function of x.

Assuming that experiments are carried out maintaining the concentration of β the same on either side of the membrane, and that measurements of flux of α, J_α^*, are obtained in the presence of vary-

ing concentrations of β on both sides of the membrane, one has from Eq. 28:

$$J_\alpha^*(C_\beta) = (q_\gamma - q_\alpha)\{1 + KK_\beta(D_\gamma/D_\alpha)\}^{-1} \quad \text{(Eq. 30)}$$

where $K = (k_1/k_2)$ and $K_\beta = C_\beta{}^l$ when $a = 0$.

If $C_\beta = 0$, then the flux of α would have been:

$$J_\alpha{}^{0*} = J_\alpha^*(C_\beta = 0) = -q_\alpha \quad \text{(Eq. 31)}$$

Thus, in the presence of β, the additional flux of α caused by the complex-forming reaction in the system, when the reaction is at equilibrium everywhere, is given by:

$$J_\alpha^*(C_\beta) - J_\alpha^*(C_\beta = 0) = \{KC_\beta D_\gamma q_\alpha - q_\gamma D_\alpha\} \times [D_\alpha + KC_\beta D_\gamma]^{-1} \quad \text{(Eq. 32)}$$

Therefore, from the measurements of fluxes J_α, under steady state in the presence of known concentrations of β on either side of the diffusional barrier, and knowledge of D_α, D_γ, q_α, and q_γ, one can compute the stability constant K of the complex using Eq. 22; q_α and q_γ are the negatives of the fluxes of species α and γ obtained for the same diffusional barrier in the absence of the other two species.

The conclusions of this section and Eq. 22 may be independently derived by the following simpler argument. Under stationary state, when the reaction rate vanishes, one has from Eqs. 4 and 6 that $C_\gamma{}'$ should be a constant when D_γ is a constant. However, from Eq. 2, one has:

$$C_\gamma{}' = (k_1/k_2)\{C_\alpha C_\beta{}' + C_\beta C_\alpha{}'\} \quad \text{(Eq. 33)}$$

The right-hand side of Eq. 33 will be a constant if and only if either $C_\alpha{}'$ or $C_\beta{}'$ vanishes. When $C_\beta{}' = 0$, one has from Eqs. 9*a–b* and 33:

$$P = (q_\alpha + q_\gamma)/D_\gamma = \{(KC_\beta D_\gamma + D_\alpha)/D_\gamma\}C_\alpha{}' \quad \text{(Eq. 34)}$$

Hence,

$$J_\alpha(C_\beta) - J_\alpha(C_\beta = 0) = q_\alpha - [PD_\gamma D_\alpha/(KC_\beta D_\gamma + D_\alpha)] \quad \text{(Eq. 35)}$$

Equation 35 leads to Eq. 32.

STATIONARY-STATE REACTION-RATE PROFILE

By assuming that the concentration of species α can be expanded in a Taylor series in terms of its concentration and derivatives about the position $x = 0$, the dependence of the reaction-rate profile in the inhomogeneous diffusional barrier on the position variable under nonsteady-state conditions was presented in Eqs. 12*a–e*. In this section, corresponding expressions will be obtained for steady states, valid when diffusion coefficients are constants and coupling between fluxes of different species is ignored. Instead of solving the nonlinear differential Eq. 7, the procedure involves finding solution of Eq. 18. Thus, both the concentration profiles and the reaction-rate profiles in the inhomogeneous region are obtained. As shown here, concentration and reaction-rate profiles can be computed to any order of powers of the position variable that one may desire, subject to an approximation presented in this section. Since the solutions on either side of the diffusional barrier are well stirred, the solutions on either side of the barrier can be regarded as homogeneous; concentrations of the species remain uniform up to a region close to what one may call the boundaries of the membrane. It is the inhomogeneous character of the diffusional barrier that is responsible for the maintenance of concentration profiles and reaction-rate profiles under steady state.

Let the plane of the barrier where inhomogeneity begins correspond to the value of the position variable $x = 0$. The boundaries of inhomogeneity extend from $x = 0$ to $x = h$, where h is the thickness of the diffusional barrier. Since the concentrations of the species are analytical functions of the position variable, one may assume that the concentrations of the three species participating in the reaction at location x may be represented in terms of the concentrations of the species at $x = 0$ as:

$$C_\alpha(x) = C_\alpha(0) + a_1x + \sum_{i=2} a_ix^i \quad \text{(Eq. 36a)}$$

$$C_\beta(x) = C_\beta(0) + b_1x + \sum_{i=2} b_ix^i \quad \text{(Eq. 36b)}$$

$$C_\gamma(x) = C_\gamma(0) + c_1x + \sum_{i=2} c_ix^i \quad \text{(Eq. 36c)}$$

$$b_i = (1/i!)[d^iC_\beta/dx^i]|_{x=0} \quad \text{(Eq. 36d)}$$

Substituting Eqs. 36*a–d* into Eq. 2, one obtains:

$$J_R^*(x) = \sum_{i=0} S_ix^i \quad \text{(Eq. 37a)}$$

$$S_0 = J_R^*(0) = k_1C_\alpha(0)C_\beta(0) - k_2C_\gamma(0) \quad \text{(Eq. 37b)}$$

$$S_1 = k_1\{C_\beta(0)a_1 + C_\alpha(0)b_1\} - k_2c_1 \quad \text{(Eq. 37c)}$$

$$S_2 = k_1\{C_\beta(0)a_2 + C_\alpha(0)b_2 + a_1b_1\} - k_2c_2 \quad \text{(Eq. 37d)}$$

$$S_3 = k_1\{C_\beta(0)a_3 + C_\alpha(0)b_3 + a_1b_2 + a_2b_1\} - k_2c_3 \quad \text{(Eq. 37e)}$$

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Validity of Eqs. 9*a–b* under steady states and the assumptions about constant diffusion coefficients requires that the Taylor expansion coefficients a_i, b_i, and c_i are related by the relations:

$$b_i = (D_\alpha D_\beta)a_i \qquad b_1 = (D_\alpha/D_\beta)a_1 + p \quad \text{(Eq. 38a)}$$

$$c_i = -(D_\alpha/D_\gamma)a_i \qquad c_1 = -(D_\alpha/D_\gamma)a_1 + q \quad \text{(Eq. 38b)}$$

$$p = (1/D_\beta)\{J_\alpha(x) - J_\beta(x)\} \quad \text{(Eq. 38c)}$$

$$q = -(1/D_\gamma)\{J_\alpha(x) + J_\gamma(x)\} \quad \text{(Eq. 38d)}$$

Both p and q are constants.

Let the solution satisfying the nonlinear differential Eq. 18 be:

$$G(x) = \sum_{i=0} m_ix^i \quad \text{(Eq. 39)}$$

If Eq. 39 satisfies Eq. 18 identically, and if one has means of evaluating the coefficients of every power of x of Eq. 39, one has solved the problem. Substitution of Eq. 39 in Eq. 18 yields the relations:

$$\mu\left\{\sum_{i=0}\sum_{j=0} m_im_jx^{i+j}\right\} + \eta^2\sum_{i=0} m_ix^i + \sigma\sum_{i=0} m_ix^{i+1} + Ax^2 + Bx + J_R^*(0) - \sum_{i=2} i(i-1)m_ix^{i-2} = 0 \quad \text{(Eq. 40a)}$$

$$J_R^*(x) = G'' = \sum_{i=2} i(i-1)m_ix^{i-2} = \sum_{i=0} S_ix^i \quad \text{(Eq. 40b)}$$

Equating the coefficients of like powers of x, one obtains:

$$\mu m_0{}^2 + \eta^2m_0 = 2m_2 - J_R^*(0) \quad \text{(Eq. 41a)}$$

$$2\mu m_0m_1 + \eta^2m_1 + \sigma m_0 + B - S_1 = 0 \quad \text{(Eq. 41b)}$$

$$\mu(m_1{}^2 + 2m_0m_2) + \eta^2m_2 + \sigma m_1 + A - S_2 = 0 \quad \text{(Eq. 41c)}$$

$$2\mu(m_0m_3 + m_1m_2) + \eta^2m_3 + \sigma m_2 - S_3 = 0 \quad \text{(Eq. 41d)}$$

$$\mu\sum_{\substack{i,j=0\\ i+j=k}}^{k} m_im_j + \eta^2m_k + \sigma m_{k-1} - S_k = 0 \quad k \geqslant 3 \quad \text{(Eq. 41e)}$$

Since, $\lim_{x\to 0} J_R^*(x) = J_R^*(0) = 2m_2$, one has from Eq. 41*a* that:

$$m_0 = -(\eta^2/\mu) \quad \text{(Eq. 42)}$$

or

$$m_0 = 0 \quad \text{(Eq. 43)}$$

One has from Eq. 41*c* that:

$$m_1 = -(\sigma/2\mu) \pm (\sigma/2\mu)[1 - (4\mu A/\sigma^2) + (4\mu/\sigma^2)\{S_2 - \eta^2m_2 - 2\mu m_0m_2\}]^{1/2}$$
$$= -(q_\alpha + q_\beta)/2 \pm {}^1/_2[(q_\alpha - q_\beta)^2 + (4/\mu)(S_2 - \eta^2m_2) - 8m_0m_2]^{1/2} \quad \text{(Eq. 44)}$$

Recall that (*cf.*, Eqs. 48*a–d*):

$$S_2 - \eta^2m_2 = S_2 - D_\alpha\eta^2a_2 = k_1a_1b_1 \quad \text{(Eq. 45)}$$

Hence, when $m_0 = 0$ or $m_2 = 0$,

$$m_1 = -(q_\alpha + q_\beta)/2 \pm {}^1/_2[(q_\alpha - q_\beta)^2 + 4D_\alpha D_\beta a_1 b_1]^{1/2} \quad \text{(Eq. 46)}$$

Upon rearrangement and squaring both sides, one obtains:

$$D_\alpha D_\beta a_1 b_1 = m_1^2 + m_1(q_\alpha + q_\beta) + q_\alpha q_\beta \quad \text{(Eq. 47a)}$$

$$m_2 \text{ or } m_0 = 0 \quad \text{(Eq. 47b)}$$

From Eqs. 38*a–d*, the constant coefficients S_i's of Eqs. 37*a–e* may be reexpressed as:

$$S_0 = k_1 K_\alpha K_\beta - k_2 K_\gamma \quad \text{(Eq. 48a)}$$

$$S_1 = D_\alpha \eta^2 a_1 + k_1 C_\alpha(0) p - k_2 q \quad \text{(Eq. 48b)}$$

$$S_2 = D_\alpha \eta^2 a_2 + k_1 a_1 b_1 \quad \text{(Eq. 48c)}$$

$$S_k = D_\alpha \eta^2 a_k + k_1(D_\alpha/D_\beta) \sum_{\substack{i,j=1 \\ i+j=k}}^{k-1} a_i a_j + k_1 p a_{k-1} \quad \text{(Eq. 48d)}$$

where $k \geq 3$, $p = (q_\beta - q_\alpha)/D_\beta$, and $q = (q_\alpha + q_\gamma)/D_\gamma$. In the limit x tending to zero, one has from Eqs. 17*a–d*, when $m_0 = -(\eta^2/\mu)$,

$$C_\alpha(0) = K_\alpha - (\eta^2 D_\beta/k_1) \quad \text{(Eq. 49a)}$$

$$C_\beta(0) = K_\beta - (\eta^2 D_\alpha/k_1) \quad \text{(Eq. 49b)}$$

$$C_\gamma(0) = K_\gamma + (\eta^2/\mu D_\gamma) \quad \text{(Eq. 49c)}$$

$$K_\sigma = C_\sigma^I \qquad \sigma = \alpha,\beta,\gamma \quad \text{(Eq. 49d)}$$

When $m_0 = 0$, one has

$$K_\sigma = C_\sigma(0) = C_\sigma^I \qquad \sigma = \alpha,\beta,\gamma \quad \text{(Eq. 50)}$$

where C_σ^I is the concentration of species σ in the bulk homogeneous region on the left-hand side of the diffusional barrier. One may verify that S_0, the reaction rate at location zero, $J_R^*(0)$, is given by Eq. 48*a*, irrespective of whether Eq. 49 or 50 is utilized in the expression 37*b*.

Utilizing the expressions for the parameters presented in Eqs. 18, 13, and 12 into Eq. 48*b*, one evaluates:

$$B - S_1 = \eta^2 D_\alpha a_1 + \eta^2 q_\beta + (2k_2 q_\gamma/D_\gamma)$$
$$= \eta^2 D_\beta b_1 + \eta^2 q_\alpha + (2k_2 q_\gamma/D_\gamma) \quad \text{(Eq. 51a)}$$

when $m_0 = -(\eta^2/\mu)$, and

$$S_1 - B = \eta^2 D_\alpha a_1 - \eta^2 q_\alpha - (2k_2 q_\gamma/D_\gamma)$$
$$= \eta^2 D_\beta b_1 - \eta^2 q_\beta - (2k_2 q_\gamma/D_\gamma) \quad \text{(Eq. 51b)}$$

when $m_0 = 0$.

From Eqs. 51*a–b* and 41*b*, one obtains:

$$m_1 = D_\alpha a_1 + (2k_2 q_\gamma/D_\gamma \eta^2) - q_\alpha \quad \text{(Eq. 52a)}^1$$
$$= D_\beta b_1 + (2k_2 q_\gamma/D_\gamma \eta^2) - q_\beta \quad \text{(Eq. 52b)}$$

when $m_0 = -(\eta^2/\mu)$, and

$$m_1 = D_\alpha a_1 - q_\alpha + (2k_2 q_\gamma/D_\gamma \eta^2) \quad \text{(Eq. 52c)}$$
$$= D_\beta b_1 - q_\beta + (2k_2 q_\gamma/D_\gamma \eta^2) \quad \text{(Eq. 52d)}$$

when $m_0 = 0$. Equations 52*a–d* yield the result: $(D_\alpha a_1 - D_\beta b_1 = q_\alpha - q_\beta)$, in agreement with Eqs. 12*a–e* and 38*a–d*.

Equations 52*a–d* yield m_1 in terms of a_1 or b_1. It is convenient to express m_1 in terms of experimentally available quantities. This is achieved by utilizing the following relation, easily derived from Eq. 46, valid when $m_0 = 0$:

$$D_\alpha D_\beta a_1 b_1 = (m_1 + q_\alpha)(m_1 + q_\beta) \quad \text{(Eq. 53)}$$

Substituting Eqs. 52*c* and 52*d* into Eq. 53, one obtains:

$$D_\alpha a_1 + D_\beta b_1 = (2k_2 q_\gamma/D_\gamma \eta^2) \qquad m_0 = 0 \quad \text{(Eq. 54)}$$

Thus, when $m_0 = 0$, one evaluates:

$$D_\alpha a_1 = (q_\alpha - q_\beta)/2 + (k_2 q_\gamma/D_\gamma \eta^2) \quad \text{(Eq. 55a)}$$

$$D_\beta b_1 = (q_\beta - q_\alpha)/2 + (k_2 q_\gamma/D_\gamma \eta^2) \quad \text{(Eq. 55b)}$$

$$m_1 = -(q_\alpha + q_\beta)/2 + (k_2 q_\gamma/D_\gamma \eta^2) \quad \text{(Eq. 55c)}$$

When $m_0 = -(\eta^2/\mu)$, one obtains from Eq. 41*c*:

$$(m_1 + q_\alpha)(m_1 + q_\beta) = D_\alpha D_\beta a_1 b_1 + (2\eta^2 m_2/\mu) \quad \text{(Eq. 56)}$$

Utilizing Eqs. 52*a* and 52*b* in Eq. 56, one evaluates:

$$D_\alpha a_1 + D_\beta b_1 = (\eta^4 D\gamma m_2/k_2 \mu q_\gamma) - (2k_2 q_\gamma/D_\gamma \eta^2) \quad \text{(Eq. 57a)}$$

$$D_\alpha a_1 - D_\beta b_1 = q_\alpha - q_\beta \quad \text{(Eq. 57b)}$$

Therefore, when $m_0 = -(\eta^2/\mu) \neq 0$, one has:

$$D_\alpha a_1 = (q_\alpha - q_\beta)/2 - (k_2 q_\gamma/D_\gamma \eta^2) + \zeta \quad \text{(Eq. 58a)}$$

$$D_\beta b_1 = (q_\beta - q_\alpha/2 - (k_2 q_\gamma/D_\gamma \eta^2) + \zeta \quad \text{(Eq. 58b)}$$

$$m_1 = -(q_\alpha + q_\beta)/2 + (k_2 q_\gamma/D_\gamma \eta^2) + \zeta \quad \text{(Eq. 58c)}$$

where $\zeta = \eta^4 D_\gamma m_2/(2\, k_2 \mu q_\gamma)$.

For Eqs. 51–58, the parameters B and η^2 are given by Eq. 18, S_1 is given by Eq. 37*c* and $K_\sigma = C_\sigma(0)$, when $m_0 = 0$, and $K_\alpha = C_\alpha(0) + (\eta^2 D_\beta/k_1)$, $K_\beta = C_\beta(0) + (\eta^2 D_\alpha/k_1)$ when $m \neq 0$.

From Eq. 40*b*, one has, by equating coefficients of like powers of position variables, the relation:

$$S_k = (k + 2)(k + 1) m_{k+2} \qquad k \geq 0 \quad \text{(Eq. 59)}$$

From the nonlinear differential equation:

$$D_\alpha C_\alpha'' = J_R^*(x) \quad \text{(Eq. 60)}$$

one has:

$$S_k = (k + 2)(k + 1) D_\alpha a_{k+2} \quad \text{(Eq. 61)}$$

Thus,

$$D_\alpha a_k = m_k \qquad k \geq 2 \quad \text{(Eq. 62)}$$

Equations 41*a–e* can be reexpressed for $k \geq 3$ as:

$$S_k - m_k(\eta^2 + 2\mu m_0) = \mu \sum_{\substack{i,j=1 \\ i+j=k}}^{k-1} m_i m_j + \sigma m_{k-1} \quad \text{(Eq. 63)}$$

Thus, knowledge of $m_0, m_1, m_2 \ldots m_{k-1}$ completely determines the right-hand side of Eq. 63. The left-hand side of Eq. 63 equals $(S_k \pm \eta^2 m_k)$, the positive sign being applicable when $m_0 = -(\eta^2/\mu)$ and the negative sign being valid when $m_0 = 0$. From Eqs. 59 and 63, one has:

$$S_k = \mp\{\eta^2/k(k-1)\} S_{k-2} + \mu \sum_{\substack{i,j=1 \\ i+j=k}}^{k-1} m_i m_j + \sigma m_{k-1} \quad \text{(Eq. 64)}$$

Equation 64 expresses the fact that knowledge of lower order coefficients, S_i's, enables one to compute higher order coefficients. For example, when $k = 4$, one has:

$$S_4 = \mp(\eta^2/12) S_2 + (\mu/4) S_0^2 + (S_1/6)\mu(q_\alpha + q_\beta + 2m_1) \quad \text{(Eq. 65a)}$$

$$\mu = (k_1/D_\alpha D_\beta) \quad \text{(Eq. 65b)}$$

In Eq. 65*a*, the negative sign is valid when $m_0 = -(\eta^2/\mu)$ and the positive sign is valid when $m_0 = 0$.

To summarize the results obtained so far, the interest is in computing the reaction-rate profiles in the inhomogeneous diffusional barrier as a function of the position variable, presented in Eqs. 37*a–e*, satisfying the differential Eq. 18. Equations 37*a–e* are of little use unless one can compute the coefficients in terms of experimen-

[1] *Note added in proof:* The value of m_1 presented in Eq. 52 is only an *approximation* and is not a unique solution. m_1 equals $-q_\alpha$, $-q_\beta$, or $\pm q_\gamma$, respectively, when a_1, b_1, or c_1 vanishes. m_1 assumes null value when diffusion coefficients vary with position. When diffusion coefficients are constants and a_1, b_1, and c_1 are nonvanishing, determination of a unique value for m_1 has not yet been achieved.

tally measurable quantities. Equation 64 enables one to compute any desired coefficient $S_k (k \geq 3)$, in terms of already obtained lower-order coefficients.

When $m_0 = 0$, one has obtained:

$$S_0 = k_1 K_\alpha K_\beta - k_2 K_\gamma \qquad K_\sigma = C_\sigma^I \qquad \text{(Eq. 66a)}$$

$$S_1 = m_1 \eta^2 + B \qquad \text{(Eq. 66b)}$$

$$S_2 = \mu\{m_1^2 + m_1(q_\alpha + q_\beta) + q_\alpha q_\beta\} + (\eta^2 S_0/2) \qquad \text{(Eq. 66c)}$$

$$S_3 = \eta^2 m_3 + (S_0\mu/2)\{q_\alpha + q_\beta + 2m_1\} \qquad \text{(Eq. 66d)}$$

$$S_k = \eta^2 m_k + \mu\{q_\alpha + q_\beta + 2m_1\} m_{k-1} + \mu \sum_{\substack{i,j=2 \\ i+j=k}}^{k-2} m_i m_j \qquad k \geq 3 \qquad \text{(Eq. 66e)}$$

The knowledge of the rate constants (k_1 and k_2), diffusion coefficients (D_α, D_β, and D_γ) of the three species participating in the reaction measured in homogeneous bulk phase, concentrations of these species (K_σ) in the left-hand side of the membrane, and the fluxes of the species (q_σ) measured in the absence of the other two species across the same diffusional barrier enables one to compute S_0, S_1, and S_2 using Eqs. 66*a–e*, valid when $m_0 = 0$. The value of m_1 required for the said computation is presented in Eq. 55*c*.

When $m_0 = -(\eta^2/\mu)$, one has:

$$S_0 = k_1 K_\alpha K_\beta - k_2 K_\gamma \qquad \text{(Eq. 67a)}$$

$$S_1 = -m_1 \eta^2 + B - \eta^2(q_\alpha + q_\beta) \qquad \text{(Eq. 67b)}$$

$$S_2 = \mu\{m_1^2 + m_1(q_\alpha + q_\beta) + q_\alpha q_\beta\} - (\eta^2 S_0/2) \qquad \text{(Eq. 67c)}$$

$$S_3 = -m_3 \eta^2 + (S_0\mu/2)\{q_\alpha + q_\beta + 2m_1\} \qquad \text{(Eq. 67d)}$$

$$S_k = -m_k \eta^2 + \mu(q_\alpha + q_\beta + 2m_1) m_{k-1} + \mu \sum_{\substack{i,j=2 \\ i+j=k}}^{k-2} m_i m_j \qquad k \geq 3 \qquad \text{(Eq. 67e)}$$

In Eqs. 67*a–e*, $K_\sigma = C_\sigma^I \neq C_\sigma(0)$. Thus, $S_0 = 2m_2$ can be computed from knowledge of rate constants k_1 and k_2 and concentrations of the species in the left-hand side of the membrane; m_1 should be computed using Eq. 44. The parameter η^2 required for the calculation is obtained from Eq. 18. Knowledge of S_0, S_1, and S_2, obtained using Eqs. 67*a–e*, can be utilized to obtain any S_k as indicated in Eqs. 64 and 65*a–b*. However, one needs knowledge of S_2 to solve for m_1 using Eq. 44, and knowledge of m_1 is required to solve for S_2 as indicated in Eq. 67*c*. Thus, in the general case, when $m_0 \neq 0$ and $m_2 \neq 0$, one must resort to an iterative procedure suggested elsewhere (12, 13). It is convenient to make the approximation that when $m_0 \neq 0$, $m_2 = 0$ and compute m_1 using Eq. 58*c*.

DISCUSSION

The analysis presented in this paper is based on the assumption that the three species transporting across a diffusional barrier can participate in a reaction of the type presented in Eq. 1. In the second section, it was shown that the reaction-rate profile in the inhomogeneous region can be expressed as a polynomial in the position variable, presented in Eqs. 12*a–e*, satisfying the dictation of equations of continuity when the three basic assumptions mentioned in the section are valid. It was shown that if the departure of the state of the system from stationary state can be regarded as small, it suffices to know the reaction-rate profile in the inhomogeneous diffusional barrier under conditions of stationary state.

In the third section, a nonlinear differential equation (Eq. 18) for a function $G(x)$, related to concentration profiles and reaction-rate profiles in the inhomogeneous region, was derived. Equation 18 is valid for stationary states when diffusion coefficients are constants and coupling between fluxes of different species can be ignored. On the basis of the solution of Eq. 18 and the usual assumption incorporated that the reaction is at equilibrium at all locations in the system, it was shown that the reaction cannot be at equilibrium in the inhomogeneous region except when the concentration gradient of one of the reactant species vanishes across the diffusional barrier.

When experiments are conducted that maintain the concentration of one of the reactant species equal on both sides of the membrane, the relation between the flux of the other reactant species and the stability constant of the complex was presented in Eq. 32.

In the fourth section, the general solution of Eq. 18, when the reaction rate is nonvanishing in inhomogeneous regions, under conditions of stationary state, was obtained. In that section, it was indicated how the reaction-rate profile can be computed to any order of accuracy one desires by using experimentally available information. Thus, when the concentrations of the species at the location where inhomogeneity sets in equal the bulk concentrations [$C_\sigma(0) = C_\sigma^I$; $m_0 = 0$], or when the reaction is at equilibrium at the location where inhomogeneity begins ($m_2 = 0$), one can safely assert that the coefficients S_i's of Eqs. 37*a–e* can be computed and thus are known.

The fluxes of the three species across the diffusional barrier under stationary-state conditions are given by:

$$J_\alpha = -q_\alpha - I(x) \qquad \text{(Eq. 68a)}$$

$$J_\beta = -q_\beta - I(x) \qquad \text{(Eq. 68b)}$$

$$J_\gamma = q_\gamma + I(x) \qquad \text{(Eq. 68c)}$$

$$I(x) = \sum_{i=0} \{S_i/(i+1)\} x^{i+1} \qquad \text{(Eq. 68d)}$$

If the diffusional barrier extends from $x = 0$ to $x = h$, and the flux is measured in the right-hand side of the membrane, the observed fluxes will be:

$$J_\alpha(h) = -q_\alpha - I(h) \qquad \text{(Eq. 69a)}$$

$$J_\beta(h) = -q_\beta - I(h) \qquad \text{(Eq. 69b)}$$

$$J_\gamma(h) = q_\gamma + I(h) \qquad \text{(Eq. 69c)}$$

$$I(h) = I(x = h) \qquad \text{(Eq. 69d)}$$

where $I(h)$ indicates the production or consumption of species in the inhomogeneous region due to the reaction, and h is of the order of about 100 Å for lipid films and many biological membranes.

The experiments of Reuning and Levy (1–3) yielded information about the rate of accumulation of, for example, species α in the second compartment, the nylon bag. Since the flux, J_α^*, indicates the number of moles of α transferred per unit area per unit time, the product J_α^* multiplied by area of the diffusional barrier should equal the rate of accumulation of the species in the nylon bag. Assuming that the area of the diffusional barrier remains constant, measured (dC_α^{II}/dt) is proportional to J_α^*, under conditions of steady state:

$$(dC_\alpha^{II}/dt) = J_\alpha(h)A \qquad \text{(Eq. 70)}$$

If the experiments are conducted with varying amounts of C_β^I in the left-hand side of the diffusional barrier and one measures (dC_α^{II}/dt) for the same diffusional barrier, one can compute $(d/dC_\beta^I)(dC_\alpha^{II}/dt)$. This quantity should equal the right-hand side of Eq. 71 according to the theory presented in this paper, subject to neglect of (h^4) order and higher order terms. The right-hand side of Eq. 71 can be computed from independently obtainable experimental quantities:

$$\frac{d}{dC_\beta^I}\left(\frac{dC_\alpha^{II}}{dt}\right) = -Ah[k_1 C_\alpha^I + h\{(q_\beta - q_\alpha)/2 - (k_2 q_\gamma/D_\gamma \eta^2)\} + (k_1/2)\{\eta^2 h^2 C_\alpha^I + (S_0 h^2/D_\alpha)\}] + 0(h^4) \qquad \text{(Eq. 71)}$$

In obtaining the right-hand side of Eq. 71, one utilizes the relations:

$$m_0 = 0;\ C_\sigma^I = K_\sigma;\ \sigma = \alpha,\beta,\gamma \qquad \text{(Eq. 72a)}$$

$$(dS_0/dK_\beta) = k_1 C_\alpha^I \qquad \text{(Eq. 72b)}$$

$$(dS_1/dK_\beta = (q_\beta - q_\alpha)/2 - (k_2 q_\gamma/D_\gamma \eta^2) \qquad \text{(Eq. 72c)}$$

$$(dS_2/dK_\beta) = (k_1/2)\{\eta^2 K_\alpha + (S_0/D_\alpha)\} \qquad \text{(Eq. 72d)}$$

$$S_0 = k_1 K_\alpha K_\beta - k_2 K_\gamma \qquad \text{(Eq. 72e)}$$

$$S_1 = m_1 \eta^2 + B \qquad \text{(Eq. 72f)}$$

$$S_2 = \mu\{m_1^2 + m_1(q_\alpha + q_\beta) + q_\alpha q_\beta\} + (\eta^2 S_0/2) \qquad \text{(Eq. 72g)}$$

If one now adopts the approximation that (h^3) order and higher order terms may be neglected, it is evident from Eq. 71 that the slope of the plot of the quantity $(Ah)^{-1}\left\{\frac{d}{dC_\beta^I}\left(\frac{dC_\alpha^{II}}{dt}\right)\right\}$ against C_α^I

will equal the negative of the association-rate constant k_1. The intercept of the said plot will enable one to compute the dissociation-rate constant k_2 from knowledge of $q_\alpha, q_\beta, q_\gamma$, and D_γ.

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Pharmacodynamics of Chemotherapeutic Effects: Dose–Time–Response Relationships for Phase-Nonspecific Agents

WILLIAM J. JUSKO

Abstract □ Pharmacodynamic relationships were developed to characterize the necrobiotic effects of phase-nonspecific chemotherapeutic agents which attach irreversibly to cell receptors. The site of drug action is considered to be a specific body compartment, and target cell inactivation by the agent results from a bimolecular drug–receptor interaction. Turnover of cells is assumed to occur by natural synthetic and degradative processes. Based on these premises, a log–linear relationship was evolved to relate the fraction of surviving cells to the drug level–time integral at the pharmacologic site. The integral was shown to be proportional to the dose and independent of the mode of administration when the entire drug level–time course is evaluated. Data from the literature for the effects of cyclophosphamide on three cell systems of mice demonstrate the usefulness and certain therapeutic implications of the equations.

Keyphrases □ Pharmacodynamics—dose–time–response relationships, phase-nonspecific agents □ Chemotherapeutic agents, phase nonspecific—pharmacodynamic model □ Dose–effect relationships—cyclophosphamide on cell systems of mice

Considerable progress has been made in the development of kinetic relationships characterizing pharmacologic effects. Levy (1) showed that the intensity *versus* time course of many clinically observable pharmacologic effects may be described adequately by mathematical expressions based on the kinetics of drug elimination and on the established relationship between amount in the body and response. In turn, the simultaneous use of pharmacologic effect and pharmacokinetic data was shown to be an added dimension in the analysis of pharmacodynamic data (2).

The pharmacologic response to most drugs can be quantitated in a log dose–linear effect manner. Such a relationship is essentially derived from the postulation of reversible interaction between drug and receptor (3, 4). The reversibility aspect of this mechanism precludes application of most classical pharmacodynamic principles to therapy with certain antibiotics, antimetabolites, and alkylating agents. The cytotoxic effects of such agents are usually dependent on the irreversible or covalent incorporation of drug into cell metabolic sites or pathways (5). The lack of a mathematical basis for predicting the clinical effects of chemotherapy and the clinical difficulties in measuring such effects have been partly responsible for the uncertainty involved in the design of appropriate dosage modes and schedules for chemotherapeutic agents (4). The purpose of this report is to develop pharmacodynamic principles that may be of quantitative and predictive value in the therapeutic use of such drugs.

THEORETICAL

A basic pharmacodynamic model for the characterization of the effects of chemotherapeutic agents is shown in Scheme I. The drug is introduced into the central compartment (X_c) using a suitable mode of administration. The site of chemotherapeutic effect (X_t) is considered to be a homogeneous compartment separate from the central volume of distribution. First-order transfer-rate constants between the two compartments are k_{12} and k_{21}, and the elimination-rate constant is k_{el}. A portion of the dose of drug that reaches the pharmacologic site is involved in an irreversible reaction (rate con-

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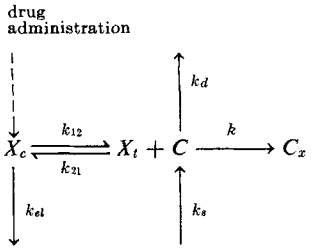

Scheme I

stant k) with receptors of the target cells, which ultimately produces mitotic arrest of these cells (C_x). It is assumed that: (*a*) viable cells increase in number at their natural mitotic rate (k_s), (*b*) viable cells are also subject to physiologic degradation (k_d), and (*c*) the number of cells is equal or directly proportional to the number of receptors.

Considering the bimolecular interaction of drug and cell receptor and the natural cell turnover, the rate of change in quantity of target cells (C) with time can be written as:

$$\frac{dC}{dt} = -k \cdot X_t \cdot C + k_s \cdot C - k_d \cdot C \qquad \text{(Eq. 1)}$$

where X_t is the amount of drug at the receptor site. If S_f is the fraction of surviving cells (C/C_0), then upon integration Eq. 1 becomes:

$$\log S_f = \frac{-k}{2.3} \cdot \int_0^t X_t \cdot dt + \frac{k_g \cdot t}{2.3} \qquad \text{(Eq. 2)}$$

where $k_g = k_s - k_d$, and which is the essential amount–time–response relationship. Since the amount of drug in the receptor compartment will vary with time due to administration and distribution factors, the integral portion of the equation is required (*Appendix*).

If a negligible fraction of the dose is consumed by the chemotherapeutic reaction or:

$$\int_0^t X_t \cdot dt \gg \int_0^t C_x \cdot dt \qquad \text{(Eq. 3)}$$

then the distribution of drug to the tissue site can be approximated by a two-compartment open model. For such a system, it can be shown (*Appendix*) that, independent of the mode of administration of drug,

$$\int_0^\infty X_t \cdot dt = \frac{k_{12} \cdot \text{dose}}{k_{21} \cdot k_{el}} \qquad \text{(Eq. 4)}$$

Therefore, if measurement of S_f is made after all potentially effective drug is eliminated ($t \rightarrow \infty$), substitution of Eq. 4 into Eq. 2 yields the useful relationship:

$$\log S_f = -K_s \cdot \text{dose} + k_g \cdot t/2.3 \qquad \text{(Eq. 5)}$$

where:

$$K_s = k \cdot k_{12}/2.3 \cdot k_{21} \cdot k_{el} \qquad \text{(Eq. 6)}$$

A lethality constant for each drug–cell system can be represented by:

$$\text{ED}_{90} = 1/K_s \qquad \text{(Eq. 7)}$$

where ED_{90} is the dose increment of drug required to reduce the fraction of surviving cells by one order of magnitude.

The exponential form of Eq. 5 is:

$$S_f = (e^{-2.3 \cdot K_s \cdot \text{dose} \cdot e^{k_g \cdot t}}) \qquad \text{(Eq. 8)}$$

If the drug is administered in a dosage regimen consisting of doses of D_0 at time intervals of τ, then the cumulative effect of n doses can be calculated from:

$$S_f^n = (e^{-2.3 \cdot K_s \cdot D_0 \cdot e^{k_g \cdot \tau}})^n \qquad \text{(Eq. 9)}$$

where cumulative time is equal to $n\tau$. Essentially complete cell loss will eventually occur if D_0 and τ are chosen so that:

$$(e^{-2.3 \cdot K_s \cdot D_0 \cdot e^{k_g \cdot \tau}}) < 1 \qquad \text{(Eq. 10)}$$

or $2.3 \cdot K_s \cdot D_0 > k_g \cdot \tau$, which fit the criterion of:

$$\lim_{n \rightarrow \infty} S_f^n = 0 \qquad \text{(Eq. 11)}$$

EXPERIMENTAL

The derived relationships can be illustrated using the data of van Putten and Lelieveld (6). These investigators obtained cell survival data in mice using colony-forming units of osteosarcoma cells, isogenic bone marrow stem cells, and chimaera spleen cells. A portion of the data obtained 18–24 hr. after intraperitoneal doses of cyclophosphamide was used in the present report.

RESULTS AND DISCUSSION

The essential dose–time–response relationship, which was evolved to quantitate the effects of chemotherapeutic agents, is Eq. 5. This equation predicts a log-linear relationship between the fraction of surviving cells and the dose of drug. The slope of the curve, K_s, will be directly dependent on the affinity of the receptor for the drug and the distribution and elimination properties of the active agent (Eq. 6). The curve will intercept the ordinate at an S_f value of unity if the generation rate of the cell system is small and the time interval between initial drug administration and cell survival measurement is soon after all active drug has been eliminated. Elimination may be due to the usual biotransformation and excretory processes; but for chemotherapeutic agents, it also may be the result of interaction of drug with cell constituents not required for the integrity or reproduction of the target cells.

The surviving fraction of cells, rather than the inactivated fraction, was chosen as the index of chemotherapeutic effect since it is usually possible to measure directly only the former (6). However, the lethality constant, ED_{90}, serves as a proportionality factor for comparing the cytotoxic effect of a drug on various cell systems or for comparison of effects of various drugs on a single cell system.

A linear log dose–effect relationship can be expected whether or not cell survival measurement is made before all effective drug is

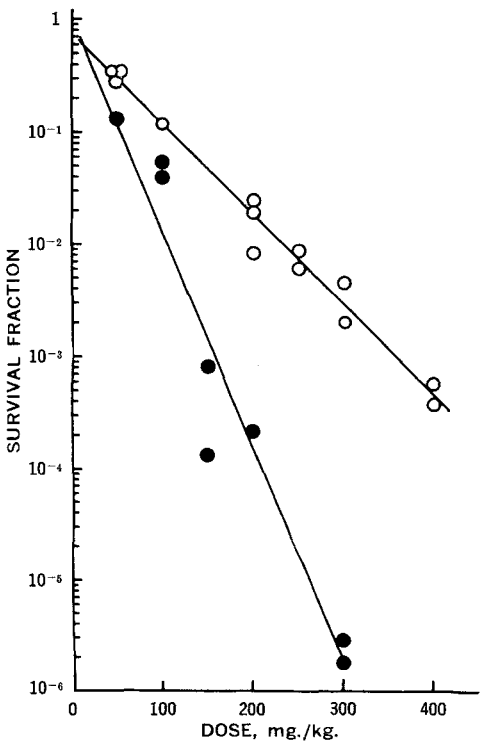

Figure 1—*Survival curves for chimaera spleen cells* (○) *and osteosarcoma cells* (●) *after intraperitoneal administration of single doses of cyclophosphamide to mice* (*data from* Reference 6).

Table I—Dose–Survival Parameters of Three Mouse Cell Systems after Cyclophosphamide Treatment

Cell Type	Mode of Drug Administration	Intercept, $k_g \cdot t/2.3$	Dose–Survival Constant, $K_s \times 10^3$	ED_{90} Dose, mg./kg.
Normal bone marrow	Single injection	0.4	3.43	292
	Multiple dose[a]	0.6[b]	2.88	347
Chimaera spleen	Single injection	0.8[b]	8.00	125
	Multiple dose[a]	0.5[b]	7.44	134
Osteosarcoma	Single injection	0.3[b]	1.88	532

[a] Four equal fractional doses at 2.5-hr. intervals. [b] Not significantly different from 1.0 ($p > 0.05$).

removed from the body. This is due to the proportionality of the drug level–time integral to dose at a specific time for each mode of drug administration. However, a constant and maximum slope, independent of the mode of drug administration, is obtained from dose–survival data only upon elimination of essentially all effective chemotherapeutic agent from the body. If cell survival analysis is performed prematurely, then the apparent slope value will underestimate the potential effectiveness of the drug.

Theoretically, it is possible to generate an apparent drug–receptor affinity constant, k, if actual tissue drug levels are directly measured. This experimental data could then be used to obtain the integral portion of Eq. 2 or to evolve the distribution- and elimination-rate constants in Eq. 6. Such pharmacodynamic refinements are most suitable for cell-culture systems, but they are also technically feasible for many tissue sites. The assumption that the receptors are contained within a homogeneous tissue compartment must be retained but on a more microscopic basis.

The log effect–dose relationship of Eq. 5 is essentially model independent. The necrobiotic effect of a drug will usually be proportional to the dose, regardless of the target site (*Appendix*). However, the proportionality constant, K_s, will vary with target site since the constant is dependent, in part, on the distribution of the agent to the site. Therefore, for drugs such as alkylating agents that bind strongly to cell receptors (5) and are usually eliminated rapidly, it is advantageous to utilize regional chemotherapeutic measures to increase distribution of the agent to localized or poorly perfused tumor sites (7, 8). This of course, also reduces exposure of the normal host cells to the dose of agent.

If the drug administered is a compound such as cyclophosphamide, which must be biotransformed to an active alkylating agent (9), then the dose factor of Eq. 5, assuming first-order kinetics, can be calculated from:

$$\text{dose} = k_f \cdot A^0/K_{el} \qquad \text{(Eq. 12)}$$

where k_f is the formation-rate constant for the alkylating agent, A^0 is the amount of precursor administered, and K_{el} is the rate constant for precursor elimination. A similar modification of Eq. 5 can be made for an absorption process where a constant fraction of nonparenterally administered drug might be considered as the dose.

Cyclophosphamide Data Analysis—Survival data for two cell systems of mice after treatment with intraperitoneal doses of cyclophosphamide are shown in Fig. 1. The sensitivity of the cell survival assay is demonstrated by the S_f values which extend over as much as six orders of magnitude. There is essentially a linear relationship between the logarithm of the fraction of surviving cells and the dosage of the drug as predicted by Eq. 5. The dose–response curves were similar in shape for vinblastine and radiation therapy (6). Furthermore, alkylating agents and radiation therapy produce log S_f–dose curves in *in vitro* cell culture systems which mimic the *in vivo* cell survival patterns (5).

Least-squares regression values for the data with cyclophosphamide were provided by van Putten and Lelieveld (6). These results were converted into the parameters of Eqs. 5 and 7 and are listed in Table I. The ordinate intercept values, except for one instance, did not differ significantly from unity. This indicates that the rate of generation of new cells (k_s) was not an appreciable factor in this set of data and that the time chosen to assay for cell survival (18–24 hr.) was sufficient to permit all drug to be eliminated. The latter is reasonable since, in mice, the alkylating metabolites of cyclophosphamide are known to be almost totally eliminated within 4 hr. of parenteral administration of 200 mg./kg. of drug (9).

The dose–survival constants (K_s) and lethality constants (ED_{90}) listed in Table I show marked differences for the three cell systems studied. These values, according to Eq. 6, reflect cell receptor differences in affinity for the drug as well as distribution factors responsible for drug accessibility to the pharmacologic sites. Also shown in Table I are K_s and ED_{90} values obtained in two of the cell systems when the drug was administered in four fractionated doses. The small differences in effect between the two modes of drug administration were not statistically significant (6).

Therapeutic Implications—The chemotherapeutic effects of multiple-dose administration of drug can be quantitated with Eq. 8. To elicit remission of the target cell mass, it is necessary to choose the dose and dosing interval so that the rate of cell kill is greater than the rate of cell regeneration, as shown by Eq. 10. This relationship also can be applied in the converse manner to protect certain host cells. To prevent inordinate loss of the most sensitive normal tissue, the dose and time interval must reflect: $2.3 \cdot K_s \cdot D_0 < k_g \cdot \tau$, where the constants apply to the host cells. The total number of doses required to eradicate completely the target cells will be dependent on the ability of a small number of such cells to resist other rejection mechanisms of the body.

The relationships evolved from the pharmacodynamic model (Eq. 5 and *Appendix*) predict that the *total* lethal effect of a given dose of drug will be independent of the mode of administration. This is, however, with the reasonable assumption that only a minute portion of the dose is sequestered at the receptor site. The relative usefulness of different modes of chemotherapeutic drug administration, such as slow infusion compared to rapid intravenous injection, has been a controversial question (10, 11). It is, therefore, of considerable interest that, as predicted by the model, subdivision of cyclophosphamide into four fractional doses did not significantly modify the dose–effect constants of the drug (Table I). Such results are most likely to occur for alkylating agents which are capable of inactivating cells during all phases of the mitotic cycle (5, 12).

These data and equations do not constitute a recommendation for an arbitrary mode of drug administration. A drug with side effects that can be quantitated in the classical log dose–effect manner should be given in fractionated doses. This is also true for chemotherapeutic agents that are cytotoxic during a limited phase of the cell mitotic cycle (12). And, not of least importance, biopharmaceutical factors may dictate use of a particular mode or route of administration.

The pharmacodynamic model proposed is a greatly simplified version of an extremely complex *in vivo* situation. Many factors can modify its direct application. For example, a general expression such as Eq. 5 will not be directly useful if the pharmacokinetics of the drug are dose dependent, if the affinity of the receptors for the drug change with time, or if distribution parameters of the target cells are perturbed during the course of drug therapy. However, the evolved mathematical relationships should prove of value for initial estimation of drug dosage schedules which maximize chemotherapeutic effect and reduce the incidence or severity of undesired effects. In addition, it is likely that a similar pharmacodynamic approach can be used to characterize the effects of other pharmacologic agents which elicit their effects through an apparently irreversible reaction with cell receptors.

APPENDIX

Definition of Tissue Level–Time Integrals—With the assumption that the receptors take up a negligible amount of drug, the time course of drug levels in compartment X_t after rapid intravenous injection can be written (10, 13) as:

$$X_t = \text{dose}\,(C_1 \cdot e^{-\alpha t} + C_2 \cdot e^{-\beta t}) \qquad \text{(Eq. A1)}$$

where $C_1 = k_{12}/(\beta - \alpha)$, $C_2 = k_{12}/(\alpha - \beta)$, and $\alpha \cdot \beta = k_{21} \cdot k_{el}$. The Laplace transform of Eq. A1 is:

$$L\{X_t\} = \text{dose}\left(\frac{C_1}{s + \alpha} + \frac{C_2}{s + \beta}\right) \qquad \text{(Eq. A2)}$$

or

$$L\{X_t\} = \text{dose} \cdot g(s) \qquad \text{(Eq. A3)}$$

If, instead of the unit dose impulse, drug is administered by a time-dependent transfer function, $F(t)$, which has a transform, $f(s)$, such that[1]:

$$\lim_{s \to 0} f(s) = \text{dose} \qquad \text{(Eq. A4)}$$

then Eq. A3, upon convolution of the transfer function, becomes:

$$L\{X_t\} = g(s) \cdot f(s) \qquad \text{(Eq. A5)}$$

Since it can be shown from Laplace theory that (13):

$$\int_0^\infty X_t \cdot dt = \lim_{s \to 0} \left[\left(\frac{C_1}{s + \alpha} + \frac{C_2}{s + \beta} \right) \cdot f(s) \right] \qquad \text{(Eq. A6)}$$

then evaluation of the limit, including substitution of Eq. A4 as well as for C_1, C_2, and $\alpha \cdot \beta$, yields the solution:

$$\int_0^\infty X_t \cdot dt = k_{12} \cdot \text{dose} / k_{21} \cdot k_{el} \qquad \text{(Eq. A7)}$$

which is also shown as Eq. 4 in the text.

Equation A7 was evolved previously by Gibaldi (10) for the instances when drug is administered by rapid intravenous injection and by constant-rate infusion. With a general proof similar to that presented here, however, it can be shown that for any multiple-compartment model describable by linear first-order differential equations with constant coefficients, each drug level–time integral (time $0 \rightarrow \infty$) will be independent of the mode of drug administration and proportional to the dose administered.

Time-Dependent Integrals—If it is necessary to use the time-dependent integral of Eq. 2, the specific equations must be evolved to characterize each mode of drug administration. Applying the Laplace theorum (13):

$$L\left\{ \int_0^t X(t) \cdot dt \right\} = X(s)/s \qquad \text{(Eq. A8)}$$

to the transform for the two-compartment system with rapid dose input (Eq. A2) yields:

$$L\left\{ \int_0^t X_t \cdot dt \right\} = \frac{\text{dose} \cdot C_1}{s(s + \alpha)} + \frac{\text{dose} \cdot C_2}{s(s + \beta)} \qquad \text{(Eq. A9)}$$

for which the antitransform is the integral:

$$\int_0^t X_t \cdot dt = \frac{\text{dose} \cdot C_1}{\alpha}(1 - e^{-\alpha t}) + \frac{\text{dose} \cdot C_2}{\beta} \times (1 - e^{-\beta t}) \qquad \text{(Eq. A10)}$$

This relationship can be substituted into Eq. 2 to calculate the time course of chemotherapeutic effect of a single intravenous dose of drug, providing the distribution and elimination constants of the system are available.

If the transfer function involves first-order input (*i.e.*, drug absorption) with the rate constant k_a:

$$F(t) = k_a \cdot \text{dose} \cdot \exp(-k_a \cdot t) \qquad \text{(Eq. A11)}$$

then the transform is:

$$f(s) = \frac{k_a \cdot \text{dose}}{s + k_a} \qquad \text{(Eq. A12)}$$

Convolution of Eqs. A12 and A3 and utilization of the principle of Eq. A8 yield the integral:

$$\int_0^t X_t \cdot dt = k_a \cdot \text{dose} \cdot C_1 \left[\frac{1}{\alpha k_a} + \frac{e^{-\alpha t}}{\alpha(\alpha - k_a)} - \frac{e^{-k_a \cdot t}}{k_a(\alpha - k_a)} \right] + k_a \cdot \text{dose} \cdot C_2 \left[\frac{1}{\beta k_a} + \frac{e^{-\beta t}}{\beta(\beta - k_a)} - \frac{e^{-k_a \cdot t}}{k_a(\beta - k_a)} \right] \qquad \text{(Eq. A13)}$$

Equations A13 and A10 may be useful for model-simulation purposes but, needless to say, are rather cumbersome to use in evaluation of experimental data. It is, therefore, advantageous to analyze chemotherapeutic effect data in terms of the total effect of a given dose of drug.

[1] For example, see Eq. A12.

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Antagonistic Activities of Derivatives of Cystinyl-tyrosyl-tyrosine to Actions of Oxytocin

Y. ISHIDA, M. ONISHI, T. HIYAMA, and Y. YABUUCHI

Abstract □ Peptides with both a tyrosyl-tyrosine and a cystine or *S*-benzylcysteine residue in the molecule (six peptides of cystinyl-tyrosyl-tyrosine derivatives and five peptides of *S*-benzylcysteinyl-tyrosyl-tyrosine derivatives) were synthesized, purified, and evaluated for antagonistic properties of oxytocin. Almost all the peptides exhibited antagonistic activities to the action of oxytocin on isolated rat uterus. The most active peptide was *N*-dicarbobenzoxy-L-cystinyl-di-(L-tyrosyl-L-tyrosine) with pA_2 of 6.06. This peptide also showed the inhibition of the responses to oxytocin on rat uterus *in situ* and on avian blood pressure. *N*-Carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine with pA_2 of 5.78 did not have the inhibitory actions of oxytocin *in situ*.

Keyphrases □ Oxytocin activity antagonists—peptides □ Cystinyl-tyrosyl-tyrosine derivatives—synthesis, oxytocin antagonists □ *S*-Benzyl-cysteinyl-tyrosyl-tyrosine derivatives—synthesis, oxytocin antagonists □ Peptides—as oxytocin antagonists

In a previous paper (1), it was reported that there are two types of oxytocin antagonists. One type consists of phenolic compounds such as peptides containing tyrosine. The other type is L-cystine diethylester, whose action may be related to the disulfide bond of cystine in the oxytocin molecule. Of the antagonists tested by Ishida and Hara (2), *N*-carbobenzoxy-L-tyrosyl-L-tyrosine ethylester (Cbz-Tyr-TyrOEt) was the most potent antagonist of the action of oxytocin on isolated rat uterus; L-cystine diethylester, which has a disulfide bond in the molecule, was a weaker inhibitor. Overweg *et al.* (3) recently reported that *S*-benzyl-tetrapeptide and *S*-benzyl-octapeptide, acyclic oxytocin intermediates, were antagonists of the uterotonic action of the hormone. Both of these peptides have a *S*-benzyl-cysteinyl residue, and the octapeptide also has a tyrosine residue. These results suggest that peptides with both a tyrosyl-tyrosine and a cystine or *S*-benzylcysteine residue in the molecule should be even more potent. The studies reported in this paper were directed to these possibilities.

It was found that *N*-dicarbobenzoxy-L-cystinyl-di-(L-tyrosyl-L-tyrosine) was more antagonistic than Cbz-Tyr-TyrOEt to the action of oxytocin on isolated rat uterus and inhibited the action of oxytocin on rat uterus *in situ* and the avian vasodepressors. *N*-Carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine was almost as antagonistic as Cbz-Tyr-TyrOEt to the action of the hormone on isolated rat uterus.

Table I—Physicochemical Properties of Peptides

Peptide	Melting Point	$[\alpha]_D^t$ (c, Solvent)	Formula	Analysis, % Calcd.	Analysis, % Found	R_f(Solvent[a])
(Cbz-Cys-Tyr-TyrOEt)$_2$	173°	−84.0[13] (1, DMF)[b]	$C_{62}H_{68}N_6O_{16}S_2$	C, 61.17 H, 5.63 N, 6.90	C, 61.32 H, 6.04 N, 6.87	0.65(a),0.96(c), 0.98(e)
(Cys-Tyr-TyrOEt)$_2$	206–207°	−60.8[13] (0.5, DMF)	$C_{46}H_{56}N_6O_{12}S_2$	C, 58.21 H, 5.95 N, 8.86	C, 57.48 H, 6.20 N, 8.89	0.72(c)
(Cbz-Cys-Tyr-TyrOH)$_2$	183–185°	−41.2[18] (0.5, EtOH)	$C_{58}H_{60}N_6O_{16}S_2$	C, 59.99 H, 5.21 N, 7.24	C, 59.59 H, 5.56 N, 7.18	0.55(e)
(Cbz-Cys-Tyr-TyrNH$_2$)$_2$	207–210°	−95.6[18] (1, DMF)	$C_{58}H_{62}N_8O_{14}S_2$	C, 60.09 H, 5.39 N, 9.67	C, 59.26 H, 5.65 N, 9.81	0.10(a)
Cbz-Cys·Bz-Tyr-TyrOEt	156.5–157°	−19.7[18] (2, EtOH)	$C_{38}H_{41}N_3O_8S$	C, 65.22 H, 5.91 N, 6.01	C, 65.01 H, 6.14 N, 5.76	0.92(d)
Cbz-Cys·Bz-Tyr-TyrOH	188–189°	−12.0[13] (0.5, EtOH)	$C_{36}H_{37}N_3O_8S$	C, 64.36 H, 5.55 N, 6.26	C, 64.22 H, 5.73 N, 6.00	0.74(d)
(Cys-Tyr-TyrOH)$_2$·2H$_2$O	168°	−53.4[13] (1, DMF)	$C_{42}H_{52}N_6O_{14}S$	C, 54.30 H, 5.65 N, 9.05	C, 54.50 H, 5.59 N, 9.18	0.65(b),0.46(c)
Cbz-Cys·Bz-Tyr-TyrNH$_2$	204°	−33.8[13] (1, DMF)	$C_{36}H_{38}N_4O_7S$	C, 64.46 H, 5.71 N, 8.35	C, 64.38 H, 5.79 N, 8.38	0.45(a)
Cys·Bz-Tyr-TyrOEt·H$_2$O	157°	−12.0[13] (1, EtOH)	$C_{30}H_{37}N_3O_7S$	C, 61.73 H, 6.39 N, 7.20	C, 61.40 H, 6.47 N, 7.65	0.75(a)
Cys·Bz-Tyr-TyrOH·HBr	148°	+22.6[18] (1, EtOH)	$C_{28}H_{32}BrN_3O_6S$	C, 54.37 H, 5.78 N, 6.79	C, 54.51 H, 5.54 N, 6.64	0.77(b)
Cys·Bz-Tyr-TyrNH$_2$	148°	+17.4[13] (1, DMF)	$C_{28}H_{32}N_4O_5S$	C, 62.66 H, 6.01 N, 10.44	C, 62.15 H, 6.16 N, 10.10	0.13(a)

[a] Developing solvents for TLC (v/v): (a) benzene–acetone (1:1); (b) *n*-BuOH–AcOH–H$_2$O (4:1:2); (c) *n*-BuOH–AcOH–H$_2$O (40:5:50); (d) *n*-BuOH–AcOH–pyridine–H$_2$O (4:1:1:2); and (e) *n*-BuOH–pyridine–H$_2$O (40:5:50). [b] DMF = dimethylformamide.

Table II—Comparison of Inhibitory Activities of Peptides to Uterotonic Response of Oxytocin on Isolated Rat Uterus[a]

Peptide	Concentration, M	pA_2	Average of All Values
(Cbz-Cys-Tyr-TyrOEt)$_2$	10^{-5}	5.06 ± 0.13 (6)	5.08 ± 0.07 (12)
	3×10^{-5}	5.09 ± 0.07 (6)	
(Cys-Tyr-TyrOEt)$_2$	10^{-5}	5.41 ± 0.10 (8)	5.54 ± 0.09 (18)
	3×10^{-5}	5.67 ± 0.13 (8)	
(Cbz-Cys-Tyr-TyrOH)$_2$	3×10^{-6}	6.09 ± 0.17 (6)	6.06 ± 0.13 (22)
	10^{-5}	5.95 ± 0.16 (8)	
	3×10^{-5}	6.15 ± 0.17 (8)	
(Cys-Tyr-TyrOH)$_2$	10^{-4}	No inhibition	
(Cbz-Cys-Tyr-TyrNH$_2$)$_2$	10^{-5}	4.80 ± 0.11 (4)	4.83 ± 0.07 (8)
	3×10^{-5}	4.86 ± 0.10 (4)	
Cbz-Cys·Bz-Tyr-TyrOEt	10^{-4}	No inhibition	
Cys·Bz-Tyr-TyrOEt	10^{-5}	5.20 ± 0.13 (4)	5.12 ± 0.11 (8)
	3×10^{-5}	5.04 ± 0.18 (4)	
Cbz-Cys·Bz-Tyr-TyrOH	10^{-5}	5.70 ± 0.10 (4)	5.78 ± 0.06 (8)
	3×10^{-5}	5.85 ± 0.07 (4)	
Cys·Bz-Tyr-TyrOH	10^{-5}	4.97 ± 0.10 (4)	5.01 ± 0.07 (8)
	3×10^{-5}	5.05 ± 0.10 (4)	
Cbz-Cys·Bz-Tyr-TyrNH$_2$	3×10^{-5}	4.76 ± 0.11 (4)	4.79 ± 0.07 (8)
	10^{-4}	4.82 ± 0.11 (4)	
Cys·Bz-Tyr-TyrNH$_2$	3×10^{-5}	4.62 ± 0.17 (4)	4.68 ± 0.10 (8)
	10^{-4}	4.74 ± 0.12 (4)	
Cbz-Tyr-TyrOEt	10^{-5}	5.70 ± 0.14 (8)	5.74 ± 0.09 (16)
	3×10^{-5}	5.79 ± 0.11 (8)	

[a] Mean ± SE, number of experiments in parentheses.

EXPERIMENTAL

Synthetic Procedure—The antagonists used were synthesized in this laboratory. *N*-Dicarbobenzoxy-L-cystinyl-di-(L-tyrosyl-L-tyrosine ethylester), (Cbz-Cys-Tyr-TyrOEt)$_2$, was synthesized by coupling 1 mole of *N*-dicarbobenzoxy-L-cystine (5.08 g.) and 2 moles of L-tyrosyl-L-tyrosine ethylester hydrobromide (4) (9.06 g.) by *N*,*N*-dicyclohexylcarbodiimide (yield 9.8 g.). From this peptide ester protected by carbobenzoxy radicals, the next three peptides were obtained as follows: L-cystinyl-di-(L-tyrosyl-L-tyrosine ethylester), (Cys-Tyr-TyrOEt)$_2$, was obtained by decarbobenzoxylation with 25% HBr in glacial acetic acid; *N*-dicarbobenzoxy-L-cystinyl-di-(L-tyrosyl-L-tyrosine), (Cbz-Cys-Tyr-TyrOH)$_2$, was obtained by hydrolysis with 2 *N* NaOH in methanol; and *N*-dicarbobenzoxy-L-cystinyl-di-(L-tyrosyl-L-tyrosine amide), (Cbz-Cys-Tyr-TyrNH$_2$)$_2$, was obtained by ammonolysis in NH$_3$-saturated methanol.

N-Carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine ethylester, Cbz-Cys·Bz-Tyr-TyrOEt, was prepared from *N*-carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosine hydrazide (6.28 g.) and L-tyrosine ethylester hydrochloride (3.04 g.) by the azide method (yield 4.2 g.). Alkaline hydrolysis gave *N*-carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine, Cbz-Cys·Bz-Tyr-TyrOH, from which L-cystinyl-di-(L-tyrosyl-L-tyrosine), (Cys-Tyr-TyrOH)$_2$, was obtained by cleavage with sodium in liquid ammonia and then by oxidation. *N*-Carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine amide, Cbz-Cys·Bz-Tyr-TyrNH$_2$, was prepared by ammonolysis from Cbz-Cys·Bz-Tyr-TyrOEt. *S*-Benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine ethylester (Cys·Bz-Tyr-TyrOEt), *S*-benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine (Cys·Bz-Tyr-TyrOH), and *S*-benzyl-L-cysteinyl-L-tyrosyl-L-tyrosine amide (Cys·Bz-Tyr-TyrNH$_2$) were obtained by treatment of the corresponding *N*-carbobenzoxypeptides with 25% HBr in glacial acetic acid.

The hydrobromide salts of (Cys-Tyr-TyrOEt)$_2$, Cys·Bz-Tyr-TyrOEt, and Cys·Bz-Tyr-TyrNH$_2$ were neutralized with sodium bicarbonate solution and free bases obtained were recrystallized from alcohol.

The structures of the peptides were confirmed by elemental analysis and TLC. Physicochemical properties of these peptides are given in Table I.

PHARMACOLOGICAL PROCEDURES

Isolated Rat Uterus—Experiments were made on strips of smooth muscle suspended in a bath containing 10 ml. of aerated physiological saline solution at 28°. Isotonic recordings were made with a light lever on a smoked drum. The uterine horns were taken from virgin rats of about 200-g. weight, preferably in the stage of natural dioestrus or dioestrus after ovariectomy. These were suspended in the physiological saline solution of Kumagai *et al.* (5) which had the following composition (in grams per liter): NaCl, 8.8; KCl, 0.4; $CaCl_2$, 0.04; $MgCl_2$, 0.018; $NaHCO_3$, 0.4; KH_2PO_4, 0.08; and glucose, 0.5. It contains a lower concentration of calcium chloride than does de Jalon's but does contain magnesium chloride. Since antagonists were insoluble in water, they were used as emulsions with gum arabic. Some acidic peptides were neutralized with sodium bicarbonate, since acidic conditions greatly inhibit the uterotonic action of oxytocin (6). Synthetic oxytocin[1] was used for the assay.

The cumulative dose procedure was used as described by van Rossum (7). The presence of a competitive antagonist results in a parallel shift of the log dose–response curve for oxytocin to higher concentrations. The pA_2 value for competitive inhibition was calculated from the following equation, according to the method of Schild (8, 9) and van Rossum (7), from the shift of the curve:

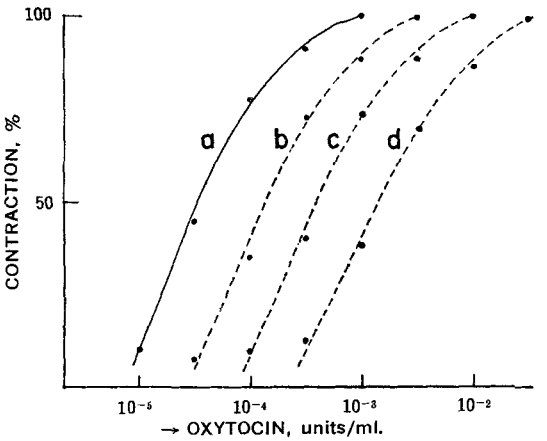

Figure 1—*Typical example of cumulative log dose–response curves of oxytocin in the presence of (Cbz-Cys-Tyr-TyrOH)$_2$ on isolated rat uterus. Key: Curve a, oxytocin alone; and Curves b, c, and d, in the presence of 3 × 10^{-6}, 10^{-5}, and 3 × 10^{-5} M of inhibitor, respectively.*

[1] Syntocinon, Sandoz AG.

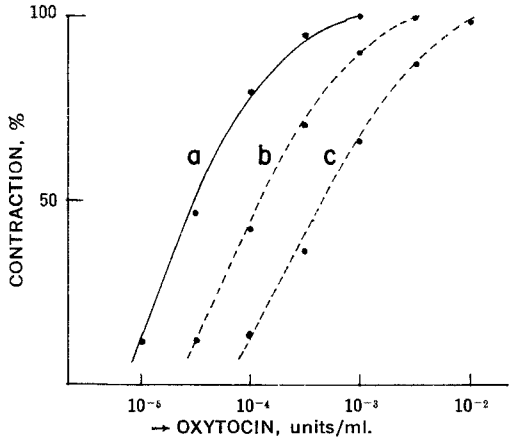

Figure 2—*Typical example of cumulative log dose–response curves of oxytocin in the presence of Cbz-Cys·Bz-Tyr-TyrOH on isolated rat uterus. Key: Curve a, oxytocin alone; and Curves b and c, in the presence of 10^{-5} and 3×10^{-5} M of inhibitor, respectively.*

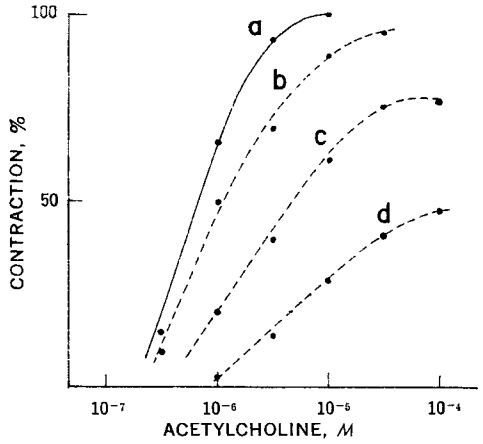

Figure 3—*Typical example of cumulative log dose–response curves of acetylcholine in the presence of Cbz-Cys·Bz-Tyr-TyrOH on isolated rat uterus. Key: Curve a, acetylcholine alone; and Curves b, c, and d, in the presence of 3×10^{-5}, 10^{-4}, and 3×10^{-4} M of inhibitor, respectively. Note the decrease of maximum height of the contractions induced by acetylcholine. This indicates noncompetitive antagonism between acetylcholine and the inhibitor.*

$pA_2 = pA_x + \log(x - 1)$, where pA_x is the negative logarithm of the concentration of the antagonist causing the shift of x. The pD_2' value for noncompetitive antogonism is the negative logarithm of the molar concentration of antagonist that may cause a 50% depression of the maximum concentration of antagonist (7). All concentrations, unless otherwise stated, are expressed in moles in a liter (M) for antagonists.

Rat Uterus *In Situ*—Rats of about 200 g., 1–2 weeks after ovariectomy, received 5 mcg./100 g. of estradiol benzoate each day for 2 days before the experiments. The animals were anesthetized with urethan given in two dosages (first, 100 mg./100 g. i.p., and second, 50 mg./100 g. s.c.). Atropine, 0.1 mg./100 g. s.c., was also administered. Polyethylene tubing filled with normal saline was inserted through a cut in the wall into the lumen of the horn near the cervix, and pressures were recorded from it with a Nihon Kohden Mp-4 pressure transducer and a polygraph, since uterine contractions result in changes in the intrauterine pressure.

Avian Blood Pressure—Leghorn cocks of about 2 kg. were anesthetized with sodium phenobarbital, 200 mg./kg. s.c. The arterial pressure was recorded on a smoked drum with a mercury manometer. Oxytocin and antagonists were injected into the femoral vein.

RESULTS

Isolated Rat Uterus—Almost all the peptides with one or two tyrosyltyrosine residues exhibited antagonistic effects on the uterotonic action of oxytocin as shown in Table II. However, two peptides, (Cys-Tyr-TyrOH)$_2$ and Cbz-Cys·Bz-Tyr-TyrOEt, did not show any antagonistic action to oxytocin even at high concentrations of 10^{-4} M. The pA_2 values were calculated from the parallel shifts of the log dose–response curve of oxytocin. The most active antagonist among these peptides was (Cbz-Cys-Tyr-TyrOH)$_2$, which was more potent than Cbz-Tyr-TyrOEt (2). The log dose–response curves of the rat uterus to oxytocin in the presence of the peptide at concentrations of 3×10^{-6}, 10^{-5}, and 3×10^{-5} M (Fig. 1, Curves b, c, and d) were parallel to that of oxytocin alone (Curve a); and at these concentrations there was no decrease in the maximal response. The pA_2 value of (Cbz-Cys-Tyr-TyrOH)$_2$ was calculated as 6.02. The second most active one was Cbz-Cys·Bz-Tyr-TyrOH with a pA_2 value of 5.78 (Fig. 2).

It was concluded that (Cbz-Cys-Tyr-TyrOH)$_2$ was the most specific antagonist of oxytocin; at higher concentrations of 3×10^{-5}, 10^{-4}, and 3×10^{-4} M, it did not inhibit uterine contractions induced by acetylcholine or bradykinin. As shown in Table III, Cbz-Tyr-TyrOEt inhibited both acetylcholine and bradykinin noncompetitively at over 3×10^{-5} M, and Cbz-Cys·Bz-Tyr-TyrOH only inhibited acetylcholine noncompetitively at over 10^{-4} M (Fig. 3) but had no effect on bradykinin.

On Rat Uterus *In Situ*—Most of the rat preparations described under experimental methods were suitable for assay of antagonism to oxytocin because they did not contract spontaneously during repeated oxytocin infusion. On these preparations, high dosages of oxytocin, 100 and 200 milliunits/kg., had to be used for inducing the uterotonic contractions. Figure 4 shows recordings in a typical experiment. The rat was given two control dosages of oxytocin, 100 and 200 milliunits/kg. When 2 mg./kg. of (Cbz-Cys-Tyr-TyrOH)$_2$ was infused, the contraction resulting from injection of 100 milliunits/kg. of oxytocin was nearly completely suppressed. Recovery was observed in less than 15 min. after cessation of infusion of the inhibitor. A dosage of 4 mg./kg. of the inhibitor also suppressed the contractions due to 200 milliunits/kg. of oxytocin. Some uteri of normal rats, without ovariectomy, in the stage of oestrus or dioestrus, did not show spontaneous contractions at the beginning of the experiments, but antagonism of the peptide to the action of oxytocin was also observed in these animals. After repeated infusions of oxytocin the uteri gradually developed spontaneous contractions during which the inhibitory activity of the peptide could not be estimated. An example of the latter part of an experiment is shown on Fig. 5. Dosages of 5–20 milliunits/kg. of oxytocin were used for the assay. Among the antagonistic peptides tested, (Cbz-Cys-Tyr-TyrOH)$_2$ was the most active and (Cys-Tyr-TyrOEt)$_2$·2HBr, another of the cystinyl derivatives, showed weak inhibition. Other peptides tested, such as Cbz-Cys·Bz-Tyr-TyrOH and Cbz-Tyr-TyrOEt did not inhibit the uterotonic action of oxytocin *in situ*.

On Avian Blood Pressure—(Cbz-Cys-Tyr-TyrOH)$_2$ also inhibited the effect of oxytocin in reducing the blood pressure of Leghorn cocks as shown in Fig. 6. (Cys-Tyr-TyrOEt)$_2$·2HBr also caused weak

Table III—Inhibitory Activities of Some Active Peptides to Oxytocin, Acetylcholine, and Bradykinin on Isolated Rat Uterus[a]

Antagonist and Concentration (M)	To Oxytocin pA_2	To Oxytocin pD_2'	To Acetylcholine pA_2	To Acetylcholine pD_2'	To Bradykinin pA_2	To Bradykinin pD_2'
(Cbz-Cys-Tyr-TyrOH)$_2$						
3×10^{-6}	6.09	(−)				
10^{-5}	5.95	(−)				
3×10^{-5}	6.16	(−)				
10^{-4}			(−)	(−)	(−)	(−)
Cbz-Cys·Bz-Tyr-TyrOH						
10^{-5}	5.70	(−)				
3×10^{-5}	5.85	(−)				
10^{-4}				3.40	(−)	(−)
3×10^{-4}				3.50	(−)	(−)
Cbz-Tyr-TyrOEt						
10^{-5}	5.70	(−)		(−)		(−)
3×10^{-5}	5.79	(—)		3.68		3.73
10^{-4}				3.88		3.85

[a] pA_2 for competitive inhibition and pD_2' for noncompetitive inhibition.

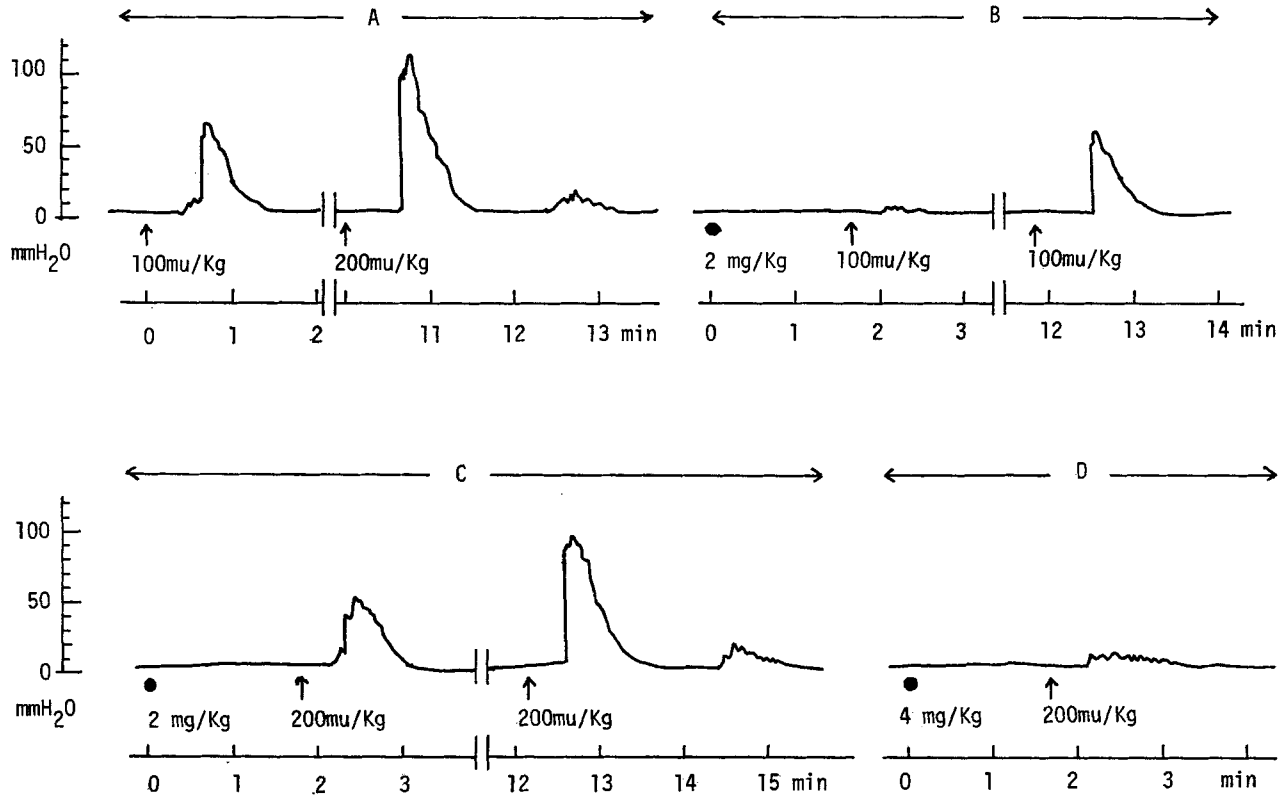

Figure 4—*Inhibition by (Cbz-Cys-Tyr-TyrOH)₂ to oxytocin on rat uterus* in situ. *The rat was in the stage of oestrus induced by injection of estradiol benzoate after ovariectomy. Column A = control contractions of two dosages of oxytocin. Columns B and C = inhibition by 2 mg./kg. of the peptide to 100 and 200 milliunits/kg. of oxytocin. Column D = 4 mg./kg. of the peptide to 200 milliunits/kg. of oxytocin. Note that inhibitions by peptide recovered in less than 15 min.*

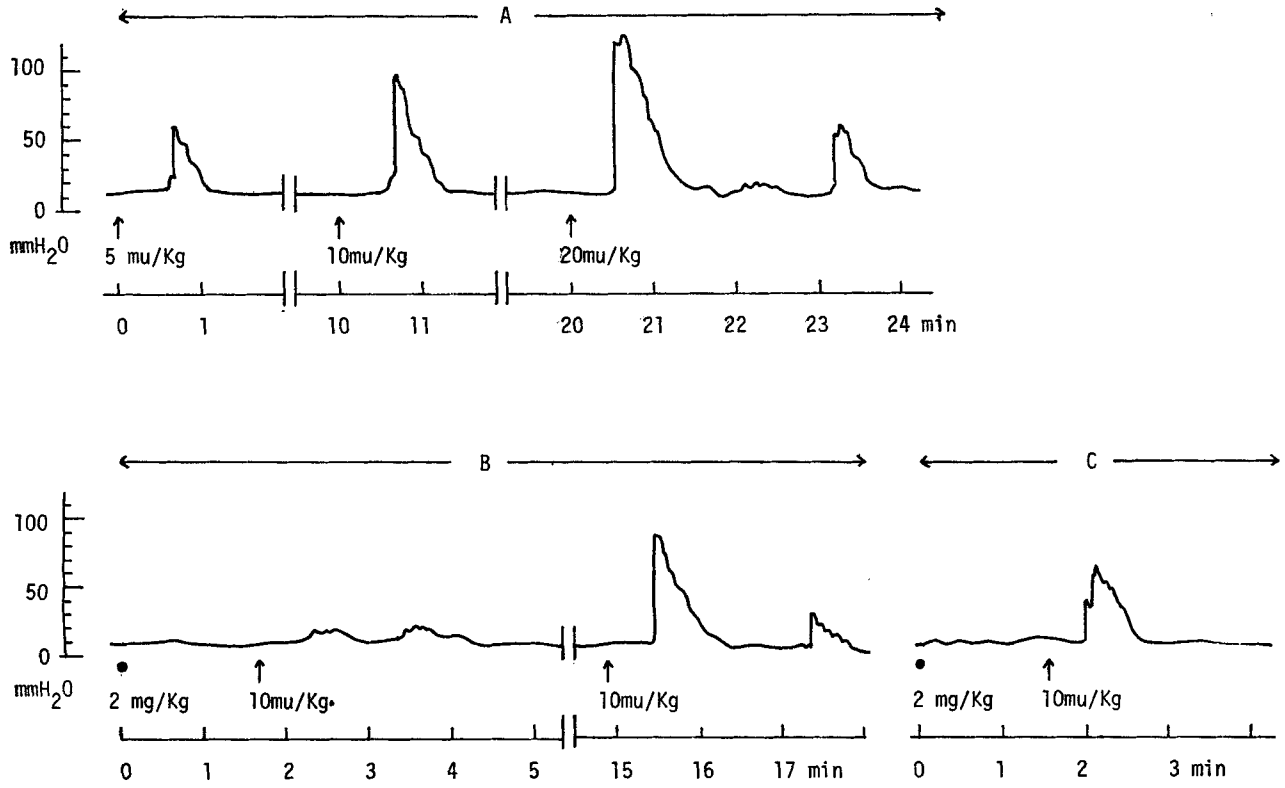

Figure 5—*Inhibition by (Cbz-Cys-Tyr-TyrOH)₂ to oxytocin on rat uterus* in situ. *The rat was in the stage of natural oestrus. Column A = control contractions of three dosages of oxytocin. Columns B and C = inhibition by 2 mg./kg. of the peptide to 10 and 20 milliunits/kg. of oxytocin. Note that dosages of oxytocin in natural oestrus for inducing contractions were smaller than those in the rat in Fig. 4.*

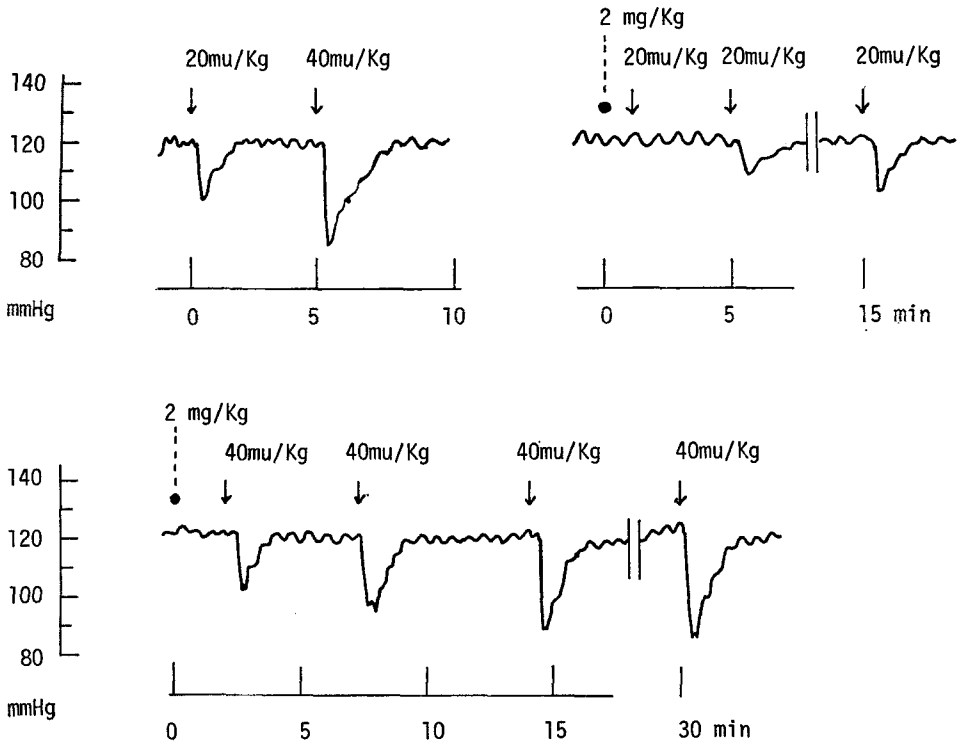

Figure 6—*Inhibition by (Cbz-Cys-Tyr-TyrOH)₂ to oxytocin on avian blood pressure. Column A = control blood depressure of two dosages of oxytocin. Columns B and C = inhibition by 2 mg./kg. of the peptide to 20 and 40 milliunits/kg. of oxytocin.*

inhibition, while Cbz-Cys·Bz-Tyr-TyrOH and Cbz-Tyr-TyrOEt had no inhibitory activity on it.

DISCUSSION

The experiments show that (Cbz-Cys-Tyr-TyrOH)$_2$ is a strong inhibitor of the uterotonic response to oxytocin of rat uterus both *in vitro* and *in situ*, and of the effect of oxytocin on avian blood pressure. The log dose–response analysis, as shown in Fig. 1, indicates that the inhibition of the response to oxytocin in the presence of the antagonist is competitive and reversible. It did not inhibit the response to acetylcholine or bradykinin, or spontaneous uterine contractions either *in vitro* or *in situ*. Chan *et al.* (10) reported that 1-L-penicillamine oxytocin and 1-deaminopenicillamine oxytocin were strong inhibitors of the actions of oxytocin both *in vitro* and *in vivo*. The inhibitory actions of (Cbz-Cys-Tyr-TyrOH)$_2$ to oxytocin might be quite similar qualitatively to those of the penicillamine analogs. But its effect is much weaker because it has been reported that the dose-response curve of oxytocin can be inhibited by 1-L-penicillamine oxytocin at 25×10^{-11} and 50×10^{-11} *M*.

Overweg *et al.* (3) reported interesting data on the inhibition of the action of oxytocin on isolated rat uterus by acyclic intermediates. Among the peptides they tested, the octapeptides, L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, which contains a tyrosine residue, was found to be a competitive inhibitor of the uterotonic response to oxytocin (pA_2 of 5.70). The tetrapeptide with a *S*-benzyl-cysteinyl residue, *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, is only a competitive inhibitor at low concentrations (pA_2 of 4.20). At higher doses of tetrapeptide ($8–10 \times 10^{-4}$ *M*), a factor other than pure competitive inhibition became evident, as indicated by the decrease in the maximal response. The *S*-benzylcysteinyl peptide, Cbz-Cys·Bz-Tyr-TyrOH (pA_2 of 5.70), considering the pA_2 value, is a more potent inhibitor than the above tetrapeptide, but at higher doses (10^{-4} and 3×10^{-4} *M*) it showed an inhibitory response to acetylcholine and not to bradykinin. Furthermore, it does not inhibit there sponse to oxytocin *in situ*. Cbz-Tyr-TyrOEt inhibits the responses to acetylcholine and bradykinin noncompetitively, as shown in Table III. When tested on isolated guinea pig ileum, none of the peptides tested showed the inhibitory responses to acetylcholine, barium chloride, and histamine.

Thus, this study shows that a peptide containing both cystine and tyrosyl-tyrosine residues, (Cbz-Cys-Tyr-TyrOH)$_2$, is a more specific and stronger antagonist of responses to oxytocin than Cbz-Tyr-Tyr-OEt or cystine diethylester.

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Effect of Temperature on Solubilization and Hydrolytic Degradation of Solubilized Benzocaine and Homatropine

I. A. HAMID* and E. L. PARROTT

Abstract □ The solubilization of benzocaine with polyoxyethylene (23) lauryl ether and polysorbate 80 was determined as a function of temperature. The enhancement of the stability of benzocaine and homatropine solubilized with from 3 to 15% polyoxyethylene (23) lauryl ether and polysorbate 80 was quantitatively determined at 30, 50, and 70°. For the alkaline hydrolysis of benzocaine and homatropine, the logarithm of the specific rate constant is linearly related to the percent of the surface-active agent. The specific rate constant for the hydrolysis of benzocaine is linearly related to the reciprocal of the apparent solubility of benzocaine in polyoxyethylene (23) lauryl ether and polysorbate 80.

Keyphrases □ Benzocaine, solubilization, hydrolysis—temperature effect □ Surfactants, nonionic, effect—solubilized benzocaine, homatropine hydrolysis □ Solubility, apparent—specific rate constant, benzocaine, homatropine □ Temperature effect—solubilized benzocaine, homatropine hydrolysis □ Homatropine, solubilization, hydrolysis—temperature effect

In a previous investigation, it was reported that in a 3% concentration four nonionic surface-active agents reduced the rate of hydrolysis of benzocaine and homatropine (1). The present study was undertaken to determine at several temperatures the effectiveness of polyoxyethylene (23) lauryl ether and polysorbate 80 in solubilizing and in retarding the alkaline hydrolysis of benzocaine and homatropine. It was assumed that concentrations as great as 15% of the nonionic surface-active agents would be acceptable in cosmetics and pharmaceuticals. The concentration of surface-active agent ranged from 3 to 15%. This investigation was carried out to determine if these concentrations of surface-active agents would slow the rate of hydrolysis of benzocaine and homatropine to an extent that would increase the shelflife significantly.

EXPERIMENTAL

Analysis and Solubility of Benzocaine—The method of analysis and the method for the determination of the solubility of benzocaine NF in various concentrations of polyoxyethylene (23) lauryl ether[1] and polysorbate 80[2] were reported previously (1, 2). The surface-active agents were commercial lots used without further purification. The apparent solubility was determined at 30, 50, and 70° and is given in Tables I and II.

Hydrolysis of Benzocaine—The rate of hydrolysis of benzocaine was determined in various concentrations of polyoxyethylene (23) lauryl ether and polysorbate 80 in 0.04 *N* hydroxyl-ion concentration at 30, 50, and 70° as previously described (1). At each temperature with each concentration of the surface-active agent, a standard absorbance–concentration curve was prepared, and the coefficients of the simultaneous equations were evaluated.

Hydrolysis of Homatropine—The method of analysis and the method for the determination of the rate of hydrolysis of homatropine[3] in various concentrations of polyoxyethylene (23) lauryl ether and polysorbate 80 were reported (1, 3). The rate of hydrolysis

[1] Brij 35 SP, Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Tween 80, Atlas Chemical Industries, Inc.
[3] Obtained from K & K Laboratories, Inc., Plainview, N. Y.

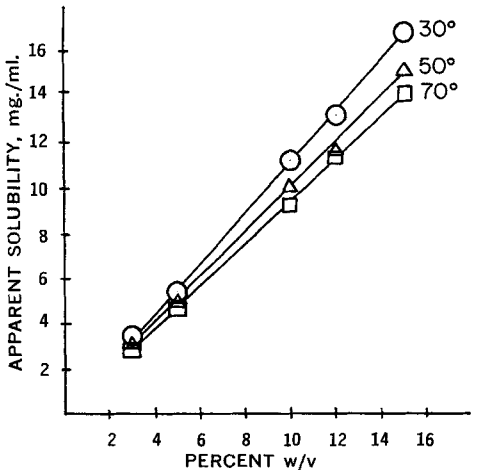

Figure 1—*Apparent solubility of benzocaine at 30, 50, and 70° in solutions of polyoxyethylene (23) lauryl ether.*

was determined in a borate buffer at pH 8 at 30, 50, and 70°. At 30°, the borate buffer consisted of 658 ml. of 0.4 *M* boric acid solution and 342 ml. of 0.1 *M* sodium borate solution. At higher temperatures, the buffer was adjusted by the addition of 0.4 *M* boric acid solution to pH 8, as measured by a Beckman model 72 pH meter.

RESULTS AND DISCUSSION

It is well known that surface-active agents in concentrations exceeding their CMC will solubilize or increase the apparent solubility of compounds of low water solubility. As shown in Figs. 1 and 2, the total or apparent solubility of benzocaine is linearly dependent on the concentration of polyoxyethylene (23) lauryl ether and polysorbate 80, respectively.

The influence of temperature on solubilization is complex, because both the structure of the micelle and the solubility of the solute may change with a change of temperature (4). This change in structure of the micelle and solubility of the solute may or may not be in the same direction. As the temperature of a solubilized system is increased, the extent of aggregation of the surface-active agent or the formation of micelles tends to be lessened due to the increased thermal activity. This phenomenon would tend to decrease the

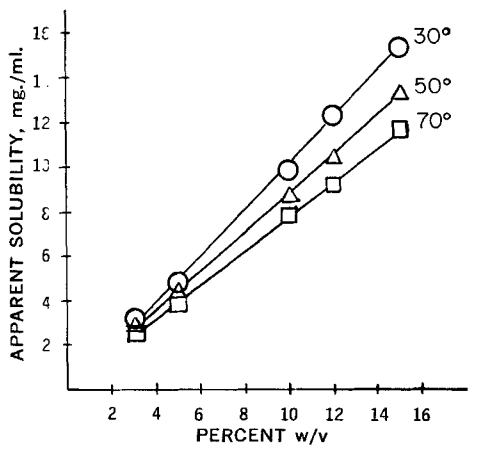

Figure 2—*Apparent solubility of benzocaine at 30, 50, and 70° in solutions of polysorbate 80.*

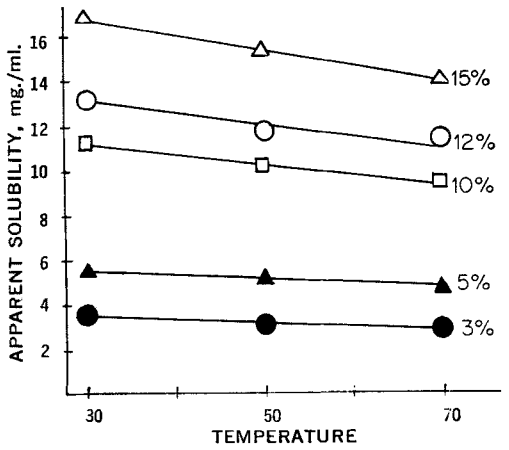

Figure 3—*Influence of temperature on apparent solubility of benzocaine solubilized with polyoxyethylene (23) lauryl ether.*

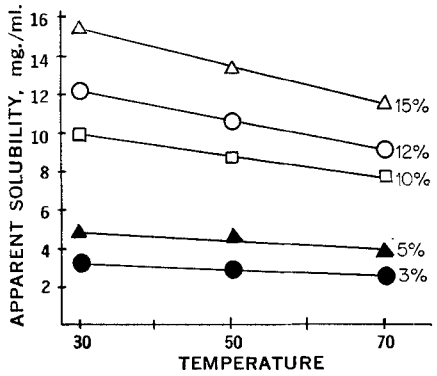

Figure 4—*Influence of temperature on apparent solubility of benzocaine solubilized with polysorbate 80.*

amount of solute solubilized. If the intrinsic solubility of the solute is decreased with an increase in temperature, the apparent solubility of the solute would be markedly lowered as both the intrinsic and micellar solubility are lessened with an increase in temperature.

If the intrinsic solubility of the solute is increased with an increase in temperature, the apparent solubility may be lowered or raised, depending on which of the opposing phenomena is predominant.

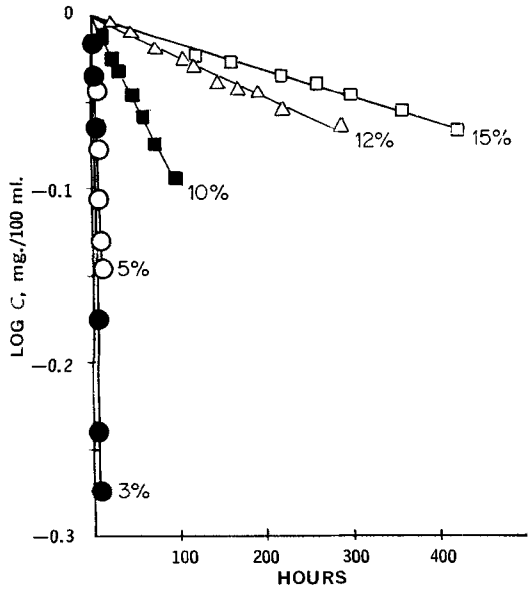

Figure 5—*Hydrolysis of benzocaine at 30° in polyoxyethylene (23) lauryl ether at 0.04* N *hydroxyl-ion concentration.*

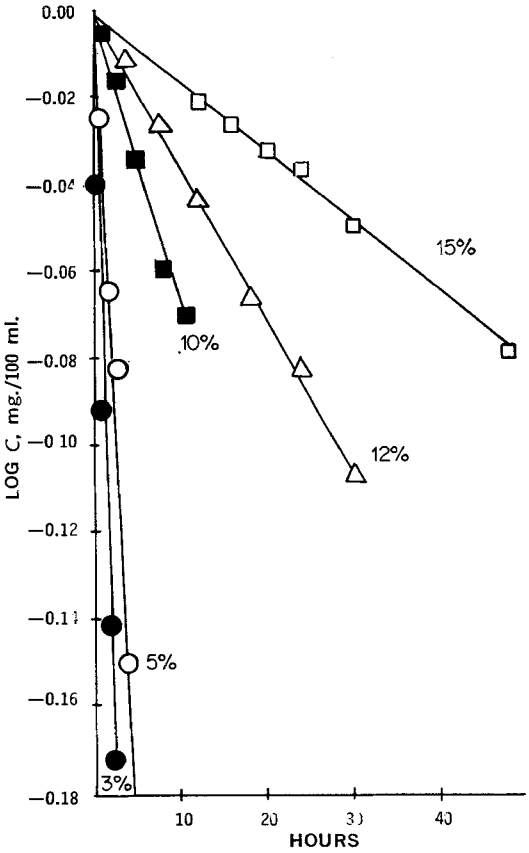

Figure 6—*Hydrolysis of benzocaine at 30° in polysorbate 80 at 0.04* N *hydroxyl-ion concentration.*

The influence of temperature on the apparent solubility of benzocaine in various concentrations of polyoxyethylene (23) lauryl ether and polysorbate 80 is shown in Figs. 3 and 4, respectively. Within the temperature range from 30 to 70° for a given concentration of polyoxyethylene (23) lauryl ether and polysorbate 80, the apparent solubility is decreased as the temperature is increased. Thus, it appears that although an increase in temperature increases the intrinsic solubility of benzocaine, the apparent solubility is due less to micellar disruption.

At high concentrations of surface-active agents, it is possible that the degree of change in the structure of the micelle is greater than the degree of change in solubility of the solute. Thus, for a given in-

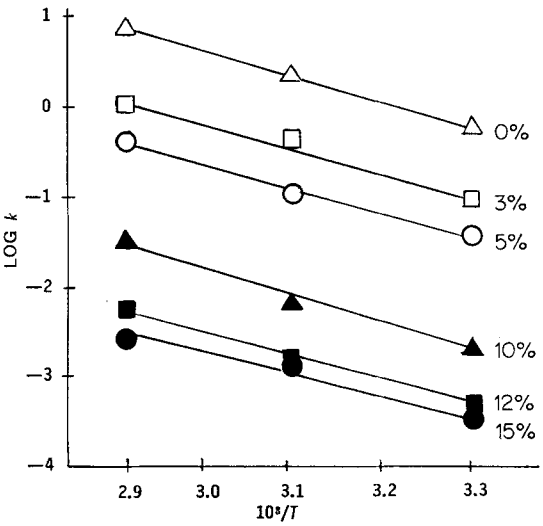

Figure 7—*Arrhenius plot showing temperature dependence of hydrolysis of benzocaine in various concentrations of polyoxyethylene (23) lauryl ether.*

Table I—Apparent Solubility, Specific Rate Constant, and Half-Life Period for the Hydrolysis of Benzocaine in 0.04 *N* Hydroxyl-Ion Concentration at 30, 50, and 70° in Various Concentrations of Polyoxyethylene (23) Lauryl Ether

Percent w/v of Polyoxyethylene (23) Lauryl Ether	k, hr.$^{-1}$	$t_{1/2}$, hr.	Apparent Solubility, mg./ml.	Reciprocal of Solubility
		30°		
0	0.63000	1.1	—	—
3	0.09840	7.0	3.56	0.280
5	0.03828	18.1	5.48	0.182
10	0.00230	301.6	11.26	0.088
12	0.00054	1283.3	13.20	0.075
15	0.00034	2008.7	16.85	0.059
		50°		
0	2.49600	0.3	—	—
3	0.45960	1.5	3.04	0.328
5	0.11220	6.2	5.12	0.195
10	0.00690	100.4	10.22	0.097
12	0.00167	415.5	11.69	0.085
15	0.00135	513.3	15.26	0.065
		70°		
0	7.88400	0.1	—	—
3	1.05600	0.6	2.84	0.352
5	0.45960	1.5	4.68	0.213
10	0.03282	21.1	9.32	0.107
12	0.00612	113.2	11.36	0.088
15	0.00276	251.1	14.10	0.070

crease in temperature the effect of diminution of micellar structure is greater than the effect of increased intrinsic solubility of the solute, and there is a marked decrease in apparent solubility. For example, in Fig. 3 with 15% polyoxyethylene (23) lauryl ether, the slope of the line obtained by plotting the apparent solubility of benzocaine against the temperature is −0.070 mg./ml./deg.

At lower concentrations of surface-active agents, the extent of aggregation and the change in intrinsic solubility of the solute are involved to a similar degree. As the temperature is increased, micellar

Table II—Apparent Solubility, Specific Rate Constant, and Half-Life Period for the Hydrolysis of Benzocaine in 0.04 *N* Hydroxyl-Ion Concentration at 30, 50, and 70° in Various Concentrations of Polysorbate 80

Percent w/v of Polysorbate 80	k, hr.$^{-1}$	$t_{1/2}$, hr.	Apparent Solubility, mg./ml.	Reciprocal of Solubility
		30°		
0	0.6300	1.1	—	—
3	0.1476	4.7	3.24	0.308
5	0.0900	7.7	4.67	0.214
10	0.0145	47.8	9.90	0.101
12	0.0076	90.9	12.28	0.081
15	0.0034	200.9	15.48	0.064
		50°		
0	2.496	0.3	—	—
3	1.254	0.5	2.92	0.342
5	0.612	1.1	4.55	0.219
10	0.138	5.0	8.72	0.114
12	0.092	7.5	10.60	0.094
15	0.048	14.3	13.40	0.074
		70°		
0	7.884	0.1	—	—
3	4.140	0.2	2.56	0.390
5	2.364	0.3	3.74	0.267
10	0.762	0.9	7.84	0.127
12	0.552	1.2	9.24	0.108
15	0.243	2.8	11.62	0.086

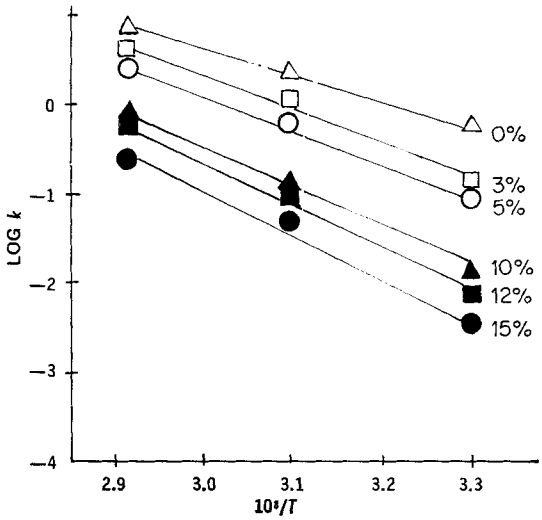

Figure 8—*Arrhenius plot showing temperature dependence of hydrolysis of benzocaine in various concentrations of polysorbate 80.*

solubilization is decreased and the intrinsic solubility of the solute is increased. Since at low concentrations of the surface-active agent these opposing effects are of a similar magnitude, the apparent solubility of the solute is not as greatly affected by a change in temperature as a solubilized system with a higher concentration of the surface-active agent. For example, in Fig. 3 with 3% polyoxyethylene (23) lauryl ether, the slope of the line is −0.018 mg./ml./deg., whereas with 15% polyoxyethylene (23) lauryl ether the slope is four times as great.

Similarly, for benzocaine solubilized with polysorbate 80, an increase in temperature results in a decrease of apparent solubility. For example, in Fig. 4 with 15% polysorbate 80, the slope of the line is −0.096 mg./ml./deg., while with 3% polysorbate 80 the slope is −0.017 mg./ml./deg.

For systems of benzocaine solubilized with polyoxyethylene (23) lauryl ether and polysorbate 80, the rate of change of apparent solubility with temperature is less at low concentrations than at high concentrations of surface-active agent.

Hydrolysis of Benzocaine in Presence of Polyoxyethylene (23) Lauryl Ether and Polysorbate 80—The rate of hydrolysis of benzocaine in 0.04 *N* hydroxyl-ion concentration was followed at 30, 50, and 70° in the presence of various concentrations of polyoxyethylene (23) lauryl ether and polysorbate 80. As demonstrated previously (1) and as shown in Figs. 5 and 6 for a given temperature and concentration of a surface-active agent, a plot of the logarithm of concentration of benzocaine against time was linear. The specific rate constant (k) for the pseudo-first-order reaction was determined

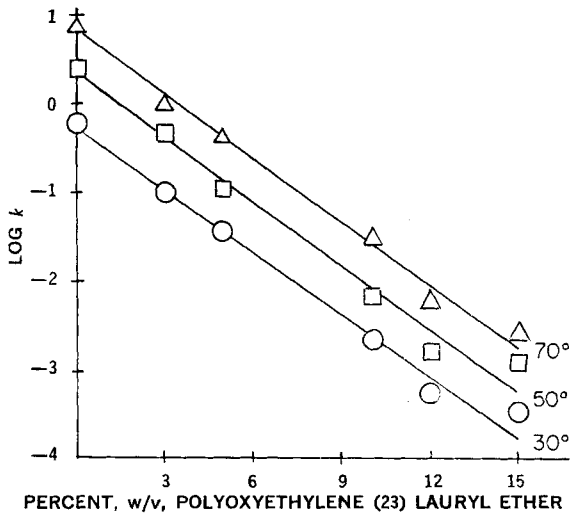

Figure 9—*Relationship of log k for hydrolysis of benzocaine at 30, 50, and 70° to concentrations of polyoxyethylene (23) lauryl ether.*

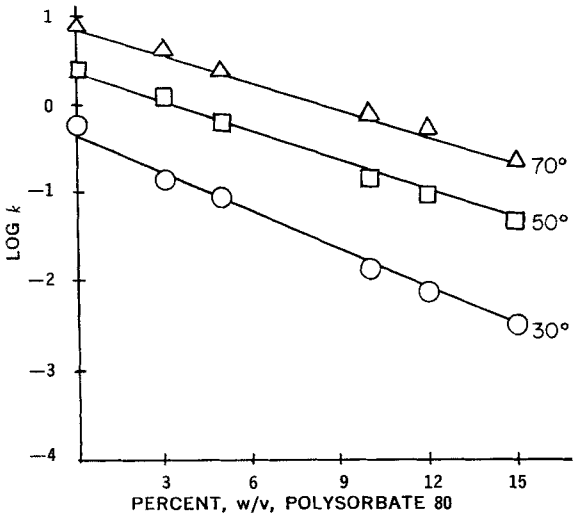

Figure 10—*Relationship of log* k *for hydrolysis of benzocaine at 30, 50, and 70° to concentrations of polysorbate 80.*

by multiplying the slope of the curve by 2.303; the half-life period ($t_{1/2}$) was calculated from the relationship: $t_{1/2} = 0.693/k$. The experimental values of k and $t_{1/2}$ at 30, 50, and 70° in the presence of various concentrations of polyoxyethylene (23) lauryl ether and polysorbate 80 are given in Tables I and II.

The dependence of k on temperature is described by an Arrhenius plot in Fig. 7 for various concentrations of polyoxyethylene (23) lauryl ether. The activation energy for the alkaline hydrolysis of benzocaine as calculated from this plot ranged from 12 to 15 kcal./mole. A similar influence of temperature on the hydrolysis of benzocaine in the presence of polysorbate 80 is shown in Fig. 8.

As shown in Table I, the $t_{1/2}$ is increased as the concentration of surface-active agent is increased. For example, at 30° the $t_{1/2}$ of benzocaine solubilized with 15% polyoxyethylene (23) lauryl ether is 1825-fold as great as that without the surface-active agent. As shown in Fig. 9, the logarithm of the rate constant is linearly related to the concentration of polyoxyethylene (23) lauryl ether. This relationship may be expressed:

$$\log k_{30°} = -0.25C - 0.201 \quad \text{(Eq. 1)}$$

$$\log k_{50°} = -0.25C + 0.398 \quad \text{(Eq. 2)}$$

$$\log k_{70°} = -0.25C + 0.892 \quad \text{(Eq. 3)}$$

where C is the percent of polyoxyethylene (23) lauryl ether. Since the slopes of the lines in Fig. 9 are the same, for the range of temperature investigated $d(\log k)/dC = -0.25$.

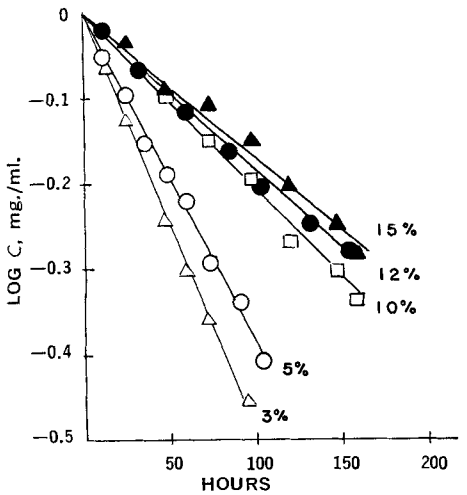

Figure 11—*Hydrolysis of homatropine at 30° and pH 8 in various concentrations of polyoxyethylene (23) lauryl ether.*

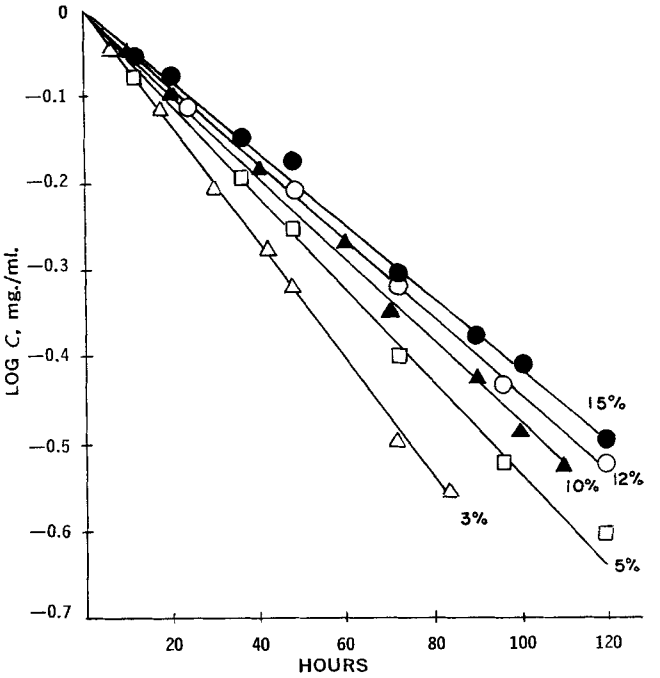

Figure 12—*Hydrolysis of homatropine at 30° and pH 8 in various concentrations of polysorbate 80.*

Inspection of Table II shows that the $t_{1/2}$ of benzocaine solubilized with 15% polysorbate 80 is increased 180-, 50-, and 30-fold at 30, 50, and 70°, respectively. The linear relationship shown in Fig. 10 between log k and the concentration of polysorbate 80 may be expressed:

$$\log k_{30°} = -0.1C - 0.2 \quad \text{(Eq. 4)}$$

$$\log k_{50°} = -0.1C + 0.4 \quad \text{(Eq. 5)}$$

$$\log k_{70°} = -0.1C + 0.9 \quad \text{(Eq. 6)}$$

Thus, the influence of concentration of polysorbate 80 on the hydrolysis of benzocaine may be expressed as $d(\log k)/dC = -0.1$.

Table III—Specific Rate Constant and Half-Life Period for the Hydrolysis of Homatropine at pH 8 at 30, 50, and 70° in Various Concentrations of Polyoxyethylene (23) Lauryl Ether

Percent w/v of Polyoxyethylene (23) Lauryl Ether	k, hr.$^{-1}$	$t_{1/2}$, hr.
	30°	
0	0.0160	43.2
3	0.0113	61.5
5	0.0091	75.9
10	0.0048	144.6
12	0.0043	162.7
15	0.0037	188.1
	50°	
0	0.0958	7.4
3	0.0828	8.4
5	0.0690	10.0
10	0.0593	11.7
12	0.0566	12.2
15	0.0531	13.1
	70°	
0	0.3162	2.2
3	0.3067	2.3
5	0.2629	2.6
10	0.2300	3.0
12	0.2156	3.2
15	0.2044	3.4

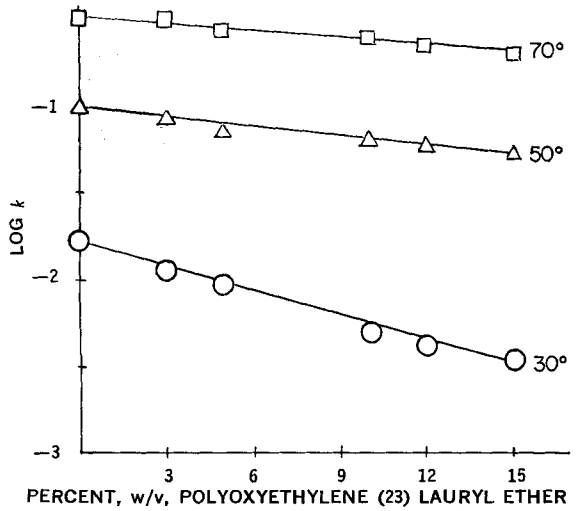

Figure 13—*Relationship of log* k *for hydrolysis of homatropine at 30, 50, and 70° to concentrations of polyoxyethylene (23) lauryl ether.*

The ratio of $d(\log k)/dC$ for polyoxyethylene (23) lauryl ether to that of polysorbate 80 is approximately 2.5. On a percentage basis, polyoxyethylene (23) lauryl ether is more effective than polysorbate 80 in retarding the hydrolytic degradation of benzocaine in the solubilized systems under the conditions studied.

Hydrolysis of Homatropine in Presence of Polyoxyethylene (23) Lauryl Ether and Polysorbate 80—The rate of hydrolysis of homatropine at pH 8 was followed at 30, 50, and 70° in the presence of various concentrations of polyoxyethylene (23) lauryl ether and polysorbate 80. Typical results for the first-order hydrolysis of homatropine are given in Figs. 11 and 12. From such plots the experimental values of k and $t_{1/2}$, as given in Tables III and IV, were determined. Plots (not shown) of log k against $1/T$ showed that the influence of temperature on the hydrolysis of homatropine in the presence of polyoxyethylene (23) lauryl ether and polysorbate 80 is described by the Arrhenius equation.

As shown in Tables III and IV, the $t_{1/2}$ is increased as the concentration of the surface-active agent is increased. For example, at 30° the $t_{1/2}$ of homatropine solubilized with 15% polyoxyethylene (23) lauryl ether is four times as great as that without the surface-active agent. The fourfold increase of the $t_{1/2}$ for homatropine is much less than the 1825-fold increase for benzocaine under the same conditions. This indicates that the solubilization technique may be used to increase stability significantly only with very slightly soluble compounds. In this case, a greater fraction of the moderately water-soluble homatropine remains in the aqueous phase, where it is readily attacked by the hydroxyl ion, than the less water-soluble benzocaine, which is associated with the micelle where it is not appreciably attacked by the hydroxyl ion, and enhanced stability is exhibited.

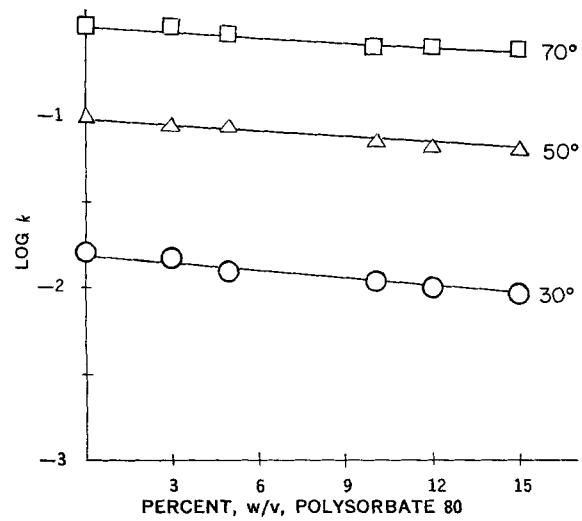

Figure 14—*Relationship of log* k *for hydrolysis of homatropine at 30, 50, and 70° to concentrations of polysorbate 80.*

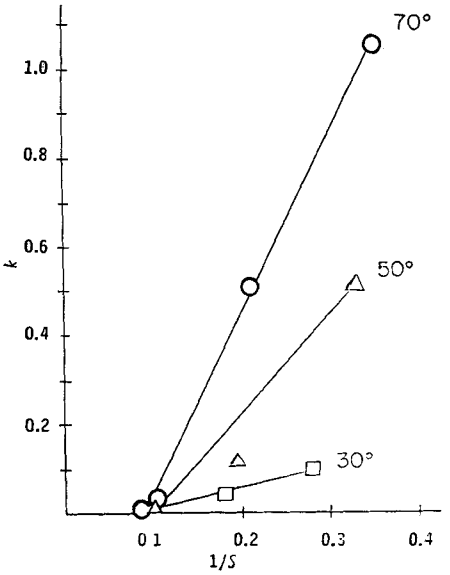

Figure 15—*Reciprocal relationship between apparent solubility of benzocaine and* k *in the presence of polyoxyethylene (23) lauryl ether at 30, 50, and 70°.*

As shown in Fig. 13, the log k is linearly related to the concentration of polyoxyethylene (23) lauryl ether. This may be expressed:

$$\log k_{30°} = -0.042C - 1.8 \quad \text{(Eq. 7)}$$

$$\log k_{50°} = -0.025C - 1.0 \quad \text{(Eq. 8)}$$

$$\log k_{70°} = -0.025C - 0.5 \quad \text{(Eq. 9)}$$

where C is the percent of polyoxyethylene (23) lauryl ether.

Inspection of Table IV shows that the $t_{1/2}$ of homatropine solubilized with 15% polysorbate 80 is only slightly lengthened, *i.e.*, 50%. The linear relationship shown in Fig. 14 between the log k and concentration of polysorbate 80 may be expressed:

$$\log k_{30°} = -0.017C - 1.8 \quad \text{(Eq. 10)}$$

Table IV—Specific Rate Constant and Half-Life Period for the Hydrolysis of Homatropine at pH 8 at 30, 50, and 70° in Various Concentrations of Polysorbate 80

Percent w/v of Polysorbate 80	k, hr.$^{-1}$	$t_{1/2}$, hr.
	30°	
0	0.0160	43.2
3	0.0149	46.3
5	0.0126	54.8
10	0.0108	63.9
12	0.0102	67.8
15	0.0094	73.6
	50°	
0	0.0958	7.4
3	0.0885	7.8
5	0.0852	8.1
10	0.0700	9.9
12	0.0657	10.5
15	0.0639	10.8
	70°	
0	0.3162	2.2
3	0.3136	2.2
5	0.2875	2.4
10	0.2421	2.9
12	0.2400	2.9
15	0.2300	3.0

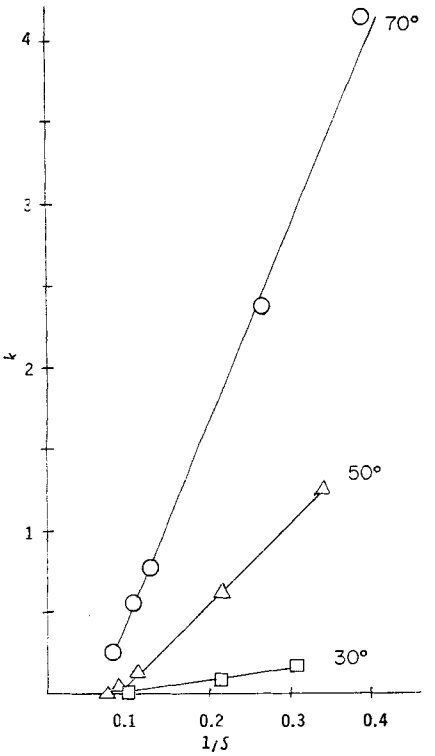

Figure 16—*Reciprocal relationship between apparent solubility of benzocaine and* k *in the presence of polysorbate 80 at 30, 50, and 70°.*

$$\log k_{50°} = -0.017C - 1.0 \qquad \text{(Eq. 11)}$$

$$\log k_{70°} = -0.017C - 0.5 \qquad \text{(Eq. 12)}$$

Thus, the influence of concentration of polysorbate 80 on the hydrolysis of homatropine may be expressed as $d(\log k)/dC = -0.017$.

The ratio of $d(\log k)/dC$ for polyoxyethylene (23) lauryl ester to that of polysorbate 80 is approximately 1.5. On a percentage basis, polyoxyethylene (23) lauryl ether is more effective than polysorbate 80 in retarding the hydrolytic degradation of homatropine in the solubilized systems under the conditions studied.

Apparent Solubility and *k*—Solubilization occurs by association with, adsorption on, and/or solution within the micelle (2). Presumably, a solubilized molecule is more stable because the attacking species cannot as readily contact the solubilized compound. The rate of hydrolysis of benzocaine in aqueous solutions is increased with an elevation of temperature. An elevation of temperature in a solubilized system, however, may affect also the degree of aggregation of the micelle, and the rate of change of diffusivity of the attacking species with temperature may be different from that in the aqueous solution. An examination of Tables I and II indicates that the apparent solubility is related to the stability. The correlation between the reciprocal ($1/S$) of apparent solubility and k is shown in Figs. 15 and 16 for benzocaine at 30, 50, and 70° in polyoxyethylene (23) lauryl ether and polysorbate 80, respectively. Thus, in the formulation of a solubilized product of benzocaine, its stability as expressed in terms of k would be anticipated to be linearly dependent on the reciprocal of the apparent solubility.

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Atomic Absorption Analysis of Sodium, Potassium, and Calcium in Ringer's Solution

ROBERT V. SMITH* and MARK A. NESSEN

Abstract □ A method was developed for the analysis of sodium, potassium, and calcium in Ringer's solution based on atomic absorption spectroscopy. Interelemental interference effects were investigated and are discussed. The proposed method was compared to NF procedures and offers some advantage in terms of accuracy and convenience.

Keyphrases □ Ringer's solution—sodium, potassium, calcium determination □ Interelemental interferences—sodium, potassium, calcium determination □ Atomic absorption spectroscopy—analysis □ Flame photometry—analysis

Various materials have been analyzed for their sodium, potassium, and calcium content by atomic absorption spectroscopy (AA). These include human serum (1, 2), cell membranes (3), milk (4), coal ash (5), cement (6), and plant tissue (7). One problem in measuring mixtures of elements by AA arises from frequent interference effects (*i.e.*, depression or enhancement of absorption by one or more atomic species) which are dependent on the nature of the elements present and their relative proportions. This is true with mixtures of sodium, potassium, and calcium; thus, each combination of these can present unique difficulties.

The solutions officially recognized as Ringer's solution (8) and Ringer's injection (9) are identically composed of sodium, potassium, and calcium as the chlorides. Sterility is a requisite of the latter preparation. For Ringer's solution, the NF (8) describes a newly introduced flame-photometric analysis for sodium and potassium while calcium is assayed by chelometric titration. The USP methods (9) for the cations in Ringer's injection involve three different titrimetric procedures which appear cumbersome. AA was proposed for the analysis of sodium, potassium, and calcium in Ringer's solution, anticipating that a procedure could be developed that might offer advantages over the official methods.

EXPERIMENTAL

Instruments—A Jarrell-Ash 82-270 Atomsorb atomic absorption spectrophotometer, equipped with a single-slot burner head (10-cm. pathlength) and sodium/potassium and calcium hollow cathode lamps, was used throughout. Instrumental parameters are listed in Table I. Flame photometric determinations were performed with Baird DB 4 and KY 2 flame photometers.

Reagents—Lanthanum chloride[1] ($LaCl_3 \cdot 6H_2O$), stated purity 99.9%, and cesium chloride[2] (CsCl), stated purity 99+%, were used as obtained. All other reagents were ACS grade. Sodium and potassium chloride were maintained in a desiccator under vacuum.

[1] Alfa Inorganics.
[2] Matheson Coleman and Bell.

Table I—AA Instrumental Parameters

Wavelengths (Å): Na, 5890; K, 7665; Ca, 4227
Hollow cathode lamp currents (mamp.): Na and K, 4; Ca, 5
Fuel: acetylene (flowmeter, 5; 10 psig.)
Oxidizer: air (flowmeter, 22; 35 psig.)
Slit widths: monochromator entrance, 50 μ; monochromator exit, 75 μ[a]
Gain: Na, 4.2; K, 7.2; Ca, 3.8
Pathlengths: Na, 0.5 mm.[b]; K and Ca, 10 cm.[c]

[a] Instrument manufacturer's recommendations. [b] Slot burner turned 90° through light source. [c] Standard burner configuration.

Deionized water was used for all solutions; reagent solutions were kept no longer than 24 hr. in glass containers.

Stock Solutions—Solutions contained: 8.11, 8.60, and 9.10 g. NaCl/l.; 240 and 360 mg. KCl/l.; and 294 and 368 mg. $CaCl_2 \cdot 2H_2O$/l.

Sodium Working Standards—Ten milliliters of the 8.11 and 9.10 g./l. NaCl stock solutions was diluted to 1000 ml. with water. Final dilutions were equivalent to 31.9 and 35.8 p.p.m. Na, respectively.

Potassium Working Standards—Into two 1000-ml. volumetric flasks, 10 ml. of the 8.60 g./l. NaCl stock solution was pipeted. Then 10 ml. of the 240 mg./l. KCl stock solution was added to one, and 10 ml. of the 360 mg./l. KCl stock solution was added to the other. They were made to volume with water. Resulting dilutions were equivalent to 1.26 and 1.89 p.p.m. K, respectively, plus 33.8 p.p.m. Na in each.

Calcium Working Standards—Ten milliliters each of the 8.60 g./l. NaCl stock solution was pipeted into two 100-ml. volumetric flasks. Then 10 ml. of the 294 mg./l. $CaCl_2 \cdot 2H_2O$ stock solution was added to one, and 10 ml. of the 364 mg./l. $CaCl_2 \cdot 2H_2O$ stock solution was added to the other and made to volume with water. Final dilutions were equivalent to 8.02 and 10.02 p.p.m. Ca, respectively, plus 338 p.p.m. Na in each.

Blank Solutions—Deionized water or appropriately diluted solutions containing NaCl were used.

PROCEDURES

Sodium, Potassium, and Calcium Working Curves—After optimizing the wavelengths indicated in Table I and zeroing (while aspirating blank solutions), percent *A* of the sodium, potassium, and calcium working standards was measured. Sodium was measured with the slot-burner turned 90° through the light source, resulting in a pathlength of 0.5 mm. Potassium and calcium were measured with the slot-burner in the standard configuration (*i.e.*, pathlength equal to 10 cm.). The *A versus* concentration curves were drawn for sodium, potassium, and calcium.

AA of Ringer's Solution—A 100-fold diluted Ringer's solution was measured for sodium and potassium; calcium was determined with 10-fold diluted solutions.

Comparison Analysis—Ringer's solution was analyzed for sodium and potassium by flame photometry and for calcium by EDTA titration, using hydroxy naphthol blue[3] as indicator, as described in the NF XIII (8). Polysorbate 80 was used as surfactant in the flame-photometric assay; where lithium internal standard was used, a 250-p.p.m. Li (as $LiNO_3$) solution was used in preparation of standards and in dilution of samples.

[3] Mallinckrodt Chemical Works.

Table II—Interelemental Interference Effects on the Absorption of Na, K, and Ca

K and Ca on Na			Na and Ca on K			Na and K on Ca		
Concentration, p.p.m. K[b]	Ca[c]	A[a] of 33.8 p.p.m. Na[d]	Concentration, p.p.m. Na[e]	Ca	A[a] of 1.57 p.p.m. K[d]	Concentration, p.p.m. Na	K	A[a] of 8.99 p.p.m. Ca[f]
Nil	Nil	0.30	Nil	Nil	0.10	Nil	Nil	0.11
1.04	Nil	0.30	32.1	Nil	0.11	300	Nil	0.14
1.57	Nil	0.30	33.8	Nil	0.11	400	Nil	0.14
2.09	Nil	0.30	35.6	Nil	0.11	Nil	12.6	0.11
Nil	0.0818	0.30	Nil	0.0818	0.10	Nil	18.9	0.11
Nil	0.0899	0.30	Nil	0.0899	0.10	338	15.7	0.14
Nil	0.0981	0.30	Nil	0.0981	0.10	—	—	—
1.57	0.0899	0.30	33.8	0.0899	0.11	—	—	—

[a] See Table I for instrumental conditions. [b] Required to meet NF specifications: 1.30–1.80 p.p.m. (in Ringer's diluted 100×'s). [c] NF specifications: 0.082–0.098 p.p.m. (in Ringer's diluted 100×'s). [d] Median concentration of 100-fold diluted Ringer's. [e] NF specifications: 32.3–35.4 p.p.m. (in Ringer's diluted 100×'s). [f] Median concentration of 10-fold diluted Ringer's.

Table III—Analysis of Na, K, and Ca in Ringer's Solution

Ringer's Solution	Element	Amount Found as Percentage of Amount Declared ± Relative *SD*[a]			
		AA	Flame Photometry A[b]	Flame Photometry B[c]	EDTA Titration
1[d]	Na	99.6 ± 0.9	—	—	—
	K	102.5 ± 1.7	—	—	—
	Ca	98.0 ± 0.0	—	—	—
2[e]	Na	101.5 ± 1.4	107.9 ± 1.4	104.0 ± 1.0	—
	K	98.9 ± 1.1	104.7 ± 1.8	97.3 ± 0.8	—
	Ca	100.3 ± 1.0	—	—	100.9 ± 0.2

[a] Based on 10 determinations. [b] Without internal standard. [c] Using Li internal standard. [d] Laboratory-prepared solution. [e] Commercially obtained solution.

RESULTS AND DISCUSSION

Enhancement or depression of the absorption of sodium, potassium, and calcium because of admixture of these elements is well described in the literature (1–3, 7, 10–12). These interactions are very dependent on relative concentrations; thus, their importance in Ringer's solution or dilutions thereof had to be determined. Initial experiments indicated that linear curves (*A versus* concentration) for sodium and potassium could be generated at concentrations present in 100-fold diluted Ringer's solution (*i.e.*, about 33.8 p.p.m. Na and 1.57 p.p.m. K); Ringer's solution diluted 10 times was suitable for measuring calcium (*i.e.*, about 9 p.p.m.).

Table II lists interelemental interference effects observed with sodium, potassium, and calcium at the concentrations indicated. For each element, consideration was given to the fact that the accompanying elements could vary over the range indicated in the official compendia. As noted in Table II, potassium and calcium exert no influence on the absorption of sodium. Therefore, the reference standards for sodium only require this cation. The absorption of potassium is affected by the relatively high concentration of sodium but is not affected by the calcium present; thus, sodium must be included in the potassium reference standards. This effect and its magnitude are in accord with the results of others (7, 10, 13). Enhancement of the absorption of calcium by sodium was also observed and necessitates preparation of calcium standards containing sodium. The enhancement effects caused by alkali metals are apparently due to their ability to increase the number of atoms (of the elements being measured) in the ground state, resulting in an increase in absorption (3). Fortuitously, these enhancement effects tend to plateau over a wide range of concentrations, which allows for compensation by appropriate addition of the interfering element to reference standards.

The use of 1% lanthanum solutions (12, 13) to suppress the enhancement of potassium and calcium absorption by sodium was not successful with the solutions studied. Cesium chloride was suggested for use in counteracting the effect of sodium on potassium (3); however, it accomplishes this by causing an analogous enhancement. Thus, its use in the present application offers no advantage over simply adding sodium to potassium reference standards.

Application of the proposed method to Ringer's solution was attempted. The best results were obtained when the two-standard or sandwiching method, discussed by Ramirez-Munoz (14) and described in the *Experimental* section of this report, was used. Results of determinations on laboratory prepared Ringer's solution and a commercially available product are given in Table III. Also included in Table III are results of NF analyses of the latter. The AA method seems more accurate than the NF flame-photometric procedures for sodium and potassium. This is in contrast to the fact that AA appears to offer no advantage in accuracy over the EDTA titration method for calcium. The AA procedures overall possess similar precision to that of the NF methods, but, because they can be performed with only one apparatus, they are more convenient than the official assays and could provide savings in time.

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GLC of Trimethylsilyl Derivatives of Pantothenyl Alcohol and Pantothenates

A. R. PROSSER and A. J. SHEPPARD

Abstract □ The trimethylsilyl ester-ethers of pantothenic acid, calcium pantothenate, and sodium pantothenate and the trimethylsilyl ethers of pantothenyl alcohol were prepared readily and quantitatively by using a mixture of bis(trimethylsilyl)acetamide, trimethylchlorosilane, trimethylsilylimidazole, and dimethyl sulfoxide. GLC column packings of 5% SE-30 and 3% OV-1 were demonstrated to yield symmetrical, resolved peaks for the trimethylsilyl derivatives. Quantitatively, 25 ng. of the trimethylsilyl derivatives, based on the starting materials, was effectively measured with the flame-ionization detector; GLC responses were linear as the quantity of the compound increased. The structures and molecular weights of the derivatives were confirmed by NMR and mass spectrometry.

Keyphrases □ Pantothenyl alcohol, pantothenates—analysis □ Trimethylsilyl esters, pantothenyl alcohol, pantothenates—synthesis, determination □ GLC—analysis □ NMR spectroscopy—structure

The naturally occurring isomer of pantothenic acid is the *d*(+)-enantiomorph. Synthetic pantothenic acid is a racemic mixture and, since only the *d*(+)-isomer is biologically active, this fact must be taken under consideration if the *dl* mixture is to be used as a source of pantothenic acid. Synthetic *d*-pantothenic acid as the calcium or sodium salt is widely used in pharmaceutical products; the calcium salt is used more often in solid products because of its ease of handling. Aqueous dispersions of combined oil- and water-soluble vitamins, in which calcium ions must be avoided to prevent precipitation of other components, contain the sodium salt.

Pantothenyl alcohol, referred to commercially as panthenol, is now widely used as a source of pantothenate activity in pharmaceutical vitamin products because it is more stable than the pantothenate salts, especially in liquid multivitamin products that must be slightly acid in order to preserve the thiamin content. Panthenol itself has no pantothenate activity; in fact, it is an inhibitor of many lactic acid-producing bacteria. The inhibitory property of panthenol was reported early in the microbiological work on pantothenic acid (1); it was made the basis of the microbiological assay for panthenol (2) and was recommended as the preferred method. Panthenol is quantitatively converted to pantothenic acid in the animal body (3) and is equivalent to pantothenic acid in man (4, 5).

As an extension of the work of this laboratory on the GLC of water-soluble vitamins (6–10) and the work of others (11–19), the behavior of the trimethylsilyl derivatives of the pantothenates and panthenol was investigated. Although a number of methods have long been available for the identification and determination of the pantothenates and panthenol (19–26), GLC has been successfully used for the analysis of ethyl pantothenate diacetate and panthenol triacetate (9, 10) and should be readily adaptable to the analysis of the diether monoester trimethylsilyl derivative of the pantothenates and the triether trimethylsilyl derivative of panthenol. This report describes a one-step procedure for the rapid and

Table I—Summary of NMR Spectrum of Tritrimethylsilyl Pantothenic Acid

Proton(s)	Band Center, p.p.m.[a]	Description	Number of Protons
$-OSi(CH_3)_3$	0–0.2	Three singlets	27
$-C(CH_3)_2-C-$	0.60, 0.72	Two singlets	6
$-C-CH_2-C(=O)-$	2.33	Triplet (J = 6 Hz.)	2
$-O-CH_2-C-$	3.13	*ab* Pattern (J = 9.5 Hz.)	2
$-N-CH_2-C-$	3.25	Triplet (J = 6 Hz.)	2
$-C(-O-)-CH-C(=O)-$	3.83	Singlet	1
$-C(=O)-NH-C-$	(Below 5.5)	(Broad)	1

[a] Referred to tetramethylsilane.

Table II—Summary of NMR Spectrum of Tritrimethylsilyl Panthenol

Proton(s)	Band Center, p.p.m.[a]	Description	Number of Protons
$-OSi-(CH_3)_3$	0.0, 0.04, 0.05	Three singlets	27
$-C(CH_3)_2-C-$	0.79, 0.84	Two singlets	6
$-C-CH_2-C-$	1.63	Quintet with fine structure (J = 6 Hz.)	2
$-O-CH_2-C-$	3.24	*ab* Pattern (J = 9 Hz.)	2
$-C-CH_2-O-$	3.22	Triplet (J = 6 Hz.)	2
$-N-CH_2-C-$	3.59	Triplet (J = 6 Hz.)	2
$-C(-O-)(-CH-)-C(=O)-$	3.94	Singlet	1
$-C(=O)-NH-C-$	6.81	(Broad)	1

[a] Referred to tetramethylsilane.

quantitative preparation of the trimethylsilyl derivatives of pantothenic acid, calcium pantothenate, sodium pantothenate, and pantothenyl alcohol. The structure confirmation and GLC determination of the trimethylsilyl derivatives of the pantothenates and panthenol are presented.

EXPERIMENTAL

Instrumentation—A model 5000 Barber-Colman gas chromatograph[1], fitted with a high temperature hydrogen flame-ionization detector (FID) and 5-mv., 2-sec., 27.9-cm. (11-in.) strip chart recorder, was used for the analysis of the trimethylsilyl derivatives.

Materials—The following were used: *d*-pantothenyl alcohol[2], *d*-pantothenic acid calcium salt[2], *dl*-pantothenyl alcohol[2], *dl*-pantothenic acid[3], *d*-pantothenic acid sodium salt[4], bis(trimethylsilyl)acetamide[5], bis(trimethylsilyl)trifluoroacetamide[5], trimethylchlorosilane[6], trimethylsilylimidazole[6], pyridoxine (pyridoxol) hydrochloride USP reference standard[7], dimethyl sulfoxide[8], and hexane (*n*-hexane, pure grade)[9]. All other reagents used were ACS reagent grade and required no further purification.

Preparation of Trimethylsilyl Derivatives—The diether monoester trimethylsilyl derivative of pantothenic acid was prepared from *dl*-pantothenic acid, *d*- and *dl*-calcium pantothenate, and *d*-sodium pantothenate. The triether trimethylsilyl derivative of panthenol was prepared from *d*- and *dl*-pantothenyl alcohol.

GLC Standards—Standard solutions of the trimethylsilyl derivatives were prepared as follows. A 50-ml. volumetric flask, with a 24/40 stopper and stirring bar, was charged with 146.9 mg. of *d*-sodium pantothenate and 125.0 mg. of *d*-pantothenyl alcohol. To these compounds was added 5 ml. of a 4:2:2 mixture of bis(trimethylsilyl)acetamide – trimethylsilylimidazole – trimethylchlorosilane or bis(trimethylsilyl)trifluoroacetamide–trimethylsilylimidazole–trimethylchlorosilane. The reaction mixture was stirred for 10 min. at room temperature, and 0.2 ml. of dimethyl sulfoxide was added. The mixture was stirred for another 5 min. and then diluted to volume with benzene. The concentration of each derivative, based on the starting material, was equivalent to 2.5 mg./ml. of panthenol.

Calibration Standards—Aliquots of 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 ml. were taken from the GLC standard solution and diluted to 10 ml. with benzene. After dilution, a standardized injection of 2 μl. contained 0.25, 0.50, 0.75, 1.00, 1.50, and 2.00 mcg., respectively, of each trimethylsilyl derivative.

Spectroscopy Standards—The trimethylsilyl derivatives for the spectroscopic studies were prepared individually in the manner described for GLC standards, except that dimethyl sulfoxide was omitted, *n*-hexane was used in place of benzene, and the mixture was heated at 60° for 10 min. The reaction solvents were removed with a stream of nitrogen; the oily residue was taken up in *n*-hexane, heated for 5 min. at 60°, cooled, and filtered to remove the NaCl. The *n*-hexane was then removed with nitrogen, and the oily residue was placed in a sealable ampul (color break) for spectroscopic analysis.

GLC—Two column packings were evaluated for their usefulness to resolve the trimethylsilyl derivatives of panthenol and the pantothenates:

Column 1—244 × 0.4 cm. i.d. packed with a liquid phase of 5% SE-30 (w/w) on 100–120-mesh Gas Chrom Q. Operating conditions for the separation of the trimethylsilyl derivatives were: column, 185°; detector, 270°; flash heater, 270°; and carrier gas, 80 ml./min. (40 p.s.i.).

Column 2—244 × 0.4 cm. i.d. packed with a liquid phase of 3% OV-1 (w/w) on 110–120-mesh Anakrom ABS. Operating conditions for the trimethylsilyl derivative separation were: column, 200°; detector, 280°; flash heater, 280°; and carrier gas, 80 ml./min. (40 p.s.i.).

All columns were U-shaped Pyrex glass and were preconditioned for 24 hr. at 295° with a carrier gas flow rate of 60 ml./min. Nitrogen was used as the carrier gas throughout the study. Compressed air and hydrogen flow rates used with the FID were 460 and 36 ml./min., respectively. Peak areas were determined by triangulation (peak width at half-height × peak height = peak area).

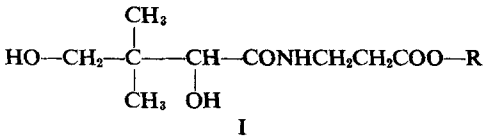
Efficiency of Derivative Preparation—During the preparation of the GLC standards, equal aliquots of the reaction mixture were taken for GLC analysis every 2 min. for 10 min. after addition of the dimethyl sulfoxide. The trimethylsilyl ether derivatives were

[1] Barber-Colman, Rockford, Ill.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] K & K Laboratories, Inc., Plain View, N. Y.
[4] Mann Research Laboratories, Division of Becton Dickinson & Co., New York, N. Y.
[5] Supelco, Inc., Bellefonte, Pa.
[6] Applied Science Inc., State College, Pa.
[7] USP Reference Standards, Bethesda, Md.
[8] Crown Zellerbach Corp., Chemical Products Division, Camas, Wash.
[9] Phillips Petroleum Co., Special Products Division, Bartlesville, Okla.

completely formed at 14 min., as indicated by constant peak height. Pantothenic acid and panthenol can also be converted to their trimethylsilyl derivatives by using a 4:1 mixture of bis(trimethylsilyl)acetamide–trimethylsilylimidazole and diluting with *n*-hexane or benzene. Trimethylchlorosilane was required to convert calcium and sodium salts of pantothenic acid to its trimethylsilyl ester derivative. The dimethyl sulfoxide was added as a solvent and catalyst.

RESULTS AND DISCUSSION

Compounds involved and their identifications are as follows:



I

Compound I—Pantothenic acid, R = H, mol. wt.: 219.23; calcium pantothenate, R = Ca/2, mol. wt.: 476.53/2; and sodium pantothenate, R = Na, mol. wt.: 241.21.

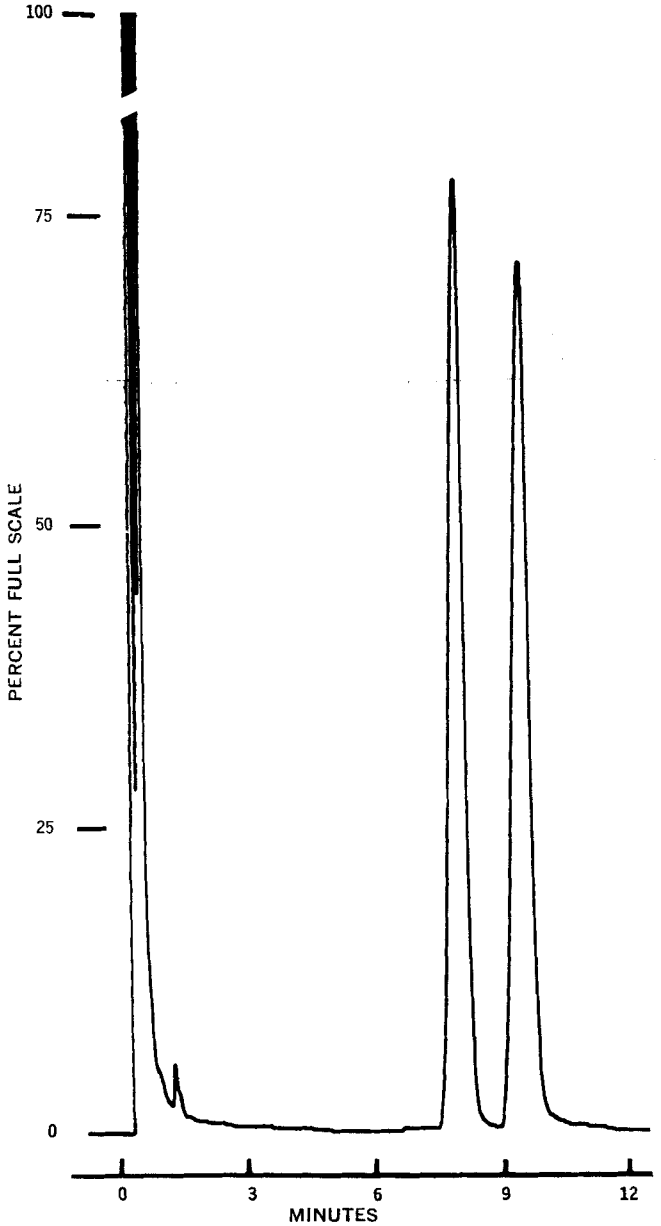
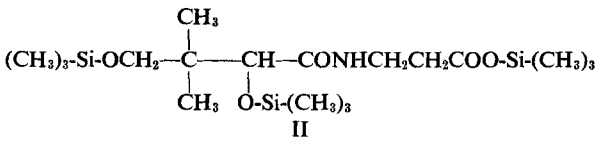


Figure 1—*Chromatogram of 1.00 mcg./μl. of panthenol as the panthenol tritrimethylsilyl derivative (8.0 min.) and 1.18 mcg./μl. of sodium pantothenate as the pantothenic acid tritrimethylsilyl derivative (9.5 min.) in* n-*hexane on a 5% SE-30 column at 185°.*

Table III—Response of Pantothenates and Pantothenol Trimethylsilyl Derivatives[a]

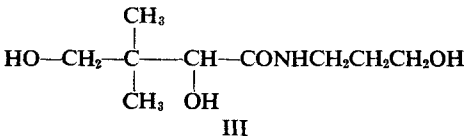
Starting Material, mcg.	Peak Area, mm.[2b] Tritrimethylsilyl Pantothenic Acid	Tritrimethylsilyl Panthenol
0.25	2.7 ± 0.1	9.3 ± 0.2
0.50	12.1 ± 0.5	19.5 ± 0.7
0.75	22.0 ± 0.8	30.3 ± 1.2
1.00	35.0 ± 1.4	42.5 ± 1.7
1.50	55.5 ± 1.6	65.0 ± 2.2
2.00	77.5 ± 1.9	86.0 ± 2.4

[a] 10× attenuation, electrometer output 1 × 10^{-10} amp., hydrogen flame detector, and a 5% SE-30 column. [b] Mean of six analyses ± standard deviation.



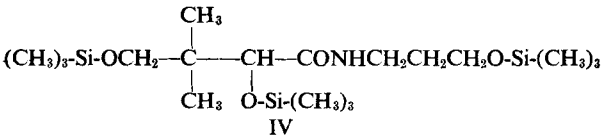
II

Compound II—Tritrimethylsilyl pantothenic acid, mol. wt.: 435.79.



III

Compound III—Panthenol, mol. wt.: 205.26.



IV

Compound IV—Tritrimethylsilyl panthenol, mol. wt.: 421.82.

After conditions of silylation were found that produced only one reaction product per compound in quantitative yield, NMR and mass spectrometry were used to confirm that the trimethylsilyl derivatives (II and IV) were actually formed from the parent compounds (I and III).

NMR Spectroscopy—The spectra of chloroform-*d* solutions of II and IV were determined at 60 and 100 MHz.[10]; internal $CHCl_3$ served as the reference standard. The spectra were compatible with the proposed structures of II and IV; they are summarized in Tables I and II.

Mass Spectrometry—An aliquot of the individually prepared spectrometry standard was prepared for mass spectrometry by breaking open the ampul and removing the contents. The oily brown liquid was subjected to mass spectroscopic analysis. The mass spectrum of IV exhibited a molecular ion of low relative abundance at *m/e* 421. An intense ion produced by the loss of a methyl group, commonly present in trimethylsilyl derivatives and often used to confirm their molecular weights, was observed at *m/e* 406. Characteristic product ions at *m/e* 277 and *m/e* 247 involved cleavage of the bonds on either side of the secondary carbon bearing the silyl ether group, with a hydrogen migration in the case of the ion at *m/e* 277. In both instances, the positive charge remained with the fragment containing the silyl ether group. These two ions substantiated the structural assignments. Product ions, attributed more specifically to the silyl ether group and the alkyl portion of the molecule, were observed at *m/e* 73, 89, 103, 117, and 131.

No molecular ion was observed in the mass spectrum of II. However, an intense ion at *m/e* 420 (M-15) supported 435 as the molecular weight of the compound. Furthermore, product ions at *m/e* 291 and *m/e* 247, occurring by processes described for IV, were consistent with the postulated structure.

[10] Varian A-60 and HA-100 NMR spectrometers.

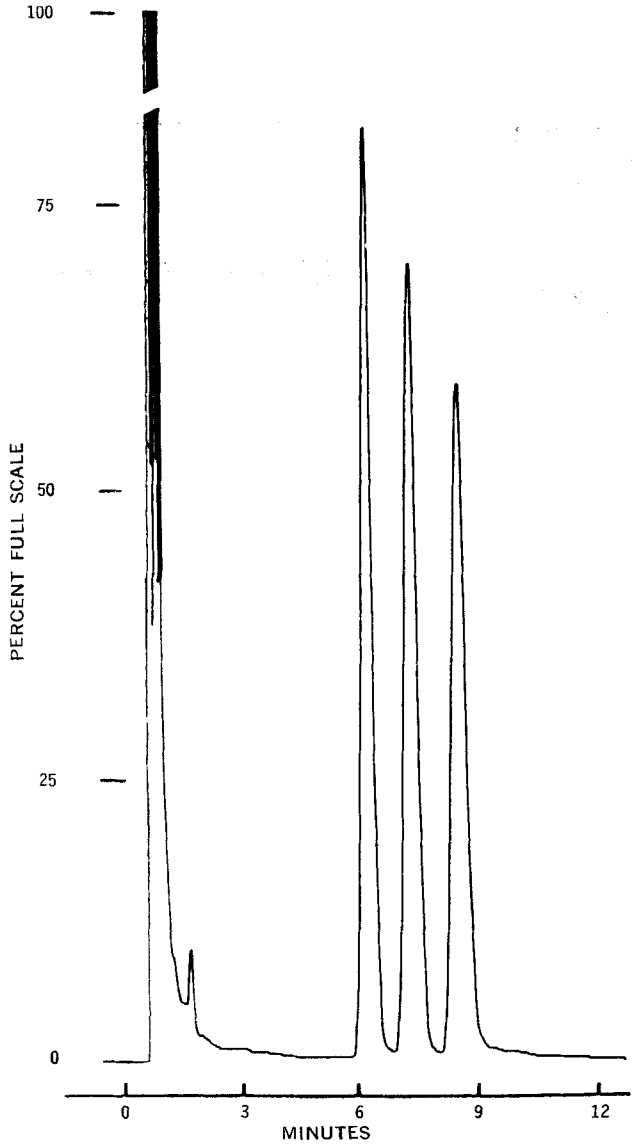


Figure 2—*Chromatogram of 1.00 mcg./μl. of pyridoxol as the pyridoxol tritrimethylsilyl ether (6.3 min.), 1.00 mcg./μl. of panthenol as the panthenol tritrimethylsilyl ether (7.5 min.), and 1.16 mcg./μl. of calcium pantothenate as the pantothenic ditrimethylsilyl ether monotrimethylsilyl ester (8.8 min.) in benzene on a 3% OV-1 column at 200°.*

The postulated structures shown for the two tritrimethylsilyl derivatives give the molecular weights expected.

Effect of Immobile Phases—The elution peaks of II and IV gave distinct peaks on a 5% SE-30 column operated at 185° and 80 ml./min. (Fig. 1). The 3% OV-1 column, operated at 200° and 80 ml./min., gave distinct elution peaks of trimethylsilyl pyridoxol, II, and IV (Fig. 2). The tritrimethylsilyl derivative of pyridoxol (6.3 min. retention time) was used as a reference standard (1.00) to calculate the relative retention times for II (1.2) and IV (1.4) and may be used as an internal standard for samples not containing pyridoxol.

Effect of Quantity Injected—Response data for II and IV, which were determined on a 5% SE-30 column, are given in Table III. These data show that the response was linear as the quantity of compound was increased.

Compound II, used for these data, was prepared from sodium pantothenate. However, the calcium and sodium pantothenate and pantothenic acid can be used interchangeably for obtaining these data when calculated on an equal mole basis.

Sensitivity—The GLC procedure for the analyses of II and IV is very sensitive. Using the FID, an attenuator setting of 1, an electrometer output of 1×10^{-10} amp., and a 0–1-mv. recorder, 2–4 ng. of I and III as Compounds II and IV was detectable. However, quantitative measurement at these low levels is difficult because of the low signal-to-noise ratio.

CONCLUSIONS

A rapid and accurate silylation procedure has been presented which will yield derivatives of all of the pantothenates (calcium pantothenate, pantothenic acid, and sodium pantothenate) and panthenol. Studies are in progress to apply the GLC procedure to pharmaceuticals containing I and III. This procedure may also find use as a monitoring device for determining pantothenic acid and its derivatives in the formation or degradation of coenzyme A.

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Assay for Vitamins D_2 and D_3 in the Presence of Tocopherols (Vitamin E) and Vitamin A

D. K. FEETER, M. F. JACOBS, and H. W. RAWLINGS

Abstract □ A GLC procedure for determination of vitamins D_2 and D_3 is described. By pretreatment consisting of saponification and subsequent esterification of all free hydroxyl groups to their respective propionates, vitamins D_2 and D_3 can be isolated from the interference usually encountered from other oil-soluble vitamins during GLC analysis. The procedure described, including the "internal standard" technique, supplies precise data rapidly, with accuracy comparable to that obtained by the vitamin D assay method given in USP XVII.

Keyphrases □ Vitamins D_2, D_3, determination, differentiation—dosage forms □ Tocopherols, vitamin A in dosage forms—vitamins D_2, D_3 determination □ Trioctanoin—internal standard ■ GLC—analysis

Much work has been done to develop a satisfactory physicochemical method for assaying vitamin D. Although biological methods for assaying vitamins D_2 and D_3 give answers of good accuracy, they are imprecise, time consuming, and expensive. The biological methods are thus impractical for use in trade negotiations or in manufacturing control.

An excellent review of the extensive work done in developing an instrumental procedure was given by Richter (1). All this work finally culminated in the adoption by USP XVII (2) of a procedure closely based on that described by Theivagt and Campbell (3). In the USP procedure, the Nield *et al.* (4) antimony trichloride reagent is used.

Although this USP procedure serves industry as the "official assay" to date, serious shortcomings still exist. Its basis is an elaborate work-up, including two column chromatographic steps to eliminate interference due mainly to vitamin A. Mixtures of vitamins A and D can be assayed with fair success up to a maximum A:D ratio of about 20:1. Serious interference has been noted when vitamin E and certain sterols (notably tachysterol) are also present. Osadca and De Ritter (5) and Mulder *et al.* (6) described additional steps recommended for overcoming these difficulties. Furthermore, this assay procedure requires a minimum of 6 hr.

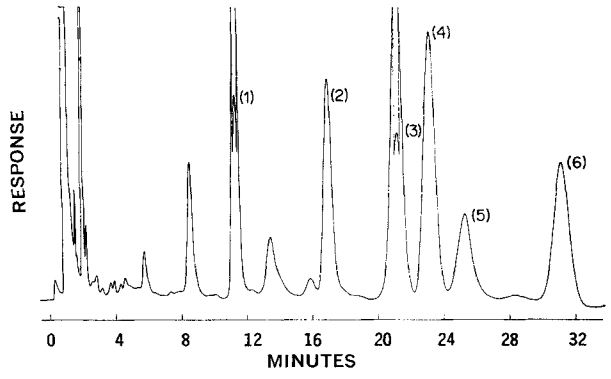

Figure 1—*Vitamin D_2–tocopherol resolution (for propionate esters). Key: (1), trioctanoin; (2), δ-tocopherol; (3), γ-tocopherol; (4) pyrovitamin D_2; (5), α-tocopherol; and (6), isopyrovitamin D_2.*

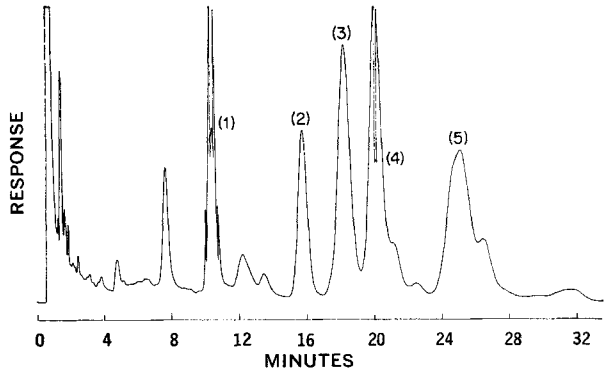

Figure 2—*Vitamin D_3–tocopherol resolution (for propionate esters). Key: (1), trioctanoin; (2), δ-tocopherol; (3), pyrovitamin D_3; (4), γ-tocopherol; and (5) isopyrovitamin D_3 + α-tocopherol.*

An analyst must be experienced in the art of preparing the critically important chromatographic columns. Under the best conditions, intralaboratory assay precision is in the range of $CV \pm 5\%$, but interlaboratory discrepancies of 10–25% are not uncommon.

The interference of vitamin A with the determination of vitamin D by GLC is widely recognized (7–9). Removal of vitamin A generally necessitates lengthy pre-GLC work-up. No mention is made in the literature of the interference of tocopherols which have the same retention times as the D vitamins when the natural vitamins or their trimethylsilyl ether derivatives are chromatographed.

To overcome these disadvantages, an improved assay procedure was developed based on GLC. The procedure consists of preliminary saponification and extraction steps to remove some interfering materials, and then isolation and concentration of vitamin D, vitamin E, and vitamin A in their alcohol forms. The mixture so obtained is treated with propionic anhydride to esterify all the alcohols to their corresponding propionates.

By using trioctanoin as an internal standard and GLC columns of SE-52 (for vitamin D_2, see Fig. 1) and

Table I—Relative Retention Data of Pertinent Propionate Esters to Trioctanoin

Compound	SE-52 Column	SE-30 Column
Trioctanoin	1.00	1.00
Propionate ester of:		
α-Tocopherol	2.38	2.38
β-Tocopherol	1.96	1.96
γ-Tocopherol	1.96	1.96
δ-Tocopherol	1.56	1.54
Pyrovitamin D_2	2.17	2.04
Isopyrovitamin D_2	2.94	2.86
Pyrovitamin D_3	1.96	1.82
Isopyrovitamin D_3	2.63	2.50

Table II—Replicate Vitamin D_2 Assays of Six Randomly Picked Commercial Blends of Vitamins A, D_2, and E

	Label Potency, Vitamin D_2 Units/g.					
	50,000	50,000	50,000	105,000	400,000	150,000
Assay 1	60,000	65,600	63,500	131,200	408,900	169,000
Assay 2	61,500	64,600	61,000	132,400	406,100	163,700
Assay 3	62,200	66,000	63,300	132,700	401,100	168,100
Assay 4	61,300	63,200	62,500	133,600	413,000	169,000
Average	61,250	64,850	62,580	132,500	407,200	168,000

SE-30 (for vitamin D_3, see Fig. 2), vitamin D and the tocopherols are separated effectively, while vitamin A pyrolysis products elute in the "solvent front."

EXPERIMENTAL

Materials—The following were used: (*a*) reference standards of activated 7-dehydrocholesterol USP and ergocalciferol USP; (*b*) vitamin D_2 for preparing known blends—ergocalciferol; (*c*) vitamin D_3 for preparing known blends—activated 7-dehydrocholesterol[1]; (*d*) trioctanoin for internal standard[2]; (*e*) Diatoport-S solid support, 80/100 mesh[3]; (*f*) silicone liquid phases SE-30 and SE-52[4]; (*g*) chloroform, B&A reagent grade; (*h*) propionic anhydride[5]; (*i*) pyridine[6]; (*j*) toluene, B&A reagent grade; (*k*) diethyl ether, B&A reagent grade; and (*l*) butylated hydroxytoluene[7].

Pretreatment of Liquid Samples—One gram of sample and 0.05 g. of butylated hydroxytoluene were weighed into a 250-ml. saponification flask. To this mixture, 6 ml. of 50% aqueous potassium hydroxide solution and 34 ml. of denatured (3A) alcohol were added. An air condenser was attached, and the mixture was placed on a hot plate to reflux for 30 min. The flask was then cooled under cold water, 100 ml. (pipet) of toluene was added, and the flask was stoppered and shaken vigorously for 1 min. The toluene solution was then transferred to a 250-ml. separator and washed once with 40 ml. of 1 *N* aqueous potassium hydroxide solution, once with 40 ml. of 0.5 *N* aqueous potassium hydroxide solution, and then with 40-ml. portions of distilled water until the water layer was neutral to phenolphthalein. The last water wash was drawn off, and the toluene solution was dried by shaking vigorously with about 20 g. of anhydrous sodium sulfate.

An aliquot of the toluene solution, containing at least 100 mcg. of vitamin D, was transferred to a suitable small flask; 5 ml. of a chloroform solution of purified trioctanoin was added (trioctanoin should not exceed 10 times the estimated weight of vitamin D).

The solvents were removed under vacuum on a 40° water bath. To the residue were added 1 ml. of pyridine and 2 ml. of propionic anhydride, and the mixture was heated for 15 min. at 80° on a hot plate. The reagents were then removed at 50–70° under a stream of nitrogen, and the residue was immediately dissolved in an appropriate volume of chloroform.

Pretreatment of Tablet Samples—A number of tablets (equivalent to at least 25 mcg. of vitamin D) and 0.050 g. of butylated hydroxytoluene were finely ground in a 250-ml. saponification flask. To this powder, 5 ml. of distilled water and 6 ml. of 50% aqueous potassium hydroxide solution were added. The mixture was warmed to make a slurry, 34 ml. of denatured alcohol was added, and the sample was saponified as with liquid samples. The flask was then cooled under cold water, and its content was transferred quantitatively to a 500-ml. separator with 150 ml. of diethyl ether. Fifty milliliters of distilled water was added to the saponification flask, which was warmed and swirled to disperse the residue. The resultant dispersion was cooled and added to the separator. The flask was then rinsed with 20–30 ml. of ether, which was also added to the separator. The water layer was extracted twice with ether, and the combined ether extracts were washed with 50-ml. portions of distilled water until the water layer was neutral to phenolphthalein.

To the ether solution, a 5-ml. chloroform solution of purified trioctanoin (not to exceed 10 times the estimated weight of vitamin D) was added. The solution was transferred to a 500-ml. conical flask and the solvents were removed under nitrogen. The esters were then prepared in a suitable small flask.

Crystalline and resin samples received no preesterification treatment.

Low-actinic glassware was used throughout the preparation of samples.

Column Preparation—Prior to coating, 15 g. of solid support was added to 90 ml. of propionic anhydride and heated at 80° for 2 hr. The bulk of the anhydride was removed by filtration through a büchner funnel, and final traces were removed in a 75° vacuum oven at 10 mm. Hg.

The vitamin D_2 column packing was prepared by adding 15 g. of propionated support to 90 ml. of toluene, containing 2.0% (w/v) of SE-52 liquid phase, and allowing the mixture to stand at room temperature for 60 min. with occasional gentle stirring. The coated support was filtered on a büchner funnel until dry. A 2.44-m. × 0.63-cm. (8-ft. × 0.25-in.) glass column was packed by applying suction and tapping gently.

The vitamin D_3 column was prepared by adding 15 g. of the propionated support to 90 ml. of chloroform, in which 0.60 g. of

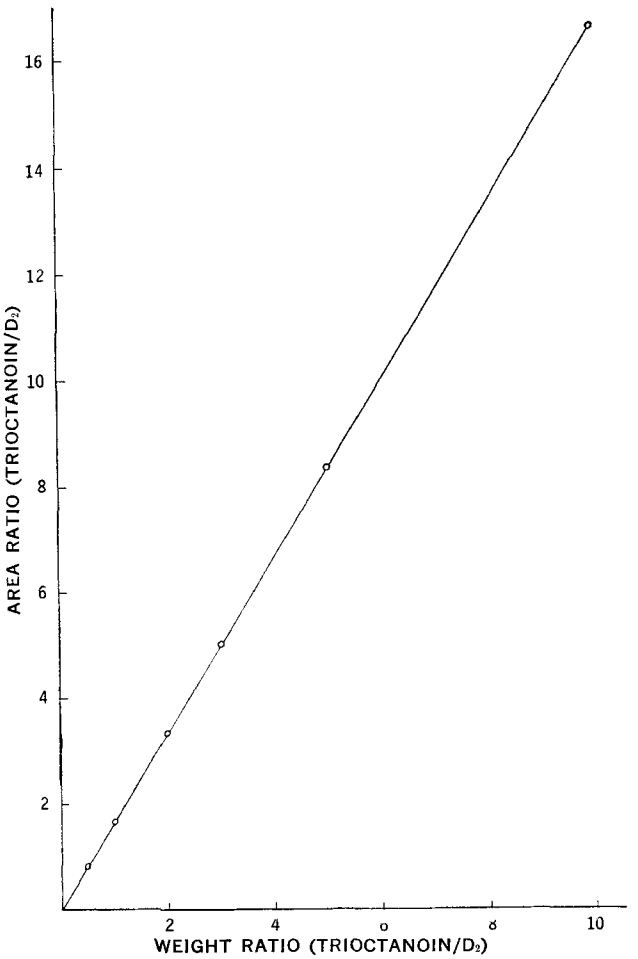

Figure 3—*Relative weight response of vitamin D_2 propionate* versus *trioctanoin:* (a) *peak areas are average of three determinations made over a period of 2 days; and* (b) *1.6 mcg. trioctanoin injected each time.*

[1] Eastman Organic Chemical 8090.
[2] Eastman Organic Chemical 2097, redistilled in laboratory.
[3] Hewlett-Packard, Avondale, Pa.
[4] Supelco Inc., Bellefonte, Pa.
[5] Eastman Organic Chemical 1291.
[6] Eastman Organic Chemical H-214.
[7] Tenox, Tennesse Eastman Co., Kingsport, Tenn.

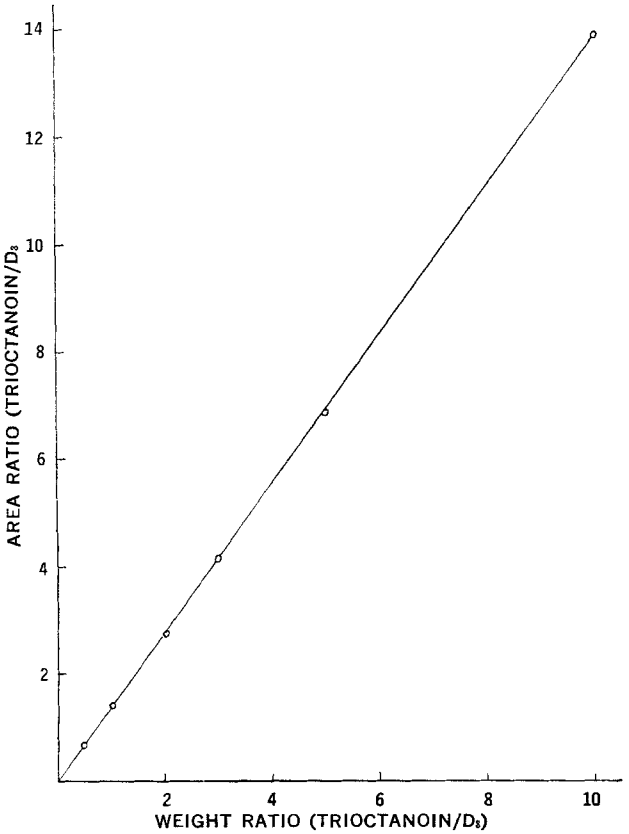

Figure 4—*Relative weight response of vitamin D_3 propionate* versus *trioctanoin:* (a) *peak areas are averages of three determinations made over a period of 3 days; and* (b) *1.6 mcg. trioctanoin injected each time.*

SE-30 liquid phase was dissolved, and allowing the mixture to stand at room temperature for 18 min. with occasional gentle stirring. The coated support was filtered, dried, and packed into a 2.44-m. × 0.63-cm. (8-ft. × 0.25-in.) column.

Columns were conditioned with the column attached to the oven inlet port only. Air was purged from the column with helium; then the helium flow was shut off. The oven temperature was programmed from ambient to 300° at 1°/min. and held for 64 hr. The oven temperature was then lowered to 275°, and the helium flow was adjusted to 75 ml./min. These conditions were maintained for 2 hr., after which the column was ready for use.

Analysis—An F&M model 810 gas chromatograph equipped with a hydrogen flame-ionization detector was used. The injection port temperature was set at 280°; the detector block temperature at 300°; and the column oven temperature at 270° for vitamin D_2 or at 240–250° for vitamin D_3. Helium flow rate was 75 ml./min.; hydrogen flow rate, 60 ml./min.; and air flow rate, 500 ml./min.

For each determination, a volume of sample solution containing approximately 1.6 mcg. of internal standard was injected at an attenuation of 10 × 8. Following elution of the internal standard, the attenuation was adjusted to give a satisfactory vitamin D peak.

Standard relative weight responses were obtained from chromatograms of the USP reference standards *versus* trioctanoin. These solutions were prepared by propionating (as before) a mixture of

Table III—Vitamin D_3 Assays of Laboratory-Prepared Blends of 7-Dehydrocholesterol, Vitamin A Palmitate, Mixed Tocopherols, and Vegetable Oil

Blend[a]	Ratio A:D	Vitamin D Potency, Units/g. Calculated	Found	
M	2.2:1	686,000	690,000	680,000
A	2.2:1	344,000	339,000	340,000
B	2.2:1	172,900	169,600	170,000
C	2.2:1	70,700	70,000	69,200
D	2.2:1	35,800	34,700	35,800

[a] Each blend contained a 1.3:1 ratio of γ-tocopherol to vitamin D_3.

Table IV—Vitamin D_2 Assays of Laboratory-Prepared Blends of Ergocalciferol, Vitamin A Palmitate, and Mixed Tocopherols

Blend[a]	A:D Ratio	Vitamin D Potency, Units/g. Calculated	Found
M	1.5:1	643,000	640,000
A	3.2:1	319,000	320,000
B	6.5:1	160,400	157,400
C	16.0:1	65,800	66,100
D	32.0:1	33,200	33,400

[a] Each blend contained a 1.5:1 ratio of γ-tocopherol to vitamin D_2.

30 mg. USP standard and 15 mg. trioctanoin, removing reagents, and diluting the residue to 10 ml. with chloroform.

The vitamin D potency in units per gram is given by the equation:

$$\text{potency (u./g.)} = \frac{Ax}{Wx} \times \frac{Ws}{As} \times F \times (40 \times 10^6) \quad \text{(Eq. 1)}$$

where Ax = area of the sample pyrovitamin peak, Wx = weight of sample, As = area of internal standard peak, Ws = weight of internal standard, $F = (As/Ws) \times (WR/AR)$ = relative weight response factor, WR = weight of *reference* standard, AR = area of reference pyrovitamin peak, and (40×10^6) = potency as units per gram of pure vitamin D. Areas of all peaks were determined by peak height times width at one-half peak height.

RESULTS AND DISCUSSION

The treatment of the solid support and the column-conditioning procedure were critical factors for eliminating on-column degradation of the vitamin D esters. The results obtained were satisfactorily reproducible; the columns so prepared exhibited efficiencies of 300–400 plates/ft. [16 $(tR/\Delta t)^2$], where tR is the uncorrected retention time and Δt is the peak width at the base.

Optimum conditions for esterification of vitamin D were determined by monitoring the rate of ester formation with GLC and by comparing the weight response of various levels of vitamin reacted. The corresponding pyro and isopyro thermal rearrangement isomers (10, 11) were observed.

The retention times of pertinent propionate esters relative to trioctanoin are given in Table I.

As noted previously (11), thermal rearrangement is only slightly dependent upon injection port temperature. For the propionate esters, the ratio of pyro to isopyro forms was constant for port temperatures between 230 and 300°. Walle *et al.* (12) reported a

Table V—Comparison of GLC and USP XVII Procedures for Various Multivitamin Preparations

Sample	Potency by USP XVII	Potency by GLC	Label Potency
Vitamin D_3 resin, units/g.	23.5×10^6 22.7×10^6	23.4×10^6 25.7×10^6	25×10^6 26×10^6
Vitamin D_3 oil, units/g.	33,000	47,000	40,000
Vitamin D_2 oil, units/g.	63,400 60,100 48,900 1.14×10^6 60,900 37,000 54,600 45,200	61,800 63,200 50,300 1.19×10^6 61,200 62,000 52,100 60,000	50,000 50,000 50,000 1.0×10^6 62,500 50,000 50,000 50,000
Vitamin D_2 crystal, units/g.	14.9×10^6	15.2×10^6	—
Vitamin D_2 beaded, units/g.	169,600 62,600 420,000 128,800	168,000 62,600 407,200 135,000	150,000 50,000 400,000 100,000
Vitamin D_2 tablet, units/tablet	16 13 12 13	12.5 14.6 11.6 11.5	10–15 10–15 10–15 10–15

contribution of vitamin A to the degree of rearrangement of the trimethylsilane derivatives. No such effect was noted in this study using the propionate esters. However, some difficulty was encountered with high proportions of vitamin A causing a large "solvent front" in the region where the internal standard was eluting. This was readily overcome by using a higher level of internal standard which allowed a higher attenuation in the region of interest and a consequent reduction of the "front" magnitude.

The response of vitamin D relative to trioctanoin was determined over the range of weight ratios used by plotting the mean ratios of the peak areas against the weight ratios and calculating the equations of the lines by the method of least squares (Figs. 3 and 4). The lines passed through the origin and had standard deviations of 0.011 and 0.013 for vitamin D_2 and vitamin D_3, respectively.

Column overloading was observed when injections of the internal standard exceeded 4 mcg.

At the start of each day's run, the chromatogram of the first injection was discarded because its value was sometimes erroneous.

The precision attained by this method is indicated by the data in Tables II and III.

Blends for accuracy analysis (Tables III and IV) contained sufficient γ-tocopherol to produce a peak height equivalent to pyrovitamin D and vitamin A in excess of the amount normally encountered in multivitamin preparations.

For comparison, assays of a variety of samples which had recently been assayed by the procedure given in USP XVII are given in Table V. The "oil," "beaded," and "tablet" samples are multivitamin preparations of various potencies.

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X-Ray Diffraction Analysis for Identification of Kaolin NF and Bentonite USP

FARID SADIK, JULIAN H. FINCHER, and CHARLES W. HARTMAN*

Abstract □ The identities of kaolin NF and bentonite USP were tested by using the identification test stated in the NF XIII monograph on kaolin. The results were inconclusive because both clays gave positive results to the test. Since kaolin NF and bentonite USP are crystalline in nature, X-ray diffraction analysis could be used for their identification. Because of its accuracy, X-ray diffraction analysis is recommended to be included in the NF and USP monographs on kaolin and bentonite, respectively, as an alternative test.

Keyphrases □ Kaolin—identification □ Bentonite—identification □ X-ray diffractometry—identification, kaolin, bentonite

Kaolin was first introduced into the USP VIII in 1905 as a constituent of Cataplasma Kaolini. Later, it was deleted from the USP IX and was introduced into the NF IV in 1916. Bentonite was first introduced into the USP XII in 1942. The NF XIII monograph on kaolin includes a specific identification test, which is merely a test for the presence of aluminum. On the other hand, the USP XVIII monograph on bentonite does not include a specific identification test but relies on tests such as gel formation and swelling power.

Since both clays are chemically similar and contain aluminum, it was obvious that methods other than the chemical analysis for aluminum needed to be included in the NF monograph on kaolin in order to distinguish between kaolin NF and bentonite USP. Furthermore, it was felt that a specific identification test should be included in the USP monograph on bentonite. The objective of this study was to show the importance of the inclusion of X-ray diffraction analysis in the NF and USP monographs on kaolin and bentonite, respectively. Since both clays are crystalline in nature (1), it was possible to utilize X-ray diffraction analysis to measure the angles of diffraction of the X-ray from the atomic planes of the crystal.

EXPERIMENTAL

NF XIII Identification Test for Kaolin—The NF XIII identification test for kaolin was conducted on kaolin NF and bentonite USP. Both clays responded positively to the test for aluminum, *i.e.*, a gelatinous, white precipitate was produced.

X-Ray Diffraction Analysis—Two types of samples were prepared. An unoriented powder sample was prepared by packing the powder in an aluminum planchet. The powder was smoothed by

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Table I—Basal (001) and Higher Order X-Ray Diffraction Spacings of Official Clays[a]

Kaolin NF	Bentonite USP	Glycolated Bentonite USP
7.1091	12.2670	16.2910
4.4445	4.4357	8.3544
4.3496	3.1163	5.6041
4.1639		4.4226
3.8176		4.2108
3.7169		3.3706
3.5587		
3.3731		

[a] Lattice spacings in angstroms.

using the flat side of a spatula to obtain a uniform level suitable to the X-ray beam. A sample of an oriented aggregate was prepared as follows: a 5% (w/w) aqueous clay suspension was prepared by sprinkling the clay over the surface of the water. The mixture was allowed to hydrate for 2 hr. and then was passed through a manual homogenizer. By means of a pipet, 2 ml. of the resultant suspension was withdrawn and allowed to drain onto a glass microscopic slide (27 × 46 mm.) which was completely covered with suspension. The slide was allowed to stand undisturbed at 22.2° (72°F.) and at a relative humidity of 52–55% for 72 hr. to permit air drying.

A Norelco X-ray diffractometer was used. Each sample was placed in the diffractometer and was exposed to a monochromatic X-ray beam generated by using a copper target X-ray tube and nickel filter operated at 40 kv. and 30 mamp. Scanning was begun at 2° (2θ) at the rate of 1°/min. The X-ray diffractogram obtained was recorded by a chart recorder. The sample was then transferred to a desiccator, in which an evaporating dish filled with ethylene glycol was placed. After exposing the sample to the ethylene glycol vapor for 24 hr., a second X-ray diffractogram was obtained as before. Table I shows the basal (001) spacings as well as the spacings of higher order X-ray reflections of each sample.

RESULTS AND DISCUSSION

The NF XIII defines kaolin as a native hydrated aluminum silicate; the USP XVIII defines bentonite as a native colloidal, hydrated aluminum silicate. Thus, the primary elements of kaolin and bentonite composition are aluminum and silicon. Because they contain aluminum in their structure, both clays gave positive results to the identification test of the NF XIII monograph on kaolin, which is basically a test for the presence of aluminum. This finding indicates that the identification test of the NF XIII is inconclusive. To distinguish between kaolin and bentonite, one needs to check other specifications in the NF and USP. The USP XVIII monograph on bentonite does not include a specific identification test.

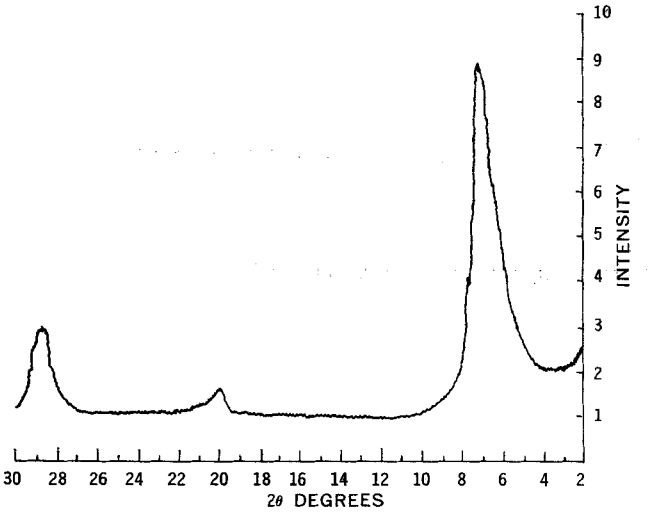

Figure 2—*X-ray diffractogram for bentonite USP.*

Although chemically similar, kaolin NF and bentonite USP differ mainly in their crystal structure. The unit cell structure of kaolin has two layers, stacked one over the other, with their flat sides together. One layer is composed of Al_2O_3 molecules and the other of SiO_2 molecules. The unit cell structure of bentonite is composed of three layers: two layers of SiO_2 molecules with one layer of Al_2O_3 molecules lying between them. This dissimilar assembly of the atoms permitted distinct identification of kaolin and bentonite by means of X-ray diffraction analysis. X-ray diffractograms for kaolin NF and bentonite USP are shown in Figs. 1 and 2, respectively. It is evident that these diffractograms differ and that each clay exhibits a characteristic series of X-ray diffraction peaks.

Because the tests of the official compendia are time consuming and obviously not conclusive, X-ray diffraction analysis was chosen as a methodology which could be conveniently and rapidly used for the identification of kaolin and bentonite. A total time of approximately 40 min. is needed to prepare an unoriented powder sample, obtain an X-ray diffractogram, and identify a sample. The purpose of exposing the clay samples to ethylene glycol is to provide added evidence as to the identity of bentonite and to distinguish it from kaolin. Bentonite is characterized by an expanding lattice. It was shown by MacEwan (2, 3) and MacKenzie (4) that ethylene glycol is adsorbed on the basal plane surface of bentonite and that the C-axis spacing is increased. Ethylene glycol, however, did not affect the nonexpanding lattice of kaolin and the basal plane remained unchanged. The basal plane of glycolated bentonite USP expanded from 7.20° (2θ) to 5.42° (2θ); kaolin NF, when glycolated, remained unchanged at 12.44° (2θ) (Figs. 1–3).

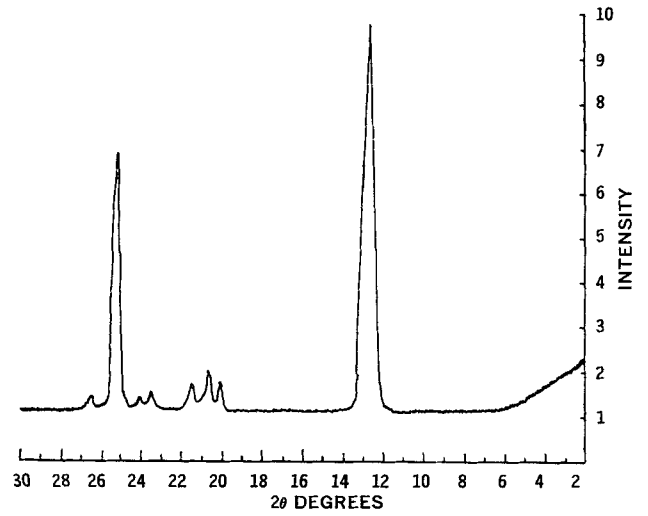

Figure 1—*X-ray diffractogram for kaolin NF.*

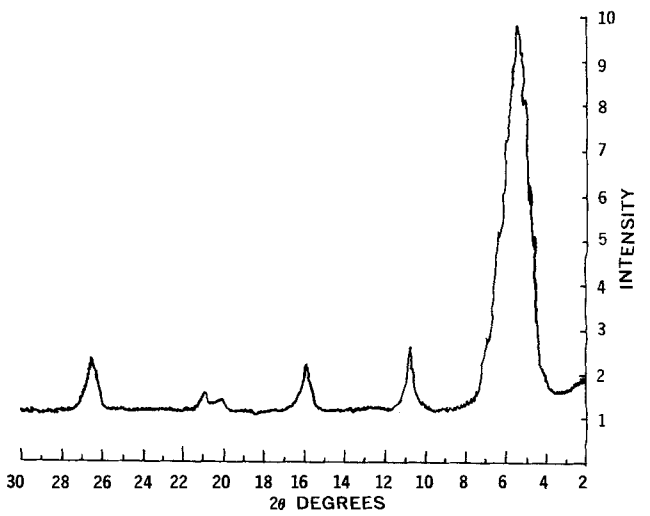

Figure 3—*X-ray diffractogram for glycolated bentonite USP.*

In conclusion, for identification of kaolin and bentonite, X-ray diffraction analysis was found to be more accurate, dependable, conclusive, and much less time consuming than the methods of identification presently employed in the NF XIII and USP XVIII monographs on kaolin and bentonite, respectively. Consequently, it is recommended that these monographs should be supplemented by X-ray diffraction analysis.

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* Deceased.

Spectrophotometric Determination of Fe(II), Fe(III), and Total Fe

CHRISTOS ZACHARIADES* and WILLIAM C. McGAVOCK†

Abstract □ The orderly use of two known reactions [Fe(II) with α,α'-dipyridyl and Fe(III) with ascorbic acid] allowed to take place successively in the same aliquot sample solution provides a procedure for the quantitative determination of Fe(II), Fe(III), and total Fe. The procedure is simple, rapid, and sensitive enough to cope with samples containing small amounts of iron or with samples of limited solubility. From a pharmaceutical point of view, the method is useful for the control of both hematinic raw materials or finished products, and it is especially useful for stability and content uniformity studies.

Keyphrases □ Fe(II), Fe(III), total Fe in aqueous solution—determination □ α,α'-Dipyridyl—color formation, Fe(II) determination □ Ascorbic acid—color formation, Fe(III) determination □ Hematinic salts—determination of iron content □ Colorimetric analysis—spectrophotometer

A simple procedure is presented for the simultaneous determination of Fe(II), Fe(III), and total Fe in a single aliquot from an aqueous solution of these components. This procedure can be readily adapted to content uniformity (1) and shelflife stability studies (2) on both hematinic raw materials and finished pharmaceutical products, especially those labeled to contain iron in its Fe(II) state. The titrimetric methods described by the USP (3) and NF (4) for total Fe on *finished products* are not feasible for single tablet or capsule analysis. Furthermore, these sources do not as yet provide methods for Fe(III) on finished products. These needs are met by the present procedure.

The procedure uses two well-known reactions [Fe(III) *versus* ascorbic acid to yield Fe(II), and Fe(II) *versus* α,α'-dipyridyl to yield a red complex] taking place in succession in the same aliquot solution. Initially, the intensity of the color produced by the reaction product of Fe(II) with α,α'-dipyridyl is measured against the blank to yield the Fe(II) content. Then solid ascorbic acid (10–15 mg.) is added to reduce any Fe(III) to additional Fe(II); the latter, in turn, reacts with the existing excess of α,α'-dipyridyl to produce an increase in color intensity. This intensity is then measured twice: once against the first colored solution yielding the Fe(III) content, and once against the blank yielding the total Fe content. Because of the small amount of solid ascorbic acid added, the volume increase of the final solution is negligible.

EXPERIMENTAL

Reagents and Instruments—The following were used: (*a*) α,α'-dipyridyl 0.1% solution in water; (*b*) buffer (pH 4.5) solution of 83 g. sodium acetate and 120 ml. acetic acid in water to a total volume of 1 l.; (*c*) ascorbic acid USP, crystalline powder; (*d*) Beckman DB-G double-beam spectrophotometer; (*e*) Beckman 25.4-cm. (10-in.) recorder for spectrophotometers; and (*f*) three matched cells, 1-cm. lightpath, marked as No. 1, No. 2, and No. 3.

Procedure—Using approximately 0.1 *N* HCl, dilute the soluble iron sample to an expected concentration of 10–300 mcg. Fe/ml. In the case of solid dosage forms, crush and powder the sample in a mortar, dissolve the iron salts with 0.1 *N* HCl, filter, and dilute as above. Transfer an aliquot, estimated to contain about 300–350

Table I—Analysis of Mixtures of Known Composition

Standard Mixtures[a]	Total Fe, —mcg./ml.— Taken	Found	Fe(II), —mcg./ml.— Taken	Found	Fe(III), —mcg./ml.— Taken	Found
Preparation A	2.60	2.58	0.81	0.82	1.79	1.76
Preparation B	2.83	2.84	1.63	1.62	1.20	1.22
Preparation C	3.19	3.16	2.26	2.21	0.93	0.95
Preparation D	2.94	2.97	1.69	1.70	1.25	1.27
Preparation E	2.43	2.45	2.43	2.44	0.00	0.01
Preparation F	2.33	2.36	0.00	0.02	2.33	2.34

[a] Prepared by dissolving six pairs of weighed amounts of ferrous and ferric ammonium sulfate. Single aliquots were then taken and diluted to indicated final concentration.

In conclusion, for identification of kaolin and bentonite, X-ray diffraction analysis was found to be more accurate, dependable, conclusive, and much less time consuming than the methods of identification presently employed in the NF XIII and USP XVIII monographs on kaolin and bentonite, respectively. Consequently, it is recommended that these monographs should be supplemented by X-ray diffraction analysis.

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Spectrophotometric Determination of Fe(II), Fe(III), and Total Fe

CHRISTOS ZACHARIADES* and WILLIAM C. McGAVOCK†

Abstract □ The orderly use of two known reactions [Fe(II) with α,α'-dipyridyl and Fe(III) with ascorbic acid] allowed to take place successively in the same aliquot sample solution provides a procedure for the quantitative determination of Fe(II), Fe(III), and total Fe. The procedure is simple, rapid, and sensitive enough to cope with samples containing small amounts of iron or with samples of limited solubility. From a pharmaceutical point of view, the method is useful for the control of both hematinic raw materials or finished products, and it is especially useful for stability and content uniformity studies.

Keyphrases □ Fe(II), Fe(III), total Fe in aqueous solution—determination □ α,α'-Dipyridyl—color formation, Fe(II) determination □ Ascorbic acid—color formation, Fe(III) determination □ Hematinic salts—determination of iron content □ Colorimetric analysis—spectrophotometer

A simple procedure is presented for the simultaneous determination of Fe(II), Fe(III), and total Fe in a single aliquot from an aqueous solution of these components. This procedure can be readily adapted to content uniformity (1) and shelflife stability studies (2) on both hematinic raw materials and finished pharmaceutical products, especially those labeled to contain iron in its Fe(II) state. The titrimetric methods described by the USP (3) and NF (4) for total Fe on *finished products* are not feasible for single tablet or capsule analysis. Furthermore, these sources do not as yet provide methods for Fe(III) on finished products. These needs are met by the present procedure.

The procedure uses two well-known reactions [Fe(III) *versus* ascorbic acid to yield Fe(II), and Fe(II) *versus* α,α'-dipyridyl to yield a red complex] taking place in succession in the same aliquot solution. Initially, the intensity of the color produced by the reaction product of Fe(II) with α,α'-dipyridyl is measured against the blank to yield the Fe(II) content. Then solid ascorbic acid (10–15 mg.) is added to reduce any Fe(III) to additional Fe(II); the latter, in turn, reacts with the existing excess of α,α'-dipyridyl to produce an increase in color intensity. This intensity is then measured twice: once against the first colored solution yielding the Fe(III) content, and once against the blank yielding the total Fe content. Because of the small amount of solid ascorbic acid added, the volume increase of the final solution is negligible.

EXPERIMENTAL

Reagents and Instruments—The following were used: (*a*) α,α'-dipyridyl 0.1% solution in water; (*b*) buffer (pH 4.5) solution of 83 g. sodium acetate and 120 ml. acetic acid in water to a total volume of 1 l.; (*c*) ascorbic acid USP, crystalline powder; (*d*) Beckman DB-G double-beam spectrophotometer; (*e*) Beckman 25.4-cm. (10-in.) recorder for spectrophotometers; and (*f*) three matched cells, 1-cm. lightpath, marked as No. 1, No. 2, and No. 3.

Procedure—Using approximately 0.1 *N* HCl, dilute the soluble iron sample to an expected concentration of 10–300 mcg. Fe/ml. In the case of solid dosage forms, crush and powder the sample in a mortar, dissolve the iron salts with 0.1 *N* HCl, filter, and dilute as above. Transfer an aliquot, estimated to contain about 300–350

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Preparation F	2.33	2.36	0.00	0.02	2.33	2.34

[a] Prepared by dissolving six pairs of weighed amounts of ferrous and ferric ammonium sulfate. Single aliquots were then taken and diluted to indicated final concentration.

Table II—Analysis of Raw Materials[a] by This Method and USP or NF Method

Substance and Lot Number	Total Fe Found, %: USP or NF	Total Fe Found, %: This Method	Fe(II) Found, %: USP or NF	Fe(II) Found, %: This Method	Fe(III) Found, %: USP or NF	Fe(III) Found, %: This Method
Ferrous gluconate NHT-404	11.05	11.10	10.30	10.30	0.75	0.80
Ferrous fumarate LBB	32.05	31.96	29.15	29.02	2.85	2.94
Ferrous gluconate NET-2667	11.00	11.13	10.39	10.45	0.61	0.67
Ferrous fumarate H2177 × A12	31.60	31.71	29.75	29.71	1.85	1.95

[a] Raw material in "as is" condition.

mcg. of total iron, into a 100-ml. volumetric flask and add water to bring the volume to about 50 ml. Mix and add 15 ml. buffer solution and 6 ml. α,α'-dipyridyl solution. Bring the volume to the mark with water, mix, fill cell No. 2, and scan this solution so as to obtain an absorbance curve from 500 to 600 nm. against a blank in cell No. 1, prepared similarly but omitting the α,α'-dipyridyl. To the remaining sample in the flask, add an estimated 10–15 mg. of ascorbic acid, shake well, and fill cell No. 3; scan this solution through the same wavelengths, once against the contents in cell No. 2, and once against the contents in cell No. 1.

Under the conditions of this procedure, it was found that Beer's law is obeyed by the system and that the absorptivity of the red complex is 158 l./g.cm. at the wavelength of maximum absorbance. Therefore, the maximum absorbance readings (at around 523 nm.) from the first, second, and third graphs divided by 0.158 represent the micrograms per milliliter of Fe(II), Fe(III), and total Fe, respectively.

Since the sum of Fe(II) and Fe(III) is that for total Fe, it is obvious that one of the graphs may be omitted and the corresponding result be obtained by adding or subtracting the other two recorded results.

Table III—Analytical Results Obtained by This Procedure on Commercial Products

Commercial Products	Dosage Form	Claim, mg./unit	Found, mg./unit	Percent Labeled Found
A	Tablet	36.9 Fe(II) / T	38.6 Fe 35.7 Fe(II) 2.9 Fe(III)	104.9 97.0 7.9
B	Liquid	44 Fe(II) / 5 ml.	43.2 Fe 40.7 Fe(II) 2.4 Fe(III)	98.1 92.6 5.5
C	Tablet	40.0 Fe(II) / T	39.6 Fe 39.6 Fe(II) 0.0 Fe(III)	99.0 99.0 0.0
D	Liquid	200 Fe(III) / oz.	196.2 Fe 192.4 Fe(II) 3.8 Fe(III)	98.1 96.2 1.9
E	Tablet	65.7 Fe(II) / T	66.2 Fe 62.8 Fe(II) 3.6 Fe(III)	100.8 95.4 5.4
F	Liquid	10 Fe / 5 ml.	10.2 Fe 0.3 Fe(II) 9.9 Fe(III)	101.8 3.1 98.7
G	Tablet	> 160 $FeSO_4$ / T	173.2 $FeSO_4$ 0.0 Fe(III)	108.2 0.0
H	Tablet	100 Fe / T	107.2 Fe 6.3 Fe(II) 100.9 Fe(III)	107.2 6.3 100.9

RESULTS

Various known concentrations of Fe(II) and Fe(III) mixtures were prepared and analyzed by this method. The results in Table I demonstrate the validity of the method and the degree of its accuracy. Furthermore, various raw material hematinic salts were analyzed; the results reflected in Table II show no appreciable difference from those obtained by the USP and/or NF methods. Finally, a variety of commercial hematinic tablets and elixirs was tested by this method at the level of their unit dosage form. The results in Table III are of comparative value only, because the manufacturer's overage and the actual input in the unit were, of course, unknown.

DISCUSSION

Many hematinic preparations tested by this procedure were colored from caramel or other sources but presented no difficulty. This was due either to the subdilutions or to the decolorizing properties of ascorbic acid. Any persisting color could be taken into account in the blank. Some other hematinic products were found to require time and warming of the solution for the reduction of Fe(III) to Fe(II) by ascorbic acid. Products with an excess of ascorbic acid or other reducing agents present will not show difference in Fe(II) and total iron content, because, if present in elixirs, they maintain the iron in the ferrous state or react with any existing Fe(III) in the tablet or capsule to yield Fe(II) during the first step of dilution in this procedure.

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Determination of Di-(2-ethylhexyl) Sodium Sulfosuccinate and Related Dialkyl Esters in Aqueous Solutions by the Ferric Hydroxamate Procedure

JOSEPH A. FELDMAN, SYLVAN G. FRANK*, and THOMAS J. HOLMES, Jr.†

Abstract □ Di-(2-ethylhexyl) sodium sulfosuccinate and its closely related calcium salt, di-(*n*-octyl) isomer, and di-(*n*-hexyl) derivative have shown excellent quantitative response to the ferric hydroxamate reaction under controlled conditions. Their diester structures make them extremely susceptible to attack by the reagent, while hydrolysis is inhibited by the reaction conditions. Optimum conditions for successful determination have been elucidated for the compounds mentioned.

Keyphrases □ Surfactant determination—aqueous solutions □ Di-(2-ethylhexyl) sodium sulfosuccinate, related esters—determination □ Ferric hydroxamate procedures—analysis □ Colorimetric analysis—spectrophotometer

The usual methods for analysis of the dialkyl sulfosuccinates (1–6) involve titration of a dye–cationic surfactant complex with the anionic surfactant in aqueous solution in a two-phase system (aqueous buffer solution–chloroform). The inherent problems of foaming, possible emulsification at the interface, and possible incomplete surfactant transfer between the phases are unavoidable disadvantages of these methods. The difficult visual detection of the end-point and standardization of the cationic surfactant present additional problems in the titration techniques. Other proposed assay procedures such as that of Stroehl and Kurzak (7), which requires chloroform extraction of a dye complex formed in an aqueous medium, or those based on reduction of surface tension (8, 9) or production of turbidity (10), or those relying on the Draves test (8) also reveal added difficulties. However, by utilizing the ferric hydroxamate reaction, the assay may be carried out in a single-phase system, with reduced foaming and direct measurement of the formed complex with a suitable spectrophotometer. The problems associated with phase transfer, extraction, possible emulsification, and foaming are eliminated in this procedure.

EXPERIMENTAL

Materials—Di-(2-ethylhexyl) sodium sulfosuccinate, di-(*n*-hexyl) sodium sulfosuccinate, and di-(*n*-octyl) sodium sulfosuccinate were synthesized according to previously recommended procedures (11, 12). Di-(*n*-hexyl) sodium sulfosuccinate and di-(*n*-octyl) sodium sulfosuccinate, which exist as crystalline compounds, unlike di-(2-ethylhexyl) sodium sulfosuccinate which is amorphous (13), were recrystallized from anhydrous methanol (9). Di-(2-ethylhexyl) calcium sulfosuccinate[1] was obtained commercially and decolorized in a methanol solution prior to use.

Reagents were prepared as follows:

1. Hydroxylamine hydrochloride solution (saturated)—dissolve 83 g. of the salt in 100 ml. of water.
2. Potassium hydroxide solution (10.5 *M*)—dissolve 58.8 g. of KOH in a sufficient quantity of water to make 100 ml. of solution.

[1] Surfak, Hoechst.

Table I—Absorbance Values Obtained with Reaction at 0°, Room Temperature, and Variable Reaction Times with Samples of Di-(2-ethylhexyl) Sodium Sulfosuccinate

	0°			Room Temperature	
	Weight of Di-(2-ethylhexyl) Sodium Sulfosuccinate per 5 ml. of Sample Preparation			Weight of Di-(2-ethylhexyl) Sodium Sulfosuccinate per 5 ml. of Sample Preparation	
Minutes	1 mg.	2 mg.	3 mg.	2 mg.	3 mg.
0	—	0.059	0.079	0.208	0.327
20	—	0.349	0.473	0.330	0.499
40	—	0.387	0.573	0.305	0.440
60	—	0.408	0.620	0.323	0.456
75	0.235	0.459	0.630	—	—
90	0.202	0.414	0.625	—	—
120	0.209	0.412	0.624	—	—

3. Hydrochloric acid solution—add 100 ml. of concentrated acid to 100 ml. of water.
4. Ferric chloride solution (0.37 *M*)—dissolve 25.004 g. of ferric chloride in a sufficient quantity of 0.1 *N* hydrochloric acid solution to make 250 ml. of solution.

Instrumentation—All absorbance readings were obtained on the Bausch and Lomb Spectronic-20 spectrophotometer at 540 nm. with 11.6-mm. (i.d.) cells.

Procedure—The procedure followed was an adaptation of the Feldman and Robb (14) method. Various concentrations of samples were prepared from initial aqueous solutions containing 2.5 mg./ml. of surfactant. Samples were brought to a total volume of 5.0 ml. by the addition of water up to a volume of 2.0 ml., followed by the addition of 2.0 ml. of methanol and 1.0 ml. of isopropanol. The samples were then placed in an ice bath to cool. Subsequently, 1.0 ml. of the hydroxylamine hydrochloride solution followed by 1.0 ml. of the potassium hydroxide solution was added to the samples; they were again placed in an ice bath for a period of 1 hr. During this time, white crystals (presumably excess hydroxylamine hydrochloride) formed in the tubes. However, when the samples were brought to room temperature (22–25°), the crystals redissolved. After 1 hr., the solutions were acidified with 2.0 ml. of the hydrochloric acid solution (pH approximately 0.5); transient gas evolution occurred at this point and the crystals gradually redissolved. The solutions were allowed to reach room temperature, and then 1.0 ml. of the ferric chloride solution was added. The solution becomes stable within 1 min.

Since the colored complex fades slightly with time, spectrophotometer readings should be taken immediately at 540 nm. Calculate the amount of surfactant present in the sample, in milligrams, from the formula $C\ (A_U/A_S)$, where C is the total weight (dry) in milligrams of a suitable reference standard (prepared in aqueous solution such that the final concentration of standard is 1.0–1.5 mg./ml. of solution) treated concurrently with the unknown solution as directed, and A_U and A_S are the absorbances obtained for the unknown sample and reference sample, respectively.

RESULTS AND DISCUSSION

The analysis is composed of two main reaction steps: (*a*) the possible formation of the dihydroxamic acid, and (*b*) the formation of a stable red to reddish-brown colored complex of ferric hydroxamate, the intensity of which is proportional to the amount of ester present. Conditions must be controlled so that the base-catalyzed

Table II—Absorbance Values Obtained for Related Dialkyl Sulfosuccinates Using the Ferric Hydroxamate Procedure

Total Milligrams Present[a]	Di-(2-ethylhexyl) Sodium Sulfosuccinate	Di-(*n*-hexyl) Sodium Sulfosuccinate	Di-(*n*-octyl) Sodium Sulfosuccinate	Di-(2-ethylhexyl) Calcium Sulfosuccinate
0.5	0.130	0.114	0.087	0.128
1.0	0.246	0.274	0.206	0.231
1.5	0.335	0.368	0.283	0.371
2.0	0.536	0.528	0.444	0.462
3.0	—	0.810	0.644	0.703

[a] Per 5 ml. of sample preparation.

hydroxamic acid formation predominates over the alkaline hydrolysis of the ester groups. To suppress hydrolysis and promote hydroxamic acid formation, the reaction is carried out in an ice bath while a high concentration of hydroxylamine is maintained in the mixture.

A hydroalcoholic solution is used to enhance the solubility of the surfactants and prevent turbidity in the solution while maintaining the solubility of the reagents. The ratio of alcohol to water utilized in the procedure was determined experimentally. Initial trials in an all-aqueous medium resulted in turbid solutions at relatively low concentrations (2–3 mg.) of surfactant. However, with the recommended system, samples containing as much as 10 mg. of surfactant produced no turbidity; however, the color density of the solution prevented accurate measurement of absorbance. Substitution of other alcohols (ethanol and *n*-propanol) into the system and adjustment of their ratios revealed no additional advantages.

The effects of temperature on the production of color and length of reaction time were also noted. Results in agreement with Beer's law were obtained with reaction at room temperature (22–25°) as well as at ice bath temperature (0°). However, it was observed (Table I) by varying the length of reaction time that although complex formation is in agreement with Beer's law, it is not directly proportional to reaction time. It is suggested that this variation results from a competitive reaction at room temperature, possibly hydrolysis of the hydroxamic acid at one or both positions as it is being formed. At 0°, this variation is not observed; complex formation increases directly with the length of reaction time (Table I). It may also be observed that according to the greater increase in absorbance between concentrations with the reaction at 0°, greater complex formation occurs at this temperature. For these reasons, the ice bath technique is preferred over reaction at room temperature. Subsequent time trials also showed (Table I) that reaction times slightly longer than 1 hr. might be advantageous; however, this supposition is inconsistent for much longer reaction times.

Absorbance values obtained using this method of analysis for various related anionic surface-active dialkyl esters are shown in Table II. Correlation with Beer's law was observed with concentrations of surfactants up to 5 mg./sample; however, instrument limitations prevented accurate measurement of absorbance near this value. Average molar absorptivities calculated for di-(2-ethylhexyl) sodium sulfosuccinate, di-(*n*-hexyl) sodium sulfosuccinate, di-(*n*-octyl) sodium sulfosuccinate, and di-(2-ethylhexyl) calcium sulfosuccinate are 960, 860, 770, and 1900, respectively. Two precautions which must be taken to achieve absorbance values that conform to Beer's law are: (*a*) volume measurements in sample dilution must be exact, and (*b*) the pH of the solution prior to addition of the ferric chloride must be sufficiently acid.

CONCLUSION

A satisfactory assay for several of the dialkyl sulfosuccinate anionic surfactants in aqueous solution was developed. The conditions necessary for reproducible reaction have been established. This procedure removes many of the difficulties and disadvantages associated with other methods of analysis for these compounds. The ferric hydroxamate procedure allows simple and accurate determination of these compounds without unnecessarily complex techniques. Additionally it allows for the analysis of low concentrations of these surfactants. It is hoped that the proposed procedure will offer a better and more practical means of analysis than those methods already in use.

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Granule Strength as a Formulation Factor I: Instrumentation

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Abstract □ Although it is recognized that granule strength is an important property of powders influencing pharmaceutical processes, little information has been published on the subject. This lack of information is due mainly to difficulty in obtaining quantitative measurement of granule strength and, therefore, in obtaining meaningful data. To meet this need, instrumentation was constructed. It consists of a mechanical linkage to apply a compressive load at a uniform rate and a strain-gauge instrumented cantilever beam to convert the load to a proportional millivolt response which is measured on a recorder. A granule is placed on the platform of the cantilever beam, and an overhead cam depresses a plunger which compresses the granule. The reaction of the granule is obtained on a recorder tracing from which the stress pattern is analyzed and the crushing strength calculated.

Keyphrases □ Granule strength determination—instrumentation □ Instrumentation—granule strength testing □ Diagram—granule strength testing instrument □ Compressive strength—granules

The effect of granule strength as a formulation factor is virtually unknown, and the literature contains little information on the subject to aid the industrial pharmacist in formulating solid-dosage formulations. The effects of granule strength on such factors as compressibility, tablet hardness, friability, dissolution of active ingredients, and mouthfeel of chewable tablets are but a few areas that need to be explored. The limited data available are difficult to interpret due to the variety and subjectivity of methods used to measure granule strength.

Friability has been used as a parameter to evaluate granule strength, with increased friability associated with softer granules. Granules are placed in a Roche Friabilator or shaken with a standardized shaking device; the weight of granules exceeding a specific mesh size, expressed as a fraction or percentage, serves as an index of granule strength. In a study designed to determine the effect of granule size upon the physical properties of the granules and the tablets produced from these granules (1), the degree of friability was found to be inversely proportional to the granule size. In another study to assess the manufacturing procedures for preparing granules suitable for pharmaceutical coating purposes, the authors reported that the hardness index increased as the density of the granule increased (2). In the absence of a definitive study relating friability to granule strength, it may only be assumed that resistance to abrasion data accurately reflects the strength of the granule.

A method for measuring the breaking strength of individual granules was recently reported (3). In this test, lead shot was poured onto a pan held directly on top of the granule until the granule broke. Breaking of the granule was determined visually and had to be coordinated with manually stopping the flow of lead shot at the breaking point. The study showed that the strength of granules was dependent on both their size and on their polyvinylpyrrolidone content. The authors attributed the increase in strength to solid bridges of polyvinylpyrrolidone which formed on evaporation of the granulating solution. They also reported considerable variation in granule strength and theorized that it was due to the brittle properties of polyvinylpyrrolidone in the solid state.

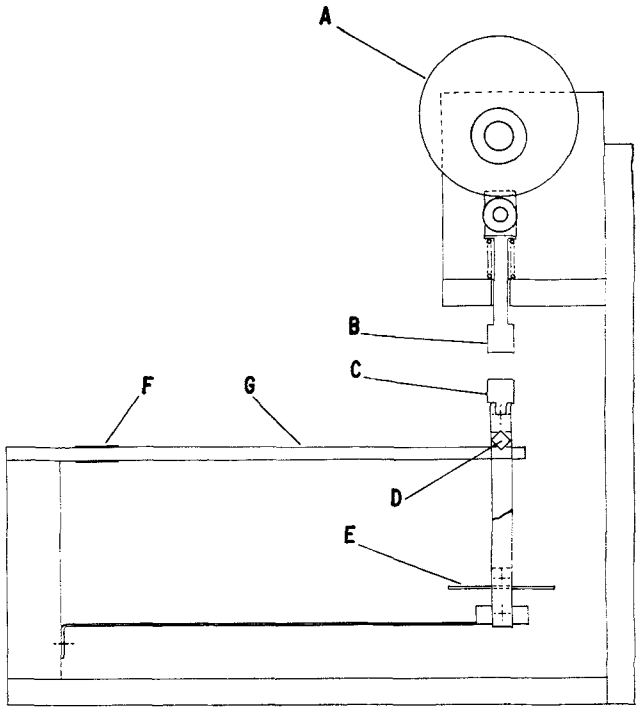

Figure 1—*Instrumentation for measuring granule strength. Key: A, motor-driven cam; B, upper jaw; C, lower jaw or anvil; D, steel pivot; E, weight platform for calibration; F, strain gauges; and G, cantilever beam.*

In another recent study (4), the authors used a simple crushing test with a spatula and observed that an increase in the amount of granulating solution increased the strength of the granules.

Other investigators (5) modified a microtensile testing machine to measure compressive strain. They calculated the elastic moduli for sodium chloride, sucrose, hexamine, salicylamide, and aspirin. Diffi-

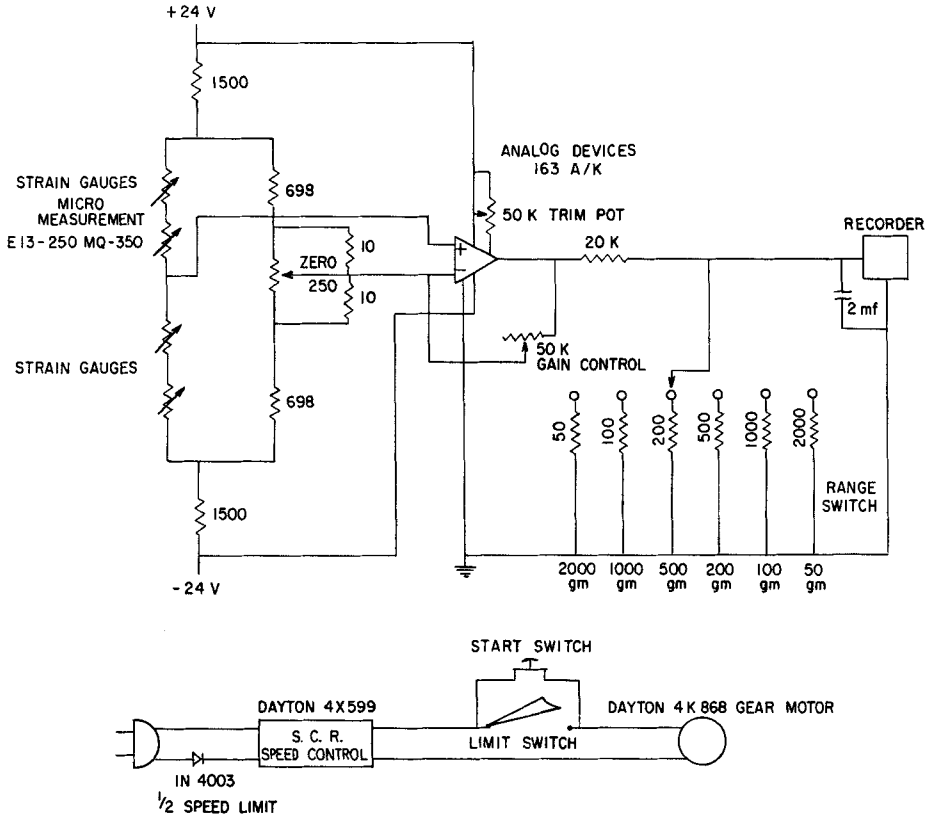

Figure 2—*Electrical diagram of strain-gauge instrumented cantilever beam and motor-driven cam.*

culties were encountered in determining the elastic modulus due to crystal defects, misorientation, slip, and cracking under load, which contributed to a scattering of results. The scattering of results was also reported in a study of tensile strength measurement on moist granules (6).

At the present time, there is no generally accepted method for measuring granule strength nor is there a method suitable for the desired purposes. The immediate objective of this study was the development of instrumentation that would yield basic information on granule strength and be practical for use in an industrial setting. This report describes the instrumentation developed.

EXPERIMENTAL

Description of Instrument—The instrumentation (Fig. 1) consists of a mechanical linkage to supply the load at a uniform rate, a strain-gauge instrumented cantilever beam to convert the compressive load to a proportional millivolt response, and a recorder to measure the millivolt response.

A single granule is selected at random from within a mesh size range and is placed on the anvil (C). The cam (A) is then rotated at a uniform rate, which may be varied from 12 sec. to 3.5 min. per revolution. Rotation of the cam, controlled by a link chain, gear motor[1], and speed control switch[2], causes the plunger to descend, compressing the granule against the anvil. The compressive load bends the cantilever beam, thereby deforming the strain gauges. By arranging a hardened steel pivot (D) in a parallelogram arrangement, the load is applied with low friction at one point on the beam. The millivolt response across the strain-gauge bridge circuit is proportional to the compressive load on the granule and is measured on the horizontal axis of a recorder. A tracing is obtained of increasing compressive load with time, with a break in the tracing indicating the compressive load at fracture. Twenty measurements are made, and the average breaking load and standard deviation are calculated.

The electrical diagram (Fig. 2) shows the strain gauges connected in series as a voltage divider, with the center point between the upper and lower gauges being fed to an operational amplifier and compared with a point on another voltage divider fed from the same power source. All components have low temperature coefficients to reduce thermal drift, and the power supply uses an integrated circuit voltage regulator with regulation of 0.025%.

Calibration of Instrument—To calibrate the instrument, weights are added to the platform and the recorder response is noted. The magnitude of the change in potential was found to be directly pro-

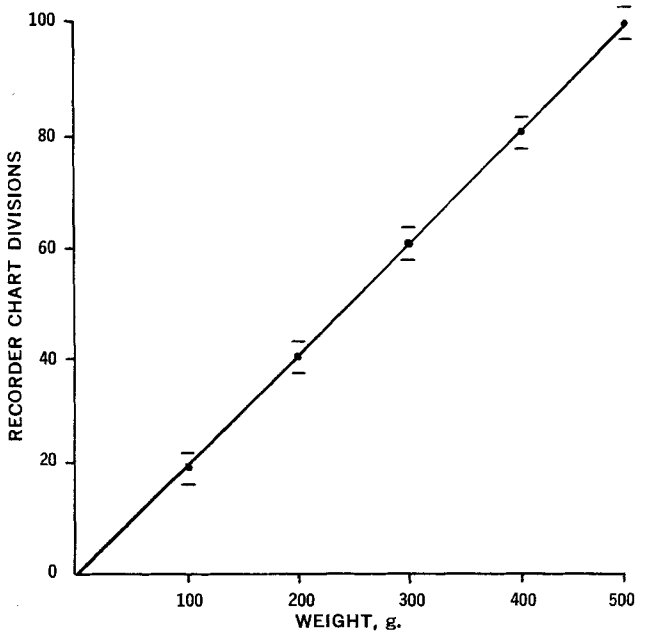

Figure 3—*Calibration curve relating weight in grams to recorder response.*

[1] Model 4K868, Dayton Electric Mfg. Co., Chicago, Ill.
[2] Model 4X599, Dayton Electric Mfg. Co., Chicago, Ill.

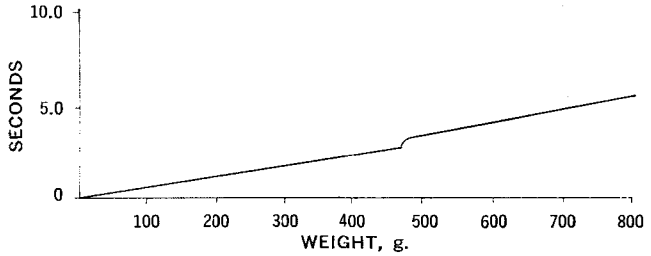

Figure 4—*Typical recorder tracing of an aspirin granule, indicating the magnitude of the breaking point and its breaking pattern.*

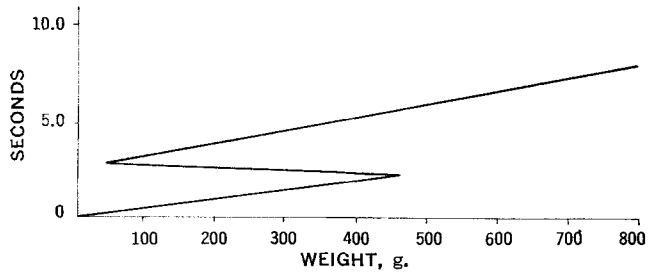

Figure 5—*Typical recorder tracing of a sugar–starch pellet, indicating the magnitude of the breaking point and its breaking pattern.*

portional to the weight added to the platform of the cantilever beam. The linear relationship for the 0–500-g. range is shown in Fig. 3; the same linear relationship was found to exist over a range of 0–2000 g. Each point represents the average of 20 measurements, and the horizontal lines above and below each point are the 95% confidence limits. The points are connected by a line calculated by the method of least squares.

Materials—Commercially available materials of either USP, NF, or pharmaceutical grade were used. Granules of lactose and dicalcium phosphate dihydrate were prepared by the following wet granulation procedure. Eleven kilograms of material was passed through a No. 12 screen on an oscillating granulator and placed in a 1.8-cu. ft. Stokes mixer. The granulating solution at room temperature was added in four equal volumes at 2-min. intervals until 2000 ml. was added. The wet mass was mixed for 20 min., wet-sieved through a No. 6 screen on an oscillating granulator, dried at 54.4° (130°F.) for approximately 20 hr., and dry-sieved through a No. 12 screen. The granules were separated into a 14–16-mesh size, using the Ro-Tap testing sieve shaker with U. S. standard sieves. Granules of aspirin[3] and pellets of sugar–starch[4] were purchased. Aspirin granules were separated into 10–12-, 14–16-, 16–18-, and 20–25-mesh ranges, and sugar–starch pellets were separated into a 14–16 range.

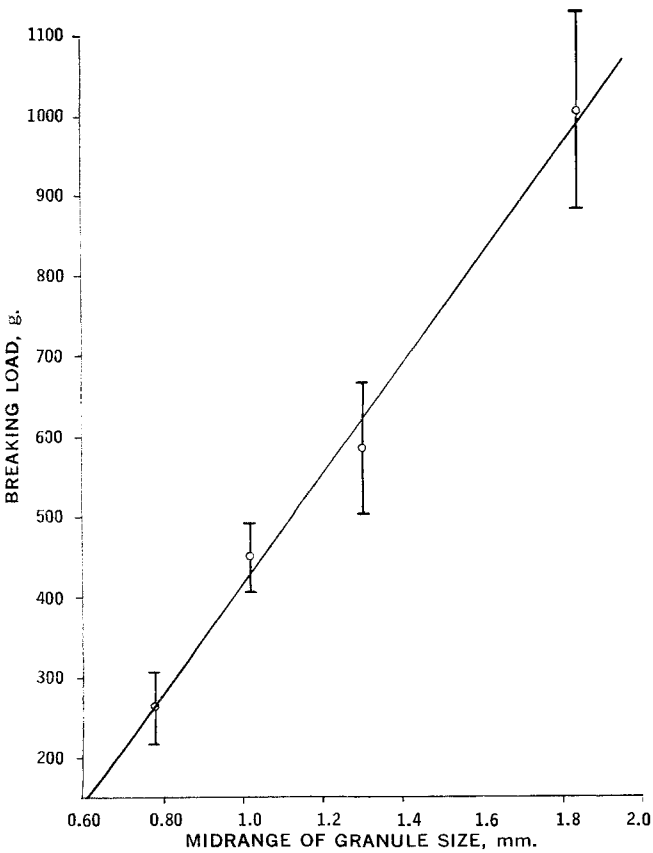

Figure 6—*Plot showing relationship of strength of aspirin granules to granule size.*

Table I—Breaking Load[a] and Standard Deviation of 14–16-Mesh Granules

Granule	Breaking Load, g.	*SD*
Aspirin with 10% starch	466.1	169.6
Sugar–starch pellets	336.0	142.5
Lactose granulations		
Water	62.4	27.0
20% Polyvinylpyrrolidone	196.3	85.5
10% Methylcellulose, 15 cps.	134.9	66.6
Dicalcium phosphate granulations		
Water	11.9	5.14
10% Methylcellulose, 15 cps.	152.9	51.1

[a] Average of 20 measurements.

RESULTS AND DISCUSSION

A typical recorder tracing for a 14–16-mesh aspirin granule is shown in Fig. 4; the horizontal axis is the compressive load and the vertical axis is the time. The break occurred at 475 g. Compressive strength may be defined as the maximum compressive load causing fracture and should not be confused with hardness, which is a measure of the resistance to indentation. Consequently, in this example the compressive strength of the granule is 475 g.

The loading rate is extremely important and is readily calculated from the tracing. As the loading rate increases, it approaches an impact load rather than a compressive load, causing the granule to exhibit different properties. It is also important that the compressive load be applied at a uniform rate to enable the granule to respond with a characteristic stress. In Fig. 4 the reciprocal of the slope of the line, 165 g./sec., is the rate at which the weight is being applied.

The pattern of the break indicates the reaction of the granule to the compressive load. The break that is characteristic for aspirin granules shows that the granule is capable of supporting additional compressive load after fracture. Examination of the fractured granule reveals that it remains intact. This is in contrast to sugar–starch pellets where the breaking pattern (Fig. 5) is characterized by a sharp break with a long backswing. The break occurs at 460 g. at a loading rate of 165 g./sec., and a backswing develops from the breaking point to 40 g. The backswing is significant because it provides insight as to the ability of the fractured particle to support a load. In this case, the fractured pellet does not retain its spherical form but rather disperses into fine particles. In effect, there is a collapse of the spherical particle. The backswing develops because the compressive load on the cantilever beam is abruptly relieved and the release in tension allows the beam to rise until the anvil makes contact with the descending plunger. In the case of aspirin granules (Fig. 4), the fractured granule remains intact between the plunger and anvil and continues to transmit the compressive load. In addition to the breaking pattern, the tracing provides a quantitative measurement of granule strength.

The average breaking load for several different strength, 14–16-mesh materials is summarized in Table I. Each value represents an average of 20 measurements. Results varied from a high granule strength of 466.1 g. for aspirin granules with a standard deviation of 169.6 to a granule strength of 11.9 g. for dicalcium phosphate

[3] Marketed as aspirin 10% starch granulation, white, 12–15 mesh, by the Monsanto Co., St. Louis, Mo.

[4] Marketed as white pellets by the Raymond Confectionery Co., Niles, Ill.

granules with a standard deviation of 5.14. The relatively high standard deviation indicates intergranule variability in strength. To meet the requirement of statistical significance, the data were evaluated by Duncan's multiple-range test (7) which classifies the means into subsets. A statistical difference exists between different subsets but not between members of the same subset. The first subset is aspirin granules; the second, sugar–starch pellets; the third, lactose with 10% methylcellulose, lactose with 20% polyvinylpyrrolidone and dicalcium phosphate with 10% methylcellulose; and the fourth, lactose granulated with water and dicalcium phosphate granulated with water. The use of 20% polyvinylpyrrolidone or 10% methylcellulose in preparing lactose granules increased granule strength over those made by aqueous granulation. Also, 10% methylcellulose increased the granule strength of dicalcium phosphate over the straight aqueous granulation. The increase in strength of 20% polyvinylpyrrolidone over 10% methylcellulose for lactose granules does not have statistical significance.

Measurements were also made on 10–12-, 16–18-, and 20–25-mesh aspirin granules; the effect of granule size on the strength of these granules is shown in Fig. 6. Each value represents the average of 20 measurements, and the vertical bracketed lines are the 95% confidence limits. A linear relationship was found to exist between the strength of these granules and granule size.

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(1) A. M. Marks and J. J. Sciarra, *J. Pharm. Sci.*, **57**, 497(1968).

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NOTES

Schiff Base Derivatives of Anti-Inflammatory *O*-Substituted Hydroxylamines

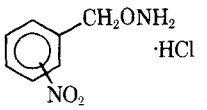
GLENN H. HAMOR and LESLIE D. WATSON*

Abstract □ The synthesis of 12 azomethine (Schiff base) derivatives by the reaction of various nitrobenzaldehydes and salicylaldehydes with the anti-inflammatory compounds, *m*-nitrobenzyloxyamine hydrochloride and 4-thiazolylmethoxyamine dihydrochloride, and with congeneric *O*-substituted hydroxylamines is described. These Schiff bases, which are structurally related to the naturally occurring pyridoxal derivatives, showed no activity in the carrageenin-induced rat paw edema test. These compounds possessed no antimalarial activity and no activity against the L-1210 mouse lymphoid leukemia test system.

Keyphrases □ Hydroxylamines, *O*-substituted—synthesis of Schiff base derivatives, pharmacological screening □ Azomethine (Schiff base) derivatives—synthesis, pharmacological screening □ Anti-inflammatory hydroxylamines—synthesis, pharmacological screening of Schiff base derivatives

Potent anti-inflammatory activity in the carrageenin-induced rat paw edema test is shown by *m*-nitrobenzyloxyamine hydrochloride (I), *p*-nitrobenzyloxyamine hydrochloride (II), and 4-thiazolylmethoxyamine dihydrochloride (III) (1). Moreover, I, II, and III also possess powerful inhibitory activity *in vitro* against the histamine-forming enzyme, specific histidine decarboxylase (1–3).

The present work describes the synthesis and biological activity of Schiff base derivatives of these anti-inflammatory *O*-substituted hydroxylamines (I and III) and congeneric compounds. These Schiff bases are



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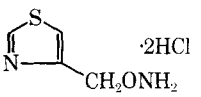

III

structurally related to naturally occurring pyridoxal derivatives.

Pyridoxal phosphate, the coenzyme of mammalian histidine decarboxylases, is attached to the apoenzyme by an azomethine linkage (Schiff base) with the ϵ-amino group of a lysine unit in the enzyme molecule (4). Schiff bases of pyridoxal phosphate are highly reactive and can react with an amino acid more rapidly than can the free aldehyde (5).

It is hoped that the synthetic Schiff bases described in this report might compete with pyridoxal phosphate for the amino group of apoenzyme-bound histidine.

The 12 Schiff bases synthesized are listed and their analytical data and melting points are given in Table I. The syntheses of the starting benzyloxyamines (1) (with the exception of 2-chloro-4,5-methylenedioxybenzyloxyamine hydrochloride which is described in the *Experimental* section) and 4-thiazolylmethoxyamine dihydrochloride (6) are reported in other publications. The Schiff bases were prepared by standard methods (7, 8)

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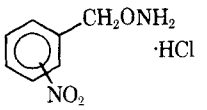

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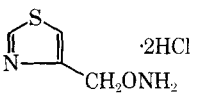

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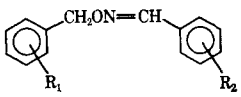
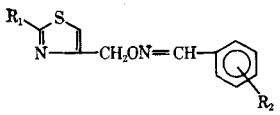
Table I—Schiff Bases

Compound	R_1	R_2	Formula	Melting Point	Recrystallization Solvent	Yield, %	Anal. Calcd.	Anal. Found
								
1	*m*-NO_2	*m*-NO_2	$C_{14}H_{11}N_3O_5$	94–96°	Me_2CO–H_2O	85	C, 55.82 H, 3.68 N, 13.95	C, 56.00 H, 4.00 N, 13.68
2	*m*-NO_2	*p*-NO_2	$C_{14}H_{11}N_3O_5$	131.5–132°	Me_2CO–H_2O	77	C, 55.82 H, 3.68 N, 13.95	C, 56.02 H, 3.69 N, 14.06
3	*m*-NO_2	*o*-OH	$C_{14}H_{12}N_2O_4$	83.5–85.5°	EtOH	75	C, 61.75 H, 4.45 N, 10.29	C, 61.97 H, 4.44 N, 10.66
4	*m*-NO_2	2-OH 5-Cl	$C_{14}H_{11}ClN_2O_4$	113–114°	Me_2CO–EtOH	88	C, 54.82 H, 3.62 N, 9.14	C, 54.87 H, 3.74 N, 9.09
5	*m*-Cl	*o*-CH	$C_{14}H_{12}ClNO_2$	36–37°	MeOH	83	C, 64.24 H, 4.63 N, 5.35	C, 64.29 H, 4.70 N, 5.22
6	*m*-Cl	2-OH 5-Cl	$C_{14}H_{11}Cl_2NO_2$	82°	MeOH	82	C, 56.77 H, 3.75 N, 4.73	C, 56.88 H, 3.75 N, 4.72
7	*p*-Cl	*o*-OH	$C_{14}H_{12}ClNO_2$	94–95°	MeOH	93	C, 64.24 H, 4.63 N, 5.35	C, 64.26 H, 4.74 N, 5.18
8	*p*-Cl	2-OH 5-Cl	$C_{14}H_{11}Cl_2NO_2$	95–98°	MeOH	90	C, 56.77 H, 3.75 N, 4.73	C, 56.83 H, 3.93 N, 4.65
9	*p*-Cl	H	$C_{14}H_{12}ClNO$	31–32°	MeOH	83	C, 68.42 H, 4.93 N, 5.70	C, 68.50 H, 5.14 N, 5.46
10	2-Cl-4,5-methylenedioxy	2-OH 5-Cl	$C_{15}H_{11}Cl_2NO_4$	130–132.5°	EtOH	60	C, 52.96 H, 3.27 N, 4.12	C, 53.06 H, 3.36 N, 3.96
								
11	H	*m*-NO_2	$C_{11}H_9N_3O_3S$	98–99°	EtOH	61	C, 50.20 H, 3.42 N, 15.96	C, 50.47 H, 3.59 N, 15.72
12	H	*p*-NO_2	$C_{11}H_9N_3O_3S$	127–128°	EtOH–H_2O	51	C, 50.20 H, 3.42 N, 15.96	C, 50.03 H, 3.54 N, 15.45

involving reaction of the appropriate aldehyde with the oxyamine hydrochloride in ethanol.

BIOLOGICAL RESULTS

Anti-Inflammatory Screening—Selected compounds were submitted for pharmacological testing; preliminary results[1] are shown in Table II. The four compounds screened, namely, *m*-nitrobenzaldehyde *O*-(*m*-nitrobenzyl)oxime (Compound 1), *p*-nitrobenzaldehyde *O*-(*m*-nitrobenzyl)oxime (Compound 2), salicylaldehyde *O*-(*m*-nitrobenzyl)oxime (Compound 3), and 5-chlorosalicylaldehyde *O*-(*m*-chlorobenzyl)oxime (Compound 6), showed no anti-inflammatory activity in the carrageenin-induced rat paw edema test. In fact, several of the compounds produced a slight increase in edema. This lack of anti-inflammatory activity is in marked contrast to the potent activity of the parent *m*-nitrobenzyloxyamine (1). These four compounds displayed no significant CNS or cardiovascular effects in animal testing. Preliminary results obtained for the related thiazole derivative, 5-chlorosalicylaldehyde *O*-(4-thiazolylmethyl)oxime, indicate that it lacks anti-inflammatory activity as well as being less toxic than the parent 4-thiazolylmethoxyamine dihydrochloride (9).

Anticancer Screening—Seven of the compounds, Compound 1 (NSC 131323), Compound 2 (NSC 131324), Compound 3 (NSC 131406), Compound 4 (NSC 132327), Compound 5 (NSC 132326), Compound 11 (NSC 132328), and Compound 12 (NSC 132329), were submitted[2] for screening against the L-1210 mouse lymphoid leukemia test system (10). None of the compounds possessed any significant antitumor activity.

Antimalarial Screening—Seven compounds (Compounds 1–5, 11, and 12) were tested for antimalarial activity against *Plasmodium bergheii* in mice[3] and were found to be inactive.

EXPERIMENTAL

The syntheses of representative compounds reported in Table I are described here. All melting points were taken on a Fisher-Johns hot stage and are uncorrected. Elemental microanalyses were performed[4]. The IR spectra of all compounds were determined on a Perkin-Elmer Infracord apparatus in mineral oil mulls and are in agreement with the assigned structures.

[1] The authors thank Riker Laboratories, Inc., Northridge, CA 91324, for performing the pharmacological testing.

[2] To the Cancer Chemotherapy National Service Center, National Cancer Institute.

[3] Under the auspices of Walter Reed Army Institute of Research.

[4] Elek Microanalytical Laboratories, Torrance, Calif.

Table II—Pharmacology[a]

Compound	LD_{50}, mg./kg. i.p., Mice	LD_{50}, mg./kg. p.o., Rat	Carrageenin-Induced Rat Paw Edema Percent Reduction, 250 mg./kg.
1	>800	>1000	(2) Increase
2	800	>1000	4
3	>800	—	2
6	>800	>1000	(18) Increase

[a] Pharmacological testing was performed by Riker Laboratories, Inc., Northridge, Calif.

***m*-Nitrobenzaldehyde *O*-(*m*-Nitrobenzyl)oxime (Compound 1)**—To 2.4 g. (0.012 mole) of *m*-nitrobenzaldehyde dissolved in 30 ml. hot 95% EtOH was added slowly 1.8 g. (0.012 mole) of *m*-nitrobenzyloxyamine hydrochloride (1) dissolved in 40 ml. 95% EtOH. The solution was stirred for 10 min. and then filtered to give 3.0 g. (85% yield) of a white crystalline solid, m.p. 93–95°. An analytical sample, m.p. 94–96°, was obtained by two recrystallizations from acetone–H_2O.

***m*-Nitrobenzaldehyde *O*-(4-Thiazolylmethyl)oxime (Compound 11)**—To 8.1 g. (0.04 mole) of 4-thiazolylmethoxyamine dihydrochloride (6) dissolved in 600 ml. warm 95% EtOH was added, in portions, 6.0 g. (0.04 mole) of *m*-nitrobenzaldehyde dissolved in 200 ml. warm 95% EtOH. The solution was refluxed for 3 hr. and let stand overnight at room temperature. A small amount (0.5 g.) of nonsulfur-containing solid, m.p. 195.5°, was filtered off and discarded. The filtrate was diluted to 2 l. with H_2O; the resultant crystalline solid was collected and recrystallized from EtOH–H_2O to give 6.4 g. (61%) of cream-colored solid, m.p. 97–99°. One additional recrystallization yielded an analytical sample, m.p. 98–99°.

2-Chloro-4,5-methylenedioxybenzyloxyamine Hydrochloride—To a solution of 16.5 g. (0.05 mole) *N*-(2-chloro-4,5-methylenedioxybenzyloxy)phthalimide (1) in 600 ml. warm anhydrous EtOH was added 2.5 ml. (0.05 mole) hydrazine hydrate (99%), and the solution was refluxed for 4 hr. The reaction mixture was cooled, and the precipitated phthalhydrazide was removed by filtration. Addition of excess ethanolic HCl to the filtrate followed by reduction in volume by evaporation gave 10 g. (84%), m.p. 191–194°. An analytical sample was obtained by dissolving the compound in H_2O, converting to the free base with Na_2CO_3, extracting with ether, drying with Na_2SO_4, and reconverting to the hydrochloride with ethanolic HCl.

Anal.—Calcd. for $C_8H_9Cl_2NO_3$: C, 40.40; H, 3.78; N, 5.88. Found: C, 40.31; H, 4.00; N, 5.73.

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* National Science Foundation Undergraduate Research Participant, GY-5829.

Worldwide Virtual Temperatures for Product Stability Testing

JOHN D. HAYNES

Abstract □ The market temperatures under which product stability expiration dates for products are to apply should be simulated by laboratory conditions. An actual simulation would involve a pattern of changing monthly temperatures for each location. For those products whose loss rate constant is related to temperature by the Arrhenius relationship, a single virtual temperature can be determined at which the loss rate is equivalent to that of this changing pattern of temperature. This has been calculated for each of 15 cities in the United States and 15 elsewhere. Tables I and II should serve as guides in considering the standard temperature to be incorporated into the laboratory stability test protocol for such products.

Keyphrases □ Product stability testing—simulation of worldwide virtual temperatures □ Expiration dating—guides for determining standard temperatures □ Virtual temperature—application to product stability testing

Drug products stored in pharmacies and warehouses for extended periods of time are exposed to a range of temperatures which, in the United States at least, is narrowed from the ambient range by the widespread use of air conditioning. Storage of some products is governed by restrictions printed on the labels, and these products are not the topic of this paper, although the principle employed may be applied to some of them. According to R. Blythe (1): "Twenty-seven or 68 percent of the reporting [pharmaceutical] companies conduct *field tests* on some of the products. . . in the warmer, more humid areas of the United States. . . Twenty-one

Table II—Pharmacology[a]

Compound	LD_{50}, mg./kg. i.p., Mice	LD_{50}, mg./kg. p.o., Rat	Carrageenin-Induced Rat Paw Edema Percent Reduction, 250 mg./kg.
1	>800	>1000	(2) Increase
2	800	>1000	4
3	>800	—	2
6	>800	>1000	(18) Increase

[a] Pharmacological testing was performed by Riker Laboratories, Inc., Northridge, Calif.

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Abstract □ The market temperatures under which product stability expiration dates for products are to apply should be simulated by laboratory conditions. An actual simulation would involve a pattern of changing monthly temperatures for each location. For those products whose loss rate constant is related to temperature by the Arrhenius relationship, a single virtual temperature can be determined at which the loss rate is equivalent to that of this changing pattern of temperature. This has been calculated for each of 15 cities in the United States and 15 elsewhere. Tables I and II should serve as guides in considering the standard temperature to be incorporated into the laboratory stability test protocol for such products.

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Drug products stored in pharmacies and warehouses for extended periods of time are exposed to a range of temperatures which, in the United States at least, is narrowed from the ambient range by the widespread use of air conditioning. Storage of some products is governed by restrictions printed on the labels, and these products are not the topic of this paper, although the principle employed may be applied to some of them. According to R. Blythe (1): "Twenty-seven or 68 percent of the reporting [pharmaceutical] companies conduct *field tests* on some of the products. . . in the warmer, more humid areas of the United States. . . Twenty-one

Table I—Temperature Values[a]

Country	City	Restricted[a] Annual Average Temperature	Virtual Temperatures: Annual Basis	Virtual Temperatures: Six Consecutive Hottest Months	Temperature Range[b]
United States	Atlanta	19.9°	20.4°	23.1°	
	Boston	18.1°	18.2°	19.3°	5.2°
	Chicago	18.5°	18.7°	20.2°	6.9°
	Cleveland	18.4°	18.6°	20.0°	6.3°
	Detroit	18.2°	18.3°	19.5°	5.8°
	Galveston	22.0°	22.7°	26.4°	
	Los Angeles	18.9°	19.0°	20.8°	5.8°
	New Orleans	21.9°	22.7°	26.2°	
	New York	18.6°	18.7°	20.3°	
	Philadelphia	19.1°	19.4°	21.5°	8.0°
	Portland, Oregon	17.4°	17.4°	17.9°	
	St. Louis	19.8°	20.3°	23.0°	10.2°
	San Francisco	17.0°	17.0°	17.0°	0.0°
	Tampa	22.5°	23.1°	26.3°	
	Washington	19.3°	19.7°	21.9°	8.6°
Australia	Sydney	18.8°	18.9°	20.6°	5.2°
Belgium	Brussels	17.1°	17.1°	17.2°	0.8°
Brazil	Porto Allegero	19.9°	20.2°	22.6°	7.6°
	Rio de Janeiro	23.0°	23.1°	24.8°	5.8°
	Salvador, Bahia	25.4°	25.4°	26.4°	3.3°
Cuba	Havana	25.1°	25.2°	27.0°	5.6°
England	London	17.0°	17.0°	17.0°	0.2°
Germany	Munich	17.0°	17.0°	17.0°	0.4°
India	Bombay	27.1°	27.1°	28.0°	5.8°
Japan	Tokyo	19.2°	19.5°	21.6°	8.8°
Pakistan	Karachi	26.0°	26.5°	29.4°	10.0°
E. Pakistan	Dacca	25.8°	26.1°	28.5°	9.7°
W. Pakistan	Lahore	24.8°	26.1°	30.7°	16.6°
Puerto Rico	San Juan	25.6°	25.6°	26.5°	3.1°
South Africa	Johannesburg	18.2°	18.2°	19.3°	2.9°

[a] 17° has replaced any lower monthly values in these computations including that for restricted annual average temperature. [b] Hottest month to coldest month (with heat, if necessary).

of the companies indicated in a Pharmaceutical Manufacturers' Association survey that they used special tests for products shipped to foreign markets." The current prevalence of air conditioning probably has led to a reduction of the amount of field testing within the United States. He also indicates that three companies use "...cyclic testing in which temperature and humidity are alternately increased and decreased in a given pattern...."

Currently, much interest is being shown in drug dating, as indicated by the 1969 conference on dating of pharmaceuticals (2, 3). Also, some laboratories store the product at elevated temperatures and/or at a "room" temperature. This widespread interest in dating makes it desirable to consider carefully the determination of a standard temperature used to establish the expiration date on a product. The purpose of this note is to present the method of calculating the virtual temperature (defined later), which will guide the choice of a standard temperature for dating purposes. Also, the virtual temperatures for 30 cities are given, and an approximate formula is given which simplifies the calculation for other places.

DISCUSSION

It is well known that changes in the actual field storage temperatures cause the reaction rate constant of some products to change according to the Arrhenius relationship (4). The mathematical development given here applies only to such products. For example, if the storage temperature were 20° for 1 year and 40° for the next year, the final concentration of the active ingredient of such a product may be 85% of the initial value; the average temperature is 30°, but 2 years of storage at 30° would not result in 85% potency (assay error assumed to be negligible). The better way to determine a single equivalent temperature would be to calculate rate constants (of the proper order) for the 1st and 2nd years and average them. This average rate constant could be substituted into the Arrhenius equation, which could then be solved for the corresponding temperature, here named the virtual temperature. Two years' storage of the product of the example at the virtual temperature (perhaps 31°, dependent upon the magitude of the heat of activation) would result in 85% potency.

The pharmacist with the tasks of exploratory work on stability of new formulations, of estimating overages for accepted formulations, and of providing data for dating purposes needs a reference storage temperature for incorporation into his laboratory stability test protocols for both "room" temperature studies and elevated temperature studies. Conceptually, the *average* of the 12 monthly reaction rate constants based on frequent observations at a given geographical location could be calculated. If this average reaction rate constant is substituted into the Arrhenius equation, it then can be solved for the single corresponding temperature, named the annual virtual temperature. The potency data for the product held in the *laboratory* at this virtual temperature for 12 months would yield, upon calculation, a reaction rate constant equal to the average mentioned previously, with due allowance for observation variation. (The annual virtual temperature value would differ to some degree from the annual temperature and usually would equal none of the monthly temperatures; the virtual temperature may be said to exist in effect but not in fact.)

Obviously that method of calculating the virtual temperature is not feasible since it would require stability studies at each location or in the laboratory with temperatures changing as at the location. Since the loss rate constant (of the pertinent order) in any month is, according to the Arrhenius relationship:

$$k_i = Ae^{-\Delta H/RT_i} \qquad i = 1, \ldots, 12 \qquad \text{(Eq. 1)}$$

with A = a constant for a given reaction; ΔH = heat of activation, calories mole^{-1} (a constant for a given reaction); R = universal gas constant, 1.987 cal. mole^{-1} degree^{-1}; and T_i = the absolute tempera-

Table II—Extreme Virtual Temperature Values from Table I

Extreme	Annual Virtual Temperature	Hot Season Virtual Temperature
Smallest in U. S. cities shown	17.0°, San Francisco	17.0°, San Francisco
Largest in U. S. cities shown	23.1°, Tampa, Florida	26.4°, Tampa, Florida
Smallest in Table I	17.0°, San Francisco, London, Munich	17.0°, San Francisco, London, Munich
Largest in Table I	27.1°, Bombay	30.7°, Lahore, West Pakistan

ture in the ith month; the average of the 12 values of k_i is proportional to the average of the 12 values of $e^{-\Delta H/RT_i}$. Monthly temperatures, rather than weekly or daily temperatures, are used because the typical storage situation prevents the product from changing temperatures rapidly; also they are more readily available. The T_i values can be obtained easily for most locations and, with a suitable value of ΔH, used to calculate the average of the 12 $e^{-\Delta H/RT_i}$; this numerical value is defined to be equal to $e^{-\Delta H/R(VT)}$, where VT stands for the virtual temperature. The solution of this for VT in degrees absolute is given in Eq. 2:

$$(VT°\text{K.}) = \left[\frac{(\Delta H/R)}{-\ln\left(\frac{e^{x_1} + e^{x_2} + \cdots e^{x_{12}}}{12}\right)}\right] \qquad \text{(Eq. 2)}$$

where $x_1 = -\Delta H/RT_1$ for the 1st month, *etc.*, and ln stands for log base e.

A heat of activation of 14 kcal. mole^{-1} represents products near the middle of the range of 10–20 kcal. mole^{-1} chosen by Kennon (5) and is appropriate for products with reaction rate constants that approximately double as the temperature increases by 10° within the normal range.

Equation 2 was applied to the monthly temperatures secured from United States Weather Bureau records (6) for 20 or more years for 15 continental United States cities and 15 other cities chosen to represent extremes (7). Temperatures below 17° (65°F.) were replaced by 17° in Eq. 1, because it was assumed the storage area would be heated; the annual temperature calculated in this fashion is called the restricted annual temperature. The annual virtual temperature values are shown in Table I as well as virtual temperature values for the six consecutive hottest months. Table II presents the extreme values of Table I.

A rule-of-thumb (obtained from a plot of the data) that gives essentially the same result as Eq. 2 uses the temperature *range* (with 17° substitution for any period if appropriate) to estimate the annual VT°C. (*a*) If the temperature range is 4.5° or less, the approximate annual VT°C. is estimated by the restricted *annual* temperature. (*b*) If the range is over 4.5°, approximate (VT°C.) = (restricted annual temperature) + 0.1 (range − 4.5°).

Equation 2 was also used with ΔH = 20 kcal. mole^{-1}, the average value quoted by Kennon (5), and ΔH = 30. The former corresponds approximately to an increase in the rate constant by a factor of 3 per 10° increase, and the latter by a factor of 5.5. The virtual temperatures obtained from these equations were practically unchanged for some cities and became larger for the rest: by less than 0.3° for 20 kcal. mole^{-1} and by less than 0.5° for 30 kcal. mole^{-1} for 29 cities. The only exception was Lahore which had increases of 0.4 and 1.0°, respectively. The slightness of these changes is understandable when it is realized that if the geometric mean k were appropriate rather than the arithmetic mean k, the corresponding "virtual" temperature would equal the harmonic mean of the 12 monthly temperatures regardless of the value of ΔH. The near equality of the arithmetic mean k and the geometric mean k for most of these cities is reflected in the slightness of the dependence of the virtual temperatures reported here upon ΔH.

The largest annual virtual temperature in these 15 United States cities is 23.1° for Tampa for the 30-kcal. mole^{-1} case. The difference between annual and virtual temperature in these 15 United States cities is largest for Tampa also: 0.8° for 14 kcal. mole^{-1} and 1.3° for 30 kcal. mole^{-1}. A 1° temperature difference translated into a difference in rate constants for 14, 20, and 30 kcal. mole^{-1} products is 8, 12, and 19%, respectively, which corresponds to sizable differences in overage.

This information provides a basis for choosing a standard stability temperature for the United States. No allowance has been made for air conditioning in pharmacies and warehouses, so these values may be considered as upper limits for virtual temperature. The oft-quoted room temperature of 25° for dating purposes (5, 8–10) is safe for the United States and is probably high for such products. An annual virtual temperature of 24° would be consonant with the data presented here. If a survey of air conditioning were undertaken, it probably would support an annual virtual temperature of 23°.

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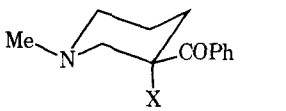
Absolute Configurations of Antipodes of γ-Chloro-β-carboxy-β-hydroxybutyric Acid, Chlorocitramalic Acid

SOON-HEE KIM SUH and G. HITE*

Abstract □ A new four-step synthesis of γ-chloro-β-carboxy-β-hydroxybutyric acid, chlorocitramalic acid, from β-carboxybut-2-enoic acid is described. The absolute configuration of the chlorocitramalic acid antipodes was established.

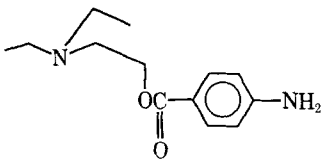
Keyphrases □ Chlorocitramalic acid—synthesis from itaconic acid □ Antipodes of γ-chloro-β-carboxy-β-hydroxybutyric acid, chlorocitramalic acid—absolute configurations □ NMR spectroscopy—structure □ IR spectrophotometry—structure

The molar potency ratio (8:1) of the local anesthetic, I*a*, relative to procaine (II) (1), the novel rotatory dispersion characteristics of I*b* (2), and the speculative nature of the interpretations of the rotatory dispersion curves of I*b*, although consistant with studies conducted to date (2–4), prompted an investigation of the absolute configuration of I. Wilcox *et al.* (5) claimed the synthesis and resolution of III and the conversion of (−)-III to asymmetrically tagged citric acid which, upon degradation by arsenite-treated rat liver homogenate, afforded α-ketoglutaric acid tagged in the γ-carboxyl group. This and later studies on the stereochemistry of intermediates and reactions of the tricarboxylic acid cycle, recently reviewed (6), permitted assignment of the R-configuration to the Wilcox citric acid precursor, which was to be used as the configurational reference in the projected investigation (Scheme I).

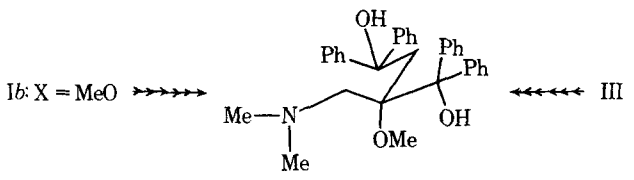


I*a*: X = *p*-H_2N-$C_6H_4CO_2$

I*b*: X = Cl, H, Me, MeO, HO, AcO, Et

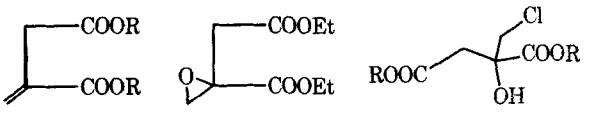


II



Scheme I

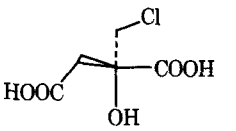
The *abbau* sequence, the subject of a later communication (7), failed. While that work was in progress, attempts to reproduce the synthesis and resolution cited by Wilcox *et al.* (5) also ended in failure (*cf.*, 8). The procedure afforded crude, viscous, acid products containing nitrogen in varying amounts (*cf.*, 8). Attempts to resolve these materials repeatedly led to optically inactive, viscous, halogen-free, acid products of indeterminate composition, containing nitrogen in varying amounts (*cf.*, 8). Because it was hoped that attempts to resolve III would prove more fruitful starting with a demonstrably pure sample of III, and because the Wilcox synthesis required the use of cyanide, powerful vesicants, and lachrymators (such as ethyl chloroacetate and γ-chloroacetoacetate) and afforded only crude acid from which it was not possible to isolate pure III, a new synthesis of III was developed. Again, pure crystalline III failed to resolve, as cited by Wilcox (5, *cf.*, 8), but the corresponding, optically active lactone (IV) was obtained. While these results do not contradict the main conclusion regarding the biochemical stereospecificity of the aconitase system or the ultimate deduction of the R-configuration for a Wilcox precursor of citric acid, there existed some doubt as to the structure of the precursor and, thus, as to the sign of rotation of R-III.



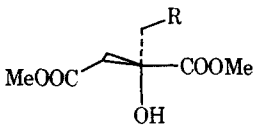
V: R = H
VI: R = Et

VII

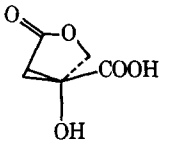
III: R = H
VIII: R = Et



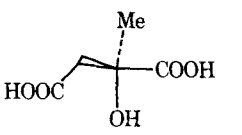
S-(+)-III



R-(+)-IX: R = OH
R-(+)-X: R = *p*-$CH_3C_6H_4SO_3$
S-(+)-XI: R = Cl
S-(+)-XII: R = I
XIII: R = SH
S-(+)-XIV: R = H



R-(+)-IV



S-(+)-XV

This paper deals with an unequivocal synthesis of III and a sequence of reactions which permitted assignment of absolute configuration to its antipodes.

After esterification of itaconic acid (V), the ester (VI) was converted to the epoxide (VII) which, as expected (9), afforded the β-hydroxy-γ-chloro ester (VIII) exclusively by treatment with dry HCl. Acid hydrolysis

gave III, a solid, in 30% overall yield. While attempts to resolve III failed using (−)-brucine, the lactone, (+)-IV, was obtained in optically pure form from the (−)-brucine salt after 11 crystallizations. Hydrolysis of (+)-IV and esterification of the dibasic acid *via* the silver salt yielded (+)-IX, which was converted to the γ-tosylate, (+)-X. After treatment with LiCl, (+)-X afforded (+)-XI and then (+)-III by hydrolysis in acid. Correlation of configuration of (+)-III with S-(+)-XV (8, 10) by catalytic reduction of III and XI or by Zn/H^+ reduction failed. The assignment of configuration was made by conversion of (+)-IV of 86 ± 4% optical purity to S-(+)-XV of about the same degree of optical purity (94 ± 6%). The tosylate, (+)-X, prepared from the partially resolved lactone, (+)-IV, was converted to the iodo analog, (+)-XII [*cf.*, (+)-IV → (+)-IX → (+)-X → (+)-XI], from which the γ-sulfhydryl compound (XIII) was obtained. Raney nickel desulfurization and hydrolysis afforded (+)-XIV and S-(+)-XV, respectively. Since the intermediates and products of this sequence were liquids that resisted all efforts to effect crystallization, X was subjected to the same sequence and afforded crystalline XV, identified by melting point and mixed melting point with an authentic commercial sample of XV. The NMR spectra of both samples of XV were identical to that of the oil assigned the structure (+)-XV.

Accordingly, R-III is the levorotatory antipode, $[\alpha]_D^{25}$ −7.5°. The results achieved by Wilcox *et al.* (5) clearly require that their resolved acid, $[\alpha]_D^{25}$ −44.9°, an oil, contain γ-halo functionality, the quantity and structure of which must remain enigmatic.

EXPERIMENTAL[1]

Melting points were measured in a Thomas-Hoover unimelt apparatus and are uncorrected. Refractive indexes were measured on a calibrated, thermostated, Zeiss-Abbe refractometer and are believed accurate to within ±2 parts per 10^4. Optical rotations were determined with a Perkin-Elmer model 141 photoelectric polarimeter, using a 1.000-dm. cell. IR spectra were determined with a Perkin-Elmer model 421 spectrophotometer, using sodium chloride plates or cells. Assignments of absorption bands, believed accurate to within ±5 $cm.^{-1}$, were made by analogy with reported values (11). Proton NMR spectra were determined on a Varian A-60A spectrometer. Assignments of absorption bands, believed accurate to within ±1 Hz., were made by analogy with reported values (12). When absorptions of exchangeable protons fell within areas of other bands of prime interest, samples were treated with D_2O. Tetramethylsilane was used as the internal reference. Drying, clarifying, and decolorizing of solutions in organic solvents were carried out simultaneously with dry Na_2SO_4 and activated charcoal[2], respectively. After suction filtration through diatomaceous earth[3] pads in sintered-glass funnels, all solvents were removed in a rotatory evaporator under reduced pressure. Oils and solids were further dried (0.1 mm. Hg, room temperature, 4–12 hr.) prior to spectral analysis. Petroleum ether refers to the fraction boiling from 30–60°.

Ethyl β-Carbethoxybut-2-enoate (VI)—Itaconic acid (V), 780 g., in 2 l. of absolute EtOH containing 1.5 g. of hydroquinone and 95 g. of concentrated H_2SO_4, was boiled (18 hr.), cooled (5°), and then alkalized to pH 6–7 with cold (5°) 20% Na_2CO_3. The EtOH was evaporated, and the residue was extracted with $HCCl_3$. The extract was dried, filtered, and fractionally distilled to give 734 g. (67%) of VI: b.p.$_{0.23}$ 58°; n_D^{20} 1.4384; IR (film) 1730 (ester C=O) and 1635 $cm.^{-1}$ (C=C); lit. (13) b.p.$_{20}$ 118°; n_D^{20} 1.4383.

(±)-Ethyl β,γ-Epoxy-β-carbethoxybutyrate (VII)—To 140 ml. of 90% H_2O_2 in 1 l. of cold (5°) H_2CCl_2 was added (90 min.) 1260 g. of $(F_3CCO)_2O$ with vigorous stirring. This solution was added with stirring (5 hr.) through a pressure-equalizing funnel to 734 g. of VI and 2260 g. of dry Na_2HPO_4 in 4 l. of boiling H_2CCl_2. After the exothermic reaction subsided, the mixture was boiled (1 hr.) and then cooled (10°) while adding enough water to dissolve the salts. The H_2CCl_2 layer was separated and combined with 1.5 l. of H_2CCl_2 used to extract the aqueous phase. The H_2CCl_2 solution was washed with saturated $NaHCO_3$, dried, filtered, and fractionally distilled to give 531 g. (66%) of VII: b.p.$_{0.15}$ 75–76°; n_D^{20} 1.4372; IR (film) 1735 (ester C=O), 925, and 900 $cm.^{-1}$ (C—O—C); NMR (CCl_4) δ 4.4–3.9 (q,q,2H,2H,OCH_2,OCH_2), 3.25–2.75 (m,2H,-H_2C—O—C), and 1.23 p.p.m. (t,6H,CCH_3,CCH_3).

Anal.—Calcd. for $C_9H_{14}O_5$: C, 53.46; H, 6.93; O, 39.61. Found: C, 53.7; H, 6.93; O, 39.42 (by difference).

(±)-Ethyl γ-Chloro-β-carbethoxy-β-hydroxybutyrate (VIII)—Dry HCl (70 g.) was bubbled into a solution of 312 g. of VII in 1.1 l. of dry, cold (5°) Et_2O. After 5 hr., the solvent and excess HCl were evaporated. The residue, dissolved in Et_2O, was washed with cold (5°) saturated $NaHCO_3$. The Et_2O phase was dried and filtered, and the oil was distilled to give 338 g. (92%) of VIII: b.p.$_{0.07}$ 81–82°; n_D^{20} 1.4510; f.p. 27°; IR (film) 3510 (OH) and 1740 $cm.^{-1}$ (ester C=O); NMR ($DCCl_3$) δ 4.6–3.9 (q,q,2H,2H,OCH_2,OCH_2), 3.73 (s,2H,$ClCH_2$), 2.88, 2.83 (s,s,H,H,CH_2COO), and 1.6–1.1 p.p.m. (t,t,3H,3H,CCH_3,CCH_3).

Anal.—Calcd. for $C_9H_{15}O_5Cl$: C, 45.43; H, 6.34. Found: C, 45.7; H, 6.52.

(±)-γ-Chloro-β-carboxy-β-hydroxybutyric Acid (III)—After 410 g. of VIII and 600 ml. of concentrated HCl were boiled (4 hr.) and cooled (room temperature, 12 hr.), the solvent and HCl were evaporated and the residue was dried and dissolved in $HCCl_3$ to give an oil which crystallized overnight. The solid was recrystallized from Et_2O and then from EtOAc. Residues obtained from evaporation of the mother liquors were recycled to give a combined yield of 226 g. (73%) of III: m.p. 153–154°; IR (Et_2O) 3500 (OH) and 1740 $cm.^{-1}$ (acid C=O), (mineral oil) 3400, 3300 (OH) and 1750, 1680 $cm.^{-1}$ (acid C=O); NMR (Me_2CO-d_6) δ 3.86 (s,2H,CH_2Cl), 2.97, and 2.90 p.p.m. (s,s,H,H,CH_2COO).

Anal.—Calcd. for $C_5H_7O_5Cl$: C, 32.9; H, 3.87. Found: C, 33.0; H, 3.88. Neutral equivalent, 92.

Attempted Resolution of III—To 146 g. of III in 150 ml. of H_2O was added 335 g. of (−)-brucine in 450 ml. of hot MeOH. To this was added 450 ml. of H_2O (20°). The solution was cooled (10°) rapidly and then refrigerated (4°, 12 hr.). The salt was harvested, washed with cold (4°) H_2O–MeOH (1:1), dried, and redissolved in 400 ml. of MeOH. To this was added 800 ml. of H_2O (20°). The solution was cooled (4°, 12 hr.). After four crystallizations, 47 g. of salt was obtained: $[\alpha]_D^{25}$ (dimethylformamide) −11.4 ± 0.3° (c 10.00); m.p. 112–120°. Ten grams of salt was dissolved in 50 ml. of 50% H_2SO_4, and the solution was extracted in a lighter-than-water continuous liquid–liquid extractor using Et_2O (2 days). The Et_2O phase was dried and evaporated to give a crude oil, 0.9 g., which was chromatographed on a silicic acid column eluting with Et_2O–petroleum ether (1:4 → 4:1). One fraction, on evaporation, afforded III: m.p. 153–154°; undepressed on admixture with an authentic sample; no detectable rotation ±0.002° (c 2.50, H_2O). Another fraction afforded an oil: $[\alpha]_D^{25}$ (H_2O) +8.6 ± 0.2° (c 1.30); IR 3400 (OH), 1780 (γ-lactone C=O), 1730 $cm.^{-1}$ (acid C=O); NMR (Me_2CO-d_6) δ 4.72, 4.56, 4.40, 4.24 (4 line *AB* pattern, CH_2O), 3.36, 3.06, 2.80, 2.51 p.p.m. (4 line *AB* pattern, CH_2COO); assigned the Structure IV.

Synthesis and Resolution of (±)-β-Hydroxy-β-carboxybutyrolactone (IV)—A slurry of 45.5 g. of III and 50 g. of $CaCO_3$ in 150 ml. of H_2O was stirred (24 hr.), concentrated (0.5 volume), mixed with 63 g. of $(COOH)_2 \cdot H_2O$ in 50 ml. of H_2O, cooled (5°), and filtered. The filtrate was evaporated to give, after drying, 21 g. (86%) of IV, an oil that resisted all attempts to effect crystallization. The spectra of a chromatographically pure sample were identical to those of IV obtained in the attempted resolution of III.

To 40 g. of IV in 100 ml. of H_2O was added 109 g. of (−)-brucine in 40 ml. of hot MeOH. After 24 hr. at room temperature, the salt was harvested, washed with cold (4°) MeOH–H_2O (4:1), and dried.

[1] Analyses were performed by Weiler and Strauss, Oxford, England.
[2] Norit.
[3] Celite.

After 10 recrystallizations, the salt (3 g.), $[\alpha]_D^{25}$ (H_2O) $-12.5 \pm 0.2°$ (c 1.00), was dissolved in H_2O and passed through an acid ion-exchange resin[4] column. Evaporating the effluent and drying the residue gave 0.57 g. of (+)-IV: m.p. 87–89°; $[\alpha]_D^{23}$ (H_2O) $+45.2 \pm 0.2°$ (c 3.1); lit. (13) m.p. 86–90°, $[\alpha]_D^{16}$ (H_2O) $-45.6°$ (c 2.43); the IR and NMR spectra were identical to those of IV obtained from III.

Anal.—Calcd. for $C_{28}H_{32}N_2O_9$: C, 62.21; H, 5.96; N, 5.18. Found: C, 61.9; H, 5.64; N, 5.00.

(±)- and R-(+)-Methyl γ-(*p*-Toluenesulfonyloxy)-β-carbomethoxy-β-hydroxybutyrate [X and R-(+)-X]—A slurry of 1 g. of IV and 1.5 g. of $CaCO_3$ in 10 ml. of H_2O was stirred and heated (70°, 24 hr.), concentrated to 5 ml., mixed with 10 ml. of MeOH, and cooled (4°). The solid was harvested, washed with cold (4°) MeOH–H_2O (1:1), and mixed with 10 ml. of H_2O and 0.75 g. of $(NH_4)_2CO_3$. After stirring (room temperature, 12 hr.), the $CaCO_3$ was filtered off, and the filtrate and H_2O washings were concentrated to 5 ml. This was treated with 2.5 g. of $AgNO_3$ in 3 ml. of H_2O. The precipitate was harvested, washed with cold (4°) H_2O, dried (50°) in the dark, suspended in 10 ml. of dry C_6H_6, and treated with 6 ml. of MeI. After stirring (room temperature, 12 hr.), the solid was filtered off and washed with C_6H_6. The filtrate and wash liquor were mixed with activated charcoal, filtered, and evaporated to give, after drying, 0.82 g. (62%) of an oil assigned as Structure IX, (±)-methyl β,γ-dihydroxy-β-carbomethoxybutyrate: IR (film) 3490 (OH) and 1740 cm.$^{-1}$ (ester C=O); NMR ($DCCl_3$) δ 3.86 (s,H, OCH), 3.80 (s,3H,$COOCH_3$), 3.68 (s,s,4H,OCH,$COOCH_3$), 2.82, and 2.78 p.p.m. (s,s,H,H,CH_2COO).

To 1.13 g. of IX in 2 ml. of $HCCl_3$ and 1 ml. of dry pyridine was added (30 min.) dropwise a solution of 1.15 g. of *p*-$CH_3C_6H_4SO_2Cl$ in 2 ml. of $HCCl_3$. The reaction was stirred (room temperature, 6 hr.), mixed with 10 ml. of *N* HCl, and extracted with two 10-ml. portions of $HCCl_3$. The extract was washed with H_2O, mixed with activated charcoal, dried, filtered, and evaporated. The residue was recrystallized three times from 1:1 EtOAc–petroleum ether to give 1.3 g. (68%) of X: m.p. 74–75.5°; IR ($HCCl_3$) 3510 (OH), 3030 (ArH), 1750 (ester C=O), and 1175 cm.$^{-1}$ ($ArSO_3$); NMR (CCl_4) δ 7.9–7.2 (m,4H,C_6H_4), 4.16 (s,2H,SO_3CH_2), 3.78 and 3.68 (s,s,3H, 3H,$COOCH_3$,$COOCH_3$), 2.81 and 2.75 (s,s,H,H,CH_2COO), and 2.46 p.p.m. (s,3H,CH_3Ar).

Anal.—Calcd. for $C_{14}H_{18}O_8S$: C, 48.54; H, 5.23. Found: C, 48.4; H, 5.29.

In like manner, 0.57 g. of optically pure R-(+)-IV afforded, after drying, 0.53 g. (72%) of R-(+)-IX, an oil: $[\alpha]_D^{23}$ (MeOH) $+5.1 \pm 0.2°$ (c 5.30), and then 0.55 g. (58%) of S-(+)-X: m.p. 70–72°. The IR and NMR spectra of these antipodes were identical to those of the respective racemates.

(±)- and (+)-Methyl γ-Chloro-β-carbomethoxy-β-hydroxybutyrate [XI and S-(+)-XI]—A solution of 0.5 g. of X and 0.5 g. of LiCl in 5 ml. of dry Me_2CO, after stirring (room temperature, 6 days), was evaporated and the residue was dissolved in 10 ml. of H_2O. This solution was extracted with two 10-ml. portions of Et_2O. The extract was dried, mixed with activated charcoal, filtered, and evaporated to give 195 mg. (65%) of XI from 1:1 EtOAc–petroleum ether: m.p. 43–45°; IR ($HCCl_3$) 3520 (OH) and 1748 cm.$^{-1}$ (ester C=O); NMR (CCl_4) δ 3.85 and 3.70 (s,s,3H,3H,$COOCH_3$, $COOCH_3$), 3.67 (S,2H,CH_2Cl), and 2.83 and 2.77 p.p.m. (s,s,H,H, CH_2COO), lit. (5) m.p. 44–45°, (8) 44°.

In like manner, 0.55 g. of R-(+)-X afforded an oil purified by chromatography on a silica gel column using Et_2O–petroleum ether (1:4) as the eluant. One fraction yielded, after evaporating the solvent and drying the residue, 0.26 g. (80%) of an oil, S-(+)-XI: $[\alpha]_D^{25}$ ($HCCl_3$) $+4.0 \pm 0.3°$; the spectra were identical to those of XI.

S-(+)-γ-Chloro-β-carboxy-β-hydroxybutyric Acid [S-(+)-III]—A solution of 0.18 g. of S-(+)-XI in 2 ml. of concentrated HCl was stirred (room temperature, 12 hr.—90°, 4 hr.) and evaporated to give a residue, which was dried and dissolved in 3 ml. of H_2O. The solution was mixed with $HCCl_3$. The residue obtained by evaporation of the aqueous solution was dissolved in EtOAc. This was mixed with activated charcoal, dried, filtered, chromatographed, and evaporated to give, after drying, 130 mg. (65%) of an oil, S-(+)-III: $[\alpha]_D^{25}$ (H_2O) $+7.5 \pm 0.5°$ (c 1.00); the spectra were identical to those of III prepared from XI and from VIII.

(±)- and S-(+)-β-Carboxy-β-hydroxybutyric Acid [XV and S-(+)-XV]—A solution of 1 g. of NaI and 0.5 g. of partially resolved tosylate, R-(+)-X {$[\alpha]_D^{25}$ ($HCCl_3$) $+11.6 \pm 0.5°$ (c 5.00), [86.5 ± 4% (+)-X], prepared from the lactone, R-(+)-IV, $[\alpha]_D^{23}$ (H_2O) $+33 \pm 1°$ (c 6.70), [86.5 ± 4% (+)-IV]} in 5 ml. of dry Me_2CO was stirred (room temperature, 24 hr.) and then evaporated. The residue was dissolved in 4 ml. of H_2O, and the solution was extracted with two 10-ml. portions of Et_2O. The extract was dried, mixed with activated charcoal, filtered, and evaporated to give, after drying, 0.434 g. (98%) of an oil assigned the structure S-(+)-XII, S-(+)-methyl γ-iodo-β-carbomethoxy-β-hydroxybutyrate: $[\alpha]_D^{25}$ ($HCCl_3$) $+5.1 \pm 0.3°$ (c 4.30); IR (film) 3500 (OH) and 1740 cm.$^{-1}$ (ester C=O); NMR (CCl_4) δ 3.86 and 3.72 (s,s,3H,3H,$COOCH_3$,$COOCH_3$), 3.45 (s,2H,ICH_2), and 2.90 and 2.86 p.p.m. (s,s,H,H,CH_2COO).

A cold (4°) solution of 0.5 g. of dried NaSH in 5 ml. of absolute EtOH was saturated with H_2S, warmed to room temperature, mixed with 0.43 g. of S-(+)-XII in 4 ml. of absolute EtOH, stirred (2 hr.), acidified with 0.01 *N* HCl, and evaporated to give a residue which was mixed with 5 ml. of H_2O. The mixture was extracted with two 10-ml. portions of Et_2O. The extract was dried, mixed with activated charcoal, filtered, and evaporated to give, after drying, 0.238 g. (82%) of a vile-smelling, halogen-free oil assigned the structure methyl γ-mercapto-β-carbomethoxy-β-hydroxybutyrate, XIII: IR ($HCCl_3$) 3508 (OH), 2590 (SH) and 1700 cm.$^{-1}$ (broad, ester C=O); NMR ($DCCl_3$) principal absorptions at δ 3.89, 3.81, 3.68 (s,s,s, SCH_2,$COOCH_3$,$COOCH_3$), 2.88 and 2.75 p.p.m. (s,s,CH_2COO).

Freshly activated Raney nickel (2 g.) was boiled in 10 ml. of Me_2CO (2 hr.). The Me_2CO was displaced by several washings and decantations with absolute EtOH. The slurry was mixed with 0.238 g. of XIII, boiled (15 min.), and filtered. The filtrate and wash liquor were evaporated to give an oil, which was dried and dissolved in Et_2O. This solution was treated with activated charcoal, filtered, and evaporated to give, after drying, 0.144 g. (57%) of a sulfur-free, dextrorotatory ($HCCl_3$) oil assigned the structure S-(+)-methyl-β-carbomethoxy-β-hydroxybutyrate, S-(+)-XIV: NMR ($DCCl_3$) δ 3.80, 3.70 (s,s,3H,3H,$COOCH_3$,$COOCH_3$), 2.89, 2.78 (s,s,H,H, CH_2COO), and 1.44 p.p.m. (s,3H,CH_3C).

A solution of 0.114 g. of S-(+)-XIV in 1.5 ml. of concentrated HCl was stirred (room temperature, 24 hr.), diluted with 1.5 ml. of H_2O, heated (90°, 4 hr.), cooled to room temperature, alkalized with saturated $NaHCO_3$, extracted with two 3-ml. portions of $HCCl_3$, acidified with 0.01 *N* HCl, and evaporated. The dried residue was mixed with 5 ml. of EtOAc. The mixture was treated with activated charcoal, filtered, and evaporated to give, after drying, 27 mg. (30%) of an oil assigned the structure S-(+)-β-carboxy-β-hydroxybutyric acid, S-(+)-XV: $[\alpha]_D^{23}$ (H_2O) $+20.9 \pm 3°$ (c 0.45); lit. (8, 14) m.p. 110°, $[\alpha]_D^{22}$ (H_2O) $+23.7°$ (c 5.0); NMR (Me_2CO-d_6) δ 2.85, 2.76 (s,s,H,H,CH_2COO), and 1.44 p.p.m. (s,3H,CH_3C).

The NMR spectrum of XV, m.p. 114–116° from EtOAc–petroleum ether, obtained in like manner from X, was identical to that of S-(+)-XV and to that of an authentic commercial sample of XV, m.p. 115–116°, mixture m.p. 115–116°, lit. (14) m.p. 116.4–117.2°. The NMR spectra of the respective racemic and optically active intermediates (XII–XIV) were also identical.

[4] Dowex W50-X4.

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Inhibitory Effect of Cholesteryl Nitrate on Interaction of Nitrogen Dioxide with Cholesterol in Monomolecular Films

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Abstract □ Cholesterol monomolecular films exhibit a loss in surface pressure on exposure to nitrogen dioxide due to the formation and subsequent desorption of cholesteryl nitrate. The cholesterol–nitrogen dioxide interaction is inhibited by the prior addition of cholesteryl nitrate, and the degree of inhibition is a direct function of the mole fraction of the nitrate ester. Total inhibition occurs when this mole fraction is about 0.75. The differential equations developed to describe the sequence of events involved in this process were programmed for solution on an analog computer. This simulation indicated that at least six molecules of cholesteryl nitrate are required for each remaining cholesterol molecule to produce a total inhibition of the nitrogen dioxide–cholesterol reaction.

Keyphrases □ Cholesteryl nitrate, inhibitory effect—nitrogen dioxide–cholesterol interaction, monomolecular films □ Monomolecular films, nitrogen dioxide–cholesterol interaction—inhibitory effect of cholesteryl nitrate

Recently, it was reported that cholesterol monomolecular films, when exposed to approximately 175 p.p.m. of nitrogen dioxide for 60 min., exhibited a condensation effect (*i.e.*, a decrease in surface area at all surface pressures tested) that corresponded to a loss of about 75% of the cholesterol (1). Moreover, continued exposure did not result in any further loss of cholesterol (1). Other studies demonstrated that the formation and subsequent desorption of cholesteryl nitrate were responsible for this loss of cholesterol from the film and the concurrent condensation effect (2).

Similarly, Bergström and Wintersteiner (3) noted that the extent of autoxidation of cholesterol dispersed in an aqueous soap solution did not exceed 70%, even after extended exposure to air. These workers demonstrated that this limiting effect was caused by the build-up of the reaction products. Furthermore, their data indicated that this effect was not due to reversibility of the reaction but rather to a physical phenomenon related to the state of the system. The accumulation of the oxidation products at the surface of the soap–cholesterol micelles apparently served to protect the remainder of the cholesterol from oxidation (4).

It is also very likely that the reaction between cholesterol and nitrogen dioxide, which leads to the formation of cholesteryl nitrate, is irreversible. Under such conditions, then, the limiting effect observed after a loss of 75% of the cholesterol on exposure to nitrogen dioxide could be due to a physical interaction between the cholesteryl nitrate and the unreacted cholesterol.

This paper presents data which support this postulation and presents a reaction scheme that accounts for the experimental observations.

EXPERIMENTAL

Materials—Cholesterol[1] and cholesteryl nitrate[2] were used for the film studies. Their purity was verified by TLC using samples of 250 mcg. Single spots were observed in both cases. Solutions of these lipids were prepared in spectroscopic grade hexane. All other chemicals were of reagent grade. The water used for the subphase was first deionized and then distilled from an all-glass still just prior to use.

A mixture of 0.5% nitrogen dioxide[3] (99.5% pure) and 99.5% prepurified grade nitrogen[3] was used as the source of nitrogen dioxide.

Apparatus and Methods—The gas mixture was allowed to pass through a flowmeter at a rate of 100 ml./min. and directed into a short length of perforated Teflon tubing, which was fixed to the underside of a Lucite trough cover, as previously described (5). This served to maintain the desired gaseous atmosphere over the film.

The film balance used to study the surface pressure–surface area (π–A) characteristics of the film was described previously (5). Surface pressures were measured by the Wilhelmy plate method (6).

An EAI TR20 analog computer[4] was used to simulate possible models descriptive of the experimental results.

Solutions of the lipids in hexane were spread on a 0.065 M phosphate buffer at pH 7.0, and the gas was allowed to flow over the film for 60 min. At the end of this time, the gas flow was discontinued; any nitrogen dioxide remaining over the surface was removed by use of the exhaust fan in the hood in which the film-balance unit was set. Manual compression of the film was then initiated, and surface pressure readings were obtained at various film areas.

[1] Mann Research Laboratories, New York, N. Y.
[2] Eastman Organic Chemicals, Rochester, N. Y.
[3] Matheson Co., East Rutherford, N. J.
[4] Electronic Associates, Inc., Long Branch, N. J.

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Abstracted from the M.S. thesis of S. K. Suh.

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Inhibitory Effect of Cholesteryl Nitrate on Interaction of Nitrogen Dioxide with Cholesterol in Monomolecular Films

N. G. LORDI*, A. FELMEISTER*, and N. D. WEINER†

Abstract □ Cholesterol monomolecular films exhibit a loss in surface pressure on exposure to nitrogen dioxide due to the formation and subsequent desorption of cholesteryl nitrate. The cholesterol–nitrogen dioxide interaction is inhibited by the prior addition of cholesteryl nitrate, and the degree of inhibition is a direct function of the mole fraction of the nitrate ester. Total inhibition occurs when this mole fraction is about 0.75. The differential equations developed to describe the sequence of events involved in this process were programmed for solution on an analog computer. This simulation indicated that at least six molecules of cholesteryl nitrate are required for each remaining cholesterol molecule to produce a total inhibition of the nitrogen dioxide–cholesterol reaction.

Keyphrases □ Cholesteryl nitrate, inhibitory effect—nitrogen dioxide–cholesterol interaction, monomolecular films □ Monomolecular films, nitrogen dioxide–cholesterol interaction—inhibitory effect of cholesteryl nitrate

Recently, it was reported that cholesterol monomolecular films, when exposed to approximately 175 p.p.m. of nitrogen dioxide for 60 min., exhibited a condensation effect (*i.e.*, a decrease in surface area at all surface pressures tested) that corresponded to a loss of about 75% of the cholesterol (1). Moreover, continued exposure did not result in any further loss of cholesterol (1). Other studies demonstrated that the formation and subsequent desorption of cholesteryl nitrate were responsible for this loss of cholesterol from the film and the concurrent condensation effect (2).

Similarly, Bergström and Wintersteiner (3) noted that the extent of autoxidation of cholesterol dispersed in an aqueous soap solution did not exceed 70%, even after extended exposure to air. These workers demonstrated that this limiting effect was caused by the build-up of the reaction products. Furthermore, their data indicated that this effect was not due to reversibility of the reaction but rather to a physical phenomenon related to the state of the system. The accumulation of the oxidation products at the surface of the soap–cholesterol micelles apparently served to protect the remainder of the cholesterol from oxidation (4).

It is also very likely that the reaction between cholesterol and nitrogen dioxide, which leads to the formation of cholesteryl nitrate, is irreversible. Under such conditions, then, the limiting effect observed after a loss of 75% of the cholesterol on exposure to nitrogen dioxide could be due to a physical interaction between the cholesteryl nitrate and the unreacted cholesterol.

This paper presents data which support this postulation and presents a reaction scheme that accounts for the experimental observations.

EXPERIMENTAL

Materials—Cholesterol[1] and cholesteryl nitrate[2] were used for the film studies. Their purity was verified by TLC using samples of 250 mcg. Single spots were observed in both cases. Solutions of these lipids were prepared in spectroscopic grade hexane. All other chemicals were of reagent grade. The water used for the subphase was first deionized and then distilled from an all-glass still just prior to use.

A mixture of 0.5% nitrogen dioxide[3] (99.5% pure) and 99.5% prepurified grade nitrogen[3] was used as the source of nitrogen dioxide.

Apparatus and Methods—The gas mixture was allowed to pass through a flowmeter at a rate of 100 ml./min. and directed into a short length of perforated Teflon tubing, which was fixed to the underside of a Lucite trough cover, as previously described (5). This served to maintain the desired gaseous atmosphere over the film.

The film balance used to study the surface pressure–surface area (π–A) characteristics of the film was described previously (5). Surface pressures were measured by the Wilhelmy plate method (6).

An EAI TR20 analog computer[4] was used to simulate possible models descriptive of the experimental results.

Solutions of the lipids in hexane were spread on a 0.065 M phosphate buffer at pH 7.0, and the gas was allowed to flow over the film for 60 min. At the end of this time, the gas flow was discontinued; any nitrogen dioxide remaining over the surface was removed by use of the exhaust fan in the hood in which the film-balance unit was set. Manual compression of the film was then initiated, and surface pressure readings were obtained at various film areas.

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[2] Eastman Organic Chemicals, Rochester, N. Y.
[3] Matheson Co., East Rutherford, N. J.
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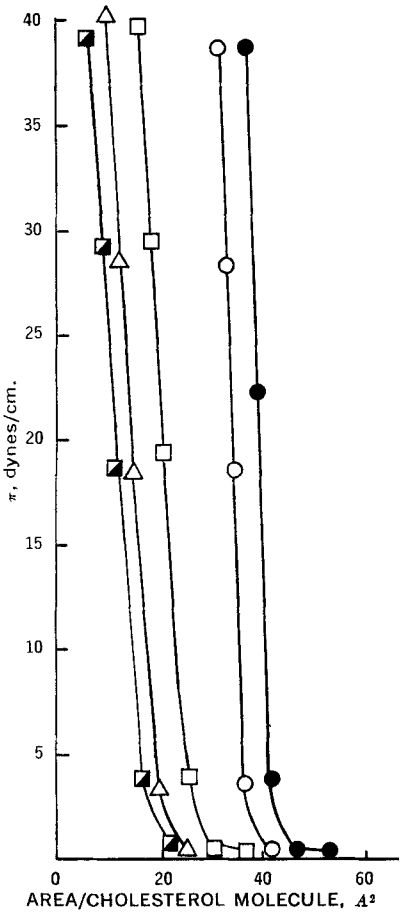

Figure 1—*Surface pressure* (π) versus *surface area per cholesterol molecule for various mole fraction mixtures of cholesterol and cholesteryl nitrate after exposure to 175 p.p.m. of nitrogen dioxide for 60 min. Key: Mole fraction of cholesteryl nitrate:* ◪, *0;* △, *0.25;* □, *0.50; and* ○, *0.75. Closed circle curve represents films of all of these mole fraction mixtures prior to exposure to nitrogen dioxide.*

The concentration of the nitrogen dioxide over the subphase during exposure was determined as follows: At periodic intervals during each experiment, 10-ml. samples of the atmosphere under the Lucite cover were drawn into a gas-tight Hamilton syringe containing 10 ml. of absorbing solution. The color of the absorbing solution was permitted to develop for 15 min., and the absorbance was measured at 550 nm. using a Spectronic-20 spectrophotometer. The concentration of nitrogen dioxide was determined from a standard nitrite curve (7).

RESULTS

As previously reported (2), cholesteryl nitrate does not form a stable monomolecular film on an aqueous subphase. Moreover, when mixtures of various mole fractions of cholesterol and cholesteryl nitrate are spread on an aqueous subphase, the π–A curves are representative only of the cholesterol fraction (2). Thus, even in the presence of cholesterol, the nitrate ester does not occupy an area-determining position in the film. Nevertheless, as seen in Fig. 1, the presence of cholesteryl nitrate does have an inhibitory effect on the nitrogen dioxide-induced condensation of cholesterol monomolecular films, and the degree of inhibition is a direct function of the mole ratio of cholesteryl nitrate.

To quantitate this inhibitory effect, an estimate of the mole fraction (F) of cholesterol that was desorbed from the film after the 60-min. exposure to nitrogen dioxide (*i.e.*, cholesterol assumed converted to the nitrate ester) was made using the following equation (1):

$$F = (1 - A/A_0) \qquad \text{(Eq. 1)}$$

where A_0 and A are the areas/molecule apparently occupied by cholesterol before and after exposure to nitrogen dioxide, respectively, at a surface pressure of 30 dynes/cm.

Equation 1 assumes that if products other than cholesteryl nitrate form during the course of the reaction, they remain in the film and occupy the same area as that of cholesterol. This is a reasonable assumption, even though some autoxidation of cholesterol will occur under these conditions (2). This is particularly so since all of the autoxidation products formed yield stable films (*i.e.*, do not desorb from the surface) and exhibit areas/molecule only slightly larger than that of cholesterol (8). Furthermore, mixed films of cholesterol with these oxidation products exhibit an average area/molecule essentially not different from that of cholesterol alone (8).

Calculated values of the fraction of cholesterol converted to the nitrate ester as a function of the mole fraction of this ester present prior to exposure are given in Table I. It is apparent that as the initial mole fraction of cholesteryl nitrate increases, the fraction of cholesterol converted (F) decreases. Furthermore, as can be seen in the third column, the limiting effect is observed when the mole fraction of the cholesteryl nitrate reaches about 0.75 and that of the cholesterol about 0.25; that is, when the system contains approximately three molecules of cholesteryl nitrate for each molecule of cholesterol, total inhibition is observed.

Table I—Estimated Mole Fraction of Cholesterol (C) Converted to Cholesteryl Nitrate (CNO_3) by NO_2[a] as a Function of the Initial Mole Fraction of CNO_3

Initial Mole Fraction, CNO_3	F (Mole Fraction C → CNO_3)	Calculated Mole Fraction after Exposure to NO_2 of: C_p[b]	CNO_3
0	0.76	0.24	0.76
0.25	0.68	0.25	0.75
0.50	0.53	0.24	0.76
0.75	0.12	0.22	0.78

[a] 60-min. exposure to 175 ± 25 p.p.m. of NO_2; 90–120-min. exposures gave essentially the same results. [b] C_p is protected cholesterol, *i.e.*, not subject to attack by NO_2.

Table II—Results of a Computer Simulation of Proposed Model for Cholesteryl Nitrate Inhibition of NO_2–Cholesterol Reaction

Initial Mole Fraction of CNO_3	Mole Fraction of C_p Determined when: $x = 1$	$x = 2$	$x = 4$	$x = 6$
0	0.25	0.25	0.25	0.25
0.25	0.23	0.245	0.25	0.25
0.50	0.18	0.215	0.24	0.25
0.75	0.10	0.135	0.17	0.205
k_2/k_1 =	0.087	0.0185	0.0055	0.003

DISCUSSION

The sequence of events on exposure of a cholesterol film to nitrogen dioxide appears to involve first the formation of cholesteryl nitrate, *i.e.*,

$$C + NO_2 \xrightarrow{\text{air}} CNO_3 \qquad \text{(Eq. 2)}$$

where C is the unreacted "free" cholesterol, and CNO_3 is cholesteryl nitrate. Since the concentration of the nitrogen dioxide is held constant throughout the reaction, the rate of disappearance of the cholesterol can be expressed by a pseudo-first-order expression.

This reaction step is followed by a rapid desorption of the cholesteryl nitrate from the film to a non-area-determining position. While this compound does not remain in the film and is not soluble in the subphase, it must nevertheless maintain a position at the interface, probably in contact with the water and in close proximity to the cholesterol film. Polycyclic hydrocarbons (*e.g.*, 1'-methyl-1,2-benzanthracene) possess similar properties and have, under certain conditions, been reported to aggregate as colloidal dispersions, without area-determining capability, below sterol monomolecular films (9).

Early in the reaction, the desorbed cholesteryl nitrate will "see" predominately cholesterol molecules and will tend to associate with them *via* van der Waal's interaction. As the reaction proceeds and more cholesteryl nitrate is formed, an accumulation of these molecules will occur around each cholesterol molecule. Eventually, as sufficient numbers of cholesteryl nitrate molecules accumulate around each remaining cholesterol molecule in the film, further attack by the nitrogen dioxide will be inhibited. The data show that this total inhibition occurs when the mole fraction of cholesteryl nitrate reaches about 0.75 (Table I).

This interaction between the "free" unreacted cholesterol and the cholesteryl nitrate can be described by:

$$C \xrightarrow{CNO_3} C_p \qquad \text{(Eq. 3)}$$

where C_p is protected cholesterol, *i.e.*, cholesterol not sensitive to attack by nitrogen dioxide. The C_p is indistinguishable from C in so far as its effect on surface pressure is concerned.

The protected cholesterol may be viewed as a probability complex involving cholesteryl nitrate. Any molecule of the nitrate ester may be associated with any molecule of the "free" unreacted cholesterol; therefore, the rate of this process becomes a function of the total amount of the cholesteryl nitrate species.

Differential equations representing Eqs. 2 and 3 can be formulated as follows:

$$-\frac{dC}{dt} = k_1(C) + k_2(C)(CNO_3)^x \qquad \text{(Eq. 4)}$$

$$\frac{d(CNO_3)}{dt} = k_1(C) \qquad \text{(Eq. 5)}$$

where x is the number of molecules of CNO_3 required to protect the unreacted cholesterol, k_1 and k_2 are the respective rate constants, and

$$C_p = C_0 - C - CNO_3 \qquad \text{(Eq. 6)}$$

where C_0 is the initial amount of cholesterol in the film. The magnitude of k_2 is a function of the rate of surface diffusion of CNO_3.

To establish the validity of the proposed mechanism, these equations were programmed for solution on an analog computer[5]. In addition, this simulation was used to determine the smallest value of x for which the amount of protected cholesterol formed would be independent of the initial amounts of cholesterol and cholesteryl nitrate.

In this simulation, k_1 was made large enough so that the reaction between cholesterol and nitrogen dioxide was completed rapidly. The ratio k_2/k_1 was adjusted so that the concentration of protected cholesterol observed in the absence of any added cholesteryl nitrate was equivalent to that observed experimentally, *i.e.*, 0.25 mole fraction.

Table II lists the mole fraction of protected cholesterol calculated at different initial mole fractions of cholesteryl nitrate for values of x from 1 to 6.

It can be seen that when the value of x is increased to 6, the mole fraction of protected cholesterol is almost independent of the initial mole fraction of the cholesteryl nitrate. This suggests that an average of at least 6 moles of the nitrate ester per mole of cholesterol is required to inhibit completely the surface reaction of nitrogen dioxide with cholesterol.

Thus, the model represented by Eqs. 4 and 5 satisfactorily reproduces the experimental observations. Furthermore, since there are only three molecules of the nitrate present for each cholesterol molecule when the limiting effect is reached, it is apparent that each molecule of cholesteryl nitrate must be associated with more than one molecule of cholesterol.

Direct complexation between the cholesterol and cholesteryl nitrate, an alternate model, is precluded on the basis of surface pressure measurements of mixed films. Under such conditions, the surface pressure of the "free" and protected cholesterol would be expected to differ.

[5] The program is available on request.

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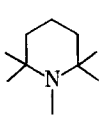
Aryl-2-piperidylcarbinols: Potential Cardiovascular Agents

JOSEPH SAM, DOROTHY NOBLES VACIK, and M. N. ABOUL-ENEIN

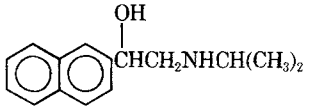
Abstract □ The syntheses of some substituted aryl-2-piperidylcarbinols are described. Weak α- and/or β-agonist and/or antagonist activity was observed in preliminary experimental studies in animals.

Keyphrases □ Aryl-2-piperidylcarbinols, substituted—synthesis, pharmacological screening □ Cardiovascular agents, potential—synthesis, pharmacological screening, aryl-2-piperidylcarbinols

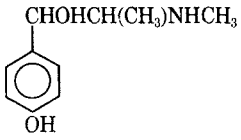
The presence of a hydrogenated benzyl piperidine system in the veratrum alkaloids (1), the hypotensive properties exhibited by some piperidine derivatives (2–6) (*e.g.*, pempidine, I), and the cardiovascular effects (7, 8) elicited by arylhydroxyalkylamines (*e.g.*, propranolol, II; and suprifen, III) prompted the investigation of some aryl-2-piperidylcarbinols (X, Table I).



I

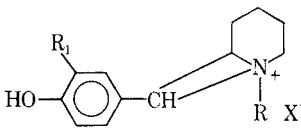


II

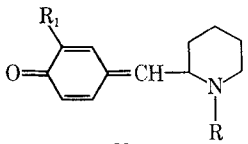


III

As the work progressed, it was anticipated that the structural characteristics of the phenyl-2-piperidylcarbinols would provide useful information regarding α- and β-agonist activity (9). Since intramolecular cyclization to the aziridine (IV) is sterically hindered, the quinone methide (V) probably would be a favored intermediate and consequently elicit β-agonist activity (9).

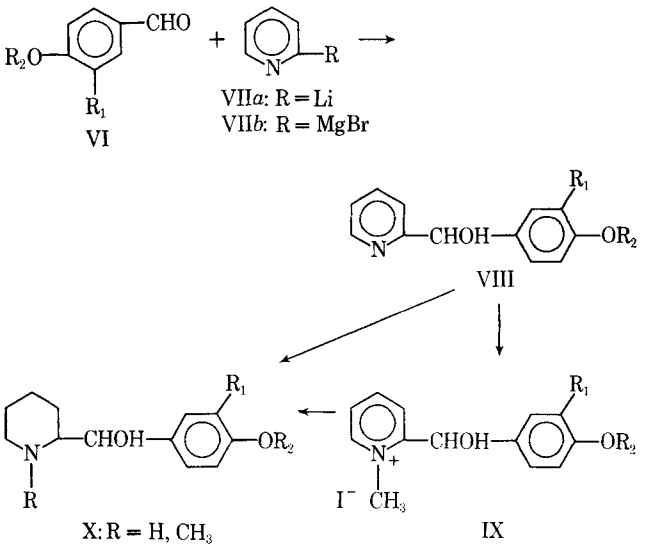


IV



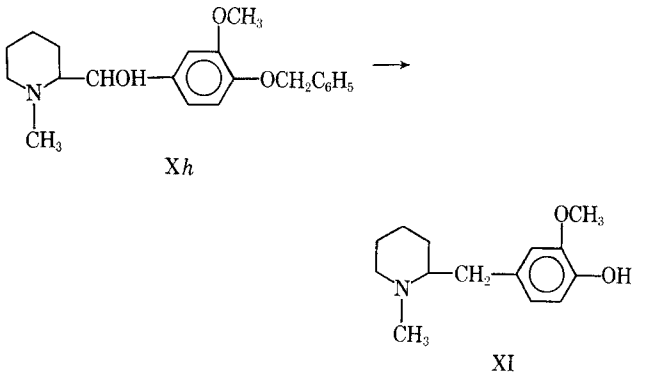
V

The preparation of aryl-2-pyridylcarbinols (VIII, Table II) was accomplished by the reaction of the appropriate benzaldehyde (VI) with either pyridyllithium (VII*a*) or pyridylmagnesium bromide (VII*b*) (Scheme I). The reaction of *p*-hydroxybenzaldehydes with an excess of the organometallic derivative provided approximately 10% yields of VIII (R_2 = H). The yields, although still low, were improved considerably by masking the phenols as the benzyl ethers. The hydrogenation of VIII or the methiodides (IX) provided the corresponding aryl-2-piperidylcarbinols (X).



Scheme I

The hydrogenolysis of 1-methyl-α-[(3-methoxy-4-benzyloxy)phenyl]-2-piperidylcarbinol hydriodide (X*h*) proceeded smoothly; however, hydrogen in excess of the amount necessary for debenzylation was consumed (Scheme II). This was not surprising inasmuch as benzyl



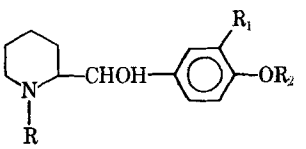
Scheme II

alcohols are known to undergo hydrogenolysis (10), especially under acidic conditions. The product was identified as the dehydrated and reduced compound, XI, *via* methylation to the dimethoxy derivative (XIV). The latter also was prepared by an alternate route from VIII*e* (Scheme III). Compound XIV from both synthetic routes was converted to the methiodide.

PHARMACOLOGY

Preliminary pharmacological data on the aryl-2-piperidylcarbinols (X) are summarized in Table III. None of the compounds showed significant adrenergic agonist activity relative to either the α- or β-receptor. The piperidines (X*a*–X*c*) without the *N*-methyl substituent exhibited very weak β-receptor blockade. In general, the

Table I—Aryl-2-piperidylcarbinols



Number[a]	R	R_1	R_2	Method	Recrystallization Solvent[b]	Melting Point	Yield, %	Molecular Formula	Analysis—Calcd.	Analysis—Found
X*a*	H	H	H	D	E	229–230°	50	$C_{12}H_{18}NO_2Cl$[c]	C, 59.14 H, 7.44 N, 5.75	C, 59.42 H, 7.53 N, 5.60
X*b*	H	OCH_3	H	D	E	250–251°	75	$C_{13}H_{20}NO_3Cl$[c]	C, 57.01 H, 7.36 N, 5.11	C, 57.16 H, 7.46 N, 4.92
X*c*	H	OCH_3	CH_3	D	E	201–202°	76	$C_{14}H_{22}NO_3Cl$[c]	C, 58.43 H, 7.70 N, 4.87	C, 58.46 H, 7.80 N, 4.68
X*d*	CH_3	H	H	E	M–Et	171–172°	52	$C_{13}H_{19}NO_2$	C, 70.56 H, 8.65 N, 6.33	C, 70.29 H, 8.71 N, 6.27
X*e*	CH_3	OCH_3	H	D	E–PE	163–164°	91	$C_{14}H_{21}NO_3$	C, 66.91 H, 8.42 N, 5.57	C, 66.81 H, 8.57 N, 5.33
X*f*	CH_3	OCH_3	CH_3	D		160–162°(0.15)[d]	70	$C_{15}H_{23}NO_3$	C, 67.90 H, 8.74 N, 5.28	C, 67.54 H, 8.85 N, 5.12
X*g*	CH_3	H	$CH_2C_6H_5$	D	E–Et	166–168°	63	$C_{20}H_{26}NO_2I$[e]	C, 54.66 H, 5.97 N, 3.19	C, 54.37 H, 6.07 N, 3.12
X*h*	CH_3	OCH_3	$CH_2C_6H_5$	D	W	207–210°	30	$C_{22}H_{27}NO_3I$[e]	C, 53.86 H, 5.83 N, 2.99	C, 53.66 H, 6.11 N, 2.95

[a] IR spectra are consistent with the assigned structures. [b] E = ethanol, Et = ether, M = methanol, PE = petroleum ether (30–60°), and W = water. [c] Hydrochloride. [d] Boiling point. [e] Hydriodide.

compounds were nonspecific, smooth muscle depressants of very low potency.

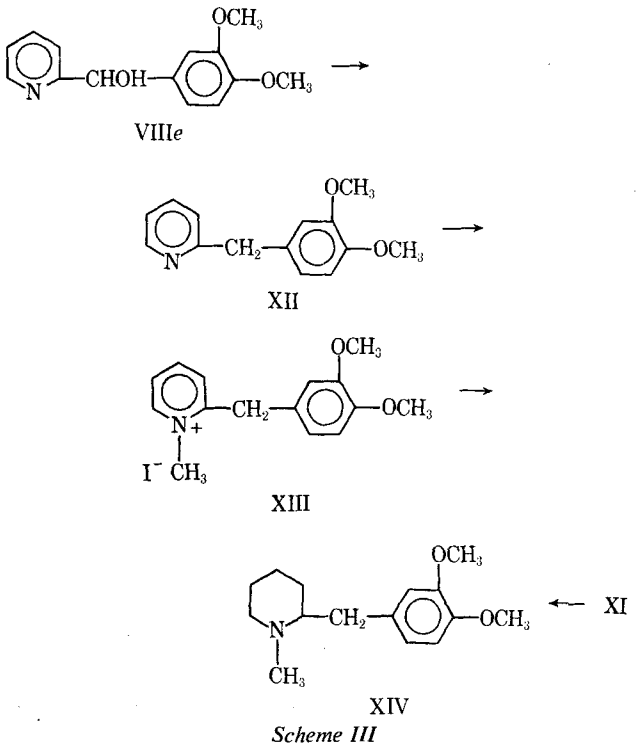
EXPERIMENTAL[1]

The procedures described by Buu-Hoi *et al.* (11) and Anker *et al.* (12) were utilized for the preparation of *p*-benzyloxybenzaldehyde and benzyloxyvanillin, respectively. Materials other than those described in the *Experimental* section were obtained from commercial sources.

Aryl-2-pyridylcarbinols (VIII, Table II)—*Method A*—Modification of the procedure of Wibaut *et al.* (13) was employed for the preparation of 2-pyridyllithium. A stirred, oxygen-free mixture of 100 ml. of anhydrous ether and 3.78 g. (0.4 mole) of lithium wire [flattened and cut into pieces approximately 0.64 cm. (0.25 in.) long], maintained at −10°, was treated dropwise with 27.4 g. (0.2 mole) of *n*-butyl bromide. The solution was stirred until the lithium disappeared; it was then cooled to −45° and maintained at this temperature while being treated dropwise with a solution of 31.6 g. (0.2 mole) of 2-bromopyridine in 80 ml. of anhydrous ether. The blood-red solution was stirred an additional hour and then treated slowly at −45° with a solution of 0.1 mole of the appropriate benzaldehyde in 100 ml. of anhydrous ether. The milky reaction mixture was stirred for an additional hour, allowed to come to room temperature, and then decomposed with cold 16% ammonium chloride. An ether or a methylene chloride extract of the mixture was dried over anhydrous sodium sulfate and evaporated. The residual material either was recrystallized or distilled.

Method B—A solution of 2-pyridylmagnesium bromide [prepared from 16 g. (0.1 mole) of 2-bromopyridine by the procedure described by Overhoff and Proost (14)] in 120 ml. of anhydrous ether was treated dropwise at 36° with a solution of 0.06 mole of the appropriate benzaldehyde in 50 ml. of anhydrous ether and thereafter stirred for 12 hr. at room temperature. The product was isolated as described under *Method A*.

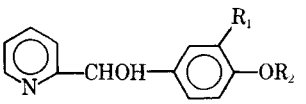
Method C—A solution of 0.016 mole of the benzyloxy derivative (VIII*a* and VIII*c*) in 230 ml. of methanol was hydrogenated in the presence of 0.6 g. of 10% palladium-on-charcoal for 28–30 hr. at room temperature at an initial hydrogen pressure of 48 p.s.i. The mixture was filtered, and the filtrate was evaporated under reduced pressure; the residual material was either recrystallized or converted to the hydrohalides or the methiodides in the usual manner and recrystallized.



Scheme III

[1] All melting points were taken on a Fisher-Johns melting-point apparatus and are corrected. IR data were obtained on all compounds with Perkin-Elmer model 137G and 257 IR spectrophotometers using KBr pellets or chloroform solutions.

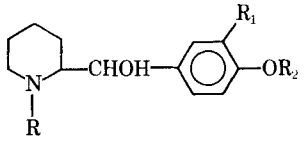
Table II—Aryl-2-pyridylcarbinols



Number[a]	R_1	R_2	Method	Recrystallization Solvent[b]	Melting Point	Yield, %	Molecular Formula	Analysis—Calcd.	Analysis—Found
VIII*a*	H	$CH_2C_6H_5$	B	A–Et	179–180°[c]	48	$C_{20}H_{20}NO_2I$[d]	C, 55.44 H, 4.63 N, 3.23	C, 55.54 H, 4.49 N, 3.36
VIII*b*	H	H	A,C	W	139–140°[e]	11,80	$C_{12}H_{11}NO_2$	C, 71.62 H, 5.50 N, 6.96	C, 71.14 H, 5.52 N, 7.12
VIII*c*	OCH_3	$CH_2C_6H_5$	A,B	E	156–158°[f]	43,40	$C_{21}H_{22}NO_3I$[d]	C, 54.44 H, 4.79 N, 3.02	C, 54.52 H, 4.85 N, 3.16
VIII*d*	OCH_3	H	A,C	A–M	171–172°[g,h]	10,90	$C_{13}H_{14}NO_3Cl$[i]	C, 58.35 H, 5.31 N, 5.24	C, 58.37 H, 5.30 N, 5.01
VIII*e*	OCH_3	CH_3	A,B	E–PE	93–94°[j,k,l]				

[a] IR spectra are in agreement with the assigned structures. [b] A = acetone, E = ethanol, EA = ethyl acetate, Et = ether, M = methanol, PE = petroleum ether (30–60°), and W = water. [c] Free base, m.p. 95–96° (E–Et). [d] Methiodide. [e] Hydrochloride, m.p. 174–175° (M–EA). [f] Free base, m.p. 124–125° (E). [g] The base was obtained as a yellow viscous oil. [h] The methiodide was obtained as a syrupy viscous oil. [i] Hydrochloride. [j] *Reference 15.* [k] Hydrochloride, m.p. 161–162° (M–EA). [l] Methiodide, m.p. 180–181° (E–W).

Table III—Adrenergic Activity



Number	R	R_1	R_2	β-Adrenergic Blockade[a,b]	β-Adrenergic Stimulation[a,c]	α-Adrenergic Blockade[d,e]	α-Adrenergic Blockade[d,f]	α-Adrenergic Stimulation[d,g]
X*a*	H	H	H	*ca.* 100	>1000	220	520	>1000
X*b*	H	OCH_3	H	320	>1000	310	820	>1000
X*c*	H	OCH_3	CH_3	52	>250	355	>1000	>1000
X*d*	CH_3	H	H	>1000	>1000	225	690	>1000
X*e*	CH_3	OCH_3	H	>1000	>1000	340	450	>1000
X*f*	CH_3	OCH_3	CH_3	>1000	>1000	130	450	>1000

[a] On guinea pig trachea. [b] Concentration (micrograms per milliliter) causing 50% inhibition of the relaxant action of isoproterenol. [c] Concentration (micrograms per milliliter) relaxing spontaneous tissue tonus by 75% of maximum. [d] On isolated rat seminal vesicle. [e] Concentration (micrograms per milliliter) causing 50% inhibition of norepinephrine. [f] Concentration (micrograms per milliliter) causing 50% inhibition of barium chloride. [g] Concentration (micrograms per milliliter) eliciting a contractile response 50% of the magnitude of epinephrine.

Aryl-2-piperidylcarbinols (X, Table I)—*Method D*—A solution or suspension of 0.032 mole of the hydrochloride or methiodide of the appropriate 2-pyridyl carbinol (VIII) in 150 ml. of water was hydrogenated in the presence of 0.5 g. of PtO_2 for 24–30 hr. at room temperature at an initial hydrogen pressure of 48 p.s.i. The mixture was filtered, and the filtrate was evaporated to dryness. The residual material was either recrystallized to yield the hydrohalide or treated with 10% sodium bicarbonate and concentrated ammonium hydroxide to pH 8, saturated with sodium chloride, and extracted with methylene chloride. The solvent was evaporated, and the residual amine was either recrystallized or distilled.

Method E—A solution of 0.04 mole of the appropriate 1-methyl-α-(4-benzyloxyphenyl)-2-piperidylcarbinol in 200 ml. of methanol was debenzylated as described under *Method C*.

2-(3,4-Dimethoxybenzyl)-1-methylpiperidine (XIV)—*Method F*—A solution of 6.9 g. (0.03 mole) of 2-(3,4-dimethoxybenzyl)pyridine (XII), prepared according to the method of Sugimoto (15), and 20 g. (0.14 mole) of methyl iodide in 100 ml. of acetonitrile was refluxed for 24 hr. The solvent was removed under reduced pressure. The residual crude methiodide (12.8 g.) was dissolved in 60 ml. of water and hydrogenated in the presence of 0.3 g. of platinum oxide at room temperature for 24–30 hr. at an initial hydrogen pressure of 48 p.s.i. The mixture was filtered and the filtrate treated with excess 10% sodium hydroxide. A chloroform extract (3 × 30 ml.) of the mixture was dried over anhydrous sodium sulfate and distilled at 126–130° (0.15 mm.) to give 4.6 g. (61%) of product. A picrate was prepared in the usual manner and recrystallized from ethanol–acetone; m.p. 150–151°.

Anal.—Calcd. for $C_{21}H_{26}N_4O_9$: C, 52.72; H, 5.48; N, 11.71 Found: C, 52.70; H, 5.45; N, 12.02.

A methiodide was prepared in the usual manner and recrystallized from ethanol; m.p. 164–165°.

Anal.—Calcd. for $C_{16}H_{26}NO_2I$: C, 49.08; H, 6.69; N, 3.58. Found: C, 48.54; H, 6.54; N, 3.49.

Method G—The methylation procedure described by Vogel (16) was followed using 2 g. (0.008 mole) of 2-(4-hydroxy-3-methoxybenzyl)-1-methylpiperidine (XI), 1 g. (0.005 mole) of sodium hydroxide, 20 ml. of water, and 1.3 g. (0.01 mole) of dimethyl sulfate. The mixture was refluxed for 2 hr. with stirring and then treated with 25 ml. of water. An ether extract of the oil was washed with water and then dried over anhydrous sodium sulfate. Evaporation of the ether left an oil, which was converted to a picrate in the usual manner and recrystallized from ethanol; m.p. 149–150°. A mixture melting point with the product obtained from *Method F* showed no depression. IR spectra were identical.

2-(4-Hydroxy-3-methoxybenzyl)-1-methylpiperidine (XI)—A solution of 13.6 g. (0.04 mole) of 1-methyl-α-(3-methoxy-4-benzyloxyphenyl)-2-piperidylcarbinol hydriodide (X*h*) in 50 ml. of methanol was hydrogenated as described under *Method C*. The hydrochloride was prepared in the usual manner and was recrystallized from acetone–methanol to yield 4.5 g. (25%) of product; m.p. 220–221°.

Anal.—Calcd. for $C_{14}H_{22}NO_2Cl$: C, 61.87; H, 8.19; N, 5.15; Cl, 13.05. Found: C, 62.18; H, 7.58; N, 4.98; Cl, 12.95.

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Abstract □ Various additives were added to the gel-like product which results from the interaction of salicylic acid with cetrimide. The additives were alcohols, acetone, water, glycerin, propylene glycol, polyethylene glycols, amyl acetate, heptane, hexadecane, dioxane, cyclohexane, benzene, methylbenzene, ethylbenzene, nitrobenzene, pyridine, tetralin, carbon tetrachloride, chloroform, liquefied phenol BP, and cresol BP. The first 10 compounds reduced the viscosity of the gel-like product to varying degrees, and the reduction generally increased with an increasing volume of the additive. The remaining 11 compounds together with the higher alcohols, from hexyl to decyl alcohol, increased the viscosity initially, followed soon after by a viscosity decrease. The instability of the system in the presence of the additives was probably due largely to their effect on the links in the network structure of the macromolecules which formed the gel-like product. The initial rise in viscosity could be due to a tendency of a small amount of the additive to bring the macromolecules closer together, thus making the system more viscous. The fall after this rise could be attributed to the breakup of the mesh with larger amounts of the additive and possibly to a change in the nature of the system.

Keyphrases □ Salicylic acid–cetrimide system—stability in presence of additives □ Cetrimide–salicylic acid interaction, stability—effect of additives □ Additives—effect on stability of salicylic acid–cetrimide system □ Viscosity—effect of additives on salicylic acid–cetrimide interaction

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EXPERIMENTAL

Materials—Salicylic acid was recrystallized, m.p. 158.5–159°. Cetrimide BP[1], which consisted chiefly of tetramethylammonium bromide, was used as supplied. The additives used were as follows: redistilled alcohols (methyl, ethyl, *n*-propyl, and *n*-butyl)[2], amyl alcohol[3], *n*-hexyl alcohol[3], *n*-heptyl alcohol[3], *n*-octyl alcohol[3], *n*-decyl alcohol[3], acetone[2], distilled water, glycerin BP, propylene glycol[3], polyethylene glycol 400 and 600[4], amyl acetate[3], heptane[3], hexadecane[3], dioxane[5], cyclohexane[3], benzene[3], methylbenzene[6], ethylbenzene[6], nitrobenzene[3], pyridine[7], tetralin[3], carbon tetrachloride[3], chloroform[8], liquefied phenol BP, and cresol BP.

Apparatus—A portable Ferranti viscometer[9] was used.

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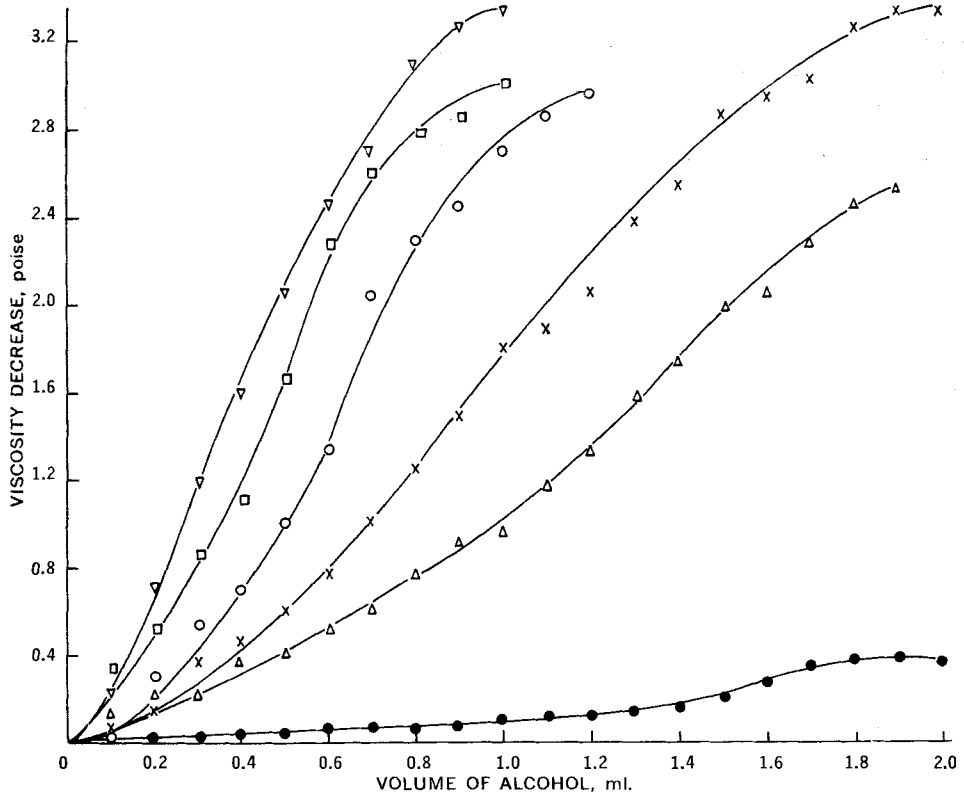

Figure 1—*Viscosity decrease of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid in the presence of various alcohols at 25°. Key: △, methyl; ×, ethyl; ○, n-propyl; □, n-butyl; ▽, n-amyl; and ●, water. Shear rate = 78.56 sec.$^{-1}$.*

viscometer. The viscosity was measured, allowing an interval of 30 sec. between readings. The shear rates were increased initially and then decreased. Successive additions of the additive to the system were continued until the viscosity was reduced to such a low value that it could not be measured accurately with the viscometer.

RESULTS AND DISCUSSION

Methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl alcohols were added to the gel-like product. All these alcohols reduced the viscosity (Fig. 1); the reduction increased with the volume of alcohol added. With

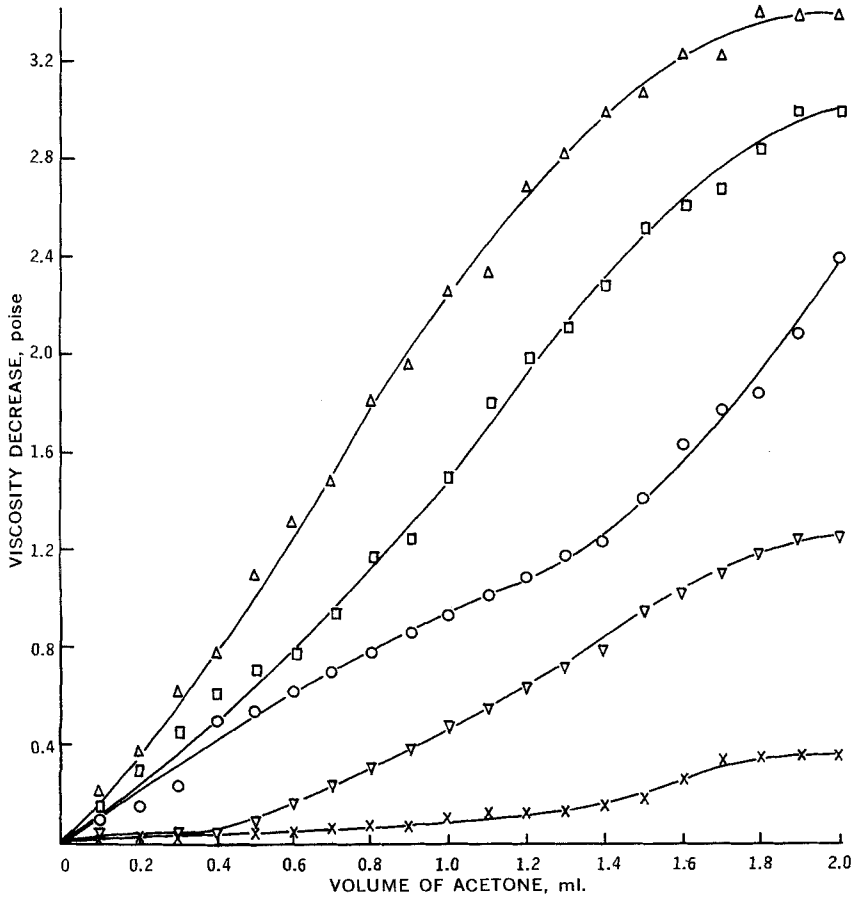

Figure 2—*Viscosity decrease of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid due to the addition of acetone of varying concentrations at 25°. Key: ×, water; ▽, 20% acetone; ○, 40% acetone; □, 60% acetone; and △, 80% acetone. Shear rate = 78.56 sec.$^{-1}$.*

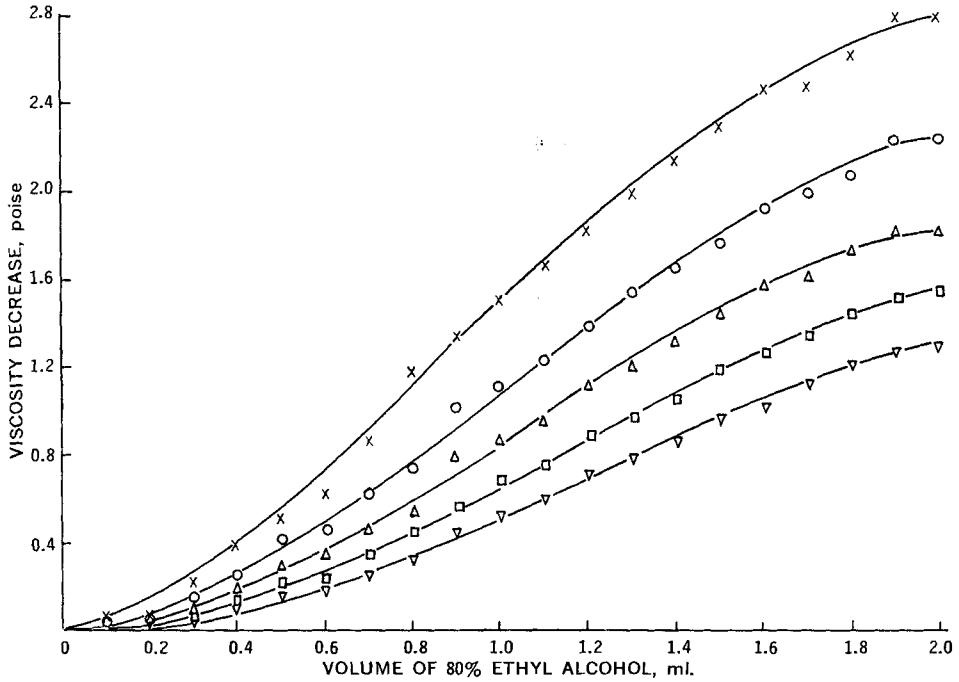

Figure 3—*Viscosity decrease of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid in the presence of 80% ethyl alcohol with different shear rates at 25°. Key: ×, 78.56 sec.$^{-1}$; ○, 117.35 sec.$^{-1}$; △, 155.1 sec.$^{-1}$; □, 195.9 sec.$^{-1}$; and ▽, 234.6 sec.$^{-1}$.*

the addition of 1 ml. *n*-amyl alcohol or 2 ml. ethyl alcohol, the viscosity was so much reduced that the gel-like property of the system was practically eliminated and it appeared fluid and watery. Only a small amount of alcohol was sufficient to produce a substantial decrease in viscosity. Addition of 0.4 ml. of *n*-amyl alcohol or *n*-butyl alcohol resulted in the reduction of viscosity from 3.4 to 1.8 and 2.3 poises, respectively, at a shear rate of 78.56 sec.$^{-1}$. The decrease in viscosity increased with the chain length of the alcohol. The addition of water caused a small change in the viscosity, since the greatest reduction in viscosity observed was not more than 0.4 poise at a shear rate of 78.56 sec.$^{-1}$. This finding demonstrated that the viscosity decrease probably was not due to a simple dilution effect of the additive.

Figure 2 shows that the higher the concentration of acetone, the more marked the viscosity reduction. This was also true for various alcohol concentrations. Since the gel-like product was a non-Newtonian system, the apparent viscosity obtained varied with different shear rates. For the same volume of additive, the change in apparent viscosity was more pronounced when a slower shear rate was applied to the system (Fig. 3). As the volume of additive increased, the non-Newtonian behavior of the system became less apparent and there was a tendency toward a Newtonian behavior.

With the exception of amyl alcohol, which is slightly soluble in water, the other alcohols are completely miscible with water. When this series of alcohols was extended to the higher alcohols, the behavior of these additives in respect to the gel-like product was different (Fig. 4). The effect of *n*-hexyl, *n*-heptyl, *n*-octyl, and *n*-decyl

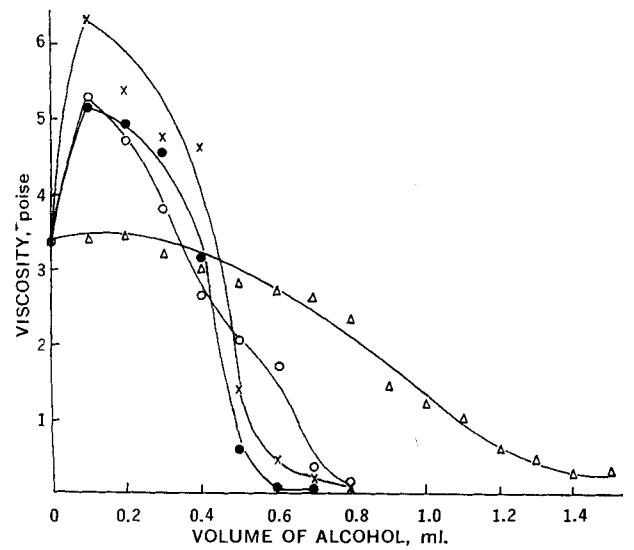

Figure 4—*Effect of water-insoluble alcohols on the viscosity of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key: △, n-hexyl; ●, n-heptyl; ×, n-octyl; and ○, n-decyl. Shear rate = 78.56 sec.$^{-1}$.*

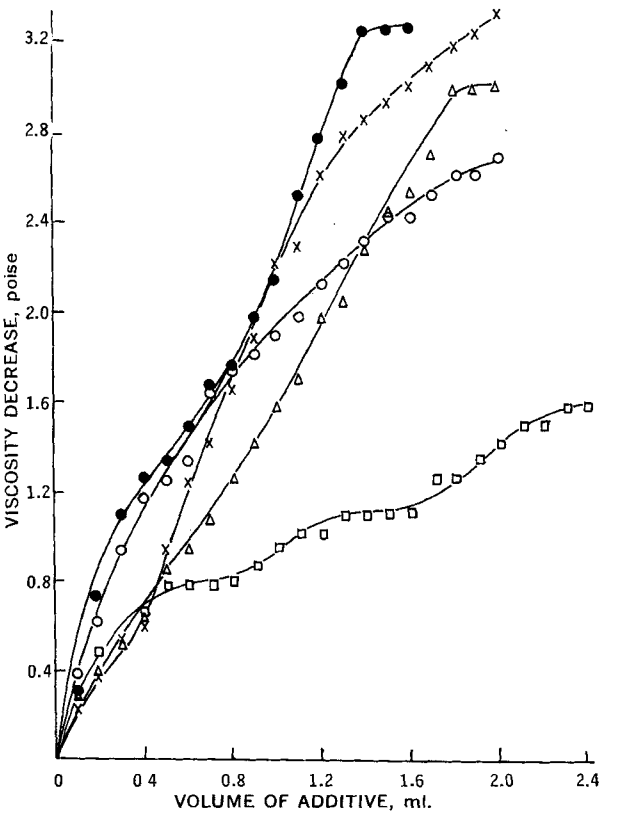

Figure 5—*Effect of polyhydric alcohols and amyl acetate on the viscosity of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key: △, polyethylene glycol 600; ○, polyethylene glycol 400; ×, propylene glycol; □, glycerin; and ●, amyl acetate. Shear rate = 78.56 sec.$^{-1}$.*

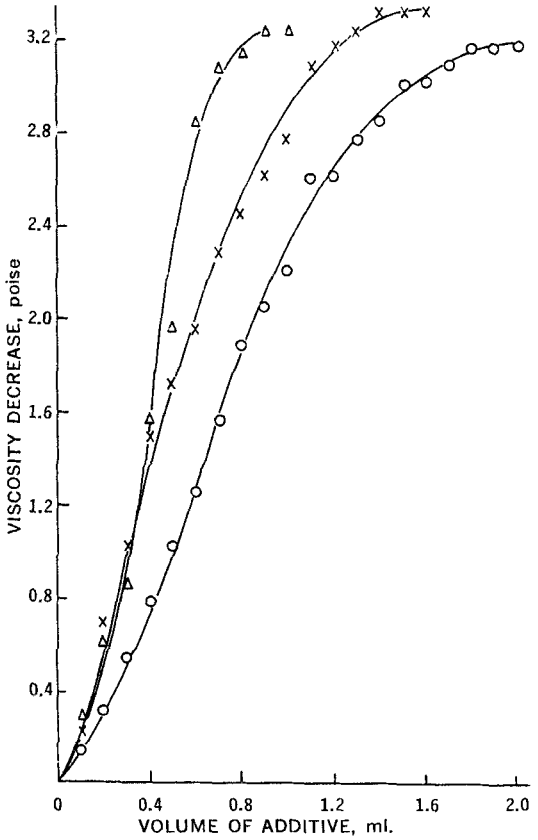

Figure 6—*Effect of hexadecane, heptane, and dioxane on the viscosity of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key: △, hexadecane; ×, heptane; and ○, dioxane. Shear rate = 78.56 sec.$^{-1}$.*

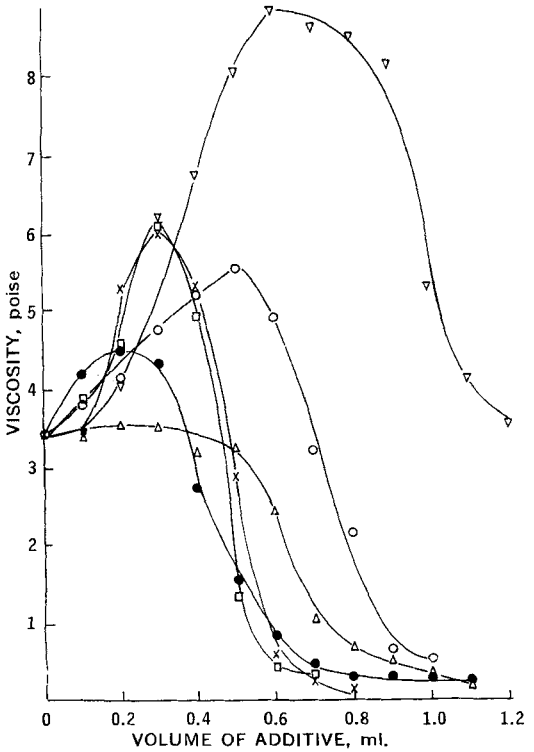

Figure 7—*Effect of benzene, substituted benzenes, pyridine, and cyclohexane on the viscosity of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key: ○, benzene; ×, methylbenzene; □, ethylbenzene; ●, nitrobenzene; ▽, pyridine; and △, cyclohexane. Shear rate = 78.56 sec.$^{-1}$.*

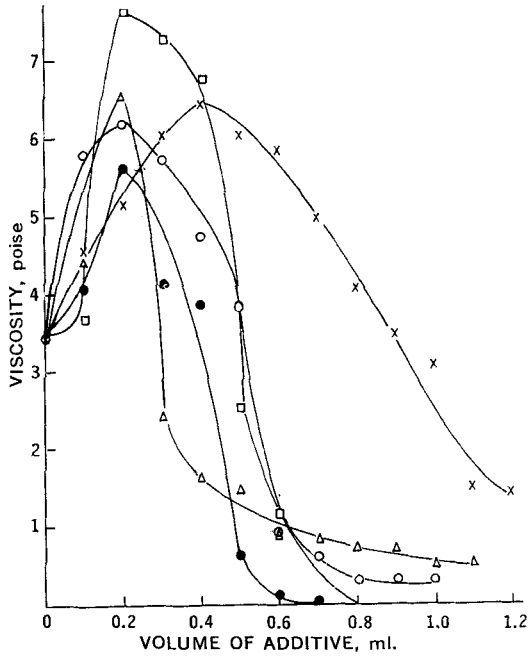

Figure 8—*Effect of various additives on the viscosity of an aqueous system (50 g.) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key: ×, liquefied phenol BP; ○, carbon tetrachloride; △, chloroform; □, cresol BP; and ●, tetralin. Shear rate = 78.56 sec.$^{-1}$.*

alcohols was an initial rise in viscosity, followed by a decrease with subsequent additions of the alcohol. All these alcohols, except decyl alcohol which is water insoluble, have very limited solubilities in water. When the viscosity commenced to fall, it was generally followed by cloudiness in the system; eventually, as more of the additive was added, droplets of the alcohol could be seen dispersed in the system. At the same time, the degree of cloudiness increased also. Not more than 0.2 ml. alcohol/50 g. product was adequate to reduce viscosity after the initial rise.

The effects of the addition of other additives such as glycerin, propylene glycol, polyethylene glycol 400 and 600, amyl acetate, hexadecane, heptane, and dioxane can be seen in Figs. 5 and 6. The decrease in viscosity was similar to that exhibited by the water-miscible alcohols and acetone (Figs. 1 and 2). Several other compounds were found to behave in like manner as the higher alcohols. Thus, cyclohexane, benzene, methylbenzene, ethylbenzene, nitrobenzene, pyridine, tetralin, carbon tetrachloride, chloroform, liquefied phenol, and cresol (Figs. 7 and 8) caused an initial increase in viscosity when incorporated in the gel-like product. This increase was temporary—a decrease in viscosity was observed with further additions of the additive.

It would be unlikely that the viscosity decrease resulted from a solution of the gel-like product in the additive since the amount of additive necessary to effect a change in viscosity was relatively small. It did not seem that the water solubility or water miscibility of the additive could account for the viscosity reduction since water-soluble or water-miscible and water-insoluble or water-immiscible compounds behaved in the same way. The question of solubility of salicylic acid or cetrimide in the additive being responsible for the breakdown of the system and, hence, a lowering of the viscosity was not probable because both the acid and the surfactant are soluble in some additives and insoluble in others belonging to the same group of compounds that produced a fall in viscosity immediately when they were added. The fact that carbon tetrachloride and chloroform did not cause a viscosity reduction immediately meant that the effect was not limited to aliphatic substances only. The fall in viscosity was not associated with the polarity of the additive because both polar and nonpolar liquids were able to produce the same effect.

It is believed that the interaction of salicylic acid with aqueous cetrimide solutions led to the formation of macromolecules, enhancing the viscosity of the system. This enhancement could be brought about not only by the size of the macromolecules but also by their approaching each other to form a sort of matted matrix of high

viscosity. When an additive was added, it would penetrate the framework of the structure and weaken or disrupt the building units. It is expected that the macromolecules are joined by weak cohesive forces and not by strong bonds. Once the structural elements were affected, the system became unstable and the viscosity began to decrease. When the volume of additive present was increased, the gel-like character of the product gradually became less apparent and finally it was converted to a system of very low viscosity.

The initial rise in viscosity could not be attributed to solubilization of the additive by the surfactant to cause a swelling of the gel-like product, because most of the cetrimide molecules would have interacted with salicylic acid already, the latter being present in sufficient quantities to saturate practically the system. In support of this, it was observed that heptane and hexadecane, which are water insoluble, and amyl acetate, which is slightly water soluble, did not produce a rise in viscosity initially, although they could be solubilized. In addition, pyridine and liquefied phenol, both of which are miscible with water and could not be solubilized, also caused an initial increase in viscosity. Apparently the additives that increased the viscosity when first added were not able to disrupt the links at once, but they instead probably tended to attract the macromolecules closer together and thereby made the system more viscous. However, this situation did not remain for long; as soon as more of the additive was present, the bridges were unable to prevent a rupture of the framework and instability of the system was produced.

The decrease in viscosity after the initial increase was also associated with the change of the nature of the system, from a gel-like product to a cloudy dispersion or emulsion containing droplets of the immiscible additive. Generally the viscosity commenced to fall when the additive present had already exceeded its solubility or miscibility limits. However, this was not always the case. Pyridine and liquefied phenol, which did not produce cloudiness in the system, showed the same rise and fall in viscosity.

The findings of this investigation showed that two effects are possible when additives, many of which are simple solvents, are added to the system. One is the effect of immediate reduction in viscosity with a small volume of the additive; the other is an initial increase with very small amounts of the additive, followed by a decrease when a larger volume is included. These results will be of value in determining the usefulness of the gel-like product when incorporated in suspension formulation.

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MASAHIRO TSUBOUCHI

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Keyphrases □ Organic cations—spectrophotometric determination by solvent extraction with tetrabromophenolphthalein ethyl ester □ Tetrabromophenolphthalein ethyl ester, solvent extraction—spectrophotometric determination of organic cations □ Spectrophotometric determination—organic cations–tetrabromophenolphthalein ethyl ester, solvent extraction

In general, acid dye is known to react with amine or quaternary ammonium salt to form a colored compound (1, 2). Bromthymol blue (3), bromcresol green (4), or bromphenol blue (5) was used for the determination of thiamine, quaternary compound, or quinine.

Tetrabromophenolphthalein ethyl ester has been used as a pH indicator. Various amines and organic cations were extracted with tetrabromophenolphthalein ethyl ester into 1,2-dichloroethane. During this study, the colors of the extracts were found to be classified into three categories: (*a*) red-violet, which is developed by the presence of diphenhydramine, pilocarpine, eserine, *N,N*-dimethylpiperazine, papaverine, or triethanolamine; (*b*) blue, which is extracted by the presence of neostigmine, benzethonium, sparteine, tetraethylammonium, acetylcholine, or thiamine; and (*c*) yellow, which is the same color as the reagent blank for the presence of aniline, 3-aminoquinoline, *N,N*-dimethylformamide, ethylenediaminetetraacetic acid, or nitrilotriacetic acid.

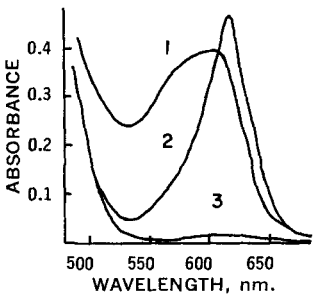

Figure 1—*Absorption spectra. Key: 1, extract with 5.0 × 10⁻⁶ M diphenhydramine; 2, extract with 2.0 × 10⁻⁶ M neostigmine; and 3, extract without neostigmine and diphenhydramine. Reference = water.*

This paper deals mainly with the determination of neostigmine with tetrabromophenolphthalein ethyl ester based on such a phenomenon; the results are then summarized for the determination of diphenhydramine, benzethonium, sparteine, or tetraethylammonium, which are on the market as medicines or disinfectants. The proposed methods have a very good reproducibility and a high sensitivity.

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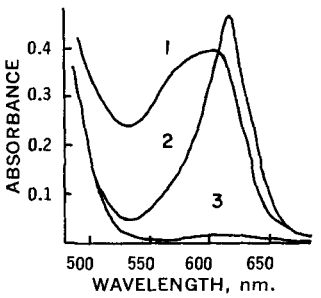

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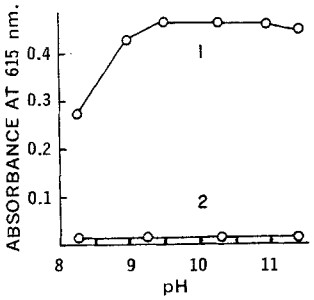

Figure 2—*Effect of pH. Key: 1, 2.0 × 10^{-6} M neostigmine; and 2, reagent blank. Reference = water.*

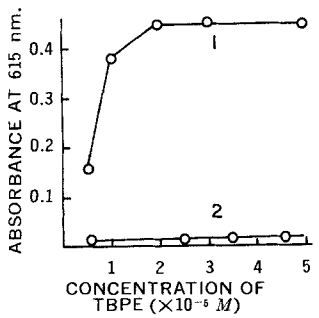

Figure 3—*Effect of concentration of tetrabromophenolphthalein ethyl ester (TBPE). Key: 1, 2.0 × 10^{-6} M neostigmine; and 2, reagent blank. Reference = water.*

The titrimetric method (6) has been used for the determination of neostigmine, which is widely used as a stimulant for parasympathetic nerves.

EXPERIMENTAL

Apparatus—The spectrophotometric measurements were made with a Shimazu model QR-50 spectrophotometer, with 10-mm. cells. An Iwaki model KM shaker with a time switch was used for the extraction. The pH measurements were made with a Toa Denpa model HM-5 pH meter.

Reagents—*Standard Solution*—A stock solution of 1.0 × 10^{-2} *M* neostigmine was prepared by dissolving 3.032 g. of neostigmine bromide[1] (dried at 105°) and diluting to 1 l. with water. The stock solution was used to prepare the standard solution with the desired concentration.

A standard solution of diphenhydramine[1], benzethonium[1], sparteine[1], or tetraethylammonium[1] was prepared by dissolving their chlorides, bromides, or sulfates in water.

Tetrabromophenolphthalein Ethyl Ester Solution—A 1.0 × 10^{-3} *M* solution of tetrabromophenolphthalein ethyl ester was prepared by dissolving 0.700 g. of tetrabromophenolphthalein ethyl ester potassium salt[2] to a volume of 1 l. with ethyl alcohol.

Buffer Solution—The borate–phosphate buffer was prepared by adding 3 *N* sulfuric acid or 3 *N* sodium hydroxide solution to the 0.3 *M* potassium dihydrogen phosphate solution containing 0.1 *M* sodium borate.

All the chemicals were of reagent grade, and the water used was passed through an ion-exchange resin.

Recommended Procedures—*Neostigmine*—Pipet 5 ml. of neostigmine solution (less than 2.0 × 10^{-5} *M*), 2 ml. of tetrabromophenolphthalein ethyl ester solution (1 × 10^{-3} *M*), and 5 ml. of borate–phosphate buffer solution (pH 9.5–11.0) into a separator. Dilute the mixture to 25 ml. with water, and shake the solution for 2 min. with 10 ml. of 1,2-dichloroethane. After separation of the two layers, run off the extract into a glass tube through a filter paper to remove droplets of water. Measure the absorbance of the extract at 615 nm., using a reagent blank as a reference.

Benzethonium, Tetraethylammonium, and Sparteine—Pipet 5 ml. of benzethonium, tetraethylammonium, or sparteine solution (less than 2.0 × 10^{-5} *M* for each solution), 2 ml. of tetrabromophenolphthalein ethyl ester solution (1 × 10^{-3} *M*), and 5 ml. of borate–phosphate buffer solution (pH 7.0–11.0 for the determination of benzethonium or sparteine, pH 9.5–10.5 for the determination of tetraethylammonium) into a separator. Treat the mixture in the same manner as described for neostigmine. Measure the absorbance at 615 nm.

Diphenhydramine—Pipet 5 ml. of diphenhydramine solution (less than 5.0 × 10^{-5} *M*), 5 ml. of tetrabromophenolphthalein ethyl ester solution (1 × 10^{-3} *M*), and 5 ml. of borate–phosphate buffer solution (pH 7.0–9.0) into a separator. Treat the mixture in the same way as for neostigmine, and measure the absorbance at 594 nm.

[1] Purchased from Tokyo Kasei Kogyo Co., Toshima, Kita-ku, Tokyo, Japan.

[2] Purchased from Wako Pure Chemical Industries, Doshu, Higashi-ku, Osaka, Japan.

Table I—Effect of Foreign Substances on the Determination of Neostigmine

Substance	Mole Ratio	Recovery of Neostigmine, %[a]
Ammonium sulfate	50000	101.0
Calcium chloride	50000	99.5
Sodium carbonate	50000	100.0
Sodium chloride	50000	100.0
Sodium nitrate	50000	100.0
Potassium bromide	50000	100.0
Aminopyrine	50	103.0
Antipyrine	40	106.0
Benzyl alcohol	50000	100.0
Caffeine	30	113.0
o-Cresol	50000	98.5
Ethyl alcohol	50000	99.7
Glucose	50000	100.0
Lactose	50000	100.0
Nicotinamide	100	107.5
Papaverine	20	105.0
Phenacetin	10	104.2
Phenol	50000	98.0
Sodium acetate	50000	100.0
Sodium citrate	50000	100.0
Sodium salicylate	50000	100.0
Sodium tannate	50000	98.5
Vitamin B_1	10	109.7
Vitamin C	50000	100.0
Starch	0.5%	100.0

[a] Neostigmine taken: 2.0 × 10^{-6} *M* as neostigmine bromide.

RESULTS AND DISCUSSION

Absorption Spectra—Figure 1 shows the visible absorption spectra of neostigmine or diphenhydramine extracts with tetrabromophenolphthalein ethyl ester. The absorbance maximum of the extracts was at 615 nm. for neostigmine and 594 nm. for diphenhydramine. The same absorption spectra as for neostigmine were obtained for sparteine, benzethonium, or tetraethylammonium extracts. The blue or red-violet color in the organic layer may be attributed to the effect of ion-pair formation or association between those substances and tetrabromophenolphthalein ethyl ester.

Effect of pH—The effect of pH on the extraction was studied by extracting the neostigmine from a series of aqueous solutions buffered at various pH values. As shown in Fig. 2, the absorbance of the extract was constant when the pH of the aqueous phase lay within the range 9.5–11.0.

An extreme pH dependence was observed for the determination of quinine (5) or thiamine (4) with bromphenol blue or bromthymol blue. Similarly, the constant absorbance in any pH range could not be obtained when bromphenol blue or bromcresol green was

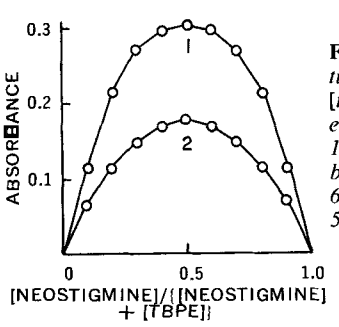

Figure 4—*Continuous variation curves. [Neostigmine] + [tetrabromophenolphthalein ethyl ester (TBPE)] = 8.0 × 10^{-6} M. Reference = reagent blank. Key: 1, measured at 615 nm.; and 2, measured at 590 nm.*

Table II—Analysis of Practical Samples

Analysis for	Sample	Titrimetric Method	Proposed Method
Neostigmine	Injection	0.367 mg./ml.	0.368 mg./ml.
	Tablet	3.69 mg./g.	3.66 mg./g.
	Powder	3.65 mg./g.	3.66 mg./g.
Benzethonium	Disinfectant solution	0.218 *M*	0.220 *M*
Sparteine	Injection	27.7 mg./ml.	27.6 mg./ml.
Tetraethyl-ammonium	Injection	61.6 mg./ml.	61.3 mg./ml.
Diphenhydramine	Ointment	9.90 mg./g.	9.87 mg./g.

used as the extractant for neostigmine. The extreme dependence of pH may be attributed to the hydrophilic radical such as sulfone or carboxylic acid in the dye molecule. The hydrophilic radicals may cause the extraction with the dye to be difficult.

Effect of Reagent Concentration—The influence of tetrabromophenolphthalein ethyl ester concentration on the extraction is illustrated in Fig. 3. It is apparent that the concentration of tetrabromophenolphthalein ethyl ester should be maintained at more than 10-fold molar excess over neostigmine to obtain a constant extraction.

Excess amounts of borate–phosphate buffer solution had no influence on the absorbance of the extract. When the addition of the buffer solution was less than 2 ml. in the aqueous layer, a good separation of the two layers was not observed.

Solvent for Extraction—The behavior of various solvents in the extraction was studied. The solvents could be classified into three categories: (*a*) those with which the presence of neostigmine led to a considerable increase in the extraction of blue tetrabromophenolphthalein ethyl ester, *e.g.*, 1,2-dichloroethane and chloroform; (*b*) those which do not extract the blue dye even in the presence of neostigmine, *e.g.*, carbon tetrachloride, cyclohexane, *n*-hexane, monochlorobenzene, and toluene; and (*c*) those with which the blue dye is extractable even without neostigmine, *e.g.*, butyl acetate, ether, ethyl acetate, isoamyl alcohol, nitrobenzene, nitromethane, and methyl isobutyl ketone.

1,2-Dichloroethane was found to be most suitable for the extraction of a neostigmine–tetrabromophenolphthalein ethyl ester system.

Other Variables—Full color development took about 1 min. of shaking. Continued shaking up to 5 min. produced no further change in absorbance. The color intensity of dichloroethane extracts remained constant for 1 hr. Normal room temperature fluctuations (16–25°) were without measurable effect in absorbance.

Calibration and Precision—The system followed Beer's law up to 4.0×10^{-6} *M* of neostigmine in aqueous layer, with a molar absorptivity of 2.25×10^{5} moles^{-1} cm.$^{-1}$ l. at 615 nm.

The reproducibility of the proposed method was estimated from the results of 10 sample solutions, each with a final neostigmine concentration of 2.0×10^{-6} *M*. The mean absorbance was 0.450, with a standard deviation of 0.005 absorbance unit.

Extraction Rate and Composition of Colored Species—A sample (25 ml.) containing 4.0×10^{-6} *M* of neostigmine was extracted with 10 ml. of dichloroethane at pH 10.0. Then the amounts of neostigmine in the aqueous layer and organic layer were determined according to the proposed method. The extraction rate was about 2% in the absence of tetrabromophenolphthalein ethyl ester and 95% in its presence.

Figure 4 shows the continuous variation curve for neostigmine. It may be suggested that a 1:1 ion-pair compound is formed in the dichloroethane phase between tetrabromophenolphthalein ethy ester and neostigmine.

Effect of Foreign Substances—Table I shows the effect of foreign substances on the determination of neostigmine. Chloride, glucose, lactose, bromide, and starch, which are apt to exist in pharmaceutical preparations with neostigmine, do not interfere.

Analysis of Practical Samples—Commercial samples as medicine or disinfectant obtained from a drugstore were analyzed according to the proposed method and titrimetric method (6–10).

Injection and Disinfectant Solution—Dilute a sample with water and treat the solution in the same manner as the recommended procedure.

Powder and Tablet—Dissolve a sample in dilute sulfuric acid. Filter the solution with a glass filter and dilute the filtrate with water. Treat the solution in the same manner as the recommended procedure.

Ointment—Place a sample in a separator and add 15 ml. of ether. Extract the contents three times with 30-ml. portions of 0.03 *N* sulfuric acid. Combine the extracts and dilute with water. Treat the solution in the same manner as the recommended procedure.

The results obtained are summarized in Table II.

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Transfer of Clindamycin and 1′-Demethyl-4′-depropyl-4′-pentylclindamycin by the Cannulated-Everted Rat Gut

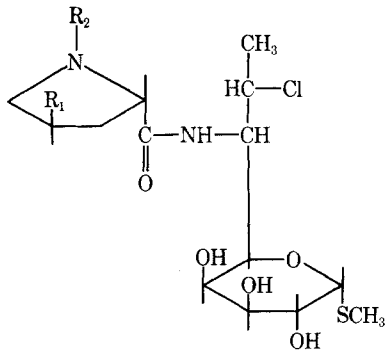
MILDRED J. TARASZKA

Abstract □ A cannulated-everted rat gut technique was used to measure the rate of clindamycin transfer to the serosal side for various mucosal concentrations of clindamycin. The rate of transfer of 1′-demethyl-4′-depropyl-4′-pentylclindamycin to the serosal side for a given 1′-demethyl-4′-depropyl-4′-pentylclindamycin mucosal concentration can be calculated from the clindamycin data if the difference in pKa between clindamycin and 1′-demethyl-4′-depropyl-4′-pentylclindamycin is taken into account. The predicted rate of transfer for 1′-demethyl-4′-depropyl-4′-pentylclindamycin is approximately one-third of the transfer rate for clindamycin and is in good agreement with the observed rate of transfer. It was assumed that only the unionized species of both compounds were transferred by similar mechanisms. No correction was made for lipid solubility, since the true partition coefficients (ether/water) for the two compounds are similar. GLC was used to assay for both compounds as their trifluoroacetate derivatives.

Keyphrases □ Clindamycin, transfer—in cannulated-everted rat gut □ 1′-Demethyl-4′-depropyl-4′-pentylclindamycin, transfer—in cannulated-everted rat gut □ Transfer rates—clindamycin, 1′-demethyl-4′-depropyl-4′-pentylclindamycin, in cannulated-everted rat gut □ GLC—analysis

It is generally believed that for most compounds only the unionized species is absorbed from the gastrointestinal tract (1–3). It has also been shown that partitioning of drugs between gastric juice and plasma is dependent on their pKa's (3). To determine whether the pH-partition hypothesis could be used predictively in the assessment of rates of transfer of ionizing compounds through biological membranes, two compounds dissimilar in pKa but similar with respect to other physical properties are needed. It is well known that compounds with higher relative lipid solubilities are generally better absorbed. Since the effect of relative lipid solubility on transfer rate has not been quantitated, compounds with similar true partition coefficients[1] are needed to make the assumption that lipid solubility would not have an appreciable effect on the transfer rate. In addition, compounds of similar molecular weight and structure are needed to assure that these compounds would be transferred by similar mechanisms with diffusion constants of similar magnitude.

Two compounds that meet these requirements fairly well are clindamycin[2] (Compound 1) and 1′-demethyl-4′-depropyl-4′-pentylclindamycin (Compound 2). Their free base molecular weights are 427 and 441, respectively. These two compounds also have similar true partition coefficients (ether/water) but different pKa's. As will be shown in this paper, correcting the concentrations of Compound 1 and Compound 2 for the amount unionized, which equals amount unprotonated, allows the prediction of the transfer rate for Compound 2 from that of Compound 1 when the cannulated-everted rat gut technique is used.



	R_1	R_2
clindamycin	C_3H_7	CH_3
1′-demethyl-4′-depropyl-4′-pentylclindamycin	C_5H_{11}	H

EXPERIMENTAL

General—Clindamycin (Compound 1) and 1′-demethyl-4′-depropyl-4′-pentylclindamycin (Compound 2) were prepared in the Upjohn Research Laboratories. Trifluoroacetic anhydride was from Eastman Kodak, white label. Tetrahydrofuran was purified and stored over alumina. All other reagents were analytical grade.

Cannulated-Everted Rat Gut Procedure—Krebs-Ringer phosphate–bicarbonate buffer was prepared according to the literature (4), except the $CaCl_2$ concentration was decreased 50% and the buffer was made 10 m*M* in glucose. The pH of the buffer at 37° in equilibrium with 95% O_2–5% CO_2 was 7.20. After 2 mg. base equivalent of the hydrochloride salt of clindamycin was added per milliliter of buffer, the pH was 7.18.

Sprague-Dawley rats, weighing 180–200 g., were killed by a blow on the head. A segment of intestine starting at the bile duct entrance was stripped from the mesentery, rinsed, and maintained as far as possible in cold, oxygenated, Krebs-Ringer phosphate–bicarbonate buffer. A 10-cm. segment was measured by hanging a 2-g. weight on the distal end from the bile duct. The segment was everted on a polyethylene cannula, with the distal end tied to a groove in the cannula (5). One milliliter of buffer was syringed into the sac, and the sac was placed into 75 ml. of oxygenated buffer at 37° containing the drug to be transferred. This large volume on the mucosal side of the intestine acted as a reservoir of drug, so the effective concentration of drug to be transferred did not change significantly with time. The entire preparation of the intestine was usually completed within 6 min. of the animal's death. Peristaltic action was present throughout the experiment.

After 30 min., serosal fluid was withdrawn by inserting a small diameter polyethylene tube, attached to a syringe, down the lumen of the cannula and aspirating. The serosal side was washed twice with 1.5 ml. of buffer at 37°; the washings, along with the sample, were collected in a centrifuge tube. One milliliter of buffer was then added to the serosal side to obtain a second 30-min. transfer sample.

After the second 30 min. of transfer, the sac was removed from the drug solution and the mucosal side (outside) was rinsed with 6 ml. of buffer. The sac was placed over a centrifuge tube, and a hole was cut

[1] The true partition coefficient equals the concentration of unionized species in the organic phase/the concentration of the unionized species in the aqueous phase.

[2] Cleocin, The Upjohn Co.

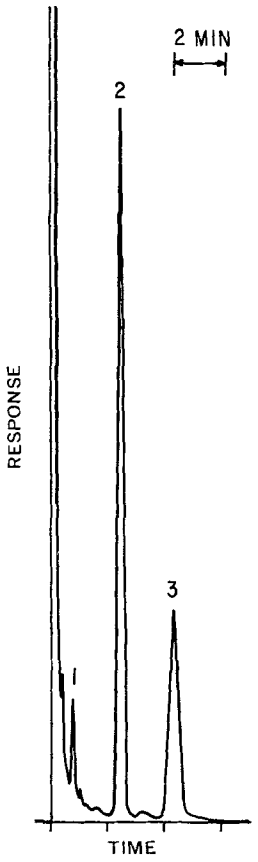


Figure 1—*Typical gas chromatogram for clindamycin. Peak 1 = impurities extracted from buffer and rat intestine. Peak 2 = internal standard: triphenylmethane. Peak 3 = clindamycin trifluoroacetate derivative.*

in the bottom to allow the serosal solution to drain. The serosal side was rinsed with 4 ml. of buffer and collected in the centrifuge tube for assay.

The experimental range of error between rats for the aspirating-sampling technique was ±23% when it was used for the second 30-min. sample, while the error for the cut-rinse-sampling technique was only ±10%. Due to this difference in experimental error, the cut-rinse-sampling technique was used to collect the data for the comparison of transfer of Compounds 1 and 2.

GLC Assay—Trifluoroacetate derivatives of Compounds 1 and 2 were used for GLC because they are readily formed, they are stable in an excess of reagent, they can be chromatographed at lower temperatures than silyl ethers or acetates, and there is no residue build-up on the detector.

For Compound 1, 2 ml. of chloroform containing 0.2 mg. or 0.02 mg. of triphenylmethane as internal standard was added to the buffer containing 0.1–1 mg. or 0.01–0.1 mg. of clindamycin base, respectively. To aid extraction, 0.2 ml. of 0.1 *N* KOH was added. The mixture was shaken vigorously and centrifuged. Most of the chloroform layer was transferred to a 2-ml. volumetric flask and evaporated to dryness under a dry stream of air. The dried residue was dissolved in 0.2 ml. of tetrahydrofuran, and 0.2 ml. of trifluoroacetic anhydride was added. The mixture was allowed to react for 30 min. at room temperature and then was gas chromatographed on a U-shaped glass column 0.61 m. × 1.27 cm. (2 ft. × 0.5 in.) packed with Gas Chrom Q, 60–80 mesh, and coated with 1% OV-17. Oven temperature was 178° and helium gas flow was 40 ml./min. A typical gas chromatogram is given in Fig. 1 and standard curve in Fig. 2. The experimental range due to the assay is ±5%.

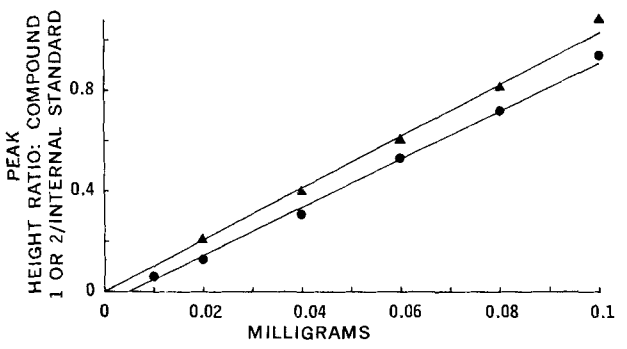


Figure 2—*Standard curve for: ▲, Compound 1; and ●, Compound 2.*

For Compound 2, 4 ml. of chloroform containing 0.06 mg. of tetraphenylethylene as internal standard was added to the buffer containing 0.01–0.1 mg. of Compound 2 as clindamycin base equivalents. After the mixture was shaken vigorously and centrifuged, it was assayed in the same manner as Compound 1, except the mixture was allowed to react for 30 min. at 47°. A typical gas chromatogram is given in Fig. 3 and standard curve for the major component of the *cis–trans*-isomer in Fig. 2. The extracted impurities from buffer and rat intestine cause no interferences in either assay.

RESULTS AND DISCUSSION

Determination of pKa and Partition Coefficient—The Radiometer titrator was used to determine the pKa's of 7.45 ± 0.02 and 7.96 ± 0.04 ($\bar{X} \pm \sigma$) for Compounds 1 and 2, respectively, at 37° in aqueous media.

The true partition coefficient for Compound 1 between diethylether and water is 9.8 at 25° (6). The apparent partition coefficient of Compound 2 between diethylether and water was measured at pH 7.70, 25°, and the true partition coefficient was calculated (7) to be 7.2. These partition coefficients for Compound 1, 9.8, and Compound 2, 7.2, are sufficiently similar and their pKa's sufficiently dissimilar to test the transfer hypothesis.

Cannulated-Everted Rat Gut Transfer of Compound 1—All weights for Compounds 1 and 2 are in terms of clindamycin base equivalents.

The transfer rate of Compound 1 was measured for glucose concentrations of 0, 10, and 20 m*M*. The transfer rate of Compound 1 in the absence of glucose was approximately twice the rate when glucose was present. With no glucose, peristaltic action was not observed and large sections of villi started to slough off during the first 30-min. interval, indicating this increase in transfer rate may be due

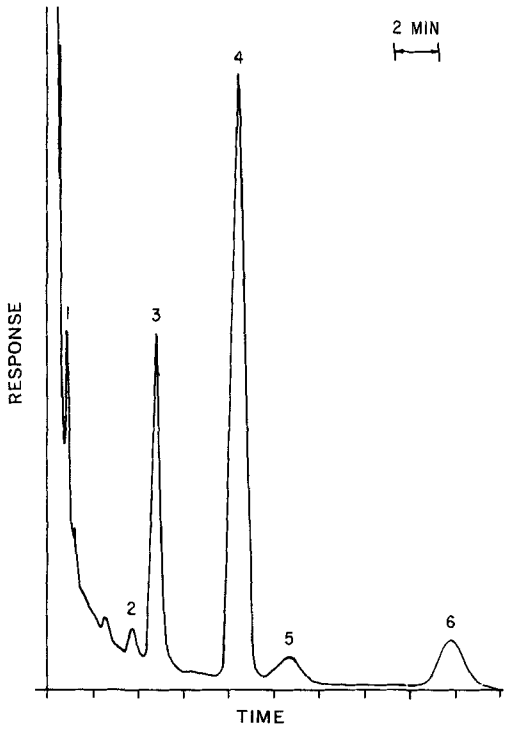


Figure 3—*A typical gas chromatogram for Compound 2. Peaks 1, 5, and 6 = impurities extracted from buffer and rat intestine. Peak 2 = minor component of* cis–trans *mixture of Compound 2. Peak 3 = major component of* cis–trans *mixture of Compound 2. Peak 4 = internal standard: tetraphenylethylene.*

Table I—Milligrams of Clindamycin Transferred for Three Consecutive 30-min. Intervals[a]

Rat	First 30 min.	Second 30 min.	Third 30 min.
	Milligrams		
1	0.035	0.167	0.330
2	0.054	0.192	0.238
3	0.058	0.245	0.257
4	0.040	0.271	0.244

[a] Samples obtained by aspirating technique.

to intestine degeneration. Since the intestine rapidly degenerates without glucose and 10 or 20 m*M* glucose gave equivalent transfer rates, the experiments were run using 10 m*M* glucose.

The amount of clindamycin base transferred in the second 30-min. interval was the same, within experimental error (range ±10%), for the first seven successive 10-cm. segments of intestine proximal to the bile duct. Thus, the first 10-cm. segment proximal to the bile duct from different rats was used in the rest of the experiments.

When the mucosal bathing solution contained 1 mg. clindamycin base/ml. of buffer, 0.041 ± 0.01 and 0.189 ± 0.02 ($\bar{X} \pm \sigma$) mg. of clindamycin base were transferred to the serosal side during the first (sampled by aspirating) and second (sampled by cutting and rinsing), 30-min. intervals, respectively. This difference in amount transferred is related to the lag time required to establish a steady state. To ensure that a steady state had been established by the second 30-min. interval, the aspirating-sampling technique was used to measure three 30-min. samples on the same intestinal segment. The results for four different rats are given in Table I. In all cases but one, the third 30-min. sample was within the experimental range (±23%) of the second 30-min. sample. This indicates that a steady state had been established for the second 30-min. sample. Since by the end of 1.5 hr., small particles of tissue were starting to appear in the bathing solution, the transfer rates for the second 30-min. interval were used in all calculations.

The amount transferred per second 30-min. interval curve for various mucosal concentrations of Compound 1 is linear within experimental error, as shown in Fig. 4. Each point is an average of at least four values, and the bars indicate the experimental range ±10% by the cut-rinse-sampling technique.

Predicted and Measured Transfer Rates for Compound 2—Due to its poor aqueous solubility, only 0.875 mg. of Compound 2 as clindamycin base per milliliter of buffer was used for the mucosal solution. At this experimental concentration, 0.875 mg./ml., only 0.057 ± 0.007 ($\bar{X} \pm \sigma$) mg. of Compound 2 as clindamycin base was transferred to the serosal side during the second 30-min. interval. Data indicate steady state had been reached.

To calculate the amount of Compound 2 that is expected to be transferred using the Compound 1 curve, the following equation is used:

$$\text{pKa} = \text{pH} - \log \frac{A}{HA_T - A} \qquad \text{(Eq. 1)}$$

where A = amount of unionized compound, and ($HA_T - A$) = amount of ionized compound or the total amount of compound added, HA_T, minus A.

The equations:

$$HA_T = 2.79A_{\text{Compound 1}} \qquad \text{(Eq. 2)}$$

$$HA_T = 6.75A_{\text{Compound 2}} \qquad \text{(Eq. 3)}$$

for Compounds 1 and 2, respectively, are obtained by substituting the pKa's and pH of the buffer at 37° into Eq. 1. When the total concentration bathing the mucosal side is the same for both compounds, Eqs. 2 and 3 are combined to give:

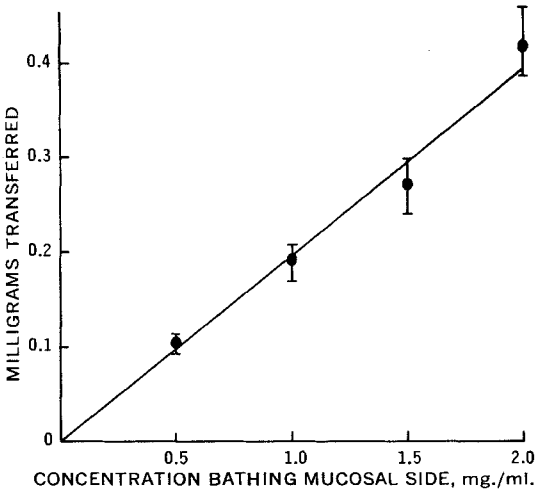


Figure 4—*Amount of clindamycin base transferred during the second 30-min. interval* versus *its mucosal concentration.*

$$A_{\text{Compound 2}} = 0.413A_{\text{Compound 1}} \qquad \text{(Eq. 4)}$$

or the effective concentration of Compound 2 is approximately four-tenths that of Compound 1. The effective Compound 2 concentration for 0.875 mg./ml. at pH 7.2 was calculated with Eq. 4 as 0.36 mg./ml. Using this effective concentration of 0.36 mg./ml. with Fig. 4, it was predicted that 0.070 ± 0.007 mg. of Compound 2 as clindamycin base should be transferred in the second 30-min. interval when 0.875 mg./ml. of Compound 2 was in the mucosal solution. The experimentally observed value of 0.057 ± 0.007 is in good agreement with this predicted value of 0.070 ± 0.007.

The experimentally observed value differs from the predicted value in the direction that is expected if the relative lipid solubilities are considered, and it appears that the *in vitro* partitioning between ether and water simulates the biophase partitioning. But due to the experimental error (±12%) involved, further corrections of the predicted value are unwarranted. If no correction was made for pKa, the predicted value for the amount to be transferred was 0.172 mg. in the second 30-min. interval, which is much higher than the observed values. Thus, it appears that transfer rates can be predicted for compounds differing in pKa if the unionized concentration is quantitated and certain other criteria, such as similar molecular weight, structure, and lipid solubility, are met.

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Microdegradation Procedure for Radioactive Peptide Ergot Alkaloids

G. BASMADJIAN and H. G. FLOSS

Abstract □ A degradation procedure for ^{14}C-labeled peptide ergot alkaloids is described; it allows the determination of the specific activities of all the products obtained in the hydrolysis of these alkaloids from a sample of not more than 10 μmoles.

Keyphrases □ Ergot alkaloids, radioactive peptides—biosynthesis, degradation, isotope distribution □ Peptide ergot alkaloids, radiolabeled—degradation, distribution □ TLC—separation, analysis

In the course of biosynthetic studies on ergot alkaloids, the need arose for a procedure that would permit the determination of the radioactivity distribution within the various portions of the molecules of ^{14}C-labeled peptide-type ergot alkaloids. To be useful, the degradation procedure had to meet the following criteria:

1. It should allow the determination of the specific radioactivity of all hydrolytic fragments of peptide ergot alkaloids.
2. The specific radioactivity of all the fragments should be determined directly, rather than by difference, in order to establish a radioactivity balance.
3. The degradation should be universally applicable to all peptide ergot alkaloids and even to simple mixtures of alkaloids like ergotoxine.
4. It should be possible to carry out the entire degradation starting with a sample of not more than 10 μmoles of radioactive alkaloid, since this is usually the amount available after extensive purification of the alkaloids from one experiment and since nonlabeled carrier material is often difficult to obtain in quantity.

The degradations used in previous biosynthetic studies on peptide ergot alkaloids (1–5) required rather large samples of alkaloid and/or were not complete since only some fragments of the molecule were isolated. This article describes a degradation procedure which meets essentially all the above criteria and which has been successfully employed in studies on the biosynthesis of ergotamine and ergotoxine (6). This degradation is applicable to all peptide ergot alkaloids containing the cyclol structure, it can be carried out with samples of 10 μmoles, and it allows the direct determination of the specific radioactivity of all hydrolytic fragments of these alkaloids.

EXPERIMENTAL

Pure samples of the alkaloids ergotamine, ergocornine, and ergocryptine were used[1]. Radioactive alkaloids, degraded, were samples obtained from feeding experiments[2] which had been purified to constant specific radioactivity. D,L-Valine-1-^{14}C was purchased[3]. Radioactivity determinations were carried out in a Beckman LS 100 liquid scintillation spectrometer, using 10 ml. of a toluene scintillator solution (7 g. 2,5-diphenyloxazole and 0.3 g. dimethyl-1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene/1000 ml. toluene) for compounds dissolved in organic solvents or 10 ml. of Bray's solution (7) for aqueous samples.

The counting efficiency for each sample was determined by recounting the sample after the addition of a known amount of toluene-^{14}C as internal standard. Colorimetric determinations were carried out in a Zeiss PMQ II spectrophotometer using cells with a 1-cm. lightpath. Standard curves were determined for all the compounds to be analyzed using analytically pure, crystalline materials; from these, factors were obtained for the conversion of extinction readings into concentrations. Ergonovine maleate[4] was used to standardize the quantitative determination of ergot alkaloids and lysergic acid by reaction with *p*-dimethylaminobenzaldehyde (8, 9). The reagent used (van Urk's reagent) consists of 200 mg. *p*-dimethylaminobenzaldehyde and 0.15 ml. of 10% $FeCl_3$ solution in 100 ml. of 65% (v/v) sulfuric acid. TLC was carried out on 0.25-mm. thick Merck precoated silica gel plates, unless stated otherwise.

Degradation of Alkaloids—*Alkaline Hydrolysis*—Ten micromoles of the alkaloid, containing between 5×10^3 and 10^5 d.p.m. of ^{14}C, is placed in a 10-ml. round-bottom flask equipped with a gas inlet and a reflux condenser which can be closed by a stopcock. The alkaloid is dissolved in 0.5 ml. of methanol (ergotamine) or chloroform (ergocornine, ergocryptine, ergotaminine, and ergotoxine), and 2 ml. of 8% aqueous KOH is added. The system is flushed with nitrogen and heated to 100° in an oil bath. After 10 min., the stream of nitrogen is stopped, the system is closed, and refluxing is continued for a total of 1 hr. The flask is then cooled and its contents acidified to pH 1–2 with concentrated HCl.

Isolation and Quantitation of Keto Acid—The hydrolysate is transferred to a 15-ml. conical centrifuge tube containing 1 ml. of a freshly prepared solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl (5 mg./ml.). The mixture is kept at room temperature for 20 min. with occasional shaking. Three milliliters of ethyl acetate is then placed on top of the aqueous solution, and the mixture is shaken on a vibromixer. The organic phase is removed with a Pasteur pipet and transferred to a 125-ml. separator. This extraction is repeated until the ethyl acetate layer is almost colorless. The combined extracts are washed with 3×3 ml. of water, the washings are combined with the original aqueous phase and freeze-dried in a 25-ml. round-bottom flask, and the residue is saved for further processing. Petroleum ether (b.p. 30–60°) is added to the organic phase to beginning cloudiness, and the solution is extracted with 5×10 ml. of a carbonate solution (5.3 g. Na_2CO_3 and 0.84 g. $NaHCO_3$ in 1000 ml. of distilled water).

The extract is washed with 3×5 ml. of ethyl acetate–petroleum ether (1:1), which is discarded, then acidified to pH 1–2 with concentrated HCl, and extracted with ethyl acetate until the organic layer is colorless. The ethyl acetate extract is washed with 3×5 ml. of water, dried over anhydrous Na_2SO_4, and taken to dryness in a rotary evaporator. The residue is dissolved in 3 ml. of ethanol, filtered through a coarse sintered-glass funnel into a centrifuge tube, and concentrated to 1 ml. in a stream of nitrogen; 2 *N* HCl is added until cloudiness results. The mixture is left in the refrigerator overnight. After centrifugation, the supernatant is removed with a Pasteur pipet; the yellow-orange crystals are washed with 2×1 ml. of distilled water and dried *in vacuo* at 50° over P_2O_5. TLC at this point [chloroform–benzyl alcohol–acetic acid (70:30:3)] always showed a single spot in the case of the dimethylpyruvate 2,4-dinitrophenylhydrazone and two spots (*cis*- and *trans*-isomers) for the derivative of pyruvate.

The crystalline dinitrophenylhydrazone is dissolved in 2 ml. of

[1] Provided by Farmitalia S.A. through the courtesy of Dr. C. Spalla.
[2] Carried out by Dr. D. Gröger, Institute for Biochemisty of Plants, Halle, Germany, or by Dr. C. Spalla, Institute for Basic Research, Farmitalia S.A., Milan, Italy.
[3] Volk Radiochemical Co.
[4] Provided by Eli Lilly and Co. through the courtesy of Dr. Gordon Svoboda.

Table I—Degradation of Two Samples of Ergotoxine (Mixture of Ergocornine and Ergocryptine from Identical, Duplicate Biosynthetic Experiments)

Degradation Number	Calculated Specific Activity of Alkaloid, d.p.m./μmole	Dimethylpyruvate	Valine	Leucine	Proline	Lysergic Acid
		d.p.m./μmole = % of Total				
1	Ergocornine 3920	58% 2280	37% 1450	— 54	3% 130	1.5% 60
	Ergocryptine 2520	90%	—	2%	5%	3%
2	Ergocornine 456	54% 247	40% 184	— 16	4% 18.5	1% 6.5
	Ergocryptine 288	86%	—	6%	6%	2%

ethyl acetate, and an aliquot of 0.5 or 1.0 ml. is counted for its radioactivity. Another aliquot, 20–50 μl. depending on the quantity, is evaporated to dryness and used for the quantitation. In the case of pyruvate dinitrophenylhydrazone, the residue is dissolved in 5 ml. of the carbonate solution and the extinction is measured at 355 nm. against a blank of carbonate solution. For dimethylpyruvate dinitrophenylhydrazone, 2 ml. of carbonate solution and 2 ml. of 2 *N* NaOH are added to the residue, and the extinction at 430 nm. is measured within 10 min. The micromoles of pyruvate or dimethylpyruvate derivative in the aliquot taken are calculated as $0.269 \times E_{355}$ or $0.252 \times E_{430}$, respectively. This figure is then used in calculating the micromoles of substance in the aliquot counted to obtain the specific radioactivity in disintegrations per minute per micromole. The remainder of the ethyl acetate solution of the dinitrophenylhydrazone is evaporated to dryness, and the residue is recrystallized from benzene–petroleum ether. The crystals are washed with 2 × 1 ml. of petroleum ether, dried, and used for another specific activity determination as described previously. The figures obtained in these two determinations in this study usually agreed within ±5%.

Formation of Dinitrophenyl Dipeptide—The freeze-dried residue from the aqueous solution obtained after the extraction of the keto acid dinitrophenylhydrazone is dissolved in 5 ml. of water; 100 mg. of $NaHCO_3$ is added, followed by a freshly prepared solution of 50 mg. of fluorodinitrobenzene in 8 ml. of ethanol. The clear solution is kept at room temperature with frequent shaking for 3 hr. in the dark, during which time its color changes from pale yellow to deep orange. All subsequent steps are performed under subdued light. The solution is evaporated to dryness in a rotory evaporator below 40°. Five milliliters of water and 50 mg. of Na_2CO_3 are added to the residue, the solution is extracted with ether until the ether layer is colorless, and the extracts are discarded. The solution is then acidified to pH 2 with concentrated HCl and again extracted with ether until the organic phase is colorless. The latter extracts are combined, dried over Na_2SO_4, and evaporated *in vacuo*. The extracted aqueous solution is freeze dried, and the residue is kept for the isolation of lysergic acid.

Isolation and Quantitation of Dinitrophenyl Amino Acids—The residue of the ether phase is dissolved in 0.5 ml. of methanol, 3 ml. of concentrated HCl is added, and the mixture is refluxed at 110° for 5 hr. After cooling, 3 ml. of water is added and the solution is extracted with ether until the ether layer is colorless. The extract is dried over Na_2SO_4 and evaporated to dryness to give a crude dinitrophenyl amino acid, *e.g.*, dinitrophenylphenylalanine from ergotamine, or a mixture of dinitrophenyl amino acids if an alkaloid mixture is degraded, *e.g.*, dinitrophenylleucine and dinitrophenylvaline from ergotoxine. The aqueous solution, which contains free proline, is freeze dried and the proline is dinitrophenylated exactly as described for the dipeptide. The crude dinitrophenylproline is obtained as described after extraction with ether. The crude dinitrophenyl amino acids are purified by TLC on 0.5-mm. thick silica gel G plates using chloroform–benzyl alcohol–acetic acid (70:30:3) as the solvent, which is allowed to travel 20 cm. (R_f values of dinitrophenyl derivatives: proline, 0.37; phenylalanine, 0.51; valine, 0.59; and leucine, 0.66).

For a better resolution of dinitrophenylvaline and dinitrophenylleucine obtained in the degradation of the alkaloid mixture ergotoxine, the solvent benzene–pyridine–acetic acid (80:20:2) is used and the plate is usually dried for 1 hr. at room temperature in the dark and developed a second time in the same system. The bands of the individual dinitrophenyl amino acids are scraped off into centrifuge tubes, 3 ml. of a 1% $NaHCO_3$ solution is added, the mixture is shaken well, and 3 ml. of ether is placed on top of it. Then 2–3 drops of concentrated HCl is added, and the contents of the tube are mixed on a vibromixer. After separation of the phases, the ether is removed with a Pasteur pipet and the extraction is repeated until a colorless ether layer results. The combined ether extracts are dried with Na_2SO_4 and evaporated to dryness in a stream of nitrogen. The residue is crystallized from methanol–water and the crystalline product is dried *in vacuo* over P_2O_5.

At this stage, the material is homogeneous as judged by TLC in the two systems mentioned previously. For the determination of the specific radioactivity, the material is dissolved in 2 ml. of ethanol, one aliquot is used for counting and another aliquot (20 μl.) for colorimetry. The latter aliquot is evaporated, the residue is dissolved in 5 ml. of a 1% $NaHCO_3$ solution, and the extinction is measured at 385 nm. (dinitrophenylproline) or 360 nm. (dinitrophenylvaline, dinitrophenylleucine, and dinitrophenylphenylalanine) against a blank of 1% $NaHCO_3$ solution. The factors for the conversion of E_{385} or E_{360} into micromoles of dinitrophenyl amino acid in the aliquot analyzed are 0.280 (dinitrophenylproline), 0.302 (dinitrophenylphenylalanine), 0.327 (dinitrophenylleucine), and 0.331 (dinitrophenylvaline). The remainder of the ethanol solution of the dinitrophenyl amino acid is taken to dryness, the residue is recrystallized from ether–ligroin, and the determination of the specific radioactivity is repeated.

Isolation and Quantitation of Lysergic/Isolysergic Acid—The freeze-dried residue of the aqueous phase obtained after the extraction of the dinitrophenyl amino acids is dissolved in 1 ml. of water and quantitatively streaked on a 46- × 57-cm. water-washed sheet of Whatman 3MM chromatography paper. The chromatogram is developed descendingly with distilled water as the solvent for 4–5 hr. (R_f values: lysergic acid, 0.30; and isolysergic acid, 0.40; varying somewhat depending on the amount of salts present). The bands of lysergic and isolysergic acid are marked quickly under UV light, cut out, and eluted together with distilled water. The eluate is evaporated to dryness *in vacuo* below 50°, and the residue is dissolved in 2 ml. of water. One aliquot is used for counting and another aliquot (usually 50 μl.) for colorimetry. To the latter, 1 ml. of a 2% succinic acid solution and 2 ml. of van Urk's reagent are added, and the extinction at 580 nm. is read after 10–20 min. against a blank of 1 ml. of 2% succinic acid and 2 ml. of van Urk's reagent. The micromoles of lysergic and isolysergic acids in the aliquot analyzed are calculated as $0.195 \times E_{580}$.

Check on Consistency of Specific Radioactivity Determinations—A sample of D,L-valine-^{14}C of an approximate specific radioactivity of 10 μc./mmole was prepared by appropriate dilution of D,L-valine-1-^{14}C (20 mc./mmole) with nonlabeled carrier material. Ten micromoles of this sample was dinitrophenylated as outlined previously, and the specific radioactivity of the dinitrophenylvaline was determined by the method used in the degradation. The average of two determinations was 1.83×10^4 d.p.m./μmole. Thirty micromoles of the same sample was dissolved in 1 ml. of 0.1 *M* phosphate buffer (pH 7.0); 10 mg. of catalase and 5 mg. of L-amino acid oxidase

were added, as well as a drop of *n*-octanol as antifoam; and oxygen was passed through the solution at 37° for 3 hr. One drop of concentrated HCl and 1 ml. of dinitrophenylhydrazine solution were then added, and the dimethylpyruvate dinitrophenylhydrazone was isolated and purified as described previously. Determination of its specific radioactivity by the method used in the degradation gave 1.76×10^4 d.p.m./μmole as the average of two determinations.

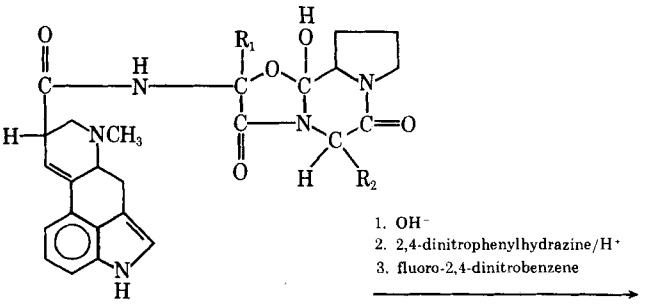
RESULTS AND DISCUSSION

Several degradations have been used in the structure elucidation of the peptide ergot alkaloids (*cf.*, 10), but some of them, *e.g.*, the cleavage with hydrazine (11), the reductive cleavage with $LiAlH_4$ (12), or the pyrolytic cleavage (12), *a priori* seemed not very suitable for this study. The requirement that it should be possible to carry out the entire degradation on a sample of not more than 10 μmoles severely limits the choice of procedures, because it requires reasonably good yields for all the products and it excludes weighing as the method for quantitating the compounds in the determination of the specific radioactivity. Rather, all the products have to be isolated in the form of a colored derivative suitable for colorimetry or have to be quantitated by a suitable color reaction.

After some trials with an acid hydrolysis method using a cation-exchange resin (13), this procedure was abandoned because of poor yields in favor of the milder alkaline hydrolysis (14). The latter makes use of the fact that the cyclol structure incorporating an α-hydroxy amino acid is much more sensitive to base hydrolysis than normal peptide bonds. Thus, hydrolysis of normal peptide ergot alkaloids like ergotamine and ergocornine with 8% KOH for 1 hr. at reflux temperature produces lysergic and isolysergic acid, an α-keto acid from the α-hydroxy amino acid, and a dipeptide containing proline at the carboxy terminal end. The latter can be further hydrolyzed with acid to give the two constituent amino acids. This procedure was adopted as the general degradation scheme (Scheme I), and the various products were isolated in the form of suitable derivatives. Treatment of the reaction mixture obtained in the alkaline hydrolysis with acidic 2,4-dinitrophenylhydrazine solution converts the keto acid into its dinitrophenylhydrazone. Control experiments showed that under these conditions no significant decomposition of the lysergic acid takes place. The keto acid dinitrophenylhydrazone can be extracted into ethyl acetate and is purified and quantitated essentially by the method of Katsuki *et al.* (15) and Kawano *et al.* (16), which consists of measuring the extinction at 355 nm. (pyruvate dinitrophenylhydrazone) or 430 nm. (dimethylpyruvate dinitrophenylhydrazone) in alkaline solution. The relation between extinction and concentration is linear over a range of 1–13 mcg./ml., and concentrations are usually adjusted to obtain extinctions between 0.3 and 0.4.

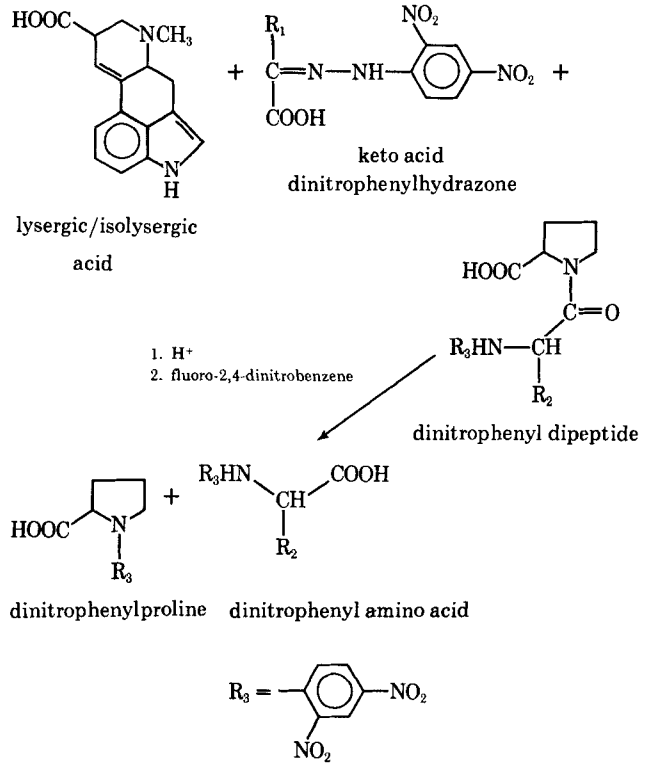
Separation of the dipeptide from the lysergic acid mixture is achieved by dinitrophenylation of the free amino group of the dipeptide, followed by extraction of the resulting dinitrophenyl dipeptide into ether at an acidic pH. From the aqueous phase remaining behind, lysergic and isolysergic acid are isolated by preparative paper chromatography. Their quantity is determined by the color reaction with van Urk's reagent, which is widely used for the quantitative determination of ergot alkaloids (8, 9). The dinitrophenyl dipeptide is further subjected to acid hydrolysis, which cleaves the peptide bond without affecting the dinitrophenyl group, giving rise to free proline and a dinitrophenyl amino acid. The two are separated by ether extraction of the latter and, subsequently, the proline is also converted into its dinitrophenyl derivative. All the dinitrophenyl amino acids are purified by preparative TLC followed by recrystallization and are quantitated colorimetrically as described by Lucas *et al.* (17). If a mixture of alkaloids is degraded, a mixture of dinitrophenyl amino acids is obtained in the acid hydrolysis, which is resolved in the TLC purification step, and the individual amino acid derivatives are then determined separately.

The recovery of the various degradation products is fairly good. The yields of the keto acid derivatives are 60–85%, lysergic/isolysergic acid 30–50%, and the dinitrophenyl amino acids 50–90%. The specific radioactivities of the products are determined by counting aliquots of the same solutions that are used for the colorimetry. No problems are encountered with the color quenching of these compounds in samples of up to 5 μmoles, and counting efficiencies between 45 and 70% are usually obtained. The radioactivity balance is very good. Thus, in four complete degradations of ergotamine-^{14}C



	R_1	R_2
ergotamine	$-CH_3$	$-CH_2-C_6H_5$
ergocornine	$-CH(CH_3)_2$	$-CH(CH_3)_2$
ergocryptine	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$

ergotoxine = mixture of ergocornine and ergocryptine



Scheme I—Degradation of peptide ergot alkaloids

from biosynthetic experiments, the sums of the specific radioactivities of the products amounted to 103, 98, 100, and 82%, respectively, of the measured specific radioactivity of the alkaloid. The low figure in the last degradation seems to be due to an impurity in the alkaloid sample degraded. These data indicate that the specific radioactivity determinations by the method employed are quite accurate.

As an additional check on this point, a control experiment was carried out. Of a sample of valine-1-^{14}C, one aliquot was converted to the dinitrophenyl derivative and its specific activity was determined by the method used in the degradation. Another aliquot was oxidized to dimethylpyruvate with L-amino acid oxidase, and its specific radioactivity was determined by colorimetry of the dimethylpyruvate dinitrophenylhydrazone. The figures obtained by the two determinations agreed within 4%, showing that the methods used for the specific activity determination of valine and dimethylpyruvate give values consistent with each other.

The reproducibility of the degradation procedure is documented by the data given in Table I. This table shows the good agreement between the results of degradations carried out on two samples of

ergotoxine, a mixture of the alkaloids ergocornine and ergocryptine, obtained from different biosynthetic experiments with the same labeled precursor, which were done under as near identical conditions as possible. In the degradation of such alkaloid mixtures, a radioactivity balance usually cannot be established. The percentage distribution of radioactivity can, however, be calculated if the identical moieties in the alkaloids have the same specific radioactivities.

The procedure described here has been used routinely for the degradation of labeled ergotamine and ergotoxine samples from biosynthetic experiments. It has been found to be reliable and easy to carry out. It requires only small quantities of material, and the amount of time needed is reasonable. One complete degradation requires about 3–4 days, but the working time per sample can be reduced considerably by degrading several samples simultaneously. Although this has not been done, the degradation procedure can undoubtedly be applied to other cyclol-type peptide ergot alkaloids.

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COMMUNICATIONS

Evaluation of Cryogenine in Rat Sympathetic Ganglia

Keyphrases ☐ Cryogenine—ganglionic activity, rats ☐ Ganglionic activity—cryogenine effect, rats

Sir:

Previous reports indicated that the various pharmacologic actions ascribed to the plant extracts of *Heimia salicifolia* Link and Otto essentially are elicited by its major alkaloidal constituent, cryogenine (1–3). Recent studies demonstrated that orally administered cryogenine is equipotent to phenylbutazone in both acute and chronic models of inflammation at doses appearing to be unrelated to any systemic toxic manifestation (3). In extensive gross observational studies by Robichaud *et al.* (1), the production of mydriasis, mucosal blanching, and pilomotor erection as well as decreased motor activity were noted in rats in response to intraperitoneal administration of cryogenine.

In addition to producing mild hypothermia at relatively low, nonataxic dosage levels, cryogenine has been shown to suppress conditioned behavior responses selectively in both discrete and continuous avoidance–escape situations (4). Moreover, cardiovascular studies in the anesthetized intact dog and cat have demonstrated a significant blockade of the pressor response to exogenous epinephrine, with doses of cryogenine having no apparent effect on resting blood pressure (2, 5). Kinetic experiments on various isolated tissue preparations have demonstrated, however, an apparent lack of specificity for the commonly employed cholinergic and adrenergic receptor systems (3, 5, 6). In view of the foregoing, it was of interest to determine whether the anti-inflammatory properties attributed to cryogenine might be mediated through alterations in ganglionic activity.

Young Charles River Wistar rats of either sex were anesthetized with a mixture[1] of allobarbital (100 mg./kg.), urethan (400 mg./kg.), and monoethylurea (400 mg./kg.) administered intraperitoneally. The surgical and recording procedures employed in this superior cervical ganglion preparation were described in detail by Hancock and his coworkers (7, 8). Unless indicated otherwise, the sympathetic trunk was stimulated at a rate of 0.3 Hz. (0.2 msec.). Ganglionic potentials evoked by drugs or preganglionic stimulation were recorded from the surface of the ganglion by means of silver–silver chloride bipolar electrodes. All drugs were administered by close intraarterial injection through a 27-gauge needle inserted into the common carotid artery. The constant volume of injection was 0.05 ml., and all drugs were dissolved in a solution of 0.9% NaCl. With the exception of cryogenine (used as the acetate),

[1] Dial with Urethane, Ciba.

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all dosages in the following text refer to the salt form of the drugs. Clotting in the needle was prevented by the prior administration of heparin (400 units/kg. intraarterially).

Cryogenine was administered intraarterially during continuous preganglionic stimulation at frequencies ranging from 0.1–1.0 Hz. Following injection of the drug, ganglionic transmission was monitored continuously for 2 hr. Cryogenine (50–750 mcg.) failed to produce any detectable changes in either spike amplitude or afterpotential contour evoked by supramaximal or submaximal preganglionic stimulation. The submaximal stimulation was determined by selecting the voltage that would produce a ganglionic spike one-half the amplitude of that obtained with supramaximal preganglionic stimulation. It was determined that cryogenine (50–750 mcg.) did not evoke ganglionic firing. Moreover, no changes in the resting demarcation potential were observed as the result of cryogenine administration. Similarly, there was no evidence of asynchronous postganglionic discharge evoked by cryogenine (750 mcg.) in ganglia conditioned by repetitive supramaximal preganglionic stimulation (30 Hz. for 30 sec.) or in ganglia pretreated with *d,l*-isoproterenol HCl (2 mcg.).

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The postganglionic firing evoked by the intraarterial administration of tetramethylammonium Cl (5–10 mcg.) was not modified in ganglia pretreated with cryogenine 15 sec. earlier. Furthermore, the biphasic ganglionic demarcation potential produced by tetramethylammonium Cl (10 mcg.) and 1,1-dimethyl-4-phenylpiperazinium iodide (5 mcg.) was unaltered by the previous administration of cryogenine (750 mcg.). The postganglionic firing produced in response to physostigmine salicylate (200 mcg.), oxotremorine picrolonate (75 mcg.), and 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium Cl (25 mcg.) was unaffected by the administration of cryogenine (750 mcg.) prior to or during the evoked asynchronous discharges. Moreover, there were no apparent alterations in the enhanced level of firing evoked by these agents in ganglia pretreated with *d,l*-isoproterenol HCl (2 mcg.) or in ganglia conditioned previously by repetitive supramaximal preganglionic stimulation (30 Hz. for 30 sec.). Cryogenine (750 mcg.) failed to prevent the complete abolition of the "muscarinic" firing that occurred almost immediately following the administration of atropine sulfate (2 mcg.).

In ganglia pretreated with cryogenine immediately prior to or 30 sec. before injection of 1,1-dimethyl-4-phenylpiperazinium iodide (5 mcg.), tetramethylammonium Cl (10 mcg.), levarterenol bitartrate (0.5 mcg.), epinephrine bitartrate (0.5 mcg.), or methacholine Cl (50 mcg.), no observable antagonism or enhancement of the ganglionic blockade produced by these compounds was noted.

The lupine alkaloids (cytisine, sparteine, *etc.*) are reputed to possess a nicotinelike common denominator pharmacologically and some structural similarities with possible metabolic degradation products of cryogenine. Previous studies demonstrated, however, distinct differences between the two groups in regard to cardiovascular, anti-inflammatory, and psychopharmacologic activity (2–4, 9). The present study showed cryogenine to be devoid of any ganglionic activity at the relatively high dose levels employed. Numerous nonsteroidal anti-inflammatory drugs presently in clinical use inhibit vasoconstriction produced by a variety of vasoactive agents (10). While the anti-inflammatory efficacy of cryogenine does not appear to be related to gangliotropic activity, its actions might still be explained by interference with inflammatory mediators or tissue reactions at the neurovascular or cellular level.

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Urinary Excretion of Chlorpromazine and Chlorpromazine Sulfoxide in Four Patients on Different Days

Keyphrases ☐ Chlorpromazine and sulfoxide—urinary excretion, human ☐ Urinary excretion, chlorpromazine—daily variations

Sir:

Urinary concentrations of chlorpromazine in individuals receiving this drug were determined (1–3). The results showed wide variations among the persons tested, in spite of the fact that the same dose was administered. However, the data do not indicate whether a patient excreting low quantities of the drug on the day of

all dosages in the following text refer to the salt form of the drugs. Clotting in the needle was prevented by the prior administration of heparin (400 units/kg. intraarterially).

Cryogenine was administered intraarterially during continuous preganglionic stimulation at frequencies ranging from 0.1–1.0 Hz. Following injection of the drug, ganglionic transmission was monitored continuously for 2 hr. Cryogenine (50–750 mcg.) failed to produce any detectable changes in either spike amplitude or afterpotential contour evoked by supramaximal or submaximal preganglionic stimulation. The submaximal stimulation was determined by selecting the voltage that would produce a ganglionic spike one-half the amplitude of that obtained with supramaximal preganglionic stimulation. It was determined that cryogenine (50–750 mcg.) did not evoke ganglionic firing. Moreover, no changes in the resting demarcation potential were observed as the result of cryogenine administration. Similarly, there was no evidence of asynchronous postganglionic discharge evoked by cryogenine (750 mcg.) in ganglia conditioned by repetitive supramaximal preganglionic stimulation (30 Hz. for 30 sec.) or in ganglia pretreated with *d,l*-isoproterenol HCl (2 mcg.).

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Keyphrases ☐ Chlorpromazine and sulfoxide—urinary excretion, human ☐ Urinary excretion, chlorpromazine—daily variations

Sir:

Urinary concentrations of chlorpromazine in individuals receiving this drug were determined (1–3). The results showed wide variations among the persons tested, in spite of the fact that the same dose was administered. However, the data do not indicate whether a patient excreting low quantities of the drug on the day of

collection will always excrete low amounts or whether he might differ in the excretion of chlorpromazine on different days. To determine if daily variations do exist, we measured the urinary concentrations of chlorpromazine and one of its metabolites, chlorpromazine sulfoxide, in four patients with schizophrenic reactions for 6–8 days.

Four male patients (identified as C, F, K, and M) with schizophrenic reactions, who had been hospitalized for more than 1 year[1] and who had been on chlorpromazine for more than 6 months, were chosen for this study. Drug therapy was discontinued for 2 days before the first urine collection. During the study, all four patients received 200 mg. chlorpromazine orally in tablet form each day at 7:45 a.m. No other medication was given, except that C received 75 mg. of desipramine hydrochloride[2] daily.

The daily routine of the patients, with the exception of the urine collections, remained undisturbed. Because of hospital routine, urine was collected at 7:00 a.m., followed by three collection periods of 7:00 to 11:00 a.m., 11:00 a.m. to 1:00 p.m., and 1:00 to 3:00 p.m. Hollister *et al.* (3) showed that, within an 8-hr. period, patients normally excrete half the daily medication. Upon collection, the urines were refrigerated, frozen, and assayed within 3 weeks.

The assay method was essentially the method described by Salzman and Brodie (4). Urines were thawed and stirred; a 10-ml. aliquot was made alkaline and heated in a boiling water bath for 15 min. After cooling, chlorpromazine and chlorpromazine sulfoxide were extracted with heptane–isoamyl alcohol. The extract from the organic phase with 0.1 *M* acetate buffer, pH 5.6, was read as chlorpromazine sulfoxide, and the 0.1 *N* HCl extraction was read as chlorpromazine in a Beckman DU spectrophotometer at the appropriate wavelengths. Calculations were based on internal standards containing both chlorpromazine and chlorpromazine sulfoxide[3].

Figure 1 shows the partial excretion patterns of chlorpromazine and chlorpromazine sulfoxide in the four patients. The urinary concentrations of chlorpromazine and chlorpromazine sulfoxide are in the same range as those reported for other patients (1–3). A comparison of the pattern of excretion of chlorpromazine and chlorpromazine sulfoxide by the same patient on different days shows that the chlorpromazine pattern was rather consistent in Patients M, F, and C, whereas K showed considerable variations. The excretion pattern of chlorpromazine sulfoxide on various days was most similar in Patients M and F and most dissimilar in Patients C and K. The ratio of the concentrations of chlorpromazine sulfoxide to chlorpromazine varied least in Patients M, F, and K and most in Patient C. Patient M received his chlorpromazine dose in liquid form for the last 4 days of this study; this did not change his typical excretion pattern. A comparison of the urinary excretion of chlorpromazine and chlorpromazine sulfoxide on a given day among the four patients shows the variations others have reported.

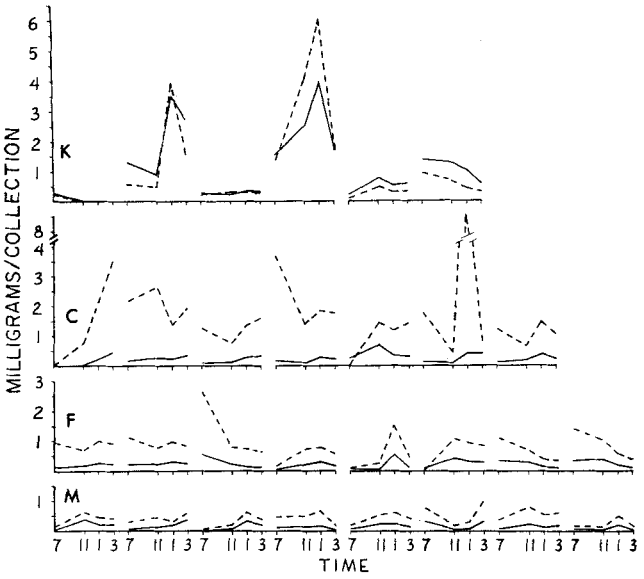

Figure 1—*Partial daily excretion pattern of chlorpromazine and chlorpromazine sulfoxide in four patients receiving 200-mg. tablets of chlorpromazine daily at 7:45 a.m. Daily collection was started at 7:00 a.m. followed by three periods of: 7:00 to 11:00 a.m., 11:00 a.m. to 1:00 p.m., and 1:00 to 3:00 p.m. Collections were on successive days with a 4-day interval indicated by space in baselines. Key: chlorpromazine, —; and chlorpromazine sulfoxide, - - -.*

In summary, the partial excretion pattern of chlorpromazine and chlorpromazine sulfoxide after administration of 200 mg. of the drug was studied in four patients with schizophrenic reactions for 6–8 days. Two patients remained rather consistent, while the other two showed relatively large daily fluctuations in chlorpromazine and chlorpromazine sulfoxide excretion during this period. The results indicate the need for more long-term studies on drug metabolism and excretion in individual patients.

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[1] At the Veterans Administration Hospital, Coatesville, Pa.
[2] Norpramine.
[3] Provided by Smith Kline and French Laboratories, Philadelphia, Pa.

Characterization of USP XVIII Content Uniformity Plan

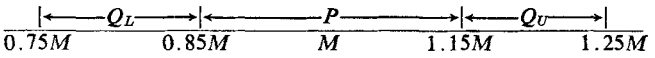
Keyphrases ☐ Tablets, content uniformity—probability equations, USP XVIII requirements ☐ Content uniformity, tablets—USP XVIII requirements, probability equations

Sir:

In our recent paper, "A Characterization of the Content Uniformity Plan" (1), a procedure was reported for determining the probabilities of tablets passing the USP XVII and NF XIII content uniformity sampling plans. At the time that paper was submitted, it was understood, based upon preliminary page proofs, that the same sampling plan would be effective in USP XVIII (2). However, the published 1970 volume contains a different requirement for tablet content uniformity which reads as follows:

> Tablets—The requirements are met if the content of each of not less than 9 of the tablets is within the limits of 85 percent and 115 percent of the average of the tolerances specified in the potency definition in the monograph, and if the content of none of the tablets falls outside the limits of **75** percent and 125 percent of that average.
>
> If the content of not more than 2 tablets falls ouside the limits of 85 percent and 115 percent, assay each of the remaining 20 tablets. The requirements are met if the content of each of the additional 20 tablets falls within the limits of 85 percent and 115 percent of the average of the tolerances specified in the potency definition in the individual monograph.

In the referenced paper, tables were presented showing the probability of meeting content uniformity requirements based upon certain combinations of four parameters of lot quality: γ_p, γ_y, δ_p, and δ_y. These tabled values were applicable to the content uniformity plans of USP XVII and NF XIII. The effect of the new sampling plan of USP XVIII on the reported probabilities of meeting content uniformity requirements has now been determined and may be developed by Scheme I.



Scheme I

In Scheme I, M represents the mean of the tolerances specified in a particular monograph. A lot of tablets meets the requirements of USP XVIII for tablet content uniformity if and only if one of the following events occurs:

1. 10 tablets have a drug content in the region P.
2. 9 tablets are in P and 1 in Q where $Q = Q_L + Q_U$.
3. 8 tablets are in P; 2 in Q from the first sample plus a second sample of 20, all 20 of which must be in P.

The probability that the lot meets requirements then becomes: Pr(lot passes) = Pr(event 1) + Pr(event 2) + Pr(event 3).

Letting p equal the proportion of single dosage units in P, q equal the proportion of units in Q, and $1 - p - q$ equal the proportion of units in neither, we can write the trinomial expression:

$$Pr(\text{Pass}) = \frac{10!}{10!0!0!}p^{10}q^0(1-p-q)^0 + \frac{10!}{9!1!0!}p^9q^1(1-p-q)^0 + \left[\frac{10!}{8!2!0!}p^8q^2(1-p-q)^0\right]\left[\frac{20!}{20!0!0!}p^{20}q^0(1-p-q)^0\right]$$
$$= p^{10} + 10p^9q + 45p^{28}q^2 \qquad \text{(Eq. 1)}$$

Lots are assumed to be of sufficient size to make the finite population correction factor negligible. Equation 1 may be evaluated for different values of p and q, where p is equal to $(1 - \pi)$ in *Reference 1*, and q is found as $Pr(0.75M, 0.85M) + Pr(1.15M, 1.25M)$ where:

$Pr(0.75M, 0.85M)$ = probability of a tablet potency failing in the region Q_L

$$= F\left[\frac{0.85 - (1+\delta_p)(1+\delta_y)}{\gamma_{py}}\right] - F\left[\frac{0.75 - (1+\delta_p)(1+\delta_y)}{\gamma_{py}}\right] \qquad \text{(Eq. 2)}$$

and, for region Q_U,

$$Pr(1.15M, 1.25M) = F\left[\frac{1.25 - (1+\delta_p)(1+\delta_y)}{\gamma_{py}}\right] - F\left[\frac{1.15 - (1+\delta_p)(1+\delta_y)}{\gamma_{py}}\right] \qquad \text{(Eq. 3)}$$

The trinomial expression (Eq. 1) was evaluated for the

Table I—Probability of Meeting USP XVIII Content Uniformity Requirement for Tablets when $\delta_p = \delta_y = 0$[a,b]

γ_p	γ_y 0.01	0.02	0.03	0.04	0.05
0.01	1.000 (1.000)	1.0000 (1.0000)	1.0000 (1.0000)	1.0000 (1.0000)	1.0000 (0.9975)
0.02		1.0000 (1.0000)	1.0000 (1.0000)	1.0000 (0.9998)	0.9998 (0.9936)
0.03			1.0000 (1.0000)	1.0000 (0.9983)	0.9989 (0.9786)
0.04				0.9994 (0.9861)	0.9938 (0.9331)
0.05					0.9729 (0.8322)

[a] When $\delta_p = \delta_y = 0$, the tables are symmetrical. [b] Probabilities for USP XVII and NF XIII are in parentheses.

Table II—Probability of Meeting USP XVIII Content Uniformity Requirements for Tablets[a]

γ_p	γ_y 0.01	0.03	0.05
	when $\delta_p = -0.04$, $\delta_y = 0.02$		
0.01	1.0000 (1.0000)	1.0000 (1.0000)	0.9999 (0.9954)
0.03	1.0000 (1.0000)	1.0000 (0.9997)	0.9979 (0.9663)
0.05	0.9996 (0.9896)	0.9966 (0.9539)	0.9600 (0.7871)
	when $\delta_p = 0.09$, $\delta_y = -0.08$		
0.01	1.0000 (1.0000)	1.0000 (1.0000)	0.9997 (0.9986)
0.03	1.0000 (1.0000)	1.0000 (1.0000)	0.9972 (0.9786)
0.05	1.0000 (0.9836)	0.9994 (0.9459)	0.9694 (0.7943)
	when $\delta_p = 0.07$, $\delta_y = -0.01$		
0.01	1.0000 (1.0000)	1.0000 (0.9986)	0.9447 (0.7794)
0.03	1.0000 (0.9996)	0.9947 (0.9560)	0.8734 (0.6310)
0.05	0.9708 (0.8593)	0.8994 (0.6823)	0.7052 (0.4063)

[a] Probabilites for NF XIII and USP XVII are in parentheses.

parameters given in Tables I and II of *Reference 1*. The results are shown here in Tables I and II. The probabilities for the NF XIII and USP XVII plans as previously reported are included in parentheses. (The new values are taken from tabled probabilities for all combinations of the four parameters where δ_p, δ_y go from -0.10 to 0.10 in steps of 0.01 and γ_p, γ_y go from 0 to 0.06 in steps of 0.005.)

As may be seen in Tables I and II, the probability of passing a lot, when the coefficients of variation increase, is greater for the new plan than for the old.

(1) C. B. Sampson, H. L. Breunig, J. P. Comer, and D. E. Broadlick, *J. Pharm. Sci.*, **59**, 1653(1970).

(2) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 930.

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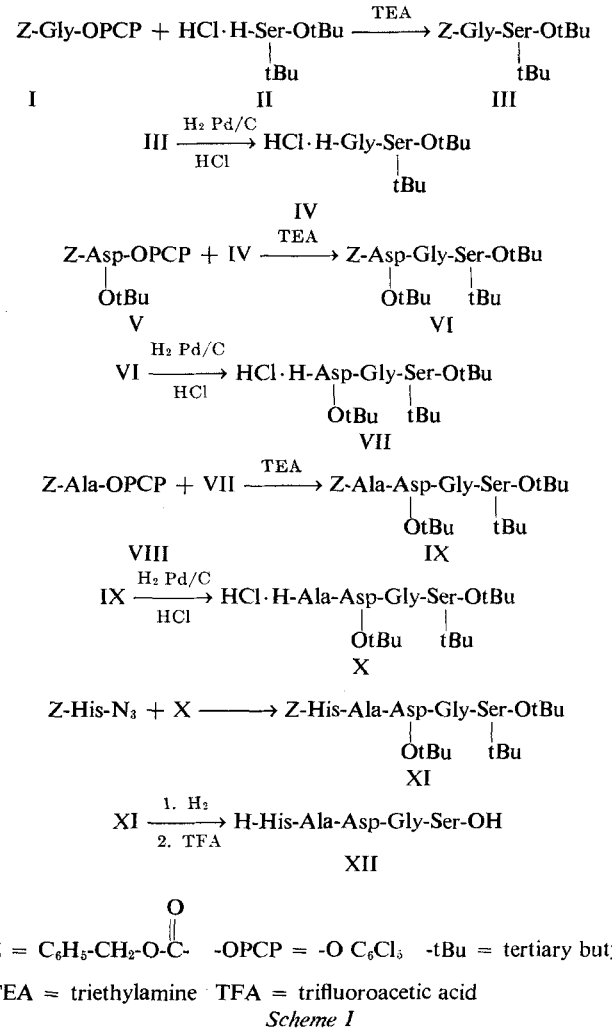
Enzyme-Active Sites: Importance of Aspartic Acid in Peptide Esterase Models

Keyphrases □ Peptide esterase models, catalytic activity—aspartic acid residue influence □ Proteolytic enzyme active sites—peptide catalytic activity determination



Scheme I

Sir:

Recent investigations point out that the serine proteinases, notably chymotrypsin and trypsin, require serine and histidine residues in some type of close steric relationship for their catalytic activity. While there are 246 amino acids in the total sequence of chymotrypsinogen, the inactive precursor of chymotrypsin, the suitable folding of this enzyme brings two histidines from positions 40 and 57 and one serine from position 195 close enough to act as the active center responsible for catalyzing the hydrolysis. Trypsin affords the same possibilities, where two histidines from positions 29 and 46 and a serine from position 183 act as the active site of the enzyme (1, 2).

One approach to studying the active sites of enzymes is the synthesis and evaluation of the catalytic activity of relatively simple peptides that embody as many known features of proteolytic enzymes as possible. A number of polymers and copolymers of histidine and serine (3) and small peptides incorporating histidine and serine were reported (4, 5). Photaki and Moschopedis (6) carried out the same type of studies with cysteine proteinases with peptides incorporating histidine and cysteine. A relatively more potent esterase model, L-histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine, was reported recently (7). The catalytic coefficient (3) for this pentapeptide was 179 l. mole^{-1} min.$^{-1}$ compared with 147 l. mole^{-1} min.$^{-1}$ for L-seryl-γ-aminobutyryl-L-histidyl-γ-aminobutyryl-L-aspartic acid (5) and 10^4 l. mole^{-1} min.$^{-1}$ for α-chymotrypsin (3). Although the catalytic coefficient of these peptides, when compared with chymotrypsin, is considerably low, further evaluation is definitely warranted of a series of peptides where the molecule can be made more flexible. Moreover, the role played by individual amino acids in the peptide esterase models should be studied.

Among a number of peptides subjected to the catalytic activity determination, the peptides incorporating histidine and serine without aspartic acid showed very little or no catalytic activity. Examples are: L-histidyl-L-alanyl-glycyl-L-serine (catalytic coefficient 15 l. mole^{-1} min.$^{-1}$), L-histidyl-glycyl-L-tyrosyl-L-serine (catalytic coefficient 21 l. mole^{-1} min.$^{-1}$), and glycyl-L-histidyl-glycyl-L-serine (catalytic coefficient 17 l. mole^{-1} min.$^{-1}$).

In this communication, we report the importance of aspartic acid in the peptide esterase models. A comparative study of catalytic activity of peptides incorporating histidine and serine, differing only in the aspartic acid residue, was conducted. To provide more or less the same physicochemical environments, the comparison was made with the peptide incorporating glutamic acid instead of aspartic acid. The

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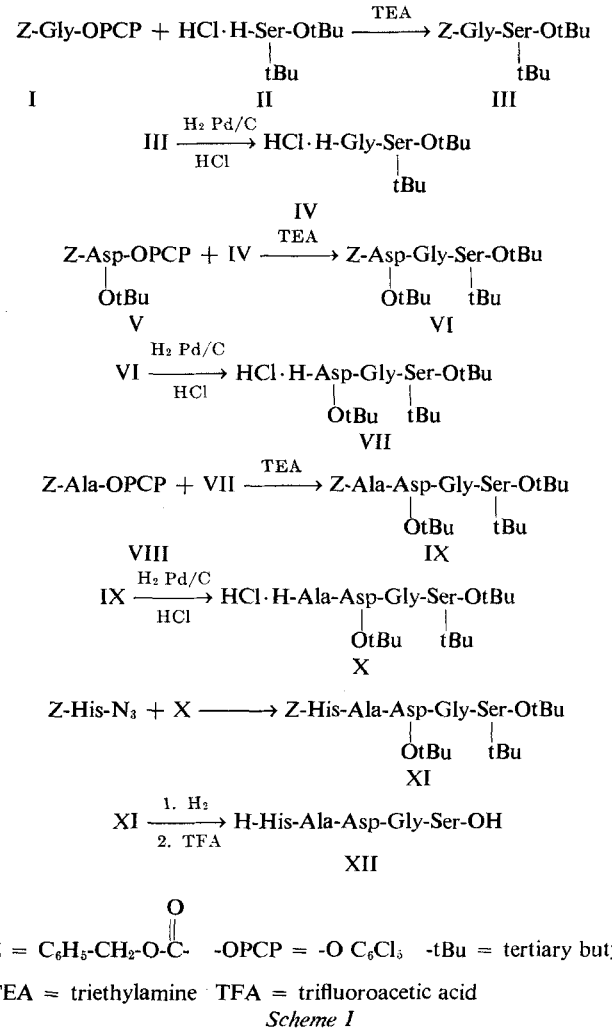

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pentapeptide, L-histidyl-glycyl-L-glutamyl-L-seryl-L-phenylalanine, had a much lower catalytic activity (93 l. mole^{-1} min.$^{-1}$) when compared with the corresponding pentapeptide containing aspartic acid in the sequence. The same observation was true in the case of two tetrapeptides, L-histidyl-L-aspartyl-glycyl-L-serine (catalytic coefficient 150 l. mole^{-1} min.$^{-1}$) and L-histidyl-L-glutamyl-glycyl-L-serine (catalytic coefficient 63 l. mole^{-1} min.$^{-1}$). The importance of aspartic acid in the peptide esterase models was further confirmed by comparing two pentapeptides, L-histidyl-L-alanyl-L-aspartyl-glycyl-L-serine (catalytic coefficient 210 l. mole^{-1} min.$^{-1}$) and L-histidyl-L-alanyl-L-glutamyl-glycyl-L-serine (catalytic coefficient 87 l. mole^{-1} min.$^{-1}$). The increased catalytic activity of peptide esterase models containing aspartic acid may be justified on the basis that, in the case of chymotrypsin, aspartic acid occupies position 194, which is adjacent to the postulated active serine at position 195. The same is true of trypsin, where aspartic acid is at position 184 adjacent to active serine at position 183.

The catalytic activity of the peptide esterase models was determined by the liberation of *p*-nitrophenol from *p*-nitrophenylacetate following the procedure used by Sheehan *et al.* (4). The synthesis of the pentapeptide, L-histidyl-L-alanyl-L-aspartyl-glycyl-L-serine, which has the highest catalytic coefficient of reported peptide esterase models, is outlined in Scheme I. To limit the degree of racemization, the peptide chains were extended from the C-terminal residues of amino acids, and all peptide bonds, except histidine, were formed using the pentachlorophenyl active ester method (8, 9). Histidine residues were incorporated in the peptide chain using the azide method (10). All the protecting groups used for unreacting functionalities during the reaction sequences were acid labile or removable by hydrogenolysis. This approach excluded the use of alkali treatment during the synthesis, thereby further limiting the degree of racemization and problems of transpeptidation (11). The elemental analyses of the intermediates and final peptides reported in this work were within experimental tolerance, and they were homogeneous to paper chromatography and paper electrophoresis under a variety of conditions.

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BOOKS

REVIEWS

Antitussive Agents, Volumes I, II, and III. *Section 27, International Encyclopedia of Pharmacology and Therapeutics.* Edited by H. SALEM and D. M. AVIADO, Pergamon Press Inc., Maxwell House, Fairview Park, Elmford, NY 10523, 1970. li + 833 pp. 16 × 23.5 cm.

All but three of the 15 chapters in this three-volume series are authored by the editors. In this ambitious undertaking, the author-editors have extensively reviewed the pharmacological and clinical literature pertaining to antitussives up through 1966 and systematically organized their findings into a readily usable format. The first portion of Volume I, an exhaustive tabular listing of patents issued for antitussive compounds, should be of great assistance to medicinal chemists as well as patent lawyers interested in searching for new chemical entities with this activity. The 453 entries include names of consignees, countries issuing, and references. A comprehensive review of antitussive testing methods in humans and animal species is also presented in tabular form. Pharmacologists should find the 100 referenced entries most useful because each includes information about the cough stimulus used, the method of assessing the cough, as well as other pertinent specifications. The last part of Volume I consists of two chapters. The first discusses physiology of the cough reflex as a basis for understanding factors causing cough and pharmacologic approaches to alleviating cough. The last chapter is an interesting historical review of the changing concepts of causes and approaches to treating pathological cough.

Volumes II and III of this series present a review of the experimental and clinical literature about specific antitussive compounds. Volume II deals with derivatives of opium (three chapters on codeine, *d*-methorphan, and miscellaneous opiates) and four chapters on classes of nonopiates with central action. Volume III considers nonopiates with peripheral actions (chapters on local anesthetics, bronchodilators, expectorants, and mucolytic agents). The format of each chapter includes chemistry and dosage, pharmacodynamics, and clinical usefulness of the compound in question, as well as a tabular listing of all domestic and foreign commercially available preparations containing those particular compounds.

The carefully designed format of these three volumes provides a comprehensive look at the presently available experimental and clinical information about antitussive compounds. Areas needing further study are clearly identified so that these volumes provide a

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BOOKS

REVIEWS

Antitussive Agents, Volumes I, II, and III. *Section 27, International Encyclopedia of Pharmacology and Therapeutics.* Edited by H. SALEM and D. M. AVIADO, Pergamon Press Inc., Maxwell House, Fairview Park, Elmford, NY 10523, 1970. li + 833 pp. 16 × 23.5 cm.

All but three of the 15 chapters in this three-volume series are authored by the editors. In this ambitious undertaking, the author-editors have extensively reviewed the pharmacological and clinical literature pertaining to antitussives up through 1966 and systematically organized their findings into a readily usable format. The first portion of Volume I, an exhaustive tabular listing of patents issued for antitussive compounds, should be of great assistance to medicinal chemists as well as patent lawyers interested in searching for new chemical entities with this activity. The 453 entries include names of consignees, countries issuing, and references. A comprehensive review of antitussive testing methods in humans and animal species is also presented in tabular form. Pharmacologists should find the 100 referenced entries most useful because each includes information about the cough stimulus used, the method of assessing the cough, as well as other pertinent specifications. The last part of Volume I consists of two chapters. The first discusses physiology of the cough reflex as a basis for understanding factors causing cough and pharmacologic approaches to alleviating cough. The last chapter is an interesting historical review of the changing concepts of causes and approaches to treating pathological cough.

Volumes II and III of this series present a review of the experimental and clinical literature about specific antitussive compounds. Volume II deals with derivatives of opium (three chapters on codeine, *d*-methorphan, and miscellaneous opiates) and four chapters on classes of nonopiates with central action. Volume III considers nonopiates with peripheral actions (chapters on local anesthetics, bronchodilators, expectorants, and mucolytic agents). The format of each chapter includes chemistry and dosage, pharmacodynamics, and clinical usefulness of the compound in question, as well as a tabular listing of all domestic and foreign commercially available preparations containing those particular compounds.

The carefully designed format of these three volumes provides a comprehensive look at the presently available experimental and clinical information about antitussive compounds. Areas needing further study are clearly identified so that these volumes provide a

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Encyclopedia of Industrial Chemical Analysis. Edited by F. D. SNELL and L. S. ETTRE, Wiley, 605 Third Ave., New York, NY 10016, 1970. xiv + 680 pp. 18.5 × 26 cm. Price $35.00 for subscription; $45.00 for single copy.

NEW JOURNAL

Pharmacology and Therapeutics in Dentistry. Edited by A. H. KUTSCHER, Association of Pharmacology and Therapeutics Teachers in Dentistry, 630 West 168th St., New York, NY 10032, 1970.

valuable point of departure for experimental or clinical pharmacologists with interests in this field. Unfortunately, this series will be of less value to the clinician who is looking for a critical appraisal of currently available antitussive compounds. Questions relating to clinical efficacy and untoward effects are left dangling in some cases. It would have been helpful to the clinician-reader if the authors had included a critique of conflicting reports presented based on their own expertise and assessment of the literature.

Each volume concludes with a complete subject index. In addition, Volume III contains an index to names of preparations and an index to authors cited in the series.

Reviewed by Robert K. Chalmers
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Permeability and Function of Biological Membranes. Edited by L. BOLIS, A. KATCHALSKY, R. D. KEYNES, W. R. LOEWENSTEIN, and B. A. PETHICA, North-Holland/American Elsevier, New York, NY 10017, 1970. vii + 364 pp. 15.5 × 22.5 cm. Price $16.25.

The papers included in this book are based on material reported at the 1969 International Conference on Biological Membranes.

While the title of the book might suggest it would have broad appeal to pharmaceutical scientists, this is not entirely accurate. The individual papers are restricted to very specific fields; the reader would have to have in-depth knowledge of many fields to benefit greatly from this book as a whole.

However, for the scientist working specifically in this area, the book does cover much of the recent work in this field and might be a useful reference source.

Staff Review ■

Advances in Drug Research, Vol. 5. Edited by N. J. HARPER and A. B. SIMMONDS, Academic Press, London, England, 1970. vii + 278 pp. 15.5 × 23.5 cm. Price $13.00.

This volume contains six comprehensive articles that summarize the progress made in certain areas of drug research.

Topics included in this volume are: Biologically Active Benzo[b]-thiophene Derivatives; Spectroscopic Techniques for the Study of Drug Interactions with Biological Systems; Endogenous Broncho-Active Substances and their Antagonism; The Role of Slow Reacting Substance in Asthma; Disodium Cromoglycate (Intal); and Recent β-Adrenoreceptor Stimulants.

Staff Review ■

Alkaloid-Bearing Plants and Their Contained Alkaloids, 1957–1968, Supplement of Lloydia—The Journal of Natural Products. By J. J. WILLAMAN and H. LI, The Lloyd Library and Museum and The American Society of Pharmacognosy, Storrs, Conn., 1970. vii + 286 pp. 17 × 25 cm. Price $6.00. (Available from The Lloyd Library and Museum, P. O. Box 484, Cincinnati, OH 45201)

This supplement to *Lloydia* contains one article that is a continuation of the compilation "Alkaloid-bearing Plants and Their Contained Alkaloids," by J. J. Willaman and Bernice G. Shubert, published in 1961 by Agricultural Research Services, Technical Bulletin No. 1234, U. S. Department of Agriculture.

The purpose of this supplement and the original compilation was to assemble in one place all the scattered information of the occurrence of alkaloids in the plant world.

The supplement is organized into two cross-referenced tables, one entered by the plant name and the second entered by using the specific alkaloid name.

Staff Review ■

Late Effects of Radiation. Edited by R. J. M. FRY, D. GRAHN, M. L. GRIEM, and J. H. RUST, Van Nostrand Reinhold, 450 West 33rd St., New York, NY 10001. 1970. ix + 298 pp. 18 × 26 cm. Price $10.95.

This book contains the proceedings of a colloquium held at the University of Chicago in May 1969. This colloquium represents the climax of a ten-year effort to study the impact of the peaceful uses of atomic energy on public health.

The concluding chapter in the book, "The degradation of our environment," was written by Lord Ritchie-Calder.

Staff Review ■

NOTICES

Teeanalyse. By L. HORHAMMER, Springer-Verlag, 1 Berlin 33, Heidelberger Platz 3, Germany, 1970. 74 pp. 21 × 29.5 cm. Price $13.00. (*German*)

Hexoprenalin: Pharmakologie und Therapeutische Anwendung Beim Asthmatischen Formendreis. Edited by E. DEUTSCH, K. IRSIGLER, and O. KRAUPP, Springer-Verlag, New York, NY 10010, 1970. viii + 127 pp. 15 × 22.5 cm. Price $5.20. (*German*)

British Veterinary Codex, Second Edition (Supplement). Prepared by The Department of Pharmaceutical Sciences of the Pharmaceutical Society of Great Britain, The Pharmaceutical Press, 17 Bloomsbury Square, W. C. 1, London, England, 1970. xxiii + 317 pp. 14.5 × 22 cm. Price 3 Pounds, 10 Shillings.

Medicaments Organiques De Synthese III. By G. VALETTE, Masson Et Cie, 120 Boulevard Saint-Germain, Paris VI, France, 1970. 341 pp. 15.5 × 22 cm. Price 120 f. (*French*)

Drug Abuse and What We Can Do About It. Edited by J. C. BENNETT and G. D. DEMOS, Charles C Thomas, Springfield, IL 62703, viii + 138 pp. 15.5 × 24 cm. Price $8.50.

Lehbuch der Pharmazeutischen Chemie. By H. AUTERHOFF, Wissenschaftliche Verlagsgesellschaft MBH, Stuttgart, Germany, 1970. xii + 536 pp. 17 × 25 cm. Price DM 54. (*German*)

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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REVIEW ARTICLE

Pharmaceutical Sciences—1970: Literature Review of Pharmaceutics

TERRY L. BENNEY* and WILLIAM E. HAMLIN†

Keyphrases ☐ Pharmaceutical sciences—1970 literature review of pharmaceutics ☐ Technology, pharmaceutical—dosage forms, sterility, packaging equipment ☐ Physical pharmacy—dissolution, solubility, complexation ☐ Surface phenomena—pharmaceutical applications ☐ Crystallization, rheology—pharmaceutical applications ☐ Antibiotics—pharmaceutical aspects ☐ Biopharmaceutics—drug properties, absorption, pharmacokinetics

CONTENTS

GENERAL PHARMACY 959
Preservatives 960
Flavor, Aroma, and Color 960
Stability 960
Stability Kinetics 961
Antibiotic Stability 963
Vitamin Stability 963
PHARMACEUTICAL TECHNOLOGY 964
Parenterals 964
Sterility 965
Tablets and Capsules 966
Suspensions 968
Emulsions 970
Ointments and Creams 970
Suppositories 971
Aerosols 972
Sustained Release 972
Cosmetics 972
Packaging 973
Equipment 974
PHYSICAL PHARMACY 974
Dissolution 976
Solubility 978
Complexation 979
Interactions of Drugs with Biological Substances 979
Interactions of Drugs with Nonbiological Substances 980
Surface Phenomena 982
Interface Studies 982
Adsorption Studies 983
Studies on General Properties of Surface-Active Agents. 984
Micelle Studies 985
Crystallization 986
Rheology 987
PHARMACEUTICAL ASPECTS: ANTIBIOTICS 988
BIOPHARMACEUTICS 989
Effects of Physicochemical Properties 991
Effects of Formulation 992
Absorption Control and Alteration 993
Absorption Mechanism 994
Drug Absorption 996
Pharmacokinetics 997
REFERENCES 998

This review of the literature represents a comprehensive cross section of the research and development efforts in various selected disciplines of the pharmaceutical sciences. It is the ninth annual survey in the series (1–8). To compile it, numerous periodicals and selected sections of *Chemical Abstracts* were abstracted.

The review was prepared to provide an opportunity for reviewing the research of the past year in specific areas of pharmaceutical sciences and to supply a convenient source of references to articles of preferred interest. The format of last year's review was retained because of its generally wide acceptance.

GENERAL PHARMACY

A comprehensive review of medicinal, chemical, and physical properties of the prostaglandins was presented. Possible pharmaceutical products of this group of potent hormones were also noted (9). The current status of drug compounds from marine sources was intensively reviewed (10). Marine organisms that showed different pharmacological effects and

Table I—Additional References on General Pharmacy

Reference	Topic
17	Method of organoleptic testing
18	Factors influencing preparation of stock tinctures
19	Review of cyclamates
20	Uses and importance of glycerol and glycerol substitutes
21	Function and composition of hormones, enzymes, and their interrelation with pharmaceuticals

potential as drugs were stressed. The potential of antimicrobial substances from the sea was also explored (11). Specifications in control of product uniformity were considered by several authors (12–14). Deficiencies in the control procedures set forth in various pharmacopeias were described, and suggestions aimed at improving the procedures were made. The neutralizing capacity of antacid drugs was evaluated by several authors (15, 16).

Other papers of interest in general pharmacy are listed in Table I.

Preservatives—A potentiating effect of phenylmercuric acetate on hexachlorophene or dichlorophene was observed in both *in vitro* and *in vivo* experiments (22). The results of a number of scientific investigations on boric acid were reported as ample evidence for eliminating this substance as a pharmaceutical agent. The risk of boric acid poisoning far outweighed its weak bactericidal power (23). Corticosteroids, such as cortisol and methylprednisolone hemisuccinate, were shown to reduce significantly the antibacterial activity of dodecyldi(β-hydroxyethyl)benzylammonium chloride and dodecyltriphenylphosphonium bromide against *Staphylococcus aureus* and, to a lesser degree, *Bacillus pyocyaneus* (24). Polyethylene glycol stearates decreased the antibacterial effects of phenols against *S. aureus*, but polyethylene glycols had little or no influence on the antibacterial activity (25, 26).

Equilibrium dialysis studies indicated a rather generalized binding of antimicrobial agents by cetomacrogol 1000 and polysorbate 80. The degree of interaction appeared to be dependent upon the type of functional group in the interacting molecule (27). The preservative activity of parabens in the presence of a polysorbate 80–water system was found to be related to the concentration of the free or unbound paraben (28). Prediction was made of the required preservative concentration for an emulsified system from a knowledge of oil–water partition coefficients, and preservative–macromolecule binding data were illustrated. In emulsions, the addition of a preservative to each phase was suggested as the best way to prevent excessive concentration of the agent in one of the phases (29). Thus, an ionizable preservative, such as a tertiary amine, is suitable for the aqueous phase; a nonionizable substance, such as benzyl alcohol, is most adequate in the oil phase. In an aqueous parenteral vehicle containing polysorbate 80 and benzyl alcohol, turbidity was found to be a function of concentration and temperature (30). The presence of electrolyte depressed the cloud point and brought the turbidity close to room temperature.

A study of the influence of dimethyl sulfoxide on the hemolytic activity of antimicrobial preservatives concluded that dimethyl sulfoxide affects the rate of preservative-induced hemolysis, probably due to a cellular mechanism rather than to an extracellular preservative–dimethyl sulfoxide chemical interaction (31). The release effect of benzalkonium chloride and hexachlorophene from various oil vehicles for the treatment of children's skin was studied (32). The best diffusion was obtained with a silicone solution and a 9:1 mixture of liquid paraffin–butyl stearate.

Different thresholds of cetyltrimethylammonium bromide uptake by *Escherichia coli* exist below which no effect on cell growth or viability could be detected (33). There was a distinct separation of the two effects. The antibacterial activity of various preservatives in several ointment bases was investigated (34). In general, the preservatives were more effective in paraffin and ophthalmic ointments. The most active preservatives were dodecyldimethylbenzylammonium chloride at 1:10,000 dilution and 0.2% sorbic acid.

Other papers of interest on preservatives are listed in Table II.

Table II—Additional References on Preservatives

Reference	Topic
35	Review of factors influencing stability and activity of antimicrobial preservatives for single-phase parenteral preparations
36	Review of antimicrobial agents in pharmaceutical products
37	Discussion of available preservatives and current practices in preservation of ophthalmic, nasal, and otic products
38	Stability and efficacy of combinations of preservatives for eye drops
39	Adsorption of preservatives on particulate solids in aqueous suspensions
40	Adsorption of various preservatives on membrane filters
41	Antimicrobial activity of some β-nitrostyrenes
42	Thermodynamic activities of monocomponent and multicomponent solutions of quaternary ammonium antibacterial agents

Flavor, Aroma, and Color—An encyclopedic evaluation of the literature in the perfumery materials field was continued by Bedoukian (43) in his 26th annual review. In a series of reports on odor measurement and its factors, additional evidence was presented on olfactory equilibrium and other aspects of odor theory (44). A primary odor, represented by isovaleric acid and its homologs and isomers, was correlated with molecular shape by a scanning computer method (45). The production of this primary odor required the carboxylic acid functional group in conjunction with a limited range of molecular shapes and sizes. A scientific evaluation of flavors was made (46), and a survey was conducted on the usefulness of fatty aliphatic compounds as components in the creation of natural flavors and fragrances (47). The effect of antioxidants on the oxidation of nine essential oils was investigated (48). α-Tocopherol citric acid was observed to be a remarkable antioxidant in lemon oil.

Stability—A set of computer programs and the system that automatically schedules all the assays necessary for a drug stability program was described (49). The effect of antioxidants on the hydrolytic and oxidative degradation of sulfacetamide in aqueous solutions

was investigated (50). Sodium metabisulfite accelerated the hydrolytic degradation of sulfacetamide to sulfanilamide, whereas sodium edetate had no effect on the rate. The sulfanilamide present in a heat-degraded solution had a major role in color formation under photolytic conditions. γ-Radiation-induced oxidation of aqueous pharmaceuticals can often be eliminated by customary antioxidants (51). Potassium bisulfite proved to be most effective in cyanocobalamin solutions and afforded suitable protective action in solutions of atropine sulfate, ephedrine HCl, hydrocodeine bitartrate, and ascorbic acid. Oxidative degradation of other drugs was presented in a series of papers (52–54).

In a study of the stability of drug solutions subjected to hydrolysis by ultrasonic irradiation, it was found that aromatic esters suffered structural changes. Aliphatic esters were resistant to such changes (55, 56). An investigation of the thermal dissociation of tolbutamide in a series of aliphatic alcohols and in polyethylene glycol 400 showed that the compound dissociates in only one fashion to give butylamine and *p*-toluenesulfonyl isocyanate (57). The thermal and radiation stability of labeled chlormerodrin and labeled diiodofluorescein also received attention (58, 59). The influence of sterilization, pH, and storage on the stability of aqueous injectable solutions of atropine (60), chlorhexidine (61), levarterenol (62), and scopolamine hydrobromide (63) was noted. The best stabilizer found for a sterile, concentrated, Ringer solution with glucose was calcium disodium ethylenediaminetetraacetate in the presence of HCl (64). The proposed acetylation of acetaminophen by acetylsalicylic acid was not evident in experimental pharmaceutical dosage forms (65).

A study of the incompatibility of 32 different amines with lactose alone or with magnesium stearate or stearic acid failed to show a dependence of the discoloration on the kind of amine (primary, secondary, tertiary, or quaternary) or on the change of pH (66). The stability of isoniazid and its derivatives was affected by blending with antacid preparations (67). Compounding aminophylline with acetylsalicylate, sulpyrin, hydralazine (Apresoline), sulfamethoxazole (Sinomin), ascorbic acid, and Panvitan should be avoided because of degradation (68). Similar compounding studies showed that when preparations of digestive enzymes are compounded with other drugs, the materials should be kept dry to preserve their activities (69).

An interesting article by Nogami *et al.* (70) was concerned with the hydrolysis stabilization of acylcholinesters by sodium lauryl sulfate at pH 7.7. Solubilization of acetylcholine–lauryl sulfate ion pairs in the micellar phase was proposed as the stabilization mechanism. A comprehensive review on the decomposition of aspirin included a complete survey of the hydrolysis studies to which this important compound has been subjected (71). The conditions for the highest stability of *Purpurea* glycosides A and B in tablet formulations were: neutral pH of the granulation mixture with a short drying time, low compression pressure of the tablets, and low temperature for storage of the tablets (72).

Table III—Additional References on Stability

Reference	Topic
73	Review of oxidative degradation of various drugs
74	Stability of a benzoyl peroxide acne cream product
75	Stability of three model suppository combinations, including appropriate excipients and additives
76	Stability study of chloramphenicol in British Pharmaceutical Codex topical formulations
77	Stability of dexamethasone ointments prepared with aquasorb or cetanol ointment
78	Thermal stability of antazoline chloride and naphazoline chloride in aqueous solution
79	Conversion of cotarnine in aqueous solutions
80	Review of stabilization of phenothiazine solutions
81	Effect of temperature and organic solvents on stability of ketazon
82	Influence of moisture, light, and air on stability of ketazon
83	Stability of benzothiadiazine derivatives in alkaline solution
84	Review of stability of morphine in opium
85	Preparation of more stable infusion solutions of sodium *p*-aminosalicylate
86	Stabilized aqueous suspensions of acetylsalicylic acid
87	Discussion of a comprehensive stability testing program for parenteral products
88	Stabilities of hexobarbital sodium and phenobarbital sodium in frozen aqueous solutions
89	Principles and techniques of accelerated storage testing and methods used for accelerating chemical and physical degradation processes
90	Stability of a prolonged-action aqueous suspension
91	Thermal stability of oxytocin in aqueous solutions and in the presence of glucose, sucrose, glycine, and sodium chloride
92	Compatibility of ointment bases with a 5% aqueous solution of methylcellulose
93	Stability of immune serum globulin during storage
94	Effect of excipients and storage conditions on stability of acetylsalicylic acid-based gels
95	Effect of chemical compounds, light, temperature, and humidity on pure gelatin capsules
96	Stability of glyceryl trinitrate tablets
97	Aminophenazone tablet decomposition during accelerated aging

Other interesting papers related to the topic of stability are listed in Table III.

Stability Kinetics—Evidence was presented to show how the salt effect in kinetic investigations may be extended to higher concentration ranges than expected by theory (not more than 0.01 *M*). Deviations from the Debye-Hückel expressions by the charged reactants and by the transition complex may be of the same magnitude and sign; this, it was claimed, may be the cause for the concentration range extension (98). The rationale of predicting the stability of pharmaceutical preparations using chemical kinetics was presented, using hydrocortisone sodium succinate as an example (99). Similarly, another author showed that in developing formulations of new compounds, kinetic studies were of great advantage (100).

A system of using computer tables and printouts for interpreting and predicting drug stability data was presented by Lintner *et al.* (101). This approach was developed to select the best prototype preparation, by giving an inkling of its chemical stability at an early date, and to estimate the shelflife of the finished dosage form. A flexible nonisothermal stability method was described, which permits the use of data from a single experiment to calculate activation energy, reaction rates, and stability predictions at any desired

temperature (102). The validity of the theory and the advantages of the method were demonstrated by a study of the inversion of sucrose and the hydrolysis of ethyl acetate. Bentley (103) presented a statistical technique for predicting thermal stability based on weighted least-squares analysis, which can easily be adapted for computer analysis.

Pawelczyk and his coworkers (104–111) investigated the kinetics of drug decomposition for a number of compounds. Investigation of the effect of 10 metal ions on the autooxidation of aqueous solutions of papaverine HCl showed that the most prominent effect was exerted by copper (Cu^{+2}) (104). The degradation was an apparent second-order reaction, and the catalytic activity was independent of the pH over the range studied (pH 2.3–4.15). The autooxidation of eupaverin in aqueous solutions was a second-order reaction and was relatively slow in the absence of traces of heavy metals. A comparison of their activation energies and 10% decomposition times indicated that eupaverin was approximately 2.5 times less stable than papaverine HCl (105).

The decomposition of antazoline in aqueous solution was first order and was independent of ionic strength and the kind and concentration of buffer (106). This drug was stable in acid solutions; the time of 10% decomposition of a pH 5.9 solution at 20° was 8.9 years. The decomposition of isonicotinic acid hydrazide in aqueous buffers between pH 3 and 7 was first order under anaerobic conditions (107). The greatest stability from solvolysis was observed at pH 6. Hydrolytic decomposition of phenylbutazone sodium was predominant in injectable preparations, and oxidative decomposition was predominant in suppositories (108). Phenylbutazone decomposition in various suppository bases followed a fractional-order reaction (109). In general, stability decreased with an increasing hydroxyl number of the suppository base. However, in aqueous buffer under nitrogen, the hydrolysis rate for sodium phenylbutazone was first order (110) and was independent of the kind and concentration of buffer, ionic strength, and hydroxyl-ion activity. The kinetics of quinidine sulfate decomposition in aqueous solutions were studied under the influence of light and heat (111). The decomposition at 353–363°K. was a first-order reaction; the light-induced decomposition was a zero-order reaction.

The stability of aminophenazone in aqueous solutions was examined in a series of papers. The decomposition of aminophenazone followed first-order kinetics, with maximum decomposition occurring at pH 6 (112). The decomposition activation energy was 9.280 kcal./mole. The characteristics of the degradation products in aqueous solutions were also examined (113). The stability of an aqueous formulation of aminophenazone stabilized with various oxidants was determined (114). The rate constants for the given stabilizers compared with a control indicated some improvement. A classical kinetic study employing the Arrhenius equation was used to predict the stability of taurinophenetidine at 25° in various pH solutions (115). The degradation kinetics were pseudo-first-order. In a similar study, 4-(aminoethanesulfonylamino)antipyrine in solution was examined at different pH's (116). By means of these kinetic studies, it was observed that the chemical in aqueous solution was practically stable at room temperature.

Kinetic examination of the stability of 2-diethylaminoethyl-3-methyl-2-phenylvalerate methobromide and its tertiary amine, 2-diethylaminoethyl-3-methyl-2-phenylvalerate hydrochloride, in aqueous solution showed that both undergo hydrolysis and follow a pseudo-first-order reaction (117). Stability of the quaternary ammonium salt and tertiary amine did not differ greatly, based on a comparison of their acid rate constants. However, comparison of their alkaline rate constants indicated that the tertiary amine was considerably less stable than the methobromide. Additional studies by Nogami *et al.* (118), with respect to the stability of some aminoethyl esters of phenylacetic acid, revealed that there was a linear relationship between λ (polar substituent constant) of R in $C_6H_5CHRCOOCH_2CH_2N(C_2H_5)$ and k_H or k_{OH}, and that the Taft formula could be applied to it. In addition, there was a linear relationship between the pKa and hydrolysis rate of the substituted esters. Additional studies of phenylacetic acid derivatives having a quaternary carbon at the 2-position indicated that the presence of steric hindrance in the position close to the ester linkage was very effective in stabilizing aminoalkyl esters (119). Rate constants were determined by Washkuhn *et al.* (120) for the alkaline hydrolysis of 36 *p*-substituted alkyl benzoates, and it was shown that these esters can be characterized on the basis of their rates of alkaline hydrolysis. Application of linear free energy relationships allowed a calculation of alkaline hydrolysis rate constants for alkyl or aromatic *p*-substituted benzoate esters not included in this study.

Murthy and Rippie (121) studied the effect of solvent anisotropicity on ester hydrolysis in lyotropic liquid crystalline phases. The reactions, as they occurred within the smectic phase, were characterized by relatively low apparent activation energies and by large negative entropies of activation. The hydrolysis of four carbonate and five carboxylic acid ester prodrugs of acetaminophen was determined in aqueous buffers at various pH's (122). The results suggested that it should be possible to formulate pharmaceutically stable suspensions of this type of drug. Mixtures of two amorphous alumina gels of different acid reactivity changed physically and chemically upon aging until a constant state was reached. The rate of conversion was found to be first order, temperature dependent, and directly dependent on the initial concentration of the less reactive gel (123).

The kinetics and mechanism of degradation of an allylic phosphate were studied as a function of pH and temperature by Oesterling and Gustafson (124). The activation energy for phosphate ester hydrolysis was 21.5 ± 0.9 kcal./mole. In the case of the solvolysis of methylprednisolone-21-phosphate, Flynn and Lamb (125) observed that there was an acceleration of reaction velocities and marked deviation from the expected pH dependency in more concentrated solutions (>0.02 *M*). This behavior was attributed to

association colloid formation. The rates of deamination of cytarabine, cytidine, and cytosine were compared in the presence and absence of catalytic buffers (126). Cytarabine exhibited only general acid catalysis, while cytidine and cytosine exhibited both general acid and general base catalyses. The hydrolysis of pilocarpine in aqueous solution was catalyzed by both hydrogen ion and hydroxide ion (127). Based on appropriate kinetic calculations, an optimum condition for the preparation of pilocarpine ophthalmic solution was suggested.

Other stability kinetic papers are listed in Table IV.

Antibiotic Stability—The kinetics and mechanism of degradation of clindamycin in buffered aqueous solutions were investigated by Oesterling (140). The antibiotic showed maximum stability at pH 3–5; however, high temperature studies indicated that not more than 10% degradation occurs in the pH range of 1–6.5 after 2 years at 25°. In a similar manner, the aqueous stabilities of lincomycin-2-phosphate and clindamycin-2-phosphate were studied at a variety of temperatures and pH values (141). Apparent first-order rate constants of phosphate ester hydrolysis, as well as activation energies (32.1 kcal./mole for lincomycin-2-phosphate and 32.9 kcal./mole for clindamycin-2-phosphate), agreed favorably. Stability studies of an antifungal antibiotic in selected ointment bases indicated glycerin monostearate was the most suitable (142). Ampicillin and carbenicillin stability in commonly used infusion solutions were investigated (143). Ampicillin showed wide variations of stability in the different vehicles, with least stability in solutions containing lactate ion; carbenicillin gave less than 10% decomposition over 24 hr. in all vehicles except 0.9% sodium chloride, in which 30% activity was lost. The degradation of a new synthetic broad-spectrum penicillin, 6-(1-aminocyclohexanecarboxamido)penicillanic acid, in aqueous solution at 37° was elucidated by the pseudo-first-order kinetics. This antibiotic was acid stable and was considered suitable for oral administration (144).

Systematic studies were carried out to prepare stable solutions of tetracycline HCl for intramuscular injection (145). A formulation containing 50–60% propylene glycol and filled under anaerobic conditions gave the most improved stability. The effects of pH, aliphatic oxyacids, vehicle type, sodium pyrosulfite, sodium bisulfite, and inert gases on the stability of tetracycline and its complexes with urea, calcium chloride, and sodium hexametaphosphate were studied (146). All showed maximum stability in weak acid media (pH 4–5), and their stability was influenced variably by lactic, citric, tartaric, and ascorbic acids: lactate gave maximum stability and ascorbic ion gave minimum stability. During microbiological determinations of chlortetracycline in riboflavin-containing solutions, the photolytic decomposition of riboflavin deactivated the antibiotic (147). Ascorbic acid or sodium thiosulfate was added to prevent this deactivation.

A study of streptomycin stability in aqueous solutions as a function of pH and temperature showed maximum stability at pH 4.0–5.0 (148). The effect of some B vitamins on the stability of dihydrostreptomycin sulfate in syrupy solutions was studied by accelerated stability methods (149). Both riboflavin and nicotinamide had a destructive effect on the antibiotic; thiamine hydrochloride had a slight destructive effect, while pyridoxine hydrochloride and pantothenate had a stabilizing effect. Dry oleandomycin phosphate, stored in evacuated containers, maintained activity within permissible limits for 1 year (150). Thermal sterilization of formulations of this antibiotic was not advised.

Additional references on antibiotic stability are presented in Table V.

Vitamin Stability—The sterically hindered vitamin A α,α-dimethyl palmitate was considered more resistant to autooxidation and more stable in the presence of the acidic emulsifier, diacetyl tartaric acid monoglycerides and diglycerides, than commercial vitamin A palmitate (159). Corn oil appeared to retard the loss of vitamin A ester potency due to acid-catalyzed degradation and isomerization. As part of a study of the stability of vitamin A, the results of a study on the stability of retrovitamin A acetate, an intermediate product of vitamin A acetate in aqueous ethanolic solution, was described (160). In a series of papers, the stability of vitamin A palmitate in selected ointments was discussed (161, 162). Generally, the vitamin was more stable in white than in yellow petrolatum; its stability in lanolin, freed of peroxides, depended on the acidity of the ointment base. Fairly stable ointments were prepared in eucerine and petroleum jelly. The stability of prepara-

Table IV—Additional References on Stability Kinetics

Reference	Topic
128	Stability of khellin
129	Effect of some hydrotropic salts on base-catalyzed degradation of khellin
130	Scheme for evaluating all new drugs by means of preformulation kinetic studies
131	Application of variable temperature kinetics with absolute reaction rate theory
132	Kinetics of hydrolysis of dimethylaminoethyl *p*-chlorophenoxyacetate hydrochloride
133	Salt effects on rates of alkaline hydrolysis of acetylsalicylate and acetylmandelate ions
134	Kinetic parameters necessary for evaluating stability of heat-sterilized pharmaceutical solutions
135	Hydrolysis of diamorphine in aqueous solutions
136	Comparison of stability programs for injectable phenobarbital solutions
137	Stability of phenylephrine hydrochloride in intravenous solutions
138	Kinetic study of aqueous solutions of sodium sulfacetamide
139	Oxidation of aqueous sulfite solutions

Table V—Additional References on Antibiotic Stability

Reference	Topic
151	Aqueous stability of triacetyloleandomycin and propionylerythromycin laurylsulfate; preparation of dry granules for reconstitution
152	Stability to heat and subsequent storage of chloramphenicol eye drops
153	Stability of mycetin D-17 during storage
154	Stability of chlortetracycline
155	Kinetics of degradation of tetracycline and 5-oxytetracycline and their initial degradation products
156	Stability of benzylpenicillin in aqueous solutions
157	Review of sodium methicillin in parenteral solutions
158	Review of factors influencing stability of penicillins

Table VI—Additional References on Vitamin Stability

Reference	Topic
178	Review of injectable solutions of water-soluble vitamins
179	Product acceptance factors in formulating a chewable multivitamin tablet
180	Stability of a vitamin A suntanning aerosol formulation
181	Improved stability of a vitamin B complex injectable by lyophilization
182	Review of kinetic studies on interactions and degradation reactions of ascorbic acid and thiamine
183	Physical stability of a tablet containing thiamine, pyridoxine, and cyanocobalamin
184	Degradation of thiamine in alkaline solution
185	Stability of vitamin C in various pharmaceutical preparations
186	Stability and mechanism of degradation of nicotinamidomethylaminopyrazolone
187	Stability of nikethamide solutions
188	Effect of copper ions on shelflife of vitamin C preparation

tions of vitamin A in a dry powdered form was studied (163). The combination of gelatin and dextrin plus 4.4% antioxidant was the most stable. In a series of papers, Bhattacharya (164–166) described the stability of various pharmaceutical vitamin A palmitate complexes in aqueous media. In general, the stability of vitamin A in an aqueous medium was much greater than in a dry state. The formation of anhydrovitamin A in these vitamin A complexes, which was dependent on the nature of the complex, was also elucidated.

The stability of thiamine hydrochloride in the presence of some antibiotics in capsules was determined by the use of accelerated stability methods (167). Tetracycline hydrochloride and chlortetracycline hydrochloride had some stabilizing action on the vitamin, but oxytetracycline hydrochloride and chloramphenicol had a destructive effect. A discussion of the mechanisms of stabilization of aqueous thiamine solutions by L-histidine indicated that the histidine complexes with metallic ions which cause the catalytic degradation (168). A kinetic study of pyridoxine hydrochloride in the presence of some antibiotics indicated that tetracycline and chlortetracycline hydrochlorides have a stabilizing effect on the vitamin, while oxytetracycline hydrochloride and chloramphenicol have a deleterious effect (169). The best container found for preserving aqueous solutions of cobamide (vitamin B_{12} coenzyme) against photolytic degradation was an amber borosilicate glass vial reheated to 650° (170). In other studies, cobamide in a lipoprotic vehicle remained more stable than when it was in an aqueous vehicle (171).

The effect of certain additives on the photochemistry of riboflavin was discussed in a paper by Shin *et al.* (172). It appeared from the compounds tested that an effective photochemical stabilizer should have a hydroxyl group, either attached to a benzene ring or in conjunction with the benzene ring. Light, pH, and heat affect the decomposition of folic acid injectable solutions (173, 174). Optimum stability was achieved at a pH value of 7.6. Vitamin K_1 in aqueous pharmaceutical preparations was relatively stable at pH 4.0–7.0 but unstable at higher pH (175). The degradation product in solution at the higher pH was isolated and identified. In addition, photolytic products of vitamin K_1 in the absence of oxygen were elucidated (176). An investigation of the influence of nonionic surfactants on the rate of degradation of menadione concluded that polysorbates (Tweens) stabilize the vitamin composition at neutral pH (177).

Other references pertaining to vitamin stability are listed in Table VI.

PHARMACEUTICAL TECHNOLOGY

The science and technology of microencapsulation, with applications in pharmaceutical and cosmetic fields, were reviewed by Luzzi (189) and Nack (190). In a series of review articles on aspects of pharmaceutical technology, a wide range of topics was discussed, including fluidization applications, pulverization with ball mills, polymeric film coating, and freeze-drying processes (191–195). Other articles outlined some effects of moisture, glidant addition, and mixing equipment on the flowability and mixing performance of bulk particle solids (196–198).

Parenterals—The effect of rubber closures on injection solutions was a topic of several interesting papers. The turbidity produced in aqueous solutions of viomycin, stored in glass vials and closed with rubber stoppers, was found by TLC and GLC to be caused by di-*tert*-butyl-*p*-cresol, tetramethylthiocarbamoyl disulfide, and mercaptobenzothiazole (199). In another similar study, stopper formulations containing zinc dithiocarbamates and thiurams caused much clouding; those containing 2-mercaptobenzothiazole, sulfenamides, or guanidines caused much less (200). Only very small amounts of turbidity were observed in solutions in contact with peroxide or phenol–formaldehyde resin-cured samples. Yanchick and Sperandio (201) studied the relative loss of benzyl alcohol-7-^{14}C when exposed to natural rubber, neoprene, and butyl rubber closures. In 1% solutions, the rate of loss depended on temperature and on the position in which the vials were stored; natural rubber gave the greatest loss and butyl rubber the least as temperature increased.

Precipitates found in admixtures of potassium chloride and 5% dextrose in water were identified as silica and alumina (202). Another study of particulate matter in intravenous solutions revealed that considerable variation existed in the same product from different manufacturers and in different solutions or different lots of the same solutions from the same manufacturer (203). The addition of devices or additives increased the number of particulates, but a final filter set was very effective. Particulate contamination in vials of sterile dry solids upon reconstitution was examined, using a membrane filtration technique and subsequent microscopic examination and counting of particles (204). Significant differences were found in particle counts, but not size distribution, in the products of different manufacturers. No significant fall in activity with time was observed in solutions of noradrenaline containing 5% dextrose, 5% dextrose in physiological saline, and acidified physiological saline (pH 3.6), but there was a significant loss of activity in physiological saline and 4.2% sodium bicarbonate solution (205). Parenteral admixtures of metaraminol

bitartrate and hydrocortisone sodium succinate with 5% dextrose in normal saline remained stable for at least 48 hr. (206). The buffer capacity and pH of a number of commercial infusion solutions were determined (207). For purposes of calculating pH after drug admixture, the buffer capacity of pure electrolyte solutions and dextrose or dextran fluids of pH 4.5 need not be considered.

A simple technique to control aseptically the required pH of solutions for the preparation of $^{113}In^{m}$-labeled ferric hydroxide particles was described (208). The advantage of the procedure was the elimination of autoclaving time, a critical factor when the nuclide has a short half-life. Residual biological activity of injectable solutions of cyano- and hydroxocobalamins after ^{60}Co γ-irradiation was higher than expected from photometric determinations (209). Solutions of high vitamin content were more resistant than low ones, hydroxocobalamin being more resistant than cyanocobalamin. A number of drugs sterilized by γ-irradiation showed little or no breakdown (210). However, in solutions of low concentration (mostly <0.5%), these compounds underwent extensive change.

Intravenous injection of 7,12-dimethylbenz[*a*]anthracene in dimethyl sulfoxide was judged to be at least as active as fatty emulsions now in use (211). Intravenously administered soybean emulsions of pentobarbital and thiopental prolonged the sleep time in mice compared with the corresponding aqueous solution (212). In another experiment, a similar effect was observed when helianthic and linseed oils, dimethyl sulfoxide, polysorbates 60 and 80 (Tweens 60 and 80), or propylene glycol was administered prior to hexobarbital treatment (213). The use of additives to enhance the stability of injectable solutions of a diethylamine salt of phenobarbital (214), reserpine (215), and esters of diphenylacetic acid derivatives (216) was reported. The effects of pH, solvent, light, metal ions, container, and oxygen content of solvent water on the stability of injectable L-ascorbic acid solutions were elucidated (217, 218). Maximum stability of the aqueous solution was found at a pH value of 6.5. Cysteine hydrochloride was used to stabilize dexamethasone-21-phosphate injectable solution sterilized by bacterial filtration (219). The best stabilizer of an injectable calcium gluconoascorbate was a mixture of 0.1% thiourea and 0.1% cysteine (220). The behavior of castor oil under conditions of thermal sterilization gave no indication that it required a more careful sterilization procedure than that used for peanut oil (221). Sterilization at 180° for 25 min. was recommended.

Other papers of interest in the category of parenterals are listed in Table VII.

Table VII—Additional References on Parenterals

Reference	Topic
222	Compilation of stability and compatibility problems of drugs in injections or intravenous infusions
223	Review of contamination of injectable solutions by perfusion and transfusion kits and other associated equipment
224	Critical discussion of particulate matter in solutions for injection and methods of determination
225	Description of apparatus for determining particulate matter originating in intravenous infusion sets
226	Effect of contact with metal equipment on stability of papaverine solutions for injection
227	Review of pharmaceutical uses of polyvinylpyrrolidone in parenterals
228	Incompatibility of drug mixtures for parenteral administration
229	Parenteral formulation procedures for investigational drugs in cancer chemotherapy
230	Compilation for preparation of solutions for injection using a weight–volume ratio
231	Incompatibility of commercial injections (298 combinations) as examined by TLC
232	Treatment of activated carbon used for purifying 40% glucose solution for injection
233	Demonstration of thermostability concept in preparation of sterilized aqueous solutions
234	Production of infusion fluids of low particle content

Sterility—The various sterilization processes employed in the pharmaceutical industry were reviewed and discussed (235). Methods of preparing sterile eye drops and ointments according to prescription were also described (236). The pattern of temperature rise in articles undergoing sterilization in an oven under normal working conditions was investigated by Peacock and Ridgway (237). Mathematical models, each expressed in terms of a set of differential equations, were fitted to the data obtained. Knowledge of the heating rate in a bottle of known volume permitted calculation of the rate in different size bottles containing the same liquid and undergoing the same heat treatment (238).

Various techniques for ethylene oxide sterilization were described and evaluated by Gunther (239). In another article, factors influencing the degassing of ethylene oxide-sterilized rubber and plastic articles and the toxicity of reaction products in these polymers were described (240). Ethylene oxide as a propellant and sterilizing agent was proposed as one method of assuring sterility in filled aerosol containers (241). An interesting paper by Chen (242) demonstrated the ability of 100% ethylene oxide to penetrate the silicone coating used on disposable hypodermic needles and syringe rubber plunger tips. Spores of *Bacillus subtilis* var. *niger*, introduced underneath the silicone coating, were killed by the ethylene oxide treatment. β-Propiolactone was put to a unique use in a method of sterilizing pig heart valves (243). An apparatus designed for sterilization with formaldehyde was described for use in pharmacy (244), and the parameters for effective formaldehyde sterilization were established.

Methods of radiation sterilization in the pharmaceutical industry were reviewed (245, 246). The effect of γ-radiation, as used in the radiosterilization of glass ampuls, on the resistance of glass containers was investigated. This radiation gave no lowering in classification of glasses in the hydrolytic resistant groups (247).

A membrane filtration procedure for testing the sterility of insulin zinc suspensions solubilized in ascorbic acid diluting fluid was a significant improvement over the direct method of sterility testing (248). The removal of abnormally large amounts of added pyrogens in 15 different standard infusion solutions by filtrations through an asbestos filter layer was investigated (249). The filtered solutions were pyrogen

Table VIII—Additional References on Sterility

Reference	Topic
250	Review of filtration methods for injection solutions
251	Review of use of gaseous sterilizing agents
252	Review of microbiological aspects of drug production
253	Discussion of factors to assure sterile water conditions
254	Significance of primary contamination testing in routine studies for rejection of ampul preparations

free, with the single exception of a high sugar content infusion.

Other papers relative to the subject of sterility are listed in Table VIII.

Tablets and Capsules—The factors affecting the compression of tablets were reviewed by Livingstone (255). Special emphasis was given to the operational steps necessary in direct compressions. In an excellent article by Fonner *et al.* (256), optimization techniques in drug product design and process analysis were presented. This method was applied to generate optimal formulations in typical tablet design problems and to locate optimizing variables in a typical encapsulation design problem.

The effect of several grinding variables on the flow characteristics of liquids and solids through a small continuous wet ball mill was investigated (257). It was found that none of the major grinding variables caused major changes in the form of the distribution (logarithmic normal) of residence time function. In one laboratory-scale fluid energy mill, a reduction in particle size to subsieve size was attained. The product particle-size distribution was very sensitive to feed rate (258). In exploratory experiments on kinetics of comminution, sodium chloride was milled in a ball mill at relative humidities of 0.5 and 4% for various periods (259). The particle size, as determined by sieve analysis, was reduced until the load no longer sufficed to break the particles. Thereafter, they agglomerated, particularly in drier air, and the agglomerate size increased with increased milling time. Some statistical methods for evaluating particle-size distribution were studied and compared (260).

The origins, nature, measurement, and effects of particle residual stresses were critically reviewed by Hirschhorn (261). The possible primary causes of residual stresses were listed as uniform and nonuniform plastic deformation, temperature and thermal strain differentials, and phase transformation in individual particles. The radiological techniques necessary to obtain quantitative geometric descriptions of particle packings (particles of any shape and a large range of sizes) were developed (262). A mathematical expression was given to the relationship between the number of equally sized spherical particles and the porosity of a unit cross section of a sedimentation bed resulting from the packing of powder (263).

Several interesting papers appeared on the mixing and blending of bulk pharmaceutical powders. The factors that contribute to the kinetic angle of repose of powders in the flights of rotary drums were shown to be particle size, drum speed, and the type of material (264). Measurements of the static angle of repose were statistically correlated with particle size. The mixing of components of similar size and density in a horizontal drum mixer were characterized by a parameter, the migration coefficient, which was used in developing a scale-up procedure (265). Segregation occurring when different particle sizes were present was investigated for a number of different drum mixers. An apparently randomized mixture was obtained when the drum was rotated at the speed at which the porosity of each component was maximum. An equation was derived for the variance of the composition of samples drawn from nonrandom binary mixtures (266). The index of mixing was equal to zero for no mixing and one for random mixing.

The present state of knowledge of continuous mixing was reviewed by Williams and Rahman (267). After considering available experimental techniques, a simple method suitable for solid mixers was described. It was shown that the performance of an inclined rotating drum mixer, investigated by the δ-input response technique, could be assessed for establishing the best operating conditions of a given system. In studies on the mixing of pharmaceutical powders, cohesive powders charged to the bottom of a V-type mixer behaved like "diffusion" from the charging position of the active ingredient (268). In an experiment to elucidate the mechanism of lubricant action in the operation of mixing and lubricating pharmaceutical powders with calcium stearate in a V-type mixer, a relationship was observed between the changing rate of apparent density of the powder and the cumulative revolution number of the mixer (269).

A good correlation was shown to exist between the flow properties of clomacran phosphate powder blend and capsule fill-weight variation when an automatic capsule-filling machine was used (270). Most of the weight variation of the finished capsules, also affected by lot-to-lot variability and concentration of the drug, was overcome by a granulation procedure. The apparent specific volume and the breaking strength of mixed systems found in granulating processes were investigated in relation to the filling properties of the systems (271). The changes in extrusion weight and other properties of the systems with the lapse of time in the process of granulation were discussed in relation to changes in particle compactness and breaking strength. The effect of properties of binder solutions was also investigated in the same mixed systems (272): the higher the viscosity of binder solution, the greater the tensile strength. In addition, it was found that the extrusion weight of granules through an extrusion-type granulator was inversely proportional to the breaking strength of the mixed system.

An investigation of the effect of particle shape on the variation of the fill in a tableting die found that the mean contained weight was greater for regular than for irregular particles (273). A maximum die fill occurred when the ratio of die diameter to particle diameter was about 20 for all particle shapes. The influence of glidants on the technical properties of tablet granulation was studied by comparing the weight variations of tablets with flow properties of the granulations, as measured by the angle of repose, the flow rate, and

the internal angle of friction (274). No correlation was observed between flow properties and weight variations of the tablets. In evaluating a glidant and in selecting its concentration in a tablet formulation, the homogeneity of the powder mixture and the possibility of segregation during preparation were considered (275).

Evaluation of a tablet-lubricating agent by electronic pressure measurements from an instrumented press was demonstrated for magnesium stearate (276). For every lubricant, a concentration could be established above which the friction conditions in the machine were not changed even with high compression numbers. Examination of a mixture of sodium bicarbonate and magnesium stearate, using an optical and a scanning electron microscope, showed that magnesium stearate formed a replicalike film over the surface of the bicarbonate particles (277). Flow regulation of powders of various particle size, form, and surface structure was studied with a preparation of aluminum hydrosilicate (278). This glidant exerted either a positive or negative flow regulation; thus, the optimum concentration must be determined for each powdered substance individually.

The effect of surface-active agents as lubricants for tablets on the release of active substances from compressed tablets was studied by comparing dissolution rates of some steroid formulations (279, 280). The concentration of the surface-active agents was not high enough to lower the surface tension of the solution significantly or to increase the solubility of the steroid, but their use as lubricants increased the release of the active ingredient. Similar effects were observed on sulfanilamide pellets and tablets (281). The differences in the minimum amount of polysorbate 80 (Tween 80) and poly(oxystearate)-40 required for the complete hydrophilization of powders for tablet manufacture were interpreted in terms of the various hydrophilic–lipophilic balance (HLB) values of the surface-active agents, different hydrophobicities of powders, and the different grain sizes of powder particles (282).

The influence of tableting pressure on the surface texture of tablets consisting of corn starch, lactose crystals, and cyproterone acetate microcrystals was examined by the scanning electron microscopic technique (283). Deformations of the starch grains in formation of solid bridges through the lactate and cyproterone crystals were observed. Tablets of magnesium carbonate placed in water disintegrated rapidly into their components, presumably due to breakdown of interparticulate bonds (284). Particles of known size were compressed and allowed to disintegrate to give evidence of fragmentation or interparticulate bonding. Moisture in tablets (2.5–6.6%) reduced the surface area. This was due to improved lubrication and, for more soluble substances, to recrystallization, which permitted the formation of interparticulate bonds (285). Similar effects of moisture on the compression of tablets were observed as a function of their apparent density (286).

The use of compression modulus to describe the effect of moisture on compression behavior was discussed by Rees (287). He presented data to show that, at higher pressures, it is insufficient to quote values of the pressing modulus unless the actual relations between pressure and relative volume are considered. Measurements of the temperature rise occurring on compression, recompression, and relaxation of tablets were investigated by means of thermocouples inserted in the compressed materials (288). With all tablets, a rise in temperature was observed on initial compression and a fall on relaxation. The temperature rise was further separated into two components: that due to compaction and a further rise due to elastic compression. In another investigation, the rise of temperature on the upper and lateral surfaces of tablets during compression was examined. The rise of temperature was linearly related to the logarithm of compression time or the logarithm of ejection force (289).

Tableting behavior of crystalline and spray-dried lactose as a function of particle size, shape, and storage of powders was examined under direct compression (290). The results indicated that particle size had little effect, but particle shape and storage influenced the strength of the tablets. Henderson and Bruno (291) found that lactose USP (beadlets) and dextrose (PAF 2011), two new agents for direct compression, were generally superior to the other materials they tested (spray-dried lactose USP and anhydrous lactose USP) for use as fillers for direct compression. Direct compression tablet formulations of phenobarbital, sodium phenobarbital, phenacetin, butyl scopolammonium bromide, and rutoside with tableting agents were described by several authors (292–294).

The rate of moisture uptake by loosely packed and tapped beds of six pharmaceutical materials was found to be independent of bulk density but dependent upon bed depth (295). The tensile strengths of the bulk solids changed when subjected to high relative humidities. The use of a microindentation apparatus to determine the hardness of tablets at various points on their diameter was described (296). This apparatus measured the total deformation at the loaded point on the tablet and also elastic recovery when the load was removed. The diametrical crushing strength of an ideal system of compressed particulate material and the relation between strength and mean compression pressure were found by Rees and Shotton (297) to depend on the time at which the strength was determined. Increases in strength of over 100% during the 1st hr. after compression were attributed to stress relief of the crystals and interparticulate bonds.

An investigation of the breaking strength of tablets containing lactose and microcrystalline cellulose showed that for tablets of 2–30-kg. nominal strength, an increase in the loading rate produced a significant increase in the breaking strength (298). These results, obtained using an Instron universal testing instrument and compared with those obtained using other instruments, indicated that discrepancies in the strength values may be partially attributed to differences in the rates of loading. The tensile strength of lactose tablets, as contrasted to crushing strength, was measured by applying the diametral compression test described by Fell and Newton (299). To obtain reproducible

results for the strength of tablets prepared at a given compression force, it was shown that the tablet must break in such a manner that the tensile strength is the major stress. Usually, there appears to be no way of quantitatively predicting the properties of tablets by considering the properties of the individual components. However, for a single material such as lactose which exists in three forms (α-anhydrous, β-anhydrous, and α-monohydrate), it was possible to predict the strength of tablets prepared from mixtures of the three forms from measurements of the strength of tablets prepared from the individual components (300).

The influence of a number of tableting parameters on the pore structure and disintegration characteristics of model tablet formulations was reported in a series of interesting papers. The addition of starch to the various tableting materials produced no significant effect on the pore structure of the dry tablet but caused disintegration of this structure when the tablet was penetrated by water (301). This effect was a function of the compressibility of the constituent materials and the pressure used to compress the tablet. In studying the effect of granule properties on the pore structure of tablets of sucrose and lactose, it was found that low pressure, high density, high strength, and large granule size promoted a more open but less uniform structure (302–304). Such conditions produced a bimodal pore-size distribution. Although conditions of wet granulation influenced the pore structure of lactose tablets prepared over a wide range of compression pressures, dry granulation only influenced pore structure when slugging pressures were high, the granules were coarse, and tableting pressures were low. The effectiveness of the disintegrating ability of starch was dependent on the solubility of the tableted drug; disintegration rates decreased with increasing water solubility of the drug (305). The beneficial influence of some crosslinked polyelectrolytes and crosslinked gum arabic in some difficult to disintegrate tablet formulations was reported by Hirata (306, 307), along with a discussion of the disintegration mechanism.

The adhesion of film coatings to surfaces of compressed tablets was examined by Wood and Harder (308) for the effect of the critical surface tension of a tablet surface and the surface tension of a coating solution and its components on the strength of the adhesive bond between the film coating and tablet surfaces. Tablets with higher critical surface-tension values had increased bond strength, but the hypothesis that decreasing the surface tension of the coating solution and balancing the surface tensions of the components to yield a positive system should result in increased bond strength was not clearly established by experimental evidence. Other experiments, conducted to characterize tablet surfaces by their critical surface-tension values, indicated that increased critical surface tension results in increased wetting by the coating solution and in an increased bonding force between the tablet surface and the polymer film coating after the solvent has evaporated (309).

A statistical analysis was made of the variation from tablet to tablet in the amount of drug in the coat of sugar-coated tablets. Although the standard deviation in a series of batches was linearly related to the square root of the number of coats, the plot failed to intersect at the origin and did not have the required slope (310). Apparently, other factors such as viscosity and surface activity of the syrup suspension, core shape, and pan build-up contribute to the variation. An improved self-programming automated tablet-coating system, based on the rate of moisture loss in the tablet mass, was designed and evaluated by Heyd and Kanig (311). A high degree of sensitivity of drying cycles to changes in environmental humidity in the coating pan was demonstrated. Methods and equipment found useful for recording temperature patterns resulting from the evaporation of volatile coating solutions applied to pellets in a rotating pan were discussed (312). These characteristic patterns were interpreted in regard to run-to-run replication, effect of application and drying rate, and coating-solution distribution. The manufacturing technique for titanium dioxide lakes, prepared from certified water-soluble dyes, and the pharmaceutical application in color-coating tablets were described and discussed in conjunction with automatic spray coating (313). Substances evaluated for enteric coating of tablets included polyglycerol stearate, poly(vinyl acid phthalate), gluten, gliadin, and glutenin (314–316). The feasibility of large-scale production of cellulose acetate phthalate enteric-coated capsules, based on the use of a fluidized-bed spray film coater, was discussed by Jones (317).

Further references of interest in the general area of tablets and capsules are listed in Table IX.

Suspensions—A model for rheological behavior of thixotropic systems was advanced for suspensions of rigid, solid particles in liquids (353), but it was not expected to be valid for materials of a polymeric nature or for suspensions of elastic, deformable particles in liquids. Some applications of rigidity and yield values in a study of gelatin and laponite gels were described (354). An important property of thixotropic hectorite gels was found to be the relatively rapid sol–gel transformation.

Depending on the concentration of a suspension, it will exhibit one of three sedimentation patterns pertaining to low, intermediate, and high concentrations of solids. The sedimentation kinetics of flocculated suspensions in the initial sedimentation region and the following intermediate region were investigated from both a theoretical and experimental standpoint by Carstensen and Su (355, 356). In the initial phase, it was shown that sedimentation data were consistent with theory. Empirically, these data can be evaluated by plotting the square root of height as a function of time; the slope of these lines appears to be proportional to the ratio of density difference to viscosity. Sedimentation of the intermediate phase presented as a theoretical equation was also supported by experimental data. A method of comparative analysis of sedimentation and Brownian motion was described for determining the maximum diameter of particles that will not sediment in an aqueous suspension (357). These calculations by a computer yielded valuable information as a guide to suspension formulation, but the presence of suspending agents nullified the method.

Table IX—Additional References on Tablets and Capsules

Reference	Topic
318	Discussion of a thixotropic agent (Aerosil) for the improvement of the flow characteristics of powders
319	Study of packing of irregular particles
320	Method for dielectric study of solid powders
321	Statistical analysis of uniformity of distribution of cyanocobalamin in tablet formulations
322	Fluidized-bed particle coating
323	Survey of research on powders done in the School of Pharmacy, University of London
324	Use of cordia myxia mucilage as a binder in tablet manufacture
325	Effect of various drying conditions on rates of drying of pharmaceutical preparations
326	Changes in physicochemical properties of a synthetic magnesium aluminum silicate due to hydrothermal treatment
327	Sampling approach to ensure tablet uniformity throughout a tableting run, and assessment of powder mixtures for cosmetics and pharmaceuticals
328	Preparation of fat-based pills using a Piluterm apparatus
329	Unique device for external lubrication of die in preparation of effervescent tablets containing nicotinic acid and sodium bicarbonate
330	Review of uses of mannitol in production of lozenges and chewable tablets
331	Preparation of sterile implants by compression
332	Formulation with microcrystalline cellulose of a morphine implantation pellet
333	Fusion for preparation of two-component tablets containing sodium salts of organic acids
334	Quality control method for determining tablet and capsule defects by visual inspection
335	Effects of cooling and granulation mixing time on amidopyrine tablets
336	Influence of humidity on granules in the compression of sodium salicylate and *p*-aminosalicylate
337	Effect of temperature and compression on tensile strength of compressed particles
338	Introduction of working bulk density as a concept in powder technology
339	Effect of methylene casein on the physical properties of tablets
340	Discussion of a disintegration apparatus for evaluation of drug availability
341	Comparative evaluation of seven dissolution apparatus for capsule dosage forms
342	Release of isoprenaline sulfate from certain sublingual tablet bases by disintegration time measurement
343	Effect of compression pressure on the disintegration of some sulfonamide tablets
344	Effect of powdered celluloses on the strength and disintegration rate of compressed tablets
345	Tabulation and discussion of tablet hardness and disintegration time measurements on 105 products
346	Principles, method, and apparatus for automatic coating of tablets
347	Review of enteric coatings
348	Influence of various additives on softening point of shellac films
349	All-glass laboratory apparatus for fluidized-bed coating of tablets or pills
350	Review of film coating of pharmaceuticals
351	Review of film moisture protective coatings for orally administered drugs
352	Manufacturing procedures of hollow gelatin capsules

Investigation of highly flocculated suspensions of clay in batch tests showed that the rate of formation of sediment of a given concentration decays exponentially with time from the start of a test (358), but established theory predicts that it should remain constant.

The influence of electrolyte, type and concentration of surfactant, and nature of vehicle upon the physical properties of sulfaguanidine suspensions was investigated (359). It was reported that acceptable suspensions of this drug were produced by controlled flocculation in the sulfaguanidine–polysorbate 80–aluminum chloride system. In a point of controversy, other authors presented data showing that flocculation cannot take place in this system (360). The effect of adding a wetting agent on the physical properties of sulfamerazine suspensions was described (361). When all the components were mixed totally and simultaneously, a more flocculated and more stable suspension was produced than when the wetting agent was added first, followed by the hydrophilic suspending agent. The effect of alcohols, surfactants, lipophilic materials, and temperature on the rate of sedimentation of aqueous talc and zinc oxide suspensions was reported (362–365).

In studies of the coagulation kinetics of mixed suspensions, Matthews and Rhodes (366) used a computer to predict the particle-size profile during the coagulation of a mixture of two normally distributed particulate suspensions; they tested the Müller equation for the coagulation of a mixture of large and small particles using the Coulter counter. A critical evaluation of the use of the Coulter counter model B in coagulation studies was made, and the implications of the effect of coarsening the suspension on the detection of small particles was discussed (367). Particle-size analyses of some pharmaceutical suspensions were made by the Andreasen pipet method, sedimentation balance, and the Coulter counter (368). As long as the particle-scattering coefficient was constant, controlled by the volume and surface area distribution functions, values from the pipet method yielded accurate results. The Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory was used by Matthews and Rhodes (369) to interpret pharmaceutical suspension stability. Practical guidelines for formulation of suspensions by a coagulation technique were presented. The dispersibility of sulfisoxazole, sulfadimethoxine, and N_1-acetylsulfisoxazole in aqueous sucrose solutions containing polyoxyethylene nonyl phenyl ether or sodium lauryl sulfate were examined (370, 371). From these results, it was concluded that the dispersive sedimentation of sulfonamides began at the polyoxyethylene nonyl phenyl ether concen-

Table X—Additional References on Suspensions

Reference	Topic
372	ζ-Potential and its use in pharmaceutical technology
373	Review of pharmaceutical suspensions
374	Effect of carboxymethylcellulose on dehydration of kaolin suspensions in the filter press
375	Properties of Spanish agar
376	Interpretation of Beer's law curves for concentrated dye suspensions
377	Review of action of dispersing agents in pigmented aqueous dispersions
378	Stabilization of calamine lotion and milk of magnesia with carboxymethylcellulose or guar gum
379	Effect of nonionic surface-active agents sorbed onto organoclays on rheological properties of oleopseudogels
380	Criteria for estimating amounts of pectins of different viscosities in the manufacture of suspensions
381	Layering phenomena in colloidal suspensions
382	Settling characteristics of precipitated magnesium hydroxide suspensions

Table XI—Additional References on Emulsions

Reference	Topic
412	Influence of some emulsifiers on stability of cosmetic emulsions
413	Effect of emulsifiers on distribution of medicinals in emulsion systems
414	Emulsion-forming characteristics of complex emulsifier systems
415	Aging processes of model emulsions with complex emulsifier systems
416	Method involving ternary phase diagrams for developing stable emulsions
417	Demonstration of a ternary system in an aerosol emulsion preparation
418	Effects of solid additives on formation and separation of emulsions
419	Factors affecting stability of concentrated emulsions
420	Influence of nonionic surface-active agents on quality of o/w emulsions
421	Stability of emulsions using sodium dodecylbenzenesulfonate
422	Emulsion and aerosol formulation
423	Formulation of castor oil emulsion
424	Mechanism of emulsification in bidisperse systems

tration of about 1×10^{-3} mole/l., and the dispersibility was decreased by the addition of sucrose. In the case of sodium lauryl sulfate, the dispersive sedimentation began at 4×10^{-3} mole/l. and was not affected by the addition of sucrose.

Other articles relating to suspensions are listed in Table X.

Emulsions—Numerous review articles were published on the theory and practice of emulsions and microemulsions and the critical factors associated with emulsion systems (383–394). Characteristics of inclusions in the dispersed phase of liquid–liquid suspensions, a structure known as a double-multiple emulsion, were determined by an investigation of the hydrodynamic formation of these structures (395). A simplified model, based on the use of spray nozzle parameters, satisfactorily correlated percent included area and volume as a function of the Reynolds number and Weber number. An investigation of the phase equilibria on properties of an emulsion showed the presence of liquid crystalline phases and also explained the sudden changes in stability, viscosity, and even inversion that can take place with small changes in emulsifier concentration (396, 397). The influence of micelles in the system was also pointed out.

An interesting paper was presented by Garrett (398) on the prediction and evaluation of emulsion stability with ultracentrifugal stress. Nonstratified creams demonstrated an initially apparent first-order process of large particle drainage from the cream simultaneously with continuous drainage of the heavier surfactant. The final rate of oil separation was constant, reflecting the rate-determining coalescence at the cream–oil interface. Use of statistical experimental design studies on emulsification with various emulsifiers indicated that the stability was affected more by the volume ratio of the water phase than by the amount of emulsifier or method of mixing (399). The stability was also influenced by temperature. The stability of w/o emulsions as a function of temperature and of the hydrophilic chain length of the emulsifier was examined (400). Relatively stable w/o emulsions were obtained when the phase-inversion temperature of the emulsions was about 10–40° lower than the storage temperature. Differential thermal analysis procedures were described for determining the phase-inversion temperature of o/w emulsions stabilized by polyoxyethylene surfactants (401). With a given oil phase, the phase-inversion temperature increased linearly as the HLB of the surfactant increased. Different methods of determining emulsion stability and HLB of surfactants were the subjects of several papers (402–404). These procedures were based on measuring the intensity of reflected β-radiation, electronic counting with a Coulter counter, and measuring a characteristic change of viscosity at the stage of phase inversion.

The autooxidation of o/w emulsions was found to depend on the hydrogen-ion concentration (405). Oxygen uptake measurements and peroxide number determinations increased with rising pH up to a maximum at about pH 10 and then fell again at a still higher pH. Hydroxycarboxylic acid antioxidants suppressed the metal-catalyzed autooxidation of o/w emulsions (406). The chelating agents, citric and tartaric acids, were shown to inhibit oxidation of oil with low peroxide numbers, but this effect was not observed for ferrous ions in samples of high peroxide number.

Liquid paraffin emulsions, stabilized by cetrimide or sodium dodecyl sulfate with long-chain alcohols, were examined by microscopy, particle-size analysis, continuous shear rheometry, and creep (407, 408). The rheological properties of each emulsion were not directly related to particle-size distribution but to the nature of a gel network present in the continuous phase. Using very similar mixed emulsifier systems, Talman and Rowan (409–411) investigated the increase in consistency of o/w emulsions. The results indicated that for a fatty alcohol or acid in conjunction with the surfactant to have any self-bodying action in an emulsion, two conditions must be met: (*a*) a portion of the alcohol or acid must migrate from the oil to the aqueous phase, and (*b*) the surfactant molecules must be of such size and shape or be present in sufficient concentration to permit penetration of the amphiphile crystal lattice, thus forming a ternary liquid crystalline phase.

Other articles related to emulsions are presented in Table XI.

Ointments and Creams—New representations of the theoretical molecular concept of ointment structure was discussed by Huettenrauch (425). The concept included molecular order, molecular geometry, supermolecular structure, and total gel structure. The release, uptake, and permeation behavior of salicylic acid in ointment bases were investigated by Nakano and Patel (426). Release of salicylic acid from five ointment bases indicated that emulsion-type ointment bases are superior to oleaginous and polyethylene glycol ointment bases. The relative importance of the factors involved in the permeation of the drug from aqueous solution through ointment bases to another aqueous solution was studied with a three-compartment diffusion cell. The effect of adding aqueous solutions of methylcellulose to hydrophobic ointment bases on the diffu-

sion of salicylic acid was studied using an agar diffusion procedure (427). Enhanced diffusion of the acid from various ointment bases was observed. A study of the effect of polyhydric alcohols in ointment bases showed that the polyols had no effect on the release of sulfathiazole and salicylic acid (428). For long-term storage of the ointment bases, glycerol and sorbitol were the best humectants, and propylene glycol tended to reverse the emulsion type. The use of polyethylene glycols as an ointment base, combined with hydroxypropylmethylcellulose and water, was tested in model preparations (429). The viscosity, flow point, and cloud point of the compositions decreased and the medication release rate increased as the proportion of the hydroxypropylmethylcellulose–water hydrogel in the compositions increased. Tertiary phosphoric acid esters of poly(ethylene oxide) fatty acid alcohol esters, as used in washable dermatological ointments, were investigated (430). The lauryl derivative, in liquid petrolatum solutions, took up water in almost equal proportions to the ester concentration without becoming turbid.

The addition of hydrogenated lanolin or propylene glycol to a mineral oil base containing β-methasone-17-valerate or 21-deoxy-β-methasone-17-propionate markedly increased the vasoconstrictor potency of the valerate but not of the propionate (431). Two *in vitro* model systems for the study of the release of valerate from various vehicles were described. It was found that prednisolone in creams undergoes undesirable crystal growth to form the stable crystalline prednisolone hydrate (432). When the hydrate was used in the cream, no crystal growth occurred. Ointments containing promethazine hydrochloride, pheniramine *p*-aminosalicylate, and chloropyramine all showed some degree of photolytic and oxidative degradation (433, 434). The least change was noted in a water-free simple ointment. The use of some antioxidants was also shown to be of value. The effects of radiosterilization on the physical, chemical, and biological properties of ophthalmic ointments containing hydrocortisone acetate or chloramphenicol were reported in a series of articles (435–437). The use of γ-rays for the sterilization of these ointments appeared satisfactory. Degradation of liquid petrolatum by *Mycobacterium rhodochrous* was reported by Myers and Leslie (438). These findings were discussed with respect to drug and cosmetic preparations containing liquid petrolatum.

Other articles of interest relating to ointments and creams are listed in Table XII.

Table XII—Additional References on Ointments and Creams

Reference	Topic
439	Study of suspending agents used in calamine lotion
440	Evaluation of new emulsifiers for ointments
441	Evaluation of stickiness as a sensory test of rheological properties of w/o type cosmetic creams
442	Characterization and evaluation of bone fat as an ingredient of ointment bases
443	Apparatus useful in surveying drug release from ointments
444	Rheological and spectroscopic investigation of ointment bases
445	Review of preparation of cream emulsions
446	Observations on suspension stability of boric acid ointments
447	Observations of particle-size change in yellow mercuric oxide eye ointments with aging

Suppositories—The value of rheological measurements for the characterization of suppository vehicles was comprehensively investigated (448). Such measurements were found useful as criteria for evaluation of lipophilic suppository vehicles. The results obtained confirmed the colloidal character of lipophilic vehicles used in the production of suppositories. Medicament release from fatty suppository bases was related inversely to the consistency of mixtures of cocoa butter with different fats in terms of the viscosity index (449). Comparative *in vitro* investigations on the release of sodium chloride from water-in-oil suppository bases showed that surface-active agents with increasing HLB values result in increasing release of the sodium chloride (450). The *in vitro* release of active ingredients from triglyceride base suppositories was investigated. The release of materials such as codeine base, carbostyrylsulfonic acid, and aminophenazone, soluble both in fat and water, occurred according to their distribution coefficients in the different phases (451).

Hardness of suppositories based on various lipophilic substances was examined as a measure of the mechanical stability of the suppositories (452). Generally, the hardness of suppositories prepared and stored at 24° decreased with time. Those containing glyceride-type bases had the greatest hardness immediately after preparation. A spreading test was described for estimating the change in physical–chemical characteristics of suppository bases due to aging (453). This spreading effect was decreased by aging. From the biopharmaceutical viewpoint, it was pointed out that as high as possible a spreading effect is desired to obtain optimum drug liberation. The relation between artificial aging of lyophilic suppositorial vehicles and the time of full deformation of the suppository was investigated (454). The most pronounced changes were observed in suppositories prepared from aged cocoa butter; these underwent full deformation in 40% less time than those prepared from nonaged vehicles.

Xerogel dosage forms, prepared by lyophilizing frozen hydrogels of various shapes, were tested for their suitability for rectal administration (455). In addition to their chemical and physical stability, these new classes of xerogel dosage forms showed advantageous properties for application in body orifices with poor liquid supply. Polytetrafluoroethylene coating

Table XIII—Additional References on Suppositories

Reference	Topic
457	Release of diazepam from suppositories
458	Solubilization of drugs in suppositories of hydrosoluble bases
459	Two-layer suppository with a gelatin–glycerol base
460	Biological efficacy of camphor and strophanthin in two-layer suppositories
461	Stability of aminophylline suppositories designed for warm climates
462	Characterization of colored degradation products from aminopyrine suppositories

as a suppository mold-releasing agent was examined (456). It was found most effective on the release of fatty base suppositories, particularly from damaged molds, but had little effect on polyethylene glycol suppositories.

Further articles of interest on suppositories are presented in Table XIII.

Aerosols—Many papers were published during the past year reviewing the theoretical principles and technological development of aerosol systems (463–473). These papers discussed the operating parameters of aerosol systems, typical aerosol formulations and methods for evaluating them, and the numerous uses of aerosol products. The stabilization of aerosol emulsions and foams was considered by Sanders (474). The hypothesis was advanced that the interfacial region around propellant droplets in aerosol emulsions stabilized with molecular complexes is polymolecular and that the molecular complex has a liquid-crystal structure, almost insoluble in either the propellant or aqueous phase, which concentrates at the interface and stabilizes the emulsion by forming essentially a solid interfacial film. The evidence that molecular complexes have liquid-crystal structures in aqueous systems and that almost all aerosol emulsions and foam stabilizers are practically insoluble in both aqueous and propellant phases was the basis given for this hypothesis.

The release of ingredients from aerosols containing selected film-forming agents was examined, using gentian violet as a model substance (475). As the polarity of the film decreased, the rate of release increased; this release from the films studied followed first-order kinetics. Hardness and tackiness of various film formers of hair sprays were studied with the aid of a pendulum hardness tester (476). The results suggested that not only moisture but also sebum, plasticizer, and fragrance can exert important effects on the hardness of hair spray film formers. The effect of the size and ratio of valve body orifice to vapor tap orifice on fractionation of propellant and concentrate in cosmetic aerosol formulations was examined (477). On the basis of the results, it was recommended that the discharge rate for cosmetic aerosols be reduced by restricting the actuator orifice rather than the valve body orifice.

A method of evaluating the amount of corrosive acidity released by a given lot of anhydrous ethanol in admixture with a hydrolizable chlorofluorocarbon aerosol propellant was developed (478). Application of the test to undenatured alcohol samples from various sources showed that they differ in the degree of chemical stability as applied to aerosol formulations.

Sustained Release—Several articles were published dealing with the theory and practice of preparing sustained-release dosage forms (479–481). Equations were presented by Robinson and Eriksen (482) to allow calculation of doses and dosing interval for multiple-dose therapy of sustained-release dosage forms. Use of the appropriate equations yielded relatively uniform blood levels of drug. A stable sustained-action suspension of dextromethorphan as a hydrate was prepared by coating crystals of a poorly water-soluble salt of dextromethorphan in a solid particle-coating device with a triglyceride fatty acid mixture and dispersing the coated crystals in an aqueous vehicle (483). Great differences in physical and release-rate stability were observed with the same salt when it was coated by different methods or crystallized from different solvent systems.

The method of bead polymerization in the aqueous phase was intensively investigated to show the applicability for embedding drugs in polymers as a sustained-release dosage form (484). The presence of drug, either soluble or insoluble in the outer aqueous phase, decreased the bead size and yield of the products; the amount of drug which could be embedded in the beads depended on the affinity and solubility of the drug for the monomers. Nylon-encapsulated sodium pentobarbital, prepared by emulsion polymerization, was shown to exhibit a considerable reduction in dissolution rates compared with the instantaneously soluble forms (485). The release rate of sodium pentobarbital from the tableted microcapsular material was observed to be inversely proportional to tablet hardness. *In vitro* release tests indicated the sustained-release properties of a number of drug formulations (486–489), many of which exhibited *in vivo* effects as well. The diffusion of various progestational steroids through capsules or chambers of silastic rubber was investigated by several authors (490, 491). Both *in vitro* and *in vivo* release rates from this type of a sustained-action dosage form were determined. In *in vitro* experiments with human plasma as the incubation medium, less steroids were resorbed in a 24-hr. period than under *in vivo* conditions. Diffusion of the steroids through the siloxane rubber depended on the solubility of the steroid in the surrounding medium. The release rate of steroid implanted in the uteri of human volunteers was found to be proportional to the capsule length and was related, but not proportional, to capsule thickness. Permeation of steroids through dimethylpolysiloxane rubber film of various thicknesses and areas was shown to follow Fick's law (492). From these data, permeability constants were calculated and used to estimate the amounts of steroids diffusing from cylindrical implants. In another study, the permeability of three types of silicone rubber to steroids was examined (493). Permeation of the steroids through the polysiloxane membranes was inversely proportional to the number of hydroxyl groups present. Dissolution of steroids from lipid pellets of various compositions was determined as a measure of sustained release (494). Values obtained in these *in vitro* studies indicated that, after an initial period, the dissolution was constant.

Other articles related to sustained release are listed in Table XIV.

Cosmetics—Many problems in cosmetic chemistry during the 1960's received much clarification by the application of conformational analysis (505). General areas reviewed were proteins, enzymes, hormones, allergy, permeability, aging, the skin and its appendages, pigmentation, antimicrobials, olfaction, and taste. Factors influencing the design and formulation of dermatological preparations were ably reviewed by Wurster (506). Justification of the use of vitamins in cosmetic preparations for external application was presented,

along with a number of recipes for such preparations (507). The magnesium salt of ascorbic acid 3-phosphate was found to be suitable in various dermatological and cosmetic uses (508). In topical therapy, the phosphate was hydrolyzed by a phosphatase of the skin to release the vitamin C activity.

The use of esters of branched-chain aliphatic fatty acids and fatty alcohols, which tend to make a cosmetic film more porous and thus allowing carbon dioxide to escape, was proposed for cosmetic formulations as a necessary improvement for skin respiration and vitality (509). A review of recent studies emphasized the need for careful design and complete testing of suntan preparations (510). Use of a solar simulator to produce erythema was described for the evaluation of various sunscreen preparations on guinea pigs, both with and without washing after application (511). In most cases, rinsing the sunscreen sites with water destroyed most of the protection. The physiological mechanism of perspiration and the effect of aluminum compounds as active compounds in antiperspirants were discussed (512). Recipes for use in various cosmetic preparations were also described. A study of aminothio ethers, such as *S*-benzylcysteamine malate, leading to interesting antiseborrheic formulations and cosmetic applications was described as a possible future development in the field of acne (513). The adherence of two vegetable oils and mineral oil to keratin was studied in a bath oil preparation at a 0.02% concentration. Results indicated that not only does more vegatable oil than mineral oil adhere to keratin, but a substantial amount of oil also appears to bind more tightly to the keratin (514). The use of pigments in cosmetic preparations and their coloring and toxicological properties were also reviewed (515). Modern perfume oil dosing in various cosmetic formulations ranged in quantities from 12–15% in aerosol perfumes to 0.3–0.5% in vanishing, face, and cleansing creams (516). Isopropyl myristate was recommended as a useful adjunct in most fluid formulations.

The use of a surface-active powder, developed from methylolurea, as a highly acceptable fabric-free cover for wounds was reported (517). Besides its use in medicine, this powder can be used in the cosmetic industry as a face, body, shaving, and foot powder or as a powder to stop bleeding. The properties and characteristics of a new cosmetic material were described (518). The advantages of this cosmetic ingredient were shown in typical formulations. A practical guide to gums and thickeners available for use in cosmetic formulations was presented by Young (519). Factors involved in producing stable products were also considered.

In a discussion of sanitation in cosmetic manufacturing, it was emphasized that a cosmetic producer must do everything he can to maintain the most stringent conditions in all aspects of manufacturing, packaging, and distribution (520). An investigation of ethylene oxide treatment of large volumes of various cosmetic ingredients demonstrated a great reduction of microorganisms in the dry ingredients (521). Dry organic materials as well as inorganic materials were favorably affected by this treatment. Various aspects

Table XIV—Additional References on Sustained Release

Reference	Topic
495	Use of glycerol palmitostearate for preparing sustained-release tablets according to embedding principle
496	Delayed-action dosage forms of chlorpromazine
497	Medicinal preparations of prolonged action
498	Preparation of sustained-release capsules by coating small tablets made by compression
499	Sustained-release tablets of cycloserine
500	Investigations of a new timed-release dosage form of propoxyphene hydrochloride
501	Sustained-release tablets with stearic acid
502	Hydrophilic gels in preparation of sustained-release tablets of ammonium chloride
503	Long-acting form of methacycline
504	Timed-release ascorbic acid capsule

of preservative failures in cosmetics resulting in microbiological spoilage were ably reviewed in several articles (522–524). The necessity for adequate preservative systems was emphasized. During the year, Gucklhorn (525–534) continued the comprehensive survey of the advantages and disadvantages of the various preservatives used in cosmetic preparations, including the updating of information on materials previously reviewed. Allergic skin reactions from cosmetic preservatives were surveyed (535). False negative allergy tests to parabens, sorbic acid, and other preservatives were reported to be prevalent when patch tests were made using the cosmetic cream or lotion supplied by the manufacturer.

In other reviews, the use of ethyl and isopropyl alcohols and other compounds that slowly release formaldehyde were considered as preservatives in cosmetic formulations (536, 537). Although the preservative action of phenylmercuric salts at 0.005–0.01% concentration was almost completely neutralized by compounds containing sulfhydryl groups, Eckardt (538) demonstrated that 2% solutions of keratin can be preserved with phenylmercuric acetate, and that the preservative activity can be improved by the addition of sodium lauryl sulfate. For the stabilization of hydrophilic bases used in cosmetic preparations, a binary preservative system composed of 0.01% hexachlorophene and one other preservative, such as 0.2% methylparaben or 0.1% benzyl alcohol, was recommended (539). Although products containing protein hydrolysates are relatively difficult to preserve, tests with 2-bromo-2-nitro-1,3-propanediol indicated it was a powerful, nontoxic preservative which was not affected by the large amounts of protein found in shampoos, rinses, conditioners, and creams (540). A new family of antimicrobial preservatives for cosmetics was described based on the development of a series of substituted imidazolidinyl urea compounds (541).

Packaging—The current status of single-unit packaging of medications was discussed in regard to the need for the availability of this package for many commonly used drugs in hospitals (542). Lists of currently available medications in single-unit packages were also presented. Drug–plastic considerations for use of plastics in packaging parenterals were reviewed by Autian (543) and included permeation, leaching, sorption, chemical reactions, and stability of the

Table XV—Additional References on Packaging

Reference	Topic
552	Packaging of compositions containing acetylsalicylic acid
553	Comparative analytical study of pharmaceutical glass containers in the establishment of Columbian National Standards
554	Release of additives from pharmaceutical containers made from polyvinyl chloride
555	Performance tests for plastic containers
556	Evaluation of extraction tests for plastics
557	Chemical resistance of carbamide plastic used for ointment containers
558	Stability of aluminum foil-sealed cycloserine capsules

materials. The sorption of *p*-hydroxybenzoic acid esters, phenols, and other preservatives by plastic materials such as poly(methyl methacrylate), polyethylene, and polyvinyl chloride were investigated by several authors (544–546). The degree of sorption apparently depended on the structure of the compound. A study of the sorption of *p*-hydroxybenzoic acid esters by capran polyamide showed that the *in vitro* biological activity of the parabens in the presence of the nylon was related to the concentration of drug in equilibrium with the plastic (547).

A procedure was proposed by which it is possible to predict, with reasonable accuracy, the change in concentration that occurs during the autoclaving of solutions in polyethylene containers (548). The prediction was based on the assumption that permeation is related to partitioning of the solute between the water and the polyethylene. Polyethylene and polypropylene containers were found to be satisfactorily inert and did not affect the stability of a 5% aqueous glucose solution; the polyvinyl chloride container was satisfactory due to the effect of an extracted plasticizer (549). Storage tests with an alcoholic camomile extract in different plastic containers indicated that high and low pressure polyethylene containers exhibited loss of chamazulene and etheric oil due to absorption into the container walls (550). No absorption was detected in the rigid polyvinyl chloride or polypropylene containers. In similar studies, the stability of eight plasma-substitute solutions in various plastic containers was investigated (551). After storage for 1 year under normal conditions, the solutions changed little in physicochemical properties aside from changes in pH.

Other articles related to packaging are described in Table XV.

Equipment—Progress in pharmaceutical engineering was reviewed by Fowler (559). The engineering aspects of size reduction, particle-size measurement or characterization, drum mixer, solids handling, and granulation were discussed. Another review discussed the advantages and applications of different systems of laminar flow and clean rooms (560). The use of Torit dust collectors on tablet compressors and mixers and in granulating areas was described (561). The main use was in product and personnel protection, but an occasional advantage was the recovery of an expensive drug. Engineering aspects of the flow of particulate materials from hoppers were presented by Miles (562). Some methods and devices for overcoming practical problems that arise from mass flow hopper design were considered, together with problems involved in designing accurate feeding systems. A description of the Marumerizer and the associated extruder, together with operating experience in the manufacture of small solid pharmaceutical spheres, was presented (563, 564). The spherical particles produced were shown to have advantages of regularity and shape, consistency of size, and definite surface characteristics in comparison with spherical or granular particles produced by other means. A general study of the technology for using a solids processer (a vacuum tumble dryer) was made to evaluate the overall applicability to the processing of pharmaceutical granulations (565). Experiments on mixing, drying, formulation factors, and processing factors were recorded.

PHYSICAL PHARMACY

The existence of six different polymorphic forms of aspirin (two of which were known) was determined by differential scanning calorimetry, hot-stage microscopy, and true density measurements (566). The thermal behaviors of four crystalline forms of sulfanilamide and of sulfanilamide-d_4 were examined, and heats of transition and fusion were determined. The deuterated modifications exhibited smaller heats of transition and heats of fusion than the corresponding undeuterated forms (567). Through a systematic investigation of the crystallization of cephaloglycin and cephalexin, a better understanding was obtained of the part that pseudopolymorphic crystal transitions play in the analysis, processing, and formulation of these compounds. A convenient and sensitive method for detecting new crystalline phases was reported which employed solubility *versus* solvent composition diagrams to detect various crystal forms of compounds. This method should find application whenever crystallizations are performed with more than one solvent and, particularly, when the instability of the compound at elevated temperatures prevents the use of conventional thermal methods or when poor crystal development limits the use of microscopic methods (568). Two polymorphic forms and a hydrate of an investigational compound were reported in which no appreciable difference in the dissolution rate was detected for the various forms in artificial gastric fluid. Dissolution studies in water indicated the formation of a hydrate, and protective colloids were shown to have an effect on the hydrate formation. In particular, methylcellulose slowed down the rate of crystal growth of Form I significantly (569). Shenouda (570) described a crystallized sulfathiazole form which consisted of varying proportions of a melting species and another species that underwent solid–solid transition. Grinding appeared to destroy the melting species and to have a significant effect on the transition temperature but not on the heat of transition. Three crystal modifications of chlordiazepoxide hydrochloride were described: a stable form, a hygroscopic form, and a monohydrate (571).

It was noted that permeability of most doublelayer films has a directional property and they can be made thinner than single films, suggesting their potential

usefulness in the manufacturing process of pharmaceuticals that are water unstable and require rapid disintegration (572). Most combinations of coating films showed "two-sided" permeability; this property was not previously reported for films composed of hydrophobic layers only. It was found that in double-layer films with "two sidedness," there is a characteristic relationship between the specificity of permeability and the humidity range (573). Since the moisture permeability of most double-layer films has a directional property, this two sidedness may be brought about mainly by a change in the permeability coefficient as a result of a change in vapor pressure (574).

Herzog and Swarbrick (575) described the development of a polymeric nonporous model membrane containing natural membrane components and its use in a two-compartment transport cell. A standard model biomembrane was designed containing 44% ethylcellulose, 44% biological materials, and 12% mineral oil. From the lack of solvent flux under experimental conditions and the first-order disappearance of salicylic acid, it appeared that the polymer membrane mimicked the functionality of natural membranes insofar as passive diffusion was concerned. A theoretical equation was developed justifying the graphical representation of vapor permeation data by 1/rate *versus* film thickness plots. The permeability coefficient for the film could be determined from the slope of the plot and had units in square centimeters per second, while the intercept at zero film thickness was dependent upon the geometry of the experimental design and the diffusion coefficient for the vapor within the diffusion cell (576). Films composed of poly(methylvinylether)-maleic anhydride copolymer, crosslinked with polysorbate 20, appeared as the most promising of the systems studied for film-controlled drug-release application. The permeability of the films could be controlled by adjusting their polysorbate 20 content and the molecular weight of the polymer and by humidity pretreatment. The permeability of the films was affected also by the pH of the diffusion medium (577). A system of molecular scale drug entrapment was developed, which provided a physicochemical and highly reproducible method of effecting drug entrapment and subsequent controlled drug release from polymeric matrixes. The flocculation of highly concentrated colloidal polymeric dispersions (latexes), in the presence of the drug in solution which is to be occluded, provides the entrapment mechanism. A significant increased duration of action and a reduction of the acute toxicity of methapyrilene in the entrapped form were established by *in vivo* effectiveness studies. The broad application of the entrapment process to acid salts of 11 widely used cationic nitrogen-containing drugs was demonstrated (578). It was shown that a suitable organic acid greatly increased the degree of interaction between the drug and the polymer and provided a mechanism in controlling both interaction and subsequent drug-release properties. *In vitro* tests indicated that the products obtained by this technique could be used in either solid or liquid dosage forms (579).

Further studies on the facilitated molecular scale entrapment technique for preparation of controlled-release pharmaceuticals were reported, including *in vitro* and *in vivo* tests of phenylephrine and phenylpropanolamine products obtained by the facilitated molecular entrapment method. This testing indicated that the process possesses considerable potential for exploitation of the sustained-action or controlled-release pharmaceutical dosage form (580).

The *in vitro* dissolution patterns of some spray-congealed products of sulfaethylthiadiazole–wax made into compressed tablets were reported. The mechanism of release of sulfaethylthiadiazole appeared to be due to erosion, solubilization, and leaching of the drug from the tablet. The Higuchi model for drug release from inert matrixes could describe the release pattern for only the initial few hours when, apparently, variables other than amount of drug released and time were essentially constant (581). The *in vitro* release behavior of chloramphenicol from four different bead polymers containing methyl methacrylate and α-methacrylic acid in various buffer solutions was studied. The concentration of α-methacrylic acid in the copolymer beads and the pH and ionic strength of the buffer solutions influenced the release rate of chloramphenicol from these beads. The beads containing no α-methacrylic acid did not release the drug in any buffer solution, and the beads containing only α-methacrylic acid released the drug at almost the same rate in all buffer solutions (582).

McGee *et al.* (583) reported on some of the factors affecting release and availability of aspirin, aspirin and lactose, and aspirin and dibasic calcium phosphate mixtures from hard gelatin capsules. Significantly better plasma levels were obtained with the more tightly compacted No. 4 capsules, as opposed to the same formula administered in a No. 3 hard gelatin capsule. This possibly was due to the diffusion of gastric juice through the gelatin, which created higher pressure within the capsules. A direct correlation of *in vitro* dissolution data and *in vivo* plasma level data could not be made. In a similar study, the effect of particle size and packing on the *in vitro* release of a water-insoluble hydrophobic drug from hard gelatin capsules was related to the liquid permeability of powder beds of similar porosities. Drug release and permeability decrease with a decrease in particle size and porosity of the powder bed. A moist granulation process transforms a nonpermeable powder bed, which allows low drug release, into one with high permeability and high drug release (584).

The *in vitro* release of medroxyprogesterone acetate from a silicone rubber matrix was studied, and a nonlinear dependence of release rate upon drug concentration within the matrix was found. Based upon a model system, equations were derived to explain this behavior and to include other parameters that may influence the release rate. The study suggested that the partition coefficient, diffusion coefficients, the medroxyprogesterone acetate concentration within the polymer, and agitation conditions play important roles in the release process (585).

The transport kinetics of salicylic acid was studied in an *in vitro* model cell containing two aqueous phases separated by a third liquid–lipid phase simulating

Table XVI—Additional References on Physical Pharmacy

Reference	Topic
596	Distribution of tetracycline antibiotics in two-phase systems
597	Characterization of the form of lactose in spray-dried lactose to show existence of α-monohydrate and α- and β-anhydrous forms
598	Review of pharmacogenetics with special emphasis on polymorphism
599	Model of a membrane structure and the basic principles of solute transport through dialysis membranes
600	Influence of some aliphatic and steroid alcohols on liberation rate of citric acid from petrolatum
601	Acid dissociation constants and zwitterionic character of 2-aminoethaneselenol
602	Review of very fine particles, including properties in the dry state and in liquids

the biomembrane. The rate constant for the transport of salicylic acid from the aqueous phase to the lipid phase always increased with increased polarity of the lipid phase; however, the transport of the drug from the lipid phase to the second aqueous phase increased or decreased, depending on the stirring condition employed in the two aqueous phases (586). The effect of surfactants on the diffusion of testosterone through cellulose acetate membranes was studied in an attempt to determine the possible mechanisms by which surfactants may affect drug transport. In all cases examined, the surfactants reduced the diffusion coefficient of testosterone (587).

By using NMR data, the hydrophobic bond-weakening effect of urea on *d*-propoxyphene hydrochloride self-association was demonstrated (588). In studying the formation of mixed ligand chelates containing penicillamine and sulfhydryl compounds, Sugiura and his coworkers (589, 590) concluded that the formation constants of mixed ligand chelates are linearly correlated with the dissociation constants of the sulfhydryl group in secondary ligands. Among the mixed ligands studied, the decrease in binding affinity of the carboxyl group in penicillamine tended to increase the formation of the mixed ligand chelate.

X-ray crystallographic procedures demonstrated that the structures of *erythro-* and *threo-α,β*-dimethylacetylcholine iodides were substantially different in conformation in that the *threo*-compound appeared to be dominated by coulombic attraction between the carbonyl oxygen and the quaternary nitrogen group. In the *erythro*-analog, the acyloxy oxygen atom was involved in a similar intramolecular interaction (591).

The effect of pH of precipitation on the physical and chemical properties of hydrous aluminum oxide was studied. During aging, changes may occur in the hydrous aluminum oxide structure which result in a loss in antacid reactivity; this loss follows apparent first-order kinetics. The rate of loss was directly dependent on the pH of precipitation and continued until a constant end-point was reached. The percentage of theoretical reactivity remaining at the end-point was inversely related to the pH of precipitation. While no differences in form were detected by X-ray diffraction, either initially or during aging, gel stability appeared to depend on the presence of anions in the gel structure; the concentration of these anions was related to the pH of precipitation. Data were presented which demonstrated that a stable, completely acid-reactive gel can be obtained if 1 mole of a monovalent anion or 0.5 mole of a bivalent anion is incorporated in the gel structure per mole of aluminum (592).

The effect of charge shielding by nonpolar groups on the partitioning of quaternized amines was studied by comparing a series of 2,6- *versus* 3,5-methyl-substituted *N*-methylpiperidine methiodides. There was little difference in their partition coefficients (593). The apparent partition coefficients of chlorpromazine and some other phenothiazine derivatives in dodecane–water and *n*-octanol–water systems were measured at 30°. Only the free base formed partitions in the dodecane system at various pH values. Intrinsic partition coefficients for all derivatives, except the very polar chlorpromazine sulfoxide metabolite, ranged from 10^4 to 10^5, indicating the remarkable hydrophobicity of these molecules. Partitioning measurements in *n*-octanol indicated significant extraction of these drugs as ion pairs, as well as higher intrinsic partition coefficients than in dodecane. From this work, it was shown that quantitative studies involving the phenothiazines in heterogeneous systems, such as membranes, must consider the extreme hydrophobicity of these compounds and the various factors that influence such behavior (594). A mathematical model was derived which permitted a quantitative evaluation of salt effects in urea–drug solutions. The theoretical calculations, based on this model, were in good agreement with the experimental values observed for neutral and pH 1 solutions of methylsalicylate and methylbenzoate in urea. Significant error in interpretation of solubility in urea may result if these salt effects are disregarded (595).

Additional references on physical pharmacy are provided in Table XVI.

Dissolution—When a commercial tablet was converted to a capsule dosage form for purposes of conducting a double-blind clinical trial, the *in vitro* dissolution times of the drugs from the capsule were strikingly prolonged. Various capsule adjuncts were examined in an attempt to decrease dissolution times of drugs from capsules. Of the series tested, lyophilized glycine had the best positive effect in shortening the dissolution times of all drugs examined (603). In studying the *in vitro* dissolution behavior of several experimental capsule formulations, it was demonstrated that the capsule lubricant magnesium stearate and the filler lactose or dibasic calcium phosphate dihydrate had the greatest influence on the disintegrations of the capsules. It was noted that drug dissolution was limited by the rate of erosion, diffusion, and/or solution of the drug and/or filler from the wet powder mass that was characteristic of slow dissolving formulations. The data suggested that the high viscosities of several of the powder packs were due to their restricted water content arising from the added hydrophobic lubricant (604). Relatively insoluble drugs formulated in soft elastic capsules were released faster than from commercially available tablets due to the more rapid dispersion of the active ingredients, which is enhanced by the use of solubilizers

and/or surfactants in the formulation. Soft elastic capsules were recommended for the formulation of low-dose medications of relatively insoluble drugs and drugs for which an early high-blood level of the drug is indicated (605).

The influence of a nonionic surface-active agent on the dissolution rate of benzoic and salicylic acids was investigated, and the dissolution mechanisms were examined as a function of the hydrodynamics of the system. The dissolution rate of a solid in a micellar solution was not proportional to the solubility of the compound in the dissolution medium. Evaluation of dissolution-rate data and theories led to the conclusion that, depending upon hydrodynamic conditions, the dissolution rate of a solid will be proportional to the effective diffusion coefficient raised to a power between 0.5 and 1.0 (606). At a very low concentration of FD&C Blue No. 1, a remarkable inhibition of dissolution was observed for single crystals of sulfathiazole, phenobarbital, thymol, and sulfaguanidine under controlled conditions. Data presented agree with the current theories concerning dissolution inhibition by small concentrations of impurities and suggest that the dye molecules are preferentially absorbed at the primary dissolution sources in the crystals investigated (607).

A three-compartment apparatus was developed for dissolution studies of slightly soluble powders under sink conditions. The apparatus could accommodate a barrier in the dissolution medium which prevented floating powders from entering and dissolving directly in the sink phase. Dissolution studies were conducted with several particle-size grades of salicylic acid under nonsink as well as sink conditions. The data indicated that with a diffusion-controlled rate of agitation, using appropriately placed propellers, it was possible to establish a rank order for *in vitro* dissolution times of different particle-size salicylic acid powders (608). An apparatus for the study of dissolution rate under conditions of continuous flow was described. Typical data were presented, demonstrating the major advantages of this method over present methods to be the following: (*a*) has greater flexibility, (*b*) produces data in a differential form, (*c*) utilizes a small volume system which assures greater homogeneity, and (*d*) prevents excessive accumulation of solute in the system and provides agitation and solvent flow in a controlled, measurable, and physically meaningful manner (609).

In a study of the influence of pulsation on the dissolution-rate measurements in column-type apparatus, it was recommended that for dissolution tests in flow-through methods, momentum pumps should be used instead of displacement pumps so that results found at different times in different laboratories can be compared (610). The dissolution of aspirin from different commercial dosage forms was evaluated by the rotating-flask method and the data were correlated with previously recorded absorption data. The dissolution rates in descending order were: buffered tablets > plain tablets > timed-release tablets. Aspirin dissolved from the buffered tablet about twice as rapidly as from the plain tablet and about eight times as rapidly as from the timed-release tablet. However, once disintegration and deaggregation took place, the dissolution of aspirin from the capsule formulation proceeded as rapidly as from the buffered tablet. Excellent single- and multiple-quantitative correlations were observed between the dissolution data and absorption data in man (611).

An investigation was conducted to analyze the dissolution behavior of a poorly water-soluble drug. Two specific methods of micronization were employed which provided drug forms of different physical characteristics, specific surface area, and particle-size range. The drug was micronized by employing spray drying and air attrition. The micron-sized drugs, pelletized pure drugs, and tablets prepared from each of these by direct compaction were subjected to dissolution-rate studies at pH 1.2 and 3. The *in vitro* dissolution data obtained demonstrated a significant variation for the pure spray-dried and air-attritioned drugs at pH 1.2, although physical specifications had suggested the contrary of that reported. When the pure drug forms were pelletized, the difference observed in the dissolution rates with the pure drug powders was eliminated. Dilution of the pure drug with tablet excipients and the subsequent direct compaction into tablets improved dissolution behavior compared with that obtained with the pure drug forms (612). The dissolution rates of phenobarbital, sodium phenobarbital, phenacetin, and prednisolone in human gastric juice were investigated. The rate of dissolution of phenobarbital from tablets prepared with gelatin as a granulating agent increased with decreasing particle size of the drug. Phenobarbital from the same tablets dissolved much faster in human gastric juice than it did from tablets prepared with sodium carboxymethylcellulose or polyethylene glycol 6000. The rate of dissolution of sodium phenobarbital in human gastric juice and in 0.1 *N* HCl was lowered by granulating a mixture of the drug and potato starch with gelatin. Compressing these granules into tablets further reduced the dissolution rate (613). Adding polysorbate 80 caused an increase in the dissolution rate of acetylsalicylic acid in 0.1 *N* HCl when the particle size of the drug was relatively small. If the particle size was larger (0.7–1.0 mm.), polysorbate 80 had no accelerating influence on the dissolution rate. This difference in the effect of the surfactant may be due to the fact that the hydrophobic properties of aspirin are so insignificant that a reduction of the surface tension between drug and liquid is of importance only when the drug's specific surface is large, *i.e.*, when the particle size is small (614).

Using a polyethylene maleic anhydride–phenylpropanolamine interaction system as a model, Heyd (615) showed that the preparation temperature of the polymer–drug has a pronounced effect on some physical–chemical properties of the polymer–drug system relative to dissolution of the drug. Factors influencing the dissolution of an ethylene–maleic acid copolymer were studied. Swelling, hydrated layer thickness, and solvent pH influenced the dissolution of the polymer. Linear dissolution rates were observed following an initial induction period, and hydrated layer thickness was a controlling factor in the dissolution process (616). Using the rotating-disk method, the dissolution

Table XVII—Additional References on Dissolution

Reference	Topic
619	Review including discussions of rates of disintegration and dissolution processes, methods of determining disintegration time *in vivo* and *in vitro*, and relations between disintegration time and physiological availability
620	Chronological bibliography on rate of dissolution of pharmaceuticals *in vitro* and *in vivo*
621	Review of historical highlights in development of rate of dissolution of drugs *in vitro* and *in vivo*
622	Theoretical and descriptive review of methods of measuring and interpreting rates of dissolution of pure compounds
623	Correlations of percent drug released *in vitro* with *in vivo* availability for various drugs
624	Differences in rate at which ferrous sulfate is released from various tablet formulations attributed to chemical reactions occurring between ferrous sulfate and other ingredients in the tablet
625	Review of intradependency of factors affecting dissolution rate, uniform dissolution-rate tests, and interpretation of dissolution-rate data, including surface area and kinetic studies
626	Influence of granulation method on the dissolution rate from tablets and tablet granules, showing significance when finely ground drug was used
627	Laboratory experiment in pharmaceutics, showing the influence of pH and agitation intensity on dissolution rate of salicylic acid
628	Influence of particle size and other technological factors on absorption and *in vitro* dissolution rate of phenytoin from tablets
629	Effect of compression force and additives in tableting on tablet dissolution *in vitro* and *in vivo*

of polyvinylpyrrolidone was investigated in an acetone–water system. Three dissolution stages were observed: the initial stage involved swelling, which induced dissolution, and the final stage involved coacervation of polyvinylpyrrolidone. When sodium chloride was added in bulk solution, the initial stage seemed unessential, and the whole dissolution rate increased. This was considered due to a suppression of the swelling of polyvinylpyrrolidone on the surface of the disk by the adsorption of sodium on polyvinylpyrrolidone (617). Similar dissolution studies of fractionated polyvinylpyrrolidone were conducted. Again, three stages of dissolution were observed; the initial stage was shortened and there was a decrease in molecular weight. The main stage was expressed as a function of the molecular weight of polyvinylpyrrolidone and the absolute temperature (618).

Additional studies on dissolution are listed in Table XVII.

Solubility—Anhydrous and dihydrate forms of an aminoalicyclic penicillin were compared for solubility in distilled water at temperatures ranging from 7 to 60°. Below the transition temperature of 61°, the anhydrous form was significantly more soluble than the dihydrate. The solubility of the anhydrous crystal was inversely related to temperature. Thermodynamic properties for the two forms of the antibiotic were experimentally evaluated (630). In a solubility study of testosterone propionate, methyltestosterone, and 19-nortestosterone, oxycholate was a better solubilizer than the aqueous sodium cholate, and both bile salts solubilized more 19-nortestosterone than the other derivatives. A possible mode of solubilization was presented (631). Using a radioactive technique, it was shown that the *in vitro* solubility of cholesterol was greatly increased in the presence of acacia and pectin. This finding is in contrast to previous reports that acacia had no effect on feeding experiments (632).

Entropy considerations showed that solvent–solute interactions occur in some solvent–testosterone propionate systems that increased solubility, resulting in deviations from regular solution behavior. It was noted that regular solutions are obtained only in saturated hydrocarbon solvents, and that solubility can be more accurately predicted as the temperature approaches the melting point and as the molar volumes of solvent approach that of the solute (633). Ostrenga and Steinmetz (634) reported solubilities for two steroids and indicated that the fractional molar attraction constant may be a useful parameter for solubility estimations, since it relates the solubilizing capacity of solvents for a given steroid.

The need for caution in interpreting solubility phenomena involving cosolvent systems in general and urea–water systems in particular was reported (635). The solubility of dodecane increased as the concentration of aqueous solutions of formamide, *N*-methylformamide, and dimethylformamide increased. Positive values of standard free energy and negative values of enthalpy and entropy decreased numerically as the concentrations of amides increased (636).

The solubilities of eight physiologically active barbiturates were determined in binary mixtures of alcohol and water. The dielectric requirement of the barbiturates illustrated an approximate inverse relationship with the number of carbon atoms in the molecule. The onset of action after administration and the duration of action of these barbiturates increased as their relative polarities decreased. An approximate correlation between activity and solubility ratios was considered (637). The effect of the hydrophobic chain length of the polysorbates on the degree of solubilization of a series of 5,5-disubstituted barbituric acid derivatives was studied. The solubilities were increased as the hydrophobic chain length increased. The number of carbon atoms of the substituents on the 5-position, as well as their inductive effects, determined the extent of solubilization (638).

A similar study of the solubilities of barbituric acid derivatives in aqueous solutions of polyoxyethylene stearates of varying polyoxyethylene chain length was conducted. Except for phenobarbital, which formed an insoluble precipitate complex with all the solubilizers, all the other barbiturates studied were micellarly solubilized. On a molar basis, solubility increased with an increase in hydrophilic chain length but decreased if the solubility was expressed in terms of the amount solubilized per ethylene oxide unit. The partition coefficient of the drug molecules between a micellar pseudophase and an aqueous phase was dependent on both the polar effect and the number of carbon atoms of the substituents on the 5-position. The formation of an insoluble precipitate complex by phenobarbital was attributed to the presence of the aromatic phenyl group in the molecule (639).

The applicability of a new technique in solubilization, using a molecular sieve, was tested on a system consist-

ing of benzoic acid and cetromacrogol 1000 in aqueous solution. The data obtained were in good agreement with those found by other methods, and the advantages of the new technique were outlined (640). The solubility features of a homologous series of alkyl *p*-hydroxybenzoates with alkyl groups, in an ascending order from methyl to *n*-butyl, were investigated together with benzylparaben and methyl *p*-methoxybenzoate. A phenomenon of mutual solubilizing potential was observed to exist when the solubility of a mixture of an alkylparaben and benzylparaben in 60% polyethylene glycol 400–water was examined. The scope of application of these esters for their antimicrobial properties was envisioned to be augmented by considering factors influencing solubility (641).

Additional studies on solubility are listed in Table XVIII.

Complexation—The many publications dealing with complexation phenomena (interaction, binding, *etc.*) have been divided into two categories: interactions dealing with biological substances such as plasma proteins, and interactions dealing with nonbiological substances.

Interactions of Drugs with Biological Substances—Mefanic acid, ethacrynic acid, nalidixic acid, and diazoxide displaced significant amounts of warfarin from human albumin *in vitro* by a noncompetitive mechanism. If this occurred *in vivo*, the clinical use of these drugs would cause an increase of 66–400% in free active warfarin, and it would be necessary to reduce the dosage of anticoagulant to prevent excessive hypoprothrombinemia and hemorrhage (664). The binding of a series of conjugated bile salts and fatty acid salt anions to cholestyramine from aqueous media was investigated; affinity constants increased as the number of hydroxyl substitutents on the bile salt ring structure decreased. An increase in the chain length of the fatty acid salt caused a corresponding increase in the affinity constant, whereas an increase in the extent of unsaturation in the fatty acid chain produced a reduction in the affinity constants for the fatty acid–cholestyramine interaction. Based on these results, it was suggested that the binding mechanism involves a primary electrostatic component reinforced by a secondary nonelectrostatic interaction, the strength of the latter force being dependent on the degree of hydrophobicity of the adsorbate molecule (665).

The *in vitro* rate of binding of several conjugated bile salt anions to cholestyramine was studied at 27°, along with and in the presence of varying concentrations of sodium chloride. The temperature and agitation studies suggested that the binding of bile salt anions to cholestyramine apparently occurs by means of a diffusion-controlled process (666). The role of hydrophobic interactions in inhibiting the relatively specific enzymatic reactions of five enzyme systems by a series of congeneric drugs was illustrated by the use of substituent constants and regression analysis. The inhibition of certain enzymes by congeneric drugs was linearly dependent on the lipohydrophilic character of the inhibitors (log P or π). The inhibition of other enzymes by drugs was linearly dependent on the log P and Hammett's σ constant. For monoamine oxidase inhibition by substituted β-carbolines, a parabolic equation of log P gave the most significant correlation. The ideal lipohydrophilic character (log P_0) for maximum inhibition was 2.74 (667). The binding of *para*-substituted acetanilides to bovine serum albumin was examined at pH 7.2; an excellent correlation was obtained between the binding enthalpy and Hammett's substituent constant, σ. This was interpreted to mean that binding is nonspecific in nature. A very good correlation was also obtained between the entropy of binding and σ, which suggests that the extent of hydration of unbound drug is a function of the charge separation within the drug molecule (668).

More than 90% of the plasma content of chlorpromazine was bound to human plasma protein, the binding being affected by the pH of the aqueous medium. Variation in binding in plasma from different humans was marked (669). Campbell and Todrick

Table XVIII—Additional References on Solubility

Reference	Topic
642	Solubility of benzoic acid in aqueous solutions of monododecyl polyoxyalkanols
643	Effects of third component on hydrophobic and hydrophilic moieties of tryptophan in aqueous solution; approach to understanding the denaturation of globular protein
644	Effect of third component on water structure around tryptophan in aqueous solution
645	Properties of mixtures of glycols and their derivatives with water, indicating that association occurs in these mixtures
646	Association in water-solubilizing agent systems in which one substance possessed both hydrophobic and hydrophilic groups; lack of association in systems having only a hydrophilic group
647	Solubilization and emulsification of hexachlorophene using nonionic emulsifiers
648	Solubility of menadione in nonionic surfactants suggested by complexation
649	Relative pharmacologic activity of insulin-inducing sulfonylureas studied as a function of their water solubility
650	Solubility of testosterone in aqueous solutions of ionic surfactants using interference refractometry
651	Solubilization of therapeutically used arylamines by *n*-propylaminoacetylation
652	Solubilization of blasticidin *S*-benzylaminobenzenesulfonate by guanidine hydrochloride–nonionic surfactant solutions
653	Laboratory experiment showing characterization of a poorly soluble complex by a solubility method
654	Solubilization of purine alkaloids by polyvinylalcohol and suggested mechanisms
655	Solubilization of *N,N*-diethylaniline by trimethylcetylammonium bromide involving formation of a dimer within the elliptical micelle; solubilization of α-naphthylamine involving an adsorption–penetration mechanism followed by dimerization
656	Solubilization of reserpine by nonionic surfactants
657	Effect of polar substances on solubility behavior of anionic, cationic, and nonionic surfactants in nonpolar solvents
658	Solubility and hydrolysis rate of 1-monolaurin in aqueous solutions, including thermodynamic parameters
659	Temperature effect on solubility of tetracycline base–water–ethylene glycol system
660	Mechanism of solubilization of khellin by sodium benzoate and salicylate
661	Successive and simultaneous solubilizations of two different water-soluble dyes by surfactants
662	Solubilization of benzene and styrene in solutions of some ionic and nonionic surfactant mixtures
663	Effects of isomeric form and length of carbon chains on solubilization of various hydrocarbons and polar compounds

Table XIX—Additional References on Interactions of Drugs with Biological Substances

Reference	Topic
675	Binding of steroid conjugates to human corticosteroid-binding globulin
676	Kinetics of dialysis for certain drugs in the presence and absence of albumin, showing estimates of unbound and bound forms of the compound
677	Physiological implications of interactions of drugs with aspirin
678	Evaluation of *in vitro* enhancement of protein binding of certain organic acids and bases
679	Studies of an *in vitro* binding reaction between thyroid microsomes and long-acting thyroid stimulator globulin; development of solid-state competitive binding radioassay methods for measurement of antimicrosomal and antithyroglobulin antibodies
680	Binding of penicillins and serum protein
681	Nature of drug interactions for use by the practicing pharmacist
682	Ion binding of penicillins to proteins
683	Measurement of binding constants of phenylbutazone and oxyphenbutazone to proteins using circular dichroism
684	Lack of enhancement by myocardial contraction on binding affinity of digitalis glycosides to myocardial receptors
685	Hypothesis of a calcium chelating mechanism for caffeine action *in vivo*
686	Binding of a diphenhydramine analog to bovine serum albumin
687	NMR study of binding of aspirin to human serum albumin
688	Complexes of certain sulfa drugs with each other being better absorbed intestinally than individual sulfa drugs
689	Induced changes in plasma protein binding of methotrexate with salicylate or sulfisoxazole being useful in cancer therapy
690	Mechanism of metachromasy of 2,4′-hydroxyphenylazobenzoic acid by serum albumin
691	Interactions of cations and local anesthetics with phospholipids
692, 693	Statistical–mechanical model for binding of flexible ligands to protein with application to various ligand–surface systems
694	Preferential binding of dipyridamole to β-globulin in plasma rather than to albumins
695	Review of plasma protein binding of pharmaceuticals
696	Interaction of antibiotics with serum and its protein fractions
697	*In vitro* effect of sodium phenobarbital and diethylnicotinamide on the protein binding of bilirubin
698	Binding of oxytetracycline to serum proteins
699	Simulation of thyroid hormone-binding protein interactions in human plasma
700	Role of plasma protein binding in inhibitory effect of nortriptyline on neuronal uptake of norepinephrine
701	Reevaluation of thyroxine binding and free thyroxine in human serum; discussion of a new free thyroxine index
702	Drug-induced reduction of urate binding *in vivo* as related to effect of some uricosuric and anti-inflammatory drugs on binding of uric acid to human serum albumin *in vitro*
703	Effect of drugs on urate binding to plasma proteins
704	Flucloxacillin binding rate and capacity of binding to horse serum protein
705	Protein binding of psychoactive drugs with a tricyclic ring system in relation to their chemical constitution
706	Plasma protein binding of amphetamine, catechol, amines, and related compounds
707	Erythrocytes infected with chloroquine-sensitive *Plasmodium falciparum* binding chloroquine, with an apparent intrinsic association constant of 1.5×10^7 l./mole
708	Small changes in protein binding of drugs in plasma and tissues causing redistribution of highly bound drugs between tissues and plasma, resulting in concentration fluctuations of drug in plasma
709	Salicylate causing a release of long-chain fatty acids from their binding sites on human plasma proteins and bovine albumin
710	Review of protein binding of rifampicin
711	Interaction of warfarin and dicoumarol with human serum albumin
712	Effects of sulfonamides on protein binding of bilirubin

(670) concluded that the therapeutic differences in potency among four tricyclic antidepressant drugs known to inhibit the uptake of 5-hydroxytryptamine by human blood platelets depended more on their individual molecular structure than on the degree of binding to circulating proteins. In a similar study, data on the binding of imipramine, desipramine, and 3-chlorodesmethylimipramine to plasma proteins were obtained over a wide range of ligand concentrations, using a modified equilibrium dialysis technique. Plasma proteins other than albumin did not appreciably bind to the drugs studied, and the association constants of the complex were low. The authors concluded that apparently atypical binding behavior was disclosed for desipramine and 3-chlorodesmethylimipramine (671). The binding to bovine albumin of the model dyes and drugs bromcresol green, eosin, imipramine, and desipramine was studied, using an improved equilibrium dialysis technique. The dyes and drugs interacted with two to four types of binding sites through hydrogen bonding, electron donator–acceptor forces, van der Waals' forces, and hydrophobic forces. In addition, the drugs and dyes interacted with up to six binding sites through electrostatic forces. Desipramine binding exhibited a more complicated mechanism, probably involving exposure of additional binding sites upon a drug-induced conformational change (672).

The effect on diphenylhydantoin binding of some other drugs, including various antiepileptics, was investigated. Of these, only salicylic acid, sulfafurazole, and phenylbutazone (in clinical concentrations) decreased diphenylhydantoin binding. The clinical significance of this effect was discussed (673). The percentage of bound testosterone was significantly increased in hyperthyroidism and decreased in hypothyroidism. The *in vitro* testosterone saturation curves showed a remarkable increase in the testosterone-binding capacity of plasma in hyperthyroidism and a decrease in hypothyroidism. The study demonstrated that the state of thyroid function has a significant effect on the binding of testosterone and plasma, and it provides an explanation for the changes in the kinetics of testosterone metabolism observed in these disorders (674).

Additional references on the interaction of drugs with biological materials are provided in Table XIX.

Interactions of Drugs with Nonbiological Substances—A 1:2 complex between borax and chloramphenicol in aqueous solution was used to explain the relative stabilities of simple aqueous solutions of chloramphenicol (713). The molecular interactions in water of phenazine and tetramethylpyrimidopteridinetetrone with alkylxanthines and benzene derivatives were studied by means of the phase-solubility technique. These results were discussed in terms of structural similarity between interacting species. Studies concerning the effects of solvent on the extent of complex formation revealed that water plays a unique role in these interactions. The observation that complexation between two structurally dissimilar compounds is favored over that between two similar compounds suggested that some forces other than mere hydrophobic bonding should be considered. It was postulated

that the results are best rationalized by hydrophobic bonding stabilized by a type of bonding similar to polarization binding (714). On the basis of a simple model of 1:1 complex formation between planar molecules, the observation was made that there is no significant difference between the average behavior of neutral and charged complexes. The maximal overlap concept provides a general first-order description of molecular complex formation in aqueous solution. Application of the maximal overlap approach leads to average complex structures without introducing arbitrary assumptions about specific group interactions. The structures thus generated are consistent with the chemical reactivity of the complexes (715).

Maulding and Zoglio investigated the interaction of dihydroergotoxine with certain xanthines (716), dihydroergocrystine with xanthine analogs (717), and methysergide maleate and caffeine (718), and, in general, they discussed investigations into the nature of ergot alkaloid–xanthine complexes (719). These workers included solubility, dissolution, and partitioning studies to evaluate the *in vitro* interaction along with *in vivo* studies.

The quaternized vinylpyridine units of synthesized alkyl copolymers constituted the binding sites for *para*-toluene sulfonate ions. The stability of the complex formed in the reaction increased with an increase in temperature from 15 to 37° but decreased at 45°. The binding process was endothermic and associated with positive entropy effects, indicating that the polymeric model system described demonstrated properties expected for hydrophobic bonding. The effect of urea on the binding of sulfonate ion by one of the alkyl copolymers was studied and was found to be in keeping with the claim that urea breaks hydrophobic bonds (720).

Equilibrium and rate constants were calculated for the reversible covalent addition of bisulfite ion to 2-aminopyrimidinium ion and to its 1-methyl and 4-methyl derivatives. The 1:1 covalent adducts had very low solubility in aqueous buffers around pH 4. This was consistent with their being zwitterions. Rate-determining steps in adduct formation appeared to involve an attack of both the bisulfite ion and sulfite ion on the pyrimidinium cation. In the reverse reactions, the zwitterionic adducts appeared to decompose by both nonbase-catalyzed and specific base-catalyzed reactions (721).

Experimental data were obtained which appear to show that the binding between organic species dissolved in water apparently takes place most effectively between members of two large, distinct classes of structures, classified arbitrarily as A and B types. Many drugs may be included in the present classification; some examples for which data are available are: caffeine, theophylline, and prednisolone in class A, and phenacetin, promethazine, and menadione in class B. The complexing tendencies of series of systems involving pairs of interacting organic molecules in aqueous solution were investigated by the phase-solubility technique. Stability constants for some caffeine interactions were evaluated by means of partitioning studies (722). The effect of different organic solvents on the extent of complex formation in binary aqueous organic solvent mixtures was studied by various techniques. In every instance, the stability constant of the complexes decreased as the ratio of organic solvent to water increased. The complexes were much less stable in aqueous dioxane mixtures than in similar mixtures of water and polyhydroxy compounds, such as glycerin and sucrose. These studies strongly indicate the significant contribution made by hydrophobic bonding to these interactions and the major role of the water structure (723).

The interaction between bentonite and microcrystalline cellulose was studied in suspension and in the solid state. The rheological behavior of the mixed suspensions and the mechanical properties of composite films prepared by drying thin layers of these suspensions were investigated as a function of composition. Based on the volume percentage of the dry components, bentonite was about five times more effective in increasing the viscosity of aqueous suspensions than cellulose. Bentonite formed strong films when its aqueous suspensions were dried, while cellulose, either pure or in a mixture, did not (724). Interactions of isoniazid with magnesium oxide and with lactose were investigated in the solid state. In the isoniazid–magnesium oxide system, chemisorption, as well as physical adsorption, of isoniazid molecules onto the magnesium oxide surface was confirmed by diffuse reflectance spectroscopic data. The mechanism of surface chemisorption was noted to be different from that of the formation of the isoniazid–metal-ion complex in solution. The browning reaction of the isoniazid–lactose system in the solid state was also studied, and the reaction products confirmed the presence of isonicotinoyl hydrazones of lactose and hydroxymethylfurfural (725).

The interaction of ascorbic acid with silicic acid in the solid state was also studied using diffuse reflectance techniques. Adsorption of ascorbic acid from methanol solution by silicic acid did not occur; however, after evaporation of the methanol, ascorbic acid did undergo strong interaction with silicic acid, probably through hydrogen bonding. Thermal degradation of ascorbic acid in the adsorbed state was found to be different from that in solution. In acid solution, furfural was a major degradation product; in the adsorbed state, furfural could not be detected (726).

The kinetics of dialysis of a number of compounds in the absence of protein were studied in some detail to characterize the nature of the dialytic process and its dependency on experimental variables. It was shown that the rate of escape of a small molecule from a dialysis cell was a first-order process, provided that sink conditions were maintained (727).

Shima and coworkers studied the interaction of some pharmaceuticals with synthetic macromolecules. Their studies included the binding of pyrazolone and phenothiazine derivatives (728); barbituric acid, hydroxybenzoic acid, and xanthine derivatives (729); and other pharmaceutically important drugs (730) with styrene–maleic anhydride copolymer. The binding equilibria fitted a Langmuir-type equation, and molec-

Table XX—Additional References on Binding of Drugs to Nonbiological Substances

Reference	Topic
731	IR study of solute–solvent interactions of testosterone propionate
732	Determination of stability constants of hydroxyindoloquinoxaline metal complexes
733	Swelling of crosslinked polyacrylic acid by adsorption of water vapor
734	Gastrointestinal absorption of *O*-benzoylthiamine disulfide–polyacid complexes
735	Loss of ascorbic acid from tablets being directly proportional to the amount of water not bound by silica gel
736	Interaction of bishydroxycoumarin and furosemide with various adjuvants due to chemisorption proceeding by a mechanism similar to that of chelation
737	Solid–solid interactions of tetracycline, bishydroxycoumarin, and methantheline bromide significantly altering availability and activity of medicinal agents in pharmaceutical dosage forms
738	Effect of molecular interaction on permeation of organic molecules through dimethylpolysiloxane membrane
739	Riboflavin tetrabutyrate photodecomposed by methyl-substituted benzene derivatives through a 1:1 molar ratio complex
740	Inhibition of photodecomposition of riboflavin by 1:1 charge-transfer complexes of pyrazolone derivatives
741	Presence of polyacrilin potassium in formulation not affecting total drug availability in gastrointestinal tract
742	Divalent metals complex with 5- and/or 6-alkyl-substituted thiouracils
743	Interaction between sodium dehydroacetate and flavins
744	Effect of added salt on interaction between polymer and detergent in aqueous solution
745	Depreciating interaction of disinfectants and preservatives with nonionic surfactants
746	Effect of a polymeric complexing agent on liberation and stability of chloramphenicol
747	Interaction of polysorbate 80 (Tween 80) and polyvinylpyrrolidone with papaverine HCl
748	Interaction of sodium dodecyl sulfate with methylcellulose and polyvinyl alcohol, showing the existence of a critical concentration of sodium dodecyl sulfate below which no interaction with the polymers occurs
749	NMR investigation of sodium-ion binding in solutions of sodium caprylate and water in caprylic acid
750	Tryptazine reacted with methyl orange at pH 3.5 to form a 1:1 color complex
751	Silicone rubber and polyvinyl chloride strongly bind lipophilic drugs, while hard plastics like Teflon do not
752	Investigation of interaction between metal ions and alkyl phosphate monolayers at air–water interface
753	Solution confirmation of valinomycin–potassium-ion complex
754	Hexachlorophene esters in pure and mixed monomolecular films interacting with fatty acids, fatty acid alcohols, and fatty acid esters
755	Interaction of macromolecular and surfactant excipients with insoluble drugs used in pharmaceutical suspensions
756	Temperature dependence of the formation constants of alkaloid–methyl orange complexes
757	Stability of dispersed systems as affected by specific chemical interaction
758	Metal complexes of selenocysteamine
759	Stability constants for some 1:1 metal–carboxylate complexes, showing stabilities of complexes to be directly related to basicities of anions
760	Preassociation and thermodynamics of hydrophobic hydration in dilute solutions of surfactants
761	Kinetics of copper (II)–ethylenediamine complex formation
762	Interaction of nonionic macromolecular agents with methylhydroxybenzoate, indicating preservative activity due to permeability of microbial membrane
763	Chromatographic evidence for interaction of certain drugs with nonionic macromolecular agents
764	Counterion binding in micellar solutions of 1-*n*-dodecylpyridinium iodide
765	Aspirin interacting with paracetamol in tablets to produce increased amounts of alicyclic acid and diacetyl-*p*-aminophenol
766	Interaction between reserpine and desmethylimipramine
767	Effect of procaine hydrochloride concentration on formation of insoluble complex with sodium lauryl sulfate
768	Interaction between procaine or tetracaine and thiamine, showing evidence of charge-transfer complex
769	Effect of varying concentrations of testosterone on CMC value of lauryltrimethylammonium bromide, noting possible effect of steroid on enthalpy and entropy of micellization and micellar structure
770	Pharmacological aspects of rauwolfia–reserpine complex
771	Effect on Donnan equilibrium, neutral salts, and varying pH on degree of binding of some drugs to macromolecules, showing degree of binding increased when direction was from uncharged to negatively charged drug molecules

ular orientation and structure were used to explain the magnitude of binding.

Additional studies involving the interaction of drugs with nonbiological substances are found in Table XX.

Surface Phenomena—The various publications dealing with surface phenomena have been subdivided to aid the reader in locating areas of specific interest. Because of the obvious overlap in this subject matter, the reader who needs a thorough review is advised to consider the entire section.

Interface Studies—Cellulose acetate butyrate, cellulose acetate stearate, and cellulose acetate phthalate were studied as monolayers at the air–water interface. Monolayers of the first two compounds were virtually unaffected by changes in subphase pH, whereas cellulose acetate phthalate exhibited large differences under the same conditions. Certain monolayer properties of the cellulose esters were used to interpret the behavior of these polymers as free films and as enteric coatings (772). At pH values of 2–4, cellulose acetate phthalate monolayers were uncharged and were arranged in a compact, coherent form. An increase in pH brought about partial ionization and marked changes in monolayer organization. The effects of charge repulsion and increased solvation of charged groups caused marked expansion and decreased the stability of cellulose acetate phthalate monolayers. The greatest effect of pH on monolayer properties appeared to occur in the vicinity of pH 6 (773).

Using the Haydon–Taylor model, in which the surfactant head groups are situated at some distance from the oil surface, the interaction energy of electrostatic repulsion between oil droplets and dilute electrolyte solutions was derived for the two cases: constant-surface potential and constant-surface charge. For comparison, the repulsive energy was also derived for the constant-surface charge case without the effect of this adsorbed layer model. The model accounted for the penetrability of the electrolyte and dielectric constant in the adsorbed surfactant layer of varying thickness, the degree of surface coverage, particle-size effect, ionic strength, and the dielectric constant in the bulk solution. In the constant-potential case, the resulting

equation is identical to the classical DLVO one. Computations showed that as flat plates and spheres approached each other, constant-surface charge with impenetrable adsorbed layer was > constant-surface charge with penetrable adsorbed layer > constant-surface potential. It was noted that because of the possible 50- to 100-fold difference in magnitude between them, the choice of the proper model is important when one considers applying the theory to the rigorous kinetic treatment of the coalescence of o/w emulsions and flocculation of suspensions (774).

The adsorption from solution onto griseofulvin powder of a series of nonionic detergents of the polyoxyethylene glycol monoalkyl ether type was described. Results were also given for polyethylene glycol 400 and a new detergent, pentaerythritol mono-*n*-octyl ether, whose synthesis and purification were reported. The effect of temperature on the adsorption was studied, and possible explanations for variations in adsorption with temperature were based on the orientation of the molecules at the interface; this was deduced from measured molecular areas (775).

The kinetics of transfer of cholesterol from an aqueous polysorbate 80 solution into hexadecane and vice versa were studied by means of the multiparticulate dispersion technique. The experimental data were quantitatively analyzed by the physical model which accounts for the effects of bulk diffusion, interfacial resistance, interfacial area, and the lipid–water partition coefficient. For the 0.1% polysorbate 80, a P value of around 1.7 to 2.2 $\times$ 10^{-7} cm. sec.$^{-1}$ was found that was consistent with all data on water-to-oil as well as oil-to-water transfer experiments. These findings suggest that such large nonspecific interfacial barriers are important in many biological and biopharmaceutical situations (776).

Ghanem *et al.* (777) investigated the effects of an interfacial barrier in interphase transport. These procedures were applied to the study of the effects of an adsorbed gelatin at the hexadecane–water interface upon the transport of diethylphthalate between the two phases. By employing diethylphthalate as the solute, the transport data were analyzed by a physical model. The two ionic surfactants, sodium lauryl sulfate and dodecylpyridinium chloride, markedly decreased the interfacial barrier, even at low concentration. The analysis also showed that neither the electrolyte type nor concentration influenced the permeability coefficients, although they significantly altered the interface transport rates themselves by changing the partition coefficients. These findings may represent types of nonspecific situations that give rise to important barriers in *in vivo* drug transport. Ghanem and coworkers studied the quantitative transport behavior of several organic solutes in matrix systems composed of micron-size hexadecane droplets dispersed in an aqueous gelatin gel in which the oil–water interfacial barrier to transport was expected to play an important role. The authors stated that the techniques developed in the investigation should be useful in the quantitation of interfacial barriers in the oil–water interface transport of solutes and in the separation of various bulk diffusional resistances from interfacial resistances in complex multiphase matrixes (778).

Table XXI—Additional References on Interface Phenomena

Reference	Topic
779	Interaction of nitrogen dioxide with cholesterol monomolecular films and the possible biological relevance
780	Peculiarities of adsorption films of macromolecules and their relation to stability of emulsions, showing their tendency to form two-dimensional networks within long periods
781	Behavior of surface-active agents and dyes at oil–water interfaces, showing influence of binding between dye and surface-active agents as influenced by the species and concentration of electrolyte in aqueous phase
782	Isoelectric points of phospholipids at oil–water interface
783	Thickness and physical properties of interfacial films stabilizing emulsions formed at oil–water interface
784	Relative adsorbability of counterions at charged interface
785	Review of solid–liquid interfaces
786	Interfacial pressures and viscoelasticity measurements of monolayers of bovine serum albumin and its derivatives, showing effect of chemic l structure of bovine serum albumin
787	Surface pressure and specific water-evaporation resistance of monolayers of aluminum stearates, demonstrating effects of intermolecular interaction
788	Review of recent developments in interfacial phenomena
789	Estimation of film thickness from cohesive pressure at air–water interface
790	Review of fundamentals of monomolecu ar films including their importan :e in biological processes

Additional references on interface studies are described in Table XXI.

Adsorption Studies—In an approach to understanding the membrane action of phenothiazines, the adsorption from solution was investigated, using such adsorbents as carbon black, graphite, silica gel, and polyethylene. The results were related to other physicochemical properties and biological activities of the drugs. The adsorbed amount was influenced by the molecular volume of R_{10}'N at the 9-position, the sulfoxide group at the 5-position, and the bulkiness of the substituent at the 2-position. The adsorbed amount increased with the pH of the buffer solution between 5 and 8, and it was considered that pH had an influence on the hydrophobic and hydrophilic balance of the molecule. The decrease in adsorption with addition of urea demonstrated that the adsorption of phenothiazines proceeded on the hydrophobic interaction (791). The adsorption of sulfonamides by carbon black from solution was considered to be a hydrophobic interaction, noting that the entropy change was positive (792). The introduction of a methyl or methoxy group into the sulfonamide molecule caused an increase in the adsorption of the sulfonamide by carbon black, an increase in the binding to bovine serum albumin, and an increase in the absorption of the sulfonamide from the rat small intestine. It was concluded that hydrophobic interaction between the various sulfonamides and the intestinal membrane formed an important factor in absorption phenomena (793).

Methylene blue was adsorbed by potato starch from aqueous solution as a monomer or dimer, depending upon its concentration. Water-miscible additives such as methanol, dioxane, sucrose, and urea shifted the monomer–dimer equilibrium of the dye in the direction

Table XXII—Additional References on Adsorption Phenomena

Reference	Topic
799	Surface chemical properties of disodium and dipotassium monoacetyl phosphates; differences in these properties being attributed to the lower water solubility of the sodium salt, resulting in easier adsorption and micelle formation
800	Influence of thixotropic agents (Aerosil and Aerosil R 972) on adsorption of water by powders
801	Effect of hydrocarbon chain length on adsorption of sulfonates at solid–water interface
802	Influence of pH on adsorption of aromatic acids on activated carbon, showing marked increase in adsorption from aqueous solutions at pH's below 7
803	Adsorption of starch and oleate and interaction between them on calcite in aqueous solutions
804	Differential heats and entropies of water adsorption on calcium and potassium montmorillonite
805	Review of adsorption of cationic surfactants by cellulosic substances
806	Adsorptive property of aluminum hydroxide for vaccine being reduced, depending on method of preparation of adsorbed vaccine, superficial structure, and crystalline form of aluminum hydroxide
807	Phase relations involving deposition of cholesterol from triglyceride solution, noting the hydrogen bonding of cholesterol to the ester bond of the triglyceride being unrelated to solubility of cholesterol in the triglyceride
808	Removal of long-chain, nonionic ethylene oxide adducts from aqueous solutions by adsorption
809	Adsorption of sodium alkyl sulfates at air–water interface, showing various stages of forces acting at interface
810	Review of theoretical approaches applied to studies of adsorption from concentrated and diluted binary mixtures
811	Adsorption of *s*-triazines by montmorillonite as a function of pH and molecular structure, showing that more basic compounds were adsorbed in greater amounts
812	Adsorption of aerosol OT at solid–liquid interface
813	Adsorption of ionized surface-active agents in solution at air–solution interface
814	Amylose–iodine–iodide complex as a model for competitive cooperative linear adsorption
815	Adsorption of bovine serum albumin at air–water interface shown to be reversible; measurement of equilibrium adsorption isotherm
816	Adsorption of ionic surface-active agents influencing stability of hydrophobic colloidal dispersions
817	Measurement of the amount of sorption water in various kinds of starch samples in equilibrium with water at 25 and 40°
818	Kinetics of adsorption from solution in which rate expression yields rate constant for adsorption and desorption simultaneously
819	Effect on adsorption of increasing the number of methyl groups in an homologous series of surface-active agents such as alcohols and alkanoic acids
820	Adsorption of polyvinyl alcohol on titanium dioxide
821	Influence of sodium dodecyl sulfate on the stability of iron (III) hydroxide and aluminum hydroxide sols
822	Adsorption of anionic surfactants on montmorillonite, as proven by X-ray diffraction data
823	Simple model for calculating heat curves for physical adsorption
824	Adsorption of dyes and their surface spectra
825	Review of mathematically treatable quasithermodynamic and statistical models in adsorption from solution
826	Review of adsorption of cationic surfactants on various solid substrates, including certain biological substances
827	Model for the adsorption of weak electrolytes on solids as a function of pH, using carboxylic acid–charcoal system
828	Adsorption equilibrium of ionic surfactants in the presence of an inorganic salt in solution
829	Adsorption of surfactant molecules at a liquid–vapor interface; variation in rates of adsorption and adsorption energies with increasing concentration of the surfactant solutions discussed in terms of detergent effectiveness of surfactant
830	Alumina surface chemical properties determined with colored indicators
831	Review of importance of physical adsorption and the information that it can provide regarding nature of a solid surface

of the monomer and reduced the extent of methylene blue adsorption without significantly affecting the maximum possible adsorption, indicating that additives act primarily on the effective concentration of the dye (794). In studying the effects of the adsorption of benzoic acid on graphite, it was concluded that the adsorbed acids were mostly oriented flat on the graphite surface; plane-to-plane stacking between the acid molecule and the fused benzene ring plane of graphite seemed to be dominant among the possible adsorption forces (795). The velocity and activation energies for adsorption and desorption of cyanocobalamin on talc from aqueous solutions were investigated. The activation energies for the adsorption and desorption were 1.16 and 3.98 kcal. mole^{-1}, respectively, using the Arrhenius equation. It was indicated that cyanocobalamin is energetically more stable in the adsorbed state than it is free in solution, because the activated states for the adsorption and the desorption were considered to be identical (796).

Intravascular thrombosis was shown to be an interfacial reaction dependent on the surface charge characteristics of the blood vessel wall, blood cells, and prosthetic materials. Positively charged prosthetic materials were thrombogenic, whereas negatively charged surfaces tended to be nonthrombogenic; the higher and the more uniform the negative charge density, the better was the chance of the materials being nonthrombogenic (797). An investigation of the interactions of the cholesterol particle surface with bile salts and alkyl surfactants was undertaken. Microelectrophoretic and adsorption techniques were used to demonstrate, in contrast to the alkyl surfactants, that the adsorption of bile salts on the cholesterol particle surfaces was much less than expected in the concentration ranges studied. While these results are initially surprising, the data are consistent with the idea that the relatively rigid bile salt molecules can adsorb only onto specific sites on the cholesterol surfaces, while the more flexible alkyl surfactants can more readily interact (798).

Additional references on adsorption studies are provided in Table XXII.

Studies on General Properties of Surface-Active Agents—The effects of pH and hexachlorophene on the net fixed-charge density of the colloids composing the epidermal surface were studied *in vivo*, employing human subjects. The results were shown to be analogous to titration curves of amphoteric macromolecules. Both the control and hexachlorophene titration curves were nonhysteretic when initiated at either extreme of the pH range, indicating reversibility of the observed pH and hexachlorophene-induced changes. The presence of hexachlorophene decreased the net fixed-

charge density of the tissue surface below pH 5.6; above this pH, a net increase was constantly observed. It was hypothesized that hexachlorophene-induced alterations in fixed-charge density result from allosterically effected changes in the dissociation constants of ionogenic protein side groups located vicinally to hexachlorophene interaction sites (832).

To elucidate the interaction of surfactants with biological membranes, the composition of epidermal phospholipids and the rate of biosynthesis of major phospholipid components were determined, using various analytical techniques. The major lipid components were cholesterol, lecithin, lysolecithin, phosphatidyl ethanolamine, and sphingomyelin. The biosynthetic and turnover rates of all identified phospholipids were greatly increased in the surfactant-treated skin. It was suggested that the tested surfactants damaged the epidermal membranes. A role of surfactants in increasing the absorption of medical substances was also proposed, in view of these results and other reports regarding the effect of surfactants on biological membranes (833). Shinoda (834) stated that, unlike ordinary solutions, an aqueous solution of nonionic surfactant resembles, in principle, a fine dispersion of two phases. One is the water phase, containing a very small amount of nonionic surfactant, and the other is the swollen surfactant phase. The thermodynamic properties, as well as the mechanism of dissolution, of biopolymers and polysoaps can be explained by the proposed model.

Although an HLB value is assigned to a definite surfactant, the real HLB of the surfactant at the oil–water interface changes with the amount and kinds of added salts in water as well as with the types of oils. On the other hand, the phase-inversion temperature of emulsions accurately reflects that the real HLB of a surfactant in a given system changes sensitively with the amount and kinds of added salts. It was found that the phase-inversion temperature of the emulsions studied was depressed about 14° in the presence of 6% sodium chloride solution, indicating that the HLB value of the surfactant was depressed about 0.8–1.0 unit in the solution (835). Solubility parameters were calculated for a homologous series of alkyl polyglycol monoethers with a variable glycol chain. These parameters were compared with the HLB values for the same surfactants. By considering the contribution made to the overall solubility parameter of a surfactant molecule by the solubility parameter of the hydrophobic portion of the molecule, a practical relationship between solubility and HLB was obtained. This relationship was valid, irrespective of the size of the hydrophobic or hydrophilic group, for compounds containing up to 20 carbon atoms or 20 ethylene oxide units in the alkyl or glycol chain, respectively. By making several assumptions, it was shown that heats of hydration and CMC's of surfactant, which have been shown to be related to HLB values, may also be related to solubility parameters of surfactant molecules (836). In studying the importance of surfactant location and required HLB in emulsification, Lin (837) noted that, for an o/w emulsion, placing the surfactants in the oil phase produced more stable emulsions than the same system prepared by initially placing the surfactants in the aqueous phase.

Table XXIII—Additional References on General Properties of Surface-Active Agents

Reference	Topic
838	Mutual relations among properties of cationic surfactants, such as solubility, micelle formation, and solubilization of water in nonpolar solvents, explained by hydration and difference in the ionic character of polar groups in surfactant molecules
839	Effect of ionic head of cationic surface-active agents on their hemolytic activity, showing that the number of carbon atoms in the alkyl radical, when less than 8, is related to hemolytic activity of cationic agent
840	Nonmicellar association of surfactant ions, noting constant enthalpy of dimerization for each chain length
841	Structure of aqueous surfactant solutions
842	New amphoteric surfactant having ether bonds in the molecule
843	Review of ethoxy sulfate-type surfactants and their colloid-chemical properties
844	Review of chemistry and technology of surfactants
845	Effects of temperature and hydrophilic chain length of nonionic surfactants on emulsion properties discussed in connection with the phase-inversion temperature of respective systems
846	Electrolyte flocculation of macromolecular stabilized emulsion in the framework of DLVO theory, with special reference to sodium alginate and gum arabic as emulsifiers
847	Review of esters of pentaerythritol and higher fatty acids, as well as their polyoxyethylene derivatives as surfactants
848	Application of nonionic surface-active agents for dispersion of pigments in aqueous media, noting that each surfactant had an optimal HLB value at which dispersion was maximum
849	Incipient flocculation properties of aqueous polymer dispersion which are stabilized solely by polyethylene oxide moieties
850	Dependency of detergency on HLB of nonionic surfactants being small for hydrophilic fabrics but large for oleophilic fabrics
851	Surfactant-ion activity of magnesium dodecyl sulfate in aqueous solution
852	Mechanism of action of a surface-active stabilizer of clay suspensions; stability of the suspension being adequate in the presence of up to 10–15% of electrolyte; at higher concentrations, addition of 1–1.5% algin being required
853	Review of classification of surfactants by HLB system
854	Review of ampholytes, their properties, and applications
855	Preparation and properties of sodium sulfoalkyl alkanoates surfactants
856	Langmuir film balance and surface-tension measurements for determining HLB of surface-active agents
857	Review of surfactant structure and performance
858	Review of application of surfactants in pharmaceuticals, including a table of toxicity of surfactants
859	Review of properties and uses in cosmetics of amphoteric surfactant cycloimidium
860	Review of effects of surface-active agents on absorption, solubility, and therapeutic utility of various drugs, including bacteriostatic agents
861	Surface properties of cellulose acetate phthalate, including claim that there is no evidence of stoichiometric complex formation with plasticizers, and interaction appearing to be completely nonspecific

Additional studies on general properties of surface-active agents are provided in Table XXIII.

Micelle Studies—The effect of change in pH on the CMC of a nonionic surfactant (polysorbate 80) was investigated; a linear relationship existed between the free energy of micellization and pH. The significance of changes in the enthalpy and entropy of micellization was discussed; both micellar molecular weight and the hydration per unit mass of surfactant decreased with an increase in pH value (862). The thermodynamics of

Table XXIV—Additional References on Micelles

Reference	Topic
868	NMR studies on micelle formation by promethazine hydrochloride
869	Properties of aqueous solutions of surface-active agents when their concentrations are below the CMC
870	Fluorescence and differential UV studies of micellar solutions of sodium phenylundecanoate
871	Biradical spin-labeled urea derivative used for studying the dynamic structure of sodium dodecyl sulfate micelles, showing the biradicals form aggregates in the micelles when present in excess in solution
872	Phospholipid bilayer–micelle transformation
873	Shift of NMR signal caused by micelle formation
874	Review of salt effects on CMC's of nonionic surfactants
875	Thermodynamics of micellar equilibrium for ionic detergents
876	Effect of change of solvent on CMC of a nonionic surfactant
877	CMC of Manoxol OT and Nonidet P40, using silver-110m as a tracer
878	Micelle size of barium dionyl naphthalene sulfonate in low polarity solvents by vapor-pressure osmometry
879	Counterion binding to reversed micelles by nuclear magnetic quadrupole relaxation of bromine-81
880	Interaction of acidic dye molecule with positive charges on micelle surface
881	Surface-active properties of ammonium salts of sulfated esters of oleic acid
882	Aggregation of local anesthetics in solution, demonstrating that hydrophobic substitution on the tetracaine *para*-amino group accounted for greater tendency to aggregate
883	Effect of temperature on micellar properties of pure nonionic surfactants; thermodynamic calculations
884	Effect of position of ester group on CMC of ester-linked sulfonates, showing that for a given number of carbon atoms in the alkyl chain, the log CMC value increases regularly with a change in the ester group away from the terminal position to more central positions in the hydrocarbon chain
885	Solubility and CMC's of fluorinated surfactants in water
886	Ultrasonic absorption in aqueous solutions of ionic amphiphiles, with interpretation of results from weakly concentrated micelle solutions
887	Salt effects on the CMC's of nonionic amphiphiles
888	Kinetics of micelle dissociation by a light-scattering temperature jump method
889	Prediction of the CMC of mixtures of alkyltrimethylammonium salts using Shinoda's equation for CMC of a multicomponent soap mixture
890	Thermodynamics of micellization and micellar molecular weights of zwitterionic *N*-alkyl betaines by light scattering and vapor-pressure osmometry
891	Potentiometric and calorimetric studies of the structure of some soap solutions with short paraffin chains
892	Influence of charge on the micelle size of a zwitterionic surface-active agent
893	Statistical treatment of micellar solutions
894	Effect of temperature and solubilization of hydrocarbons on mean micellar weights in aqueous sodium oleate solutions
895	Effects of counterion and temperature on micellar properties of dihydroxy and trihydroxy bile salts
896	Effect of temperature on CMC of tetradecylpyridinium bromide, tetradecylbenzyldimethylammonium bromide, and tetradecyltrimethylammonium bromide

micellization of three nonionic surfactants in mixed solvents were reported. The contribution of the alkyl chains of the surfactant molecules to micellization was estimated from solubility data (863). The optical rotatory dispersion of a nonionic surfactant, β-D-octyl glucoside, was investigated in aqueous solutions in the UV region. The specific rotation at any wavelength showed an increase at the CMC, which could be determined reliably from the change in specific rotation. The rotatory dispersion curve for the surfactant in micellized form was derived and compared with that of the nonmicellized surfactant below the CMC. The change was small and was ascribed to a "medium" effect, arising from the difference in the local refractive index at the micelle surface as compared with the bulk solvent. This interpretation is compatible with currently accepted ideas on the fluid nature of the micelle core, and it suggests a lack of any conformational restraint at the micellar interface (864). The temperature dependence of the CMC of decyl-, dodecyl-, and tetradecyl-α-picolinium bromides was determined by measuring conductance. Constants for the equation relating log CMC and alkyl chain length were calculated for the temperature range 5–70°. An estimate of the effect of temperature upon the degree of counterion binding was recorded. Thermodynamic parameters were calculated using the uncharged phase-separation model, and a theoretical interpretation of the process of micellization was given (865).

The weight-average aggregation numbers and micellar molecular weights for homologous zwitterionic *N*-alkyl betaines were determined by light scattering. A linear relationship was found between the logarithm of the weight-average aggregation number and the alkyl chain length for C_{10}, C_{11}, C_{13}, and C_{15} homologs. As the temperature was raised, a trend was observed in that the weight-average aggregation numbers and the micellar molecular weights for the homologs increased, reached a maximum, and then decreased. This behavior was related to changes in the CMC with temperature (866). The acid dissociation constants of long-chain esters of carnitine above the CMC were determined potentiometrically at several concentrations of added KCl. As the degree of protonation increased, the apparent pK values decreased, owing to the increased positive charge on the micelle. The difference in pK between the neutral (zwitterionic) micelle and the value at any given protonation was used to determine the surface potential of the micelle at that degree of protonation. From the partial molal volume of each surfactant in the micelle and the calculated surface charge density, it was possible to calculate the aggregation number of the micelle. Good agreement was noted between the calculated values of the micelle and the values obtained from light-scattering experiments at several ionic strengths and degrees of protonation (867).

Table XXIV provides additional references on micelle studies.

Additional studies of a general nature involving surface phenomena are provided in Table XXV.

Crystallization—The results of crystal growth-rate studies, using single crystals of sulfathiazole and methylprednisolone, were presented. The growth rate of sulfathiazole crystals growing in a supersaturated aqueous solution showed stirring rate dependence from 10 to 400 r.p.m. for all three faces studied. In alcohol solutions, the stirring rate dependence appeared to disappear above 150 r.p.m., suggesting that at the higher stirring rates the rate of crystal growth of sulfathiazole was surface controlled. A plot of the growth-rate *versus* the supersaturation ratio appeared to be linear for all studies, with intercepts exhibited on the supersaturation ratio axis. The intercept appeared

Table XXV—Additional References on Surface Phenomena

Reference	Topic
	General Studies on Colloids, Gels, and Sols
897	Interaction of two identical spherical colloidal particles at great distances, using the Derjaquin equation
898	Electric conductivity of gels of natural macromolecular substances, showing that three types of water structure exist in various gels
899	Review of properties of association colloids, isolated thin film, surface forces, and disperse systems
900	Review of stability and flocculation of typical lyophobic and lyophilic colloids, solutions containing simple ions, and nonionic surface-active agents
901	Adsorption of water vapor by gelatins being independent of molecular weight of the gelatin but dependent on ionization state of free carboxyl groups
902	Pulsed NMR study of temperature hysteresis in agar–water system, indicating that the major portion of water molecules in this system is not in an "icelike" or modified state
903	Review of methods for determining force of adhesion between colloidal particles and factors that influence adhesion between a sphere and a plane and between a plate and a plane
	Foam Studies
904	Foaming characteristics of anionic surfactants, volume of foam increasing with concentration of surfactant up to a certain value and then leveling
905	Action of electrolytes on foam stability of a nonionic surface-active agent, showing the increasing stability as a function of the concentration, as it relates to the surface tension and the CMC
906	Surface hydrolysis enhancing the inevitable pH changes in foam separation of anionic surfactants
907	Effects of surface-active agents on bubble formation
	Surface Area Studies
908	Orientation of monolayers of half-esters of polymethylvinyl ether maleic anhydride, showing effects of subphase pH and certain water-soluble plasticizers on surface pressure–area isotherms
909	Proposed methods for surface area determination based on behavior of adsorption isotherms of completely miscible liquid pairs
	Wetting and Contact Angle Studies
910	Effect of ionic-type surfactant pretreatment in contact angle studies on viable human skin
911	Adsorption and wetting phenomena associated with Graphon in aqueous surfactant solutions
912	Wettability of chloramphenicol palmitate, demonstrating lack of apparent relation between HLB values and wetting efficiency
913	Spreading coefficients, wetting energies, and adsorption of surfactants
914	Review of wetting phenomena of solid surfaces
	Surface-Tension Studies
915	Effect on surface activity of some phenothiazine derivatives due to changes in number of alkyl groups, degree of branching, and number of dissociable groups on alkylamino portion of the molecule
916	Surface activity of water–1,4-dioxane system
917	Electric surface potentials and surface tension of organic bases in aqueous solutions
918	Dynamic surface tensions of surfactants in natural hard waters
919	Comparative studies on interfacial tension of different ointment bases with water–oil emulsifiers
920	Surface activity of aqueous solutions of chlorohydrins depending mainly on the weight ratio of hydrophilic to hydrophobic groups
	Diffuse (Electrical) Double-Layer Studies
921	Approximate methods of determining double-layer free energy of interaction between two charged colloidal spheres
922	Review of Helmholtz free energy for electrochemical double layers of charged colloidal particles in aqueous medium
923	Interfacial instability due to electrical forces in double layers—general considerations
924	Interfacial instability due to electrical forces in double layers—stability of interfaces with diffuse layers
925	Electrical double layer at the graphite–surfactant interface

to be a function of its solvent, varying from 1.07 to 1.43, and to be a function of the polarity of the alcohol. The crystal growth rate of methylprednisolone, on the other hand, showed no stirring rate dependence in the range from 20 to 400 r.p.m. and appeared to be surface controlled even at low stirring rates (926).

The results of polyvinylpyrrolidone inhibition of sulfathiazole single-crystal growth were reported. The minimum concentration of polyvinylpyrrolidone required to inhibit crystal growth completely was a linear function of the supersaturation ratio, with an intercept of approximately 1.15 exhibited on the latter axis. The minimum concentration of polyvinylpyrrolidone required for complete inhibition of crystal growth was a function of the molecular weight of polyvinylpyrrolidone. The results of using polyvinylpyrrolidone molecular weights of 10,000, 40,000, and 360,000 are presented. The data suggest that the inhibition point depends on the relative rates of transport of polyvinylpyrrolidone and sulfathiazole to the crystal surface from the bulk of the solution. A model is presented that appears consistent with the data (927).

The crystal structure of the 2:1 complex of barbital with caffeine was determined by X-ray diffraction methods. The block-diagonal least-squares refinement of 496 atomic positional and thermal parameters, based on 4665 X-ray intensity data, gave a final R factor of 0.05. The structure consisted of ribbons of barbital molecules linked by NH---O=C hydrogen bonds. Caffeine molecules were bound to the ribbon by an NH---N(9) hydrogen bond and by an unusual interaction involving C(8)H with two barbital oxygen atoms. Weak interactions of nonhydrogen-bonded caffeine carbonyl groups with barbital carbonyl groups may also be important in this crystal. There was minimal overlap of the flat ring systems of the component molecules (928).

Additional references on crystallization are found in Table XXVI.

Rheology—A chemical balance provided a simple, cheap, and readily available method of assessing the consistency of biological fluids such as sputum. A scale reading at an arbitrary time provided a useful empirical parameter which could be employed in routine testing of chemical samples and in the assessment of mucolytic agents. This reading also correlated well with data obtained using a conventional cone and plate viscometer. The change of scale reading with time could be analyzed in a fundamental rheological manner, using the linear viscoelastic model. The method of measure-

Table XXVI—Additional References on Crystallization

Reference	Topic
929	Macroscopic appearance of frozen and dried samples in connection with growth of eutectic crystals; an optimum temperature range to promote the eutectic crystallization for each solute
930	Change in the macroscopic appearance during freezing, and the critical temperature necessary for freeze drying of pharmaceuticals
931	Review of recent theories of growth and dissolution of crystals, their importance in biological and physical systems, and the role of foreign substances as inhibitors of these processes

ment was nondestructive and allowed repeated measurements to be made on the same sample (932).

The rheology of six grades of white soft paraffin was investigated, using continuous shear viscometry and a creep viscometer. Working the samples on a triple roller mill decreased the apparent viscosity and, initially, increased the yield stress. Five of the grades were linear viscoelastic and one was nonlinear viscoelastic. The nature of ductility and its relation to measured rheological parameters were discussed (933). The viscoelastic gel in the continuous phase of a liquid paraffin–water emulsion stabilized by a mixed emulsifier of cetrimide and cetostearyl alcohol was similar to that formed by dispersing cetrimide and cetostearyl alcohol in water. The effects of temperature changes on the rheological properties of the emulsion and the ternary system were examined in continuous shear and in creep, and variations in the viscosities and compliances were correlated with the thermal phase transitions determined microscopically. The emulsion and the ternary systems were of maximum consistency at approximately 38 and 43°, respectively. These temperatures represent the transition from the frozen smectic to the liquid crystalline phase. At higher temperatures, the compliances rose and the viscosities fell as the network weakened and finally dissolved to form an isotropic solution (934).

Additional studies on rheology are provided in Table XXVII.

PHARMACEUTICAL ASPECTS: ANTIBIOTICS

Some studies of the oral absorption of dicloxacillin from various pharmaceutical formulations in beagle dogs suggest that the female of this species shows consistently higher and more prolonged blood serum levels of the antibiotic than the male. These studies suggest that there are variations in the level of monobasic penicillin molecules in blood serum after oral administration that are related to the sex of the animal, while a similar correlation does not appear to exist for the amphoteric penicillins. Determination of the biological half-life of the various penicillins in the male and female after intravenous administration indicated that there was no difference in the sexes with respect to the disappearance of active drug from the blood (956).

The absorption of tetracycline from the rat stomach was investigated at acid pH values. The absorption was dependent upon the anion and the surface activity of the buffer (957).

Spectinomycin slowly inhibited the steady-state growth of *E. coli* to a new steady state with a new rate constant that was linearly dependent, above a certain minimum, on the concentration of spectinomycin. This minimum concentration is a function of the concentration of the media and can be assigned to binding or removal of microbiologically effective spectinomycin as protonated material by the components of the media. The logarithm of the inhibitory constant increases linearly with the pH of the media to pH 7.6, which implies that only uncharged material is biologically active. The slow rate of achievement of a drug-equilibrated, steady-state, microbial generation rate can be reconciled with a relatively rapid reequilibrated rate on dilution with fresh media by postulating depletion of a cell-generated vital metabolite linked to the growth rate of microorganism (958).

The closely related antibiotics diumycin A and B, with monomeric molecular weights of approximately 1800 daltons in ethanol, aggregate in aqueous buffers to form particles with a molecular weight of at least 32,000 daltons. The aggregate of diumycin was essentially unaffected by esterification of the acid, acetylation

Table XXVII—Additional References on Rheology

Reference	Topic
935	Rheological measurements of semisolids, including evaluation of relationship between shear stress and shear rate, determination of product behavior during continuous shear, and characterization of static structure of the product
936	Shear-dependent interaction of plasma protein with erythrocytes in blood
937	Review of inorganic thickening, suspending, and emulsion-stabilizing agents
938	Use of rheology in pharmaceutical technology
939	Measurement of properties of semisolids, with emphasis on viscoelasticity, continuous shear, creep, and oscillation
940	Changes in rheological properties of vaselines connected with their content of cycloparaffinic and low paraffinic fraction
941	Viscosity of oxyethylated alcohols of wool fat
942	Rheological properties and composition of petrolatums
943	Importance of rheological parameters of bases for ointments in determining rate of liberation and sorption of drugs incorporated in them
944	Effect of pharmaceutical additives on rheological characteristics of dispersions of methylhydroxyethylcellulose
945	General flow characteristics in rheological evaluation of dispersions of methylhydroxyethylcellulose
946	Rheology of clay mineral suspensions
947	Review of rheology of disperse systems, dealing mainly with pigments and paints
948	Influence of particle-size distribution on apparent viscosity of non-Newtonian dispersed systems
949	Effect of glyceride distribution on rheological properties of fats
950	pH dependence of rheological properties of agar–agar hydrogels
951	Rheological properties of alginic acid gels
952	Rheological properties of hydrogels of agar–agar and stress relaxation of concentrated agarose gels
953	Rheological properties of concentrated pectin gels made through diffusion of alcohol at room temperature in concentrated subacid pectin solution
954	Review of mixing of Newtonian and non-Newtonian fluids
955	Influence of production scale-up upon quality of a cream, including changes occurring in hardness, particle-size distribution, and rheological properties

of hydroxyl groups, high ionic strength buffer, or variations in pH from 2.2 to 12.4. These results indicate that salt linkages and hydrogen bonds contribute only slightly to stabilize the aggregate. The aggregate may be disrupted by: (*a*) the addition of such hydrophobic bond-breaking agents as buffered aqueous solutions of guanidinium chloride, urea, or formamide; (*b*) the hydrolytic loss of a lipid side chain (molecular weight about 400 daltons); or (*c*) the addition of alcohols. The ability of an alcohol to disrupt the aggregate increases with its hydrocarbon content. From these data, it was concluded that lipid–lipid hydrophobic interactions are responsible for the self-association of diumycin. The aggregate is spherical with a mantle of hydrophilic sugars, including glucose and glucosamine, surrounding a tangle of lipid side chains (959).

The deuterium isotope effect on the biological activity of penicillin G was investigated by comparing the biological activity of the deuteriopenicillin with that of the protioanalog by a turbidimetric assay procedure using *S. aureus*. The biological assay indicated that a significant deuterium isotope effect operates in the antistaphylococcal action of benzylpenicillin. With the test organism chosen, the ratio of the antibiotic potencies was 125% H/D (960).

The pharmacokinetic profile of coumermycin A_1 was determined in man following intravenous and oral administration. The antibiotic was eliminated slowly from the bloodstream and appeared to be highly biotransformed. The plasma level *versus* time curve after intravenous injection was consistent with a two-compartment open system, containing a primary compartment with a volume equivalent to the volume of plasma water. The design of a pharmacokinetic model was discussed (961). As the free acid or simple salt, coumermycin A_1 is poorly absorbed from, and is not appreciably degraded in, the fluids of the gastrointestinal tract of animals and humans. A mixture of coumermycin with certain additives, such as sugar amines, can significantly enhance oral absorption in dogs, as indicated by the higher blood levels. With a mixture of antibiotic and *N*-methylglucamine in a 1:4 ratio, blood levels in both dogs and humans were enhanced 5- to 15-fold over that obtained with the antibiotic alone (962).

Additional references on antibiotics are found in Table XXVIII.

Table XXVIII—Additional References on Antibiotics

Reference	Topic
963	Increased absorption in dogs and humans after oral administration of coumermycin monosodium salt in a 1:4 w/w mixture with *N*-methylglucamine
964	Stabilities, modes of binding, and methods of disruption of aggregates of saramycetin and prasinomycin
965	Inhibitory effect of tetracyclines on rate of hydrolysis of an olive oil emulsion by pancreatic lipase
966	*In vitro* antibiotic activity of aminodeoxykanamycin
967	Stability of tetracycline hydrochloride in various ointment bases
968	Absorption, elimination, and *in vitro* sensitivity of *Pseudomonas pyocyanea* strains to carbenicillin
969	Hygroscopic and nonhygroscopic forms of oxytetracycline hydrochloride
970	Influence of storage conditions and pharmaceutical additives on stability of stamycin dragees
971	Influence of storage conditions on triacetyloleandomycin tablets
972	Metabolism of various antibiotics in hepatic dysfunction
973	Effect of adding bovine serum to various antibiotics on their *in vitro* bactericidal action
974	Review of polyene antibiotics and macrocyclic antibiotics and their effect on bilayer model membrane
975	Employment of hydron polymer antibiotic vehicle in otolaryngology
976	Biological activity of cephalexin in children
977	Pharmacokinetics of ampicillin and hetacillin
978	Stability of aqueous solutions of viomycin sulfate
979	Antibacterial activity produced in animals by coadministration of two antibiotics, demonstrating that the combination had significantly greater protective action than did individual components
980	Absorption, elimination, and metabolism of aminocyclohexylpenicillin
981	Review of structure–activity relationships of semisynthetic penicillins
982	Preparation of oxytetracycline in a dry powder for reconstitution

BIOPHARMACEUTICS

The various publications dealing with biopharmaceutics have been subdivided to aid the reader in locating areas of specific interest. Because of the obvious overlap in subject matter, the reader who needs a thorough review is advised to consider the entire section.

Schneller (983) reviewed the developments in biopharmaceutics with regard to the effectiveness of drug products. Included in his review is a table of 40 drug substances with proven, or definitely suspected, intrinsic susceptibility to bioavailability problems. The author noted that the subject of biological availability of a drug from its dosage form presents the pharmacist with challenges and opportunities which he is uniquely qualified to meet.

Numerous articles were published dealing with theoretical models as a means of correlating *in vitro* data with the therapeutic activity of a drug. The simultaneous chemical equilibria and mass transfer of basic and acidic drugs through a two-phase compartment model were theoretically investigated. The model consisted of a well-stirred bulk aqueous phase, an aqueous diffusion layer, and a lipid barrier for perfect and imperfect sink cases. The nonsteady- and quasi-steady-state changes in the concentration–distance distributions in the lipid phase were also considered. The rate of change of the total drug concentration in the bulk aqueous phase was described in the general form of a first-order equation useful for evaluating experiments. A limiting steady-state relationship involving the transport rate with the partition coefficient, pH at the aqueous–lipid interface, dissociation constant of the drug, aqueous and lipid diffusion coefficients, and thickness of the diffusion layer was derived. Increasing the agitation rate in the aqueous phase markedly affected the pH profiles for the rate of transport. The pH-partition theory was shown to be a limiting case of the more general approach presented (984).

In a similar study, multicompartment diffusional models for the absorption of neutral, acidic, basic, and amphoteric drugs were investigated. The general model consisted of a bulk aqueous phase, an aqueous

diffusion layer, *n*-compartments of homogeneous and heterogeneous phases, and a perfect sink. Equations were derived in general terms for the nonsteady- and steady-state periods. Utilizing the steady-state diffusion efficiency function of the barrier systems, the first-order rate constants for various examples of two- and three-compartment models were obtained from the general model, and some computations were given. Various sets of *in situ* experimental rat data were analyzed by means of the different models. These included the intestinal, gastric, and rectal absorption of sulfonamides and barbituric acid derivatives. Self-consistent dimensional constants and diffusion coefficients were arrived at, and the correlations obtained with the models were found to be generally satisfactory (985).

A general solution was presented for solving the pharmacokinetic parameters that describe a drug and its metabolite in a two-compartment open model. The method was specifically applied to the treatment of plasma data for acetylsalicylic acid and its metabolite, salicylic acid, after intravenous administration of the drug. The acetylsalicylic acid data were adequately described by a model in which elimination occurred solely from the central compartment (986). In the formulation of compartment models for describing biological phenomena, two separate approaches usually have been employed for isotope dilution systems and systems for which no tracers are introduced. For linear systems that can be described by first-order kinetics, it was shown that the isotope dilution problem can be cast into the more general approach by simultaneous linear differential equations, and the restriction of steady state can be removed. Solutions to these equations were shown to be easily obtainable, using the state-space approach. For systems in which linearity cannot be assumed, digital computer techniques were presented which greatly facilitated numerical solutions. These concepts were demonstrated with two examples, and a third example showed how these concepts and others could be employed with isotope dilution to find the initial pool sizes and rate constants in a six-compartment system (987). A six-compartment open-system model was presented to elucidate the physiological disposition of chlordiazepoxide and its two pharmacologically active biotransformation products by intravenous administration of the drug in dogs. The pharmacokinetic parameters used in the model were obtained by administering each of the three compounds separately. Excellent agreement was obtained between the levels of intact drug and the two metabolites found in the plasma of the dogs and the calculated levels of each generated from the model (988). A pharmacokinetic model was presented to describe the distribution of methotrexate in mice. The model was used to simulate methotrexate concentrations in plasma, lean tissue, liver, kidneys, and gastrointestinal tract following a single intravenous injection (989).

Loo and Riegelman (990) noted that if it is not feasible to administer a drug by a single bolus, it may be possible to administer the drug intravenously at a slower rate of infusion. A mathematical equation was presented which enabled one to determine the parameters identical to an intravenous bolus injection curve by utilizing the postinfusion blood curve. The equation is applicable to all compartmental models that may be described by linear first-order differential equations with constant coefficients. The transfer of salicylic acid and three sulfonamides from an aqueous phase of pH 2 or 5 through an intervening organic phase to an aqueous phase of pH 7.4 was studied, using a rotating cell. The manner of operation of the cell promoted rapid drug transfer without vortexation or emulsification of the phases. The rates of transfer of the drug showed the anticipated pH dependence (991). A transport cell was devised which consisted of three compartments, partitioned with two semipermeable cellulose membranes; the central compartment was thin and held a solution of nonpermeable material which acted as the barrier to drug transport. Transport of barbital and benzoic acid through the central compartment of aqueous polyvinylpyrrolidone was examined, and it was noted that the interaction of polyvinylpyrrolidone–benzoic acid was greater than that of polyvinylpyrrolidone–barbital, which consequently affected the transfer rates of these two drugs through the various compartments (992). A three-compartment *in vitro* transport model, consisting of two aqueous compartments buffered at pH 1.2 and 7.4 and separated by a thin, olive oil-impregnated Millipore membrane, was employed to study the transfer kinetics of four drugs in the absence and presence of the complexing agent caffeine. The apparent first-order transfer-rate constants obtained for salicylic acid, acetylsalicylic acid, dehydroacetic acid, and ethyl *p*-hydroxybenzoate correlated well with reported *in vivo* apparent first-order absorption-rate constants. In the presence of caffeine, the transfer rate of all of the drugs was significantly reduced, and calculated *in vitro* transport-rate constants were found to correlate well with reported *in vivo* absorption-rate constants for the drug–caffeine complexes (993).

For interpretation of blood concentration data to be meaningful, careful evaluation of the basic aspects of collection, description, and analysis of the data is essential. The importance of these considerations was illustrated by the magnitude of differences in rate constants obtained when the data were interpreted by different methods. The method of curve fitting presented minimized squared logarithmic deviations and offered a different approach by utilizing relative error rather than absolute error. The authors claimed that if truly equal weights are desired for data points, this is the more appropriate definition of best fit. In any case, however, no mathematical technique for fitting a model to the data can compensate for an inadequate description of drug activity (994). The concentration of drug in plasma after continuous administration has been defined as the relationship between dosage per unit time and the half-life of elimination to the volume of distribution. The plateau concentration determinants, elimination half-life, and volume of distribution were determined using a single-dose–continuous infusion technique. The data derived were used to predict the plateau concentration for a series

of substances administered by continuous intravenous infusion. Alterations in the volume of distribution and the elimination half-life of a drug may occur under clinical situations. This is reflected in changes in the plateau concentration, despite a constant dosage per unit time. An experimental example was described in which deoxycholic acid decreased the volume of distribution of bromsulfophthalein (995).

Additional references on biopharmaceutics are found in Table XXIX.

Effects of Physicochemical Properties—In addition to the effects of physical and chemical properties of a drug on its absorption, this section also includes references concerned with structure–activity relationships.

There is an often unrecognized distinction between the use of multiple-regression analysis as a predictive tool and as a means of investigating controlling physical characteristics in structure–activity studies. Three examples of complications that can arise with either of these applications were discussed. The first illustrates a "false" parabolic dependence of activity on lipophilicity; the second deals with unrecognized relationships between certain physical parameters; the third illustrates a situation wherein a number of statistically significant correlations can be presented, each of which may be given a different physical interpretation (1013). Using data previously reported, Martin (1014) pointed out the necessity of testing apparent structure–activity relationships with statistical methods. The inhibition potencies against *E. coli* W. of 11 structurally related tetracyclines, having a single substitution on the D ring, were correlated with substituent indexes, using three alternative analytical approaches. The *in vitro* activity of a compound not included in one of the analyses was predicted satisfactorily. The three approaches produced equivalent results, and correlations were applied to predict activities for tetracyclines having multiple substitutions on the D ring. Many of the predicted inhibition potencies against *E. coli* W. were of the same relative order of potencies as was observed when *S. aureus* was the test organism (1015).

Higuchi and Davis (1016) presented a new quantitative and comprehensive approach relating structures of congeneric drugs to their relative biological activities. The analysis was based on the assumption that changes in structure affect a large number of time-dependent processes, such as the absorption, transport, transformation, and excretion of a drug, and that it appears highly unlikely that any single relationship accounts for all structure–activity relationships. Relationships under equilibrium or quasiequilibrium conditions were considered, thus permitting rigorous thermodynamic treatment. On the basis of the effect of structural changes on the distributive tendencies of the drug in various body tissues, including the receptor site, relationships were derived which are, surprisingly, in good agreement with available experimental data. The approach suggested a rational way to predict the degree of lipophilicity that would result in maximal activity. Based on experimental observation that concentrations of ionized and unionized molecules of sulfonamides at the bacteriostatis are linearly related to the drug's pKa, equations of the relation of pKa to bacteriostatic activity of sulfonamides against *E. coli* were proposed; the optimal pKa of sulfonamides, which are active at a minimum concentration, was calculated (1017). In a similar article, the Hansch–Fujita equation was applied to an analysis of the natriuretic activity of sulfonamide carbonic anhydrase inhibitors, using certain hydrophobic and electronic parameters. Sixteen benzenesulfonamide derivatives were satisfactorily applied to the structure–activity analysis of heterocyclic sulfonamides. It was concluded that a strong natriuretic activity was observed for sulfonamides, which had an optimal hydrophobicity and low electronegativity at the sulfamoyl groups or a strong inhibitory activity against carbonic anhydrase (1018).

The apparent partition coefficients of 48 alkylsulfates of substituted quinolinium compounds and 19 alkylsulfates of substituted pyridinium derivatives were correlated with Bondi's group contribution to the surface area, Hansch's π constant, and Hammett's σ constant. The surface area of the ring substituent and the alkyl group appeared to be the most important factors governing the apparent partition coefficient (1019). Substitution of a methyl group at one or both of the *ortho*-positions of the benzene ring in procaine amide and procaine provided analogs that were more active in prolonging the refractory period of isolated rabbit atria than procaine amide itself. Reversal of the amide in procaine amide significantly reduced the activity by prolonging the refractory period of cardiac

Table XXIX—Additional References on Biopharmaceutics

Reference	Topic
996	Comparison of *in vivo* and *in vitro* absorption from gastrointestinal tract
997	Rank-order correlations of *in vivo* data with *in vitro* rate of dissolution, showing the variety of *in vivo* parameters employed in correlations and the many different types of *in vitro* tests employed to obtain *in vitro* parameters
998	Influence of dissolution rate of sulfadimidine tablets on absorption and excretion, using a urinary excretion method
999	Biomedical model for absorption studies from various gastrointestinal sites in dogs
1000	Correlation between *in vitro* disintegration tests and therapeutic efficacy of compressed pharmaceutical tablets
1001	Relation between drug solubility in lipids and their pharmacokinetic behavior
1002	Review of physicochemical parameters in drug design
1003	Generalized solution to linear two-compartment open model for drug distribution
1004	Pharmacokinetic model of elimination of endogenous substances such as bilirubin, iron, and glucose
1005	Pharmacokinetic studies in animals as model systems for human therapy
1006	Biopharmaceutical and pharmacokinetic aspects of drug safety and efficacy
1007	Transfer and transit as pharmacokinetic relations in a multicompartment model
1008	Lipid models of drug resorption, permeation, and penetration
1009	Review of pharmaceutics, pharmacokinetics, and biopharmaceutics
1010	Physicochemical parameters in drug design
1011	Gastrointestinal bleeding resulting from oral administration of aspirin not due to precipitation of a protective glycoprotein component of gastric juice
1012	Evaluation of the dog as an experimental model for study of insulin distribution and degradation in man

Table XXX—Additional References on the Influence of Physicochemical Properties on Drug Absorption

Reference	Topic
1021	Benzoic acid being percutaneously absorbed at 200 times the amount of its glycine conjugate, hippuric acid; nicotinic acid being insignificantly penetrated, with 10% of its amide, nicotinamide, being absorbed; suggesting that molecules may be tailored to decrease or increase penetration as needed for the most suitable biological function
1022	Relationship between lipophilic character and biological activity of cephalosporins and penicillins suggesting certain structure–activity correlations
1023	Relation of structure to the bacteriostatic activity of sulfonamides using Hansch's hydrophobicity constant
1024	Soluble methylprednisolone sodium succinate being effective more rapidly in clinical conditions responsive to steroids than the less soluble methylprednisolone acetate
1025	Potassium ampicillin being absorbed more rapidly and achieving higher peak blood levels than the trihydrate form
1026	Species differences in absorption, metabolism, and excretion illustrated by psychoactive drugs from the dibenzobicyclooctadiene series
1027	Importance of cationic charge and steric factors on cholinergic properties of three noracetylcholine derivatives
1028	Pharmacodynamic evaluation of an acetylenic derivative of gallamine
1029	Pamoates, a class of oral drugs with delayed and prolonged effects
1030	Steric models of drugs for prediction of psychedelic activity
1031	Differences in human blood levels after administration of two different L-asparaginases
1032	Importance of guanido group in position 4 and the influence of the isopropyl group in position 13 for antibacterial activity of dehydroabietylamine derivatives
1033	Review of structure–activity relationships of five tetracycline antibiotics
1034	Review of problems of establishing structure–activity relationships in drug research
1035	Molecular approach to modulation of pharmacokinetics and modification of metabolic conversion by molecular manipulation
1036	Molar attraction constants applied to structure–activity relationships
1037	Correlation between activity and electronic state of hallucinogenic amphetamines

tissue and did not seem to improve the antiarrhythmic activity of the parent compounds (1020).

Additional studies describing the influence of physicochemical properties on drug absorption are provided in Table XXX.

Effects of Formulation—The effects of different vehicles on excretion and metabolism of serotonin and imipramine were studied. Rats receiving labeled serotonin in dimethyl sulfoxide or propylene glycol excreted radioactive material in the urine slower and in lesser amounts when water was the vehicle. Dimethyl sulfoxide and propylene glycol not only caused an increase in the amount of urinary 5-hydroxytryptophol and *N*-acetylserotonin but also in the conjugated form of these two metabolites as well as that of 5-hydroxyindoleacetic acid. The two nonaqueous vehicles also caused a diminished demethylation of imipramine compared with the water vehicle (1038).

The highest blood levels of both phenacetin and free acetaminophen were observed after oral administration of a fine particle-size suspension of phenacetin with polysorbate 80 (Tween 80), followed, in decreasing order, by a fine particle-size suspension without polysorbate 80, medium particle-size suspension, and coarse particle-size suspension. This indicates that particle size is an important factor in absorption of phenacetin and that the absorption is apparently enhanced by polysorbate 80 (1039). Sodium deoxycholate markedly enhanced the absorption of phenol red in the rat by altering the permeability of the intestinal membrane. While this was true for the isolated everted rat intestine, in the intact rat these effects appeared to be reversible (1040). Certain surface-active agents placed in a Thomas canine fundic pouch in buffered solutions influenced the absorption of soluble antibiotic drugs. Such agents induced a rapidly reversible hyperabsorptive state of the organ, resulting in blood levels of absorbed drug many times greater than control values. The influence of the surface-active agent, be it nonionic, anionic, or zwitterionic, is upon the organ and not upon the drug, as evidenced by the efficiency of the absorption promoter when it is employed and removed before the drug is introduced (1041). Low concentrations of polysorbate 80 and oleic acid, which enhance drug absorption across the external membranes of goldfish, have no apparent effect on the absorption of salicylate, salicylamide, and 4-aminoantipyrine from the *in situ* rat small intestine (1042).

Below the certain critical concentration of polysorbate 80, the activity of chlorpromazine hydrochloride in solution in goldfish was enhanced in unbuffered drug solutions. Above this concentration, the activity was diminished, possibly due to some association between surfactant micelles and drug molecules. The rate of solution of chlorpromazine hydrochloride from coated tablets was increased in 2% aqueous polysorbate 80, but the activity was decreased compared with that in a simple aqueous dissolution medium, thus providing direct evidence of the erroneous conclusions that can be obtained if dissolution measurements alone are used in assessing the effect of additives on drug performance (1043).

The rectal absorption of aspirin, aluminum aspirin, and calcium carbaspirin was studied in dogs using bases of cocoa butter, polysorbate 61, polyethylene glycol mixture, and a mixture of natural saturated vegetable fatty acid glycerides. The absorption of aluminum aspirin from cocoa butter and the polyethylene glycol mixture was poor. Plasma salicylate levels from aspirin and calcium carbaspirin in the polysorbate 61 base were minimal. The greatest absorption was seen with calcium carbaspirin in vegetable fatty acid glyceride base. The authors could draw no conclusions regarding any differences in absorption of aspirin or calcium carbaspirin in the various bases or the commercial aspirin product (1044). Different oral dosage forms of griseofulvin were administered to dogs; absorption was complete for griseofulvin dissolved in polyoxyethylene glycol 400, 88% complete when dispersed in polyoxyethylene glycol 6000 contained in a capsule, 45% complete for the commercial capsule, and 33% complete for the commercial tablet (1045).

An insoluble drug–polysalt complex of chlorpromazine hydrochloride, sodium carboxy methylcellulose, and protamine sulfate was selected as a model to evaluate the effects of these macromolecular constituents on the *in vitro* and *in vivo* availability of the interacted

drug. The *in vitro* drug-release studies suggested that the product possesses prolonged-release properties, while the *in vivo* studies with rats revealed a promoted bioavailability of the drug in the presence of the polysalt complex. Protamine sulfate, a known pinocytotic inducer, was specifically implicated in this phenomenon (1046). By using a prebuffer technique or by adding buffer and/or viscolizer to certain dilute mydriatic solutions, similar physiological effects were obtained as were by using unbuffered solutions 10 times more concentrated. The addition of 0.5% hydroxypropylmethylcellulose, 4000 cps., to buffered mydriatic solutions did not further increase the pupil size of Caucasians, compared with the effects from the same buffered solution without viscolizer; a more marked dilating effect was produced in the pupils of Orientals (1047).

Additional studies on the effects of formulation on drug absorption are provided in Table XXXI.

Absorption Control and Alteration—This section of biopharmaceutics considers articles dealing with absorption control and alteration as influenced by the coadministration of drugs, disease, blood flow, fasting, route of administration, and age.

The effects of six parameters on the physiological availability of orally administered griseofulvin formulations were studied by the use of urinary metabolite excretion data from a single subject. The first parameter, time of administration, had an effect on absorption in the manner of a circadian rhythm, the absorption being least in the morning and maximum at noon. The second parameter, high-fat meal, appeared to be equivalent to a delay in administration; consequently, its effect depended on the previous factor. The third parameter, dosage, indicated that the percentage absorption of drug was independent of the amount administered. The fourth parameter, particle size, showed that twice as much micronized griseofulvin was absorbed as nonmicronized griseofulvin. The fifth parameter, dissolution rate, had little effect on absorption as long as the dissolution rate was above a given value. The sixth parameter, gastrointestinal transit time, could not be varied enough by dietary means except in the cases of greasy fried foods and nuts, to affect significantly the residence time of griseofulvin at the absorption sites (1080).

The effect of probenecid on the renal clearance of riboflavin was determined in three human subjects at various serum concentrations of the vitamin. Renal clearances of riboflavin exceeded the endogenous creatinine clearances, which indicated that riboflavin excretion involved renal tubular secretion. The clearance of riboflavin was less at low than at high serum concentrations of the vitamin, which is characteristic of a saturable tubular reabsorption process. Probenecid decreased the renal clearance of riboflavin, and this effect was directly related to the serum concentration of the inhibitor, The serum protein binding of the vitamin was essentially constant over the concentration range encountered and was unaffected by the presence of probenecid (1081). Pretreatment with heptabarbital had no effect on prothrombin complex activity before warfarin administration and did not affect the apparent first-order rate constant for prothrombin complex activity degradation or the relationship between plasma warfarin concentration and prothrombin complex activity synthesis rate. The decreased response to warfarin during barbiturate administration was, therefore, due only to the inductive effect of the barbiturate on the warfarin-metabolizing enzyme system. There was no evidence that the barbiturate affected the distribution of warfarin in the body, the inherent ac-

Table XXXI—Additional References on Effects of Formulation on Drug Absorption

Reference	Topic
1048	Blood levels of chlorphentermine in man after oral administration of the drug in solution, as a prolonged-release formulation, and as an intravenous injection
1049	Review of mechanisms of surfactant effects on drug absorption
1050	Absorption of acetylsalicylic acid from unbuffered and buffered gastric contents
1051	Influence of dimethyl sulfoxide on percutaneous migration of potassium dodecyl sulfate
1052	Review of the role of surface-active agents in drug absorption
1053	Extent of *in vivo* absorption of insoluble streptomycin in various suppository bases
1054	Release of sulfonamides from certain ointment bases
1055	Effect of syrup on the absorption of drugs from gastrointestinal tract
1056	Relation between percutaneous absorption and stratum corneum retention of drugs
1057	Percutaneous absorption of certain corticosteroid hormones
1058	Dimethyl sulfoxide markedly increased the diffusion rate of tritiated water through human abdominal autopsy skin
1059	Effect of dimethyl sulfoxide on absorption of iron applied to piglets
1060	Effect of excipients on appearance of xanthotoxin erythemia in guinea pigs upon irradiation
1061	Effects of surfactants and solvents on permeability of epidermal tissue
1062	Factors affecting drug concentration delivered from nasal inhalers, noting the importance of studying vapor pressure characteristics of the volatile drug
1063	Effect of tablet dosage form on absorption of aspirin
1064	Comparison of profiles of gastrointestinal absorption of theophylline from various vehicles
1065	Salicylate blood levels in rabbits after oral administration of aspirin in different dosage forms, showing phosphate increasing the rate of absorption
1066	Comparison of blood levels after administration of theophylline in tablet and liquid dosage forms
1067	Factors influencing release of active substances from suppositories, demonstrating advantageous effects of surfactants
1068	Influence of tablet, suspension, and syrup dosage forms on absorption of tetracycline
1069	Review of significance of particle size in relation to drug absorption
1070	Urinary elimination of micronized and crystalline nitrofurantoin, showing the micronized form is excreted more rapidly
1071	Difference in intestinal absorption of acetylsalicylic acid in dogs due to particle size
1072	Review of influence of formulation on drug action
1073	Dependence of biological activity on the degree of dispersion, showing intensity and duration of drug action are a function of particle size
1074	Review of formulation factors influencing therapeutic effect of pharmaceuticals
1075	Review of effects of pharmaceutical formulations on biological actions of drugs
1076	Effect of compression pressure on the activity of anti-inflammatory enzyme tablets
1077	Effect of various suppository bases on rectal absorption of choline salicylate in the rabbit
1078	Effect of eye drop adjuvants on the responses of human eye to some autonomic drugs
1079	Diazepam blood levels after administration as solution or syrup

tivity of the blood coagulation process, or the response of the appropriate receptor to warfarin (1082).

The absorption rate of taurine and glycine in the presence and absence of aspirin was examined in the rat digestive tract. Neither was absorbed in the stomach, either alone or with aspirin. Alone, both were absorbed to a certain extent from the small intestine, but glycine behaved differently when aspirin was present; that is, taurine was absorbed alone and in the presence of aspirin, and the absorption of glycine was increased in the presence of aspirin. These results indicate that neither compound is absorbed in the stomach, but both promote the absorption of aspirin from the stomach and the small intestine as they themselves are being absorbed. In the small intestine, absorption of glycine seems to be accelerated by aspirin (1083). In the presence of tetracycline, sulfanilic acid disappeared faster from the small intestinal recirculating solution at pH 8.0. At pH 6.0, however, this absorption enhancement was not observed. In the presence of tetracycline, the blood level of sulfanilic acid increased at pH 8.0 but not at 6.0; this result agreed with that obtained from the intestinal recirculation method. These results suggest that the increased absorption of sulfanilic acid is not due to complex formation with tetracycline in the gut lumen but to the direct effect of tetracycline on the absorptive membrane (1084).

Walker (1085) studied the influence of protein binding on the excretion of some sulfanilamidopyrimidines in man. The plasma protein binding data included the percentage bound at various concentrations of the drugs, the number of binding sites, the binding capacity of plasma albumin, and the dissociation constant of the sulfonamide–albumin complex. It was noted that the short-acting sulfasomidine was bound to a much lesser extent, at the concentration obtained clinically, in the plasma than the long-acting sulfa drugs. In addition, sulfasomidine occupied only one binding site on plasma albumin, while the other sulfonamides occupied two sites. This may partly account for the rapid excretion of sulfasomidine in man. Absorption rates of carbazochrome and nicotinamide in the presence of caffeine and hydroxyethyltheophylline were determined to ascertain whether the increase or decrease of absorption rate in the presence of the complexing agent was due to the intraluminal complex formation. The results suggested that the effect of caffeine and hydroxyethyltheophylline on the absorption rate of the drug was apparently due to complex formation (1086). Complexation influence on the rate of absorption of actively transported drug was investigated in the perfused small intestine, employing L-tryptophan as a model drug and caffeine, 8-chlorotheophylline, 8-methoxycaffeine, 7-hydroxyethyltheophylline, and flavin mononucleotide as complexing agents. Flavin mononucleotide reduced the rate of absorption of L-tryptophan—not by directly affecting the absorptive membrane or by impairing the neutral amino acid transport system but by decreasing the thermodynamic activity of L-tryptophan resulting from molecular complexation with flavin mononucleotide in solution (1087).

Nondissociable substances such as tritiated water, methanol, ethanol, urea, ethylene glycol, glycerol, and erythritol showed increased absorption rates as the intestinal blood flow in rats increased (1088). Similarly, by using sulfaethylthiadiazole in dogs, it was confirmed that the intestinal drug absorption process is hindered by a decrease in vascular profusion (1089). Chung *et al.* (1090) showed that cations play a definite role in the transport of weak organic acids in the rabbit kidney slice, although the mechanisms of action are entirely unknown at present. Dose for dose, the concentration in blood and the urinary excretion rate of pentazocine are much lower after oral than after intravenous administration (1091). Absorption of D-glucose and 3-*O*-methylglucopyranoside was enhanced significantly in both short-term and long-term alloxan diabetes, while active transport of both monosaccharides was increased by fasting for 24–72 hr. (1092).

To exclude the effect of probenecid on renal excretion, the distribution and elimination of riboflavin with and without probenecid were studied in two functionally anephric patients during hemodialysis. Using a two-compartment open model, a pharmacokinetic analysis was made of the time course of the plasma levels of the vitamin. Probenecid had no appreciable effect on the distribution- and transfer-rate constants of riboflavin. Riboflavin was eliminated more slowly by hemodialysis of the patients than by renal excretion in normal subjects (1093). The development of gastrointestinal absorption function in humans was studied using riboflavin, which is absorbed by a site-specific and saturable transport process. The urinary recovery of the vitamin ranged from 6 to 12% of the dose in subjects ranging in age from 3 months to 40 years, respectively. The kinetics of riboflavin elimination were independent of age. These observations suggest that in the age range studied, younger subjects retain the vitamin at intestinal absorption sites for a shorter period than do older subjects, due to a decreased intestinal transit rate with increasing age. Therefore, prompt release of drugs from pharmaceutical dosage forms seems even more important in children than in adults to assure adequate absorption (1094).

After administration of 20 mg. of thiamine hydrochloride, a maximum of 4.77 mg. was absorbed in normal subjects, whereas only 1.5 mg. was absorbed in malnourished alcoholic patients with fatty liver or cirrhosis. Ethanol, given either parenterally or orally, caused a 50% reduction in thiamine absorption in 4 of 12 alcoholic patients without liver disease (1095).

Additional studies on absorption control and alteration are given in Table XXXII.

Absorption Mechanism—Mechanisms of intestinal salicylamide glucuronide formation and transport were studied in the rabbit *in vitro* with cannulated everted intestines and *in vivo* with perfused and closed intestinal loops with complete mesenteric venous blood collection. Both techniques indicated that glucuronide formation is capacity limited when the lumen concentration exceeds 10^{-3} *M*. The appearance of glucuronide across the basal barrier is limited by the transport step rather than by the rate of glucuronide synthesis, leading to accumulation of the compound in the tissue compartment (1146). There are three likely mechanisms by which bile salts can affect the absorption from the rat small

Table XXXII—Additional References on Absorption Control and Alteration

Reference	Topic
1096	Drug absorption as affected by interactions mediated by binding displacement, changes in drug metabolism, interactions at specific sites of drug action, and interactions which affect drug excretion
1097	Effect of phenobarbital on disappearance of diphenylhydantoin from serum of children
1098	Enhanced penetration of testosterone by dimethyl sulfoxide through the stratum corneum of human skin
1099	Absorption and topical distribution of oxytetracycline in healthy and experimentally infected animals
1100	Increased absorption of prednisone from rat intestine using *N,N*-di-*n*-propylpropionamide
1101	Review of the effect of food on drug absorption
1102	Rate of absorption of oral thyroxine appearing to be as rapid in hypothyroid patients as in normal patients
1103	Effect of dose and sodium and potassium concentration on the absorption of digoxin from the perfused intestinal loop of the rat
1104	Comparative studies on cephaloridine and cephalothin in patients with renal insufficiency treated with or without dialysis
1105	Serum and liver levels of vitamin A after a single massive dose
1106	Effect of chlorpromazine on disposition and excretion of amphetamine in the rat, suggesting that chlorpromazine impairs the hydroxylation of amphetamine supposedly at the level of liver microsomes
1107	Mechanism of action of accelerants on skin penetration, using urea and dimethyl sulfoxide
1108	Influence of drugs on tissue permeability
1109	Comparative elimination of glyceryl guaiacol administered rectally and cutaneously as an ointment
1110	Review of reduced action of drugs in combination
1111	Effect of phenobarbitone pretreatment on the metabolism of neostigmine *in vivo* and *in vitro*
1112	Paired comparisons of absorption between three topically applied drugs
1113	Enzyme induction by drugs in humans, resulting in increased metabolism and lower blood levels
1114	Physiological factors affecting intestinal drug absorption
1115	Serum concentration and urinary excretion of ouabain and digitoxin in patients suffering from hyperthyroidism or hypothyroidism
1116	Inhibition of subcutaneous absorption in rats by local anesthetics
1117	Review of drug latentiation
1118	Review of enhanced action of drugs in combination
1119	Inhibition of activity of isoniazid in the presence of chloroquine diphosphate
1120	Evidence for significant nonperipheral effects of phenformin on glucose metabolism in normal subjects, suggesting that phenformin inhibits intestinal absorption of glucose
1121	Effect of phenobarbital on plasma disappearance and biliary excretion of drugs in rats
1122	Pharmacokinetics of *N*-acetyl-*p*-aminophenol as affected by phenylbutazone
1123	Review of interactions of drugs with other drugs or other therapeutic devices
1124	Malabsorption due to effect of paromomycin on protein synthesis in the small intestine
1125	Effect of 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride on drug concentration in the blood
1126	Effect of bile salts on calcium absorption
1127	Effect of antibiotics on hexobarbital sleeping time in rats
1128	Influence of magnesium and some other divalent cations on hepatic microsomal drug metabolism *in vitro*
1129	Synergistic effect noted when sulfamethoxazole and trimethoprim are combined in treatment of gonorrhea
1130	Synergism of tetracycline and amphotericin B *in vitro*
1131	Increased ileal absorption of salicylic acid induced by carbonic anhydrase inhibition
1132	Altered absorption of drugs from the rat small intestine by carbonic anhydrase inhibition
1133	Inhibition of tolbutamide decomposition in man by sulfaphenazole
1134	Inhibition of binding of glycine to rat brain cortex membrane by strychnine
1135	Increased urinary salicylate excretion in rats following administration of acetylsalicylic acid in combination with glucuronamide
1136	Drugs, toxins, and dietary amino acids affecting vitamin B_{12} or folic acid absorption or utilization
1137	Ineffectiveness of succinic acid and ascorbic acid in increasing the absorption of iron from a tablet dosage form
1138	Influence of succinic acid on absorption of iron studied by whole-body monitoring; iron utilization not being increased by addition of free succinic acid
1139	Review of the pharmaceutical and absorption aspects of drug interactions
1140	*In vitro* changes of the serum binding capacity of insulin-^{131}I under influence of tolbutamide and glybenclamide in normal subjects, young and old diabetics, and in insulin resistance
1141	Alterations in deoxyribonucleic acid (DNA)-bound amino acids after the administration of DNA-binding drugs
1142	Effect of administering other drugs during oral anticoagulant therapy
1143	Review of substances that facilitate sorption of pharmaceuticals by the skin
1144	Effect of renal insufficiency on behavior of deoxycycline
1145	Interactions between sympathomimetic amines and a monoamine oxidase inhibitor, showing marked potentiation of certain physiological properties of the amines

intestine: (*a*) the loss of thermodynamic activity of a drug due to the formation of the micellar complex, (*b*) the local concentration buildup effect such as an accumulation on the absorptive surface, and (*c*) the direct effect on the permeability characteristics of the intestinal mucosa (1147). Warfarin was eliminated more rapidly, but its anticoagulant effect was increased by concomitant administration of phenylbutazone. Pharmacokinetic analysis showed that the prewarfarin synthesis rate and the normal degradation of prothrombin complex activity are not affected by phenylbutazone, but this drug has a pronounced effect on the relationship between the synthesis rate of prothrombin complex activity and plasma-warfarin concentration. These observations are consistent with the assumption that phenylbutazone competitively displaces warfarin from nonspecific binding sites in the plasma and tissues and thereby increases the interaction of the anticoagulant with its pharmacologic receptor and metabolizing enzyme system (1148).

It was demonstrated that in the very early stage of drug absorption, vitamins solubilized in micelles are adsorbed onto the membrane, which are favored by the surface-active agent; in the slow phase, absorption now becomes rate limited by the partition or release of the vitamins from adsorbed micelles to the mucosal layer of the membrane (1149). The distribution and metabolism of vitamin K and warfarin were studied in the warfarin-resistant rat. The data obtained were consistent with the hypothesis that warfarin resistance results from a mutation that caused the synthesis of a protein with a lowered binding affinity for both vitamin K and its antagonist, warfarin (1150).

The demonstration that a capacity-limited system for acetylation of sulfanilamide exists in the rat is important for drug metabolism studies in this species but may not

Table XXXIII—Additional References on Mechanisms of Absorption

Reference	Topic
1155	Metoclopramide-N^4-glucuronide may be absorbed from the intestine not only in the parent compound, namely, metoclopramide, resulting from hydrolysis but in the intact form as well as the sulfonate
1156	Elucidation of mechanism of change of methanesulfonated drug *in vivo* on derivatives of substituted anilines
1157	Mechanism of inhibition of *in vitro* glucose transport by oral administration of phenformin HCl
1158	Review of stimulation and inhibition of drug metabolism
1159	Mechanism of abnormal response to oral metyrapone showing absorption and conjugation of metyrapone during diphenylhydantoin therapy
1160	Inhibition of protein synthesis as a mechanism for interfering with intestinal lipid transport
1161	Differences in the rate of biotransformation of glyceryl guaiacolate to inactive products being responsible for half-life differences of this drug in stallions and mares
1162	Degree of protection to the convulsant action of metrazole being most closely related to the rate of disappearance of the *N*-demethyl derivative
1163	Review of factors that permit availability of a drug to the absorption site in a complex organism
1164	Demonstration of a nonsaturable transport process for intestinal absorption of tritiated ouabain

be necessarily relevant to man. Based on experimental data obtained, it was concluded that drug metabolism studies must be carried out at multiple-dose levels and that the linearity of the semilogarithmic plots of drug elimination as a function of time is frequently insufficient to discriminate between dose-independent or dose-dependent biotransformations (1151). It was found that the cause of the poor absorbability of sulfaguanidine could not be attributed to its dimer formation or to the iceberg formation in aqueous solution. Since bile salts did not affect the physicochemical properties of sulfaguanidine, such as apparent partition coefficient and diffusion constant, and did not form a micellar complex with the drug, it was evident that the absorption enhancement was not caused by their intraluminal effect. On the absorptive surface, bile salts did not increase the affinity of the sulfa drug for the intestinal mucosal surface. An exsorption study demonstrated that such enhancement was caused by the direct action of bile salts on the structure of the absorptive surface (1152).

A micropuncture study of the effects of urea infusion on tubular reabsorption in the rat indicated that, at the plasma levels achieved in the study, urea did not significantly decrease water and solute reabsorption in the proximal convolutions but did inhibit water, but not sodium, reabsorption in the more distal portions of the nephron (1153). In a study of the *in vitro* human percutaneous penetration and epidermal-dermal retention of benzyl alcohol and testosterone, it was suggested that the dermis may exhibit a role in the retention and penetration of these chemicals (1154).

Additional references on the mechanism of absorption are provided in Table XXXIII.

Drug Absorption—Two experimental methods for use in kinetic studies on a compartment model for intestinal metabolism and absorption were evaluated. The *in vitro* cannulated everted intestinal sac and the *in vivo* intestinal loop with complete mesenteric venous collection were compared in the same region of rabbit intestine. These methods were used to study the effects of blood flow on the transport of salicylamide across the basal barrier and to provide experimental evidence to support the cell compartment model. The rate of transport of free drug across the basal barrier is blood flow rate limited, while the transport of glucuronides is essentially independent of blood flow. There was a lag time of 4 min. in the appearance of free salicylamide into mesenteric blood *in vivo* and a lag time of about 10 min. into serosal fluid *in vitro*. The steady-state rate of appearance of free drug into the plasma, *in vivo*, was 5–10 times greater than the rate of appearance of free drug into the serosal fluid at similar mucosal concentrations. The *in vivo* intestinal loop with complete venous collection provided many advantages in studying physiological factors of intestinal drug absorption (1165).

Diffusion rates of fatty acid in bile salt solution and in polyoxyethylene–polyoxypropylene copolymer solution depended on the amount of fatty acid solubilized and also on any physical restrictions to flow. Micelles of the two types of surfactant were significantly different in size. Assuming a passive process for the uptake of fatty acid, diffusion rates correlated with the observed uptake of fatty acid into the everted intestinal sac of the rat (1166).

Clindamycin, which is known to be absorbed from the gastrointestinal tract, was absorbed extremely slow, or possibly not at all, from the buccal cavity at various pH values. This finding indicated that buccal absorption alone cannot be used to predict the gastrointestinal absorption of a compound (1167).

A comparison was made of the gastric absorption and distribution of tritiated sodium acetylsalicylate with the absorption and distribution of sodium salts of other weakly acidic compounds. Each of the other compounds has a characteristic absorption pattern. Only sodium acetylsalicylate caused gastric lesions in the rat. These observations do not rule out the possibility that the absorption characteristics of acetylsalicylic acid and its salts may be associated with their ability to cause gastric ulcers (1168).

A simple method for the detection of chromonar and its acid metabolite by fluorescence techniques was described. Following oral or intravenous administration, chromonar was rapidly hydrolyzed to its acid metabolite. The metabolite distributes according to a single-compartment model; the plasma half-life is 1 hr. in man and dog. Excretion of the metabolite into the bile accounts for approximately 25% of the dose, and excretion into the urine accounts for the remainder (1169).

Blood concentrations of carbenoxolone generally exhibit two maxima: at 1–2 and 3–6 hr. after dosage. Because absorption of orally administered drug is so rapid and does not occur when the gastric contents have a pH greater than 2, it was inferred that the major site of absorption is the stomach. The high blood concentrations of carbenoxolone are probably due to a high degree of binding of the drug to plasma proteins. Gastric absorption of the drug may be necessary for the increased production of gastric mucus and, hence, for gastric ulcer-healing activity (1170).

Additional references on drug absorption are provided in Table XXXIV.

Pharmacokinetics—It was shown from apparent first-order urinary excretion studies with D-(−)-mandelic acid and certain of its homologs, DL-tropic acid, D-(−)-4-hydroxy-4-phenylbutanoic acid, DL-phenyllactic acid, and D-(−)-benzyllactic acid, that the biological half-lives of the homologs are significantly shorter than the half-life of D-(−)-mandelic acid in rats. Since these compounds, which differ from each other in their content of methylene groups around the carboxyl group, exhibit negligible metabolism and protein binding, low pKa values (3.3–4.7), and low lipid solubility at the physiological pH, and are recovered primarily in the urine in the intact form, they were utilized to study the effect of hydrophobic group(s) on the rate of secretion. The compounds exerted a mutual inhibitory effect on their renal tubular secretion, indicating a common "carrier" mechanism for their secretion. The addition of methylene group(s) in the vicinity of the carboxyl group of the mandelic acid molecule increased its affinity for the carrier molecules of the renal tubular secretion system in rats (1194). From the pseudo-first-order urinary excretion studies of D-(−)-mandelic acid and DL-tropic acid in rats, data regarding the apparent initial secretion rate *versus* dose (intravenous) were obtained and treated according to Michaelis–Menton kinetics. Although the maximum apparent initial secretion rate determined for these compounds was similar, the amount of substrate required to produce one-half the maximum apparent initial secretion rate determined for D-(−)-mandelic acid was found to be about twice that determined for DL-tropic acid. Data were obtained to demonstrate that these compounds competitively inhibit the renal tubular secretion of each other, thereby strongly indicating that these compounds share a common carrier transport system for their secretion. Data obtained in these studies and those described earlier were utilized to distinguish a certain structural characteristic around the cationic site of the "carrier" molecules of the renal tubular secretion system in rats (1195). The kinetics of metabolism and urinary excretion of the optical isomers of mandelic acid were studied in three human subjects. The rate constants were determined for a model consisting of parallel apparent first-order processes for excretion of intact mandelic acid and metabolism to benzoylformic acid. The kinetic studies revealed no significant difference in the rate of urinary excretion of L-(+)- and D-(−)-mandelic acid, but there was a difference in the metabolic metabolism of these two isomers in the subjects. The rate constant for metabolism of L-(+)-mandelic acid was approximately twice that for the metabolism of D-(−)-mandelic acid. The inhibitory effect of probenecid on the urinary excretion of the optical isomers of mandelic acid suggests that both isomers are involved in the active renal tubular secretion (1196).

Pharmacokinetic relationships were developed to characterize a multicompartment drug distribution and elimination model, which included a saturable renal tubular reabsorption process. The derived expressions were applied to serum concentration and urinary excretion data obtained after rapid intravenous administration of riboflavin to man and dog. The mathematical relationships and experimental data demonstrate the dependence of renal clearance on the serum concentration of the drug and on urine flow rate. The results of this study indicate that the renal excretion of riboflavin, like that of several other water-soluble vitamins, involves saturable tubular reabsorption as well as tubular secretion (1197).

The steady-state growth of *E. coli* in broth cultures was inhibited by erythromycin with a new steady-state growth-rate constant that was linearly related to drug concentrations in the range 0–10.0 mcg. ml.$^{-1}$. The growth-rate constant at drug concentrations greater than 10 mcg. ml.$^{-1}$ adhered to a kinetic model, which implies the saturation of a limited number of receptor sites in accordance with an equation. The dependence of *E. coli* growth rate on drug concentrations was invariant with the organism population or broth concentrations. However, values of k_a increased 10-fold as the pH of broth was increased from 6.8 to 7.8, while k_0 remained constant. This indicates that the unprotonated fraction of the drug concentration contributes to the activity. Lincomycin in Phase I-affected growth

Table XXXIV—Additional References on Drug Absorption

Reference	Topic
1171	Absorption, distribution, excretion, and metabolism of oxymethebanol
1172	Absorption of ^{64}Cu from the gastrointestinal tract of the rat
1173	Effect of pH of solution on percutaneous absorption of acidic and basic drugs
1174	Laboratory experiment demonstrating percutaneous absorption
1175	Intestinal absorption of cardiac glycosides by measurement of tritiated glycosides in the blood of the portal vein and in the intestinal lymph
1176	Investigation of percutaneous absorption of antibacterial agents
1177	Evaluation of drug absorption by measurement of urinary excretion
1178	Laboratory experiment demonstrating *in vitro* release of medicament from ointment bases and its application to drug absorption
1179	Oral absorption and disposition kinetics of lidocaine hydrochloride in dogs
1180	Percutaneous absorption of antibacterial substances
1181	Review of factors affecting gastrointestinal absorption of drugs
1182	Effect of various cations on the passive transfer of drugs across everted rat intestine
1183	Intestinal absorption of tritiated chymotrypsin in dogs
1184	Local and systemic absorption, excretion, and tolerance of clindamycin hydrochloride after intramuscular administration
1185	Review of absorption after oral administration of drugs in sustained-action dosage forms
1186	Relation between clinical effects of lithium and its absorption, distribution, and excretion
1187	Methods for measuring cutaneous permeability
1188	Absorption and excretion of tritiated dihydrostreptomycin in cattle and swine
1189	Blood levels and urinary excretion of harmine and its metabolites in man and rats
1190	Resorption of drugs from the gastrointestinal tract as predicted by an *in vitro* model
1191	Absorption, distribution, and metabolic fate of psychotropic drugs
1192	Modern view of the absorption, distribution, and elimination of inhalatory anesthetics
1193	Absorption and blood level values for ethosuximide in children and young adults having petit mal or mixed seizure disorders

Table XXXV—Additional References on Pharmacokinetics

Reference	Topic
1204	Pharmacokinetic profile of trimethoprim in man and dog, with the dog absorbing the drug completely
1205	Blood levels of guaiacol glycerol ether mononicotinate in animals, showing a pseudo-first-order absorption by passive transport
1206	Pharmacokinetic aspects of biliary excretion and dose dependence of riboflavin in rat
1207	Pharmacokinetic methods for evaluation of intestinal absorption
1208	Application of Laplace transform for solving differential rate equations in pharmacokinetics
1209	Kinetics of anticoagulant effect of bishydroxycoumarin in man
1210	Relation between drug-elimination kinetics in intact animals and isolated perfused liver systems, using phenylbutazone and bishydroxycoumarin
1211	Review of methods of determining biological half-lives of drugs; comprehensive tabulation of half-lives for many drug substances
1212	Rate of decline of diphenylhydantoin in human plasma
1213	Absorption, blood concentrations, and excretion of pentazocine after oral, intramuscular, and rectal administration in man
1214	Kinetics of glucose transport in isolated dog brain
1215	Absorption, distribution, and excretion of 3-trifluoromethyl-*s*-triazolo(3,4-α)isoquinoline
1216	Kinetics and drug metabolism of sympathomimetic amines in man
1217	Importance of steric, stereochemical, and physicoorganic features in drug metabolism and drug action
1218	Quasilinearization and fitting of nonlinear models of drug metabolism to experimental kinetic data
1219	Pharmacokinetics of urinary excretion of chlorphentermine in man
1220	Pharmacokinetics of dimethylaminophenazone, 4-aminophenazone, and phenazone in rats of various ages
1221	Apparent dose-dependent elimination kinetics as an experimental artifact
1222	Pharmacokinetics of haloperidol, showing the maximum blood level was reached within 15 min. with rapid elimination after subcutaneous administration
1223	Blood and urinary clearance of exogenous serotonin in relation to time and route of administration
1224	Pharmacokinetic and bacteriological properties of new oral cephalosporin compounds
1225	Review of pharmacokinetics and therapeutic importance
1226	Pharmacokinetics of cephalexin
1227	Pharmacodynamics and metabolism of propranolol in man
1228	Pharmacokinetics of trimethoprim in man and animals
1229	Review of pharmacokinetics of chemotherapeutic agents
1230	Kinetics of distribution and metabolism of ataractics of the meprobamate group in mice
1231	Pharmacokinetics of DL-ethyl *trans*-2-dimethylamino-1-phenyl-3-cyclohexene-*trans*-1-carboxylate hydrochloride in man, including blood level and excretion in urine and feces after a single oral dose of tritiated compound
1232	Kinetics of diphenylhydantoin disposition in man
1233	Kinetics of radiohippuran
1234	Kinetics of chloral hydrate metabolism in mice and the effect of ethanol
1235	Kinetics of amylobarbital metabolism in healthy men and women
1236	Kinetics of the anticoagulant effect of heparin

had the same formal dependency on concentration as did erythromycin, with a potency ratio of 6.68:1, erythromycin base to lincomycin base, on a weight basis. The combined effect of erythromycin and lincomycin in Phase I did not antagonize the growth rate of *E. coli* in the subinhibitory range and could be predicted by adding equivalent amounts in accordance with the coincident response–dose curves of erythromycin and lincomycin (Phase I) (1198).

A simple model was proposed and used, in conjunction with an analog computer, to determine the rate constants of buccal absorption of 10 carboxylic acids from solutions of pH 4.0 using a single subject. The rate constants gave a positive correlation with previously determined logarithms of *n*-heptane–0.1 *N* hydrochloric acid partition coefficients (1199).

Monoexponential and biexponential disappearance of drug was observed both from the simulated gut phase of a three-phase *in vitro* model for drug absorption and from the lumen of an *in situ* rat gut. Monoexponential disappearance occurred when accumulation of drug in the membrane phase was low or absent, whereas biexponential disappearance occurred when membrane accumulation was appreciable. In all cases, overall transfer of drug to blood was essentially irreversible. Kinetic analysis of the biexponential lumen-phase data was presented (1200).

Under multiple-dosing conditions at a subcutaneous site, equations were derived which permitted one to estimate the number of doses, *n*, required to approach within $\pm 1\%$ of the asymptotic minimum level. The equations, with numerous term definitions, explained that benzyl alcohol disappears from the subcutaneous cell in an apparent monoexponential manner (1201).

The *in vitro* absorption kinetics for nine drugs were followed, using a perfusion apparatus. Identical perfusion runs were made on everted and noneverted segments of the same rat intestine so that the ratio of directional permeability constants could be calculated. Both negatively and positively charged drug ions exhibited permeability coefficient ratios of around 1.3, while completely unionized drugs showed the expected ratio of 1.0. In light of the similarity in the ratio of permeability constants for drug ions and sodium ions, salicylate was tested in a sodium-free buffer, resulting in a ratio of 1.08. It appeared that the difference in directional permeability constants observed with ionized drugs in the intestine may be explained in relation to sodium transport. It was pointed out that *in vitro* intestinal transport studies could lead to erroneous conclusions concerning the degree of absorption of ionizable drugs *in vivo* (1202).

The urinary excretion rate was studied by administering sulfafurazole under normal physiological conditions and after an alkali load (to eliminate diurnal fluctuations in the urinary pH over a 48-hr. period). The controlled alkaline condition had a marked effect on the urinary excretion of sulfafurazole. The elimination half-life was reduced from a mean 6.3 hr. under normal conditions to a mean 4.4 hr. under alkaline conditions. Similarly, under normal and alkaline conditions, the percentage of drug acetylated was reduced from an average of 34.9% to 25.0%, respectively, and the percentage of total drug excreted increased from a mean 82.7% to 89.9%, respectively (1203).

Additional studies on pharmacokinetics are listed in Table XXXV.

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RESEARCH ARTICLES

Kinetics of Absorption and Excretion of Levodopa in Dogs

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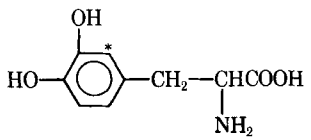
Abstract □ The rate and extent of levodopa absorption and excretion following intravenous and oral administration of 2-^{14}C-levodopa to acute and chronically treated dogs were investigated. Plasma levels of intact levodopa following the intravenous administration declined rapidly during the first 4–6 hr. The elimination-rate constant of intact levodopa from the plasma ranged between 0.73 and 0.99 hr.$^{-1}$, which corresponds to a half-life of approximately 40–60 min. The elimination-rate constant of total plasma ^{14}C ranged between 0.007 and 0.085 hr.$^{-1}$, which corresponds to a half-life of 8–9 hr., indicating that the total ^{14}C was eliminated at one-tenth the rate of intact levodopa. Peak plasma levels of intact levodopa and of total plasma ^{14}C following oral administration were attained 25–30 min. after dosing. Approximately 70–75% of the intravenous and 57–70% of the oral radioactive doses were excreted in urine over a 72-hr. period. Levodopa and dopamine accounted for a very small percentage of the radioactivity in the urine. Approximately 3.0–7.0% of the intravenous or oral radioactive dose was excreted in the feces. The efficiency of absorption of total radioactivity was calculated to range between 83.0 and 92.0%. Analysis of the ratio of intact levodopa levels to total ^{14}C levels indicated that only 22.0–30.0% of the administered dose reached the general circulation as intact levodopa, suggesting that the remainder of the absorbed dose, approximately 60.0%, is biotransformed in the gastrointestinal tract prior to absorption and/or in the liver during its "first passage" to the general circulation.

Keyphrases □ Levodopa, absorption and excretion, dogs—pharmacokinetics □ Pharmacokinetics—labeled levodopa absorption and excretion, dogs □ Absorption kinetics, labeled levodopa—oral, intravenous administration □ Excretion kinetics, labeled levodopa—oral, intravenous administration

Discovery of the presence of dopamine in the striatum and substantia nigral areas of the brain (1, 2) and of its depletion in patients with Parkinson's disease (3, 4) and in experimental animals following nigral lesions (5) provided new therapeutic approaches to Parkinsonism. Among the several means considered for correcting dopamine areas was the administration of the immediate precursor of dopamine, levodopa[1].

Consideration of the need for large doses of levodopa to attain therapeutic efficacy when given orally led to this investigation of the absorption and excretion of levodopa. The study was conducted under two experimental conditions: after the administration of a single dose and following chronic treatment of animals with levodopa. It was expected that the data obtained following the intravenous and oral administration of 2-^{14}C-levodopa (I) to dogs would yield the following information: (*a*) total plasma ^{14}C and intact levodopa levels and corresponding half-lives of elimination, (*b*) rate and extent of absorption from the gastrointestinal tract following oral administration of drug, (*c*) percent recovery of drug in urine, and (*d*) rate and extent of levodopa metabolism.



2-^{14}C-levodopa (*denotes position of radioactive label)
I

MATERIALS AND METHODS

Acute Single-Dose Study—Two beagle dogs, weighing 8–10 kg., were each given a single intravenous and oral 50-mg./kg. dose of 2-^{14}C-levodopa (Lot B-1, specific activity 0.19 μc./mg.). The dose (50 mg./kg.) selected was within the average range of the therapeutic dose administered to humans with Parkinson's disease, and it was

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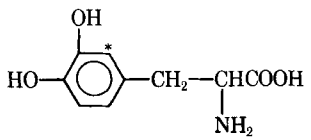

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[1] L-3,4-Dihydroxyphenylalanine.

Table I—Doses of ^{14}C-Levodopa as Altered by Emesis

Dog Number and Route of Administration	Total Dose 2-^{14}C-Levodopa, mg.	^{14}C Content of Syringe Washings, mg.	Time of Emesis, min.	^{14}C Content of Emesis Fluid, mg.	Percent Dose Vomited	Actual Dose Received, mg.
Acute Single-Dose Study						
345, i.v.	465.00	25.16	2–6	0.21	0.05	439.63
1810, i.v.	410.15	15.78	2–6	0.15	0.04	394.22
345, p.o.	410.30	—	20	167.17	62.33	154.53
			50	88.60		
1810, p.o.	429.20	—	220	271.82	63.33	157.38
Chronic Multiple-Dose Study						
346, i.v.	481.70	9.08	4	0.04	0.01	472.58
349, i.v.	349.35	16.44	9	0.07	0.02	332.84
346, p.o.	441.00	—	20	2.68	0.61	438.92
349, p.o.	350.40	—	30	1.88	0.54	348.52

200 times below the acute oral toxic dose (LD_{50}) in rats. The intravenous dose was administered as a suspension in 50% propylene glycol, while the oral dose was given to the same animals in a gelatin capsule 2 weeks later.

Following intravenous injection of the drug, the syringe used to administer the dose was rinsed; the radioactive contents of these washings were determined by liquid scintillation counting techniques. Both animals vomited between 2 and 6 min. after dosing. The emesis fluid from each animal was collected, and the radioactive content of each specimen was determined[2] (Table I).

The administration of a single 50-mg./kg. oral dose of 2-^{14}C-levodopa had an emetic effect on both dogs. Dog 345 vomited on two separate occasions after dosing, at 20 and at 50 min. Dog 1810 vomited once, 220 min. after drug administration. The emesis fluid discharged by each animal was collected, and the radioactive content of each specimen was determined (Table I),

Chronic Multiple-Dose Study—Two beagle dogs, weighing 7–8 kg., were each given a single 50-mg./kg. daily oral dose of nonradioactive levodopa (Lot 114096) in a gelatin capsule.

On the 74th day of daily dosing, each of these primed animals received a single 50-mg./kg. oral dose of 2-^{14}C-levodopa, and the ^{14}C-plasma and urinary excretion patterns of the radioactive dose were monitored. As in the case of the single-dose study, both animals vomited, 20 and 30 min., respectively, after dosing. The emesis fluid of each animal was collected, and the radioactive content of each specimen was determined (Table I).

Daily oral dosing (50 mg./kg.) with nonradioactive levodopa was continued to the 87th day. On the 88th day, both animals were given a single 50-mg./kg. i.v. dose of 2-^{14}C-levodopa; once again the ^{14}C-plasma and urinary excretion patterns of the ^{14}C labeled drug were monitored. Both animals vomited, 4 and 9 min., respectively, after dosing. Although the volumes of the emesis fluid discharged were extremely small, the radioactive content of each specimen was determined (Table I).

Both chronically treated animals vomited daily during the first 4 weeks of oral dosing. Subsequently, from the 4th through the 10th week, both the emetic response to daily dosing and the volume of emesis fluid discharged gradually diminished. Inasmuch as emesis occurred infrequently after 10 weeks, the animals appeared to have developed a substantial degree of tolerance to the 50-mg./kg. oral dose of levodopa.

Collection of Specimens—Heparinized blood samples were collected from each animal at 0 hr. (control), at 1, 5, 10, 20, 30, and 45 min., and at 1, 1.5, 2, 3, 4, 6, 8, and 24 hr. after intravenous dosing. After oral administration, the first sample collected was at 10 min., followed by the schedule as indicated after intravenous dosing. All samples were centrifuged at 2000 r.p.m. in the cold (4°), and the ^{14}C content of the separated plasma was determined. Four milliliters of each plasma sample was added to 20.0 ml. of 0.4 *N* perchloric acid; the mixture was shaken for 15 min. on a mechanical shaker, allowed to stand overnight at 0°, and then centrifuged at 2000 r.p.m. for 20 min. at 4°. The perchloric acid extract obtained was decanted, and the radioactive contents of both the extract and residual precipitate were determined.

A 0–24-hr. urine specimen was collected from each animal prior to dosing (control) and from 0–24, 24–48, and 48–72 hr. after dosing. Each sample was collected in the cold at an acid pH (by inserting 1% of 6 *N* HCl in the collection flasks), and the ^{14}C content of each sample was determined.

Fecal specimens were collected from each animal 0–24 hr. prior to dosing (control) and from 0–24, 24–48, and 48–72 hr. after dosing. The samples were homogenized in aqueous acidic medium, and the ^{14}C content of each sample was determined.

Analyses of Specimens—Differential separation and quantitation of intact levodopa in the plasma extracts and in urine were conducted. The catecholamines were separated on alumina by the method of Anton and Sayre (6), while separation of levodopa from dopamine was achieved by the method of Sedvall *et al.* (7). Quantitation of intact levodopa and dopamine followed the spectrofluorometric method of Sourkes and Murphy (8).

RESULTS

Plasma levels of intact levodopa and total plasma ^{14}C (intact levodopa plus biotransformation products), following the administration of a single 50-mg./kg. i.v. dose of the labeled compound to two dogs, are presented in Fig. 1. The corresponding data obtained following chronic treatment are shown in Fig. 2. In both cases, these

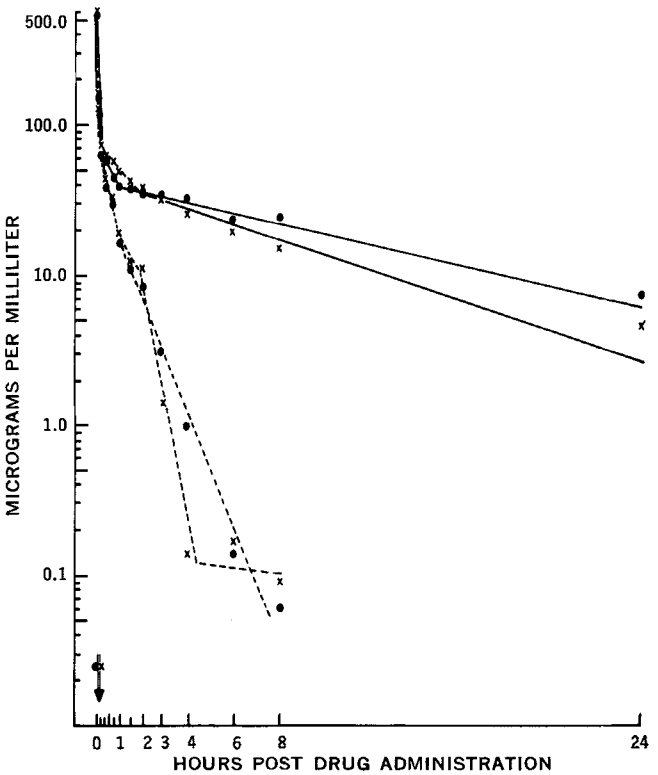

Figure 1—*Total plasma ^{14}C and intact levodopa levels following administration of a single 50-mg./kg. i.v. dose of 2-^{14}C-levodopa to dogs. Key: ●, Dog 345; ×, Dog 1810; —, total plasma ^{14}C; - - -, intact levodopa; and ↓, time of emesis.*

[2] Nuclear-Chicago Mark I scintillation spectrophotometer.

Table II—Percent of Levodopa Dose Excreted in Dog Urine following Administration of a 50-mg./kg. i.v. Dose of 2-^{14}C-Levodopa to Acute and Chronically Treated Animals

Dog Number	Time of Sample Collection, hr.	Percent of Levodopa Dose[a] Excreted			
		^{14}C Excreted	Intact Levodopa[b]	Intact Dopamine[b]	Nonidentified Components
		Acute Single-Dose Study			
345	0–24	66.520	0.040	0.410	66.100
	24–48	4.530	0.002	0.000	4.680
	48–72	2.210	0.001	0.001	2.200
	Total	73.300	0.043	0.411	72.980
1810	0–24	52.680	0.040	0.170	52.460
	24–48	20.710	0.020	0.030	20.660
	8–72	2.760	0.060	0.002	2.700
	Total	76.150	0.120	0.202	65.820
		Chronic Multiple-Dose Study			
346	0–24	56.030	0.026	1.806	56.002
	24–48	14.200	0.012	3.278	14.187
	48–72	1.660	0.101	2.486	1.560
	Total	71.890	0.139	7.570	71.749
349	0–24	65.920	1.617	0.037	64.300
	24–48	4.830	1.122	0.000	3.712
	48–72	0.920	1.286	5.224	—
	Total	71.670	4.025	5.261	—

[a] Dose of ^{14}C-levodopa as altered by emesis. [b] The percentage of these components excreted between 24 and 72 hr. in the multiple-dose study includes residual amounts from the nonradioactive daily doses.

levels fall rapidly during the 1st hr. Thereafter, the rate of decline of the total plasma ^{14}C becomes relatively slower compared to that of the intact levodopa.

During the first 4–6 hr., the plasma levels of intact levodopa fall approximately 5000-fold, after which they remain at a low level for the remainder of the experimental period.

The urinary excretion data presented in Table II show that approximately 75% of the radioactive dose is excreted during a 0–72-hr. period following the intravenous administration of 2-^{14}C-levodopa to the acute and chronically treated animals. Under both conditions, levodopa and dopamine account for a very small percentage of the total radioactive dose. The high percentage of nonidentified components present 24 hr. after dosing and the decrease in total ^{14}C excreted between 24–48 and 48–72 hr. suggest that the biotransformation products persist in the body considerably beyond 72 hr. after drug administration.

Plasma levels of total ^{14}C and of intact levodopa following the oral administration of a single 50-mg./kg. dose of 2-^{14}C-levodopa to two dogs are presented in Fig. 3, while the corresponding data obtained following chronic treatment are shown in Fig. 4. As previously mentioned, both dogs receiving the single dose underwent an emetic response, vomiting approximately 62% of the oral dose. From the total plasma ^{14}C and intact levodopa levels obtained in one of the two dogs, Dog 345 (Fig. 3), it appears that the dose of levodopa available in the gastrointestinal tract after emesis was rapidly absorbed. No detectable plasma ^{14}C levels were obtained in Dog 1810 until 1.5 hr. after oral administration.

From the plasma level data obtained following administration of a 50-mg./kg. oral dose of 2-^{14}C-levodopa to two chronically treated dogs (Fig. 4), it is apparent that the absorption of levodopa from the gastrointestinal tract of both animals is extremely rapid. Peak levels of total plasma ^{14}C and of intact levodopa are obtained 20–30 min

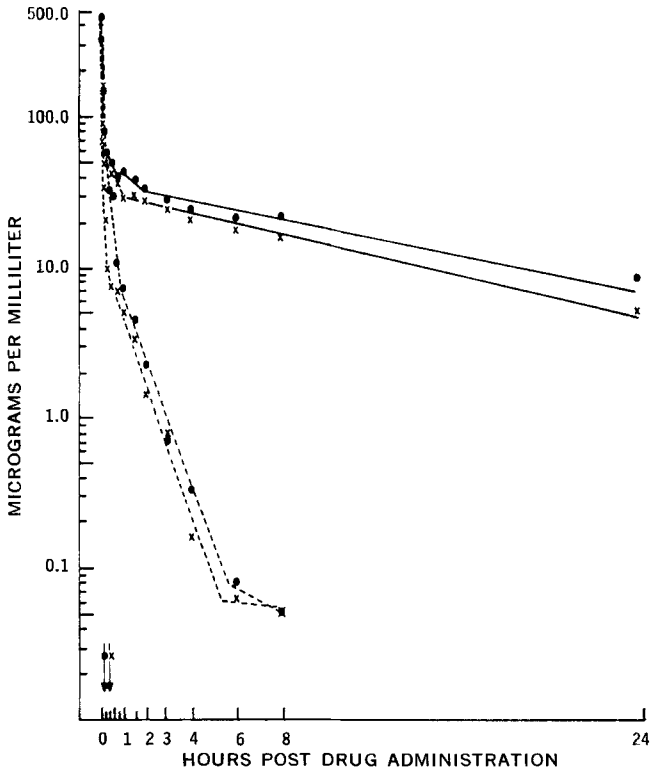

Figure 2—*Total plasma ^{14}C and intact levodopa levels following administration of a 50-mg./kg. i.v. dose of 2-^{14}C-levodopa to chronically treated dogs. Key: ●, Dog 346; ×, Dog 349; —, total plasma ^{14}C; - - -, intact levodopa; and ↓, time of emesis.*

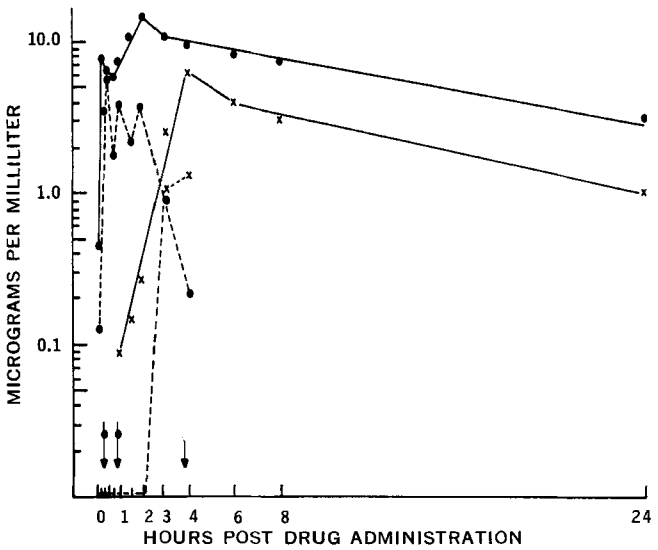

Figure 3—*Total plasma ^{14}C and intact levodopa levels following administration of a single 50-mg./kg. oral dose of 2-^{14}C-levodopa to dogs. Key: ●, Dog 345, ×, Dog 1810; —, total plasma ^{14}C; - - -, intact levodopa; and ↓, time of emesis.*

Table III—Percent of Levodopa Dose Excreted in Dog Urine following Administration of a 50-mg./kg. Oral Dose of 2-^{14}C-Levodopa to Acute and Chronically Treated Animals

Dog Number	Time of Sample Collection, hr.	Percent of Levodopa Dose[a] Excreted			
		^{14}C Excreted	Intact Levodopa[b]	Intact Dopamine[b]	Nonidentified Components
		Acute Single-Dose Study			
345	0–24	45.06	0.021	n.e.	45.036
	24–48	13.27	0.011	n.e.	13.259
	48–72	2.52	0.000	n.e.	2.520
	Total	60.85	0.032	—	60.815
1810	0–24	48.83	1.376	0.464	46.992
	24–48	5.37	0.039	0.030	5.300
	48–72	0.66	0.026	0.000	0.635
	Total	54.86	1.441	0.494	52.927
		Chronic Multiple-Dose Study			
346	0–24	61.19	0.209	1.819	60.984
	24–48	4.73	0.000	2.304	4.730
	48–72	1.60	0.528	2.055	1.069
	Total	67.52	0.737	6.178	66.783
349	0–24	65.39	1.354	0.224	64.039
	24–48	5.92	0.920	0.042	5.002
	48–72	1.21	0.552	6.260	0.661
	Total	72.52	2.826	6.526	69.702

[a] Dose of ^{14}C-levodopa as altered by emesis. [b] The percentage of these components excreted between 24 and 72 hr. in the multiple-dose study includes residual amounts from the nonradioactive daily doses; n.e. = not estimated.

after dosing. In addition, as in the case of the intravenous dose, the rate of decline of intact levodopa is considerably faster than that of the total plasma radioactivity.

The urinary excretion data presented in Table III show that 55–73% of the radioactive dose is excreted over a 0–72-hr. period following oral administration of 2-^{14}C-levodopa to both acute and chronically treated dogs. As was observed following intravenous dosing, intact levodopa and dopamine (measured spectrofluorometrically) account for only a small percentage of the dopamine and its metabolites (total radioactivity) excreted in the urine of these animals.

Table IV shows the amount of total ^{14}C excreted in dog feces following intravenous and oral administration of 2-^{14}C-levodopa to acute and chronically treated animals. No major differences appear to exist in the percent of the ^{14}C dose excreted following intravenous and oral dosing of 2-^{14}C-levodopa.

DISCUSSION

The results obtained following acute and chronic intravenous administration of levodopa to dogs (Figs. 1 and 2) compare favorably with the data of Guldberg and Yates (9) and indicate that intact drug is eliminated from plasma much faster than the total plasma radioactivity. In view of the fact that the difference between the levels of intact levodopa and total plasma ^{14}C is a function of levodopa absorption, tissue distribution, biotransformation, plasma-tissue recycling, and finally elimination, this difference serves as an indication of the presence, relative concentration, and rate of formation of biotransformation products. Some biotransformation products were identified and reported (9–11) to include homovanillic acid, *O*-methyldopa, and catechol acids.

In addition, the ratio of the percent of intact levodopa to the nonidentified components in urine (Table II) is indicative of the almost complete biotransformation of levodopa. These nonidentified components could include phenylacetic acid, phenylpyruvic acid, catecholamines, dihydroxymandelic acid, monophenolic acids, and *O*-methylated derivatives. The suggestion that the biotransformation products persist in the body considerably beyond 72 hr. after drug administration was confirmed by measurements for the presence of residual radioactivity in urine. These measurements showed that approximately 0.1–0.2% of the ^{14}C dose was excreted over a 24-hr. period at 5 days following intravenous administration of the radioactive dose to both acute and chronically treated animals.

Although the plasma data obtained following acute oral administration of levodopa to dogs (Fig. 3) do not lend themselves to interpretation of the absorption characteristics of levodopa from the gastrointestinal tract, the recovery of approximately 57% of the orally administered dose over a 72-hr. period (Table III), as compared to 75% of the intravenous dose (Table II), would indicate at least 76% efficiency of absorption.

As in the case of the intravenously dosed dogs, the ratio of the percentage of nonidentified components to that of intact levodopa in the urine suggests that levodopa undergoes extensive and rapid biotransformation following oral administration.

The plasma level curves following intravenous administration appear to be triexponential. However due to the limited data, only certain pharmacokinetic parameters could be determined (Table V). The elimination-rate constant, β, of intact levodopa from the plasma ranged between 0.73 and 1.02 hr.$^{-1}$ in the four dogs studied. This corresponds to a half-life range of approximately 40–60 min. The elimination-rate constant, β, of total plasma ^{14}C, which includes intact levodopa plus biotransformation products, ranged from 0.077 to 0.085 hr.$^{-1}$, which corresponds to a half-life range of 8–9 hr. Comparison of the intact and total ^{14}C elimination-rate constants indicates that the latter is eliminated approximately 10 times slower than the former. This marked difference in elimination-rate constants emphasizes the need for studying intact levodopa as opposed to total ^{14}C levels in defining the physiological disposition of the drug.

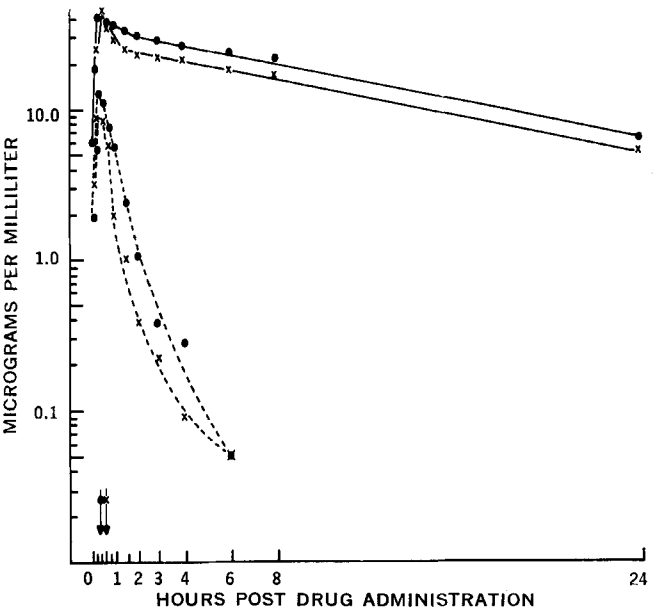

Figure 4—*Total plasma ^{14}C and intact levodopa levels following administration of a 50-mg./kg. oral dose of 2-^{14}C-levodopa to chronically treated dogs. Key: ●, Dog 346; ×, Dog 349; —, total plasma ^{14}C; - - -, intact levodopa; and ↓, time of emesis.*

Table IV—Percent of Levodopa Dose Excreted in Dog Feces following Intravenous and Oral Administration of a 50-mg./kg. Dose of 2-^{14}C-Levodopa to Acute and Chronically Treated Animals

Administration Route	Time of Sample Collection, hr.	Acute Single-Dose Study				Chronic—Multiple-Dose Study			
		Dog 345		Dog 1810		Dog 346		Dog 349	
		mg.	% Dose	mg.	% Dose	mg.	% Dose	mg.	% Dose
Intravenous	0–24	5.76	1.31	n.s.[a]	n.s.	2.96	0.63	8.82	2.65
	24–48	3.22	0.73	9.53	2.42	3.43	0.72	3.78	1.14
	48–72	3.94	0.90	0.72	0.18	0.23	0.05	0.81	0.24
	Total	12.92	2.94	10.25	2.60	6.62	1.40	13.41	4.03
Oral	0–24	0.08	0.05	2.03	1.29	17.09	3.90	23.42	6.72
	24.48	3.96	2.48	0.71	0.45	2.23	0.51	2.70	0.77
	48–72	1.04	0.65	0.16	0.10	0.43	0.10	0.65	0.19
	Total	5.08	3.18	2.90	1.84	19.75	4.51	26.77	7.68

[a] n.s. = no sample defecated.

Table V—Pharmacokinetic Parameters Describing Physiological Disposition of Intravenously and Orally Administered Levodopa in the Dog

	Single Dose		Chronic Dose	
	Dog 345	Dog 1810	Dog 346	Dog 349
Intravenous Administration				
Weight, kg.	9.3	8.2	9.8	7.0
Dose, mg.	440.0	394.0	473.0	333.0
Dose, mg./kg.	47.3	48.0	48.0	47.6
Intact levodopa:				
β, hr.$^{-1}$	0.986	0.948	0.726	1.018
0.693/β, hr. (half-life)	0.70	0.73	0.95	0.68
Percent dose excreted as intact L-dopa in 0–72-hr. urine	0.043	0.12	0.139	4.025
Total ^{14}C:				
β, hr.$^{-1}$	0.077	0.083	0.085	0.079
0.693/β, hr. (half-life)	9.0	8.3	—	8.8
Percent dose excreted in 0–72-hr. feces as total ^{14}C	2.94	2.60	1.40	4.03
Oral Administration				
Dose retained, mg.	159.50	157.40	438.30	348.50
Dose retained, mg./kg.	19.50	19.20	44.70	49.90
Ratio of area under plasma level curve[a] (p.o./i.v.)	0.83	—	0.92	0.92
Percent dose excreted in 0–72-hr. urine as total ^{14}C	60.90	54.90	67.50	72.50
Ratio of dose recovered in 0–72-hr. urine[a] (p.o./i.v.)	0.83	0.72	0.94	1.01
Percent dose excreted in 0–72-hr. feces as total ^{14}C	3.18	1.84	4.51	7.68

[a] Oral area corrected for dose administered.

The route of administration, intravenous or oral, may influence the magnitude of the area under a blood level curve for a rapidly and extensively biotransformed drug. When this occurs, the corresponding areas of intact drug cannot be used directly to assess the extent of drug absorption from the gastrointestinal tract. Comparison of the areas under the total ^{14}C oral and intravenous plasma level curves, however, indicates an efficiency of availability of total ^{14}C from the orally administered dose. This area comparison indicates an efficiency of absorption ranging from 83 to 92% of administered dose (Table V). To assess the proportion of orally administered dose absorbed into the general circulation as intact levodopa, assuming the remainder is degraded in the gastrointestinal tract and/or biotransformed during "first passage" through the liver, it is necessary to compare the ratio of intact levodopa levels to the total ^{14}C levels following each administration. Only the data in two dogs, Dog 345 and Dog 346, one singly dosed and one chronically treated, would lend themselves to this type of an evaluation.

It was estimated that 83% of the administered dose of ^{14}C was absorbed in Dog 345. However, only 22% of the administered dose reached the general circulation as intact levodopa. In Dog 346, 92% of the administered dose of ^{14}C was absorbed, with only 30% of the administered dose reaching the general circulation as intact levodopa. The remainder of the absorbed dose, approximately 60% in both dogs, may have been degraded in the gastrointestinal tract prior to absorption and/or biotransformed in the liver during its "first passage" on its way to the general circulation.

A further measure of the availability of administered ^{14}C following oral administration would be a comparison of the total percent of label (^{14}C) excreted following intravenous and oral administration. These values (Table V) indicated that a range of 72–100% of the administered ^{14}C was absorbed.

In conclusion, the data presented suggest that it is the low availability of intact levodopa following oral administration, coupled with its rapid elimination rate, that results in the need for large and frequent doses to achieve and maintain clinical efficacy.

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Kinetics and Mechanisms of Lactonization of Coumarinic Acids and Hydrolysis of Coumarins II

BERNHARD C. LIPPOLD and EDWARD R. GARRETT*

Abstract □ The kinetics of lactonization of α-chloro-, α-bromo-, α-phenyl-, α-methyl-, and β-methylcoumarinic acids and the hydrolyses of their respective 3- and 4-substituted coumarins were studied at all pH values and at various temperatures. The order of hydrogen-ion-catalyzed lactonizations is consistent with α-substituted steric effects which destroy the acid-weakening resonance of the conjugated carboxylic system by disrupting the coplanarity of the carboxylic oxygen. The order of reactivity in alkaline hydrolysis is consistent with inductive effects, *i.e.*, accelerated by electron-withdrawing substituents. The order of reactivity for other derived microscopic rate constants is consistent with the model proposed previously to rationalize the apparent discrepancies between the apparent kinetic and spectral pKa′ values. The intramolecular formation of an anionic orthoacid lactonization intermediate by the attack of a phenate anion on the substituted coumarinic acid carboxyl, its subsequent protonation to the orthoacid $H_2C‡$, and its possible loss of a hydroxyl ion to form coumarin are inhibited by electron-withdrawing groups that modify carboxyl carbon electrophilicity. This order of reactivity for these rate constants are as expected for such mechanisms. The ratio of rates of spontaneous dehydration to hydrogen-catalyzed dehydration of the neutral orthoacid lactonization intermediate, $H_2C‡$, to yield coumarin decreases with electron-donating substituents, as expected by the proposed mechanism since hydrogen-ion attack should be inhibited by electron-withdrawing substituents such as halogens.

Keyphrases □ Coumarin hydrolysis—kinetics, mechanism □ Lactonization, coumarinic acids—kinetics, mechanism □ pKa′ values, apparent—coumarins, coumarinic acids □ Substituent effect—coumarinic acid lactonization, coumarin hydrolysis

It was shown (1) that the log k–pH profile for the lactonization of coumarinic acid and the hydrolysis of its lactone, coumarin, and the discrepancy between the spectral (or potentiometric) and apparent kinetic pK_{a_1}' values can be rationalized by a proposed mechanism. This mechanism assumes the intramolecular formation of an orthoacid, $H_2C‡$, which dehydrates both spontaneously and by hydrogen-ion catalysis to give coumarin. The steady-state assumption for $H_2C‡$ permitted the fitting of the log k–pH profile consistent with the analytical pK_{a_1}' value and was consistent with the proposed mechanism.

These present investigations were conducted to determine the pKa′ values, the log k–pH rate profiles, and temperature effects for the hydrolysis of variously 3- and 4-substituted coumarins and for the lactonization of their respective coumaric acids. The purposes were: (*a*) to compare their relative reactivities, (*b*) to compare the extent of the equilibria among all charged forms and the corresponding lactones, and (*c*) to test the proposed mechanism by the expected substituent effects.

EXPERIMENTAL

3-Chlorocoumarin[1] and 3-methylcoumarin[2] were used as received. The 3-phenylcoumarin[3] was recrystallized from dioxane–water and acetone–water mixtures. The preparation of 3-bromocoumarin and 4-methylcoumarin was described previously (1). The reactions were investigated at various pH values between −1 and 13 in hydrochloric acid, phosphate buffer, and sodium hydroxide solutions at temperatures between 8.5 and 50.5° (Tables I–III). All solutions were made up with nitrogen-purged distilled water and, if possible, adjusted to an ionic strength of 0.1 with sodium chloride. The pH at the temperatures of the kinetic runs was read with a Radiometer pH meter and a Sargent combination electrode, or it was calculated (1) from the known activities (2) in strong acid and alkaline solutions. The compositions of the buffer solutions are listed in Tables I–III. Details of specific procedures were the same as those given previously (1).

Lactonization—Generally, 0.05 ml. of about 4×10^{-3} *M* solution of a coumarin that had been completely solvolyzed in 0.01 *M* NaOH was added to 3.0 ml. of the appropriate buffer solution to produce a final concentration of about 6.5×10^{-5} *M*. Only 0.02 ml. of the 4×10^{-3} *M* solution of 3-phenylcoumarin was used due to its low solubility. The stock solutions of solvolyzed 3-bromocoumarin and 3-chlorocoumarin were prepared immediately before the lactonization studies, since small amounts of bromide and chloride ions were detected in the alkaline solutions after several weeks, even when they were stored under refrigeration. The ring closure of the coumarin derivatives was monitored from the change of the UV absorbance in the manner described previously for coumarin (1), and lactonization was complete in the absence of significant concentrations of coumarinate dianion. The wavelengths used for the kinetic studies were 280 nm. for 3-chlorocoumarin and 3- and 4-methylcoumarin; 280 and 295 nm. for 3-bromocoumarin; and 280 and 310 nm. for 3-phenylcoumarin.

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Table I—Apparent First-Order Rate Constants, k, in sec.$^{-1}$ for Lactonization of 6.5×10^{-5} M Coumarinic Acids[a] to Their Respective 3- and 4-Substituted Coumarins in HCl Solution

10^2[HCl][b]	pH[c]	3-Chloro	3-Bromo	3-Phenyl	3-Methyl	4-Methyl
				10^3k, 25.5°		
300	−0.59	—	—	—	—	140
200	−0.30	—	—	—	—	77.5
100	0.10	148	—	—	171	35.8
75	0.24	113	144	—	110	21.0
50	0.43	95.1	114	77.8	—	14.0
25	0.73	77.6	80.3	—	55.4	8.70
11	1.05	—	—	27.5	35.1	—
8.2	1.21	54.2	46.9	—	27.0	4.7
4.15	1.44	—	—	17.8	21.8	—
2.9	1.60	35.5	29.2	—	—	—
1.03	2.03	—	—	9.21	—	—
0.97	2.10	15.5	—	—	12.5	2.18
0.425	2.46	8.20	—	4.38	10.5	—
0.265	2.60	—	4.61	—	—	2.03
0.13	2.91	2.34	3.60	—	—	2.02
0.033	3.49	0.733	—	0.329	3.71	—
				10^2k, 17.5°		
400	−0.87	—	—	—	—	13.9
200	−0.31	14.2	19.6	15.3	30.3	4.64
100	0.08	8.03	8.35	8.16	12.8	1.65
50	0.42	4.64	—	—	6.23	—
				10^2k, 12.5°		
400	−0.88	—	—	—	—	9.0
200	−0.32	8.67	12.7	10.5	20.6	2.59
100	0.08	5.42	—	5.2	8.0	1.12
50	0.42	—	2.6	2.89	4.04	—
				10^2k, 8.5°		
400	−0.88	12.7	—	22.8	—	5.54
200	−0.33	5.46	7.52	8.5	16.2	—
100	0.08	3.78	2.95	—	6.39	0.81
50	0.41	—	—	2.37	2.94	0.36

[a] Phenylcoumarinic acid solutions were 2.60×10^{-5} M. [b] NaCl was added to maintain the ionic strength at $\mu = 0.1$ when possible. [c] pH values in HCl at concentrations greater than 0.1 M were calculated from $pH = -\log f[HCl]$, where the activity coefficient, f, was obtained from the literature (2). All other values were measured by a pH meter.

metric cells by mixing 0.05 ml. (0.02 ml. for phenylcoumarin) of the 4×10^{-3} M alkaline solutions of the completely solvolyzed coumarins with 3.0 ml. 0.1 N HCl.

This ring closure was completed in a short time (see apparent first-order rate constants of Table I for lactonization in HCl solution), and a 6.5×10^{-5} M solution (2.6×10^{-5} M for phenylcoumarin) of the substituted coumarin was formed and stayed in solution. A calculated amount of 1 M NaOH was then added to give the desired alkaline pH on the basis of the known activities of HCl and NaOH (2). The resultant pH values were checked with a Radiometer pH meter and a Sargent combination electrode, and good agreements were found. The hydrolyses were investigated by recording the change in the UV absorbance at 310 nm. for phenylcoumarin and at 280 nm. for all the other coumarins. The apparent first-order rate constants for the alkaline hydrolysis were calculated in the manner described for the lactonization and are listed in Table III.

Spectrophotometric Determination of the Apparent pKa′ Values—The pKa_1' values of the carboxylic acid groups were determined by adding 0.05-ml. aliquots of the approximately 4×10^{-3} M solution of the completely solvolyzed substituted coumarin to 3.0 ml. of an appropriate buffer solution in the pH range 0–7. The rapid change in the absorbance at a given wavelength due to the ring closure was automatically monitored as a function of time with a Cary 15 re-

Table II—Apparent First-Order Rate Constants, k, in sec.$^{-1}$ for Lactonization of 6.5×10^{-5} M Coumarinic Acids[a] to Their Respective 3- and 4-Substituted Coumarins in Phosphate Buffers[b]

$[H_2PO_4^-]$	$[HPO_4^{-2}]$	pH[c]	3-Chloro, 10^4k	3-Bromo, 10^4k	3-Phenyl, 10^4k	3-Methyl, 10^3k	4-Methyl, 10^3k
				25.0°			
0.065	0.008	5.09	0.503	—	—	—	1.18
0.065	0.011	6.08	0.332	0.162	0.107	0.348	0.915
0.0167	0.0277	7.02	0.296	0.155	0.115	0.342[d]	0.823
0.00125	0.0329	8.26	—	—	—	0.335[d]	—
				34.5°			
0.065	0.011	6.16	0.75	0.461	0.378	0.915	2.03
0.0167	0.0277	7.15	0.757	0.460	0.377	0.913	2.04
				43.5°			
0.065	0.011	6.18	2.16	1.24	1.19	2.12	4.62
0.0177	0.0277	7.2	2.17	1.24	1.20	2.20	4.63
				50.5°			
0.065	0.011	6.21	4.10	2.67	2.25	4.10	7.88
0.0167	0.0277	7.22	4.24	2.79	2.28	4.00	7.62

[a] Phenylcoumarinic acid solutions were 2.60×10^{-5} M. [b] NaCl was added when needed to maintain the ionic strength at $\mu = 0.1$. [c] pH was measured with a Radiometer pH meter and a Sargent combination electrode. [d] $10^3k = 0.339$ (pH 7.02) and 0.341 (pH 8.26) when half the buffer concentrations was used.

Table III—Apparent First-Order Rate Constants, k, in sec.$^{-1}$ for Hydrolysis of 6.5 × 10^{-5} M 3- and 4-Substituted Coumarins[a] in Aqueous Solutions

10^3[NaOH][b]	9.0° pH[c]	9.0° 10^3k	13.0° pH[c]	13.0° 10^3k	17.5° pH[c]	17.5° 10^3k	25.0° pH[c]	25.0° 10^3k
				3-Chloro				
2.0	11.38	14.2	11.25	14.3	11.35	3.11	11.20	25.2
5.0	11.65	27.9	11.62	31.9	11.64	62.5	11.68	102
12.5	—	—	—	—	—	—	12.08	194
20.0	12.23	115	12.22	136	12.20	207	12.3	333
				3-Bromo				
2.0	11.34	10.7	11.40	14.3	11.30	20.5	11.20	18.2
5.0	11.64	24.4	11.68	32.3	11.66	46.7	11.65	74.0
7.9	—	—	—	—	—	—	11.80	110
10.0	—	—	—	—	—	—	11.95	180
20.0	12.22	99.3	12.28	110	12.17	165	12.30	347
				3-Phenyl				
4.5	11.47	0.573	11.55	1.20	11.55	1.95	11.55	2.40
5.5	11.58	0.695	11.74	1.70	11.82	2.32	11.70	4.34
20.0	12.17	3.46	12.22	5.45	12.25	7.50	12.18	13.0
	17.5° pH[c]	17.5° 10^3k	25.0° pH[c]	25.0° 10^3k	32.5° pH[c]	32.5° 10^3k	39.0° pH[c]	39.0° 10^3k
				3-Methyl				
1.5	—	—	—	—	11.23	2.65	11.20	4.41
4.5	11.52	0.870	—	—	11.52	4.87	11.46	6.90
5.5	11.67	1.47	11.88	3.95	—	—	—	—
20.0	12.25	5.15	12.27	12.2	12.18	17.5	12.21	32.0
25.0	—	—	12.33	12.5	—	—	—	—
				4-Methyl				
1.5	—	—	—	—	—	—	11.10	1.73
2.0	11.52	0.355	—	—	11.30	0.878	11.38	2.10
20.0	12.05	0.700	12.08	1.07	12.13	2.95	12.12	5.10
25.0	—	—	12.30	—	—	—	—	—
30.0	—	—	12.40	2.46	—	—	—	—
40.0	—	—	12.53	2.90	—	—	—	—

[a] 3-Phenylcoumarin solutions were 2.60 × 10^{-5} M. [b] NaCl was added when possible to maintain an ionic strength of μ = 0.1. [c] pH values at NaOH concentrations greater than 0.01 were calculated from pH = pK_w − log f'[NaOH] where the activity coefficient, f', was obtained from the literature (2). All other values were measured by a pH meter.

cording spectrophotometer immediately after mixing in the spectrophotometric cell. The absorbance at time zero, A_0, was obtained by graphical extrapolation (1) for the different pH values. This procedure was carried out at several wavelengths (Table IV) at 25°.

Since the rates of lactonization at pH values >7 were sufficiently slow (Table II), the entire spectra could be monitored at 25° and at a given pH immediately after the solutions of the completely solvolyzed coumarins and the appropriate buffer solutions were mixed at 25°. The change of the UV spectrum of 3-bromocoumarinic acid as a function of pH is shown in Fig. 1 for an example. From these data, the pKa_2' of the phenolic group would be estimated (1).

Spectra of Undissociated Coumarinic Acids and Their Anions—Aliquots of 0.022 ml. (0.011 for phenylcoumarin) of a 1.0 × 10^{-2} M alkaline solution of a completely solvolyzed substituted coumarin were mixed with 3.0 ml. of 0.5 N HCl. The rapid change in the absorbance of the 7.30 × 10^{-5} M solutions was automatically recorded with time at each 10-nm. interval between 220 and 380 nm. at 25°. The absorbances at time zero, A_0, were then obtained by graphical extrapolation (1), and these extrapolated spectra of the substituted undissociated coumarinic acids are given in Figs. 2–6.

Table IV—Spectrophotometrically Determined pKa′ Values of 6.5 × 10^{-5} M Coumarinic Acids at 25°

Derived from the Substituted Coumarin	Wavelength, nm. for pKa_1'	Wavelength, nm. for pKa_2'	pKa_1'	pKa_2'
3-Bromo	255, 330	230, 295, 340	1.80	9.35
3-Chloro	255, 330	230, 295, 335	2.05	9.60
3-Phenyl	280, 310	245, 310	3.15	9.72
3-Methyl	255, 330	235, 330	3.63	10.00
H[b]	250, 280, 320	238, 290, 335	4.00[b]	9.95[b]
4-Methyl	270, 305, 310	270, 300, 330	4.80	10.70

[a] Phenylcoumarin solutions were 2.60 × 10^{-5} M. [b] See *Reference 1;* potentiometric titration gave 4.00 and 9.90 (1).

The spectra of their monoanions were obtained in phosphate buffer at pH 7.2. The spectra of their dianions were obtained in 0.1 N NaOH. These latter spectra (Figs. 2–6) did not require an extrapolation procedure since there was no significant lactonization during the time interval required to read such spectra under these experimental conditions. The spectral curves for the unsubstituted coumarinic acid were given previously (1).

RESULTS AND DISCUSSION

Estimation of Apparent pKa′ Values—The apparent pKa′ values for the variously substituted coumarinic acids of Table IV were

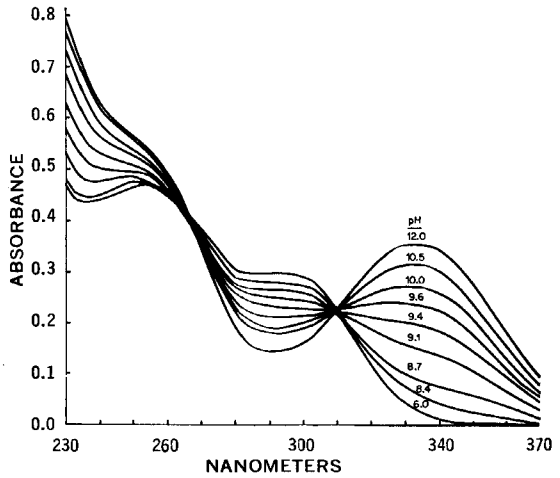


Figure 1—*Typical spectral changes for determination of pKa_2' as a function of pH for 6.25 × 10^{-5} M bromocoumarinic acid (derived from 3-bromocoumarin) in phosphate, borate, and carbonate buffers and in NaOH. The pH values are as indicated.*

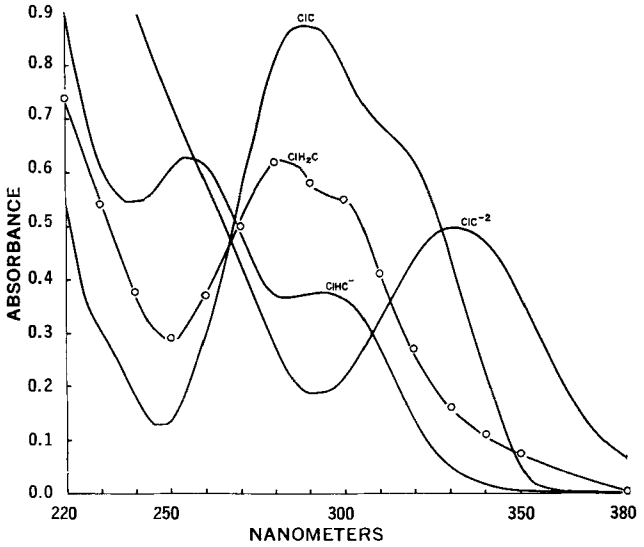


Figure 2—*UV spectra of 7.30 × 10⁻⁵ M undissociated 3-chlorocoumarinic acid, ClH_2C (based on extrapolated time-zero absorbances after introduction of the sodium 3-chlorocoumarinate into 0.5 N HCl); 3-chlorocoumarinate monoanions, $ClHC^-$ (in phosphate buffer, pH 7.2); 3-chlorocoumarinate dianions, ClC^{-2} (in 0.1 N NaOH); and 3-chlorocoumarin, ClC (in 0.5 N HCl). The numbering system used refers to the parent coumarin.*

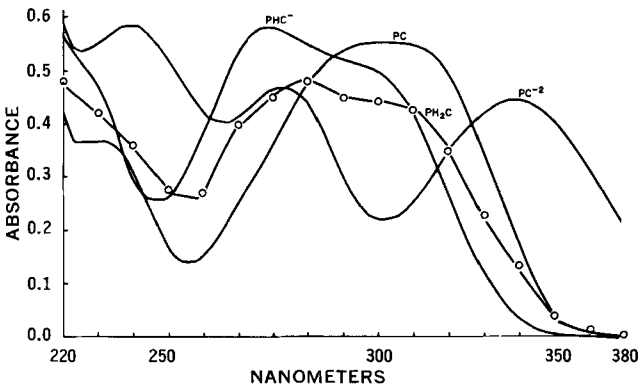


Figure 4—*UV spectra of 3.65 × 10⁻⁵ M undissociated 3-phenylcoumarinic acid, PH_2C (based on extrapolated time-zero absorbances after introduction of the sodium 3-phenylcoumarinate into 0.5 N HCl); 3-phenylcoumarinate monoanions, PHC^- (in phosphate buffer, pH 7.2); 3-phenylcoumarinate dianions, PC^{-2} (in 0.1 N NaOH); and 3-phenylcoumarin, PC (in 0.5 N HCl). The numbering system used refers to the parent coumarin.*

estimated in the manner discussed previously (1) for the several cited wavelengths by use (3) of Eq. 2:

$$\log \left| \frac{A_{HC^-} - A_0}{A_0 - A_{H_2C}} \right| = pKa' - pH \qquad \text{(Eq. 2)}$$

where the A_0 values are the time-zero estimates of absorbances at a given pH value, the A_{H_2C} value is the asymptotic absorbance in increasingly acidic solutions and is assigned to the absorbance of the 6.5 × 10^{-5} *M* undissociated substituted coumarinic acid (except at a concentration of 2.6 × 10^{-5} *M* for the phenyl derivative), and A_{HC^-} is the asymptotic absorbance achieved in neutral solution and is assigned to the absorbance of the respective substituted coumarinate monoanion of these same concentrations. These absorbances, A_{HC^-} and A_{H_2C}, at different wavelengths also may be obtained from Figs. 2–6.

It is evident (Table IV) that substituents with a negative inductive effect (−I), such as halogens, increase the acidity of the carboxylic acid groups in the acids derived from the 3-substituted coumarins. The methylcoumarinic acids have higher pKa_1' values because of lessened electron-withdrawing properties (+I) of the methyl group. However, the acid derived from 3-methylcoumarin has a lower pKa_1' than the unsubstituted coumarinic acid, whereas the acid derived from 4-methylcoumarin has a higher pKa_1'. A similar effect has been observed for *o*-methylbenzoic acid (pKa′ 3.91) and *m*-methylbenzoic acid (pKa′ 4.27) as compared to benzoic acid (pKa′ 4.20) and may imply that the disruption of resonance effects, which normally stabilizes the undissociated acid of a conjugated carboxyl, relates the pKa′ values of acids derived from the 3-substituted coumarins (4–6) to these of *ortho*-substituted aromatic acids (7–11). In essence, this implies that these α-substituted coumarinic acids disrupt the coplanarity of the carboxyl with the conjugated systems and disrupt the acid-weakening resonance which can occur more readily in the absence of such vicinal substituents (8).

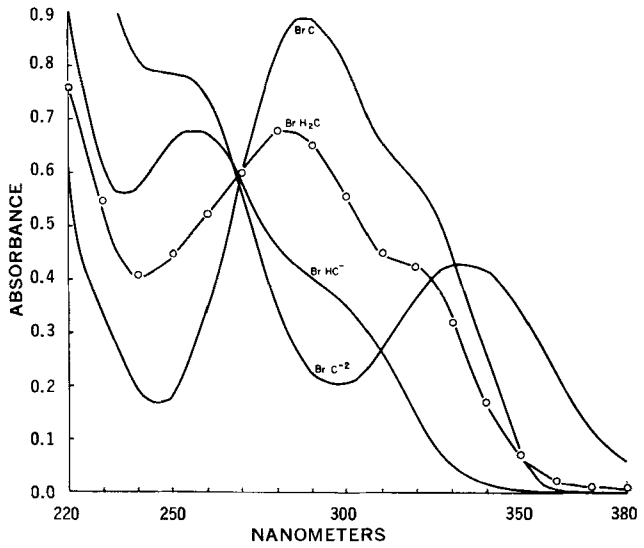


Figure 3—*UV spectra of 7.30 × 10⁻⁵ M undissociated 3-bromocoumarinic acid, BrH_2C (based on extrapolated time-zero absorbances after introduction of the sodium 3-bromocoumarinate into 0.5 N HCl); 3-bromocoumarinate monoanions, $BrHC^-$ (in phosphate buffer, pH 7.2); 3-bromocoumarinate dianions, BrC^{-2} (in 0.1 N NaOH); and 3-bromocoumarin, BrC (in 0.5 N HCl). The numbering system used refers to the parent coumarin.*

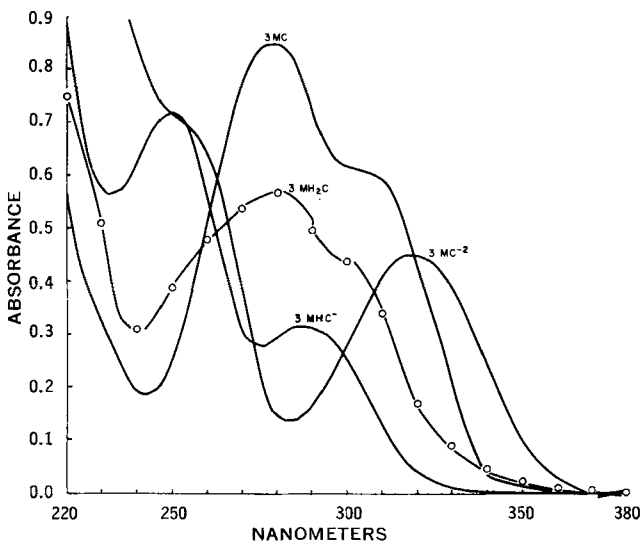


Figure 5—*UV spectra of 7.30 × 10⁻⁵ M undissociated 3-methylcoumarinic acid, $3MH_2C$ (based on extrapolated time-zero absorbances after introduction of the sodium 3-methylcoumarinate into 0.5 N HCl); 3-methylcoumarinate monoanions, $3MHC^-$ (in phosphate buffer, pH 7.2); 3-methylcoumarinate dianions, $3MC^{-2}$ (in 0.1 N NaOH); and 3-methylcoumarin, 3MC (in 0.5 N HCl). The numbering system used refers to the parent coumarin.*

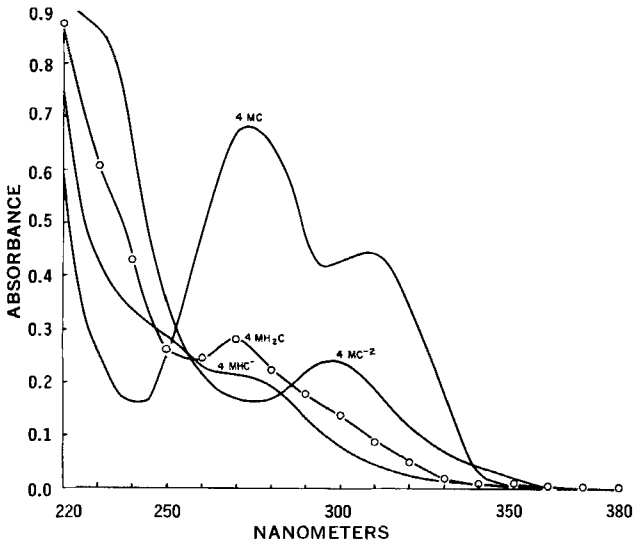


Figure 6—*UV spectra of 7.30 × 10⁻⁵ M undissociated 4-methylcoumarinic acid, 4MH₂C (based on extrapolated time-zero absorbances after introduction of the sodium 4-methylcoumarinate into 0.5 N HCl); 4-methylcoumarinate monoanions, 4MHC⁻ (in phosphate buffer, pH 7.2); 4-methylcoumarinate dianions, 4MC⁻² (in 0.1 N NaOH); and 4-methylcoumarin, 4MC (in 0.5 N HCl). The numbering system used refers to the parent coumarin.*

The pKa_1' values of the coumarinic acids derived from the 3-substituted coumarins of Table IV are plotted against the pKa′ values of the comparably *ortho*-substituted benzoic acids (4–6) in Fig. 7 [the pKa_1' value of the coumarinic acid derived from 4-methylcoumarin is plotted against that of *m*-methylbenzoic acid (4–6)]. The reasonably linear relation is demonstrated in Fig. 7 and can be fitted by the expression:

$$pKa_1' \text{ (coumarinic acid from 3-substituted coumarin)} = 1.85\ pKa' \text{ (comparably substituted } o\text{-benzoic acid)} - 3.5 \quad \text{(Eq. 3)}$$

The spread among the pKa′ values of the *meta*- (Br, 3.81; Cl, 3.83; and methyl, 4.27) and *para*-substituted benzoic acids (Br, 3.97; Cl, 3.98; and methyl, 4.37) is much less, so that plots of the pKa_1' values of the coumarinic acids derived from 3-substituted coumarins against the pKa′ of comparably substituted *meta*- and *para*-benzoic acids have considerably greater slopes. The slope is 3.64 with an intercept value of −11.9 for the *meta*-substituted benzoic acids, and the point for unsubstituted benzoic and coumarinic acids is widely displaced from the plot.

The pKa_2' values assigned to the phenolic groups of the coumarinic acids derived from the 3-substituted coumarins (Table IV) are also reasonably, linearly related to the pKa_1' of coumarinic acid and to pKa′ values of the similarly substituted benzoic acid, no matter which series of the latter (*ortho*, *meta*, or *para*) is used for the plot. The only widely outlying value in such plots is for the compound derived from 4-methylcoumarin. This implies that the inductive effect of the substituents is readily transmitted through the aryl conjugated system to the phenolic position on the ring (12), since the decrease in pKa_2' cannot be assigned to the increased interaction of the less basic carboxylate anion produced by electron-withdrawing substituents with a phenolic group of invariant acidity.

Log *k*–pH Profiles for Lactonization of Substituted Coumarinic Acids and Their Anions—No significant buffer catalytic effects were observed in phosphate buffer (1) (see *Footnote c* in Table II). The apparent first-order rate constants for the lactonization of the substituted coumarinic acids and their anions should conform to the previously derived (1) equation:

$$k = \{k_{H^+}[H^+] + k_{H_2O}\}f_{H_2C} + \{k_{H^+}'[H^+] + k'_{H_2O}\}f_{HC^-} \quad \text{(Eq. 4)}$$

where the k_i are the individual microscopic rate constants, and

$$f_{H_2C} = \frac{[H_2C]}{[H_2C] + [HC^-]} = \frac{[H^+]}{[H^+] + K_{a_1}'} \quad \text{(Eq. 5)}$$

and

$$f_{HC^-} = \frac{[HC^-]}{[H_2C] + [HC^-]} = \frac{K_{a_1}'}{[H^+] + K_{a_1}'} \quad \text{(Eq. 6)}$$

are the fractions of the substituted undissociated acid, H_2C, and its monoanion, HC^-, respectively.

Since $k_{H_2O}f_{H_2C}$ and $k_{H^+}'[H^+]f_{HC^-}$ are kinetically equivalent, Eq. 4 can be reduced to:

$$k = \{k_{H^+}[H^+] + k_{H_2O}\}f_{H_2C} + k'_{H_2O}f_{HC^-} \quad \text{(Eq. 7)}$$

An asymptotic slope of unity is approached with decreasing pH in the log *k*–pH profile for each compound (Figs. 8–10) since, with increasing acidity or $[H^+]$, Eq. 7 approaches

$$k = k_{H^+}[H^+] \quad \text{(Eq. 8)}$$

and the second-order rate constants, k_{H^+} (Table V), can be estimated from the intercepts of log *k versus* pH plots (Figs. 8–10) of negative unit slopes in the low pH regions. The value for k_{H_2O} in Eq. 7 was estimated in the pH region below neutrality, where the contribution of the term $k'_{H_2O}f_{HC^-}$ is negligible, by subtracting the calculated $k_{H^+}[H^+]f_{H_2C}$ values from the observed first-order rate

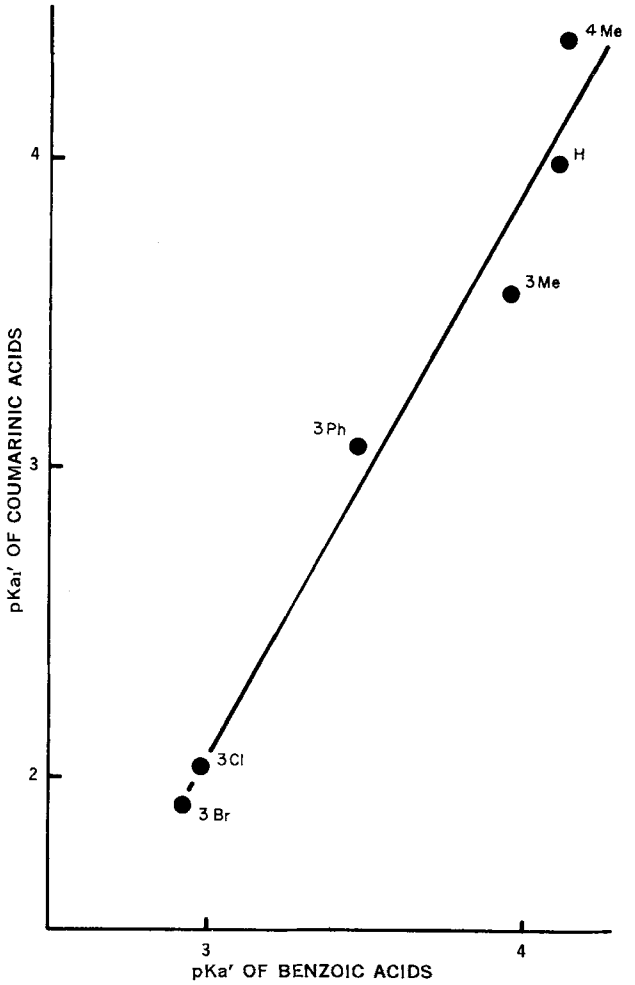
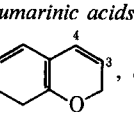
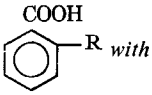


Figure 7—*Plot of pKa′ values of coumarinic acids derived from the 3- or 4-substituted coumarins,* i.e.,  *against the pKa′ values of* ortho-*benzoic acids*  *with the same substituents. The exception is that the pKa′ for the acid derived from 4-methylcoumarin is plotted against the pKa′ for* m-*toluic acid.*

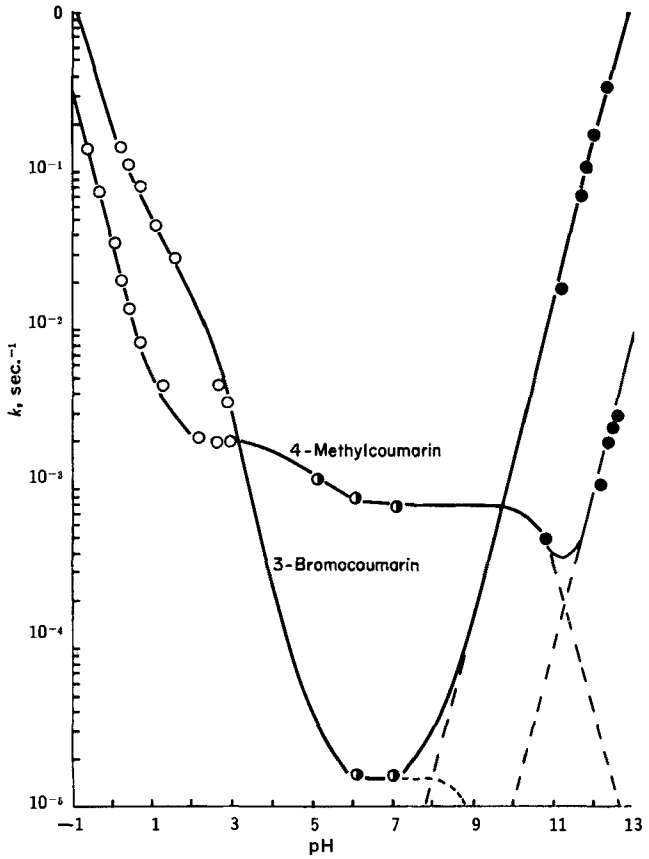


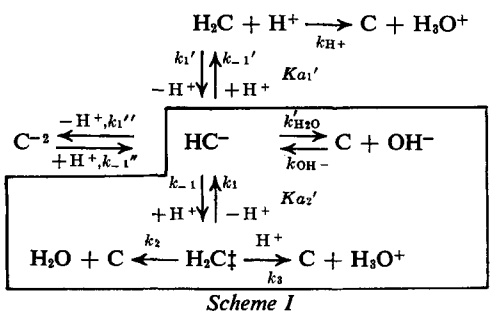
Figure 8—*Log* k–*pH profiles at 25° for hydrolyses of 3-bromocoumarin and 4-methylcoumarin and lactonization of their respective coumarinic acids. Key:* ○, *lactonization in hydrochloric acid;* ◑, *lactonization in phosphate buffer; and* ●, *hydrolysis in sodium hydroxide solutions.*

constants, so that for pH values less than 7

$$k - k_{H^+}[H^+]f_{H_2C} = k_{H_2C}f_{H_2C} \qquad \text{(Eq. 9)}$$

Various pKa_1' values were assumed for each compound for the calculations of the f_{H_2C} and f_{HC^-} values (Eqs. 5 and 6) to give the best fits for the log *k*–pH plots of Figs. 8–10. The apparent kinetic pKa_1' values for the coumarinic acids derived from the substituted coumarins were: 3-chloro, 1.52; 3-bromo, 1.7; 3-phenyl, 1.8; 3-methyl, 2.96; and 4-methyl, 3.67. The rate constants, k_{H^+}, k_{H_2O}, and k'_{H_2O}, of Eq. 7 that give best fits in Figs. 8–10 are given in Table V. The apparent kinetic pKa_1' values differ markedly from the respective spectral pKa_1' values (Table IV), in the same manner that the apparent kinetic pKa_1' of 3 and the spectral and potentiometric pKa_1' values of 4 differed for unsubstituted coumarinic acid (1). These discrepancies can be rationalized (1) on the basis of Scheme I for the lactonization of a coumarinic acid, H_2C, in equilibrium with its monoanion, HC^-, and dianion, C^{-2}, to its respective coumarin, C, where $H_2C‡$ is the monoester of an orthoacid in Scheme II. The blocked area in Scheme I includes the transformations expanded in Scheme II.

The transformations of Scheme I were given explicitly in the previous publication (1) and were shown on the basis of the steady-



Scheme I

state assumption, $d[H_2C‡]/dt \sim 0$, to result in the kinetic dependency:

$$k = k_{H^+}[H^+]f_{H_2C} + k'_{H_2O}f_{HC^-} + \frac{(k_2 + k_3[H^+])K_{a_1}'k_{-1}}{k_1 + k_2 + k_3[H^+]}f_{H_2C} \qquad \text{(Eq. 10)}$$

which, on comparison to Eq. 7, states that

$$\frac{(k_2 + k_3[H^+])K_{a_1}'k_{-1}}{k_1 + k_2 + k_3[H^+]} = k_{H_2O} \qquad \text{(Eq. 11)}$$

The values of k_{H^+} were readily obtained from the intercepts of the linear asymptotes of the log *k*–pH plots, with a slope of −1 at the pH values below 2 (Figs. 8–10). The value of k'_{H_2O} was estimated from the plateau in the log *k*–pH profile reached in the neutral pH region, where only the coumarinate monoanion, HC^-, exists and before the formation of a significant amount of nonlactonizable coumarinate dianion, C^{-2}, lowers the monoanion's apparent rate of lactonization. Thus, an apparent rate constant, *k'*, can be defined (1) in the mildly acidic pH region from consideration of Eqs. 7 and 11 as:

$$k' = k - (k_{H^+}[H^+]f_{H_2C} + k'_{H_2O}f_{HC^-}) = \frac{(k_2 + k_3[H^+])k_{-1}K_{a_1}'}{(k_1 + k_2) + k_3[H^+]}f_{H_2C}$$

$$= \frac{Q + P[H^+]}{1 + P[H^+]}k_{-1}K_{a_1}'f_{H_2C} \qquad \text{(Eq. 12)}$$

where

$$Q = k_2/(k_1 + k_2) \qquad \text{(Eq. 13)}$$

and

$$P = k_3/(k_1 + k_2) \qquad \text{(Eq. 14)}$$

Thus, at high $[H^+]$ concentrations or at pH values less than 2, where

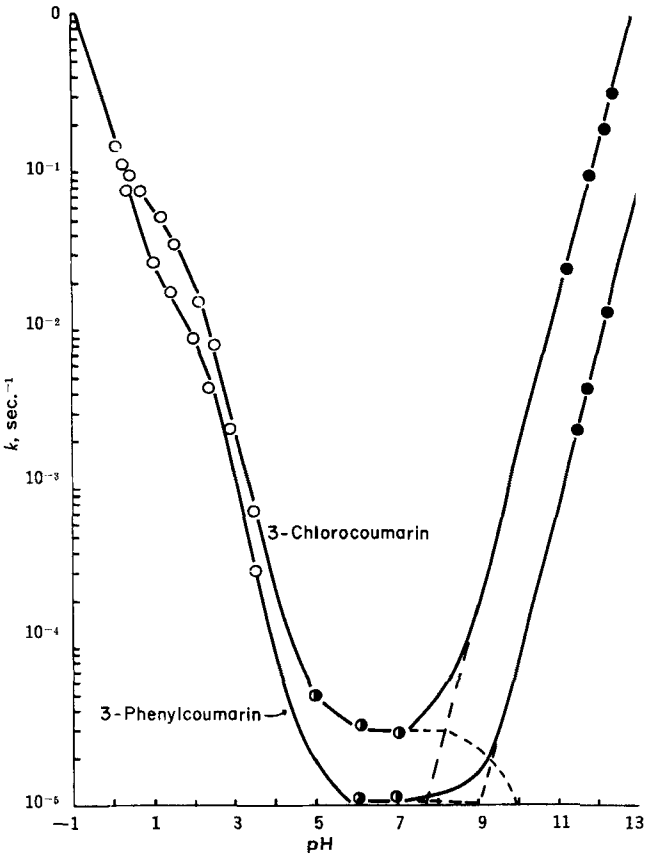
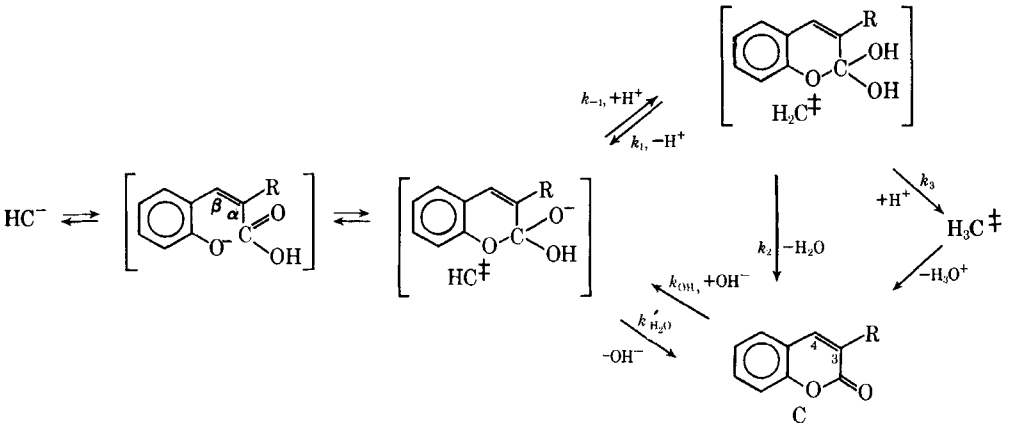


Figure 9—*Log* k–*pH profiles at 25° for hydrolyses of 3-chlorocoumarins and 3-phenylcoumarins and lactonization of their respective coumarinic acids. Key:* ○, *lactonization in hydrochloric acid;* ◑, *lactonization in phosphate buffer; and* ●, *hydrolysis in sodium hydroxide solutions.*



Scheme II

$f_{H_2C} \rightarrow 1$ and

$$(Q + P[H^+])/(1 + P[H^+]) = (k_2 + k_3[H^+])/(k_1 + k_2 + k_3[H^+]) \rightarrow 1 \quad \text{(Eq. 15)}$$

then,

$$\lim_{[H^+]\rightarrow\infty} k' = k_{-1}K_a' = k_{H_2O} \quad \text{(Eq. 16)}$$

and k_{-1} may be estimated (Table V) from the known K_{a_1}' and the apparent k_{H_2O} values, where the latter was obtained from the best fit of Eq. 7.

Thus, at low $[H^+]$ concentrations, possibly at pH values greater than 6, where $k_3[H^+] \ll k_2$,

$$\lim_{[H^+]\rightarrow 0} k' = \left(\frac{k_2}{k_1 + k_2}\right) k_{-1}K_{a_1}'f_{H_2C} = Qk_{-1}K_{a_1}'f_{H_2C} \rightarrow Qk_{-1}[H^+] \quad \text{(Eq. 17)}$$

since, from Eq. 4, $f_{H_2C} \rightarrow [H^+]/K_{a_1}'$ at $[H^+] \rightarrow K_{a_1}'$. Thus, Q, as defined in Eq. 13, may be estimated (Table V) since k_{-1} (Eq. 16), K_{a_1}', and $[H^+]$ are known.

At intermediate pH values, possibly at pH values in the range of the pK_{a_1}', the more exact Eq. 12 holds, where k_{-1} (Eq. 16) and $Q = k_2/(k_1 + k_2)$ (Eq. 17) have been estimated (Table V) and all other factors such as $[H^+]$, K_{a_1}', and f_{H_2C} (Eq. 5) are known. The values of P may be calculated from

$$P = \frac{k' - Qk_{-1}K_{a_1}'f_{H_2C}}{(k_{-1}K_a'f_{H_2C} - k')[H^+]} \quad \text{(Eq. 18)}$$

and are given in Table V.

The curves drawn through the points in Figs. 8–10 are based on the evaluated values of Q, P, k_{-1}, k_{H^+}, and k'_{H_2O} as given in Table V and may be compared with the curve previously published for coumarin (1).

Estimates of k_{H^+} and k'_{H_2O} (Table V) were determined from studies at various temperatures (Tables I and II). The thermodynamic parameters obtained from the slopes and intercepts of the Arrhenius plots are given in Table VI.

Hydrolyses of Substituted Coumarins and Monoanion and Dianion–Coumarin Equilibria—The plots of log k *versus* pH for the hydrolyses of the coumarins to coumarinate dianions are linear and of the slope of unity above the pH = pK_{a_2}' + 1.5 (Figs. 8–10) in accordance with

$$-\frac{d[C]}{dt} = \frac{d[H_2C]_T}{dt} = k[C] = k_{OH^-}[OH^-][C] \quad \text{(Eq. 19)}$$

Thus, after logarithmic transformation,

$$\log k = \log k_{OH^-} - pK_w + pH \quad \text{(Eq. 20)}$$

The values of k_{OH^-} (Table V) can be estimated from the extrapolated intercepts of the alkaline branch of the profiles (Figs. 8–10) for the several temperatures studied. The thermodynamic parameters (Table V) were estimated from the slopes and intercepts of the Arrhenius plots.

In the pH region of the pK_{a_2}' of the various coumarinic acids (Table IV), the apparent first-order rate constant, k, was expected to be the sum or the backward and forward rate constants (1) for hydrolysis and lactonization, respectively, since k was estimated from Eq. 1 and in this region the asymptotic absorbance is the sum of the equilibrated lactone, monoanion, and dianion. Thus,

$$k = k'_{H_2O}f_{HC^-} + k_{OH^-}[OH^-] \quad \text{(Eq. 21)}$$

where hydroxide-ion attack on coumarin may form HC‡ (Scheme II) and thus HC^- (Schemes I and II) in the reverse reaction of the net pH-independent loss of hydroxide ion for the coumarinate monoanion to yield coumarin.

The dashed lines coming from the left in Figs. 8–10 show the decrease in the $k'_{H_2O}f_{HC^-}$ term for the lactonization of the mono-

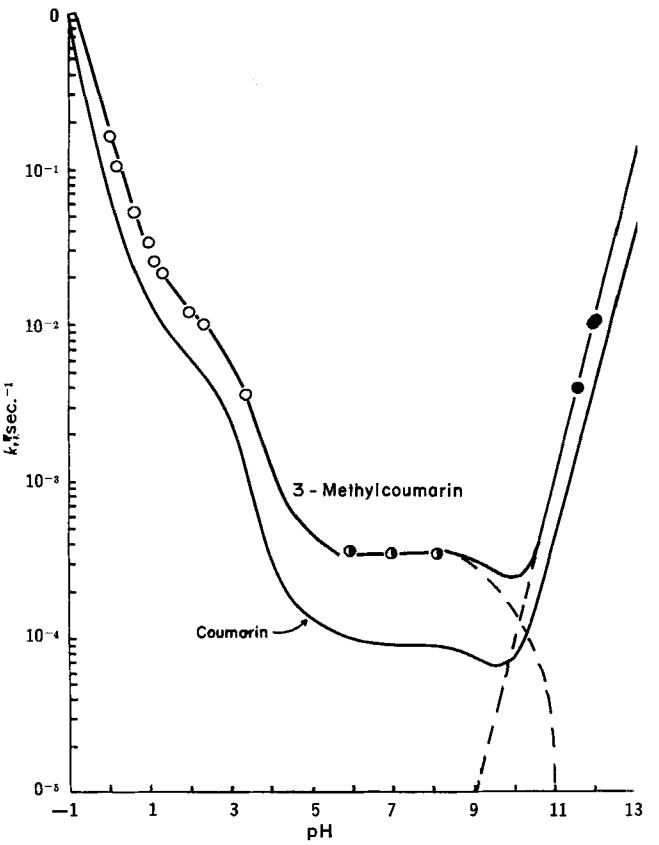


Figure 10—*Log k–pH profiles at 25° for hydrolyses of 3-methylcoumarin and lactonization of its coumarinic acid. Key:* ○, *lactonization in hydrochloric acid;* ◑, *lactonization in phosphate buffer; and* ●, *hydrolysis in sodium hydroxide solutions. The solid line is given for coumarin (1) to serve as a reference.*

Table V—Microscopic Rate Constants[a] for Lactonization of Coumarinic Acids[b] and Hydrolysis of Coumarins

Temperature	3-Chloro	3-Bromo	3-Phenyl	3-Methyl	H[c]	4-Methyl
			$10^2k_{H^+}$			
8.5°	2.60	3.50	3.75	7.60	—	0.890
10.0°	—	—	—	—	2.20	—
12.5°	4.20	5.50	5.50	9.75	—	1.20
17.5°	7.00	9.30	8.40	15.8	4.50	2.00
25.0°	14.0	17.0	18.0	25.0	10.0	3.60
			$10^3k_{H_2O}$			
25.0°	65.0	50.0	18.0	14.0	5.0	2.1
			$10^5k'_{H_2O}$			
10.0°	—	—	—	—	0.80	—
17.5°	—	—	—	—	2.40	—
25.0°	3.00	1.50	1.10	34.2	9.20	85.0
34.5°	7.53	4.60	3.78	91.5	—	203
43.5°	21.6	12.4	11.9	259	—	462
50.5°	41.7	27.3	22.6	405	—	775
			k_{OH^-}			
9.0°	6.40	5.50	0.220	—	—	—
10.0°	—	—	—	—	0.140	—
13.0°	8.00	6.20	0.340	—	—	—
17.5°	12.5	10.3	0.400	0.290	0.250	0.060
25.0°	18.0	16.0	0.800	0.520	0.450	0.100
32.5°	—	—	—	1.20	—	0.220
39.0°	—	—	—	2.10	—	0.300
			k_{-1}			
25.0°	7.30	3.14	25.5	59.8	50.0	133
			Q			
25.0°	0.274	0.795	0.0392	0.217	0.080	0.90
			$10^{-2}P$			
25.0°	0.23	0.44	0.66	6.07	8.8	92
			$10^3Q/P = 10^3k_2/k_3$			
25.0°	12	18	0.59	0.36	0.09	0.10

[a] Apparent first-order rate constants, k, in sec.$^{-1}$ for lactonization can be defined in terms of the various constants as:

$$k = k_{H^+}[H^+]f_{H_2C} + k_{H_2O}f'_{H_2C} + k'_{H_2O}f_{HC^-} = k_{H^+}[H^+]f_{H_2C} + \frac{Q + P[H^+]}{1 + P[H^+]}k_{-1}K_{a_1}'f_{H_2C} + k'_{H_2O}f_{HC^-}$$

where

$$\frac{Q + P[H^+]}{1 + P[H^+]} = \frac{(k_2 + k_3[H^+])}{k_1 + k_2 + k_3[H^+]}$$

$Q = k_2/(k_1 + k_2)$, and $P = k_3/(k_1 + k_2)$. The f'_{H_2C} is an artificial expression of the fraction undissociated consistent with the log k–pH profiles on the presumption of an apparent kinetic pK_{a_1}' different from the spectral pK_{a_1}' (Table V). $f_{H_2C} = [H^+]/([H^+] + K_{a_1}')$ and $f_{HC^-} = K_{a}'/([H^+] + K_{a_2}')$ in the pH region $< (pK_{a_1}' + pK_{a_2}')/2$ and $f_{HC^-} = [H^+]/([H^+] + K_{a_2}')$ in the pH region $> (pK_{a_1}' + pK_{a_2}')/2$. All rate constants are in l./mole-sec., except k_{H_2O} and k'_{H_2O} which are in sec.$^{-1}$. [b] The substituents are numbered with respect to the parent coumarins to avoid confusion. If numbered with respect to the coumarinic acid series, the numbers would have to be diminished by a unit, or α and β could be used for the coumarinic acids derived from 3- and 4-substituted coumarins, respectively. [c] Included for comparison; see *Reference 1*.

anion, HC^-, where

$$f_{HC^-} = \frac{[H^+]}{[H^+] + K_{a_2}'}, \qquad \text{(Eq. 22)}$$

for pH values $> (pK_{a_1}' + pK_{a_2}')/2$.

The dashed lines coming from the right in Figs. 8–10 show the decrease of the $k_{OH^-}[OH^-]$ term of Eq. 21 with decreasing pH. The equilibrium constant, K, for the monoanion and dianion–coumarin equilibria was defined (1) as

$$K = \frac{[C]_{eq.}}{[HC^-]_{eq.} + [C^{-2}]_{eq.}} = \frac{k'_{H_2O}f_{HC^-}}{k_{OH^-}[OH^-]} \qquad \text{(Eq. 23)}$$

where the subscript "eq." refers to the respective equilibrium concentrations and where the amount of undissociated acid, H_2C, is assumed to be negligible in the pH region where the coumarins exist in equilibrium with their nonlactonized open forms. Some values of this equilibrium constant at various pH values at 25° are given in Table VII.

Substituent Effects on Microscopic Rate Constants—Substituent Effects on Acid-Catalyzed Lactonization, k_{H^+}—It appears that any of the studied substituents in the α-position of coumarinic acid (*i.e.*, derived from coumarins substituted in the 3-position) accelerates hydrogen-ion-catalyzed lactonization (k_{H^+}, Table V). This contrasts with the steric inhibition of the rates of acid-catalyzed esterifications of *ortho*-substituted benzoic acids (9) but is consistent with the acceleration by similar *ortho*-substituents of the lactonization of 6- (or 3-) substituted 2- (hydroxymethyl-)benzoic acids (11).

Reactivity in acid-catalyzed ester formation is decreased by conjugation of the carbonyl group of the esterifiable carboxyl with aromatic rings or double bonds (13) and is a phenomenon similar to the decreased acidity of a conjugated carboxyl group by such resonance interaction (8). Space-filling substituents on adjacent carbons (as in α-substituted coumarinic acids) or on neighboring

Table VI—Arrhenius Energies of Activation, ΔE_a, and Entropies of Activation, $\Delta S‡$

	k_{H^+}	k'_{H_2O}	k_{OH^-}	k_{H^+}	k'_{H_2O}	k_{OH^-}
	ΔE_a(kcal.)[a]			$\Delta S‡$(e.u.)[b]		
3-Chloro	16.5	21.2	11.8	−9.0	−11.2	−15.0
3-Bromo	16.4	22.3	12.9	−8.8	−7.7	−15.0
3-Phenyl	15.3	23.2	10.6	−11.8	−5.2	−28.7
H[c]	17.8	22.8	13.2	−5.5	−3.0	−18.0
3-Methyl	12.6	19.1	16.3	−25.6	−12.4	−23.3
4-Methyl	13.9	17.3	14.8	−20.5	−16.9	−28.5

[a] ΔE_a is obtained from the slopes of the Arrhenius plots of log k *versus* $1/T$, where T is the absolute temperature. [b] Where $k = (kT/h)e^{\Delta S‡/R}e^{-\Delta H‡/RT}$, and $\Delta H‡ = \Delta E_a - 0.6$. [c] Included for comparison; see *Reference 1*.

Table VII—Equilibrium Constants[a] for Monoanion and Dianion–Coumarin Equilibria as Function of pH at 25°

pH	3-Chloro	3-Bromo	3-Phenyl	Coumarin	3-Methyl	4-Methyl
8.0	1.62	8.95×10^{-1}	13.5	2.10×10^{2}	6.16×10^{2}	8.50×10^{3}
9.0	1.28×10^{-1}	6.50×10^{-1}	1.15	1.83×10^{1}	4.10×10^{1}	8.33×10^{2}
9.5	2.86×10^{-2}	1.99×10^{-2}	2.70×10^{-1}	4.80	7.08	2.53×10^{2}
10.0	4.73×10^{-3}	1.69×10^{-3}	4.62×10^{-2}	9.60×10^{-1}	9.24×10^{-1}	7.06×10^{1}
10.5	5.8×10^{-4}	1.96×10^{-4}	4.05×10^{-3}	1.40×10^{-1}	1.04×10^{-1}	1.65×10^{1}
11.0	6.22×10^{-6}	2.03×10^{-5}	6.61×10^{-4}	1.70×10^{-2}	1.08×10^{-2}	2.80
12.0	6.03×10^{-8}	2.10×10^{-7}	7.15×10^{-6}	1.30×10^{-4}	1.09×10^{-4}	4.08×10^{-2}

[a] $$K = \frac{[C]_{eq.}}{[HC^-]_{eq.} + [C^{-2}]_{eq.}} = \frac{k'_{H2O} f_{HC^-}}{k_{OH^-}[OH^-]}$$

where $[C]_{eq.}$ is the equilibrium concentration of coumarin; $[HC^-]_{eq.}$ and $[C^{-2}]_{eq.}$ are the equilibrium concentrations of the coumarinate monoanion and dianion, respectively; and the fraction of the total coumarinic acid, $[HC^-] + [C^{-2}]$, as the monoanion is $f_{HC^-} = ([H^+]/[H^+] + K_{a_2}')$ and the fraction as the dianion is $f_{C^{-2}} = K_{a_2}'/([H^+] + K_{a_2}')$.

carbons (as in *ortho*-substituted benzoic acids) interfere with the coplanarity of carboxyl oxygens and the conjugated system, where such coplanarity is so necessary for resonance interaction. The result is to force these carboxyl oxygens out of the plane of the conjugated system, thus diminishing "the acid-weakening" resonance effect (8), and to make it more difficult to produce the alternative protonated resonant forms which serve to decrease the concentrations of protonated carboxyl forms necessary for the mechanistic sequence in acid-catalyzed esterification.

This steric effect that weakens resonance is not widely different from that which would be predicted (14) and is consistent with the order of reactivity for k_{H^+} (Table V), the rate constant for the acid-catalyzed lactonization of coumarinic acids derived from substituted coumarins: 3-methyl > 3-phenyl ~ 3-bromo > 3-chloro > H > 4-methyl. The 3-methyl and 3-phenyl compounds may be transposed from the prediction.

Substituent Effects on k_{-1}, the Rate Constant Representative of Both Degree of Cyclization of Coumarinate Monoanion (HC^-) and Rate of Proton Association of Resultant Anionic Orthoacid Intermediate ($HC\ddagger + H \rightarrow H_2C\ddagger$)—The observed sequence for k_{-1}, representative of the degree of cyclization of the coumarinate monoanion, HC^-, and the rate of association of the resultant orthoacid monoanion, $HC\ddagger$, with a proton to form the reactive orthoacid intermediate, $H_2C\ddagger$ [Schemes I and II and (1)], can be attributed mainly to inductive effects. The k_{-1} sequence (Table V) for the coumarinic acids derived from substituted coumarins is: 4-methyl > 3-methyl ≥ H > 3-phenyl ≫ 3-chloro > 3-bromo (where the double inequality represents a 10-fold greater magnitude). Assuredly, electron-withdrawing groups such as halogens in the α-position (derived from 3-substituted coumarins) would inhibit the phenate-anion attack on a more electronegative carboxyl carbon and also would increase the acidity of the resultant orthoacid ($H_2C\ddagger$) to result in lessened associations of the orthoacid anions ($HC\ddagger$) with protons. Both of these effects in the sequence of Schemes I and II would result in lessened amounts of reactive intermediate, $H_2C\ddagger$, with increasing electron-withdrawing substituents affecting the electronegativity of the carboxyls of the coumarinic acids.

Substituent Effects on k'_{H2O}, the Rate Constant for Lactonization of Coumarinate Monoanion—The sequence of reactivities for k'_{H2O} for coumarinate monoanions derived from the substituted coumarins is (Table V): 4-methyl > 3-methyl > H > 3-chloro > 3-bromo ≥ 3-phenyl and is not too different than the sequence for k_{-1}. This is reasonable since both rate constants may be affected by electron-withdrawing groups inhibiting the phenate-anion attack on a less positive carboxyl carbon (Schemes I and II). The subsequent step in the solvolytic process characterized by k'_{H2O}, the possible loss of hydroxyl ion from the resultant anionic orthoacid to give the respectively substituted coumarin (see Schemes I and II), will certainly be more favored by more electron-donating groups such as alkyls.

Substituent Effects on $Q/P = k_2/k_3$, the Ratios of Rate Constants of Dehydration, $H_2C\ddagger \xrightarrow[-H_2O]{k_2} C$, to Protonation, $H_2C\ddagger \xrightarrow[H^+]{k_3} H_3C\ddagger \xrightarrow{-H_3O^+}$ C, for Reactions of Orthoacid Reaction Intermediate—The magnitudes of these ratios decrease in the order: 3-bromo ≥ 3-chloro ≫ 3-phenyl > 3-methyl ≥ H ~ 4-methyl (Table V). This sequence is consistent with the fact that with constant rates of dehydration, the reaction pathway (Schemes I and II) dependent on the ease of protonation of the orthoacid, $H_2C\ddagger$, should increase with the greater electron-donating properties of substituents such as alkyls. The phenyl compound is slightly displaced from prediction in this sequence, but its lessened k_2/k_3 ratio may be rationalized. Although the phenyl substituent decreases the rate of protonation, k_3, of the orthoacid, $H_2C\ddagger$, the possible spontaneous dehydration, k_2, may be retarded by an intramolecular association of the carboxyl carbon's alcohol groups and the adjacent phenyl ring and thus minimize the k_2/k_3 ratio change.

Substituent Effects on k_{OH^-}, the Bimolecular Rate Constant for Hydroxyl-Ion-Catalyzed Solvolysis of Coumarins—These rate constants decrease in the order: 3-chloro ≥ 3-bromo ≫ 3-phenyl > 3-methyl ~ H > 4-methyl (Table V). The electron-withdrawing halogen and phenyl substituents facilitate the attack of hydroxyl ions on the coumarin carboxyl carbon, whereas electron-donating alkyl substituents are less favorable. Steric hindrance of this attack may be significantly important only for the 3-phenylcoumarin and could account for the relatively large decrease in its solvolytic rate constant (Table V). As has been already found for hydrolysis in dioxane (30%) and water mixtures (1), the 3-methyl derivative appears to hydrolyze faster than coumarin at 17.5 and 25.0°. However, it has the higher heat of activation; at lower temperatures, *e.g.*, < 15°, the 3-methylcoumarin is relatively much less susceptible to hydroxyl-ion attack.

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Sustained-Release Formulation of Prednisolone Administered Orally to Man

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Abstract □ A new method of formulation of prednisolone phosphate into a sustained-release tablet is presented. The sustained-release tablet consists of a porous plastic matrix in which the corticosteroid is dispersed. In the gastrointestinal tract, the active ingredient is gradually leached from the matrix and absorbed systemically. The small plastic skeleton is insoluble and is passed with the feces. *In vitro* experiments demonstrated the prolonged action of prednisolone phosphate in this form, and in man the sustained-release formulation gives a more uniform blood level of prednisolone over a longer time than prednisolone alcohol in conventional compressed tablets. The evidence for this is based on plasma prednisolone estimations and reduction of plasma cortisol in normal subjects. The sustained-release formulation also avoids high peaks of plasma prednisolone. Collection of matrixes from the feces showed that the bulk of the administered dose was released into the gastrointestinal tract.

Keyphrases □ Prednisolone phosphate sustained-release tablets—formulation □ Polyvinyl chloride matrix—prednisolone phosphate tablets □ Plasma levels—prednisolone phosphate sustained-release, nonsustained-release tablets □ Adrenocortical suppression—prednisolone phosphate dosage form comparison □ UV spectrophotometry—analysis

The therapeutic efficacy of corticosteroids has been established by years of clinical experience. The potential hazards of corticosteroid therapy are well known and well documented; they occur with prolonged usage, usually at high dosage. Numerous other corticosteroids have been developed, giving equal therapeutic effects with reduced dosage. However, to a large extent, prednisolone, which is one of the earliest and simplest derivatives of cortisol, has remained a standard corticosteroid used when oral dosage is required.

Treatment with prednisolone requires the administration of frequent doses, which can lead to peaking of plasma steroid concentrations and uneven blood levels of the steroid throughout the treatment. Therefore, a study was made of the effects in healthy volunteer subjects of a new sustained-release formulation of prednisolone, with particular reference to plasma prednisolone levels and to effects on the pituitary–adrenal axis as shown by reduction of plasma cortisol levels. The degree and duration of these effects produced by administration of the sustained-release formulation of prednisolone were compared with similar criteria after administration of the same amounts of prednisolone as conventional compressed tablets.

EXPERIMENTAL

Materials—*Sustained-Release Formulation*—The principle of the sustained-release formulation[1] is that the water-soluble prednisolone disodium phosphate is dispersed within a polyvinyl chloride framework or mesh in a compressed tablet. On contact with the gastrointestinal secretions, the prednisolone is gradually leached from the pores of the matrix and is absorbed systemically. The small plastic skeleton of the tablet is insoluble and is passed with the feces. This method of sustained release has the advantage that the formulation can be modified to provide various release patterns in order to achieve the optimal pharmacological action of the water-soluble active ingredient.

[1] The Duretter *R* sustained-release formulation is the invention of A. B. Hässle, Göteborg, Sweden, in collaboration with the Galenical Department of the Royal Pharmaceutical Institute, Stockholm, Sweden.

Table I—Sustained-Release Prednisolone Tablet Formulations

Composition	Formulation A (Milligrams per Tablet)	Formulation B (Milligrams per Tablet)	Formulation C (Milligrams per Tablet)
Prednisolone disodium phosphate BP (equivalent to 7.5 mg. of anhydrous prednisolone alcohol)	10.5	10.5	10.5
Insoluble tablet matrix	193.0	290.2	368.0
Binding agent	26.0	31.9	60.0
Lubricant	0.5	2.4	2.0
Solubilizing agent	20.0	—	—
Tablet weight	250.0	335.0	440.5

The particles of polyvinyl chloride are held together for compression by a binding agent. The porosity of the tablet matrix, upon which the rate of release of prednisolone depends, may be increased by the addition to the formula of a soluble substance. In the present work, sucrose was used.

The general principles involved in the sustained-release formulation have been described (1–5), and this method of achieving sustained release has been used in a number of experimental and proprietary tablets (6–8).

Volunteer Subjects—Ten healthy volunteers took part in one or more of the *in vivo* studies (tablet egestion, plasma prednisolone, and cortisol estimations). Seven of these subjects were male, age 22–42 years, and weighed 64–93 kg.; three were female, age 22–38 years, and weighed 64–82 kg. All tests were carried out under medical supervision.

Methods—*Measurement of* In Vitro *Release of Prednisolone from Sustained-Release Tablets*—For each tablet formulation, 20 tablets were placed in a 100-ml. beaker of 5.5-cm. diameter; 35 ml. of distilled water was added at 37°. The contents were stirred gently so that the tablets were continuously in motion. Temperature was maintained at 37 ± 1°. After 1 hr. ± 1 min., the liquid was transferred into a 100-ml. volumetric flask. The tablets were washed twice with separate 5-ml. quantities of distilled water and the washings were transferred to the flask. The prednisolone content of the solution was determined by UV spectrophotometry. A further volume of 35 ml. of distilled water at 37° was added to the tablets in the beaker, and the procedure was repeated after intervals of 1 hr. for 12 hr. and thereafter at 24 and 30 hr.

Measurement of Prednisolone Remaining in Egested Sustained-Release Tablets—The matrix of the sustained-release tablet was composed of polyvinyl chloride and did not disintegrate in the intestine. Tablet matrixes, therefore, were collected in the feces, washed, and assayed for residual prednisolone content by UV spectrophotometry after each individual tablet was ground up.

Table II—*In Vitro* Release of Prednisolone from Sustained-Release Tablet Formulations

Formulation	Cumulative Percentage Prednisolone Released 1 hr.	2 hr.	4 hr.	6 hr.
A	42.4	60.3	82.6	95.1
B	32.0	45.2	61.2	72.6
C	6.2	13.4	29.7	44.3

Table III—*In Vivo* Release of Prednisolone from Sustained-Release Tablets as Indicated by Percentage of Prednisolone Content Retained in Tablet Matrix

	Formulation A, Subjects				Formulation B, Subjects				Formulation C, Subject
	1	2	3	4	1	2	3	4	4
Percentage prednisolone content retained in sustained-release tablet matrix	3.9	10.8	21.5	27.3	14.9	28.5	33.0	52.4	60.3
	5.7	9.3	22.5	36.0	14.4	15.5	29.0	55.3	84.0
	3.5	7.2	7.1	12.0	11.7	N.C.	28.0	56.5	75.6
	5.1	8.3	8.3	17.3	13.6	N.C.	35.0	44.5	69.1
	3.5	6.1	6.9	14.1	13.7	N.C.	43.0	56.7	N.C.
	3.9	10.3	8.9	32.4	13.9	N.C.	42.5	N.C.	N.C.
	3.1	N.C.[a]	N.C.	N.C.	15.3	N.C.	34.0	N.C.	N.C.
	3.6	N.C.	N.C.	N.C.	15.5	N.C.	30.0	N.C.	N.C.
					12.9	N.C.	N.C.	N.C.	N.C.
					17.7	N.C.	N.C.	N.C.	N.C.
					13.3	N.C.	N.C.	N.C.	N.C.
					15.6	N.C.	N.C.	N.C.	N.C.
Mean	4.0	8.7	12.5	23.2	14.4	22.0	34.3	53.1	72.5
±*SE*	0.31	0.46	3.01	4.12	0.46	—	2.02	2.27	—
Number of tablets taken	8	8	8	8	12	12	12	12	12
Number of tablets recovered	8	6	6	6	12	2	8	5	4

[a] N.C. = not collected.

Plasma Prednisolone Estimations—A 20-ml. sample of venous blood was taken from each subject before administration of the prednisolone and at specific time intervals (1, 2, 3, 5, 7, 9, 12, and 24 hr.) after the corticosteroids had been taken. Blood samples were heparinized after collection and centrifuged to separate the plasma. Five milliliters of heparinized plasma was extracted with 25 ml. of methylene chloride; the extract was washed first with 2 ml. of 0.1 *N* sodium hydroxide, followed by 0.1 *N* acetic acid, and finally with water. The extract was evaporated to dryness, dissolved in a small volume of a mixture of methanol and methylene chloride, and applied to Whatman No. 1 chromatography paper. Chromatograms were run for 4 hr. in a benzene–methanol–water (100:50:50) system (9). The prednisolone area was located by UV light and eluted with methanol from the chromatogram. The eluate was taken to dryness and redissolved in 0.5 ml. of methylene chloride, and the steroid was estimated. The phenylhydrazine–sulfuric acid reagent (10) was used according to the technique of Peterson *et al.* (11). Standard amounts of prednisolone were taken through the whole procedure.

This method for measuring plasma prednisolone concentrations

Table IV—Plasma Prednisolone Levels (mcg./100 ml.) in Six Subjects after Taking 90-mg. Equivalent of Prednisolone Alcohol in a Single Dose of 12 Sustained-Release Tablets (7.5 mg.) in Formulation A, B, or C or 18 Conventional Compressed Tablets (5 mg.)

Subject	Sex	Zero	1 hr.	2 hr.	3 hr.	5 hr.	7 hr.	9 hr.	12 hr.	24 hr.
			Sustained-Release Prednisolone (Formulation A)							
			Blood Level, mcg./100 ml.							
3	M	0	28	—	82	62	44	22	14	0
6	F	0	82	—	104	90	76	64	30	6
5	F	0	58	—	64	63	51	38	30	4
4	M	0	44	—	54	20	22	12	8	16
7	M	0	63	—	70	64	33	24	10	5
1	M	0	50	—	29	—[a]	20	10	0	0
Mean		—	54.2	—	67.3	59.8	41.0	28.3	15.3	5.2
±*SE*		—	7.5	—	10.4	11.2	8.6	8.2	5.0	2.4
			Sustained-Release Prednisolone (Formulation B)							
			Blood Level, mcg./100 ml.							
3	M	0	48	—	49	44	—	37	48	4
6	F	0	46	—	62	71	54	39	33	16
5	F	0	44	—	38	22	45	38	28	0
4	M	0	53	—	44	34	20	19	3	4
7	M	0	69	—	44	56	29	0	0	0
1	M	0	21	—	36	36	28	29	14	5
Mean		—	46.8	—	45.5	43.8	35.2	27.8	21.0	4.8
±*SE*		—	6.4	—	3.8	7.3	5.6	5.7	7.6	2.4
			Sustained-Release Prednisolone (Formulation C)							
			Blood Level, mcg./100 ml.							
7	M	0	21	—	17	17	26	13	—	11
			Prednisolone in Conventional Compressed Tablets							
3	M	0	123	104	81	45	29	—	1	0
6	F	0	160	118	106	50	41	—	3	1
5	F	0	165	109	126	71	43	—	—	0
4	M	0	118	96	72	36	30	15	7	0
7	M	0	92	93	71	28	8	3	0	0
1	M	0	148	102	83	56	33	—	2	0
Mean		—	134.3	103.7	89.8	47.7	30.7	9.0	2.6	0.17
±*SE*		—	11.5	3.7	8.9	6.2	5.1	6.0	1.2	—

[a] Technical difficulties were experienced in the extraction of this sample.

Table V—Plasma Cortisol Levels (mcg./100 ml.) in Six Subjects after Taking 90-mg. Equivalent of Prednisolone Alcohol in a Single Dose of 12 Sustained-Release Tablets (7.5 mg.) in Formulation A, B, or C or 18 Conventional Compressed Tablets (5 mg.)

Subject	Sex	Control	Blood Levels (mcg./100 ml.) of Cortisol							
			1 hr.	2 hr.	3 hr.	5 hr.	7 hr.	9 hr.	12 hr.	24 hr.
Sustained-Release Prednisolone (Formulation A)										
3	M	11.7	5.1	—	1.7	0.3	2.0	0.6	1.4	3.1
4	F	15.1	4.3	—	1.1	0.0	0.0	0.0	0.0	3.5
5	F	9.0	4.7	—	3.0	1.7	1.7	1.7	2.0	4.3
6	M	11.3	3.2	—	2.6	1.3	0.0	1.7	0.0	8.1
7	M	8.9	2.7	—	0.5	1.1	0.0	0.0	0.3	1.4
8	M	12.3	3.3	—	1.8	1.3	0.5	0.0	0.0	7.7
Mean		11.4	3.90	—	1.80	0.95	0.70	0.66	0.60	4.7
±*SE*		0.86	0.39	—	0.37	0.26	0.24	0.34	0.39	0.11
Sustained-Release Prednisolone (Formulation B)										
3	M	16.3	4.4	—	1.6	2.5	2.2	2.2	1.9	15.9
4	F	30.0	14.3	—	5.4	5.0	2.5	4.3	2.5	3.9
5	F	10.6	5.0	—	3.2	2.9	2.6	2.6	2.4	2.4
6	M	23.9	6.6	—	3.8	2.1	3.8	2.4	2.9	26.7
7	M	12.3	6.8	—	4.2	3.5	4.5	4.8	4.2	7.7
8	M	28.0	6.0	—	1.7	3.0	1.3	1.0	0	15.7
Mean		20.10	7.10	—	3.36	3.10	2.80	2.80	2.30	12.00
±*SE*		3.4	1.5	—	0.60	0.43	0.47	0.57	0.47	3.72
Sustained-Release Prednisolone (Formulation C)										
7	M	17.5	7.0	—	2.1	0.7	0.4	0.7	—	5.6
Prednisolone in Conventional Compressed Tablets										
3	M	18.6	6.1	3.9	2.5	2.1	1.8	—	—	16.1
4	F	24.5	5.8	5.2	6.4	3.0	2.4	—	—	4.8
5	F	15.0	5.3	4.7	3.8	3.1	1.9	—	—	8.8
6	M	7.3	3.5	2.7	2.4	4.6	—	—	—	20.4
7	M	8.3	3.7	4.0	4.0	3.0	—	—	—	11.3
8	M	26.9	11.2	6.5	3.5	3.1	3.8	—	—	28.1
Mean		16.70	15.90	4.50	3.80	3.20	2.40	—	—	14.90
±*SE*		3.20	1.10	0.50	0.57	0.33	0.38	—	—	3.46

was used by Jenkins and Sampson (12); it measures the level following the oral administration of 90 mg. of prednisolone but is not sensitive enough to determine the concentrations achieved following normal therapeutic doses (5–15 mg.).

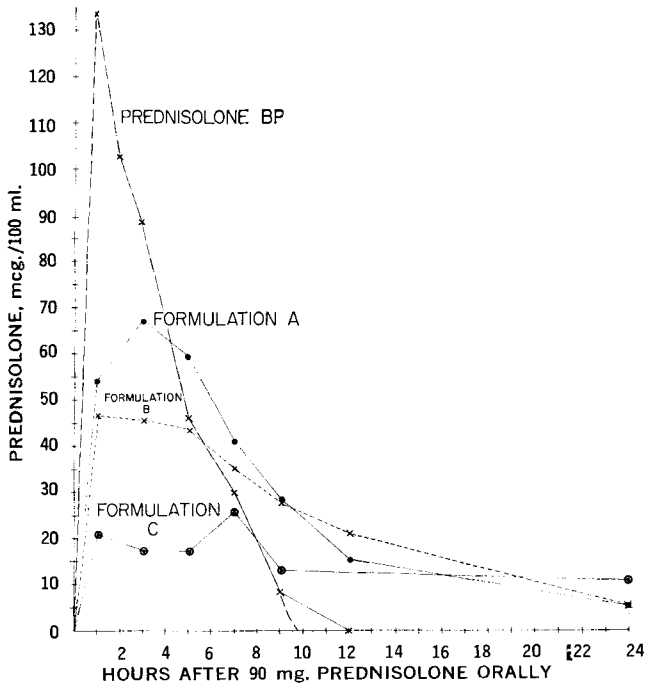

Figure 1—*Comparative plasma prednisolone levels in six subjects after taking 90-mg. equivalent of prednisolone alcohol in three sustained-release formulations, A, B, or C, and conventional compressed tablets. Key:* ×—×, *prednisolone BP compressed tablets;* ●—●, *Formulation A;* ×- - -×, *Formulation B; and* ⊙—⊙, *Formulation C.*

Plasma Cortisol Estimations—A fluorometric technique (13) was used to measure cortisol in plasma without interference from cortisone or other metabolites.

RESULTS

Effect of Formulation on *In Vitro* and *In Vivo* Release of Prednisolone—Three sustained-release tablet formulations (A, B, and C) were investigated in this study; their compositions are shown in Table I. *In vitro* release rates of prednisolone were determined for each formulation (Table II). It was apparent that the *in vitro* release rates of prednisolone from sustained-release tablet Formulation C would be too slow, since the matrix would be past the ileocecal junction within 6 hr., and that the choice of an acceptable tablet probably lay between Formulations A and B.

In the *in vivo* studies, four volunteers each took eight sustained-release tablets of Formulation A and at a later time 12 sustained-release tablets of Formulation B while one subject took 12 tablets of Formulation C. Egested tablet matrixes were collected from their feces. A total of 32 tablets of Formulation A was taken, of which 26 were egested; 48 tablets of Formulation B were taken, of which 27 were egested; and 12 tablets of Formulation C were taken, of which four were egested. In these and other related studies, there was evidence that some of the matrixes were present in the feces as small particles; it was not possible to collect these and measure their prednisolone content with any degree of accuracy. This accounts for the discrepancy of some matrixes uncollected in the present study.

The amount of residual prednisolone in each recovered matrix is shown in Table III. Results are expressed as percentages of the original prednisolone content of the tablets. The amount of prednisolone remaining in the recovered tablet matrixes of Formulation C was 72.5%, and that of Formulation B was 28.0 ± 2.35 (*SE*) %. Formulation A had the smallest residuum of prednisolone: 11.5 ± 2.48 (*SE*) %.

Plasma Prednisolone Levels in Man following Administration of Sustained-Release Tablets—The concentration of prednisolone in plasma (mcg./100 ml.) at specific intervals following the oral administration in six subjects of 12 sustained-release tablets at one time (equivalent to 90 mg. of prednisolone alcohol) of Formulation A, B, or C, or of 18 conventional compressed tablets, each of 5 mg

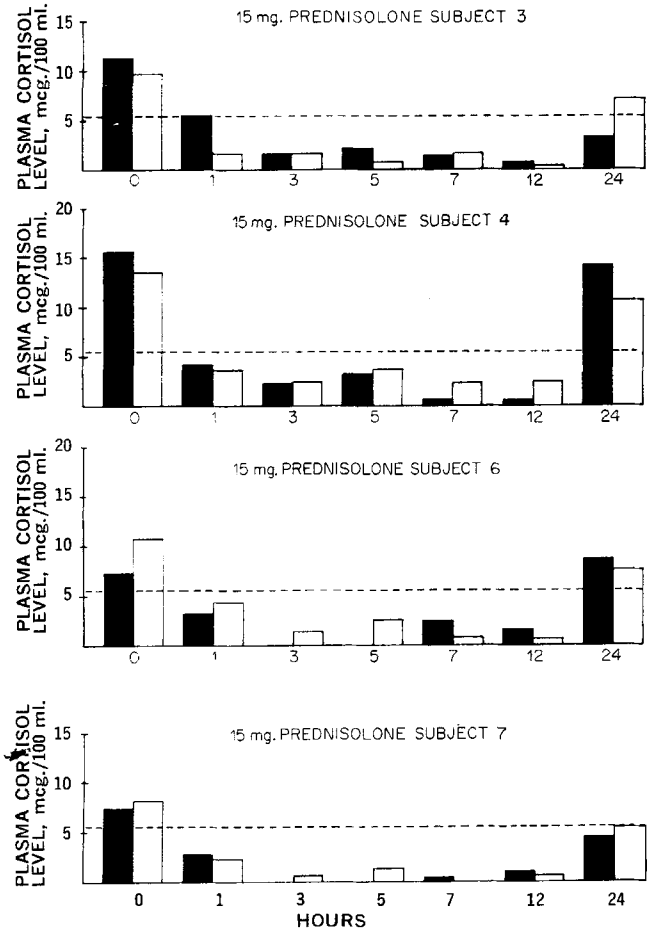

Figure 2—*Plasma cortisol levels (mcg./100 ml.) in four subjects after taking 15-mg. equivalent of prednisolone alcohol in sustained-release tablets (black columns) or conventional compressed tablets (open columns). The broken line at 5.5 mcg./100 ml. represents the lower limit for the range of normal blood cortisol levels.*

prednisolone alcohol, is shown in Table IV. Each subject received both sustained-release Formulations A and B, and one subject also received Formulation C. The conventional compressed tablet and the three sustained-release treatments were given in random sequence. An interval of 14 days was allowed between administrations to permit full recovery of adrenocortical function. The large dose of prednisolone was chosen so that levels in the blood could be measured readily by the method used.

Comparative plasma prednisolone levels produced by the three tablet formulations (sustained-release and conventional compressed tablets) are shown in Table IV. The data are presented graphically in Fig. 1, from which it can be seen that Formulation A gave the most desirable blood levels.

The peaking of plasma prednisolone shown for 3 hr. after conventional tablet administration was almost absent with sustained-release Formulation A. But the sustained-release formulation produced an appreciable plasma prednisolone level for longer than conventional compressed tablets; the increase in duration was greater than twofold. The two calculated regression lines so obtained for Formulation A were $r = -0.786$, $m = -0.0578$, and $c = 1.881$, and for conventional tablets $r = -0.9216$, $m = -0.099$, and $c = 2.116$. These differed significantly in gradient ($t = 4.43$; $p < 0.001$). It is apparent, therefore, that the difference in blood levels with time with these two formulations are statistically different.

Plasma Cortisol Levels in Man following Administration of Sustained-Release Tablets—It was expected that the high dose of prednisolone (equivalent to 90 mg. prednisolone alcohol) taken in the previous study would depress the pituitary release of corticotrophin and suppress the adrenocortical secretion. The blood samples taken from the volunteers in the previous study on plasma prednisolone levels were, therefore, also examined for their cortisol

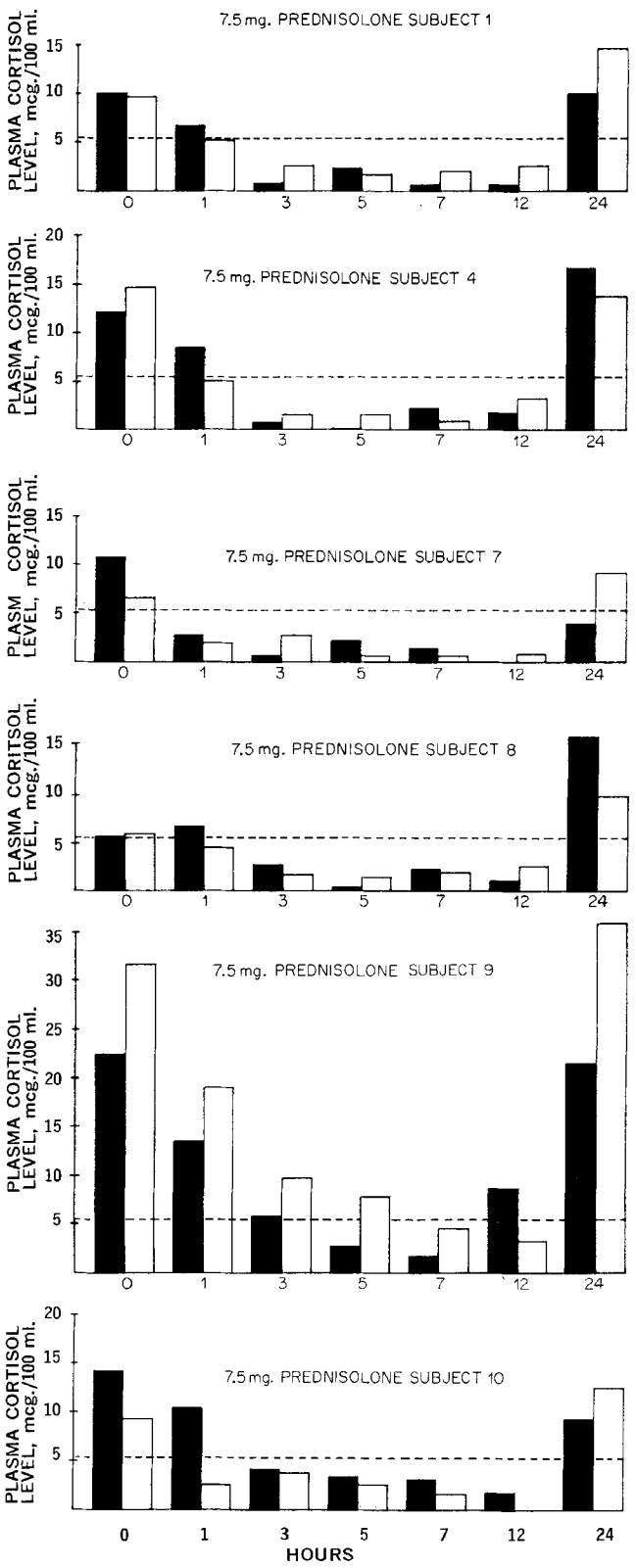

Figure 3—*Plasma cortisol levels (mcg./100 ml.) in six subjects after taking 7.5-mg. equivalent of prednisolone alcohol in sustained-release tablet formulation (black columns) or conventional compressed tablets (open columns). The broken line at 5.5 mcg./100 ml. represents the lower limit for the range of normal blood cortisol levels.*

level to determine the degree and duration of suppression produced by the two tablet formulations (sustained-release and conventional compressed tablets). Table V shows the plasma cortisol level for

each of the six subjects at specific times after taking 90 mg. of prednisolone in each of the three sustained-release formulations and after the same dose of conventional compressed tablets.

Blood cortisol levels of below 5.5 mcg./100 ml. were taken as evidence of adrenocortical suppression[2]. The range of normal blood cortisol levels is 5.5–23.5 mcg./100 ml.; wide individual and day-to-day variations within these limits were considered acceptable.

All four tablet formulations of prednisolone suppressed the adrenocortical secretion, as evidenced by a fall in plasma cortisol levels, in all six subjects. Plasma cortisol levels at 24 hr. were lower with the sustained-release formulations than corresponding levels with the conventional tablets in the six subjects. A very high dose of 90 mg. was given and, in consequence, a high level of prednisolone was maintained for a long time with the sustained-release preparation. The longer duration of adrenocortical suppression with the sustained-release tablet was thus a result of its prolonged action and supports the other evidence for such an action.

In these studies, the doses of prednisolone given were so enormous that it was unreasonable to correlate plasma prednisolone levels with the degree of cortisol suppression. It was, therefore, decided that the relative adrenocortical suppressant effects of a sustained-release formulation and conventional compressed prednisolone tablets should be investigated. The suppressant effects of Formulation A, as the most promising sustained-release preparation, were compared with conventional compressed tablets at therapeutic or near therapeutic dose levels (*i.e.*, 15 and 7.5 mg. prednisolone alcohol equivalents) at specific times after ingestion of the corticosteroid.

The results of these two latter studies on four and six subjects, respectively, are presented graphically in Figs. 2 and 3. Comparative results for the two formulations were drawn as histograms, showing plasma cortisol levels for the individual subjects.

At the 15-mg. level, both formulations of prednisolone suppressed adrenocortical secretion, as shown by the fall in plasma cortisol levels (Fig. 2). At 24 hr., Subject 3 showed adrenocortical suppression with the sustained-release preparation but not with the conventional tablets; Subjects 4 and 6 showed plasma cortisol levels within the normal range (5.5–23.5 mcg./100 ml.) for both formulations, while Subject 7 showed adrenocortical suppression with both formulations at 24 hr.

At the 7.5-mg. dosage level (Fig. 3), both formulations suppressed adrenocortical secretion as evidenced by a fall in plasma cortisol levels in all six subjects. However, at 24 hr., only one subject (Fig. 3, Subject 7) showed a blood cortisol level below 5.5 mcg./100 ml. and this was with the sustained-release preparation.

Adrenocortical suppression caused by therapeutic or near therapeutic doses of the sustained-release preparation was no greater than that produced by the same doses of prednisolone administered in conventional compressed tablets, but it was of longer duration.

DISCUSSION

A great disadvantage with conventional methods of preparing oral dosage forms has been that many of the drugs often rapidly attain a high blood concentration; side effects are, therefore, common when dosage is increased only slightly above the normal level. Thus, one goal of current pharmacological and pharmaceutical research is to produce a more even and prolonged absorption and blood level of drugs. In some instances, this can be achieved by formulating the active substance into oral preparations giving sustained release; peaking of drug plasma levels are avoided and the incidence of side effects can be reduced (1, 3, 6).

Studies of drug-release rates from plastic matrixes have been made by a number of investigators. In particular, techniques for the investigation of factors influencing *in vitro* release rates have been described (14–17), and comprehensive investigations of *in vivo* blood levels of drugs administered in sustained-release tablets of the plastic matrix type have been made (18). It has been shown that the average effective blood level of a drug administered by either a conventional compressed tablet or by a sustained-release tablet was approximately the same. However, while the conventional tablet produced variations from nil to twice the average level, the sustained-release tablet administered three times a day maintained the blood level of the drug within $\pm 20\%$ of the average throughout a 24-hr. period, and within $\pm 50\%$ of the average when taken twice a day.

Evidence from the literature suggests that the formulation of corticosteroids in sustained-release tablets may reduce the incidence of side effects. Studies, both in dogs and in man (19), showed that a very simple sustained-release formulation of prednisolone was less likely to depress the pituitary–adrenal axis than was an equal dosage of prednisolone formulated as a conventional compressed tablet. Also, although the plasma 17-hydroxycorticosteroid levels did not reach as high a peak with the sustained-release preparation as with the conventional tablet, they were more uniform and of greater duration. Furthermore, these authors reported that the total amount of 17-hydroxycorticosteroid that was available to the human subjects in the 24-hr. period following the administration of the sustained-action dosage form was equivalent to that available following administration of conventional compressed tablets of the steroid at the same dosage.

A number of workers investigated the possibilities of intermittent corticosteroid dosage with the object of minimizing side effects (20–22). Dubois and Adler (23), while accepting the advantages of alternate-day administration, advocated the use of a long-acting preparation of methylprednisolone in daily doses in the treatment of rheumatoid arthritis and other collagen disorders. The long-acting preparation was more effective and had a lower incidence of side effects than did intermittent administration of methylprednisolone in conventional tablets.

The results of the present study suggest that prednisolone in this form should have a reduced incidence of side effects. Obviously, this is a matter for clinical determination; however, the main indication to support this suggestion is that following the administration of sustained-release tablets to volunteers at a dosage equivalent to 90 mg. of prednisolone alcohol, there was an appreciable and relatively uniform plasma prednisolone level for significantly longer than with equivalent dosage given in conventional compressed tablet form. The peaking of plasma prednisolone level shown for 3 hr. after conventional tablet administration was almost absent with the sustained-release formulation. Furthermore, adrenocortical suppression caused by therapeutic or near therapeutic doses of the sustained-release preparation was no greater than that produced by the same dose of prednisolone given in conventional tablets. Release of the prednisolone from the plastic matrix was demonstrated by the initial egestion studies.

Initial clinical trials (24) in patients with rheumatoid arthritis or asthma have suggested that a reduced dosage of prednisolone in sustained-release form would control symptoms as well as conventional doses in compressed tablet form.

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Behavior of Erythrocytes in Phosphate Buffer Systems

JANIS R. PHILLIPS* and DONALD E. CADWALLADER

Abstract □ Hemolytic behavior of human erythrocytes in sodium and potassium phosphate buffer systems was investigated. The data were used to calculate *hemolytic i* values for the buffer components at various pH values. The experimental *i* values were lower than those predicted by a theoretical equation, and the deviations were attributed to changes in pH causing alterations in the permeability of the red cell membrane to the anions and/or cations in solution or changes in the red cell contents. Alkaline solutions appear to be more favorable environments for the red blood cell under the conditions studied. The increased osmotic fragility (low *hemolytic i* values) at lower pH values was attributed to an increase in the osmotic activity of the cell contents and subsequent movement of water into the cell.

Keyphrases □ Erythrocytes, hemolysis—phosphate buffer solutions □ Isotonic coefficients, phosphate buffer solutions—erythrocytes hemolysis □ Hemolysis curves—phosphate buffer solutions □ Osmotic fragility, erythrocytes—low pH effect

Phosphate buffer systems are used in parenteral solutions primarily to stabilize the active ingredient against chemical degradation. Those buffer systems employed should normally have as low a buffer capacity as possible so that body buffer systems will not be significantly disturbed when the solution is injected. Phosphate buffers are routinely used in the compounding of intravenous solutions when adjustments between pH 6 and 8 are needed.

The purposes of this investigation were to study the effect of phosphate buffering agents on red blood cells and to determine tonicity values based on measurements of fragility of human red cells in various phosphate buffer systems. The hemolytic method was employed, and isotonic coefficients were calculated by comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium and potassium phosphate buffer solutions. Experiments were designed to determine isotonic coefficients of buffer components at various pH values.

EXPERIMENTAL

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of a 22-year-old male Caucasian donor. Fresh blood samples were used in all experiments. Approximately 10 ml. of blood was obtained from the donor and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min. and then the blood was decanted into a 50-ml. conical flask and aerated by swirling the flask gently for about 5 min.

Preparation of Buffer Solutions and Determination of pH—All chemicals employed were reagent grade quality, and distilled water was used to prepare all solutions. Stock solutions were prepared (approximately 0.133 *M*); from these, quantitative amounts were taken in the desired ratios and diluted with water to produce 50-ml. samples. The pH was checked using a pH meter (Corning model 7).

Quantitative Determination of Percent Hemolysis—The hemolytic method was used in each experiment to determine the extent of hemolysis of erythrocytes in the phosphate buffer solutions. This method is a quantitative one, being based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed. Into each of two test tubes were transferred 5 ml. of the standard sodium chloride solution (0.06, 0.062 ... 0.07, 0.072 *M*) and 5 ml. of the buffer system being tested. After the test tubes were brought to a constant temperature by being placed in a water bath (37 ± 0.5°), 0.05 ml. of blood was pipeted into each tube. The tubes were then inverted several times to ensure thorough mixing and allowed to remain 45 min. at 37°. After centrifuging, the absorbance of the supernatant liquid was measured using a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter.

To find the percent hemolysis, the absorbance readings were divided by the absorbance readings for 0.05 ml. of blood in 5 ml. of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml. of blood in 5 ml. of 0.9% sodium chloride solution, was used to cancel any light absorbance inherent to the blood sample. Both the standard and the blank were subjected to the same conditions of standing for 45 min. at 37° followed by centrifuging.

Calculation of *i* Values—Through the use of the hemolytic method, concentrations of sodium chloride and the buffer solutions giving the same degree of hemolysis could be determined. Knowledge of these concentrations made it possible to calculate isotonic coefficients (*i* values) through the use of the following equation:

$$\begin{pmatrix} i \text{ value for} \\ \text{NaCl in water} \end{pmatrix} \begin{pmatrix} \text{molar concentration of} \\ \text{NaCl causing} \\ 25\% \text{ hemoly is} \end{pmatrix} = \begin{pmatrix} i \text{ value of} \\ \text{buffer components} \\ \text{in solution} \end{pmatrix} \begin{pmatrix} \text{molar concentration} \\ \text{of buffer solution} \\ \text{causing } 25\% \\ \text{hemolysis} \end{pmatrix} \quad \text{(Eq. 1)}$$

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.154 *M* (0.9%) sodium chloride in water (1).

Curves showing the degree of hemolysis in sodium chloride-water solutions and phosphate buffer solutions were plotted on

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Behavior of Erythrocytes in Phosphate Buffer Systems

JANIS R. PHILLIPS* and DONALD E. CADWALLADER

Abstract □ Hemolytic behavior of human erythrocytes in sodium and potassium phosphate buffer systems was investigated. The data were used to calculate *hemolytic i* values for the buffer components at various pH values. The experimental *i* values were lower than those predicted by a theoretical equation, and the deviations were attributed to changes in pH causing alterations in the permeability of the red cell membrane to the anions and/or cations in solution or changes in the red cell contents. Alkaline solutions appear to be more favorable environments for the red blood cell under the conditions studied. The increased osmotic fragility (low *hemolytic i* values) at lower pH values was attributed to an increase in the osmotic activity of the cell contents and subsequent movement of water into the cell.

Keyphrases □ Erythrocytes, hemolysis—phosphate buffer solutions □ Isotonic coefficients, phosphate buffer solutions—erythrocytes hemolysis □ Hemolysis curves—phosphate buffer solutions □ Osmotic fragility, erythrocytes—low pH effect

Phosphate buffer systems are used in parenteral solutions primarily to stabilize the active ingredient against chemical degradation. Those buffer systems employed should normally have as low a buffer capacity as possible so that body buffer systems will not be significantly disturbed when the solution is injected. Phosphate buffers are routinely used in the compounding of intravenous solutions when adjustments between pH 6 and 8 are needed.

The purposes of this investigation were to study the effect of phosphate buffering agents on red blood cells and to determine tonicity values based on measurements of fragility of human red cells in various phosphate buffer systems. The hemolytic method was employed, and isotonic coefficients were calculated by comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium and potassium phosphate buffer solutions. Experiments were designed to determine isotonic coefficients of buffer components at various pH values.

EXPERIMENTAL

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of a 22-year-old male Caucasian donor. Fresh blood samples were used in all experiments. Approximately 10 ml. of blood was obtained from the donor and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min. and then the blood was decanted into a 50-ml. conical flask and aerated by swirling the flask gently for about 5 min.

Preparation of Buffer Solutions and Determination of pH—All chemicals employed were reagent grade quality, and distilled water was used to prepare all solutions. Stock solutions were prepared (approximately 0.133 *M*); from these, quantitative amounts were taken in the desired ratios and diluted with water to produce 50-ml. samples. The pH was checked using a pH meter (Corning model 7).

Quantitative Determination of Percent Hemolysis—The hemolytic method was used in each experiment to determine the extent of hemolysis of erythrocytes in the phosphate buffer solutions. This method is a quantitative one, being based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed. Into each of two test tubes were transferred 5 ml. of the standard sodium chloride solution (0.06, 0.062 ... 0.07, 0.072 *M*) and 5 ml. of the buffer system being tested. After the test tubes were brought to a constant temperature by being placed in a water bath (37 ± 0.5°), 0.05 ml. of blood was pipeted into each tube. The tubes were then inverted several times to ensure thorough mixing and allowed to remain 45 min. at 37°. After centrifuging, the absorbance of the supernatant liquid was measured using a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter.

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Calculation of *i* Values—Through the use of the hemolytic method, concentrations of sodium chloride and the buffer solutions giving the same degree of hemolysis could be determined. Knowledge of these concentrations made it possible to calculate isotonic coefficients (*i* values) through the use of the following equation:

$$\begin{pmatrix} i \text{ value for} \\ \text{NaCl in water} \end{pmatrix} \begin{pmatrix} \text{molar concentration of} \\ \text{NaCl causing} \\ 25\% \text{ hemoly is} \end{pmatrix} = \begin{pmatrix} i \text{ value of} \\ \text{buffer components} \\ \text{in solution} \end{pmatrix} \begin{pmatrix} \text{molar concentration} \\ \text{of buffer solution} \\ \text{causing } 25\% \\ \text{hemolysis} \end{pmatrix} \quad \text{(Eq. 1)}$$

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.154 *M* (0.9%) sodium chloride in water (1).

Curves showing the degree of hemolysis in sodium chloride-water solutions and phosphate buffer solutions were plotted on

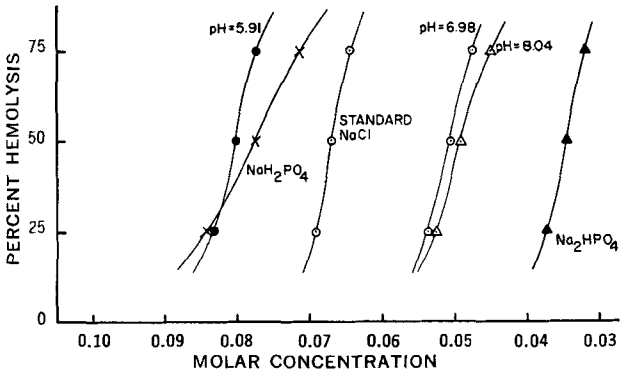

Figure 1—*Hemolysis of human erythrocytes after 45 min. at 37° in various NaH_2PO_4–Na_2HPO_4 buffer solutions.*

rectangular coordinate graph paper. From these curves, it was possible to determine the molar concentrations of sodium chloride and the buffer solutions causing 25, 50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of i for the particular phosphate buffer solution at concentrations producing 25, 50, and 75% hemolysis. The various i values for the buffer solutions tested in this study are shown in Tables I and II.

Preparation of Hemolysis Curves—Approximately 20 experiments employing human blood were performed. Standard hemolysis curves (Figs. 1 and 2) were constructed from the average readings of these experiments. Hemolysis curves for the various phosphate buffer solutions were constructed using the i values previously calculated (Tables I and II). Through a rearrangement of Eq. 1:

$$\begin{pmatrix}\text{molar concentration of}\\ \text{buffer components}\\ \text{in solution causing}\\ 25\%\text{ hemolysis}\end{pmatrix} = \frac{\begin{pmatrix}1.86\ i\text{ value for}\\ \text{NaCl in water}\end{pmatrix}\begin{pmatrix}\text{molar concentration of NaCl}\\ \text{causing 25\% hemolysis}\end{pmatrix}}{\begin{pmatrix}i\text{ value for buffer}\\ \text{components}\end{pmatrix}} \quad \text{(Eq. 2)}$$

the molar concentration of the buffer components in solution causing 25% hemolysis was calculated. Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various phosphate buffer solutions were constructed (Figs. 1 and 2).

THEORETICAL

The van't Hoff i is a correction factor used to account for the deviations of electrolytes and nonelectrolytes in solution from the laws of ideal solution. The isotonic coefficient, van't Hoff i, may be defined as the ratio of the colligative effect produced by a given molality of an electrolyte to the effect observed for the same molality of a nonelectrolyte (2). The term i as used for nonelectrolytes and electrolytes in solution indicates the degree of dissociation. In solutions of electrolytes, the value of i is determined principally by the

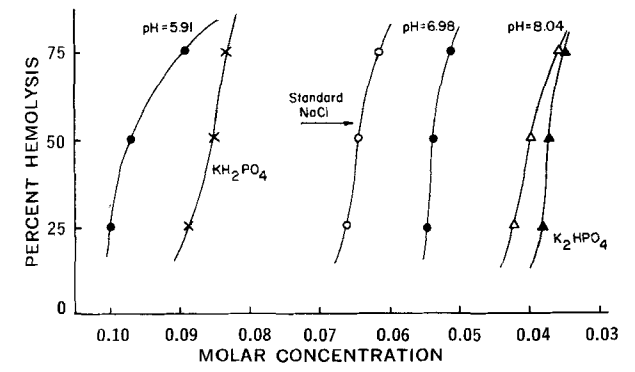

Figure 2—*Hemolysis of human erythrocytes after 45 min. at 37° in various KH_2PO_4–K_2HPO_4 buffer solutions.*

Table I—Values of i for Sodium Phosphate–Sodium Acid Phosphate Buffer Components, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes[a]

Buffer Solution	Hemolysis, % 25	50	75	Average
Sodium phosphate:				
pH = 8.8	3.42	3.43	3.52	3.46
pH = 8.04	2.54	2.40	2.56	2.50
pH = 6.98	2.38	2.38	2.48	2.41
pH = 5.91	1.48	1.43	1.53	1.48
Sodium acid phosphate:				
pH = 4.8	1.46	1.52	1.58	1.52

[a] Each i value represents an average of at least two blood samples.

number of ions into which the molecule can dissociate and the charge on these ions. At infinite dilution, the value of i is identical with the number of ions into which a molecule of the solute can dissociate.

The Debye–Hückel limiting law states that the departure from ideal behavior in a given solvent is governed by the ionic strength of the medium and the valences of the ions of the electrolyte but is independent of their chemical nature (3). The valence type of the electrolyte should thus be the essential factor in determining the activity coefficient at a given ionic strength. For a solution containing more than one electrolyte, the overall i value of the components in solution should be governed by the proportional amount of each individual electrolyte. The overall i value (i_{sum}) of two components (electrolytes A and B) in solution would be described by the equation:

$$i_{sum} = i_A f_A + i_B f_B \quad \text{(Eq. 3)}$$

where i_A and i_B represent the i values of A and B determined in solutions having approximately the same molar strength as the mixture of electrolytes; f_A and f_B are the fractional amounts of A and B, respectively, of the total molar concentration and $f_A + f_B = 1$. Then:

$$i_{sum} = f_A(i_A - i_B) + i_B \quad \text{(Eq. 4)}$$

and a straight line should result when the i_{sum} values of the components in solution are plotted against the fraction of one of the components. For Eqs. 3 and 4 to be valid, there must be no chemical reaction between the electrolytes and no additional significant ionic interactions between the ions of the individual components.

Using vapor-pressure osmometer data, Cutie and Sciarrone (4) determined the NaCl equivalents of sodium acid phosphate and sodium phosphate and tonicity values of phosphate buffer mixtures (Sorensen buffer) at 37°. When these values were converted to their corresponding i values and plotted against the proportional amounts of salts in solution (NaH_2PO_4/Na_2HPO_4), a straight line resulted, showing excellent agreement with Eq. 4 (Fig. 3).

Equation 3 would be valid for activity coefficients calculated from physicochemical data such as vapor-pressure changes and freezing-

Table II—Values of i for Potassium Phosphate–Potassium Acid Phosphate Buffer Components, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes[a]

Buffer Solution	Hemolysis, % 25	50	75	Average
Potassium phosphate:				
pH = 8.6	3.33	3.42	3.32	3.35
pH = 8.04	3.07	2.89	2.96	2.97
pH = 6.98	2.20	2.16	2.08	2.15
pH = 5.91	1.38	1.43	1.38	1.40
Potassium acid phosphate:				
pH = 4.5	1.24	1.25	1.24	1.24

[a] Each i value represents an average of at least two blood samples.

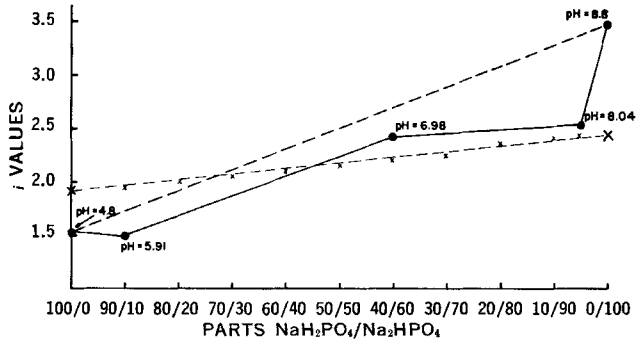

Figure 3—*Relationship between buffer composition and* i *values of* NaH_2PO_4–Na_2HPO_4 *buffer solutions. Key: - - -, theoretical line between* hem-i *values of* NaH_2PO_4 *and* Na_2HPO_4*; ——, experimental* hem-i *values; and* ×- -×*, theoretical line between* i *values of* NaH_2PO_4 *and* Na_2HPO_4 [i *values calculated from sodium chloride equivalents determined from vapor-pressure osmometer data (4)]. The small* ×*'s represent points calculated from tonicity values determined from vapor-pressure osmometer data (4).*

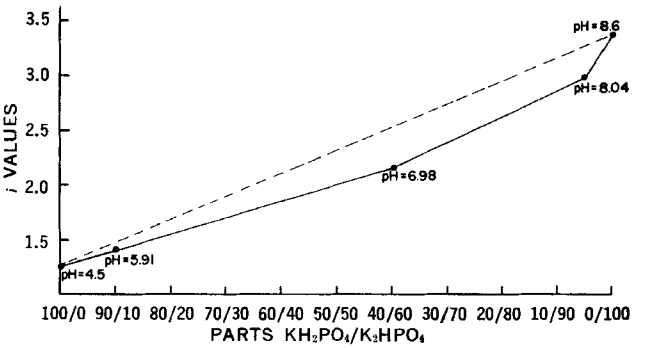

Figure 4—*Relationship between buffer composition and* i *values of* KH_2PO_4–K_2HPO_4 *buffer solutions. Key: - - -, theoretical line between* hem-i *values of* KH_2PO_4 *and* K_2HPO_4*; and ——, experimental* hem-i *values.*

point depression. For Eq. 3 to be extended to *i* values determined from data based on the ability of a substance to hemolyze red blood cells, an additional necessary requirement would be that the components in solution have no additional effect on the red blood cell than either of them alone in solution. Then Eq. 3 could be written:

$$hem\text{-}i_{sum} = hem\text{-}i_A f_A + hem\text{-}i_B f_B \qquad \text{(Eq. 5)}$$

where $hem\text{-}i_{sum}$ is the overall *hemolytic i* value for electrolytes *A* and *B*, and $hem\text{-}i_A$ and $hem\text{-}i_B$ are the *hemolytic i* values of *A* and *B* determined in solutions having approximately the same molar strength as the mixture of electrolytes. If $hem\text{-}i_A$ and $hem\text{-}i_B$ are determined for *A* and *B*, then Eq. 5 predicts that a straight line can be drawn between these *hem-i* values as shown in Fig. 3, and that any point on this line relates the $hem\text{-}i_{sum}$ to the proportional amounts of *A* and *B* in solution. If this line is the same as the line obtained using *i* values (determined from vapor-pressure or freezing-point data), then it could be assumed that the red cell membrane is acting as a perfect osmometer and the components in solution do not affect the membrane structure or the cell's contents.

A different straight line would result if either or both of the *hem-i* values were appreciably different than the *i values* (from colligative properties). Higher or lower *hem-i* values could be attributed to either the selective permeability of the red cell membrane to the anions and/or cations in solution, or to the alteration of integrity of the cell by some action of the substance on the erythrocyte membrane. If the experimental data do not fall on the predicted straight line drawn between $hem\text{-}i_A$ and $hem\text{-}i_B$, then the irrational behavior can be attributed to some change in the solvent environment brought about by mixing the electrolytes or to some unusual effect on the cell membrane and/or cell contents by the mixture. In the case where the electrolytes are buffer components, the main environmental change would be one of pH.

RESULTS AND DISCUSSION

When blood was added to 0.09–0.03 *M* phosphate buffer solutions, typical sigmoidal hemolysis curves resulted (Figs. 1 and 2). These curves were constructed in the manner described in the *Experimental* section of this report, utilizing the data presented in Tables I and II.

Tables I and II list the calculated values of *hem-i* for sodium and potassium phosphate buffer systems at various pH values. The *hem-i* values for sodium and potassium phosphates *per se* were higher than expected, whereas the *hem-i* values for the sodium and potassium acid phosphates were lower than their theoretical value of 2. As expected, the *hem-i* values of the various buffer solutions were between the values of their acid and basic components. Sodium phosphate had an unusually higher *hem-i* value (3.46) compared to the *i* value determined from vapor-pressure data (2.43). When the pH was changed from 8.8 to 8.04 by a small change in the NaH_2PO_4/Na_2HPO_4 ratio from 0/100 to 5/95, there was a dramatic decrease in the *hem-i* value. A similar type of behavior was not exhibited by the potassium salts, although there was some decrease in the *hem-i* value.

The experimental *hem-i* values were lower than those predicted by Eq. 5 for both sodium and potassium phosphate buffer systems (Figs. 3 and 4). Apparently the changes in pH cause alteration in the permeability of the red cell membrane to the anions and/or cations in solution or alter the red cell contents in some way. At alkaline pH values, both sodium and potassium buffer systems exhibited higher *hem-i* values than the corresponding physicochemical *i* values; at acid pH values, the *hem-i* values were lower (Figs. 3 and 4). The alkaline solutions appear to be more favorable environments for the red blood cell under the conditions studied.

The osmotic fragility of erythrocytes is known to be altered by changes in pH (5, 6). Parpart *et al.* (6) showed that changes in pH, from 7.8 to 6.4, of hypotonic solutions resulted in an increase in osmotic fragility. The changes in volume and, thus, water of cells suspended in isotonic media at different pH values have been well documented (7). Murphy (8, 9) recently showed that decreasing pH, from 8.0 to 6.2, increases cell water and osmotic fragility. His data indicated that the influence of pH on osmotic fragility was through changes in both the amount of cell water and the osmotic behavior of cell water. The increase in cell water, with the decrease in pH, probably results from an increase in the osmotic activity of the cell contents and subsequent movement of water into the cell. In the present studies, the increased osmotic fragility at lower pH values was manifested in lower *hem-i* values than *i* values predicted by colligative property data.

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Determination of Organically Bound Fluorine Using Sodium Biphenyl Reagent and a Fluoride-Specific Ion Electrode

B. C. JONES, J. E. HEVERAN, and B. Z. SENKOWSKI

Abstract □ A simple, accurate, and specific method for direct determination of carbon-bonded fluorine is described for various aromatic compounds of pharmaceutical interest, containing as little as 4% fluorine. Sodium biphenyl reagent was utilized for quantitative liberation of bound fluorine; a fluoride-specific ion electrode was used, in conjunction with a high ionic strength buffer solution, for direct measurement of the liberated fluoride. Incorporation of a subsequent "oxidation" step, using hydrogen peroxide, resulted in quantitative determination of fluorine for certain organo-fluoro compounds which were difficult to ionize completely using a modified oxygen flask combustion technique. It was possible to determine fluorine routinely for up to 20 samples in an 8-hr. period. Precision and accuracy studies are included.

Keyphrases □ Fluorine, organically bound—determination □ Oxidation with hydrogen peroxide—fluorine analysis □ Sodium biphenyl reagent—bound fluorine liberation □ Fluoride-specific ion electrode—organically bound fluorine determination

Many suitable methods are available for determining organically bound fluorine. These methods involve ionization of the bound fluorine using: sodium in liquid ammonia (1, 2); sodium peroxide fusion (3, 4); sodium or potassium fusion (5–9); combustion (10–30); "reductive cleavage" with a stabilized, highly reactive, ether–sodium–aromatic hydrocarbon complex (31–34); and potassium hydroxide in dimethyl sulfoxide (35). The ionization is followed by colorimetric, gravimetric, titrimetric, potentiometric, or fluoride-specific ion electrode measurement of the liberated fluoride (36–40).

Difficulties generally encountered when the widely used oxygen-flask combustion method is utilized for liberation of the bound fluorine include: incomplete combustion, loss of liberated fluoride, and loss of accuracy for compounds having low fluorine content. Consequently, the oxygen-flask combustion method may not be generally suitable for routine determination of bound fluorine in a significant number of pharmaceutical substances with the precision and accuracy expected for compendia methods.

Of the various alternate ionization procedures, reductive cleavage with a stabilized, highly reactive, ether–sodium–aromatic hydrocarbon complex appeared most suitable for rapid, convenient, and accurate liberation of the bound fluorine. These complexes have been utilized for rapid decomposition of organic halogens, including those containing fluorine (41–46). Reportedly, the most effective complex in terms of stability, reactivity at room temperature, and active sodium concentration is the diphenyl–sodium–dimethoxy–ethane complex (sodium biphenyl reagent).

This report describes the conditions generally applicable for the quantitative ionization of bound fluorine in pharmaceutical substances with sodium biphenyl reagent and for the subsequent rapid determination of the liberated fluoride with a fluoride-specific ion electrode.

EXPERIMENTAL

Reagents—The following were used: sodium biphenyl reagent (premixed solution in 15-ml. vials)[1]; ethylene glycol dimethyl ether, b.p. 83–85°[2]; spectroquality dimethyl sulfoxide[2]; certified tetrahydrofuran[3]; reagent grade hydrogen peroxide (30%)[3]; certified ACS grade sodium chloride[3]; certified ACS grade 2-propanol[3]; ACS reagent grade glacial acetic acid[4]; analytical reagent grade sodium hydroxide[5] and sodium citrate[5]; analytical reagent grade sodium fluoride[6]; and 1 *M* sodium hydroxide USP.

2-Propanol Solution (59% v/v)—Dilute 590 ml. of 2-propanol to 1 l. with demineralized water. Mix and allow the solution to cool to room temperature. Adjust final volume to 1 l. with demineralized water and mix well.

Alcoholic Acetate Buffer Solution—In a 2-l. volumetric flask, dissolve 110 g. of sodium chloride and 1 g. of sodium citrate in 600–800 ml. of demineralized water. Add and dissolve 150 g. of sodium hydroxide. Cool to room temperature and, with stirring, cautiously add 450 ml. of glacial acetic acid. Cool to room temperature, add 600 ml. of 2-propanol, and dilute to volume with demineralized water. The pH of this solution should be 5–5.5 with an ionic strength of 4 *M*.

Standard Fluoride Stock Solution (1.0 mg. Fluoride/ml.)—Weigh 2.211 g. of sodium fluoride, previously dried at least 24 hr. in an oven at 100–110°, into a 1-l. volumetric flask and dissolve in 200 ml. of demineralized water. Add 1.0 ml. of 0.1 *N* NaOH and dilute to volume with demineralized water.

Working Standard Fluoride Solution—Dilute the stock fluoride solution with alcoholic acetate buffer solution to prepare solutions equivalent to 0.02, 0.05, 0.10, 0.15, and 0.20 mg. of fluoride/100 ml. Add 10 ml. of reagent blank, as prepared for the respective assay procedure, to each working standard solution before final dilution. Prepare fresh solutions for each analysis.

Apparatus—An Orion fluoride-specific ion electrode, model 94-09, in conjunction with a modified Sargent (glass-sleeve) calomel reference electrode, model S-30084-15, was used with an Orion model 801 research digital pH meter for potential measurements. The reference electrode was modified with a mixture of 70 ml. of saturated KCl solution (freshly prepared or precooled to at least 9°) and 30 ml. of 2-propanol. The electrode was filled with the clear supernatant solution and conditioned in the 2-propanol–saturated KCl solution (30:70) for a minimum of 2 hr. before use.

Assay Procedure—*Sample Preparation*—Type A compounds (soluble without heating, requiring little or no shaking for 0.1–0.2% solubility, w/v)—Accurately weigh a sample equivalent to 16 mg. of fluorine, dissolve in 70–80 ml. of tetrahydrofuran or ethylene glycol dimethyl ether, and dilute to 100 ml. Pipet exactly 15 ml. of the solution into a 200-ml. volumetric flask. Add the contents of a 15-ml. vial of sodium biphenyl reagent and mix. After 5–10 min., destroy the excess reagent with 5–10 ml. of 2-propanol and dilute to volume with 2-propanol. Dilute 10.0 ml. of this solution to 100 ml. with alcoholic acetate buffer solution.

Prepare a reagent blank by diluting 15.0 ml. of sample solvent and 15 ml. of sodium biphenyl reagent to 200 ml. with 2-propanol (as

[1] Southwestern Analytic Chemicals.
[2] Matheson, Coleman and Bell.
[3] Fisher Scientific.
[4] Allied Chemical.
[5] Mallinckrodt.
[6] J. T. Baker Chemical.

per sample preparation). Use this solution in preparing working standard fluoride solutions.

Type B compounds (soluble with heating and/or prolonged shaking for 0.1–0.2% solubility, w/v)—Accurately weigh a sample equivalent to 20 mg. of fluorine; dissolve in 70–80 ml. of tetrahydrofuran or ethylene glycol dimethyl ether and dilute to 100 ml. (Compounds that were not readily soluble in tetrahydrofuran or ethylene glycol dimethyl ether were predissolved in 5.0 ml. of dimethyl sulfoxide with heating on a steam bath and diluted to 100 ml. with tetrahydrofuran or ethylene glycol dimethyl ether.) Pipet a 15-ml. aliquot of sample solution into a 500-ml. round-bottom flask, add the contents of a 15-ml. vial of sodium biphenyl reagent, and mix. After letting the flask stand at room temperature for 5–10 min., add 50.0 ml. of 2-propanol, 10.0 ml. of 30% H_2O_2 (reagent grade), and 4.0 ml. of 1 *M* NaOH, respectively. Connect the flask to a clean, dry, water-cooled condenser and reflux, using a preheated hot plate set for medium heat (about 250°), until the H_2O_2 is completely destroyed. (This is indicated by irregular boiling with bumping and cessation of rapid boiling and usually requires 50–60 min.) Cool the sample to room temperature, quantitatively transfer to a 250-ml. volumetric flask, and dilute to volume with 59% (v/v) 2-propanol. Dilute 10.0 ml. of sample solution to 100 ml. with alcoholic acetate buffer solution.

Prepare a reagent blank by pipeting 15 ml. of sample solvent into a 500-ml. round-bottom flask and treating in the same manner as the sample. For samples predissolved with dimethyl sulfoxide, prepare the reagent blank by diluting 5 ml. of dimethyl sulfoxide to 100 ml. with the sample solvent. Transfer 15.0 ml. of this solution to a 500-ml. round-bottom flask and treat in the same manner as the sample. After diluting to 250 ml. with 59% (v/v) 2-propanol, use this solution in preparing the working standard fluoride solutions.

Potential Measurements—Transfer the solution to be measured to a 150-ml. beaker containing a Teflon-coated stirring bar. (Although plastic labware is not required, it is recommended, particularly for sample containment during potential measurements.) Immerse the specific fluoride and modified reference electrodes, and measure the millivolt potential with constant stirring. Use an electric stirrer with an insulated top or with an asbestos pad to reduce heat transfer to the stirred solution. Take a final reading when the potential has stabilized within ±0.1 mv. or allow 1–2 min. before each measurement. Measure the working standard solutions beginning with the lowest fluoride concentration, *i.e.*, 0.02 mg./100 ml.

Plot fluoride concentration (mg./100 ml. *versus* millivolts for each working standard solution on semilogarithmic paper (1 cycle). The fluoride concentration in the measured sample solution is determined directly from this graph.

Alternately, the potentials observed for the standard solutions can be used to obtain a least-squares line represented by the equation:

$$y = mx + b \qquad \text{(Eq. 1)}$$

where y = potential in millivolts, x = log of fluoride concentration in mg./100 ml., m = slope, and b = intercept.

The potential obtained for the sample solution is substituted in Eq. 1, and the equation is solved for x. The antilog of x equals the fluoride concentration of the measured sample solution.

Single-Point Standard Comparison—If the slope of the concentration curve remains constant within ±2 mv., the standard curve or the corresponding slope (m) and intercept (b) values may be used as a fixed calibration for single-point reference standardization as follows. Prepare a standard fluoride solution as previously described, equivalent to 0.10 mg. fluoride in 100 ml. With an Orion digital pH meter or equivalent, set the dial (functional switch) in the relative millivolt position and measure the potential of this solution as previously described. Adjust the meter, using the calibration control to read the exact potential (y), calculated from Eq. 1 or read directly from the fluoride concentration curve.

To obtain the fluoride concentration in the measured sample solution, take the antilog of x calculated for the corresponding y using Eq. 1 or read fluoride concentration directly from the standard curve.

RESULTS AND DISCUSSION

Sample Dissolution—The choice of sample solvents was limited to those that were water miscible, did not cause instant decomposition of the sodium biphenyl reagent, and had at least a 0.1% sample solubility by weight. Of the various solvents examined, tetrahydrofuran, ethylene glycol dimethyl ether, and dimethyl sulfoxide were found to be suitable. Most of the compounds tested were readily soluble in dimethyl sulfoxide. However, the sodium biphenyl reagent was slowly decomposed by dimethyl sulfoxide, and fluorine recoveries were not quantitative. Therefore, compounds that required heating to attain at least 0.1% solubility by weight in tetrahydrofuran or ethylene glycol dimethyl ether were first dissolved in a small amount of dimethyl sulfoxide and then diluted with tetrahydrofuran or ethylene glycol dimethyl ether.

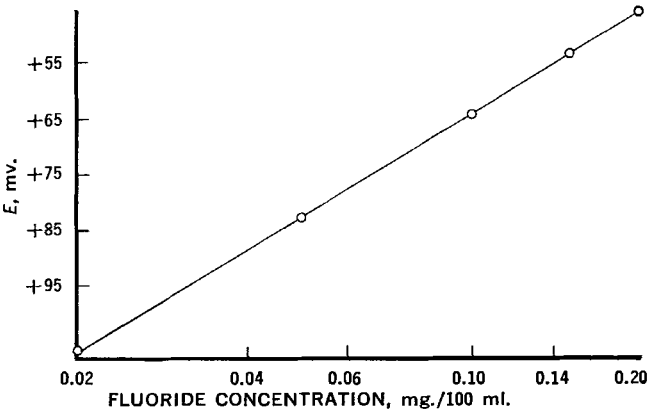

Figure 1—*Standard fluoride calibration curve.*

Sodium Biphenyl Reaction—Although the mechanism of the reaction is not well documented, it is believed that decomposition of organo-fluoro compounds with sodium biphenyl reagent involves reductive cleavage of the bound fluorine (31, 37). The reaction is apparently instantaneous at room temperature, since no significant differences in fluorine recoveries were observed for reaction periods of 1–30 min. Although the reaction between sodium biphenyl reagent and the various organo-fluoro compounds was apparently instantaneous, the reaction time for this procedure was standardized at 5–10 min.

For the 12 compounds examined, the need for subsequent oxidation for quantitative fluorine recoveries was apparently not structurally dependent. However, the relative solubility of the compounds in the sample solvents appeared to be a determining factor in the need for subsequent oxidation. All compounds that did not require subsequent oxidation for quantitative fluorine recoveries were readily soluble in the sample solvents (soluble without heating and requiring little or no shaking for 0.1–0.2% solubility, w/v). Correspondingly, six of the nine compounds that required subsequent oxidation were difficult to dissolve in the sample solvents.

"Oxidation" of Reaction Mixture with Hydrogen Peroxide—For most of the compounds tested, it was necessary to "oxidize" the reaction mixture with hydrogen peroxide to obtain quantitative fluorine recoveries. The oxidation step involved the addition of an excess of hydrogen peroxide to the reaction mixture after destroying the excess sodium biphenyl with 2-propanol. A small amount of sodium hydroxide was added to the mixture to ensure basic pH conditions, and the entire reaction mixture was refluxed until the hydrogen peroxide was completely destroyed. This usually required about 1 hr. of total refluxing time. Since hydrogen peroxide was a sufficient oxidant for all compounds investigated, no other oxidants were tested.

When subsequent oxidation was required for quantitative fluorine recovery after reaction of the compound with sodium biphenyl reagent, it was suspected that an insoluble fluoro-reaction product or an insoluble sodium compound was formed (42, 44). It is also possible that biphenyl fluoride was formed; however, this seems improbable since acidic reaction conditions would be necessary for this reaction to take place (47).

Dilution of Reaction Mixture—The oxidized reaction mixture was diluted with 59% (v/v) 2-propanol. When ethylene glycol dimethyl ether was used as the sample solvent, the 2-propanol–water ratio of the solution, used to dilute the oxidized reaction mixture, was critical. A variation of less than ±1% in the indicated ratio resulted in either precipitation or biphase formation in the resultant sample solution. However, this was not the case when tetrahydrofuran or

Table I—Determination of Organically Bound Fluorine in Various Organo-Fluoro Compounds

Compound	Empirical Formula	Fluorine Found, %	Theoretical Fluorine, %	Ratio Recovered, %	Absolute Error, %	Standard Deviation	Precision[a]
5-Fluorouracil	$C_4H_3FN_2O_2$	14.52	14.6	99.5	0.08	0.62	1.43
Flurazepam hydrochloride	$C_{21}H_{23}ClFN_3O \cdot 2\,HCl$	4.14	4.12	100.5	0.02	0.45	1.25
Chloro-fluoro	$C_{21}H_{23}ClFN_3O$	4.93	4.90	100.6	0.03	0.42	1.02
Nitro	$C_{16}H_{12}FN_3O_3$	6.07	6.07	100.0	0.00	0.16	0.39
Chloro-fluoro	$C_{17}H_{14}ClFN_2O_2$	5.72	5.71	100.2	0.01	0.29	0.80
Chloro-fluoro HCl salt	$C_{17}H_{14}ClFN_2O_2 \cdot HCl$	5.16	5.15	100.2	0.01	0.61	1.49
Chloro-fluoro HCl salt	$C_{10}H_{11}ClF_3N \cdot HCl$	20.82	20.79	100.2	0.03	0.33	0.92
Chloro-fluoro H_2SO_4 salt	$[C_{10}H_{11}ClF_3N]_2 \cdot H_2SO_4$	19.71	19.88	99.2	0.17	0.32	0.82
5-Fluorouridine	$C_9H_{11}FN_2O_6$	7.17	7.25	99.0	0.08	0.25	0.65
Dihydroxy-fluoro	$C_9H_{11}FN_2O_5$	7.75	7.72	100.4	0.03	0.34	0.86
Amino	$C_4H_4FN_3O$	14.69	14.72	99.8	0.03	0.33	0.76
Bromo-fluoro	$C_{10}H_{14}BrFN_2O_6$	5.32	5.32	100.0	0.00	0.42	1.08
Average					0.04	±0.38	±0.96

[a] Based on four to eight determinations per compound at the 95% confidence level.

dimethyl sulfoxide was the sample solvent. Samples that did not require subsequent oxidation after the sodium biphenyl reaction were diluted directly with 2-propanol.

Choice of Buffer Solution—To determine the liberated fluoride directly, without separation from the reaction mixture, a high ionic strength buffer system which was compatible with the reaction mixture was required. It had previously been determined that the optimum pH range for fluoride determination in the 10^{-4}–10^{-5} *M* range was 4.5–8.0 (48). Therefore, various alcoholic buffer systems covering the desired pH range were used to dilute the oxidized reaction mixture from 15 to 100 ml. Methanolic and ethanolic buffer systems were not suitable, since precipitation occurred even when 40% alcoholic buffer systems were used to dilute the sample solution. However, it was found that the alcoholic acetate buffer solution containing sodium chloride (pH 5–5.5) was compatible with the reaction mixture. A uniform solution resulted when the sample solution was diluted as much as 1 to 4 with this buffer solution, and the pH of this solution was equal to the initial buffer solution.

Electrode Response and Potential Measurements—Electrode response to fluoride ion was linear throughout the working range of 10^{-4}–10^{-5} *M* or 0.02–0.20 mg. F^-/100 ml. (Fig. 1). The slope of the response curve was 58.1 mv. Electrode response was rapid and, in most cases, equilibrium was reached in less than 30 sec. in stirred solutions. Since potential measurements were made in an alcoholic solution, the type and condition of the reference electrode was a critical factor. Excessive electrode drift, exceeding 0.5–1 mv./min., was encountered when measurements were made using a conventional calomel reference electrode. Therefore, it was necessary to use a modified calomel reference electrode. Potential measurements were reproducible within ±0.1 mv. and were relatively drift-free with the modified reference electrode.

Sample Analysis—The average standard deviation, based on percent sample fluorine, was ±0.38% for 12 organo-fluoro compounds containing from 4 to 21% organically bound fluorine. The average absolute error, assuming 100% purity, was less than 0.17% for all compounds tested (Table I).

CONCLUSION

The combination of the sodium biphenyl reagent to liberate bound fluorine and a fluoride-specific ion electrode for direct determination of the ionized fluoride resulted in a convenient and accurate method for determining bound fluorine in various organo-fluoro compounds of pharmaceutical interest.

By using the developed method, it was possible to determine accurately fluorine for 8–10 different samples in an 8-hr. period. For compounds that did not require subsequent hydrogen peroxide oxidation, a single analyst could analyze 16–20 samples in an 8-hr. period.

Although only a limited number of organo-fluoro compounds containing carbon–fluorine bonds were tested, it is believed that the method can be used to determine organically bound fluorine routinely in other types of organo-fluoro compounds. The method should prove especially advantageous when erratic results, due to incomplete sample combustion, are obtained when using one of the more conventional means of fluorine ionization.

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Drug-Absorption Analysis from Pharmacological Data II: Transcorneal Biophasic Availability of Tropicamide

RONALD D. SCHOENWALD and VICTOR F. SMOLEN*

Abstract □ Following an ophthalmic dose, the mydriatic drug, tropicamide, may traverse the cornea to reach its vicinal sites of action (biophase) directly or indirectly gain access subsequent to its systemic absorption. The latter route of entry into the biophase may be attributed to fluid volume loss and scleral absorption. A means by which the relative quantities of the drug absorbed transcorneally directly into the biophase and indirectly following systemic absorption at any time can be discerned from concomitant measurements of pupillary diameters in drug-treated and untreated eyes is described. However, except at very high dosage, the observed magnitude of effects in the control eye were insufficient for this method to be practical for tropicamide. The determination of the total relative quantities of drug ultimately dissipated from the absorption site by routes other than transcorneal absorption into the biophase was, however, approximated from measurements performed on the treated eye alone. Semilogarithmic plots of the time course of transcorneal drug passage were linear, indicating the biophasic availability of tropicamide to occur through the operation of apparent first-order processes. A comparison of pharmacological and biokinetic parameters characterizing the mydriatic behavior of tropicamide administered in vehicles having a pH of 5.0 and 7.4 is presented.

Keyphrases □ Tropicamide mydriasis—transcorneal biophase parameters, vehicle pH influence, rabbits □ Pharmacokinetic parameters, biophase—transcorneal tropicamide mydriasis, vehicle pH influence □ Mydriatic response behavior, tropicamide—vehicle pH influence, rabbits

The influence of formulation factors on such pharmacological response characteristics as onset and duration of response, peak response intensity, time of peak response, and rate(s) of dissipation of effect is an important consideration in the development and evaluation of pharmaceutical products. A drug induces its biological effects when it enters its biophase compartment where it interacts with its receptor sites. The rates at which a drug enters and is subsequently dissipated from its biophase determine the time course of the induced response intensity and, therefore, the characteristics of its pharmacologic behavior. At the two extremes, the availability of a drug to its biological sites of action is either limited by its release from its dosage form or by such biological factors as the permeability of tissue barriers, metabolism, distribution of the drug into tissue depots, and excretion. When the drug is administered by other than parenteral routes, its biophasic availability, *i.e.*, the total quantity of drug that has penetrated to the biophase at any time, may also be severely affected by peripheral losses from the site(s) of absorption. The relative quantities of drug that are absorbed to contribute subsequently to the pharmacologic effect and the quantities that are peripherally eliminated from the site of administration are dependent upon the relative rates of the competing, simultaneously operative processes.

As is commonly the case with ophthalmic preparations, drugs are generally most rapidly available for absorption when administered in the form of their aqueous solutions. The composition of such solutions can influence the rates at which the drug becomes available to its biophase because it can determine the form of the drug and, therefore, its tissue permeability. Alternatively, the vehicle may exert an influence directly on the permeability properties of the tissue barriers which the

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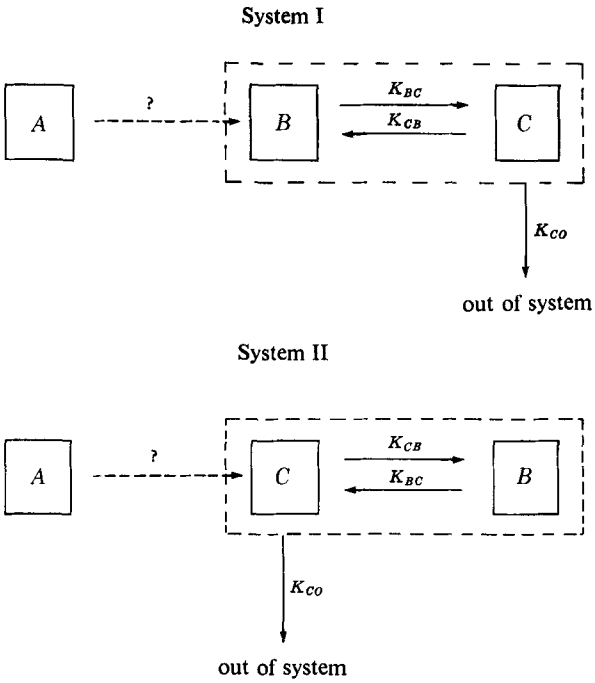


Scheme I—Resolution of a compartment model of ophthalmic drug transference into two systems which is possible through use of a control eye. System I depicts transcorneal passage of the drug from the site of administration, A, *directly into the biophase,* B. *System II is appropriate for the entry of the drug into the systemic circulation,* C, *prior to penetrating to the biophase. Although both absorptive processes are operating simultaneously, they can be studied individually. The enclosure of* B *and* C *within the broken lines is indicative of the kinetic indistinguishability of these compartments, as was found to be the case for tropicamide.*

drug must penetrate (1). The rates of peripheral drug loss can also be influenced by formulation factors, *e.g.*, by the inclusion of viscosity-imparting agents which affect the residence time of the drug vehicle at the absorption site(s) (2).

The application of conventional approaches utilizing direct assay methods in the study of *in vivo* transcorneal drug absorption can present intractable difficulties, while *in vitro* experimentation utilizing excised corneas may be poorly reflective of *in vivo* ophthalmic drug availability because of the difficulty of simulating the peripheral drug loss. Such peripheral dissipation of the drug occurs by fluid loss through the lacrimal drainage duct, scleral absorption into blood and lymphatic vessels, and aqueous flow following transcorneal passage. The purpose of the present study was to investigate the applicability of a previously described (3, 4) pharmacologic method of drug-absorption analysis to the elucidation of the biophasic availability and peripheral loss behavior of the mydriatic drug tropicamide following its administration into the eye.

THEORETICAL

A basis for the transformation and subsequent utilization of pharmacological data, derived from monitoring the temporal dependency of the drug-response intensity following dosing, for the performance of compartmental analysis and the elucidation of drug bioavailability was described and confirmed for tropicamide in previous reports (3, 4). The applicability of the method to any particular drug can be determined from pharmacological data alone; therefore, the detection of the drug by direct assay is entirely obviated. With the exception of volumes of distribution, the method can be applied to determine all the types of pharmacokinetic results calculable from data obtained by directly sampling body fluids or tissue drug levels. Drug-absorption analysis using drug level in corporeal fluid data is limited to those routes of administration (*e.g.*, oral and parenteral) by which the drug is absorbed systemically, concomitantly, or prior to reaching its sites of action. The pharmacological method, when applicable, is not disadvantaged in this manner; it can be implemented for any route of administration to determine the time dependency of blood and/or biophasic drug levels as well as the quantities of drug absorbed from the site of administration into the blood and/or biophase for any route of administration. The method is particularly advantageous in its application to drugs that cannot readily be assayed and to the study of the bioavailability of drugs administered topically for localized effects.

When drugs are administered for local effects, the intended site of their action (biophase) is obviously located vicinally to the site of administration. The absorption of the drug and its subsequent disposition in the body may be envisaged ideally to be occurring as depicted in System I of Scheme I. Inevitably, however, some drug is absorbed systemically, as shown in System II of Scheme I; it directly enters the systemic circulation by the various previously mentioned routes prior to distributing into the biophase. This latter mode of entry is inefficient and generally undesirable. It may contribute to the occurrence of systemic toxicity and should be prevented as far as possible; this is important with local anesthetics, potent alkaloidal drugs used ophthalmically, *etc.*

The pharmacological effects and the relative quantities of the drug absorbed systemically and biophasically can be distinguished when the pharmacological effects can be simultaneously recorded from an untreated site of action symmetrical with the treated site to which the drug was administered; the untreated site serves as a control for comparison to the treated site. It can be assumed that the drug that is directly absorbed systemically, *i.e.*, without first passing through a biophase, gains equal access to both the symmetrical control biophase and the biophase located vicinally to the site of administration. This is well exemplified with the ophthalmic dosing of a mydriatic drug into one eye where the systemically absorbed drug may be expected to gain equal access to the iris in both eyes. The transcorneally absorbed drug may be assumed to enter the biophase (which anatomically may be constituted by a region of the iris) directly and prior to entering the systemic circulation.

The level of drug in the biophase that is responsible for the observed intensity of pharmacological response in the treated eye at any time arises from the sum of the quantities of drug absorbed directly into the biophase and that which has arrived *via* the systemic route. Since this systemic contribution can be assumed to be the same for both the treated and control eyes, the pharmacological response intensity in the treated eye that is attributable to the level of drug in its biophase arising through direct absorption from the site of administration can be ascribed at any time to the difference in the intensities observed in the treated and control eyes. The quantities of drug reaching the biophase directly or *via* the systemic circulation can, therefore, be discerned.

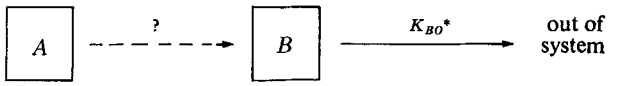
These considerations allow the resolution of the absorptive processes into the two systems depicted in Scheme I. By utilizing a treated and a control eye, each system can be studied individually, although experimentally the data for each study are obtained simultaneously. The relative quantity of drug in the biophase at any time resulting from direct biophasic absorption is simply obtained by converting the simultaneously observed values of I for both eyes to corresponding quantities of the drug in the biophase and taking their difference. As previously established (3, 4), the quantity of drug (Q_B) in the biophase compartment at any time following dosing by any route is provided by Eq. 1 where β is a constant. The details of the origin and evaluation of β were described in earlier reports (3, 4).

$$Q_B = \beta f(I) \qquad \text{(Eq. 1)}$$

The functional relationship between Q_B/β and I, *i.e.*, $f(I)$, is provided by an intravenous dose–effect curve, which is employed in the manner of a calibration curve. The curve for tropicamide was given previously (4), where it was also shown that the value of β for tropicamide is unity and Q_B may be read directly from the abscissa of the curve. As was discussed (4), this situation arises as a con-

sequence of the biophase and central systemic compartments for tropicamide being kinetically indistinguishable. This behavior can be attributed to the apparent rapidity with which tropicamide can permeate tissue barriers.

A simplified compartment model for drug absorption directly into the biophase is illustrated in Scheme II; its use can be justified



Scheme II—Simplified compartment system for the description of ophthalmic drug transference directly into, as well as removal from, the biophase. In referring to Scheme I, the elimination constant from the biophase, K_{BO}*, is seen to be related to other transfer constants by the equation:* $K_{BO}^* = K_{BO} + K_{BC}$.

by the following considerations. By excluding the volume of the biophase, the remaining volume of distribution for the drug in the animal may be assumed very large in comparison to the volume of the biophase and, therefore, provides a sink for the drug into which it is effectively eliminated upon leaving the biophase. The backflow into the biophase can then be neglected to simplify the model (Scheme II). In the event the backflow is appreciable, it can be assumed that the quantity of drug influxed from this source at any time is equal for both the experimental and reference biophases. Therefore, the correction obtaining from the control eye also takes account of the backflow of drug from the central compartment. The use of this correction, when necessary, allows the retention of the simplified model even in the event of an appreciable backflow of drug. However, when this is the case, the ability to distinguish between drug absorbed from the site of administration into the biophase and that reaching it by systemic absorption from the site of administration is vitiated. The absorption analysis then could only be performed for the direct transference of drug into the biophase. However, since the biophase and systemic compartments have been found to behave identically in the case of tropicamide, such complications are of no further concern in this analysis.

These considerations justify the adoption of the single-compartment model shown in Scheme II. The quantity of drug that has been absorbed directly into the biophase from the site of administration up to a time, t, is given by Eq. 2. The elimination constant, K_{BO}^*, from the biophase, in accordance with Scheme I is given by $K_{BO}^* = K_{BO} + K_{BC}$. It can be evaluated from the postabsorptive time course of biophasic drug levels following the administration of an ophthalmic dose of the drug.

$$A_t = \beta_{t_0}\left[f(I) + K_{BO}^* \int_0^t f(I)dt\right] \qquad \text{(Eq. 2)}$$

Equation 2 is independent of any compartment models for the description of the biokinetic behavior of the drug in the system. The bioavailability characteristics of drugs directly into a vicinal biophase can, therefore, be determined with the use of Eq. 2 without any need for developing compartment models such as are necessary for the study of systemic drug absorption (3, 4). Only the intravenous dose-effect curve, used to transform I values into $f(I)$ values, and the data obtaining from the local administration of the drug are required. However, by considering that Eq. 1 is valid irrespective of the route of administration, it is conceivable that for drugs such as tropicamide, for example, the intravenous dosing necessary to construct the dose–effect curve could be obviated. Provided that the biophase and systemic compartments are known *a priori* to be identical (β, therefore, equals unity when maximum I values corresponding to zero time are used), the functional relationship given in Eq. 1 and between Q_B and I can be defined from data obtained by simultaneously monitoring the time course of the pharmacological response intensity and the corresponding blood levels of the drug following a single dose given by any route, irrespective of the kinetics and mechanisms involved in the absorption of the drug. The result of plotting values of I as a function of corresponding blood levels would be theoretically equivalent to an intravenous dose–effect curve and could be used in a similar manner for the conversion of I values into Q_B values, provided an appropriate volume of distribution could also be computed from the data. When the volume of distribution is not readily calculable, *e.g.*, when the actual dose of drug absorbed is not known, the dose–effect relationship would still provide relative values of Q_B. This method of determining the relationship between Q_B and I has the advantage of not requiring multiple intravenous dosing. On the other hand, it requires the detection of the drug in the blood. It will be further treated in a subsequent report.

If the quantity of drug absorbed systemically before entering into the biophase relative to that absorbed directly is appreciable, it may be an important factor contributing to any observed systemic side effects in addition to the intensity of the intended local pharmacological effect. Under such circumstances, the study of the kinetic processes involved in the systemic absorption route(s) may be as important as those operative in the direct absorption of the drug into the biophase. As discussed previously, operation of the processes responsible for the systemic absorption of an ophthalmically administered drug, *i.e.*, its peripheral loss, is reflected in the response observed in the untreated control eye. Irrespective of the complexity of the involved processes, expressions for A_t, such as Eq. 2, are independent of the kinetics and mechanisms by which the drug becomes systemically absorbed. However, expressions for A_t are dependent upon the compartment models determined to describe best the biokinetic behavior of the drug in the body. These models are best determined from the results of intravenous dosing. In the simplest case, in which a single-compartment model is found adequate, Eq. 2 would be appropriate for the computation of the quantities of drug absorbed directly into the biophase as well as those amounts peripherally dissipated and subsequently absorbed systemically. Although the assumption of a single-compartment model was shown to be a reasonable approximation of the biokinetic behavior of tropicamide (4), this may not generally be the case (3).

MATERIALS AND METHODS

Materials—Tropicamide[1] solutions were prepared in concentrations of 0.010, 0.015, 0.020, and 0.030% w/v in an isotonic, pH 7.4 phosphate-buffered vehicle. A 0.015% w/v tropicamide solution, having a pH equal to 5.0, was also employed. It contained 1.313% w/v $NaNO_3$; the pH was adjusted by the dropwise addition of an appropriate volume of 2 N HNO_3. Four 3–4-month-old, white, New Zealand, male rabbits were chosen from among 20 rabbits screened on the basis of the similitude of their pupillary response to light intensity and to the mydriatic activity of tropicamide. These same rabbits were used throughout the study and were the same animals employed for the determination of the intravenous dose curve.

Methods—The technique employed for the measurement of pupillary diameters was described earlier (4). The mydriatic response intensity, I, is defined in terms of observed pupillary diameters by Eq. 3, where d_0 and d_t refer to values observed at time zero and t following dosing, respectively. The dose–effect curve, determined for the rabbits used in the present study, was reported (3). It was utilized for the conversion of I values into their corresponding $f(I)$ values.

$$I = \frac{d_t - d_0}{d_0} \qquad \text{(Eq. 3)}$$

The ophthalmic administration of tropicamide was performed by carefully pipeting 0.020-ml. volumes of the solutions into the lower conjunctival cul-de-sac of the right eyes of each of the four rabbits. The left eyes served as controls.

RESULTS

Mydriatic Response to Tropicamide—The control eyes of the rabbits were found to be entirely devoid of a detectable mydriatic response to the drug. This was generaly found to be the case, except at drug solution concentrations inordinately higher than those employed in the present experiment. The observed time and dose dependency of the mydriatic response in the tropicamide-treated eyes at pH 7.4 is graphically shown in the semilogarithmic plot in Fig. 1.

The time scale in Fig. 1, as well as that in the remaining figures, was corrected from real time by the subtraction of a consistently observed lag time of 6 min. This time likely corresponds to the ini-

[1] Supplied by Alcon Laboratories, Fort Worth, Tex.

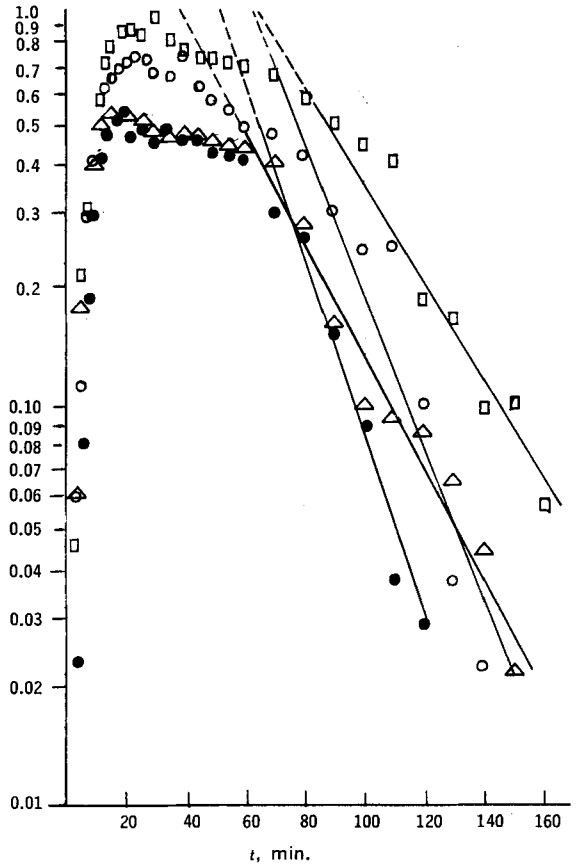


Figure 1—*Time course of mydriatic response following the ophthalmic administration of 0.02 ml. of 0.010* (●), *0.015* (△), *0.020* (○), *and 0.030* (□) % *w/v, pH 7.4, solutions of tropicamide. Each point represents the average of four determinations on different rabbits.*

tial loading of the drug into the biological barriers it must traverse, as well as the development of a level of drug in the biophase sufficient to elicit an observable threshold response. Each point in Fig. 1 represents the average of four determinations on different rabbits. The intrasubject variation of such results was previously discussed (3, 4) to be of the same magnitude as the intersubject variation, therefore justifying the averaging of the values replicated on different rabbits. The intersubject reproducibility of response may be attributed to the careful screening of the rabbits, which was performed prior to their selection for this study. The relative error of the measurements of pupillary diameter diminishes with the magnitude of the values. The overall average error was approximately 10%.

Time Course of Biophasic Drug Levels—Transformation of the I values, plotted in Fig. 1, into corresponding biophasic drug levels was accomplished through the implementation of the intravenous dose–effect curve as previously described (3). The results, replotted for each dose, are shown in Fig. 2. Relative to Fig. 1, the transformation of the data can be observed to have resulted in a somewhat enhanced linearity of the latter postabsorptive segments of the curves.

Biophasic Drug-Elimination Rates—Adopting the compartment model depicted in Scheme II permits the computation of apparent first-order biophasic elimination half-lives from the slopes of the latter linear segments of the semilogarithmic plots contained in Fig. 2. Linear least-squares regression values are listed in Table I. The expected independence of these values on dose and vehicle composition is indicated by their observed similarity, which lends credence to the assumption of the linear compartment model shown in Scheme II. The plot of dose normalized, *i.e.*, Q_B/D, values[2] shown in Fig. 3 provides a least-squares best value for K_{BO}* equal to 0.051 min.$^{-1}$.

[2] Since $\beta = 1$ and $f(I)_{max.}$ = dose, Q_B/D in reference to tropicamide is tantamount to $f(I)/f(I)_{max.}$. See *Reference 4* for further details.

Table I—Biophasic Elimination (t_B) and Apparent Mydriatic Response Dissipation (t_M) Half-Lives Observed for Ophthalmically Administered Tropicamide

pH of Vehicle	Tropicamide Concentration, % w/v	t_B[a]	t_M[a]
7.4	0.010	17.7	13.3
7.4	0.015	15.7	21.4
7.4	0.020	19.8	16.0
7.4	0.030	13.3	24.6
5.0	0.015	21.8	47.3

[a] Linear least-squares regression values.

Dissipation of Peak Mydriatic Effect—A semilogarithmic plot of averaged $I/I_{max.}$ values for each pH 7.4 solution is also shown in Fig. 3 for comparison to the corresponding plot of averaged Q_B/D values. The dissimilarity in the $I/I_{max.}$ and Q_B/D *versus* time curves emphasizes the necessity of transforming observed I values into Q_B or $f(I)$ values in order to draw conclusions concerning the biotransference behavior of a drug from pharmacological data. For example, assuming a linearity for the latter part of the $I/I_{max.}$ *versus* t plot allows a half-life of 50 min. to be ascribed to the dissipation of the peak mydriatic response intensity as compared to a value of 13.6 min. for the biophasic drug-elimination half-life. If the two quantities were directly related, the half-lives would be identical. The relationship, however, is again provided in this case by the intravenous dose–effect curve, as can be seen from the following considerations. The biophasic elimination constant, K_{BO}*, and the

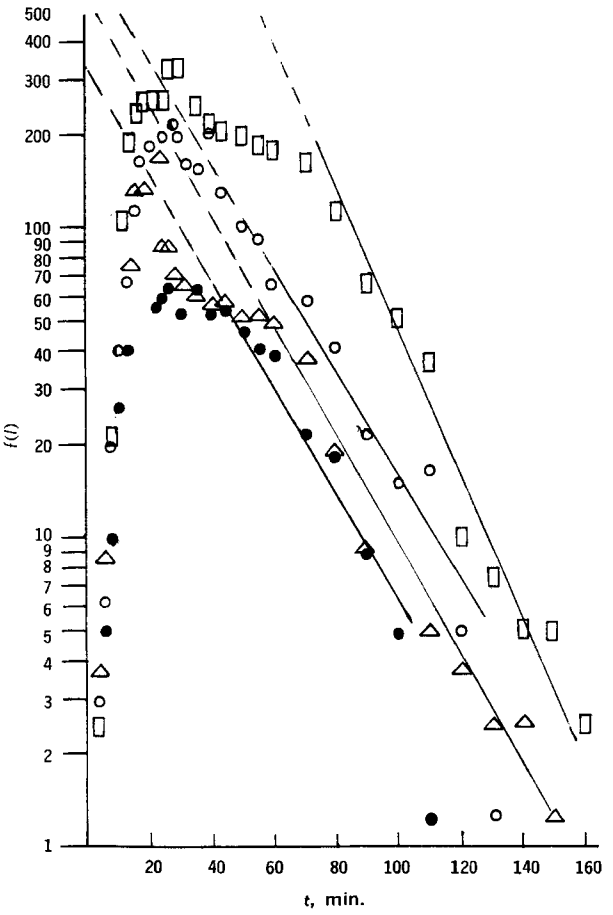


Figure 2—*Temporal variation of biophasic drug levels following ophthalmic dosing of 0.020 ml. of 0.010* (●), *0.015* (△), *0.020* (○), *and 0.030* (□) % *w/v, pH 7.4, tropicamide solutions. Each point is the average of four determinations performed on different rabbits. The time axis is corrected from real time by the subtraction of a 6-min. lag time.*

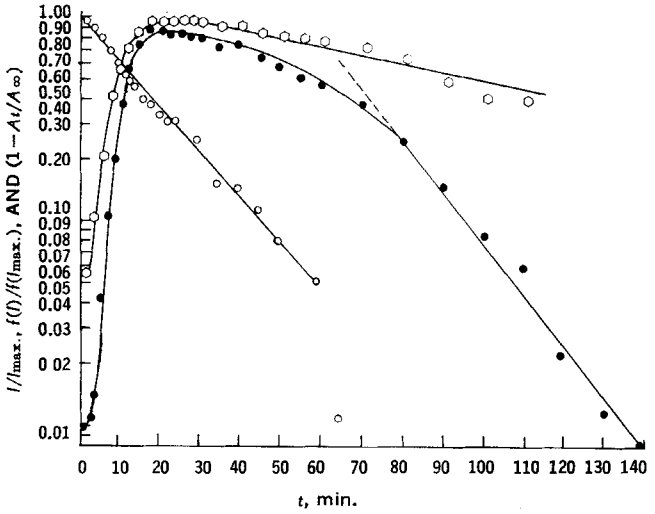


Figure 3—*Semilogarithmic plots of normalized values of* $I/I_{max.}$, *the relative mydriatic response intensity* (○); $f(I)/f(I_{max.})$, *the relative amount of drug in the biophase* (●); *and* $(1 - A_t/A_\infty)$, *the fraction of drug remaining to be transcorneally absorbed into the biophase* (○); *as a function of time following the ophthalmic administration of 0.020 ml. of 0.010, 0.015, 0.020, and 0.030% tropicamide solutions in pH 7.4 isotonic buffer to rabbits. Each point is the average of 16 determinations.*

apparent mydriatic response dissipation constant, K_M, may be defined by Eqs. 4 and 5, respectively, and related by Eq. 6:

$$\frac{dQ_B}{dt} = -K_{BO}^* Q_B \qquad \text{(Eq. 4)}$$

$$\frac{dI}{dt} = -K_M I \qquad \text{(Eq. 5)}$$

$$K_M/K_{BO}^* = \frac{d(\log I)}{d(\log Q_B)} \qquad \text{(Eq. 6)}$$

It is readily derived, as given by Eq. 6, that the ratio of the two constants at any value of I is directly equal to the instantaneous slope of a log–log plot of the dose–effect curve which, as discussed previously, is tantamount to a plot of log I *versus* log Q_B. The value of K_M may be expected to be constant and directly related to K_{BO}^* only for values of I corresponding to linear regions of such a plot. The log–log plot of the previously reported (4) dose–effect curve for tropicamide, shown in Fig. 4, approximates linearity for the majority of its extent. As expected from considering the deviations from linearity, the slope of the least-squares line drawn in Fig. 3, having a

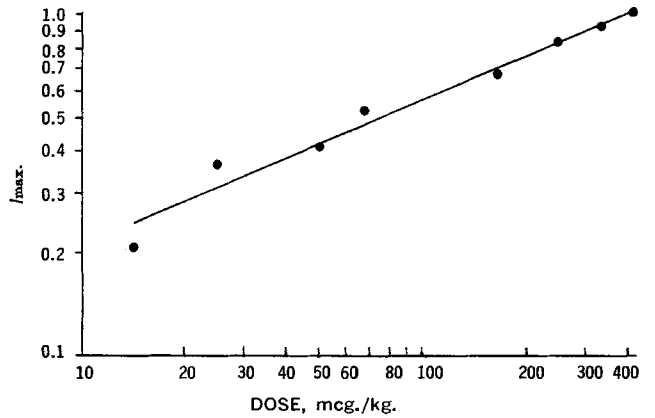


Figure 4—*Log* $I_{max.}$ versus *log dose plot for the rapid intravenous administration of tropicamide. The maximum response* ($I_{max.}$) *was observed at 2 min. following dosing. Each point is the average of four determinations on different rabbits.*

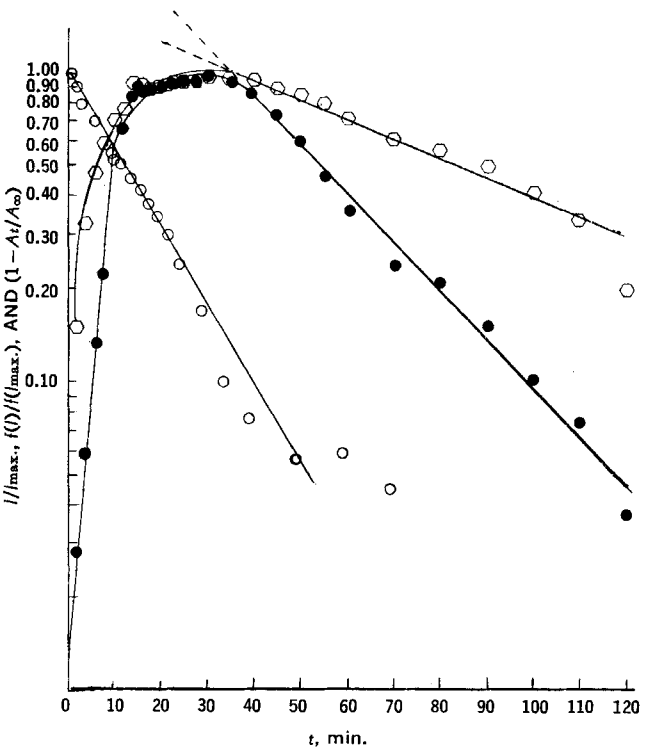


Figure 5—*Semilogarithmic plots of the time dependency of the relative mydriatic response intensity,* $I/I_{max.}$ (○); *the relative quantities of drug in the biophase,* $f(I)/f(I_{max.})$ (●); *and the fraction of drug remaining to be transcorneally unabsorbed,* $(1 - A_t/A_\infty)$ (○), *following ophthalmic dosing of 0.02 ml. of 0.015% w/v tropicamide in a pH 5.0 vehicle. Each point is the average of four determinations replicated on different rabbits.*

value of 0.43, only approximates the ratio of K_M/K_{BO}^*, equal to 0.72, calculated from the average half-lives listed in Table I.

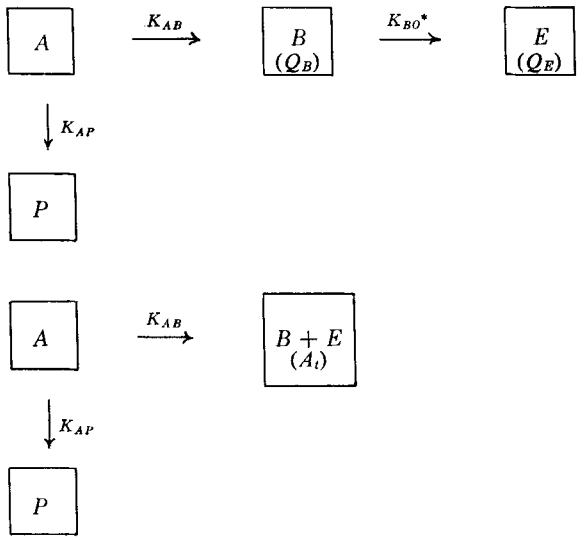
Biophasic Availability and Peripheral Loss of Ophthalmically Administered Tropicamide—Figures 3 and 5 present plots of log $(1 - A_t/A_\infty)$ *versus* t for the results obtained at pH 7.4 and pH 5.0, respectively. Values of A_t were computed numerically, using Eq. 2, with the aid of a CDC 6500 digital computer. The linearity of the plots is indicative that the clearance of the drug from the ophthalmic site of administration through both peripheral loss and absorption into the biophase can be described to occur by apparent first-order processes such as passive diffusion. Following this consideration, the compartment models, shown in Scheme III, can be applied to describe these processes. On the basis of these models, Eq. 7 can readily be derived:

$$\log (1 - A_t/A_\infty) = -\left(\frac{K_{AP} + K_{AB}}{2.303}\right)t \qquad \text{(Eq. 7)}$$

Table II—Pharmacokinetic Parameters Characterizing the Transcorneal Biophasic Availability (BA) of Tropicamide[a]

pH	Tropicamide Concentration, % w/v	$K_{AB} + K_{AP}$	r^b	K_{AB}	K_{AP}	Percent BA[c]
7.4	0.010	0.0687	0.9766	0.0348	0.0340	50.3
7.4	0.015	0.0528	0.7465	0.0230	0.0300	43.3
7.4	0.020	0.0613	0.9065	0.0454	0.0159	74.9
7.4	0.030	0.0387	0.9905	0.0294	0.0093	57.4
5.0	0.015	0.0667	0.9937	0.0461	0.0206	73.8

[a] The solutions were approximately isotonic; 0.02 ml. of each were instilled into the cul-de-sac of rabbits. Each parameter listed in the table is the average of four determinations on different rabbits. [b] Pearson r linear correlation coefficients for plots of log $(1 - A_t/A_\infty)$ *versus* time. [c] The values of percent BA represent the percent of the dose of drug transcorneally absorbed directly into the biophase.



Scheme III—Equivalent compartment models pertaining to the dissipation of drug from its site of administration in the eye. The symbol P *designates the compartment into which peripheral loss of the drug occurs;* B *represents the biophase;* E *is a compartment constituted by the combination of all body compartments other than those shown;* A *is the site of administration;* A_t *represents the quantity of drug transcorneally absorbed from* A *into the biophase; and* $A_t = Q_B + Q_E$, *where* $Q_E + Q_B$ *is the sum of the quantities of drug present and having been eliminated from the biophase at any time.*

The sum of the constants, $K_{AP} + K_{AB}$, represents the total proportional rate of loss of the drug from the site of administration. In accordance with Eq. 2, $K_{AP} + K_{AB}$ values can be obtained from the slopes of log $(1 - A_t/A_\infty)$ *versus* time plots; values obtained at pH 7.4 and 5.0 are compared in Table II.

If a mydriatic response attributable to peripheral drug loss had been detectable in the control eye, a similar plot of log $(1 - A_t/A_\infty)$ *versus* t performed for the control eye would be describable by Eq. 7 and identical to the results observed for the treated eye. Values of A_∞ would be calculable for both eyes, and their sum would be expected to equal the dose. Since Q_B values were obtained from the use of an intravenous dose-effect curve, the dose, D_e, computed from the sum of A_∞ values would correspond to an ophthalmically equivalent intravenous dose, which may not necessarily be the same as the actual ophthalmic dose administered. In any event, having values for D_e allows the resolution of individual values for K_{AP} and K_{AB} and a computation of the relative quantities of the drug absorbed directly into the biophase and dissipated peripherally from the application of Eq. 8 and 9:

$$\frac{K_{BO}^* \int_0^\infty Q_B\, d}{D_e} = \frac{A_\infty}{D_e} \qquad \text{(Eq. 8)}$$

$$\frac{K_{BO}^* \int_0^\infty Q_B\, dt}{D_e} = \frac{K_{AB}}{K_{AB} + K_{AP}} \qquad \text{(Eq. 9)}$$

Since no response was observed in the control eyes, D_e obviously cannot be computed from the sum of the A_∞ values computed separately for the two routes of drug loss. However, taking advantage of the kinetic indistinguishability of the biophase and central systemic compartments, as previously described for tropicamide (4), permits values of D_e to be obtained by extrapolating the latter linear segment of the log Q_B *versus* time curves forward to a value of the ordinate that corresponds to a real time, $t_{max.}$, equal to 1.88 min. The value of the ordinate at 1.88 min. on the extrapolated line provides the value of D_e. This can be understood by noting that 1.88 min. is the time corresponding to the maxima in the curves of I *versus* time obtained for rapid intravenous dosing, and that by definition: $Q_{B,\,max.} = f(I_{max.}) = \text{dose}$ (3).

Table III—Pharmacologic Response Parameters Characterizing Tropicamide-Induced Mydriasis following Ophthalmic Dosing[a]

pH of Vehicle	Tropicamide Concentration, % w/v	Onset[b]	$t_{max.}$[b]	Maximum Intensity	Duration[b]
7.4	0.010	10.5	17	0.603	58.5
7.4	0.015	8.8	20.5	0.722	61.0
7.4	0.020	9.2	22.5	0.7803	73.3
7.4	0.030	10.2	27.0	0.9363	86.0
5.0	0.015	6.1	14.5	0.695	79.9

[a] Each value represents the average of four replications on different rabbits. [b] Time in minutes.

In addition to the fact that some of the administered drug may not be absorbed either peripherally or transcorneally but simply washed out of the eye by tear fluids, the use of D_e instead of the actual ophthalmic dose is necessary because the mixing of the drug between tissues that actually comprise the biophase and the remainder of the body is less than instantaneous. When the drug is administered vicinally to the biophase, the drug is initially concentrated at the receptor sites before becoming mixed and diluted throughout the volume of the compartment. The actual ophthalmic dose absorbed, D_a, can be related to D_e by the equation: $D_e/V_s = D_o/V_B$, where V_B describes the volume of distribution of the biophase containing the drug receptor sites while V_s includes the entire distribution volume of the body that is being assumed to behave indistinguishably from the biophase. Since $V_s > V_B$, an intravenous dose hypothetically equivalent to an ophthalmic dose in producing the same sequence of mydriatic activity is correspondingly larger than an ophthalmic dose by a factor of V_s/V_B. Since the Q_B values measured for ophthalmic dosing are obtained from the intravenous dose–effect curve, they correspond to equivalent intravenous doses rather than the actual ophthalmic doses. In terms of ideal compartment theory, which assumes instantaneous mixing in all compartments, this treatment obviously presents a paradox. However, the justification for this approach is that it provides an explanation for the observed behavior and allows the calculation of quantities otherwise unattainable.

Pharmacologic Parameters Characteristic of Tropicamide Mydriasis—The onset of mydriasis (defined as the time following dosing required for the development of one-half the peak response intensity), the duration of mydriasis (defined as the time interval between one-half maximal responses), the peak response intensity, and the time of peak response are listed in Table III for each ophthalmic solution employed in the present study. The duration and maximum intensity exhibit a consistent increase with the concentration of tropicamide in the pH 7.4 solutions. Values for the onset and time of maximum intensity appear randomly variable.

Biokinetic Parameters Characterizing Tropicamide Mydriasis—The results of the biokinetic analysis of tropicamide-induced mydriasis are listed in Table II. In agreement with the assumed linear compartment models, no consistent pattern of dose dependency is apparent for the pH 7.4 buffers. The differences in the tabulated values of $K_{AP} + K_{AB}$, K_{AP}, K_{AB}, and the percent biophasic availability were found by t-tests to be statistically insignificant at well above the 5% level of confidence.

Influence of Vehicle pH on Mydriatic Response Behavior—Reference to Figs. 1 and 5 allows a comparison of the time dependency of biophasic drug levels following dosing with 0.015% w/v solutions of the drug in vehicles having a pH of 7.4 and 5.0, respectively. As indicated by the similarity in biophasic elimination half-lives listed in Table I, the postabsorptive phases of the curves are quite similar. The absorptive behavior of tropicamide at pH 7.4 and 5.0 is reflected in the log $(1 - A_t/A_\infty)$ plots shown in Figs. 4 and 5, respectively. A comparison of the pharmacological and biokinetic parameters for the 0.015% solutions of tropicamide of pH 5.0 and 7.4 showed them to be statistically insignificant at the 95% level of confidence. However, the onset, percent biophasic availability, and time of maximum effects exhibited statistical differences between confidence levels of 75 and 92%.

At pH 7.4, the drug exists in an essentially unionized form, whereas at pH 5.0 it is approximately 50% ionized. On this basis, a more rapid penetration of the drug into the cornea may be expected to occur at pH 7.4, relative to pH 5.0, due to the larger concentration of the more permeable unionized form of the drug (5, 6). If the pH of the vehicle can be assumed to have an influence at all, it can be

concluded from the values of K_{AB} and percent biophasic availability listed in Tables II and III that it is opposite to that expected on the basis of this consideration alone. The increased percent biophasic availability observed at pH 5.0 as equal to 73.8%, relative to 43.3% at pH 7.4, may be speculatively attributed to the interaction of tropicamide cations with anionic binding sites affixed to the colloids composing the corneal tissue; the tissue binding of the drug may function to retard peripheral drug loss relative to transcorneal absorption and provide a reservoir for the drug from which it may be subsequently more efficiently biophasically available. A similar mechanism was found responsible for the pH-enhanced effectiveness of procaine as a corneal anesthetic (1). The small magnitude of the effect of administering the drug at 5.0 relative to 7.4 may be a consequence of the dilution of the unbuffered pH 5.0 vehicle with lacrimal fluid, causing its rapid buffering to a physiological pH of 7.4.

SUMMARY AND CONCLUSIONS

A basis for the resolution of the rates and relative amounts of ophthalmically administered tropicamide which are transcorneally absorbed and peripherally dissipated by other routes has been described and applied using temporal pharmacological data. The previously established kinetic indistinguishability of the biophase and systemic compartments permitted the resolution to be accomplished despite the absence of a detectable mydriatic effect in the control eye. The biokinetic analysis of the pharmacological results permitted the mydriatic behavior of tropicamide to be described in terms of quantitative parameters. Although an insufficient number of replications (four replications on each of five ophthalmic solutions) was performed to draw firm, statistically based, conclusions regarding the effects of vehicle pH, the present study does exemplify a quantitative approach to the evaluation and design of ophthalmic drug vehicles.

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Cardiovascular Effects of Bulbocapnine

T. FAKOUHI, T. D. DARBY*, and R. S. McCUTCHEON†

Abstract □ Upon intravenous injection of bulbocapnine into the dog, myocardial ventricular contractile force was altered and the mean arterial blood pressure was markedly lowered. Following a 50-mg./kg. dose of bulbocapnine, the blood pressure and contractile force effects of 5-hydroxytryptamine became negligible. Injections of norepinephrine, epinephrine, and isoproterenol in doses of 1 mcg./kg. and of ethylnorepinephrine in a dose of 50 mcg./kg. showed reduced effects on mean arterial blood pressure and contractile force. The effects of none of these were reversed by bulbocapnine. Animals treated with bulbocapnine, 25 mg./kg. i.p. for 5 days, became more sensitive to injections of 5-hydroxytryptamine, norepinephrine, epinephrine, and isoproterenol, as was seen in consistently altered diastolic blood pressure and contractile force effects.

Keyphrases □ Bulbocapnine—cardiovascular effects, dogs □ Catecholamine cardiovascular activity—bulbocapnine effect □ 5-Hydroxytryptamine cardiovascular activity—bulbocapnine effect

This investigation examines the changes produced in some of the cardiovascular responses of 5-hydroxytryptamine and representative catecholamines in the dog following treatment with bulbocapnine, an alkaloid, 3,4-methylenedioxy-6-apomorphine, found in the tubers of *Corydalis cava* (1). The use of the last-named agent was of interest following the reports of Walaszek and Chapman (2) that bulbocapnine depresses

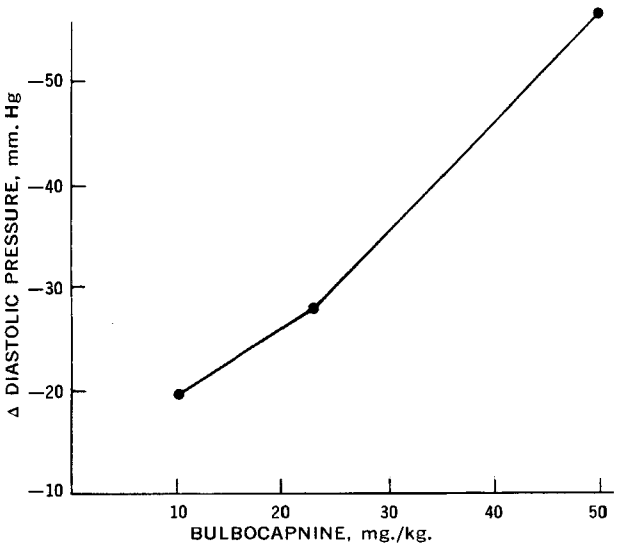

Figure 1—*Blood pressure responses to bulbocapnine. Key:* ●, *diastolic change.*

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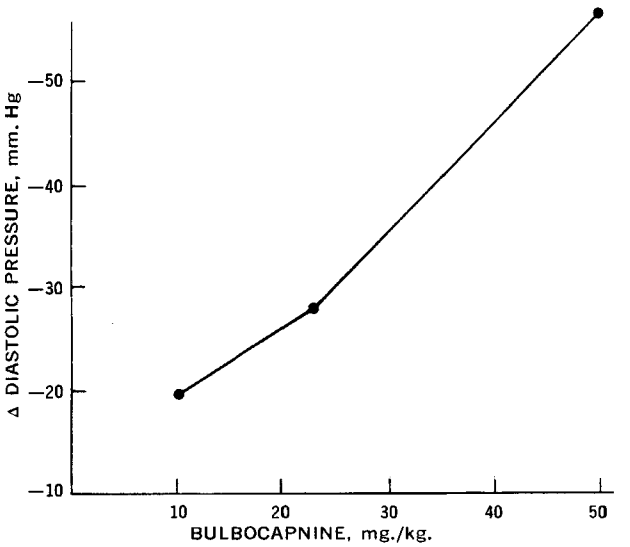

Figure 1—*Blood pressure responses to bulbocapnine. Key:* ●, *diastolic change.*

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Table I—Drug Combinations and Doses Used

Drug or Combination	Dose	No. of Dogs
Series 1[a]		
Bulbocapnine	10 mg./kg.	5
	25 mg./kg.	5
	50 mg./kg.	5

Series 2[a]

Control Agents and Dose	Followed by Bulbocapnine, 50 mg./kg.	Followed by Challenging Agent and Dose	No. of Dogs
5-Hydroxytryptamine, 5, 10, 15, or 20 mcg./kg.	↓	5-Hydroxytryptamine, 5, 10, 15, or 20 mcg./kg.	6
Norepinephrine, 1 mcg./kg.	↓	Norepinephrine, 1 mcg./kg.	6
Epinephrine, 1 mcg./kg.	↓	Epinephrine, 1 mcg./kg.	6
Isoproterenol, 1 mcg./kg.	↓	Isoproterenol, 1 mcg./kg.	6
Ethylnorepinephrine, 50 mcg./kg.	↓	Ethylnorepinephrine, 50 mcg./kg.	6

Series 3[a,b]

	Dose	No. of Dogs
Chronic bulbocapnine	50 mg./kg.	6
Followed in separate random steps by:		
5-Hydroxytryptamine	5, 10, 15, or 20 mcg./kg.	
Norepinephrine	1 mcg./kg.	
Epinephrine	1 mcg./kg.	
Isoproterenol	1 mcg./kg.	
Ethylnorepinephrine	50 mcg./kg.	

[a] The responses measured were mean arterial blood pressure (systolic + diastolic/2) or diastolic pressure, contractile force, electrocardiogram, serum and tissue potassium. [b] Followed in all experiments by a 25-mg./kg. i.v. dose of bulbocapnine. 5-Hydroxytryptamine, norepinephrine, epinephrine, isoproterenol, and ethylnorepinephrine in doses mentioned above, respectively.

same authors reported that bulbocapnine also potentiates some of the actions of histamine (4), it was useful to check further on its relationship with various amines. The responses produced by the interactions of these agents may increase understanding of the mechanism of action of bulbocapnine. They may also elucidate the

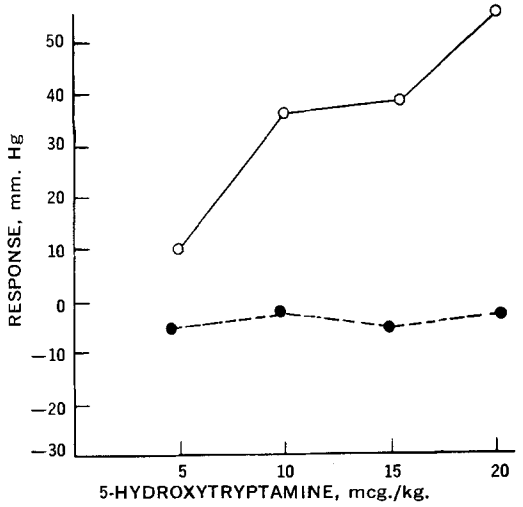

Figure 2—*Blood pressure responses to 5-hydroxytryptamine. Key: O, diastolic change, control; and ●, diastolic change after 50 mg./kg. bulbocapnine.*

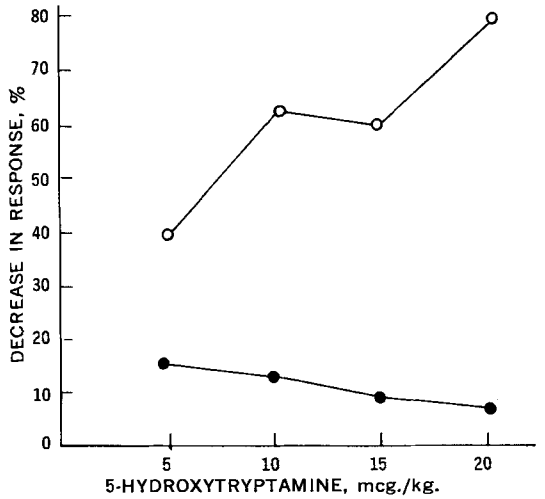

Figure 3—*Contractile force responses to 5-hydroxytryptamine. Key: O, control; and ●, after 50 mg./kg. bulbocapnine.*

cardiovascular physiology of 5-hydroxytryptamine and aid in a logical interpretation of some of its effects.

EXPERIMENTAL

Healthy mongrel dogs of either sex, weighing between 7 and 15 kg., were anesthetized with pentobarbital sodium, 30 mg./kg. i.v., and ventilated with a Harvard respirator attached through a tracheal cannula. Mean arterial blood pressure was recorded from a polyethylene catheter inserted in the carotid artery and connected to a Sanborn model 150 amplifier system or a Grass model 5 polygraph through either a Statham or Sanborn transducer. Myocardial contractile force was measured by means of a Walton-Brody strain gauge arch sutured to the right ventricle through a right thoractomy (5) and was recorded with the Sanborn or Grass instrument. Electrocardiographic recordings were made during each experiment using lead (II). The external jugular vein was cannulated for administration of drugs, all of which were washed-in with 2–5 ml. of normal saline. Serum and tissue potassium determinations were made with the Beckman 21 flame photometer.

Measurements of mean arterial blood pressure, contractile force, and EKG were made in several different series of animals, according to the schedule shown in Table I. Of the three series of experiments, the first is a determination of the responses to bulbocapnine alone;

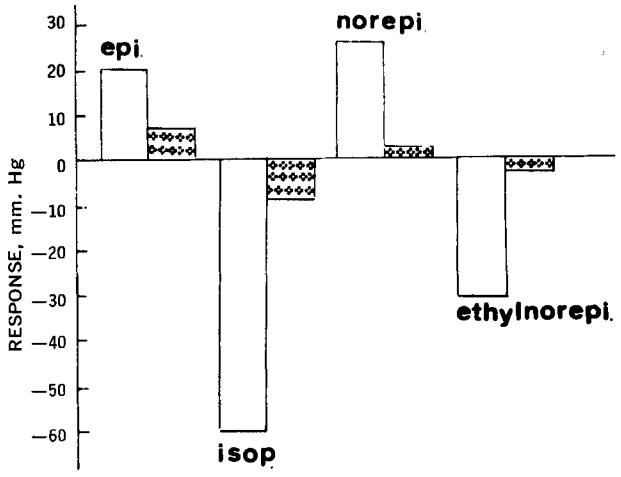

Figure 4—*Blood pressure responses to: epinephrine, 1 mcg./kg. (epi.); isoproterenol, 1 mcg./kg. (isop.); norepinephrine, 1 mcg./kg. (norepi.); and ethylnorepinephrine, 50 mcg./kg. (ethylnorepi.). Key: □, diastolic change, control; and ⊠, diastolic change after 50 mg./kg. bulbocapnine.*

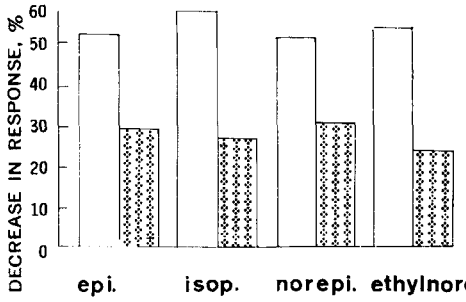

Figure 5—*Contractile force responses to: epinephrine, 1 mcg./kg. (epi.); isoproterenol, 1 mcg./kg. (isop.); norepinephrine, 1 mcg./kg. (norepi.); and ethylnorepinephrine, 50 mcg./kg. (ethylnorepi.). Key: □, control; and ⊠, contractile force after 50 mg./kg. bulbocapnine.*

the second measures the responses produced by the amines listed, given 30 min. after bulbocapnine; and the third gives the responses to the amines in the chronically bulbocapninized dog. In this last series, bulbocapnine was given to the animals for a 5-day period. On the 6th day, the animal was anesthetized with pentobarbital sodium, 30 mg./kg. i.v., and prepared surgically to record mean arterial blood pressure and myocardial contractile force. In this way it was hoped to obtain additional evidence to determine whether or not bulbocapnine was exerting its effects by releasing 5-hydroxytryptamine or catecholamine centrally, peripherally, or both.

Controls were obtained for every dose level of each drug used and consisted of an average of no less than five determinations. All responses were allowed to return to normal before subsequent injections.

All drugs were administered intravenously unless otherwise noted. The amines used in the study were dissolved in a solution of 0.1% each sodium bisulfite and chlorbutanol as preservative. Bulbocapnine was prepared just before use in each experiment. To determine the significance of the data, standard error and *t*-tests were performed (6).

RESULTS

In the first series of animals, the different levels of bulbocapnine caused no significant change in the contractile force but did produce a consistent, marked depression of diastolic blood pressure, which varied in proportion to the dosage used (Fig. 1). Electrocardiographic changes produced by the drug were seen as a prolongation of the P wave and broadening and lengthening of the QRS complex. These changes resembled those commonly seen with alterations of serum potassium levels. The level of serum potassium, determined every 10 min. during the experiment, did not show any significant change.

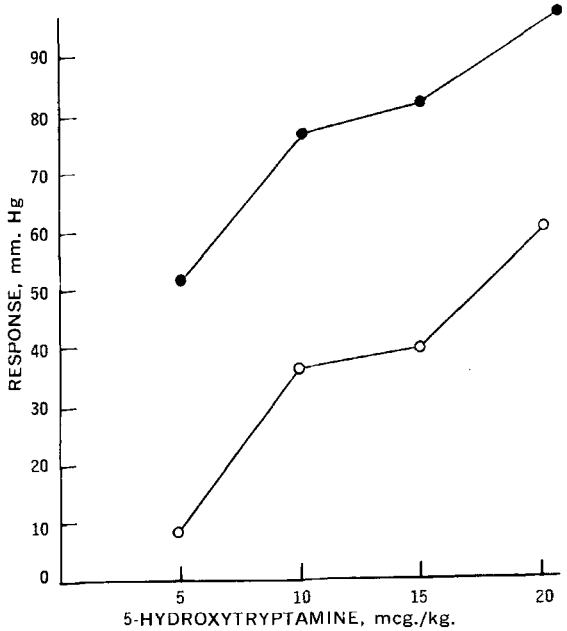

Figure 6—*Blood pressure responses to 5-hydroxytryptamine. Key: ○, diastolic change, control; and ●, diastolic change after chronic administration of bulbocapnine, 25 mg./kg. for 5 days.*

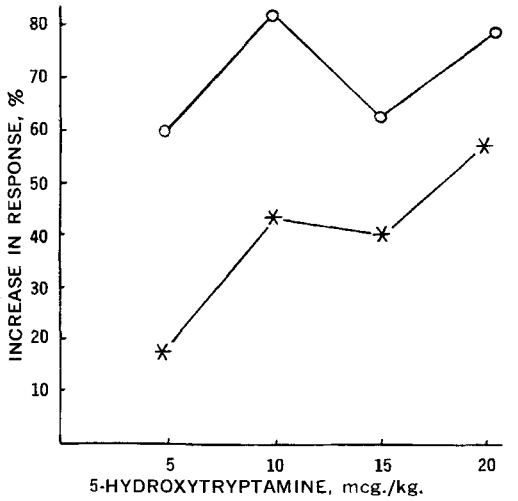

Figure 7—*Contractile force responses to 5-hydroxytryptamine. Key: *, control; and ○, contractile force after chronic administration of bulbocapnine, 25 mg./kg. for 5 days.*

The second series of experiments showed that the pressor responses produced by 5-hydroxytryptamine in the control studies were abolished by pretreatment with bulbocapnine (Figs. 2 and 3). The responses normally produced by each of the other amines were inhibited, but at no time was there evidence of a reversal of the effect of epinephrine or ethylnorepinephrine (Fig. 4). The contractile force responses of each of these agents were also reduced in every case (Fig. 5).

In the third series of experiments, the chronically bulbocapninized animals showed no physical signs or symptoms on the 6th day. The mean arterial blood pressure and contractile force responses were not different than normal. In the third series of experiment, it was found that mean arterial blood pressure and contractile force responses to 5-hydroxytryptamine given to the chronically bulbocapninized dog, on the 6th day, were greatly enhanced (Figs. 6 and 7). The action of the catecholamine, following the chronic administration of bulbocapnine, showed no significant change in

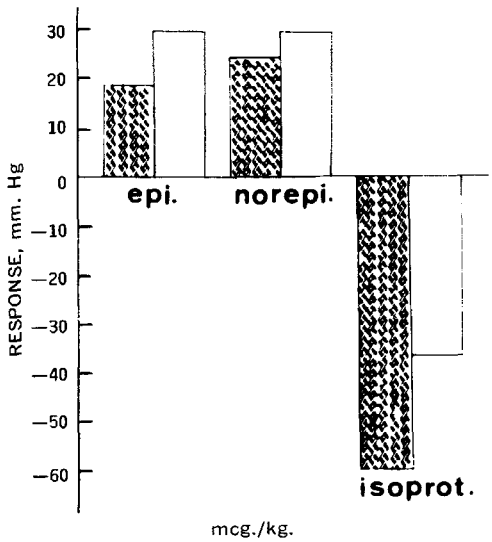

Figure 8—*Blood pressure responses to: epinephrine, 1 mcg./kg. (epi.); norepinephrine, 1 mcg./kg. (norepi.); and isoproterenol, 1 mcg./kg. (isoprot.). Key: ■, diastolic change, control; and □, diastolic change after chronic administration of bulbocapnine, 25 mg./kg. for 5 days.*

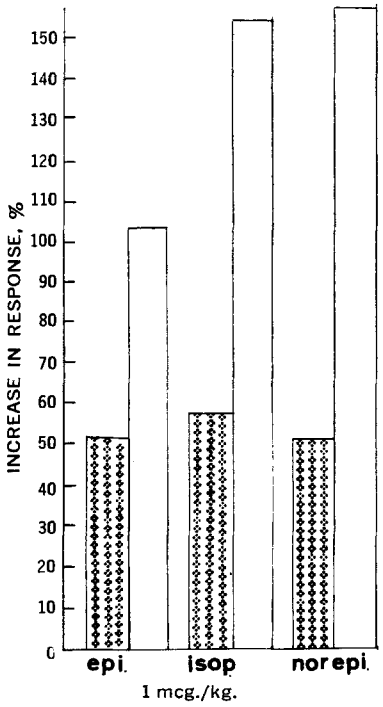

Figure 9—*Contractile force responses to: epinephrine, 1 mcg./kg. (epi.); isoproterenol, 1 mcg./kg. (isop.); and norepinephrine, 1 mcg./kg. (norepi.). Key: ▨, control; and □, contractile force after chronic administration of bulbocapnine, 25 mg./kg. for 5 days.*

regard to mean arterial blood pressure, but the contractile force effects were greatly potentiated (Figs. 8 and 9). An acute injection of 25 mg./kg. of bulbocapnine given to the chronically treated animals established a complete inhibition of the pressor response of 5-hydroxytryptamine, as well as partial inhibition of catecholamine responses, as seen in the earlier experiments.

DISCUSSION

Although the role of 5-hydroxytryptamine is still relatively unknown, evidence is accumulating to suggest that it may be a mediator in various nervous systems. The explanation of the interaction of bulbocapnine and 5-hydroxytryptamine is also equivocal. Walaszek and Chapman (2) based some of their interpretation of the action of bulbocapnine on the mediator concept and suggested that it may be an α-adrenergic blocking agent. This conclusion followed the reversal of the blood pressure response to epinephrine (7, 8). The present work failed to confirm their explanation because the reversal of epinephrine was not seen, the reduced response generally appearing to be a nonspecific depressant effect of bulbocapnine. Bulbocapnine also failed to have the characteristic blocking effect on ethylnorepinephrine (9), which one would expect of an α-adrenergic blocking agent. Consequently, the mechanism of effects of bulbocapnine on blood pressure must be explained in some other way; but the results of the present experiments and of others may indicate a rationale for its action.

It is possible that bulbocapnine may have a relatively nonspecific effect. This may be a direct action or be mediated by means of the inactivation of histaminase (4).

The present thinking relates to the peripheral storage of 5-hydroxytryptamine and its release by certain agents from storage sites (10), as well as the histaminase-inactivating action of bulbocapnine. One can postulate that bulbocapnine, by allowing an accumulation of histamine in the system, could cause an increased cellular permeability of the storage sites of 5-hydroxytryptamine as well as catecholamines. This may initiate an inhibitory action by increasing the uptake of these agents by tissue stores (11). Such uptake may more likely be selective for 5-hydroxytryptamine than for catecholamines. The blood pressure and contractile force, ordinarily stimulated by the effects of the catecholamines, are consistently inhibited but not reversed after bulbocapnine.

The responses produced by 5-hydroxytryptamine and catecholamines in chronically treated bulbocapninized animals resembled those produced by catecholamines in reserpinized animals (12, 13). Here, however, the depletion seems to be of 5-hydroxytryptamine as well as catecholamines. It is possible that bulbocapnine, acting for a prolonged period, enhances the depletion of 5-hydroxytryptamine from its receptors and then, through a sustained uptake of 5-hydroxytryptamine by tissue storage sites, makes the peripheral receptors sensitive to subsequent doses of free 5-hydroxytryptamine. Whatever the explanation, such an action by bulbocapnine has not been reported previously.

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The bulbocapnine used in this study was obtained from Merck and Co. and was prepared fresh for each experiment.

This study was initiated at the Department of Pharmacology, School of Medicine, University of West Virginia, Morgantown, W. Va., and was conducted at the Department of Pharmacology, Pahlavi University, Shiraz, Iran.

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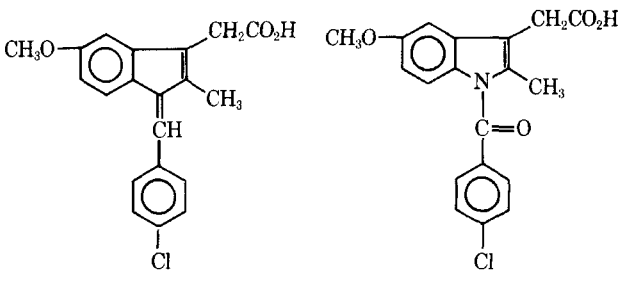
Physiologic Disposition and Metabolic Fate of Indomethacin Analogs I: 1-*p*-Chlorobenzylidenyl-5-methoxy-2-methylindene-3-acetic Acid

HOWARD B. HUCKER and ELIZABETH A. HOFFMAN

Abstract □ The title compound was well absorbed after oral administration to the dog and rat. About 70–90% of a single dose was excreted in the urine and feces in 4 days, with fecal elimination being predominant. Peak plasma levels were observed in both species at 1–2 hr. after oral administration. The plasma half-life was 1–2 and 4 hr. in the dog and rat, respectively. The compound was widely distributed but not concentrated in rat tissues and was highly bound to plasma proteins. About 35–44% of the dose was excreted in dog and rat bile, with excretion in the dog appearing to be inversely proportional to dose. The acyl glucuronide of the title compound was identified as a major metabolite in dog bile. Significant localization of the title compound and/or its metabolites in inflamed rat tissue was also demonstrated.

Keyphrases □ Indomethacin, indene analogs—physiologic disposition and metabolites, dogs, rats □ 1-*p*-Chlorobenzylidenyl-5-methoxy-2-methylindene-3-acetic acid—physiologic disposition and metabolites, dogs, rats □ GLC—determination

Several pharmacologically active compounds similar to indomethacin[1] have been discovered to date. An indene analog, 1-*p*-chlorobenzylidenyl-5-methoxy-2-methylindene-3-acetic acid (I), synthesized by Shen *et al.* (1–3), has anti-inflammatory activity nearly the same as indomethacin but reduced gastrointestinal effects[2]. Pharmacologic activity of I is at least partly stereospecific, since the *trans*-isomer, in which the *p*-chlorophenyl ring is opposite the phenyl ring of the indole nucleus, is only one-fifth as active as the *cis*-form. I is isosteric with indomethacin. Unlike the *N*-acyl indoles, it is stable under acidic and alkaline conditions. The present report describes studies on the absorption, excretion, tissue distribution, and metabolic fate of the *cis*-isomer of I.



I indomethacin

METHODS AND MATERIALS

I labeled with ^{14}C in the benzylidene bridge carbon atom, with a specific activity of 1.7 μc./mg., was used. The melting point (168–169°) and UV absorption spectrum in methanol (λ_{max}. 238, 288, and 340 nm.) were identical with the values observed for the authentic drug. Radioactive I showed a single component after paper chromatography.

Absorption and Excretion Studies—Mature male Sprague-Dawley rats and beagle dogs of both sexes were fasted overnight prior to dosing. I was administered to rats in solutions prepared by dissolving the compound in a small volume of dimethylacetamide. A solubilizing agent[3] was added to this solution before diluting with saline. The ratio of the three solvents was approximately 1:3:100 (v/v) at drug concentrations of about 3 mg./ml. I was administered to dogs in methylcellulose[4] suspensions for excretion studies and in capsules for plasma level determination. After drug administration, animals were placed in individual stainless steel cages designed for separate collection of urine and feces. Urine was frozen as collected in dry ice-cooled containers. Rats were allowed standard laboratory chow and water *ad libitum*. Dogs were fed commercial dogfood once daily with water available *ad libitum*.

Biliary Excretion Studies—Bile was collected from unanesthetized dogs 1 week after surgical preparation of bile fistulas, as previously described (4). The common bile duct of a rat was also cannulated with polyethylene tubing under methohexital anesthesia. The incision was closed, and the animal was allowed to recover before dosing.

Inflamed Tissue Studies—Left hind feet of six male rats were injected with 0.1 ml. of a 10% suspension of brewer's yeast. After 2 hr., the animals were given I-^{14}C orally (10 mg./kg). One hour later, the rats were sacrificed and both hind feet were cut off, weighed, and dissolved in 9 volumes of 1 *N* KOH with heat. After cooling, the solutions were assayed for radioactivity content.

Determination of Radioactivity—Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Aliquots of plasma and urine (0.1–0.5 ml.) were added directly to the scintillation fluid for assay of ^{14}C. The scintillation solution consisted of 80 g. of naphthalene, 7 g. of 2,5-diphenyloxazole, 0.3 g. of 1,4-bis-2-(5-phenyloxazolyl)-benzene, and 30 g. of a thixotropic gel[5] in 1 l. of dioxane. Bile was diluted 200 times with water before counting. Feces and tissues were homogenized in a suitable volume of water, and an aliquot was decolorized prior to counting (5). Red cells were diluted 15 times with water, and an aliquot was decolorized prior to counting. The counting efficiency was determined by internal standardization. In the case of particulate samples, the counting efficiency was also estimated by the channels ratio method (6).

Determination of I—I was measured in biological samples by either of the following procedures.

Procedure A—One milliliter of plasma or urine was added to 2 ml. of 1 *N* hydrochloric acid and 25 ml. of heptane (containing 3% isomayl alcohol) in a 45-ml. glass-stoppered centrifuge tube. The tube was shaken for 10 min. and centrifuged, and 20 ml. of the organic phase was transferred to a similar tube containing 5 ml. of 0.1 *N* sodium hydroxide. After shaking for 5 min., the tube was centrifuged and the organic phase was removed by aspiration. Two milliliters of the aqueous phase was counted if radioactive samples were assayed. Otherwise, the absorbance of the alkaline solution was measured at 337 nm. and used to calculate I in the sample. I added to plasma and urine in amounts of 1–25 mcg. was recovered with adequate precision (mean ± *SE* = 82 ± 2% for plasma; mean ± *SE* = 90 ± 2% for urine). Recoveries of 5–100 mcg. amounts of I added to tissue homogenates were somewhat lower but of adequate precision (mean ± *SE* = 68 ± 3%).

Procedure B—Nonradioactive drug was also measured by GLC. The 5-fluoro analog of I was used as a mass internal standard as described by Sparagana (7). The instrument, Hewlett-Packard model 810 with dual-flame detectors, was equipped with coiled glass columns [1.8 m. × 0.64 cm. (6 ft. × 0.25 in.)] packed with 3%

[1] Registered under the tradename of Indocin, Merck & Co., Inc.
[2] Unpublished data of Dr. C. A. Winter and Dr. D. A. Brodie.
[3] Emulphor-EL620, General Aniline and Film Corp.
[4] Methocel.
[5] Cab-O-Sil.

Table I—Excretion of Radioactivity by Rats and Dogs after Administration of Labeled I[a]

Sample	Day	Rat		Dog
		Oral Dose	Intravenous Dose	Oral Dose
Urine	1	4.2 (2.6–5.7)	6.4 (5.3–8.0)	4.9 (3.0–6.8)
	2	3.2 (1.8–5.3)	3.0 (2.2–3.6)	1.4 (0.3–3.7)
	3	1.2 (0.7–1.5)	1.5 (1.0–2.0)	0.3 (0.1–0.6)
	4	0.8 (0.4–1.2)	0.4 (0.3–0.5)	—
	Total	9.4 (6.9–13.1)	11.3 (8.8–12.8)	6.6 (3.4–10.7)
Feces	1	1.4 (0.5–2.8)	3.6 (1.3–5.2)	44.8 (0.4–81.6)
	2	36.3 (33.9–39.2)	35.1 (27.3–41.8)	25.8 (2.4–73.0)
	3	15.7 (7.9–21.3)	17.1 (11.1–21.8)	12.0 (1.9–42.0)
	4	8.2 (4.3–16.7)	5.9 (3.2–8.0)	—
	Total	61.6 (59.0–63.8)	61.7 (57.5–66.9)	82.6 (75.4–91.5)
Total		71.0 (69.1–72.1)	73.0 (66.6–78.6)	89.2 (78.8–96.7)

[a] Four rats per group were given 3 mg./kg., and four dogs received 0.1 mg./kg. Results are expressed as percent of administered dose, with range shown in parentheses.

QF-1 on Chromasorb G, 100–120 mesh[6]. Operating conditions were as follows: oven, 260°; injection port, 295°; detector, 305°; and helium flow, 50 ml./min.

The procedure used was as follows. One milliliter of plasma was added to a 15-ml. glass-stoppered centrifuge tube with a finely tapered tip[7] containing 5 mcg. of 5-fluoro-I (in 0.05 ml. of ethanol) and 0.1 ml. of 6 *N* HCl. The tube was mixed for 1 min. (Vortex mixer) and centrifuged. The heptane phase was transferred to a similar tube, mixed for 1 min. with 1 ml. of 1 *N* sodium hydroxide, and centrifuged. The aqueous phase was transferred to a third tube, mixed for 1 min. with 0.1 ml. of 6 *N* HCl and 0.1 ml. chloroform, and centrifuged. After careful removal of the aqueous phase by aspiration, the chloroform extract was evaporated to dryness in a stream of nitrogen with the tube immersed in warm water. The residue was dissolved in 30 μl. of ether and treated with 3 μl. of an ethereal solution of diazomethane (generated from *N*-nitrosomethylurea). After 5 min., the ether and excess reagent were removed by placing the tube in warm water. The residue was dissolved in 10 μl. of ether, and 2 μl. was injected into the chromatograph. The retention times of the methyl esters of I and the 5-fluoro internal standard were 5.7 and 3.5 min., respectively.

A standard curve was constructed by plotting the peak height ratios *versus* concentration ratios of I to the internal standard. The peak height ratio obtained from unknowns was then used to estimate the amount of I present.

TLC—TLC was performed on commercially prepared fluorescent silica gel GF-coated glass plates. The coating thickness was 250 μ. Reference compounds were located as dark spots under UV light. Radioactive compounds were detected with a Packard model 7201 radiochromatogram scanner.

RESULTS

Absorption and Excretion—The excretion of radioactive material in the urine and feces by rats after oral and intravenous administration of I (3 mg./kg.) is shown in Table I. A major fraction (61%) of the dose was excreted in the feces, with only minor amounts (10%) appearing in the urine. The similarity in excretory patterns of oral and intravenous doses demonstrated that I was completely absorbed in the rat. Rats given a higher dose of I orally (25 mg./kg.) excreted 15 and 29% of the dose in the urine and feces, respectively, in 72 hr.

An even higher fraction (82%) of an oral dose was excreted in dog feces (Table I), but this finding did not indicate poor oral absorption since the drug was at least partly excreted in dog bile. The excretory pattern in the dog was similar to that obtained in rats. The drug was also well absorbed in guinea pigs, since 24% of orally administered I was excreted in the urine in 72 hr. compared to 37% after intraperitoneal administration.

Plasma Levels of I—The concentrations of I in plasma of rats given labeled I orally and intravenously (3 mg./kg.) are shown in Table II. When the plasma was extracted as described in *Procedure A*, all of the radioactivity was recovered. TLC of these extracts gave only a single radioactive peak, which was identical with that of authentic I, as shown in Table III. These results indicated that only unchanged I was present in rat plasma. Levels reached a maximum at 1–4 hr. after oral administration. The half-life of drug in the plasma after an intravenous dose was approximately 4.5 hr.

Plasma levels of I in dogs given a single dose of the drug orally (20 mg./kg.) are shown in Table II. Concentrations were maximal after 1–2 hr. and were nearly equal to those found in rats given a much lower dose. The levels declined thereafter, with an apparent half-life of 1–2 hr. Plasma levels of I in dogs given a single oral dose (20 mg./kg.) of the drug as the sodium salt were significantly lower at 2 hr. than levels in those animals given I as the free acid.

Plasma concentrations of I after administration of multiple doses to rats and dogs are shown in Table IV. As indicated, plasma levels in rats given multiple doses of I were significantly lower than concentrations obtained after single doses. Levels in dogs given multiple doses of I were not significantly different from those observed after a single dose, except at 2 hr. when the multiple-dose level was significantly lower ($p < 0.05$).

Table II—Plasma Concentrations of I in Rats and Dogs after a Single Dose of Labeled I[a]

Hours	Rat		Dog	
	Intravenous Dose	Oral Dose	Oral Dose (FA)	Oral Dose (Na)
0.25	23.4 ± 1.3	—	—	—
0.5	21.1 ± 1.4	6.3 ± 0.2	—	—
1	17.6 ± 1.4	12.4 ± 2.0	10.6 ± 3.1	10.2 ± 0.9
2	15.7 ± 1.0	11.6 ± 1.0	11.7 ± 2.0	4.8 ± 0.3
4	13.1 ± 1.3	11.9 ± 0.1	1.4 ± 0.4	1.0 ± 0.1
6	11.4 ± 1.0	9.4 ± 2.0	1.7 ± 0.7	1.9 ± 0.2
16	—	7.3 ± 0.3	—	—
24	6.1 ± 1.0	10.2 ± 2.4	0.4 ± 0.1	0
48	2.4 ± 0.3	2.4 ± 1.0	—	—

[a] Approximately 20 rats in each group received 3 mg./kg. Eight dogs received 20 mg./kg. as the free acid (FA) or sodium salt (Na). Results are expressed as micrograms of I per milliliter (mean ± *SE*). Plasma of rats was assayed by *Procedure A* (*Methods*) and that of dogs by *Procedure B* (*Methods*).

Table III—TLC of Apparent I Extracted from Rat Tissues and Authentic Drug[a]

Compound	R_f				
	Solvent A	Solvent B	Solvent C	Solvent D	Solvent E
I	0.51	0.74	0.59	0.33	0.54
Apparent I	0.51	0.74	0.59	0.33	0.54

[a] Apparent I was extracted (*Procedure A*) from the liver, small intestine, stomach, and plasma of rats 2 hr. after administration of I-^{14}C. The solvent systems used were as follows: A, 2-propanol–15 *N* ammonia (8:2); B, 2-propanol–acetic acid (91:5); C, chloroform–acetic acid (95:5); D, 1-butanol saturated with water; and E, ethyl acetate–2-propanol–5 *N* ammonia (5:4:3).

[6] Applied Science Labs, Inc.
[7] Pesce Glass Co., Kennett Square, Pa.

Table IV—Plasma Levels of I in Rats and Dogs after Multiple Daily Doses[a]

Hours	Rat		Dog	
	Single Dose	Multiple Dose	Multiple Dose (20)	Multiple Dose (40)
0.5	—	—	2.8 ± 2.0	7.1 ± 3.8
1	—	—	6.2 ± 3.1	8.0 ± 3.6
2	128.2 ± 7.0	66.1 ± 18.1	3.9 ± 0.8	5.1 ± 1.3
4	97.3 ± 2.9	66.2 ± 5.4	3.0 ± 2.1	2.1 ± 1.4
6	99.9 ± 1.2	63.7 ± 1.4	0	0
24	—	—	0	0

[a] Twelve rats received 30 mg./kg. of I orally daily for 10 days, after which they received, along with 12 additional rats, a single oral dose (30 mg./kg.) of I-^{14}C. Differences between groups were significant ($p < 0.05$) at all times. Four dogs in each group received daily oral doses of 20 or 40 mg./kg. for 6 weeks. Results are expressed as micrograms per milliliter (mean ± *SE*). Levels in rats were determined by counting whole plasma; levels in dogs were measured by *Procedure A* (*Methods*) spectrophotometry.

Tissue Distribution—The distribution of radioactivity and apparent I in tissues of the rat 1 and 24 hr. after a single oral dose of I is shown in Table V. Only the stomach and small intestine showed levels of radioactivity greater than that in plasma after 1 hr. Since these measurements included the contents of each organ, the high levels present are undoubtedly associated, at least in part, with unabsorbed drug. However, the finding that only a fraction of the radioactivity present could be accounted for as apparent unchanged I (Table III) is of interest. This was true of most other tissues as well at 1 hr. after drug administration, except for plasma which contained a high percent of unchanged I. After 24 hr., tissue levels had declined markedly in most instances, but the fraction of apparent I present after 24 hr. was higher than at 1 hr. The decline in plasma levels was smaller than that observed in tissues.

Binding of I to nondiffusible constituents of human plasma was measured by equilibrium dialysis for 18 hr. at 37°. The drug was completely bound over a concentration range of 1–25 mcg./ml.

Localization in Inflamed Tissue—Concentrations of radioactive material were measured in normal and inflamed hind limbs of rats given labeled I as described in the *Methods* section. Inflamed tissue contained 5.5 ± 1.3 mcg./g. (mean ± *SE*) of radioactivity (expressed as I) compared to a concentration of 3.1 ± 0.6 mcg./g. in normal tissue. The difference was significant ($p < 0.05$) as shown by the paired *t*-test; levels in inflamed tissue were higher than those of control tissues in each of six rats studied.

Biliary Excretion—The excretion of radioactive material in the bile of the dog and rat after oral administration of labeled I is shown in Table VI. As shown, biliary excretion appeared to be highly dose dependent in the dog; 44% of a very low dose (0.02 mg./kg.) was recovered in the bile in 7 hr., whereas only 5% of a relatively high dose (120 mg./kg.) was thus excreted. About 19% of an intermediate dose (20 mg./kg.) was recovered in the bile, whereas 34% of a similar dose to rats (30 mg./kg.) was present in bile collected for 7 hr.

Table VI—Biliary Excretion of Radioactivity in the Dog and Rat after Oral Administration of I-^{14}C[a]

Hours	Dose Recovered, %			
	Dog A	Dog B	Dog C	Rat
1	4.8	4.9	0.0	4.1
2	7.6	7.0	0.6	4.8
3	7.1	5.3	2.0	5.6
4	10.2	1.8	0.7	6.1
5	4.5	0.2	0.6	5.2
6	5.3	0.2	1.3	4.6
7	4.4	—	—	3.9
1–7	43.9	19.4	5.2	34.3

[a] Drug was administered to Dog A in methylcellulose suspension (0.02 mg./kg.), to Dog B in saline–dimethylacetamide (20 mg./kg.), and to Dog C in capsule (120 mg./kg.). The rat received the drug in saline–dimethylacetamide (30 mg./kg.).

Metabolites of I—Analysis of urine of rats given I-^{14}C orally (25 mg./kg.) indicated that 30, 20, and 15% of the urinary radioactivity in the 0–24-hr., 24–48-hr., and 48–72-hr. samples were present as apparent unchanged I, respectively. Analysis of feces of rats given I-^{14}C orally or intravenously (3 mg./kg.) indicated that 50–95% of the fecal radioactivity was present as apparent unchanged I.

No labeled carbon dioxide was detected in the expired air of rats given I-^{14}C (3 mg./kg. i.v.), suggesting that the benzylidene bridge portion of the molecule was stable to further oxidation.

Bile of a dog given I-^{14}C orally (20 mg./kg.; 1–2-hr. sample) was examined for the presence of metabolites by TLC. Analysis of this bile sample for I showed that unchanged drug accounted for about 20% of the radioactivity in the sample. A 50-μl. aliquot was spotted on a silica gel plate and developed in chloroform–acetic acid (95:5). Two peaks were revealed on radiometric scanning, a major one at R_f 0.00 and a minor one at R_f 0.75. The R_f of authentic I in this system was 0.62.

Extraction with ethyl acetate of dog bile adjusted to pH 5 removed 82% of the radioactivity present. TLC of the ethyl acetate extract gave the results shown in Table VII.

Two radioactive peaks (three with Solvent B) were detected, one of which corresponded to unchanged I. In addition to scanning for radioactivity, plates developed in the five solvent systems were subjected to one or both of the following tests: (*a*) spraying with naphthoresorcinol (8), and (*b*) scraping off the radioactive metabolite area and testing for glucuronic acid (9). Control bile was also chromatographed and subjected to tests, with negative results in all cases.

Plates developed in the alkaline Solvent Systems B, C, and E gave a positive test on spraying with naphthorescorcinol, whereas plates developed in the acidic Solvent Systems A and D gave negative results. The blue spot produced, characteristic of glucuronic acid, had an R_f value similar to that of authentic glucuronic acid in the three solvents (0.14, 0.03, and 0.02, respectively, compared to 0.06, 0.66, and 0.08 for glucuronic acid). These results indicated

Table V—Tissue Distribution of Radioactivity after Oral Administration of I-^{14}C to Rats[a]

Tissue	^{14}C Levels			
	1 hr.		24 hr.	
	Total[b]	I[c]	Total[b]	I[c]
Liver	79 (62–95)	17 (16–18)	13 (12–14)	8 (3–12)
Lung	37 (36–38)	13 (12–14)	10 (10–10)	12 (8–15)
Heart	35 (34–36)	13 (10–16)	9 (8–9)	7 (5–8)
Testes	15 (14–16)	6 (4–8)	6 (5–6)	5 (5–5)
Spleen	18 (16–18)	6 (6–7)	4 (4–4)	2 (2–2)
Kidney	51 (48–54)	26 (24–27)	16 (14–17)	8 (6–9)
Stomach	509 (477–542)	156 (112–199)	20 (14–26)	6 (4–8)
Muscle	16 (14–18)	9 (8–10)	4 (3–4)	3 (2–4)
Fat	22 (17–27)	12 (10–15)	4 (4–6)	6 (4–7)
Small intestine	159 (127–191)	66 (36–96)	32 (26–33)	20 (14–27)
Brain	9 (8–10)	4 (4–4)	2 (2–2)	2 (2–2)
Red cells	44 (40–49)	15 (12–18)	4 (3–4)	5 (3–6)
Plasma	134 (132–137)	107 (104–110)	52 (51–53)	34 (34–35)

[a] Average and range for two rats at each time interval; dose was 32 mg./kg. p.o. [b] Total radioactivity, expressed as micrograms of I per gram tissue. [c] Apparent I, isolated as described in *Methods*, *Procedure A*. Values are corrected for recovery obtained with each tissue and expressed as micrograms per gram tissue.

Table VII—TLC of Bile of Dog Given I-^{14}C (20 mg./kg.) Orally[a]

Sample	R_f Solvent A	Solvent B	Solvent C	Solvent D	Solvent E
Bile	0.00; 0.58	0.48; 0.57; 0.89	0.68; 0.45	0.74; 0.23	0.0; 0.33
I	0.58	0.55	0.54	0.71	0.26

[a] The solvent systems used were as follows: A, chloroform–acetic acid (95:5); B, ethyl acetate–isopropyl alcohol–20% ammonia (5:4:3); C, isopropyl alcohol–15 *N* ammonia (8:2); D, isopropyl alcohol–acetic acid (95:5); and E, *n*-butanol saturated with ammonia.

that glucuronic acid was released from the metabolite on exposure to alkali.

The metabolite spots at R_f 0.00 and 0.23 in Solvents A and D, respectively, were scraped into 0.1 *N* NaOH and kept for 10 min. at room temperature. The mixture now gave a positive test for glucuronic acid by the second method.

In addition, the metabolite present in dog bile was shown to be converted to I on exposure to dilute alkali in the following experiment. Bile (0.2 ml.) from a dog given 20 mg./kg. of I (Table VI) was added to 0.8 ml. of 0.1 *N* NaOH, and the solution was kept at room temperature for 10 min. The sample was then acidified and assayed for I as described in the *Methods* section. Samples not treated with base were assayed concurrently. The results showed that the percent of apparent I in the bile increased markedly after exposure to the base in all samples, except the 1-hr. sample which apparently contained mainly unchanged I. The fraction of the total radioactivity present represented by I increased from 24 to 94% in the 2-hr. sample, from 19 to 90% in the 3-hr. sample, from 28 to 71% in the 4-hr. sample, and 53 to 93% in the 6-hr. sample.

Likewise, bile exposed to dilute alkali (2-hr. sample) gave only one radioactive peak, at R_f 0.63, when subjected to TLC in Solvent A, which corresponded in R_f value to I (R_f 0.66). The radioactive peak at R_f 0.00 (Table VII) was no longer observed.

DISCUSSION

The title compound, an indene isostere of indomethacin, was well absorbed after oral administration to the rat, excretion patterns after oral and intravenous doses being nearly identical. Drug was detected in plasma 0.5–1 hr. after oral administration and reached maximal levels at 1–2 hr. Absorption appeared to be more prolonged after administration of higher doses to rats.

Plasma half-lives of 4.5 and 1–2 hr. in the rat and dog indicated that I was metabolized more rapidly in the dog. The drug was widely distributed throughout rat tissues.

A large fraction of either an oral or intravenous dose of I was excreted in the feces of rats and dogs. Biliary excretion of the compound was extensive and appeared to be inversely proportional to the dose; saturation of the biliary excretion mechanism is possible (10, 11). The acyl glucuronide of I was apparently a major metabolite of the drug in dog bile. I was not localized to a significant extent in inflamed tissues of rats.

Comparison of the results reported here for I with those previously reported for indomethacin (12) permits estimation of the effect of: (*a*) substitution of an indene nucleus for the indole, and (*b*) substitution of an easily hydrolyzed *N*-acyl group by a stable benzylidene group. Indomethacin was extensively hydrolyzed in the rat (13) but excreted intact as the acyl glucuronide in the dog (12).

The excretion pattern was markedly different in the rat for the two compounds, with a much greater fraction of the dose being eliminated in feces with I than with indomethacin. In the dog, however, the excretion pattern for both compounds was similar, a high percent being eliminated in feces.

The plasma half-life of I after an intravenous dose to rats was 4.5 hr., the same as that of indomethacin. Apparently, the two compounds were metabolized at the same rate in this species. *O*-Demethylation is a possible metabolic pathway used for both I and indomethacin.

Neither drug was found to be concentrated in rat tissues but both were widely distributed. The indene analog appeared to be less extensively excreted in dog bile than indomethacin when the comparison was made at equivalent dose levels. Excretion in rat bile was nearly equivalent for I and indomethacin.[8]

A major metabolite of both compounds in dog bile was the corresponding acyl glucuronide, indicating that the indolyl and indenylacetic acid moieties are readily conjugated with glucuronic acid.

I was localized to a significant extent in inflamed tissue of rats. Such localization was reported for several other compounds (14–16); indomethacin has also been shown to be localized significantly in inflamed tissue of rats[8].

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[8] Unpublished data of the authors.

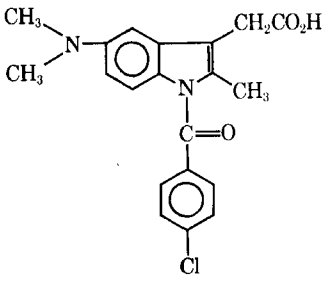
Physiologic Disposition and Metabolic Fate of Indomethacin Analogs II: 1-(*p*-Chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic Acid

HOWARD B. HUCKER, ALICE HOCHBERG, and ELIZABETH A. HOFFMAN

Abstract □ The physiologic disposition and metabolic fate of 1-(*p*-chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic acid, an analog of indomethacin, were studied in several animal species and in man. This compound was well absorbed after oral administration and widely distributed in tissues. The drug was extensively metabolized in all species by hydrolysis of the *p*-chlorobenzoyl substituent, which was excreted in the urine as *p*-chlorobenzoylglycine. In this respect, the title compound differed from indomethacin in that the latter did not undergo hydrolysis in the dog. The acyl glucuronide was a metabolite of both drugs in man.

Keyphrases □ Indomethacin, indene analogs—physiologic disposition and metabolites, animals, man □ 1-(*p*-Chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic acid—physiologic disposition and metabolites, animals, man □ Anti-inflammatory agents, potential—1-(*p*-chlorobenzoyl)-5-dimethylamino-2-methylindole-3-acetic acid, pharmacological evaluation □ GLC—determination

The anti-inflammatory activity of indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, was previously reported (1). The 5-dimethylamino analog (I) was one of several analogs synthesized (2) that retained pharmacologic activity comparable to that of indomethacin[1]. Replacement of the methoxy group by a dimethylamino substituent produced a more polar compound, with different ionization characteristics and a higher degree of water solubility. Insight as to the effect of this structural alteration on the physiologic disposition and metabolic fate of compounds in this series may be gained by comparison of such parameters for I and indomethacin. Results of such studies on I in several animal species and in man are described in the present report.



I

METHODS AND MATERIALS

I labeled with ^{14}C in the carbonyl carbon of the *p*-chlorobenzoyl group was prepared with a specific activity of 7.6 μc./mg.; it was >99% pure by TLC and paper chromatography. I labeled in the indole nucleus (2-position) was used in two experiments in rats. The specific activity was 6.8 μc./mg. *p*-Chlorobenzoic acid-7-^{14}C, with a specific activity of 37 μc./mg., was obtained[2]. *p*-Chlorobenzoylglycine was synthesized by reaction of *p*-chlorobenzoyl chloride with glycine as previously described (3). The crude product was washed with water and ether and recrystallized from hot water, m.p. 147–148°.

Anal.—Calc. for $C_9H_8ClNO_3$: C, 50.60; H, 3.78. Found: C, 50.90; H, 3.88.

Absorption and Excretion Studies—Adult male Sprague-Dawley rats, purebred beagle dogs of both sexes, male New Zealand white rabbits, male Carworth Farms mice, and male Hartley guinea pigs were used. Drug was administered as a solution of the hydrochloride in normal saline or water to animals and in gelatin capsules to normal human subjects. Doses were calculated in terms of free drug. Animals and human subjects were fasted overnight prior to oral administration of I. Urine was collected from animals housed individually in glass or stainless steel metabolism cages and was frozen as collected in dry ice-cooled containers. Urine was frozen after voiding in human experiments. Blood was collected in heparinized syringes, and the plasma was separated by centrifugation and frozen until assayed.

Tissue Distribution Studies—Tissues were obtained and prepared for analysis as previously described (4). Bile was collected from unanesthetized dogs by a technique previously described (4, 5).

Radioactivity Assay—Radioactive samples were assayed in a Tri-Carb liquid scintillation spectrometer[3] at 8° under conditions appropriate for measuring ^{14}C. All samples were counted in plastic vials containing 20 ml. of dioxane containing 8% naphthalene, 0.7% 2,5-diphenyloxazole, and 1,4-bis(5-phenyl-2-oxazolyl)-benzene. Plasma, urine, and aqueous extracts were counted directly; bile was diluted with water before counting. Feces were homogenized in a Waring blender with approximately 500 ml. of water and then diluted to 1 l. Aliquots of fecal and tissue homogenates were decolorized prior to counting by heating in alkaline peroxide (6). Counting was performed in the above medium containing 3% of a thixotropic gel[4]. Counting efficiency was determined by the internal standards method.

Measurement of I in Biological Samples—I was isolated from samples adjusted to pH 5 by extraction into heptane. The drug was back-extracted into 0.1 *N* NaOH, and the concentration was measured by spectrophotofluorometry. The procedure was as follows: One milliliter of plasma or urine was pipeted into a glass-stoppered centrifuge tube containing 2 ml. of 0.5 *M* citrate buffer, pH 5.0. Twenty-five milliliters of heptane, containing 3% isoamyl alcohol, was added, and the tube was shaken for 15 min. After centrifuging, 20 ml. of the organic phase was transferred into a second tube containing 5 ml. of 0.1 *N* NaOH. The tube was shaken for 5 min. and centrifuged. After removing the organic phase by aspiration, 2.5 ml. of the aqueous solution was transferred to a tube containing 0.5 ml. of 0.5 *M* citrate buffer, pH 5.0, and 0.2 ml. of 1 *N* HCl. The final pH was thereby brought to pH 5.0–5.5. The fluorescence of the solution was measured with an Aminco-Bowman spectrophotofluorometer (activation maximum, 295 nm.; fluorescence maximum, 385 nm., uncorrected).

The fluorescence reading was corrected for a "blank" value, obtained by analyzing control plasma or urine. The amount of drug present in the sample (assayed in duplicate) was calculated by comparison with the fluorescence obtained on assay of control plasma or urine to which known amounts of I were added. The fluorescence reading was proportional to the concentration from 0.1 to 10 mcg./ml. Recoveries of I added to plasma or urine in amounts

[1] Unpublished observations of Dr. C. A. Winter.

[2] New England Nuclear Corp.

[3] Model 314 EX or 3375, Packard Instrument Co.

[4] Cab-O-Sil.

Table I—TLC of Authentic I and Apparent Drug Isolated from Plasma and Urine

Solvent System	R_f Authentic I	R_f Apparent I
n-Butanol saturated I with 1 *N* ammonia	0.28	0.27
Ethyl acetate–isopropanol–10% ammonia (5:4:3)	0.48	0.46
Acetic acid–chloroform (5:95)	0.00	0.00
Isopropanol–7.5 N ammonia (4:1)	0.46	0.47
Acetic acid–isopropanol (5:95)	0.22	0.18

of 1–100 mcg. ranged from 75 to 93% for analyses performed over several months; excellent precision was obtained for any given day's analyses ($SD = \pm 2.3\%$).

Samples containing higher concentrations of I were assayed spectrophotometrically by a similar procedure, differing only in that the drug was back-extracted from the heptane into 5 ml. of 0.1 *N* HCl. The absorbance of the acidic solution at 265 nm. was measured, and the concentration was determined by comparison with readings from assay of known amounts of I. The absorbance (10-mm. lightpath) of a solution of 10 mcg./ml. of I in 0.1 *N* HCl was 0.55. Recoveries from urine were of adequate precision (85.3% ± 7.5 *SD*).

Possible interference in the assay by metabolites of the drug was studied by several techniques. Apparent I, isolated by the spectrophotometric procedure from plasma and urine obtained from various species, was reextracted from the dilute acid into ether after adjusting the pH to 5. The concentrated extract was subjected to TLC in several solvent systems. In all cases, only one spot was observed on the developed chromatoplate, corresponding in R_f value to that of authentic drug (Table I).

In addition, when heptane extracts of plasma from human subjects given I were assayed by GLC a major peak corresponding in retention time to authentic I methyl ester was observed.

Likewise, the excitation and fluorescence spectra of apparent drug extracted from plasma and urine were identical with those of authentic I.

GLC—GLC was performed on an F&M model 810 instrument, equipped with flame-ionization detectors and a column containing 3% QF-1 on Chromasorb G. The column, detector, and flash heater temperatures were 260, 280, and 280°, respectively. Helium was used as a carrier gas at a flow rate of 50 ml./min.

I was isolated from biological samples as was described for the spectrophotometric assay. The acid extract was shaken for 5 min. with heptane to remove isoamyl alcohol. The pH was then adjusted to 5 by addition of solid sodium acetate, and the solution was shaken with 10 ml. of chloroform for 10 min. The organic phase was evaporated to dryness *in vacuo*, and the residue was dissolved in 0.03 ml. of ether. About 0.05 ml. of an ethereal solution of diazomethane (prepared from 100 mg. of *N*-nitrosomethylurea) was added. After 5 min. the tube was placed in warm water in a hood, all solvent and unreacted reagent were allowed to evaporate, and the residue was dissolved in 0.01 ml. of ether. Approximately 3 μl. was injected into the chromatograph. The retention time (T_R) for authentic I methyl ester was 9.4 min.

TLC—Commercial silica gel-coated (250 μ) glass plates were used. I was visualized as a yellow spot after development by exposure of the plate to ammonia vapor. Radioactive compounds were located by scanning with a Packard model 7201 radiochromatogram scanner.

RESULTS

Oral Absorption and Plasma Levels—Plasma concentrations of I at various times after oral and intravenous administration of a single 5-mg./kg. dose of the ^{14}C-labeled drug were measured in rats (Table II). The plasma levels after either route of administration were comparable, indicating that oral absorption of drug was rapid and complete in this species.

Radiometric assay of plasma after extraction of I revealed that essentially all radioactivity had been extracted, indicating that only unchanged I was present. The half-life of I at this dose level in rat plasma was approximately 4 hr. A similar value was obtained after a lower oral dose of I (1 mg./kg.).

Plasma concentrations of I were also measured in dogs at various times after oral and intravenous administration of drug (Table II).

Plasma levels after oral administration were comparable to those after an intravenous dose, indicating that the drug was also well absorbed in the dog. Rapid drug absorption was indicated by the appearance of a peak plasma concentration 30 min. after oral administration. The plasma half-life was approximately 4 hr. in this species. The plasma contained only unchanged I, as shown by solvent extraction and TLC.

Plasma concentrations of I were determined in six human subjects given the drug orally (Table II). Levels were maximal 1 hr. after drug administration in five of the six subjects; the remaining subject had a peak concentration 2 hr. after drug administration. These results indicate that the drug was rapidly absorbed in man. The half-life was approximately 1 hr. Low, but measurable, concentrations of drug were found in plasma 25 hr. following drug administration. Similar results were obtained in four subjects after oral administration of I-^{14}C (300 mg. of I, including 25 μc. of I-^{14}C). Only I was present in plasma during the first 2–4 hr. after drug administration. At 6–8 hr., however, about 50% of the plasma radioactivity was not extractable into heptane and presumably represented a metabolite(s) of the drug.

Tissue Distribution—Binding of I to nondialyzable constituents of plasma was measured, using ^{14}C-labeled drug, by equilibrium dialysis at 22° for 18 hr. Under these conditions, approximately 97% of the compound was bound in human plasma when added in concentrations ranging from 1 to 25 mcg./ml. Extensive binding was also noted in similar experiments with plasma from rats (96%), dogs (92%), rabbits (98%), and guinea pigs (93%).

Distribution of radioactivity in rat tissues after intraperitoneal administration of I labeled in either the chlorobenzoyl carbonyl-^{14}C or the 2-indole-^{14}C position was determined after 1 and 24 hr. (Table III). After 1 hr., radioactivity was widely distributed among the various tissues, with the possible exception of the brain. Radioactivity was, however, not concentrated, relative to plasma, in any organ. It was localized, to some extent, in the liver, kidney, small intestine, and red blood cells. After 24 hr., radioactivity levels in the liver, kidney, and large intestine had increased relative to plasma, whereas levels in the other tissues had declined at nearly the same rate as in plasma.

Table II—Plasma Levels of I in Various Species following a Single Oral or Intravenous Dose[a]

Hours	Plasma Concentration, mcg./ml. Rat Intravenous	Rat Oral	Dog Intravenous	Dog Oral	Man Oral
0.25	25.0 ± 3.6	23.4 ± 3.4	8.5 ± 0.8	—	—
0.5	19.3 ± 1.2	24.0 ± 0.4	3.6 ± 0.6	2.3 ± 0.2	—
1.0	16.4 ± 0.2	19.9 ± 0.1	1.7 ± 0.2	2.2 ± 0.9	13.6 ± 1.2
2.0	11.1 ± 0.4	19.4 ± 0.8	1.6 ± 0.7	1.5 ± 0.5	8.2 ± 1.3
3.0	—	—	—	—	2.9 ± 0.5
4.0	11.4 ± 1.6	12.0 ± 1.0	1.0 ± 0.1	1.3 ± 0.6	1.4 ± 0.4
5.0	—	—	—	—	1.0 ± 0.4
6.0	7.6 ± 0.8	9.9 ± 0.3	1.1 ± 0.1	1.1 ± 0.4	0.9 ± 0.3
8.0	—	—	—	—	0.9 ± 0.2
24.0	0.5 ± 0.0	0.1 ± 0.0	—	0.1 ± 0.1	0.6 ± 0.3

[a] Values represent the mean ± *SE* for three rats, four dogs, and six human subjects by each route; animals received 5 mg./kg. of I-^{14}C, and human subjects received 300 mg.

Table III—Tissue Distribution of Radioactivity in Rats after a Single Intraperitoneal Dose of I-^{14}C (3 mg./kg.)[a]

Tissue	Tissue Radioactivity			
	1 hr.		24 hr.	
	Group A	Group B	Group A	Group B
Liver	0.60 (0.55–0.64)	0.58 (0.48–0.67)	1.06 (0.86–1.26)	0.98 (0.90–1.07)
Testes	0.15 (0.13–0.16)	0.08 (0.07–0.08)	0.12 (0.10–0.14)	0.17 (0.15–0.19)
Fat	0.17 (0.17–0.17)	0.12 (0.11–0.13)	0.13 (0.09–0.16)	0.16 (0.14–0.18)
Kidney	0.41 (0.39–0.43)	0.28 (0.25–0.31)	1.26 (1.12–1.40)	1.18 (1.12–1.25)
Spleen	0.10 (0.09–0.10)	0.14 (0.13–0.14)	0.08 (0.08–0.08)	0.16 (0.13–0.19)
Small intestine	0.39 (0.29–0.48)	0.62 (0.41–0.92)	0.39 (0.26–0.48)	1.64 (0.68–2.59)
Brain	0.02 (0.02–0.03)	0.02 (0.02–0.02)	0.02 (0.00–0.03)	0.05 (0.03–0.08)
Lung	0.28 (0.24–0.31)	0.29 (0.27–0.31)	0.24 (0.23–0.24)	0.19 (0.17–0.22)
Muscle	0.10 (0.09–0.11)	0.04 (0.04–0.04)	0.06 (0.05–0.06)	0.10 (0.10–0.11)
Heart	0.19 (0.18–0.20)	0.17 (0.13–0.21)	0.19 (0.15–0.22)	0.34 (0.34–0.35)
Red cells	0.63 (0.57–0.69)	—	0.72 (0.63–0.80)	—
Stomach	0.16 (0.09–0.23)	0.15 (0.10–0.20)	0.13 (0.12–0.14)	0.43 (0.25–0.60)
Large intestine	0.17 (0.12–0.21)	—	0.45 (0.29–0.57)	—
Plasma	13.2 (12.3–14.2)	17.3 (17.1–17.4)	0.7 (0.3–1.1)	1.50 (1.23–1.77)

[a] Group A represents four rats given I-chlorobenzoyl carbonyl-^{14}C; Group B represents four rats given I-indole-2-^{14}C. Two rats of each group were sacrificed at each time. Plasma levels are given as micrograms per milliliter; tissue levels are expressed as the ratio of tissue concentration to the corresponding plasma concentration. Average values are shown with individual figures in parentheses.

Analyses of plasma, liver, kidney, and small intestine for I after administration of I labeled in the chlorobenzoyl carbonyl group revealed that virtually all of the radioactivity in these tissues after 1 hr. was present as unchanged drug.

Tissue levels after administration of drug labeled in different positions were generally comparable after 1 hr. This also suggested that tissue radioactivity at that time was mainly present as I, since greater discrepancies presumably would have appeared if the *p*-chlorobenzoyl moiety of I were cleaved and distributed in tissues. After 24 hr., the only marked difference between the two labeled forms was in the small intestine, suggesting that a metabolite(s) was present.

Urinary and Fecal Excretion—Urinary and fecal excretion of I was measured in rats and dogs after single oral and intravenous doses of labeled drug (Table IV). A total of 45.7% of an oral dose to rats was recovered in the urine, and approximately 26% of the dose was recovered in the feces. Intravenous administration to rats resulted in an almost identical excretion pattern, 49.5% being found in the urine and 28.4% in feces after 4 days. The results demonstrated that the drug was well absorbed in the rat.

Approximately 32% of the dose of I-^{14}C to dogs was excreted in the urine in 24 hr. after either route of administration; excretion in the feces amounted to 52.6 and 40.7% of the dose after intravenous and oral administration, respectively.

Urinary and fecal excretion of radioactivity was also measured in several other species after a single oral dose of labeled drug (Table V). Mice given I orally excreted 66% of the dose in the urine and 9.0% in feces. Guinea pigs given labeled I intracardially excreted 76% of the dose in urine and 12% in the feces. Rabbits given labeled I intravenously excreted 88% of the dose in the urine

Excretion of total radioactivity in the urine and feces of four human subjects was measured after oral administration of a single dose of 300 mg. of I (including 25 μc. of ^{14}C-labeled I) (Table V). The drug was excreted primarily (62–75% of the dose) in the urine, chiefly in the first 24 hr. These findings indicate that I was extensively absorbed after oral administration to man and that it was excreted relatively rapidly and completely, mainly in urine. The excretion pattern found in man resembled that found in mice, rabbits, and guinea pigs more closely than the pattern found in rats and dogs.

Biliary Excretion—Biliary excretion of radioactivity and of free I was determined in a dog given a single 5-mg./kg. dose of I (including about 14 μc. of ^{14}C drug) intravenously. The results (Table VI) indicated that the drug was extensively excreted in bile (94.7% of the dose in 6 hr.). However, only 1.3% of the dose was present in the bile as apparent free drug. Plasma levels of ^{14}C declined very rapidly after intravenous administration to a dog with a bile duct fistula. The estimated half-life was <30 min. Almost all of the radioactive material (>90%) in plasma was apparent free drug.

A second experiment, in which the same dose was given orally to this dog, showed extensive biliary excretion as well, but somewhat less than after the intravenous dose. Approximately 10% of the total

Table IV—Urinary and Fecal Excretion of Radioactivity in Rats and Dogs after Administration of I-^{14}C[a]

Day	Dose Recovered, %							
	Rat				Dog			
	Oral		Intravenous		Oral		Intravenous	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
1	39.5 ± 2.0	14.9 ± 0.4	38.1 ± 1.6	6.9 ± 4.8	24.6 ± 1.1	26.1 ± 9.0	26.8 ± 1.4	36.8 ± 4.6
2	5.4 ± 0.9	13.1 ± 0.9	6.5 ± 1.9	14.3 ± 1.4	4.9 ± 0.6	12.9 ± 4.3	4.6 ± 1.6	10.2 ± 1.2
3	2.6 ± 0.2	0.3 ± 0.0	1.1 ± 0.4	4.5 ± 2.2	2.1 ± 0.6	1.7 ± 1.0	1.6 ± 0.6	4.2 ± 1.4
4	2.0 ± 0.6	0.1 ± 0.1	—	—	—	—	—	—
Total	49.5 ± 3.1	28.4 ± 0.6	45.7 ± 3.1	25.7 ± 1.8	31.6 ± 1.3	40.7 ± 4.3	33.0 ± 3.2	51.2 ± 4.0

[a] Values represent the mean ± *SE* for three rats and four dogs given 5 mg./kg. of I-^{14}C.

Table V—Urinary and Fecal Excretion of Radioactivity in Mouse, Rabbit, Guinea Pig, and Man after Administration of I-^{14}C[a]

Day	Dose Recovered, %							
	Mouse		Rabbit		Guinea Pig		Man	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
1	60.0	4.0	51.9 ± 18.4	0.0	68.0 ± 3.2	11.0 ± 2.2	58.6 ± 6.4	2.2 ± 2.0
2	6.3	5.0	28.6 ± 13.5		6.0 ± 0.7	0.6 ± 0.1	11.4 ± 3.8	1.9 ± 1.2
3	—	—	7.0 ± 6.8		1.7 ± 0.2	—	—	—
Total	66.3	9.0	87.5 ± 8.5	0.0	75.7 ± 3.8	11.6 ± 1.0	70.0 ± 5.2	4.1 ± 3.0

[a] Values represent the mean ± *SE* for three rabbits, three guinea pigs, and four human subjects and pooled excreta from eight mice. I was administered orally to mice (5 mg./kg.), intravenously to rabbits and guinea pigs (5 mg./kg.), and orally to man (300 mg., equivalent to 273 mg. of free I).

Table VI—Biliary Excretion of Radioactivity in Dogs Given I-^{14}C[a]

Hours	Dose Excreted in Bile, %—Dog 1	Dose Excreted in Bile, %—Dog 2	^{14}C Plasma Concentration, mcg./ml.—Dog 1
0.25	—	—	15.0
0.50	—	—	9.1
1.0	52.4	13.7	2.9
2.0	32.3	22.4	0.8
3.0	6.5	11.5	—
4.0	1.8	3.9	0.1
5.0	1.2	0.9	—
6.0	0.5	1.8	<0.1
24.0	—	—	<0.1
Total	94.7	54.2	—

[a] Dose = 5 mg./kg., including approximately 10 μc. of I-^{14}C. Dog 1 received the drug intravenously, and Dog 2 received the drug orally.

radioactivity excreted after oral administration was apparent free drug.

These results are consistent with the appearance of a significant fraction of an intravenous dose of I-^{14}C in feces (Table IV). Whether or not labeled material excreted in the bile was reabsorbed and reexcreted in the bile is not known. These results also suggest that the excretion average of 40.7% of an oral dose of I-^{14}C in feces did not indicate incomplete absorption. The more likely explanation is that the drug was extensively excreted in the bile and then eliminated in the feces.

Urinary Metabolites of I in Various Species—Only about 5–15% of the administered dose of radioactive I (Tables IV and V) was excreted in the urine of all species as free drug, indicating that the drug was extensively metabolized. No labeled carbon dioxide was detected in the expired air of rats given labeled I, indicating that the administered radioactivity was not lost during these experiments. The following metabolites of I were isolated and identified in the excreta of various species.

p-*Chlorobenzoic Acid*—This metabolite was isolated, identified, and determined in urine of various species given labeled I. A suitable aliquot was adjusted to pH 5 with 1 *N* HCl and extracted with heptane–3% isoamyl alcohol (5 volumes). The heptane extract was back-extracted with 0.1 *N* HCl ($^1/_5$ volume) to remove I and then shaken with 0.1 *N* NaOH. Aliquots of the base were counted to determine the *p*-chlorobenzoic acid content. Recovery of added *p*-chlorobenzoic acid was quantitative. For chromatographic studies, the 0.1 *N* NaOH extract was acidified and extracted with ether and the concentrated ether extract was used.

Apparent *p*-chlorobenzoic acid was identical with authentic compound by TLC as shown in Table VII. Cochromatography gave only a single radioactive peak. This metabolite represented about 2–6% of the urinary radioactivity in all species examined.

p-*Chlorohippuric Acid*—This metabolite was isolated from urine by the following procedure. Urine from animals and human subjects given I-^{14}C was adjusted to pH 5 and extracted with heptane to remove I and *p*-chlorobenzoic acid-^{14}C. After adjusting to pH 3, the residue was extracted three times with equal volumes of ether. About 45% of the urinary radioactivity was found in the ether extract.

The ether extract was subjected to TLC, with the results shown in Table VIII. As indicated, the metabolite was identical with *p*-chlorohippuric acid. The R_f value of the metabolite was unaltered after

Table VII—TLC of Authentic *p*-Chlorobenzoic Acid and Metabolite Isolated from Urine of Human Subjects Given I-^{14}C

Solvent System	R_f—Authentic *p*-Chlorobenzoic Acid	R_f—Metabolite
Ethanol–water–ammonia (100:12:16)	0.52	0.52
Ethyl acetate–methanol–acetic acid (7:4:1)	0.72	0.72
Benzene–dioxane–ammonia (10:80:10)	0.06	9.06

Table VIII—TLC of Authentic *p*-Chlorohippuric Acid and Metabolite Isolated from Urine of Human Subjects Given I-^{14}C[a]

Solvent System	R_f—Authentic *p*-Chlorohippuric Acid	R_f—Metabolite
Ethyl acetate–methanol–acetic acid (7:4:1)	0.79	0.81
Butanol–acetic acid–water (4:1:1)	0.73	0.68
Acetic acid–isopropanol (5:95)	0.73	0.74
Ether (saturated with water)–methanol–88% formic acid (95:1:4)	0.77	0.77

[a] Spots were located by radiometric scanning and by spraying with 4% *p*-dimethylaminobenzaldehyde in acetic anhydride (7).

treatment with 0.1 *N* NaOH, also indicating that the *p*-chlorobenzoylindole group was not present in this metabolite since I was deacylated by dilute alkali.

The metabolite was also identified by reverse-isotope dilution in the following experiment. Authentic *p*-chlorohippuric acid (200 mg.) was dissolved in 150 ml. of I-^{14}C human urine. The urine was extracted twice with equal volumes of ether, the solvent was evaporated, and the residue was crystallized from hot water to constant melting point and specific activity. The results shown in Table IX indicate that the metabolite was identical to *p*-chlorohippuric acid.

In addition, the metabolite was also subjected to GLC after conversion to its methyl ester by means of diazomethane (Table X). Under the conditions used, *p*-chlorohippuric acid and hippuric acid were well separated. The metabolite had a retention time identical with that of authentic *p*-chlorohippuric acid. Cochromatography of the metabolite and authentic *p*-chlorohippuric acid gave no additional peaks but enlarged the metabolite peak. This metabolite represented 35–48% of the urinary radioactivity in four human subjects.

p-Chlorohippuric acid was also identified in guinea pig, rabbit, dog, and rat urine by TLC, GLC, and reverse-isotope dilution analyses. This metabolite accounted for approximately 59, 75, 80, and 78%, respectively, of the urinary radioactivity in these species.

I-Glucuronide—Incubation of human urine with β-glucuronidase (6 mg./ml.; pH 7.0, 37° for 18 hr.) released additional quantities of I. The amount of I liberated in this manner was proportional to the enzyme concentration over a range of 25–1000 mcg./ml. Addition of saccharolactone (10 mg.) to the incubation mixture resulted in almost complete inhibition (90%) of I release by 100 mcg. of β-glucuronidase.

Extraction of urine with ethyl acetate at pH 3, following removal of I and *p*-chlorohippuric acid with heptane and ether, removed about 35% of the urinary radioactivity. TLC of the extract in isopropanol–acetic acid (95:5) revealed three peaks on scanning: R_f 0.03, 0.28, and 0.61. Similarly, paper chromatography in methanol–

Table IX—Reverse-Isotope Dilution Analysis of Apparent *p*-Chlorohippuric Acid Isolated from Human Urine

Crystallization Number	Melting Point	Specific Activity, d.p.m./mg.
1	147.5–148.5°	1833
2	147.5–148.5°	1818
3	147.5–148.0°	1820

Table X—GLC of Authentic *p*-Chlorohippuric Acid and Metabolite Isolated from Urine of Human Subjects Given I[a]

Compound	T_R, min.
Hippuric acid, methyl ester	2.6
p-Chlorohippuric acid, methyl ester	4.3
Metabolite, methyl ester	4.3

[a] Column was packed with 1% QF-1 on Chromasorb G and operated at 170°. All compounds were esterified with diazomethane prior to chromatography.

benzene–water–butanol (2:1:1:1) revealed three peaks, suggesting that the extract contained three metabolites.

The ethyl acetate extract was incubated with β-glucuronidase, and the incubation mixture was extracted sequentially with heptane, ether, and ethyl acetate. TLC of the ethyl acetate fraction now yielded only two spots: R_f 0.03 and 0.61. Evidently, the spot at R_f 0.28 was labile to β-glucuronidase.

On the basis of this evidence, this metabolite was tentatively identified as the acyl glucuronide of I. No synthetic sample was available as a reference. Urine from four human subjects given labeled I was assayed for this glucuronide by analysis of I present before and after enzymatic hydrolysis. The results indicated that 20–32% of the urinary radioactivity was present as the acyl glucuronide of I.

Analysis of urine of dogs, guinea pigs, and rabbits for I-glucuronide showed that the metabolite was not present in dog or rabbit urine. Approximately 20% of the radioactivity present in guinea pig urine was present as apparent I-glucuronide.

Other Metabolites—The remaining metabolites in the ethyl acetate extract of human urine apparently retained the *p*-chlorobenzoylindole structure, since treatment with 0.1 *N* NaOH altered their solvent partitioning behavior as well as TLC pattern. GLC of the base-treated metabolites revealed five peaks, two of which corresponded to *p*-chlorobenzoic acid and 4-chlorosalicylic acid. *p*-Chlorobenzoic acid was identified in the base-treated material by reverse-isotope dilution analysis. However, 4-chlorosalicylic acid was shown not to be present.

The unknown metabolites in the ethyl acetate extract were treated with acetic anhydride, since, if they were *N*-demethylated derivatives of I (5-position), acetylation should influence their solvent partitioning and TLC patterns. Marked changes were noted in these criteria after acetylation, suggesting that the metabolites may be *N*-demethylated structures.

Larger quantities of the metabolites were prepared by TLC in isopropanol–acetic acid (95:5). Major radioactive peaks at R_f 0.02 and 0.56 were eluted and rechromatographed in *n*-propanol–1 *N* acetic acid (3:1) and isopropanol–acetic acid (95:5), respectively. The 0.02 peak was run a second time in this system and then in ethyl acetate–methanol–acetic acid (7:4:1) on a cellulose plate. The original 0.56 peak was eluted and run twice in ether (saturated with water)–methanol–88% formic acid, followed by elution. Eluates of these TLC plates contained approximately 40 mcg. of the 0.02 peak present and 200 mcg. of the 0.56 peak.

An aliquot of each fraction was subjected to GLC following treatment with diazomethane. The 0.82 R_f metabolite gave a peak at 3.7 min. (170°, 1% QF-1 on Chromasorb Q); the 0.56 R_f metabolite gave a peak at 2.6 min. No further conclusions as to the structure of these metabolites were possible.

Since I was shown to be extensively debenzoylated in the rat, a preliminary experiment was performed in which rats were given I labeled in the 2-carbon of the indole nucleus. Excretion of label in this case would reflect primarily the physiologic disposition of the indole nucleus of I. In this experiment, 23% of the dose (5 mg./kg. p.o.) was excreted in the urine and 52% in feces (average of four rats) compared to 49.5 and 28.4%, respectively, when I labeled in the chlorobenzoyl-carbonyl carbon was given (Table IV).

Metabolites in Bile—Bile from a dog given I-^{14}C containing 95% of the administered radioactivity was assayed for I. The results showed that only approximately 1% of the biliary radioactivity was present as free I. The bile was incubated with β-glucuronidase and again analyzed for I. The results indicated that approximately 90% of the biliary radioactivity was now accounted for by I. The amount released was proportional to the enzyme concentration, and release was inhibited by addition of saccharolactone.

The apparent I released after incubation was identified as I by TLC and GLC of the heptane extract. Only one spot was seen after TLC in the systems described in Table I, and only one peak after GLC (after methylation) as described in the *Methods* section. In each case, the metabolite was identical with authentic I.

In a separate experiment, bile was extracted with ethyl acetate at pH 5. The extract, containing 92% of the biliary label, was subjected to TLC in two solvent systems. In isopropanol–acetic acid (95:5), only one radioactive spot was observed: R_f 0.08. No free drug (R_f 0.20) or glucuronic acid (R_f 0.0) was present. The spot was eluted with 0.1 *N* NaOH, and the eluate gave a positive test for glucuronic acid (8).

In ethyl acetate–isopropanol–10% ammonia (5:4:3), two radioactive components were observed: R_f 0.51 and 0.88. The lower R_f spot corresponded with I; free glucuronic acid was detected at R_f 0.05.

DISCUSSION

Comparison of I with the closely related structure of indomethacin offers an opportunity to observe the effect of replacing the 5-methoxy substituent by a dimethylamino group on the physiologic disposition and metabolic fate of compounds in the *N*-benzoylindole acetic acid series. These parameters were previously described for indomethacin (4, 9). Both I and indomethacin were well absorbed after oral administration. The rate of metabolism of I, as defined by the plasma half-life, appeared to be more rapid than indomethacin in man but slower in the dog. Rates were equivalent in the rat.

Both compounds were widely distributed in rat tissues but highest concentrations of drug remained in plasma, presumably a reflection of the extensive binding of both drugs to plasma protein. I was excreted more rapidly and more extensively in rat feces than was indomethacin, possibly due to more extensive biliary excretion of I. I labeled with ^{14}C in the *p*-chlorobenzoyl carbonyl carbon was used in most of these experiments. A different excretion pattern was obtained in rats given I labeled in the indole-2 carbon, which suggested that hydrolysis of the amide bond was an important metabolic route.

A major metabolite of I in human urine was *p*-chlorohippuric acid. This metabolite accounted for approximately 40% of the urinary radioactivity and resulted from hydrolysis of the *p*-chlorobenzoyl substituent of I and subsequent conjugation with glycine. Although it was originally reported (9) that only free indomethacin and its acyl glucuronide were excreted by man, more recent data (10) show that this drug is also extensively metabolized in man by *O*-demethylation and *N*-debenzoylation. In the dog, however, a marked difference in the metabolic fate of I and indomethacin existed. The latter compound was rapidly excreted in dog bile as the acyl glucuronide and excreted almost completely in feces (4), whereas I in dogs was extensively *N*-debenzoylated and excreted in urine, *p*-chlorohippuric acid accounting for about 80% of the urinary radioactivity.

The acyl glucuronide of I was also identified as a metabolite of I in man and accounted for about 25% of the urinary radioactivity. Unknown metabolites, possibly *N*-dealkylated derivatives of I, represented about 25% of the urinary radioactivity in man. Minor amounts of unchanged I and *p*-chlorobenzoic acid were also identified in human and animal urine.

McChesney *et al.* (11) reported that *p*-chlorobenzoic acid, formed as a metabolite of chlormezanone in the dog and man, was also excreted in the urine as *p*-chlorohippuric acid. Benzoic acid, however, is excreted largely as benzoylglucuronic acid in the dog (12) but as hippurate in man, suggesting that the *p*-chloro substituent, in view of the results with I, caused increased conjugation with glycine in the dog. Quick (13) reported that a *p*-chloro substituent had little effect on the extent of glycine conjugation in the benzoic acid series. The dose administered is, however, important in selection of the means of conjugation, but it is possible that *p*-chlorobenzoic acid formed *in situ* is conjugated differently than when administered *per se*.

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Keyphrases □ Lactams, medium ring derivatives—CNS activities, mice, dogs □ CNS depressants, stimulants—medium ring lactams, mice, dogs □ Tonic-clonic convulsions—lactam derivatives, subcortical, supraspinal CNS effects □ Resonating structure, lactams—CNS activities, mice, dogs

Lien and Kumler (1) pointed out that many CNS depressants and CNS stimulants have a common resonating structure: $\rangle\ddot{N}-C(=A)- \leftrightarrow \rangle\overset{+}{N}=C(-\bar{A})-$, where A = O or S. Since lactams are simple compounds possessing this resonating structure, and since very few studies on the biological activities of these compounds have been reported (2), it was decided to study the physicochemical properties and pharmacological activities of these compounds in the CNS. The purpose of this report is to present results of the preliminary screening of some compounds containing the lactam moiety. γ-Thiobutyrolactone, which has the same resonance structure as lactams, is also included in this study. Also, as general representatives of these drugs, 2-azacyclooctanone and 2-azacyclononanone were subjected to pharmacological investigation.

MATERIALS AND METHODS

γ-Thiobutyrolactone was provided by Kharasch and Langford (3) and was purified before use by distillation under atmospheric pressure (b.p. 198°). δ-Valerolactam (m.p. 35–39°), ε-caprolactam (m.p. 69–70°), 2-azacyclooctanone (m.p. 36°), 2-azacyclononanone (m.p. 77–79°), 1-methyl-2-piperidone (n_D^{20} = 1.4823), and 1-butyl-2-pyrrolidinone (n_D^{20} = 1.4640) were purchased[1] and used without further purification.

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Partition Coefficient—The *n*-octanol–water partition coefficients of γ-thiobutyrolactone and *N*-ethyl-ε-caprolactam were measured using a Carey-14 spectrophotometer. γ-Thiobutyrolactone was found to have an absorption maximum ($\lambda_{max.}$) of 2340 Å and a molar absorptivity ($\epsilon_{max.}$) of 4.358×10^3. The partition coefficients of 2-azacyclononanone and *N*-ethyl-ε-caprolactam were measured by GLC[3]. The logarithms of the partition coefficients (log P) are summarized in Table I.

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Estimation of ED_{50} and LD_{50} of 2-Azacyclooctanone—Male Swiss albino mice, weighing 20–30 g., were used. The experiments were carried out in a room constantly maintained at 26°. Graded doses of 2-azacyclooctanone dissolved in physiological saline were administered intraperitoneally. For a given experiment,

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Table I—CNS Activity of Lactams and γ-Thiobutyrolactone



Compound	*n*	R	Observation	LD_{50} (mg./kg.) C = (mole/kg.)	log 1/C	log P
δ-Valerolactam	4	H	6/6[a] slight sedation[b] at 750 mg./kg.	>1000 (>1.01 × 10^{-2})	<2.00	−0.62[c]
ε-Caprolactam	5	H	6/6 slight sedation[b] at 300 mg./kg. 6/6 Straub tail and clonic and tonic convulsion at 500 mg./kg.	650 (5.74 × 10^{-3})	2.24	−0.19[c]
2-Azacyclooctanone	6	H	6/6 normal at 100 mg./kg. 4/6 Straub tail at 200 mg./kg. 6/6 clonic and tonic convulsions at 300 mg./kg.	270 (2.13 × 10^{-3})	2.67	0.24 ± 0.3[d]
2-Azacyclononanone	7	H	6/6 mild sedation[b] at 100 mg./kg. 5/6 Straub tail at 200 mg./kg. 3/6 clonic and tonic convulsions at 200 mg./kg.	185 (1.31 × 10^{-3})	2.88	0.67 ± 0.01[d]
1-Methyl-2-piperidone	4	CH_3	6/6 mild sedation[b] at 500 mg./kg. 1000 mg./kg.	>1000 (>8.83 × 10^{-3})	—	−0.32[c]
N-Ethyl-ε-caprolactam	5	C_2H_5	6/6 clonic and tonic convulsion at 300 mg./kg.	550 (3.89 × 10^{-3})	2.41	0.65 ± 0.1[d]
1-Butyl-2-pyrrolidinone	3	*n*-C_4H_9	6/6 sedation[b] and ataxia at 300 mg./kg.	500–750[e] (3.54–5.31 × 10^{-3})	—	0.79[c]
2-Azacyclotridecanone	11	H	5/6 side position at 500 mg./kg. 6/6 trembling at 500 mg./kg.	500–750[e] (2.55–3.80 × 10^{-3})	—	1.72[c]
γ-Thiobutyrolactone			5/6 tonic and clonic convulsions at 100 mg./kg.	199 (1.95 × 10^{-3})	2.71	0.60 ± 0.01[d]

[a] Ratio of number of animals responding to number of animals tested. [b] The word "sedation" is used here to indicate diminution of physical activity. Some signs suggested the possibility of a catatonic state. [c] Calculated value taking advantage of the additivity of Hansch's π-constant (see *Reference 5*); π_{-CH_2-} = 0.43 and $\pi_{C_2H_5-(on\ N)}$ = 0.84 obtained in this study were used in the calculations. [d] Standard deviation from three to four separate experimental measurements. [e] Two percent suspension of this drug in 5% acacia solution was used for this injection.

a group of four or five mice received each appropriate dose to be tested. Tonic-clonic convulsions or death within 1 hr. after injection were used to determine the effective convulsant or the lethal doses. Experiments were repeated until 25 animals had been challenged at each dose level. The ED_{50} for convulsion and LD_{50} were estimated by the method of Litchfield and Wilcoxon (6).

Effects of Anticonvulsants on CNS Activity of 2-Azacyclooctanone—A group of 12 mice was injected intraperitoneally with 100 mg./kg. of diphenylhydantoin and a second group of 12 with 40 mg./kg. of sodium pentobarbital. Each group was then challenged with the ED_{50} and the LD_{50} of 2-azacyclooctanone. The number of animals that convulsed or died at each dose was recorded. In a few animals injected with the LD_{50} doses, death was not preceded by frank convulsions. Death in these animals might have been due to toxic effects on a vital organ such as the heart. But in all animals that convulsed, the convulsive event was preceded by signs of CNS stimulation such as rigidity and tremor, staring eyes, and fully extended hindlegs. Since these signs were observed in the few animals that died abruptly, it was presumed that they convulsed also prior to death; these animals were included in the statistical estimation of the protective effects of diphenylhydantoin and sodium pentobarbital against the convulsive effects of the drug.

Activity Measurements in Mice—By using a motility meter[4], activities of mice were compared before and after injection of 2-azacyclooctanone. Six mice were challenged individually with the convulsant ED_{50} (37 mg./kg.) and another group of six with the LD_{50} (300 mg./kg.). The total activity of each animal for 30 min. before injection was used as the control and compared with the activity for 30 min. after the injection of the drug. Values were read directly from the meter at intervals of 5 min.

Electroencephalographic Evidence for CNS-Stimulant Activity of 2-Azacyclooctanone—Three dogs were anesthetized with intravenous doses of 30 mg./kg. sodium pentobarbital. Doses of 2-azacyclooctanone were then injected, and the EEG, ECG, and blood pressure changes were monitored on an E&M physiograph. Three dogs were anesthetized with 15 mg./kg. sodium thiopental slowly injected by the intravenous route. A tracheotomy was rapidly performed and a tube was inserted for artificial ventilation. At the first sign of recovery from surgical anesthesia, an adequate dose of curare (4.5 mg. total) was administered to paralyze the animal, after which enough time was allowed to elapse (usually 30 min.) for full recovery from the anesthesia. This procedure immobilized the animal and enabled changes in EEG to be recorded without interfering signals from associated muscle movements. Doses of 2-azacyclooctanone were injected, and the EEG, ECG, and blood pressure changes were monitored on an E&M physiograph.

Effect of 2-Azacyclooctanone on Sleeping Time—Three groups of 12 mice each were used. The control group received 40 mg./kg. of sodium pentobarbital intraperitoneally, and the experimental group received both pentobarbital and either the ED_{50} or the LD_{50} of 2-azacyclooctanone. The sleeping time was then determined, which is defined as the total time elapsed between the loss and the return of the righting reflex

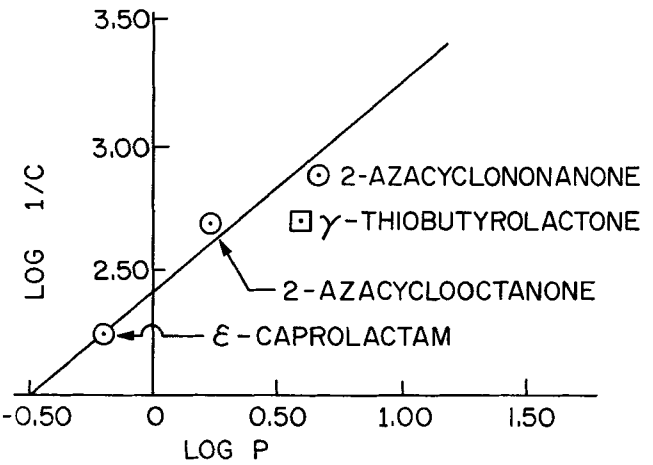

Figure 1—*Plot of 1/C against log* P, *where* C *is concentration expressed in moles per kilogram mouse, and* P *is the partition coefficient (1-octanol–water).*

[4] Motron Produkter, Stockholm, Sweden.

Table II—Influence of 2-Azacyclononanone on Respiratory Rate and Sleeping Time of Mice Pretreated with Sodium Pentobarbital[a]

Minutes after Injection of Pentobarbital	Respiratory Rate/Minute—Sodium Pentobarbital (60 mg./kg.)	Sodium Pentobarbital (60 mg./kg.) and 2-Azacyclononanone (30 mg./kg.)	Percent Increase	$N = 6, df = 5, t (t_{0.95} = 2.02)$
20	53 ± 13	81 ± 18	53	2.86
30	47 ± 14	65 ± 13	40	2.22
40	42 ± 18	74 ± 24	76	2.42
50	47 ± 14	81 ± 28	71	2.42
Mean sleeping time	86 ± 11	68 ± 16	−20	2.03

[a] 2-Azacyclononanone injected 10 min. after sodium pentobarbital.

RESULTS AND DISCUSSION

Results of the preliminary screening for activity and the partition coefficients of eight lactams and the structurally related γ-thiobutyrolactone are shown in Table I. From this table, it can be seen that most of the lactams studied had CNS-stimulating activity, although a few had CNS-depressant components. For the lactams with small ring size (n = 5, 6, 7, 11; R = H, C_6H_5), CNS stimulation appeared to be dominant. This is especially true for n = 6, which did not show any sedative effect. This finding is similar to what was observed in the case of cyclic thioureas. *N,N'*-Dimethyltrimethylenethiourea was found to be the most potent respiratory stimulant among the homologs reported (1). This finding suggests that 2-azacyclooctanone and 2-azacyclononanone may be potential analeptic agents with fairly low toxicity. γ-Thiobutyrolactone, which has a similar resonating structure: —S—C=O↔ —$\overset{+}{S}$=C—$\overset{-}{O}$, is a potent convulsant while γ-butyrolactone is known to be a CNS depressant (7–9).

The plot of log $1/C$ against log P in Fig. 1 shows that there is a high degree of correlation between the acute lethal toxicity and the lipophilic–hydrophilic character of the *N*-unsubstituted lactams: 2-azacyclononanone, 2-azacyclooctanone, and ε-caprolactam. This is true for γ-thiobutyrolactone also. The following regression is obtained by the method of least squares: $\log 1/C = 0.655 \log P + 2.409$, $n = 4$, $r = 0.949$, $s = 0.106$, where n is the number of the data points, r is the correlation coefficient, and s is the standard deviation. This correlation is significant at the 90 percentile level ($F_{1,2} = 17$; $F_{1,2_{0.90}} = 8.5$).

The toxicity of *N*-alkylated lactams is appreciably lower as compared with that of the nonalkylated lactams with the same log P value. The reason why the *N*-alkylated lactams are less toxic than the nonalkylated ones may be due to the interference of dipole–dipole interactions at the receptor sites by the *N*-alkyl group.

In estimating the ED_{50} of 2-azacyclooctanone, 25 mice were used at each dose and the doses ranged from 10 to 200 mg./kg. In estimating the LD_{50} of 2-azacyclooctanone, 25 mice were also used at each dose, but the doses ranged from 50 to 5000 mg./kg. The convulsant ED_{50} was estimated to be 37 mg./kg. with 95% confidence limits of 24–59 mg./kg., and the LD_{50} was estimated to be 300 mg./kg. with 95% confidence limits of 240–375 mg./kg. This gives a ratio of LD_{50} to convulsant ED_{50} of about 8.

Eighty percent of the mice pretreated with diphenylhydantoin and only 20% of the animals treated with sodium pentobarbital convulsed when challenged with the ED_{50} dose of 2-azacyclooctanone. Since diphenylhydantoin does not protect against midbrain and medullary convulsions, the findings suggest that 2-azacyclooctanone has the midbrain and medulla oblongata as the main sites of action. In this respect, the drug is similar to nikethamide and pentylenetetrazole. Although the other CNS-stimulant drugs discussed in this report were not tested as extensively as 2-azacyclooctanone, the results of the preliminary screening and the physicochemical and molecular considerations led to the suspicion that

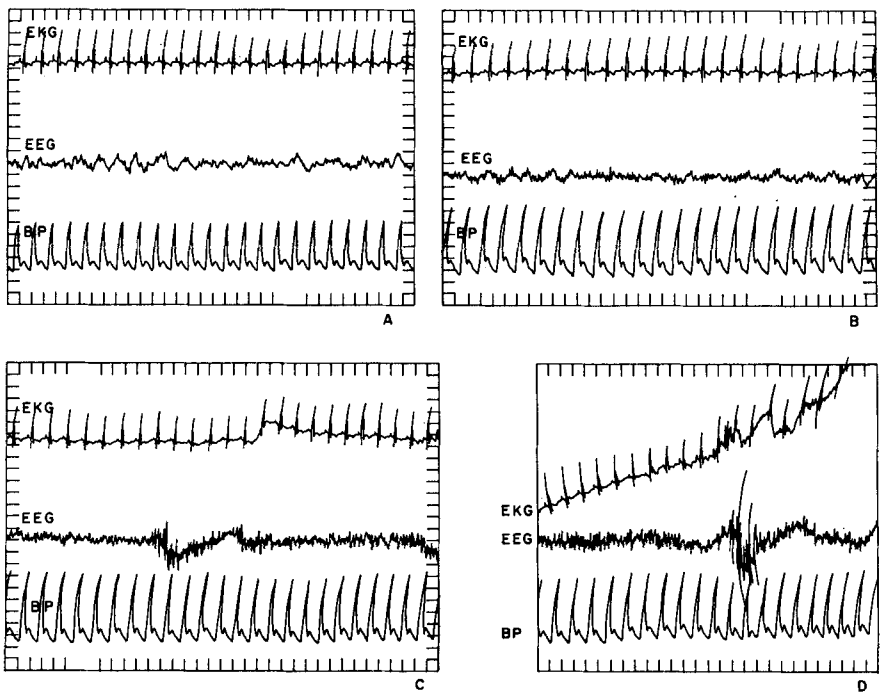

Figure 2—*EEG, ECG (lead I), and blood pressure tracings (right femoral artery) of a male dog 40 min. after the intravenous injection of 30 mg./kg. sodium pentobarbital (A); EEG, ECG (lead I), and blood pressure tracings (femoral artery) 40 min. after the recordings of A were taken (B) and immediately after injection of a total of 1600 mg. of 2-azacyclooctanone. The tracings of C were taken 3 min. after B; the tracings of D were taken 4 min. after B. Note the gradual appearance of an alerted EEG culminating in full-blown convulsion.*

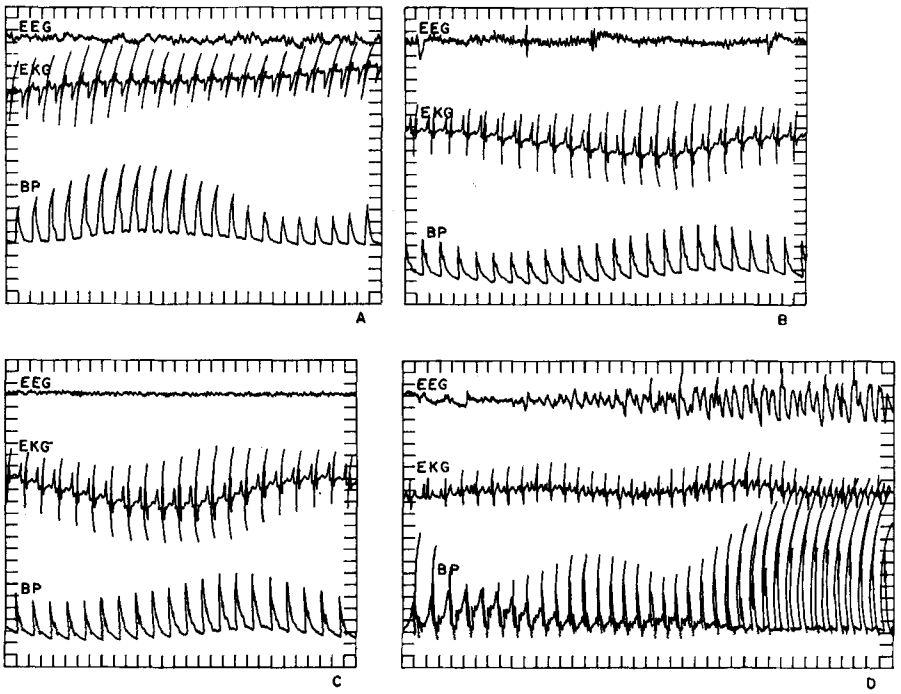

Figure 3—*ECG (lead I), EEG, and blood pressure tracings (right femoral artery) of a male dog anesthetized with 15 mg./kg. thiopental, artificially ventilated through a tracheotomy, paralyzed with* d-*tubocurarine, and injected intravenously with 1600 mg. of 2-azacyclooctanone (A). After 2 min., the record of B was seen showing spikes in the EEG. After 4 min., the record of C was seen showing the high frequency, low amplitude waves of an alert EEG. D shows evidence of a full-blown convulsion.*

they too have the same neuroanatomical areas as their sites of action.

As can be seen from the results in Table II, the respiratory rate of the animals receiving a combination of 2-azacyclononanone and pentobarbital increased by 40–76% and the sleeping time decreased by about 20% when compared with the animals receiving pentobarbital alone. Using the same standard *t*-test, these differences were found to be significant at the 95% level.

Figure 2A shows the EEG, ECG, and blood pressure recordings from a dog anesthetized with 30 mg./kg. sodium pentobarbital 40 min. after injection. The EEG shows the low frequency, high amplitude waves of surgical anesthesia of approximately Stage III Plane III (10). Figure 2B shows the EEG obtained 40 min. after injection of 2-azacyclooctanone to a total dose of 1600 mg. The appearance of the high frequency, low amplitude waves is consistent with an alerted CNS. The ability of 2-azacyclooctanone to produce changes in the EEG from a sleeping to an alerted pattern is taken to mean that the compound has a strong CNS-stimulant effect. No significant changes in the ECG and the blood pressure tracings were noted. Both depth and rate of respiration were increased. Figure 2C shows the recording obtained 43 min. after the injection of a total dose of 1600 mg. 2-azacyclooctanone. Convulsive waves began to appear, and 1 min. later a full-blown convulsion set in, as shown in Fig. 2D. Except for an increase in pulse pressure, no significant changes in blood pressure were seen.

Figure 3 shows EEG, ECG, and blood pressure recordings obtained from a dog which was paralyzed with *d*-tubocurarine and artificially ventilated. This dog was anesthetized with just sufficient thiopental to produce anesthesia to enable performance of tracheostomy and insertion of a cannula for the artificial ventilation; the effects of the anesthetic were then allowed to wear off. Upon injection of 2-azacyclooctanone in several doses to this alert, paralyzed animal, the gradual stimulation of the CNS was noted until a total dose of 1600 mg. was given and a full-blown convulsion was seen (Figs. 3A–D). In spite of the marked CNS activity, again as before, no significant changes in blood pressure were noted and the ECG patterns appeared normal.

The effect of 2-azacyclooctanone on the time elapsed between loss and return of the righting reflex (sleeping time) in mice treated with 40 mg./kg. of sodium pentobarbital is shown in Table III. The convulsant ED_{50} caused a reduction of greater than 50%. This effect is also reflected in corresponding changes in the ranges of sleeping time. The findings provide further evidence for the CNS-stimulant activity of this compound.

The activity measurements with the motility meter showed a peculiar course which was not in accord with predictions based on the known activities of the compound, namely CNS stimulation. Injection of effective doses of the drug to mice produced a diminution of activity which may terminate in recovery, convulsions, or convulsions and death. Prior to these end-points, at no time did the animals show any increase in activity above the control levels. But, even when the activities appeared depressed, the animals appeared somewhat "stunned," with some tremor and a posture as if faced with impending danger. Their behavior may be a manifestation of a catatonic state rather than a true CNS depression. Respiratory depth and rate were considerably higher. Convulsions set in abruptly and may continue until recovery or death. In spite of this unexplainable effect of the drug, sufficient CNS analeptic stimulant activity has been demonstrated by other experiments to conclude that 2-azacyclooctanone is a CNS stimulant.

The experimental findings and observations presented in this report confirm to a large degree the prediction, based on the earlier observations of Lien and Kumler (1), that many CNS stimulants and depressants contain the common resonating structures:

Table III—Effect of 2-Azacyclooctanone on Sleeping Time of Pentobarbital-Treated Mice (40 mg./kg.)

	Sodium Pentobarbital Alone, min.	*Sodium* Pentobarbital and Convulsant ED_{50} of 2-Azacyclooctanone, min.	*Sodium* Pentobarbital and LD_{50} of 2-Azacyclooctanone, min.
Mean sleeping time	25	11.8	0.5
Range of sleeping times	5–47	2–22	0–5
Number of animals	12	12	12

$\rangle\ddot{N}-C(=O)- \leftrightarrow \rangle\overset{+}{N}=C(-\overset{-}{O})-$. In general, of the compounds studied, the lactams possess predominantly CNS-stimulant activities. This effect is especially pronounced with 2-azacyclooctanone and 2-azacyclononanone. While many compounds were studied in order to make a generalization with regard to the effects of lipophilic–hydrophilic character and alkyl substitutions on the general biological behavior of these drugs, the majority of the report converges on the most active stimulants, namely 2-azacyclooctanone and 2-azacyclononanone. By various methodologies, sufficient data and information were obtained to conclude that they are CNS stimulants with the predominant site of action located in the midbrain and the medulla, that is, supraspinal and subcortical.

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Keyphrases □ *dl*-2-(3-Phenoxyphenyl)propionic acid—GLC analysis in human plasma □ Fenoprofen—GLC analysis in human plasma □ Plasma levels—fenoprofen, GLC analysis □ GLC—analysis, *dl*-2-(3-phenoxyphenyl)propionic acid (fenoprofen) in human plasma

Since Northover (1) showed that aryl- and alkyl-substituted phenoxyacetic acids exhibit anti-inflammatory properties, several structurally related chemicals have been synthesized and marketed. These include ibufenac or 2-(4-isobutylphenyl)acetic acid (2), ibuprofen or 2-(4-isobutylphenyl)propionic acid, and namoxyrate or 2-dimethylaminoethanol salt of 2-(4-biphenyl)butyric acid (3). In general, sensitive, simple, specific, and reproducible methods have not been readily available to determine quantitatively this class of chemical compounds in biological fluids. To determine blood levels of ibufenac, Adams and Cliffe (2) developed a method based upon paper chromatographic separation and reaction of the drug with bromcresol purple. The method was quantitative above 7.5 mcg./ml. serum, with a precision of ±15%; unfortunately, the analysis required 48 hr. Bergen *et al.* (3) used ^{14}C-namoxyrate to study the absorption, distribution, and metabolism of the drug.

Nickander *et al.* (4) reported on the synthesis and pharmacologic effects of a series of structurally related chemicals, one of which is fenoprofen, *dl*-2-(3-phenoxyphenyl)propionic acid. A procedure is described herein for its quantitative determination in plasma using GLC. This method should be useful in quantitating other structurally related compounds in biological media.

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Reagents—Sodium fenoprofen dihydrate and *dl*-2-(4-phenoxyphenyl)valeric acid were synthesized[1]. The latter compound served as a mass internal standard (MIS). All solvents were analytical reagent quality, except spectroquality hexane, which was used at the concentration stage of the procedure. Diatoport-S (80–100 mesh)

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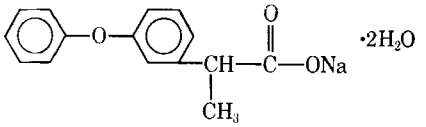
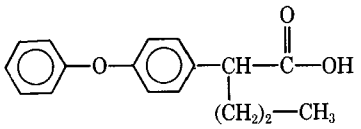


sodium *dl*-2-(3-phenoxyphenyl)propionate dihydrate



dl-2-(4-phenoxyphenyl)valeric acid

Structural formulas of phenoxyphenyl alkanoic acids

and methylvinyl silicone gum rubber (UCCW-982) were purchased[2]. Dimethyldichlorosilane and hexamethyldisilazane were also used[3].

Apparatus—An F&M[4] model 402 dual-column gas chromatograph, equipped with hydrogen flame-ionization detectors and −0.2 to 1.0-mv. Honeywell Electronik 16 recorders, was used. Samples were evaporated to dryness under nitrogen on a model 106 N-Evap[5]. Dissolution of the dried samples in a solution of hexamethyldisilazane in carbon disulfide was aided by ultrasonic vibration[6]. Tapered glass centrifuge tubes (12- and 15-ml.), fitted with Teflon-lined screw caps, were used[7].

Chromatographic Conditions—The Honeywell Electronik recorder was operated at a chart speed of 0.63 cm./min.

Helium carrier gas, flowing at 90 ml./min., was filtered through a 1.83-m. × 1.27-cm. (6-ft. × 0.5-in.) copper tubing filled with pellets of molecular sieve 5A. The filter was preconditioned overnight at 200° while flushing with dry nitrogen.

A glass column, 0.92-m. (3-ft.) × 3.0-mm. i.d., which was packed with 3.8% UCCW-982 (80–100 mesh) on Diatoport-S and operated at a temperature of 175°, was used in the analyses. To condition newly packed columns, carrier gas flow was turned on for 2–5 min. and then turned off. The oven temperature was raised to 250° without carrier flow for 1 hr. and then cooled to room temperature. The oven was reheated to 250° with carrier gas flowing overnight. The columns were then "loaded" with repeated injections of 3 mcg. of fenoprofen as the trimethylsilyl ester and 0.5 mcg. of the trimethylsilyl ester of the MIS in a chloroform solution until: (*a*) peak

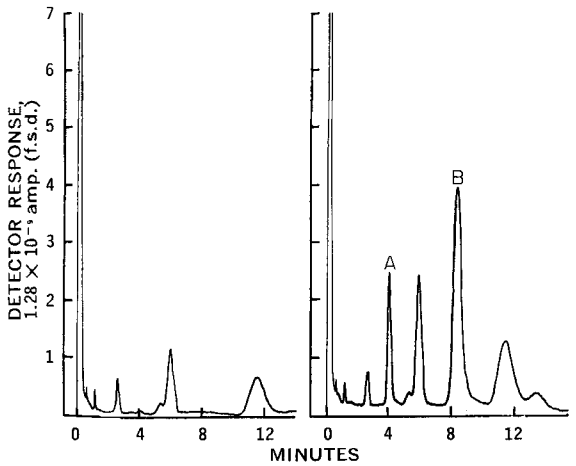


Figure 1—*Gas chromatogram of human plasma assayed as described. Left: chromatogram from normal plasma. Right: chromatogram from normal plasma with 5 mcg./ml. fenoprofen (A) plus 12 mcg./ml. MIS (B) added.*

[2] F&M Scientific Co., Avondale, Pa.
[3] From Pierce Chemical Co., Rockford, Ill.
[4] Hewlett Packard, Skokie, Ill.
[5] Organomation Associates, Shewsbury, Mass.
[6] Using a model D-100 Sonogen, Cole-Parmer, Chicago, Ill.
[7] Matheson Scientific Co., Chicago, Ill.

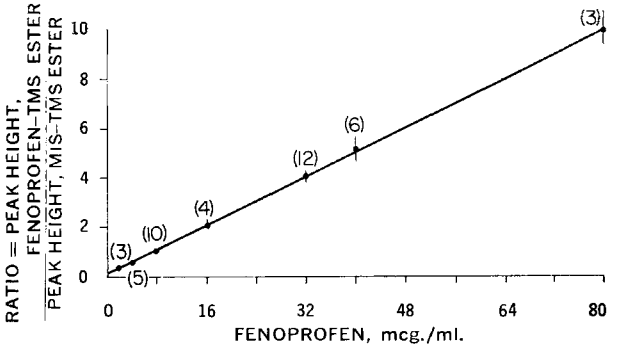


Figure 2—*Calibration curve. Relationship between the ratio of peak height of fenoprofen–trimethylsilyl ester/MIS–trimethylsilyl ester and fenoprofen concentration in plasma. Parentheses enclose the number of observations. The bar represents the range of values.*

heights were stable, and (*b*) injection of the ester of the internal standard 10 min. after injection of the ester of fenoprofen did not produce a peak of the ester of fenoprofen. This latter observation is important because occasionally injections of the trimethylsilyl ester of the internal standard appear to displace residual amounts of the ester of fenoprofen from column binding sites if the columns are not conditioned and repeatedly "loaded" as described. This "knock-off" phenomenon may reappear after 5–7 months of column usage; in this case, it is convenient to replace the column because the problem has not been eliminated in aged columns.

The glass column and glass wool used to prepare the column were siliconized with dimethyldichlorosilane before use (5). The injection port and detector block were at 220°. Under these conditions, the retention times of the trimethylsilyl esters were 4 min. (19.1 methylene units) for fenoprofen and 8.5 min. (20.8 methylene units) for the MIS (Fig. 1)[8]. At a sensitivity of 1.28×10^{-9} amp./mv., 1 mcg. of fenoprofen as its trimethylsilyl ester produced a full-scale deflection.

Procedure—The glassware was washed with chromic acid cleaning solution, rinsed with water, dried, and siliconized using a 1% solution of dimethyldichlorosilane in toluene[9].

Heparinized (or citrated) blood was centrifuged to separate the plasma[10]. Plasma samples from fenoprofen-treated subjects and control samples for calibration curves were analyzed similarly, except that fenoprofen was added in known amounts to the latter. Calibration curves were prepared daily as follows: 1 ml. of human plasma was pipeted into each of five 12-ml. screw-cap centrifuge tubes. By using a 50-μl. syringe, 0.0, 5.0, 10.0, 20.0, or 40.0 μl. of a standard aqueous solution of fenoprofen sodium dihydrate (1 mcg. equivalent acid/μl.) was added in separate tubes. To each tube was added 5 μl. (15 mcg.) of MIS dissolved in chloroform. The mixture was shaken to ensure uniform distribution of its constituents.

Two milliliters of an aqueous solution of trichloroacetic acid (10%) was added to each tube, and the contents were mixed and allowed to stand for 10 min. Five milliliters of hexane was added to the tubes; each tube was shaken vigorously for 2 min. and centrifuged at 2000 r.p.m. for 2 min. Approximately 4 ml. of the hexane extract was transferred to a 15-ml. screw-cap centrifuge tube. After redispersing the protein precipitate, the hexane extraction was repeated; approximately 5 ml. of hexane extract was withdrawn and added to the first hexane extract[11].

To the combined hexane extract was added 5 ml. of 0.1 *N* sodium hydroxide. The tubes were shaken for 2 min. and centrifuged, and the organic phase was discarded by aspiration. After adjusting the

[8] The extraction procedure effectively separates fenoprofen and the internal standard from normally appearing plasma constituents and from a primary metabolite of fenoprofen, 4′-hydroxyfenoprofen.
[9] No experimental evidence is available to indicate that siliconization of the glassware is necessary. Glassware is routinely siliconized in this laboratory, unless the procedure is specifically contraindicated. If glassware requires additional cleaning after siliconization, rinse with chloroform before use.
[10] Samples of fresh plasma as well as plasma frozen at −20° for 3–4 weeks were successfully used. However, blood bank plasma that has been frozen for more than 3 months was unsatisfactory for this assay due to interference from silylatable contaminants.
[11] Samples may be stored in a refrigerator for at least 3 days at this stage.

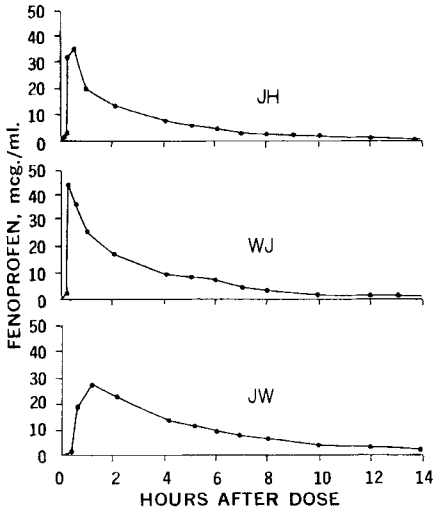


Figure 3—*Plasma concentrations of fenoprofen in three male subjects administered 250 mg. p.o. fenoprofen.*

pH of the aqueous phase to approximately 3 with 1.2 ml. of glacial acetic acid, extraction was performed again using 8 ml. of spectroquality hexane. The hexane phase[11] was transferred to a 12-ml. centrifuge tube and was concentrated to dryness on the N-Evap at 50° under a gentle stream of dry nitrogen. The sides of the tubes were washed with 0.5 ml. of spectroquality hexane, which was again evaporated to dryness[11]. The washing and evaporating were repeated. To the residue[12] was added 20 μl. of 1:20 hexamethyldisilazane in carbon disulfide to form the trimethylsilyl esters of fenoprofen and of internal standard, thus facilitating their chromatography. The samples were sonicated for 30 sec. One microliter of each sample was injected onto the GLC column.

Following chromatography, the baseline was drawn and the heights of the peaks corresponding to the fenoprofen ester and internal standard ester were measured[13]. The ratio of the peak heights was plotted on the ordinate of Cartesian graph paper, and the concentration in micrograms of fenoprofen per milliliter plasma was plotted on the abscissa. Using the peak height ratio of an unknown sample and the calibration curve (Fig. 2), the concentration of fenoprofen in a sample was determined.

RESULTS AND DISCUSSION

To obtain the precision and accuracy of the assay method, plasma samples, prepared to contain known amounts of fenoprofen, were randomized and assayed with the collected plasma samples (Table I).

Using 1-^{14}C-fenoprofen, 4.2 μc./mg., the extraction efficiency for the drug from plasma was studied. The data show that the drug was extracted quantitatively after an initial loss in the protein precipitate fraction (Table II).

A 1:20 dilution of hexamethyldisilazane in carbon disulfide was optimal to provide quantitation of fenoprofen below 200 mcg./ml. plasma[14]. Reacting 1-^{14}C-fenoprofen with hexamethyldisilazane in carbon disulfide yielded a product which, when chromatographed by thin layer, had an R_f 0.07 and contained 90% of the radioactivity. A standard of fenoprofen had an R_f 0.30. The silation reaction appears to have proceeded quantitatively. The F-254 plate was developed in a vapor-saturated tank of benzene–ethanol (95:5 v/v) until the solvent front ascended 15 cm. The small amount of radioactivity at the R_f of the fenoprofen may have been due to an on-the-plate hydrolysis of the silyl ester.

Table I—Precision and Accuracy in Measurement of Fenoprofen Added to Human Plasma

Added, mcg./ml.	Measured[a], mcg./ml.	*RSD*	*RE*
3.3	3.2 (2.9–4.1)	±12.0%	−4.5%
4.9	5.0 (4.8–5.4)	±5.5%	±2.2%
8.2	8.4 (8.0–9.6)	±7.9%	±1.6%

[a] Mean of five replicate samples (range).

Table II—Recovery of 1-^{14}C-Fenoprofen from Human Plasma

Added[a], mcg./ml.	Percent of Radioactivity Remaining in Fractions after Extraction of 1-^{14}C-Fenoprofen: Protein Precipitate	Supernatant after TCA Precipitation	Combined Hexane Extracts of Supernatant	Acidified Aqueous Phase	Percent 1-^{14}C-Fenoprofen in Final Hexane Extract
12.5	—[b]	<1	<0.5	3	71
25	14	<1	—[b]	4	77

[a] Means of three replicate determinations. [b] Not analyzed.

Application of GLC Assay to Biological Samples—The plasma concentrations of fenoprofen in three human subjects following oral administration of 250 mg. fenoprofen (312 mg. sodium fenoprofen dihydrate) illustrates the clinical utility of this method (Fig. 3). The results indicate that the drug is absorbed rapidly from the gastrointestinal tract and disappears rapidly from the plasma, with only trace amounts present after 10 hr.[15].

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[12] This residue was analyzed in a TLC system using glass, precoated silica gel plates (20 × 20 cm., Brinkmann F-254). A solvent system of benzene–glacial acetic acid–methanol (95:5:25) was allowed to develop 10 cm. Visual observation in the short UV range revealed a spot at R_f 0.53, corresponding to a similar spot obtained using authentic fenoprofen chromatographed concurrently.

[13] Results were virtually unaffected when relative peak areas were used instead of relative peak heights.

[14] By diluting hexamethyldisilazane, interference with the GLC analysis by the "pure" silylating agent was eliminated. Silation was also attempted using bis(trimethylsilyl)acetamide, trimethylchlorosilane, and bis(trimethylsilyl)trifluoroacetamide. These reagents were unsatisfactory, causing interference with the GLC analysis.

[15] A pharmacodynamic profile of fenoprofen in man will be published in the future.

Accelerated Peritoneal Dialysis of Barbiturates, Diphenylhydantoin, and Salicylate

RONALD M. KUDLA*, EMAD A. EL-BASSIOUNI, and ALBERT M. MATTOCKS

Abstract □ A combination of anthranilic acid (1%) and *N*-myristyl-β-aminopropionic acid (0.25%), which had been previously found to accelerate peritoneal dialysis of salicylate, was found also to accelerate the removal of diphenylhydantoin, phenobarbital, pentobarbital, and amobarbital. *N*-Myristyl-β-aminopropionic acid alone was equally or nearly as effective as the combination for the barbiturates. Studies indicate that displacement of the drugs from binding sites on plasma proteins may be involved in the mechanism of action of these accelerators.

Keyphrases □ Peritoneal dialysis, acceleration—barbiturates, diphenylhydantoin, and salicylate □ Barbiturates, diphenylhydantoin, and salicylate—accelerated peritoneal dialysis □ Anthranilic acid and *N*-myristyl-β-aminopropionic acid—peritoneal dialysis acceleration □ *N*-Myristyl-β-aminopropionic acid—peritoneal dialysis acceleration □ Plasma protein, drug displacement—anthranilic acid peritoneal dialysis effect □ Drug displacement from plasma protein binding sites—peritoneal dialysis accelerators

In previous work in this laboratory (1), anthranilic acid and *N*-myristyl-β-aminopropionic acid[1], when added to the dialysis fluid, was found to accelerate the removal of salicylic acid by peritoneal dialysis. A combination of the two agents, called M-1 fluid, containing 1% anthranilic acid and 0.25% *N*-myristyl-β-aminopropionic acid was tested further and found to give clearances more than four times the control fluids. Anthranilic acid was initially selected for test due to its similarity in structure to salicylate. In the same laboratory, other studies of peritoneal dialysis of barbiturates and diphenylhydantoin were being conducted; although these compounds have little structural similarity to anthranilic acid, the M-1 fluid was tested on these drugs. It was found to be effective; these tests, along with similar tests of the *N*-myristyl-β-aminopropionic acid alone, are reported in this paper.

MATERIALS AND METHODS

Radioactive Injections—^{14}C-Diphenylhydantoin was synthesized and purified by a published method (2). Solutions for injection were freshly made to contain 10 mg. diphenylhydantoin as the sodium salt and 1 μc. radioactivity/ml. This was injected at a level of 10 mg./kg.

^{14}C-Phenobarbital was obtained commercially, and the injection was prepared to contain 50 mg. phenobarbital as the sodium salt and 0.1 μc. radioactivity/ml. It was injected at a level of 50 mg./kg.

^{14}C-Pentobarbital was obtained commercially, and the injection contained 18 mg. pentobarbital as the sodium salt and 4 μc. radioactivity/ml. It was injected at a level of 18 mg./kg.

^{14}C-Amobarbital and ^{14}C-butabarbital were synthesized from the appropriate malonic esters and ^{14}C-urea. Their identity and purity were checked by IR spectra and paper chromatography. The R_f values agreed with those of Smith (3). The injections were prepared and used in the same manner as pentobarbital.

^{14}C-Salicylic acid was obtained commercially, and the injection contained 50 mg. salicylic acid as the sodium salt and 1 μc. radioactivity. It was injected at a level of 100 mg./kg.

[1] A mixture of *N*-myristyl- and *N*-lauryl-β-aminopropionic acids as their sodium salts, marketed in 50% solution by General Mills, Kankakee, Ill., under the trade name Deriphat 170-C. Percentages used in this work are of the 50% solution as supplied.

Table I—Results of Intermittent Dialysis

Dialysis Fluid	Clearance, ml./min.
Diphenylhydantoin	
Control fluid	0.47
	0.39
	0.89
	0.36
Av.	0.53
M-1 fluid	1.94
	1.57
	2.05
	1.66
Av.	1.81
N-Myristyl-β-aminopropionic acid (0.25%)	1.35
	1.54
	0.96
Av.	1.28
Phenobarbital	
Control fluid	0.93
	1.12
	1.01
	0.86
	1.37
	1.06
Av.	1.06
M-1 fluid	2.48
	2.18
	2.02
	1.83
	2.35
	1.97
Av.	2.14
N-Myristyl-β-aminopropionic acid (0.25%)	2.06
	1.44
	1.90
Av.	1.80
Butabarbital	
Control fluid	1.66
	1.94
Av.	1.80
N-Myristyl-β-aminopropionic acid (0.25%)	3.26
	3.18
Av.	3.22
Pentobarbital	
Control fluid	0.94
	0.77
	1.07
	0.98
	1.16
	1.09
Av.	1.00
M-1 fluid	1.74
	2.35
	2.51
	1.98
	1.87
	1.95
Av.	2.06
N-Myristyl-β-aminopropionic acid (0.25%)	2.91
	2.62
Av.	2.77
Amobarbital	
Control fluid	0.70
	0.48
	1.06
	1.25
	1.05
Av.	0.91
M-1 fluid	2.35
	2.11
	2.58
	2.58
	2.49
Av.	2.36
N-Myristyl-β-aminopropionic acid (0.25%)	2.21
	2.16
Av.	2.19
Salicylate	
Control fluid[a]	0.44
	0.52
	0.46
Av.	0.47
M-1 fluid[a]	2.54
	1.88
	2.38
Av.	2.27
N-Myristyl-β-aminopropionic acid (0.25%)	0.99
	1.03
	0.97
Av.	1.00

[a] From previous work (1).

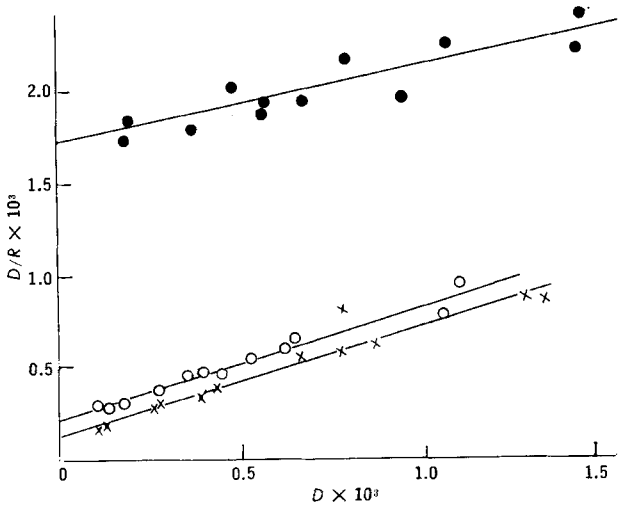

Figure 1—*Typical plots of protein binding data for salicylate. Key: ×, control points; ○, points with 0.25% N-myristyl-β-aminopropionic acid added; and ●, points with 1% anthranilic acid added. Lines are computed by least squares.*

Dialysis Fluids—The control dialysis fluid was a typical formula with 377 milliosmoles/l. It contained dextrose (1.5%), sodium lactate (0.5%), sodium chloride (0.56%), calcium chloride (0.026%), and magnesium chloride hexahydrate (0.015%). The M-1 fluid was prepared by dissolving 1% anthranilic acid and an equivalent amount of sodium bicarbonate in sufficient water to make it isotonic with the control fluid, then dissolving 0.25% of the commercial *N*-myristyl-β-aminopropionic acid solution, and making up to volume with control fluid. *N*-Myristyl-β-aminopropionic acid dialysis fluid was prepared simply by dissolving 0.25% of the commercial solution in the control fluid.

Intermittent Dialysis—Healthy, mature, male, albino rabbits were used as the test animals. They were administered the ^{14}C-tagged drug intravenously, and 1 hr. was allowed for distribution in the body before beginning dialysis. Then 60 ml./kg. of dialysis fluid was introduced into the peritoneum *via* a pediatric size catheter over a period not exceeding 1 min. The fluid was allowed to remain in the peritoneum for 19 min. and to drain by gravity for 10 min., thus giving a cycle time of 30 min. The fluid was drained into a graduated cylinder, its volume was read, it was then mixed, and a sample was taken for counting. Blood samples were taken from the marginal ear vein at the time of introduction of the dialysis fluid and at the completion of drainage of each dialysate, thus affording calculation of an average plasma concentration for each 30-min. cycle. Five such cycles were performed on each animal.

Measurements of Drug Concentrations—For salicylate and phenobarbital, the amount of drug metabolized in the 3-hr. experiment was negligible; 0.5-ml. samples of plasma and dialysate were added directly to the counting fluid [0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene and 0.4% 2,5-diphenyloxazole in a solvent of 33% nonionic surfactant[2] and 67% toluene]. Samples thus prepared were counted in a liquid scintillation counter[3] using the channels ratio method for quench correction.

Pentobarbital and its metabolites were not extracted completely by conventional methods, and a new procedure was developed for these experiments. A 0.3-ml. sample of dialysate or 50-μl. sample of plasma was counted directly for total radioactivity. A second sample of 2 ml. dialysate or 1 ml. plasma was transferred to a vial, acidified with 0.4 ml. 0.5 *N* HCl, and extracted with 4 × 3 ml. ether. The aqueous residue, containing only metabolites, was counted directly. The ether residue, containing all the unchanged drug and small amounts of metabolite, was spotted on a 20 × 20-cm. sheet of Whatman No. 2 chromatography paper. The chromatogram was developed in an Eastman chromatogram chamber with ammonium hydroxide–amyl alcohol (10:90). This gave average R_f values of 0.90 for pentobarbital and 0.10 for metabolites. After drying, the chromatograms were cut into strips, taped together, and scanned on a radiochromatogram scanner. The ratio of the area of pentobarbital peak to the sum of the two areas gave the fraction of unchanged drug present in the ether extract. This ratio, when multiplied by total count of the sample after correction for that remaining in the aqueous residue, gave the concentration of unchanged drug in the original sample. Application of this method to samples of known concentrations gave recoveries of 97% or better.

Amobarbital and butabarbital were extracted completely from acidified samples with 4 × 5 ml. ether. Contamination by metabolites was less than 3%. Extracts were evaporated directly in counting vials.

For diphenylhydantoin, the samples were acidified with 0.5 *N* HCl and extracted with 2 × 5 ml. chloroform, which was evaporated in counting vials and counted.

In each case, a sample of the radioactive injection was counted with the samples as a standard.

Binding Studies—The degree of binding of the drugs to plasma proteins was measured by equilibrium dialysis. One milliliter of pooled human plasma was placed in a bag made of 8-mm. dialysis tubing, which had been soaked overnight in pH 7.4 isotonic buffer. The plasma bags were tied and placed in 12 × 100-mm. test tubes. Outside the bags was added 6 ml. of isotonic phosphate buffer, pH 7.4, to which had been added a given concentration of ^{14}C-tagged drug. The tubes were allowed to stand at refrigerator temperature (to avoid bacterial putrefaction) for 48 hr. Each drug had been previously tested for the time required to establish equilibrium, and between 24 and 48 hr. was required in each instance. At the end of this period, the bags were removed, quickly rinsed with distilled water, wiped, and then cut open. Samples of 0.5 ml. plasma and outer fluid were pipeted into vials and counted.

For displacement tests, the agent, anthranilic acid or *N*-myristyl-β-aminopropionic acid, was dissolved in water to make an isotonic solution. Then the drug was added, and the solution was made to volume with isotonic buffer.

RESULTS

The M-1 fluid was tested for removal of diphenylhydantoin, phenobarbital, pentobarbital, and amobarbital. *N*-Myristyl-β-aminopropionic acid was tested with these drugs and also with butabarbital and salicylate. Previously reported results for control and M-1 fluids on salicylate were added for comparison. Intermittent dialysis results are shown in Table ', where each entry represents the average clearance for five exchanges on one animal.

From Table I, it is seen that the M-1 fluid accelerated removal of diphenylhydantoin to about three times the control; it accelerated phenobarbital, pentobarbital, and amobarbital to about twice the control. These results were quite unexpected, since the M-1 formula had been developed specifically for salicylate, and anthranilic acid bears little structural resemblance to diphenylhydantoin and the barbiturates. When anthranilic acid alone had been tested previously, the results were quite variable; thus the wetting agent, *N*-myristyl-β-aminopropionic acid, was tested alone to see if it might account for the full activity of the M-1 fluid. Although the wetting agent acted as an accelerator in each case, it appeared to represent the full effect of M-1 fluid only with pentobarbital and amobarbital and nearly the full effect with diphenylhydantoin and phenobarbital (Table I).

One possible effect of anthranilic acid might be the displacement of bound drug from binding sites on plasma proteins; thus the protein binding studies were performed. Because of the availability of human plasma, it was used for the binding studies, although rabbits were used for the dialyses. There may be some species differences in the degree of binding to plasma, but experiments in this laboratory demonstrated close agreement in binding of salicylate to bovine serum albumin and human plasma and in binding of pentobarbital to rabbit and human plasma. Thus, the species differences probably are small with these drugs and the displacing agents would have similar effects, since the proteins involved are the same in the different species.

The ranges of drug concentration used in plasma binding tests were selected to represent the plasma levels encountered in therapeutic doses and toxic reactions of the drugs. With diphenylhydantoin, the range that could be studied was limited by the solubility of the drug in pH 7.4 buffer. In all cases except salicylate, the fraction of drug bound was essentially constant throughout the range studied; a simple average represents adequately the degree of

[2] Triton X-100, Rohm & Haas, Philadelphia, Pa.
[3] Packard 3320 scintillation spectrometer, Packard Instrument Co., LaGrange, Ill.

Table II—Effects of Anthranilic Acid and *N*-Myristyl-β-aminopropionic Acid on Binding of Drugs to Plasma Proteins

Drug	Plasma Concentration Range, mcg./ml.	Displacement Agent	Fraction Bound	$K^a \times 10^4$	n
Diphenylhydantoin	2–11	None	0.86		
		Anthranilic acid (1%)	0.71		
		N-Myristyl-β-aminopropionic acid (0.25%)	0.70		
Phenobarbital	40–300	None	0.32		
		Anthranilic acid (1%)	0.16		
		N-Myristyl-β-aminopropionic acid (0.25%)	0.26		
Pentobarbital	6–40	None	0.47		
		Anthranilic acid (1%)	0.37		
		N-Myristyl-β-aminopropionic acid (0.25%)	0.47		
Amobarbital	6–40	None	0.41		
		Anthranilic acid (1%)	0.35		
		N-Myristyl-β-aminopropionic acid (0.25%)	0.40		
Butabarbital	6–40	None	0.30		
		Anthranilic acid (1%)	0.20		
		N-Myristyl-β-aminopropionic acid (0.25%)	0.30		
Salicylate	130–370	None	0.45–0.80	1.87	1.40
		Anthranilic acid (1%)	0.22–0.30	40.8	2.23
		N-Myristyl-β-aminopropionic acid (0.25%)	0.42–0.70	3.91	1.61

[a] Binding constants were calculated only for salicylate, since the other drugs showed no significant difference in fraction bound within the range studied.

binding. With salicylate, the fraction bound varied throughout the range studied, and the data were plotted by an equation attributed to Klotz and cited by Goldstein (4):

$$D/r = K/n + 1/n(D) \qquad \text{(Eq. 1)}$$

where D is the molar concentration of drug in plasma, r is the ratio of moles of drug bound per mole of protein, n is the number of binding sites on the protein molecule, and K is the binding constant. This equation was found, in this laboratory, to give better definition of binding curves with a number of drugs, probably because of the range of drug concentrations utilized.

Binding results are presented in Table II, and the salicylate binding curves are illustrated in Fig. 1.

Table II shows that anthranilic acid reduced the protein binding of each of the drugs studied. *N*-Myristyl-β-aminopropionic acid reduced the plasma binding of diphenylhydantoin and phenobarbital but had no significant effect on the binding of the other drugs.

DISCUSSION

The broad effect of the M-1 fluid as an accelerator for peritoneal dialysis of drugs makes the understanding of its mode of action important in order to devise more effective and less toxic accelerators. The general effect of anthranilic acid to reduce plasma binding is of interest but is not yet conclusively shown to represent its action as an accelerator.

If displacement of the drug from protein occurred equally throughout the circulatory system, it would be expected that the free drug would rapidly be equilibrated throughout its compartments of distribution, possibly most of the body water, giving a rapid drop in plasma level and a rather small increase in free drug concentration in plasma. If this occurred, the increase in dialysis rate would be smaller than that observed in the laboratory. Thus, it is believed more likely that the displacement from binding sites occurs chiefly in the vessels of the mesentery where the agent is chiefly absorbed and where dialysis is taking place. Therefore, the full effect of drug displacement is reflected in dialysis rate increase, and results might certainly be as high as those observed. This concept is supported in part by an experiment conducted in this laboratory. Anthranilic acid was infused intravenously while dialyzing salicylate with control fluid. No acceleration of dialysis could be detected. Other evidence is needed, of course, to determine whether this is truly the mode of action of these accelerators.

It is conceivable that the displacement of drugs from plasma proteins might reflect a more general effect, including displacement from attachment to the peritoneal membrane. This points to another possible mechanism by which accelerators may act.

Preliminary evidence found in this laboratory indicates that the amounts of anthranilic acid and *N*-myristyl-β-aminopropionic acid needed for the full accelerating effect may be greatly reduced by special techniques. This work is continuing and offers promise of making these agents quite useful with greatly reduced danger of toxicity.

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GLC Assay Method for Neomycin in Petrolatum-Based Ointments

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Abstract □ A GLC assay method is described for neomycin in various petrolatum-based ophthalmic and topical ointments. Other components such as bacitracin, polymyxin, and steroids do not interfere significantly in the assay. The ointment is dissolved in chloroform, and the neomycin is removed by centrifugation. The chloroform layer is discarded, and the neomycin is dissolved in water and freeze dried. The neomycin is then silylated and chromatographed. The relative standard deviation of the method is approximately 2%, with a recovery of neomycin from spiked samples of 98–100%.

Keyphrases □ Neomycin, in petrolatum-based ointments—GLC analysis □ Ointments, neomycin in petrolatum—GLC analysis □ Antimicrobial activity—neomycin in petrolatum-based ointments □ GLC—assay method, neomycin

Neomycin has been incorporated into many topical and ophthalmic ointments because of its wide spectrum of antimicrobial activity. The ointments are generally petrolatum-based, with some containing antibiotics such as bacitracin and polymyxin or anti-inflammatory agents such as methylprednisolone acetate.

The microbiological agar diffusion assay method (1) for determination of neomycin in petrolatum-based ointments has been somewhat troublesome, often giving low results with a relative standard deviation larger than desired. In addition, it has not permitted the quantitation of neomycins B and C which have different biopotencies (2).

The GLC determination of neomycins B and C, developed by Tsuji and Robertson (3), provided the first rapid and precise means of quantitating neomycins B and C and encouraged the development of assays for neomycin in petrolatum-based ointments.

EXPERIMENTAL

Apparatus—An F&M model 402 gas chromatograph with flame-ionization detector was used. The gas flow rates were: hydrogen, 50 ml./min.; air, 600 ml./min.; and helium, 70 ml./min. The chart speed was 0.64 cm. (0.25 in.)/min. Operating conditions were: oven temperature, 290°; detector temperature, 310°; and flash heater, 290°. Since the trimethylsilyl-neomycin is extremely sensitive to moisture, it is important that the carrier gas be dried through an efficient drying agent, *e.g.*, 4A molecular sieve.

Column—Glass, 3-mm. i.d. × 61 cm. (2 ft.) packed with 3% OV-1 on Gas Chrom Q, 100–120 mesh[1], was used. To precondition the column before packing, fill the empty column with a 50% solution of dimethyldichlorosilane[2] in hexane and allow to stand for 5 min. Empty the column and wash with 50 ml. of hexane followed by 50 ml. of chloroform. Dry the column with a stream of dry air. Pack the column to within 8–9 mm. of each end, and fill the remaining portions of the column with a small piece of silylated glass wool.

[1] Applied Science Laboratories, Inc., State College, Pa.
[2] Pierce Chemical Co., Rockford, Ill.

Table I—Recovery of Spiked Neomycin from Topical Ointment Containing Bacitracin and Polymyxin[a]

Milligrams Spiked per Gram of Ointment	Milligrams Found per Gram of Ointment	Percent Recovery
3.053	3.010	98.6
	2.926	95.9
3.452	3.327	96.4
	3.376	97.8
3.852	3.730	96.8
	3.862	100.3
4.234	4.184	98.8
	4.084	96.5
4.625	4.523	97.8
	4.592	99.3
	Average	97.8

[a] Mycitracin, The Upjohn Co.

The trimethylsilyl-neomycin must be injected on the column packing to minimize degradation of the trimethylsilyl-neomycin and to prevent tailing of the solvent.

Heat the column at 350° for 30 min. with the carrier gas off. Cool the column to room temperature and turn on the carrier gas. Heat the column to 300° with a flow of 70 ml./min. When the oven reaches about 250°, inject 40 μl. of Silyl-8[3]. At 300°, inject 50 μl. of trimethylsilyl-neomycin. An injection of trimethylsilyl-neomycin, which is an essential part of the column conditioning, should be made before each day's run to minimize column adsorption. Approximately 265 theoretical plates per foot for trimethylsilyl-neomycin are normally obtained with this column.

Internal Standard-Silylation Reagent—Add 50 μl. *N*-trimethylsilyldiethylamine[2] and 2 mg. trilaurin[3]/ml. TRI-SIL Z[2]. Store the solution in a serum vial tightly capped with a rubber septum and a metal retainer.

Reference Standard—Prepare a water solution containing 5.0 mg./ml. of neomycin sulfate, USP Lot I Reference Standard. Before using, dry the neomycin sulfate standard at 60° in a vacuum oven (< 5 mm. Hg) for 3 hr. Pipet 1-ml. aliquots into 2-ml. serum vials[4] and freeze dry. Cap the vials using red rubber closures[5] and metal retainers[6].

Chloroform—Analytical reagent grade was used. Wash with two portions of water to remove any ethyl alcohol present.

Sample Extraction Procedure—Accurately weigh 5 g. of ointment or the equivalent of 25 mg. neomycin sulfate into a 35-ml. centrifuge tube. Add 25 ml. of chloroform and stopper. Place in a 60° water bath for 3 min. and then shake vigorously until the ointment is well dispersed. Centrifuge at 2000×*g* for 10 min. Remove the chloroform by aspiration; the neomycin remains in the bottom of the tube. Wash the neomycin with 15 ml. of chloroform, centrifuge, and remove the chloroform. Add 5.0 ml. of water and 15 ml. of *n*-heptane to the tube and shake until the neomycin is dissolved in the water. Centrifuge at 220×*g* for 3 min. and discard the heptane. Pipet 1 ml. of the water solution into a 2-ml. serum vial and freeze dry. Cap the vial with a red rubber closure and metal retainer.

[3] Supelco, Inc., Bellefonte, Pa.
[4] Kimble Glass No. 62113-3174.
[5] No. V-35, West Co., Phoenixville, Pa.
[6] No. 12-FA-003, West Co., Phoenixville, Pa.

Table II—Recovery of Spiked Neomycin from Ophthalmic Ointment Containing Cortisone Acetate[a]

Milligrams Spiked per Gram of Ointment	Milligrams Found per Gram of Ointment	Percent Recovery
3.088	3.089	100.0
	3.122	100.1
3.516	3.557	101.2
	3.581	101.9
3.833	3.872	101.0
	3.886	101.5
4.203	4.189	99.7
	4.230	100.6
4.617	4.606	99.8
	4.506	97.6
	Average	100.4

[a] Neosone, The Upjohn Co.

Silylation Procedure—Add 1 ml. of internal standard–silylation mixture to each vial, using a 1-ml. tuberculin syringe. Heat the vials in a 75° oil bath for 35 min., swirling occasionally.

Calculation of Biopotency—Measure each peak area. Add one-third of the area of neomycin C[7] to the area of neomycin B, and determine the peak area ratio of neomycin to trilaurin. The biopotency is determined by comparing area ratios between sample and standard solutions.

RESULTS

The topical ointments used in this work contained white petrolatum, microcrystalline wax, mineral oil, methylparaben, butyl-*p*-hydroxybenzoate, cholesterol, and neomycin sulfate. The ophthalmic ointments were prepared from white petrolatum, mineral oil, anhydrous lanolin, and neomycin sulfate. In addition, some ointments contained bacitracin and polymyxin or an anti-inflammatory steroid such as prednisolone acetate and methylprednisolone acetate.

The method described in the "Code of Federal Regulations" for the extraction of neomycin from ointments (1) uses ethyl ether to dissolve the ointment, followed by extraction of the neomycin with water. Unfortunately, the ethyl ether does not completely dissolve the ointment, which results in an incomplete extraction of the neomycin and a recovery of about 90% by GLC. Other solvents were tested, such as hexane, heptane, toluene, and chloroform, with chloroform proving the most efficient in dissolving all of the ingredients except the neomycin. If water is used to extract the neomycin from the chloroform, a precipitate forms in the ophthalmic ointment extracts. The precipitate comes from the lanolin and hinders the extraction of neomycin, giving recoveries of about 90% by GLC.

Ointments containing bacitracin and polymyxin in addition to neomycin[8] pose another problem. With this product the bacitracin and polymyxin are extracted along with the neomycin and interfere with the silylation of neomycin. In the presence of these two antibiotics, only 80–90% of the neomycin is recovered. Increasing the amount of *N*-trimethylsilyldiethylamine or TRI-SIL Z did not improve the recovery of neomycin.

Since ointments are usually anhydrous, and the neomycin is insoluble in chloroform, the neomycin can be removed physically by suspending the ointments in chloroform and centrifuging. Centrifugation separates the neomycin from bacitracin and polymyxin; the bacitracin floats on the top of the chloroform, the polymyxin remains suspended, and the neomycin settles on the bottom.

A series of recovery studies was conducted on a variety of topical and ophthalmic ointments. Table I shows recovery data for a topical ointment that contained bacitracin, polymyxin, and neomycin. The ointment was spiked with five different levels of neomycin, and assays were performed in duplicate. Table II shows recovery data for an ophthalmic ointment that contained cortisone acetate and neomycin. The ointment was spiked with five levels of neomycin. Recoveries better than 98% were obtained from topical and ophthalmic ointments containing either hydrocortisone, prednisolone, methylprednisolone, or fluorometholone (topical only). The grand average recovery was 99.6%. A production lot of each type of ointment was assayed six times to give a grand average relative standard deviation of 1.5%.

[7] The antimicrobial activity of neomycin C is 33% of neomycin B (2).
[8] Mycitracin is the registered trademark of The Upjohn Co. for the mixture of these three antibiotics.

Table III—Assay of Production Lots of Topical Ointment Containing Prednisolone Acetate[a]

	GLC			Microbiological		
Lot	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*
1	2	4.02	0.007	2	3.88	0.042
2	2	3.60	0.035	2	3.71	0.057
3	2	3.48	0.035	2	3.74	0.127
4	2	3.56	0.156	4	3.45	0.119
5	2	3.79	0.099	4	4.21	0.210
6	2	3.86	0.120	2	3.85	0.028
7	2	4.00	0.113	2	4.08	0.035
8	2	3.98	0.050	2	3.86	0.014
9	2	3.84	0.113	2	3.76	0.050
All	18	3.79[c]	0.094	22	3.84[c]	0.114
		RSD = 2.47%[d]			*RSD* = 2.97%[d]	

[a] Neo-Delta-Cortef, The Upjohn Co. [b] Average of replicate assays within a lot. [c] Label is 3.5 mg. neomycin base/g. ointment. [d] A pooled estimate of the within-sample variance.

Production lots of each type of ointment were assayed by GLC and by microbiological assay. The microbiological data were obtained using the extraction method as described in the "Code of Federal Regulations" (1), with *Staphylococcus aureus* ATCC 6538P as the test organism. The data for a topical and an ophthalmic ointment are shown in Tables III and IV. No significant differences were found in the potencies obtained by the two assay methods when they were compared using an approximate paired *t*-test ($p < 0.05$). A typical chromatogram of neomycin extracted from a topical ointment is shown in Fig. 1.

Several 5-year-old ointments were assayed to see if aging caused any interferences in the performance of this GLC method. None was observed. The degradation of neomycin can be followed by the appearance of neamine and neobiosamine, which are silylated and chromatographed along with the neomycin (3).

DISCUSSION

The ointments used in this study contained 3.5–4.2 mg. neomycin base/g. ointment. A straight-line standard curve was obtained over

Table IV—Assay of Production Lots of Ophthalmic Ointment Containing Hydrocortisone Acetate[a]

	GLC			Microbiological		
Lot	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*	Number of Assays	$\bar{X}$, mg./g.[b]	*SD*
1	2	3.90	0.042	4	4.14	0.209
2	2	3.76	0.049	2	3.84	0.021
3	2	3.97	0.014	2	3.80	0.050
4	2	3.93	0.057	3	3.76	0.156
5	2	4.00	0.042	2	3.66	0.177
6	2	4.12	0.078	5	3.70	0.512
7	2	3.78	0.042	4	3.83	0.298
8	2	3.65	0.092	2	3.92	0.092
9	2	4.03	0.042	2	3.92	0.092
10	2	3.72	0.049	2	3.71	0.042
All	20	3.89[c]	0.055	28	3.84[c]	0.268
		RSD = 1.41%[d]			*RSD* = 7.16%[d]	

[a] Neo Cortef, The Upjohn Co. [b] Average of replicate assays within a lot. [c] Label is 3.5 mg. neomycin base/g. ointment. [d] A pooled estimate of the within-sample variance.

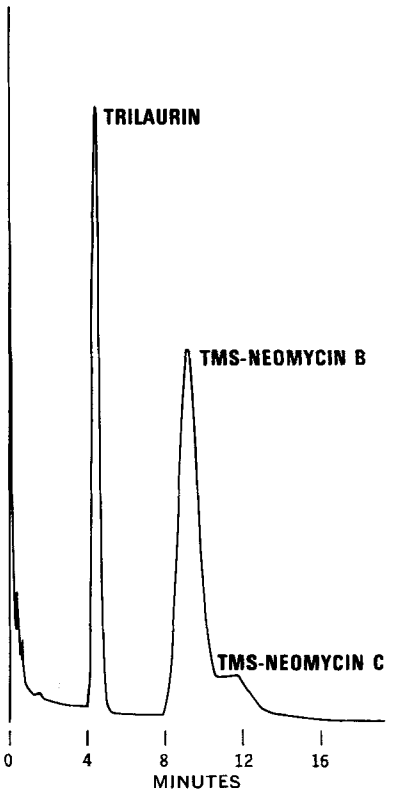

Figure 1—*Typical chromatogram of neomycin extracted from a topical ointment.*

the range of 3.0–4.6 mg. when a silylation mixture of 50 μl. of *N*-trimethylsilyldiethylamine in 1 ml. of TRI-SIL Z was used. The amount of *N*-trimethylsilyldiethylamine was found to be quite critical in obtaining a sharp symmetrical peak of trimethylsilyl-neomycin. If the amount of *N*-trimethylsilyldiethylamine was increased to 60 μl., a trailing shoulder on the neomycin peak was produced. Mass spectrometric data on the major trimethylsilyl-neomycin peak indicated all 13 sites on the neomycin molecule were silylated (3). It is possible that the trailing shoulder was due to silylation of an additional hydrogen of one or more nitrogen groups. Increasing the concentration of *N*-trimethylsilyldiethylamine or silylating reagent did not produce a single peak with the retention time of the shoulder. The amount of *N*-trimethylsilyldiethylamine must be determined experimentally if samples outside the range of 3.0–4.6 mg. neomycin base are to be analyzed.

A 3-day stability study at room temperature was conducted with trimethylsilyl-neomycin contained in sealed vials. The silylated samples were stable for 1 day at room temperature, with an estimated degradation of 3% after 8 hr. Degradation was characterized by the broadening of the trimethylsilyl-neomycin peak. Samples may be kept for longer periods with a minimum of degradation if they are refrigerated at 5° or placed at a freezer temperature of −18°. After 7 days at 5°, the trimethylsilyl-neomycin degraded 10%. After 7 days at −18°, 5% degradation was detected. The silylated samples must always be protected from moisture. Differences in the rate of degradation of neomycins B and C were not detectable.

The most reliable results were obtained with a 61-cm. (2-ft.) column packed with 3% OV-1 on Gas Chrom Q, 100-120 mesh. A 122-cm. (4-ft.) column of 3% OV-1 gave better separation of neomycins B and C, but the increased column temperature needed to chromatograph neomycin reduced column life, with a higher frequency of repair of the gas chromatograph. Increasing column adsorption was noted with 2% OV-1 or less. Column life using the 61-cm. (2-ft.), 3% OV-1 column is around 3 months.

REFERENCES

(1) "Code of Federal Regulations, Title 21, F.D.A.," U. S. Government Printing Office, Washington, D. C., 1967, pp. 67, 71.

(2) K. Tsuji, J. H. Robertson, R. Baas, and D. J. McInnis, *Appl. Microbiol.*, **18**, 396(1969).

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Cathode Ray Polarography of Riboflavin, Thiamine Hydrochloride, and Niacinamide Content of Pharmaceutical Preparations

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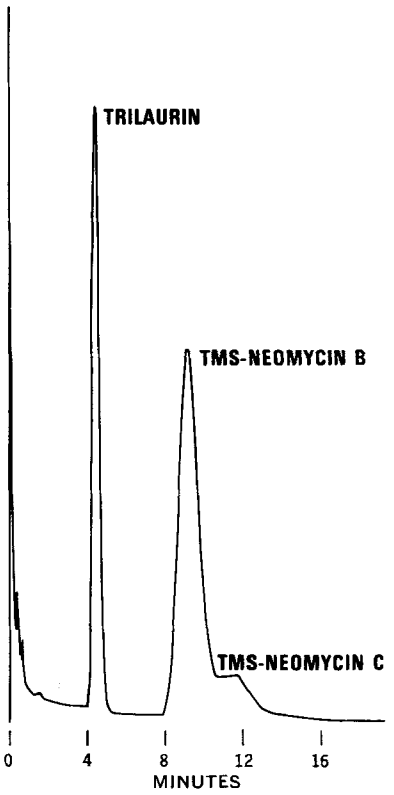
Abstract □ A polarographic method for the determination of riboflavin, thiamine hydrochloride, and niacinamide in multivitamin preparations was evaluated. Distinct polarographic waves of the three vitamins from single extracts were obtained after adjusting the pH to 5.7–6.0. Polarographic results were compared with official chemical analyses. Results indicate that the polarographic method has potential for rapidly screening the vitamin content of multivitamin preparations.

Keyphrases □ Riboflavin, thiamine HCl, niacinamide in dosage forms—determination □ Multivitamin products—riboflavin, thiamine HCl, niacinamide determination □ Polarography, cathode ray fast sweep—analysis

Several investigators described the basic design, application, and advantages of single-sweep cathode ray polarography (1, 2). Several polarographic methods for vitamins require solutions between pH 7 and 10 because of the interference from hydrogen and its catalytic wave in acid solution, especially with thiamine and niacinamide (3–7). In preliminary studies, using conventional polarography, waves of several vitamins in



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a dilute KCl extracting solution, pH 5.7–6.0, were resolved and measured from a single extract in the presence of foreign substances extracted along with the vitamins from commercial preparations in the authors' laboratory. This latter method used 0.05 *M* KCl as both extracting solution and base electrolyte for polarography of thiamine hydrochloride ($B_1 \cdot HCl$), riboflavin (B_2), and niacinamide in a single determination. Stability tests with standard solutions indicated that these vitamins were all stable in the boiling 0.05 *M* KCl used in this latter method. The pH range of 5.7–6.0 is more advantageous than the higher pH range previously used because B_1 and B_2 are more stable in acid solution. Also, this procedure forms a basis for possible determinations of three of the B vitamins in a single extract.

This study deals with the application of the single-sweep cathode ray polarograph combined with a 0.05 *M* KCl extraction–electrolyte system for the analysis of $B_1 \cdot HCl$, B_2, and niacinamide in pharmaceuticals. Data are presented regarding the general performance and operating characteristics of the instrument under the conditions imposed during this investigation.

EXPERIMENTAL

Apparatus—A twin-cell Davis differential cathode ray polarograph[1] was used. The instrument was equipped with a dropping mercury electrode, a mercury pool, and an oscilloscope camera[2].

Reagents—USP reference standards of thiamine hydrochloride, riboflavin, and niacinamide were used. All other reagents (analytical grade) were used without further purification. The samples were standard Triasyn B and hexavitamin NF pharmaceutical products and one miscellaneous formulation, all available on the open market. The NF samples were from different manufacturing lots representing three different manufacturing sources.

Standard Solutions—*Polarography*—Thiamine and niacinamide standard solutions were prepared by dissolving 50 mg. of thiamine hydrochloride or niacinamide in 40 ml. of 0.05 *M* KCl; the pH was adjusted to 5.7–6.0 with 0.2 *M* KOH, and the solution was diluted to 50 ml. with 0.05 *M* KCl. Each milliliter of the final standard solution contained 1000 mcg. of vitamin.

The riboflavin standard solution was prepared by dissolving 50 mg. of riboflavin in 100 ml. of 0.1 *M* HCl by heating in an autoclave at 121° for 20 min. The pH of the digest was adjusted to 5.7–6.0 with 2 *M* KOH and diluted to 200 ml. with 0.05 *M* KCl. Each milliliter of the final standard solution contained 250 mcg., which is about the practical limit of solubility for riboflavin.

Chemical Assays—The standards for the chemical assays were prepared according to official methods as follows: thiamine, USP (8); riboflavin, AOAC[3] (9); and niacinamide, AOAC (10).

Sample Preparation—Tablets, capsules, and liquid samples were dissolved in 100 ml. of boiling 0.05 *M* KCl. Once the sample dissolved (usually within 20 min.), the pH was adjusted to a final pH of 5.7–6.0 with 0.2 *M* KOH. If solids were a problem, they were removed by passing the solution through double-washed, medium filter paper[4] before adjusting the pH. Generally, the filtration step was not necessary. The final dilution with 0.05 *M* KCl was made so that the assay solution contained the minimum concentration to give a usable wave based on the label claim for the lowest ingredient of interest, which was thiamine in all of the products used in this study; *i.e.*, 30–45 mcg. of riboflavin, 20 mcg. of thiamine, or 20 mcg. of niacinamide per ml.

Polarography—Five milliliters of sample solution (pH 5.7–6.0) was added to each of three cells. When measuring thiamine or niacinamide, 0.05–0.2 ml. of standard solution was added to Cells 2 and 3, respectively. In the riboflavin analysis, 0.50 and 1.0 ml. of the standard solution were added to Cells 2 and 3, respectively. The three cells were deaerated with water-pumped high purity nitrogen for 8 min. The dropping mercury electrode was lowered into the cell solution, deaerated 1 additional minute, and polarographed. The sweep was from −0.0 to −0.5 v. *versus* the mercury pool reference electrode for riboflavin, with a peak potential of −0.30 ± 0.05 v. The next sweep was from −1.0 to −1.5 v. *versus* the mercury pool reference electrode for thiamine, with a peak potential of −1.25 ± 0.5 v. The final sweep was from −1.5 to −2.0 v. *versus* the mercury pool reference electrode for niacinamide, with a peak potential of −1.65 ± 0.5 v. The temperature was maintained at 25 ± 1°. Sensitivity settings and slope compensation were adjusted for proper wave height and shape, respectively.

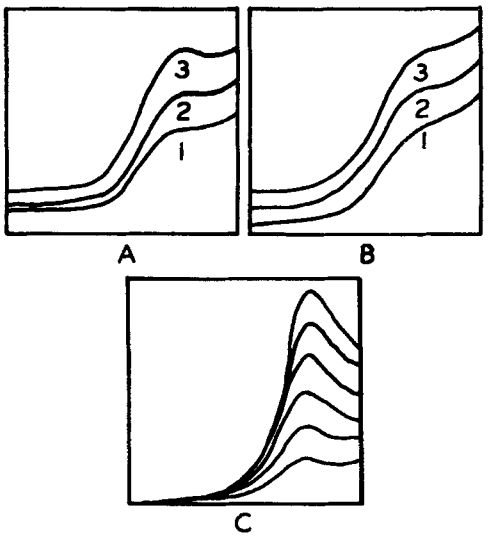

Figure 1—*Polarograms of niacinamide from a multivitamin sample extract (A1 and B1); extract plus 10 mcg. of niacinamide/ml. (A2 and B2); and extract plus 20 mcg. of niacinamide/ml. (A3 and B3). The sample was diluted with 0.05 M KCl to about 20 mcg. of niacinamide/ml. (estimation based on declared value). C shows six exposures of 10-mcg./ml. increments of niacinamide standard ranging from 10 to 60 mcg./ml. on one film. Slope setting is 60 for A and 0 for B and C.*

Three waves, representing the sample (Cell 1), sample + standard (Cell 2), and sample + standard (Cell 3; standard concentration twice that in Cell 2), were photographed[5] by triple exposure on one film at a setting of 0.2 sec. and between lens opening f5.6 and f8 with 3000 speed, 10-sec. developing time. As many as six exposures were satisfactorily developed on a single print (Fig. 1C); in this case, the brilliance of the cathode ray trace was adjusted at about midpoint on the scale to prevent overexposure of the film, and the screen lighting switch was set on visual.

The wave heights were then measured from the photographs or directly from the oscilloscope screen as the differences between the top and bottom of the wave on the vertical scale. Sample concentrations were then calculated from the direct proportion between the wave height of the sample alone and the wave height contribution by the internal standard, allowing for a slight volume correction as a result of the standard addition.

RESULTS AND DISCUSSION

A single cell was used with the Davis differential cathode ray polarograph. The standard addition method was used throughout the procedure described in this study. It is suitable when the preparation of a calibration curve would be too time consuming. It is especially useful for biological fluids that cannot be obtained vitamin free and for commercial samples of unknown composition, such as those analyzed in these experiments. Standard and sample can be analyzed in about the same medium. Except where designated otherwise, 0.05 *M* KCl (pH 5.7–6.0) was used as the supporting electrolyte.

Figure 1 shows typical niacinamide waves at slopes 60 (A) and 0 (B), obtained from an extract of a Triasyn B capsule. The wave

[1] Type A 1660, The Bendix Corp., Cincinnati, OH 45241
[2] Fairchild Polaroid model F-286.
[3] Association of Official Agricultural Chemists.
[4] Schleicher & Schüll No. 589, White Ribbon.
[5] Polaroid Land Pictures Roll (film).

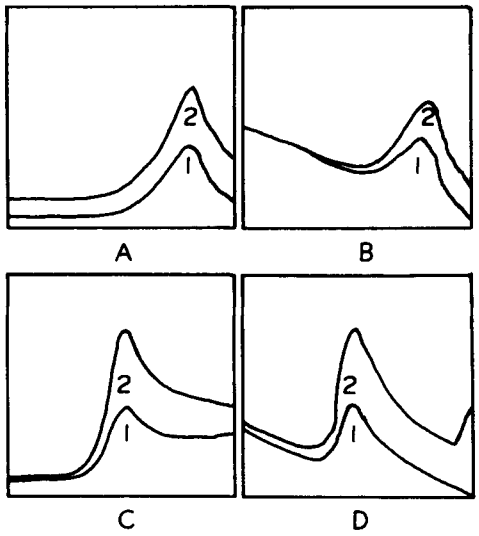

Figure 2—*Polarograms of 25 and 50 mcg. of riboflavin standard/ml. in 0.05 M KCl at slope 0 (A1 and A2, respectively); 25 and 50 mcg. of riboflavin standard/ml. in 0.05 M KCl at slope 60 (B1 and B2, respectively); 10 and 20 mcg. of Cd^{+2}/ml. in 1 M HCl at slope 0 (C1 and C2, respectively); and 10 and 20 mcg. of Cd^{+2}/ml. in 1 M HCl at slope 60 (D1 and D2, respectively).*

heights can be accurately read at slope 60. No slope adjustment was required to obtain the six exposures on a single film (C), which represent six amounts of niacinamide standard from 10 to 60 mcg./ml. in 10-mcg./ml. increments.

The curves of sample plus standard (concentration *versus* wave height) did not always pass through zero in these experiments. This was true even of the standard curves of some vitamins in pure electrolyte. As shown in Fig. 2A, doubling the concentration of riboflavin standard did not precisely double the wave height. However, with the slope compensator properly adjusted, the standard curve (concentration *versus* wave height) obtained by the standard addition method could be made to pass approximately through zero (Fig. 2B). The tendency was to obtain high results when the slope compensator was at zero. Figure 3 also illustrates the wave changes effected by adjusting the slope compensator. Thiamine showed a double wave in pure electrolyte (C and D) and a single wave in impure extract (A and B).

Figure 4 shows graphically the relationship between the slope setting and the calculated value of thiamine in an unknown Triasyn B formulation by the standard addition method. According to the

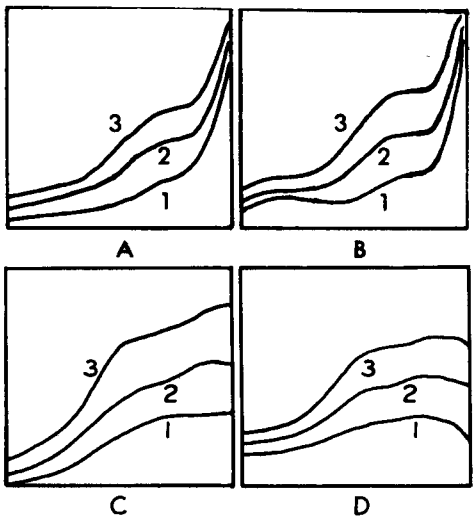

Figure 3—*Polarograms of thiamine HCl. A and B are sample extracts (Curve 1), sample extract plus 10 mcg. (Curve 2), and sample extract plus 20 mcg. (Curve 3). Concentrations are 20, 40, and 60 mcg./ml. in C and D for waves 1, 2, and 3, respectively. Slope settings are 0 for A and C, 15 for B, and 20 for D.*

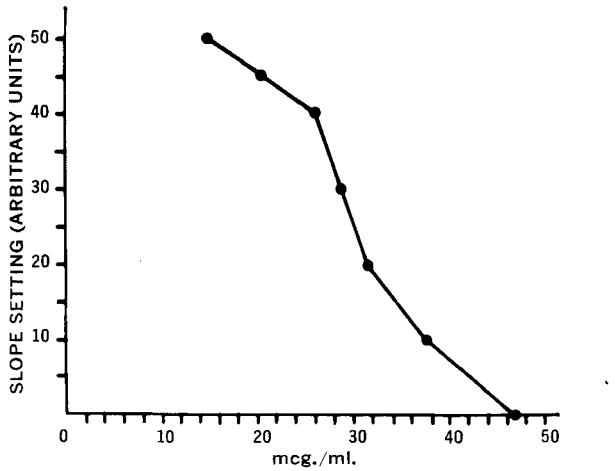

Figure 4—*Results of polarographic assays of thiamine by the standard addition method at various slope settings. The sample concentration was 25.8 mcg. of thiamine/ml. of extract of Triasyn B capsules (according to the USP fluorometric assay). The best empirical setting is found in the steep portion of the curve.*

fluorometric assay, this sample extract should contain 25.8 mcg./ml. An intermediate slope setting gave a comparable polarographic value, while slope settings higher or lower gave unrealistic results when measuring the waves as described previously. The wave heights at lower slope settings could not be read accurately due to poorly shaped waves. Any change in sensitivity and/or slope setting, type of compound assayed, concentration of the compound, and background interference altered the configuration of the graph in Fig. 4 and the shape of the polarographic waves. Wave shape and the polarographic behavior of each vitamin in pure electrolyte differed from those in the same electrolyte containing extracted sample materials.

Under certain polarographic conditions, a slope setting of 0 was satisfactory. In Fig. 2C, for example, the concentrations of 10 and 20 mcg. Cd^{+2}/ml. in 1 *N* HCl are directly proportional at slope 0, but a slight divergence from proportionality is noted at slope 60.

Figure 5 shows polarograms of thiamine hydrochloride present in two batches of the same formulation (Samples E and F). The lack of thiamine in Fig. 5A indicates that it may have decomposed during storage. Both the chemical and polarographic results indicate that this vitamin also may have partially decomposed in other samples.

Slope compensation was usually needed to produce waves with well-defined crests for accurate measurement, with the exception of riboflavin whose waves were well defined at slope 0. In most assays, well-shaped waves were produced for all three vitamins except in the presence of excessive interference. The thiamine wave was not resolved with the sugar-coated, chocolate-flavored Triasyn B tablets and the hexavitamin tablets, and niacinamide was not resolved in the same hexavitamin assay. In five other cases, waves for all three vitamins could be resolved and measured. It should be emphasized that changing the slope setting appears to change the calculated values when assaying by standard addition.

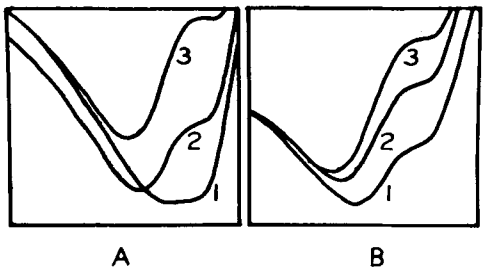

Figure 5—*A and B represent two separate batches of Triasyn B tablets. A1 shows that no thiamine is present, and B1 shows that an appreciable amount is present. Key: A2 and B2, addition of 10 mcg. of thiamine/ml.; and A3 and B3, addition of 20 mcg. of thiamine/ml. Slope setting is 40.*

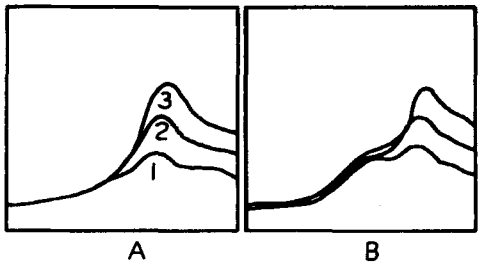

Figure 6—*Polarograms of riboflavin at slope 0. Key: A1, multiple-vitamin sample extract (about 45 mcg./ml.); A2, addition of 25 mcg. of riboflavin/ml.; and A3, addition of 50 mcg./ml. B shows that adsorption prewave height did not change significantly with two increments of standard added internally to sample extract.*

Figure 6B shows the only case in this investigation in which a double riboflavin wave occurred, the prewave being an adsorption wave (4, 7). The height of the adsorption wave remained constant with added increments. The riboflavin wave was displaced about 0.2 v. toward zero potential, possibly indicating some electrode interaction with the sample impurities. The three riboflavin waves in Fig. 6A are typical of most other riboflavin assays at slope 0. The assay results were slightly lower when the peak voltage was displaced.

Figure 7 demonstrates a timesaving step which may be of practical value. Four Triasyn B capsules were extracted and diluted to 400 ml. (See *Sample Preparation.*) In this experiment, the three waves were produced consecutively from a single 5-ml. aliquot by selecting appropriate slope settings, starting voltages, and sensitivity settings in each case and were exposed consecutively on a single frame.

Increasing or decreasing the sample concentrations served no apparent advantage. A silver chloride anode gave about the same

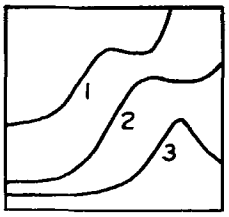

Figure 7—*Polarograms of: 1, 20 mcg. of thiamine hydrochloride/ml.; 2, 200 mcg. of niacinamide/ml.; and 3, 30 mcg. of riboflavin/ml. from one 5-ml. aliquot of an extract of Triasyn B capsules.*

results as the mercury pool anode. Some observations encountered in these experiments may be unique for this type of polarography, arising, for example, from the change in drop area of the mercury cathode during voltage sweep and current interferences.

The results of polarographic and official chemical analysis of thiamine hydrochloride, riboflavin, and niacinamide content of commercially available multivitamin preparations are summarized in Table I. The interference produced by the sugar caused polarography difficulties, precluding the determination of thiamine in Samples B and D. Additional sample clean-up techniques must be developed before samples containing sugar can be analyzed for thiamine by polarography. Another complicating factor is that the described polarographic method does not respond well to thiamine mononitrate, which was the thiamine form present in Sample D. No usable signal could be obtained on Sample D for niacinamide due to unknown interferences.

An analysis of variance was used to evaluate the data obtained by the two methods. The mean values for thiamine, riboflavin, and niacinamide were 1.27:3.48, 3.32:3.68, and 20.20:21.07, respectively, for the polarographic and chemical methods. The within-sample variations for thiamine, riboflavin, and niacinamide were 0.10:0.11, 0.10:0.40, and 0.78:0.71, respectively, for the polarographic and chemical methods. A statistically significant ($p < 0.01$) interaction occurred for riboflavin and niacinamide between formulations and methods. There was no statistical significant difference ($p < 0.05$) between the polarographic and chemical method means for ribo-

Table I—Comparison of Data Obtained Polarographically and Chemically for Seven Vitamin Formulations

Formulation	Vitamin	Found, mg./Capsule or Tablet					
		Polarographic			Chemical		
		S[a]–1	S–2	$\bar{X}$[b]	S–1	S–2	$\bar{X}$[b]
A							
Triasyn B (gelatin capsules)[c]	B_2	3.17	3.19	3.18	3.55	3.64	3.60
	B_1	1.97	1.92	1.95	2.57	2.60	2.59
	Niacin	21.2	19.5	20.4	21.0	20.0	20.5
B							
Triasyn B (sugar-coated, chocolate-flavored tablets)	B_2	4.25	4.23	4.24	4.02	4.07	4.05
	B_1[d]	—[e]	—	—	2.00[f]		
	Niacin	20.2	20.8	20.5	23.8	22.6	23.2
C							
Triasyn B (gelatin capsules)	B_2	2.98	3.17	3.08	3.41	3.66	3.54
	B_1	0.49	0.52	0.51	0.95	0.96	0.96
	Niacin	22.3	20.9	21.6	20.4	21.6	21.0
D							
Hexavitamin tablets NF (sugar-coated)	B_2	2.72	2.89	2.81	3.52	3.80	3.66
	B_1[d]	—[e]	—	—	2.10[f]		
	Niacin	—[e]	—	—	18.8	19.8	19.0
E							
Triasyn B (gelatin capsules)	B_2	3.92	3.89	3.81	3.84	3.75	3.80
	B_1	1.43	1.45	1.44	1.98	1.98	1.98
	Niacin	26.9	26.0	26.5	21.2	20.8	21.0
F							
Triasyn B (gelatin capsules)	B_2	3.33	3.14	3.24	3.45	3.45	3.45
	B_1	0	0	0	0.37	0.40	0.39
	Niacin	19.5	18.4	19.0	18.2	18.2	18.2
G							
Liquid, orange-flavored, multivitamin formula[g]	B_2	2.91	2.75	2.83	3.78	3.64	3.71
	B_1	2.28	2.60	2.44	2.84	2.73	2.79
	Niacin	20.3	20.4	20.4	21.8	23.2	22.5

[a] Subsamples. [b] Mean. [c] Triasyn B NF formulations contain 20 mg. of niacinamide, 2 mg. of B_1, and 3 mg. of B_2/capsule or tablet. [d] B_1 as mononitrate. [e] No usable signal obtained due to interferences. [f] Single analysis run to determine presence of B_1. [g] Non-NF formulation containing 3 mg. of B_1, 3 mg. of B_2, 1 mg. of B_6, 20 mg. of nicotinamide, 5 mg. of pantothenic acid, 5 mcg. of B_{12}, 1.5 mcg. of A, 50 mg. of C, and 25 mcg. of D/5 ml.

flavin and niacinamide. The high degree of interaction between formulations and methods probably accounts for the inability to detect any significant differences between the method means of riboflavin and niacinamide. However, the thiamine method means were significantly different ($p < 0.01$) from the chemical method, giving a consistently higher result. These higher results may be due to a reagent or background blank not detected by the polarographic procedure, as indicated in Sample F where no thiamine was found by the polarographic method but 0.4 mg. was found by the chemical procedure. By assuming that the 0.4 mg. was a background blank and subtracting this value from the chemical result obtained on all samples, good agreement with the polarographic data is obtained.

CONCLUSIONS

It was established that it is possible to detect and analyze most multivitamin pharmaceutical preparations for their riboflavin, thiamine hydrochloride, and niacinamide content by cathode ray fast sweep polarography. Preliminary results indicate that the cathode ray fast sweep polarographic instrumentation is adaptable and has several readily apparent advantages for the analysis of multivitamin products containing riboflavin, thiamine hydrochloride, and niacinamide. Some problems and their subsequent solution associated with the utilization of this type of analytical instrumentation are provided. The method appears to have an excellent potential for becoming a practical analytical routine with further refinements.

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(10) *Ibid.*, sections 39.037 and 39.039.

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Keyphrases □ Suppositories, commercial—GLC drug assay □ Antipyretic drug assay, suppositories—GLC □ Drug assay, multicomponent suppositories—GLC method □ GLC—analysis

It is well known that drug assay in suppositories following the classical techniques (*e.g.*, spectrophotometric and colorimetric methods) is cumbersome and often leaves much to be desired, both in accuracy and precision, due to interference of the excipients and other additives. It seemed particularly desirable to have a rapid, specific, and precise technique, such as GLC, for the assay of some suppository formulations marketed for the symptomatic treatment of the common cold and related diseases. According to the different formulations, the main antipyretic drug is either alone or, more often, associated with antihistamines and/or analgesics and other components (*e.g.*, sulfa drugs and caffeine).

Few papers have been published concerning the determination of active ingredients in suppositories by GLC (1–4). Reportedly, only single-drug (chlordantoin, 7-chlor-4-hydroxyindan, vitamin K_3, and prenylamine) determinations in single- (1, 2) and dual- (3, 4) component suppositories are performed. In the present report, data are presented regarding the GLC assay of suppositories containing: (Type I) aminophenazone in association with sodium sulfadimethoxine (not determined); (Type II) chlorpheniramine maleate, *d*-propoxyphene hydrochloride, lidocaine, and caffeine in association with paracetamol and calcium urea acetylsalicylate (not determined); and (Type III) aminophenazone, phenacetin, chlorpheniramine maleate,

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Table I—Composition of Formulations of Suppositories Examined

Suppository Type	Active Ingredients	Amount Declared, mg.	Additives and Excipients
I	Aminophenazone	300	Butylhydroxytoluene
	Sodium sulfadimethoxine	250	Butylhydroxyanisole
			Saturated fatty acids glycerides
			Polysorbate 61
II	*d*-Propoxyphene hydrochloride	60	Butylhydroxytoluene
			Butylhydroxyanisole
	Caffeine	50	Saturated fatty acids glycerides
	Chlorpheniramine maleate	3	Aluminum hydroxide gel
	Lidocaine	20	
	Paracetamol	250	
	Calcium urea acetylsalicylate	650	
III	Phenacetin	300	Saturated fatty acids glycerides
	Aminophenazone	400	
	Caffeine	60	
	Chlorpheniramine maleate	4	
	Fenalamide	30	
	Sodium benzoate	60	

fenalamide[1], caffeine, and sodium benzoate. Although GLC determination of these compounds in other pharmaceutical forms as components of different associations (5–15) and/or as single drugs (16–26) was already reported, the procedures described could not be utilized in the present case.

The procedure adopted for sample preparation is very simple, involving only a dissolution step, with consequent time saving and a better warranty of quantitative recovery. The choice of columns and preparation were accomplished on the basis of the literature indications. Due to the presence of very polar groups in the compounds studied (basic nitrogenous and amidic functions and, in one case, a carboxylic group), problems were encountered in finding suitable inert solid supports. Concerning basic nitrogenous compounds, three suggestions came from the literature: (*a*) use of support-coating with alkali to neutralize the "acid sites" and thus prevent adsorption (8, 21, 27–33) [actually, the amount of alkali used is far greater than the stoichiometric amount necessary for the neutralization process and, therefore, it modifies the retention characteristics of the stationary phases (28)]; (*b*) use of suitably silanized supports (12–18, 34, 35); and (*c*) use of inert supports such as glass beads or Teflon (5, 7, 22, 36, 37).

Two columns were prepared following suggestions (*a*) and (*b*). One, Column A, was a mixture of methyl silicone gum rubber[2] and polyethylene glycol[3] (20,000) on silanized diatomite[4] impregnated with KOH instead of acid- and base-washed silanized diatomite[5] (12) because of its failure in terms of column efficiency. No difference was observed either by varying the KOH concentration (from 2 to 10%) or by treating the support with KOH before or after coating with the stationary phase (29). No appreciable troubles, as cited by some authors (38–40), were met. Aminophenazone alone (Type I) and chlorpheniramine maleate, *d*-propoxyphene hydrochloride, lidocaine, and caffeine (Type II) were determined simultaneously on this column.

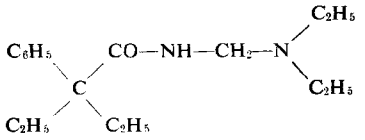
The other column (B) was prepared using silanized diatomite[6] coated with methylvinyl silicone gum rubber[7]. This column was found suitable both for basic and amidic compounds and was consequently used for simultaneous determination of aminophenazone, phenacetin, chlorpheniramine maleate, and fenalamide (III). For the carboxylic compound, benzoic acid in III, Nikelly's work (26) was taken as a basis for preparing an adequate column (C). Accordingly, carboxylic acids (also the aromatic ones) are chromatographed without preliminary esterification. This column, prepared by coating glass beads with polyethylene glycol (20,000) and isophthalic acid, was found to be somewhat cumbersome to reproduce.

All the columns used were glass, and the on-column injection technique was adopted; however, metallic columns and the metallic injector were also found suitable. Injector port temperature was maintained at 230–250°, sufficient to dissociate the salts (19).

EXPERIMENTAL

Column Preparation—Three columns (2-m. Pyrex glass U-tubes 4-mm. i.d.) were prepared: (Column A) a mixed column of 2%[8] polyethylene glycol (20,000) + 2% methyl silicone gum rubber on 100–120-mesh silanized diatomite impregnated with 5% KOH, (Column B) a column of 10% methylvinyl silicone gum rubber on 80–100-mesh silanized diatomite, and (Column C) a mixed column of 0.4% isophthalic acid[9] +0.25% polyethylene glycol (20 *M*) on 80–120-mesh glass beads[9] (26). The column packings were prepared by the standard procedure by dissolving a weighed amount of

[1] Fenalamide is the common name of *N*-(2-diethylaminoethyl)-2-phenyl-2-carbethoxybutyramide [A. Buttini, M. M. Melandri, and P. Galimberti, *Boll. Chim. Farm.*, **107**, 362(1968)]:



[2] SE 30; Carlo Erba, Milan, Italy.
[3] Carbowax (20,000); Carlo Erba, Milan, Italy.
[4] Gas Chrom P; Carlo Erba, Milan, Italy.
[5] Anakrom ABS.
[6] Diatoport S; Hewlett Packard, Avondale, Pa.
[7] UCC W 98; Hewlett Packard, Avondale, Pa.
[8] Percentage of stationary phase or KOH refers to the weight of support.
[9] Applied Science Laboratories, Inc., State College, Pa.

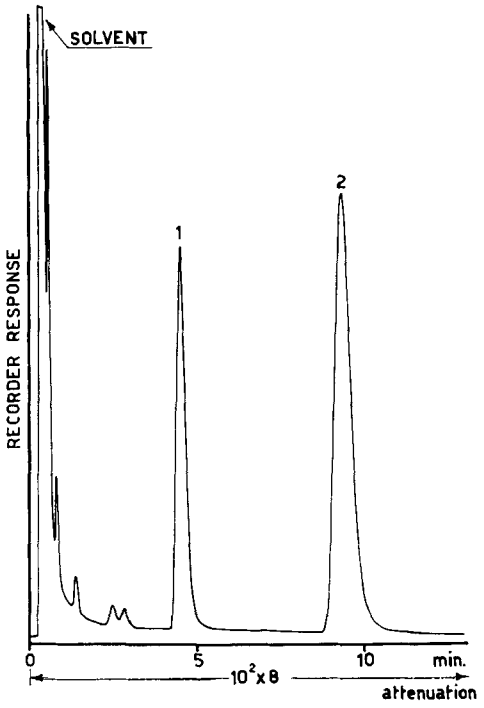


Figure 1—*Representative chromatogram for suppository Type I (Column A; temperature, 200°; flow, 100 ml./min.). Key: 1, lidocaine (internal standard); and 2, aminophenazone.*

stationary phase in a volatile solvent, adding a weighed amount of solid support, and evaporating the solvent to dryness with frequent manual stirring and mild heating. In the case of Column A, the support was impregnated beforehand with KOH, applied by means of a methanol solution, and rescreened to remove crushed particles (19). The columns were filled in the conventional manner using vacuum and tapping until no more packing was accepted (10, 8, and 35 g. for Columns A, B, and C, respectively) and conditioned for 24 hr. with a nitrogen flow rate of about 80 ml./min. at 210, 250, and 180° for Columns A, B, and C, respectively.

Apparatus and Operating Conditions—A Carlo Erba model Fractovap GV 200 gas chromatograph, equipped with a flame-ionization detector and a 2.5-mv. Leeds & Northrup Speedomax model W recorder, was used.

In all cases, hydrogen and air flow rates were 50 and 300 ml./min., respectively. The on-column injection technique was used. Column conditions were as follows:

1. *Column A;* oven, injector, and detector temperatures were 200, 210, and 210°, respectively; nitrogen flow rate was 100 ml./min.
2. *Column A;* oven, injector, and detector temperatures were

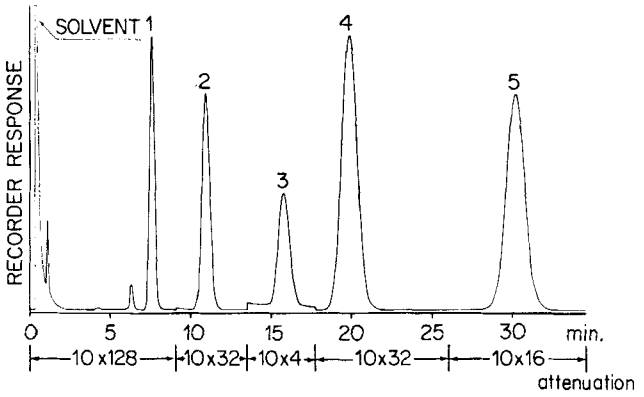


Figure 2—*Representative chromatogram for suppository Type II (Column A; temperature, 195°; flow, 75 ml./min.). Key: 1, diphenylamine (internal standard); 2, lidocaine; 3, chlorpheniramine; 4, d-propoxyphene; and 5, caffeine.*

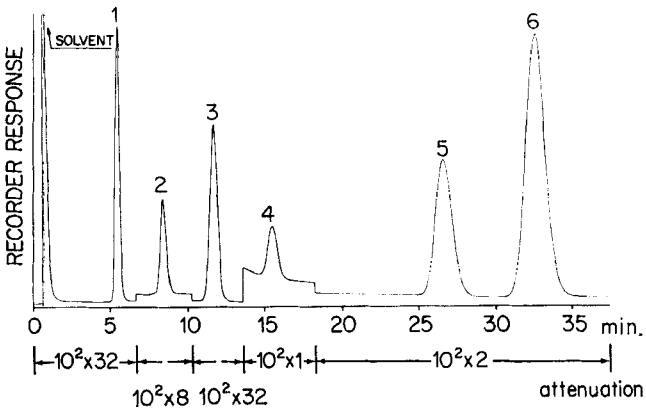


Figure 3—*Representative chromatogram for suppository Type III (Sample IIIa) (Column B; temperature, 205°; flow, 60 ml./min.). Key: 1, phenacetin; 2, caffeine; 3, aminophenazone; 4, chlorpheniramine; 5, fenalamide; and 6, 2-chlorothioxantone (internal standard).*

195, 210, and 210°, respectively; nitrogen flow rate was 75 ml./min.

3. *Column B;* oven, injector, and detector temperatures were 205, 250, and 250°, respectively; nitrogen flow rate was 60 ml./min.
4. *Column C;* oven, injector, and detector temperatures were 165, 190, and 190°, respectively; nitrogen flow rate was 60 ml./min.

PROCEDURE

Three types (I, II, and III) of commercially available suppositories, whose compositions are shown in Table I, were examined.

Internal Standard Solutions—Lidocaine hydrochloride and diphenylamine were used as internal standards for Types I and II, respectively; 2-chlorothioxantone and *p*-toluic acid were used for Type III. Internal standard solutions, I, II, III*a*, and III*b*, were prepared by dissolving about 600, 200, 350, and 240 mg., exactly weighed, of lidocaine hydrochloride, diphenylamine, 2-chlorothioxantone, and *p*-toluic acid in 250 ml. of absolute ethanol, 100 ml. of chloroform, 50 ml. of chloroform, and 100 ml. of 95% ethanol, respectively.

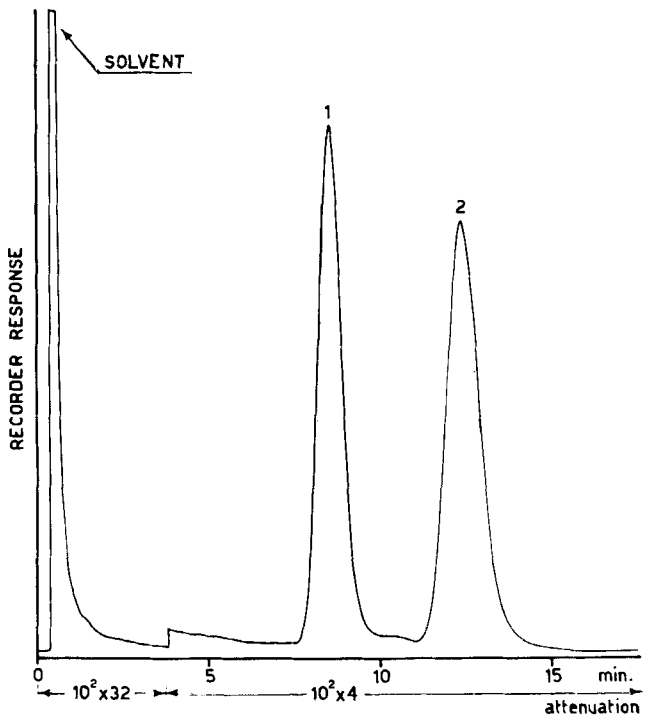


Figure 4—*Representative chromatogram for suppository Type III (Sample IIIb) (Column C; temperature, 165°; flow, 60 ml./min.). Key: 1, benzoic acid; and 2, p-toluic acid (internal standard).*

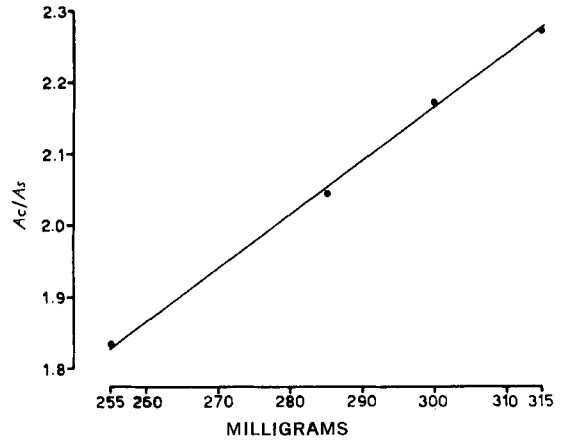


Figure 5—*Calibration curve for aminophenazone in suppository Type I.* (Ac *and* As *equal component peak area and internal standard peak area, respectively.*)

Sample Preparation—One suppository for Types I and II, and two suppositories, separately treated, for Type III were used for the analysis. The suppositories were melted in a suitable flask by heating in a water bath at 50–60° and treated as described here.

Sample I—Internal Standard Solution I (50 ml.) was added to one melted suppository of Type I. Heating was continued with shaking until complete dissolution. About 2.5 μl. of the clear solution, still hot, was injected into the gas chromatograph under operating conditions No. 1. Aminophenazone was the active ingredient determined (Fig. 1).

Sample II—Internal Standard Solution II (25 ml.) was added to one melted suppository of Type II. Heating was continued with shaking for a few minutes. The suspension was filtered, and about 4 μl. of the filtrate was injected into the gas chromatograph under operating conditions No. 2. *d*-Propoxyphene hydrochloride, caffeine, chlorpheniramine maleate, and lidocaine were the active ingredients determined (Fig. 2).

Sample IIIa—Internal Standard Solution III*a* (10 ml.) and about 40 ml. of chloroform were added to one melted suppository of Type III. Heating was continued until complete dissolution. About 4 μl. of the clear solution, still hot, was injected into the gas chromatograph under operating conditions No. 3. Phenacetin, aminophenazone, caffeine, chlorpheniramine maleate, and fenalamide were the active ingredients determined (Fig. 3).

Sample IIIb—Internal Standard Solution III*b* (25 ml.) and 0.5 ml. of concentrated hydrochloric acid were added to one melted suppository of Type III. The mixture was completely dissolved by heating in a water bath at 50–60° with shaking. About 4 μl. of the still hot solution was injected into the gas chromatograph under operating conditions No. 4. Sodium benzoate was the active ingredient determined (Fig. 4). After every four consecutive sample injections, a break of about 90 min. must be made for the elution of the sample components not determined.

Standard Solutions—Quantities of the sample components to be determined, corresponding to about 85, 95, 100, and 105% of the declared amount, were weighed for each of the four samples. Corresponding internal standard solution and solvent, in the same amount used for the sample preparation, were added; shaking and heating was performed as for the sample. The resulting solutions were then analyzed under the same operating conditions as for the corresponding samples. The data obtained were used to set up calibration curves for each component in each type of suppository. These curves were obtained by plotting the ratio of the component peak area to the corresponding internal standard peak area against the amount of the components. Each point represents the average of three determinations.

Simulated Suppository Preparations—To simulate the suppositories, known mixtures were prepared by mixing all the active ingredients, additives, and excipients in quantities corresponding to the declared amounts. These mixtures were then treated and analyzed exactly as the corresponding samples.

RESULTS AND DISCUSSION

Typical chromatograms for the three types of suppositories are shown in Figs. 1–4. All peaks are well resolved and symmetrical. The technique used for the quantitative analysis was "internal standardization." Quantitation of the peaks was based on peak area, obtained by multiplying the peak height by width at half-height. The calibration curves shown in Figs. 5–7 were utilized to calculate the amount of each ingredient; the range examined was limited to 85–105% of the declared amount as a reasonable range for covering the expected deviations for commercial preparations. These curves appear to be linear for the examined interval. Frequent check of the curves is, however, necessary if the highest accuracy is required.

The major problem encountered in obtaining and evaluating quantitative data was due to the possible interferences. Three types of interference were considered: (*a*) chromatographic interferences due to overlapping between extraneous peaks and those to be measured; (*b*) incomplete availability of the active ingredients in the solution to be chromatographed, owing to physicomechanical hindrance exerted from the undissolved portion of the sample (*e.g.*, excipients); and (*c*) interferences due to physicochemical interaction between dissolved components under the GLC operating conditions. The (*a*) and (*c*) types of interference were checked by analyzing the simulated preparations and were ruled out on the basis of the accuracy data obtained by these analyses. Interference of type (*b*) was to be considered only in the case of suppository Type II, where some

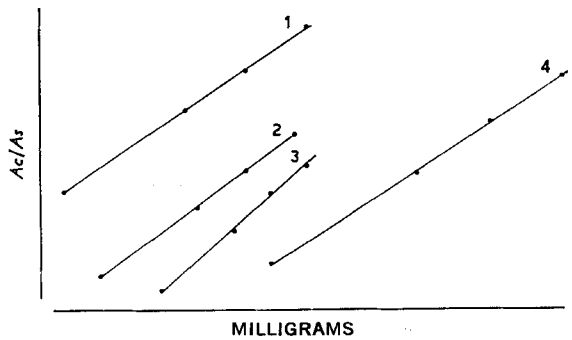


Figure 6—*Calibration curves for suppository Type II components. Key: 1, caffeine (for milligram range of 52.5–42.5,* Ac/As *range of 0.357–0.289); 2, lidocaine (for milligram range of 21–17,* Ac/As *range of 0.313–0.254); 3, chlorpheniramine maleate (for milligram range of 3.15–2.55,* Ac/As *range of 0.0299–0.0248); and 4,* d-*propoxyphene hydrochloride (for milligram range of 63–51,* Ac/As *range of 0.823–0.668).* (Ac *and* As *equal component peak area and internal standard peak area, respectively.*)

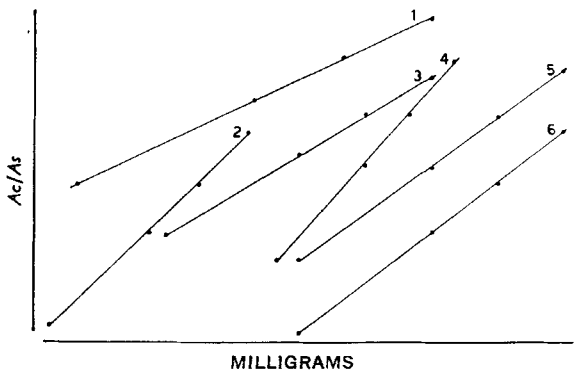


Figure 7—*Calibration curve for suppository Type III components. Key: 1, aminophenazone (for milligram range of 420–340,* Ac/As *range of 4.070–3.315); 2, fenalamide (for milligram range of 31.5–25.5,* Ac/As *range of 0.435–0.347); 3, phenacetin (for milligram range of 315–255,* Ac/As *range of 3.801–3.083); 4, chlorpheniramine maleate (for milligram range of 4.2–3.4,* Ac/As *range of 0.0467–0.0377); 5, sodium benzoate (for milligram range of 63–51,* Ac/As *range of 0.887–0.714); and 6, caffeine (for milligram range of 63–51,* Ac/As *range of 0.832–0.648).* (Ac *and* As *equal component peak area and internal standard peak area, respectively.*)

Table II—Analysis of Simulated and Commercial Suppository Preparations

Suppository Type	Weight Declared and Range Found	Component Determined	Simulated Preparation: Weighed[a] or Declared[b], mg.	Simulated Preparation: Found, Average, mg.	Simulated Preparation: Number of Replicate Analyses	Simulated Preparation: Relative *SD* (p = 0.05)	Simulated Preparation: Relative Error	Commercial Suppository: Found, Average, mg.	Commercial Suppository: Number of Lots Examined	Commercial Suppository: Relative *SD* (p = 0.05)	Commercial Suppository: Relative Error
I	1.65 ± 0.02	Aminophenazone	300	299.8	6	1.01	−0.07	304.2	29	2.38	+1.40
II	3.30 ± 0.03	*d*-Propoxyphene hydrochloride	60	60.4	6	0.67	+0.67	61.3	25	2.56	+2.17
		Caffeine	50	50.5		1.62	+1.00	51.9		3.33	+3.80
		Chlorpheniramine maleate	3	3.04		2.58	+1.33	3.08		5.16	+2.67
		Lidocaine	20	20.2		2.40	+1.00	20.6		2.98	+3.00
III	2.65 ± 0.02	Phenacetin	300	298.8	6	0.97	−0.40	299.3	20	2.20	−0.23
		Aminophenazone	400	399.4		1.25	−0.15	397.7		2.61	−0.58
		Caffeine	60	59.9		1.18	−0.17	59.5		3.53	−0.83
		Chlorpheniramine maleate	4	3.99		2.72	−0.25	3.94		5.90	−1.50
		Fenalamide	30	29.9		1.45	−0.33	29.6		3.46	−1.33
		Sodium benzoate	60	59.6		1.32	−0.67	59.0		6.18	−1.67

[a] For simulated preparation. [b] For commercial suppository.

of the components were not soluble under the operating conditions and some trapping action on the active components to be determined might have been suspected. In this case the absence of the interference in the sample cannot be directly inferred from the ascertained absence in the simulated preparation, because it was practically impossible to reproduce exactly in the latter the dissolution process of the suppository components concerned. Therefore, the accuracy estimated by analysis of the simulated preparation, although it proved acceptable, cannot be strictly considered true. Accordingly, Table II shows precision and accuracy data, both obtained on the simulated preparations, for each component, as well as the results on several lots of each commercial preparation examined. As is evident, both precision and accuracy also are excellent for components (chlorpheniramine maleate and lidocaine) present in very small quantities. However, this precision and accuracy require optimum experimental conditions, particularly a frequent check of the calibration curves. For the commercial samples, as expected, the relative standard deviations are higher, especially for chlorpheniramine maleate and sodium benzoate, due to the added error associated with the manufacturing process.

In conclusion, it can be stated that the GLC analysis appears to be particularly suitable for the routine control of preparations of the types examined. In fact, this technique provides a simple way of obtaining precise and specific results with noticeable time and manpower savings.

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Kinetic Simulation by Digital Computer Using Graphic Display Techniques

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Abstract □ A method of simulating chemical and biological processes by means of computer-generated plots is described, and two examples are given. The essence of the procedure is the successive approximation of smooth computed curves to the experimental data points, by means of systematized parameter variation. The curves generated during the simulation procedure are monitored on a video display screen, and instantaneous human direction or intervention may be made by means of numerical command signals from a coupled keyboard-printer. Knowledge of the parameter values for the simulation curves best fitting the original data points provides valuable insight into the principles underlying the experimental process under investigation and furnishes a predictive and manipulative tool of some precision.

Keyphrases □ Pharmacokinetic, chemical kinetic simulation—graphic display method □ Computerized methods—kinetic analysis, simulated □ Drug distribution, *in vivo*—computerized simulation □ Diagram, computer program—kinetic simulation

Previous papers from these laboratories illustrated the utilization of computer procedures for on-line data acquisition and control of pharmacological experiments (1–3). The present paper reports on the use of computer procedures as a means of simulating experimental conditions, with the aim of gaining an insight into the mechanisms underlying specified chemical, physical, and biological processes, with a maximum of accuracy and a minimum of routine and repetitive experimentation.

The simulation procedure involves the building up of a series of smooth computer-generated curves to find those most closely approximating the line of best fit through the data values determined experimentally, when the latter are presented as a graphic plot. The simulation curves are built up and manipulated by means of systematic variation of the parameters in a series of specification equations (4–7).

Digital computer procedures offer the advantage of large internal data storage capacity and high speed of decision-making and calculation (8). Analog computer procedures, on the other hand, offer the advantage of a direct human interaction facility, favoring optimum parameter variation control (9, 10). Hybrid computer systems utilize the advantages of both procedures: the parameter optimization strategy is performed in the digital control unit, whereas the actual solution of the specification equations is developed in the analog component (11). The present system achieves this dual capacity by means of an interactive simulation program on a digital computer equipped with graphic display devices.

SYSTEM DESIGN

A flow chart of the basic operations performed in the simulation procedure is given in Fig. 1.

The problem is defined for the computer as a series of FORTRAN IV subroutines and is entered into the system on punched cards. These statements include the differential equations governing the curve forms specified and the techniques necessary for their solution (12–15). The fixed data (notably the experimental results) are also entered *via* punched card input.

Such factors as selection of the parameters to be considered, initial values for all variables, increment sizes to be adopted when changing variables, type of scale to be used for each graphic axis (*i.e.*, log or linear), range and limits of the axis-scale values, and required precision of numerical integration are entered by means of numerical command signals from the keyboard-printer (Fig. 1). For example, numerical instruction "08–100.0" specifies that the upper limit of the horizontal axis will be set equal to 100.0. These commands may be varied instantaneously and at will during the actual simulation procedure by entering new instructions *via* the keyboard.

The keyboard may also be used to take a shortcut in parameter variation in the light of the simulation results already displayed, or to change the graphic output from video display to hard copy on the mechanical plotter, or vice versa (Fig. 1).

EQUIPMENT

The simulations are performed on an IBM-1800 digital computer with 32K (16 bits) core storage and 2-μ sec. cycle time. The computer is equipped with digital input and output capabilities, including programmable electronic contacts, process interruption signal lines, and digital input sense lines. A Calcomp 565 incremental drum plotter is attached to the computer for hard copy output of graphical information. The plotter may be operated in on-line mode or by tape-spooling on one of four available tape drives.

The IBM-1800 is a disk-oriented data acquisition system and ideally suited for on-line simulations when time shared with other batch or process operations (16).

A Tektronix 611 bistable storage oscilloscope is used as the graphical display device in kinetic simulations (Fig. 2). The cathode ray tube has a resolution of 20 stored line pairs/cm. and 0.5-sec. erase time. The device is remotely controlled by programmable electronic contacts which command the write (Z-axis signal) and erase functions. Horizontal (X-axis) and vertical (Y-axis) deflections are obtained from 2 × 10 programmable electronic contacts, which are connected to the input of a digital-to-analog converter.

The converter was developed in this laboratory: it is of a simple ladder circuit design, giving a full-scale deflection of 1 v. and compatible with the standard IBM digital-to-analog converter.

PROCEDURE

The simulation program is written entirely in FORTRAN IV and is available on request.

The arithmetic statements specifying the set of differential equations are inserted at the start of the fourth-order Runge–Kutta subroutine (17). Where the statements refer to special subroutine functions, these functions are also compiled before initiating the

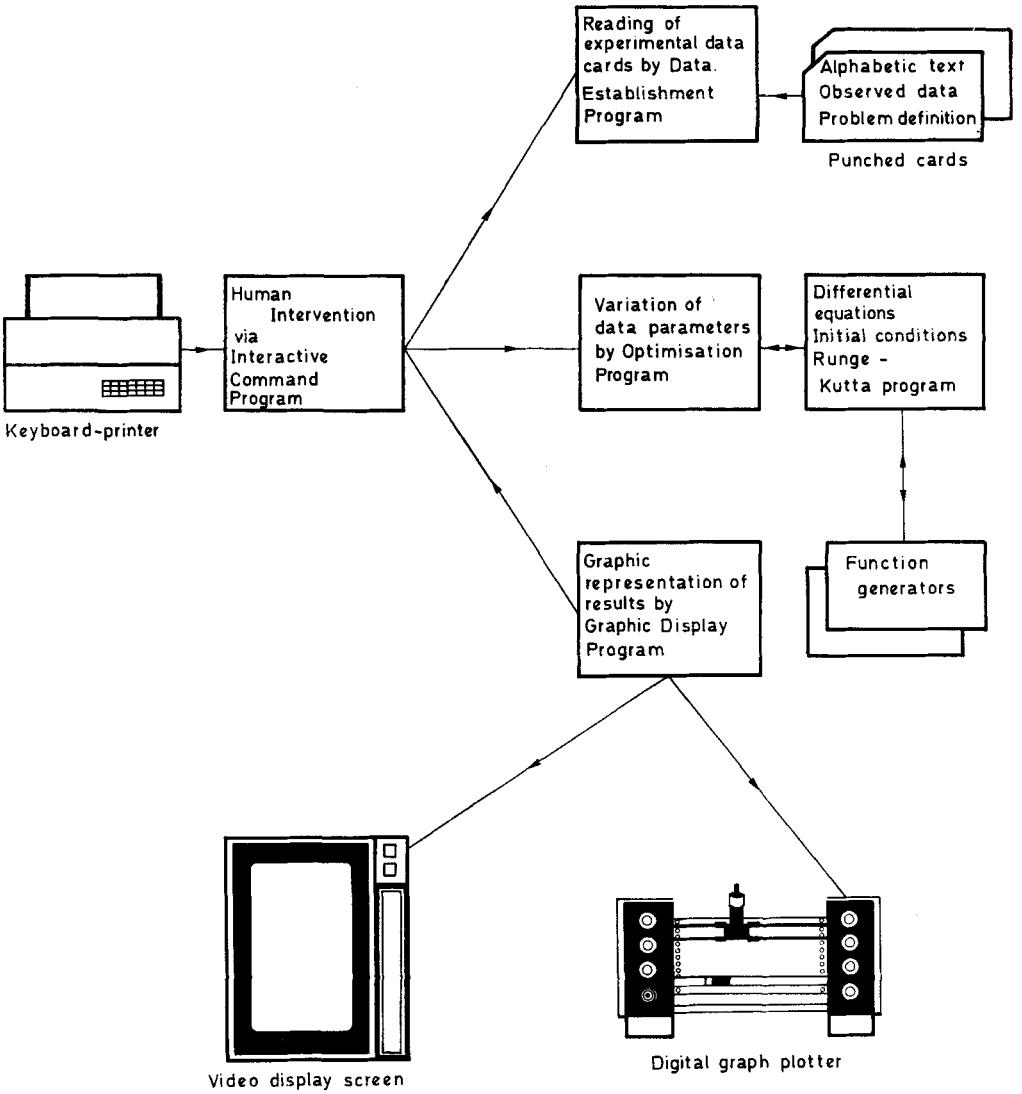


Figure 1—*Basic flowchart of the simulation procedure.*

simulation. Special nonlinear functions are available as in the Continuous Systems Modeling Program (CSMP) (18). The CSMP, however, does not provide for optimization of the parameters of a model.

For a problem involving the concentration of Compound X in a number of different intercommunicating compartments as a function of time, the fixed data input includes an alphabetic header (as in Figs. 3 and 4), the number of parameters (*NP*), the number of time values (*NT*), the number of compartments (*NC*) and all the time and concentration values, together with their standard errors and weight coefficients when these are available. The data are entered in the form of three matrixes of dimensions $NT \times NC$.

For each concentration value obtained experimentally (C^e), solution of the differential equations gives a computed value (C^c). The sum of the squares of the differences between C^e and C^c gives a measure of the goodness of fit of the simulation curve to the original data (C^e) values. The optimization program, therefore, computes the sum (Q) of the squared residuals, based on the initial (or previous) estimates of the parameter set $P_1, \ldots, P_K, \ldots, P_{NP}$, according to the formula:

$$Q = \frac{\sum_j \sum_i W_{ij}(C^c_{ij} - C^e_{ij})^2}{\sum_j \sum_i W_{ij}} \qquad \text{(Eq. 1)}$$

where i denotes, in turn, each compartment specified; j denotes, in turn, each experimental time value specified; and W_{ij} represents the weight coefficient associated with the value C^e_{ij}. (The weight coefficients may be obtained by taking the reciprocals of the observed concentration values or of the standard errors, or by using the weights specified on punched card input.)

If required, the optimization may be obtained from the sum of the squared *logarithmic* residuals:

$$Q = \frac{\sum_j \sum_i W_{ij} \left(\log \frac{C^c_{ij}}{C^e_{ij}}\right)^2}{\sum_j \sum_i W_{ij}} \qquad \text{(Eq. 2)}$$

This procedure has the merit of minimizing the squared relative deviations between fitted and observed values, and it represents a special method of weighting the data. The adequacies of the different weighting strategies were discussed recently by Mueller and Lieberman (19).

After calculation of the initial value for Q, the parameters selected for optimization are incremented by an amount ΔP_K (of which the magnitude may be varied by means of an appropriate command signal).

The new sum of squared residuals thus obtained is used for the estimation of the components of the gradient vector:

$$\frac{\partial Q}{\partial P_K} \cong \frac{Q(P_1,\ldots,P_K + \Delta P_K,\ldots,P_{NP}) - Q(P_1,\ldots,P_K,\ldots,P_{NP})}{\Delta P_K} \qquad \text{(Eq. 3)}$$

The components of the gradient vector (μ_k) are normalized by



Figure 2—*Keyboard entry device, incremental plotter, and visual display console used in connection with the IBM-1800 computer.*

means of the equation:

$$\mu_K = \frac{\partial Q}{\partial P_K} \Big/ \left[\sum_k \left(\frac{\partial Q}{\partial P_K} \right)^2 \right]^{1/2} \qquad \text{(Eq. 4)}$$

and new values of the parameters are computed from the relationship:

$$P_K' = P_K - \mu_K \beta \qquad \text{(Eq. 5)}$$

where β represents the step size in the parameter space. This procedure for automatic variation of the parameters was proposed by Giloi *et al.* (11) for a hybrid computer system.

Figure 3—*Computer-generated simulation plot for the progress of a chemical reaction (benzoylation of naphthalene). The vertical axis represents the concentration of naphthalene (moles/liter), and the horizontal axis represents the time (T) in hours. Crosses indicate the data values obtained experimentally. For explanation of alpha-numeric symbols, see text. The initial curve is uppermost, and each subsequent curve was computed with successively improved values of the parameters.*

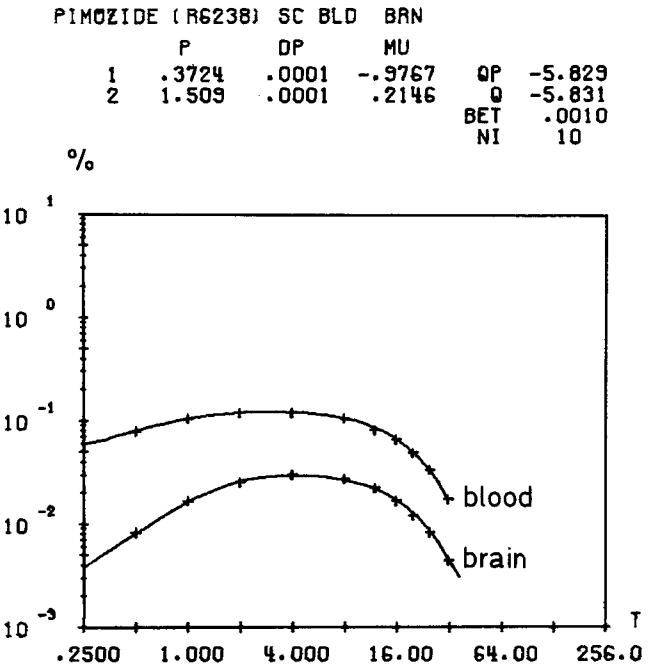
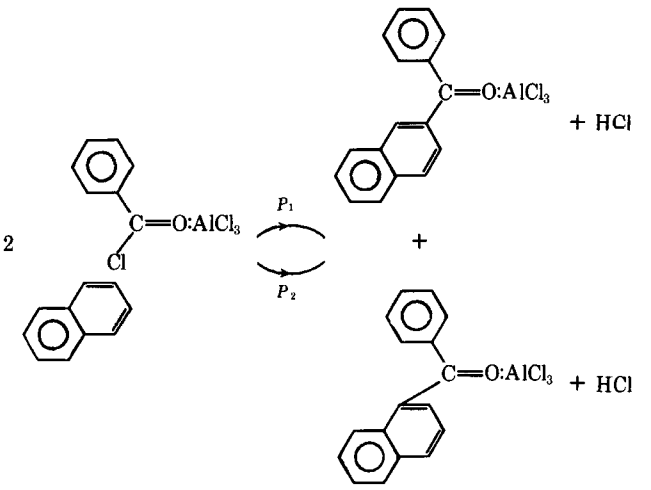


Figure 4—*Pharmacokinetic simulation of the level of the neuroleptic drug pimozide (R6238) in the brain (BRN) of rats, based on the known levels of drug in the blood (BLD). The vertical axis represents the brain level (as a percentage of the initial dose), and the horizontal axis represents the time in hours after subcutaneous (s.c.) injection of the drug. Crosses indicate the data values obtained experimentally. For explanation of alpha-numeric symbols, see text.*

Each parameter may be varied sequentially according to the Gauss-Seidel procedure (4). Alternatively, two or more of the available parameters may be varied simultaneously. The different weighting strategies and objective functions available in the optimizing algorithm permit optimization over several different compartments.

When the time–concentration functions of one or more compartments can be adequately approximated from the observed data by utilizing sums of exponential functions (20), gamma variates, polynomial expressions (21, 22), or other procedures, these functions and their derivatives may be used in the Runge–Kutta algorithm to develop solutions for other compartments.

When a solution has been obtained, it can be presented on a graphic display frame on the cathode ray tube or on the plotter as shown in Fig. 2. The display area includes an alphabetic zone (top line) and two numerical zones (next five lines)—one containing the actual parameter values (*P*), their increments (*DP*), and the



Scheme I—*Benzoylation of naphthalene by means of the Friedel–Crafts reaction in chlorobenzene*

normalized gradients (*MU*), and the other containing logarithmic values of the previous (*QP*) and actual (*Q*) sums of squared residuals, the step size in the parameter space (*BET*), and the number of integrations (*NI*) in the Runge–Kutta procedure.

The system permits visualization, on the same frame, of successive solutions for one compartment (Fig. 3) or parallel solutions for different compartments (Fig. 4).

Example 1—*Benzoylation of Naphthalene (Fig. 3)*—The simulation program was used to study the kinetics of the benzoylation of naphthalene in chlorobenzene (Scheme I) by the Friedel–Crafts reaction (23). The initial concentrations of the two reactants were both 0.488 mole/l. Experimental data on the progress of the reaction were obtained by withdrawing and analyzing samples of the reaction mixture at different times.

A good fit between the simulation curves and the experimental data was obtained using a third-order main reaction rate constant (P_1) of 21.10/hr. and a second-order parallel reaction rate constant (P_2) of 0.8995/hr. (Fig. 3).

Example 2—*Drug Distribution within the Body (Fig. 4)*—It is possible to study more complex kinetic models than the chemical reaction described previously by resolving them into a number of separate simulations, each involving only some of the compartments. An example is given here.

The distribution of the neuroleptic drug pimozide (R6238) was studied extensively in rats by radioisotopic labeling (24). Radioactivity levels of the nonmetabolized drug were recorded for the brain, blood, liver, urine, and feces at a number of time intervals after subcutaneous administration of the drug.

The first step in the solution of the blood–brain relationship was to derive a polynomial approximation by a least-squares method between blood concentration (C_{b1}) and log time (T) values. This function $F(T)$ was entered as a subroutine program and used to simulate the brain concentration (C_{br})–time function.

The problem was thus defined by the statement:

$$\dot{C}_{br} = P_1F(T) - P_2C_{br} \qquad \text{(Eq. 6)}$$

where P_1 represents the rate constant of uptake from the blood compartment, and P_2 represents the rate constant of elimination from the brain compartment. The initial concentrations were set equal to the best-fit concentration values for the smallest time interval after pimozide administration (0.25 hr.).

As Fig. 4 shows, the rate constant for the transfer of pimozide from blood to brain (P_1) was 0.3724/hr., and that for the reverse transfer (P_2) was 1.509/hr.

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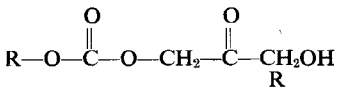
Unsymmetrical Carbonates as Potential Long-Lasting Insect Repellents

LORRIN R. GARSON and DANIEL D. GARNER

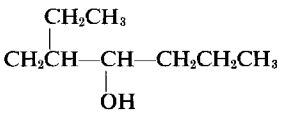
Abstract □ The synthesis of unsymmetrical carbonates of 1,3-dihydroxy-2-propanone with mono- and difunctional insectifugal alcohols is described. The compounds were designed to provide perdurable insect repellency upon topical application.

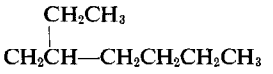
Keyphrases □ Repellents, insect, potential—1,3-dihydroxy-2-propanone carbonates, synthesis □ 1,3-Dihydroxy-2-propanone carbonates—synthesis, insect repellents, potential □ Carbonates, unsymmetrical, 1,3-dihydroxy-2-propanone—insect repellents, potential

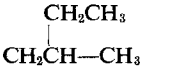
Due to the insect repellent efficacy of several monoesters of 1,3-dihydroxy-2-propanone[1] (1, 2), this study sought to incorporate the standard insect repellent 2-ethyl-1,3-hexanediol (Rutgers 612) and other insectifugal alcohols (3) into "precursor molecules" through a carbonate-connecting linkage with 1,3-dihydroxy-2-propanone. The rationale of this approach (4) and the structural features of several monoacyl and diacyl derivatives of 1,3-dihydroxy-2-propanone were previously discussed in detail (5).



Compound

I 

II 

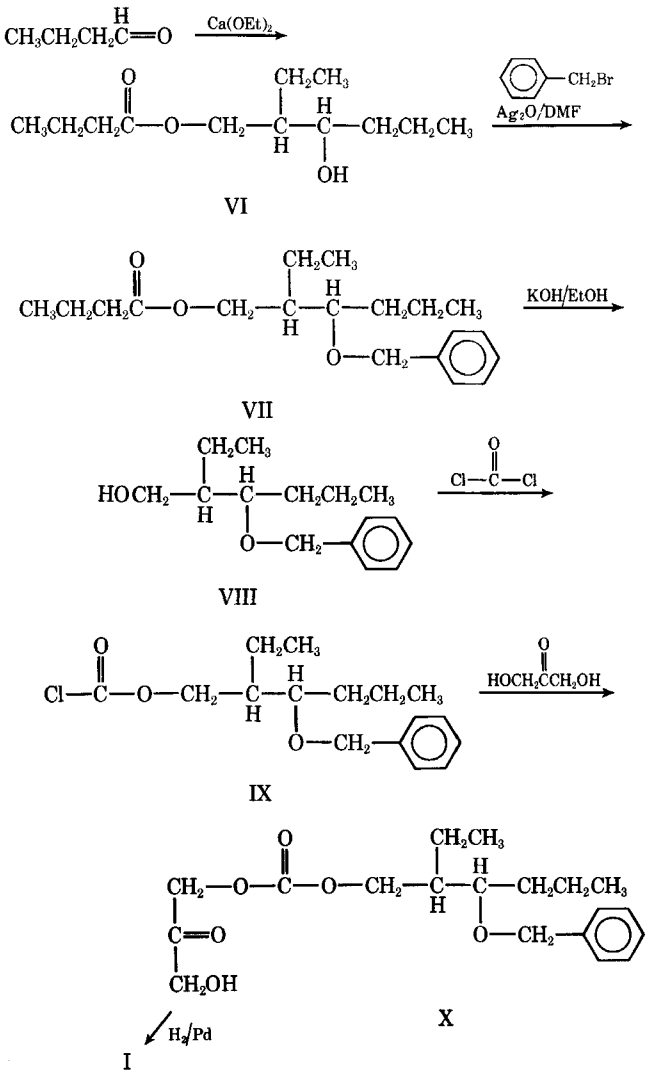
III 

IV $CH_2CH_2—O—C_6H_5$

Compounds I–IV are designed to release the respective repellent alcohols *via* hydrolysis of the carbonate function subsequent to dermal application. Cleavage should be facilitated by the influence of both the secondary alcohol function in I (6, 7) and the ketone group (8) present in each of the precursor molecules. Compounds II and III are, obviously, structural relatives of I. They were designed to ascertain the influence of the hydroxyl function and that of the chain length in the repellent component.

The preparation of Compounds II–IV is accomplished by reacting ROH with phosgene in ether to give the corresponding chlorocarbonate R—O—CO—Cl, which in turn is allowed to react with an excess of 1,3-dihydroxy-2-propanone in pyridine. The resulting unsymmetrical carbonates are sufficiently stable to permit purification, and separation from the accompanying biscarbonates, by distillation. In preparing IV, the bisester [1,3-di(2-phenoxyethylcarbonato)-2-propanone, V] was isolated and fully characterized.

The synthesis of I, however, does not lend itself to the same reaction sequence. While, as in the preparation of mephenesin carbamate (9), it may be possible to obtain selectively the chlorocarbonate of the primary alcohol of



Scheme I

[1] The term dihydroxyacetone frequently appears in the literature.

2-ethyl-1,3-hexanediol, cyclization to the corresponding 1,3-dioxane-2-one would appear to be a serious side reaction; furthermore, such a synthesis would not be unambiguous. Accordingly, Compound I was prepared by protecting the secondary alcoholic function of 2-ethyl-1,3-hexanediol by conversion to a benzyl ether moiety (Scheme I). All intermediates in this sequence, being oily liquids, were subjected to distillation at reduced pressures. Compound X was readily purified by high-vacuum distillation (molecular still); however, Compound I was not amenable to this procedure. A small sample (about 0.4 ml.) of I was successfully distilled using this technique, but decomposition occurred when attempts were made to distill larger quantities.

Compounds I–IV were tested for insectifugal activity against *Aedes aegypti* (L.) mosquitoes subsequent to application on the forearms of human volunteers. Experimental details were previously described (1). In comparing the insect repellency of these agents with the standard repellent *N,N*-diethyl-*m*-toluamide ("deet"), the compounds elicited an unexpected low level of activity; they provided 10% or less duration of protection than that afforded by the standard. The length of protection for these agents was not significantly increased upon subjecting the treated volunteers to conditions inducing sweating (27° at 80% relative humidity). The data reflect a lack of significant repellency for the intact precursor molecules and suggest that the desired hydrolytic cleavage of the carbonate linkage does not occur at a sufficiently rapid rate.

EXPERIMENTAL[2]

2-Ethyl-1,3-hexanediol 1-Butanoate (VI)—By employing a modification of the Villani and Nord method (10), 15.4 g. (0.384 mole) of calcium and a few crystals of mercuric chloride and iodine were added to 650 ml. of absolute EtOH. The ensuing exothermic reaction, which required heating to initiate, was occasionally quenched in an ice bath; the reaction mixture was heated at reflux 2 hr. and allowed to stand at ambient temperatures for 20 hr. The excess solvent was removed by distillation *in vacuo*, and the calcium ethoxide was dried for 16 hr. (25°/1.5 mm.). To the ethoxide was *cautiously* added 1000 ml. (810 g., 11 moles) of freshly distilled (b.p. 74.5°) cold butanal[3]; the mixture was cooled, with difficulty, in an ice–EtOH bath and allowed to stand at −10 to 15° for 3 hr. and then at room temperature for 4.5 hr. After removal of the metal catalyst by filtration, the reaction mixture was distilled *in vacuo*, employing an 80-cm. Vigreaux column, affording 168 g. (20.6%) of VI: b.p. 95–96° (0.2 mm.); n_D^{25} 1.4406; ρ^{25} 0.9405 [lit. (1) b.p. 100–102° (0.5 mm.); n_D^{21} 1.4462; ρ^{21} 0.9492]; $\nu_{max.}^{CHCl_3}$ 3571, 3460 (OH), and 1727 cm.$^{-1}$ (carbonyl).

Anal.—Calc. for $C_{12}H_{24}O_3$: C, 66.63; H, 11.18. Found: C, 66.58; H, 11.09.

3-Benzyloxy-2-ethyl-1-hexanol Butanoate (VII)—A mixture of 40.4 g. (0.187 mole) of VI, 63.9 g. (0.374 mole) of benzyl bromide, 43.4 g. (0.187 mole) of silver oxide, and 100 ml. of anhydrous *N,N*-dimethylformamide was protected from light and stirred at room temperature for 1 week. The reaction mixture was filtered through a bed of Celite, the residue was washed with *N,N*-dimethylformamide (2 × 20 ml.), and the filtrate was distilled *in vacuo* (40-cm. Vigreaux column). The fraction boiling at 154–159° (0.7 mm.) was redistilled, yielding pure VII; consistent yields of 66–73% were obtained: b.p. 123–127° (0.15 mm.); n_D^{25} 1.4791; ρ^{25} 0.9700; $\nu_{max.}^{CHCl_3}$ 1724 cm.$^{-1}$ (carbonyl); $\lambda_{max.}^{EtOH}$ 210 nm. (ϵ 7320).

Anal.—Calc. for $C_{19}H_{30}O_3$: C, 74.47; H, 9.87. Found: C, 74.42; H, 9.88.

3-Benzyloxy-2-ethyl-1-hexanol (VIII)—A solution of 5.00 g. (0.0163 mole) of VII in 16.3 ml. of 2 *N* alcoholic KOH was heated at reflux for 3 hr., cooled, treated with charcoal, and filtered; the filtrate was distilled *in vacuo*, leaving a brown, semisolid material. The residue was partitioned between H_2O (50 ml.) and $CHCl_3$ (50 ml.), and the aqueous phase was extracted with $CHCl_3$ (3 × 30 ml.). The combined $CHCl_3$ phases were dried ($MgSO_4$), filtered, concentrated, and distilled *in vacuo*, yielding 2.50 g. (64.8%) of VIII: b.p. 121-122° (0.05 mm.); n_D^{25} 1.4954; $\nu_{max.}^{CHCl_3}$ 3472 cm.$^{-1}$ (OH); $\lambda_{max.}^{EtOH}$ 210 nm. (ϵ 6920).

Anal.—Calc. for $C_{15}H_{24}O_2$: C, 76.23; H, 10.23. Found: C, 76.10; H, 10.35.

3-Benzyloxy-2-ethyl-1-hexyl Chlorocarbonate (IX)—To a solution of 29.6 g. (0.299 mole) of phosgene in 100 ml. of anhydrous ether was added 29.3 g. (0.124 mole) of VIII. After heating at reflux for 12.5 hr., a stream of dry air was passed through the system, removing excess phosgene and HCl; the residue was concentrated and dried overnight *in vacuo*. No attempt was made to purify IX before subsequent use: $\nu_{max.}^{CHCl_3}$ 1773 cm.$^{-1}$ (carbonyl), OH bands not present.

1-(3-Benzyloxy-2-ethylhexyl-1-carbonato)-3-hydroxy-2-propanone (X)—To a cooled (−8 to −1°), well-stirred solution of 44.7 g. (0.496 mole) of dry 1,3-dihydroxy-2-propanone in 200 ml. of anhydrous pyridine was slowly added a solution of 37.1 g. (0.124 mole) of chlorocarbonate IX in 200 ml. of freshly distilled (from P_2O_5) $CHCl_3$. After warming to room temperature and stirring for an additional hour, the reaction mixture and 500 ml. of 6 *N* HCl were simultaneously added dropwise to 250 ml. of 3 *N* HCl and 500 g. of ice, taking care to maintain a reaction temperature of −10 to −1°. After separating the organic phase, the aqueous layer was extracted with $CHCl_3$ (3 × 50 ml.); the combined organic phases were dried ($MgSO_4$), filtered, and concentrated *in vacuo*, affording a yellow liquid. Purification of the oil by molecular distillation (130–135° at 0.010–0.015 mm.) yielded a viscous yellow product in 20.5 g. (46.9%) yield: n_D^{25} 1.4982; $\nu_{max.}^{CHCl_3}$ 3472 (OH) and 1742 cm.$^{-1}$ (carbonyl); $\lambda_{max.}^{EtOH}$ 208 nm. (ϵ 7520).

Anal.—Calc. for $C_{19}H_{28}O_6$: C, 64.75; H, 8.01. Found: C, 64.67; H, 8.13.

1-(2-Ethyl-3-hydroxyhexyl-1-carbonato)-3-hydroxy-2-propanone (I)—With a Brown hydrogenator (12), 410 mg. (1.16 mmoles) of X in 20 ml. of 95% EtOH was catalytically reduced (24 hr.), employing 500 mg. of 10% palladium on charcoal. After removing the catalyst by filtration (Celite), the filtrate was concentrated *in vacuo* and the residue was purified by molecular distillation (135° at 0.004 mm.) to yield 97.0 mg. (31.8%) of I: n_D^{25} 1.4630; $\nu_{max.}^{CHCl_3}$ 3460 (OH) and 1745 cm.$^{-1}$ (carbonyl).

Anal.—Calc. for $C_{12}H_{22}O_6$: C, 54.95; H, 8.45. Found: C, 54.98; H, 8.93.

1-(2-Ethylhexyl-1-carbonato)-3-hydroxy-2-propanone (II)—In a manner analogous to the preparation of IX, 36.2 g. (0.278 mole) of freshly distilled [b.p. 60-61° (1.6 mm.)] 2-ethyl-1-hexanol was converted to the chlorocarbonate by reaction with 83.6 g. (0.845 mole) of phosgene. As in the preparation of X, the chlorocarbonate was allowed to react with 1,3-dihydroxy-2-propanone (100 g., 1.11 moles) to yield 36.4 g. (53.1%) of analytically pure II after molecular distillation (107° at 0.020 mm.): n_D^{25} 1.4565; $\nu_{max.}^{CHCl}$ 3472 (OH) and 1748 cm.$^{-1}$ (carbonyl).

Anal.—Calc. for $C_{12}H_{22}O_5$: C, 58.52; H, 9.00. Found: C, 58.61; H, 9.20.

1-(2-Methylbutyl-1-carbonato)-3-hydroxy-2-propanone (III)—Similarly, 24.5 g. (0.278 mole) of 2-methyl-1-butanol and 50.1 g. (0.506 mole) of phosgene afforded the desired chlorocarbonate. This, in turn, was allowed to react with 100 g. (1.11 moles) of 1,3-dihydroxy-2-propanone to yield, after molecular distillation (93° at 0.005–0.010 mm.), 36.7 g. (64.6%) of III: n_D^{25} 1.4460; $\nu_{max.}^{CHCl_3}$ 3497 (OH) and 1745 cm.$^{-1}$ (carbonyl).

Anal.—Calc. for $C_9H_{16}O_5$: C, 52.93; H, 7.90. Found: C, 53.08; H, 7.99.

1-(2-Phenoxyethylcarbonato)-3-hydroxy-2-propanone (IV) and 1,3-Di(2-phenoxyethylcarbonato)-2-propanone (V)—By employing the procedures used in the preparation of IX and X, the chlorocarbonate from 42.1 g. (0.305 mole) of 2-phenoxyethanol and 31.3 g.

[2] Melting points are corrected; they were determined with a Büchi melting-point apparatus. Boiling points are uncorrected. UV and IR spectra were obtained with the Perkin-Elmer model 202 and 137B spectrophotometers, respectively. Molecular distillations were performed employing a Rota-Film model 50-2 apparatus. The mass spectral analysis was carried out by the Morgan–Schaffer Corp., Montreal, Canada. Combustion analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

[3] Addition of the catalyst to butanal, as described by Villani and Nord (10), frequently led to an uncontrollable exothermic reaction.

(0.316 mole) of phosgene, when allowed to react with 100 g. (1.11 moles) of 1,3-dihydroxy-2-propanone, afforded 61.2 g. of a mixture of IV and V. The crude product was extracted with boiling ether (6 × 200 ml.) to remove the bisester. The ether-insoluble material was recrystallized three times from 95% EtOH, yielding 25.0 g. (32.3%) of analytically pure IV: m.p. 121–126°; $\nu_{max.}^{mineral\ oil}$ 3356 (OH) and 1739 cm.$^{-1}$ (carbonyl); $\lambda_{max.}^{EtOH}$ 219, 266, 273, and 280 nm. (ϵ 7570, 1220, 1630, and 1390).

Anal.—Calc. for $C_{12}H_{14}O_6$: C, 56.69; H, 5.55. Found: C, 56.64; H, 5.69.

The combined ethereal extracts were evaporated *in vacuo*. The solid residue was recrystallized five times from 95% EtOH, affording pure V: m.p. 101–102°; $\nu_{max.}^{CHCl_3}$ 1751 cm.$^{-1}$ (carbonyl); $\lambda_{max.}^{EtOH}$ 218, 264, 271, and 278 nm. (ϵ 1420, 2320, 3100, and 2590); mass spectrum, *m/e* (relative intensity) 418 (6.3), 325 (5.6), 237 (50), 143 (41), 121 (92), and 94 (100); isotope distribution calc. for $C_{21}H_{22}O_9$, *m/e* (relative intensity) 418 (100), 419 (23.3), 420 (4.38), found 418 (100), 419 (23.5), 420 (4.30).

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Structure Elucidation of Maytenonic Acid, a New Triterpene from *Maytenus senegalensis* (Celastraceae)

D. J. ABRAHAM, J. TROJÁNEK, H. P. MÜNZING, H. H. S. FONG, and N. R. FARNSWORTH

Abstract □ Evidence for the structure of a new triterpene from *Maytenus senegalensis*, named maytenonic acid, is presented. The structure was postulated mainly on the basis of interpretation of IR, NMR, and mass spectral data.

Keyphrases □ Maytenonic acid, triterpene from *Maytenus senegalensis*—structure elucidation □ *Maytenus senegalensis* (Celastraceae)—structure elucidation of maytenonic acid □ Spectroscopy, IR, NMR, mass—structure elucidation of maytenonic acid from *Maytenus senegalensis*

Previously, the isolation and identification of β-amyrin, lupenone, β-sitosterol, β-sitosterol xyloside, wilforine, dulcitol, and two new triterpenes from *Maytenus senegalensis* (Lam.) Excell. were reported (1). Extracts and fractions from the plant were shown to have reproducible activity against the 9KB carcinoma of the nasopharynx in cell culture, as well as the L-1210 leukemia and PS leukemia tumor systems in mice. Dulcitol was shown to be responsible for a part of the antitumor activity. At this time, evidence is presented for the structure elucidation of one of the triterpenes previously isolated, triterpene A (1).

DISCUSSION

Maytenonic acid (I) (triterpene A), m.p. 262°, $[\alpha]_D^{26}$ −32 (c, 0.5, MeOH) was shown to have a molecular formula of $C_{30}H_{48}O_3$ by

Table I—Mass Spectrometric Data for Maytenonic Acid[a]

m/e	Percent	*m/e*	Percent
456	8.7	163	32.6
441	6.5	155	63.5
410	4.3	135	22.3
395	2.0	121	33.4
371	4.3	109	100.0
273	32.6	95	61.8
250	16.2	81	52.7
221	8.7	69	42.8
218	13.0	55	60.5
205	6.5	43	52.7
189	10.0	41	34.5

[a] Spectra were determined using a mass spectrometer LKB-9000 (LKB Produkter, Stockholm, Sweden) at 70 e.v.

(0.316 mole) of phosgene, when allowed to react with 100 g. (1.11 moles) of 1,3-dihydroxy-2-propanone, afforded 61.2 g. of a mixture of IV and V. The crude product was extracted with boiling ether (6 × 200 ml.) to remove the bisester. The ether-insoluble material was recrystallized three times from 95% EtOH, yielding 25.0 g. (32.3%) of analytically pure IV: m.p. 121–126°; $\nu_{max.}^{mineral\ oil}$ 3356 (OH) and 1739 cm.$^{-1}$ (carbonyl); $\lambda_{max.}^{EtOH}$ 219, 266, 273, and 280 nm. (ϵ 7570, 1220, 1630, and 1390).

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Abstract □ Evidence for the structure of a new triterpene from *Maytenus senegalensis*, named maytenonic acid, is presented. The structure was postulated mainly on the basis of interpretation of IR, NMR, and mass spectral data.

Keyphrases □ Maytenonic acid, triterpene from *Maytenus senegalensis*—structure elucidation □ *Maytenus senegalensis* (Celastraceae)—structure elucidation of maytenonic acid □ Spectroscopy, IR, NMR, mass—structure elucidation of maytenonic acid from *Maytenus senegalensis*

Previously, the isolation and identification of β-amyrin, lupenone, β-sitosterol, β-sitosterol xyloside, wilforine, dulcitol, and two new triterpenes from *Maytenus senegalensis* (Lam.) Excell. were reported (1). Extracts and fractions from the plant were shown to have reproducible activity against the 9KB carcinoma of the nasopharynx in cell culture, as well as the L-1210 leukemia and PS leukemia tumor systems in mice. Dulcitol was shown to be responsible for a part of the antitumor activity. At this time, evidence is presented for the structure elucidation of one of the triterpenes previously isolated, triterpene A (1).

DISCUSSION

Maytenonic acid (I) (triterpene A), m.p. 262°, $[\alpha]_D^{26}$ −32 (c, 0.5, MeOH) was shown to have a molecular formula of $C_{30}H_{48}O_3$ by

Table I—Mass Spectrometric Data for Maytenonic Acid[a]

m/e	Percent	*m/e*	Percent
456	8.7	163	32.6
441	6.5	155	63.5
410	4.3	135	22.3
395	2.0	121	33.4
371	4.3	109	100.0
273	32.6	95	61.8
250	16.2	81	52.7
221	8.7	69	42.8
218	13.0	55	60.5
205	6.5	43	52.7
189	10.0	41	34.5

[a] Spectra were determined using a mass spectrometer LKB-9000 (LKB Produkter, Stockholm, Sweden) at 70 e.v.

Table II—High-Resolution Mass Spectral Measurements of Selected Ions of Maytenonic Acid[a]

Formula	Calculated *m/e*	Observed *m/e*
$C_{30}H_{48}O_3$	456.3603	456.3628
$C_{29}H_{46}O$	410.3548	410.3573
$C_{28}H_{43}O$	395.3314	395.3315
$C_{25}H_{39}O_2$	371.2950	371.2968
$C_{19}H_{29}O$	273.2218	273.2229
$C_{16}H_{26}O_2$	250.1933	250.1952
$C_{15}H_{25}O$	221.1905	221.1943
$C_{14}H_{21}O_2$	221.1541	221.1580
$C_9H_{15}O_2$	155.1072	155.1074
C_9H_{13}	121.1017	121.1023
C_8H_{13}	109.1017	109.1003

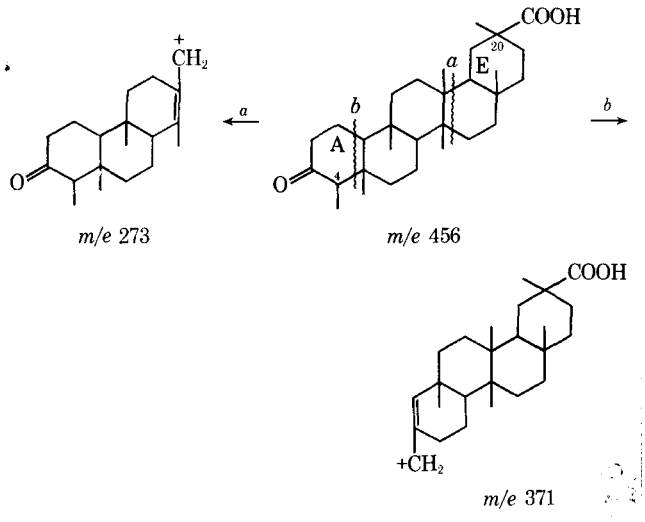
[a] Spectra were obtained on a double-focusing mass spectrometer MS 9 (Allied Electrical Industries Ltd., Manchester, England).

high-resolution mass spectrometric measurements (Tables I and II). These data, together with a positive Liebermann–Burchard color test, showed the compound to be a triterpene. The IR spectrum of I in mineral oil exhibited two separate bands of nonconjugated carbonyl groups at 5.7 and 5.83 μ, together with a broad absorption band between 2.8 and 3.8 μ, corresponding to hydrogen bonding of an OH group. The IR spectrum of I in chloroform was similar to that in mineral oil but the broad band was shifted toward higher wavelengths (3.0–4.0 μ) and both carbonyl bands appeared at about 5.81 and 5.85 μ.

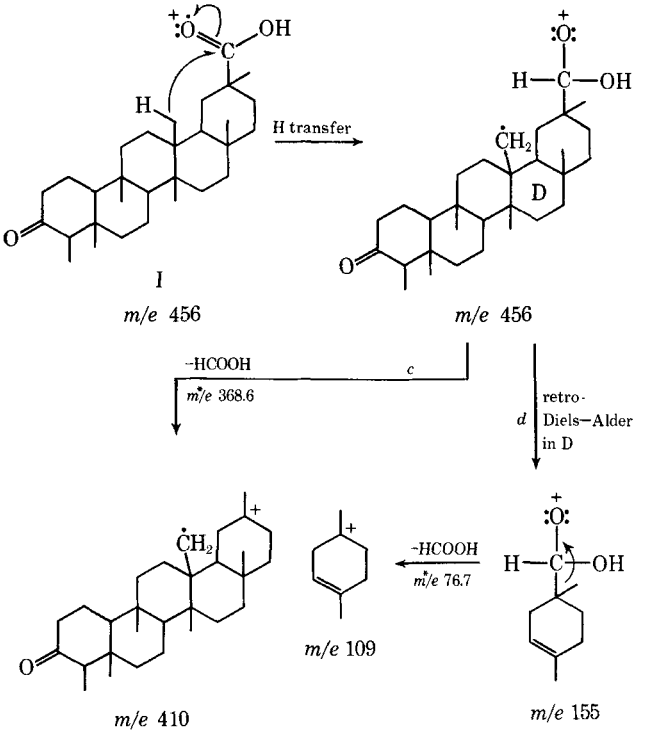
These spectral characteristics indicated the presence of either two keto and one hydroxyl group, or a keto and a carboxy group. To distinguish between these two possibilities, an attempt was made to extract I from chloroform solution with a 5% solution of sodium hydroxide; however, the compound did not appear in the aqueous fraction after acidification as would be expected of an acid. That, in fact, a carboxy group was present in the molecule was later discovered by gently warming I in dimethyl sulfoxide with aqueous sodium hydroxide followed by cooling. The material that precipitated from solution was the water-insoluble sodium salt of an acid, m.p. >300°, as shown by its IR spectrum (mineral oil) in which the original carbonyl band at 5.83 μ was shifted to 6.2 μ.

An examination of the mass spectrum of maytenonic acid (Table I) afforded evidence that I belonged to the friedelane series of triterpenes. Earlier, it was shown (2, 3) that the peaks at *m/e* 273 and 341 for 3-keto friedelane arise by retro-Diels–Alder reaction, together with a loss of one hydrogen, and they are very characteristic for this series of triterpenes. The presence of peaks at *m/e* 273 and 371 (Table II) in the mass spectrum of I provided not only the evidence concerning the carbon skeleton of I but also indicated that one oxygen function, *i.e.*, the carbonyl group, was in the A ring and that the acid function was in the terminal E ring (Scheme I).

The NMR spectrum of I confirmed the mass spectral evidence for the friedelane type of triterpene since it lacked the signals for



Scheme I



Scheme II

olefinic protons and it exhibited a methyl doublet at 0.85 δ due to the methyl group at C_4. Moreover, integration showed the presence of seven methyl groups, again confirming that I was a keto carboxylic acid, since there would be eight methyl groups present if I were a diketo hydroxy compound or it would show a CH_2OH absorption if the hydroxyl group was located at one of the methyl groups. Finally, the NMR spectrum also gave evidence concerning the position of the acid group on ring E. Tursch *et al.* (4, 5) studied the NMR spectra of a large series of triterpenes and found that the absorption of a C-methyl group geminal to a carboxyl group occurs downfield from all other C-methyl groups at about 1.25 δ. The NMR spectrum of I contained one methyl absorption at 1.25 δ, thus placing the carboxylic function with all due probability at C_{20}.

With this structure in hand, it was possible to interpret further the strong peaks in the mass spectrum of maytenonic acid (Tables I and II) as shown in Scheme II.

If the interpretation of the transfer of one hydrogen from the methyl group at C_{13} (pathways *c* and *d*) is correct, then the carboxyl group at C_{20} should be *cis* oriented with respect to the methyl group at C_{13}.

Further studies are underway to confirm the structure of maytenonic acid as I, as well as to establish the stereochemistry of the molecule.

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Collaborative Study of Aerobic Media for Sterility Testing by Membrane Filtration

FRANCES W. BOWMAN, MACK WHITE, and MIRIAM P. CALHOUN

Abstract □ The USP XVIII and NF XIII (first supplement) have replaced the sterility test medium, fluid Sabouraud, with a soybean-casein digest medium. A collaborative study performed by 12 laboratories showed that soybean-casein digest medium is superior to fluid Sabouraud medium for sterility testing by membrane filtration.

Keyphrases □ Aerobic media for membrane filtration sterility testing—collaborative study □ Soybean-casein digest medium—collaborative study of use in membrane filtration sterility tests □ Membrane filtration sterility testing—collaborative study on soybean-casein digest medium

The Food and Drug Administration has been using Sabouraud liquid medium USP (pH 5.7) for testing the sterility of antibiotics since 1964 (1). Prior to that time, a modified Sabouraud medium (also pH 5.7) was used (2). The primary reason for using this medium in the sterility test was to detect the presence of fungi rather than bacteria. A few bacteria found as contaminants of antibiotics did not grow at pH 5.7 but were recovered in thioglycollate medium (pH 7.1). Since the sterility test is intended to detect as many microorganisms as possible, it is patently undesirable to use a medium, such as fluid Sabouraud at pH 5.7, that inhibits certain bacteria. To eliminate the use of a selective medium for the sterility test, the USP XVIII and the first supplement to NF XIII replaced fluid Sabouraud USP XVII with a soybean-casein digest (SBCD) medium which has a pH of 7.3 ± 0.2. However, before implementing any change in the sterility tests for antibiotics as required in the *Antibiotic Regulations*, a collaborative study was performed to compare the growth-promoting qualities of SBCD medium to those of fluid Sabouraud. Twelve laboratories participated in this study[1].

Since preliminary work indicated that fluid Sabouraud medium at pH 7.0 supports the growth of fungi without inhibiting bacteria, this medium was also included in the study. All three media were manufactured by each of two companies (designated as Manufacturers A and B) for use in this study. A protocol containing detailed instructions and the media to be used in the study were supplied to each collaborator (3).

[1] Biological Safety Control, Becton, Dickinson and Co., Raleigh, N. C.; Bristol Laboratories, Syracuse, N. Y.; Difco Laboratories, Detroit, Mich.; Food and Drug Administration, National Center for Antibiotic Analysis, Sterility Testing Branch; Food and Drug Directorate Laboratories, Ottawa, Canada; Lederle Laboratories, Pearl River, N. Y.; Eli Lilly and Co., Indianapolis, Ind.; Laboratory of Control Activity, Division of Biological Standards, National Institutes of Health, Bethesda, Md.; Parke, Davis & Co., Detroit, Mich.; Chas. Pfizer & Co., Inc., Brooklyn, N. Y.; E. R. Squibb & Sons, Inc., New Brunswick, N. J.; and Wyeth Laboratories Inc., West Chester, Pa.

EXPERIMENTAL

The procedure used was essentially the same as that used for testing the sterility of antibiotics (4). The design of the study was similar to that used in *Reference 5*.

Organisms—Cultures or spore suspensions of the nine microorganisms used in the study were supplied by the authors. Dilutions of inocula were selected to simulate low levels of contamination that might possibly be encountered in a contaminated pharmaceutical preparation being tested for sterility. The following microorganisms were employed: *Bacillus subtilis* ATCC 6633 (spores), *Staphylococcus aureus* ATCC 6538 P; *Aspergillus niger* ATCC 6275 (spores), *Bacillus circulans* PCI 260 (spores), *Bacillus* sp. PCI 208 (spores), *Saccharomyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 10536, and *Bacillus* sp. PCI 258 (spores).

Media—Instructions for reconstitution and sterilization of the media were as follows: Weigh 120 g. of the dehydrated medium and rehydrate by adding 4 l. of distilled water. If necessary, warm the mixture to complete solution. When the medium is in solution, dispense it in 100-ml. portions to each of 36 (38 × 200-mm.) screw-capped tubes. Label each tube as to contents. Sterilize the tubes of media in an autoclave at 121° for 20 min. The autoclave temperature should be reached within 10 min.

Each collaborator prepared a total of 216 labeled tubes, comprising 36 tubes each of six media (SBCD medium A and B, Sabouraud medium pH 5.7 A and B, and Sabouraud medium pH 7.0 A and B).

Incubation Time—Incubation time was limited to 2, 5, and 7 days since the collaborative study of insulin (4), in which samples were incubated 7, 10, and 14 days, had shown that 7 was the maximum number of days required for the recovery of microorganisms where the membrane filtration procedure was used.

The following protocol was supplied to and followed by the collaborators: For each of the nine microorganisms, prepare about 150 ml. of a stock organism suspension containing approximately 100 colony-producing units (CPU) per 20 ml. of sterile 0.1% peptone solution and dispense 20 ml. into seven sterile bottles. From the remaining stock, prepare a low-level inoculum of approximately 5 CPU/20 ml. by adding 1 ml. to 19 ml. of sterile 0.1% peptone solution in each of seven bottles. Immediately perform the membrane filtration test on the contents of each of the 14 bottles as follows: Filter the entire contents of one bottle through a 0.45-μ membrane filter. Rinse the filter by filtering 100 ml. of sterile 0.1% peptone solution through it. Cut a circular disk approximately 17.5

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Table I—Number of Collaborators Reporting Positive Tests in the Recovery of Microorganisms in Several Aerobic Media

Microorganism	Inoculum Expressed[a] CPU's	Sabouraud pH 5.7 Manufacturer A—Days of Incubation 2	5	7	Sabouraud pH 5.7 Manufacturer B—Days of Incubation 2	5	7	Sabouraud pH 7.0 Manufacturer A—Days of Incubation 2	5	7	Sabouraud pH 7.0 Manufacturer B—Days of Incubation 2	5	7	Soybean-Casein Digest Medium Manufacturer A—Days of Incubation 2	5	7	Soybean-Casein Digest Medium Manufacturer B—Days of Incubation 2	5	7
Bacillus subtilis	1–5	1	4	6	1	2	7	4	5	5	6	10	11	8	11	11	12	12	12
ATCC 6633 (spores)	50–100	5	7	8	4	10	11	8	11	12	9	11	11	12	12	12	12	12	12
Staphylococcus aureus	1–5	0	0	0	12	12	12	0	0	1	8	12	12	5	10	10	9	11	12
ATCC 6538	50–100	0	1	2	1	6	10	1	4	4	9	11	12	9	12	12	10	11	11
Aspergillus niger	1–5	6	9	9	6	10	11	6	10	10	6	10	10	6	12	12	6	10	10
ATCC 6275 (spores)	50–100	10	12	12	10	11	11	9	12	12	10	12	12	11	12	12	11	12	12
Bacillus circulans	1–5	0	1	2	1	3	4	0	0	1	3	8	10	1	2	4	3	5	7
PCI 260 (spores)	50–100	0	0	1	0	2	4	1	2	3	7	9	10	4	6	7	6	9	10
Bacillus sp.	1–5	1	3	7	1	3	8	0	4	7	1	4	7	1	2	4	2	5	7
PCI 208 (spores)	50–100	1	4	8	1	5	7	0	6	9	0	8	8	1	3	5	2	7	8
Saccharomyces cerevisiae	1–5	8	12	12	8	10	10	4	9	10	7	10	10	6	12	12	9	12	12
ATCC 9763	50–100	12	12	12	10	11	11	11	12	12	10	11	11	10	11	12	12	12	12
Candida albicans	1–5	7	11	11	7	10	12	7	10	10	7	11	12	7	11	11	8	11	11
ATCC 10231	50–100	10	11	11	9	10	10	10	11	11	11	12	12	10	12	12	12	12	12
Escherichia coli	1–5	9	11	11	6	10	11	10	12	12	5	7	7	9	9	9	10	11	11
ATCC 10536	50–100	11	12	12	8	11	11	11	12	12	11	12	12	10	10	10	11	11	11
Bacillus sp.	1–5	0	0	1	1	1	1	1	1	1	1	3	6	4	11	11	5	11	11
PCI 258	50–100	0	3	3	0	0	2	1	1	1	0	3	10	6	10	10	8	11	11

[a] Number of colony-producing units.

mm. in diameter from the center of the membrane, and transfer it to a 38 × 200-mm. tube containing 100 ml. of SBCD broth from Manufacturer A. Using sterile forceps, transfer the outer portion of the membrane into a second similar tube also containing SBCD broth from Manufacturer A. Repeat the above test for the low and high microorganism populations for the other five media.

As a control on each inoculum for each microorganism, filter the contents of the seventh bottle but do not cut the membrane. Instead, place the entire membrane on the surface of a peptone-casein agar plate. Incubate the plates in an inverted position at 22–25° until colonies are visible. Count the number of colonies and record the number of CPU's. This confirms whether the expected inoculum is obtained. If the inoculum is found to be incorrect for any one of the microorganisms, repeat the test. Incubate all of the tubes at 22–25° and read them at 2, 5, and 7 days. Record the presence or absence of growth of each microorganism in each of the 36 tubes of each medium containing either the periphery or the disk portion of the membrane. (Growth in a tube containing either the periphery or the disk or both will be reported as a positive test in the final analysis.) Confirm all isolates from observable growth as being the same organisms as the original inoculum.

RESULTS AND CONCLUSIONS

The average inocula, as confirmed by plate counts, were 4.7 CPU/20 ml. for the low level and 83 CPU/20 ml. for the high level. As shown in Table I, the test results from 12 laboratories verified among the collaborators that, for the recovery of the nine microorganisms, SBCD medium is the best of the media tested when a membrane filtration sterility test is performed. A 7-day incubation period gives the highest recovery of organisms. On statistical analysis, the collaborator times media interaction suggested that the results with a given medium may be dependent on the experience of the collaborator with the particular method. This is in agreement with the assessment by Streeter and Robertson (6) that a laboratory needs at least 3 months of experience with the filtration technique before reproducible and certain results can be expected.

The effect of the individual collaborator's experience or lack of it was borne out by the results reported on *B. circulans* PCI 260 and *Bacillus* sp. PCI 258 in Sabouraud medium pH 5.7. These organisms were selected for the study because they do not grow at pH 5.7 but do grow at approximately pH 7.0; nevertheless, 18 recoveries were reported at pH 5.7 out of a possible 48. Also, several collaborators commented that Manufacturer B's SBCD medium presented a problem in reading when the growth was sparse. The difficulty was apparently due to a precipitate in the medium which macroscopically simulated sparse growth. This problem could be solved in an actual sterility test by subculture. However, in this study a subculture in pH 7.0 medium would merely have shown growth of the dormant spores which had been added to the samples being tested.

As a result of this collaborative study, the *Antibiotic Regulations* will be amended to change Medium E from fluid Sabouraud medium USP to soybean-casein digest medium USP, thus assuring a high recovery of microorganisms and improving the sensitivity of the antibiotic sterility test.

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Agents for Alkylating Steroid Hormone Receptors III: ω-Substituted Esters of 17α-Hydroxyprogesterone

A. J. SOLO and JOHN O. GARDNER

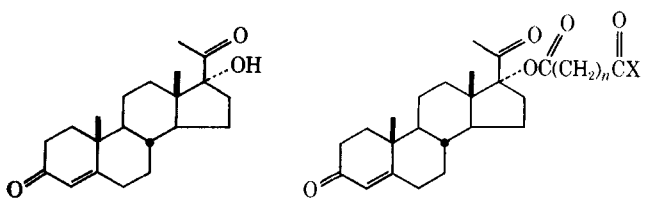
Abstract □ The synthesis and Clauberg activity of the 8-carbomethoxyoctanoate (II*e*), 9-carbomethoxynonanoate (II*f*), 9-carboethoxynonanoate (II*g*), 9-carbo-*tert*-butoxynonanoate (II*h*), 8-carboxyoctanoate (III*d*), 9-oxo-10-diazodecanoate (IV*d*), 5-oxo-6-hydroxyhexanoate (V*a*), and 6-oxo-7-hydroxyheptanoate (V*b*) esters of 17α-hydroxyprogesterone are reported and compared to the activity of related compounds.

Keyphrases □ 17α-Hydroxyprogesterone ω-substituted esters—synthesis, Clauberg activity □ Progestational activity—17α-hydroxyprogesterone-substituted esters □ Steroid hormone receptors—alkylating agents, synthesis of ω-diazoketones, activity □ NMR spectroscopy—structure □ IR spectrophotometry—structure

This study is an attempt to utilize the principles set forth by Baker (1) for the design of active-site-directed irreversible enzyme inhibitors to find compounds capable of selectively tagging the receptor sites of the steroid hormones (2, 3). Initially, analogs were chosen for the study, including, as potential alkylating agents, the ω-diazoketones derived from esters of 17α-hydroxyprogesterone (2). The results of a considerable extension of the study are reported here.

CHEMISTRY

Condensation of 17α-hydroxyprogesterone (I) with the mixed anhydrides derived from trifluoroacetic anhydride and various monoesters of dibasic aliphatic acids afforded II*a*–II*h*. Selective hydrolyses of the esters II*a*, II*b*, II*c*, and II*e* gave the carboxylic acids III*a*–III*d*. Attempted hydrolysis of II*f* and II*g*, under the conditions used to hydrolyze II*a*, II*b*, II*c*, and II*e* failed because of low solubility of II*f* and II*g* in cold methanol. Other attempts to hydrolyze selectively II*f* and II*g* with base or II*h* with trifluoroacetic acid also failed to produce significant amounts of the acid III*e*. The carboxylic acids III*a*–III*d* were converted to diazoketones IV*a*–IV*d* by the method of Wilds and Shunk (4). Diazoketones IV*a* and IV*b* were transformed to the corresponding ketols, V*a* and V*b*, by stirring their ether solutions with aqueous sulfuric acid.



I

II*a*: n = 3, X = OMe
II*b*: n = 4, X = OMe
II*c*: n = 5, X = OMe
II*d*: n = 5, X = OEt
II*e*: n = 7, X = OMe
II*f*: n = 8, X = OMe
II*g*: n = 8, X = OEt
II*h*: n = 8, X = O-*t*-Bu

III*a*: n = 3, X = OH
III*b*: n = 4, X = OH
III*c*: n = 5, X = OH
III*d*: n = 7, X = OH
III*e*: n = 8, X = OH
IV*a*: n = 3, X = CHN_2
IV*b*: n = 4, X = CHN_2
IV*c*: n = 5, X = CHN_2
IV*d*: n = 7, X = CHN_2
V*a*: n = 3, X = CH_2OH
V*b*: n = 4, X = CH_2OH

EXPERIMENTAL[1]

17α-Hydroxypregn-4-ene-3,20-dione 8-Carbomethoxyoctanoate (II*e*)—A mixture of 4.0 g. of azelaic acid monomethyl ester and 4 ml. of trifluoroacetic anhydride in 60 ml. of dry benzene was refluxed for 20 min. After cooling, 3 g. of 17α-hydroxyprogesterone and 1.0 g. of sodium carbonate were added, and reflux was resumed for 20 hr. The mixture was concentrated. The residue was diluted with ether and then extracted with aqueous sodium hydroxide. The organic phase was washed (water) and dried (magnesium sulfate), and the solvent was distilled. The residue was chromatographed over 120 g. of acid-washed alumina. Benzene eluted 2.8 g. of an oil, which crystallized from hexane to give II*e*, in a yield of 70%: m.p. 88–91°; ν(CCl_4)1738, 1720, 1688, and 1680 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.68, 1.26, 2.04, 3.67, and 5.75 corresponding to hydrogens on C-18, C-19, C-21, OMe, and C-4, respectively.

Anal.—Calc. for $C_{31}H_{46}O_6$: C, 72.34; H, 9.01. Found: C, 72.46; H, 9.20.

17α-Hydroxypregn-4-ene-3,20-dione 9-Carbomethoxynonanoate (II*f*)—Monomethyl sebecate was treated with trifluoroacetic anhydride and then reacted with 17α-hydroxyprogesterone under conditions similar to those described under II*e*. Crystallization occurred from acetone–hexane to afford II*f*, in a yield of 80%: m.p. 144–145°; ν($CHCl_3$) 1730 and 1660 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.70, 1.21, 2.05, 3.71, and 5.80 corresponding to the hydrogens on C-18, C-19, C-21, OMe, and C-4, respectively.

Anal.—Calc. for $C_{32}H_{48}O_6$: C, 72.69; H, 9.15. Found: C, 72.79; H, 9.09.

17α-Hydroxypregn-4-ene-3,20-dione 9-Carboethoxynonanoate (II*g*)—Monomethyl sebecate was treated with trifluoroacetic anhydride and then reacted with 17α-hydroxyprogesterone essentially as described under II*e*. Crystallization occurred from ether–hexane to give II*g*, in a 62% yield: m.p. 96–97°; ν (CCl_4) 1730, 1720, and 1677 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.63, 1.21, 2.10, and 5.61 and a quartet (J = 7 Hz.) at δ 4.07 corresponding to the hydrogens at C-18, C-19, C-21, C-4, and —OCH_2Me, respectively.

Anal.—Calc. for $C_{33}H_{50}O_6$: C, 73.03; H, 9.29. Found: C, 73.21; H, 9.41.

***tert*-Butyl 9-Carboethoxynonanoate (VI)**—To 8 g. of ethyl hydrogen sebecate in 200 ml. of methylene chloride was added 0.8 ml. of concentrated sulfuric acid. The mixture was cooled in an ice bath, and 200 ml. of liquid isobutylene was added. The vessel was sealed and shaken at room temperature for 20 hr. The product, VI, after purification by filtration through alumina, was obtained as an oil.

Anal.—Calc. for $C_{16}H_{30}O_4$: C, 67.10; H, 10.56. Found: C, 67.26; H, 10.68.

***tert*-Butyl 9-Carboxynonanoate (VII)**—A solution of 2 g. of VI in 50 ml. of methanol containing 0.4 g. of sodium hydroxide in 5 ml. of water was stored at 0–5° for 20 hr. The mixture was then concentrated and partitioned between ether and water. The aqueous layer was washed with ether, acidified with hydrochloric acid, and extracted with ether. The product, VII, was obtained as an oil in a yield of 780 mg.

Anal.—Calc. for $C_{14}H_{26}O_4$: C, 65.09; H, 10.14. Found: C, 65.25; H, 10.19.

17α-Hydroxypregn-4-ene-3,20-dione 9-Carbo-*tert*-butoxynonanoate (II*h*)—A solution of 0.75 g. of VI in methanol was titrated to

[1] Melting points were determined in open-capillary tubes on a Mel-Temp apparatus and are uncorrected. IR spectra were determined on a Perkin-Elmer Infracord model 137 or on a Beckman IR-8 spectrophotometer. NMR spectra were determined in $CDCl_3$ on a Varian A-60 spectrometer and are reported in parts per million downfield from a tetramethylsilane internal standard. Elemental analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Table I—Activity of 17α-Acyloxy-Substituted Progesterones in a Modified Clauberg Assay (Subcutaneous Injection)

Compound	17α-Side Chain $(CH_2)_n$	17α-Side Chain End	Total Dose, mg.	Number of Rabbits	Mean Body Weight, g. Initial	Mean Body Weight, g. Final	Mean Ovarian Weight, mg.	Mean Uterine Weight, g.	Mean Proliferation Index	Range
Progesterone			0.2	14	1084	1332	39.7	1.19	0.9^+	$0–2.0^+$
			0.5	8	1102	1357	47.2	2.26	3.1^+	$2.5–3.5^+$
IIa	3	COMe	5.0	4	1109	1260	31.5	1.18	0	0
			10.0	2	1080	1165	25.5	1.25	0.8^+	$0.5^+–1.0^+$
IIb	4	COMe	1.0	2	1136	1204	110.6	1.40	0.3^+	$0–0.5^+$
			2.0	2	1043	1042	24.4	1.18	0.8^+	$0–1.5^+$
			5.0	4	1112	1230	30.8	1.74	2.5^+	$2.0–3.0^+$
IIc	5	COMe	2.0	2	1136	1423	44.7	1.51	1.5^+	$1.0^+–2.0^+$
			5.0	2	1014	1425	41.9	2.25	3.8^+	$3.5–4.0^+$
			10.0	2	1111	1240	71.7	3.14	4.0^+	4.0^+
IIe	7	COMe	1.0	2	991	1344	35.8	1.30	0	0
			2.0	6	1178	1362	44.0	1.37	0.6^+	$0–1.5^+$
			5.0	6	1120	1458	37.4	1.92	3.4^+	$2.5^+–4.0^+$
			20.0	2	1069	1341	32.3	3.32	4.0^+	4.0^+
IIj	8	COMe	2.0	2	1284	1244	38.3	1.05	0	0
			5.0	4	1044	1321	58.1	1.46	0.9^+	$0–2.5^+$
			10.0	4	1100	1358	45.1	1.53	1.6^+	$0–3.0^+$
			15.0	2	1094	1507	24.3	1.46	2.0^+	2.0^+
			20.0	2	1036	1226	79.6	1.50	3.3^+	$3.0^+–3.5^+$
IIg	8	COEt	2.0	4	1089	1444	44.8	1.39	0.8^+	$0.5^+–1.0^+$
			5.0	4	1222	1415	50.2	2.21	3.8^+	$3.5^+–4.0^+$
			10.0	2	1067	1156	44.7	2.38	4.0^+	4.0^+
IIh	8	COtBu	5.0	2	916	1072	40.8	1.04	0	0
			10.0	2	1082	1315	41.4	1.52	0.8^+	$0.5^+–1.5^+$
IIIa	3	COH	2.0	2	1209	1511	38.6	1.10	0	0
			20.0	2	1020	1466	43.5	1.36	0.8^+	$0.5^+–1.0^+$
IIIb	4	COH	2.0	4	1078	1330	48.0	1.17	0	0
			5.0	2	1210	1389	42.7	1.40	1.0^+	1.0^+
			10.0	2	1210	1377	53.0	1.98	2.0^+	2.0^+
			20.0	2	1156	1443	58.4	1.96	2.3	$2.0–2.5^+$
IIIc	5	COH	2.0	2	1077	1068	31.8	1.53	1.0^+	1.0^+
			20.0	2	1103	1384	40.6	2.10	2.3^+	$2.0^+–2.3^+$
IIId	7	COH	1.0	2	1100	1450	47.2	1.12	0	0
			2.0	2	997	1184	36.1	0.96	0.3^+	$0–0.5^+$
			5.0	2	979	1413	52.4	1.25	0.5^+	$0–1.0^+$
			10.0	2	1175	1496	68.5	1.74	0.3^+	$0–0.5^+$
IVa	3	$CCHN_2$	1.0	2	1066	1314	32.2	1.10	0	0
			2.0	2	1072	1257	30.2	1.40	2.3^+	$2.0–2.5^+$
			5.0	4	1078	1257	36.8	1.53	2.6^+	$2.5^+–3.0^+$
			10.0	2	1141	1365	35.6	1.98	3.8^+	$3.5^+–4.0^+$
IVb	4	$CCHN_2$	1.0	2	807	1046	29.2	1.29	2.0^+	2.0^+
			2.0	2	868	1050	27.6	1.40	3.0^+	$2.5^+–3.5^+$
			5.0	2	852	1039	45.0	2.10	2.8^+	$1.5^+–4.0^+$
IVc	5	$CCHN_2$	1.0	2	1078	1333	33.6	1.12	1.0^+	1.0^+
			2.0	2	1290	1557	47.8	1.55	2.3^+	$1.5^+–3.0^+$
			5.0	2	1260	1427	38.6	2.12	3.5^+	$3.0^+–4.0^+$

Table I (*Continued*)

Compound	—17α-Side Chain— $(CH_2)_n$	End	Total Dose, mg.	Number of Rabbits	Mean Body Weight, g. Initial	Final	Mean Ovarian Weight, mg.	Mean Uterine Weight, g.	Mean Proliferation Index	Range
IV*d*	7	$\overset{O}{\overset{\|\|}{C}}CHN_2$	1.0	2	1118	1213	43.6	0.89	0	0
			2.0	4	1090	1300	35.2	1.12	0.3+	0–0.5+
			5.0	2	1276	1436	34.1	2.52	4.0+	4.0+
V*a*	3	$\overset{O}{\overset{\|\|}{C}}CH_2OH$	1.0	2	1182	1508	49.0	1.56	0	0
			5.0	2	976	1336	59.6	1.76	2.8+	2.5+–3.0+
			20.0	2	1052	1404	55.8	2.78	4.0+	4.0+
V*b*	4	$\overset{O}{\overset{\|\|}{C}}CH_2OH$	1.0	2	968	1278	39.8	1.36	0.5+	0.5+
			2.0	2	1054	1382	32.0	1.61	0.8+	0.5+–1.0+
			5.0	2	1150	1445	28.6	1.92	2.5+	2.0+–3.0+

the phenolphthalein end-point with aqueous sodium hydroxide. The resulting salt was rigorously dried. It was then suspended in 20 ml. of dry glyme, and trifluoroacetic anhydride was added (approximately 0.42 ml.) until a homogeneous solution was obtained. The solution was refluxed for 1 hr., and then 0.6 g. of I was added. After the mixture was refluxed for 20 hr., it was worked up in the usual way. Chromatography over neutral alumina afforded II*h* as crystals from hexane, in a yield of 34%: m.p. 77–79°; ν (CCl_4) 1722 and 1675 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.68, 1.21, 1.50, and 2.03 corresponding to the hydrogens at C-18, C-19, O-*tert*-Bu, and C-21, respectively.

Anal.—Calc. for $C_{35}H_{54}O_6$: C, 73.65; H, 9.54. Found: C, 73.47; H, 9.35.

17α-Hydroxypregn-4-ene-3,20-dione 8-Carboxyoctanoate (III*d*)—A solution of II*e* in 50 ml. of methanol containing 5 ml. of 2 *N* sodium hydroxide was kept under nitrogen at 0–5° for 24 hr. The methanol was distilled under reduced pressure, and the residue was partitioned between ether and water. The aqueous layer was acidified and extracted with ether. The latter extract was washed with water, dried with magnesium sulfate, and concentrated to leave a residue which crystallized from ether–hexane to give III*d*, in a 42% yield: m.p. 140–141°; ν ($CHCl_3$) 3250, 1720, 1710, 1700, and 1660 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.69, 1.21, 2.04, and 5.76 corresponding to the hydrogens at C-18, C-19, C-21, and C-4, respectively.

Anal.—Calc. for $C_{30}H_{44}O_6$: C, 71.97; H, 8.86. Found: C, 72.15; H, 9.05.

17α-Hydroxypregn-4-ene-3,20-dione 4-Oxo-10-diazodecanoate (IV*d*)—A solution of 400 mg. of III*d* in methanol was titrated to the phenolphthalein end-point with aqueous sodium hydroxide. The resulting salt was dried and then slurried in 15 ml. of dry benzene. Three milliliters of pyridine was added. The mixture was cooled in an ice bath, and 4 ml. of oxalyl chloride was added. After stirring for 30 min. in the cold, the mixture was concentrated under reduced pressure. The residue was three times mixed with 10-ml. portions of dry benzene and distilled to dryness under reduced pressure. After the residue was dried at high vacuum, it was dissolved in 10 ml. of benzene and filtered. The filtrate was added with cooling to an ethereal, alcohol-free solution of diazomethane, which had previously been prepared from 10 mmoles of *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide. The solvent was removed under vacuum, and the residue was crystallized from ether–hexane to give IV*d*, in a yield of 62%: m.p. 60–62.5°; ν (CCl_4) 2100, 1720, and 1675 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.68, 1.20, 2.03, 5.29, and 5.87 corresponding to the hydrogens on C-18, C-19, C-21, $\overline{C}HN_2$, and C-4, respectively.

Anal.—Calc. for $C_{31}H_{44}N_2O_5$: C, 70.96; H, 8.54; N, 5.34. Found: C, 71.07; H, 8.54; N, 5.11.

17α-Hydroxypregn-4-ene-3,20-dione 5-Oxo-6-hydroxyhexanoate (V*a*)—To a solution of 150 mg. of IV*a* in 20 ml. of ether and a few drops of methylene chloride was added a solution of 0.5 ml. of concentrated sulfuric acid in 10 ml. of water. The mixture was stirred at room temperature for 48 hr. and was then diluted with ether, extracted with aqueous sodium bicarbonate, washed with water, and dried. The solvent was distilled. The residue crystallized from hexane–acetone to afford V*a*, in a 56% yield: m.p. 114–115°; ν ($CHCl_3$) 3500, 1715, and 1660 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.68, 1.21, 2.07, 4.22, and 5.78 corresponding to the hydrogens at C-18, C-19, C-21, C(=O)$\overline{C}H_2$OH, and C-4, respectively.

Anal.—Calc. for $C_{27}H_{38}O_6$: C, 70.72; H, 8.35. Found: C, 70.53; H, 8.52.

17α-Hydroxypregn-4-ene-3,20-dione 6-Oxo-7-hydroxyheptanoate (V*b*)—A solution of 110 mg. of IV*b* in ether was treated under essentially the same conditions as described for V*a*. The product, V*b*, crystallized from ether–hexane, in a yield of 84%: m.p. 100–104° dec.; ν ($CHCl_3$) 3500, 1720, and 1660 cm.$^{-1}$. The NMR spectrum had singlets at δ 0.68, 1.21, 2.07, 4.22, and 5.78 corresponding to the hydrogens at C-18, C-19, C-21, C(=O)$\overline{C}H_2$OH, and C-4, respectively.

Anal.—Calc. for $C_{28}H_{40}O_6$: C, 71.16; H, 8.53. Found: C, 71.07; H, 8.66.

RESULTS AND DISCUSSION

As shown in Table I, the esters II*a*–II*c* and II*e*–II*h* were tested by the McPhail modification of the Clauberg assay[2]. The progestational activity increased with chain length for the glutarate ester II*a* through the pimelate ester II*c*. It then decreased slightly for the azelate ester II*e* and decreased further for the methyl and *tert*-butyl sebacates II*f* and II*h*. However, the activity of the ethyl sebacate II*g* appears to be at least as great as that of the methyl adipate II*b* or of the methyl azelate II*e*. This anomaly appears to rule out the possibility of correlating the variation in activity of these esters solely with a single empirical parameter, such as Hansch's π (5). The acid III*d* and the diazoketone IV*d*, which have more than seven contiguous carbon atoms in their side chains, also are less active than would be expected on the basis of the activities noted for lower members of the respective homologous series.

n-Alkyl esters of 17α-hydroxyprogesterone show maximal Clauberg activity when six or seven contiguous carbon atoms are present in the side chain and then show a progressive decrease both in intensity and in duration of Clauberg activity as the acid moiety of the esters is homologated (6). A number of β-(*p*-alkoxyphenyl)-propionate esters of 17α-hydroxyprogesterone are reported to retain high Clauberg activity (7), but the ether oxygen in these compounds occupies essentially the position that would be occupied by the eighth carbon in a straight-chain ester. This results both in an increase in hydrophilic character and a bend in the molecule at that position.

The reported data are in accord with the view that straight-chain esters of 17α-hydroxyprogesterone, which have eight or more contiguous carbon atoms in the side chains, undergo an additional interaction with some biological receptor.

The diazoketones IV*a*–IV*d* are all at least as active as (in some cases, more active than) the esters from which they are derived.

[2] Clauberg assays were performed at the Endocrine Laboratories, Madison, Wis.

Of particular significance is the fact that diazoketone IV*b* is more active than ketol V*b* which is derived from it. This strongly supports the assumption that the Clauberg activity measured for the diazoketones is a measure of their inherent activity and not simply a measure of the activity of their hydrolysis products.

It was also of interest to observe that the carboxylic acids III*a*–III*d* showed a low, but real, Clauberg activity. The limited data available suggest that although these acids may not elicit a high level of response in this assay, up to a response level of 2.0+, their activity is equal to, or slightly greater than, that of the isomeric methyl esters. The calculated π-values (5) are also slightly greater for the acids than for the isomeric methyl esters.

None of the diazoketones has an activity so abnormal, either in comparison with the other members of this series or with that of the analogous methyl esters, as to suggest that it has alkylated the Clauberg receptor. However, the activity of diazoketones IV*a*, IV*b*, and, to a lesser extent, IV*c* seems surprisingly high, and it may warrant a reinvestigation with an assay based on intrauterine application of these compounds.

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Spectrophotofluorometric Determination of Salicylamide in Blood Serum and Urine

STEPHEN A. VERESH, FOO SONG HOM, and JOHN J. MISKEL

Abstract □ A spectrophotofluorometric method is reported for the determination of salicylamide in blood serum and urine. The procedure involves the simultaneous determination of salicylamide and salicylic acid at pH 11 after the acid hydrolysis of the salicylamide metabolites. The precision and accuracy of the method are comparable to the regular ferric-ion complex procedure. The present method has the sensitivity and specificity desired in the assay of blood serum and urinary salicylamide concentrations after the oral ingestion of a 325-mg. dose of the drug. The method may be used in monitoring bioavailability of salicylamide provided by different dosage forms.

Keyphrases □ Salicylamide—spectrophotofluorometric determination, in blood serum, urine □ Bioavailability evaluation—application of spectrophotofluorometric salicylamide determination □ Spectrophotofluorometry—salicylamide determination, blood serum, urine

The absorption and pharmacokinetics of salicylamide in humans were found to be both dose and dosage form dependent (1–3). However, most of the salicylamide blood level results reported were elicited with larger than normal doses of the drug. Therefore, a sensitive yet specific analytical method is needed to study the blood serum levels of salicylamide in humans after the ingestion of as little as 325 mg. of the drug. The ferric-ion complex colorimetric method is not sufficiently sensitive for this purpose.

In basic solution, salicylamide is fluorescent. Based on this property, several methods were reported for the assay of free salicylamide in biological fluids. Lange *et al.* (4) employed a fluorometric technique to determine salicylamide and other salicylates following a gel filtration separation. Barr and Riegelman (3) detailed a spectrophotofluorometric procedure for the determination of salicylamide and its metabolites in plasma and other biological fluids. Unfortunately, the body metabolizes salicylamide; hence, very little exists as free salicylamide in the blood or urine (1, 5, 6). For this reason, total salicylamide is determined as free salicylamide after acid hydrolysis. Barr and Riegelman (3)

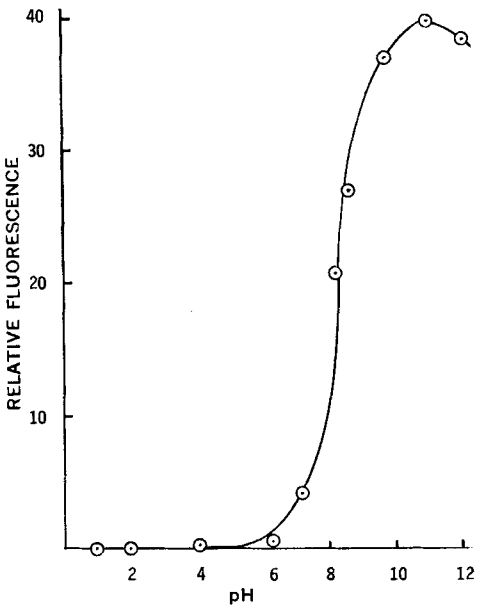

Figure 1—*Relative fluorescence of 0.1 mcg./ml. of salicylamide as a function of pH at the activation and emission wavelengths of 340 and 435 nm., respectively.*

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Keyphrases □ Salicylamide—spectrophotofluorometric determination, in blood serum, urine □ Bioavailability evaluation—application of spectrophotofluorometric salicylamide determination □ Spectrophotofluorometry—salicylamide determination, blood serum, urine

The absorption and pharmacokinetics of salicylamide in humans were found to be both dose and dosage form dependent (1–3). However, most of the salicylamide blood level results reported were elicited with larger than normal doses of the drug. Therefore, a sensitive yet specific analytical method is needed to study the blood serum levels of salicylamide in humans after the ingestion of as little as 325 mg. of the drug. The ferric-ion complex colorimetric method is not sufficiently sensitive for this purpose.

In basic solution, salicylamide is fluorescent. Based on this property, several methods were reported for the assay of free salicylamide in biological fluids. Lange *et al.* (4) employed a fluorometric technique to determine salicylamide and other salicylates following a gel filtration separation. Barr and Riegelman (3) detailed a spectrophotofluorometric procedure for the determination of salicylamide and its metabolites in plasma and other biological fluids. Unfortunately, the body metabolizes salicylamide; hence, very little exists as free salicylamide in the blood or urine (1, 5, 6). For this reason, total salicylamide is determined as free salicylamide after acid hydrolysis. Barr and Riegelman (3)

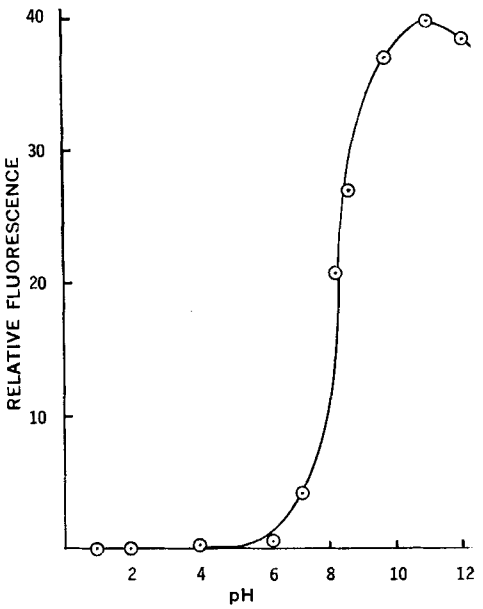

Figure 1—*Relative fluorescence of 0.1 mcg./ml. of salicylamide as a function of pH at the activation and emission wavelengths of 340 and 435 nm., respectively.*

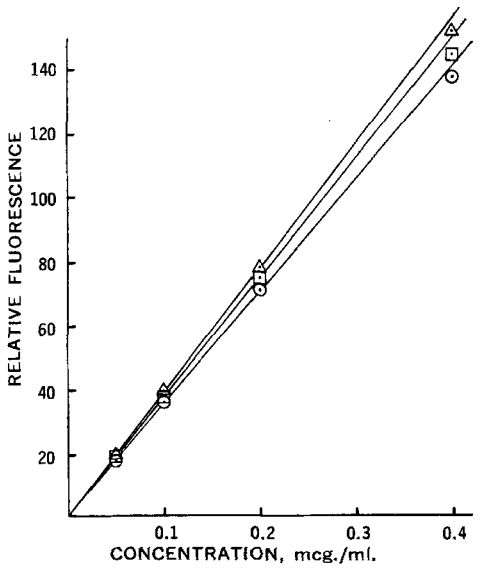

Figure 2—*Standard curves of the sum of relative fluorescences at 435 nm. from activations at 310 and 340 nm.* versus *total amounts of salicylamide and/or salicylic acid. Key:* ⊙, *salicylic acid;* △, *salicylamide; and* ⊡, *1:1 mixture of salicylic acid and salicylamide.*

employed hydrolysis methods similar to the one described by Levy and Matsuzawa (1). Total salicylamide was determined by hydrolyzing all metabolites to salicylic acid. The fluorescence of salicylamide in the aqueous basic solution was determined directly at the maximum activation and emission wavelengths of 350 and 430 nm., respectively. However, the use of strongly basic aqueous solutions, such as 0.2 *N* NaOH, may lead to erroneous results due to the hydrolysis of salicylamide, particularly when under irradiation at the activation wavelength. Under such conditions, it is possible for a reference standard solution to change its value during an analysis.

The present method is based on the simultaneous spectrophotofluorometric determination of salicylamide and salicylic acid at pH 11. It is only necessary to hydrolyze the salicylamide metabolites to a resultant mixture of salicylamide and salicylic acid.

EXPERIMENTAL

Reagents and Chemicals—The chemicals used were: salicylamide powder[1], salicylic acid crystals USP[2], gentisamide[3], and gentisic acid[4]. The pH 11 buffer solution was made by dissolving 1.0 g. of sodium bicarbonate and 4.0 g. of sodium carbonate in water to make 100 ml. of solution.

Instrumentation—The fluorescence was determined by use of an Aminco-Bowman spectrophotofluorometer[5]. The Teflon-lined, screw-cap, 15-ml. capacity centrifuge tubes[6] were hand ground and polished to fit tightly.

Human Blood and Urine Samples—Blood samples were withdrawn into sterile, 7-ml. "Vacutainers"[7] at 0, 1, 2, 3, and 6 hr. after the ingestion of 312 mg. of salicylamide. Blood samples were stored in a refrigerator pending assay. The urine samples were collected into bottles at 0, 1, 2, 3, 4, 5, and 7 hr. and stored in a refrigerator pending assay.

[1] New York Quinine and Chemical Works.
[2] S. B. Penick and Co.
[3] USV Pharmaceutical Corp.
[4] Eastman Kodak Co.
[5] American Instrument Co.
[6] Matheson Scientific.
[7] Becton-Dickinson.

Table I—Determination of Total Salicylamide from Known Mixtures of Salicylamide and Salicylic Acid, Using a 1:1 Mixture as Reference

Concentration, mcg./ml.		Relative Fluorescence				
Salicylamide	Salicylic Acid	310–435 nm.	340–435 nm.	Sum	Found, mcg./ml.	Percent of Theory
0.00	0.20	69.0	7.8	76.8	0.196	98.1
0.04	0.16	57.5	19.8	77.3	0.197	98.7
0.08	0.12	46.7	31.1	77.8	0.199	99.4
0.12	0.08	36.2	42.6	78.8	0.201	100.6
0.16	0.04	24.4	56.7	81.1	0.207	103.6
0.20	0.00	12.9	68.9	81.8	0.209	104.5

Procedure—Pipet 1.0 ml. of blood serum or an appropriately diluted urine sample (0–12 mcg./ml. of salicylamide) into a Teflon-lined screw-cap centrifuge tube. Add 1.0 ml. of a 2 *N* HCl solution. Secure cap tightly, and heat sample in a boiling water bath for 2.5 hr. At the end of the heating period, cool to room temperature with cold water. Add 5.0 ml. of chloroform and shake well. Centrifuge and then carefully aspirate off the upper aqueous phase. Extract a 1.0-ml. aliquot of the chloroform phase with 4.0 ml. of 0.1 *N* NaOH. Dilute a 1.0-ml. aliquot of the aqueous phase with 4.0 ml. of the pH 11 buffer solution. Determine the fluorescence of the resulting solution directly with the spectrophotofluorometer at the activation and emission wavelengths of 340 and 435 nm. and then at 310 and 435 nm., respectively. Add the two corresponding relative fluorescence readings together, and then refer to a standard curve treated in the same manner as the samples. Estimate the amount of salicylamide present in the sample from the standard curve.

RESULTS AND DISCUSSION

The activation and emission wavelengths of salicylic acid at the optimum pH of 11 are listed as 310 and 435 nm., respectively (7). The relative fluorescence as a function of pH for salicylamide is shown in Fig. 1. It can be seen that salicylamide at activation and emission wavelengths of 340 and 435 nm., respectively, also has maximum fluorescence at pH 11. The standard curves of relative fluorescence *versus* concentration of each component for salicylamide, salicylic acid, and a 1:1 mixture of the two are shown in Fig. 2. A study of these preliminary data indicates that it is possible to determine salicylamide and salicylic acid at pH 11 and calculate a value for total salicylamide. This may be accomplished since both compounds have maximum fluorescence (435 nm.) at pH 11 of nearly equal intensity at their respective activation and similar but lesser fluorescence (435 nm.) at each other's activation. Hence, in a mixture of both compounds, the sum of the fluorescence (435 nm.) at the two activation wavelengths will be directly proportionate to the sum of their concentrations and independent of their ratios. This postulation was tested by running known mixtures of salicylamide and salicylic acid in several different proportions. Table I shows the magnitude of the actual deviation in a series of mixtures, using a 1:1 mixture as the reference.

Upon examination of the Percent of Theory column in Table I, one observes the greatest accuracy when the ratios of salicylamide to

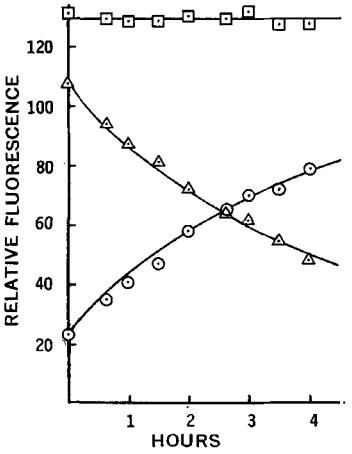

Figure 3—*Plot of relative fluorescence* versus *time in hours for the hydrolysis of salicylamide in 1 N HCl at 100°. Key:* △, *Curve A, 340-nm. activation;* ⊙, *Curve B, 310-nm. activation; and* ⊡, *Curve C, sum of Curves A and B.*

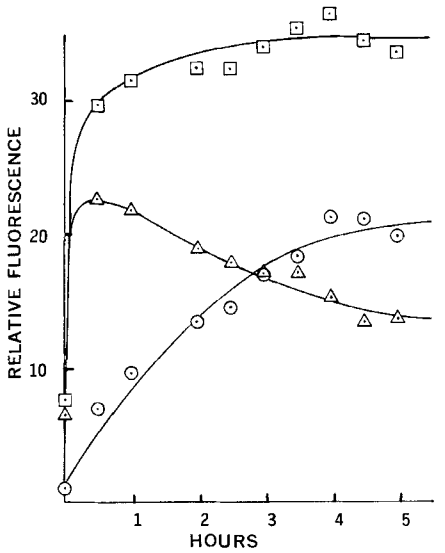

Figure 4—*Plot of relative fluorescence at 435 nm.* versus *time in hours for the hydrolysis of a blood serum sample of salicylamide metabolites in 1* N *HCl at 100°. Key:* △, *Curve A, 340-nm. activation;* ⊙, *Curve B, 310-nm. activation; and* ⊡, *Curve C, sum of Curves A and B.*

salicylic acid are close to the reference 1:1 mixture. In actual practice, the sample and standard are subjected to the same hydrolysis conditions so the resultant mixtures are of nearly equal proportions. Under the conditions of the present method, gentisic acid and gentisamide have relatively low fluorescences and they do not interfere. Since gentisic acid, gentisamide, and its glucuronide are present in small amounts in both the blood serum and urine (1), they are not considered in the present study.

Hydrolysis of Salicylamide—Figure 3 shows the hydrolysis of salicylamide in 1 *N* HCl at the boiling water bath temperature as a function of time. The relative fluorescence of Curve A (340–435 nm.) drops with time, whereas Curve B (310–435 nm.) rises with time. Curve C is the sum of Curves A and B. It can be seen that Curve A intersects Curve B at about the 2.5-hr. point. This point corresponds to approximately the optimum time for this set of hydrolysis conditions.

Hydrolysis of Salicylamide Metabolites—The blood serum and urine samples, after appropriate dilutions with water, were hydrolyzed in the manner described previously. Figure 4 shows the hydrolysis of serum salicylamide metabolites, and Fig. 5 shows the hydrolysis of a urine sample as a function of time. The reason the

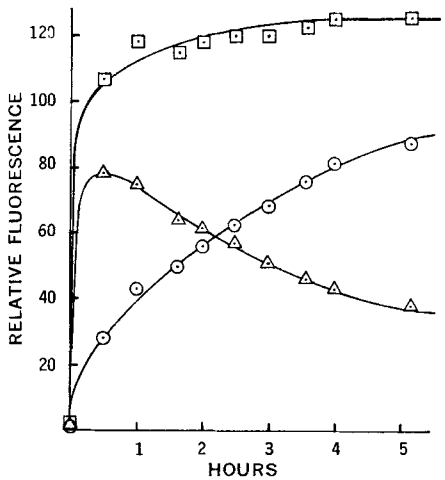

Figure 5—*Plot of relative fluorescence at 435 nm.* versus *time in hours for the hydrolysis of a sample of urinary salicylamide metabolites in 1* N *HCl at 100°. Key:* △, *Curve A, 340-nm. activation;* ⊙, *Curve B, 310-nm. activation; and* ⊡, *Curve C, sum of Curves A and B.*

Table II—Repetitive Assays of Two Urine Samples as Compared to Data Obtained with Ferric-Ion Complex Colorimetric Method

Sample	Present Method, mcg./ml.	Colorimetric Method, mcg./ml.
Urine A	243	
	240	248
	244	259
	239	249
Average	242 ± 2.5 or ± 1.0%	252 ± 6.1 or ± 2.4%
Urine B	217	219
	209	216
Average	213 ± 4.0 or ± 1.9%	218 ± 2.0 or ± 0.9%

Table III—Repetitive Assays of Pooled Blood Serum Sample and Recoveries of 10 mcg. of Salicylamide Added per Milliliter of Urine Blank Sample

Salicylamide Found in Serum, mcg./ml.	Salicylamide Found in Urine, mcg./ml.
3.02	
3.03	10.3
3.02	10.2
3.02	10.7
3.21	10.2
Average 3.07 ± 0.08	10.35 ± 0.25

lines curved at the beginning of hydrolysis is because the salicylamide metabolites have relatively low fluorescence. Curves A and B intersect between the 2.25–2.85-hr. marks. The metabolite curves, C, in Figs. 4 and 5 level off slowly as compared to a pure salicylamide sample. This apparently does not negate the method in any way as shown by comparison recovery studies with the usual colorimetric method.

Precision and Accuracy of Method—Repetitive assays of an appropriately diluted urine sample are listed in Table II along with results from a colorimetric method (1). Table III records repetitive determinations of 10 mcg. of salicylamide added to 1 ml. of urine blank samples (zero-hr. sample), as well as the results from five replicate assays of a blood serum sample. No attempts were made to assay this blood serum sample by the colorimetric method. However, when 8.0 mcg. of salicylamide was added per milliliter of blood serum blank (zero-time sample), 8.19 mcg./ml. was found which is a 102% recovery. This is comparable to recoveries from urine blank samples which have an accuracy of 103.5% with a precision of ±2.4%. The colorimetric assay appeared to give higher results as shown in Table II; however, its precision of ±2.4% is identical to the present method.

CONCLUSIONS

A spectrophotofluorometric method has been developed to determine salicylamide in blood serum and urine samples. The procedure is more sensitive than previously reported methods. The accuracy and precision of the method are 103.5 ± 2.4%, respectively. These results are comparable to the colorimetric ferric-ion complex method. However, the present method is capable of estimating a concentration of salicylamide and/or its metabolites as low as 0.1 mcg./ml. in blood serum or other biological fluids. The use of Teflon-lined screw-cap centrifuge tubes instead of ampuls facilitated the operating procedures and the use of small sample sizes. The method requires milder hydrolysis conditions and shorter time. The present method has been applied satisfactorily in this laboratory in bioavailability evaluation of various dosage form designs, employing salicylamide as the test drug.

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Sulfur-Containing Derivatives of β-Phenethylamine: Methanesulfonamides

WILLIAM O. FOYE, JON C. ANDERSON, and JAYANT N. SANE

Abstract □ *N*-Methanesulfonyl derivatives of β-phenethylamine, (+)-amphetamine, β-4-aminophenethylamine, and 4-(2-trifluoroacetamidoethyl)aniline have been prepared. *N*-Trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine significantly lowered blood pressure in hypertensive rats, and the methanesulfonamide of (+)-amphetamine lowered blood pressure in the dog.

Keyphrases □ Methanesulfonamides—synthesis, pharmacological screening as antihypertensives □ β-Phenethylamine, sulfur-containing derivatives—synthesis, pharmacological evaluation □ Antihypertensive agents, potential—synthesis, evaluation of *N*-methanesulfonyl derivatives of β-phenethylamine

In continuation of a study of the effects of sulfur-containing functional groups on the biological activity of β-phenethylamine, it was desired to observe the effects of including the alkanesulfonamide function in the molecule. Larsen and Lish (1) already pointed out that incorporation of the alkanesulfonamide group into the ring of phenethanolamines gives compounds having similar acidities to phenolic structures. Their phenethanolamine sulfonamides either showed the same biological profile or were antagonists to the catecholamines. According to the list of compounds subsequently reported from their laboratory, numerous alkanesulfonamides and arenesulfonamides of the phenethanolamines were synthesized (2, 3), but no alkanesulfonamides of β-phenethylamine itself were prepared. Previously, the *N*-benzenesulfonyl derivatives of β-phenethylamine (4) and β-4-nitrophenethylamine (5), and the 4-methanesulfonamido derivative (6) of 1-phenyl-2-propylamine, were described. 1- and 2-*p*-Methanesulfonylphenethylamines were synthesized and found to have some antibacterial activity *in vitro* (7).

The *N*-methanesulfonyl derivative of β-phenethylamine was readily prepared from the sulfonyl chloride. To introduce the methanesulfonamido group into the 4-position of the ring, β-phenethylamine was first nitrated in the 4-position by the method of Ehrlich and Pistschimuka (8). The amino group was converted to the trifluoroacetamido group, and the nitro group was reduced to give the 4-amino derivative. This was sulfonated by methanesulfonyl chloride, giving *N*-trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine. An attempt to obtain the corresponding molecule having a free aliphatic amino group by lithium aluminum hydride reduction of 4-methanesulfonamidobenzyl cyanide failed to give the desired 1-(4-methanesulfonamidophenyl)-2-ethylamine.

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Pharmacological tests for effects on blood pressure in rats and dogs revealed that *N*-trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine caused significant and consistent reductions in the blood pressure when administered orally to rats. The methanesulfonamide of (+)-amphetamine also produced a significant lowering of blood pressure after intravenous injection in a dog. Whereas the previous results of Uloth *et al.* (2) and Larsen *et al.* (3) showed that inclusion of the alkanesulfonamide group in the aromatic ring of phenethanolamines conferred either adrenergic stimulant or adrenergic-blocking activity (the latter generally in conjunction with an isopropyl or larger group on the aliphatic nitrogen), it is apparent from the results reported here that conversion of the aliphatic amino group to an acidic methanesulfonamide or even trifluoroacetamide group can lead to a depressor response from the simpler phenethylamine structure.

PHARMACOLOGICAL RESULTS

Tests for blood pressure effects in unanesthetized neurogenic hypertensive dogs (10) and in metacorticoid hypertensive rats (11) were performed[1].

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N-Trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine produced significant reductions in the mean systolic blood pressure of four test rats following oral administration of 80 mg./kg. The test animals were dosed on 2 consecutive days; significant reductions were seen on Day 1, 5 hr. postdrug, and on Day 2, both 5 and 24 hr. postdrug. Three dogs were administered oral doses of 5, 10, and 20 mg./kg. on Days 1, 2, and 3, respectively, and a fourth dog received doses of 10, 20, and 40 mg./kg. over the 3-day period. A significant hypotensive effect was seen on Days 2 and 3 in only one dog receiving the lower dose.

N-Methanesulfonyl-1-methyl-2-phenethylamine produced a significant reduction in blood pressure when a dose of 0.5 mg./kg. i.v. was administered to a normotensive dog[2]. *N,N'*-Bis(methanesulfonyl)-1-(4-aminophenyl)-2-ethylamine exerted no significant effect on blood pressure of either dogs or rats.

ANTIMICROBIAL RESULTS

Tests for antimicrobial activity (9) revealed that *N*-trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine and the bis(methanesulfonyl) derivative of β-4-aminophenethylamine have some activity with minimum inhibitory concentrations of <1/2000 *M versus Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Candida albicans*.

EXPERIMENTAL[3]

Melting points were taken on a Mel-Temp block and are uncorrected. IR absorption spectra were obtained with a Perkin-Elmer model 137 B spectrometer.

N-Methanesulfonyl-2-phenethylamine—To a cooled solution of 2.42 g. (0.02 mole) of β-phenethylamine[4] in 30 ml. of methylene chloride were added dropwise, with stirring, 1.67 ml. (0.022 mole) of methanesulfonyl chloride in 20 ml. of methylene chloride and 3 ml. (0.022 mole) of triethylamine in 20 ml. of methylene chloride. The mixture was allowed to come to 25° and was stirred for 15 hr. The resulting solution was washed with 1 *N* hydrochloric acid and water, and the solvent was distilled *in vacuo*. The product was air-dried and recrystallized from aqueous ethanol, giving a 55% yield; m.p. 50–52°. IR(KBr) 1160 (SO_2), 1325 (SO_2), 1600 (NH), and 3300 (NH) $cm.^{-1}$.

Anal.—Calc. for $C_9H_{13}NO_2S$: C, 54.26; H, 6.58; N, 7.03. Found: C, 54.51; H, 6.40; N, 7.20.

N-Methanesulfonyl-1-methyl-2-phenethylamine—To a cooled solution of 2.45 g. (0.02 mole) of (+)-amphetamine[4] in 10 ml. of methylene chloride were added dropwise, with stirring, 1.67 ml. (0.022 mole) of methanesulfonyl chloride in 30 ml. of methylene chloride and 3 ml. (0.022 mole) of triethylamine in 30 ml. of methylene chloride. The mixture was allowed to come to 25° and was stirred for 15 hr. The resulting solution was washed with 1 *N* hydrochloric acid and water, dried over Na_2SO_4, and evaporated in a rotary evaporator. The residue was distilled, and 2.10 g. was collected at 190–195°/1.9 mm.; IR(film) 1150(SO_2), 1315 (SO_2), 1600 (NH), and 3300 (NH) $cm.^{-1}$; $[\alpha]_D^{20} + 51°$(CH_2Cl_2).

Anal.—Calc. for $C_{10}H_{15}NO_2S$: C, 56.33; H, 7.04; N, 6.54. Found: C, 56.45; H, 7.40; N, 6.38.

N-Trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine—To a cooled solution of 0.696 g. (0.003 mole) of 4-(2-trifluoroacetamidoethyl)aniline (9) in 5 ml. of methylene chloride were added dropwise, with stirring, 0.3 ml. (0.0035 mole) of methanesulfonyl chloride in 5 ml. of methylene chloride and 0.5 ml. of triethylamine in 5 ml. of methylene chloride. The mixture was allowed to reach 25°, was stirred for 3 hr., and was washed with 1 *N* hydrochloric acid and water. The solvent was distilled *in vacuo*, and a 70% yield of product remained; m.p. 155–160°. IR(KBr) 1160 (SO_2), 1325 (SO_2), 1700 (C=O), and 3250 (NH) $cm.^{-1}$.

Anal.—Calc. for $C_{11}H_{13}F_3N_2O_3S$: C, 42.58; H, 4.22; N, 9.03. Found: C, 42.70; H, 4.34; N, 9.04.

***N,N'*-Bis(methanesulfonyl)-1-(4-aminophenyl)-2-ethylamine**—To a cooled solution of 2.71 g. (0.02 mole) of β-(4-aminophenyl)-ethylamine[4] in 25 ml. of benzene were added dropwise, with stirring, 3.4 ml. (0.041 mole) of methanesulfonyl chloride in 30 ml. of methylene chloride and 6 ml. (0.044 mole) of triethylamine in 30 ml. of methylene chloride. The mixture was allowed to reach 25° and was stirred for 3 hr. The precipitate was collected, washed with 1 *N* hydrochloric acid and water, and dried *in vacuo*. Additional product was obtained from the filtrate, and the combined product was recrystallized from ethanol, giving a 92% yield; m.p. 190–192°. IR(KBr) 1150 (SO_2), 1320 (SO_2), 1600 (NH), and 3250 (NH) $cm.^{-1}$.

Anal.—Calc. for $C_{10}H_{16}N_2O_4S_2$: C, 41.09; H, 5.52; N, 9.58. Found: C, 41.06; H, 5.59; N, 9.70.

4-Methanesulfonamidobenzyl Cyanide—To a cooled solution of 2.64 g. (0.02 mole) of 4-aminobenzyl cyanide[4] in 25 ml. of benzene were added 1.67 ml. (0.022 mole) of methanesulfonyl chloride in 15 ml. of methylene chloride and 3 ml. (0.022 mole) of triethylamine in 15 ml. of methylene chloride. The mixture was allowed to reach 25° and was stirred for 4 hr. Precipitated triethylamine hydrochloride was removed, and the filtrate was washed with dilute hydrochloric acid and water and dried (Na_2SO_4). The solvent was distilled under reduced pressure, and the residue was dried *in vacuo* and recrystallized from ethanol, giving a 93% yield of product; m.p. 113–114°.

Anal.—Calc. for $C_9H_{10}N_2O_2S$: C, 51.42; H, 4.79; N, 13.32. Found: C, 51.35; H, 4.84; N, 12.99.

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[2] The authors are indebted to Mr. V. K. Sharma for this result.

[3] Analyses for carbon, hydrogen, and nitrogen were done by Weiler and Strauss, Oxford, England.

[4] Aldrich Chemical Co.

Occurrence and Distribution of Phenoxazinone Pigments in the Genus *Pycnoporus*

GERALD SULLIVAN and ERNEST D. HENRY

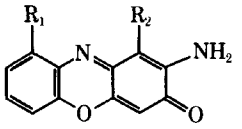
Abstract □ A TLC procedure was developed for the separation of phenoxazinone-type pigments. By employing silica gel G as the adsorbent and a solvent system of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1), the separation of the following reference compounds was accomplished: cinnabarinic acid (R_f 0.35), cinnabarin (0.47), 2-amino-3-oxo-3*H*-phenoxazine (0.57), tramesanguin (0.67), and phenoxazine (0.99). Examination of the individual acetone extracts of *Pycnoporus cinnabarinus* (Jacq. ex Fr.) Karst., *P. coccineus* (Fr.) Bond. and Sing., and *P. sanguineus* (L. ex Fr.) Murr. by this technique revealed that all three species contained the same pigments. In addition to cinnabarinic acid, cinnabarin, and tramesanguin, two additional phenoxazinone-type pigments (R_f 0.53 and 0.90) were found in the respective carpophores.

Keyphrases □ *Pycnoporus* genus—occurrence, distribution of phenoxazinone pigments □ Phenoxazinone pigments—separation, identification from *Pycnoporus* genus □ Spectrophotometry, visible—analysis □ TLC—analysis, identification

Nobles and Frew (1) presented genetical and morphological evidence which supports the validity of three *Pycnoporus* species: *P. cinnabarinus* (Jacq. ex Fr.) Karst., which occurs in the North Temperate Zone; *P. coccineus* (Fr.) Bond. and Sing., which occurs in the South Temperate Zone and in countries bordering on the Indian and Pacific Oceans; and *P. sanguineus* (L. ex Fr.) Murr., found in tropical and subtropical regions of the Northern and Southern Hemispheres.

Three phenoxazinone-type pigments were isolated from *Pycnoporus* species and their structures elucidated by Gripenberg *et al.* (2–5) and Cavill *et al.* (6, 7). The chemical structures for cinnabarinic acid (2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) (I), cinnabarin (2-amino-9-hydroxymethyl-3-oxo-3*H*-phenoxazine-1-carboxylic acid) (II), and tramesanguin (2-amino-1-formyl-3-oxo-3*H*-phenoxazine-9-carboxylic acid) (III) are illustrated here.

Gripenberg (5) stated that three different types of phenoxazinone-producing fungi appeared to exist: one producing only cinnabarin, *Trametes cinnabarina* (Jacq.) Fr. (= *P. cinnabarinus*); one producing cinnabarin and cinnabarinic acid, *Polystictus sanguineus* L. (= *P. sanguineus*); and one producing cinnabarin and tramesanguin, *T. cinnabarina* var. *sanguinea* (L.) Pilat (= *P. sanguineus*). Lemberg (8) reported the isolation and characterization of polystictin (cinnabarin) from carpophores of *Coriolus sanguineus* (Fr.) Cunningham (= *P. sanguineus*) and also the presence of an uncharacterized pigment, polystictinin. Cavill *et al.* (6) worked with both carpophores and cultures of *C. sanguineus* and only cinnabarin was reported to be present.



I: R_1 = COOH, R_2 = COOH
II: R_1 = CH_2OH, R_2 = COOH
III: R_1 = COOH, R_2 = CHO

In 1962, Madhosingh (9) worked with cultures of all three species of *Pycnoporus*. By utilization of paper chromatographic techniques, eight different pigments were observed, one of which was thought to be identical with cinnabarin. The remaining seven pigments were not characterized or identified. Consequently, the chromophore of these compounds and their relationship to the phenoxazinone-type pigments are unknown.

Prior to the present study, cinnabarin, cinnabarinic acid, and tramesanguin were reported to be present in carpophores of *P. sanguineus*, whereas only cinnabarin was reported in *P. cinnabarinus*. No report was found concerning the pigment content of carpophores of *P. coccineus*. Therefore, the present study represents an effort to establish the occurrence and distribution of phenoxazinone-type pigments as they exist in the carpophores of the three *Pycnoporus* species.

EXPERIMENTAL

Several investigators (10–12) reported the successful separation of phenoxazinone-type pigments by paper chromatography; Gerber (13) used TLC for the separation and purification of several phenoxazinone-type pigments. None of these techniques was found satisfactory for this investigation.

Table I—Collection Data and Weight of Powdered Carpophores

Pycnoporus Species	Collection Site	Collection Date	Weight, g.	Source
P. cinnabarinus	Long Branch, N. Y.	Oct. 27, 1915	1.82	—[a]
P. cinnabarinus	Washington, D. C.	Aug. 19, 1912	1.44	—[a]
P. cinnabarinus	Ontario, Canada	Sept. 22, 1955	1.84	DAOM 31954[b]
P. coccineus	Bougainville, Solomon Islands	Nov. 6, 1959	1.76	DAOM 72107[b]
P. sanguineus	Westland, New Zealand	April 1963	1.75	DAOM 99448[b]
P. sanguineus	Kingston, Jamaica	Jan. 18, 1966	0.62	—[c]

[a] Obtained from Dr. J. L. Lowe, Department of Botany, New York State College of Forestry, Syracuse, N. Y. No herbarium number given. [b] Obtained from Mycological Herbarium, Plant Research Institute, Department of Agriculture, Ottawa, Canada. [c] Obtained from Dr. C. J. Alexopoulos, Department of Botany, University of Texas at Austin, Austin, Tex. No herbarium number given.

Preparation of TLC Plates—TLC plates were prepared by placing 20 g. of silica gel G[1] in a 250-ml. conical flask with 40 ml. of distilled water and shaking vigorously for 1 min. A uniform 200-μ layer was applied to five 20 × 20-cm. glass plates. The plates were allowed to stand for 30 min. and activated at 110° for 30 min.

Developing Solvent—Solvents used were: benzene, chloroform, methanol, ethanol, and a number of solvent mixtures including ethanol–water (1:1), methanol–water (1:1), methanol–10% HCl (1:1), chloroform–ethyl acetate (9:1), chloroform–glacial acetic acid (9:1), chloroform–ethanol (99:1), chloroform–acetone (95:5), ethyl acetate–glacial acetic acid (9:1), butanol–glacial acetic acid–water (4:1:1), toluene–ethanol–water (4:17:1), benzene–ethyl acetate–diethylamine (6:3:1), benzene–ethyl acetate–glacial acetic acid (6:3:1), benzene–ethyl acetate–ethyl formate (6:3:1), benzene–ethyl acetate–formic acid (6:3:1), and benzene–ethyl acetate–glacial acetic acid–formic acid (60:30:1:1 and 12:6:1:1). Excellent separation of compounds was obtained when plates were developed with a solvent system composed of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1).

Reference Compounds—Cinnabarin[2], tramesanguin[2], cinnabarinic acid[3], 2-amino-3-oxo-3*H*-phenoxazine[3], and phenoxazine[4] were dissolved in minimal amounts of methanol–pyridine (3:1) and were employed as standard spotting solutions.

Extraction of Carpophores—Individual carpophores were ground to a 20-mesh powder in a Wiley-laboratory mill, placed in a continuous extraction apparatus, and exhaustively extracted with acetone. Each acetone extract was evaporated to dryness under reduced pressure, and the residual pigments were dissolved in a methanol–pyridine solution.

Species designation, collection data, and weights of powdered carpophores are presented in Table I.

Detection—Reference compounds and pigments isolated from the carpophores were detected after TLC separation by visual inspection. Less than 0.02 mcg. could be detected in this manner. Iodine vapors intensified the colors.

Preparative TLC—The methanol–pyridine solution containing the extracted pigments was streaked in a continuous band 2 cm. from the lower edges of the plates, prepared as described previously. These plates were developed in a solvent system composed of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1) for a distance of 15 cm., removed from the developing chamber, and allowed to air dry. The corresponding individual bands on each plate were removed, combined, placed in a continuous extraction apparatus, and exhaustively extracted with acetone. Each acetone extract was taken to dryness under reduced pressure and dissolved in ethanol.

Visible Spectra—The visible spectral characteristics of the reference compounds and the respective pigments obtained from the preparative TLC bands were determined with a spectrophotometer[5]. Ethanol was employed as the solvent in all spectrophotometric determinations.

RESULTS AND DISCUSSION

Satisfactory separation of the reference compounds into discrete spots was accomplished using silica gel G as the adsorbent and any of the following solvent systems: benzene–ethyl acetate–ethyl formate (6:3:1), benzene–ethyl acetate–formic acid (6:3:1), and benzene–ethyl acetate–glacial acetic acid–formic acid (60:30:1:1). Excellent separation of cinnabarinic acid (R_f 0.35), cinnabarin (R_f 0.47), 2-amino-3-oxo-3*H*-phenoxazine (R_f 0.57), tramesanguin (R_f 0.67), and phenoxazine (R_f 0.99) resulted when a solvent system of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1) was employed. Chromatographic systems and R_f values are listed in Table II.

Examination of the acetone extracts of all three *Pycnoporus* species by preparative TLC resulted in the formation of seven distinct colored bands: bands 1 (origin), 2 (R_f 0.35), 3 (R_f 0.47), 4 (R_f 0.53), 5 (R_f 0.67), 6 (R_f 0.90), and 7 (R_f 0.99). The pigments eluted from bands 2 through 6 each exhibited identical visible spectral characteristics, with maximum absorption at 430 nm., and were identical to the spectra obtained with the reference phenoxazinone-type compounds. Since the pigments obtained from the origin and at R_f 0.99 did not exhibit absorbance in this region, they were of no further interest in this investigation. The pigments isolated from bands 2, 3, and 5 were chromatographically indistinguishable from cinnabarinic acid, cinnabarin, and tramesanguin, respectively. Since the pigments eluted from bands 4 (R_f 0.53) and 6 (R_f 0.90) exhibited spectral characteristics identical to the reference compounds, they were assumed to be phenoxazinone derivatives. Due to the limited supply of these compounds, no further characterization was attempted.

It is apparent from this study that carpophores of all three *Pycnoporus* species contain identical pigments and that species differentiation cannot be supported by the presence of these secondary metabolites.

Table II—Chromatographic Separation of Reference Compounds

Compound	Chromatographic System[a] and R_f Values A	B	C	D	E
Cinnabarinic acid	0.01	0.44	0.28	0.26	0.35
Cinnabarin	0.27	0.54	0.48	0.42	0.47
2-Amino-3-oxo-3*H*-phenoxazine	0.51	0.43	0.78	0.68	0.57
Tramesanguin	0.37	0.79	0.73	0.66	0.67
Phenoxazine	0.95	0.97	0.97	0.98	0.99

[a] Silica gel G thin layer. System A: benzene–ethyl acetate–ethyl formate (6:3:1); System B: benzene–ethyl acetate–formic acid (6:3:1); System C: benzene–ethyl acetate–glacial acetic acid (6:3:1); System D: benzene–ethyl acetate–glacial acetic acid–formic acid (60:30:1:1); and System E: benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1).

SUMMARY

Cinnabarinic acid, cinnabarin, tramesanguin, and two additional phenoxazinone-type pigments, which are as yet uncharacterized, were detected by TLC in the carpophores of *P. cinnabarinus*, *P. coccineus*, and *P. sanguineus*.

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[1] E. Merck AG, Darmstadt, Germany.
[2] Obtained from Dr. Jarl Gripenberg, Department of Chemistry, Technical University Finland, Otaniemi, Finland.
[3] Obtained from Dr. L. C. Vining, Atlantic Regional Laboratory, National Research Council of Canada, Halifax, Nova Scotia, Canada.
[4] Obtained from K & K Laboratories of California, Inc., Hollywood, Calif.
[5] Beckman model DB.

Microbial Contamination of Nonsterile Antibiotic Market Samples: A Survey

FRANCES W. BOWMAN, MACK WHITE, and RONALD L. LYLES

Abstract □ Nonsterile antibiotic samples on the U. S. market were examined for microbial contamination in a survey that included 261 batches from 38 different manufacturers. All the drugs tested were of acceptable hygienic quality in that their microbial content was minimal and innocuous.

Keyphrases □ Antibiotics, nonsterile market samples—microbial contamination, survey □ Microbial contamination—survey of nonsterile antibiotic market samples □ Contaminants, in nonsterile antibiotics—survey of market samples

Since the 1964 inquiry by the Royal Swedish Medical Board into microbiological contamination of medical preparations (1), there has been growing concern about the possibility of excessive and undesirable microbial contamination of nonsterile drugs. In Sweden, severe eye disorders caused by cortisone ointments contaminated with *Pseudomonas aeruginosa* were among the human infections found due to contaminated nonsterile drugs. Cases of salmonellosis due to thyroid tablets contaminated with *Salmonella* were also reported. These findings led to official Swedish recommendations in 1966 on "Production, Hygiene, and Bacteriological Control in the Manufacture of Pharmaceuticals" (2).

In other countries, infections in man caused by contaminated oral or topical pharmaceutical preparations have been reported. Phillips (3) described four cases of lung infection caused by contaminated lignocaine ointment used for the lubrication of endotracheal catheters. Hospital outbreaks of *Salmonella cubana* infections in both the United States and the United Kingdom in 1966 were traced to the administration of capsule products containing contaminated carmine (4).

Since contamination of nonsterile drugs has been found to be widespread, other countries have issued regulations designed to improve the sanitary quality of these drugs. The Czechoslovakian Pharmacopeia (5) sets a total count limitation on the number of permissible microorganisms per gram or milliliter of various preparations. The *General Notices on Added Substances* in the 1968 BP (6) contains a warning that such substances should be free from harmful organisms. USP XVIII provides tests for estimating the numbers of viable aerobic microorganisms present and for freedom from designated microbial species in pharmaceutical articles of all kinds, from raw materials to the finished dosage forms (7). The International Pharmacopeia (8) specifies that microbiological contamination of nonsterile drugs should be no greater than that permitted for foods under the law of the country concerned.

The USP and NF defer to the Antibiotic Regulations for standards of potency and purity for antibiotic drugs. In these Regulations, there are no specified tolerances for microbial contamination of nonsterile antibiotics, except for one topical preparation (9). The microorganism count for that preparation must not exceed 10 per gram of powder, or per container if it is packaged with inert gases. Even though other certifiable antibiotic products are not regularly examined, they are required under Section 501(a) of the Federal Food, Drug, and Cosmetic Act to be free from harmful microorganisms. The Good Manufacturing Practice Regulations (10) stipulate that a drug must possess the quality and purity characteristics it purports or is represented to possess. An antibiotic and all its dosage forms must, therefore, be manufactured in a facility that provides adequate microbiological controls to preclude contamination by extraneous adulterants and prevent the dissemination of microorganisms from one area to another.

In 1968, several batches of an antifungal antibiotic powder were found to be grossly contaminated with microorganisms (11). Investigational studies revealed that the use of this grossly contaminated powder in the formulation of tablets or ointments resulted in highly contaminated products. In addition, an antifungal topical lotion was found to be contaminated with *P. aeruginosa* (12). The raw materials used in the formulation of this preparation were tested, and these organisms were not detected; therefore, the manufacturing facilities or the personnel were suspected as the source of the contamination. As a result of these findings, six other antibiotic powders were screened for microbial contamination. No gross contamination was detected in this limited number of samples of nafcillin, bacitracin, zinc bacitracin, neomycin, penicillin G, and tyrothricin. However, with the increased concern about the microbial purity of nonsterile drugs, a more extensive survey was made to determine the level of contamination in nonsterile antibiotic drugs on the market.

EXPERIMENTAL

Since antibacterial and antifungal antibiotics prevent the growth of some microorganisms, these products cannot be effectively tested for microbial contamination by the usual procedures used for other drugs, cosmetics, or foods. Special methods (11, 13, 14) have been developed for testing antibiotic preparations for sterility and microorganism count. Therefore, for the initial screening of the antibiotic samples tested in this survey, a sterility test was performed by the method described in the Antibiotic Regulations (15). The antibiotic products were divided into two categories: those that could be tested by the membrane filtration method after dissolution in water or another solvent, and those insoluble products for which the direct method was used. If no growth was observed after the 7-day incubation period at the appropriate temperatures, the product was reported as sterile. If contamination was found by the sterility test, additional tests were performed to identify and quantitate the number of viable microorganisms.

Precautionary measures, including performing the tests under a laminar flow hood, were taken to avoid adventitious contamination. A total aerobic microbial count was performed on all preparations found contaminated in the original screening test. All the products were sufficiently soluble or translucent to permit the use of the plate method for total aerobic microbial count.

Table I—Results of Survey of Marketed Nonsterile Antibiotic Samples for Microbial Contamination

Sample	Number of Batches Tested	Number of Batches Contaminated[a]	Types of Organisms Recovered from Contaminated Batches[b]	Number of Batches per Contaminant Type
Oral preparations:				
Potassium penicillin G tablets	5	3	*Staphylococcus epidermidis*	2
			Bacillus subtilis	1
Ampicillin capsules	4	2	*Bacillus circulans*	2
Tetracycline hydrochloride capsules	26	10	*S. epidermidis*	7
			Penicillium sp.	1
			Saccharomyces cerevisiae	2
Sodium novobiocin capsules	4	0		
Oxytetracycline hydrochloride capsules	4	0		
Trioleandomycin capsules	2	0		
Methacycline hydrochloride capsules	2	1	*S. epidermidis*	1
Lincomycin hydrochloride capsules	2	0		
Tetracycline syrup	6	0		
Erythromycin syrup	4	0		
Chloramphenicol palmitate syrup	2	0		
Lotions and topical ointments:				
Nystatin lotion	15	2	*S. epidermidis*	2
Nystatin ointment	33	17	*S. epidermidis*	10
			Bacillus sp.	7
Bacitracin–neomycin–polymyxin ointment	7	1	*Aspergillus niger*	1
Ophthalmic ointments:				
Neomycin–polymyxin	1	0		
Potassium penicillin G	1	1	*S. epidermidis*	1
Oxytetracycline hydrochloride–polymyxin B	1	0		
Bulk antibiotic powders:				
Sodium ampicillin	4	0		
Chloramphenicol	2	1	*S. cerevisiae*	1
Dihydrostreptomycin sulfate and streptomycin sulfate	10	0		
Potassium penicillin G	18	5	*Bacillus* sp.	3
			Mold (unidentified)	1
			S. cerevisiae	1
Oxytetracycline base or hydrochloride	18	2	*Rhizopus* sp.	2
Tetracycline base	35	9	*S. epidermidis*	8
			Mold (unidentified)	1
Neomycin sulfate	7	0		
Nystatin	2	2	Gram-negative coccus bacillus	1
			Bacillus sp.	1
Griseofulvin	2	1	*Bacillus* sp.	1
Amphotericin	2	1	*Bacillus* sp.	1
Lincomycin hydrochloride	4	1	*Bacillus* sp.	1
Gramicidin	3	0		
Bacitracin	2	0		
Sodium cloxacillin	1	0		
Kanamycin sulfate	3	0		
Tyrothricin	1	1	*Penicillium* sp.	1
Sodium novobiocin	1	1	Mold (unidentified)	1
Chlortetracycline hydrochloride	2	0		
Streptomycin sulfate	3	1	*Bacillus subtilis*	1
Erythromycin	8	1	*Mucor* sp.	1
Polymyxin B sulfate	2	0		
Penicillin G diethylaminoethyl ester hydrochloride	1	0		
Dihydrostreptomycin sulfate	5	0		
Triacetyloleandomycin	3	0		
Sodium penicillin O	1	0		
Zinc bacitracin	1	0		
Ampicillin trihydrate	1	1	*Penicillium chrysogenum*	1
Total	261[c]	64		

[a] All contaminated batches had less than 50 microorganisms per gram or milliliter. [b] All contaminated samples were further examined and found negative for the presence of *Pseudomonas, E. coli, S. aureus* (coagulase-positive), and *Salmonella*. The 261 batches were made by 38 different manufacturers.

Materials—The soybean–casein digest agar medium was prepared according to the USP XVIII. Peptone water was prepared by dissolving 1.0 g. of peptic digest of animal tissue USP in sufficient distilled water to make 1000 ml., and 99-ml. portions were dispensed into flasks and sterilized at 121° for 20 min. The final pH was 7.1 ±0.1.

Method I: Insoluble Antibiotics—A 1.0-g. portion of powder or 1 ml. of a liquid sample was added to 9 ml. of sterile peptone water. A number of 10-fold serial dilutions were prepared by aseptically transferring 1 ml. of the sample suspension to 9 ml. of sterile peptone water. From each dilution, 1 ml. was removed and added to separate sterile 15 × 150-mm. petri dishes; 40 ml. of sterile melted soybean–casein digest agar, which had been cooled to approximately 45°, was then added to each dish. The agar was swirled in the plate, covered, and allowed to harden. The plates were inverted and incubated at 35–37° for 48–72 hr., and the colonies on each plate

were then counted. If a count of less than 10/ml. was expected, the measured quantity of sample was added directly to the agar without making the dilutions in peptone water.

Method II: Soluble Antibiotics—A membrane filter of 0.45-μ porosity was placed in a filter funnel attached to a vacuum flask. Then 1.0 g. of powder or 1 ml. of a solution was added aseptically to 200 ml. of sterile peptone water; powders were allowed to dissolve. The solution was immediately vacuum filtered through the membrane. The membrane was washed three times with 100 ml. of sterile peptone water and allowed to dry by continuing the vacuum for 5 min. after filtration. The membrane was then placed on the surface of 20 ml. of solidified soybean–casein digest agar in a petri dish. The plate was inverted and incubated for 48–72 hr. at 35–37°, and any colonies that appeared on the membrane were counted.

Method III: Petrolatum-Based Antibiotic Ointments—The test procedure described for ointments in the USP XVIII (7) was used with the following exceptions. If the ointment contained penicillin, approximately 10,000 Levy units of penicillinase was added to both the thioglycollate and soybean–casein digest test media; if the ointment contained neomycin, 27.5 g. of sodium chloride and 1 g. of ascorbic acid were added to the thioglycollate test medium, and 30 g. of sodium chloride and 1.0 g. of ascorbic acid were added to the soybean–casein digest broth.

As stated previously, the concept of monitoring nonsterile pharmaceutical products was introduced in the USP XVIII as a means of ensuring that the manufacturing process is under control and the product will not contain excessive amounts of either bacteria or molds. To implement this concept, specific microbiological monitoring procedures were prescribed in the USP for the first time. In addition to providing tests for estimating the number of viable aerobic microorganisms, the USP XVIII also describes procedures for detecting four pathogenic organisms: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* species, and *Escherichia coli*. Since the USP XVIII designated these four indicator organisms as undesirable contaminants, the contaminating microorganisms recovered in this survey were submitted to microbiological tests for identity. If the contaminant was found to be a Gram-negative bacillus or Gram-negative coccus-bacillus, it was transferred to the selective agars described in the USP XVIII for the tests for *Salmonella* species, *P. aeruginosa*, and *E. coli*. Upon examination of the colonies on the selective agars, if none showed colonial morphology characteristic of the respective organisms, the product was reported to be free from these bacteria. If a Gram-positive coccus was recovered in the sterility test, it was transferred to the selective Vogel–Johnson agar medium. Colonies on this medium suspected of being *S. aureus* were tested for coagulase production. If no coagulation was observed, the sample was reported to be free from coagulase-positive *S. aureus*.

RESULTS

A total of 261 batches of nonsterile antibiotics from 38 different manufacturers were examined for microbial contamination; all were found to be of satisfactory quality. As shown in Table I, no microbial growth was observed in 197 (75.5%) of the batches tested. For the 64 samples in which some growth was detected, the total aerobic counts were made and, in all cases, were less than 50 organisms per gram or milliliter. Of the 64 batches in which contamination was detected, only seven of the contaminants required plating on differential media. Six contaminants were tested to assure freedom from coagulase-positive *S. aureus* and one contaminant was tested on the various differential media to assure freedom from *P. aeruginosa*, *E. coli*, and *Salmonella*. All these batches were found to be free from coagulase-positive *S. aureus*, *E. coli*, *Salmonella* sp., and *P. aeruginosa* when tested by the USP XVIII microbial limit tests.

DISCUSSION

Since it has been shown that contaminated drugs can mediate infection in man, harmful organisms should be absent from nonsterile antibiotic preparations. Therefore, this survey was conducted to obtain general information on the microbial content of nonsterile antibiotic market samples. Experience has shown that antibiotic preparations can be produced with a low microbial content if the raw materials are not grossly contaminated and the manufacturer adheres to strict industrial hygienic procedures. Good Manufacturing Practice Regulations (9) consider that microbiological quality controls of water, air, equipment, and personnel are essential for all drugs.

Although these drugs have no sterility requirement, since they are not purported to be sterile, approximately 75% of the 261 batches tested were found to be sterile. In the remainder that were found contaminated, the number of microorganisms per gram or milliliter was extremely small. This survey showed that nonsterile antibiotic drugs sold in the United States are generally of acceptable hygienic quality in that their microbial content is minimal and innocuous. However, manufacturers should continue to monitor these preparations since past experience has shown that samples may occasionally become heavily contaminated.

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Spasmolytics IV: Azo and Azoxy Derivatives of 3-Tropanyl 2,3-Diarylacrylates

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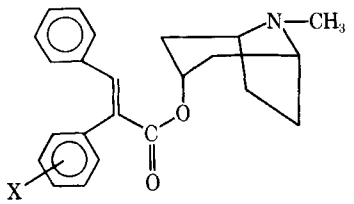
Abstract □ Some derivatives of potent spasmolytic aminoesters were made and tested to determine if they were satisfactory prodrug forms. These azo- and azoxyesters were much less active than the aminoesters in a 5-hr. spasmolytic test in mice, and results suggest that these potential aminoesters are not satisfactory prodrugs.

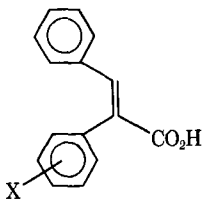
Keyphrases □ 3-Tropanyl 2,3-diarylacrylates, azo and azoxy derivatives—synthesis, evaluation as potential spasmolytic prodrug forms □ Spasmolytics, synthesis, evaluation—3-tropanyl 2,3-diarylacrylate derivatives

It was recently reported that a series of 3-tropanyl 2,3-diarylacrylates possessed spasmolytic activity without anticholinergic effect (1). Spasmolytic test data for nine of the first 10 of the 2-aryl-3-phenyl esters were subjected to multiple-parameter analysis[1]. It was found that the linear equation: $-\log ED_{50} = -1.624\ \sigma_o - 0.796$, where σ_o refers to the electron density in the 2-phenyl ring at the position *ortho* to the side chain, accounted for 95% of the variability in the test data for seven of the compounds. In contrast, the linear equation: $-\log ED_{50} = -1.077\ \sigma_{sc} - 0.906$, where σ_{sc} refers to the electron density in the 2-phenyl ring at the carbon attached to the side chain, accounted for only 54% of the variability in the test data for the seven compounds.



I: X = 4-NO_2
II: X = 4-NH_2
III: X = 3-NO_2
IV: X = 3-NH_2
V: X = 3-N=N-3
VI: X = 3-N=N-3 (↓ O)

XII: X = 4-N=N-4
XIII: X = 4-N=N-4 (↓ O)
XIV: X = 4-N=N-φ
XV: X = 3-N=N-φ
XVI: X = 4-OH, 3-N=N-φ



VII: X = 3-NO_2
VIII: X = 4-NO_2
IX: X = 3-N=N-3
X: X = 3-N=N-3 (↓ O)
XI: X = 4-N=N-4

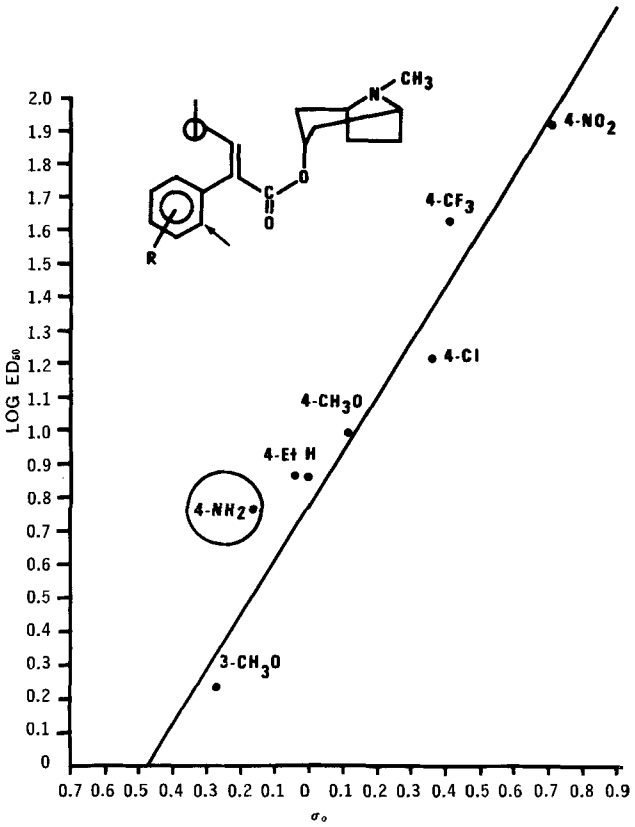


Figure 1—*Relationship between spasmolytic potency and sigma at the* ortho*-position (arrow). The circled compound provided a test of this relationship. Its actual potency was close to the potency predicted by the line.*

A plot of σ_o *versus* ED_{50} (Fig. 1) shows that compounds with negative σ_o values are more potent than those with positive σ_o values. Test results for the 4-amino compound available after the Hansch analysis provided a test of the ability of the equation to predict activity. It fell very close to the predicted curve. Figure 1 suggests that compounds with more highly negative σ_o values will be more potent.

To follow this lead, the 3-amino, 3-isobutylamino, and 3-hydroxy compounds were made. Subsequent to this work, data on these and certain newer com-

[1] By Professor C. Hansch.

Table I—Azo and Azoxy Compounds

	V	VI	IX	X				
	←M+→						Azo-benzene[a]	Azoxy-benzene[b]
	720	736	474	490	XII	XIII		
λEtOH (log $E_{max.}$)	220(4.56) 288(4.63) 440(2.73)	220(4.55) 286(4.63) 440(0)	274(4.55) 440(2.85)	272(4.53) 440(0)	223(4.42) 234(shoulder) 282(4.54) 344(4.38) 440(3.08)	221(shoulder) 282(4.60) 342(4.56) 440(0)	230(4.2) 320(4.3) 438(2.9)	231(3.92) 260(3.85) 323(4.16)
Color	Yellow-orange	Light-yellow	Yellow-orange	Tan	Orange	Yellow	Orange-red	Pale-yellow
Type	3-Azoester	3-Azoxyester	3-Azoacid	3-Azoxyacid	4-Azoester	4-Azoxyester	Azo	Azoxy

[a] W. S. McGuire, T. F. Izzo, and S. Zuffanti, *J. Org. Chem.*, **21**, 632(1956). [b] P. H. Gore and O. H. Wheeler, *J. Amer. Chem. Soc.*, **78**, 2160(1956).

pounds in the series were analyzed and it was found that the equation: $\log 1/c = 4.744 - 1.215\ \sigma_o^2 - 0.842\ \sigma_o$, accounted for 86% of the test data for 14 of the compounds. A plot of $\log 1/c$ *versus* σ_o gave a parabola with an ideal σ_o of −0.346, which suggested that the two most potent compounds in the series had been made (2). These compounds, 3-tropanyl 2-(2- and 3-methoxyphenyl)-3-phenylacrylate, were as potent as atropine in the fecal pellet inhibition test.

The purposes of this article are to: (*a*) describe the chemistry that led to the 3-amino compound and to some potential precursor amines, and (*b*) report pharmacological test results on these compounds.

DISCUSSION

The potent spasmolytic, 3-tropanyl 2-(4-aminophenyl)-3-phenylacrylate hydrochloride hydrate (II), was first made in 56% yield by hydrogenation of the 4-nitroester hydrochloride hydrate (I) over platinum on charcoal. The same procedure was used to reduce the isomeric 3-nitroester hydrochloride (III) to give a hydrated compound in low yield with identical elemental composition as II; this was assumed to be 3-tropanyl 2-(3-aminophenyl)-3-phenylacrylate hydrochloride hydrate (IV). Initial test results looked interesting, so more tests were planned; for these, more supplies were required. This time the reduction was run over Raney nickel in an attempt to improve the yield. But the two reduction products of III were different. The original melted at 283.5–284.5° dec. while the latter melted at 230–231° dec. (1). NMR showed the original had no NH protons, while the latter had two NH protons. Thus, the latter was established as the authentic IV, and the original was probably an azoester (V) or azoxyester (VI).

Authentic V was made by the sequence: nitroacid (VII) → azoacid (IX) → azoester (V), and VII (1 mole) was reduced with zinc dust (2 moles) and sodium hydroxide (5 moles) by the procedure for nitrobenzene → azobenzene (3). But the product was the azoxyacid (X) instead of IX. When the nitroacid, zinc dust, and sodium hydroxide ratios were changed to 1, 4, and 9.6, respectively, the hydrazoacid was obtained and air oxidized (4) to the azoacid (IX), which was converted to the authentic azoester (V), m.p. 288–289° dec., M^+ 720. The original reduction product was assigned Structure VI because it had M^+ of 736 and had no UV absorption at 440 nm. (azo band). Additional data are listed in Table I. VI was subsequently made in 67% yield by reducing the free base III over platinum on carbon.

The formation of IV, V, and VI from III in alkaline media is explained in Scheme I (5). Evidence in the literature suggests that a similar reductive sequence might occur *in vivo*. For example, *m*-nitrosonitrobenzene (RNO), *m*-nitrophenylhydroxylamine (RNHOH), and *m*-nitroaniline (RNH_2) are found in the urine of rabbits dosed with *m*-dinitrobenzene (6). Azoxy compounds were isolated in the urine of man and rabbits, although these might be artifacts which appear on standing or during isolation procedures because aromatic hydroxyamines are rapidly converted to azoxy compounds in neutral or alkaline solution (7). Azo compounds are reduced *in vivo* to hydrazo compounds, which are cleaved to amino compounds (8, 9). Salicylazosulfapyridine is an example (10). Also a variety of aromatic nitro compounds, including chloramphenicol, are reduced to the corresponding aromatic amines (11).

Thus, the nitro, azoxy, and azo compounds might be active *per se* or they might be active only after *in vivo* conversion to the amino compound. In either case, the authors wanted to pursue this in the *m* and *p* series and in related compounds.

The isomeric 4-azoester (XII) was made by the sequence: nitroacid (VIII) → azoacid (XI) → azoester (XII), and the 4-azoxyester (XIII) was made by reducing the 4-nitroester base (II). The azophenylesters (XIV and XV) were made from the aminoesters (II and IV) and nitrosobenzene (12). The benzeneazophenol ester (XVI) was made from the phenol ester and benzenediazonium chloride (13).

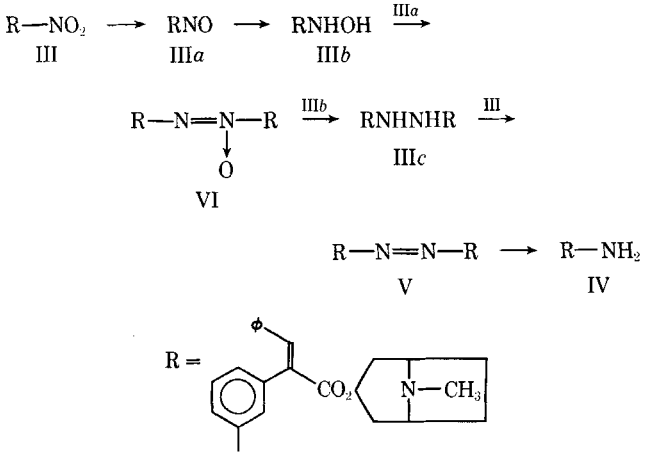
EXPERIMENTAL

Chemistry—Melting points were determined in open capillary tubes, using the Thomas-Hoover Uni-Melt, and are uncorrected.

3,3'-Diphenyl-2,2'-[azoxydi-(m-phenylene)]-diacrylic Acid (X)—This was made by the general method Bigelow and Robinson (3) used to prepare azobenzene. 2-(*m*-Nitrophenyl)-3-phenylacrylic acid (VII, 5.39 g., 0.02 mole) was suspended in methanol (35 ml.), and NaOH (4.04 g., 0.10 mole) in water (8 ml.) was added. Zinc dust (2.68 g., 0.041 mole) was added, and the mixture was heated and stirred at reflux for 11 hr. The solution was filtered, and the filtrate was acidified with hydrochloric acid. Crystallization (dioxane–water) gave 4.4 g. (90%) of a tan solid, m.p. 288–290° dec.

Anal.—Calc. for $C_{30}H_{22}N_2O_5$: C, 73.46; H, 4.52; N, 5.71. Found: C, 73.73; H, 4.70; N, 5.64.

3,3'-Diphenyl-2,2'-[azodi-(m-phenylene)]-diacrylic Acid (IX)—This was made by the general method of Bigelow and Robinson (3), but the nitro compound–zinc–sodium hydroxide ratio was changed from 1:2:5 to 1:4:9.6. 2-(*m*-Nitrophenyl)-3-phenylacrylic acid (VII, 20.2 g., 0.075 mole) was suspended in methanol (200 ml.), and NaOH (28.8 g., 0.72 mole) in water (60 ml.) was added to form a solution. Zinc dust (10 g.) was added, and the



Scheme I

Table II—Lethal, Spasmolytic, and Mydriatic[a] Effects in Mice of 3-Tropanyl 2,3-Diarylacrylates (Dose, mg./kg. p.o.)

Compound (Free Base)	X	MLD	Fecal Pellet, ED_{50}
I[b]	4—NO_2	162	82
II[b]	4—NH_2	512	5.7
III	3—NO_2	—	—
IV[b]	3—NH_2	256	8.2
V	3—N=N—3	256	>63
VI	3—N=N—3 (↓ O)	>1024	52
XII	4—N=N—4	768	>63
XIII	4—N=N—4 (↓ O)	>1024	>100
XIV	4—N=N—ϕ	>1024	82
XV	3—N=N—ϕ	768	60
XVI	4—OH, 3—N=N—ϕ	>1024	46
Atropine[b]	—	620	2.5

[a] Only atropine produced mydriasis. [b] These data were reported in *Reference 1*.

mixture was heated and stirred at reflux. After 1 hr., additional zinc dust (9.6 g.) was added; the blood-red solution turned orange and then pale yellow-green in 2.5 hr. The hydrazoacid solution was filtered, and air was passed through the warmed filtrate for about 20 hr. until the solution was red and the azoacid salt started to precipitate (4). The mixture was cooled, the orange precipitate was dissolved in aqueous methanol, and the solution was acidified with hydrochloric acid. Crystallization from dioxane–water gave, after drying *in vacuo*, 12 g. (68%) of a yellow-orange product, m.p. 299.5–300.5° dec.

Anal.—Calc. for $C_{30}H_{22}N_2O_4$: C, 75.94; H, 4.67; N, 5.90. Found: C, 75.76; H, 4.55; N, 5.73.

*3,3'-Diphenyl-2,2'-[azodi-(*p*-phenylene)]-diacrylic Acid (XI)*—This was made from 2-(*p*-nitrophenyl)-3-phenylacrylic acid (VIII, 11 g.) by the method described for IX. Crystallization from methanol–water gave 65% of a tan product, m.p. 329° dec.

Anal.—Calc. for $C_{30}H_{22}N_2O_4$: C, 75.94; H, 4.67; N, 5.90. Found: C, 75.58; H, 4.97; N, 5.83.

3,3'-Diphenyl-2,2'-[azodi-(m-phenylene)]-diacrylic Acid, Bis(3-tropanyl) Ester Dihydrochloride, Dihydrate (V)—A mixture of IX (5.6 g.), thionyl chloride (7 g.), dry benzene (56 ml.) and pyridine (1 drop) was heated at reflux for 2 hr. Excess thionyl chloride and benzene were removed, and three separate portions of dry benzene were added and removed in the same way. The residue was dissolved in dry benzene (60 ml.), and dry tropine (8.33 g.) in dry benzene (55 ml.) was added slowly with stirring. The mixture was heated at reflux temperature for 24 hr. and filtered. The filtrate was washed with water until neutral and dried, and the solvent was removed. The residue was dissolved in methanol, and excess methanolic HCl was added. Crystallization from ethanol gave 4.9 g. (51%) of an orange product, m.p. 288–289° dec.

Anal.—Calc. for $C_{46}H_{48}N_4O_4 \cdot 2HCl \cdot 2H_2O$: C, 66.58; H, 6.56; N, 6.75; Cl, 8.55. Found: C, 66.44; H, 6.61; N, 6.78; Cl, 8.37.

3,3'-Diphenyl-2,2'-[azodi-(p-phenylene)]-diacrylic Acid, Bis(3-tropanyl) Ester Dihydrochloride, 1.5 Hydrate (XII)—This was made from XI (6 g.) by the method described for V. Crystallization from methanol–isopropanol gave an orange solid (39% yield), m.p. 300° dec.

Anal.—Calc. for $C_{46}H_{48}N_4O_4 \cdot 2HCl \cdot 1.5H_2O$: C, 67.31; H, 6.51; N, 6.83. Found: C, 66.91; H, 6.48; N, 6.50.

3,3'-Diphenyl-2,2'-[azoxydi-(m-phenylene)]-diacrylic Acid, Bis(3-tropanyl) Ester Dihydrochloride, 1.5 Hydrate (VI)—A solution of 3-tropanyl 2-*m*-nitrophenyl-3-phenylacrylate (III, 12.9 g.) in methanol (150 ml.) was hydrogenated over platinum on carbon. The reaction mixture was warmed to dissolve the formed solid, and the solution was filtered. Excess HCl was bubbled into the filtrate, and the solvent was removed. The residue was crystallized from isopropanol–methanol to yield 8.4 g. (67%) of a light-yellow solid, m.p. 283.5–284.5° dec.

Anal.—Calc. for $C_{46}H_{48}N_4O_5 \cdot 2HCl \cdot 1.5H_2O$: C, 66.01; H, 6.38; N, 6.70; Cl, 8.47. Found: C, 65.85; H, 6.51; N, 6.71; Cl, 8.37.

3,3'-Diphenyl-2,2'-[azoxydi-(p-phenylene)]-diacrylic Acid, Bis(3-tropanyl) Ester (XIII)—This was prepared from 3-tropanyl 2-(*p*-nitrophenyl)-3-phenylacrylate (I, 10 g.) by the procedure described for VI. The yellow base (23%) melted at 251–251.5°.

Anal.—Calc. for $C_{46}H_{48}N_4O_5$: C, 74.98; H, 6.57; N, 7.60. Found: C, 74.89; H, 6.59; N, 7.53.

3-Phenyl-2-(p-phenylazophenyl)-acrylic Acid, 3-Tropanyl Ester Hydrochloride (XIV)—This was made by Anspon's method (12). Nitrosobenzene (1.7 g., 0.016 mole) was added to 3-tropanyl 2-(*p*-aminophenyl)-3-phenylacrylate hydrochloride (II, 6.5 g., 0.016 mole) in 39 ml. of cooled glacial acetic acid. The resulting solution was allowed to stand for 16 hr. at room temperature. The product was collected and crystallized from ethanol to give an orange solid (39% yield), m.p. 265.5–267° dec.

Anal.—Calc. for $C_{29}H_{29}N_3O_2 \cdot HCl$: C, 71.37; H, 6.20; N, 8.61; Cl, 7.27. Found: C, 71.38; H, 6.30; N, 8.64; Cl, 7.21.

3-Phenyl-2-(m-phenylazophenyl)-acrylic Acid, 3-Tropanyl Ester Hydrochloride (XV)—This was prepared from 3-tropanyl 2-*m*-aminophenyl-3-phenylacrylate (IV, 6.5 g., 0.016 mole) and nitrosobenzene (1.7 g., 0.016 mole) in glacial acetic acid (39 ml.) by the procedure described for XIV. It was recrystallized twice from isopropanol to give a red-brown solid (59%), m.p. 202–202.5°.

Anal.—Calc. for $C_{29}H_{29}N_3O_2 \cdot HCl$: C, 71.37; H, 6.20; N, 8.61; Cl, 7.27. Found: C, 71.27; H, 6.24; N, 8.39; Cl, 7.30.

3-Phenyl-2-(p-hydroxy-m-phenylazophenyl)-acrylic Acid, 3-Tropanyl Ester, 0.25 Hydrate (XVI)—Benzenediazonium chloride (0.02 mole) was added in six portions to a cooled solution of 3-tropanyl 2-(*p*-hydroxyphenyl)-3-phenylacrylate (7.36 g., 0.02 mole) and 10% NaOH solution (16 ml.) in dimethylformamide (100 ml.). The mixture was stirred for 30 min. and the product was filtered. It was dissolved in tetrahydrofuran, converted to the hydrochloride salt, dissolved in dimethylformamide, neutralized with NaOH solution, and extracted into chloroform. It was recrystallized from methanol to give a red-orange solid (28%), m.p. 188.5–189.5°.

Anal.—Calc. for $C_{29}H_{29}N_3O_3 \cdot 0.25H_2O$: C, 73.78; H, 6.30; N, 8.90. Found: C, 73.85; H, 6.37; N, 8.31.

Pharmacological Methods—The drugs were administered orally by stomach tube in all cases.

Minimum Lethal Dose and Observation of Overt Effects—Groups of three mice were administered various doses of the test drug. Observations for behavioral changes, impairment of reflexes, mydriasis, and lethality were made. The lowest dose causing death was defined as the MLD. Measurements of pupil diameter served to indicate possible anticholinergic activity.

Spasmolytic Activity—A modification of the Janssen *et al.* (14) method was used. Potency was expressed as the dose that reduced the 5-hr. fecal pellet count in mice by 50% as compared to a control group (ED_{50}).

RESULTS

The pharmacological results are shown in Table II. All the compounds were active. In the series with substituents in the 3-position, the 3-aminoester (IV) was six to seven times more potent in the spasmolytic test than the 3-azoester (V), the azoxyester (VI), and the 3-azophenyl ester (XV). Likewise the 4-amino ester (II) was 11–17 times more potent than the 4-nitroester (I), the 4-azoxyester (XIII), the 4-azoester (XII), and the 4-azophenyl ester (XIV).

These data may be interpreted in two ways. The potential aminoester intermediates may be less active *per se* than the amino compounds, or they may appear less active because of the 5-hr. time limit of the spasmolytic test used. In either case, these potential intermediates of the potent aminoesters do not appear to be satisfactory prodrugs.

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Estimation of Diffusion Coefficients and Molecular Weights of Interacting Colloids from Dissolution-Rate Data

STUART FELDMAN*, MILO GIBALDI†, and MARK REINHARD*‡

Abstract □ Based on dissolution-rate theory, diffusion coefficients for the salicylamide–micelle species were calculated from dissolution and solubility data obtained in micellar solutions of sodium taurodeoxycholate and sodium taurocholate at 20°. The calculated values were in good agreement with those determined experimentally using a diffusion cell and silver membrane. By assuming that the solubilized drug does not interfere with the organization of the micelle, the Stokes–Einstein equation was employed to estimate the molecular weight of the micelle. Excellent agreement was found between values calculated from dissolution data and those determined directly by ultracentrifugation or light scattering.

Keyphrases □ Diffusion coefficients, salicylamide–micelle species—calculated from dissolution, solubility data □ Molecular weight determinations, micellar—sodium taurodeoxycholate and sodium taurocholate with salicylamide □ Bile salt solutions—diffusion coefficients, molecular weight estimations, from dissolution-rate data

Numerous methods have been proposed to measure diffusion coefficients of colloids (1–5). These methods often involve rather complex experimental procedures, and much time may be required before experimental results can be obtained.

The most common method of determining diffusion coefficients of colloids has been the use of a diffusion cell and some means to separate the drug solution from the solvent "sink." Desai *et al.* (3) utilized a sintered-glass disk to separate the two solutions. An improvement on the glass disk was reported by Singh *et al.* (4), who used a filter made of glass reinforced with epoxy. Recently, Goldberg and Higuchi (5) successfully measured diffusion coefficients utilizing a silver membrane filter. These workers found that the silver filter produced good data precision and resulted in a fairly rapid method of determining diffusion coefficients.

A number of different investigators (4, 6) confirmed that dissolution from a rotating disk conforms to the theoretical equation presented by Levich (7) for that system. Since the dependence of the dissolution rate on the diffusion coefficient is known for the rotating-disk system, one should be able to use a reversal of the usual procedure—that is, measure dissolution rate of a drug in colloidal and aqueous systems and then calculate the diffusion coefficient of the drug in the system utilizing the Levich equation. This approach could lead to an improvement over the usual methods to determine diffusion coefficients because dissolution-rate measurements are rapid and reproducible. Moreover other factors such as changes in the membrane or cell constant or the incomplete washing out of the diffusion cell would not be problems in the dissolution-rate determination.

THEORY

Levich (7) proposed a convective diffusion theory for the rate of mass transport to or from the face of a rotating disk. The equation for the dissolution rate of a solid in a medium containing a colloidal solubilizing agent according to the rotating disk theory is (4):

$$DR = 0.621\gamma^{-1/6}\omega^{1/2}(D_{\text{eff.}})^{2/3}C_T \qquad \text{(Eq. 1)}$$

where DR is the dissolution rate per unit area of dissolving solid, C_T is the total solubility of the drug in the dissolution medium, γ is the viscosity of the medium, ω is the angular velocity of the rotating disk, and $D_{\text{eff.}}$ is the effective diffusion coefficient. The

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Table I—Solubility and Dissolution Rate of Salicylamide at 50 m*M* Concentration of Each Bile Salt at 20°

	Solubility, mg./ml.[a]	Dissolution Rate[b], mcg./ml./min.	Ratio[c]
Control	2.04 ± 0.08	4.36 ± 0.05	1.00
Sodium taurodeoxycholate	3.24 ± 0.14	5.51 ± 0.03	1.26
Sodium taurocholate	3.21 ± 0.14	5.77 ± 0.05	1.32
Equimolar mixture	3.24 ± 0.10	5.60 ± 0.03	1.28

[a] Mean and standard deviation of five determinations. [b] Mean and standard deviation of three determinations. [c] Ratio of dissolution rate of salicylamide in surfactant solution to that in 0.15 *M* sodium chloride.

effective diffusion coefficient is defined as:

$$D_{\text{eff.}} = \frac{DC_s + D_mC_m}{C_T} \qquad \text{(Eq. 2)}$$

where D and D_m are the diffusion coefficients of the free and colloid solubilized drug, respectively. The solubility of the drug in the absence of the colloidal solubilizing agent is denoted by C_s, and C_m is the increase in solubility of the drug due to the presence of the colloid. (Therefore, $C_T = S_s + C_m$.)

By taking the ratio of the dissolution rate of the solid in a colloidal solution to that in a noncolloidal aqueous solution, Eq. 3 results:

$$\text{ratio} = \frac{(D_{\text{eff.}})^{2/3}C_T}{D^{2/3}C_s} \qquad \text{(Eq. 3)}$$

Thus, by determining the dissolution rate of a drug with a known diffusion coefficient in a colloidal solution and pure aqueous solution, and by measuring the equilibrium solubility of the drug in each solution, it is possible by means of Eqs. 2 and 3 to calculate the colloidal diffusion coefficient of the drug molecule.

EXPERIMENTAL

Materials—Taurodeoxycholic acid and taurocholic acid were obtained[1] as the sodium salt (A grade) and used as such. All other chemicals were analytical reagent grade and were used without further purification.

Dissolution-Rate Determination—The dissolution rate of salicylamide from nondisintegrating tablets, prepared by compression of 600 mg. of pure drug in a Carver model B hydraulic press at 10,000 p.s.i. for 10 sec., was determined by the rotating-disk method (8). The salicylamide tablets were mounted in Plexiglas holders with the aid of paraffin, and the apparatus was attached to a Servodyne constant torque unit. A 150-ml. water-jacketed beaker, containing 50 ml. of 50 m*M* sodium taurodeoxycholate, 50 m*M* sodium taurocholate, or a 50 m*M* equimolar mixture of the two bile salts (sodium taurodeoxycholate/sodium taurocholate) in 0.15 *M* sodium chloride was maintained at 20 ± 0.1° by circulation of water through the beaker. Control dissolution-rate determinations were run in 0.15 *M* NaCl. The Plexiglas holder and tablet were rotated at 100 r.p.m. and placed in the dissolution fluid. A 1-ml. sample was withdrawn at 5-min. intervals, diluted appropriately with 0.1 *N* HCl, and read spectrophotometrically at 299 nm.

Solubility Determination—The solubility of salicylamide was determined in solutions of 50 m*M* sodium taurodeoxycholate, sodium taurocholate, or sodium taurodeoxycholate–sodium taurocholate in 0.15 *M* NaCl and in 0.15 *M* NaCl alone by adding an excess of solid salicylamide to 5 ml. of the respective solution in 10-ml. capped culture tubes. The solutions were maintained at 20 ± 0.1° and shaken for 24–48 hr. Equilibrium was established by repetitive sampling.

Diffusion-Coefficient Measurements—Diffusion coefficients were measured directly by a method similar to that described by Goldberg and Higuchi (5). The silver membrane, 47-mm. diameter, 1.2-μ pore size, was prepared according to Goldberg and Higuchi (5). At time zero, 40 ml. of the appropriate solution (50 m*M* surfactant in 0.15 *M* NaCl or 0.15 *M* NaCl alone) containing 1 mg./ml. of salicylamide was placed in the left chamber of the apparatus.

[1] Calbiochem, Los Angeles, Calif.

Table II—Effective Diffusion Coefficients and Micellar Diffusion Coefficients of Salicylamide in 50 m*M* Bile Salt Determined from Dissolution-Rate Data and Direct Experimental Measurement

	$D_{\text{eff.}}$, cm.²/sec. × 10^6 Calculated[a]	$D_{\text{eff.}}$, cm.²/sec. × 10^6 Measured	D_m, cm.²/sec. × 10^6 Calculated[b]	D_m, cm.²/sec. × 10^6 Measured
Sodium taurodeoxycholate	7.79	7.80	2.33	2.36
Sodium taurocholate	8.46	8.39	4.03	3.84
Equimolar mixture	7.97	8.00	2.82	2.90

[a] Calculated by means of Eq. 3. [b] Calculated by means of Eq. 2.

At the same time, an identical solution without drug was introduced into the right chamber. The stirring motors were started, and 1-ml. samples were withdrawn from the right chamber at appropriate intervals, diluted, and assayed spectrophotometrically for salicylamide. A volume of solution was added to the right chamber to replace that withdrawn by the sample. This was corrected for in subsequent calculations. The temperature was maintained at 21 ± 1°.

The cell was calibrated by assuming the diffusion coefficient of benzoic acid in distilled water at 21° to be 1.11 × 10^{-5} cm.²/sec. (9). The cell constant thus obtained was then used to calculate the diffusion coefficient of salicylamide in the presence and absence of 50 m*M* bile salt.

RESULTS AND DISCUSSION

The solubility of salicylamide in 50 m*M* solutions of sodium taurodeoxycholate or 50 m*M* sodium taurocholate and in a 50 m*M* solution of an equimolar mixture of the two bile salts at 20° is shown in Table I. The solubility of salicylamide in 0.15 *M* sodium chloride also appears in this table. The increased solubility of salicylamide in each of the micellar bile salt solutions was in the order of 57–59%.

The results obtained from the dissolution-rate experiments utilizing the rotating disk are also presented in Table I. The presence of the micellar bile salt solution resulted in an increase in the rate of salicylamide dissolution as compared to the rate in 0.15 *M* sodium chloride. The ratio of dissolution rate of salicylamide in bile salt solution to that of control also appears in Table I. The differences in dissolution rates between each bile salt solution were small, but each result was statistically different[2] from any other as determined by the Student t test (10). The rate of salicylamide dissolution in the various surfactant solutions decreased in the following order: sodium taurocholate > equimolar mixture > sodium taurodeoxycholate.

The results of the diffusion-coefficient measurements appear in Table II. The diffusion coefficient of salicylamide in 0.15 *M* sodium chloride was found to be 11 × 10^{-6} cm.²/sec. This value was used in all subsequent calculations. Included in this table are the effective diffusion coefficients ($D_{\text{eff.}}$) determined experimentally and the $D_{\text{eff.}}$ calculated from the dissolution-rate data by means of Eq. 3. The micellar coefficient (D_m) of salicylamide in 50 m*M* of the various bile salt solutions was calculated by means of Eq. 2 from both the diffusion-coefficient measurement data and the dissolution-rate data (Table II). As can be seen from the data, the agreement between the two methods of analysis is quite good. However, caution must be exercised in the use of this method if the dissolution rate is not entirely diffusion controlled.

It was previously shown that the Stokes–Einstein relationship gives a good estimation of the micellar molecular weight of the drug–micelle complex based upon the diffusion coefficient (6). According to the Stokes–Einstein relationship, the diffusion coefficient of a solute may be calculated by means of the following equation (11):

$$D = \frac{RT}{6\pi\eta N} \cdot \sqrt[3]{\frac{4\pi N}{3\bar{M}\bar{v}}} \qquad \text{(Eq. 4)}$$

[2] $p < 0.05$.

Table III—Micellar Molecular Weight of Each Bile Salt Determined from Literature Data and Calculated Using Eq. 5 from Dissolution-Rate Data and Measured Diffusion-Coefficient Data

	—Micellar Molecular Weight—		
	Dissolution-Rate Data	Diffusion Coefficient Data	Literature[a]
Sodium taurodeoxycholate	14,148	13,306	12,500
Sodium taurocholate	2,662	3,072	2,689
Equimolar mixture	7,848	7,189	7,945

[a] From *Reference 15.*

where R is the molar gas constant, T is the absolute temperature, η is the viscosity of the solvent, N is Avogadro's number, M is the molecular weight, and $\bar{v}$ is the partial specific volume. Equation 4 predicts an inverse cube-root relationship between the diffusion coefficient and molecular weight. Therefore, estimation of micellar molecular weight can be made by means of the following relationship:

$$(M)^{1/3}\text{micelle-drug} = \frac{(M)^{1/3}\text{drug} \cdot D}{D_m} \qquad \text{(Eq. 5)}$$

The calculations were made for each bile salt solution studied from both diffusion and dissolution data (Table III). Several studies in the literature report the micellar molecular weights of a number of bile salts (12–15). Carey and Small (16) presented a detailed study of the micellar properties of sodium taurocholate and sodium taurodeoxycholate. By using the molecular weight of each individual bile salt and the rounded-off aggregation number at 20° and 0.15 M sodium chloride concentration, it was possible to calculate the molecular weight of each bile salt micelle. An estimation of the molecular weight of the mixed micelle of the equimolar mixtures of the bile salts was obtained by multiplying the aggregation number by the mean of the bile salt molecular weights. Since the molecular weights of each bile salt are similar, little error should be involved in this estimation. The molecular weight contributed by the moles of salicylamide solubilized by each mole of bile salt was then subtracted from the experimentally measured values of micellar weights to obtain the molecular weight of micelle (Table III). As can be seen in Table III, the agreement between the literature values and those calculated from Eq. 5 is excellent.

The cube-root relationship may only be used for an estimate of colloid molecular weight under those conditions where the interaction between the colloid and the drug molecule does not interfere with the organization of the colloid. If this is the case, one can determine the molecular weight of the colloid independent of the drug molecule, which is being used strictly as a probe.

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Aminotetrahydrofuranol Derivatives

C. M. DARLING* and HERNDON JENKINS

Abstract □ Some esters of 4-dimethylamino-3-tetrahydrofuranol are reported along with a methyl and benzyl quaternary ammonium salt. Compound VI possessed about one-fifth the anticholinergic activity of atropine sulfate.

Keyphrases □ 4-Dimethylamino-3-tetrahydrofuranol, esters—synthesis as possible anticholinergic agents, pharmacological evaluation □ Aminotetrahydrofuranol derivatives—synthesis, pharmacological screening as possible anticholinergic agents □ Anticholinergic agents, potential—synthesis of aminotetrahydrofuranol derivatives, pharmacological evaluation

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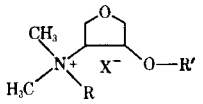
Table I—Derivatives of 4-Dimethylamino-3-tetrahydrofuranol



Compound	R	R′	X	Melting Point	Yield, %	Formula	Analysis[a], % Calc.	Found
I[b]	H	CH_3CO	Cl	159–160.5°	82	$C_8H_{16}ClNO_3$	C, 45.83 H, 7.69 N, 6.68	C, 45.83 H, 7.96 N, 6.72
II[b]	CH_3	CH_3CO	Br	184.5–186°	94	$C_9H_{18}BrNO_3$	C, 40.31 H, 6.76 N, 5.22	C, 39.86 H, 6.82 N, 5.17
III[c]	$C_6H_5CH_2$	CH_3CO	Br	170–171°	76	$C_{15}H_{22}BrNO_3$	C, 52.33 H, 6.44 N, 4.07	C, 51.97 H, 6.19 N, 4.01
IV[c]	H	p-$O_2NH_6H_4CO$	Cl	188.5–190°	38	$C_{13}H_{17}ClN_2O_5$	C, 49.30 H, 5.41 N, 8.84	C, 49.38 H, 5.53 N, 8.70
V[b]	H	p-$H_2NC_6H_4CO$	Cl	180.5–182°	80	$C_{13}H_{19}ClN_2O_3$	C, 54.45 H, 6.68 N, 9.77	C, 54.59 H, 6.69 N, 9.80
VI[c]	H	Benziloyl	Cl	204–206°[d]	65[e]	$C_{20}H_{24}ClNO_4$	C, 63.57 H, 6.40 N, 3.71	C, 63.87 H, 6.46 N, 3.54

[a] Elemental analyses for C and H were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Elemental analyses for N were performed by the Analytical Department, Research Laboratories, A. H. Robins Co., Inc., Richmond, Va. [b] Recrystallized from ethanol. [c] Recrystallized from a mixture of methylethylketone and methanol. [d] Lit. (2) m.p. 186° dec. [e] The reported yield is for the free base, m.p. 78–80°.

hydrofuranol or by the method of Reppe *et al.* (1) using 3,4-epoxytetrahydrofuran. The aminoalcohol was then esterified with the appropriate reagents. Both methods of preparation are considered stereospecific routes leading to *trans*-3,4-disubstituted analogs[1].

The acetate ester was easily quaternized with methyl bromide or benzyl bromide. Unsuccessful attempts to quaternize the benzilate ester led to the respective quaternary ammonium alcohol.

None of the compounds reported in Table I exhibited cholinergic activity in the isolated guinea pig ileum screen. All the compounds showed negligible anticholinergic activity, except Compound VI which possessed about one-fifth the potency of atropine sulfate. Compound V, an analog of procaine, possessed no significant local anesthetic activity.

EXPERIMENTAL[2]

4-Dimethylamino-3-tetrahydrofuranol—A solution of dimethylamine (162 g., 3.6 moles) and 4-chloro-3-tetrahydrofuranol (116 g., 0.94 mole) in absolute ethanol (250 ml.) was heated in a steel bomb at 126° for 18 hr. The cooled solution was concentrated *in vacuo* to an oil, which was added to a 25% solution of sodium hydroxide (200 ml.). The basic solution was extracted with chloroform (2 × 100 ml.), and the combined chloroform extracts were concentrated *in vacuo*. The residue was distilled, 74.5 g. (60.5%), b.p.$_{0.6}$ 77–80° [lit. (2) b.p.$_{0.7}$ 87–88°], and used without further purification.

Esters of 4-Dimethylamino-3-tetrahydrofuranol—The benzilate ester was prepared by transesterification (2). The acetate and *p*-nitrobenzoate esters were prepared by refluxing a chloroform solution of 4-dimethylamino-3-tetrahydrofuranol and an excess of either acetic anhydride or *p*-nitrobenzoyl chloride for 2–3 hr. After a routine acid–base wash, the respective free base was converted to the hydrogen chloride salt and recrystallized.

4-Dimethylamino-3-tetrahydrofuryl *p*-aminobenzoate was prepared by hydrogenating the corresponding nitro compound in the presence of a catalytic amount of 10% palladium-on-charcoal in methanol.

Quaternary Ammonium Bromides—A solution of the appropriate amine in methylethylketone was stirred while a solution of excess bromo compound in methylethylketone was added. After the suspension was stirred at ambient temperature for several hours, the product was isolated by filtration and recrystallized.

[1] While this paper was in preparation, the paper of Nelson *et al.* (3) was published.

[2] Melting points are uncorrected. Spectral data, NMR and IR, are in agreement with the proposed structures. Carbon-hydrogen analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich., and nitrogen analyses were performed by Analytical Department, Research Laboratories, A. H. Robins Co., Inc., Richmond, Va.

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Effect of Calcium Ions on the Scattering of Light by Heparin

S. P. LOUCAS, K. C. YEH, and H. M. HADDAD

Abstract □ The light-scattering patterns of sodium heparin under the influence of calcium ions were investigated using unpolarized light of 436 nm. A weight average molecular weight on the order of 17,500 was obtained for samples with varying degrees of sulfation. The interaction constant was found to be influenced by the ionic strength of the solution. At fixed electrolyte concentrations, changes in the second virial coefficient were governed by the charge density of the mucopolysaccharide.

Keyphrases □ Heparin light scattering—effect of calcium^{+2} □ Calcium^{+2}—effect on heparin light scattering □ Molecular weight determination—sulfated heparin

Since its discovery by McLean (1), heparin has been used as a blood anticoagulant. Previous investigations attempted to relate bioactivity with molecular weight from diffusion (2) and sedimentation velocity (3) studies. Using weight average molecular weight, Barlow *et al.* (4) found no correlation with biological activity.

In connecting the anticoagulant properties of heparin with its chemical structure, Foster and Huggard (5) suggested that three effects should be considered: degree and distribution of sulfate groups, molecular size, and shape.

Since highly charged polyions in solution are known to be influenced by the presence of electrolyte (6), the present study was undertaken to examine the scattering patterns of heparin samples, with varying sulfur content and potency, as a function of ionic strength. The data were examined in terms of the interaction constant B relative to bioactivity. Calcium chloride was selected as the damping electrolyte since the heparin–calcium–water model reflects similarity to the blood-clotting system.

MATERIAL

Samples of sodium heparin were obtained[1]. Elemental analysis, on an anhydrous basis, gave sulfur contents of 10.10, 11.92, and 12.99%. Sodium was determined with a Baird Atomic flame photometer, using LiCl and NaCl as internal and reference standards. Triple-distilled sterile water was used as solvent for all solutions whose apparent optical turbidity was found to be on the order of 10^{-5} cm.$^{-1}$. Reagent grade $CaCl_2$ was employed as the damping electrolyte, and all solutions were calculated on the dried basis. Three or four solutions strengths were prepared for each phase of study. Solution clarity was achieved by filtration through 0.2-μ presolution washed Metricel[2] 3-mm. GA-8 disks and subsequently centrifuged for 30 min. at 10,000 r.p.m.[3].

LIGHT SCATTERING

The specific refractive increment, $(n - n_0)c$, where n and n_0 are the refractive indexes for solution and solvent, c is the concentration (g./ml.), and τ (the turbidity) (cm.$^{-1}$) $= 1.17[n^2(Rw/Rc)][\alpha(Gs/Gw)F]$, were obtained with a Brice Phoenix differential refractometer and modified dual photomultiplier-type photometer, model 2000, using 40 × 40-mm. semioctagonal cells and unpolarized incident light of 436 nm. Instrument calibration factors were supplied by the manufacturer with respect to incomplete compensation of refractive effects Rw/Rc and working to opal reference standard α. Photomultiplier responses Gs and Gw were recorded with either no neutral filters F or a combination of filters and α to arrive at the scattering ratio Gs/Gw. Reagent grade NaCl was used in all solutions for dn/dc calibration. The experimentally determined constant, 9.245×10^{-4}, was used to convert readings to refractive index increments and found to be applicable for solutions containing up to 3% sodium heparin.

Table I—Refractive Index Increment and Interaction Constant Data for Sodium Heparin (Sulfur = 12.99%) at 436 nm. and 25°

Solvent	dn/dc(ml./g.) × 10^{-1}	B(mole-ml./g.^{2}) × 10^3
H_2O	1.28	18.00
0.05 M $CaCl_2$	1.29	1.88
0.10 M $CaCl_2$	1.23	1.10
1.00 M $CaCl_2$	1.16	0.22

Weight average molecular weight Mw, defined as $\Sigma N_i M_i^2 / \Sigma N_i \Sigma M_i$, and solution interaction constant B were obtained from the Debye (7) equation $Hc/(\tau_s - \tau_0) = 1/Mw + 2Bc$, in which $(\tau_s - \tau_0)$ is the difference between solution (τ_s) and solvent (τ_0) turbidity reflecting solute or apparent excess turbidity, and c is in terms of grams per milliliter. A plot of the function $Hc/(\tau_s - \tau_0)$ against c when extrapolated to infinite dilution yields the reciprocal molecular weight. The term H is an optical constant equal to $32\pi^3 n^2(dn/dc)^2/3N\lambda^4$, where N is the Avogadro number, λ the wavelength of light in vacuum, and n the refractive index parameter previously described. The B term is the second virial coefficient and reflects deviations from ideality and a measure of the interactions in solution as governed by the thermodynamic properties of the system studied (8). Conditions rendering the sign of this constant positive indicate that net electrostatic charges reside on solute molecules with negative heats of mixing and good solvent properties. Negative values suggest that a high degree of solute–solute interactions is preferred with poor solvation. At $B = 0$, phase separation may ensue. Under these circumstances, for a highly charged macromolecule, the presence of swamping electrolyte can influence the slope of the $Hc/(\tau_s - \tau_0)$ concentration plots.

RESULTS AND DISCUSSION

Heparin was studied under conditions of low (0.05–1.00 M) and high ionic strength, as well as in the absence of electrolyte.

Refractive index increments, given in Table I for heparin, show a distinct dependency on $CaCl_2$ concentration. The data indicate that changes in refractive index, relative to electrolyte concentration changes, are not identical. Previous findings (4) reported constant dn/dc in NaCl.

Average values of $Hc/(\tau_s - \tau_0)$ were plotted against concentration, and lines were fitted by the method of least squares. Each point represents an average of triplicate readings on singularly prepared solutions. Recorder response was found to be within a range of ±1%.

Figures 1 and 2 show all samples per group, irrespective of ionic strength or degree of sulfation, to exhibit close extrapolation. Mean Mw values on the order of 17,600 and 17,200 with a range of ±5%, respectively, were obtained after taking into account a 12% sodium

[1] Eli Lilly & Co. (Lot SO 1292; 138.5 USP units/mg.) and Abbott Laboratories (Lot 807-7199; 157 USP units/mg.).
[2] Gelman Instrument Co.
[3] International Equipment Co., model B-20.

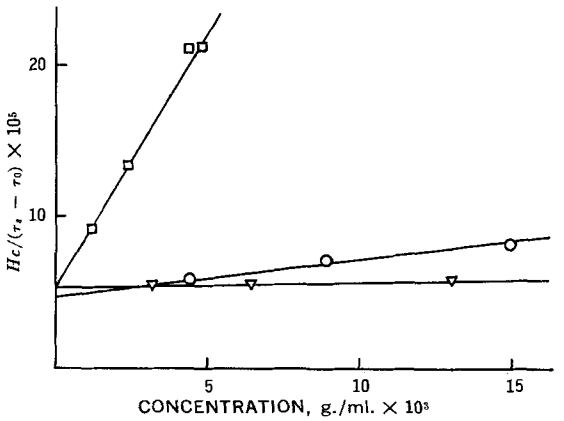

Figure 1—*Effect of ionic strength on the scattering pattern of heparin at 436 nm. and 25°. Key:* □, *H_2O;* ○, *0.1* M *$CaCl_2$; and* ▽, *1.0* M *$CaCl_2$.*

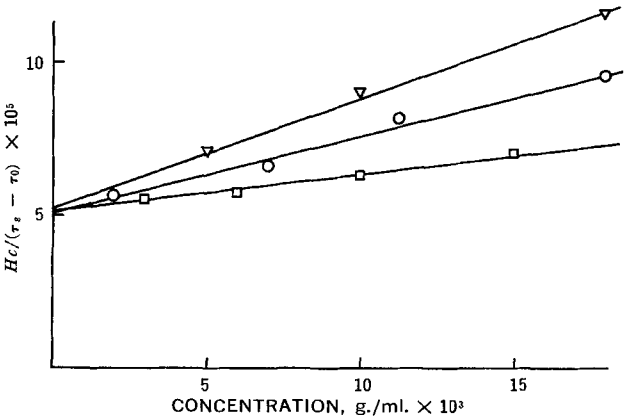

Figure 2—*Scattering of light by heparin samples with varying degrees of sulfation at constant ionic strength* ($\Gamma/2$ = *0.15 $CaCl_2$);* λ = *436 nm.; 25°. Key:* ▽, *12.99% sodium;* ○, *11.92%; and* □, *10.10% sodium.*

contribution. These values are in agreement with those reported from diffusion and sedimentation velocity (2, 3) studies. The results suggest that weight average particle distribution of the polyanion is not influenced by the degree of sulfation in the presence of added electrolyte but is governed by the structural saccharidic backbone. This contention also is supported by the results of Barlow *et al.* (4), who obtained a *Mw* of 16,600 in NaCl for heparin with bioactivities on the order of 5 and 153 units/mg. It appears, therefore, that high ionic strengths are not necessary nor do they greatly influence the extrapolation.

Further scrutiny of the data in Fig. 2, under conditions of constant ionic strength, reveals a progressive decrease in the slopes with diminishing potency. The observed behavior reflects screening of gegenions and seems to denote that with changing polyelectrolyte charge density, the electrical double layer is progressively altered, with less collapse noted in the more highly sulfated species. These findings indicate that the second virial coefficient ($B = 1.83 \times 10^{-3} - 0.68 \times 10^{-3}$ mole-ml./g.^{2}; 157.0–138.5 USP units/mg.) can possibly serve as a physicochemical measure for screening biological potency and may reflect relative differences in potency to a greater degree than weight average molecular weight, which depends on aggregate distribution.

The large positive interaction constant obtained in water is further indication that net electrostatic negative charges reside on the molecules, giving rise to high solute–solvent interactions. Studies (9) with its antagonist, protamine sulfate, showed a negative second virial coefficient on the order of 2.13×10^{-3} mole-ml./g.^{2}.

By contrasting $CaCl_2$ to NaCl, it appears from the present findings that electrolyte valence governs the magnitude of the slopes in accord with theoretical considerations (10). Calcuim chloride at 0.05 *M* was found to influence the slope to the same extent as 0.5 *M* NaCl for heparin samples with reported similar *Mw*. While significant solute–solvent interactions are present in 1.0 *M* NaCl, the present findings in $CaCl_2$ show a drastic decrease in the *B* terms, approximating zero at comparable concentration. Compared to sodium ions, calcium appears to be more strongly bound to heparin, decreasing the ionic character of the mucopolysaccharide to a greater degree. Strong interactions between the two species would be expected to play an important role in the complex anticoagulation mechanism.

Positive slopes noted for all plots also suggest that the particles in solution may exist in the form of flexible chains, as reported by Stones and Moss (11). While heparin has a specific conformation, a degree of flexibility within the molecule can be expected.

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Tablet Absorption in the Rat

Keyphrases ☐ Pharmacokinetics, bioavailability—4-(3′-pyridyl)-bicyclo[2.2.2]octane-1-amine maleate tablets, rats ☐ Tablet absorption—rats ☐ 4-(3′-Pyridyl)bicyclo[2.2.2]octane-1-amine maleate—tablet absorption, rats

Sir:

The current interest in bioavailability data and their requirement by the FDA have necessitated increased effort in the development of biopharmaceutical programs by the pharmaceutical industry. The absorption of a drug from a given dosage form is one important criterion that must be studied early in the development process. Since many products are marketed in tablet form, this dosage form should be compared to parenteral and oral solution administrations. Usually, the dog is the animal of choice for tablet studies because of convenience in dosing with tablets. However, the dog may sometimes be a poor model for preliminary absorption studies because of low excretion of unchanged drug or metabolic differences which do not permit a readily monitored system.

In the present study, the drug under investigation, 4-(3′-pyridyl)bicyclo[2.2.2]octane-1-amine maleate, was not sufficiently recovered from the urine of the dog to permit tablet absorption studies. Since the rat differed from the dog in excreting a significant amount of unchanged drug in the urine, it was used in such a study.

Standard tableting equipment was used to prepare 21-mg., 0.32-cm. (0.125-in.) diameter tablets. The tablets were administered to untrained rats by a relatively simple technique which required two technicians. The equipment required was minimal and readily available.

The special tablets for this rat study were prepared from tablets produced for human clinical use. The tablets for clinical use contained 50 mg. of active ingredient in a tablet of 175-mg. total weight, prepared by a wet granulation process. The special tablets were prepared by comminuting the original tablets in a mortar and then recompressing tablets on a Stokes A-3 single-punch tablet press equipped with a standard concave, 0.32-cm. (0.125-in.) punch and die set. The newly produced tablets contained 6.7 mg. (range 6.4–6.9 mg.) of active ingredient (as assayed by GLC) in a tablet of 21-mg. total weight.

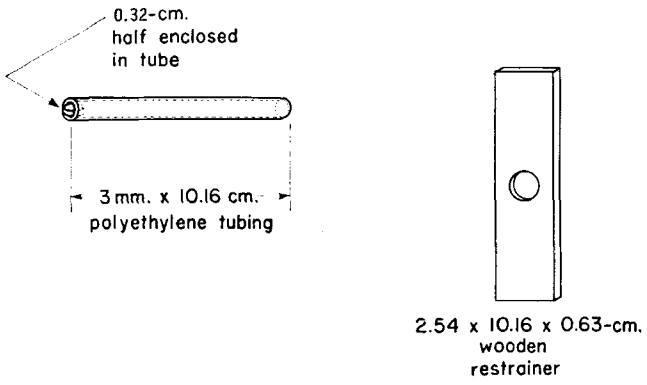

Figure 1—*Rat tablet-dosing apparatus.*

Table I—Relative Absorption Data

Hours Posttreatment	Percent Dose Excreted in Urine as Unchanged Drug		
	Intravenous	Oral Solution	Tablet
0–5	15.6	14.7	9.1
5–12	0.4	1.4	7.8
12–24	0.5	0.4	1.1
24–29	0.1	0.3	0.1
29–48	0.0	0.0	0.1
Total	16.6	16.8	18.2

n = 24 rats/dosage form

It was found that 200-g. rats were the minimum size necessary for dosing with the 0.32-cm. (0.125-in.) tablet. The tablet was partially secured inside a 3-mm. i.d. × 10.16-cm. (4-in.) section of polyethylene tubing (Fig. 1) by pressing the tubing down over about one-half of the tablet. The rat was held in the technician's left hand, and the mouth of the rat was held open by use of a 2.54 × 10.16 × 0.63-cm. (1 × 4 × 0.25-in.) section of wood with a 1.27-cm. (0.5-in.) hole drilled in the center. The hole was centered in the rat's mouth. The second technician slid the polyethylene tubing containing the tablet through the hole in the wooden guide and into the esophagus (similar to using an intubation cannula). A silver cannula was inserted into the tubing to push the tablet away and down the esophagus of the rat. The rat showed no sign of discomfort during the treatment. No sign of rupture or internal bleeding was seen. Necropsy of the rat immediately after dosing showed that the tablet reached the stomach intact.

Table I lists relative absorption data for three different dosage forms of the compound. The data show that the tablet was well absorbed in the rat. Despite the relatively low excretion of unchanged drug in the urine, the data demonstrate that the tablet dosage form can be successfully used in the rat.

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Unsaturated compounds have been added to most of the functional group classes. The amides have been increased from two to nine, and the number of sulfur compounds has been doubled. The number of properties tabulated has been increased by twelve, which does not include references to spectra.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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Journal of Pharmaceutical Sciences

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REVIEW ARTICLE

Organosilicon Entities as Prophylactic and Therapeutic Agents

LORRIN R. GARSON and LUCILLE K. KIRCHNER*

Keyphrases ☐ Organosilicon compounds—review of literature ☐ Silicon compounds, organic—properties, toxicology, potential prophylactic or therapeutic agents ☐ Prophylactic agents, potential—organosilicon compounds ☐ Toxicology—organosilicon compounds

CONTENTS

CHEMISTRY 1113
GENERAL BIOLOGICAL PROPERTIES 1114
Disease and Siliceous Materials 1114
Detoxification Patterns 1115
Naturally Occurring Organosilicon Compounds 1116
TOXICOLOGY 1116
Silicones 1116
Nitrogenous Organosilicon Compounds 1117
Silatranes 1118
Haloorganosilicon Compounds 1119
Alkoxysilanes 1120
Miscellaneous Compounds 1120
AGENTS ELICITING BIOLOGICAL ACTIVITY 1120
Compounds Affecting Metabolism 1120
Cardiovascular Agents 1121
Agents Affecting Nervous System 1122
Compounds Effective against Bacterial and Fungal Infections 1124
Prophylactics against UV and Ionizing Radiation 1125
Insecticides and Insect Repellents 1125
Compounds Affecting Plant Growth 1125

Within the past 5 years, the synthesis and biological evaluations of organosilicon agents have rapidly developed into a potentially explosive area of medicinal chemistry. It has repeatedly been demonstrated that silicon analogs of recognized medicinal agents exhibit biological activity. Even more significant is the activity elicited by several types of organosilicon entities that are not isosteric to biologically active organic compounds. Ample evidence is also now available to establish that organosilicon compounds, *per se*, are not toxic. While certain classes of these compounds produce toxic responses, indeed, a few are highly poisonous, they too are not merely silicon congeners of toxic organic compounds. The diverse employment of these compounds—from therapeutic agents to agricultural chemicals—evidences the importance of, and exceptional potential for, these materials.

CHEMISTRY

The substitution of silicon for carbon in a specific molecule represents more than the replacement of one atom for a larger one. Even though the chemistry of carbon and silicon has a certain degree of similarity, the differences are significant and of paramount importance. The present discussion of silicon chemistry is of necessity brief; the reader is referred to the excellent books by Sommer (1) and Eaborn (2) and the reviews of Müller and Rochow (3) and Haiduc (4) for a comprehensive treatment of this topic.

The utilization of silicon in congeners of prophylactic and therapeutic agents is limited, to some extent, by the instability of certain silicon bonds. For example, nitrogenous compounds containing an Si—N bond do not form salts like organic amines but undergo cleavage: $R_3SiNH_2 + 2HX \rightarrow R_3SiX + NH_4X$.

Silylamines also undergo hydrolysis or alcoholysis, affording the corresponding silanol or alkyloxysilane

and amine. Those entities possessing silicon-to-sulfur and silicon-to-pseudohalide bonds are also susceptible to hydrolysis. Organosilicon compounds with Si—X bonding act more like acid halides than alkyl halides; they readily react with water, affording silanols and mineral acid. Silanols themselves do not behave like alcohols: $R_3SiX + H_2O \rightarrow R_3SiOH + HX$; they are much more acidic and readily lose water to form disiloxanes: $R_3SiOH \rightarrow R_3Si—O—SiR_3 + H_2O$. Indeed, the most characteristic chemical property of organosilicon compounds is their tendency to form Si—O bonds. Silyl ethers are chemically more similar to carboxylic acid esters than ethers in that the former readily react with alcohol under mild acidic catalysis: $R_3SiOR + R'OH \overset{H^+}{\rightleftarrows} R_3SiOR' + ROH$.

Organosilicon compounds with an Si—H bond are easily oxidized to silanols by Tollen's reagent. The silanes, which are hydrocarbon isosteres, readily react with water under basic conditions to give the corresponding silanol and hydrogen; however, they are reasonably stable under acidic or neutral conditions. Silicon-to-carbon bonding is generally stable, but the Si—C bond of aromatic moieties is broken in the presence of aqueous acid: $R_3SiAr + H_2O \xrightarrow{H^+} R_3SiOH + ArH$.

Compounds in which silicon forms a double bond (sp^2 bonding) are apparently unknown, thus precluding the incorporation of silicon in an aromatic ring or the preparation of silaketones, *etc.* Silicon-to-silicon bonding, while known, is weak and readily broken.

The differences in physical and chemical properties between carbon and silicon can be rationalized from the characteristics of their respective valence shells. Although both elements participate in sp^3 bonding, silicon also possesses vacant *d*-orbitals. In addition, both the difference in electronegativity (2.5 for carbon and 1.8 for silicon on the Pauling scale) and covalent radius (0.77 Å for carbon and 1.17 Å for silicon) contribute to their respective bonding characteristics.

In silicon, the vacant *d*-orbitals can participate in ($p \rightarrow d$) π-bonding with Si—N, Si—O, and Si—X (X = F, Cl) bonds. For example, π-bonding would account for: (*a*) the relatively high acidity of silanols as compared to that of alcohols, (*b*) an Si—O—Si bond angle of 145°, (*c*) the low basicity of siloxanes compared to that of ethers, and (*d*) the difference in the measured and calculated bond length of the Si—O bond (5). Interaction between π-aromatic electrons and silicon *d*-orbitals is reflected in the Hammett substituent constants for the trimethylsilyl group ($\rho = -0.07$) as compared to the *tert*-butyl moiety ($\rho = -0.20$) (5). On the basis of electronegativity alone, one would expect trisilylamine to be a stronger Lewis base than triethylamine. In fact, the silylamine is a poor donor; the lone electron pair on nitrogen is apparently engaged in π-bonding with the *d*-orbitals of silicon (2). The bonding and structural characteristics of silicon were recently reviewed (6), and the utilization of its *d*-orbitals was discussed in depth (7).

Although certain structural limitations are imposed in the employment of silicon in medicinal agents, the distinctive bonding characteristics of this element would appear to permit the preparation of agents with truly unique chemical, physical, and biological properties.

GENERAL BIOLOGICAL PROPERTIES

Disease and Siliceous Materials—Frequently, the element silicon is automatically associated with the chronic lung disease silicosis, one of several pneumoconioses caused by dust inhalation. The fibrogenic pneumoconioses (caused by free silica, asbestos, some talcs, and diatomaceous earth) are characterized by widespread fibrosis and are clinically manifested by shortness of breath and a greatly increased susceptibility to tuberculosis. In the case of asbestosis, the incidence of tuberculosis, although high, is lower than that observed with silicosis; however, exposure to asbestos has been implicated as a major factor in the causation of carcinoma of the lung (diffuse mesothelioma) (8).

Pulmonary response to accumulations of siliceous material involves four basic processes: (*a*) necrosis of microphages which had taken up and subsequently liberated silicon, (*b*) continued production of fresh phagocytes to reingest the silica, (*c*) formation of collagen, and (*d*) hyalinization (9). Numerous theories have been proposed to account for the mechanism of silicotic fibrogenesis. The earliest, the "mechanical theory," suggested that silicosis is caused by a sharp particle irritation; this theory has been discarded since other sharp particles (silicon carbide) do not lead to the pneumoconiosis. Current textbooks of medicine (*e.g.*, 10) state that silica is a chemical rather than a mechanical irritant and that the observed tissue changes are caused by soluble silicic acid produced by silica reacting with body fluids, or by some other physical–chemical reaction.

Recently, Heppleston (9) suggested that silica appears to exert its fibrogenic effect by virtue of its surface properties, which are not necessarily connected with solubility or surface area; he suggested that silica acts by a "surface effect" on the macrophages. Stalder (11) postulated that the effect of silica can be explained by damage to biological membranes, the injury being the result of a surface interaction—mainly an absorption process—between the mineral and membrane lipids or lipoproteins. There is considerable evidence that an immunological response is associated with silicosis. As pointed out in a recent review by Fessenden and Fessenden (12), there is a high content of γ-globulin (up to 50%) in the hyaline substance of the silicotic nodule and an increase in blood serum levels of globulins and histamine in silicotic individuals.

Clearly, the precise nature of the chemical and/or physical reaction of silica implicated with silicosis is yet unknown. It is most important to recognize that although the inorganic substance silica is a causative factor in this malady, *there is no evidence to suggest that organosilicon compounds evoke this condition.*

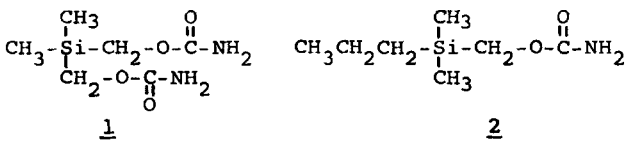
Current therapy for silicosis primarily consists of treatment of its concomitant conditions, namely, tuberculosis, emphysema, and other complications. At present, once fibrosis has developed in the lungs, there is no way to remove it. Within the past decade,

numerous therapeutic agents have been described; unfortunately, they do not exhibit a high degree of efficacy, nor are any widely accepted. These agents include: (*a*) inhalation of aluminum powder (10) and certain aluminum salts (13); (*b*) administration of trypsin as an inhaled aerosol (14); (*c*) steroid therapy with prednisolone (15, 16), cortisone (17), and other steroids (18); (*d*) subcutaneously administered $Na_2MgEDTA$ (19); and (*e*) inhalation (silicotic rats) of a poly(vinylpyridine *N*-oxide) aerosol (20–24). In the last instance, the antisilicotic action may be species and tissue specific (25). Ashbel *et al.* (26), in treating silicotic patients with an ascorbic acid aerosol[1], reported a normalization of capillary permeability and a distinct decrease in the intensity of infectious inflammation. Other investigators (28, 29), however, showed that the vitamin elicits a deleterious effect.

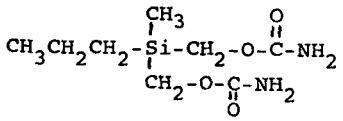
In addition to the pneumoconioses, it has been suggested that silicon compounds play a direct role in other pathological conditions (30, *cf.*, 31): "Numerous pathological processes, including cancer, atherosclerosis, tuberculosis, and diabetes, goiter, certain types of dermatitis, stone formations in the urethra, *etc.*, are associated with the breakdown of the metabolism of silicon compounds." These silicon-containing entities supposedly function by facilitating the urinary elimination of metabolites of foreign and toxic derivatives. Because of inadequate confirming evidence to the contrary, one cannot categorically dismiss the statement *in toto;* however, due to the absence of documentation and lack of independent verifying evidence, it is suggested that the generalizations are unwarranted.

Silicon is essential for reproduction in marine plankton and for nutrition in higher plants, although its precise function is little understood (32). The element is also present in mammals, including man, in different amounts in each organ (33). Silicic acid has been shown to stimulate *in vitro* collagen biosynthesis in chicken lung tissue (34), and Fregert (35) found relatively large amounts of silicon (presumably as organosilicon material) in keratinous tissue. Fregert suggested that these materials may be of biochemical significance to this specific tissue and that they may play a role in formation of the absorption barrier in skin and contribute to the solidity and chemical resistance of keratinous structures. Recently, in reporting the results of approximately 5000 quantitative, electron-probe microanalyses for calcium, phosphorus, and silicon in normal tibia of young mice and rats, Carlisle (36) showed silicon to be localized in active calcification sites; she implied that silicon may be linked to the initiation of mineralization of preosseus tissue.

Detoxification Patterns—When employing organosilicon compounds as medicinal agents, their fate in the treated subject is of major interest and importance. Not only is metabolism relevant to detoxification, but one might expect a difference in metabolic rates between silicon and carbon congeners (if not a completely different metabolic route) and thereby observe dramatic dissimilarities in physiological response. Fessenden and Ahlfors (37) recently published an account of the metabolic fate of three silicon-containing carbamates: bis(hydroxymethyl)dimethylsilane dicarbamate (*1*)[2], (hydroxymethyl)dimethyl-*n*-propylsilane carbamate (*2*), and bis(hydroxymethyl)methyl-*n*-propylsilane dicarbamate (silameprobamate, *3*). After

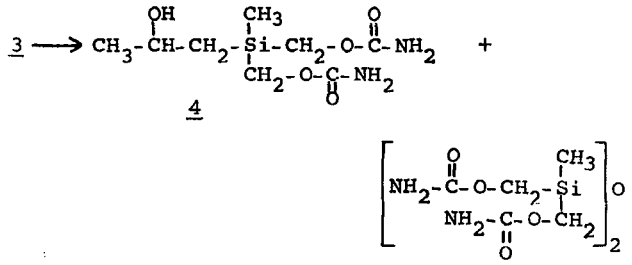


1 *2*



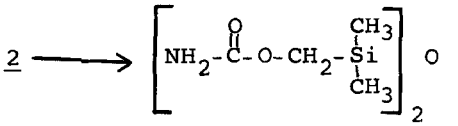
3

oral administration in rats, analysis of the urine showed that *1* was eliminated unaltered (53% recovery) whereas Compounds *2* and *3* afforded metabolic products. Oxidation of the propyl group in *3* produced its major metabolite, Compound *4;* a minor product, disiloxane *5*, was also isolated[3]. The formation of *4* parallels that for the metabolism of meprobamate itself. In



4 *5*

the case of *2*, no unchanged material could be detected in the urine; the disiloxane metabolite (*6*) was isolated and fully characterized. No other metabolic product of *2* was detected. The formation of disiloxanes *5* and *6* suggests that dealkylation may be a pathway in the



6

detoxification of organosilicon compounds.

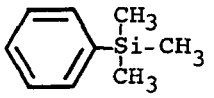
In another study, Fessenden and Hartman (38) described the urinary metabolic products of methyl-^{14}C-labeled phenyltrimethylsilane (*7*) and phenyldimethylsilane (*8*) upon oral (stomach tube) administration in rats. These compounds possess two key structural features common to many organosilicon entities,

[1] The specific medicament is galascorbin, a mixture of vitamin C and neutralized tannin in water (27).

[2] In the text, compound numbers appear in italic type. In the structures, these numbers are underscored.

[3] As pointed out by the investigators (37), Structures *4* and *5* must be considered tentative because they were unable to obtain the compounds analytically pure; the structure assignments were based on spectral evidence and qualitative tests.

the phenyl—silicon and the silicon—hydrogen bonds.



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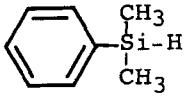

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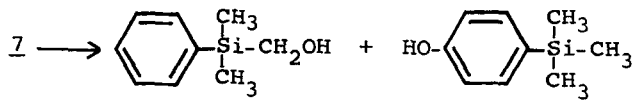
Compound *7* was oxidized to give three metabolites: (hydroxymethyl)dimethylphenylsilane (*9*), *p*-trimethylsilylphenol (*10*), and an unknown conjugate of *9;* the metabolites represent 31, 17, and 35% of the radioactive activity, respectively. No radioactive carbon dioxide from respiration was detected. In the metabolism of Compound *8*, approximately 90% of the



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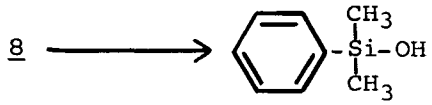
radioactivity was accounted for as phenyldimethylsilanol (*11*), which is unstable and readily loses water to give the corresponding disiloxane. From these data,



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it has been concluded that the Si—H bond is not stable *in vivo* and that this bond is the principal site for metabolic attack.

Naturally Occurring Organosilicon Compounds—The literature contains several reports on the existence of organosilicon compounds in animal tissue. Drechsel and Winogradow (39) described the isolation and partial characterization of a mixture of orthosilicate esters of cholesterol and cholesterol analogs, Isaacs (40) alluded to the presence of silicolipins in brain tissue, Holzapfel reported the existence of organosilicon compounds in silicotic lungs (41–43) and blood (44), Holt and Yates (45) quantitatively determined the silicon content of the ethanol-soluble portion of egg yolk, and Viehoever and Prusky (46) compiled an impressive amount of data from the older literature on the silicon content of various animal and plant tissues. However, in no instance has a pure organosilicon compound been isolated and fully characterized. An additional degree of uncertainty is introduced because most of the cited investigators employed ethanol for extraction, and ethyl silicate may presumably be formed during this procedure (45).

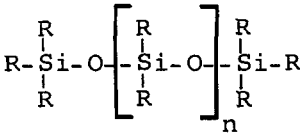
In a more recent investigation, Holt and Yates (47) determined the distribution of ^{31}Si injected in rats as soluble silica. They observed that the most radioactivity was present in the kidney and the least in muscle tissue. Part of the ^{31}Si could be extracted from the kidney with ethanol or dioxane; however, the greater portion could not be removed and, therefore, was probably present as inorganic silicate. These investigators suggested that the ethanol-soluble organosilicon materials may be micelles, consisting of hydrogen-bonded complexes between the hydroxyl functions of polymeric silicic acid and the hydroxyl groups of organic molecules such as cholesterol, or between other polar moieties in lecithin and choline.

TOXICOLOGY

Silicones—It is beyond the scope of the present communication to discuss in depth this important group of polymers. Nevertheless, their wide usage in medicine and pharmacy requires brief comment. The chemistry, technology, and medical use of these macromolecules have been exhaustively described elsewhere (48–50).

Although the term "silicone" is sometimes employed to encompass all organosilicon compounds with Si—C bonds, both monomeric and polymeric, the word is generally understood to refer only to organosilicon polymers containing Si—O—Si bonding. The preferred, more precise term for this class of polymers is polyorganosiloxane; however, because of the wide acceptance of the term silicone, it will be used interchangeably with polyorganosiloxane in the present discussion.

Being partially organic in constitution, the silicones exhibit structural and physical features of organic macromolecules; in addition, they also resemble the inorganic polymeric silicic acids and silicates. It is this dual nature, intermediate between inorganic and organic, that provides the silicones with their unique characteristics. A hypothetical silicone fluid is illustrated here. The representation illustrates only mono-



and difunctional structural units; branched subunits would be representative of a crosspolymerized silicone or silicone rubber.

The most striking biological feature of these substances is their general physiological inertness. Various studies in animals (*e.g.*, 51–56) and man (*e.g.*, 57–59) demonstrated a singular lack of pathogenic tissue response to systemically or orally administered silicones[4]. In one study, in which massive doses of dimethylpolysiloxane fluid were injected into rats (up to 540 ml. per animal), the only untoward effect reported was difficulty in locomotion (61).

The one exception to the biological inertness of silicones is eye irritation. In rabbits, a transitory conjunctival irritation develops within a few hours after contact with these polymers and usually completely disappears within 24 hr. (56). The human eye reacts to contact with a painful burning sensation (48), characterized by an erythema of the conjunctival membranes and frequently accompanied by edema

[4] This esoteric topic is not without its humorous facets. Helal (60), in rebuttal to a report citing an untoward effect allegedly caused by a silicone, quipped: "This statement, like the topless waitresses, is completely unsupported."

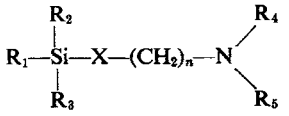


Table I—Mammalian Toxicity of Nitrogenous Organosilicon Compounds

Compound	R_1	R_2	R_3	X	n	R_4	R_5	LD_{50}[a], mg./kg.	Reference
12	C_2H_5O	C_2H_5O	CH_3	CH_2	3	H	H	0.045[b]	2
13	C_2H_5O	C_2H_5O	C_2H_5O	CH_2NH	6	H	H	1.5[c]	75
14	C_2H_5O	C_2H_5O	CH_3	CH_2NH	6	H	H	1.6[c]	75
15	C_2H_5O	C_2H_5O	CH_3	CH_2	0	H	C_2H_5	3.0[c]	75
16	C_2H_5O	C_2H_5O	C_2H_5O	CH_2	0	C_2H_5	C_2H_5	3.0[c,d]	75
17	C_2H_5O	C_2H_5O	C_2H_5O	CH_2	2	H	H	>4.0[c,e]	75
18	C_2H_5O	C_2H_5O	CH_3	CH_2	2	—$CH_2CH_2CH_2CH_2CH_2$—		6.6	30
19	C_2H_5O	C_2H_5O	CH_3	CH_2	2	H	H	40	30
20	C_2H_5	C_2H_5	CH_3	CH_2	2	H	H	47	30
21	C_2H_5	C_2H_5	C_2H_5	CH_2	2	—$CH_2CH_2CH_2CH_2CH_2$—		47	30
22	*n*-C_3H_7	*n*-C_3H_7	*n*-C_3H_7	CH_2	2	H	H	75	30
23	C_2H_5O	C_2H_5O	CH_3	CH_2	2	—CH_2CH_2—O—CH_2CH_2—		86	30
24	C_2H_5	C_2H_5	C_2H_5	CH_2	2	H	H	90	30
25[f]	C_2H_5	C_2H_5	C_2H_5	CH=CH	1	—$CH_2CH_2CH_2CH_2CH_2$—		110	30
26	C_6H_5	C_6H_5	C_2H_5	CH_2	2	H	H	120	30
27[f]	C_2H_5	C_2H_5	C_2H_5	CH_2	2	—$CH_2CH_2CH_2CH_2CH_2$—		120	30
28	C_6H_5	C_2H_5	C_2H_5	CH_2	2	H	H	140	30
29	CH_3	CH_3	CH_3	CH_2	2	H	H	155	30
30	C_2H_5	C_2H_5	C_2H_5	O	2	H	H	165	30
31	C_2H_5	C_2H_5	C_2H_5	CH_2	2	H	—[g]	185	30
32[f]	C_2H_5	C_2H_5	C_2H_5	C≡C	1	—$CH_2CH_2CH_2CH_2CH_2$—		195	30
33	C_2H_5O	C_2H_5O	C_2H_5	CH_2	2	H	H	215	30
34	C_2H_5O	C_2H_5O	C_2H_5O	CH_2	2	H	H	260	30
35	C_2H_5	C_2H_5	C_2H_5	O	2	—$CH_2CH_2CH_2CH_2CH_2$—		280	30
36	CH_3	CH_3	CH_3	O	2	H	H	320	30
37	C_2H_5	C_2H_5	C_2H_5	O	2	H	*n*-C_4H_9	360	30
38	CH_3	CH_3	CH_3	O	2	—$CH_2CH_2CH_2CH_2CH_2$—		400	30
39	C_2H_5	C_2H_5	C_2H_5	O	2	C_2H_5	C_2H_5	400	30
40	C_2H_5O	C_2H_5O	CH_3	CH_2	2	—$CH_2CH_2N(CH_3)CH_2CH_2$—		400	30
41	C_2H_5O	C_2H_5O	CH_3	CH_2	2	—$CH_2CH_2SCH_2CH_2$—		470	30
42	CH_3	CH_3	CH_3	O	2	C_2H_5	C_2H_5	500[h]	30
43	CH_3	CH_3	CH_3	O	2	CH_3	CH_3	620	30
44	CH_3	CH_3	CH_3	O	2	*n*-C_4H_9	*n*-C_4H_9	640	30
45	C_2H_5	C_2H_5	C_2H_5	O	2	CH_3	CH_3	670	30
46	C_2H_5	C_2H_5	C_2H_5	O	2	*n*-C_4H_9	*n*-C_4H_9	1650	30

[a] Tests by intraperitoneal injection of mice, rats, or rabbits. [b] Skin absorption (ml./kg.). [c] Atmospheric concentration (mg./l.). [d] LD_{100}, 10 mg./kg. (administered in a single dose *via* stomach tube). [e] Maximum allowable concentration 0.001 mg./l. (165). [f] Administered as the hydrochloride salt. [g] 2-Furanylmethyl. [h] A value of 630 is also reported (30).

of the lids. Interestingly, however, dimethylpolysiloxane fluids have been injected into the eye of animals and man as a replacement for aqueous and vitreous humor. The fluid is apparently well tolerated, with no tissue or inflammatory response. The varied use of liquid silicones in medicine and pharmacy is due largely to their low surface tension and antisurfactant activity. Selected silicones have been used with success as antiflatulents in the treatment of meteorism (62, 63) and for foam modulation in extracorporeal devices such as bubble oxygenators (64); others have been employed as antifoaming agents to control pulmonary edema (65–68). Because of their water-repellent characteristics, these polymers have been: (*a*) incorporated into ointments and pastes for skin protection; (*b*) proposed as additives for nail polishes, lipsticks, and antisunburn preparations; and (*c*) included in dentifrices (48). The silicone oils (69) and colloidal silicates (70) have also been employed in burn treatment. The use of silicone fluids in soft tissue augmentation (57) and the importance of resinous silicones to dentistry and surgery were recently discussed in depth (48).

Dimethylpolysiloxane and phenylmethylpolysiloxane were evaluated as potential agents for the treatment of atherosclerosis in rabbits (71–73). While the former may possibly exert a minor antihypercholesterolemic effect, the aromatic polymer definitely results in lowered blood cholesterol levels in animals fed high cholesterol diets.

Nitrogenous Organosilicon Compounds—This class of compounds, whose chemistry was recently reviewed (74), comprises the largest single group of entities for which toxicological data are available (Tables I–III).

Inspection of Table I reveals specific trends within the aminosilane series: (*a*) compounds containing alkoxy substituents on the silicon atom (R_1, R_2, and R_3) are more toxic than those with corresponding alkyl substitution (*cf.*, Compounds *18* with *21* and *19* with *20*); (*b*) compounds containing an oxygen atom between the silicon and amino moieties, where X equals O, are noticeably less toxic than the corresponding carbon analogs in which X equals C (*cf.*, Compounds *24* with *30*, *21* with *35*, and *29* with *36*); and (*c*) in comparing those entities where X is oxygen, the molecules containing a primary amino function are more toxic than those possessing a tertiary amino group. Voronkov and Lukevics (30) pointed out that the hydrochloride salts of the γ-aminopropylsilanes (*e.g.*, Compounds *17–29*) are less toxic than their corresponding free bases. Furthermore, in the instance of Compounds *85*, *86*, and *87*, it is interesting

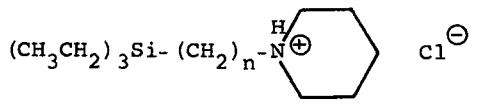
Table II—Mammalian Toxicity of Nitrogenous Organosilicon Compounds[a]

$(R_1)_{4-n}$—Si(O—CH_2CH_2—$NR_2R_2)_n$

Compound	R_1	R_2	n	LD_{50}, mg./kg.
47	C_2H_5	H	2	125
48	—	C_2H_5	4	230
49	CH_3	H	3	400
50	C_2H_5	C_2H_5	3	490
51	C_2H_5	H	3	500
52	C_6H_5	H	3	500
53	C_6H_5	C_2H_5	3	560
54	CH_3	H	2	570
55	CH_3	C_2H_5	3	600
56	C_2H_5	C_2H_5	2	600
57	CH_3	C_2H_5	2	720
58	C_2H_5	*n*-C_4H_9	2	2400
59	$O[Si(CH_3)_2CH_2CH_2CH_2NH_2]_2$			30
60	$C_6H_4[Si(CH_3)_2CH_2CH_2CH_2NH_2]_2$			74
61	$[(CH_3)_3SiOCH_2CH_2]_2NH$			1250
62	$[(C_2H_5)_3SiOCH_2CH_2]_2NH$			1850
63	$[(CH_3)_3SiOCH_2CH_2]_3N$			3500
64	$[(C_2H_5)_3SiOCH_2CH_2]_3N$			4000
65	$(CH_3)_3Si$—O—$Si(CH_3)_3$			4500

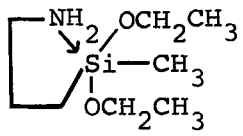
[a] Tests by intraperitoneal injection in mice, rats, and rabbits (30).

to note a decrease in toxicity (LD_{50} 60, 88, and 120 mg./kg., respectively) as the amino group is further removed from the silicon atom (30).



85, n=1
86, n=2
87, n=3

Compound *12* also elicits an extremely toxic response upon dermal application and was reported to cause serious injury in contact with the eye (2). Voronkov (76) suggested that the high physiological activity of *12* may be ascribed to intramolecular coordination of the γ-amino group with the silicon atom; this is

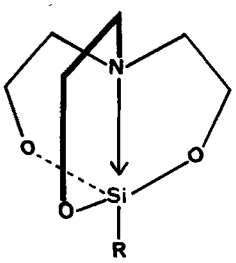


12

consistent with the observation that several compounds (*17–24*) containing an amino group gamma to the silicon atom are relatively toxic. Nevertheless, the exceedingly high toxicity of *12* remains anomalous and cannot be totally rationalized in terms of chemical structure.

Nitrogenous silanes containing more than one amino function appear to be relatively nontoxic (Table II). It is not immediately apparent why the introduction of a second amino group should have this effect.

Silatranes—This group of caged pentacovalent organosilicon compounds, with 1-alkyl- and 1-aryl-substituents, has been observed to elicit striking biological effects. In these compounds, which were studied by spectral and dipole moment techniques (76), silicon does not participate in sp^3 bonding but in sp^3d coordination with a tetrahedral nitrogen atom as shown here:



The 1-substituted silatranes can be readily prepared by several methods which were described in detail by Voronkov (76) and Zelchan (77). A number of 1-alkyloxy- and 1-acyloxysilatranes, which undoubtedly have similar bonding characteristics, have also been synthesized (77); while the former are gradually hydrolyzed in the atmosphere, the latter are extremely unstable and very difficult to purify. Neither has apparently been tested for physiological activity.

The most prominent physiological feature of the silatranes is the remarkable mammalian toxicity exhibited by some of the 1-aryl derivatives (Table III). 1-*p*-Tolyl- and 1-phenylsilatrane (Compounds *66* and *67*, respectively) are even more toxic than strychnine [LD_{100} (mice) 0.5–1.25 mg./kg.] or hydrocyanic acid [LD_{100} (mice) 3–10 mg./kg.] (78). When sublethal doses (0.20–0.25 mg./kg.) of *67* are intraperitoneally administered to white mice, the agent elicits a strong analeptic action as evidenced by motor excitation and accelerated breathing; at a slightly higher dose (0.35 mg./kg.), alternating clonic and tonic convulsions occur (79). The effect of *67* upon unanesthetized cats is similar to that with mice; however, when the compound is intravenously administered in a 10-fold lethal dose to cats anesthetized with ethyl carbamate or chloralose, the only effect is convulsive contraction of the skeletal muscles followed by intensive breathing. The same effect is observed upon anesthetized and unanesthetized rabbits.

Table III—Toxicity of Silatranes[a]

$RSi(OCH_2CH_2)_3N$

Compound	R	LD_{50}, mg./kg.
66	*p*-Tolyl	0.20
67	Phenyl	0.43
68	*m*-Chlorophenyl	4.4
69	Phenyl[b]	8.1
70	3,5-Dimethylphenyl	14.7
71	Hydro	100
72	Cyclohexyl	150
73	Phenoxy	200
74	*p*-Nitrophenoxy	700
75	*p*-Chlorophenoxy	1050
76	Benzyl	1115
77	*p*-Tolyloxy	1270
78	Methoxy	2100
79	5-Methyl-2-isopropylphenoxy	4000
80	Ethoxy	5000
81	Methyl	5000
82	Ethyl	5000
83	Isopropyl	5000
84	Allyl	5000

[a] Tests by peritoneal injection in white mice (30). [b] Compound *69* contains a 2-carbo substituent in the parent silatrane moiety.

Table IV—Mammalian Toxicity of Haloorganosilicon Compounds[a]

Compound	Structure	LD_{100}[b], g./kg.	LD_{100}[c], g./kg.	LD_{50}[d], mg./l.	Maximum Tolerance Dose, mg./l.	Maximum Allowable Concentration, mg./l.	Lethal[d] Concentration	Reference
88	$(CH_3)_3SiCl$	—	—	4	—	—	—	31
89	$(C_2H_5O)_2Si(H)Cl$	6.30[e]	—	—	—	—	—	85
90	$ClCH_2Si(CH_3)(H)Cl$	—	—	—	—	0.001	—	86
91	$ClCH_2Si(CH_3)_2Cl$	—	—	—	—	0.001	—	86
92	$(CH_3)_2SiCl_2$	1.0	0.10	—	—	—	—	56
—		—	—	—	—	0.02	0.44	87
—		—	—	10	—	—	—	31
93	$(C_2H_5)_2SiCl_2$	1.0	0.10	—	—	—	—	56
94	$C_6H_5Si(CH_3)Cl_2$	0.1–0.25 mg./l.[f]	—	—	—	—	—	88
—		—	0.25 mg./l.	0.15	—	0.08 (1.0)	0.01	89 (90)
95	$C_2H_5Si(C_6H_5)Cl_2$	—	—	—	—	0.005	—	91
96	*x*—$FC_6H_4Si(CH_3)Cl_2$	—	—	—	—	0.001	—	92
97	*x*—$ClC_6H_4Si(CH_3)Cl_2$	—	—	—	—	0.005	—	93
98	$(C_3H_4F_3)CH_3SiCl_2$	—	—	—	—	—	8.0	94
99	$SiHCl_3$	—	—	—	—	0.001	—	95
—		1.03[e]	—	—	—	—	—	85
100	CH_3SiCl_3	1.0	0.30	—	—	—	—	56
—		—	—	10	—	—	—	31
—		—	0.12–0.14[f]	—	0.08–0.1	0.02	—	87
101	$C_6H_5SiCl_3$	—	0.12–0.14[f]	—	0.08–0.1	0.05	0.60	87
—		2.39	—	—	—	—	—	96
102	$ClCH_2SiCl_3$	—	0.15–0.2 mg./l.	0.06	—	0.025 (1.0)	0.04	89 (90)
—		—	—	—	—	0.001	—	97
103	$C_2H_5SiCl_3$	1.0	0.03	—	—	—	—	56
—		—	0.08	—	0.05	—	0.44	87
104	*x,y*—$Cl_2C_6H_3SiCl_3$	—	—	—	—	0.001	0.08–0.1	98
—		—	0.1[f]	0.04	—	0.02	0.03	89
105	$C_2H_3SiCl_3$	1.28	—	—	—	—	—	96
106	$SiCl_4$	—	0.1[f]	5.0	—	—	—	31

[a] Tests performed on mice, rats, or rabbits. [b] Administered in a single dose *via* stomach tube. [c] Injected intraperitoneally. [d] Atmospheric concentration. [e] LD_{50}. [f] Administration route not specified.

Frogs are exceptionally resistant to 1-phenylsilatrane; doses of 30–40 mg./kg. (higher doses not being practical due to the compound's low water solubility) have no effect. Compound *67* does not markedly change the reaction of isolated m. rectus abdominis muscle (frog) to acetylcholine, nor does it cause contraction of perfused frog's hind legs. Thus, a direct influence on the peripheral neuromuscular mechanism is excluded (76). Accelerated breathing and convulsions are observed at low doses in decerebrated cats, and spasmodic twitching also is observed in spinally anesthetized cats; cats with "destroyed nervous systems" do not react to intravenous injections. Voronkov and Lukevics (30, 76) thus concluded that 1-phenylsilatrane acts upon the CNS and that its effect upon the spinal column is of great importance. They further suggest that the compound acts on an enzyme system, although it is not a cholinesterase inhibitor (76). The toxicity of 1-phenylsilatrane is highly species dependent, being extremely toxic to mammals and essentially nontoxic to frogs, bacteria, and fungi (75, 80).

Examination of the effect of 1-substitution upon silatrane toxicity (Table III) reveals that *only* the 1-arylsilatranes exhibit high toxicity; the 1-aryloxy-derivatives, 1-benzylsilatrane, and silatrane itself are only moderately toxic, and 1-alkyl- and 1-alkyloxysilanes are remarkably inert. It is known that silatranes undergo hydrolysis in aqueous solution and exhibit a first-order reaction (76, 77, 81); consequently, some physiological activity may be due to hydrolysis product(s). Because 1-phenylsilatrane is highly toxic and poorly soluble (82), it is suggested that distribution coefficients may be of major importance to toxicity with these compounds.

Haloorganosilicon Compounds—The chlorosilanes, important intermediates in the commercial production of silicones, must be considered a highly toxic class of compounds (Table IV). Materials such as tetrachlorosilane or tetrabromosilane and the hydrosilanes in general are reported to exhibit severe acute toxicity upon ingestion, inhalation, or application to the skin (83). Silicon tetrafluoride, a colorless toxic gas which evolves hydrogen fluoride in the presence of moisture, is known to cause acute fluoride poisoning by inhalation (84). Rowe *et al.* (56) cautioned that particular care must be exercised with these compounds, because even one small drop splashed into the eye could cause its loss.

Severe skin burns occur if halosilanes come into contact with dermal tissue; however, death is not likely unless a large portion of the body is involved. Voluntary inhalation of lethal concentrations of chlorosilanes is prevented by the marked irritation caused by these agents. Both alkyl- and arylchlorosilanes most often affect the upper respiratory tract; aryl compounds (such as the phenylchlorosilanes) cause infections of the mucous lining of the mouth. It was reported that people working with these agents can develop chronic occupational intoxication, characterized by chronic laryngotracheitis, and rhinitis accompanied by atrophic processes in the mucous lining of the nasopharynx (99). As an additional hazard, the volatile chlorosilanes are flammable; those entities containing a SiH moiety liberate hydrogen upon treatment with a protic solvent (100). The aryl-

Table V—Mammalian Toxicity of Alkyloxysilanes

Compound	Name	LD_{50}, g./kg.[a]	LD_{100}, g./kg.[b]		Maximum Allowable Concentration[c]
107	Ethoxytrimethylsilane	—	0.1	2.0	—
108	Diethoxydimethylsilane	—	0.4	10.0	—
109	Triethoxymethylsilane	—	0.4	10.0	—
110	Tetraethoxysilane	—	15	5.0	0.02
111	Triethoxysilane	—	—	—	0.001
112	Vinyltriethoxysilane	22.5	—	—	—
113	Amyltriethoxysilane	19.6	—	—	—
114	Tris(2-chloroethoxy)silane	0.19	—	—	—
115	Ethyltriethoxysilane	13.72[d]	—	—	—

[a] Oral administration in rats (96). [b] Oral administration in rats; the data in the left column are from Voronkov (31), and those on the right are from Rowe *et al.* (56). [c] Atmospheric concentration (milligrams per liter) according to Kulagina and Kochetkova (107). [d] Smythe *et al.* (85).

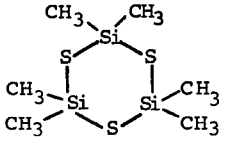
chlorosilanes are generally more toxic than the alkyl derivatives, although the former are less volatile (101). The presence of chloro or amino substituents on the alkyl or aryl group intensifies the toxicity, whereas the introduction of a cyano substituent decreases this effect (102).

It has generally been accepted that the toxicity of the chlorosilanes is due to the hydrochloric acid produced upon hydrolysis (12, 100, 102). Golubev (103), in investigating the inhalation toxicity of trifluoropropylmethyldichlorosilane, found that not only was the clinical symptomatology in mice the same for the halosilane and its hydrolysis product hydrochloric acid, but also that their lethal concentrations (LD_{50} 8.12 and 2.51 mg./l., respectively) were in good agreement for the calculated amount of acid released by hydrolysis. However, Voronkov (31) pointed out that dimethyldichlorosilane (*92*) and methyltrichlorosilane (*100*) are less toxic than trimethylchlorosilane (*88*). Not only do *92* and *100* produce more acid upon hydrolysis, but the two compounds hydrolyze more rapidly than *88*. This would suggest that the *in toto* chlorosilanes may be responsible for some of the toxic effects.

All of the values of maximum allowable atmospheric concentration (Table IV) were published in the Soviet literature; to the authors' knowledge, standards for these specific agents have not been established in the United States.

Alkoxysilanes—Very little data concerning the toxicological properties of the alkyloxysilanes are available. Tetraethoxysilane (*110*) was reported to be highly toxic, its vapor being irritating to the respiratory tract and affecting CNS function (104). Animals exposed to *110* expire with grave damage to lung, liver, and kidney tissue. It has been recognized for some time that the industrially important ethyl silicates, a mixture of *110* and various ethyl polysilicates, may cause serious damage to the lungs and kidneys (105). Indeed, the toxicological properties of ethyl silicates have long been recognized (85, 106). It can be seen from Table V that toxicity decreases with an increase in the number of alkoxy substituents.

Miscellaneous Compounds—Moody (108) described the synthesis and properties of hexamethylcyclotrisilthiane (*116*) and tetraphenylcyclodisilthiane (*117*). Both of these unique organosilicon heterocyclics readily produce hydrogen sulfide in contact with water and other protic substances.



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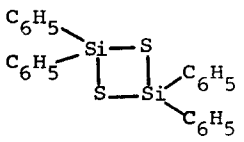
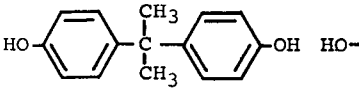


117

An interesting instance of allergy to an organosilicon compound was reported by Fregert and Rorsman (109). Four individuals, hypersensitive to 2,2-bis(4-hydroxyphenyl)propane (*118*), exhibited the identical response in a patch test with dimethyldi-(4-hydroxyphenyl)silane (*119*) as with *118*. None of the subjects had previously been exposed to Compound *119*.



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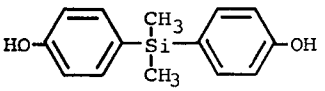
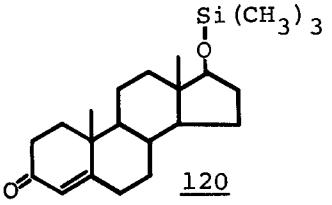


119

AGENTS ELICITING BIOLOGICAL ACTIVITY

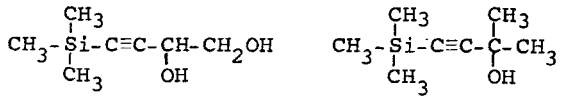
Compounds Affecting Metabolism—Chang and Jain (110) recently described six testosteroxysilanes and their respective androgenic and myotropic activities upon subcutaneous injection in rats. Within the series, only testosteroxytrimethylsilane (*120*) exhibited greater activity than testosterone itself. Observing that *120* was the most susceptible to hydrolysis (88% conversion to testosterone when incubated in water at 37° for 24 hr.) and least polar, as demonstrated by paper chromatography, it was postulated that its elevated activity was the result of rapid transport across the lipid membrane barrier and facile cleavage to the parent steroid. Brown and Laos (111), in de-



120

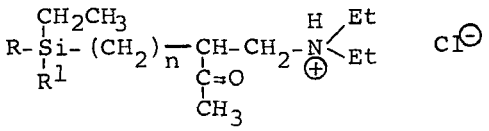
scribing another group of 17-trialkylsilyloxy steroids, also discussed the elevated androgenic activity of *120*. However, no comment was made regarding its mechanism of action, nor were any specific biological data reported. The fate of the trimethylsilyl residue from these compounds has apparently not been investigated; one may speculate on the formation of the corresponding innocuous disiloxane (*65*). The elaboration of other organosilicon entities and their potential toxicity must obviously not be discounted.

Cardiovascular Agents—Numerous silicon-containing compounds have been reported which lower blood pressure. For example, Ignat'eva and Kuznetsov (112) observed that 4-trimethylsilyl-3-butyne-1,2-diol (*121*) and 2-methyl-4-trimethylsilyl-3-butyne-2-ol (*122*)



121 122

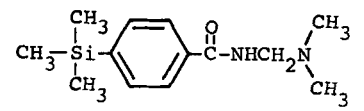
effected a decrease in the blood pressure of rats upon intravenous or intraabdominal injection; in greater doses, the compounds sporadically caused a rise in blood pressure. The magnitude of blood pressure change was unfortunately not reported. Toxic effects (spasms and miosis) were observed for *121* at doses up to 10 mg./kg.; at higher doses, general spasms and death occurred. In the case of *122*, subcutaneous administration caused a rise in respiration, and higher doses resulted in death (cessation of breathing). Shostakovskii *et al.* (113) reported that silyl ketoamines (*123–125*) reduce blood pressure, stimulate breathing, and elicit a spasmolytic effect; however, specific data were not given.



Compd.	R	R^1	n
123	C_2H_5	C_2H_5	1
124	C_2H_5	CH_3	2
125	CH_3	CH_3	2

Belsky *et al.* (114) reported that several amides of trimethylsilyl-substituted aromatic acids cause a lowering of blood pressure in anesthetized cats upon intravenous injection; a concomitant increase in respiration without a change in heart rate was also observed. The most efficacious of these agents (*126*) elicited the following effects [dose (mg./kg.), decrease in blood pressure (mm.), duration of decrease (min.), respiration rate increase (% of control)]: 1, 30, 4, 22; 2, 40, 4, 35; 4, 70, 20, 83; and 6, 100, 60, 81. In another study (115), these investigators showed that under the same conditions, at a dose of 10 mg./kg., *n*-(*p*-trimethylsilyl-*β*-phenethyl)morpholine hydrochloride (*127*) lowers arterial blood pressure 120 mm. for 40 min.

Frankel *et al.* (116) prepared and pharmacologically evaluated a group of *p*-trimethylsilyl- and *p*-trimethylsilylmethylphenethylamines. Those agents having the silyl substituents in the *para*-position elicit a blood pressure-lowering response, whereas the *ortho*- and *meta*-derivatives exhibit a hyperpressor effect. In comparing *o*-trimethylsilylphenethylamine hydrochloride and its *meta*-isomer, the pressor activity of the former was found to be greater. Testing these agents in anesthetized cats, the heart rate was not affected while respiration rate increased with the *para*-substituted compounds and decreased with the corresponding *ortho*- and *meta*-isomers. Among the compounds exhibiting depressor action, *p*-triethylsilylphenethylamine hydrochloride exhibited the greatest activity on both lowering blood pressure and duration (dose 5 mg./kg., −130 mm. for 150 min.). Neither introduction of a methyl group in the carbon atom of the aliphatic side chain nor addition of a methylene substituent between the silyl moiety and the benzene ring caused a significant change in blood pressure.



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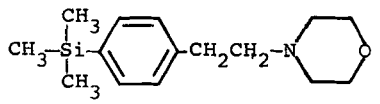

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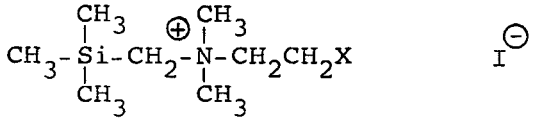
A series of trialkylsilyl derivatives of choline has been found to exhibit a hypotensive effect (*30*). Compounds *128* and *129* were particularly efficacious,



128, X=$OCOCH_3$
129, X=I

causing a decrease of 70–90 and 50–60 mm. Hg, respectively, at doses of 1 mg./kg. A number of the *O*-silyl derivatives caused an increase in blood pressure of short duration prior to the hypotensive action.

Loeper and his colleagues (*117–122*)[5] presented convincing evidence that silicon, or the lack of this element, probably plays an important role in atherosclerosis. In contrast to the earlier finding of Kvorning (123), these investigators showed that the silicon content of the aorta in normal man decreases with age. Expressing the aortic silicon content in mcg./100 mg. of tissue nitrogen, the following data were presented:

Age, Year	Silicon Content
Infants	205 ± 44
10–20	160 ± 43
20–30	125 ± 30
40	86 ± 16

[5] *References 117* and *118*, except for a slight modification in title and the introductory sentence, *are identical*, although published in two different journals; the reference with the most recent publication date (117) has the caption: "article original."

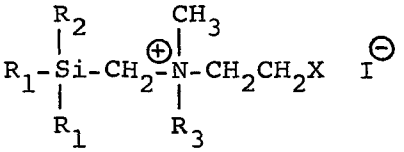


Table VI—Mammalian Toxicity of Organosilicon Derivatives of Choline[a]

Compound	R_1	R_2	R_3	X	ED_{50}[b], mg./kg.	EC_{50}[c], mg./kg.	LD_{50}[d], mg./kg.
142	C_2H_5	CH_3	CH_3	Cl	0.35	3.2×10^{-6}	71
143	CH_3	CH_3	CH_3	Cl	0.50	9.1×10^{-5}	124
144	CH_3	CH_3	CH_3	OH	0.56	8.0×10^{-4}	266
145	CH_3	CH_3	CH_3	CH_3COO	0.62	2.4×10^{-4}	328
146	CH_3	CH_3	CH_3	I	0.69	2.5×10^{-5}	40
147	C_2H_5	C_2H_5	CH_3	OH	0.85	4.9×10^{-6}	53
148	CH_3	CH_3	CH_3	C_6H_5COO	1.26	2.3×10^{-5}	465
149[e]	CH_3	CH_3	H	Cl	—	1.3×10^{-5}	137
150[f]	—	—	—	—	—	4.5×10^{-4}	238

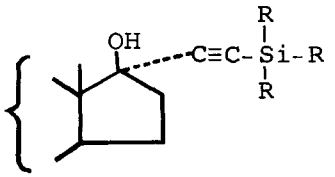
[a] Voronkov and Lukevics (30). [b] Ganglionic blocking in cats. [c] Cholinolytic action in frog muscle. [d] Intraperitoneal injection in white mice. [e] With chloride anion in place of iodide. [f] $[(CH_3)_3SiOCH_2CH_2N(CH_3)]I$.

Loeper and Polet (120) showed that normal human aorta and carotid arteries contain approximately 10 times more silicon than atherosclerotic arteries. In a more refined study (121), in which the sclerotic lesions were classified according to their severity, a much more striking correlation between silicon content of the aorta and disease condition was noted: (*a*) in those arteries free of atheromatous deposits, the value for silicon was found to be 180 ± 21 (mcg. silicon/100 mg. tissue nitrogen); (*b*) in arteries moderately altered with light subendothelial lipid deposits, discrete rarefaction of elastic fibers, and metachromasia of the ground substance, a silicon value of 105 ± 12 was obtained; and (*c*) in the instance of lesions where the arteries were greatly altered with lipid deposits uniting the media and intima, rarefaction and displacement of elastic fibers occurred, and calcium excess and augmentation of the ground substance were observed, the value for silicon was found to be 63 ± 8. The findings were experimentally verified in rabbits fed on high cholesterol diet. Here, again, the course of atheroma was marked by a significant decrease in aortic silicon level, the initial decrease being quite early in the disease process.

It has been suggested that silicon elicits its antisclerotic action by decreasing the permeability to lipid infiltration (121). More recently (118), evidence has been presented associating protection and development of elastic fibrils of the arterial wall with fibrillogenesis stimulation. Holt and Osborne (124) found the permeability of rat subcutaneous tissue membrane to be reduced after treatment with silicic acid, and Loeper *et al.* (121) showed that diffusion of a dye across rabbit skin is inhibited by small amounts of sodium silicate.

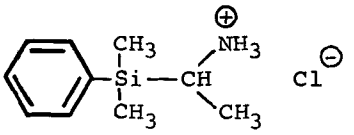
Rager (125, 126) was able to prevent the development of atherosclerosis in rabbits, fed an atherogenic diet, by intravenously administering "sodium monomethyltrisilanolorthoxybenzoate"—a complex of methylsilanetriol and sodium salicylate in an aqueous solution (127). The complex was also used with ionokinetic treatments (repeated daily for 16–20 days) in patients with coronary insufficiency and muscular ischemia. Although these individuals had previously been refractory to more conventional treatment, 75% of those with coronary insufficiency and 35% of those with ischemia were reported to have rapid improvement. Gendre recently described the effect of the complex upon the ultrastructure of rabbit arterial wall (128) and its action on lipid plaque deposits (129). Recently, Loeper and Loeper (118) investigated the prophylactic effects of sodium silicate, lysyl silicate, and the salicylate and citrate of methylsilanetriol upon experimental atheroma in rabbits. All of the compounds decreased the macroscopic atheromatous lesions, the incidence of plaques being 80% for the controls and 28% for the treated animals. The organosilicon entities were reported to be the most active.

Brown *et al.* (130) described a number of acetylenic silyl steroids with the D-ring substituted as shown here:



These compounds, in which R is methyl, ethyl, propyl, or phenyl, and ring A reflects the incorporation of several steroidal entities, are reported to elicit estrogenic, progestational, and hypocholesterolemic activity as well as counteracting the development of exogenously reduced hypercholesterolemia.

Agents Affecting Nervous System—The compound (α-aminoethyl)dimethylphenylsilane hydrochloride (*130*) and its carbon isostere have been compared in



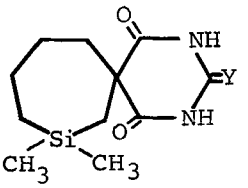
130

terms of their physiological activities (131). Their toxicities (LD_{50} 105–113 and 102–107 mg./kg., respectively) in white mice are nearly equivalent, and both compounds cause an increase in the rate and depth of respiration. ECG and EEG studies in rats anesthe-

tized with sodium pentobarbital revealed no analeptic activity at toxic doses; mice given sublethal doses exhibited intense excitement. No gross difference in pharmacological activity was observed between Compound *130* and its carbon analog. Several trimethylsilylphenethylamines were reported (132), but their biological activities were not described.

Compound *121* (4-trimethylsilyl-3-butyne-1,2-diol) was reported to induce spasms and miosis in white rats at doses up to 10 mg./kg.; higher doses caused general spasms and death (112). Animal tests of silyl ketoamines *123–125* demonstrated their spasmolytic effects (113).

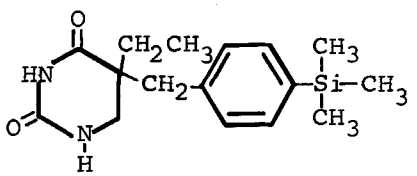
In the pharmacological evaluation of a group of silabarbiturates, Compound *131* gave the most rapid



Compd.	Y
131	O
132	S

onset of action and possessed the highest therapeutic ratio, whereas Compound *132* elicited a belated onset of action and the highest toxicity (133). Although these compounds did not give high therapeutic ratios, their level of biological activity is promising.

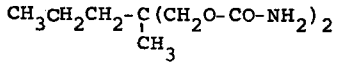
Belsky *et al.* (114, 115, 134) synthesized and evaluated numerous compounds which exhibit anticonvulsive activity. Upon oral administration of *p*-trimethylsilylphenylacetylurea to fasted male mice, the agent was more effective in protection against supramaximal electroshock than its carbon analog, phenylacetylurea (114); the compound was also the only member of a group of amides of silicon-containing aromatic carboxylic acids that exhibited an anticonvulsive effect against maximal pentylenetetrazole (115). The same investigators reported 5-(*p*-trimethylsilylbenzyl)-5-acetamidobarbituric acid (*133*) to show anticonvulsive



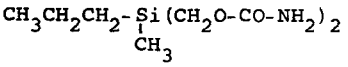
133

activity in male mice against pentylenetetrazole (134). The activity of Compound *133* was similar to, but lower than, that observed for phenobarbital. The preparation of several barbiturates, 2-thiobarbiturates and 2-aminobarbiturates substituted in the 5-position with one or two *p*-trimethylsilylbenzyl moieties, was reported (135); however, no data regarding their biological properties were presented.

An investigation of 24 alkanediol carbamates, monocarbamates, and their silicon isosteres revealed essentially equivalent muscle-relaxant activity, as measured in the rotating rod test (136). In intraperitoneal administration, meprobamate and silameprobamate (*134*) differed only in duration of activity (meprobamate acting four times longer than *134*) and not in their

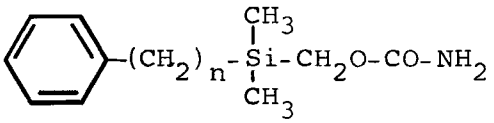


Meprobamate



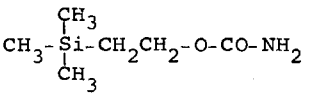
134

effective dose. However, upon oral administration, silameprobamate exhibited essentially no activity. Fessenden and Coon (137) reported that phenyl-substituted silacarbamates, Compounds *135–137*, exhibited muscle-relaxant properties with activity of short duration (10–20 min.). Evaluation of 2-trimethylsilyl-1-ethanol carbamate (*138*) and its carbon analog *139*



135, n=0
136, n=1
137, n=2

in the guinea pig ileum assay revealed that both compounds lack muscarinic activity; however, they were



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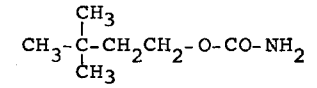
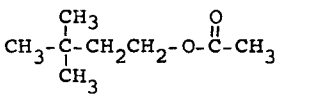


139

antagonistic to the muscarinic activity of both acetylcholine and carbachol (138). From these data, it has been inferred that although the compounds do not possess muscarinic activity, they do have an affinity for the muscarinic site.

Henderson *et al.* (139) studied the cholinergic effect of acetyl "carbocholine" (*140*) and its silicon isostere, acetyl "silicocholine" (*141*). These investigators concluded that both *140* and *141* should be considered as indirectly acting cholinergic (nonnicotinelike)



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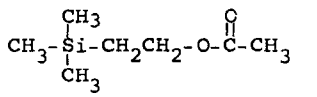


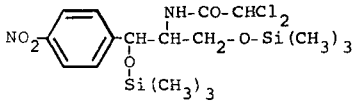
141

compounds, with their action being based on the stimulation of the release of acetylcholine from the presynaptic nerve terminals.

In studies employing frog stomach muscle, organosilicon derivatives of choline, acetylcholine, "chlorocholine," and "iodocholine" were shown to have a cholinolytic action (140). Voronkov and Lukevics (30) reported that other organosilicon derivatives of choline

(*142–150*, Table VI) exhibit cholinolytic activity. *N*-Trimethylsilylacetamide was claimed to exhibit hypnotic and anticonvulsant properties, but no specific pharmacological data were presented (141).

Compounds Effective against Bacterial and Fungal Infections—Improved properties over chloramphenicol have been claimed for its bis- and mono(trimethylsilyl) ethers (*151* and *152*, respectively) (142). The ethers are reported to possess a nonbitter taste and



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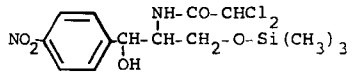
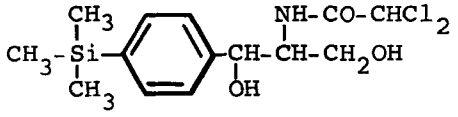


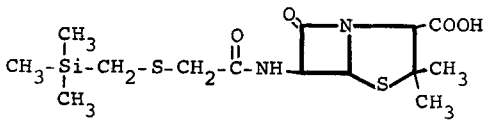
152

exert a prolonged activity in parenteral administration. Upon subcutaneous administration (as well as oral dosage for *151*), *152* demonstrated a substantial superiority over chloramphenicol. Other bis- and monotrialkyl ethers were described, but no pharmacological data were given. Frankel *et al.* (143) prepared an interesting analog of chloramphenicol, namely, *threo*-2-dichloroacetamide-1-(*p*-trimethylsilylphenyl)propan-1,-3-diol (*153*), but did not report it to possess antibacterial activity.



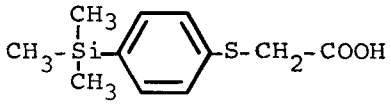
153

Voronkina *et al.* (144) synthesized trimethylsilylmethylmercaptomethylpenicillin (*154*) in an impure state and reported its antibiotic properties to be similar to those for benzylpenicillin. Gueyne and Duffaut



154

(145, 146) reported potassium "siliconate" complexes of penicillic acid and of benzyl penicillinate as therapeutic agents against infections. The mercaptoacetic acids (*e.g.*, Compound *155*) were found to be readily utilized by penicillin-producing molds in the biochemical formation of aliphatic-type penicillin (147).

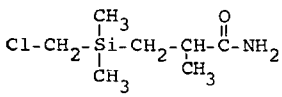


155

Both *p*-trimethylsilylphenethylamine hydrochloride and *p*-trimethylsilylmethylphenethylamine hydrochloride were evaluated against various types of bacteria and fungi (116). Inhibition of bacterial growth at concentrations of 500 and 100 mcg./ml. were observed for the compounds, respectively, whereas phenethylamine hydrochloride failed to inhibit growth even at 1000 mcg./ml. However, the presence of blood in the growth media significantly reduced the antibacterial activity of the latter organosilicon agent.

Cason and Rhone (148) described the synthesis of a large group of *ortho*- and *para*-trimethylsilylbenzoates of aminoalcohols. The compound β-diethylaminoethyl *p*-trimethylsilylbenzoate hydrochloride, for example, was found to exhibit antibacterial activity against *Streptococcus hemolyticus*. Other esters were reported to elicit analgesic and local anesthetic properties.

Twenty-eight agents, representative of different classes of nitrogen-containing organosilicon compounds, were tested against eight strains of bacteria and fungi (149). The most potent fungistatic activity was observed for *156* and *157*, both being less active than nystatin by a factor of 1.8 against *Trichophyton gypseum 4/3;* Compound *156* elicited activity against



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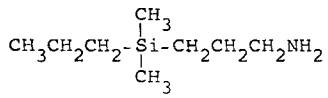
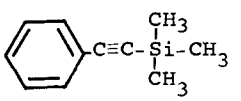


157

Epidermophyton Kaufmann-Wolf comparable to that of the standard. Activity against bacteria was, in all cases, markedly less than that for nystatin.

Growth inhibition of the fungi *Macrosporium* and *Cladosporium*, equivalent to that of mercuric chloride, was reported for several thiocyanato-substituted silanes (150). A concentration of 1–3% of these agents (*e.g.*, trimethylsilyl thiocyanate) in nutrient media infected with the fungi strongly suppressed or completely prevented fungal growth for a year or more. It does not appear possible to correlate toxic action with chemical structure; however, since some of the least active compounds are most readily hydrolyzed to thiocyanic acid, toxicity must not be only a result of hydrolysis. A group of isothiocyano-substituted silanes was also reported (151), but biological data were not included.

Some mercury-containing organopolysiloxane materials have been shown to provide bactericidal and fungicidal properties. For example, the growths of *Staphylococcus aureus* and *Salmonella dublin* were completely inhibited at 1:27,000 and 1:9000 dilutions, respectively, by a mercury-containing methyl(phenylethyl)-siloxane (152). The acetylenic organosilicon compounds *158* and *159* have been reported to inhibit the growth of *S. aureus*, *Trichophyton mentagrophytes*, and the mushroom fungus *Hormodendrum* sp. (153). Claims of bactericidal and fungicidal activity have been made



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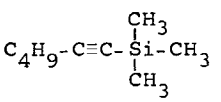


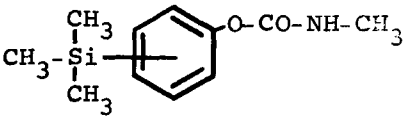
159

for numerous other organosilicon compounds (154–160).

Prophylactics against UV and Ionizing Radiation—Because of the water-repellent characteristics of polysiloxanes, several of these materials have been employed as skin-protective creams. For example, Black (161) and Morehouse (162) prepared polyphenylcarbamoylalkyl silicon compounds as sunscreen agents which absorb UV light in the 260–310-nm. range. Other polyethylsiloxanes were suggested by Bruevich as protective creams (163).

Silicon-substituted thiophene derivatives of mercaptoethylamine have been synthesized in anticipation of effecting protection against ionizing radiation (164). The silyl compounds were prepared in order to ascertain the effects of the trialkylsilyl moiety upon radioprotective activity; however, biological evaluation of these compounds has not been reported.

Insecticides and Insect Repellents—In an attempt to develop biodegradable insecticides, Metcalf and Fukuto (165) prepared the three (*ortho*, *meta*, and *para*) isomeric trimethylsilylphenyl *N*-methylcarbamates (*160*). The *meta*-isomer was the most potent inhibitor

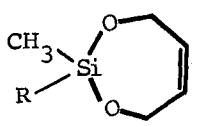


160

of fly-head cholinesterase, being nearly twice as potent as its carbon isostere and also the most active isomer against the larva and adult mosquito *Culex pipiens quinquefasciatus* Say.

Insecticidal properties have been reported for organosilicon polymers containing the sulfonamido moiety (156) and the dithiocarbamyl group (157) linked to the silicon atom through a polymethylene chain of at least three carbon atoms. The sulfonamido-containing polymers are also claimed to possess sunscreening properties and fungicidal activity. Silicon oils are also reported to exert a synergistic effect with other insecticidal agents (166–170).

Insecticidal, bactericidal, and antifungal properties have been claimed for some 2,2-dialkyl-1,3-dioxa-2-silacyclohept-5-ene derivatives (*161*), where R is methyl, ethyl, or vinyl (154). Similar biological properties have also been claimed for a group of alkylfluorosilanes (171). In the latter instance, toxicity is probably

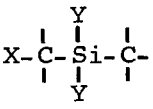


161

due, in part, to the hydrofluoric acid released by hydrolysis. Marova *et al.* (150) reported dimethyldiisothiocyanosilane to be a potent insecticide against insects of the Pentatomidae family. Several chlorinated arylsilanes with DDT-like structures have been synthesized (172) and tested against *Tribolium castaneum* (Hbn.) and *Oncopeltus fasciatus* (Dallas) but did not exhibit significant insecticidal activity (173).

Various cyclic silyl ethers, both polymeric and nonpolymeric, incorporating the well-known insect repellent 2-ethyl-1,3-hexanediol ("612"), have been found to be approximately equal in potency to the standard repellents "612" and *N,N*-diethyl-*m*-toluamide ("deet") (174). The high level of activity and known lability of silyl ethers to hydrolysis would suggest that the repellency may be due to hydrolytic release of the repellent diol.

Compounds Affecting Plant Growth—Leasure and his coworkers (175–177) reported that a group of haloalkylsilicon compounds possess strong herbicidal activity in both pre- and postemergent screening tests. For example, structures like *162*, where X is halide and Y is any of the groups commonly known



162

to be prone to hydrolysis from silicon (*e.g.*, alkoxy), are reported to exhibit strong herbicidal action (177). The greatest activity was observed when X was chlorine; changing the number of X groups on carbon to zero or two or the number of Y groups on silicon to zero, one, or three destroyed the activity. These investigators (178) also prepared several chloromethyl- and α-chloroethylsilanes which modify the growth characteristics of plants.

Bennett and Lee (179) described a group of acetylenic silanes, corresponding to formula $R_nSi(C{\equiv}CH)_{n-4}$ (where n = 0, 1, 2, or 3 and R is aryl or alkyl), which exhibit herbicidal activity; the highest potency is observed for those compounds in which n is three. Durquety and Magimel (180) claimed that an aqueous emulsion of methylpolysiloxane sprayed on leaves or buds delays the water and salt metabolism of the sprayed organs. They suggested that method for delay of flowering in plants, thus affording protection from spring frost.

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Methotrexate Pharmacokinetics

K. B. BISCHOFF*‡, R. L. DEDRICK*, D. S. ZAHARKO†, and J. A. LONGSTRETH*

Abstract □ A pharmacokinetic model is presented to predict the detailed distribution and excretion of methotrexate in several mammalian species over a wide range of doses. Anatomical compartments include liver, kidneys, gastrointestinal tract, muscle, and plasma; multicompartment representations simulate biliary excretion and movement of drug through the gastrointestinal tract with partial reabsorption. Tissue-to-plasma distribution coefficients include linear and strong saturable (presumed dihydrofolate reductase) effects. Required parameters are measured directly or estimated on the basis of physiological principles for mice, rats, dogs, and man.

Keyphrases □ Methotrexate pharmacokinetics—model, mammals □ Tissue/plasma distribution coefficients—methotrexate, mammals □ Pharmacokinetic parameters—methotrexate absorption, distribution, excretion □ Dihydrofolate reductase binding—methotrexate □ Model, methotrexate distribution, mammals—one, two compartments

A previous paper (1) described a preliminary mathematical model for methotrexate pharmacokinetics in mice. Since it was based on only one dose level, many of its conclusions were, necessarily, tentative. This paper describes much more extensive work at a variety of dose levels with several species, including man. The same general rationale proposed in previous studies (1–3) is utilized, namely, basing the model, as much as possible, on established, independently verifiable, physiological concepts. It is important to emphasize this approach since, in this fashion, one can obtain pharmacokinetic models that provide generally applicable *a priori* predictions of drug distribution behavior. Specific details will be given with the model description.

Cancer chemotherapeutic agents are usually screened for activity against a limited number of tumor systems in the mouse and rat. If their activity is sufficient to warrant further investigation, they are tested for toxicity in a variety of animal species. Detailed information is developed on quantitative and qualitative toxicology as well as the pharmacologic distribution and metabolic fate. The most promising drugs are then evaluated clinically in man.

The fundamental issues of drug toxicity include both the physical aspects of drug distribution within the body and drug effect at particular sites of action. Experience to date indicates that the dynamics of drug distribution in the body can often be *predicted* on the basis of fundamental information on physicochemical properties, transport, metabolism, and various excretory mechanisms. The method is analogous to that used for "scale-up" for a chemical plant where rational design can be based on laboratory data applied according to reasonably well-established techniques. Application of this concept to pharmacokinetic modeling should permit the quantitation of interspecies differences with respect to drug distribution and prediction of drug concentration at specific sites as functions of dose schedule and route of administration. Reliable prediction can enable more effective drug screening and enhance the ability of the clinician to devise optimum therapeutic regimens. The latter is of particular concern for antineoplastic drugs for which therapeutic effect and toxicity must be balanced carefully.

BASIS AND DEVELOPMENT OF MODEL

The model employs the lumped compartmental approach with the restriction that the volumes, flows, and other properties be physiologically meaningful and usually independently measured. The concept of flow-limited conditions is used for all body regions. If quantitative membrane permeabilities were more readily available, other cases could be considered, but this does not seem feasible at present.

Many of the broad features described earlier (1) are valid following intravenous injection of methotrexate: (*a*) a very rapid drop in plasma concentration and an increase in gut-lumen concentration, suggesting the importance of tissue uptake and biliary secretion; (*b*) a peak gut-lumen/plasma concentration ratio of about 100; and (*c*) linear binding of methotrexate by tissues at plasma concentrations above 0.1 mcg./ml. At low concentration levels, the binding became nonlinear (4), presumably because of the strong binding to dihydrofolate reductase.

The basic model, incorporating the important enterohepatic circulation, is shown in Fig. 1. The various symbols are defined in the

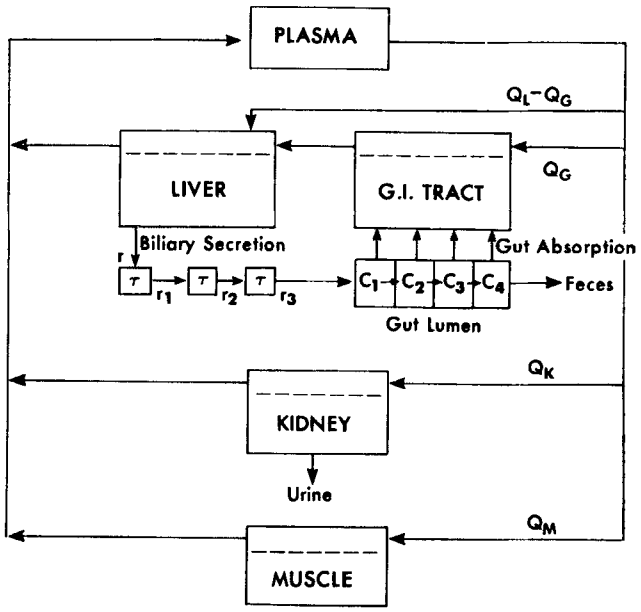

Figure 1—*Body compartments important in methotrexate distribution.*

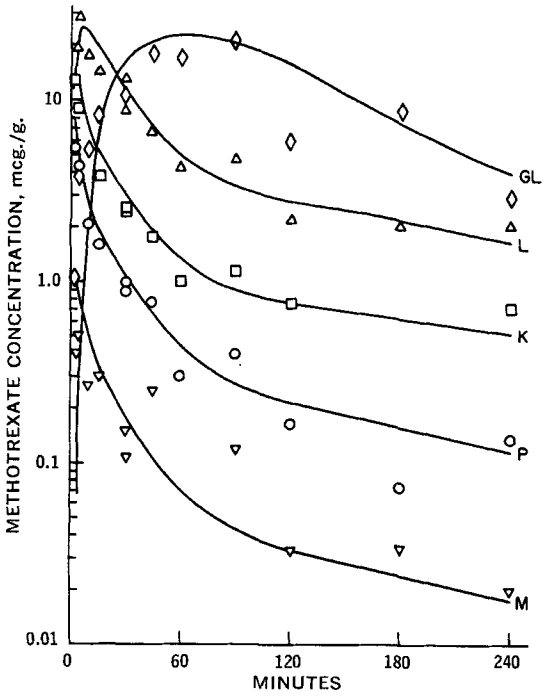

Figure 2—*Model prediction* versus *experimental results, mice, 3 mg./kg. i.v. Solid lines are model predictions; symbols are experimental data. Key:* GL(◇), *small intestine;* L(△), *liver;* K(□), *kidney;* P(○), *plasma; and* M(▽), *muscle.*

Nomenclature section. Two rather unique features need to be described in more detail—the "multicompartment" models used for the biliary secretion process and for the gut lumen. In the previous study (1), a mathematical time-delay or step function was used to simulate the bile formation and secretion time in the liver. In other words, a period of about 5 min. (in mice) was allowed before the drug in the bile was introduced into the gut lumen. This abrupt introduction caused this portion of the gut-lumen-predicted curve to overshoot the actual data, and a smoother representation of the real "S-shaped" bile concentration efflux curve was desired. A finite series of discrete compartments is able to do this, as is commonly done in chemical engineering for similar situations (5). A more extensive description and test of this scheme to represent bile function are given elsewhere (6), and it was found materially to improve the present model predictions.

The transit of drug down the gut is handled similarly, except that provision for transport through the intestinal wall is made. This concept, rather than a single compartment with time delay, is used again to provide a smooth response. Also, rather than a well-mixed uniform region, the tubular nature of the gut lumen must be accounted for to simulate the proper concentration appearing in the feces. This is done by having the feces "exit" from the last gut-lumen region. The rigorous details of properly accounting for binding, *etc.*, were given previously (1). The complete set of mass balance equations for the various anatomical body regions can be written as follows.

Plasma:

$$V_P \frac{dC_P}{dt} = Mg(t) + Q_L \frac{C_L}{R_L} + Q_K \frac{C_K}{R_K} + Q_M \frac{C_M}{R_M} - (Q_L + Q_K + Q_M)C_P \quad \text{(Eq. 1)}$$

Muscle:

$$V_M \frac{dC_M}{dt} = Q_M \left(C_P - \frac{C_M}{R_M}\right) \quad \text{(Eq. 2)}$$

Kidney:

$$V_K \frac{dC_K}{dt} = Q_K \left(C_P - \frac{C_K}{R_K}\right) - k_K \frac{C_K}{R_K} \quad \text{(Eq. 3)}$$

Liver:

$$V_L \frac{dC_L}{dt} = (Q_L - Q_G)\left(C_P - \frac{C_L}{R_L}\right) + Q_G \left(\frac{C_G}{R_G} - \frac{C_L}{R_L}\right) - r \quad \text{(Eq. 4)}$$

where:

$$r \equiv \frac{k_L(C_L/R_L)}{K_L + (C_L/R_L)} \quad \text{(Eq. 5)}$$

Equation 5 defines the secretion rate (mcg./min.) of methotrexate out of the liver cells into the bile ducts, and it allows for potential saturation effects (which were not observed at any dose levels studied).

$$\tau \frac{dr_1}{dt} = r - r_1 \quad \text{(Eq. 6}a\text{)}$$

$$\tau \frac{dr_2}{dt} = r_1 - r_2 \quad \text{(Eq. 6}b\text{)}$$

$$\tau \frac{dr_3}{dt} = r_2 - r_3 \quad \text{(Eq. 6}c\text{)}$$

Equations 6*a–c* are balanced directly on the secretion rate; the only model parameter involved is the holding time, τ. Three compartments are adequate to model separate experiments where the bile duct is cannulated and the bile concentration of methotrexate is directly measured.

Gut tissue:

$$V_G \frac{dC_G}{dt} = Q_G \left(C_P - \frac{C_G}{R_G}\right) + \sum_{i=1}^{4} \frac{1}{4} \left(\frac{k_G C_i}{K_G + C_i} + bC_i\right) \quad \text{(Eq. 7}$$

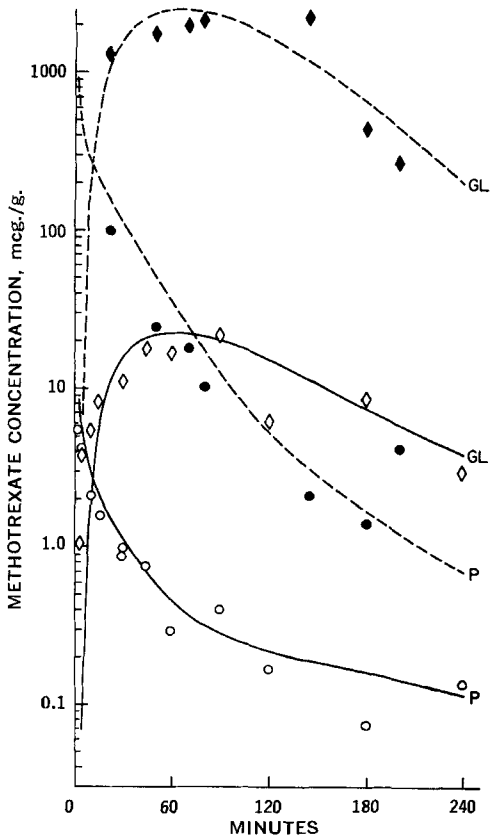

Figure 3—*Model prediction* versus *experimental results, mice. Empty symbols are 3 mg./kg.; solid symbols are 300 mg./kg. i.v. See Fig. 2 for symbol key.*

Table I—Model Parameters for Methotrexate in Several Species

Parameter	Mouse	Rat	5 kg., Dog/Monkey	17 kg., Dog	Man
Body weight, g.[a]	22	200	5000	17,000	70,000
Volume, ml.[b]					
V_P	1.0	9.0	220	650	3,000
V_M	10.0	100	2500	7,500	35,000
V_K	0.34	1.9	30	76	280
V_L	1.3	8.3	135	360	1,350
V_G[c]	1.5	11.0	230	640	2,100
V_{GL}[c]	1.5	11.0	230	640	2,100
Plasma flow rate, ml./min.[b]					
Q_M	0.5	3.0	50	140	420
Q_K	0.8	5.0	74	190	700
Q_L	1.1	6.5	92	220	800
Q_G	0.9	5.3	75	190	700
Tissue/plasma equilibrium distribution ratio for linear binding[d,e]					
R_M	0.15	0.15	(0.15)	(0.15)	(0.15)
R_K	3.0	3.0	(14)	14	(3.0)
R_L	10	3.0	(2.0)	2.0	(3.0)
R_G	1.0	(1.0)	(1.0)	(1.0)	(1.0)
Strong specific binding[e]					
a_M	0.0	0	(0)	(0)	(0)
a_K	0.3	0.3	(0.3)	(0.3)	(0.3)
a_L	0.3	0.5	(0.4)	(0.4)	(0.4)
a_G	0.1	(0.1)	(0.1)	(0.1)	(0.1)
Kidney clearance, ml./min.[d,e]					
k_K	0.2	1.1	(20)	56	190[f]
Bile secretion parameters[d,e]					
Clearance = k_L/K_L, ml./min.[g]	0.4	3.0	(2.0)	8	200[h]
τ, min.	2.0	2.0	(6)	8.0	(10)
Gut-lumen parameetrs[e]					
Transit time, min.	100	100[i]	(450)	(650)	(1,000)
Reciprocal = k_F, min.$^{-1}$	0.01	0.01	(0.0022)	(0.0015)	(0.001)
k_G, mcg./min.	0.20	(20)	(340)	(1,000)	(1,900)
K_G, mcg./ml.	6.0	(200)	(200)	(200)	(200)
b, ml./min.	0.001	—	—	—	—

[a] "Standard" values (average). [b] Based on physiological information; see *Appendix*. [c] Gut and gut-lumen volumes not independently measured, *i.e.*, gut-lumen "concentrations" are effective values. [d] Measured experimentally; see text. [e] Values in parentheses are estimates. [f] Liegler *et al.* (15). [g] Only the ratio k_L/K_L is needed, since no saturation was observed; so K_L is very large. [h] Conjectural value to create best fit to data. [i] Compare Sikov *et al.* (16).

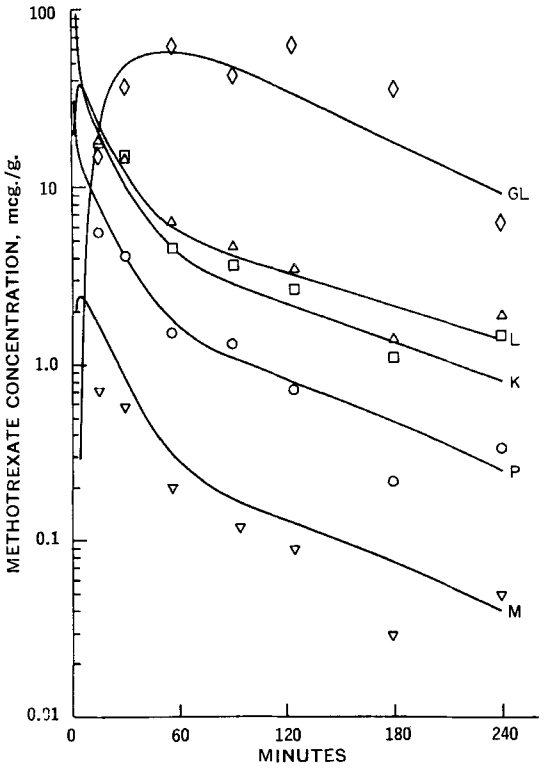

Figure 4—*Model prediction* versus *experimental results, rats, 6 mg./kg. i.p. See Fig. 2 for symbol key.*

Gut lumen:

$$\frac{dC_{GL}}{dt} = \frac{1}{4}\sum_{i=1}^{4}\frac{dC_i}{dt} \qquad \text{(Eq. 8}a\text{)}$$

$$\frac{V_{GL}}{4}\frac{dC_1}{dt} = r_3 - k_F V_{GL} C_1 - \frac{1}{4}\left(\frac{k_G C_1}{K_G + C_1} + bC_1\right) \qquad \text{(Eq. 8}b\text{)}$$

$$\frac{V_{GL}}{4}\frac{dC_i}{dt} = -k_F V_{GL}(C_{i-1} - C_i) - \frac{1}{4}\left(\frac{k_G C_i}{K_G + C_i} + bC_i\right) \quad (i = 2,3,4) \qquad \text{(Eq. 8}c\text{)}$$

In Eqs. 7 and 8, the last terms represent gut absorption from the lumen to the tissue (and blood flow), with the possibility of both saturable $[(k_G C_i/K_G + C_i)]$ and nonsaturable (bC_i) effects. Both effects may be important for folic acid (7, 8) and, therefore, may apply to methotrexate, since both substrates were reported to use the same transport system (9). The four regions are judged sufficient to model the gut-lumen response. The absorption characteristics are assumed the same for all segments because of a lack of more detailed information; other complications, such as location-dependent absorption, could be accounted for. Upon substitution of suitable numerical values for the various parameters, these equations can be solved by a computer.

PARAMETER VALUES

Many of the parameters for mice, as given earlier (1), are retained here, except for the case of the zero-order gut absorption. This was reasonable for the one dose of 3 mg./kg., but the simulation was inadequate for a wider range of dose levels. The justification for the assumption of zero-order absorption (1) was the apparent leveling of

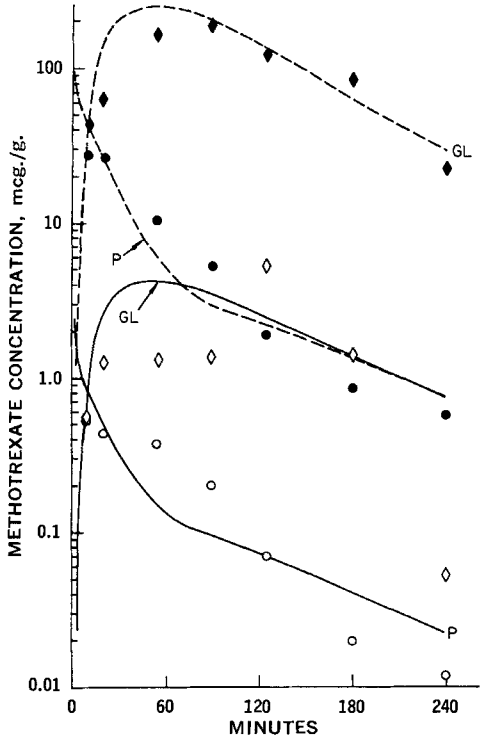

Figure 5—*Model prediction* versus *experimental results, rats. Empty symbols are 0.5 mg./kg.; solid symbols are 25 mg./kg. i.p. See Fig. 2 for symbol key.*

the plasma concentration after 2–3 hr. However, since it was found (10) that significant intestinal metabolism eventually occurs, the reliability of the measured radioactivity at these longer times is questionable. Strong binding to dihydrofolate reductase is also a complication. Thus, even though a definitive model for gut absorption is still not available, it is apparently not readily saturable. Therefore, the model accounts for both saturable and nonsaturable absorption in Eqs. 7 and 8.

The simple physiological parameters of organ volumes and flows for other animal species are detailed in the *Appendix*. Although the

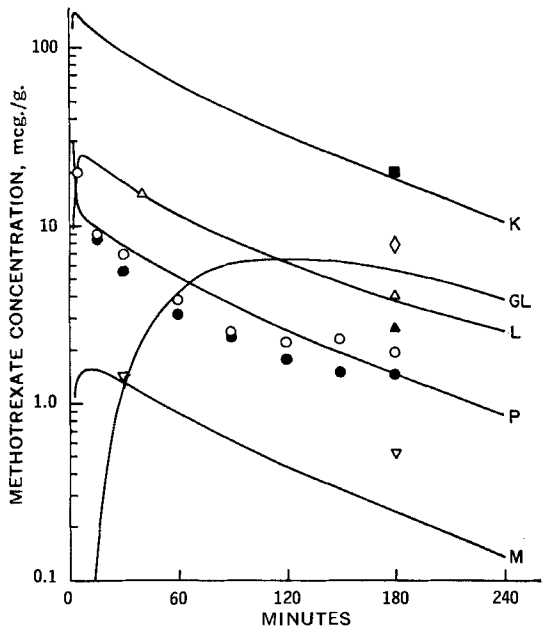

Figure 6—*Model prediction* versus *experimental results. Two experiments, dogs, 3 mg./kg. i.v., represented by empty and solid symbols. See Fig. 2 for symbol key.*

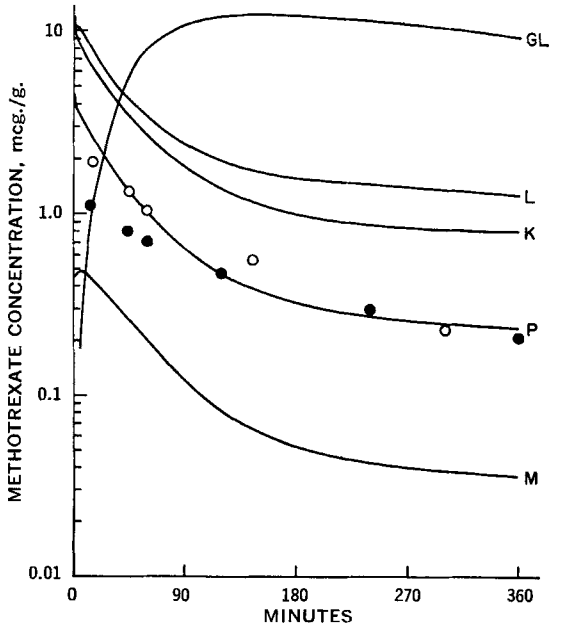

Figure 7—*Man, 1 mg./kg. i.v. Dark and clear symbols are results of two separate experiments. See Fig. 2 for symbol key (12).*

kidney clearance is close to inulin, it is specifically determined for methotrexate by comparing the time integral of the plasma concentration with cumulative urine formation after intraperitoneal or intravenous injections of the drug. The tissue/plasma equilibrium ratios are derived by some constant-infusion experiments and/or the portion of the intravenous pulse injection curve after the initial redistribution. For high concentrations, they are constants, indicating linear binding; for low concentrations, they are given by the sum of this linear nonspecific binding (RC_P) and strong binding (a), presumed to be associated with dihydrofolate reductase, according to:

$$C_{\text{tissue}} = RC_P + \frac{aC_P}{\epsilon + C_P} \qquad \text{(Eq. 9)}$$

The magnitude of ϵ may be estimated from the work of Werkheiser (11), who found that the dissociation constant of the enzyme–drug complex is less than 1.5×10^{-10} M for a noncompetitive inhibitor or

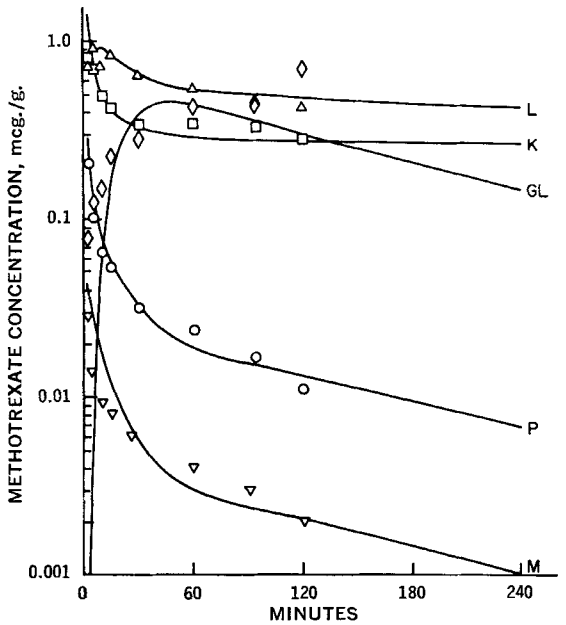

Figure 8—*Model prediction* versus *experimental results, mice, 0.12 mg./kg. See Fig. 2 for symbol key.*

less than 3×10^{-11} M for a competitive inhibitor. Thus, ϵ is of the order of 10^{-5} mcg./ml. or less. At the plasma concentrations studied, therefore, the binding to dihydrofolate reductase may be considered stoichiometric.

For high values of C_P, the second term in Eq. 9 is much smaller than the first, since a is much less than RC_P and ϵ is very much less than C_P. Thus, RC_P dominates, and the binding may be considered linear. For small values of C_P, however, the second term in Eq. 9 becomes important.

The bile clearance is determined by separate experiments, except where indicated, and the complete set of parameter values used in the simulations to be described is given in Table I.

SIMULATIONS AND COMPARISONS WITH DATA

The model parameters in Table I are used in Eqs. 1–9 for simulations of various dose levels and animal species. The values presented in Table I were used for *all* dose levels so that the model would achieve real physiological meaning.

Figure 2 compares the model results in several body regions with experimental data for 3 mg./kg. in the mouse. The results are in good agreement, similar to those reported earlier (1) for the preliminary model, except for a better gut-lumen simulation; also, the curves do not level out. Figure 3 gives the results for two high doses, 3.0 and 300 mg./kg. Other tissues are not presented in order to simplify the figure; however, they were also predictable and followed the same time course as plasma (Fig. 2).

The same types of results are given for rats in Figs. 4 and 5, showing fair agreement. Although the trend of the gut-lumen concentration is predictable, there is more variability in the data obtained at the low dose. Figure 6 illustrates the comparisons for a small amount of dog data, and the results are reasonably good. Finally, Fig. 7 compares the plasma results for man with model predictions and also gives predicted values for the other body regions. The prediction of 57% of the total dose in the urine at 4 hr. may be compared with the observed value range of 38–75% observed by Henderson *et al.* (12) at the same dose level; corresponding values for 24 hr. are: predicted, 87%; and observed, 75–88%. Based on these illustrations, the model does a reasonable job of predicting the methotrexate distribution for all the species at the fairly high dose levels ($\geq$1 mg./kg.). More extensive specific clinical experiments would be useful for more confidence in the parameter values for man.

The case of very low doses is of interest here because it illustrates the effect of the nonlinear strong binding in Eq. 9. The kinetics of distribution in various body tissues are also of practical importance to dermatologists, since such low doses are used to treat psoriasis (13). Figure 8 shows the predicted and measured tissue concentrations following an intravenous dose of 0.12 mg./kg. in the mouse. Comparison with Fig. 2 (3 mg./kg.) shows clearly the effect of the strong binding in the liver and kidney at the low dose. The plasma concentration falls rapidly in very short times, while a large fraction of the dose is bound to these tissues. As the plasma concentration continues falling, the concentrations in the liver and kidney remain high. As suggested by Werkheiser (11), other dihydrofolate reductase-containing tissues no doubt behave in a similar manner. There is evidence that dihydrofolate reductase inhibitors exhibit strong saturable binding in human liver, kidney, and small intestine which persists for days but which can be dissociated by a high dose of nonlabeled drug (14).

CONCLUSIONS

A mathematical model is presented which predicts detailed distribution of methotrexate in the tissues of several mammalian species. It is based on anatomical compartments and intercompartment flow transport. Tissue-to-plasma distribution coefficients include provision for strong binding to dihydrofolate reductase. The drug is cleared from the plasma by the kidney and excreted in the bile, with subsequent partial intestinal reabsorption. At present, the greatest uncertainty is in the exact kinetics to describe intestinal absorption in the intact animal. The model contains multicompartment descriptions of the biliary system and the intestine based on physiologic principles. Phenomena, such as local differences in intestinal absorption, could be incorporated if sufficient data were available.

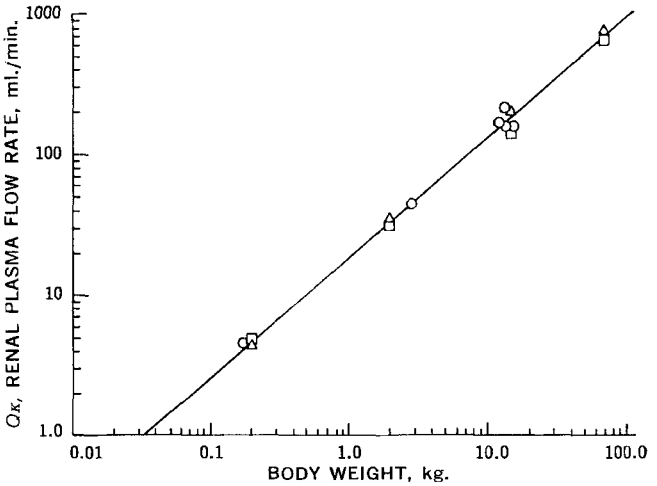

Figure A1—*Renal plasma flow rate. Data sources:* ○, Reference 20, *p. 127; and* △, □, Reference 21, *p. 1483.*

The same model is used for the mouse, rat, dog, and human, with specific parameters chosen for the species to be simulated. The model predicts tissue concentrations in the subhuman species and also predicts observed plasma concentrations and urinary and fecal excretion in man.

NOMENCLATURE

a	= strong specific binding, mcg./g.
b	= rate constant for nonsaturable gut absorption, ml./min.
C	= drug concentration, mcg./g. or mcg./ml.
$g(t)$	= injection function, min.$^{-1}$
k_F	= reciprocal of nominal transit time in small intestine, min.$^{-1}$
k_G	= saturable rate of intestinal absorption, mcg./min.
k_K	= clearance by kidney, ml./min.
k_L	= saturable rate of drug transport into bile, mcg./min., Eq. 5
K	= constant of Michaelis form for intestinal absorption or bile formation, mcg./ml.
M	= amount of drug, mcg.
Q	= plasma flow rate, ml./min.
r	= drug-transport rate in bile, mcg./min.
R	= tissue-to-plasma equilibrium distribution ratio for linear binding, dimensionless
R'	= variable tissue-to-plasma equilibrium distribution ratio including effect of strong specific binding
t	= time, min.
V	= volume of compartment, ml.
W	= body weight, kg.
ϵ	= dissociation constant of enzyme–inhibitor complex, mcg./ml., Eq. 9
τ	= nominal residence time in bile subcompartment, min.

Subscripts

G	= gastrointestinal tract
GL	= gut lumen
K	= kidney
L	= liver
M	= muscle
P	= plasma
tissue	= G, K, L, or M
1, 2, . . .	= bile or gut-lumen subcompartments

APPENDIX

This section briefly summarizes the literature data used for the physiological values of organ volumes and flows.

The volumes are essentially taken from the correlations of Adolph (17) and spot-checked with some of the authors' own organ weight data and that of Mapleson (18) and Crile and Quiring (19). Adolph (17) gives $(V_{Bl})_{Adolph} = 0.055\ (1000W)^{0.99}$, where the volume is in milliliters (grams for density of 1.0 g./ml.) and the body weight, W, is in kilograms. For a standard man of 70 kg., $(V_{Bl})_{Adolph} = 3500$.

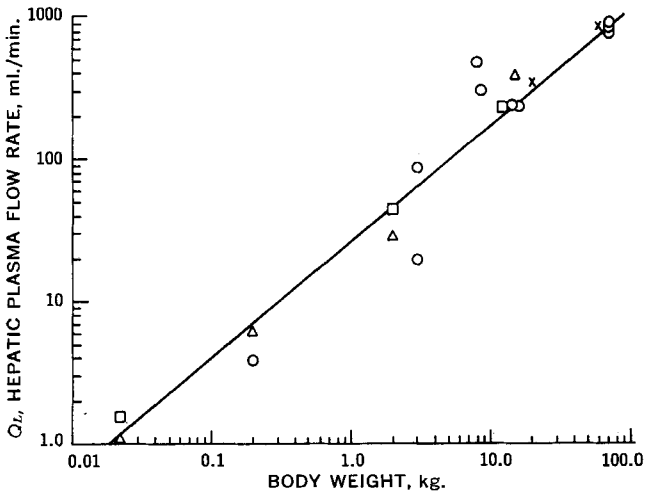

Figure A2—*Hepatic plasma flow rate. Data sources:* ○, Reference 20, *pp. 128, 129;* ×, Reference 22, *p. 244;* □, Reference 21, *p. 641; and* △, Reference 21, *p. 1405.*

This seemed too small compared to the more common 5000 ml., so the final equation used is adjusted by the ratio 5000/3500. For an average hematocrit of 40%, then:

$$V_P = (0.6)(5000/3500)(0.055)(1000W)^{0.99} = 44W^{0.99} \quad \text{(Eq. A1)}$$

At the other end of the scale, V_P for a 22-g. mouse ($W = 0.022$) is then 1 ml., which is the value used in the previous work (1).

The other relations are:

$$V_L = 0.082(1000W)^{0.87} = 34W^{0.87} \quad \text{(Eq. A2)}$$

$$V_K = 0.0212(1000W)^{0.85} = 7.5W^{0.85} \quad \text{(Eq. A3)}$$

$$V_M \simeq {}^1/_2(1000W) = 500W \quad \text{(Eq. A4)}$$

Adolph (17) gave the stomach and intestine weight as $0.112(1000W)^{0.94}$. Since the present model considers the small intestine only as the participating organ, two-thirds of the weight was used:

$$V_G = {}^2/_3(0.112)(1000W)^{0.94} = 49W^{0.94} \quad \text{(Eq. A5)}$$

These obviously somewhat arbitrary relations correspond reasonably well with commonly accepted values. It was found in previous work that consistency of values for the organ volumes is more important than absolute accuracy, which is the reason for devising the general equations.

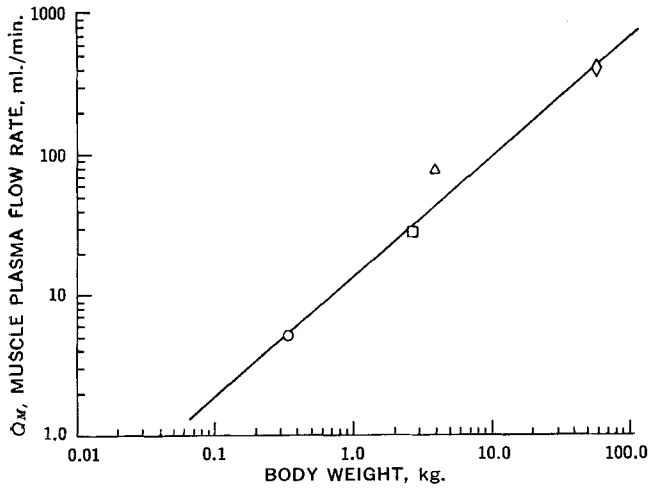

Figure A3—*Muscle plasma flow rate. Data sources:* ○, Reference 23; □, Reference 22, *p. 861;* △, Reference 24; *and* ◇, Reference 18.

With regard to flows, Fig. A1 shows data on renal plasma flow rate as a function of body weight on log–log paper to illustrate relationships in the form used by Adolph (17). The line is arbitrarily drawn through the data; it is also bracketed by Adolph's relations of *para*-aminohippuric acid and iodopyracet[1] clearances and should be reliable.

Figure A2 presents hepatic plasma flow rate. The data here are more scattered, but a reasonably good general estimate is evident. Limited available information on muscle perfusion rate is shown in Fig. A3. Much less data were readily available for the gut flow rate, and they were all for large animals. Based on these, a rough estimate of 0.8–0.85, or 0.82, of the hepatic flow rate was used (*Reference 20*, p. 1443; *Reference 22*, p. 244).

In the calculation of the flow rates for Figs. A1–A3, total plasma flow rates to the three compartments are calculated, as required, on the basis of a hematocrit of 0.40 and organ weight-to-body weight relationships.

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Clinicopathologic Effects of Chronic Reserpine Administration in Mongrel Dogs

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Abstract □ Twenty-four mongrel dogs were given low oral doses of reserpine twice daily for periods of 1–12 months, and certain clinicopathologic examinations were compared with findings from an equal number of control dogs which received placebos. Prolapsed nictitating membrane, miosis, and diarrhea were the initial clinical signs observed, and most reserpine-treated dogs exhibited the former throughout the study. CNS depression, muscle tremors, and a pronounced Parkinsonism-like syndrome were observed in the reserpine-treated population; these central effects, in contrast to alterations in peripheral autonomic activity, appeared to increase as the duration of reserpine administration lengthened. The hematocrit, hemoglobin content, and total leukocyte counts decreased in the reserpine-treated population, and the mean values were routinely significantly lower ($p < 0.05$) than in the control group. Right ventricular dilatation was diagnosed upon gross necropsy in five of nine dogs that received reserpine for 2–8 months and in three of four dogs that received the drug for 1 year. These findings suggest that the chronic administration of relatively low dose levels of reserpine in dogs may induce marked behavioral and ambulatory abnormalities and possible detrimental effects upon hematologic components and the myocardium.

Keyphrases □ Reserpine, chronic—clinicopathologic effects, dogs □ Antihypertensive agent—chronic reserpine administration, myocardial surveillance □ CNS depression, Parkinsonism-like syndrome—chronic reserpine, dogs □ Myocardial effect—chronic reserpine, dogs

The long-term administration of various dose levels of rauwolfia compounds for the therapeutic management of hypertension and other chronic ailments is well documented (1–4). Treatment periods are often a year in duration and may continue throughout the life of the patient. However, few experimental studies have simulated this chronic treatment schedule, even though the potential toxicity of large acute doses of reserpine is well recognized. Such studies would be important since, in certain circumstances, the prolonged administration of low dose levels of reserpine may also induce toxic responses. For example, clinical studies in man indicated that antihypertensive therapeutic doses of reserpine can adversely alter myocardial function (5) and induce signs of congestive heart failure (6, 7). Premature ventricular contractions and disruption of normal electrocardiographic patterns were likewise associated with therapeutic regimens of reserpine (8, 9). In experimental animals, alterations in myocardial function and structure were observed following the administration of relatively small quantities of reserpine (10, 11). It appeared, therefore, that a long-term study might elucidate certain effects of reserpine that may not be apparent upon short-term administration.

The purposes of the present study were to investigate certain effects of reserpine administered for 1–12 months in mongrel dogs and to correlate these data with information currently available from acute animal experiments as well as clinical studies in man. Relatively low doses of reserpine, less than those suggested for chronic clinical maintenance in the dog (12), were utilized in an attempt to simulate chronic administration of clinical dose levels in man.

MATERIALS AND METHODS

Animals and Treatment Schedule—The dogs used were mongrels received from a commercial supplier. Each dog selected was subjected to the following standards: (*a*) body weight between 8 and 12 kg. or appear as though, upon conditioning, it would be within this range; (*b*) age approximately between 1 and 3 years as determined by dentition; and (*c*) heart and lung sound, with no detectable abnormalities upon auscultation.

After selection, each accepted dog was vaccinated against common canine infectious diseases and treated for endo- and ectoparasites. Forty-eight dogs were selected, half males and half females, and placed in quarantine for at least 30 days prior to the beginning of the study. Approximately 500 g. of Purina dog chow was given once daily and water was available as needed. The dogs were randomly placed into four groups: male or female treated and male or female control. Each dog was maintained in an individual cage, and males and females were housed in separate rooms which were maintained at a constant temperature and a 12-hr. dark and 12-hr. artificial light cycle throughout the study.

A random sample of the tablets[1] contained 0.137 mg. of reserpine per tablet. Preliminary studies, conducted over an 8-week period, indicated that the minimal effective oral dose of reserpine in 10-kg. mongrel dogs (using relaxation of the nictitating membrane as the criterion) was approximately 0.15 mg., b.i.d. In the present study, each treated dog received 0.137 mg. of reserpine *per os*, b.i.d. through Day 21 of the test. At this point, the daily dose was reduced in half to 0.137 mg. per dog, which was continued through Day 62; however, at this time the original dose was reinstituted and maintained throughout the remainder of the study. The average daily dose of reserpine administered to each dog was approximately 26 mcg./kg. The dose varied from 18 to 39 mcg./kg.; however, the outside dose levels represent only a few animals since body weight was similar among the reserpine-treated dogs with little deviation from the group mean (see *Results*). The control dogs received a placebo tablet on the same schedule as the treated animals. The clinical condition of each dog was assessed daily, and body weights were determined every 2 weeks and body temperature twice a week.

Clinical Chemistry—The following clinical hematologic tests were conducted at 2–4-week intervals on most of the dogs: blood urea nitrogen, erythrocyte count, leukocyte count (total and differential), hemoglobin, hematocrit, bilirubin, and blood glucose. Serum glutamic-oxalacetic transaminase was assayed utilizing a radioisotopic method developed in this laboratory[2]. A sample of serum (0.05 ml.) was incubated for 1 hr. at 37° in a phosphate buffer medium containing aspartic acid and α-ketoglutaric acid-U-^{14}C. The incubation mixture was then acidified and passed over a 0.5 × 3-cm. column of Dowex-50 (hydrogen form) exchange resin. The product of the reaction, glutamic acid-U-^{14}C, was retained on the column, subsequently eluted with alkali, and quantitated in a liquid scintillation counter.

Twelve of the reserpine-treated and 12 control dogs (six of each sex) were randomly selected for glucose tolerance tests, and the re-

[1] Supplied and assayed by Ciba Pharmaceutical Co., Summit, N. J.
[2] Unpublished study of R. J. Ertel *et al.*

maining 12 treated and 12 controls were employed every 4 weeks for adrenocortical function tests utilizing β^{1-24}-corticotrophin[3]. For glucose tolerance determinations, the dogs received an intravenous dose of 1 g./kg. of sterile glucose solution (50%) following the collection of the initial preglucose-load blood sample. Venous blood samples were withdrawn at 15 and 60 min. after loading, and blood glucose was determined utilizing prepared reagents[4].

The analysis of adrenocortical function was performed essentially as described by Wood *et al.* (13). The animals received a single dose of 250 mcg. of β^{1-24}-corticotrophin after a predrug blood sample was obtained. Thirty minutes after the administration of the synthetic corticotrophin, an additional blood sample was withdrawn, and the adrenal function was evaluated on the basis of the mcg./100 ml. increase in serum corticosteroids. Fluorometric analysis of serum corticosteroids was performed according to the method of Nielsen and Asfeldt (14), which is based on the principle that cortisol, the major corticosteroid, and corticosterone have the same fluorescent intensity 5 min. after development of fluorescence by the addition of ethanolic sulfuric acid solution in the final step. The effects of reserpine on serum sodium and potassium were determined utilizing an atomic absorption spectrophotometer (Perkin-Elmer).

Gross and Histopathologic Studies—The animals were sacrificed using pentobarbital administered by intravenous injection. Two dogs, one male and one female, were sacrificed from the treated and control groups each month for the first 5 months following the initiation of reserpine treatment, except at 3 months when one treated and two controls were studied. In addition, two treated and two control dogs were sacrificed after 8 months, and the remaining animals were sacrificed at the termination of the study. Gross necropsy examinations were conducted on the 11 reserpine-treated dogs that were sacrificed through the 8th month, on four dogs treated with reserpine for 1 year, and on control dogs. Changes in cardiac structure were determined utilizing a method that involves macroscopic evaluation of the relative size and consistencies of myocardial areas during postmortem examination (15). The following tissues were fixed in 10% buffered formalin and prepared for histopathologic examination utilizing hematoxylin and eosin staining: heart, lung, stomach, small intestine, adrenals, kidneys, liver, testes, ovaries, and pituitary glands.

Additional cardiovascular and biochemical studies were conducted throughout the investigation; details of these phases will be reported elsewhere.

RESULTS

Clinical Signs—Minimal variations were observed in body temperature and body weights in the control and treated populations throughout the 1-year study. The mean body temperature varied between 38.2 and 39.0° in both groups. The mean body weight ($\bar{X} \pm SE$) in the reserpine-treated group was 9.9 ± 0.57 kg. at the beginning of the study and 10.38 ± 0.57 kg. at the termination. The mean value was below 10 kg. on only five occasions throughout the study, and the maximum value did not exceed 10.73 ± 0.83 kg. In the control group, similar variations were observed, and the body weight was 10.2 ± 0.4 kg. at the beginning and 11.63 ± 0.69 kg. at the termination of the study. The differences between the mean body weights of the treated and control populations at the beginning and at the termination of the study were not significant ($p > 0.98$ and $p > 0.1$, respectively).

Cardiovascular studies[5] showed no significant differences in peripheral arterial pressure, heart rate, or blood volumes between control and treated groups prior to initiation of reserpine administration. Chronic administration of reserpine did not induce significant changes in the indirectly measured (obtained from the left forelimb using a Korotkoff microphone) arterial blood pressure of unanesthetized dogs. The heart rate was significantly depressed in the treated dogs after 2 weeks of reserpine administration, and this effect persisted throughout the study.

Relaxation of the nictitating membrane was the initial detectable clinical sign observed in the reserpine-treated dogs beginning on the 3rd day of reserpine administration. Within 2 months, 70–95% of the treated dogs exhibited this sign, which persisted throughout the study (Fig. 1). In the initial 6 months of the study, 5–8 mm. of the third eyelid was usually visible; however, after the 6th or 7th month, less of the structure was observed. Persistent prolapsed nictitating membrane was not seen in control dogs.

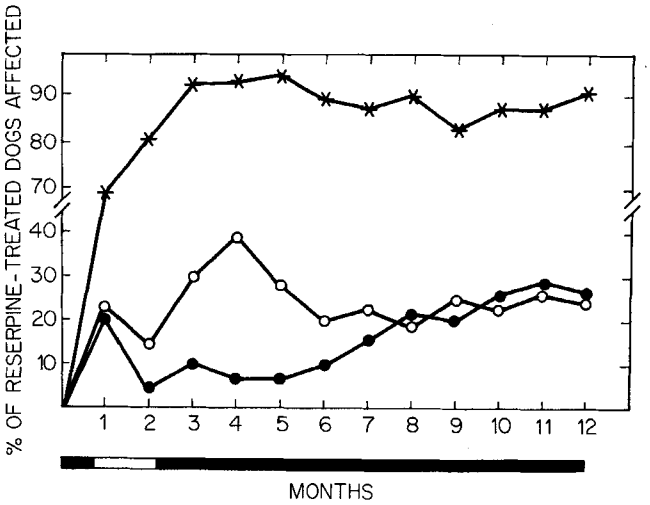

Figure 1—*Clinical signs observed in reserpine-treated dogs. Each value represents a monthly mean of clinical signs observed daily. Key:* *—*, *relaxed nictitating membrane;* ○—○, *tremors;* ●—●, *depression;* ■, *0.137 mg. reserpine, p.o., b.i.d.; and* □, *0.137 mg. reserpine, p.o., daily.*

The general behavioral patterns of the dogs demonstrated a form of sedation–depression within the initial month of the study, characterized by inactivity, recumbency, frequent narcosis, and minimal interest in stimuli from other activity within the animal room. The sedation–depression state was initially observed in three of the 24 treated dogs after 8 days of reserpine administration; however, the incidence of this condition rapidly increased through the 3rd and 4th weeks, when 33% of the treated dogs were affected. (As shown in Fig. 1, the monthly mean was 20%.) The incidence of sedation decreased, after a 1-week delay, during the 6-week period of reduced dosage (see *Methods*). However, upon reinstitution of the original dose of reserpine, the condition was again observed within a week and increased in incidence through the remainder of the study (Fig. 1).

Pupillary size determinations, measured with a millimeter rule under constant illumination, revealed miosis in four treated dogs after 4 days of reserpine administration. This condition was observed in 20–80% of the reserpine-treated population through 2 months of treatment; after this time, reliable results could not be determined due to individual variation. During this same period, miosis was diagnosed in less than 15% of the control dogs.

Blepharoptosis was occasionally observed in treated dogs only after the initial 3–4 months of reserpine administration; this sign was neither persistent nor pronounced, and it became unnoticeable upon stimulation of the animal.

Diarrhea was observed in the first 2 weeks of the study in six of the reserpine-treated dogs. It lasted only a few days and recurred in only one treated dog throughout the 1-year study.

Resting muscle tremors were noticed after a week of reserpine administration in approximately 25% of the reserpine-treated dogs. After a reduction in the incidence and severity of tremors during the period of decreased dosage, the incidence again increased through the 4th month when approximately 40% of the treated dogs were affected. By the 6th month and throughout the remainder of the study, between 20 and 30% of the reserpine-treated population exhibited resting tremors (Fig. 1). A larger percentage of reserpine-treated dogs often exhibited physiological tremors upon removal from their cages; however, only those individuals exhibiting resting tremors while in the cage undisturbed were classified as positive for this sign.

A prominent Parkinsonism-like syndrome was observed in three dogs after 3, 7, and 8 months, respectively, of reserpine treatment. This condition was characterized by severe skeletal muscle tremors, head and limb shaking, ataxia, difficulty in ambula-

[3] Synacthen, Cortrosyn.
[4] Diagnostest, Dow Chemical Co.
[5] Unpublished data of B. S. Jandhyala *et al.*

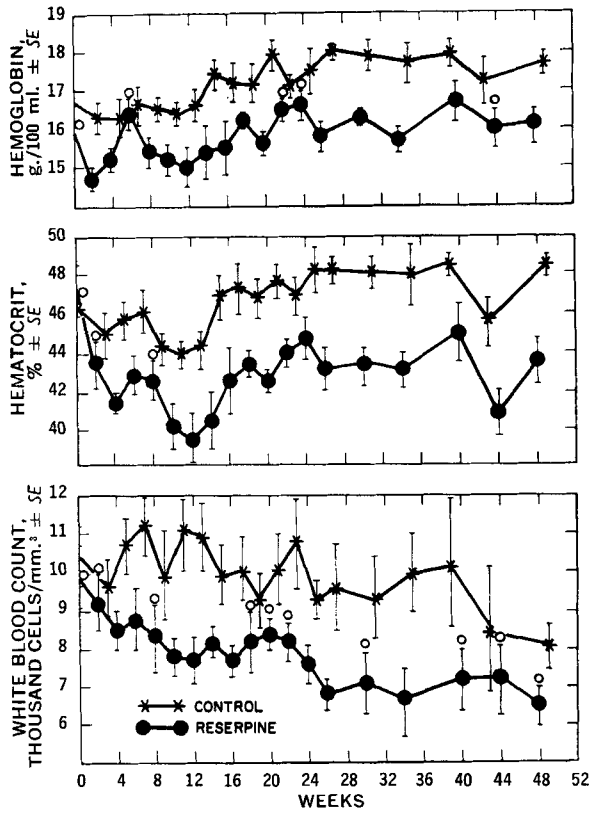

Figure 2—*Effects of chronic reserpine administration on certain hematologic components in dogs.* O = *not significantly different from respective control values* ($p > 0.05$). *All other values of the reserpine-treated group were significantly less than respective control values* ($p < 0.05$).

tion, and severe depression. A similar, though less severe, syndrome was periodically observed in other dogs. Reserpine administration was stopped in the dog exhibiting the Parkinsonism-like signs after 3 months of treatment, and the dog returned to normal behavioral patterns in approximately 10 days. However, when the original dose of reserpine was reinstituted, the animal again exhibited marked depression, severe tremors, head shaking, diarrhea, and ambulatory difficulties within 10 days. Reserpine administration was again halted, and a complete remission of clinical signs was observed in 2 weeks after which the animal was deleted from the study.

Reserpine administration was continued in the dog exhibiting Parkinsonism-like signs after 7 months of treatment. After 1 month of this syndrome, the dog became even more unresponsive. It was persistently recumbent and semicomatose in appearance, and extreme coaxing was necessary to induce standing or walking. Walking appeared very difficult and was stilt-legged and shuffling in manner and extremely slow. Additional withdrawal, nonambulation, and depression occurred until death after 9 months of reserpine administration. Autolytic changes observed upon necropsy precluded pathologic evaluation of this animal. The reserpine dosage was decreased by 50% in the dog exhibiting the Parkinsonism-like syndrome after 8 months of treatment, and a gradual decrease in severity of the syndrome was observed. However, the dog continued to exhibit pronounced depression and tremors while regaining body weight.

Hematologic Studies—The data summarized in Fig. 2 show that the most significant alterations produced by chronic reserpine administration were in the hemoglobin content, hematocrit, and total leukocyte count. Throughout the study, the hemoglobin content and hematocrit content were consistently and significantly lower than corresponding control values, although total erythrocyte counts were not significantly altered. The mean total leukocyte count of the reserpine-treated group was consistantly lower than the total leukocyte count of the control group. This reduction in the total leukocyte count was not associated with a significant alteration of the relative percentages of monocytes, lymphocytes, or granulocytes.

Analysis of blood urea nitrogen, glucose, and bilirubin revealed no significant differences in these values throughout the study. The mean serum glutamic-oxalacetic transaminase activity ranged between 7.5 and 11 μM/min./l. in both groups of dogs throughout the period of study, and no significant differences were observed. Aside from an initial rise in serum K^+ observed in the reserpine group during the first 4 weeks of the program, the mean serum K^+ ranged between 4 and 5 meq./l. in both groups of dogs through the remainder of the study. Serum Na^+ levels in both the reserpine and control groups ranged between a mean of 135 and 145 meq./l. throughout the experimental period, and there were no consistently significant differences. The glucose tolerance tests were compared statistically on the basis of the sum of the three glucose values; that is, the sum of 0-time, 15-min., and 1-hr. postload glucose values. Aside from a significantly lower ($p < 0.05$) glucose tolerance sum (GTS) in the reserpine group at Weeks 4 and 6, there were no significant differences in the GTS throughout the remainder of the experimental period. There were no significant differences between the reserpine-treated and respective control group values either in the resting corticosteroid levels or in the increase in serum levels following administration of synthetic corticotrophin. The resting mean ($\pm SE$) serum corticosteroid level varied from 3.75 ± 0.5 to 4.70 ± 0.7 mcg./100 ml. in the control group and from 2.93 ± 0.4 to 4.22 ± 0.3 mcg./100 ml. in the reserpine-treated dogs. Following the administration of β^{1-24}-corticotrophin, the mean ($\pm SE$) increase in serum corticosteroid levels varied from 7.76 ± 0.8 to 8.87 ± 1.24 mcg./100 ml. in the control dogs and from 6.03 ± 0.7 to 7.24 ± 0.7 mcg./100 ml. in the reserpine-treated group.

Pathologic Findings—Right ventricular dilatation was diagnosed in eight of the 15 reserpine-treated dogs that were necropsied. Abnormal changes in other cardiac structures and chambers were not noticed. The right ventricular finding was observed initially in two dogs after 2 months of treatment, in one dog after 3 months, and again in two dogs after 8 months of reserpine administration. This lesion was also observed in three of four dogs treated with reserpine for 1 year (Table I). The gross diagnosis of right ventricular dilatation was based primarily upon the enlarged, rounded, and flaccid appearance of the right ventricle (Fig. 3), although a relatively thin wall and enlarged lumen were also usually noted. The degree of right ventricular involvement in this example is representative of the condition observed in other treated dogs and differs markedly from the normal right ventricle (16). The gross appearance of right ventricular dilatation was observed in only two of 24 control dogs (one at 4 and one at 12 months), and the degree of flaccidity observed in the right ventricular wall of reserpine-treated dogs was not seen in the hearts from these two controls. The incidence of this lesion was significantly greater ($p < 0.01$) in the reserpine-treated group when the data obtained from the sacrifice periods were pooled (Table I). In the dogs treated with reserpine for 2–8 months, focal fatty infiltration was often observed upon histopathologic examination of the right-side dilated hearts. However, control dogs occasionally exhibited the same findings, and this histologic lesion was, therefore, classified as incidental. Chronic interstitial nephritis and chronic peribronchial pneumonia also were occasionally seen in the control and treated dogs. These findings did not appear to be correlated with the incidence of right ventricular changes and were considered incidental findings. All other tissues examined appeared normal.

Table I—Incidence of Right Ventricular Dilatation in Hearts of Dogs Treated Chronically with Reserpine

Months	Treated (n = 15) Positive	Treated (n = 15) Negative	Control (n = 24) Positive	Control (n = 24) Negative
1	0	2	0	2
2	2	0	0	2
3	1	0	0	2
4	0	2	1	1
5	0	2	0	2
8	2	0	0	2
12	3	1	1	11
Total	8[a]	7	2	22

[a] Significant difference between the reserpine-treated and control group, $p < 0.01$ (χ^2-analysis with Yates correction factor).

DISCUSSION

One purpose of the present investigation was to simulate chronic administration of clinical dose levels of reserpine in man; therefore, the dosages utilized bear discussion. Various quantities of reserpine have been studied in experimental and clinical situations in both man and animal. Following early experimental studies in animals, Earl (17) advocated the clinical use of 10–20 mcg./kg. for producing tranquilizing effects in dogs. Repeated doses of 50–100 mcg./kg. were reported to produce good effects in a much shorter time, with only an increased incidence of intestinal disturbances mentioned. Rauwolfia alkaloids have not been used extensively in the clinical practice of veterinary medicine, but 10–20 mcg./kg. of reserpine was suggested as an optimal daily oral dosage for ataractic effects in dogs (12). Gassner *et al.* (18), however, concluded that greater quantities of reserpine, 22–66 mcg./kg., were the most effective dosages in treating hypertension and other disorders in the same species. It was also mentioned that up to 35 mcg./kg. was well tolerated by dogs and that they could be chronically maintained on a daily oral dosage of up to 100 mcg./kg. of reserpine (12). It seems justifiable, therefore, to consider the daily oral administration of 18–39 mcg./kg., as in the present investigation, as a relatively low dose level in the dog.

In man, small amounts of reserpine, 0.1–0.6 mg., were administered daily in fixed-ratio combination with other antihypertensive agents for prolonged treatment of high blood pressure (2–4). Increased dosages, up to 1–2 mg. per day, however, were administered chronically when reserpine was the sole antihypertensive agent studied (1, 19). Also, daily doses of 10 mg. and often more were used in the management of mental disorders (1, 7). These reported quantities represent, considering a 70-kg. individual, a dose range of approximately 1.5–138 mcg./kg. per day. It is submitted, therefore, that the dose level used in the present investigation represents relatively low daily oral dosages.

The daily oral administration of 0.274 mg./dog of reserpine induced clinical evidence of the pharmacologic effects of the compound upon adrenergic neuronal function. The signs observed in the initial weeks of the investigation included a relaxed nictitating membrane, a decreased pupillary diameter, increased intestinal activity, and CNS depression. These effects can be attributed to the well-documented depletion of endogenous amines by reserpine (1, 20–22) with, in some instances, resulting parasympathetic dominance (23). These observations are in accord with Plummer *et al.* (23), who stated that the pupil and nictitating membrane of the dog are rapidly responsive to small amounts of reserpine, and with Earl (17), who suggested diarrhea as an indication of the attainment of effective reserpine dosage in the dog. However, with the exception of CNS depression, which steadily increased in incidence after a daily dosage of 0.274 mg. was reinstituted at the end of the 2nd month (Fig. 1), the severity of reserpine-induced effects upon adrenergic activity appeared to wane. For instance, the incidence of diarrhea was of short duration, and a decreased pupillary diameter was not detectable after the initial 2 months of the investigation due to the introduction of individual variability. Also, the extent of relaxation of the nictitating membrane appeared to decrease after 6–7 months of reserpine administration, even though the percentage of dogs affected remained the same. It is possible, therefore, that the peripheral effects of reserpine decline even though daily administration of the agent is continued. In contrast, adverse clinical manifestations of reserpine administration appeared to increase with the chronic daily administration of the drug.

Adverse clinical effects observed in this study were primarily severe CNS depression and a pronounced Parkinsonism-like syndrome. The presence of these effects in response to large doses of reserpine is well documented; however, this study demonstrates that the chronic daily administration of relatively small amounts of reserpine can likewise induce this characteristic syndrome in certain instances. A pronounced Parkinsonism-like condition with severe CNS depression was consistently present in only three dogs after 3, 7, and 8 months, respectively, of reserpine administration, although other dogs periodically exhibited less severe but similar signs after the initial few months of the study. This obvious clinical syndrome indicates the potentiality of toxic responses in certain, apparently susceptible, individuals following prolonged administration of relatively small amounts of the drug, even

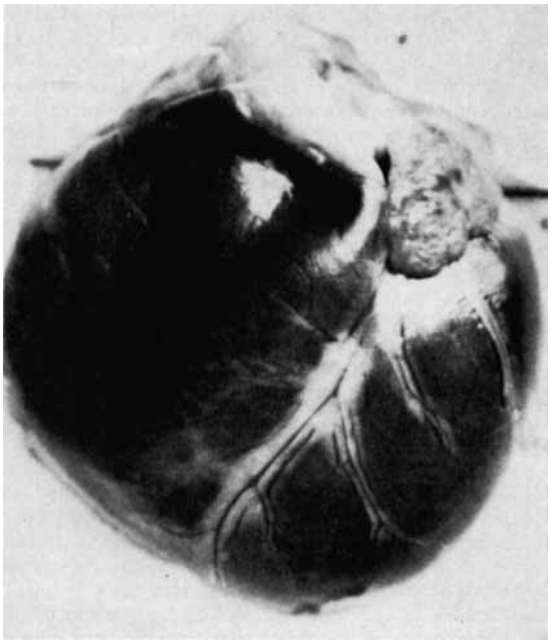

Figure 3—*Right ventricular dilatation observed in a reserpine-treated dog.*

though the mean group values for muscle tremors and CNS depression were not excessively high. Since both reserpine-induced CNS depression and Parkinsonism are probably related to the balance of brain amine levels, it appears that central pharmacologic actions of the drug, as determined by clinical evaluation, may be augmented by repeated administration whereas peripheral autonomic effects appear to decline during repeated dosage.

A possible adverse myocardial effect was another finding in the present investigation. In dogs necropsied at intervals throughout the 1-year study, right ventricular dilatation was observed in eight of 15 reserpine-treated dogs, whereas the lesion was seen in only two of 24 controls. Ventricular dilatation may be occasionally observed in clinically normal dogs following sacrifice, and this may explain the incidence of the finding in the two control dogs. However, in the present study, this represents less than 10% of the control population and, as previously mentioned, the lesion was diagnosed in approximately 50% of the treated dogs. Furthermore, pooled data obtained from all sacrifice periods indicate that the incidence in the two groups was statistically different ($p < 0.01$). This finding is in agreement with previous reports that reserpine can induce adverse alterations in myocardial function and structure in animals and, by inference, in man. Cohen *et al.* (5) examined cardiac effects of the chronic administration of 0.25 and 0.5 mg. of reserpine in patients with essential hypertension. Their study indicated that therapeutically administered reserpine may decrease cardiac output, increase atrioventricular conduction, and augment second-degree heart block during induced tachycardia in a hypertensive population. Similar antihypertensive–therapeutic doses of reserpine induced signs of cardiac failure in man (6, 7). Disruption of normal electrocardiographic patterns and premature ventricular contractions likewise were associated with therapeutic dose levels of reserpine (8, 9).

In dogs with experimental heart block, Roberts and Modell (24) observed that reserpine (0.1 mg./kg.) resulted in a high incidence of fatalities. These authors concluded that death was probably due to heart failure since cardiac enlargement, ascites, and pulmonary edema were observed upon gross necropsy examination. Withrington and Zaimis (25) and Zaimis (26) reported that circulatory failure was present in cats 24 hr. following the administration of 1 mg./kg. of reserpine. Furthermore, upon thoracotomy of these animals, the authors observed that the hearts were enlarged, flabby, and soft, and the right side appeared more severely affected. In later experiments, Withrington and Zaimis (10) studied the effects of chronic administration of doses of reserpine "of a similar magnitude to those administered to man." They concluded that the daily intraperitoneal injection of 10 mcg./kg. of reserpine in

cats for 5–26 weeks induced evidence of alterations in myocardial function.

Direct evidence of impairment of myocardial function by relatively high concentrations of reserpine, which appeared unrelated to catecholamine depletion, was presented by Nayler (27), utilizing the isolated toad ventricular strip. Histopathologic evidence of alterations of myocardial structure in cats treated with large doses of reserpine was reported by Scott and Zaimis (28). Also, marked mitochondrial swelling and fragmentation were seen upon electron microscopy of the myocardium following the daily administration of only 25 mcg./kg. of reserpine in dogs (11). It appears, therefore, that various dose levels of reserpine are capable of inducing deleterious changes upon the heart of man and animal. The present finding of right ventricular dilatation agrees with prior reports of similar lesions observed in dogs and cats. More important, it indicates that the long-term administration of relatively low doses of reserpine may introduce the possibility of adverse myocardial effects.

The hematologic determinations conducted in the present investigation generally remained within the normal limits of recognized canine blood values. However, in reserpine-treated dogs, the hematocrit and hemoglobin content were consistently and significantly lower ($p < 0.05$) than control determinations, and the total leukocyte count was consistently lower in the treated population. These findings are in contrast with the report by Earl (17), who observed no effect of chronic reserpine administration upon hematologic components in dogs; however, reserpine was administered for only 5 days a week rather than daily, as in the present study. The present hematologic findings are in agreement with Marley and Pare (7), who observed a reduced hemoglobin value and an increased plasma portion of the hematocrit following reserpine treatment in man, and, by inference, with West *et al.* (29), who observed a reserpine-mediated lack of terminal leukocytosis in leukemic mice.

Marley and Pare (7) suggested that reserpine produced a hemodilution *via* fluid retention and they rejected the possibility of altered hematopoiesis. However, impaired hematopoietic processes could induce a microcytic condition which would have given similar results and may explain the current findings. Also, the peripheral edema observed by these investigators may have been a sequela to the reserpine-induced cardiac failure rather than an indication of fluid retention leading to cardiac decompensation, as suggested. Blood volumes were unaffected by reserpine in the present study[6]; it appears, therefore, that fluid retention is an insignificant factor in reserpine-induced hematologic alterations. It is possible that the present hematologic findings induced by reserpine can be explained on the basis of an overall direct or indirect cytotoxic action of the agent, which has been suggested by previous reports (29–31). Likewise, a direct cytotoxic action of reserpine could explain the mechanism of its myocardial effect, which remains undetermined.

Reserpine is commonly administered on a chronic basis for the treatment of hypertension in man. Thus, a pathologic condition(s) of the cardiovascular system already exists upon initiation of reserpine therapy. In the present study, clinical evidence of cardiac failure was not observed. However, the incidence of cardiopathy, as detected by macroscopic examination, supports previous reports which suggest that rauwolfia preparations should be used cautiously in patients with preexisting cardiac irregularities (5–7). The findings of the present study, therefore, reinforce the need for patient surveillance during reserpine therapy. Furthermore, it is suggested that particular emphasis on surveillance of myocardial characteristics be considered during prolonged periods of administration of even small doses of reserpine.

[6] Unpublished data of B. S. Jandhyala *et al.*

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Antihypertensive Properties of Cryptenamine Used with Reserpine and Methyclothiazide

CHARLES C. KENNEDY*, RALPH E. SPIEKERMAN*, and LILA ELVEBACK†

Abstract □ By a double-blind crossover method, the hypotensive effects and side effects of cryptenamine were studied in 22 patients with Group 1 or 2 essential hypertension. The dosage administered was 2.5 mg. of methyclothiazide alone and a combination of 2.5 mg. of methyclothiazide and: (*a*) 2.0 mg. of cryptenamine, (*b*) 0.1 mg. of reserpine, and (*c*) 1.0 mg. of cryptenamine and 0.1 mg. of reserpine. Each preparation was administered four times each day for 8 weeks. The mean decrease in diastolic blood pressure with methyclothiazide alone was 10.5 mm. Hg; with methyclothiazide and cryptenamine, 15 mm. Hg; with methyclothiazide and reserpine, 21.6 mm. Hg; and with methyclothiazide, reserpine, and cryptenamine, 23.5 mm. Hg. Two patients receiving methyclothiazide and cryptenamine were unable to complete the study because of nausea or depression. Mild side effects were more frequent with the combinations. Hypokalemia was noted in 50% of the patients, and small increases in levels of uric acid, urea, and blood sugar were without complication.

Keyphrases □ Antihypertensive activity and side effects—cryptenamine, methyclothiazide, reserpine combinations □ Cryptenamine, methyclothiazide, reserpine combinations—hypotensive effects and side effects □ Methyclothiazide, cryptenamine, reserpine combinations—hypotensive effects and side effects □ Reserpine, cryptenamine, methyclothiazide combinations—hypotensive effects and side effects □ Veratrum alkaloids, cryptenamine—hypotensive effects, side effects, in combination with methyclothiazide and reserpine

When diuretics alone do not restore satisfactory blood pressure levels, an antihypertensive agent must be added. For many years, veratrum viride has been considered as a significant hypotensive agent. However, it has not been used extensively because of the small range between hypotensive and emetic doses. By means of an extraction process using nonaqueous benzene triethylamine, an alkaloid preparation called cryptenamine has been obtained from veratrum viride. With cryptenamine, studies in animals have demonstrated an appreciable difference between hypotensive and emetic doses.

The principal objective of the study was to determine the hypotensive effects and side effects of cryptenamine. As an aid in evaluating the effectiveness, a comparison was made with a drug of established efficacy, methyclothiazide. The agents used in combination with methyclothiazide were: (*a*) cryptenamine, (*b*) reserpine, and (*c*) cryptenamine and reserpine.

MATERIAL

Twenty-two ambulatory patients, who had essential hypertension of Group 1 or 2, were selected. Their average blood pressures all exceeded 160 mm. Hg systolic and 90 mm. Hg diastolic during a 1-month period in which they were receiving a placebo. Eighteen patients satisfactorily completed the study. No patient received specific antihypertensive medication other than that administered during the study, and each had clinical records verifying the presence of chronic and sustained hypertension that was not secondary to any known cause. Thirteen of the patients were women, five were men, and all were Caucasians. Their ages ranged from 41 to 71 years with an average of 55 years. The average blood pressure of these patients before treatment ranged from 120 to 240 mm. Hg systolic, with a mean systolic pressure of 179 mm., and from 76 to 126 mm. Hg diastolic, with a mean diastolic pressure of 104 mm.

Examination of the ocular fundi revealed that all 18 patients had hypertensive changes of Group 1 or 2 severity, according to the Keith–Wagener–Barker classification; that is, none had retinal exudates or hemorrhages. Renal function was apparently adequate, the concentration of blood urea being within or close to normal limits in all patients. None of the patients had congestive heart failure or edema, and none was taking digitalis. No patient had symptomatic cerebrovascular impairment or residue from previous injury to the brain.

METHODS

All patients underwent complete physical and appropriate laboratory examinations before beginning the study. Thereafter, they came to the clinic at weekly intervals at their convenience, almost invariably at the same time of the day each week. The examining physician did not know which preparation the patient was receiving. After the patient rested for a short period, the blood pressure was taken three times, first with the patient in the sitting position and then twice after the patient had been standing for 1 min. The two lowest values for blood pressure (sitting and standing), the pulse rate, and body weight were recorded, along with relevant notes regarding possible side effects.

At regular monthly intervals during the entire study, fasting blood samples were obtained in the clinic laboratory for determination of levels of serum sodium, serum potassium, serum chloride, carbon dioxide, blood sugar, urea, and uric acid. An excretory urogram was obtained on all patients prior to beginning the study, and none gave evidence of a renal cause for hypertension. At the beginning and at the end of the study, the following laboratory tests were obtained: leukocyte and differential counts, hemoglobin, serum GOT, serum alkaline phosphatase, urinalyses, urine amylase, and urine metanephrines. In addition, chest roentgenograms for each patient were obtained before and after the study. Additional examinations were done when needed.

The study was carried out as a double-blind crossover procedure. The medications and placebo were taken four times each day at approximately 4-hr. intervals. The use of all previous antihypertensive medications was stopped, and the patient was given a placebo during the 1st month of the study. Each of the four medications was then given for a period of 2 months. The active medication was dispensed under a code number in a sequence determined by a person who did not have contact with the patients. The study lasted 9 months. Blood pressure recordings during the first 2 weeks of each 2-month period were disregarded because of the possible sustained effect of reserpine. The amount of drug per dose was 2.5 mg. of methyclothiazide; 2.5 mg. of methyclothiazide with 0.1 mg. of reserpine; 2.5 mg. of methyclothiazide with 2.0 mg. of cryptenamine; and 2.5 mg. of methyclothiazide, 0.1 mg. of reserpine, and 1.0 mg. of cryptenamine.

As mentioned previously, 22 patients were initially included in the study. One patient was excluded from the study because of inconsistencies in taking the medication regularly. Another was excluded because of the need for more vigorous antihypertensive treatment. Two others experienced drug intolerance and were unable to finish the study. After the completion of the study, the code was revealed; the values obtained during the pretreatment, placebo, and treatment periods were tabulated.

RESULTS

Effect on Blood Pressure—All medications, when compared to methyclothiazide, produced an increased hypotensive effect. The

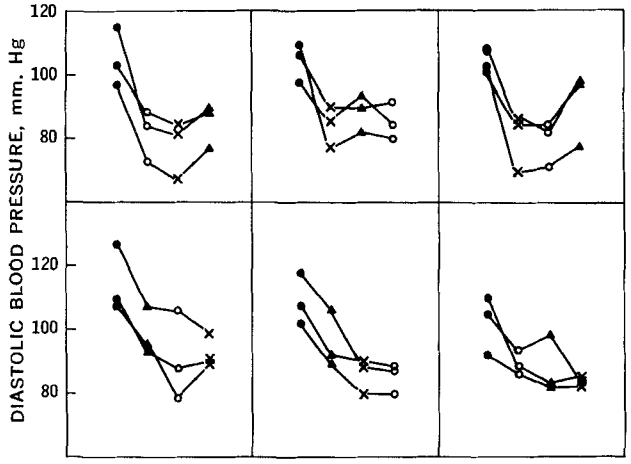

Figure 1—*Distribution of diastolic blood pressures for 18 patients in six orders of drug administration. Each of the six orders is represented by values from three patients. Each of the four medications was given for a period of 2 months. Key: ●, methyclothiazide alone; ▲, cryptenamine with methyclothiazide; ○, reserpine with methyclothiazide; and ×, combination of cryptenamine, reserpine, and methyclothiazide.*

mean decrease in diastolic blood pressure with methyclothiazide when compared with control levels was 10.5 mm. Hg. The effectiveness of the other drugs was compared with methyclothiazide. The mean diastolic blood pressure decreased an additional 5 mm. during treatment with methyclothiazide and cryptenamine (15 mm., as contrasted to control levels). The mean diastolic blood pressure decreased 11 mm. with methyclothiazide and reserpine (21.6 mm., as contrasted to control levels). The mean diastolic blood pressure decreased 13 mm. when the combination of methyclothiazide, reserpine, and cryptenamine was employed (23.5 mm., as compared to the control levels).

The sequence in which the medication was administered did not affect the therapeutic effectiveness (Fig. 1).

Effect on Pulse and Body Weight—The mean pulse rate was slowed with the methyclothiazide that contained cryptenamine, reserpine, or reserpine and cryptenamine, as contrasted to the rate

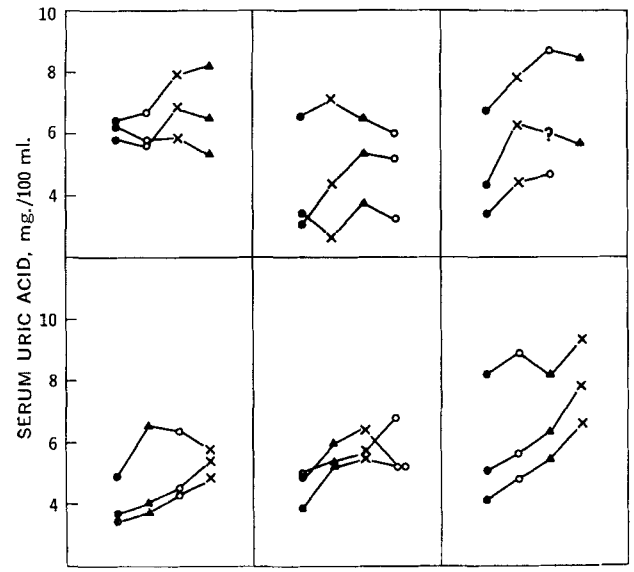

Figure 2—*Distribution of serum uric acid levels for 18 patients in six orders of drug administration. Each of the six orders is represented by values from three patients. Each of the four medications was given for a period of 2 months. Key: ●, methyclothiazide alone; ▲, cryptenamine with methyclothiazide; ○, reserpine with methyclothiazide; and ×, combination of cryptenamine, reserpine, and methyclothiazide.*

with methyclothiazide alone. The decrease ranged from 4 to 16 beats/min. The mean body weight was essentially unchanged with all medications.

Effect of Constituents on Blood—*Serum Potassium*—Nine of the 18 (50%) patients required an orally administered potassium supplement to maintain their serum potassium level in a normal range of 4–5 meq./l. Hypokalemia was consistently present with all medications in patients who required the supplemental use of potassium. The mean decrease in patients who required potassium was 0.5 meq., and the lowest potassium value was 2.9 meq./l. No symptoms could be attributable to hypokalemia in any of these patients. One patient was withdrawn from the study for evaluation of hyperaldosteronism, but the diagnosis could not be established, and antihypertensive treatment was reinstituted.

Serum Sodium—There was no significant change in mean serum sodium value during treatment with any of the medications, except for one patient. This patient persistently had a serum sodium level that ranged from 125 to 130 meq./l.

Serum Chloride—All patients tended to have serum chloride levels that were at the lower limits of normal. Four had mild hypochloremia in the range of 90–97 meq./l. The one patient with low levels of serum sodium also had slightly reduced levels of serum chloride; the values usually varied from 82 to 91 meq./l. No one medication consistently produced hypochloremia.

Plasma CO_2-Combining Power—Compared to that of patients taking methyclothiazide alone, CO_2-combining power was generally elevated a slight amount. In 60 of 144 determinations of CO_2-combining power, the values were elevated slightly above the normal range. The two highest levels were 37 and 36 meq./l. All medications produced mild elevation with equal frequency.

Serum Uric Acid—The serum uric acid levels increased during treatment in nine of the 18 patients, as compared with uric acid levels obtained when the patient was taking methyclothiazide alone (Fig. 2). Only three female patients and no male patient had mild elevations of the serum uric acid level while taking methyclothiazide alone. Six women and three men had mild hyperuricemia during combination treatment. The elevation in uric acid levels occurred with equal frequency with all three drugs given. The highest level of uric acid found was 9.5 mg./100 ml. in a female. There was no incidence of gouty arthritis or renal calculus during the study.

Blood Urea—Mild elevation of blood urea levels occurred in seven patients. The highest value was 55 mg./100 ml. (normal 20–40 mg./100 ml.). The majority of values ranged from 41 to 46 mg./100 ml. All medications produced this degree of elevation with equal frequency.

Blood Sugar—Nine patients had mild hyperglycemia which was not increased by the medication and which occurred consistently with all medications in the same patient.

Other Blood Counts—No abnormalities or significant changes were found in hemoglobin concentration, leukocyte counts, or differential blood cell counts when values before and after treatment were compared.

Side Effects—Two patients were unable to complete the study because of side effects. One of these patients had moderately severe nausea while taking methyclothiazide and cryptenamine. The nausea subsided after the use of the medication was discontinued and recurred when the dosage was resumed. The other patient had an agitated depression while taking this same combination of medication. The symptoms subsided within 10 days after the medication was discontinued and during the coincident administration of amytriptyline[1]. None of the other side effects was severe enough to preclude taking the drugs four times each day. A summary of side effects is listed in Table I. Three patients had no side effects during the entire study. Some of the more nonspecific complaints which could be associated with minor coincidental acute infections were not included as side effects.

The major side effects were weakness, fatigue, nausea or anorexia, and lightheadedness. Weakness and fatigue were complaints of equal frequency with any combination containing cryptenamine or reserpine or both and may well have been dose related inasmuch as these compounds were taken four times daily. Lightheadedness and nausea or anorexia occurred equally with all medications. These symptoms generally subsided in 2–3 weeks and would seem to be

[1] Elavil.

Table I—Side Effects in Drug Administration for Essential Hypertension

Side Effect	Placebo	Methyclothiazide	Methyclothiazide and Cryptenamine	Methyclothiazide and Reserpine	Methyclothiazide, Reserpine, and Cryptenamine
Lightheadedness	1	2	2	0	2
Nausea or anorexia	0	2	3	2	1
Weakness and fatigue	1	0	3	3	3
Nasal congestion	0	0	0	1	2
Thirst	0	1	0	0	0
Bloated	1	0	0	1	1
Total	3	5	8	7	9

related to the diuretic effect of methyclothiazide after the placebo period. These patients had a weight reduction of 1.49–2.98 kg. (4–8 lb.), and the symptoms may have been caused by a sudden decrease in plasma volume. Nasal congestion was experienced by three patients while receiving medication containing reserpine. No patients complained of diarrhea, palpitations, or blurred vision. In patients who were free of side effects, the medications containing reserpine or reserpine and cryptenamine in combination were considered to be the most satisfactory antihypertensive medications.

COMMENT

The pharmacodynamics of the hypotensive action of veratrum or of its active ester alkaloids have been considered by many investigators to constitute an ideal mechanism because blood pressure is lowered by dilatation of the arterioles without concomitant peripheral adrenergic or ganglionic blockade. Furthermore, the functioning of the autonomic nervous system remains unimpaired, even though the means by which the arterioles are dilated is essentially neurogenic. Thus, there is little likelihood of peripheral pooling of the blood (and orthostatic hypotension), and all or most of the normal vascular pathways remain intact. The basic mechanism by which the veratrum alkaloids produce their hypotensive responses was long believed to be primarily reflex in nature, whereby the reflex actions were initiated by stimulation of afferent fibers which run in the vagi aortic nerves (von Bezold-Jeriah) reflex. Although this mechanism undoubtedly has a role, several studies showed that the primary mechanisms of action are related to more direct central effects of these alkaloids, such as stimulation of the carotid sinus receptors, depression of the central vasomotor mechanism, or both of these mechanisms (1–3). More recently, studies by Jandhyala and Buckley (4) demonstrated that cryptenamine significantly decreased the epinephrine content of adrenal venous blood while increasing the norepinephrine content, thus suggesting that the drug may inhibit the methylation of norepinephrine in the adrenal medulla.

The data from the present study are interpreted as showing that in a wide range of patients with Group 1 or 2 essential hypertension, cryptenamine in combination with methyclothiazide was significantly effective in lowering blood pressure. Only a minimal additive hypotensive effect was obtained by adding cryptenamine to the reserpine–methyclothiazide combination. However, the dose of cryptenamine used was reduced to 1 mg. when given with reserpine. If the dose had been maintained at 2 mg., perhaps a more significant response would have been obtained. Although the drugs were administered four times each day in this study, a significant number of patients with essential hypertension of Grade 1 or 2 could be controlled by a twice daily dose. It would seem justifiable to make two similar trials of cryptenamine in patients with moderate hypertension, one utilizing a higher dose in combination with methyclothiazide (or ideally a potassium-sparing diuretic), given two to four times a day, and another utilizing a higher dose in combination with 0.1 mg. of reserpine and methyclothiazide (or a potassium-sparing diuretic), given two or four times a day. The optimal well-tolerated oral dose of cryptenamine should be determined. A better decision could then be made regarding the usefulness of the three drugs (methyclothiazide, cryptenamine, and reserpine) in combination.

The response of patients to methyclothiazide alone was similar to that previously reported by Spiekerman *et al.* (5). The changes in serum electrolyte levels were comparable to those reported previously with the administration of thiazide diuretics. In the present study, half of the patients required the supplemental use of oral potassium. Elevations of serum uric acid occurred in 50% of the patients (9 of 18) who were taking methyclothiazide with cryptenamine or reserpine, as compared to those taking methyclothiazide alone. The specific mechanism by which uric acid levels were increased is not known. However, it may be that cryptenamine and reserpine interfere with the reabsorption of filtered urate in the tubules or with the active secretion of urate in a manner such as that postulated for other drugs including the thiazide diuretics or pyrazinoid acid amide. Symptomatic gout did not develop in any of the patients who had hyperuricemia. Other small electrolyte changes noted were mild elevations of CO_2-combining power (60 of 144 determinations).

The medications generally were tolerated well. The two patients who stopped taking the combination of methyclothiazide and cryptenamine had a definite abrupt onset of symptoms (nausea and depression) which subsided when the use of the medication was withdrawn. These were the only serious side effects noted in the study and seemed to be drug related rather than dose related.

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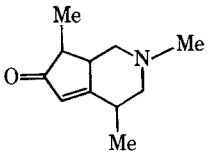
Stability of Tecomine, the Major Antidiabetic Factor of *Tecoma stans* (Juss.) f. Bignoniaceae

YOUSSEF HAMMOUDA and NAWAL KHALAFALLAH

Abstract □ Tecomine, a recently isolated alkaloid with considerable hypoglycemic activity in experimental animals but apparently low stability, was subjected to stability study. The results indicate that the degradation of the alkaloid is dependent on the pH of its solution and that antioxidants are beneficial in delaying its deterioration. Apart from the stability study, a spectrophotometric method was established for the assay of tecomine in the presence of its degradation products, the dissociation constant of the alkaloid was determined, and a rapid and efficient method was introduced for quantitative recovery of tecomine (in a pure form) from tecomine picrate, the stable crystalline form of the alkaloid.

Keyphrases □ Hypoglycemic activity—tecomine, stability evaluation □ Tecomine—degradation rates, temperature and pH effects, hypoglycemic activity □ Tecomine picrate—ion-exchange isolation of tecomine □ UV spectrophotometry—analysis, tecomine

Tecomine, the major alkaloid of *Tecoma stans* (Juss.), was isolated for the first time by Hammouda and Motawi (1) in 1959. In 1963, Jones *et al.* (2) isolated, from the same plant, a similar alkaloid for which they proposed Structure I. The identity of the two compounds was established in the same year by direct comparison of their IR spectra and mixed melting point of their picrates (3).

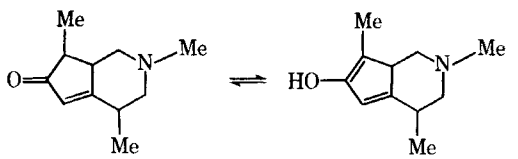


I

In 1964, Hammouda *et al.* (4, 5) showed that tecomine possesses valuable hypoglycemic activity, with a wide margin of safety, when tested on experimental animals (4, 5), thus confirming the wide reputation of the plant as an antidiabetic drug used by the Mexican people (6).

The instability of the antidiabetic factor of the leaves of Tecoma, expressed in the precautions taken by the Mexican pharmacopeia (6) for the drying of the leaves, is again confirmed by the rapid discoloration of tecomine; the colorless liquid alkaloid suffers rapid darkening in color on standing at room temperature.

This apparent instability of tecomine, which would probably affect its antidiabetic activity, made it necessary to subject the alkaloid to a stability study to establish the best conditions of formulation of the alkaloid for medicinal use. In the present work, the degradation rates of tecomine under varying conditions of temperature



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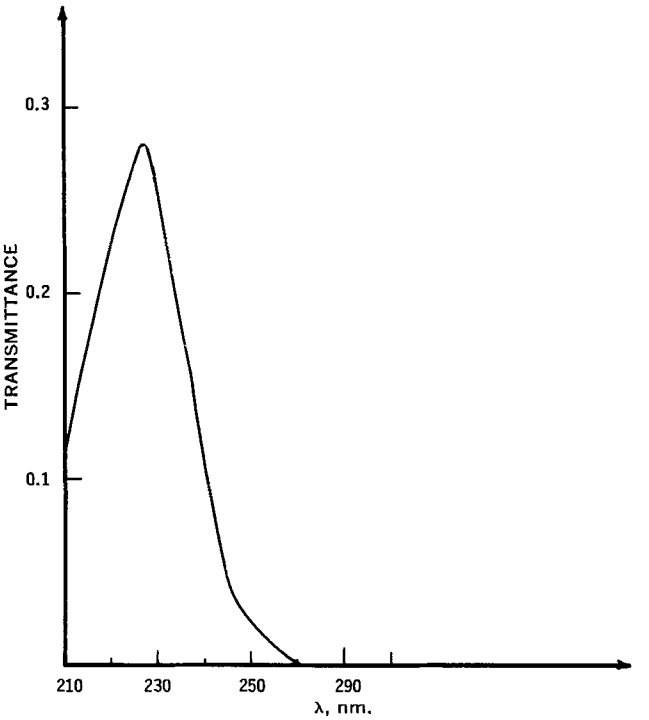

Figure 1—*UV spectrum of tecomine.*

and pH were investigated, together with the role of antioxidants in preventing or delaying deterioration. In planning the stability study, an oxidation reaction was anticipated to contribute largely to the overall degradation of tecomine; this assumption was based on the presence in tecomine of a ketonic group, which undergoes enolization to a partially phenolic group (Structure II).

EXPERIMENTAL

Materials—The following were used: tecomine[1] picrate, m.p. 176° (uncorr.); Amberlite resin, IRA 400 (Cl)[2]; and buffer solutions (7) consisting of suitable mixtures of analytical grades of hydrochloric acid, citric acid, phosphoric acid, boric acid, and sodium hydroxide.

Apparatus—A Unicam Sp 500 spectrophotometer and a pH meter, PYE model 75, were used.

Assay Method—Tecomine in EtOH was reported to exhibit a peak at 226 nm. (2). In the present study, tecomine picrate was used as the starting material for the stability study owing to the ease of crystallization and good keeping quality. Figure 1 represents the UV spectrum of tecomine picrate in water, using as blank an equivalent aqueous solution of picric acid; a peak at 227 nm. is apparent for tecomine (ϵ at 227 nm. = 18.02×10^3). Aqueous solutions of tecomine were found to obey the Beer–Lambert law in concentrations reaching 1 mg. % (calculated as tecomine picrate), thus permitting

[1] Extracted from freshly collected, shed dried leaves, using the method described by Hammouda and Motawi (1).
[2] British Drug House, analytical grade.

Table I—Effect of pH on Peak Position of Tecomine

pH	Peak Position, nm.
2.2	227
3.3	227
5.5	227
7.8	229
11.5	233

the application of UV spectroscopy in the assay of pure samples of tecomine. In constructing the standard curve of tecomine used in the assay, each dilution of tecomine picrate solution was measured spectrophotometrically at 227 nm., using as blank an equivalent aqueous solution of picric acid.

Examination of Possible Interference of Degradation Products of Tecomine with Assay of Undecomposed Tecomine—During the stability study, the samples of tecomine solution withdrawn at increasing time intervals showed a gradual decrease in the magnitude of absorption at 227 nm. Examination of the UV spectrum of solutions containing tecomine together with its degradation products did not reveal any additional new peaks other than that at 227 nm. Solutions in which the extent of degradation reached ≈20% of the initial tecomine concentration were characterized by the appearance of a yellow color, increasing in intensity as degradation proceeded. This color presented no problem in the assay of undecomposed tecomine since, in the dilution reached for tecomine measurement, the yellow color visually disappeared and the solution was found transparent apart from the peak at 227 nm.

Effect of pH on Peak Position of Tecomine—Buffered aqueous solutions of tecomine, prepared according to the procedure outlined in the next paragraph, were examined spectrophotometrically; the results are recorded in Table I. The variation in the peak position of tecomine with pH made it necessary to adjust the pH below 5 prior to assay. Hence, all samples of tecomine solution withdrawn during the stability study were diluted to a known volume with buffer (pH 4) and then read at 227 nm.

Preparation of Aqueous Tecomine Solution for Stability Study—The anion-exchange resin, Amberlite IRA 400 (Cl), after treatment with NaOH, proved efficient in liberating tecomine from tecomine picrate and in retaining the picrate anion while the tecomine base was eluted with a suitable solvent. Three grams of the resin packed in a glass column, 1.5 cm. in diameter, was treated with 2 *N* NaOH until almost free from chloride; it was then washed with distilled water followed by EtOH. Tecomine picrate was applied to the column as an alcoholic solution (5 ml.). Elution was performed with EtOH; tecomine was recovered in the first 50 ml. eluent. Three grams of the resin was used for the transformation of up to 200 mg. of tecomine picrate into an equivalent amount of tecomine. To the

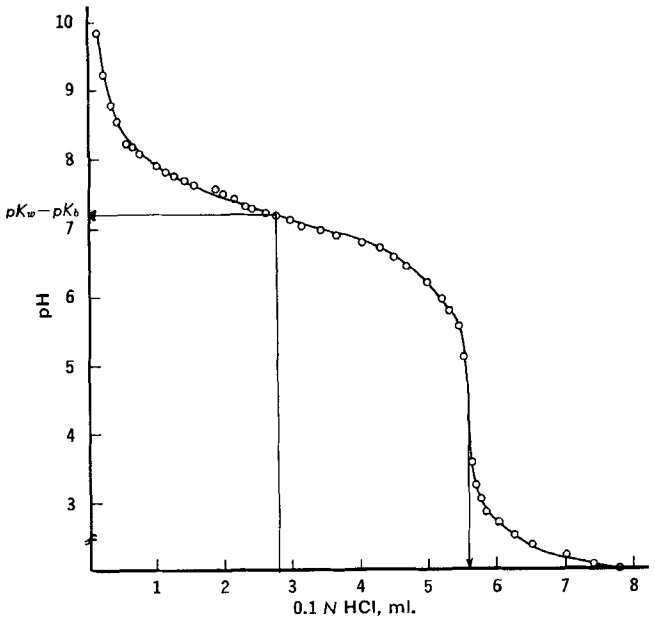

Figure 2—*Neutralization curve of tecomine.*

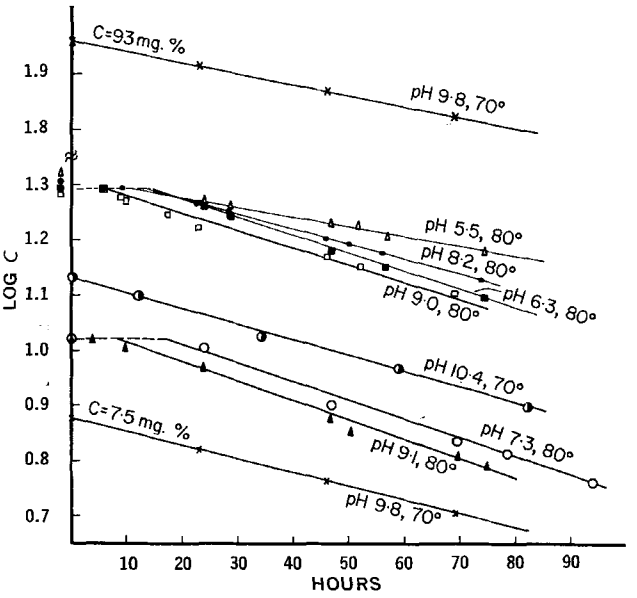

Figure 3—*Effect of pH on degradation of tecomine at 70 and 80°.*

eluent, a slight excess of 0.1 *N* HCl was added; EtOH was removed by distillation, and the resulting tecomine hydrochloride solution was adjusted with distilled water to a suitable volume. Spectrophotometric assay indicated that ≈90% of the amount of tecomine applied to the column was recovered in the first 50 ml. eluent. The subsequent eluent recovered was free from any tecomine base or tecomine picrate, as indicated by the absence of color and transparency at 227 nm.

Stability Study—Ten milliliters of the standardized tecomine hydrochloride solution was added to 80 ml. of buffer solution, previously heated to the desired temperature, in a 100-ml. volumetric flask. The volume was adjusted with buffer solution, and the flask was placed in a thermostatically controlled water bath. Samples were withdrawn at 0 hr. and subsequently at suitable time intervals while the flask remained in the water bath. Each sample (3 ml.) was cooled before withdrawing 1 ml. for analysis. This procedure was used in the study of the effect of pH and temperature variation on the rate of tecomine degradation, as well as in the study of the effect of 0.1% w/v sodium sulfite as antioxidant on the degradation rate of the alkaloid in alkaline medium. In the latter case, 0.1 g. of sodium sulfite was accurately weighed and added to the contents of the flask before completing to volume; one flask, devoid of tecomine, was included as a blank for spectrophotometric analysis to overcome the absorption of sodium sulfite at 227 nm.

Determination of pKb of Tecomine—This experiment was undertaken to identify the various tecomine species present along the

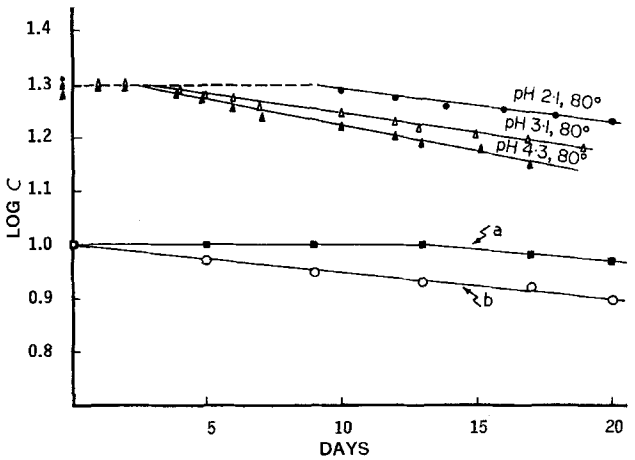

Figure 4—*Effect of pH on degradation of tecomine at 80°. Effect of 0.1% w/v Na_2SO_3 on the degradation rate of tecomine at pH 10.4 and 40° with: (a) Na_2SO_3 present, and (b) Na_2SO_3 absent.*

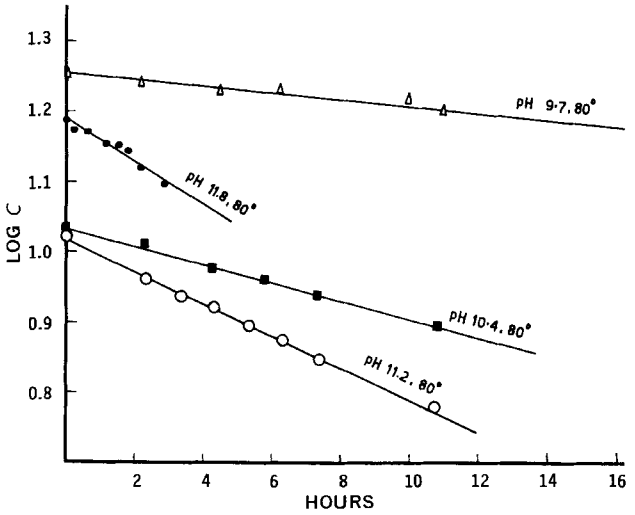

Figure 5—*Effect of pH on degradation of tecomine at 80°.*

entire pH range and to assist in the interpretation of results obtained on the effect of pH variation on the rate of tecomine degradation. The pKb value of tecomine was determined by titrating an aqueous solution of the base with 0.1 *N* HCl and recording pH changes during the neutralization reaction, using a glass electrode maintained dipped in the solution. Continuous agitation was performed with a magnetic stirrer. The solution of tecomine base was prepared by applying 5 ml. of tecomine hydrochloride solution (90 mg. tecomine) to 3 g. of the anion-exchange resin, pretreated in the usual manner with 2 *N* NaOH and washed with distilled water. Elution was performed with distilled water; the first 25 ml. eluent was found to contain 85 mg. of tecomine in the form of free base. Twenty-four milliliters of the collected eluent (81.6 mg. tecomine) was used for the determination of pKb. The results are illustrated in Fig. 2. The pH corresponding to half-neutralization was found to be 7.2, giving a pKb of 6.8.

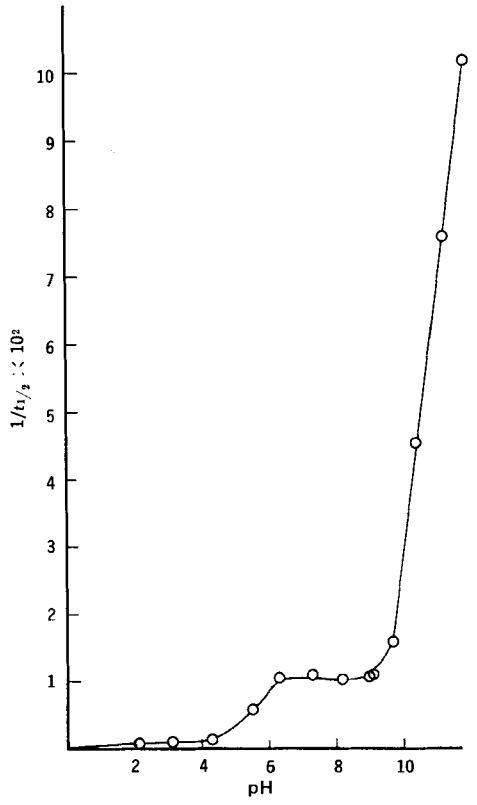

Figure 6—*Plot of $1/t_{1/2}$ as a function of pH.*

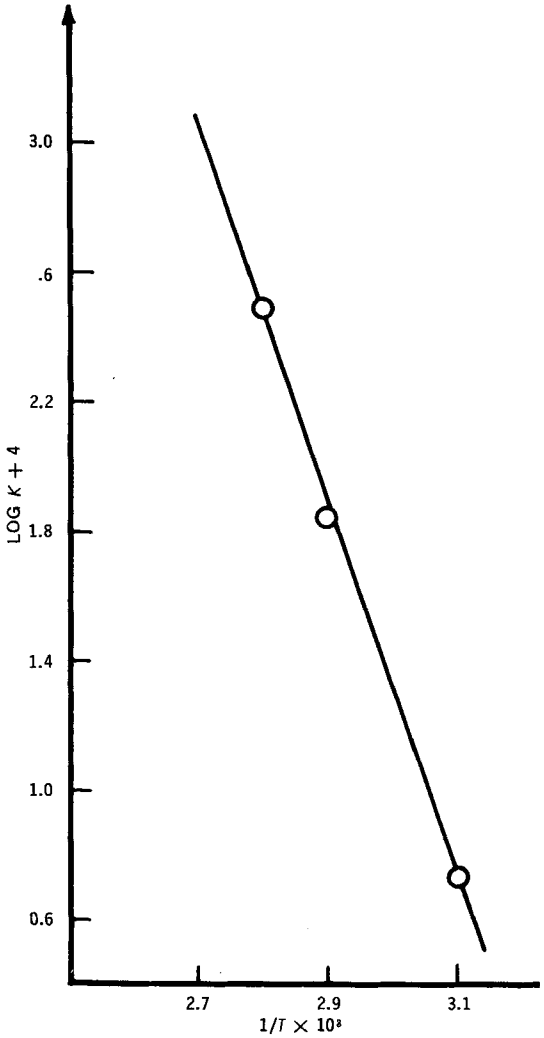

Figure 7—*Arrhenius plot of degradation of tecomine at pH 10.4.*

RESULTS AND DISCUSSION

Reaction Order of Tecomine—The rates of tecomine degradation in the various buffered media followed a first-order reaction with respect to tecomine concentration, as is apparent in the linear logarithmic plots (Figs. 3–5)[3]. *K*, the rate constant, was found to be unaffected by the initial tecomine concentration, as is evident from the identical slopes (Fig. 3, pH 9.8, 70°); this lends further proof to the observed order of the degradation reaction.

Effect of pH on Tecomine Degradation—The rate of tecomine degradation was determined at a temperature of 80° and at pH values ranging from 2 to 12 (Figs. 3–5). It is apparent that the degradation rate is mainly dependent on the hydroxyl-ion concentration. At pH values between 2 and 9, the degradation pattern was characterized by the presence of an initial lag period, during which no degradation could be detected; the lag period was found to decrease with a rise in pH and disappeared above pH 9. The shape of the pH–rate profile (Fig. 6) indicates that the rate of degradation is influenced by the type of tecomine species present.

In the neutralization curve (Fig. 2), it is apparent that tecomine is present in the protonated form at pH values below 5; above pH 9, it occurs in the free base form. The results expressed in Figs. 3–6 indicate that both the protonated and free base forms of tecomine undergo degradation proportional to the hydroxyl-ion concentration but at different rates. At pH values between 5 and 9, where tecomine is present as a mixture of both species, the rates of degradation are apparently not proportional to the hydroxyl-ion concen-

[3] *C*, the concentration of tecomine, is expressed as milligrams percent of tecomine picrate.

tration. The overall shape of the pH–rate profile obtained for tecomine is not uncommon among degradation patterns of weak bases involving either oxidation or hydrolysis; similar profiles were obtained in the oxidative degradation of morphine (8) and in the hydrolysis of homatropine (9). From the study of the effect of pH on the rate of tecomine degradation, it can be deduced that acid medium, below pH 4, is appropriate for obtaining a stable tecomine preparation.

Effect of Temperature on Tecomine Degradation—The rate of tecomine degradation was determined at pH 10.4 and temperatures of 40, 70, and 80° (Figs. 3–5). Figure 7 represents the Arrhenius plot from which the value of k at 25° was found to be 1.995×10^{-3} hr. and $t_{1/2}$ = 14 days at pH 10.4. The energy of activation and the frequency factor were calculated and found to be 26.67 kcal./mole and 1.099×10^{13}/min., respectively.

Effect of Antioxidants on Tecomine Degradation—The rate of tecomine degradation in alkaline medium was significantly decreased in the presence of 0.1% sodium sulfite (Fig. 4); furthermore, the presence of antioxidant delayed the onset of degradation for almost 2 weeks at pH 10.4 and 40°. The results obtained increase the probability of oxidation being the mechanism responsible for degradation.

CONCLUSION

The results of the stability study performed on tecomine point to an oxidation reaction, accompanied by the formation of highly conjugated colored oxidation products, as the most probable degradation route. The decrease in the degradation rate in the presence of antioxidant, the appearance of colored degradation products, and the presence in tecomine of a carbonyl group susceptible to enolization all point to the probability of oxidation. The changes observed in the UV spectrum of tecomine during degradation are in accordance with the postulated degradation route. Theoretically, the peak at 227 nm. resulting from the presence in tecomine of a conjugated system may be subject to a bathochromic shift if further conjugation is added to the structure. Or it may disappear without the appearance of additional peaks if the original conjugation is destroyed. In the present study, the disappearance of the peak at 227 nm. during degradation was accompanied by the occurrence of absorption in the visible range, thus favoring the formation of highly conjugated colored oxidation products.

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Kinetics of Hydrolysis of Barbituric Acid Derivatives

EDWARD R. GARRETT, JACEK T. BOJARSKI*, and GERALD J. YAKATAN

Abstract □ The neutral and alkaline hydrolyses of barbituric acid and some of its substituted derivatives were followed spectrophotometrically. The rate–pH profiles for all of the 5,5-disubstituted barbiturates were similar. A different profile was observed for barbituric acid because of its comparatively low pKa_1'. The rate–pH profile of metharbital showed no curvature at high pH values, since the 1-methyl substituent prevents the second possible proton dissociation. All profiles could be explained by hydroxyl-ion attack on the undissociated and monoanion forms of the barbiturate, whereas the dianions are unreactive. The reactivity of the barbiturates was correlated with the Newman rule of six as a measure of steric influence on hydrolysis. A previously unmentioned, reversible system between the barbiturate and its ring-opened malonuric acid derivative was observed and may be used to postulate that the fraction decomposing *via* 1,6 ring opening is a function of pH rather than a direct consequence of a unique degradation pathway for the ionic form of the barbiturate. Ionic strength effects in the alkaline hydrolyses of amobarbital and phenobarbital increase the rate of barbiturate degradation by the attack of hydroxyl ion on the monoanion and/or its kinetic equivalent. The Arrhenius parameters for all compounds were determined.

Keyphrases □ Barbituric acid and 5,5-disubstituted barbiturates—hydrolysis kinetics, pH effect □ Hydrolysis kinetics, parameters—barbituric acid and derivatives □ UV spectrophotometry—monitoring, barbituric acid hydrolysis

Interest in the kinetics of barbiturate solvolysis arose from studies on halouracils and halouridines which showed that barbituric acid and ribosylbarbituric acid, respectively, were formed as hydrolytic intermediates in strong alkali (1, 2). Although the decomposition of barbiturate salts in aqueous solution or in the presence of alkali from room to autoclaving temperatures was reported in a descriptive manner (3–12), systematic kinetic approaches to the hydrolysis of barbiturates were undertaken in only a few instances (13–20). These studies include the excellent work of Eriksson and coworkers (16–19) on the hydrolysis of 1,5,5-trisubstituted barbiturates and the only published report on thiobarbiturate solvolysis by Goto *et al.* (20). Nevertheless, quantitative kinetic data pertaining to the stability of many pharmaceutically useful barbiturates are still lacking. The literature indicates that many questions regarding the alkaline hydrolysis of barbituric acid derivatives, such as mechanism, ionic strength effects, rate–pH profiles,

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Keyphrases □ Barbituric acid and 5,5-disubstituted barbiturates—hydrolysis kinetics, pH effect □ Hydrolysis kinetics, parameters—barbituric acid and derivatives □ UV spectrophotometry—monitoring, barbituric acid hydrolysis

Interest in the kinetics of barbiturate solvolysis arose from studies on halouracils and halouridines which showed that barbituric acid and ribosylbarbituric acid, respectively, were formed as hydrolytic intermediates in strong alkali (1, 2). Although the decomposition of barbiturate salts in aqueous solution or in the presence of alkali from room to autoclaving temperatures was reported in a descriptive manner (3–12), systematic kinetic approaches to the hydrolysis of barbiturates were undertaken in only a few instances (13–20). These studies include the excellent work of Eriksson and coworkers (16–19) on the hydrolysis of 1,5,5-trisubstituted barbiturates and the only published report on thiobarbiturate solvolysis by Goto *et al.* (20). Nevertheless, quantitative kinetic data pertaining to the stability of many pharmaceutically useful barbiturates are still lacking. The literature indicates that many questions regarding the alkaline hydrolysis of barbituric acid derivatives, such as mechanism, ionic strength effects, rate–pH profiles,

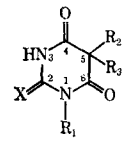


Table I—Compounds Used in This Study

Number	Compound	Substituents R1	R2	R3	X	λmax. (nm.) of Monoanion at pH 10.1	Source
1	Barbituric acid	H	H	H	O	256	Eastman Organic Chemicals
2	Amobarbital	H	$-CH_2-CH_3$	$-CH_2-CH_2-CH(CH_3)_2$	O	239	Eli Lilly and Co.
3	Barbital	H	$-CH_2-CH_3$	$-CH_2-CH_3$	O	238	Retort Pharmaceutical Co.
4	Butabarbital	H	$-CH_2-CH_3$	$-CH(CH_3)-CH_2-CH_3$	O	239	McNeil Laboratories
5	Butethal	H	$-CH_2-CH_3$	$-CH_2-CH_2-CH_2-CH_3$	O	238	Abbott Laboratories
6	Cyclobarbital	H	$-CH_2-CH_3$		O	233	Sterling Winthrop Research Institute
7	Pentobarbital	H	$-CH_2-CH_3$	$-CH(CH_3)-CH_2-CH_2-CH_3$	O	239	Abbott Laboratories
8	Phenobarbital	H	$-CH_2-CH_3$		O	240	Mallinckrodt Chemical Co.
9	Vinbarbital	H	$-CH_2-CH_3$	$-C(CH_3)=CH-CH_2-CH_3$	O	236	Merck Sharp & Dohme Laboratories
10	Secobarbital	H	$-CH_2-CH=CH_2$	$-CH(CH_3)-CH_2-CH_2-CH_3$	O	239	Eli Lilly and Co.
11	Thiamylal	H	$-CH_2-CH=CH_2$	$-CH(CH_3)-CH_2-CH_2-CH_3$	S	303	Parke, Davis & Co.
12	Metharbital	CH_3	$-CH_2-CH_3$	$-CH_2-CH_3$	O	245	Abbott Laboratories

and structure–hydrolytic activity relationships, have not been completely answered. These present studies on therapeutically useful barbiturates (Table I) were begun with the expectation that a systematic quantitative approach to the problem would help answer some of these questions.

EXPERIMENTAL

Materials—The barbiturates studied were supplied or purchased from the manufacturers listed in Table I and were used without further purification. Compounds 1, 5, 9, and 12 were obtained as the free acids; the others were available as the sodium salts. Diethylmalonuric acid (m.p. 159–161° dec.; after solidifying, m.p. 206–207°) and diethylacetylurea (m.p. 208–209°) were obtained by hydrolysis of barbital according to the method of Aspelund and Skoglund (21). Silica gel GF_{254}[1] and cellulose powder MN 300 UV_{254}[2] were used for TLC experiments. All other materials employed in this study were of analytical reagent grade.

Kinetic Procedures—Appropriate quantities of the barbiturates to produce final concentrations of 10^{-2} *M* were weighed into volumetric flasks and diluted to volume with distilled water. From these stock solutions, aliquots were taken and diluted with alkali of appropriate concentration or with a buffer solution of known pH to produce, in general, a final barbiturate concentration of about 10^{-4} *M*. The solutions of alkali and buffer were equilibrated at the temperature of the study prior to initiation of the reaction.

The reaction flasks were maintained in constant-temperature oil baths, controlled within 0.1°, at selected temperatures between 60 and 80°. Samples were withdrawn at suitable time intervals and cooled to room temperature. Spectrophotometric readings at the absorption maximum ($\lambda_{max.}$) were taken on the Beckman model DU-2 spectrophotometer, or the entire spectrum was recorded on the Cary model 15 spectrophotometer. Matched spectrophotometric cells (10 mm.) were used for all measurements, and a slit width of 0.10 mm. was employed. Since the $\lambda_{max.}$ values of the barbiturates vary with pH, the appropriate values for each compound were experimentally determined in each alkali and buffer solution prior to reaction. Table I lists some pertinent $\lambda_{max.}$ values. Samples in the lower pH buffer solutions, where the barbiturates exist predominantly in the nonchromophoric unionized form, were adjusted to about pH 11.5 by the addition of an appropriate volume of sodium hydroxide solution immediately before spectrophotometric measurements.

Alkali solutions were prepared by dilution of a standardized sodium hydroxide solution[3] with distilled water. The effect of ionic strength on the reaction rate in alkaline solutions was checked by adding varying amounts of potassium chloride to the reaction flasks. All reactions run in buffer solutions were maintained at a constant ionic strength of 0.15, where possible.

pH Measurements—The pH values of the sodium hydroxide solutions at the temperature of study were calculated from activity coefficient data in the literature (22). The pH values of the buffer solutions were measured directly at the temperature of study on a Photovolt pH meter equipped with high-temperature electrodes. The pH meter was standardized with standard buffer solutions at the same temperature.

TLC—Thin-layer chromatograms were made by coating 20 × 20-cm. glass plates with a 250-μ layer of either silica gel GF_{254} or cellulose MN 300. The chromatograms were usually spotted with 10–20 μl. of sample (10^{-2} *M*) and developed in closed tanks for the time required for the solvent front to travel 15 cm. from the starting line. After drying, the plates were viewed under a

[1] E. Merck AG, Darmstadt, Germany.
[2] Machery, Nagel & Co., Düren, Germany.
[3] Acculute, Anachemia Chemicals Ltd., Champlain, N. Y.

Table II—Apparent First-Order Rate Constants, 10^6k in sec.$^{-1}$, for Hydrolysis of Some Barbiturates at 80.0°

pH	Buffers [H_2PO_4]	Buffers [HPO_4^{-2}]	Barbital	Phenobarbital	Amobarbital	Metharbital	Thiamylal	Barbituric Acid	Vinbarbital	Butabarbital	Pentobarbital	Cyclobarbital	Secobarbital
6.01	0.100	0.0166	—	—	0.253	—	6.23	1.36	—		—	—	—
6.49	0.0582	0.0306	1.41	1.38	1.20	—	15.0	—	—	—	—	—	—
6.98	0.0250	0.0416	3.49	2.58	2.96	—	20.9	1.47	—	—		—	—
7.45	0.00894	0.0470	6.34	3.14	5.58	9.09	26.2	1.61	—	—	—	—	—
7.91	0.00187	0.0493	8.10	4.41	6.64	11.1	27.8	2.71	—	—	—	—	—
	[HCO_3^-]	[CO_3^{-2}]											
9.08	0.0460	0.0040	10.7	6.80	7.02	13.2	35.9	3.04	1.50	—	—	—	—
9.58	0.0340	0.0160	11.3	12.6	8.03	13.9	40.1	4.9	1.38	—	—	—	—
9.89	0.0226	0.0276	13.7	23.8	9.05	18.7	38.7	7.33	2.65	—	—	—	—
10.12	0.0116	0.0386	18.8	41.3	11.0	23.7	47.2	11.1	3.95	—	—	—	—
	—	[NaOH]											
10.54	—	0.010	24.7	72.0	16.4	32.1	70.7	17.9	4.18	0.697	2.28	6.46	1.40
11.20	—	0.050	101	270	72.2	100	190	64.3	14.1	2.29	2.84	9.85	3.50
11.49	—	0.100	143	363	90.5	169	257	78.4	24.1	3.90	4.77	16.5	7.21
11.75	—	0.200	208	358	142	283	355	100	34.8	10.8	7.57	21.4	—
12.05	—	0.400	261	391	211	530	400	119	40.5	14.5	11.5	33.4	—

short wavelength UV light and sprayed with various reagents to visualize the spots.

Barbituric acid degradation products were separated on cellulose TLC plates, using a solvent system consisting of *n*-butanol–acetic acid–water (6:3:1). TLC of the degradation products of thiamylal and barbital utilized silica gel plates and isopropanol–chloroform–ammonium hydroxide (25%) (9:9:2) as the solvent system. Spray reagents included: 0.1% aqueous solution of potassium permanganate for thiamylal and its degradation products; freshly prepared acidic *p*-dimethylaminobenzaldehyde (23) for barbituric acid, urea, and its derivatives; bromcresol green solution[4] for acidic compounds; and saturated aqueous solution of mercurous nitrate (24) for barbital. Identification of spots was made by comparison of R_f values and by color reactions for unknown compounds and standard substances spotted on the same plate.

RESULTS

Rate Constants and Spectral Changes—The kinetic parameters describing the hydrolysis of the barbituric acid derivatives studied were determined by following the loss of absorbance, A, of the UV chromophore as a function of time. All of the compounds studied lose their UV absorbance in the strongly alkaline and carbonate buffer regions without any significant perturbation in the chromophoric absorption band. A small residual absorbance, A_∞, was noted in some cases at the completion of the reaction. Typical spectral changes as a function of time are given for butethal in 0.4 *N* NaOH at 70° in Fig. 1.

Plots of ln $(A - A_\infty)$ at the $\lambda_{max.}$ *versus* time were reasonably linear for all studies in the alkaline pH region according to the first-order expression:

$$\ln (A - A_\infty) = \ln (A_0 - A_\infty) - kt \qquad \text{(Eq. 1)}$$

where A_0 is the absorbance at zero time, and k is the apparent first-order rate constant. The rate constants and the conditions under which they were determined are listed in Tables II and III. Several typical first-order plots for the hydrolysis of barbituric acid at various alkali concentrations and for barbital in buffers (after alkaline adjustment for spectrophotometric measurements) according to Eq. 1 are shown in Figs. 2 and 3.

Some minor spectral changes, *i.e.*, slight bathochromic shifts in the $\lambda_{max.}$, were noted for the degradation of metharbital in phosphate buffers at pH values less than 7 after approximately two half-lives. Nevertheless, the semilogarithmic plots of the data were representative of good first-order reactions, and good estimates of the apparent first-order rate constants were still obtainable from estimates of a slightly variable A_∞ for application of Eq. 1. However, when reactions were run in buffers[5], pH 6.4–8.2, significant residual absorbances which changed with time were observed after considerable decrease in the barbiturate chromophore. This anomalous behavior, which prohibited estimates of rate constants, was characteristic of all the barbiturates studied; typical spectra for the hydrolysis of metharbital in tromethamine buffer are shown in Fig. 4. As metharbital degrades in this buffer, the chromophore having a $\lambda_{max.}$ at 244 nm. decreases until it reaches an apparent asymptote. This is followed by the relatively rapid rise of a new peak, showing a $\lambda_{max.}$ at 275 nm. and a shoulder at 290 nm. No isosbestic point was observed and, thus, it can be argued that this new peak is not the direct result of the opening of the barbiturate ring; *i.e.*, there is no 1:1 correspondence between the loss of the barbiturate chromophore and the formation of the new peak. A plausible explanation is that there is a reaction between one or more of the ring-opened barbiturate hydrolysis products and the buffer to produce the new chromophore. Reactions between tromethamine and other compounds have been noted in the literature, together with a caution on the use of these buffers in studying reaction kinetics (25–27).

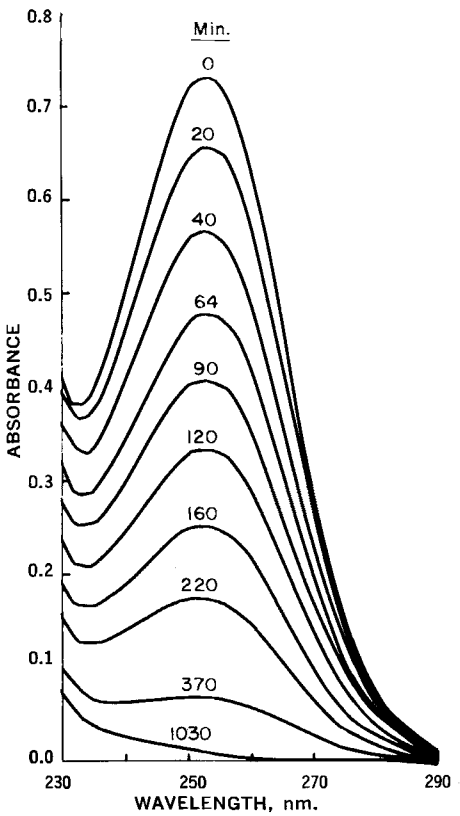


Figure 1—*Typical spectral changes for hydrolysis of 10^{-4} M butethal in 0.40 N NaOH at 70.0°. The curves are labeled as to minutes after the start of the reaction.*

[4] Applied Science Labs., Inc. TLC spray reagent according to Stahl (41).

[5] Tromethamine [TRIS, tris(hydroxymethyl)aminomethane].

Table III—Apparent First-Order Rate Constants, 10^4k in sec.$^{-1}$, and Thermodynamic Parameters for Hydrolysis of Some Barbiturates

Temperature	Barbital			Phenobarbital			Amobarbital		Butethal		Thiamylal			Metharbital		Barbituric Acid		
	0.4 *N* NaOH	pH 10.12	pH 7.37	0.4 *N* NaOH	pH 10.12	pH 7.37	0.4 *N* NaOH	pH 10.12	0.4 *N* NaOH	pH 10.12	0.4 *N* NaOH	pH 10.12	pH 7.37	0.4 *N* NaOH	pH 10.12	0.4 *N* NaOH	pH 10.12	pH 7.37
80.0°	261	18.8	6.34	391	41.3	3.14	211	11.0	199	14.3	400	47.2	26.2	530	23.7	119	11.1	1.61
75.0°	180	12.3	3.83	311	27.6	1.67	130	7.81	137	9.98	324	33.2	16.5	405	18.7	76.9	6.86	0.84
70.0°	141	8.01	2.48	237	19.1	1.41	—	5.98	109	5.93	195[a]	22.8	11.5	326	12.8	49.9	4.48	0.47
60.0°	54.0	3.15	—	115	8.17	—	4.58	2.22	51.8	2.08	—	10.4	4.25	193	5.06	19.6	1.38	0.15
ΔH_a[c], kcal./mole	18.2	20.8	22.4	14.4	18.8	19.1	17.4	18.6	15.4	22.8	19.2	16.2	17.7	21.0	17.7	18.4	21.0	243	27.4
ln P	17.8	18.8	20.0	12.8	16.47	14.5	16.3	15.2	13.5	21.4	15.0	15.4	15.3	19.4	9.08	15.6	20.9	23.2	25.7
ΔS_a[b,d], cal./°K-mole	−23.6	−21.5	−19.1	−33.5	−25.6	−30.1	−26.5	−28.7	−32.1	−16.2	−29.0	−28.3	−28.5	−20.2	−40.8	−27.8	−17.3	−12.8	−7.76

[a] At 68°. [b] At 80°. [c] In accordance with the expression: $\ln k = \ln P - \Delta H_a/RT$. [d] In accordance with the expression: $\Delta S_a = R \ln P - [\ln (\bar{k}T/h)]$.

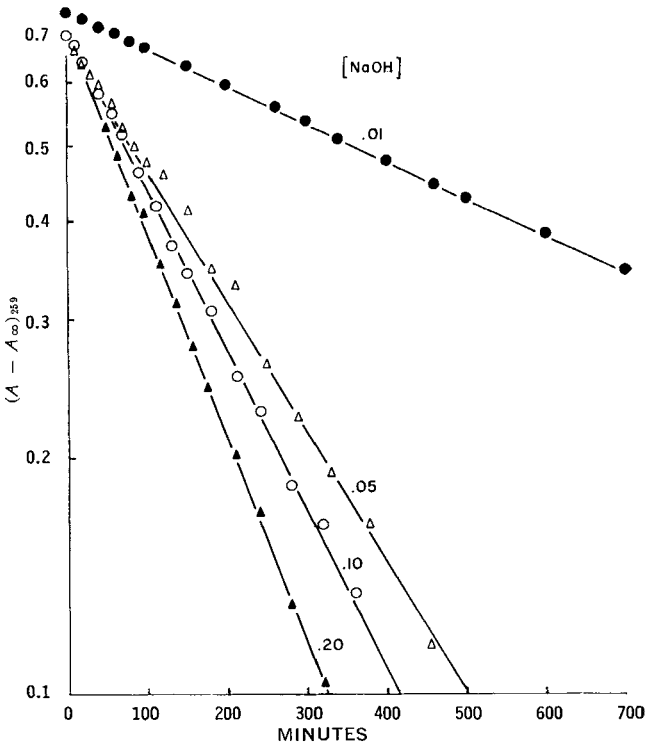


Figure 2—*Typical apparent first-order plots for hydrolysis of 10^{-4} M barbituric acid in alkali at 80.0°. The plots are labeled with the NaOH concentrations used. The absorbance values,* A, *were measured at 259 nm., and* A_∞ *is the final asymptotic absorbance.*

Significant spectral changes, other than decreases in absorbance, were observed during the hydrolysis of the thiobarbiturate, thiamylal. The decrease in absorbance with time at the $\lambda_{max.}$ (305 nm.) of this compound in sodium hydroxide solutions was accompanied by the formation of a new peak having a maximum at 235 nm. The presence of an isosbestic point at 252 nm. and the observation that the rate constant for the formation of the new peak closely matches that for the disappearance of the original chromophore are strong evidence for the occurrence of a 1:1 transformation. The spectra for the degradation of thiamylal in 0.1 *N* NaOH are shown in Fig. 5. During the hydrolysis, a low intensity, broad absorbance band appeared in the 300–400-nm. region. Higher concentrations of degraded solution allowed this absorbance band to be assigned a $\lambda_{max.}$ of 357 nm. Both new peaks, 237 and 357 nm., increased with time initially and then decreased, following apparent first-order kinetics. The spectrum of the 237-nm. chromophore is consistent with that of thiourea, one of the possible hydrolysis products of thiamylal.

Dependence of Rate on Temperature—The Arrhenius parameters for the hydrolysis of several barbiturates were calculated from the slopes and intercepts of plots of the logarithm of the apparent first-order rate constants, k, *versus* the reciprocal of the absolute temperature, T, in accordance with the expression:

$$\ln k = \ln P - \Delta H_a/RT \qquad \text{(Eq. 2)}$$

where R is 1.987 cal. deg.$^{-1}$. Typical Arrhenius plots are shown in Fig. 6. From a consideration of absolute rate theory (28),

$$k = \frac{\bar{k}T}{h} e^{\Delta S_a/R} \cdot e^{-\Delta H_a/RT} \qquad \text{(Eq. 3)}$$

and Eq. 2, the entropy of activation can be calculated by:

$$\Delta S_a = R \ln P - [\ln (\bar{k}T/h)] \qquad \text{(Eq. 4)}$$

where ΔS_a is the entropy of activation, $\bar{k}$ is the Boltzman constant, and h is the Planck constant. The k, ΔH_a, ln P, and ΔS_a values at several pH values are given in Table III.

Rate–pH Profiles—The rate–pH profiles for the hydrolysis of barbiturates were constructed from the logarithm of the apparent

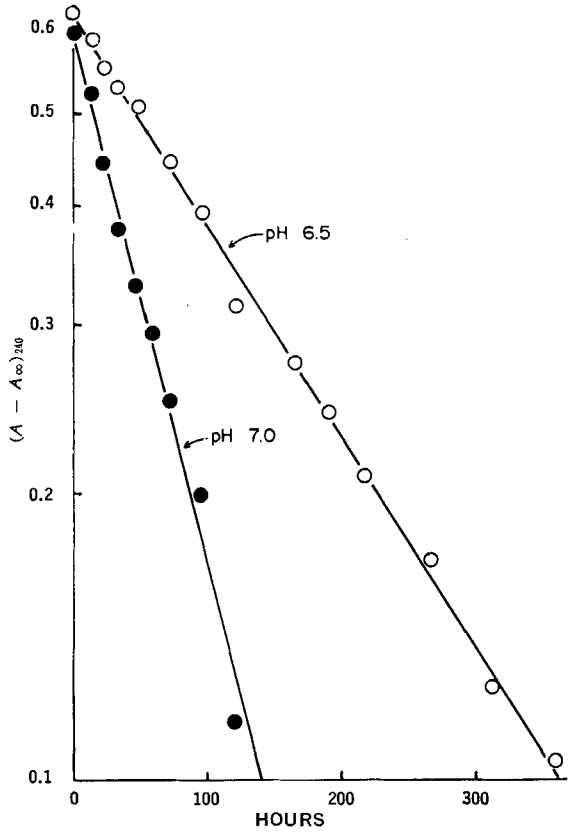


Figure 3—*Typical apparent first-order plots for hydrolysis of 10^{-4} M barbital in phosphate buffers at 80.0°. The samples were adjusted to an alkaline pH of 11.5 by diluting 1:1 with an appropriate NaOH solution immediately prior to spectrophotometric measurement.*

first-order rate constants, k, and the pH values at 80° (Table II). The profiles were similar for all of the 5,5-disubstituted barbituric acid derivatives studied (Fig. 7). The shape of these curves indicates hydroxyl-ion-catalyzed degradation of the unionized and monoionized species or the kinetically equivalent water attack on the mono- and dianionic species. Accordingly, the apparent first-order rate constant at a given pH can be defined as:

$$k = k_{OH}[OH^-]f_{BA} + k_{OH}'[OH^-]f_{BA}^- \qquad \text{(Eq. 5)}$$

or as its kinetic equivalent,

$$k = k_0 f_{BA}^- + k_0' f_{BA}^{-2} \qquad \text{(Eq. 6)}$$

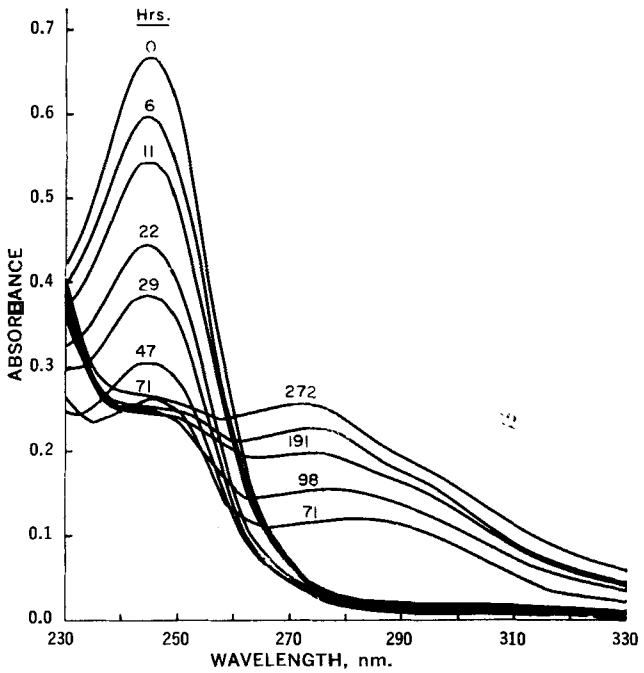


Figure 4—*Typical spectral changes for hydrolysis of 10^{-4} M metharbital in pH 8.8 tromethamine buffer at 80.0°, μ = 0.15. The curves are labeled as to minutes after the start of the reaction.*

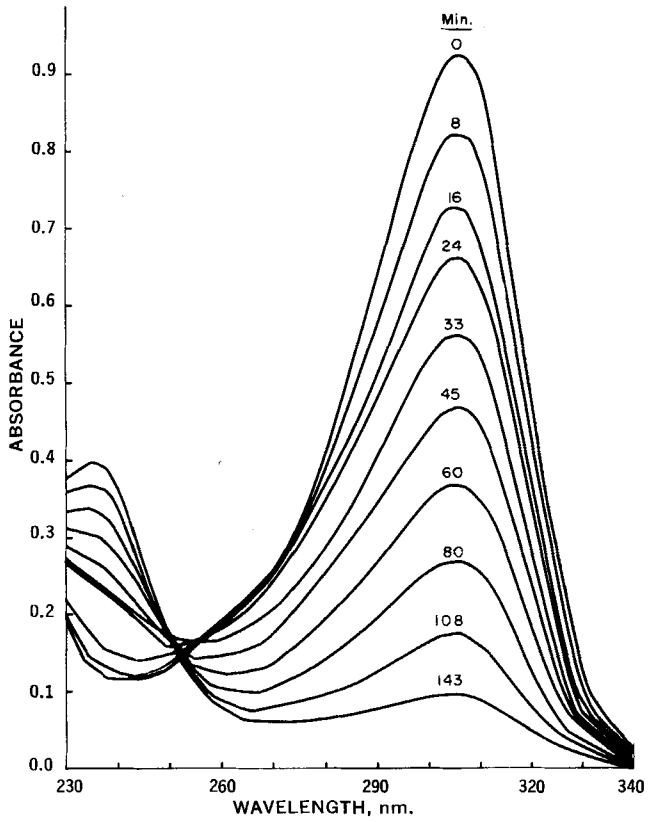


Figure 5—*Typical spectral changes for hydrolysis of 5 × 10^{-5} M thiamylal in 0.10 N NaOH at 80.0°. The curves are labeled as to minutes after the start of the reaction.*

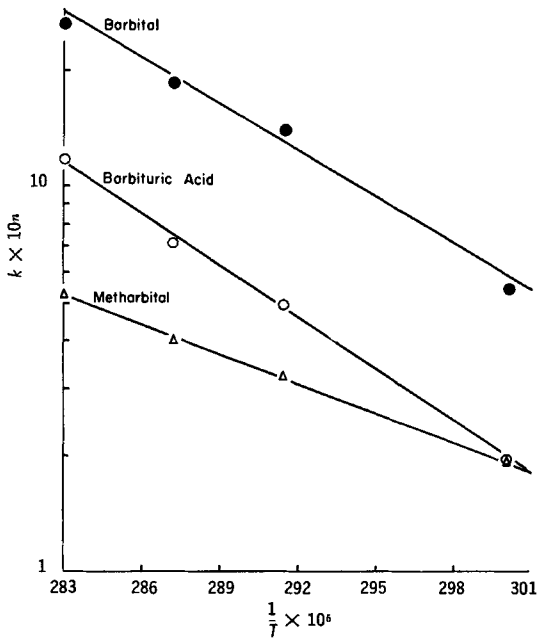


Figure 6—*Typical Arrhenius plots for apparent first-order rate constants for several barbiturates in 0.40 N NaOH; n = 4 for methorbital and 5 for barbital and barbituric acid.*

Table IV—Catalytic Rate Constants and Kinetic pKa′ from the Hydrolysis of Some Barbiturates at 80.0°

	Barbital	Amobarbital	Phenobarbital	Thiamylal	Metharbital	Barbituric acid
pKa_1'[a]	7.35	7.26	6.31	6.64	7.25	—
pKa_2'[a]	12.01	12.15	10.76	11.58	—	11.20
k_{OH}[b]	2.17	2.06	8.94	32.85	3.34	—
$10^3 k_{OH}'$[b]	2.14	1.45	35.2	6.04	270	3.39
$10^6 k_0$[c]	11.4	8.76	4.27	33.6	13.9	1.40
$10^5 k_0'$[c]	51.2	46.8	4.73	53.7	—	12.6

[a] Values reported are determined from best fit of rate–pH profile. [b] l. mole^{-1} sec.$^{-1}$. [c] sec.$^{-1}$.

where k_{OH} and k_{OH}' are the bimolecular rate constants for hydroxyl-ion attack on the undissociated (f_{BA}) and the monoanion (f_{BA^-}) forms of the barbiturate, respectively, and k_0 and k_0' are the first-order rate constants for water attack on the monoanion and dianion ($f_{BA^{-2}}$) forms, respectively.

Equation 5 can be rewritten by substituting for f_{BA} and f_{BA^-} in terms of the equilibrium constants, K_{a1} and K_{a2}, for the dissociation steps of the 5,5-disubstituted barbiturates (2):

$$k = \frac{k_{OH}K_w[H^+] + k_{OH}'K_wK_{a_1}'}{[H^+]^2 + K_{a_1}'[H^+] + K_{a_1}'K_{a_2}'} \qquad \text{(Eq. 7)}$$

The bimolecular rate constants that produced the best fits of the observed rate–pH profiles and the kinetic pKa′ values determined are given in Table IV.

The rate–pH profile obtained for thiamylal has the same shape as those for the 5,5-disubstituted barbituric acids (Fig. 7). Thus, the expressions given in Eqs. 5–7 also hold for this thiobarbiturate. The differences observed with thiamylal are in the magnitudes of the bimolecular rate constants. These data are also given in Table IV.

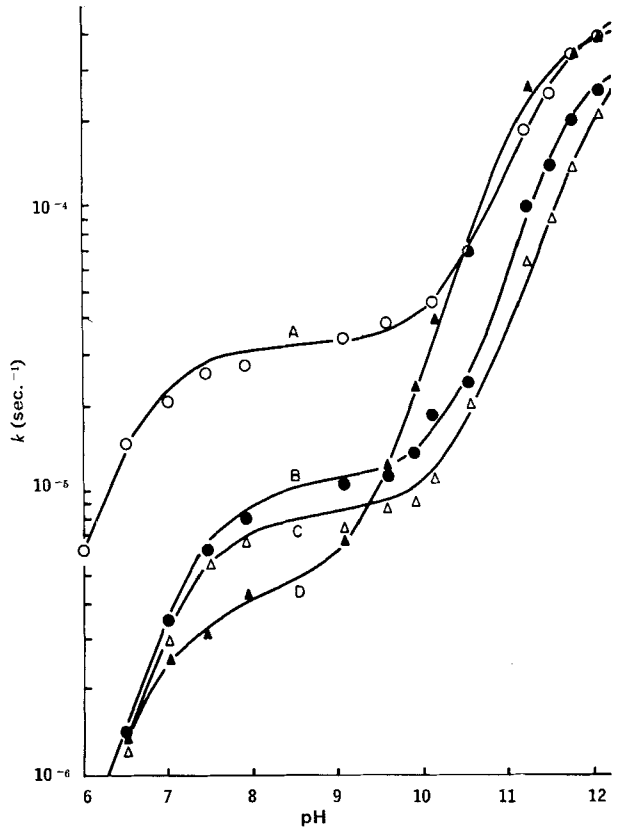


Figure 7—*Rate–pH profiles for 5,5-disubstituted barbiturates at 80.0°. The lines represent the theoretical curves drawn from Eq. 7 and the constants in Table IV. The points are the experimental values. Key: Curve A, thiamylal; Curve B, barbital; Curve C, amobarbital; and Curve D, phenobarbital.*

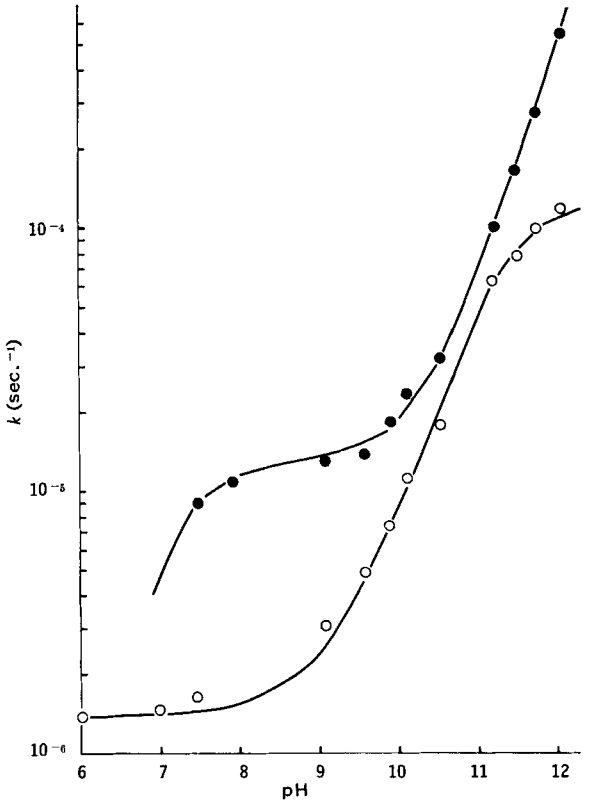


Figure 8—*Rate–pH profiles for some barbiturates. Key: ●, metharbital; and ○, barbituric acid. The metharbital curve is drawn from Eq. 8 and Table IV, and the barbituric acid curve from Eq. 10 and Table IV. The circles represent the experimentally determined values.*

Two rate–pH profiles which differ from those of the 5,5-disubstituted barbituric acids are shown in Fig. 8. The profile for the 1,5,5-trisubstituted barbiturate, metharbital, shows no curvature at the high pH values, since the 1-methyl substituent blocks the second possible dissociation; thus, hydroxyl-ion attack on the monoanion is not perturbed by the formation of a doubly negatively charged ion. An expression that fits the metharbital data is:

$$k = \frac{k_{OH}K_w + k_{OH}'[OH^-]K_a'}{[H^+] + K_a'} \qquad \text{(Eq. 8)}$$

or its kinetic equivalent:

$$k = \{k_0 + k_{OH}'[OH^-]\}\frac{K_a'}{[H^+] + K_a'} \qquad \text{(Eq. 9)}$$

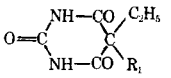
where the latter quotient is the fraction of metharbital monoanion. The catalytic constants and pKa′ estimates are given in Table IV.

The other rate–pH profile shown in Fig. 8 was obtained from studies on the hydrolysis of the unsubstituted parent barbiturate, barbituric acid. A bending of the profile at the higher pH values indicated the presence of a pKa in this region just as is found with the 5,5-disubstituted compounds. However, contrary to findings with the other barbiturates, the profile does not bend in the region of pH 7–8. This is consistent with the fact that the first pKa′ for barbituric acid is 3.9 at 20.0° (29)—well below that of the other barbiturates. One kinetically equivalent expression that fits the barbituric acid data for pH values >6 is:

$$k = \{k_0 + k_{OH}'[OH^-]\}\frac{[H^+]}{[H^+] + K_{a_2}'} \qquad \text{(Eq. 10)}$$

where the latter quotient is the fraction of barbituric acid monoanion. Values of the constants providing the best fit for this profile are also found in Table IV.

Table V—Effect of Substituents on Rate of Hydrolysis and Relationship of Order of Reactivity to the Newman Rule of Six



R_1	$k \times 10^5$[sec.$^{-1}$] 0.4 N NaOH, 80°	Total "Six Number"
C_2H_5—	26.10	6
CH_3—CH_2—CH_2—CH_2—	23.80	6
CH_3 \ CH—CH_2— / CH_2	21.08	6
	3.33	8
	2.58	8
CH_3—CH_2—CH(—CH_3)—	1.46	9
CH_3—CH_2—CH_2—CH(—CH_3)—	1.16	9
CH_3—CH_2—CH=C(—CH_3)—	4.07	8
	39.13	7

Effect of Substituents on Rate of Hydrolysis—Several barbituric acid derivatives were hydrolyzed in 0.4 N NaOH at 80° to determine the effect of various substituents on the rates of hydrolysis. Tables V and VI show the quantitative results obtained.

Separation and Identification of Degradation Products of Barbital—Barbital as free acid (1.6 g.), pKa 7.86 (29), was refluxed with 100 ml. of phosphate buffer (pH 5.8) composed of 2.76 g. of sodium phosphate monobasic $NaH_2PO_4 \cdot H_2O$ and 0.47 g. of sodium phosphate dibasic (anhydrous) for 4 days. The final pH of the reaction mixture was 6.15. The solution was cooled, and the crystals were filtered and dried, yielding 0.6 g. of white needles, m.p. 209–210°. This corresponded to the literature value of 207–208° for diethylacetylurea (7), one of the reported hydrolysis products of barbital. No melting-point depression was observed on mixing with an authentic sample of diethylacetylurea. After prolonged cooling in the refrigerator, another portion (0.1 g.) of diethylacetylurea was obtained. No attempt was made to recover unhydrolyzed barbital.

TLC—TLC was used to separate and identify some of the degradation products of barbituric acid and thiamylal. After degrading a 0.1% solution of barbituric acid in phosphate buffer (pH 8.2) at 80° for 97 hr., only unhydrolyzed starting material (R_f 0.37) and urea (R_f 0.65) could be identified.

Five different compounds were separated by TLC after the hydrolysis of a 0.1 M solution of thiamylal in 0.4 N NaOH for 3 hr. at 80°. Two of these compounds were identified as unhydrolyzed thiamylal (R_f 0.76) and thiourea (R_f 0.56). Two others (R_f 0.24 and 0.67) showed an acidic character by giving yellow spots with bromcresol green. Attempts to isolate these compounds on a larger scale were unsuccessful.

Hydrolysis of Diethylmalonuric Acid—Diethylmalonuric acid has been shown to be one of the products of barbital hydrolysis (7). The hydrolysis of diethylmalonuric acid in phosphate and carbonate buffers was monitored by UV spectroscopy, and an unexpected increase in absorbance was observed. The new peak formed showed the same spectral characteristics as barbital. The absorbance decreased after reaching a maximum ($\lambda_{max.}$ = 238 nm.) by an apparent first-order process. These spectral changes were followed over the pH region 7.45–10.12 at 80.0°. Typical examples are shown in Figs. 9 and 10. The data of Fig. 10 were quantitated by substracting the apparent A_∞ values from each absorbance reading and mathematically evaluating the resulting data by the "feathering" technique to obtain the two apparent

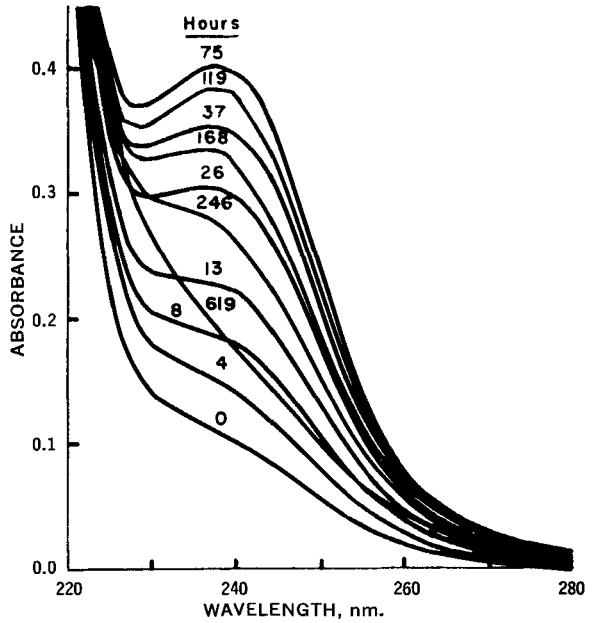
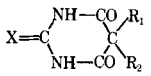


Figure 9—*Typical spectral changes for hydrolysis of 5 × 10^{-4} M diethylmalonuric acid in pH 6.98 phosphate buffer at 80.0°; μ = 0.15. The samples were adjusted to an alkaline pH of 11.5 by diluting 1:1 with an appropriate NaOH solution immediately prior to spectrophotometric measurement. The curves are labeled as to hours after the start of the reaction.*

first-order rate constants governing the increase and subsequent decrease of absorbance at 238 nm. with time. The larger rate constant was designated as k_1, while the smaller rate constant was called k_2. The values of k_1 and k_2 for each pH studied are given in Table VII. The reaction products were identified by dissolving 0.023 g. of diethylmalonuric acid in 100 ml. of phosphate buffer (pH 6.49), heating at 80° for 75 hr., cooling, and extracting the solution with two 25-ml. portions of ether. The ether was evaporated and the residue dissolved in 1.5 ml. of acetone. This solution (30 μl.) was spotted on a TLC plate along with barbital and diethylacetylurea standards (50 μl. of about 10^{-2} M solutions in acetone). The hydrolysis reaction products had R_f values identical to the barbital (0.65) and diethylacetylurea (0.82) standards. This suggests that diethylmalonuric acid simultaneously degrades to diethylacetylurea and cyclizes to form barbital.

Ionic Strength Effects—Variations in the apparent first-order rate constants with ionic strength changes were observed for the hydrolysis of amobarbital and phenobarbital at pH 11.20 where the barbiturate anion would exist (Fig. 11). The use of the Bronsted–

Table VI—Effect of Substituents on Rate of Hydrolysis



X	R_1	R_2	$k \times 10^5$ [sec.$^{-1}$] 0.4 N NaOH, 80°
O	CH_3—CH_2—CH(—CH_3)—	CH_3—CH_2—	1.46
		CH_2=CH—CH_2—	1.59
O	CH_3—CH_2—CH_2—CH(—CH_3)—	CH_3—CH_2—	1.16
		CH_2=CH—CH_2	1.52
S	CH_3—CH_2—CH_2—CH(—CH_3)—	CH_3—CH_2—	15.5
		CH_2=CH—CH_2—	40.02

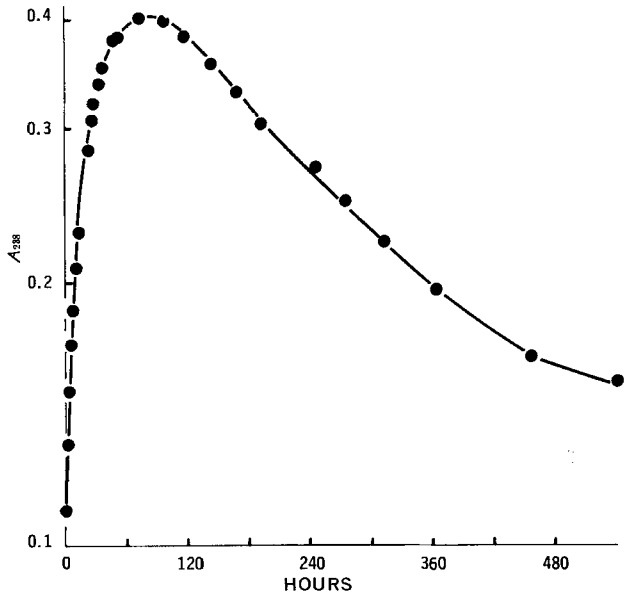


Figure 10—*Logarithm of absorbance changes at 238 nm.* versus *time for hydrolysis of* 5×10^{-4} M *diethylmalonuric acid in pH 6.98 phosphate buffer at 80.0°;* $\mu = 0.15$. *The samples were adjusted to an alkaline pH of 11.5 by diluting 1:1 with an appropriate NaOH solution immediately prior to spectrophotometric measurement.*

Bjerrum equation, together with the extended Debye–Huckel equation, results in the relationship (30):

$$\log k = \alpha + 2QZ_AZ_B \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \qquad \text{(Eq. 11)}$$

A plot of the logarithm of the apparent first-order rate constant *versus* $\sqrt{\mu}/(1 + \sqrt{\mu})$ was linear for both barbiturates (Fig. 11). The plots had significant positive slopes of 1.1507 for phenobarbital and 1.0666 for amobarbital. The value of $2Q$ at 80° is 1.145 (30).

DISCUSSION

General Structure–Hydrolytic Reactivity Correlations—Nonkinetic information has been used as a basis for qualitative appreciation of substituent effects on the hydrolysis of barbituric acid derivatives (6, 7, 12, 32, 33). Lamb (31) claimed a correlation between the rates of base-catalyzed decomposition of some 5,5-disubstituted barbiturates and the Taft E_s steric substituent constants. He stated that the variation in rate of decomposition in the barbiturate series appears to be only a function of the steric effects of the substituents and argued that the steric effects on the mechanism of phenobarbital decomposition were similar to the related steric effects for ester hydrolysis proposed by Pavelich and Taft (36). Eriksson (16, 17) also pointed out the importance of steric effects on substituent-modified hydrolyses of 1-methyl-5,5-disubstituted barbituric acids. He stated his belief that Taft-type parameters cannot be used to evaluate substituent effects for barbiturate-type compounds; instead, he utilized isokinetic relationships to group equivalent degrees of steric hindrance in the transition state. In contrast, Carstensen *et al.* (37) showed the validity of prediction of stability for a series of 5-substituted allylbarbituric acids on the basis of the Hammet $\rho\sigma$ linear free energy relation, in which the pKa values of the unsubstituted and substituted compounds were used to determine the reaction constant.

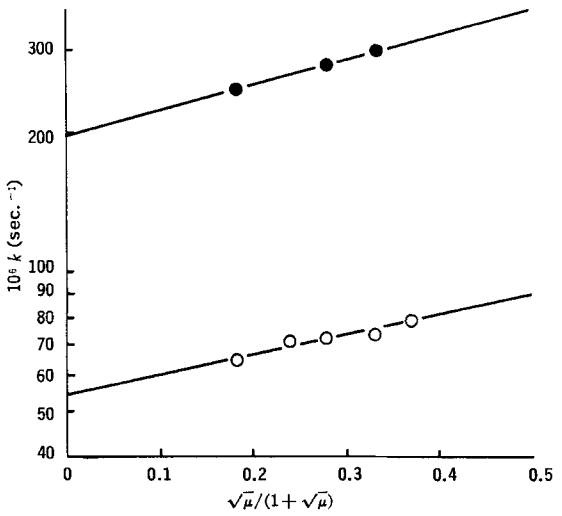


Figure 11—*Effect of varying ionic strength on apparent first-order rate constant at pH 11.20 for hydrolysis of some barbiturates at 80.0°. Key:* ●, *phenobarbital; and* ○, *amobarbital.*

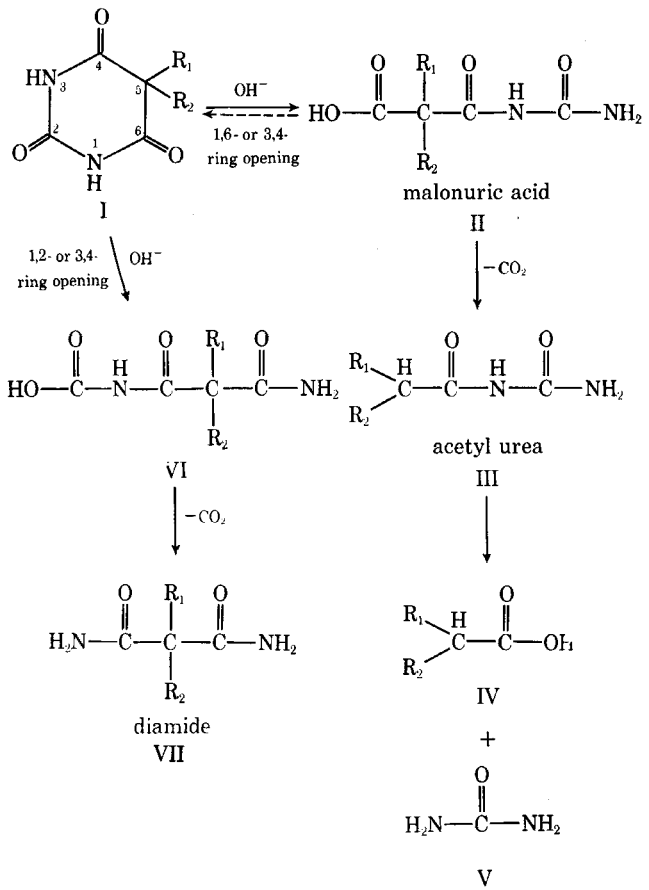
Table VII—Apparent First-Order Rate Constants[a] for Hydrolysis of Diethylmalonuric Acid at Various pH Values at 80.0°

pH	10^6k_1[b]	10^6k_2[c]
10.12	37.6	8.94
9.89	17.6	6.85
9.58	10.9	6.83
9.08	12.0	4.13
7.91	13.0	1.47
7.45	11.4	1.81

[a] Apparent first-order rate constants, k_1 and k_2, were determined by the "feathering" technique based on the assumption of an $A \rightarrow B \rightarrow C$ model. The faster rate constant was designated as k_1 and the smaller rate constant as k_2, and they are purely arbitrary estimates as described in the text. The true model may be $A \rightleftharpoons B \rightarrow C$, and k_1 (or k_2) may be more complex functions of the contant for the reversible equilibrium between A and B. [b] k_1 is the rate constant assigned to the cyclization equilibrium between I and II (Scheme I). [c] k_2 is the rate constant assigned to the decarboxylation of the malonuric acid derivative II (Scheme I).

Studies using the rate constants for the hydrolysis of many commonly used barbiturates under constant conditions (Tables V and VI) also show the effects of substituents in the 5-position. Substitution of sulfur for oxygen in the 2-position and replacement of one of the imide protons with a methyl group increase the susceptibility to hydrolysis. Increased alkyl group length decreases the rate of hydrolysis slightly, but branching at the first carbon of the alkyl chain or 5-substitution of a bulky cyclic group markedly decreases hydrolytic rate.

Newman Rule of Six and Hydrolytic Reactivity—The Newman rule of six (38), which essentially states that those atoms most effective in sterically hindering addition are separated from the oxygen of the attacked carbonyl in the transition state by a chain of four atoms, provides further evidence of the importance of steric effects in the hydrolysis of barbiturates. The order of reactivity (Table V) follows the "six number," *i.e.*, the number of atoms in the 6-position where the carbonyl oxygen is considered number "one." A more systematic approach would be to relate the bimolecular rate constants to the Newman "six numbers." Our data could not be subjected to this type of analysis due to the limited number of compounds for which the necessary complete rate–pH profiles were studied to determine exactly the bimolecular rate constant for hydroxide-ion attack on the monoanion. However, the excellent data of Eriksson (16, 17) were utilized to show this relationship since he studied derivatives that do not undergo a second dissociation; therefore, the bimolecular rate constants could be calculated from one first-order rate constant at a given alkali concentration. The use of the "six number" to classify relative reactivities provides interesting relations when Eriksson's detailed data of second-order rate constants for the alkaline hydrolysis of 1-methyl-5,5-disubstituted barbituric acids at 25° (16, 17) are considered. When the logarithms of the second-order rate constants are plotted against the total "six number," *i.e.*, for both 5-substituents, three distinct linear relations can be observed (Fig. 12). Curve A represents those compounds in which only one of the 5-substituents is exerting steric influence since the other group is a methyl which, in the barbiturate system, has a "six number" of zero. Curve B represents those compounds in which both substituents are interacting, and Curve C shows those compounds in which one of the substituents is a phenyl. Curves B and C, both of which are for compounds containing two interacting groups at the 5-position, have essentially the same slopes but different intercepts. Since the slopes of Curves B and C differ from that of Curve A,



Scheme I—Simplified scheme for hydrolysis of barbituric acid derivatives

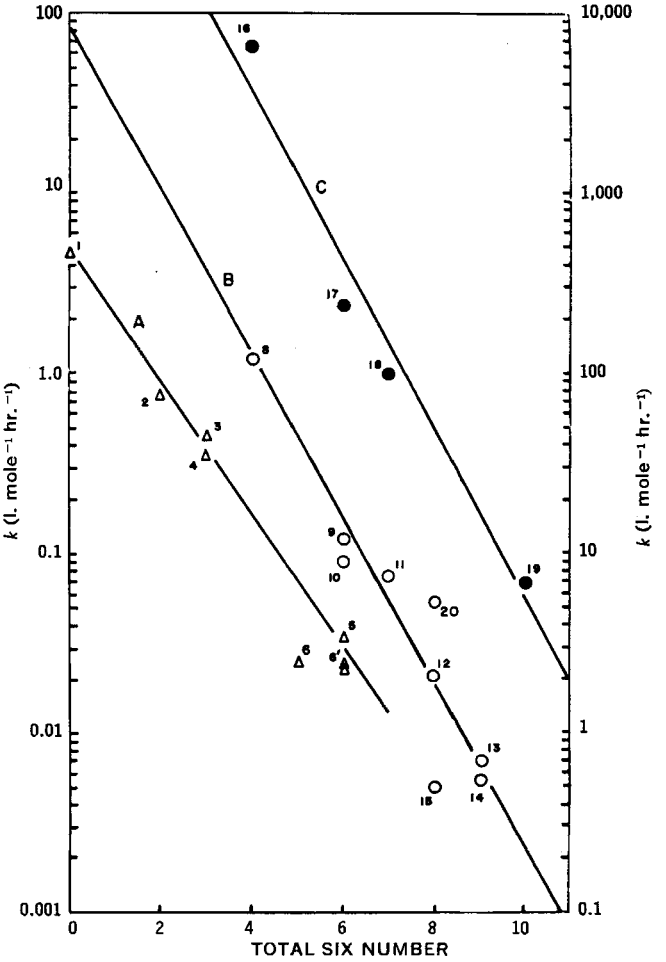


Figure 12—*Logarithm of bimolecular rate constant for hydrolysis of various substituted barbituric acid derivatives* versus *the total "six number" for the compound. The rate constants are taken from* References 16 *and* 17. *The numbers next to the various points represent 1-methyl barbituric acids substituted at the 5-position in the following manner: (1) methyl, methyl; (2) methyl, allyl; (3) methyl, ethyl; (4) methyl, propyl; (5) methyl, isopropyl; (6) methyl, 1-cyclohexenyl; (6′) methyl, cyclohexyl; (7) methyl,* sec*-butyl; (8) allyl, allyl; (9) ethyl, ethyl; (10) ethyl, butyl and propyl, propyl; (11) allyl, 1-methyl-2-pentynyl; (12) allyl, isopropyl; (13) propyl, isopropyl and ethyl, cyclohexyl; (14) propyl, 1-methylpentyl; (15) isopropyl, 2-bromoallyl; (16) phenyl, methyl; (17) phenyl, allyl; (18) phenyl, ethyl; (19) phenyl, isopropyl; and (20) ethyl, 1-cyclohexenyl. Curve A should be read using the ordinate on the right-hand side of the plot; for Curves B and C, use the ordinate on the left.*

it may indicate that the rate of hydrolysis of barbiturates is not a function of steric effects alone but that electronic effects also play some role. In line with this, the only two compounds that fall markedly off their respective lines are the cyclohexenyl and bromoallyl derivatives. More derivatives with electronegative groups in the 5-position should be studied to confirm this finding.

Mechanism of Barbiturate Hydrolysis—Considerable work has been done on the isolation and identification of the products of barbiturate hydrolysis (7–10, 33–35). The most comprehensive studies were published by Fretwurst (7) and Aspelund and coworkers (33–35). Barbiturate hydrolytic pathways are complex due to the possible alternative routes of sequential hydrolyses of the intermediates (39). The simplified scheme shown in Scheme I includes the major postulated routes.

Studies with the hydrolysis of barbital and its degradation product, diethylmalonuric acid, have demonstrated a previously unmentioned reversible system between the barbiturate and the ring-opened malonuric acid derivative (Figs. 9 and 10)[6]. The malonuric acid hydrolysis product can simultaneously degrade to the acetylurea derivative and cyclize to form the barbiturate (Scheme I).

Several investigators argued or stated that the hydrolysis of barbiturates proceeds through two different pathways (13, 15, 16). The unionized form of the barbiturate has been said to hydrolyze *via* ring opening at the 1,2- or equivalent 2,3-position to yield a substituted diamide, VII, and the ionized form to hydrolyze *via* opening at the 1,6- or equivalent 3,4-position to produce a substituted acetylurea derivative, III (Scheme I). There are rate–pH profiles in uridine solvolysis (2), similar to those of the barbiturates, in which different products do indeed result from hydroxyl-ion attack on the undissociated and dissociated species. The mechanism proposed by Hasegawa *et al.* (14) did not allow for production of the diamide and was criticized by the proponents of the dual pathways (13).

The argument (13) for different products of hydrolysis from the ionic and nonionic forms was based on two factors: (*a*) analysis of equilibria phenomena and series first-order reaction kinetics showed that the fraction decomposing by the ureide path (1,6-ring opening) was dependent on pH changes which could be related to the concentration of the ionic form of 1, and (*b*) the diamide, VII (1,2-ring opening) was isolated as a hydrolysis product by two investigators (7, 9). However, the diamide, supposedly a product of nonionized barbiturate solvolysis, was isolated by Fretwurst (7) from alkaline solutions of the ionized barbiturate. No mention of diamide isolation was made by Kapadia *et al.* (9).

The hydrolysis of barbital in phosphate buffer at a pH value (6.15) well below the first pKa′ (7.86) of the compound, where the barbiturate was largely unionized, yielded diethylacetylurea as 84% of the hydrolyzed barbital. The extent of hydrolysis at 100° to correct for unreacted barbital present was calculated for the time of reaction on the basis of the determined heat of activation (Table III) and the catalytic constants in Table IV. This diethylacetylurea

[6] Subsequent to submission of this manuscript for pu lication, the authors learned that Aspelund recently described the cyclization of a malonuric acid derived from a tetrasubstituted barbituric acid. [H. Aspelund, *Acta Acad. Aboensis Math. Phys., Ser. B*, **29** (8), (1969).]

cannot be formed if ring opening occurs at the 2-position of the barbiturate ring. This strongly identifies an acetylurea as the major hydrolysis product of *both* the ionized and unionized form of the barbiturates. A close look at the Fretwurst data (7) indicates that the diamide is most easily formed from those barbiturates with a 1-methyl substituent or with bulky groups substituted at the 5-position. This argues that 1,6-ring opening would be favored in all barbiturates and that 1,2-splitting is of minor importance except when 1,6-opening is sterically hindered by a bulky 5-substituent or the combination of a 5-substituent and a 1-methyl group.

These observations are difficult to reconcile with the proposed sole dependence of phenylethylacetylurea formation on the concentration of the ionized parent barbiturate, phenobarbital (13). The basis of this proposal (13) was the postulated instability of the presumed irreversibly formed malonuric acid intermediate, II (Scheme I), so that the analyses of the acetylurea, III, with time could be taken as measures of the rate of hydroxyl-ion attack on the ionized form. The determination of the total rate of loss of barbiturate, I, permitted the assumption that the difference between this rate and the rate of acetylurea, III, formation could be attributed to the rate of formation of intermediate VI and the diamide VII. The resultant observed pH dependence (13) of the fraction of acetylurea, III, formed was assigned to the attack of hydroxyl ion on the ionized barbiturate where the diamide was considered to result from the attack of hydroxyl ion on the uncharged barbiturate.

The data in Table VII indicate that the cyclization of II to I occurs predominantly with the anion of the malonuric acid derivative since the rate of formation and amount of chromophore assigned to the cyclized product, *i.e.*, barbital, decrease with decreasing pH. In addition, a study of the cyclization of II to I at pH 1.1 indicated that no chromophore at 238 nm. was formed with time, while a similar study at pH 4.55 showed the slow formation of this chromophore assigned to barbital. Since decarboxylation of the anion of the malonuric acid derivative II is hydroxide-ion catalyzed (40) and the cyclization of II to I, if assignable to a function of the magnitude of k_1, appears to be pH independent up to a pH of 9.5 (Table VII), it is possible that the rate of formation of the acetylurea derivative, III, decreases with decreasing pH. The net effect would be a buildup of II and an increase in the rate of cyclization to the parent barbiturate. The result would be an apparent decrease in acetylurea III formation and an apparent increase in hydrolysis *via* 1,2-ring opening which is not related to any relative increase in hydroxyl-ion attack on one position of the barbiturate ring over another for the ionized or nonionized state.

Thus, it is possible that the data showing that the fraction decomposing by the ureide path (1,6-ring opening) is a function of pH in accordance with the observed experimental data (13) is not a direct consequence of a unique degradation pathway for the ionic form of the barbiturate, as was implied. The prime factor responsible for a pH-dependent path for barbiturate hydrolysis would then be the balance struck between hydroxyl-ion-catalyzed decarboxylation of the malonuric acid anion and the pH-independent recyclization below pH 9.5 (Scheme I).

Steric effects of the substituents may further affect the yield through each path of hydrolysis. Thus, barbital, with very little steric hindrance to 1,6-ring opening, produces primarily the acetylurea derivative even from the unionized form (84% of reacted barbital). The data of Fretwurst (7) were confirmatory. 1-Methyl barbiturates with bulky 5-substituents give the highest yields of diamide, even from alkaline solutions of ionized compounds (7).

These observations indicate the need for further studies to determine the rates of formation and relative amounts of barbiturate hydrolysis products as a function of pH. Various chromatographic techniques could be employed to separate and quantitate the reaction products. In addition, studies should be attempted to isolate the malonuric acids of various barbiturates and to investigate the rates of cyclization as functions of the 5-substituents and pH.

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Dissociation Constants of Sparingly Soluble Substances: Nonlogarithmic Linear Titration Curves

RENÉ H. LEVY* and MALCOLM ROWLAND

Abstract □ A potentiometric method, employing nonlogarithmic linear titration curves, is proposed for the accurate determination of dissociation constants of acids and bases whose aqueous solubilities are less than 2×10^{-4} mole/l. In this method the substance is allowed to precipitate, and pH measurements are made throughout the titration. An equation is derived to describe the titration of the unknown after precipitation. For a base, the pKa is calculated from the slope (equal to solubility/K_a) of the straight line obtained when the number of millimoles of unknown neutralized is plotted *versus* the hydrogen-ion concentration and the solubility, determined independently of the uncharged species. This method extends the ability to measure aqueous dissociation constants and can avoid the determination of pKa's in nonaqueous solvents. The applicability of the method was demonstrated with lidocaine and two structurally related, sparingly soluble, local anesthetic amines. The limitations of the existing and proposed methods are discussed.

Keyphrases □ Dissociation constants—sparingly soluble substances □ Anesthetics, sparingly soluble—dissociation constants, determination □ Potentiometric titration—dissociation-constant determination □ UV spectrophotometry—analysis

Potentiometric methods are the most generally applicable to the determination of aqueous dissociation constants of compounds. Coupled with the use of nonlogarithmic titration plots (1, 2), which offer a significant advantage over previous techniques for handling the data, accurate dissociation constants are possible using very low concentrations of acids and bases (from 5×10^{-4} *M* to 1×10^{-3} *M*). However, for many compounds the uncharged species is so insoluble that precipitation occurs during the titration even at these concentrations. Generally, at this point the titration is stopped and the method is thought to preclude an accurate pKa determination. This problem of low aqueous solubility is very prevalent among drugs and is attested by the scarcity of reliable pKa values for therapeutic agents. To overcome this problem, pKa determinations based on changes in the spectrum, using concentrations below the solubility of the uncharged moiety, or in the solubility as a function of pH have been very useful; however, these methods either lack general applicability or are very tedious. Several other approaches utilizing potentiometric data have been taken to obtain aqueous dissociation constants for sparingly soluble compounds (3, 4) but they lack adequate accuracy.

To circumvent the problem of low aqueous solubility, Mizutani (5), as early as 1925, originated the practice of determining pKa's in several concentrations of ethanol and water. From a plot of the pKa values *versus* the percent ethanol, the aqueous pKa is taken as the value when the line is extrapolated to 0% ethanol. However, Albert and Serjeant (6) warned that: "This temptation [of extrapolation] should be resisted for reasons which will shortly be discussed." This was further stressed by Benet and Goyan (7) who, after a careful analysis of the theoretical and practical considerations involved in determining the pKa by titration and semiaqueous solvents, concluded that: "There is presently no completely satisfactory method for converting p_sK_a values to pKa values. Therefore, although good accuracy can be realized in determining p_sK_a values, this accuracy cannot be carried over to the aqueous dissociation constants." Nonetheless, several authors (8–10) faced with the need to know the pKa of some sparingly soluble drugs have utilized Mizutani's approach.

In the present study, the authors attempted to extend the applicability of the existing nonlogarithmic titration plots to the determination of dissociation constants of compounds having solubilities less than 5×10^{-4} *M*. In addition, a new method for the determination of dissociation constants of acids and bases is proposed and several of its aspects are discussed. This method involves the titration of the weak acid or base during precipitation, followed by the determination of the solubility of the unionized species. The pKa is then calculated from the slope of a nonlogarithmic plot of the data.

THEORETICAL

Titration during Precipitation: Plot of Z' *versus* $[H^+]$—Consider the titration of a weak acid, like the salt of a strong acid and a weak amine BH^+X^-, with a strong base *M*OH. When the unionized species is sparingly soluble, it precipitates out very early during the titration. From this point, the concentration of the free amine in the solution remains constant. In the experiments to be described, it will be shown that several factors in the experimental design predispose toward maintaining a saturated solution.

At any time after precipitation, three equations have to be simultaneously satisfied:

1. The equilibrium expression:

$$K_a^c = \frac{[B]_{sol}[H^+]}{[BH^+]} \qquad \text{(Eq. 1)}$$

which can be rewritten:

$$K_a^c = \frac{B_{sol}}{BH^+} \cdot [H^+] \qquad \text{(Eq. 2)}$$

using relations adapted from the terminology of Leeson and Brown (2), $[B]_{sol} = B_{sol}(10^3/V)$ and $[BH^+] = BH^+(10^3/V)$, where:

- K_a^c = stoichiometric ionization constant of conjugate acid, BH^+
- $[B]_{sol}$ = molar concentration of free base in saturated solution
- $[H^+]$ = molar concentration of hydrogen ion in saturated solution
- $[BH^+]$ = molar concentration of acid
- V = volume of solution in milliliters
- B_{sol} = absolute number of moles of unionized species (free amine) present in saturated solution
- BH^+ = absolute number of moles of acid present in saturated solution

From Eq. 2, it can be seen that:

$$BH^+ = \frac{B_{sol}}{K_a^c}[H^+] \qquad \text{(Eq. 3)}$$

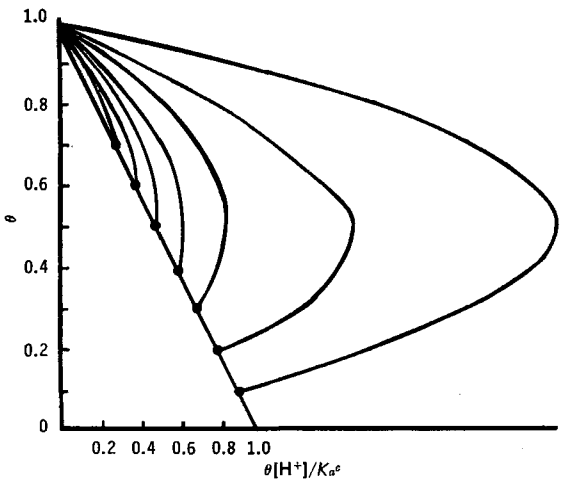

Figure 1—*Analog computer plot of* θ versus $\theta[H^+]/K_a^c$ *for various values of* θ_p.

2. Conservation of the weak acid in solution requires that at any time:

$$X^- = A^0 \qquad \text{(Eq. 4)}$$

where A^0 = absolute number of moles of acid originally added to the solution.

3. Electrical neutrality in the saturated solution requires that:

$$BH^+ + H^+ + M^+ = OH^- + X^- \qquad \text{(Eq. 5)}$$

where:

H^+ = absolute number of moles of hydrogen ion present in solution
M^+ = absolute number of moles of base added to solution
OH^- = absolute number of moles of hydroxyl ion present in solution
X^- = absolute number of moles of acid originally added to solution

The Z' term defined by Leeson and Brown (2) as:

$$Z' = M^+ + H^+ - OH^- \qquad \text{(Eq. 6)}$$

can also be written in terms of Eq. 5:

$$Z' = X^- - BH^+ \qquad \text{(Eq. 7)}$$

Substituting from Eqs. 4 and 3 into Eq. 7 yields:

$$Z' = A^0 - \frac{B_{sol}}{K_a^c} \cdot [H^+] \qquad \text{(Eq. 8)}^1$$

If the concentration of free amine in the solution remains constant throughout the precipitation phase, the B term in Eq. 8 will assume a constant value. Therefore, a plot of Z' *versus* $[H^+]$ should yield a straight line with a slope of B_{sol}/K_a^c and an intercept[2,3] of A^0.

Equation 8 is very similar to the following equation developed by Leeson and Brown (2):

$$Z' = A^0 - \frac{1}{K_a^c} Z'[H^+] \qquad \text{(Eq. 9)}$$

[1] For the titration of the salt of a strong base and a weak acid (A^-M^+), Eq. 8 would read:

$$Z' = A^0 - K_a^c A_{sol} \cdot \frac{1}{[H^+]}$$

[2] When only the amine is available, a slight molar excess of acid (HX) can be added to dissolve the amine. The intercept A^0 is still the number of moles of acid (in this case HX) added but now no longer equals the moles of amine titrated.

[3] The intercept on the $[H^+]$ axis has a value of $A^0K_a^c/B_{sol}$, from which K_a^c could be calculated if B_{sol} is known. However, the accuracy of this method is no greater than determining K_a^c from the slope.

However, the differences are important and warrant some discussion. Equations 8 and 9 apply to two different systems. Equation 8 utilizes only the data obtained during the precipitation phase; when Eq. 9 is applied to these data, it yields a curvilinear function which cannot be used to calculate a pKa value. Conversely, Eq. 8 is inapplicable when no precipitation occurs during a titration.

The B_{sol}/K_a^c term in Eq. 8 is a constant, which is not the case for the Z'/K_a^c term in Eq. 9. The slope of the plot of Z' *versus* $Z'[H^+]$ can be immediately converted into a pKa value. In Eq. 8, two separate determinations are needed before a pKa can be calculated. First, the slope B_{sol}/K_a^c is determined. Then the amount of base in the saturated solution is measured.

Titration before and during Precipitation—As was shown in the preceding section, Eq. 9 yields a linear plot as long as the concentration of the unionized amine is below the saturation point; Eq. 8 applies from the start of precipitation and onward. A more general equation applicable to either phase can be written as:

$$\theta = 1 - \theta' \frac{[H^+]}{K_a^c} \qquad \text{(Eq. 10)}$$

where:

$\theta = \dfrac{Z'}{A^0}$
θ' = θ before precipitation
θ' = θ_p at and after precipitation
$\theta_p = \dfrac{B_{sol}}{A^0}$

By using an analog computer, the characteristics of the plot of θ *versus* $\theta[H^+]/K_a^c$ (analogous to plotting Z' *versus* $Z'[H^+]$) were examined before and during precipitation. To examine these relationships, it was necessary to reexpress Eq. 10. This was accomplished in the following manner.

The ratio θ/θ' can be expressed as:

$$\theta/\theta' = 1 + (\theta - \theta')1/\theta_p \qquad \text{(Eq. 11)}$$

By rearranging Eq. 11,

$$\theta = \left[1 + (\theta - \theta')\frac{1}{\theta_p}\right]\theta' \qquad \text{(Eq. 12)}$$

and, therefore,

$$\theta \frac{[H^+]}{K_a^c} = \left[1 + (\theta - \theta')\frac{1}{\theta_p}\right]\theta' \frac{[H^+]}{K_a^c} \qquad \text{(Eq. 13)}$$

From Eq. 10,

$$\theta' \frac{[H^+]}{K_a^c} = 1 - \theta \qquad \text{(Eq. 14)}$$

Substituting Eq. 14 into Eq. 13 yields:

$$\theta \frac{[H^+]}{K_a^c} = \left[1 + (\theta - \theta')\frac{1}{\theta_p}\right](1 - \theta) \qquad \text{(Eq. 15)}$$

Hence, a plot of θ *versus* the right-hand side of Eq. 15 is equivalent to plotting θ *versus* $\theta[H^+]/K_a^c$. The results are shown in Fig. 1 for various fractions of neutralization at which precipitation occurs. Before precipitation ($\theta < \theta_p$), the plot gives a straight line with a slope of -1 and an intercept of 1. This is analogous to the plot of Z' *versus* $Z'[H^+]$ in Eq. 9, where a straight line with a slope of $1/K_a^c$ is obtained. After precipitation ($\theta > \theta_p$), the plot yields a sickle-shaped curve which eventually intercepts the ordinate axis at a value of 1. It will be shown that the same type of curve is obtained with lidocaine and other sparingly soluble compounds when the data are plotted according to Eq. 9 (Fig. 2). As such, the sickle-shaped curve cannot be used to calculate a pKa unless the data are plotted according to Eq. 8, as shown in Fig. 3.

EXPERIMENTAL

Materials—In the present work, the hydrochloride salts of a homologous series of local anesthetics of the amide type were investigated. Structurally, these are *meta*-substituted 2-diethylamino

acetanilides with solubilities ranging from 1.3×10^{-2} to 6×10^{-5} M.

Lidocaine HCl·H_2O was used[4]. The pH measurements were made on the expanded scale of a Beckman Expandomatic pH meter with an accuracy of ±0.005 pH unit. The meter was equipped with a Beckman glass electrode and a Beckman fiber junction calomel electrode.

Carbonate-free sodium hydroxide was also used[5]. The additions of sodium hydroxide solution were made with a Gilmont micropipet-buret[6].

Ion-free water was prepared using the Crystalab "Deeminizer" water demineralizer[7]. During the titration, the temperature of the solution was maintained constant using a Tamson precision circulation bath[8].

Methods—The sample of base hydrochloride was dissolved in 60 ml. of freshly deionized water or extemporaneously prepared KCl solution. The titration was performed under nitrogen in a water-jacketed thermostated vessel (24 ± 0.1°). Sodium hydroxide was added from a 1-ml. microburet calibrated to 0.0002 ml. In most titrations, the amounts of acid were calculated such that 0.5–1.0 ml. of 0.1 *N* NaOH was consumed. A minimum of 10 additions was made in a single run. During the precipitation phase, 6–10 min. after each addition of base ensured a stable and accurate pH reading.

At the end of those experiments where precipitation occurred during the titration, the solution containing an excess of amine was either filtered or centrifuged at $8000 \times g$ for 10 min. When the filtrate or supernatant was not clear, these operations were repeated. The concentration of the substance in the solution was then determined spectrophotometrically by reference to a previously established calibration curve. All measurements were made using a Beckman DBG spectrophotometer.

Several precautions were taken to avoid changes in the solubility to ensure accuracy in the solubility determination. First, the ultracentrifugation was performed immediately following the titration at a temperature identical to the temperature of the water-jacketed vessel. Next, an appropriate spectroscopic dilution bar, rather than dilution of the solution, was employed with those bases whose saturated solution gave too high an absorbance in the normal 1-cm. cell. Last, all amine solutions were maintained at a pH above 11 because acidification presents the danger of dissolving particles.

Examination of Nonlogarithmic Titration Curves prior to Precipitation—For the most soluble compounds of the series (solubility ranging from 1.3×10^{-2} *M* to 4.6×10^{-4} *M*), the pKa was obtained by using the lowest concentration (5×10^{-4} *M*) recommended by Benet and Goyan (7) and calculating the data according to the method of Leeson and Brown (2). When these same experimental conditions (concentration of drug 5×10^{-4} *M*) were utilized for the rest of the series, precipitation of the unionized species occurred during the titration. In such cases the titration was stopped, and several attempts to use lower concentrations were made. At concentrations below 5×10^{-4} *M*, the pH range covered during the titration is such that unavoidable errors in pH measurements become significant. This represents a serious limitation since the accuracy of the method using nonlogarithmic titration curves depends on the ability to distinguish between small [H^+] differences.

Those compounds with a solubility ranging between 2×10^{-4} *M* and 4×10^{-4} *M* were singled out for further examination. At the concentration used, the fraction neutralized before precipitation (θ_p) ranged between 0.4 and 0.8. The titration was pursued until complete neutralization, *i.e.*, throughout the precipitation phase. The data were treated according to the method of Leeson and Brown (2); the plot Z' *versus* $Z'[H^+]$ presents two distinct regions, the expected linear region (slope $1/K_a^c$), followed by the nonlinear portion of the graph obtained by continuing the titration during precipitation. As previously shown, this sickle-shaped curve cannot be used to calculate the pKa automatically. Therefore, it became necessary to ascertain whether in such cases ($0.4 < \theta_p < 0.8$) the linear portion of the graph could be used to compute the pKa. This was investigated in the following manner.

Lidocaine (2-diethylamino-2′,6′-acetoxylidide) is a fairly water-soluble weak base with an apparent pKa of 7.86 at 25° (11). Using concentrations below the solubility, the pKa was measured in this laboratory and the average of five determinations at 24° was found to be 7.86 (*SD* 0.03). Then duplicate runs were performed at two concentrations of lidocaine which were chosen such that the fraction neutralized before precipitation varied between 0.2 and 0.6. The data from those experiments are summarized in Table I, and Fig. 2 is a plot of Z' *versus* $Z'[H^+]$ for one titration. Note that even at the same concentration (especially at 0.05 *M*), the fraction neutralized at the point of visual precipitation varied between experiments. Also, when $\theta_p > 0.4$, the linear portion of the plot led to a fairly accurate measure of the pKa of lidocaine, and the A^0 value (numbers of moles of acid originally added to the solution) obtained by extrapolation of the linear portion of the graph was within a few percent of the theoretical value. When $\theta_p < 0.3$, the pre-precipitation curve was thereby reduced, and it became difficult to obtain an accurate value of the pKa. In Fig. 2, $\theta_p = 0.24$, and the pKa value was 7.98 instead of 7.85. Furthermore, the extrapolated A^0 value was off the theoretical value (number of moles of acid originally added) by more than 25%.

Table I—Extrapolated pKa of Lidocaine for Different Values of θ_p

Lidocaine Concentration, *M*	Fraction Neutralized at Beginning of Visual Precipitation	A^0 Extrapolated[a]	pKa
0.050	0.520	106.7	7.875
0.050	0.383	105.3	7.871
0.0775	0.258	109.2	7.909
0.0775	0.241	125.8	7.895

[a] Percent of theoretical value.

These inaccuracies would occur for compounds with an unionized species in the solubility range of 5×10^{-4} *M*. In those situations such an extrapolation would be more difficult than has been shown with lidocaine, because the effective pH range of the titration would be very small. Such an example is the 2-diethylamino-3′-phenylacetanilide, which has a water solubility of 7×10^{-5} *M* for the free amine. When the titration was performed at a concentration of 5×10^{-4} *M*, θ_p was approximately 0.1 and almost no linear portion of the plot of Z' *versus* $Z'[H^+]$ was obtained. In an attempt to increase the linear portion, the titration was performed at 1.7×10^{-4} *M*. Even though θ_p was equal to 0.4, it was not possible to obtain a straight line for the pre-precipitation curve because the pH range was too small.

From the foregoing experiments, the authors believe that when $\theta_p < 0.6$ the experimentor should be fully aware of the lack of accuracy inherent in the pKa measurement determined by the procedure of Leeson and Brown (2). Preferably, under these circumstances, Eq. 8 should be utilized as shown in the next section.

EXAMINATION OF NONLOGARITHMIC LINEAR TITRATION CURVES UTILIZING DATA OBTAINED DURING PRECIPITATION

Practical Evaluation of Method Using Lidocaine—Before using Eq. 8 to measure the pKa of the less soluble amines of the series, a preliminary test of that equation and the method was undertaken. Lidocaine was used because its pKa is already known and its solubility can be easily measured by UV spectrophotometry. Figure 3 is a plot of Z' *versus* [H^+] for lidocaine (0.775 *M*, 24°) with a slope of 0.69×10^8. At the end of the experiment, the solution was pipeted through a glass wool plug at the tip of a pipet. Several samples were taken and the absorbance measured. From the solubility obtained with the calibration curve, the B_{sol} term was calculated to be 0.9350 m*M* in the 60 ml. of solution; consequently, the pKa of lidocaine was found to be 7.869. In a duplicate experiment, the following results were obtained: slope = 0.666×10^8, B_{sol} = 0.9184 m*M*, and pKa = 7.861. The pKa values are in good agreement with those obtained in this laboratory using Eq. 9.

Examination of Method Using Less Soluble Compounds (Solubility 7×10^{-5} *M*)—For the foregoing experiments, although the B_{sol} term was constant throughout the titration, it cannot be assumed that this value equals the thermodynamic solubility of the compound. Generally, this will not be so because theoretically in-

[4] Supplied by Astra Pharmaceutical Products Inc.
[5] J. T. Baker Chemical Co.
[6] Scientific Products, Evanston, Ill.
[7] Crystal Research Laboratories, Inc., Hartford, Conn.
[8] Neslab Instruments, Portsmouth, N. H.

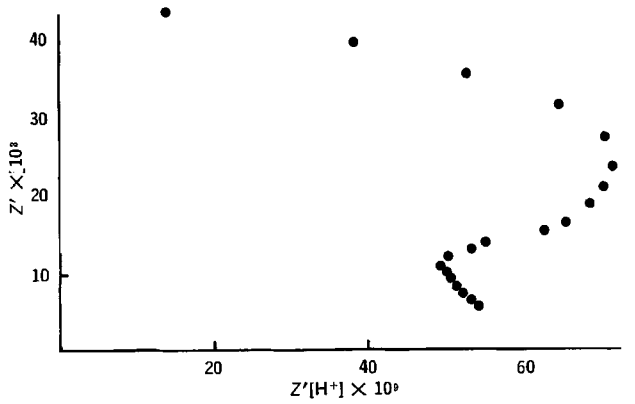

Figure 2—*Plot of* Z′ versus Z′ [H^+] *for lidocaine (0.775* M, *24°) when* $\theta_p = 0.24$.

finite time is required before thermodynamic equilibrium is achieved. However, Eq. 8 only requires that the change in concentration of the unionized species during the titration is nil or negligibly small.

Several factors in the experimental conditions favor constancy of the solubility. First, the solution is maintained at a constant temperature and continually stirred. Next, the unionized species is gradually liberated until the concentration in solution exceeds the saturation point. Finally, as one approaches saturation, there is almost inevitably a local precipitation of unionized species at the tip of the buret caused by the transient high concentration of titrant. This phenomenon tends to prevent supersaturation and favors constancy of the solubility. However, it is useful to know if B_{sol} is reproducible and independent of the concentration of compound. 2-Diethylamino-3′-phenylacetanilide has a water solubility of 7×10^{-5} *M* at 24°. Its absorption at 243 nm. is sufficiently high that it allows direct measurement of the saturated solution. The compound was titrated at three different concentrations (1.67×10^{-4} *M*, 5.22×10^{-4} *M*, and 1.52×10^{-3} *M*) but at constant ionic strength. The plots of *Z′ versus* [H^+] at these concentrations are shown in Fig. 4. The three lines are practically parallel to one another, which implies that the B_{sol} term did not significantly vary between the runs. Furthermore, differences between the absorbances measured at the end of each run were no greater than such variations in repeated runs on the same solution.

From Eq. 8 and a plot of *Z′ versus* [H^+], the dissociation constant is given by:

$$\frac{1}{K_a} = \frac{\text{slope}}{B_{sol}} \qquad \text{(Eq. 16)}$$

This relationship requires that when B_{sol} varies, the slope should vary in the same direction such as to maintain their ratio constant. The extent to which this holds true is a measure of the precision of this method. Twelve consecutive determinations were performed

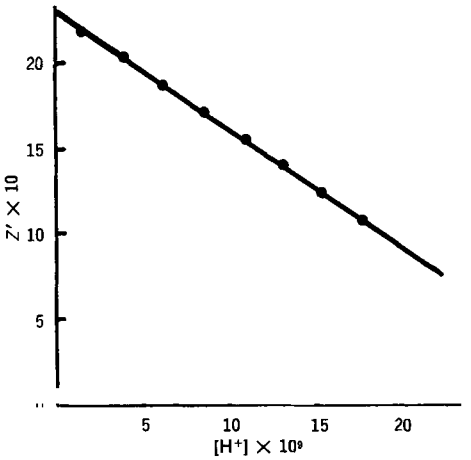

Figure 3—*Plot of Eq. 8* (Z′ versus [H^+]) *for lidocaine (0.775* M, *24°). Intercept* = A^0, *slope* = B_{sol}/K_a^0.

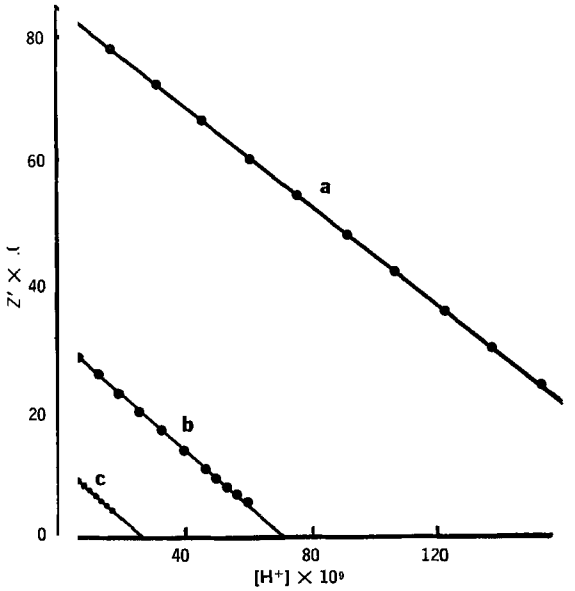

Figure 4—*Plot of* Z′ versus [H^+] *for 2-diethyl-3′-benzyloxyacetanilide showing that* B_{sol} *is not affected by a 10-fold change in concentration:* $a = 1.52 \times 10^{-3}$ M; $b = 5.22 \times 10^{-4}$ M; *and* $c = 1.67 \times 10^{-4}$ M.

with the 2-diethylamino-3′-benzyloxyacetanilide at a concentration of 10^{-3} *M*. At the end of each run, the supernatant was separated by ultracentrifugation and the solubility was measured by UV spectrophotometry. These data are shown in Table II. One can see that the solubility and the slope can vary by as much as 10–15% from run to run, which points out that only the values obtained for B_{sol} and the slope in a particular experiment may be used to calculate the pKa. To evaluate the significance of those variations, the mean and the standard deviation were computed for pKa, solubility, and slope. Table II also shows some data on the precision of the procedure of Leeson and Brown (2) obtained with lidocaine. It is quite common to express the precision of a pKa ±1 *SD*. Although it would be more correct to calculate the standard deviation of K_a's rather than pKa's, experience in this work has shown that the difference between the two is negligible.

It has been estimated that by using Eq. 9, a pKa can be determined with a precision of ±0.03 unit (6). The results with lidocaine agree very closely with those estimations. Table II also shows that this new procedure yields a pKa with a precision of ±0.04 unit. Although (as expected) this standard deviation is larger, it is less than one would anticipate knowing that this procedure has an additional source of error in the solubility measurement. Indeed, the overall precision of the pKa is intimately associated with the precision and, hence, the method of solubility determination of the substance under investigation.

PRACTICAL ASPECTS OF THE METHOD

Solubility Determination—Inherent in this method is the measurement of the concentration of unionized acid or base in a saturated solution of the compound[9]. As previously indicated, the first step involves separation of the solid from the solution; occasionally this presents a serious problem. Several techniques have been tried: centrifugation, filtration through a glass wool plug, filtration using millipore filters, and ultracentrifugation.

In this work, two main criteria, the particle size of the precipitate and the chemistry of the compound, proved useful in choosing a particular procedure. Systems containing large-size crystals afford an easy separation with centrifugation at the usual speeds, although one should watch for temperature changes during centrifugation. However, certain systems will not separate under these conditions. Filtration presents the advantage of simplicity, but adsorption can be a serious limitation. For example, extensive adsorption of 2-di-

[9] Attempts to correlate the fraction of the substance neutralized at the point of visual precipitation with independent solubility measurements proved unsuccessful, probably because of some supersaturation at this point.

Table II—Precision of the Proposed Method Using 2-Diethylamino-3′-benzyloxyacetanilide

2-Diethylamino-3′-benzyloxyacetanilide				Lidocaine	
Experiment	Slope $\times 10^5$	Absorbance	pKa	Experiment	pKa
1	3.36	0.599	7.946	A	7.850
2	3.77	0.540	8.039	B	7.825
3	3.75	0.535	8.041	C	7.907
4	3.87	0.700	7.939	D	7.835
5	3.87	0.710	7.933	E	7.884
6	3.85	0.620	7.990		
7	3.16	0.575	7.998		
8	4.03	0.543	8.061		
9	4.04	0.585	8.034		
10	4.01	0.605	8.016		
11	4.13	0.650	7.999		
12	3.90	0.610	8.001		
Average	3.81	0.606	8.00		7.860
SD	0.29	0.058	0.042		0.034

ethylamino-3′-benzyloxyacetanilide onto a glass wool plug at the tip of a pipet became apparent when successive samples drawn from the same mother solution gave widely different absorbances. Millipore filters did not solve the problem. After one filtration the absorbance of a solution of 2-diethylamino-3′-benzyloxyacetanilide was reduced from 0.577 to 0.385. Ultimately the problem was solved using ultracentrifugation (at $8000 \times g$ for 10 min.). Satisfactory results were consistently achieved with this method. However, a temperature control device is necessary to prevent excessive temperature variations which could affect the solubility.

Calculation of Eq. 8—The $[H^+]$ term is calculated by converting the hydrogen-ion activity (a_{H^+}) to hydrogen-ion concentration. As shown by Leeson and Brown (2), one has the choice between the activity coefficient of the hydrogen ion calculated by Kielland (12) and the experimentally determined $\gamma \pm$ values of Lewis and Randall (13). By knowing a_{H^+}, the K_w at the temperature of the experiment gives the hydroxide-ion activity (a_{OH^-}). The activity coefficient needed to calculate the hydroxide-ion concentration can be either computed from the Guggenheim equation (14) or taken from the tables of Harned and Owen (15). At the concentrations used in this work, all the titration occurred in the pH range 5–10, and H^+ and OH^- became negligible terms in the calculation of Z' in Eq. 6.

Influence of Ionic Strength—Ionic strength had practically no effect on the acidic dissociation constant of lidocaine and 2-diethylamino-3′-benzyloxyacetanilide. Consequently, all the pKa values were pooled together, regardless of the ionic strength at which they were determined (Table II). However, the ionic concentration may affect the solubility (B_{sol}), and careful consideration should be given to this fact. The mechanism involved is the salting-out effect where salt ions withdraw water molecules and cause the unionized species to reach saturation earlier. This would affect the B_{sol} term and the slope without changing the K_a^c. This effect was not noticeable in the case of the local anesthetic amines studied (Fig. 4). However, it should be kept in mind when working with weak acids for which the only way to obtain an accurate thermodynamic pKa is to extrapolate a plot of pK_a^c *versus* $\sqrt{\mu}/(1 + \sqrt{\mu})$.

Limits of Method—For obvious reasons, the proposed method is not suited to the direct titration of an acid by a base and vice versa. Rather, one should use a soluble salt of the sparingly soluble acid or base under investigation. Moreover, in the case of monoprotic species, this system allows the ionic strength to remain constant during all the titration. The concentration at which to run the titration deserves careful consideration. Using very low concentrations (5×10^{-4} *M*) is advantageous when the availability of substance is limited. Also, it circumvents activity effects and enables one to obtain thermodynamic dissociation constants. However, one should also consider the interplay of titration range, pKa, and solubility. In the titration of the salt of weak base $BH^+ X^-$, Eq. 1 can be rearranged to give:

$$\frac{K_a^c}{[H^+]} = \frac{[B]_{sol}}{[BH^+]} \qquad \text{(Eq. 17)}$$

It becomes apparent that for 90% of the titration after precipitation, the pH will change only 1 unit. Moreover, it is desirable that this pH change be within the range 5–9. (In that case, H^+ and OH^- are negligible terms in the calculation of Z'.) What determines the pH at which the base begins to precipitate (pH_p) is the solubility, pKa, and concentration of salt used (A^0), since:

$$pH_p = pKa + \log \frac{[B]_{sol}}{[A^0] - [B]_{sol}} \qquad \text{(Eq. 18)}$$

By choosing $[A^0] = 10^{-3}$ *M* for a base whose solubility is 10^{-5} *M*, it is clear that this method is satisfactory if the pKa is within the range 7–10. Similarly, for a weak acid this corresponds to a pKa range of 4–7. The proposed method would reach its limits of applicability in the case of a base that is very weak and at the same time very insoluble. Then, the unionized species precipitates as soon as the salt is put into water because water is competing with the weak base for the proton. However, most pharmacological agents appear to fall within the practical limits of the method.

In the case of polyprotic species, the relationship between pKa and solubility is even of more consequence. Consider the titration of a dibasic acid where the two pKa's are separated by 2 units. At a pH equal to the second pKa, the unionized species constitute 1% of the total concentration in solution. If the solubility is so small that it is already exceeded at that pH, precipitation with titration of the first hydrogen takes place as the second hydrogen is only half-neutralized. Therefore, the degree of separation between any two pKa's needs to be larger than required for soluble polyprotic species.

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Drug Absorption IV: Influence of pH on Absorption Kinetics of Weakly Acidic Drugs

W. G. CROUTHAMEL, G. H. TAN, L. W. DITTERT, and J. T. DOLUISIO

Abstract □ This paper reports the absorption kinetics of sulfaethidole and barbital from the rat stomach and rat small intestine *in situ*. The absorption rates varied with pH for both drugs. With both drugs, absorption from the intestines proceeded 10 times faster at a given pH than from the stomach. Rate constants for the transport of unionized and ionized drug moieties were determined from the stomach and from the intestine; in all cases, significant transport of ionized drug was noted.

Keyphrases □ Pharmacokinetics, gastrointestinal absorption, pH effect—sulfaethidole and barbital, rats □ Drug absorption, gastrointestinal, pH effect—sulfaethidole and barbital, rats □ pH-partition coefficients—gastrointestinal drug absorption *in situ*, rats □ Membrane transport, gastrointestinal drug absorption—ionized and unionized sulfaethidole rate constants, pH effect

Since the pH-partition hypothesis of gastrointestinal drug absorption was first proposed by Shore *et al.* (1), reports have appeared both agreeing and disagreeing with the theory. For example, Hogben *et al.* (2) found that the gastric absorption of several drugs appeared to obey the pH-partition theory, whereas the intestinal absorption of the same drugs deviated from the theory. The latter deviations were explained by hypothesizing the existence of a layer at the intestinal mucosal surface with a pH different from that of the bulk intestinal lumen fluid. If the "virtual pH" at the membrane surface was assumed to be 5.3, the compounds studied by Hogben *et al.* (2) obeyed the pH-partition theory. Other investigators (3, 4) postulated virtual pH's at 6 or 7 to explain intestinal absorption data.

Factors such as drug binding to intestinal mucosa or the absorption of ionized drug may also be responsible for deviations from the pH-partition hypothesis. For example, Kakemi *et al.* (5) found that the absorption-rate constants for a series of barbituric acids in an *in situ* rat intestinal preparation did not always follow the expected changes when pH was altered; they suggested that binding to intestinal mucosa also had to be taken into account.

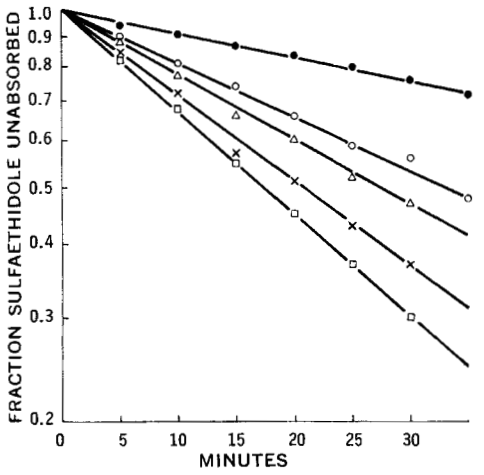

Figure 1—*Semilogarithmic plots of the disappearance of sulfaethidole from the lumen of rat intestine* in situ *at various lumen solution pH's. Key:* ●, *pH 8.6,* $t_{1/2}$ = *81 min.;* ○, *pH 6.1,* $t_{1/2}$ = *34 min.;* △, *pH 5.75,* $t_{1/2}$ = *28 min.;* ×, *pH 5.45,* $t_{1/2}$ = *20 min.; and* □, *pH 5.1,* $t_{1/2}$ = *17 min.*

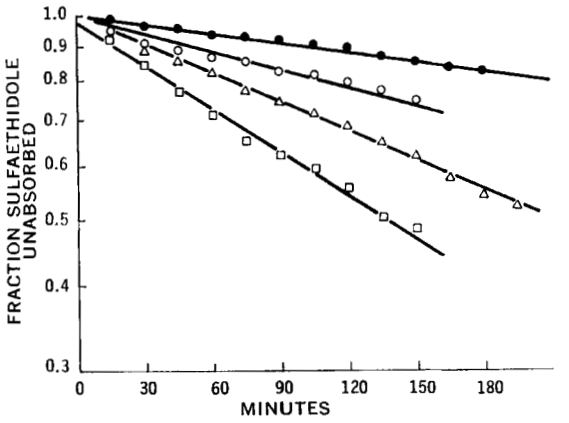

Figure 2—*Semilogarithmic plots of the disappearance of sulfaethidole from the lumen of rat stomach* in situ *at various lumen solution pH's. Key:* ●, *pH 8.3,* $t_{1/2}$ = *637 min.;* ○, *pH 5.9,* $t_{1/2}$ = *325 min.;* △, *pH 5.35,* $t_{1/2}$ = *210 min.; and* □, *pH 3.45,* $t_{1/2}$ = *143 min.*

Nogami *et al.* (6) showed that the ionized as well as the unionized forms of sulfonamides were absorbed through isolated rat intestinal segments *in vitro*, and Turner *et al.* (7) tested the pH-partition hypothesis experimentally in everted intestinal sacs *in vitro*. The latter authors concluded that the virtual pH hypothesis of Hogben *et al.* (2) appeared to be inoperative in *in vitro* preparations, and such preparations do not always mimic *in vivo* behavior, especially with regard to the transport of ions which occurs to a much greater degree *in vitro* than *in vivo*.

If the pH-partition hypothesis is correct, acidic drugs might be expected to be absorbed preferentially from the stomach and basic drugs preferentially from the intestines, since at these sites the respective drug classes would be ionized to a lesser degree than at other gastrointestinal sites. However, anatomical and functional differences such as total surface area, blood supply, and transit time greatly influence the rate and extent of absorption at the two sites; in actual practice, the intestines may be the primary absorption site for both weak acids and weak bases. The purpose of the present investigation was to study the influence of pH on the rates of absorption of weakly acidic drugs from *in situ* rat stomach and small intestinal preparations (8). It was expected that the results would reveal differences between the two absorption sites with regard to kinetics of drug transfer of unionized and ionized drug species.

Table I—Sulfaethidole Absorption Rates ($k_{app.}$) and Fraction of Drug Unionized (f_U) at Various pH Values

Average pH (Range)	Buffer[a]	Fraction Unionized (f_U)	Half-Life, min.	$k_{app.}$, min.$^{-1}$
Rat Intestine *In Situ*				
5.20 (5.0–5.4)	C	0.67	17	0.0408
5.10 (5.0–5.2)	C	0.72	17	0.0408
5.45 (5.3–5.6)	P	0.53	21	0.0330
5.45 (5.3–5.6)	P	0.53	20	0.0346
5.75 (5.7–5.8)	P	0.36	28	0.0248
5.80 (5.7–5.9)	P	0.33	29	0.0239
6.05 (6.0–6.1)	P	0.22	30	0.0231
6.10 (6.0–6.2)	P	0.20	34	0.0204
8.60 (9.0–8.2)	B	0.001	81	0.0086
8.45 (9.0–7.9)	B	0.001	79	0.0088
Rat Stomach *In Situ*				
3.45 (3.0–3.9)	C	0.99	143	0.00485
5.15 (5.3–5.0)	C	0.69	156	0.00444
4.65 (5.3–4.0)	C	0.88	150	0.00462
4.90 (5.3–4.5)	C	0.80	150	0.00462
5.50 (5.7–5.3)	C	0.50	246	0.00282
5.45 (5.7–5.2)	C	0.53	243	0.00285
5.35 (5.7–5.0)	C	0.59	210	0.00330
6.40 (6.5–6.3)	C	0.11	338	0.00205
6.35 (6.5–7.2)	C	0.12	323	0.00215
5.90 (6.0–5.8)	C	0.28	325	0.00213
5.85 (6.0–5.7)	C	0.31	275	0.00252
8.20 (9.0–7.5)	B	0.002	690	0.00100
8.30 (9.0–7.6)	B	0.002	637	0.00109

[a] C = McIlvaine's citrate–phosphate buffer (14), P = 0.09 *M* phosphate buffer made isotonic with NaCl, and B = Clark and Lubs borate buffer (14).

EXPERIMENTAL

Apparatus and Reagents—All chemicals were reagent grade except sulfaethidole[1] and sodium barbital[2]. A Beckman Zeromatic II pH meter and a Cary model 15 spectrophotometer were used. The perfusion and drug solutions used were described in a previous communication (8). The initial concentrations of sulfaethidole and sodium barbital were approximately 2.5×10^{-3} and 5×10^{-3} *M*, respectively.

Test Animals—Male Sprague-Dawley albino rats, weighing 220–260 g., were fasted for 15–20 hr. prior to use.

Procedure—The procedure for studying drug disappearance from the *in situ* rat stomach and rat small intestinal lumen was described previously (8).

Analytical Methods—Barbital was extracted from the samples with chloroform and then from the chloroform into a pH 9.4 borate buffer; the absorbance of the buffer solution was determined at 242 nm. Sulfaethidole was analyzed by the method of Bratton and Marshall (9).

RESULTS AND DISCUSSION

Semilogarithmic plots, showing the disappearance of sulfaethidole from rat intestinal lumen *in situ* at average pH's between 5.15 and 8.52 and from rat gastric lumen *in situ* at average pH's between 3.45 and 8.30, are shown in Figs. 1 and 2. In both preparations, all disappearances followed first-order kinetics, and the disappearance-rate constants increased (half-lives decreased) as the pH was lowered. Similar results were obtained with barbital. With both drugs, absorption from the intestines proceeded about 10 times faster at a given pH than from the stomach, reflecting the difference in surface area of these two organs. The half-lives and apparent first-order rate constants for the disappearances of sulfaethidole and barbital are summarized in Tables I and II. In all cases, the pH of the lumen solutions shifted several tenths of a unit during the experiment, and the average of the initial and terminal pH's was used to calculate the fraction of drug in the unionized form during the experiment. These fractions are also shown in Tables I and II.

[1] Smith Kline & French Laboratories.
[2] Merck & Co.

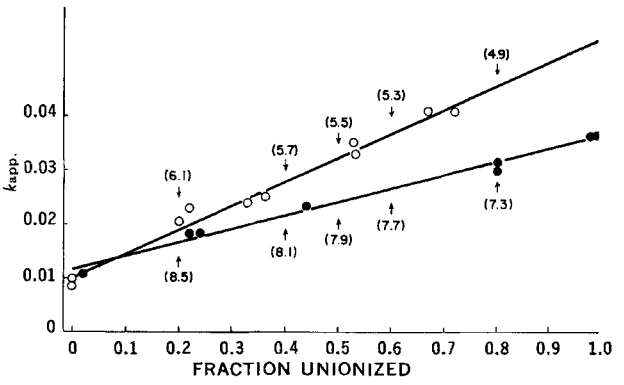

Figure 3—*Plots showing the relationship between* $k_{app.}$, *the rate constant controlling overall disappearance of drug from rat intestinal lumen* in situ, *and the fraction of drug present in the unionized form. Key:* ○, *sulfaethidole; and* ●, *barbital. The numbers in parentheses are pH's at various points on the respective curves.*

If it is assumed that both the unionized and the ionized drug species are capable of being absorbed and that the apparent first-order absorption-rate constant ($k_{app.}$) at a given pH is the sum of the rate constants attributable to the two species at that pH, then the influence of pH on the rate of absorption of a weak electrolyte can be expressed as (10):

$$k_{app.} = f_U k_U + f_I k_I \qquad \text{(Eq. 1)}$$

where k_U and k_I are the rate constants controlling absorption of the unionized and ionized species, respectively, and f_U and f_I are the fractions of the drug present in the unionized and ionized forms, respectively. Since:

$$_I = (1 - f_U) \qquad \text{(Eq. 2)}$$

Eq. 1 can be rewritten:

$$k_{app.} = k_I + f_U(k_U - k_I) \qquad \text{(Eq. 3)}$$

Table II—Barbital Absorption Rates ($k_{app.}$) and Fraction of Drug Unionized (f_U) at Several pH Values

Average pH (Range)	Buffer[a]	Fraction Unionized (f_U)	Half-Life, min.	$k_{app.}$, min.$^{-1}$
Rat Intestine *In Situ*				
6.10 (6.0–6.2)	P	0.98	19	0.0365
6.10 (6.0–6.2)	P	0.98	19	0.0365
6.05 (6.0–6.1)	P	0.99	19	0.0365
7.30 (7.5–7.1)	P	0.80	23	0.0301
7.30 (7.5–7.1)	P	0.80	22	0.0315
7.70 (8.1–7.3)	P	0.44	30	0.0231
8.40 (8.5–8.3)	P	0.24	38	0.0182
8.45 (8.5–8.4)	B	0.22	38	0.0182
9.70 (10.0–9.4)	B	0.02	65	0.0107
Rat Stomach *In Situ*				
3.20 (3.00–3.40)	C	1.00	222	0.00312
3.20 (3.00–3.40)	C	1.00	201	0.00345
5.95 (6.00–5.90)	P	0.99	232	0.00299
5.90 (6.00–5.80)	P	0.99	240	0.00289
6.00 (6.00–6.00)	P	0.99	260	0.00267
6.40 (7.00–5.80)	B	0.97	232	0.00299
6.45 (7.00–5.90)	B	0.96	242	0.00289
7.45 (8.00–6.90)	B	0.74	290	0.00239
7.45 (8.00–6.90)	B	0.74	315	0.00220
7.50 (8.00–7.00)	B	0.71	375	0.00185
7.85 (8.50–7.20)	B	0.53	340	0.00204
7.85 (8.50–7.20)	B	0.53	330	0.00210
7.85 (8.50–7.20)	B	0.53	380	0.00182
7.85 (8.50–7.20)	B	0.53	320	0.00217
7.85 (8.50–7.20)	B	0.53	390	0.00180
8.20 (9.00–7.40)	B	0.33	390	0.00178
8.40 (9.00–7.80)	B	0.24	465	0.00149

[a] C = McIlvaine's citrate–phosphate buffer (14), B = Clark and Lubs borate buffer (14), and P = 0.09 *M* phosphate buffer made isotonic with NaCl.

Table III—Rate Constants (min.$^{-1}$) for Absorption of Unionized (k_U) and Ionized (k_I) Sulfaethidole and Barbital from Rat Gastrointestinal Segments *In Situ*

Drug	pKa	Gastrointestinal Segment	Range of f_U Studied	k_U	k_I	k_U/k_I
Sulfaethidole	5.5	Intestine	0.002–0.72	0.055	0.0099	5.55
		Stomach	0.002–0.99	0.0051	0.0012	4.25
Barbital	7.9	Intestine	0.02–0.99	0.037	0.012	3.08
		Stomach	0.24–1.00	0.0029	0.0009	3.22

According to Eq. 3, a plot of $k_{app.}$ *versus* f_U is a straight line, with intercepts of k_I (at $f_U = 0$) and k_U (at $f_U = 1.0$) and a slope equal to $(k_U - k_I)$.

Recently Suzuki *et al.* (11, 12) proposed physical models for drug absorption. The data shown in Tables I and II are consistent with their Model III (12) where the membrane phase is heterogeneous (aqueous and lipid), simulating a membrane consisting of lipoidal cells in an aqueous intercellular fluid environment. For this model, it would be expected that the unionized moiety could pass with differing ease through both the aqueous and nonaqueous environments while the ionized moiety could pass through only the aqueous. Hence, Eq. 1 can be modified to:

$$k_{app.} = f_U(k_{U,lipid} + k_{U,aqueous}) + f_I k_I \qquad \text{(Eq. 4)}$$

Equation 4 could then be modified to:

$$k_{app.} = k_I + f_U(k_{U,lipid} + k_{U,aqueous} - k_I) \qquad \text{(Eq. 5)}$$

Thus, a plot of $k_{app.}$ *versus* f_U should yield a straight line with intercepts of k_I (at $f_U = 0$) and $k_{U,lipid} + k_{U,aqueous}$ (at $f_U = 1.0$), and a slope of $(k_{U,lipid} + k_{U,aqueous} - k_I)$ if the physical model is correct. Equations 3 and 5 both yield linear plots, and the present data fit both equations. Equation 5 assumes a heterogeneous physical model where ionized drug transport occurs through aqueous portions of the membrane. Equation 3 assumes that ionized drug passes the same barrier(s) as the unionized moiety but with differing ease. Consequently, Eqs. 3 and 5 lead to slightly different interpretations, but both cases provide for absorption of the ionized drug moiety. Equation 3 is probably the more correct expression. For the data reported in this paper, the value of $k_{U,lipid}$, calculated from Eq. 5 by assuming that $k_{I,aqueous} = k_{U,aqueous}$, would be approximately 26% greater than the values of k_U calculated with Eq. 3 and reported in Table III.

Plots according to Eq. 3 for the absorption of sulfaethidole and barbital from the rat intestine *in situ* are shown in Fig. 3. Similar plots for the rat stomach are shown in Fig. 4. The rate constants, k_U (Eq. 3) and k_I, for the two drugs were calculated by regression analysis and are summarized in Table III. In all cases, the correlation coefficient was greater than 0.96. The ionized forms of both sulfaethidole and barbital are absorbed at significant rates from both the rat gastric and intestinal *in situ* preparations. The unionized forms of these drugs are absorbed 3–5 times faster than the ionized forms at both sites. The ratios of k_U/k_I shown in Table III for sulfaethidole are greater than those for barbital, suggesting that pH would have a greater influence on the absorption rate of sulfaethidole than on that of barbital.

The larger ratio for sulfaethidole is probably due to a greater partition coefficient for the drug into the absorbing membrane. True partition coefficients for the two drugs were calculated from apparent coefficients at the indicated pH's using a separator shake-out method (13). The chloroform–water true partition coefficients were: sulfaethidole, 2.7 (pH 5.6 and 6.6); and barbital, 0.8 (pH 7.7 and 8.5). The *n*-octanol–water true partition coefficients were: sulfaethidole, 7.5 (pH 6.0 and 7.0); and barbital, 3.4 (pH 8.0 and 9.0). Thus, partitioning data in chloroform–water and *n*-octanol–water systems agree with kinetic data obtained in the *in situ* preparation.

The results shown in Figs. 3 and 4 and in Table III can be rationalized completely on the basis of the pKa's of the drugs and the pH's of the bulk lumen solutions as these factors affect the fractions of the drugs present in their unionized forms. For sulfaethidole and barbital in the *in situ* rat gastric and intestinal preparations, there is no evidence of the existence of a "virtual pH" layer at the absorbing membrane surface different from the pH of the bulk lumen solution. If such a layer exists, it is likely that changes in the pH of the bulk solution would have little or no effect on the absorption rate. On the other hand, transfer of sulfaethidole and barbital ions made significant contribution to the overall absorption of these drugs, which suggests that in rationalizing deviations from the traditional pH-partition hypothesis, serious consideration must be given to absorption of drug ions.

The significance of the absorption of the ionized moieties can be illustrated by the relatively rapid intestinal absorption of sulfaethidole at pH 6.1. At this pH, $f_I = 0.80$ and $f_U = 0.20$ for sulfaethidole. Since the apparent rate constants for drug absorption are equal to $f \times k$, it can be calculated that the apparent rate constants for the intestinal absorption of ionized and unionized sulfaethidole are 0.0079 and 0.011 min.$^{-1}$, respectively. Consequently, the absorption of sulfaethidole ions accounts for 42% of the total absorption at this pH. Thus, even though k_U and k_I greatly favor the preferential absorption of unionized drug, under certain pH conditions a rather significant degree of absorption may be due to the transport of ionized drug.

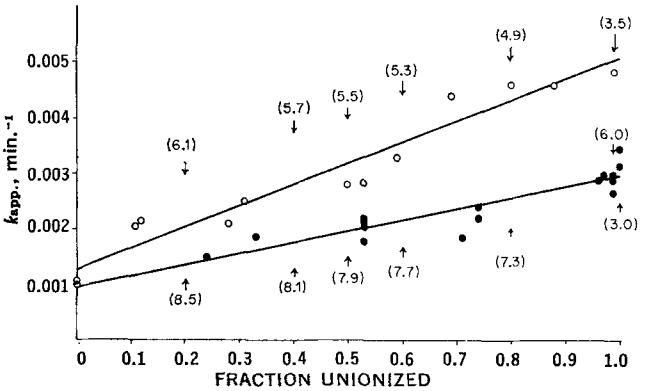

Figure 4—*Plots showing the relationship between* $k_{app.}$, *the rate constant controlling overall disappearance of drug from rat gastric lumen* in situ, *and the fraction of drug present in the unionized form. Key:* ○, *sulfaethidole; and* ●, *barbital. The numbers in parentheses are pH's at various points on the respective curves.*

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Toxicity of Ethylene Chlorohydrin II: Subacute Toxicity and Special Tests

W. H. LAWRENCE, K. ITOH*, J. E. TURNER, and J. AUTIAN

Abstract □ Ethylene chlorohydrin may be found as a reaction product when plastic items are sterilized with ethylene oxide. A previous publication presented results of an acute toxicity study of ethylene chlorohydrin in animals and tissue culture. This paper presents additional toxicity information derived from a study of rats receiving the compound intraperitoneally three times a week for 3 months, and from a similar study in which the compound was administered daily for 1 month. No adverse effects were observed in the 1-month study from 6.4 mg./kg. daily of ethylene chlorohydrin (one-tenth of the acute LD_{50}), or from 12.8 mg./kg. administered three times a week for 3 months. Additionally, special tests were included to determine the effect of the compound upon: cardiovascular, respiratory, and neuromuscular functions in rabbits; sensitizing potential in guinea pigs; and pentobarbital sleeping time in mice.

Keyphrases □ Ethylene chlorohydrin—subacute toxicity, cardiovascular, respiratory, neuromuscular, sensitizing potential, sleeping time effects □ Ethylene oxide sterilization—ethylene chlorohydrin toxicity evaluation □ Toxicity, subacute—ethylene chlorohydrin evaluation, rats

During the late sixties, a growing awareness of a lack of pertinent information relating to the toxicity of ethylene chlorohydrin (2-chloroethanol) prompted a number of studies. For many years, industrial workers have experienced local and systemic adverse reactions from improper handling of this compound; more recently, the treatment of foods and the sterilization of polymeric substances with ethylene oxide have resulted in the formation of ethylene chlorohydrin when chlorides were present even in minute amounts in the exposed material. The acquisition of additional toxicological data is essential to alert medical care personnel to the potential hazards associated with ethylene chlorohydrin when hospitalized patients come in contact with ethylene oxide-sterilized devices, to warn the general public of the dangers of consuming ethylene oxide-treated foods, and, finally, to inform those who come in contact with large quantities of the chemical in industry.

In a previous paper (1), results of an acute toxicity study of ethylene chlorohydrin gave LD_{50} values within a rather narrow range (64–98 mg./kg.) when the compound was administered to mice, rats, rabbits, and guinea pigs, which were independent of the route of administration. The compound was easily absorbed through the intact skin; thus, the LD_{50} produced by topical administration was comparable to that from intraperitoneal or oral administration. Its vapors, when mixed with air, killed 50% of the test animals after 13.3 min. of exposure. The compound was found to be highly irritating intradermally or from ophthalmic application. Little irritation was noted, however, when the compound was applied to the skin of rabbits.

Because it is possible for people to be repeatedly exposed to ethylene chlorohydrin in very small concentrations by the parenteral, dermal, or oral[1] routes, it was deemed appropriate to extend the previous studies into an investigation of subacute toxicity and to conduct certain other special studies.

EXPERIMENTAL

Materials—Ethylene chlorohydrin[2] was used.

Subacute Toxicity Studies—*Phase One: 12-Week Study*—In these experiments, male Sprague-Dawley rats were used, having initial weights of 57–64 g. In the first series of experiments, two dose levels were administered intraperitoneally to groups of 12 rats. The dose levels were based upon one-fifth and one-half the acute LD_{50} dose or 12.8 and 32.0 mg./kg., respectively. When possible, the compound was administered as such or, when necessary, it was diluted in distilled water and administered using microliter syringes. The respective dose levels were administered three times a week (Monday, Wednesday, and Friday) over a 12-week period. A control group of rats received normal saline in a volume equivalent to the test samples and at the same time intervals. The animals were housed two in a cage and given food and water *ad libitum*.

All animals were observed daily to assess general health and mortalities; they were weighed three times per week, just prior to injection of the chlorohydrin, and the weights were recorded. Food consumption was measured during the 1st, 2nd, 5th, 9th, and 12th weeks. Hematologic tests were performed on six rats selected at random from each test group and saline controls at the end of the 12th week. Several drops of blood were obtained from each animal by clipping the end of the tail for determination of the following:

[1] In addition to the possibility that it may be contained in certain foods, some hospitals sterilize plastic drinking tumblers and pitchers with ethylene oxide and then fill these with drinking water, which, of course, contains chlorides and possibly fluorides.

[2] Matheson, Coleman, and Bell, East Rutherford, N. J. The liquid was established as being more than 99% ethylene chlorohydrin.

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(12) *Ibid.*, **59**, 651(1970).
(13) D. R. Reese, G. M. Irwin, L. W. Dittert, C. W. Chong, and J. V. Swintosky, *J. Pharm. Sci.*, **53**, 591(1964).
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Toxicity of Ethylene Chlorohydrin II: Subacute Toxicity and Special Tests

W. H. LAWRENCE, K. ITOH*, J. E. TURNER, and J. AUTIAN

Abstract □ Ethylene chlorohydrin may be found as a reaction product when plastic items are sterilized with ethylene oxide. A previous publication presented results of an acute toxicity study of ethylene chlorohydrin in animals and tissue culture. This paper presents additional toxicity information derived from a study of rats receiving the compound intraperitoneally three times a week for 3 months, and from a similar study in which the compound was administered daily for 1 month. No adverse effects were observed in the 1-month study from 6.4 mg./kg. daily of ethylene chlorohydrin (one-tenth of the acute LD_{50}), or from 12.8 mg./kg. administered three times a week for 3 months. Additionally, special tests were included to determine the effect of the compound upon: cardiovascular, respiratory, and neuromuscular functions in rabbits; sensitizing potential in guinea pigs; and pentobarbital sleeping time in mice.

Keyphrases □ Ethylene chlorohydrin—subacute toxicity, cardiovascular, respiratory, neuromuscular, sensitizing potential, sleeping time effects □ Ethylene oxide sterilization—ethylene chlorohydrin toxicity evaluation □ Toxicity, subacute—ethylene chlorohydrin evaluation, rats

During the late sixties, a growing awareness of a lack of pertinent information relating to the toxicity of ethylene chlorohydrin (2-chloroethanol) prompted a number of studies. For many years, industrial workers have experienced local and systemic adverse reactions from improper handling of this compound; more recently, the treatment of foods and the sterilization of polymeric substances with ethylene oxide have resulted in the formation of ethylene chlorohydrin when chlorides were present even in minute amounts in the exposed material. The acquisition of additional toxicological data is essential to alert medical care personnel to the potential hazards associated with ethylene chlorohydrin when hospitalized patients come in contact with ethylene oxide-sterilized devices, to warn the general public of the dangers of consuming ethylene oxide-treated foods, and, finally, to inform those who come in contact with large quantities of the chemical in industry.

In a previous paper (1), results of an acute toxicity study of ethylene chlorohydrin gave LD_{50} values within a rather narrow range (64–98 mg./kg.) when the compound was administered to mice, rats, rabbits, and guinea pigs, which were independent of the route of administration. The compound was easily absorbed through the intact skin; thus, the LD_{50} produced by topical administration was comparable to that from intraperitoneal or oral administration. Its vapors, when mixed with air, killed 50% of the test animals after 13.3 min. of exposure. The compound was found to be highly irritating intradermally or from ophthalmic application. Little irritation was noted, however, when the compound was applied to the skin of rabbits.

Because it is possible for people to be repeatedly exposed to ethylene chlorohydrin in very small concentrations by the parenteral, dermal, or oral[1] routes, it was deemed appropriate to extend the previous studies into an investigation of subacute toxicity and to conduct certain other special studies.

EXPERIMENTAL

Materials—Ethylene chlorohydrin[2] was used.

Subacute Toxicity Studies—*Phase One: 12-Week Study*—In these experiments, male Sprague-Dawley rats were used, having initial weights of 57–64 g. In the first series of experiments, two dose levels were administered intraperitoneally to groups of 12 rats. The dose levels were based upon one-fifth and one-half the acute LD_{50} dose or 12.8 and 32.0 mg./kg., respectively. When possible, the compound was administered as such or, when necessary, it was diluted in distilled water and administered using microliter syringes. The respective dose levels were administered three times a week (Monday, Wednesday, and Friday) over a 12-week period. A control group of rats received normal saline in a volume equivalent to the test samples and at the same time intervals. The animals were housed two in a cage and given food and water *ad libitum*.

All animals were observed daily to assess general health and mortalities; they were weighed three times per week, just prior to injection of the chlorohydrin, and the weights were recorded. Food consumption was measured during the 1st, 2nd, 5th, 9th, and 12th weeks. Hematologic tests were performed on six rats selected at random from each test group and saline controls at the end of the 12th week. Several drops of blood were obtained from each animal by clipping the end of the tail for determination of the following:

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Table I—Mortalities in Subacute Toxicity Studies of Ethylene Chlorohydrin in Rats

Dose[a]	Ratio (Deaths/Total)
Phase One	
0.20 LD_{50}	4/12[b]
0.50 LD_{50}	6/12
Normal saline (control)	0/12
Phase Two	
0.10 LD_{50}	0/12
0.20 LD_{50}	0/12
Normal saline (control)	0/12

[a] LD_{50} in rats intraperitoneally is 64.0 mg./kg. [b] These rats died in the first 1–2 weeks and were most likely due to errors in dosage. The experiment was repeated in Phase Two with no deaths.

hemoglobin, hematocrit, erythrocytes, total leukocytes, platelets, and differential leukocyte counts.

At the end of the study, six animals in each group were sacrificed and autopsied for gross pathologic lesions. Organ-to-body weights were determined for the adrenals, brain, gonads, heart, kidneys, liver, lungs, and spleen; these organs were preserved in 10% buffered formalin, sectioned, and stained with hematoxylin-eosin for histopathologic evaluation.

Phase Two: 12-Week Study—During the early course of the subacute toxicity study reported in Phase One, four rats in the 0.20 LD_{50} group died but it was believed that these deaths may have been caused by an overdose of chlorohydrin; thus the experiment was repeated following the procedures already described. A second 12-week subacute toxicity study was also conducted in a manner similar to the previous studies except that a lower dose of chlorohydrin was employed: 0.10 LD_{50} (6.4 mg./kg.). In each of these studies, an additional test was utilized to ascertain if possible liver damage may have occurred by selecting six animals from each group and evaluating the rate of disappearance of sulfobromophthalein[3] from serum. The method of Gaebler (2) was used for sulfobromophthalein determination.

Phase Three: 30-Day Cumulative Study—Another study was undertaken to determine if cumulative toxic effects might be seen when animals were administered daily doses of the test compound for 30 days. Two dose levels were administered to male rats of the same strain. Doses of 0.10 LD_{50} (6.4 mg./kg.) and 0.20 LD_{50} (12.8 mg./kg.) were employed, and a control group of 12 rats received a similar volume of normal saline. All animals were observed daily for mortalities and general health, and weights of the animals were recorded daily. At the end of the 30-day period, representative animals from each group were sacrificed and autopsied for gross pathological lesions. The adrenals, brain, gonads, heart, liver, kidneys, lungs, and spleen were removed from each animal and weighed to permit calculation of the organ-to-body weight ratio. Sections of each organ were prepared for histopathologic examination.

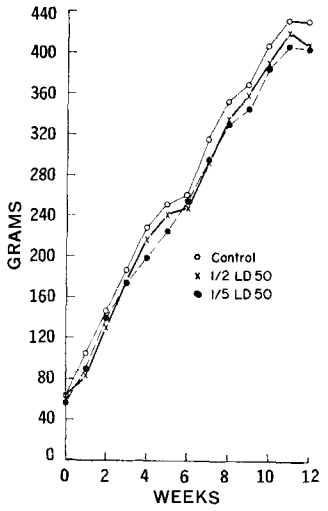

Figure 1—*Effect of ethylene chlorohydrin on growth rate of rats (injected three times a week).*

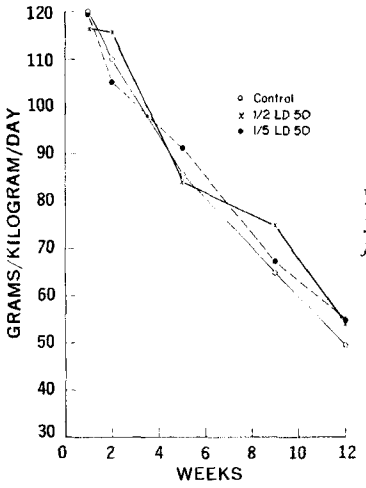

Figure 2—*Effect of ethylene chlorohydrin on food consumption in rats.*

Special Tests and Studies—*Sensitization Test*—Sensitization tests were performed on Hartley strain guinea pigs, using the "guinea pig maximization test" of Magnusson and Kligman (3) with 2,4-dinitrochlorobenzene as the positive control and cottonseed oil and petrolatum as the negative control. The test, as conducted here, involved an intradermal injection of 0.1 ml. of 10% ethylene chlorohydrin in one group of guinea pigs along with Freund's Complete Adjuvant[4], as described by Magnusson and Kligman (3), and 0.1 ml. of 5% ethylene chlorohydrin in a second group. This was followed in 7 days with a topical application of the same quantity of test compound. Two weeks later, the hair was shaved from another site and the test material was applied topically with an occluding bandage for 24 hr. The bandage then was removed, and the area was cleansed with alcohol. After an additional 24 hr., the test sites were evaluated for response to the challenging test agent. The results were scored as follows: 0 = no reaction; 1 = scattered, mild redness; 2 = moderate and diffuse redness; and 3 = intense redness and swelling. The challenging dose of the positive control (25% 2,4-dinitrochlorobenzene in petrolatum) produced a 3 reaction. Five animals per group were used in this test.

Cardiovascular, Respiratory, and Neuromuscular Effects—Rabbits, of approximately 2 kg., were anesthetized by intravenous injection of 30–40 mg./kg. of pentobarbital sodium approximately 15 min. after an intraperitoneal injection of 500 mg./kg. of urethan (ethyl carbamate). All recordings were made with a Grass model 7 polygraph in conjunction with appropriate preamplifiers[5]. Standard surgical procedures were employed in cannulation or isolation of various anatomical structures. Systemic blood pressure was determined by cannulating a branch of the left femoral artery and connecting it to a Statham pressure transducer. Electrical activity of the anterior tibialis and/or gastrocnemius muscle was monitored on the oscilloscope of a dual-beam Neurophysiograph[6], while the mechanical response was recorded using a Grass force-displacement transducer. Shielded electrodes were placed on the sciatic nerve, and the stimulus (voltage, frequency, duration, and delay) was supplied and controlled by a Grass S-4 stimulator through a matching stimulus isolation unit. Respiration was recorded using an impedance pneumograph, and the ECG was obtained from appropriately placed subdermal electrodes.

In these experiments, each rabbit was administered a specific dose of chlorohydrin solution intravenously. The doses administered were as follows: 38.3, 78.9, 157.7, 606.5, and 1577 mg./kg. Cardiovascular, respiratory, and neuromuscular effects were recorded before and after injections.

Sleeping Time—Male, ICR albino mice, weighing 25–30 g., received intraperitoneal injections of ethylene chlorohydrin at three dose levels (0.10, 0.20, and 0.50 LD_{50}) 24 and 48 hr. prior to sleeping time determinations. Pentobarbital sodium, 50 mg./kg., was injected intraperitoneally into each mouse; the mice were observed

[3] Bromsulphalein, brand of sulfobromophthalein sodium injection, by Hynson, Westcott & Dunning, Inc., Baltimore, Md.

[4] Difco Laboratories, Detroit, Mich.
[5] Grass Instruments Co., Quincy, Mass.
[6] E. & M. Instrument Co., Inc., Houston, Tex.

Table II—Subacute Toxicity of Ethylene Chlorohydrin in Rats[a], Hematologic Values[b] (Mean ± *SE*)

Item	Saline Control	0.20 LD_{50} (12.8 mg./kg.)	0.50 LD_{50} (32.0 mg./kg.)
Hemoglobin, g./100 ml.	17.73 ± 0.72	15.67 ± 0.18	16.76 ± 0.65
Hematocrit, %	54.83 ± 1.22	52.17 ± 1.42	55.25 ± 1.59
Erythrocytes, $\times 10^6$/mm.^{3}	9.965 ± 0.616	9.465 ± 0.259	9.625 ± 0.669
Total leucocytes, $\times 10^3$/mm.^{3}	15.81 ± 2.26	17.74 ± 3.18	13.33 ± 1.54
Platelets[c]	185.00 ± 30.72	233.00 ± 43.56	189.00 ± 25.95
Differential leucocyte count, as %			
Segs	9.25 ± 1.48	8.42 ± 0.98	12.50 ± 1.79
Lymphs	83.42 ± 1.35	87.75 ± 1.20	80.33 ± 1.67
Monocytes	1.83 ± 0.60	0.833 ± 0.146	1.33 ± 0.16
Eosinophils	1.417 ± 0.25	1.50 ± 0.36	1.42 ± 0.33
Meta	0.250 ± 0.145	0.083 ± 0.076	0.083 ± 0.076
Juvenile	1.00 ± 0.26	0.033 ± 0.172	0.58 ± 0.13
Bands	2.33 ± 0.73	0.917 ± 0.249	3.92 ± 0.69

[a] Drug injected intraperitoneally three times per week for 12 weeks. [b] Based on six rats per group. $SE = \sqrt{\frac{(X - \bar{X})^2}{n - 1}} \cdot \frac{1}{\sqrt{n}}$. [c] Number observed per 10 oil immersion fields.

closely for loss and then return of the righting reflex. Sleeping time was considered as that time period during which the righting reflex was absent. Ten mice were used for each dose level.

RESULTS

Subacute Toxicity Studies—*Phase One and Two Studies*—Table I summarizes the mortalities that occurred in the Phase One and Two studies. Four animals out of 12 died in Phase One at a 0.20 LD_{50} dose. As was already mentioned, these deaths occurred in the early portion of this study and some suspicion was cast upon the dose actually administered. When this experiment was repeated (Phase Two), no deaths occurred. As will be seen from Table I, when the dose was increased to 0.50 LD_{50}, six animals died during the 12-week period. No deaths were seen for animals administered the 0.10 LD_{50} dose nor for the saline-treated controls. The remaining animals in all groups remained in good health throughout the study.

Figure 1 graphically represents the weekly weight gain (mean value) throughout the 12-week study for those rats receiving 12.8 and 32 mg./kg. of chlorohydrin as well as for the saline controls. Animals receiving 6.4 mg./kg. of chlorohydrin (not included in Fig. 1) showed weight gains similar to the control animals. A careful examination of the weight gain–time curve reveals a very slight decrease in the mean weight gains for the experimental animals *versus* the controls. No significant difference (by *t*-test) was apparent in the average weight gain over the 12-week period for the experimental animals when compared to the controls.

There was no appreciable difference in food consumption between the saline-treated controls and those receiving ethylene chlorohydrin.

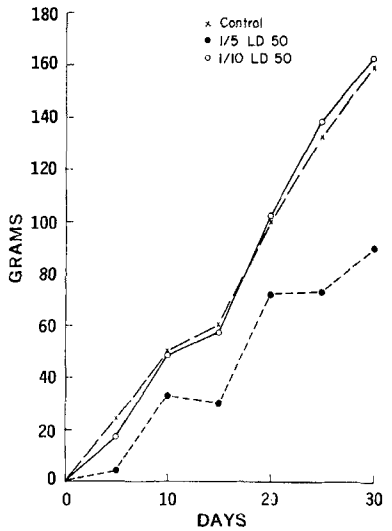

Figure 3—*Effect of daily administration of ethylene chlorohydrin on weight gain in rats.*

This may be seen in Fig. 2, where the amount of food consumed, as grams/kilogram/day, is plotted against weeks of the study. This figure shows data for the two higher dose groups (12.8 and 32 mg./kg.) and the controls. Data for the group receiving 6.4 mg./kg. were not included; however, this group was not significantly different from the saline-treated controls.

Results of hematologic tests conducted on chlorohydrin-treated (12.8 and 32 mg./kg.) rats and controls are shown in Table II. There appears to be no remarkable dose-related effects among the hematologic parameters tested. In the instances of differences between the test animals and the controls, the variations did not follow a dose–response pattern. Results of hematology tests for the animals treated with 6.4 mg./kg. of chlorohydrin did not differ significantly from the control animals.

Table III includes organ-to-body weight ratios from rats administered chlorohydrin over the 12-week period. The data include the average values for animals receiving 0.20 and 0.50 LD_{50} and for the control group (saline treated). Since the ratios for the animals treated with 0.10 LD_{50} were similar to the control group (saline treated), these values were not included in the table.

Histopathologic evaluation of the various organs studied in both the experimental animals (treated with the various dose levels of chlorohydrin) and the control animals did not reveal any significant pathologic features which could be attributed to the administration of the compound.

Phase Three (30-Day Cumulative Study)—During the 30-day period, no deaths occurred in the saline-treated controls or in the group receiving 0.10 of the LD_{50} (6.4 mg./kg.) dose of chlorohydrin (Table IV). At the higher daily dose (0.20 LD_{50}), seven of the 12 animals died (58.3%). Figure 3 presents the weight gain of both groups of experimental animals and the control animals. The curves reveal that the treated group on the highest dose (0.20 LD_{50}) exhibited a marked reduction in the rate of growth. These differences were statistically significant at 15 days ($p = < 0.20 > 0.10$) and at 30 days ($p = 0.01$).

Table III—Organ-to-Body Weight Ratio of Rats Administered Ethylene Chlorohydrin over 12-Week Period[a] (Expressed as Percent ± *SE*)

Organ	Control (Normal Saline)	Ethylene Chlorohydrin 0.20 LD_{50} (12.8 mg./kg.)	Ethylene Chlorohydrin 0.50 LD_{50} (32.0 mg./kg.)
Adrenals	0.019 ± 0.005	0.013 ± 0.001	0.010 ± 0.002
Brain	0.340 ± 0.018	0.350 ± 0.014	0.410 ± 0.015
Gonads	0.780 ± 0.023	0.800 ± 0.029	0.780 ± 0.021
Heart	0.290 ± 0.012	0.290 ± 0.009	0.310 ± 0.008
Kidneys	0.650 ± 0.008	0.660 ± 0.022	0.680 ± 0.037
Liver	3.550 ± 0.146	3.580 ± 0.215	3.700 ± 0.131
Spleen	0.180 ± 0.033	0.210 ± 0.018	0.200 ± 0.016

[a] Animals in each group were administered, intraperitoneally, control solution or test solutions three times a week up to and including the 12th week. Values are based upon results from six rats per group.

Table IV—Mortalities in 30-Day Cumulative Studies of Ethylene Chlorohydrin in Rats

Dose	Ratio (Deaths/Total)
0.10 LD_{50} (6.4 mg./kg.)	0/12
0.20 LD_{50} (12.8 mg./kg.)	7/12
Normal saline (control)	0/12

Histopathologic examinations of the adrenals, brain, gonads, heart, kidneys, liver, lungs, and spleen did not reveal any unusual pathologic lesions which could be attributed to the administration of the compound. Organ-to-body weights, expressed as percentages, are shown in Table V. Several of the organs (adrenals, brain, gonads, kidneys, and lungs) tended to show a relative increase in weight with an increase in the dose of chlorohydrin.

Special Studies—*Sensitization Test*—Chlorohydrin did not produce a sensitizing response in guinea pigs under the experimental conditions employed.

Cardiovascular, Respiratory, and Neuromuscular Effects—The acute effects of chlorohydrin upon cardiovascular function of the anesthetized rabbit may be divided into two categories, depending upon the quantity administered. With the lower doses (38.8, 78.9, and 157.7 mg./kg.), intravenous administration was followed by a fall in diastolic pressure, presumably from vasodilation since heart rate, systolic pressure, and the ECG pattern remained essentially unchanged. About 2–2.5 hr. following the administration of the agent, systolic pressure in some, but not all, of the animals declined. Higher doses (606.5 mg./kg. or more) produced a dose-related reduction in both systolic and diastolic pressures, culminating in death of the animal usually between 1 and 2 hr. after the agent was administered. With a dose of 1577 mg./kg., the largest dose used, marked dyspnea was produced and a precipitous fall in both systolic and diastolic pressure. Death usually occurred in about 5 min., which was preceded by respiratory failure.

It is difficult to pinpoint the exact cause of death in these experiments, but it is believed to be due to failure of the respiratory center of the medulla; however, one should not rule out a direct cardiotoxic or neuromuscular effect. The effects on blood pressure are shown diagrammatically in Fig. 4.

The respiratory system of the rabbit is susceptible to the action of chlorohydrin. Low doses, as used, had litle or no effect upon the rate or depth of respiration, while moderate doses tended to diminish it and larger doses to arrest it.

The response of skeletal muscle (anterior tibialis and gastrocnemius) of the rabbit to electrical stimulation of the sciatic nerve was impaired and eventually blocked by the administration of ethylene chlorohydrin. Electrical stimuli were supplied to the nerve at a frequency of one per second and a duration of 0.3 msec., with a voltage sufficient to produce nearly a maximum twitch response. After injection of the compound, the latency period was prolonged, followed by an increase in threshold and finally failure to respond to 5–10 times the initial voltage. Observation of the electrical activity of the muscle, displayed on an oscilloscope, showed the development of

Table V—Organ-to-Body Weight Ratio of Rats Administered Ethylene Chlorohydrin over 30-Day Period[a] (Expressed as Percent ± *SE*)

Organ	Control (Normal Saline)	Ethylene Chlorohydrin— 0.10 LD_{50} (6.4 mg./kg.)	0.20 LD_{50} (12.8 mg./kg.)
Adrenals	0.010 ± 0.001	0.012 ± 0.001	0.022 ± 0.004
Brain	0.387 ± 0.018	0.496 ± 0.013	0.715 ± 0.011
Gonads	0.906 ± 0.059	0.990 ± 0.038	1.130 ± 0.069
Heart	0.263 ± 0.009	0.291 ± 0.016	0.291 ± 0.008
Kidneys	0.634 ± 0.022	0.719 ± 0.061	0.782 ± 0.030
Liver	3.850 ± 0.132	4.218 ± 0.278	3.855 ± 0.211
Lungs	0.303 ± 0.013	0.368 ± 0.041	0.448 ± 0.067
Spleen	0.244 ± 0.014	0.263 ± 0.028	0.249 ± 0.012

[a] Animals in each group were administered, intraperitoneally, saline or test solutions each day up to and including the 30th day. Values are based upon six rats per group, except in the 0.20 LD_{50} group in which only five rats survived to the end of the study.

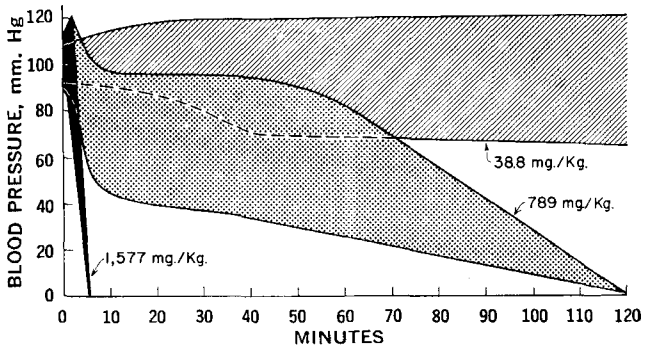

Figure 4—*Representative effects of intravenous ethylene chlorohydrin upon blood pressure of rabbits at three dosage levels.*

fasciculations within the muscle which persisted until it completely lost its contractile response to nerve stimulations. This syndrome was elicited in many of the animals, even at the low range of the doses used. The effect produced was not one of total skeletal muscle paralysis, as might be produced by succinylcholine or tubocurarine, since stimulation of a quiescent muscle group resulted in a prompt response.

Intravenous injection of the middle and high doses (*i.e.*, 606.5 mg./kg. and above) evoked a pain response (a cry and movement) in the rabbit even though anesthesia was satisfactory for surgical procedures.

Sleeping Time—As may be noted from Fig. 5, there was a dose-related increase in mean sleeping time of the treated animals. The mean sleeping time of the mice receiving 0.20 LD_{50} of chlorohydrin was significantly different from the saline control group, according to Student's t test, at the 90% level ($p = 0.10$), while that for the group receiving 0.50 LD_{50} was significant at the 99% level ($p = 0.01$).

DISCUSSION

Subacute Toxicity—Ambrose (4), in a 7-month feeding study in rats, noted that there were no apparent ill effects when the concentration of ethylene chlorohydrin in the diet was 0.08% or less. When the concentration was increased to 0.12 or 0.16%, the weight gain of the treated animals was less than the controls, but no animals died during the experimental period. However, when the quantity was increased to 0.24% or more, mortalities ranged from 40 to 100% of the test groups. Histopathology of organs from all surviving animals was essentially negative. From the data presented by Ambrose (4), it is impossible to determine accurately the quantity of ethylene chlorohydrin each rat consumed in these experiments; however, this may be approximated based upon daily food consumption (per rat per day) and concentration of the toxicant.

The highest concentration of ethylene chlorohydrin that produced no inhibition of growth was 0.08% in food, or approximately 34.6 mg./kg. of rat/day; the highest concentration at which there were no fatalities was about 73.4 mg./kg. of rat/day (0.16% in food). In previous studies (1) in this laboratory, the acute, oral LD_{50} of ethylene chlorohydrin was 71.3 mg./kg. for rats, while Johnson (5) reported the acute, oral LD_{50} to be 77 mg./kg. The "no effect" daily dose in the feeding studies of Ambrose (4) was found to be approximately one-half the acute LD_{50} of ethylene chlorohydrin. A reduced rate of weight gain, but no mortalities, was noted by Ambrose (4) in the group of rats consuming a daily quantity of ethylene chlorohydrin essentially equal to the acute LD_{50}.

More recently, Carson and Oser (6) studied the oral toxicity of ethylene chlorohydrin in the food of rats, dogs, and monkeys for 90 days. They indicated that a "no adverse effect" could be estimated to be 45 mg./kg./day, which, for the rat, represented about 60% of the acute LD_{50}.

The results of the study reported here showed a "no adverse effect" in a group of rats who received 10% of the acute LD_{50} dose (6.4 mg./kg. i.p.) daily for 30 days, which is, in essence, in agreement with that of Johnson (7) who found that a life-long oral intake of 12% of the acute, oral LD_{50} (about 9 mg./kg.) produced no adverse effects.

A review of the oral toxicity data of Ambrose (4), Carson and Oser (6), and Johnson (7), as well as some parenteral data reported

here, shows several common features. First, the initial sign of toxicity (other than death) manifests itself by a reduced rate of growth which is dose dependent. Second, relatively large doses given daily over extended periods of time tend to produce no histological evidence of toxicity. Even in groups of rats receiving quantities of the compound that were lethal to some of the group, the surviving animals did not show unusual histopathology of organs or of tissues.

Apparently, ethylene chlorohydrin is detoxified rapidly in animals, thus preventing a build-up to toxic levels over these periods of time. Johnson (7), based upon considerable biochemical studies, explained that the rather innocuous effects of daily amounts of ethylene chlorohydrin are due to the compound being absorbed through the portal system and being detoxified rapidly in the liver by enzymatic conversion to *S*-carboxymethylglutathione. More reduced glutathione is then synthesized to replace the depleted glutathione, thus tending to protect more sensitive (or critical) organs from the very toxic metabolite, chloroacetaldehyde. At these intracellular sites, the formed chloroacetaldehyde is conjugated with glutathione and removed to prevent a toxic biologic response. If the concentration of chloroacetaldehyde is increased, however, it will conjugate more or all of the glutathione, thereby destroying the body's ability to protect itself from the toxic action of chloroacetaldehyde.

In the subacute toxicity study reported here, ethylene chlorohydrin was administered intraperitoneally three times a week over a 3-month period; the results showed no lethality or adverse effects at one-tenth and one-fifth the acute LD_{50} dose in rats. Increasing the dose to one-half the acute LD_{50}, however, produced death in six out of the 12 animals in the test group. When the compound was administered daily for 30 days, no apparent adverse effects were noted for the group receiving the 0.10 LD_{50} dose. Very toxic effects (deaths and impaired weight gain), however, resulted when the daily dose was increased to one-fifth the LD_{50}. Comparisons of these results with those of other investigators who used the oral route indicate that the parenteral subacute toxicity of ethylene chlorohydrin is considerably greater than that seen from incorporation of the compound into the animal's food (4, 6). Since acute toxicity experiments have shown the compound to have essentially the same toxicity orally as it does intraperitoneally in rats, factors other than inherent toxicity, must be responsible for this apparent discrepancy. First, when a dose is injected, the animal receives its "daily dose" all at once; when it is contained in food, the animal receives the compound gradually over a period of several hours, thus allowing more time for metabolic transformation and glutathione replenishment before the cells become unprotected from glutathione depletion. Second, when the compound is absorbed from the gastrointestinal tract, it enters the portal circulation to permit the liver to remove much of it, thereby helping to protect cells of other organs. Third, the daily consumption of the compound by the animal is less certain and more difficult to control when the material is contained in the diet. Finally, due to the volatility of the compound, special feeding techniques may be needed to ensure that the compound is not lost from the diet.

When, in spite of the body's attempts to detoxify it, sufficient quantities of ethylene chlorohydrin reach the intracellular sites, the compound is converted to chloroacetaldehyde, as postulated by Johnson (7). If the quantity of chloracetaldehyde accumulation is sufficient to deplete the pool of glutathione (by conjugation) within the cell, toxic manifestations result.

Hayes (8) introduced the term "chronicity factor" and equated this factor to the ratio of a one-dose LD_{50} to a repeated-dose LD_{50} given over a specific time period. Even though Hayes suggested that the denominator be the LD_{50} dose for a 90-day period (daily doses), he indicated that other time periods can be used. If the data from this study are used, and it can be assumed that in the 30-day study the LD_{50} is 12.8 mg./kg. (actually, as will be seen from Table I, this dose killed 58% of the animals), an approximate chronicity factor of 5 (64.0/12.8) would result. Hayes stated that a chronicity factor of 2 or greater indicates a cumulative effect of the compound and, as this number increases, there is an increase in the cumulative effect. With a slight modification, the chronicity factor for the 3-month study (with 36 doses administered three times a week) can be estimated to be approximately 2 (64.0 mg./32.0 mg.), thus indicating much less of a cumulative toxic effect than produced by the 30 daily doses of ethylene chlorohydrin. This would indicate that its toxic biologic half-life (*i.e.*, cumulative toxic effect) is rather short, a matter of hours rather than days or weeks.

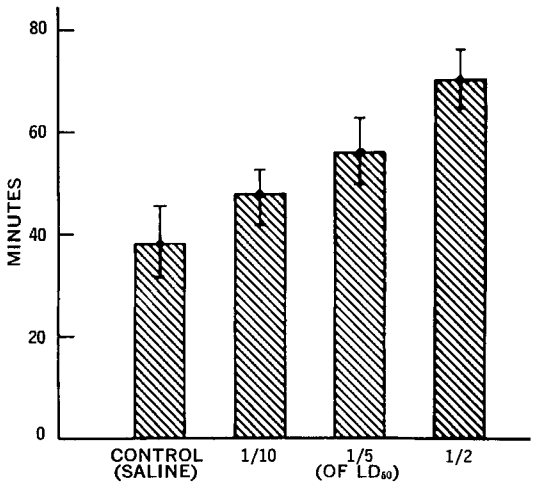

Figure 5—*Effect of ethylene chlorohydrin pretreatment upon pentobarbital sleeping time in mice (mean ± SE).*

Special Tests and Studies—Ethylene chlorohydrin was found not to be a sensitizing agent, according to the test method of Magnusson and Kligman (3). This method of testing was found to be a more sensitive test (3) than the method of Draize (9), which admittedly may not detect weak sensitizing agents.

Results from acute cardiovascular, respiratory, and neuromuscular studies did show that relatively high doses (606.5 mg./kg.) produced a dose-related reduction in both systolic and diastolic pressure, leading to death. Lesser doses produced a slight decline in diastolic pressure and, later, systolic pressure. Low doses (<40 mg./kg.) appeared to have little effect upon the rate or depth of respiration; but as the dose was increased, rate and depth were diminished and, eventually, at higher doses, completely arrested. Death of animals was generally attributed to failure of the respiratory center of the medulla, even though there may be a direct cardiotoxic or neuromuscular action. Ethylene chlorohydrin showed an effect upon the *in vivo* nerve–muscle complex, impairing and eventually blocking nerve–muscle function. Goldblatt (10) demonstrated that ethylene chlorohydrin in cats led to a fall in blood pressure and inhibition of respiration. He found that a 1% solution of the compound applied to an isolated nerve of a frog or cat could completely block the response to electrical stimulation of the nerve. The block, however, was reversible.

The sleeping time experiment in mice demonstrated that ethylene chlorohydrin can increase the mean sleeping time, suggesting that the parent compound or a metabolic product inhibits or antagonizes one or more enzymes or enzyme systems concerned with the metabolic detoxification of pentobarbital or, alternatively, the compound or metabolite produces more generalized liver damage which, in turn, would impede metabolic detoxification of pentobarbital. If, however, generalized hepatic damage is produced, it has not been apparent from histopathologic examination of hematoxylin-eosin-stained sections of liver from ethylene chlorohydrin-treated rats.

Ethylene Chlorohydrin and Devices—It has been demonstrated that repeated parenteral administration of ethylene chlorohydrin to rats over periods of time is more toxic than similar quantities given by the oral route (feeding experiments). Since it is possible that plastic and rubber items that have been ethylene oxide sterilized could contain ethylene chlorohydrin, the question may be asked as to what might constitute a "safe concentration" of ethylene chlorohydrin per day parenterally in man. If a dose of 6.4 mg./kg./day is taken as that dose which has been found to produce a "no adverse effect" in rats (for 30 days), then no medical device should be capable of releasing to a 70-kg. man an amount equal to 448 mg. of the agent.

Since it is dangerous to extrapolate rodent data to man, every attempt should be made to reduce the ethylene chlorohydrin concentration in plastic and rubber items to a minimum and, if possible, to a zero level. One must also consider that a plastic item may contain ethylene oxide and ethylene glycol as well as other reaction products, including ethylene chlorohydrin; thus, the subacute and chronic toxicity profile may be quite different from such combinations than when ethylene chlorohydrin is considered alone. It should

also be apparent that plastic or rubber devices most likely should not be repeatedly sterilized with ethylene oxide if there is any possibility that chlorides are present. Many hospitals apparently reuse disposable devices a number of times and feel that general washing of the item and sterilization with ethylene oxide ensure the safety of the item. This practice should be discouraged.

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Relative Substituent Effects on Alkaline Solvolysis of β-Lactams (2-Azetidinones) and Amides

RICHARD J. WASHKUHN* and JOSEPH R. ROBINSON†

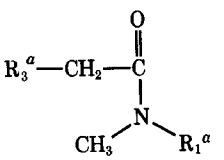
Abstract □ The effect of aliphatic and aromatic substituents, in the 1- and 3-positions of β-lactams, on the rate of solvolysis were examined and compared to analogous substituent effects in the model linear amides. It was found that substituent effects in the β-lactams could be quantitated using the Taft equation, and that a greater sensitivity to polar effects exists in the β-lactams as compared to the model linear amides. In addition, for single substituents in the 1- and 3-positions of the lactams, there is less steric influence on the rate of solvolysis as compared to linear amides. The mechanism of alkaline hydrolysis of these β-lactams appears to be the rate-limiting attack of hydroxide ion at the carbonyl carbon of the amide group and not the breakdown of an ionized tetrahedral intermediate as has been proposed for γ-lactams. Hydrolytic studies on β-lactams at 80° yield a different pathway of degradation, because deamination of the amino acid appears to be rate limiting; thus, elevated temperature studies on β-lactams should be carried out with caution. Finally, it is concluded that β-lactams are not unusually stable in their reactivity toward nucleophiles, such as hydroxide and methoxide ions, and their lability closely follows the parent linear amides.

Keyphrases □ 2-Azetidinones and linear amides—solvolysis mechanism, substituent effects □ β-Lactams, unfused, and linear amides—solvolysis mechanism, substituent effects □ Solvolysis (alkaline) mechanism, substituent effects—unfused β-lactams and linear amides

An enormous effort has been expended investigating various physicochemical and biological aspects of penicillins and cephalosporins (1–4), but comparatively little work has been done on the physicochemical properties of β-lactams. This is somewhat surprising, considering that present thinking (5–8) concerning the mode of action of the penicillins and cephalosporins centers around the acylating potential of the β-lactam moiety in these compounds. Recent work (9) demonstrated that fusion of the β-lactam ring to other rings can introduce considerable strain into the β-lactam ring which, when coupled with polar inductive effects arising from the C-3 side chain and the prevention of amide ground-state resonance, helps explain the unusual lability of these antibiotics to nucleophilic attack. However, the question still remains whether β-lactams are unusual in their stability toward nucleophiles, such as hydroxide and methoxide ions, as compared to corresponding linear amides. In addition, a mechanism of solvolysis of these agents has not been reported.

A summary of the effect of substituents on reactivity for a variety of β-lactams was reported (10), and these effects are all qualitative in nature. The only quantitative results of substituent effects on reactivity appears to be the work by Holley and Holley (11–13). These investigators examined the solvolysis of some substituted β-lactams, amides, and penicillins in 85% ethanolic solutions. They discussed, in qualitative terms, the effect of structural variation on reactivity. Recent work concerning the effect of ring size in fused ring β-lactams on the rate of hydrolysis was published by Earle *et al.* (9) and Moll (14).

The purposes of the present study were: (*a*) to investigate substituent effects on the rates of alkaline hydrolysis and solvolysis of unfused β-lactams and (*b*) to compare these effects to analogous substituent effects exhibited in normal amide solvolysis to determine if β-lactams are



amide structure

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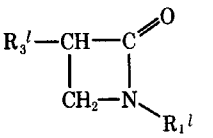

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II

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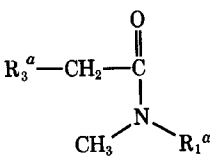

amide structure

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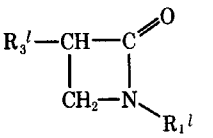


β-lactam structure

II

Table I—β-Amino Acids Prepared by the Blicke and Gould Method (19)

R[a]	Yield, %	Melting Point[b]
C_6H_5—	70	125–126°
$C_6H_5CH_2CH_2$—	95	194°
CH_2=CH—CH_2—	80	165°
$(CH_3)_3C$—	90	150–152°
$C_6H_5CH_2$—	97	195–196°
$(CH_3)_2CH$—	70	183°
Cyclo-C_6H_{11}—	90	193–194°
$CH_3CH_2CH_2$—	70	172°
CH_3CH_2—	50	191–192°
CH_3—	95	200–201°
H—	25	224–225°

[a] $RNHCH_2CH(C_6H_5)CO_2H$. [b] All temperatures are corrected.

substantially different from their corresponding linear amides. Structures I and II show the structural similarities between the amides and the β-lactams. In addition, since the mechanism for solvolysis of β-lactams has apparently not been reported, the possible pathways for solvolysis of these agents are explored here.

EXPERIMENTAL

Reagents—All chemicals and solvents were of analytical grade. Nevertheless, some had to be further purified prior to use since trace impurities and small moisture content can seriously affect the product yield in the various syntheses performed in this study. Methanol and ethanol were purified by distillation from metallic sodium and the corresponding formate ester, as described by Vogel (15). To ensure complete dryness in all solvents, an all-glass distillation apparatus was used and distillations were carried out immediately prior to use. Diethyl ether and benzene were purified by distillation from sodium wire as described by Vogel (15). Ethyl bromide and methyl iodide, used in the prepartion of Grignard reagents, were purified by distillation.

Analytical grade dimethylaniline was purified by distillation, using the acetic anhydride method (16) to remove any secondary amines present, and then dried with anhydrous potassium carbonate, filtered, and redistilled from sodium hydroxide. Pyridine was purified in an analogous manner as described by Vogel (16). Thionyl chloride was purified by procedures set forth by Friedman and Wetter (17); acetyl chloride was purified by fractional distillation, and propionyl and phenylacetylchloride were purified by methods described by Fieser and Fieser (18). Magnesium turnings were washed thoroughly with anhydrous diethyl ether, dried, and stored in a desiccator prior to use. Water was doubly distilled from alkaline permanganate in an all-glass distillation apparatus.

Synthesis—The various β-lactams were prepared by one of two methods: Blicke and Gould's method (19), involving an amine hydrochloride acid chloride intermediate, or Holley and Holley's method (11–13), involving a Grignard reagent with a β-amino ester. Tables I and II present the various β-amino acids and esters synthesized in this study, together with yields and melting or boiling points. The β-lactams prepared by both methods are presented in Table III, together with yields and melting or boiling points. Structures of β-amino acids, β-amino esters, and β-lactams were confirmed by IR and PMR spectra as well as elemental analysis.

Table II—β-Amino Esters Prepared by the Holley and Holley Method (11–13)

R_1[a]	R_2[a]	R_3[a]	Yield, %	Boiling Point[b] (°/mm. Hg)
H—	CH_3CH_2—	CH_3—	50	63–64°/13
H—	CH_3CH_2—	CH_3CH_2—	70	70–72°/14
H—	CH_3CH_2—	$(CH_3)_2CH$—	52	125°/0.3
H—	CH_3CH_2—	$C_6H_5CH_2$—	55	131°/2.7
H—	CH_3CH_2—	Cyclo-C_6H_{11}—	54	119°/0.5
CH_3—	CH_3—	CH_3—	65	68–70°/18
CH_3—	CH_3—	CH_3CH_2—	81	67–68°/13
CH_3—	CH_3—	$(CH_3)_2CH$—	50	63–65°/2
CH_3—	CH_3—	$C_6H_5CH_2$—	41	104–106°/0.4
CH_3—	CH_3—	Cyclo-C_6H_{11}—	65	83–85°/0.4

[a] R_3—$NHCH_2C(=R_1)H$—CO_2R_2. [b] All temperatures are corrected, and pressures were determined using a Todd Universal vacuum gauge.

Table III—β-Lactams Prepared in This Study[a]

R_3[l]	R_1[l]	Yield, %	Melting Point[b] (°) or Boiling Point[b] (°/mm. Hg)
H—	CH_3—	12	68–69°/18
H—	CH_3CH_2—	12	67–69°/14
H—	$C_6H_5CH_2$—	15	110°/2
CH_3—	CH_3—	20	65–66°/12
CH_3—	CH_3CH_2—	22	76–78°/14
CH_3—	$C_6H_5CH_2$—	50	100–102°/0.7
CH_3—	$(CH_3)_2CH$—	20	38–40°/1
CH_3—	Cyclo-C_6H_{11}—	40	72–75°/0.3
C_6H_5—	CH_3—	30	106–107°/0.4
C_6H_5—	CH_3CH_2—	75	140–142°/2
C_6H_5—	$CH_3CH_2CH_2$—	50	102–105°/0.1
C_6H_5—	$C_6H_5CH_2$—	70	72°
C_6H_5—	$(CH_3)_2CH$—	80	130–133°/0.2
C_6H_5—	Cyclo-C_6H_{11}—	50	60–61°

[a] The first eight compounds were prepared by the Holley and Holley method (11–13) and the remainder by the Blicke and Gould method (19). [b] All temperatures are corrected, and pressures were determined using the Todd Universal vacuum gauge.

Amides of acetic, propionic, and phenylacetic acids were prepared either by direct addition of an ethereal solution of an amine to the acid chloride solution or by the conventional method employing pyridine as a catalyst. The resulting amides were then purified by filtration, followed by repeated extraction with 5% hydrochloric acid and 5% sodium hydroxide solutions. The ethereal solution of amide was washed with water and dried with $MgSO_4$; finally the solvent was evaporated at reduced pressures. The residue was then either vacuum distilled or recrystallized to yield the pure amide. Melting and boiling points of these amides coincided with those available in the literature (Table IV). IR and PMR spectra, as well as elemental analysis, were used to confirm structures of these compounds.

Apparatus—IR spectra for all synthesized compounds were obtained using a Beckman IR-5A or IR-9. PMR spectra were taken with a Varian A-60 spectrometer. Measurements and adjustments of pH were made with a Corning model 12 pH meter equipped with an expanded scale, using a Beckman type E3 wide-range glass electrode. The pH meter and electrode system was standardized at 25° against a phosphate buffer as described by Bates (20). Water and oil bath temperatures were maintained to 0.1° with Sargent Thermonitor electronic relays. Reaction progress was monitored on a Cary 11, 14, or 15 recording spectrophotometer with thermostated cell compartments.

Procedures—The following procedure was used for reactions of convenient rapidity to warrant direct measurement of absorbance change as a function of time. A 2- or 5-cm. spectrophotometer cell, containing an alkaline solution at the desired pH, was temperature equilibrated in the cell compartment. After equilibration, 1 or

Table IV—Melting and Boiling Points of Some Amides

R_3[a]	R_1[a]	Melting Point[a] (°) or Boiling Point[a] (°/mm. Hg)	
H—	H—	203°/760	(27–28°)
H—	CH_3—	165°/760	
H—	$C_6H_5CH_2$—	90–91°/1.0	(25°)
H—	Cyclo-C_6H_{11}—	86–87°/1.0	
CH_3—	H—	205–207°/760	
CH_3—	CH_3—	175°/760	
CH_3—	$C_6H_5CH_2$—	111–112°/1.0	
CH_3—	Cyclo-C_6H_{11}—	90–91°/1.0	
C_6H_5—	H—	58°	
C_6H_5—	CH_3—	40–41°	
C_6H_5—	$C_6H_5CH_2$—	175–176°/1.0	(63–64°)
C_6H_5—	Cyclo-C_6H_{11}—	161–163°/1.0	

[a] All temperatures are corrected, and pressures were determined using the Todd Universal vacuum gauge.

Table V—Second-Order Rate Constants for Alkaline Hydrolysis of Some Disubstituted β-Lactams in Water at 25°

Compound	$k_{OH} \times 10^4 (M^{-1}$ min.$^{-1})^a$	*SD*	Number of Determinations
1,3-Dimethyl-2-azetidinone	10.3	0.4	3
1-Methyl-3-hydrogen-2-azetidinone	22.6	0.15	6
1-Methyl-3-phenyl-2-azetidinone	27.2	0.17	4
1-Ethyl-3-methyl-2-azetidinone	3.70	0.25	2
1-Ethyl-3-hydrogen-2-azetidinone	8.32	0.20	3
1-Ethyl-3-phenyl-2-azetidinone	9.51	0.11	3
1-Benzyl-3-methyl-2-azetidinone	12.9	0.8	3
1-Benzyl-3-hydrogen-2-azetidinone	32.0	0.2	6
1-Benzyl-3-phenyl-2-azetidinone	35.0	0.5	5

[a] Determined on the basis of hydroxide-ion activity.

2 μl. of pure liquid was injected into the cell and mixed thoroughly. With low melting solids, the compound was first melted and then the liquid was injected. With high melting solids, a given amount of solid was accurately weighed corresponding to a 10^{-3} *M* concentration and placed into 200 ml. of alkaline solution at the desired temperature, mixed rapidly, and equilibrated; absorbance changes were monitored as a function of time.

Wavelengths chosen to monitor absorbance changes were obtained by comparison of UV spectra of the β-lactam with that of the corresponding amino acid or, in the case of amides and the methoxide-catalyzed methanolysis studies, by comparison of the initial UV spectrum with that obtained 48 hr. later. All the wavelengths used varied between 225 and 230 nm., depending upon substituents, temperature, and nucleophiles involved in the reaction.

For reactions with exceedingly slow rates at 25, 35, and 45°, the procedure described earlier was followed except that absorbances were determined in an intermittent manner. For reactions at 80°, the following procedure was employed. A 2.0×10^{-3} *M* solution of the β-amino acid or β-lactam was prepared in an alkaline 25% v/v methanol–water solution, mixed well, and placed into 5-ml. ampuls. The ampuls were sealed and placed into an oil bath at 80°. At appropriate time intervals, samples were removed from the oil bath and quenched in an ice–brine mixture, and absorbances were measured.

Table VI—Second-Order Rate Constants for Alkaline Hydrolysis of Amides in Water at 25°

Compound	$k_{OH} \times 10^4 (M^{-1}$ min.$^{-1})^a$	*SD*	Number of Determinations
N,*N*-Dimethylacetamide	11.0	0.5	3
N,*N*-Dimethylpropionamide	11.3	0.4	3
N,*N*-Dimethylphenylacetamide	8.0	0.4	3
N-Methylacetamide	4.08	0.25	2
N-Methylpropionamide	3.50	0.31	2
N-Methylphenylacetamide	4.57	0.43	2
N-Methyl-*N*-benzylacetamide	2.35	0.16	2
N-Methyl-*N*-benzylpropionamide	1.18	0.20	2
N-Methyl-*N*-benzylphenylacetamide	3.0	0.4	2

[a] Determined on the basis of hydroxide activity.

Table VII—Pseudo-First-Order Rate Constants for Alkaline Hydrolysis of 1-Benzyl-2-azetidinone as a Function of pH at 25°

Molar Concentration of NaOH	$pH_{app.}$[a]	$pH_{corr.}$[b]	$k_{obs.} \times 10^3$ min.$^{-1}$
3 *M*	13.94	14.54	11.3
2 *M*	13.82	14.20	5.3
1.5 *M*	13.71	14.00	3.3
1.0 *M*	13.59	13.80	2.0
0.5 *M*	13.36	13.46	0.99
0.1 *M*	13.00	13.00	0.32
	12.55	12.55	0.13
	12.00	12.00	0.031

[a] Measured pH. [b] pH corrected for sodium-ion concentration.

Methoxide-catalyzed methanolysis of the β-lactams was carried out in a methanolic solution of sodium methoxide prepared by reacting freshly cut sodium with anhydrous methanol, followed by filtration through a fine-sintered-glass funnel, and diluting to the desired concentration of sodium methoxide with anhydrous methanol. The final concentration of sodium methoxide in methanol was determined by accurately pipeting a small volume of the basic solution into 50 ml. of water and titrating this solution with standardized 0.1 *N* hydrochloric acid aqueous solution to a mixed indicator end-point. The mixed indicator was composed of a neutralized 0.1% bromcresol green solution and a 0.1% solution of sodium alizarin sulfonate.

Pseudo-first-order conditions were maintained in all kinetic experiments, since a large excess of hydroxide or methoxide ion was present at all times. Pseudo-first-order rate constants were converted to second-order rate constants using hydroxide or methoxide concentration or activity. For methoxide-catalyzed methanolysis, the rate constants were calculated on the basis of concentration rather than activity; the aqueous solution studies were determined on the basis of either concentration or activity, depending upon temperature. At 25°, activity measurements were used; at elevated temperatures, concentration was employed. Many pH measurements required inclusion of a sodium-ion correction factor.

Identification of Reaction Products—*Alkaline Hydrolysis of β-Lactams at 25°*—Identification of the reaction products following alkaline hydrolysis of the β-lactams was accomplished in three ways: (*a*) comparison of molar absorptivities to the amino acids, (*b*) IR spectral data, and (*c*) TLC. The molar absorptivity of the reaction products was calculated and coincided with the theoretical value for the amino acid. Comparison of the IR spectrum of the reaction product with that of the amino acid indicated they were

Table VIII—Effect of Temperature on Second-Order Rate Constants for Alkaline Hydrolysis of β-Lactams and Linear Amides in 1.0 and 2.0 *M* NaOH

	$k_{OH} \times 10^4\ M^{-1}$ min.$^{-1}$[a]				
	45°		35°	25°	
Compound	2 *M* NaOH	1 *M* NaOH	2 *M* NaOH	2 *M* NaOH	1 *M* NaOH
1-Benzyl-2-azetidinone	105.0	102.0	57.1	25.2	20.2
1-Methyl-2-azetidinone	109.0	67.3	39.9	18.0	21.3
1-Methyl-3-methyl-2-azetidinone	32.3	44.2	18.0	8.39	6.13
1-Methyl-3-phenyl-2-acetidinone	89.7	73.5	42.5	19.6	18.0
N,*N*-Dimethylacetamide	39.8	37.4	20.4	9.8	6.1
N,*N*-Dimethylpropionamide	36.8	34.0	21.8	10.1	6.2
N,*N*-Dimethylphenylacetamide	28.5	21.2	15.2	7.61	3.67

[a] The second-order rate constants are based on concentration of hydroxide ion as determined by titration at 25°.

Table IX—Activation Parameters for Alkaline Hydrolysis of β-Lactams and Linear Amides

Compound	$\Delta H^{\neq}_{25°}$, kcal./mole	$\Delta G^{\neq}_{25°}$, kcal./mole	$\Delta S^{\neq}_{25°}$, e.u.
1-Benzyl-2-azetidinone	13.9	23.4	−32.2
1-Methyl-2-azetidinone	13.4	23.6	−34.3
1-Methyl-3-methyl-2-azetidinone	15.2	24.2	−30.1
1-Methyl-3-phenyl-2-azetidinone	13.3	23.6	−34.7
N,N-Dimethylacetamide	14.6	24.1	−31.9
N,N-Dimethylpropionamide	13.7	24.1	−34.9
N,N-Dimethylphenylacetamide	13.9	24.3	−35.2

identical compounds. TLC provided further verification that the amino acid was the sole product.

The chromatographic procedure was as follows. A 5×10^{-2} *M* solution of the β-lactam 1-benzyl-2-azetidinone, a 5×10^{-2} *M* solution of its corresponding amino acid, and a 1:1 mixture of the two solutions were prepared. Preliminary investigations with these solutions suggested that a 1:1 mixture of methanol and chloroform could efficiently separate the amino acid from the β-lactam on a 5 × 20-cm. silica gel TLC plate. The average R_f values associated with each are 0.18 and 0.73, respectively, based on six determinations. A 5×10^{-2} *M* solution of the β-lactam was then prepared in 2 *M* NaOH solution, reacted for 2 days, neutralized with concentrated HCl to pH = 7.0, and then placed on the TLC plate together with the three known solutions. Following elution, the R_f values were calculated, and the R_f value for the reaction product was identical to that of the amino acid in the two known solutions. Only one spot appeared from the reaction products.

Alkaline Hydrolysis of β-Lactams at 80°—At 80°, different reaction products are involved in the alkaline hydrolysis of β-lactams, since the final absorbance after complete reaction does not coincide with the theoretical based on the molar absorptivities of the corresponding amino acid. The final absorbances were significantly higher than predicted, suggesting that a conjugated system is present in the reaction product. Isolation of the reaction products was not carried out; however, some inference concerning their identity can be made. In the case of the β-lactam 1-propyl-3-phenyl-2-azetidinone, the reaction was allowed to proceed to completion at 80° and then the system was acidified with concentrated hydrochloric acid. The acidified reaction solution was extracted with chloroform and a UV spectrum was taken. Comparison of this spectrum with that of atropic acid in chloroform showed that the molar absorptivities and maximum wavelengths were the same, suggesting that the major reaction product is atropic acid. Further evidence supporting this was derived from investigation of the alkaline-catalyzed deamination of the corresponding amino acid under identical conditions. The rates of reaction for the amino acid were approximately the same as the lactam, under identical conditions, suggesting that this is indeed what is occurring. The fact that β-amino acids undergo a deamination process is well documented in the literature (21, 22).

RESULTS

Second-order rate constants for the alkaline hydrolysis of a variety of 1,3-disubstituted 2-azetidinones are presented in Table V, together with standard deviations and number of determinations associated with each 2-azetidinone. Alkaline hydrolytic second-order rate constants for some linear amides are presented in Table VI. Included in the table are standard deviations and number of determinations associated with each amide. For both the β-lactams and the amides, a pH range of 12.0–14.54 was used in the hydrolytic studies. Below pH 12.0, the reaction is inconveniently slow; above pH 14.54, the sodium-ion correction factor becomes exceedingly large. In addition, the uncorrected pH measurement is no longer on the scale of the pH meter.

Pseudo-first-order rate constants for the alkaline hydrolysis of 1-benzyl-2-azetidinone were observed as a function of hydroxide-ion activity over a moderate range of pH values (Table VII). Each pseudo-first-order rate constant as a function of pH was determined from at least two determinations at the specific pH. The standard deviations of these rate constants were all less than 5% of the average and generally in the range of 2–3% of the average. The lower limit of pseudo-first-order conditions is likely to be approached at pH's less than 13.0, so a buffer was added to ensure constant pH. A variety of buffer concentrations was used to establish that no buffer catalysis occurred in the system.

Table X—Methoxide-Catalyzed Methanolysis of β-Lactams and Linear Amides at 45°

Compound	$k_{OCH_3} \times 10^3$ M^{-1} min.$^{-1}$[a]
1-Benzyl-2-azetidinone	11.1
1-Methyl-2-azetidinone	12.6
1-Methyl-3-methyl-2-azetidinone	3.0
1-Methyl-3-phenyl-2-azetidinone	5.6
N,N-Dimethylacetamide	0.25
N,N-Dimethylpropionamide	0.10

[a] Second-order rate constants are based on concentration of methoxide ion as determined by titration at 25°.

Salt effects on the rate of alkaline hydrolysis were also investigated; small changes in the rate constant occurred over substantial salt concentrations. Since this study was carried out in 1.0 *M* sodium hydroxide solution, it was necessary to use salt concentrations in the range of 2–4 *M*.

The effect of temperature on the second-order rate constants for the alkaline hydrolysis of various 1,3-disubstituted 2-azetidinones and some of their linear amide analogs are shown in Table VIII. These second-order rate constants are based on concentration rather than activity, since accurate estimates of activity could not be readily obtained at temperatures other than 25°. The second-order rate constants were converted to units of M^{-1} sec.$^{-1}$, and the activation parameters were then evaluated for each compound at each concentration of hydroxide ion. The energies of activation were determined from Arrhenius plots and converted to enthalpies of activation at 25°; the entropies of activation were calculated using the equations involved in transition state theory. The values of $\Delta H^{\neq}$, $\Delta S^{\neq}$, and $\Delta G^{\neq}$ at each sodium hydroxide concentration were then averaged (Table IX). The values of $\Delta G^{\neq}$ in the two concentrations of sodium hydroxide were quite close to the average for each compound, suggesting that these averages are reasonably accurate. The values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$, on the other hand, fluctuated to a much greater extent from their respective averages. These fluctuations were random in nature and a result of the large standard deviations associated with the rate constants at the specific temperature and concentration of sodium hydroxide.

Second-order rate constants at 45° for the methoxide-catalyzed methanolysis of a variety of 1,3-disubstituted 2-azetidinones and some corresponding linear amides are shown in Table X. The second-order rate constants were determined on the basis of concentration of methoxide ion.

Some β-lactams were subjected to hydrolysis at elevated temperatures (80°). All the β-lactams tested appeared to undergo a relatively

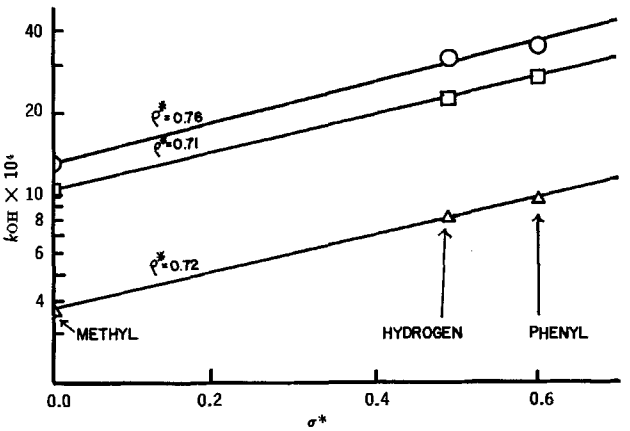


Figure 1—*Plot of second-order rate constants for alkaline hydrolysis at 25° of some 2-azetidinones, with substituent variations in the 3-position,* versus *Taft's substituent constant,* σ*. Key:* ○, *1-benzyl series;* □, *1-methyl series; and* △, *1-ethyl series.*

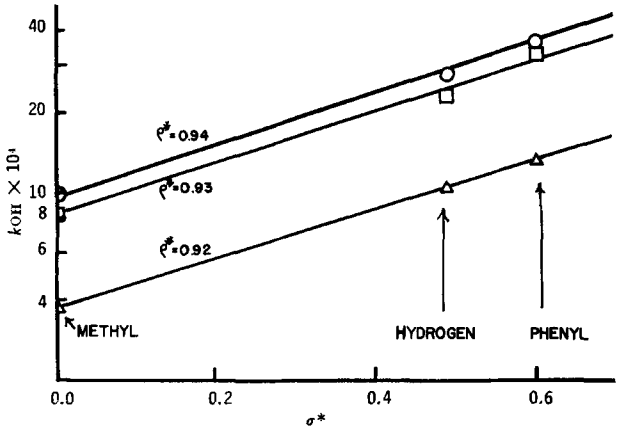


Figure 2—*Plot of second-order rate constants for alkaline hydrolysis at 25° of some 2-azetidinones, with substituent variations in the 1-position,* versus σ^*. *Key:* O, *3-phenyl series;* □, *3-hydrogen series; and* △, *3-methyl series.*

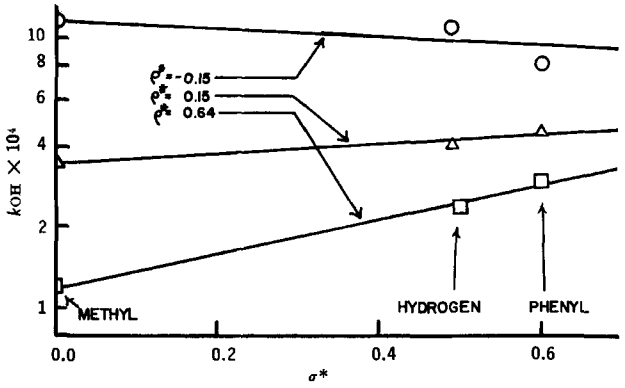


Figure 3—*Effect of substituent variations in the acyl position on the second-order rate constants for alkaline hydrolysis of some amides* versus σ^*. *Key:* O, N,N-*dimethylamides;* □, N-*methyl,* N-*benzylamides; and* △, N-*methylamides.*

rapid cleavage of the β-lactam ring followed by a slower deamination step. Earlier workers (11–13) used elevated temperatures in their studies of β-lactam solvolysis. Since their method of following the reaction could not detect this elimination, their results could be specious. This points out once again that elevated temperature studies to measure stability of a compound must be made with caution.

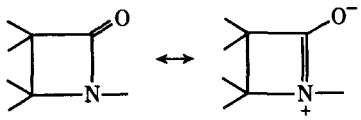
DISCUSSION

β-Lactams—*Substituent Effects in 3-Position*—A number of qualitative and semiquantitative comparisons of the rates of hydrolysis of a series of β-lactams with structures were made during the penicillin program and were summarized by Ballard and Melstrom (10). The general opinion was that substituent effects in the 3-position of 2-azetidinones governed the rates of hydrolysis primarily through a steric influence (21); this conclusion resulted from observations for introduction of two groups into the 3-position. For introduction of a single substituent, this is not the case since substituent effects in the 3-position may be correlated using Taft's polar substituent constant (22), as demonstrated in Fig. 1 for the data obtained in this study[1]. The slopes of these plots are not influenced by the substituent in the 1-position since each series of β-lactams, with variations in the 3-position, has very nearly parallel sensitivities to polar effects in the alkaline hydrolysis as judged by the similar ρ^* values. Insertion of an additional substituent in the 3-position introduces a considerable steric factor; a 25-fold decrease in rate is observed for the 3,3-dimethyl lactam relative to its monomethyl analog (23).

The effects of temperature variation on the ρ^* values obtained from the Taft plot for substituent variations in the 3-position of 1-methyl-2-azetidinones were negligible, suggesting that the isokinetic temperature for this reaction series might be significantly different from the temperature utilized in this study[2].

Substituent Effects in 1-Position—Holley and Holley (11, 12) observed a retardation of an order of magnitude in reactivity when comparing the 1-alkyl-substituted 2-azetidinones to the unsubstituted compound, and they suggested that this was primarily due to a steric effect. In the present studies, it was found that within a series of alkyl substituents in the 1-position, the substituent effects are dependent upon both polar and steric parameters since neither Taft's polar substituent constant, σ^*, nor the steric parameter, E_s, alone could effectively correlate substituent effects on the rates of hydrolysis. It was necessary to define the substituent as $-CH_2R$ to linearize the Taft plot (Fig. 2). Interpretation is not quite clear when a methylene group is inserted between the substituent and the nitrogen, because both steric and polar effects of the substituent are altered by insertion of the methylene group. Here again, the slopes of the Taft plot appear to be independent of the substituent in the 3-position, as suggested by the similar values exhibited for each series.

Comparison of the ρ^* values for the substituent effects in each position reveals that the 1-position is influenced by polar effects to a greater degree than the 3-position. The ρ^* values for variation in the 1-position could conceivably be even greater than the observed 0.93 for all series since insertion of a methylene group diminishes transmission of the polar effects of the substituent due to the greater distance involved between substituent and the reactive site. The enhanced sensitivity observed for the 1-alkyl substituents may be due to enhanced transmission of polar effects by the free pair of electrons present on the nitrogen since these are more fluid than the sigma electrons in the carbon backbone. Another possible explanation may involve amide resonance in the β-lactam molecule (Scheme I), which would be affected to a greater



Scheme I

degree by polar effects in a 1-position than in the 3-position.

Amides—*Substituent Effects in Acyl Portion*—Substituent effects on the alkaline hydrolysis of acyl substituted amides may also be correlated by the Taft relationship (Fig. 3). The substituent constants were defined on the basis of the substituent on the α-carbon rather than directly attached to the carbonyl group so that direct comparison of inductive effects may be made. In this instance, the slopes of the Taft plots for each series are independent of the substituent on the nitrogen and appear to be associated with the inductive effect of the substituent on the nitrogen rather than a steric effect. The negative slope for the alkaline hydrolysis of the *N,N*-dimethylamides is unexpected since alkaline hydrolysis of amides and esters is usually facilitated by lower electron density at the carbonyl carbon. Interpretation of this negative value must be made with caution because the magnitude is very small. An explanation for the negative value might be that the reaction temperature may be near the isokinetic temperature of the reaction for this series. A similar situation may exist for the *N*-methylamides since the magnitude of ρ^* is also very small.

The effect of temperature on the rates of hydrolysis of the *N,N*-dimethylamide series was investigated and does not appear to influence the values of ρ^* appreciably. However, the magnitudes of the

[1] The polar substituent constant (σ^*) expresses the purely polar effects of a substituent in an aliphatic reaction. Utilization of this substituent constant is generally through the following equation:

$$\log k/k_0 = \sigma^*\rho^*$$

where one plots the logarithm of the ratio of the rate constants of the particular reaction at hand against σ^*, presumably yielding a straight line with a slope equal to ρ^*. The sign and magnitude of the slope are interpreted as the electronic requirements for that particular reaction, *e.g.*, a negative slope indicates that the reaction is favored by high electron density.

[2] The isokinetic temperature is the temperature when $\delta\ \Delta G^{\neq} = 0$; that is, substituent effects on the free energy change disappear at this temperature. Thus, the $\Delta H^{\neq}$ and $T\ \Delta S^{\neq}$ terms exactly offset each other at the isokinetic temperature.

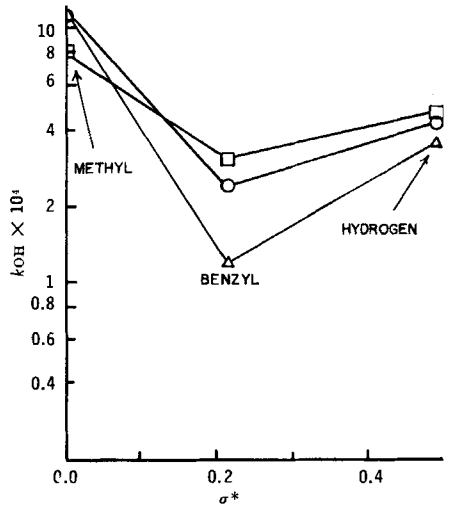


Figure 4—*Plot of second-order rate constants for alkaline hydrolysis at 25° of some amides, with substituent variations on the nitrogen,* versus σ^*. *Key:* ○, *acetamides;* □, *phenylacetamides; and* △, *propionamides.*

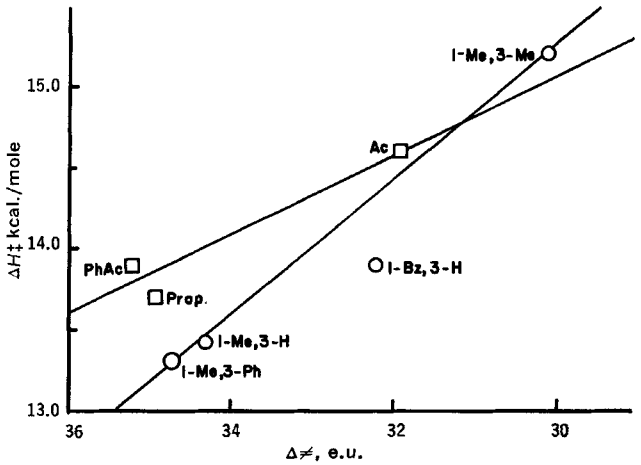


Figure 5—*Enthalpies of activation* versus *entropies of activation for the alkaline hydrolysis of amides and β-lactams. The lines drawn have a slope value of 300°K. for amides and 525°K. for β-lactams. Key:* ○, *β-lactams with variations in the 3-position; and* □, *acyl variations of amides.*

ρ^* values are difficult to estimate due to the significant standard deviations (10–20%) associated with the rate constants.

Substituent Effects on Amide Nitrogen—Variation of substituents on the amide nitrogen could be successfully correlated with Taft substituent constants, as illustrated in Fig. 4. This may conceivably be explained by a change in the rate-determining step; however, this interpretation must be made with caution since only three substituents are involved and no ^{18}O-exchange studies were done. Biechler and Taft (24) observed that alkaline hydrolysis of anilides included a term in the rate expression that was second order with respect to hydroxide ion and attributed this to a rate-determining base-catalyzed expulsion from an anionic tetrahedral intermediate. Others (25–28) reported that the hydrolysis of anilides is subject to general base catalysis, suggesting a similar mechanism. Bender and Thomas (29) observed that ^{18}O-exchange with the solvent occurs more rapidly than hydrolysis, thereby suggesting that breakdown of the tetrahedral intermediate is the rate-determining step. Other ^{18}O-exchange studies (29) suggest that the ratio of hydrolysis to exchange varies with the number of substituents on the nitrogen.

Comparison of β-Lactams with Their Acyclic Analogs—Comparison of the substituent effects on the 3-position of β-lactams with that in the acyl portion of linear amides reveals that both are correlatable with Taft's substituent constants. However, unlike the β-lactams, the ρ^* values are greatly influenced by substituents on the nitrogen in the case of linear amides. The sensitivities of the alkaline hydrolysis of β-lactams to substituent effects in the 3-position also appear to be greater than those observed in the alkaline hydrolysis of linear amides.

The effect of 1-position substituents on the alkaline hydrolysis of β-lactams could also be correlated with σ^*, but similar substituents on the nitrogen in linear amides could not, suggesting that the mechanisms of hydrolysis are different and that direct comparisons may not be readily made. The sensitivities of alkaline hydrolysis of β-lactams to polar substituents in the 1-position are not influenced by substituent variations in the 3-position, unlike those of linear amides where variations on the nitrogen are influenced by the acyl portion of the molecule.

The activation parameters for the alkaline hydrolysis of some β-lactams and their acyclic analogs are presented in Table IX. No significant differences in the values for β-lactams and the amides are apparent. The free energies of activation for the alkaline hydrolysis of β-lactams are slightly less than those of the corresponding amides. This may be due to ring strain in the β-lactams. A plot[3] of $\Delta H^{\neq}$ *versus* $\Delta S^{\neq}$ for the alkaline hydrolysis of both series is illustrated in Fig. 5. By considering only the variations in the 3-position of the β-lactams, a line with a slope of 525°K., corresponding to the isokinetic temperature for this series, may be drawn through the three points, whereas a much smaller value (275–300°K.) is observed for similar acyl variations in the amide series. The large difference in the isokinetic temperature for each series does coincide with the expected differences based on the ρ^* values in Figs. 1 and 3.

The methoxide-catalyzed methanolysis of β-lactams and amides was studied for two reasons: (*a*) to note the effect of a different nucleophile on the rates of reaction of β-lactams and amides, and (*b*) to establish steric requirements involved in both series since the methoxide ion is much larger than the hydroxide ion. The different reaction complexities involved in alkaline hydrolysis and methoxide solvolysis, such as different nucleophilicities and solvent composition, are recognized; this simple interpretation is only for comparison of β-lactams and amides. These studies on the relative alkaline hydrolysis to methoxide-catalyzed methanolysis of β-lactams and amides reveal that the β-lactams exhibit approximately the same reactivity to either nucleophile, while linear amides are significantly less reactive to the methoxide ion, suggesting that steric hindrance to nucleophilic attack is much less in the β-lactams.

Mechanism—Vinnik and Moiseyev (30) speculated on the hydrolytic mechanism for lactams and amides in aqueous solutions of potassium hydroxide and concluded that formation of a stable tetrahedral intermediate was the appropriate mechanism. This mechanism was based primarily on IR spectral evidence exhibited by a γ-lactam, γ-butyrolactam, in concentrated aqueous solutions of

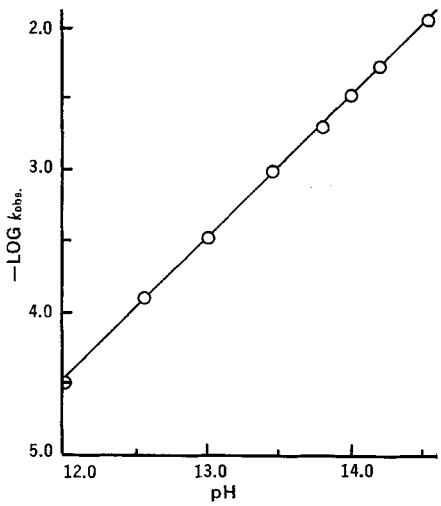


Figure 6—*Partial pH–rate profile for alkaline hydrolysis of 1-benzyl-2-azetidinone at 25°.*

[3] This treatment and interpretation are highly speculative considering the small number of points, the large error associated with each value, and the relatively small differences in $\Delta H^{\neq}$ and $\Delta S^{\neq}$.

Table XI—Some Physicochemical Constants Associated with Amides, β-Lactams, and Antibiotics

Compound	Bond[a] Lengths, Å C—N	Bond[a] Lengths, Å C=O	Distance of[a] Nitrogen from Plane of Ring, Å	C=O[a] Stretching Frequencies, cm.$^{-1}$	$k_{OH}(M^{-1}$ sec.$^{-1})$[b]	Antibiotic[a] Activity
Penicillins	1.34–1.37	1.17–1.20	0.40	1795–1775	10^{-1}–1	Yes
Δ^3-Cephalosporins	1.38–1.48	1.21–1.28	0.22–0.24	1790–1765	10^{-1}	Yes
Δ^2-Cephalosporins	1.34	1.22	0.065	1760–1755	10^{-3}–10^{-2}	No
Fused β-lactams[b,c]	—	—	—	1750	10^{-3}–10^{-2}	No
Unfused β-lactams	1.33	—	0	1760–1730	10^{-6}–10^{-5}	No
Amides[b]	1.32	—	0	1690–1670	10^{-6}–10^{-5}	No

[a] From *Reference 31.* [b] This work. [c] *References 11–13.*

potassium hydroxide. They noted the appearance of several bands with frequencies of 1555, 1740, and 1395 cm.$^{-1}$, the intensities of which increased as the alkali concentration became greater. The band with frequency at 1555 cm.$^{-1}$, appearing at low concentrations of potassium hydroxide, was ascribed to the monoionized intermediate; the frequencies of 1740 and 1395 cm.$^{-1}$, which appeared at approximately 30% potassium hydroxide, were ascribed to the doubly ionized species. IR spectral studies for 1-benzyl-2-azetidinone were performed in a similar fashion. The appearance of spectral bands was not evident as a function of potassium hydroxide concentrations up to 25% (w/v) KOH. In addition, kinetic studies performed by simultaneously measuring the disappearance of the carbonyl-stretching band for the β-lactam and the appearance of the carbonyl-stretching band for the amino acid suggested that the rates associated with each were quite similar. This indicates that a mechanism involving a stable charged intermediate would not be applicable in this case.

Further evidence inconsistent with a mechanism involving a charged intermediate was derived from the partial pH–rate profile and the salt effect on the rates of alkaline hydrolysis for 1-benzyl-2-azetidinone. The partial pH–rate profile is shown in Fig. 6. The slope of this plot indicates that the alkaline hydrolysis is first order with respect to hydroxide ion over a pH range of 12.0–14.54. Since linearity is observed, no charged intermediate is apparently participating in the reaction, a requirement implicit in the mechanism of Vinnik and Moiseyev (30), further discounting charged intermediate formation as a rate-determining factor. Therefore, it appears, for the β-lactams employed in this study, that the rate-determining step is attack of hydroxide ion rather than breakdown of the tetrahedral intermediate.

Comparison of β-Lactams and Amides with Penicillins and Cephalosporins—Sweet and Dahl (31) published data dealing with some structural parameters associated with various penicillins and cephalosporins and related them to biological activity. Table XI, a summary of present observations in conjunction with their results, indicates that chemical reactivity and biological activity parallel a variety of structural parameters associated with the β-lactam ring. The apparent differences in reactivity and biological activity from the penicillins to linear amides is reflected by the degree of planarity of the nitrogen in the β-lactam ring. As the nitrogen atom becomes more pyrimidal in nature, the chemical reactivity increases almost six orders of magnitude. Woodward (32) suggested that normal amide resonance occurs in β-lactams which are not fused to another ring and, consequently, the C—N and C=O bond lengths would be very similar to those of a linear amide provided the resonance involved in both instances occurs to the same extent. Decreasing the resonance capabilities of the β-lactam ring would then result in a decrease in the C=O bond length and an increase in the C—N bond length. In penicillins, changes in bond lengths coincide with the expected changes in reactivity, both being sensitive to the planar configuration of the nitrogen since less overlap between the π-electron orbitals of the carbonyl group and the lone pair of electrons on the nitrogen should exist.

In addition to the lack of planarity of the nitrogen in the β-lactam ring, the biologically active cephalosporins have another factor, enamine resonance, which may account for the unusual lability of the β-lactam amide bond. This, in addition to the nonplanarity of the nitrogen, would result in diminished amide resonance; consequently, the bond lengths would be lengthened to an even greater extent. On the other hand, Δ^2-cephalosporins have a nitrogen in the ring which is nearly planar and have no enamine resonance. This suggests that the bond lengths should be very similar to the unfused β-lactams and linear amides and this does coincide with the observed values (33).

CONCLUSIONS

The enhanced sensitivity to alkaline hydrolysis of penicillins and Δ^3-cephalosporins relative to unfused β-lactams is not primarily due to substituent effects, considering the relatively small changes in the rate constants for alkaline hydrolysis observed as the polarity of the substituent on the ring is varied. Similarly, strain induced into the β-lactam ring associated with fusion to another ring may not totally account for the unusual lability. However, fusion of another ring to the β-lactam ring in conjunction with inhibition of normal amide resonance does effectively account for the five or six orders of magnitude difference in reactivity. Unfused β-lactams are more closely associated with linear amides than penicillins or Δ^3-cephalosporins, as evidenced by the similarities exhibited in kinetic, structural, and spectral properties.

Even though unfused β-lactams are very similar in most respects to amides, some dissimilarities are apparent when comparisons are made. The sensitivities of alkaline hydrolysis to substituent effects are very different for β-lactams and amides, and this difference may be a result of the differences in isokinetic temperatures. For the temperature range employed in this study, alkaline hydrolysis of various unfused β-lactams has an isokinetic temperature which is far removed from the experimental temperature, unlike that observed for amide hydrolysis. Differences in steric hindrance to nucleophilic attack are also apparent, as suggested by reactivities observed in the methoxide-catalyzed methanolysis of amides and β-lactams relative to hydroxide-catalyzed hydrolysis. Differences in mechanism of alkaline hydrolysis may also be involved. In the case of alkaline hydrolysis of β-lactams, the rate-determining step is the attack of the nucleophile. For the alkaline hydrolysis of linear amides, the rate-determining step is dependent upon the number of substituents on the amide nitrogen and may involve either nucleophilic attack or decomposition of the tetrahedral intermediate. These differences, however, are not sufficient to warrant classification of the unfused β-lactams with penicillins and Δ^3-cephalosporins solely on the basis of ring strain.

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Keyphrases □ Fluocinolone acetonide, fluocinonide topical gels—skin penetration □ Topical gels, fluocinolone acetonide, fluocinonide—release, penetration, *in vivo*, *in vitro* data □ Vehicle composition profiles, *in vivo*, *in vitro*—fluocinolone acetonide, fluocinonide topical gels □ Pharmacokinetics, skin penetration—fluocinolone acetonide, fluocinonide topical gels, drug efficacy, vehicle and drug physical properties, effects

In the formulation of vehicles for topical drugs, the efficacy of such dosage forms is often dependent on the composition of the vehicle. The ability of a drug in a topical formulation to penetrate the skin and exert its effect is dependent on two consecutive physical events. The drug must first diffuse out of the vehicle to the skin surface, and then it must penetrate this natural barrier en route to the site of action. Many so-called "vehicle effects" reported in the literature are consequences of these two diffusional processes. Depending on which process proceeds slower, either event could determine the overall effectiveness of the topical dosage form. These two processes are intimately related, and both are dependent upon the physical properties of the drug, vehicle, and barrier.

The physical picture is one in which a single molecular species, the drug, experiences a changing environment as it diffuses out of the vehicle and across the skin. Poulsen *et al.* (1) reported on vehicle effects regarding the relative release characteristics of gels with varying compositions. The main objectives of this study were to explore the second process of diffusion, penetration of the drug through human skin, and to show that it is rate controlling when a topical dosage form is applied to normal skin. A more general objective was to provide insight into the manner in which the physical–chemical properties of drug and vehicle can be utilized to increase a formulation's effectiveness. Such information hopefully should aid in the intelligent design of topical dosage forms.

THEORETICAL CONSIDERATIONS

The transport of drugs across the skin barrier may be considered a process of passive diffusion. The flux, J (moles cm.$^{-2}$ sec.$^{-1}$), for

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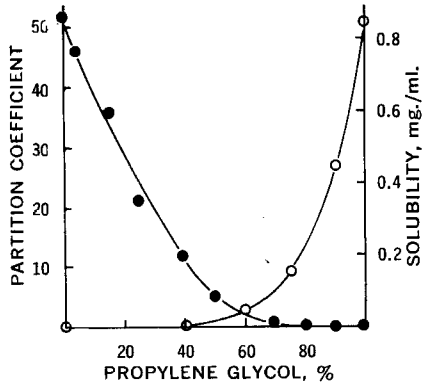

Figure 1—*Isopropyl myristate/propylene glycol–water partition coefficients* (●) *and propylene glycol–water solubilities* (○) *for fluocinonide.*

transport across a membrane is proportional to the product of force and concentration. That is,

$$J = \frac{DC}{RT}\left[\frac{-d\mu}{dx}\right] \quad \text{(Eq. 1)}$$

$$= -DC\frac{d\ln a}{dx}\ (\mu = \mu_0 + RT\ln a) \quad \text{(Eq. 2)}$$

$$= -DC\left[\frac{d\ln C}{dx} + \frac{d\ln\gamma}{dx}\right]\ (a = \gamma C) \quad \text{(Eq. 3)}$$

$$= -D\left[\frac{dC}{dx} + \frac{Cd\ln\gamma}{dx}\right] \quad \text{(Eq. 4)}$$

$$= \frac{-D\,dC}{dx}\ (\text{for a constant } \gamma) \quad \text{(Eq. 5)}$$

In Eqs. 1–5, D is the diffusion coefficient for the drug in the barrier, R is the gas constant, T is the absolute temperature, $d\mu/dx$ is the chemical potential gradient across the barrier, a is the thermodynamic activity, and γ is the activity coefficient. The rate of penetration, dQ/dt, then is given by:

$$\frac{dQ}{dt} = \frac{D(C_2 - C_1)}{h} \quad \text{(Eq. 6)}$$

where h is the effective thickness of the barrier, and Q is the amount penetrated per unit area. Since the concentration of drug in the membrane surface on the vehicle side, C_2, is related to the concentration in the vehicle, C_v, by $PC = C_2/C_v$, and if the concentration at the membrane surface on the opposite side, C_1, is maintained at zero concentration such that the concentration gradient for diffusion, dC/dx, is equal to the ratio of C_2 over the thickness of the membrane, C_2/h, then:

$$\frac{dQ}{dt} = \frac{D(PC)C_v}{h} \quad \text{(Eq. 7)}$$

In Eq. 7, the parameters that are alterable and may be modified by vehicle composition to facilitate skin penetration are D, PC, and C_v. The diffusion coefficient, D, of the drug in the barrier can be modified by the influence of the vehicle components on the nature of the barrier. The partition coefficient, PC, of the drug between the skin and the vehicle can be optimized by decreasing its solubility in the vehicle. The concentration of diffusible drug in the vehicle, C_v, for a given labeled strength can be optimized by ensuring that all of the drug is in solution. Experimentally, dQ/dt can be obtained from penetration studies by calculating the slope in the steady-state region from plots of amount of drug penetrated *versus* time. PC can be estimated by partitioning the drug between the vehicle and a phase thought to be representative of the barrier material. C_v either is known (if all of drug is in solution) or can be obtained by determining the solubility of the drug in the vehicle (if a fraction of drug is suspended). D can be obtained from Eq. 7 if the true partition coefficient is known, or it can be approximated indirectly from the lag time, L:

$$L = \frac{h^2}{6D} \quad \text{(Eq. 8)}$$

obtained by extrapolation of the pseudo-steady-state portion of a plot of amount of drug penetrated *versus* time to the time axis.

To characterize the relationships between a diffusing drug and its environment (vehicle and barrier), two steroids with substantially different physical properties were utilized. The steroids were fluocinolone acetonide[1] and its 21-acetate ester, fluocinonide[2].

EXPERIMENTAL

Materials—Propylene glycol[3], carboxypolymethylene[4], isopropyl myristate[5], diisopropanolamine[3], fluocinolone acetonide[1], and fluocinonide[2] were used as received. All other chemicals were of analytical reagent grade. The skin specimens were whole human abdominal sections obtained from autopsy and stored in the frozen state until utilized.

Solubility—The method employed was essentially the same as that reported previously (2). The solubility data correspond to the most stable polymorph at equilibrium (25°) for each solvent mixture.

Partition Coefficients—Isopropyl myristate and the various propylene glycol–water mixtures were saturated with respect to each other. Ten milliliters of the presaturated isopropyl myristate phases, containing approximately 0.02 mg./ml. of radioactive steroid (^{14}C) of known specific activity, was added to 10 ml. of the respective propylene glycol–water phases in a separator at room temperature. After equilibration, the phases were separated and assayed individually for steroid content. Partition coefficients were calculated as the ratio of steroid concentration in the isopropyl myristate phase to that in the propylene glycol–water phase.

Preparation of Vehicles—An appropriate amount of radioactive steroid (see *Release Rates* section) was dissolved in each propylene glycol–water mixture with the aid of heat and stirring. These solutions were gelled by first dispersing 1% carboxypolymethylene resin and then neutralizing to a pH of approximately 6.5 by adding a sufficient quantity of either diisopropanolamine or concentrated aqueous sodium hydroxide solution with proper mixing.

Release Rates—Initially, it was found that manufacturing difficulties caused a variation in release rates for different batches of the same gel composition. This variability was attributed to supersaturation effects during the heating step of manufacture. The problem was overcome by first preparing each gel with the steroid at saturation concentration in each propylene glycol–water solution for those preparations in which the solubility was less than 0.25 mg./ml. and then adding additional steroid (the insoluble portion) to the gelled preparation to give a final concentration of 0.025% (w/w). Release data were then obtained for each gel in a manner similar to that reported by Poulsen *et al.* (1) to verify a proper manufacturing technique.

Preparation of Membranes—Whole human abdominal skin, obtained at autopsy, was frozen on a glass plate with the epidermal surface flat in contact with the plate. Just prior to an experiment, the frozen skin was dislodged by warming the plate with water. A layer of skin, 0.076-cm. (0.030-in.) thick, was removed from the epidermal side with an Electro-Dermatome[6]. This procedure allowed removal of the subcutaneous fat without contamination of the intact epidermal surface. The epidermal surface was washed with hexachlorophene[7] and warm water; circular sections, having a diameter of 2.21 cm. (0.87 in.), were cut. Each section was then moistened with a few drops of distilled water and placed on a Whatman No. 1 filter paper of equal size with the dermal side in contact. The prepared sections were then positioned between the two Teflon disks of the diffusion cell. To ensure a good seal, the surfaces of both disks were coated

[1] 6α,9α-Difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide, Syntex Laboratories, Inc., Palo Alto, Calif.
[2] 6α,9α-Difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide 21-acetate, Syntex Laboratories, Inc., Palo Alto, Calif.
[3] Union Carbide Co., New York, N. Y.
[4] Carbopol 934, B. F. Goodrich Co., Cleveland, Ohio.
[5] General Aniline & Film Corp., New York, N. Y.
[6] Model B, Padgett-Hood, Division of Kansas City Assemblage Co., Kansas City, MO 64111
[7] pHisohex.

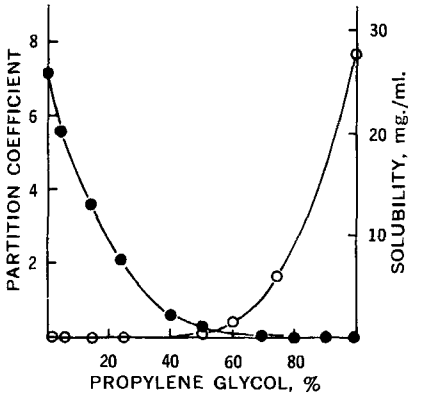

Figure 2—*Isopropyl myristate/propylene glycol–water partition coefficients* (●) *and propylene glycol–water solubilities* (○) *for fluocinolone acetonide.*

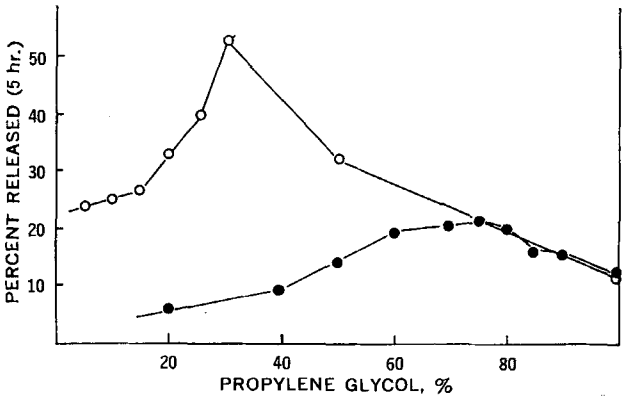

Figure 4—*Release profile for 0.025% fluocinolone acetonide* (○) *and 0.025% fluocinonide* (●) *at 25° (propylene glycol–water gels).*

with a thin film of silicone grease prior to positioning of the membrane.

Diffusion Cells—The cell design and operation were the same as these reported by Coldman *et al.* (3), except that the exposed area for penetration had a diameter of 1.33 cm. and an area of 1.41 cm.[2] The gels were applied to the epidermal surface, and the upper Teflon disk reservoir was filled completely. The surface of each gel was smoothed over with a spatula to make it flush with the disk surface. Each cell accommodated 0.83 g. of gel. To prevent evaporation, a glass coverslip was placed over the gel and sealed with silicone grease. A Teflon-coated stirring bar attached to a polyethylene sail provided efficient mixing in the sampling chamber, which contained 9–10 ml. of a 30% propylene glycol–water solution. The sampling arm was stoppered with a cork to prevent evaporation. One-milliliter samples were withdrawn for analysis at appropriate times over approximately 2 weeks (330–360 hr.).

Radiochemical Assays—A radiochemical assay using ^{14}C steroids was employed throughout the experimental work. The radiochemical purities of the steroids were checked by spotting an aliquot of each on a silica gel TLC plate, developing with CCl_4–dioxane–water (280:160:1), and scanning on a Vanguard model 880-D scanner. The resultant chromatograms indicated that the purity was ≥98% for both steroids. The radioactive steroids were prepared from solutions of high specific activity by diluting with nonradioactive steroid to about 2 μc./mg. and recrystallizing from ethanol and water. The analytical methodology was the same as that given previously (2).

***In Vivo* Assay**—Each gel was tested in an *in vivo* human assay procedure, a modified Stoughton–McKenzie assay (4), where the degree of blanching (vasoconstriction) of the skin was taken as a measure of efficacy or penetrability. Forty normal adult subjects (male and female) were employed in the study. All subjects had 3 mg. of each gel preparation applied to two sites on each forearm, so the total number of sites over all subjects was 160 per preparation.

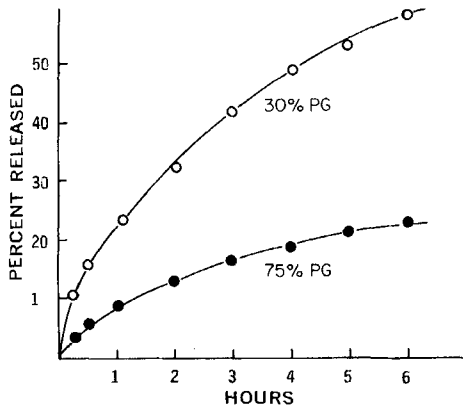

Figure 3—*Release for 0.025% fluocinolone acetonide* (○) *and 0.025% fluocinonide* (●) *from carboxypolymethylene gels (propylene gycol–water), giving maximum release at 25°.*

Each subject accommodated 32 sites per arm, and 16 gel preparations were studied in the same subject group at one time. The study was conducted under conditions of occlusion (6 hr.), and the number of sites showing vasoconstriction at 24 hr. was recorded.

RESULTS AND DISCUSSION

To demonstrate the significance of vehicle composition on both release and penetration, several carboxypolymethylene gels of propylene glycol–water mixtures in varying proportions, which contained either 0.025% fluocinolone acetonide or 0.025% fluocinonide separately, were evaluated. The physical constants of the two steroids applicable to this study are the solubilities of the steroids in the vehicles under consideration and the partition coefficients of the steroids between the barrier (stratum corneum) and these same vehicles. Isopropyl myristate was chosen to represent the barrier for the partition process[8]. The solubilities (25°) and partition coefficients (room temperature) for the two steroids are given in Figs. 1 and 2.

Although release data were reported previously (1), the rates of release into isopropyl myristate were obtained to ensure proper manufacturing of the gels as discussed in the *Experimental* section. Figure 3 gives the percent of drug released as a function of time for each

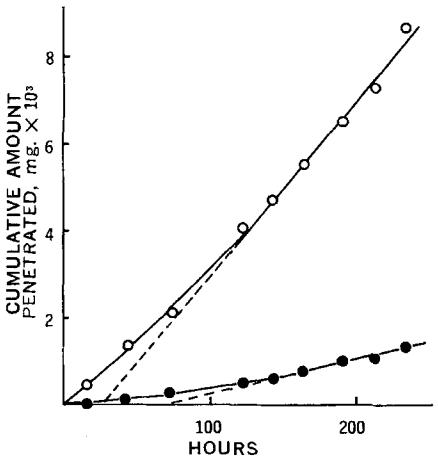

Figure 5—*Typical steroid penetration through human abdominal skin at RT (optimal vehicle). Key: ○, fluocinolone acetonide, 30% glycol; and ●, fluocinonide, 75% glycol.*

[8] Isopropyl myristate was arbitrarily chosen to represent the barrier since evidence has suggested that the barrier contains a lipid–protein matrix (5, 6) and that the lipid solubility of steroids is a significant factor in determining their penetrability (7).

Table I—Individual and Average Accumulative Amount(s) (mg. × 10^3) Penetrated[a]

Propylene Glycol, %	1	2	3	4	Av. ± $\bar{\sigma}_x$
			Fluocinolone Acetonide[b]		
5	11.80	1.55	4.65	7.60	6.40 ± 2.18
10	6.00	0.93	3.60	6.00	4.13 ± 1.21
15	4.05	1.05	5.70	—	3.60 ± 1.36
20	4.65	0.85	5.45	5.60	4.14 ± 1.12
25	7.85	1.90	8.25	7.70	6.43 ± 1.51
30	7.50	1.75	9.50	8.50	6.81 ± 1.74
50	4.65	1.35	4.25	2.50	3.19 ± 0.77
100	2.35	0.55	0.75	0.40	1.01 ± 0.45
			Fluocinonide[b]		
40	1.15	0.21	0.65	0.50	0.63 ± 0.20
60	1.90	0.18	1.30	0.46	0.96 ± 0.39
70	3.50	0.42	1.45	0.93	1.58 ± 0.68
75	4.05	0.61	2.05	1.31	2.01 ± 0.74
80	5.55	0.61	2.50	0.99	2.41 ± 1.12
90	4.95	0.48	0.95	0.82	1.80 ± 1.06
100	3.80	0.53	0.80	0.57	1.43 ± 0.79

[a] Whole abdominal human skin, 230 hr., room temperature. [b] The standard errors, $\bar{\sigma}_x$, give the variation between skins and do not necessarily reflect the significance of differences for amounts penetrated at different compositions. For this reason, standard errors are not indicated in Figs. 4–6.

steroid for the gel from which the corresponding release rate was maximal. Figure 4 gives the percent of drug released for each steroid after 5 hr. as a function of the percent glycol in the gel, and it clearly demonstrates the dependence of release on vehicle composition. In Fig. 4, a maximum is exhibited in the release profile for each steroid. These maxima correspond to that vehicle composition at which each steroid is completely solubilized and at its saturation concentration. Following each release profile from left to right, the increase in the amount released as the glycol content increases to the left of the maximum is due to an increase in the concentration of drug in solution, while the decrease in the amount released as the glycol content increases to the right of the maximum is due to a less favorable partition coefficient[9].

In Fig. 5, typical penetration curves (amount penetrated *versus* time) are shown for the same vehicle compositions that gave maximal release for each steroid. These penetration curves depict the usual behavior for passive diffusion through a membrane because there is a lag time before the penetration rate becomes constant. The amounts penetrated at 230 hr. for each steroid through four different skin specimens are given in Table I for the individual experiments as well as the average of these values. All 15 gel preparations were studied on each specimen, so the resultant data are comparable. The total number of gel preparations used in the penetration studies was limited to 15 initially because of the skin specimen size. Subsequently, however, it was thought advisable to obtain additional data points for the fluocinonide gels at low proportions of glycol. The penetration of fluocinonide from gels containing 1, 5, and 10% glycol was studied in a single and different skin specimen along with four gels from the original set. The results were normalized to make the data comparable[10].

The relationship between amount penetrated at a particular time (including the three normalized data points) and composition is shown in Fig. 6. There is an obvious dependence of penetrability on vehicle composition. As can be ascertained from Table I, comparable profiles can be obtained utilizing data from any single experimental set. Figure 6 not only demonstrates the importance of vehicle composition but also that the effect of vehicle composition is dependent on the nature of the drug, as indicated by the difference in the profiles for the two drugs. The maximum for fluocinolone acetonide occurs at a gel composition of about 30% propylene glycol while the corresponding maximum for fluocinonide occurs at about 75%. These maxima correspond to that minimal glycol composition necessary to solubilize each steroid, as may be ascertained from the solubility data in Figs. 1 and 2.

The data in Table I were analyzed statistically *via* a repeated measurement design (8), which partitions out the variation due to people from the error term. The standard errors, $\bar{\sigma}_x$, given in Table I represent the skin-to-skin variation which has been partitioned out. Significant differences were shown for the amount penetrated for both fluocinolone acetonide ($p < 0.01$) and fluocinonide ($p < 0.05$). The maximum amount penetrated for fluocinolone acetonide (30% gel) was shown to be significantly different at the 5% level from all other compositions except 5 and 25%. For fluocinonide, the maximum amount penetrated (75% gel) was significantly different at the 5% level from compositions with 60% or less glycol. This analysis supports the existence of real maxima for the dependence of penetration on composition. An equally significant result is that the data obtained on each skin specimens give similar profiles.

The results obtained when these same gels were tested in an *in vivo* human vasoconstrictor assay are shown in Fig. 7. The percent of sites responding 24 hr. after application is plotted as a function of gel composition. The similarity between the profiles for vasoconstriction and penetration is striking[11]. These data suggest that the rate-controlling step is in the skin barrier and that the efficacy of a topical preparation is directly related to the ability of a drug in that vehicle to penetrate the skin barrier. The results for both *in vitro* penetration and *in vivo* vasoconstriction in the case of fluocinolone acetonide (Figs. 6 and 7) show that the 5% glycol gel, which contained most of the steroid in suspension, performed as well as the 30% glycol gel where the steroid was just solubilized. This can be attributed to a very high partition coefficient of the 5% glycol gel offsetting the decrease in concentration of steroid in solution. Thus, it is possible that in certain instances a vehicle in which the drug is suspended will be most efficacious.

The results in Fig. 7 should not be interpreted to judge the relative potency of the two steroids. Although the *in vivo* data for the gels indicate that the steroids are nearly equivalent when comparing their optimum gels, fluocinonide releases and penetrates at about

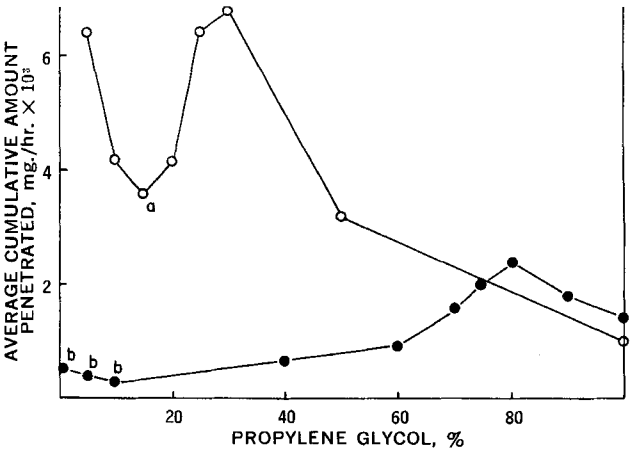

Figure 6—*Average cumulative amount penetrated at RT as a function of vehicle composition (human abdominal skin, 230 hr.). Key: ○, fluocinolone acetonide; and ●, fluocinonide.* a = *average of three data points.* b = *normalized data points from a single experiment (see text).*

[9] A change in partition coefficient may also be viewed as a change in thermodynamic activity. That is, the tendency of the drug to leave (partition from) the vehicle decreases as the glycol content increases.

[10] The amounts penetrated after 230 hr. for these three gels were 0.24, 0.21, and 0.13 mg. (× 10^{-3}) for the 1, 5, and 10% gels, respectively. To make these data comparable to those for the other compositions, the data for the four controls (40, 70, 75, and 100% gels) on the same single specimen were compared to the average values obtained for the four other specimens at these gel compositions. Proportionality factors were calculated at each of the four compositions, and the average was used to normalize the single values for the 1, 5, and 10% gels. The resultant normalized values were 0.48, 0.42, and 0.26 mg. (× 10^{-3}) for the 1, 5, and 10% gels, respectively.

[11] Although it was suggested by the reviewer to make a linear correlation between the *in vivo* and *in vitro* data, this was not feasible since the two maxima in the composition profiles do not occur at exactly the same composition. The difference in the position of these maxima, however, can be rationalized on the basis that the gel during *in vitro* penetration may incorporate some water from the receptor, whereby a higher percentage of glycol is required to solubilize the drug, thus placing this profile to the right of the composition profile for *in vivo* response.

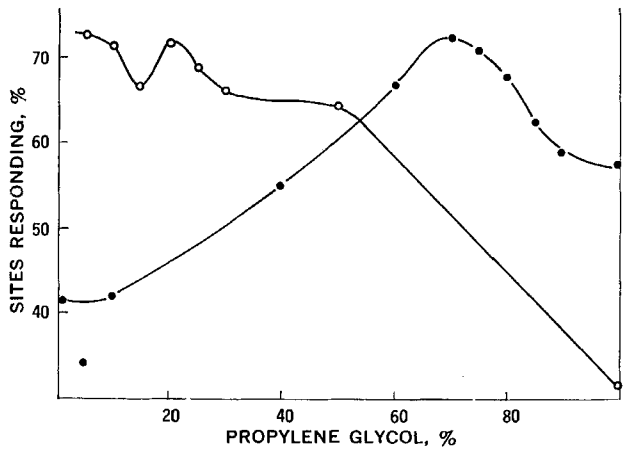

Figure 7—In vivo *response (24-hr. vasoconstriction) as a function of vehicle composition. Key:* ○, *fluocinolone acetonide; and* ●, *fluocinonide.*

one-third the rate of fluocinolone acetonide. One may conclude then that fluocinonide has an *intrinsic* potency three times that of fluocinolone acetonide. The relative potencies are also undoubtedly dependent on the vehicle type (gel, cream, ointment, *etc.*), and studies with other formulations could give different relative clinical efficacies.

The similarity between the *in vitro* penetration and *in vivo* vasoconstriction profiles for each steroid suggests a working hypothesis which can be used to describe by *in vitro* techniques the relationship between clinical efficacy, penetrability, and physical parameters of the drug, vehicle, and skin. In this study, increasing the proportion of glycol increases the concentration of drug in solution but, at the same time, creates a less favorable partition coefficient. Consequently, a proper balance of PC, C_v, and D is required to obtain the optimal composition and thereby produce the best conditions to facilitate penetration and clinical response. Equation 1 predicts that the ability of a drug to penetrate a barrier is proportional to the term $(PC)DC_v$. A subsequent report (9) will deal with the relative significance of the physical parameters (PC, D, and C_v), the predictive capability of the term $(PC)DC_v$, and the possible changes in barrier properties due to vehicle composition.

SUMMARY

1. Data were obtained which illustrated the dependence of release rate, skin penetration, and *in vivo* vasoconstrictor response on vehicle composition for two steroids with significantly different physical properties. The vehicles studied were various propylene glycol–water gels.

2. The relationships between the physical properties of the drug, vehicle, and barrier were shown to correlate well with the nature of the profiles of release, penetration, and vasoconstriction. The efficacy of a topical preparation was shown to be directly related to the ability of a drug to penetrate the skin barrier.

3. The correlations have led to a working hypothesis for vehicle formulation which describes by *in vitro* experimentation the relationship between vehicle composition and clinical efficacy and may aid in the design of efficacious topical dosage forms.

4. In general, an efficacious topical gel preparation is one in which: (*a*) the concentration of diffusible drug in the vehicle, C_v, for a given labeled strength is optimized by ensuring that all of the drug is in solution, (*b*) the minimum amount of solvent is used to dissolve the drug completely and yet maintain a favorable partition coefficient, and (*c*) the vehicle components affect the permeability of the stratum corneum in a favorable manner.

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Significance of Vehicle Composition II: Prediction of Optimal Vehicle Composition

JAMES OSTRENGA, CAROLYN STEINMETZ, BOYD POULSEN, and SUSAN YETT

Abstract □ Terms from the flux equation describing the passive transport of two steroids across a membrane were shown to be useful in predicting the optimal vehicle composition of gels. Calculations based on the increase in lag time for steady-state penetration with vehicle composition were made to explore the possible effects of vehicle composition on barrier permeability and drug binding within the barrier. Additional penetration studies were done to determine solvent effects on barrier permeability. These combined results indicated that the variation in barrier permeability was associated with a vehicle effect rather than binding.

Keyphrases □ Permeability, membrane—propylene glycol solvent effects □ Membrane permeability, fluocinolone acetonide and fluocinonide topical gels—predicting optimal vehicle composition □ Topical gels—optimal vehicle composition, theoretical □ Vehicle composition, membrane permeability—steroidal topical gels

A previous report (1) demonstrated that, for normal skin, the clinical efficacy of a series of topical gels containing fluocinolone acetonide or its 21-acetate ester (fluocinonide) was directly dependent on the ability of the drug to penetrate the stratum corneum. In turn, the penetrability was shown to be highly dependent upon the composition of the propylene glycol–water gels studied. This report: (*a*) illustrates the predictability of vehicle efficacy based on the physical parameters used to describe the diffusion of drugs across the stratum corneum, and (*b*) explores the possible effects of vehicle composition on barrier permeability and drug binding within the barrier.

Applicable theoretical considerations were described in the first paper of this series (1), where the terms in the following two equations were defined:

$$\frac{dQ}{dt} = \frac{D(PC)C_v}{h} \qquad \text{(Eq. 1)}$$

$$L = \frac{h^2}{6D} \qquad \text{(Eq. 2)}$$

Equation 1 suggests that the ability of a drug to penetrate the stratum corneum can be predicted by the term $D(PC)C_v$. If Eq. 2 is applicable, the term $(PC)C_v/L$ is equally useful. If barrier permeability is not altered by vehicle composition, then penetration should be proportional to $(PC)C_v$ for various vehicle compositions.

Equation 2 is valid only if the penetrant does not bind to components of the barrier. If binding occurs, L can be defined approximately by Eq. 3 (2):

$$L = \frac{h^2}{4D} + \frac{h^2 A_b}{2D(PC)C_v} \qquad \text{(Eq. 3)}$$

where A_b is the amount of drug bound per unit volume of barrier. Equation 3 gives reasonable estimates of L only when the degree of binding is significant. If the term A_b is small, however, Eq. 3 gives $L \simeq h^2/4D$, which differs substantially from the theoretical value $(L = h^2/6D)$.

Table I—Individual and Average Steady-State Penetration Rate(s) (mg./cm.^{2}/hr. × 10^6)[a]

Propylene Glycol, %	1	2	3	4	Av. ± $\bar{\sigma}_x$
Fluocinolone Acetonide[b]					
5	38.3	4.3	23.4	31.2	24.3 ± 7.3
10	19.2	2.8	19.1	27.0	17.0 ± 5.1
15	12.8	3.8	22.7	—	13.1 ± 5.5
20	14.9	2.1	24.1	22.7	16.0 ± 5.0
25	27.0	4.2	32.6	28.4	23.1 ± 6.4
30	26.2	3.5	34.8	39.7	26.1 ± 8.0
50	17.0	3.6	18.4	9.2	12.1 ± 3.5
100	12.1	1.8	5.0	2.8	5.4 ± 4.3
Fluocinonide[b]					
40	5.0	1.0	3.1	2.8	3.0 ± 0.8
60	7.8	1.4	6.5	2.5	4.6 ± 1.5
70	10.6	2.3	7.2	4.3	6.1 ± 1.8
75	31.9	5.4	15.4	6.2	14.7 ± 6.2
80	27.0	6.6	20.0	3.1	14.2 ± 5.6
90	27.7	4.5	7.7	4.3	11.1 ± 5.6
100	19.9	5.7	6.5	2.8	8.7 ± 3.8

[a] Whole abdominal skin, room temperature. [b] The standard errors, $\bar{\sigma}_x$, give the variation between skins and do not necessarily reflect the significance of differences for penetration rates at different compositions. For this reason, standard errors are not indicated in Fig. 1.

EXPERIMENTAL

The experimental results pertaining to this work were reported previously (1).

RESULTS AND DISCUSSION

The steady-state penetration rates for 0.025% fluocinolone acetonide and 0.025% fluocinonide in various propylene glycol–water gels are given in Table I. The average rates are also given in Table I. Each set of gels was studied in four different abdominal human skin sections. A plot of average penetration rate *versus* percent glycol is given in Fig. 1 to show the dependence of penetrability on vehicle composition[1]. Figure 1 includes three data points for fluocinonide penetration (1, 5, and 10% glycol) which are not included in Table I. These data were obtained on a fifth skin specimen, and the data were normalized to make the rates comparable[2].

The data in Table I were analyzed statistically *via* a repeated measurement design (3) in the same manner as in the first paper of this series (1). Significant differences were shown for the penetration data for both fluocinolone acetonide ($p < 0.01$) and fluocinonide ($p < 0.05$). The maximum penetration rate for fluocinolone acetonide (30% gel) was shown to be significantly different at the 5% level from the 15, 50, and 100% gels. For fluocinonide, that

[1] As may be ascertained from Table I, similar profiles can be obtained utilizing data from any single experimental set.

[2] The steady-state penetration rates for these three gels were 2.4, 1.2, and 0.9 mg. cm.$^{-2}$ hr.$^{-1}$ (× 10^{-6}) for the 1, 5, and 10% gels, respectively. To make these data comparable to those for the other compositions, the data for four controls (40, 70, 75, and 100% gels) on the same single specimen were compared to the average values obtained for the four other specimens at these gel compositions. Proportionality factors were calculated at each of the four compositions, and the average was used to normalize the single values for the 1, 5, and 10% gels. The resultant normalized values were 3.5, 1.7, and 1.4 mg. cm.$^{-2}$ hr.$^{-1}$ (× 10^{-6}) for the 1, 5, and 10% gels, respectively.

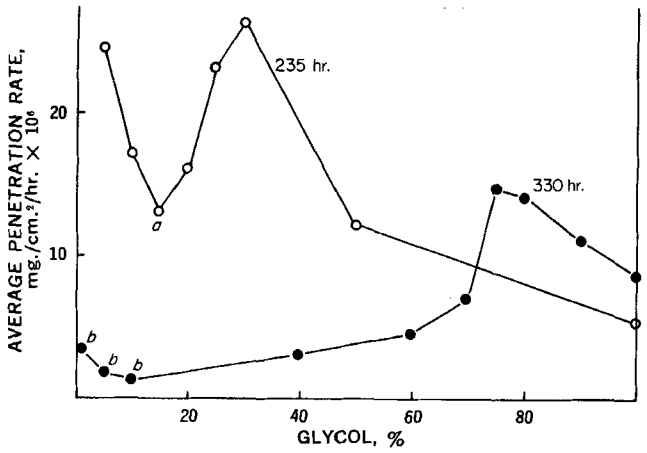

Figure 1—*Average penetration rate at RT as a function of vehicle composition (human abdominal skin). Key:* O, *fluocinolone acetonide; and* ●, *fluocinonide.* a = *average of three data points.* b = *normalized data points from a single experiment (see text).*

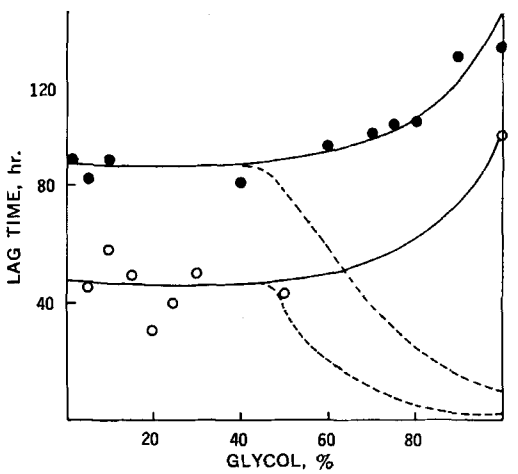

Figure 2—*Experimental* (——) *and calculated* (- - -) *lag times as a function of gel composition. Key:* O, *fluocinolone acetonide; and* ●, *fluocinonide.*

maximum penetration (75% gel) was significantly different at the 5% level from compositions with 70% or less glycol. This analysis supports the existence of real maxima for the dependence of penetration on composition. An equally significant result is that the data obtained on each skin specimen give similar profiles.

Figure 1 not only demonstrates the importance of vehicle composition but also that the effect of vehicle composition is dependent on the nature of the drug, as indicated by the difference in the profiles for the two steroids. A previous report (1) illustrated the similarity of the profile in Fig. 1 to that for the dependence of *in vivo* vasoconstrictor response on composition.

The lag times for penetration were estimated by extrapolation of the pseudo-steady-state portion in plots of amounts penetrated *versus* time to the time axis. The average lag time in hours as a function of gel composition is given in Fig. 2 for both steroids (solid line). The data points corresponding to the 1, 5, and 10% gel compositions were normalized in the same manner described for penetration rates. For both steroids, the lag times are invariant at gel compositions containing less than about 50% propylene glycol and increase substantially as this percentage approaches 100. Since the diffusivity (D) of the drug within the barrier is inversely related to the lag time (Eq. 2), this result suggests that the permeability of the barrier decreased at high proportions of glycol.

From Eq. 1, it can be predicted that the ability of a drug to penetrate a barrier is proportional to $(PC)DC_v$ or, perhaps, $(PC)C_v/L$. The physical parameters PC, C_v, and D all may be altered by vehicle composition so as to facilitate skin penetration. The quantity $D_p(PC)C_v$ was calculated at each gel composition, using experimental data previously reported (1). D_p refers to the diffusion coefficient calculated by Eq. 2 (no binding). Experimental values for C_v were used directly, and values for PC and D_p were obtained by interpolation from plots of PC or D_p *versus* glycol content. The partition coefficient data were obtained by partitioning the steroids between various glycol–water mixtures and isopropyl myristate. The relationship between the quantity $D(PC)C_v$ and vehicle composition is given in Fig. 3 for both steroids. Figure 3 also includes the relationship between $(PC)C_v$ and composition (broken lines) to demonstrate the usefulness of this approximation in the event that L values for the calculation of D_p are not available. The similarity between the calculated profile and that for penetration rates (and *in vivo* responses) demonstrates the predictive nature of the terms $(PC)C_v/L$ and $(PC)C_v$. Since the PC terms used in the calculations were not the true values, deviations in the calculated profile are understandable. Other partitioning systems could accentuate the profile for the calculated values of $D(PC)C_v$ at either low or high proportions of glycol in the gel, depending on the relative shift in the partition coefficient scale for these other systems. Ideally, partition coefficients between the stratum corneum and the vehicle are required.

By examining the dependence of the parameters PC, C_v, and D (or L) on gel composition, the shape of the profile for both steroids in Fig. 3 can be interpreted. At proportions of glycol from 0 to 50%, D is essentially constant for both steroids, so the nature of the profile can be attributed to the terms PC and C_v. In the case of fluocinolone acetonide, at proportions of glycol less than 10%, PC is decreasing faster than C_v is increasing to produce a negative slope; at glycol proportions between 10 and 30%, the converse relationship exists to give a positive slope. Above 30% glycol, all of the steroid is in solution, C_v remains constant, and the term $D(PC)C_v$ decreases markedly, mainly due to a decreasing PC. Above 50% glycol, some of the decrease in $D(PC)C_v$ may also be attributed to an apparent decrease in permeability. In the case of fluocinonide, the profile can be interpreted in the same manner, the only difference being that the maximum occurs at a higher proportion of glycol due to the poorer solubility of this steroid in these vehicles. That is, at proportions of glycol from 0 to 50%, C_v is increasing faster than PC is decreasing; from 50 to 75% glycol, C_v is increasing faster than PC and D combined are decreasing. At 75% glycol, all of the steroid is in solution, C_v remains constant, and the term $D(PC)C_v$ decreases, mainly due to a decreasing PC.

The shape of the composition profiles for penetration and *in vivo* response, in the region where the gels are saturated with respect to steroid, deserves some comment. Theoretically, for all the gels with undissolved drug present, the driving force (chemical potential) for diffusion is the same. Consequently, one would predict, contrary to the experimental results, that the penetration rate would remain constant with composition in this region. This was not the case. The calculated profiles obtained from experimental solubilities and partition coefficients show a similar deviation. The discrepancy between theory and experimental results is particularly obvious with fluocinolone acetonide gels containing 0–30% glycol. The variation in rate is apparently not due

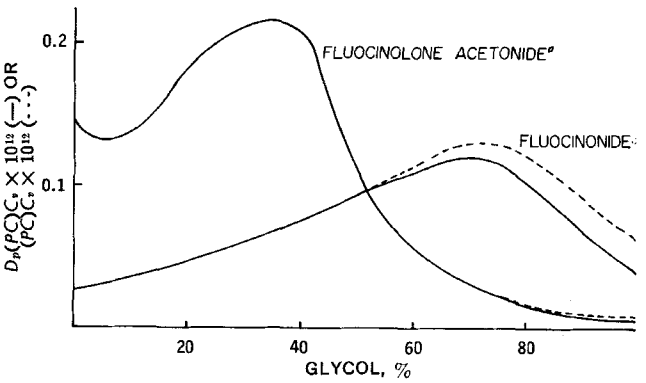

Figure 3—*Relationship between* $D_p(PC)C_v$ *and gel composition.* [a] *Values for* $D_p(PC)C_v$ *were divided by 2.21, which corresponds to the average* D *value for compositions with less than 50% glycol.* [b] *Values for* $D_p(PC)C_v$ *were divided by 1.20.*

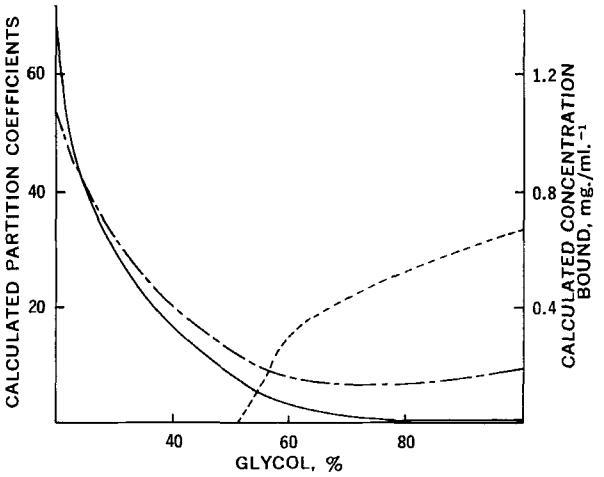

Figure 4—*Apparent partition coefficients* (- - -), *the theoretical partition coefficients* (——), *and fluocinolone acetonide concentration bound* (- - -) *as a function of gel composition.*

to the influence of the vehicle on D, since the lag time appears to be invariant in this composition range. One explanation is the possible alteration of barrier composition due to partial miscibility of the vehicle in the barrier lipid fraction without an alteration in barrier permeability (D remains the same). This would yield a barrier with varying composition as the glycol content varies. Such an alteration of barrier composition would then allow an effective drug concentration in the barrier different from the theoretical. In other words, the activity of the drug within the barrier may vary because of these barrier composition changes. Model systems which could include such an explanation have been discussed (4). Nonetheless, one can predict what the optimal vehicle composition for skin penetrability and clinical efficacy will be from a knowledge of drug solubilities and partition coefficients. Hopefully, these findings will be applicable to other topical vehicle types as well.

The increase in lag time to achieve steady-state penetration for both steroids at gel compositions of glycol above 50% suggests that the change in vehicle composition may be associated with two phenomena. These are: (*a*) a change in membrane permeability (D) due to the direct effect of vehicle components on the disposition of the barrier, and/or (*b*) the binding of the steroid within the barrier during transport. It is conceivable, for example, that as the glycol content increases, the barrier tends to dehydrate and becomes less permeable. Upon completion of the penetration studies, it was observed that the membranes exposed to high concentrations of glycol had become less pliable[3]. Also, it is logical that incorporation of some glycol by the membrane would alter its barrier properties in addition to a dehydration effect. Alteration of the barrier by glycol could conceivably increase or decrease its permeability. If binding or adsorption of the steroid on polar sites within the barrier occurs, one would expect an increase in binding tendency as the polarity of the vehicle decreases, corresponding to less competition for binding sites by the solvent. With binding playing a role, the lag time would tend to increase as the propylene glycol content increases because a longer period of time would be required to establish a steady-state concentration gradient across the barrier.

Thus, one can take two approaches in examining the data to speculate on the significance of the varying lag time with composition.

Case I: No Binding—If no binding occurs, the interpretation is simple. For this case, $D_p = h^2/6L$ is valid, and D_p can be calculated at each gel composition. By using 15 μ as an estimate of the effective thickness of the barrier (stratum corneum), the calculated values for D_p fall in the range of $1.1\text{–}2.2 \times 10^{-12}$ cm.^{2} sec.$^{-1}$ for fluocinolone acetonide and $0.75\text{–}1.2 \times 10^{-12}$ cm.^{2} sec.$^{-1}$ for fluocinonide. These D_p values are in the range of those reported for other steroids (5). This simple interpretation suggests that the membrane becomes less permeable as the glycol content increases above 50%.

Case II: Binding—In this case, the increase in lag time can be caused by both binding and a permeability change. Equation 3 may be used to estimate the extent of binding (A). This, however, requires a knowledge of the diffusivity of the steroids in the barrier and the barrier/vehicle partition coefficient at the various gel compositions. Although barrier/vehicle partition coefficients were not determined, they can be estimated in the following manner. The expression $D = h^2/6L$ is applicable in this case for gel compositions with less than 50% glycol where the lag time is invariant. Substitution for D in the flux equation (Eq. 1) allows one to calculate apparent partition coefficients, PC_p, over the 0–50% glycol range. The PC_p values over the 50–100% range can be obtained in a similar manner for the case where binding is insignificant.

The solubilities of the steroids in the barrier can then be estimated from Eq. 4, utilizing PC_p values for the 0–50% range:

$$PC_p \simeq \frac{S_b}{S_v} \qquad \text{(Eq. 4)}$$

where S_b and S_v are the steroid solubilities in the barrier and vehicle, respectively. Equation 4 should provide fairly good estimates of S_b since the gels are at or near saturation in the 0–50% glycol range. With S_b and S_v known, Eq. 4 can then be used to calculate theoretical partition coefficients, PC_b, over the whole glycol range. This requires the assumption that the vehicle does not alter the steroid solubility in the barrier at high glycol concentrations. Values for PC_p and PC_b so calculated are given in Figs. 4 and 5 for fluocinolone acetonide and fluocinonide, respectively, where they are plotted as a function of glycol content. The calculated solubility of fluocinolone acetonide in the barrier ranged from 2.5 to 6.4 mg./ml., with an average value of 4.1. The calculated fluocinonide solubility ranged from 0.66 to 1.7 mg./ml., with an average value of 1.1. The finding that the calculated solubility of fluocinolone acetonide in the barrier is greater than that of fluocinonide agrees with solubility data reported previously (6). Yet the lipid/water partition coefficient of fluocinonide is larger. This result corresponds to the markedly reduced solubility of fluocinonide (relative to fluocinolone acetonide) in the polar phase.

Using the values for PC_b, one can now calculate theoretical D values (D_b) over the entire glycol range using Eq. 1, assuming

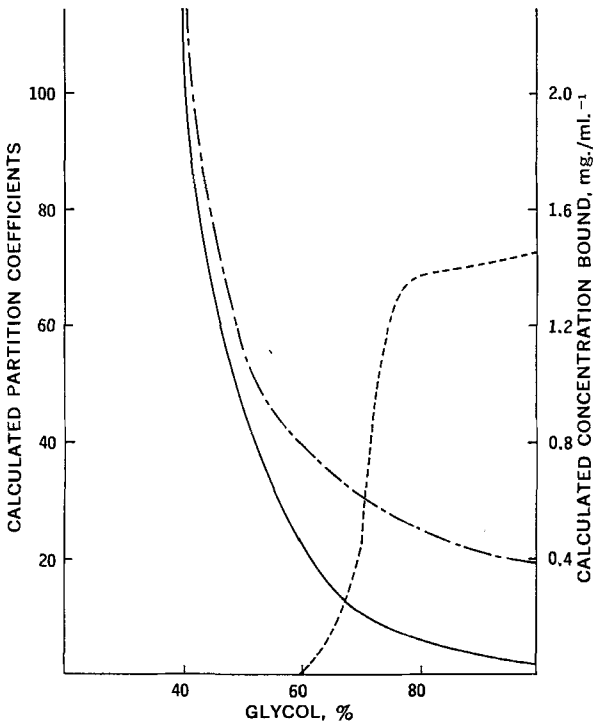

Figure 5—*Apparent partition coefficients* (- - -), *theoretical partition coefficients* (——), *and fluocinonide concentration bound* (- - -) *as a function of gel composition.*

[3] A decrease in pliability need not necessarily mean the membrane became less permeable. Other investigators (4) reported that dimethyl sulfoxide causes a hardening of skin and yet dimethyl sulfoxide is known to increase skin permeability.

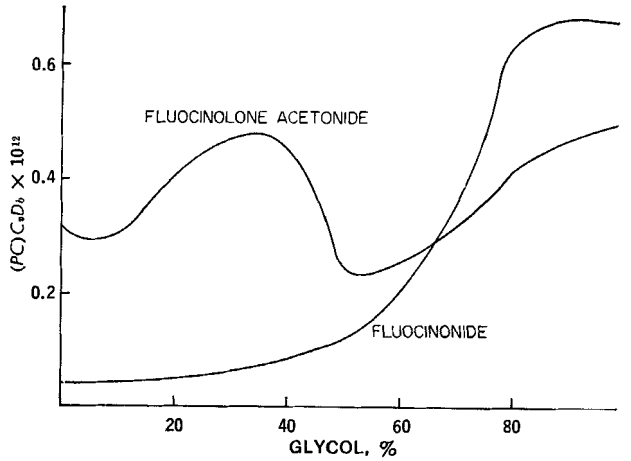

Figure 6—*Profile for the prediction of penetrability for steroid binding within the barrier.*

that binding does not affect diffusivity. Subsequently, lag times calculated from Eq. 2 using these D_b values will give estimates of L that correspond to the hypothetical situation where no binding occurs. The L values as a function of composition are given in Fig. 2 (dashed lines). The difference between experimental and calculated lag times (or D_p and D_b values) is actually a reflection of the difference between apparent and theoretical partition coefficients. Figure 2 indicates that glycol concentrations above 50% actually increase the permeability of the barrier (decreased lag time) and that the increase in experimental lag times for glycol concentration above 50% corresponds to a hypothetical binding phenomenon.

By using PC_b and D_b values calculated in the manner described and experimental L values, the hypothetical concentration of steroid bound to the barrier, A, was calculated using Eq. 3. A is plotted *versus* gel composition in Figs. 4 and 5. For each steroid, the hypothetical concentration of steroid bound is seen to increase rapidly at gel compositions above 50% glycol. For both steroids, the dependence of A on gel composition is linear above 75% glycol.

The extent of binding may be dependent on several factors. These include the steroid concentration in the barrier, the affinity of the steroid for the solvent mixture, and the relative affinity of the steroid and solvent mixture for the binding sites. The steroid concentration is directly related to the term $PC \times C_v$. The steroid affinity terms would be difficult to describe except that as the proportion of glycol increases, they increase. Also, different solvent compositions most likely change the nature of the barrier such that the number or type of binding sites varies.

This analysis of the data, of course, does not necessarily tell one what actually happens during skin penetration. However, one can gain some insight into which of the two cases more closely corresponds to the experimental situation for skin penetration with these gel formulations. Case I gave D_p values based on the experimental lag times and the use of Eq. 2, while Case II gave D_b values based on a theoretical partition coefficient and the use of Eq. 1. $(PC)C_vD_b$ values were calculated using isopropyl myristate/glycol–water partition coefficients and vehicle solubilities. A plot of $(PC)C_vD_b$ *versus* percent glycol is given in Fig. 6. The profile using $(PC)C_vD_p$ is the same as that in Fig. 3. Since the profile generated by using D_p (or experimental lag times in Fig. 3) more closely resembles the penetration profile (Fig. 1) for both steroids, Case I is probably more representative of the experimental situation.

To help ascertain the significance of the apparent permeability changes at high glycol concentrations, the following penetration experiments were done. Five membranes were prepared from a single skin specimen and placed in diffusion cells in the same manner described for previous experiments (1). Three membranes were pretreated with water saturated with isopropyl myristate, and two membranes were pretreated with propylene glycol saturated with isopropyl myristate. The pretreatment procedure was simply immersion of the membranes in the solvents for 36 hr. One-half milliliter of a solution of 0.025% w/v fluocinolone acetonide in isopropyl myristate saturated with either water or propylene glycol was placed on the epidermal side of the membranes. Isopropyl myristate saturated with either water or propylene glycol was used as the receptor from which samples were withdrawn. The penetrant solution and receptor were saturated with water or propylene glycol to ensure that solvent extraction from the membrane would be minimal during the penetration experiments. The average rate of penetration for the membranes pretreated with water was 0.121 mg. cm.$^{-2}$ hr.$^{-1}$; for the membranes pretreated with propylene glycol, the average rate was 0.006 mg. cm.$^{-2}$ hr.$^{-1}$. The average lag times were 89 and 256 hr., respectively. Thus, propylene glycol does decrease the permeability of the barrier significantly. Compared to water, the rate was reduced 20-fold. This result, coupled with the finding that the term $D_p(PC)C_v$ [rather than $D_b(PC)C_v$] as a function of composition gave a profile more similar to that for penetration rates as a function of composition, strongly suggests that: (*a*) propylene glycol causes the barrier to become less permeable[4], and (*b*) there is little or no binding of the steroid within the barrier.

The observation that propylene glycol causes the barrier to become less pliable probably corresponds to a dehydration of the barrier by the vehicle. Since there appears to be no binding, the difference between the apparent and theoretical partition coefficients is apparently fictitious. The apparent partition coefficients are most likely real. This result implies that the vehicle affects the barrier to give partition coefficients that vary from predicted ones. This alteration of the barrier by the vehicle could involve incorporation of vehicle components by the barrier or extraction of barrier components by the vehicle at high proportions of glycol.

SUMMARY

The dependence of skin penetrability and *in vivo* response on vehicle composition was demonstrated for two topical steroids in propylene glycol–water gels. In this report, it was shown that the terms $(PC)C_v/L$ and $(PC)C_v$ from the diffusion equation had a similar dependence on vehicle composition and that these terms may be useful in predicting the optimal vehicle composition for vehicle and clinical efficacy of topical drugs. Calculations based on the increase in lag time with vehicle composition were made to explore the possible effects of vehicle composition on barrier permeability and steroid binding within the barrier. Additional penetration studies were also performed to determine the relative solvent effect of propylene glycol and water on barrier permeability. The combined results suggests that propylene glycol decreases the permeability of the barrier and that there is little or no binding of steroid within the barrier.

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[4] Permeability refers to the diffusivity of the steroid within the barrier. Penetration rates are not necessarily indicative of permeability, because partition coefficients usually vary from one set of experimental conditions to another. A change in barrier permeability implies an actual change in the nature of the barrier (*e.g.*, a swelling effect); the best measure of this for a particular penetrant is the diffusion coefficient, D.

Enzyme Inhibitors XXIII: Syntheses of 9-(Substituted Aralkyl)-6-Substituted Purines as Inhibitors of Adenosine Deaminase

HOWARD J. SCHAEFFER*, EUGENE ODIN, and SHMUEL BITTNER

Abstract □ A series of 9-(substituted aralkyl)-6-substituted purines was prepared as potential inhibitors of adenosine deaminase. The adenine derivatives were reversible inhibitors of adenosine deaminase, with potencies not strikingly different than the corresponding benzyl derivatives. However, 9-(*p*-bromoacetamidophenethyl)-adenine (XV) and 9-(3-*o*- and *p*-bromoacetamidophenylpropyl)-adenines (XXIX*a* and XXIX*b*) were incapable of causing irreversible inhibition of adenosine deaminase, whereas the corresponding *ortho*- and *para*-benzyl derivatives (I and II) and 9-(*m*-bromoacetamidophenethyl)adenine (IV) caused rapid irreversible inactivation of this enzyme. These experiments demonstrated the selectivity of irreversible inhibition that can be obtained by site-directed irreversible enzyme inhibitors.

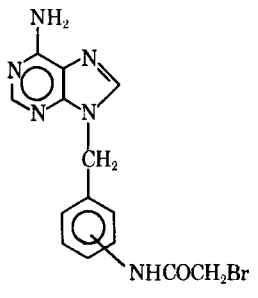
Keyphrases □ Adenosine deaminase inhibitors, irreversible—9-(substituted aralkyl)-6-substituted purines □ Purines, 9-(substituted aralkyl)-6-substituted—syntheses, adenosine deaminase irreversible inhibitors □ Enzymes, irreversible site-directed inhibitors—9-(substituted aralkyl)-6-substituted purines

Previous studies on the irreversible inactivation of adenosine deaminase established that the *para*- and *ortho*-isomers of 9-(bromoacetamidobenzyl)adenines (I and II) were excellent irreversible inhibitors, whereas the corresponding *meta*-isomer (III) was a poor irreversible inhibitor (1–3). In contrast to III, the *meta*-isomer of 9-(bromoacetamidophenethyl)adenine (IV) was capable of causing rapid, irreversible inactivation of adenosine deaminase (4). With a view toward further exploration of the hydrophobic region and the irreversible inhibition of adenosine deaminase, a series of 9-(*para*-substituted phenethyl)-6-substituted purines and 9-(*ortho*- and *para*-substituted phenylpropyl)-6-substituted purines was synthesized and evaluated.

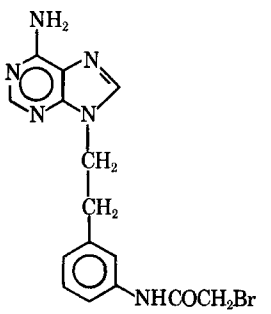
CHEMISTRY

The synthesis of these compounds was accomplished by a modification of procedures previously employed (1–6) (Schemes I and II).

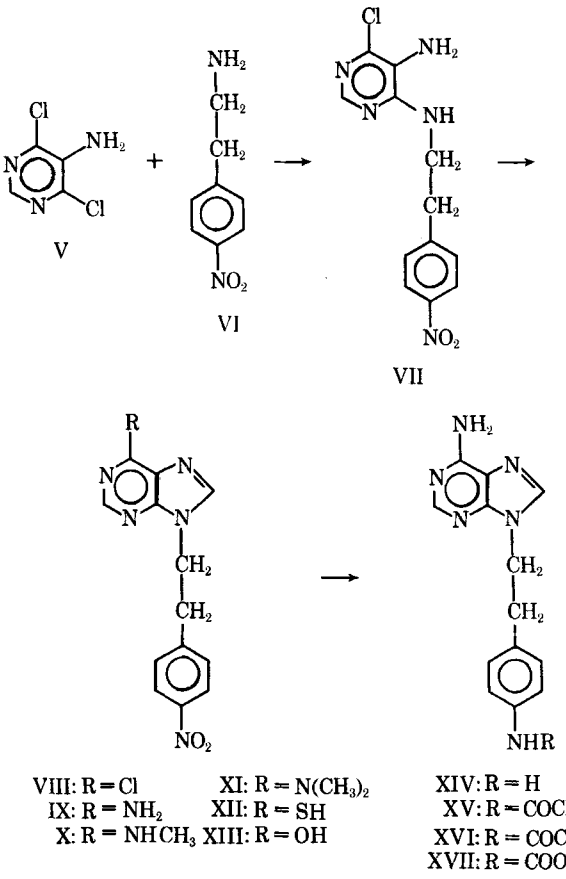
Condensation of 5-amino-4,6-dichloropyrimidine (V) with *p*-nitrophenethylamine (VI) gave the appropriately substituted pyrimidine (VII) which, upon treatment with triethyl orthoformate



I = *para*
II = *ortho*
III = *meta*



IV



VIII: R = Cl
IX: R = NH_2
X: R = $NHCH_3$
XI: R = $N(CH_3)_2$
XII: R = SH
XIII: R = OH
XIV: R = H
XV: R = $COCH_2Br$
XVI: R = $COCH_3$
XVII: R = $COOC_6H_5$

Scheme I

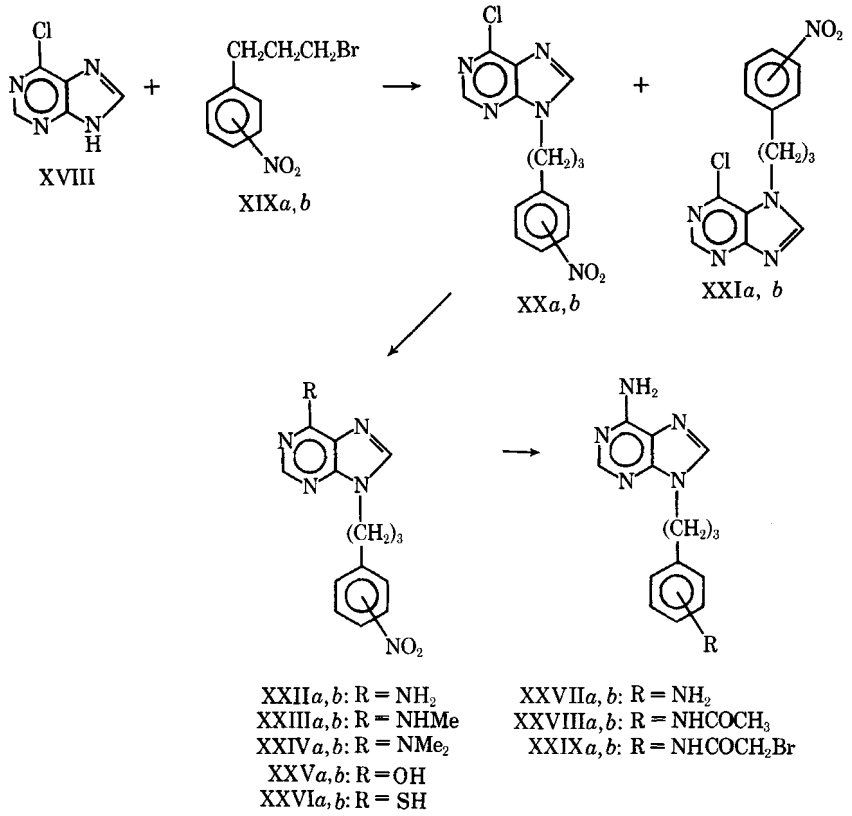
and ethanesulfonic acid, gave 9-(*p*-nitrophenethyl)-6-chloropurine (VIII). Treatment of the 6-chloro derivative (VIII) with ammonia, methylamine, dimethylamine, thiourea, and aqueous hydrochloric acid gave the corresponding 6-substituted derivatives (IX–XIII). Catalytic hydrogenation of IX gave 9-(*p*-aminophenethyl)adenine (XIV) which, upon reaction with bromoacetic anhydride, acetic anhydride, and phenyl chloroformate, gave the *para*-*N*-bromoacetamido, acetamido, and phenoxycarbonyl derivatives (XV, XVI, and XVII).

The 9-(*o*- and *p*-substituted-3-phenylpropyl)-6-substituted purines were prepared by condensation of the appropriately substituted 3-phenylpropyl bromide (XIX*a* and XIX*b*) with 6-chloropurine (XVIII). The resultant mixture of 9- and 7-isomers (XX and XXI) was separated, and XX was converted into XXII through XXVI by reaction with the appropriate nucleophile. Finally, the nitro group of XXII was reduced with hydrogen using a palladium catalyst, and the amino group was acetylated and bromoacetylated to give XXVIII and XXIX.

By a similar procedure, several 6-substituted-9-(3-phenylpropyl)-purines were prepared.

RESULTS AND DISCUSSION

When these compounds were tested as reversible inhibitors of adenosine deaminase, it was found that compounds with an amino group at the 6-position of the purine nucleus caused reversible



XVIII

XIX*a*, *b*

XX*a*, *b*

XXI*a*, *b*

XXII*a*, *b*: R = NH_2
XXIII*a*, *b*: R = NHMe
XXIV*a*, *b*: R = NMe_2
XXV*a*, *b*: R = OH
XXVI*a*, *b*: R = SH

XXVII*a*, *b*: R = NH_2
XXVIII*a*, *b*: R = $NHCOCH_3$
XXIX*a*, *b*: R = $NHCOCH_2Br$

a series = *ortho* substituted
b series = *para* substituted

Scheme II

inhibition of the enzyme (Table I), whereas compounds (VIII and X–XIII) with substituents at the 6-position other than an amino group were either noninhibitory or too weakly inhibitory to allow an accurate evaluation of $[I/S]_{0.5}$.

The lack of irreversible inhibition by the *p*-phenoxycarbonylamino derivative (XVII) is not surprising since irreversible inhibition with this type of compound had not been observed previously (1–4). However, even though 9-(*p*- and *o*-bromoacetamidobenzyl)adenines (I and II) and 9-(*m*-bromoacetamidophenethyl)adenine (IV) are capable of causing rapid irreversible inactivation of adenosine deaminase (1, 4), none of the structurally similar bromoacetamido derivatives (XV, XXIX*a*, and XXIX*b*) is capable of causing irreversible inhibition. Thus, the lack of irreversible enzyme inactivation by XV, XXIX*a*, and XXIX*b* represents another example of the selectivity in active-site-directed irreversible enzyme inhibitors (7).

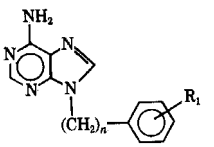
EXPERIMENTAL[1]

5-Amino-6-chloro-4-(*p*-nitrophenethylamino)pyrimidine (VII)—A solution of 405 mg. (2.00 mmoles) of *p*-nitrophenethylamine hydrochloride, 328 mg. (2.00 mmoles) of V, and 1.21 g. (12.0 mmoles) of triethylamine in 10 ml. of *n*-propyl alcohol was heated under reflux for 6 hr. The solvent was evaporated *in vacuo;* recrystallization of the residue from methanol and water gave the analytical sample; yield: 321 mg. (54.8%), m.p. 205–207°.

Anal.—Calc. for $C_{12}H_{12}ClN_5O_2$: C, 49.15; H, 4.12; Cl, 12.05; N, 23.80. Found: C, 49.22; H, 4.12; Cl, 12.21; N, 24.04.

6-Chloro-9-(*p*-nitrophenethyl)purine (VIII)—To a mixture of 150 mg. (0.510 mmole) of VII in 2 ml. of triethyl orthoformate w s added 13.3 mg. (0.121 mmole) of ethanesulfonic acid, and the mixture was stirred at 50° for 1.5 hr. The crystalline material which precipitated was collected by filtration; after recrystallization from

Table I—Inhibition of Adenosine Deaminase by:



Compound[a]	*n*	R_1	$[I/S]_{0.5}$[b]
IX	2	NO_2	6.9 ± 0.4[c]
XIV	2	NH_2	4.1 ± 0.1
XV	2	$NHCOCH_2Br$	1.9 ± 0.2
XVI	2	$NHCOCH_3$	2.5 ± 0.2
XVII	2	$NHCOOC_6H_5$	1.4 ± 0.1
XXX	2	H	5.0 ± 0.6
XXII*a*	3	NO_2	0.94 ± 0.06
XXVII*a*	3	NH_2	4.6 ± 0.2
XXIX*a*	3	$NHCOCH_2Br$	6.3 ± 1.3
XXII*b*	3	NO_2	5.3 ± 0.1
XXVII*b*	3	NH_2	1.4 ± 0.1
XXVIII*b*	3	$NHCOCH_3$	0.59 ± 0.1
XXIX*b*	3	$NHCOCH_2Br$	0.45 ± 0.02
XXXI	3	H	0.78 ± 0.02

[a] *a* series = *ortho*-substituted; *b* series = *para*-substituted. [b] The concentration of adenosine in all experiments was 0.066 m*M*. In no experiment of reversible inhibition did the concentration of inhibitor exceed 0.12 m*M*. The inhibition index $[I/S]_{0.5}$ is the ratio of the millimolar concentration of the inhibitor giving 50% inhibition to the millimolar concentration of the substrate. The inhibitor solutions were prepared in 0.05 *M* phosphate buffer (pH 7.6) containing 10% dimethyl sulfoxide. [c] Average of two determinations of the I_{50} obtained from a plot of V_0/V_i *versus* [I] with five different inhibitor concentrations. V_0 = initial velocity of the uninhibited enzymatic reaction, and V_i = initial velocity of the inhibited enzymatic reaction at various inhibitor concentrations.

[1] The melting points, unless noted otherwise, were taken in open capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples had IR and UV spectra compatible with their assigned structures and moved as a single spot on TLC on Brinkmann silica gel. The analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

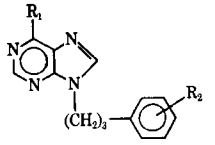


Table II—Physical Constants and Analytical Data of:

Compound	R_1	R_2	Method	Melting Point	Yield, %	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
XXII*a*	NH_2	NO_2	A	216–217°	76	MeOH	$C_{14}H_{14}N_6O_2$	C, 56.37 H, 4.73 N, 28.17	C, 56.19 H, 4.90 N, 28.21
XXIII*a*	NHMe	NO_2	B	159–160°	49	2-PrOH	$C_{15}H_{16}N_6O_2$	C, 57.68 H, 5.16 N, 26.91	C, 57.78 H, 5.24 N, 27.31
XXIV*a*	NMe_2	NO_2	C	119–120°	85	H_2O	$C_{16}H_{18}N_6O_2$	C, 58.87 H, 5.55 N, 25.74	C, 59.02 H, 5.55 N, 25.87
XXV*a*	OH	NO_2	E	203–204°	57	H_2O–EtOH	$C_{14}H_{13}N_5O_3$	C, 56.18 H, 4.38 N, 23.40	C, 56.25 H, 4.54 N, 23.56
XXVI*a*	SH	NO_2	D	269–270°	88	Precipitate	$C_{14}H_{13}N_5O_2S$	C, 53.28 H, 4.15 N, 22.21 S, 10.02	C, 53.27 H, 4.13 N, 22.12 S, 10.26
XXVII*a*	NH_2	NH_2	F	165–166°	69	H_2O	$C_{14}H_{16}N_6$	C, 62.67 H, 6.01 N, 31.32	C, 62.87 H, 5.88 N, 31.14
XXVIII*a*	NH_2	$NHCOCH_3$	H	231–232°	48	Tetrahydrofuran	$C_{16}H_{18}N_6O$	C, 61.93 H, 5.85 N, 27.08	C, 62.05 H, 5.77 N, 27.24
XXIX*a*	NH_2	$NHCOCH_2Br$	G	230°[a]	58	MeOH–H_2O	$C_{16}H_{17}BrN_6O$	C, 49.36 H, 4.40 N, 21.56 Br, 20.52	C, 49.12 H, 4.50 N, 21.39 Br, 20.42
XXII*b*	NH_2	NO_2	A	223–224°	78	MeOH	$C_{14}H_{14}N_6O_2$	C, 56.37 H, 4.73 N, 28.17	C, 56.19 H, 4.73 N, 28.37
XXIII*b*	NHMe	NO_2	B	175–176°	82	2-PrOH	$C_{15}H_{16}N_6O_2$	C, 57.68 H, 5.16 N, 26.91	C, 57.54 H, 5.42 N, 26.70
XXIV*b*	NMe_2	NO_2	C	168–169°	76	H_2O–MeOH	$C_{16}H_{18}N_6O_2$	C, 58.87 H, 5.55 N, 25.74	C, 58.89 H, 5.73 N, 25.59
XXV*b*	OH	NO_2	E	249–250°	72	H_2O–MeOH	$C_{14}H_{13}N_5O_3$	C, 56.18 H, 4.38 N, 23.40	C, 56.03 H, 4.45 N, 23.19
XXVI*b*	SH	NO_2	D	281–288°	91	Precipitate	$C_{14}H_{13}N_5O_2S$	C, 52.38 H, 4.15 N, 22.21 S, 10.02	C, 53.40 H, 4.16 N, 22.23 S, 9.96
XXVII*b*	NH_2	NH_2	F	235–236°	75	H_2O	$C_{14}H_{16}N_6$	C, 62.67 H, 6.01 N, 31.32	C, 62.71 H, 6.24 N, 31.22
XXVIII*b*	NH_2	$NHCOCH_3$	H	220–221°	67	H_2O[b]	$C_{16}H_{18}N_6O$	C, 61.93 H, 5.85 N, 27.08	C, 61.84 H, 5.65 N, 27.38
XXIX*b*	NH_2	$NHCOCH_2Br$	G	240–275°[a]	61	MeOH–H_2O	$C_{16}H_{17}BrN_6O$	C, 49.36 H, 4.40 N, 21.56 Br, 20.52	C, 49.32 H, 4.60 N, 21.48 Br, 20.44
XXXI[c]	NH_2	H	A	185–186°	65	MeOH	$C_{14}H_{15}N_5$	C, 66.40 H, 5.93 N, 27.67	C, 66.47 H, 6.08 N, 27.74
XXXIV	NHMe[d]	H	B	207–208°	54	2-PrOH	$C_{15}H_{18}ClN_5$	C, 59.29 H, 5.97 N, 23.05 Cl, 11.67	C, 59.21 H, 6.09 N, 22.88 Cl, 11.75
XXXV	NMe_2[d]	H	C	220–221°	81	2-PrOH	$C_{16}H_{20}ClN_5$	C, 60.46 H, 6.34 N, 22.04 Cl, 11.15	C, 60.30 H, 6.34 N, 22.04 Cl, 11.09
XXXVI	OH	H	E	256–257°	56	2-PrOH	$C_{14}H_{14}N_4O$	C, 66.12 H, 5.51 N, 22.05	C, 65.97 H, 5.48 N, 21.90
XXXVII	SH	H	D	318–319°	88	Precipitate	$C_{14}H_{14}N_4S$	C, 62.18 H, 5.21 N, 20.72 S, 11.86	C, 62.14 H, 5.19 N, 20.95 S, 11.96

[a] No well-defined melting point, decomposition temperature given. [b] Recrystallized from H_2O which was adjusted to pH ≃8 with sodium bicarbonate. [c] B. R. Baker and E. H. Erickson prepared XXXI by a different procedure and reported m.p. 184–186°[*J. Pharm. Sci.*, **56**, 1075(1967)]. [d] Isolated as the hydrochloride salt.

methanol and then from 2-propanol, it gave the analytical sample; yield: 98 mg. (63%), m.p. 167–168°.

Anal.—Calc. for $C_{13}H_{10}ClN_5O_2$: C, 51.41; H, 3.32; Cl, 11.67; N, 23.06. Found: C, 51.65; H, 3.51; Cl, 11.46; N, 22.89.

Method A: 9-(*p*-Nitrophenethyl)adenine (IX)—A mixture of 1.00 g. (3.29 mmoles) of VIII in 40 ml. of methanolic ammonia (20%) was heated at 95° in a stainless steel bomb for 24 hr. After the reaction mixture was cooled, the insoluble material was collected by filtration. Two recrystallizations of the crude material from methanol gave the analytical sample; yield: 615 mg. (56.9%), m.p. 260–261°.

Anal.—Calc. for $C_{13}H_{12}N_6O_2$: C, 54.92; H, 4.26; N, 29.57. Found: C, 55.16; H, 4.33; N, 29.58.

Method B: 6-Methylamino-9-*p*-nitrophenethylpurine (X)—A mixture of 304 mg. (1.00 mmole) of VIII in 10 ml. of ethanol and 10 ml. of aqueous methylamine (40%) was heated in a stainless steel bomb at 90° for 22 hr. The volatile material was evaporated *in vacuo*, and the crystalline residue was recrystallized from 2-propanol. Another recrystallization from methanol gave the analytical sample; yield: 153 mg. (51.4%), m.p. 220–222°.

Anal.—Calc. for $C_{14}H_{14}N_6O_2$: C, 56.37; H, 4.73; N, 28.18. Found: C, 56.11; H, 4.80; N, 28.45.

Method C: 6-Dimethylamino-9-*p*-nitrophenethylpurine (XI)—A mixture of 304 mg. (1.00 mmole) of VIII in 10 ml. of ethanol and 10 ml. of aqueous dimethylamine (25%) was heated in a stainless steel bomb at 90° for 22 hr. The volatile material was evaporated *in vacuo*, leaving a yellow crystalline material which was recrystallized with 2-propanol. Another recrystallization from methanol gave the analytical sample; yield: 215 mg. (69.0%), m.p. 169–170°.

Anal.—Calc. for $C_{15}H_{16}N_6O_2$: C, 57.68; H, 5.16; N, 26.92. Found: C, 57.42; H, 5.19; N, 26.76.

Method D: 6-Mercapto-9-*p*-nitrophenethylpurine (XII)—A solution of 304 mg. (1.00 mmole) of VIII and 84 mg. (1.10 mmoles) of thiourea in 15 ml. of *n*-propyl alcohol was heated under reflux for 1 hr. and then cooled in an ice bath. The precipitate which formed was collected by filtration. Recrystallization of the solid material from methanol gave the analytical sample; yield: 179 mg. (59.7%), m.p. 300–301° dec.

Anal.—Calc. for $C_{13}H_{11}N_5O_2S$: C, 51.81; H, 3.68; N, 23.25; S, 10.64. Found: C, 51.69; H, 3.80; N, 23.06; S, 10.65.

Method E: 6-Hydroxy-9-*p*-nitrophenethylpurine (XIII)—A solution of 304 mg. (1.00 mmole) of VIII in 25 ml. of 3 *N* hydrochloric acid was heated under reflux for 1.5 hr. and then evaporated *in vacuo*. Recrystallization of the solid residue from methanol gave the analytical sample; yield: 200 mg. (70.3%), m.p. 294–295° dec.

Anal.—Calc. for $C_{13}H_{11}N_5O_3$: C, 54.73; H, 3.89; N, 24.55. Found: C, 54.62; H, 3.96; N, 24.51.

Method F: 9-(*p*-Aminophenethyl)adenine (XIV)—A solution of 988 mg. (3.48 mmoles) of IX in 150 ml. of glacial acetic acid was added to 400 mg. of 5% palladium on carbon, and the mixture was hydrogenated at room temperature in a Parr hydrogenator at an initial pressure of 4 kg./cm.[2]. After 1 hr., the catalyst was removed by filtration through a diatomaceous earth[2] pad, and the solvent was concentrated to 50 ml. *in vacuo*. The solution was cooled in an ice bath, and the pH was adjusted to 13 with 1 *N* sodium hydroxide. The insoluble material was collected by filtration; yield: 714 mg. (80.8%), m.p. 219–221°. The crude product recrystallized from water; yield: 548 mg. (62.1%), m.p. 221.5–223°.

Anal.—Calc. for $C_{13}H_{14}N_6$: C, 61.39; H, 5.55; N, 33.05. Found: C, 61.28; H, 5.56; N, 32.83.

Method G: 9-(*p*-Bromoacetamidophenethyl)adenine (XV)—To a cold solution of 153 mg. (0.60 mmole) of XIV in 5 ml. of tetrahydrofuran and 0.6 ml. of 10% aqueous acetic acid was added dropwise 271 mg. (1.05 mmoles) of bromoacetic anhydride in 1 ml. of tetrahydrofuran. After 1 hr. of stirring at 0°, the insoluble material was collected by filtration and dissolved in hot methanol to which sodium bicarbonate solution (2%) was added until the solution was basic. Upon cooling in an ice bath, a precipitate formed which, after recrystallization from methanol, gave the analytical sample as the hemisolvate; yield: 44 mg. (20%), m.p. 230–240° dec. with evolution of gas.

Anal.—Calc. for $C_{15}H_{15}BrN_6O \cdot 1/2CH_4O$: C, 47.58; H, 4.38; Br, 20.43; N, 21.48. Found: C, 47.72; H, 4.25; Br, 19.99; N, 21.14.

[2] Celite.

Method H: 9-(*p*-Acetamidophenethyl)adenine (XVI)—To a cold solution of 152 mg. (0.60 mmole) of XIV in 0.6 ml. of 10% aqueous acetic acid and 5 ml. of tetrahydrofuran was added dropwise 602 mg. (5.90 mmoles) of acetic anhydride in 4 ml. of tetrahydrofuran. The mixture was stirred in an ice bath for 1 hr. and the insoluble material which precipitated was collected by filtration and dried *in vacuo* at 100°; yield: 105 mg. (59.4%), m.p. 237–238°. Recrystallization of the crude product from chloroform gave the analytical sample; yield: 73.4 mg. (41.4%), m.p. 239–240°.

Anal.—Calc. for $C_{15}H_{16}N_6O$: C, 60.79; H, 5.44; N, 28.26. Found: C, 60.65; H, 5.39; N, 28.19.

9-(*p*-Phenoxycarbonylaminophenethyl)adenine (XVII)—To a cold solution of 152 mg. (0.60 mmole) of XIV and 60.0 mg. (0.60 mmole) of triethylamine in 150 ml. of *p*-dioxane was added slowly 366 mg. (2.35 mmoles) of phenyl chloroformate, and the mixture was stirred at room temperature for 26 hr. The insoluble material was collected by filtration and dried *in vacuo* at 100° for 48 hr. Recrystallization of the residual solid from methanol and water gave the analytical sample; yield: 99.5 mg. (44.4%), m.p. 163° softened with decomposition, solidified, melted 310–312° with decomposition and evolution of gas.

Anal.—Calc. for $C_{20}H_{18}N_6O_2$: C, 64.20; H, 4.85; N, 22.44. Found: C, 63.92; H, 4.96; N, 22.32.

Method I: 6-Chloro-9- and 7-*p*-nitrophenylpropylpurines[3] (XX*b* and XXI*b*)—6-Chloropurine (2.90 g., 0.019 mole) was dissolved in dimethylformamide (50 ml.), and 3-*p*-nitrophenylpropyl bromide (6.1 g., 0.025 mole) and triethylamine (2.52 g., 0.025 mole) were added to the solution. The yellow reaction mixture was left at room temperature for 24 hr. and then poured into a cooled mixture of 2-propanol–water (1:2) (300 ml.) and stirred vigorously. The insoluble white material was filtered off (4.7 g., 84%). This material was dissolved in chloroform (50 ml.) and introduced on a column (29.5 mm. i.d.) of silica gel (140 g. of 100–200 mesh) in chloroform. The column was eluted with chloroform, and 60–125-ml. fractions were collected. Then a 1.5% solution of methanol in $CHCl_3$ was used to elute the column, and 11–125-ml. fractions were collected. Eluent fractions 18–67 were combined and evaporated *in vacuo*, which gave 3.2 g. (57%) of XX*b*. Recrystallization from 2-propanol gave pure XX*b* (2.57 g., 50%), m.p. 138–139°.

Anal.—Calc. for $C_{14}H_{12}ClN_5O_2$: C, 52.92; H, 3.80; Cl, 11.16; N, 22.04. Found: C, 52.90; H, 3.82; Cl, 10.99; N, 21.91.

Eluent fractions 69–71 were combined and evaporated *in vacuo*, which gave the crude 7-isomer. This material was recrystallized from methanol and gave 70 mg. (1.5%) of pure XXI*b*, m.p. 152–153°.

Anal.—Calc. for $C_{14}H_{12}ClN_5O_2$: C, 52.92; H, 3.80; Cl, 11.16; N, 22.04. Found: C, 53.02; H, 3.90; Cl, 11.00; N, 21.97.

6-Chloro-9- and 7-(3-*o*-nitrophenylpropyl)purines (XX*a* and XXI*a*)—These were prepared from 6-chloropurine and 3-*o*-nitrophenylpropyl bromide by Method I; yield of XX*a*: 21%, m.p. 141–142°/2-propanol.

Anal.—Calc. for $C_{14}H_{12}ClN_5O_2$: C, 52.92; H, 3.80; Cl, 11.16; N, 22.04. Found: C, 53.05; H, 4.04; Cl, 11.04; N, 21.94.

Yield of XXI*a*: 11%, m.p. 142–143°/2-propanol.

Anal.—Found: C, 53.06; H, 3.69; Cl, 11.40; N, 22.11.

6-Chloro-9- and 7-(3-phenylpropyl)purines (XXXII and XXXIII)—These were prepared by Method I from 6-chloropurine and 3-phenylpropyl bromide; yield of XXXII: 49%, m.p. 62–63°/hexane.

Anal.—Calc. for $C_{14}H_{13}ClN_4$: C, 61.65; H, 4.80; Cl, 13.00; N, 20.55. Found: C, 61.70; H, 4.95; Cl, 12.91; N, 20.41.

Yield of XXXIII: 4%, m.p. 68–69°/hexane–methanol. Found: C, 61.80; H, 4.93; Cl, 13.17; N, 20.75.

ENZYME ASSAY

Adenosine deaminase (Type I, calf intestinal mucosa) was purchased[4]. The assay procedure for the study of reversible inhibitors was previously described (3) and is a modification of the procedure of Kaplan (8) based on the method of Kalckar (9).

[3] The structures of the isomers were assigned by analogy to similar alkylations. See, for example, *Reference 2*.

[4] Sigma Chemical Co.

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(1) H. J. Schaeffer and E. Odin, *J. Med. Chem.*, **9**, 576(1966).
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* Present address: Wellcome Research Laboratories, Burroughs Wellcome & Co., Research Triangle Park, NC 27709

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JOHN H. BILLMAN and JOHN A. TONNIS*

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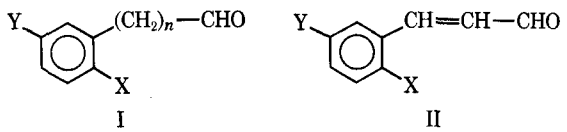
Keyphrases □ Antitumor activity evaluation—substituted aralkyl aldehydes □ Phenylacetaldehydes, substituted—synthesis, antitumor activity evaluation □ Hydrocinnamaldehydes, substituted—synthesis, antitumor activity evaluation □ Cinnamaldehydes, substituted—synthesis, antitumor activity evaluation

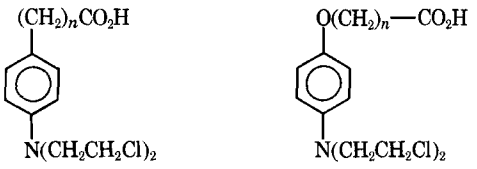
A considerable number of aliphatic and aromatic aldehydes and their derivatives were shown to possess appreciable antitumor activity (1–6). Several of these aldehydes and their derivatives were also used in the clinic as antitumor agents (7–9). All of the aldehydes that have shown activity have been alkyl or aromatic aldehydes. No substituted aralkyl aldehydes of general types I and II have been tested as antitumor agents and, indeed, few have even been synthesized.



I II

n = 1–2
X = OH, OCH_3, $-OCOCH_3$
Y = electron-donating or withdrawing groups

Various studies showed that the length of the alkyl chain in aralkyl compounds similar to I and II plays an important role in the antitumor activity. Some



III IV

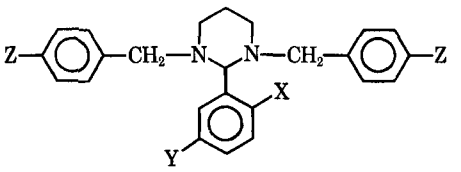
n = 0–3

investigators (10–12) prepared a series of *N*,*N*-bis(2-chloroethyl)phenylalkanoic acids (III) and *N*,*N*-bis-(2-chloroethyl)phenoxyalkanoic acids (IV).

In type III compounds, they found that when n is 0, the compound was only slightly active; as n increased the activity likewise increased rapidly and reached a maximum at n = 3. The compound of Structure III, when n is 3, is called chlorambucil and has been used clinically for treatment of chronic lymphocytic leukemia (13, 14).

Type IV compounds also show the same increase of activity as the alkyl chain length increases and reaches a maximum when n is 2.

This relationship of antitumor activity to alkyl chain length has been demonstrated on other compounds similar to type I and II compounds.



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X = OH, OCH_3
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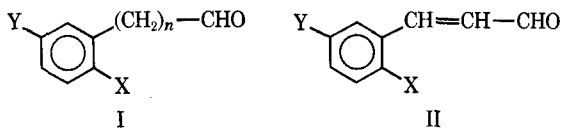

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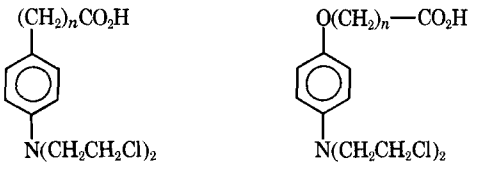

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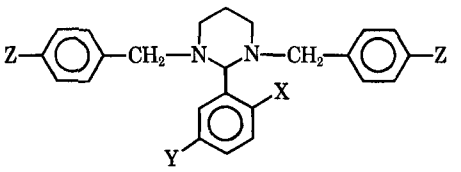

V

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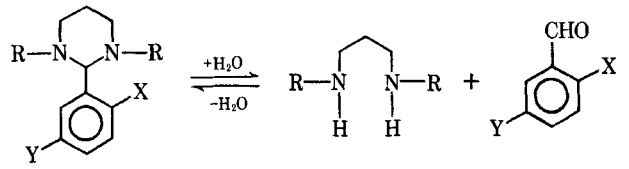
Z = OCH_3, $-N(CH_3)_2$, $-N(CH_2CH_2Cl)_2$



Scheme I

This variance of the activity with the length of the alkyl side chain may also be operative in aralkyl aldehydes of general types I and II. If so, the antitumor activity of aldehydes of types I and II should be greater than that of the corresponding substituted benzaldehydes.

It was previously found that many hexahydropyrimidines (V) show extremely high antitumor activity (15). The hexahydropyrimidines can be looked upon as aldehyde derivatives which are extremely susceptible to hydrolysis, releasing the free aldehyde according to Scheme I.

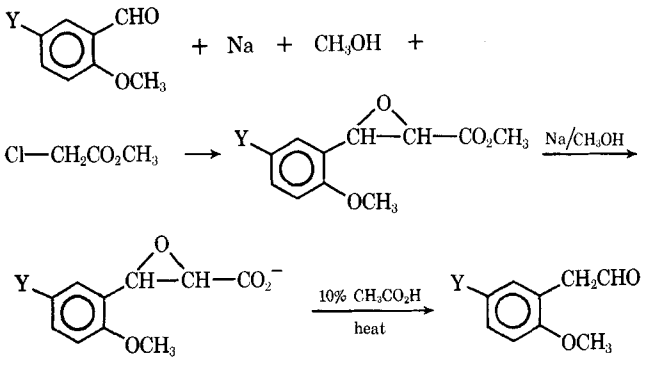
The hexahydropyrimidine may be serving as a carrier for the aldehyde which, upon hydrolysis, liberates the free aldehyde at the tumor site. Thus, part of the high activity may be due to the liberation of the free aldehyde at the slightly acidic tumor site.

The aralkyl aldehydes of type I, when *n* is 1 (substituted phenylacetaldehydes), were prepared from the corresponding substituted benzaldehydes using Darzen's glycidic ester synthesis, followed by hydrolysis of the ester and decarboxylation of the acid intermediate (Scheme II).

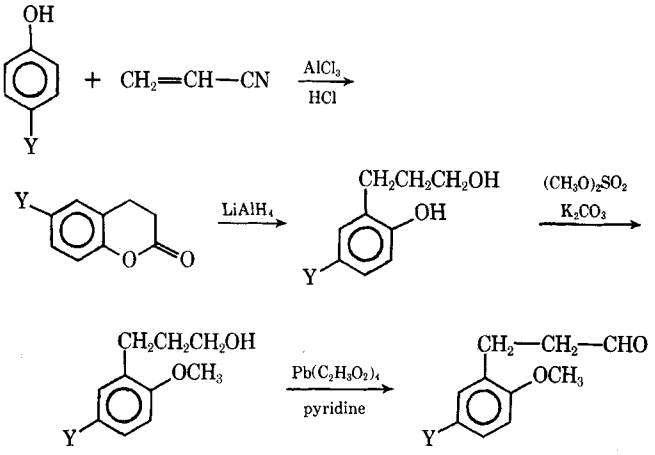
The decarboxylation of the intermediate glycidic acid gave a higher yield of the aldehyde when an equal molar quantity of 10% aqueous acetic acid was used than when using an equal molar quantity of glacial acetic acid.

The yield of the aldehyde was very dependent on the nature of the substituent Y. If Y was an electron-donating group, a good yield of the aldehyde was obtained. When Y was a strong electron-withdrawing group (—NO_2) or two chloro groups such as (3,5-dichloro-), the glycidic acid would not decarboxylate even at elevated temperatures.

Aldehydes of type I, where *n* is 2 (dihydrocinnamaldehydes), were prepared from the corresponding *p*-substituted phenols according to Scheme III. The 6-substituted dihydrocoumarins were prepared according to the procedure of Sato *et al.* (16). The alcohols were oxidized to the aldehydes in generally good yields,



Scheme II



Scheme III

using a procedure similar to the one of Partch (17). The 5-acetyl-2-methoxyhydrocinnamyl alcohol could not be oxidized to the hydrocinnamaldehyde using this reagent.

The substituted cinnamaldehydes of type II were prepared from the corresponding substituted benzaldehydes according to Scheme IV (18).

The preparation of cinnamaldehydes of type II, which contain the *o*-phenolic group, is somewhat complicated by the presence of the phenol group. The phenol group must be protected as the acetate before reaction with the ethyl vinyl ether. The acetate-protecting group can then be removed by anhydrous Na/CH_3OH hydrolysis.

BIOLOGICAL DATA

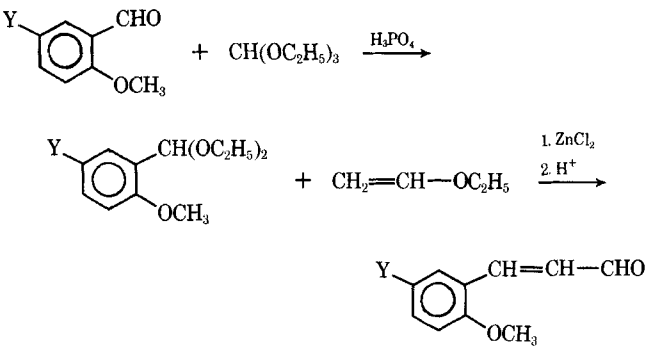
These compounds were screened primarily against the tumor system leukemia L-1210[1], for which they did not show significant antitumor activity.

EXPERIMENTAL[2]

Methyl-3-(3,5-disubstituted-2-methoxyphenyl)glycidates—These were prepared according to the procedure of Henecka (19) (Table I).

5-Substituted-2-methoxyphenylacetaldehydes—These were prepared according to the procedure of Ban and Oishi (20) (Table II).

6-Nitrodihydrocoumarin—This was prepared according to the procedure of Ingle and Bhide (21).



Scheme IV

[1] By the Cancer Chemotherapy National Service Center, National Institutes of Health.

[2] The starting 5-substituted salicylaldehydes were purchased from either Aldrich Chemical Co. or Eastman Organic Chemicals. Melting points, obtained on a Thomas-Hoover apparatus, are uncorrected. Microanalyses were carried out by Midwest Microlab, Indianapolis, Ind., and Alfred Bernhardt, Muhlheim, Germany.

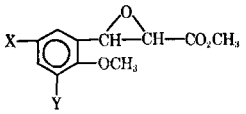


Table I—Methyl-3-(3,5-disubstituted-2-methoxyphenyl)glycidates

X	Y	Yield, %	Melting or Boiling Point	Formula	Analysis, % Calc.	Analysis, % Found	IR, μ(C=O)
H	H	79	49–50.5°	$C_{11}H_{12}O_4$	C, 63.45 H, 5.77	C, 63.94 H, 5.92	5.74
Cl	H	70	58–59.5°	$C_{11}H_{11}ClO_4$	C, 54.44 H, 4.57	C, 54.69 H, 4.71	5.73
Br	H	66	68–69.5°	$C_{11}H_{11}BrO_4$	C, 46.01 H, 3.86	C, 46.28 H, 4.12	5.74
—OCH_3	H	70	161–163°/0.3 mm.	$C_{12}H_{14}O_5$	C, 60.50 H, 5.92	C, 60.11 H, 6.27	5.78
NO_2	H	37	144–145°	$C_{11}H_{11}NO_6$	C, 52.17 H, 4.38	C, 52.23 H, 4.37	5.82
Cl	Cl	69	84–85°	$C_{11}H_{10}Cl_2O_4$	C, 47.68 H, 3.64	C, 47.96 H, 3.93	5.77

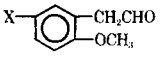


Table II—5-Substituted-2-methoxyphenylacetaldehydes

X	Yield, %	Boiling Point	Formula	Analysis, % Calc.	Analysis, % Found	IR, μ(C=O)
H	66	65–67°/0.3 mm.	$C_9H_{10}O_2$	C, 71.98 H, 6.71	C, 71.98 H, 6.72	5.82
Cl	45	115–117°/0.6 mm.	$C_9H_9ClO_2$	C, 58.55 H, 4.91	C, 58.63 H, 5.04	5.80
Br	47	124–126°/0.5 mm.	$C_9H_9BrO_2$	C, 47.18 H, 3.96	C, 47.12 H, 3.95	5.81
—OCH_3	22	112–113°/0.7 mm.	$C_{10}H_{12}O_3$	C, 66.65 H, 6.72	C, 66.62 H, 6.71	5.82

6-Bromodihydrocoumarin—This was prepared according to the procedure of Barnes *et al.* (22).

6-Substituted Dihydrocoumarins—The other 6-substituted dihydrocoumarins were prepared according to the procedure of Sato *et al.* (16).

5-Substituted-2-hydroxyhydrocinnamyl Alcohols—By use of a continuous extraction apparatus, 36.3 g. (0.18 mole) of 6-bromodihydrocoumarin was added to a refluxing suspension of 6.70 g. (0.18 mole) of lithium aluminum hydride in 400 ml. of diethyl ether. After complete addition (6 hr.), the mixture was refluxed an additional 1 hr. and the excess reducing agent was destroyed by the addition of water. The resulting mixture was poured into 300 ml. of cold 4 *N* sulfuric acid. The ether layer was separated, and the aqueous layer was extracted with ether. The ether extracts were combined and dried over anhydrous sodium sulfate, and the ether was removed *in vacuo*. The resulting white solid was recrystallized from benzene, yielding a white solid (Table III).

5-Substituted-2-methoxyhydrocinnamyl Alcohols—To a warm (80°) solution of 5-bromo-2-hydroxyhydrocinnamyl alcohol (23.1 g., 0.1 mole), dissolved in 80 ml. of 10% NaOH solution, was added dropwise 25.2 g. (0.2 mole) of dimethyl sulfate. After complete addition, the reaction mixture was heated at 90° for 3 hr., then rendered basic by the addition of 50 ml. of 10% NaOH solution, and then heated at 90° for an additional 30 min. The mixture was extracted with diethyl ether, and the ether extracts were dried over sodium sulfate. Removal of the ether *in vacuo* gave a light-yellow oil, which was distilled under reduced pressure. The pure product was obtained as a clear oil (Table IV).

5-Substituted-2-methoxyphenylhydrocinnamaldehydes—To a cooled (5°) solution of 12.3 g. (0.05 mole) of 5-bromo-2-methoxyhydrocinnamyl alcohol dissolved in 150 ml. of anhydrous pyridine was added 22.2 g. (0.05 mole) of lead tetraacetate. The resulting dark-red mixture was stirred at 5° for 4 hr. and then at 25° for 20 hr. The pyridine was removed *in vacuo*. The resulting brown residue was extracted with three 150-ml. portions of diethyl ether. The ether extracts were dried over anhydrous Na_2SO_4, and the ether was removed *in vacuo*. The resulting brown liquid was fractionally distilled under reduced pressure, and the fraction boiling at 124°/0.5 mm. was collected. The aldehyde was obtained as a clear liquid (Table V).

5-Acetyl-2-methoxyhydrocinnamyl Alcohol—A mixture of 2-methoxyhydrocinnamyl alcohol (16.6 g., 0.1 mole), acetic anhydride (20.4 g., 0.20 mole), and $ZnCl_2$ (0.1 g.) was heated at gentle reflux for 6 hr. After cooling, the reaction mixture was poured into 400 ml. of cold water. The oil, which separated, was extracted with four 150-ml. portions of diethyl ether. The ether extracts were washed with saturated $NaHCO_3$ solution and then with water. The ether extracts were dried over anhydrous sodium sulfate, and the ether was re-

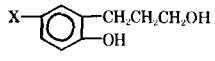
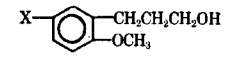


Table III—5-Substituted-2-hydroxyhydrocinnamyl Alcohols

X	Yield, %	Melting or Boiling Point	Formula	Analysis, % Calc.	Analysis, % Found
H	95	177–179°/12 mm.	$C_9H_{12}O_2$[a]		
Cl	94	79–80°	$C_9H_{11}ClO_2$	C, 57.91 H, 5.95	C, 58.09 H, 5.94
Br	88	86–87°	$C_9H_{11}BrO_2$	C, 46.77 H, 4.76	C, 46.96 H, 5.05
CH_3	92	145–147°/1 mm.	$C_{10}H_{14}O_2$[b]		
OCH_3	94	63–65°	$C_{10}H_{14}O_3$	C, 65.91 H, 7.74	C, 65.96 H, 7.67

[a] *J. Chem. Soc.*, **1939**, 787. [b] *J. Amer. Chem. Soc.*, **62**, 3067(1940).

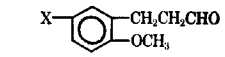
Table IV—5-Substituted-2-methoxyhydrocinnamyl Alcohols



X	Yield, %	Melting or Boiling Point	Formula	Analysis, % Calc.	Found
H	86	84–87°/0.2 mm.	$C_{10}H_{14}O_2$[a]		
Cl	91	108–111°/0.2 mm.	$C_{10}H_{13}ClO_2$	C, 59.85 H, 6.53	C, 59.49 H, 6.62
Br	85	124–127°/0.4 mm.	$C_{10}H_{13}BrO_2$	C, 49.00 H, 5.34	C, 49.39 H, 5.40
$-CH_3$	84	96–99°/0.4 mm.	$C_{11}H_{16}O_2$	C, 73.28 H, 8.98	C, 73.58 H, 8.86
$-OCH_3$	82	105–108/0.1 mm.	$C_{11}H_{16}O_3$	C, 67.32 H, 8.22	C, 67.54 H, 8.02
$-COCH_3$	88	46.5–48°	$C_{12}H_{16}O_3$	C, 69.27 H, 7.75	C, 69.40 H, 7.57

[a] *J. Chem. Soc.*, **1939**, 787.

Table V—5-Substituted-2-methoxyphenylhydrocinnamaldehydes



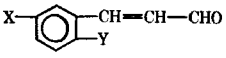
X	Yield, %	Boiling Point	Formula	Analysis, % Calc.	Found	IR, μ(C=O)
H	57	77–80°/0.4 mm.	$C_{10}H_{12}O_2$	C, 67.32 H, 8.22	C, 67.54 H, 8.02	5.83
Cl	42	115–116°/0.5 mm.	$C_{10}H_{11}ClO_2$	C, 60.46 H, 5.58	C, 60.65 H, 5.72	5.81
Br	54	120–124°/0.5 mm.	$C_{10}H_{11}BrO_2$	C, 49.40 H, 4.56	C, 49.71 H, 4.89	5.80
$COCH_3$	31		$C_{12}H_{14}O_3$	C, 69.88 H, 6.84	C, 69.72 H, 7.01	5.82 (aldehyde) 5.98 (ketone)
$-CH_3$	52	108–111°/0.2 mm.	$C_{11}H_{15}O_2$	C, 74.13 H, 7.92	C, 73.98 H, 8.00	5.82
$-OCH_3$	28	114–115°/0.2 mm.	$C_{11}H_{15}O_3$	C, 68.02 H, 7.26	C, 68.28 H, 7.25	5.82

moved *in vacuo*. The resulting brown oil was distilled *in vacuo*, giving the 5-acetyl-2-methoxyhydrocinnamyl acetate as a clear oil. The acetate ester and 100 ml. of 10% NaOH were heated at gentle reflux for 5 hr. The mixture was extracted with diethyl ether, and the ether extracts were dried over anhydrous sodium sulfate. Removal of the ether gave an oil which was distilled under reduced pressure. The product was obtained as a clear oil which crystallized to a white solid.

5-Substituted-2-methoxycinnamaldehydes—A mixture of 5-bromo-2-methoxybenzaldehyde (18.0 g., 0.084 mole), triethyl orthoformate (13.6 g., 0.092 mole), and 0.2 ml. of 85% phosphoric acid was stirred at 25° under a nitrogen atmosphere for 24 hr. The resulting diethyl acetal was cooled to 0°, and 2.2 ml. of 10% $ZnCl_2$–ethyl acetate solution was added. Freshly distilled ethyl vinyl ether (12.2 g., 0.17 mole) was then added dropwise at a rate to maintain the temperature below 0°. After complete addition, the solution was stirred at room temperature for 12 hr. A solution of 83 ml. of glacial acetic acid, 8.7 g. of sodium acetate, and 7.7 ml. of water was added, and the solution was heated at 95° for 2 hr. The solution was then poured into 700 ml. of cold water where a brown solid formed. Recrystallization of the solid from a 1:1 benzene–petroleum ether mixture gave the product as a light-yellow solid (Table VI).

5-Substituted-2-acetoxycinnamaldehydes—To a cooled solution (0°) of 5-methoxysalicylaldehyde (38.0 g., 0.25 mole) in 100 ml. of

Table VI—2,5-Disubstituted Cinnamaldehydes



Y	X	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found	IR, μ(C=O)
OCH_3	H	75	46–47°	$C_{10}H_{10}O_2$[a]			5.99
OCH_3	Cl	65	78–79.5°	$C_{10}H_9ClO_2$	C, 61.08 H, 4.61	C, 60.93 H, 4.62	6.02
OCH_3	Br	54	89–90.5°	$C_{10}H_9BrO_2$	C, 49.81 H, 3.73	C, 50.07 H, 3.87	5.97
OCH_3	NO_2	40	143–144°	$C_{10}H_9NO_4$	C, 57.97 H, 4.38	C, 58.17 H, 4.61	5.98
OCH_3	OCH_3	59	86–87°	$C_{11}H_{12}O_3$	C, 68.45 H, 6.69	C, 68.38 H, 6.40	6.00
$OCOCH_3$	H	63	81–82°	$C_{11}H_{10}O_3$	C, 69.41 H, 5.30	C, 69.60 H, 5.07	5.76 (ester) 6.01 (aldehyde)
$OCOCH_3$	Cl	47	112–113.5°	$C_{11}H_9ClO_3$	C, 58.81 H, 4.04	C, 58.65 H, 4.14	5.71 (ester) 5.98 (aldehyde)
$OCOCH_3$	OCH_3	63	70.5–71.5°	$C_{12}H_{12}O_4$	C, 65.44 H, 5.45	C, 65.34 H, 5.46	5.71 (ester) 5.96 (aldehyde)
OH	H	88	132–133°	$C_9H_8O_2$[a]			6.02
OH	Cl	87	155–156°	$C_9H_7ClO_2$	C, 59.20 H, 3.87	C, 59.48 H, 3.88	6.05
OH	OCH_3	85	124–125°	$C_{10}H_{10}O_3$	C, 67.96 H, 5.66	C, 68.13 H, 5.89	6.03

[a] *Ber.*, **56**, 606(1923).

pyridine was added slowly 25.5 g. (0.25 mole) of acetic anhydride. The solution was stirred at 0° for 5 hr. and diluted with 1.0 l. of cold water. The organic layer was separated, and the aqueous layer was extracted with diethyl ether. The organic layer and ether extracts were combined and dried over anhydrous Na_2SO_4, and the ether was removed *in vacuo*. The resulting yellow oil was distilled *in vacuo*, and the collected fraction distilled at 113–116°/0.2 mm. The 2-acetoxy-5-methoxybenzaldehyde was collected as a white solid (TableVI).

2-Acetoxy-5-substituted Cinnamaldehydes—The procedure described for 5-substituted-2-methoxycinnamaldehydes was followed.

5-Substituted-2-hydroxycinnamaldehydes—To a cooled (10°) solution of 2-acetoxy-5-methoxycinnamaldehyde (6.6 g., 0.03 mole) in 60 ml. of $CHCl_3$ was added a solution of Na (0.69 g., 0.03 mole) in 25 ml. of CH_3OH. After complete addition (30 min.), the solution was stirred at 10° for 15 min. and then at room temperature for 1 hr. The reaction mixture was diluted with 100 ml. of water, and the $CHCl_3$ layer was separated. The aqueous layer was rendered acidic by the addition of dilute H_2SO_4. The solid that formed was collected and washed thoroughly with water. Recrystallization from benzene gave the product as a bright-yellow solid (Table VI).

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Triazenes of Phenylbutyric, Hydrocinnamic, Phenoxyacetic, and Benzoylglutamic Acid Derivatives

Y. FULMER SHEALY, CHARLES A. KRAUTH, CLYDE E. OPLIGER, H. WAYNE GUIN, and W. RUSSELL LASTER, Jr.

Abstract □ *p*-Dialkyltriazeno derivatives of the ethyl esters and acid hydrazides of phenylbutyric, hydrocinnamic, phenoxyacetic, and benzoylglutamic acids were synthesized. The triazenophenylbutyric acid derivatives are structurally related to chlorambucil, and the hydrocinnamic and phenoxyacetic acid derivatives are related to other antineoplastic aromatic nitrogen mustards. All of the triazeno groups contained unsubstituted alkyl groups (except for a hydroxyethyl group in a derivative of benzoylglutamic acid). In initial tests of these compounds *versus* mouse lymphatic leukemia L-1210, ethyl *p*-(3-butyl-3-methyl-1-triazeno)hydrocinnamate (VI*b*) was the most effective compound in increasing the survival time of treated animals. Certain other hydrocinnamic and phenylbutyric acid derivatives caused small increases in survival time.

Keyphrases □ Triazenes of phenylbutyric, hydrocinnamic, phenoxyacetic, and benzoylglutamic acid esters and hydrazides—synthesis, antileukemic activity □ Antileukemic activity—*p*-dialkyltriazene derivatives

After antineoplastic activity was found among triazenoimidazoles (*e.g.*, 1, 2), it seemed reasonable to suppose that combining substituted triazeno groups with structural moieties that can presumably serve as carrier groups might also produce derivatives having antineoplastic activity. Studies of aromatic nitrogen mustards included a series of phenylalkanoic acid derivatives. Of the initial series, chlorambucil, 4-{*p*-[bis(2-chloroethyl)-amino]phenyl}butyric acid, was the most effective derivative in inhibiting the transplanted Walker rat carcinoma (3, 4) and proved to be a clinically useful agent (*e.g.*, 5, 6). The enhanced activity of chlorambucil was attributed (3, 4) to the constitution of its phenylbutyric acid moiety rather than to its chemical reactivity or physical properties, and the phenylbutyric acid portion was used as a carrier for other cytotoxic groups (7).

Nitrogen mustard derivatives of hydrocinnamic acid (3) and of phenoxyalkanoic acids (8, 9)—notably the phenoxypropionic acid derivative—were also active antineoplastic agents, and activity was retained in certain ester derivatives (3, 9). The ethyl esters of phenylbutyric acid, hydrocinnamic acid, and phenoxyacetic acid were chosen, therefore, for attachment of triazeno

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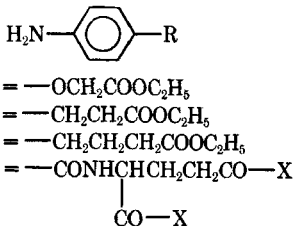

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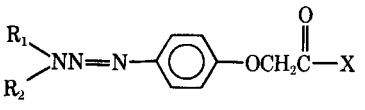
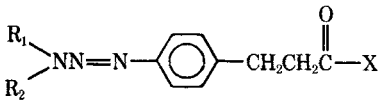
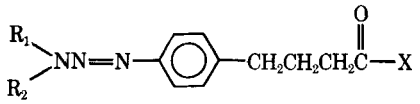
II: R = —$CH_2CH_2COOC_2H_5$
III: R = —$CH_2CH_2CH_2COOC_2H_5$
IV: R = —$CONHCHCH_2CH_2CO—X$ (CO—X)



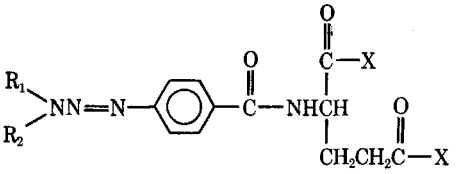
X = —OC_2H_5 or —$NHNH_2$
V*a-g*



X = —OC_2H_5 or —$NHNH_2$
VI*a-g*



X = —OC_2H_5 or —$NHNH_2$
VII*a-h*



X = —OC_2H_5, —COOH, or —$NHNH_2$
VIII*a-h*

groups at the *para*-position. Diethyl *p*-aminobenzoylglutamate, the ester of a folic acid moiety, was also chosen for conversion to triazeno derivatives[1].

Representative triazeno derivatives (V–VIII, Table I) of the four aromatic moieties were prepared by diazotizing an aromatic amine (I–IV) and then adding an aliphatic amine in sufficient quantity to raise the pH to 9–10 or above. Certain liquid triazenes were sufficiently volatile and stable to be distilled; the other triazenes were purified by other conventional methods. Compound VIII*f*, the only triazeno derivative of an acid, precipitated from a slightly acidic solution as the mono(dibutylammonium) salt. The acid hydrazides (V*f–g*, VI*f–g*, VII*g–h*, and VIII*g–h*) were obtained by reactions of hydrazine with the dimethyltriazeno or butylmethyltriazeno derivatives of the ethyl esters.

All of the triazenes except V*c* and VIII*h* were tested on a daily treatment schedule against lymphatic leukemia L-1210 in mice in accordance with previously described procedures (13, *cf.*, footnotes *a–d* of Table I in *Reference 12*). The highest increases in lifespan resulted from treatment with ethyl *p*-(3-butyl-3-methyl-1-triazeno)hydrocinnamate (VI*b*, NSC-77587). In several separate experiments, increases in lifespan of 40–80% were recorded at doses of 250–600 mg./kg./day (Table II). The highest dose was acutely toxic in one of two tests, and the average difference in weight change between treated and untreated animals was about 3–4 g. in most tests at these dose levels.

Initial tests of the phenylbutyric acid derivatives and of the remaining hydrocinnamic acid derivatives indicated that some of these compounds can also cause small increases in lifespan. Thus, modest increases in survival time were observed in two or more tests of the dimethyltriazeno derivatives of the acid hydrazides (VI*f* and VII*g*) and after administration of ethyl 4-[*p*-(3,3-dimethyl-1-triazeno)phenyl]butyrate (VII*a*) at three dosages. Data from tests of these and of other compounds that produced an increase of 25% or more in lifespan in at least one test are also summarized in Table II. Although Compound VI*c* caused a significant increase in lifespan in a single test at 375 mg./kg./day, this apparent activity was not confirmed by a second test at a slightly higher dose (400 mg./kg./day).

Extensive testing of the derivatives not included in Table II might reveal activity, but none of these compounds caused significant increases in lifespan in the preliminary standard L-1210 tests. When only one dose of a compound is administered and the ratio of survival times is near 100%, a possible explanation is that the potential increase in survival time of treated animals due to cell kill is counterbalanced by chronic toxicity to the host. A lower dose might then produce a higher *T/C* ratio (*e.g.*, VI*f*, Table II). Except for those compounds specifically mentioned here, the remaining hydrocinnamic (VI) and phenylbutyric (VII) acid derivatives, the phenoxyacetic acid derivatives (V), and the benzoylglutamic acid derivatives (VIII) were tested at two or more daily dosages; the highest dose was either a toxic dose or the maximum administered dose (400 or 500 mg./kg./day). The exceptions were: Compounds VI*d* and VIII*e* were tested at only one dose (400 mg./kg./day), but the slight difference in weight change between treated and control animals indicated that their failure to increase lifespan was not due to toxicity; the highest doses of Compounds V*g* and VI*g* administered were just below toxic doses in tests *versus* adenocarcinoma 755 (Ca 755); the only dose (30 mg./kg./day) of VII*h* was one-half the Ca 755 toxic dose; and of the two doses (200 and 100 mg./kg./day) of VIII*g* administered, the highest dose was the same as that which caused a large difference in weight change in a Ca 755 test.

EXPERIMENTAL[2]

Aromatic Amine Precursors (I–IV)—Ethyl *p*-nitrophenoxyacetate [m.p. 74–75°; lit. 75–76° (14), 74–75° (15)] was prepared by esterifying the nitro acid in ethanolic hydrogen chloride. Catalytic hydrogenation (5% palladium-on-charcoal in ethanol) of the nitro ester and recrystallization of the product from a benzene–petroleum ether mixture gave ethyl *p*-aminophenoxyacetate (I) (16) in yields of 85–97%: m.p. 56–57°.

Ethyl *p*-aminohydrocinnamate (II) (17, 18) was prepared by two methods. First, *p*-aminohydrocinnamic acid (m.p. 133–134°), obtained in yields of 80–95% by reduction of *p*-nitrocinnamic acid by

[1] This type of structure was previously utilized for the attachment of the nitrogen mustard group (10, 11).

[2] Unless otherwise stated, melting temperatures were determined on a Kofler Heizbank apparatus (gradiently heated bar). Those designated "cap." were determined in a capillary tube.

Table I—Triazenes (V–VIII)

Number	Compound R_1	Compound R_2	Compound X	Method of Purification[a]	Yield, %	Melting Point or Boiling Point[b]	n_D (°C) or[c] $[\alpha]_D^{25}$	Empirical Formula	Analysis, % Calc.	Analysis, % Found
Phenoxyacetic Acid Derivatives										
V*a*	CH_3	CH_3	OC_2H_5	C	86	126–127°[d] (0.15 mm.)	1.5748 (25°)	$C_{12}H_{17}N_3O_3$	C, 57.36 H, 6.82 N, 16.72	C, 57.54 H, 6.96 N, 16.83
V*b*	CH_3	*n*-C_4H_9	OC_2H_5	A	88		1.5543 (20°)	$C_{15}H_{23}N_3O_3$	C, 61.41 H, 7.91 N, 14.32	C, 61.37 H, 7.83 N, 14.34
V*c*	CH_3	iso-C_4H_9	OC_2H_5	F	72		1.5517 (26°)	$C_{15}H_{23}N_3O_3$	C, 61.41 H, 7.91 N, 14.32	C, 61.48 H, 7.89 N, 14.63
V*d*	CH_3	C_6H_{11}	OC_2H_5	F	40	40–41°	1.5679 (26°)[e]	$C_{17}H_{25}N_3O_3$	C, 63.92 H, 7.89 N, 13.15	C, 63.97 H, 7.90 N, 13.34
V*e*	R_1R_2N = pyrrolidinyl		OC_2H_5	D[f](EtOH–H_2O)	89	50–51°		$C_{14}H_{19}N_3O_3$	C, 60.64 H, 6.91 N, 15.15	C, 60.87 H, 6.88 N, 15.16
V*f*	CH_3	CH_3	—$NHNH_2$	D(EtOH)	92	145–146°		$C_{10}H_{15}N_5O_2$	C, 50.65 H, 6.37 N, 29.52	C, 50.51 H, 6.34 N, 29.46
V*g*	CH_3	*n*-C_4H_9	—$NHNH_2$	D(C_6H_6–hexane)	90	78–80°		$C_{13}H_{21}N_5O_2$	C, 55.89 H, 7.58 N, 25.07	C, 56.02 H, 7.47 N, 25.16
Hydrocinnamic Acid Derivatives										
VI*a*	CH_3	CH_3	OC_2H_5	C	85	128–130° (0.05 mm.)	1.5645 (25°)	$C_{13}H_{19}N_3O_2$	C, 62.62 H, 7.68 N, 16.86	C, 62.62 H, 7.74 N, 16.90
VI*b*	CH_3	*n*-C_4H_9	OC_2H_5	E	74		1.5468 (23.5°)	$C_{16}H_{25}N_3O_2$	C, 65.94 H, 8.65 N, 14.42	C, 65.65 H, 8.63 N, 14.21
VI*c*	CH_3	*sec*-C_4H_9	OC_2H_5	E	79		1.5401 (26°)	$C_{16}H_{25}N_3O_2$	C, 65.94 H, 8.65 N, 14.42	C, 65.49 H, 8.87 N, 14.47
VI*d*	CH_3	C_6H_{11}	OC_2H_5	A	85		1.5627 (24°)	$C_{18}H_{27}N_3O_2$	C, 68.11 H, 8.58 N, 13.25	C, 67.95 H, 8.58 N, 13.23
VI*e*	R_1R_2N = pyrrolidinyl		OC_2H_5	A	96	39–40°		$C_{15}H_{21}N_3O_2$	C, 65.43 H, 7.69 N, 15.26	C, 65.62 H, 7.80 N, 15.23
VI	CH_3	CH_3	—$NHNH_2$	D(EtOH–hexane)	83	94–95°		$C_{11}H_{17}N_5O$	C, 56.14 H, 7.28 N, 29.76	C, 56.08 H, 7.09 N, 29.72
VI*g*	CH_3	*n*-C_4H_9	—$NHNH_2$	D(C_6H_6–hexane)	87	51–52°		$C_{14}H_{23}N_5O$	C, 60.62 H, 8.36 N, 25.25	C, 60.52 H, 8.32 N, 25.10
Phenylbutyric Acid Derivatives										
VII*a*	CH_3	CH_3	OC_2H_5	A	97		1.5603 (21°)	$C_{14}H_{21}N_3O_2$	C, 63.85 H, 8.04 N, 15.96	C, 64.07 H, 8.08 N, 16.10
VII*b*	CH_3	*n*-C_4H_9	OC_2H_5	B	84		1.5427 (24°)	$C_{17}H_{27}N_3O_2$	C, 66.85 H, 8.91 N, 13.76	C, 66.62 H, 8.73 N, 13.93
VII*c*	CH_3	iso-C_4H_9	OC_2H_5	E	71		1.5418 (24°)	$C_{17}H_{27}N_3O_2$	C, 66.85 H, 8.91 N, 13.76	C, 67.06 H, 8.50 N, 13.90

Table I—(*Continued*)

Number	Compound R_1	Compound R_2	Compound X	Method of Purification[a]	Yield, %	Melting Point or Boiling Point[b]	n_D (°C) or[c] $[\alpha]_D^{25}$	Empirical Formula	Analysis, % Calc.	Analysis, % Found
VII*d*	CH_3	*sec*-C_4H_9	OC_2H_5	E	76		1.5416 (26°)	$C_{17}H_{27}N_3O_2$	C, 66.85 H, 8.91 N, 13.76	C, 66.67 H, 8.84 N, 14.14
VII*e*	CH_3	C_6H_{11}	OC_2H_5	E	82[g]		1.5555 (24°)	$C_{19}H_{29}N_3O_2$	C, 68.85 H, 8.81 N, 12.68	C, 69.08 H, 8.92 N, 12.60
VII*f*	*n*-C_4H_9	*n*-C_4H_9	OC_2H_5	B	93		1.5319 (25°)	$C_{20}H_{33}N_3O_2$	C, 69.12 H, 9.58 N, 12.10	C, 69.25 H, 9.36 N, 12.20
VII*g*	CH_3	CH_3	—$NHNH_2$	D(EtOH–P.E.)	68	118–120°		$C_{12}H_{19}N_5O$	C, 57.81 H, 7.68 N, 28.09	C, 58.08 H, 7.71 N, 27.90
VII*h*	CH_3	*n*-C_4H_9	—$NHNH_2$	D(C_6H_6–hexane)	92	56–58°		$C_{15}H_{25}N_5O$	C, 61.83 H, 8.65 N, 24.04	C, 62.02 H, 8.55 N, 23.91
Benzoylglutamic Acid Derivatives[h]										
VIII*a*	CH_3	CH_3	OEt	D(EtOH–H_2O)	73	77°	−12.7	$C_{18}H_{26}N_4O_5$	C, 57.12 H, 6.93 N, 14.80	C, 56.98 H, 6.79 N, 15.00
VIII*b*	CH_3	CH_3	OEt	D(EtOH–H_2O)	78	114–115°	DL	$C_{18}H_{26}N_4O_5$	C, 57.12 H, 6.93 N, 14.80	C, 57.16 H, 7.01 N, 14.83
VIII*c*	CH_3	*n*-C_4H_9	OEt	D(C_6H_6–hexane)	80	46–48°	−10.0	$C_{21}H_{32}N_4O_5$	C, 59.98 H, 7.67 N, 13.32	C, 59.80 H, 7.49 N, 13.27
VIII*d*	*n*-C_4H	*n*-C_4H_9	OEt		44	44–46°	−9.3	$C_{24}H_{38}N_4O_5$	C, 62.31 H, 8.28 N, 12.11	C, 62.59 H, 8.08 N, 11.95
VIII*e*	CH_3	—CH_2CH_2OH	OEt	E	73		−10.6	$C_{19}H_{28}N_4O_6$	C, 55.88 H, 6.91 N, 13.72	C, 55.59 H, 6.91 N, 13.54
VIII*f*	*n*-C_4H_9	*n*-C_4H_9 mono(dibutylammonium) salt	OH		71	136–142°	+17.2	$C_{20}H_{30}N_4O_5 \cdot C_8H_{19}N$	C, 62.78 H, 9.21 N, 13.08	C, 63.12 H, 9.22 N, 12.91
VIII*g*	CH_3	CH_3	—$NHNH_2$	D(DMF–H_2O)	64	208–210°	+32	$C_{14}H_{22}N_8O_3$	C, 47.98 H, 6.33 N, 32.01	C, 48.84 H, 6.37 N, 31.96
VIII*h*	CH_3	*n*-C_4H_9	—$NHNH_2$	D(EtOH)	53	192°	+29	$C_{17}H_{28}N_8O_3$	C, 52.03 H, 7.21 N, 28.55	C, 51.99 H, 7.17 N, 28.44

[a] Methods of purification are defined in the general experimental procedure for the triazenes of phenoxyacetic acid ester, *etc.*; further details for the benzoylglutamate triazenes are given in the *Experimental* part. P.E. = petroleum ether (b.p. 30–60°); DMF = dimethylformamide. [b] Boiling point when pressure in the distillation apparatus is stated. Melting points of V*d*, VI*e*, VI*g*, VII*h*, VIII*c*, and VIII*e* were determined in capillary tubes; *cf. Footnote 2*. [c] $[\alpha]_D^{25}$ for benzoylglutamic acid derivatives. EtOH was the solvent for VIII*a–f* and dimethylformamide for VIII*g–h*; c = 1.5–1.6. [d] Obtained as a liquid; solidified during storage at low temperatures (m.p. 29–31°). [e] Obtained as a liquid; solidified during storage at low temperatures. [f] Product precipitated from reaction mixture; ethyl acetate extraction omitted. [g] Yield after Method A only; IR spectrum and analyses satisfactory. [h] Derivatives of L-glutamic acid except for VIII*b*.

Table II—Results of Tests of Some Triazenes against L-1210[a,b]

Compound	Dose, mg./kg./day	Schedule[c]	Mortality	Difference in Average Weight Change, *T/C*	Survival Time, *T/C*, %
Ethyl *p*-(3-butyl-3-methyl-1-triazeno)hydrocinnamate (VI*b*)	400	1	0/6	−2.1	108
	400	1–9	0/6	−4.0	161
	400	1–9	0/6	−6.0	130
	375	1–d	0/6	−4.4	137
	250	1–d	0/6	−2.5	142
	187	1–d	0/6	−2.4	117
	600	1–9	1/6	−4.3	179
	400	1–9	0/6	−3.4	162
	266	1–9	0/6	−2.1	128
	177	1–9	0/6	−1.3	117
	600	1–7	5/6		
	400	1–7	0/6	−3.3	171
	266	1–7	0/6	−3.3	160
	177	1–7	0/6	−3.1	144
p-(3,3-Dimethyl-1-triazeno)-hydrocinnamic acid hydrazide (VI*f*)	67	1–d	0/6	−6.4	91
	45	1–d	0/6	−4.2	141
	45	1–d	0/6	−5.4	132
	30	1–d	0/6	−1.7	114
4-[*p*-(3,3-Dimethyl-1-triazeno)-phenyl]butyric acid hydrazide (VII*g*)	85	1–d	0/6	−5.9	82*t*
	45	1–d	0/6	−3.3	128
	45	1–d	0/6	−3.4	142
	42	1–d	0/6	−4.5	129
	42	1–d	0/6	−3.3	130
	63	1–9	0/6	−4.3	146
	42	1–9	0/6	−2.9	127
	28	1–9	0/6	−2.7	111
	18	1–9	0/6	−1.6	103
Ethyl 4-[*p*-(3,3-dimethyl-1-triazeno)phenyl]butyrate (VII*a*)	250	1–d	5/6		*t*
	125	1–d	0/6	−2.8	92
	100	1–9	1/5	−3.3	130
	75	1–9	0/6	−2.7	134
	50	1–9	0/6	−1.7	133
Ethyl *p*-(3,3-dimethyl-1-triazeno)-hydrocinnamate (VI*a*)	400	1–9	0/6	−2.9	90
	250	1–d	0/6	−1.8	105
	125	1–9	0/6	−1.6	115
	83	1–9	0/6	−1.8	125
Ethyl 4-[*p*-(3-butyl-3-methyl-1-triazeno)phenyl]butyrate (VII*b*)	500	1–d	0/6	−4.1	97
	250	1–d	0/6	−3.9	121
	187	1–9	0/6	−1.4	128[d]
	125	1–d	0/6	−4.2	134
	125	1–9	0/6	−1.7	119
	84	1–9	0/6	−2.0	115
Ethyl 4-[*p*-(3-*sec*-butyl-3-methyl-1-triazeno)phenyl]-butyrate (VII*d*)	400	1–d	1/6	−2.8	112
	200	1–8	0/6	−2.0	130
Ethyl *p*-(3-*sec*-butyl-3-methyl-1-triazeno)hydro-cinnamate (VI*c*)	375	1–d	0/6	−2.6	147
	400	1–d	0/6	−1.0	119
	250	1–d	0/6	−1.6	108
Diethyl *N*-[*p*-(3-butyl-3-methyl-1-triazeno)benzoyl]glutamate (VIII*c*)	500	1–d	0/6	−0.6	126
	375	1–d	0/6	−0.4	108
	250	1–d	0/6	−1.2	121

[a] Derivatives of V–VIII that increased lifespan by 25% or more in at least one test; *t* = toxic. Except as noted, fresh solutions or suspensions of the triazenes were prepared each day before injection. [b] Further explanation of testing *versus* L-1210 is given in *References 12* (footnotes *a–d* of Table I) and *13*. [c] 1 means administration of a single dose on Day 1 (24 hr. after implantation of 10^6 L-1210 cells); 1–9, 1–8, 1–7, and 1–d mean daily administration on Days 1–9, 1–8, 1–7, and from Day 1 until Day 15 or until death, respectively. [d] Administered as a stable compound at this dose; solutions prepared on Days 0 and 6 (13).

the method of Skinner *et al.* (19), was esterified with refluxing (2 hr.) ethanolic hydrogen chloride. The solvent was evaporated, the residue was dissolved in dilute aqueous sodium hydroxide (pH 9), and the ester was extracted with ethyl acetate. Removal of the solvent and distillation of the residue gave the amino ester (II) as a colorless oil which solidified during storage at about 5°; yield, 83%; b.p. 99–100° (0.09 mm.); n_D^{25} 1.5360. The second method consisted of catalytic hydrogenation (5% palladium-on-charcoal) of ethyl *p*-nitrocinnamate in ethanol and distillation after removal of the solvent: yield of II, 80–90%.

Ethyl *p*-aminophenylbutyrate (III) (20) was also prepared by two routes. First, *p*-nitrophenylbutyric acid was obtained by the nitration procedure of Reppe (21) in 22% yield after two recrystallizations: m.p. 92–93° [lit. 95° (21), 92° (3), 92–93° (22)]. (Some of the *ortho*-isomer was also isolated.) The nitro acid was esterified with refluxing (1.5 hr.) ethanolic hydrogen chloride, the solvent was removed *in vacuo*, and the residual oil was distilled: yield, 76%; b.p. 121–123° (0.09 mm.); n_D^{25} 1.5238. The boiling point, refractive index, and IR spectrum were unchanged after redistillation; only the *para*-isomer was observable in the NMR spectrum, and assay by VPC showed the redistilled product to be 99.5% *para*-isomer.

Anal.—Calc. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.91. Found: C, 60.84; H, 6.21; N, 5.96.

Catalytic hydrogenation (palladium-on-charcoal in ethanol) of the nitro ester and distillation of the crude product gave a yield of 92% of III: b.p. 104–106° (0.1 mm.); n_D^{25} 1.5297.

Anal.—Calc. for $C_{12}H_{17}NO_2$: C, 69.54; H, 8.27; N, 6.76. Found: C, 69.81; H, 8.24; N, 6.92.

The second route to III consisted of the synthesis of *p*-aminophenylbutyric acid (m.p. 130–132°) from succinic anhydride and acetanilide (23, 24) and esterification of the amino acid in the usual manner. The IR spectrum and refractive index were identical with those of III obtained by the first route.

DL-*p*-Aminobenzoylglutamic acid [m.p. 197–198° (cap.); lit. (25) 198–199°] was prepared by hydrogenation (5% palladium-on-charcoal in ethanol) of the nitro derivative (25). Esterification of the acid with refluxing (3.5 hr.) ethanolic hydrogen chloride gave diethyl

DL-*p*-aminobenzoylglutamate[3] (DL-IV, X = OC_2H_5): m.p. 117–119° (cap.).

Anal.—Calc. for $C_{16}H_{22}N_2O_5$: N, 8.66. Found: N, 8.76.

Diethyl L-*p*-aminobenzoylglutamate (L-IV, X = OC_2H_5) (26, 27) was prepared similarly from L-*p*-aminobenzoylglutamic acid (L-IV, X = OH).

Triazenes of Esters of Phenoxyacetic, Hydrocinnamic, and Phenylbutyric Acids (V*a–e*, VI*a–e*, and VII*a–f*)—The triazenes were routinely protected from light during the preparation and purification procedures and were prepared under an atmosphere of nitrogen. The aromatic amine (I–III) in 1 *N* hydrochloric acid (3–4 ml./mmole) was diazotized at 0–5° by adding, dropwise during 5–30 min., a solution of sodium nitrite (1.1 mmoles/mmole of aromatic amine) in a volume of water convenient for the size of the preparation (usually 10–20 ml./g.). The solution was stirred at 0–5° for 0.5–1 hr. before the excess nitrite was decomposed with sulfamic acid. The amine to be coupled was then added quickly in sufficient quantity to raise the pH of the solution to 9–10 or above, and stirring at 0–5° was continued for 0.5–2 hr. (typically 1 hr.). The triazene was extracted from the aqueous mixture with ethyl acetate. The ethyl acetate extract was washed well with aqueous sodium chloride solution or successively with 1 *N* hydrochloric acid (to facilitate removal of extracted amine), aqueous sodium bicarbonate, and aqueous sodium chloride. The ethyl acetate solution was dried ($MgSO_4$), treated with activated carbon, and freed of solvent *in vacuo*. The residual triazene was dried (P_2O_5) *in vacuo* at room temperature.

The products of some preparations were pure, as adjudged by analytical data and TLC, without further treatment; other compounds were obtained analytically pure after redissolution of the products in ethyl acetate or ether and repetition of the washing and drying operations. The purification procedures (Table I) to which the initially isolated triazenes were subjected were the following: (A) no further treatment; (B) redissolution in ethyl acetate or ether and repetition of the washing and drying operations; (C) distillation under reduced pressure; (D) recrystallization; (E) chromatography on magnesia–silica gel[4] and elution with petroleum ether, hexane, or cyclohexane and then with one of these solvents containing small percentages of acetone; (F) stirring of the dry ethyl acetate extract with a mixture of magnesia–silica gel and activated carbon followed by treatment of the ethyl acetate residue in hexane solution with a portion of magnesia–silica gel (V*d*) or in methanol with activated carbon (V*c*).

The preparation of ethyl 4-[*p*-(3-*sec*-butyl-3-methyl-1-triazeno)-phenyl]butyrate (VII*d*) illustrates both the general procedure and purification by chromatography. A solution of 2.8 g. of sodium nitrite in 50 ml. of water was added during 0.5 hr. to a cold (0–5°) solution of 7.5 g. of ethyl 4-(*p*-aminophenyl)butyrate in 150 ml. of 1 *N* hydrochloric acid; then the following operations were performed successively: the mixture was stirred for 0.5 hr., sufficient sulfamic acid was added to destroy excess nitrite, 45 ml. of *N*-methyl-*sec*-butylamine was added, and the resulting solution (still at 0–5°) was stirred for 1 hr. and then extracted with two 250-ml. portions of ethyl acetate. The ethyl acetate solution (combined portions) was washed successively with two 100-ml. portions of 1 *N* hydrochloric acid, saturated aqueous sodium bicarbonate, and aqueous sodium chloride and was then dried ($MgSO_4$) and treated with activated carbon. Evaporation of the solvent *in vacuo* left 10.6 g. (96%) of an orange oil; TLC (80 mcg. of sample on silica gel, 95:5 benzene–ethyl acetate, detection by UV and UV-Ultraphor[5]), prominent spot of the triazene plus a small amount of a contaminant at the origin. A hexane solution of the crude product was chromatographed on a column prepared from 100 g. of magnesia–silica gel and hexane. Elution with 500 ml. of hexane gave 8.4 g. (76%) of faintly yellow oil; n_D^{26} 1.5416. Further elution with 500 ml. of 99.5:0.5 hexane–acetone gave 0.89 g. (8%) of orange oil that was identical, according to TLC and IR, with the hexane-eluted product.

***N*-[*p*-(3,3-Dialkyl-1-triazeno)benzoyl]glutamates (VIII*a–f*)**—The procedure was similar to that described for the triazenes of ethyl hydrocinnamate and related esters, except that: (*a*) the solution of diethyl *p*-aminobenzoylglutamate (IV, X = OC_2H_5) or the parent acid (IV, X = OH) in 1 *N* hydrochloric acid was added (during approximately 1 hr.) to the aqueous solution of sodium nitrite, and (*b*) the amount of water used in the sodium nitrite solution was larger (typically, 35 ml./g.). Except for Compound VIII*f*, the triazenes precipitated from the reaction mixtures. Compound VIII*a* precipitated as a solid, and Compound VIII*b* was induced to solidify by adding ethanol to the reaction mixture. These two compounds were isolated by filtration and washed with water. The others precipitated as gums or oils and were extracted from the reaction mixtures with ether (VIII*c* and VIII*d*) or ethyl acetate (VIII*e*). The reaction mixture containing VIII*f* was washed with ether, and VIII*f* was precipitated by lowering the pH. Precipitation began at pH 6.0–5.8, and the pH was not allowed to fall below 5.2. Compounds VIII*a–c* were recrystallized (Table I).

The ether extract (preceding paragraph) containing VIII*d* was freed of solvent, the residual oil was stirred with two portions of cold hexane, the hexane layers were decanted, the oil layer was stirred with a third portion of hexane, and this mixture was then stored overnight at 5°. The solidified triazene was removed by filtration, washed with hexane, and treated in ether solution with activated carbon. The viscous oil remaining after evaporation of the ether was leached with hot petroleum ether. Evaporation of solvent from the petroleum ether extract left a light-yellow oil that solidified: m.p. 44–46°.

The ethyl acetate extract containing VIII*e* was washed successively with 1 *N* hydrochloric acid, aqueous sodium bicarbonate, and aqueous sodium chloride and was dried ($MgSO_4$). The light-brown oil remaining after evaporation of the solvent was reprecipitated from benzene solution with hexane and treated in methanol solution with activated carbon; the residual oil (5.6 g.) obtained by evaporation of the methanol was chromatographed on magnesia–silica gel (60 g.). Elution with 400-ml. portions of hexane containing 0.5, 1, 2, 4, 8, and 16% acetone gave negligible amounts of product; elution with two 400-ml. portions of 32% acetone–hexane then gave 4.70 and 0.67 g., respectively, of a pale-yellow oil (VIII*e*, 73% yield): TLC (silica gel, 1:3 benzene–ethyl acetate), 1 spot; $UV_{max.}$ ($\epsilon \times 10^{-3}$) in phosphate buffer (pH 7) 223 (9.6), 322 (18.1).

Hydrazide Derivatives (V*f–g*, VI*f–g*, VII*g–h*, and VIII*g–h*)—The ester derivative of a triazene was cooled in an ice bath, hydrazine was added, and ethanol was then added in sufficient quantity to form a homogeneous reaction mixture. The solution was stirred at room temperature, and the disappearance of the ester was monitored by TLC. During the preparation of the higher melting acid hydrazides, the reaction mixture became a solid mass. The usual reaction times were 24 or 48 hr. The excess hydrazine and the ethanol were then evaporated *in vacuo* (regardless of whether the reaction mixture was solid or liquid), and the residue was recrystallized (Table I). The triazene derivatives were protected from light during the preparation and purification operations.

[3] This compound is reported in the English translation of a paper by Alekseeva and Pushkareva (10); however, the melting points tabulated for the diethyl ester and its two precursors are in agreement with those of the L-isomers.

[4] Florisil, The Floridin Co.

[5] Ultraphor WT, an optical whitening agent, BASF Colors and Chemicals, Inc., Charlotte, N. C.

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Rheological and Sensory Evaluation of Work Softening and Recovery of Pharmaceutical White Soft Paraffins

B. W. BARRY and A. J. GRACE

Abstract □ Continuous shear and creep viscometry were used to investigate the effect of work softening and recovery on the rheological properties of four grades of white soft paraffin BP. In continuous shear, using a cone and plate viscometer, the apparent viscosities of two samples increased with recovery time after working, which indicated partial structural recovery. Apparent viscosities of unworked samples were generally lower than those of the worked samples due to elastic recovery and/or sample fracture of the unworked samples during shear in the viscometer. Viscoelastic results were analyzed to obtain initial elastic compliances and residual viscosities. These data indicated that the loss of consistency during working and recovery after working were mainly viscous phenomena. Continuous spectra of retardation times derived for worked and unworked soft paraffins showed that working caused little irreversible structural breakdown. Structural recovery, indicated by concentrations of retardation mechanisms in the continuous spectra, occurred for at least 240 hr. after work softening. A sensory evaluation of white soft paraffin in different states of working was correlated with discrete viscoelastic parameters and continuous shear yield stresses of the material; no correlation was found with continuous shear apparent viscosity data.

Keyphrases □ White soft paraffin, rheology—work softening and recovery effects □ Rheological properties, white soft paraffin—continuous shear and creep viscometry □ Viscometry, continuous shear, creep—white soft paraffin rheology □ IR spectrophotometry—structure, work softening of soft paraffins

White soft paraffin BP, which is used as an excipient in many pharmaceutical and cosmetic ointments, consists of a three-dimensional crystalline matrix embedded in a colloidal gel of liquid and amorphous hydrocarbons (1). When the material shears, whether during manufacture, viscometry, or in application to the skin, the structure is broken down by rupture of bonds within the material and the consistency decreases as the material work softens. Few experiments have been reported concerning the effect of work softening on the rheological properties of white soft paraffin. Haighton (2) and Shama and Sherman (3) investigated work softening of margarine and butter, which are similar in consistency to white soft paraffin. Tsagareishvili *et al.* (4) reported that work softening of soft paraffin caused up to 90% breakdown of bonds within the material, that it reduced the viscosity, and that the cycloparaffin content, measured by IR spectroscopy, increased.

In manufacturing processes involving cold working of materials containing significant amounts of white soft paraffin, the final product will not have its maximum possible structure because of the working procedure. The product consistency will vary, depending on: (*a*) the initial structural condition of the material, for example, melted and cooled or worked when cold; (*b*) the degree of work softening; (*c*) the type or grade of material; and (*d*) the age of the finished product. The first two variables are, to some extent, under the control of the manufacturer. The effect of grade variation on the rheological properties of unworked white soft paraffin has been investigated by continuous shear and creep viscometry (5). The present study investigated the effect of work softening and recovery on the rheological properties of four grades of white soft paraffin (factors *c* and *d*).

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EXPERIMENTAL

Materials—Four grades of white soft paraffin of BP quality were used[1]. For previous work on these samples, see Barry and Grace (5, 6).

Procedure—The white soft paraffins were worked in the standard manner using a grease worker (7). This consisted of one double stroke per second for 1 min. at approximately 25°, in a cup 6.35 cm. deep and 7.60 cm. in diameter, using a perforated plate 7.45 cm. in diameter containing 51 apertures of diameter 0.635 cm.

A Ferranti-Shirley viscometer with automatic flow curve recorder unit and *X-Y* plotter was employed for the continuous shear investigation. The viscometer, with medium cone, was used in two test regimes; rates of shear varied linearly from: (*a*) 0.0 to 1754.0 to 0.0 sec.$^{-1}$, and (*b*) 0.0 to 175.4 to 0.0 sec.$^{-1}$. Each cycle was 1200-sec. duration at 25 ± 0.1°. Apparent viscosity data were derived by point determinations (8) at 1754.0 and 175.4 sec.$^{-1}$, and shear stresses were derived from the spur points where the slopes of the flow curves became infinite for the first time.

Creep experiments at 25 ± 0.2° were of short duration (1200 sec.) except with unworked samples, because restricted time studies were necessary with work-softened materials due to their rapid rate of recovery. Discrete parameters, J_0 (initial elastic compliance) and η_0 (residual viscosity), were derived from each curve. Since the creep curves of worked samples did not reach a steady-state flow condition, an approximate residual viscosity was obtained using the terminal 300 sec. of each curve. Such approximate values are represented in the text by the symbol η_0'.

Continuous spectra of retardation times were determined from each creep curve using the second approximation method of Schwarzl and Staverman (9):

$$L(\tau) \approx \frac{d}{d \ln t}\left[J(t) - \frac{d}{d \ln t}\frac{J(t)}{2}\right]\Bigg|_{t = 2\tau} \qquad \text{(Eq. 1)}$$

where $L(\tau)$ is the value of the retardation spectrum and $J(t)$ is the total compliance at time t.

IR spectra were determined with a Perkin-Elmer 257 grating spectrophotometer with materials mounted between rock salt plates. Worked white soft paraffin samples were prepared in the grease worker, using one double stroke per second for 300 sec. at approximately 25°.

In the sensory evaluation of worked and unworked white soft paraffin, 12 persons were required to assess samples of soft paraffin by rubbing them into the skin of the back of the hand and ranking the materials in order of ease of rubbing in. A point system was used, 4 points for the easiest and 1 point for the most difficult. The materials were all Sample 1: (*a*) unworked; (*b*), (*c*), and (*d*), worked and aged 0.5, 24.0, and 240.0 hr., respectively. To avoid the subjects gaining sensory impressions during removal of material from containers, each subject was given 0.2-ml. samples of the materials using a disposable syringe modified so that the sample was not sheared on delivery. The panel members were academic and technical staff of the School of Pharmacy with no previous experience of this type of test; they were given no training in this type of evaluation so that they would react as typical consumers.

RESULTS

Continuous Shear—Table I contains the continuous shear data for worked and unworked white soft paraffins. Samples 3 and 4 in the unworked state were grossly ejected from the cone-plate gap of the viscometer during shearing, and their apparent viscosity data were derived by extrapolation of Arrhenius-type curves (5). Sample 4 was grossly ejected from the viscometer gap after work softening, and apparent viscosity data were not obtained. Apparent viscosity data for Samples 1 and 3 indicated that structural recovery occurred to some degree after working, which is further evidence for partial thixotropic breakdown in white soft paraffin (6). Little recovery of apparent viscosity was detected for Sample 2. The manufacturers stated that this sample was susceptible to shear breakdown (*cf.*, viscoelastic results). Thus, any recovered structure was probably destroyed during development of the upcurve in viscometry. The yield stresses of each sample increased with age after working.

[1] Sample 1 was produced by Printar Industries; Samples 2, 3, and 4 were, respectively, grades 892/2/148, 783/L, and 783/L/40A produced by Dalton & Co. Ltd.

Table I—Continuous Shear Data for White Soft Paraffins at 25°[a]

Sample	Recovery Time after Working, hr.	—0.0–1754.0 sec.$^{-1}$— η', poise	σ, dyne cm.$^{-2}$	—0.0–175.4 sec.$^{-1}$— η', poise	σ, dyne cm.$^{-2}$
1	0.5	10.0	5550	28.9	2150
	24.0	10.2	5600	28.4	2700
	120.0	10.6	5750	29.9	3400
	Unworked	10.2	7700	61.1	5100
2	0.5	1.9	1250	8.5	700
	24.0	2.0	1500	8.4	750
	Unworked	1.2	3550	6.5	2600
3	0.5	6.3	3000	19.0	1550
	24.0	7.5	3800	20.9	1850
	Unworked	6.8	3350	12.1	2600
4	0.5	—	1850	—	1750
	24.0	—	2150	—	2600
	Unworked	6.2	4500	19.0	2800

[a] Apparent viscosity, η', and yield stress, σ, determined as stated in the *Experimental* section. Unworked apparent viscosity data for Samples 3 and 4 were determined by extrapolation of Arrhenius-type curves of η' *versus* $1/T$°K.

The apparent viscosities of unworked samples at 1754.0 and 175.4 sec.$^{-1}$ were often lower than those of the worked samples after 24 hr. recovery, although the yield stresses were greater. The flow curves obtained for Sample 3 were complex, as shown in Fig. 1, and indicated that some shear thickening may occur at high rates of shear, which correlates with the low ductility of this sample (5).

Discrete Viscoelastic Parameters—Figure 2 indicates the changes that occurred in the viscoelastic properties of Sample 1 with aging after work softening. A plot of total compliance [$J(t)$] at 1200 sec. after onset of stress against recovery time was linear on double logarithmic axes. Discrete viscoelastic parameters derived from the creep curves are given in Table II. The decrease in consistency after working was shown by an increase in total compliance. The largest relative initial compliance, J_{rel} (0.5/∞), after working occurred in Sample 2; J_{rel} (0.5/∞) is defined as J_0 at 0.5 hr. after working/J_0 of the unworked sample (Table III). The largest relative residual viscosity, η_{rel}'(∞/0.5) (defined as η_0 of the unworked sample/η_0' at 0.5 hr. after working) occurred in Sample 3. Recovery of J_0 and η_0' after working is shown in Table III, using relative parameters similar to those above but defined as J_0 for sample aged 0.5 hr./J_0 for sample aged 24.0 hr. [J_{rel}(0.5/24.0)], and η_0' for sample aged 24.0 hr./η_0' for sample aged 0.5 hr. [η_{rel}'(24.0/0.5)]. The relative recovery of J_0 and η_0' was greatest for Sample 2; the other samples were similar. The increase in η_0' with recovery time after working was linear when plotted on double logarithmic axes, but no simple relationship was found for recovery of J_0. Sample 4, which was nonlinear viscoelastic in the unworked state (5), was still nonlinear after working.

Continuous Retardation Spectra—The continuous retardation spectra for worked and unworked white soft paraffins are shown in Figs. 3 and 4. Unworked Samples 2 and 3 had bimodal retardation

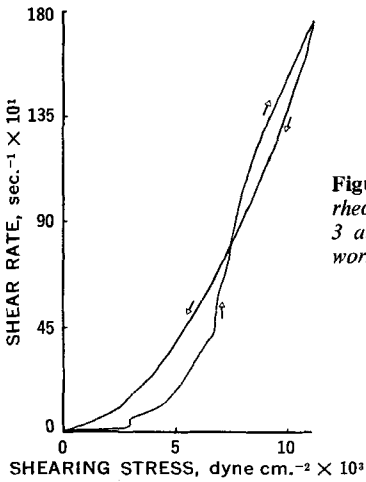

Figure 1—*Continuous shear rheogram for worked Sample 3 at 25 ± 0.1°, 0.5 hr. after working.*

Table II—Discrete Viscoelastic Data for Worked and Unworked White Soft Paraffins at 25°[a]

Sample	Recovery Time after Working, hr.	J_0, dyne^{-1} cm.^{2} × 10^{-7}	η_0', poise × 10^7
1	0.5	28.7	3.4
	2.0	20.9	7.5
	6.0	18.6	8.1
	24.0	16.4	19.8
	72.0	16.3	22.0
	120.0	14.7	43.4
	240.0	8.4	50.6
	Unworked	7.6	246.0
2	0.5	51.9	4.1
	2.0	43.5	6.5
	6.0	29.9	13.5
	24.0	9.5	40.9
	Unworked	4.3	315.0
3	0.5	28.5	3.4
	2.0	16.4	6.2
	6.0	13.3	10.6
	24.0	12.5	19.9
	Unworked	3.5	2780.0
4	0.5	16.2	1.0
	2.0	12.9	1.7
	6.0	11.7	2.5
	24.0	9.6	5.9
	Unworked	3.5	104.0

[a] Sample 4 (nonlinear viscoelastic): unworked, creep shearing stress, 120 dynes cm.$^{-2}$; and worked, creep shearing stress, 15 dynes cm.$^{-2}$.

spectra, while the curves for unworked Samples 1 and 4 contained three maxima. In general, the largest peak was broad and occurred at large values of t (2000–4000 sec.). The retardation spectrum curves for the worked samples decreased in height with recovery time after working and, with one exception, the peaks moved to larger values of t. Worked Samples 1 and 4 exhibited three maxima in their retardation spectra. Only part of the terminal peak, *C*, at large values of t was determined due to the restricted time of the creep tests (see *Experimental* section). The movement of the maxima to larger values of t with recovery time after working may be seen easily (*e.g.*, Fig. 3A).

Sample 2 was exceptional because the retardation distribution of the worked material did not initially show any distinct maxima but sloped up toward one general peak at large values of t. After 24.0 hr. recovery, a single peak appeared in the spectrum which was correlated with a peak in the unworked sample spectrum. Work-softened Sample 3 had three maxima in its retardation spectra compared to two in the spectrum of the unworked sample. Two maxima, *B* and *C*, of the worked Sample 3 behaved normally since they decreased in height and moved to larger values of t as the recovery time

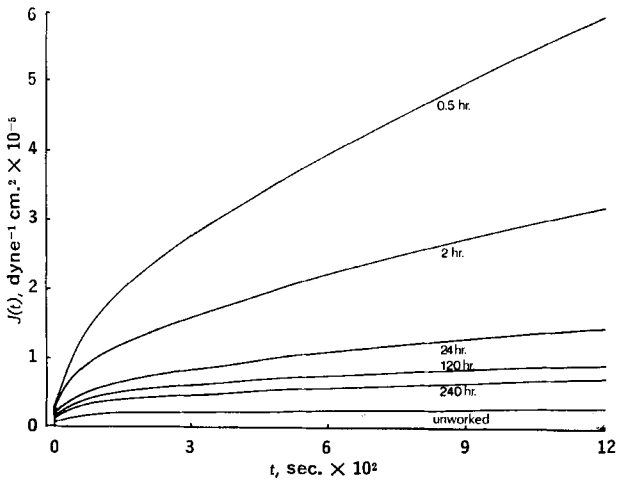

Figure 2—*Creep compliance curves, indicating changes in the viscoelastic behavior at 25 ± 0.2°, of white soft paraffin Sample 1 with working. Aging time after working is shown in the figure.*

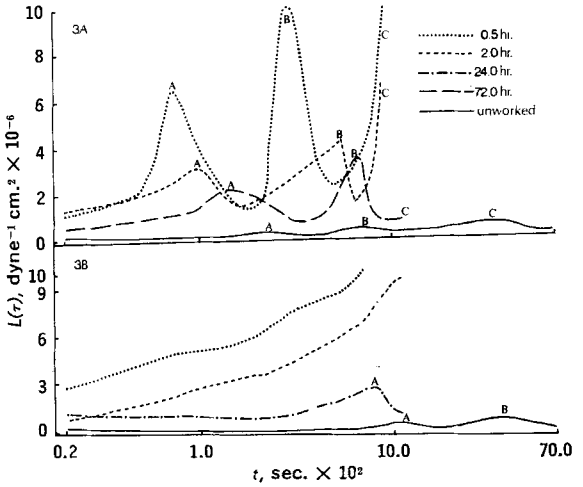

Figure 3—*Continuous spectra of retardation times, L(τ), for worked and unworked white soft paraffins. Key: A, Sample 1; and B, Sample 2. Aging time after working is shown in the figure.*

increased. Peak *A* decreased in height with recovery but moved to smaller values of t as the material aged after work softening. Peak *B* was not resolved by analysis for the 0.5-hr. sample, but its presence was indicated by the shoulder on peak *C*.

IR Spectroscopy—No significant differences were found between IR absorption spectra for unworked and heavily worked white soft paraffins.

Sensory Investigation—The mean panel score derived in the sensory evaluation of Sample 1 in various states of work softening gave a linear plot against η_0' data using semilogarithmic axes (Fig. 5). A similar linear relationship was found with J_0, but the data were more scattered. When the test was repeated using fresh samples, the slopes of the linear plots differed by 9 and 3%, respectively, for η_0' and J_0. A linear relationship was found between the logarithms of the yield stresses determined in continuous shear and the sensory data (Fig. 6). No correlation was found between the sensory data and the apparent viscosities of the materials used (apparent viscosities determined at various rates of shear from less than 30 to 1754 sec.$^{-1}$). The linearity of the single logarithmic graphs is in accord with the Weber–Fechner equation described by Scott Blair (10).

DISCUSSION

The continuous shear viscosity data derived concerning the effect of work softening and recovery of white soft paraffin were of limited practical use; changes which were determined were generally small. An increase in apparent viscosity with age after work softening occurred for Samples 1 and 3. Barry and Grace (6) previously

Table III—Relative Compliance and Relative Viscosity Data for White Soft Paraffins[a]

	Worked		Recovered	
Sample	$J_{rel}\left(\frac{0.5}{\infty}\right)$	$\eta_{rel}'\left(\frac{\infty}{0.5}\right)$	$J_{rel}\left(\frac{0.5}{24.0}\right)$	$\eta_{rel}'\left(\frac{24.0}{0.5}\right)$
1	3.8	72.3	1.7	5.8
2	12.0	76.8	5.5	10.0
3	8.1	817.0	2.3	5.8
4	4.6	104.0	1.7	5.9

[a] Derived from Table II, where:

$$J_{rel}\left(\frac{0.5}{\infty}\right) = \frac{J_0\ (0.5\ \text{hr.})}{J_0\ (\text{unworked})}$$

$$\eta_{rel}'\left(\frac{\infty}{0.5}\right) = \frac{\eta_0\ (\text{unworked})}{\eta_0'\ (0.5\ \text{hr.})}$$

$$J_{rel}\left(\frac{0.5}{24.0}\right) = \frac{J_0\ (0.5\ \text{hr.})}{J_0\ (24.0\ \text{hr.})}$$

$$\eta_{rel}'\left(\frac{24.0}{0.5}\right) = \frac{\eta_0'\ (24.0\ \text{hr.})}{\eta_0'\ (0.5\ \text{hr.})}$$

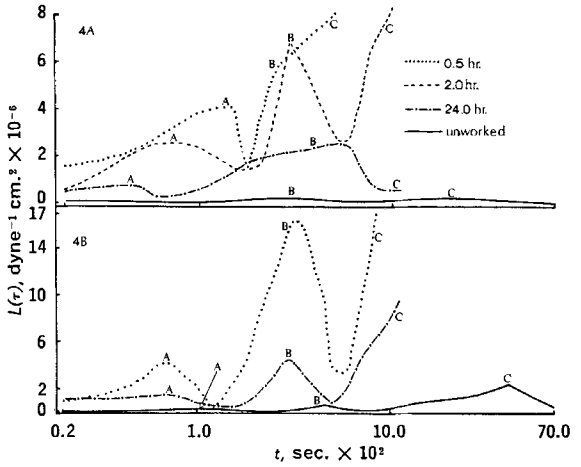

Figure 4—*Continuous spectra of retardation times,* L(τ), *for worked and unworked white soft paraffins. Key: A, Sample 3; and B, Sample 4. Aging time after working is shown in the figure.*

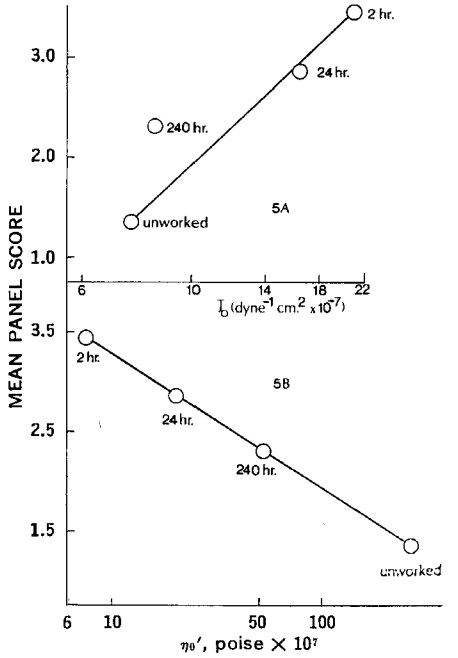

Figure 5—*Correlation of sensory assessment test data with discrete viscoelastic data; mean panel score* versus: *A, logarithmic initial elastic compliance,* J_0; *and B, logarithmic residual viscosity,* η_0'. *Aging time after working is shown in the figure.*

reported that Sample 1 may undergo partial thixotropic breakdown during shear. The manufacturer states that Sample 2 is susceptible to shear breakdown, a fact confirmed by the small strain experiments, which also indicated that Sample 2 recovered considerably with time after working. The lack of measured recovery detected using continuous shear viscometry was probably due to breakdown of any recovered structure during development of the upcurve of the rheogram. This indicates that continuous shear rheological testing is too drastic a technique for materials containing labile structures.

The continuous shear viscometry data (Table I) seem to indicate that work-softened materials aged 24 hr. often increased in apparent viscosity when compared with unworked samples. This anomalous result is probably in part an instrument artifact and may be explained by: (*a*) elastic recovery or fracture of the sample during shear, causing ejection of sample from the cone-plate gap of the viscometer; or (*b*) fracture of the material in the sample gap of the viscometer during shear but with no visible ejection of the material from the cone-plate gap; or (*c*) fracture of the adhesive forces between the sample and the viscometer elements leading to slippage (11–13). For example, gross expulsion of sample occurred during viscometry of unworked white soft paraffins 3 and 4, and apparent viscosities were derived indirectly. No noticeable expulsion of material occurred for unworked Samples 1 and 2, but it is possible that the samples fractured in the viscometer gap during shear and/or a small amount of each sample was ejected from the gap. Loss or fracture of the samples during shear in the viscometer would reduce the measured torque and, hence, the derived apparent viscosities at any given rate of shear. Apparent viscosity data derived would thus be lower than their correct values. Work-softened materials with lower elastic moduli would not be expelled or fractured to the same extent, and derived data would be nearer to the correct apparent viscosity values. Using this theory, it is apparent that a degree of fracture and/or sample ejection may have occurred with Samples 1 and 2, although there was no indication of the phenomena in the rheograms obtained or at the cone-plate gap. Preliminary experiments showed that a concentric cylinder viscometer (Rotovisko) could not be used because the initial shear stress often exceeded the capacity of the torque scale and sample cleavage occurred at higher rates of shear. This situation clearly indicates one disadvantage of continuous shear viscometry of viscoelastic semisolid materials.

In general, the yield stresses obtained in continuous shear were lower after work softening than in the unworked state, even after recovery. This indicates that sample ejection from the viscometer gap or fracture within the gap occurs after formation of the spur point in the rheogram. It is thus possible that yield stresses derived from spur points may be utilized in quality control procedures, especially if they can be correlated with other parameters such as sensory data (Fig. 6) or viscoelastic retardation times (5, 12).

The discrete viscoelastic parameters derived from the creep curves (Table II) were mainly of qualitative value, although correlations were found with sensory data. The elastic properties of Sample 2 were the most affected by working, in agreement with the manufacturer's data, but this sample also recovered to a greater extent, or at a faster rate, within 24 hr. than the other materials. Thus, worked and unworked penetration tests (7) may yield misleading data since the official method does not include penetration after recovery. Sample 4, which was nonlinear viscoelastic before working (5), was still nonlinear viscoelastic after working because the material work hardened in creep. This correlates with the short fiber length of the material (5). The comparison of worked and unworked Sample 4 is complicated; the materials were sheared at different stresses, because the compliance of the unworked sample was too small to be measured at the lower shearing stress employed for the worked sample. The parameters quoted for this nonlinear viscoelastic sample are thus specific for the shearing stresses given in Table II.

The relative residual viscosity data are not fundamental because the method used to derive η_0' was an approximation. The accuracy of the approximation depended on the degree of work softening, that is, on how near to a steady state of flow the material was be-

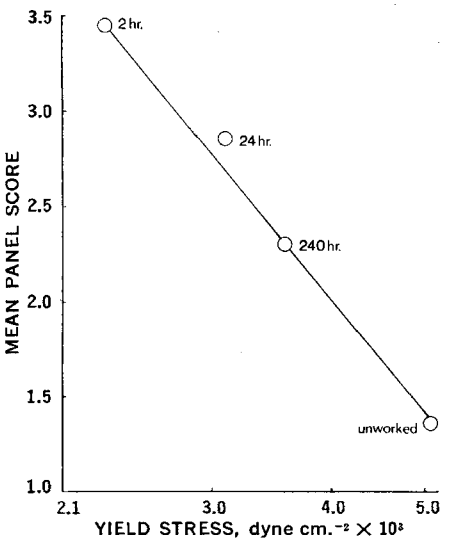

Figure 6—*Correlation of sensory assessment test data with the logarithm of yield stresses determined in continuous shear viscometry using shear rate regime (b). Aging time after working is shown in the figure.*

tween 900 and 1200 sec. after the onset of stress, since the part of the curve obtained during this time interval was used in the determination of η_0'. The large variation between relative residual viscosities after working, η_{rel}' (∞/0.5), may thus correctly represent material variations or be due to varying degrees of approximation in the determination of η_0'. The similar values obtained, except for Sample 2, for the relative residual viscosity on recovery, η_{rel}' (24.0/0.5) (Table III), indicated that variation in the degree of approximation between worked samples was small. Comparison of residual viscosity data for worked and unworked samples of white soft paraffins is not as useful as comparing the recovery data.

The continuous retardation spectrum, L, is defined by Eq. 2 (14, 15):

$$J(t) = J_0 + \int_{-\infty}^{\infty} L(\tau)\,(1 - e^{-t/\tau})\,d \ln \tau + t/\eta_0 \quad \text{(Eq. 2)}$$

where $J(t)$ is total compliance at time t, J_0 is the initial elastic compliance, η_0 is the residual Newtonian viscosity, and τ is a retardation time. Although L has the dimensions of a compliance, it is in the nature of a distribution function of retardation times. Maxima in the distribution indicate concentrations of retardation processes (time-dependent reversible strain mechanisms at molecular level) measured by their contribution to compliance.

The three maxima in the retardation spectra of Samples 1 and 4 probably represent similar retardation mechanisms; indeed, the terminal peak, C, at large values of t is common to each sample, which indicates that they have at least one type of retardation mechanism of a similar nature. The three maxima in work-softened Samples 1 and 4 (Figs. 3A and 4B) relate to those in the unworked samples since they approach to the size and position of the maxima of the unworked samples with recovery time after working. It is possible that the maxima represent three distinct types of molecular interactions or retardation processes within the materials which are removed from an equilibrium state by the working process. This leads to an increase in the elastic compliance, indicated by the height of the curves, and an increase in the rate at which strain occurs, indicated by the shifting of the maxima to smaller values of t. The gradual recovery to an equilibrium state (which is not necessarily identical with the former state, as primary bonds may have been irreversibly ruptured during working) begins immediately after working ceases. The actual molecular retardation mechanisms are not known; however, the recovery of the samples indicates that the majority of work softening was due to reversible rupture of weak secondary bonds. An almost infinite array of such bond strengths is possible within white soft paraffin because the strength of the bonds depends on particle size and shape (16), the distance between particles, and the medium separating them (17).

Examples of the types of major interactions which could cause the three maxima in the retardation spectra of Samples 1 and 4 are given here. The examples are hypothetical since there is no direct evidence to suggest that the interactions cited contribute to the viscoelastic properties of white soft paraffin. Three types of material compose white soft paraffin: crystalline, liquid, and amorphous. Assuming that the liquid hydrocarbons do not contribute significantly to the elastic properties of the soft paraffin, that is, their contribution is mainly viscous, three major types of interaction may occur: (*a*) crystal–crystal, (*b*) amorphous–amorphous, and (*c*) crystal–amorphous. Within each group of interactions, there would be variation in bond strengths due to the factors mentioned previously. Crystal–crystal cohesive bonds, formed during cooling of a molten white soft paraffin by the crystals growing together, are examples of bonds that may rupture irreversibly during shear in a working procedure. It may prove possible in future work to alter the ratio of crystalline to amorphous material and thus deduce the contribution of the two forms to the bulk rheological properties.

The distribution of retardation times of worked Sample 2 provided a relatively smooth curve, with no distinct maxima formed until after 24 hr. recovery (Fig. 3B). Since this sample was susceptible to shear breakdown, it is probable that working altered the internal structure to such a degree that the retardation processes producing peaks in the spectra of the unworked sample were entirely disrupted. The retardation spectra thus became very broad distributions in which no distinct maxima appeared until the material aged sufficiently to reconstitute structures that caused concentrations of retardation mechanisms.

Peak A in the spectra of worked Sample 3 was anomalous in tha it was not present in the spectrum of the unworked sample and moved to smaller values of t as the sample recovered after working. It is possible that a labile structure was imposed upon the natural structure of the material by the working process; a similar phenomenon was reported previously (5). Movement of the peak to shorter time intervals indicated that the structure causing it gradually decayed with age after work softening.

It was reported that mechanical treatment of white soft paraffin in a viscosimeter increased the content of cycloparaffins, as detected by IR spectroscopic analysis (4). The same authors also reported up to 95% breakdown of internal bonds among hydrocarbon molecules, which caused changes in the rheological properties of their materials. However, in the present work, the IR absorption spectra for worked and unworked white soft paraffins showed that no changes occurred during working that were detectable spectrophotometrically.

In the sensory evaluation of consistency, the simple test used correlated rheological data with sensory data (Figs. 5 and 6), indicating that the changes in consistency of Sample 1 caused by working and subsequent aging may be detected by the consumer. The material used in this test was selected because it was *not* the most affected by working and recovery; it is probable that a consumer could detect differences in other samples with equal or greater facility. Panel members were asked to rank the samples in order, using a specific test; they were not indicating a preference for a material of a given consistency. The work is of significance for manufacturers of semisolid pharmaceutical and cosmetic ointments and creams whose production involves a cold-working procedure. Katz (18) show edthat consumers are unable to differentiate between elasticity and viscosity in semisolid materials but tend to measure the *ratio* of these properties. The product of an elastic compliance (inverse of an elastic modulus) and its associated viscosity is a retardation time. Therefore, it is possible that continuous retardation spectra may be useful in textural studies of semisolid viscoelastic materials where the test panel is required to evaluate products in order of preference rather than to use a simple ranking test as in this work.

The loss of consistency of white soft paraffin on working and subsequent recovery were not easily or clearly determined using continuous shear viscosity data derived by cone and plate viscometry, a method frequently employed as a routine testing procedure. However, yield stresses determined from the spur points in the continuous shear rheograms did indicate such changes, as did the viscoelastic data, and these parameters were correlated with the sensory test data (Figs. 5 and 6). The cause and significance of yield stresses are not fully known. Davis (12) and Barry and Grace (5) correlated yield stresses with retardation times determined in creep; a linear relationship was found between yield stress and temperature (6). Yield stresses are a function of some property of the test material and not simply an instrumental artifact (6). A yield stress probably represents the maximum stress that a test material may support before gross structural deformation occurs, and the spur points from which yield stresses are derived form at low rates of shear. It is thus apparent that, for the materials used here, low shear rate data are more valuable for correlation with sensory data than are high shear rate data. Continuous shear techniques at relatively high shear rates are often assumed to be more valuable than small strain experiments in textural tests, because the shearing conditions are reported to approximate closely to consumer usage (19, 20). However, the present work indicates that this correlation is not necessarily valid. The rate of shear attained during application of a material to the skin depends inversely on the consistency of the material. Thus, low shear rate studies are necessary for correlation with sensory evaluations of preparations of high consistency. For example, recent data on sensory testing of semisolid creams (21, 22) indicated that the sensory determination of consistency was best correlated with instrumental data using the initial shear stress (a parameter derived with viscometers limited to discontinuous shear rate variation and which is equivalent to a yield stress).

SUMMARY AND CONCLUSIONS

The consistency of four grades of white soft paraffin was reduced by working and increased on aging after working. Apparent viscosity data derived in continuous shear using a cone and plate viscometer were not suitable for following such changes, partly due

to the phenomena of sample fracture and/or sample expulsion from the cone-plate gap of the viscometer. Continuous shear yield stresses, which are formed at low rates of shear, did indicate the breakdown of structure within soft paraffin caused by working and the subsequent structural recovery with time.

Significant structural–rheological changes were found after working by utilizing small strain viscometry. Discrete viscoelastic parameters, J_0 and η_0', were used to describe the changes in consistency of the materials and to determine whether these changes were due mainly to elastic or viscous processes. These parameters were not useful for studying changes in structure at a molecular level, but they were correlated with sensory parameters for one grade of white soft paraffin. Continuous spectra of retardation times, which are more fundamental viscoelastic functions, indicated changes that occurred in the molecular retardation processes. The advantage of continuous spectra is that they may be derived from creep curves which have not attained a steady state of flow; they may also be used as an approximate measure of the viscoelastic behavior of nonlinear viscoelastic materials. To obtain a full discrete spectrum analysis, using, for example, the method of Warburton and Barry (23), the creep curve must attain a steady state of flow, which may take up to 4 hr. for white soft paraffin under certain conditions. In systems that are changing rapidly with time, it is thus not feasible to use full discrete spectra to characterize their rheological properties.

The investigation has shown that manufacturers of semisolid materials could standardize the structural state of their products more effectively using viscoelastic techniques rather than continuous shear viscometry; thus, they could control more closely the rheological and sensory properties of their products. An alternative method for determination of viscoelastic properties of creams, ointments, pastes, and other semisolids using penetration testing has been suggested (24). The combination of viscoelastic and textural studies may be valuable for determining rheological properties of semisolids which will ensure maximum consumer acceptance of a product.

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Enzyme Inhibitors XXIV: Bridging Hydrophobic and Hydrophilic Regions on Adenosine Deaminase

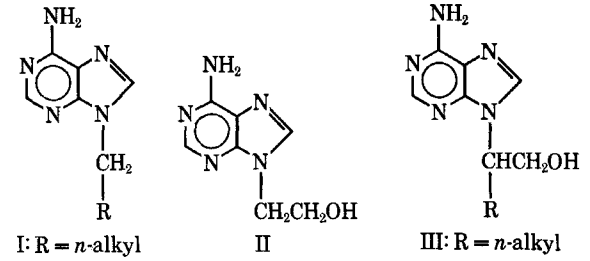
HOWARD J. SCHAEFFER* and CHARLES F. SCHWENDER†

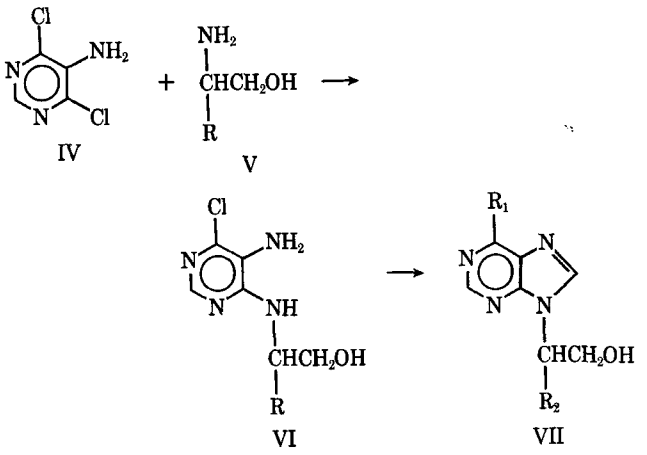
Abstract □ Previous studies have shown that adenosine deaminase has a large hydrophobic region which is important for enzyme inhibitor complex formation with some 9-*n*-alkyladenines. Further, the enzyme has a hydroxyl binding site which makes a contribution to enzyme inhibitor complex formation in a compound such as 9-(2-hydroxyethyl)adenine. In an attempt to gain information concerning the spatial relationship of these two binding regions, a variety of 9-(1-hydroxy-2-alkyl)adenines were prepared and found to be potent inhibitors of adenosine deaminase. These data argue strongly for the presence of a single binding site for the adenine moiety of the inhibitors and for the close spatial relationship of the hydrophobic region and the hydroxyl binding region on the enzyme.

Keyphrases □ Adenosine deaminase inhibitors—9-(1-hydroxy-2-alkyl)adenines, single binding site–bridging mechanism, theoretical □ Enzyme inhibitors—single binding site–hydrophobic–hydrophilic bridging, theoretical □ 9-(1-Hydroxy-2-alkyl)adenines—synthesis, adenosine deaminase inhibitors

Calf intestinal mucosal adenosine deaminase was found to possess several different types of binding regions for the 9-substituent of some 6,9-disubstituted purines (1). By the use of different inhibitors, it was possible to show the presence on adenosine deaminase of a specific methyl binding region (2), a hydroxyl binding site (3, 4), and a large hydrophobic region (5) which are important in forming complexes with the 9-substituent of the 9-substituted adenines.

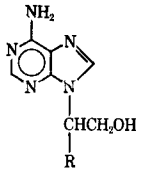
Since there are several different binding regions for the 9-substituent, it is possible that there are, in fact, three different binding sites for the three classes of inhibitors. However, the possibility also exists that the adenine moiety of these inhibitors always complexes to the same site on the enzyme and the 9-substituent bridges to the appropriate accessory binding region. It should be possible to obtain information concerning the identity or nonidentity of inhibitor binding sites by incorporating in one inhibitor the moieties that contribute to binding in two different inhibitors. 9-*n*-Alkyladenines (I) constitute a class of inhibitors in which the 9-substituent forms complexes with the large hydrophobic region on adenosine deaminase (5) and the hydroxyl group of 9-(2-hydroxyethyl)adenine is important in the formation of complexes with the hydroxyl binding site on the enzyme (2, 4). If the adenine moiety of these two types of inhibitors (I and II) complexes to the same site on the enzyme, and the accessory binding regions (hydrophobic and hydroxyl binding sites) are spatially closely associated with the site to which the adenine moiety binds, one would predict that an inhibitor bearing an alkyl chain and the 2-hydroxyethyl group at the 9-position of adenine (III) would be a potent inhibitor of adenosine deaminase. The purposes of this paper are: (*a*) to describe the synthesis of some 9-(1-hydroxy-2-alkyl)-





Scheme I

Table I—Inhibition of Adenosine Deaminase by:



Compound[a]	R	$(I/S)_{0.5}$[b]	$\Delta G/CH_2$[c] (Calc.)
II[d]	H	1.1 ± 0.05[e]	—
VIII	CH_3	1.2 ± 0.03	—
IX	C_2H_5	0.49 ± 0.02	−521
X	C_3H_7	0.071 ± 0.004	−1140
XI	C_4H_9	0.033 ± 0.006	−451
XII	C_5H_{11}	0.015 ± 0.001	−467
XIII	C_6H_{13}	0.0062 ± 0.0002	−521
XIV	C_7H_{15}	0.0047 ± 0.00003	−163
XV	C_8H_{17}	0.0039 ± 0.00002	−110
XVI	C_9H_{19}	0.0030 ± 0.00006	−155

[a] None of these compounds served as substrates of adenosine deaminase. [b] The inhibition index $(I/S)_{0.5}$ is the ratio of the millimolar concentration of the inhibitor for 50% inhibition to the millimolar concentration of the substrate. The concentration of adenosine in all experiments was 0.066 m*M*. [c] Calculated by the equation

$$\Delta G = -RT \ln \left[\frac{(I/S)_{0.5} \text{ for Compound A}}{(I/S)_{0.5} \text{ for Compound B}}\right]$$

where A and B differ by one methylene group. [d] Data taken from *Reference 3*. [e] Average of two determinations.

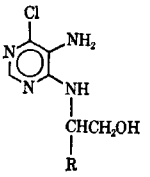


Table II—Physical Constants and Analytical Data of:

Compound[a]	R	Melting Point	Yield, %	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
XVII	CH_3	74–77°	82	H_2O	$C_7H_{11}ClN_4O$	C, 41.50 H, 5.47 Cl, 17.50 N, 27.66	C, 41.34 H, 5.51 Cl, 17.33 N, 27.63
XVIII	C_2H_5	164°[b]	47	H_2O	$C_8H_{13}ClN_4O$	C, 44.34 H, 6.05 Cl, 16.36 N, 25.86	C, 44.19 H, 6.15 Cl, 16.15 N, 25.88
XIX	C_3H_7	89–90°	48	MeOH–H_2O	$C_9H_{15}ClN_4O$[c]	C, 43.46 H, 6.87 Cl, 14.26 N, 22.53	C, 43.33 H, 6.85 Cl, 14.66 N, 22.40
XX	C_4H_9	102–109°	88	MeOH–H_2O	$C_{10}H_{17}ClN_4O$[d]	C, 46.67 H, 7.44 Cl, 13.15 N, 20.75	C, 47.08 H, 7.50 Cl, 13.37 N, 20.68
XXI	C_6H_{13}	102–105°	75	MeOH–H_2O	$C_{12}H_{21}ClN_4O$	C, 52.83 H, 7.77 Cl, 12.99 N, 20.54	C, 52.62 H, 7.73 Cl, 13.23 N, 20.61
XXII	C_7H_{15}	105–108°	71	MeOH–H_2O	$C_{13}H_{23}ClN_4O$	C, 54.44 H, 8.08 Cl, 12.36 N, 19.61	C, 54.34 H, 7.83 Cl, 12.52 N, 19.36
XXIII	C_8H_{17}	103–107°	70	MeOH–H_2O	$C_{14}H_{25}ClN_4O$	C, 55.90 H, 8.37 Cl, 11.79 N, 18.63	C, 56.00 H, 8.30 Cl, 11.66 N, 18.82
XXIV	C_9H_{19}	109–113°	64	MeOH–H_2O	$C_{15}H_{27}ClN_4O$	C, 57.21 H, 8.63 Cl, 11.26 N, 17.79	C, 57.26 H, 8.68 Cl, 11.41 N, 17.56

[a] These compounds were all prepared by minor modification of Method A from 5-amino-4,6-dichloropyrimidine and the appropriate 2-amino-1 alkanol. [b] Melting point taken on Kofler Heizbank. [c] Isolated as the monohydrate. [d] Isolated as $\frac{1}{2}$ $H_2O \cdot \frac{1}{2}$ CH_4O.

adenines (III), and (*b*) to provide evidence that, for adenosine deaminase, there is a single inhibitor binding site for, at least, several classes of inhibitors.

CHEMISTRY

The compounds required for this study were synthesized by the general procedure (1–7) of condensation of an appropriate amino alcohol (V) with 5-amino-4,6-dichloropyrimidine (IV) (Scheme I). The resultant substituted pyrimidine VI was cyclized with triethyl orthoformate to the 6-chloropurine intermediate (VII: R_1 = Cl), which, upon treatment with a variety of nucleophilic agents, gave the desired 6-substituted purines (VII). In all cases, the adenine derivative was prepared. In selected cases, the 6-substituent was methylamino, dimethylamino, hydroxy, mercapto, methoxy, hydrazino, or hydrogen. The amino alcohols (V) employed were the homologs from 2-amino-1-propanol through 2-amino-1-undecanol; *i.e.*, R equals CH_3 through C_9H_{19}.

DISCUSSION

Previous studies have shown that the hydroxyl binding site and the large hydrophobic site of adenosine deaminase cannot be bridged by adenine derivatives which are substituted at the 9-position by a straight-chain alkyl group bearing a hydroxyl group on carbon 2 (8). Examination of Table I, however, clearly demonstrates the dramatic increase in potency of inhibition that can result by incorporation of several moieties previously found to facilitate enzyme inhibitor complex formation into a single inhibitor molecule. For example, II has an $(I/S)_{0.5}$ of 1.1 and 9-*n*-heptyladenine (5) has an $(I/S)_{0.5}$ of 0.32, but the $(I/S)_{0.5}$ for XIII is 0.0062. The enhanced inhibitory properties of the 9-(1-hydroxy-2-alkyl)adenines are interpreted to be the results of a single binding site for the adenine moiety in compounds of general structure I, II, or III and of the close spatial relationship of the large hydrophobic region and the hydroxyl binding site to the site to which adenine is bound on the enzyme[1].

For this potent series of compounds, it was hoped that the effect of the 6-substituent on the magnitude of inhibition of adenosine deaminase could be accurately assessed. Therefore, several series were prepared with different substituents at the 6-position of the purine nucleus, particularly the 9-(1-hydroxy-2-octyl)- and the 9-(1-hydroxy-2-undecyl)-6-substituted purines. Unfortunately, those analogs substituted at the 6-position by dimethylamino, methoxy, mercapto, hydroxy, or chloro were either inactive or too weakly inhibitory to allow an accurate evaluation of $(I/S)_{0.5}$. The 6-hydrazino and 6-hydrogen analogs were sufficiently insoluble that reliable data on inhibition could not be obtained, although both substituents did cause weak inhibition when compared to the 6-amino derivative. The 6-methylamino derivatives exhibited 9–15 times less inhibition of adenosine deaminase when compared to the corresponding 6-amino analogs.

Two other points require brief comment. Normally, hydrophobic forces are considered to be relatively weak interactions. If one considers a single methyl or methylene, it is generally true that hydrophobic forces are relatively weak (9, 10). However, if the macromolecular system possesses a large hydrophobic region, the sum of

[1] The close spatial relationship of the hydrophobic region and the hydroxyl binding region may be native or may be the result of a conformational change in the enzyme induced by the enzyme inhibitor complex.

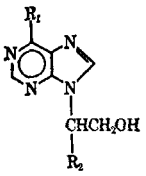


Table III—Physical Constants and Analytical Data of:

Compound	R_1	R_2	Melting Point	Yield, %	Recrystallization Solvent[a]	Method	Formula	Analysis, % Calc.	Analysis, % Found
XXV	Cl	CH_3	201–204°	47	$CHCl_3$	B	$C_8H_9ClN_4O$	C, 45.19 H, 4.22 Cl, 16.68 N, 26.35	C, 45.20 H, 4.40 Cl, 16.58 N, 26.13
VIII	NH_2	CH_3	209–212°	50	EtOH	C	$C_8H_{11}N_5O$	C, 49.73 H, 5.74 N, 36.25	C, 50.06 H, 6.02 N, 36.31
XXVI	NHMe	CH_3	158–160°	60	A	D	$C_9H_{13}N_5O$	C, 52.17 H, 6.33 N, 33.80	C, 51.91 H, 6.39 N, 34.07
XXVII	NMe_2	CH_3	106.5–108.5°	78	B	E	$C_{10}H_{15}N_5O$	C, 54.27 H, 6.83 N, 31.65	C, 54.35 H, 6.97 N, 31.90
XXVIII	OH	CH_3	232.5–235.5°	72	H_2O	F	$C_8H_{10}N_4O_2$	C, 49.47 H, 5.18 N, 28.85	C, 49.48 H, 5.37 N, 29.00
XXIX	SH	CH_3	272–274°[b]	44	iso-PrOH	G	$C_8H_{10}N_4OS$	C, 45.69 H, 4.81 N, 26.64 S, 15.24	C, 45.92 H, 5.00 N, 26.52 S, 15.30
XXX	Cl	C_2H_5	136°[c]	26	A	B	$C_9H_{11}ClN_4O$	C, 47.68 H, 4.89 Cl, 15.64 N, 24.71	C, 47.89 H, 4.69 Cl, 15.43 N, 24.83
IX	NH_2	C_2H_5	185–188°	26	EtOH	C	$C_9H_{13}N_5O$	C, 52.16 H, 6.32 N, 33.80	C, 52.22 H, 6.43 N, 33.93
XXXI	NHMe	C_2H_5	123–125°	52	B	D	$C_{10}H_{15}N_5O$	C, 54.27 H, 6.83 N, 31.65	C, 54.06 H, 6.86 N, 31.60
XXXII	NMe_2	C_2H_5	112–115°	73	B	E	$C_{11}H_{17}N_5O$	C, 56.14 H, 7.28 N, 29.77	C, 56.19 H, 7.29 N, 29.63
XXXIII	OH	C_2H_5	196–198°	44	MeOH	F	$C_9H_{12}N_4O_2$	C, 51.92 H, 5.81 N, 26.91	C, 51.63 H, 5.52 N, 26.70
XXXIV	SH	C_2H_5	280–285°	47	*n*-PrOH	G	$C_9H_{12}N_4OS$	C, 48.19 H, 5.39 N, 24.98 S, 14.30	C, 48.40 H, 5.49 N, 24.76 S, 14.47
XXXV	Cl	C_3H_7	112–114°	62	A	B	$C_{10}H_{13}ClN_4O$	C, 49.90 H, 5.43 Cl, 14.73 N, 23.28	C, 50.04 H, 5.52 Cl, 15.05 N, 23.29
X	NH_2	C_3H_7	147–148°	58	C	C	$C_{10}H_{15}N_5O$	C, 54.27 H, 6.83 N, 31.65	C, 54.03 H, 6.51 N, 31.59
XXXVI	Cl	C_4H_9	77–81°	70	A	B	$C_{11}H_{15}ClN_4O$	C, 51.87 H, 5.88 Cl, 13.92 N, 22.00	C, 51.85 H, 5.93 Cl, 14.09 N, 21.82
XI	NH_2	C_4H_9	149–152°	44	A	C	$C_{11}H_{17}N_5O$	C, 56.10 H, 7.28 N, 29.77	C, 56.36 H, 7.11 N, 29.65
XII	NH_2	C_5H_{11}	158–161°	47	D	C	—[d]	C, 53.05 H, 6.87 N, 23.80	C, 52.91 H, 6.86 N, 23.68
XXXVII	Cl	C_6H_{13}	72–74°	87	A	B	$C_{13}H_{19}ClN_4O$	C, 55.21 H, 6.77 Cl, 12.54 N, 19.81	C, 55.09 H, 6.84 Cl, 12.79 N, 20.08
XIII	NH_2	C_6H_{13}	150–152°	49	E	C	$C_{13}H_{21}N_5O$	C, 59.30 H, 8.04 N, 26.60	C, 59.31 H, 7.93 N, 26.54
XXXVIII	NHMe	C_6H_{13}	108–112°	68	B	D	$C_{14}H_{23}N_5O$	C, 60.61 H, 8.37 N, 25.21	C, 60.45 H, 8.45 N, 25.10
XXXIX	NMe_2	C_6H_{13}	183–184°	85	D	E	—[e]	C, 53.57 H, 7.19 N, 18.35	C, 53.31 H, 7.03 N, 18.28
XL	OH	C_6H_{13}	223–227°	54	E	F	$C_{13}H_{20}N_4O_2$	C, 59.07 H, 7.65 N, 21.16	C, 58.88 H, 7.58 N, 20.99

Table III—(*Continued*)

Compound	R_1	R_2	Melting Point	Yield, %	Recrystallization Solvent[a]	Method	Formula	Analysis, % Calc.	Found
XLI	SH	C_6H_{13}	278–280°[b]	20	F	G	$C_{13}H_{20}N_4OS$	C, 55.68 H, 7.22 N, 19.98 S, 11.44	C, 55.67 H, 7.40 N, 19.79 S, 11.19
XLII	OMe	C_6H_{13}	132–134°	68	A	H	$C_{14}H_{22}N_4O_2$	C, 60.42 H, 7.99 N, 20.13	C, 60.40 H, 8.04 N, 20.27
XLIII	$NHNH_2$	C_6H_{13}	141–144°	88	F	I	$C_{13}H_{22}N_6O$	C, 56.08 H, 7.98 N, 30.19	C, 56.10 H, 8.03 N, 30.30
XLIV	H	C_6H_{13}	65–67°	64	B	J	$C_{13}H_{20}N_4O$	C, 62.88 H, 8.14 N, 22.57	C, 63.05 H, 8.27 N, 22.51
XLV	Cl	C_7H_{15}	85–89°	65	A	B	$C_{14}H_{21}ClN_4O$	C, 56.65 H, 7.13 Cl, 11.95 N, 18.88	C, 56.48 H, 7.01 Cl, 12.11 N, 18.60
XIV	NH_2	C_7H_{15}	150–153°	77	F	C	$C_{14}H_{23}N_5O$	C, 60.61 H, 8.38 N, 25.25	C, 60.70 H, 8.65 N, 25.56
XLVI	OH	C_7H_{15}	224–228°	72	F	F	$C_{14}H_{22}N_4O$	C, 60.42 H, 7.98 N, 20.13	C, 60.27 H, 7.99 N, 20.34
XLVII	Cl	C_8H_{17}	87–90°	75	C_6H_{14}	B	$C_{15}H_{23}ClN_4O$	C, 57.93 H, 7.46 Cl, 11.41 N, 18.03	C, 58.17 H, 7.50 Cl, 11.52 N, 17.94
XV	NH_2	C_8H_{17}	148–152°	70	F	C	$C_{15}H_{25}N_5O$	C, 61.82 H, 8.66 N, 24.04	C, 61.58 H, 8.61 N, 23.93
XLVIII	NHMe	C_8H_{17}	107–110°	85	B	D	$C_{16}H_{27}N_5O$	C, 62.92 H, 8.91 N, 22.93	C, 62.78 H, 8.90 N, 22.90
XLIX	OH	C_9H_{17}	226–230°	63	F	F	$C_{15}H_{24}N_4O_2$	C, 61.61 H, 8.28 N, 19.16	C, 61.87 H, 8.47 N, 19.25
L	Cl	C_9H_{19}	91–93°	68	C_6H_{14}	B	$C_{16}H_{25}ClN_4O$	C, 59.16 H, 7.76 Cl, 10.92 N, 17.25	C, 59.26 H, 7.90 Cl, 11.13 N, 17.02
XVI	NH_2	C_9H_{19}	147–150°	73	F	C	$C_{16}H_{27}N_5O$	C, 62.92 H, 8.92 N, 22.93	C, 63.16 H, 9.17 N, 22.63
LI	NHMe	C_9H_{19}	105–110°	81	F	D	$C_{17}H_{29}N_5O$	C, 63.92 H, 9.15 N, 21.93	C, 64.02 H, 9.12 N, 22.02
LII	NMe_2	C_9H_{19}	182–185°	75	D	E	—[f]	C, 56.72 H, 7.85 N, 16.54	C, 56.66 H, 7.84 N, 16.71
LIII	OH	C_9H_{19}	229–231°	60	F	F	$C_{16}H_{16}N_4O_2$	C, 62.71 H, 8.56 N, 18.29	C, 62.54 H, 8.70 N, 18.31
LIV	SH	C_9H_{19}	233–235°	39	F	G	$C_{16}H_{26}N_4OS$	C, 59.58 H, 8.14 N, 17.37 S, 9.94	C, 59.37 H, 7.88 N, 17.19 S, 10.06
LV	$NHNH_2$	C_9H_{19}	138–140°	74	F	I	$C_{16}H_{28}N_6O$	C, 59.98 H, 8.82 N, 26.23	C, 59.78 H, 8.68 N, 25.99

[a] Solvents: A, chloroform and hexane; B, benzene and hexane; C, acetone and hexane; D, isopropyl alcohol containing 1% oxalic acid; E, ethanol and water; and F, methanol and water. [b] Melts with decomposition. [c] Melting point taken on Kofler Heizbank. [d] Isolated as oxalate salt, $C_{12}H_{19}N_5O \cdot {}^1/_2\, C_2H_2O_4$. [e] Isolated as oxalate salt, $C_{15}H_{25}N_5O \cdot C_2H_2O_4$. [f] Isolated as oxalate salt, $C_{18}H_{31}N_5O \cdot C_2H_2O_4$.

the interaction forces due to hydrophobic binding can be considerable. For example, comparison of VIII with XIII; *i.e.*, the change of R from methyl to hexyl results in a change of ΔG of -3.1 kcal. In terms of the enzyme inhibitor dissociation constant, the change in free energy corresponds to a *Ki* of approximately 5×10^{-3} *M*, a considerable effect due mainly to the summation of hydrophobic forces.

Finally, the large hydrophobic region on adenosine deaminase, which is important in enzyme inhibitor complex formation for the hydrocarbon chain in compounds of type III, is most probably the same region involved in complex formation of the 9-*n*-alkyl group in compounds of type I. This statement is based on the observation that the dimension of the hydrophobic region on the enzyme is the same for both sets of compounds. Both sets of compounds (I and III) exhibit significant changes in $\Delta G/CH_2$ as the hydrocarbon chain is lengthened from propyl through heptyl[2]. Further extension of the hydrocarbon chain in either series resulted in minimal changes in $\Delta G/CH_2$. Note the unusually large change in free energy for the additional methylene group when comparing IX to X. The magnitude of this change is beyond simple hydrophobic transfer forces (10) and may reflect a conformational change in the enzyme (1, 11).

[2] For the purpose of comparing chain length of the 9-(1-hydroxy-2-alkyl)adenines to the 9-*n*-alkyladenines, the hydroxymethyl group has been ignored; *i.e.*, XIII has an alkyl chain length of seven. The listing of $\Delta G/CH_2$ for compounds of type I is found in *Reference 5* and for compounds of type III in Table I of this paper.

In summary, the combination of a properly positioned hydrophobic group and hydroxyl group at the 9-position of adenine has resulted in inhibitors of adenosine deaminase with greatly enhanced inhibitory activities.

EXPERIMENTAL[3,4]

Method A: 5-Amino-4-chloro-6-(1-hydroxy-2-propylamino)pyrimidine (XVII)—A mixture of 322 mg. (1.97 mmoles) of 4,6-dichloro-5-aminopyrimidine, 174 mg. (2.29 mmoles) of 1-hydroxy-2-propylamine, and 303 mg. (3.00 mmoles) of triethylamine in 15 ml. of 1-butanol was heated at reflux temperature for 22.5 hr. The reaction mixture was evaporated *in vacuo* to a liquid residue, which crystallized upon trituration with water. The crude solid material was recrystallized from water, giving the pure product.

Method B: 6-Chloro-9-(1-hydroxy-2-propyl)purine (XXV)—To a suspension of 626 mg. (3.08 mmoles) of 5-amino-4-chloro-6-(1-hydroxy-2-propylamino)pyrimidine in 25 ml. of triethyl orthoformate was added 53.4 mg. (0.485 mmole) of ethanesulfonic acid; the mixture was stirred at room temperature for 19 hr. The reaction mixture was cooled, and the precipitate was collected by filtration; the filtrate was evaporated *in vacuo* to a liquid residue which was dissolved in chloroform. *n*-Hexane was added, which gave a second crop of crude material. Recrystallization of the combined fractions from chloroform gave the pure product.

Method C: 9-(1-Hydroxy-2-propyl)adenine (VIII)—A mixture of 367 mg. (1.72 mmoles) of 6-chloro-9-(1-hydroxy-2-propyl)purine and 23 ml. of methanolic ammonia was heated at 90° for 18 hr. in a steel bomb. The reaction mixture was evaporated *in vacuo* and gave a residual solid which was extracted with hot acetone. The acetone extract was evaporated *in vacuo* to give the crude product, which gave the pure crystalline product upon recrystallization from ethanol.

Method D: 6-Methylamino-9-(1-hydroxy-2-propyl)purine (XXVI)—A steel bomb containing 404 mg. (1.90 mmoles) of 6-chloro-9-(1-hydroxy-2-propyl)purine and 20 ml. of aqueous methylamine (40%) was heated at 92° for 16 hr. The reaction mixture was evaporated *in vacuo* and gave a glassy residue which was extracted with hot chloroform. The chloroform extract was concentrated to a small volume, and hexane was added. Cooling gave the white crystalline product, which gave the analytically pure product upon recrystallization from chloroform–hexane.

Method E: 6-Dimethylamino-9-(1-hydroxy-2-propyl)purine (XXVII)—In a steel bomb was heated a mixture of 490 mg. (2.30 mmoles) of 6-chloro-9-(1-hydroxy-2-propyl)purine and 20 ml. of aqueous dimethylamine (25%) for 17 hr. at 100°. The reaction mixture was evaporated *in vacuo* and gave a liquid residue which was extracted with hot benzene. Upon addition of hexane to the extract and cooling, a crystalline precipitate formed. The solid material was recrystallized from benzene–hexane, and a white needlelike solid was obtained as the analytical sample.

Method F: 6-Hydroxy-9-(1-hydroxy-2-propyl)purine (XXVIII)—A reaction mixture of 510 mg. (2.24 mmoles) of 6-chloro-9-(1-hydroxy-2-propyl)purine and 10 ml. of 3 *N* hydrochloric acid was heated at reflux for 10 min. After the reaction mixture had been evaporated *in vacuo* to a residual oil, concentrated ammonium hydroxide (3 ml.) was added to the crude material. This mixture was evaporated *in vacuo*, giving a white solid material which was recrystallized from water, yielding the white crystalline product.

Method G: 6-Mercapto-9-(1-hydroxy-2-propyl)purine (XXIX)—A mixture of 325 mg. (1.52 mmoles) of 6-chloro-9-(1-hydroxy-2-propyl)purine, 116 mg. (1.52 mmoles) of thiourea, and 10 ml. of 1-propanol was heated at reflux for 15 min. The reaction mixture was allowed to cool, and the precipitate which formed was collected by filtration. The material was recrystallized from 1-propanol, giving a white crystalline product as the analytical sample.

Method H: 6-Methoxy-9-(1-hydroxy-2-octyl)purine (XLII)—To a solution of 1.06 g. (3.74 mmoles) of 6-chloro-9-(1-hydroxy-2-octyl)-purine in 20 ml. of methanol, 0.540 g. (10.0 mmoles) of sodium methoxide was added, and the mixture was refluxed 1 hr. The reaction mixture was then cooled in an ice bath and adjusted to neutral pH with 6 *N* hydrochloric acid. The mixture was then evaporated *in vacuo* to a residual solid which was triturated in water (10 ml.). The remaining solid material was collected by filtration. The product was recrystallized from chloroform–hexane, and the analytical product was obtained.

Method I: 6-Hydrazino-9-(1-hydroxy-2-octyl)purine (XLIII)—A mixture of 12 mg. (3.22 mmoles) of 6-chloro-9-(1-hydroxy-2-octyl)-purine and 15 ml. of hydrazine (95%) was cooled at 0° for 10 min. and stirred at room temperature for 2 hr. Cooling the reaction mixture gave a solid precipitate which was collected by filtration. The material was recrystallized from methanol–water and gave the analytical sample.

Method J: 9-(1-Hydroxy-2-octyl)purine (XLIV)—A mixture of 1.01 g. (3.56 mmoles) of 6-chloro-9-(1-hydroxy-2-octyl)purine, 202 mg. (5.00 mmoles) of magnesium oxide, and 200 mg. of 5% palladium-on-charcoal in 200 ml. of ethanol was hydrogenated over 2 hr. until an uptake of the theoretical amount of hydrogen had occurred. The mixture was filtered through a Celite pad, and the catalyst was washed with 100 ml. of hot ethanol. The combined ethanol filtrates were evaporated *in vacuo* to 50 ml. in volume, and 20 ml. of sodium carbonate solution (5%) was added. The mixture was evaporated *in vacuo* to an oily residue. The crude product was dissolved in hot hexane. Cooling gave a white crystalline product. Further recrystallizations of the material from benzene–hexane gave the analytical sample.

2-Amino-1-alkanols—Most of the amino alcohols required for this study were either commercially available or had been described in the literature. The following general procedure was employed for the new amino alcohols.

2-Amino-1-decanol—To a suspension of 13.4 g. (71.7 mmoles) of 2-aminodecanoic acid in 250 ml. of absolute ethanol, cooled at 0°, was added 10.7 g. (90.0 mmoles) of thionyl chloride. The resulting mixture was heated at reflux temperature for 8 hr. The reaction mixture was evaporated *in vacuo* and gave crude solid ethyl 2-aminodecanoate hydrochloride, 17.0 g. (94.2%), which was dissolved in 100 ml. of chloroform and washed with excess cold 5% sodium hydroxide solution and with water (1 × 5 ml.). After the chloroform solution was dried with anhydrous magnesium sulfate, it was evaporated *in vacuo* and gave a quantitative yield of crude ethyl 2-aminodecanoate; ν in cm.$^{-1}$ (film): 1735 (ester).

To a suspension of 3.79 g. (100 mmoles) of lithium aluminum hydride in 2 l. of anhydrous ether was added an ethereal solution (1 l.) of ethyl 2-aminodecanoate (67.5 mmoles). The reaction mixture was refluxed for 7 hr., after which time 25 ml. of water was added slowly. The suspension was filtered, and the solid residue was washed with ether (500 ml.). The ether solutions were combined and evaporated *in vacuo* to give a liquid residue, which was then crystallized and recrystallized from hexane to give white crystalline 2-amino-1-decanol; yield 7.81 g. (67.2%), m.p. 41–46°.

Anal.—Calc. for $C_{10}H_{23}NO$: C, 69.30; H, 13.38; N, 8.09. Found: C, 69.19; H, 13.35; N, 8.17.

By a similar procedure, the following amino alcohols were synthesized.

2-Amino-1-heptanol—This was isolated as the oxalate salt and recrystallized from 2-propanol, m.p. 150–151°.

Anal.—Calc. for $C_7H_{17}NO \cdot C_2H_2O_4$: C, 48.86; H, 8.67; N, 6.34. Found: C, 48.57; H, 8.48; N, 6.55.

2-Amino-1-nonanol—This was isolated as the hydrochloride salt and recrystallized from chloroform–hexane, m.p. 94–96°.

Anal.—Calc. for $C_9H_{21}NO \cdot HCl$: C, 55.15; H, 11.33; N, 7.15; Cl, 18.11. Found: C, 55.40; H, 11.28; N, 6.98; Cl, 18.23.

2-Amino-1-undecanol—This was isolated as the free base and recrystallized from hexane, m.p. 48–52°.

Anal.—Calc. for $C_{11}H_{25}NO$: C, 70.53; H, 13.40; N, 7.48. Found: C, 70.60; H, 13.42; N, 7.29.

Enzyme Assay—Adenosine deaminase (Type I, calf intestinal mucosa) was purchased[5]. The assay procedure for the study of reversible inhibitors was previously described (2–5) and is a modification of the procedure of Kaplan (12) based on the method of Kalckar (13).

[3] General procedures are given for the synthesis of the various types of compounds. The physical constants and analytical data for the compounds are given in Tables II and III.

[4] The melting points, unless noted otherwise, were taken in open capillary tubes on a Mel-Temp apparatus and are uncorrected. All analytical samples had IR and UV spectra compatible with their assigned structures and moved as a single spot on TLC on Brinkmann silica gel. Analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

[5] Sigma Chemical Co.

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Chemical and Pharmacological Evaluation of *Banistereopsis argentea* Spring ex Juss

S. GHOSAL*, U. K. MAZUMDER, and S. K. BHATTACHARYA†

Abstract □ Seven simple indole bases—*viz.*, *N,N*-dimethyltryptamine (I), its N_b-oxide, harmine (II), harmaline (III), (+)-N_b-methyltetrahydroharman (IV), (+)-tetrahydroharmine (V), and (+)-5-methoxytetrahydroharman (VI)—were isolated from the leaves and stems of *Banistereopsis argentea* Spring ex Juss (Malpighiaceae) and their identity established by physical and chemical methods. In addition to these alkaloids, two partially characterized minor indole-3-alkylamines, together with choline and betaine, were obtained from the same parts of the plant. This was the first demonstration of the occurrence of N_b-methyltetrahydroharman in the family Malpighiaceae and of a 5-methoxytetrahydro-β-carboline alkaloid in nature. The total alkaloids from the leaves have shown three main pharmacological actions: nonspecific spasmolytic action against acetylcholine-, histamine-, and serotonin-induced spasm; histamine release-mediated depressor response; and pronounced behavioral effects.

Keyphrases □ *Banistereopsis argentea* Spring ex Juss—isolation, identification, pharmacological evaluation of seven indole alkaloids □ N_b-Methyltetrahydroharman—isolation, identification from *B. argentea* □ 5-Methoxytetrahydro-β-carboline alkaloid—occurrence in nature, *B. argentea* □ CNS activity—indole alkaloids in *B. argentea* □ TLC—separation, isolation, *B. argentea* alkaloids □ Mass spectrometry—identification, structure

The use of members of the genus *Banistereopsis* (family Malpighiaceae) in the preparation of narcotic and hallucinogenic decoctions by certain Colombian and Peruvian Indian tribes is well known (1). Earlier chemical investigations with a number of *Banistereopsis* species showed (2–5) the presence of the following four indole bases in their various parts: *N,N*-dimethyltryptamine (I), harmine (II), harmaline (III), and (+)-tetrahydroharmine (V). Since then, there has been much speculation (6, 7) as to the contribution of the individual alkaloidal entities to the hallucinogenic activity of the crude preparations. Although the hallucinogenic activity of harmine was demonstrated earlier, the crude preparations of plants containing harmine have, however, appeared to produce greater psychotropic action than pure harmine (6). Moreover, the well-documented hallucinogenic activity of *N,N*-dimethyltryptamine (7) cannot alone account for the strong behavioral changes caused by the plant extracts, because the indole base is only present as a minor constituent in the genus *Banistereopsis*. It seems likely that the strong hallucinogenic activity of the crude plant preparations is due to some unidentified, more potent, extraneous materials introduced or generated chemically during preparation, to other natural products, or to a synergistic action of the mixture.

To extend the knowledge of the content and distribution of the aforementioned and related alkaloids in the genus *Banistereopsis*, the phytochemical and pharmacological evaluation of a rare species, *B. argentea* Spring ex Juss[1], was undertaken. Some recent investigations were directed toward the study of Indian medicinal plants used as psychotherapeutic agents in the Ayurvedic system of medicine. The results, with a number of species belonging to the families Leguminosae and Gramineae, showed the presence of about two dozen indole-3-alkylamines and β-carbolines, several of which were either hallucinogens or MAO inhibitors (8–14). The

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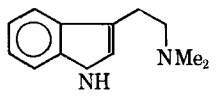
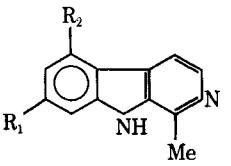
present paper deals with isolation, identification, and pharmacological studies of alkaloids from the stems and leaves of *B. argentea*.

CHEMISTRY

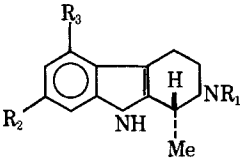
From the leaves of the plant, seven indole-3-alkylamine and β-carboline bases have been isolated to date. These are identified as *N,N*-dimethyltryptamine (I) and its N_b-oxide, harmine (II), harmaline (III), (+)-N_b-methyltetrahydroharman (IV), (+)-tetrahydroharmine (V), and a new tetrahydro-β-carboline alkaloid, (+)-5-methoxytetrahydroharman (VI). The structure of VI was established from its color reactions with alkaloidal reagents; determinations of optical rotation, UV, and mass spectra; and dehydrogenation with palladium-on-charcoal to 5-methoxyharman (VII) which had been confirmed by comparison with a synthetic sample.



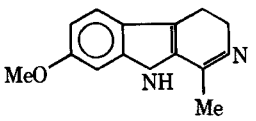
I



II: $R_1 = OMe$; $R_2 = H$
VII: $R_1 = H$; $R_2 = OMe$



IV: $R_1 = Me$; $R_2 = R_3 = H$
V: $R_1 = R_3 = H$; $R_2 = OMe$
VI: $R_1 = R_2 = H$; $R_3 = OMe$



III

This was the first demonstration of the occurrence of a 5-methoxytetrahydro-β-carboline (VI) in nature and of (+)-N_b-methyltetrahydroharman in the family Malpighiaceae. The new naturally occurring base, which was obtained in the (+)-form, was tentatively assigned the R-configuration by analogy with the established steric configuration of (+)-tetrahydroharmine (V) (15) and (+)-tetrahydroharman (IV) (16).

In addition to the aforementioned alkaloids, two partially characterized quaternary indole-3-alkylamines and a liberal amount of choline and betaine were isolated from the leaves of this plant. The stems contain the same alkaloids but in poorer yields.

The presence of both 5- and 7-methoxytetrahydro-β-carbolines in a single plant species such as this is of considerable biogenetic and chemotaxonomic significance. In view of the currently accepted hypothesis regarding the genesis of hydroxy- (or methoxy)-indole-3-alkylamines, involving *N,N*-dimethyltryptamine (17, 18), it would seem likely that the corresponding hydroxylation in β-carbolines, derived from tryptophan (or tryptamine) (19), would take place after the piperidine–pyridine ring (C-ring) formation.

PHARMACOLOGY

Detailed pharmacological study was made only with the total alkaloids (consisting of Fractions A, B, and D). The crude plant extracts elicited only feeble activity, while the individual bases were not available in quantities sufficient for complete screening. The behavioral effects were, however, studied with both total alkaloids and with the tetrahydro-β-carbolines (obtained from Fraction B).

Effect on Smooth Muscles—*Guinea Pig Ileum*—The total alkaloids (10–20 mcg./ml.) produced an initial spasm, which was followed by tachyphylaxis after a second or third dose of the drug. In the same concentrations, the drug showed spasmolytic activity against equispasmodic doses of acetylcholine (0.1 mcg./ml.), histamine (0.1 mcg./ml.), and serotonin (0.5 mcg./ml.). The antagonism followed the order: acetylcholine < histamine < 5-hydroxytryptamine. The initial inhibition of the spasmogens was subsequently followed by marked potentiation. In case of the tetrahydro-β-carbolines, more powerful antagonism to serotonin and subsequent potentiation were observed.

Rat Uterus—The total alkaloids (20–50 mcg./ml.) elicited spasm in isolated estrus uterus of the rat. The spasm was antagonized by pretreatment of the isolated tissue with lysergic acid diethylamide (1 mcg./ml.). In smaller concentrations (0.2–0.5 mcg./ml.), the drug caused facilitation of the serotonin response.

Effect on Skeletal Muscle—*Frog Rectus Abdominis Muscle*—The total alkaloids and those from Fraction D blocked specifically acetylcholine-induced (1 mcg./ml.) spasm in isolated frog rectus abdominis muscle. The drug did not alter the KCl-induced (3 mg./ml.) spasm. The antagonism to acetylcholine was reversible; the spasmolytic ED_{50}'s were 35 (for total alkaloids) and 8.5 (for alkaloids from Fraction D), bufotenidine control (11) being used.

Effect on Heart—*Frog Heart*—No significant action was observed with the total alkaloids (up to a dose of 200 mcg./ml.) on perfused frog heart preparation.

Effect on Blood Pressure, Respiration, and Intestine *In Situ*—The total alkaloids (0.5–1.0 mg./kg. i.v.) produced an initial transient rise and then a sharp fall in blood pressure of the pentobarbital sodium- (35 mg./kg. i.p.) anesthetized dog. The recovery was slow and gradual. Marked bronchoconstriction was observed, which was preceded by a transient respiratory stimulation. In dogs pretreated with polysorbate 80 (0.01 ml./kg.), the depressor response and bronchoconstriction were very much less marked. These observations indicated that the depressor effect was histamine-release mediated (11, 13). When the respiration was artificially maintained, the total alkaloids (1–2 mg./kg.) produced a pressor response which was followed by tachyphylaxis on repeated drug administrations. The pressor response was antagonized by tolazoline[2] (10 mg./kg.) and was absent in reserpinized dogs. The pressor response was, therefore, catecholamine-release mediated.

The intestinal tone was increased during the depressor response; in artificially respired dogs, intestinal relaxation was followed by tachyphylaxis on repeated drug administrations.

Effect on Frog Melanophores—Histamine-releasing activity of the total alkaloids was studied according to the method of Bhide and Gupta (20). *In vivo* administration of the drug (0.5–1.0 mg./kg.) produced, within 15–20 min., yellow coloration of the dorsal skin, intense red coloration of the ventral skin, and punctation of melanophore granules in the web melanophores. The effect was over within 60–90 min. In the case of the water-soluble alkaloids (Fraction D), the histamine-releasing activity was more pronounced and the changes were caused by much lower concentrations (0.2–0.4 mg./kg.).

Hallucinogenic Activity—The initial testing for the psychotropic activity of the total alkaloids and of the tetrahydro-β-carbolines (from Fraction B) was done on estrus rat uterus according to the method of Costa (21)[3]. The test compounds, in low concentrations (0.1–0.2 mcg./ml.), caused, in varying degrees, direct potentiation of serotonin-induced spasm. In higher concentrations, only the total alkaloids caused an initial antagonism to serotonin, while the tetrahydro-β-carbolines had no significant effect on serotonin response.

The total alkaloids, in low concentrations (1–3 mg./kg. i.p.), produced excitation, fine tremors, salivation, and hypermotility in mice and rats. In higher concentrations (5–10 mg./kg.), the drug caused jumping movements, rapid tapping of the forelimbs, paralysis of the hindlimbs, convulsions, and respiratory arrest. In the case of 5-methoxy-*N,N*-dimethyltryptamine, these effects were observed in similar concentrations. A laparotomy, after the respiratory arrest, showed that the heart was still beating although the lungs had collapsed.

These effects, with the exception of the hindlimb paralysis in rats (which might be partly associated with the neuromuscular blocking activity of the alkaloids), were almost completely antagonized by pretreatment with chlorpromazine (5 mg./kg. s.c.) but not by atropine (100 mg./kg.). The effects, in the case of the total alkaloids, were potentiated by nialamide (10 mg./kg.), a known MAO inhibitor. Further, the total alkaloids also antagonized chlorpormazine- and reserpine-induced (5 mg./kg.) sedation in mice.

[2] Priscol.

[3] Costa (21) reported that the facilitation of the spasmogenic activity of serotonin on rat uterus by a drug, in low concentration, was a typical effect of compounds that cause hallucination, *e.g.*, mescaline and lysergic acid diethylamide.

EXPERIMENTAL[4]

The general procedure for the isolation of the alkaloids involved gradient-pH extraction of the $CHCl_3$-soluble bases; gradient-pH precipitation of the reineckates of water-soluble bases (22); regeneration of water-soluble bases by passing the reineckate salts in acetone or alcohol over De-Acidite FF resin column (pH 8); and preparative chromatography over neutral alumina (S. Merck, according to Brockmann specification) and magnesia-silica gel[5] columns and over silica gel G chromatoplates. Identification of the individual entities involved determination of melting point; mixed melting point; co-TLC in the presence of marker compounds; UV, IR, and mass spectra; and preparation of picrate, hydrochloride, and methiodide where possible. In the TLC, methyl alcohol–$CHCl_3$–acetic acid (75:25:15) was used as the developer; Dragendorff (D), Ehrlich (E), and α-nitroso-β-naphtholnitrous acid (N) reagents were used for the staining purposes. Indole-3-alkylamines and tetrahydro-β-carbolines showed light-blue fluorescence under UV light on papers, while aromatic β-carbolines showed violet and 3,4-dihydro-β-carbolines showed sea-green fluorescence. None of the alkaloids from this investigation responded to the Jepson and Stevens test (23) for N_b-unsubstituted tryptamines.

Isolation of Alkaloids—Dried and milled leaves (1.8 kg.) were defatted with petroleum ether (60–80°) and then extracted with ethyl alcohol in a soxhlet for 16 hr. The ethyl alcohol extract was concentrated under reduced pressure to give a viscous brown consistency (78 g.), which was poured into 2% acetic acid (200 ml.) with stirring. The mixture was kept at ordinary temperature overnight. Suspended impurities were filtered off; the clarified solution was shaken with $CHCl_3$ (three 50-ml. portions), which removed the weakly basic $CHCl_3$-soluble acetates (11 g.) (Fraction A). The aqueous acidic solution was basified with NH_4OH, and the liberated bases were extracted with $CHCl_3$ (150 ml.). The $CHCl_3$ extract was washed (H_2O) and dried ($CaCl_2$), and the solvent was removed under reduced pressure. The residue (4.2 g.), a brown basic gum, was marked Fraction B. The aqueous alkaline mother liquor was treated with a saturated aqueous solution of ammonium reineckate, and the light-pink reineckate salt (2.7 g.) was marked Fraction C. The filtrate was acidified (6 *N* H_2SO_4), and more ammonium reineckate solution was added to ensure complete precipitation of the complex salt. The reineckate salt (1.8 g.), obtained under acidic conditions, was marked Fraction D.

Fraction A—The residue from this fraction, containing a mixture of alkaloids, was dissolved in benzene and chromatographed over alumina (35 × 2.5 cm.). Elution was carried out with petroleum ether, benzene, $CHCl_3$, methyl alcohol, and different proportions of mixtures thereof. Forty-milliliter fractions were collected.

***N*$_b$-Methyltetrahydroharman (IV)**—The first few fractions of the $CHCl_3$ eluates gave a pale-yellow oil (37 mg.); R_f 0.51; D, orange, E and N, negative; $[\alpha]_D^{25}$ + 26° (c, 0.72; $CHCl_3$); $\lambda_{max.}$ 225–228, 278, and 293 nm. (log ϵ, 4.50, 3.83, and 3.81); *m/e* 200 (M^+, 58%), significant peaks at *m/e* 199 (100) and 197 (72); yellow picrate from ethyl alcohol–H_2O as needles, m.p. 181–183°; the identity was established by superimposing IR spectra of the base with an authentic specimen and also by determining the mixed melting point of the picrate, which was not depressed.

***N*,*N*-Dimethyltryptamine (I)**—The $CHCl_3$–methyl alcohol (99:1) eluates gave *N*,*N*-dimethyltryptamine as a hygroscopic oil (48 mg.); R_f 0.47; D, orange; E, blue; N, negative; $\lambda_{max.}$ 224, 274, and 294 nm. (log ϵ, 4.48, 3.77, and 3.75); as yellowish-orange picrate needles from methyl alcohol–H_2O, m.p. 166–167°; as colorless methiodide needles from acetone–methyl alcohol, m.p. 216°; mixed melting point of the derivatives with authentic specimens remained undepressed.

***N*,*N*-Dimethyltryptamine-*N*$_b$-oxide**—The *tert*-amine oxide migrated from the column as a hygroscopic solid (26 mg.) with methyl alcohol eluates; R_f 0.88; D, dirty orange; E, cherry red; N, negative; reduction with Zn dust and acetic acid gave *N*,*N*-dimethyltryptamine (co-TLC, mixed melting point of the picrate).

Fraction B—The basic gum showed six Dragendorff-positive spots, R_f 0.32–0.95. The gum was dissolved in 1 *N* HCl (60 ml.), and the acidic solution was extracted with ethyl acetate (three 50-ml. portions). The organic layer was washed and dried (Na_2SO_4), and the solvent was removed under reduced pressure. The residue (1.3 g.) was dissolved in benzene and chromatographed over Florisil (50 g.) using $CHCl_3$, $CHCl_3$–methyl alcohol, and methyl alcohol as eluents.

Harmine (II)—The middle $CHCl_3$ eluates afforded a brown amorphous solid (0.8 g.); R_f 0.73 and 0.86. The major base crystallized from methyl alcohol–H_2O as colorless needles, m.p. 258–260°, mixed melting point with an authentic synthetic sample of harmine (m.p. 260–261°) remained undepressed; R_f 0.73; D, orange; E, negative; N, faint violet; $\lambda_{max.}$ 240, 303, 323, and 338 nm. (log ϵ, 4.61, 4.20, 3.62, and 3.68). The base hydrochloride crystallized from ethyl alcohol as colorless needles, m.p. and mixed m.p. 268–270°.

The mother liquor, after separation of harmine hydrochloride (ethyl acetate), was evaporated to dryness. The residue was taken in H_2O (10 ml.) and basified (NH_4OH). The liberated bases were extracted with $CHCl_3$; the $CHCl_3$ extract showed four Dragendorff-positive spots on TLC. The extract was evaporated to dryness, and the residue was dissolved in benzene (12 ml.) and chromatographed over Florisil (38 g.). $CHCl_3$ and $CHCl_3$ with increasing proportions of methyl alcohol were used as eluents. The first three fractions of the $CHCl_3$ eluates afforded a further crop of harmine (72 mg.). The $CHCl_3$–methyl alcohol (1:1) eluates gave a brown gum (97 mg.) consisting of two bases, R_f 0.62 and 0.66.

(+)-Tetrahydroharmine (V)—The above-mentioned basic gum was dissolved in xylene containing traces of ether; the solution, on keeping at ordinary temperature, gave (+)-tetrahydroharmine as straw-colored microneedles, m.p. 195–198° (16 mg.) [lit. (24) m.p. 199°]; R_f 0.66; D, orange; E, negative; N, dull violet; Fröhde, navy blue; Hopkin–Cole's glyoxalic reagent, purple; $[\alpha]_D^{25}$ + 28° (c, 0.516; $CHCl_3$); $\lambda_{max.}$ 217 and 268–272 nm. (log ϵ, 4.53 and 3.72); *m/e* 216 (M^+, 42%), significant peaks at *m/e* 215 (100), 213 (47), 200 (52), 197 (18), 170 (14), and 169 (52).

(+)-5-Methoxytetrahydroharman (VI)—The xylene–ether mother liquor showed two spots, R_f 0.62 (major component) and 0.66 (traces). The two components were separated by preparative TLC on H_2SO_4 (5%)-treated silica gel G chromatoplates. The major component was obtained as a pale-brown gum (66 mg.), and the corresponding base was obtained in the usual way. It could not be induced to crystallize. The base showed alkaloidal color reactions typical of tetrahydro-β-carbolines: Fröhde, navy blue; Hopkin–Cole, purple; $[\alpha]_D^{25}$ + 34° (c, 0.83; $CHCl_3$); $\lambda_{max.}$ 215–217 and 290 (sh) nm. (log ϵ, 4.55 and 3.66); *m/e* 216 (M^+, 72%), significant peaks at *m/e* 215 (100), 213 (16), 201 (35), and 169 (64).

Dehydrogenation of (+)-5-Methoxytetrahydroharman—A suspension of 10% palladium-on-charcoal catalyst (0.1 g.) and the alkaloid (24 mg.) in xylene (40 ml.) was refluxed for 8 hr., and the mixture was filtered hot. The solvent was removed from the filtrate under reduced pressure. The amorphous brown residue (19 mg.) showed $\lambda_{max.}$ 242–244, 286, 332, and 344 nm. (log ϵ, 4.62, 4.03, 3.71, and 3.73); *m/e* 212 (M^+, 100%), significant peaks at *m/e* 197 (16) and 169 (84), metastable peak observed at *m/e* 145 (197 → 169 transition requires m* at *m/e* 145). The methosulfate crystallized from acetone–methyl alcohol as needles, m.p. 285° [lit. (24) m.p. 285°]; $\lambda_{max.}$ 248–252, 288, 338, 342, and 358 nm. (log ϵ, 4.59, 4.11, 3.68, 3.71, and 3.71); $\lambda_{max.}$ (ethyl alcohol–NaOH) 255, 305, and 340 nm. (ϵ not accurate). The UV spectral data are in excellent agreement with those reported in the literature (24).

Harmaline (III)—The methyl alcohol eluates (from Fraction B) on evaporation gave a brown solid (21 mg.), which was further purified by sublimation *in vacuo*. The pale-yellow prisms sublimed at 110–120°/0.01 mm. and had a melting point of 236–238°. Mixed melting point with an authentic sample of harmaline[6] (m.p. 236–238°) remained undepressed. The identity of the alkaloid as harmaline was further established from co-TLC, R_f 0.38; *m/e* 214 (M^+), and by superimposing the IR spectra on the spectrum of an authentic specimen.

Fraction C—The reineckate complex was dissolved in ethyl alcohol (25 ml.) and was passed through a column of De-Acidite FF (pH 8). Elution was done with the same solvent. The total eluates (120 ml.), on evaporation under reduced pressure, gave a hygroscopic

[4] Melting points were determined on a Toshniwal melting-point apparatus, in open capillary, and are uncorrected. UV spectra were recorded in a Carl Zeiss PMQ II spectrophotometer in spectral EtOH. IR spectra were recorded with a Perkin-Elmer 137 instrument in KBr and mineral oil. Mass spectra were determined with an AEI MS 9 instrument operated at 70 ev. Microanalyses were performed by the Analytical Section, Central Drug Research Institute, Lucknow.

[5] Florisil.

[6] The authors are grateful to Prof. (Mrs.) A. Chatterjee, Calcutta University, for an authentic specimen of harmaline.

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Fraction D—The reineckate salt, precipitated under acidic conditions, was treated in the same manner; a brown basic gum (82 mg.) was obtained. The gum showed three Dragendorff-positive spots on TLC, R_f 0.09, 0.12, and 0.28.

Betaine—An ethyl alcohol solution of the above base was treated with a solution of picric acid in the same solvent, and the mixture was concentrated. Betaine picrate separated as yellow needles, m.p. and mixed m.p. 188–190°.

The ethyl alcohol mother liquor, after separation of betaine picrate, was passed through a column of De-Acidite FF. The regenerated mixture of bases was separated by preparative TLC.

Quaternary Indole-3-alkylamines—The major component (R_f 0.09; D, orange, E, blue; N, violet) showed UV $\lambda_{max.}$ 218, 272 (sh), and 285 nm., characteristic of indole-3-alkylamines. The minor component, (R_f 0.12; D, orange; E, blue; N, dull violet) showed $\lambda_{max.}$ 224–226, 272, 294, and 305–310 (sh) nm., characteristic of 5-oxy-indole-3-alkylamines.

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HARRY L. WELLES, ALEXANDER R. GIAQUINTO, and RICHARD E. LINDSTROM*

Abstract □ The degradation of urea in 2.00, 4.00, 6.00, and 8.00 *M* aqueous solutions was studied at 25.0, 35.0, and 45.0°. Data were obtained by measuring the specific conductivity of the solutions at 6-hr. intervals over 3.5 days. The results show that the degree of degradation is extremely small and that the overall process conforms to a first- and second-order reversible reaction. Rate constants were determined for the forward and reverse reactions and compare favorably to values reported by other workers for the separate reactions.

Keyphrases □ Urea—degradation in concentrated aqueous solution, temperature control □ Conductivity—urea degradation monitoring □ Rate constants—aqueous urea degradation □ Specific conductivity—urea degradation monitoring

The hydrolysis of urea in various aqueous media has been studied extensively. An excellent review and bibliography on the subject are given by Frost and Pearson (1). However, these earlier investigations, including a more recent study by Lynn (2), were concerned with the nature of the hydrolysis reaction as it occurs in relatively dilute solutions of urea and, for the most part, at temperatures between 60 and 100°. As a consequence, little information exists relative to the degradation process under conditions of current interest. Thus, workers who are studying solubility (3–5) and denaturation (6, 7) phenomena in solutions ranging up to 9 *M* in urea, and at temperatures between 25 and 50°, are unable to appraise the untoward effects produced by this reaction. Instead, and because degradation is known to occur, investigators are compelled to initiate their studies with freshly prepared urea solutions in the hope that these effects will be minimized. Since solubility studies, for example, characteristically require days for equilibration, even this technique offers little in the way of reduced uncertainty.

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The hydrolysis of urea in various aqueous media has been studied extensively. An excellent review and bibliography on the subject are given by Frost and Pearson (1). However, these earlier investigations, including a more recent study by Lynn (2), were concerned with the nature of the hydrolysis reaction as it occurs in relatively dilute solutions of urea and, for the most part, at temperatures between 60 and 100°. As a consequence, little information exists relative to the degradation process under conditions of current interest. Thus, workers who are studying solubility (3–5) and denaturation (6, 7) phenomena in solutions ranging up to 9 *M* in urea, and at temperatures between 25 and 50°, are unable to appraise the untoward effects produced by this reaction. Instead, and because degradation is known to occur, investigators are compelled to initiate their studies with freshly prepared urea solutions in the hope that these effects will be minimized. Since solubility studies, for example, characteristically require days for equilibration, even this technique offers little in the way of reduced uncertainty.

Table I—Density and Viscosity of Urea Solutions

Temperature	[Urea], *M*	ρ, g. ml.$^{-1}$	η, cps.
25.0°	0.00	0.997[a]	0.89[a]
	2.00	1.032[b]	0.98
	4.00	1.062[b]	1.09
	6.00	1.090[b]	1.25
	8.00	1.112[b]	1.44
35.0°	0.00	0.994[a]	0.72[a]
	2.00	1.024	0.79
	4.00	1.054	0.89
	6.00	1.083	1.02
	8.00	1.112	1.11
45.0°	0.00	0.990[a]	0.60[a]
	2.00	1.020	0.66
	4.00	1.059	0.74
	6.00	1.078	0.84
	8.00	1.106	0.97

[a] From the "Handbook of Chemistry and Physics," 47th ed., Chemical Rubber Co., Cleveland, Ohio, 1966. [b] Derived from data of F. T. Gucker, Jr., F. W. Gage, and C. E. Moser, *J. Amer. Chem. Soc.*, **60**, 2528(1938).

Bull *et al.* (8), presumably measuring the extent of the reaction:

$$CO(NH_2)_2 \xrightarrow{k_1} NH_4^+ + CNO^- \qquad \text{(Eq. 1)}$$

observed that the specific conductivity of concentrated urea solutions increased linearly with time. Further, they noted that the rate of increase in specific conductivity was proportional to the product *relative viscosity* × *urea concentration*. It appears, then, that urea degradation follows first-order kinetics in both dilute (9) and concentrated solutions. Schwartz and Nelson (10), on the other hand, suggested that the degradation in concentrated solutions is more accurately described as:

$$CO(NH_2)_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} NH_4^+ + CNO^- \qquad \text{(Eq. 2)}$$

The present study was undertaken to: (*a*) test the validity of the suggestion made by Schwartz and Nelson (10), and (*b*) obtain more extensive, quantitative information relative to the degradation of urea in the temperature and concentration ranges frequently cited in the current literature. Because of the relative ease with which conductivity data may be obtained, and in view of the problems certain to arise in a chemical analysis for ammonium cyanate, it was decided to monitor the degradation process by observing the change in the specific conductance of the urea solutions with time.

EXPERIMENTAL

Solutions—Reagent grade urea, without further purification, was dissolved in distilled water having a specific conductance of 1.1 μmho cm.$^{-1}$, to make solutions containing 2.00, 4.00, 6.00, and 8.00 moles/l. of solute, respectively. A fresh set of solutions was prepared for the study at each of the three temperatures used. Each set was prepared in 500-ml. volumetric flasks and at the particular temperature of interest.

Conductivity Measurements—At 6-hr. intervals, 10-ml. samples were withdrawn from the flasks and transferred to a fill-type conductivity cell suspended in the same bath. The cell had been previously calibrated against standard potassium chloride solutions. The conductance was measured using a Beckman model RC-19 bridge, and the observed values were converted to specific conductances using standard procedures. These measurements, at each of the three temperatures, were continued into the 4th day.

Thermostating—All density, viscosity, and conductance measurements were made in a thermostated bath. Temperatures were adjusted to 25.0, 35.0, and 45.0° with the aid of a quartz thermometer. The same instrument indicated a maximum variation of ± 0.01° at all three temperatures.

Densities—The density of each solution was determined at 35.0 and 45.0° using a 50-ml. pycnometer.

Viscosities—Kinematic viscosities of freshly prepared solutions were measured at the three temperatures using an Ostwald viscometer. Viscosities were then calculated relative to the density and flow time of distilled water at the corresponding temperature.

RESULTS

The density and viscosity data obtained in the present investigation are shown in Table I. The densities are in accord with values given by Stokes (11). The viscosity data agree with literature values at 25 (12) and 35° (13). Since they were obtained in the same fashion, it is assumed that the data at 45° are equally valid, at least for the purposes of this work.

The specific conductivity *versus* time data were subjected to a polynomial regression analysis using an IBM 360/60 O.S. computer. The results of the analysis, given in Table II, are of the form:

$$K = a + bt + ct^2 + dt^3 \qquad \text{(Eq. 3)}$$

where K is the specific conductivity, and t is the elapsed time in hours. In view of the corresponding uncertainties in the concentrations, temperatures, densities, and viscosities, the observed conductances were rounded to four significant figures. The data for the 8.00 *M* urea solutions are illustrated in Fig. 1. Note the departure from linearity as the temperature increases and the apparent equilibrium condition approached at 45°. Data for the other solutions describe a similar, though proportionately less pronounced, departure from linearity. In the time allowed, only the 8.00 *M* at 45° reached the equilibrium state.

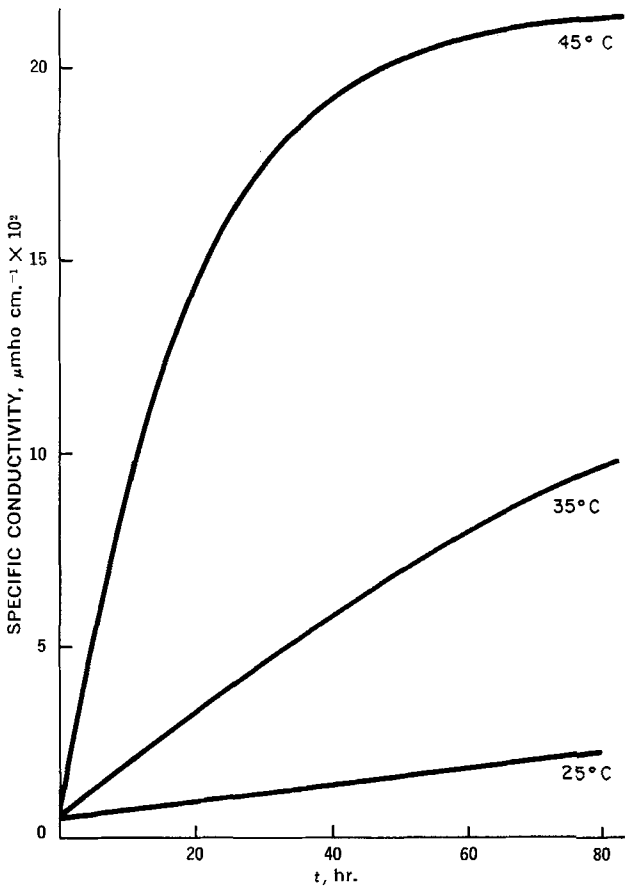

Figure 1—*Specific conductivity of 8.00* M *urea solutions as a function of time at 25, 35, and 45°.*

Table II—Constants for Eq. 3: $K = a + bt + ct^2 + dt^3$

Temperature	[Urea], M	a	b	10^2c	10^4d	Average Deviation, Observed–Calculated, μmho
25.0°	2.00	17.23	0.759			±3.50
	4.00	29.47	1.367			±5.69
	6.00	40.64	1.805			±7.16
	8.00	51.88	2.037			±7.66
35.0°	2.00	22.61	5.111	−0.6416		±1.63
	4.00	17.07	9.781	−1.944		±1.85
	6.00	16.68	14.01	−4.311		±8.88
	8.00	35.48	15.43	−4.794		+4.69
45.0°	2.00	16.19	31.77	−16.76	0.0440	±3.58
	4.00	31.51	61.47	−62.44	21.41	±6.49
	6.00	40.57	78.21	−99.70	44.62	±5.99
	8.00	71.17	94.58	−153.4	85.52	±6.84

DISCUSSION

The data in Table II describe kinetic systems of greater complexity than was indicated by Bull *et al.* (8). At 35 and 45°, for example, the rate of degradation is a decreasing function with time. This suggests that the reverse reaction is a factor in the degradation of concentrated urea solutions, in accordance with Eq. 2. In this event, the net rate expression is given by:

$$dC/dt = k_1M - k_2C^2 \qquad \text{(Eq. 4)}$$

where dC/dt is the rate of appearance of ammonium cyanate, k_1 is the rate constant for the degradation of urea, M is the concentration of urea, k_2 is the rate constant for the formation of urea from ammonium cyanate, and C is the concentration of ammonium cyanate.

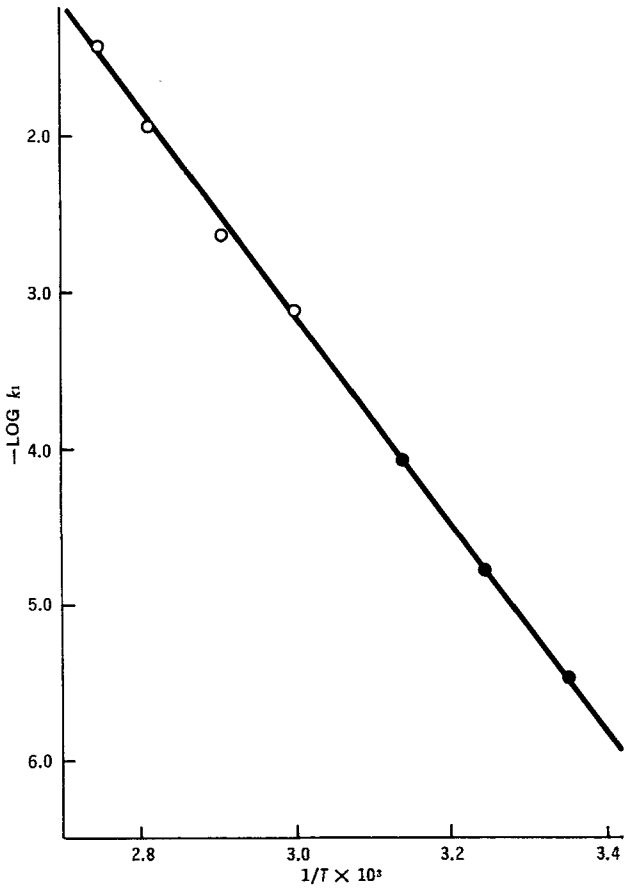

Figure 2—*Arrhenius plot for decomposition of urea in water. Points obtained in the present investigation* (●) *are compared with those reported by Shaw and Bordeaux* (9) (○). *The slope of the line corresponds to* $E_a = 31.6$ *kcal.*

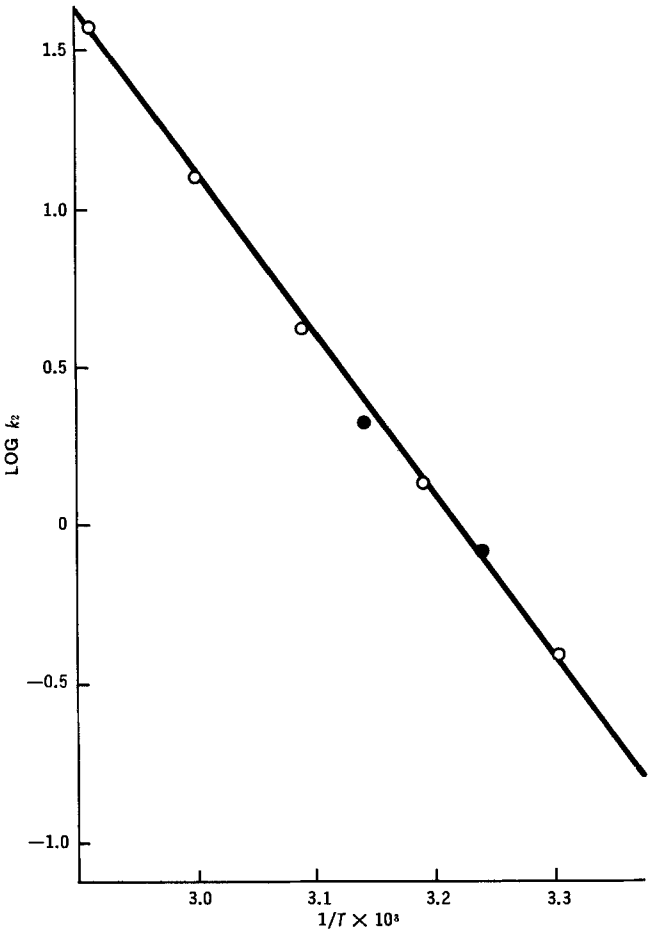

Figure 3—*Arrhenius plot for formation of urea from ammonium cyanate in water. Points obtained in the present investigation* (●) *are compared with those reported by Svirbely and Warner* (18) (○). *The slope of the line corresponds to* $E_a = 23.6$ *kcal.*

Qualitatively, Eq. 4 satisfies the two most obvious criteria established by the experimental observations. First, it can account for the decrease in the overall rate of urea degradation with time at 35 and 45°. Second, it can become a linear function at 25°, where, presumably, the concentration of ammonium cyanate is not great enough to cause a noticeable reverse reaction.

An interpretation of Eq. 4, using the present data, must rely on the relationship:

$$C = 1000K/\Lambda \qquad \text{(Eq. 5)}$$

where C, as before, is the concentration of ammonium cyanate, K is the specific conductance of the solution, and Λ is the equivalent conductance for the conditions that pertain at the time of the measurement. Since Λ will vary among the systems, the solutions to Eq. 5 are not straightforward. Nevertheless, realistic estimates of C can be obtained if two simplifying assumptions are made regarding Λ. These are: (*a*) that Walden's conductance–viscosity rule is applicable, and (*b*) that the equivalent conductance is essentially constant over the range of electrolyte concentrations encountered in this study.

The assumption relative to Walden's rule requires acceptance of a condition in which the movement of ions is a function of viscosity only. However, in view of the variety of solvent systems traditionally used to illustrate this theorem (14), it seems reasonable that the rule will provide good estimates for the purpose at hand. Accordingly, limiting equivalent conductances, Λ_0, for each urea solution at 25, 35, and 45°, were calculated using the relationship:

$$\Lambda_0\eta = \Lambda_0'\eta' \qquad \text{(Eq. 6)}$$

Table III—Limiting Equivalent Conductance of Ammonium Cyanate in Various Solutions of Urea[a]

Temperature	[Urea], M				
	Λ_0, ohm^{-1} cm.^{2}				
	0.00	2.00	4.00	6.00	8.00
25.0°	138[b]	127	113	99	86
35.0°	170	158	139	120	102
45.0°	206	187	166	146	127

[a] Calculated according to Walden's rule. [b] Value obtained from *Reference 15*.

where Λ_0 and η are the limiting equivalent conductance and viscosity, respectively, in one solvent system, and Λ_0' and η' are the corresponding values in a second system. The calculations were based on the viscosities given in Table I and the limiting equivalent conductance of ammonium cyanate in water at 25° (15). Thus, the limiting equivalent conductance of ammonium cyanate in a 2 M urea solution at 25° is given by:

$$\Lambda_0(2\ M\ \text{urea})_{25°} = \frac{\eta(\text{water})_{25°}}{\eta(2\ M\ \text{urea})_{25°}}\ \Lambda_0(\text{water})_{25°} \qquad \text{(Eq. 7)}$$

Table III is a compilation of the Λ_0 values derived in this manner. The values at 25°, when plotted as Λ_0 *versus* M, yield a line with a slope of 6.5 Λ_0/M. This value compares favorably with the 7.0 Λ_0/M value obtained by plotting the experimental data of Bull *et al.* (8) for ammonium chloride at 30° in solutions of the same urea concentration.

The second assumption implies that $\Lambda \approx \Lambda_0$ when, in fact, the two conductances are related by the expression:

$$\Lambda = \Lambda_0 - SC^{1/2} \qquad \text{(Eq. 8)}$$

where S is a function of viscosity, temperature, and dielectric constant; and C is the concentration of the conducting species (16). It is possible to evaluate the corrective term on the right by estimating: (*a*) S using the viscosity data from the present study and dielectric constants for urea solutions (17), and (*b*) C using the specific conductance data in Table II and the corresponding limiting equivalent conductance values in Table III. In so doing, it is found that the slope, S, decreases as temperature and urea concentration increase. However, this is offset by the proportionately higher values of C under the same conditions. The net result is that the maximum error in assuming $\Lambda \approx \Lambda_0$ occurs at the limit of the study involving the 8 M urea solution at 45°. Since the error at that point was calculated to be less than 4%, and because the uncertainty in Λ is lower as the time interval, temperature, and urea concentration are decreased, the assumption was thought to be reasonable. The alternative was to treat Λ as a function of time. However, it was felt that neither the conditions nor the objectives of the investigation warranted this step.

On the basis of these two assumptions, therefore, for a solution of given urea concentration and temperature,

$$dC/dt = (1000/\Lambda_0)\ (dK/dt) \qquad \text{(Eq. 9)}$$

where Λ_0 is the appropriate limiting equivalent conductance given in Table III, and dK/dt is the first derivative of Eq. 2.

Combining Eqs. 4, 5, and 9 yields the expression:

$$dK/dt = \frac{k_1\Lambda_0 M}{1000} - \frac{k_2 1000}{\Lambda_0} K^2 \qquad \text{(Eq. 10)}$$

which predicts a plot of dK/dt *versus* K^2 to be linear, with a slope equal to $-k_2 1000/\Lambda_0$, and an intercept of $k_1 \Lambda_0 M/1000$. While Λ_0 and M will vary from one urea solution to the next, k_1 and k_2 should be constant at any one temperature. Seven values for dK/dt and K^2 were determined at corresponding time intervals for each system studied, using Eq. 3 and the appropriate coefficients in Table II. Each set of dK/dt and K^2 values was then subjected to a linear regression analysis to evaluate the respective slopes and intercepts predicted by Eq. 10. Subsequently, k_1 and k_2 values were determined using the appropriate M and Λ_0. The results are shown in Table IV.

Actual plots of dK/dt *versus* K^2 displayed some scatter. The average deviation between the calculated dK/dt and those predicted by the equations representing the regression analyses ranged from 1 to 4% of the corresponding dK/dt. Because the points were scattered, in contrast to a smooth deviation from linearity, a treatment of Λ as a function of time would seem to offer little in the way of enhanced precision. As might be expected, however, the percent average deviation was observed to increase with urea concentration and temperature.

Table IV—Rate Constants for the Reaction: $(NH_2)_2CO \underset{k_2}{\overset{k_1}{\rightleftharpoons}} NH_4^+ + CNO^-$

Temperature	[Urea], M		$k_1 \times 10^5$, hr.$^{-1}$	k_2, 1 mole^{-1} hr.$^{-1}$
25.0°	2.00		0.298	—
	4.00		0.302	—
	6.00		0.303	—
	8.00		0.292	—
		Av.	0.299 ± 0.004	(0.200)[a]
35.0°	2.00		1.53	0.71
	4.00		1.53	0.73
	6.00		1.83	1.02
	8.00		1.81	0.83
		Av.	1.68 ± 0.14	0.82 ± 0.10
45.0°	2.00		7.92	1.98
	4.00		8.88	1.94
	6.00		7.37	1.92
	8.00		8.00	2.32
		Av.	8.04 ± 0.42	2.04 ± 0.14

[a] Estimated from Fig. 3.

In general, the precision in k_1 is considered to be less as the temperature increases, *i.e.*, as the plots of K *versus* t become more curvilinear. The opposite is true for k_2, since the reverse reaction is more pronounced at the higher temperatures. In the latter regard, a plot of dK/dt *versus* K^2 for the data at 25° has no noticeable slope. The value for k_2 given in Table IV was estimated from Fig. 3.

Figures 2 and 3 compare the rate constants obtained in this investigation with those observed by other workers who studied the forward (9) and the reverse (18) reactions separately and in relatively dilute solutions. In view of the approximations used in the present study, the agreement is excellent, suggesting that the mechanisms involved in the respective reactions are independent of the urea concentration.

CONCLUSIONS

The results of this investigation appear to substantiate the proposal by Schwartz and Nelson (10) that the degradation of urea in concentrated solutions is a reversible reaction. Accordingly, it is to be expected that the overall process will reach an equilibrium at a point in time that is dependent upon the temperature and urea concentration. The indications are that the amount of contaminant produced, presumably in the form of ammonium cyanate, is negligible for many current applications of concentrated urea solutions. Furthermore, unless the experiment demands, and unless the experiment is of relatively short duration, it is pointless to strive for ultrapure urea to be used in preparing concentrated solutions. The observations made in this study show that at 25, 35, and 45°, it would take but 23, 2.5, and 1 hr., respectively, for a solution of ultrapure urea to reach the same specific conductance level as the solutions prepared from reagent grade unrecrystallized urea.

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Combined GLC and High-Resolution Mass Spectroscopic Analysis of Diphenylhydantoin

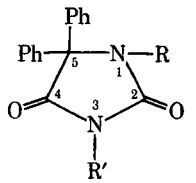
KHALID SABIH* and KHAWLA SABIH*

Abstract □ A sensitive GLC method for the analysis of diphenylhydantoin in biological material is described. This method involves the conversion of diphenylhydantoin to its methyl derivative. The identity of the methyl derivative was determined by combined GLC–mass spectroscopy. The high-resolution mass spectra of diphenylhydantoin methyl derivatives indicate that the methyl groups were introduced at the nitrogen atoms of the hydantoin ring to produce 3-methyl- and 1,3-dimethyldiphenylhydantoin.

Keyphrases □ Diphenylhydantoin and methyl derivatives, analysis in biological material—GLC and mass spectroscopy □ GLC—analysis, diphenylhydantoin and methyl derivatives □ Mass spectroscopy—analysis, diphenylhydantoin and methyl derivatives

Several spectrophotometric, colorimetric, and UV methods to determine levels of diphenylhydantoin (I) in biological material were published (1–5). However, most of these methods are nonspecific and time consuming, and they require many selective extractions to avoid interference of other drugs, *e.g.*, barbiturates. Recently, a GLC method was reported (6) which involved the conversion of diphenylhydantoin to its methoxy derivative by treatment with diazomethane. However, the structure of the methylated derivative has not been determined. In a recent report (7), a direct GLC method was described for the determination of the drug at therapeutic levels in blood. Another recent report (8) described a GLC method for the determination of diphenylhydantoin in which the drug was methylated with tetramethylammonium hydroxide. The corresponding peak of the methylated drug was identified by NMR and mass spectroscopy. However, no discussion of the mass spectra was included.

This report describes a more sensitive GLC method which measures even subtherapeutic levels of the drug in biological material. However, when using GLC for qualitative determination of drugs in biological material, it is necessary to have parameters other than the retention time of the drug in order to make a more certain identification of the drug. This is especially true if derivatives of the drugs are being analyzed when more than one reactive center is available in the drug molecule. Mass spectroscopy, therefore, was utilized as a tool for identification of diphenylhydantoin derivatives.



I: R = R′ = H; diphenylhydantoin
II: R = H, R′ = CH_3; 3-methyldiphenylhydantoin
III: R = R′ = CH_3; 1,3-dimethyldiphenylhydantoin

EXPERIMENTAL

Reagents—Diphenylhydantoin[1] and dimethyl sulfate[2] were used. Heptane and chloroform were redistilled before use. Acetate buffer (0.2 *M*, pH 5.6) was made by mixing 4.8 ml. of acetic acid solution (0.2 *M*, 11.55 ml. in 1000 ml. water) and 45.2 ml. of sodium acetate solution (0.2 *M*, 16.4 g. in 1000 ml. water), and the mixture was diluted with water to 100 ml. Methanolic potassium carbonate solution was made by mixing 1 ml. of 2–5% aqueous solution of potassium carbonate and 9 ml. of methanol (analytical reagent).

Preparation of Methyl Derivatives of Diphenylhydantoin—*Monomethyl Derivative*—A solution of 1.0 g. diphenylhydantoin in 30 ml. methanolic potassium carbonate was placed in a three-necked, 150-ml. flask fitted with a reflux condenser and a magnetic stirrer. Twenty milliliters of dimethyl sulfate was added, and the reaction was allowed to proceed for 15 min. at 70°. The reaction mixture was then cooled, and methanol was removed under reduced pres-

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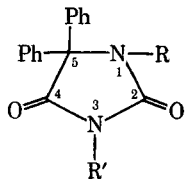

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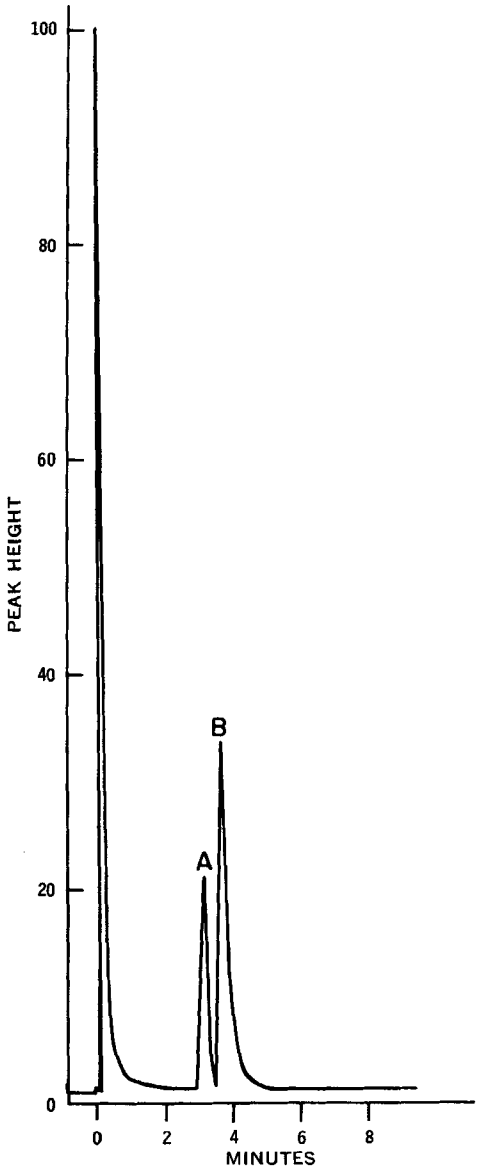


Figure 1—*Gas chromatogram of: (A) dimethyldiphenylhydantoin and (B) monomethyldiphenylhydantoin. The curve represents 5 μl. of a solution of 40 mcg. diphenylhydantoin products in 100 μl. chloroform.*

sure. The resulting mixture was extracted three times with 150-ml. portions of heptane and once with chloroform (100 ml.). The organic layers were mixed and dried over sodium sulfate, and the solvents were removed under reduced pressure to give a white solid material. This material was recrystallized from benzene, m.p. 223°.

Anal.—Calc. for $C_{16}H_{14}N_2O_2$: C, 72.18; H, 5.26; N, 10.52. Found: C, 72.29; H, 5.29; N, 10.62.

The yield was 95% of the monomethyl derivative. GLC of this material, using the DC-200 column, showed the presence of one major component.

Dimethyl Derivative—The procedure described above was used to methylate diphenylhydantoin, except sodium hydroxide was utilized instead of potassium carbonate in concentration ranging between 2.5 and 10%. The procedure gave a mixture of two components, as shown by the presence of two spots on silica gel TLC. Attempts to separate the two components on a silica gel column were not successful. The gas chromatogram of this product, using the DC-200 column, showed the presence of two major peaks (Fig. 1).

Mass Spectra—The high-resolution mass spectra of diphenylhydantoin and its methyl derivatives were determined using an A.E.I. MS-9 double-focusing mass spectrometer. The MS-9 was

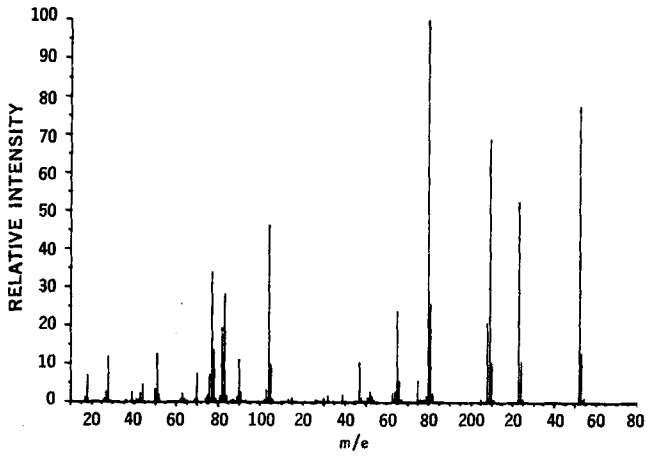


Figure 2—*Mass spectrum of diphenylhydantoin.*

coupled to a digital data system[3]. Computer processing of the digitalized data was carried out on Battelle's CDC-6400 computer. Diphenylhydantoin was introduced by a direct probe operating at 220°, while the methyl derivatives were introduced through a gas chromatograph connected to the MS-9. These derivatives were chromatographed using 0.61-cm. (0.125-in.) stainless steel tubing [3.05 m. (10 ft.) long] packed with 2% OV-17 on Gas Chrom Q. The gas chromatograph (Varian Aerograph model 1740) was connected to the mass spectrometer by means of a McCloskey-type separator (9).

Each component emerging from the column of the gas chromatograph goes directly to the ion source of the mass spectrometer where it is ionized by electron impact. Helium was used as the carrier gas at a rate of 30 ml./min. with the column temperature programmed from 210 to 280 at 2°/min. All of the mass spectra were obtained at 70 e.v. Other operating parameters were: injection port, 210°; ion source, 200°; separator temperature, 250°; and accelerating voltage, 8 kv.

Gas Chromatography—An F&M model 5755 B gas chromatograph, equipped with a flame-ionization detector, was used for routine analyses. The column was 1.4-m., 0.61-cm. (4.5-ft., 0.125-in.) stainless steel tubing packed with Gas Chrom Q 80–100 mesh coated with 3% DC-200. The operating temperatures used for analysis were: column, 200°; and injection port, 310°. Helium was used as the carrier gas with a flow rate of 40 ml./min.

Extraction Procedure—*Standards*—A stock solution of 5 mg. diphenylhydantoin in 10 ml. chloroform was prepared. Aliquots containing 0, 2.5, 5, 10, 20, 50, 80, and 100 mcg. were placed in separate glass-stoppered centrifuge tubes, and solvent was removed

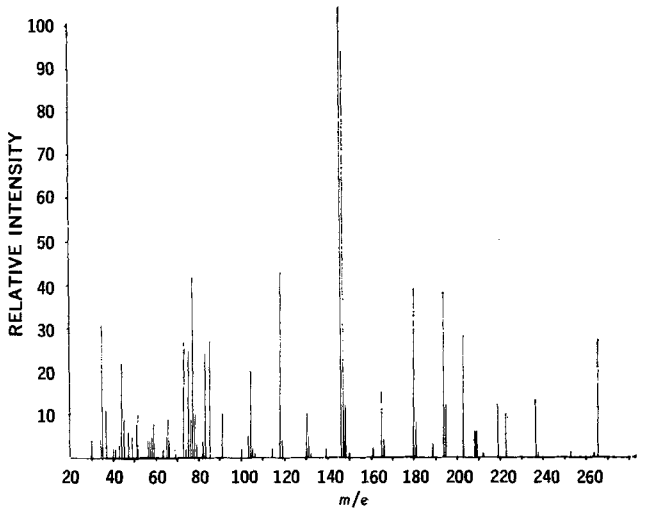


Figure 3—*Mass spectrum of monomethyldiphenylhydantoin.*

[3] Designed and built at Battelle.

Table I—High-Resolution Mass Measurement of Diphenylhydantoin

Measured Mass	Relative Intensity	Calculated Mass	Composition
103.0433	3.24	103.0422	C_7H_5N
104.0513	46.67	104.0500	C_7H_6N
104.5423	3.59	209.0814	Peak doubly ionized
105.0346	2.09	105.0340	C_7H_5O
163.0542	2.42	163.0548	$C_{13}H_7$
165.0678	24.03	165.0664	$C_{13}H_9$
180.0823	100.00	180.0813	$C_{13}H_{10}N$
209.0814	69.31	209.0841	$C_{14}H_{11}NO$
252.0922	78.00	252.0939	$C_{15}N_{12}N_2O_2$ (M^+)

under a stream of air. Each residue was dissolved in 1 ml. of methanolic potassium carbonate followed by 0.1 ml. of dimethyl sulfate. The mixtures were then heated for 5 min. at 70° on a water bath followed by removal of methanol at the same temperature by applying a stream of air. Each residue was extracted with 10 ml. of heptane by shaking on a vortex mixer. Eight milliliters of the heptane layer was taken to dryness, and each residue was dissolved in 100 μl. of chloroform. Samples (1–5 μl.) were injected onto the column under the conditions mentioned previously.

Biological System—From Plasma—A stock solution of 5 mg. diphenylhydantoin in 10 ml. of 5% sodium hydroxide was prepared. The sodium salt of diphenylhydantoin was added to plasma samples in varying amounts (0–40 mcg./ml. plasma). Two milliliters of the resulting mixtures was acidified with 1 ml. of 1 *N* HCl followed by extraction with 10 ml. of chloroform and shaking for 2 min. Layers were separated by centrifugation, and 5–8 ml. of the chloroform solution was taken to dryness. The residues were treated with dimethyl sulfate in the presence of base and chromatographed as mentioned previously.

Blood samples from patients taking the drug were treated similarly, using 1–2 ml. of plasma.

Biological System—From Tissues—Liver samples (0.5–2.0 g.) were weighed and placed in a graduated cylinder; water was added to give a final volume 10-fold greater than the weight of the tissue. The content of the cylinder was transferred to a blending tube and homogenized for 2 min. A 5–10-ml. portion was then transferred to a glass-stoppered centrifuge tube and acidified with 5 ml. of 1 *N* HCl. The mixture was extracted by shaking with 25 ml. of chloroform for 5 min. Layers were separated by centrifugation, and the water layer was aspirated. Chloroform was removed under a stream of air, and the residue was treated with dimethyl sulfate and analyzed using the procedure described previously.

RESULTS AND DISCUSSION

Methylation—The product obtained from the methylation of diphenylhydantoin was dependent on the strength and type of the base used in the reaction. Lower concentrations of methanolic potassium carbonate (2–5%) gave solely the monomethyl derivative of the drug. The gas chromatogram obtained on this product showed one major peak. Microanalysis of this product showed that it was the monomethyl derivative. When higher concentrations of potassium carbonate were used, two products were obtained,

Table II—High-Resolution Mass Measurement of 3-Methyldiphenylhydantoin

Measured Mass	Relative Intensity	Calculated Mass	Composition
103.0417	4.50	103.0422	C_7H_5N
104.0500	19.70	104.0500	C_7H_6N
105.0338	1.65	105.0340	C_7H_5O
146.0591	100.00	—	Background
147.0668	89.95	147.0684	C_9H_9NO
165.0770	14.75	165.0704	$C_{13}H_9$
180.0786	39.17	180.0813	$C_{13}H_{10}N$
208.0722	6.04	208.0762	$C_{14}H_{10}NO$
209.0784	6.00	—	$C_{14}H_{11}NO$
266.0674	31.00	266.0653	$C_{16}H_{14}N_2O_2$ (M^+)

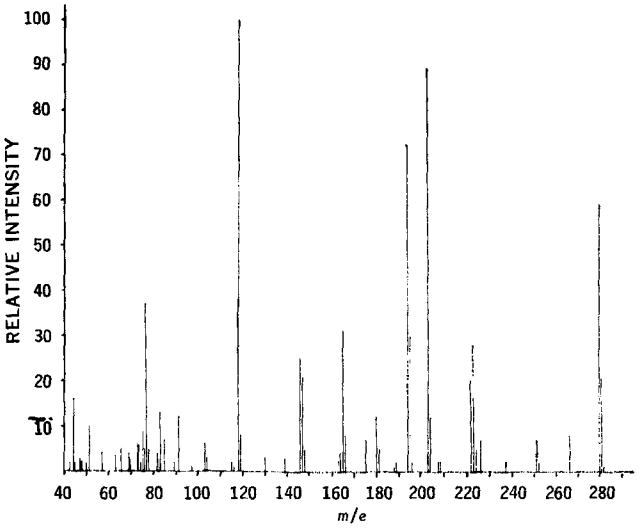


Figure 4—*Mass spectrum of dimethyldiphenylhydantoin.*

with the monomethyl derivative predominant. The use of sodium hydroxide also produced two products as indicated by the presence of two peaks in the chromatogram obtained on this mixture. The first product was predominant and had a shorter retention time (peak A, Fig. 1) relative to the second product (peak B). This second product was identical to that obtained with lower concentrations of potassium carbonate; both were the monomethyl derivative of diphenylhydantoin.

The product that corresponded to peak A (Fig. 1) was unstable if kept in organic solvents, such as chloroform, and decomposed to the methyl derivative corresponding to peak B. This was indicated by a change in peak intensity of Components A and B when the chromatograms of the same solution were obtained on separate days. This may indicate that Component A undergoes decomposition in solution to Component B. For this reason, methylation of diphenylhydantoin in the presence of potassium carbonate for routine GLC purposes is preferred over sodium hydroxide. However, for the determination of diphenylhydantoin metabolites, mainly the *p*-hydroxy derivative, sodium hydroxide is preferred over potassium carbonate for methylation. This is due to the presence of the phenolic hydroxyl group which was also methylated with dimethyl sulfate in the presence of sodium hydroxide but not potassium carbonate.

Mass Spectra of Methyl Derivatives—Samples of the mixture of methylated diphenylhydantoin were studied by the combination of GLC–mass spectroscopy. The two derivatives were separated by GLC, using OV-17 as the stationary phase, and were identified by mass spectroscopy.

The molecular ion (M^+) corresponding to peak A (Fig. 1) was at *m/e* 280 (60%). This molecular ion corresponds to the formula

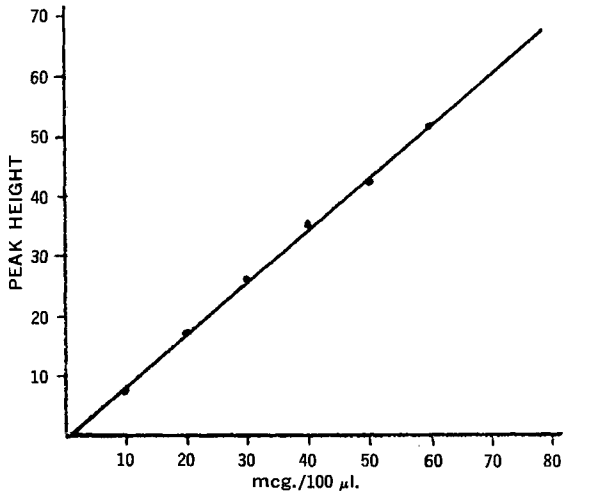


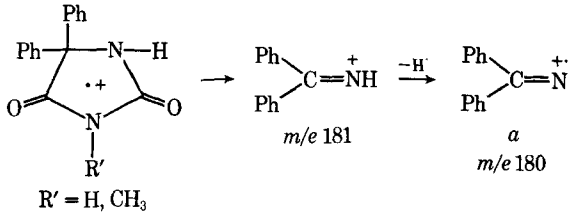
Figure 5—*Relationship between amount of monomethyldiphenylhydantoin injected and peak height. Five microliters injected in each case.*

Table III—High-Resolution Mass Measurement of 1,3-Dimethyldiphenylhydantoin

Measured Mass	Relative Intensity	Calculated Mass	Composition
103.0418	3.90	103.0422	C_7H_5N
104.0512	3.20	104.0500	C_7H_6N
105.0365	0.50	105.0340	C_7H_5O
118.0660	100.00	118.0657	C_8H_8N
165.0684	31.15	165.0704	$C_{13}H_9$
180.0834	12.05	180.0813	$C_{13}H_{10}N$
194.0940	72.94	194.0970	$C_{14}H_{12}N$
209.0777	1.68	—	$C_{14}H_{11}NO$
223.1006	27.58	223.0997	$C_{15}H_{13}NO$
280.1180	60.00	280.1212	$C_{17}H_{16}N_2O_2$ (M^+)

$C_{17}H_{16}N_2O_2$, which indicates the addition of two methyl groups to diphenylhydantoin. The molecular ion coresponding to peak B (Fig. 1) was at *m/e* 266 (27%) which corresponds to the formula $C_{16}H_{14}N_2O_2$. This formula indicates the addition of one methyl group to diphenylhydantoin.

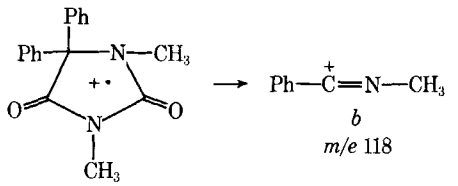
The mass spectra of diphenylhydantoin (Fig. 2) and of the monomethyl derivative (Fig. 3) show a very intense peak at *m/e* 180 ($C_{13}H_{10}N$), but it is very weak in the mass spectrum of the dimethyl derivative (Fig. 4). This peak may correspond to ion *a*. The formation of ion *a* upon electron impact of diphenylhydantoin monomethyl derivative may indicate that the methyl group may be attached to N-3 of the hydantoin ring (Scheme I) and that N-1 has no



Scheme I

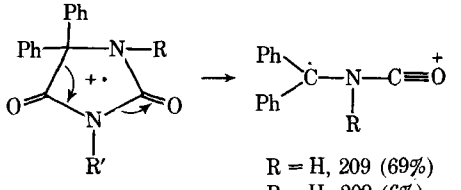
substituent as is the case with diphenylhydantoin itself. Similar results were reported by Grimmer *et al.* (10).

The presence of a very intense peak at *m/e* 118, C_8H_8N in the mass spectrum of the dimethyl derivative (Fig. 4), probably corresponds to ion *b* (Scheme II). Therefore, the additional methyl group



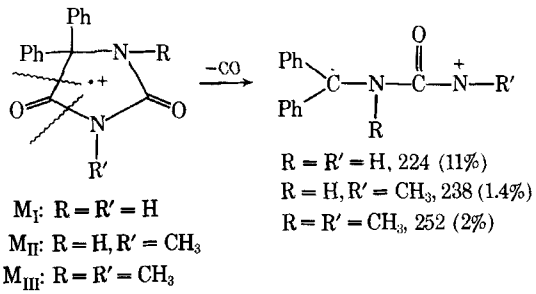
Scheme II

present in the compound corresponding to peak A is undoubtedly located on the nitrogen adjacent to the carbon carrying the two phenyl groups. Thus, it appears that the methyl groups were introduced at the nitrogen atoms of the hydantoin ring to form 3-methyl- and 1,3-dimethyldiphenylhydantoin. These two compounds have some common fragmentation patterns upon electron impact. Both compounds undergo loss of the C_2H_3NO fragment to give peaks at *m/e* 209 and 223, respectively. The most probable structure that corresponds to these ions is shown in Scheme III.



Scheme III

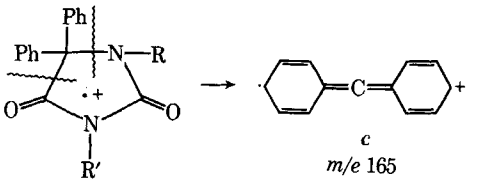
Both derivatives exhibit M—CO and M—HCO similar to that of diphenylhydantoin. Since there are two carbonyl groups in the hydantoin ring, either one could be lost upon electron bombardment. Thus, more than one possible structure could be drawn for such ions. However, loss of the carbonyl group at position 4 would give rise to a radical at C-5, which is highly stabilized by the two phenyl rings at that position. Thus, the possible structures of M—CO of the three compounds is shown in Scheme IV.



Scheme IV

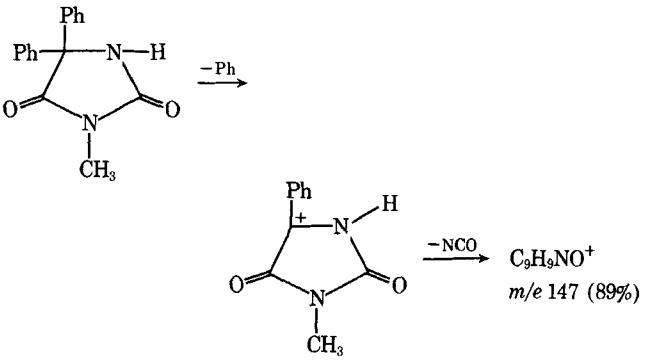
The mass spectra of diphenylhydantoin and its methylated derivatives show a peak at *m/e* 105, which corresponds to C_7H_5O, which may correspond to benzoyl ion (Ph—C≡O$^+$). This ion may originate only if one of the two phenyl groups migrates to the adjacent carbonyl group to produce a benzoyl urea derivative which may give rise to the benzoyl ion. However, the intensity of the peak at *m/e* 105 is relatively weak. Skeletal rearrangements of this type were reported for several other compounds (11–16).

The mass spectra of diphenylhydantoin and its methyl derivatives show a peak at *m/e* 165 ($C_{13}H_9$). This peak may correspond to ion *c*. The possible origin of this ion is shown in Scheme V.



Scheme V

The mass spectrum of 3-methyldiphenylhydantoin shows an intense peak at *m/e* 147 (89%). The possible origin of this peak is shown in Scheme VI.

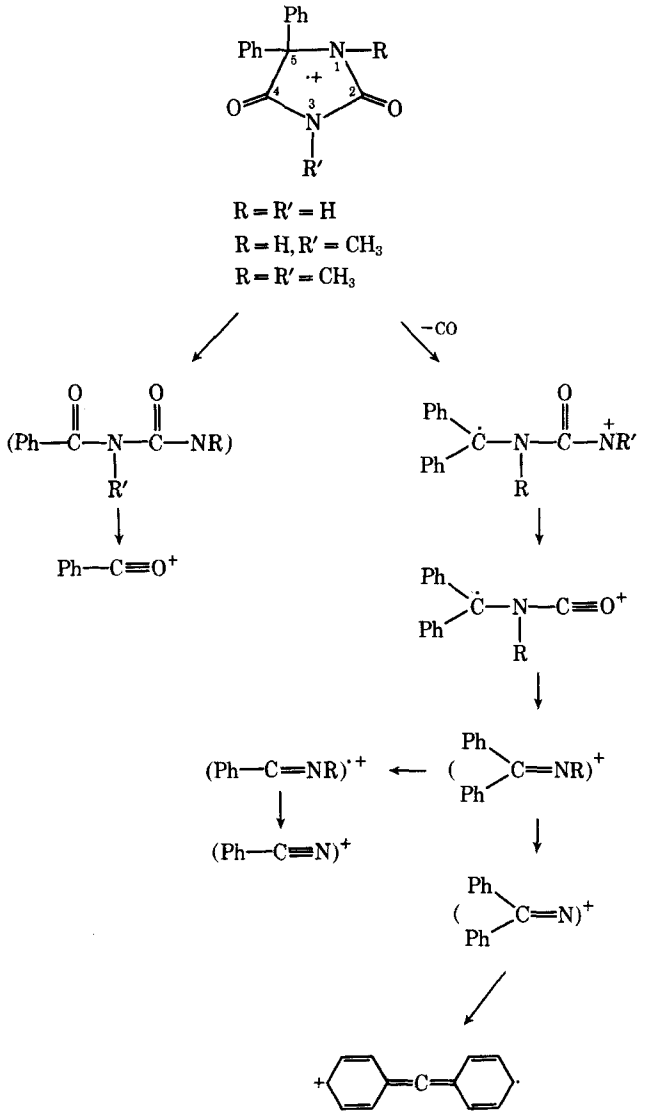


Scheme VI

The possible overall common fragmentation pattern of diphenylhydantoin and its methyl derivatives is shown in Scheme VII.

GLC—For routine work, methanolic potassium carbonate was utilized to methylate diphenylhydantoin. This procedure gave the monomethyl derivative in yields ranging from 90 to 100%. Analysis of the methyl derivative was carried out on the DC-200 column. Figure 1 shows a typical chromatogram of the monomethyl and dimethyl derivatives of diphenylhydantoin.

The methylation procedure rendered the GLC method more sensitive over the published method (7). Subtherapeutic levels were easily detected using the procedure mentioned here.



Scheme VII

The method is applicable to determine diphenylhydantoin in biological fluids, such as blood, and is also applicable to tissues such as liver.

The extraction procedure of diphenylhydantoin from plasma and tissues was adequate and gave maximum recovery, as reported earlier (7). A plot of peak height against the amount of the drug injected (0.1–1.0 mcg.) gave a straight line passing through the origin (Fig. 5), indicating that the method is suitable for quantitative analysis.

Other commonly used drugs, *e.g.*, barbiturates and glutethemide, showed no interfering peaks in the same region of diphenylhydantoin. These drugs can also be methylated and analyzed using the procedure described in this article.

CONCLUSIONS

Methylation of diphenylhydantoin with dimethyl sulfate in the presence of a base produces monomethyl and dimethyl derivatives of the drug, depending on the reaction conditions. The structure of the derivatives was identified by high-resolution mass spectrometry and was found to be *N*(3)-methyl- and *N,N'*-dimethyldiphenylhydantoin. Similar results were obtained when other methylating agents, such as diazomethane, were used (9).

These derivatives, together with diphenylhydantoin itself, undergo mainly ring cleavage upon electron impact, accompanied by loss of groups such as CO, HCO, RNCO, or phenyl groups. Skeletal rearrangements were also observed.

The GLC method described is very sensitive and can be used for clinical and toxicological studies of diphenylhydantoin.

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Selected Pharmacological Studies of an Alkaloidal Extract of *Banisteriopsis quitensis*

R. E. STULL, N. M. FERGUSON, and G. G. FERGUSON

Abstract □ The preparation and pharmacological testing of an alkaloidal extract of *Banisteriopsis quitensis* are presented. The extract was found to exert numerous effects on the CNS in mice, including sedation, transient paralysis, ataxia, prolongation of hexobarbital "sleeping" times, and prevention of reserpine-induced hypothermia. The preparation was without effect on the ECG, blood pressure, or respiration in unanesthetized rats. A testing method for analgesia is presented, utilizing a grid-shock apparatus. The extract produced an analgesic effect which was dependent upon intact supplies of serotonin but not norepinephrine.

Keyphrases □ *Banisteriopsis quitensis*, alkaloid extract—pharmacological screening, mice, rats □ CNS activity—*B. quitensis* extract, mice

In past years, studies involving various species of *Banisteriopsis* developed primarily in two directions: elucidation of the chemical composition of the crude drugs and observation of the psychological and somatic effects after administration of *Banisteriopsis* preparations to humans. Several investigators (1–3) reported that the majority of the alkaloids isolated from *B. caapi* and *B. inebriens* are harman derivatives. *B. rusbyana* was reported by Poisson (4) to contain primarily *N,N*-dimethyltryptamine, and this finding was confirmed by other investigators (5–7). *B. quitensis* contains both tryptamine derivatives and at least one carboline-type structure, and it is considered by some to be identical to *B. caapi* (8).

Considerable information exists concerning the effects of *Banisteriopsis* preparations on human subjects. Der Marderosian *et al.* (5) reported that *B. caapi*, *B. inebriens*, *B. longialata*, and other species have been used among South American natives for purposes of divination, prophesy, and increased bravery. The preparations are known as "caapi," "yaje," "ayahuasca," and "Natem," depending upon the area of South America where they are used. Among the effects reported are: hallucinations, in which a bluish "aureole" is present; prolonged, intense dreams of unusual clarity; immobility; and anesthesia.

Naranjo (9) studied the effects of harmala alkaloids contained in *Banisteriopsis* species used in the "montaña" region of South America. He found harmine ("telepathine," "yageine," and "banisterine") and harmaline to produce paresthesias, numbness, isolated physical symptoms, hallucinations, superposition of images, passivity of movement, and lethargy, and he noticed a distinct difference between the effects of these drugs and those of mescaline and lysergic acid diethylamide. He regarded the drugs as stimulants of the midbrain reticular formation. Udenfriend *et al.* (10) found the harmala alkaloids to have strong MAO-inhibitory effects. Harman, harmine, harmaline, and α-carboline produced 50% inhibition of serotonin degradation at a concentration of 10^{-6} *M* in rat brain tissue preparations. Reference was made to the use of the harmala alkaloids as far back as 1920 in the treatment of disorders now treated with MAO inhibitors.

In this paper, selected pharmacological studies of an alkaloidal extract of *B. quitensis* are presented, emphasizing the CNS effects of the drug.

EXPERIMENTAL

Preparation of Alkaloidal Extract—Powdered leaves and stems of *B. quitensis*[1] were placed in 70% ethanol (10 ml./g. powdered drug), stoppered, agitated for 4 hr., and then centrifuged at 3000 r.p.m. for 15 min.[2]. The supernatant was decanted, tested for the presence of alkaloids with Mayer's reagent, and evaporated to dryness in a vacuum oven at 45°[3]. The residue was dissolved in 70% ethanol (1 ml./5 g. original powdered drug) and diluted with distilled water to a final concentration representing 1 g. original powdered drug/5 ml. 7% ethanol[4]. A solution of 7% ethanol of equal volume to the extract was used for all "control" animals. To eliminate effects of ethanol at very high doses of extract, a suspension of the residue in distilled water was used for LD_{50} determinations.

Animals—Male Swiss-Webster albino mice, having an average weight of 25 g., were used for all studies except ECG, blood pressure, and respiration studies where male Wistar rats, having an average weight of 150 g., were employed.

Preliminary Screening Tests—Observational studies, rotarod performance, acute LD_{50}, and effects on ECG, blood pressure, and respiration[5], were used initially to establish both a range of active dosages and a direction of pharmacological activity.

Hexobarbital "Sleeping" Time—Mice were pretreated with extract (2 ml./100 g. i.p.) 1 hr. prior to administration of hexobarbital (85 mg./kg. i.p.). The length of time between losing and regaining the righting reflex ("sleeping" time) was then determined for each animal, and the results were compared to controls (11).

Prevention of Reserpine Hypothermia—Mice were restrained in plastic holders, and their rectal temperatures were monitored using electronic thermometers equipped with temperature-sensing probes[6]. The rectal temperatures were recorded immediately after reserpine administration and at hourly intervals for 4 hr. following these zero-time readings. In two studies, reserpine was not used, and the zero time began 1 hr. after pretreatment. Treatments were as follows:

Group 1: reserpine (2.5 mg./kg. i.p.)
Group 2: 7% ethanol (2.4 ml./100 g. i.p.) 1 hr. prior to injection of reserpine (2.5 mg./kg. i.p.)
Group 3: extract (2.4 ml./100 g. i.p.) 1 hr. prior to injection of reserpine (2.5 mg./kg. i.p.)
Group 4: *p*-chlorophenylalanine (350 mg./kg. i.p.) 3 days prior to injection of 7% ethanol (2.4 ml./100 g. i.p.), followed in 1 hr. by injection of reserpine (2.5 mg./kg. i.p.)
Group 5: *p*-chlorophenylalanine (350 mg./kg. i.p.) 3 days prior to injection of extract (2.4 ml./100 g. i.p.), followed in 1 hr. by injection of reserpine (2.5 mg./kg. i.p.)
Group 6: 7% ethanol (2.4 ml./100 g. i.p.) 1 hr. prior to zero time
Group 7: extract (2.4 ml./100 g. i.p.) 1 hr. prior to zero time

Testing for Analgesic Effect—A 50.8 × 50.8-cm. (20 × 20-in.) wooden box was constructed and divided into two portions by a

[1] Identified by Dr. John D. Dwyer, Henry Shaw School of Botany, St. Louis, Mo.
[2] International model SBV, Rotor No. 823.
[3] Precision Scientific Co., No. 31468.
[4] Further characterization of active materials is being undertaken at this time.
[5] Physiograph Small Animal Study Unit, E&M Instrument Co.
[6] Tele-Thermometer model 43TA, with probes model 402, Yellow Springs Instrument Co.

Table I—Effects of Various Treatments[a] on Rectal Temperature in Mice

Treatment	Number of Mice	Difference at 4 hr.
Reserpine	9	−4.6 ± 0.6°
Ethanol, then reserpine	6	−4.0 ± 0.3°
Extract, then reserpine	9	−0.8 ± 0.9°
p-Chlorophenylalanine, then ethanol, then reserpine	9	−6.2 ± 0.8°
p-Chlorophenylalanine, then extract, then reserpine	10	−5.4 ± 0.6°
Ethanol	8	−1.4 ± 0.6°
Extract	8	−1.8 ± 0.4°

[a] For details of treatment, see text.

strip of plywood so that mice could be tested in both sides simultaneously. This box was prepared as follows. Strips of 0.96-cm. (0.38-in.) galvanized mesh wire were attached to the floor of the box in rows 0.32 cm. (0.125 in.) apart. Alternate rows were connected in series, so that a mouse placed into the box would touch two or more strips and complete an electrical circuit with his paws. Voltage was controlled by a rheostat[7], having a fixed amperage output within the range of voltages used, and was monitored by a voltmeter[8]. The grid floor of the box was lightly sanded, cleaned, and tested prior to each experiment.

The pain threshold was determined by slowly increasing the voltage from zero to the point at which the mouse began rapidly lifting and lowering a rear foot. (Front-foot lifting, while occurring at slightly lower voltages, is not a dependable end-point, since it is commonly employed in preening or washing movements.) Mice were tested before and immediately after administration of the extract or control solution (zero time) and at 5-min. intervals for 60 min. following injection; the voltage required to elicit the pedal response was noted. The box was standardized prior to use by measuring the pain threshold in 200 untreated, male, Swiss-Webster mice; the mean threshold was found to be 11.4 ± 1.6 v. Standard analgesics such as morphine had been found previously to produce a dose-related elevation of analgesic threshold in mice when using this system. Treatments were as follows:

Group M1: extract (2.4 ml./100 g. i.p.)
Group M2: reserpine (2.5 mg./kg. i.p.) 16 hr. prior to administration of extract (2.4 ml./100 g. i.p.)
Group M3; *p*-chlorophenylalanine (350 mg./kg. i.p.) 3 days prior to administration of extract (2.4 ml./100 g. i.p.)
Group M4: *p*-chlorophenylalanine (350 mg./kg. i.p.) 3 days prior to administration of extract; reserpine (2.5 mg./kg. i.p.) 16 hr. prior to administration of extract (2.4 ml./100 g. i.p.)
Group M5: α-methyltyrosine (80 mg./kg. i.p.), three doses, at 24, 18, and 4 hr. prior to administration of extract (2.4 ml./100 g. i.p.)
Group M6: 7% ethanol (2.4 ml./100 g. i.p.)

Statistical Analysis—For comparing similar experimental and control groups, the difference between the means and the standard error of the difference between the means were determined and Student's *t* test was applied; *p* values of 0.05 or smaller were considered significant. For experiments involving multiple treatments or dosages, Duncan's New Multiple Range Test was employed, using an IBM 1130 computer, with a completely randomized block design.

The procedure involved was: (*a*) determine standard error, $s_{\bar{x}} = \sqrt{(\text{error mean square})/r}$, where r = replicates within each treatment; (*b*) obtain significant studentized ranges from appropriate tables and multiply these figures by $s_{\bar{x}}$ to give the least significant ranges; (*c*) rank the means; and (*d*) test the differences in the following order: largest minus smallest, largest minus second smallest, and so on until a difference is obtained from each mean utilizing every other mean. Each difference is declared statistically significant if it exceeds the corresponding least significant range; otherwise, it is declared not statistically significant.

[7] "Powerstat" type 116, Superior Electric Co.
[8] Triplett model 666-R.

Table II—Effects of Extract on Threshold Voltages in Mice[a]

Minutes Elapsed	Treatment[b] (Eight Mice/Group)			
0	M4 10.25	M1 10.38	M2 10.50	M3 11.00
5	M1 10.88	M4 12.88	M2 14.63	M3 18.38
10	M1 11.38	M4 14.63	M2 15.13	M3 20.13
15	M1 11.33	M2 14.25	M4 15.50	M3 22.63
20	M1 12.13	M2 14.13	M4 19.63	M3 20.25
25	M1 11.25	M2 16.00	M4 19.63	M3 23.25
30	M1 11.75	M2 15.88	M3 20.50	M4 21.50
35	M1 11.63	M2 16.13	M3 23.38	M4 24.13
40	M1 11.63	M2 16.88	M4 23.75	M3 24.75
45	M1 11.50	M2 16.75	M3 23.75	M4 24.25
50	M1 11.75	M2 14.38	M3 21.50	M4 23.25
55	M1 11.75	M2 13.50	M3 19.38	M4 22.00
60	M1 11.25	M2 13.00	M4 20.38	M3 20.50

[a] Mean threshold voltages are ranked beginning with the lowest value. Means for a given time interval not underlined by the same line are significantly different ($p = 0.05$). Standard error values are incorporated into the analysis (Duncan's New Multiple Range Test). [b] Treatments were as follows: M1, 0.3 ml./100 g. extract; M2, 0.6 ml./100 g. extract; M3, 1.2 ml./100 g. extract; and M4, 2.4 ml./100 g. extract. For details of treatment, see text.

RESULTS AND DISCUSSION

In simple observational screening tests, the alkaloidal extract was found to produce sedation, ataxia, lordosis, and transient paralysis of the rear limbs which were dose related (0.3–4.8 ml./100 g.). Rotarod performance was also impaired at dosage levels of 0.3 ml./100 g. and above. These effects were not unlike those reported following ingestion of *Banisteriopsis* preparations by South American natives, in which anesthesia, passivity of movement, and paresthesias were noted (5, 9). Control animals (receiving an identical quantity of 7% ethanol) were unaffected.

The preparation was without effect on blood pressure, ECG, or respiration in unanesthetized rats at dosage levels of 0.4–1.6 ml./100 g. i.v. Repeated injections were likewise ineffective.

The acute LD_{50} in mice was found to be 2.5 ml./100 g. i.p. of a suspension representing 800 mg. original powdered drug/ml. distilled water. This dosage is equivalent to 10.0 ml./100 g. of the final 7% ethanol preparation.

The hexobarbital "sleeping" time was prolonged significantly ($p = 0.05$) in animals pretreated with 2 ml./100 g. i.p. extract. The value for controls (10 mice) was 37.4 ± 7 min., and it was 59.1 ± 7 min. for extract-treated animals (10 mice). This effect is seen with many types of compounds, including certain sedatives, tranquilizers, and MAO inhibitors (12).

Reserpine-induced hypothermia was prevented to a significant degree ($p = 0.01$) in animals pretreated with the extract (2.4 ml./100 g. i.p.) as compared to those pretreated with control solution (Table I). Hypothermia produced in animals pretreated with control solution was not significantly different from that in animals with no pretreatment. Animals pretreated with *p*-chlorophenylalanine (350 mg./kg. i.p.) and control solution exhibited a significantly greater degree ($p = 0.05$) of hypothermia after reserpine treatment than was exhibited by animals receiving only control solution prior to reserpine treatment. This effect was not prevented to a significant degree

Table III—Effects of Various Treatments on Threshold Voltages in Mice[a]

Minutes Elapsed	Treatment[b] (Eight Mice/Group)					
0	M1 10.25	M3 10.25	M6 10.25	M4 12.00	M2 12.13	M5 12.25
5	M6 10.63	M3 11.25	M4 12.50	M1 12.88	M5 12.88	M2 13.38
10	M6 11.13	M3 11.88	M4 12.75	M5 14.13	M2 14.50	M1 14.63
15	M6 11.25	M3 12.00	M4 13.50	M2 15.00	M1 15.50	M5 16.63
20	M6 11.50	M3 12.75	M4 13.88	M2 15.13	M5 16.63	M1 19.63
25	M6 11.50	M3 13.13	M4 14.13	M2 16.00	M5 18.75	M1 19.63
30	M6 11.63	M3 12.75	M4 14.13	M2 15.75	M5 19.50	M1 21.50
35	M6 12.00	M3 12.75	M4 13.50	M2 15.00	M5 20.38	M1 24.13
40	M6 11.63	M3 12.63	M4 13.38	M2 15.00	M5 21.00	M1 23.75
45	M6 11.38	M3 12.38	M4 13.25	M2 14.88	M5 20.50	M1 24.25
50	M6 10.88	M4 12.13	M3 12.50	M2 15.00	M5 19.13	M1 23.25
55	M6 11.50	M3 11.75	M4 12.25	M2 14.00	M5 17.75	M1 22.00
60	M3 11.25	M6 11.50	M4 12.00	M2 13.63	M5 17.13	M1 20.38

[a] Mean threshold voltages are ranked beginning with the lowest value. Means for a given time interval not underlined by the same line are significantly different ($p = 0.05$). Standard error values are incorporated into the analysis (Duncan's New Multiple Range Test). [b] Treatments were as follows: M1, extract; M2, reserpine, then extract; M3, *p*-chlorophenylalanine, then extract; M4, *p*-chlorophenylalanine, then reserpine, then extract; M5, α-methyltyrosine, then extract; and M6, control solution. For details of treatment, see text.

by pretreatment with extract. Treatment with extract alone (without reserpine) and with control solution alone (without reserpine) did not produce significant alterations in rectal temperature. These findings are interpreted to indicate that the alkaloidal extract acts by inhibiting MAO, since good correlation exists between prevention of reserpine-induced hypothermia and MAO inhibition (12). It also seems apparent that serotonin participates in the maintenance of body temperature, since the pretreatment that produced the greatest restriction on the availability of serotonin [*i.e.*, *p*-chlorophenylalanine pretreatment, which strongly inhibits serotonin synthesis but not norepinephrine synthesis to any significant extent (13)] produced the greatest degree of hypothermia after reserpine treatment. Similar effects have been reported for the harmala alkaloids by Udenfriend *et al.* (10).

When tested in the grid-shock apparatus, the extract was found to have analgesic effects which were dose related for most values (Table II). Equivalent volumes of control solution were without effect. Pretreatment with reserpine, *p*-chlorophenylalanine, or a combination of the two markedly inhibited the analgesic effect of the extract (Table III). Pretreatment with α-methyltyrosine, a known inhibitor of norepinephrine synthesis which has essentially no effect on serotonin synthesis (14), had considerably less effect on the analgesic threshold produced by the extract. Thus, it is suggested that serotonin, rather than norepinephrine, is the more important intermediary in pain response of this type.

SUMMARY

A study of selected pharmacological effects of an alkaloidal extract of *B. quitensis* was presented. The extract produced ataxia, lordosis, sedation, and transient paralysis; it impaired rotarod performance; and it significantly prolonged the hexobarbital "sleeping" time in mice. Reserpine-induced hypothermia was prevented to a significant degree by pretreatment with the preparation. An analgesic effect was observed with the preparation when a grid-shock apparatus was employed. This effect was prevented to a significant degree by pretreatment with reserpine, *p*-chlorophenylalanine, or both, but was modified considerably less by pretreatment with α-methyltyrosine. A relationship of serotonin to temperature maintenance and perception of pain stimuli is suggested.

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Intramuscular and Intravenous Administration of Small Doses of 2-Pyridinium Aldoxime Methochloride to Man

FREDERICK R. SIDELL and WILLIAM A. GROFF

Abstract □ Small doses (2.5, 5.0, 7.5, and 10.0 mg./kg.) of 2-pyridinium aldoxime methochloride were given intramuscularly and intravenously to groups of volunteers. After intramuscular administration, oxime plasma concentrations of greater than 4 mcg./ml. were attained only with the two higher doses and these concentrations were maintained for less than 1 hr. The half-times for elimination were almost identical (74 min. after intravenous and 77 min. after intramuscular administration), and the oxime was rapidly eliminated in the urine. Very transient symptoms were reported by the subjects receiving 2-pyridinium aldoxime methochloride intravenously.

Keyphrases □ 2-Pyridinium aldoxime methochloride—intramuscular, intravenous administration, man □ Anticholinesterase poisoning—administration of 2-pyridinium aldoxime methochloride

The efficacy of the pyridinium oximes as adjuvants to atropine in the therapy of anticholinesterase poisoning has been recognized for over a decade. Several early studies (1–3) and one recent study (4) demonstrated the value of small doses (5–7.5 mg./kg.) of 2-pyridinium aldoxime methochloride, but most recent publications recommended much higher doses (1–2.5 g./man) (5–7) intravenously. Although this may be optimal therapy, intravenous 2-pyridinium aldoxime methochloride should be given only by medical personnel, and this requirement may often preclude its immediate or early administration.

Atropine sulfate, the pharmacological mainstay in anticholinesterase poisoning, is available in self-injection (intramuscular) devices (containing 2 mg. of atropine sulfate)[1], which can be used immediately by an individual accidentally exposed to an anticholinesterase compound. Such devices possibly could also be used for oximes, so a poisoned individual or his coworker could administer an oxime intramuscularly before medical personnel arrive.

The dose contained in such a device would be limited by the volume of solution in the injector (most contain less than 2 ml.) and the concentration of 2-pyridinium aldoxime methochloride (concentrations above 30% w/v produce muscle necrosis in animals)[2]. It is, therefore, desirable to know the blood levels of oxime, the rapidity with which the oxime enters the circulation, and the side effects produced when low doses of 2-pyridinium aldoxime methochloride are administered intramuscularly to man. This study was undertaken to provide such information. In addition, low doses of 2-pyridinium aldoxime methochloride were administered intravenously, and the time course of the disappearance of oxime from the blood was compared with that noted in the intramuscular study.

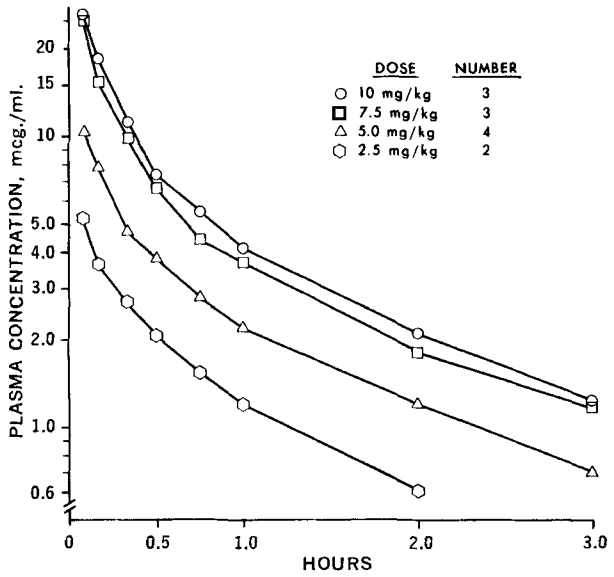

Figure 1—*Plasma concentration of 2-pyridinium aldoxime methochloride after intravenous administration.*

EXPERIMENTAL

Subjects[3]—The subjects were U. S. Army enlisted men who volunteered for this test after a thorough physical examination and laboratory examinations[4] had shown them to have no abnormalities. They were told they would receive a previously tested antidote for nerve agent poisoning and might experience mild symptoms.

Drug—The 2-pyridinium aldoxime methochloride was obtained commercially[5]. Each day a fresh solution (containing 300 mg./ml. for the intramuscular study and 100 mg./ml. for the intravenous study) was prepared from the powder, using isotonic saline as the diluent. The doses, given both intramuscularly and intravenously, were 2.5, 5.0, 7.5, and 10.0 mg./kg. The intramuscular injection was into the deltoid muscle.

Procedure—The subjects were admitted to the test ward the evening before the test. They were awakened about 2 hr. before receiving the drug; after they ate a light breakfast, they drank fluids to promote an adequate urinary output. For the first 3 hr. of the test, they drank at least 950 ml. hourly. Except to void, they remained in bed for this period.

Blood was drawn for oxime analysis just before injection of the drug and at frequent intervals thereafter (Figs. 1 and 3). An attempt was made to obtain urine specimens at half-hourly intervals for the first 3 hr., and all the urine collected in 12 hr. was analyzed for oxime.

Oxime Measurement—Plasma and urinary oxime concentrations were measured by the method described previously (8). Plasma

[1] Autoinjector, Rodana Research Corp., Bethesda, Md.; Ampin, Strong, Cobb and Co., Cleveland, Ohio; and atropine injection, E. R. Squibb and Sons, New York, N. Y.

[2] Unpublished data of J. E. Martin and J. F. Ferrell of this laboratory.

[3] These tests were governed by the principles, policies, and rules for medical volunteers as established in AR 70–25.

[4] Urine analysis, hematocrit, total and differential white blood count, blood urea nitrogen, serum glutamic oxaloacetic transaminase, alkaline phosphatase, total bilirubin, thymol turbidity, red blood cell and plasma cholinesterase, chest X-ray, and ECG.

[5] Protopam chloride, Ayerst Laboratories, New York, N. Y.

Table I—Urinary Excretion of 2-Pyridinium Aldoxime Methochloride

Dose, mg./kg.	Number of Subjects	Total Excreted by 12 hr., % of Dose	Amount Excreted, % of Total — Hours 0.5	1.0	1.5	2.0	2.5
			Intramuscular				
2.5	2	99	29	46	55	61	65
5.0	2	81	37	56	69	76	80
7.5	4	96	36	54	70	76	80
10.0	4	90	30	48	60	69	72
Mean ± *SD*		91 ± 10	33 ± 5	51 ± 6	64 ± 8	71 ± 8	75 ± 8
			Intravenous				
2.5	2	82	66	83	89	92	94
5.0	4	75	58	73	78	82	90
7.5	3	77	52	69	78	85	88
10.0	1[a]	94.5	53	66	71	74	77
Mean ± *SD*		80 ± 11	57 ± 8	73 ± 7	80 ± 6	84 ± 6	89 ± 5

[a] One subject accidentally discarded an early specimen; another had an unusual excretion pattern. Neither is included in this tabulation.

concentration was measured the day blood was drawn; urine samples were refrigerated and analyzed the following day. (Preliminary studies showed that oxime deteriorates less than 2% in refrigerated urine over a week's time.) In the study after intravenous administration, whole blood and red blood cell oxime concentrations were also measured.

Physiological Measures—Three baseline measurements of heart rate and blood pressure (supine) were taken in the morning before drug injection. Thereafter, heart rate and blood pressure were measured just before each blood sample was taken over a period of 2 hr. (intramuscular injection) or 3 hr. (intravenous injection).

RESULTS

Intravenous Administration—*Plasma Levels*—Mean plasma levels for each dose group are plotted against time in Fig. 1. In each case, the whole blood oxime concentration was roughly 45–50% of the plasma concentration, and the results are not shown. The studies on the red blood cell oxime concentration will be the subject of a separate report.

Urinary Excretion—Within the 12-hr. collection period, 80.4% of the dose was found unchanged in the urine. Fifty-seven percent of this amount (or 45.8% of the dose) was excreted within the first 30 min. and 73% (or 58.7% of the dose) within the 1st hr. (Table I). The total amount found in the urine was not as great as that found after intramuscular administration, but the difference is probably within the limits of experimental error.

One subject in the highest dose group had an unusual urinary excretion pattern. In contrast to the asymptotic line found with all the other subjects when percentage excreted was plotted against time (both on linear scales), a plot of the percentage of oxime he excreted against time (Fig. 2) was linear. No explanation is offered for this pattern.

Kinetic Considerations—The pattern of oxime disappearance from the plasma is described by the biexponential equation:

$$C_1 = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}^6$$

Each of the constants, A, B, α, and β, was estimated graphically for each subject and is shown in Table II along with the biological half-time ($+0.693/\beta$), also known as the disposition half-time (10). Although the constants were estimated by graphic means, the error from the experimental values introduced by these estimations is not great, as indicated in Fig. 3 where the experimental values for one subject are plotted with a calculated line using the graphically estimated values for that subject.

The percent of the dose to be excreted in the urine that has not been excreted by time t is:

$$100 \times F \times \frac{A\beta e^{-\alpha t} + B\alpha e^{-\beta t}}{A\beta + B\alpha} \qquad \text{(Eq. 2)}^7$$

where F is the fraction of the total dose eventually eliminated in the urine. In Fig. 4, this relationship is shown for two subjects and for two dose groups, using mean values for the constants in the latter instance[8].

Physiological Measures and Side Effects—There was a slight and transient increase in heart rate, with a maximal increase of 10 beats/min. and a duration of less than 30 min. There was no significant change in blood pressure.

All subjects reported mild and very transient symptoms just after injection. Those at the lowest dose had slight dizziness; those receiving 5.0 mg./kg. had dizziness, and three complained of blurred vision. All at the 7.5-mg./kg. dose noted dizziness and blurred vision, and one complained of diplopia. Two at the highest dose complained of double and blurred vision, and the third stated he felt dizzy. All symptoms subsided within 3–4 min.

Intramuscular Administration—*Plasma Levels*—Figure 5 shows the mean plasma levels of oxime for each dose group plotted against time. By 5 min., the levels were more than 50% of the highest levels. If 4 mcg./ml. is needed for a therapeutic effect (13), only the two highest doses (7.5 and 10 mg./kg.) would be adequate. This level is reached rather rapidly (5–10 min.) with these doses.

Urinary Excretion—Overall, 91% of the oxime administered was excreted unchanged in the urine within the 12-hr. collection period.

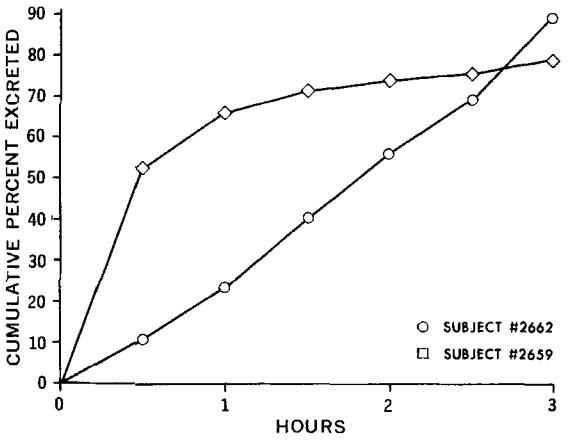

Figure 2—*Urinary excretion patterns of 2-pyridinium aldoxime methochloride in two subjects (dose 10 mg./kg.).*

[6] This equation was adequately discussed elsewhere (9–12), and only the definitions are given here. C_1 is the plasma level at time t; β is -2.303 times the slope of the terminal linear segment of the plot of plasma level on a logarithmic scale *versus* time on a linear scale, and B is the extrapolated zero-time intercept of this line. α is -2.303 times the slope of a line plotted by the method of residuals from the initial plasma level values, and A is the extrapolated zero-time intercept of this line. α and β are hybrid rate constants incorporating the rate constants for absorption, distribution, and metabolism or excretion in and out of the central (plasma) and peripheral (tissue) compartments. For further details, see pp. 124–133 of *Reference 12*.

[7] The derivation of this equation is given in the *Appendix* (Eq. A26).

[8] Note that there were inadequate data to determine A and α for one subject in the 5-mg./kg. group; his excretion data were also excluded for purposes of the figure.

Table II—Biological Half-Times and Kinetic Constants for 2-Pyridinium Aldoxime Methochloride Administered Intravenously

Subject Case Number	Dose, mg./kg.	$t_{1/2}$ (0.693/α), min.	$t_{1/2}$ (0.693/β), min.	A, mcg./ml.	B, mcg./ml.	α, min.$^{-1}$	β, min.$^{-1}$
2644	2.5	11	73	4.3	1.1	0.063	0.009
2653	2.5	11	87	5.0	2.1	0.063	0.008
Mean		11	80	4.7	1.6	0.063	0.0085
2645	5.0	7	63	8.7	4.3	0.099	0.011
2654	5.0	11	67	7.6	3.1	0.063	0.010
2651	5.0	7	69	14.7	4.6	0.099	0.010
2646	5.0	—	56	—	5.6	—	0.012
Mean		8.3	64	10.3	4.4	0.087	0.011
2652	7.5	6	60	25.5	7.2	0.115	0.012
2660	7.5	6	59	28.5	6.2	0.115	0.012
2661	7.5	10	112	29.5	4.9	0.069	0.006
Mean		7.3	77	27.8	6.1	0.100	0.010
2663	10	11	81	25.5	5.6	0.063	0.009
2659	10	8	71	24.0	7.0	0.087	0.010
2662	10	8.5	86	34.0	5.5	0.082	0.008
Mean		9.2	79	27.8	6.0	0.077	0.009
Mean of all subjects		8.8	73.7	—	—	0.083	0.010
± *SD*		2.0	15.2	—	—	0.020	0.002

Of this, 75% (or 69% of the dose) was excreted within the first 2.5 hr. As seen in Table I, about 50% of the total amount excreted (or 46% of the dose) was eliminated within the 1st hr., indicating a very rapid renal excretion of this compound.

Kinetic Considerations—Although the data fit a two-compartment model, the plasma level values after intramuscular administration were too meager to permit graphic dissection of similar constants (α, β, and, possibly, *k*, the rate constant for absorption) which might define a kinetic model for these data. [Wagner (14) recently commented on this problem, but the data were inadequate to pursue his suggested solution.]

By using the terminal points on a plasma concentration (logarithmic scale) *versus* time (on a linear scale) plot, the mean half-times by dose group were found to be 75 min. (2.5 mg./kg.), 72 min. (5 mg./kg.), 77 min. (7.5 mg./kg.), and 83 min. (10 mg./kg.). The corresponding rate constants for elimination were 0.0096, 0.0096, 0.0097, and 0.0084 min.$^{-1}$.

Theoretically, the urinary data should fit a plot calculated from the constants *k*, α, and β. Since the former two constants could not be separated on the basis of the data, this could not be done. The general shape of the curves, when amount not excreted (logarithmic) is plotted against time (linear), suggests a two-compartment model (Fig. 6).

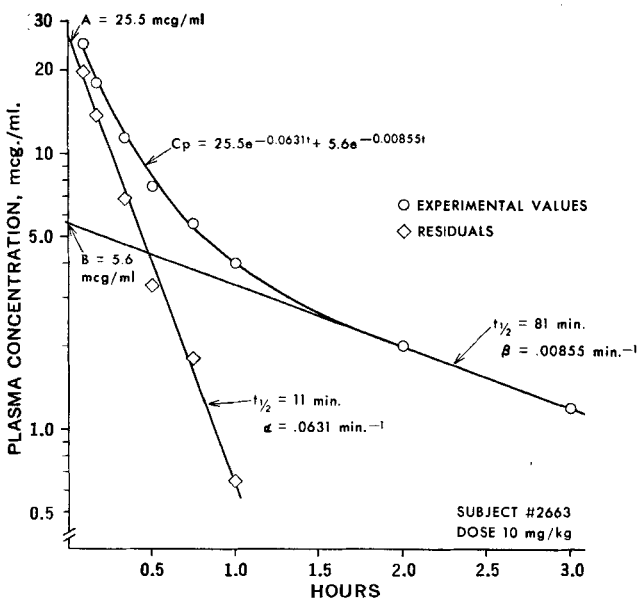

Figure 3—*Experimental values and calculated disappearance curve of 2-pyridinium aldoxime methochloride from plasma.*

Physiological Measures and Side Effects—There were no changes in heart rate or blood pressure. No subject had any signs or symptoms except mild pain at the site of injection which persisted for a few hours. There was slight tenderness to fist percussion at the injection site 24 hr. later.

DISCUSSION

Barkman *et al.* (15) found that 2-pyridinium aldoxime methanesulfonate, 10 mg./kg. i.m., produced maximal plasma concentra-

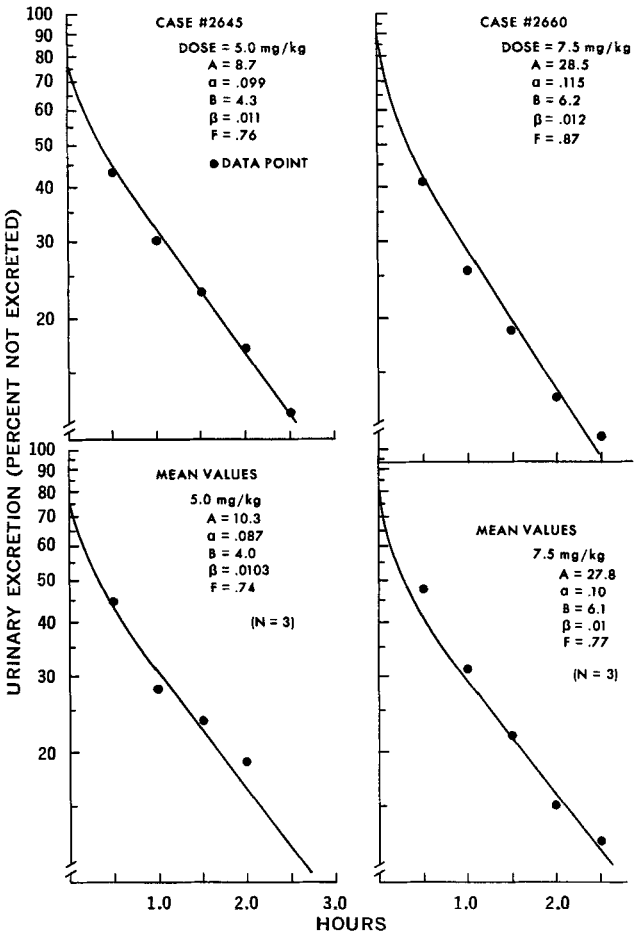

Figure 4—*Urinary excretion* versus *time: calculated and actual amounts.*

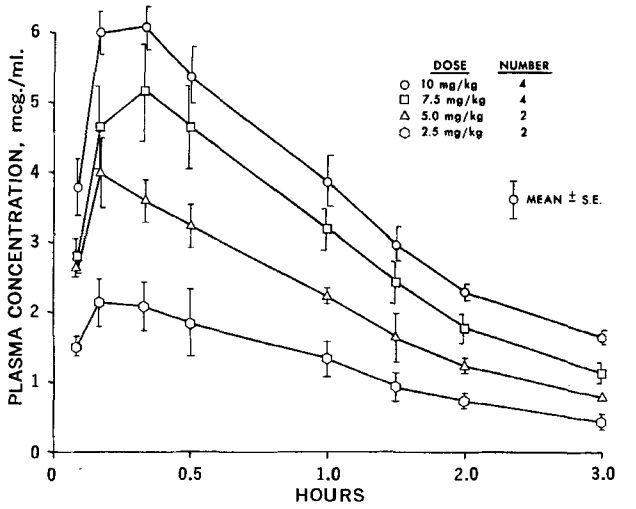

Figure 5—*Plasma concentration of 2-pyridinium aldoxime methochloride after intramuscular administration.*

tions of about 7 mcg./ml. and that a concentration of 4 mcg./ml. was maintained for about 90 min. A similar dose of 2-pyridinium aldoxime methochloride produced maximal concentrations of about 6 mcg./ml., and a concentration of greater than 4 mcg./ml. was maintained for only 50–55 min. Barkman *et al.* (15) did not measure urinary excretion; in the present study, it was found that 2-pyridinium aldoxime methochloride was eliminated in the urine very rapidly. If it is desired to maintain a high plasma level for a long period of time after a single intramuscular injection, it would appear that 2-pyridinium aldoxime methanesulfonate is preferable. However, because repeated injections would probably be given in practice and because intravenous therapy should be started within an hour under most conditions, it is doubtful if the difference between 2-pyridinium aldoxime methanesulfonate and 2-pyridinium aldoxime methochloride is of practical significance. Both oximes are absorbed rapidly and produce maximal plasma levels in about 5–15 min.

The rapid and rather complete urinary excretion of 2-pyridinium aldoxime methochloride was reported previously (16, 17). It is possible that 2-pyridinium aldoxime methochloride is not only not reabsorbed to any degree by the renal tubules after filtration but may be secreted by tubular mechanisms. Studies on the exact mechanisms by which the kidneys handle 2-pyridinium aldoxime methochloride are underway.

The half-life of 2-pyridinium aldoxime methochloride in the body is about the same whether administered by the intravenous or intramuscular route (74 min. *versus* 77 min.). This short half-life is the basis for the recommendation (5–7) that repeated doses be administered at intervals of an hour or so should the clinical circumstances warrant.

APPENDIX

If D is the total dose of drug administered intravenously, material balance at time t will be:

$$D = D_b + D_e \qquad \text{(Eq. A1)}$$

where D_b is the amount of drug in the body at time t, and D_e is the cumulative amount of drug eliminated from the body (by any means) by time t.

The fit of the plasma data to the biexponential equation infers a two-compartment model of the type described elsewhere (12). In this case, the total amount of drug in the body is the sum of the amount (D_1) in Compartment 1 (or central compartment) and the amount (D_2) in Compartment 2, or the peripheral (tissue) compartment. Thus,

$$D_b = D_1 + D_2 \qquad \text{(Eq. A2)}$$

In each case, the amount present is the product of concentration (C) times the volume of distribution (V) of that compartment, or:

$$D_1 = C_1 \times V_1 \qquad \text{(Eq. A3)}$$

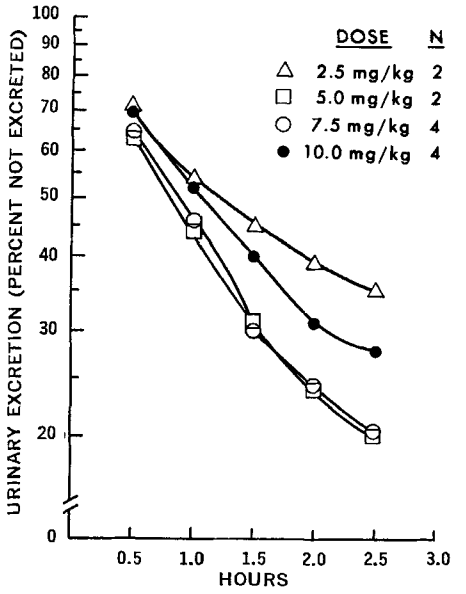

Figure 6—*2-Pyridinium aldoxime methochloride intramuscularly. percent not excreted* versus *time.*

and

$$D_2 = C_2 \times V_2 \qquad \text{(Eq. A4)}$$

In this case, C_1 is the plasma concentration; hence:

$$D_1 = V_1(Ae^{-\alpha t} + Be^{-\beta t}) \qquad \text{(Eq. A5)}$$

Since (12):

$$V_1 = \frac{D}{A + B} \qquad \text{(Eq. A6)}$$

this becomes:

$$D_1 = \frac{D}{A + B}(Ae^{-\alpha t} + Be^{-\beta t}) \qquad \text{(Eq. A7)}$$

C_2 was described (12) as:

$$C_2 = \frac{(K_1)(D)}{V_2(\alpha - \beta)}(e^{-\beta t} - e^{-\alpha t}) \qquad \text{(Eq. A8)}$$

where K_1 is the rate constant for transfer from Compartment 1 to Compartment 2.

The further definitions (12):

$$K_1 = \alpha + \beta - K_2 - K_{-1} \qquad \text{(Eq. A9)}$$

$$K_{-1} = \frac{A\beta + B\alpha}{A + B} \qquad \text{(Eq. A10)}$$

$$K_2 = \frac{\alpha\beta}{K_{-1}} \qquad \text{(Eq. A11)}$$

permit C_2 to be expressed in terms of the graphically defined α and β. Hence:

$$C_2 = \frac{(D)(AB)(\alpha - \beta)}{(A\beta + B\alpha)(A + B)(V_2)}(e^{-\beta t} - e^{-\alpha t}) \qquad \text{(Eq. A12)}$$

and

$$D_2 = \frac{D(AB)(\alpha - \beta)}{(A\beta + B\alpha)(A + B)}(e^{-\beta t} - e^{-\alpha t}) \qquad \text{(Eq. A13)}$$

By combining, $D_b = D_1 + D_2$, or:

$$D_b = \frac{D}{(A + B)}(Ae^{-\alpha t} + Be^{-\beta t}) + \frac{D}{(A + B)}\frac{(AB)(\alpha - \beta)}{(A\beta + B\alpha)} \times (e^{-\beta t} - e^{-\alpha t}) \qquad \text{(Eq. A14)}$$

By rearranging and combining, this becomes:

$$D_b = D\frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha} \qquad \text{(Eq. A15)}$$

The amount of drug eliminated from the body by time t can be represented by the expression:

$$D_e = Du + Dx \qquad \text{(Eq. A16)}$$

where Du is the cumulative amount of drug eliminated in the urine by time t, and Dx is the cumulative amount eliminated by other routes or means (metabolism, *etc.*).

If F is the fraction of the dose eliminated in the urine by $t = \infty$, or:

$$F = \frac{Du_\infty}{D} \qquad \text{(Eq. A17)}$$

then:

$$Du = F \times D_e \qquad \text{(Eq. A18)}$$

or since $D_e = D - D_b$ (from Eq. A1):

$$Du = F(D - D_b) \qquad \text{(Eq. A19)}$$

The fraction of drug eliminated by other means is $(1 - F)$ and:

$$Dx = (1 - F)(D - D_b) \qquad \text{(Eq. A20)}$$

By combining Eqs. A16, A19, and A20:

$$D_e = F(D - D_b) + (1 - F)(D - D_b) \qquad \text{(Eq. A21)}$$

and by combining A21 and A1:

$$D = D_b + F(D - D_b) + (1 - F)(D - D_b) \qquad \text{(Eq. A22)}$$

By dividing by D, this becomes:

$$1 = \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha} + F\left(1 - \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha}\right) + (1 - F)\left(1 - \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha}\right) \qquad \text{(Eq. A23)}$$

The fraction excreted into the urine by time t is:

$$\frac{Du}{D} = F\left(1 - \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha}\right) \qquad \text{(Eq. A24)}^9$$

and the fraction yet to be excreted is the total fraction excreted by $t = \infty$, F, minus this, or:

$$F - F\left(1 - \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha}\right) \quad \text{or}$$

$$F \times \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha} \qquad \text{(Eq. A25)}$$

Thus the percent yet to be excreted is:

$$100 \times F \times \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha} \qquad \text{(Eq. A26)}$$

If the total dose is eliminated unchanged into the urine, $F = 1$, and the percent remaining to be excreted at time t is $100 \times [A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]/A\beta + \beta\alpha$, which is the percent remaining in the body.

[9] A slightly different derivation of this is to combine Eq. A19, where $Du = F(D - D_b)$, and Eq. A17, $F = Du_\infty/D$, producing:

$$Du = \frac{Du_\infty}{D}(D - D_b) \qquad \text{(Eq. A27)}$$

or

$$Du = Du_\infty\left(1 - \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha}\right) = Du_\infty - \left(Du_\infty \times \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha}\right) \qquad \text{(Eq. A28)}$$

When $F = 1$, this becomes:

$$Du = D\left(1 - \frac{[A\beta e^{-\alpha t} + B\alpha e^{-\beta t}]}{A\beta + B\alpha}\right) \qquad \text{(Eq. A29)}$$

This states that the cumulative amount of drug in the urine at time t is the total amount excreted in the urine ($t = \infty$) minus the fraction of this amount still in the body.

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Analysis of Phenylephrine and Phenylpropanolamine Hydrochlorides in Combination

NEIL H. BROWN and GLENN A. PORTMANN

Abstract □ An accurate and precise method for determination of phenylephrine hydrochloride and phenylpropanolamine hydrochloride is described. This method is based on measuring the aryl aldehydes, which are selectively extracted after periodate oxidation of the two phenylethanolamines. It is sufficiently sensitive for individual dosage unit measurements. No assay interferences were apparent with a stressed mixture where phenylephrine was acetylated by aspirin.

Keyphrases □ Phenylephrine–phenylpropanolamine combination—assay in aspirin mixture □ Phenylpropanolamine–phenylephrine combination—assay in aspirin mixture □ Aspirin mixture—determination of phenylephrine and phenylpropanolamine □ UV spectrophotometry—determination, phenylethanolamines in aspirin mixture □ Periodate oxidation—assay, separation of phenylethanolamines

Periodate oxidation of vicinal glycols, α-hydroxyketones, and α-primary or secondary aminoalcohols is a well-known reaction. Heimlich *et al.* (1) utilized this reaction for the measurement of phenylpropanolamine in urine by spectrophotometric analysis of the oxidation product, benzaldehyde. Alkaline periodate oxidation also was used by Wallace (2) to determine phenylpropanolamine and related compounds in biologic specimens. The assay of phenylephrine hydrochloride and phenylpropanolamine hydrochloride in separate formulations using periodate oxidation was described by Chafetz (3). A modified version of this oxidative technique is reported here for the assay of these two drugs in combination in a mixture with aspirin. By careful adjustment of pH, selective separation of the two oxidation compounds, benzaldehyde and *m*-hydroxybenzaldehyde, is quantitative.

EXPERIMENTAL

Apparatus—A Beckman DBG with a 25.4-cm. (10-in.) recorder was used for UV scans and absorbance measurements.

Reagents—Analytical grade reagents were used.

0.3 N Sodium Metaperiodate—Dissolve 3.2 g. of sodium metaperiodate in 50 ml. of water.

pH 7.5 Buffer (1.0 M)—Dissolve 69.0 g. of monobasic sodium phosphate in 250 ml. water. Adjust the pH to approximately 7.5 using 10% NaOH. The final adjustment can be made with 0.1 *N* NaOH. Then add water to make 500 ml. total volume.

1.0 N Sodium Hydroxide—Dissolve 40.0 g. of sodium hydroxide in 1.0 l. of water.

1.0 N Hydrochloric Acid—Add 83 ml. of concentrated hydrochloric acid to enough water to make 1.0 l.

Standard Solutions—Prepare three solutions containing both phenylephrine hydrochloride[1] and phenylpropanolamine hydrochloride[2] in the following amounts, respectively: 25 and 60 mcg./ml., 50 and 120 mcg./ml., and 75 and 180 mcg./ml.

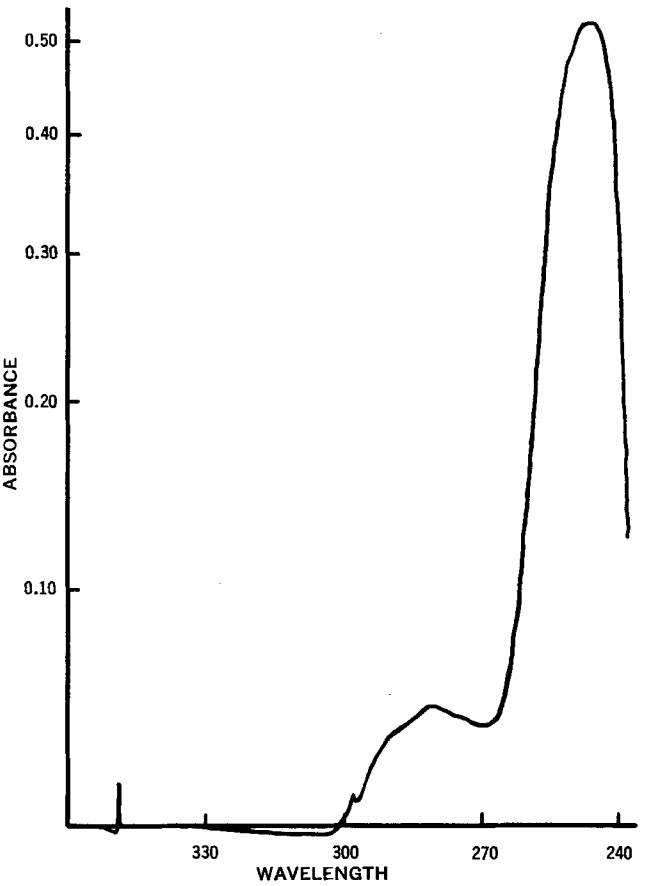

Figure 1—*Absorption spectrum of benzaldehyde in chloroform (8.0 mcg./ml.).*

Periodate Oxidation Method—A 10-ml. aliquot of a filtered solution containing not more than 0.75 mg. of phenylephrine hydrochloride and 1.8 mg. of phenylpropanolamine hydrochloride is pipeted into a 60-ml. separator. To the sample aliquot and to a 10-ml. aliquot of distilled water (reagent blank), add 1.5 ml. 1.0 *N* sodium hydroxide and mix. Then add 1.5 ml. of 0.3 *N* sodium metaperiodate, mix, and let stand 10–30 min. (The oxidation is complete at 10 min. and remains stable for at least 30 min.). Extract three times with 20 ml. of chloroform. Filter the combined chloroform extract through chloroform-washed cotton into a 200-ml. volumetric flask. Wash the cotton with a small amount of chloroform and then bring the total volume up to 200 ml. with chloroform. Measure the absorbance of this solution at 247.5 nm. *versus* the reagent blank and use it for the calculation of the concentration of phenylpropanolamine hydrochloride. The UV spectrum obtained by this method is characteristic of benzaldehyde (Fig. 1).

[1] Winthrop Laboratories.

[2] Penick.

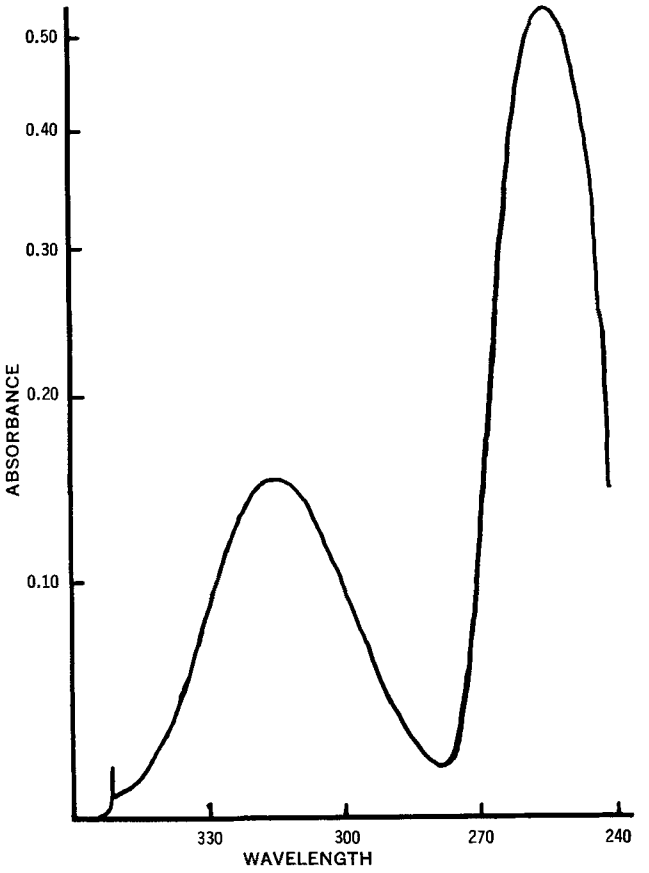

Figure 2—*Absorption spectrum of* m-*hydroxybenzaldehyde in chloroform (12.5 mcg./ml.).*

Adjust the pH of the aqueous phase of the extraction to 7.5 ± 0.5 by adding 5 ml. of the pH 7.5 phosphate buffer (1 *M*) and 1.0 ml. of 1.0 *N* hydrochloric acid. Extract three times with 15 ml. of chloroform. Filter the combined chloroform extract through chloroform-washed cotton into a 50-ml. volumetric flask. Wash the cotton with less than 5 ml. of chloroform and then bring the total volume up to 50 ml. with chloroform. Measure the absorbance of this solution at 252.5 nm. *versus* the reagent blank and use it for the calculation of the concentration of phenylephrine hydrochloride. The UV spectrum of this chloroform solution is characteristic of *m*-hydroxybenzaldehyde (Fig. 2).

For calculations, standard curves are obtained by assaying 10.0-ml. aliquots of the three standard solutions. From these curves, the measured absorbances can be converted to micrograms per milliliter; then, by multiplying by the appropriate dilution factor, the assay values of the phenylephrine hydrochloride and phenylpropanolamine hydrochloride are determined.

TLC—This procedure is a modified form of the method used by Troup and Mitchner (4). The absorbent used is silica gel F-254[3]. The solvent system is the chloroform layer from a mixture of chloroform–glacial acetic acid–methanol–water (85:20:8:20) which has been equilibrated for 16 hr. The spotting solution is a 4:1 mixture of ethanol–acetone, of which enough is spotted to leave 10 mcg. of phenylephrine hydrochloride and 25 mcg. of phenylpropanolamine hydrochloride on the plate. Detection is made by spraying the plate with a diazo reagent (25 ml. of 0.3% *p*-nitroaniline plus 1.5 ml. of 5% sodium nitrite). The plate is then heated at 70° for 15 min., after which the plate is sprayed with 20% sodium carbonate. Acetylated phenylpropanolamine and the mono-, di-, and triacetyl derivatives of the phenylephrine can be detected in this manner (Fig. 3). For reference standards, three acetylated forms of phenylephrine hydrochloride were synthesized according to the methods reported by Troup and Mitchner (4). Phenylpropanolamine hydrochloride was readily acetylated by heating it on a steam table in the presence of acetic anhydride.

[3] Merck.

Aspirin Assay—The aspirin was assayed by a nonaqueous titration method, using dimethylformamide as the solvent and lithium methoxide (0.1 *N*) as the titrant. Since both phenylephrine hydrochloride and phenylpropanolamine hydrochloride are also titrated by the lithium methoxide, a correction factor was used in the calculations.

Free Salicylic Acid Assay[4]—A weighed sample, equivalent to 400 mg. of aspirin, is transferred to a 125-ml. separator. To this, add 50.0 ml. of solvent (1:1 *n*-pentane–ether)[5] and shake for 1 min. Draw off the solids and wash the solvent two times with 10 ml. of distilled water, shaking each for 15 sec. Discard the water washes. Evaporate a 20-ml. aliquot of the pentane–ether extract in a stream of air or nitrogen. Dissolve the residue in 2.0 ml. of 95% ethanol. To this, add 20 ml. of distilled water, mix, and immediately add 0.4 ml. of iron reagent (to 8.0 ml. of 10% ferric ammonium sulfate, add 7.0 ml. of 1 *N* hydrochloric acid and dilute to 100 ml. with distilled water). Mix and immediately determine the absorbance *versus* a reagent blank in a suitable spectrophotometer at 526 nm. Compare with a standard curve prepared by assaying 200, 300, and 400 mcg. of salicylic acid in 2.0 ml. of 95% ethanol with water and iron reagent as described previously.

RESULTS AND DISCUSSION

Both phenylephrine hydrochloride and phenylpropanolamine hydrochloride, together in the presence of aspirin, can be quantitatively measured by this periodate assay. The standard curves obtained are linear and reproducible over the ranges indicated. Absorbances representative of the solutions used for the standard curves are as follows: phenylephrine hydrochloride—5 mcg./ml.

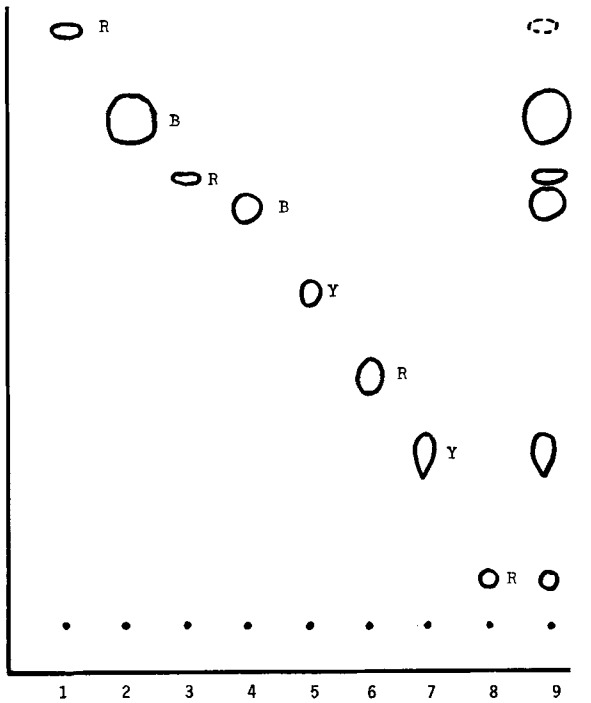

Figure 3—*TLC of components of stability sample with their respective degradation products and a typical stressed stability sample. Key: 1, triacetylphenylephrine* [N-(m,β-*diacetoxyphenethyl*)-N-*methylacetamide*]; *2, aspirin; 3, diacetylphenylephrine* [N-(m-*acetoxy*-β-*hydroxyphenethyl*)-N-*methylacetamide*]; *4, salicylic acid; 5, diacetylated phenylpropanolamine; 6,* N-*monoacetylphenylephrine* [N-(m,β-*dihydroxyphenethyl*)-N-*methylacetamide*]; *7, phenylpropanolamine hydrochloride; 8, phenylephrine hydrochloride; and 9, sample stressed at 70° for 42 days. Colors: Y = yellow, R = red, and B = brown.*

[4] This method was developed by the Analytical Chemistry Department, Sterling-Winthrop Research Institute.

[5] The *n*-pentane should be passed through a column of silica gel before it is used.

Table I—Results of Stressing Mixture of Phenylephrine Hydrochloride, Phenylpropanolamine Hydrochloride, and Aspirin at 70°

Assay	Original	21 Days	42 Days
Phenylephrine hydrochloride	5.0 mg.	3.7 mg.	2.3 mg.
Phenylpropanolamine hydrochloride	12.6 mg.	12.5 mg.	11.4 mg.
Aspirin	325.8 mg.	—	—
Free salicylic acid	0.03%	0.63%	2.74%
Free salicylic acid (theoretical)[a]	—	0.88%	2.67%
Moisture (Karl Fischer titration)	0.25%	—	—

TLC

21 Days

1. Trace amount of mono- and triacetylated phenylephrine
2. Moderate amount of diacetylated phenylephrine
3. No detectable amount of acetylated phenylpropanolamine

42 Days

1. No detectable amount of monoacetylated phenylephrine
2. Large amount of diacetylated phenylephrine
3. Trace amount of triacetylated phenylephrine
4. No detectable amount of acetylated phenylpropanolamine

[a] Calculated by using the assayed amount of phenylephrine hydrochloride and by assuming that salicylic acid is formed only during the acetylation of phenylephrine by aspirin and that this acetylation is the only cause of phenylephrine decrease in potency.

(0.209), 10 mcg./ml. (0.424), and 15 mcg./ml. (0.634); phenylpropanolamine hydrochloride—3 mcg./ml. (0.209), 6 mcg./ml. (0.411), and 9 mcg./ml. (0.612). The assays and their standard deviations, based on six replicate values for the low and high quantities of phenylephrine hydrochloride, are 0.249 ± 0.011 mg. and 0.745 ± 0.006 mg. Likewise, for phenylpropanolamine hydrochloride, they are 0.567 ± 0.011 mg. and 1.81 ± 0.01 mg.

To obtain an indication of the validity of the assay, the following mixture was stressed at 70°: phenylephrine hydrochloride, 5.0 mg., phenylpropanolamine hydrochloride, 12.5 mg.; and aspirin, 325 mg. The mixture was assayed at 0, 21, and 42 days for phenylephrine hydrochloride, phenylpropanolamine hydrochloride, and free salicylic acid. Aspirin was assayed in only the unstressed mixture. TLC was used to detect any acetylated forms of phenylephrine or phenylpropanolamine.

The results of the stressing test (Table I) indicate that the periodate assay is capable of accurately measuring the decomposition of phenylephrine hydrochloride and phenylpropanolamine hydrochloride. In this case, the phenylephrine hydrochloride is being acetylated, as indicated by TLC (Fig. 3). Also, the increase in free salicylic acid corresponds to the theoretical amount that would be formed by the acetylated amount of phenylephrine hydrochloride, as indicated by the assay results, assuming that acetylation of phenylephrine is the only reaction. The phenylpropanolamine hydrochloride proves to be quite stable under these conditions as compared to phenylephrine hydrochloride. The acetylated phenylephrine and phenylpropanolamine compounds were tested for possible interferences in the assay procedure. As expected, none of the acetylated compounds was oxidizable with the periodate, and no absorbance was obtained in the range of interest (360–240 nm.) under the conditions of assay. Also, there were no interferences due to the presence of the excipients: starch, sugar, and talc.

SUMMARY

A periodate oxidation assay method for phenylephrine hydrochloride and phenylpropanolamine hydrochloride in combination with aspirin was shown to be accurate, sensitive, and precise. This assay is based on the oxidation of the two phenylethanolamines to their respective aryl aldehydes, which are selectively extracted by pH manipulation. The assay was also proven to be stability indicating by following the decomposition of a stressed mixture of phenylephrine hydrochloride, phenylpropanolamine hydrochloride, and aspirin. For the usual dosages used in capsule and tablet forms of these two drugs, this assay procedure is sufficiently sensitive for individual dosage unit measurements.

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Analysis of NMR Spectra for Rotamer Populations of Dimethoxyamphetamines

K. BAILEY

Abstract □ The side chain of amphetamines gives an NMR spectrum analyzed as an *ABX* pattern. The method used for obtaining relative populations of rotamers about the α,β-bond from the spectrum is described, and results are presented for dimethoxyamphetamines. The data do not show any correlation between psychotropic activity and relative rotamer populations.

Keyphrases □ Amphetamines, dimethoxy-, rotamers—NMR spectroscopy, analysis □ Dimethoxyamphetamine rotamers—NMR spectroscopy, analysis, *ABX* pattern □ Psychotropic activity—dimethoxyamphetamine conformational behavior □ Conformational analysis, NMR spectroscopy—dimethoxyamphetamine rotamers □ NMR spectroscopy—analysis, dimethoxyamphetamine rotamers

Methods for the conformational analysis of organic molecules by consideration of their NMR spectra are well known (1, 2). Among biologically important open-chain compounds, the conformations of acetylcholine (3) and some isologs (4) have been studied. Diastereoisomers with protons on adjacent asymmetric carbons have received attention (5–11); for such compounds, the ratio of that rotamer with *trans*-disposition of the vicinal protons to the two rotamers with *gauche*-arrangement may be estimated from the observed mean vicinal coupling constant (1, 5). The effects of intramolecular hydrogen bonding on conformational behavior have been considered for ethane-1,2-diols (7), ephedrines (9, 10), and chloramphenicols (11). Interest in amphetamines led to the attempt to measure their relative rotamer populations using NMR data. Intramolecular hydrogen bonding between an *ortho*-methoxyl oxygen atom and the amino protons has been suggested (12) as modifying the conformational behavior of such psychotropic compounds.

DISCUSSION

In principle, it is possible to estimate relative populations of the three rotamers, 1, 2, and 3, shown in Newman projection (Fig. 1) for amphetamines $\mathrm{ArCH_2CH(CH_3)NH_2}$, provided that the diastereotopic methylene protons can be identified and distinguished from one another in the NMR spectrum. Let the mole fraction of Rotamer 1 be a, of 2 be b, and of 3 be c (Fig. 1). In every rotamer, all *trans*-$J\alpha,\beta$ are approximately equal (to Jt), and all *gauche*-$J\alpha,\beta$ are approximately equal (to Jg) (2). Then, where $J\alpha_1,\beta$ and $J\alpha_2,\beta$ are the two observed (mean) vicinal couplings:

$$J\alpha_1,\beta = a \cdot Jt + b \cdot Jg + c \cdot Jg \quad \text{(Eq. 1)}$$

$$J\alpha_2,\beta = a \cdot Jg + b \cdot Jt + c \cdot Jg \quad \text{(Eq. 2)}$$

$$1 = a + b + c \quad \text{(Eq. 3)}$$

Rearrangement gives:

$$a = \frac{J\alpha_1,\beta - Jg}{Jt - Jg} \quad \text{(Eq. 4)}$$

$$b = \frac{J\alpha_2,\beta - Jg}{Jt - Jg} \quad \text{(Eq. 5)}$$

$$c = \frac{Jt + Jg - (J\alpha_1,\beta + J\alpha_2,\beta)}{Jt - Jg} \quad \text{(Eq. 6)}$$

Using values for Jt and Jg obtained from suitable model compounds, c may be estimated; but unless the two observed $J\alpha,\beta$ couplings can be unambiguously assigned to the protons α_1H and α_2H, as depicted in Fig. 1, the values obtained for a and b can be assigned to either Rotamers 1 and 2 or 2 and 1. Secondary information from known steric preferences and/or observed chemical shifts assists in the definition. Data from the NMR spectra of five

Table I—NMR Spectral Data[a] for Dimethoxyamphetamines

Compound	β-CH_3 (Doublet)	NH_2	2αH (2 Double Doublets)	β-H (Multiplet)	$ArOCH_3$
2,3-Dimethoxyamphetamine	8.88 (6.0)	8.12	7.44 (−12.7, 8.7) 7.30 (−12.7, 4.1)	6.81	6.18, 6.16
2,4-Dimethoxyamphetamine	8.92 (6.0)	8.35	7.54 (−13.0, 8.2) 7.36 (−13.0, 4.6)	6.84	6.22, 6.22
2,5-Dimethoxyamphetamine	8.91 (5.8)	8.08	7.51 (−12.5, 8.4) 7.35 (−12.5, 4.4)	6.83	6.29, 6.29
3,4-Dimethoxyamphetamine	8.89 (6.0)	7.90	7.53 (−13.0, 8.4) 7.35 (−13.0, 4.5)	6.85	6.15, 6.15
3,5-Dimethoxyamphetamine	8.88 (6.0)	8.09	7.57 (−13.0, 8.5) 7.37 (−13.0, 4.3)	6.83	6.25, 6.25

[a] τ-values, measured with a Varian A-60A spectrometer using solutions about 15% in $CDCl_3$ at 40° containing tetramethylsilane as internal standard. Coupling constants (Hz., signs by analogy with known systems) calculated as described are thought accurate ±0.1 Hz. The NH_2 protons (τ-concentration dependent) exchanged with D_2O with no other change in τ or J values. Appropriate aromatic signals and integration ratios were observed. The (unreported) 2,6-dimethylamphetamine has not yet been obtained.

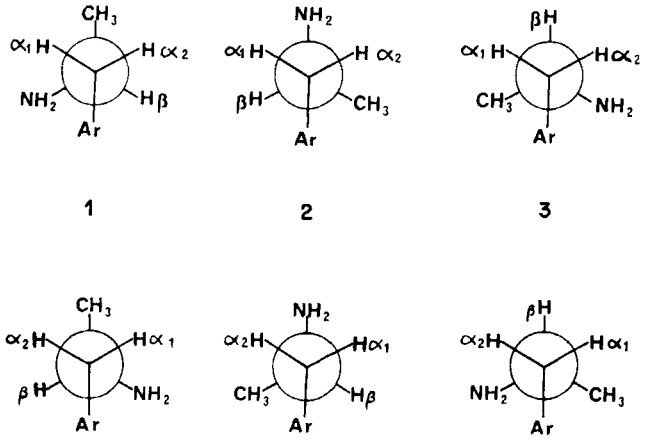
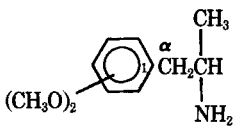


Figure 1—*Staggered rotamers for R (lower) and S (upper) amphetamines.*

dimethoxyamphetamines (two aromatic methoxyl groups) are sum-



dimethoxyamphetamine

marized in Table I. The side-chain spectral type is that for fast rotation, unequal populations (2). The two α- (benzylic) protons and the β-proton give signals ($\alpha_1\alpha_2\beta$) analyzable as *ABX* (at 60 MHz., $\nu\alpha_1 - \nu\alpha_2 \simeq 20$ Hz.; $J\alpha_1, \alpha_2 \simeq -13$ Hz.; $\nu\alpha - \nu\beta \simeq 30$ and 40 Hz.). Only the eight-line *AB* ($\alpha_1\alpha_2$) portion was used here because the *X* (β-proton) part is complicated by further coupling with the β-CH_3 protons. The calculated *a*, *b*, and *c* are sensitive to $J\alpha_1\beta$ and to $J\alpha_2\beta$ (and to *Jt* and *Jg*), so a first-order analysis of the spectrum would give results in some error; thus, the two first-order $J\alpha,\beta$ are about 7.7 and 5.2 Hz., but calculation of the *ABX* type gives values of about 8.5 and 4.3 Hz. (Table I). The calculated *a*, *b*, and *c* of Table II (at once approximate because of the approximations made in deducing Eqs. 1 and 2) were obtained using 12 and 2 Hz. for *Jt* and *Jg*, respectively (5). Even if these chosen values are in error, the results will be of the right order and the direction of any observed changes will be correct[1]. If α_1H as shown is the proton with greater $J\alpha,\beta$ (observed at higher field), then on the average $a = 0.65$, $b = 0.23$, and $c = 0.12$ (Rotamer 1 dominates); if α_1H as shown is the proton with lower $J\alpha,\beta$ (observed at lower field), then $a = 0.23$, $b = 0.65$, and $c = 0.12$ (Rotamer 2 dominates). A *gauche*-β-CH_3 will have a weakly shielding effect (compared with H), and a *gauche*-β-NH_2 will have a more strongly deshielding effect on the α-protons (9, 13). Inspection of Rotamers 1, 2, and 3 (Fig. 1) and summing effects of either $b > a$ or $a > b$ shows that α_1H will be that at lower field *on this basis;* therefore, $J\alpha_1,\beta < J\alpha_2,\beta$ and, hence, $b > a > c$. The dominance of Rotamer 2 may be due to electrostatic repulsion between the electronegative amino and electronegatively substituted aryl groups, since CH_3 and NH_2 exert similar steric effects; this work seems to provide the first experimental confirmation of theoretical predictions (14) that the nitrogen atom of these compounds is antiplanar to the phenyl ring.

According to the model of Snyder and Richelson (12) for psychotomimetic activity of methoxyamphetamines, hydrogen bonding of the amino protons with a 2-methoxyl group favors a side-chain conformation approximating ring C of LSD. Such hydrogen bonding may occur in Rotamers 1 and 3 (possibly with some deformation) but not 2. An additional 3-methoxyl group will hinder this (12). Consequently, the model predicts that Rotamers 1 and 3 may contribute more to the mean for both 2,4- and 2,5-dimethoxyamphetamine than they do for 2,3-, 3,4-, or 3,5-dimethoxyamphetamine. Any marked changes in the true rotamer populations would be reflected in the observed coupling constants and, hence, the calculated rotamer populations in Table II (*e.g.*, a 10% change in Rotamer 1 or 2 would alter the corresponding *J* by about 1 Hz.). Differences in these figures are very small and about the same as the estimated error (arising in $J\alpha,\beta$) using the present technique; the results indicate little difference in the conformations of the dimethoxyamphetamines as free bases in an aprotic medium. This approach will be extended using different amphetamines, solvents, pH's, *etc.* Since the benzylic protons are distinguishable, such analyses may help in evaluating structure–activity relationships in the nature of the enzymic process in amine-β-hydroxylation of amphetamines to norephedrines, *etc.*

[1] During the preparation of this communication, J. E. Forrest, R. A. Heacock, and T. P. Forrest, *J. Pharm. Pharmacol.*, **22**, 512(1970), reported data for some adrenalin derivatives, neglecting rotamers of type 3. A first-order analysis was made, although $\nu\beta_1 - \nu\beta_2 \simeq 5$ Hz. and $J\beta_1,\beta_2$ (not reported) would be $\simeq -13$ Hz. Values of 9 and 2.5 Hz. for *Jt* and *Jg*, respectively, were obtained from the spectrum of adrepine which was assumed to exist as one rotamer of the 1 or 2 type.

Table II—Calculated Rotamer Populations (Percent) for Dimethoxyamphetamines[a]

Compound	Rotamer 1	Rotamer 2	Rotamer 3
2,3-Dimethoxyamphetamine	21 (67)	67 (21)	12
2,4-Dimethoxyamphetamine	26 (62)	62 (26)	12
2,5-Dimethoxyamphetamine	24 (64)	64 (24)	12
3,4-Dimethoxyamphetamine	25 (64)	64 (25)	11
3,5-Dimethoxyamphetamine	23 (65)	65 (23)	12

[a] Solutions about 15% in $CDCl_3$ at 40°. Figures in parentheses apply if α_1H and α_2H, as shown in Fig. 1, are oppositely assigned.

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4,17α-Dimethyl-4-aza-5α-androstan-17β-ol Acetate and Related Azasteroids

NORMAN J. DOORENBOS and SISTER JANE MARIE BROWN*

Abstract □ 4,17α-Dimethyl-4-aza-5-androsten-17β-ol-3-one, 4,17α-dimethyl-4-aza-5α-androstan-17β-ol-3-one, 4,17α-dimethyl-4-aza-5α-androstan-17β-ol, and their corresponding acetate esters were synthesized and compared for antimicrobial activity. 4,17α-Dimethyl-4-aza-5α-androstan-17β-ol acetate exhibited significant activity.

Keyphrases □ Azasteroids—synthesis, antimicrobial activity evaluation □ 4,17α-Dimethyl-4-aza-5α-androstan-17β-ol acetate, related compounds—synthesis, screening as antimicrobial agents □ Antimicrobial agents, potential—synthesis, screening, 4,17α-dimethyl-4-aza-5α-androstan-17β-ol acetate, esters

Antimicrobial activity was found in 4-azacholestanes (1–6), 4-aza-androstanes (7), 4-azapregnanes (7), 17a-aza-D-homoandrostanes (8), and 2-azacholestane and 3-azacholestane (9) lacking hydroxyl substituents, while corresponding 4-aza-androstanes (2, 4, 5, 7), 4-azapregnanes (4, 7), and 17a-aza-D-homoandrostanes (10) with hydroxy substituents possessed very little activity.

The objective of this study was to determine if esterification of these hydroxyl groups would restore antimicrobial activity. A comparison of the antimicrobial properties of 4,17α-dimethyl-4-aza-5α-androstan-17β-ol (III) and its acetate ester (VI) was selected for this study.

4,17α-Dimethyl-4-aza-5-androsten-17β-ol-3-one (I) (11) was hydrogenated to obtain 4,17α-dimethyl-4-aza-5α-androstan-17β-ol-3-one (II) in 91% yield and reduced with lithium aluminum hydride to give 4,17α-dimethyl-4-aza-5α-androstan-17β-ol (III) in 71% yield (Scheme I). Acetate esters IV, V, and VI were prepared by treatment of I, II, and III with acetic anhydride. Attempts to prepare carbamate esters by reaction of I, II, and III with dimethylcarbamyl chloride and phenyl isocyanate were unsuccessful, except for the preparation of the *N*-phenylcarbamate (VIII) of I following a procedure developed for preparing methyltestosterone *N*-phenylcarbamate (VII) from methyltestosterone.

Each of these azasteroids was compared with *N*-methyl-4-aza-5α-cholestane (ND-502) against a representative group of microorganisms previously found useful in screening azasteroids for activity using described procedures (2). VI, the only active compound, is compared with ND-502 in Table I.

Table I—Antimicrobial Activity

Microorganisms	Minimum Inhibitory Concentration of Steroids (2)[a] VI	ND-502
Staphylococcus aureus	—[b]	5
Escherichia coli	—	—
Candida albicans	50	10
Aspergillus niger	5	5

[a] All values are expressed in micrograms per milliliter. [b] Inactive (no inhibition at 100 mcg./ml.).

EXPERIMENTAL[1]

4,17α-Dimethyl-4-aza-5α-androstan-17β-ol-3-one (II)—To a solution of 1.50 g. (4.7 mmoles) of 4,17α-dimethyl-4-aza-5-androsten-17β-ol-3-one (I) (11) in 50 ml. of glacial acetic acid was added 1.0 g. of platinum dioxide. The mixture was hydrogenated at 70° and 60 p.s.i. for 20 hr. The catalyst was filtered, and the solvent was evaporated *in vacuo*. The residue was crystallized from ether, and the resulting white crystals were used without further purification in the next step. The filtrate was concentrated twice, giving a total yield of 1.37 g. (91%), m.p. 210–211°; $\lambda_{max.}^{EtOH}$ no absorption at 235 nm.; $\lambda_{max.}^{CHCl_3}$ no shoulder at 5.96 μ (hence, —C=C— was reduced).

4,17α-Dimethyl-4-aza-5α-androstan-17β-ol (III)—A solution of 2.00 g. (0.006 mole) of 4,17α-dimethyl-4-aza-5α-androstan-17β-ol-3-one (II) in dry tetrahydrofuran was added dropwise to a cooled suspension of 3.0 g. (0.09 mole) of lithium aluminum hydride in 100 ml. of dry tetrahydrofuran under a nitrogen atmosphere. The mixture was allowed to reflux with stirring for 18 hr. The reaction vessel was cooled in an ice bath while moist tetrahydrofuran was added slowly. The mixture was filtered, the inorganic salts were washed with additional tetrahydrofuran, the solvent was removed *in vacuo*, and the residue was crystallized from acetone to yield 1.44 g. (71%) of III; m.p. 154–156°; $\lambda_{max.}^{CHCl_3}$ no absorption at 6.1 μ (hence, C=O was reduced).

4,17α-Dimethyl-4-aza-5-androsten-17β-ol-3-one Acetate (IV)—4,17α-Dimethyl-4-aza-5-androsten-17β-ol-3-one (I) (1.0 g., 0.03 mole) and 10 ml. of acetic anhydride were refluxed for 30 min. The hot mixture was poured into ice water with stirring. Crystals formed rapidly from the original yellow oil. Recrystallization from acetone yielded white crystals, 970 mg. (87%); m.p. 198–199°; $\lambda_{max.}^{CHCl_3}$ 5.8 and 6.1 μ.

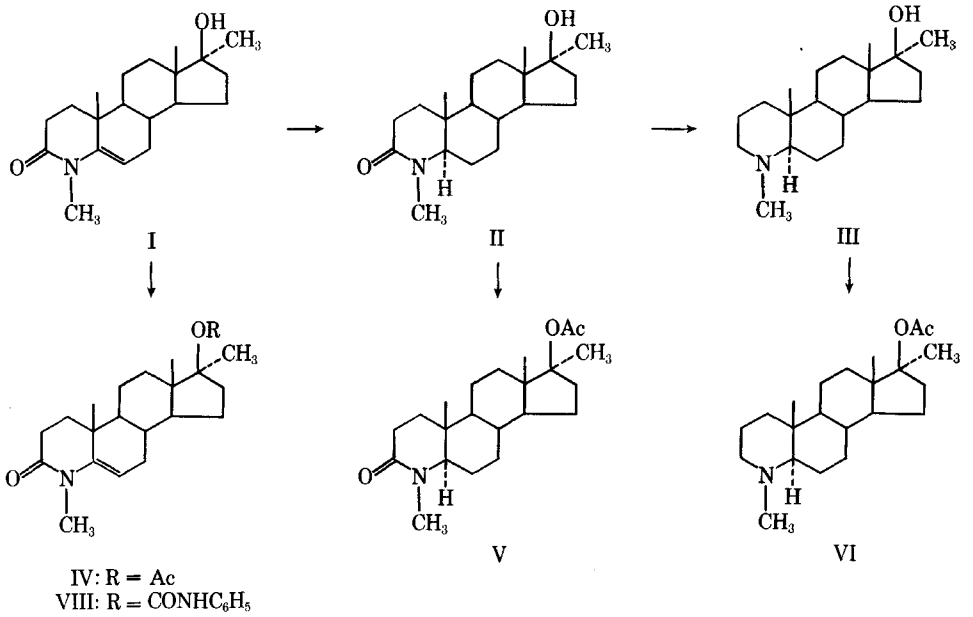
Anal.—Calc. for $C_{22}H_{31}NO_3$: C, 73.50; H, 9.25; N, 3.90. Found: C, 73.90; H, 9.26; N, 4.03.

4,17α-Dimethyl-4-aza-5α-androstan-17β-ol-3-one Acetate (V)—A solution of 1.00 g. of 4,17α-dimethyl-4-aza-5α-androstan-17β-ol-3-one (II) in 80 ml. of acetic anhydride was refluxed 1 hr. The acetic anhydride was evaporated *in vacuo*, and the residue, a yellow oil, was dissolved in ethyl ether. The solution was washed with a saturated sodium bicarbonate solution until effervescence ceased. After washing with water, the ether layer was dried over anhydrous sodium sulfate and taken to dryness *in vacuo*. Crystallization from acetone yielded 585 mg. (54%); m.p. 203–204°; $\lambda_{max.}^{CHCl_3}$ 5.8 and 6.1 μ.

Anal.—Calc. for $C_{22}H_{35}NO_3$: C, 73.09; H, 9.76; N, 3.87. Found: C, 73.18; H, 9.55; N, 3.86.

4,17α-Dimethyl-4-aza-5α-androstan-17β-ol Acetate (VI)—4,17α-Dimethyl-4-aza-5α-androstan-17β-ol (III) (100 mg., 0.3 mmole) was dissolved in 15 ml. of freshly distilled acetic anhydride, and the solution was refluxed for 30 min. The hot reaction mixture was poured into ice water with stirring. The crystals were filtered and recrystallized from methanol to yield 86 mg. (83%) of VI; m.p. 119.5–120°; $\lambda_{max.}^{CHCl_3}$ 5.78 μ.

[1] All melting points are uncorrected and were observed on a Thomas-Hoover or a Fisher-Johns melting-point apparatus. IR spectra were recorded by a Perkin-Elmer IR recording spectrophotometer. The UV spectra were recorded by a Beckman DB spectrophotometer. The elemental analyses were determined by Schwarzkopf Microanalytical Laboratory.



Scheme I

Anal.—Calc. for $C_{22}H_{37}NO_2$: C, 76.03; H, 10.73; N, 4.03. Found: C, 75.68; H, 10.83; N, 3.98.

17α-Methyl-4-androsten-17β-ol-3-one *N*-Phenylcarbamate (VII)—To a chilled solution of 1.00 g. (3.3 mmoles) of methyltestosterone in 25 ml. of dry ethylbenzene was added 1.0 ml. (9 mmoles) of phenyl isocyanate. Vigorous refluxing under a nitrogen atmosphere was maintained for 30 hr. The solvent and excess phenyl isocyanate were completely removed *in vacuo*. The residue was crystallized from acetone to obtain 946 mg. (68%); m.p. 296–298°; λ_{max}^{KBr} 3.0, 5.78, 6.0, 13.1, and 14.3 μ.

Anal.—Calc. for $C_{27}H_{35}NO_3$: C, 76.92; H, 8.37; N, 3.32. Found: C, 76.89; H, 8.61; N, 3.72.

4,17α-Dimethyl-4-aza-5-androsten-17β-ol-3-one *N*-Phenylcarbamate (VIII)—To a chilled solution of 1.00 g. (3.2 mmoles) of 4,17α-dimethyl-4-aza-5-androsten-17β-ol-3-one (I) in dry ethylbenzene was added 1.0 ml. (9 mmoles) of phenyl isocyanate. Vigorous refluxing under a nitrogen atmosphere was maintained for 30 hr. The solvent and excess phenyl isocyanate were removed *in vacuo*, and the residue was crystallized from ethyl acetate–benzene to obtain 74 mg. (60%) of VIII as white crystals; m.p. 278–280°; λ_{max}^{KBr} 2.98, 5.75, 6.10, 13.10, and 14.25 μ.

Anal.—Calc. for $C_{27}H_{36}N_2O_3$: C, 74.28; H, 8.31; N, 6.42. Found: C, 74.02; H, 8.92; N, 6.31.

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Derivatives of 17a-Aza-D-homo-5-androsten-3β-ol

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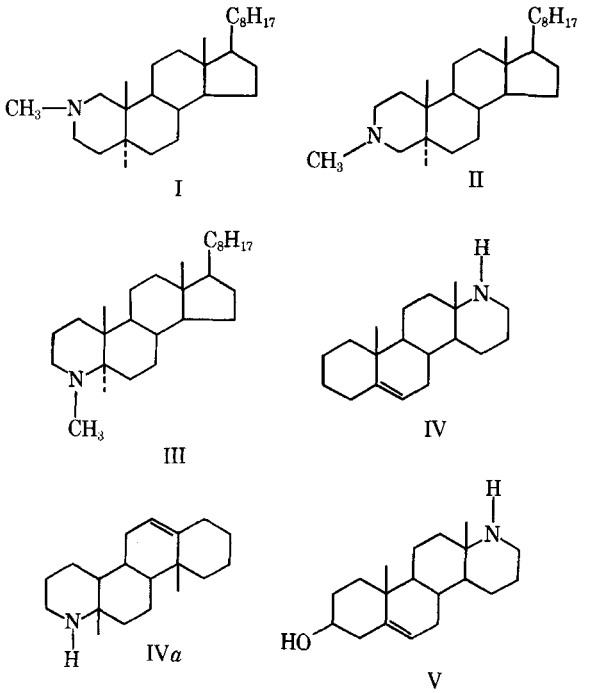
Abstract □ The synthesis of several types of *N*-substituted derivatives of 17a-aza-D-homo-5-androsten-3β-ol as potential antimicrobial agents is reported. Methyl and propyl groups were added by reductive alkylation, and β-dialkylaminoethyl and γ-dialkylaminopropyl groups were introduced by the reaction of dimethylamine or piperidine with the appropriate 17a-(ω-chloroacyl) derivative of VI. None of these derivatives exhibited significant antibacterial or antifungal activity.

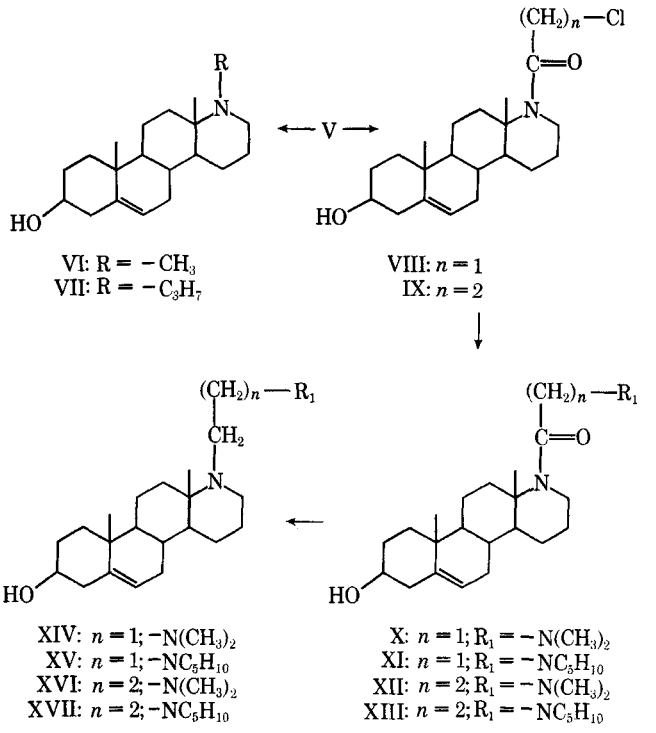
Keyphrases □ 17a-Aza-D-homo-5-androsten-3β-ol derivatives—synthesis, screening as possible antimicrobial agents □ Azasteroids—synthesis, pharmacological evaluation of 17a-aza-D-homo-5-androsten-3β-ol derivatives □ Antimicrobial agents, potential—synthesis, pharmacological screening, 17a-aza-D-homo-5-androsten-3β-ol derivatives

Earlier investigations (1–6) in this laboratory established that certain 4-azasteroids possessed substantial antimicrobial activity. Active derivatives inhibit the growth of most Gram-positive bacteria, molds, and yeasts at concentrations of 0.1–10 mcg./ml. and kill at higher concentrations. More recently, *N*-methyl-2-aza-5α-cholestane (I) and *N*-methyl-3-aza-5α-cholestane (II) were reported to possess antimicrobial properties similar to *N*-methyl-4-aza-5α-cholestane (III) (7). Thus, it appears that 2-azasteroids and 3-azasteroids have antimicrobial properties similar to 4-azasteroids.



This investigation was begun with the objective of determining if 17a-aza-D-homosteroids also possess antimicrobial properties. 17a-Aza-D-homo-5-androstene (IV*a*) resembles a 4-azasteroid in shape and location of nitrogen, as illustrated when its structure is drawn in the unconventional manner (IV*b*). Since the ring fusions are *trans*, the overall shapes are similar. The double bond at position 5 in IV does not produce a major change in the shape of the steroid molecule.

This paper describes the synthesis and preliminary antimicrobial study of derivatives of 17a-aza-D-homo-5-androsten-3β-ol (V). V was prepared in four synthetic steps from androstenolone, as previously described (8–10). 17a-Methyl-17a-aza-D-homo-5-androsten-3β-ol (VI) was prepared by the Leuckart reaction (Method A) or reductive methylation (Method B) of V. The yields were 72% by each method. 17a-Propyl-17a-aza-D-homo-5-androsten-3β-ol (VII) was synthesized in 75% yield by reductive alkylation of V using propionaldehyde (Scheme I).



Scheme I

β-Dialkylaminoethyl (XIV and XV) and γ-dialkylaminopropyl (XVI and XVII) substituents were introduced at position 17a in V through the 17a-(ω-chloroacyl) derivatives (VIII and IX) by reaction with dimethylamine or piperidine, in the presence of alkali, and subsequent reduction of the resulting amides with lithium aluminum hydride. Representative synthetic procedures are described in the *Experimental* section, and data on these steroids are presented in the *Experimental* section and Table I.

Azasteroids V, VI, VII, XIV, XV, XVI, and XVII were screened by serial dilution (1) for antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*,

Table I—*N*-Substituted Derivatives of 17a-Aza-D-homo-5-androsten-3β-ol

Compound	R	Method	Recrystallization Solvent[a]	Yield, %	Melting Point	Molecular Formula	Analysis, % Calc.	Analysis, % Found
VII	C_3H_7	B	Ac	75	308–309°	$C_{22}H_{38}BrNO$[b,c]	C, 64.06 H, 9.28 N, 3.39	C, 64.60 H, 9.31 N, 3.32
XI	$COCH_2NC_5H_{10}$	C		70	302–303°	$C_{26}H_{43}BrN_2O_2$[b,d]	C, 63.01 H, 8.75 N, 5.65	C, 62.87 H, 8.66 N, 5.84
XV	$(CH_2)_2NC_5H_{10}$	D	Et–W	95	123–125°	$C_{26}H_{44}N_2O$	C, 77.95 H, 11.07 N, 6.99	C, 77.69 H, 11.02 N, 6.90
						$C_{38}H_{50}N_8O_{15}$[e]	C, 53.15 H, 5.86 N, 13.04	C, 53.19 H, 5.72 N, 12.89
XII	$CO(CH_2)_2N(CH_3)_2$	C	Et	89	210–212°	$C_{24}H_{40}N_2O_2$	C, 74.18 H, 10.38 N, 7.21	C, 74.32 H, 10.56 N, 7.24
					233–234°	$C_{30}H_{43}N_5O_9$[e]	C, 58.33 H, 7.02 N, 11.34	C, 58.40 H, 7.08 N, 11.49
XVI	$(CH_2)_3N(CH_3)_2$	D	Et	88	146–147.5°	$C_{24}H_{42}N_2O$	C, 76.95 H, 11.30 N, 7.48	C, 76.99 H, 11.32 N, 7.21
XVII	$(CH_2)_3NC_5H_{10}$[f]	D	Et–W	75	156–158°	$C_{27}H_{46}N_2O$	C, 78.21 H, 11.18 H, 6.76	C, 78.39 H, 11.40 H, 6.84
					227–229°	$C_{38}H_{50}N_8O_{15}$[e]	C, 53.67 H, 6.00 N, 12.83	C, 53.06 H, 5.79 N, 12.67

[a] Ac = acetone; Et = ethanol; W = water. [b] HBr salt. [c] Calc. for Br: 19.38. Found: 19.25. [d] Calc. for Br: 16.31. Found: 15.91. [e] Picrate salt. [f] Intermediate XIII, prepared by Method C, was used without complete purification.

Aspergillus niger, and *Candida albicans*. None of these azasteroids exhibited activity at concentrations as high as 100 mcg./ml. and, hence, are considered inactive.

EXPERIMENTAL

17a-Methyl-17a-aza-D-homo-5-androsten-3β-ol (VI)—*Method A*—A mixture of 10.0 g. of 17a-aza-D-homo-5-androsten-3β-ol (V) (8–10) in 50 ml. of 37% formaldehyde and 50 ml. of formic acid was refluxed 5 hr. The reaction mixture was cooled, basified with ammonia solution, and extracted with ether. The ether extract was washed with water and dried, and the solvent removed *in vacuo*. Crystallization from acetonitrile gave white platelets; yield 7.5 g. (72%), m.p. 170–172° (reported 170–172°). The picrate salt crystallized as yellow needles, m.p. 264–265°, from ethanol.

Anal.—Calc. for $C_{26}H_{36}N_4O_8$: C, 58.63; H, 6.81; N, 10.52. Found: C, 58.92; H, 7.00; N, 10.66.

The methiodide of the 3-acetyl derivative of VI was prepared by treating VI with acetic anhydride and pyridine. The oily product thus obtained was treated in ethanol solution with methyl iodide. The solvent was removed *in vacuo*, and the residue was crystallized from ethanol to give white platelets, m.p. 287–289°

Anal.—Calc. for $C_{23}H_{38}INO_2$: C, 56.67; H, 7.86; I, 26.04. Found: C, 56.62; H, 7.89; I, 26.47.

Method B—A mixture of 2.0 g. of 17a-aza-D-homo-5-androsten-3β-ol (V), 30 ml. of ethanol, 10 ml. of 37% formaldehyde, and 30 mg. of platinum oxide was treated with hydrogen at 60° and 60 p.s.i. for 15 hr. The catalyst was filtered and the solvent evaporated. Crystallization of the residue from acetone yielded 1.5 g. (72%) of white platelets, m.p. 170–172°. This sample was identical to the sample prepared by Method A.

17a-Propyl-17a-aza-D-homo-5-androsten-3β-ol (VII)—17a-Aza-D-homo-5-androsten-3β-ol (V) (2.0 g.) was dissolved in a mixture of 30 ml. of ethanol and 10 ml. of propionaldehyde, and 30 mg. of platinum oxide was added. The mixture was hydrogenated at 60° and 60 p.s.i. for 15 hr. The catalyst was filtered from the mixture, and the filtrate was evaporated to dryness. The residue (1.5 g.) was chromatographed on 40 g. of neutral alumina (activity grade I). Elution with benzene–ether (1:1) gave a substance which, on crystallization from acetone, gave white needles of VII, m.p. 151–153°. The hydrobromide salt of VII was obtained as light-brown platelets, m.p. 308–309°.

Anal.—Calc. for $C_{22}H_{37}NO \cdot HBr$: C, 64.06; H, 9.28; N, 3.39; Br, 19.38. Found: C, 64.60; H, 9.31; N, 3.32; Br, 19.25.

17a-(Chloroacetyl)-17a-aza-D-homo-5-androsten-3β-ol (VIII) and 3β-(Chloroacetoxy)-17a-(chloroacetyl)-17a-aza-D-homo-5-androstene—A mixture of 2.00 g. of 17a-aza-D-homo-5-androsten-3β-ol (V), 50 ml. of methylene chloride, 100 ml. of 10% sodium hydroxide, and 10 ml. of chloroacetyl chloride was stirred 2 hr. at room temperature. The organic layer was separated, washed with water, and dried, and the solvent was removed *in vacuo*. The solid residue was crystallized from ethanol to yield 1.8 g., m.p. 170–220°. Two recrystallizations from ethanol gave 200 mg. (6%) of 3β-(chloroacetoxy)-17a-(chloroacetyl)-17a-aza-D-homo-5-androstene as white crystals, m.p. 175–177°; $\nu_{max.}^{CHCl_3}$ 1754 cm.$^{-1}$ (ester C=O) and 1656 cm.$^{-1}$ (amide C=O).

Anal.—Calc. for $C_{23}H_{33}Cl_2NO_3$: C, 62.44; H, 7.52; Cl, 16.03; N, 3.17. Found: C, 61.88; H, 7.51; Cl, 15.89; N, 3.08.

The ethanolic mother liquor was concentrated to give a substance, m.p. 240–247°. Two crystallizations from ethanol yielded 1.60 g. (64%) of VIII as white crystals, m.p. 260–261°; $\nu_{max.}^{KBr}$ 1650 cm.$^{-1}$ (amide C=O).

Anal.—Calc. for $C_{21}H_{32}ClNO_2$: C, 68.92; H, 8.81; Cl, 9.69; N, 3.38. Found: C, 68.78; H, 8.69; Cl, 9.94; N, 3.95.

17a-(3-Chloropropionyl)-17a-aza-D-homo-5-androsten-3β-ol (IX)—A solution of 2.0 g. of V in 100 ml. of methylene chloride was treated with 10 ml. of β-chloropropionyl chloride and 100 ml. of 10% sodium hydroxide solution. The mixture was stirred for 15 hr. The methylene chloride layer was separated, and the aqueous layer was extracted with methylene chloride. The combined methylene chloride extract was washed with water and dried with anhydrous sodium sulfate; the residue obtained on evaporating the solvent was 1.5 g. of IX as a white substance, m.p. 210–225°. Attempts to purify this substance further were unsuccessful. It was used as such for further reaction.

17a-(Dimethylaminoacetyl)-17a-aza-D-homo-5-androsten-3β-ol (X)—*Method C*—To a solution of 3.00 g. of VIII in 100 ml. of ethanol, 3.0 g. of dimethylamine hydrochloride and 3 g. of potassium hydroxide were added. The mixture was left at room temperature for 48 hr. It was filtered and evaporated to dryness under reduced pressure. The solid residue was washed with water, dried, and crystallized from ethanol to yield 2.7 g. (88%) of white crystals of X, m.p. 174–176°; $\nu_{max.}^{CHCl_3}$ 1645 cm.$^{-1}$ (amide C=O). The picrate of X was prepared and crystallized from ethanol to give yellow needles, m.p. 201–202°.

Anal.—Calc. for $C_{29}H_{41}N_3O_3$: C, 57.70; H, 6.85; N, 11.60. Found: C, 58.07; H, 7.37; N, 12.03.

17a-(2-Dimethylaminoethyl)-17a-aza-D-homo-5-androsten-3β-ol (XIV)—*Method D*—Compound X (2.0 g.) was dissolved in 30 ml. of dioxane, a solution of 1.0 g. of lithium aluminum hydride in 300 ml. of dioxane was added, and the mixture was refluxed for 48 hr. Excess hydride was destroyed with water. The inorganic salts were filtered and washed with dioxane. The dioxane solution was evaporated to dryness, and the residue was crystallized from ethanol to give 1.8 g. (94%) of white crystals of XIV, m.p. 195–196°. The hydrobromide salt of XIV was prepared by passing HBr gas into its acetone solution and crystallizing from ethanol to give white needles, m.p. 309–310°.

Anal.—Calc. for $C_{23}H_{40}N_2O_2 \cdot HBr$: C, 52.88; H, 8.10; N, 5.36; Br, 30.60. Found: C, 52.45; H, 7.95; N, 5.09; Br, 31.22.

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Unexpected Formation of Thioacridone and Its Spectral Properties

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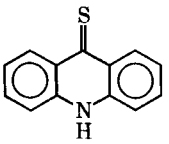
Abstract □ The isolation of thioacridone as the major product in the literature synthesis of 9-(*p*-aminophenyl)acridine and from reaction of 9-aminoacridine with *n*-butylisothiocyanate is described. Chromatographic and spectral properties of thioacridone, useful in its identification, are reported.

Keyphrases □ Thioacridone—isolation, characterization as a reaction product, spectral properties □ 9-Aminoacridine and *n*-butylisothiocyanate—isolation, characterization of thioacridone as a reaction product □ 9-(*p*-Aminophenyl)acridine with acridine, aniline, and sulfur—isolation, characterization of thioacridone as a reaction product

Current interest in these laboratories in the use of acridine derivatives as fluorescent tagging agents has led to the isolation of thioacridone [9(10*H*)-acridinethione] (I) in two separate reactions. This article describes these isolations and reports spectral data of value in the characterization of thioacridone.

Chupakhin *et al.* (1) reported the synthesis of 9-(*p*-aminophenyl)acridine in a yield of 12–13% by heating acridine, aniline, and sulfur at 185°. This yield was confirmed under similar conditions in these laboratories, but thioacridone in 83% yield also was isolated from the same reaction mixture. The formation of the thioacridone is not surprising in light of its synthesis in 85%



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yield by reaction of acridine and sulfur in a sealed tube at 190° (2) and the reported (3) high yield of 3,6-bis(dimethylamino)-9(10*H*)-acridinethione from the fusion of 3,6-bis(dimethylamino)acridine and sulfur. It is interesting that Chupakhin *et al.* (1) were able to prepare 9-(*p*-aminophenyl)-10-methylacridinium iodide from 10-methylacridinium iodide and aniline in satisfactory yield (75%), apparently without appreciable thioacridone formation. This result is in agreement with Elsager's suggestion (3) that thioacridone formation in a sulfur fusion reaction proceeds through an *N*-sulfide intermediate.

The second unexpected isolation in these laboratories of thioacridone resulted from reactions involving 9-aminoacridine and *n*-butylisothiocyanate. The result is not readily explainable based upon prior reports in the literature. Formation of thioacridone under mild conditions in this reaction, together with no indication of isothiocyanate decomposition, speaks against involvement

Anal.—Calc. for $C_{29}H_{41}N_3O_3$: C, 57.70; H, 6.85; N, 11.60. Found: C, 58.07; H, 7.37; N, 12.03.

17a-(2-Dimethylaminoethyl)-17a-aza-D-homo-5-androsten-3β-ol (XIV)—*Method D*—Compound X (2.0 g.) was dissolved in 30 ml. of dioxane, a solution of 1.0 g. of lithium aluminum hydride in 300 ml. of dioxane was added, and the mixture was refluxed for 48 hr. Excess hydride was destroyed with water. The inorganic salts were filtered and washed with dioxane. The dioxane solution was evaporated to dryness, and the residue was crystallized from ethanol to give 1.8 g. (94%) of white crystals of XIV, m.p. 195–196°. The hydrobromide salt of XIV was prepared by passing HBr gas into its acetone solution and crystallizing from ethanol to give white needles, m.p. 309–310°.

Anal.—Calc. for $C_{23}H_{40}N_2O_2 \cdot HBr$: C, 52.88; H, 8.10; N, 5.36; Br, 30.60. Found: C, 52.45; H, 7.95; N, 5.09; Br, 31.22.

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Unexpected Formation of Thioacridone and Its Spectral Properties

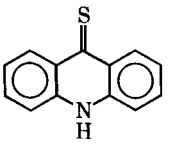
ANDRÉ DeLEENHEER*, Y. Y. SHUM, J. E. SINSHEIMER, and J. H. BURCKHALTER

Abstract □ The isolation of thioacridone as the major product in the literature synthesis of 9-(*p*-aminophenyl)acridine and from reaction of 9-aminoacridine with *n*-butylisothiocyanate is described. Chromatographic and spectral properties of thioacridone, useful in its identification, are reported.

Keyphrases □ Thioacridone—isolation, characterization as a reaction product, spectral properties □ 9-Aminoacridine and *n*-butylisothiocyanate—isolation, characterization of thioacridone as a reaction product □ 9-(*p*-Aminophenyl)acridine with acridine, aniline, and sulfur—isolation, characterization of thioacridone as a reaction product

Current interest in these laboratories in the use of acridine derivatives as fluorescent tagging agents has led to the isolation of thioacridone [9(10*H*)-acridinethione] (I) in two separate reactions. This article describes these isolations and reports spectral data of value in the characterization of thioacridone.

Chupakhin *et al.* (1) reported the synthesis of 9-(*p*-aminophenyl)acridine in a yield of 12–13% by heating acridine, aniline, and sulfur at 185°. This yield was confirmed under similar conditions in these laboratories, but thioacridone in 83% yield also was isolated from the same reaction mixture. The formation of the thioacridone is not surprising in light of its synthesis in 85%



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yield by reaction of acridine and sulfur in a sealed tube at 190° (2) and the reported (3) high yield of 3,6-bis(dimethylamino)-9(10*H*)-acridinethione from the fusion of 3,6-bis(dimethylamino)acridine and sulfur. It is interesting that Chupakhin *et al.* (1) were able to prepare 9-(*p*-aminophenyl)-10-methylacridinium iodide from 10-methylacridinium iodide and aniline in satisfactory yield (75%), apparently without appreciable thioacridone formation. This result is in agreement with Elsager's suggestion (3) that thioacridone formation in a sulfur fusion reaction proceeds through an *N*-sulfide intermediate.

The second unexpected isolation in these laboratories of thioacridone resulted from reactions involving 9-aminoacridine and *n*-butylisothiocyanate. The result is not readily explainable based upon prior reports in the literature. Formation of thioacridone under mild conditions in this reaction, together with no indication of isothiocyanate decomposition, speaks against involvement

by elemental sulfur. Also, there was no indication of SH^- arising from *n*-butylisothiocyanate under the reaction conditions. Direct attack by SH^- is the basis of several syntheses of thioacridones (4) and, indeed, an extension of such a process described by Cherntsov (5) was employed in the present investigation to obtain reference material. These syntheses, however, involve the facile replacement of chlorine in 9-chloroacridine.

The reaction may proceed by an attack of *n*-butylisothiocyanate, either through the nucleophilic sulfur upon the 9-position of aminoacridine or through an electropositive isothiocyanate carbon attack at the 10-position of 9-aminoacridine. In the latter possibility, a nucleophilic sulfur could result and serve in an attack upon a second 9-aminoacridine molecule at the 9-position.

The presence of thioacridone accompanying the synthesis of 9-(*p*-aminophenyl)acridine was first assumed on the basis of the reaction conditions used in the presence of sulfur and was confirmed by comparison to reference material. In the case of the *n*-butylisothiocyanate reaction, the presence of thioacridone was deduced from IR, NMR, and mass spectral data and was confirmed by comparison with a reference sample.

EXPERIMENTAL[1]

GLC was performed on a Hewlett-Packard model 5750 instrument, equipped with a 3.8% UCW-98 on 80/100 mesh Diatoport S in a glass column, 1.22 m., 0.64 cm. (4 ft., 0.25 in.). Thioacridone chromatographed at a retention time of 20 min. 24 sec., with helium as the carrier gas at 11 cm. $sec.^{-1}$. Operating temperatures were: injector, 227°; oven, 202°; and flame-ionization detector, 217°.

Isolation of Thioacridone during Synthesis of 9-(*p*-Aminophenyl)-acridine—Aniline (10.08 g., 0.106 mole) was added to a mixture of 4.52 g. (0.025 mole) of acridine and 3.76 g. (0.014 mole) of sulfur and refluxed at 190° for 1.5 hr. The brown-red reaction mixture was washed three times with ether, and the ether solutions were discarded. 9-(*p*-Aminophenyl)acridine was recovered from the washed residue by extraction with 0.5 *N* HCl as previously described (1).

The acid-insoluble residue (5.37 g.) was dissolved in 100 ml. of acetone, the solution was filtered to remove sulfur, and an equal volume of water was added to deposit orange crystals. Two recrystallizations from acetone–water produced 3.75 g. (83%) of fine orange crystals, m.p. 262°. These crystals did not depress the melting point of thioacridone, and they possessed the same GLC characteristics and IR spectra of a sample of thioacridone synthesized by the procedure of Cherntsov (5).

Thioacridone from 9-Aminoacridine and *n*-Butylisothiocyanate—9-Aminoacridine (0.5 g.) was refluxed in 10 ml. of *n*-butylisothiocyanate, which produced a red solution in 10 min. and was followed by the precipitation of red crystals. The mixture was refluxed for 1.5 hr. before excess reagent was removed *in vacuo* in a rotary evaporator. The crude compound was triturated with petroleum ether (30–60°), and the residue was recrystallized twice from acetone–pyridine to give 351 mg. (58%) of a product which chromatographed as a single compound by GLC and TLC: R_f 0.72 on silica gel G (250 μ) with a acetone, benzene, and ammonia system (74:74:2), and fluorometric detection.

The reaction product was deduced to be thioacridone on the basis of the following data: m.p. 265° [lit. (3) 266°]; IR (KBr) 3250 (NH), 3070 (aromatic CH), 1620, 1575, 1480, and 1455 (phenyl), 1292 and 1175 (NH), 1220 (C=S), 999 and 752 $cm.^{-1}$ (1,2-disubstituted phenyl); NMR (DMSO-d_6, tetramethylsilane) 8.61 (d, 2, J = 8.0 Hz., aromatic H), 8.17 (s, 1, D_2O shift, N—H), 7.0–7.7 (m, 6, aromatic H); mass spectrum (50 ev., 180°) *m/e* (relative intensity) 211 (80) M, 167 (21) M—CS, 105.5 (5) M/2, 83.5 (4) M—CS/2.

Identification was confirmed by comparison of GLC, mixed melting point, and identical IR spectrum with thioacridone synthesized by the procedure of Cherntsov (5).

Progress of this reaction at room temperature was also followed by GLC. In this manner, a yield of 5.2% of thioacridone was noted after 4 hr. and 10.2% after 48 hr. When a mixture of 9-aminoacridine (1.94 g.) and *n*-butylisothiocyanate (1.44 g.) was refluxed in 50 ml. of toluene, a 2% yield of thioacridone was detected by GLC after 30 min. and a 2.2% yield after 1 hr.

[1] Melting points, observed by means of a Mel-Temp apparatus, are uncorrected. Spectrophotometers used were Perkin-Elmer model 337 IR, Varian A-60A NMR, and A.E.I. MS 902 mass spectrometer.

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Pharmacological Evaluation of 3,4-Dimethoxyphenylacetamide

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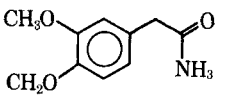
Abstract □ Studies on the pharmacological activity of 3,4-dimethoxyphenylacetamide revealed that the compound produced definite signs of CNS depression. A hypocholesterolemic effect was also demonstrated, plus tendencies toward a reduction in plasma glucose levels.

Keyphrases □ 3,4-Dimethoxyphenylacetamide—pharmacologically evaluated for CNS depression, hypocholesterolemic activity □ Hypocholesterolemic activity—3,4-dimethoxyphenylacetamide from *Catharanthus lanceus* □ CNS depression—3,4-dimethoxyphenylacetamide from *Catharanthus lanceus*

Recent work in these laboratories (1) led to the isolation of a crystalline compound, 3,4-dimethoxyphenylacetamide (I), from leaves of *Catharanthus lanceus* (Apocynaceae). This amide is the first reported example of the occurrence of a dialkoxyarylacetamide-type compound in nature.



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Data on the pharmacological activity of 3,4-dimethoxyphenylacetamide are completely lacking in the reported literature; however, there are reports on the hypocholesterolemic properties of phenylethylacetic acid and other compounds closely related to 3,4-dimethoxyphenylacetamide in experimental animals and man (2–12). In addition to the hypocholesterolemic effect of these compounds, several investigators have shown: (*a*) that cardiovascular and CNS changes in mice, pigeons, and chickens were noted with phenylmethylacetic and phenylethylacetic acids when administered at extremely high doses (13); (*b*) anti-inflammatory, local anesthetic, and diuretic activity for a series of α,α-disubstituted phenylacetamides (14, 15); and (*c*) anticonvulsant activity for certain amides of trialkoxyphenylacetic acids (16).

Since 3,4-dimethoxyphenylacetamide had not been pharmacologically evaluated and since many related compounds have been reported to be biologically active, it was considered worthwhile to determine the pharmacological profile of 3,4-dimethoxyphenylacetamide.

EXPERIMENTAL

Animals—A total of 234 male albino mice (Swiss-Webster strain, weighing 16–25 g.) and 24 male albino rats (Wistar strain, weighing 250–260 g.) were used[1]. The animals were allowed at least 3 days to acclimatize to the laboratory environment before being used in any experiment.

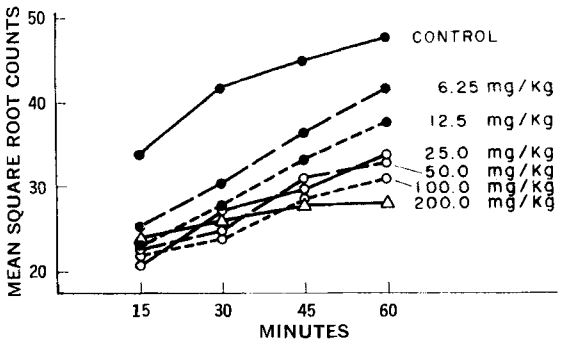

Figure 1—*Effects of 3,4-dimethoxyphenylacetamide on spontaneous activity in mice.*

Drug Preparation and Administration—3,4-Dimethoxyphenylacetamide was synthesized and purified by standard procedures as previously described (1). Since solubility of this compound was incomplete even at a concentration of 1 mg./ml. of distilled water, the compound was suspended in a 0.25% agar solution just prior to administration. Subsequently, drug administration was by the intraperitoneal route in all experiments. Mice received injections in a volume of 0.1 ml./10 g. of body weight, while rats were administered a volume of 0.2 ml./100 g. of body weight; all control animals received an equivalent amount of vehicle.

Determination of LD_{50}—Forty mice were divided into four groups of 10 each. Doses of 3,4-dimethoxyphenylacetamide ranging from 50 to 400 mg./kg. were administered. All groups were kept for 5 days, at which time the total number of dead mice per group was tabulated. This length of time was selected to determine whether the test compound caused any delayed deaths upon acute dosing due to metabolites that might possibly be formed and accumulated.

Gross Observations—Gross observations were made in clear plastic cages. Groups of two mice each were placed in these cages 30 min. prior to drug administration for general gross observations. This period was designated as the control phase of the experiment. Subsequently, these same groups were given either 50, 100, or 200 mg./kg. i.p. of 3,4-dimethoxyphenylacetamide; gross behavioral and physiological changes were recorded at 15, 30, 60, and 120 min. following injection.

Spontaneous Motor Activity—Sixty mice were tested, using three actophotometers[2], 15 min. after the administration of doses of 3,4-dimethoxyphenylacetamide ranging from 6.25 to 200 mg./kg. i.p. according to the method described by Dews (17). The cumulative number of counts for each group was converted into square roots, and statistical differences among them were determined using the paired *t*-test of the means.

Hexobarbital Sleeping Time—Forty mice, divided into four groups of 10 mice each, were used. One group received only the vehicle, while the three remaining groups received 25, 50, and 100 mg./kg. i.p. of 3,4-dimethoxyphenylacetamide, respectively. Thirty minutes later, all groups were administered 100 mg./kg. i.p. of hexobarbital sodium. The time to complete loss of the righting reflex (induction time) and the time at which each animal regained its righting reflex were recorded, and statistical evaluation of the data was made using Student's *t* test.

Anticonvulsant Activity—Forty mice were divided into four groups of 10 mice each. Three groups were given 25, 50, and 100 mg./kg. i.p. of 3,4-dimethoxyphenylacetamide, while the last group received vehicle. Thirty minutes after drug administration, all mice were subjected to maximal electroshock, *i.e.*, 50 ma. of current for

[1] All animals were obtained from Hilltop Lab Animals, Inc., Scottdale, Pa.

[2] Metro Industries.

Table I—Effects of 3,4-Dimethoxyphenylacetamide on Plasma Glucose and Cholesterol Levels in Rats

Minutes following Injection	Glucose, mg. %		Cholesterol, mg. %	
	Control	Drug	Control	Drug
30	70.4 ± 2.15	70.7 ± 1.96	70.1 ± 3.39	62.4 ± 3.86
60	74.3 ± 1.22	70.7 ± 1.87	65.2 ± 3.06	51.2 ± 3.40[a]

[a] Significantly different from control at $p > 0.01$.

0.2-sec. duration *via* corneal electrodes as described by Swinyard *et al.* (18). Anticonvulsant activity was considered when protection against the tonic extension of both front and hindlimbs was afforded the animals.

Anticholinergic Activity—Four groups of six mice each were administered vehicle or 50, 100, or 200 mg./kg. i.p. of 3,4-dimethoxyphenylacetamide 30 min. prior to 1 mg./kg. i.p. oxotremorine. For a 30-min. period following oxotremorine injection, the mice were observed for the occurrence of tremors and salivation.

Antidepressant Activity—The reversal of hypothermia and the incidence of ptosis induced by reserpine were used as measures of antidepressant activity for 3,4-dimethoxyphenylacetamide according to a modification of the method described by Vernier *et al.* (19). A total of 24 mice (four groups of six each) was used. After assignment to groups, the degree of ptosis and rectal body temperature were measured. Ptosis was qualitatively evaluated, and the degree of severity was given the following scores: 0 = none, 1 = partial, and 2 = complete. Immediately following this initial testing, all mice were given 5 mg./kg. i.p. of reserpine. Twenty-four hours later, the degree of ptosis and rectal temperatures of all mice were again recorded. Immediately after the second temperature reading, each group received either 50, 100, or 200 mg./kg. i.p. of 3,4-dimethoxyphenylacetamide or its vehicle. All mice were again graded as to their degree of ptosis and rectal temperature was taken 30, 60, and 120 min. later. At each testing interval, ptosis was graded before body temperature was taken to minimize the effects of handling on the former. Antidepressant activity was considered when ptosis was either partially or completely reversed and rectal temperatures showed a statistically (Student's *t* test) significant elevation.

Plasma Glucose and Cholesterol Levels—Four groups of six rats each were used in this study; two groups served as controls, receiving vehicle only, and two groups were given 100 mg./kg. 3,4-dimethoxyphenylacetamide. This dose of 3,4-dimethoxyphenylacetamide was chosen since data from the LD_{50} study indicated that this compound was relatively nontoxic. In addition, the 100-mg./kg. dose of 3,4-dimethoxyphenylacetamide is in the dose range exhibited by closely related compounds (2–12) for hypocholesterolemic activity. A group of control rats and one group of drug-treated animals were studied at 30-min. postinjection as were two separate groups at 60 min. At the preset absorption time, each animal was sacrificed by decapitation and blood samples (2–4 ml.) were collected in heparinized beakers. Immediately following the collections, each blood sample was centrifuged at $2000 \times g$ for 20 min. Plasma samples were taken and frozen until glucose and cholesterol determinations could be made using the Dow Diagnostest colorimetric technique[3]. Statistical analyses of the data were done using Student's *t* test.

RESULTS AND DISCUSSION

3,4-Dimethoxyphenylacetamide, in a dose range of 50–400 mg./kg., caused no deaths in mice for a period of up to 5 days following a single intraperitoneal injection. Therefore, it can be calculated that the LD_{50} is greater than 400 mg./kg.

Definite signs of CNS depression were observed in the gross observation studies: decreased motor activity, ataxia, passivity, and decreases in limb tone, body tone, and grip strength. The severity of these symptoms appeared to be dose dependent, with an onset of action of 15–30 min. and a duration of at least 60 min. Moreover, the autonomic nervous system (ANS) appeared to be slightly affected. Blockade of the sympathetic division of the ANS might have been manifested since signs of flushing, a slight degree of ptosis, and a slight amount of diarrhea were evident, indicating dominance of the cholinergic division. Compounds related to 3,4-dimethoxyphenylacetamide possessed similar effects on the cardiovascular system (13); however, no diuretic or local anesthetic effect was observed with 3,4-dimethoxyphenylacetamide (14, 15).

Data obtained in the spontaneous motor activity studies indicated a dose-related depressant effect through 25 mg./kg. of 3,4-dimethoxyphenylacetamide (Fig. 1). Above this dose, *i.e.*, 50, 100, and 200 mg./kg., there was no statistically reliable difference ($p > 0.1$) among the doses. However, whenever the lowest dose (6.25 mg./kg.) was compared with the control responses, the depressant action was markedly significant ($p < 0.001$); that is, differences were significant at each 15-min. interval. Both the 6.25- and 12.5-mg./kg. doses exhibited a significantly lesser amount of depression when compared with all the higher doses tested.

The onset and duration of action of hexobarbital sleeping time were unaffected by 25–100-mg./kg. doses of 3,4-dimethoxyphenylacetamide, since the effects among the vehicle and drug-treated groups did not differ significantly from one another ($p > 0.10$). In addition, 3,4-dimethoxyphenylacetamide did not display anticonvulsant activity in doses of 25–100 mg./kg. when tested against maximal electroshock-induced seizures. Likewise, 3,4-dimethoxyphenylacetamide did not exhibit either anticholinergic or antidepressant activity in doses of 50–200 mg./kg.

Table I summarizes the effects of 100 mg./kg. 3,4-dimethoxyphenylacetamide on plasma glucose and cholesterol levels. There was no statistically significant effect of 3,4-dimethoxyphenylacetamide on plasma glucose levels either 30 or 60 min. following drug administration; however, at 60 min., there was a tendency toward a decrease. Cholesterol levels were reduced at both testing times, with a significant reduction ($p < 0.01$) occurring only at the 60-min. postinjection time.

3,4-Dimethoxyphenylacetamide appears to have selective hypocholesterolemic activity; however, the compound also exhibited definite signs of CNS depression. Additional studies are underway in these laboratories to explore further the selectivity and potency of the cholesterol-reducing properties of 3,4-dimethoxyphenylacetamide.

[3] The Dow Chemical Co., Diagnostic Products, Indianapolis, Ind.

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Structure and Activity in Molluscicides III: Enzymatic Peroxidation of the Molluscicidal Agent Pentachlorophenol

I. NABIH and J. METRI

Abstract □ Pentachlorophenol, a widely used molluscicidal and herbicidal agent, is biologically oxidized through the catalytic effect of the protoporphyrin enzyme peroxidase, detected within the snail's body, to give 2,2′,3,3′,5,5′,6,6′-octachlorobiphenylquinone. This compound showed potent molluscicidal activity. The reaction goes through the symmetrical pairing of the initially formed chlorophenoxy radical, accompanied by the rupture of the *para*- (C—Cl) link. The eliminated chlorine ion was determined in the reaction medium.

Keyphrases □ Pentachlorophenol—enzymatic peroxidation, structure–activity relationships of molluscicides □ Molluscicidal agents —pentachlorophenol enzymatic peroxidation, structure–activity relationships □ Structure–activity relationships—molluscicidal agents

A previous communication (1) explained the structure–activity relationship in molluscicides of the phenolic type. Biological findings reported by various investigators (2, 3) showed the existence of the structural specificity that governs the biological activity as molluscicides within these types of compounds. The activity depends on the nature of substituents on the aromatic ring. Those bearing bulky groups were of least activity, while those carrying smaller substituents were of significant activity. This specificity in structure with relation to the biological activity parallels the ability of the phenoxy radical, the intermediate stage in the chemical oxidation of phenols, to undergo subsequent transformation to the corresponding quinoid system, which is similarly dependent on the nature of the substituent on the ring. Compounds belonging to this system are biologically active as molluscicides.

Previous studies revealed the presence of the protoporphyrin enzyme, peroxidase, in the snail's body. The biochemical assay for this enzyme was determined in some species of snails of economic importance (4).

MECHANISM

The effect of the enzyme peroxidase on some types of phenols and amines has been studied. Of these, 2,6-dimethylphenol could be oxidized by this system to give 3,5,3′,5′-tetramethyldipheno-4,4′-quinone; 2,6-dimethoxyphenol could be similarly changed to the corresponding diphenoquinone: 3,5,3′,5′-tetramethoxy-4,4′-quinone (5). Oxidation may also proceed to give a mesomeric quinoid structure as 2-hydroxy-1,3,5-trimethylbenzene, with which the reaction gives 2,6-dimethyl-1,4-benzoquinone (6, 7). With polysubstituted alkylphenols such as 1-hydroxy-2,3,5,6-tetramethylbenzene, the corresponding 2,3,5,6-tetramethyl-1,4-benzoquinone was obtained. With *para*-halogenated amines, such as *p*-chloroaniline, elimination of the chlorine ion takes place, thus causing a drop in the pH of the reaction medium and resulting in the formation of 2-amino-5-(*p*-chloroanilino)-benzoquinone-di-*p*-chloranil (8). Other aromatic amines with halogens as *para*-substituents could be oxidized in a similar fashion to give quininoid systems with the displacement of the halogen atom at the *para*-position. In the case of fluorine, the reaction is toxified through the hydrofluoride which acts as an enzyme poison.

Mechanistically, it has been suggested that the oxidation through peroxidase involves free radical formation either by direct loss of a hydrogen atom or by an electron removal followed by a loss of a proton; this may be followed by the symmetrical pairing of two such radicals (9).

The phenoxy radical is a monovalent oxygen radical species, which can be formed through the homolysis of the O—H bond. The delocalization of the unpaired electron over the aromatic ring and its substituents was proved (10, 11); thus the radical is stabilized by resonance. For the radical to exist other than as a transient intermediate, it is necessary that the ring be substituted with bulky groups, which would give steric hindrance to block further decomposition of the radical by slow dimerization or disproportionation. The polyhalophenoxy radical usually undergoes dimer formation readily. The stability of these radicals is due to the mesomeric contribution of halogen on the ring. The pentachlorophenoxy radical has been studied (12, 13); carbon–carbon dimerization of the radical may proceed with the loss of the *para*-substituent. The formation of such dimers was reported for a large number of these phenols (14, 15).

In this work, the catalytic oxidation of the enzyme peroxidase and hydrogen peroxide on pentachlorophenol (I), a widely used molluscicide and herbicide, was investigated. It was submitted, as its sodium salt, to the action of the enzyme in a water–phosphate buffer solution at pH 7 and 37°. The hydrogen peroxide solution was added to the sodium pentachlorophenate in the phosphate buffer, followed by the addition of the enzyme peroxidase solution; the temperature of the mixture was kept constant. The isolated product was identified as 2,2′,3,3′,5,5′,6,6′-octachlorobiphenylquinone (II). IR spectral analysis showed an absorption band at 1610 cm.$^{-1}$ referring to the quinoidal structure (16). Oxidation of II

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Structure and Activity in Molluscicides III: Enzymatic Peroxidation of the Molluscicidal Agent Pentachlorophenol

I. NABIH and J. METRI

Abstract □ Pentachlorophenol, a widely used molluscicidal and herbicidal agent, is biologically oxidized through the catalytic effect of the protoporphyrin enzyme peroxidase, detected within the snail's body, to give 2,2′,3,3′,5,5′,6,6′-octachlorobiphenylquinone. This compound showed potent molluscicidal activity. The reaction goes through the symmetrical pairing of the initially formed chlorophenoxy radical, accompanied by the rupture of the *para*- (C—Cl) link. The eliminated chlorine ion was determined in the reaction medium.

Keyphrases □ Pentachlorophenol—enzymatic peroxidation, structure–activity relationships of molluscicides □ Molluscicidal agents —pentachlorophenol enzymatic peroxidation, structure–activity relationships □ Structure–activity relationships—molluscicidal agents

A previous communication (1) explained the structure–activity relationship in molluscicides of the phenolic type. Biological findings reported by various investigators (2, 3) showed the existence of the structural specificity that governs the biological activity as molluscicides within these types of compounds. The activity depends on the nature of substituents on the aromatic ring. Those bearing bulky groups were of least activity, while those carrying smaller substituents were of significant activity. This specificity in structure with relation to the biological activity parallels the ability of the phenoxy radical, the intermediate stage in the chemical oxidation of phenols, to undergo subsequent transformation to the corresponding quinoid system, which is similarly dependent on the nature of the substituent on the ring. Compounds belonging to this system are biologically active as molluscicides.

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MECHANISM

The effect of the enzyme peroxidase on some types of phenols and amines has been studied. Of these, 2,6-dimethylphenol could be oxidized by this system to give 3,5,3′,5′-tetramethyldipheno-4,4′-quinone; 2,6-dimethoxyphenol could be similarly changed to the corresponding diphenoquinone: 3,5,3′,5′-tetramethoxy-4,4′-quinone (5). Oxidation may also proceed to give a mesomeric quinoid structure as 2-hydroxy-1,3,5-trimethylbenzene, with which the reaction gives 2,6-dimethyl-1,4-benzoquinone (6, 7). With polysubstituted alkylphenols such as 1-hydroxy-2,3,5,6-tetramethylbenzene, the corresponding 2,3,5,6-tetramethyl-1,4-benzoquinone was obtained. With *para*-halogenated amines, such as *p*-chloroaniline, elimination of the chlorine ion takes place, thus causing a drop in the pH of the reaction medium and resulting in the formation of 2-amino-5-(*p*-chloroanilino)-benzoquinone-di-*p*-chloranil (8). Other aromatic amines with halogens as *para*-substituents could be oxidized in a similar fashion to give quininoid systems with the displacement of the halogen atom at the *para*-position. In the case of fluorine, the reaction is toxified through the hydrofluoride which acts as an enzyme poison.

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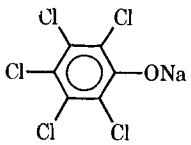

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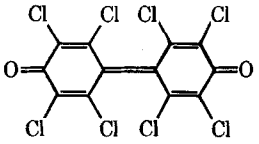

II

with lead dioxide in dilute sulfuric acid gave 2,3,5,6-tetrachlorobenzoquinone (chloranil). Oxidation of diphenoquinone by the same method usually gave the free *p*-benzoquinone (17).

An alternative synthesis for II was reported as a patent (18). This included the oxidation of 4,4′-dihydroxy-2,2′,3,3′,5,5′,6,6′-octachlorobiphenyl obtained by oxidation of decachlorobiphenyl in presence of excess alkali. Compound II was reported to be useful as an insecticide, fungicide, herbicide, fire-proofing compound, stabilizer for dielectric compounds, additive for oils, pigment for plastics and coating compounds, and dye intermediate (19).

In this biological oxidation, an initial formation of the polyhalophenoxy radical apparently took place, accompanied with the repulsion of the *p*-located chlorine followed by the dimerization of the radical. The liberated chlorine ion was quantitatively determined (20), and it corresponded to the calculated value.

EXPERIMENTAL[1]

Reagents—*Sodium Pentachlorophenate Solution* (7×10^{-3} M)—Five grams was dissolved by warming in 2500 ml. of 0.1 *M* phosphate buffer of pH 7.

Phosphate Buffer Solution (0.1 M pH 7)—Stock Solution A consisted of 0.2 *M* solution of monobasic sodium phosphate (55.6 g. of NaH_2PO_4) in 2000 ml. bidistilled water. Stock Solution B consisted of 0.2 *M* solution of dibasic sodium phosphate (107.3 g. of $Na_2HPO_4 \cdot 7H_2O$) in 2000 ml. bidistilled water. Then 487.5 ml. of Solution A and 762.5 ml. of Solution B were diluted to 2500 ml. with bidistilled water.

Hydrogen Peroxide Solution (3×10^{-2} M)—Hydrogen peroxide (30% w/v), 0.33 ml., was diluted to 100 ml. with bidistilled water.

Peroxidase[2] *Solution*—Thirty-five milligrams of the enzyme was dissolved in 50 ml. 0.05 *M* phosphate buffer.

Procedure—To 2500 ml. of sodium pentachlorophenate solution, 1 ml. of hydrogen peroxide solution was added in 15-min. intervals, followed by the addition of 1 ml. of the enzyme solution. Addition of both reagents to the phenate solution was continued until 50 ml. of each reagent was added. The temperature of the reaction mixture was kept at 37° during the addition period. A yellow precipitate was formed. This was left overnight at 37°, filtered, washed with bidistilled water several times, and dried. Upon recrystallization from dilute alcohol, the product melted at 154–156° and weighed 2.6 g. (65% yield).

Anal.—Calc. for $C_{12}Cl_8O_2$: C, 31.34; Cl, 61.69. Found: C, 30.78; Cl, 61.10.

Chlorine-Ion Determination in Reaction Medium (20)—The phosphate buffer was precipitated with concentrated nitric acid and ammonium molybdate solution and removed by filtration. To 100 ml. of the filtrate was slowly added 0.1 *N* silver nitrate solution during stirring. A slight excess of silver nitrate was added until no precipitate was formed. The mixture was heated to coagulate the precipitate; the solution was allowed to stand in the dark for 2 hr. and filtered in a weighed crucible. The precipitate was then washed with 0.02 *N* nitric acid solution until 3–5 ml. of the washing gave no turbidity with a drop of 0.1 *N* hydrochloric acid. It was dried at 130–150° for 1 hr. and weighed to give 0.044 g. of silver chloride.

The calculated amount of chlorine present in that portion equaled 0.011 g. The total amount of chlorine present in the whole filtrate and washings was:

$$\frac{0.011 \times 3250}{100} = 0.3575 \text{ g.} \qquad \text{(Eq. 1)}$$

The theoretical value of chlorine was 0.3630 g.

Oxidation of II to Produce 2,3,5,6-Tetrachlorobenzoquinone (Chloranil)—To a hot solution of II (0.5 g.) in 100 ml. (20%) sulfuric acid was added 2 g. lead dioxide in small portions. The mixture was refluxed for 2 hr. and filtered; then the precipitate was recrystallized from alcohol, m.p. 280° dec. (no depression in mixed melting point with 2,3,5,6-tetrachlorobenzoquinone). UV for both compounds in *n*-hexane showed identical maxima absorption at 286 nm.

Biological Screening—Five snails were placed with 200 ml. of the solution. Compound II was dissolved in the smallest possible amount of alcohol and diluted with bidistilled water to form the solution. All the snails died after the exposure period of 24 hr. (21).

[1] Melting points were uncorrected and were taken in open capillary tubes by the use of a Gallenkamp electric melting-point apparatus. UV spectra were determined in *n*-hexane and a Unicam spectrophotometer, model 500. The IR spectra were recorded with a Carl Zeiss Infra-cord spectrophotometer, model "UR 10." Microanalyses were performed by the microanalytical laboratory, National Research Centre, Cairo, U.A.R., and the Spang Microanalytical Laboratory, Ann Arbor, Mich.

[2] Horse-radish peroxidase type II, provided by Sigma Chemical Co., St. Louis, Mo., approximately 110 purpurogallin units/mg.

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Synthesis and Biological Evaluation of Substituted α-Phenylcinnamonitriles

J. T. STEWART

Abstract □ The synthesis of some new α-phenylcinnamonitriles is reported along with biological results in the areas of chemotherapy, gastrointestinal activity, fertility control, parasitology, and agricultural screening. The compounds showed negative or marginal biological activity in the areas tested.

Keyphrases □ α-Phenylcinnamonitriles, substituted—synthesis, biological evaluation □ Pharmacological, biological screening—substituted α-phenylcinnamonitriles

Interest in this laboratory in the use of α-phenylcinnamonitriles as potential analytical reagents resulted in the synthesis of some α-phenylcinnamonitrile derivatives (Table I). A literature search revealed that derivatives of this type have been shown to possess some antibacterial, antifungal, hypocholesterolemic, and estrogenic activity (1–4).

In this paper, the synthesis of some new α-phenylcinnamonitriles is reported, along with biological results for these compounds in the areas of chemotherapy, gastrointestinal activity, fertility control, parasitology, and agricultural screening.

The α-phenylcinnamonitriles were synthesized *via* base-catalyzed condensations of substituted phenylacetonitriles and benzaldehydes (5, 6). Starting materials required for the synthesis either were available commercially or were readily obtained in a few synthetic steps from commercial materials.

The nitro, isothiocyanate, amine, and quaternary amino compounds which were synthesized are shown, together with their melting points and elemental analysis data, in Table I. Compounds 3, 4, and 6–13 are new compounds. Compounds 1, 2, and 5 are known in the literature, but no biological data have been reported for them.

BIOLOGICAL SCREENING[1]

Compounds 1, 2, 4–10, and 13 were evaluated using the assay method of Conkey and Carlson (7) in a chemotherapy screen, involving a variety of Gram-positive and Gram-negative bacteria and fungi of industrial importance. The bacteria and fungi used were *Aerobacter aerogenes* (IPC 500)[2], *Bacillus mycoides* (IPC 509), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 10145), *Saccharomyces cerevisiae*[3], *Rhizoctonia solani* (ATCC 16115), and *Pullularia pullulans* (ATCC 9348). None of the substances tested showed any activity in this preliminary screen.

The gastrointestinal assay on Compounds 1 and 5 evaluated the ability of these substances to depress the volume of gastric secretions to one-half or less of the control value and/or raise the pH of the secretions above 2.5. Male rats, starved for 24 hr., were given a suspension of the test compound in 1% methylcellulose solution by stomach tube. After 30 min., the stomach was exposed under light anesthesia and the pyloric end tied off. The incision was closed, and the animals were allowed to recover from the anesthesia. Two hours later, the animals were killed and the volume and pH of the stomach secretions were measured. The compounds were tested in this assay at a standard dose level of 16 mg./kg., and both were inactive.

Two milligrams each of Compounds 1, 2, 4, 7, and 12 was tested in a fertility control assay. Each compound was administered orally once a day for 12 days to adult female mice. The suspending medium was carboxymethylcellulose. On the 4th treatment day, a fertile male was placed with the female, where he remained for 8 days. On the 5th day after cessation of treatment, the females were sacrificed and the uteri examined for implants. A positive response was obtained when none of the six treated animals showed implants. Estrone was used as a reference compound, and an untreated control group of six animals was used to establish fertility of the group of animals. In this screen, Compounds 1, 2, 4, 7, and 12 showed negative or marginal activity.

Compounds 1–13 were evaluated in *in vitro* and *in vivo* parasitology screens (8, 9). The *in vitro* screen consisted of an anthelmintic test in which larvae or eggs of trichostrongyle nematodes were exposed to the test drug at a concentration of 100 mcg./ml. Efficacy was determined by direct microscopic inspection of the condition of the parasites. Any active compounds were then retested in 10-fold serial dilutions to determine the end-point. None of the compounds, 1–13, was active in this test.

The *in vivo* parasitology screen tested the compounds in a coccidiosis screen at a dose level of 0.05% of the diet. For measurement of anticoccidial effects of drugs, 2-week-old-sex and weight-balanced "white-cross" chicks in groups of 10 were fed a standard laboratory ration to which drugs were added just prior to use. The uninfected and infected control birds were fed the basal ration. On the 2nd day of the test, the chicks were inoculated orally with 50,000 sporulated oocysts of *Eimeria tenella*. The chicks were held for 6 days following inoculation. Several criteria were employed for evaluation of drug efficacy. These included observations and records on the mortality rate, growth, severity of pathological lesions, and number of oocysts produced. All of the compounds, 1–13, were inactive in this test.

Several of the compounds were also tested in a liver fluke assay at a dosage level of 200 mg./kg. *in vivo*. In this assay, immature rodents were infected with 5–8 *Fasciola hepatica* metacercaria by the oral route. After 3 weeks, the test drug was administered orally (two doses spaced 2 days apart). The animals were sacrificed 1 week after initial dosing, the results being estimated from the presence or absence of living or dead flukes in the liver.

The agricultural screen consisted of testing Compounds 2, 7, and 10 in a herbicide test at a dose level of 15 lb./acre and against mosquito larvae at 10-p.p.m. concentration. In both assays, Compounds 2 and 7 were inactive. Compound 10 was inactive in the herbicide test but produced an 80% kill of larvae at 10 p.p.m. and a 20% kill at 1 p.p.m. This level of activity was, however, not considered promising enough to pursue.

EXPERIMENTAL[4]

Reduction of Nitro to Amino Compounds (Procedure A)—A suspension or solution of a nitro compound and 100 mg. of Adams' catalyst/10 g. of compound in absolute ethanol was allowed to consume a calculated amount of hydrogen in a Parr hydrogenator. The

[1] Tests for the various biological activities were carried out by the Merck Sharp and Dohme Research Laboratories, Rahway, N. J.

[2] IPC = Institute of Paper Chemistry, Appleton, Wis.

[3] Isolate obtained from Carolina Biological Supply Co., Burlington, N. C.

[4] Catalytic hydrogenation reactions were carried out at room temperature and 60 p.s.i. by means of a Parr hydrogenator. Melting points were taken in open capillary tubes with a Thomas-Hoover apparatus; they are corrected. IR spectra were obtained with a Perkin-Elmer model 237B grating spectrometer and were as expected. Microanalyses were performed by Atlantic Microlab, Atlanta, Ga.

Table I—α-Phenylcinnamonitrile Derivatives



Compound	R_1	R_2	Melting Point	Method of Synthesis[a]	Yield, %	Analysis, % Calc.	Analysis, % Found
1	4-Methoxyphenyl	3-Aminophenyl	107–108° (lit.)	—[b]	—	—	—
2	4-Methoxyphenyl	4-Dimethylaminophenyl	148–150° (lit.)	—[c]	—	—	—
3	Phenyl	2-Methoxy-5-nitrophenyl	158–160°	—[c,d]	92	C, 68.56 H, 4.31 N, 9.99	C, 68.69 H, 4.46 N, 9.92
4	Phenyl	2-Methoxy-5-aminophenyl	101–103°	A	85	C, 76.77 H, 5.63 N, 11.19	C, 76.88 H, 5.67 N, 11.06
5	4-Aminophenyl	2-Methoxyphenyl	105° (lit.)	—[e]	—	—	—
6	Phenyl	4-Trimethylammonium-phenyl iodide	189–190°	B	80	C, 55.38 H, 4.90 N, 7.17	C, 55.51 H, 5.05 N, 7.39
7	4-Isothiocyanatophenyl	2-Methoxyphenyl	193–194°	C	86	C, 69.84 H, 4.13 N, 9.58	C, 69.44 H, 4.31 N, 9.36
8	3,4-Dimethoxyphenyl	3-Nitro-4-methoxyphenyl	195–197°	—[f]	93	C, 63.52 H, 4.73 N, 8.23	C, 63.58 H, 4.89 N, 8.17
9	3,4-Dimethoxyphenyl	3-Amino-4-methoxyphenyl	184–186°	A	88	C, 69.66 H, 5.84 N, 9.02	C, 69.75 H, 5.97 N, 9.11
10	3,4-Dimethoxyphenyl	3-Trimethylammonium-4-methoxyphenyl iodide	158–162°	B	75	C, 52.49 H, 5.24 N, 5.83	C, 52.31 H, 5.37 N, 5.78
11	3,4-Dimethoxyphenyl	2-Methoxy-5-nitrophenyl	205–207°	—[f]	92	C, 63.52 H, 4.73 N, 8.23	C, 63.67 H, 4.85 N, 8.29
12	3,4-Dimethoxyphenyl	2-Methoxy-5-aminophenyl	85–88°	A	88	C, 69.66 H, 5.84 N, 9.02	C, 69.62 H, 5.95 N, 9.09
13	3,4-Dimethoxyphenyl	2-Methoxy-5-trimethylammoniumphenyl iodide	164–168°	B	80	C, 52.49 H, 5.24 N, 5.83	C, 52.63 H, 5.48 N, 5.88

[a] Superscripts are for general literature procedures and key intermediates. Letters refer to the author's experimental procedures. [b] See *Reference 10*. [c] Synthesized according to general procedure described in *Reference 5*. [d] See *Reference 11*. [e] See *Reference 12*. [f] Synthesized according to general procedures described in *References 5* and *6*.

catalyst was removed by filtration through Celite 545, and the ethanol was evaporated, The residue was recrystallized from aqueous ethanol or another appropriate solvent.

Quaternary Salt Formation (Procedure B)—A suspension of 0.01 mole of amino compound, 0.03 mole of dimethyl sulfate, 5 ml. of water, and 5 g. of potassium carbonate in 60 ml. of acetone was heated on a steam bath for 2 hr. Filtration of the hot suspension followed by air evaporation of the filtrate (hood) gave the crude methosulfate salt. Addition of potassium iodide to an aqueous solution of the methosulfate formed the iodide, which was collected by filtration and dried. The iodide salts were recrystallized from absolute ethanol and/or water.

Formation of Isothiocyanate from Amino Compound (Procedure C)—An acetone solution of the amino compound and an excess of thiophosgene (hood) was refluxed on a steam bath for 2 hr. Removal under reduced pressure at room temperature of the unreacted thiophosgene (hood) and acetone left a material, which was recrystallized from glacial or aqueous acetic acid.

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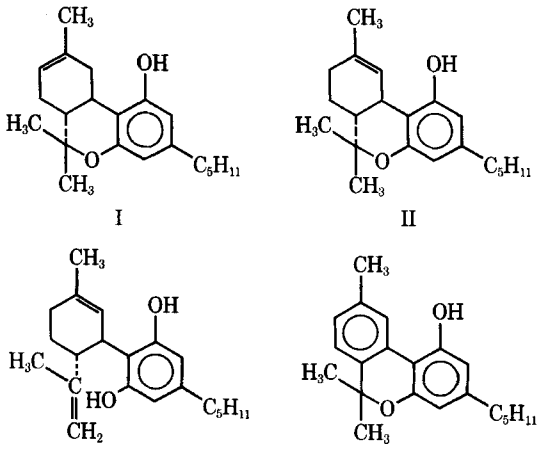
Mississippi-Grown *Cannabis sativa* L.: Preliminary Observation on Chemical Definition of Phenotype and Variations in Tetrahydrocannabinol Content *versus* Age, Sex, and Plant Part

PATRICIA S. FETTERMAN, ELIZABETH S. KEITH, COY W. WALLER, OSWALDO GUERRERO, NORMAN J. DOORENBOS, and MAYNARD W. QUIMBY

Abstract □ Nine strains of *Cannabis sativa* L. (marijuana) were grown for research by the University of Mississippi. The seeds for these strains were obtained from Iowa, Minnesota, Mexico, Turkey, Italy, France, and Sweden. The cannabinoid content was determined using GLC, and the material was divided into two chemical phenotypes according to cannabinoid content. These phenotype categories are used to differentiate between drug-type and fiber-type *Cannabis sativa*. In addition, the (−)-Δ⁹-*trans*-tetrahydrocannabinol content was determined for both male and female plants, various plant parts, and a Turkish variety during various stages in its growth.

Keyphrases □ *Cannabis sativa*, nine strains—phenotype definition, tetrahydrocannabinol content *versus* age, sex, plant part □ Tetrahydrocannabinol, *Cannabis sativa*—content compared by age, sex, plant part □ Phenotypes, chemical—drug and fiber *Cannabis sativa* □ GLC—analysis, tetrahydrocannabinol

It is generally known that two types of marijuana exist. One type is used principally for drug purposes, while the other type is used mainly for its fiber. So far, there are no botanical methods by which the two types of plant material may be distinguished. The pharmacological effects usually associated with marijuana are produced by (−)-Δ⁸-*trans*-tetrahydrocannabinol[1] (I) and (−)-Δ⁹-*trans*-tetrahydrocannabinol (II) (1, 2). The other main cannabinoids present are cannabidiol (III) and cannabinol (IV). Cannabidiol is generally considered to be the precursor in the metabolic pathway to (−)-Δ⁹-*trans*-tetrahydrocannabinol (3, 4). It has not been found to have any psychotomimetic activity. According to Levine (5), cannabinol is the degradation product of (−)-Δ⁹-*trans*-tetrahydrocannabinol. Grlić (3)[2] differentiated samples of *Cannabis sativa* on the basis of the cannabinoids by means of combined spectrophotometric methods. He classified his samples into various "ripening" stages. Test animals may be used to approximate the tetrahydrocannabinol content of marijuana (6, 7).



GLC was used for the quantitation of the samples reported in this paper. Studies and analyses of *Cannabis sativa*, as reported here, indicated that marijuana may be classified into two chemical phenotypes according to cannabinoid content.

METHODS

Samples used were either grown in Mississippi or received from the National Institute of Mental Health (NIMH) or various individual sources. The seeds for the plants grown in Mississippi in 1968 and 1969 were obtained from Mexico, Turkey, Italy, France, Sweden, and wild stands in Iowa and Minnesota. This material was harvested and manicured[3] so that analyses could be made according to the sex of the plant and the various plant parts as well as the source of the seeds. The seeds[4], roots, various sizes of stems, leaves, bracts, and male flowers were separated and analyzed for cannabinoid content. To eliminate cannabinoids from outside sources, the seeds were washed in chloroform prior to extraction. Then they were crushed and extracted according to the procedure described here. The Turkish variety was collected after sexual differentiation at intervals during the growing season so the presence of cannabinoids could be traced through the growth of the plant.

The extraction was basically that used by Lerner (8) as modified by those working with analysis of tetrahydrocannabinol, both natural and synthetic. An analysis consisted of 1 g. of material in 40 ml. of chloroform. This solution was kept in the refrigerator and shaken at 10-min. intervals for 1 hr. The plant material was then removed by filtration, and the chloroform was removed *in vacuo* at 40°. The remaining residue was dissolved and filtered in 25 ml. ethanol (5 × 5-ml. aliquots). After evaporation under reduced pressure, the residue was dissolved in 1 ml. of ethanol containing a known concentration of 4-androstene-3,17-dione as the internal standard. One microliter of this solution was injected into the chromatograph.

Analyses were performed using Beckman GC-5 and GC-45 gas chromatographs equipped with flame-ionization detectors and operated isothermally at 210° with an inlet temperature of 230°. The columns were 0.41-cm., 3.04-m. (0.125-in., 10-ft.) stainless steel packed with 2% OV-17 (phenyl methyl silicone) on 100/120 mesh Gas Chrom Q[5]. Nitrogen was used as the carrier gas at a flow of 30 ml./min.[6]. Peak area measurements were made using the method of

[1] Naturally occurring (−)-Δ⁸-*trans*-tetrahydrocannabinol was first isolated by R. L. Hively, W. A. Mosher, and F. W. Hoffman, *J. Amer. Chem. Soc.*, **88**, 1832(1966). For a summary of the comparison of natural and synthetic materials, see R. Mechoulam, *Science*, **168**, 1159 (1970).

[2] Grlić proposed a coding system containing 32 coded numbers, each corresponding to one of the parameters he used. Each sample has at least six "coded characteristics," which are necessary to classify marijuana into one of his "ripening" stages: "unripe, intermediate, ripe, overripe, and altered."

[3] Manicured material is devoid of seeds and large stems. This was accomplished by passing the material through a 10-mesh sieve.

[4] The seeds of marijuana are really fruits which serve as seeds.

[5] Obtained from Applied Science Laboratories, State College, Pa.

[6] Under the conditions of analysis, all cannabinoid acids would be converted to their respective phenols.

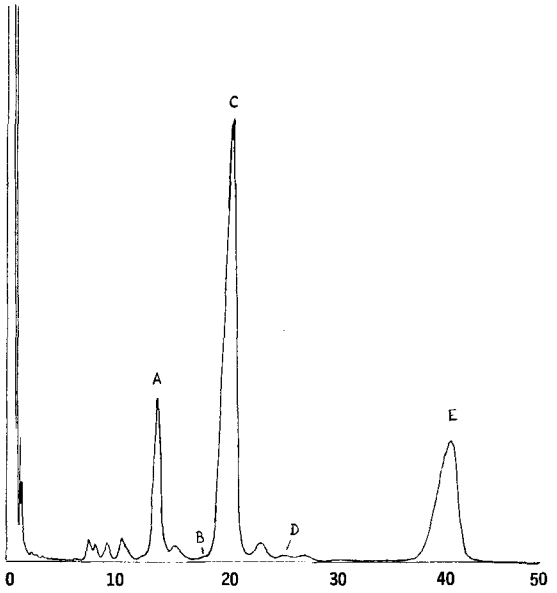


Figure 1—*Chromatogram of 1969 male Mexican marijuana grown in Mississippi. Phenotype I. Peaks: A = cannabidiol, B = (−)-Δ⁸-trans-tetrahydrocannabinol, C = (−)-Δ⁹-trans-tetrahydrocannabinol, D = cannabinol, and E = internal standard.*

peak height times width at half-height. The peak area of each cannabinoid was compared with the peak area of the internal standard; using the appropriate correction factors, the cannabinoid concentration then was determined. Through NIMH, synthetic (−)-Δ⁸-*trans*-tetrahydrocannabinol[7] and (−)-Δ⁹-*trans*-tetrahydrocannabinol[7] and cannabidiol[8] and cannabinol[8] were obtained.

RESULTS AND DISCUSSION

Phenotype—Recently, Grlić classified *Cannabis sativa* into five "ripening" groups (*Footnote 2*) (3). Also an attempt was made to classify the plant into fiber and drug types on the basis of the cannabinoid acids as well as the phenols[9]. The following ratio is proposed as a means of classifying marijuana into chemical phenotypes:

$$\text{phenotype ratio} = \frac{\%\,(-)\text{-}\Delta^9\text{-}trans\text{-tetrahydrocannabinol} + \%\,\text{cannabinol}}{\%\,\text{cannabidiol}} \quad \text{(Eq. 1)}$$

Grlić proposed a similar ratio: (tetrahydrocannabinol + cannabinol)/(cannabidiol acid + cannabidiol), using UV and IR spectral data, along with other ratios with which to classify marijuana into "ripening" groups. This ratio does not take into account the acids of cannabinol and tetrahydrocannabinol. The phenotype ratio makes use of data obtained from a method in which the cannabinoid acids were converted into their corresponding cannabinoid phenols, similar to the results of storage or smoking.

Analytical work indicates that some plant materials have a very high cannabidiol content while others have a high (−)-Δ⁹-*trans*-tetrahydrocannabinol content. Limited experience in handling the individual cannabinoids shows (−)-Δ⁹-*trans*-tetrahydrocannabinol to be the least stable. According to Levine (5), loss of potency of marijuana is accompanied by conversion of (−)-Δ⁹-*trans*-tetrahydrocannabinol to cannabinol. This leads to the suggestion that (−)-Δ⁹-*trans*-tetrahydrocannabinol + cannabinol would approximate the (−)-Δ⁹-*trans*-tetrahydrocannabinol content, irrespective of degradative changes.

[7] From Arthur D. Little, Inc., Cambridge, Mass.

[8] From Research Triangle Institute, Research Triangle Park, N. C.

[9] Toffoli *et al.* (9) attempted, by a complicated alkaline extraction and subsequent GLC analysis, to classify *Cannabis sativa* into fiber and biologically active types. They concluded that in the fiber type the cannabidiol was present largely as the acid. From the acid analyses in this study, it was concluded that the acids themselves are not indicative of the type of plant material but rather of the age after harvesting, the drying conditions, or the storage of the plant material. The fiber and drug types can be ascertained by a simple analysis and comparison of the ratio of the cannabinoids, without regard to their acids as such.

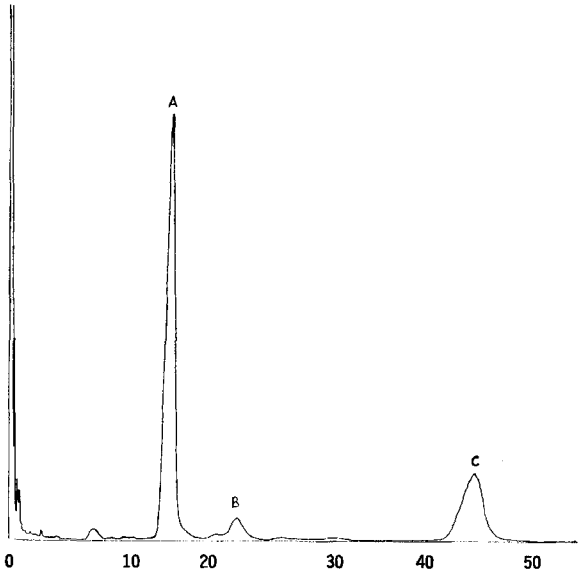


Figure 2—*Chromatogram of 1969 female Turkish marijuana grown in Mississippi. Phenotype II. Peaks: A = cannabidiol, B = (−)-Δ⁹-trans-tetrahydrocannabinol, and C = internal standard.*

Marijuana samples with phenotype ratios greater than 1.0 are classified into Phenotype I, which represents the biologically active type of marijuana. Samples having ratios less than 1.0 are classified into Phenotype II or the fiber-type marijuana. Figure 1 is a chromatogram produced by an extract of Phenotype I marijuana; Fig. 2 was produced by an extract of Phenotype II marijuana.

The data in Table I are the results of various studies of marijuana. The table shows the extensiveness with which the phenotype system applies to the marijuana plant. For instance, this system classifies all the various plant parts, including those found underground, of each plant or variety into the same phenotype. All parts of the Mexican plant are Phenotype I material, while all the Turkish plant parts are in Phenotype II. This also is the case for the male and female plants of each species. A survey of the Turkish plant showed that the plant, when analyzed during various stages of its growth, was always Phenotype II. The phenotype of one variety remains the same each year. For example, samples of Mexican marijuana of 1968 and 1969 were analyzed and both were found to be Phenotype I. The place of growth of the plant material makes no difference in the phenotype classification. The Minnesota and Iowa samples grown both at their origin and in Mississippi are Phenotype II. From the data, therefore, the phenotype of one variety of marijuana remains the same regardless of the plant part, sex, age, year, or place of growth of the sample analyzed.

The only exceptions to this classification system were samples having unknown histories. These unknown samples were either confiscated or supplied without protocol, and all could have contained mixtures of various types of marijuana. All samples of known origin and history fit into the phenotype classification system. Therefore, as a preliminary observation, it seems that the chemical phenotype classification proposed here is a relatively simple and useful means of distinguishing between the drug and fiber types of marijuana.

Male *versus* Female—It was believed previously that the male plant contained little or no resin and, by definition, marijuana consisted of the female parts of *Cannabis sativa* L., the male plants being grown mainly for fiber (10). When marijuana was last classified as an official drug in 1936, it was defined as "the dried flowering tops of the pistillate plants" (11). Recently, Valle *et al.* (6) showed, through pharmacological studies, that male marijuana is as potent as the female. However, there have been no published data as to the actual (−)-Δ⁹-*trans*-tetrahydrocannabinol content of male and female marijuana[10]. Data from this study (Table I) show that the male

[10] Private communication with Dr. Stig Agurell, Department of Pharmacognosy, Faculty of Pharmacy, Uppsala University, Stockholm, Sweden, confirms the data that male and female marijuana contains roughly similar amounts of cannabinoids.

Table I—GLC Analysis and Phenotype Classification of Various Samples of Marijuana[a]

Sample	From	Percent Cannabidiol	Percent (−)-Δ^9-*trans*-Tetrahydrocannabinol	Percent Cannabinol	Phenotype Ratio	Phenotype
Mexican, 1968:						
Female	UM	0.075	1.0	0.54	21	I
Male	UM	0.32	1.2	0.59	5.6	I
Mexican, 1969:						
Female	UM	0.12	1.4	0.073	12	I
Male	UM	0.40	1.5	0.070	3.9	I
Immature	UM	0.063	0.60	0.002	9.5	I
Mexican female plant parts:						
Bracts	UM	0.15	3.7	0.18	26	I
Small leaves	UM	0.085	1.4	0.051	17	I
Seeds	UM	t	0.01	0.01	$\gg$1.0	I
Mexican male plant parts:						
Flowers	UM	0.88	1.6	0.078	1.9	I
Leaves	UM	0.079	1.0	0.047	13	I
Stems	UM	0.055	0.89	0.076	18	I
Turkish, 1968:						
Female†	UM	$\sim$6 $\times$ Δ^9-THC	0.059	0.023	N.Q.	II
Male	UM	0.24	0.0070	t	0.029	II
Turkish, 1969:						
Female	UM	1.7	0.18	0.062	0.14	II
Turkish female plant parts:						
Bracts†	UM	$\sim$15 $\times$ Δ^9-THC	0.37	0.038	N.Q.	II
Leaves†	UM	$\sim$5 $\times$ Δ^9-THC	0.32	0.088	N.Q.	II
Stems:						
1-mm. diameter	UM	0.19	0.02	t	0.11	II
2–4 mm.	UM	0.03	0.007	t	0.23	II
10–15 mm.	UM	0.003	t	t	$<$1.0	II
Roots	UM	0.015	0.0020	0.00074	0.18	II
Seeds	UM	0.0087	0.00057	t	0.066	II
Turkish male at various weeks after planting:						
8 weeks	UM	0.11	0.02	0.02	0.36	II
11 weeks	UM	0.21	0.03	0.04	0.33	II
15 weeks	UM	0.28	0.02	0.02	0.14	II
18 weeks	UM	0.53	0.04	0.01	0.094	II
Turkish female at various weeks after planting:						
8 weeks	UM	0.15	0.02	0.02	0.27	II
11 weeks	UM	0.21	0.03	0.04	0.33	II
15 weeks	UM	0.28	0.02	0.03	0.18	II
18 weeks	UM	0.87	0.04	0.04	0.092	II
19 weeks	UM	1.00	0.05	0.02	0.07	II
Minnesota, 1968:						
1	Minn.	0.77	0.073	0.028	0.13	II
2	Minn.	1.2	0.074	0.016	0.075	II
Minnesota, 1969	UM	0.71	0.054	0.0095	0.089	II
Minnesota female plant parts:						
Bracts	UM	1.3	0.054	0.0033	0.044	II
Leaves	UM	1.0	0.043	t	0.043	II
Iowa, 1968	Iowa	0.95	0.061	0.026	0.092	II
Des Moines, 1968	Des Moines, Iowa	1.2	0.071	0.010	0.068	II
Illinois male, 1968*	Dr. Susiana and Dr. Dunbar, Samford Univ.	0.26	1.1	0.085	4.6	I
Seized marijuana*	Dr. L. Way, Univ. of California	0.88	0.084	t	0.095	II
Carmagnola, 1968	UM	1.2	0.32	0.085	0.34	II
Fibranova, 1969	UM	1.55	0.11	0.040	0.097	II
Unknown*	Dr. A. Yuwiler, Vet. Hosp., Los Angeles, Calif.	0.71	0.077	t	0.11	II
Confiscated cigarette*:						
a	Dr. H. Isbel,	1.08	0.15	0.049	0.18	II
b	Univ. of Kentucky	0.48	0.51	0.10	1.3	I
Monophyllous bracts	UM	6.1	0.5	t	0.082	II
Charas tincture*	Dr. C. C. Pfeiffer, Princeton	3.8	1.4	4.0	1.4	I
Unknown—laboratory grown*	UM, grown by Dr. Walter	2.1	0.19	t	0.091	II
Hashish cake and powder*	Athens, Greece, Dr. M. Fink, New York Univ.	9.8	2.1	3.5	0.57	II
Red oil or marijuana extract distillate	Research Triangle Institute	0.88	10	3.5	15	I
NIMH 1 (confiscated)*	NIMH	0.095	0.58	0.37	10	I

Table I—(*Continued*)

Sample	From	Percent Cannabidiol	Percent (−)-Δ^9-*trans*-Tetrahydrocannabinol	Percent Cannabinol	Phenotype Ratio	Phenotype
NIMH 12*	NIMH	1.0	0.052	0.020	0.072	II
USP fluid extract* of *Cannabis sativa*, manufactured by H. K. Mulford Co., 40 years old	Dr. K. Redman, South Dakota State Univ.	2.7	0.43	5.2	2.1	I
Thailand QCD-65472, 1969	Dr. R. Forney, School of Medicine	0.16	2.2	t	14	I
Thailand QCD-65169, 1969	Indianapolis, Ind.	0.11	1.3	t	12	I
Carmagnola (Italy), 1969	UM	1.4	0.37	0.077	0.32	II
Fibranova (Italy), 1969	UM	1.6	0.11	0.04	0.094	II
Unknown*		0.19	0.025	0.018	0.23	II
Turkish extract, 1969	UM	28	1.4	t	0.050	II

[a] UM = University of Mississippi. † = cannabidiol peak offscale, roughly estimated. * History unknown. t = trace. ~ = approximately. N.Q. = not quantitative.

and female of the same variety contain similar amounts of (−)-Δ^9-*trans*-tetrahydrocannabinol.

Various Plant Parts—A GLC survey of the various plant parts showed that the parts decrease in (−)-Δ^9-*trans*-tetrahydrocannabinol content in the following order: bracts, flowers, leaves, smaller stems, larger stems, roots, and seed. All plant parts contain cannabinoids. The data from this study are reported in Table I.

Plant Growth—From the preliminary study of the growth of the Turkish plant, it was found that young plants contain cannabinoids. No other conclusions were made. Some of the data from this study appear in Table I.

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Analgesics: Synthesis of 1-Dioxolanoalkylnormeperidines and 1-Dioxanoalkylnormeperidines

DOUGLAS C. KRIESEL* and OLE GISVOLD†

Abstract □ The syntheses of the maleate salts of *N*-(2-methylene-1,3-dioxolano)normeperidine (II), *N*-(2-methylene-1,3-dioxano)-normeperidine (III), *N*-(2-ethylene-1,3-dioxolano)normeperidine (IV), and *N*-(2-ethylene-1,3-dioxano)normeperidine (V) are reported. The dioxolane derivatives (II and IV) possessed analgesic activity, with II being slightly more active than meperidine. None of these compounds antagonized the analgesic effect of meperidine.

Keyphrases □ 1-Dioxolanoalkylnormeperidines—synthesis as possible analgesics □ 1-Dioxanoalkylnormeperidines—synthesis as possible analgesics □ Analgesics, potential—synthesis, pharmacological evaluation of 1-dioxolanoalkylnormeperidines, 1-dioxanoalkylnormeperidines

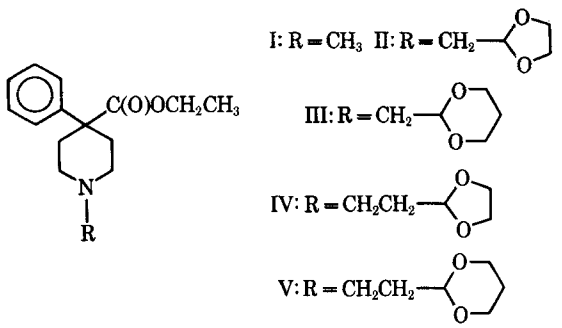
Replacement of the *N*-methyl in meperidine with heterocyclic-containing alkyls has sometimes given rise to compounds that are more potent analgesics than meperidine (I) itself (1–4). Potent analgesic derivatives of meperidine also occur when the nitrogen is substituted with groups that contain ether linkages in the chain (3–9). In the continuation of a study designed to explore the potential of substituted dioxolanes and dioxanes as medicinal agents, some *N*-dioxolanoalkylnormeperidines and *N*-dioxanoalkylnormeperidines were prepared and screened for analgesic activity (10, 11).

Alkylation of normeperidine with the appropriate 2-bromoalkyl-1,3-dioxolane or 2-bromoalkyl-1,3-dioxane in dimethylformamide at 100° or refluxing toluene gave the desired meperidine analog. The products were characterized as their maleate salts. The synthesis of ethyl 4-phenylpiperidine-4-carboxylate (normeperidine) was accomplished through *N*-tosylation, nitrile hydrolysis, and detosylation and esterification of 4-phenyl-4-cyanopiperidine according to the procedure of Thorp and Walton (12).

Following a method described by Bedoukian (13), the cyclic acetals, 2-bromomethyl-1,3-dioxolane and 2-bromomethyl-1,3-dioxane, were synthesized by adding 1,2-dibromoethyl acetate to a solution of the appropriate glycol and distilling the resulting mixture. The preparation of 2-(2-bromoethyl)-1,3-dioxolane and 2-(2-bromoethyl)-1,3-dioxane was carried out according to an established procedure that required the addition of acrolein to a cold solution of anhydrous hydrogen bromide in an appropriate glycol (14).

PHARMACOLOGICAL RESULTS

The *N*-substituted normeperidine salts were subjected to a preliminary analgesic screen, using a modification of the Eddy and Leimbach method (15). Male and female white mice were observed for signs of pain after being placed on a metal plate floating on a circulating hot water (53°) bath. Those animals whose reaction time increased to at least twice the mean reaction time of the control animals were assumed to show significant pain relief. The test was conducted 15 min. after subcutaneous administration of the required dose. The ED_{50} levels were calculated according to the method of Miller and Tainter (16). Meperidine, according to this method, had an ED_{50} of 9 mg./kg., while *N*-(2-methylene-1,3-dioxolano)normeperidine and *N*-(2-ethylene-1,3-dioxolano)normeperidine had ED_{50}'s of 20 and 6 mg./kg., respectively. The dioxane derivatives did not possess any analgesic activity at doses of 50 mg./kg.



Using the general procedure of Harris and Pierson (17), Compounds II, III, IV, and V were assayed in rats for their ability to antagonize the normal analgesic response of meperidine. A dose of 100 mg./kg. of the test compound was administered subcutaneously 10 min. prior to a 100-mg./kg. subcutaneous dose of meperidine. Analgesia was determined using the D'Amour–Smith tail-flick method (18). Pain measurements at 30- and 60-min. intervals showed that the *N*-dioxolanoalkylnormeperidines and the *N*-dioxanoalkylnormeperidines did not reverse the analgesic effect of meperidine and possessed no narcotic antagonist effect. In fact, they potentiated the analgesic effect of meperidine; rats receiving both meperidine and the test compound were much more lethargic and unresponsive than the control animals who were given only meperidine.

EXPERIMENTAL[1]

2-Bromomethyl-1,3-dioxolane—Following the procedure of Bedoukian (13), 2-bromomethyl-1,3-dioxane was isolated as colorless liquid distilling at 70–76° at 18–20 mm. [lit. (19) 63–65° at 12 mm.]; yield: 72%.

2-Bromomethyl-1,3-dioxane—Following the procedure of Bedoukian (13), 2-bromomethyl-1,3-dioxane was isolated as a colorless liquid distilling at 67–72° at 6 mm. [lit. (19) 80-86° at 12 mm.]; yield: 70%.

2-(2-Bromoethyl)-1,3-dioxolane—Following the procedure of Hill and Potter (14), 2-(2-bromoethyl)-1,3-dioxolane was isolated as a colorless oil distilling at 68–73° at 10 mm. [lit. (14) 72–73° at 10 mm.]; yield: 30%.

2-(2-Bromoethyl)-1,3-dioxane—Following the procedure of Hill and Potter (14), 2-(2-bromoethyl)-1,3-dioxane was isolated as a colorless oil distilling at 68–74° at 4.8 mm.

Anal.—Calc. for $C_6H_{11}BrO_2$: C, 36.94; H, 5.69. Found: C, 37.16; H, 5.77.

***N*-(2-Methylene-1,3-dioxolano)normeperidine Maleate**—Normeperidine (3.0 g., 0.013 mole) and 2-bromomethyl-1,3-dioxolane (4.3 g., 0.026 mole) were added to 25 ml. of toluene. Five grams of anhydous sodium carbonate was added, and the mixture was refluxed for 18 hr. After filtration, the filtrate was extracted twice with 30 ml.

[1] Melting points were determined on a Thomas-Hoover melting apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis, Minn., with the exception of *N*-(2-methylene-1,3-dioxano)normeperidine maleate which was analyzed by Galbraith Laboratories, Inc., Knoxville, Tenn.

of 0.5 *N* aqueous hydrochloric acid. The aqueous layer was basified immediately with aqueous sodium carbonate, and the solution was extracted three times with ether. The combined ether extracts were dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to a yellow oil, which was distilled to yield 1 g. (24%) of a colorless oil, b.p. 134–137° at 0.2 mm. This material was dissolved in 5 ml. of anhydrous ether; to it was added maleic acid (0.38 g., 0.0032 mole) dissolved in 2 ml. of ethanol. The white crystals which formed were recrystallized from ethanol–ether; yield: 1.0 g. (74%); m.p. 116–119°.

Anal.—Calc. for $C_{22}H_{29}NO_8$: C, 60.88; H, 6.71. Found: C, 61.05; H, 7.05.

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Anal.—Calc. for $C_{23}H_{31}NO_8$: C, 61.45; H, 6.95. Found: C, 61.32; H, 7.16.

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Abstract □ Structural combination between the two moieties necessary for the biological effectiveness in both oral schistosomicidal agents, 1-(2-diethylaminoethylamino)-4-methylthiaxanthone and 1-(5-nitro-2-thiazolyl)-2-imidazolidinone, namely, the substituted aromatic amine and aminothiazole, respectively, were tried. The combination between both systems was suggested to be through amide linkage.

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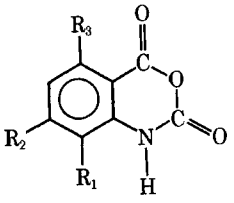

V

V*a*: $R_1 = R_2 = R_3 = H$

V*b*: $R_1 = CH_3$; $R_2 = R_3 = H$

V*c*: $R_1 = R_3 = H$; $R_2 = CH_3$

V*d*: $R_1 = Cl$; $R_2 = R_3 = H$

V*e*: $R_1 = R_3 = H$; $R_2 = Cl$

V*f*: $R_1 = CH_3$; $R_2 = H$; $R_3 = Cl$

V*g*: $R_1 = R_3 = H$; $R_2 = NO_2$

V*h*: $R_1 = R_2 = H$; $R_3 = NO_2$

cidal activity (6). But this agent is insoluble in water and it produces toxic effects during treatment.

The purpose of the present work was to synthesize compounds containing both essential systems required for the biological activity, namely the substituted aromatic amine and 2-amino-5-nitrothiazole or 2-aminothiazole. An amide linkage between the groups was sought, because it could be hydrolyzed in the body to free both entities, which could then act either independently or additively upon the parasite within the host.

Condensation of 2-amino-5-nitrothiazole with 4,6-dichloro-*m*-tolylchloride (I) gave *N*-(5-nitro-2-thiazolyl)-4,6-dichloro-3-methylbenzamide (II). Trials to replace the chlorine atom in position 6 in the amide (II) with the 2-diethylaminoethylamine side chain failed.

In an attempt to activate the 6-chlorine atom in the 4,6-dichloro-*m*-toluic acid system for replacement with the amino side chain, the corresponding 4,6-dichloro-*m*-toluamide (III) and 4,6-dichloro-*m*-tolunitrile (IV), through dehydration of the former, were prepared. But replacement of the 6-chlorine atom with the amino side chain in either the amide or the nitrile also failed to occur.

Due to the resistance of the chlorine atom in these structures for replacement by the amines, the condensation of 2-aminothiazole was attempted with systems that would furnish an amino group on the aromatic part along with the desired amide linkage. Thus, the tendency of isatoic anhydride and its derivatives to react with amines of different types to form amide linkage was investigated.

The condensation of isatoic anhydride with primary amines was found to yield the corresponding substituted anthranilamides and, concurrently, the corresponding ω-substituted *o*-ureidobenzoic acid (7–11).

In this work, 2-aminothiazole and 2-amino-5-nitrothiazole were used as the primary amine components in the condensation reaction with isatoic anhydride and derivatives.

The isatoic anhydrides, V*a–h*, were prepared through oxidation of the isatins using chromic trioxide (11). For comparison, the oxidation was also attempted using monoperphthalic acid. This was more fruitful because the yields of the anhydrides were higher (about 85%) and because of the high extent of purity. With chromic trioxide, the anhydrides came in yields of about 65%.

The condensation of the isatoic anhydride (V*a*), or any of its derivatives (V*b–h*), with 2-amino-5-nitrothiazole failed under different experimental conditions. But it readily occurred with 2-aminothiazole, and the products were *N*-(2-thiazolyl)-*o*-aminobenzamides (VI*a–f*). In the presence of nitro groups on the aromatic parts of the anhydrides (V*g–h*), the condensation reaction between 2-aminothiazole and the corresponding anhydride failed to occur.

EXPERIMENTAL[3]

4,6-Dichloro-*m*-tolyl Chloride (I)—A solution of 5 g. (0.024 mole) of pure 4,6-dichloro-*m*-toluic acid (12) in 10 ml. of dry benzene was treated with 3 g. (0.025 mole) of freshly distilled thionyl chloride. The reaction mixture was heated on a steam bath for 1 hr. and then allowed to cool at room temperature. The solvent and the unreacted thionyl chloride were removed by distillation. The liquid residue was distilled under reduced pressure at 150° (11 mm.) to give 3.2 g. (60%).

***N*-(5-Nitro-2-thiazolyl)-4,6-dichloro-3-methylbenzamide (II)**—To a suspension of 2.5 g. (0.015 mole) of 2-amino-5-nitrothiazole in 5 ml. of dry benzene was added, dropwise under stirring, 3 g. (0.013 mole) of I dissolved in 5 ml. of dry benzene. The mixture was refluxed on a steam bath for 3 hr.; then the solvent was distilled, and the residue was poured on ice-cold water with stirring. The solid product was collected, washed with water, and dried. After recrystallization from methanol, it provided 3.2 g. (74%) as yellow crystals, m.p. 210–212°.

Anal.—Calc. for $C_{11}H_7Cl_2N_3O_3S$: C, 39.75; H, 2.10; N, 12.55. Found: C, 39.65; H, 2.24; N, 12.87.

4,6-Dichloro-*m*-toluamide (III)—A stream of dry ammonia was passed through a cold solution of 2 g. (0.0089 mole) of the acid chloride, I, in 20 ml. of dry benzene for a few minutes. The solid material that separated was filtered, washed with water, dried, and recrystallized from an ethanol–water mixture to yield 1.58 g. (83%) as shiny white needles, m.p. 164–165°; IR: 1675 cm.$^{-1}$ (—CO·NH_2).

Anal.—Calc. for $C_8H_7Cl_2NO$: C, 47.05; H, 3.43. Found: C, 46.82; H, 3.61.

4,6-Dichloro-*m*-tolunitrile (IV)—To a suspension of 1 g. (0.005 mole) of the amide III in 2 ml. of dry pyridine was added 1 g. of phosphorus oxychloride under good cooling. The mixture was left at room temperature for 2 hr. and then heated on a steam bath for 20 min. Ice-cold water was added, and the formed precipitate was filtered, washed with cold water, and recrystallized from ethanol. The obtained white crystals were further purified through sublimation to give 0.75 g. (80%), m.p. 111–112°; IR: 2232 cm.$^{-1}$ (—CN).

Anal.—Calc. for $C_8H_5Cl_2N$: C, 51.61; H, 2.70; N, 7.52. Found: C, 51.34; H, 2.87; N, 7.18.

Isatoic Anhydrides (V*a–h*)—These anhydrides were prepared through the oxidation of the corresponding isatins by two different methods: one using chromic trioxide (11) and the other using monoperphthalic acid (13). The obtained melting points were: V*a*, 243° dec. [lit. (14) m.p. 240–243° dec.]; V*b*, 273° dec. [lit. (15) m.p. 277° dec.]; V*c*, 245° dec. [lit. (16) m.p. 245° dec.]; V*d*, 237° dec. [lit. (15) m.p. 232° dec.]; V*e*, 268° dec. [lit. m.p. (17) 268° dec.]; V*g*, 215° dec. [lit. (18, 19) m.p. 215° dec.]; and V*h*, 243° dec. [lit. (11) m.p. 244° dec.]. Compound V*f* was recrystallized from acetic acid and had m.p. 310° dec.

Anal.—Calc. for $C_9H_6ClNO_3$: C, 51.06; H, 2.87. Found: C, 51.43; H, 3.24.

[3] All melting points were taken in open glass capillaries, using a Gallenkamp melting-point apparatus, and are uncorrected. Microanalyses were performed by Micro-analytical Laboratory, National Research Centre, Cairo, U.A.R., and Spang Microanalytical Laboratory, Ann Arbor, Mich. IR spectra were determined with a Carl-Zeiss model IR 10 infracord spectrophotometer and were determined in chloroform.

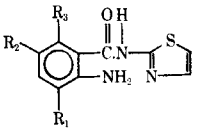

Table I—*N*-(2-Thiazolyl)-*o*-aminobenzamides (VI)

Compound	R_1	R_2	R_3	Yield, %	Melting Point[a]	Formula	Analysis, % Calc.	Analysis, % Found
VI*a*	H	H	H	73	164°	$C_{10}H_9N_3OS$	C, 54.79 H, 4.14 N, 19.17	C, 54.52 H, 4.18 N, 18.88
VI*b*	CH_3	H	H	83	192°	$C_{11}H_{11}N_3OS$	C, 56.56 H, 4.75 N, 18.02	C, 56.41 H, 4.80 N, 18.08
VI*c*	H	CH_3	H	79	198°	$C_{11}H_{11}N_3OS$	C, 56.65 H, 4.75	C, 56.35 H, 4.85
VI*d*	Cl	H	H	88	195°	$C_{10}H_8ClN_3OS$	C, 47.33 H, 3.18 N, 16.56	C, 47.28 H, 3.18 N, 16.76
VI*e*	H	Cl	H	80	210°	$C_{10}H_8ClN_3OS$	C, 47.33 H, 3.18 N, 16.56	C, 46.97 H, 3.16 N, 16.36
VI*f*	CH_3	H	Cl	81.5	243°	$C_{11}H_{10}ClN_3OS$	C, 49.34 H, 3.76 N, 15.69	C, 48.85 H, 3.63 N, 15.59

[a] Recrystallization solvent was ethanol.

***N*-(2-Thiazolyl)-*o*-aminobenzamides (VI*a–f*, Table I)**—A mixture of 0.005 mole from the isatoic anhydride and 0.43 g. (0.005 mole) of 2-aminothiazole in 5 ml. of isopropanol was refluxed for 10 hr. The alcohol then was distilled off and the residue crystallized from the proper solvent.

IR for VI*f*: 1640 cm.$^{-1}$ (—CO·NH—), 807 cm.$^{-1}$ (two adjacent ring hydrogen atoms in a 1,2,3,4-tetrasubstituted aromatic ring in the molecule), 1570 cm.$^{-1}$ (thiazole moiety) (20).

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Tumor Inhibitors II: Cytotoxic Alkaloids from *Annona purpurea*

PHILIP E. SONNET and MARTIN JACOBSON

Abstract □ Investigation of the cytotoxic principles of *Annona purpurea* L. resulted in the isolation and characterization of four new alkaloids of the aporphine variety. One of the four is an oxoaporphine which shows borderline activity *in vitro* against the 9-KB tumor test system. The known aporphine isocorydine, oxoaporphine *O*-methylatheroline, and the proaporphines glaziovine and stepharine were also obtained. Glaziovine and *O*-methylatheroline also showed limited tumor inhibition.

Keyphrases □ *Annona purpurea*—isolation, characterization of four aporphine alkaloids □ Tumor inhibitors—isolation, characterization of four aporphine alkaloids from *Annona purpurea* □ Aporphine alkaloids, *Annona purpurea*—potential tumor inhibitors

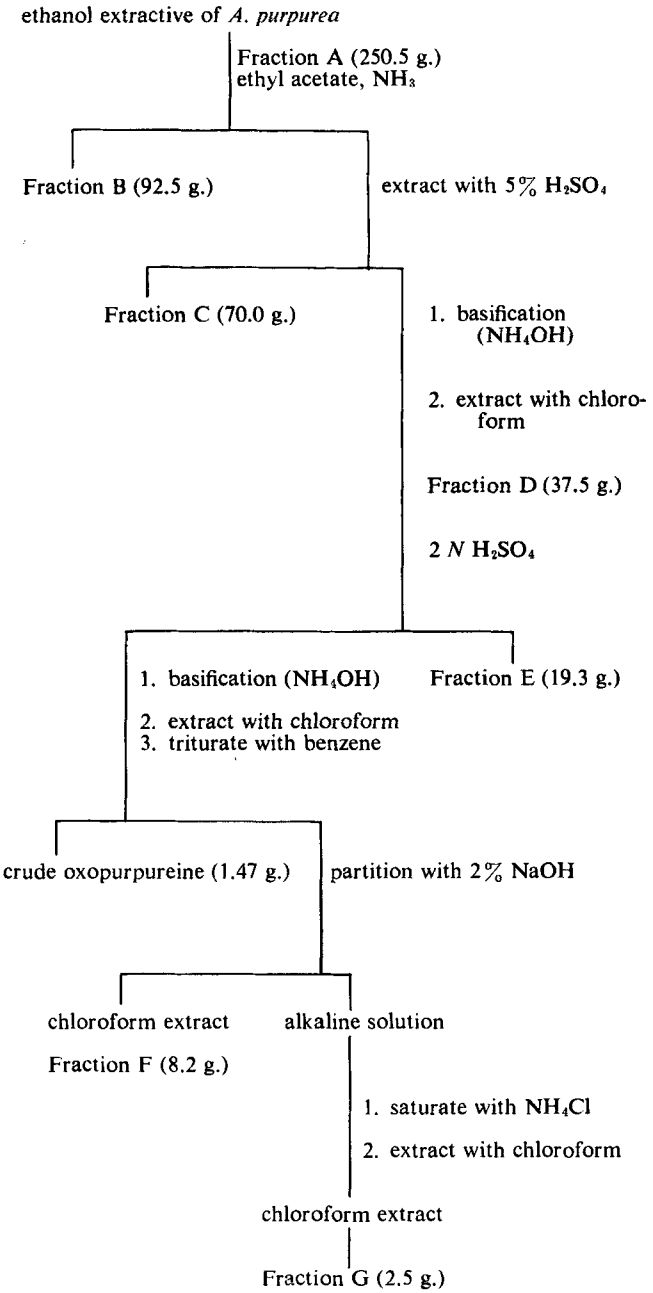
The first paper in this series (1) reported the isolation and identification of liriodenine from wood and stem bark of *Annona glabra* L. The present paper reports the continuation of this search for tumor inhibitors from plant and insect sources. Ethanol extracts of ground stems and leaves of *Annona purpurea* L.[1] have been found to inhibit tumor activity *in vitro* in the test system 9-KB[2]; several cytotoxic alkaloids from these extracts have been isolated and characterized.

EXPERIMENTAL

The procedure was as follows: 10.9 kg. (24 lb.) of dried plant material was steeped with hexane and extracted with ethanol. The concentrated ethanol extract was then extracted with ammoniacal ethyl acetate. This extract was washed repeatedly with 5% sulfuric acid and then made basic (pH 10) with ammonium hydroxide. It was then extracted with chloroform. The activity proved to be concentrated in the acid-soluble fraction (Scheme I).

The crude alkaloids were triturated with benzene, and the triturate was separated into base-soluble alkaloids (Fraction E) and base-insoluble alkaloids (Fraction F). Each group was then further fractionated with McIlvaine buffer solutions. Finally, each was subjected to column chromatography on various grades of neutral alumina until pure compounds, judged by TLC, were obtained.

An orange solid was obtained from the benzene-insoluble fraction, which was resolved by further column chromatography on Activity I alumina with benzene and chloroform into an orange compound and a small amount of a yellow compound. The yellow compound proved to be identical with *O*-methylatheroline (I*a*). The sample melted at 220–226°[3]; reported melting points are 225–227° (2), 227–229° (3), and 235–236° (4). The IR spectrum[4] was identical to the published spectrum for I*a* (4), and the UV (Table I) and NMR spectral data were, likewise, in accord with published data (3–5).



Fraction	ED_{50}, mcg./ml.
A	100
B	>100
C	>100
D	20.0
E	21.0
F	25.0
G	8.2

Scheme I—Fractionation of cytotoxic principles of A. purpurea *and cytotoxicity of the fractions*

[1] Collected in Puerto Rico in 1969.

[2] Cytotoxicity was assayed, under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, against Eagle's KB strain of cells derived from human carcinosarcoma of the nasopharynx [H. Eagle and G. E. Foley, *Amer. J. Med.*, **21**, 739(1956); *Cancer Res.*, **18**, 1017(1958)].

[3] Melting points are uncorrected and were obtained on a Fisher-Johns melting-point apparatus. Mention of a proprietary item in this paper does not constitute an endorsement of this product by the U. S. Department of Agriculture.

[4] IR spectra were obtained with Perkin-Elmer 137 and 521 spectrophotometers.

Table I—UV Spectral Data[a]

Compound	$\lambda_{max.}$, nm.	Log ε	Reference
Ia: *O*-Methylatheroline	246, 277, 363	4.59, 4.58, 4.16	—[b]
	242, 272, 355	4.52, 4.53, 3.99	3
Ia: H^+	260, 284, 385	4.65, 4.63, 4.20	—[b]
Ib: Oxopurpureine	251, 282, 354, 392, 456	4.37, 4.54, 3.86, 3.94, 3.78	—[b]
II: Cassamedine	252, 281, 324, 364, 460	4.47, 4.53, 4.12, 3.97, 3.76	6
Ib: H^+	260, 287, 425	4.40, 4.49, 3.86	—[b]
II: H^+	272, 286, 408, 534	4.49, 4.50, 4.10, 3.40	6
IIIa: Glaziovine	286, 315sh	3.59, 2.93	—[b]
	288	3.57	9
IIIb: Stepharine	278	3.78	—[b]
	284	3.48	14
IVa: Norpurpureine	272sh, 280, 300, 311sh	4.13, 4.20, 4.13, 4.07	—[b]
IVb: Purpureine	273sh, 282, 303, 312sh	4.26, 4.36, 4.33, 4.29	—[b]
V: Isocorydine	267, 271sh, 305	4.03, 4.02, 3.69	—[b]
	266, 302	4.26, 3.82	—[7]
VI: *O*-Demethylpurpureine	272sh, 283, 302, 312sh	4.11, 4.25, 4.21, 4.15	—[b]
VIIa: *O*-Methylcassyfiline	283, 302, 312	4.31, 4.30, 4.27	6
VIIb: Ocoteine	283, 302	4.24, 4.25	14

[a] Spectra were determined in ethanol solvent with a Cary 14 recording spectrophotometer. [b] This work.

After recrystallization from toluene, the orange compound was analyzed for $C_{21}H_{19}NO_6 \cdot {}^1/_2\, C_7H_8$, m.p. 198–202° dec. The absence of NH and alkyl CH_2 stretching absorptions in its IR spectrum, the presence of a strong 1640 $cm.^{-1}$ band, and the formation of a cherry-red solution in dilute acid suggested an oxoaporphine nucleus. The NMR spectrum (Table II) indicated five methoxyl groups; the UV spectra, both in neutral and in acidic solutions, showed some similarity to those of cassamedine (II) (6). From the NMR spectrum, positions C-4 and C-5 were clearly unsubstituted. The remaining pair of aryl protons was unsplit and could, therefore, be present *para* to each other at positions C-8 and C-11 as in Structure I*b*. Further evidence for this structure is presented later.

Investigation of the base-insoluble Fraction F provided two new aporphine alkaloids (IV*a* and IV*b*) and the known proaporphine, stepharine (III*b*). Stepharine was identified by direct comparison with authentic (+)-stepharine (IR and mixed melting point). Compound IV*a*, m.p. 115–117°, was identified as a pentamethoxynoraporphine from its NMR spectrum; it also gave an *N*-methyl methiodide, m.p. 227–229°, identical with the methiodide of the pentamethoxyaporphine (IV*b*). Oxidation of Compound IV*b* with chromium trioxide pyridine provided a small sample (~2 mg.) of the corresponding oxoaporphine which was identical to Compound I*b* (TLC and IR).

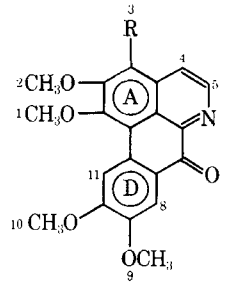
The base-soluble Fraction G provided the phenolic aporphine, isocorydine (V), m.p, 183–185°: [lit (7) m.p. 186°]. The NMR, UV, and IR data (7) were identical with those obtained for the alkaloid isolated from *A. purpurea*. The methiodide showed m.p. 231–232° (darkening at 224°) [lit. (8) m.p. 231–232° when heated rapidly]. This alkaloid was finally identified with a known sample of (+)-isocorydine (IR and mixed melting point). A phenolic proaporphine, m.p. 227–231° dec., was also obtained; its NMR and UV spectra compared well with those of glaziovine (III*a*), m.p. 235–237° dec. (9). The picrate of Compound III*a* had m.p. 196.5–200° [lit. (9) m.p. 199–203°]. This alkaloid was also identified with an authentic sample of (+)-glaziovine (IR).

A phenolic tetramethoxyaporphine (VI) was also isolated; complete methylation generated the same methiodide described for Compounds IV*a* and IV*b*. The pattern of oxygenation must, therefore, be the same for the phenol, Compounds IV*a* and IV*b*, and for the oxoaporphine, Compound I*b*. The phenol (VI) did not incorporate deuterium when treated under conditions that normally cause protons *ortho* and *para* to phenolic hydroxyl groups to be exchanged

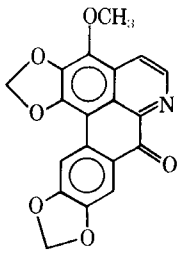
Table II—NMR Data[a]

Compound	Solvent	Aryl Protons C4	C5	C8	C9	C11	Other Protons	Reference
Ib: Oxopurpureine	$CDCl_3$	8.13d(5.4)	8.91d	7.94s	—	8.65s	OCH_3: 4.03, 4.06, 4.07, 4.10, 4.15	—[b]
	TFA	8.87d(6.3)	9.01d	8.08s	—	8.98s	OCH_3: 4.18, 4.26, 4.34, 4.38, 4.43	—[b]
II: Cassamedine	TFA	7.83d	8.85d	8.19s	—	8.85s	OCH_3: 4.48; OCH_2O: 6.23, 6.62	6
IVa: Norpurpureine	$CDCl_3$	—	—	6.74s	—	7.98s	OCH_3: 3.72, 3.90(3), 3.94	—[b]
IVb: Purpureine	$CDCl_3$	—	—	6.77s	—	7.95s	NCH_3: 2.54; OCH_3: 3.71, 3.88, 3.91(2), 3.94	—[b]
IVb: Methiodide	Dimethyl sulfoxide	—	—	7.02s	—	7.79s	NCH_3: 3.36, 3.40; OCH_3: 3.73, 3.78, 3.82, 3.89(2)	—[b]
VI: *O*-Demethylpurpureine	$CDCl_3$	—	—	6.76s	—	7.89s	NCH_3: 2.54; OCH_3: 3.70, 3.90(2), 3.96	—[b]
	Dimethyl sulfoxide	—	—	6.90s	—	7.76s	NCH_3: 2.44; OCH_3: 3.66, 3.77(3)	—[b]
	Dimethyl sulfoxide–NaOMe	—	—	6.75	—	7.70		—[b]
V: Isocorydine	Dimethyl sulfoxide	—	—	6.84,6.87[c]	6.84,6.87[c]	—	C3H: 6.83; NCH_3: 2.49; OCH_3: 3.58, 3.76, 3.82	—[b]
	Dimethyl sulfoxide–NaOMe	—	—	6.44[d]	5.89[d]		C3H: 6.53	—[b]

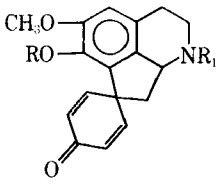
[a] NMR spectra were recorded with a Varian HA-100 spectrometer. Data are given in parts per million measured from tetramethylsilane as an internal standard. Standard abbreviations used are s = singlet, d = doublet. [b] This work. [c] Coupling constant J_{AB} 8 Hz. [d] J_{AX} 8 Hz.



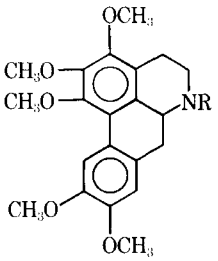
Ia: R = H
Ib: R = OCH_3



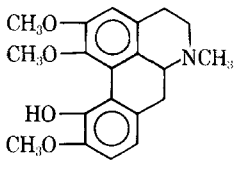
II



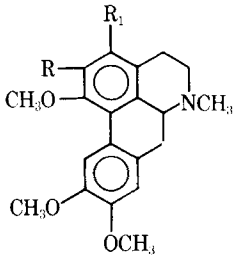
IIIa: R = H, R_1 = CH_3
IIIb: R = CH_3, R_1 = H



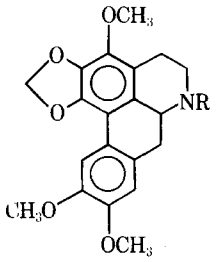
IVa: R = H
IVb: R = CH_3



V



VIa: R = OH, R_1 = OCH_3
VIb: R = OCH_3, R_1 = OH



VIIa: R = H
VIIb: R = CH_3

(10). A comparison of the NMR spectra of the phenol in dimethyl sulfoxide, with and without added base, showed no large upfield shift of either aryl proton in the basified solution, although a shift of 0.4–0.6 p.p.m. can be expected for protons *meta* to a phenolic hydroxyl (11). An example of such a shift is provided in Table II (isocorydine). The observations, therefore, indicated that the phenolic hydroxyl was situated on a fully substituted ring so the two aryl protons must be on the same ring. Since they are not visibly coupled, they must be *para* to one another and can only be assigned to positions C-8 and C-11 in ring D. Then, since the oxygenation pattern of the four new compounds is the same, the structures of the nonphenolic alkaloids, Compounds I*b*, IV*a*, and IV*b*, were determined. The hydroxyl group of the new phenol must be present in ring A. Position C-1 can be eliminated since no downfield shift of the proton at C-11 occurred in the NMR spectrum of the phenol anion (11). Structure VI*a* or VI*b*, in which the hydroxyl group is either on C-2 or C-3, can be assigned to this phenol. Insufficient material prevented a degradative study to distinguish the two alternatives. The UV spectra of *O*-methylcassifiline (VII*a*) and ocoteine (VII*b*) were included in Table I for comparison with IV*a* and IV*b*.

Table III—Inhibition Test Data

Compound	Amount Isolated, mg.	ED_{50}, mcg./ml.
I*a*: *O*-Methylatheroline	21	5.1
I*b*: Oxopurpureine	300	5.8
III*a*: Glaziovine	159	2.6
III*b*: Stepharine	107	17
IV*a*: Norpurpureine	33	22
IV*b*: Purpureine	293	>100
V: Isocorydine	163	87
VI: *O*-Demethylpurpureine	104	22

The results of the cytotoxicity tests for the isolated alkaloids are summarized in Table III. The oxoaporphines, Compounds I*a* and I*b*, and the proaporphine, glaziovine, showed borderline inhibitory activity. Corydine hydrochloride, an aporphine, was reported to be active against Sarcoma 37 when intramuscularly implanted in hybrid mice (12); liriodenine, an oxoaporphine, is also cytotoxic (1). The proaporphines, which have the susceptibility to nucleophilic attack inherent in the cyclohexadienone structure, are therefore good candidates for tumor screening (13) and should be further investigated. The following names are suggested for the new alkaloids: I*b*, oxopurpureine; IV*a*, norpurpureine; IV*b*, purpureine; and VI, *O*-demethylpurpureine.

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Synthesis of Compounds Related to Metabolites of Trimetazidine in Rabbits

SHUN-ICHI NAITO, SEIMEI OSUMI, KAZUHIKO KITAO, MATAICHI TETSUO*, MASAKI KITAURA*, and TAKESHI FUJITA*

Abstract □ Synthetic methods of trimetazidine metabolites in rabbits are described. One of the metabolites, 3-hydroxy-2,4-dimethoxybenzylpiperazine, is a new compound and its chemical structure is established.

Keyphrases □ Trimetazidine metabolites, in rabbits—synthesis of related compounds □ 3-Hydroxy-2,4-dimethoxybenzylpiperazine—synthesis, structure determination □ NMR spectroscopy—structure determination □ IR spectrophotometry—structure determination

In a previous paper (1), absorption, excretion, and distribution of trimetazidine [1-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride] in animals were investigated. The present work was performed to synthesize several compounds related to trimetazidine metabolites in rabbits. The compounds synthesized are shown here.

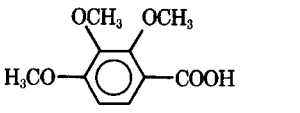
3-Hydroxy-2,4-dimethoxybenzylpiperazine is a new compound, and the chemical structure was established in the present work.

EXPERIMENTAL

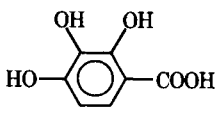
2,3,4-Trimethoxybenzoic acid, 2,3,4-trihydroxybenzoic acid, 2,3,4-trimethoxytoluene, 2,3,4-trihydroxytoluene, 1,3-dimethylpyrogallol, 2-ethyl-1,3-dimethylpyrogallol, 3-ethoxy-2,4-dimethoxy benzaldehyde, and 3-ethoxy-2,4-dimethoxybenzoic acid were synthesized by methods described in the literature (cited in Table I).

1-(2,3,4-Trimethoxybenzyl)-N^4-acetylpiperazine—To 10 g. of trimetazidine, a mixture of 20 ml. of acetic anhydride and 50 ml. of acetic acid was added and the reaction mixture was refluxed on a steam bath for 15 min. After cooling, 10% sodium carbonate solution was added to make the reaction mixture alkaline. The alkaline solution was extracted with ether, and the ethereal solution was evaporated on a steam bath. The residue was distilled *in vacuo*. Three grams of a colorless oily fraction, boiling at 175–185°/5 mm. Hg, was obtained.

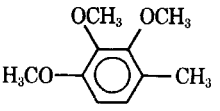
Anal.—Calc. for $C_{16}H_{24}N_2O_4$: C, 62.34; H, 7.79; N, 9.09. Found: C, 62.40; H, 7.81; N, 8.87.



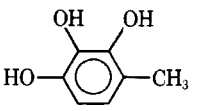
2,3,4-trimethoxybenzoic acid



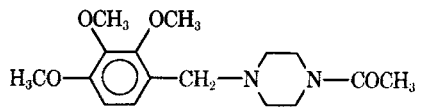
2,3,4-trihydroxybenzoic acid



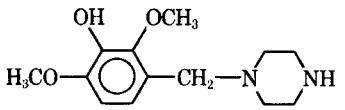
2,3,4-trimethoxytoluene



2,3,4-trihydroxytoluene



1-(2,3,4-trimethoxybenzyl)-N^4-acetylpiperazine



3-hydroxy-2,4-dimethoxybenzylpiperazine

1-(Monohydroxydimethoxybenzyl)piperazine Dihydrochloride—A mixture of 10 g. of trimetazidine dihydrochloride and 24 g. of anhydrous aluminum chloride in 250 ml. of dried nitrobenzene was stirred at 50–55° for 7 hr. After cooling, the reaction mixture was poured into ice water and the nitrobenzene layer was removed. The water layer was filtered, and the filtrate was treated with 96 g. of sodium bicarbonate and 52 g. of ethylenediaminetetraacetic acid to make the medium alkaline. This mixture was extracted with 2 l. of chloroform, and the chloroform was washed with water and dried with anhydrous sodium sulfate. After the solvent was removed from the chloroform layer, the residue was dissolved in carbon tetrachloride. Hydrochloric acid gas was conducted into the carbon tetrachloride solution under cooling, and the precipitate that separated out was recrystallized from a mixed solvent of methanol and ether and then methanol. Colorless crystals (1.4 g.,

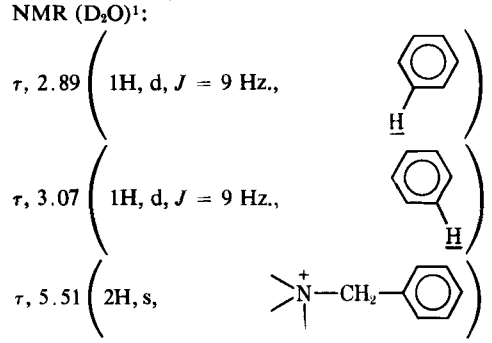
Table I—Syntheses of Several Compounds

Compound	Yield, %	Melting Point	Formula	Anal., % Calc.	Anal., % Found	References
2,3,4-Trimethoxybenzoic acid		98–100°				(2–6)
2,3,4-Trihydroxybenzoic acid	22	198–201°	$C_7H_6O_5$	C, 49.41 H, 3.53	C, 49.60 H, 3.44	(7, 8)
2,3,4-Trimethoxytoluene	52	B.p. 97–98° /1 mm. Hg	$C_{10}H_{14}O_3$	C, 65.93 H, 7.69	C, 65.82 H, 7.91	(9)
2,3,4-Trihydroxytoluene	14	94–97°	$C_7H_8O_3$	C, 60.00 H, 5.71	C, 60.18 H, 5.85	(7, 8)
1,3-Dimethylpyrogallol	23	B.p. 133–134°/17 mm. Hg				(10)
2-Ethyl-1,3-dimethylpyrogallol	88	B.p. 98–99° /16 mm. Hg				(8)
3-Ethoxy-2,4-dimethoxybenzaldehyde	61	Not purified				(8)
2,4-Dinitrophenylhydrazone of 3-ethoxy-2,4-dimethoxybenzaldehyde		170°	$C_{17}H_{18}N_4O_7$	C, 52.30 H, 4.65 N, 14.36	C, 52.36 H, 4.60 N, 14.46	(8)
3-Ethoxy-2,4-dimethoxybenzoic acid	14	91–92°	$C_{11}H_{14}O_5$	C, 58.39 H, 6.24	C, 58.55 H, 6.34	(8)

14.6% in theoretical amount), melting at 240–245° dec., were obtained.

Anal.—Calc. for $C_{13}H_{22}Cl_2N_2O_3$: C, 48.01; H, 6.82; Cl, 21.80; N, 8.61. Found: C, 48.13; H, 7.00; Cl, 21.94; N, 8.56.

NMR (D_2O)[1]:



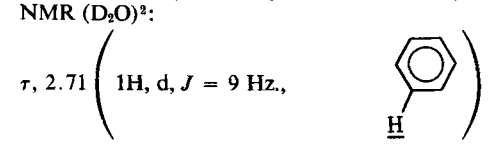
τ, 6.05 (3H, s, —OCH_3)
τ, 6.08 (3H, s, —OCH_3)
τ, 6.32 (8H, s, 4z —CH_2—)

NMR(dimethyl sulfoxide)[1]:, τ, −0.04 (1H, broad s, —OH). IR cm.$^{-1}$: δ_{C-H} 820 (KBr); $\nu_{C=C}$ 1620 (KBr); ν_{O-H} 3400 (KBr).

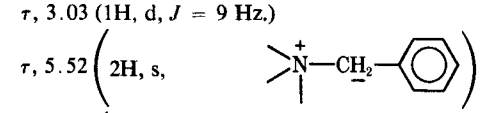
Ethylated Compound of 1-(Monohydroxydimethoxybenzyl)piperazine Dihydrochloride—Ethylsulfate (3.9 ml.) and then 12.7 ml. of 10% sodium hydroxide solution were added dropwise successively to a mixture of 0.7 g. of sodium hydroxide and 2.85 g. of the compound, 1-(monohydroxydimethoxybenzyl)piperazine, in 7.7 ml. of 10% sodium hydroxide solution, under refluxing and stirring. The reaction was continued for an additional hour under refluxing and stirring. After 2.5 ml. of diethylsulfate and then 3.9 ml. of 20% sodium hydroxide solution were added dropwise to the reaction mixture, stirring and refluxing were still continued for 20 min. After cooling, the reaction mixture was extracted with chloroform, and the chloroform layer was washed with water and dried with anhydrous sodium sulfate. The residual oily substance, after evaporation of chloroform, was dissolved in methanol; hydrochloric acid gas was conducted into this solution. The crystalline substance that separated out after the addition of a small amount of ether to the methanol solution was recrystallized from a mixed solvent of methanol and ether. A colorless crystal (0.6 g., 18.0% in theoretical amount), melting at 211–213° dec., was obtained.

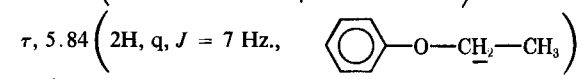
Anal.—Calc. for $C_{17}H_{30}Cl_2N_2O_3$: C, 53.54; H, 7.93; Cl, 18.59; N, 7.35. Found: C, 53.45; H, 8.02; Cl, 18.79; N, 7.22.

NMR (D_2O)[2]:



τ, 3.03 (1H, d, J = 9 Hz.)





τ, 6.01 (3H, s, —OCH_3)
τ, 6.07 (3H, s, —OCH_3)
τ, 6.32 (8H, s, 4x —CH_2—)
τ, 6.62 (2H, q, J = 7 Hz., >N^+—CH_2—CH_3)
τ, 8.61 (6H, t, J = 7 Hz., >N^+—CH_2—CH_3 and —O—CH_2—CH_3)

IR cm.$^{-1}$: δ_{C-H} 830 (KBr); $\nu_{C=C}$ 1615 (KBr).

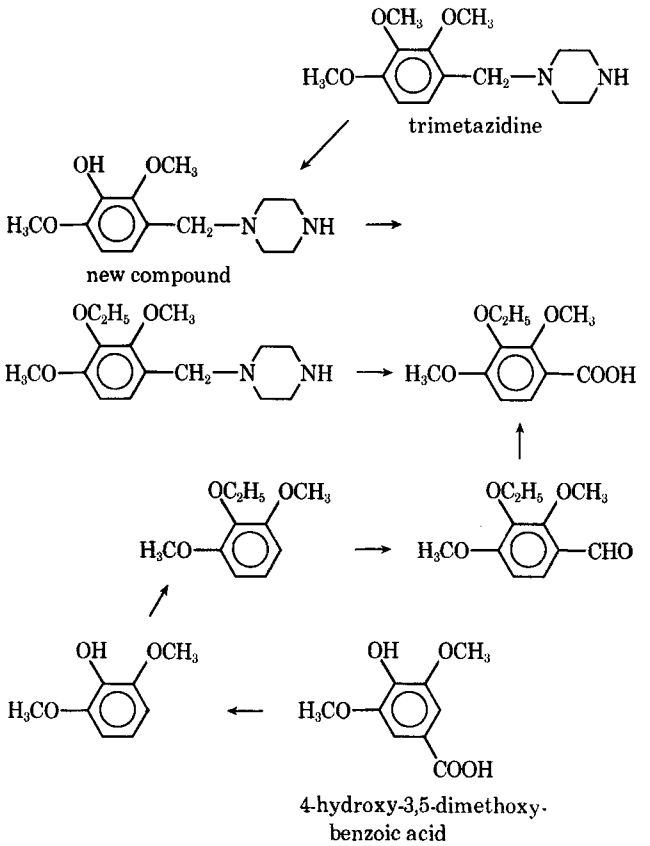
Potassium Permanganate Oxidation Product of Ethylated 1-(Monohydroxydimethoxybenzyl)piperazine Dihydrochloride—A mixture of 1.2 g. of potassium permanganate and 0.2 g. of the compound, ethylated compound of 1-(monohydroxydimethoxybenzyl)-piperazine, in 20 ml. of water was stirred at 85–90° until the color of potassium permanganate solution disappeared. Manganese dioxide that precipitated was dissolved into the reaction mixture by the introduction of sulfur dioxide gas; this reaction mixture was extracted with ether. An acidic component was extracted with saturated sodium bicarbonate solution from the ethereal solution after washing with water. The water layer was extracted with ether after acidification with hydrochloric acid, and this ethereal solution was washed with water and dried with anhydrous sodium sulfate. Crystals, after evaporation of the ether, were recrystallized from petroleum ether (b.p. 60–70°). A colorless crystal (16 mg., 13.5% in theoretical amount), melting at 89°, was obtained and coincided with the compound 3-ethoxy-2,4-dimethoxybenzoic acid in its melting point and IR spectrum.

[1] Internal standard: tetramethylsilane.
[2] Internal standard: dioctyl sodium sulfosuccinate.

RESULTS AND DISCUSSION

The compounds, 2,3,4-trimethoxybenzoic acid, 2,3,4-trihydroxybenzoic acid, 2,3,4-trimethoxytoluene, 2,3,4-trihydroxytoluene, 1-(2,3,4-trimethoxybenzyl)-N^4-acetylpiperazine, and 3-hydroxy-2,4-dimethoxybenzylpiperazine, were used as the authentic samples for identification of trimetazidine metabolites in rabbit urine.

One of the trimetazidine monohydroxydimethoxybenzyl piperazine metabolites (3-hydroxy-2,4-dimethoxybenzylpiperazine) is a new compound and was obtained from demethylation of trimetazidine with anhydrous aluminum chloride. The chemical structure of this new compound was established as shown in Scheme I.



Scheme I

The results of instrumental analysis of the new compound reveal the following facts:

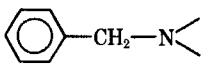
1. *AB*-type couplings at τ 2.89 and 3.07 were observed in the NMR spectrum of the new compound. J_{AB} = 9 Hz. in the NMR (D_2O) existed in the range of 5–10 Hz. in *J*-value, corresponding to 2H adjoined with each other. The signals of τ 6.05 and 6.08 came from 3H and showed the existence of two methoxy groups on a benzene skeleton

2. Absorptions corresponding to $\nu_{C=C}$ at 1620 cm.$^{-1}$ and δ_{C-H} at 820 cm.$^{-1}$ were recognized in the IR spectrum of the new compound.

3. In the NMR (dimethyl sulfoxide) spectrum of the new compound, the signal of τ −0.04, which disappeared in the NMR (D_2O), was detected.

4. The IR absorption of ν_{O-H} at 3400 cm.$^{-1}$, which disappeared when the compound was ethylated, was detected.

These facts support an existence of a phenol group in the new compound. It is reasonable to consider that the signal of τ 5.51 comes from 2H, is not coupling, and is shifted differing from that of τ at about 6.7 in usual absorption due to:



Also, it can be interpreted that a benzyl skeleton having two methoxys, one phenolic hydroxy, and two hydrogens adjoined with each other is bound with piperazine at the position of one nitrogen atom, N^+, in the piperazine skeleton. The signal of τ 6.32 due to 8H was observed as a sharp singlet in the NMR spectrum of the new compound.

The chemical structure of the new compound was established also from the IR spectrum of the compound; that is, the potassium permanganate oxidation product of ethylated 1-(monohydroxydimethoxybenzyl)piperazine coincided with that of the compound 3-ethoxy-2,4-dimethoxybenzoic acid synthesized separately, and no depression in a mixed melting-point determination was observed.

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Color Reaction of Thyroxine and Its Derivatives with Nitrous Acid

S. E. SAHEB and M. A. SANASSIAN

Abstract □ The 3′,5′-diiodo-4′-hydroxydiphenylether system in thyroxine was shown to be indispensable for the success of the color reaction with nitrous acid. This reaction was also successful with sodium hypochlorite and chlorine gas. The products were separated, and evidence is presented to show that this reaction involves an oxidation of the 3′- and/or 5′-position to iodoso or iodoxy derivatives while the amino acid side chain remains intact.

Keyphrases □ Thyroxine, derivatives—chemical nature of color reaction with nitrous acid □ 3′,5′-Diiodo-4′-hydroxydiphenylether system—role in color formation of thyroxine and nitrous acid

Thyroxine (I), when treated with nitrous acid, is reported to give a characteristic red color, even in a concentration of 1 in 40,000 (1). This standard procedure is described in various pharmacopeias (2) for the identification of thyroxine. Roche and Michel (3) reported on the analytical aspect of this reaction and suggested its use as a quantitative method for the determination of thyroxine. The absorption of the color formed obeyed Beer–Lambert's law within a certain concentration range, 100–300 mcg. This paper reports on the chemical nature of this reaction, which has not been investigated previously.

EXPERIMENTAL

Thyroxine (I), tetraiodothyropropionic acid (II), 3,5,3′-triiodo- and 3,5-diiodothyronine, 3,5-di- and 3-iodotyrosine, and tyrosine were used[1]. The purity of the commercially available thyroxine was checked by UV, TLC, and IR and was found acceptable. However, C, H analyses purveyed by any manufacturer were beyond the accepted limits. It was used as such without further purification. Both the D- and L-forms were used whenever available without any difference in results. Spectrophotometric readings were taken on Perkin-Elmer 202 (UV, visible) and 237 (grating IR) instruments with KBr pellets. Silica gel G was used[2].

Color Formation (1, 3)—To a solution of 5 mg. of thyroxine or its derivatives in 5 ml. of 95% acidic ethanol (enough hydrochloric acid to bring the solid in solution), a freshly prepared $NaNO_2$ (1%) solution was added dropwise until the color of the starch iodide paper became blue. The yellow color produced was intensified on boiling. When the cooled solution was made alkaline (pH 9) with ammonia, an intense red color developed, which became yellow on acidification (pH 1.5). Similarly, when the acidic solution of thyroxine was treated with NaOCl, it turned green, then yellow upon heating, and red with dilute alkali. With Cl_2 gas, the solution remained colorless until made alkaline; then it became red, and it turned yellow with acids. When the procedure was applied to Compound VI, molecular iodine and *p*-methoxyphenol were obtained (*cf.*, 4). With 3,5,3′-triiodo- and 3,5-diiodothyronine, 3,5-di- and 3-iodotyrosine, and tyrosine, the reaction mixture acquired a yellow color but did not turn red with base (*cf.*, 3).

Preparation of *N,O*-Diacetylthyroxine[3] (IV)—One gram of the sodium salt of thyroxine or its analogs was dissolved in 150 ml. of freshly distilled, cold acetic anhydride. The solution was left to stand overnight at room temperature. The solvent was evaporated to dryness under vacuum and the white amorphous residue, A, was

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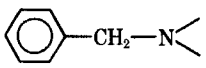

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Table I—UV and Chromatographic Values of Thyroxine and Its Reaction Products

Compounds	UV Absorption in Aqueous Ethanol, nm. Acid	Neutral	Base	R_f
Thyroxine I	210, 225, 290	210, 225, 300	330	0.33
Band a	210, 225	210, 225, 280, 460	465	0.3
Band b	210, 225, 290	202, 225	225	0.23
Band c	202, 225, 290	202, 225, 290, 460	225, 460	0.2
Band d	202, 225	202, 225, 290	225	0.16
Band e	210, 225, 290	210, 225, 290	225	0.14

collected. It was shown by TLC and IR (absorptions at 1730 and 1765 cm.$^{-1}$) to be almost all IV. Impurities, as revealed by TLC, consisted only of traces of *N*-acetylthyroxine, generated probably by the alkalinity of the eluent (see *Chromatography*). Residue A did not have a definite melting point but decomposed over a range (188–210°) and could not be recrystallized. The impurity in residue A could be partly removed when it was dissolved in absolute ethanol and reprecipitated by the addition of water; yield: 0.87 g.

Preparation of *N*-Acetylthyroxine[3] (III)—Residue A (1 g.) was dissolved in about 100 ml. of 1 *N* NaOH; to this, 50 ml. of 10 *N* NaOH was added. The precipitate formed was filtered and washed with a saturated NaCl solution and dried. The amorphous powder could not be recrystallized from the common organic solvents and had no definite melting point. Its purity was checked by TLC (see *Chromatography*) and IR, where only one peak appeared in the carbonyl region at 1720 cm.$^{-1}$; yield: 0.83 g.

Chromatography—The reaction product was dried under vacuum and washed several times with distilled water and dried. The brown amorphous powder was dissolved in methanol and spotted on preparatory TLC slides (0.5 mm., silica gel G). The only eluent that gave a good separation was the organic layer of an *n*-butanol–concentrated ammonia–water mixture (200:27:23) (5). Elution was allowed for 4 hr., after which the colored bands were scraped, the silica was extracted with acidulated (HCl) methanol, and the extract was dried under vacuum and washed several times with distilled water until the washings gave a negative test for chlorides.

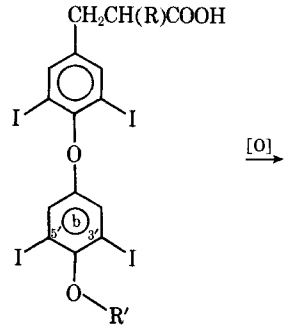
All five bands of the reaction product of I, II, or III were colored and traveled less than the starting material (R_f 0.33, 0.5, and 0.4, respectively), except the first spot which had the same R_f as the starting material but differed from it in IR and UV.

RESULTS AND DISCUSSION

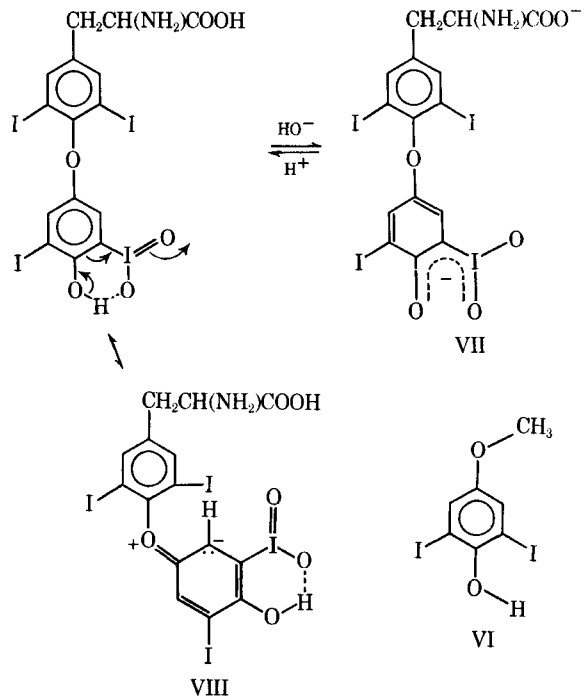
In addition to thyroxine, several of its derivatives, *e.g.*, its methyl ester (6), tetraiodothyropropionic acid (II), and *N*-acetylthyroxine (III), were found to give a positive reaction with nitrous acid, whereas *N,O*-diacetylthyroxine (IV), the 4′-*O*-methyl ether derivative of thyroxine (7) (V), 3,5,3′-tri- and 3,5-diiodothyronine, 3,5-di-[4] and 3-iodotyrosine, tyrosine, and 2,6-diiodo-4-methoxyphenol (8) (VI) gave negative results with nitrous acid.

The reaction product of I, II, and III was unextractable with lipophilic solvents (ether, ethylacetate, *etc.*) and would shift from wine-red to yellow upon changing the pH of the medium from the basic (pH 9) to the acidic side (pH 1.5) and vice versa. Besides, no iodide or elemental iodine, which may have arisen from deiodination, could be detected (9) in the medium of the reaction product[5].

Upon chromatography, five colored spots of small and very close R_f values (Table I) were obtained with the reaction products of I, II, and III with an *n*-butanol–ammonia system on silica plates. These spots ranged in color from yellow to violet and had the same R_f value whether prepared from I or III; the ones prepared from II remained close together but invariably traveled faster, presumably due to the absence of the aliphatic amino group. When these bands were isolated and extracted from preparatory TLC, they: (*a*) gave a positive ninhydrin test; (*b*) contained nitrogen (as determined by elemental analysis), except those isolated from thyropropionic acid (II); (*c*) had no absorption around 1500 (nitroso) or 1523–1544 cm.$^{-1}$



I: R = NH_2, R′ = H
II: R = H, R′ = H
III: R = $NHCOCH_3$, R′ = H
IV: R = $NHCOCH_3$, R′ = $COCH_3$
V: R = NH_2, R′ = CH_3



Scheme I

(nitro) (10); (*d*) had superimposable IR spectra through the whole range; (*e*) had different $\lambda_{max.}$ and common peaks at 225 and 290 nm. (Table I); (*f*) had no melting points; and (*g*) were insoluble in the common organic solvents and changed in color as the medium was shifted from alkaline to acidic.

Such evidence as described in the preceding paragraphs allowed for the following conclusions:

1. The integrity of the 3′,5′-diiodo-4′-hydroxydiphenylether moiety is a necessary and sufficient requirement for the success of this reaction[6].

[4] Roche and Michel (3) reported that this compound gave a positive reaction with nitrous acid, but the compound did not develop a red color in the present study.

[5] G. M. De Escobar, P. Llorente Rodriguez, T. Jolin, and F. Escobar del Rey, *Biochem. J.*, **88**, 526(1963), reported on the deiodination of thyroxine.

[6] It was reported to be specific for *o*-diiodophenols (3).

2. The R—CH(NH_2)COOH of the thyroxine side chain is unnecessary and remains intact in the product (1150 and 2800 $cm.^{-1}$ when in the zwitterion form).

3. There is no displacement of the aromatic-bound iodine (4).

Furthermore, when one of the pure bands isolated from the reaction product of I, II, or III was respotted a few hours after its isolation, it gave two to three spots. These new spots had R_f values similar to one or more of the bands present in the original reaction mixture[7]. This strongly implies the presence in the reaction product of iodoso derivatives, which are known (11) to disproportionate on standing or on TLC to the iodoxy and the original iodo compound.

Further spectral studies, such as mass spectrum, electron spin resonance, and NMR, could not be used in the elucidation of the structure, mainly because of a lack of volatility and insolubility problems. Although it is attractive in the case of thyroxine to speculate that the red color in the product is due to a stable free radical (12), limited electron spin resonance experiments did not substantiate this proposition.

Other agents, *e.g.*, chlorine and sodium hypochlorite, gave results similar to the ones obtained with nitrous acid.

Diphenyl ethers are reported to be stable systems (13). However, autoxidation of thyroxine with oxygen in dimethyl sulfoxide, followed ultimately by rupture of the diphenyl ether linkage, was shown (14) very recently to give rise to quinones[8], while nitrous acid gave rise to quinones with 1,2,3-trimethoxybenzene (15).

In this study, however, the facts that: (*a*) the use of different oxidizing agents such as nitrous acid, sodium hypochlorite, and chlorine gave the same products as determined spectrophotometrically and by TLC; (*b*) the fractions isolated from any of these oxidations were identical to one another in IR, different in UV, and gave a positive ninhydrin test, except those isolated from II; (*c*) the physical behavior of all fractions (ease of decomposition, color, lack of solubility, *etc.*) are characteristic of polyvalent iodine compounds (11); (*d*) II and III gave rise to products similar to the ones obtained from thyroxine; and (*e*) there was the indicatorlike behavior of all fractions towards acid and bases (Scheme I), can best be reconciled by visualizing an oxidation of the iodine at the 3′- and/or 5′-positions of thyroxine to the iodoso or more probably to the iodoxy compound (or both). At this point, it is proposed that the *N,O*-diacetylthyroxine (IV) and the 4′-*O*-methylether derivative (V) of thyroxine were not readily oxidized because a substituent on the oxygen atom at C-4′ interferes sterically or mesomerically with the formation of Structure VII.

The hindrance to free rotation around the ether oxygen in the diphenyl ether system was studied (16). Moreover, in the case of thyroxine, the bulk of the iodine at positions 3 and 5 (17), plus the greater participation of the *p*-electrons of the ether oxygen into ring b (4) of thyroxine (see Structure VIII), makes the 2′ and 6′- as well as the 3′- and 5′-positions, nonequivalent[9] (18). This may be used to explain the number of fractions appearing on TLC by suggesting that an oxidation at 3′ by nitrous acid, sodium hypochlorite, or chlorine gas does not give rise to the same compound obtained by oxidation at 5′.

[7] Two-dimensional chromatography was performed with no further separation.

[8] Which, contrary to a popular theory, may not be involved in the metabolism of thyroxine in humans, *cf.*, C. S. Pittman, V. H. Read, and J. B. Chambers, *J. Clin. Invest.*, **49**, 373(1970); E. Gruenstein and J. Wynn, *J. Theoret. Biol.*, **26**, 343(1970).

[9] For further substantiation, see the system described by J. H. Ackerman and G. M. Laidlow, *Tetrahedron Lett.*, **1969**, 4487.

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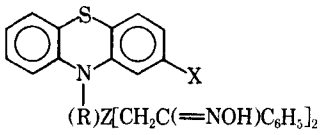
Phenothiazines as Anticholinergics

V. N. SHARMA, N. S. PANCHOLI, H. L. SHARMA, R. L. MITAL*, and DHARMA CHANDRA*

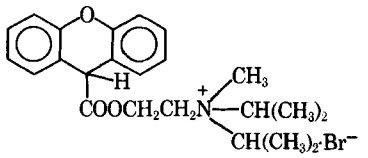
Abstract □ The synthesis and anticholinergic activity of a new series of quaternary oximes of some 10-*N*-substituted phenothiazines are reported.

Keyphrases □ Phenothiazine oximes—synthesis, pharmacological screening as possible anticholinergic agents □ Anticholinergics, potential—synthesis, pharmacological evaluation of quaternary oximes of 10-*N*-substituted phenothiazines □ Quaternary phenothiazine oximes—anticholinergic activity

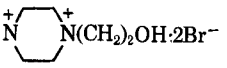
Earlier attempts (1, 2) showed that one way to reduce the Parkinsonism liability of phenothiazine drugs, with little loss of tranquilizing efficacy, is to increase the anti-acetylcholinelike activity. Since chemically related quaternary salts such as banthine and probanthine are well-known anticholinergic drugs, the authors speculated that quaternary oximes of phenothiazines might also exhibit enhanced anticholinergic activity. The CNS effects of a few phenothiazine oximes were already reported (1, 2). Some other quaternary oximes of various 10-*N*-substituted phenothiazines (I–IX) synthesized in the authors' laboratory recently were, however, found to be devoid of any significant CNS-depressant activity (3). When assayed for their activity as acetylcholine inhibitors on frog rectus abdominis muscle preparation (4), they exhibited pronounced anticholinergic activity as

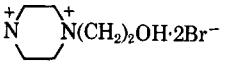


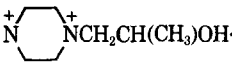
I–IX

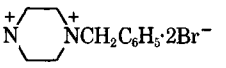


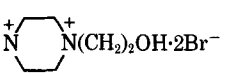
X: Probanthine

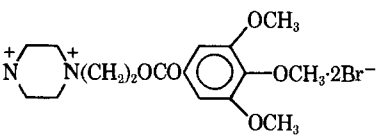
I: X = H, R = $COCH_2$, Z =  $N(CH_2)_2OH \cdot 2Br^-$

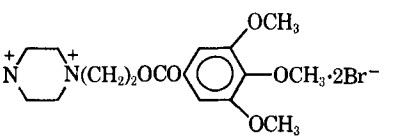
II: X = Cl, R = $COCH_2$, Z =  $N(CH_2)_2OH \cdot 2Br^-$

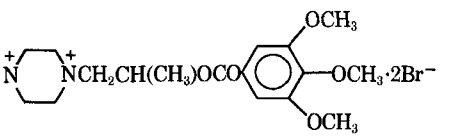
III: X = Cl, R = $COCH_2$, Z =  $NCH_2CH(CH_3)OH \cdot 2Br^-$

IV: X = Cl, R = $(CH_2)_3$, Z =  $NCH_2C_6H_5 \cdot 2Br^-$

V: X = Cl, R = $CH_2CH(CH_3)CH_2$, Z =  $N(CH_2)_2OH \cdot 2Br^-$

VI: X = Cl, R = $CH_2CH(CH_3)$, Z =  $OCH_3 \cdot 2Br^-$

VII: X = OCH_3, R = $CH_2CH(CH_3)$, Z =  $OCH_3 \cdot 2Br^-$

VIII: X = Cl, R = $(CH_2)_3$, Z =  $OCH_3 \cdot 2Br^-$

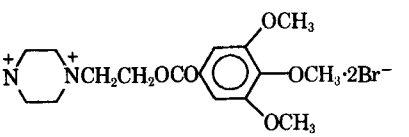
IX: X = Cl, R = $(CH_2)_4$, Z =  $OCH_3 \cdot 2Br^-$

Table I—Phenacyloxime (Phenothiazine) Dibromides and Their Pharmacological Test Data

Product[a]	Melting Point	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found	Dose, mcg./ml.	Reduction[b], %	Recovery, min.
I	163–165°	70	$C_{36}H_{39}Br_2N_5O_4S$	C, 54.20 H, 4.89 N, 8.78	C, 53.80 H, 4.72 N, 8.70	1 2	68.8 82.7	25 25
II	163–165°	80	$C_{36}H_{38}Br_2ClN_5O_4S$	C, 51.71 H, 4.57 N, 8.50	C, 51.52 H, 4.52 N, 8.40	1 2	50.6 56.4	15 40
III	163–165°	72	$C_{37}H_{40}Br_2ClN_5O_4S$	C, 52.51 H, 4.73 N, 8.23	C, 52.41 H, 4.28 N, 8.13	1 2	42.9 71.8	40 40
IV	143–144°	70	$C_{42}H_{44}Br_2ClN_5O_2S$	C, 57.43 H, 5.01 N, 7.97	C, 56.92 H, 4.82 N, 7.78	1 2	5.06 5.90	Immediate Immediate
V	143–145°	72	$C_{38}H_{44}Br_2ClN_5O_3S$	C, 56.05 H, 5.40 N, 8.60	C, 55.74 H, 5.00 N, 8.52	1 2	24.3 68.3	35 No recovery[c]
VI	150–153°	70	$C_{47}H_{52}Br_2ClN_5O_7S$	C, 54.98 H, 5.07 N, 6.82	C, 55.12 H, 4.85 N, 6.71	1 2	13.30 13.04	15 20
VII	148–153°	80	$C_{48}H_{55}Br_2N_5O_8S$	C, 56.41 H, 5.40 N, 6.85	C, 56.25 H, 5.12 N, 6.52	1 2	40.2 59.0	55 55
VIII	128–133°	75	$C_{48}H_{54}Br_2ClN_5O_7S$	C, 55.41 H, 5.10 N, 6.73	C, 55.31 H, 5.00 N, 6.51	1 2	32.1 49.4	35 35
IX	138–140°	80	$C_{48}H_{54}Br_2ClN_5O_7S$	C, 55.41 H, 5.10 N, 6.73	C, 55.22 H, 4.92 N, 6.67	1 2	31.3 40.3	40 40
X: Probanthine	—	—	—	—	—	1 2	57.0 59.0	Immediate 7

[a] The compounds were dissolved in ethanol, treated with carbon, and diluted with large volume of ether. The crystallized products were isolated by filtration and dried, and furnished satisfactory elemental analysis. [b] The height of contraction (millimeters) due to acetylcholine (0.1 mcg./ml.) was measured before and after each addition of different doses of compounds. The percent reduction in contraction produced by each dose of individual drug was thus determined. [c] Up to 1 hr.

expected. The results were compared with probanthine (X). The chemical and pharmacological test data of these compounds appear in Table I. Their structural formulas are shown (I–X).

It seems difficult to correlate the chemical structures of these compounds with their actual potencies from the results recorded in Table I. However, all these compounds were longer acting than the standard drug, in this case probanthine. The 10-*N*-acylaminophenothiazines (I–III) were more potent than the 10-*N*-alkylaminophenothiazines (IV–IX). Compound I was exceptionally more potent, while II and III were almost equipotent to probanthine. Since some reported acylaminophenothiazines were found to exhibit a spasmolytic effect (5), their enhanced activity might be attributed to an additive effect, exerted by the —CO— group in the presence of the oxime moiety.

EXPERIMENTAL

Melting points are uncorrected and were taken in open capillary tubes. IR and UV absorption spectra were measured on a Perkin-Elmer model 137 and Beckman quartz spectrophotometer model DU, respectively. Corex cells (1 cm.) were used to run UV spectra in ethanol, while IR spectra were run in KBr disks. These spectra were run in conjunction with melting points to ascertain the structures of the compounds. Where analyses are indicated only by symbols of the elements, analytical results for those elements were within ±0.4% of the theoretical values. For pharmacological studies, the compounds were dissolved in a minimal amount of warm ethanol and then the required dilution was made with distilled water. Studies were always conducted with freshly prepared solutions of each drug. Since the control studies with solvent alone did not exhibit any antiacetylcholine activity, the activities of the compounds are reported here. Reaction to acetylcholine by the muscle was determined; then the test drug was allowed to act on the muscle preparation for 2 min., and acetylcholine (0.1 mcg./ml.) was added.

10-*N*-Acyl- or Alkylaminophenothiazines—10-[4′-(β′-Hydroxyethyl)piperazinyl-1′]acetylphenothiazine (Compound A), 2-chloro-10-(β′-hydroxyethyl)piperazinyl-1′]acetylphenothiazine (Compound B), 2-chloro-10-[4′-(β′-hydroxypropyl)piperazinyl-1′]acetylphenothiazine (Compound C) (6), 2-chloro-10-γ-[4′-(benzyl)piperazinyl-1′]propylphenothiazine difumarate (Compound D)[1], 2-chloro-10-β-methyl-[4′-(β′-hydroxyethyl)piperazinyl-1′]propylphenothiazine difumarate (Compound E)[1], 2-chloro-10-β-[4′-(β′-hydroxyethyl)-piperazinyl-1′]propylphenothiazine 3′,4′,5′-trimethoxybenzoic ester difumarate (Compound F), 2-methoxy-10-β-[4′-(β′-hydroxyethyl)piperazinyl-1′]propylphenothiazine 3′,4′,5′-trimethoxybenzoic ester difumarate (Compound G) (6), 2-chloro-10-γ-[4′-(β′-hydroxypropyl)piperazinyl-1′]propylphenothiazine 3′,4′,5′-trimethoxybenzoic acid ester dimaleate (Compound H) (6), and 2-chloro-10-δ-[4′-(β′-hydroxyethyl)piperazinyl-1′]butylphenothiazine 3′,4′,5′-trimethoxybenzoic ester (Compound J) (7) were prepared by adopting the procedures reported earlier.

Phenacyl Bromide Oxime—This was synthesized by the method of Sharma *et al.* (1).

Phenacyloxime (Phenothiazine) Dibromides—The procedure employed was essentially the same as reported earlier (1). The free base was first libera ed from its respective salt by 20% aqueous NaOH, taken up by $CHCl_3$, and worked up as usual. The solution of the free base (0.005 mole) in anhydrous ether, when treated with phenacyl bromide oxime (0.0115 mole), resulted in an immediate precipitation of quaternary oxime (I–IX) from the respective compound (A–J).

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[1] These compounds were supplied by Dr. Toldy of the Institut für Arzneimittelforschung, Budapest, Hungary.

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* Present address: Department of Chemistry, University of Rajasthan, Jaipur, India.

Detection of CL-912, a 1,3-Dioxalane, in Collapsible Tube Liner by Attenuated Total Reflection Spectroscopy

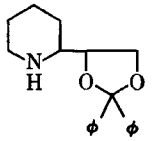
S. C. MUTHA

Abstract □ An attenuated total internal reflection technique was used to demonstrate the migration of the free base of CL-912C from a urological jelly to the lining of collapsible tubes during the sterilization cycle. This observation was substantiated by examination of isooctane extract of the sample liner by TLC and attenuated total internal reflection.

Keyphrases □ CL-912, a 1,3-dioxalane, in urological jelly—migration to collapsible tube liner, attenuated total internal reflection □ Attenuated total internal reflection—migration of CL-912 from urological jelly to collapsible tube lining □ Sterilization—migration of a 1,3-dioxalane from urological jelly to tube lining

Attenuated total internal reflection (ATR) has been established as a useful analytical tool. Briefly, the principles of ATR are as follows. When a beam of radiation enters a prism, it is reflected internally if the angle of incidence at the interface between the sample and prism is greater than the critical angle (Fig. 1). The internally reflected beam appears to penetrate slightly beyond the reflecting surface. If a sample that absorbs IR radiation is placed against the reflecting surface, the beam loses energy at those wavelengths where the sample absorbs.

A plot of intensity of reflected radiation as a function of wavelength resembles the absorption spectrum obtained by the transmission method. The apparent depth to which the radiation penetrates the sample is limited to a few microns and is dependent on the ratio of refractive indexes and the angle of incidence. Consequently, this technique has made possible the applications of IR spectroscopy in areas where it was not previously possible, such as with polymers, resins, leather, rubber, thin coatings, condensed GC fractions, fibers, and fabrics (1–3). Authentication of delicate objects like postage stamps have been carried out using ATR (4, 5); induced changes in biological tissues can also be detected (6). In this study, its usefulness in demonstrating the migration of (−)-2,2-diphenyl-4-(2-piperidyl)-1,3-dioxalane (CL-912) from a jelly to the lining of collapsible tubes is discussed.



CL-912

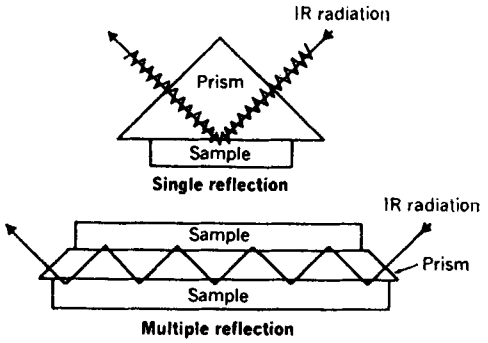


Figure 1—*Schematic diagram of single and multiple reflections.*

A prior-to-fill assay on urological jelly containing (−)-2,2-diphenyl-4-(2-piperidyl)-1,3-dioxolane hydrochloride (CL-912C)[1] indicated that the calculated amount of CL-912C was present in the formulation, but the assay immediately after sterilization of samples showed an 8–10% loss of the drug (7). Either the drug was degraded during the sterilization cycle or was bound to the liner. Hydrolysis of CL-912 gives benzophenone and 2-piperidyl-1,2-ethanediol, but no appreciable quantities of these were found in the jelly (7). The present studies were undertaken to determine if intact CL-912C, its free base (CL-912), or the degradation products are adsorbed on the linear surface.

EXPERIMENTAL

Materials—In all experiments, CL-912C[2] was used. The free base (CL-912) was prepared by dissolving the hydrochloride in a quantity of distilled water, liberating it by addition of 5 *N* sodium hydroxide, and extracting with isooctane. The solvent was removed under reduced pressure.

[1] Levoxadrol hydrochloride, Cutter Laboratories, Berkeley, CA 94710
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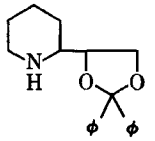
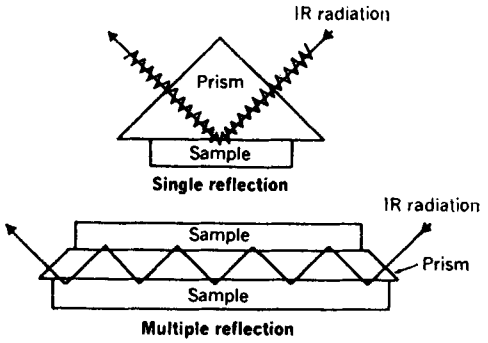


CL-912



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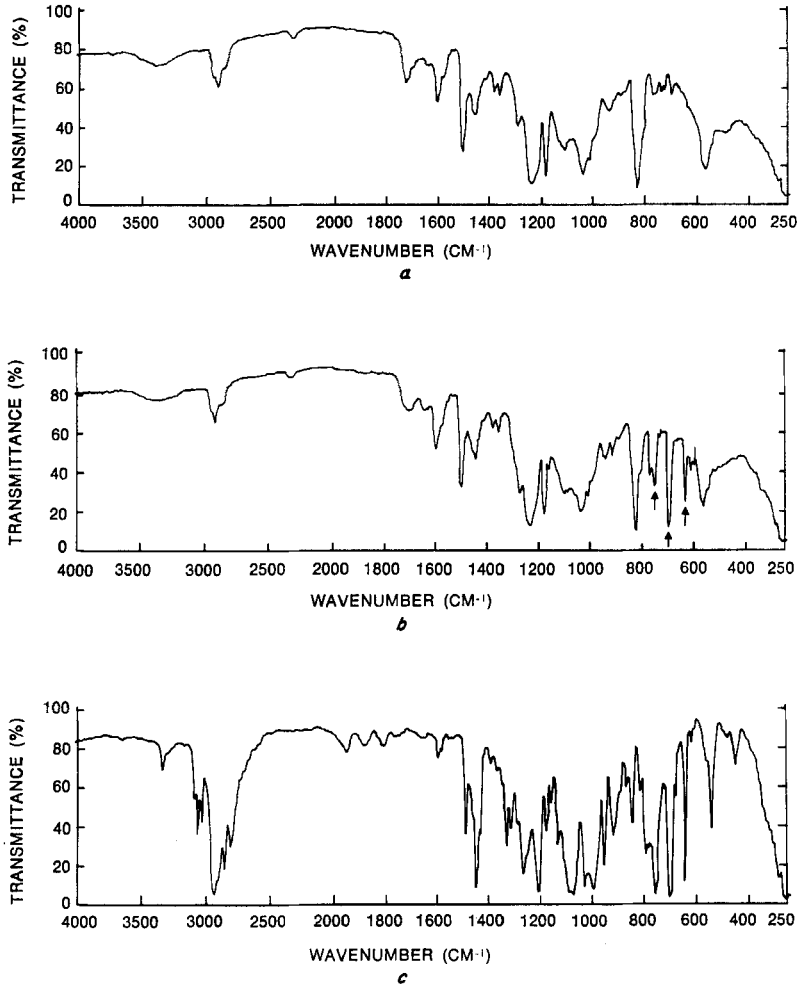

Figure 2—*ATR spectra of reference liner* (a), *sample liner* (b), *and CL-912* (c). *Arrows in Fig. 2b show major changes in the spectrum of sample liner due to migration of CL-912.*

A urological jelly containing 0.25% CL-912C in a water-soluble base of hydroxyethylcellulose[3] (1.85%), polyethylene glycol 400 (10.0%), sodium succinate (0.27%), and methylparaben (0.015%) was used. The jelly was filled in collapsible tin tubes lined with phenol–formaldehyde–epoxy resin and steam sterilized.

TLC was carried out on precoated silica gel plates without fluorescent indicator[4] using hexane. Iodine vapors were used to locate the spots.

Equipment—ATR spectra were recorded on a Perkin-Elmer IR grating spectrophotometer, model 457, using the Wilk's ATR accessory model 50 with a 45°-2 mm. KRS-5 plate and 45° angle of incidence.

Procedure—Empty tubes were used to obtain the spectrum of the unused liner (reference liner). Tubes containing CL-912C jelly were emptied, flushed well, and washed with distilled water. The tubes were cut open with scissors and blotted dry, and an ATR spectrum of the liner surface was obtained (sample liner).

RESULTS

Comparison of the ATR spectra of reference and sample liners reveals an overall similarity, except in the 800–600-cm.$^{-1}$ region. The reference liner (Fig. 2*a*) shows weak bands in this region (765, 755, 725, 698, and 638 cm.$^{-1}$), whereas sample liners (Fig. 2*b*) show intense bands at 770, 755, 700, and 638 cm.$^{-1}$. In addition, change in contours of the bands at 1458, 1235, 1038, and 935 cm.$^{-1}$ are notable. An examination of an ATR spectrum of CL-912 (Fig. 2*c*) indicates that these differences are attributable to the adsorption of free base on the liner. In addition, isooctane extraction of the sample liners and removal of solvent afford an oil identified as CL-912 by comparing its ATR spectrum and TLC (single spot) with an authentic sample. Migration of CL-912 can be rationalized as follows. The jelly contains CL-912C, a salt of a weak base and strong acid in an aqueous buffered medium of pH 6.4. The pH of buffers can change markedly owing to significant changes in K_w with temperature. Thus, the salt is subject to hydrolysis and capable of giving rise to the free base CL-912, which is preferentially adsorbed on the liner.

[3] Natrosol 250H, Hercules Powder Co., Wilmington, DE 19800
[4] Merck, Darmstadt, West Germany.

SUMMARY

A comparison of ATR spectra of reference liner, sample liners, and CL-912 revealed the migration of free base to the liner. Awareness of such migration could lead to a more rational and scientific approach to selection of liners for collapsible tubes.

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New Compounds: Preparation of Oxime Esters from 1-Azabicyclo[4.4.0]decane-4-one

R. V. SAENZ and R. A. CROCHET*

Abstract □ The synthesis of two new oxime esters is described. Preliminary results of pharmacological tests are also reported.

Keyphrases □ Oxime esters—synthesis from 1-azabicyclo[4.4.0]-decane-4-one, pharmacological evaluation as anticholinergic agents □ Anticholinergic agents, potential—synthesis, pharmacological evaluation of oxime esters from 1-azabicyclo[4.4.0]decane-4-one

Many of the presently available anticholinergic compounds possess an esteratic function. Recently, it was shown that the esteratic function is not absolutely essential (1). Numerous modifications have been studied, and recent reports indicated that esters derived from appropriate oximes possess anticholinergic activity (2). A greater activity in the tropane derivatives was reported relative to 1-methyl-4-piperidine. This was explained partially on the basis of a greater rigidity of the tropane system.

It was also reported (3–5) that the esters of amino-alcohols with a bridgehead nitrogen possess anticholinergic activity. It appeared that oximino esters derived from bridgehead nitrogen compounds might have some merit. 1-Azabicyclo[4.4.0]decane-4-one was prepared and derivatized to two oxime esters, 4-(benzoyloximino)-1-azabicyclo[4.4.0]decane and 4-(xanthene-9-acyloximino)-1-azabicyclo[4.4.0]decane. These oximino esters should possess the rigidity of a bicyclic system as well as characteristics of oximino esters which may be responsible for the activity.

Preliminary pharmacological testing was performed on isolated rabbit ileum.

EXPERIMENTAL

1-Azabicyclo[4.4.0]decane-4-one—The synthetic procedure employed was described by Rhodes and Soine (3). The initial synthesis commenced with bromobenzene. During the course of the work, the intermediate ethyl(2-pyridyl) acetate became commercially available; subsequent syntheses began with this intermediate.

1-Azabicyclo[4.4.0]decane-4-oxime—The oxime was prepared by the method described by Vogel (6). Characteristic IR bands for the oxime and carbon–hydrogen analytical data substantiated the structure: IR bands at 6.02 and 6.22 μ; m.p. 162–163°.

4-(Benzoyloximino)-1-azabicyclo[4.4.0]decane—This compound was prepared from 1-azabicyclo[4.4.0]decane-4-oxime (0.84 g., 0.005 mole) and benzoyl chloride (0.70 g., 0.005 mole) in anhydrous benzene. The reaction period was 48 hr., at which time the benzene was removed. Upon addition of a 3% solution of sodium bicarbonate, an oily layer separated. The oily layer was extracted into benzene and dried. The desired compound was identified as the methyl iodide: IR bands at 5.70–5.85 and 6.0–6.2 μ.

4-(Xanthene-9-acyloximino)-1-azabicyclo[4.4.0]decane—This compound was prepared using 1-azabicyclo[4.4.0]decane-4-oxime (0.84 g., 0.005 mole) and xanthene-9-acyl chloride (12 g., 0.005 mole) in anhydrous benzene. The conditions for isolation were analogous to the benzoate. The desired product was identified as the methyl iodide salt: IR bands at 5.70–5.85 and 6.0–6.2 μ.

Table II—Relative Potencies of Esters Derived from 1-Azabicyclo[4.4.0]decane-4-oxime

Compound	Effect	Relative Potency Compared with Atropine Sulfate
Benzoate	Cholinomimetic	—
Xanthene-9-acyl	Anticholinergic	1/46

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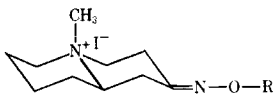
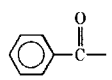
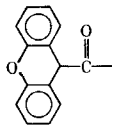
(3) H. J. Rhodes and T. O. Soine, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 746(1956).

Table I—Physical Constants and Analytical Data



R	Recrystallized from	Melting Point[a]	Empirical Formula	Analysis, %[b] Calc.	Found
	Ethanol–isopropyl ether	152.5–153°	$C_{17}H_{23}IN_2O_2$	C, 48.93 H, 5.56	C, 49.12 H, 6.20
	Isopropyl alcohol Isopropyl ether	171.5–172°	$C_{24}H_{27}IN_2O_3$	C, 55.60 H, 5.25	C, 55.48 H, 5.42

[a] All melting points were taken on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. [b] Carbon–hydrogen analyses were conducted by Schwarzkopf Microanalytical Laboratory, Woodside, NY 11377

(4) R. E. Counsell and T. O. Soine, *ibid.*, **49**, 289(1960).

(5) L. H. Sternbach and S. Kaisen, *J. Amer. Chem. Soc.*, **74**, 2219 (1952).

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COMMUNICATIONS

NMR Evidence for Self-Association of Theophylline in Aqueous Solution

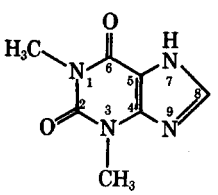
Keyphrases ☐ Theophylline, association in aqueous solution—determination, NMR spectroscopy ☐ Dimers, theophylline—in aqueous solution, NMR spectroscopy ☐ NMR spectroscopy—determination, theophylline self-association ☐ Association, theophylline—aqueous solution

Sir:

In 1957, Guttman and Higuchi (1) observed that the partition coefficient of theophylline between water and an organic phase remained unaltered (at ~23) over a concentration range (in the aqueous phase) of 2.3–28 × 10^{-3} *M*. This observation and the assumption that theophylline would exist only as the monomer in the nonaqueous phase (chloroform, 90%; isooctane, 10%) led them to conclude that: "for all practical purposes the partition coefficient of theophylline remains a constant over the concentration range studied, indicating the absence of association tendencies." In this respect, theophylline was unique; all the other xanthines examined, including several theophylline derivatives, were found to associate. The idea that theophylline does not self-associate in aqueous solution appears to have been generally accepted (2–5).

We present NMR spectroscopic evidence showing that theophylline does, in fact, associate in aqueous solution. This communication is part of a larger NMR investigation of the various association properties of xanthines.

The high-resolution 100-MHz. NMR spectrum of a 0.004 *M* D_2O solution of theophylline (I) has three



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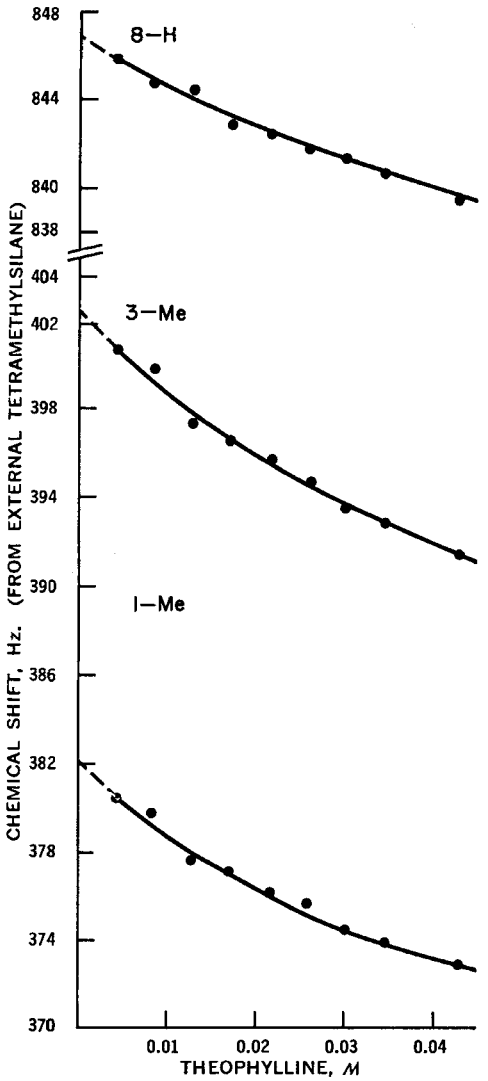

Figure 1—*Concentration dependence of theophylline proton chemical shifts at 30° in D_2O.*

signals of relative intensities, 1:3:3, at about 846, 401, and 380 Hz. (from external tetramethylsilane), corresponding to the proton at C-8 and to the 3- and 1-

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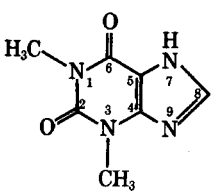

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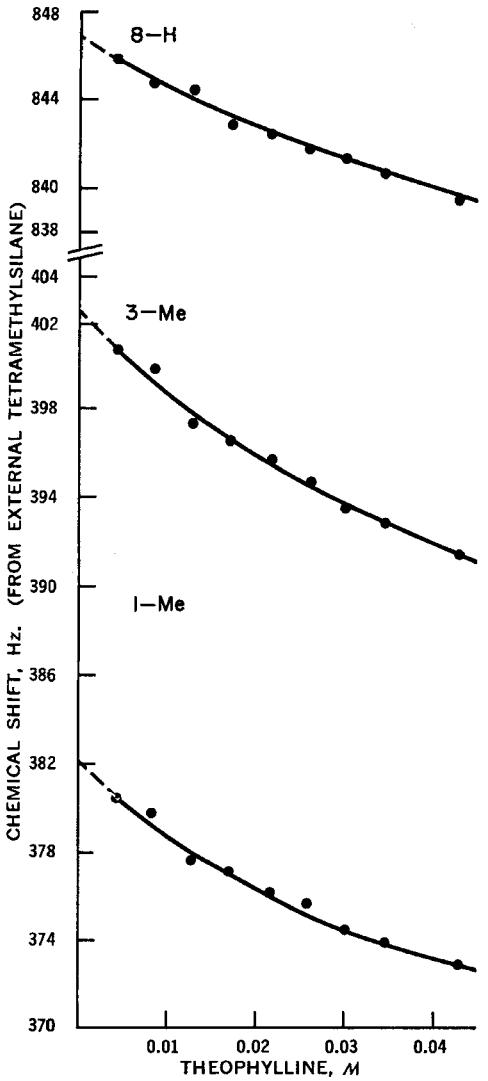

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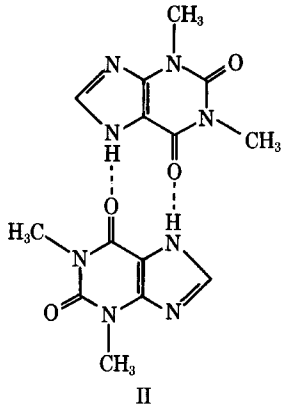

II

methyl protons, respectively. Figure 1 shows the concentration dependence of chemical shifts. All the signals move significantly upfield with an increase in concentration. This is in contrast to the unchanging chemical shift to be expected in nonassociating systems. It is well established that upfield changes in chemical shift, as observed here, indicate plane to plane or vertical stacking (6). We have observed a similar chemical shift dependence for caffeine (7). Previous studies, using methods other than NMR, unequivocally showed that caffeine self-associates (1, 8, 9).

For a dimerization process, it can be shown that the observed chemical shift, $\delta_{obsd.}$, is:

$$\delta_{obsd} = \frac{1 + 4MK - (1 + 8MK)^{1/2}}{4MK} \cdot (\delta_D - \delta_M) + \delta_M \quad \text{(Eq. 1)}$$

where M is the molar concentration of theophylline in D_2O; K is the dimerization constant in liter · mole^{-1}; and δ_M and δ_D are chemical shifts of the monomer and dimer, respectively. Because δ_M and δ_D cannot be measured directly, the best values for K, δ_M, and δ_D were obtained simultaneously by fitting these parameters to the experimental data[1]. The computed values of K and the dimer shift, $\delta_D - \delta_M$, are listed in Table I.

The values of K and the dimer shifts calculated by the three methods show excellent agreement for a given proton group. The best value of K, 6.0 M^{-1}, for theophylline can be compared with that for caffeine, 8.6 M^{-1}, also obtained by NMR (7). Both these values are smaller than the values of 11.7 M^{-1} at 30° (1) and 19 M^{-1} at 25° (2) obtained for caffeine by partition methods. The lower value of the dimerization constant for theophylline compared to that for caffeine can be readily understood, considering that the theophylline molecule has one methyl group less than does caffeine. The free proton at N-7 in theophylline provides one more site for hydration. Theophylline is, therefore, less hydrophobic than caffeine and self-associates to a lesser degree in water. A parallel situation is reported by Chan *et al.* (11) who found that the dimerization constant of purine, 2.1 M^{-1}, is smaller than that of 6-methylpurine, 6.7 M^{-1}.

[1] Three computer programs, based upon different methods of minimizing error functions, were used. Two of the programs minimize different error functions and calculate all three parameters. A third program, due to Bangerter and Chan (10), requires a predetermined value of δ_M, which can be obtained from the other two programs or by visual extrapolation. Mathematical details of these methods will be presented in a subsequent paper.

Table I—Dimer Shifts and Dimerization Constant for Theophylline in D_2O at 30°

Protons	$\delta_D - \delta_M$, Hz. Method I	Method II	Method III	K, M^{-1} Method I	Method II	Method III
1-Me	34.6	34.5	34.6	5.9	5.9	5.9
3-Me	41.8	41.8	42.0	6.0	6.0	6.0
8-H	35.8	35.7	38.3	4.0	3.9	3.4

As suggested here, the upfield dimer shifts indicate vertical stacking similar to that found for caffeine (7) and for various structurally analogous purines (6, 11, 12). The experimental errors to which the values in Table I are subject and their confidence limits will be discussed in a subsequent communication. Suffice it to say here that the 8-H signal is quite broad in the very dilute solutions used in this study and, therefore, the K and dimer shifts derived from the observed chemical shifts are considerably less precise than those for the methyl signals.

It seems worthwhile to consider the reasons why partition studies (1) did not reveal the self-association of theophylline. If theophylline were to associate to the same extent in the nonaqueous phase as in the aqueous phase over the limited concentration range examined, then no detectable change in the partition coefficient would occur. Several purine derivatives with a free proton at N-7, as in theophylline, were shown (13) by NMR and IR studies to self-associate by hydrogen bonding in nonaqueous solvents such as chloroform. In the partition studies, the assumption was made that xanthines exist only in the unassociated monomeric form in nonaqueous solutions, and it was tested only for caffeine. This assumption probably is valid for all the xanthines with alkyl substituents at N-7 but not for theophylline. One possible way in which the free proton at N-7 in theophylline could act as a hydrogen-bond donor in intermolecular hydrogen-bond formation is shown in II.

In the crystalline state, theophylline occurs in such a hydrogen-bonded form (14) while, of course, caffeine does not (15). The stronger intermolecular bonding of theophylline in the crystalline state as compared with caffeine is reflected in the higher melting point of theophylline and its more limited solubility in water (1). We have attempted, using both IR and NMR techniques, to observe spectral changes due to hydrogen bonding of theophylline in nonaqueous solvents. The very low solubility of theophylline, however, presents practical problems; as yet, we have not obtained conclusive data.

In summary, NMR data provide evidence that theophylline self-associates to a significant degree in aqueous solution, as do caffeine and other xanthines. The self-associated complexes of theophylline and caffeine in aqueous solution are alike in configuration and have similar association constants.

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(15) *Ibid.*, **11**, 453(1958).

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Note Added in Proof: While this paper was being considered for publication, S. Ng [*Mol. Pharmacol.*, **7**, 177(1971)] presented IR evidence that theophylline does indeed self-associate in nonaqueous (chloroform) solution. The association is quite strong—even stronger than the association in water—the dimerization constant being 313 M^{-1} at 25°. The structure of the hydrogen-bonded dimer given by Ng is essentially identical to that postulated by us (II). Ng's data confirm our conclusion that our NMR data, when examined with the partition coefficient data of Guttman and Higuchi (*1*), point to association of theophylline in the nonaqueous phase.

NMR Evidence for Self-Association of Theophylline in Aqueous Solution: A Response

Keyphrases ☐ Theophylline, association in aqueous solution—nonquantitation ☐ Association, theophylline—aqueous solution

Sir:

In all probability, theophylline does dimerize or associate in some fashion in aqueous solution, as suggested by Thakkar *et al.* (1). A total lack of associative tendency is quite unlikely, since it was shown conclusively that closely related derivatives of theophylline readily form not only dimers but also more highly associated species (2). This is evident in Fig. 1, reproduced from our earlier partitioning studies, the increase in distribution coefficients toward water being attributed to formation of multimolecular species in the aqueous phase.

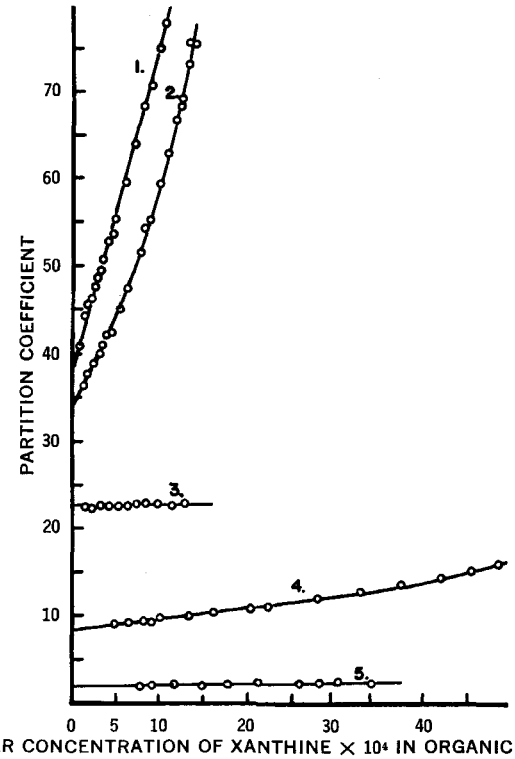

Figure 1—*A plot of the partition coefficients of a number of xanthines between water and an organic solvent at 30°. The organic solvent used for all of the studies except theophylline was isooctane. In the case of theophylline, chloroform–isooctane (90:10) was used. Key: 1, ethyltheobromine; 2, 7-ethyltheophylline; 3, theophylline; 4, 7-propyltheophylline; and 5, butyltheophylline.*

There are two reasons why the associative tendency of theophylline can usually be neglected. As is evident from the NMR data of Thakkar *et al.* (1), even if all of the observed upfield shift is ascribed to dimerization, their calculated dimerization constant (3.4–6.0 l./mole at 30°) is significantly smaller than that for caffeine (12 l./mole). The other reason is that the solubility of theophylline in water is relatively low and does not permit concentration levels conducive to high degrees of association.

If we accept the NMR values, we can calculate the ratio of dimer to monomer for the average concentration of theophylline used in the study shown in Fig. 1. This ratio comes to 1 in 14 for K_D = 6.0 l./mole or 1 in 23 for the lower K_D = 3.4 l./mole. These values contrast with the associative tendency of caffeine, where more than two-thirds of the total caffeine is present by weight in associated forms at half-saturation in water at 30°. Such small amounts of associated species appear to be nearly undetectable and within experimental error.

Furthermore, in the NMR study, neither δ_M nor δ_D is obtained directly but is estimated along with K by simultaneous best fit to the experimental data; therefore, any slight perturbation arising from formation of higher species would produce an out of proportion error in these results. Although the exact methods of mathematical analysis employed are not provided, it would appear that contributions from tetramer formation would tend to yield too low δ_D values and, hence, falsely high dimer constants.

(5) J. L. Cohen and K. A. Connors, *ibid.*, **34**, 197(1970).
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Note Added in Proof: While this paper was being considered for publication, S. Ng [*Mol. Pharmacol.*, **7**, 177(1971)] presented IR evidence that theophylline does indeed self-associate in nonaqueous (chloroform) solution. The association is quite strong—even stronger than the association in water—the dimerization constant being 313 M^{-1} at 25°. The structure of the hydrogen-bonded dimer given by Ng is essentially identical to that postulated by us (II). Ng's data confirm our conclusion that our NMR data, when examined with the partition coefficient data of Guttman and Higuchi (*1*), point to association of theophylline in the nonaqueous phase.

NMR Evidence for Self-Association of Theophylline in Aqueous Solution: A Response

Keyphrases ☐ Theophylline, association in aqueous solution—nonquantitation ☐ Association, theophylline—aqueous solution

Sir:

In all probability, theophylline does dimerize or associate in some fashion in aqueous solution, as suggested by Thakkar *et al.* (1). A total lack of associative tendency is quite unlikely, since it was shown conclusively that closely related derivatives of theophylline readily form not only dimers but also more highly associated species (2). This is evident in Fig. 1, reproduced from our earlier partitioning studies, the increase in distribution coefficients toward water being attributed to formation of multimolecular species in the aqueous phase.

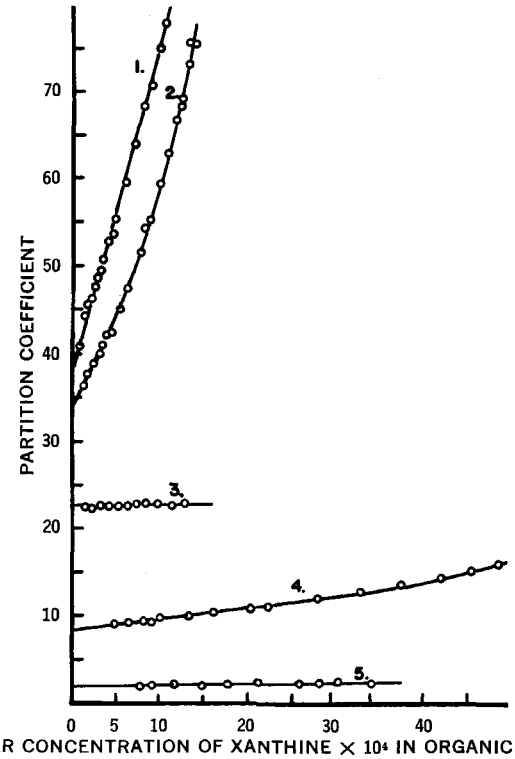

Figure 1—*A plot of the partition coefficients of a number of xanthines between water and an organic solvent at 30°. The organic solvent used for all of the studies except theophylline was isooctane. In the case of theophylline, chloroform–isooctane (90:10) was used. Key: 1, ethyltheobromine; 2, 7-ethyltheophylline; 3, theophylline; 4, 7-propyltheophylline; and 5, butyltheophylline.*

There are two reasons why the associative tendency of theophylline can usually be neglected. As is evident from the NMR data of Thakkar *et al.* (1), even if all of the observed upfield shift is ascribed to dimerization, their calculated dimerization constant (3.4–6.0 l./mole at 30°) is significantly smaller than that for caffeine (12 l./mole). The other reason is that the solubility of theophylline in water is relatively low and does not permit concentration levels conducive to high degrees of association.

If we accept the NMR values, we can calculate the ratio of dimer to monomer for the average concentration of theophylline used in the study shown in Fig. 1. This ratio comes to 1 in 14 for K_D = 6.0 l./mole or 1 in 23 for the lower K_D = 3.4 l./mole. These values contrast with the associative tendency of caffeine, where more than two-thirds of the total caffeine is present by weight in associated forms at half-saturation in water at 30°. Such small amounts of associated species appear to be nearly undetectable and within experimental error.

Furthermore, in the NMR study, neither δ_M nor δ_D is obtained directly but is estimated along with K by simultaneous best fit to the experimental data; therefore, any slight perturbation arising from formation of higher species would produce an out of proportion error in these results. Although the exact methods of mathematical analysis employed are not provided, it would appear that contributions from tetramer formation would tend to yield too low δ_D values and, hence, falsely high dimer constants.

In conclusion, we believe that: (*a*) the NMR data suggest that theophylline does associate to a limited degree in water but do not permit useful quantitative estimates; and (*b*) for the most practical range of concentrations, the associative tendency of theophylline still appears to be negligible.

(1) A. Thakkar, L. G. Tensmeyer, and W. L. Wilham, *J. Pharm. Sci.*, **60**, 1267(1971).

(2) D. Guttman and T. Higuchi, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 4(1957).

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Interference in GLC Determination of Iodoamino Acids in Hydrolysis Products of Thyroid Extracts

Keyphrases ☐ Iodoamino acids—GLC analysis, thyroid extracts ☐ Thyroid extracts—GLC analysis of iodoamino acids ☐ GLC—assay, iodoamino acids

Sir:

The quantitative determination of iodoamino acids in dried thyroid extract is a problem of current interest in many laboratories. The separation and quantitative determination of standard mixtures of the iodoamino acids by GLC were reported by many authors (1–8). However, the extension of these methods to the determination of the hydrolysis products of dried thyroid extract has not been reported.

To shed light on the problems encountered in this determination, we wish to make the following report. In our work, we noticed that our standard mixture of trimethylsilyl derivatives of iodoamino acids did not yield the usual peak areas when injected onto a column that had been used for repeated injections of derivatives of hydrolysis products of dried thyroid extract. However, on injection onto a second column used previously only in the temperature-programming sequence, a return to normal peak areas was obtained (Fig. 1).

Our GLC investigation followed the method of Hansen (7), modified by substituting 1% dimethyl silicone[1] for the stationary phase coated onto a support of acid–base washed, silane-treated diatomaceous earth[2], mesh size 80/100. Three-foot glass U-shaped columns were utilized in this part of the study. A standard derivative mixture was prepared by heating 1.017 mg. of liothyronine (T_3) and 1.510 mg. of thyroxine (T_4) in 100 μl. of anhydrous pyridine and 300 μl. of *N,O*-bis(trimethylsilyl)-acetamide[3] for 2 hr. at 50°.

The first column had some carbon present at the point of injection. To determine if the carbon was interfering with the complete elution of the iodoamino acids, three columns were prepared: one containing the normal

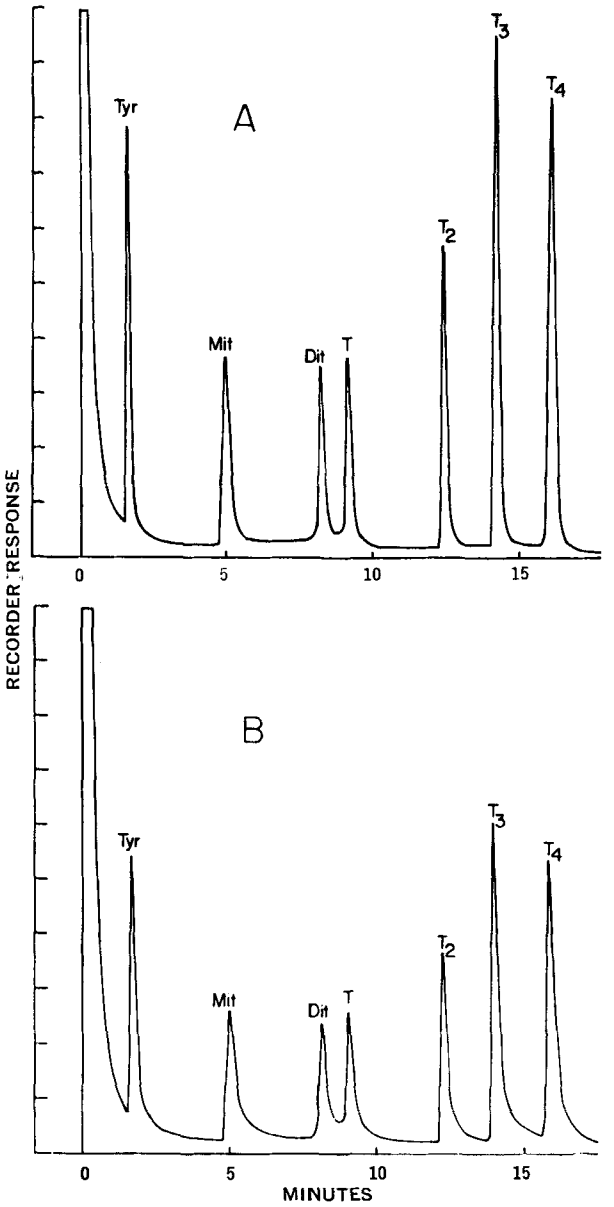

Figure 1—*GLC detection of a synthetic mixture of iodoamino acids on a new column (A) and a used column (B). Key: Tyr, tyrosine; Mit, monoiodotyrosine; Dit, diiodotyrosine; T, thyronine; T_2, diiodothyronine; T_3, liothyronine; and T_4, thyroxine. Chromatographic conditions were: temperature programmed at 165–285°, 3 min. isothermal followed by a programmed rate of 10°/min.; nitrogen carrier gas flow, 100 ml./min. at 165° (40 p.s.i.); injector temperature, 235°; detector temperature, 315°; attenuation, 9 × 10^{-10} amp.; and chart speed, 0.5 in./min.*

[1] OV-1, Applied Science Laboratories, Inc., State College, Pa.

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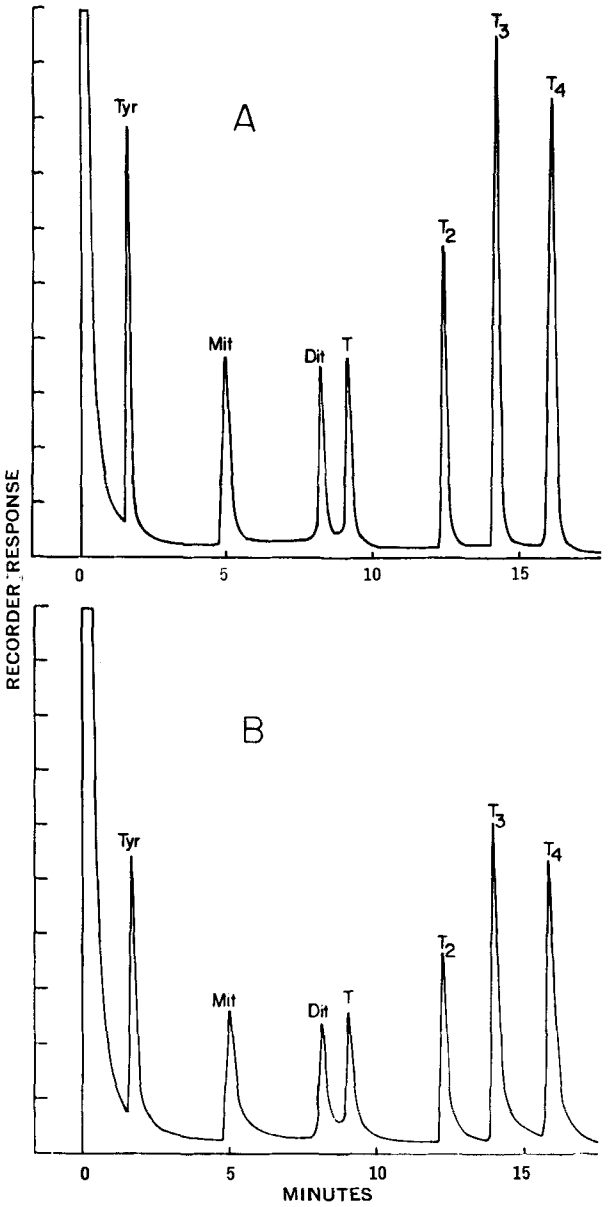

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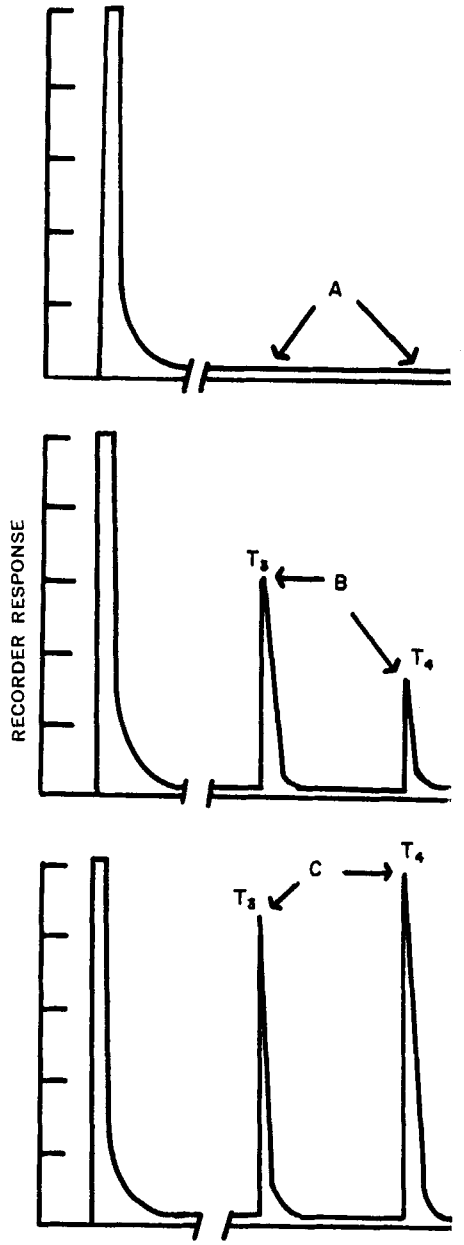

Figure 2—*Composite representation of recovery of a mixture of liothyronine (T_3) and thyroxine (T_4) from GLC columns containing 1.25 mg. (A), 0.125 mg. (B), or no (C) activated charcoal.*

support system previously described, a second with 1.25 mg. of activated charcoal[4] mixed with 50 mg. of this support and placed on top of the column on the injection site, and a third containing 0.125 mg. of activated charcoal mixed with 50 mg. of support and placed on top of the column. All columns were conditioned at 300° for at least 18 hr.

The peak heights obtained on injection of 4 μl. of the derivative mixture into the normal column 1, at an attenuation of 3×10^{-9} amp., were comparable to those expected from previous new columns. No response was observed with the column containing 1.25 mg. of activated charcoal (Fig. 2).

These results appear to substantiate the reasoning that this amount of carbon on the column significantly reduces the ability to detect the iodoamino acids. It was determined that each injection of 3 μl. of hydrolyzed sample deposited at least 1.5 mg. of material on the column. After several such injections, carbon deposits could reach the levels used in the trials. Thus, it may be reasonable to assume that the presence of nonvolatile materials in the protein hydrolysates causes charring on the GLC column and could account for the incomplete recovery of iodoamino acids. This observation may be of some benefit to laboratories involved in this work.

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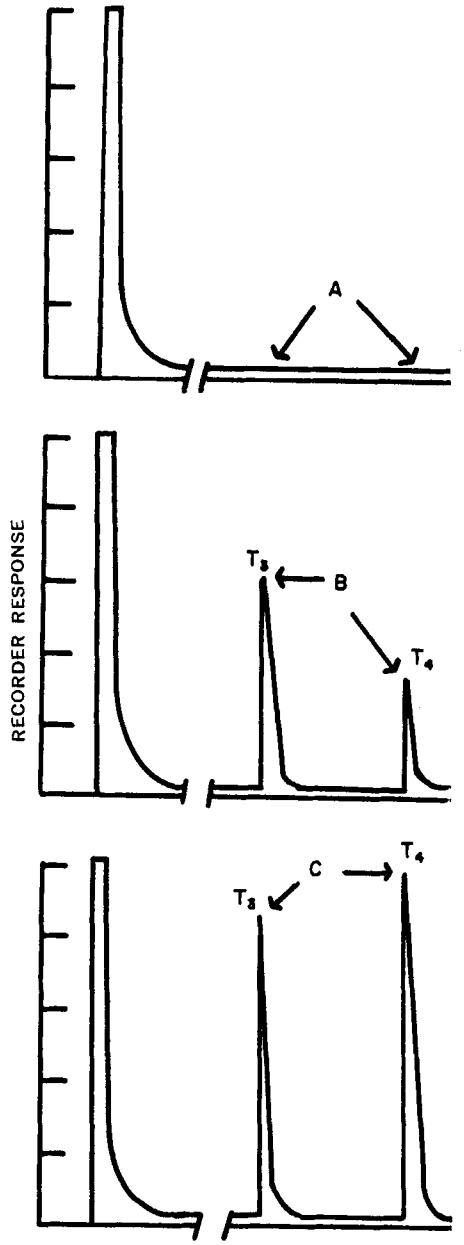
Assay of Warfarin

Keyphrases ☐ Warfarin—plasma levels, TLC and spectrophotometric assays ☐ Plasma levels—warfarin, assay ☐ TLC—assay, warfarin

Sir:

For the benefit of other investigators interested in the determination of the concentration of warfarin in plasma or stool samples, some comments are in order regarding the work of Welling *et al.* (1). Two of their findings are at variance with our observations.

First, these investigators were unable to detect the presence of metabolites of warfarin in the plasma. We have no experience with the TLC procedure described by Welling *et al.* (1). This procedure utilizes quenching, rather than fluorescence, as the marker for chromatographic loci of interest. In their hands, the same quenching loci were observed in blank plasma and in plasma from subjects receiving warfarin. Our work has been based on a procedure that was outlined in abstract form (2) and subsequently reported in detail (3). After TLC of several hundred extracts of plasma standards (plasma to which warfarin was added), we never observed loci with blue fluorescence with R_f values corresponding to warfarin metabolites. On occasion, with any plasma samples, a clump of fluorescent material runs at the



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Second, these authors (1) contend that an assay of warfarin based on chromatographic isolation of unchanged drug offers no advantage over methods relying solely on differential extraction. The interested reader should make reference to our formal report on the assay of warfarin (3). The most pertinent aspects of this paper noted consistent (five of five subjects) differences when data from a fluorimetric assay (6) or a spectrophotometric assay (7) were used to determine apparent plasma clearance rates. When the spectrophotometric method was compared to the TLC method as we described it, the former indicated slower clearance rates in two of four subjects: $T_{1/2}$ of 57.5 hr. *versus* 43.1 hr. and $T_{1/2}$ of 36.2 hr. *versus* 30.5 hr. were determined by the least-squares best fit line relating log warfarin concentration to time.

Since we have no experience with the TLC assay of Welling *et al.* (1), it is impossible to assess adequately the possible sources of error. In common with these investigators, we noted that outdated blood bank plasma gives higher blank values in the spectrophotometric assay than does freshly drawn citrated plasma. For this reason, we prepare all plasma ourselves. The high blank values may have contributed to the discrepancy in their results. In Table I of their report (1), the slope of the line relating net absorbance to warfarin concentration is 0.182. Blank plasma in their hands resulted in a net absorbance of 0.216 ± 0.056 or 0.261 ± 0.024, the equivalent of 1.2 ± 0.3 or 1.4 ± 0.1 mcg. "warfarin"/ml. plasma. Since warfarin has an apparent volume of distribution of 8–9 l. calculations from the 25-mg. dose would indicate that the maximum plasma concentration of their samples would be about 3 mcg./ml. at T_0. Subsequent values would be much less. Thus, these investigators were working at plasma levels at most only 2–3 times their blank. In our studies, we employed a dose of 1.5 mg. warfarin/kg. body weight, which produced T_0 concentrations of about 12 mcg./ml. Our values for blank plasma were the equivalent of 0.15 mcg./ml. plasma.

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Assay of Warfarin: A Rebuttal

Keyphrases ☐ Warfarin—small, single oral dosage plasma levels, TLC and spectrophotometric assays ☐ Plasma levels—warfarin, small oral dosage ☐ TLC—assay, warfarin

Sir:

Dr. Lewis' communication (1) refers to the warfarin report of Welling *et al.* (2) and his own published reports (3, 4). Concerning the application of our methods, Dr. Lewis and I are not nearly so far apart as his communication tends to indicate. We clearly indicated that our results were obtained following oral administration of 10- and 25-mg. single doses of sodium warfarin. We clearly stated that our results may not apply after administration of large single doses nor after multiple doses of sodium warfarin. However, under the conditions of our studies, metabolites of warfarin were not present in detectable amounts in plasma. Furthermore, the modified O'Reilly assay and TLC did give equivalent results, as shown in Fig. 1 of our paper (2). I have no doubt that following large single doses, such as 1.5 mg./kg. as administered by Lewis and Trager (5), or when assaying plasma samples obtained after multiple doses of warfarin, warfarin metabolites are detectable as shown by Lewis *et al.* (3, 4).

The TLC procedure that we used was almost identical to the method used by Lewis *et al.* (3, 4). The adsorbent in both cases was silica gel, but they used Eastman No. 6061 silica gel G TLC sheets, while we used our own 20 × 20-cm. plates with a 250-μ thickness of silica gel GF 254. The solvent was 9:1 (v/v) 1,2-dichloroethene–acetone in both cases. Our silica gel contained a fluorescent indicator, while theirs did not. However, both we and they reported essentially the same R_f value (0.50–0.54) for unchanged warfarin. We reported clean separations of the 4′-OH, 6-OH, and 7-OH metabolites of warfarin, with R_f values of 0.22, 0.18, and 0.23, respectively. Lewis and Ilnicki (3), following an unspecified dose of warfarin, reported separation of two metabolites from plasma (R_f 0.23 and 0.19). Lewis *et al.* (4) displayed a photograph showing separation of a mixture of diasterisomeric warfarin alcohols from warfarin on their plates. The evidence suggests strongly that after 10- and 25-mg. doses of warfarin, metabolites are not detectable in plasma as we concluded, whereas after higher doses the metabolites are obviously detectable as Lewis *et al.* (4) showed. The discrepancy appears not to lie in any basic difference in the TLC procedures employed but rather in the doses administered. The reason why our blank plasma samples gave spots on the thin-layer plates, whereas Lewis *et al.* (4) never observed such spots, is unknown. The difference may or may not be due to the fluorescent indicator.

Two other comments made by Lewis (1) deserve special attention. Lewis stated: "When the spectrophotometric method was compared to the TLC method as we described it, the former indicated slower clearance rates in two of four subjects: $T_{1/2}$ of 57.5 hr. *versus* 43.1 hr. and $T_{1/2}$ of 36.2 hr. *versus* 30.5 hr. were determined

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The TLC procedure that we used was almost identical to the method used by Lewis *et al.* (3, 4). The adsorbent in both cases was silica gel, but they used Eastman No. 6061 silica gel G TLC sheets, while we used our own 20 × 20-cm. plates with a 250-μ thickness of silica gel GF 254. The solvent was 9:1 (v/v) 1,2-dichloroethene–acetone in both cases. Our silica gel contained a fluorescent indicator, while theirs did not. However, both we and they reported essentially the same R_f value (0.50–0.54) for unchanged warfarin. We reported clean separations of the 4′-OH, 6-OH, and 7-OH metabolites of warfarin, with R_f values of 0.22, 0.18, and 0.23, respectively. Lewis and Ilnicki (3), following an unspecified dose of warfarin, reported separation of two metabolites from plasma (R_f 0.23 and 0.19). Lewis *et al.* (4) displayed a photograph showing separation of a mixture of diasterisomeric warfarin alcohols from warfarin on their plates. The evidence suggests strongly that after 10- and 25-mg. doses of warfarin, metabolites are not detectable in plasma as we concluded, whereas after higher doses the metabolites are obviously detectable as Lewis *et al.* (4) showed. The discrepancy appears not to lie in any basic difference in the TLC procedures employed but rather in the doses administered. The reason why our blank plasma samples gave spots on the thin-layer plates, whereas Lewis *et al.* (4) never observed such spots, is unknown. The difference may or may not be due to the fluorescent indicator.

Two other comments made by Lewis (1) deserve special attention. Lewis stated: "When the spectrophotometric method was compared to the TLC method as we described it, the former indicated slower clearance rates in two of four subjects: $T_{1/2}$ of 57.5 hr. *versus* 43.1 hr. and $T_{1/2}$ of 36.2 hr. *versus* 30.5 hr. were determined

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Subject	Treatment A (5 5-mg. Tablets) TLC	O'Reilly	Normalized Difference, %[c]	Treatment B (1 25-mg. Tablet) TLC	O'Reilly	Normalized Difference, %
1	33.6	28.8	−15.4	35.1	34.3	−2.3
2	24.6	27.1	9.7	29.9	29.7	−0.7
3	34.8	47.2	30.2	42.7	55.0	25.2
4	26.8	33.7	22.8	29.5	26.2	−11.8
5	40.8	31.1	−27.0	41.4	56.9	31.5
6	—	—	—	33.7	34.5	2.3
6a	49.5	44.2	−11.3	—	—	—
Averages	35.0[d]	35.4[d]	1.5	35.4[e]	39.4[e]	7.4

	TLC	O'Reilly	Normalized Difference, %
Overall averages	35.2[f]	37.4[f]	4.5

[a] Half-lives estimated by obtaining the slope of line, by the method of least squares, when ln C_p plotted *versus* t and dividing the absolute value of the slope into 0.693. Only plasma concentrations corresponding to times equal to or greater than 24 hr. were employed. [b] Data plotted in Fig. 4 of Welling *et al.* (2). [c] Normalized difference = half-life from O'Reilly assays − half-life from TLC assays/average half-life from O'Reilly and TLC assays × 100. [d] Difference in averages is not significant by paired t-test ($t = 0.097$, $p > 0.25$). [e] Difference in averages is not significant by paired t-test ($t = 1.27$, $p > 0.10$). [f] Difference in averages is not significant by paired t-test ($t = 0.95$, $p > 0.25$).

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A key paper cited by Lewis (1) (his Reference 4) is still not published and has not been available to us.

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Kinetic Demonstration of a Metastable Intermediate in Isomerization of Penicillin to Penicillenic Acid in Aqueous Solution

Keyphrases □ Benzylpenicillin methyl ester, isomerization—metastable intermediate kinetics □ Penicillin to penicillenic acid isomerization—metastable intermediate demonstrated

Sir:

Although several kinetic stability studies on penicillins have been performed [*e.g.*, benzylpenicillin (1, 2), phenethicillin (3), methicillin (4), ampicillin (5), and cloxacillin (6)], only a few studies have dealt with the mechanism of the hydrolytic reactions. As a part of a study concerning chemical reactions possibly involved in penicillin allergy, this paper reports preliminary results about the mechanism by which benzylpenicillin methyl ester in aqueous solution isomerizes to methyl benzylpenicillenate.

On the basis of experimental data by Krejci (7), Schwartz (8) showed that the degradation of benzylpenicillin in acidic aqueous solution is characterized by two parallel reactions. The formation of penicillenic acid is thought to be a result of the hydrogen-ion-catalyzed hydrolysis of the penicillinate ion or the kinetically equivalent uncatalyzed rearrangement of undissociated penicillinic acid.

It has now been found that the formation of penicillenic acid from the penicillin molecule goes through a metastable intermediate and that both undissociated and dissociated penicillinic acid are isomerized but to a different extent. In this brief report, only the results obtained with benzylpenicillin methyl ester are presented;

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Keyphrases □ Benzylpenicillin methyl ester, isomerization—metastable intermediate kinetics □ Penicillin to penicillenic acid isomerization—metastable intermediate demonstrated

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On the basis of experimental data by Krejci (7), Schwartz (8) showed that the degradation of benzylpenicillin in acidic aqueous solution is characterized by two parallel reactions. The formation of penicillenic acid is thought to be a result of the hydrogen-ion-catalyzed hydrolysis of the penicillinate ion or the kinetically equivalent uncatalyzed rearrangement of undissociated penicillinic acid.

It has now been found that the formation of penicillenic acid from the penicillin molecule goes through a metastable intermediate and that both undissociated and dissociated penicillinic acid are isomerized but to a different extent. In this brief report, only the results obtained with benzylpenicillin methyl ester are presented;

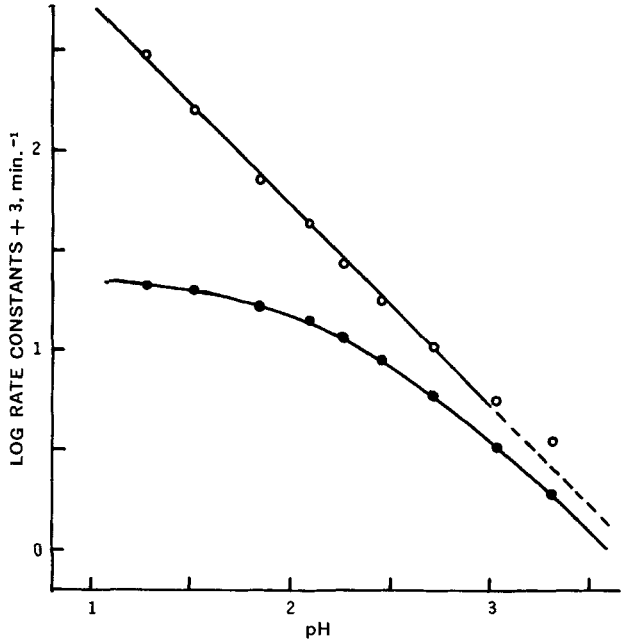


Figure 1—*pH–rate profile of the total degradation of benzylpenicillin methyl ester* (○) *and of the formation of methyl benzylpenicillenate* (●) *at 30°.*

as expected, however, benzylpenicillinic acid was found to give qualitatively similar results.

The hydrolysis of benzylpenicillin methyl ester was carried out at 30° in the pH range 1.28–3.33. The solutions were buffered with 0.02 *M* phosphate and adjusted to an ionic strength of 0.2 with KCl. It has been determined that there is no catalytic effect of the phosphate buffer in this concentration. Mercuric chloride was added to the buffers in a concentration five times as great as the initial benzylpenicillin methyl ester concentration ($\sim 5 \times 10^{-5}$ *M*) to stabilize the very labile methyl benzylpenicillenate (9). It was confirmed (10) that mercuric chloride in the given concentration has no effect upon the rate of methyl benzylpenicillenate formation or the rate of degradation of benzylpenicillin methyl ester. The rates of formation of methyl benzylpenicillenate from benzylpenicillin methyl ester were measured by following the increase in absorbance at 322 nm. as a function of time on a Zeiss PMQ II spectrophotometer, with thermostated cell compartment, connected with a Servogor recorder. Both the rate constants for total degradation of benzylpenicillin methyl ester (K) and for methyl benzylpenicillenate formation (k_{PNCE}) were obtained from the recorded curves by the Guggenheim method and were treated in the manner recently described by Niebergall and Sugita (11). The molar absorptivity of methyl benzylpenicillenate at 322 nm. is 26,600 (12).

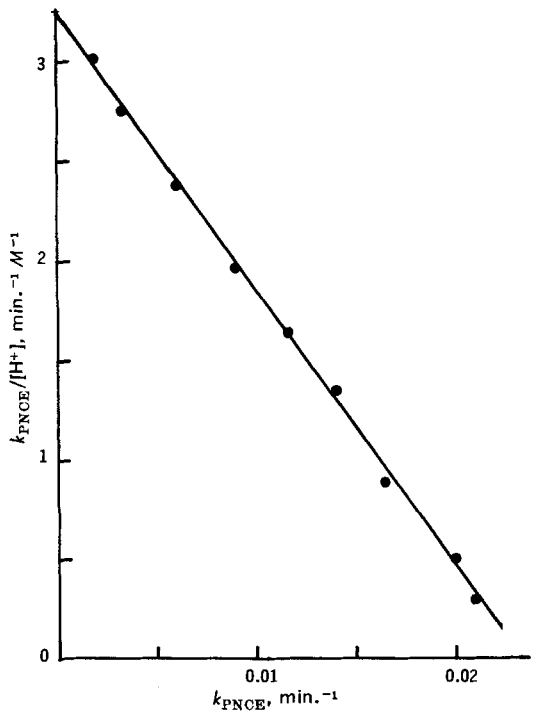


Figure 2—*Plot showing the fulfillment of Eq. 2.*

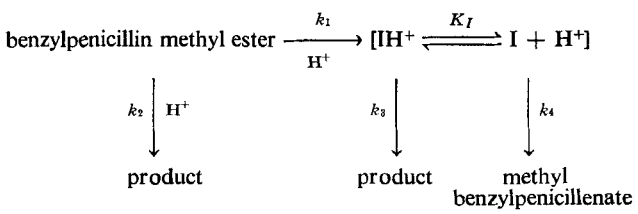
At constant pH, the hydrolysis could be described by pseudo-first-order kinetics. Figure 1 shows a plot of the logarithm of the observed pseudo-first-order rate constants K and k_{PNCE} *versus* pH; the hydrolysis of benzylpenicillin methyl ester at pH < 3 exclusively consists of a specific hydrogen-ion-catalyzed reaction (slope = −1). On the basis of the connection between pH and concentration of hydrogen ion (13) ($\log [H^+] = -pH + 0.12$), an average value of the specific hydrogen-ion-catalytic rate constant, K_{H^+}, was calculated to 4.01. mole^{-1} min.$^{-1}$. However, the value of k_{PNCE} does not increase in a linear fashion when pH is lowered, as anticipated for a reaction undergoing hydrogen-ion catalysis. Instead, as the value of hydrogen-ion concentration increases, the value of k_{PNCE} first increases rapidly and then slowly, becoming an independent function of hydrogen-ion concentration at the higher acid concentrations. Therefore, as the acidity increases, the rate-determining step in the formation of methyl benzylpenicillenate changes from one strongly dependent on acidity to one that is independent. The data were found to fit a curve of the form of Eq. 1:

$$k_{PNCE} = \frac{a \cdot [H^+]}{b + [H^+]} \qquad \text{(Eq. 1)}$$

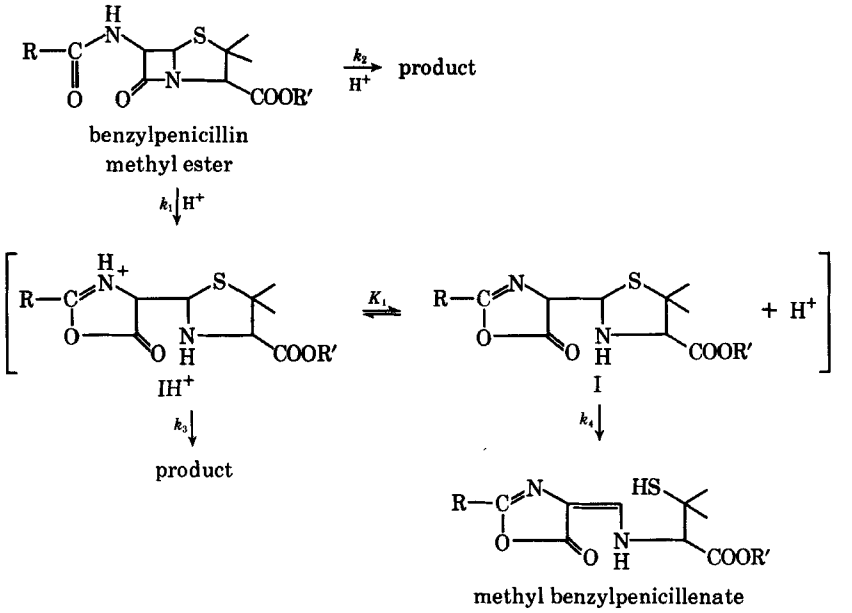
where a and b are constants. Rearrangement of Eq. 1 leads to Eq. 2:

$$\frac{k_{PNCE}}{[H^+]} = -\frac{1}{b} \cdot k_{PNCE} + \frac{a}{b} \qquad \text{(Eq. 2)}$$

As seen in Fig. 2, a plot of $k_{PNCE}/[H^+]$ *versus* k_{PNCE} is linear. From the slope and intercept, the constants a and b were derived: $a = 0.0234$, $b = 0.0072$.



Scheme I



Scheme II—R = $C_6H_5CH_2$—, R′ = CH_3—

The most reasonable mechanism which fits the determined kinetics involves metastable intermediates that are in acid–base equilibrium. Such a mechanism is shown in Scheme I. The IH^+ and I represent intermediates formed from benzylpenicillin methyl ester by a specific hydrogen-ion-catalyzed reaction, K_I represents the dissociation constant of IH^+, and $K = k_1 + k_2$.

Assuming steady state in the metastable intermediates IH^+ and I, the following expression can be derived for the observed rate constant, k_{PNCE}:

$$k_{PNCE} = \frac{[(k_4 \cdot K_I \cdot k^1_{H^+})/k_3] \times [H^+]}{[(k_4 \cdot K_I)/k_3] + [H^+]} \qquad \text{(Eq. 3)}$$

where $k^1_{H^+} = k_1/[H^+]$, since benzylpenicillin methyl ester degrades by a specific hydrogen-ion-catalyzed reaction. This equation possesses the same mathematical form as Eq. 1, showing that the proposed mechanistic scheme fits the experimental data. A fact supporting the metastability of the intermediates is the lack of a lag period in the production of methyl benzylpenicillenate. The proposed mechanism implies that the rate constants k_3 and k_4 are of the same magnitude. If $k_3 = k_4$, the apparent dissociation constant K_I equals $b \sim K_I = 10^{-2.1}$. From Eqs. 2 and 3, the value of $k^1_{H^+}$ is obtained: $k^1_{H^+} = a/b = 3.25$ l. mole^{-1} min.$^{-1}$.

It is suggested that the intermediate is a compound of the oxazolone–thiazolidine structure (Scheme II). Some factors which support this suggestion are: (*a*) compounds of the oxazolone–thiazolidine structure are known to be so unstable that they are capable of only transitory existence (14); (*b*) the β-lactam structure of benzylpenicillin methyl ester is so closely related to the oxazolone–thiazolidine that interconversion is brought about by a simple proton transfer; (*c*) there is a close relation between the oxazolone–thiazolidine structure and methyl benzylpenicillenate; and (*d*) the basicity of the nitrogen atom in the oxazolone is of a size corresponding to a pKa value of about 2 (14). Recently, on theoretical grounds the presumption was made (15) that the oxazolone–thiazolidine structure ("pseudopenicillin") is a probable intermediate in the isomerization of penicillin to penicillenate.

The suggestion previously was made (16) that a reactive structure such as the oxazolone–thiazolidine—if it exists—should be a powerful sensitizer. Further studies are underway to evaluate the significance of the reported findings in the penicillin allergy together with an evaluation of the other degradation products.

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Possible Reaction Pathway for Acylation of Coenzyme A

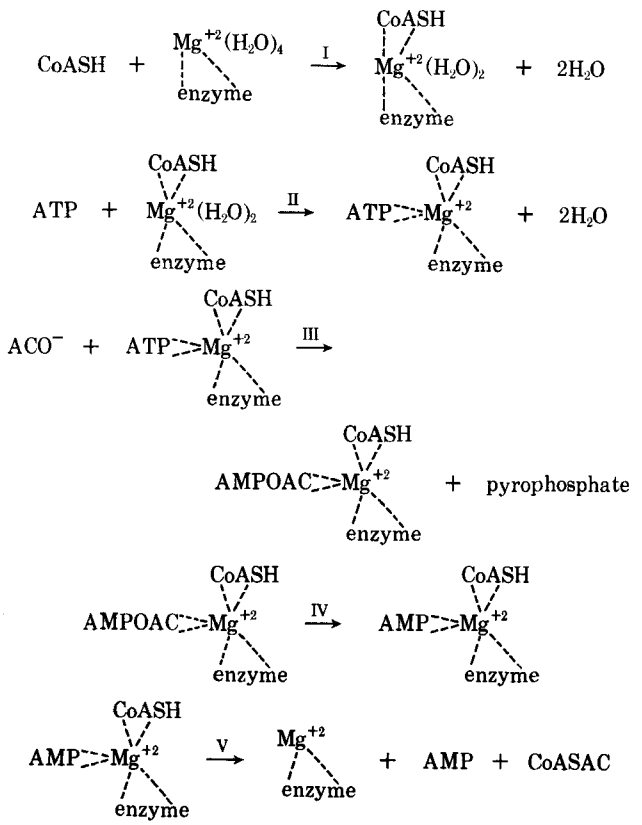
Keyphrases ☐ Coenzyme A, acylation—mechanism, anhydride intermediate ☐ Enzyme catalysis—formation of acyl coenzymes A ☐ Anhydride intermediates—coenzyme A acylation

Sir:

It is well known that acyl coenzymes A (CoA) such as acetyl, succinyl, and propionyl CoA act as catalysts in numerous enzymatic synthetic processes It is also known that the acylation process involves the sulfhydryl group of CoA.

Abundant evidence indicates that the overall reaction for the formation of an acyl CoA proceeds as follows: ATP + organic acid + CoA $\xrightarrow[\text{enzyme}]{Mg^{+2}}$ acyl CoA + AMP + pyrophosphate. However, the exact mechanism by which the acylation reaction takes place is still controversial.

Ingraham and Green (1) postulated a five-step mechanism for the formation of acetyl CoA from CoA ATP, acetate, and magnesium ions. They indicated, based on theoretical considerations, that the first step in the synthesis involves formation of a complex between CoA, Mg^{+2}, and the enzyme, and that this step is followed by reaction with ATP and acetate to form an acetyl–AMP–magnesium–enzyme complex. The final step involves the transfer of the acetyl group from the complex to the sulfhydryl group of CoA, as shown here:



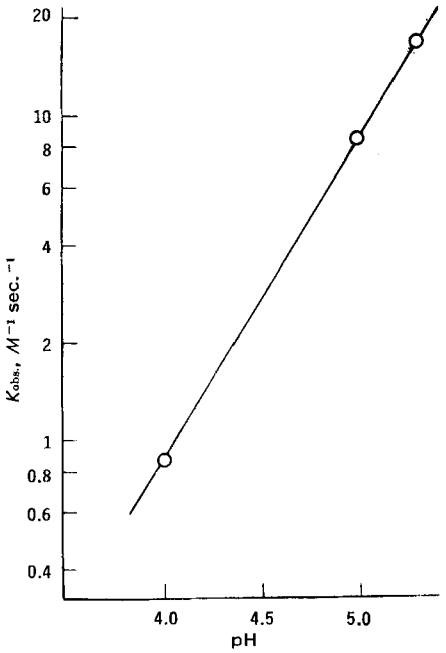


Figure 1—*The pH–rate profile of the reaction of succinic anhydride with cysteine at 25°.*

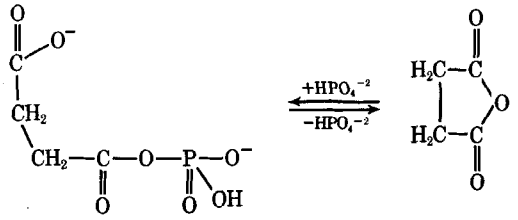
In their proposed theoretical mechanism, Ingraham and Green (1) hypothesized that the first two steps of the reaction, *i.e.*, the formation of the chelate complex between ATP, CoA, Mg^{+2}, enzyme, and ACO^- are the rate-limiting steps.

Berg (2), however, disagreed with Ingraham and Green's proposed mechanism. Supported by experimental evidence, Berg (2) indicated that the overall reaction between ATP, Mg^{+2}, acetate, and CoA occurs in at least two discrete steps:

$$\text{ATP} + \text{acetate} \underset{\text{Reaction I}}{\overset{Mg^{+2}\ \text{enzyme}}{\rightleftharpoons}} \text{acetyl AMP} + \text{pyrophosphate}$$

$$\text{acetyl AMP} + \text{CoA} \underset{\text{Reaction II}}{\rightleftharpoons} \text{acetyl CoA} + \text{AMP}$$

Reaction I, Berg (2) indicated, requires the presence of Mg^{+2} but not the presence of CoA. He also indicated that not only is CoA not required for Reaction I to take place, but if it is added, the rate of exchange is actually decreased. Reaction II, which is measured by the conversion of acetyl AMP to acetyl CoA, proceeds by the same rate in the absence of Mg^{+2}. However, a further observation made by Berg (2), which is inconsistent with this two-step reaction mechanism, is the fact that when acetyl AMP is added to the complete system (ATP, acetyl, CoA, Mg^{+2}, and enzyme), acetyl CoA synthesis continues but pyrophosphate is decreased to about one-tenth the rate found in the absence of acetyl AMP. For Berg's two-step reaction mechanism to be valid, the presence of acetyl AMP should not only affect the rate of formation of pyrophosphate, but it should also affect the rate of formation of acetyl CoA, since the addition of acetyl AMP should have an effect on both Reactions I and II.



Scheme I

A more attractive mechanism that is consistent with Berg's observations, I believe, involves another step in the reaction sequences. This step probably involves a rapid formation of acid anhydride intermediate from acyl AMP. The final step of the overall reaction then involves the reaction between the anhydrides and the sulfhydryl group of CoA to form the acyl CoA:

$$\text{ATP} + \text{organic acid} \xrightleftharpoons{Mg^{+2}\ \text{enzyme}} \text{acyl AMP} + \text{pyrophosphate}$$

$$\text{acyl AMP} \xrightarrow{\text{very fast}} \text{acid anhydride} + \text{AMP}$$

$$\text{acid anhydride} + \text{CoA} \longrightarrow \text{acyl CoA}$$

For this proposed mechanism to be valid, the following two requirements must be met.

1. *Anhydrides form as intermediates in the biosynthetic processes*—Anhydrides have not been isolated as products from biochemical reactions involving CoA. This may be due to their spontaneous hydrolysis in aqueous media which makes their isolation extremely difficult. However, anhydrides have been postulated as intermediates in both enzymatic and nonenzymatic reactions.

In enzymatic reactions (3), anhydrides were proposed as intermediates in the formation of succinyl CoA from succinate 18 and acetoacetyl CoA. In nonenzymatic reactions (4–6) involving the formation of anilidine in aqueous solution from aniline and succinate or tartrate buffers, the reaction was shown unequivocally to proceed *via* formation of acid anhydride intermediates:

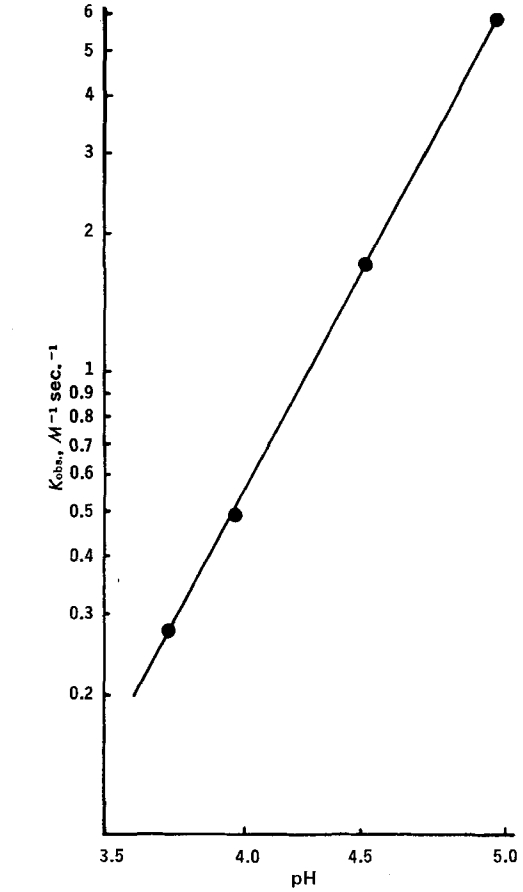
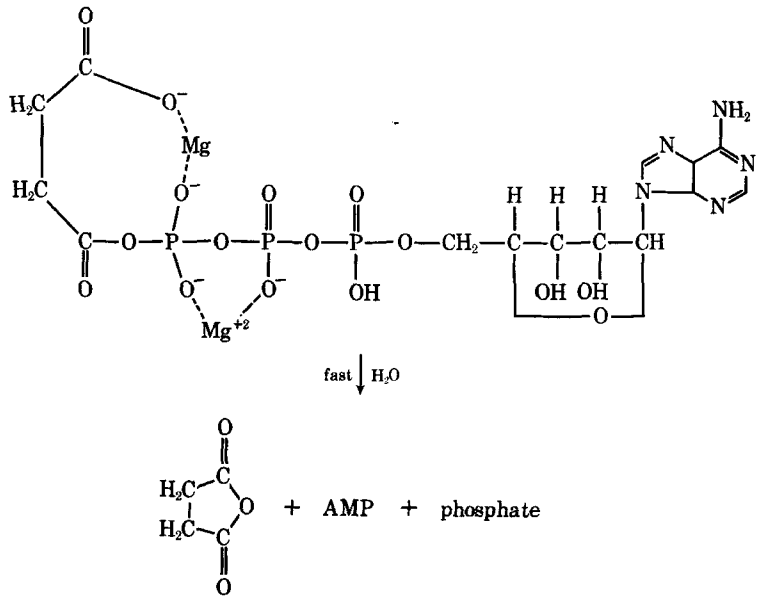


Figure 2—*The pH–rate profile of the reaction of propionic anhydride and 2-dimethylaminoethanethiol at 25°.*

$$\text{succinic acid} \rightleftharpoons \text{succinic anhydride} \xrightarrow{\text{aniline}} \text{anilide}$$

It was also shown (7) that anhydrides, such as succinic or phthalic anhydrides, could form with an ex-



Scheme II

tremely fast rate from the corresponding succinyl or phthalyl phosphate (Scheme I). Since ATP possesses phosphate groups in its molecule, it is conceivable that succinic anhydride is formed from succinyl ATP–magnesium-ion complex (Scheme II). A similar reaction pathway could be postulated for the formation of acetic anhydride.

2. *The rate of reaction between the sulfhydryl group of CoA and the anhydrides is much greater than the rate of hydrolysis of the anhydrides*—I have determined the rate of reaction between succinic anhydride and L-cysteine, a thiol-containing amino acid, and between propionic anhydride and 2-dimethylaminoethanethiol. The pH–rate profiles for these reactions are shown in Figs. 1 and 2.

The reaction rates were too rapid to be measured conveniently at pH's above 6. Thus, it would appear from these experiments that the reaction between a sulfhydryl group and anhydrides would proceed extremely fast under the conditions existing in the body, whereas the rates of hydrolysis of the anhydrides are relatively slow.

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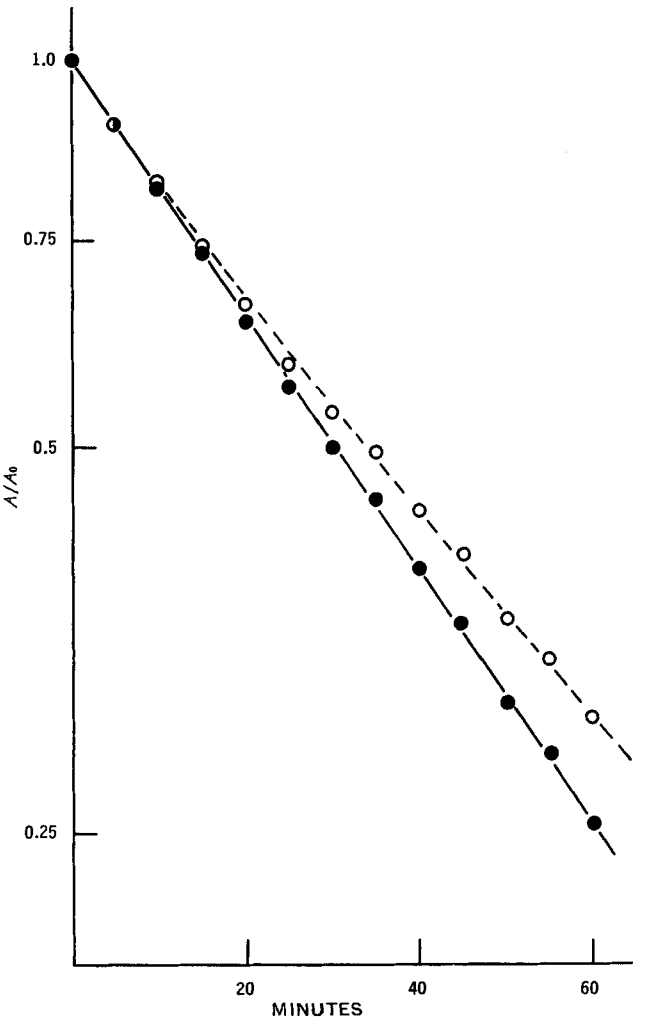

Figure 1—*Effect of dilution on diffusion of* p-*methoxyacetanilide from aqueous buffer to 1-octanol. Key:* ——, *in the absence of dilution;* - - -O- - -, *with dilution; and* —●—, *corrected for dilution.*

Correction for Effect of Dilution on Diffusion through a Membrane

Keyphrases ☐ Absorption kinetics, buccal—saliva dilution effect ☐ Dilution effect correction—first-order diffusion equation ☐ Diffusion equation, first order—dilution correction

Sir:

A number of steps in drug absorption and excretion involve simultaneous dilution of the fluid containing the drug. There does not, however, appear to be any published information concerning the effect of such dilution on diffusion rates. Beckett and Moffat (1) recognized that dilution by saliva affected buccal absorption, but they used an analog computer technique to allow for this condition. Our own work on buccal absorption, which showed that the rate of saliva production was constant during the period of a test (10 min.), led us to develop an equation to correct for the effect of such dilution on first-order diffusion.

The rate at which drug molecules enter the membrane is proportional to their concentration. Let A be the amount of drug in solution at time t, A_0 the initial amount of drug in solution, V_0 the initial volume of drug solution, and v the rate at which diluent is added.

For first-order diffusion in the absence of dilution, we thus have:

$$\frac{dA}{dt} = \frac{-kA}{V_0} \qquad \text{(Eq. 1)}$$

which on integration gives:

$$\ln \frac{A}{A_0} = \frac{-kt}{V_0} \qquad \text{(Eq. 2)}$$

When dilution occurs, we must write:

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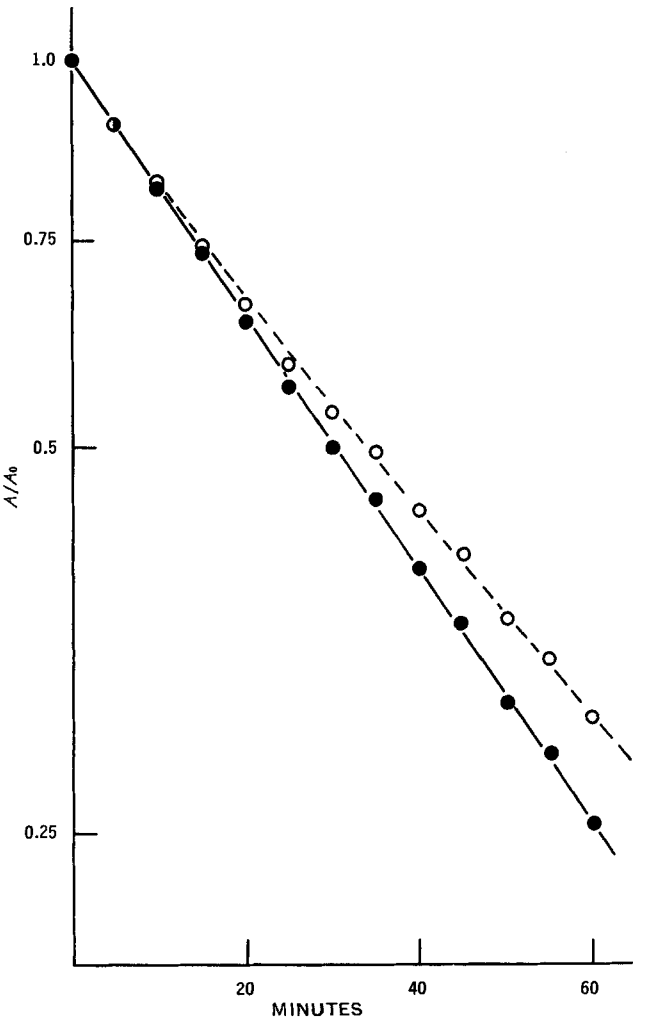

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which on integration gives:

$$\ln \frac{A}{A_0} = \frac{-k}{v} \ln \left(1 + \frac{vt}{V_0}\right) \quad \text{(Eq. 4)}$$

Provided that vt/V_0 is <1, this expands to:

$$\ln \frac{A}{A_0} = \frac{-k}{v} \left(\frac{vt}{V_0} - \frac{v^2t^2}{2V_0^2} + \frac{v^3t^3}{3V_0^3} - \ldots\right) \quad \text{(Eq. 5a)}$$

$$= \frac{-kt}{V_0} + \frac{kvt^2}{2V_0^2} - \frac{kv^2t^3}{3V_0^3} + \ldots \quad \text{(Eq. 5b)}$$

Thus, to a good approximation, deviations from rectilinearity in the ln (A/A_0) *versus* t plot can be accounted for by the terms $[(kvt^2/2V_0^2) - (kv^2t^3/3V_0^3)]$.

We demonstrated the validity of the correction by examining the effect of dilution on the diffusion of *p*-methoxyacetanilide from 0.2 *M* phosphate buffer[1], pH 7.2, into 1-octanol. The compound is virtually completely unionized at this pH, since its pKb is about 13.

The octanol phase was stirred mechanically and the aqueous phase magnetically. The area of the interface was 81 cm.^{2}, and the temperature was 22°. Samples were taken from and returned to the aqueous phase with a syringe. Concentrations were determined on a Unicam SP.800 spectrophotometer. The initial volume of the aqueous phase was 350 ml., and the volume of octanol was 500 ml. The partition coefficient of *p*-methoxyacetanilide between these phases is 10.5, so that there was negligible back-diffusion, as shown by the straight-line logarithmic plot of diffusion in the absence of dilution (Fig. 1), up to test times of 1 hr.

When the aqueous phase was diluted with buffer at a rate of 3 ml. min.$^{-1}$, the rate of diffusion of drug decreased (Fig. 1). Applying the correction brought the loss curve back to the first-order line.

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BOOKS

REVIEWS

Animal Experiments in Pharmacological Analysis. By Floyd R. Domer, Charles C Thomas, Springfield, IL 62707, 1971. 669 pp. Price $26.50.

This treatise on the quantitative aspects of pharmacology stems from the teaching program of the author, who has taught this phase of pharmacology to graduate students for many years. It is therefore expressly intended to be of service to pharmacologic specialists who are constantly confronted with the problem of conducting quantitative evaluation of drug activity on specific organ systems.

The text is divided into chapters dealing with the evaluation of drugs on the various organ systems, such as the Neuromuscular Junction, the Autonomic Ganglic, Smooth Muscle, and others. These specific topic chapters are preceded by three general introductory chapters on the Five Areas of Pharmacology, Initial Screening Experiments, and Toxicity Determination.

The author develops the subject matter of each chapter with broad introductory comments regarding the purpose of the experiment. Detailed description is included of operational procedures, often well illustrated with pictures or diagrams. Constant-temperature baths, stimulating electrodes, and pieces of equipment used in many pharmacologic procedures are shown in the diagrams of experiments.

Chapter 11, Techniques for Evaluation of Anesthetics, is especially commendable and is abreast of progress in the field. This, like the other chapters of the book, is provided with an adequate bibliography indicating an extensive knowledge of the various areas of pharmacologic techniques with which the author is acquainted.

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Reviewed by John C. Krantz, Jr.
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In this monograph, Meyers presents an excellent comparative review of the current law in the U. S. and Britain, with comments on the law in other parts of the world.

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The author's treatment of organ transplantation and euthanasia provides fascinating reading for those interested in the subject. The legal aspects of both the subjects are of current social concern and Meyers' treatment of them is exceptionally well done.

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Meyers points out that medical experimentation has been practiced through the centuries since the early days of Galen, yet it has yet to be clearly recognized legally as a legitimate part of medical practice. The law on human experimentation, or lack of it, stems from the fact that there is no set of specific guidelines available to ensure that human research may go forward on the highest scientific and ethical planes with legal protection for both the subject and the investigator.

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In his conclusion, Meyers deplores the fact that, while so many private codes governing medical experimentation have been promulgated in recent years, few of these pronouncements have found their way into recognized legal expressions of the courts or legislatures.

Taken as a whole, the Meyers monograph is a worthwhile contribution to the medico-legal literature.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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Journal of Pharmaceutical Sciences

SEPTEMBER 1971
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REVIEW ARTICLE

Pharmaceutical Applications of Solid Dispersion Systems

WIN LOUNG CHIOU*‡ and SIDNEY RIEGELMAN†

Keyphrases ☐ Solid dispersion systems—review ☐ Dispersions, solid systems—review ☐ Absorption kinetics—solid dispersion systems, review ☐ Dosage forms, fast-release—solid dispersion systems, review

CONTENTS

HISTORICAL BACKGROUND 1281
DEFINITION AND METHODS OF PREPARATION OF SOLID DISPERSIONS 1283
Definition 1283
Methods of Preparation 1283
Melting Method 1283
Solvent Method 1283
Melting–Solvent Method 1283
CLASSIFICATION AND FAST-RELEASE MECHANISMS 1284
Simple Eutectic Mixtures 1284
Solid Solutions 1286
Continuous Solid Solution 1287
Discontinuous Solid Solution 1287
Substitutional Solid Solution 1288
Interstitial Solid Solution 1288
Glass Solutions and Glass Suspensions 1290
Amorphous Precipitations in a Crystalline Carrier 1293
Compound or Complex Formations 1293
Combinations and Miscellaneous Mechanisms 1293
METHODS OF DETERMINATION OF TYPES OF SOLID DISPERSION SYSTEMS 1294
Thermal Analysis 1294
Cooling-Curve Method 1294
Thaw-Melt Method 1294
Thermomicroscopic Method 1294
DTA 1294
Zone Melting Method 1295
X-Ray Diffraction Method 1295
Microscopic Method 1296
Spectroscopic Method 1296
Dissolution-Rate Method 1296
Thermodynamic Method 1297
AGING OF SOLID DISPERSIONS 1297
Aging Effects of Eutectic Mixture 1297
Aging Effects of Solid Solution 1297
Aging Effects of Glass Solution 1298
Aging Effects of Metastable Polymorphic Forms in Solid Dispersions 1299
REVIEW OF *In Vivo* STUDIES 1299
Sulfathiazole–Urea Systems 1299
Chloramphenicol–Urea Systems 1299
Reserpine–Bile Acid Coprecipitates 1299
Griseofulvin–Polyethylene Glycol Polymers 1300
MISCELLANEOUS APPLICATION 1301

HISTORICAL BACKGROUND

The effect of the particle size of drugs on their dissolution rates and biological availability was reviewed comprehensively by Fincher (1). For drugs whose GI absorption is rate limited by dissolution, reduction of the particle size generally increases the rate of absorption and/or total bioavailability. This commonly occurs for drugs with poor water solubility. For example, the therapeutic dose of griseofulvin was reduced to 50% by micronization (2), and it also produced a more constant and reliable blood level. The commercial dose of spironolactone was also decreased to half by just a slight reduction of particle size (3). Such enhancement of drug absorption could further be increased several fold if a micronized product was used (3, 4).

Particle-size reduction is usually achieved by: (*a*) conventional trituration and grinding; (*b*) ball milling; (*c*) fluid energy micronization; (*d*) controlled precipitation by change of solvents or temperature, application of ultrasonic waves (5–7), and spray drying (8); (*e*)

administration of liquid solutions from which, upon dilution with gastric fluids, the dissolved drug may precipitate in very fine particles (9); and (*f*) administration of water-soluble salts of poorly soluble compounds from which the parent, neutral forms may precipitate in ultrafine form in GI fluids. Although the reduction of particle size can be easily and directly accomplished by the first four methods (*a–d*), the resultant fine particles may not produce the expected faster dissolution and absorption. This primarily results from the possible aggregation and agglomeration of the fine particles due to their increased surface energy and the subsequent stronger van der Waals' attraction between nonpolar molecules. This was demonstrated by Lin *et al.* (10), who showed that the *in vitro* dissolution rates of micronized griseofulvin and glutethimide were slower than those of their coarser particles. However, the opposite finding for griseofulvin was reported by Chiou and Riegelman (11, 12). Another inherent disadvantage of these pure fine powders of poorly soluble drugs is their poor wettability in water. The wetting of powders is the first step for them to dissolve and sometimes disperse in fluids (13). Furthermore, drugs with plastic properties are difficult to subdivide by methods *a–c*. They have more tendency to stick together, even if fine powders can be produced by controlled precipitation.

Theoretically, the solvent method (*c*) seems to be an ideal approach in achieving particle-size reduction. However, it is not frequently employed in the commercial market due to such reasons as selection of a nontoxic solvent, limitation to drugs with a low dose, and high costs of production. The water-soluble salts of many poorly soluble acidic or basic drugs have been widely used clinically as solid dosage forms. Indeed, they have been shown frequently to produce better absorption than their parent forms. It has been shown that the potassium or sodium salts may react with atmospheric carbon dioxide and water to precipitate out poorly soluble parent compounds. This occurs especially on the outer layer of a dosage form and thereby retards rates of dissolution and absorption. This precipitation effect is believed to be responsible for the slower *in vitro* dissolution rates and the lower novobiocin plasma levels in dogs following the oral administration of its soluble sodium salt rather than the less soluble amorphous form of the parent compound (14). The reported failure of the clinical response from three commercial capsule dosage forms containing sodium diphenylhydantoin may be caused by the same reason (15). In addition, the alkalinity of some salts may cause epigastric distress following administration (16).

In 1961, a unique approach of solid dispersion to reduce the particle size and increase rates of dissolution and absorption was first demonstrated by Sekiguchi and Obi (17). They proposed the formation of a eutectic mixture of a poorly soluble drug such as sulfathiazole with a physiologically inert, easily soluble carrier such as urea. The eutectic mixture was prepared by melting the physical mixture of the drug and the carrier, followed by a rapid solidification process. Upon exposure to aqueous fluids, the active drug was expected to be released into the fluids as fine, dispersed particles because of the fine dispersion of the drug in the solid eutectic mixture and the rapid dissolution of the soluble matrix. Levy (9) and Kanig (18) subsequently noted the possibility of using a solid solution approach in which a drug is dispersed molecularly in a soluble carrier. In a series of reports in 1965–1966, Goldberg *et al.* (19–22) presented a detailed experimental and theoretical discussion of advantages of the solid solution over the eutectic mixture.

In 1965, Tachibana and Nakamura (23) reported a novel method for preparing aqueous colloidal dispersions of β-carotene by using water-soluble polymers such as polyvinylpyrrolidone. They dissolved the drug and the polymer carrier in a common solvent and then evaporated the solvent completely. A colloidal dispersion was obtained when the coprecipitate was exposed to water. In 1966, Mayersohn and Gibaldi (24) demonstrated that the dissolution rate of griseofulvin could be markedly enhanced when dispersed in polyvinylpyrrolidone by the same solvent method. The mechanisms of increased dissolution rates of drugs, solid dispersed in polyvinylpyrrolidone carriers, were thoroughly discussed by Simonelli *et al.* (25, 26). Chiou and Riegelman (11) recently advocated the application of glass solutions to increase dissolution rates. The significance of the solid dispersion technique was strengthened by the demonstration of Chiou and Riegelman (27–29) of the fast and almost complete absorption of the insoluble griseofulvin in man and dogs while the commercial micronized griseofulvin was incompletely absorbed (30–60%). They used polyethylene glycol 6000 as a dispersion carrier. The main advantages of using water-soluble polymers as carriers are their nontoxicity and general applicability to most drugs.

It is believed that this relatively new field of pharmaceutical technique and principles will play an important role in increasing dissolution, absorption, and therapeutic efficacy of drugs in future dosage forms. Therefore, a thorough understanding of its fast-release principles, methods of preparation, selection of suitable carriers, determination of physical properties, limitations, and disadvantages will be essential in the practical and effective application of this approach. The main purpose of this article is to review critically the hitherto limited pharmaceutical literature pertinent to this area. Since a great amount of excellent work on solid dispersion systems has been accumulated in the sciences of metallurgy, geology, and chemistry, a brief summary of some of these findings would be extremely helpful in the future study and understanding of pharmaceutical applications of solid dispersion systems. One major objective of this review article is to introduce and correlate these works to possible pharmaceutical applications.

In addition to absorption enhancement, the solid dispersion technique may have numerous pharmaceutical applications which remain to be further explored. It is possible that such a technique can be used to obtain a homogeneous distribution of a small amount of drugs at solid state, to stabilize unstable drugs, to dispense liquid or gaseous compounds, to formulate a fast-release priming dose in a sustained-release dosage form, and to formulate sustained-

release or prolonged-release regimens of soluble drugs by using poorly soluble or insoluble carriers. It is hoped that this review paper will stimulate interest and research in these unexplored areas.

DEFINITION AND METHODS OF PREPARATION OF SOLID DISPERSIONS

Definition—It seems suitable here to define the term "solid dispersions" as used in this paper. The term refers to the dispersion of one or more active ingredients in an inert carrier or matrix at solid state prepared by the melting (fusion), solvent, or melting–solvent method. The dispersion of a drug or drugs in a solid diluent or diluents by traditional mechanical mixing is not included in this category. The solid dispersions may also be called solid-state dispersions, as first used by Mayersohn and Gibaldi (24). The term "coprecipitates" has also been frequently used to refer to those preparations obtained by the solvent methods such as coprecipitates of sulfathiazole–polyvinylpyrrolidone (25) and reserpine–polyvinylpyrrolidone (30). Since the dissolution rate of a component from a surface is affected by the second component in a multiple-component mixture (31), the selection of the carrier has an ultimate influence on the dissolution characteristics of the dispersed drug. Therefore, a water-soluble carrier results in a fast release of the drug from the matrix, and a poorly soluble or insoluble carrier leads to a slower release of the drug from the matrix. This review paper primarily deals with fast-release solid dispersions, although some principles discussed later may also be applied to slow-release solid dispersion systems. To achieve a faster release of a drug from the matrix, it is generally necessary that the active drug be a minor component in the dispersion system in terms of the percent weight (not on molar basis).

Methods of Preparation—*Melting Method*—The melting or fusion method was first proposed by Sekiguchi and Obi (17) to prepare fast-release solid dispersion dosage forms. The physical mixture of a drug and a water-soluble carrier was heated directly until it melted. The melted mixture was then cooled and solidified rapidly in an ice bath under rigorous stirring. The final solid mass was crushed, pulverized, and sieved. Such a technique was subsequently employed with some modification by Goldberg *et al.* (20–22) and Chiou and Riegelman (11). To facilitate faster solidification, the homogeneous melt was poured in the form of a thin layer onto a ferrite plate or a stainless steel plate and cooled by flowing air or water on the opposite side of the plate. The solidified masses of drug–polyethylene glycol polymer systems were often found to require storage of 1 or more days in a desiccator at ambient temperatures for hardening and ease of powdering (11). Some systems, such as griseofulvin and citric acid, were found to harden more rapidly if kept at 37° or higher temperatures (11, 32).

The main advantages of this direct melting method are its simplicity and economy. In addition, a supersaturation of a solute or drug in a system can often be obtained by quenching the melt rapidly from a high temperature (34). Under such conditions, the solute molecule is arrested in the solvent matrix by the instantaneous solidification process. Similarly, a much finer dispersion of crystallites was obtained for systems of simple eutectic mixtures if such quenching techniques were used (34, 35). The disadvantage is that many substances, either drugs or carriers, may decompose or evaporate during the fusion process at high temperatures. For example, succinic acid, used as a carrier for griseofulvin (21), is quite volatile and may also partially decompose by dehydration near its melting point (36). However, this evaporation problem can be avoided if the physical mixture is heated in a sealed container. Melting under vacuum or a blanket of an inert gas such as nitrogen may be employed to prevent oxidation of the drug or carrier (37).

The melting point of a binary system is dependent upon its composition, *i.e.*, the selection of the carrier and the weight fraction of the drug in the system (33). By proper control, the melting point (the temperature at which the mixture completely melts) of a binary system may be much lower than the melting points of its two components. Under such a condition, this simple melting method can still be used to prepare solid dispersions, even if the pure drug may undergo decomposition at or near its melting point. This principle was used to prepare solid dispersions of steroids and a cardiac glycoside in polyethylene glycol 6000 (38) and that of griseofulvin in pentaerythritol (11).

Solvent Method—This method has been used for a long time in the preparation of solid solutions or mixed crystals of organic or inorganic compounds (33). They are prepared by dissolving a physical mixture of two solid components in a common solvent, followed by evaporation of the solvent. This method was used to prepare solid dispersions of β-carotene–polyvinylpyrrolidone (23), griseofulvin–polyvinylpyrrolidone (24), sulfathiazole–polyvinylpyrrolidone (25), steroid–polyvinylpyrrolidone (26), reserpine–polyvinylpyrrolidone (30), and reserpine–deoxycholic acid (39).

The main advantage of the solvent method is that thermal decomposition of drugs or carriers can be prevented because of the low temperature required for the evaporation of organic solvents. However, some disadvantages associated with this method are the higher cost of preparation, the difficulty in completely removing liquid solvent, the possible adverse effect of the supposedly negligible amount of the solvent on the chemical stability of the drug, the selection of a common volatile solvent, and the difficulty of reproducing crystal forms. In addition, a supersaturation of the solute in the solid system cannot be attained except in a system showing highly viscous properties, as is discussed later. It must be emphasized that the suitability of the solvent method to prepare simple eutectics or partial solid solutions remains to be studied further because their final physical properties may be quite different from those obtained by the melting method.

Melting–Solvent Method—It was shown recently that 5–10% (w/w) of liquid compounds could be incorporated into polyethylene glycol 6000 without significant loss of its solid property (40). Hence, it is

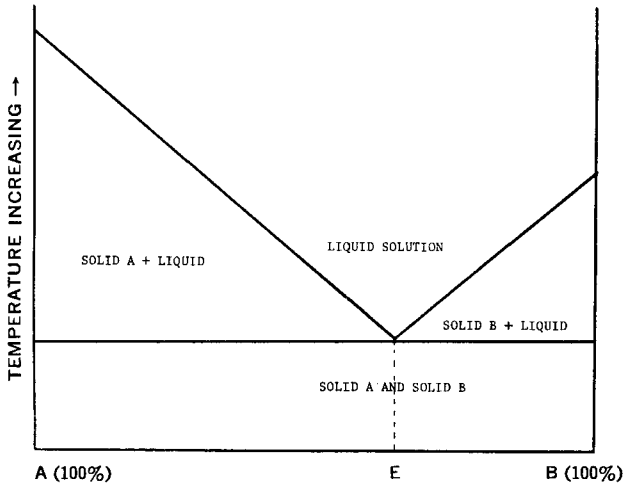


Figure 1—*Phase diagram of a simple eutectic mixture with negligible solid solubility.*

possible to prepare solid dispersions by first dissolving a drug in a suitable liquid solvent and then incorporating the solution directly into the melt of polyethylene glycol, obtainable below 70°, without removing the liquid solvent. It is possible that the selected solvent or dissolved drug may not be miscible with the melt of polyethylene glycol. The polymorphic form of the drug precipitated in the solid dispersion may be affected by the liquid solvent used. Such a unique method possesses the advantages of both the melting and solvent methods. Unfortunately, from a practical standpoint, it is only limited to drugs with a low therapeutic dose, *e.g.*, below 50 mg. The feasibility of this method was demonstrated on spironolactone–polyethylene glycol 6000 and griseofulvin–polyethylene glycol 6000 systems (41). Its application to other drugs and carriers, however, remains to be explored.

CLASSIFICATION AND FAST-RELEASE MECHANISMS

Although solid dispersion systems may include more than two components, for the sake of simplicity and practicality, this article is primarily limited to binary systems. As a measure of the interaction between the two components, 30 different phase diagrams were proposed for binary alloy systems (42). Vasil'ev (43) further classified phase diagrams according to: (*a*) the relative strength of interaction between similar and different atoms, and (*b*) the limiting permissible degree of deformation of the energy field of the liquid solvent or its crystal lattice in the solid state. While it is believed that these classifications can also be applied to most organic drugs, in this article it is felt more appropriate to classify various systems of solid dispersions on the basis of their major fast-release mechanisms. Accordingly, they are discussed in the following six groups: Group 1, simple eutectic mixtures; Group 2, solid solutions; Group 3, glass solutions and glass suspensions; Group 4, amorphous precipitations of a drug in a crystalline carrier; Group 5, compound or complex formations between the drug and the carrier; and Group 6, any combinations among Groups 1–5. The methods used to identify these systems are reviewed in the next section.

Simple Eutectic Mixtures—The simple eutectic mixture is usually prepared from the rapid solidification of the fused liquid of two components which show complete liquid miscibility and negligible solid–solid solubility (33). These properties can be illustrated in a phase diagram (Fig. 1). Thermodynamically, such a system is regarded as an intimately blended physical mixture of its two crystalline components (19, 33, 34).

When a eutectic (Composition E in Fig. 1) composed of a poorly soluble drug is exposed to water or GI fluids, the carrier may be released into aqueous medium in fine crystalline form (17). This is based on the assumption that both components may simultaneously crystallize out in very small particulate sizes (33). The increase of the specific area due to this reduction of particle size generally increases rates of dissolution and oral absorption of poorly soluble drugs. Ultrafine or colloidal crystallites of eutectics can be found in such examples as tin–lead (34) and naphthalene–phenanthrene (44) systems. In addition to the reduction of the crystallite size, the following factors may contribute to the faster dissolution rate of a drug dispersed in the eutectic:

1. An increase in drug solubility may occur if the majority of its solid crystallites are extremely small (45).

2. A possible solubilization effect by the carrier may operate in the microenvironment (diffusion layer) immediately surrounding the drug particle in the early stage of dissolution since the carrier completely dissolves in a short time. This was demonstrated by the faster dissolution rate of acetaminophen from its physical mixture with urea than that of the pure compound with comparable particle size (22). This hypothesis was further supported by a marked increase of acetaminophen solubility in the presence of urea in water (22). A similar rationale was also given to the enhancement of dissolution rates of reserpine from a physical mixture of reserpine and polyvinylpyrrolidone (30).

3. The absence of aggregation and agglomeration between fine crystallites of the pure hydrophobic drug may play a far more important role in increasing rates of dissolution and absorption than is presently recognized by research workers in this field. An aggregate is defined as a particle or an assembly of particles held together by strong inter- or intramolecular or atomic cohesive forces (46). Usually the aggregate is stable to high-speed mixing or ultrasonic forces. An agglomerate is defined as a gathering of two or more particles and/or aggregates held together by relatively weak cohesive forces. In many cases, these forces are due to an electrostatic surface charge generated during handling or processing operations (46). It is also likely that these electrostatic forces may be involved only in bringing particles together, but they are not responsible for holding them together. Such agglomeration is more severe for very finely divided particles (about 0.1 μ) due to the greater specific surface charge. Although the agglomerates may be broken, their dispersion in the mildly stirred GI fluids may not be very efficient. As mentioned previously, these problems of aggregation and agglomeration are most

detrimental to the application and efficacy of pure fine particles because their effective specific surface area is markedly reduced[1]. Serious drawbacks of aggregation and agglomeration and lumping in the dissolution medium between pure drug particles are, however, rarely present in most solid dispersion systems because the individually dispersed particles are surrounded in the matrix by carrier particles. It must be emphasized that the aggregation and agglomeration of the solid dispersion powders may not significantly affect the dissolution of the drug, which can still disintegrate quickly due to the more rapid dissolution of the soluble carrier. Such a unique advantage of solid dispersion systems was demonstrated in the *in vivo* absorption (28, 29) of griseofulvin when dispersed in polyethylene glycol 6000 (10% w/w) and compressed into a hard tablet. As discussed later, the 10% griseofulvin dispersion in polyethylene glycol 6000 contains at least half of the griseofulvin in the finely dispersed crystalline form. The dissolution rates of the pure and dispersed griseofulvin are shown in Fig. 2.

4. Excellent wettability and dispersibility of a drug from a eutectic or other solid dispersion system prepared with a water-soluble matrix result in an increased dissolution rate of the drug in aqueous media. This is due to the fact that each single crystallite of the drug is very intimately encircled by the soluble carrier which can readily dissolve and cause the water to contact and wet the drug particle. As a consequence, a fine homogeneous suspension of a drug can be easily obtained with minimum stirring (17). These striking advantages were observed by the authors with various drug–polyethylene glycol solid dispersions. In contrast, the aggregates and agglomerates of poorly soluble pure powders are surrounded by the nonpolar air, which is hard to penetrate or displace by water.

5. An increased rate of dissolution and absorption may also occur if a drug crystallizes in a metastable form after solidification from the fused solution. A metastable, crystalline form has a higher solubility which, in turn, leads to a faster dissolution rate according to the well-known Noyes–Whitney equation. Interested readers should consult an excellent review paper by Haleblian and McCrone (47) on the pharmaceutical applications of polymorphism. The high possibility of the polymorphic crystallization during the preparation of solid dispersions can be seen from the facts that many compounds can exhibit polymorphism. For example, 67% of steroids, 43% of sulfonamides, and 63% of barbiturates were shown to exhibit polymorphism in an extensive survey by Kuhnert-Brandstätter (48). It must be noted that the existence of a different polymorphic form or forms results in a phase diagram differing from that shown in Fig. 1 (47).

[1] Peculiar examples were encountered by the authors when 125 mg. of the pure micronized griseofulvin and 100–200-mesh griseofulvin, loosely packed in a No. 3 gelatin capsule, were studied for dissolution rates in 18 l. of water at 37° under a fairly vigorous stirring condition (*Reference 11;* the capsule kept in a cylindrical container, 5 × 3 cm., made of No. 8 mesh stainless steel screen and moved by a standard USP disintegration apparatus). The griseofulvin lumped together as a single mass even after 4–6 hr. of study, and dissolution only took place at the surface of the mass. This phenomenon was also noted in a commercial capsule product of griseofulvin.

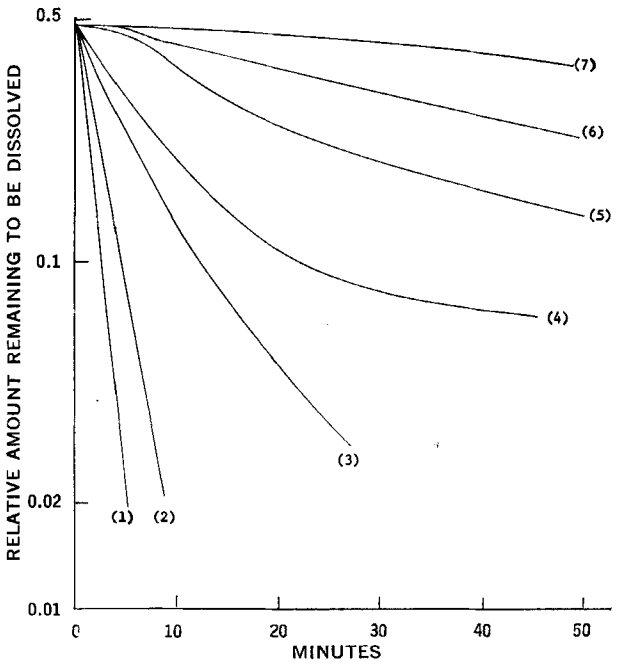


Figure 2—*Griseofulvin dissolution-rate data (amount remaining to be dissolved) from 125 mg. in 18 l. of water at 36.7°. Key: (1), 10% griseofulvin–polyethylene glycol 6000 powder; (2), 20% griseofulvin–polyethylene glycol 6000 powder; (3), 40% griseofulvin–polyethylene glycol 6000 powder; (4), wetted, micronized griseofulvin powder; (5), nonwetted, micronized griseofulvin powder; (6), micronized griseofulvin in capsule; and (7), 100–200-mesh griseofulvin powder in capsule.*

In addition to the possible aforementioned differences between the eutectics and the physical or mechanical mixtures, the rapidly crystallized (quenched) eutectics are characterized by increased hardness (49). This was explained on the basis of a high degree of strain resulting from the action of mechanical forces. The effect of such increased hardness on the dissolution rate, however, remains to be explored. Savchenko (49) advocated that a eutectic is formed by some sort of loose molecular or atomic interaction which does not involve the formation of a chemical bond. This is thought to relate to some of the changes in physical properties of eutectic alloys such as a reduction in electrical conductivity, vapor pressure, and thermal effects. It must be emphasized that a slow process of cooling and solidification from the melt may not result in fine dispersion of the phases (49), which is primarily responsible for the higher dissolution rate of the drug.

The composition of a eutectic may have a significant effect on the particle size of the crystallite. If it is made up of a high weight fraction of drug, an ultrafine crystallization of the drug may not be obtained. This is logical if one expects that the higher the dilution, the finer the crystalline size of its precipitate. This probably accounts for the failure to find an increased dissolution rate of acetaminophen from the eutectic with urea which contains 52% of the acetaminophen (20). It is believed that the hardening effect of the eutectic may also play a role in retarding its dissolution.

Recently, Chiou (50) contended that the system of chloramphenicol–urea should be described as a simple eutectic mixture with negligible solid–solid solubility

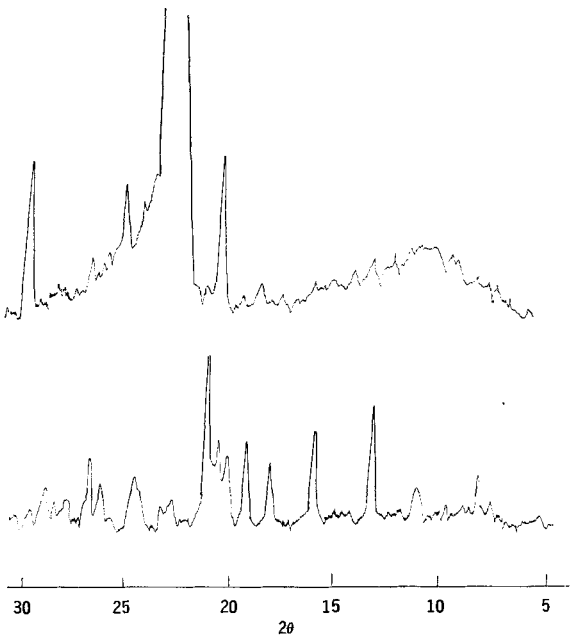


Figure 3—*X-ray diffraction spectra of pure chloramphenicol (bottom) and pure urea (top).*

rather than an extensive, partial solid solution as previously proposed by Sekiguchi *et al.* (51) and Goldberg *et al.* (22). This appears to be supported by differential thermal analysis (DTA) and X-ray diffraction data. The endothermic peaks of 2.5 and 97% chloramphenicol resolidified samples at the eutectic temperature (51) indicate that the samples started to thaw at that temperature. If their compositions did not belong to a simple eutectic system, then the thaw points should begin at a higher temperature (52).

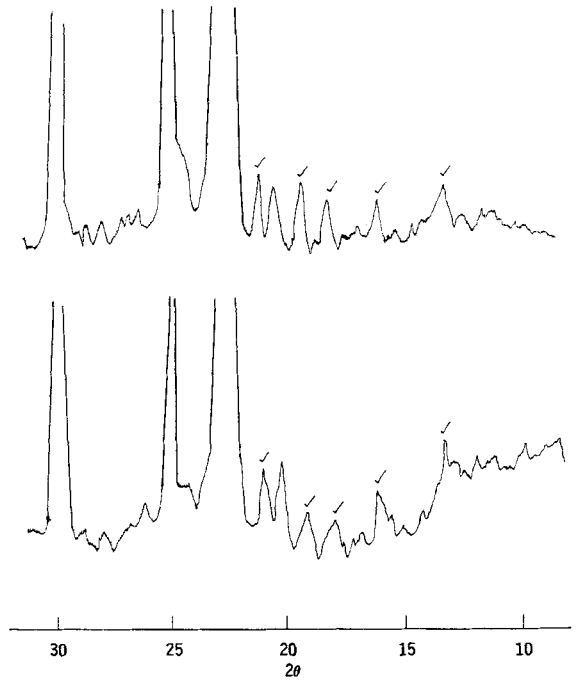


Figure 4—*X-ray diffraction spectra of a physical mixture of 10% chloramphenicol–90% urea (bottom) and resolidified fused mixture of 10% chloramphenicol–90% urea (top). Arrows indicate diffraction peaks due to the presence of chloramphenicol crystallites.*

In the previously proposed phase diagram, chloramphenicol was shown to dissolve in the solid urea at a concentration of 25% (w/w). To investigate this system further, diffraction spectra were obtained from a Norelco X-ray diffractometer using CuKα radiation. The spectra of pure chloramphenicol, pure urea, and the physical mixture and resolidified mixture of 10% chloramphenicol are shown in Figs. 3 and 4. The presence of the typical X-ray diffraction peaks of chloramphenicol in the freshly prepared, quenched sample of 10% chloramphenicol unmistakably indicates that the sample is not a solid solution but a eutectic mixture. The height of these peaks, which are comparable with those obtained from the physical mixture, also indicates the negligibility of solid solubility. The slight increase in dissolution rate of the eutectic (75% chloramphenicol w/w) over the pure chloramphenicol (22) may be due to a coarser particle size of chloramphenicol crystallization and the hardening effect of the eutectic. The small particle size of the precipitate at the lower concentration of the chloramphenicol, however, may be primarily contributory to the reported attainment of supersaturation and marked enhancement of dissolution rate from 25% solid dispersion (22, 51). It is further expected that a much faster dissolution rate may be obtained from the lower concentrations of the chloramphenicol in such a eutectic mixture.

From their microthermal microscope studies, Goldberg *et al.* (21) reported that griseofulvin, a water-insoluble antibiotic, forms a solid solution with succinic acid at a concentration of 25% w/w. The dissolution rate from such dispersions was found to be several times higher than that of the micronized griseofulvin. Furthermore, a supersaturation of about 250% of the solubility was also observed. Chiou and Niazi (53) recently concluded from their DTA and X-ray diffraction studies that such a binary system is a simple eutectic mixture with negligible solid solubility. The dissolution rates of griseofulvin were found to increase as the concentration of griseofulvin in the solid dispersion decreased.

Solid Solutions—A solid solution, compared to a liquid solution, is made up of a solid solute dissolved in a solid solvent. It is often called a mixed crystal because the two components crystallize together in a homogeneous one-phase system (33). In their theoretical paper, Goldberg *et al.* (19) suggested that a solid solution of a poorly soluble drug in a rapidly soluble carrier achieves a faster dissolution rate than a eutectic mixture because the particle size of the drug in the solid solution is reduced to a minimum state, *i.e.*, its molecular size. In other words, the dissolution of the drug takes place in the solid state prior to its exposure to the liquid medium. In addition to such maximum size reduction, other factors such as Factors 1–4 discussed under *Simple Eutectic Mixtures* may contribute to increased rates of dissolution and absorption of drugs dispersed in solid solutions. It must be emphasized that the advantage of a solid solution may not be so significant if the solid solution is exposed to a medium with a volume much less capable to dissolve all the drug. Under these conditions, a drug may precipitate. However, due to the maximum particle-size reduction

in the solid solution and to the possible solubilization effect of the carrier in the microenvironmental diffusion layer of bulk fluids, the drug may temporarily result in a high supersaturation of the bulk fluid. Obviously, this is temporary and would lead to precipitation if the drug is not being absorbed or removed by other processes.

Solid solutions can generally be classified according to the extent of miscibility between the two components or the crystalline structure of the solid solution (33, 34, 54). Based on the former criterion, they can be divided into two groups: continuous (or isomorphous, unlimited, complete) solid solutions and discontinuous (or limited, restricted, partial, incomplete) solid solutions. According to the latter criterion, they can also be classified into two groups: substitutional solid solutions and interstitial solid solutions. The important physical properties of each group are reviewed briefly.

Continuous Solid Solution—In this system, the two components are miscible or soluble at solid state in all proportions (Fig. 5). No established solid solution of this kind has been shown to exhibit fast-release dissolution properties, although it is theoretically possible. It is obvious that a faster dissolution rate would be obtained if the drug is present as a minor component. However, the presence of a small amount of the soluble carrier in the crystalline lattice of the poorly soluble drug may also produce a dissolution rate faster than the pure compound with similar particle size. This may be due to a small number of the neighboring drug molecules holding the dissolving drug molecule after the rapid dissolution of the neighboring water-soluble carrier. The total lattice energy of the continuous solid solution at various compositions theoretically should be greater than that of either pure component, because the strength of bond between the two different components at the solid state, U_{AB}, should be greater than that between the same species of molecules, U_{AA} and U_{BB}, in order to form a continuous solid solution (33). The solid solution above the temperature of the miscibility gap, as shown in Fig. 5, is also thermodynamically stable, with a free energy lower than that anticipated from the mixture law (54, 55). The miscibility gap noted in Fig. 5 may occur as a result of limited solid-state solubility at lower temperatures. The implication of this phenomenon is discussed later in this article.

Discontinuous Solid Solution—In contrast to the continuous solid solution, there is only a limited solubility of a solute in a solid solvent in this group of solid solutions. This can be best depicted in a standard phase diagram (Fig. 6). The regions of solid solutions in this diagram are shown as the α and β regions. Each component shown is capable of dissolving the other component to a certain degree above the eutectic temperature. However, as the temperature is lowered, the solid solution regions become narrower. The implication of the decreasing solubility with declining temperature is discussed later. The free energy of a stable, limited solid solution is also lower than that of the pure solvent (55).

In reality, some solid-state solubility can be expected for all two-component systems (19, 34). However, the degree of solubility is usually small enough to be considered negligible. Goldberg *et al.* (19) suggested that, for practical purposes, solubility of greater than 5% of one component in the other could be considered to be a solid solution. It is felt that such a criterion is not adequate. Sensitive instruments now allow the detection of solid solution formation below a 5% level. Furthermore, many drugs with low therapeutic doses (*e.g.*, below 25 mg.) can be practically incorporated into solid solutions at concentrations of less than 5%.

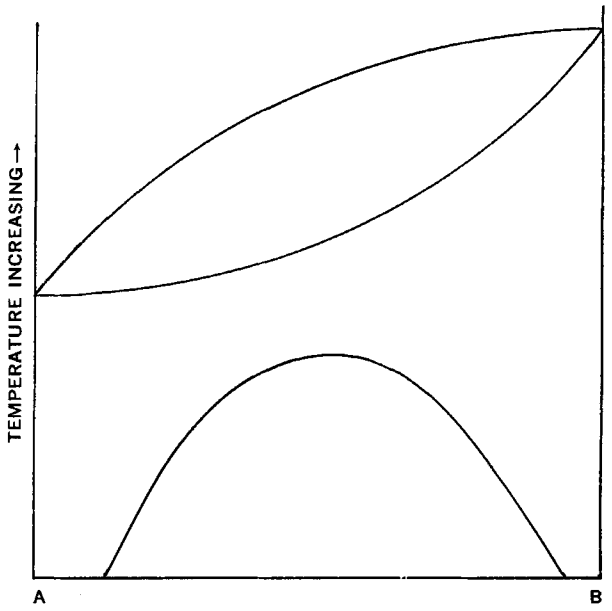


Figure 5—*A typical phase diagram of continuous solid solution of a binary system, A and B. The lowest curve indicates a solubility gap at lower temperatures.*

The phase diagram of a sulfathiazole–urea binary system was studied by thermal analysis (17). It was interpreted as a system of limited solid solution, in which the maximum solubility of sulfathiazole is about 10% w/w and that of urea is about 8% w/w (19). The eutectic composition is located at 52% of sulfathiazole. Therefore, the eutectic of this system is

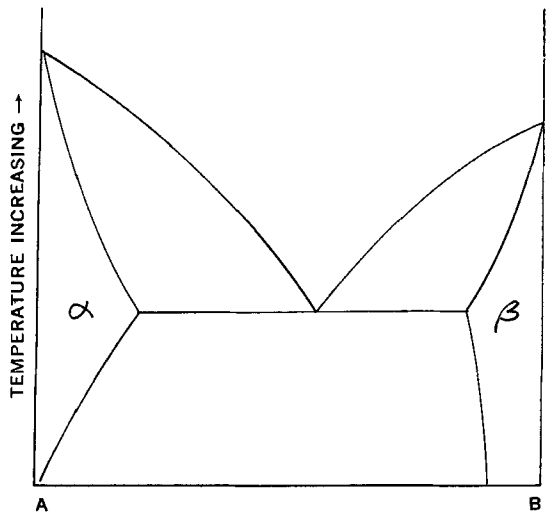


Figure 6—*A typical phase diagram of a discontinuous solid solution of a binary system, A and B. α and β are regions of solid solution formation.*

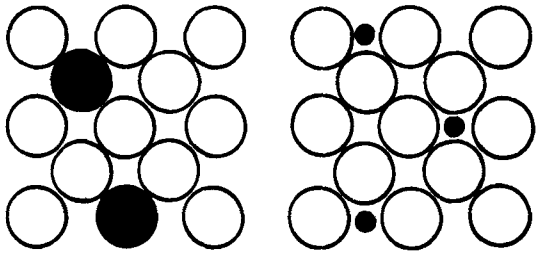


Figure 7—*Right diagram shows the formation of an interstitial solid solution, and left diagram shows the formation of a substitutional solid solution. Dark circles indicate solute atoms or molecules, while open circles indicate solvent atoms or molecules (from* Reference 54, *reprinted with permission).*

theoretically a physical mixture of two solid solutions, α and β. The faster absorption rates found in man with this eutectic mixture were claimed to be primarily due to these solid solutions. However, it was also recently noted by Chiou and Niazi (56), from their X-ray diffraction studies, that sulfathiazole is mainly present as an amorphous form (more correctly a glass solution) in the freshly prepared eutectic. No significant amount of sulfathiazole was found to crystallize when kept at 27° for 2 weeks. It was proposed that such an amorphous form, with a solubility much greater than the crystalline form (25), was an important contributing factor to the increased rate of dissolution and absorption.

Substitutional Solid Solution—In this type of solid solution, the solute molecule substitutes for the solvent molecule in the crystal lattice of the solid solvent. A schematic diagram is shown in Fig. 7. It can form a continuous or discontinuous solid solution. The size and steric factors of the solute molecule were shown to play a decisive role in the formation of solid solutions (33, 34, 43, 54). The size of the solute and the solvent molecule should be as close as possible. According to the Hume-Ruthery rule (54, 57), an extensive solid solution can be formed only when the effective diameter of the solute differs less than 15% from that of the solvent. This has been experimentally proven in a variety of solid solutions of metals and inorganic compounds.

Timmermans (60) proposed a term called the degree of molecular isomorphism to express the degree of similarity of the shape of the two components. He superimposed the two molecules and calculated the overlapping volume, r, and the nonoverlapping volume, Δ. The degree of molecular isomorphism, e, is then equal to $1 - \Delta/r$. From his extensive studies of phase diagrams of organic compounds, he found that wide or complete solubility required the value of e to be around 0.9. Examples of continuous solid solutions of systems include mixtures of *p*-dibromobenzene–*p*-chlorobromobenzene (61) and anthracene–acenaphthene (62).

The distortion of the crystal lattice of the solvent by the steric effect or chemical interaction (63) is also important. The solubility of the solute increases until the distortion of the lattice field of the solvent by the solute molecule can no longer be tolerated. For example, naphthalene (59) can form solid solutions with its β-derivatives substituted with halogens, hydroxyl, or amino groups but it only forms eutectic mixtures with its α-substituted derivatives. However, e values are the same for the pairs of α- and β-derivatives with naphthalene.

Frequently, water-insoluble drugs contain halogens, hydroxyl, methyl, methoxy, or other small functional groups. It might be possible to synthesize relatively inert soluble congeners by substituting a specific functional group which will change the physical properties with minimal changes in the degree of molecular isomorphism. It is expected that the insoluble drugs and the congeners can possibly form wide ranges of solid solutions due to their similarity in size and shape. Under such conditions, the relatively inactive soluble derivatives can serve as carriers for the active drugs. Such a combination may result in more rapid dissolution and absorption.

It is well known that globular or plastic compounds form a wide range of solid solutions above their plastic points. For example, pairs of cyclopentane–2,2-dimethylbutane (64) and chemically unrelated methane and argon (65) form continuous solid solutions at appropriate temperatures. Typical properties of globular or plastic compounds are (64): (*a*) low entropy of fusion, usually less than 5 e.u.; (*b*) high triple-point temperature and pressure; (*c*) crystals, usually of cubic or hexagonal symmetry, which are clear (almost glasslike), tacky, and easily deformed; and (*d*) one or more energetic transitions in the solid state. The reasons for their mutual solubility are the similarity in their symmetry and almost free rotation (hence, low lattice energy) above their plastic points. Since plastic compounds have the lowest lattice energy and strain, it is reasonable to expect that they will more easily accommodate all kinds of molecules in their crystal lattice.

Pentaerythritol, a typical plastic compound (64) with an entropy of fusion of 3.2 e.u., was selected as a carrier to disperse griseofulvin (11). The 10% griseofulvin dispersion was found to dissolve much faster than micronized griseofulvin. In addition, a supersaturation was rapidly obtained when an excess amount was studied. Its potential usage as a carrier for other drugs, however, remains to be further explored. A similar carrier, pentaerythritol tetraacetate, was also shown to enhance the dissolution rate of griseofulvin (11). The phase diagrams of both systems have not been established. A comprehensive listing of globular molecules and some skeleton structures with low entropies of fusion was compiled by Ubbelohde (66). Interested readers should consult it for their possible applications.

Interstitial Solid Solution—In this type of solid solution, the solute (guest) molecule occupies the interstitial space of the solvent (host) lattice. A schematic diagram is shown in Fig. 7. It usually forms only a discontinuous (limited) solid solution. The size of the solute is critical in order to fit into the interstices (34, 54). It was found that the apparent diameter of the solute atom should be less than 0.59 that of the solvent (54) in order to obtain an extensive interstitial solid solution of metals. From this, one may calculate that the volume of the solute should be less than 20% of

the solvent. It is likely that the principle can also be applied to organic compounds. Water-soluble crystalline polymers of high molecular weight appear to be logical choices for this type of solid solution of insoluble drugs, since the molecular weight of most organic drugs is usually less than 1000. Low toxicity and lack of absorption from the GI tract are the advantages of polymer carriers.

Polyethylene glycols of 4000, 6000, and 20,000 molecular weights are crystalline, water-soluble polymers with two parallel helixes in a unit cell (67). It is predicted that significant amounts of drug can be trapped in the helical interstitial space when polyethylene glycol–drug melts are solidified. Such systems were prepared using griseofulvin, digitoxin, methyltestosterone, prednisolone acetate, and hydrocortisone acetate in the matrix of polyethylene glycol 6000. They all possess a fast rate of dissolution (11, 38). The results of these dissolution studies, except for griseofulvin, are summarized in Table I. The griseofulvin dispersed in polyethylene glycol 4000 and 20,000 was also shown to have a marked increase in dissolution rate (11). Indomethacin dispersed in polyethylene glycol 6000 was also shown to produce a faster dissolution rate (68).

In addition to the large molecular size of the polymers favoring the formation of thermodynamically stable interstitial solid solutions, other factors such as high viscosity, supercooling, and physical–chemical interaction between the drugs and the polymers may contribute to the formation of metastable solid solutions if the drug–polyethylene glycol melt is solidified rapidly. The melt of polyethylene glycol polymers is highly viscous, even at a temperature of 200° (67). Furthermore, the viscosity increases rapidly with the decrease in temperature. Therefore, as drug–polyethylene glycol melt is allowed to solidify quickly, the crystallization of the drug is retarded due to reduced solute migration and the difficulty in nucleation of the drug in the viscous medium (11, 64, 69).

Although the melting points of some polyethylene glycol polymers are higher than 50°, they can often be supercooled to below 40° (11). Such supercooling phenomena were also observed with the drug–polyethylene glycol mixture. For example, it was found feasible to supercool 10, 20, and even 40% of griseofulvin in polyethylene glycol 4000 or 6000 to about 40° before solidification started, although their upper melting points (when mixtures completely melt) ranged from about 150 to 200°. The possible physical or chemical interaction between drugs and polyethylene glycol polymers has been well documented, as demonstrated by their solubilization effect in the aqueous medium (45, 72). It is believed that such interaction may also exist in the drug–polyethylene glycol melt and may contribute to the retardation of crystallization of the pure drugs. In the case of griseofulvin, its solubility was found to increase onefold in the 7% (w/w) polyethylene glycol 6000 aqueous solution (41).

The possibility of the existence of a metastable solid solution of a drug in polyethylene glycol was investigated in quenched 5% griseofulvin–95% polyethylene glycol 4000 and 5% griseofulvin–95% polyethylene glycol 6000 (41). The freshly quenched samples of both systems showed no noticeable X-ray diffraction peaks of the crystalline griseofulvin, while their powdered samples exhibited such peaks. It was suggested that the powdering process might cause some of the supersaturated griseofulvin in the metastable solid solution to precipitate out. Therefore, the solid solubility of griseofulvin in polyethylene glycol 4000 or 6000 is much less than 5%. The X-ray diffraction spectra of the griseofulvin–polyethylene glycol 6000 system are shown in Figs. 8 and 9. Similar findings were also reported for the 10% indomethacin–90% polyethylene glycol 6000 solid dispersion (68). In 10 and 20% griseofulvin dispersed in polyethylene glycol 6000, both the pulverized and nonpulverized quenched samples showed the diffraction spectra of crystalline griseofulvin. This is because the concentrations of griseofulvin now exceeded its maximum solid solubility in the polyethylene glycol.

In addition to working as a universal solvent for the formation of stable or metastable limited solid solutions of most drugs, the polyethylene glycol can also be expected to produce an ultrafine or colloidal crystallization of the pure drug if its concentration is much greater than its solid solubility and the drug–polyethylene glycol melt is solidified rapidly (11). This is mainly due to the difficulty of growth of the crystallite in a highly viscous medium and the short time interval for the completion of solidification. This is often referred to by some surface chemists as the transition from primary to secondary nucleation. The phenomenon is well known and is taken advantage of in the preparation of single crystals in microelectronics. It is also the method by which doped crystals are prepared to render specific physical properties in a system in which a material is crystallized in a retarded manner due to solute depletion in the immediate environment affecting crystal growth. The highly possible physical–chemical interaction between the drug and polyethylene glycol may also play a role in preventing the crystalline growth. Such a contention is indirectly supported by a recent study of the ability of polyvinylpyrrolidone to inhibit the crystalline growth of sulfathiazole and methylprednisolone in

Table I—Twenty, Fifty, and Seventy Percent Dissolution Times for Selected Drugs in Various Physical Forms in Half-Saturation Dissolution Test

Preparations	T_{20}, min.	T_{50}, min.	T_{70}, min.
Pure prednisolone acetate[a]	8.0	45.0	—
Fused mixture of prednisolone[a] acetate–polyethylene glycol 6000 (5:95 w/w)	≪1.0	≪1.0	~0.6
Pure 17-methyltestosterone	2.0	12.0	28.0
Fused mixtures of 17-methyltestosterone–polyethylene glycol 6000 (5:95 w/w)	≪1.0	≪1.0	~0.6
Pure hydrocortisone acetate	20.0	—	—
Fused mixture of hydrocortisone acetate–polyethylene glycol 6000 (5:95 w/w)	≪1.0	≪1.0	1.5
Pure microcrystalline digitoxin	15.0	80.0	—
Fused mixture of digitoxin–polyethylene glycol 6000 (2:98 w/w)	≪1.0	≪1.0	0.3–0.5

[a] This test system utilized only 30% saturation.

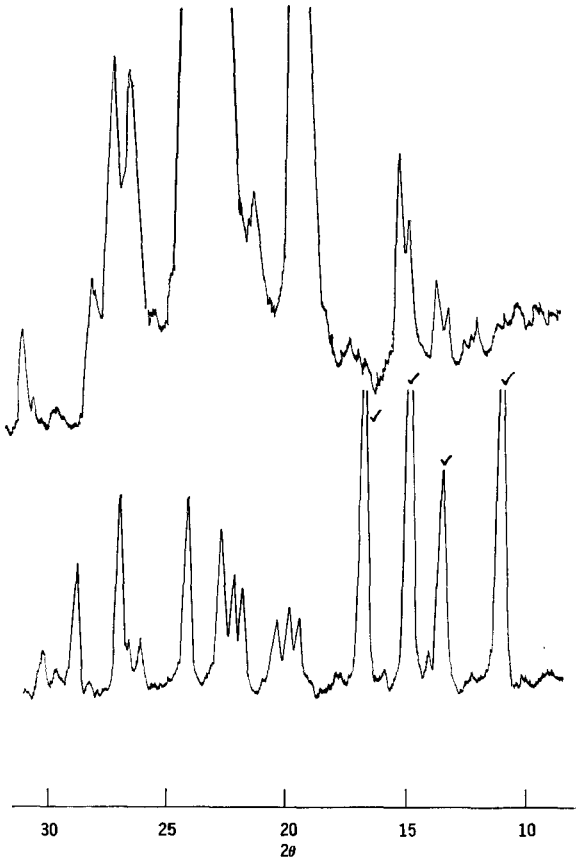


Figure 8—*X-ray diffraction spectra of pure griseofulvin (bottom) and pure polyethylene glycol 6000 powders (top).*

water, even at a very low concentration (73). The adsorption of the polyvinylpyrrolidone on the crystalline surface was used to explain such a phenomenon. It seems logical to assume that the polyethylene glycol polymer may also act as a protective colloid in retarding the coagulation, aggregation, or coarsening of the fine crystallites before solidification. The possibility of an ultrafine or colloidal dispersion of drugs in polyethylene glycol polymers is demonstrated by the fact that even the solid dispersion of 40% griseofulvin–60% polyethylene glycol 6000 showed a faster dissolution rate than the wetted micronized griseofulvin (11). It is believed that this rationale for employing polyethylene glycol polymers as ideal solid-dispersing carriers may also be applied to other soluble polymers. As mentioned, the short interval of solidification is critical in the formation of metastable solid solutions from the viscous melt of drug–polyethylene glycol systems. Therefore, in the solvent method of preparation, the control of temperature and time of evaporation are very important to the final physical properties of the solid dispersions (11). It was found that big crystals of griseofulvin were formed if the griseofulvin–polyethylene glycol 6000–ethanol mixture was kept at high temperatures (*e.g.*, 120°) for a relatively long period (0.5–2 hr.).

A patent was obtained for the use of water-soluble polymers such as polyethylene glycol, polyoxyethylene esters or ethers, polyoxyethylene sorbitan esters, or their mixtures that form solid solutions of insoluble estrogens for preparation of pessary dosage forms (74). The estrogen was claimed to be precipitated in an extremely fine state of subdivision when the preparation was placed in water. The concentration of the drug preferred was below 20%. This patent may not be known to many research workers in this area, and no experimental data in the pharmaceutical literature could be found to support the claim. One interesting suggestion in the patent is that the inclusion of effervescent materials, such as combinations of sodium bicarbonate and citric or tartaric acid, would increase the distribution (or dispersion) of the drug upon exposure to an aqueous medium. No oral application of such dosage forms was advocated.

Glass Solutions and Glass Suspensions—The concept of formation of a glass solution (75) was first introduced by Chiou and Riegelman (11) as another potential modification of dosage forms in increasing drug dissolution and absorption. Since physical–chemical properties of glass solutions have not been adequately discussed in the pharmaceutical literature, they are briefly reviewed in this article. A glass solution is a homogeneous, glassy system in which a solute dissolves in a glassy solvent. The familiar term "glass," however, can be used to describe either a pure chemical or a mixture of chemicals (window glass is a mixture of inorganic oxides) in a glassy or vitreous state. The glassy or vitreous state is usually obtained by an abrupt quenching of the melt (76, 77). It is characterized by transparency and brittleness below the glass-transforming temperature, T_g. On heating, it softens progressively and continuously without a sharp melting point. This is primarily due to the facts that the chemical bonds in the glass differ considerably in length and, therefore, in strength and that there is no one temperature at which all the bonds become loosened simultaneously (34). The glassy form of pure compounds can often be transformed to a crystalline state upon heating. It is likely that such transformation may also

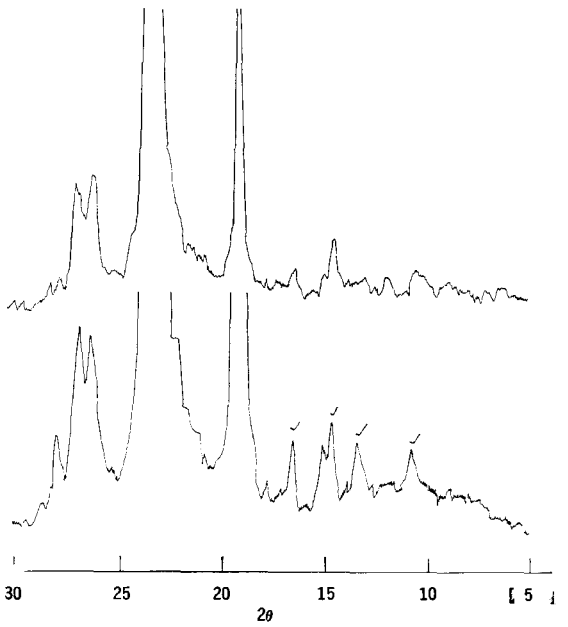


Figure 9—*X-ray diffraction spectra of solid dispersion of 5% griseofulvin–95% polyethylene glycol 6000. The top spectrum was obtained from a nonpowdered sample, and the bottom spectrum was obtained from a powdered sample.*

occur in some glassy solutions. Usually the thermodynamic properties of a glass, such as specific volume, specific heat, viscosity, refractive index, compressibility, and thermal conductivity, all show critical change around the temperature T_g.

The relation of the volume between the glassy, liquid, and solid states is shown in Fig. 10 (76). As the liquid is cooled through the freezing point, T_f, it may either freeze into a crystalline solid, with a discontinuous change in volume, or it may continue as a supercooled liquid below this temperature. Many substances may behave in either way, according to circumstances. For example, supercooling is increasingly likely to occur if the presence of any nuclei is carefully avoided. The viscosity of a supercooled liquid may be so great that the behavior of the material starts to appear indistinguishable from that of an ordinary solid. If the liquid is further cooled rapidly, a change in slope of the volume–temperature curve occurs and the new slope is often nearly the same as that of the corresponding curve for the crystal. The temperature at which the curve changes slope is called the glass-transforming temperature, T_g. Below T_g, the curve is no longer an equilibrium curve. Therefore, a glass or glass solution is metastable. It is also interesting to note that any liquid or supercooled liquid whose viscosity is greater than 10^{13} poises is generally called a glass (75).

A crystalline solid possesses both long-range and short-range orders of structure, whereas a glass or liquid has a structure only with a short-range order (76, 78). This can be differentiated easily by X-ray diffraction methods. A glass or liquid can only produce weak and diffuse diffraction effects, while crystallites can give strong and sharp diffraction effects (76, 79). In this sense, a glass is also amorphous to X-ray diffraction.

Many compounds have been shown to be able to form glasses readily upon cooling from the liquid state. These compounds include sucrose, glucose, ethanol, and 3-methylhexane (66). Glass formation is common in many polyhydroxyl molecules such as sugars, presumably due to their strong hydrogen bonding which may prevent their crystallization (64). Polymers possessing linear, flexible chains can freeze into a glassy state of transparency and brittleness (66). Glass formation can occur for the pure substance itself or when in the presence of other components. If a water-insoluble drug forms a glass solution with a water-soluble, glass-forming carrier, then the *in situ* dissolved drug is released into the aqueous medium rapidly because the carrier quickly dissolves upon exposure to the aqueous medium (11).

There is usually a relatively strong chemical binding between the solute and the solvent in the solid solution (4), while the lattice energy in the glass solution is expected to be much less because of its similarity with the liquid solution. Similarly, the dissolution rate from a crystal is usually faster than from an amorphous or glassy solid of the same chemical identity. Therefore, if everything is equal, the dissolution rate of drugs in the glass solution should be theoretically faster than that in the solid solution. There is another important advantage of glass solutions over solid solutions. When

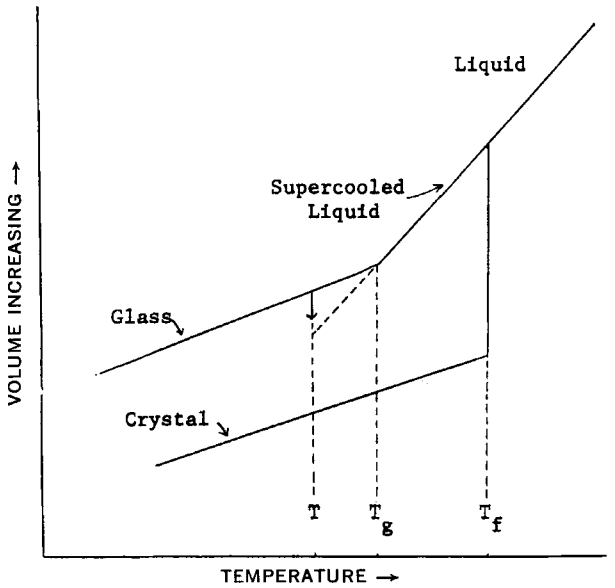


Figure 10—*Relation between the glassy, liquid, and solid states (from* Reference 76, *reprinted with permission).*

the content of the solute exceeds the solubility in both solutions at ambient temperatures, the particle size of crystallization of the solute is much smaller in the glass solution due to the difficult growth of the crystal in its viscous medium. A higher supersaturation of the drug in the glass solution is also more likely to take place if the extremely viscous melt is cooled rapidly.

Citric acid, a normal constituent of animals, was found capable of glass formation (11). The melt is highly viscous and can be drawn into a thread or sheet[2]. After standing at 37° for a few days, a hard, brittle, and transparent glass can be obtained. However, this glassy state was transformed into a crystalline state after months of standing at room temperature. Glassy solutions were obtained after the cooling of melts of 5 and 20% griseofulvin (11), 10% phenobarbital, and 10% hexobarbital (12). A marked increase in the dissolution rate of griseofulvin in the citric acid glass solution was reported (11). The potential usage of citric acid and the previously mentioned glass-forming polyhydroxyl compounds as water-soluble carriers remains to be investigated.

The properties of a glass may be related to the method of solidification or cooling (79). The particle-size distribution in the crystallization of benzophenone in hydrocarbon glass was shown to be a function of the cooling rate, ranging from being invisible to opaque in appearance as the rate of cooling was prolonged (80). A term of "glass suspension" is proposed here to refer to a mixture in which precipitated particles are suspended in a glassy solvent.

Pure polyvinylpyrrolidone and some other polymers dissolved in the organic solvents may become glassy after the evaporation of the solvents. It is possible that the precipitation of drugs introduced into the system is inhibited due to the increase in viscosity as the solvents evaporate. Such inhibition may also be

[2] It is entirely possible that the formation of the citric acid glass is partially due to decomposition of some molecules by dehydration into aconitic acid.

Table II—Dissolution Studies of Griseofulvin[a]

Sample	Relative Dissolution Rate 1 min.	4 min.
Micronized griseofulvin	1.0	1.0
Griseofulvin–chloroform solvate	0.5	0.4
Griseofulvin–polyvinylpyrrolidone (1:5)	6.1	5.1
Griseofulvin–polyvinylpyrrolidone (1:10)	7.2	6.1
Griseofulvin–polyvinylpyrrolidone (1:20)	11.0	7.3

[a] Obtained from *Reference 24*.

facilitated by the possible complexation between the drug and the polymer. Thereby, a transparent, brittle, glassy solution is formed. This principle of glass formation probably best explains the rationale behind the polymer approach suggested by Tachibana and Nakamura (23) and Mayersohn and Gibaldi (24). The amorphous and glassy property of polyvinylpyrrolidone is also evidenced by its diffuse, broadening, X-ray diffraction spectra (25, 41). Evidence for molecular dispersion of drugs in polyvinylpyrrolidone (*i.e.*, glass solution) is provided by use of the UV method for β-carotene (23), high-resolution electron microscope method for iopanoic acid (41), and X-ray diffraction method for sulfathiazole (25) and iopanoic acid (41). By the same reasoning as was discussed for the polyethylene glycol carrier, the crystallite size of the drug may also be very fine if the drug concentration greatly exceeds its solubility in polyvinylpyrrolidone. The crystallization was found to occur at the higher concentration of sulfathiazole by the X-ray diffraction method (25). Amorphous precipitation of iopanoic acid was also found in the 50% iopanoic acid–50% polyvinylpyrrolidone 10,000 coprecipitate by the electron microscope technique (41). These systems also appear to be metastable since crystallization has been initiated in fissures or cracks in the glass on standing.

Due to the chemical stability of polyvinylpyrrolidone to heat (81) and its high melting point (probably decomposing before melting at a temperature beyond 250°), the drug–polyvinylpyrrolidone solid dispersions can only be prepared by the solvent method. Polyvinylpyrrolidone is also soluble in a variety of organic solvents (81), an advantage in accommodating various drugs which possess limited solubility properties. The marked enhancement of griseofulvin dissolution from the coprecipitate is shown in Table II (24). Almost 100% supersaturation was also obtained in 1 min. Such a striking effect is also reported for reserpine (30). For a 1:6 reserpine–polyvinylpyrrolidone coprecipitate, a 200-fold increase in dissolution was found in comparison with the equal particle size of the pure drug. The dissolution rates of the drugs decreased as the concentrations of the drugs in the coprecipitates increased in both systems. Probably this is mainly due to the increase of particle size of the drugs in the higher concentration compositions (30).

Table III—Experimental Relative Release Rates of Sulfathiazole as a Function of Polyvinylpyrrolidone Weight Fraction[a]

Polyvinylpyrrolidone Weight Fraction	Absolute Sulfathiazole Release Rate		Relative[b] Sulfathiazole Release Rate	
	Initial	Limiting	Initial	Limiting
0.25 (3:1)	0.135	—	—	—
0.40 (1.5:1)	0.510	0.140	3.78	1.02
0.50 (1:1)	0.520	0.140	3.85	1.04
0.60 (1:1.5)	0.520	—	3.85	—
0.67 (1:2)	0.680	—	5.04	—
0.75 (1:3)	1.155	—	8.90	—
0.83 (1:5)	1.100	—	8.15	—
0.91 (1:10)	0.934	—	6.91	—
0.95 (1:20)	0.450	—	3.33	—

[a] Obtained from *Reference 25*. [b] Relative to a pure sulfathiazole crystalline Form I tablet.

Simonelli *et al.* (25) presented thorough experimental studies to elucidate the dissolution mechanisms from a constant surface for compressed tablets of polyvinylpyrrolidone–sulfathiazole coprecipitates. The enhancement of dissolution rate was found to be a function of the molecular weight of polyvinylpyrrolidone, the concentration of sulfathiazole in the coprecipitates, and, in some instances, the dissolution medium and time. A model was presented to describe dissolution mechanisms of the coprecipitates and physical mixtures over a wide range of composition. For the coprecipitates, it was concluded that the sulfathiazole was the controlling external layer at lower polyvinylpyrrolidone weight fractions and the polyvinylpyrrolidone at higher weight fractions. For details, interested readers are urged to consult this detailed original paper. The relative release rates of sulfathiazole as a function of the polyvinylpyrrolidone weight fraction are shown in Table III. In 40 and 50% polyvinylpyrrolidone samples, the release rates were not linear but changed with time.

Several points arising from the Simonelli *et al.* (25) paper seem to warrant further discussion. The possible effect of molecular dispersion (in this case, glass solution) and colloidal dispersion of sulfathiazole in the polyvinylpyrrolidone on the dissolution rate of sulfathiazole was ignored by the authors. The necessity of taking the molecular dispersion into account for the enhancement of dissolution rate from tablet forms with a constant surface was clearly demonstrated by an approximately 10-fold increase in dissolution rate from a solid solution of 10% indomethacin–90% polyethylene glycol 6000 and also a threefold increase from a solid solution of 5% sulfathiazole–95% urea over the physical mixtures with the same chemical composition (68). A tablet made of 10% griseofulvin–90% succinic acid eutectic mixture was also found to dissolve about threefold faster than the mechanical mixture of 10% micronized griseofulvin–90% succinic acid (53). Such effects are more likely to take place at the higher weight fractions of a carrier.

In their dissolution model, Simonelli *et al.* (25) proposed polyvinylpyrrolidone as the controlling external layer at higher polyvinylpyrrolidone fractions. The identity of the controlling layer can easily be determined by comparing the relative movement of the solid–liquid boundary of each component (25, 31). On the basis of the dissolution data shown in the original article, in the first 20 min. the ratios of the movement of polyvinylpyrrolidone over sulfathiazole at compositions of 1:20, 1:10, and 1:5 (sulfathiazole–polyvinylpyrrolidone) were found to be all close to 1. These ratios indicate that both components were released

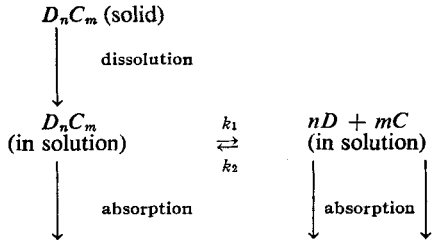
almost simultaneously from the tablets. This finding is contradictory to the dissolution model proposed by Higuchi (31), which defines a congruent dissolution from a binary mixture tablet only taking place at a single, fixed composition. This is valid only when the solubilities of the two components remain constant. It is well known that the magnitude of solubility increases as the particle size reduces to submicron or colloidal range (45). In the solid solution or glass solution of a drug in the soluble carrier, the maximum concentration of a drug at the dissolution interface is undoubtedly much higher than the regular solubility. Furthermore, colloidal or molecular particulates probably cannot aggregate or agglomerate into bigger particles in the short time that they exist at the dissolution surface. If this is true, it is difficult to define the solubility value at different weight fractions of solid dispersions.

The theoretical dissolution rates of sulfathiazole in the higher polyvinylpyrrolidone fractions, calculated according to the model proposed by Simonelli *et al.* (25), imply that similar dissolution rates also can be obtained from the physical mixtures. Although this has not been proved experimentally, it is regarded as unlikely in light of the striking increase of dissolution rates of the drugs dispersed in polyvinylpyrrolidone in powdered forms (24, 30).

Amorphous Precipitations in a Crystalline Carrier—Instead of forming a simple eutectic mixture in which both the drug and the carrier crystallize simultaneously from a melting or a solvent method of preparation, the drug may also precipitate out in an amorphous form in the crystalline carrier. Since the amorphous form is the highest energy form of a pure drug, it should, under almost all conditions[3], produce faster dissolution and absorption rates than the crystalline form whether the crystals are or are not dispersed in a carrier. Amorphous novobiocin has 10-fold higher solubility than its crystalline form (82). A much faster dissolution rate and higher blood levels were also found for the amorphous form of novobiocin (82). As discussed previously, the amorphous sulfathiazole dispersed in the crystalline urea was believed to be a primary contributing factor in increasing its oral absorption in man (17). It is postulated that a drug with a high supercooling property has more tendency to solidify as an amorphous form in the presence of a carrier.

Compound or Complex Formations—In a strict sense, the modification of a dosage form by a compound or complex formation (D_nC_m) between a drug (D) and an inert soluble carrier (C) should not be classified under the applications of solid dispersion systems. Nevertheless, due to their frequent occurrence during preparation of solid dispersions by the standard methods, it seems worthwhile to review them here briefly.

The dissolution and absorption of a drug into the body from a complex or a compound are schematically shown in Scheme I. It is clear from Scheme I that the availability of a drug depends on the solubility, the dissociation constant, and the intrinsic absorption rate



Scheme I

of the complex. Although the water-soluble polymers have been considered as ideal carriers for the solid dispersion of poorly soluble drugs, the implication of the possible complexation should not be overlooked. Polyvinylpyrrolidone was shown to retard the pharmacological action of numerous compounds such as penicillin, novocaine, prostigmine, hexobarbital, quinine (83), and hexylresorcinol (84). The formation of an insoluble complex between phenobarbital and polyethylene glycol 4000 or 6000 was shown to reduce rates of dissolution and permeation of phenobarbital through everted guts of rats (85). The complexation between griseofulvin and polyethylene glycol 6000 may be thought to occur on the basis of the traditional solubility study. (The solubility is increased onefold by the presence of 7% polyethylene glycol 6000 in water.) Such a water-soluble weak complex apparently did not retard the oral absorption of griseofulvin in man and dogs (27–29). It is believed that in comparison with pure, insoluble, solid drugs, the rates of dissolution and GI absorption can be increased by the formation of a soluble complex with a low association constant.

The compound formation among simple organic chemicals seems more common than expected. Among 12 phase diagrams, Sekiguchi *et al.* (51) found 11 cases of compound formations. Guillory *et al.* (86) reported four compound formations out of nine phase diagrams studied. However, the occurrence of these compound formations, which previously took place at melt state, does not necessarily mean that they will also take place in a liquid medium. On the other hand, the existence of compound or complex formation in a liquid medium does not predicate its occurrence in the solid state. This is shown in the griseofulvin–succinic acid system. Although the solubility of griseofulvin was increased markedly by the succinic acid in water (approximately onefold per 1.5% succinic acid), their interaction could not be detected by the phase diagram study (53).

Combinations and Miscellaneous Mechanisms—Quite often a solid dispersion does not entirely belong to any of the four groups discussed but is made up of combinations of different groups. Therefore, the observed increase in dissolution and absorption rates may be the contribution of different mechanisms. The griseofulvin dispersed at high concentrations in polyethylene glycol may exist as individual molecules and as microcrystalline particles. The sulfathiazole dispersed at high concentrations in polyvinylpyrrolidone may be present as individual sulfathiazole and sulfathiazole–polyvinylpyrrolidone complex molecules, amorphous and polymorphic sulfathiazole, and possibly an amorphous sulfathiazole–polyvinylpyrrolidone complex.

[3] A large amorphous mass with entrapped air probably will not dissolve faster than microcrystals dispersed in a water-soluble carrier.

The coprecipitates of reserpine with bile steroids such as deoxycholic acid (39), cholic acid, lithocholic acid, and 3,12,24-trihydroxycholane (87) were shown to increase blepharoptotic activity of reserpine in mice. The exact physical properties of such systems have not been elucidated. A decrease in the particle size of reserpine in the coprecipitates was proposed from the *in vitro* dissolution studies (88, 89). The ability of these carriers to reduce the surface tension of aqueous fluids led Stoll *et al.* (89) to propose that the carriers may also facilitate the wetting and, hence, the dissolution rate of reserpine. Since these bile steroids can form clathrate compounds (inclusion compounds) with a variety of organic molecules (90), it is possible that this may also occur with reserpine and thus cause molecular or ultrafine dispersion of reserpine in the hollow channels of the clathrates.

METHODS OF DETERMINATION OF TYPES OF SOLID DISPERSION SYSTEMS

Many methods are available that can contribute information regarding the physical nature of a solid dispersion system. In many instances, a combination of two or more methods is required to study its complete picture. The advantages and disadvantages of each method are briefly expounded here.

Thermal Analysis—This is the most common approach used to study the physicochemical interactions of two or more component systems. Several modified techniques utilizing the principle of change of thermal energy as a function of temperature are discussed separately.

Cooling-Curve Method—In this method, the physical mixtures of various compositions are heated until a homogeneous melt is obtained. The temperature of the mixture is then recorded as a function of time. From a series of temperature–time curves, the phase diagram can be established (33, 34). The method suffers from many inherent disadvantages. It is time consuming, it requires a relatively large amount of sample, and changes in slopes can be missed, especially if cooling takes place rapidly (86). In addition, the method cannot be applied to samples that decompose after melting. It is also difficult to detect samples with small solid–solid solubility. This method was recently used to determine phase diagrams of deoxycholic acid–menadione and caffeine–phenobarbital (86).

Thaw-Melt Method—In this method, a sample of a solidified mixture in a capillary melting-point tube is heated gradually. The thaw point is referred to a temperature on crossing a solidus line (33). This simple method was used extensively by Rheinboldt (91), Rheinboldt and Kircheisen (92, 93), and Guillory *et al.* (86). A stirring device in the capillary tube was employed for more accurate results by Sekiguchi *et al.* (94). The stirring facilitates the attainment of a homogeneous system; however, such stirring only affects the melting point and not the thaw point. In differentiating between a simple eutectic system and a limited solid solution, the diagnostic point lies at the thaw point. Therefore, the usage of this more complicated device is not necessary for such a purpose. The principal drawback of this thaw-melt method is that it depends on a subjective observation and, thereby, is not highly reproducible (86). This is especially serious for the determination of thaw points. A range of six degrees of variation was reported in the study of thaw points of a chloramphenicol–urea system (51). Furthermore, a suitable, upper range of melting points is only limited to about 300° due to the problem associated with capability of visualization (86, 94). The sample used for study may also be prepared from merely the physical mixture or the evaporated mixture obtained after removing the liquid solvent from the solution (94). Thaw points are often found at lower temperatures from the samples of physical mixtures, while the melting points are not affected (94). A special quenching method is proposed for samples exhibiting supercooling properties (33). A mixture that has not completely solidified results in lower thaw and melting points upon reheating. This was observed in the eutectic composition of a sulfathiazole–urea system (56).

Thermomicroscopic Method—Goldberg *et al.* (20) used polarized microscopy with a hot stage to study phase diagrams of binary systems. The physical mixture is placed on a slide covered with a cover slip and sealed with silicone grease to prevent sublimation. The mixture is heated until it completely liquifies. After cooling, the mixture is heated at the rate of 4°/min. The thaw and melting points are then determined by visual observation. The advantages of this method are that it is simple and it requires only a small amount of sample. However, it suffers some disadvantages by often being subjective, limited to thermally stable compounds, and potentially inhomogeneous in distribution after resolidification. Furthermore, the melting of isotropic crystals often cannot be detected accurately under a polarizing microscope (95). The existence of a limited solid solution of griseofulvin in succinic acid determined by this method (21) appears to have been disproved by the DTA and X-ray diffraction method (53). The Köfler contact method, also utilizing polarizing microscopes, was proposed to establish various forms of phase diagrams (95). However, the usage of such a technique seems to require a good knowledge of crystallography.

DTA—DTA is an effective thermal method for studying phase equilibria of either a pure compound or a mixture. Differential effects, associated with physical or chemical changes, are automatically recorded as a function of temperature or time as the substance is heated at a uniform rate (96). In addition to thawing and melting, polymorphic transitions, evaporation, sublimation, desolvation, and other types of decomposition can be detected. Apparatus permitting direct observation of samples during heating were used to facilitate the observation of any physical–chemical changes (97).

The greatest advantage of using this technique is in constructing phase diagrams of high reproducibility; a higher temperature range is permitted, and greater resolution results (52). A sample size of less than 1 mg. can be used for measurement with some commercial instruments. Although the sensitivity and accuracy of the DTA thermograms can be influenced by many

factors such as sample size, heating rate, sample geometry, thermal conductivity of the sample container, and method of measurement of the sample temperature, these variables can be adjusted to optimize the desired characteristics of the DTA apparatus (52).

The DTA method was used extensively to construct phase diagrams of a number of binary systems (51, 52, 86, 98–110). The correlation of DTA data with most frequently encountered phase diagrams is shown in Figs. 11 and 12. This technique is especially valuable in detecting the presence of a small amount of eutectic in the mixture, because its melting at the eutectic temperature can be sensitively detected (98). The observation of such small fractions of melting at eutectic temperature can often be missed when employing thaw-melt or thermomicroscopic methods.

Zone Melting Method—This technique was first introduced in 1952 (111). It has been primarily used for ultrapurification of metals and inorganic and organic compounds. The phase diagram can be constructed for metals and inorganic and organic compounds. A molten zone effected by a heater traverses a cylindrical ingot or solidified melt at a rate of about 0.5–0.001 cm./hr. A mechanical stirring device is also required for the mixing of the liquid in the molten zone. After zone melting is finished, the bar is sectioned and analyzed for its chemical composition. From their chemical compositions and freezing temperatures of the corresponding sections, a phase diagram of a binary or multicomponent system can be constructed. This method is limited to compounds with high thermal stability and low volatility (111, 112). It is especially valuable in determining the exact chemical composition of a eutectic and the minute solid–solid solubility at the eutectic temperature by merely a single pass. The solubility of InSe in InSb was found to be less than 1%; that of InSb in InSe was also found to be less than 1% by this method (113). Many phase diagrams of metal systems have been determined by this method (114–118).

X-Ray Diffraction Method—In this method, the intensity of the X-ray diffraction (or reflection) from a sample is measured as a function of diffraction angles. Counter and film methods detect the diffraction intensity. The advantages and disadvantages of these two methods were well discussed (119, 120). In the former method, a better resolution of diffraction peaks can be obtained, and it is also easier to compare their relative diffraction intensity. However, it requires more sample and has less reliability and more sensitivity to sample preparation and position. The latter method is more sensitive for the detection of weak lines.

The diffraction method is a very important and efficient tool in studying the physical nature of solid dispersions. Recently, it was used to study binary eutectic systems of chloramphenicol–urea (50) and griseofulvin–succinic acid (53). Many phase diagrams of inorganic and metal compounds were also determined by this method (121–125).

In simple eutectic systems, diffraction peaks of each crystalline component can be found in the diffraction spectra. In a substitutional solid solution, the lattice parameter of the solvent crystal is either increased, unchanged, or decreased, depending on the relative

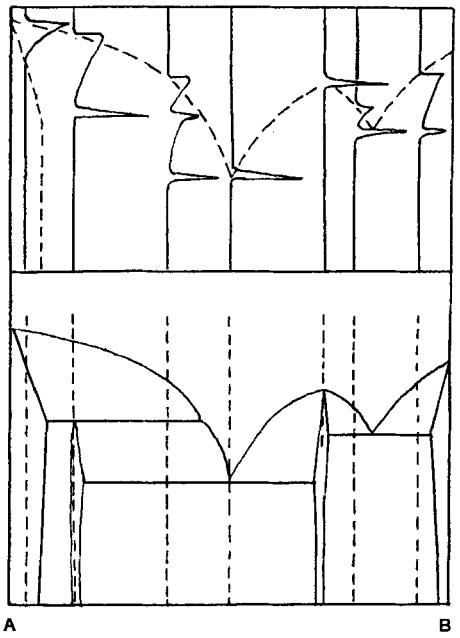


Figure 11—*Typical DTA thermograms corresponding to a hypothetical binary system (from* Reference 52, *reprinted with permission).*

size of the solute atom or molecule (55). However, a gradual shift in the positions of the diffraction lines with changes in composition, which reflects the resulting change in the lattice parameter, is accepted generally as sufficient evidence for the existence of solid solutions. In a system of a continuous solid solution, there will be a shift from the position in one pure component to those in the other (126). The interruption of this smooth change is indicative of immiscibility in the system. The change of lattice parameter, unit cell volume, and density in a continuous solid solution of ammonium chloride–ammonium bromide is shown in Fig. 13. In an interstitial solid solution, the diffraction spectra of the solvent component may or may not

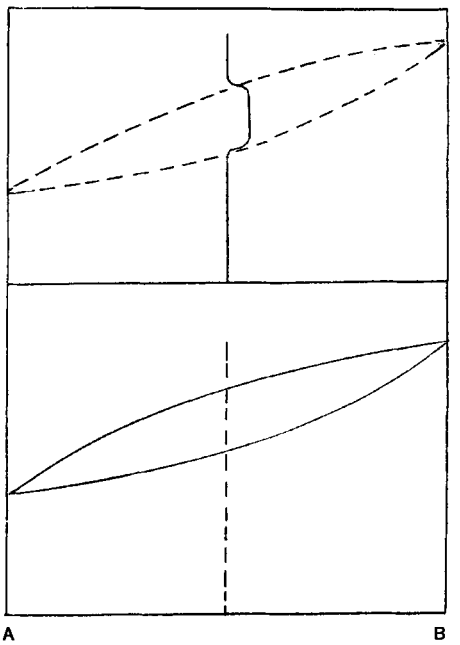


Figure 12—*A DTA thermogram of a continuous solid solution system (from* Reference 52, *reprinted with permission).*

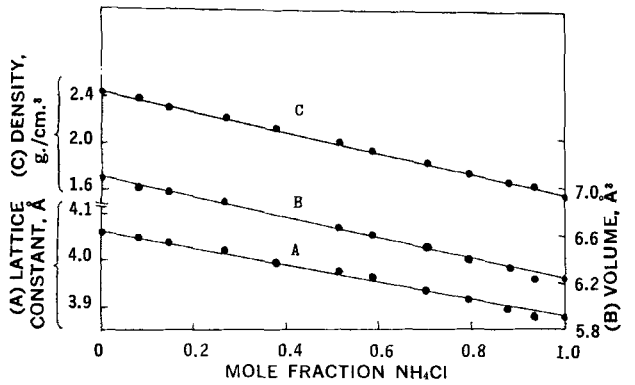


Figure 13—*Variation of composition of continuous solid solution of NH_4Cl–NH_4Br system with: (A) lattice constant, Å; (B) unit cell volume, Å³; and (C) density, g./cm.³; low temperature, high-angle data only (from* Reference 125, *Fig. 3, reprinted with permission).*

be changed, while those of the solute component disappear.

The diffraction method is also particularly valuable in detecting compound or complex formation since its spectra or lattice parameters are markedly different from those of pure components. It has been used to disprove the existence of a patented salt formation between penicillin V and tetracycline (126). The biggest drawback of using the diffraction method to study dispersion systems is its frequent inability to differentiate amorphous precipitation from molecular dispersion if the lattice parameter of the solvent component is not changed. This is because of the disappearance of the diffraction peaks or lines of the crystalline solute compound in both systems. This problem is encountered in the lower concentrations of drugs dispersed in polyethylene glycol (41) or polyvinylpyrrolidone (25) polymers. The solidified eutectic of sulfathiazole–urea has a broad (instead of sharp melting point as found for its physical mixture) and lower melting range. This is attributed to the presence of amorphous sulfathiazole. The amorphous form is transformed into a crystalline form after annealing at high temperature, as shown by the appearance of its sharp diffraction peaks (56).

The diffraction method has been used to study quantitatively the concentration of a crystalline component in the mixture (126–128). The ability of this method to quantitate the crystalline component in solid dispersion systems may be limited by its low concentration or weak intrinsic intensity of diffraction. The height of diffraction peaks may be attenuated by a reduction of crystallite size, usually below 0.2 μ. This is also accompanied by a broadening of the peaks (126). An extremely fine crystalline dispersion of sulfathiazole in polyvinylpyrrolidone has also been considered one reason leading to the disappearance of sulfathiazole diffraction peaks (25). Integrated diffraction peak areas were used to study particle-size distribution between 0.002 and 0.2 μ (125).

Microscopic Method—Microscopy has been used quite often to study the polymorphism (47) and morphology of solid dispersions (34, 44, 51, 54, 55, 124, 129). The fine particles of crystallization in the glassy polyvinylpyrrolidone matrix can be readily detected by the polarizing microscope (41). The high resolution of an electron microscope was used to study the dispersed particle size of iopanoic acid in polyvinylpyrrolidone (41). The application of the electron microscope technique is, however, usually limited to chemicals with high atomic numbers (130).

Spectroscopic Method—Visible absorption spectroscopy was used to study the low concentration dispersion of β-carotene in polyvinylpyrrolidone (23). The spectrum of the dispersed β-carotene resembles that of β-carotene dissolved in organic solvents but not that of β-carotene particles. These results indicated that β-carotene is dispersed molecularly in the polymer. The undetected shift of IR bands of the dispersed β-carotene was thought to indicate the absence of the marked interaction between β-carotene and polyvinylpyrrolidone. IR spectroscopy was also used to study the solid solutions of nitrite ion in many inorganic halides such as KBr, NaCl, and KI (131, 132).

Dissolution-Rate Method—The dissolution-rate method was recently proposed by Allen and Kwan (68) to study the degree of crystallinity in solid–solid equilibria, especially in temperature regions below solid–liquid equilibria. The method involves comparing the *in vitro* dissolution rates of the solute component from a constant-surface tablet made from molecular dispersion (*i.e.*, solid or glass solution) with a physical mixture of the same chemical composition. The technique is simple to perform, except that in some binary systems the tablet surface may not remain constant due to the leaching of particles into the dissolution medium. Such difficulty was encountered in the mechanical mixture of the high sulfathiazole to polyvinylpyrrolidone ratio tablets (25), solid dispersion of barbital–polyethylene glycol 6000 system (41), and physical mixture of 10% griseofulvin–90% polyethylene glycol 6000 (41). Tablets made up of 10% sulfathiazole–90% urea physical mixture under various pressures were also found to disintegrate almost immediately in the aqueous medium (56). This was primarily due to the almost instantaneous dissolution of urea into water because the solubility of this small molecule compound in water is very high, approximately 1 g. in 1 ml. The dissolution of 10% sulfathiazole–90% urea solid solution from 10–20-mesh granules was also found to be complete almost immediately upon their exposure to water (56). The almost instantaneous dissolution from such dispersion systems will make them difficult to compare quantitatively with the dissolution from physical mixtures.

The application of this method also requires: (*a*) the observed dissolution rate to be proportional to the surface area, (*b*) a reasonably large difference between the dissolution rate of the physical mixture and the corresponding solid solution, and (*c*) the use of the same polymorphic form of a drug in the tablet of the physical mixture as that precipitated out from the solid dispersion (68). Most commercially available sulfathiazole, which was often used to prepare solid dispersions, is polymorphic Form I, while the precipitated sulfathiazole in the sulfathiazole–urea system is polymorphic Form II (56). The dissolution rate of Form II was found to be 1.6 times higher than that of Form I (56).

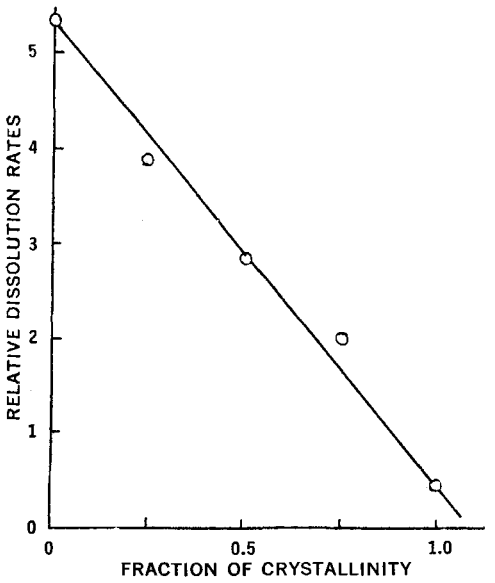


Figure 14—*Dissolution rate* versus *degree of crystallinity of indomethacin in indomethacin–polyethylene glycol 6000 system (from* Reference 68, *reprinted with permission).*

Furthermore, one must assume in this dissolution method that the distribution of particle size (maybe as small as in the subcolloidal range) precipitated from the solid solution or glass solution does not affect the dissolution rate. Such assumption needs to be proved experimentally.

The dissolution-rate method has been shown to be applicable to simulated systems of indomethacin–polyethylene glycol 6000 and sulfathiazole–urea. The data on 10% indomethacin–90% polyethylene glycol 6000 are shown in Fig. 14. The validity of this principle, however, needs further confirmation by other methods.

Thermodynamic Method—The phase diagrams of eutectic and solid solution systems can be constructed on the basis of some thermodynamic parameters (34, 54, 62, 121, 133, 134). A knowledge of heats of fusion, entropies, and partial pressures at various compositions enables one to determine the solubility gap below the solid–liquid equilibrium temperature (133). A solubility gap in the continuous solid solution of the AgBr–NaBr system was also found from thermodynamic data obtained from an electromotive force study by galvanic cells (121). The detailed mathematical discussion of such an approach is beyond the scope of this article.

AGING OF SOLID DISPERSIONS

The solid dispersion appears to be a potential dosage form modification for increasing dissolution and absorption rates of poorly soluble drugs. However, the result of aging or storage under various conditions and the effects on the fast-release characteristics and chemical stabilities have not been reported extensively. Undoubtedly, this will be an interesting and important research subject for pharmaceutical scientists before the wide and long-range practical applications of this unique approach are feasible. The effects of aging in many nonpharmaceutical systems such as alloys and inorganic compounds have been well studied. The purpose of this section is to review these studies with a hope that similar principles and methodologies can be utilized to apply to our systems.

Aging Effects of Eutectic Mixture—It is well known that the dispersed-phase particles tend to coarsen on aging because the interfacial energy of the system is reduced by the concomitant reduction in interface area (129). The phenomenon of particle coarsening was extensively studied both theoretically (135, 136) and experimentally (137–140). This phenomenon occurs in eutectic systems with or without solid solution formation. The extent of coarsening increases with time and aging temperature. The morphology and transparency of a freshly prepared eutectic mixture of naphthalene–phenanthrene were found to change after standing primarily due to recrystallization of fine grains (44).

The increased hardness of freshly prepared eutectics of Pb–Sn systems was found to decrease considerably after annealing (49). Eutectic alloys are more sensitive to corrosion, because in the eutectics the metals are in a somewhat activated or reactivated state (49). It is thought that the displacement of the electrons into higher orbitals facilitate their transfer to a third component, such as oxygen, which is an active agent in corrosion. One should also bear in mind that different polymorphic forms in the solid dispersion may also have different chemical stabilities (47).

Aging Effects of Solid Solution—The most important aging effect from solid solutions is the precipitation from supersaturated solid solutions along with the subsequent changes of physical–chemical properties (33, 34, 54, 55, 63).

The precipitation (also called decomposition or demixing) from a solid solution occurs when the concentration of the solute exceeds its equilibrium solubility. As shown in Figs. 5 and 6, the solubility in the continuous or discontinuous solid solution may decrease with decreasing temperature. When a mixture within the solid solution range at high temperature is quenched from the melt to ambient temperature, a

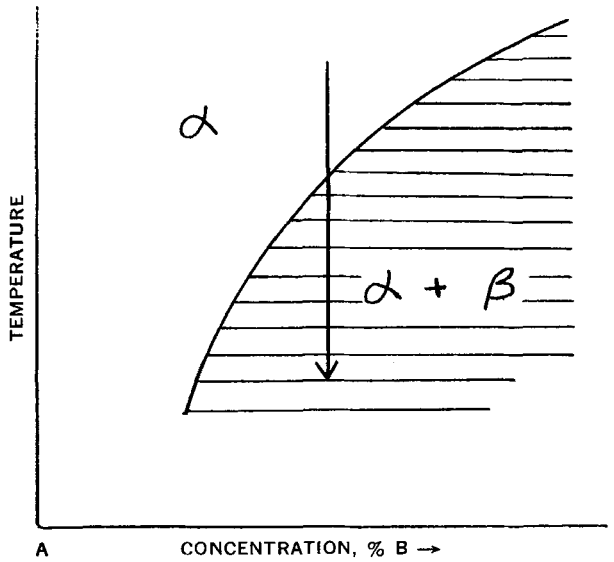


Figure 15—*Phase relation for precipitation. The solid phase, β, precipitates from the solid solution, α, on cooling (arrow) (from* Reference 55, *p. 392, reprinted with permission).*

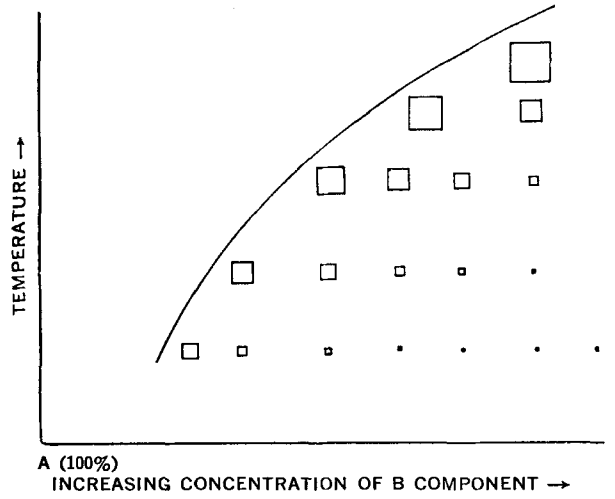


Figure 16—*Diagram illustrating relative nuclei and particle size of precipitation from supersaturated solid solutions at various temperatures and compositions (from* Reference 55, *p. 398, reprinted with permission).*

metastable solid solution is usually obtained. Such excess solute is bound to precipitate out in order to reduce the total free energy of the mixture to a minimum. The phase relations for precipitation are schematically shown in Fig. 15, in which the supersaturated α-phase is transformed into the saturated α-phase and β-solid phase. The β-phase may be a pure crystalline solute, *B*, or a saturated solid solution of the other component, *A*, in the *B* component. The percentage of precipitation can be calculated according to the tieline or lever rule (34, 54, 55).

The particle size and the rate of precipitation certainly have a critical influence upon the dissolution behavior of the dispersed drug. Based on nucleation and growth theory, the relative size of stable nuclei and subsequent precipitation are expected to vary with the composition and storage temperature (Fig. 16). The rate of precipitation is a function of time. After an initial delay of nucleation, it usually proceeds rapidly and finishes slowly (54, 124). A typical example

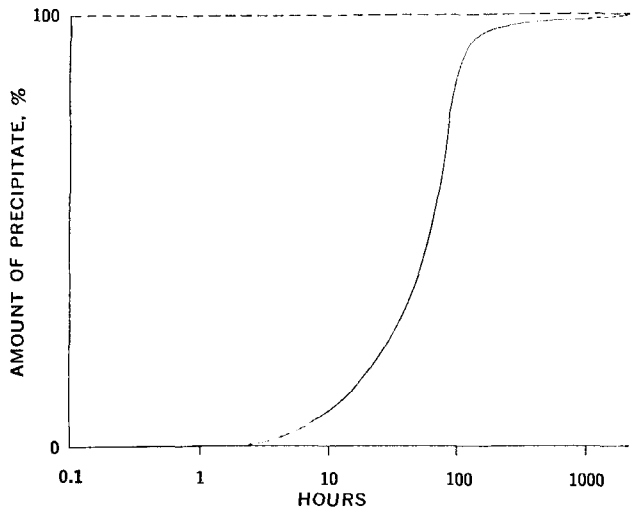


Figure 17—*Amount of precipitate as a function of time in an iron-carbon alloy (0.018% carbon) allowed to precipitate from a supersaturated solution at 76° (from* Reference 54, *p. 239, reprinted with permission).*

of the precipitation of carbon from an iron–carbon alloy annealed at 76° is shown in Fig. 17. The rate of precipitation also varies with temperature (Fig. 18). The rate is slow at very low temperatures because the diffusion rate of molecules is very low. The precipitation rate is also very low at temperatures just below the solvus line. In this case, the solution is only slightly supersaturated, and the free energy decrease resulting from the precipitation is very small. The nucleation rate is accordingly slow, although the diffusion rate at these high temperatures is high. The maximum precipitation rate, therefore, lies at an intermediate temperature as a compensated result of moderate diffusion and nucleation rates.

The presence of precipitation is usually detected by X-ray diffraction (54, 55, 122, 124, 129, 141–143), X-ray small-angle scattering (141), and electron microscopy (54, 55, 124, 129, 141, 143, 144). A change of lattice parameter of the solvent component after aging is considered as definite evidence of precipitation (55). As discussed previously, the capability of X-ray diffractometry may be handicapped by small particle-size effects. Diffraction from particle sizes well below 0.01 μ may not be detected (145). The appearance of second-phase particles in electron microscopy is also indicative of the occurrence of precipitation. The dissolution-rate method was also recently proposed to study precipitation (68).

The effect of precipitation from supersaturated solid solutions on the age-hardening of alloys is well known (34, 54, 55). The extent of this effect is proportional to the amount precipitated. Therefore, the hardening effect is also a function of composition, aging temperature, and time. Holding or aging the preparations for too long a period at a given temperature may also cause them to lose their hardness. This effect is known as overaging (54). The implications of age-hardening on the overall performance criteria (such as dissolution, disintegration, and tableting) of pharmaceutical solid dispersions remain to be further investigated. In addition to the hardening effect, the precipitation also has caused intergranular corrosion with changes in electrical properties, heat resistance, and specific density (55).

Aging Effects of Glass Solution—Since a glass solution is a metastable form, it may be subjected to aging transformation, yielding a more stable form. This may take place rapidly or extremely slowly, as in the case of untreated ordinary window glass kept at room temperature. Small-angle X-ray scattering and electron microscope methods were used to study the kinetics of a metastable amorphous phase separation from CaO–MgO–SiO_2 glass at 825° (146). The growth of amorphous particles was found to be rate limited by the diffusion process. Their average radius is proportional to the square root of annealing time. The crystallization of iopanoic acid and chloramphenicol palmitate dispersed in polyvinylpyrrolidone 10,000 (5% w/w) was detected by polarizing microscope visualization of crystal needles in unpulverized and pulverized samples. These samples were kept at ambient temperature for several months (41). The effect of such precipitation on the dissolution rate should be further studied.

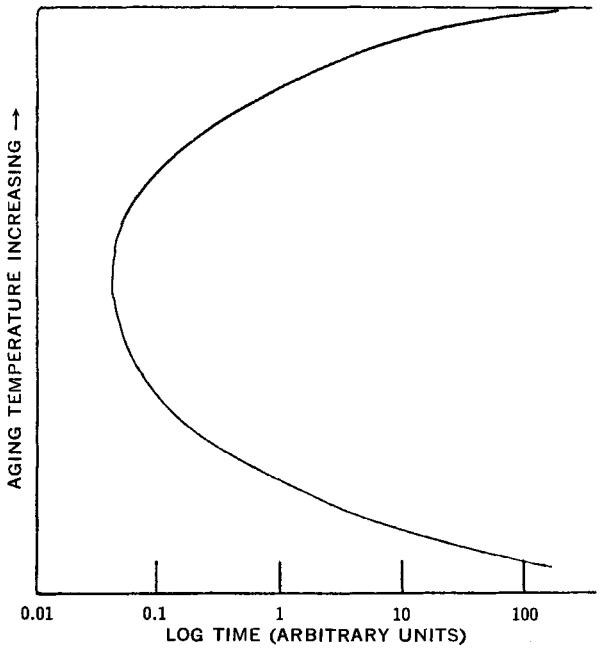


Figure 18—*Diagram illustrating the time for 100% of precipitation from a supersaturated solid solution as a function of aging temperature (from* Reference 54, *p. 240, reprinted with permission).*

Aging Effects of Metastable Polymorphic Forms in Solid Dispersions—The amorphous and other metastable crystalline forms of the dispersed drug in solid dispersions are also subject to aging changes. The importance of this aspect can be seen from the marked difference of dissolution and absorption characteristics between various polymorphic forms of drugs (47). Metastable forms may range from being extremely stable to extremely unstable. Diamond, a crystalline form of carbon, is a good example of the first case. Amorphous form and Form C of chloramphenicol palmitate are examples of the latter case (127).

The methodology for the detection of polymorphic transitions was well reviewed (47). Recently, X-ray diffraction techniques were utilized to study the kinetics of the transformation of amorphous sulfathiazole dispersed in urea at eutectic compositions and their effect on the dissolution rate (56).

REVIEW OF *IN VIVO* STUDIES

Sulfathiazole–Urea Systems—The potential of pharmaceutical applications of solid dispersions was early demonstrated in the human studies of the sulfathiazole–urea system (17). The oral administration of the solidified "eutectic mixture" resulted in a faster and higher rate of absorption than the 50–100-mesh sulfathiazole particles alone on the basis of blood levels and urinary excretion data. The cumulative excretion of the drug and its metabolites in 8 hr. was also 23% higher from the "eutectic mixture." The excretion rate data are shown in Fig. 19. The presence of the urea was found not to interfere with the absorption of the sulfathiazole. The *in vitro* dissolution rate of sulfathiazole will probably be diminished in the presence of urea due to its decreasing solubility in the aqueous solution of urea (17).

Chloramphenicol–Urea Systems—In oral suspension studies in rabbits (51), the solid dispersion of 20% chloramphenicol–80% urea produced a faster and higher absorption in the 1st hr. than the pure chloramphenicol with a similar particle-size distribution (50–100 mesh). The peak value was about 70% higher for the solid dispersion. However, the total areas under the blood level–time curve from both dosage forms were almost the same. When administered in capsule form, the solid dispersion produced a much higher blood level in the first 4 hr. In the first 2 hr., the ratio of blood levels gave a threefold difference. Such marked difference in absorption characteristics obtained in both suspension and capsule forms has not been entirely explained. It is believed that in the capsule case, this difference is a reflection of better wetting and dispersion of solid in the urea system than in the pure, poorly soluble chloramphenicol system. These advantages would become less significant when administered in suspension form. The solid dispersion with eutectic composition (76% chloramphenicol–24% urea) was shown to be inferior in absorption than the pure compound when studied in either the capsule or suspension form.

The finer particle sizes of chloramphenicol obtained in the low concentration of the mixture were proposed to have contributed to its better absorption and the attainment of supersaturation from the lower concentration dispersed form (50, 51). Unfortunately, these studies were conducted on rabbits whose rate of stomach emptying in the feeding and fasting state differs markedly from man. The lack of suitability of using rabbits in evaluating drug absorption was recently raised by Chiou *et al.* (147).

Reserpine–Bile Acid Coprecipitates—A more rapid onset of blepharoptotic activity as well as a significantly increased potency relative to reserpine base was shown in mice after oral administration of reserpine–deoxycholic acid coprecipitates (39). The enhancement generally increased as the concentration of reserpine in the coprecipitates decreased. The only exception was that of the lowest concentration dispersion studied (1:32 molar amounts of reserpine–desoxycholic acid). The physical mixture was also more potent than the reserpine base. These findings were attributed to the enhancement of oral absorption of the drug dispersed

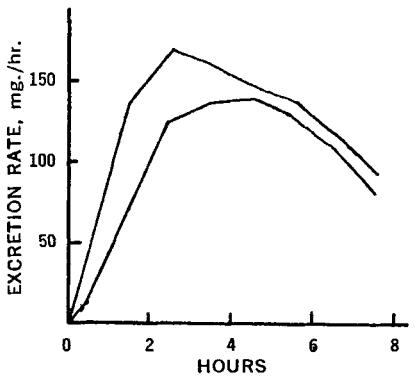


Figure 19—*Average excretion rates of total sulfathiazole in urine after administration of 2 g. of sulfathiazole as a eutectic mixture (top curve) and pure compound (lower curve) to a human subject (from* Reference 17, *Fig. 11, reprinted with permission).*

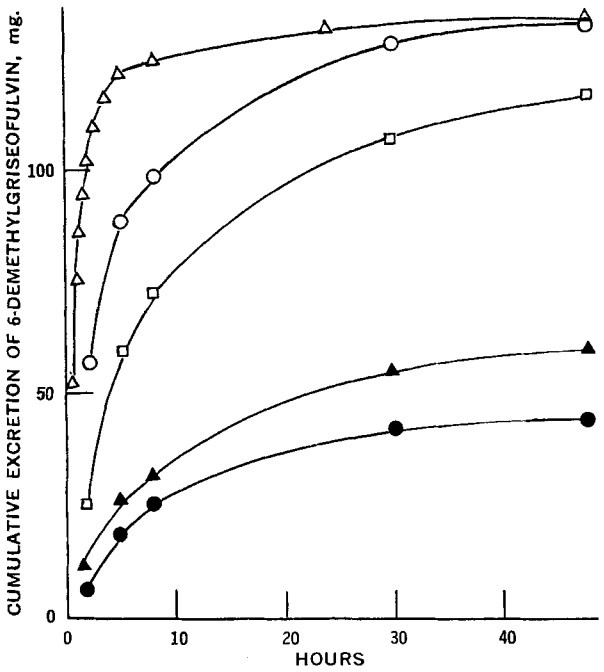


Figure 20—*Average cumulative urinary excretion of 6-demethylgriseofulvin, a major metabolite, after oral and intravenous doses of griseofulvin in dogs. Key: △, intravenous dose; ○, griseofulvin in polyethylene glycol 400 solution; □, griseofulvin dispersed in polyethylene glycol 6000 (10% w/w); ▲, commercial capsule of micronized griseofulvin; and ●, commercial tablet of micronized griseofulvin (all data corrected for 250-mg. dose) (from* Reference 27, *Fig. 2, reprinted with permission).*

in the bile acid. A rank correlation with the *in vitro* dissolution rate was found (88). Similar phenomena of increased blepharoptotic activity in mice were also reported for the reserpine coprecipitates with other bile acids (87). The general application of drug coprecipitates in increasing drug absorption remains to be explored.

Griseofulvin–Polyethylene Glycol Polymers—In none of the *in vivo* studies of three solid dispersion systems discussed here were comparisons made with micronized or microcrystalline powders of pure drugs. The solid dispersion approach will certainly appear unique and valuable if it proves to yield better oral absorption than that obtainable with the commercially available micron-size powders. Such critical evaluation was first carried out in dogs (27) and man (28, 29) for micronized griseofulvin and griseofulvin dispersed in polyethylene glycol.

In the dog studies, the total areas under the blood concentration curves in the first 8 hr. for the micronized griseofulvin, either in tablet or capsule form, were found to be approximately only 25% of those obtained from capsule forms of 10% griseofulvin–90% polyethylene glycol prepared by melting methods. By analyzing the total excretion of the major metabolite in 48 hr., it was found that approximately 88% of dispersed griseofulvin, 45% of micronized griseofulvin in capsule form, and 33% of micronized griseofulvin in tablet form were absorbed. The griseofulvin dissolved in polyethylene glycol 400 was found to be completely absorbed. Their cumulative excretion plots are shown in Fig. 20. From the analysis of the excretion rate data, it was found that oral absorption of griseofulvin in dogs could proceed for more than 40 hr. The amounts absorbed were shown to correlate linearly with the logarithm of the *in vitro* dissolution rates. The solid dispersion of 5% griseofulvin–95% polyethylene glycol 4000 also produced about fourfold the blood area in the first 8 hr. than did the micronized griseofulvin in a dog (12). In a preliminary study, the presence of polyethylene glycol 4000 in a physical mixture was found not to affect the oral absorption of micronized griseofulvin (12).

To test its practical application, the absorption studies were further carried out in human subjects. The 10 and 20% griseofulvin dispersions in polyethylene glycol 6000 were found to be almost completely absorbed in eight trials, while only 43% of micronized griseofulvin was absorbed. More strikingly, the absorption from dispersed forms was almost complete within 2 hr. after administration. The absorption from the micronized product was found to continue for 30–80 hr. after dosing. The average cumulative excretion of urinary metabolites (6-demethylgriseofulvin and its glucuronide) obtained from administration of various forms is plotted in Fig. 21. The rapid and complete absorption of the insoluble antibiotic in man was mainly attributed to the molecular and colloidal dispersion of the drug in a highly water-soluble carrier. It is predicted that the polyethylene glycol can act as one

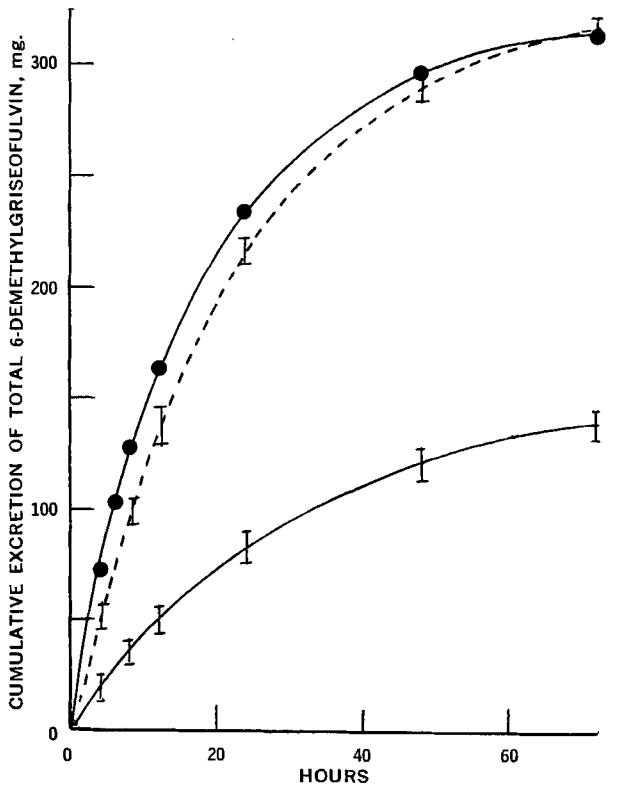


Figure 21—*Cumulative total 6-demethylgriseofulvin urinary excretion data after intravenous and oral administration of griseofulvin to two human subjects (intravenous data only for a subject; others are mean values of eight trials). Key: ●, intravenous dose; - - -, griseofulvin dispersed in polyethylene glycol 6000 (10 and 20% w/w); and ——, tablets of micronized griseofulvin (all data corrected for 500-mg. dose) (from* Reference 28, *reprinted with permission).*

of the ideal universal carriers for most poorly soluble drugs.

MISCELLANEOUS APPLICATION

A unique method in formulating a liquid drug or chemical in a solid dosage form was recently introduced by Chiou and Smith (40). A liquid drug such as methyl salicylate, vitamin E, clofibrate, benzyl benzoate, and benzonatate was mixed by mechanical stirring with the melted liquid of polyethylene glycol 6000 at a temperature below 70°. The mixture was then rapidly cooled, and the resultant "solid" mass was pulverized, encapsulated, and tableted. The method is particularly valuable for drugs with low therapeutic doses because the maximum concentration that can be incorporated into a solid form only ranged between 5 and 10% (w/w). It is believed that other thermoplastic polymers with low melting points can also function as carriers for such purposes.

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Determination of Flurazepam and Its Major Biotransformation Products in Blood and Urine by Spectrophotofluorometry and Spectrophotometry

J. ARTHUR F. de SILVA and NORMAN STROJNY

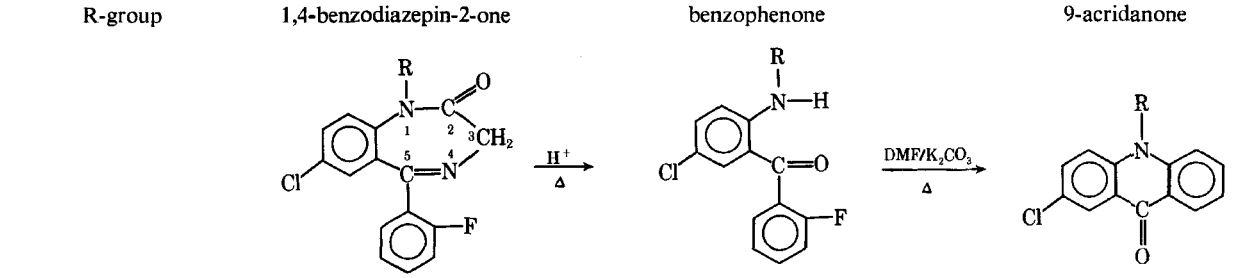
Abstract □ A spectrophotofluorometric assay for the determination of flurazepam and its major metabolites in blood and in urine was developed. It was applied to the determination of blood levels and urinary excretion in a dog following intravenous and oral administration and in two human subjects following the oral administration of flurazepam · 2HCl. In the two human subjects, 51 and 56% of the administered doses were recoverable in the 0–48-hr. urine as known metabolites.

Keyphrases □ Flurazepam and metabolites—spectrophotofluorometric assay, blood, urine, dog, human □ Spectrophotofluorometry—assay, flurazepam

Flurazepam [7-chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one· 2HCl] (I) was synthesized by Sternbach *et al.* (1) and is marketed as a hypnotic[1]. The major metabolites of flurazepam in human and dog urine were characterized by Schwartz *et al.* (2) using TLC and mass spectrometry. The chemical synthesis of these compounds was reported by Earley *et al.* (3). Studies on the metabolism of ^{14}C-flurazepam · 2HCl (4) showed that it was extensively metabolized in both species to yield measurable amounts of the hydroxyethyl (II) and *N*-desalkyl metabolite (III) in the blood, which was further metabolized to the 3-hydroxy analog (IV) present in trace amounts in urine as a glucuronide conjugate. In addition, they showed that both I and II were metabolized to an acidic compound (V) in the dog by the oxidation of the alcohol side chain to a carboxylic acid.

The chemical structures of flurazepam and its major metabolites are given in Scheme I and Table I. The chemical synthesis of the acidic metabolite (V) was reported by Fryer and Earley (5). The 1,4-benzodiazepin-2-ones all undergo acid hydrolysis to yield their respective benzophenones (5–7), which in turn are cyclized in an alkaline medium to yield the 9-acridanone derivatives (8) (Scheme I).

The spectrophotofluorometric assay described for the determination of flurazepam and its major metabolites in blood and urine involves the following: selective extraction into diethyl ether from blood buffered to pH 9.0 or urine made alkaline with NaOH, and back-extraction into 4 *N* HCl followed by hydrolysis at 100° for 2 hr.

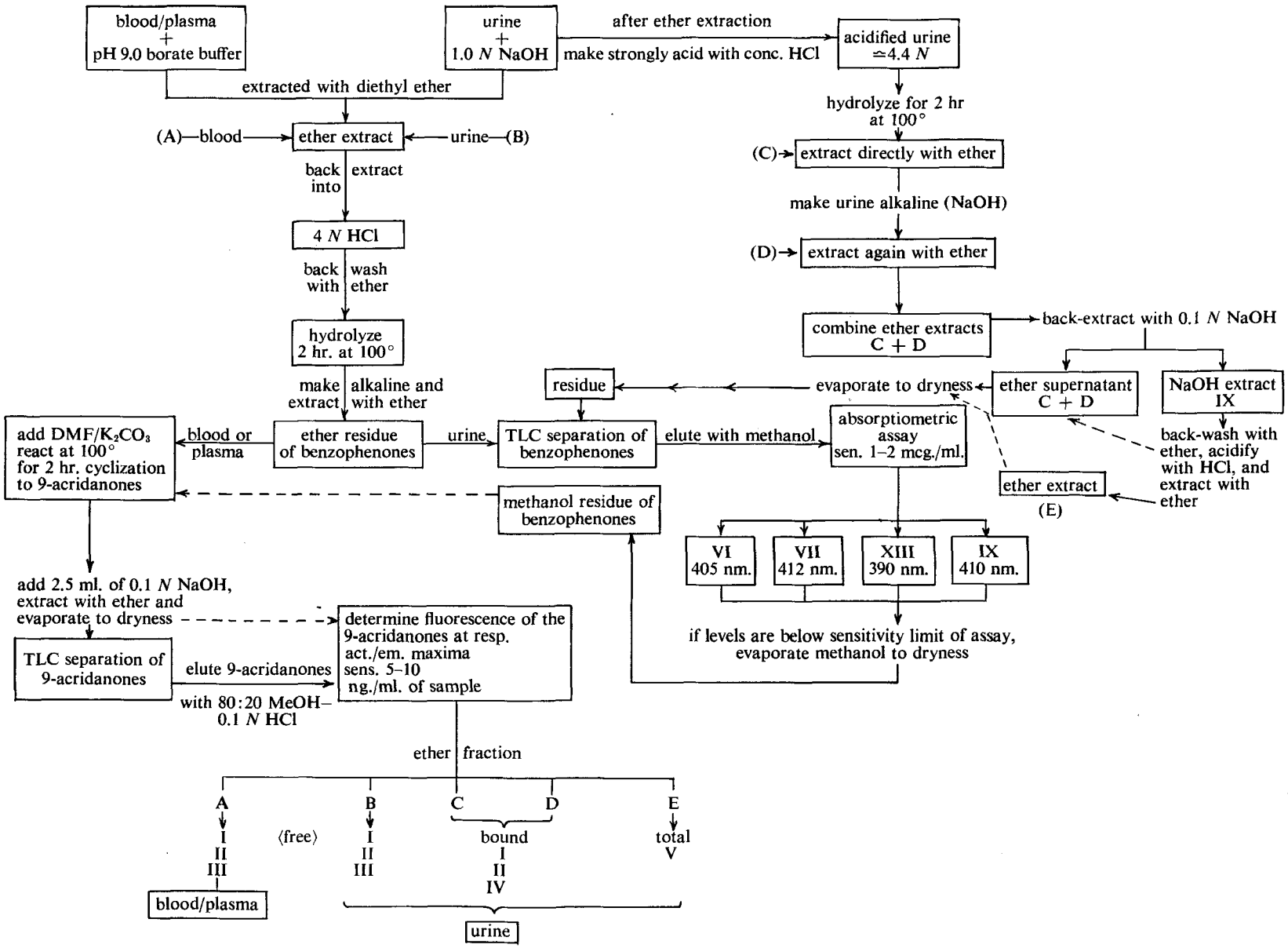


Scheme I—Chemical reactions of N-*1-alkyl-substituted 2'-fluoro-1,4-benzodiazepin-2-ones*

to yield the respective benzophenones.

The benzophenones are extracted into diethyl ether (from the hydrolysate after making it alkaline with NaOH), the residue of which is reacted in dimethyl formamide, using K_2CO_3 as a catalyst at 100° for 2 hr. to effect the cyclization to the 9-acridanone derivatives. These derivatives are extracted into diethyl ether and separated by TLC, and their fluorescence is determined in 80:20 methanol–0.1 N HCl (after elution from the silica gel) at their respective maxima of activation and emission (Fig. 1). The fluorescence yield of the 9-acridanones is sufficiently high and linear with concentration to enable their quantitation in the range of 0.003–10.0 mcg. of compound/ml. of blood or urine (Fig. 2).

[1] Flurazepam · 2HCl is the active drug substance in the pharmaceutical formulation Dalmane, marketed by Hoffmann–La Roche Inc., Nutley, N. J.



Scheme II—Flow diagram of the differential extraction procedure

Table I—Chemical Names and Compound Reference Nomenclature of the Benzodiazepin-2-ones, Benzophenones, and 9-Acridanones Referred to in Scheme I[a]

Compound	Generic Name	Chemical Name	Molecular Weight	Melting Point	Reference to Synthesis
1,4-Benzodiazepin-2-ones:					
I	Flurazepam	7-Chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one·2HCl	460.83	190–220° (dec.)	1
II	*N*-1-Hydroxyethylflurazepam	7-Chloro-1-(2-hydroxyethyl)-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one	332.76	110–115°	3
III	*N*-1-Desalkylflurazepam	7-Chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one	288.5	205–206°	1
IV	*N*-1-Desalkyl-3-hydroxyflurazepam	7-Chloro-5-(2-fluorophenyl)-3-hydroxyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one	304.72	195–198°	3
V	Flurazepam-*N*-1-yl-acetic acid	2-[7-Chloro-5-(2-fluorophenyl)-1,3-dihydro-2-oxo-2*H*-1,4-benzodiazepin-1-yl]acetic acid	346.76	216–225° (dec.)	5
Benzophenones:					
VI	—	5-Chloro-2-(2-diethylaminoethylamino)-2′-fluorobenzophenone·HCl	385.30	134–143°	5
VII	—	5-Chloro-2′-fluoro-2-(2-hydroxyethyl)aminobenzophenone	293.73	96–101°	5
VIII	—	2-Amino-5-chloro-2′-fluorobenzophenone	250.00	94–95°	5
IX	—	*N*-[4-Chloro-2-(2-fluorobenzoyl)]glycine	307.72	162–168°	5
9-Acridanones:					
X	—	2-Chloro-10-(2-diethylaminoethyl)-9-acridanone·HCl	365.32	265–275°	5
XI	—	2-Chloro-10-(2-hydroxyethyl)-9-acridanone	273.70	216–219°	5
XII	—	2-Chloro-9-acridanone	229.66	>350°	5
XIII	—	2-Chloro-10-(carboxymethyl)-9-acridanone	287.71	335–345° (dec.)	5

[a] The benzophenones were prepared by the acid hydrolysis of their respective benzodiazepin-2-ones and were recrystallized as their hydrochloride salts or bases (5) [using modifications of published procedures (6, 7)]. The 9-acridanones were prepared from their respective benzophenones (5) using the published procedure (8).

The assay was modified to employ selective extraction procedures for the determination of flurazepam and its major metabolites in the free and bound (conjugated) forms in urine, either by spectrophotometry as their benzophenones or by spectrophotofluorometry as their respective 9-acridanones.

TLC was used to separate the different reaction products, thus imparting the required specificity for the quantitation of each compound. A flow diagram of the differential extraction procedure is shown in Scheme II.

The method was applied to the determination of the blood levels and urinary excretion of flurazepam and its major metabolites in a dog following the oral and intravenous administration of a 5-mg./kg. dose and in man following the administration of single 90-mg. oral doses of flurazepam·2HCl.

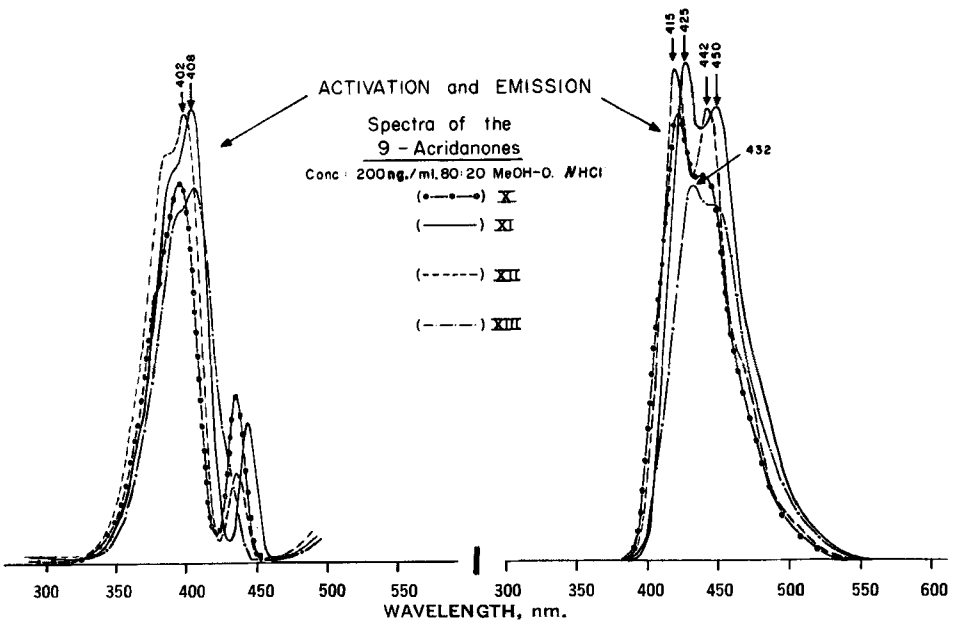


Figure 1—*Activation/emission spectra of the 9-acridanones.*

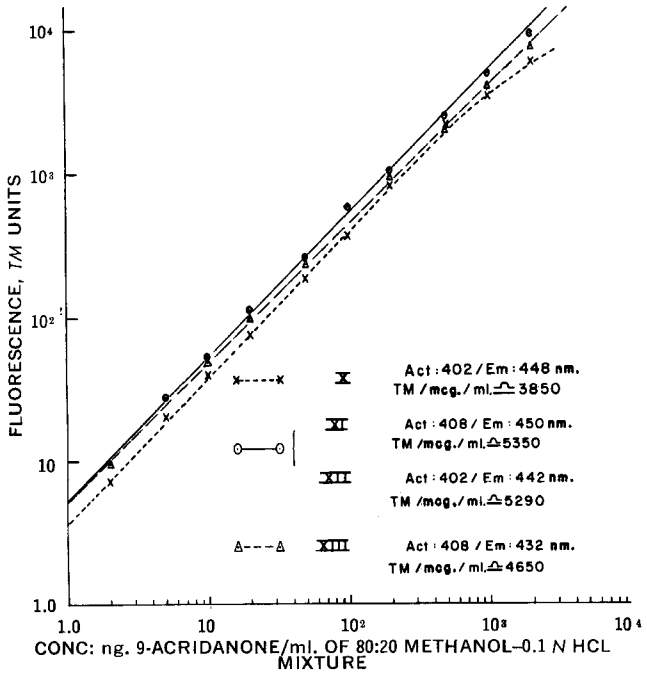


Figure 2—*Linear dynamic range of fluorescence* versus *concentration of the 9-acridanones.*

EXPERIMENTAL

Preparation of Standard Solutions—The authentic compounds required as analytical standards are listed in Table I and are of pharmaceutical grade purity (>98%).

1,4-Benzodiazepin-2-ones—Weigh out 10 mg. of each of the 1,4-benzodiazepin-2-ones (free base) (I–V) or an equivalent amount of the hydrochloride salt (where applicable) and dissolve in 100 ml. of absolute ethanol in low actinic volumetric flasks. These stock solutions contain 100 mcg./ml. Make suitable serial dilutions of these stock solutions in absolute ethanol to yield working solutions containing 1 mcg./ml., aliquots of which are used for recovery determination from blood or urine.

9-Acridanones—Weigh out 10 mg. of each of the 9-acridanones (free base) (X–XIII) or an equivalent amount of the hydrochloride salt (where applicable) and dissolve in 100 ml. of an 80:20 (v/v) mixture of methanol–0.1 *N* HCl in low actinic volumetric flasks. Heating the solution at 50–55° for 60 min. with intermittant high-speed mixing in an ultrasonic cleaner[2] expedites complete dissolution. These stock solutions contain 100 mcg./ml. Make serial 1:10 dilutions of these solutions in 80:20 methanol–0.1 *N* HCl to yield working solutions covering a concentration range of 1.0–1000 ng./ml. These solutions are used for the preparation of calibration curves for recovery determination.

Spectrofluorometric Determination of Flurazepam and Its Major Metabolites (II and III) in Blood or Plasma—Into a 50-ml. centrifuge tube, add 1.0-ml. aliquots of blood or plasma specimen and 5 ml. of pH 9.0, 1 *M* borate–KCl–Na_2CO_3 buffer[3]. Along with the samples, run a 1.0-ml. specimen of control blood or plasma (taken from the subject prior to medication or from a control source) and duplicate 1.0-ml. specimen of control blood or plasma added to mixtures of 100 and 300 ng. each of Compounds I–III, respectively. Extract the samples with two successive 10-ml. portions of diethyl ether[4] on a reciprocating shaker for 10 min., centrifuge for 10 min. at 2100 r.p.m. (preferably at 0–5° in a refrigerated centrifuge), and combine the ether extracts in another 50-ml. tube. (The sensitivity of the assay may be increased, if needed, by using a 4-ml. specimen of blood and 10 ml. of borate buffer. In this event, extract the sample with 2 × 15-ml. portions of ether, combine the ether extracts in a 50-ml. centrifuge tube, and evaporate the combined ether extract to about 20 ml.)

Add 5 ml. of 4 *N* HCl to the combined ether extract and back-extract by mechanical shaking for 10 min. Centrifuge the samples and aspirate off the ether. Wash the acid extract with two 10-ml. portions of diethyl ether by shaking for 10 min. on a reciprocating shaker (to remove any lipid material present at the interphase), centrifuge, and aspirate off the ether. Equilibrate the acid extract in a hot water bath to expel any residual ether, stopper the tubes, and hydrolyze the acid extract in a boiling water bath (100°) for 2 hr. to convert quantitatively the benzodiazepin-2-ones to their benzophenones.

After hydrolysis, cool the samples in ice; add 1 drop of bromthymol blue indicator (0.1% in ethanol) and neutralize with 4 *N* NaOH to a blue-colored end-point. Extract the sample successively with two 10-ml. portions of ether, shaking for 10 min. on a reciprocating shaker per extraction. Centrifuge and combine the ether extracts in a 15-ml. tube by evaporating the ether extracts successively to dryness in a hot water bath (35–40°). To this residue containing the benzophenones (VI–VIII), add 0.5 ml. of dimethylformamide and about 300 mg. of anhydrous K_2CO_3. Seal the stopper with a drop of dimethylformamide and place the tubes in a 110° oven. Maintain at 110° for 2 hr. to effect the cyclization of the benzophenones to the 9-acridanones and then cool to room temperature. Add 2.5 ml. of 0.1 *N* NaOH and 2 ml. of distilled water; mix to dissolve the K_2CO_3 and dimethylformamide. Add 1 drop of bromthymol blue indicator and extract the sample successively with two 5-ml. portions of ether, shaking for 5 min. on a reciprocating shaker. Centrifuge the samples after each extraction and combine the ether extracts in a 15-ml. centrifuge tube.

Centrifuge the combined ether extracts for 5 min. and carefully remove any aqueous contamination present with a hypodermic syringe fitted with a cannula (B-D-20, 15 cm. in length). Evaporate the ether to dryness in a hot water bath at 35–50° under a stream of nitrogen to remove traces of dimethylformamide. Dry in a vacuum desiccator for 10 min. to remove any moisture and dimethylformamide present and then dissolve the residues in 100 μl. of $CHCl_3$, mixing the sample on a vortex high-speed supermixer to ensure uniform solution. Transfer the $CHCl_3$; extract onto a 20 × 20-cm. Brinkmann precoated (F_{254}) silica gel thin-layer chromatoplate, rinsing the tubes with another 50-μl. portion of $CHCl_3$ to ensure quantitative transfer. Develop the plate twice for 12–15 cm., ascending in a vapor-saturated tank in 85:15 chloroform–acetone (v/v). This TLC step separates the three 9-acridanones (X, XI, and XII) derived from their respective benzophenones (VI, VII, and VIII) (Scheme I). The plate is examined under shortwave and longwave UV light, and the respective 9-acridanones (X, R_f 0.15–0.28; XI, R_f 0.28–0.47; and XII, R_f 0.42–0.67) are identified as intensely fluorescent blue-green spots by reference to the R_f values of 1.0-mcg. amounts of the authentic standards run alongside the sample extracts.

The areas on the TLC plate corresponding to the three acridanones are carefully scraped off, transferred into 15-ml. conical centrifuge tubes, and eluted directly with 4 ml. of 80:20 methanol–0.1 *N* HCl by slurrying for 1 min. on a vortex supermixer. The samples are then centrifuged and the supernatant is transferred into a 1-cm. path cell, using a capillary pipet and taking care not to disturb the silica gel at the bottom of the tube.

The fluorescence of the solution containing X is determined at 448 nm., activating at 402 nm., while XI is determined at 450 nm., activating at 408 nm.; that of XII is determined at 442 nm., activating at 402 nm. The fluorescence of XIII (derived from the acidic metabolite in urine) is read at 432 nm., activating at 408 nm. (Figs. 1 and 2).

Calculations—The fluorescence readings[5] [transmittance (T) × meter multiplier factor (M) = TM] of all the samples and internal standards are corrected for control (blood or plasma) readings but are uncorrected with respect to activation energy. The amounts of the intact drug (I) and its major blood metabolites (II and III) in

[2] Bronson Automatic Ultrasonic Cleaner, model D-100.

[3] Dissolve 61.8 g. of orthoboric acid (H_3BO_3) and 74.6 g. of KCl/l. of distilled water. Add 630 ml. of this solution to 370 ml. of solution containing 106 g. Na_2CO_3/l. of distilled water. Shake well and adjust to pH 9.0 with Na_2CO_3, if necessary. Store this stock solution at 37°.

[4] Analytical grade reagent, Mallinckrodt.

[5] All fluorescence measurements were made on a Farrand spectrofluorometer equipped with a xenon arc energy source and an RCA IP-28 photomultiplier. The 10-nm. slit arrangement was used at all four positions. The monochromators were corrected for the fundamental Hg lines, but the fluorescence measurements were not corrected for energy and other instrumental artifacts. The instrument sensitivity was adjusted for constant energy for each day of operation, using a reference standard of quinine sulfate (0.1 mcg./ml. in 0.1 *N* H_2SO_4).

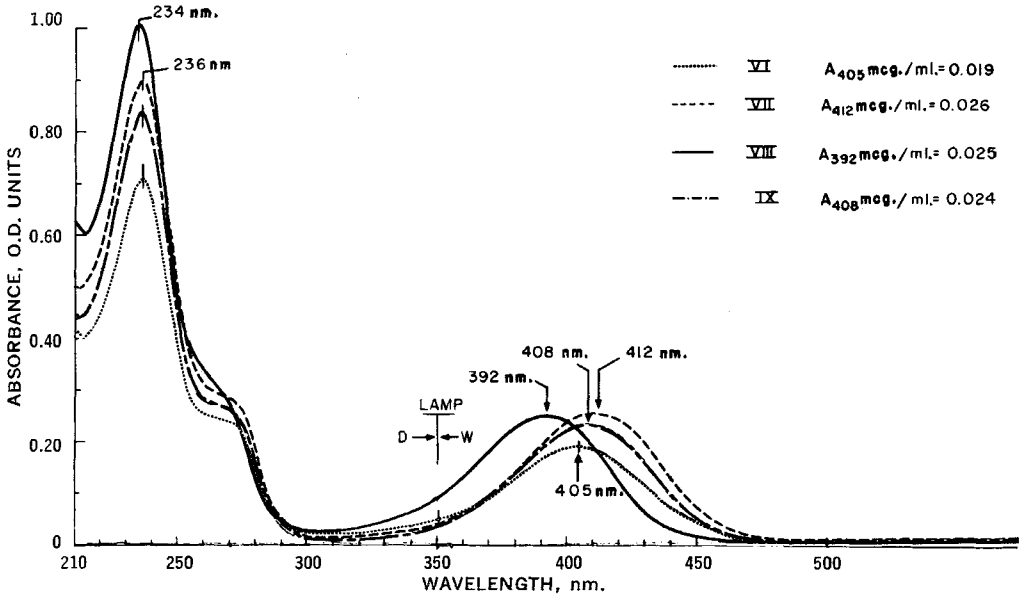


Figure 3—*Absorption spectra of the benzophenones.*

the unknowns is determined by direct comparison of the corrected fluorescence of the unknowns against that of their respective internal standards.

Determination of Percent Recovery—The overall percent recovery of internal standards of I, II, and III, determined as their respective 9-acridanone derivatives, is calculated routinely as a check on analytical precision and accuracy, using the appropriate molecular weight conversion factors for I, II, III, IV, and V of 1.180, 1.216, 1.256, 1.327, and 1.205, respectively.

Spectrophotometric Determination of Flurazepam and Its Major Metabolites in Urine—The measurable drug-related compound in the free or directly ether-extractable fraction includes trace amounts of flurazepam (I), with measurable amounts of II, III, and IV. Their concentrations are usually below the sensitivity limit of the absorptiometric assay (<1–2 mcg./ml. of urine); consequently, they have to be quantitated using the spectrofluorometric assay described for blood (Scheme II).

In man the major urinary metabolite is the hydroxyethyl compound (II) present as a conjugate, along with trace amounts of the intact drug, the *N*-desalkyl compound (III), and its 3-hydroxy analog (IV). The urines from treated dogs contain measurable amounts of II, III, and IV mainly in the conjugated form, whereas the major metabolite is the carboxylic acid derivative (V). Since the directly extractable fraction does not yield a significant amount of the dose, it can be combined with the bound fraction and determined as "total" metabolites instead of using separate extraction procedures for the free and conjugated forms. Therefore, after strong acid hydrolysis of the urines to deconjugate the metabolites and hydrolyze them to their respective benzophenones, a differential extraction procedure is employed (Scheme II) which, when combined with TLC, imparts the required specificity of the assay for the respective metabolites. The individual benzophenones are quantitated in methanol by UV/visible absorption spectrophotometry (Fig. 3)[6].

Standard Solutions—In addition to the solutions of the 1,4-benzodiazepin-2-ones required for the fluorometric assay, prepare stock solutions of the *o*-aminobenzophenones (VI–IX) listed in Table I. Weigh out 10 mg. of each of the benzophenones (free base) or an equivalent amount of the hydrochloride salt (where applicable), and prepare the standard solutions as described for the 1,4-benzodiazepin-2-ones in the spectrofluorometric assay.

Procedure for Urine—Into a 50-ml. centrifuge tube, add 5 ml. of urine sample and 3.0 ml. of 12 *N* HCl (end normality ≃4.5). Along with the samples, run a 5-ml. sample of control urine and duplicate 5-ml. samples of control urine containing 25.0 mcg. each of Compounds I–V (0.25 ml. of their respective stock solutions evaporated to dryness under nitrogen) as internal standards. Transfer all the samples (stoppered) directly into a 100° water bath and hydrolyze for 2 hr. to convert the benzodiazepin-2-ones to their respective benzophenones. Cool the samples to room temperature, and extract with 2 × 15-ml. portions of ether by shaking for 15 min. on a reciprocating shaker. Centrifuge and combine the ether extracts in another 50-ml. centrifuge tube. This is the acidic extract C in Scheme II. Then make the urine specimen strongly alkaline with 5 ml. of 10 *N* NaOH, add 15 ml. of ether, and extract again as before. Centrifuge and combine this alkaline extract (D in Scheme II) with the acidic extract (C). Discard the urine specimen.

Extraction at both acidic and alkaline pH's is necessary for complete recovery of the four benzophenones (VI–IX). However, it does not result *per se* in a quantitative separation of the acidic benzophenone (IX) from the three basic benzophenones (VI–VIII). Therefore, to the combined ether extract (C + D), add 5 ml. of 0.1 *N* NaOH and extract for 10 min. by shaking on a reciprocating shaker. Centrifuge, transfer the supernatant ether (which now contains only the basic benzophenones) into another 50-ml. tube, and evaporate to dryness. Back-wash the NaOH phase, containing only IX, with 10 ml. of ether, shaking for 10 min. Centrifuge and combine this ether wash with the residue of ether extact (C + D); evaporate again to dryness. Process the ether-washed NaOH solution separately for IX. Dissolve the ether residue (C + D) in 100 μl. of acetone, transfer quantitatively onto a 20 × 20-cm. Brinkmann (F_{254}) silica gel chromatoplate, and develop the plate in a vapor-saturated chamber to 12–15 cm., ascending in benzene–methanol–glacial acetic acid (90:10:10 v/v). The three benzophenones (VI, $R_f \simeq 0.3$; VII, $R_f \simeq 0.5$; VIII, $R_f \simeq 0.7$) produced by acid hydrolysis of their respective benzodiazepin-2-ones (Scheme I) are identified either under shortwave UV light as dark absorbing areas or under visible light as intensely yellow spots by reference to the R_f of 25-mcg. amounts of the authentic benzophenone standards run alongside the sample extracts. The plate may be redeveloped in the same solvent system to obtain greater resolution if needed.

The ether-washed NaOH solution containing the acidic benzophenone (IX) is made strongly acidic with 1 ml. of 4 *N* HCl and extracted with two 15-ml. portions of ether by shaking for 10 min. Centrifuged, the ether supernatants are combined and evaporated to dryness. Discard the NaOH solutions at this stage. Dissolve the ether residues in 100 μl. of methanol, transfer quantitatively onto a 20 × 20-cm. Brinkmann (F_{254}) chromatoplate, and develop in the same solvent system as described for the basic benzophenones. The acidic benzophenone (IX, $R_f \simeq 0.47$) is identified by reference to the R_f of 25 mcg. of the authentic standard run alongside the sample extracts. Besides IX, the acidic fraction contains a considerable amount of impurities, probably phenolic and indolic acids, which contribute to high blank values in spectrophotometric analysis. These impurities can be adequately separated from IX by TLC due to their higher R_f values. Consequently, it is advantageous to separate the basic benzophenones first from the acidic fraction by selective extraction prior to TLC separation.

[6] The UV absorption spectra were obtained using a Coleman-Hitachi EPS-3 double-beam ratio recording spectrophotometer.

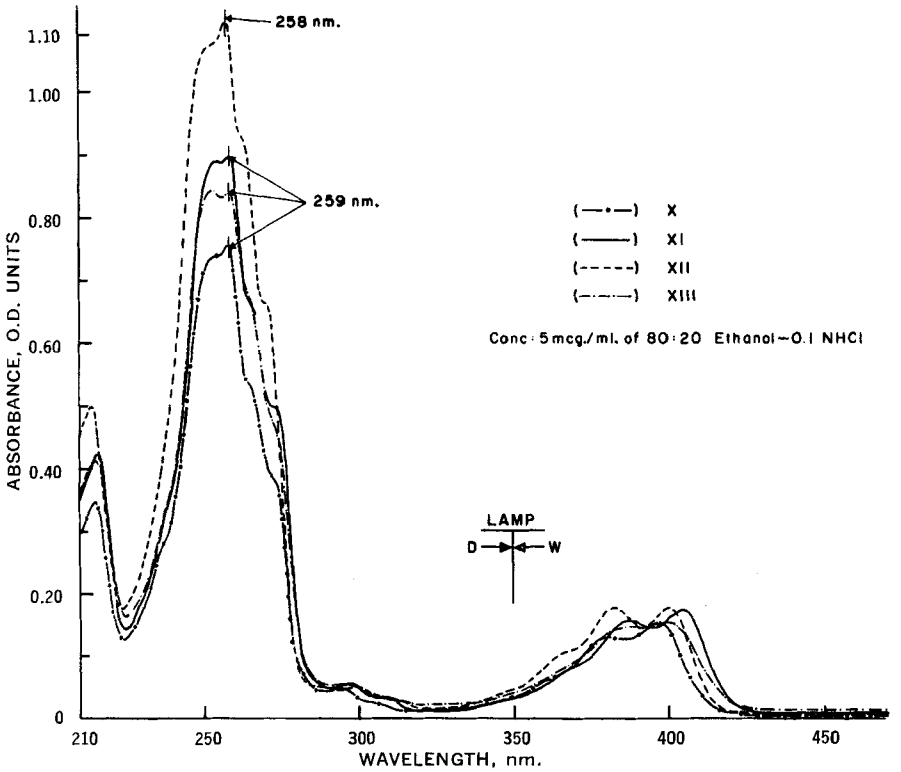


Figure 4—*Absorption spectra of the 9-acridanones.*

The silica gels from the respective benzophenone areas are carefully scraped off into separate 15-ml. centrifuge tubes, eluted with 4 ml. of methanol by slurrying for 1 min. on a vortex high-speed supermixer, and centrifuged. The supernatant is decanted into a 1-cm. path cell, and the absorbance is determined at their respective maxima (VI at 405 nm., VII at 412 nm., VIII at 392 nm., and IX at 408 nm.) (Fig. 3).

The amount of each compound (I–V) in the unknowns is calculated by direct comparison to the absorbance values of their respective internal standards.

Determination of Percent Recovery—The overall recovery of the internal standards is determined by comparing the absorptivity of the recovered internal standards measured as the benzophenones directly to that of an external standard of the respective authentic benzophenones using the appropriate molecular weight conversion factors for Compounds I, II, III, IV, and V of 1.112, 1.132, 1.156, 1.220, and 1.127, respectively.

If the concentration of the benzodiazepin-2-ones is below the limit of detectability of the absorptiometric assay (<1–2 mcg. of compound/ml.), the limit of detection can be extended by employing the fluorometric assay. A 2-ml. aliquot of the methanol solution (from the spectrophotometric assay) of the control, the unknowns, and a 0.01-ml. aliquot of the internal standards (containing $\triangleq$ 0.625 mcg. of compound) are evaporated to dryness; the benzophenone is cyclized in dimethylformamide/K_2CO_3 to the 9-acridanone as described in the blood assay. However, when extracting the acidic acridanone (XIII), the extraction procedure is modified. For the acidic acridanone, *slowly* add 2.5 ml. of 4 *N* HCl, mix (end pH $\sim$ 1.0–2.0), and extract the sample successively with 3 × 5-ml. portions of ether, which are combined and evaporated to dryness. The TLC separation of these 9-acridanone derivatives is not necessary. The final ether residues containing the respective 9-acridanones are dissolved in 4 ml. of 80:20 methanol–0.1 *N* HCl and quantitated fluorometrically as described.

RESULTS AND DISCUSSION

Several analytical parameters were investigated during the development of a suitable assay procedure for flurazepam and its major metabolites. The UV absorption spectrum of the pure compound (I) in 0.1 *N* HCl showed a maximum at 240 nm., with an absorptivity (*A*/mcg./ml.) of 0.070. In 0.1 *N* NaOH, the UV maximum was at 234 nm., with an absorptivity of 0.076. The potential sensitivity limit was of the order of 2–3 mcg./ml. of sample, but its usefulness as an absorptiometric assay was limited due to high blank values obtained from the biological specimen.

A new synthesis of 9-acridanones from *o*-amino-2′-fluorobenzophenones was reported by Fryer *et al.* (8). According to these investigators, the fluorine was sufficiently activated by the *ortho*-carbonyl group to react readily with the aromatic amine, thus undergoing internal nucleophilic exchange to give high yields of the corresponding 9-acridanones. This procedure was successfully scaled down to enable the preparation of these derivatives in high yield in the submicrogram range.

The major metabolites of flurazepam (I) are the hydroxyethyl analog (II) and the *N*-dealkylated analog (III) present in measurable amounts in the blood. The 3-hydroxy-*N*-dealkylated analog (IV) is present in the urine mainly as the glucuronide conjugate, as is the hydroxyethyl analog (II). The carboxylic acid (V) is found mainly in the dog and to lesser amounts in man (4). All these compounds undergo strong acid hydrolysis to their respective benzophenones, which can then be cyclized to their respective 9-acridanones as indicated in Scheme I. The same sequence of reactions also takes place under strongly alkaline conditions but is not analytically useful due to the formation of several undesirable by-products which greatly reduces the overall yield of the 9-acridanones.

The spectrofluorometric assay initially developed for the determination of intact I and its metabolites in blood was modified, using differential extraction procedures for the determinations of the metabolites (II–V) in urine. Several TLC systems (Table II) were employed to separate the different metabolites either as their respective benzophenones or as their 9-acridanones prior to quantitation, thus ensuring the specificity of the assay for each compound (Scheme II). The high fluorescence intensity of each of the 9-acridanones in 80:20 methanol–0.1 *N* HCl was sufficient for the determination of about 3.0–5.0 ng. of 9-acridanone/ml. of final solution. This property was used by Sawicki *et al.* (9) in the determination of trace amounts of 9-acridanone (0.4 ng./m^3 of air) as an atmospheric pollutant. The specific fluorescence intensity of each compound, determined at its respective activation/emission maxima[6], was of about the same order of magnitude, with *TM*/mcg./ml. values of 3850, 5350, 5290, and 4650 for X, XI, XII, and XIII, respectively.

The absorption spectra of the 9-acridanones in 80:20 methanol–0.1 *N* HCl (Fig. 4) show great similarity in their spectral characteristics, despite the presence of a different alkyl side chain in each compound. The main absorption bands due to the acridanone ring

Table II—TLC Systems for the Separation of the 1,4-Benzodiazepin-2-ones, Their Respective Benzophenones, and 9-Acridanones Using Brinkmann (F_{254}) Silica Gel G Plates in Vapor-Saturated Chambers

Compound	R_f in Solvent System[a]					Detection by[c]		
	1[b]	2	3[b]	4a[b]	4b[b]	Visible	Shortwave UV	Longwave UV
1,4-Benzodiazepin-2-ones:								
I	0.14	0.38	0.26	0.00	0.05	—	Abs.	Abs.
II	0.23	0.52	0.47	0.15	0.25	—	Abs.	Abs.
III	0.40	0.60	0.50	0.25	0.42	—	Abs.	Abs.
IV	0.20	0.42	0.42	0.15	0.50	—	Abs.	Abs.
V	0.00	0.00	0.34	0.00	0.00	—	Abs.	Abs.
Benzophenones:								
VI	0.36	0.57	0.30	0.05	0.12	Yellow	Abs.	Abs.
VII	0.40	0.64	0.50	0.35	0.58	Yellow	Abs.	Abs.
VIII	0.56	0.72	0.70	0.60	0.83	Yellow	Abs.	Abs.
IX	0.00	0.00	0.47	0.00	0.00	Yellow	Abs.	Abs.
9-Acridanones:								
X	0.16	0.45	0.27	0.15	0.28	—	Blue/fluor.	Fluor.
XI	0.27	0.52	0.45	0.28	0.47	—	Blue/fluor.	Fluor.
XII	0.40	0.64	0.30	0.42	0.67	—	Blue/fluor.	Fluor.
XIII	0.00	0.00	0.40	0.00	0.00	Faint yellow	Blue/fluor.	Fluor.

[a] Developing solvent systems were: 1, 100:0.5 ethyl acetate–NH_4OH; 2, 100:10:0.3 ethyl acetate–ethanol–NH_4OH; 3, 90:10:10 benzene–MeOH–HAc; and 4, 85:15 chloroform–acetone (*a*, first development, and *b*, second development). [b] These systems gave the best overall resolution. [c] Abs. = absorption. Fluor. = fluorescence.

appears to be unaffected by the alkyl substituents. A strong UV band ($\lambda_{max.}$ = 258–260 nm.) offers good potential for absorptiometric analysis. The absorption in the visible region reveals maxima at 380, 390, 400, and 405 nm. due to the yellow color of these compounds. These bands also correspond to the wavelengths of activation selected for the fluorometric assay. The spectral resolution of the monochromators of the Farrand spectrofluorometer used in this study is sufficiently narrow that these activation bands, which differ from the fluorescence emission bands selected by 30 nm. or more, can be used without interference from Raman scatter. Although the strong UV absorption bands at 258–260 nm. would theoretically be superior for purposes of activation, the low energy output of the xenon arc continuum in this region greatly reduces the intensity of fluorescence emission (and, therefore, sensitivity) when the samples are activated at 258–260 nm. However, with a mercury vapor lamp as an energy source, these UV bands would be preferred for activation of the 9-acridanones.

The fluorescence intensity of each compound was also linear with concentration in the range of 2.5 to 2.0 $\times$ 10^3 ng./ml. of final solution (Fig. 2). The recovery of I and II is linear and reproducible in the concentration range of 10 ng.–10 mcg. of compound/ml. of blood or urine. The sensitivity limits of the assay are of the order of 3.0–10.0 ng. of compound/ml. of blood or urine, using a 4-ml. specimen per assay and a sample–blank (*TM*) ratio of 2:1 as the limit of detectability.

The overall recovery of the spectrofluorometric assay for flurazepam and its metabolites is based on four separate steps: extraction, hydrolysis, cyclization, and elution from the TLC plate prior to fluorometric determination, and it represents the product of quantitative extraction from blood or urine >95% followed by a >95% hydrolysis to the benzophenone, a >85% cyclization to the 9-acridanone, and a >90% recovery from the TLC plate. Therefore, the expected overall recovery from blood and urine is of the order of 70%. Optimal reaction conditions for each step were established initially for flurazepam (I) and the hydroxyethyl compound (II) and applied either as such or with minor modifications for the other compounds. The actual recoveries obtained, however, fell short of the expected value because of interference incurred in the cyclization and TLC separation steps due to the presence of impurities extracted from blood or urine.

The overall recovery of flurazepam (I) and its major metabolite (II) obtained from blood was 50 ± 9.0 and 71 ± 4.0%, respectively, while that of the metabolite (II) from urine was 64 ± 5.0%. The recovery of II is in acceptable agreement with the theoretically expected value. The poor overall recovery of flurazepam (I) from blood appears to be due to a decrease in the efficiency of the cyclization reaction in the presence of blood-extracted impurities as compared against a direct reaction of the pure benzophenone to the 9-acridanone where the efficiency was >75%. The overall recovery of the other three metabolites (III–V) determined by the present procedure was somewhat less than that of II (Table III). However, the recoveries were sufficiently reproducible to permit their quantitation in biological specimens.

The identification of the major urinary metabolites of flurazepam in man and in the dog using ^{14}C-labeled flurazepam·2HCl was reported by Schwartz and Postma (4). The major urinary metabolites in man and in the dog were II and V, respectively, and were assayed as their respective benzophenones by spectrophotometry since their concentrations were sufficiently high (>1–2 mcg./ml.) for their quantitation by the less sensitive but simpler assay. The absorption spectra (Fig. 3) indicated specific absorbance (*A*/mcg./ml.) values and resulting sensitivity limits of detection (1–2 mcg./ml. of final solution) of the same order of magnitude for each benzophenone, measured at its respective maximum in the visible region. The absorbance of each compound was linear with concentration in the range of 1.0–50.0 mcg. of benzophenone/ml. of final solution (methanol). Although the absorbance in the UV at 234–236 nm. was greater than that in the visible region and provided greater potential sensitivity, the high blank values obtained in this region of the UV from biological extracts read in methanol offset this advantage. The

Table III—Determination of Percent Recovery of Flurazepam and Its Metabolites in the Separate Steps Involved in the Overall Spectrofluorometric and Spectrophotometric Assays

Parent Compound	Spectrofluorometric Assay in Blood and Urine						Spectrophotometric Assay in Urine, Actual Recovery from Urine
	Hydrolysis to Benzophenone	Cyclization to 9-Acridanone	Elution from Silica Gel	Overall Recovery Expected	Actual Recovery from Blood	Actual Recovery from Urine	
I	93 ± 2.0	74 ± 4.0	98 ± 2.0	67	50 ± 9.0	60 ± 4.0	90 ± 5.0
II	95 ± 5.0	90 ± 6.0	94 ± 4.0	80	70 ± 4.0	65 ± 4.0	83 ± 2.0
III	95 ± 5.0	63 ± 3.0	93 ± 4.0	56	38 ± 7.0	60 ± 6.0	70 ± 15.0
IV	83 ± 5.0	63 ± 3.0	93 ± 4.0	49	N.D.[a]	35 ± 5.0	35 ± 4.0
V	86 ± 4.0	74 ± 3.0	98 ± 2.0	63	N.D.	45 ± 5.0	78 ± 8.0

[a] N.D. = not determined as such in blood.

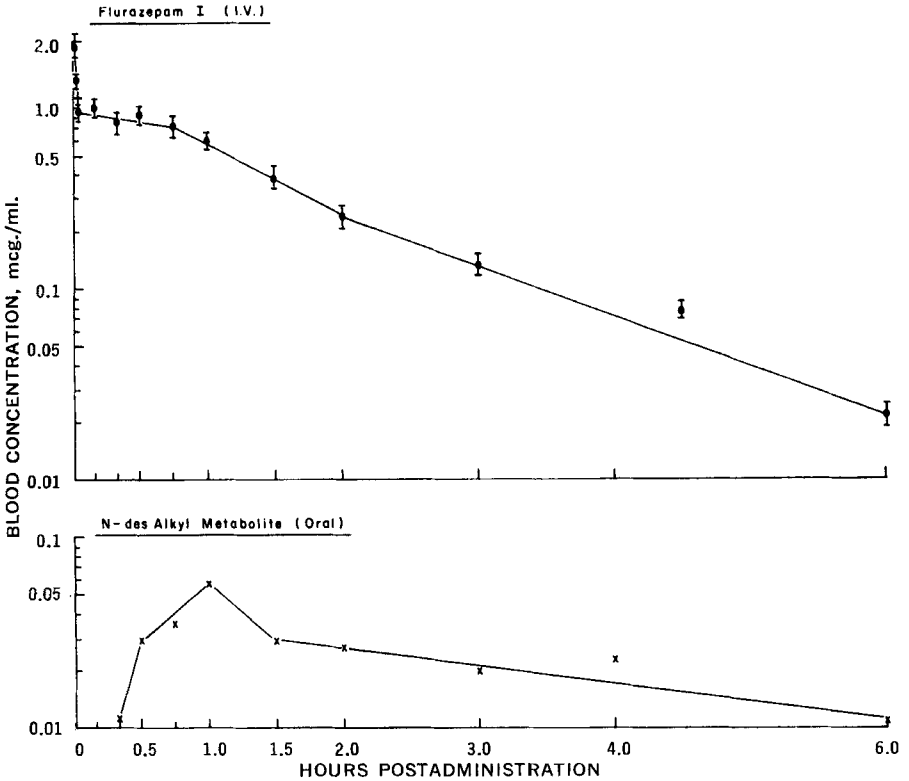


Figure 5—*Blood level fall-off curves of flurazepam and its major metabolites in a dog following the administration of a 5-mg./kg. dose by (top) intravenous and (bottom) oral routes.*

overall recovery from urine of added internal standards of II, IV, and V determined by the absorptiometric assay was of the order of 85, 35, and 78%, respectively (Table III). The sensitivity limits of the absorptiometric assay can be extended by cyclization of the respective benzophenone to its 9-acridanone derivative, which is then quantitated by the more sensitive fluorometric assay (Scheme II).

Application of Methods in Biological Specimens—*Determination of Blood and Urine Levels in the Dog*—Blood level fall-off curves of flurazepam and its major metabolites were determined in a dog following intravenous and oral administrations of 5-mg./kg. doses (50 mg. of flurazepam·2HCl) given 2 weeks apart. Following intravenous administration, only the intact drug (I) was seen in the blood (Fig. 5) over an 8-hr. period. No measurable amounts of metabolites II–V were seen. Following oral administration, however, no measurable levels of flurazepam (I) were noted. The only measurable compound in the blood was the *N*-desalkyl metabolite (III), which was present in low amounts and measurable up to 6 hr. postdosing. The data indicate that the drug was rapidly and extensively metabolized.

The urinary excretion data following both intravenous and oral doses are given in Table IV. Following intravenous administration, approximately 4.58% of the dose was recovered as the intact drug and 10.9% of the dose was recovered as the acidic compound (V) (the major urinary metabolite in the dog) over a 24-hr. period. The other metabolites (II–IV) accounted for only 1.88% of the dose in the same period. The 24–48-hr. period yielded only 0.44% of the dose in approximately equal amounts of II, IV, and V. Following oral administration, the acidic metabolite (V) accounted for 10.1% of the dose in the 0–24-hr. period. The other metabolites accounted for a total of 2.54% of the dose over the same time period. The 24–48-hr. period yielded negligible amounts (0.7%) of the dose, mainly as V. The total amount of the administered dose recovered in the 0–48-hr. excretion period following intravenous and oral administrations was 17.8 and 13.34%, respectively.

These experiments indicate that the rapid elimination of flurazepam from the blood following intravenous administration, the absence of measurable intact drug in the blood following oral administration, and the excretion of several metabolites in the urine are due to tissue distribution and rapid biotransformation of the drug.

The presence of the hydroxyethyl compound (II), the *N*-desalkyl compound (III), the *N*-desalkyl-3-hydroxy compound (IV), and the *N*-1-acetic acid metabolite (V) suggest that two pathways of biotransformation are possible, arising from a common intermediate such as an aldehyde (Scheme III) as proposed by Schwartz and Postma (4).

According to these authors, II is the major metabolite in man whereas V is the major metabolite in the dog. Further metabolic studies for the elucidation of the biotransformation of II were conducted in a dog following the oral administration of a 20-mg./kg.

Table IV—Urinary Excretion Data on Flurazepam and Its Major Metabolites in a Dog following Intravenous and Oral Administration[a]

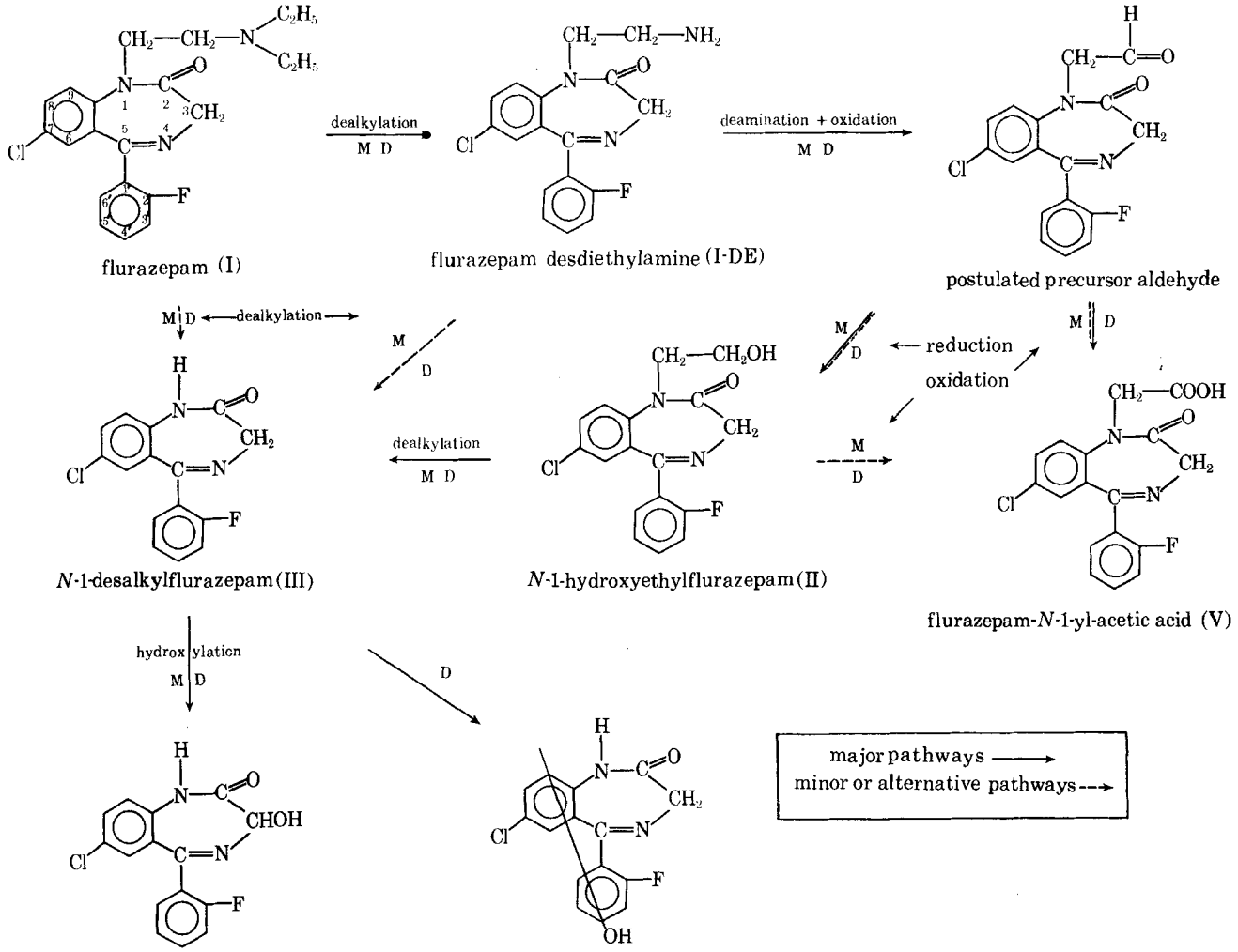
Compound Excreted[b] as Percent of Dose	Route of Administration: Intravenous	Oral
I	4.58	0.37
II	0.37	0.29
III	0.11	0.04
IV	1.40	1.84
V	10.90	10.10
Total percent of dose recovered[c]	17.36	12.64

[a] Dose: 50 mg. flurazepam·2HCl = 42.10 mg. of free base (5 mg./kg.). Excretion period: 0–24 hr. postdosing. [b] Calculated as the flurazepam equivalent. [c] As "free" (directly extractable) and "bound" (conjugated) fractions.

Table V—Urinary Excretion Data on the Hydroxyethyl Metabolite (II) in a Dog following Administration of a Single Oral 20-mg./kg. Dose of the Authentic Compound as the Hydrochloride Salt *versus* the Free Base[a]

Hours Postdosing	Hydrochloride Salt —270 mg.— Free	Hydrochloride Salt —270 mg.— Bound	Free Base —256 mg.— Free	Free Base —256 mg.— Bound
	Percent of Dose Recovered in Urine[b]			
0–24	0.15	11.00	1.20	9.70
24–48	0.22	1.40	0.13	1.10
48–72	0.01	0.44	0.02	0.24
	0.38	12.84	1.35	11.04
Percent total dose recovered	13.23		12.39	

[a] Dose: 20 mg./kg. = 256 mg. of free base. [b] Free = directly extractable II. Bound = glucuronide/sulfate conjugate of II.



Scheme III—Postulated metabolic pathways of flurazepam in man (M) and in dog (D)

dose of II as the hydrochloride salt *versus* the free base. The two forms of the compound were administered 7 days apart in a gelatin capsule. Specimens of whole blood and urine were collected over a 72-hr. period. The blood level fall-off curves (Fig. 6) showed that both forms were rapidly absorbed and produced a blood level peak between 30 and 60 min. The areas under the curves in the 0.24-hr. period were found to be equivalent (base: HCl = 4.99/4.55 = 1.097).

The urinary excretion data on free and conjugated II are given in Table V. The data show that the major portion of the administered dose that was recoverable was excreted in the 0–24-hr. period. The major excretion product recovered was the glucuronide and/or sulfate conjugate of II, accounting for 9.7–11% of the dose recovered in the first 24 hr. The amount of free II excreted in the same period was 0.15% of the dose from the hydrochloride salt and 1.20% of the dose from the free base. Approximately equal amounts of II (*i.e.*, free and conjugated), 13.23% from the hydrochloride salt and 12.3% from the free base, were recovered in the 0–72-hr. period. The low percentage of the dose recovered again indicates either extensive biotransformation and/or alternate routes of excretion.

Due to the low recovery of the administered dose as free II in the treated dog urines, the samples were reanalyzed by the differential spectrophotometric assay for the quantitation of IV and V, the other known metabolites of flurazepam. The data in Table VI show that approximately 8% of the dose was recovered as IV and V, respectively. The total amount of the dose administered either as the free base or the hydrochloride salt of II recovered in the 0–24-hr. period

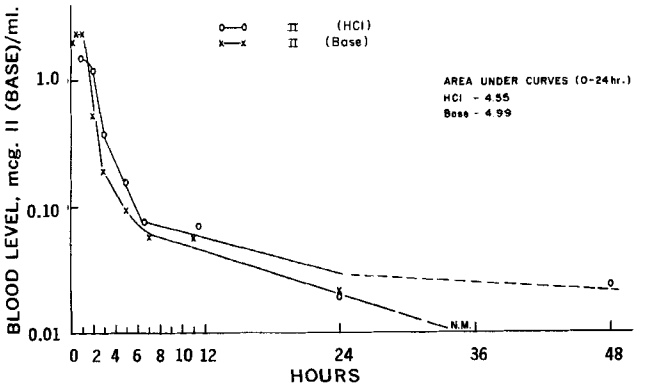


Figure 6—*Blood level fall-off curves of II in a dog following the oral administration of a single 20-mg./kg. dose of the hydrochloride salt* versus *the free base.*

Table VI—Urinary Excretion Data on the Metabolites of II in a Dog following the Oral Administration of a 20-mg./kg. Dose of the Free Base (256 mg.)

Excretion Period	Metabolite Measured	Milligrams of Metabolite Recovered	Calculated as II Equivalent	Percent of Dose Excreted
0–24 hr.	IV	19.0	21.0	8.16
0–24 hr.	V	22.0	21.8	8.50
Total recovered in 0–24 hr.:		41.0	42.8	16.66

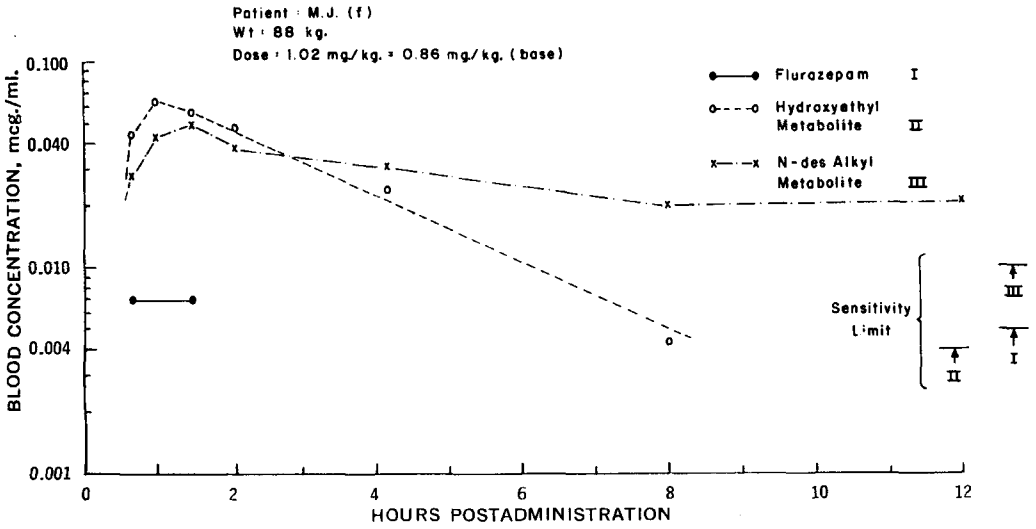


Figure 7—*Blood level fall-off curves of flurazepam and its major biotransformation products in man following a single 90-mg. oral dose of flurazepam·2HCl.*

was 27.6%. These experiments also indicate that the preferred metabolic pathway of flurazepam in the dog leads to the formation of the acidic metabolite (V) and appears to be *via* an intermediate such as an aldehyde rather than *via* the hydroxyethyl compound (II).

Determination of Blood and Urine Levels in Man—Blood level fall-off curves and the urinary excretion of flurazepam and its major metabolites were determined in two human subjects: in Patient M. J. (f) following a single oral 90-mg. dose of flurazepam·2HCl (3 × 30-mg. capsules) and in Patient H. R. (m) following two oral 90-mg. doses of the drug given 21 days apart.

The blood level curves in Patient M.J. (Fig. 7) show data points at 40 and 90 min., at which measurable levels of flurazepam were seen. The major blood components in this subject were: (*a*) the hydroxyethyl metabolite (II), which reached a maximum concentration of 0.064 mcg./ml. at 1 hr. and declined over the next 7 hr., with a half-life of about 2 hr.; and (*b*) the *N*-desalkyl metabolite (III), which reached a peak level of 0.049 mcg./ml. at 1.5 hr. and appeared to plateau thereafter.

The blood level curves in Patient H. R. (Fig. 8A) following the first 90-mg. dose showed low but measurable amounts of flurazepam, which reached a maximum of 0.02 mcg./ml. at 1 hr. and were detectable up to 12 hr. The metabolite (II), on the other hand, showed a peak concentration of 0.072 mcg./ml. at 40 min. and declined over the next 11 hr., with a half-life of about 2 hr. The *N*-desalkyl metabolite (III) showed a peak level of 0.056 mcg./ml. at 1 hr. and after 2 hr. declined with an apparent half-life of about 19 hr.

Upon administration of the second 90-mg. dose to the same subject (H. R.) 21 days later, the blood levels of flurazepam (I) (Fig. 8B) again reached a peak at 1 hr. of about 0.012 mcg./ml. and declined rapidly to the detectable limit at 4 hr. The blood level curves of metabolites II and III almost paralleled those obtained following the first dose.

The urinary excretion data on flurazepam and its metabolites in man are summarized in Table VII. In both subjects, the amount of intact drug and the major blood metabolites (II and III) excreted in the free or directly extractable fraction was very small (1% or less). The major urinary metabolite was the hydroxyethyl compound (II), which was excreted mainly in the bound form as a glucuronide/sulfate conjugate, accounting for more than 40% of the administered dose in the 0–24-hr. excretion period and about 4% of the dose in the 24–48-hr. period. Trace amounts of metabolites IV and V were also seen, collectively accounting for about 4% of the

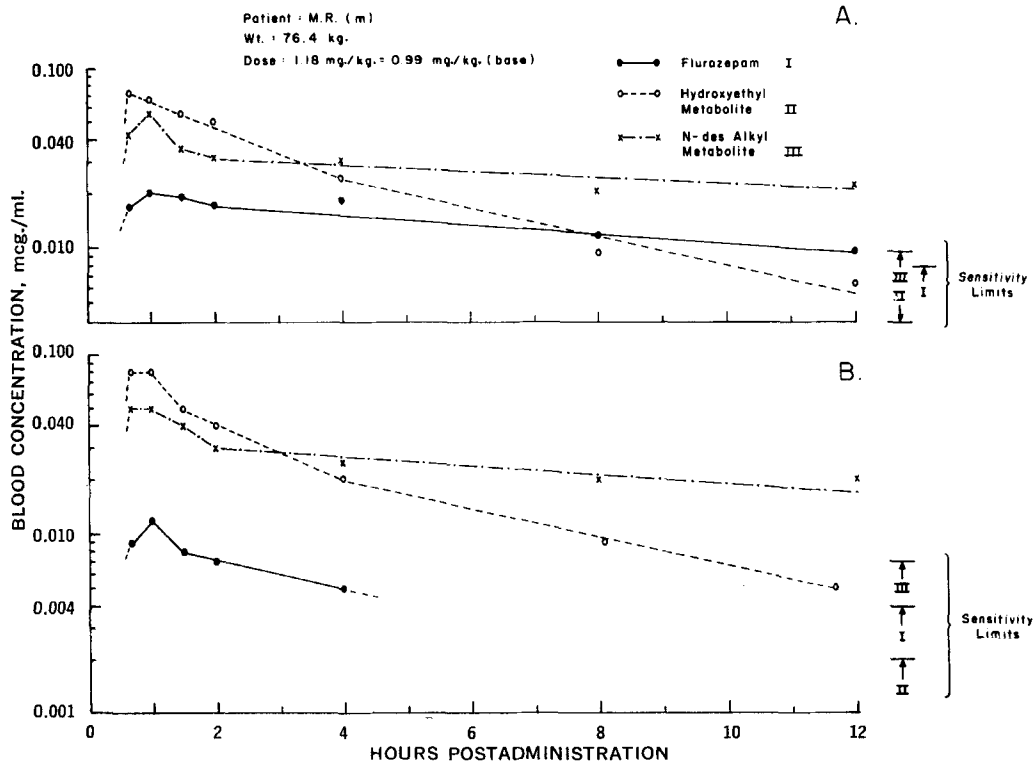


Figure 8—*Blood level fall-off curves of flurazepam and its major biotransformation products in man following single 90-mg. oral doses of flurazepam·2HCl administered 21 days apart.*

Table VII—Urinary Excretion Data on Flurazepam and Its Major Metabolites in Humans following Administration of Single Oral 90-mg. (3 × 30-mg.) Doses of Flurazepam·2HCl

Hours	Form Excreted	Compound Measured	Micrograms per Milliliter	Total, mg.	Milligrams Equivalent as Flurazepam	Percent of Dose Excreted	Total Recovered as Free and Bound	Percent Total Dose Recovered
A: Subject M. J. (f) (Dose: 90 mg. Salt = 75.76 mg. of Flurazepam Free Base)								
0–8	Free[a]	I	0.14	0.08	0.08	0.10		
		II	0.15	0.09	0.10	0.13		
		III	0.65	0.38	0.50	0.66	0.89	
	Bound[b]	II	45.00[c]	26.30	30.60	40.40		
		IV	0.96	0.56	0.70	0.93		
		V	1.20	0.67	0.75	1.00	42.33	43.22
8–24	Free	I	0.03	0.05	0.05	0.08		
		II	0.02	0.04	0.04	0.05		
		III	0.10	0.16	0.21	0.28	0.41	
	Bound	II	2.75	4.10	4.80	6.30		
		IV	0.10	0.15	0.19	0.25		
		V	0.09	0.14	0.16	0.21	6.76	7.17
24–48	Free	I	0.03	0.08	0.08	0.10		
		II	0.02	0.05	0.05	0.07		
		III	0.14	0.38	0.50	0.66	0.83	
	Bound	II	1.00	2.90	3.40	4.40		
		IV	0.06	0.16	0.20	0.26		
		V	0.06	0.16	0.18	0.24	4.90	5.73
								56.12
B: Subject H. R. (m) (Dose I: 90 mg. Salt = 75.76 mg. Free Base)								
0–24	Free	I	0.14	0.16	0.16	0.21		
		II	0.07	0.08	0.10	0.12		
		III	0.63	0.72	1.00	1.30	1.63	
	Bound	II	24.20[c]	27.80	32.40	42.80		
		IV	0.64	0.74	0.97	1.26		
		V	0.26	0.30	0.34	0.45	44.51	46.14
24–48	Free	I	0.05	0.09	0.09	0.12		
		II	0.01	0.02	0.03	0.04		
		III	0.22	0.40	0.50	0.70	0.86	
	Bound	II	1.41	2.54	2.96	3.91		
		IV	0.07	0.12	0.16	0.21		
		V	0.05	0.09	0.10	0.14	4.26	5.12
								51.26
Determination of Only the Total Major Urinary Metabolite (II) (Free and Bound) by Spectrophotometry								
C: Subject H. R. (m) (Dose II: 90 mg. Salt = 75.76 mg. of Free Base)								
0–8	Total[d]	II	74.1	14.8	17.3	22.8		
8–24	Total	II	15.4	8.5	9.9	13.0		
24–48	Total	II	n.m.[e]	—	—	—		
								35.8

[a] Free = directly extractable. [b] Bound = conjugated. [c] Determined by spectrophotometry, all other data by spectrofluorometry. [d] Total = free and conjugated forms. [e] n.m. = not measurable < 1–2 mcg./ml.

dose in the 0–48-hr. period. When the same dose was repeated in the same subject (H. R.) after a wash-out period of 21 days, the 0–24-hr. urine specimen yielded 36% of the administered dose as the hydroxyethyl metabolite (II) in both free and conjugated forms. The total urinary excretion of flurazepam and its metabolites in both subjects in the 0–48-hr. period following the oral administration of 90-mg. doses of flurazepam·2HCl appears to be consistent and reproducible, with a total of 56% of the dose eliminated by Patient M. J. (f) and 51% of the dose eliminated by Patient H. R. (m).

The administration of flurazepam by either intravenous or oral routes in the dog and orally in man results in rapid and extensive biotransformation prior to elimination. According to Schwartz and Postma (4), flurazepam (I) was metabolized by successive *N*-dealkylation of the side chain (Scheme III), which appeared to be the preferred pathway to direct hydroxylation of the benzodiazepin ring. In the dog, the major urinary metabolite was the acidic compound V, while the hydroxyethyl compound II was a minor metabolite. In man, the converse was true; II was the major blood and urinary metabolite and V was a minor metabolite seen only in the urine. The presence of small amounts of III and IV in the urine of both species lends support to the postulation of the existence of a common precursor (metabolite), such as an aldehyde produced by *N*-dealkylation and oxidative deamination of the terminal (—N<) in the alkyl side chain of flurazepam. In the dog, oxidation of the aldehyde as the preferred pathway would result in the excretion of V as the major metabolite; in man, reduction of the aldehyde to an alcohol would result in the excretion of II as the major metabolite. The monodesethyl and desdiethylamine (I-DE) metabolites detected in trace amounts in both man and dog and the phenolic analog (III-A) (2) seen only in the dog, as reported by Schwartz and Postma (4), were detected in this study but were not quantitated.

The results of this study, using flurazepam·2HCl in the dog as a pure chemical and in man as the pharmaceutical formulation, are in agreement with the findings of the authors (4) who used ^{14}C-labeled flurazepam·2HCl exclusively.

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New Method for Evaluating Topical Action of Substances on Gastric Mucosa

R. S. ALPHIN and D. A. DROPPLEMAN

Abstract □ A new method is described to evaluate the topical action of drugs on the gastric mucosa of laboratory animals. Of distinct advantage is the ability to assess the effects of more than one drug simultaneously on adjacent mucosal areas. The method also has proven to be of value in studying the effects of such variables as duration of exposure, pH, and pepsin.

Keyphrases □ Gastric mucosa injury—multidrug simultaneous evaluation, animals □ Aspirin—gastrotoxic effects, cats □ Phenylbutazone—gastrotoxic effects, cats

An important and well-known facet of the pharmacology of various substances, particularly anti-inflammatory drugs, is their injurious topical action upon the GI tract. Most methods used to determine such action have involved assessing the gastric effects following the oral administration of drugs to guinea pigs (1–3), rats (4), dogs (5, 6), rabbits (3), and man (7). Some investigators introduced the substances under investigation directly into the stomach of several species of animals (3, 8, 9). Direct application of anti-inflammatory drugs on the buccal mucosa of man (9) and the explanted gastric mucosa of dogs (10) also were studied. Few, if any, of these methods provide for the simultaneous assessment of the damage produced by two or more agents on adjacent areas of the gastric mucosa. The apparatus and procedure described in this report make such an evaluation possible and, in addition, allow the experimenter to observe the progression of some events as they occur.

EXPERIMENTAL

Apparatus—A 10.2-cm. round bar of clear Plexiglas was bored to 6.5-cm. i.d. and cut into pieces 5.6 and 2.4 cm. in length for the two upper pieces of the apparatus. Two pieces of Plexiglas tubing (0.5 × 2.5 cm.) were glued into holes bored into opposite sides of the upper portion using Plexiglas solvent MC-25. Three holes were drilled down through the top ring and into the bottom ring; the bottom ring was threaded to receive 10–32 SS machine screws. Three machine screws were ground to a point for easier penetration of the mucosa.

A continuous groove, 0.3-cm. deep and 0.4-cm. wide, was cut in the center of the bottom surface of the upper ring where it contacts

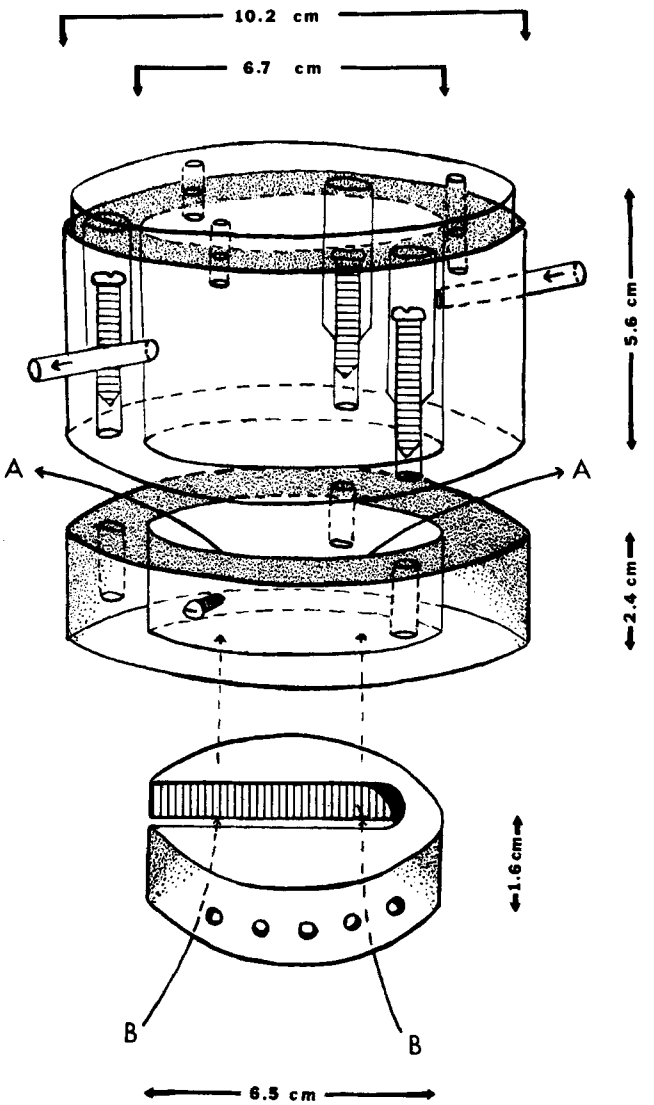

Figure 1—*Diagram of the Plexiglas chamber used for assessment of topical effects of substances on the gastric mucosa. Key: A, position of the stomach wall and mucosa; B, direction of major blood vessels. The lower Plexiglas disk, when placed into position, provides a relatively flat mucosal surface.*

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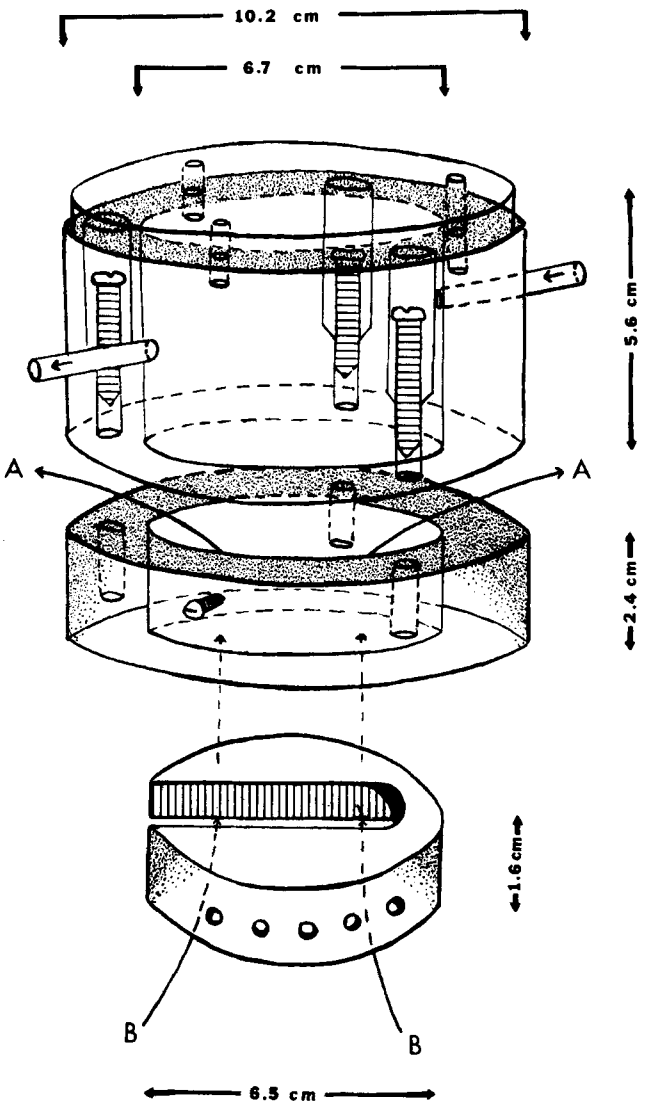

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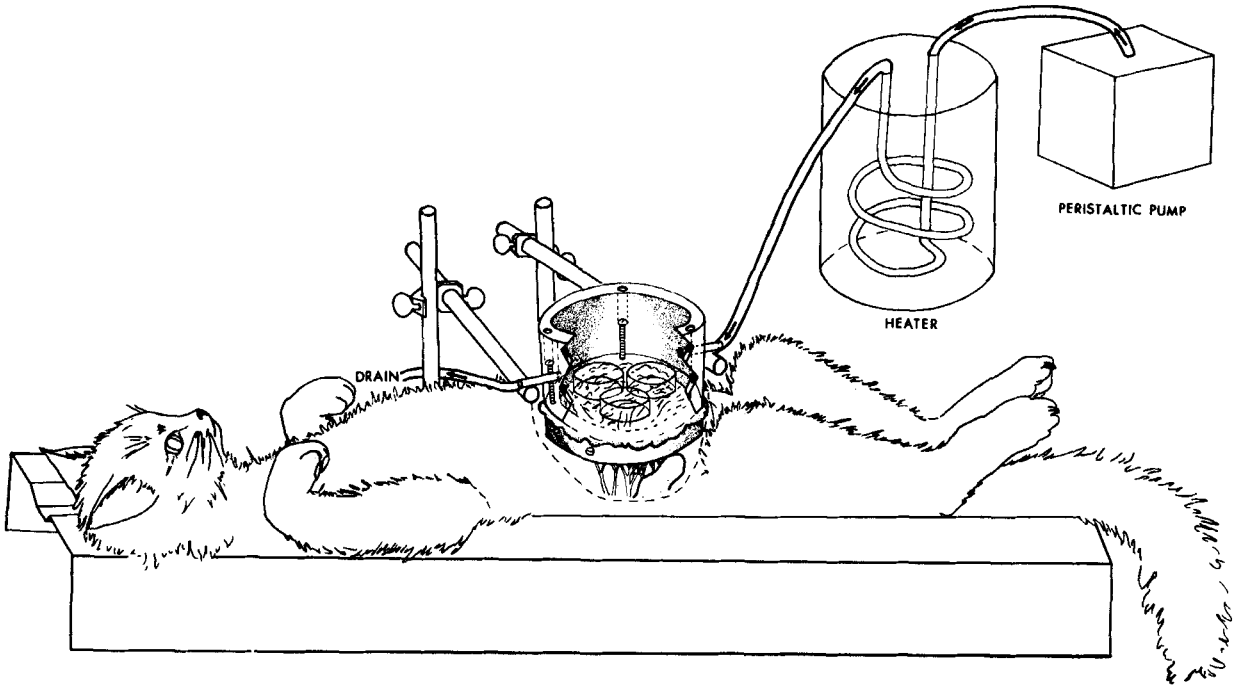

Figure 2—*Schematic drawing of the apparatus in position in the cat for assessing the topical action of substances on the gastric mucosa. Cutaway view of the chamber shows the position of the glass containers into which the test materials are placed.*

the mucosa. Likewise, a continuous tongue was cut in the top surface of the lower ring to fit into the groove. Gaskets of 0.2-cm. gum rubber were glued over the tongued surface and the grooved surface of the rings. A Plexiglas disk, 1.6-cm. thick, was made to fit inside the lower ring. A slot, 2-cm. wide, was cut into the disk to accommodate the vessels of the mucosa. The disk was held in place by a set screw. Holes were drilled into the sides of the disk so that the set screw could hold the disk more firmly. A diagram of the apparatus is shown in Fig. 1.

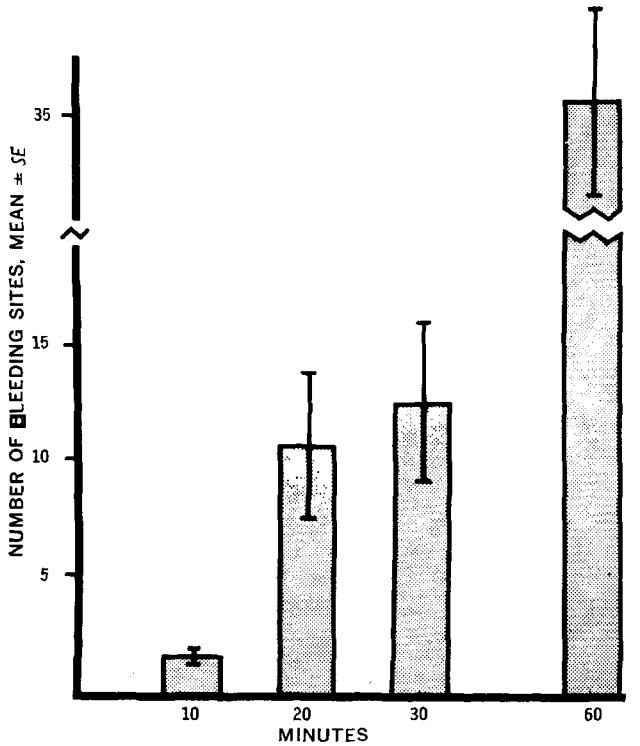

Figure 3—*Time–bleeding response pattern of aspirin (10 mg.) applied to the gastric mucosa of cats. The pH of the artificial gastric juice was 1.3. Results shown were obtained from 66 exposures in 11 cats.*

Procedure—Fasted cats of either sex, 2.5–5.0 kg., were anesthetized with phenobarbital sodium (130 mg./kg. i.p.). The stomach was exposed through a midline abdominal incision. The right gastric artery and vein were included with the ligature placed around the pyloric–duodenal juncture (excluding the right gastroepiploic artery and vein). Additional ligatures were placed around the esophagus, the left gastric artery and vein, and the larger vessels adjoining the lesser curvature. The stomach was further isolated by cutting through the esophagus and the pyloric ring distal and proximal to the respective ligatures. Eversion of the mucosa was made through an incision along the entire length of the lesser curvature.

Eight Allis clamps were attached to the stomach wall in a circular fashion and brought up through the Plexiglas ring (A in Fig. 1). The top part of the Plexiglas chamber was placed on the mucosa directly over the bottom ring and secured with screws. Blood vessels along the greater curvature were placed through the slot of the Plexiglas disk designed to provide a reasonably flat mucosal surface. Tissue blood supply was thus maintained in a similar fashion to that described by Rehm (11). In most of the experiments, physiological saline was allowed to remain on the mucosa for 1–2 hr. prior to perfusion of the chamber with artificial gastric juice USP.

The temperature within the chamber was maintained at approximately 37° by preheating the gastric juice prior to its entry into the chamber. This was accomplished by means of a Harvard peristaltic pump (model 1202) and a water bath (Blue M, model 120). The Tygon tubing leading from the water bath to the chamber was carefully insulated with asbestos tape to avoid heat loss. During the perfusion of artificial gastric juice, test substances were applied directly on the gastric mucosa with a tuberculin syringe (0.1 ml.) and confined to relatively small areas by means of glass rings (2.5 × 1.2 cm.). A schematic drawing of the apparatus in position in the cat is shown in Fig. 2. During the exposure to various substances, any visible bleeding sites on the mucosa were counted as they appeared.

Following termination of the experiment, the gastric mucosa was examined macro- and microscopically for injury, *i.e.*, erosions and ulceration[1].

[1] When it was desirable to record the progression of events, an EXA 35-mm. single-reflex camera (Exakta Camera Co., New York, N. Y.) was mounted directly above the chamber; Ektachrome EHB-135 film was used along with a 3200°K Photo-Flood lamp. A Polaroid Land camera (model Ed-10, film 108), mounted on a Bausch and Lomb Stereozoom 7 microscope, was also used successfully.

Table I—Comparative Topical Effects of Aspirin and Phenylbutazone on the Gastric Mucosa of Cats[a,b]

Drug	Exposure Time, min. 10	20	30	60
Aspirin	1.8 ± 1.8	14.3 ± 4.6	25.2 ± 4.9	44.9 ± 2.7
Phenylbutazone	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.3
p-Value	>0.05	<0.01	<0.001	<0.001

[a] Results expressed as the mean number ± *SE* of bleeding sites following the exposure of both phenylbutazone and aspirin to a total of 17 areas each in six cats. [b] Equimolar amounts of aspirin (18 mg.) and phenylbutazone (30.8 mg.) suspended in 20% arabic gum were applied to gastric mucosa perfused with artificial gastric juice (pH 1.3).

To illustrate the utility of the method, the gastric effects of the topical application of suspensions of aspirin and phenylbutazone are presented.

RESULTS AND DISCUSSION

The results of the application of equimolar amounts of aspirin and phenylbutazone on a total of 34 sites of the gastric mucosa in six cats are depicted in Table I. Both drugs were suspended in 20% arabic gum (USP powder), with the initial pH of the aspirin suspension being 3.3 and of the phenylbutazone being 4.4 prior to application on the gastric mucosa. Of 17 sites exposed to aspirin (18.0 mg.) for 60 min., 16 showed gastric bleeding and lesions; in the phenylbutazone (30.8 mg.)-treated areas, only slight bleeding and no lesions were observed. Four of the sites exposed to aspirin showed frank ulceration. The findings that phenylbutazone produced significantly less acute local gastric injury than aspirin are in agreement with those of Hitchens *et al.* (12); they found that upon oral administration to rats, phenylbutazone was less ulcerogenic than aspirin. Comparative clinical studies by Wood (13) indicated that, following oral administration of aspirin, 70% of the subjects studied showed occult bleeding whereas no reproducible bleeding occurred following phenylbutazone.

Quantitative assessment of the bleeding produced by 10 mg. of aspirin (0.1 ml. of a suspension with purified starch, pH 2.7) applied topically to the mucosa is shown in Fig. 3. The pH of the artificial gastric juice was 1.3 in all of these experiments. It is evident that the number of bleeding sites increased with time.

Another application of the method was shown by determining the effect of the pH of the artificial gastric juice on the bleeding produced by aspirin. The results obtained in 27 cats (Fig. 4) show that when the pH of the juice was maintained above 2.0, little or no bleeding resulted from the topical application of 10 mg. of aspirin. In contrast, as the pH decreased from 2.0, there was a marked increase in gastric bleeding.

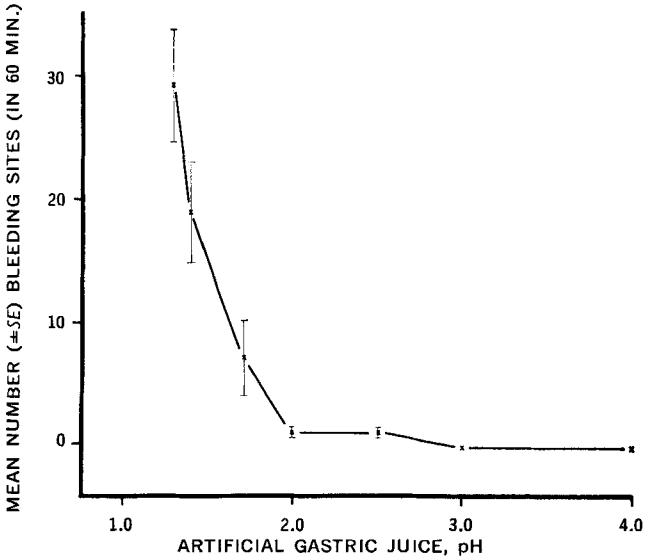

Figure 4—*Effect of pH of artificial gastric juice on bleeding produced by aspirin. The results were obtained from an average of 17 exposure sites to aspirin (initial pH of 2.7 in a suspension of 20% purified starch) with a minimum of three cats at each pH, with the exception of pH 4.0 where two cats were used.*

It appeared to be of interest at this point to determine whether or not, and if so to what extent, exogenous pepsin contributes to the bleeding response observed with aspirin. Following 1 hr. of exposure to aspirin (artificial gastric juice USP, pH 1.3), 18 areas in six cats showed a mean number of bleeding sites of 28.2 ± 4.5 *SE*. When under similar conditions 18 sites in five cats were exposed to aspirin with deletion of pepsin from the artificial gastric juice, the mean number of bleeding sites was 12.3 ± 3.7 *SE*. The difference between the number of bleeding sites with and without pepsin was significant ($p < 0.01$). Under the conditions used, pepsin appeared to contribute to the bleeding observed with aspirin.

The method as described has been used routinely in this laboratory for the assessment of the topical action of drugs on the gastric mucosa of laboratory animals. In one series of experiments, 10 mg. of aspirin (applied to 350 different areas of the gastric mucosa in 154 cats) produced 15.82 ± 1.00 *SE* bleeding sites within 30 min. When the time was extended to 60 min., the number of sites increased to 38.02 ± 1.11 *SE* (266 exposures in 134 cats).

Experience with the method has demonstrated that it offers the following advantages: (*a*) one or more substances may be evaluated simultaneously on adjacent mucosal areas; (*b*) the quantity of material applied and exposure time may be easily varied; (*c*) gastrotoxic effects can be observed as they occur; and (*d*) the apparatus may be adapted for use in large or small animals.

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Use of Thermal Gravimetric Analysis in Sorption Studies I: Interaction of Selected Liquid Compounds with Nylon

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Abstract □ Thirty-nine liquid compounds were studied as to their sorption by nylon. Each liquid was kept in contact with test samples of nylon until equilibrium was established, and then the amount of compound sorbed was determined in a thermogravimetric analysis instrument. The same instrument was then used to desorb the compound from the nylon at a specified rate of heating. The resulting thermogram permitted the evaluation of the activation energy of desorption. Twenty compounds were found to be significantly sorbed by nylon, while the remaining compounds were not significantly sorbed. It was apparent that compounds having hydrogen-bonding potential would interact with nylon in most instances. Several compounds not possessing hydrogen-bonding properties were also sorbed but to a much lesser extent. The size of the molecule in a homologous series also played a part in the extent of sorption and in the energy requirement for sorption. Thermal gravimetric analysis appears to be a practical method for conducting sorption studies when large numbers of compounds are to be studied or screened for a packaging application in a polymeric material.

Keyphrases □ Sorption equilibria—liquids sorbed by nylon □ Nylon–liquid sorption equilibrium—thermal gravimetric analysis □ Thermal gravimetric analysis—liquid–nylon sorption □ Activation energy, desorption—liquids from nylon □ Containers, plastic—liquids–nylon sorption

The packaging of pharmaceutical products in plastic containers and in various types of packaging devices requires that little or no interaction takes place between the component in the product and the plastic. Such drug–plastic interaction can lead to a reduction in the potency of the product, negating its value. One method of ascertaining if potential drug–plastic interactions will occur is to undertake sorption studies at one or more isothermal conditions with the test compound and the specific plastic. When properly conducted, the equilibrium sorption studies can lead to data which can then be treated on a thermodynamic basis. Studies of this type have appeared in the literature in recent years (1–5).

While sorption studies at isothermal conditions are excellent for generating both kinetic and thermodynamic data, they are generally quite time consuming when a large number of compounds are to be studied with a specific polymer. For this reason, attention was directed toward the use of thermogravimetric analysis (TGA) in which one experiment could be utilized to determine the equilibrium sorption of the test compound as well as the activation energy of desorption. This latter value can also be considered as approximating the heat of desorption which, in turn, should be equal to the magnitude heat of sorption. In the report presented here, 39 compounds as liquids, representing a broad spectrum of molecular properties and functional groups, were studied as to their equilibrium sorption with nylon.

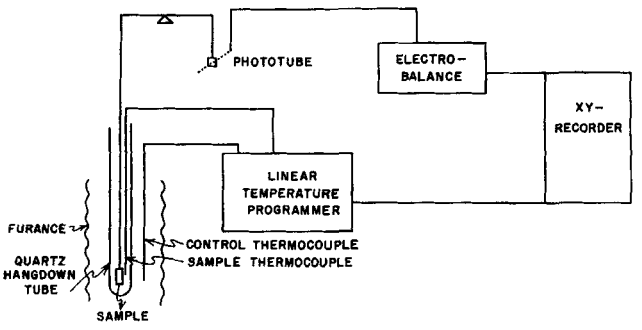

Figure 1—*Schematic diagram of TGA instrument for desorption studies.*

EXPERIMENTAL

Materials and Equipment—The compounds used in the study were either reagent grade or of a similar purity and were used as such. Nylon-6,6[1] was used as the film, having an average thickness of 0.013 cm. (0.005 in.).

A schematic diagram of the TGA instrument[2] is shown in Fig. 1. A straight nichrome wire, 0.1 mm. diameter and 33 cm. long, was used to load the sample on the balance beam. Both the sample weight change and temperature could be recorded simultaneously.

Preparation of Sample—Nylon film was soaked in 95% (v/v) alcohol for a period of 24 hr. and then rinsed with distilled water a total of six times to remove contaminants. After drying for 12 hr. at 50°, the sample was placed into a desiccator until needed for a sorption experiment. Prior to an experiment, the film was removed from the desiccator and cut into sections measuring 0.63 × 1.27 cm. (0.25 × 0.5 in.)[3]. One section (or sample) was then placed into the TGA instrument and heated to 120° at a programmed heating rate of 5°/min. to remove any sorbed water from the sample. Preliminary experiments indicated that this treatment would be adequate in removing sorbed water from the film.

At the end of the heating cycle, the exact weight of the test sample was recorded at 25°, and the sample was then immersed into 10 ml. of one of the test liquids previously placed into an all-glass conical flask (25-ml. size). After the flask was sealed with a ground-glass stopper, it was stored at a temperature of 25 ± 0.5° in a constant-temperature room for 1 week or until it was apparent that equilibrium was obtained. The test sample was then removed by blotting the film with tissue paper. Immediately, the sample was introduced into the TGA, the sample weight was recorded, and a thermogram was determined.

TGA Procedure for Desorption Studies—In any desorption run for the liquids in this study, the electrobalance was set at a sensitivity of 0.01 mg. and recorded on a 1-mv. instrument. Temperature was programmed to increase at a rate of 5°/min. To prevent chemical oxidation of the nylon and to sweep out volatile substances not sorbed, nitrogen gas was passed through the heating chamber at a rate of 0.1 l./min. The weight loss of the sample was recorded in the temperature range from 25 to 200°. In this temperature range, nylon is not thermally degraded. It was ascertained that the in-

[1] Polypenco 101, Polymer Corporation of Pennsylvania, Reading, Pa.

[2] The TGA instrument consisted of the following: (*a*) Fisher model 100 TGA System, Fisher Scientific Co., Pittsburgh, Pa.; (*b*) Cahn RG electrobalance, Cahn Instrument Co., Paramount, Calif.; and (*c*) Servo/Riter II potentiometric XY recorder, Texas Instrument Co., Houston, Tex.

[3] Approximately 20 mg.

Table I—Compounds Studied in Sorption Experiments with Nylon[a]

Compounds Not Significantly Sorbed	Compounds Significantly Sorbed
Carbon tetrachloride	Water
Ethylene dichloride	Methanol
Ethyl bromide	Ethanol
Methyl benzoate	Propanol
Aniline	Butanol
Anisole	Pentanol
Benzonitrile	Acetic acid
Ethynyl benzene	Chloroform
Chlorobenzene	Ethylene glycol
Phenyl isothiocynate	2-Chloroethanol
o-Nitroanisole	Ethyl iodide
Methyl salicylate	Acetone
2-Hydroxyacetophenone	Methyl acetate
Benzaldehyde	Phenol
p-Isopropylbenzaldehyde	Benzyl alcohol
p-Tolualdehyde	Benzyl chloride
Anisoaldehyde	Benzaldoxime
o-Chlorobenzenaldehyde	Benzene
trans-Cinnamaldehyde	Toluene
	Acetophenone

[a] The weight of sample used in the sorption studies was approximately 20 mg. Since the instrument used was accurate to ±1.0 mg., "significant sorption" occurred when the amount of compound sorbed was greater than 0.2 mg.

strument as used in this study produced an error of less than ±1% in the recording of weights.

Treatment of Data—Numerous methods have been proposed for deriving kinetic parameters from TGA thermograms. Quantitative aspects of this subject dealing with degradation of polymers were presented by Madorsky (6). Doyle (7) and Freeman and Carroll (8) presented equations which permit the calculations of activation energies of degradation and orders of reaction. More recently, Broido (9) reported a simplified method for evaluating the activation energy of degradation of polymers. Broido's method (9) was found to be more sensitive to small weight changes than previous methods. Even though these aforementioned authors used their respective expressions for evaluating the energy requirement for degradation of polymers, the same expressions should also be applicable for approximating the energy requirement for binding a molecule to a polymer. In this case, the activation energy of desorption may be considered as approaching in magnitude the thermodynamic constant referred to as the "heat of sorption."

Preliminary treatments of thermogram data established that Broido's method (9) was the most suitable for calculating the activation energy. His expression may be written as:

$$\ln \ln (1/Y) = -\frac{E}{RT} + \text{constant} \qquad \text{(Eq. 1)}$$

where Y is further defined as:

$$Y = \frac{W_t - W_\infty}{W_0 - W_\infty} \qquad \text{(Eq. 2)}$$

and E, R, and T are the activation energy of desorption, the universal gas constant, and the absolute temperature, respectively. The terms in Eq. 2 are further defined as: W_t = weight at time t; W_0 = weight at beginning of experiment (start of desorption); and W_∞ = weight at end of experiment (end of desorption).

A plot of ln ln $(1/Y)$ *versus* $1/T$ yields a linear relationship from which E can be calculated by knowing the slope of the line.

RESULTS AND DISCUSSION

Equilibrium sorption experiments were run on 39 liquid compounds at 25°, and the final weights of sorption were determined in the TGA apparatus prior to conducting the desorption experiments. Since the accuracy of the method used was ±1% or less, equilibrium sorption weights of less than 0.2 mg. (for 20-mg. sample of nylon) were considered as "not significantly sorbed." Of the 39 compounds studied, 20 compounds showed significant sorption. Table I lists both the compounds not sorbed and those which were sorbed by nylon. The weights of compounds sorbed at equilibrium are shown in Table II.

Table II—Equilibrium Sorption Concentrations of Compounds Sorbed by Nylon

Compound	Milligrams per Gram	Millimoles per Gram
Water	79.2	4.4
Methanol	83.3	2.6
Ethanol	96.7	2.1
Propanol	85.9	1.43
Butanol	24.4	0.33
Pentanol	14.1	0.16
Acetic acid	252.2	4.2
Chloroform	182.7	1.53
Ethylene glycol	105.5	1.7
2-Chloroethanol	262.5	3.3
Ethyl iodide	62.4	0.4
Acetone	21.5	0.37
Methyl acetate	21.1	0.27
Phenol[a]	—	—
Benzyl alcohol	205.4	1.9
Benzyl chloride	60.8	0.48
Benzaldoxime	70.3	0.58
Benzene	10.1	0.13
Toluene	12.0	0.13
Acetophenone	132.5	0.11

[a] Phenol dissolves the nylon.

Activation energies of desorption for the compounds studied are listed in Table III. An example of a Broido (9) plot for benzyl alcohol is depicted in Fig. 2, from which the activation energy was calculated. For each compound, two thermograms were prepared and the average slope was then used in the calculation of the activation energy. All of the compounds, when plotted as shown in Fig. 2, were linear, with all of the points falling on the line. Table III also contains the heats of vaporization for each compound.

A knowledge of the structures of the compounds (Table I), the amount of sorption (Table II), the activation energies of desorption (Table III), and the heats of vaporization (Table III) can help to present some tentative suggestions as to the mechanism of interaction of the compounds studied with nylon.

Nylon has three polar sites in its structure where binding may occur with various types of molecules: (*a*) an end amino group, (*b*) an end carboxylic group, and (*c*) repeating amide groups in the polymer chain. By far the most frequent groups are the amide linkages, thereby implying that this site is perhaps the most significant for interactions. Previous reports suggested that for unionized molecules having acidic hydrogens or hydrogens capable of forming hydrogen bonds, an interaction of the molecule with the carbonyl

Table III—Activation Energies of Desorption and Heats of Vaporization for Compounds Sorbed by Nylon

Compounds	Energy of Activation, kcal./mole	Heat of Vaporization, kcal./mole
Water	8.7	9.7
Methanol	8.6	8.4
Ethanol	8.1	9.4
Propanol	9.0	9.9
Butanol	10.3	10.5
Pentanol	12.9	10.7
Acetic acid	5.2	5.8
Chloroform	7.2	6.96
Ethylene glycol	7.3	11.8
2-Chloroethanol	5.1	9.8
Ethyl iodide	9.0	7.1
Acetone	7.0	7.65
Methyl acetate	9.6	7.25
Phenol[a]	—	—
Benzyl alcohol	6.3	12.1
Benzyl chloride	8.1	—[b]
Benzaldoxime	8.6	—[b]
Benzene	8.1	7.35
Toluene	12.5	8.0
Acetophenone	11.5	7.7

[a] Phenol dissolves nylon. [b] Reported value not found.

group in the amide structure in nylon can take place through a hydrogen-bond formation (10).

The derivation of activation energy of desorption, which is achieved by the method described in this paper, permits the consideration of the possible mechanisms of interaction between the compound and the substrate. It is recognized, of course, that thermal measurements by themselves do not offer sufficiently conclusive evidence in this regard. An analysis of the nature of the bonding interaction, however, is possible, at least as an initial step, leading to the design of adequate experiments by suitable techniques to pursue mechanistic details in greater depth if required.

An examination of the compounds not significantly sorbed (Table I) shows that only two of the series have hydrogens capable of forming hydrogen bonds with nylon. These are methyl salicylate and 2-hydroxyacetophenone. Their lack of significant sorption with nylon may be due to the intrahydrogen bond formation preventing the OH groups from interacting with nylon.

As Table I reveals, 20 of the 39 compounds studied were sorbed by nylon. Of these 20 compounds, 12 have OH groups capable of hydrogen-bond formation; therefore, it appears reasonable to assume that these compounds will hydrogen bond with the amide linkages in the nylon. Since hydrogen in chloroform is sufficiently positive, the sorption of this compound may also take place through a hydrogen bond. Acetone, methyl acetate, and acetophenone have carbonyl oxygens and thus can act as sites of interaction with other molecules possessing acidic hydrogens. This suggests, but does not prove, that sorption of these three compounds may take place *via* the amide hydrogen of nylon interacting with the carbonyl oxygen of the compounds.

Sorption through hydrogen bonding cannot explain the interaction of four compounds (ethyl iodide, benzyl chloride, benzene, and toluene) with nylon since these compounds do not have hydrogen-bonding capabilities. A suggested mechanism for their interactions with nylon cannot be given from the experimental data, even though it is attractive to conjecture that probably the interaction is primarily through dipole or Van der Waals' forces.

Water, the smallest molecule studied in the series, showed the greatest equilibrium sorption (4.4 mmoles/g.). The large number of moles of water sorbed is more than would be expected. Here it is assumed that a water molecule hydrogen bonds to the amide groups in the nylon. Since the activation energy of desorption is slightly less than the heat of vaporization (Table III), the additional water molecules sorbed by the nylon may be due to association of water molecules sorbed by the nylon matrix. Puffr and Sebenda (11), in fact, suggested from their studies that three molecules of water interact at two neighboring amide groups in nylon. As may be noted from Table II, acetic acid is also sorbed to a great extent by nylon (4.2 mmoles/g.). A comparison of the activation energy of desorption with the heat of vaporization of acetic acid (Table III) suggests that acetic acid may also be in an associated state within the nylon matrix and thus the interaction may be similar to the water–nylon interaction.

The aliphatic alcohols reveal that the size of the molecule plays an important role in the quantity of sorption occurring. As is clear from Table II, the equilibrium sorption decreases as the homologous series is ascended from methyl alcohol (2.6 mmoles/g.) to pentanol (0.16 mmole/g.). With these alcohols, the activation energies of desorption have values close to their heats of vaporization. This implies that the state of these molecules in nylon is similar to their liquid state. It is also interesting to observe from Table III that as the alcoholic series is ascended, the energy of activation increases from a value of 8.7 kcal./mole for methanol to 12.9 kcal./mole for pentanol. As was indicated previously, the activation energy of desorption may be considered as approximating the heat of sorption and, thus, as an indication of the strength of interaction. These results indicate the importance of the hydrophobic portion of the compounds in acting as secondary sources of interaction forces with the nylon, a suggestion consistant with hydrophobic bonding (12, 13). In the alcohol series, methanol has an activation energy of desorption similar to that of water.

Ethylene glycol, having a similar molecular size to propyl alcohol, was sorbed to the same extent as *n*-propyl alcohol (Table II). The activation energy of desorption (7.3 kcal./mole), however, is lower than the heat of vaporization (11.8 kcal./mole). It is possible that the molecule is interacting at an amide site through only one of its hydroxyl groups (one hydrogen bond rather than two hydrogen bonds which may occur in the liquid state). Introduction of a chlorine atom to ethanol produces the compound 2-chloroethanol and increases the sorption from 2.1 mmoles/g. (for ethanol) to 3.3 mmoles/g. The activation energy of desorption for 2-chloroethanol was 5.1 kcal./mole, while its heat of vaporization was 11.8 kcal./mole. With the data available, it is difficult to postulate the reason or reasons for the high degree of sorption or to rationalize in a satisfactory manner the large difference between the activation energy of desorption and the heat of vaporization.

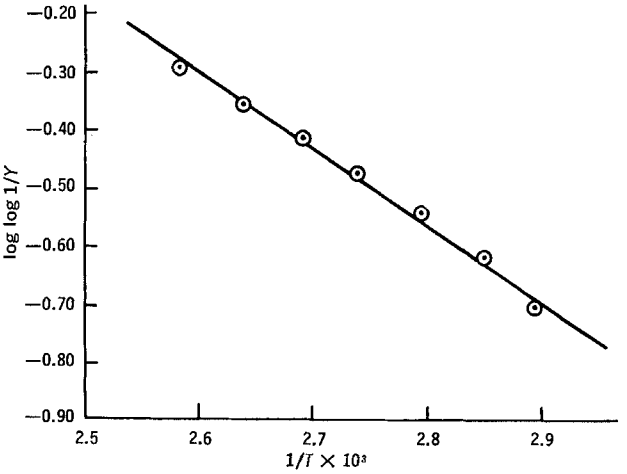

Figure 2—*An example of a Broido (9) plot for benzyl alcohol.*

Chloroform is sorbed to a reasonable extent (1.53 mmoles/g.), similar to *n*-propanol (1.43 mmoles/g.). The bond strength of chloroform in nylon is approximately the same as the bond energy of chloroform in the liquid state.

Ethyl iodide was sorbed to a small extent by nylon, while ethyl bromide showed no significant sorption. Small sorption levels were also found for acetone and methyl acetate, but the rationale for these interactions cannot be presented in a satisfactory manner.

In general, the aromatic compounds, with the exception of phenol and benzyl alcohols, showed less tendency to be sorbed by nylon than the aliphatic compounds. Phenol interacted so strongly with nylon that it dissolved the polymer. Benzyl alcohol was sorbed to nearly the same extent as ethyl alcohol (Table II). The activation energy of desorption for benzyl alcohol was approximately one-half that of its heat of vaporization, indicating that the bond formation of the molecules in the liquid state is much stronger than exists when the molecules are in nylon. It may be assumed that both phenol and benzyl alcohol interact primarily through hydrogen-bond formation with nylon. Benzaldoxime is also sorbed but to a much lesser extent; since it also has an acidic hydrogen, the interaction may be through a hydrogen bond with nylon. Benzyl chloride, benzene, toluene, and acetophenone sorb to a small extent, but their mode of interaction is not clear.

Even though dipole moment values for the compounds are not referred to in this report, they were obtained for most of the compounds and were studied to see if any general relationship could be detected between the constant and sorption. No significant relationship was noted.

In reviewing the sorption behavior of the 39 compounds in this study, it is apparent that compounds having hydrogen-bonding capabilities (*i.e.*, OH or COOH groups) are most likely sorbed by nylon. Other structural features in the molecule can, however, increase or decrease the extent of sorption. Size of the molecule in a homologous or related series also plays an important role in the final equilibrium sorption process. Other compounds not having hydrogen-bonding capabilities may also be sorbed but generally to a much lesser extent than the hydrogen-bonding molecules.

SUMMARY

In the studies reported here, 39 compounds were investigated as to their interaction with nylon. Twenty of the compounds were significantly sorbed by the nylon. TGA permitted the determination of the equilibrium sorption weights of the compounds sorbed and the activation energies of desorption. A knowledge of these properties was helpful in drawing some general conclusions as to the mechanism of the interactions. TGA may be of practical value in

obtaining both kinetic and approximate thermodynamic data from sorption experiments when there is a need to evaluate or screen a large number of compounds as to their potential interaction with a polymeric material. Research in this field is continuing, and the results will be reported in subsequent publications.

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Many researchers have related changes in cerebral metabolism to changes in oxygen metabolism, cerebral blood flow, the electroencephalogram, and enzymes of the cerebrospinal fluid, for example. The present study was undertaken to investigate the action of lysergide, ephedrine, and nimergoline on these same parameters. To eliminate any interference from systemic response, the action of the drugs was investigated during a direct perfusion into the circle of Willis.

METHOD

The experiments were performed in the dog using two techniques: (*a*) the isolated perfused brain *in situ*, and (*b*) depression of cerebral activity by progressive hypoxia.

Isolated Perfused Brain *In Situ* Technique—*Animals and Anesthesia*—The experiments were carried out on 40 beagle dogs (10.7–14.6 kg. body weight), which were preanesthetized with urethan (0.4 g./kg. i.p.). Anesthesia was induced and maintained by nitrous oxide, cyclopropane, or ethyl ether in closed circuit. The animals were given artificial ventilation after tracheal intubation with a Warne tube, following succinylcholine chloride (1 mg./kg. i.v.) administration.

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Both of the isolated jugular veins were ligated, cannulated, and connected to the venous reservoir of the pump-oxygenator system by gravitational flow. Both of the isolated carotid arteries also were cannulated and connected to the pump-oxygenator system. The isolated right thyroidea superior artery was cannulated by a polystan tube and connected to a perfusor apparatus (Palmer). Arterial blood pressure was measured from a cannula inserted into a femoral artery. Extradural electrodes were set in place some days before the experiments; during the experiments the electroencephalogram was

obtaining both kinetic and approximate thermodynamic data from sorption experiments when there is a need to evaluate or screen a large number of compounds as to their potential interaction with a polymeric material. Research in this field is continuing, and the results will be reported in subsequent publications.

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recorded continuously with both systemic and cerebral pressure on a 12-channel polygraph.

Brain Pump-Oxygenator System—The brain perfusion apparatus employed consists of a venous reservoir, an oxygenator with gasmeter, a roller-type pump with a flowmeter, two blood filters, an apparatus to eliminate blood foam, a perfusion pressure regulator with manometer, and a blood exchanger with telethermometer. Before the extracorporeal perfusion, the pump-oxygenator system was filled with 500 ml. of heparinized blood diluted with Tyrode solution (3:1) added to glucose (10%). The blood was obtained 20 min. prior to use to prevent the accumulation of lactic acid, which would subsequently require a considerable degree of neutralization. Before the perfusion, the diluted blood was filtered through glass wool and polyester staple and adjusted to pH 7.35 using 1 *M* sodium bicarbonate.

The priming blood, which was fully oxygenated and warmed, was circulated through the pump-oxygenator system. A flow of O_2 + CO_2 mixture (95:5), maintained at the rate of 5–8 l./min., was passed into the oxygenator during the extracorporeal brain perfusion; the blood flow rate was kept between 8 and 12 ml./min./kg., a pressure equal to the initial systemic pressure of the animal. The time of brain perfusion was limited to 60 min. and was related to the presence of a considerable cerebral electric activity. The eventual leakage of perfusate into the systemic circulation was evaluated: (*a*) during the metabolic research by taking samples from the general circulation of the blood and of the lymph to verify the absence of the tested substance or its metabolites, and (*b*) at the end of the metabolic research by adding either a dye or a radiopaque substance to the blood of the extracorporeal circuit.

Cerebral Metabolizing Activity—The metabolizing activity of the brain was investigated *in situ* by: (*a*) demethylation, by evaluating the transformation of aminopyrine to 4-aminoantipyrine using the method of Brodie and Axelrod (3) after addition of aminopyrine to the extracorporeal circuit; (*b*) acetylation, by evaluating the transformation of 4-aminoantipyrine to *N*-acetyl-4-aminoantipyrine under the above-mentioned conditions; and (*c*) glucuronoconjugation, by evaluating the transformation of oxazepam to glucuronide, using the method of Walkenstein *et al.* (4) after addition of oxazepam to the extracorporeal circuit.

Blood samples of the extracorporeal circuit were collected every 10 min. for determination of tested substances and their metabolites. Hematocrit, clotting time, artereovenous blood oxygen, pH, and lactic acid values were also measured. The brain concentrations of both oxazepam or aminopyrine and their metabolites were evaluated at the end of the perfusion.

Evaluation of Drug Action—Thirty minutes after the addition of aminopyrine or oxazepam to the extracorporeal circuit, the perfusion was started through the thyroidea superior artery (at the rate of 0.5 ml./min. for 30 min.) with: (*a*) saline solution; (*b*) *N,N*-diethyl-D-lysergamide (lysergide), 5×10^{-7} *M;* (*c*) 1-ephedrine chloride, 1×10^{-4} *M;* and (*d*) 1,6-dimethyl-8β-(5-bromonicotinoyloxymethyl)-10α-methoxyergoline tartrate (nimergoline), 5×10^{-6} *M.*

Depression of Cerebral Activity by Hypoxia—*Animals and Anesthesia*—The experiments were carried out on 36 mongrel dogs (8.5–14.4 kg. body weight) which were preanesthetized with urethan (0.4 g./kg. i.p.). Anesthesia was induced and maintained by nitrous oxide, cyclopropane, or ethyl ether in closed circuit. The animals were given artificial ventilation after tracheal intubation with a Warne tube, following succinylcholine chloride (1 mg./kg. i.v.) administration. The general condition of the animals was investigated by evaluation of the systemic arterial blood pressure (from a cannula inserted into a femoral artery) and the tone and motility of both the duodenum and small intestine (by a rubber balloon).

Operative Procedure—By drilling, monopolar electrodes were set in place in the left and right frontal, parietal, and occipital areas. Heated-type thermocouples were set in place on the ventral and dorsal surfaces of the brain, and electrical activity and cerebral blood flow were recorded continuously using a 12-channel polygraph.

Benzi *et al.* (5) stated that the operative procedure for arterial injection into the brain consists primarily of isolation of the common carotid arteries and of ligature of all their branches, except the internal carotid arteries and the right thyroidea superior artery. The right external jugular vein was isolated, and the vertebral vessels were ligated before their entrance into the transverse foramen of C2 or C3. The numerous muscular branches arising from the vertebral vessels, the anastomosis between vertebral and carotid arteries, the muscular vessels of the neck, the vessels running under the carotid arteries and vagus nerves, and the zygomatic, maxillary, auricolar,

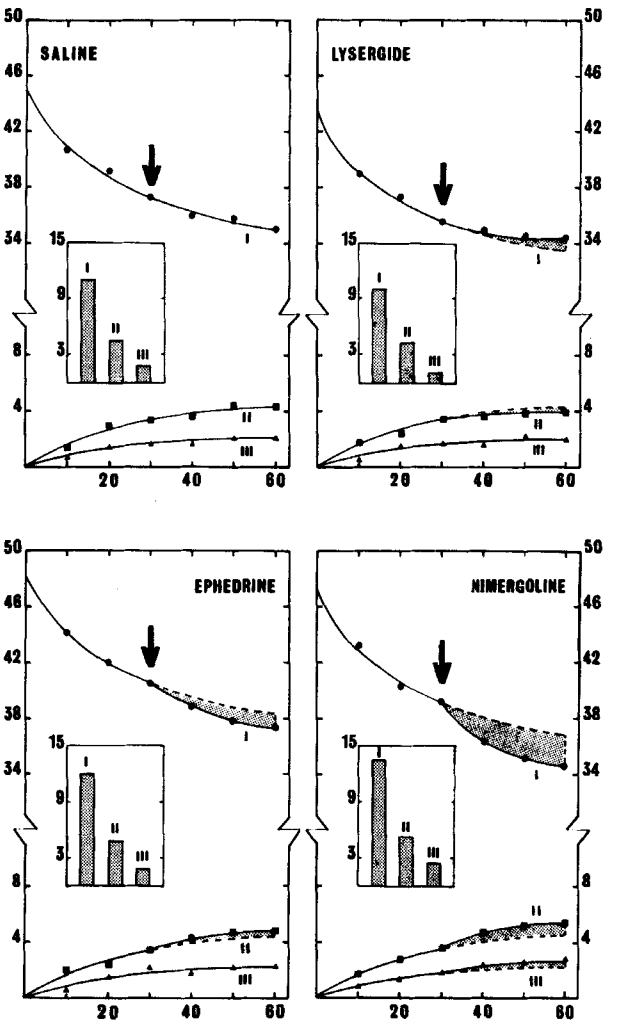

Figure 1—*Action of lysergide* (5×10^{-7} M), *ephedrine* (1×10^{-4} M), *and nimergoline* (5×10^{-6} M) *on aminopyrine metabolism investigated by the isolated perfused brain* in situ *of the beagle dog. Blood in the extracorporeal circuit = 500 ml.; blood flow rate of extracorporeal circuit = 10–12 ml./min./kg. On the ordinates are the plasma concentrations (micrograms per milliliter) of aminopyrine (I), 4-aminoantipyrine (II), and N-acetyl-4-aminoantipyrine (III), assayed for 60 min. at intervals of 10 min. (abscissae) after aminopyrine addition to the extracorporeal cerebral circuit; 30 min. after aminopyrine addition (at the arrow) cerebral perfusion with saline or drug solution (0.5 ml./min. for 30 min.) started. The unbroken line represents the experimental results, the broken line represents the expected results, and the hatched area depicts the difference between the expected and experimental results. There are no broken lines for III in the graphs for lysergide and ephedrine, indicating that the experimental and "expected" lines cross. Inserts show the brain concentrations (micrograms per gram ordinate) of aminopyrine (I) and its two metabolites (II and III), assayed after 60 min. of the extracorporeal brain perfusion with addition of aminopyrine.*

and supraorbital vessels were occluded by ligature or compression. The isolated thyroidea superior artery was cannulated by a polystan tube and connected to a perfusor (Palmer). The isolated right external jugular vein was cannulated by a polystan catheter. The basal electroencephalographic pattern of anesthesia, after the surgical procedure, was maintained at a fourth of Faulconer's level (6).

Depression of Cerebral Activity—Benzi *et al.* (5) showed that depression of the cerebral activity is produced during treatment with tubocurarine (0.1 mg./kg. i.v. every 30 min.) by repeated suppression of artificial ventilation. This condition induced a series of burst suppression patterns in the electroencephalogram. Myocardial depression began, and the animals were given artificial ventilation.

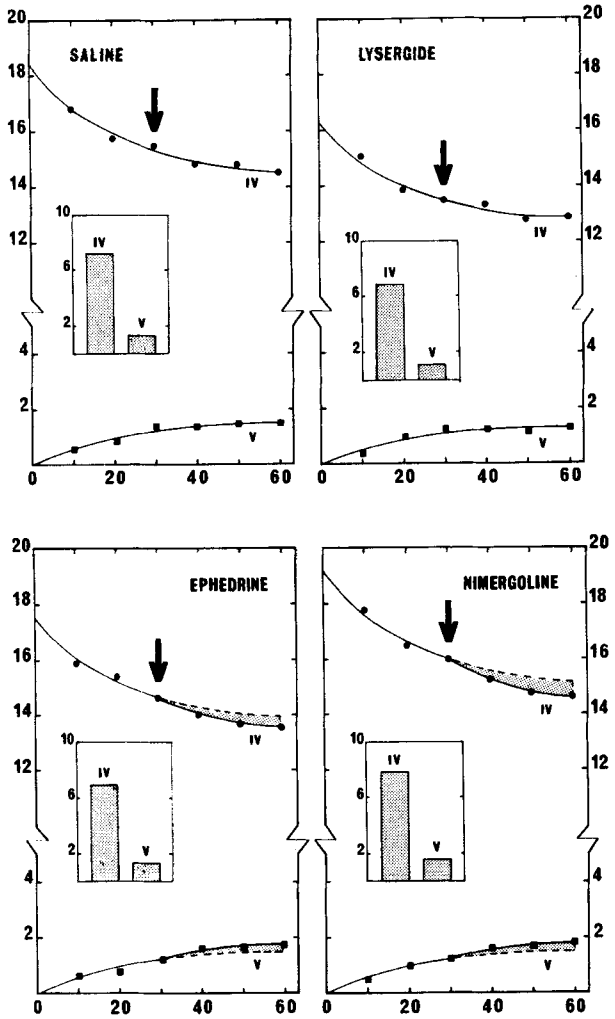

Figure 2—*Action of lysergide (5×10^{-7} M), ephedrine (1×10^{-4} M), and nimergoline (5×10^{-5} M) on oxazepam metabolism studied by the isolated perfused brain* in situ *of the beagle dog. Blood in the extracorporeal circuit = 500 ml.; blood flow rate of extracorporeal circuit = 10–12 ml./min./kg. On the ordinates are the plasma concentrations (micrograms per milliliter) of oxazepam (IV) and its glucuronide (V), assayed for 60 min., at intervals of 10 min. (abscissae) after oxazepam addition to the extracorporeal cerebral circuit; 30 min. after oxazepam addition (at the arrow) cerebral perfusion with saline or drug solution (0.5 ml./min. for 30 min.) started. The unbroken line represents the experimental results, the broken line represents the "expected" results, and the hatched area depicts the difference between the expected and experimental results. There are no broken lines for oxazepam and its glucuronide in the lysergide graph because the experimental and "expected" lines cross. Inserts show the brain concentrations (micrograms per gram, ordinate) of oxazepam (IV) and its glucuronide (V) assayed after 60 min. of the extracorporeal brain perfusion with addition of oxazepam.*

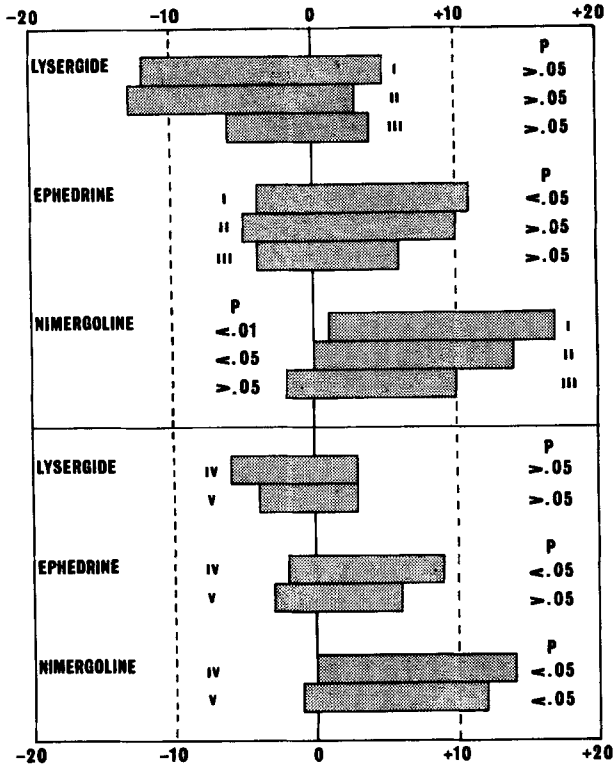

Figure 3—*Cerebral metabolizing activity investigated by the isolated perfused brain* in situ *of the beagle dog. Range of the percent variations (with probability levels; n = 5) between the expected and experimental values induced by the cerebral perfusion (0.5 ml./min. for 30 min.) of lysergide (5×10^{-7} M), ephedrine (1×10^{-4} M), and nimergoline (5×10^{-5} M) upon the decrease of aminopyrine (I) and oxazepam (IV) concentrations in the extracorporeal circuit, and the appearance of their metabolites 4-aminoantipyrine (II), N-acetyl-4-aminoantipyrine (III), and oxazepam glucuronide (V).*

Spontaneous partial recovery occurred in both electroencephalographic and myocardial activity; after 5–15 min., artificial ventilation was suppressed. This procedure was repeated many times (4–8). After the last myocardial depression, without cardiac arrest, artificial ventilation was given for 1 hr.

Cerebrospinal Fluid Enzyme Activity—Before and after induction of hypoxia and after perfusion with the tested drugs, the cerebrospinal fluid was evaluated for glutamic oxalacetic transaminase, lactate dehydrogenase, and alkaline phosphatase enzyme activity. These particular enzymes were chosen because of the well-known changes they undergo during experimental cerebral injury in laboratory animals and in neurological diseases in man.

Drug Tested—After the first 0.5 hr. of natural recovery, the perfusion through the thyroidea superior artery was started (at the rate of 0.5 ml./min. for 30 min.) with: (*a*) saline solution; (*b*) *N,N*-diethyl-D-lysergamide (lysergide), 5×10^{-7} *M*; (*c*) 1-ephedrine chloride, 1×10^{-4} *M*; and (*d*) 1,6-dimethyl-8β-(5-bromonicotinoyloxymethyl)-10α-methoxyergoline tartrate (nimergoline), 5×10^{-5} *M*.

RESULTS AND DISCUSSION

Isolated Perfused Brain *In Situ*—As observed previously (1), the *in situ* isolated brain shows a metabolizing activity; Figs. 1 and 2 show typical examples of the transformation rate of aminopyrine to the demethylated and acetylated products and of oxazepam to the glucuronoconjugated metabolite. In Figs. 1 and 2, it is possible to observe the effects, on this metabolizing ability, of the perfusion with drugs (lysergide, ephedrine, and nimergoline) compared with control conditions (perfusion with saline solution). The range of the percent variations between the expected values and the experimental values, induced by the perfusion with the drugs, is summarized in Fig. 3.

The perfusion (0.5 ml./min. for 30 min.) with lysergide (5×10^{-7} *M*) tends to decrease the cerebral metabolizing activity but without significant statistical differences; on the other hand, perfusion with ephedrine (1×10^{-4} *M*) significantly increases the disappearance of aminopyrine and oxazepam from the extracorporeal cerebral circuit without significant increase in the metabolites appearance. When nimergoline is perfused (5×10^{-5} *M*), there is a significant increase both in the disappearance of aminopyrine and oxazepam from the extracorporeal cerebral circuit and in the appearance of the demethylated and glucuronoconjugated metabolites.

As indicated in Fig. 4, during the cerebral perfusion with saline solution (control condition), at the constant rate of the blood flow into the extracorporeal circuit, the cerebral blood pressure increases significantly, without significant change in oxygen consumption. The cerebral perfusion (0.5 ml./min. for 30 min.) with lysergide (5×10^{-7}

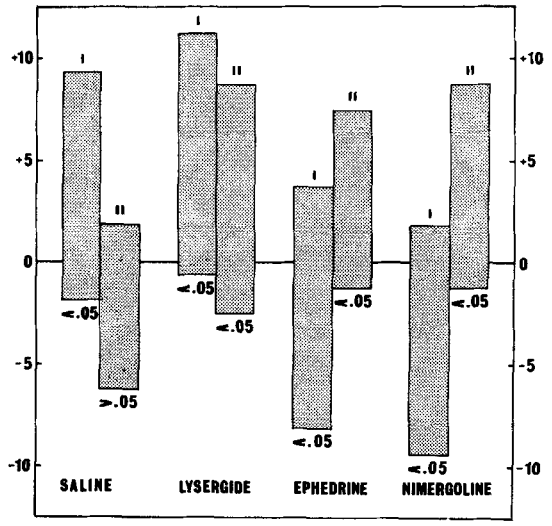

Figure 4—*Isolated perfused brain* in situ *of the beagle dog. Range of the percent variations from the initial values (with probability levels; n = 10) of the cerebral blood pressure (I) and oxygen utilization (II), induced by the cerebral perfusion (0.5 ml./min. for 30 min.) of saline solution, lysergide (5 × 10⁻⁷ M), ephedrine (1 × 10⁻⁴ M), and nimergoline (5 × 10⁻⁵ M).*

M) significantly increases this last parameter, and both ephedrine (1×10^{-4} *M*) and nimergoline (5×10^{-5} *M*) increase oxygen utilization and decrease cerebral blood pressure.

The increase in oxygen consumption by ephedrine and nimergoline can probably be related to the activation of specific biotransformations, such as the tested demethylation and acetylation of aminopyrine and the glucuronate conjugation of oxazepam. The increase in oxygen utilization by lysergide may be dependent, at least in part, on activation of energetic and kinetic reactions involved in processes inducing a pyretogenic effect. In fact, according to Horita and Dille (7), small doses of lysergide produce a hyperpyretic response in the trained rabbit; larger doses produce a fatal hyperpyrexia.

The activation of the tested biotransformation by ephedrine and, particularly, by nimergoline is accomplished by the decrease in cerebral vascular resistance, which can be dependent on: (*a*) direct action of the drugs on the blood circulation (8), and (*b*) indirect activation of the ganglion cell metabolism with detumescence of the glial process, because of the importance of astrocite pressure on the capillaries for vascular resistance (9–12).

Depression of Cerebral Activity by Hypoxia—The repeated suppression of artificial ventilation in curarized mongrel dogs induces a depression of the spontaneous electrical activity of the cortex, with only a partial natural reversion after 30 min. of recovery of artificial respiratory conditions; the "before" column of Fig. 5 shows the residual depression of the electroencephalogram pattern at this time. The subsequent infusion (0.5 ml./min. for 30 min.) with saline solution or lysergide (5×10^{-7} *M*), directly into the circle of Willis, does not induce any effects on electroencephalographic activity; ephedrine (1×10^{-4} *M*) and nimergoline (5×10^{-5} *M*) significantly improve the partial spontaneous reversion of the electroencephalogram depression, as summarized in the "after" column of Fig. 5.

As indicated in Fig. 6, during the induction of the severe hypoxia it is possible to observe that the depression of the spontaneous electrical activity is accomplished by an increase of: (*a*) some enzymes in the cerebrospinal fluid, at first glutamic oxalacetic transaminase and lactate dehydrogenase and subsequently alkaline phosphatase, and (*b*) the cerebral blood flow during the first phases, with a subsequent decrease at the terminal tested phase of hypoxia itself.

The increase of cerebrospinal fluid enzymes during experimental cerebral injury in laboratory animals and in neurological diseases in man has been demonstrated (13–30). In experimental conditions of this study, this increase can be explained at least in five different ways: (*a*) damage to the cerebral tissue with liberation of intracellular enzymes which, in the CNS, primarily pass into the cerebrospinal fluid space, as occurs in some neurological conditions (31); (*b*) vascular disturbance, with an increase of enzyme levels in damaged vascular endothelium and of their transmitting function across the barrier (32); (*c*) an increase of the permeability of the blood–cerebrospinal fluid barrier so that the enzymes, normally confined in serum in higher levels than in cerebrospinal fluid (33–35), pass into this fluid from the blood (36, 37); (*d*) a change in cerebral metabolic pathways with a decrease of metabolic activity of the ganglion cells accomplished both by an inverse proportional rise in enzyme concentration of astroglia and by accumulation of polysaccharides, mainly in the glial processes of astrocytes (38–41); and (*e*) a change in sodium and potassium brain homeostasis, dependent on oxidative metabolism and related to the electric activity (11, 12, 42, 43).

The change in cerebral blood flow can also be related to these five conditions, in particular to the metabolic change, with a reduction of cerebral metabolic rate for oxygen. The increase in cerebral blood flow is related to the increased cerebral production of CO_2 and acid metabolites, such as lactate (44). The subsequent moderate reduction of the cerebral blood flow is probably dependent on the initial condition of a general brain edema or on the local tumescence of particular cerebral cells (*e.g.*, astrocytes) related to the remarkable fluctuation in the degree of the activity of some enzymes (38, 45, 48).

The perfusion into the circle of Willis with ephedrine (1×10^{-4} *M*) and, particularly, with nimergoline (5×10^{-5} *M*) induces a partial

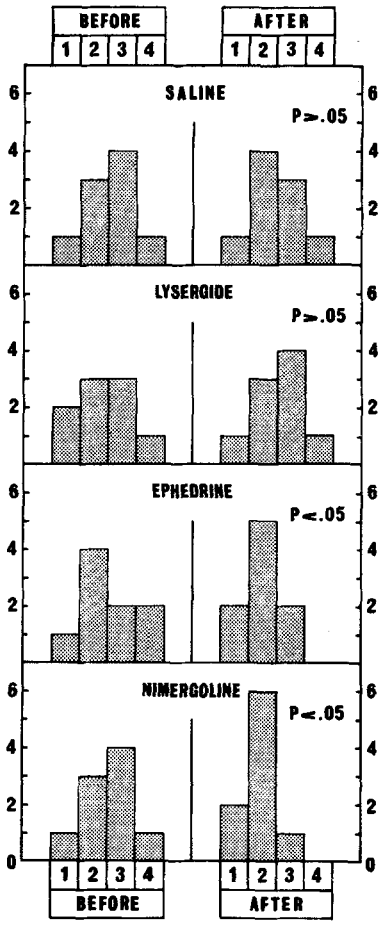

Figure 5—*Degree of the depressed spontaneous electrical activity of the cerebral cortex in the curarized mongrel dog obtained by severe hypoxia, before and after the direct perfusion into the circle of Willis (0.5 ml./min. for 30 min.) with saline solution, lysergide (5 × 10⁻⁷ M), ephedrine (1 × 10⁻⁴ M), and nimergoline (5 × 10⁻⁵ M). On the abscissae is the electroencephalogram pattern scale (related to control values = 100, corresponding to the fourth of Faulconer's levels): 1 = reduction <15%; 2 = reduction from 15 to 30%; 3 = reduction from 30 to 45%; and 4 = reduction from 45 to 60%. On the ordinate is the number of dogs. The figure indicates also the probability level (n = 9) of statistical difference between the distributions before and after the perfusion with drug. Note that the perfusion started 30 min. after the recovery of artificial respiratory ventilation.*

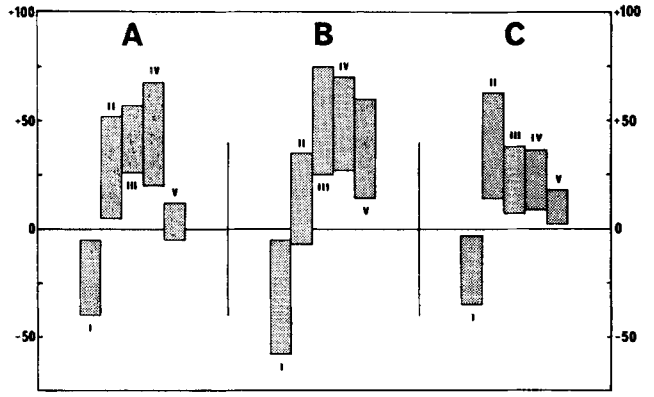

Figure 6—*Range of percent variations of electroencephalogram pattern (I), cerebral blood flow (II), and cerebrospinal fluid glutamic oxalacetic transaminase (III), lactate dehydrogenase (IV), and alkaline phosphatase (V) activity, from the control conditions before the induction of the severe hypoxia in mongrel dogs* (n = 9). *A = 40 ± 4 min. after hypoxia induction; B = at the end of the hypoxia condition; C = at the end of the perfusion (0.5 ml./min. for 30 min.), directly into the circle of Willis with nimergoline* (5×10^{-5} M). *Note that the perfusion started 30 min. after the recovery of artificial respiratory ventilation.*

recovery of the altered parameters (Fig. 6) by means of an activation of the cerebral metabolic activity and/or lowering the vascular resistance related to the change in cerebral blood flow (49).

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Mechanism of Action of Starch as a Tablet Disintegrant IV: Effect of Medicaments and Disintegrants on Mean Pore Diameter and Porosity

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Abstract □ Two full factorial experiments were designed. The first one had three disintegrants (corn starch, cation-exchange resin, and waxy maise starch) at four different concentrations (0, 5, 10, and 15%), and three levels of compression pressure (1000, 3000, and 6000 psig.) with aspirin. The second experiment had four different medicaments (aspirin, MgO, Mg trisilicate, and salicylamide), three levels of pressure, and corn starch at the four concentration levels. Mean pore diameter, porosity, and disintegration time were the measured parameters. Mean pore diameters were measured in an air permeability apparatus and calculated from:

$$D_c = \frac{2}{7}\sqrt{\frac{\eta \cdot L \cdot Q}{\Delta P \cdot A \cdot t \cdot \epsilon}}$$

Dried corn starch with 2% moisture was compared to regular corn starch containing 11% moisture. The effect of colloidal silicon dioxide was also studied. Broad generalities as to the effect of the variables on the measured parameters could not be made because of the complexities and interactions of the variables; *i.e.*, disintegrant concentration effect on mean pore diameter varied with the medicament used, the pressure level, and even the specific disintegrant. Neither the effect on mean pore diameter nor porosity was found to be the mechanism of action of starch and other disintegrants in disintegrating tablets under the conditions of these experiments.

Keyphrases □ Starch—mechanism as tablet disintegrant □ Tablet disintegration—mean pore diameter, porosity, disintegration time parameters □ Disintegration, tablets—medicaments, disintegrant effects on mean pore diameter and porosity

Theories of the mechanism of action of starch as a tablet disintegrant were proposed with increasing frequency in the past 10 years. There is as yet no general agreement on what constitutes the mechanism of action. The knowledge of how starch acts as a disintegrant could lead to the development of more effective and reliable disintegrants.

Although starch grains swell to a significant degree, they do not swell until a temperature of about 65° is reached. At 37°, only a 5–10% increase in mean grain size of corn starch was observed (1, 2). Starch grains damaged by ball milling did increase in mean grain diameters by 40–80% (3). Manudhane *et al.* (4) showed that amylose, which does not swell in water, may be an effective tablet disintegrant.

Führer (5) found no swelling in potato starch grains. He claimed that the starch grains that were deformed under pressure did swell in water. Ingram and Lowenthal (3) could find no evidence of corn starch grain damage when pressures as high as 24,000 psig. were used and when a hard, abrasive material was added to the starch and the mixture was compressed.

The apparent ability of substances to absorb water is another proposed theory. It was suggested that water absorption was the mechanism by which colloidal silicon dioxide and rice starch, which swell very little, disintegrated tablets of calcium lactate and of a simple granulation. A capillary system in the tablets was also postulated (6). Jaminet *et al.* (7) claimed disintegration time was proportional to the amount of water absorbed by the disintegrant. Using sodium carboxymethylcellulose, sodium alginate, and potato starch, they found that the starch absorbed less water but was a good disintegrant. The adhesiveness and high viscosity of the gums may have hindered better tablet disintegration. Bánó *et al.* (8) showed that potato starch in a tablet absorbed a methylene blue solution more rapidly than did theophylline, corn starch, triticum starch, or theobromine. Since potato starch was a good disintegrant, it was theorized that water absorption is a primary factor in tablet disintegration. Potato starch also produced the largest porosity. As force increased from 400 to 800 kg., the porosity decreased from 7 to 3%. They felt that water decreases the adhesive energy between particles and thereby causes disintegration. Nogami *et al.* (9) found that even though microcrystalline cellulose had greater water uptake than potato starch, it was not a better disintegrant.

It has been proposed that the heat of wetting when water enters a tablet results in warming the entrapped air, with a subsequent increase in air volume which disintegrates the tablet (10).

Nogami *et al.* (11) postulated that immersional wetting may be the controlling factor in tablet disintegration. The wetting depends upon the material, moisture content, crystal structure, and compression conditions. They claimed that potato starch was hydrophilic with a high heat of wetting and that penetration of water into a packed bed of powder depends on the density, viscosity, and surface tension of the liquid; the contact angle of the liquid with the solid; and radii of the pores. In another study (12), it was stated that surfactants only decreased disintegration time if both surface tension and contact angle were decreased and if the contact angle was initially greater than 90°. This may help to explain the widely different results due to addition of surfactants on tablet disintegration.

Higuchi *et al.* (13) found that the logarithm of compression force is linearly related to tablet porosity, up to about 5000 lb. of force. Furthermore, tablet porosity is sensitive to the material used. Later, Higuchi *et al.* (14) reported that as porosity decreased, surface area in the tablet increased up to about 10% porosity. After this maximum, surface area decreased as porosity decreased. Matsumaru (15) determined the effect of pressure on pore radius and pore volume from the absorption isotherm of tablets. He reported that the peak of the pore radius distribution at 0.76 ton/cm.^{2} was 62.4 Å,

and the pore volume was 148 × 10^{-4} ml./g. Å; at 1.5 tons/cm.^{2}, 42.4 Å and 81 × 10^{-4} ml./g. Å; at 2.9 tons/cm.^{2}, 21 Å and 60.4 × 10^{-4} ml./g. Å; and at 4.2 tons/cm.^{2}, 21 Å and 35.9 × 10^{-4} ml./g. Å, respectively. At the last two pressures, the pore radius did not change. By using the Brunauer, Emmett, and Teller method, it was reported that aluminum silicate tablets contained bottleneck pores (16). As force increased, specific surface area, heat of absorption, and void space slowly decreased. The largest pore diameter in aluminum silicate tablets was found to be 124.8 Å (17). Wurster and Seitz (18) found that 0.10-cm. (0.04-in.) diameter pores drilled into benzoic acid compacts may be occluded by air preventing liquid from entering. Using a solution of 0.2% sodium lauryl sulfate resulted in the pores being filled with solution.

Using an air permeability apparatus, Nogami *et al.* (12) obtained pore diameters with magnesium oxide tablets made with 5% potato starch as disintegrant and 3% starch paste as binder. They found pore diameters decreased with increasing pressure; an increase from 0.5 to 2.0 tons/cm.^{2} resulted in a decrease from 0.197 to 0.069 μ. They obtained an increase in pore diameters with an increasing concentration of potato starch. At 0.5 ton/cm.^{2}, aspirin tablets without starch had pore diameters of 0.36–0.47 μ; with 10% potato starch, the diameters went up to 1.14, 1.43, and 1.23 μ. In magnesium oxide tablets without disintegrant, diameters were 0.07 and 0.09 μ; with 10% potato starch, they were 0.30 and 0.32 μ. There were no linear correlations between compression force and pore diameter or pore diameter and disintegration time.

Later, Nogami *et al.* (19) measured pore diameters, porosity, and dissolution and disintegration times. The variables were aspirin particle size, pressure, starch concentration, and potato and corn starches. Porosity decreased with increasing pressure. With large aspirin particles (d_{vs} = 0.953 mm.) and 1 ton pressure, pore diameter increased (from 0.05 to 0.38 μ) with corn starch concentration (from 0 to 20%). Pore diameter also increased with particle size of aspirin. With small aspirin particles (d_{vs} = 0.0119 mm.), pore diameter did not increase with starch concentration. Tablets made from small- or medium-sized aspirin particles and starch "dissolved" rather than disintegrated. The difference, if any, between corn and potato starches was not made clear, although the investigators claimed that potato starch was better under certain conditions. It appeared as if not all of the data from the 87 experiments were given or analyzed. The authors postulated a critical amount of starch, depending on the drug and starch particle sizes or relative surface areas. This was related to the interfacial characteristics of the capillary walls. When sufficient starch is added, the pores consist of starch or starch and some aspirin. An inadequate amount of starch results in pores consisting mainly of aspirin. Aspirin was reported to have a contact angle greater than 90° and starch as having one less than 90°, so that starch is more easily wetted. For medium- and large-sized aspirin particles, 5–10% starch is required for disintegration. That there is critical starch concentration for drug particle size for minimum disintegration times agrees with the observations of other investigators (20–22).

Nogami *et al.* (9) reported, in a study of aspirin using microcrystalline cellulose or potato starch as disintegrants, that even though the former produced equal or larger pore diameters in some cases, the starch was a better disintegrant. The writers felt that the cellulose allowed water only to travel into the pores, whereas the potato starch also absorbed water. The cellulose could also act as a binder. Interestingly, the writers claimed that the cellulose had a contact angle smaller than potato starch.

Reich and Gstirner (22) also studied tablet pore radius and pore volume. They verified that pore volume and radius decreased as pressure increased. As tablet height increased, both volume and radius increased, but both parameters decreased as moisture content of starch increased. At 950 lb./cm.^{2} force, they reported radii in the 1–2-μ range. Borzunov and Shevchenko (23) reported that potato starch increased the porosity of both bromisovalum and terpin hydrate tablets. They also postulated a microcapillary system due to the addition of the starch.

That pores or capillaries exist in tablets is undeniable. When the drug particles or granules are mixed with starch as the last step before compression, one would expect to find the disintegrant around the granules and even appearing to adhere to the granules. Hence, when the mixture is compressed, the starch appears to form "pores" around the granules. The pores appear to be connected because the starch surrounding the particles now comes together as the mixture is compressed. The evidence of Patel and Hopponen (24) and the rationalization of Reich and Gstirner (22) that the pores are lined with starch grains requires verification. Why are not the pores lined with lubricant?

Whether the major mechanism of starch in tablet disintegration is by starch grain deformation, heating or wetting, or rate of fluid penetration into the tablet, it seems logical that the disintegrating fluid (*i.e.*, water or gastric juice) must get into the tablet. Pharmaceutical tablets are known to be porous to a varying degree, depending on the material, method of granulating, and compression force used. It is necessary to extend the work of Nogami *et al.* (19) to determine the existence of statistical correlations between such variables as types of active ingredients, chemically different disintegrants, disintegrant concentrations, and compression forces, using mean pore diameter, porosity, and disintegration times as the measurable parameters. Interactions among single factors should be determined for more subtle relationships and to see if there are any optimum conditions for tablet disintegration. Finally, if it is true that aspirin–starch tablets compressed to maximum density still have good disintegration times (24), then one can ask if pores in tablets are necessary for disintegration.

EXPERIMENTAL

Tablet Compression—The proper quantities of drug and disintegrant were mixed on a roller mill for 15–30 min. to obtain a uniform mixture. A roller mill was used so there would be a minimum of change in the particle-size distribution. Amounts (0.5 g.) for the tablets, weighed to the nearest 0.1 mg., were placed in a

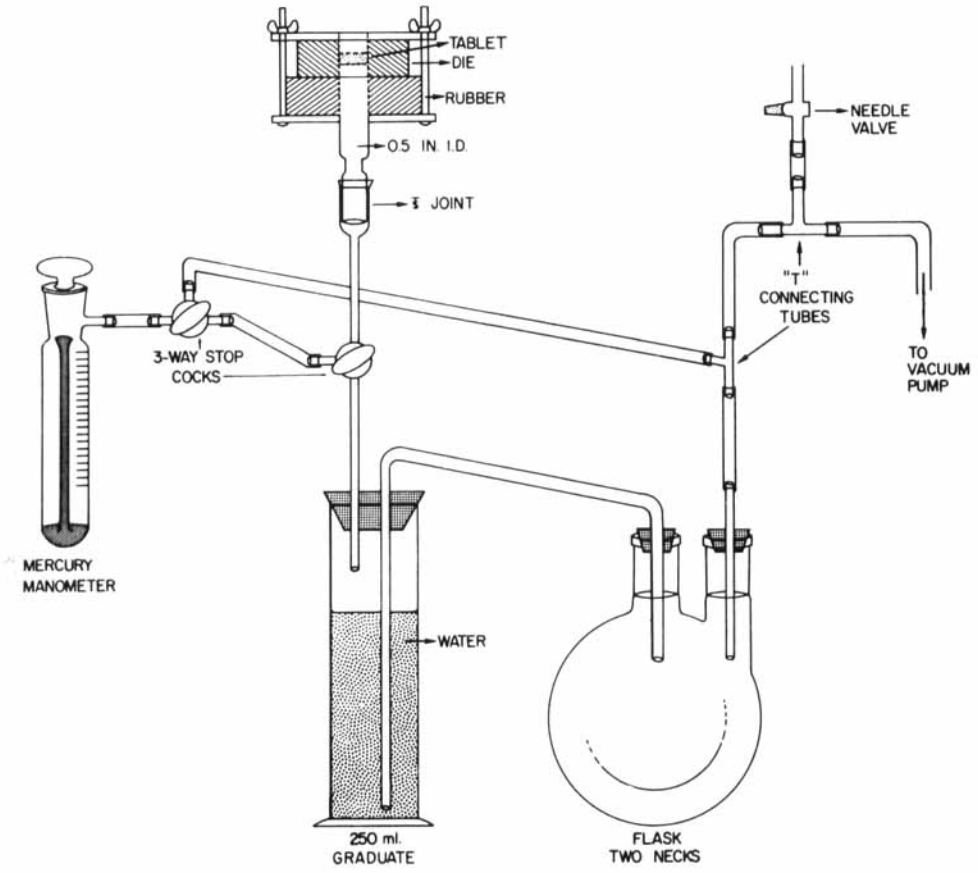

Figure 1—*Tablet air permeability apparatus.*

tablet die held in a special holder on the Carver laboratory press[1] and compressed with 1.27-cm. (0.5-in.) diameter, flat-face punches to 1000, 3000, or 6000 psig. This corresponds to 5093, 15,279, and 30,558 lb. when the ram pressure is converted to the pressure on the 1.27-cm. (0.5-in.) diameter punches. The tablet was left in the die for the pore diameter and porosity measurements. The tablets for the disintegration studies were compressed similarly but removed from the die. The die was not lubricated.

Predominant Disintegration Times—The USP tablet disintegration apparatus (25) was used, with distilled water as the disintegrating fluid, to determine tablet disintegration times.

Porosity Measurement—Freshly boiled, cooled water was used in the graduated cylinder in Fig. 1. The stoppers were sealed into place, and the threeway stopcocks were opened. The tablet in its die was sealed onto the rubber stopper in the tablet die holder. The equilibrium vacuum of 10 cm. Hg used in this study was obtained after about 10 min. with a vacuum pump[2]. Prior work showed that 10 cm. Hg pressure below the tablet gave reasonable rates of air flow through the tablets so that accurate readings of water displacement and of time could be obtained. When the vacuum below the tablet became constant and the rate of water displacement uniform, its level in the graduate was noted and the timer[3] was started. When the pressure below the tablet changed 0.1 cm., as seen on the manometer (Fig. 1), the water level was again noted and the timer stopped. The amount of water displaced in the cylinder, in the given time interval, was equal to the amount of air drawn through the tablet. Two sets of readings were taken on each tablet. The barometric pressure and room temperature were also recorded at this time.

Air was used as the fluid in these measurements to prevent any effect due to dissolution of the ingredients and to minimize any change in packing within the tablets that may occur if even a nonpolar solvent is used.

[1] Model B, Fred S. Carver, Inc.
[2] Duo Seal Vacuum Pump, model 1405, The Welch Scientific Co.
[3] Precision Scientific Co., Catalog No. 69239.

Porosity–Porosity was determined by compressing the powder mixture to 24,000 psig. (122,231 lb.) to eliminate all void spaces. Tablet thicknesses and diameter measurements were made by a 25-mm. micrometer[4].

Particle Size—The mean particle sizes of the four different drugs were determined microscopically with a calibrated eyepiece. The mean particle size was calculated from 100 particles measured at random orientation. In this manner, all particle dimensions were eventually measured.

Materials—Corn starch USP[5] (11% moisture), waxy maise starch[6], a potassium salt of a carboxylic cation-exchange resin[7] dried at 60° to 6.1% moisture, and colloidal silicon dioxide[8] were used as disintegrants. The waxy maise starch was used because it has been recommended as a tablet disintegrant and consists entirely of amylopectin. The cation-exchange resin was previously reported as a disintegrant that may swell when hydrated (26). The colloidal silicon dioxide was included in this study to see if the high bulk volume of this material would affect tablet pore diameter. It had been reported to increase tablet porosity (27). The percent disintegrant added was based on the weight of the drug.

Aspirin USP[9] comminuted through an 80-mesh screen, magnesium oxide USP[10] (heavy), magnesium trisilicate USP[11], and salicylamide powder NF[12] were used as examples of drugs. Aspirin was used because of its wide commercial use and because several previous studies employed this drug so that it serves as a standard for comparison with other studies. Magnesium oxide (MgO) was also used in previous tableting investigations (12). Magnesium trisilicate and salicylamide served as additional examples of insoluble inorganic and organic drugs of pharmaceutical importance.

[4] Catalog No. 436 MRL, L. S. Starrett Co.
[5] STR-R, A. E. Staley Manufacturing Co.
[6] Amioca, National Starch and Chemical Corp.
[7] Amberlite IRP-88, lot 0430, Rohm & Haas Co., formerly XE-88.
[8] Cab-O-Sil M-5, Cabot Corp.
[9] Merck & Co., Inc., lot 60706.
[10] J. T. Baker Chemical Co.
[11] Mallinckrodt Chemical Works, Control SAS1.
[12] S. B. Penick & Co., lot 15-NEO-209.

Table I—Analysis of Variance of Pore Diameters in Tablets Containing Various Disintegrants

Source	*df*	*SS*	*MS*	*F*
Disintegrants (*D*)	2	2.0688	1.0344	3.42[a]
D_1 *versus* D_2	1	1.5000	1.5000	4.96[b]
D_1 *versus* D_3	1	0.001665	0.001665	0.0055[a]
Pressure (*P*)	2	804.0538	402.0269	1328.57[c]
P_1 *versus* P_3	1	734.8252	734.8252	2428.37[c]
P_1 and P_3 *versus* P_2	1	69.2271	69.2271	228.77[c]
Disintegrant concentration (*C*)	3	56.4452	18.8150	62.18[c]
C_2 *versus* C_3	1	0.3472	0.3472	1.15[a]
C_2 and C_3 *versus* C_1	1	11.1157	11.1157	36.73[c]
C_2 and C_3 *versus* C_4	1	27.1646	27.1646	89.77[c]
$D \times C$	6	19.2288	3.2048	10.59[c]
$P \times C$	6	12.7905	2.1317	7.04[c]
Error[d]	16	4.8421	0.3026	—

[a] Not significant. [b] Significant at $p = 0.05$. [c] Significant at $p = 0.001$. [d] Term is sum of the three-factor interaction and $D \times P$ interaction which was not statistically significant.

CALCULATIONS

Porosity—Tablet porosities were calculated from the following equation:

$$\text{porosity} = 1 - \frac{\text{apparent density}}{\text{true density}} \qquad \text{(Eq. 1)}$$

The true density of the powder mixture was determined by compressing to eliminate all void spaces. Since the weight of each tablet was kept constant, as was the radius, only the tablet height varied; therefore, the ratio of densities in Eq. 1 becomes a ratio of heights. Thus,

$$\text{porosity} = 1 - \frac{\text{height of tablet at true density}}{\text{height of tablet under study}} \qquad \text{(Eq. 2)}$$

Pore Diameters—Mean tablet pore diameters were calculated from an equation derived from specific surface areas, developed from Darcy's law by Kozeny and Carman (12, 28–31):

$$S_w = \frac{14}{\rho}\frac{\Delta P \cdot A \cdot t}{\eta \cdot L \cdot Q}\frac{\epsilon^2}{(1-\epsilon)^2} \qquad \text{(Eq. 3)}$$

where S_w is the specific surface area in the tablet, ρ is the density of the powder, A is the cross sectional area of the tablet surface, Q is the volume of air that permeated through the tablet in time t, ΔP is the pressure difference across the thickness (L) of the tablet, η is the viscosity of air at the temperature of the experiment, and ϵ is the porosity.

Pore diameter, D_c, is found from:

$$D_c = \frac{4}{S_w}\frac{\epsilon}{1-\epsilon} \qquad \text{(Eq. 4)}$$

Combining Eqs. 3 and 4 gives:

$$D_c = \frac{2}{7}\sqrt{\frac{\eta \cdot L \cdot Q}{\Delta P \cdot A \cdot t \cdot \epsilon}} \qquad \text{(Eq. 5)}$$

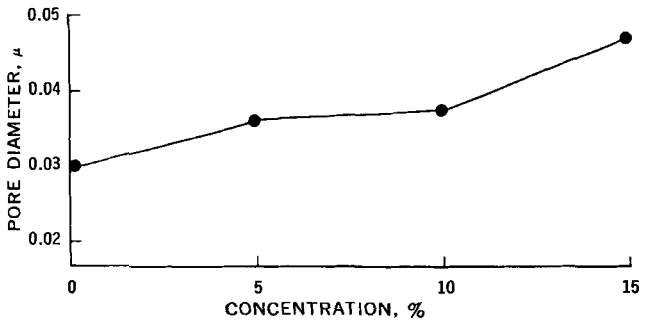

Figure 2—*Effect of disintegrant concentration on mean pore diameter in aspirin tablets.*

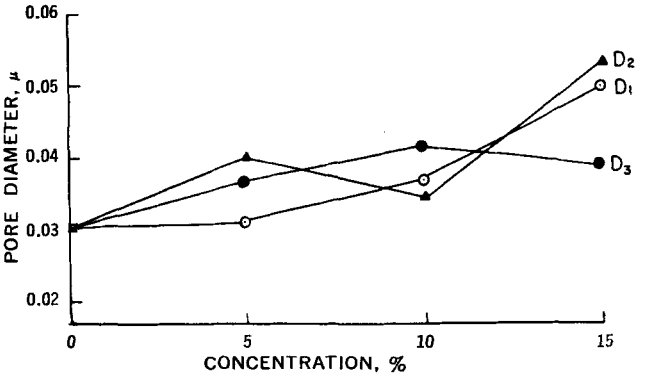

Figure 3—*Effect of distintegrant concentration on mean pore diameter in aspirin tablets. Key:* D_1, *corn starch;* D_2, *cation-exchange resin; and* D_3, *waxy maise starch.*

RESULTS AND CONCLUSIONS

Using aspirin, the first experiment was designed to determine the effect of compression force at three levels (P_1 = 1000 psig., P_2 = 3000 psig., and P_3 = 6000 psig.), disintegrant concentration at four levels (C_1 = 0%, C_2 = 5%, C_3 = 10%, and C_4 = 15%), and three different disintegrants (D_1 = corn starch, D_2 = cation-exchange resin, and D_3 = waxy maise starch) on mean pore diameter and porosity. This gave a 3 × 4 × 3 full factorial design experiment. The analysis of variance is given in Table I.

The analysis of variance showed that there was no significant difference in the mean pore diameters between the two starches. The cation-exchange resin produced larger mean pore diameters than either of the two starches.

Mean pore diameters decreased from 0.070 to 0.028 to 0.015 μ as compression pressure increased from 1000 to 3000 to 6000 psig., respectively. The decrease was of the same order of magnitude for each individual disintegrant. The relationship of diameters to compression pressure was not logarithmic.

Mean pore diameters increased with increasing concentration of the disintegrants (Fig. 2). There was no significant difference in mean pore diameters at 5 and 10% concentrations, but these diameters were significantly different from those at both 0 and 15%. The $D \times C$ interaction disclosed that the increase in mean pore diameters with increasing concentration occurred only with corn starch. The resin produced a decrease at 10%, while the waxy maise starch had a slight decrease at the 15% level (Fig. 3). The significance of the $C \times P$ interaction is shown in Fig. 4. At 1000 psig., there was an increase in mean pore diameters with concentration. At 3000 psig., there was a decrease in diameters at 10% concentration; at 6000 psig., the 5 and 10% levels had the same mean pore diameters. Moreover, the rate of diameter increase dropped as the pressure

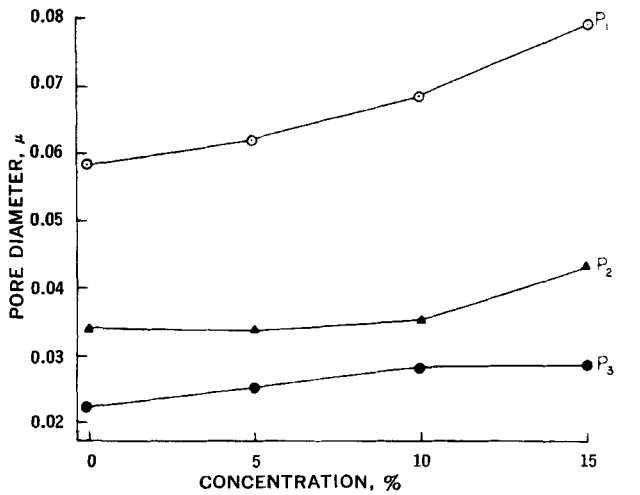

Figure 4—*Effect of disintegrant concentration on mean pore diameter in aspirin tablets. Key:* P_1, *1000 psig.;* P_2, *3000 psig.; and* P_3, *6000 psig.*

Table II—Analysis of Variance of Porosities of Aspirin Tablets Containing Various Disintegrants

Source	*df*	*SS*[a]	*MS*	*F*
Disintegrants (*D*)	2	5.6504	2.8252	3.51[b]
D_1 *versus* D_3	1	0.7820	0.7820	0.971[b]
D_1 and D_3 *versus* D_2	1	5.5722	5.5722	6.92[c]
Concentration (*C*)	3	48.8714	16.2904	20.23[d]
C_1 *versus* C_2	1	0.1250	0.1250	0.155[b]
C_1 and C_2 *versus* C_3	1	25.8476	25.8476	32.09[d]
C_1 and C_2 *versus* C_4	1	7.9349	7.9349	9.95[e]
Pressure	2	617.1066	308.5533	383.11[d]
P_1 *versus* P_3	1	592.1251	592.1251	735.19[d]
P_1 and P_3 *versus* P_2	1	24.9806	24.9806	31.02[d]
$D \times C$	6	27.4533	4.5755	5.68[e]
$C \times P$	6	16.2144	2.7024	3.36[e]
Error[f]	16	12.8868	0.8054	—

[a] The sum of squares (*SS*) of the individual effects may not add up to the *SS* of the total effect because the comparisons are not quite orthogonal but were the comparisons of interest according to the data. [b] Not significant. [c] Significant at $p = 0.05$. [d] Significant at $p = 0.001$. [e] Significant at $p = 0.01$. [f] Term is the sum of the three-factor interaction and $D \times P$ interacting which was not statistically significant.

increased. Although overall there was a decrease in mean pore diameters as pressure increased and an increase in diameters as disintegrant concentration increased (Fig. 2), these effects actually varied with the specific disintegrant and the compression pressure used to form the tablets. The broad generalities based on averaging data over different variables may at times be misleading.

The same experimental design was used to determine the effect of the variables on tablet porosities. The analysis of variance is shown in Table II.

Overall there was no significant difference between the disintegrants, although a comparison of the two starches with the resin showed that the starches produced significantly higher tablet porosities in aspirin tablets than the resin. The resin produced larger mean pore diameters in aspirin tablets.

Porosity decreased with increasing compression force. The porosities at 1000, 3000, and 6000 psig. were 0.127, 0.0600, and 0.0280, respectively. The relationship was not logarithmic. The $C \times P$ interaction indicated that the decrease of porosity at each concentration level was at about the same rate for each pressure, but the porosities *per se* at the 10 and 15% levels were significantly different from 0 and 5% levels. The porosity also decreased with increasing pressure at the same rate with all three disintegrants.

The disintegrant concentration effect on tablet porosity was variable (Fig. 5). This is the reason for the statistical significance of the $D \times C$ interaction.

The next experiment was devised to determine the effect on mean pore diameter and porosity of the four different medicaments (M_1 = aspirin, M_2 = MgO, M_3 = Mg trisilicate, and M_4 = salicylamide), four concentrations of corn starch, and three different

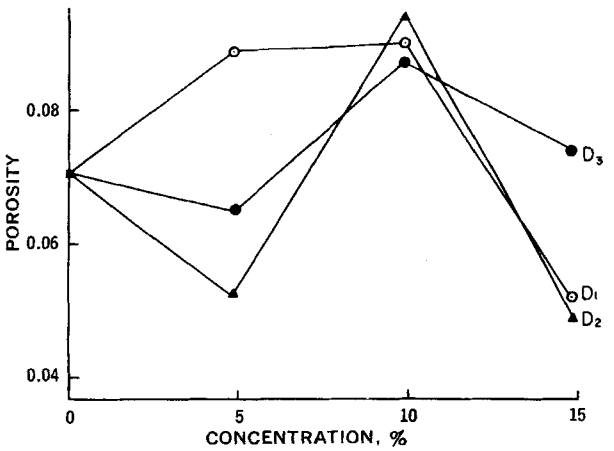

Figure 5—*Effect of disintegrant concentration on porosity in aspirin tablets. Key:* D_1, *corn starch;* D_2, *cation-exchange resin; and* D_3, *waxy maise starch.*

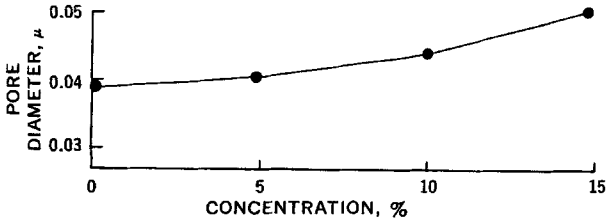

Figure 6—*Effect of corn starch concentration on mean pore diameter in four medicaments.*

compression forces. This resulted in a $4 \times 4 \times 3$ full factorial design experiment.

The analysis of variance in Table III showed that the medicaments gave significantly different pore diameters. Salicylamide had a mean pore diameter of 0.076 μ, aspirin 0.037 μ, MgO 0.030 μ, and Mg trisilicate 0.030 μ. Mean pore diameters decreased as compression increased. Thus, at 1000, 3000, and 6000 psig., mean pore diameters were 0.067, 0.037, and 0.027 μ, respectively. Mean pore diameters increased with increasing corn starch concentration (Fig. 6).

The significance of the $M \times P$ interaction supports the fact that each medicament behaved differently with respect to mean pore diameters and pressure (Fig. 7). Salicylamide and aspirin, which had the largest mean pore diameters, also had the largest decrease with increasing pressure. The two magnesium compounds showed the least effect. The greatest change in diameters occurred between 1000 and 3000 psig., probably due to the greater compressibility of the powders. Although the overall effect of starch concentration is shown in Fig. 6, a statistically significant $M \times C$ interaction implies the concentration effect is not simple. This is demonstrated in Fig. 8 where only aspirin (M_1) showed an increase in mean pore diameter with increasing corn starch concentration. The other three medicaments had a variable effect; *i.e.*, there was a slight increase in mean pore diameter with three out of four medicaments at the 5% level and three of the four medicaments showed an increase in diameters at 15%, but the drugs differed. The significance of the $P \times C$ interaction is similar to the one previously shown in Fig. 4.

The second experimental design was also used to determine the effects of the variables on tablet porosity.

The analysis of variance presented in Table IV disclosed that the porosities of aspirin and salicylamide tablets were similar, even though the mean pore diameters were different. The two magnesium compounds had significantly higher porosities than aspirin or salicylamide. In this experiment, the concentration of corn starch had no effect on porosity. The $M \times C$ interaction disclosed the reasons for this situation. All four medicaments behaved differently, as shown in Fig. 9.

Porosities decreased as compression pressure increased, but the rate of change was not as large as in the previous experiment. The

Table III—Analysis of Variance of Pore Diameters of Tablets Containing Various Medicaments

Source	*df*	*SS*	*MS*	*F*
Medicaments (*M*)	3	715.5500	238.5166	320.76[a]
M_1 *versus* M_2	1	10.6666	10.6666	14.35[b]
M_1 and M_2 *versus* M_3	1	4.4005	4.4005	5.92[c]
M_1 and M_2 and M_3 *versus* M_4	1	700.4839	700.4839	942.02[a]
Pressure (*P*)	2	567.5419	283.7709	381.62[a]
P_1 *versus* P_3	1	523.2593	523.2593	703.68[a]
P_1 and P_3 *versus* P_2	1	44.2814	44.2814	59.55[a]
Starch concentration (*C*)	3	35.4701	11.8233	15.90[a]
C_1 *versus* C_2	1	0.2816	0.2816	0.38[d]
C_1 and C_2 *versus* C_3	1	5.6674	5.6674	7.62[c]
C_1 and C_2 and C_3 *versus* C_4	1	29.5211	29.5211	39.70[a]
$M \times P$	6	272.3713	45.3952	61.05[a]
$M \times C$	9	37.3322	4.1480	5.58[b]
$P \times C$	6	14.1540	2.3590	3.17[c]
Error[e]	18	13.3842	0.7435	—

[a] Significant at $p = 0.001$. [b] Significant at $p = 0.01$. [c] Significant at $p = 0.05$. [d] Not significant. [e] Term is the three-factor interaction.

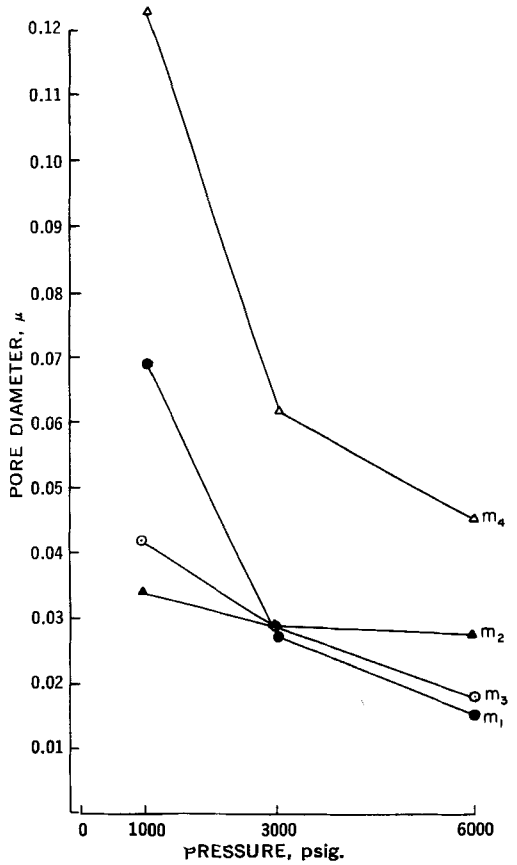

Figure 7—*Effect of pressure on mean pore diameter. Key:* M_1, *aspirin;* M_2, *MgO;* M_3, *Mg trisilicate; and* M_4, *salicylamide.*

porosities at 1000, 3000, and 6000 psig. were 0.261, 0.175, and 0.123, respectively. The average porosities were also generally higher than previously described, possibly due to the very high porosities of the two magnesium compounds. The importance of the $M \times P$ interaction is illustrated in Fig. 10, where the difference in porosities of the four medicaments becomes evident. The general shape of the curves is similar, indicating a parallel rate of porosity decrease with pressure.

Only qualitative correlations of tablet disintegration times with mean pore diameter or porosities could be made because tablets

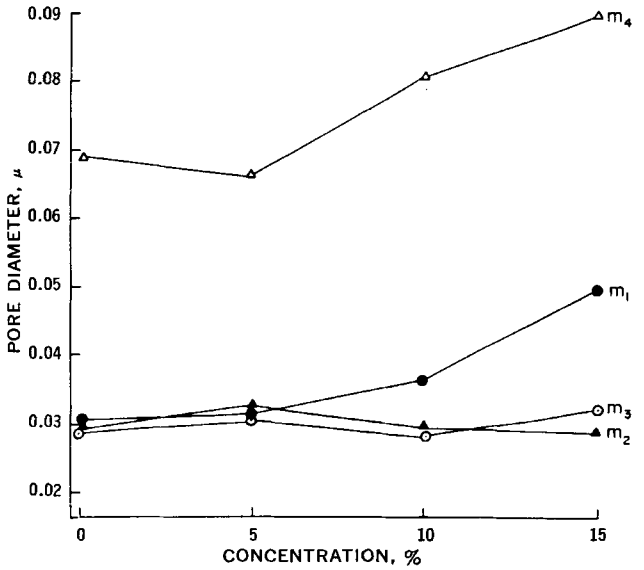

Figure 8—*Effect of corn starch concentration on mean pore diameter. Key:* M_1, *aspirin;* M_2, *MgO;* M_3, *Mg trisilicate; and* M_4, *salicylamide.*

Table IV—Analysis of Variance of Porosities of Tablets Containing Various Medicaments

Source	*df*	*SS*	*MS*	*F*
Medicaments (*M*)	3	7026.7104	2342.2368	993.78[a]
M_1 *versus* M_4	1	5.2266	5.2266	2.22[b]
M_1 and M_4 *versus* M_2	1	2663.2764	2663.2764	1129.99[a]
M_1 and M_4 *versus* M_3	1	6311.2354	6311.2354	2677.77[a]
Concentration (*C*)	3	8.0228	2.6742	1.13[b]
Pressure (*P*)	2	1567.0254	783.5127	332.43[a]
P_1 *versus* P_3	1	1538.1792	1538.1792	652.63[a]
P_1 and P_3 *versus* P_2	1	28.8422	28.8422	12.24[a]
$M \times C$	9	226.1976	25.1330	10.66[a]
$M \times P$	6	135.0128	22.5021	9.55[a]
Error[c]	24	56.5653	2.3569	—

[a] Significant at $p = 0.001$. [b] Not significant. [c] Term is sum of the three-factor interactions and $C \times P$ interaction which was not statistically significant.

did not disintegrate within 1 hr. in many instances (Tables V and VI).

Figures 7 and 8 show that MgO exhibited the least decrease in mean pore diameter with pressure and corn starch concentration, and Fig. 9 shows that MgO tablet porosity increased steadily above 5% corn starch concentration. Table VI indicates that at 5% and greater disintegrant concentration, MgO tablets disintegrated in 5–10 sec. at all pressures. This would seem to indicate that the small change in mean pore diameter is reflected in a constant disintegration time above 5% starch concentration, but it does not explain the porosity effect. The decrease in mean pore diameter and a decrease in porosity (Figs. 7 and 10) with pressure for Mg trisilicate tablets corresponded to an increase in disintegration times (Table VI). The concentration of starch had no effect on mean pore diameter and a variable effect on porosity in Mg trisilicate tablets, which could not be correlated to disintegration times.

The decrease of mean pore diameters with pressure (Fig. 7) corresponded to an increase in disintegration times with pressure at 10 and 15% corn starch concentrations in aspirin and salicylamide. Mean pore diameters in aspirin and salicylamide tablets (Fig. 8) increased above 5% starch concentration and this correlates with the disintegration data in Table VI. While there was some correlation between mean pore diameters and disintegration times, the

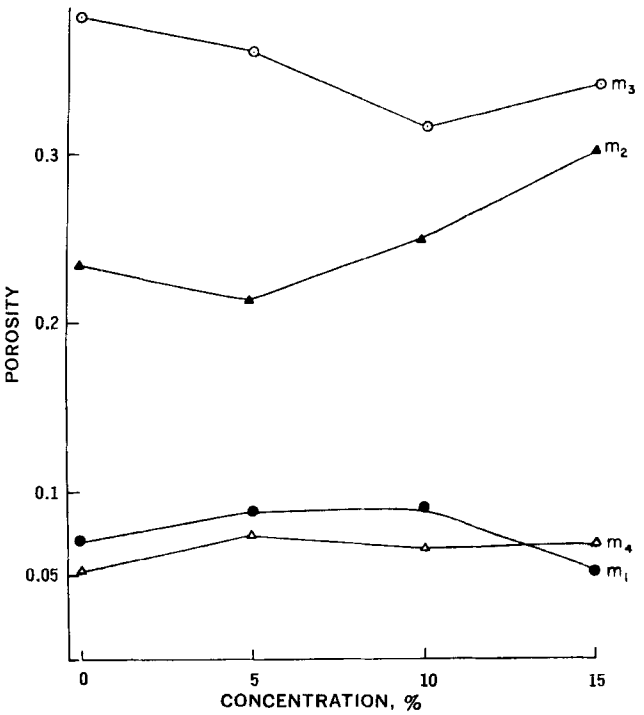

Figure 9—*Effect of corn starch concentration on porosity. Key:* M_1, *aspirin;* M_2, *MgO;* M_3, *Mg trisilicate; and* M_4, *salicylamide.*

Table V—Predominate Disintegration Times of Aspirin Tablets[a]

Disintegrant	Concentration, %	Pressure, psig. 1000	3000	6000
Corn starch	0	>3600	>3600	>3600
	5	>3600	>3600	>3600
	10	550	970	>3600
	15	38	49	25
Cation-exchange resin	0	>3600	>3600	>3600
	5	25[b]	25[b]	25[b]
	10	25[b]	25[b]	25[b]
	15	15	12	25[b]
Waxy maise starch	0	>3600	>3600	>3600
	5	>3600	>3600	>3600
	10	39	50[b]	50[b]
	15	50[b]	50[b]	50[b]

[a] Seconds. [b] Chips remained in basket for long time.

porosity of aspirin tablets and salicylamide tablets in Fig. 9 could not be correlated to disintegration times.

The resin at 5% and greater concentrations and at all pressures caused tablets to break apart into small chips within 25 sec., yet this is not reflected in its effect on mean pore diameters or porosity (Figs. 3 and 5).

The medicaments in Fig. 8 that had the least change in mean pore diameters also exhibited minimal changes in disintegration times, while those with the largest change in mean pore diameters with starch concentration had the greatest change in disintegration times.

The degree of change in mean pore diameters at the three pressure levels illustrated in Fig. 4 was reflected in the corresponding degree of change in disintegration times at the various pressures.

The two magnesium salts had the largest porosities (Fig. 9) and the best disintegration times, but the changes in porosity with starch concentration were not correlated to disintegration times. Tablets of the two organic compounds had the lowest porosities and the poorest disintegration times (Table VI).

Porosity decreased and, in general, disintegration times decreased with increasing pressure. Taking the data in Fig. 6, one could qualitatively say that the increase in mean pore diameters with increasing starch concentration correlates with the data in Table VI. Yet to conclude that disintegration times decrease as porosity decreases or starch concentration increases is an oversimplification (Fig. 10). MgO tablets also showed a decrease in porosity with increasing pressure; but above 5% starch concentration, pressure had no apparent effect on disintegration times.

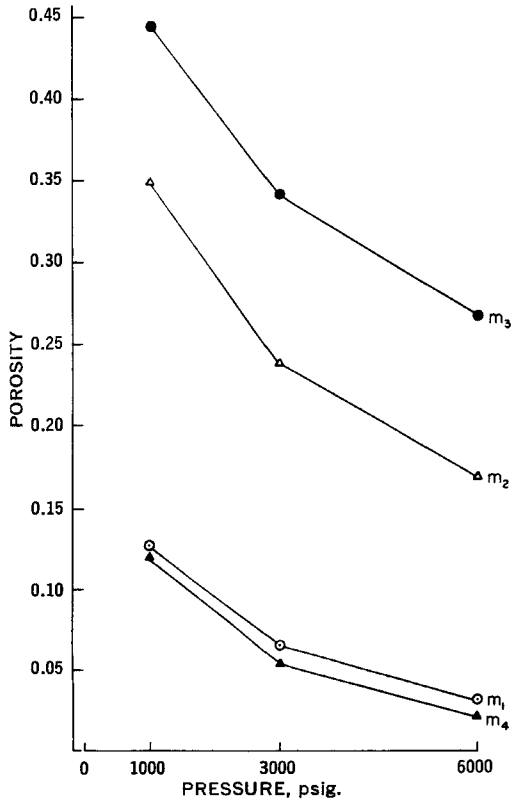

Figure 10—*Effect of pressure on porosity. Key:* M_1, *aspirin;* M_2, *MgO;* M_3, *Mg trisilicate; and* M_4, *salicylamide.*

Table VI—Predominate Tablet Disintegration Times[a]

Medicament	Starch Concentration, %	Pressure, psig. 1000	3000	6000
Aspirin	0	>3600	>3600	>3600
	5	>3600	>3600	>3600
	10	550	970	>3600
	15	38	49	25
MgO	0	>3600	>3600	>3600
	5	9	5	5
	10	5	9	9
	15	7	8	10
Mg trisilicate	0	228	>3600	>3600
	5	12	728	>3600
	10	16	23	90
	15	11	8	24
Salicylamide	0	>3600	>3600	>3600
	5	1300	>3600	>3600
	10	356	65	211
	15	30	100	87

[a] Seconds.

The suggestion of Nogami *et al.* (19) that there may be a critical amount of disintegrant could not be verified for different medicaments and particle sizes in this study. The MgO used had a particle size of 23 ± 8 μ, which was similar to that of aspirin (28 ± 19 μ), but the MgO tablets disintegrated more rapidly (Table VI) at the same starch concentrations. Mg trisilicate, with a particle size less than 2 μ, had faster disintegration times than either aspirin or salicylamide (particle size 61 ± 28 μ) at the same starch concentrations.

The mean pore diameters and porosities of aspirin tablets made with colloidal silicon dioxide are shown in Table VII. Concentration greater than 5% colloidal silicon dioxide could not be used because the increase in bulk density gave difficulty in filling the material into the die cavity. The 0.5% concentration resulted in larger mean pore diameter than the 5% level. The increase due to the addition of 5% colloidal silicon dioxide was the same order of magnitude as, for example, 15% corn starch. The porosities decreased as pressure increased and the 5% concentration had higher porosities than the 0.5% level. The order of magnitude corresponded to the porosities with 5–10% concentration of the other disintegrants. Since the aspirin tablets with colloidal silicon dioxide did not disintegrate, the mean pore diameter and the porosity were not determining factors in this instance.

Table VII—Aspirin–Colloidal Silicon Dioxide Tablets

Pressure, psig.	Concentration, %	Pore Diameter, μ	Porosity
1000	0.5	0.120	0.120
	5.0	0.088	0.158
3000	0.5	0.054	0.530
	5.0	0.040	0.084
6000	0.5	0.047	0.0305
	5.0	0.034	0.0473

Table VIII—Porosity of Magnesium Oxide Tablets Made with Regular and Dried Corn Starches

Pressure	Dried Starch	Regular Starch
1000	0.294	0.350
3000	0.207	0.240
6000	0.142	0.169

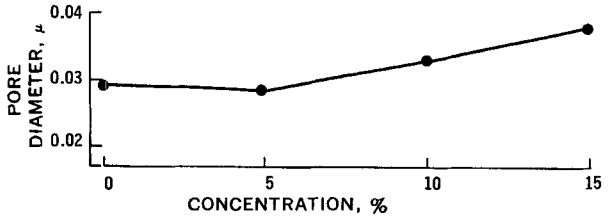

Figure 11—*Effect of dried corn starch concentration on mean pore diameter of MgO tablets.*

The effect on mean pore diameters, porosity, and disintegration times of corn starch dried to 2% moisture in MgO tablets was investigated. The mean pore diameters for MgO tablets with dried starch were similar to that of MgO tablets plus the "wet" starch, except at the 15% concentration. This occurred at all three compression forces, as can be seen by comparing curve M_2 in Fig. 8 with Fig. 11. The decrease in mean pore diameters due to increasing pressure was similar to the regular starch.

The porosities of MgO tablets containing dried starch were similar to those due to the addition of regular starch at all concentrations, except 15%, when the dried starch had a porosity of 0.205 and the "wet" starch had a porosity of 0.307. Table VIII shows that the effect of pressure on the porosities of MgO tablets containing the two starches was different in that the dried starch addition resulted in lower porosities.

There was no significant difference in disintegration times of MgO tablets made with the dried or "wet" starches except at the 5% concentration. The MgO dried starch tablets did not disintegrate within 10 min., whereas the MgO "wet" starch tablets disintegrated in 5–9 sec.

SUMMARY

Two full factorial experiments were designed. The first one had three disintegrants at four different concentrations and three levels of compression pressure with aspirin. The second experiment had four different medicaments, three levels of pressure, and corn starch at four concentration levels. Mean pore diameter, porosity, and disintegration time were the measured parameters. Under the conditions of these experiments, the following conclusions could be made.

1. The apparent decrease in mean pore diameter with increasing pressure and the apparent increase in mean pore diameter with increasing disintegrant concentration that were observed in both experiments actually varied with the particular disintegrant, pressure, medicament, and disintegrant concentration. For example, of the three disintegrants, only corn starch with aspirin gave a constant increase in mean pore diameter with increasing concentration. Only at 1000 psig. did the mean pore diameter consistently increase with the disintegrant concentration. The rate of mean pore diameter increase due to increasing corn starch concentration dropped as pressure increased.

2. The cation-exchange resin produced larger mean pore diameters and lower porosities than the two starches but could not be correlated with disintegration times.

3. Apparent porosity decreased with increasing pressure.

4. Starch concentration had a different effect on porosity for all four medicaments.

5. Porosities decreased with increasing pressure for all four medicaments at about the same rate.

6. Medicaments that had the least change in mean pore diameters exhibited minimal changes in disintegration time; the converse was also true.

7. Broad generalities as to the effect of pressure, disintegrant, disintegrant concentration, and medicament on mean pore diameter, porosity, and disintegration time of tablets cannot be made because of the complexity of the interactions of the variables.

8. Because apparent mean pore diameter and porosity decreased with increasing pressure under the conditions of these experiments, which only correlates with increasing disintegration time in specific instances, it cannot be concluded that the mechanism of starch as a tablet disintegrant is by its effect on mean pore diameter or porosity. Large mean pore diameter or porosity did not always give rapid disintegration time, nor did small mean pore diameter or porosity imply poor disintegration.

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Phase Diagram and Dissolution-Rate Studies on Sulfathiazole–Urea Solid Dispersions

WIN LOUNG CHIOU* and SARFARAZ NIAZI

Abstract □ The phase diagram of the sulfathiazole–urea binary system was determined from the physical mixtures of sulfathiazole Form I–urea and sulfathiazole Form II–urea through differential thermal analysis (DTA) and conventional capillary tube methods. The eutectic temperature and the final melting points were found to be different for the two polymorphic systems. The mutual solid solubilities in solid dispersion forms were confirmed by DTA and X-ray diffraction methods. The characteristics of DTA thermogram, X-ray diffraction spectrum, and *in vitro* dissolution rates of the eutectic mixture containing 52% (w/w) of sulfathiazole were found to change with aging. This may have a significant effect on the clinical application of sulfathiazole. The dissolution rates of sulfathiazole in solid solutions of urea were found to be extremely fast, over 700 times higher than the pure compound.

Keyphrases □ Sulfathiazole–urea solid dispersions—phase diagrams, *in vitro* dissolution rates, aging effect □ Urea–sulfathiazole solid dispersions—phase diagrams, *in vitro* dissolution rates, aging effect □ X-ray diffraction—monitoring, aging sulfathiazole–urea solid dispersions □ Differential thermal analysis—monitoring, aging sulfathiazole–urea solid dispersions

The historical background, classifications, and pharmaceutical applications of solid dispersion systems were thoroughly reviewed by Chiou and Riegelman (1). The application to increase rates of dissolution and GI absorption of poorly water-soluble drugs was first introduced in 1961 by Sekiguchi and Obi (2). They showed a faster and more complete absorption of sulfathiazole in man when it was administered as a eutectic mixture with a water-soluble and physiologically inert carrier, urea. The eutectic mixture contained 52% (w/w) of sulfathiazole and 48% (w/w) of urea. The enhancement of the absorption was attributed primarily to the achievement of crystal size reduction of sulfathiazole in the eutectic mixture. After reexamining the phase diagram reported by Sekiguchi and Obi (2), Goldberg *et al.* (3) found that there existed a limited solid solution formation in both extremes of the phase diagram. Since, in a solid solution, the particle size of the solute will be reduced to its minimum state (*i.e.*, at the molecular level), they proposed that the existence of the solid solutions, rather than the reduced crystal size in the eutectic mixture, was the primary factor in producing enhanced dissolution and GI absorption of sulfathiazole.

In an excellent review article on the pharmaceutical applications of polymorphism, Haleblian and McCrone (4) pointed out that different polymorphs in a given system may result in isomorphous solid solutions, eutectics, or molecular addition compounds. Since sulfathiazole has been known to exist at least in two polymorphic forms, such as Forms I and II (5–10), it is logical to expect that the phase diagram of sulfathiazole–urea binary system may not be as simple as reported previously. This article conveys the results of such investigations. Attention was also directed to the effect of aging on the dissolution rate of sulfathiazole from the eutectic mixture. This aspect may be of extreme importance in the evaluation of potential solid dispersion dosage forms. The possible effects of aging on physicochemical properties of solid dispersion dosage forms were extensively reviewed (1). The *in vitro* dissolution rates of sulfathiazole in solid solutions and other possible mechanisms of dissolution enhancement from solid dispersion systems are also discussed.

EXPERIMENTAL

Materials—Form I[1] of sulfathiazole was confirmed by the X-ray diffraction spectrum (8, 9) and differential thermal analysis (DTA) (7, 10). Form II of sulfathiazole was obtained by heating sulfathiazole Form I to 180° (7), and the complete conversion to Form II was confirmed by the same methods (7–10). Both forms of sulfathiazole showed a melting range from 201 to 203°. The resolidified fused urea was prepared by carefully melting the pure urea[2] (m.p. 133–136°) in an oil bath and pouring the melt onto a stainless steel plate to induce rapid solidification. The resolidified fused urea has a melting range from 129 to 136°. The major portion of the X-ray diffraction spectrum was found to be essentially the same as that of the untreated urea.

Solid dispersions of the sulfathiazole–urea system were prepared by employing the fusion method (11). The concentration of sulfathiazole in the physical mixtures and the solid dispersions was confirmed by assaying the preparations spectrophotometrically at 227 nm., using a Beckman DBG spectrophotometer. The presence of urea does not show any interference in this range.

Thermal Analyses—The DuPont 900 thermal analyzer[3], attached with a standard DTA cell (500° model), was used for DTA studies. The samples were placed in microcapillary tubes and heated at a constant rate of either 5 or 10°/min., using glass beads as the reference material. The two different rates gave almost identical thermograms. The instrument was calibrated against two standard compounds, acetanilide (m.p. 115°) and sulfapyridine (m.p. 192°), supplied by the manufacturer of the instrument. In addition to the DTA analysis, the conventional capillary tube method, using a Thomas-Hoover capillary melting-point apparatus[4], was employed to study the thaw-melt points of certain samples.

X-Ray Diffraction Studies—Samples for X-ray diffraction studies were prepared as follows. A thin rectangular metal plate (28 × 70 × 2 mm.), with a cavity of 20 × 10-mm. dimension, was placed over a glass slide; the fine powder of the sample was packed fully and firmly into the cavity. The sample was then covered firmly with another glass slide, which was fastened to the metal plate with adhesive tape. The plate was reversed, the glass slide base was removed, and the flat surface of the sample was exposed for the diffraction study using a Norelco X-ray diffractometer[5]. All diffraction spectra were run at 2°/min. in terms of a 2θ angle.

Dissolution-Rate Studies—Unless otherwise specified, the dissolution studies were carried out on powdered samples, equivalent to 15 mg. of sulfathiazole, in 500 ml. of water in a 600-ml. water-

[1] Sulfathiazole, Eli Lilly & Co., Indianapolis, Ind.
[2] Urea NF, purified from methanol, J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] E. I. duPont de Nemours & Co., Wilmington, DE 19898
[4] Arthur H. Thomas Co., Philadelphia, Pa.
[5] Philip Electronic Instrument Co.

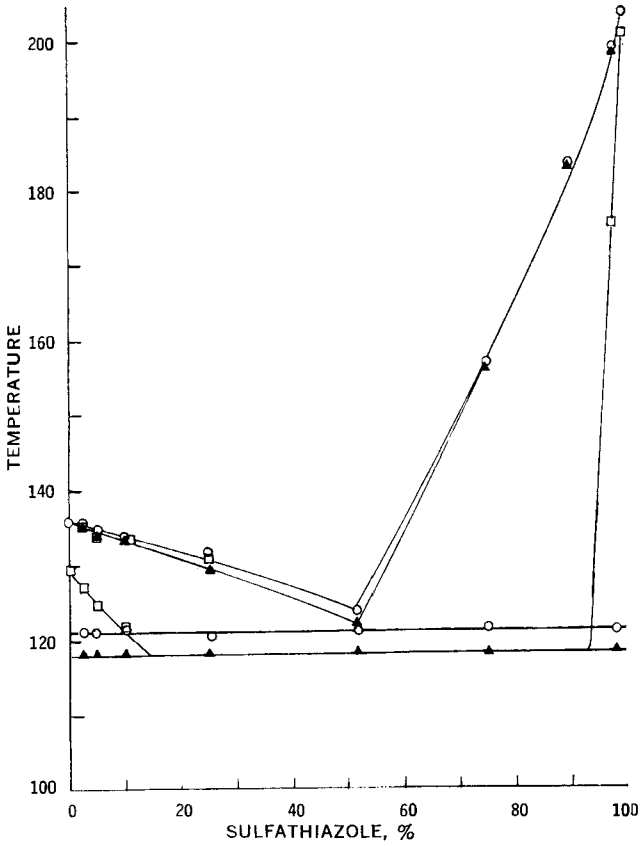

Figure 1—*Phase diagrams of the sulfathiazole–urea binary system determined from different mixtures. Key: ▲, sulfathiazole Form I–urea physical mixture; ○, sulfathiazole Form II–urea physical mixture; and □, solid dispersion.*

jacketed beaker being kept at 37°. A stirring rate of 60 r.p.m. and a recirculating flow rate of 70 ml./min. were employed (12). The dissolution rates of sulfathiazole were calculated from direct measurements of absorbance of the solution at 227 nm. All samples were run at least in duplicate. Highly reproducible results were obtained throughout the studies.

RESULTS AND DISCUSSION

Phase Diagram Determination from Physical Mixtures—It is regrettable that the methods of sample preparation and thaw-melt point determination were not clearly stated in the original article of Sekiguchi and Obi (2) for the construction of their phase diagrams. Phase diagrams erected from data on physical mixtures obtained through thermal analysis were often found to correlate well with those determined from the fused or evaporated mixtures (13, 14). The phase diagrams for the two polymorphic forms of sulfathiazole determined from homogeneous physical mixtures with nonfused urea are shown in Fig. 1. The thaw points were determined from DTA thermograms, since the DTA instruments are generally thought to be more sensitive and objective than the visual capillary tube method in detecting the beginning of melting (*i.e.*, thawing point) of a small fraction of a sample (15–17). The final melting points were, however, determined from both the DTA and capillary tube methods.

DTA thermograms may show some ambiguity as to the final melting range of certain samples. In such instances, the melting points determined from the capillary tube method were used to construct the phase diagrams.

As shown in Fig. 1, there are indeed some differences between the phase diagrams of two polymorphic forms. The eutectic temperature for sulfathiazole Form I–urea system is 118°, which is in good agreement with the previously reported data (2). The eutectic temperature for sulfathiazole Form II–urea system is, however, 3° higher than that of the Form I system. The final melting points of the eutectic mixture (52% sulfathiazole) determined from DTA thermograms are also higher by 2° for the sulfathiazole Form II–urea system. The differences of the melting points become smaller as the concentration of sulfathiazole decreases. The differences become almost negligible when the samples contain more than 75% of sulfathiazole. This may be due primarily to the transition of sulfathiazole from Form I to Form II at higher temperatures (around 150°), thus eliminating the original polymorphic difference of sulfathiazole.

Although there actually exists some degree of mutual solid solubilities in this binary system, samples of physical mixtures containing from 1 to 98% sulfathiazole showed their thaw points starting at the eutectic temperature. On the basis of the studies on the physical mixtures alone, this binary system may be mistakenly considered as a simple eutectic mixture with negligible solid solubilities. The DTA thermograms of 5% sulfathiazole–95% urea physical mixtures are shown in Fig. 2.

Phase Diagram Determination from Solid Dispersions—The DTA thermograms of 2.5 and 5% sulfathiazole solid dispersions (Fig. 2) show that the samples started to thaw at temperatures higher than the eutectic temperatures. This is indicative of solid solution formation (16). The solid solution formation in these samples is further

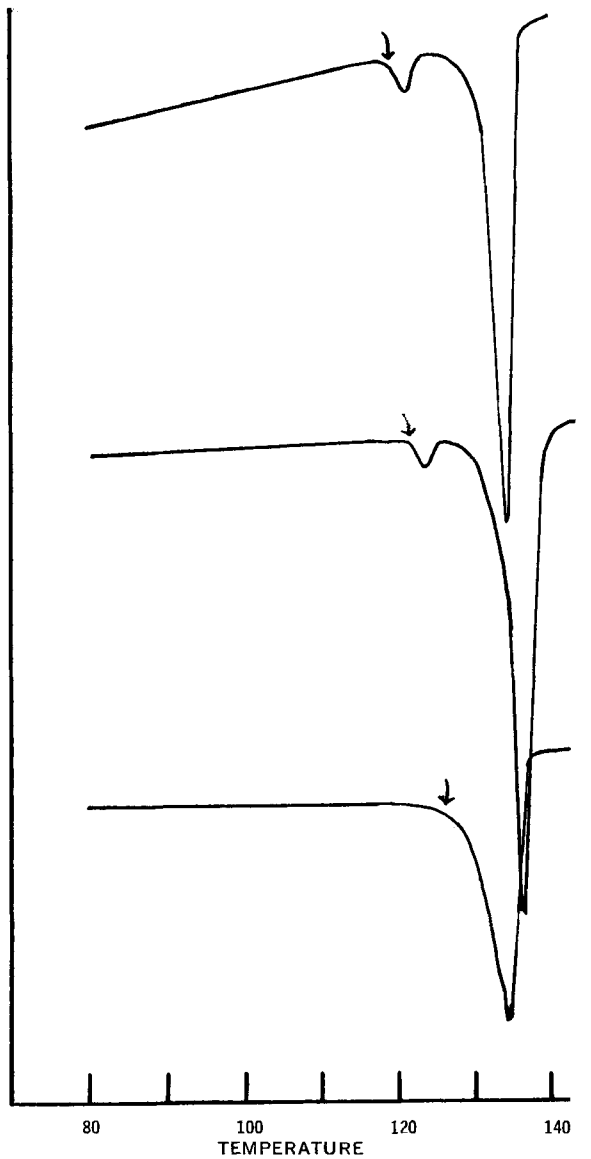

Figure 2—*DTA thermograms of various 5% sulfathiazole–95% urea systems. Different thaw points are indicated by the arrows. Key: top thermogram, sulfathiazole Form I–urea physical mixture; middle thermogram, sulfathiazole Form II–urea physical mixture; and bottom thermogram, solid dispersion.*

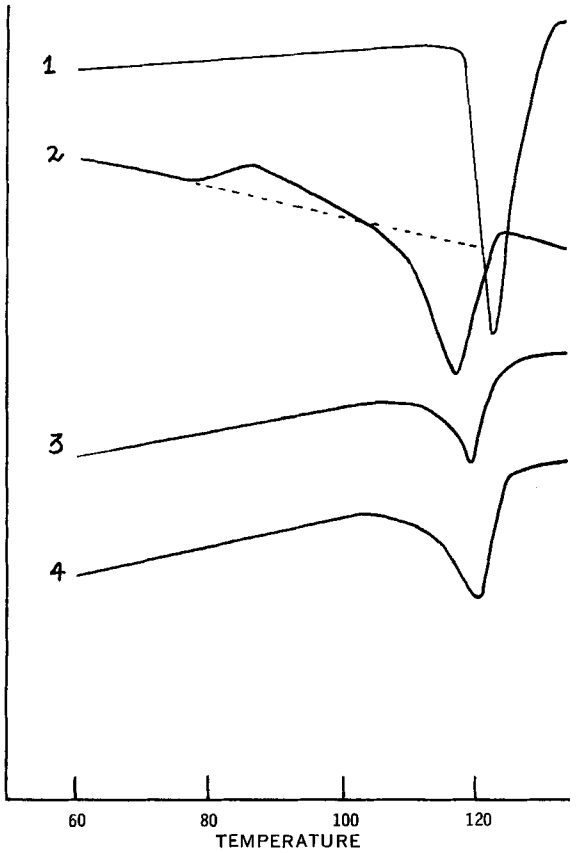

Figure 3—*DTA thermograms of the sulfathiazole–urea system with eutectic composition (52% sulfathiazole–48% urea). Key: 1, physical mixture of sulfathiazole Form I–urea; 2, freshly prepared solid dispersion (melting method); 3, solid dispersion kept at ambient temperature for 1 week; and 4, solid dispersion kept at ambient temperature for 2 months.*

substantiated by the absence of sulfathiazole X-ray diffraction peaks in their diffraction spectra.

As shown in Fig. 1, the extent of the maximum solid solution formation of sulfathiazole in urea depends on the type of polymorphic form of sulfathiazole precipitated from the solid solution. By intersecting the solidus line with the two eutectic isothermal lines, one can calculate that the solubility of sulfathiazole in the binary system will be 10% (w/w) at 121°, when the sulfathiazole Form II precipitates, and 15% (w/w) at 118°, when the sulfathiazole Form I precipitates. The solid solubility of sulfathiazole in urea at the eutectic temperature was previously reported to be 10% (w/w); that of urea in sulfathiazole was reported to be around 8% (w/w) (3).

Solid dispersions containing more than 80% (w/w) of sulfathiazole, prepared by the melting method, were intensively discolored (light to dark brown) and somewhat soft and sticky. This may be due to the thermal decomposition of sulfathiazole at higher temperatures. Obviously, these samples could not be used to obtain the pertinent data. Such a problem has not been reported previously in the literature. An alternative solvent method was then used to prepare intimately mixed mixtures. Generally speaking, a common solvent is preferred to dissolve the two solid components initially, followed by the evaporation of the solvent (18). It is, however, difficult to find a truly common solvent because of the extreme difference in solubility properties between the polar urea and nonpolar sulfathiazole. Nevertheless, absolute ethanol was chosen as a solvent, although solubilities of the two components were about 20 times different in this solvent (19). The sample of 98% sulfathiazole–2% urea solid dispersion prepared by the solvent method did show, indeed, on the DTA thermogram the thaw point higher than the eutectic temperature. The extrapolated solubilities of urea in both polymorphic forms of sulfathiazole at the eutectic temperatures were found to be around 7% (Fig. 1). This value is in good agreement with the previously reported figures. Ironically, 96% sulfathiazole–4% urea solid dispersion prepared by the same solvent method showed a eutectic peak on the DTA thermogram. This is due to the independent crystallization of both components from the nonideal solvent. Theoretically, the two components should crystallize simultaneously as mixed crystals if they form a solid solution (1, 18).

The DTA thermograms of 25, 52, and 75% sulfathiazole solid dispersions prepared by the fusion method (in the case of 75% sulfathiazole solid dispersion, the DTA rerun of its physical mixture was used) show irregular and lower thaw points ranging from 90 to 110°. Furthermore, the thermal properties of these samples were found to change after aging. Typical DTA thermograms of the 52% sulfathiazole (eutectic composition) solid dispersion are shown in Fig. 3. The X-ray diffractometry was employed subsequently to reveal this peculiarity. As shown in Fig. 4, the diffraction spectrum of the freshly prepared 52% sulfathiazole solid dispersion contains none of the diffraction peaks of sulfathiazole but only those of the resolidified fused urea. The complete absence of any sulfathiazole diffraction peaks may be due to the presence of sulfathiazole in an amorphous form (more correctly, the glass solution of urea in sulfathiazole) or in extremely fine crystallites (9) (more correctly the solid solution of urea in sulfathiazole). In the light of the ability of sulfathiazole to supercool to a glassy, amorphous form and the transparent nature of the freshly prepared mass containing more than 75% sulfathiazole, one can conclude more likely that the sulfathiazole was present as an amorphous form in the solid dispersion. Such contention is also substantiated by the presence of an exothermic peak in its DTA thermogram (Fig. 3), which probably is due to the transition of the higher energy amorphous form to the lower energy crystalline form of sulfathiazole. Furthermore, the presence of crystalline forms of both urea and sulfathiazole in the solid dispersion should not theoretically result in a considerable decrease in the thaw point and a wider range of melting. This phenomenon, therefore, may be attributed to the presence of the metastable amorphous form.

As found in the DTA studies, the diffraction spectrum of 52% sulfathiazole solid dispersion also changed with time. After storage at 27° for 2 weeks, weak diffraction peaks of the sulfathiazole Form II appeared (Fig. 5). Storage at 5° for the same period of time caused no change in the spectrum, indicating a slower rate of conversion of the amorphous form to Form II at lower temperatures. Interestingly, an incubation at 105° for only 1 hr. resulted in the appearance of strong sulfathiazole Form II peaks (Fig. 6). The sulfathiazole Form II diffraction peaks were present in the freshly prepared 25% sulfathiazole solid dispersion, even though it showed an earlier thawing and melting in the DTA thermogram.

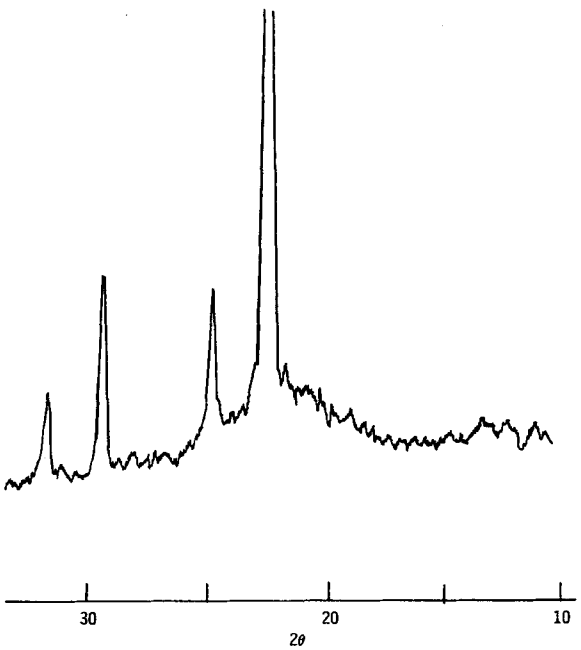

Figure 4—*X-ray diffraction spectrum of the freshly prepared sulfathiazole–urea eutectic mixture (52% sulfathiazole).*

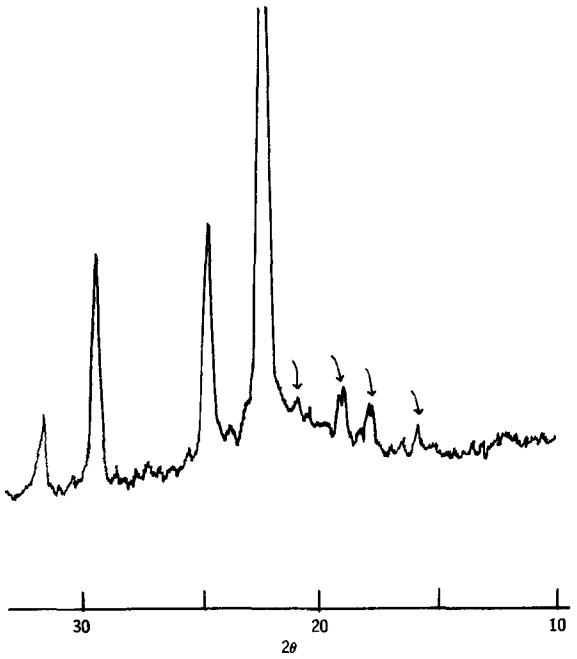

Figure 5—*X-ray diffraction spectrum of the sulfathiazole–urea eutectic mixture kept at 27° for 2 weeks. Peaks indicated by arrows correspond to sulfathiazole Form II diffraction spectrum.*

The positions of the diffraction peaks of either sulfathiazole or urea in all of the solid dispersions studied were not altered as compared with that of pure compounds, albeit there existed certain degrees of solid–solid solubilities which might affect the crystalline lattice parameters of the pure compounds (20, 21).

Effect of Aging on Dissolution Rates of Sulfathiazole Solid Dispersions—Sekiguchi *et al.* showed that the GI absorption of sulfathiazole could be enhanced when it was administered as a 52% solid dispersion. Since the history of the solid dispersion used for oral studies was not specified in their article and the possible conversion of the amorphous sulfathiazole to crystalline sulfathiazole is not a rapid process at ambient temperature, it will be of interest to know how the aging of the sample can affect the *in vitro* dissolution rate of sulfathiazole. The data on the freshly prepared sample and the sample kept at 105° for 1 hr. are shown in Fig. 7. The aged sample shows a slower dissolution rate, especially during the first few minutes of the study. Its effect on the *in vivo* absorption of the drug, however, remains to be studied. Furthermore, since the Form I of sulfathiazole is the stable form, the ultimate conversion of the metastable Form II to Form I also may have a significant effect on the dissolution rate and bioavailability of the preparation.

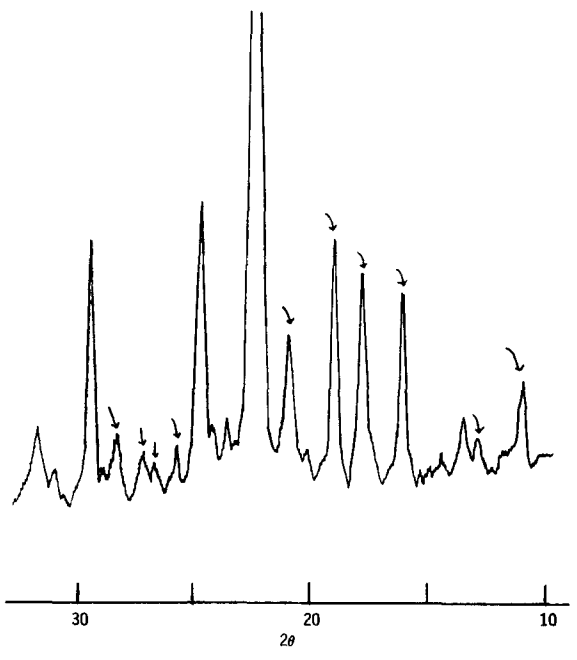

Figure 6—*X-ray diffraction spectrum of the sulfathiazole–urea eutectic mixture kept at 105° for 1 hr. Peaks indicated by arrows correspond to sulfathiazole Form II diffraction spectrum.*

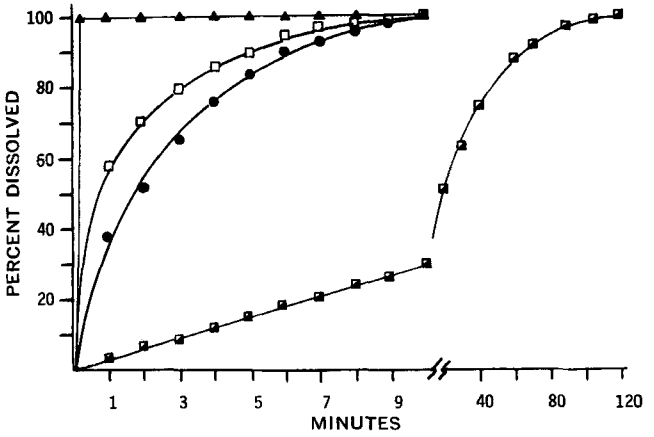

Figure 7—*Dissolution rates of sulfathiazole in various forms in 500 ml. water at 37°. Key: ▲, 5 or 10% sulfathiazole solid solution (60–100 or 10–20 mesh); □, freshly prepared eutectic mixture (52% sulfathiazole, 60–100 mesh); ●, eutectic mixture kept at 105° for 1 hr. (60–100 mesh); and ◪, pure sulfathiazole Form I (60–100 mesh).*

Dissolution Rates of Pure Sulfathiazole—Although the administration of the 52% sulfathiazole solid dispersion was shown to increase the physiological availability of the drug, no rank correlation between its *in vivo* availability and *in vitro* dissolution rate was reported. The *in vitro* dissolution rates of sulfathiazole powder with the same particle size are shown in Fig. 7. Comparison of the time required for the complete dissolution of sulfathiazole reveals that the solid-dispersed form is about 12 times faster than the pure Form I. Since the coadministration of urea was not found to interfere with the GI absorption of sulfathiazole in a pure form (2), these data clearly indicate that the increased dissolution rate of sulfathiazole in a solid-dispersed form is the principal factor contributing toward its faster absorption in man.

Dissolution Rates of Sulfathiazole in Solid Solutions—Although the solid solution approach has been advocated to obtain faster dissolution rates of poorly soluble drugs since 1965 (3), no *in vitro* dissolution-rate studies of the powder of a drug in a complete solid solution form have been reported so far. The DTA and X-ray diffraction methods were recently employed to disprove (22, 23) the previous studies on the existence of extensive solid solution formation in the chloramphenicol–urea (3, 24) and griseofulvin–succinic acid systems (25). Both of these systems appear to be simple eutectic mixtures with negligible solid solubilities.

The dissolution rates of sulfathiazole in the 5 and 10% solid solutions were found to be extremely fast (Fig. 7). After about 15 sec. of dissolution in a recirculating flowcell system, the absorbance of the dissolution media reached a constant, indicating complete dissolution of sulfathiazole. More surprisingly, no noticeable differences in dissolution rates between the fine 60–100-mesh powders and the coarse 10–20-mesh granules were found. Visual observation revealed that all of the powder disappeared into dissolution media in about 10 sec. Furthermore, the big 10–20-mesh particles were found to disappear completely (probably dissolve completely) before they settled down to the bottom of the beaker in an unstirred condition (in the order of seconds). Such an almost instantaneous dissolution is not surprising if one considers that sulfathiazole has already dissolved *in situ* in the urea matrix and urea is extremely and readily water soluble (19). The solubility of urea in water at room temperature is about 1 g. in 1 ml. In these solid solutions, the rate-limiting step of the dissolution of sulfathiazole in water is mainly the dissolution of the matrix. The observed fast dissolution is also in analogy with the mixing of a sulfathiazole solution in a water-miscible organic solvent such as ethanol or polyethylene glycol 300 with water.

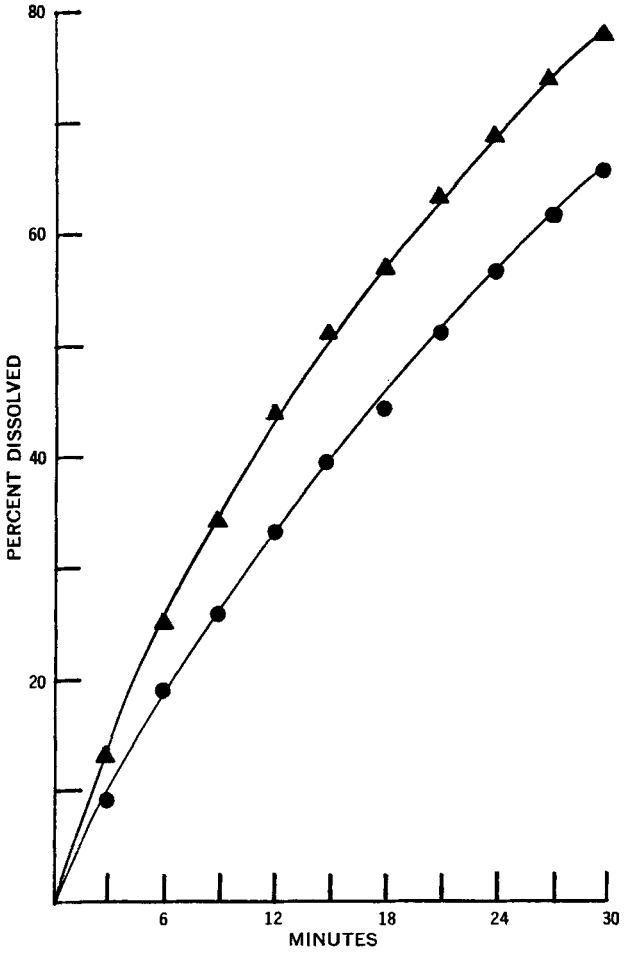

Figure 8—*Dissolution rates of sulfathiazole Form I in distilled water* (●) *and in 10% aqueous urea solution* (▲).

In comparing the time required to obtain 100% dissolution of sulfathiazole in solid solutions with that of the 60–100-mesh sulfathiazole Form I, the dissolution rates from the solid solutions are estimated to be about 700 times faster. If one compares dissolution rates of 10–20-mesh powders, the ratio would be in the order of thousands; the particle size has very slight effect on the dissolution rate of sulfathiazole in the solid solutions, but it has a great effect on the dissolution rate of the pure sulfathiazole. Such marked enhancement of *in vitro* dissolution rates was not found in dispersion systems of griseofulvin–succinic acid (25) and chloramphenicol–urea (24, 26). By using constant-surface tablets, Allen and Kwan (27) found that the dissolution rate of sulfathiazole in 5% sulfathiazole–95% urea solid solution was only about three times higher than that in the 5% sulfathiazole–95% urea physical mixture. Their finding seems to have no correlation in terms of the degree of enhancement with the present results using the powder method. Attempts to compare the dissolution rate of 10% sulfathiazole–90% urea systems using the constant surface method had to be given up since the hard tablets of the physical mixture compressed at even 62,500 p.s.i. or 94,750 p.s.i. started to disintegrate immediately when the assembly containing the tablet was moved down to the bottom of the aqueous dissolution medium at 37°.

Effect of Urea on Dissolution Rate of Sulfathiazole—Solubilization of drugs in carriers often was thought to be one factor to increase dissolution rates of the drugs in solid-dispersed forms (1, 9, 11, 12, 23–25). Since the solubility of sulfathiazole in water, on the contrary, was found to be decreased by the presence of urea (2), it will be interesting to know whether such desolubilization has any effect on the sulfathiazole dissolution. The result of the dissolution study on the powder of the pure Form I in a 10% (w/v) aqueous urea medium is shown in Fig. 8. Surprisingly, the dissolution rate also was increased by urea. It is postulated that such an enhancement must be due to better wetting of the powder in the urea solution, presumably with a lower surface tension. Such effect also may operate significantly in the microenvironment (diffusion layer) immediately surrounding the drug particles, especially in the early stage of dissolution from solid-dispersed forms since the carrier will completely dissolve in a short period of time. The lower surface-tension effect also has been thought to contribute to the increased dissolution rate of reserpine from reserpine–cholanic acid precipitates (29).

CONCLUSION

Different phase diagrams of the sulfathiazole–urea binary system were obtained for the two polymorphs of sulfathiazole. The mutual solid solubilities at the eutectic temperatures were found to be dependent upon the presence of a specific polymorphic form of sulfathiazole in the solid dispersion. The effect of aging on the eutectic mixture (52% sulfathiazole–48% urea) was studied by noting the changes in DTA thermograms, X-ray diffraction patterns, and *in vitro* dissolution rates. The dissolution rate of sulfathiazole in a solid solution was found to be almost instantaneous, more than 700 times the dissolution rate of the pure compound. Although urea decreases the solubility of sulfathiazole, its presence in the dissolution medium was shown to increase the dissolution rate, probably due to the lowering of interfacial tension.

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Influence of First-Pass Effect on Availability of Drugs on Oral Administration

M. GIBALDI, R. N. BOYES*, and S. FELDMAN†

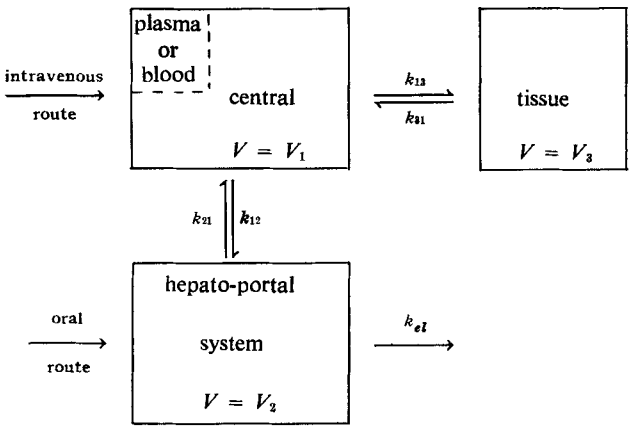
Abstract □ Currently used pharmacokinetic models assume that drug administered both intravenously and orally initially enters the same vascular pool. However, literature data suggest that although a drug is completely absorbed, the area under the plasma level–time curve after oral administration may be considerably less than the corresponding area following intravenous therapy. This has been explained on the basis of a "first-pass" effect in the liver. Simple equations have been derived, allowing prediction of the extent of this first-pass effect for a particular drug. Plasma level data for propranolol in man have been used to indicate the utility of these equations. The significance of these calculations to the design of clinical studies with new drugs intended for oral use is discussed.

Keyphrases □ Bioavailability, estimate calculations—from plasma levels, first-pass effect □ Absorption kinetics, oral—plasma level data, first-pass effect

Pharmacokinetic analysis of plasma level data for drugs generally assumes that the site of elimination is an integral part of the same compartment as the sampled plasma. For drugs eliminated by hepatic metabolism, this assumption may not be valid under all circumstances. Two recent papers (1, 2) indicated that the areas under the blood level–time curves for aspirin and lidocaine were considerably greater when a dose of the drug was infused into a peripheral vein as compared to results observed upon infusion of an equal dose into the portal vein of the dog. Administration of a drug directly into the portal vein is, in most instances, equivalent to the pathway followed after oral administration. The reduction in area under the blood level–time curves following portal vein infusion has been attributed to the fact that the drugs were exposed to the liver before reaching the vascular site being sampled. This phenomenon has been commonly termed the "first-pass" effect. Clearly, then, differences in areas under blood level–time curves as a function of route of administration may reflect not only differences in the amount of drug absorbed but the first-pass phenomenon as well. Based on these considerations, a somewhat different model or a correction factor may be required to compare plasma levels of certain drugs following oral and intravenous administration. The purpose of this communication is to present a simple method of calculation which can be used to predict, from plasma levels following intravenous or oral administration, the approximate reduction in area under the curve due to the first-pass phenomenon.

THEORETICAL

In a previous report (3), a linear three-compartment open model was proposed to explain the influence of route of administration (*i.e.*, intravenous *versus* oral) on the area under the plasma concentration–time curve. A modification of the model is shown in Scheme I. The essential feature of this model is that the hepatoportal system is treated as being, or being within, a compartment distinct from the compartment containing the vascular site sampled. Moreover, it was suggested that it often is exceedingly difficult to justify the existence of three distinct compartments solely on the basis of curve-fitting plasma concentration–time data after intravenous administration. Hence, although the plasma concentration data suggest simply a two-compartment model, an additional, rapidly accessible compartment might well exist and, in fact, must exist, from a mathematical point of view to explain certain pharmacokinetic anomalies (1, 2).



Scheme I—Three-compartment open model

If the vascular system being sampled is a component of the central compartment (inset of Compartment 1 of Scheme I), a difference will indeed occur with respect to the area under the drug concentration *versus* time curve as a function of route of administration (3). When the drug is given directly into Compartment 1, a situation comparable to intravenous administration, the total area under the plasma concentration–time curve is given by:

$$(\text{area})_1 = \text{dose}\ (k_{21} + k_{el})/(V_1 k_{12} k_{el}) \qquad \text{(Eq. 1)}$$

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Influence of First-Pass Effect on Availability of Drugs on Oral Administration

M. GIBALDI, R. N. BOYES*, and S. FELDMAN†

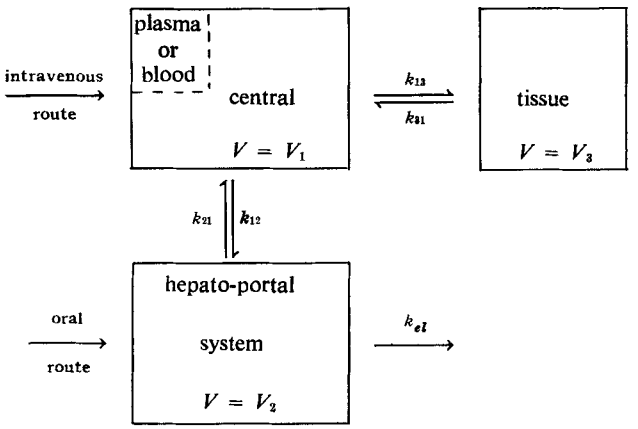
Abstract □ Currently used pharmacokinetic models assume that drug administered both intravenously and orally initially enters the same vascular pool. However, literature data suggest that although a drug is completely absorbed, the area under the plasma level–time curve after oral administration may be considerably less than the corresponding area following intravenous therapy. This has been explained on the basis of a "first-pass" effect in the liver. Simple equations have been derived, allowing prediction of the extent of this first-pass effect for a particular drug. Plasma level data for propranolol in man have been used to indicate the utility of these equations. The significance of these calculations to the design of clinical studies with new drugs intended for oral use is discussed.

Keyphrases □ Bioavailability, estimate calculations—from plasma levels, first-pass effect □ Absorption kinetics, oral—plasma level data, first-pass effect

Pharmacokinetic analysis of plasma level data for drugs generally assumes that the site of elimination is an integral part of the same compartment as the sampled plasma. For drugs eliminated by hepatic metabolism, this assumption may not be valid under all circumstances. Two recent papers (1, 2) indicated that the areas under the blood level–time curves for aspirin and lidocaine were considerably greater when a dose of the drug was infused into a peripheral vein as compared to results observed upon infusion of an equal dose into the portal vein of the dog. Administration of a drug directly into the portal vein is, in most instances, equivalent to the pathway followed after oral administration. The reduction in area under the blood level–time curves following portal vein infusion has been attributed to the fact that the drugs were exposed to the liver before reaching the vascular site being sampled. This phenomenon has been commonly termed the "first-pass" effect. Clearly, then, differences in areas under blood level–time curves as a function of route of administration may reflect not only differences in the amount of drug absorbed but the first-pass phenomenon as well. Based on these considerations, a somewhat different model or a correction factor may be required to compare plasma levels of certain drugs following oral and intravenous administration. The purpose of this communication is to present a simple method of calculation which can be used to predict, from plasma levels following intravenous or oral administration, the approximate reduction in area under the curve due to the first-pass phenomenon.

THEORETICAL

In a previous report (3), a linear three-compartment open model was proposed to explain the influence of route of administration (*i.e.*, intravenous *versus* oral) on the area under the plasma concentration–time curve. A modification of the model is shown in Scheme I. The essential feature of this model is that the hepatoportal system is treated as being, or being within, a compartment distinct from the compartment containing the vascular site sampled. Moreover, it was suggested that it often is exceedingly difficult to justify the existence of three distinct compartments solely on the basis of curve-fitting plasma concentration–time data after intravenous administration. Hence, although the plasma concentration data suggest simply a two-compartment model, an additional, rapidly accessible compartment might well exist and, in fact, must exist, from a mathematical point of view to explain certain pharmacokinetic anomalies (1, 2).



Scheme I—Three-compartment open model

If the vascular system being sampled is a component of the central compartment (inset of Compartment 1 of Scheme I), a difference will indeed occur with respect to the area under the drug concentration *versus* time curve as a function of route of administration (3). When the drug is given directly into Compartment 1, a situation comparable to intravenous administration, the total area under the plasma concentration–time curve is given by:

$$(\text{area})_1 = \text{dose}\ (k_{21} + k_{el})/(V_1 k_{12} k_{el}) \qquad \text{(Eq. 1)}$$

Table I—Ratio of Areas (f)[a] after Oral and Intravenous Administration of Propranolol Obtained Experimentally and Calculated by Means of Eq. 8

Subject[b]	Experimental[b]	Calculated: Blood Flow Model[c]	Calculated: Plasma Flow Model[d]
O. F.	0.60	0.64	0.50
D. S.	0.30	0.38	0.25
G. Y.	0.32	0.39	0.26
J. C.	0.20	0.27	0.17
J. F.	0.17	0.22	0.13

[a] Ratio of area under the plasma curve after oral administration to that after intravenous administration, assuming equivalent doses. [b] Data obtained from *Reference 7*, intravenous dose = 10 mg., oral dose = 80 mg. [c] Based on hepatic blood flow of 1.7 l./min. [d] Based on hepatic plasma flow of 0.94 l./min.

On the other hand, when the drug is given directly into Compartment 2, a situation comparable to administration *via* the hepatic portal vein and often comparable to oral administration, then the total area under the plasma level–time curve is given by:

$$(\text{area})_2 = \text{dose}\ (k_{21})/(V_1 k_{12} k_{el}) \qquad \text{(Eq. 2)}$$

By combining Eqs. 1 and 2, it can been seen (Eq. 3) that the relative area under the plasma level curve, when a dose of drug is administered into Compartment 2 as compared to an equivalent dose into Compartment 1, is given by the following relationship:

$$f = \frac{(\text{area})_2}{(\text{area})_1} = \frac{k_{21}}{k_{21} + k_{el}} \qquad \text{(Eq. 3)}$$

In Eq. 3, f represents the proportion of the drug administered into Compartment 2 that actually reaches the plasma or central compartment.

Although Eq. 3 provides an exact solution to the ratio of areas, it is of little practical value since the rate constants cannot be determined. If certain assumptions are made, however, a general relationship, analogous to Eq. 3, may be written which may be quite useful in a predictive sense for a given drug.

Multiplying the numerator and denominator of Eq. 3 by V_2 yields:

$$f = (k_{21}V_2)/(k_{21}V_2 + k_{el}V_2) \qquad \text{(Eq. 4)}$$

By assuming that clearance from one compartment to another is equal in both directions (4), $k_{21}V_2 = k_{12}V_1$. Substitution of $k_{12}V_1$ for $k_{21}V_2$ in Eq. 4 yields:

$$f = k_{12}V_1/(k_{12}V_1 + k_{el}V_2) \qquad \text{(Eq. 5)}$$

If one assumes further that transfer between Compartments 1 and 2 is blood or plasma flow rate limited (5, 6), then $k_{12}V_1 \cong$ flow rate and:

$$f = (\text{flow rate})/(\text{flow rate} + k_{el}V_2) \qquad \text{(Eq. 6)}$$

The product of k_{el} and V_2 may be evaluated from Eq. 2.

Since $V_1k_{12} = V_2k_{21}$, then:

$$k_{el}V_2 = \text{dose}/(\text{area})_2 \qquad \text{(Eq. 7)}$$

In the previous report (3), it was suggested that $k_{el}V_2$ could be approximated by the term dose/(area)$_1$. As demonstrated here, this is clearly not the case.

Substituting Eq. 7 into Eq. 6 yields:

$$f = \frac{\text{flow rate}}{\text{flow rate} + [\text{dose}/(\text{area})_2]} \qquad \text{(Eq. 8)}$$

If plasma level data are available following oral administration of a drug, substitution of the oral dose and respective plasma level–time curve area, as well as the appropriate flow rate, into Eq. 8 should yield a reasonable estimate of f, provided the dose of the drug is completely absorbed. If the latter assumption is not correct, then Eq. 8 must be modified to take into account the differences in areas under the plasma concentration–time curve after oral and intravenous administration that are due to a difference in the amount absorbed as well as the first-pass effect. In this case, one may write:

$$f = \frac{F\ (\text{flow rate})}{\text{flow rate} + [(F \cdot \text{dose})/(\text{area})_2]} \qquad \text{(Eq. 9)}$$

where F is the fraction of the administered oral dose absorbed. Under certain conditions, F may be determined independently from urinary excretion data based on total metabolites or an isotopic tag.

Perhaps a more realistic approach is the evaluation of f from plasma concentration–time data obtained after intravenous administration of the drug. This is readily accomplished by rearranging Eq. 8. Since $f = (\text{area})_2/(\text{area})_1$, then:

$$\frac{(\text{area})_2 \cdot \text{flow rate}}{(\text{area})_1} + \frac{\text{dose}}{(\text{area})_1} = \text{flow rate} \qquad \text{(Eq. 10)}$$

or

$$f = 1 - \frac{\text{dose}}{(\text{area})_1 \cdot \text{flow rate}} \qquad \text{(Eq. 11)}$$

Substitution of the intravenous dose and respective plasma level–time curve area, as well as the appropriate flow rate, into Eq. 8 should yield an estimate of the extent to which the first-pass effect is contributing to a reduction in the area under the curve after oral administration relative to that observed after peripheral intravenous administration. If one finds experimentally a value of f that is lower than that calculated by Eq. 11, it would suggest that oral administration does not lead to complete absorption. In this case, the difference in areas under the plasma level–time curve as a function of route of administration would reflect differences in the amount absorbed as well as the first-pass effect.

APPLICATIONS

The value of flow rate to be used in solving Eq. 8 depends upon the physiologic significance of the various compartments. For example, if one views Compartment 2 as simply the hepatoportal system, it is appropriate to use an hepatic flow rate. If the drug in the blood is restricted to the plasma, or if the portion of drug in the blood that is partitioned in the erythrocytes is not "instantaneously" exchanged with drug in the plasma, then the transfer process is best described in terms of plasma flow rates. This approach was used successfully by Bellman *et al.* (6). If, on the other hand, rapid transport could be envisioned between red cells and plasma, then whole blood flow rates may be useful, as suggested by Bischoff and Dedrick (5).

Application of the calculation presented here to recently reported plasma level data for propranolol leads to some interesting results. Shand *et al.* (7) estimated the total area under the plasma level *versus* time curve in fasting adult volunteers after oral administration of 80 mg. propranolol in tablet form and after intravenous administration of 10 mg. of the drug. These data permit a direct and independent test of Eqs. 8 and 11 for the estimation of f.

Substitution of the area under the plasma level–time curve after oral administration for each subject reported by Shand *et al.* (7), the

Table II—Ratio of Areas (f)[a] after Oral and Intravenous Administration of Propranolol Obtained Experimentally and Calculated by Means of Eq. 11

Subject[b]	Experimental[b]	Calculated: Blood Flow Model[c]	Calculated: Plasma Flow Model[d]
O. F.	0.60	0.66	0.29
D. S.	0.30	0.55	0.19
G. Y.	0.32	0.51	0.11
J. C.	0.20	0.46	0.03
J. F.	0.17	0.44	—[e]

[a] Ratio of area under the plasma curve after oral administration to that after intravenous administration, assuming equivalent doses. [b] Data obtained from *Reference 7*, intravenous dose = 10 mg., oral dose = 80 mg. [c] Based on hepatic blood flow of 1.7 l./min. [d] Based on hepatic plasma flow of 0.94 l./min. [e] f calculated to be a negative member.

oral dose (80 mg.), and either hepatic blood flow (1.7 l./min.) (8) or hepatic plasma flow (0.94 l./min.) into Eq. 8 yields the values of f shown in Table I. Equation 8, based either on hepatic plasma or hepatic blood flow rate, predicts a substantial first-pass effect with propranolol. The blood flow model predicts that if the oral dose is completely absorbed, the area under the plasma level–time curve will only be, on the average, 38% of that observed after intravenous administration of an equivalent dose. The plasma flow model suggests an even greater effect, yielding a mean f-value of 0.26. The experimentally determined mean f-value of 0.32 falls midway between these estimates (7). While either a plasma or blood flow model provides an excellent estimate of the experimental results, it is suggested that the hepatic blood flow model is the more realistic. It must be stressed that Eq. 8 provides a minimum estimate of $(1 - f)$, since it is assumed that the oral dose is completely absorbed. If this is not the case, then the experimentally determined value of f will be smaller than that generated by Eq. 8. In fact, if one assumes that only 70% of the oral propranolol dose is actually available to the hepatoportal system, *i.e.*, only 70% is absorbed as such, then according to Eq. 9, using hepatic blood flow, the mean value of f is 0.32, which is identical to the experimental observation.

A similar approach was employed to test Eq. 11. Estimates of the area under the plasma level *versus* time curve after intravenous administration for each subject, as reported by Shand *et al.* (7), the intravenous dose (10 mg.), and the value for either hepatic blood or plasma flow rate were substituted into Eq. 11, and f was determined for each individual. These data are reported in Table II. The blood flow model predicts that the oral administration of an equivalent dose of propranolol will at best provide a plasma level–time curve area that is 45–65% of that determined after intravenous administration. The difference in area predicted by Eq. 11 based on blood flow is smaller than that actually observed. On the other hand, the plasma flow model predicts substantially larger differences than are actually observed and, in this particular case, is unrealistic. Again, it must be noted that Eq. 11 provides a minimum estimate of $(1 - f)$, since it does not take into account possible differences in the amount absorbed as a function of route of administration. Accordingly, if one assumes, as before, that only 70% of the oral propranolol dose is actually available to the hepatoportal system, then using the hepatic blood flow model with the intravenous data, one estimates a mean f-value of 37%, which is in excellent agreement with the experimental value of 32%.

Application of Eq. 11 to preclinical and early phase I clinical studies with new drugs intended for oral administration is of considerable practical importance. Reasonable predictions can be made from plasma level data following intravenous administration as to the minimum relative dose required when the drug is to be given orally. Stated another way, one can estimate the maximum physiologic availability that can be anticipated from oral administration of a drug regardless of how well it is absorbed. Viewed in this light, the inability of intact drug to reach the central compartment and tissues of distribution results in decreased physiologic availability and the net effect is completely equivalent to that observed when a compound is poorly absorbed from the GI tract because of its intrinsic physical–chemical properties or because of faulty formulation.

Several significant clinical problems may arise with compounds that are subject to substantial first-pass effects. Since the intersubject dose variation is expected to be large, it would be important to titrate each patient to his effective dose. In clinical studies carried out double-blind against a placebo, this could be very awkward. Another problem arises because even though a small amount of the dose is available for pharmacologic effect, in general, it is expected that the total dose will be largely absorbed and produce metabolites. If these metabolites have little desired effectiveness but do, in fact, produce untoward responses, patients receiving high doses of the drug necessary for pharmacological results may also have a higher incidence of side effects due to the large amounts of metabolites formed.

Hence, for a variety of reasons, it would seem desirable to screen new compounds with respect to the first-pass phenomenon. By carrying out a few well-controlled intravenous experiments with a new drug and then applying a calculation similar to Eq. 11, it may be possible to save considerable time and expense in terms of extensive clinical trials in the evaluation of new compounds intended for oral use.

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In Vivo Conversion of ^{14}C-Labeled Cyclamate to Cyclohexylamine

L. PROSKY and R. G. O'DELL

Abstract □ To determine the effects of long-term, low-level feeding of cyclamate on cyclamate metabolism, two groups of weanling rats were fed a chow diet and a chow diet containing 0.1% calcium cyclamate, respectively, for 8 months. Twelve rats from each group were then intubated with ^{14}C-cyclamate; urine was collected for analysis by a paper electrophoretic method which separates cyclamate, cyclohexylamine, dicyclohexylamine, and *N*-hydroxycyclohexylamine. Elution of the radioactive areas from the paper indicated that none of the controls converted ^{14}C-cyclamate to additional products, but seven of 11 cyclamate-fed rats converted cyclamate to cyclohexylamine. The labeled cyclohexylamine accounted for 12–25% of the total radioactivity in urine. Two of these urine samples contained traces of radioactive dicyclohexylamine, but *N*-hydroxycyclohexylamine was not found. Isolated liver perfusion studies indicated that the liver is probably not the site of conversion.

Keyphrases □ Cyclamate, labeled—conversion to cyclohexylamine and other metabolites, *in vivo*, rats □ Excretion, urinary—^{14}C-cyclamate, cyclohexylamine and other metabolites, rats

Until Kojima and Ichibagase (1) reported the presence of cyclohexylamine in urine and feces of one man and one dog ingesting cyclamate orally, the preponderance of evidence indicated a complete and rapid excretion of dietary cyclamate as unchanged cyclamate (2–4). Since that time, several reports supported the presence of metabolic products of cyclamate in urine and feces of man (5–11) and animals (11–13).

The present study was undertaken to ascertain the effects of long-term, low-level feeding of cyclamate on cyclamate metabolism in rats, with special emphasis placed on the separation, identification, and assay of its metabolites in urine. The liver was also studied as a possible site for these conversions.

EXPERIMENTAL

Male Holtzman weanling rats, weighing 40–60 g., were divided into two groups of 24 animals each and were kept in a room maintained at 24° and 50% humidity. The control group was fed a commercial chow diet[1], and the treated group was fed the chow diet supplemented with 0.1% calcium cyclamate[2] for 8 months. Twelve animals from each group were orally intubated with a known amount (ranging from 2 to 3 μmoles) of an aqueous solution of ^{14}C-cyclamate[3] (uniformly labeled; specific activity 2.6 μc./μmole); the rats were then placed in individual metabolism cages. Eighteen-hour urine specimens were collected in aluminum foil-wrapped plastic tubes, which were submerged in dry ice during the collection period and kept frozen until analysis. These precautions minimized the conversion of cyclamate to cyclohexylamine in urine specimens.

Metabolites of cyclamate in urine were separated by two TLC systems and one paper electrophoretic system. The urine and appropriate standards (2 μl. of aqueous solutions containing 1 mg./ml. of cyclamate[4], cyclohexylamine[5], dicyclohexylamine[5], or *N*-hydroxycyclohexylamine[5]) were applied to silica gel plates[6]. The plates were developed for 45 min. in acetone–absolute ethanol (1:1), dried in an air stream at room temperature, sprayed with 0.2% 2,7-dichlorofluorescein in methanol, and examined under a UV lamp. The R_f values of the compounds were: cyclamate, 0.71; cyclohexylamine, 0.22; dicyclohexylamine, 0.22; and *N*-hydroxycyclohexylamine, 0.49. A typical chromatogram is illustrated schematically in Fig. 1A.

The second chromatographic system utilized cellulose F plates[7] and a 2.5-hr. developing time in *n*-butanol–acetic acid–water (8:2:1). As shown in Fig. 1B, the metabolic products of cyclamate migrated closer to the solvent front, and the R_f values were: cyclamate, 0.56; cyclohexylamine, 0.73; dicyclohexylamine, 0.94; and *N*-hydroxycyclohexylamine, 0.89. The second method made use of the difference in electrophoretic mobilities of cyclamate and its metabolites in a pyridine–acetic acid–water (8:15:977) system run at 300 mv. for 2 hr. (Fig. 1C). Areas on the paper corresponding to the standards (10 μl. of aqueous solutions containing 1 mg./ml.) were eluted with water and counted for radioactivity in a Nuclear Chicago Mark I scintillation counter[8] using Bray's solution (14). Cyclamate and cyclohexylamine were assayed by previously described colorimetric methods (15, 16).

The liver, suspect as a site of cyclamate metabolism, was perfused in the dark by using the apparatus and procedure described by Green and Miller (17). The livers from three rats known to convert cyclamate to cyclohexylamine were used in conjunction with blood drawn from remaining animals in the group. ^{14}C-Calcium cyclamate (100 μmoles and 10 μc.), dissolved in 10 ml. of Ringer's solution[9], was infused into the blood supply at a constant rate of 0.16 ml./min. immediately before the blood entered the liver. Aliquots of blood were withdrawn for analysis from the pool formed at the exit from the inferior vena cava at 15-min. intervals for the 2-hr. period of perfusion. At the end of perfusion, the livers were removed from the apparatus, frozen in Freon, and extracted with perchloric acid (18) for later determination of cyclamate and its metabolites.

RESULTS AND DISCUSSION

In a previous study (19), it was demonstrated that calcium cyclamate at dietary levels not exceeding 1% had little or no effect on a variety of biochemical parameters in the rat. Indeed, others reported that cyclamate fed at a level of 0.43% seemed to stimulate appetite and to cause an increase in body weight (20). In the present study, rats were fed 0.1% cyclamate to eliminate changes in growth or energy metabolism that might be associated with the extremely high doses used by other investigators (12, 21). A working hypothesis was developed which indicated that long-term feeding of cyclamate at low levels would increase its absorption and circulation in the blood and present optimum conditions for cyclamate conversion to cyclohexylamine.

Eighteen-hour urine specimens were collected after intubation of ^{14}C-cyclamate; previous investigators showed that 70–90% of the radioactivity in cyclamate is excreted in urine and feces in this period (4, 22). Control rats excreted approximately 40% of the radioactive dose in the urine during the 18-hr. collection period

[1] Ralston Purina Co., St. Louis, Mo.
[2] Cyclohexanesulfamic acid calcium salt, Matheson, Coleman & Bell, East Rutherford, N. J.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Abbott Laboratories, North Chicago, Ill.
[5] Obtained from the Wisconsin Alumni Research Foundation, Madison, Wis.
[6] TLC plates, silica gel (without fluorescent indicator), Brinkmann Instruments, Inc., Westbury, N. Y.
[7] TLC plates, cellulose F, Brinkmann Instruments, Inc., Westbury, N. Y.
[8] Nuclear Chicago Corp., Des Plaines, Ill.
[9] Cutter Laboratories, Berkeley, Calif.

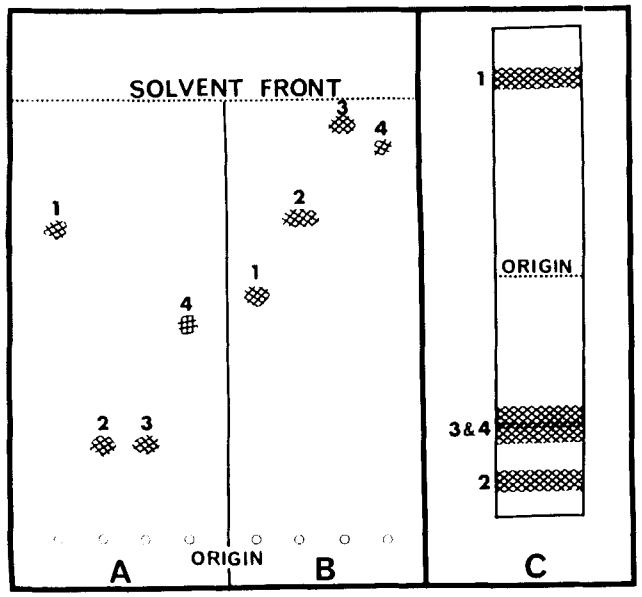

Figure 1—*Thin-layer and electrophoretic patterns of cyclamate and its metabolites. Key: A, silica gel plates developed in acetone–absolute ethanol (1:1); B, cellulose F plates developed in butanol–acetic acid–water (8:2:1); and C, paper electrophoresis in pyridine–acetic acid–water (8:15:977), run at 300 mv. The compounds were: 1, cyclamate; 2, cyclohexylamine; 3, dicyclohexylamine; and 4, N-hydroxycyclohexylamine.*

with a range of 22–58%, while treated rats excreted 18–100% during the same period. The wide range of radioactivity excreted in the urine of treated rats was probably related to the size of the cyclamate pool in each rat, *i.e.*, the larger the pool, the smaller the amount of radioactivity in the urine. The erratic excretion of cyclamate, varying from day to day, also tends to spread excretion values.

The distribution of the radioactivity excreted in urine was determined by eluting the areas on paper that corresponded to the authentic compounds after electrophoresis and counting the eluates. As shown in Table I, the radioactivity in the urine of control rats was detected mainly as unchanged ^{14}C-cyclamate (98.2%). The spot corresponding to cyclohexylamine contained 1.8% of the radioactivity; most of this amount (1.3%) could be attributed to a cyclohexylamine impurity in the original ^{14}C-cyclamate intubated in the rat. Seven of the 11 treated rats were converters. In the urine of these rats, cyclohexylamine accounted for an average of 18.4% of the radioactivity; the values ranged from 12 to 25%. Two of the seven rats that converted cyclamate to cyclohexylamine also had trace amounts of dicyclohexylamine present in the urine, but this compound accounted for less than 0.1% of the radioactivity excreted. The amount of dicyclohexylamine was too small to assay chemically but could be detected by the dichlorofluorescein spray and was verified by the differential TLC procedures. No *N*-hydroxycyclohexylamine could be detected by either electrophoresis or TLC.

Table I—Distribution of Radioactivity Excreted in 18-hr. Urine Collections of Control and Treated Rats Given ^{14}C-Cyclamate[a]

Group	Number of Rats	^{14}C-Cyclamate, c.p.m.	^{14}C-Cyclohexylamine, c.p.m.	^{14}C-Cyclohexylamine, %[b]
Control (chow)	12	3570 ± 469	61 ± 11	1.81 ± 0.23
Treated (chow + 0.1% calcium cyclamate)	7[c]	5240 ± 1294	1209 ± 377	18.36 ± 2.00

[a] Dosage was a known amount of ^{14}C-cyclamate containing 2–3 μmoles; specific activity 2.6 μc./μmole. Values are given as means ± *SE*. [b] Calculated as cyclohexylamine ÷ (cyclohexylamine + cyclamate) × 100. [c] Rats that converted cyclamate to cyclohexylamine.

Table II—Chemical Determination of Cyclamate and Cyclohexylamine in 18-hr. Urine Collections of Rats[a]

Group	Number of Rats	Cyclamate, μmoles	Cyclohexylamine, μmoles
Control (chow)	12	0.92 ± 0.08	0.02 ± 0.002
Treated (chow + 0.1% calcium cyclamate)	7[b]	16.82 ± 5.51	3.82 ± 1.63

[a] Values are given as means ± *SE*. [b] Rats that converted cyclamate to cyclohexylamine.

The concentrations of cyclamate and cyclohexylamine in the 18-hr. urine specimens, as determined by chemical methods, are shown in Table II. In the case of the treated rats, the specific activity of the intubated ^{14}C-cyclamate was lowered by the body pool of cold cyclamate and was variable because of different rates of cyclamate absorption and excretion; therefore, excretion of smaller amounts of radioactivity represented larger concentrations of both cyclamate and cyclohexylamine. The percent radioactivity excreted as cyclohexylamine was 10-fold higher for treated rats (Table I), while the actual amount of cyclohexylamine excreted was increased 190 times (Table II). The treated animals, intubated with 2–3 μmoles of ^{14}C-cyclamate, excreted 16.8 μmoles of cyclamate and 3.8 μmoles of cyclohexylamine, with the difference in amount being supplied by the pool.

Other investigators suggested the intestine as the site of conversion of cyclamate to cyclohexylamine (9, 11, 13). The differences in concentration of cyclohexylamine in urine and feces would indicate that cyclamate is converted to cyclohexylamine in the intestine, with most cyclohexylamine being excreted in the urine; some cyclohexylamine is not absorbed and is excreted in the feces. This type of distribution could also occur if the liver was the site of conversion. The cyclamate could be absorbed from the intestine, converted to cyclohexylamine in the liver, and excreted in urine *via* blood passage through the kidney and in feces *via* the bile. During the isolated perfusion of rat livers of known converters, no metabolites of cyclamate could be detected in blood, liver, or bile; unchanged ^{14}C-cyclamate was the single radioactive component detected in these tissues. Thus, the liver is probably eliminated as the site of conversion.

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Polymer–Drug Interaction: Stability of Aqueous Gels Containing Neomycin Sulfate

ALLEN HEYD

Abstract □ A method for preparing aqueous gels containing neomycin sulfate is described. Gels of synthetic polymers containing polyionic or polar reactive sites were prepared and stabilized by adsorbing the drug molecule onto a cationic-exchange resin prior to incorporation into the bases. The binding capacity of the drug for the polymers was determined, and the activity of the drug–resin was substantiated and compared relative to an inert gel containing the free drug.

Keyphrases □ Neomycin sulfate aqueous gels—preparation, polymer–drug interaction, viscosity and drug activity □ Polymer–drug interaction—neomycin sulfate gels

Neomycin, an epimeric drug molecule, has been shown to interact with numerous macromolecules of biological importance (1–6). Inhibitory activity of the antibiotic toward DNA-dependent RNA polymerase of *Escherichia coli* was shown (1), and formation of a DNA–neomycin complex was found to be a linear function of the DNA concentration (2). Gubernieva and Silaev (3), using heparin as a model, concluded that interaction with basic antibiotics was due to complex formation. They also demonstrated the complex-forming ability of polymyxin, neomycin, and albamycin with plasma proteins, albumin, and γ-globulin in man, rabbits, and rats (4).

Steiner *et al.* (5), using the property of antibiotic complexation, studied the effect of antibiotics on serum cholesterol levels when given orally. When injected, neomycin had no cholesterol-lowering effect. However, when taken orally, the serum cholesterol level was lowered; this suggests a local effect in the GI tract. Oral aureomycin and bonamycin had similar but lesser lowering effects; achromycin, bacitracin, chloromycetin, streptomycin, mycostatin, and penicillin failed to cause any significant lowering of serum cholesterol (6).

Polymeric antibiotics such as neomycin, viomycin, and polymyxin were studied for their interaction with condensed phosphates. The binding capacity of neomycin for these higher phosphates was the strongest (7).

Table I—Aqueous Gel Systems

Polymer	Concentration, %	pH	Gel–Resinate Viscosity, cps.
Methylcellulose 400 cps.[a]	5	7.00	36,850
Ethylene maleic anhydride	4	2.55	27,410
Carboxyvinyl polymer	1	3.58	42,120
Ethylene oxide copolymer	5	7.20	35,381
Heteropolysaccharide	2	6.78	31,560
Sodium carboxymethylcellulose	5	6.80	51,840

[a] Reference standard.

Many naturally occurring clays or earths of pharmaceutical importance in liquid or semisolid dosage forms are anionic polyelectrolytes in nature and, therefore, strongly bind medicaments cationic in nature. Physicochemical studies of antibiotic–clay complexes were carried out by Pinck *et al.* (8). They studied the chemical and physical properties of 10 antibiotics; their adsorption by montmorillonite, vermiculite, illite, and kaolinite; and the X-ray diffraction patterns of some of the formed complexes. Streptomycin sulfate, dihydrostreptomycin sulfate, and neomycin sulfate formed complexes to varying degrees with the four clays; X-ray diffraction studies of the complexes indicated monolayer adsorption for strongly basic and dilayer adsorption for amphoteric antibiotics. Danti and Guth (9) studied cation-saturated bentonites and showed that neomycin sulfate was inactivated as a result of an ionic incompatibility with this pharmaceutical agent. Nakashima and Miller (10) qualitatively demonstrated ionic incompatibilities of 18 suspending agents of pharmaceutical importance with several therapeutic agents. Neomycin sulfate showed an immediate precipitating or coagulating action with more than 50% of the agents studied.

A review of the literature, therefore, supports the proposal that neomycin has the greater binding capacity of the antibiotics in common use and presents a more

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A review of the literature, therefore, supports the proposal that neomycin has the greater binding capacity of the antibiotics in common use and presents a more

Table II—Viscosity Measurements for Polymer–Drug Interaction[a]

Polymer	Concentration, mg./ml.	Viscosity, cps. Polymer Solution	Polymer–Resinate	Polymer–Drug
Carboxyvinyl polymer	1.5	13.5	13.5	0.695
Heteropolysaccharide	0.5	3.13	3.13	0.704
Sodium carboxymethylcellulose	1.0	2.67	2.67	0.695
Methylcellulose 400 cps.	1.0	1.13	1.17	1.17
Ethylene oxide copolymer	0.5	0.956	0.956	0.956
Ethylene maleic anhydride	1.0	0.776	0.776	0.695

[a] Viscosity of water at 37° is 0.6947 cps.

serious problem when considering drug activity and the physical stability of gel vehicles.

Gels prepared with polyionic macromolecules can be used as vehicles for complex-forming drugs. This may be accomplished by adsorbing the drug onto a stronger complexing agent prior to incorporating it into the gel vehicle. The agent of choice might be ion-exchange resins, which have been used effectively as carriers for drugs. Baruffini (11) initially indicated their use. He utilized hydrated resins in ointments to control ionic variations, to absorb noxious skin secretions, and as carriers of medicinal substances. The resinates were studied in water-in-oil and oil-in-water emulsion vehicles. The resin (Amberlite IRC-50) was shown to give the most effective adsorption of neomycin; a prolonged and uniform release of the drug was also found. Fiedler and Sperandio (12) further studied the activity of ion exchangers in hydrophilic bases and suggested their use for controlled drug release in topical ointments (13).

The objectives of this investigation were, therefore, to: (*a*) study the stabilization of neomycin-containing gels prepared from a selected group of synthetic macromolecules, each possessing reactive sites; (*b*) determine the binding capacity of neomycin sulfate with the polymers; and (*c*) determine the relative activity of the resinate in the gel bases as compared to a free neomycin-containing gel base.

EXPERIMENTAL

Materials—The polymers used in this study were: (*a*) the free acid derivative of ethylene maleic anhydride[1]; (*b*) a carboxyvinyl polymer[2]; (*c*) an ethylene oxide copolymer[3]; (*d*) an anionic heteropolysaccharide[4]; and (*e*) sodium carboxymethylcellulose[5], a natural polymer chemically modified by carboxymethylation. Each polymer was of commercial grade and used as received from the suppliers.

The resin employed for adsorbing the antibiotic was a pharmaceutical grade sulfonated polystyrene-type cation-exchange resin[6]. The resin is a micropowder, has a particle-size specification of 95% through a 325-mesh screen, and is a very strong acid having an apparent pKa of 1.3.

Neomycin sulfate, commercial (pharmaceutical) grade, was used[7]. All other chemicals were of reagent grade.

Drug Assay and Microbial Activity Measurement—A spectrophotometric method of drug assay, developed by Dutcher *et al.* (14), was used in portions of this study. The method is based on the observation that neomycin, when heated with strong mineral acid, yields furfural as one of the decomposition products. The quantitative measurement of the furfural formed is made by measurement of the intensity of absorption at 280 nm.

The absorption densities of the standard and unknowns were determined in the Beckman DU[8] spectrophotometer with 40% sulfuric acid in the reference cell. The standard solutions were found to follow the Beer–Lambert law at concentrations of 25–500 mcg./ml.

The microbiological activity of the neomycin-containing gels was determined using the official cylinder-plate method, as described in USP XVIII (15), with several adaptations. The test organism was *Micrococcus pyogenes* var. *aureus* (ATCC 6538P) obtained commercially[9]. Nutrient agar was prepared with an artificial plasma electrolyte solution as described by Fiedler and Sperandio (13). This provided a simulated system for intact skin and allowed an electrolyte exchange with the resinate in the aqueous gels and subsequent release of drug.

Gels of the synthetic polymers described were prepared in the concentrations shown in Table I. A gel of methylcellulose 400 cps., which by preliminary experimentation did not show any interaction with neomycin sulfate, was prepared as a reference standard to determine the relative activity of the resinate-containing gels. The six gels were freshly prepared. Neomycin sulfate and drug–resin equivalent to 1% neomycin were incorporated into separate portions of the methylcellulose gel. Drug–resin was also incorporated into the remaining gels in an amount equivalent to 1% neomycin.

Samples of standard and unknown containing free neomycin sulfate and drug resinate, respectively, were transferred asceptically with 10-ml. sterile syringes for filling the stainless steel cylinders. The plates were incubated for 20 hr. at 37°, and the diameters of the zones of inhibition were measured.

Resin–Drug Preparation—Neomycin was adsorbed onto the cation-exchange resin by agitating a solution of the drug containing an equivalent of 800 mg. base wth 1 g. of resin. The resin and drug mixture was placed in 4-oz. ointment jars which were tightly sealed with plastic tape. The jars were then agitated in a rotating-bottle-type water bath at 37°. Studies showed that 1 g. of the resin was capable of adsorbing 790 mg. of neomycin base after an equilibration period of 24 hr. under the conditions described.

Polymer–Drug Interaction Studies—Fresh aqueous gels were prepared with each of the synthetic polymers. Initial qualitative tests were carried out with 50-g. samples of these gels by adding 300 mg. of neomycin sulfate and making observations for physical gel breakdown.

The degree of polymer–drug interaction was then determined quantitatively by preparing a 100-mg. solution of each polymer, adding to this a solution containing 250 mg. of neomycin sulfate[10], and assaying for remaining drug. The resulting precipitates were centrifuged, the supernatant was decanted, and the precipitates were washed. The washings were mixed with each respective decanted solution, and then each solution was assayed spectrophotometrically for loss of neomycin.

[1] EMA-71, Monsanto Co., St. Louis, Mo.
[2] Carbopol 934, B. F. Goodrich Chemical Co., Cleveland, Ohio.
[3] Polyox, Union Carbide Chemicals Co., New York, N. Y.
[4] Biopolymer XB-23, General Mills, Inc., Minneapolis, Minn.
[5] Cellulose Gum, Hercules Powder Co., Wilmington, Del.
[6] Amberlite IRP-69M, Rohm & Haas Co., Philadelphia, Pa.
[7] Supplied by The Upjohn Co.
[8] Beckman Instruments, Fullerton, Calif.
[9] American Type Culture Collection, Rockville, Md.
[10] An arbitrary amount chosen but in excess, as substantiated in the results.

Table III—Relative Activity of Neomycin Ointments

Polymer–Resinate	Inhibition Diameter, mm.
Ethylene maleic anhydride	15
Methylcellulose 400 cps.	24
Carboxyvinyl polymer	25
Sodium carboxymethylcellulose	28
Heteropolysaccharide	30
Ethylene oxide copolymer	35
Methylcellulose-free drug[a]	50

[a] Reference standard.

Gel Stability Studies—Six gels (Table I) were freshly prepared, and drug–resin was incorporated into each in an amount equal to 1% neomycin base. Immediately after preparation, the viscosity was taken of each gel–resinate at 27° using a Haake Roto-Visco rotating bob viscometer. Twenty-five grams of gel was used for each measurement. The gels were stored in tight containers at 27°, and their viscosity was measured weekly for a period of 6 weeks to determine physical stability. Fresh solutions of each polymer were prepared in concentrations shown in Table II. Resinate equivalent to 1% neomycin was added to 100 ml., and 1% of neomycin sulfate was added to another 100 ml. of each solution. Viscosity measurements were taken of the pure polymer, the resinate–polymer, and the drug–polymer at 37° in an Ostwald–Fenske viscometer, using 20 ml. of test solution. Distilled water was used as the reference standard. Observations for change in viscosity were made.

RESULTS AND DISCUSSION

Polymer–Drug Interaction Studies—The general reaction observed upon incorporating the drug into the gels was immediate loss of structure and formation of stringy, opaque masses which were insoluble except for the interaction with ethylene oxide copolymer. In this case, structure breakdown was evidenced by a decrease in viscosity from a gel to a free-flowing liquid. Initial qualitative tests showed that neomycin sulfate in 300-mg. amounts was sufficient to cause complete breakdown of 50 g. of gel.

Assay of neomycin remaining in the supernatant after having interacted an excess with 100 mg. of polymer indicated that 128.5, 102.5, 66.5, and 55 mg. of drug had interacted with 1 g. of carboxyvinyl polymer, sodium carboxymethylcellulose, ethylene maleic anhydride, and heteropolysaccharide, respectively. The amount interacting with ethylene oxide copolymer was indeterminable because no precipitate was formed.

Gel Stability Studies—The initial viscosity found for each freshly prepared gel–resinate (Table I) did not appreciably change over the 6-week period of observation, therefore suggesting no interaction between polymeric gel and drug. On the other hand, the viscosities determined for the polymer solutions (Table II) showed that for those polymers forming insoluble interaction systems, there was complete precipitation of polymer–drug from solution. The viscosities of the supernatent liquids were the same as that of the standard, distilled water. Ethylene oxide copolymer in this low concentration and methylcellulose showed no change in viscosity, indicating no drug interaction. The drug–resinate system was successful in stabilizing each polymer system that was sensitive to interaction with neomycin, as evidenced by no change in the viscosities of the polymer solutions.

Drug Activity—Results of the bacteriological studies indicated that neomycin was available for activity from the cation-exchange resin. The average diameters of the zones of inhibition (Table III) indicate a varying degree of drug availability from the ointment and/or resinate. Methylcellulose, which has no reactive functional groups, released the free drug to the largest extent. The release of drug in the methylcellulose–resinate mixture was approximately half that of the free drug, indicating the general effect that could be expected in the remaining group of gels which showed an activity of approximately one-third to three-quarters that of the standard gel, methylcellulose. Ethylene oxide copolymer, which indicated little or no interaction with neomycin, showed the second highest activity over that of the polymers containing active polyfunctional groups.

The viscosity of the gels apparently had little or no effect on drug diffusion to the surface of the simulated skin. The ethylene maleic anhydride–resinate system was the least viscous and also showed the lowest activity. However, the pH of the gel systems may have had an effect on neomycin activity. Neomycin shows optimum activity at about pH 7.0; therefore, one would expect the ethylene maleic anhydride gel (pH 2.65) to show the least activity and ethylene oxide copolymer (pH 7.2) to show the greatest activity. Drug activity appeared to be related to the binding capacity of the polymers. Sodium carboxymethylcellulose and carboxyvinyl polymer interacted with the largest amount of drug, as indicated here, but showed a smaller inhibition diameter (Table III) than the heteropolysaccharide which interacted with about half as much drug.

This study shows that a much lower amount of antibiotic than is necessary to interact ultimately with all the reactive portions of the polymer molecules is sufficient to break down the gel structure. Breaking of gel structure is, therefore, not dependent upon the degree of polymer–drug interaction. This was evidenced by 6 mg. of neomycin sulfate being capable of completely precipitating 1 g. of each polymer studied, whereas 55–128.5 mg. of drug was found to have interacted with each polymer.

A cation-exchange resin with a low pKa is readily capable of forming a complex with neomycin strong enough to prevent further interaction with polyfunctional polymers. The resin, therefore, serves to stabilize aqueous gels until they are applied topically. Recognizing the general principles of ion exchange, one could suggest that the resin exchanges the drug moiety for the electrolytes present in the aqueous portion of the skin or the exudate of open wounds.

Aqueous gel systems, which are becoming increasingly more popular in use, are not limited to use with nonreactive drugs whenever an ion-exchange resin can be used to stabilize them. In addition, this affords the formulator a greater number of possible materials to choose from in developing efficacious semisolid dosage forms.

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Determination of Phenothiazine Compounds in Biologic Specimens by UV Spectrophotometry

JACK E. WALLACE and JOHN D. BIGGS

Abstract □ A UV spectrophotometric technique for specific determination of drugs that have the phenothiazine group as part of their molecular structure was developed. The method utilizes the oxidation capability of the cobalt (III) ion. The phenothiazines and their sulfoxide metabolites are oxidized to a product that is stable in a hexane–tertiary butanol mixture. No other group of alkaline-extractable compounds gives a UV-absorbing product by the conditions of the proposed method. The procedure is sensitive, and the spectra of the products are highly specific for the phenothiazine drugs. Analysis is linear over the concentration range of 0.5–50.0 mcg./ml. The distribution pattern of certain phenothiazine drugs in the rat is presented.

Keyphrases □ Phenothiazines, cobalt (III) oxidation products—UV spectrophotometric determination □ UV spectrophotometry—determination, phenothiazines in blood, urine, tissues □ TLC—identification, phenothiazines

Phenothiazine-type drugs have a high sensitivity for the absorption of UV radiations in the 250–260-nm. region of the spectrum. Numerous other alkaline-extractable drugs, as well as certain normal tissue components, also absorb radiations in this area with high efficiency. Therefore, direct UV spectrophotometric methods for the determination of phenothiazine drugs have little or no specificity (1–3). These drugs are often metabolized to their corresponding sulfoxide derivatives. Such metabolites have a high molar absorptivity for UV radiations but at wavelength maxima different from that of unchanged phenothiazine.

The sulfoxides were used by several investigators to establish, either by colorimetric or UV spectrophotometric methods, the presence of phenothiazine-type compounds in urine (1, 4, 5). Unless an elaborate separation of the phenothiazines from their metabolites is accomplished, the methods have limited application to quantitative analysis of the unchanged drugs. For specimens other than urine, identification of the sulfoxide as a mechanism to determine specifically the presence of phenothiazine compounds is unrewarding.

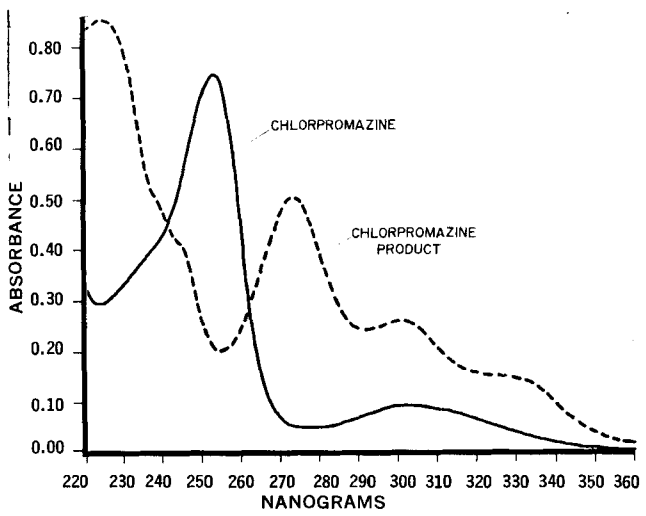

Figure 1—*UV absorption spectra of chlorpromazine in 0.5 N HCl (7.5 mcg./ml.) and of the chlorpromazine product in hexane containing 20% tertiary butanol (10.0 mcg./ml.).*

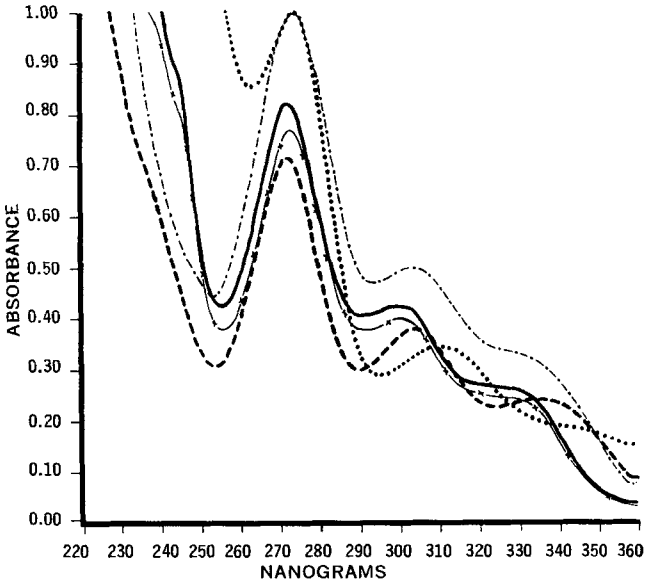

Figure 2—*UV absorption spectra of the prochlorperazine product (——), perphenazine product (X-X), trimeprazine product (-··-), propiomazine product (. . .), and the trifluoperazine product (- - -), all in hexane–tertiary butanol mixture, each corresponding to a phenothiazine concentration of 20 mcg./ml.*

The sulfoxide metabolites have excellent fluorescent characteristics, and several spectrophotofluorometric methods utilizing that property were described (6–9). The drug and the primary sulfoxide metabolite can be extracted simultaneously, the former oxidized by agents such as hydrogen peroxide, and the total phenothiazine content of the specimen measured spectrophotofluorometrically. Such procedures have proven to be useful and have improved the sensitivity for analysis of these compounds. However, only approximate identity for phenothiazine compounds can be established by that technique. In addition, previous reactions for the oxidation of phenothiazines to their corresponding sulfoxides are time and temperature dependent and nonstoichiometric. In a recent report, Kaul *et al.* (10) described a fluorometric method for determining chlorpromazine and its metabolites at the nanogram level in plasma and urine. This method, which utilizes a reaction of the phenothiazine with 5-dimethylaminonaphthylene-1-sulfonyl chloride, is apparently not applicable to the analysis of tissues or phenothiazines other than chlorpromazine.

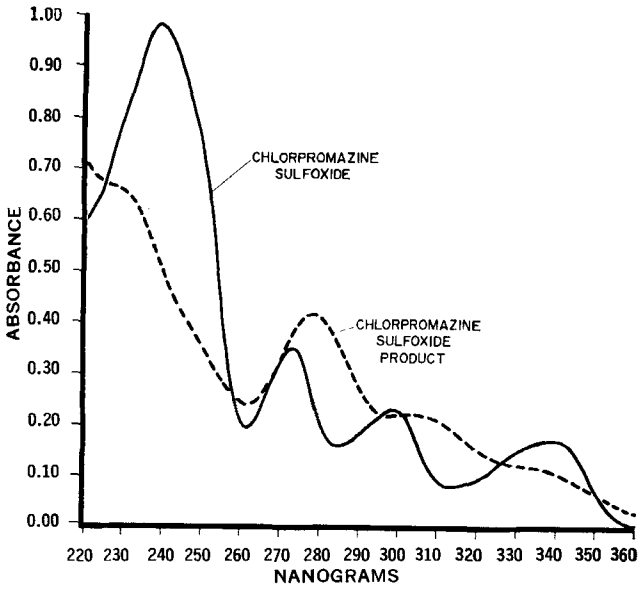

Figure 3—*UV absorption spectra of chlorpromazine sulfoxide in 0.5 N HCl and of the chlorpromazine sulfoxide product in hexane–tertiary butanol mixture, both corresponding to a chlorpromazine sulfoxide concentration of 10 mcg./ml.*

Methods for specific determination of amitriptyline[1] and imipramine[2], two drugs that have molecular structures closely related to that of the phenothiazine compounds, were published (11, 12). In extracts from biologic samples, the three pharmacologic agents are difficult to analyze by direct UV spectrophotometric scanning. In an intoxication involving more than one of the compounds, specific quantitative analysis in the past has been extremely complex. Prior to the series of investigations initiated at this laboratory, elaborate GLC techniques had to be employed for quantitative analysis of biologic extracts containing two or more of the compounds. Such an analysis is limited since many of the phenothiazines and, especially, sulfoxides are not easily made volatile (3, 13). In addition, a certain degree of specificity is lacking in GLC techniques. TLC analysis of urine samples can be misleading because of the variable chromogenic nature of the major metabolites of the phenothiazine that may appear on the chromatographic plate. In practice, phenothiazine substances are converted into sulfoxides during solvent-induced migration on the chromatographic plate (13). This method has been used primarily for identification of the drugs in dosage forms and has little applicability to the analysis of biologic specimens.

The method proposed in this report is a specific and quantitative UV spectrophotometric technique for determining phenothiazine compounds in blood, urine, and tissues. The drugs are oxidized to sulfone or similar type derivatives whose spectra are different from that of the original compounds. No other drugs have been observed to interfere. Each phenothiazine and its sulfoxide metabolite give the desired product in reproducible amounts. Quantitative results are obtained if the type of phenothiazine is known. Rapid qualitative identification is best achieved by TLC (14).

[1] Elavil.
[2] Tofranil.

Table I—Standard Curve Data of Phenothiazine Reaction Products

Compound	$\lambda_{max.}$, nm.	Concentration, mcg./ml.	Absorbance[a]	Absorbance/Concentration
Chlorpromazine	273	5	0.24	0.048
		10	0.48	0.048
		15	0.72	0.048
		20	0.94	0.047
Perphenazine	272	5	0.20	0.040
		10	0.39	0.040
		15	0.61	0.041
		20	0.77	0.039
Prochlorperazine	272	5	0.21	0.042
		10	0.43	0.043
		15	0.63	0.042
		20	0.82	0.041
Propiomazine	273	5	0.26	0.052
		10	0.52	0.052
		15	0.76	0.051
		20	1.00	0.050
Pyrathiazine	273	5	0.15	0.030
		10	0.29	0.029
		15	0.44	0.029
		20	0.56	0.028
Thioridazine	276	5	0.15	0.030
		10	0.28	0.028
		15	0.43	0.029
		20	0.54	0.027
Trifluoperazine	272	5	0.19	0.038
		10	0.37	0.037
		15	0.60	0.040
		20	0.71	0.036
Trimeprazine	273	5	0.27	0.054
		10	0.51	0.051
		15	0.75	0.050
		20	0.98	0.049

[a] In hexane–tertiary butanol.

EXPERIMENTAL

Instrumentation—UV absorption data were obtained with a Beckman DK-2A ratio-recording spectrophotometer with linear presentation of the wavelength. A Beckman IR-9 spectrophotometer was used for IR absorption measurements.

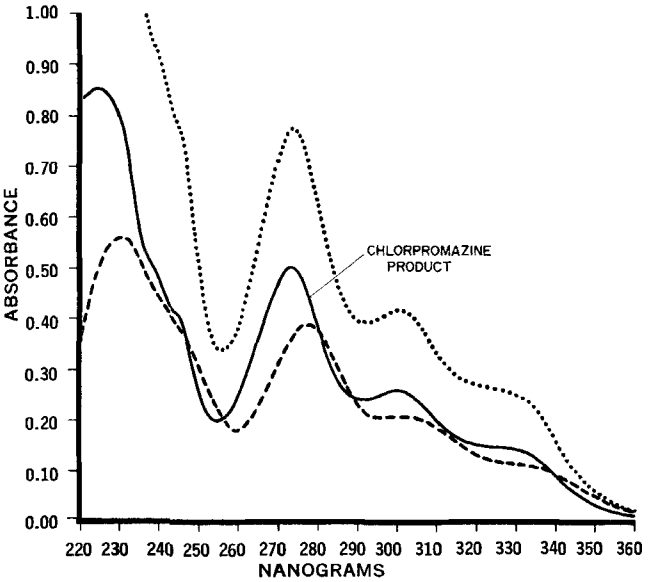

Figure 4—*UV absorption spectra of the chlorpromazine product, 10 mcg./ml., and of the chlorpromazine product from oxidation of drug isolated from rat liver (. . .) and rat urine (- - -), each in hexane–tertiary butanol mixture.*

Table II—Drugs Found Not to Interfere[a] with the Cobalt (III) Oxidation Method for Determining Phenothiazine Compounds[b]

Amitriptyline	Imipramine	Phenethylamine
Amphetamine	Isoniazid	Phenelzine
Atropine	Lidocaine	Phenindamine
Azacyclonol	Meclizine	Phenmetrazine
Benzphetamine	Mepenzolate	Phenylpropanolamine
Brucine	Meperidine	Phenyltoloxamine
Captodiamine	Meprobamate	Phenyramidol
Carphenazine	Mescaline	Picrotoxin
Carbinoxamine	Methadone	Pilocarpine
Chlophedianol	Methapyrilene	Pipenzolate
Chlorcyclizine	Methoxamine	Procaine
Chlordiazepoxide	Methoxyphenamine	Procyclidine
Chlorpheniramine	4-Methyl-2,5-di-methoxy-amphetamine	Propoxyphene
Chlorphentermine		Pyrrobutamine
Clemizole		Quinidine
Cyclizine	Methylphenidate	Reserpine
Dextromethorphan	Morphine	Strychnine
Diethylpropion	Nicotine	Sulfadiazine
Diphenhydramine	Orphenadrine	Thenyldiamine
Diphenylpyraline	Papaverine	Thonzylamine
Doxylamine	Pargyline	Trichlormethiazide
Ephedrine	Phenacemide	Trimethadione
Epinephrine	Phenacetin	Tripelennamine
Ethoheptazine	Phenaglycodol	Triprolidine
Hydroxyzine	Phendimetrazine	Yohimbine

[a] Optical density less than 0.01 for 10 mcg./ml. of each drug in solvent mixture of 20% tertiary butanol in hexane. [b] Absorbance measured at 273–275 nm.

Method—Five- to ten-milliliter volumes of blood, serum, or urine are placed in a separator and made strongly alkaline (pH 10.0) by the addition of 6 *N* NaOH. For tissue analysis, 5–10-g. amounts are homogenized and 10 *N* NaOH is added to achieve a normality of 2.0 (8). The tissue mixture is hydrolyzed in a boiling water bath for 5 min., removed, and allowed to cool in an ice bath. At this point, all specimens are extracted by vigorous shaking for 3 min. with 50–200 ml. of a solvent system of *n*-heptane (ACS grade) containing 4% tertiary butanol[3]. The aqueous phase is removed and discarded, after which the organic solvent is filtered through a fast-flowing filter paper and washed with a 0.5 *N* NaOH solution. Complete recovery of the heptane–alcohol mixture is not necessary, but the recovered volume is recorded and the solvent loss is included in the final calculations.

Ten milliliters of 2.5 *N* hydrochloric acid solution is added to the recovered solvent phase, and the mixture is shaken vigorously in a separator for 3 min. After phase separation, 9 ml. of the aqueous acid layer is transferred to a 250-ml. round-bottom flask with a ground-glass neck for attachment to a water-cooled reflux condenser. To the flask is also added a magnetic Teflon-coated stirring bar, 10 ml. of an 80:20 mixture of spectroquality *n*-hexane[4] and tertiary butanol (specifications as listed previously), and 250 mg. of cobalt (III) oxide, Co_2O_3[5]. The mixture is refluxed by utilization of a heating mantle[6] with 40 v. applied. Constant stirring of the flask contents is maintained by means of a magnetic mixer.

After 30 min. of refluxing, the reaction flask is cooled to room temperature and the solvent layer is removed. The latter is scanned in a recording UV spectrophotometer between the wavelengths of 360 and 220 nm. Two or more maxima are identified if the drug product concentration in the final solution is equivalent to at least 2.5 mcg./ml. of phenothiazine. To enhance sensitivity further, the hexane–tertiary butanol mixture can be concentrated to 0.5–1.0 ml., utilizing a rotary vacuum evaporator, and the absorption determined in a microcell.

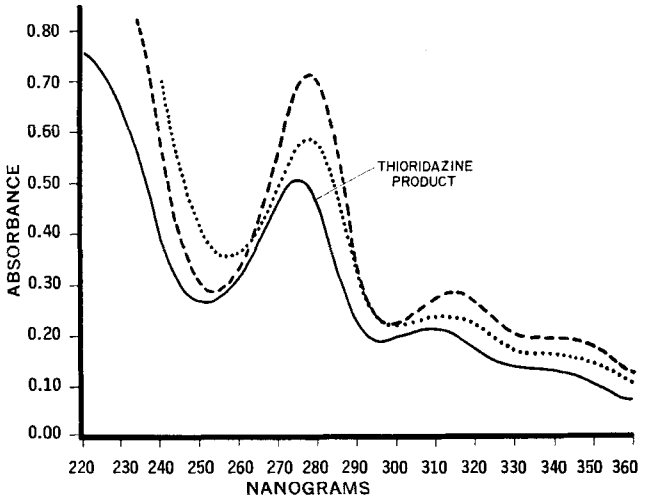

Figure 5—*UV absorption spectra of thioridazine product, 18 mcg./ml., and of the thioridazine product from oxidation of drug isolated from human liver (. . .) and human lung (- - -), each in hexane–tertiary butanol mixture; liver level, 21 mcg./g.; lung level, 23 mcg./g.*

[3] Baker Analyzed Reagent, No. 9056 or equivalent.

Table III—Recovery of Chlorpromazine after *In Vitro* Addition to Serum, Urine, and Homogenized Liver

Amount Added, mcg./ml. (g.)	Recovery, Mean ± Standard Deviation (mcg./ml.)[a] Serum	Urine	Homogenized Liver
50	—[b]	49.0 ± 0.9	48.9 ± 0.8
25	24.7 ± 0.7	24.5 ± 0.6	24.7 ± 0.5
10	9.4 ± 0.5	9.3 ± 0.4	9.7 ± 0.4
5	4.8 ± 0.3	4.5 ± 0.2	—[b]
2.5[c]	2.4 ± 0.2	2.4 ± 0.3	2.4 ± 0.2
1.0[c]	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
0.5[c]	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.05
Average recovery	94.1%	93.6%	95.8%

[a] Six determinations for each mean value. [b] Not determined. [c] Tertiary butanol–hexane mixture concentrated.

Table IV—Distibution of Phenothiazine Compounds[a] in the Rat

Tissue[b,c]	Chlorpromazine	Prochlorperazine	Propiomazine	Thioridazine	Trifluoperazine
	mcg./ml. or g.				
Blood	1.23	0.86	0.20	0.84	0.77
Brain	8.85	4.58	1.64	6.50	8.27
Fat	4.24	3.47	5.00	4.40	4.10
Kidney	37.83	17.21	10.65	20.17	16.56
Liver	58.48	19.08	14.71	26.43	27.60
Lung	85.45	43.93	29.20	52.64	50.81
Muscle	4.54	2.79	2.51	1.48	3.05

[a] Each phenothiazine was administered in oral doses at 25 mg./kg. body weight. [b] Animals sacrificed after 4 hr. [c] For each drug, the results are based on analysis of specimens from the same rat.

RESULTS

Phenothiazine compounds, after oxidation with cobalt (III) in dilute hydrochloric acid, give reproducible amounts of a product with unique UV absorption spectra. Figure 1 illustrates the spectrum of the product from chlorpromazine. Spectra of the oxidation products obtained from several phenothiazine compounds (Fig. 2) have only minor differences. Standard curve data for eight frequently utilized phenothiazines are shown in Table I. The sulfoxides are also oxidized to similar products, as demonstrated by the oxidation of the sulfoxide of chlorpromazine (Fig. 3). Phenothiazines oxidized after extraction from the tissues and urine of animals that have received the drug yield spectra nearly identical to those observed for the product from the pure drug (Figs. 4 and 5).

IR spectra of chlorpromazine, chlorpromazine sulfoxide, and the chlorpromazine product show several distinct differences (Figs. 6–8). Most significant are the IR absorption changes in the 1000–1200-

[4] Fisher Scientific Co., No. H-334 or equivalent.
[5] Alfa Inorganics, Ventron Division, Beverly, Mass., No. 23129.
[6] Glas-Col Apparatus Co., Terre Haute, Ind.

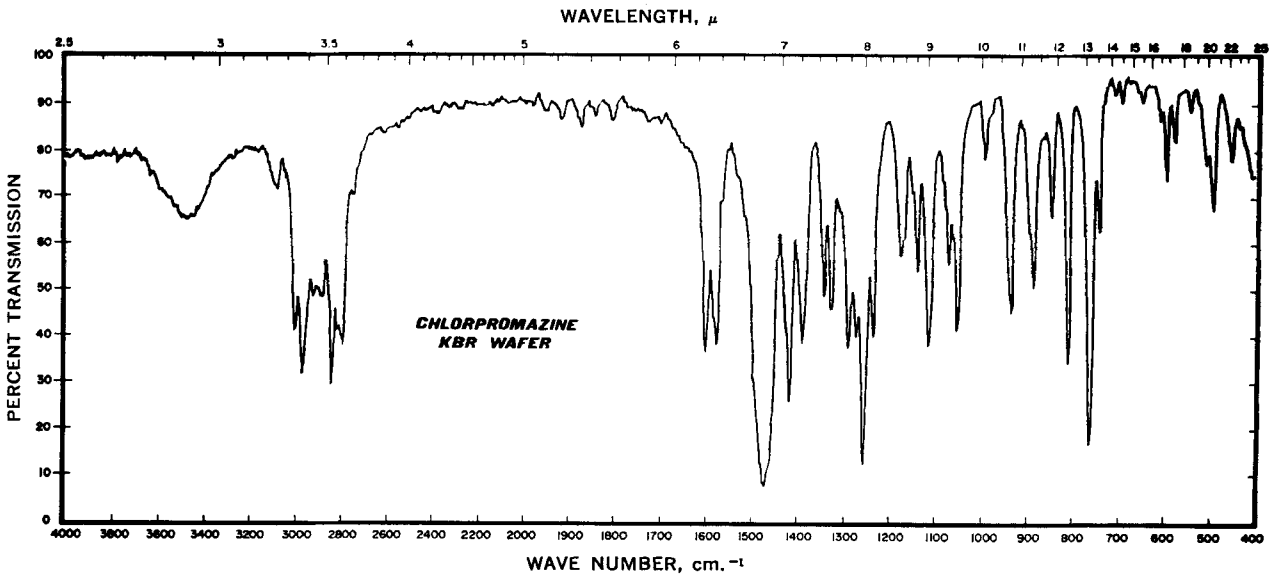

Figure 6—*IR spectrum of chlorpromazine, 2 mg. in 400 mg. potassium bromide.*

cm.$^{-1}$ region. The spectra for the sulfoxide show the characteristic strong absorption for that functional group at 1040–1050 cm.$^{-1}$ (15); yet in the cobalt oxidation product, a strong band at 1130–1150 cm.$^{-1}$ (15) characteristic of sulfone absorption is observed.

Many nitrogen-containing organic bases were investigated for possible interference with qualitative or quantitative determination of phenothiazines by the proposed method. After cobalt oxidation, the compounds listed in Table II provided no significant absorption (less than 0.02 optical density) within the wavelength range of 240–360 nm.

The recovery of chlorpromazine after *in vitro* addition to whole blood, urine, and homogenized liver is summarized in Table III. Freshly made standard aqueous solutions of chlorpromazine hydrochloride were added to the biologic material to provide concentrations of 5, 10, 25, and 50 mcg./ml. (g.). The mixtures were allowed to incubate at room temperature for 30 min. prior to analysis. Ten milliliters of blood, 20 ml. of urine, or 10 g. of homogenized liver with added drug was carried through the procedure as described previously. Recoveries greater than 92% were obtained.

The distribution of the drugs among the various tissues of the rat, 4 hr. after the animals received orally 25 mg./kg. of body weight, is shown in Table IV. The levels of the drugs in the tissues, in decreasing order, were: lung, liver, kidney, brain, fat, muscle, and blood. The procedure of this report gives, after oral ingestion, distribution ratios in agreement with those previously published (16) for intramuscular injection of the drug. With the exception of the brain, the findings are in agreement also with levels observed by Salzman and Brodie (1) in dog tissues after intravenous injection of chlorpromazine.

DISCUSSION

Cobalt (III) oxidation permits rapid identification of phenothiazine drugs, even in a biologic extract containing a mixture of several pharmacologic compounds. It affords a useful UV spectrophotometric technique for measuring phenothiazines as characteristic oxidation products. In certain instances, some indication of the type of phenothiazine can be elucidated. With each drug the product can be distinguished from the unoxidized phenothiazine. The efficiency of the products for absorbing UV radiations is slightly less than that of the original phenothiazines, yet the specificity of the spectrophotometric analysis is greatly enhanced. Concentration of the hexane–tertiary butanol mixture permits the method to approach a sensitivity limit of 0.25 mcg./ml. (g.) if 10 ml. of urine, bile, or blood or 10 g. of tissue are utilized (Table III). Extracts from urine or partially decomposed tissues containing considerable amounts of compounds absorbing in the 250–270-nm. region can be analyzed with no difficulty. Systems that utilize hydrogen peroxide oxidation for fluorometric analysis are more sensitive, but considerable interference due to Raman scattering (7) is encountered when low con-

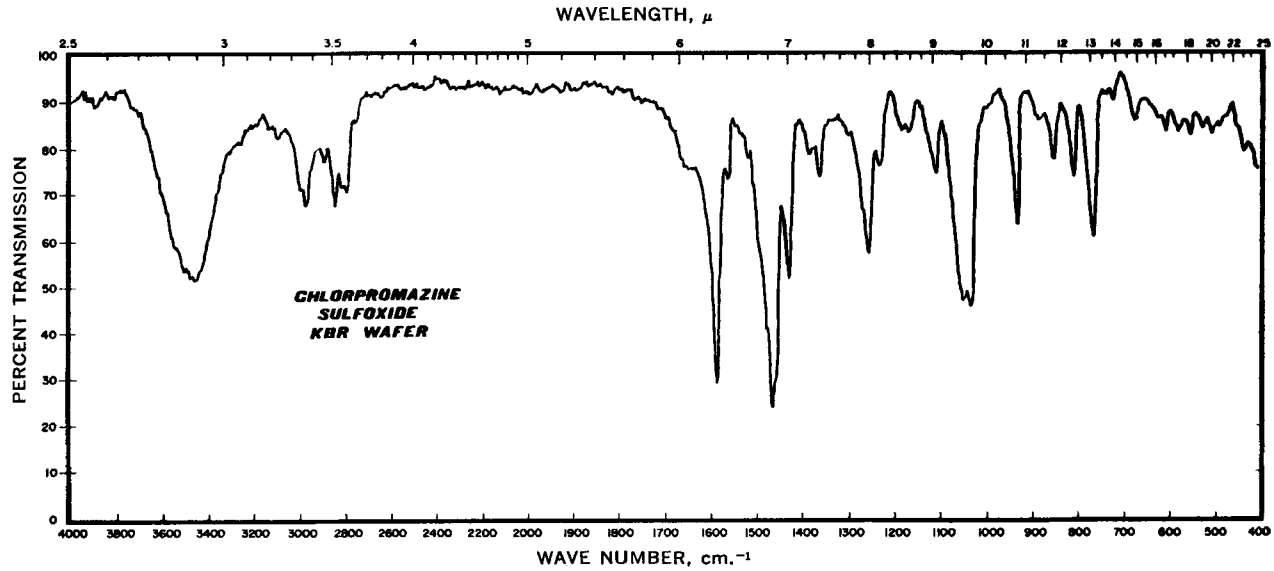

Figure 7—*IR spectrum of chlorpromazine sulfoxide, 1 mg. in 400 mg. potassium bromide.*

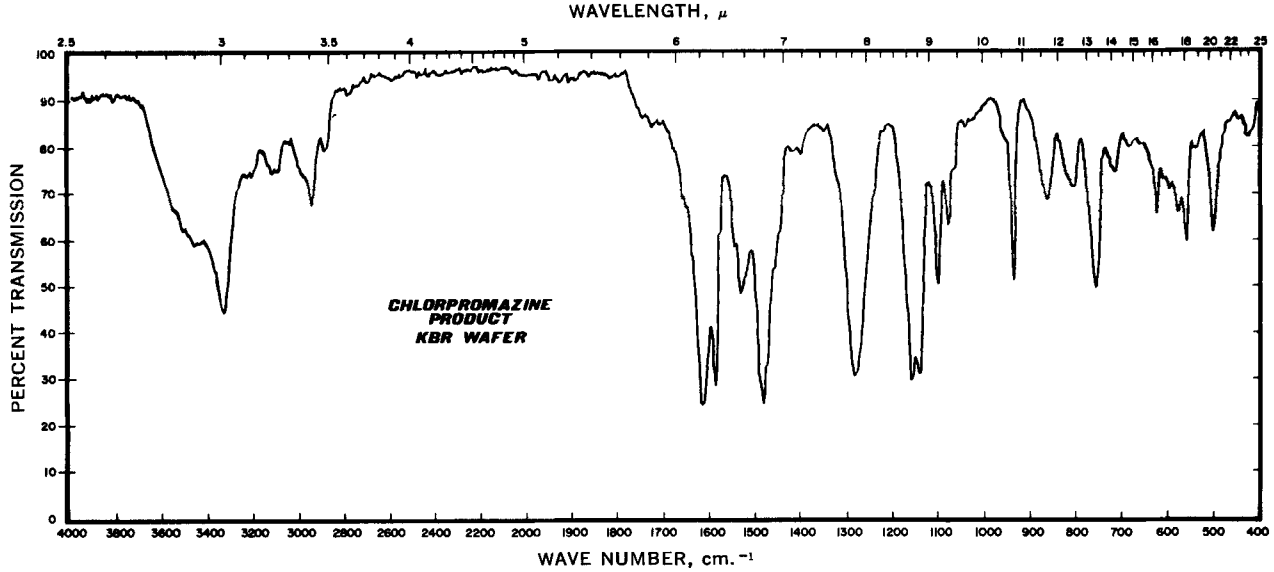

Figure 8—*IR spectrum of chlorpromazine oxidation product, 1 mg. in 400 mg. potassium bromide.*

centrations of the drugs are determined. This type of interference is detrimental in the determination of certain 2-position-substituted phenothiazines which have emission maxima near 380 nm. and excitation maxima in the range of 350 nm. (17). The close proximity of the two maxima can be separated by lowering the excitation wavelength but this significantly decreases sensitivity. The fluorescent characteristics of the products from cobalt (III) oxidation of the phenothiazines will be presented in a subsequent report.

In contrast to phenothiazine compounds and their sulfoxide derivatives, it has been impossible to obtain GLC data on the cobalt (III) oxidation products, using OV-1 or OV-17 liquid phases at temperatures up to 340°. This is apparently the result of the low volatility and high polarity of the sulfone compounds.

Since concentrations of the drug are much higher in urine than in blood, the former is routinely used for toxicologic analysis. With the proposed technique, the analyst is not hindered by variable amounts of sulfoxide derivative in the urine. A high order of specificity is provided by the inability of other alkaline-extractable drugs to form UV-absorbing derivatives after oxidation with the cobalt system (Table II).

IR data suggest the oxidized products to be sulfone derivatives. Their exact chemical structures are not known, but this information is not essential for the effectiveness of the procedure. The structural elucidation of the products and the cobalt (III) reaction mechanism will be the subjects of a subsequent report.

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Viscoelastic Properties of Pharmaceutical Semisolids III: Nondestructive Oscillatory Testing

STANLEY S. DAVIS

Abstract □ Semisolid ointments and creams were examined by nondestructive oscillatory methods, using the Weissenberg rheogoniometer and a digital transfer function analyser. Fundamental rheological parameters (η', G', and G'') were measured over the frequency range from 2.5×10^{-4} to 25 Hz. The variation with frequency of the loss tangent (a ratio of viscous to elastic contributions) provides a useful "consistency spectrum" for characterization of pharmaceutical products. The shapes of spectra and the magnitudes of the loss tangent are discussed with relation to seven ointments and four creams. A comparison of oscillatory and transient (creep) methods is also made.

Keyphrases □ Ointments, creams, semisolid—viscoelastic properties □ Creams, ointments, semisolid—viscoelastic properties □ Viscoelasticity, nondestructive oscillatory testing—semisolid formulations □ Rheological parameters, oscillatory method—semisolid systems □ Oscillatory testing, nondestructive—semisolid rheological parameters

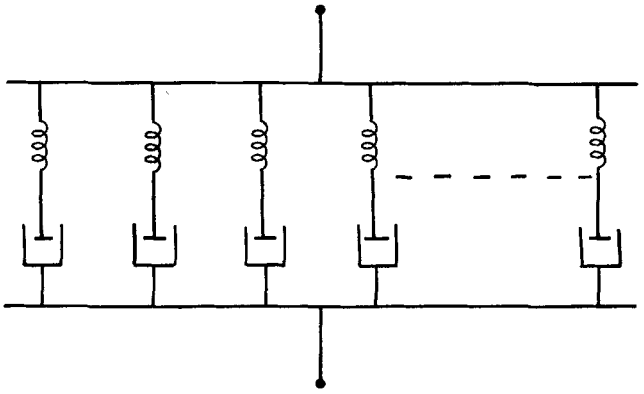

Figure 1—*Generalized Maxwell model representing behavior of a viscoelastic material.*

It is becoming increasingly important to be able to characterize the "consistency" of semisolid gels, ointments, and creams in a meaningful fashion. Rheological parameters are now required in various pharmaceutical areas, including: (*a*) quality control of semisolid products; (*b*) storage stability of semisolid products; (*c*) correlation of physical parameters with sensory assessment and consumer evaluation; (*d*) effects of consistency on the percutaneous absorption of drugs; (*e*) effects of formulation on consistency (*e.g.*, action of self-bodying agents); (*f*) prediction of flow behavior under the shear conditions met in manufacture (*e.g.*, pumping, milling, and packaging); (*g*) effects of mucolytic agents on properties of mucoid body fluids; and (*h*) effects of radiation sterilization on the physical properties of topical products.

Moreover, in a recent review, Boylan (1) considered that rheological characterization is a must when developing semisolid dosage formulations.

The important question that one must answer is: "What are suitable rheological parameters?"

For many years, the usual approach to the rheological testing of pharmaceutical semisolids has been some form of continuous shear viscometry or penetrometry. Unfortunately, such methods have a number of basic disadvantages and limitations (2, 3), and derived consistency values have been of limited application. In parts I and II (4, 5), it was shown that creep testing, along with the subsequent derivation of fundamental viscoelastic parameters, provides a suitable method for the rheological evaluation of semisolids. However, to obtain complete information about viscoelastic behavior and the molecular properties it reflects, it is often necessary to make measurements over a wide range of the time scale (6). Transient methods, such as creep, are limited at short times by inertial effects and by the impossibility of having an instantaneous application of stress at the beginning of the experiment. Creep tests can be supplemented by oscillatory (dynamic) tests to provide information at short times, since a periodic experiment of frequency ω (rad. sec.$^{-1}$) ($\omega = 2\pi n, n$ = frequency in hertz) is qualitatively equivalent to a creep experiment of time $t = 1/\omega$ (6). Therefore, the semisolid systems of parts I and II (4, 5) were studied using oscillatory methods and the theory of linear viscoelasticity.

It is possible in many cases to derive full dynamic data from creep experiments by using suitable formulas (4, 7). Therefore, the methods of interconversion and their limitations for pharmaceutical systems were examined.

THEORY

The behavior of a semisolid material under oscillation can best be considered in terms of a mechanical model made up from a combination of springs (elastic elements) and dashpots (viscous elements) (Fig. 1). At high frequency, the springs can elongate and contract under imposed shear but the dashpots have very little time in which to move. The system, therefore, behaves essentially as an elastic solid of modulus G. At low frequency, the springs also can extend, but in this case the dashpots have ample time to move and their extension greatly exceeds that for the springs. The system, therefore, behaves essentially as a viscous fluid of viscosity η. The interrelationship between G and η is the relaxation time, τ ($= \eta/G$). If $1/\omega \gg \tau$, the dashpots can keep up with the oscillation, and energy stored in each cycle is almost completely dissipated. But if $1/\omega \ll \tau$, almost no energy is dissipated and solid behavior dominates. When $1/\omega \cong \tau$, both G and η have definite contributions; one is then dealing with the so-called viscoelastic region.

Any experimental system can be considered to be made up from an infinite number of such springs and dashpots. Nonetheless, the manner in which G and η change with frequency is often highly characteristic and can be correlated with definite structures or molecular entities (6, 8).

The equation of state, for a linear viscoelastic material undergoing harmonic oscillation, can be written in the form (9):

$$\sigma = 2\eta^*\dot{\gamma} \qquad \text{(Eq. 1)}$$

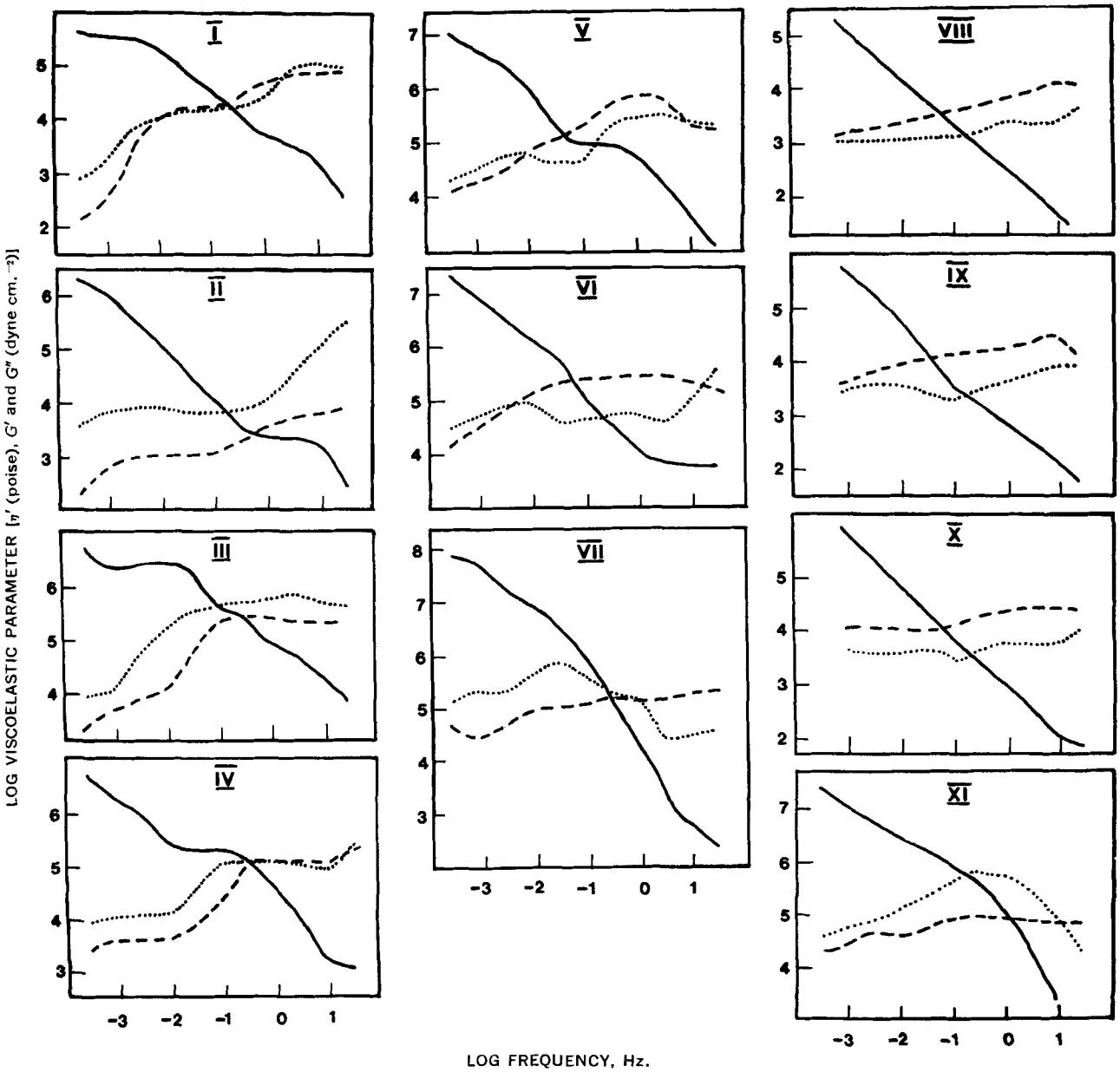

Figure 2—*Dynamic viscoelastic data for ointments and creams. Key:* ——, η'; - - -, G'; . . ., G''; *I, wool fat; II, lanolin; III, paraffin ointment; IV, wool alcohols ointment; V, white soft paraffin; VI, simple ointment; VII, emulsifying ointment, VIII, chlorhexidine cream; IX, aqueous cream; X, cetrimide cream; and XI, oily cream.*

where σ is the shear stress, $\dot{\gamma}$ is the shear rate, and η^* is the complex dynamic viscosity; η^* is usually expressed as real and imaginary parts:

$$\eta^* = \eta' - iG'/\omega \qquad \text{(Eq. 2)}$$

where η' is the dynamic viscosity, and i is the square root of -1. For a purely viscous liquid, η' is constant with change in frequency and G' is zero. G' is also known as the storage modulus and is a measure of the energy stored and recovered per cycle. The loss modulus:

$$G'' = \eta'\omega \qquad \text{(Eq. 3)}$$

is a measure of the energy dissipated or lost as heat per cycle (6).

Experimentally, the material under test is subjected to a small sinusoidal shear strain, and the subsequent shear stress is measured. If the behavior is linear viscoelastic, the stress also is sinusoidal but is out of phase with the input strain. This response can be resolved vectorially into two components, one in phase with the input (real) and one 90° out of phase (imaginary).

EXPERIMENTAL

The sample under test is contained in a suitable geometry; by measuring the amplitudes of stress and strain and their phase difference, G' and η' can be obtained at selected frequencies. Different geometries have been reported in the literature, but parallel plates are the most convenient on theoretical grounds (10). This system also has the advantages that the gap between the plates can be altered without difficulty and a series of experiments can be performed on the same sample.

A Weissenberg rheogoniometer (R 16) was used for all experiments[1]. Data collection was greatly simplified by the use of a digital transfer function analyzer (DTFA) (11–13).

Seven ointments and four creams were examined in their linear viscoelastic region (11), over the frequency range from 2.5×10^{-4} to 25 Hz. Their formulations were presented previously (4, 5).

[1] One of the few commercial instruments with facilities for oscillatory testing (11).

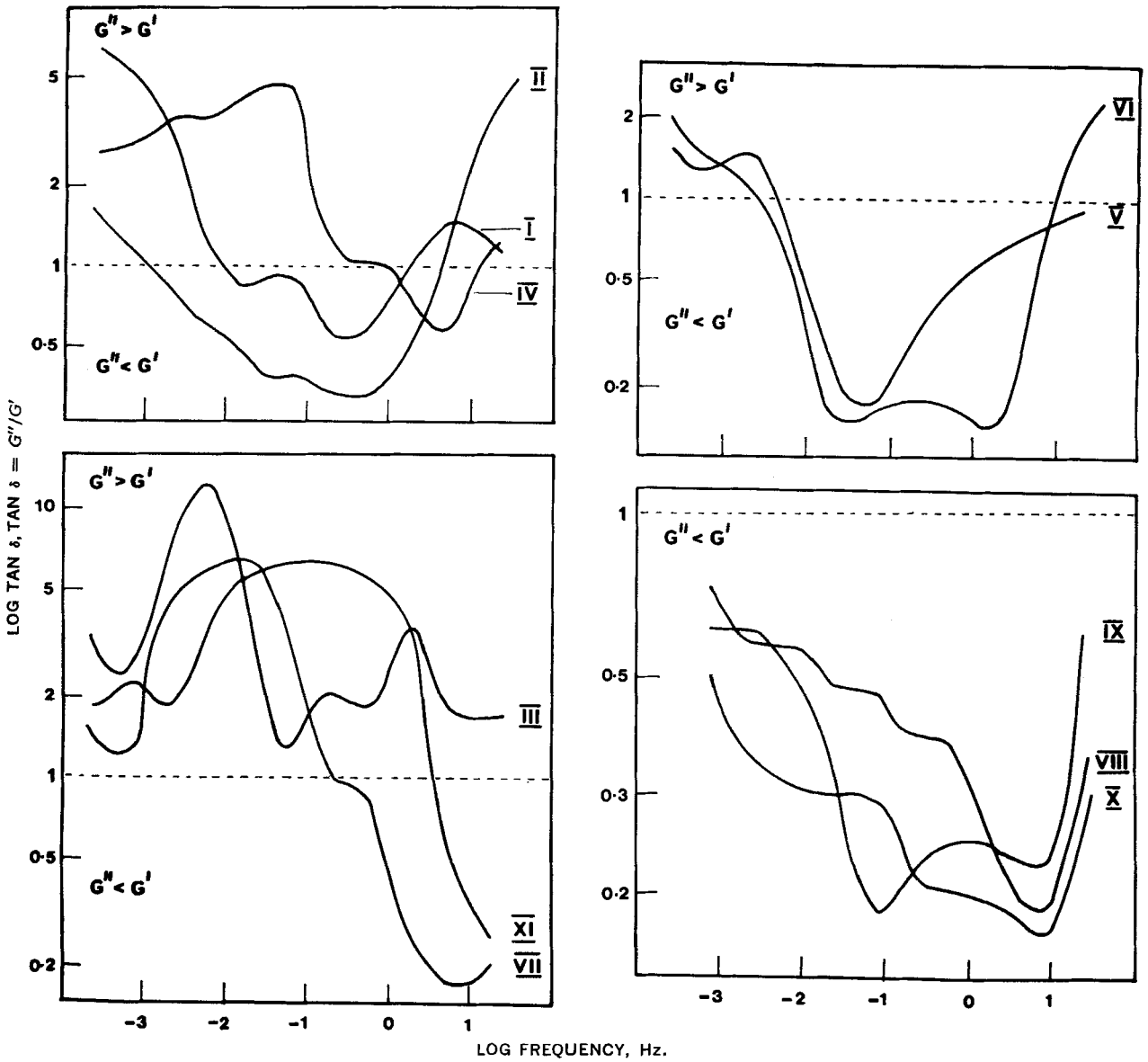

Figure 3—*Variation of loss tangent with frequency. Numerals refer to the systems listed in Fig. 2.*

RESULTS AND DISCUSSION

Calculated Viscoelastic Parameters—Values for dynamic viscosity (η'), storage modulus (G'), and loss modulus (G'') were calculated from the raw data, using the equations of Walters and Kemp (10), and are presented in Fig. 2. A useful dimensionless parameter, tan δ, the loss tangent, was also calculated. It is a measure of the ratio of energy lost to energy stored in a cyclic deformation (G''/G'); it has considerable practical interest because it provides a comparative parameter that combines both the elastic and viscous contributions (Fig. 3).

The parameters η', G', and G'' change characteristically with frequency. At low frequency, η' is high but it falls more or less monotonically as the frequency is increased. At very low frequencies, η' approaches η_0, the steady-state viscosity obtained in the creep studies described previously (4, 5).

It is clear from Eq. 3 that when η' is inversely proportional to frequency (*e.g.*, Systems VIII, IX, and X), the G'' *versus* n relation is almost flat. Alternatively, when η' flattens out, G'' rises steeply.

The storage modulus G' increases gradually as the frequency is increased. As already discussed, G' should approach zero at low frequency (the terminal zone), but this condition was not reached for any of the systems. It requires that the difference in phase angle approaches 90° and that the energy stored per cycle of deformation becomes negligible compared with that dissipated as heat (6). Wool fat (I) is the only system that comes close to this condition. At high frequencies, G' approaches a limiting value for all systems. In regions where G' changes slowly with frequency, the behavior can be considered as being nearly perfectly elastic (6). Therefore, G'' tends to be considerably less than G' (Systems VIII, IX, and X). At high frequency, as the movement of the dashpots in Fig. 1 becomes negligible to that for the springs, G'' should approach zero.

The more or less flat relation between G' and n is often called the plateau region of viscoelastic behavior and can exist for up to 10 decades of frequency. Here, viscoelastic properties are dominated by the presence of a network arrangement such as cross-linkages or the entanglement of polymeric fibers. The smaller the ratio of G''/G' (= tan δ), the more rubbery or elastomeric is the behavior (6). Plots of tan δ *versus* frequency (Fig. 3) are reminiscent of spectra from UV spectrophotometry. Each system has a characteristic curve containing maxima and minima. I suggest that this ratio of viscous to elastic contribution provides an extremely useful rheological parameter and will subsequently refer to the change of tan δ with frequency as a consistency spectrum.

By examining the results presented in Figs. 2 and 3 in some detail, characteristic behavior for the different semisolid systems can be distinguished. Wool fat (I) and lanolin (II), although of similar formulation, have quite distinct behavior. For wool fat, $G' \simeq G''$ (tan δ = 1), except at the lowest frequency. However, the addition of 30% water to produce lanolin increases the elastic contribution and re-

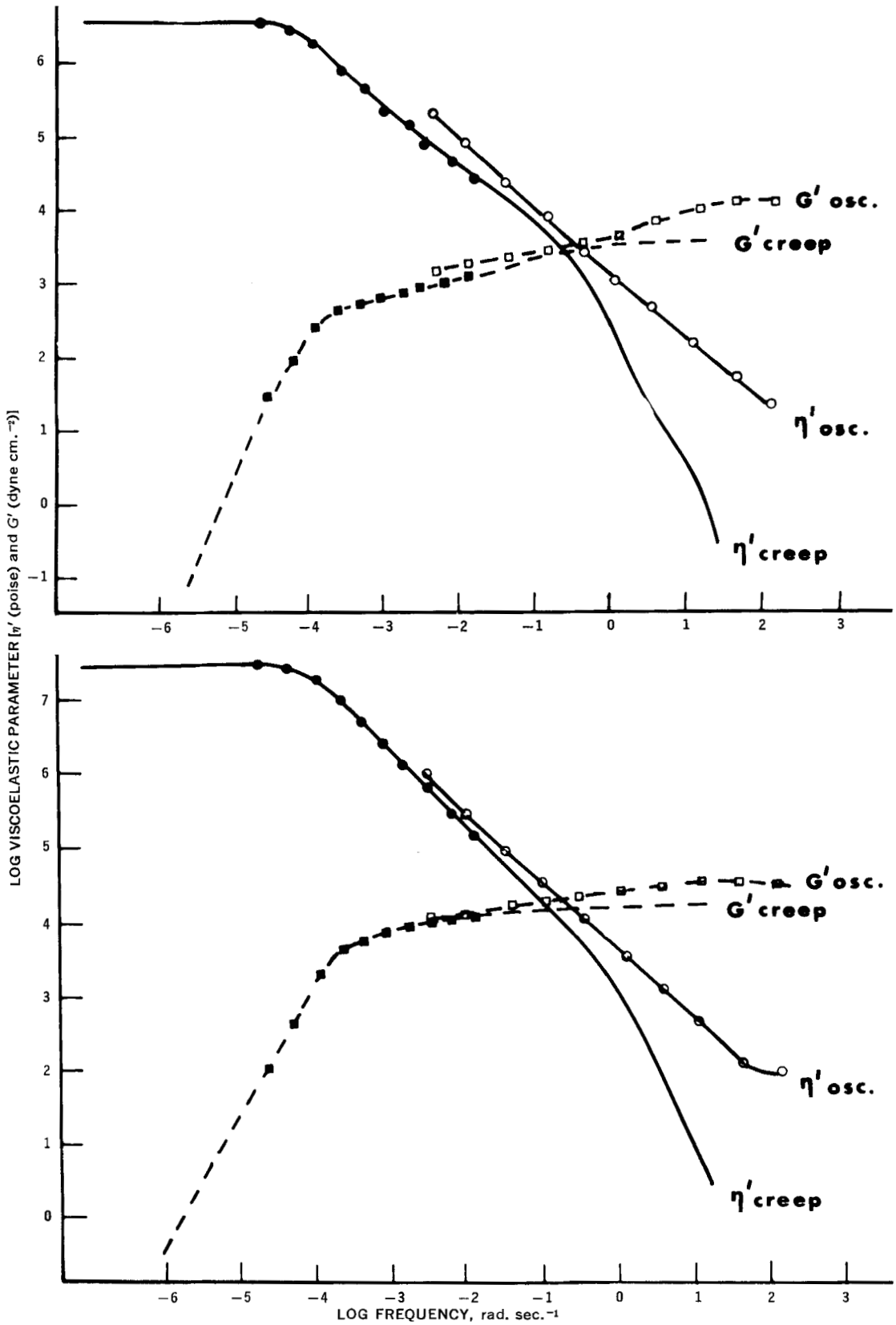

Figure 4—*Comparison of creep and oscillatory techniques using data for chlorhexidine cream (top) and cetrimide cream (bottom). Key: - - -, dynamic viscosity and shear modulus derived from the creep model using Ferry's equations (6) and the data in* Reference 5; ●, ■, *dynamic viscosity and storage modulus values calculated from raw creep data using Schwarzl's equations (7); and* ○, □, *dynamic viscosity and storage modulus values obtained from oscillatory experiments (Fig. 2).*

duces the viscous contribution, with $G' \gg G''$. The presence of water modifies the shape of the consistency spectrum slightly, but the minimum occurs at the same frequency for both systems. The water droplets increase the elastic nature of wool fat by forming an interlinked network of emulsion droplets. In general, the ointment systems are characterized by $G'' \geqslant G'$ (as is also the case with oily cream). The change of η' with n is far from linear, and some plateau regions are evident. The sizes of the plateaus in the log G' *versus* log n plots are related to a distribution of relaxation times; in polymeric systems, they can be used to estimate the molecular weight between entanglement points (6). Systems III, IV, VII, and XI all show pronounced maxima in tan δ at intermediate frequencies. This condition can be associated with an amorphous structure containing some primary linkages (cross-linkages). On the other hand, Systems V and VI have pronounced minima in their consistency spectra at intermediate frequencies. These can be tentatively associated with a lack of primary linkages.

It is somewhat surprising to find that System V, white soft paraffin, has viscoelastic properties, since it was purely elastic in previous creep investigations. However, the samples of paraffin used in the present work were different from those studied in creep, although they were both of BP specification. Different paraffin samples can have differing viscoelastic properties (14, 15), depending upon the method of manufacture and the microcrystalline makeup.

System III, paraffin ointment, contains 90% white soft paraffin and only 3% hard paraffin, 2% beeswax, and 5% cetostearyl alcohol. Nevertheless, these small quantities of additional materials can profoundly alter the viscoelastic properties of the paraffin base. Figure 3 shows that white soft paraffin (V) has a pronounced minimum in its spectrum and $G'' \ll G'$, whereas paraffin ointment has a large maximum in its spectrum and $G'' > G'$.

Those systems containing long-chain wool alcohols (I, II, IV, and VI) all have pronounced minima in their spectra. This may be characteristic for these materials.

System VII shows a complete reversal in G' and G'' at around 1 Hz. The fall in η' with frequency is very steep, and the relative change in η', over the five decades of frequency, is 200 times greater than for any other system. This is clearly due to the presence of a highly shear-sensitive viscous contribution.

The oil-in-water creams (VIII, IX, and X) have $G' \gg G''$ in all cases, indicating that the elastic properties greatly exceed the viscous. All three systems have formulations based on surfactant–long-chain alcohol complexes, which give a self-bodying action (15–19). It is known that a viscoelastic gel network links the emulsion droplets and entraps continuous phase, thereby giving the high elastic component. The differences in consistency between the three systems can be rationalized on the basis of differing oil contents and the natures of the emulsifying agents (5). The consistency spectra are of similar shapes, all with minima at around 10 Hz. The products tested (aqueous cream, cetrimide cream, and chlorhexidine cream) are all popular pharmaceuticals in the United Kingdom. In future work, it is the author's hope to correlate viscoelastic parameters with sensory assessment and consumer evaluation. At that time, it may become apparent that a "satisfactory" cream has $G' \gg G''$ and a consistency spectrum similar to those in Fig. 3.

Oily cream (XI) is a water-in-oil emulsion. The continuous phase consists of liquid and soft and hard paraffins; it is, therefore, not surprising that this material is unlike the oil-in-water creams and behaves rheologically in a similar fashion to the ointments.

Calculation of Dynamic Data from Creep Experiments—Although creep experiments give viscoelastic data at long time periods, it is possible to convert these to dynamic data. These data can be used either to supplement oscillatory experiments and to cover as many decades of frequency as possible or to provide a single source of dynamic data.

Previously in creep (4, 5), the systems were examined over a wide range of temperature; few, if any were examined specifically at 25°. Quite small changes in temperature were shown to lead to large changes in viscoelastic parameters. The comparison of creep and oscillatory results is, therefore, somewhat limited, except for the oil-in-water creams, which are only slightly affected by temperature changes (5). Little error will be introduced if data for chlorhexidine and cetrimide creams (VIII and X) are used for comparison.

The creep data were analyzed using a line spectrum Voigt model representation for viscoelastic parameters (4, 5), and dynamic data can be calculated using the equations of Ferry (6).

If only the raw creep compliance values are available, the approximate equations of Schwarzl (7) can be employed. Data derived, using both the methods, are shown in Fig. 4.

In general, the results show that the agreement between creep and oscillation at intermediate frequency is very good, if one bears in mind that the values refer to slightly different temperatures and that the samples were examined on widely differing occasions. As expected, at low frequency, η' levels off and approaches the steady-state η_0 value and G' falls rapidly and eventually becomes less than G''.

It is clear from Fig. 4 that derived data from creep will be unreliable at times shorter than about 5 sec. due to inertial effects, the response time of the recording system, and the impossibility of applying a truely instantaneous stress. Thus, the extrapolation of creep results to frequencies greater than those covered by creep itself is liable to considerable error. As a result, three different frequency regions can be defined for the testing of semisolid ointments and creams: (*a*) $\omega > 10^{-1}$, oscillatory testing only; (*b*) $4 \times 10^{-3} < \omega < 10^{-1}$, creep or oscillatory methods; and (*c*) $\omega < 4 \times 10^{-3}$, creep testing only [$\omega$ — rad. sec.$^{-1}$ ($= 2\pi n$)].

The judicious combination of creep and oscillatory methods will provide dynamic data over at least nine decades of frequency. This should be ample to characterize any pharmaceutical semisolid formulation in detail.

CONCLUSIONS

The testing of pharmaceutical semisolids by an oscillatory method provides a wealth of fundamental rheological information. It has the advantage over transient (creep) methods of covering a far wider time scale; therefore, it will allow one to postulate, with more confidence, processes occurring at a molecular level. There are various ways of presenting the data, but a "consistency spectrum" in the form of log tan δ *versus* log frequency is the most suitable for pharmaceutical systems. It provides a measure of the ratio of viscous to elastic contributions. Maxima and minima in the spectra are characteristic for given systems, and they can be changed markedly by changes in formulation. The author strongly recommends the use of such spectra when correlating experimental data on semisolids with rheological parameters. For example, if one returns to the list in the introduction, it is apparent that the consistency spectrum approach can be applied directly to follow rheological changes in formulation, quality control, storage stability, the action of mucolytic agents, and radiation sterilization. Its direct use in percutaneous absorption, product assessment, and shear behavior is not as yet clear, but it will certainly provide a convenient starting point for further investigations. In part IV, the author examines destructive oscillatory testing and its importance in product assessment (20).

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Viscoelastic Properties of Pharmaceutical Semisolids IV: Destructive Oscillatory Testing

STANLEY S. DAVIS

Abstract □ Rheogoniometer oscillatory tests of large amplitude were used to simulate the conditions of dermatological usage of semisolid ointments and creams. The onset of nonlinear viscoelastic behavior can be correlated with the yield stress found from continuous shear experiments. The rate of breakdown of viscoelastic structure follows a first-order process and is dependent on the oscillatory frequency and amplitude, product formulation, and, above all, sample thickness. Reformation of structure (thixotropy) was also studied for representative materials. The importance of these tests in product assessment and consumer evaluation is discussed.

Keyphrases □ Ointments, creams, semisolid—viscoelastic properties □ Creams, ointments, semisolid—viscoelastic properties □ Viscoelasticity, destructive oscillatory testing—semisolid formulations □ Oscillatory shear—semisolid breakdown parameters □ Thixotropy—semisolids □ Topical formulations—viscoelastic properties

In part III (1), the viscoelastic properties of pharmaceutical semisolids were examined using nondestructive oscillatory techniques. The imposed shear strains were such that the ointments and creams were measured in their linear viscoelastic region. The calculated parameters (dynamic viscosity, storage modulus, and loss tangent) and their change with frequency were suitable for fundamental rheological characterization. However, it is realized that in actual product usage, such as the rubbing of an ointment onto the skin, the imposed shear strains and stresses are large and the materials are nonlinear in their response. The essential difference between linear and nonlinear behavior is that the former is nondestructive while the latter is destructive, and viscoelastic structures are broken down either reversibly or irreversibly.

The mathematical interpretation of nonlinear viscoelasticity is extremely complex (2, 3); as yet, no formalized treatment exists that can be usefully applied to pharmaceutical semisolids. Even so, it is rewarding to enlarge deliberately the shear strain in an oscillatory experiment, and to penetrate the nonlinear region, to reveal new sets of shear properties which govern the effects of second and higher degrees (3).

The rubbing of a semisolid onto the skin was considered by Henderson *et al.* (4), who obtained a rough approximation of the possible shear rates involved. Their model consisted of two parallel plates, with a gap of 1–3 mm., with a stroke length of 6 cm. and four strokes performed per second. All these variables were highly arbitrary but reasonably described the rubbing process within an order of magnitude. By considering constant shearing conditions, they arrived at a shear rate value of 120 sec.$^{-1}$. Setnikar *et al.* (5) also reported values ranging from 3 to 250 sec.$^{-1}$ for dermatological lotions of 0.2–3-mm. thickness rubbed in at a velocity of 1–5 cm./sec. And, in extreme cases, values as high as 10,000 sec.$^{-1}$ were reported (6).

A more detailed examination of the Henderson *et al.* (4) model should be made. If the reasonable assumption is made that most rubbing processes are periodic in nature (*i.e.*, so many strokes per second), then an oscillatory motion rather than continuous shear should be considered. The shear rate, therefore, varies during the stroke and is not constant as usually assumed. It is zero at each end of the stroke and has a maximum value in the middle. Therefore, to test a semisolid material rheologically under conditions similar to those in usage, oscillatory testing is far more realistic than continuous shear. The skin and hand surfaces can be idealized in the

Table I—Correlation between Maximum Input Strain for Linearity and Yield Stress from Continuous Shear Experiments[a]

System	Input Strain (Maximum)	Yield Stress, Dynes cm.$^{-2}$
Chlorhexidine cream	0.05	1.0×10^3
Aqueous cream	0.16	3.0×10^3
Cetrimide cream	0.20	3.0×10^3
Oily cream	0.23	6.1×10^3
White soft paraffin	0.08	2.0×10^3
Lanolin	0.9	—
Wool fat	0.9	—
Paraffin ointment	1.0	1.5×10^4
Wool alcohols ointment	0.13	3.0×10^3
Emulsifying ointment	1.2	7.6×10^3
Simple ointment	0.13	1.2×10^5
Shortening (7)	1.1	7.0×10^3
Lard (7)	1.5	1.4×10^4

[a] $n = 7.9 \times 10^{-2}$ Hz. Gap $= 6.4 \times 10^{-2}$ cm.

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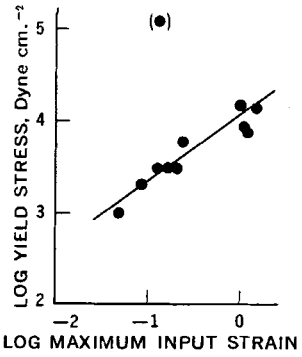

Figure 1—*Correlation between yield strain in oscillation and yield stress in continuous shear (cone and plate) studies.*

form of circular parallel plates (available with the Weissenberg rheogoniometer), and the gap between them can be altered at will to cover a range of usage conditions.

By reexamining the Henderson *et al.* (4) model in these terms, maximum shear rates can be calculated. Four strokes per second is equivalent to an oscillatory frequency of 2 Hz., and a 6-cm. stroke gives an amplitude of 3 cm. Six centimeters can be taken as the suitable plate diameter. The shear rate ($\dot{\gamma}$) alters throughout the cycle of deformation, but for small gaps its maximum value is given by (3):

$$\dot{\gamma} = n\theta_I Ka/h \qquad \text{(Eq. 1)}$$

where n is the frequency of oscillation (in Hz.), θ_I is the input amplitude, K an apparatus constant, a the plate radius, and h the gap width. Using the values quoted, the approximate relation is:

$$\dot{\gamma} \sim 4/h \qquad \text{(Eq. 2)}$$

so that for the Henderson *et al.* (4) values of h = 0.1–0.3 cm., $\dot{\gamma}$ has maximum values of 40 and 13 sec.$^{-1}$, respectively. Likewise, for the smallest thickness quoted by Setnikar *et al.* (5) of 0.02 cm., $\dot{\gamma}$ = 200 sec.$^{-1}$.

EXPERIMENTAL

The Weissenberg rheogoniometer in parallel plate geometry (plate diameter = 7.5 cm.) provides an extremely useful means of studying the behavior of semisolids under dermatological usage conditions. Equation 1 shows that the maximum shear rate depends on the frequency of oscillation, the gap width between the plates, and the strain amplitude. Experiments were performed to investigate each of these factors in turn, as well as the effect of formulation. As already discussed, fundamental rheological parameters such as elasticities and viscosities are difficult, if not impossible, to obtain in nonlinear testing. However, one is able to study the rate of breakdown of viscoelastic structure under various imposed shear conditions. At high frequencies, this breakdown was followed using a digital transfer function analyzer (DTFA) (1); at low frequency, an *X-Y* plotter was used.

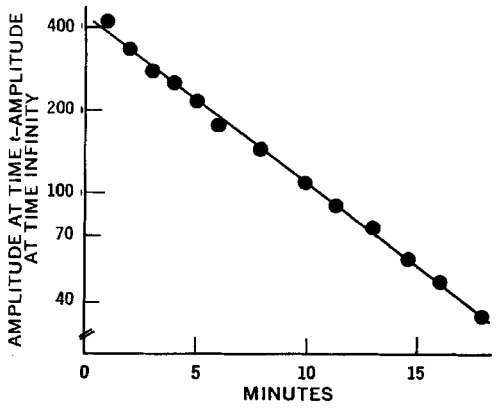

Figure 3—*First-order representation of data in Fig. 2.*

RESULTS AND DISCUSSION

Nonlinear Strain—The onset of nonlinearity at a given frequency is shown by a sudden change in a previously constant amplitude ratio and phase angle (1). The maximum input strain can be calculated by integrating Eq. 1, and some representative values are listed in Table I. The range of strain is quite extensive, with values from 0.05 to 1.5. This range is related to the shear sensitivities of the various semisolids which, in turn, depend on their molecular makeup. Microcrystalline materials, such as white soft paraffin, wool alcohols ointment, and simple ointment, are very susceptible to breakdown under shear and, therefore, have a small linear region. On the other hand, materials made from long viscoelastic threads (*e.g.*, wool fat and lanolin) or large bundles of long acicular crystals (*e.g.*, lard) have much larger linear regions.

The more shear sensitive a material, the less work is needed to cause it to undergo structural breakdown. Thus, in continuous shear, one would expect that the yield stress (minimum shear stress to cause observable flow) would be lower for a shear-sensitive material than for less shear-sensitive material. Yield values from Ferranti–Shirley cone and plate viscometer studies are listed in Table I (8, 9); there appears to be a reasonable rank-order correlation between strain and yield value (Fig. 1), except for the case of simple ointment.

Rate of Breakdown of Viscoelastic Structure—The rate of breakdown of viscoelastic structure was followed by measuring the change in the rheogoniometer output amplitude (stress) with time under an imposed sinusoidal strain. At high frequencies, the output signal voltage from the DTFA was plotted directly against time from the onset of the shearing procedure (Fig. 2). The samples were sheared until a constant stress was obtained, and the data were then plotted in the form of a conventional first-order representation (Fig. 3). In general, the semisolid systems all gave reasonable first-order plots from which rate constants for breakdown could be calculated. This type of first-order change in shear stress was presented in earlier studies on thixotorpic materials, where a constant shear rate

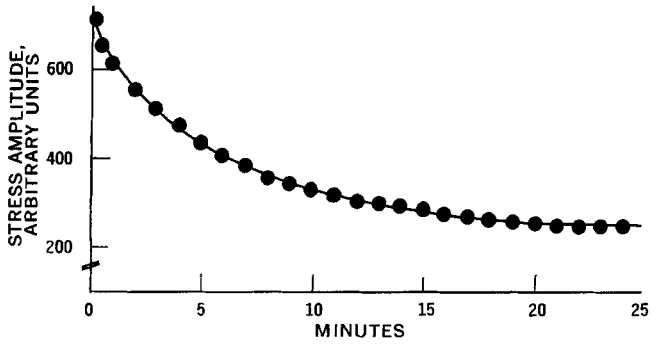

Figure 2—*Breakdown of viscoelastic structure in oily cream by oscillation (nonlinear region),* n = 2.5 × 10^{-1} *Hz. Strain amplitude* = 2.0 × 10^{-2} *rad. Gap* = 6.4 × 10^{-2} *cm.*

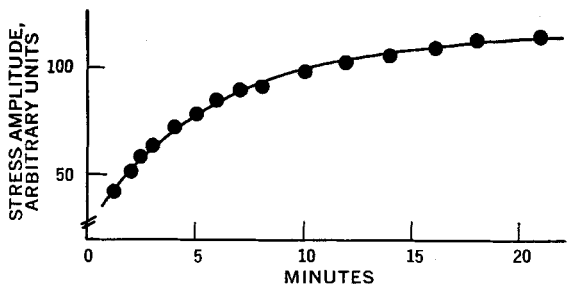

Figure 4—*Reformation of viscoelastic structure in oily cream. Testing in linear viscoelastic region,* n = 2.5 × 10^{-1} *Hz. Strain amplitude* = 2.0 × 10^{-3} *rad. Gap* = 6.4 × 10^{-2} *cm.*

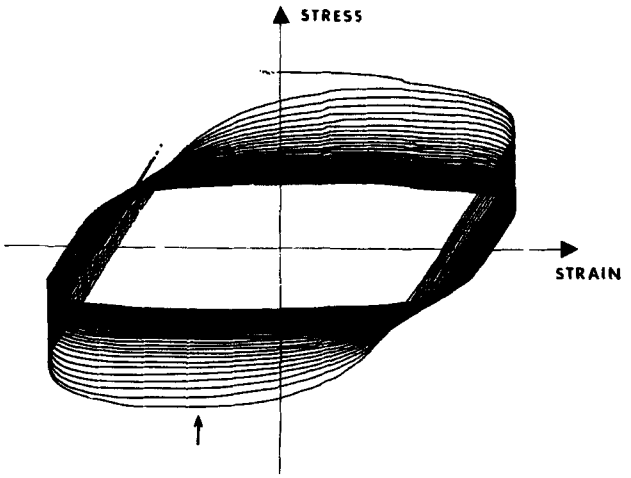

Figure 5—*Stress/strain hysteresis loop for oily cream undergoing structural breakdown,* n = 7.9×10^{-3} *Hz. Strain amplitude* = 8.1×10^{-3} *rad. Gap* = 6.4×10^{-2} *cm.*

was suddenly imposed and the fall in shear stress with time was followed (10).

In some cases, after stress reached a constant value, the input strain could be reduced to a value in the linear viscoelastic region (nondestructive testing), and the slow build-up of structure was followed by measuring the increase in output stress (Fig. 4). Structural build-up is discussed later in this report.

The DTFA requires three input cycles to perform its data analysis and integration. Therefore, at periods longer than 15 sec., much of the structure was broken down before the instrument could give its first result. An alternative method of data collection was therefore used. If, in an oscillatory experiment, one arranges that the stress and strain signals are placed at right angles to each other, using an *X-Y* plotter or oscilloscope, then for a phase-angle difference of 0°, a straight line at 45° to the axis results. But for a 90° phase difference, an ellipse or circle is produced (stress/strain hysteresis loop). A viscoelastic material, undergoing structural breakdown, gives a series of ellipses of changing shapes; a typical result, obtained with an *X-Y* plotter for oily cream, is shown in Fig. 5. The strain amplitude (*X*-axis) is constant, but the stress amplitude (*Y*-axis) gradually decreases as structure is broken down. By taking a suitable point in the cycle (arrow), the change in amplitude per period can be calculated and compared to the final infinity value to give a first-order representation (Fig. 6). In effect, the *X-Y* plotter provides an automatic method of following structure loss, which would be useful in quality control processes. (The DTFA also has provision for automatic data collection, but the DTFA is many times more expensive than an *X-Y* plotter.)

Formulation—The change of the rate constant for breakdown with formulation was investigated at a constant frequency of 7.9×10^{-3} Hz. The use of a constant input amplitude (strain) would naturally give different results, because each sample had different linearity characteristics. To compare the samples under the same conditions, they were examined at an input strain four times that in Table I for the onset of nonlinearity. The results (Table II) for those systems that could be examined by the rheogoniometer under such conditions show that the ointments (and oily cream) have similar rate constants (in the region of 8×10^{-4} sec.$^{-1}$) while the creams have higher values (around 2.5×10^{-3} sec.$^{-1}$). The creams are more susceptible to breakdown, under shear at the given frequency, than are the ointments.

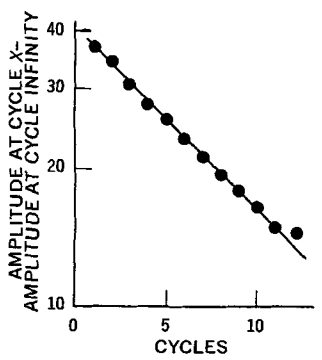

Figure 6—*First-order representation of data in Fig. 5.*

Table II—Effect of Formulation on Rate Constant for Breakdown of Viscoelastic Structure[a]

System	Input Strain (Maximum)	Rate Constant, sec.$^{-1}$
White soft paraffin	0.32	7.8×10^{-4}
Oily cream	0.92	7.8×10^{-4}
Simple ointment	0.52	7.2×10^{-4}
Wool alcohols ointment	0.52	9.0×10^{-4}
Aqueous cream	0.64	2.0×10^{-3}
Cetrimide cream	0.80	2.5×10^{-3}
Chlorhexidine cream	0.20	3.4×10^{-3}

[a] $n = 7.9 \times 10^{-3}$ Hz. Strain amplitude = four times maximum strain values in Table I. Gap = 6.4×10^{-2} cm.

Each system could, of course, be examined over a wide range of frequency (including the usage frequency of about 2 Hz.) and a "spectrum" for each presented.

Amplitude—The effect of strain amplitude was studied, using oily cream as a representative material (Table III). The change in rate constant is not excessive for a four-times change in input strain and indicates that the predominant breakdown processes involve structures and mechanisms that change little with the input strain at constant frequency.

Gap—Decreasing the gap thickness increases the rate of breakdown quite markedly (Table IV). The maximum shear rate per cycle increases with a decrease in the gap thickness, as predicted by Eq. 1. The smaller the gap, the smaller is the shear wave between the plates and, thus, more energy is available for destructive breakdown. When the gap is very small, the rate of breakdown becomes high; in any usage test or model, the sample thickness is the most important variable. Any rheological method of simulating product usage must, therefore, have provision for altering sample thickness.

Frequency—The rate constant for breakdown is directly related to the frequency of oscillation. At high frequency, more material is broken down in unit time than at low frequency. However, this can be allowed for if the rate constant for breakdown is normalized in terms of each cycle. If the same destructive processes occur at different frequencies, the amount of breakdown per cycle should be constant. The results in Fig. 7 show that the rate constant *per cycle* falls as frequency increases. That is, less structure is broken down per cycle at high frequency than at low; the mechanisms of breakdown are not frequency independent. At high frequency, there is less time in each cycle for breakdown to occur than at low frequency. The Maxwell model and linear behavior were discussed previously (1). At high frequency, a viscoelastic material is essentially elastic in nature and viscous effects have insufficient time to manifest themselves. At low frequency, the viscous contribution has more than enough time to respond to the imposed strain; as a result, the material appears to be essentially viscous. When dealing with nonlinear behavior, one must not use model interpretations too widely. But, in a similar manner, one can postulate that at low frequency both elastic and viscous contributions are affected by the imposed nonlinear strain, while at high frequency the elastic contribution protects the system from rapid loss of structure.

Table III—Effect of Input Strain Amplitude on Rate Constant for Breakdown of Viscoelastic Structure in Oily Cream[a]

Input Strain (Maximum)	Shear Rate (Maximum), sec.$^{-1}$	Rate Constant, sec.$^{-1}$
0.35	1.7×10^{-2}	8.1×10^{-3}
0.70	3.4×10^{-2}	1.6×10^{-2}
1.05	5.1×10^{-2}	2.3×10^{-2}
1.38	6.8×10^{-2}	3.1×10^{-2}

[a] $n = 7.9 \times 10^{-3}$ Hz. Gap = 6.4×10^{-2} cm.

Table IV—Effect of Gap Thickness on Rate Constant for Breakdown of Viscoelastic Structure in Oily Cream[a]

Gap, cm.	Input Strain (Maximum)	Shear Rate (Maximum), sec.$^{-1}$	Rate Constant, sec.$^{-1}$
0.25	0.22	1.1×10^{-2}	6.6×10^{-4}
0.15	0.35	1.8×10^{-2}	1.0×10^{-3}
0.10	0.53	2.7×10^{-2}	1.4×10^{-3}
0.06	0.86	4.3×10^{-2}	1.5×10^{-3}
0.04	1.40	7.1×10^{-2}	1.7×10^{-3}
0.025	2.14	1.1×10^{-1}	2.0×10^{-3}

[a] $n = 7.9 \times 10^{-3}$ Hz. Strain amplitude = 2.0×10^{-2} rad.

For white soft paraffin, a plot of the rate constant against log frequency (Fig. 7) gives a reasonably straight line and suggests that quality control experiments could be performed at more convenient low frequencies and an extrapolation then made to the frequency of usage. The total fall in amplitude (percent) follows a more curved relation, but it indicates that less structure is destroyed at high frequency than at low, even at infinite time. Hence, different mechanisms of breakdown are occurring at different frequencies.

Recovery (Thixotropy)—Besides following the rate of breakdown of viscoelastic structure, one can also examine the rate of structure recovery. The increase in stress for an oily cream sample is presented in Fig. 4. The reformation of structure with time is a demonstration of the phenomenon of thixotropy, which is usually defined as the time-dependent reversible breakdown of structure upon shear. Attempts to measure thixotropy using continuous shear methods, such as the Ferranti–Shirley viscometer, have been limited in their success (11). Highly viscoelastic materials exhibit shear fracture when subjected to high shear rates, with the result that material is expelled from the measuring surfaces of the viscometer. This, of course, gives an apparent irreversible loss of structure.

With nonaqueous systems, the recovery of the structure broken down under oscillatory shear can be studied by allowing the material to rest in the rheogoniometer for periods of up to 1 week before retesting it in the linear or nonlinear regions. However, in some cases, such as with white soft paraffin (Fig. 8), there is little evidence of any reformation after the initial breakdown process (except for a stress peak in the first test cycle). In addition, highly viscoelastic materials may show little structural breakdown in oscillation, even under the severest oscillatory conditions available with the rheogoniometer. However, the continuous shear facility of the instrument provides an alternative method.

A sample of wool fat was examined for thixotropy in this manner by first shearing it at a constant shear rate until the shear stress reached an equilibrium value. It was then allowed to recover and was tested at intervals, in oscillation, in its linear viscoelastic region. Immediately after shearing (Fig. 9), the stress/strain hysteresis loop was of small amplitude and large phase angle. However, as the structure rebuilt, the phase angle decreased and the stress amplitude increased. After 2 days of storage, the material was gradually returning to its unsheared condition (phase angle 5° at 2.5×10^{-2} Hz.).

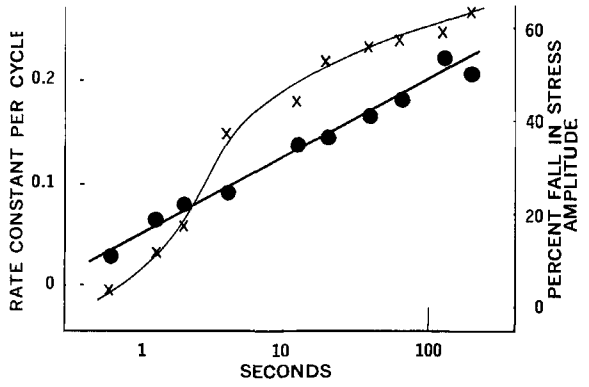

Figure 7—*Change of rate constant for breakdown and total breakdown with frequency for white soft paraffin. Rate constant per cycle,* ●, *and percent fall in stress amplitude,* ×. *Strain amplitude = 2.0 × 10^{-2} rad. Gap = 6.4 × 10^{-2} cm.*

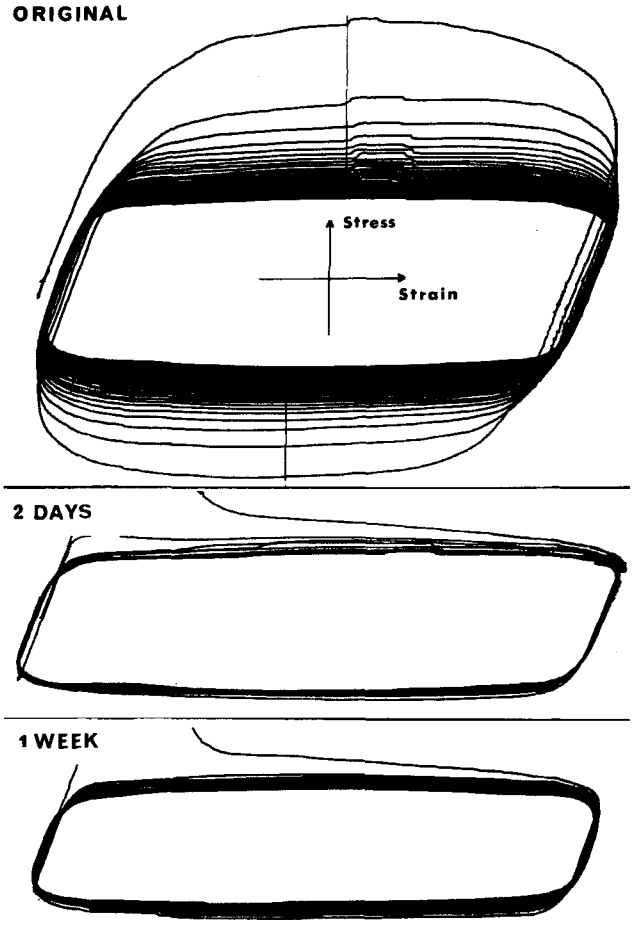

Figure 8—*Effect of storage on stress/strain hysteresis loop for white soft paraffin, nonlinear testing,* n = *5 × 10^{-3} Hz. Strain amplitude = 2.0 × 10^{-2} rad. Gap = 6.4 × 10^{-2} cm.*

CONCLUSIONS

Although destructive oscillatory testing does not provide fundamental rheological parameters, it does provide extremely useful information for quality control and for the correlation of physical properties with product assessment and consumer evaluation. The rate of breakdown of viscoelastic structure is approximately first order, and rate constants can be determined using the rheogoniometer in parallel plate geometry. Variables, such as amplitude and frequency of oscillation and the sample thickness, can be altered to determine the characteristic behavior of a given formulation. In

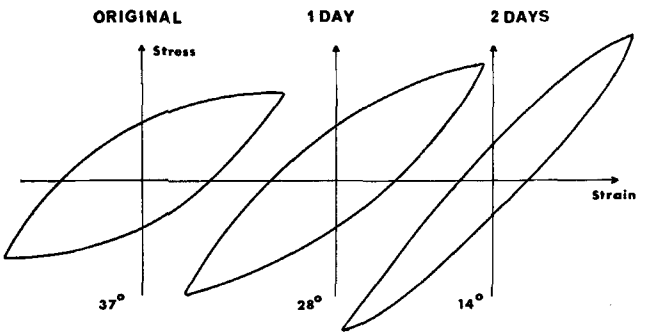

Figure 9—*Effect of storage on stress/strain hysteresis loop for wool fat, linear testing,* n = *2.5 × 10^{-2} Hz. Strain amplitude = 2.3 × 10^{-2} rad. Gap = 6.4 × 10^{-2} cm. Phase angle calculated from graphical method of Lammiman and Roberts (12).*

general, materials that have high elastic contributions (1) (*e.g.*, o/w creams) break down more rapidly than ointments and w/o creams, which are more viscous (1).

The rate constant for breakdown under oscillatory shear is an important parameter when assessing consumer utilization of topical products. In future studies, both nondestructive and destructive oscillatory tests and consumer panels will be used to arrive at a detailed viscoelastic specification for a range of semisolid products.

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CHARLES H. NIGHTINGALE* and MILO GIBALDI†

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Keyphrases □ Goldfish, overturn—4-aminoantipyrine absorption–exsorption kinetics □ Pharmacokinetics, 4-aminoantipyrine absorption–exsorption—goldfish □ 4-Aminoantipyrine, absorption–exsorption kinetics—goldfish

The kinetics of drug absorption in goldfish were recently studied by several workers (1–5). One model, describing the uptake of drugs and toxic chemicals by fish, was developed by Levy and Gucinski (1). As noted by these authors, this theoretical treatment possesses certain limitations due to the simplifying assumptions. The model predicts that a plot of reciprocal time of occurrence of pharmacological effect (time of death or overturn time) *versus* drug concentration in the solution in which the fish are immersed will be linear and pass through the origin. Although the model successfully described the time course of pharmacologic effect of several drugs in the goldfish, some exceptions to the model also were reported. For example, Powers (6) found a number of positive concentration intercept values in plots of reciprocal time of death *versus* concentration of a variety of drug and toxic substances. Powers proposed that there is a concentration of every toxic substance below which no pharmacologic response is observed, regardless of the exposure time. This concentration was designated as a threshold of toxicity. Hall and Hayton (5) subsequently arrived at a similar conclusion by placing goldfish in dilute ethanol solutions for over 24 hr. without observing overturn. In their experiments, it was found that in ethanol-induced overturn and death of goldfish, a plot of reciprocal time of occurrence of the pharmacological end-point *versus* ethanol concentration resulted in a 1% (v/v) intercept. This finding was verified by Gibaldi and Nightingale (2), using the overturn end-point at low ethanol concentrations.

The purpose of the present study was to extend the Levy–Gucinski kinetic model of pharmacologic effect in the goldfish to situations where an apparent threshold concentration does exist.

THEORETICAL

The model of Levy and Gucinski (1) describes the relationship between the drug concentration in the fluids bathing the fish, the absorption rate, and the time of occurrence of pharmacologic effect in the goldfish. The basis of this model is Fick's first law of diffusion:

$$\frac{dA_B}{dt} = \frac{DA}{l}(C_o - C_i) \qquad \text{(Eq. 1)}$$

where A_B is the amount of drug absorbed by the goldfish, (dA_B/dt) is the rate of drug absorption, D is the diffusion coefficient, A is the surface area of biologic membrane, l is the thickness of the biologic membrane, C_o is the drug concentration outside the membrane (the solution bathing the fish), and C_i is the drug concentration on the inside of the membrane.

If one assumes that $C_o \gg C_i$ and integrates Eq. 1 between the limits of zero time and the time necessary to produce a pharmaco-

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If one assumes that $C_o \gg C_i$ and integrates Eq. 1 between the limits of zero time and the time necessary to produce a pharmaco-

logic response, *e.g.*, time of death (T_d) or time of overturn (T_o) (2), one obtains:

$$1/T_d = \frac{DAC_o}{l \cdot A_B} = K \cdot C_o \qquad \text{(Eq. 2)}$$

where K is a hybrid constant.

Equation 2 is based upon the following assumptions:

1. Absorption occurs by passive diffusion and, therefore, is not a saturable process.
2. The drug concentration gradient across the absorbing membrane remains essentially constant during the experiment.
3. The permeability characteristics of the membrane do not change with time or drug concentration over the time and concentration range of the experiment.
4. Drug elimination is negligible during the time of the experiment.
5. The pharmacologic effect occurs without significant delay after a given amount of drug has been absorbed and does not involve mechanisms requiring inductive or other delayed effects (1, 2).

Under these conditions, Eq. 2 predicts that a plot of $1/T_d$ *versus* C_o should be linear with a slope of K and pass through the origin. Levy and Gucinski (1) found this to be true for both ethanol and pentobarbital, using death as the pharmacologic end-point. According to Levy and Gucinski (1), if any of the required conditions used in deriving Eq. 2 are not present, a plot of $1/T_d$ *versus* C_o will not be linear.

It was of interest to modify the Levy–Gucinski model by considering the existence of a threshold concentration. If one assumes that the occurrence of a pharmacologic effect is absorption rate limited, *i.e.*, distribution of drug to the drug receptor, and combination with the receptor to produce the pharmacologic response is very rapid, the following model is applicable:

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B \qquad \text{(Eq. 3)}$$

where compartment A is the fluid bathing the fish and compartment B is the fish. The volumes of compartment A (V_A) and compartment B (V_B) are constant. Under these conditions, the following differential equations are appropriate:

$$\frac{dX_A}{dt} = k_{21}X_B - k_{12}X_A \qquad \text{(Eq. 4)}$$

and

$$\frac{dX_B}{dt} = k_{12}X_A - k_{21}X_B \qquad \text{(Eq. 5)}$$

where (dX/dt) is the change in the amount of drug in the respective compartments with respect to time, and the k's are constants with dimensions of reciprocal time.

Solving Eqs. 4 and 5 for X_B, one obtains:

$$X_B = \frac{k_{12}X_A^o}{(k_{12} + k_{21})}\left[1 - e^{-(k_{12}+k_{21})t}\right] \qquad \text{(Eq. 6)}$$

where X_A^o is the initial amount of drug in compartment A (the bathing solution). Changing the amounts of drug (X) to concentration terms ($C = X/V$) and rearranging, one obtains:

$$e^{-(k_{12}+k_{21})t} = \frac{KC_A^o - C_B}{KC_A^o} \qquad \text{(Eq. 7)}$$

where C is the concentration of drug in the respective compartment, V is the volume of the respective compartment, and $K = k_{12}V_A/(k_{12} + k_{21})V_B$.

Assuming that the concentration of drug in the fish (C_B) reaches the level necessary to produce a pharmacologic response (C_B^*) at time t^*, then:

$$e^{-(k_{12}+k_{21})t^*} = \frac{KC_A^o - C_B^*}{KC_A^o} \qquad \text{(Eq. 8)}$$

Equation 8 may be rewritten as follows:

$$1/t^* = \frac{k_{12} + k_{21}}{\ln\left(\dfrac{KC_A^o}{KC_A^o - C_B^*}\right)} \qquad \text{(Eq. 9)}$$

The denominator on the right side of Eq. 9 may be approximated by the following logarithmic series:

$$\ln Z = 2\left[\frac{Z-1}{Z+1} + \frac{1}{3}\left(\frac{Z-1}{Z+1}\right)^3 + \frac{1}{5}\left(\frac{Z-1}{Z+1}\right)^5 + \cdots\right] \qquad \text{(Eq. 10)}$$

where Z is greater than zero and equals: $KC_A^o/(KC_A^o - C_B^*)$.

If all terms of Eq. 10 except the first are assumed to be negligible, then:

$$1/t^* \cong \frac{k_{12} + k_{21}}{2\left(\dfrac{Z-1}{Z+1}\right)} \qquad \text{(Eq. 11)}$$

and upon substitution for Z,

$$1/t^* \cong \frac{K(k_{12} + k_{21})C_A^o}{C_B^*} - \frac{(k_{12} + k_{21})}{2} \qquad \text{(Eq. 12)}$$

Hence a plot of $1/t^*$ *versus* C_A^o should have a linear portion with a slope of $(k_{12} + k_{21}) \cdot K/C_B^*$ and an intercept of $-(k_{12} + k_{21})/2$.

The Levy–Gucinski model can be readily shown to be an approximation of the proposed absorption-rate-limited model. This can be seen from Eq. 5, assuming that X_A is large and constant and X_B is negligible:

$$\frac{dX_B}{dt} = k_{12}X_A^o \qquad \text{(Eq. 13)}$$

Upon integration:

$$X_B^* = k_{12}X_A^o \cdot t^* \qquad \text{(Eq. 14)}$$

where X_B^* is the amount of drug in the fish after exposure to the bathing solution containing X_A^o drug for a period of time (t^*) necessary to cause the pharmacologic response. Equation 14 may be rewritten as:

$$X_B^* = k_aC_A^ot^* \qquad \text{(Eq. 15)}$$

where $k_a = k_{12}V_A$. Upon rearrangement:

$$1/t^* = K \cdot C_A^o \qquad \text{(Eq. 16)}$$

where $K = k_a/X_B^*$.

Equation 16 is similar to Eq. 2 derived by Levy and Gucinski (1) using a different approach. While the hybrid rate constant, K, has a different meaning in each model, they are kinetically indistinguishable.

Since Eq. 12 is not exact, it will only approximate the kinetics of drug transfer in the proposed model. To gain insight into the exact relationship between reciprocal overturn time ($1/t^*$) and drug concentration in the fish bathing solution (C_A^o), it is necessary to solve Eq. 9 using reasonable estimates of the critical parameters. When this is done, as can be seen from Fig. 1, hyperbolic curvature occurs at low drug concentrations. Since this type of curvature has not been previously reported, the purposes of the *Experimental* portion of this report were to characterize the $1/t^*$ *versus* C_A^o plot at low drug concentrations and to calculate the constants k_{12}, k_{21}, and C_B^* for 4-aminoantipyrine from Eq. 9.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing about 8–10 g., served as the test animal. All fish used in the experiment were from the same lot. The test drug, 4-aminoantipyrine[1], was dissolved in distilled water. The final solutions were adjusted to pH 6.1 with either 1 *N* HCl or NaOH. The fish were immersed in 250 ml. of drug solution at 26–28°, and the overturn time was determined as previously described (2, 7).

RESULTS AND DISCUSSION

The results of the present study indicate that 4-aminoantipyrine-induced overturn apparently follows the Levy and Gucinski model

[1] Eastman.

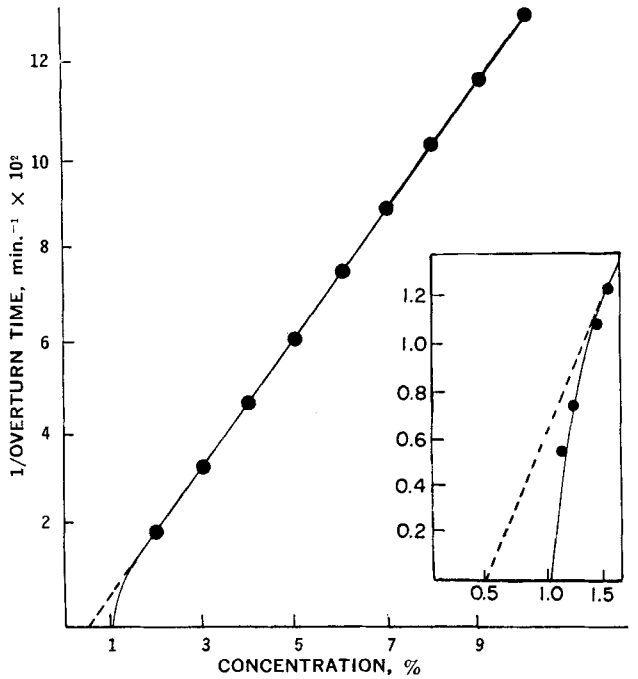

Figure 1—*Reciprocal times of overturn of goldfish as a function of concentration. The data were simulated from the proposed drug absorption-rate-limited model, using a Control Data Corp. 6400 digital computer (9). The parameters were:* $k_{12} = 2.8 \times 10^{-4}$ *min.*$^{-1}$, $k_{21} = 1.4 \times 10^{-3}$ *min.*$^{-1}$, $X_B^* = 0.05$ *mg.*, $V_B = 5$ *ml., and* $V_A = 250$ *ml. Inset represents data at low concentrations.*

at high drug concentrations. This is clearly shown in Fig. 2, where a plot of reciprocal overturn time *versus* concentration is linear over a concentration range from 40 to 800 mg. %. Analysis of the data using an IBM 360 digital computer resulted in a regression equation of:

$$1/T_o = 0.00042X - 5.13 \times 10^{-6} \qquad \text{(Eq. 17)}$$

where X is concentration in mg. %, and T_o is the overturn time in minutes. The correlation coefficient of the line was 0.999. The mean data obtained at each concentration of 4-aminoantipyrine, along with standard deviations, are summarized in Table I. These findings (concentration range: 40–800 mg. %) agree with the data of Anello and Levy (4) in that the curve is linear and apparently passes through the origin. Examination of Table I reveals, however, that the mean overturn times become disproportionately large at

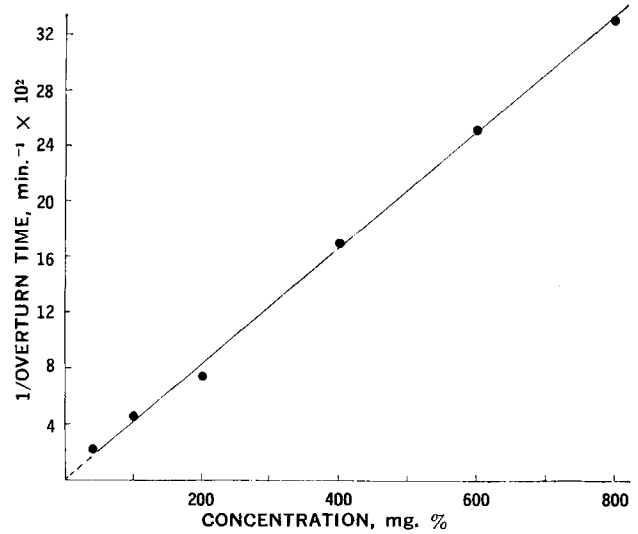

Figure 2—*4-Aminoantipyrine-induced overturn data at high concentrations. Regression equation* $1/T_0 = 0.00042X - 5.13 \times 10^{-6}$, *where X is concentration in milligrams percent. Data are from Table I.*

Table I—Overturn Times of Goldfish in Various Concentrations of 4-Aminoantipyrine

Concentration, mg. %	Number of Animals	Mean Overturn Time, min.[a]	*SD*
800	8	2.99	0.083
600	8	3.95	0.104
400	8	5.86	0.130
200	8	13.30	0.131
100	8	22.00	1.260
50	16	45.06	1.372
40	8	58.14	0.793
30	12	86.02	2.052
25	8	106.70	6.139
20	8	194.95	3.738
15	8	∞[b]	
10	8	∞[b]	

[a] All values different by t-test, $p < 0.01$. [b] > 24 hr.

low drug concentrations. In fact, concentrations of 4-aminoantipyrine of 15 mg. % and lower do not cause overturn. The data at low drug concentrations are shown in Fig. 3. It is apparent that the curve does not pass through the origin but has a finite intercept on the positive X-axis, with a definite deviation from linearity as predicted by the proposed model.

An attempt was made to utilize the experimental data to calculate the forward and reverse rate constants, k_{12} and k_{21}, using Eq. 9. By setting V_A, the volume of the bathing solution, as 250 ml. and V_B, the volume of the fish, as 10 ml., it was possible to write a program for the digital computer capable of calculating k_{12}, k_{21}, and C_B^* (8). This program calculates the sum of squared deviations of the experimental data from those calculated according to Eq. 9. This sum was obtained by setting initial conditions for k_{12}, k_{21}, and C_B^* and incrementing these parameters in a stepwise fashion so that every possible combination of the three constants was tested. Each set of parameters was tested by calculating a $1/t^*$ value for each pair of data points, taking the absolute value of the difference between the calculated $1/t^*$ and experimental $1/t^*$, squaring, and summing. This value was the sum for the function. Each sum was compared to the previously calculated sum, and the larger value was rejected. The parameters giving the smallest sum were taken as the best set of constants for the experimental data. Only one such set was ever found utilizing this program, although it is theoretically possible to have more than one "best" set.

The constants were incremented as follows: k_{12} from 0.0 to 0.01 in increments of 0.0001; k_{21} from 0.0 to 0.1 in increments of 0.001; and C_B^* from 10.0 to 20.0 in increments of 0.1. All other possibilities were previously rejected using this program with larger increments. Computation time can be quite lengthy due to the large number of calculations involved. For example, using 20 data points, 1×10^8 calculations are needed, resulting in computer usage of approximately 60 min. This time can be appreciably reduced to about 10 min. by using a smaller number of increments.

After the critical parameters were obtained, another digital computer program was written to solve Eq. 9 for $1/t^*$, given concentrations of drug from 5 to 800 mg. %.

The results of these calculations are shown in Fig. 3, where the solid line is calculated and the points are experimental. The overturn data used to generate the critical parameters and the theoretical curve ranged from 15 to 100 mg. %. The data at high drug concentrations were not used because they were found to bias the results; their inclusion introduced a predominance of linear data which effectively linearized the curve. Experimental variation tends to influence the slope of the line appreciably at high drug concentrations and to bias, through an averaging process, the curvature at low drug concentrations. The use of low drug concentration data did not appreciably affect the slope of the curve. The slope of the linear portion of the curve was found to be 5.1×10^{-2} ml./min. mg. and compares favorably to the least-squares regression slope of 4.2×10^{-2} ml./min. mg. obtained at high drug concentrations (Fig. 2).

Anello and Levy (4) found that goldfish, when exposed to a 250-mg. % 4-aminoantipyrine solution, overturned in approximately 37 min. (as estimated from their Fig. 3). Using this overturn time, it was possible to calculate the corresponding amount of drug pres-

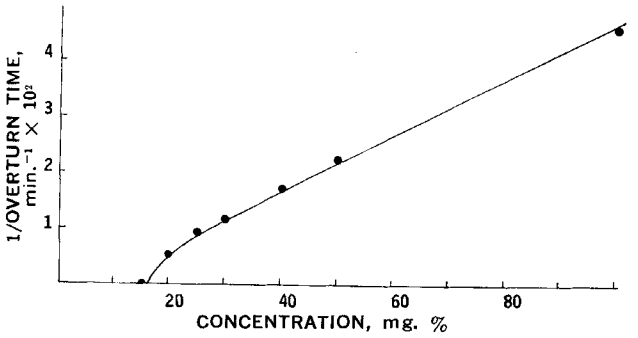

Figure 3—*4-Aminoantipyrine-induced overturn data at low concentrations. The parameters are:* $k_{12} = 4.0 \times 10^{-4}$ *min.*$^{-1}$, $k_{21} = 7.8 \times 10^{-3}$ *min.*$^{-1}$, $C_B^* = 19.5$ *mg.%*, $V_A = 250.0$ *ml., and* $V_B = 10.0$ *ml.*

ent in the goldfish at overturn from their zero-order absorption-rate plot. The amount of drug in the fish at overturn was estimated to be 2.03 mg./10-g. fish. Assuming that a 10-g. fish has a volume of 10 ml., the drug concentration at overturn is 20.3 mg. %, which is in excellent agreement with the theoretical computer-generated C_B^* value of 19.5 mg. % found for the same drug in the present study.

Figure 3 shows the curvature at low 4-aminoantipyrine levels. The shape of the curve is a hyperbola whose general equation, written in terms of reciprocal time and drug concentration, is:

$$1/t^* = \frac{b}{a}\sqrt{(C_A^o)^2 - a^2} \qquad \text{(Eq. 18)}$$

where a and b are constants and correspond to the distance from the origin to the point of intersection of the curve with the X-axis (point P) and the distance from point P to the extrapolated straight-line portion of the graph (asymptote), respectively. Using the "Step-Function" program (8), a and b were calculated from Eq. 18; the equation was then solved for reciprocal overturn time at various 4-aminoantipyrine concentrations. The generated data were plotted and found to yield a curve superimposable to that shown in Fig. 3, which indicates that Eq. 9 is a hyperbolic function.

Based on the data obtained with 4-aminoantipyrine, one can visualize the process of drug absorption in the goldfish. Drug is passively absorbed from the bathing solution. The rate of drug absorption is a function of the rate constant k_{12} and the concentration of drug in the bathing solution (C_A^o). Initially and at high drug concentration, $C_A^o \cdot k_{12} \gg C_B \cdot k_{21}$, and diffusion out of the fish is negligible. Under these conditions the Levy–Gucinski model is applicable, and a plot of $1/t^*$ *versus* C_A^o is linear and should intersect the origin. As C_B becomes larger or as C_A^o becomes smaller, the product $C_B \cdot k_{21}$ approaches $C_A^o \cdot k_{12}$ in magnitude, and deviations from linearity in a $1/t^*$ *versus* C_A^o plot are observed. The curve is hyperbolic and may have a finite positive X-axis intercept. This will occur if X_B^* has a relatively high value or if $k_{21} \gg k_{12}$. When $C_B^* \cdot k_{21} > C_A^* \cdot k_{12}$, the critical concentration necessary for pharmacologic effect in the goldfish will not be reached. The relationship between X_B^* and overturn times has not been experimentally determined; however, these relationships are under study at present.

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Conformation of a *trans*-Diaxial Decalin Analog of Acetylcholine

ELI SHEFTER* and E. E. SMISSMAN†

Abstract □ The three-dimensional structure of 3(a)-dimethylamino-2(a)-acetoxy-*trans*-decalin methiodide was determined by X-ray crystallographic analysis. The spatial disposition of the N^+—C—C—O grouping of atoms was found to be anticlinal (147°). A conformation of $+150 \pm 15°$ for this linkage is felt to be one of a number of factors important in influencing the muscarinic potency and hydrolytic activity of a cholinergic ligand.

Keyphrases □ 3(a)-Dimethylamino-2(a)-acetoxy-*trans*-decalin methiodide—conformational X-ray analysis, muscarinic potency □ X-ray crystallography—conformation, 3(a)-dimethylamino-2(a)-acetoxy-*trans*-decalin methiodide □ Muscarinic potency—acetylcholine-decalin analog conformation

The use of conformationally constrained analogs of a drug entity for the elucidation of structure–activity relationships is a popular technique. Smissman *et al.* (1) synthesized and pharmacologically tested a series of *trans*-decalin analogs of acetylcholine to develop a relationship with regard to the muscarinic receptor. Of the four diastereoisomers studied (I, II, III, and IV), the *trans*-diaxial molecule (I) proved to be the most potent agonist. This isomer was the only one to be hydrolyzed by acetylcholinesterase at a substantial rate when compared to acetylcholine. The observations led these investigators to conclude that muscarinic response and hydrolytic activity require a *trans*-arrangement of the quaternary nitrogen relative to the acetoxy oxygen. Chothia (2) recently proposed that the *trans*-conformation of I makes it a relatively inactive cholinergic molecule. A question arises as to the validity of either idea, because structural data on these molecules were not utilized in the arguments. An X-ray structure analysis of I was undertaken to clear the record on the conformation of this molecule; the results are reported in this article.

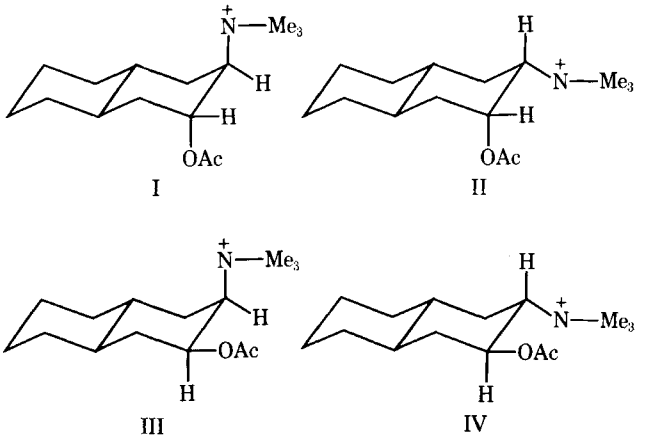
EXPERIMENTAL

Crystals of 3(a)-dimethylamino-2(a)-acetoxy-*trans*-decalin methiodide (I) have the following measured parameters:

$a = 15.976\ (6)$ Å	density (calculated) $= 1.459$ g. cm.$^{-3}$
$b = 9.047\ (4)$ Å	density (measured) $= 1.457$ g. cm.$^{-3}$
$c = 12.752\ (5)$ Å	$Z = 4$
$\beta = 109.66\ (3)°$	space group $P2_1/c$

Intensity data were collected by the stationary crystal–stationary counter technique using Ni filter Cu$K\alpha$ radiation. The reflections between 0 and 100° in 2θ were measured: a total of 1854 independent reflections. Corrections to these measurements were made for absorption (approximately), α_1–α_2 splitting, and the Lorentz-polarization phenomena.

A trial structure was obtained using the "heavy atom" technique. The positional and thermal parameters of all the nonhydrogen atoms were refined by least squares to an R index of 0.12. With the exception of iodine, the atoms were assigned isotropic thermal parameters in the refinement. No attempt was made to locate the hydrogens in the structure[1].



RESULTS

The final atomic parameters from the least-squares refinement are given in Table I. The intramolecular bond distances and angles obtained with these parameters are shown in Fig. 1. The estimated standard deviations in the lengths and angles denoted in this figure are on the average 0.04 Å and 2°. Within the limits of error, the lengths and angles found agree with expected values (3).

The spatial disposition of atoms or groups of atoms about a particular bond is best discussed in terms of torsion angles. The Klyne and Prelog (4) recommendations for describing such angles are adhered to in this paper. The bulky trimethylammonium group attached to C(3) causes a significant degree of distortion in a portion of the decalin system. The optimum conformation angle about a ring bond is 60°; with the exception of those about the C(2)—C(3) and C(3)—C(4) bonds, there is a reasonable agreement. The torsion angles about these two bonds are (+) 31 and (−) 39°, respectively. In parentheses are the signs of these angles for the 2(S)—3(S) enantiomer.

The acetylcholine portion of the molecule can be spatially specified (5) by the torsion angles for the N^+—C(3)—C(2)—O(1), C(3)—C(2)—O(1)—C(11), and C(2)—O(1)—C(11)—C(12) groupings of atoms. The values for these angles in the 2(S)—3(S) enantiomer are 147, −89, and 179°, respectively. The 2(R)—3(R) isomer has torsion angles that are opposite in sign. A drawing of the 2(R)—3(R) enantiomer, as seen down the C(2)—C(3) bond, is shown in Fig. 2. The displacement of the N—C(3)—C(2)—O(1) torsion angle from 180° results from the repulsion between the C(1) and C(10) *axial* hydrogens and the hydrogen attached to C(13). This can be verified by molecular models[2], which show that an antiperiplanar (180°) model cannot be constructed without great strain.

DISCUSSION

Scientists interested in structurally describing the active site(s) of the cholinergic receptors have principally resorted to studying the comparative activities of ligands having constrained conformations. Numerous studies along these lines have been reported, but one of their principal drawbacks has been the syntheses of conformation-

[1] A tabulation of the observed and calculated structure factors will be supplied on requests directed to E. Shefter.

[2] CPK Space Filling, Ealing Corp., Cambridge, MA 02140

Table I—Positional and Thermal Parameters[a]

Atom	$x/a \cdot 10^4$	$y/b \cdot 10^4$	$z/c \cdot 10^4$	B^b, Å^2
I	1555(1)	1123(2)	−313(1)	
C(1)	3028(15)	7461(26)	4812(19)	5.4(0.4)
C(2)	2344(17)	8525(27)	4957(21)	6.3(0.5)
C(3)	1992(14)	9698(25)	3919(18)	5.1(0.4)
C(4)	2729(15)	10048(24)	3427(19)	5.4(0.5)
C(5)	4080(19)	9413(33)	2933(24)	7.5(0.6)
C(6)	4732(22)	8323(38)	2860(28)	8.9(0.8)
C(7)	5146(20)	7536(36)	4035(25)	8.1(0.6)
C(8)	4462(21)	6922(37)	4444(27)	8.6(0.7)
C(9)	3778(16)	8105(27)	4538(20)	6.0(0.5)
C(10)	3331(15)	8820(23)	3324(19)	5.1(0.4)
C(11)	2301(14)	10124(23)	6443(17)	4.7(0.4)
C(12)	2834(24)	11133(35)	7340(30)	8.8(0.8)
C(13)	1148(16)	7870(28)	2438(21)	6.4(0.6)
C(14)	436(22)	8998(32)	3686(28)	8.3(0.7)
C(15)	806(21)	10469(37)	2264(27)	8.6(0.7)
N	1096(13)	9205(20)	3057(17)	5.8(0.4)
O(1)	2786(10)	9518(17)	5868(12)	5.5(0.3)
O(2)	1534(11)	9804(19)	6223(14)	6.6(0.4)

[a] Their estimated standard deviations are in parentheses. [b] Iodine refined anisotropically; final values of the coefficients ($\times 10^4$) are: B_{11} = 88(1), B_{22} = 203(3), B_{33} = 100(1), B_{12} = 2(3), B_{13} = 83(2), and B_{23} = −5(3).

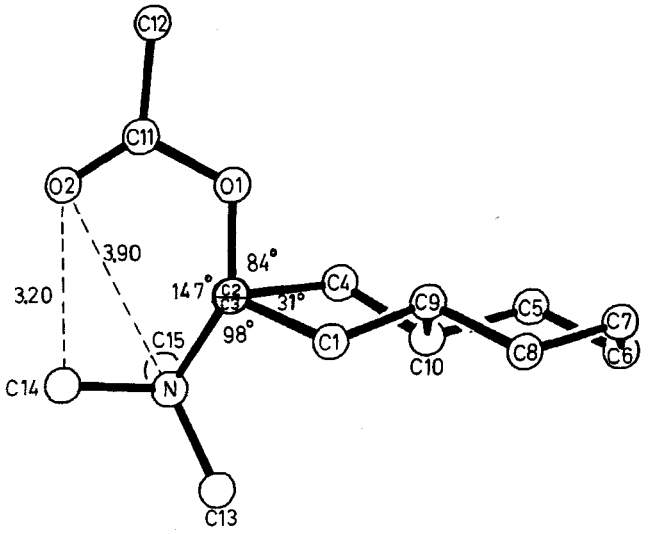


Figure 2—*A view of I showing conformation about the C(2)—C(3) bond.*

ally "rigid" analogs which are similar in "binding" and "efficacy" to acetylcholine. Two other important factors, which have not been considered to any great extent in such studies, concern the differences in electronic features and the thermodynamic properties of these ligands when the acetylcholine framework is chemically altered.

The "structure–activity relationship" formulator usually works under the premise that there is a simple "lock-and-key" mechanism operative for the various cholinergic receptors. The studies of Moran and Triggle (33) on the muscarinic "receptor" indicate a dual mode of agonist binding, thus questioning the validity of a single active site for this receptor. Multiple modes of ligand binding at the catalytic site of acetylcholinesterase also were demonstrated (34, 35). One should, therefore, measure "binding" and "efficacy" of a ligand to make more sense out of comparative pharmacological testing of constrained molecules.

Any consideration of the architecture of the various cholinergic receptors must be concerned with the spatial disposition of the pertinent chemical entities necessary to produce a significant pharmacological response. With regard to the muscarinic activity of acetylcholine and its hydrolysis by acetylcholinesterase, the spatial arrangement of the quaternary nitrogen relative to the acetate group appears to be important. Two conformation angles are sufficient in describing their spatial relationship in acetylcholine and most cholinergic ligands (5); these are the twist angles about the C(2)—C(3) and C(2)—O(1) bonds. Comparison of the pertinent torsion angles obtained from crystallographic studies on a number of cholinergic ligands are presented in Table II and Fig. 3, along with data on their overall rates of acetylcholinesterase hydrolysis and muscarinic potency (on concentration level). Since "binding" data and detailed kinetic data are essentially nonexistent for the compounds tabulated (a few exceptions do exist), a crude activity scale was devised to show the variability in the observed biological activities of the compounds. One must not consider these biological data as directly correlateable with conformation; in general, they are a function of a complex series of events dependent on many other chemical factors.

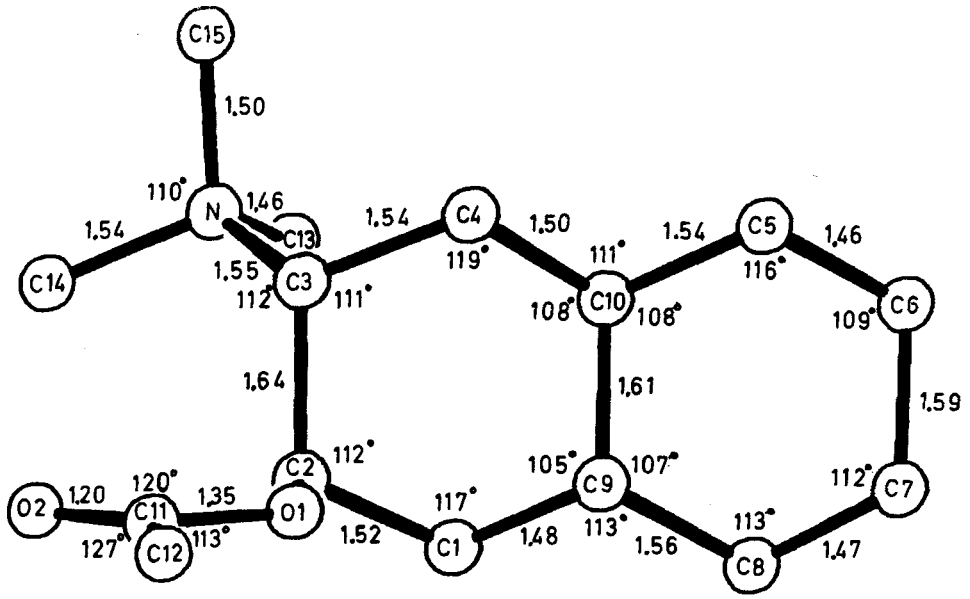


Figure 1—*Bond lengths and angles.*

C12-C11	1.49	C2-O1-C11	118°
C2-O1	1.45	C1-C2-O1	108
		C3-C2-O1	101
		C4-C3-N	115
		C3-N-C13	115
		C3-N-C15	105°
		C13-N-C15	109
		C13-N-C14	111
		C3-N-C14	107

Table II—Comparison of Conformation and Relative Pharmacological Data for Some Cholinergic Ligands

Compound	Torsion Angles: N^+—C(3)—C(2)—O(1)	Torsion Angles: C(3)—C(2)—O(1)—C(11)	Muscarinic Activity[a]	Acetylcholinesterase Hydrolytic Rate[a]	Reference
3(a)-Dimethylamino-2(a)-acetoxy-*trans*-decalin methiodide [3(S)—2(S)] (1)	147°	−89°	+[b]	++[b]	1[d]
Acetylcholine bromide (chloride) (1)	77(85)°	79(167)°	++++	++++	8[c], 9[c]
Acetylthiolcholine bromide (2)	171°	129°	++	+++	10[c], 11[d]
Acetylselenolcholine iodide (3)	175°	123°	+	+++	12[c]
Acetylthionocholine bromide (4)	92°	76°	[g]	[g]	—
erythro-α,β-Dimethylacetylcholine iodide (5) α(R)-β(S)	76°	−156°	++[b]	0[b]	13[c], 1[d]
threo-α,β-Dimethylacetylcholine iodide (6) α(S)-β(S)	143°	−95°	+[b]	++[b]	13[c], 1[d]
(+)-*trans*-2-Acetoxycyclopropyl-trimethylammonium iodide (7)	137°	−151°	++++	++++	14[c], 15[d]
L(−)*S*-α-Methylacetylcholine iodide					
Molecule A [8*a*-α(R)]	90°	170°	—	—	—
Molecule B (8*b*)	148°	−176°	—	—	—
L(+)*S*-β-Methylacetylcholine iodide (9)	85°	−147°	++++(+++[b])	++(++[b])	17[c], 6[d]
Carbamoylcholine bromide (10)	178°	−174°	+++	+[e]	18[c], 19[d]
L(+)-Muscarinic iodide (11)	73°	144°	+++++	—	20[c], 7[d]
Lactoylcholine iodide, L(+)-isomer (12)	85°	157°	+(+[b])	+++++(+++[b])	21[c], 22[d]
L(+)-*cis*-2(S)-Methyl-4(R)-trimethyl-ammonium methyl-1,3-dioxolan iodide (13)	68°	100°	+++++	—	23[c], 24[d]
1-Methyl-3(a)-acetoxy-*trans*-decahydro-quinoline methiodide [3(R)] (14)	74°	153°	+	0[f]	25[d]
Propionylthiolcholine iodide (15)	176°	108°	—	—	10[c]
2(*N*,*N*-Diethyl-*N*-benzylammonium)-ethyl carbamate bromide (16)	81°	163°	0	—	26[c], 19[d]
2(*N*,*N*-Dimethyl-*N*-benzylammonium)-ethyl carbamate bromide (17)	162°	−107°	0	—	27[c], 19[d]
Choline chloride (18)	84°	—	0–+	—	28[c], 29[d]

[a] Relative scale: ++++++, greater than acetylcholine; ++++, equivalent to acetylcholine; +++, one-third to two-thirds that of acetylcholine; ++, a tenth to a third the activity; +, very weak response; and 0, inactive. [b] Measured for racemic mixture. [c] Structural reference. [d] Biological reference. [e] Hydrolysis of carbamylated enzyme takes place much more slowly than the acetylated enzyme. [f] Inhibitor. [g] Chu and Mautner (30) reported that the depolarizing ability (measured on the electroplax) of this compound is 1.7 times less potent than acetylcholine on a molar basis. The hydrolysis rate in the presence of acetylcholinesterase was reported to be significantly lower than that for acetylcholine, but their K_m values were similar.

The two receptors of concern here (nicotinic receptor neglected) were clearly demonstrated to be stereoselective (6, 7, 15). The conformational parameters given in Table II and Fig. 3 are for the enantiomer of a compound that corresponds to the highly active compounds: L(+)-muscarine and (+)-*trans*-2-acetoxycyclopropyltrimethylammonium iodide. Molecular models show that steric factors influence the chirality attainable by a particular enantiomorph (5, 32).

A great deal of variability is found for the C(3)—C(2)—O(1)—C(11) arrangements of the cholinergic ligands tabulated. It is sterically feasible for primary and secondary esters to adopt a wide gamut of conformations about the C(2)—O(1) bond, with the exception of the *synplanar* angle (0 ± 30°). This is evident in Fig. 3. The rotational energy barrier about this bond is, however, greater for secondary esters than for primary esters. Steric factors also restrict the torsion angle range that secondary esters are able to achieve (5). L(+)-Muscarine and *cis*-2(S)-methyl-4(R)-trimethylammonium methyl-1,3-dioxolan are much more constrained about the C—O bond than in the ester acetylcholine analogs. Whether the +144 and +103° values in these two crystal structures represent the boundary condition on this conformation that will produce a muscarinic response remains to be answered. A suggestion that the relevant C(2)—O(1) conformations with regard to muscarinic activity (2) and acetylcholinesterase hydrolysis (32) is antiperiplanar (180 ± 30°) is based on inadequate evidence.

Molecular models of a large variety of cholinergic ligands show that if either of the two α-carbon hydrogens, *i.e.*, C(3), is replaced by bulky substituents, the conformations about C(2)—O(1) are further restricted. Wijngaarden *et al.* (31) postulated that a potent muscarinic agent should have an α-carbon hydrogen 2.3 Å away from the quaternary nitrogen which may bind to the receptor surface (based on the low potency of α-methyl acetylcholine). Specific receptor binding of an α-hydrogen is unlikely; it is more reasonable to consider that the substitution by other groups on the α-carbon influences the important C(2)—O(1) conformation. This ester conformation angle directly affects the position of the methyl group of the acetyl moiety in relation to the cationic head of the molecule. The importance of the corresponding methyl group in muscarine relative to muscarinic activity was shown by Waser (7). In such a saturated system, the C(2)—O(1) conformation is not enough to fix the position of the methyl group. In such cases, the conformation of the C(12)—C(11)—O(1)—C(2) atoms should be defined. This conformation in the case of esters is *antiperiplanar* (180°) but varies for the other muscarinic agents. Pauling and Petcher (39) suggested a value of −137° for nonester cholinergics.

The acetylcholine–decalin analogs were synthesized with the idea of spatially constraining the acidic (N^+) and basic (ester) portions of the cholinergic ligand acetylcholine. With the exception of the *trans*-diaxial isomer (I), space-filling molecular models show that their sterically preferred N^+—C—C—O— conformations are between 70 and 90°. The racemate of I, although substantially less potent a muscarinic agent compared to acetylcholine (on concentration basis), is much more active than the other isomers. Similarly, its hydrolytic behavior is greater.

Much more "rigid" N^+—C—C—O— conformations are found in the *cis*- and *trans*-cyclopropane analogs of acetylcholine (15). Only the *trans*-isomer was found to be a potent muscarinic agent. It was also hydrolyzed by acetylcholinesterase at an overall rate similar to acetylcholine. Its N—C—C—O torsion angle is quite similar to that of the *trans*-decalin analog. None of the other highly active cholinergic ligands has such a constrained conformation, but models show that they are all capable of attaining this spatial arrangement. Studies on conformationally constrained ligands other than those tabulated clearly suggest that conformations between 135 and 165° for the N^+—C—C—O atoms will impart higher activity, whether it be muscarinic or hydrolytic (36–38).

It is felt that there is good indication that a N^+—C—C—O— conformation of 150 ± 15° gives the optimal separation between the acid and basic portions of a cholinergic ligand leading to a substantial biological response at the muscarinic receptor or at the active site of acetylcholinesterase. There is also a preference by these receptors for the (+)-chiral conformation (5, 32, 36, 39) over the (−)-chiral. The ester conformation at the receptor sites remains to be disclosed, but without a doubt it is of equal importance with the N^+—C—C—O— conformation. Other factors, such as the basicity of the ester (10), hydrophobic nature of the ligand, and general size of the ligand, can

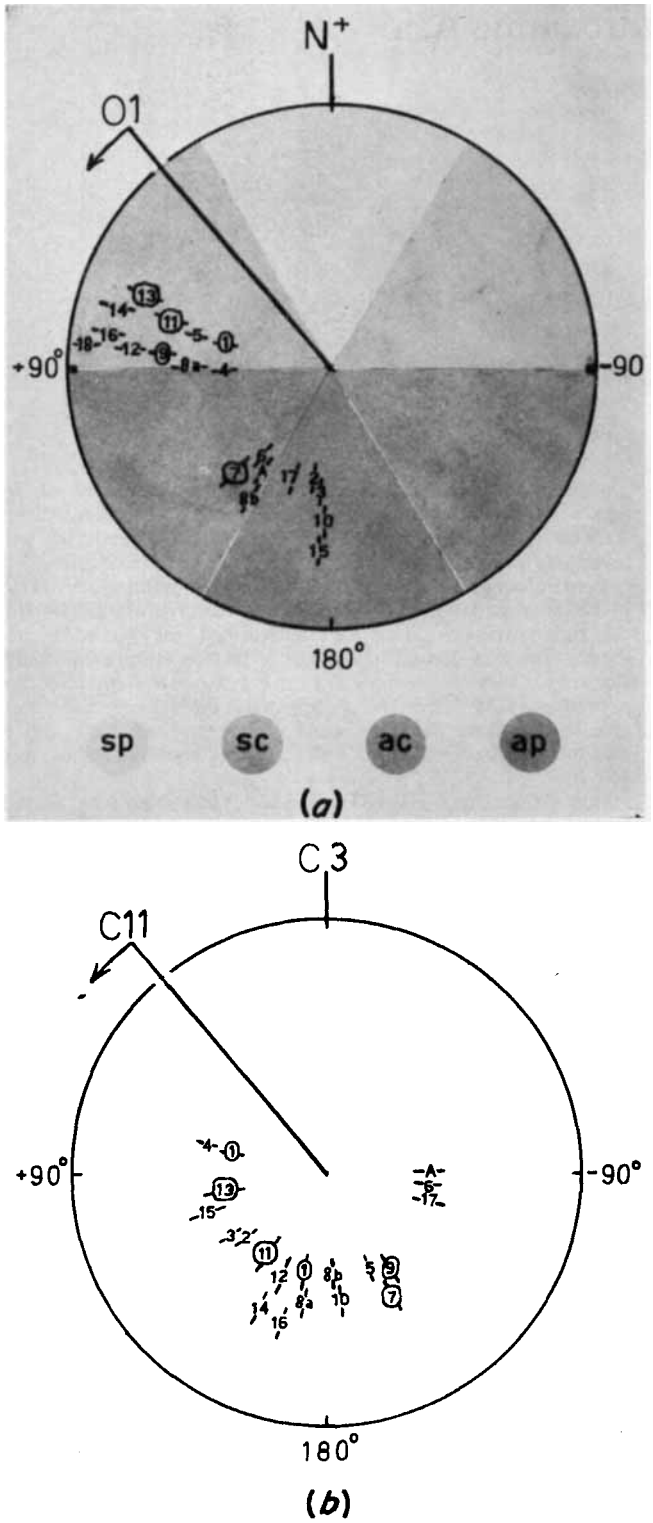


Figure 3—*A plot of the torsion angles of compounds listed in Table II. Key: (a), N+—C(3)—C(2)—O(1); and (b), C(3)—C(2)—O(1)—C(11). Circled numbers refer to most potent agonists listed.*

be considered to influence significantly the responses observed (Table II).

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Estimation of 4-Butoxyphenylacetohydroxamic Acid Utilizing the Lossen Rearrangement

D. DELL, D. R. BOREHAM, and B. K. MARTIN

Abstract □ A sensitive and specific GLC method for the determination of 4-butoxyphenylacetohydroxamic acid (bufexamac) is described. The trimethylsilyl derivative of bufexamac is chromatographed with docosane as the internal standard. This derivative of bufexamac undergoes the Lossen rearrangement on the column to give a quantitative yield of 4-butoxybenzylisocyanate. Possible metabolites of the drug, 4-butoxyphenylacetic acid and 4-butoxyphenylacetamide, can be simultaneously estimated.

Keyphrases □ 4-Butoxyphenylacetohydroxamic acid—Lossen rearrangement, GLC analysis, plasma, urine □ Bufexamac—Lossen rearrangement, GLC analysis, plasma, urine □ Lossen rearrangement—4-butoxyphenylacetohydroxamic acid (bufexamac) □ GLC—analysis, bufexamac

Bufexamac (4-butoxyphenylacetohydroxamic acid[1]) was shown to possess anti-inflammatory properties (1–4). Lambelin *et al.* (5, 6) used the colorimetric method of Bergmann and Segal (7) to determine the hydroxamic acid function in the serum of patients dosed with bufexamac. This method lacks specificity because it detects not only bufexamac but also hydroxy derivatives (substitution in the aromatic ring and/or the butoxy side chain) of bufexamac. The lower limit of sensitivity was reported as 3 mcg.

The present paper describes a GLC method which utilizes the Lossen rearrangement and is capable of determining bufexamac independently of metabolites.

Esters of hydroxamic acids readily undergo a Lossen rearrangement (8) on heating to form the corresponding isocyanates, a reaction that was reviewed by Yale (9) and Franklin (10). By applying this method to bufexamac, the reaction in Scheme I may be written. The free acids also undergo this reaction but only very slowly and usually not quantitatively.

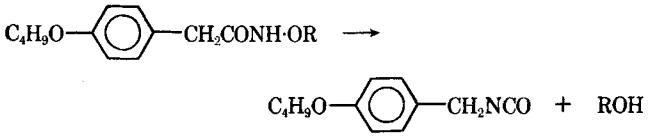
The method described involves the application of this rearrangement to the analysis of bufexamac in plasma and urine *via* the trimethylsilyl ether derivative.

EXPERIMENTAL

Reagents—All solvents used were of A.R. grade purity. Bufexamac dissolved in chloroform (114 mcg./ml.) was a stock solution for calibration, as was 4-butoxyphenylacetic acid in chloroform (99 mcg./ml.). The derivatization reagent was bistrimethylsilylacetamide[2] dissolved in tetrahydrofuran (1:2 v/v). The internal standard was docosane[3] dissolved in carbon tetrachloride (124 mcg./ml.).

Apparatus—A Philips PV-4000 gas chromatograph[4] with a flame-ionization detector was used. The column was a 3.0-m. × 4-mm. (i.d.) glass coil with internal walls silanized; it was packed with Gas Chrom Q[5] (80–100 mesh) and coated with 9% OV-17[6].

[1] Droxaryl, Continental Pharma S. A., Belgium.
[2] Phase Separations Ltd., Flintshire, Scotland.
[3] Koch-Light Labs. Ltd., Colnbrook, Bucks, England.
[4] Pye-Unicam Ltd., Cambridge, England.
[5] Applied Science Labs Inc., State College, PA 16801
[6] Perkin-Elmer Ltd., Beaconsfield, Bucks, England.



Scheme I

The column temperature was 220°; the flow rate was 40 ml. N_2/min. Under these conditions, the trimethylsilyl derivatives of bufexamac and 4-butoxyphenylacetic acid gave single peaks with retentions 0.356 and 0.516, respectively, relative to docosane.

Preparation of 4-Butoxybenzylisocyanate—4-Butoxyphenylacetohydroxamic acid (2.2 g.) was dissolved in tetrahydrofuran (140 ml.) containing pyridine (2 ml.); acetic anhydride (2 ml.) was added, and the solution was allowed to stand at room temperature for 10 min. The solution was concentrated *in vacuo* (15 mm. of mercury) and poured into cold water. The precipitated solid was collected, dried, and recrystallized from hot ethyl acetate–acetone (50:1 v/v) to give the acetyl derivative of bufexamac as colorless flakes, m.p. 160–161°.

Anal.—Calc. for $C_{14}H_{19}NO_4$: C, 63.6; H, 7.18; N, 5.29. Found: C, 63.7; H, 7.25; N, 5.18.

The acetyl derivative was heated at 180° at 0.5-mm. pressure to provide a colorless liquid as distillate, which was characterized as 4-butoxybenzylisocyanate by IR spectroscopy and elemental analysis.

Anal.—Calc. for $C_{12}H_{15}NO_2$: C, 70.2; H, 7.32; N, 6.83. Found: C, 69.7; H, 7.55; N, 6.53.

The IR spectrum showed strong absorption at 2270 $cm.^{-1}$, indicative of an isocyanate. This compound was unstable and changed very rapidly to a white solid.

METHODS

General Procedure—Various volumes of the standard chloroform solution of bufexamac, containing 1–50 mcg., were evaporated to dryness under reduced pressure (15 mm. of mercury) at room temperature in pear-shaped flasks. Tetrahydrofuran (100 μl.) was added, followed by bistrimethylsilylacetamide (50 μl.). The solution was allowed to stand at room temperature for 10 min. with occasional shaking. It was shown that silylation of bufexamac, under these conditions, is complete after 5 min. The internal standard solution (0.5 ml.) was then added, and 1 μl. of the final solution was injected onto the column. The ratios of peak heights (4-butoxybenzylisocyanate–docosane) were plotted against the amount of bufexamac.

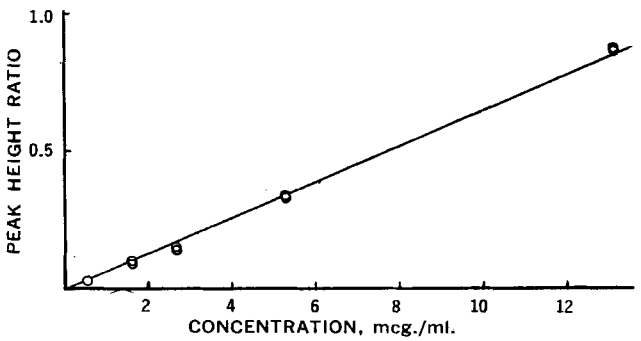


Figure 1—*Relationship between relative peak height and bufexamac concentration in urine.*

Table I—Recovery of Bufexamac from Urine and Plasma

Urine			Plasma		
Micrograms per Milliliter		Mean Percentage Recovery	Micrograms per Milliliter		Mean Percentage Recovery
Added	Found		Added	Found	
16.40	14.50, 14.40	88.1	16.40	13.70, 13.70	83.3
6.57	5.77, 5.60	86.5	6.57	5.60, 5.60	85.2
3.28	2.66, 2.54	79.3	3.28	2.71, 2.67	82.0
1.97	1.63, 1.72	84.9	1.97	1.50, 1.33	71.8
0.66	0.55, 0.58	86.0	0.66	0.54, 0.54	82.7
Overall recoveries		85.0 ± 3.4			81.0 ± 5.3

A similar procedure was adopted for 4-butoxyphenylacetic acid, using the standard choloroform solution.

Extraction from Urine—*Bufexamac*—Two aqueous solutions of bufexamac, containing 19.7 mcg./ml. (I) and 1.97 mcg./ml. (II), were used. From Solution I, 2.5-, 1.0-, and 0.5-ml. samples were taken in duplicate. From Solution II, 3.0- and 1.0-ml. samples were taken, also in duplicate. Each solution was mixed with urine (3 ml.) and 1 *N* HCl (1 ml.), and the volume was adjusted to 9 ml. by the addition of distilled water. Chloroform (25 ml.) was added, and the mixture shaken mechanically for 10 min. An aliquot (20 ml.) of the chloroform extract was evaporated and treated as described previously to provide a calibration curve.

4-Butoxyphenylacetic Acid—Two aqueous solutions, containing 12.5 mcg./ml. (I) and 1.25 mcg./ml. (II) of the acid, were used. From Solution I, 2.0- and 1.0-ml. samples were taken in duplicate; from Solution II, 4.0- and 2.0-ml. samples were taken in duplicate. The subsequent procedure was as already described.

Extraction from Plasma—Various volumes of the standard aqueous bufexamac solutions were added to samples of fresh pig plasma (3 ml.) containing heparin. Tartaric acid (0.5 *M*, 3 ml.) was added to each sample, followed by distilled water to provide a final volume of 9 ml. The subsequent procedure was as described for urine.

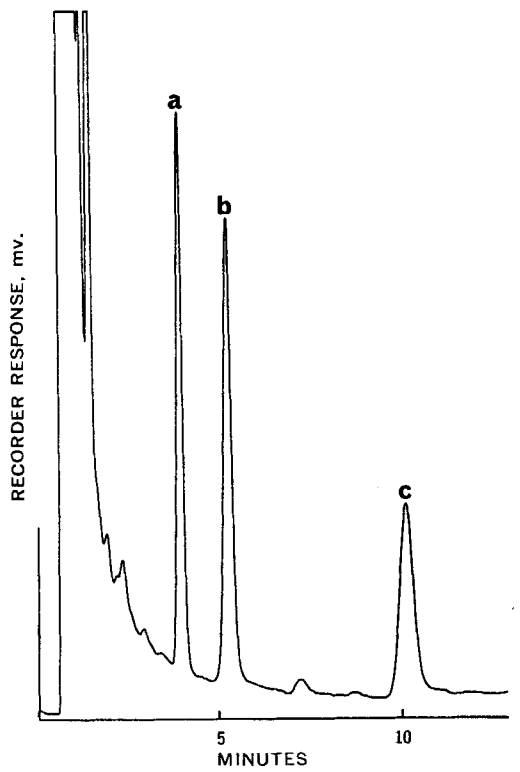


Figure 2—*Gas chromatogram of a mixture of bufexamac, 4-butoxyphenylacetic acid, and 4-butoxyphenylacetamide as their trimethylsilyl derivatives. Conditions were as described in text. Key: a, 4-butoxybenzylisocyanate; b, trimethylsilyl derivative of 4-butoxyphenylacetic acid; and c, trimethylsilyl derivative of 4-butoxyphenylacetamide.*

RESULTS AND DISCUSSION

The relationship between peak height ratio (4-butoxybenzylisocyanate–docosane) and amount of bufexamac was linear within the range studied (2–80 ng. *injected*). This relationship was used to calculate the recovery of bufexamac from urine and plasma, and a similar relationship for 4-butoxyphenylacetic acid was used to calculate the recovery of the acid from urine.

The acetyl, trifluoroacetyl, and trimethylsilyl derivatives of bufexamac, when chromatographed, all gave a single peak, coincident with that obtained from a freshly prepared sample of 4-butoxybenzylisocyanate. This finding suggests that these derivatives, when injected onto the GLC column, undergo the Lossen rearrangement spontaneously, giving 4-butoxybenzylisocyanate. Further evidence to this effect was obtained by interposing a splitter between the end of the column and the detector, so that 80% of the effluent was directed into a trap containing powdered KBr. Repeated injections of a solution of the acetyl derivative of bufexamac in acetone were carried out, and the component with a relative retention of 0.356 was collected. The KBr was pressed into a disk, and the IR spectrum obtained was identical to that of 4-butoxybenzylisocyanate. This appears to be the first example of a trimethylsilyl derivative of a hydroxamic acid undergoing the Lossen reaction. When bufexamac itself is injected, a very small isocyanate peak is obtained.

Vagelos *et al.* (11) reacted hydroxylamine with mixtures of acyl coenzyme A compounds to obtain the corresponding hydroxamic acids. The mixtures of hydroxamates were then acetylated and analyzed as isocyanates by GLC. Several workers observed that the facility of this rearrangement varies according to the nature of the hydroxamic acid derivative (12–15).

In the present study, it was observed that the trimethylsilyl derivative of bufexamac gave twice the response for the isocyanate peak compared with that from the acetyl and trifluoroacetyl derivatives. The trimethylsilyl derivative gave the same response as an equimolar amount of the freshly prepared isocyanate. This indicates that the conversion of this derivative to the isocyanate is virtually quantitative. Variation of the column and injection port temperature between 150 and 250° did not significantly affect the rearrangement. The conditions selected (column and injection heater temperature 220°) were those that gave a retention time (4 min.) suitable for routine analyses. The silylation method has the additional advantage of permitting the derivatization of other substances which might be present in the analytical sample. In this instance, a mixture of bufexamac, 4-butoxyphenylacetic acid, and 4-butoxyphenylacetamide may be estimated separately by the same silylation procedure.

An important advantage of this procedure over current colorimetric methods of analysis for hydroxamates is that any hydroxy metabolite of the drug would probably be silylated and determined

Table II—Recovery of 4-Butoxyphenylacetic Acid from Urine

Micrograms per Milliliter		Mean Percentage Recovery
Added	Found	
16.60	17.70, 17.30	105.4
8.32	8.33, 8.37	100.4
4.16	4.20, 4.29	102.1
1.66	1.46, 1.58	91.7
	Overall recovery	99.9 ± 5.9

as the corresponding isocyanate. The colorimetric methods cannot distinguish between bufexamac and any hydroxy derivatives of bufexamac. No evidence was, in fact, obtained for the presence of a hydroxy derivative of bufexamac as a metabolite of the drug in blood or urine.

The smallest amount of bufexamac that could be assayed following extraction with a standard deviation within the limits reported here corresponded to a concentration of about 0.6 mcg./ml. in urine or plasma. This amount is equivalent to about 2 ng./μl. injected, giving a peak height of about 60 mm. (1×10^{-11} amp. full scale deflection).

An investigation into the general applicability of the Lossen rearrangement to the analysis of hydroxamic acid derivatives of other compounds of pharmaceutical interest is currently being undertaken.

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Reaction of 3-(Chloroalkyl)-2-benzoxazolinones with Amines: Formation of 3-(Aminoalkyl)-2-benzoxazolinones and 5-Substituted-2,3,4,5-tetrahydro-1,5-benzoxazepines

J. SAM, J. L. VALENTINE, and M. N. ABOUL-ENEIN

Abstract □ The reactions of 3-(chloroalkyl)-2-benzoxazolinones with various bases are described.

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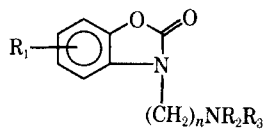
The observed CNS depressant activity associated with some 3-(aminoalkyl)-2-benzoxazolinones (I) (1, 2), as well as parenthetic analogy with unbranched aminopropyl side chains in the 10-position of phenothiazines (II) (3), prompted the investigation of other 3-(aminoalkyl)-2-benzoxazolinones.



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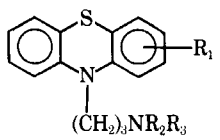
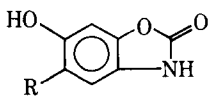


II

Metabolic studies by Bray *et al.* (4) on 2-benzoxazolinone and by Conney and Burns (5) on 5-chloro-2-benzoxazolinone indicated that the major urinary metabolites of both are the 6-hydroxylated compounds



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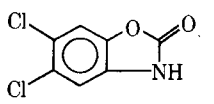

IV

(III). To determine if a hindrance of metabolic detoxication would enhance biological activity of type I compounds, 5,6-dichloro-2-benzoxazolinone (IV) was selected for further study. The introduction of the aminopropyl side chain of the phenothiazines into the 3-position of the parent system was undertaken in anticipation of obtaining interesting CNS depressants.

The preparation of I ($n = 2$ or 3) by the reaction of an appropriately substituted 2-benzoxazolinone with an aminoalkyl halide was described (1, 2, 6). Such synthetic pathways occasionally, however, involve troublesome preparations of the intermediate aminoalkyl halides, thus subjecting the overall synthetic pathways to poor

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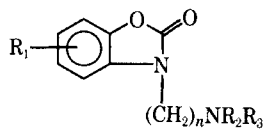
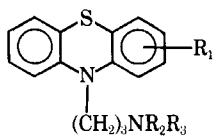
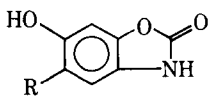


I

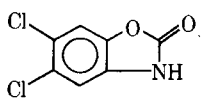


II

Metabolic studies by Bray *et al.* (4) on 2-benzoxazolinone and by Conney and Burns (5) on 5-chloro-2-benzoxazolinone indicated that the major urinary metabolites of both are the 6-hydroxylated compounds



III

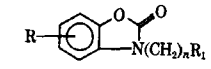


IV

(III). To determine if a hindrance of metabolic detoxication would enhance biological activity of type I compounds, 5,6-dichloro-2-benzoxazolinone (IV) was selected for further study. The introduction of the aminopropyl side chain of the phenothiazines into the 3-position of the parent system was undertaken in anticipation of obtaining interesting CNS depressants.

The preparation of I ($n = 2$ or 3) by the reaction of an appropriately substituted 2-benzoxazolinone with an aminoalkyl halide was described (1, 2, 6). Such synthetic pathways occasionally, however, involve troublesome preparations of the intermediate aminoalkyl halides, thus subjecting the overall synthetic pathways to poor

Table I—3-Substituted-2-benzoxazolinones



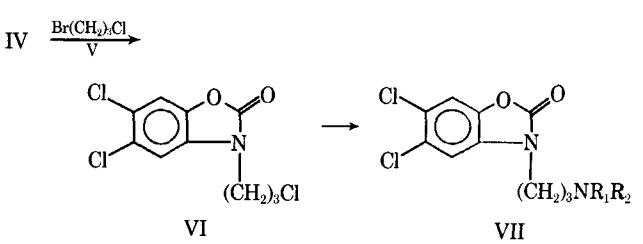
Number[a]	R	R_1	n	Method	Recrystallization Solvent[b]	Yield, %	Melting Point	Molecular Formula	Analysis, % Calc.	Found
I*a*	5,6-DiCl	$(CH_3)_2N$	3	A	E	48	62–63°	$C_{12}H_{14}Cl_2N_2O_2$	C, 49.84 H, 4.87 N, 9.05	C, 50.00 H, 5.03 N, 9.25
I*b*	5,6-DiCl	C_4H_8N[c]	3	D	E–W	63	243–245°	$C_{14}H_{17}Cl_3N_2O_2$[d]	C, 47.81 H, 4.87 N, 7.96	C, 47.92 H, 5.02 N, 7.89
I*c*	5,6-DiCl	$C_5H_{10}N$[e]	3	C	E	64	123–124°	$C_{15}H_{18}Cl_2N_2O_2$	C, 54.73 H, 5.51 N, 8.51	C, 54.91 H, 5.47 N, 8.34
I*d*	5,6-DiCl	$C_6H_{12}N$[f]	3	C	E	62	88–89°	$C_{16}H_{20}Cl_2N_2O_2$	C, 55.98 H, 5.87 N, 8.15	C, 56.17 H, 5.82 N, 8.05
I*e*	5,6-DiCl	C_4H_8NO[g]	3	C	C	53	128–129°	$C_{14}H_{16}Cl_2N_2O_3$	C, 50.76 H, 4.86 N, 8.46	C, 50.91 H, 4.75 N, 8.35
I*f*	5,6-DiCl	$C_5H_{11}N_2$[h]	3	C	I	55	101–102°	$C_{15}H_{19}Cl_2N_3O_2$	C, 52.33 H, 5.56 N, 12.26	C, 52.43 H, 5.65 N, 12.11
I*g*	5-Cl	C_4H_8N[c]	3	C	E–Et	74	194–196°	$C_{14}H_{18}Cl_2N_2O_2$[d]	C, 53.06 H, 5.71 N, 8.83	C, 52.86 H, 5.71 N, 8.63
I*h*	6-Cl	C_4H_8N[c]	3	C	E	54	220–222°	$C_{14}H_{18}Cl_2N_2O_2$[d]	C, 53.06 H, 5.71 N, 8.83	C, 53.20 H, 5.87 N, 8.67
I*i*	5-Cl	C_4H_8N[c]	2	C	E		188–190°	$C_{19}H_{18}ClN_5O_9$[i,j]	C, 46.02 H, 3.65 N, 14.12	C, 46.54 H, 4.56 N, 13.50
I*j*	5,6-DiCl	C_4H_8N[c]	2	C	E	35	273–274°	$C_{13}H_{15}Cl_3N_2O_2$[d]	C, 46.24 H, 4.44 N, 8.30	C, 46.43 H, 4.63 N, 8.11
I*k*	5-Cl	$C_5H_{10}N$[e]	2	C	E	45	268–269°	$C_{14}H_{18}Cl_2N_2O_2$[d]	C, 53.06 H, 5.71 N, 8.83	C, 53.46 H, 6.00 N, 8.78
I*l*	5-NO_2	C_4H_8N[c]	3	C	E	77	226–228°	$C_{20}H_{20}N_6O_{11}$[i]	C, 46.15 H, 3.87 N, 16.11	C, 46.05 H, 3.92 N, 15.45
XII*a*	6-NO_2	Cl	3	A	E	40	93–94°	$C_{10}H_9ClN_2O_4$	C, 46.74 H, 3.53 N, 10.91	C, 46.61 H, 3.79 N, 10.97
XII*b*	5-NO_2	Cl	3	A		61	135–140°(0.1 mm.)[k]	$C_{10}H_9ClN_2O_4$[l]	C, — H, — N, —	C, — H, — N, —
XII*c*	6-Cl	Cl	3	A	E	90	95–96°	$C_{10}H_9Cl_2NO_2$	C, 48.70 H, 3.69 N, 5.65	C, 48.22 H, 3.64 N, 5.95
XII*d*	5-Cl	Cl	2	A	E	81	84–85°	$C_9H_7Cl_2NO_2$	C, 46.19 H, 3.04 N, 6.03	C, 46.72 H, 2.83 N, 6.31
XII*e*	5-Cl	Cl	3	A,B	E	59,81	85–86°	$C_{10}H_9Cl_2NO_2$[m]	C, — H, — N, —	C, — H, — N, —

[a] IR and NMR spectra are in accordance with the structures. [b] E = ethanol, C = cellosolve, Et = ether, I = isopropyl ether, and W = water. [c] Pyrrolidino. [d] Hydrochloride. [e] Piperidino. [f] Hexamethyleneimino. [g] Morpholino. [h] *N*-Methylpiperazino. [i] Picrate. [j] Hydrochloride, m.p. 195–196° (ethanol). [k] Boiling point. [l] Characterized through II. [m] Characterized through Ig and XIV*b*.

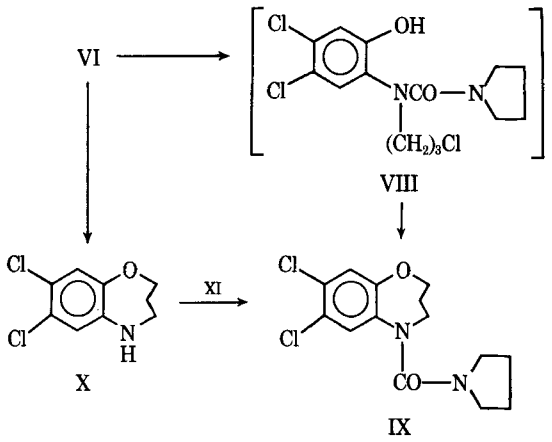
yields. One objective of the present work, therefore, was to investigate an alternate route for the preparation of I.

The use of 1-bromo-3-chloropropane (V) as an alkylating agent is well documented (7, 8). Consequently, V was utilized for the preparation of 3-(3-chloropropyl)-5,6-dichloro-2-benzoxazolinone (VI) (Scheme I). Subsequent reaction of VI with appropriate secondary amines gave VII (I, Table I).

The aminoalkylbenzoxazolinones (I) show a common IR carbonyl absorption at 1750 cm.$^{-1}$. The reaction of pyrrolidine with VI, however, gave a product insoluble in acids and showing IR absorption at 1650 cm.$^{-1}$, indicative of an amide. The IR and NMR spectra and elemental analyses were consistent with the structure of 7,8-dichloro-5-pyrrolidinocarbonyl-2,3,4,5-tetrahydro-1,5-benzoxazepine (IX). Compound IX also was prepared by the alkaline hydrolysis of VI to X and subsequent treatment of the latter with pyrrolidinocarbonyl chloride (XI) (Scheme II).

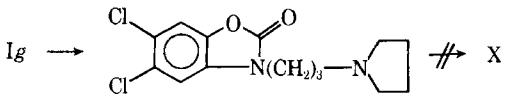


Scheme I



Scheme II

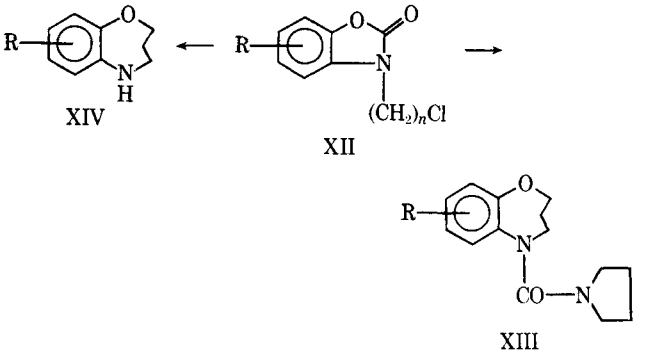
The pyrrolidino derivative (VII*b*) (I*b*, Table I) was prepared by the chlorination of 5-chloro-3-(3-pyrrolidinopropyl)-2-benzoxazolinone (I*g*) (Scheme III). Refluxing I*b* with pyrrolidine under the conditions utilized for the preparation of IX from VI resulted in the recovery of unchanged I*b*. The formation of IX from VI apparently proceeds *via* VIII and not I*b*.



Scheme III

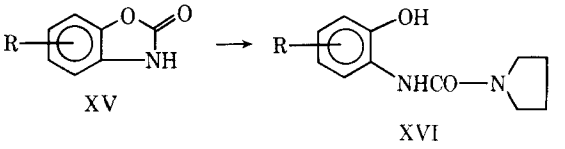
The rearrangement of 3-(3-chloropropyl)-2-benzoxazolinones (XII, $n=3$) to XIII (Table II) occurred only when a strong electron-withdrawing group (6-nitro or 5,6-dihalo) was present in the benzoxazolinone ring and only when the amine was pyrrolidine. However, XII ($n=3$) undergoes rearrangement to XIV when heated with alkali hydroxides. A similar rearrangement of 3-(β-chloroethyl)-2-benzoxazolinone (XII; R = H, $n=2$) to 2,3,4-trihydro-1,4-benzoxazine was noted by Fujii (9).

The reaction of the chloroethyl derivatives (XII, $n=2$) with amines provided the anticipated products (I, $n=2$) (Scheme IV). Moreover, the chloropropyl derivatives (II, $n=3$), containing a monohalo aromatic substituent or a 5-nitro group, also produced I ($n=3$).



Scheme IV

Further evidence that the course of the rearrangement proceeds *via* an intermediate such as VIII was obtained by treating 2-benzoxazolinones (XV) with pyrrolidine under the same experimental conditions as utilized for the preparation of IX. When R = H, rearrangement failed to occur; however, when R = 5,6-dichloro, XVI was obtained (Scheme V).



Scheme V

Attempts to prepare XIV from XIX *via* *o*-aminophenol and XVIII were unsuccessful. Efforts to cyclize XVIII by the method of Sidhu *et al.* (10) resulted only in the unsaturated derivative (XX) (Scheme VI). Attempts to cyclize XX also were unsuccessful. The results are in agreement with the report of Loudon and Ogg (11).

A number of derivatives (XXIII, Table III) of XIV were prepared and subjected to pharmacological evaluation. Except for XXIII*v*, XXIII*w*, and XXIII*x* (Table

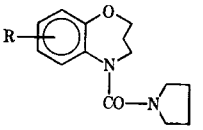
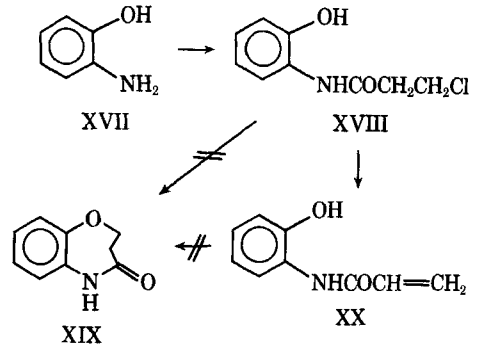


Table II—5-Pyrrolidinocarbonyl-2,3,4,5-tetrahydro-1,5-benzoxazepines

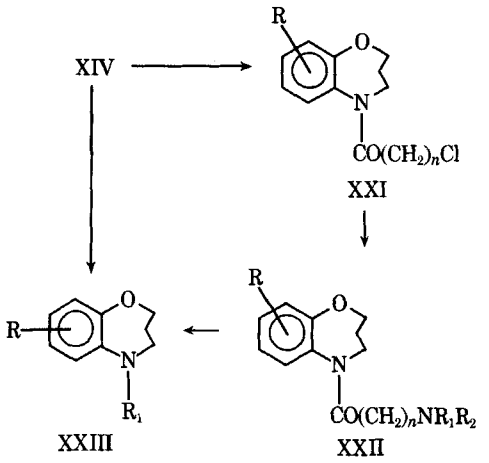
Number[a]	R	Recrystallization Solvent[b]	Method	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
XIII*a*	8-NO_2	E	A	71	148–150°	$C_{14}H_{17}N_3O_4$	C, 57.72 H, 5.88 N, 14.42	C, 57.59 H, 5.66 N, 14.48
XIII*b*	7-Cl-8-Br	E	A	67	167–168°	$C_{14}H_{16}BrClN_2O_2$	C, 46.77 H, 4.48 N, 7.79	C, 46.61 H, 4.49 N, 7.65
XIII*c*	7,8-DiCl	E–W	A,B	62,53	153–154°	$C_{14}H_{16}Cl_2N_2O_2$	C, 53.34 H, 5.12 N, 8.89	C, 53.37 H, 5.24 N, 8.71
XIII*d*	8-NH_2	E	—[c]	43	227–228°	$C_{14}H_{19}N_3O_2$	C, 64.35 H, 7.33 N, 16.08	C, 63.90 H, 7.19 N, 15.97

[a] IR and NMR spectra are in accordance with the structures. [b] E = ethanol, and W = water. [c] By platinum oxide reduction of XIII*a* in ethanol at 3 kg./cm.^{2} at room temperature.



Scheme VI

III), which were prepared directly from XIV, all were synthesized *via* XXI and XXII (Scheme VII).



Scheme VII

EXPERIMENTAL[1]

3-Substituted-2-benzoxazolinones (Table I)—References to the preparation of 2-benzoxazolinones utilized in this study are cited in the review by Sam and Valentine (12).

Method A—To a solution of 11.2 g. (0.1 mole) of potassium hydroxide in 200 ml. of ethyl cellosolve was added 0.1 mole of the appropriate 2-benzoxazolinone (XV). The mixture was stirred at room temperature for 1 hr. and thereafter heated to reflux and treated with 0.2 mole of the requisite alkyl halide. The mixture was stirred and refluxed for an additional 4 hr. The solution was filtered and then concentrated under reduced pressure. The resultant material either was distilled or recrystallized.

Method B—To a stirred and refluxing (for 1 hr.) solution of 4.6 g. (0.2 atom) of sodium and 34 g. (0.2 mole) of 5-chloro-2-benzoxazolinone in 240 ml. of absolute ethanol was added 94.6 g. (0.6 mole) of 1-bromo-3-chloropropane in one portion. Refluxing was continued for 24 hr. The reaction mixture was cooled, filtered, and evaporated under reduced pressure. An ether solution (500 ml.) of the residual material was washed with 10% potassium hydroxide (5 × 20 ml.) and water (3 × 50 ml.) and dried ($MgSO_4$). Evaporation of the ether gave a crystalline mass, which was recrystallized.

Method C—To 200 ml. of ethyl cellosolve was added 0.05 mole of the appropriate 3-(chloroalkyl)-2-benzoxazolinone (XII, Table I; also *References 1* and *9*). The mixture was stirred and refluxed until solution occurred (30 min.). In one portion, 0.1 mole of the appropriate secondary amine was added; the mixture then was refluxed for 6 hr. The solvent was distilled under reduced pressure; the resultant oily crystalline mass was treated with 500 ml. of 5% sodium hydroxide and extracted with ether. The ether extract was dried (Na_2SO_4) and concentrated under reduced pressure. The residual solid was washed with acetonitrile and then recrystallized from a suitable solvent.

Method D—A solution of 14.0 g. (0.05 mole) of 3-(3-pyrrolidinopropyl)-5-chloro-2-benzoxazolinone (Ig) in 200 ml. of dry chloroform was treated dropwise with a solution of 4.3 (0.06 mole) of chlorine in 100 ml. of dry chloroform. Then the mixture was stirred and refluxed for 24 hr. The solvent was distilled under reduced pressure; the resultant solid was treated with 500 ml. of 10% sodium hydroxide and extracted with ether (3 × 100 ml.). The ether extract was dried (Na_2SO_4) and converted to a hydrochloride in the usual manner.

5-Pyrrolidinocarbonyl-2,3,4,5-tetrahydro-1,5-benzoxazepine (XIIIa–XIIIc, Table II)—*Method E*—The procedure described under Method C was followed using the appropriate 3-(3-chloropropyl)-2-benzoxazolinone and pyrrolidine.

Method F—A mixture of 4.0 g. (0.019 mole) of 7,8-dichloro-2,3,4,5-tetrahydro-1,5-benzoxazepine (X) and 2.5 g. (0.019 mole) of pyrrolidinocarbonyl chloride (13) was fused in an oil bath at 200° for 4 hr. The resultant charred mass was treated with 200 ml. of 10% sodium hydroxide and extracted with ether. The ether was dried (Na_2SO_4) and then distilled under reduced pressure. The residue was washed with acetonitrile and recrystallized from ethanol: IR $\nu_{max.}^{CHCl_3}$ 1650 cm.$^{-1}$ (C=O); NMR(CCl_4) δ 1.63–2.25 (m, 6, —CH_2—), 2.91–3.14 (m, 4, —CH_2—N—CH_2—), 3.60–3.78 (t, 2, —N—CH_2—), 3.96–4.14 (t, 2, —OCH_2), and 7.12 (s, 2, aromatic protons). The product was identical in every respect to that obtained in Method E.

2-Hydroxy-4,5-dichloro-*N*-pyrrolidinocarbonylaniline (XVI)—A mixture of 20.6 g. (0.1 mole) of XV (R = 5,6-dichloro) and 14.2 g. (0.2 mole) of pyrrolidine in 200 ml. of methyl cellosolve was stirred and refluxed for 4 hr. The solvent was distilled under reduced pressure; the resulting solid was washed with 5% hydrochloric acid and then recrystallized from methyl cellosolve to give 25 g. (91%) of product, m.p. 219–221°: IR $\nu_{max.}^{KBr}$ 3400 cm.$^{-1}$ (NH) and 1650 cm.$^{-1}$ (C=O); NMR (d_5-pyridine) δ 1.55–1.78 (m, 4, —CH_2—), 3.29–3.51 (t, 4, N—CH_2), 7.23 (s, 1, aromatic proton), 8.71 (s, 1, aromatic proton), 7.90 (s, 1, NH), and 10.71 (s, 1, OH).

Anal.—Calc. for $C_{11}H_{12}Cl_2N_2O_2$: C, 48.01; H, 4.39; N, 10.18. Found: C, 48.27; H, 4.57; N, 10.12.

2,3,4,5-Tetrahydro-1,5-benzoxazepines (X, XIV*a*, and XIV*b*, Table III)—A solution of 33 g. (0.156 mole) of 3-(3-chloropropyl)-2-benzoxazolinone and 35 g. (0.625 mole) of potassium hydroxide in 400 ml. of methyl cellosolve was refluxed for 48 hr. The solvent was removed *in vacuo;* the residue was treated with 500 ml. of cold water and extracted with ether (3 × 100 ml.). The ethereal solution was extracted with 20% hydrochloric acid (3 × 30 ml.). The acidic solution was basified with 15% sodium hydroxide, extracted with ether (3 × 100 ml.), and dried ($MgSO_4$). Distillation of the ether extract gave 12.6 g. (55%) of XIV*a*, b.p. 90–94° (0.6 mm.), m.p. 51.5–52.5°: IR $\nu_{max.}^{CHCl_3}$ 3360 cm.$^{-1}$ (NH); NMR ($CDCl_3$) δ 2.00 (m, 2, —CH_2—), 3.20 (t, 2, N—CH_2), 4.05 (t, 2, O—CH_2), 3.80 (s, 1, NH), and 6.85 (m, 4, aromatic protons). The picrate was prepared in the usual manner and recrystallized from ethanol.

The procedure described here also was used for the preparation of X and XIV*b* (Table III); however, methylene chloride was used as the solvent for the extraction of X.

5-Chloroacyl-2,3,4,5-tetrahydro-1,5-benzoxazepines (XXIII*a*–XXIII*d*, Table III)—To a stirred mixture of 0.1 mole of XIV and 12 g. of anhydrous sodium carbonate in 300 ml. of dry benzene was added dropwise at room temperature 0.11 mole of the appropriate chloroacyl chloride in 50 ml. of dry benzene. The mixture was refluxed overnight, filtered, and concentrated under reduced pressure at 100°. The residue was recrystallized from benzene–methanol.

5-Aminoacyl-2,3,4,5-tetrahydro-1,5-benzoxazepines (XXIII*e*–XXIII*o*, Table III)—A solution of 0.1 mole of XXIII*a*–XXIII*d* and 0.2–0.5 mole of the appropriate amine in 80 ml. of dry benzene was heated in a sealed tube at 110–115° for 20 hr. The mixture was filtered and concentrated under reduced pressure. The residue was dissolved in 50 ml. of dilute hydrochloric acid and extracted with ether (3 × 30 ml.). The acidic solution was basified with 10% sodium hydroxide and extracted with ether (3 × 40 ml.). The extract was dried (Na_2SO_4), evaporated, and purified either by distillation or recrystallization.

5-Aminoalkyl-2,3,4,5-tetrahydro-1,5-benzoxazepines (XXIII*p*–XXIII*u*, Table III)—A solution of 0.027 mole of XXIII*e*–XXIII*o*

[1] Melting points were determined on a Fisher-Johns melting-point apparatus and are corrected. IR spectra were obtained on Perkin-Elmer 137G and 257 IR spectrophotometers. NMR spectra were determined on a Varian A-60-A spectrometer, using Me_4Si as an internal standard.

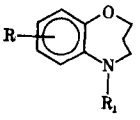


Table III—5-Substituted-2,3,4,5-tetrahydro-1,5-benzoxazepines

Number[a]	R	R′	Melting Point or Boiling Point (mm.)	Yield, %	Molecular Formula	Analysis, % Calc.	Found
X	7,8-DiCl	H	84–85°[b]	66	$C_9H_9Cl_2NO$	C, 49.56 H, 4.11 N, 6.42	C, 49.20 H, 4.59 N, 6.47
XIV*a*	H	H	90–94°(0.6) 51–52°	55	$C_{15}H_{14}N_4O_8$[c]	C, 47.62 H, 3.70 N, 14.81	C, 47.93 H, 3.84 N, 15.55
XIV*b*	7-Cl	H	115–116°(0.5) 70–72°	57	$C_{15}H_{13}ClN_4O_8$[d]	C, 43.63 H, 3.15 N, 13.57	C, 43.69 H, 3.24 N, 13.72
XXIII*a*	H	$ClCH_2CO$	101–102°[e]	94	$C_{11}H_{12}ClNO_2$[f]	C, — H, — N, —	C, — H, — N, —
XXIII*b*	H	$Cl(CH_2)_2CO$	90–91°[e]	72	$C_{12}H_{14}ClNO_2$[f]	C, — H, — N, —	C, — H, — N, —
XXIII*c*	7-Cl	$Cl\text{-}CH_2CO$	129–130°[e]	76	$C_{11}H_{11}Cl_2NO_2$[f]	C, — H, — N, —	C, — H, — N, —
XXIII*d*	7-Cl	$Cl(CH_2)_2CO$	82–83°[e]	89	$C_{12}H_{13}Cl_2NO_2$[f]	C, — H, — N, —	C, — H, — N, —
XXIII*e*	H	$(CH_3)_2NCH_2CO$	120–122°(0.1)	67	$C_{13}H_{18}N_2O_2$	C, 66.64 H, 7.74 N, 11.96	C, 66.18 H, 7.70 N, 11.85
XXIII*f*	H	$(C_2H_5)_2NCH_2CO$	128–132°(0.1)	79	$C_{15}H_{22}N_2O_2$	C, 68.67 H, 8.45 N, 10.68	C, 68.62 H, 8.06 N, 10.92
XXIII*g*	H	C_4H_8NO[g]CH_2CO	114–115°[h]	82	$C_{15}H_{20}N_2O_3$	C, 65.20 H, 7.30 N, 10.14	C, 65.11 H, 7.31 N, 10.14
XXIII*h*	H	$(CH_3)_2N(CH_2)_2CO$	128–130°(0.25)	69	$C_{14}H_{20}N_2O_2$	C, 67.72 H, 8.12 N, 11.28	C, 67.25 H, 8.09 N, 11.29
XXIII*i*	H	$(C_2H_5)_2N(CH_2)_2CO$	130–134°(0.2)	72	$C_{16}H_{24}N_2O_2$	C, 69.53 H, 8.75 N, 10.14	C, 68.94 H, 8.75 N, 10.10
XXIII*j*	H	C_4H_8N[i]$(CH_2)_2CO$	152–154°(0.45)	74	$C_{16}H_{22}N_2O_2$	C, 70.04 H, 8.08 N, 10.21	C, 69.55 H, 8.08 N, 9.56
XXIII*k*	H	C_4H_8NO[g]$(CH_2)_2CO$	154–156°(0.15)	56	$C_{16}H_{22}N_2O_3$[f]	C, — H, — N, —	C, — H, — N, —
XXIII*l*	7-Cl	$(CH_3)_2NCH_2CO$	138–140°(0.2)	74	$C_{13}H_{17}ClN_2O_2$	C, 58.10 H, 6.33 N, 10.42	C, 57.87 H, 6.49 N, 10.46
XXIII*m*	7-Cl	$(C_2H_5)_2NCH_2CO$	148–152°(0.6)	58	$C_{15}H_{21}ClN_2O_2$	C, 60.70 H, 7.08 N, 9.44	C, 60.55 H, 7.16 N, 9.27
XXIII*n*	7-Cl	$(CH_3)_2N(CH_2)_2CO$	148–150°(0.25)	84	$C_{14}H_{19}ClN_2O_2$	C, 59.46 H, 6.72 N, 9.91	C, 59.00 H, 7.07 N, 10.05
XXIII*o*	7-Cl	$(C_2H_5)_2N(CH_2)_2CO$	158–160°(0.05)	84	$C_{16}H_{23}ClN_2O_2$	C, 61.83 H, 7.41 N, 9.01	C, 62.37 H, 7.69 N, 9.54
XXIII*p*	H	$(CH_3)_2N(CH_2)_2$	100–102°(0.2)	69	$C_{13}H_{20}N_2O$	C, 70.87 H, 9.15 N, 12.72	C, 70.51 H, 9.03 N, 12.47
XXIII*q*	H	$(C_2H_5)_2N(CH_2)_2$	100–105°(0.15)	50	$C_{15}H_{26}Cl_2N_2O$[j]	C, 56.10 H, 8.16 N, 8.72	C, 56.61 H, 8.48 N, 8.70
XXIII*r*	H	C_4H_8NO[g]$(CH_2)_2$	130–134°(0.15)	60	$C_{15}H_{22}N_2O_2$	C, 68.67 H, 8.45 N, 10.68	C, 68.06 H, 8.58 N, 10.69
XXIII*s*	H	$(CH_3)_2N(CH_2)_3$	120–122°(0.06)	82	$C_{14}H_{22}N_2O$	C, 71.76 H, 9.46 N, 11.95	C, 71.51 H, 9.36 N, 11.93
XXIII*t*	H	$(C_2H_5)_2N(CH_2)_3$	116–120°(0.3)	98	$C_{16}H_{26}N_2O$	C, 73.24 H, 9.99 N, 10.68	C, 73.05 H, 10.06 N, 10.66
XXIII*u*	H	C_4H_8NO[g]$(CH_2)_3$	138–140°(0.2)	60	$C_{16}H_{24}N_2O_2$	C, 69.53 H, 8.75 N, 10.14	C, 69.87 H, 8.92 N, 9.77
XXIII*v*	H	$3,4(CH_3O)_2C_6H_3(CH_2)_2$	172–175°(0.15)	40	$C_{19}H_{23}NO_3$	C, 72.83 H, 7.40 N, 4.47	C, 72.30 H, 7.67 N, 4.36

Table III—(*continued*)

Number[a]	R	R′	Melting Point or Boiling Point (mm.)	Yield, %	Molecular Formula	Analysis, % Calc.	Found
XXIII*w*	H	$C_4H_7N_2$[k]	102–103°	97	$C_{13}H_{18}ClN_3O$[l]	C, 58.31 H, 6.35 N, 15.70	C, 58.52 H, 6.63 N, 15.84
XXIII*x*	H	NH_2	190–192°[m]	18	$C_9H_{13}ClN_2O$[m]	C, 53.86 H, 6.03 N, 13.96	C, 53.51 H, 6.44 N, 13.65

[a] IR and NMR spectra are consistent with the assigned structures. [b] Recrystallized from ethanol. [c] Picrate (ethanol), m.p. 193–194°. [d] Picrate (ethanol), 189–190°. [e] Recrystallized from benzene–methanol. [f] Characterized through their derivatives which appear in Table III. [g] Morpholino. [h] Recrystallized from ether. [i] Pyrrolidino. [j] Dihydrochloride (isopropanol), m.p. 190–201°. [k] 2-Imidazolin-2-ylmethyl. [l] Hydrochloride (isopropanol–ethyl acetate), m.p. 220–221°. [m] Hydrochloride (isopropanol).

in 100 ml. of anhydrous ether was added with stirring over 1.5 hr. to a suspension of 2.2 g. (0.057 mole) of lithium aluminum hydride in 100 ml. of anhydrous ether. The mixture was refluxed for 3 hr., cooled to 0–5°, and treated with 10 ml. of a saturated sodium sulfate solution. The precipitate was removed by filtration and washed thoroughly with ether. The combined filtrates were dried ($MgSO_4$) and evaporated. The residue was purified by distillation.

5-(3,4-Dimethoxyphenethyl)-2,3,4,5-tetrahydro-1,5-benzoxazepine (XXIII*v*, Table III)—A mixture of 4.5 g. (0.03 mole) of XIV*a* and 4 g. of anhydrous sodium carbonate in 150 ml. of dry benzene was treated dropwise at room temperature with a solution of 7.1 g. (0.035 mole) of 3,4-dimethoxyphenylacetyl chloride (prepared from 5.9 g. of 3,4-dimethoxyphenylacetic acid and 25 ml. of thionyl chloride) in 100 ml. of dry benzene. The mixture was refluxed for 24 hr., filtered, and concentrated under reduced pressure. The residue was dissolved in 200 ml. of ether and washed with 15% sodium hydroxide (3 × 30 ml.), water (3 × 20 ml.), and 20% hydrochloric acid (3 × 30 ml.), respectively. The extract was dried (Na_2SO_4) and evaporated to give 9.8 g. of a dark-green viscous oil, which was dissolved in 100 ml. of dry tetrahydrofuran and added dropwise with stirring to a suspension of 4 g. (0.105 mole) of lithium aluminum hydride in 200 ml. of dry tetrahydrofuran. The mixture was refluxed for 24 hr. and then decomposed at 0–5° with a saturated sodium sulfate solution. The mixture was filtered and evaporated under reduced pressure. The residue (8 g.) was dissolved in 50 ml. of 20% hydrochloric acid, washed with ether (3 × 30 ml.), rendered alkaline with 15% sodium hydroxide, and extracted with ether (3 × 60 ml.). The ethereal extract was dried (Na_2SO_4) and distilled.

5-(2-Imidazolin-2-ylmethyl)-2,3,4,5-tetrahydro-1,5-benzoxazepine (XXIII*w*, Table III)—The procedure of Werner *et al.* (14) was followed. A solution of 7.5 g. (0.05 mole) of XIV*a* in 26 ml. of glacial acetic acid was treated with 1.52 g. (0.05 mole) of paraformaldehyde. The reaction mixture was stirred and cooled to 15° while adding dropwise a solution of 3 g. (0.06 mole) of sodium cyanide in 7.5 ml. of water. The temperature was raised to 45° over a 30-min. period and maintained at 45–50° for 3 hr. After cooling to 35°, 2.2 ml. of 37% formaldehyde was added; after 20 min., 6 ml. of water was added and the precipitated oil was extracted with ether (3 × 30 ml.). The ether solution was washed with 10% sodium hydroxide (3 × 20 ml.), dried (Na_2SO_4), and evaporated under reduced pressure to give 6.6 g. (69%) of the cyanomethyl derivative, b.p. 120–124° (0.25 mm.). A mixture of 6.6 g. (0.035 mole) of the nitrile, 2.6 g. (0.043 mole) of anhydrous ethylenediamine, and 0.08 ml. of carbon bisulfide was heated for 6 hr. at 128–130°. After cooling, the crystalline mass was triturated with 45 ml. of hot water and extracted with ethyl acetate (3 × 50 ml.). The extract was dried (Na_2SO_4) and evaporated to give 7.9 g. (97%) of green viscous oil, which solidified on standing to a pale-yellow crystalline mass, m.p. 102–103°. The hydrochloride was prepared in the usual manner and recrystallized.

5-Amino-2,3,4,5-tetrahydro-1,5-benzoxazepine (XXIII*x*, Table III)—To a solution of 4 g. (0.026 mole) of XIV*a* in 60 ml. of isopropanol and 50 ml. of 10% hydrochloric acid was added at 0–5° a solution of 3.2 g. of sodium nitrite in 15 ml. of water. The mixture was heated at 75° for 3 hr. and treated with another 3.2 g. of sodium nitrite. The red solution was kept at 75° for 12 hr., diluted with 100 ml. of cold water, and concentrated under reduced pressure to 100 ml. An ether extract (3 × 40 ml.) was dried (Na_2SO_4) and evaporated to give 3.2 g. of red oil (*N*-nitroso derivative), which was dissolved in 30 ml. of dry tetrahydrofuran and added dropwise with stirring into a suspension of 1.5 g. (0.04 mole) of lithium aluminum hydride in 100 ml. of tetrahydrofuran. The mixture was heated at reflux for 3 hr., cooled, and decomposed with saturated sodium sulfate solution. After filtration and evaporation under reduced pressure, the residue was converted to a hydrochloride in the usual manner and recrystallized.

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Absorption Characteristics of Solid Dispersed and Micronized Griseofulvin in Man

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Abstract □ Griseofulvin dispersed in polyethylene glycol 6000, prepared by the melting or solvent method, was found to be completely and rapidly absorbed after oral administration to two human subjects. The absorption from the commercially available, micronized griseofulvin was irregular and incomplete (and the average was only 45% of the dose absorbed). In addition, the absorption process from the commercial product was found to continue for up to 3 days after administration.

Keyphrases □ Griseofulvin, solid dispersion—absorption kinetics, man □ Absorption kinetics, oral—griseofulvin solid dispersion, man

Although application of solid dispersion systems to increase rates of dissolution and oral absorption of poorly water-soluble or insoluble drugs was proposed in 1961 (1), its potential and practical application in clinical therapeutics was only recently demonstrated in this laboratory in dogs (2). The drug tested was griseofulvin, which is a water-insoluble and neutral antibiotic. The dispersion of griseofulvin in a solid matrix of polyethylene glycol 6000 was found to give rise to a much higher and more constant absorption than that of the micronized products. This paper reports on the verification of these results in man. Not only does this finding have potential importance to griseofulvin therapy, it also may lead to a new mechanism for the formulation of other poorly soluble or insoluble drugs.

EXPERIMENTAL

Preparation of Dosage Forms—The parenteral solution of griseofulvin, 25 mg./ml. in polyethylene glycol 300, was prepared by a method similar to that described by Rowland *et al.* (3). Dosage forms for the oral administration included micronized griseofulvin in tablet form[1] and griseofulvin dispersed in polyethylene glycol 6000 as described later. Griseofulvin–polyethylene glycol 6000 (1:9 w/w) solid dispersions, prepared by both fusion and ethanol solvent methods, were described previously (4). The 80–200-mesh powders were collected to prepare two final dosage forms: capsule and tablet. Each capsule, size 00, contained 500 mg. mixture, which was equivalent to 50 mg. of griseofulvin. Nondisintegrating tablets, containing 500 mg. of the mixture, were prepared by direct tableting of the powder using an IR pellet-making machine[2]. Griseofulvin–polyethylene glycol 6000 (2:8 w/w) mixture was prepared by the ethanol method (4) and was administered in a size 00 gelatin capsule containing 625 mg. of the mixture (80–200-mesh powders).

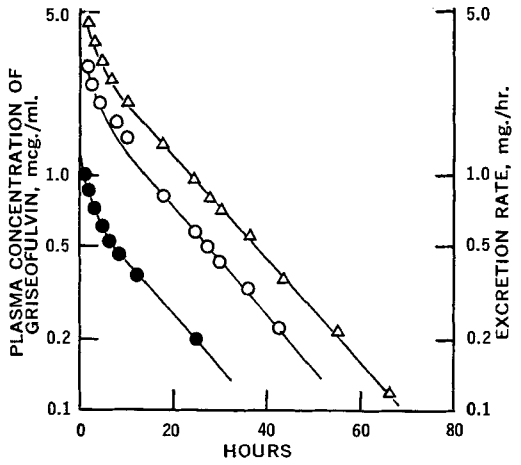

Figure 1—*Plasma griseofulvin levels and 6-demethylgriseofulvin urinary excretion-rate data after intravenous administration of 108 mg. griseofulvin to Subject A. Key:* ●, *plasma griseofulvin levels;* O, *free 6-demethylgriseofulvin; and* △, *total 6-demethylgriseofulvin.*

Procedures for Intravenous Studies—Griseofulvin, 108 mg., was administered through the cephalic vein to a 68-kg. (150-lb.) male subject (Subject A). The method of administration was the same as that described by Rowland *et al.* (3). To prevent the possible precipitation of griseofulvin in the vein and possible hemolysis, the parenteral solution was slowly injected over a 4-min. period, using a Harvard infusion pump[3], and diluted into rapidly flowing saline drip (15 ml./min.) before entering the vein.

Blood samples (5 ml.) were drawn from the opposite arm at 0, 0.5, 1, 2, 3, 4, 5, 8, 12, 24, and 28 hr. Blood samples collected in heparinized test tubes were centrifuged, and the plasma was stored at 4° before assayed. Food and drugs that interfere with the fluorescent assay of griseofulvin in the plasma (especially salicylates) were avoided.

Procedures for Oral Studies—Micronized griseofulvin tablets (500 mg.) and griseofulvin dispersed in polyethylene glycol 6000 (250 mg.) were administered to two normal subjects[4], on empty stomachs, with approximately 200 ml. of water. No food was eaten for at least 2 hr. postadministration. A minimum of 1 week separated each experiment. Both subjects received four studies on the micronized griseofulvin, while Subject A received five studies and Subject B received three studies on the dispersed griseofulvin. The numerical number for each experiment listed in Tables I and II indicates the relative order of each absorption study.

Seven studies were conducted on the 10% griseofulvin dispersion. Among them, the capsule form was used in six studies and the tablet form (nondisintegrating) was used only in one study. The dispersed griseofulvin was all prepared by the melting method, with only one exception (Experiment A-13 in Table II). The 20% griseofulvin dispersion prepared by the solvent method and administered in the capsule form was studied only once (Experiment B-7 in Table II). Urine samples were usually collected at 4, 8, 12, 24, 48, and 72 hr. and occasionally at 2, 6, 36, 60, 84, 96, and up to 102 hr. The volume of urine was recorded, and an aliquot was stored in a plastic container at 4° prior to assay. The urine pH was also measured by a pH meter[5].

Assay of Plasma and Urine Samples—The spectrophotofluorometric assay of griseofulvin in the plasma (3) and UV spectrophotometric assay (5) for the two main metabolites, 6-demethylgriseofulvin and 6-demethylgriseofulvin glucuronide, were previously reported. Duplicate assay was done only on a selective basis.

RESULTS AND DISCUSSION

Intravenous Data—The pharmacokinetic study of griseofulvin in man after intravenous administration was first reported by Rowland

[1] Grifulvin V, 500 mg., Product No. 21470, McNeil Laboratories Inc.
[2] Carver Laboratory Press, model B, Fred S. Carver Inc., Summit, N. J.
[3] Multispeed transmission, mold model 600, Dover, Mass.
[4] Subject A: male, 30 yr., 68 kg. (150 lb.); Subject B: female, 30 yr., 45.3 kg. (100 lb.).
[5] Corning, model 7, Matheson Scientific, Inc.

Table I—Cumulative Total 6-Demethylgriseofulvin Urinary Excretion Data[a] after Oral Administration to Humans of 500-mg. Griseofulvin[b] Tablet

Experiment	Hours 4	8	12	24	48	72	96	192
A-2	—	—	—	—	—	153	—	—
A-5	19	37.8	53.0	85.0	134.7	150.7	—	—
A-7	13.2	27.0	43.7	85.9	139.9	158.4	—	—
A-8	—	17.4	24.8	49.7	72.6	79.1	—	—
B-1	17.8	31.0	43.2	76.8	103.1	124.5	—	—
B-4	17.3	30.5	50.9	92.9	128.8	154.9	173.2	175.2
B-5	25.0	48.8	64.2	88.7	119.9	132.3	135.2	—
B-6	—	46.5	74.2	115.2	151.2	163.2	166.3	—
Average ± *SEM*	18.5 ± 1.9	34.1 ± 4.1	50.6 ± 6.0	84.9 ± 7.4	121.5 ± 9.9	139.5 ± 9.8	158.2 ± 11.7	175.2

[a] Milligrams of total 6-demethylgriseofulvin. [b] Grifulvin.

et al. (3) from this laboratory. The data obtained on Subject A in this study show a biexponential decay of the plasma concentration of griseofulvin, which corresponds to a two-compartment open model with a half-life for the slower disposition phase of 14 hr. (Fig. 1).

The excretion rate of 6-demethylgriseofulvin and total 6-demethylgriseofulvin (summation of 6-demethylgriseofulvin and its glucuronide) peaked during the 1st hr., after which time it was found proportional to the blood level of griseofulvin except at 15 min. These data are shown in Fig. 1. (The initial point at 15 min. is not shown.) The urinary excretion rate of weak acids like 6-demethylgriseofulvin (pKa = 4.5, *Reference 6*) may depend upon the pH of the urine. A higher urinary pH will favor excretion because the renal reabsorption will be minimized by the ionization of the molecule. The urinary pH was found to be within the range of 5.3–6.9, which may play a possible role in the urinary excretion rate in various samples.

It is usually expected that the excretion rate of a compound is proportional to its concentration in the blood. However, in some circumstances the excretion rate of a metabolite can reflect the blood level of its precursor. Such a situation is seen in Fig. 1, where the excretion rates of 6-demethylgriseofulvin, free and total, have the identical slope as that obtained from the blood level of griseofulvin data. One must assume that the 6-demethylgriseofulvin excretion rate is several orders of magnitude faster than the formation rate; therefore, the appearance of 6-demethylgriseofulvin in the urine is rate limited by the formation-rate constant of 6-demethylgriseofulvin. This is in marked contrast to the experiments done in dogs (7) where the excretion data were triexponential, with a slowest phase showing a half-life ranging from 3 to 5 hr., while the griseofulvin disposition half-life was normally less than 1 hr. Unfortunately, it was not feasible to administer 6-demethylgriseofulvin intravenously to man to confirm this contention.

The recovery of 6-demethylgriseofulvin in 72 hr. was 42.5%, and that of the 6-demethylgriseofulvin glucuronide was 22.8%. The total 6-demethylgriseofulvin excretion exceeded five half-lives. Further, when one considers the α-phase, it is inclusive of better than 99% of the infinite excretion values. Therefore, the total recovery from these two known metabolites in this subject is 65.3% of the dose. No intact griseofulvin was detected. The excretion of the intact griseofulvin was reported to be negligible in man (8, 9) and in other animals (8–14). The metabolites were shown to be excreted through the bile after intravenous administration of radioactive griseofulvin-^{14}C to rats and rabbits (14) but not dogs (15). Only about 42% of the radioactivity was recovered in the urine of rats and 77% in rabbits. In dogs, about 60% of 6-demethylgriseofulvin and its glucuronide was recovered from the intravenous dose (7). Hence, fecal excretion and, conceivably, biliary excretion may also be present in man.

Oral Data—The cumulative excretion of total 6-demethylgriseofulvin after oral adminstration of micronized griseofulvin and dispersed griseofulvin in polyethylene glycol 6000 is shown in Tables I and II. The average cumulative excretion of 6-demethylgriseofulvin and total 6-demethylgriseofulvin is shown in Figs. 2 and 3.

The amount of 6-demethylgriseofulvin and total 6-demethylgriseofulvin after oral administration of griseofulvin–polyethylene glycol dispersion was remarkably constant within each subject. In Subject A, 65% of the administered dose was excreted in these experiments, which is identical to the percent excreted after intravenous administration. Therefore, it appears that the amount of metabolites excreted can serve as a basis of evaluating the availability of griseofulvin from oral dosage forms. Almost complete

Table II—Cumulative Total 6-Demethylgriseofulvin Urinary Excretion Data[a] after Oral Administration to Humans of 250 mg. Griseofulvin–Polyethylene Glycol 6000 Solid Dispersion in Capsule or Tablets

Experiment	Formulation	Hours 4	8	12	24	48	72
A-1	10% Griseofulvin–polyethylene glycol 6000	—	—	—	—	—	315.6
A-3	10% Griseofulvin–polyethylene glycol 6000	59.8	112.8	150.6	233.0	292.4	316.6
A-6	10% Griseofulvin–polyethylene glycol 6000	56.8	100.6	139.2	218.0	277.8	310.8
A-10	10% Griseofulvin–polyethylene glycol 6000[b]	50.0	93.4	125.6	200.0	284.8	312.8
A-13	10% Griseofulvin–polyethylene glycol 6000[c]	47.0	88.0	112.0	191.6	285.0	315.2
B-2	10% Griseofulvin–polyethylene glycol 6000	66.4	128.4	167.8	230.4	305.4	324.2
B-3	10% Griseofulvin–polyethylene glycol 6000	38.0	105.2	129.8	225.2	305.6	334.8
B-7	20% Griseofulvin–polyethylene glycol 6000[c]	62.0	110.6	150.6	228.6	303.4	322.0
Average ± *SEM*		54.3 ± 3.7	105.6 ± 5.1	139.4 ± 7.0	218.1 ± 6.1	293.6 ± 4.3	319.0 ± 2.7

[a] All values, milligrams of total 6-demethylgriseofulvin, corrected to an equivalent excretion as if 500-mg. dose administered. [b] Administered in tablet form, while administered in capsule form in all other experiments. [c] Prepared by the ethanol solvent method, while others were prepared by direct fusion method.

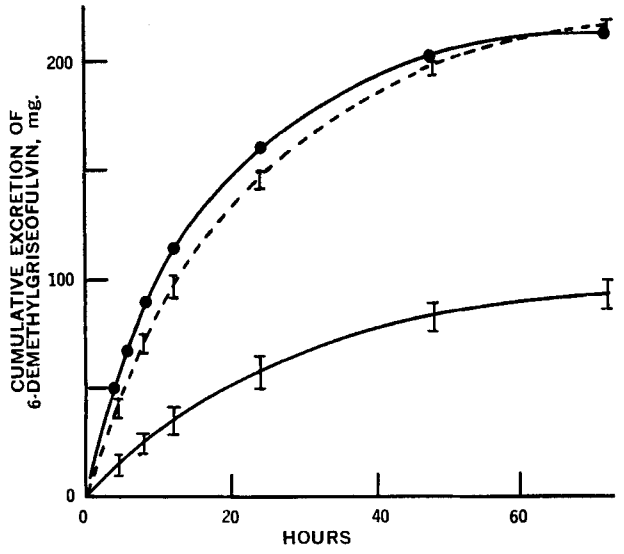

Figure 2—*Cumulative free 6-demethylgriseofulvin urinary excretion data after intravenous and oral administration of griseofulvin to Subjects A and B (intravenous data only for Subject A; others are mean data). Key: ●, intravenous griseofulvin to Subject A; - - -, griseofulvin dispersed in polyethylene glycol 6000; and ——, micronized griseofulvin (all data corrected for 500 mg. griseofulvin).*

absorption was found in all five trials of the dispersed griseofulvin and only 43% from four studies of micronized griseofulvin in Subject A. The cumulative excretion of 6-demethylgriseofulvin from intravenous and oral administration (Figs. 2 and 3) indicates fast and complete absorption from the dispersed griseofulvin in these subjects. The incomplete and rather irregular absorption from the micronized griseofulvin also confirms the findings by

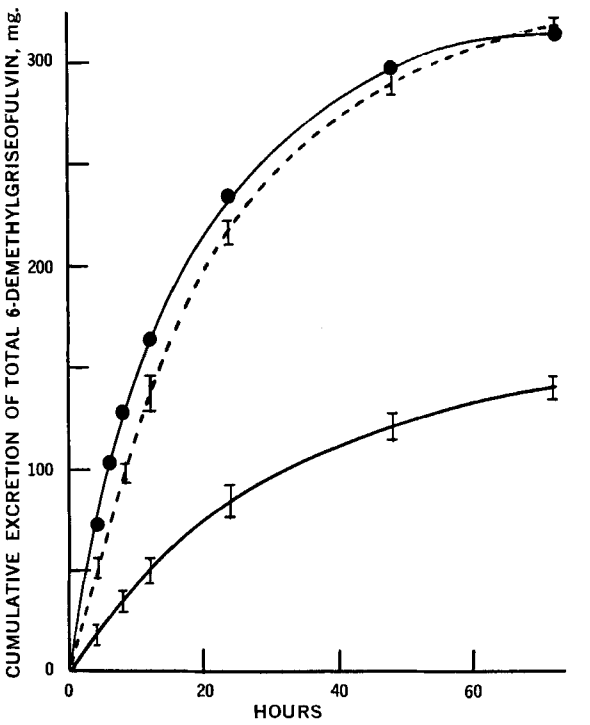

Figure 3—*Cumulative total 6-demethylgriseofulvin urinary excretion data after intravenous and oral administration of griseofulvin to Subjects A and B (intravenous data only for Subject A; others are mean data). Key: ●, intravenous griseofulvin to Subject A; - - -, griseofulvin dispersed in polyethylene glycol 6000; and ——, micronized griseofulvin (all data corrected for 500 mg. griseofulvin).*

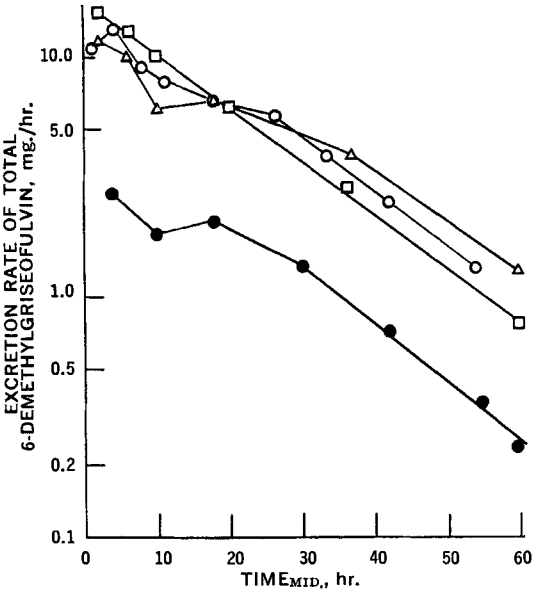

Figure 4—*Plots of urinary excretion rate of total 6-demethylgriseofulvin after oral administration of different dosage forms of griseofulvin to Subject A (all data corrected for 500 mg. griseofulvin). Key: △, 10% griseofulvin–polyethylene glycol 6000 tablet (A-10, melt method); ○, 10% griseofulvin–polyethylene glycol 6000 capsule (A-13, solvent method); □, 10% griseofulvin–polyethylene glycol 6000 capsule (A-3, melt method); and ●, commercial tablet of micronized griseofulvin (A-8).*

Rowland *et al.* (3) that somewhat less than 50% of the micronized drug is absorbed orally in man.

The semilog plots of the excretion rate of total 6-demethylgriseofulvin reveal interesting results (Figs. 4 and 5). For the fused mixture of griseofulvin–polyethylene glycol 6000 administered to Subject A in capsule forms, all the points from 2 to 60 hr. fall on the straight line, with the half-life exactly the same as that obtained from the intravenous study. This indicates that griseofulvin was probably completely absorbed in 2 hr. or less. The same excretion pattern from this dosage form also occurred in Subject B. Although the intravenous study was not carried out in this subject to obtain the disposition half-life and fraction of metabolites excreted, it is believed that the half-life, 13 hr., obtained from the dispersed griseofulvin study reflects the slower disposition half-life of griseofulvin. Furthermore, the total urinary metabolite recovery from three trials (B-2, B-3, and B-7) is very constant. It is, therefore, believed that the absorption from the dispersed system was also fast and complete in this subject.

The absorption of the dispersed griseofulvin in dogs was not as fast and complete as in man. This may be due to the following two reasons. First, the dose given to dogs was about threefold higher than in man on the basis of weight. Since griseofulvin is water insoluble and, therefore, requires a high amount of GI fluids to dissolve 250 mg. of the dose before being absorbed, the absorption of such a high dose in dogs may be partly rate limited by the intrinsic absorption process. Second, it is possible that absorption also may be related to the motility of the GI tract. Since the dogs remained almost stationary during the first few hours postadministration, the expected lower motility of the gut may, therefore, affect the absorption rate.

Analysis of the excretion rate from the micronized preparation shows slow and prolonged absorption. Absorption appeared to continue for 30–40 hr. in Subject A (Fig. 4) and 40–84 hr. in Subject B (Fig. 5), as indicated by the similar slope of the excretion rate after these times with that from the intravenous or dispersed griseofulvin dose. The urinary assay is accurate enough to confirm these data (optical density ranging from 0.3 to 0.7). The absorption process was also found to continue for 40 hr. in dogs, as noted previously (2). It is believed that this was the first instance where absorption was found to continue for more than 3 days for any drug.

An interesting comparison of the data on the dispersed and micronized griseofulvin can be made when the relative availability

Table III—Ratio of Cumulative Total 6-Demethylgriseofulvin Urinary Excretion between Dispersed Griseofulvin and Micronized Griseofulvin after Oral Administration

Griseofulvin	Hours					
	4	8	12	24	48	72
Micronized	1.0	1.0	1.0	1.0	1.0	1.0
Dispersed	2.9	3.1	2.8	2.6	2.4	2.3

is compared for these two different dosage forms, using cumulative excretion of metabolites at different times. As noted in Table III, the relative availability of the dispersed griseofulvin compared to the micronized griseofulvin is about threefold during the first 8 hr. and decreases to 2.3-fold by 72 hr. owing to the prolonged absorption of the micronized form. Symchowicz and Katchen (16) gave three griseofulvin preparations with different dissolution rates to 18 normal subjects for 7 days (500 mg./day). If one utilizes the area under the plasma concentration–time curve reported by these workers to evaluate the relative drug availability, the availability changes with time as shown in Table IV. The availability of Preparation 6 with the slowest dissolution rate was assumed as a standard at all the times. The better availability from Preparations 2 and 6 with faster dissolution rates is quite impressive in the first 8 hr.; however, they become much less significant if one compares the data through to 173 hr.

The complete and fast absorption of the 250-mg. dose of griseofulvin dispersed in polyethylene glycol 6000 is quite striking considering the volume of water required to dissolve this amount of drug. One might ask: Should not the solid dispersion precipitate free griseofulvin and lead to results much like those obtained from the micronized preparation? Chiou and Riegelman (4) pointed out that the solid dispersion can be classified into three groups, namely, eutectic mixture, solid solution, and glass solution, since the fused mixture probably belongs to a combination type. Even if these systems are predominantly solid solutions, the limited amount of the available GI fluids would cause precipitation before

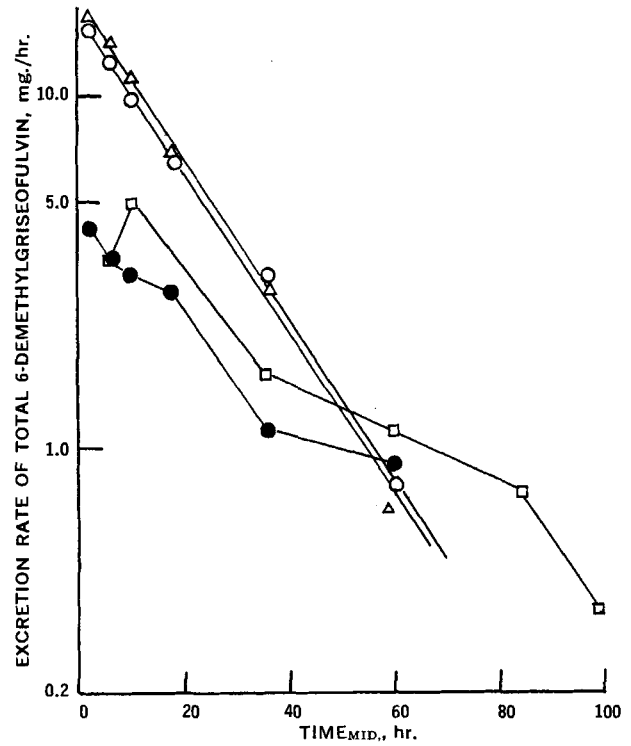

Figure 5—*Plots of urinary excretion rate of total 6-demethylgriseofulvin after oral administration of different dosage forms of griseofulvin to Subject B (all data corrected for 500 mg. griseofulvin). Key: △, 10% griseofulvin–polyethylene glycol 6000 capsule (B-2, melt method); ○, 20% griseofulvin–polyethylene glycol 6000 capsule (B-7, solvent method); □, commercial tablet of micronized griseofulvin (B-4), and ●, commercial tablet of micronized griseofulvin (B-1).*

Table IV—Comparative Availability after Repeated Doses of Griseofulvin in Different Dosage Forms[a]

Preparation	*In Vitro* Dissolution Data[b]	Comparative Areas		
		0–8 hr.	0–25 hr.	0–173 hr.
6	33	1.00	1.00	1.00
2	50	1.22	1.15	1.06
5	94	1.48	1.36	1.19

[a] Data calculated from *Reference 12.* [b] Percent dissolved in 30 min.

absorption. However, the precipitated griseofulvin might be in the colloidal or submicron range which will redissolve rapidly. The system might also form a supersaturated solution, as was found in the *in vitro* studies (4). Griseofulvin dispersed in polyethylene glycol 6000, prepared by the ethanol solvent method, not only showed visible and "colloidal" precipitate but also exhibited a much slower dissolution rate than the fused mixture (Fig. 2 in *Reference 4*). However, *in vivo* tests in man on these mixtures indicated that they were rapidly and completely absorbed (with the exception of A-10 and A-13 studies, which showed slower but complete absorption). Their excretion data are shown in Tables I and II and Figs. 4 and 5. The fusion method appears to give consistently rapid and efficient absorption from capsule form in Subject A. One can conclude, therefore, that fast-release solid solution is not absolutely required, but readily wetted and dispersed particles, colloidal or microfine, are essential for the complete and fast absorption of griseofulvin. In other words, fast and complete absorption of insoluble drugs should be obtained from fast-release solid dispersions: eutectic mixture, solid solution, or glassy solution. This was even noted when the dispersed griseofulvin was compressed into a hard tablet. It, nevertheless, was completely absorbed (Table II and Fig. 4).

Several authors have discussed how griseofulvin is absorbed in man and animals (3, 11, 17). The fast and complete absorption from the dispersed griseofulvin, with its maximum absorption rate in the first 2–3 hr., supports the idea that the upper small intestine is the major absorption site. It appears that there may be a limited time of sojourn through a limited region of the GI tract where conditions for optimum absorption exists. In spite of this, absorption continued for more than 20 hr. in one of the tests conducted on the dispersed griseofulvin preparation (Fig. 4, A-13). This could possibly be explained by a certain fraction of griseofulvin passing through the main absorption site without being absorbed, possibly due to a variation in motility such that a shorter time was available at the optimum absorption sites. Nevertheless, in this instance the drug was ultimately absorbed completely, probably due to the microfine size of the precipitated griseofulvin. In the case of the micronized preparation, the poorly wetted and agglomerated griseofulvin granules probably limit the surface available to the dissolution process; this, therefore, becomes rate limiting. The continuous absorption for more than 3 days might be rationalized by the supposition that the aggregated or agglomerated coarse particles are adsorbed to the intestinal invaginations where the particles slowly dissolve and are absorbed.

Since the percent of absorption from the dispersed griseofulvin is more than two times higher than the commercial micronized product, it is suggested that the present dose, 500 mg./day, can be reduced to 50%, *i.e.*, 250 mg./day, with a solid dispersion system. It is also believed that results similar to those from this study might be achieved with other insoluble drugs.

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J. A. MOLLICA, C. R. REHM, J. B. SMITH, and H. K. GOVAN

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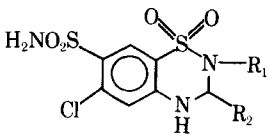
Keyphrases □ Benzothiadiazines—hydrolysis, mechanism, pH–rate profiles □ Hydrochlorothiazide, 3- and 2-ethyl analogs—hydrolysis, mechanism, pH–rate profiles □ Hydrolysis, rates and mechanism—benzothiadiazines

Benzothiadiazines form one of the most important classes of compounds used as diuretics. They have been divided into two main categories: "thiazides," which are 2*H*-1,2,4-benzothiadiazine-1,1-dioxides, and "hydrothiazides," which are 3,4-dihydro-[2*H*]-1,2,4-benzothiadiazine-1,1-dioxides. The former is represented by chlorothiazide, which is formed by condensing 5-chloro-2,4-disulfamylaniline (hereafter referred to as "disulfonamide") with formic acid; the latter is represented by hydrochlorothiazide, which is formed by condensing disulfonamide with formaldehyde (1).

Although these compounds form an important class of drugs, few publications concerning their routes and mechanisms of degradation have appeared. Yamana *et al.* (2) reported on the hydrolysis of chlorothiazide, and the stability of this compound was also studied by Charnicki *et al.* (3) and Baer *et al.* (4). Hydrothiazides, however, have not been studied in detail. Previous reports from this laboratory considered the analysis of hydrochlorothiazide (5) and presented the pH–rate profile for its hydrolysis (6). Yamana *et al.* (7) also published a similar report covering the pH–rate profile and reported on the hydrolysis in 1 *N* NaOH (8).

The study of the hydrolysis of hydrochlorothiazide was extended in this laboratory to explore the validity of the hypotheses that the reaction is reversible and that the extent of reaction is independent of pH. In addition, factors that influence the rate of reaction were investigated in order to postulate a possible mechanism for the hydrolysis. The present article is an extensive report on the hydrolysis of hydrochlorothiazide and its 3-ethyl (Su 9604) and 2-ethyl (Su 6835) analogs.



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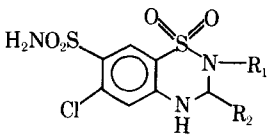

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Formaldehyde, 37%, reagent grade, was assayed according to USP XVI; ethylene glycol monomethyl ether, reagent grade, was used directly.

Buffers—Reagent grade chemicals were used without further purification in the preparation of buffer solutions, with the exception of imidazole which was recrystallized from benzene, using Norit as a decolorizing adsorbent, m.p. 89–90°. The following buffers were used: pH 1–2.5, hydrochloric acid; 2.5–4, formate; 4–6, acetate; 5–7.5, imidazole; 5–11, phosphate; 9–10, borate; and 11–13, hydroxide. All pH measurements of buffer solutions were made at ambient temperatures, except for imidazole buffers which were also measured at 60°. The meter–electrode system was standardized against the standard buffers recommended by Bates (10).

Ionization Constants—The dissociation constants of hydrochlorothiazide were determined by potentiometric titration, using a Radiometer titrator and a thermostated titration cell at 60°. Calculations following the method of Noyes (11), for substances with two ionizing groups with pKa values less than 2.7 units apart, were made. Values of 8.6 and 9.9 were obtained, respectively, for pKa_1, and pKa_2. The Merck Index gives values of 7.9 and 9.2, respectively (12). Spectrophotometric measurements yielded only one pKa value for the compounds: hydrochlorothiazide, 8.7; *N*-ethyl hydrochlorothiazide, 9.5; and *C*-ethyl hydrochlorothiazide, 8.8, all at 25°.

Equipment—The pH values were measured with a Radiometer model 25SE meter, using a K401 calomel electrode and a G202B glass electrode. A Radiometer titrator type TTTla and titragraph type SBR2C were used for the determination of the ionization constants by potentiometric titration. Spectrophotometric measurements were made using a Beckman model DU, and spectra were obtained with a Cary model 11 or 14 spectrophtometer. Constant-temperature baths were maintained to within 0.05° of specified temperatures.

Procedures—Several procedures were utilized to monitor the reactions:

1. Colorimetric determination of 5-chloro-2,4-disulfamylaniline by reaction with nitrous acid and coupling with chromotropic acid, according to the previously published procedure (5), was the primary method used in the kinetic studies.

2. There was sufficient difference in the UV absorption spectra of hydrothiazides and disulfonamide so that rate data could be obtained by monitoring the decrease in absorbance at constant wavelength (λ = 272 nm.) as a function of time.

3. Formaldehyde was determined colorimetrically with phenylhydrazine and potassium ferricyanide, utilizing a modified procedure of Tanenbaum and Brecker (13).

The determination of formaldehyde was the primary method employed to monitor the hydrolysis of *N*-ethyl hydrochlorothiazide. In addition, the 2-(*N*-ethylsulfamyl)-4-sulfamyl-5-chloroaniline produced upon hydrolysis was determined by the colorimetric procedure. This yields a colored compound with essentially the same spectrum as that produced by the unsubstituted disulfonamide. Rate constants calculated by following the appearance of formaldehyde or of the aniline agree to within 5%.

Pseudo-first-order rate constants were calculated by taking the initial slopes of plots of log $(a - x)$ *versus* time, where a is the initial concentration of hydrothiazide and x is the concentration of disulfonamide produced, and/or from plots of:

$$\log \frac{1 - (1 - Xe)(1 - X)}{(1 - X) - (1 - Xe)} \textit{ versus } \text{time}$$

where X is the fraction reacted at time, t, and Xe is fraction reacted at equilibrium. In alkaline solution, rate constants were evaluated by determining the initial rate of formation of disulfonamide produced over the first few percent of reaction.

RESULTS AND DISCUSSION

The hydrolysis of hydrochlorothiazide and *C*-ethyl hydrochlorothiazide approaches a state of equilibrium which is invariant with pH. The fraction reacted, Xe, defined as $1 - C_e/C_0$, where C_e and C_0 are, respectively, the equilibrium and initial concentration of hydrothiazide, as a function of pH is given in Table I. The extent of reaction does vary with initial concentration, yet the equilibrium constant remains invariant within the experimental error as expected. *N*-Ethyl hydrochlorothiazide hydrolyzes very slowly, and it was not possible to obtain equilibrium values in all cases. For example, at pH 7.9 the compound is only 12% hydrolyzed after 2 months at 60°.

Table I—Extent of Reaction and Equilibrium Constant as a Function of pH and Concentration

Compound	pH	C_0(× 10^4 *M*)[a]	*Xe*[b]	*K*(× 10^4 *M*)[c]
Hydrochlorothiazide	1.50	6.74	0.41	1.9
	4.61	6.65	0.41	1.9
	4.62	16.8	0.28	1.9
	4.60	67.2	0.16	2.2
	7.41	6.72	0.40	1.8
	8.18	67.2	0.16	2.0
	4.38[d]	6.78	0.42	2.1
C-Ethyl hydrochlorothiazide	3.60	6.14	0.80	20
	5.29	6.14	0.80	20
	7.75	6.14	0.81	21
	9.80	6.14	0.79	18
N-Ethyl hydrochlorothiazide	3.56	6.14	0.43[e]	—
	4.44	6.14	0.43	—
	5.86	6.14	0.50	—

[a] Initial concentration of hydrothiazide. [b] $Xe = 1 - C_e/C_0$, where C_e and C_0 are, respectively, the equilibrium and initial concentration of hydrothiazide. [c] K = (aldehyde) (disulfonamide)/(hydrothiazide). [d] Formation of hydrochlorothiazide from equimolar (6.78×10^{-4}) concentrations for disulfonamide and formaldehyde. [e] Extent of reaction calculated at 1728 hr.

Formaldehyde is essentially completely hydrated in aqueous solution; however, its rate of dehydration should not be rate controlling under the conditions of these experiments (14). In alkaline solution, however, the disproportionation of formaldehyde into methanol and formic acid becomes significant and the reaction can be driven to completion in basic media. Hence, rate constants for alkaline hydrolysis were obtained from the initial slopes of plots of disulfonamide produced as a function of time (Fig. 3), and at other pH

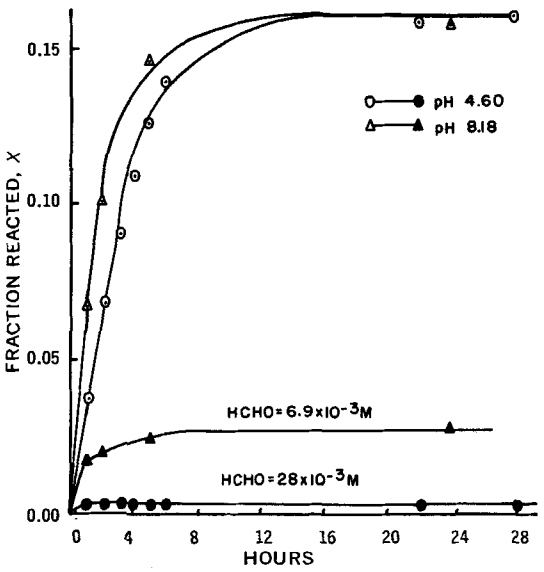


Figure 1—*Plot showing the extent of reaction,* X, *for the hydrolysis of hydrochlorothiazide in the presence of an added equimolar amount and an excess of formaldehyde.* $C_0 = 6.71 \times 10^{-3}$ M *hydrochlorothiazide;* T = 60°.

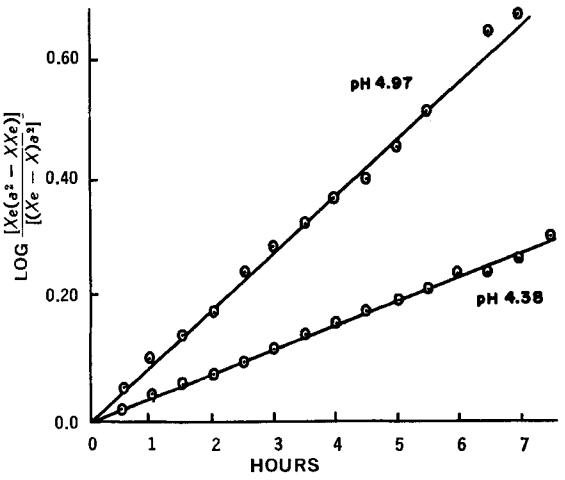


Figure 2—*Plots of the integrated equation for the formation of hydrochlorothiazide from equimolar concentrations of disulfonamide and formaldehyde at two pH values.* $C_0 = 6.78 \times 10^{-4}$ M; T = 60°.

values they were obtained by plotting the data as described previously. Figures 4 and 5 show typical plots of the integrated equation.

Although propionaldehyde does not undergo the Cannizzaro reaction, it can undergo base-catalyzed condensation. However, hydrolysis of *C*-ethyl hydrochlorothiazide was only studied in the pH range 3–10 and was carried to equilibrium at all pH values studied.

The effect of ionic strength on the hydrolysis of hydrochlorothiazide at pH 4.6 was determined. Variation of ionic strength from 0.2 to 1.3 had no effect on the rate of reaction when the rate constants, $k_{obsd.}/a_{OH^-}$, were compared.

Arrhenius plots were made at pH 1.47 and 4.01. The activation energy was 25.3 kcal. at pH 1.47 and 30.6 kcal. (18.7 kcal. when corrected for the ΔH of water) at pH 4.01. This may be compared to the value of 27.7 kcal. reported for the hydrolysis of hydrochlorothiazide in 1 *N* sodium hydroxide solution (8). The extent of reaction is relatively insensitive to temperature, as can be seen from the data in Table II. The rate constant extrapolated to 25° is approximately 4×10^{-5} hr.$^{-1}$ at pH 4. This is in fair agreement with a value calculated utilizing the equilibrium constant of 2×10^{-4} M^{-1} and a half-life of 70 min. for the reaction of disulfonamide with 0.1 *M* formaldehyde at 25° (the reverse reaction follows pseudo-first-order kinetics in the presence of excess formaldehyde).

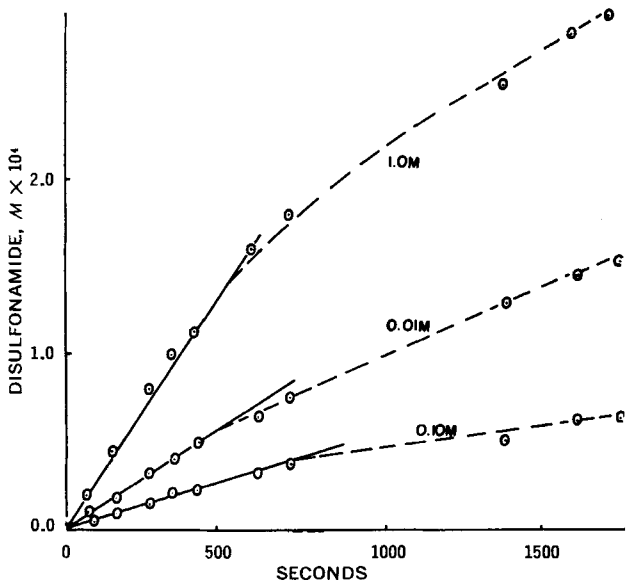


Figure 3—*Concentration of 5-chloro-2,4-disulfamylaniline produced from the hydrolysis of hydrochlorothiazide at several hydroxide-ion concentrations;* T = 60°. *Rate constants were obtained from the initial slopes.*

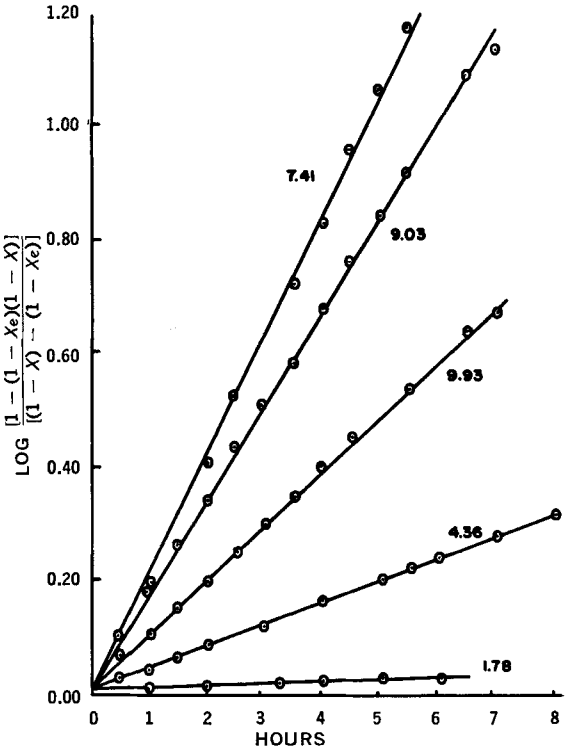


Figure 4—*Plot of the integrated form of the rate equation for an equilibrium reaction of the type* A = B + C *for the hydrolysis of hydrochlorothiazide at several pH values;* T = 60°.

The data in Table III indicate that there is only a slight buffer catalysis. This is in accord with observations made by Yamana *et al.* (7). Analogous reactions such as the base-catalyzed decomposition of formocholine chloride (15) and the hydrolysis of benzylidene-*tert*-

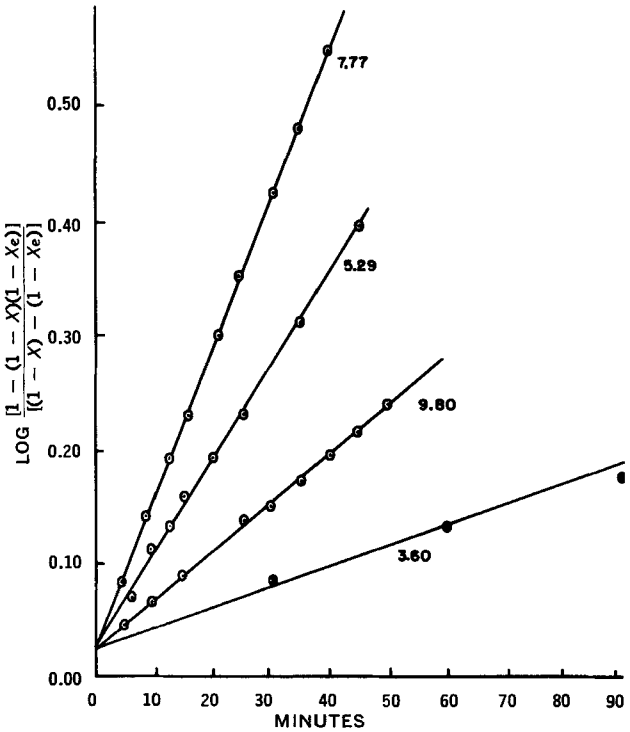


Figure 5—*Plot of the integrated form of the rate equation for an equilibrium reaction of the type* A = B + C *for the hydrolysis of* C*-ethyl hydrochlorothiazide at several pH values;* T = 60°.

Table II—Rate Constants and Extent of Reaction for Hydrolysis of Hydrochlorothiazide at Several Temperatures and at pH 1.47 and 4.01

Temperature	pH 1.47 10^3k, hr.$^{-1}$	pH 1.47 Xe^a	pH 4.01 10^3k, hr.$^{-1}$	pH 4.01 Xe^a
45°	1.46	0.35	1.20	0.44
53°	3.98	0.37	4.03	0.38
62°	10.8	0.43	14.5	0.42
70°	26.7	0.47	42.3	0.47

[a] $C_0 = 6.7 \times 10^{-4}$ *M*.

butylamine (16) are also not subject to buffer catalysis. The change in the extent of reaction for the hydrolysis of hydrochlorothiazide with imidazole buffers was at first surprising. Although one expects a primary amine or other carbonyl reagent to react readily with formaldehyde [it was reported (5) previously that hydroxylamine will bring the reaction to completion by reacting with the formaldehyde produced], the apparent reaction of formaldehyde with a secondary and/or a tertiary amine was at first unexpected. However, Kallen and Jencks (17) reported the formation of protonated hydroxymethylamines of secondary amines and their data strongly suggest that imidazole reacts with formaldehyde. The increase in extent of reaction, therefore, with both increasing buffer concentration and pH agrees with the conclusions in their study regarding the complex equilibrium between imidazole and formaldehyde.

The bell-shaped pH–rate profiles obtained (Fig. 6) are relatively complex and not amenable to simple interpretation. The discussion will be of the pH–rate profile for hydrochlorothiazide, the compound is studied in greatest detail. However, the behavior of the other two compounds is analogous.

Although the inflection point on the descending portion of the curve is close to the ionization value for hydrochlorothiazide (hydrothiazides and the disulfonamides behave as monoprotic or diprotic acids, depending on the extent of substitution with pKa values in the range 8–10), the left inflection point (pH 4.5) does not correspond to a dissociation constant. The bell-shaped profile must be, therefore, an expression of the kinetics of the reaction rather than of the ionization constants of the reactants. This behavior has been recognized in a number of reactions, particularly those involving carbonyl groups such as Schiff base formation and hydrolysis (18–20) and condensation of formaldehyde with tetrahydrofolic acid (21) and other tetrahydroquinoxalines (22), and may be described as a change in the rate-determining step of the reaction with changing pH.

A change in the rate-determining step implies the presence of at least two steps and one intermediate. No intermediate was isolated or observed by spectroscopic, chromatographic, or polarographic methods. However, it is reasonable to postulate the presence of an imine or a more reactive species, the cationic imine, $R—\overset{+}{N}(—H)=CH_2$, which upon hydration gives the hydroxymethylamine, $R—NH—CH_2OH$. Decomposition of this carbinolamine then yields formaldehyde and disulfonamide or the appropriate aldehyde and sulfonamide.

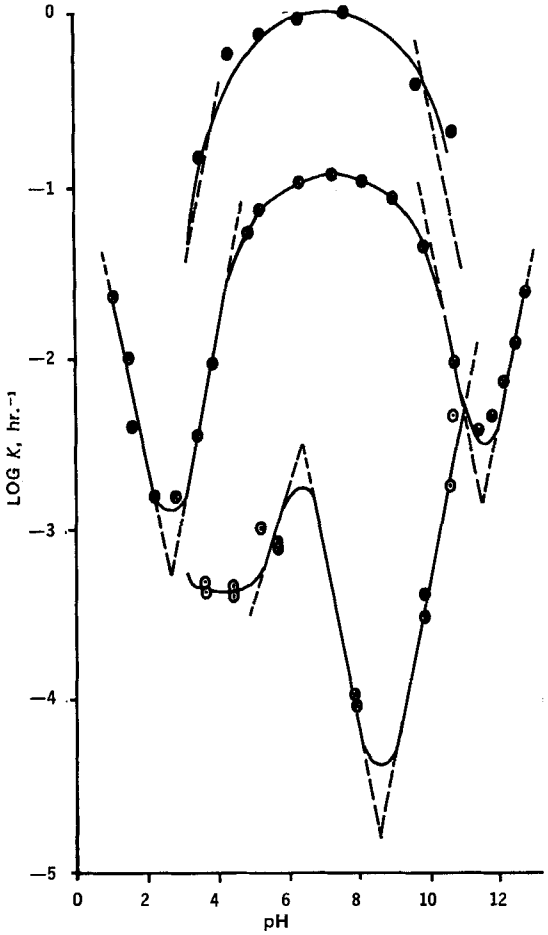


Figure 6—*The pH–rate profiles for the hydrolysis of* C*-ethyl hydrochlorothiazide, hydrochlorothiazide, and* N*-ethyl hydrochlorothiazide at 60° (top to bottom). Dashed lines are drawn with slope of +1 or −1.*

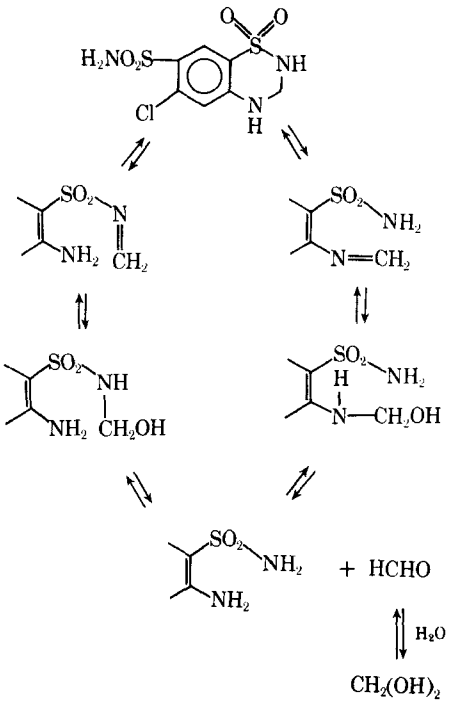
This type of scheme has been presented to account for the observed pH–rate profiles of other carbonyl group reactions and appears reasonable in this case also. Although there is no direct evidence of an intermediate in this case, formamide intermediates were reported in the hydrolysis of chlorothiazide (2), and analogous compounds were reported from the reaction of *o*-aminosulfonamides with various aldehydes, formic acid, and *ortho*-esters (23–25).

The pH–rate profile, the lack of significant buffer catalysis, the reports that aldehydes react with both the aniline nitrogen and the sulfonamide nitrogen, and the other evidence cited here all support the overall reaction scheme shown in Scheme I.

Table III—Effect of Various Buffers and Concentration on Extent and Rate of Reaction[a]

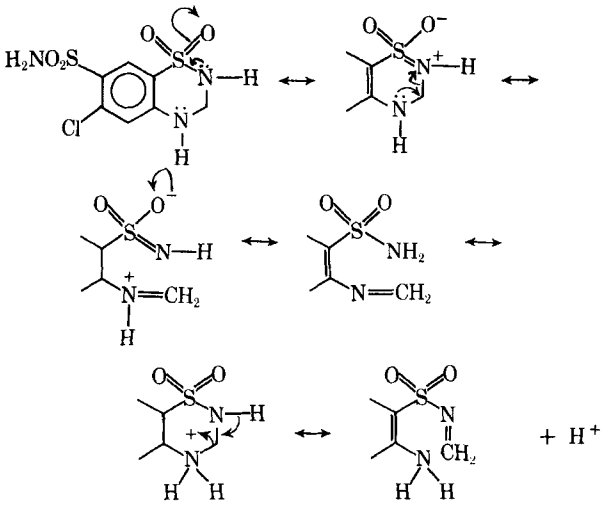
Compound	pH	Buffer, *M*	10^3k, hr.$^{-1}$	*Xe*
Hydrochlorothiazide	4.49	Acetate 0.12	2.76	0.42
	4.49	Acetate 0.24	2.74	0.41
	4.49	Acetate 0.36	2.76	0.41
	4.49	Acetate 0.48	2.79	0.41
C-Ethyl hydrochlorothiazide	5.20	Imidazole 0.10	59.8	0.80
	5.20	Imidazole 1.0	61.8	0.78
	7.75	Imidazole 0.10	87.1	0.81
	7.75	Imidazole 1.0	98.1	0.78
Hydrochlorothiazide	5.19	Imidazole 1.0	5.66	0.54
	5.31	Imidazole 0.10	6.27	0.43
	8.35	Imidazole 0.10	78.0	0.47
	8.35	Imidazole 1.0	77.9	0.70
C-Ethyl hydrochlorothiazide	10.78	Phosphate 0.05	20.4	0.81
	10.80	Phosphate 0.50	19.2	0.78

[a] $T = 60°$; hydrochlorothiazide $C_0 = 6.7 \times 10^{-4}$ *M*; *C*-ethyl hydrochlorothiazide $C_0 = 6.1 \times 10^{-4}$ *M*.



Scheme I

It can be shown that ring opening can occur *via* several pathways. Two examples are given in Scheme II. Either pathway yields an



Scheme II

imine intermediate and could account for the observed behavior.

The *N*-ethyl substitution in Su 6835 undoubtedly accounts for its relative unreactivity, being several orders of magnitude less reactive than hydrochlorothiazide or *C*-ethyl hydrochlorothiazide, since the replacement of the acidic proton suppresses ring opening. The electron-donating effect of the ethyl substitution at the 3-position, however, does accelerate the overall rate relative to hydrochlorothiazide; the observed effects of ethyl substitution in the 2- and 3-positions are, therefore, in accord with what one would predict and support the postulated mechanism.

It is postulated, therefore, that the overall reaction occurs as shown in Scheme I. The rate-determining steps could vary from acid/base-catalyzed ring opening through hydration of the free or cationic imine by water and/or hydroxide ion to the decomposition of the carbinolamine to yield disulfonamide and aldehyde.

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Separation and Quantitative Assay of Furazolidone and Nifuroxime in Suppositories by Magnesium Silicate Adsorption Column Chromatography

PAMELA F. WOJTOWICZ and JOSEPH J. DILIBERTO*

Abstract □ A rapid and quantitative column chromatographic separation method, in which a patented activated magnesium silicate is used as the adsorbent, is presented for the analysis of a suppository containing furazolidone and nifuroxime. Nifuroxime is eluted from the column with chloroform, and furazolidone is eluted with 5% glacial acetic acid in dimethylformamide. The separated nitrofurans are then compared spectrophotometrically to respective standards.

Keyphrases □ Suppositories, furazolidone–nifuroxime—separation, analysis □ Furazolidone–nifuroxime suppositories—separation, analysis □ Nifuroxime–furazolidone suppositories—separation, analysis □ Column chromatography—separation, furazolidone–nifuroxime suppositories □ UV spectrophotometry—analysis, furazolidone–nifuroxime suppositories

The current official assay procedure (1) for furazolidone and nifuroxime in furazolidone and nifuroxime suppositories NF[1] involves the column chromatographic separation of the nitrofurans, using a mixture of an acid-activated montmorillite[2] and a purified siliceous earth[3] as the adsorbent, followed by UV spectrophotometric quantitation. In this procedure, the variability of the montmorillite presents several problems (2). It is difficult to obtain the suggested elution rate of 0.5 ml./min., even when vacuum is applied. The assay time is critical because furazolidone and nifuroxime undergo degradation through hydrolysis and exposure to light. When vacuum is applied, fine particles of adsorbent accompany the active ingredient effluents and cause high background interference, making quantitation difficult, particularly for furazolidone. Reportedly, up to 20% of the furazolidone may be irreversibly adsorbed onto the column packing.

Methods for the detection and quantitation of furazolidone and nifuroxime, utilizing TLC, were reported. Zoni and Lauria (3) and Bortoletti and Perlotto (4) eluted the separated nitrofurans from a developed silica gel plate and determined their concentrations spectrophotometrically. Boice *et al.* (2) determined the concentrations of furazolidone and nifuroxime on a developed plate with a recording densitometer.

The need for a rapid, accurate, and reliable method for determining furazolidone and nifuroxime in combination, without the use of highly specialized equipment, prompted this investigation.

EXPERIMENTAL

Apparatus—The following were used: UV-visible spectrophotometer; and glass column, 250 mm. long by 10 mm. i.d., fitted with a fritted-glass disk and a Teflon stopcock.

Solvents—Chloroform, methanol, and dimethylformamide, all spectral grade, were used.

Adsorbent—Patented activated magnesium silicate[4] was used.

Acid–Dimethylformamide Solution—Dilute 10 ml. of reagent grade glacial acetic acid to 200 ml. with dimethylformamide.

Standard Solutions—Dissolve NF reference standard furazolidone, 5 mcg./ml., in the acid–dimethylformamide solution; dissolve NF reference standard nifuroxime, 7.5 mcg./ml., in methanol–chloroform (1:9).

Sample Preparation—Dilute a sample equivalent to approximately 37.5 mg. of nifuroxime to 500 ml. with methanol.

Chromatographic Column Preparation—Transfer 5.0 g. of the adsorbent to a chromatographic column, and tap gently to settle the adsorbent. Insert a small glass wool plug above the adsorbent.

Caution: Protect chromatographic column, standard solutions, and all sample solutions from light.

Assay Procedure—Place a 50-ml. volumetric flask under the column, and pipet a 5.0-ml. aliquot of the sample preparation onto the column. Allow the solution to pass into the glass wool, and wash the inner wall of the column with a little chloroform. Allow this chloroform wash to pass into the column, and then elute with 50 ml. of chloroform at a rate of 1–2 drops/sec. Discontinue the elution, wash the column tip, and dilute to volume with chloroform. Reserve this eluate for the quantitative determination of nifuroxime.

Add the acid–dimethylformamide solution to the column, and collect 5 ml. of the column eluate in a graduated cylinder. Replace the graduated cylinder with a 50-ml. volumetric flask, and continue the elution with the dimethylformamide solution until approximately 48 ml. has been collected[5]. Dilute to volume with acid–dimethylformamide, and reserve for the quantitative determination of furazolidone.

Quantification—Protect eluted solutions from light; read absorbances within 4 hr., using 1-cm. quartz cells.

Nifuroxime—Record the UV absorbances of the reference standard and sample solutions from 430 nm. with methanol–chloroform (1:9) as the reference solution, reading the absorbance maximum at 338 nm.

Furazolidone—Record the UV absorbances of the reference standard and sample solutions from 470 nm. with the acid–dimethylformamide solution as the reference, reading the absorbance maximum at 370 nm.

Calculate the percentages of furazolidone and nifuroxime present in the sample.

[1] Tricofuron is a registered trademark of Eaton Laboratories, Division of Norwich Pharmacal Co., Norwich, N. Y.
[2] Filtrol Grade No. 19 supplied by Filtrol Corp., Los Angeles, Calif.
[3] Celite 545, supplied by Johns-Manville, New York, N. Y.
[4] Florisil, 60–100 mesh, Fisher Chemical Co., Catalog No. F-100.
[5] The exact volume of eluting solvents is not critical. Elution patterns indicate that 100% of the nifuroxime present is recovered from the column in the first 10 ml. of chloroform and 100% of the furazolidone in the first 25 ml. of acid–dimethylformamide.

Table I—Recovery Data for Mixtures of Nifuroxime and Furazolidone

Analysis Number	Milligrams Recovered—Nifuroxime	Milligrams Recovered—Furazolidone
Recoveries for Synthetic Mixture		
1	36.3	25.2
2	36.8	25.0
3	36.5	25.2
4	36.5	25.1
5	36.3	24.9
6	36.4	24.9
7	36.3	25.1
8	36.4	24.8
9	36.2	25.0
10	36.3	25.0
Milligrams added	37.5	25.6
Milligrams recovered ± *SD*	36.4 ± 0.2	25.0 ± 0.1
Percent recovered ± *SD*	97.1 ± 0.4	97.7 ± 0.6
Recoveries for Drugs Added to Commercial Product		
1	18.6	12.3
2	18.8	12.1
3	18.7	12.0
4	18.6	11.7
5	18.7	12.3
Milligrams added	19.2	12.2
Milligrams recovered ± *SD*	18.7 ± 0.1	12.1 ± 0.2
Percent recovered ± *SD*	97.3 ± 0.4	99.0 ± 1.6

RESULTS AND DISCUSSION

The accuracy of the proposed method is based upon the results of 10 replicate analyses of a synthetic solution, containing 37.5 mg. of nifuroxime and 25.6 mg. of furazolidone in 500 ml. of methanol, and the results of five replicate determinations of a previously analyzed commercial preparation, with 19.2 mg. of nifuroxime and 12.2 mg. of furazolidone added to the sample solution. Better than 97% of each ingredient was recovered. The results of these analyses and statistical treatment of the data are presented in Table I.

Quintuplicate assays were performed on four lots of a commercial product, declared to contain 0.375% nifuroxime and 0.25% furazolidone. The results of these determinations and the statistical treatment of the data are presented in Table II. The replicate results from the recovery analyses and from the product assays were used to determine statistically the precision of the data.

In a limited study, the product was exposed to heat and UV radiation to force degradation of the active ingredients. In the original assay, the nifuroxime concentration was 0.367 and 0.337% after degradation. Furazolidone was quantified at 0.239% originally and 0.141% after degradation.

No change in peak shape or shift in absorbance maxima was noted in the spectra of degraded sample ingredients. This indicates that the column procedure presented is capable of distinguishing between active ingredients and degradation products. The higher degree of furazolidone decomposition indicates that its instability probably accounts for the lower original assay results in the commercial product.

Table II—Analysis of Commercial Samples of Nifuroxime and Furazolidone[a]

Sample	Analysis Number	Percent Found—Nifuroxime	Percent Found—Furazolidone
A	1	0.365	0.237
	2	0.367	0.238
	3	0.368	0.239
	4	0.368	0.240
	5	0.368	0.240
	Percent ingredient ± *SD*	0.367 ± 0.001	0.239 ± 0.001
	Percent declaration ± *SD*	97.9 ± 0.3	95.6 ± 0.5
B	1	0.352	0.233
	2	0.352	0.236
	3	0.354	0.233
	4	0.356	0.231
	5	0.355	0.230
	Percent ingredient ± *SD*	0.354 ± 0.002	0.233 ± 0.002
	Percent declaration ± *SD*	94.4 ± 0.5	93.0 ± 0.9
C	1	0.362	0.233
	2	0.364	0.233
	3	0.362	0.231
	4	0.363	0.233
	5	0.365	0.231
	Percent ingredient ± *SD*	0.363 ± 0.001	0.232 ± 0.001
	Percent declaration ± *SD*	96.8 ± 0.4	92.9 ± 0.4
D	1	0.362	0.237
	2	0.363	0.234
	3	0.365	0.235
	4	0.365	0.233
	5	0.364	0.235
	Percent ingredient ± *SD*	0.364 ± 0.001	0.235 ± 0.002
	Percent declaration ± *SD*	97.0 ± 0.3	93.9 ± 0.6

[a] Declared to contain 0.375% nifuroxime and 0.25% furazolidone.

The results of this study indicate that an activated magnesium silicate adsorbent may be used successfully to separate nifuroxime and furazolidone quantitatively. The column chromatographic procedure is sufficiently accurate and precise for routine pharmaceutical analysis.

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Colorimetric Determination of Physostigmine in Pharmaceuticals

S. K. WAHBA KHALIL and B. D. PATTERSON

Abstract □ A colorimetric method for determination of physostigmine is suggested based on measurement of the yellow color produced when physostigmine dithiocarbamate reacts with copper. The color obeys Beer's law in the range of 65–400 mcg. The reaction was found to be specific for physostigmine in the presence of other alkaloids and nitrogen-containing substances, using a pH 8.6 buffer and sodium bicarbonate.

Keyphrases □ Physostigmine, analysis—colorimetric measurement of physostigmine dithiocarbamate–copper complex □ Colorimetry —analysis, physostigmine

A number of methods have been proposed for the quantitative determination of physostigmine in solution. The majority of the older methods have not proved completely satisfactory, either because of a lack of specificity for physostigmine or because of a lack of sensitivity for microquantities of the alkaloid.

Teare and Borst (1) recently developed a gas chromatographic method using the trimethylsilyl derivative of physostigmine. This method requires the use of equipment not always readily available, but it does represent a procedure for accurate determination of small quantities of physostigmine. In 1968, Fletcher and Davis (2) developed a method based on measuring the intensity of the yellow color of a physostigmine nitroso compound.

An earlier procedure, of Teare and Taylor (3), is an indirect titration method involving measurement of methylamine both before and after alkaline hydrolysis. Thus, physostigmine can be determined in the presence of its degradation products. However, the possibility exists that other ingredients in a preparation may yield methylamine on alkaline hydrolysis to interfere with the determination.

This paper describes a procedure based on the formation of a copper dithiocarbamate complex which is quantitative for microquantities of physostigmine. The method is a modification of a basic method for determination of primary and secondary amines (4, 5, 6). The modification permits measurement of physostigmine in the presence of its degradation products and other chemicals commonly included with physostigmine in pharmaceutical products. This procedure was applied to several commercial preparations containing physostigmine.

EXPERIMENTAL

Reagents—The following were used (all of analytical grade): physostigmine salicylate; saturated sodium bicarbonate solution; barbiturate buffer, pH 8.6, or an alkaline borate buffer, pH 8.6; copper sulfate 5% solution; benzene; and carbon disulfide.

Procedure—To 1 ml. of an aqueous solution of physostigmine salicylate, 0.05%, in a test tube, add 2 ml. of the buffer solution, 1 ml. saturated sodium bicarbonate solution, 1 ml. copper sulfate 5% solution, and 5 ml. carbon disulfide–benzene mixture (1:3).

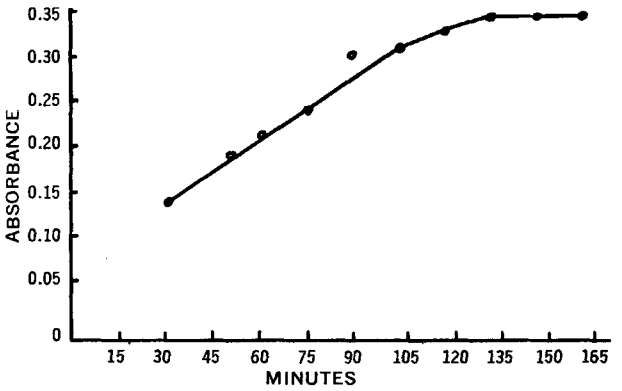

Figure 1—*Time for maximum color development.*

Shake the mixture periodically for 2.25 hr. Centrifuge the tube for 2 min. at 6000 r.p.m. to separate the two phases. Decant the organic layer immediately and measure the intensity of the yellow color using a Beckman DB spectrophotometer at 444 nm. against a blank, prepared under the same conditions using 1 ml. of water in place of the physostigmine solution.

To determine the amount of intact alkaloid present, the procedure must be run on two 1-ml. samples of physostigmine salicylate. In one sample the sodium bicarbonate solution is eliminated and 1 ml. of distilled water is added. In this case, the methylamine present due to decomposition of physostigmine reacts to give the copper dithiocarbamate complex while the intact physostigmine salicylate does not react. The difference in absorbance between the two sample tubes is a measure of intact physostigmine present in solution.

This procedure was also applied to solutions of other ingredients (atropine, pilocarpine, adrenaline, and benzethonium chloride) commonly included with physostigmine in commercial preparations at a variety of concentration levels. Finally, the procedure was applied to three commercial preparations to ascertain whether it is suitable as a possible assay procedure for physostigmine salicylate in commercial preparations. A comparison between the proposed method and Annino's method (7) was made.

RESULTS AND DISCUSSION

A standard curve was plotted using various concentrations of physostigmine salicylate solution. The color obeys Beer's law over a concentration range of 65–400 mcg. pure alkaloid and is stable indefinitely at room temperature in the organic layer after separation from the aqueous layer.

It was found, as shown in Fig. 1, that a period of 2.25 hr. was required to achieve maximum absorbance. However, the intensity of the color can be measured at any time after 15 min. if a standard is run concurrently using the same shaking conditions. Once the organic phase is separated from the aqueous phase, no further color development takes place and the color is stable for at least 24 hr.

This procedure is a modification of the copper dithiocarbamate method for detection of secondary amines. Although the previous

Table I—Recovery of Physostigmine Salicylate

Physostigmine, mcg./ml.	Physostigmine Found, mcg./ml.		Percent Recovery
100	99.7		99.7
200	201.8		100.9
300	301.2		100.4
400	398.6		99.6
		Average	100.2%

Table II—Comparison between Dithiocarbamate Method and Annino's Method (7).

Preparation	Dithiocarbamate Method: Labeled Amount, mg./ml.	Dithiocarbamate Method: Amount Found, mg./ml.	Dithiocarbamate Method: Percent Recovery	Annino's[a] Method, Percent Recovery
1. Eye drops containing physostigmine salicylate, methylcellulose, sodium bisulfite, sodium chloride, chlorobutanol, and citric acid	5	5.046	102.0	100.0
2. Injection containing physostigmine salicylate, sodium bisulfite, and benzyl alcohol	4	3.41	85.5	86.9
3. Injection containing physostigmine salicylate, l-hyoscyamine hydrobromide, benzyl alcohol, and sodium chloride	0.6	0.591	98.66	98.5

[a] Calculated as physostigmine.

reference (4) stated that tertiary aliphatic amines, aromatic amines, and secondary aliphatic–aromatic amines give a negative test, several alkaloids and other substances with primary, tertiary, and quaternary nitrogen groups were found that give a positive test, including atropine, pilocarpine, benzethonium chloride, urea, and some amino acids. Substitution of sodium bicarbonate for ammonium hydroxide in the procedure increased the specificity, eliminating positive reactions with all these chemicals except pilocarpine. The use of the pH 8.6 buffer further increased the specificity, with only physostigmine giving the color.

Different amounts of physostigmine were determined using this method. Recovery data are listed in Table I.

Three commercial preparations containing physostigmine were analyzed using this method. The results are listed in Table II.

REFERENCES

(1) F. W. Teare and S. I. Borst, *J. Pharm. Pharmacol.*, **21**, 277 (1969).

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(6) B. M. Phillips, P. J. Kraus, and M. E. Stratmeyer, *J. Pharm. Sci.*, **54**, 803(1965).

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Abstract □ Quingestanol acetate, norethindrone acetate 3-cyclopentyl enol ether, was not separable from its sesame oil dosage form vehicle as the intact compound using chromatography procedures that depend on differences in polarity. The steroid can be retained quantitatively on a Florisil column treated with a relatively high concentration of ammoniacal silver nitrate and then quantitatively eluted with ethanolic ammonium chloride. The progestin can be determined selectively in the presence of its products of solvolytic or oxidative degradation by UV spectrophotometry after treatment with sodium borohydride; the chromophore of the decomposition products is eliminated by this process while that of the intact compound is unaffected. Quinestrol or ethinyl estradiol, which may be coformulated with the progestin, does not interfere with its determination. Methods for the quantitative determination of the declared steroids in quingestanol acetate formulations and for the estimation of degradation products of the progestin are presented.

Keyphrases □ Quingestanol acetate capsules, combinations with ethinyl estradiol or quinestrol—separation, analysis □ Sesame oil formulations—separation of quingestanol acetate □ Column chromatography—separation, quingestanol acetate in capsules □ UV spectrophotometry—analysis, quingestanol acetate □ TLC—monitoring, quingestanol acetate separation

Quingestanol acetate (I), the cyclopentyl enol ether of norethindrone acetate (II), was shown to be a potent progestational and antiestrual agent (1, 2). As is typical of enol ethers, quingestanol acetate is sensitive to acid-catalyzed solvolysis. Both norethindrone acetate, the solvolysis product, and 6-hydroxynorethindrone acetate (III), formed by autoxidation, have been detected in aged samples of the drug and its dosage forms, but no other decomposition products have been observed. Optimum stability and activity of quingestanol acetate

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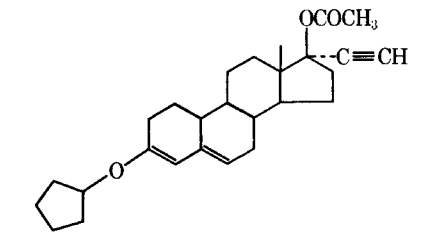
have been achieved by formulating it as sesame oil solutions, stabilized with piperidine (3) and enclosed in soft gelatin capsules.



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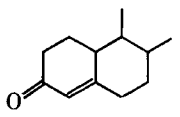
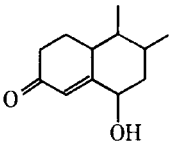


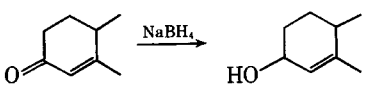
II



III

Because of the very lipophilic character imparted to the progestin molecule by the cyclopentyl enol ether function, all previous attempts to separate quingestanol acetate from sesame oil were unsuccessful. The drug migrated with the oil in some systems and decomposed in others. Its more polar decomposition products, however, could be separated from the oil by any of several paper chromatographic or TLC methods (4–9). A paper chromatographic method, similar to that described by Talmage *et al.* (4) for progesterone 3-cyclopentyl enol ether, was used in these laboratories for several years. In this procedure, two aliquots of diluted sesame oil solution are subjected to paper chromatography, one directly and the other after acid hydrolysis. The difference in the values obtained by elution and colorimetric measurement of the zones corresponding to norethindrone acetate in the two chromatograms is taken as the measure of intact quingestanol acetate, and the decomposition products are measured by elution and colorimetry of the appropriate zones in the chromatogram from the dilution obtained without preliminary hydrolysis. Although this method affords reliable results in the hands of an experienced worker, it is time consuming and requires meticulous technique.

Görög (10) described a differential UV spectrophotometric procedure for Δ^4-3-ketosteroids, applicable in the presence of other types of steroids and some oil vehicles. The absorbance of an aliquot of an alcoholic dilution of steroid added to decomposed sodium borohydride is determined *versus* another where the UV chromophoric system has been destroyed by reduction with sodium borohydride (Scheme I).



Scheme I

Quingestanol acetate has a UV chromophore system similar to those of its decomposition products (Fig. 1). The enol ether function, however, is stable in the alkaline medium afforded by the reducing agent. Unlike its decomposition products, its UV absorption is unaffected by treatment with borohydride. Thus, the intact drug can be determined selectively in the presence of its degradation products. Unfortunately, sesame oil also exhibits a differential spectrum in the borohydride procedure, so the method is inapplicable to quingestanol acetate dosage forms. A simple procedure was developed in which the oil solution was hydrolyzed with alcoholic hydrochloric acid; the norethindrone acetate, formed by hydrolysis of the enol ether, was separated from the oil by partition between aqueous methanol and isooctane, and it was determined by Görög's method. This assay surmounted many of the problems encountered with the paper chromatographic procedure, but it shared the failing that it provided only an indirect measure of intact drug.

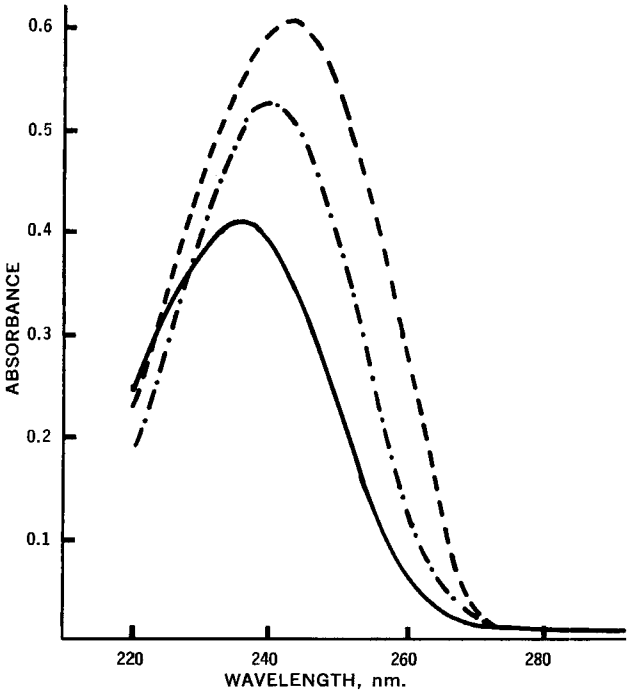


Figure 1—*Spectra of quingestanol acetate (- - -), norethindrone acetate (·-·), and 6β-hydroxynorethindrone acetate (——), all at concentrations of about 10 mcg./ml. in alcohol.*

In contradistinction to chromatographic methods dependent on polarity differences for separation, the chromatographic methods for 17-ethynyl steroids reported by Ercoli *et al.* (11) depend on a specific structural feature. They reported that chromatographic adsorbents impregnated with metallic silver or silver nitrate bound 17α-ethynyl steroids so that other lipoidal materials could be washed away. They described silvered silica gel G for TLC or Florisil for column chromatography, prepared by reducing ammoniacal silver nitrate with glucose, and silica gel G plates impregnated with silver nitrate solution. The silvered materials gave variable results in a number of trials in this laboratory. Kulkarni and Goldzieher (12), however, reported that the silvered Florisil column provided quantitative recoveries of ethinyl estradiol after elution with ethanolic ammonium chloride solution. We reasoned that the efficiency of the preferential adsorption of ethynyl steroids on silvered chromatographic adsorbents depends on the number of sites of silver ion present, and

that variable results in adsorbing these compounds could be ascribed to too efficient reduction of silver ion to metallic silver. Based on this rationale, we found that quingestanol acetate could be retained quantitatively on a Florisil column impregnated with ammoniacal silver ion, desorbed by elution with ammonium chloride in alcohol, and determined by an extension of the Görög method. This report describes methods for the determination of quingestanol acetate and its combinations with ethinyl estradiol or quinestrol in soft gelatin capsule dosage forms.

EXPERIMENTAL

Reagents and Supplies—The absolute methanol and ethanol, acetone, sodium borohydride, sulfuric acid, silver nitrate, ammonium chloride, ammonium hydroxide, and sodium sulfate used were reagent grade chemicals. Phillips "pure" grade isooctane was found satisfactory for use in colorimetry of the estrogens. The steroids used were quingestanol acetate and quinestrol reference standards[1], ethinyl estradiol reference standard USP, norethindrone acetate NF[2], and 6β-hydroxynorethindrone acetate[2]. Dosage forms declaring quingestanol acetate alone, quingestanol acetate with ethinyl estradiol, and progestin with quinestrol were available as clinical test supplies and stability samples.

The separators used were fitted with Teflon stoppers and stopcocks. Disposable glass columns[3], 0.65 cm. (0.25 in.) i.d. and 30.5 cm. (12 in.) long, were used for chromatography. Confirmatory TLC experiments were run using chromatoplates[4] coated with 0.25-mm. layers of alumina F. The plates were activated before use by heating at 105° for 30 min. UV spectrophotometry was performed using 1-cm. silica cells in a Beckman DU fitted with the Gilford model 222 modification or a Cary model 14 recording instrument.

Assay of Quingestanol Acetate Capsules—*Column Adsorbent Preparation*—Transfer about 10 g. of Florisil, 100–200 mesh, to a 250-ml. conical flask containing 100 ml. of distilled water. Add 50 ml. of a 2 in 100 solution of silver nitrate in equal volumes of water and concentrated ammonium hydroxide; stopper the mixture, and shake it mechanically for 30 min. Filter it through a büchner funnel, transfer the solid to a crystallizing dish, and dry it overnight at 105°. Protect the adsorbent from light by storage in a low actinic vessel.

Ethanolic Ammonium Chloride—Dissolve 100 mg. of ammonium chloride in 5 ml. of water. Dilute the solution to 500 ml. with absolute ethanol.

Benzene–Acetone Solvent—Mix equal volumes.

Standard Preparation—Dissolve an accurately weighed quantity of quingestanol acetate reference standard in benzene–acetone. Dilute the solution quantitatively and stepwise with this solvent mixture to obtain a concentration of about 0.6 mg./ml.

Assay Preparation—Determine the average weight of the contents of not fewer than 20 capsules. Weigh an amount equivalent to about 3 mg. of quingestanol acetate into a 5-ml. volumetric flask, and dilute the sample to volume with benzene–acetone.

Procedure—Place a pledget of glass wool at the bottom constriction of each of two chromatography columns. Percolate slurries of 1.0 g. of adsorbent in 15 ml. of benzene–acetone into each of the columns, allowing the solvent to descend into the top of the adsorbent beds. Wrap the columns with aluminum foil to shield them from light.

Concomitantly transfer 2.0 ml. of the assay preparation and 2.0 ml. of the standard preparation to the columns. Allow them to percolate into the upper portion of the adsorbent; successively wash the columns with single 50-ml. portions of benzene–acetone and 10-ml. portions of absolute ethanol. Discard the washings. Elute the columns with ethanolic ammonium chloride, receiving the eluates in 50-ml. volumetric flasks, until the receiving vessels are nearly to volume. Adjust the eluates to the mark with eluant.

[1] Warner-Lambert.
[2] Warner-Vister Steroid Research Institute, Casatenovo (Como), Italy.
[3] Bio-Rad Laboratories.
[4] Analtech, Inc.

Table I—Spectral Data for Quingestanol Acetate and Its Decomposition Products in Ethanol

Steroid	$\lambda_{max.}$, nm.	a	ϵ
Quingestanol acetate	243	48.8	20,000
Norethindrone acetate	238	50.1	17,000
6β-Hydroxynorethindrone acetate	236	41.2	14,660

Pipet 10-ml. portions of the eluates from the assay preparation and the standard preparation and 10 ml. of ethanolic ammonium chloride into each of three 25-ml. volumetric flasks charged with 10 mg. of sodium borohydride. Allow the mixtures to stand for 30 min.; then dilute the solutions to the mark with absolute alcohol. Determine the absorbance of the solutions from the assay preparation and the standard preparation in 1-cm. cells at the wavelength of maximum absorbance (about 243 nm.), using the diluted ethanolic ammonium chloride solution as the blank. Calculate the quantity of $C_{27}H_{36}O_3$, in milligrams per capsule, from the formula $5C(A_U/A_S)(B/SW)$, where C is the concentration, in milligrams per milliliter, of quingestanol acetate reference standard in the standard preparation; A_U and A_S are the absorbances of the solutions from the assay preparation and the standard preparation, respectively; B is the average weight, in milligrams, of the capsules; and SW is the weight, in milligrams, of the sample taken for assay.

Assay of Quingestanol Acetate–Quinestrol Capsules—*Methanol–Sulfuric Acid*—Cautiously add sulfuric acid, in small increments and with mixing, to 30 ml. of chilled anhydrous methanol in a 100-ml. volumetric flask. Adjust to room temperature, dilute with sulfuric acid to volume, and mix.

Standard Preparation—Dissolve accurately weighed amounts of quingestanol acetate and quinestrol reference standards in benzene–acetone solvent, and dilute the solution quantitatively and stepwise to obtain concentrations of about 0.5 mg./ml. of quingestanol acetate and 0.4 mg./ml. of quinestrol.

Assay Preparation—Determine the average weight of the contents of not fewer than 20 capsules. Weigh an amount of capsule contents equivalent to about 5 mg. of quingestanol acetate into a 10-ml. volumetric flask, and dilute the sample to volume with benzene–acetone solvent.

Procedure for Quingestanol Acetate Determination—Follow the *Procedure* given under *Assay of Quingestanol Acetate Capsules*, through: "Discard the washings." Elute the columns with ethanolic ammonium chloride, receiving the eluates in 100-ml. volumetric flasks, until the receiving vessels are nearly to volume. Adjust the eluates to the mark with eluant.

Pipet 10-ml. portions of the eluates from the assay preparation and the standard preparation and 10-ml. of ethanolic ammonium chloride into each of three 25-ml. volumetric flasks charged with 10 mg. of sodium borohydride. Allow the solutions to stand for 30 min.; then determine the absorbance of the solutions from the assay preparation and the standard preparation in 1-cm. cells at the wavelength of maximum absorbance (about 243 nm.), using the ethanolic ammonium chloride solution as the blank. Calculate the quantity of $C_{27}H_{36}O_3$, in milligrams per capsule, from the formula $10C(A_U/A_S)(B/SW)$, where the symbols have the definitions given previously.

Procedure for Quinestrol Determination—Pipet 3.0-ml. portions of the eluates from the assay preparation and the standard preparation into 125-ml. separators. Add 25 ml. of water and 4 drops of concentrated hydrochloric acid to each, and extract the mixtures with 30

Table II—Recovery of Quingestanol Acetate Added to Sesame Oil

Milligrams Added	Milligrams Found	Percent Recovered
3.30	3.22	97.6
	3.26	98.6
	3.37	102.0
3.09	3.09	100.0
	3.08	99.6
3.05	3.00	98.3
	3.08	100.9
	3.02	99.1
		Mean = 99.7
		RSD = ±1.4%

ml. of isooctane. Filter the upper layers through beds of anhydrous sodium sulfate supported on a filter funnel, wash the sodium sulfate layer with an additional 5 ml. of isooctane, and collect the filtrates in scrupulously dry 60-ml. separators. Add 5.0 ml. of methanol–sulfuric acid, and shake vigorously for 2 min. Allow the layers to separate, and withdraw about 4.5 ml. of the colored lower phase. Transfer 4.0-ml. portions to dry glass-stoppered centrifuge tubes, add exactly 0.5 ml. of anhydrous methanol to each, mix well, and clarify by centrifugation. Concomitantly determine the absorbances of the solutions from the assay preparation and from the standard preparation in 1-cm. cells in a suitable spectrophotometer at the wavelength of maximum absorbance (about 540 nm.). Calculate the quantity of $C_{25}H_{32}O_2$, in milligrams per capsule, by the formula $10C(A_U/A_S)(B/SW)$, where C is the concentration, in milligrams per milliliter, of quinestrol in the standard preparation, and the other symbols have the definitions given previously.

Assay of Quingestanol Acetate–Ethinyl Estradiol Capsules—*Standard Preparation*—Dissolve accurately weighed amounts of quingestanol acetate reference standard and ethinyl estradiol reference standard USP in benzene–acetone solvent. Dilute the solution quantitatively and stepwise to obtain concentrations of about 0.8 mg./ml. of quingestanol acetate and about 0.08 mg./ml. of ethinyl estradiol.

Assay Preparation—Determine the average weight of the contents of not fewer than 20 capsules, and weigh an amount equivalent to about 4 mg. of quingestanol acetate into a 5-ml. volumetric flask. Dilute the contents to volume with benzene–acetone solvent.

Procedure for Quingestanol Acetate Determination—Follow the *Procedure* given under *Assay of Quingestanol Acetate Capsules*, but transfer 3.0-ml. volumes of the assay preparation and the standard preparation to the columns. Continue through: "Adjust the eluates to the mark with eluant."

Pipet 5-ml. portions of the eluates from the assay preparation and the standard preparation and 5-ml. of ethanolic ammonium chloride into each of three 25-ml. volumetric flasks. Follow the *Procedure* given under *Assay of Quingestanol Acetate Capsules*, beginning with: "Allow the mixtures to stand for 30 min...."

Procedure for Ethinyl Estradiol Determination—Pipet 4.0-ml. portions of the eluates from the assay preparation and the standard preparations into 125-ml. separators. Add 25 ml. of water and 2 ml. of 12 N sulfuric acid to each, and extract with 25 ml. of carbon tetrachloride. Filter the lower layers through beds of anhydrous sodium sulfate supported on a filter funnel, wash the funnels with 25-ml. portions of isooctane, and combine the filtrates in clean, dry, 125-ml. separators. Follow the *Procedure for Quinestrol Determination*, beginning with: "Add 5.0 ml. of methanol–sulfuric acid...." Calculate the quantity of $C_{20}H_{24}O_2$, in milligrams per capsule, by the formula $5C(A_U/A_S)(B/SW)$, where C is the concentration, in milligrams per milliliter, of ethinyl estradiol in the standard preparation; A_U and A_S are the absorbances of the solutions from the assay preparation and the standard preparation, respectively; and B and SW have the definitions given previously.

Estimation of Norethindrone Acetate and 6β-Hydroxynorethindrone Acetate—*Standard Preparation*—Dissolve equal and accurately weighed quantities of authentic specimens of norethindrone acetate and 6β-hydroxynorethindrone acetate in a mixture of equal volumes of acetone and methanol. Dilute the solution quantitatively and stepwise to obtain a series of solutions containing 100-, 80-, 60-, 40-, and 20-mcg./ml. concentrations of each of the decomposition products of quingestanol acetate.

Assay Preparation—Transfer an aliquot of the eluant obtained in the assay, equivalent to about 1 mg. of quingestanol acetate, to a 50-ml. glass-stoppered, round-bottom flask; evaporate it to dryness under reduced pressure. Carefully dissolve the residue in 0.5 ml. of a mixture of equal volumes of acetone and methanol.

Procedure—At equidistant points along a line 2.5 cm. from the lower edge of a 20 × 20-cm. TLC plate, coated with a 0.25-mm. layer of aluminum oxide F_{254} and previously activated by heating at 105° for 30 min., spot 50 μl. each of the standard preparations (corresponding to 1, 2, 3, 4, and 5 mcg. of each standard) and 50 μl. of the assay preparation (corresponding to 100 mcg. of quingestanol acetate). Place the plate in a chamber lined with filter paper on three sides and previously equilibrated with a 5 in 200 solution of anhydrous methanol in benzene. Allow the solvent front to ascend about 15 cm. above the line of application. Remove the plate from the chamber, mark the front, and dry it at 105° for 5 min. View the chromatogram under shortwave UV light in a suitable apparatus; compare the intensity of the norethindrone acetate and 6β-hydroxynorethindrone acetate zones, if present in the lanes for the assay preparation spot, with the zones for the standard preparations. Norethindrone acetate and 6β-hydroxynorethindrone acetate appear as UV-absorbing zones at R_f values of about 0.55 and 0.25, respectively.

Table III—Recovery of Quingestanol Acetate and Quinestrol Added to Sesame Oil

Quingestanol Acetate			Quinestrol		
Milligrams Added	Milligrams Found	Percent Recovered	Milligrams Added	Milligrams Found	Percent Recovered
5.26	5.30	100.9	4.12	4.19	101.7
5.12	5.13	100.1	4.10	4.17	101.9
	5.13	100.1		4.21	102.5
5.11	5.13	100.3	4.12	4.18	101.6
	5.13	100.3		4.22	102.3
	5.09	99.7		4.12	100.0
	5.09	99.7		4.14	100.4
	Mean =	100.2		Mean =	101.5
	RSD =	±0.4%		*RSD* =	±0.9%

RESULTS AND DISCUSSION

Selectivity of the Method—The elution of the column was monitored by TLC. No steroid could be detected in the washings of the column until it was eluted with ethanolic ammonium chloride. Quingestanol acetate was eluted as a single compound, having identical R_f with quingestanol acetate reference standard. Subjecting a portion of the column effluent to hydrolysis before TLC led to formation of norethindrone acetate, a further confirmation that quingestanol acetate was eluted as the intact enol ether.

UV spectra of quingestanol acetate and its two degradation products are shown in Fig. 1. The spectral data obtained for the three compounds are presented in Table I. It is evident from these data that decomposition of quingestanol acetate could be detected by direct UV only if it had proceeded to a significant extent, and the amount of decomposition would be underestimated. Use of sodium borohydride reduction, however, eliminates interference by the degradation products without affecting the spectra of the enol ether. Neither of the estrogens, ethinyl estradiol or quinestrol, interferes at the wavelength of maximum absorbance for quingestanol acetate. Since the UV spectra of estrogens are not affected by treatment with borohydride, one could eliminate interferences by such compounds, if they were observed, by differential measurement. That is, pipet two aliquots of column effluent into separate volumetric flasks. Then:

1. Add sodium borohydride to the first, destroy excess reducing agent, and dilute to volume.
2. Hydrolyze quingestanol acetate to norethindrone acetate with alcoholic hydrochloric acid, neutralize, add sodium borohydride, destroy excess borohydride, and dilute to volume.
3. Determine the absorbance of the solution prepared in Step 1 *versus* the solution prepared in Step 2.

The selectivity of the methods used for the estrogens was reported previously by Chafetz *et al.* (13). The TLC procedure provided for estimation of the quingestanol acetate decomposition products was employed to confirm decomposition and to determine whether degradation, when observed, was due to hydrolysis or oxidation.

Table IV—Recovery of Quingestanol Acetate and Ethinyl Estradiol Added to Oil

Quingestanol Acetate			Ethinyl Estradiol		
Milligrams Added	Milligrams Found	Percent Recovered	Milligrams Added	Milligrams Found	Percent Recovered
3.51	3.45	98.1	0.361	0.361	100.0
	3.51	100.0		0.367	101.8
4.08	4.07	99.7	0.400	0.393	98.4
	4.04	98.9		0.402	100.4
	4.02	98.6		0.395	98.7
	Mean =	99.1		Mean =	99.9
	RSD =	±0.8%		*RSD* =	±1.4%

Table V—Assay Values on Some Quingestanol Acetate Formulations

Steroid Declared	mg./Capsule	Storage	Found, mg./Capsule
Quingestanol acetate	0.750	Initial	0.772, 0.769, 0.775, 0.766
Quingestanol acetate	0.30	19 months, RT	0.305, 0.308, 0.305, 0.305
Quingestanol acetate	2.5	Initial	2.64, 2.56, 2.45
With quinestrol	2.0		2.09, 2.08, 2.08
Quingestanol acetate	0.50	28 months, RT	0.474, 0.470
With ethinyl estradiol	0.05		0.054, 0.055

Precision and Recovery—Analytical samples for each formulation were prepared by dissolving accurately weighed amounts of the steroids in benzene–acetone, pipeting aliquots to volumetric flasks containing 1 ml. of sesame oil, and diluting the solutions to volume with the benzene–acetone solvent mixture. The procedures described were used to obtain the data in Tables II, III, and IV. Overall, the recovery of quingestanol acetate was 99.7%. Recoveries of the estrogens were 101.5% for quinestrol and 99.9% for ethinyl estradiol.

Application to Laboratory Formulations—Typical data obtained with formulations available as clinical supplies or as stability testing samples are presented in Table V.

SUMMARY

The problem of separating intact quingestanol acetate from its sesame oil vehicle had proved intractable owing to the very lipophilic character imparted to the steroid molecule by its 3-cyclopentyl enol ether function. A reinvestigation of the chromatographic methods described by Ercoli *et al.* (11), which provided selective adsorption of 17α-ethynyl steroids on silvered or silver nitrate impregnated supports, led to the finding that reliable separations of 17α-ethynyl steroids from other lipids could be obtained using a relatively high concentration of ammoniacal silver nitrate adsorbed on Florisil. After desorption with ethanolic ammonium chloride, quingestanol acetate is determined selectively by UV spectrophotometry, using a modification of the Görög technique (10). The UV chromophore of the degradation products is destroyed by reduction with sodium borohydride without affecting the spectra of the enol ether conjugated system in the intact compound. Coformulated quinestrol or ethinyl estradiol is not affected by this treatment; however, they do not interfere. Procedures for the determination of quingestanol acetate, estimation of its products of hydrolytic and oxidative decomposition, and selective determination of coformulated estrogens in dosage forms are presented.

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Nonlinear Regression Applied to Non-Newtonian Flow

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Abstract □ Modifications of the Gauss–Newton method are among the most widely used methods for nonlinear regression analysis. One such modification, which the authors found applicable to a wide variety of pharmaceutical systems, is described. Its application in describing the flow behavior of non-Newtonian systems and the FORTRAN IV program utilized are presented. One of the more useful, accurate, and physically significant equations for describing non-Newtonian flow is the structure equation: $F = f + \eta_\infty S - b_v e^{-aS}$, in which F is shear stress, S is shear rate, and the other terms are constants. The equation was originally evaluated through the use of multiple regression, with the constant a assumed to be equal to 0.001, which gave good fit to a variety of flow systems. Since the original equation was developed, nonlinear regression techniques have appeared which make it possible to examine the structure equation in greater detail. It was found, for example, that for dispersions of a wax, consisting of a mixture of polyethoxylated higher fatty alcohols, in water the constant a varied from 0.013 to 0.049 rather than remaining fixed at 0.001. Some of the original data, upon which the structure equation was based, were reevaluated using nonlinear regression analysis. These data were for suspensions of salicylamide (varying concentration) in methylcellulose solutions of varying concentration. The constant a was found to vary from 0.00109 to 0.00172 as the concentration of methylcellulose increased and was independent of salicylamide concentration. In all instances, allowing a to vary as an adjustable parameter gave a better fit to the data than assuming it to be constant at 0.001. The use of nonlinear regression analysis served to emphasize the usefulness of the structure equation.

Keyphrases □ Regression analysis, nonlinear—non-Newtonian flow systems □ Non-Newtonian flow systems—application of nonlinear regression analysis □ NONLIN, subroutine—digital computer program □ Digital computer programs—subroutine NONLIN

Although nonlinear regression analysis techniques (1–3) have been available for a number of years, they have received little attention in the pharmaceutical sciences except for the area of pharmacokinetics (4–7). The techniques of nonlinear regression analysis, except for practical difficulties which may occasionally arise, should be capable of curve fitting any system that may be of interest to pharmaceutical scientists. The authors, for example, successfully applied these techniques to studies of protein binding, the determination of dissociation constants for polyprotic acids, complexation studies, Michaelis–Menten kinetics, and the investigation of rheological systems. One dramatic example of the application of nonlinear regression analysis was the analysis of one of the more useful, accurate, and physically significant equations for describing non-Newtonian flow. This equation, designated as the structure equation (8), is:

$$F = f + \eta_\infty S - b_v e^{-aS} \qquad \text{(Eq. 1)}$$

in which F is the shear stress, S is the shear rate, and the other terms are constants. At the time the equation was developed, the only manner in which it could be analyzed was to assume a value for the constant a and treat the equation as a multiple-regression problem in the form:

$$F = f + \eta_\infty S - b_v X \qquad \text{(Eq. 2)}$$

in which:

$$X = e^{-aS} \qquad \text{(Eq. 3)}$$

An arbitrary value of 0.001 was found to give very good fit to a wide variety of systems. Data from the authors' laboratories, as well as some of the data upon which the equation was based originally (9)[1], were analyzed using the techniques of nonlinear regression analysis, in which the constant a was allowed to be an adjustable parameter. The results and a discussion of the techniques utilized are presented in this article.

Table I—Constants of Structure Equation for Dispersions of a Mixture of Polyethoxylated Higher Fatty Alcohols[a] in Water at 25°

Percent Wax	f	η_∞	b_v	a
1	25.2	0.1040	19.55	0.013
2	41.7	0.3146	17.39	0.044
3	110.4	0.7101	45.68	0.044
4	232.7	1.0350	131.80	0.049

[a] Polawax (Croda).

THEORETICAL

To utilize the techniques of nonlinear regression, initial estimates, $P_1^0, P_2^0, P_i^0, \ldots, P_k^0$, are needed for the k adjustable parameters, and correction vectors defined such that:

$$P_1 = P_1^0 - \Delta P_1 \qquad \text{(Eq. 4a)}$$

$$P_2 = P_2^0 - \Delta P_2 \qquad \text{(Eq. 4b)}$$

$$\vdots$$

$$P_k = P_k^0 - \Delta P_k \qquad \text{(Eq. 4c)}$$

in which ΔP_i represents a correction vector that will enable P_i to be a "better" estimate of the true parameter than P_i^0. The function:

$$Y = f(X; P_1, P_2, P_i, \ldots, P_k) \qquad \text{(Eq. 5)}$$

in which Y is the dependent variable, X is the independent variable, and P_i represents the current estimate of the true parameter, is

[1] The authors are grateful to Dr. Wayne Grim for his permission to use the data from his doctoral thesis. All of the data on salicylamide in methylcellulose referred to in this publication were obtained from this thesis.

Table II—Nonlinear Regression Analysis on 10.29% v/v Salicylamide in 1.59% w/w Methylcellulose, 1500 cps., Solutions

Constant	Value	Standard Deviation	95% Confidence Limits
f	1.4082×10^{3}	3.058×10^{1}	$1.348 \times 10^{3} - 1.468 \times 10^{3}$
η_∞	6.3453×10^{-1}	1.298×10^{-2}	$6.091 \times 10^{-1} - 6.600 \times 10^{-1}$
b_v	1.2887×10^{3}	2.645×10^{1}	$1.237 \times 10^{3} - 1.340 \times 10^{3}$
a	1.8900×10^{-3}	7.037×10^{-5}	$1.752 \times 10^{-3} - 2.028 \times 10^{-3}$

$Y \times 10^{-3}$	Y Calculated $\times 10^{-3}$	Percent Difference
0.2587	0.2846	−10.003
0.3317	0.3414	−2.926
0.4709	0.4695	0.296
0.6235	0.6014	3.552
0.7628	0.7377	3.285
0.8756	0.8610	1.660
1.008	1.006	0.213
1.154	1.142	1.009
1.267	1.273	−0.471
1.393	1.403	−0.700
1.512	1.528	−1.009
1.625	1.631	−0.393
1.711	1.725	−0.809
1.784	1.804	−1.088
1.857	1.872	−0.786
1.930	1.937	−0.350
1.990	1.994	−0.187
2.050	2.047	0.117
2.109	2.097	0.586
2.149	2.147	0.077
2.202	2.196	0.300
2.249	2.243	0.270
2.295	2.285	0.415
2.335	2.327	0.348
2.388	2.374	0.587
2.428	2.425	0.115
2.467	2.466	0.068
2.521	2.515	0.209
2.560	2.561	−0.039
2.613	2.614	−0.020
2.673	2.669	0.139
2.733	2.727	0.220
2.773	2.778	−0.180
2.826	2.829	−0.112
2.865	2.867	−0.054
2.912	2.907	0.178
2.938	2.942	−0.122
2.958	2.975	−0.553

expanded in a Taylor series retaining only the first-order terms to give:

$$Y = \hat{Y} + \Delta P_1 F_1 + \Delta P_2 F_2 + \Delta P_i F_i + \ldots + \Delta P_k F_k \quad \text{(Eq. 6)}$$

in which:

$$F_i = \partial Y / \partial P_i \quad \text{(Eq. 7)}$$

and $\hat{Y}$ represents the theoretical value of Y calculated from the current set of estimated parameters.

Equation 6 is linear in the correction vectors and can be treated using the techniques of multiple regression in which Eq. 6 can be represented by:

$$Y = a_1 X_1 + a_2 X_2 + a_i X_i + \ldots + a_k X_k + \epsilon \quad \text{(Eq. 8)}$$

in which ϵ represents the residual for a given point. Taking the partial derivative of ϵ^2 with respect to each ΔP_i, setting each resulting equation equal to zero to minimize the sum of squares of residuals, and summing over the entire set of experimental points yield a set of "normal equations":

$$(\Sigma F_1 F_1)\Delta P_1 + (\Sigma F_1 F_2)\Delta P_2 + (\Sigma F_1 F_i)\Delta P_i + \ldots + (\Sigma F_1 F_k)\Delta P_k = \Sigma F_1 F \quad \text{(Eq. 9a)}$$

$$(\Sigma F_2 F_1)\Delta P_1 + (\Sigma F_2 F_2)\Delta P_2 + (\Sigma F_2 F_i)\Delta P_i + \ldots + (\Sigma F_2 F_k)\Delta P_k = \Sigma F_2 F \quad \text{(Eq. 9b)}$$

$$(\Sigma F_i F_1)\Delta P_1 + (\Sigma F_i F_2)\Delta P_2 + (\Sigma F_i F_i)\Delta P_i + \ldots + (\Sigma F_i F_k)\Delta P_k = \Sigma F_i F \quad \text{(Eq. 9c)}$$

$$\ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots$$

$$(\Sigma F_k F_1)\Delta P_1 + (\Sigma F_k F_2)\Delta P_2 + (\Sigma F_k F_i)\Delta P_i + \ldots + (\Sigma F_k F_k)\Delta P_k = \Sigma F_k F \quad \text{(Eq. 9d)}$$

in which:

$$F = Y - \hat{Y} \quad \text{(Eq. 10)}$$

and the subscripted F_i's refer to the partial derivatives as given by Eq. 7. The initial estimates, $P_1^0, P_2^0, P_i^0, \ldots, P_k^0$, are used to solve for $F, F_1, F_2, F_i, \ldots, F_k$ at each data point: the sums of the products are inserted into the set of normal equations (Eqs. 9). This set of simultaneous equations can be solved in a wide variety of ways for $\Delta P_1, \Delta P_2, \Delta P_i, \ldots, \Delta P_k$. The Crout reduction method (10) is used here. These correction vectors are then substituted into Eqs. 4 to

Table III—Reduction in Sums of Squares of Residuals between Values Obtained Using Multiple Regression with $a = 0.001$, and Nonlinear Regression in Which a Was Considered an Adjustable Parameter. Data for Varying Concentrations of Salicylamide in 1.59 % w/w Methylcellulose, 1500 cps., Solutions

Percent Salicylamide, v/v	Sums of Squares of Residuals $\times 10^{-4}$ — Multiple Regression	Nonlinear Regression
0.00	1.00	0.26
5.40	3.65	0.37
10.29	4.57	0.45
14.59	4.58	0.52
19.69	4.26	1.00
24.21	6.49	0.93
28.07	11.91	2.91
34.39	24.03	5.41

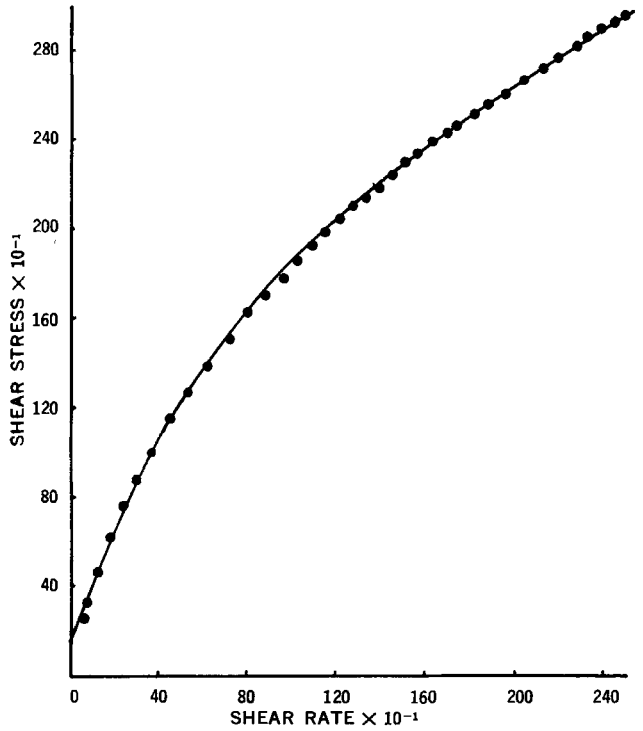


Figure 1—*Comparison between calculated and experimental values of shear stress. The solid line represents the calculated values of shear stress, while the closed circles represent the actual experimental points. The system represented is 10.29% v/v salicylamide in 1.59% w/w methylcellulose, 1500 cps.*

give new estimates of the parameters P_1, P_2, P_i, ..., P_k, which together with the experimental values of Y and X are used to solve for F, F_1, F_2, F_i, ..., F_k for each data point. The sums of squares are again tabulated, and the set of Eqs. 9 is solved for the correction vectors. This iterative procedure is continued until the desired degree of accuracy is obtained.

Although the set of Eqs. 4 can be used successfully for fitting many functions, convergence may not be obtained in others. An alternate procedure, which converges more frequently and which is used in this computer program, is as follows.

1. After each titration, correct the estimated parameters:

$$P_1 = P_1^0 - R\,\Delta P_1 \quad \text{(Eq. 11}a\text{)}$$

$$P_2 = P_2^0 - R\,\Delta P_2 \quad \text{(Eq. 11}b\text{)}$$

$$P_i = P_i^0 - R\,\Delta P_i \quad \text{(Eq. 11}c\text{)}$$

$$\cdots \quad \cdots \quad \cdots$$

$$P_k = P_k^0 - R\,\Delta P_k \quad \text{(Eq. 11}d\text{)}$$

using values of $R = 0$, 0.5, and 1 on each of the parameters.

2. Calculate the sum of squares of residuals (ΣF^2) using the three estimates of P_1, P_2, P_i, ..., P_k, designating S_0 as the result for $R = 0$, $S_{0.5}$ for the set in which $R = 0.5$, and S_1 as the result for $R = 1$.

3. Calculate $R_{\min.}$:

$$R_{\min.} = 0.5 + \frac{(S_0 - S_1)}{4(S_0 - 2S_{0.5} + S_1)} \quad \text{(Eq. 12)}$$

4. Now correct each parameter:

$$P_1 = P_1^0 - R_{\min.}\,\Delta P_1 \quad \text{(Eq. 13}a\text{)}$$

$$P_2 = P_2^0 - R_{\min.}\,\Delta P_2 \quad \text{(Eq. 13}b\text{)}$$

$$P_i = P_i^0 - R_{\min.}\,\Delta P_i \quad \text{(Eq. 13}c\text{)}$$

$$\cdots \quad \cdots \quad \cdots \quad \cdots$$

$$P_k = P_k^0 - R_{\min.}\,\Delta P_k \quad \text{(Eq. 13}d\text{)}$$

Table IV—Differences between Constants Obtained *via* Multiple Regression with $a = 0.001$, and Nonlinear Regression in Which a Was Considered an Adjustable Parameter. Data for Varying Concentrations (v/v%) of Salicylamide in 1.59 % w/w Methylcellulose, 1500 cps., Solutions

Percent Salicylamide	f	η_∞	b_v	$a \times 10^3$	Yield Value ($f - b_v$)
0.00%:					
Literature[a]	1634.0	0.2800	1507.0	1.00	127.0
This work	1086.0	0.4638	1021.0	1.72	65.0
Percent difference	33.5	65.6	32.2	72.0	48.8
5.40%:					
Literature	1981.0	0.2965	1806.0	1.00	175.0
This work	1356.0	0.5046	1248.0	1.65	18.0
Percent difference	31.5	70.2	30.9	65.0	38.3
10.29%:					
Literature	2205.0	0.3624	1985.0	1.00	220.0
This work	1408.0	0.6345	1289.0	1.89	119.0
Percent difference	36.1	75.1	35.1	89.0	45.9
14.59%:					
Literature	2766.0	0.3611	2509.0	1.00	257.0
This work	1736.0	0.7144	1611.0	1.93	125.0
Percent difference	37.2	97.8	35.8	93.0	33.2
19.69%:					
Literature	3511.0	0.3937	3199.0	1.00	312.0
This work	2416.0	0.7579	2220.0	1.64	196.0
Percent difference	31.2	92.5	30.6	64.0	37.2
24.21%:					
Literature	3773.0	0.5492	3415.0	1.00	358.0
This work	2477.0	0.9884	2271.0	1.79	206.0
Percent difference	34.3	80.0	33.5	79.0	42.5
28.07%:					
Literature	5021.0	0.7281	4528.0	1.00	493.0
This work	3333.0	1.2976	3036.0	1.76	297.0
Percent difference	33.6	78.2	33.0	76.0	39.8
34.39%:					
Literature	7454.0	0.9837	6754.0	1.00	700.0
This work	4969.0	1.8206	4552.0	1.74	417.0
Percent difference	33.3	85.1	32.6	74.0	40.4

[a] Reference 9.

Table V—Listing for the Main Program

```
      IMPLICIT REAL*8 (A-H,O-Z)
      INTEGER NAME(60)
      COMMON/SET1/P,NAME
      COMMON/SET2/Y(100),YCALC(100),X(100),F(10)
      DIMENSION P(10)
    1 READ(5,10)NAME
   10 FORMAT(60A1)
      READ,N,NOCON,TS
      READ,(P(I),I-1,NOCON)
      READ,(X(I),I=1,N)
      READ,(Y(I),I=N)
      NO = N
      WRITE(6,15)NAME
   15 FORMAT('1',T3,60A1,///)
      CALL NONLIN(NO,NOCON,TS)
      READ,MORE
      GO TO(1,20),MORE
   20 STOP
      END
```

5. Continue the iteration procedure as before.

If the right side of the set of Eqs. 9 is replaced by the unit matrix (one in which all of the diagonal elements are equal to 1 and all other elements are equal to zero), the covariance matrix can be obtained by solving Eqs. 9 using each column of the unit matrix in succession. The diagonal elements of the covariance matrix, designated as V_{11}, V_{22}, V_{tt}, . . . , V_{kk}, are used to obtain estimates of the standard deviation for each parameter as follows:

$$\text{VAREXT} = S_0/(N - \text{NOCON}) \qquad \text{(Eq. 14)}$$

in which VAREXT is an external estimate of the unit variance, N is the number of data points, and NOCON is the number of parameters being fitted. The standard deviation for each parameter is defined as follows:

$$\text{STNDV}_1 = (V_{11} \cdot \text{VAREXT})^{1/2} \qquad \text{(Eq. 15}a\text{)}$$

$$\text{STNDV}_2 = (V_{22} \cdot \text{VAREXT})^{1/2} \qquad \text{(Eq. 15}b\text{)}$$

$$\text{STNDV}_i = (V_{ii} \cdot \text{VAREXT})^{1/2} \qquad \text{(Eq. 15}c\text{)}$$

It is necessary at times to evaluate a constant, for example G, which is a function of the adjustable parameters of a particular equation:

$$G = f(P_1, P_i, \ldots, P_n) \qquad \text{(Eq. 16)}$$

in which n may be equal to or less than k, the total number of adjustable parameters of a particular equation. The variance, STNDV_g^2, for G can be obtained from:

$$\text{STNDV}_g^2 = \text{VAREXT} \sum_{i=1}^{n} V_{ii}F_i^2 + 2\text{VAREXT} \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \times V_{ij}F_iF_j \qquad \text{(Eq. 17)}$$

in which F_i represents the partial derivative of G with respect to P_i.

An estimate of the variance for each calculated value of the dependent variable, $\hat{Y}$, as calculated from the adjusted parameters can be obtained from:

$$\sigma_{\hat{Y}}^2 = \text{VAREXT}\left(\sum_{i=1}^{k} V_{ii}F_i^2 + 2\sum_{i=1}^{k-1} \sum_{j=i+1}^{k} V_{ij}F_iF_j\right) \qquad \text{(Eq. 18)}$$

and the 95% confidence interval for $\hat{Y}$ would be:

$$\text{limits} = \hat{Y} \pm t\sigma_{\hat{Y}} \qquad \text{(Eq. 19)}$$

ANALYSIS OF THE STRUCTURE EQUATION

To use the computer program (*Appendix*), the structure equation, Eq. 1, has to be converted into terms of $\hat{Y}$, X, and P_4:

$$\hat{Y} = P_1 + P_2X - P_3e^{-P_4X} \qquad \text{(Eq. 20)}$$

The appropriate partial derivatives, as described by Eq. 7, are:

$$F_1 = \partial\hat{Y}/\partial P_1 = 1.0 \qquad \text{(Eq. 21}a\text{)}$$

$$F_2 = \partial\hat{Y}/\partial P_2 = X \qquad \text{(Eq. 21}b\text{)}$$

$$F_3 = \partial\hat{Y}/\partial P_3 = -e^{-P_4X} \qquad \text{(Eq. 21}c\text{)}$$

$$F_4 = \partial\hat{Y}/\partial P_4 = XP_3e^{-P_4X} \qquad \text{(Eq. 21}d\text{)}$$

The conversion of Eqs. 20 and 21 into the FORTRAN IV language used in the computer program is described in the *Appendix*.

According to Eq. 1, when the shear rate approaches zero:

$$F = f - b_v = \text{yield value} \qquad \text{(Eq. 22)}$$

The estimated variance for the yield value, which is obtained from parameters P_1 and P_3 of Eq. 20, can be obtained using Eq. 17 to give:

$$\text{variance for yield value} = \text{VAREXT}(V_{11}F_1^2 + V_{22}F_2^2) + 2\text{VAREXT}(V_{12}F_1F_2) \qquad \text{(Eq. 23)}$$

RESULTS AND CONCLUSIONS

A series of dispersions, containing varying amounts of a mixture of polyethoxylated higher fatty alcohols[2], in water at 25° was analyzed using an Epprecht-Rheomat 15. Initial estimates of P_1 and P_2 in Eq. 20 were obtained from the approximately linear portion of the plot of shear stress *versus* shear rate. The standard "feathering" technique was used to obtain initial estimates of P_3 and P_4. The data were analyzed using the computer program given in the *Appendix* on an IBM 360-75 system using the WATFOR compiler. The results are shown in Table I. The initial estimated parameters differed from the final adjusted parameters by 30–50% in all instances. Since the values of the constant a of Eq. 1 were 13–49 times greater than the value of 0.001, the data of Grim (9) were reanalyzed in an effort to determine whether the wax dispersions showed abnormal behavior or whether the nonlinear regression analysis was indeed indicating that the constant a was capable of being treated as an adjustable parameter.

The results for a typical set of data are shown in Table II, which was adapted from the computer printout. The same set of data is shown in Fig. 1 in an effort to demonstrate the close agreement between the theoretical line drawn from the computer-adjusted constants and the experimental data, In a least-squares regression analysis, the line giving the best fit to a set of experimental data is that line for which the sum of squares of residuals, SS, is a minimum in which:

$$SS = \Sigma(Y - \hat{Y})^2 \qquad \text{(Eq. 24)}$$

and $\hat{Y}$ represents the theoretical value of Y, calculated from the adjusted parameters obtained in the least-squares analysis, and the experimental values of X. The structure equation, Eq. 1, was used to calculate the SS for the set of data represented in Table II, using the constants obtained by multiple regression (9) and those obtained by nonlinear regression analysis, respectively. It was found that the SS obtained using nonlinear regression analysis was 32.6% lower than the SS obtained using multiple regression and assuming a equal to 0.001.

Table III shows the marked reduction in SS for a series containing varying amounts of salicylamide suspended in 1.59% w/w methylcellulose, 1500 cps. It should be noted that both sets of constants, that obtained assuming a was equal to 0.001 and that obtained with nonlinear regression analysis, gave good fit to the expeimental data. The nonlinear regression analysis simply expands the inherent usefulness of the structure equation by now allowing it to be analyzed in such a manner as to take full advantage of its ability to fit non-Newtonian flow data. The differences obtained between the constants obtained using both techniques for the set of data shown in Table III are shown in Table IV. Although the values of the constants changed markedly in some instances, the overall conclusions initially drawn (9) as to effects of concentration, *etc.*, on the constants are fully substantiated, with an even greater degree of con-

[2] Polawax (Croda).

Table VI—Necessary Input Data; This Program Uses Unformatted Input

Input Record	Reference Line Number	Input Variable
1	6	NAME = any 60 characters for identification of the problem
2	8	N = number of data points NOCON = number of constants to be fitted TS = Student's t for N − NOCON degrees of freedom
3	9	P(I) = initial estimates of constants $P_1 \ldots P_{NOCON}$
4	10	X(I) = independent variable. Enter $X_1, X_2 \ldots, X_N$
5	11	Y(I) = dependent variable. Enter $Y_1, Y_2 \ldots, Y_N$
6	16	MORE = code for running more than one set of data. Enter 1 if you wish to run another set, and the number 2 if you wish to stop.

Table VII—Input Program in Which Raw Data Are Transformed into the Master Variables for Curve Fitting and in Which the Initial Estimates of the Constants Are Taken from the Raw Data

```
1                    IMPLICIT REAL*8 (A-H,O-Z)
2                    INTEGER NAME(60)
3                    COMMON/SET1/P,NAME
4                    COMMON/SET2/Y(100),YCALC(100),X(100),F(10)
5                    DIMENSION P(10)
6           1        READ(5,10)NAME
7           10       FORMAT(60A1)
8                    READ,N,NOCON,TS
9                    READ,TAU,ATAU,P(4)
10                   READ,(X(I),I=1,N)
11                   READ,(Y(I),I=1,N)
12                   DO 20 I = 1,N
13                   X(I) = ATAU*X(I)
14                   Y(I) = TAU*Y(I)
15          20       CONTINUE
16                   P(2) = (Y(N)-Y(N-1))/(X(N)-X(N-1))
17                   P(1) = Y(N)-P(2)*X(N)
18                   P(3) = P(1) − (Y(1)-(Y(2)-Y(1))/(X(2)-X(1))/(X(2)-X(1))*X(1))
19                   WRITE(6,30)NAME
20          30       FORMAT('1',T3,60A1,///)
21                   WRITE(6,40)
22          40       FORMAT(5X,'SHEAR STRESS',5X,'SHEAR RATE',/)
23                   DO 50 I = 1,N
24                   WRITE(6,45)Y(I),X(I)
25          45       FORMAT(5X,1PD10.3,12X,D10.3)
30          50       CONTINUE
31                   WRITE(6,60)
32          60       FORMAT(//5X,'INITIAL ESTIMATES OF CON. FOR STRUCTURE EQUATION'/)
33                   WRITE(6,70)(P(I),I=1,NOCON)
34          70       FORMAT(5X,'F=',1PD12.5,2X,'ETA INF. =',D12.5,2X,'BV=',D12.5,
35                   *2X,'A=',D14.7,//)
36                   NO = N
37                   CALL NONLIN(N,NOCON,TS)
38                   READ,MORE
39                   GO TO(1,80),MORE
40          80       STOP
41                   END
```

fidence using the nonlinear regression estimates for the parameters of the structure equation.

In summary, the structure equation was found to give excellent fit to a variety of systems, and a nonlinear regression analysis of the equation showed that the constant a of Eq. 1 is an adjustable parameter. In systems containing varying amounts of salicylamide suspended in solutions of varying concentrations of methylcellulose, the constant a varied from 0.00109 to 0.00172 as the w/w% of methylcellulose increased, but it seemed to be relatively insensitive to changes in salicylamide concentration. The full physical significance of the constant a cannot be ascertained at this time, due to the relatively small amount of data analyzed. Studies are in progress to determine the rheological significance and potential usefulness of this parameter.

APPENDIX

The double-precision FORTRAN IV program for nonlinear regression analysis developed by the authors has proven to be easy to use, readily adaptable to a number of digital computer systems (IBM 360-75, Sigma 7, and Burroughs 5500), and rather inexpensive. Although the cost of running the program will vary from system to system, the IBM 360-75 system[3] will run five sets of data, each with 30–50 experimental points, for a total cost of about $0.55 if the WATFOR compiler is used. The exact same material will cost approximately $0.85 using the G level standard compiler. The entire nonlinear regression analysis program is written in three sections. The only section that needs revision from problem to problem is the third section, a subroutine called FUNC.

Main Program—This program is used for entering all of the required data. The basic routine is shown in Table V. The line numbers on the extreme left of the listing are not part of the program *per se* but are included as reference points into the program. The necessary input data are described in Table VI. This basic program can be readily modified to manipulate input data into master variables for the curve-fitting procedure; it may be used to obtain initial estimates for some or all of the parameters to be fitted; and it may be modified to include additional printout of information.

The modification statements would normally be placed between lines 12 and 13 of the basic routine. One such modification that the authors currently are using for evaluation of the structure equation

[3] At the University of Pennsylvania.

Table VIII—Listing of Subroutine NONLIN

```
1.000          SUBROUTINE NONLIN(NO,NOCON,TS)
2.000          IMPLICIT REAL*8(A-H,O-Z)
3.000          INTEGER NAME (60)
4.000          DIMENSION P(10),SP(10),STNDV(10),DEL(10),A(10,21),
5.000         *B(10,21),V(10,10),SB(10,21),PRCNT(100),SYCALC(100),
6.000         *PONE(10),PHALF(10),HILIM(10),HILIMY(100)
7.000          REAL*8 LOLIM(10),LOLIMY(100)
8.000          COMMON/SET1/P,NAME
9.000          COMMON/SET2/Y(100),YCALC(100),X(100),F(10)
10.000         SIGMA = 0.0
11.000    5    NUM = (2*NOCON)+ 1
12.000         N = NOCON + 1
13.000         L = NOCON + 2
14.000    C INITIALIZE ARRAYS
15.000         DO 10 I = 1,NOCON
16.000         DO 10 J = 1,NUM
17.000         A(I,J) = 0.0
18.000    10   CONTINUE
19.000         DO 30 I = 1,NOCON
20.000         J = I + N
21.000         A(I,J) = 1.0
22.000    30   CONTINUE
23.000    C CALCULATE VALUES FOR THE "A" ARRAY
24.000         DO 35 I = 1,NO
25.000         CALL FUNC(I,P,N)
26.000         DO 35 K = 1,N
27.000         DO 35 J = 1,N
28.000         A(K,J) = A(K,J) + F(K)*F(J)
29.000    35   CONTINUE
30.000    C CALCULATE "B" MATRIX
31.000         SUM = 0.0
32.000         DO 85 I = 1,NOCON
33.000         DO 85 J = 1,NUM
34.000         IF(I - J) 60,40,40
35.000    40   L = J - 1
36.000         IF(L)65,55,65
37.000    55   B(I,J) = A(I,J) - SUM
38.000         GO to 80
39.000    60   L = I - 1
40.000         IF(L)65,75,65
41.000    65   DO 70 K = 1,L
42.000         SUM = SUM + B(I,K)*B(K,J)
43.000    70   CONTINUE
44.000         IF(I - J)75,55,55
45.000    75   B(I,J) = (A(I,J) - SUM)/B(I,I)
46.000    80   SUM = 0.0
47.000    85   CONTINUE
48.000    C CALCULATE CORRECTION VECTORS
49.000         DO 130 I = 1,NOCON
50.000         J = N - I
51.000         IF(J - NOCON)115,125,125
52.000    115  L = J + 1
53.000         DO 120 K = L,NOCON
54.000         SUM = SUM + B(J,K)*DEL(K)
55.000    120  CONTINUE
56.000    125  DEL(J) = B(J,N) - SUM
57.000         SUM = 0.0
58.000    130  CONTINUE
59.000         DO 135 I = 1,NOCON
60.000         PONE(I) = P(I) - DEL(I)
61.000         PHALF(I) = P(I) - 0.5*DEL(I)
62.000    135  CONTINUE
63.000         RONE = 0.0
64.000         RHALF = 0.0
65.000         RZERO = 0.0
66.000         DO 137 I = 1,NO
67.000         CALL RESID(I,PHALF)
68.000         RHALF = RHALF + F(N)*F(N)
69.000         CALL RESID(I,PONE)
70.000         RONE = RONE + F(N)*F(N)
71.000         CALL RESID(I,P)
72.000         RZERO = RZERO + F(N)*F(N)
73.000    137  CONTINUE
74.000         RMIN = 0.5 + (0.25*(RZERO - RONE))/
75.000        *(RONE - 2.0*RHALF + RZERO)
76.000         DO 145 I = 1,NOCON
77.000         PRINT 140,I,P(I)
78.000    140  FORMAT(2X,'P(',I2,') = ',1PD14.7)
79.000    145  CONTINUE
80.000         PRINT 150,RZERO
81.000    150  FORMAT(2X,'RESIDUAL = ',1PD14.7,//)
82.000         IF(SIGMA - 0.0)155,160,155
83.000    155  RATIO = RZERO/SIGMA
84.000         IF(ABS(RATIO) - 0.999999)160,185,270
```

Table VIII—(*Continued*)

```
85.000                C SAVE "B" MATRIX, "P" MATRIX AND "YCALC" MATRIX
86.000           160      DO 165 I = 1,NOCON
87.000                    DO 165 J = 1,NUM
88.000                    SB(I,J) = B(I,J)
89.000           165      CONTINUE
90.000                    DO 170 I = 1,NOCON
91.000                    SP(I) = P(I)
92.000           170      CONTINUE
93.000                    DO 172 I = 1,NO
94.000                    SYCALC(I) = YCALC(I)
95.000           172      CONTINUE
96.000                    SRZERO = RZERO
97.000                C CALCULATE NEW VALUES FOR THE PARAMETERS
98.000                    DO 175 I = 1,NOCON
99.000                    P(I) = P(I) - RMIN*DEL(I)
100.000          175      CONTINUE
101.000          180      SIGMA = RZERO
102.000                   GO TO 5
103.000               C CALCULATE INVERSE MATRIX
104.000          185      NUM = NOCON + 1
105.000                   SUM = 0.0
106.000                   DO 205 M = 1,NOCON
107.000                   KO = NUM + M
108.000                   DO 205 I = 1,NOCON
109.000                   J = NUM - I
110.000                   IF(J - NOCON)190,200,200
111.000          190      L = J + 1
112.000                   DO 195 K = L,NOCON
113.000                   SUM = SUM + B(J,K)*V(K,M)
114.000          195      CONTINUE
115.000          200      V(J,M) = B(J,KO) - SUM
116.000                   SUM = 0.0
117.000          205      CONTINUE
118.000               C CALCULATE STANDARD DEVIATION ON EACH PARAMETER
119.000                   VAREXT = RZERO/(FLOAT(NO) - FLOAT(NOCON))
120.000                   DO 210 I = 1,NOCON
121.000                   J = I
122.000                   STNDV(I) = DSQRT(V(I,J)*VAREXT)
123.000          210      CONTINUE
124.000                   PRINT 211, NAME
                  a
125.000          215      FORMAT(///,T3,'CONSTANT NO.',T18,'STNDRD. DEV.',
126.000                 *T32,'95% LOW LIM.',T47,'95% HI LIM.')
127.000               C CALCULATE AND PRINT CONFIDENCE LIMITS
128.000                   DO 225 I = 1,NOCON
129.000                   ABLE = TS*STNDV(I)
130.000                   HILIM(I) = P(I) + ABLE
131.000                   LOLIM(I) = P(I) - ABLE
132.000                   PRINT 220,I,P(I),STNDV(I),LOLIM(I),HILIM(I)
133.000          220      FORMAT(T2,0PI1,T5,1PD12.5,T19,D10.3,T32,
134.000                 *D10.3,T47,D10.3)
135.000          225      CONTINUE
136.000                   PRINT 230,RZERO
137.000          230      FORMAT(///,' RESIDUAL = ',1X,1PD14.7)
138.000                   PRINT 235,VAREXT
139.000          235      FORMAT(////,' VAREXT = ',1X,1PD14.7)
140.000               C CALCULATE CONFIDENCE LIMITS ON YCALC
141.000                   DO 250 I = 1,NO
142.000                   CALL FUNC(I,P,N)
143.000                   SUM1 = 0.0
144.000                   SUM2 = 0.0
145.000                   DO 240 L = 1,NOCON
146.000                   J = L
147.000                   SUM1 = SUM1 + V(L,J)*F(L)**2
148.000          240      CONTINUE
149.000                   KO = NOCON - 1
150.000                   DO 245 L = 1,KO
151.000                   M = L + 1
152.000                   DO 245 J = M,NOCON
153.000                   SUM2 = SUM2 + 2.0*(V(L,J))*F(L)*F(J)
154.000          245      CONTINUE
155.000                   BAKER = DABS(VAREXT*(SUM1*SUM2))
156.000                   DOG = TS*DSQRT(BAKER)
157.000                   HILIMY(I) = SYCALC(I) + DOG
158.000                   LOLIMY(I) = SYCALC(I) - DOG
159.000                   DIF = Y(I) - SYCALC(I)
160.000                   PRCNT(I) = (DIF/Y(I))*100.0
161.000          250      CONTINUE
162.000                   PRINT 255
163.000          255      FORMAT(///,T6,'Y',T15,'YCAP',T27,'% DIFF.',
164.000                 *T39,'LOLIM',T50,'HILIM')
165.000                   DO 265 I = 1,NO
166.000                   PRINT 260,Y(I),SYCALC(I),PRCNT(I),LOLIMY(I),
167.000                  *HILIMY(I)
```

(*Continued*)

Table VIII—(*Continued*)

```
168.ØØØ          26Ø     FORMAT(T2,1PD1Ø.3,2X,D1Ø.3,2X,D1Ø.3,2X,D1Ø.3,
169.ØØØ                  *2X,D1Ø.3)
17Ø.ØØØ          265     CONTINUE
171.ØØØ          C CALCULATE AND PRINT THE CORRELATION COEFFICIENT
172.ØØØ                  SUMY = Ø.Ø
173.ØØØ                  DO 267 I = 1,NO
174.ØØØ          267     SUMY = SUMY + Y(I)**2
175.ØØØ                  R2 = (SUMY - RZERO)/SUMY
176.ØØØ                  R = DSQRT(R2)
177.ØØØ                  PRINT 268, R2,R
178.ØØØ          268     FORMAT(///,T3,'R SQUARED =',1X,D12.3,3X,
179.ØØØ                  *'R =',1X,D12.3,//)
18Ø.ØØØ          C PRINT THE CO-VARIANCE MATRIX
181.ØØØ                  DO 269 I = 1,NOCON
182.ØØØ                  DO 269 J = I,NOCON
183.ØØØ                  PRINT 271,I,J,V(I,J)
184.ØØØ          271     FORMAT(2X,'V(',I1,',',I1,') = ',2X,1PD14.7)
185.ØØØ          269     CONTINUE
186.ØØØ                  GO TO 285
187.ØØØ          C RECALL "B" MATRIX AND "P" ARRAY FROM STORAGE
188.ØØØ          270     IF(SIGMA - RZERO)272,185,185
189.ØØØ          272     NUM = (2*NOCON) + 1
19Ø.ØØØ                  DO 275 I = 1,NOCON
191.ØØØ                  DO 275 J = 1,NUM
192.ØØØ                  B(I,J) = SB(I,J)
193.ØØØ          275     CONTINUE
194.ØØØ                  DO 28Ø I = 1,NOCON
195.ØØØ                  P(I) = SP(I)
196.ØØØ          28Ø     CONTINUE
197.ØØØ                  RZERO = SRZERO
198.ØØØ                  GO TO 185
199.ØØØ          285     RETURN
2ØØ.ØØØ                  END
```

[a] At this point the following two lines should be added:

```
125.ØØØ      211     FORMAT (///,T3,60A1;//)
126.ØØØ              PRINT 215
```

All subsequent lines should then be renumbered.

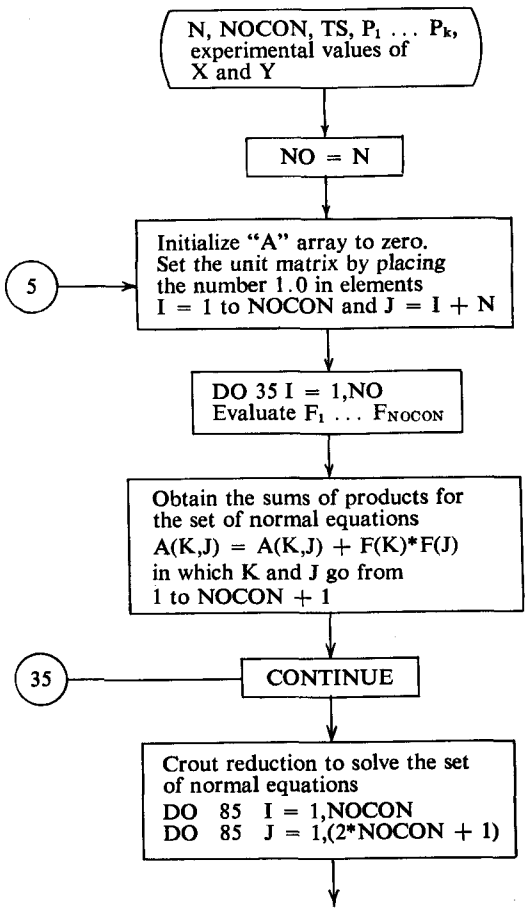
is given in Table VII. Instead of inputting initial estimates of the parameters in reference line 9, the variables TAU and ATAU are input, along with an estimate of P (4) which corresponds to the constant a of Eq. 1. The variables TAU and ATAU are used to convert the rheometer readings into shear stress and shear rate, respectively. This is done in reference lines 12–15. Initial estimates of the constants P_1, P_2, and P_3 of Eq. 20 are obtained in lines 16–18. Reference lines 21–35 are used for printing out additional information generally not included in the basic program.

Subroutine NONLIN—This subroutine is used for the actual curve-fitting procedure and is shown in Table VIII. It does not have to be altered and remains constant regardless of the function to be analyzed. It does call the subroutine FUNC, which must be defined for each function that is to be analyzed.

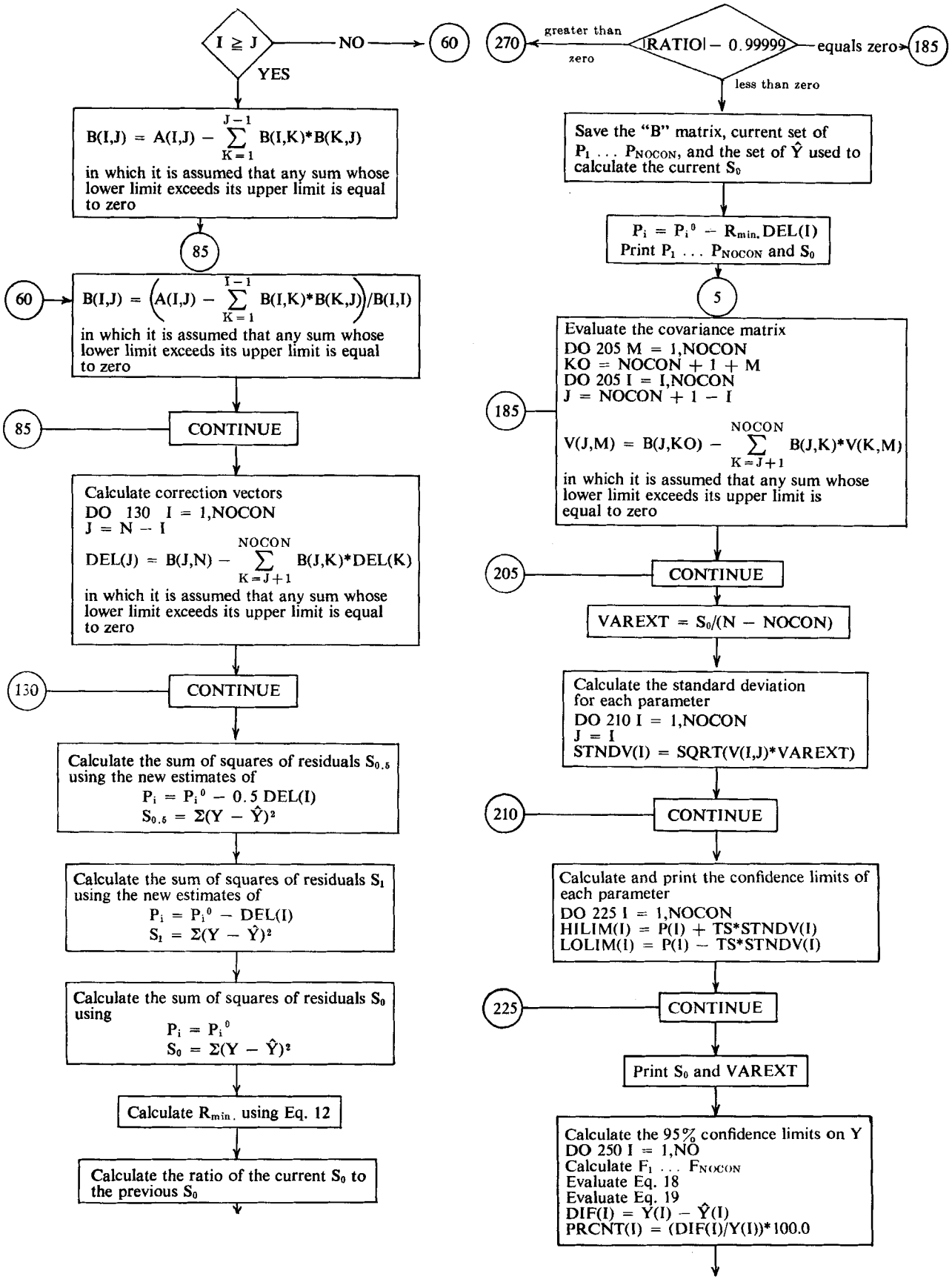
Subroutine FUNC—This subroutine calculates the values of the partial derivatives, as defined in Eq. 7, for each set of experimental data points. The listing of this subroutine is given in Table IX. Reference lines 1–4 should remain unchanged from function to function. The statement immediately following the statement on reference line 9 must contain the definition for YCALC (that is, the function to be fitted must be defined mathematically). The partial derivatives of YCALC with respect to P_1, P_2, *etc.*, are defined starting with reference line 5. This particular listing for subroutine

Table IX—Program Listing for Subroutine FUNC, Which Converts Eqs. 18 and 19 into Computer Language

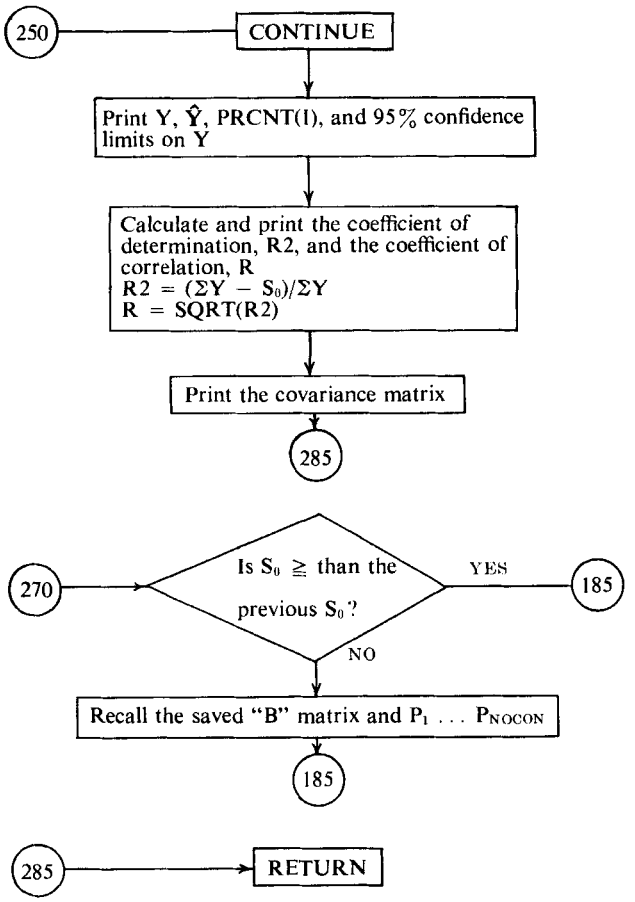
```
1  SUBROUTINE FUNC(I,P,N)
2  IMPLICIT REAL*8 (A-H,O-Z)
3  COMMON/SET2/Y(100),YCALC(100),X(100),F(10)
4  DIMENSION P(10)
5  F(1) = 1.0
6  F(2) = X(I)
7  F(3) = -DEXP(-P(4)*X(I))
8  F(4) = X(I)*P(3)*DEXP(-P(4)*X(I))
9  ENTRY RESID(I,P)
10 YCALC(I) = P(1) + P(2)*X(I) - P(3)*DEXP(-P(4)*X(I))
11 F(N) = Y(I) - YCALC(I)
12 RETURN
13 END
```



Scheme I—Flow Diagram for Subroutine NONLIN



Scheme I—(Continued)



Scheme I—(Continued)

FUNC shows the appropriate definitions for the structure equation and converts Eqs. 20 and 21 into computer language.

Computer Printout—The first set of printout data gives the estimates of each parameter and the sum of squares of residuals for each iteration. When the iteration process is satisfied, the printout skips to the next page and prints the title of the problem, the final value for each constant, the estimated standard deviation for each constant, and the 95% confidence interval for each constant.

The next section of printout gives the final sum of squares of residuals and the estimate of the unit variance, VAREXT. This section is followed by one which gives the experimental and calculated values for the dependent variable (in this instance, shear stress), the relative difference between the two, and the 95% confidence interval for the calculated values. This section is followed by an estimate of the coefficient of determination, which gives the fraction of the variance accounted for by regression, and the correlation coefficient.

The final section of printout gives the elements of the covariance matrix. These elements are useful for determining the variance on any constant that is a function of the parameters adjusted through the nonlinear regression analysis (Eqs. 16 and 17).

A flow diagram for the subroutine NONLIN as presented in this article is given in Scheme I. This should prove useful in the event modifications are needed for a particular computer system.

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Identification of a Major Metabolite of Nortriptyline in Human Urine

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Abstract □ Urine samples taken from a person who ingested nortriptyline·HCl (4 × 25 mg. daily) were extracted by a two-phase procedure with ether. A first screening by UV spectrophotometry showed the presence of compound(s) with basic character but different from free nortriptyline. The TLC separation of aliquots of extracts revealed traces of free nortriptyline, three minor metabolites, and a more polar major metabolite. These results were confirmed by GLC, which was performed on the acetylated extracts using two different columns. Elucidation of the structure of the major metabolite was attempted in two ways. A 1-hr. treatment of the extract with 1 *N* HCl at 100° caused a β-elimination and transformed the compound to a polyene structure demonstrated by the typical UV spectral shift. A microcollection technique for trapping GLC effluents was developed to obtain the acetylated derivative of the metabolite in a highly purified form. From the trapped compound, a micro-IR and low- and high-resolution mass spectra were recorded; these permitted a thorough interpretation. These experiments indicated that 10-hydroxynortriptyline is excreted in human urine as a major metabolite from nortriptyline. Desmethylnortriptyline was not detected in the urine.

Keyphrases □ Nortriptyline—identification of 10-hydroxynortriptyline as major metabolite in human urine □ 10-Hydroxynortriptyline—identification as major metabolite of nortriptyline in human urine, GLC

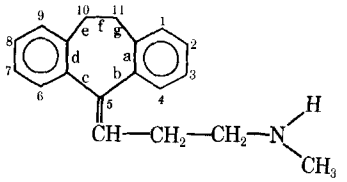
Nortriptyline (I) is a dibenzocycloheptadiene antidepressant (1). It is the monodesmethyl derivative of amitriptyline and is a metabolite of amitriptyline in animals as well as in man (2). From the structural formula, it may be deduced that the free base should be extractable from alkalized biological fluids with a nonpolar organic solvent such as ether. The less polar metabolites may also be expected in the same extract.

McMahon *et al.* (3) reported that nortriptyline is metabolized in the rat to 10-hydroxynortriptyline. Amundson and Manthey (4) published an excellent study on nortriptyline excretion by man. With a different experimental approach, the objective of the present study was to obtain corresponding information about the metabolism of nortriptyline in man.

EXPERIMENTAL

The total urine output from a 50-year-old man, who ingested 4 × 25 mg. of nortriptyline·HCl daily for 7 months, was collected on 4 successive days. Each urine sample was immediately placed in the refrigerator, and a 200-ml. aliquot of the 24-hr. collection was extracted prior to analysis without any delay.

Extraction Procedure—A 200-ml. aliquot of each urine sample was acidified with 2 *N* HCl and extracted twice under nitrogen atmosphere with 400 and 200 ml. of peroxide-free ether (freshly distilled over hydroquinone); the ether phases were rejected. The aqueous phase was alkalized with 10 *N* NaOH and again extracted twice under nitrogen atmosphere with 200 ml. of ether. The ether layers, which contained nortriptyline and less polar metabolites,



5-(3-monomethylaminopropylidene)-5*H*-dibenzo[*a*,*d*][1,4]-cycloheptadiene

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were washed with 5-ml. portions of 0.001 *N* NaOH until the aqueous layer remained colorless. The washed ether was evaporated in a rotatory evaporator to a final volume of about 100 ml., saturated with nitrogen, and extracted twice with 10 ml. of 0.1 *N* HCl. These acid extracts were first used for UV analysis. The total acid extracts were made basic with 1 ml. of 10 *N* NaOH, saturated with nitrogen, and extracted twice with 40 ml. of peroxide-free ether. The combined ether phases were dried over Na_2SO_4, filtered, and evaporated on a water bath at 40° under a slow stream of nitrogen. The residue was dissolved in 10 ml. of ethanol and kept in a well-closed tube at −15° for further analysis ("alcoholic extraction liquid").

UV Spectrophotometry—The UV spectra of the acidic extracts were recorded with a double-beam Unicam SP 800 spectrophotometer in the wavelength range of 450–190 nm.

TLC—A 1-ml. aliquot of each alcoholic extract was evaporated on a water bath at 40° to a small volume with the aid of a stream of nitrogen. The extract, together with 10 mcg. of the reference compounds nortriptyline·HCl and desmethylnortriptyline·HCl (0.1% ethanolic solutions), was spotted separately on a 250-μ thin-layer plate prepared with equal parts of silica gel H 254 and cellulose MN 300. Chloroform, acetone, and 25% ammonia (50:50:1 in milliliters) was used for development (5). Then the plate was viewed under 254-nm. light to determine R_f values. A more selective visualization was performed by spraying the plate with iodoplatinate reagent, prepared by mixing chloroplatinic acid 5% (w/v), potassium iodide 10% (w/v), and distilled water (5:45:100 in milliliters) (6).

GLC—GLC analysis was performed with Hewlett-Packard research gas chromatograph 5750, equipped with dual flame-ionization detectors (FID). Two packed columns of different polarity were installed for simultaneous single-column operation. One was prepared with 2% FFAP (a reaction product of carbowax 20M and *m*-dinitroterephthalic acid) and the other with 5% OV-1 (a methylsilicone polymer) by coating the liquid phases on Diatoport S (acid and silane treated), 80–100 mesh, as a support.

The working conditions were: glass spiral columns, 1.80-m. length, 4-mm. i.d.; carrier gas, nitrogen, 70 ml. $min.^{-1}$ (bubble flowmeter); oven temperature, 240° for analysis on 2% FFAP (polar column), 215° for analysis on 5% OV-1 (apolar column); injector block temperature, 245° (FFAP), 220° (OV-1); detector block temperature (FID), 245° (FFAP), 220° (OV-1); and air and hydrogen, calibrated for optimum sensitivity.

The compounds were transformed to their respective acetylated derivatives before injection, because the presence of a secondary amine function in the side chain causes irreversible retention on the polar 2% FFAP column. For that reason, 1-ml. aliquots of the individual extracts were transferred to conical siliconized tubes of 15-ml. capacity and evaporated on a water bath at 40° under a slow stream of nitrogen. To the final residues, 0.2 ml. of pyridine (refluxed and distilled over KOH) and 0.2 ml. acetic acid anhydride

Table I—Volume and pH of Collected Urine Samples

Sample	Volume of Urine, ml./24 hr.	pH
1 (1st day)	1650	6.0
2 (2nd day)	1950	5.5
3 (3rd day)	1800	6.5
4 (4th day)	1950	6.5

(refluxed and distilled over CaC_2) were added. After a 1-hr. reaction time in a P_2O_5 desiccator, the reaction mixtures were evaporated again under the mentioned conditions. The residues obtained were dissolved in a small volume of ethyl acetate (50 μl.) with the aid of a Whirlmixer. The standards, nortriptyline·HCl and desmethylnortriptyline·HCl (10-mg. amounts), were treated in the same way, but the residues obtained were taken up in 10 ml. of ethyl acetate (0.1% solutions).

Trapping Technique of GLC Effluents on Microscale—A simple system for trapping GLC effluents arising from 20–200 mcg. of injected samples was developed. A splitting system[1] was installed at the exit of each column, splitting the effluent gas so that 10 parts went to the FID and 100 parts entered the catharometer block. This split ratio was obtained by passing the effluent through two parallelly mounted capillaries of 101.6- (FID) and 10.16- (catharometer) cm. (40- and 4-in.) lengths, respectively.

The catharometer detector was heated at the same temperature as the oven and only used as a transfer zone (bridge current switched off). As a collection device, a Pyrex capillary of 12.5-cm. length and 1.5-mm. i.d., fixed with two Teflon O-rings in a brass tube of 10.5-cm. length, 7-mm. o.d., and 4-mm. i.d., provided with a 0.42-cm. (0.125-in.) swagelok nut, was used. For each collection, a brass tube was first loaded with a carefully cleaned capillary (stored overnight in chromic acid, rinsed six times with distilled water, and dried at 120° in a drying stove), and the assembly was attached to the exit of the catharometer block approximately 1 min. before the desired compound was eluted. The simultaneous recording of the chromatogram allowed us to follow the chromatographic process and indicated the moment at which the collection device could be unscrewed from the chromatograph. The brass tube served as a temperature gradient which avoided high losses of trapped compounds through aerosol formation.

IR Spectrophotometry—A 1-ml. aliquot of the alcoholic extract was acetylated, and the residue was dissolved in 50 μl. of ethyl acetate. This solution was at once preparatively chromatographed on the 5% OV-1 column at 215°. After collection, the condensed parent metabolite was rinsed from the capillary with 2 × 10 μl. of ethanol. The ethanol solutions were poured on 27 mg. of KBr in an agate mortar, and the solvent was allowed to evaporate. A micropellet was prepared by means of a punched disk (2 × 10-mm. hole); the IR spectrum was recorded from 625 to 4000 $cm.^{-1}$ with a Perkin-Elmer 257 spectrophotometer, equipped with microbeam condenser RIIC C-41.

Degradation Reaction—The product, 10-hydroxyamitriptyline, is known to give the reaction product 10,11-dehydroamitriptyline when heated in 1 *N* HCl (3). Two solutions were prepared containing, respectively, 100 mcg. of 10α-hydroxyamitriptyline and 100 mcg. of 10β-hydroxyamitriptyline in 10 ml. of 1 *N* HCl. After heating the solutions for 1 hr. on a boiling water bath, the UV spectra of the reaction mixtures were recorded. The same reaction was carried out on 1 ml. of the alcoholic extracts.

Mass Spectrometry—A low-resolution mass spectrum was obtained using a fragment of the KBr micropellet (containing the acetylated compound) with an AEI MS 902 mass spectrometer. The remaining part of the pellet was eluted with ethyl acetate. The solution was employed for recording high-resolution mass spectral data by means of the same spectrometer, provided with the AEI DS 10 data acquisition system.

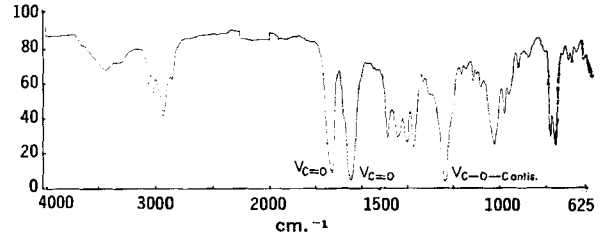


Figure 1—*IR spectrum of trapped major metabolite (acetylated compound).*

[1] Hewlett-Packard.

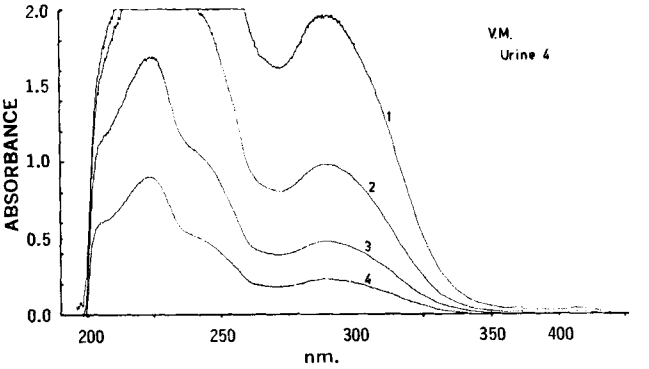


Figure 2—*UV spectrum of the extract of Sample 4 after treatment with 1 N HCl at 100° for 1 hr.*

RESULTS AND DISCUSSION

The volume and pH of the urine samples were determined just after each 24-hr. collection. These data are summarized in Table I.

The UV spectra of all samples showed a striking correspondence with the reference spectra of 10α-hydroxyamitriptyline ($\lambda_{max.}$ 209 and 239 nm. with ε 37,378, and 14,885; $\lambda_{min.}$ 230 nm.) and β-hydroxyamitriptyline ($\lambda_{max.}$ 209 and 239 nm. with ε 40,355 and 15,878; $\lambda_{min.}$ 229 nm.).

More details concerning the value and interpretation of UV spectra obtained on biological extracts were published earlier (7).

A small amount of free nortriptyline was detected by TLC only in the extract of Sample 4 (R_f 0.25 brown). A constant metabolic pattern was found in all extracts; three minor metabolites (R_f 0.04 brown, 0.50 blue-gray, and 0.53 brown) besides one major metabolite (R_f 0.16 brown) were present. No spots occurring in the extracts could be assigned to desmethylnortriptyline (R_f 0.62 brown).

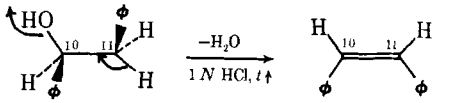
GLC analysis on the 2% FFAP column (240°) showed the presence of free nortriptyline (R_t 23 min. 25 sec., theoretical plates 2500) in the fourth urine sample. On the 5% OV-1 column (215°), free nortriptyline (R_t 12 min. 18 sec., theoretical plates 2100) was also revealed in Sample 4; one minor metabolite, with a relative retention time of 1.65, and one major metabolite with a relative retention time of 1.94, were noted in all extracts examined. These results correlate with the TLC analysis, except that the later technique detected two more metabolites which probably are adsorbed irreversibly on the GLC columns.

The IR spectrum (Fig. 1) of the acetylated metabolite isolated by GLC gave the following structural information:

1. A strong absorption band at 1650 $cm.^{-1}$ corresponds to the ketone-stretching vibration ($\nu_{C=O}$) of a tertiary amide structure (6); the last was caused by the acetylation treatment which converted the secondary amine group of the side chain to the respective acetamide function [—N(—CH_3)—C(=O)—CH_3].

2. Two other strong absorption bands occur at 1735 and 1240 $cm.^{-1}$. The former is explained as a carbonyl-stretching vibration ($\nu_{C=O}$) of an alcoholic acetylester, which is further confirmed by the second band owing to an ester antisymmetrical stretching vibration (ν_{C-O-C} stronger and broader than $\nu_{C=O}$ of the same group) (8). Here the acetylation reaction transformed the alcoholic function to the acetylester group [≡C—O—C(=O)—CH_3].

The degradation reaction can be understood as a *trans*-diaxial β-elimination, which results in the formation of a supplementary double bond between C_{10} and C_{11} (Scheme I). The 10,11-dehydro derivative possesses an extensive conjugation system, which may be



10-hydroxy compound 10,11-dehydro compound

Scheme I

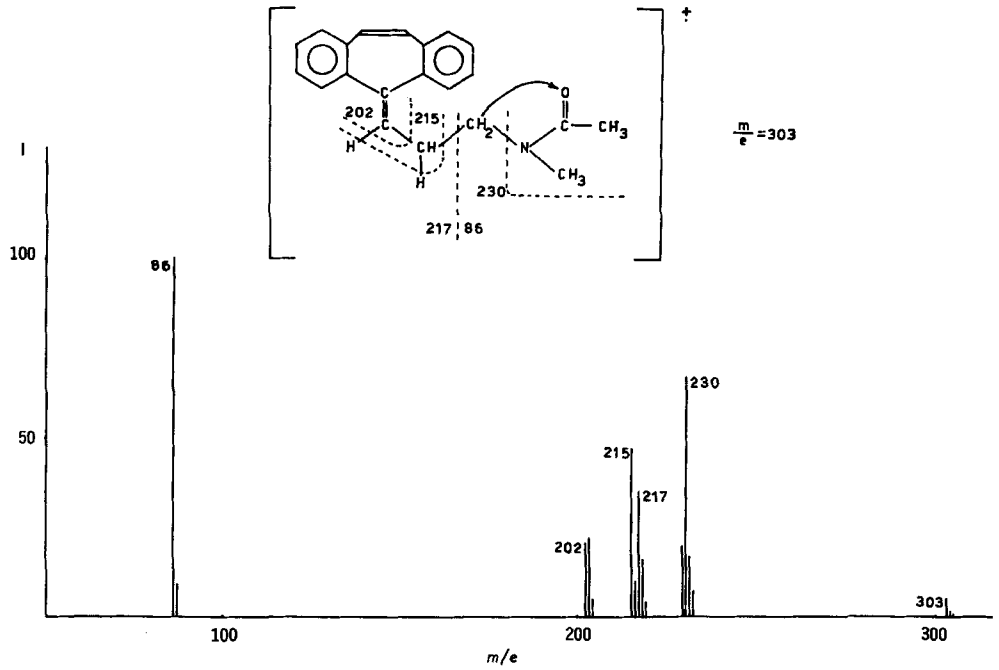
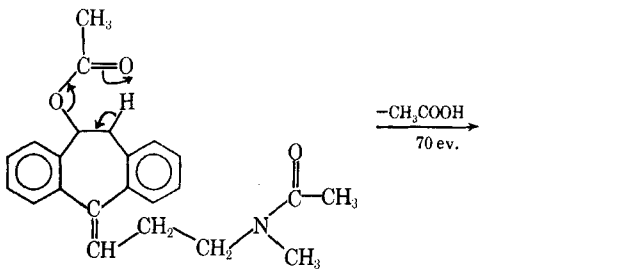


Figure 3—*Mass spectrum of trapped major metabolite (acetylated compound).*

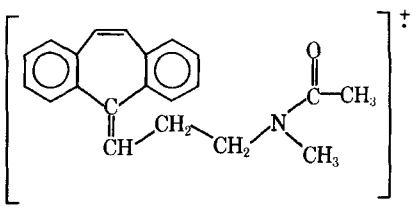
observed as a typical UV spectral shift. In this way, the UV spectra from 10α- and 10β-hydroxyamitriptyline reference solutions were highly different from those obtained before reaction, and both showed two absorption maxima at 225 and 290 nm. and an absorption minimum at 266 nm. A completely similar effect was found with the extracts of Samples 1 and 4 (Fig. 2).

Final evidence was derived from following mass spectral data (70 ev., 150°): *m/e* (relative intensity) 303(5) $C_{21}H_{21}NO$, 230(67) $C_{18}H_{14}$, 217(37) $C_{17}H_{13}$, 215(47) $C_{17}H_{11}$, 202(21) $C_{16}H_{10}$, 86(100) C_4H_8NO. The peak at 303 corresponded to nortriptyline *O,N*-diacetate minus a molecule of CH_3COOH. Loss of the molecular ion peak is known for acetates with long alkyl chains (9, 10). The process occurring for the unknown apparently may be envisioned as shown in Scheme II.

The mass spectral fragmentation pattern (Fig. 3) is conclusive for accepting an unchanged side chain of nortriptyline metabolite. All these experiments indicate the presence of a parent metabolite with a similar basic structure but of higher polarity than the free compound nortriptyline. The IR spectrophotometric analysis of the acetylated and trapped metabolite led to the conclusion of an *O,N*-diacetate structure. Therefore, the metabolite had to possess an alcoholic OH function besides its original secondary amine group.



expected *m/e* = 363



m/e = 303

Scheme II

The location of this OH function could not be on one of the two phenyl groups as seen from the IR spectrum [that is, absorption at 1735 cm.$^{-1}$ is typical for saturated acetylesters as compared to 1760 cm.$^{-1}$ reported for phenol acetate esters (8)]. The remaining positions were then C_{10}, C_{11} or the alkyl side chain. The former possibility was strongly supported by a β-elimination reaction followed by UV spectrophotometric analysis, which gave exactly the same result for the major metabolite as for the model compounds 10α- and 10β-hydroxyamitriptyline. Complete confirmation of former interpretations was given by mass spectral analysis. Therefore, it was concluded that 10-hydroxynortriptyline is the structure of the major metabolite.

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Mechanism of Increased Rates of Dissolution and Oral Absorption of Chloramphenicol from Chloramphenicol–Urea Solid Dispersion System

WIN LOUNG CHIOU

Abstract □ The phase diagram of the chloramphenicol–urea binary system is reexamined using the differential thermal analysis and X-ray diffraction methods. It is concluded that the system is a simple eutectic mixture rather than a partial solid solution as previously proposed by other authors. The increased rates of *in vitro* dissolution and *in vivo* absorption of chloramphenicol solid dispersed in urea are explained mainly on the basis of a change of particle size of the chloramphenicol crystals.

Keyphrases □ Chloramphenicol–urea solid dispersion system—mechanism of increased dissolution, absorption rates of chloramphenicol □ Phase diagram, chloramphenicol–urea binary system—reexamined using differential thermal analysis, X-ray diffraction □ Dissolution, absorption, chloramphenicol solid dispersed in urea—mechanism of increased rates

In 1964, Sekiguchi *et al.* (1) showed that the rates of *in vitro* dissolution and *in vivo* oral absorption of chloramphenicol could be markedly increased by solid dispersions of the drug in urea at certain weight fraction (20% chloramphenicol–80% urea). They attributed such striking findings mainly to the particle-size reduction of chloramphenicol in the solid dispersed form as the eutectic grains of the chloramphenicol–urea mixture might be in the colloidal or micron range.

After reexamining the phase diagram (Fig. 1) reported by Sekiguchi *et al.* (1), Goldberg *et al.* (2, 3) proposed that the binary system of chloramphenicol–urea should be classified as a partial solid solution rather than a simple eutectic mixture. They further proposed that the attainment of supersaturation and the observed increase of dissolution and absorption rates were primarily due to the molecular dispersion of chloramphenicol in the carrier, urea, as they formed a solid solution. Their papers were cited by numerous authors (4–6).

A careful analysis of the differential thermal analysis (DTA) data of this binary system by Sekiguchi *et al.* (1) revealed, however, a possible error in their construction of the phase diagram. Additional X-ray diffraction studies of the system in these laboratories substantiated the DTA evidence. The following is a brief report of this study.

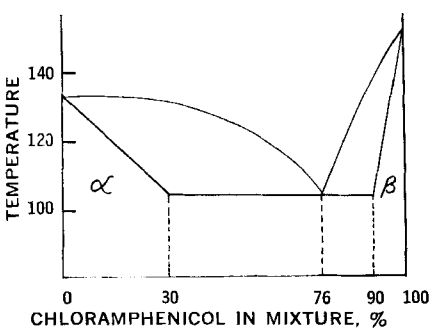

Figure 1—*Phase diagram for chloramphenicol–urea system (from* Reference 1).

EXPERIMENTAL

Materials—Chloramphenicol USP[1] and urea[2], reagent grade, were used without further purification.

Sample Preparation—The physical mixtures of various compositions of chloramphenicol–urea in suitable beakers were heated in an oil bath with stirring until melted. After solidification by shock cooling on a stainless steel plate, the masses were pulverized into fine powders.

X-Ray Diffraction Studies—The fine powders of samples were evenly laid as thin layers on glass slides with the aid of a small amount of Duco Cement[3]. The X-ray diffraction spectra of these preparations were obtained by scanning at 2°/min. in terms of a 2θ angle by a Norelco X-ray diffractometer.

DTA Study—The DTA thermograms of finely powdered samples were obtained from a Dupont 900 thermal analyzer attached with a standard DTA cell (500°). A sample size of 2–3 mg. of powders in a 2-mm. diameter sample tube and a heating rate of 5°/min. were employed. Glass beads were used as a reference.

RESULTS AND DISCUSSION

X-Ray Diffraction Studies—The X-ray diffraction technique was recently used to study the physical nature of polyvinylpyrrolidone–sulfathiazole coprecipitates (7). The crystalline precipitates of the sulfathiazole in the coprecipitates were easily detected by the presence of the typical spectra of the crystalline forms. Goldberg *et al.* (2, 3) suggested that an α-phase solid solution exists up to 30% chloramphenical in 70% urea and that a β-phase solid solution exists from 10% urea in 90% chloramphenicol. Therefore, to assess the validity of this postulate, the X-ray technique was used in this investigation to see if chloramphenicol crystals could be detected in the resolidified fused masses containing 2, 5, and 10% chloramphenicol.

The X-ray diffraction spectra of the pure chloramphenicol and urea [melting point and DTA thermograms of both compounds used in this study are almost identical to those reported by Sekiguchi *et al.* (1)] are shown in Fig. 2. The diffraction spectra of the mechanical (or physical) and dispersed mixtures of 10% chloramphenicol–90% urea are shown in Fig. 3. By comparing these spectra with the diffraction spectrum of pure chloramphenicol, the peaks, due to chloramphenicol crystals in the physical mixture, can be readily identified, as shown by checkmarks in the spectrum. The presence of these identical chloramphenicol diffraction peaks in terms of diffraction angles in the spectrum of the resolidified fused mass unmistakably show that the mass contains some separated chloramphenicol crystallites. From a theoretical point of view, if the chloramphenicol dissolved completely as a minor component in the urea at the solid state, *i.e.*, solid solution formation, one should not be able to find these typical chloramphenicol diffraction peaks. On the contrary, the lattice parameters, such as diffraction peak angles of the solvent crystal, can be either increased, unchanged, or decreased in the formation of a solid solution (8, 9). No noticeable change in the urea crystalline lattice could be detected in the resolidified fused

[1] Supplied by Parke, Davis and Co.
[2] Mallinckrodt Chemical Works.
[3] Dupont Co.

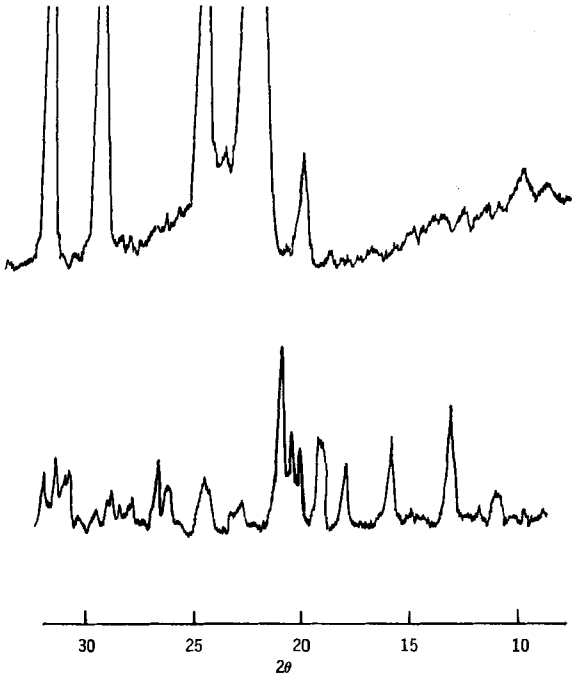

Figure 2—*X-ray diffraction spectra of pure chloramphenicol (bottom) and resolidified pure urea (top).*

mixture, as indicated by the same diffraction peaks of the urea present in the mixture as in the pure urea sample.

The aforementioned diffraction peaks of chloramphenicol are also present in the 5 and 2% resolidified samples, but with a decreasing intensity. These diffraction spectra were run within 2 hr. after the resolidification of the melt by the rapid cooling process. It is well known that a supersaturated solid solution can be often obtained at room temperature by the quenching method, as employed in this study (10). Based on these data, one can, therefore, conclude that the solid solubility of chloramphenicol in this binary system at the eutectic temperature is probably less than 2% w/w, and its solid solubility at ambient temperatures (less than 30°) is certainly less than 2% w/w.

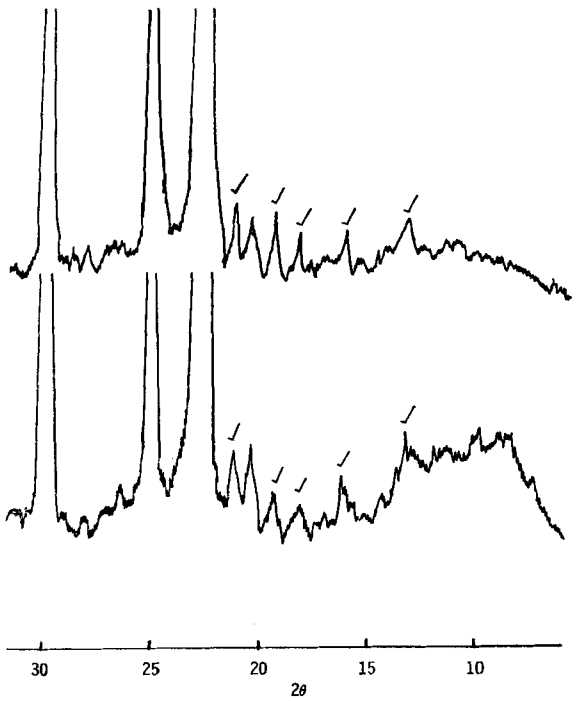

Figure 3—*X-ray diffraction spectra of physical mixture of 10% chloramphenicol–90% urea (bottom) and resolidified fused mixture of 10% chloramphenicol–90% urea (top). Arrows indicate diffraction peaks due to the presence of chloramphenicol crystallites.*

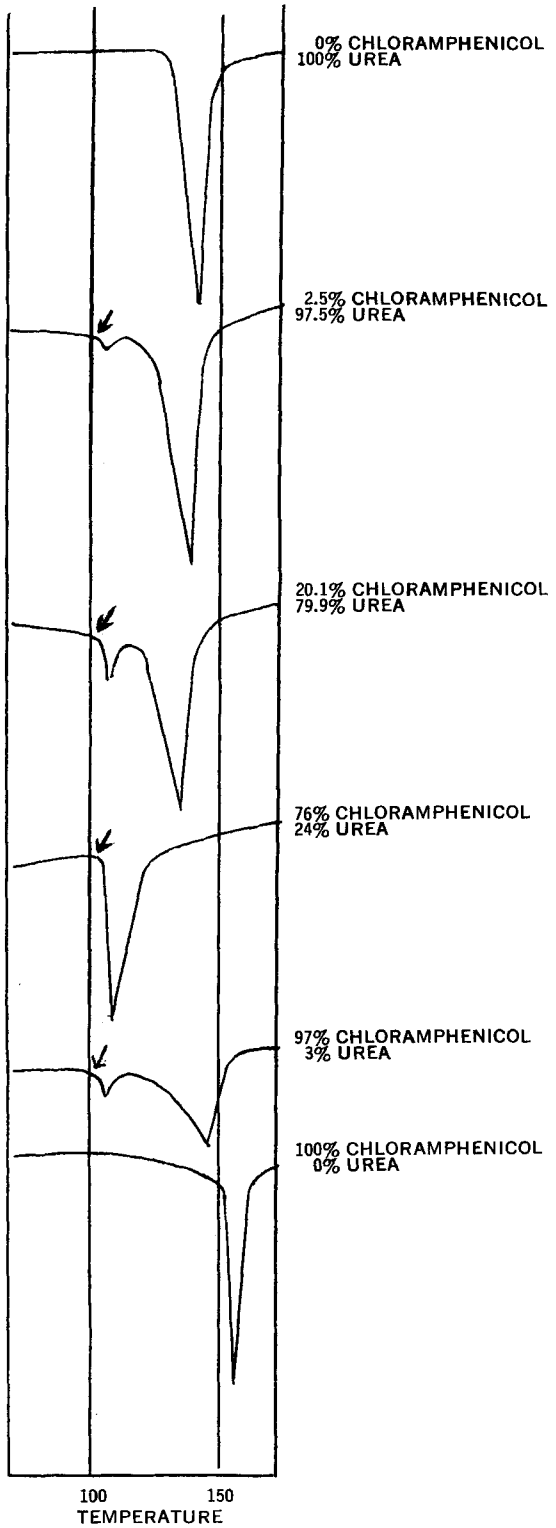

Figure 4—*DTA thermograms of chloramphenicol–urea mixtures redrawn from* Reference 1.

DTA Studies—Figure 4 includes a modified drawing of the original DTA data of Sekiguchi *et al.* (1) for the fused mixtures of chloramphenicol–urea. The top and bottom curves represent the DTA data on the pure compounds. The four intermediate curves are on the respective mixtures noted to the right of the curves. All four of these curves show clear evidence of a eutectic peak (e.p.)

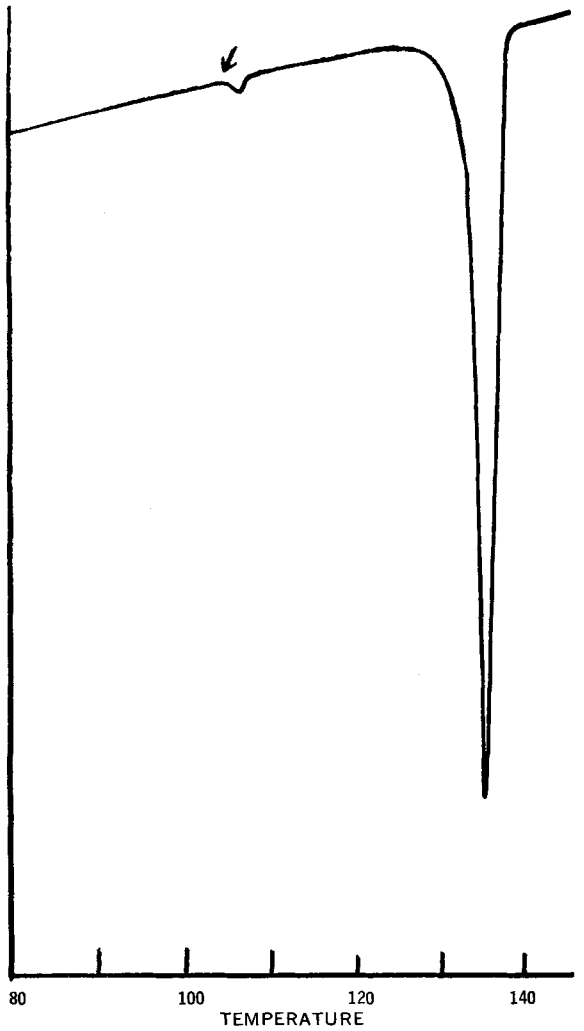

Figure 5—*DTA thermogram of 2% chloramphenicol–98% urea physical mixture run at 5°/min. (arrow indicates the thaw point of the mixture which is equal to the eutectic temperature).*

starting approximately at the same eutectic temperature, as indicated by an arrow (equivalent to e.p. used in the original reference) (11). The data of these samples, ranging from 2.5 to 97% chloramphenicol (w/w), indicate that they all start to thaw at the eutectic temperature. In other words, the eutectic isothermal line in Fig. 1 should extend to at least the 2.5% chloramphenicol–97.5% urea on the left and to at least the 97% chloramphenicol–3% urea on the right (12). Therefore, the solid solubility for chloramphenicol at the eutectic temperature is less than 2.5% w/w, and that for urea is less than 3% w/w in this binary system on the basis of the DTA data.

The original Sekiguchi *et al.* (1) chloramphenicol–urea phase diagram was the result of a thaw–melt study utilizing capillary melting-point tubes. It may have been a difficult task to detect visually the thaw point at the extreme ends of the curve by this method. However, the DTA apparatus is extremely sensitive to endothermic and exothermic reaction, and minute amounts of impurities in samples can be detected by this method (12). The average thaw points from capillary studies reported by Sekiguchi *et al.* (1) for 10 and 5.6% chloramphenicol physical mixtures are 107 ± 1.5 and 113 ± 3°, respectively. The thaw point for 2% chloramphenicol physical mixture might then be expected to be much higher than 113°. Nevertheless, the thaw points of 2, 5, and 10% chloramphenicol physical mixtures are all at 104 ± 1° according to the DTA studies conducted in this laboratory. The value, 104°, is in good agreement with the reported eutectic temperature. The thermogram of 2% chloramphenicol–98% urea run at 5°/min. is shown in Fig. 5.

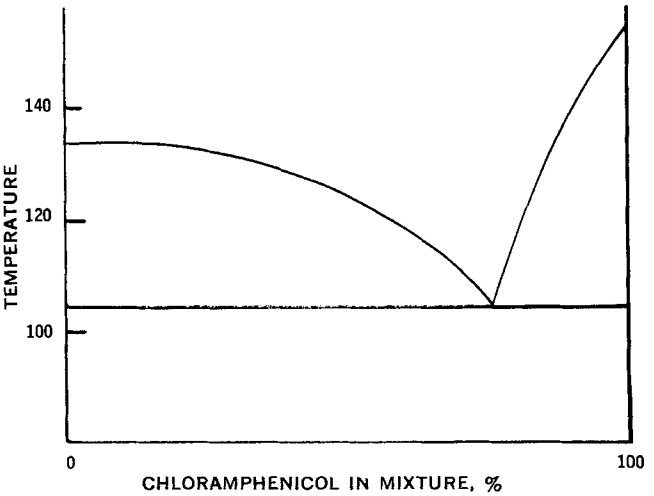

Figure 6—*Phase diagram of a simple eutectic mixture of chloramphenicol–urea system. Negligible solubilities at solid state are not shown in the diagram.*

Mechanisms of Increased Rate of Dissolution—From the DTA and X-ray diffraction evidence, one can conclude that the mutual solid solubility of chloramphenicol and urea is very limited and such a binary system can be more correctly described as a simple eutectic mixture with negligible solid solubility (Fig. 6). Goldberg *et al.* (2, 3) based their postulate on the extremes of the phase diagram, which are clearly not in accord with the DTA or X-ray spectral data reviewed here. It would appear that the original suggestion of Sekiguchi *et al.* (1) of a particle-size effect in increasing rates of dissolution and absorption of chloramphenicol is more in accord with the physical–chemical facts discussed here.

There is little doubt that a solid solution of a poorly water-soluble drug in a rapidly dissolving matrix would result in a rapid availability and absorption of a drug such as chloramphenicol. However, no data are available at present to allow a comparison of a solid solution *versus* a dispersion system as represented by chloramphenicol–urea.

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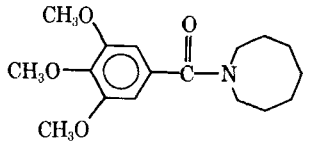
Structure–Activity Studies of New Potential Depressants 3,4,5-Trimethoxybenzamides II

HEINO A. LUTS

Abstract □ A group of 3,4,5-trimethoxybenzamides of various amines was synthesized and subjected to general pharmacological screening. The structures of Compounds 7, 8, 9, and 10 show strong depressant properties. A hypothesis is provided for the possible difference in activity of the various substituents on the amide nitrogen.

Keyphrases □ 3,4,5-Trimethoxybenzamides—synthesis, evaluation as potential depressants, structure–activity relationships □ Depressants, new potential—structure–activity relationships, synthesis, evaluation, 3,4,5-trimethoxybenzamides □ Structure–activity relationships—3,4,5-trimethoxybenzamides, potential depressants

In 1963, 3,4,5-trimethoxybenzoylheptamethyleneimine was synthesized and tested and, subsequently, the results were published (1). The compound was shown to



3,4,5-trimethoxybenzoylheptamethyleneimine

be a stimulant-antidepressant, and it exhibited no analgesic action. Since that time, several other workers have repeated the studies with the same findings (2, 3).

In continuation of these studies, some new structures were prepared and pharmacologically evaluated (Table I). The compounds prepared all have —N(—C)—C linkage; no —N(—H)—C linkage was prepared because of possible hydrogen bonding formation *in vivo*. The findings indicate that in cases where the imine ring was either decreased in size (Compound 3) or enlarged (Compound 4), the compounds exhibited depressant activity. Of the structures examined, Compounds 7, 8, 9, and 10 are strong depressants, while 3 and 5 possess only medium depressant activity. In general, it can be stated that if the compounds have nitrogen atoms outside the ring, and the amine function is nonaromatic, the depressant activity is stronger. The fact that the heptamethyleneimine derivative is an antidepressant and the hexa- and octamethyleneimine relatives are not prompted a look at other possible differences. By building the molecular models (Figs. 1–3), it was noted that the hydrogen atom from the number 5 carbon atom was overlapping the nitrogen atom and likely altering the electron density.

In the canonical form, the overlapping is expected to be even more pronounced. Possibly because of this, two five-membered ring structures are observed. This is not possible with the hexa- and octamethyleneimine derivatives, and it is postulated that the proximity effect is the reason for the difference in biological activity.

The compounds were prepared according to the method previously described (1).

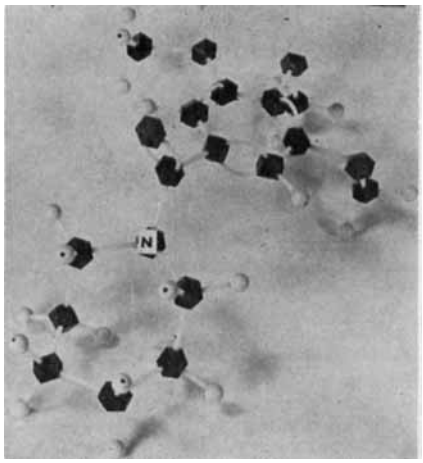


Figure 1—*3,4,5-Trimethoxybenzoylhexamethyleneimine.*

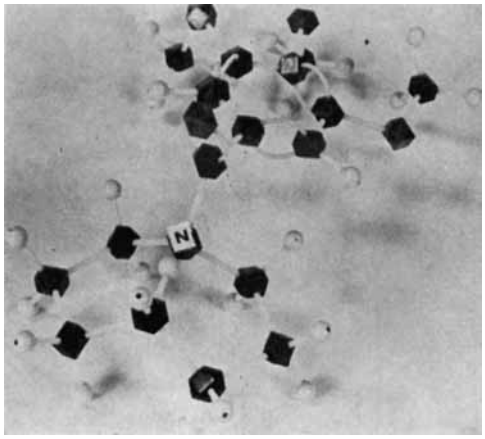


Figure 2—*3,4,5-Trimethoxybenzoylheptamethyleneimine.*

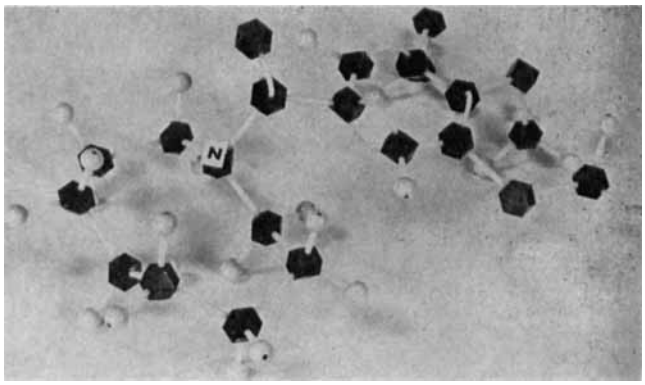
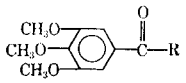
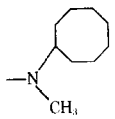
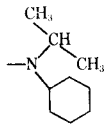
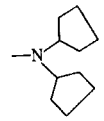
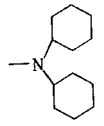
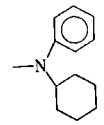


Figure 3—*3,4,5-Trimethoxybenzoyloctamethyleneimine.*

Table I—Physical Data for 3,4,5-Trimethoxybenzamide Derivatives



Compound Number	R	Formula	Melting Point	Yield, %	Analysis, % Calc.	Analysis, % Found
1		$C_{19}H_{27}NO_4$	135°	78	C, 68.44 H, 8.16 N, 4.19	C, 68.32 H, 8.16 N, 4.14
2		$C_{18}H_{25}NO_4$	111°	83	C, 67.63 H, 7.89 N, 4.38	C, 67.66 H, 7.85 N, 4.37
3		$C_{18}H_{27}NO_4$	111°	86	C, 73.73 H, 9.28 N, 4.77	C, 73.76 H, 9.30 N, 4.70
4		$C_{16}H_{23}NO_4$	75°	42	C, 41.12 H, 7.21 N, 4.35	C, 41.04 H, 7.27 N, 4.36
5		$C_{19}H_{21}NO_4$	95°	84	C, 69.70 H, 6.46 N, 4.27	C, 69.74 H, 6.49 N, 4.36
6		$C_{19}H_{21}NO_4$	98°	86	C, 69.70 H, 6.46 N, 4.27	C, 69.76 H, 6.51 N, 4.26
7		$C_{19}H_{29}NO_4$	79°	78	C, 68.03 H, 8.71 N, 4.17	C, 68.12 H, 8.69 N, 4.19
8		$C_{19}H_{29}NO_4$	69°	72	C, 68.03 H, 8.71 N, 4.17	C, 68.06 H, 8.74 N, 4.24
9		$C_{29}H_{29}NO_4$	184°	91	C, 69.13 H, 8.41 N, 4.02	C, 69.01 H, 8.37 N, 4.01
10		$C_{22}H_{33}NO_4$	172°	77	C, 70.36 H, 8.85 N, 3.72	C, 70.29 H, 8.89 N, 3.78
11		$C_{22}H_{27}NO_4$	134°	84	C, 71.52 H, 7.36 N, 3.78	C, 71.43 H, 7.88 N, 3.67

EXPERIMENTAL

General Procedure—To a solution of 0.05 mole of appropriate acid chloride in 150 ml. of benzene solution was added dropwise 0.1 mole of appropriate amine. The reactants were then refluxed for 30 min., and the crystals thus formed were separated by filtration. The benzene solution was then evaporated to dryness, and the crystalline product obtained was washed twice with petroleum ether, b.p. 30–60°.

Pharmacological Procedures—*Reserpine Antagonism*—The method utilized is a modification of that of Randall and Baydon (5). Fasted male mice were injected with 2.5 mg./kg. i.p. reserpine. After 1 hr., the mice were injected subcutaneously with each of the drugs and were observed hourly for 4 consecutive hr. The neurological deficits observed were general motor activity, miosis, and ptosis.

Hexobarbital Potentiation—A modification of the Kuhn and Van Maanen (6) method was used. Fasted male mice (S.W. strain) were administered the drugs intraperitoneally 15 min. prior to the injection of hexobarbital sodium, 100 mg./kg. i.p., and observed for their sleeping time.

Phenylquinone Writhing—The method employed was that of Hendershot and Forsaith (7). Fasted male mice (S.W. strain) were administered intraperitoneally with 0.2 ml. of phenylquinone solution (0.02%). The mice were placed in a single 1-l. beaker and ob-

Table II—Partial Pharmacological Data

Tests	Compounds, in Descending Order, Best in Each Test
Hexobarbital potentiation	9, 8, 7, 6
Phenylquinone writhing	5, 9, 8
Randall–Sellito pressure paw	9, 8, 7, 3, 10, 5
Reserpine antagonism	None
Antipyresis	7, 10, 5, 9, 8, 3

served for 10 min. The test drugs were given 20 min. prior to injection of phenylquinone.

Randall–Sellito Pressure Paw—The method employed was adapted from that of Randall and Sellito (8). Inflammation of the hindpaw of fasted Sprague-Dawley rats (body weight 100–125 g.) was induced by an injection of 0.1 ml. of 20% brewer's yeast suspension into the plantar surface. The ability of the subcutaneously administered test drugs (administered concomitantly with phlogistic agent) to affect the pressure pain interval was determined 1, 2, 3, and 4 hr. after drug administration. This test was modified to determine antipyretic activity; thus, rectal temperatures were taken prior to drug–phlogistic administration and at the designated time intervals after administration.

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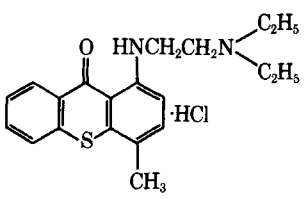
Condensed *p*-Toluidines with Aminothiazoles as Schistosomicidal Agents II

I. NABIH and M. ABBASI

Abstract □ Compounds representing structural combinations between the two moieties necessary for the biological activity in both schistosomicidal agents, 1-(β-diethylaminoethylamino)-4-methylthiaxanthone and 1-(5-nitro-2-thiazolyl)-2-imidazolidinone (*p*-toluidine and 5-nitrothiazole, respectively), have been synthesized. For the condensation reaction, aromatic azido acids were used followed by selective reduction of the azido groups through metallic hydrides.

Keyphrases □ *p*-Toluidines, 5-nitrothiazole—synthesis, potential schistosomicidal agents □ Schistosomicidal agents, potential—synthesis of condensed *p*-toluidines, aminothiazoles

Investigations carried out since the discovery of the schistosomicidal activity of 1-(β-diethylaminoethylamino)-4-methylthiaxanthone[1] (I) and its analogs showed that the presence of an amino side chain located on C-1 in *para*-position to the methyl group on C-4 is necessary for the biological activity of these agents (1, 2). In the recent schistosomicidal agent, 1-(5-nitro-2-thiazolyl)-2-imidazolidinone[2] (II), the presence of the nitrothiazole group was confirmed to be an essential feature for its activity (3, 4).



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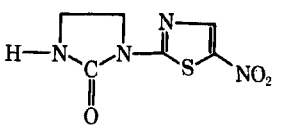
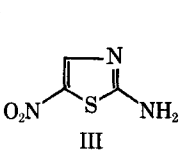
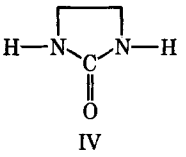


II

[1] Lucanthone or Miracil D.
[2] Ambilhar or Ciba 32644-Ba.



III



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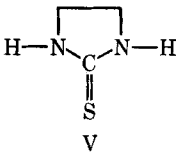
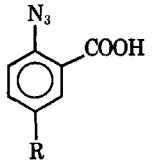


V

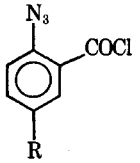
These considerations suggested the synthesis of compounds containing these necessary moieties. Both systems were designed to be connected through an amide linkage, because this may be biologically hydrolyzed to free both parts. Thus, the molecules may act as a whole or as separate components in the presence of one another.

Aromatic azido acids were used in the condensation reaction with 2-amino-5-nitrothiazole (III), 2-imidazolidinone (IV), and its thione analog, 2-imidazolidinethione (V). 2-Imidazolidinone is structurally included with 5-nitrothiazole in the schistosomicidal agent II.

The azides of anthranilic acid (VI*a*) and its 3-methyl derivative (VI*b*) were prepared by a procedure involving the addition of hydrazoic acid to the corresponding diazonium salt. The azido acid chlorides (VII*a* and VII*b*) prepared through the action of thionyl chloride upon the acids VI*a* and VI*b*, respectively, reacted readily



VI*a*: R = H
VI*b*: R = CH_3



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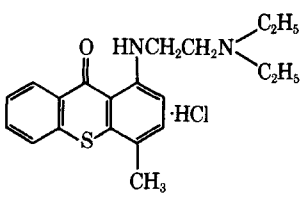

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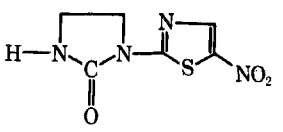

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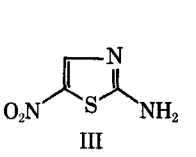
[1] Lucanthone or Miracil D.
[2] Ambilhar or Ciba 32644-Ba.



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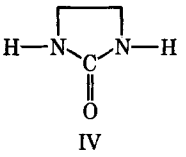

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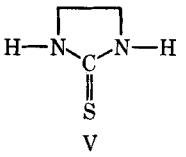
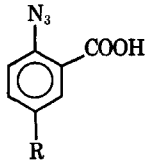


V

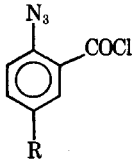
These considerations suggested the synthesis of compounds containing these necessary moieties. Both systems were designed to be connected through an amide linkage, because this may be biologically hydrolyzed to free both parts. Thus, the molecules may act as a whole or as separate components in the presence of one another.

Aromatic azido acids were used in the condensation reaction with 2-amino-5-nitrothiazole (III), 2-imidazolidinone (IV), and its thione analog, 2-imidazolidinethione (V). 2-Imidazolidinone is structurally included with 5-nitrothiazole in the schistosomicidal agent II.

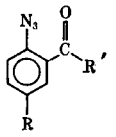
The azides of anthranilic acid (VI*a*) and its 3-methyl derivative (VI*b*) were prepared by a procedure involving the addition of hydrazoic acid to the corresponding diazonium salt. The azido acid chlorides (VII*a* and VII*b*) prepared through the action of thionyl chloride upon the acids VI*a* and VI*b*, respectively, reacted readily



VI*a*: R = H
VI*b*: R = CH_3

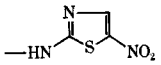
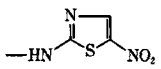
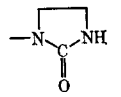
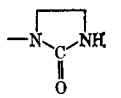
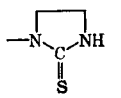
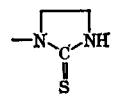


VII*a*: R = H
VII*b*: R = CH_3



VIII

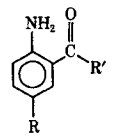
Table I—2-Azidobenzoyls

Number	R	R′	Yield, %	Melting Point (Recrystallization Solvent)[a]	Molecular Formula	Analysis, % Calc.	Analysis, % Found
a	H		64	208–210°(D)	$C_{10}H_6N_6O_3S$	C, 41.37 H, 2.08 N, 28.96 S, 11.03	C, 41.37 H, 2.36 N, 29.01 S, 11.14
b	CH_3		70	214–215°(D)	$C_{11}H_8N_6O_3S$	C, 43.42 H, 2.63 N, 27.63	C, 43.67 H, 2.34 N, 27.61
c	H		73	170–172°(E)	$C_{10}H_9N_5O_2$	C, 51.94	C, 51.99
d	CH_3		63	174–175°(E)	$C_{11}H_{11}N_5O_2$	C, 53.87 H, 4.52 N, 28.57	C, 54.19 H, 4.79 N, 28.62
e	H		50	176–177°(D)	$C_{10}H_9N_5OS$	C, 48.58 H, 3.64 N, 28.34	C, 48.59 H, 3.57 N, 28.04
	CH_3		53	170–171°(D)	$C_{11}H_{11}N_5OS$	C, 50.57 H, 4.24 N, 26.81	C, 50.79 H, 5.06 N, 26.81

[a] D = dioxane, E = ethanol.

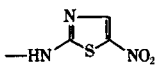
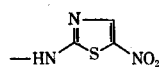
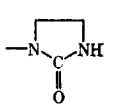
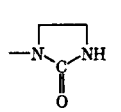
with III, IV, and V to give the azidobenzoyls (VIII*a–f*), respectively (Table I). The reaction was accelerated through the addition of dry sodium carbonate.

The lower reactivity of sodium borohydride compared to that of lithium aluminium hydride was advantageous in permitting the selective reduction of the azides. Although the former could reduce amides slowly (5), it was possible to reduce the azido groups in the amides VIII*a–d* selectively, giving the amines IX*a–d*, respectively (Table II). The reduction occurred readily in methanol or ethanol, provided the reaction mixture was kept at a temperature less than 25°.



IX

Table II—2-Aminobenzoyls

Number	R	R′	Yield, %	Melting Point (Recrystallization Solvent)[a]	Molecular Formula	Analysis, % Calc.	Analysis, % Found
a	H		34	127–130°(E–P)	$C_{10}H_8N_4O_3S$	C, 45.45 H, 3.05 N, 21.21 S, 12.12	C, 46.07 H, 3.40 N, 21.16 S, 12.84
b	CH_3		36	167–168°(E–P)	$C_{11}H_{10}N_4O_3S$	C, 47.48 H, 3.59 N, 20.14 S, 11.51	C, 47.16 H, 3.39 N, 19.88 S, 11.40
c	H		70	137–138°(B)	$C_{10}H_{11}N_3O_2$	N, 20.48	N, 20.29
d	CH_3		74	93–94°(B–P)	$C_{11}H_{13}N_3O_2$	N, 19.16	N, 18.77

[a] E–P = ethyl acetate–petroleum ether, B = benzene.

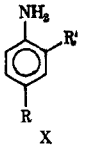


Table III—Amino Alcohols

Number	R	R′	Melting Point (Recrystallization Solvent)[a]	Molecular Formula	Analysis, % Calc.	Found
a	H	—CH_2OH	80–81°[b] (*n*-H)	C_7H_9NO	C, 68.29 H, 7.37 N, 11.38	C, 67.86 H, 7.79 N, 11.90
b	CH_3	—CH_2OH	121–122°[c] (*n*-H)	$C_8H_{11}NO$	C, 70.07 H, 8.08 N, 10.21	C, 69.66 H, 8.30 N, 10.10

[a] *n*-H = normal hexane. [b] Lit. (6) m.p. 82°. [c] Lit. (7) m.p. 122°.

Similar treatment of VIII*e* and VIII*f* caused hydrolysis of the amide linkage, along with the reduction of the azido groups, and yielded the alcohols X*a* and X*b*, respectively (Table III). Alternatively, the reduction of both azides was attempted through hydrobromic acid, which selectively reduced the azido groups, giving the corresponding amines XI*a* and XI*b*, respectively.

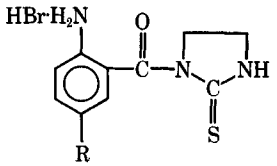
EXPERIMENTAL[3]

2-Azidobenzoyl Chloride (VII*a*) and 2-Azido-3-methylbenzoyl Chloride (VII*b*)—The azido acid (0.0023 mole) was mixed in 20 ml. of dry benzene with 0.025 mole of redistilled thionyl chloride. The mixture was gently heated on a steam bath with occasional shaking until the evolution of hydrogen chloride and sulfur dioxide ceased. The excess of thionyl chloride and benzene was distilled under reduced pressure, and the residual acid chloride was used without any further treatment due to explosion upon distillation.

(2′-Azidobenzoyl)-2-amino-5-nitrothiazole (VIII*a*), (2′-Azidobenzoyl)-2-imidazolidinone (VIII*c*), (2′-Azidobenzoyl)-2-imidazolidinethione (VIII*e*), and Their (3′-Methyl) Derivatives (VIII*b*, *d*, and *f*) (Table I)—To a solution of 0.018 mole of the azido acid chloride in 10 ml. of dry benzene was added 0.02 mole of the desired amine: III, IV, or V. The mixture was heated gently on a steam bath for 12 hr.; then the benzene was distilled, and the residue was poured into water. The product formed was collected, washed with water, and recrystallized from a proper solvent.

Reduction of the 2-Azidobenzoyls (VIII*a*–*f*) (Tables II and III)—To a cold suspension of 0.005 mole of the azidobenzoyl in 10 ml. of absolute ethanol was added 0.095 g. (0.0026 mole) of sodium borohydride portionwise, with stirring for 1 hr. Ethanol was then evaporated under reduced pressure, and the residue was decomposed by addition of 5 ml. of water followed by adjustment to pH 8.0 with diluted hydrochloric acid. The solution was extracted with hot ethyl acetate. The ester layer was separated, dried over sodium sulfate, and filtered; the filtrate was concentrated and then refrigerated. The product that separated was collected, dried, and recrystallized from a proper solvent.

(2′-Aminobenzoyl)-2-imidazolidinethione Hydrobromide (XI*a*)—To a suspension of 1.22 g. (0.005 mole) of VIII*e* in 10 ml. of absolute ethanol, 5 ml. of hydrobromic acid (48%) was added. This was refluxed for 3 hr. until the suspension went into solution. The solution was refrigerated overnight; the precipitated hydrobromide was collected, washed with cold alcohol, dried, and recrystallized from ethanol to give 0.6 g. (40% yield) as yellow crystals, m.p. 202–203°.



XI*a*: R = H
XI*b*: R = CH_3

Anal.—Calc. for $C_{10}H_{12}BrN_3OS$: N, 13.90; S, 10.59. Found: N, 14.50; S, 10.39.

(2′-Amino-5′-methylbenzoyl)-2-imidazolidinethione Hydrobromide (XI*b*)—Similar reaction of 1.5 g. (0.005 mole) of VIII*f* with hydrobromic acid gave 0.95 g. (50% yield) of XI*b*, which was recrystallized from ethanol as yellow crystals, m.p. 250–251°.

Anal.—Calc. for $C_{11}H_{14}BrN_3OS$: C, 41.45; H, 4.43; N, 13.29. Found: C, 40.90; H, 4.38; N, 12.84.

BIOLOGICAL TESTING

Compounds IX*a*, IX*b*, IX*d*, and XI*b*, in suspension or in water solution, were submitted to biological screening. Groups of mice, six each, of 29–31-g. average weight and infected with *Schistosoma mansoni*, were used for testings. All mice in each group were showing viable ova in their stools.

Group A was kept as the control. Group B was given oral doses of 75 mg./kg. of body weight from Compound IX*a*. Oral administration of the compound continued for 10 consecutive days. Three other groups (C, D, and E) were similarly given Compounds IX*b*, IX*d*, and XI*b*, respectively, at the same dosage levels as in Group B (75 mg./kg. of body weight) for the same period. After 1 week from the end of treatment, ova excretion in each group was examined once weekly for 6 weeks. Examinations showed a gradual decrease in the rate of ova excretion until it ceased in both Groups C and E given Compounds IX*b* and XI*b*, respectively.

In Groups B and D, given Compounds IX*a* and IX*d*, respectively, no remarkable decrease in the rate of ova excretion was observed compared to the control group A.

These biological findings suggest that the presence of the methyl group *para* to the amino group (*p*-toluidine type) is necessary for the activity, as shown by Compounds IX*b* and XI*b*.

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[3] Melting points are uncorrected and were taken in open capillaries, using a Gallenkamp melting-point apparatus. Microanalyses were performed by the Microanalytical Laboratory, National Research Centre, Cairo, Egypt, and Spang Microanalytical Laboratory, Ann Arbor, Mich.

Relative Enzymatic Hydrolysis Rates of Lincomycin Esters I: Soluble Esters

MILDRED J. TARASZKA

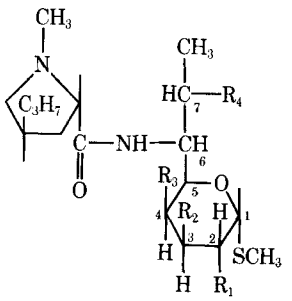
Abstract □ Enzymatic hydrolysis rates were determined for different positional and structural esters of lincomycin in dog serum and simulated intestinal fluid USP. In general, the hydrolysis rates were faster in simulated intestinal fluid than in dog serum, indicating a higher esterase activity in simulated intestinal fluid. The order of hydrolysis rates for positional lincomycin monohexanoate esters was $2 \gg 3 > 4 \sim 7$ in simulated intestinal fluid and $2 > 3 \sim 4 > 7$ in dog serum. The 2-propionate ester of lincomycin was hydrolyzed slower than the longer chain 2-hexanoate ester, with the greatest difference in rates occurring in simulated intestinal fluid. Sterically hindered esters, *e.g.*, lincomycin-2-pivalate and lincomycin-2-(3,3-dimethyl)butyrate, were hydrolyzed at extremely slow rates. The significance of the relative hydrolysis rates when compared to the *in vivo* activity of the esters is discussed.

Keyphrases □ Lincomycin esters, dog serum, simulated intestinal fluid—enzymatic hydrolysis rates □ Hydrolysis rates, *in vivo* enzymatic—lincomycin esters

Monoesters of the antibiotic lincomycin were synthesized to improve the pharmaceutical (taste, solubility, *etc.*) and biological (absorption, *etc.*) properties of lincomycin (1–4). Since most monoesters showed poor *in vitro* bioactivity on the standard curve plate bioassay (4, 5), it is conceivable that hydrolysis of these compounds in the body to lincomycin may be essential for *in vivo* antibacterial activity. The hydrolyses of several monoesters of lincomycin in fresh dog serum and simulated intestinal fluid were studied to determine the effects of: (*a*) derivative position and structure, and (*b*) differences in body fluid esterase specificity on the *in vitro* enzymatic hydrolysis rates of the esters.

Fresh dog intestinal fluid was unavailable so simulated intestinal fluid was used to approximate the relative intestinal fluid enzymatic hydrolysis rates. If there are appreciable differences in esterase specificity between dog serum and simulated intestinal fluid, it might be possible to design a reversible drug derivative which would be inactive *in vitro* and also tasteless, better absorbed, *etc.* However, once the drug derivative is absorbed and/or transported to its receptor site, it would be enzymatically converted to the active form of the drug. From the monoesters studied, it appears that the faster hydrolyzing monoesters have greater *in vivo* antibacterial activity and that no gross differences in esterase specificity exist between dog serum and simulated intestinal fluid.

The structures of lincomycin and its monoesters studied are given here.



Compound	R_1	R_2	R_3	R_4
Lincomycin	—OH	—OH	—OH	—OH
Lincomycin-2-hexanoate	—O—C(=O)—C_5H_{11}	—OH	—OH	—OH
Lincomycin-3-hexanoate	—OH	—O—C(=O)—C_5H_{11}	—OH	—OH
Lincomycin-4-hexanoate	—OH	—OH	—O—C(=O)—C_5H_{11}	—OH
Lincomycin-7-hexanoate	—OH	—OH	—OH	—O—C(=O)—C_5H_{11}
Lincomycin-2-propionate	—O—C(=O)—C_2H_5	—OH	—OH	—OH
Lincomycin-2-pivalate	—O—C(=O)—C(CH_3)$_2$—CH_3	—OH	—OH	—OH
Lincomycin-2-(3,3-dimethyl)butyrate	—O—C(=O)—CH_2—C(CH_3)$_2$—CH_3	—OH	—OH	—OH

structures of lincomycin and its monoesters

EXPERIMENTAL

Enzymatic Hydrolysis—The pH of fresh-pooled dog serum at 37° was checked and adjusted to pH 7.4, if needed, at the start of each experiment. At the end of the experiment, the pH was in the range 7.4–8.0. One milliliter of a concentrated lincomycin ester solution was added to 15 ml. of the pH-adjusted dog serum to give an initial

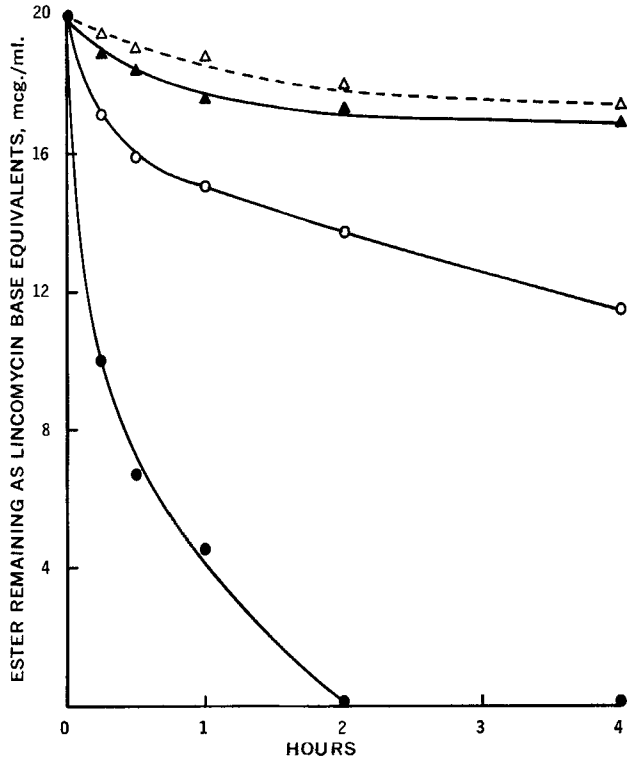


Figure 1—*Simulated intestinal fluid hydrolysis at pH 7.4 and 37° of lincomycin monoesters. Key: 2-hexanoate, ●; 3-hexanoate, ○; 4-hexanoate, △; and 7-hexanoate, ▲.*

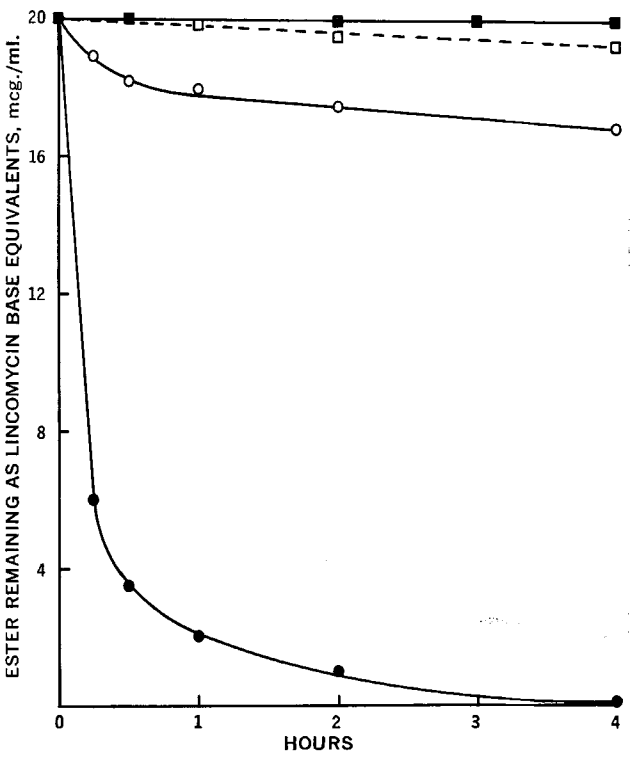


Figure 2—*Simulated intestinal fluid hydrolysis at pH 7.4 and 37° of lincomycin monoesters. Key: 2-hexanoate, ●; 2-propionate, ○; 2-pivalate, □; and 2-(3,3-dimethyl)butyrate, ■.*

ester concentration of 20 mcg. of lincomycin base/ml. At given time intervals, samples of the reaction mixture were assayed for the amount of lincomycin present. All dog serum hydrolysis experiments were started within 2.0–2.5 hr. after the blood was withdrawn from the dogs.

Simulated intestinal fluid USP was prepared from analytical grade buffer components and USP grade pancreatin[1]. Pancreatin was added to the buffer solution 1 hr. before the beginning of the hydrolysis experiment. The same procedure was used as for dog serum. Due to the better buffering capacity of simulated intestinal fluid, the pH of the solution was 7.4 ± 0.1 throughout the experiment.

Since enzyme concentration or degradation could not be controlled, lincomycin-2-hexanoate was chosen as the standard to measure the enzyme activity of dog serum and simulated intestinal fluid on different days. All enzymatic hydrolysis experiments were done at 37°.

Lincomycin Assay—To use the standard curve plate bioassay (6) for lincomycin in the reaction solutions, the esters of lincomycin first had to be extracted since they also showed some bioactivity. Two milliliters of the reaction solution was added to 2 ml. of water-saturated ether in a 15-ml. stoppered centrifuge tube. The tube was shaken vigorously and centrifuged, and the ether layer was discarded. The ether extraction process was repeated a total of three times immediately after sampling the reaction solution. After the last centrifugation, 1–1.5 ml. of the aqueous layer was transferred to a 2-ml. volumetric flask. The dissolved ether in the reaction mixture was evaporated by placing the samples under house vacuum for 1 hr. The samples were held in a frozen state and assayed collectively by spotting 0.08 ml. on 1.27-cm. (0.5-in.) diameter paper disks[2], using *Sarcina lutea* (ATCC-9341) inoculated agar (6). The assay values were determined graphically with 10 standard points for each set of unknowns.

The ether extractions did not interfere with the standard curve plate bioassay for lincomycin, since the same inhibition zone diameters were obtained when lincomycin solutions were spotted before and after the three ether extractions. Ester bioactivity was completely removed with the three ether extractions in the plate bioassay for lincomycin-2-hexanoate, lincomycin-3-hexanoate, lincomycin-2-pivalate, lincomycin-4-hexanoate, and lincomycin-2-(3,3-dimethyl)butyrate at concentrations up to 50 mcg./ml. The ether extractions, however, did not completely remove the bio-

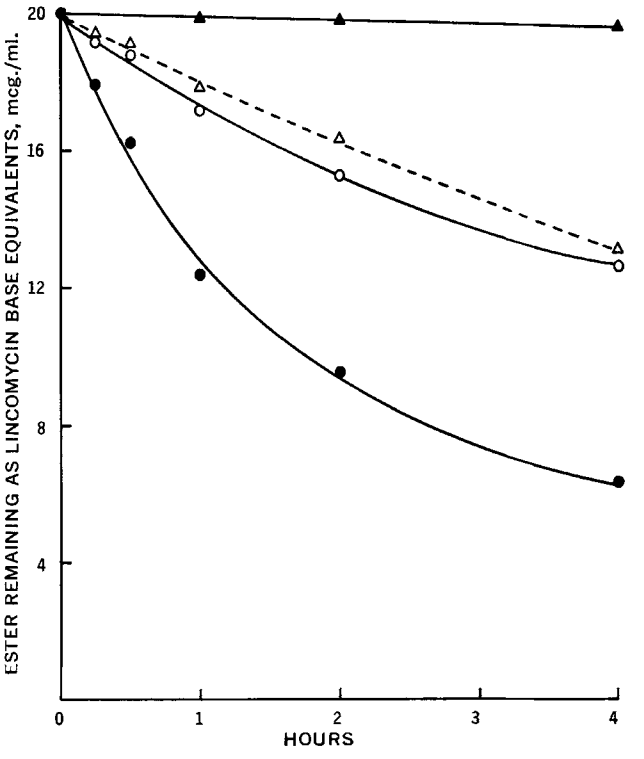


Figure 3—*Fresh dog serum hydrolysis at pH 7.4 and 37° of lincomycin monesters. Key: 2-hexanoate, ●; 3-hexanoate, ○; 4-hexanoate, △; and 7-hexanoate, ▲.*

[1] Nutritional Biochemicals Corp.
[2] Schleicher and Schuell.

Table I—Antibacterial Activity and Relative Enzymatic Hydrolysis Rates of Lincomycin Monoesters

Lincomycin Esters	Antibacterial Activity: *In Vitro* Activity, mcg./mg.[a]	Antibacterial Activity: Relative *In Vivo* Activity[b]: Subcutaneous Route	Antibacterial Activity: Relative *In Vivo* Activity[b]: Oral Route	Enzymatic Rates[c]: Dog Serum	Enzymatic Rates[c]: Simulated Intestinal Fluid
4-Hexanoate	86	0.28 (0.13–0.45)	0.15 (0.09–0.21)	0.33 (0.22–0.50)	0.07 (0.05–0.10)
7-Hexanoate	59	0.34 (0.17–0.55)	0.33 (0.18–0.51)	<0.02	0.13 (0.11–0.15)
3-Hexanoate	490	0.50 (0.28–0.78)	1.27 (0.81–2.10)	0.42 (0.34–0.53)	0.31 (0.28–0.32)
2-Hexanoate	1050	0.70 (0.34–1.29)	1.37 (0.86–2.34)	1.00	1.0
2-(3,3-Dimethyl)-butyrate	16	0.45 (0.20–0.78)	0.86 (0.59–1.22)	0.06 (0.03–0.09)	<0.02
2-Pivalate	<4	0.68 (0.35–1.13)	1.23 (0.78–1.94)	0.16 (0.14–0.18)	0.03 (0.01–0.04)
2-Propionate	245	0.76 (0.37–1.35)	1.21 (0.75–2.02)	0.61 (0.54–0.70)	0.13 (0.08–0.16)

[a] As measured on a standard curve agar assay *versus Sarcina lutea*. Results expressed as micrograms of lincomycin base per milligrams of ester (4, 5). [b] Median protective dose or CD_{50} relative to that of lincomycin as 1. The numbers in parentheses are the 95% confidence intervals (4, 5). [c] Enzymatic hydrolysis rates relative to that of lincomycin-2-hexanoate as 1. The numbers in parentheses are the ranges.

activity due to lincomycin-7-hexanoate and lincomycin-2-propionate. The error due to the incomplete extraction of these two esters was, therefore, corrected by using the equation:

$$X(20) + (1 - X)1 = A \quad \text{when } A \leq 20 \text{ mcg./ml.} \quad \text{(Eq. 1)}$$

$$\begin{pmatrix}\text{contribution due}\\ \text{lincomycin}\end{pmatrix} + \begin{pmatrix}\text{contribution due to}\\ \text{lincomycin ester}\end{pmatrix} = \text{total value of lincomycin obtained in bioassay (mcg./ml.)}$$

where X is the fraction present as lincomycin.

RESULTS AND DISCUSSION

The amount of monoester remaining *versus* time is plotted in Figs. 1 and 2 for simulated intestinal fluid hydrolysis and in Figs. 3 and 4 for fresh dog serum hydrolysis. No attempt was made to analyze these plots into kinetic hydrolysis mechanisms since no correction was made to account for any loss of esterase activity during the hydrolysis runs.

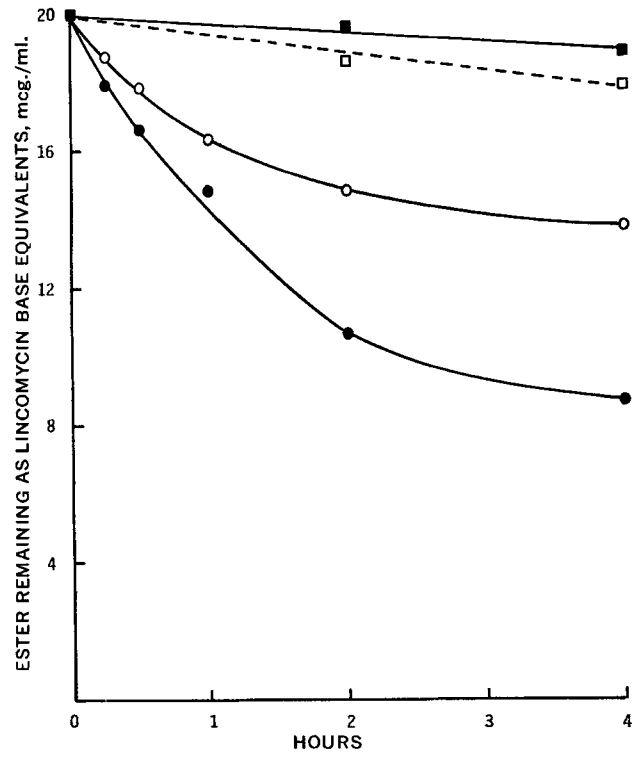


Figure 4—*Fresh dog serum hydrolysis at pH 7.4 and 37° of lincomycin monoesters. Key: 2-hexanoate, ●; 2-propionate, ○; 2-pivalate, □; and 2-(3,3-dimethyl)butyrate, ■.*

Positionally Isomeric Esters—The four positionally isomeric hexanoate esters of lincomycin were enzymatically hydrolyzed in the order $2 \gg 3 > 4 \sim 7$ in simulated intestinal fluid (Fig. 1) and $2 > 3 \sim 4 > 7$ in fresh dog serum (Fig. 3). The difference in hydrolysis rates of corresponding esters in simulated intestinal fluid and dog serum was probably due to a difference in concentration or activity of specific esterases in these two media.

Molecular models show that the four hydroxyl groups in lincomycin have different steric and electronic environments. These nonsymmetrical environments probably contribute to the rather large differences observed in enzymatic hydrolysis rates for the positionally isomeric esters. The large differences in hydrolysis rates were not observed for the nonenzymatic hydrolyses of the isomeric lincomycin hexanoate esters, where the order was $2 \sim 3 \sim 4 > 7$ (7, 8). The steric and electronic differences appear to have a larger effect on the enzymatic rates than on the nonenzymatic rates of hydrolysis. This would be anticipated since the steric requirements for enzymatic reactions are usually more stringent than for hydrolytic reactions.

The *in vitro* and *in vivo* bioactivities of the isomeric hexanoate esters of lincomycin are reproduced in Table I (4), along with the relative enzymatic hydrolysis rates. While the error is rather large, there still is some indication of a correlation between the bioactivity and the enzymatic hydrolysis rate for the hexanoate positional isomers, with the faster hydrolyzing positional isomers having the larger *in vivo* antibacterial activity.

Sterically Hindered Esters—Although lincomycin-2-hexanoate and lincomycin-2-(3,3-dimethyl)butyrate have the same number of carbon atoms in the acid moiety, the sterically hindered 2-(3,3-dimethyl)butyrate ester was enzymatically hydrolyzed much slower than the 2-hexanoate ester (Figs. 2 and 4). The sterically hindered 2-pivalate ester was also hydrolyzed extremely slowly. These results are in agreement with literature data on crude enzyme mixtures (9).

Lincomycin-2-propionate was enzymatically hydrolyzed slower than lincomycin-2-hexanoate, with the greatest difference in rates occurring in simulated intestinal fluid (Figs. 2 and 4). The dependence of enzymatic hydrolysis rate on chain length of the acid moiety is in agreement with the literature (10). The greatest rate for the longer chain ester (hexanoate) might be expected for simulated intestinal fluid, since it contains digestive enzymes which are responsible for the hydrolysis of fats (glyceryl esters of long-chain fatty acids).

The correlation between bioactivity and enzymatic hydrolysis rate for the sterically hindered esters is not as good as for the positionally isomeric esters of lincomycin (Table I). Possible reasons for this lack of correlation are: (*a*) the crude enzyme mixture hydrolysis rates are not representative of the *in vivo* fluid enzymatic hydrolysis rates; and (*b*) other factors such as solubility and absorption, as well as enzymatic rates, are important for *in vivo* antibacterial activity. For the monoesters of lincomycin studied, the enzymatic rates of hydrolysis were generally faster in simulated intestinal fluid than in dog serum. This finding indicates that if an ester derivative is resistant to enzymatic hydrolysis in the intestinal tract and absorbed intact, it probably will be resistant to hydrolysis in the blood. However, with the large variety and high concentration of enzymes in the liver, the hydrolysis in the total vascular system may be sufficiently rapid to yield activity comparable to the parent

drug. Possibly this is the explanation for the good *in vivo* antibacterial activity of the 2-propionate, 2-pivalate, and 2-(3,3-dimethyl)-butyrate monoesters of lincomycin, even though they are enzymatically hydrolyzed *in vitro* relatively slowly.

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(3) A. A. Sinkula, W. Morozowich, C. Lewis, and F. A. MacKellar, *J. Pharm. Sci.*, **58**, 1389(1969).

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Free and Bound Water in Crude Drugs: Effect of Extraction Method on Subsequent Analysis by GLC

NOURI Y. MARY

Abstract □ The effect of the extraction procedure on the recovery of water from crude drugs was related with its quantitative analysis by GLC. Some crude drugs seem to retain part of their water in a bound form, which is not available for extraction with methanol by disintegration in a blender but can be readily removed by boiling the product with the solvent under reflux for 1 hr. A number of crude drugs, representing a variety of plant parts and products, were extracted with methanol by the two procedures; the extracts were subsequently analyzed for their total water by GLC. Reflux extraction is the method of choice for the preparation of extracts of crude drugs that contain bound water prior to their analysis by this technique.

Keyphrases □ Water, recovery from crude drugs—effect and comparison of extraction procedures □ Reflux extraction—preparation of crude drug extracts □ Crude drugs—extraction procedures, effect on GLC analysis □ GLC—analysis of crude drugs, effect of extraction procedures

A previously published paper from this laboratory (1) described a GLC method for the quantitative determination of water in natural products by reaction with 2,2-dimethoxypropane. In the course of analysis by this method, it was observed that the extraction procedure normally used to remove water from these products (disintegration with methanol in a blender) gave, in one instance (pectin), results that markedly deviated from the values given by the official methods. Pectin seems to retain part of its water in a bound form which does not lend itself readily to extraction by this procedure.

Further studies on this problem showed that some degree of water binding also exists in other crude drugs and natural products besides pectin. For accurate quantitative analysis, water in this form must be completely extracted from such products prior to determination by GLC. The purposes of this paper are to record the effect of extraction on water recovery from crude drugs and to relate this effect with quantitative analysis by this technique.

EXPERIMENTAL

The plant materials used in this investigation were obtained, in powdered form, from a commercial source[1].

Water Determination by Direct GLC

Extraction and Sample Preparation—*Procedure A: Blender Extraction*—In a typical analysis, 10.00 g. of the crude drug was placed in a blender jar[2] containing 100.00 ml. of anhydrous methanol[3] and 3.00 ml. of *n*-propanol[4] as the internal standard. After blending for 5 min., the mixture was allowed to settle; then a sample of the clear supernatant was drawn into a vial, and 4.00 μl. was injected with a microliter syringe[5] into a gas chromatograph[6] equipped with thermistor detector.

Procedure B: Reflux Extraction—A 10.00-g. sample of the plant material was heated for 1 hr. under reflux with 100.00 ml. of anhydrous methanol and 3.00 ml. of *n*-propanol as the internal standard. After cooling the mixture, a sample of the clear supernatant was transferred into a vial, and 4.00 μl. was injected into a gas chromatograph equipped with thermistor detector.

Calculations—The percent water in the original sample of the crude drug was determined by computing the ratio of peak height of water to *n*-propanol from the chromatogram, obtaining the corresponding weight ratio of water to *n*-propanol from a standard curve prepared by chromatographing samples containing various amounts of water in mixtures of 100.00 ml. anhydrous methanol and 3.00 ml. *n*-propanol, and multiplying by the weight of *n*-propanol[7].

[1] S. B. Penick and Co., New York, N. Y.
[2] Waring Products Co., Winsted, Conn.
[3] Reagent grade, Merck and Co., Inc., Rahway, N. J.
[4] Matheson, Coleman and Bell, East Rutherford, N. J.
[5] Hamilton No. 701, Hamilton Co., Whittier, Calif.
[6] Perkin-Elmer model 154 vapor fractometer, Norwalk, Conn.
[7] Water and *n*-propanol were measured by volume and converted to weight using specific gravity calculations.

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[7] Water and *n*-propanol were measured by volume and converted to weight using specific gravity calculations.

Table I—Percent Water in Crude Drugs That Do Not Retain Bound Water as Determined by GLC, Gravimetric Analysis, and Azeotropic Distillation

Crude Drug	GLC[a] Direct Analysis: Blender Extraction	GLC[a] Direct Analysis: Reflux Extraction	GLC[a] Indirect Analysis: Blender Extraction	GLC[a] Indirect Analysis: Reflux Extraction	Gravimetric Analysis[b]	Azeotropic Distillation[b]
Areca	8.22	8.34	8.22	8.61	7.94	7.17
Belladonna leaf	6.35	6.40	6.88	6.82	7.17	6.70
Cascara sagrada	7.11	7.35	8.41	8.00	7.24	7.75
Ipecac	8.10	8.22	8.66	8.96	8.50	8.08
Podophyllum	8.08	8.00	8.60	9.25	8.76	8.50
Tragacanth	8.26	9.31	8.37	9.20	8.03	8.38

[a] Each value is the average of results obtained from six chromatographic injections, representing two or more extractions of the crude drug. [b] Each value is the average of two or more determinations.

Table II—Percent Water in Crude Drugs That Retain Part of Their Water in a Bound Form as Determined by GLC, Gravimetric Analysis, and Azeotropic Distillation

Crude Drug	GLC[a] Direct Analysis: Blender Extraction	GLC[a] Direct Analysis: Reflux Extraction	GLC[a] Indirect Analysis: Blender Extraction	GLC[a] Indirect Analysis: Reflux Extraction	Gravimetric Analysis[b]	Azeotropic Distillation[b]
Acacia	7.33	12.37	8.10	12.70	12.95	11.90
Colchicum corm	5.38	8.40	5.76	8.88	8.75	8.50
Pectin (Lot 1)	—	—	7.35	13.17	12.33	13.97
Pectin (Lot 2)	4.65	9.53	4.00	9.20	9.61	9.54
Nux vomica	6.44	8.82	6.22	8.65	8.02	8.16

[a] Each value is the average of results obtained from six chromatographic injections, representing two or more extractions of the crude drug. [b] Each value is the average of two or more determinations.

The column, instrumentation, and chromatography conditions used in this study were described previously (2).

Water Determination by Indirect GLC

Extraction—*Procedure A: Blender Extraction*—A 10.00-g. sample of the crude drug was disintegrated with 100.00 ml. methanol[8] for 5 min. in a stainless steel blender. After allowing the mixture to settle, a 10.00-ml. aliquot of the clear supernatant was transferred with a pipet to a 25-ml. volumetric flask.

Procedure B: Reflux Extraction—A 10.00-g. sample of the plant material was refluxed for 1 hr. with 100.00 ml. methanol. After cooling the mixture, a 10.00-ml. aliquot of the clear supernatant was transferred to a 25-ml. volumetric flask.

Sample Preparation and Calculations—The extract of the crude drug prepared by each of the two procedures was analyzed for its water content by the GLC method previously reported (1). The analytical method is based upon the quantitative acid-catalyzed conversion of water to acetone by 2,2-dimethoxypropane; the acetone formed in the reaction is determined by GLC and related to the amount of water present in the original sample.

Determination of Accuracy of GLC Analysis

The water content of three of the crude drugs was assayed by direct and indirect GLC. Known quantities of water were then added to the plant materials prior to extraction by the reflux procedure, and the extracts were reassayed to estimate the recovery of the added water.

Water Determination by Gravimetric Method

The water content of the plant products was determined by the NF XIII procedure (3) for vegetable drugs containing no constituents volatile at 105°.

Water Determination by Azeotropic Distillation

The toluene distillation method given in NF XIII (3) was followed in determining the water content of the plant materials.

[8] Spectrograde, Distillation Products Ind., Eastman Organic Chemicals Department, Rochester, N. Y.

RESULTS AND DISCUSSION

Water is believed to exist in several forms in organic matter. As a constituent of materials of plant origin, it may be present free or partially bound to macromolecular components of the cells. A reliable estimate of the moisture content in crude drugs by GLC can be made when the extraction procedure assures the total removal of water from these products. When present in the free form, water can be extracted readily by comminution of the crude drug with methanol in a suitable blender for few minutes. Bound water, on the other hand, is tightly linked into the complex structure of the larger molecules and is mechanically, although not chemically, a part of the structure (4); in this form, it is difficult to remove or extract by the usual blending procedure. When a more rigorous extraction procedure (refluxing with methanol) is used, however, the removal of bound water from the crude drug is found to be more efficient and complete.

In the case of crude drugs that do not retain bound water (Table I), the moisture values obtained by GLC, either by blender or reflux extraction, are quantitatively in accord with those obtained by gravimetric analysis and azeotropic distillation. Water in these products exists entirely in a free form, which can be quantitatively extracted by either procedure prior to analysis by GLC.

On the other hand, some crude drugs and similar natural products retain part of their water in a bound form (Table II). Water in this form is not amenable to blender extraction but is readily and efficiently removed by reflux extraction. When blender extraction is used in the GLC analysis of moisture in such products, low and erratic results are obtained if bound water represents a significant portion of the total water content. This is clearly evident from an examination of the results shown in Table II, where blender extrac-

Table III—Effect of Reflux Time on Water Extraction[a]

Crude Drug	Water, %: Direct GLC, 1 hr. Reflux	Direct GLC, 3 hr. Reflux	Indirect GLC, 1 hr. Reflux	Indirect GLC, 3 hr. Reflux
Acacia	12.37	12.06	12.70	12.74
Colchicum corm	8.40	8.61	8.88	8.70
Ipecac	8.22	8.18	8.96	8.37
Tragacanth	9.31	9.11	9.20	9.48

[a] Mean results of two or more estimations.

Table IV—Recovery Studies of GLC Determination (Reflux Extraction), Gravimetric Analysis, and Azeotropic Distillation[a,b]

Crude Drug	Water, Percent in Crude Drug: Originally Present	Added	Total Calculated	Total Found	Recovery Difference, %
Direct GLC					
Belladonna leaf	6.40	5.00	11.40	10.50	−7.90
Pectin	9.53	3.00	12.53	12.33	−1.60
Nux vomica	8.82	7.00	15.82	16.68	+5.44
Indirect GLC					
Belladonna leaf	6.82	5.00	11.82	12.30	+4.06
Pectin	9.20	3.00	12.20	12.81	+5.00
Nux vomica	8.65	7.00	15.65	15.00	−4.16
Gravimetric analysis					
Belladonna leaf	8.07	5.00	13.07	12.55	−3.98
Pectin	12.41	3.00	15.41	15.29	−0.78
Nux vomica	9.45	7.00	16.45	16.04	−2.50
Azeotropic distillation					
Belladonna leaf	8.00	5.00	13.00	11.50	−11.54
Pectin	12.50	3.00	15.50	15.00	−3.23
Nux vomica	9.00	7.00	16.00	15.00	−6.25

[a] Each recovery value is the mean of two determinations. [b] Gravimetric and azeotropic analyses were made on lots of belladonna leaf, pectin, and nux vomica other than those used in Tables I and II.

tion, in each case, gave a discernible difference in the GLC values as compared to gravimetric analysis and azeotropic distillation. Reflux extraction, however, gave values for water content by GLC that are in good agreement with those given by the other two methods. The results in Table III show that an increase in the reflux time from 1 to 3 hr. did not substantially increase the values for water content.

Water binding is known to exist in natural products in which the carbohydrate content is characteristically high (4). In this study, a number of such products appeared to contain bound water in amounts ranging from 30 to 55%.

The good agreement obtained between the GLC technique and official procedures for water determination in crude drugs suggests that the results represent an accurate assessment of the actual water content of these materials. The accuracy of the GLC procedure was further established by determining the recovery of added water to three selected crude drugs. Table IV shows a comparison of the recovery data obtained by GLC with those given by gravimetric analysis and azeotropic distillation. The results indicate that the accuracy of analysis by direct and indirect GLC (5 and 4.40%, respectively) is within the accuracy range obtained by the official methods (2.42% for gravimetric analysis and 7% for azeotropic distillation). The reproducibility of the GLC analysis is satisfactory. This can be readily seen from the results shown in Table V; the maximum standard deviation obtained for direct and indirect procedures after reflux extraction was 0.20 and 0.31, respectively.

Table V—Reproducibility of GLC Analysis (Reflux Procedure)[a]

Crude Drug	Water, %: Direct GLC	Indirect GLC
Acacia	11.93, 12.81	12.62, 12.78
Nux vomica	8.73, 8.80, 8.92	8.31, 8.68, 8.97
Pectin	9.10, 9.45, 10.04	9.01, 9.15, 9.44
Tragacanth	9.30, 9.33	9.02, 9.14, 9.45

[a] Each value recorded represents the mean of six chromatographic injections from two aliquots of an extract of the crude drug.

SUMMARY

The data obtained in this study underline the fact that crude drugs bind water to different degrees within their cellular composition. Water in this form cannot be completely removed by blender extraction for subsequent analysis by GLC. To assess accurately the moisture content of such products by GLC, the reflux procedure described herein should be used. For crude drugs that do not retain part of their water in a bound form, the blending and reflux procedures appear to be equally accurate.

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Microscope Observations of Aspirin Crystallization from Ethanol

R. TAWASHI

Abstract □ Experimental observations of growth steps on aspirin crystals grown from a saturated solution in ethanol are described. The extensive examination of the surface feature of aspirin crystals indicates that the step system is the normal process of growth. From a saturated solution in ethanol, two-dimensional spherulites similar to those in polymers were also obtained; on heating (at 10°/min.), they transformed into aspirin crystals, melting at 125°.

Keyphrases □ Aspirin, crystallization, spherulites—saturated aspirin in ethanol □ Photomicrographs—aspirin spherulites □ Crystallization—saturated aspirin from ethanol

In previous studies, two crystal forms of aspirin were prepared and their dissolution and absorption rates were studied (1, 2). Summers *et al.* (3) recently confirmed these forms, and they characterized four other aspirin polymorphs. Pfeiffer (4) questioned the use of the term polymorphism in aspirin and asked for explicit directions for the preparation and identification of the crystal forms. The purpose of this report is to show that the growth of aspirin form I from saturated solution in ethanol can be understood in terms of Frank's (5) growth mechanism. In addition, it will be shown that two-dimensional spherulites could be prepared from a saturated aspirin solution in ethanol.

EXPERIMENTAL

Materials and Equipment—The materials used in this study were: commercial aspirin USP, ethanol, FD&C Violet No. 1, and 1% gold chloride[1] solution. Photomicrographs were taken with a Polaroid MP-3 Land camera mounted on top of a polarizing microscope. The Mettler FP-2 hot stage was used for determining the melting points.

Observation of Growth Steps—Monoclinic aspirin crystals grow from filtered saturated ethanol solution when left to evaporate slowly (2–3 days) at room temperature (no stirring). These crystals are elongated prismatic tablets, identical to the aspirin form I previously reported (1). Photomicrographs were taken for aspirin crystals still growing in a saturated solution in ethanol (95%) at room temperature. Figure 1 shows a single spiral step originating from the point of emergence of screw dislocation in the aspirin crystal. The step system in the form of a growth hill is seen in Fig. 2. The spiral step system was rendered visible on a number of crystals after etching, for a few seconds, with 1% gold chloride or 5 p.p.m. FD&C Violet No. 1 aqueous solutions. The effect of these etchants on the crystal surface is consistent with findings on crystal poisoning (6, 7) and suggests preferential adsorption of these impurities on the step system of aspirin crystals. The presence of visible growth steps on the surface of aspirin crystals indicates that the step system is the normal process of growth and supports the conclusion made by Glasby and Ridgway (8) that surface reaction could be the rate-controlling step in the crystallization of aspirin from ethanol.

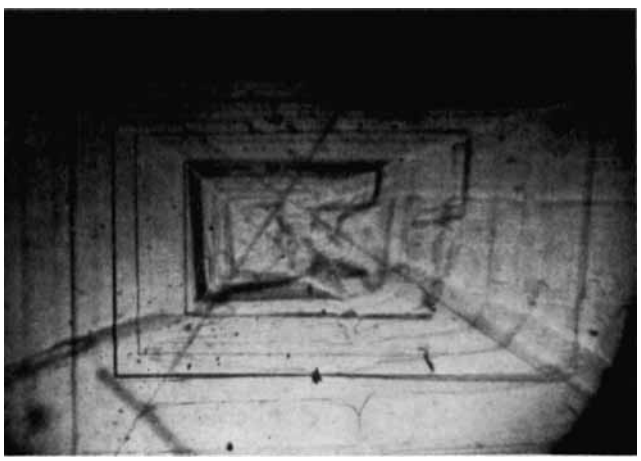

Figure 1—*Growth step system originating from the points of emergence of screw dislocations* (×125).

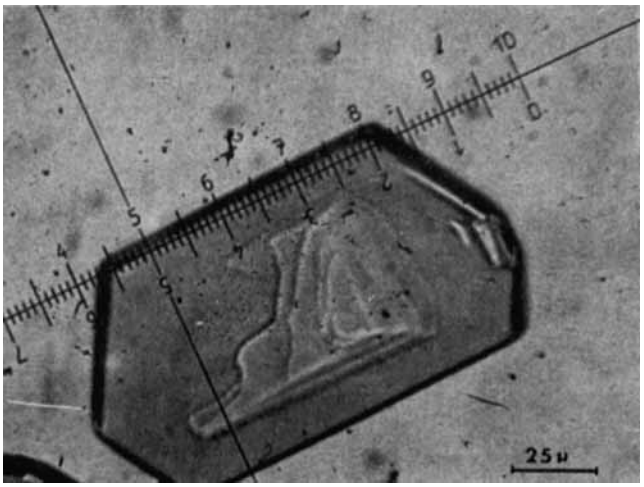

Figure 2—*A growing aspirin crystal showing step system in the form of a growth hill* (×500).

Growth of Aspirin Spherulites (a Mesomorphic Phase)—Spherulites of aspirin were observed under a polarizing microscope when one drop of a fresh, filtered, saturated solution of aspirin USP in ethanol (95%) was spread as a thin film on a clean glass slide, previously washed with ethanol. The spherulites were observed to grow after a few seconds, filling the microscopical field (Fig. 3). The structure was identical in appearance to spherulites obtained from high polymers (9).

Aspirin spherulites exhibited a maltese cross when viewed between crossed polars, where the dark bands were parallel to the polarization directions, indicating that the optical axes were either parallel or perpendicular to the spherulite radii. Larger spherulites (Fig. 4) showed the appearance of fibrillar structures much like those seen for cholesteryl acetate (10) and 2,4,6-trinitrotoluene grown in thin films (11). Qualitative microscopic observation of aspirin spherulites revealed that the spherulites grow radially outward from their center at a rate that increases with decreasing temperature.

Structural changes accompanying the change of temperature were studied, using the Mettler FP-2 hot-stage microscope. On heating at 10°/min., the two-dimensional spherulites were transformed at 124° into needle-like crystals starting from the center of the spherulite. This transformation did not reverse on cooling, and the solid product obtained melted at 125° (10°/min.; Fig. 5).

The growth of aspirin into two-dimensional spherulites is an interesting phenomenon, because substances of low molecular

[1] British Drug House Pharmaceuticals, Dorval, Quebec, Canada.

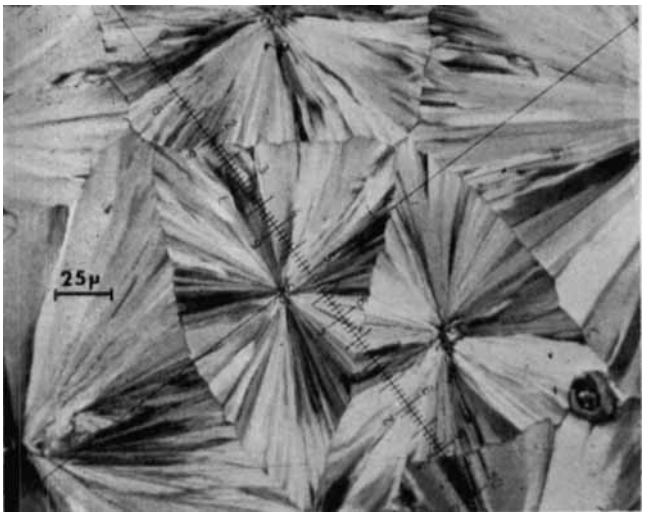

Figure 3—*Photomicrograph between crossed polars for spherulites of aspirin at room temperature (×500).*

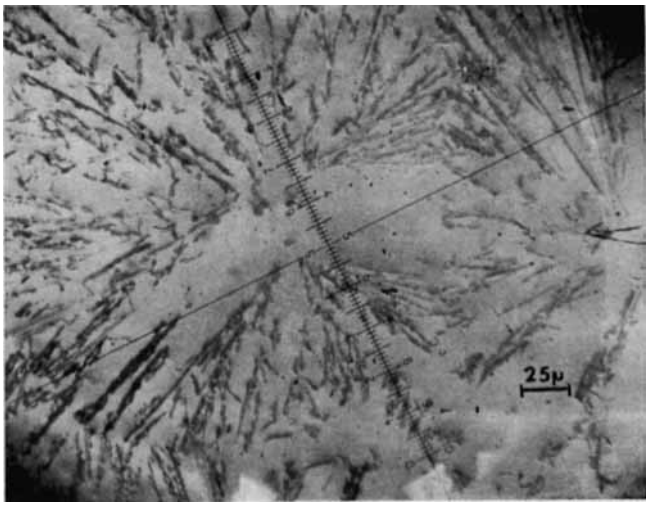

Figure 5—*Photomicrograph from the same field (Fig. 4) after heating to 124° (at 10°/min.), showing the transformation to solid crystalline needles of aspirin.*

weight like aspirin do not normally crystallize in this way. However, there are special circumstances when high supersaturation and a high viscosity of the crystallization medium can favor the formation of spherulites (12). These conditions are likely to be met when the aspirin solution is spread as a thin film on the glass slide, where the available surface for evaporation increases, and the solvent mass transfer rate becomes an important factor in crystallization.

On the basis of this observation, one might be tempted to conclude that variation in thermal and dissolution characteristics of commercial aspirin could be attributed to the presence of aspirin spherulites on the surface of commercial aspirin crystals. The formation of these spherulites on the surface of aspirin crystals takes place immediately after separation of the crystals from the crystallizing solvent and/or during the drying period. The entirely different grain structure on the surface of aspirin crystals can lead to an appreciable change in the response of commercial aspirin to thermal stress and to mass transfer in dissolution. This view is supported by the following:

1. Commercial aspirin USP has a long history of giving trouble in melting-point determination. Freshly crystallized aspirin from ethanol is a monoclinic prismatic crystal, which melts at 143–144° (2°/min.) if the crystal is washed with hexane before the melting point is determined. This value agrees with the work of Wheatley (13) and with the previously reported value (1).

2. There is a depression in the melting point of at least 4° if the same crystals are left for 48 hr. in the solution or if the crystals are not washed from the adhering mother liquor before determination of the melting point. The formation of aspirin spherulites on the crystal surface and the appearance of a new type of grain structure could explain the wide variation in melting point.

3. A recent study on the dissolution behavior of commercial aspirin USP by Griffiths and Mitchell (14) demonstrated that bulk concentration increased rapidly and showed an initial peak before the equilibrium solubility was reached. The authors suggested that phase change in the surface layers of aspirin crystals is responsible for the unusual dissolution patterns obtained and indicated that the nature of surface transformation cannot be established from the kinetic and thermodynamic data.

Experimental study on spherulite growth in aspirin and a complete understanding of spherulite formation will be helpful in interpreting the dissolution characteristics and thermal properties of commercial aspirin crystals.

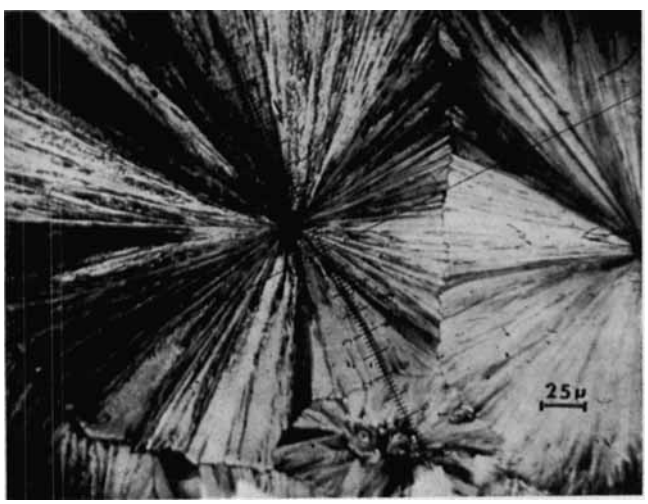

Figure 4—*Center of aspirin spherulites with a higher growth rate obtained by cooling.*

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Dialysis Method for Determining Preservative Distribution in Emulsions

S. J. A. KAZMI and A. G. MITCHELL

Abstract □ A three-chambered dialysis cell was used to estimate the distribution of preservative between the oil phase and the aqueous phase of an emulsion. The method also differentiated between preservative that was bound, or solubilized, by the surfactant and preservative that was free in the aqueous phase. The distribution data were plotted on a three-dimensional graph from which the total concentration of preservative needed to provide a given free concentration in the aqueous phase could be determined. Results from the dialysis method were compared with those calculated, using mathematical models for preservative distribution.

Keyphrases □ Preservative distribution, emulsions—determination, dialysis method □ Dialysis—preservative distribution determination, emulsions, comparison with mathematical models

Garrett (1) developed a basic integrated model for the evaluation of preservative action in complex disperse systems. Bean *et al.* (2) and Patel and Romanowski (3) correlated simplified forms of the equation with the observed antimicrobial activity of preservatives in oil-in-water emulsions. The equation may be expressed:

$$[D] = [D_f]\{1 + nK[M]/(1 + K[D_f]) + K_w^oQ\}/(Q + 1) \quad \text{(Eq. 1)}$$

where:

- $[D]$ = total concentration of preservative
- $[D_f]$ = concentration of free preservative in the aqueous phase; for an acid preservative, $[D_f] = [D_f'](1 + ka/[H^+])$, where $[D_f']$ is the concentration of unionized preservative in the aqueous phase and ka is the ionization constant
- $[M]$ = concentration of surfactant, n = maximum number of independent binding sites on the surfactant, and K = association constant for the binding of a molecule of preservative to one of these sites
- K_w^o = observed pH-dependent, concentration-dependent oil–water partition coefficient; $K_w^o = K_w'^o/(1 + ka/[H^+])$, where $K_w'^o$ is the pH-independent partition coefficient
- Q = oil–water phase volume ratio = V_o/V_w, where V_o and V_w are the volumes of the oil and aqueous phases, respectively.

In the equation of Bean *et al.* (2) and Patel and Romanowski (3), the term $1 + nK[M]/(1 + K[D_f])$ was replaced by R, where $R = 1 + nK[M]$; that is, for a given macromolecule concentration, the R value, or binding or solubilization constant, was assumed to be independent of $[D_f]$. As discussed elsewhere, this is true only in special cases (4).

Determination of the necessary factors in Eq. 1 is a lengthy process, particularly if, as is usual in most pharmaceutical and cosmetic emulsions, more than one type of macromolecule is present. Moreover, even a slight change in the formulation of the emulsion necessitates a reevaluation of the various terms. From a practical viewpoint, it would be of advantage to have a direct method to measure the amount of preservative in each "phase" of the emulsion and, hence, the total concentration required to provide the desired concentration in the aqueous phase. Garrett (1) suggested an ultracentrifuge technique. In this article, a three-chambered dialysis method is described. Results obtained using the dialysis method are compared with predictions made with Eq. 1, using experimentally determined physicochemical parameters.

EXPERIMENTAL

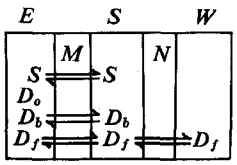
Reagents and Solutions—Peanut oil[1] and mineral oil[2] were used as the disperse phase in emulsions stabilized with the nonionic surfactant cetomacrogol 1000 BPC[3]. Analytical quality benzoic acid was used as the preservative. Aqueous solutions were made in citrate–phosphate buffer, pH 3.0 and ionic strength 0.2. Oil–water partition coefficients, the interaction between the preservative and the surfactant, and the distribution of preservative in emulsions were all studied at 30 ± 0.1° in a thermostatically controlled water-bath fitted with a tumbling device.

Oil–Water Partition Coefficients—Equal volumes of solutions of benzoic acid in oil and aqueous buffer were pipeted into glass-stoppered cylinders and agitated by a wrist-action shaker for about 1 hr. at room temperature. The cylinders were then tumbled in the water bath until equilibrium was reached (about 7 days). The aqueous phase was separated by centrifugation, and the benzoic acid concentration was analyzed. The concentration of benzoic acid in the oil phase was calculated by difference.

Two-Chambered Dialysis Cell—The interaction between benzoic acid and cetomacrogol was studied using equilibrium dialysis cells made according to the design of Patel and Foss (5). The two chambers of the cell were separated by a nylon membrane[4]. Details of the method and the results were described previously (4).

An emulsion containing various amounts of benzoic acid was pipeted into one compartment of a dialysis cell fitted with a nylon membrane. An aqueous buffer containing various amounts of benzoic acid was pipeted into the other compartment. A few glass beads were added to each compartment to ensure continuous mixing. The cells were equilibrated (about 4 days), and the concentration of benzoic acid in the aqueous compartment was analyzed.

Three-Chambered Dialysis Cell—The two-chambered dialysis cell was enlarged by the addition of an extra spacer in the center (Scheme I). A Millipore VS membrane[5] was used between compartments *E* and *S* and a nylon membrane between compartments *S* and *W*. The Millipore membrane is permeable to surfactant and benzoic acid but not to oil, while the nylon membrane is permeable to the benzoic acid only. Twenty milliliters of emulsion was pipeted into compartment *E*, 20 ml. of cetomacrogol solution was pipeted into compartment *S*, and 20 ml. of aqueous buffer solution was pipeted into compartment *W*. Known amounts of benzoic acid were



Scheme I—Three-chambered dialysis cell. Key: E, *emulsion;* S, *surfactant;* W, *water;* M, *Millipore VS membrane;* N, *nylon membrane;* D, *preservative;* D_o, *preservative in oil phase;* D_b, *preservative bound to surfactant; and* D_f, *free preservative in the aqueous phase*

[1] Planters Peanut Oil, Standard Brands Ltd., Canada.
[2] Primol 355, Imperial Oil Ltd., Canada.
[3] Glovers Chemicals Ltd., England.
[4] Capran 77C, Allied Chemical Corp., Morristown, N. J.
[5] Millipore Ltd., Montreal, Canada.

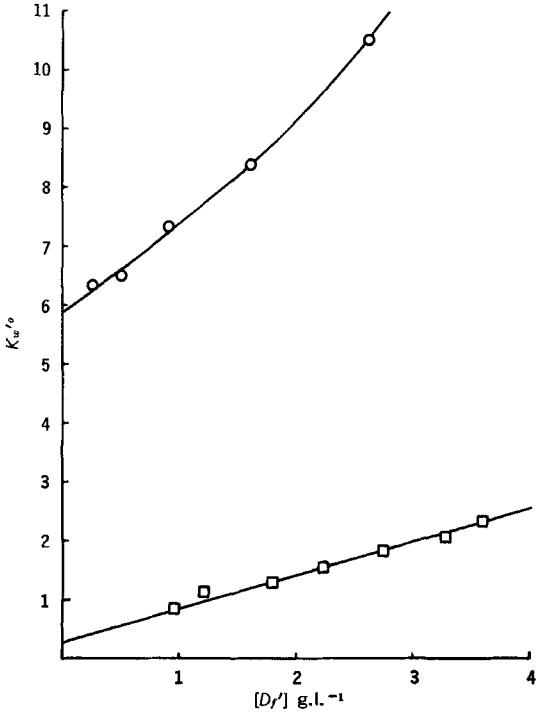


Figure 1—*pH-independent partition coefficient of benzoic acid* versus *the aqueous concentration of unionized benzoic acid. Key:* O, *peanut oil; and* □, *mineral oil.*

included in each chamber to accelerate equilibrium. The concentration of cetomacrogol in S was the same as used in the emulsion. Glass beads were added to each chamber, and the cell was tumbled until equilibrium was reached (about 7 days). The concentration of benzoic acid was determined in compartments S and W, and compartment S was analyzed for cetomacrogol.

Analytical Methods—Benzoic acid was analyzed spectrophotometrically at 273 nm., using a Coleman-Hitachi 124 spectrophotometer. Control experiments without the addition of benzoic acid were performed to provide blank solutions for use in the reference cell. Cetomacrogol was analyzed by the method of Crabb and Persinger (6). Preliminary experiments were carried out to determine the extent of binding of preservative and surfactant to the dialysis membranes. The values obtained were used to correct results from the interaction studies.

RESULTS AND DISCUSSION

Oil–Water Distribution Coefficient—Figure 1 shows the pH-independent partition coefficients, $K_w'^o$, plotted as a function of unionized benzoic acid concentration, $[D_f']$, in peanut oil and mineral oil. The distribution does not obey the simple partition law, and it is apparent that benzoic acid associates in both oils. According to the treatment of Gross and Schwarz (7), the equation of the line is:

$$K_w'^o = K_{dw}^o + mk'(K_{dw}^o)^m[D_f']^{m-1} \qquad \text{(Eq. 2)}$$

where K_{dw}^o is the distribution coefficient for the monomer, m is the number of molecules in an m-mer, and k' is the association equilibrium constant for monomer and m-mer. The linear relation between $K_w'^o$ and $[D_f']$ for mineral oil (Fig. 1) indicates dimerization of the benzoic acid, where $m = 2$, while the curve for peanut oil indicates m-merization in the oil phase. The intercept on the $K_w'^o$ axis at $[D_f'] = 0$ gives K_{dw}^o values of 5.84 and 0.23 for peanut oil and mineral oil, respectively. For substitution into Eq. 1, the pH-dependent observed partition coefficient, K_w^o, is more convenient than $K_w'^o$ or K_{dw}^o. Appropriate values of K_w^o were determined, therefore, from a plot of K_w^o against $[D_f]$.

Interaction between Preservative and Surfactant—Appropriate values of n and K for the interaction of benzoic acid with cetomacrogol were determined from a Scatchard plot of $[D_b]/[M][D_f]$ *versus* $[D_f]$, as described elsewhere (4), where $[D_b]$ is the concentration of preservative bound to the surfactant.

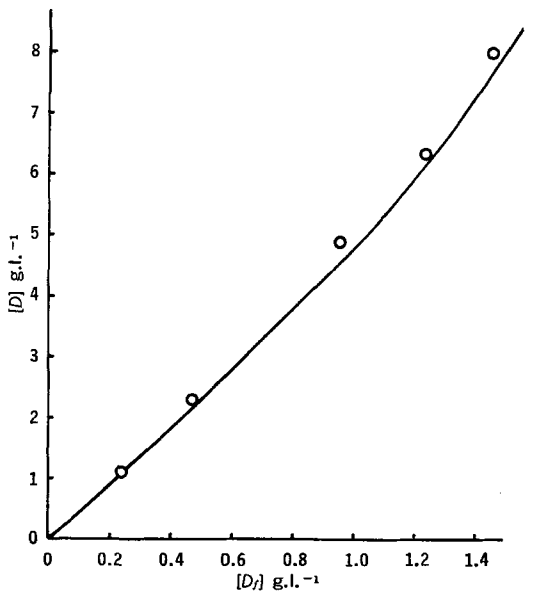


Figure 2—*Variation of free benzoic acid concentration,* $[D_f]$, *in the aqueous phase of an emulsion with total benzoic acid concentration,* [D], *for an o/w emulsion containing 50% v/v peanut oil emulsified with 4.0% w/v cetomacrogol. Key:* O, *observed* $[D_f]$; *curve calculated from Eq. 1.*

Estimation of Free Preservative Concentration in an Oil-in-Water Emulsion—Analysis of the concentration of benzoic acid in the aqueous compartment of the two-chambered dialysis cell enables the free benzoic acid concentration, $[D_f]$, to be determined. From $[D_f]$ and the total amount of benzoic acid added, $([D_o] + [D_b])$ can be determined, although it is not possible to separate these quantities. Figure 2 shows the total preservative concentration, $[D]$, plotted as a function of $[D_f]$.

Distribution of Preservative between the Oil, Surfactant, and Aqueous "Phases" of an Oil-in-Water Emulsion—Analysis of compartments W and S of the three-chambered dialysis cell after

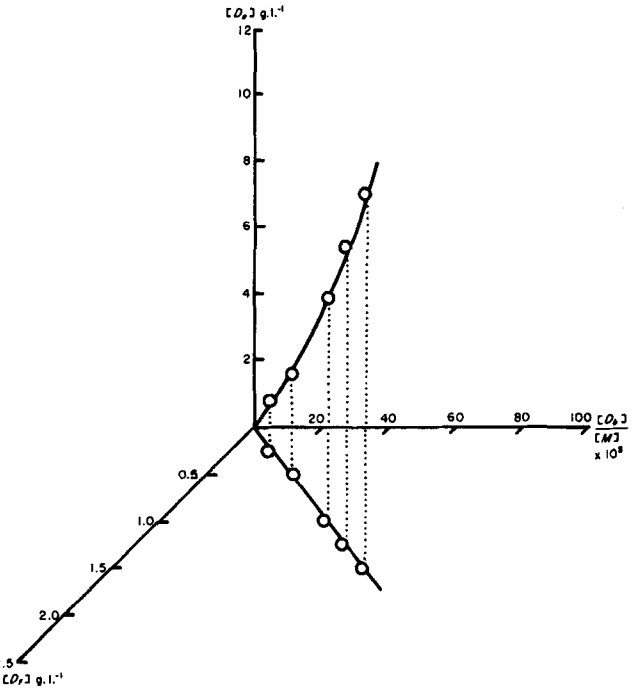


Figure 3—*Three-dimensional graph of the distribution of benzoic acid in an o/w peanut oil emulsion stabilized with cetomacrogol. Key:* O, *experimental values; curves calculated using Eq. 1.*

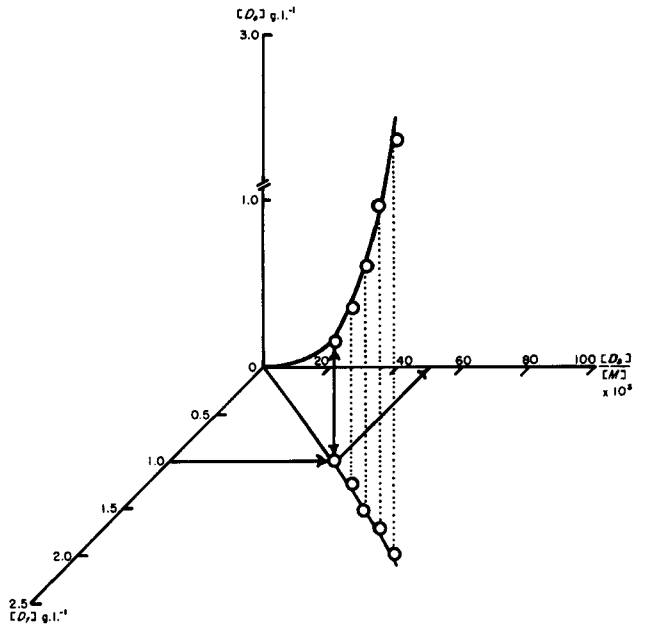


Figure 4—*Three-dimensional graph of the distribution of benzoic acid in an o/w mineral oil emulsion stabilized with cetomacrogol. Key: O, experimental values; curves calculated using Eq. 1.*

equilibrium gives $[D_f]$ and $([D_f] + [D_b])$, respectively, and, hence, $[D_b]$. From these terms and the amount of preservative added initially, $[D_o]$ can be obtained. Figures 3 and 4 show three-dimensional plots of $[D_o]$, $[D_b]/[M]$, and $[D_f]$ for the distribution of benzoic acid between peanut oil–cetomacrogol–aqueous buffer and mineral oil–cetomacrogol–aqueous buffer, respectively. There is close agreement between the experimentally determined values and the curves predicted using the oil–water distribution coefficient, K_w^o, and the preservative–surfactant binding constants, n and K.

The preservation of an emulsion requires that there must be a minimum inhibitory concentration of free preservative in the aqueous phase. The total amount of preservative required in the emulsion to provide a minimum inhibitory concentration of free preservative in the aqueous phase can be calculated from Eq. 1, where $[D_f]$ is the minimum inhibitory concentration. Determination of the various physicochemical parameters for substitution into Eq. 1 is a time-consuming process, however. Agreement between the predicted and observed values shows that the three-chambered dialysis technique provides a relatively simple method by which the total concentration of preservative necessary can be estimated. The first step is to construct three-dimensional calibration curves using the values of $[D_o]$, $[D_b]/[M]$, and $[D_f]$ obtained by dialyzing the emulsion containing varying known concentrations of preservative in the three-chambered cell. The graph is entered at a $[D_f]$ value corresponding to the minimum inhibitory concentration, and the corresponding values of $[D_b]/[M]$ and $[D_o]$ are determined. These terms are then used to calculate the total concentration of preservative necessary.

The procedure is illustrated using the mineral oil emulsion, containing benzoic acid as preservative (Fig. 4). Let the minimum inhibitory concentration = 1.0 g. l.$^{-1}$ = $[D_f]$ (8). The corresponding value of $[D_b]/[M]$ = 0.05. The corresponding value of $[D_o]$ = 0.75 g. l.$^{-1}$.

The total preservative concentration, $[D]$, is given by:

$$[D] = \{([D_f] + [D_b])V_w + [D_o]V_o\}/1000 \text{ g. .}^{-1} \quad \text{(Eq. 3)}$$

For an emulsion containing 4% cetomacrogol with an oil–water ratio of 1.0:

$$[D] = [(1.0 + 0.05 \times 40)500 + 0.75 \times 500]/1000 = 1.88 \text{ g. l.}^{-1} \quad \text{(Eq. 4)}$$

For a given oil–water–surfactant system, the three-dimensional calibration curves are theoretically independent of the oil–water ratio and the surfactant concentration. This was confirmed experimentally for oil–water ratios from 0.18 to 1.0 and for cetomacrogol concentrations from 1 to 4%.

In addition to the simple emulsions used in this work, the method should be applicable to more complex emulsions in which the existence of liquid crystalline phases and the presence of reversed micelles in the oil phase (9) would make the use of a mathematical model difficult or impossible.

The total preservative concentration can also be determined from a calibration curve of $[D]$ *versus* $[D_f]$ (Fig. 2), constructed using the two-chambered dialysis technique. However, separate calibration curves would be required for each oil–water ratio and for each surfactant concentration. Moreover, the three-chambered dialysis method provides more information because it differentiates between preservative in the oil phase and preservative associated with the surfactant. The effect of modification to the formulation on the distribution of preservative between the oil, surfactant, and water "phases" of the emulsion can, therefore, be readily assessed.

In an emulsion, some surfactant is adsorbed at the oil–water interface and, depending on its oil solubility, some partitions into the oil phase. Both factors reduce the amount of surfactant available for interaction with the preservative and affect the oil–water partition coefficient. Analysis of compartment S of the three-chambered dialysis cell for surfactant enables the distribution of surfactant between the oil and aqueous phases to be estimated. In this work, no change could be detected in the amount of cetomacrogol in compartment S after equilibration, which indicates that little surfactant was lost from the aqueous phase by adsorption or partition. This observation was supported by the close agreement between the predicted and observed values of $[D_b]$.

Where appreciable amounts of surfactant are adsorbed or partitioned into the oil, determination of the parameters necessary for substitution into Eq. 1 becomes difficult. This problem is avoided by using the three-chambered dialysis technique which permits direct observations to be made of the preservative distribution under conditions existing in the actual emulsion.

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Photolysis of 5-Nitrofuramides in Methanol

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Abstract □ A preliminary investigation of the photolysis of *N*-butyl-5-nitro-2-furamide in methanol is reported. The photolysis product was identified as *N*-butyl-5-methoxy-2-furamide, corresponding to the displacement of the nitro group by a solvent molecule. The nitro group apparently gives rise to nitrite ion, which was qualitatively detected in the photolysis mixture.

Keyphrases □ 5-Nitrofuramides—photolysis in methanol □ Photolysis—5-nitrofuramides in methanol □ *N*-Butyl-5-methoxy-2-furamide—identification as photolysis product

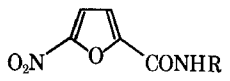
Nitrofuran antibacterial agents undergo two reactions *in vitro*, which have been proposed by various workers to account for their detoxification and/or antibacterial activity *in vivo*. The reduction of the nitro group to a hydroxyl amine has been proposed to be important in both the detoxification (1) and the antibacterial activity (2) of the nitrofurans. The second reaction, which has been observed *in vitro*, is the displacement of the nitro group by a nucleophile. This reaction was proposed by Yoneda and Nitta (3) to account for the antibacterial activity of nitrofurans and by Boyland and Speyer (4) to account for the detoxification of the nitrofurans.

DISCUSSION

As part of an investigation into the antibacterial activity of nitrofurans, the nitrofuramides (I) were synthesized (5). These compounds were previously prepared by Snyder (6) and shown to be approximately as effective *in vitro* as nitrofurazone. Since it was observed that the aliphatic amides decomposed on exposure to light while the aromatic amides were stable, an attempt to isolate and identify the photoproduct of this reaction was undertaken.

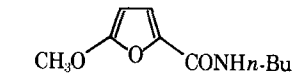
The lack of decomposition in the absence of light was verified using TLC and UV spectroscopy. *N*-Butyl-5-nitro-2-furamide (I*a*) was chosen for the studies aimed at the isolation and identification of the photoproducts because of its adequate solubility and rapid decomposition. Examination of the UV spectrum of the solution of I*a* in methanol and in water before and after photolysis revealed that the nature of the photoproduct was solvent dependent (Fig. 1). Lack of absorption in the UV spectrum above 220 nm. indicates that the photolysis in aqueous solution leads to the destruction of the furan ring (Fig. 1, Curve C). The photolysis of the nitrofuramide in methanol apparently leads to the formation of another furan derivative, as evidenced by the absorption maximum at 283 nm. (Fig. 1, Curve B). For this reason, the reaction in methanol was chosen for this initial investigation of the photolysis reaction.

The nitrofuramide I*a* was dissolved in methanol and stirred under fluorescent lighting in a Pyrex flask for 1 week. Evaporation of the solvent and column chromatography yielded one photoproduct and recovered starting material. The UV spectrum of the isolated photoproduct was as expected from the preliminary observations (Fig. 1, Curve C). The IR spectrum indicated that the amide portion of the molecule was still intact (2.80 and 5.99 μ) but that the nitro group had been lost (lack of absorption at 7.40 μ). In the NMR spectrum, the signals for the butyl protons were as expected. A three-proton singlet at 3.9 δ suggests the presence of a methoxy group. The only other signals in the spectrum were doublets centered at 5.3 and 7.0 δ and a broad singlet at 6.2 δ. These signals were assigned to two furan ring protons and an amide proton, respectively. The coupling constant (3.5 Hz.) of the furan ring protons indicates that the furan ring has 2,5-substitution (7, 8). Based on this evidence, the structure of the photoproduct was assigned as *N*-butyl-5-methoxy-2-furamide (II), corresponding to the displacement of the nitro group by a solvent molecule. The elemental analysis supports this structure, and the UV spectrum ($\lambda_{max.}$ = 283 nm., log ϵ = 4.10) is in good agreement with the UV data reported by Manly and Amstutz (9) for methyl 5-methoxy-2-furoate ($\lambda_{max.}$ = 279 nm., log ϵ = 4.10).



I

Ia: R = *n*-butyl



II

The detection of nitrite ion in the reaction mixture using Ilosvay's diazotization method (10) indicates that the displacement of the nitro group by a solvent molecule is the general pathway to the photoproduct. Boyland and Speyer (4) previously observed that glutathione transferase catalyzes the displacement of the nitro group of nitrofurans as nitrite by glutathione.

If the photolysis of the furamide I*a* in aqueous solution gives rise to a corresponding displacement of the nitro group by a solvent molecule, a 5-hydroxyfuramide would result. Since this 5-hydroxyfuramide would probably enolize and hydrolyze in the aqueous solution, a displacement reaction by a solvent molecule would account for the changes in the UV spectrum of I*a* on photolysis in aqueous solution as well as in methanolic solution. The photodecomposition of the nitrofurans in both aqueous and methanolic solutions is currently being investigated in greater detail in the hope of determining the importance, if any, of displacement reactions in the physiological effects of nitrofurans.

EXPERIMENTAL[1]

***N*-Butyl-5-nitrofuramide**—This compound was synthesized from 5-nitrofuroic acid[2] using the procedure of Snyder (6); m.p. 92–93° [lit. (6) 92.5–93.5°]. The NMR, IR, and UV spectra of the compound were as expected.

Photolysis of *N*-Butyl-5-nitro-2-furamide—I (2.0 g., 0.0093 mole) was dissolved in 50 ml. of methanol, and the solution was stirred in a nitrogen atmosphere for 1 week under fluorescent lighting. The solvent was evaporated, and the residue was chromatographed on silica gel (100 g.) using ether–benzene (10–15% ether) as the eluting solvent system. Early fractions contained unchanged starting material (1.8 g.). Subsequent fractions yielded 0.2 g. of a light-yellow powder, which was recrystallized from hexane–benzene to give light-yellow crystals: m.p. 59–60°; IR (CCl_4): 2.80 μ (N—H), 5.99 μ (C=O), 6.40 μ (N—H + C—N); NMR ($CDCl_3$): 0.7–1.0 (m, 3, CH_3C), 1.1–1.5 (m, 4, CH_2), 3.2–3.5 (m, 2, N—CH_2), 3.9

[1] Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. NMR spectra were determined in $CDCl_3$ (tetramethylsilane) solution on a Varian A-60A analytical spectrometer. Resonances are expressed as δ values. IR spectra were determined in CCl_4 solution (0.2 mm.) on a Perkin-Elmer model 137 spectrophotometer. UV spectra were determined on a Perkin-Elmer 202 spectrophotometer. Nitrite ion was qualitatively determined using a standard procedure (10).

[2] Eastern Chemical Co.

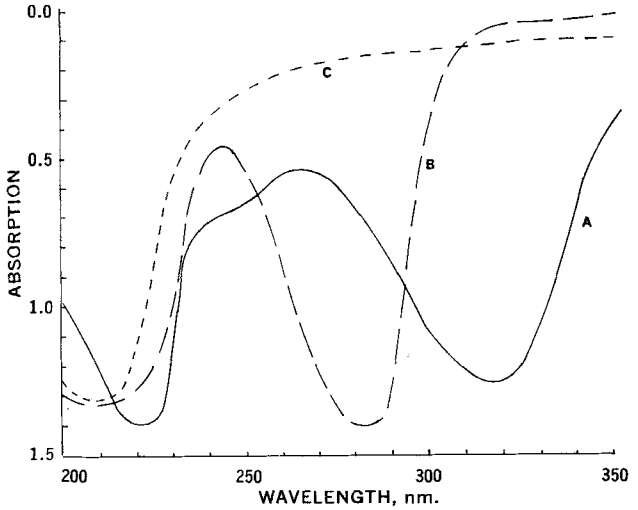


Figure 1—*Changes in UV absorption spectra of aqueous and methanolic solutions of Ia on exposure to light. All solutions are 1 × 10⁻⁴ mole/l. Key: Curve A, UV spectrum of Ia in water prior to exposure to light (the spectrum in methanol is essentially the same); Curve B, methanolic solution after exposure to light for 24 hr.; and Curve C, aqueous solution after exposure to light for 24 hr.*

(s, 3, O—CH_3), 5.3 (d, 1, J = 3.5 Hz., H—C4), 7.0 (d, 1, J = 3.5 Hz., H—C3), and 6.2 (s, 1, N—H).

Anal.—Calc. for $C_{10}H_{15}NO_3$: C, 60.88; H, 7.68; N, 7.10. Found: C, 61.03; H, 7.68; N, 7.07.

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COMMUNICATIONS

Benzene Analogs of Triazenoimidazoles

Keyphrases ☐ Triazenoimidazoles, benzene analogs—synthesis, antileukemic activity ☐ Triazenobenzamides—synthesis, light stability, antileukemic activity ☐ Antileukemic activity—triazenoimidazoles, benzene analogs

Sir:

The fact that 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (I*a*) (NSC-45388) increased survival time in mouse lymphatic leukemia L-1210 and inhibited other neoplasma (1) led to the synthesis of similar triazenoimidazole amides (2–6), triazenoimidazole esters (I*b*) (7), heterocyclic ring analogs (8–11), and certain phenyltriazenes with *para*-attached chains selected for presumed carrier properties (12). Benzene analogs (II–IV) of the triazenoimidazole amides and esters, as well as similar *para*-substituted derivatives (V–VIII), were also synthesized for antineoplastic evaluation; investigations of such derivatives were further stimulated by demonstrations of clinical activity by Compound I*a* (13). This communication is a preliminary account of some of the structural changes made in the aryl moiety and of antileukemic activity by certain of these derivatives.

The *p*-benzamides (V), the *o*- and *p*-benzoates (IV and VII), and the *p*-benzamidines (VIII) were prepared by diazotizing the appropriate aromatic amine derivative and coupling with an aliphatic amine by the general procedure described for certain other phenyltriazenes (12). The *o*- and *p*-benzoic acid hydrazides (III and VI) were obtained by treating the analogous esters (IV and VII) with hydrazine. Since it is well known that diazotization of 2-aminobenzamides gives 1,2,3-benzotriazin-4(3*H*)-ones (14), the *o*-benzamides (II) were synthesized by first isolating *o*-carbamoylbenzenediazonium tetrafluoroborate [m.p. 114–115° dec., IR band at 2290 cm.$^{-1}$ (N_2^+)]. Calc. for $(C_7H_6N_3O)^+BF_4^-$: C, 35.78; H, 2.57; N, 17.88. Found: C, 35.77; H, 2.61; N, 17.75. Coupling with aliphatic amines was then performed in anhydrous media to minimize intramolecular cyclization to 1,2,3-benzo-

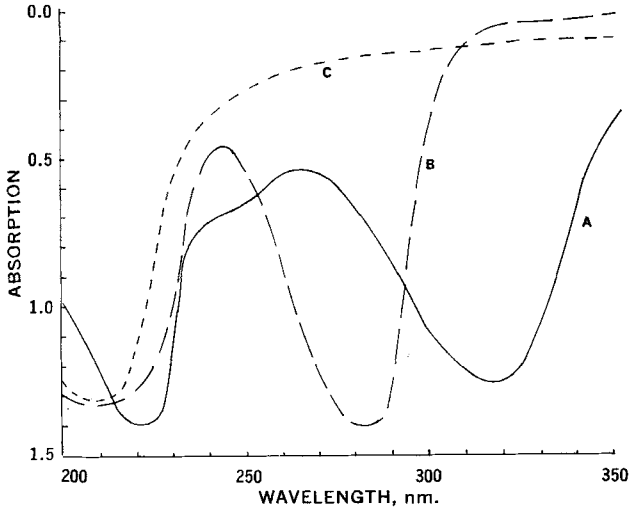

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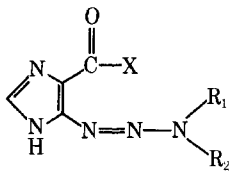
Table I—Increases in Survival Time in L-1210 Tests[a]

Compound	Dose, mg./kg., and Schedule[b]	Survival Time, T/C[c], %
o-(3,3-Dimethyl-1-triazeno)-benzamide (II, $R_1 = R_2 = CH_3$)	225, A	139
	150, A	127,162,150[d]
	100, A	137
o-(3-Butyl-3-methyl-1-triazeno)-benzamide (II, $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$)	200, A	150,144
	200[e], A	108,137
	133[e], A	122,126
	89[e], A	108,134
p-(3,3-Dimethyl-1-triazeno)-benzamide (V, $R_1 = R_2 = CH_3$)	100, B	146
	75, B	144
	60, B	121
	50, B	132
p-(3,3-Dimethyl-1-triazeno)benzoic acid hydrazide (VI, $R_1 = R_2 = CH_3$)	100, B	129
	75, B	134
	50, B	119
Ethyl *p*-(3-butyl-3-methyl-1-triazeno)-benzoate (VII, $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$)	400, B	137
	200, B	92
p-(3,3-Dimethyl-1-triazeno)-benzamidine (VIII, $R_1 = R_2 = CH_3$)	52, A	128
	35, A	133

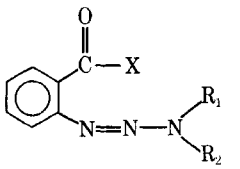
[a] Explanations of testing *versus* L-1210 are given in *References 11* (Footnotes *a–d* of Table I) and *17*. [b] Schedule A = daily administration of the stated dose on Days 1–9 (q.d. 1–9); Schedule B = administration on Days 1–30 or to death. [c] Ratio in percent of average survival time of treated mice (*T*) with L-1210 leukemia to untreated leukemic control mice (*C*). [d] Results of three separate tests. [e] Two separate dose–response tests. The value of T/C at 200 mg./kg./day in the first test in comparison with the T/C ratio in three other tests at this dose indicates that the first dose–response test was anomalous.

triazin-4(3*H*)-one. The following compounds[1] are illustrative of several derivatives that have been prepared with the Structures II–VIII.

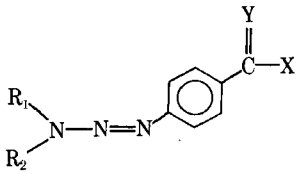
Benzamides—Compound II, $R_1 = R_2 = CH_3$: m.p. 124–126° (ethyl acetate–cyclohexane); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 242 (10.7), 280–290 (sh), and 318 nm. (12.8) at pH 7 and 13. Calc. for $C_9H_{12}N_4O$: C, 56.23; H, 6.29; N, 29.15. Found: C, 56.18; H, 6.50; N, 29.50. Compound II, $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$: m.p. 83–86° (cyclohexane); $UV_{max.}$ 242 (10.6), 280–290 (sh), and 320 nm. (13.4) at pH 7 and 13. Calc. for $C_{12}H_{18}N_4O$: C, 61.51; H, 7.74; N, 23.92. Found: C, 61.58; H, 7.80; N, 23.97. Compound V, $R_1 = R_2 = CH_3$: m.p. 171–172° (ethanol–ethyl acetate–hexane); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 221 (10.6) and 322 nm. (21.6) at pH 7. Calc. for $C_9H_{12}N_4O$: C, 56.23; H, 6.29; N, 29.15. Found: C, 56.42; H, 6.15; N, 29.05. Compound V, $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$: m.p. 101–102° (ethanol–hexane). Calc. for $C_{12}H_{18}N_4O$: C, 61.51; H, 7.74; N, 23.92. Found: C, 61.62; H, 7.64; N, 23.97.



Ia: X = NH_2, $R_1 = R_2 = CH_3$
Ib: X = OR



II: X = NH_2 (*o*-benzamides)
III: X = —$NHNH_2$ (*o*-benzoic acid hydrazides)
IV: X = OCH_3 (*o*-benzoates)



V: X = NH_2, Y = O (*p*-benzamides)
VI: X = —$NHNH_2$, Y = O (*p*-benzoic acid hydrazides)
VII: X = OC_2H_5, Y = O (*p*-benzoates)
VIII: X = NH_2, Y = NH (*p*-benzamidines)

Benzoic Acid Hydrazides—Compound III, $R_1 = R_2 = CH_3$: m.p. 128° (ethanol); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 223 (11.6), 247 (sh), 296 (sh), and 316 nm. (12.5) at pH 7. Calc. for $C_9H_{13}N_5O$: C, 52.16; H, 6.32; N, 33.80. Found: C, 52.06; H, 6.46; N, 34.06. Compound III, $R_1 = CH_3$, R_2 = cyclohexyl; m.p. 110° (ethanol–hexane); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 225 (11.3), 250 (sh), 297 (sh), and 321 nm. (14.1) at pH 7. Calc. for $C_{14}H_{21}N_5O$: C, 61.07; H, 7.69; N, 25.44. Found: C, 61.25; H, 7.65; N, 25.71. Compound VI, $R_1 = R_2 = CH_3$: m.p. 136–137° (ethanol–hexane). Calc. for $C_9H_{13}N_5O$: C, 52.16; H, 6.32; N, 33.80. Found: C, 51.96; H, 6.13; N, 33.77. Compound VI, $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$; oil that solidified after storage at low temperature; m.p. 37°. Calc. for $C_{12}H_{19}N_5O$: C, 57.81; H, 7.68; N, 28.10. Found: C, 57.66; H, 7.56; N, 27.94.

Benzoates—Compound IV, $R_1 = R_2 = CH_3$[2]: oil purified by chromatography on magnesia–silica gel (Florisil), elution with cyclohexane–acetone; $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 235 (10.7), 278 (9.8), and 312 nm. (10.8) at pH 7. Calc. for $C_{10}H_{13}N_3O_2$: C, 57.96; H, 6.32; N, 20.28. Found: C, 57.87; H, 6.33; N, 20.28. Compound IV, $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$: pale-yellow oil (purified by chromatography on Florisil, elution with petroleum ether–acetone); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 235 (10.6),

[1] Solvents used for recrystallization are given in parentheses after the melting points.

[2] This compound was reported by Elks and Hey (15), who stated that their specimen did not give satisfactory analytical data. It also was used in studies of benzyne formation (16).

280 (9.9), and 312 nm. (11.1) at pH 7 and 13. Calc. for $C_{13}H_{19}N_3O_2$: C, 62.62; H, 7.68; N, 16.86. Found: C, 62.78; H, 7.92; N, 17.15. Compound VII, R_1 = *n*-C_4H_9, R_2 = CH_3: oil, n_D^{25} 1.5799. Calc. for $C_{14}H_{21}N_3O_2$: C, 63.85; H, 8.04; N, 15.96. Found: C, 63.64; H, 7.92; N, 15.99.

Benzamidines—Compound VIII, R_1 = R_2 = CH_3: m.p. 113° dec. (hexane–chloroform); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 226 (10.9) and 327 nm. (22.4) at pH 7. Calc. for $C_9H_{13}N_5$: C, 56.52; H, 6.85; N, 36.63. Found: C, 56.32; H, 6.59; N, 36.53. Compound VIII, R_1 = methyl, R_2 = cyclohexyl: m.p. (hydrochloride) 235° dec. (ethanol–ethyl acetate); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 228 (12.0) and 333 nm. (25.0) at pH 7. Calc. for $C_{14}H_{21}N_5 \cdot HCl$: C, 56.84; H, 7.50; N, 23.68; Cl, 12.00. Found: C, 56.64; H, 7.43; N, 23.59; Cl, 12.0.

In standard tests against mouse lymphoid leukemia L-1210, *o*-(3,3-dimethyl-1-triazeno)benzamide, the analogous *o*-(3-butyl-3-methyl-1-triazeno) derivative, and *p*-(3,3-dimethyl-1-triazeno)benzamide increased lifespan by 40–60%. Dosages of these compounds that prolonged survival time are summarized in Table I; additional data in Table I indicate that a benzoic acid hydrazide, a benzoate ester, and a benzamidine also cause modest increases in lifespan. Although additional testing is required to delineate the degree and scope of activity of Compounds II–VIII, the available biological data indicate that at least some of the derivatives represented by Structures II–VIII can cause significant increases in average survival time of mice bearing leukemia L-1210. Previously, it was reported (18) that certain benzenoid triazenes, which lack the amide or carboxyl-type groups and which are inhibitory to certain experimental tumors (18–20), are not active against lymphatic leukemia L-1210.

The stability to sunlight of the benzamide analog (II, R_1 = R_2 = CH_3) of I*a* and of *p*-(3,3-dimethyl-1-triazeno)benzamide (V, R_1 = R_2 = CH_3) is a point of additional interest. In a prior study (11), it was shown that I*a* decomposes more rapidly in direct sunlight than does its pyrazole analog, 3-(3,3-dimethyl-1-triazeno)pyrazole-4-carboxamide. Solutions (4×10^{-5} *M*) of the two dimethyltriazenobenzamides and of the pyrazole analog in 50% aqueous ethanol were placed side-by-side in direct sunlight which passed through window glass and through the Pyrex glass of the containers. Under the prevailing exposure conditions, the UV absorbance of the pyrazole analog decreased by 40% within 3 hr. and by 85% within 10 hr., whereas the UV spectra of the two benzamide derivatives were unchanged during the same periods.

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Assessment of Compression Characteristics of Powders

Keyphrases ☐ Powders—compression characteristics ☐ Tablets—compression characteristics ☐ Compression properties—powders, tablets

Sir:

The characterization of the compression properties of powders by the "modulus of pressing," *i.e.*, the slope of the log of the applied pressure against the log of the relative density of the compact, has been criticized by Jones (1). As an alternative, he suggested that a plot of the power expended in pressing a powder against the volume displacement would show differences in the pressing qualities of various powders (1). Rather than using the power in forming the tablet, *i.e.*, the energy expended in unit time, we consider that the work done in forming the tablet, *i.e.*, the force times the distance moved by the punch, would be a more useful characterization.

Varsano and Lachman (2) described how the work done in forming a tablet can be obtained from the area under the load/displacement curves when powders are compressed at a constant rate on an Instron physical

280 (9.9), and 312 nm. (11.1) at pH 7 and 13. Calc. for $C_{13}H_{19}N_3O_2$: C, 62.62; H, 7.68; N, 16.86. Found: C, 62.78; H, 7.92; N, 17.15. Compound VII, R_1 = *n*-C_4H_9, R_2 = CH_3: oil, n_D^{25} 1.5799. Calc. for $C_{14}H_{21}N_3O_2$: C, 63.85; H, 8.04; N, 15.96. Found: C, 63.64; H, 7.92; N, 15.99.

Benzamidines—Compound VIII, R_1 = R_2 = CH_3: m.p. 113° dec. (hexane–chloroform); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 226 (10.9) and 327 nm. (22.4) at pH 7. Calc. for $C_9H_{13}N_5$: C, 56.52; H, 6.85; N, 36.63. Found: C, 56.32; H, 6.59; N, 36.53. Compound VIII, R_1 = methyl, R_2 = cyclohexyl: m.p. (hydrochloride) 235° dec. (ethanol–ethyl acetate); $UV_{max.}$ ($\epsilon \times 10^{-3}$) at 228 (12.0) and 333 nm. (25.0) at pH 7. Calc. for $C_{14}H_{21}N_5 \cdot HCl$: C, 56.84; H, 7.50; N, 23.68; Cl, 12.00. Found: C, 56.64; H, 7.43; N, 23.59; Cl, 12.0.

In standard tests against mouse lymphoid leukemia L-1210, *o*-(3,3-dimethyl-1-triazeno)benzamide, the analogous *o*-(3-butyl-3-methyl-1-triazeno) derivative, and *p*-(3,3-dimethyl-1-triazeno)benzamide increased lifespan by 40–60%. Dosages of these compounds that prolonged survival time are summarized in Table I; additional data in Table I indicate that a benzoic acid hydrazide, a benzoate ester, and a benzamidine also cause modest increases in lifespan. Although additional testing is required to delineate the degree and scope of activity of Compounds II–VIII, the available biological data indicate that at least some of the derivatives represented by Structures II–VIII can cause significant increases in average survival time of mice bearing leukemia L-1210. Previously, it was reported (18) that certain benzenoid triazenes, which lack the amide or carboxyl-type groups and which are inhibitory to certain experimental tumors (18–20), are not active against lymphatic leukemia L-1210.

The stability to sunlight of the benzamide analog (II, R_1 = R_2 = CH_3) of I*a* and of *p*-(3,3-dimethyl-1-triazeno)benzamide (V, R_1 = R_2 = CH_3) is a point of additional interest. In a prior study (11), it was shown that I*a* decomposes more rapidly in direct sunlight than does its pyrazole analog, 3-(3,3-dimethyl-1-triazeno)pyrazole-4-carboxamide. Solutions (4×10^{-5} *M*) of the two dimethyltriazenobenzamides and of the pyrazole analog in 50% aqueous ethanol were placed side-by-side in direct sunlight which passed through window glass and through the Pyrex glass of the containers. Under the prevailing exposure conditions, the UV absorbance of the pyrazole analog decreased by 40% within 3 hr. and by 85% within 10 hr., whereas the UV spectra of the two benzamide derivatives were unchanged during the same periods.

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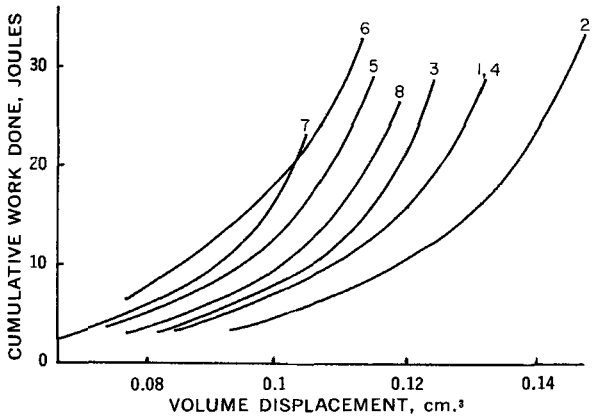

Figure 1—*Relationship between work done and the displacement of the powder bed. Key: 0–32-μ size fraction, 1 = crystalline and 2 = spray dried; 75–104-μ size fraction, 3 = crystalline and 4 = spray dried; 210–295-μ size fraction, 5 = crystalline and 6 = spray dried; and unfractionated, 7 = crystalline and 8 = spray dried.*

testing instrument. This method of determining work done has applied when known particle-size fractions of crystalline and spray-dried lactose were compressed, as described by Fell and Newton (3). These two materials were chosen because they have different direct compression characteristics (4). The modulus of pressing is given in Table I; the work done, calculated from the area under the load/displacement curve, for loads greater than 200 kg. is shown in Fig. 1. The former treatment shows slight differences between the two materials, the spray-dried lactose being more compressible. The latter treatment, however, shows clearly that, for a given value of work, there is a greater displacement of spray-dried lactose than crystalline lactose. Similarly, the differences between the various size fractions are clearly distinguishable by the latter treatment of results.

The ability of a powder mass to reduce in volume when compressed does not ensure the formation of a tablet. It is essential that the powder cohere into a suitable form after removal of the applied load. The tensile strength of the various tablets, as determined by the diametral compression test (3), provides a

Table I—Modulus of Pressing of Crystalline and Spray-Dried Lactose

Particle-Size Fraction, μ	Modulus of Pressing	
	Crystalline Lactose	Spray-Dried Lactose
0–32	0.11	0.13
75–104	0.11	0.12
295–410	0.10	0.11
Unfractionated	0.09	0.10

measure of the utilization of the work done in forming the tablet. The results presented in Fig. 2 show that there is a linear relationship between the work done and the tensile strength of the tablet for both forms of lactose and all the particle-size fractions tested. Although crystalline lactose is less compressible, it forms stronger tablets for an equal amount of work, except the 0–32-μ size fraction. For spray-dried lactose, the tablet strength increases as the particle size decreases for the same amount of work expended. For crystalline lactose this is not the case; the tablets prepared from the 75–104-μ size fraction are the strongest of the three size fractions. Thus, the work done in compressing a powder mass is not necessarily utilized in the same manner for volume reduction and particle bonding.

The application of these concepts can provide useful characterization of the compression properties of pharmaceutical materials.

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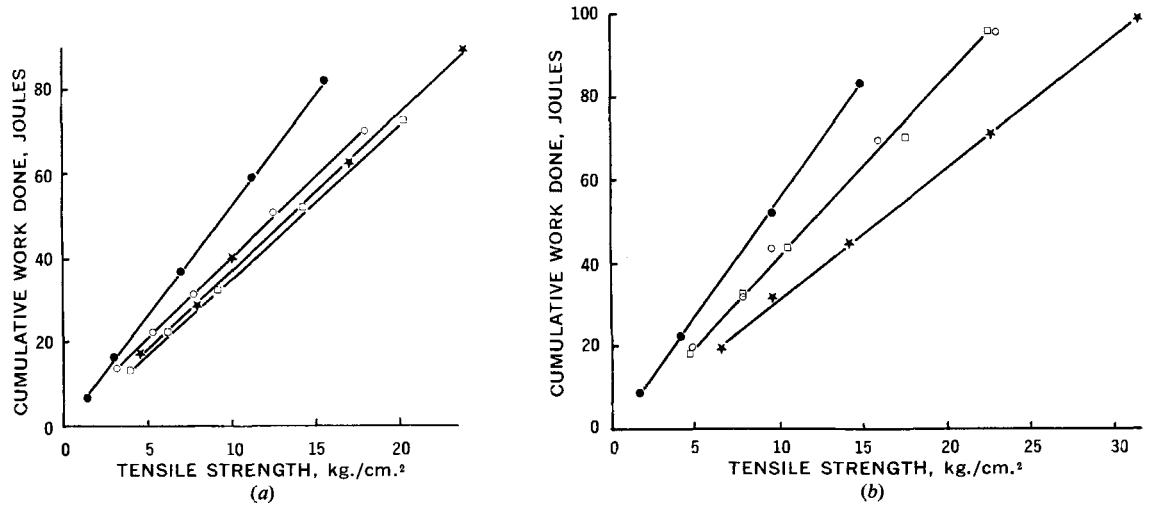


Figure 2—*Relationship between work done and the tensile strength of tablets:* a = *crystalline lactose and* b = *spray-dried lactose. Key:* ●, *210–295 μ;* □, *75–104 μ;* ∗, *0–32 μ; and* ○, *unfractionated.*

Fluorescence Techniques in Drug–Plasma Protein Binding

Keyphrases ☐ Drug–protein binding—determination, fluorescent probe indicators ☐ Protein–drug binding—determination, fluorescent probe indicators ☐ Fluorometry—determination, drug–protein binding

Sir:

Various experimental methods have been employed for the study of drug–protein interactions (1). These include equilibrium dialysis, ultrafiltration, ultracentrifugation, solubility measurement, and, more recently, nuclear magnetic relaxation measurements and fluorescence techniques. Although fluorescence techniques have proved useful for following enzyme–substrate interactions (2), they have not been adequately studied as a tool for elucidating drug–plasma protein interactions. The fluorescence techniques reported here employ fluorescent probes as indicators of binding. The usefulness of this technique for the study of drug–protein binding comes from the fact that fluorescent probes are practically nonfluorescent in aqueous solutions but fluoresce strongly when bound to protein molecules (3). The enhancement of fluorescence intensity of a probe as a result of its interaction with protein is taken as an indication of the extent of binding. If a drug competes at the binding site or sites on a protein molecule, the decrease in fluorescence is commensurate with the decrease in bound probe molecules. The difference in fluorescence with and without a drug can then be related to a measurement of binding of the drug molecules to proteins (2).

Fluorescence intensities of the bound probes were measured at two protein concentrations (0.2 and 1.0 mg. protein/2 ml. solution). Two milliliters of each protein solution was titrated with the addition of successive 2 μl. of a 1×10^{-3} *M* solution of the probes in methanol. The fluorescence titrations were carried out manually with Hamilton microsyringes. Experiments were carried out at room temperature and at pH 7.4.

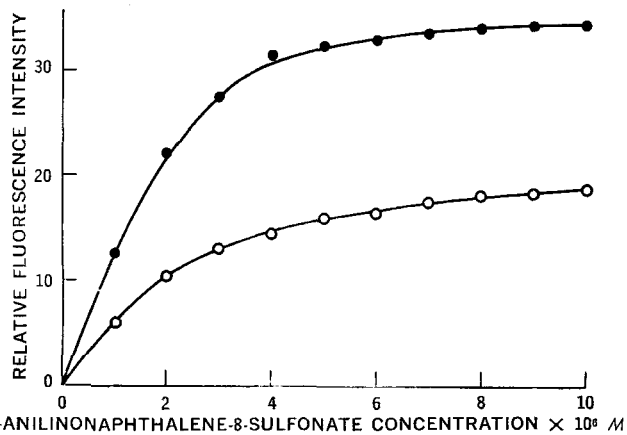
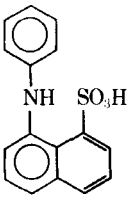


Figure 1—*Fluorescence titration of bovine serum albumin (0.2 mg. bovine serum albumin/2 ml. solution) with 1-anilinonaphthalene-8-sulfonate. The upper curve (●) represents the fluorescence intensity of 1-anilinonaphthalene-8-sulfonate complex. The lower curve shows the decreased intensity of the 1-anilinonaphthalene-8-sulfonate–bovine serum albumin complex due to bound phenylbutazone.*

Fluorometric measurements were made with a Perkin-Elmer model 203 fluorometer. The relative fluorescence intensities were obtained directly from fluorometer readings at uncorrected excitation and emission wavelengths for the probes.

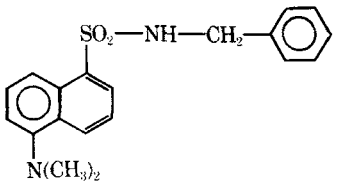
The probes used in this study were as follows:

Probe I—1-Anilinonaphthalene-8-sulfonate



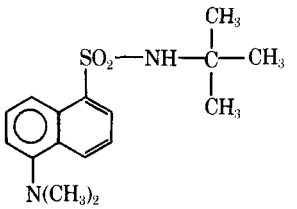
excitation, 350 nm.; emission, 465 nm.

Probe II—*N*-Benzyl-(5-dimethylaminonaphthalene)-1-sulfonamide



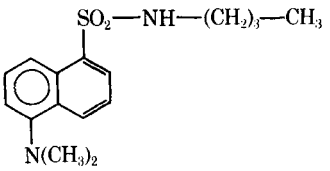
excitation, 320 nm.; emission, 495 nm.

Probe III—*N-tert*-Butyl-(5-dimethylaminonaphthalene)-1-sulfonamide



excitation, 320 nm.; emission, 495 nm.

Probe IV—*N-n*-Butyl-(5-dimethylaminonaphthalene)-1-sulfonamide



excitation, 325 nm.; emission, 500 nm.

Bovine serum albumin, Fraction V and crystalline, was purchased[1]. Acetylated bovine serum albumin was prepared according to the procedure of Fraenkel-Conrat *et al.* (5). Percentage acetylation was estimated to be 2.5% of the terminal amino groups[2].

Figure 1 shows the application of this technique for the binding of phenylbutazone to bovine serum albumin, and binding is indicated by the decreased

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] By Galbraith Laboratories, Inc., Knoxville, Tenn.

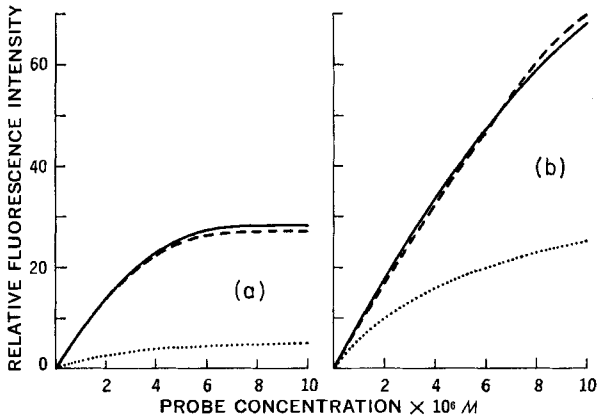


Figure 2—*Fluorescence titration of crystalline bovine serum albumin (—), Fraction V bovine serum albumin (- - -), and acetylated bovine serum albumin (. . .) with 1-anilinonaphthalene-8-sulfonate. Key: (a) lower bovine serum albumin concentration; and (b), higher bovine serum albumin concentration.*

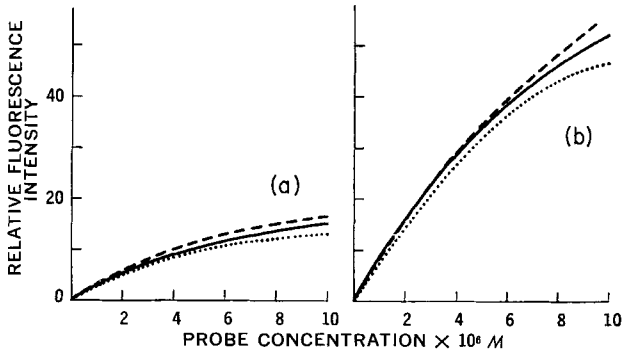


Figure 3—*Fluorescence titration of crystalline bovine serum albumin (—), Fraction V bovine serum albumin (- - -), and acetylated bovine serum albumin (. . .) with Probe II. Key: (a), lower bovine serum albumin concentration; and (b), higher bovine serum albumin concentration.*

fluorescence intensities of a probe in the presence of the drug. Phenylbutazone is known to bind to bovine serum albumin; with this technique binding is further demonstrated by displacement of the bound probe by the drug.

During these studies, using this technique, it was found that the binding of the probes to Fraction V and crystalline bovine serum albumin appeared to be nearly identical. This observation supports recent findings (4) of identical binding properties of the two bovine serum albumin fractions for sulfaethidole. Figures 2 and 3 show the relative fluorescence intensities of probes bound to bovine serum albumin fractions. All curves labeled "a" indicate a saturation of binding sites of the proteins at relatively low fluorescence intensities, and this was due to the low concentration of protein (0.2 mg. protein/2 ml. solution). The "b" curves show that more binding sites were available (1.0 mg. protein/2 ml. solution), and this was indicated by a saturation at higher intensities. The "a" and "b" curves showed no significant difference in the binding of the probes to Fraction V and crystalline bovine serum albumin fractions.

To establish the validity of the experiment, a comparison was made of the binding of the probes for acetylated bovine serum albumin and the bovine serum albumin fractions. A significant difference was found between the binding of 1-anilinonaphthalene-8-sulfonate to acetylated bovine serum albumin and the crystalline and/or Fraction V bovine serum albumin (Fig. 2). The difference in binding affinity for 1-anilinonaphthalene-8-sulfonate between acetylated bovine serum albumin and the bovine serum albumin fractions could be due to the increased net negative charges on the acetylated bovine serum albumin molecule as a result of acetylation of the terminal amino groups of bovine serum albumin (5). This could cause a reduced affinity for the more negatively charged 1-anilinonaphthalene-8-sulfonate for acetylated bovine serum albumin when compared to the three amide probes (see structures of probes) which would be expected to be more hydrophobic than 1-anilinonaphthalene-8-sulfonate. This finding seems to indicate that 1-anilinonaphthalene-8-sulfonate tends to interact with terminal amino groups of the bovine serum albumin, while the other probes bind preferably at the more hydrophobic environments of the bovine serum albumin molecules.

The explanation as to why the more negatively charged acetylated bovine serum albumin has less binding affinity for the three amide probes than do the intact bovine serum albumin fractions (see typical Fig. 3) is not immediately apparent from experimental data. An electrostatic interaction between the proteins and the probes may be a factor. Additional work in these laboratories is aimed at extending the significance of these observations to understand better the drug–protein and drug–drug interactions through elucidation of the nature of the binding using these techniques.

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REVIEWS

Chemical Influences on Behaviour. Ciba Foundation Study Group No. 35. Edited by R. PORTER and J. BIRCH. J. & A. Churchill, 104 Gloucester Place, London, England, 1970. ix + 221 pp. 12.5 × 18.5 cm. Price $7.50. (Williams & Wilkins, Baltimore, MD 21202, U. S. agents)

Behavioral effects related to chemicals have been a major area of interest for scientists for some time. Recently, the role of environmental contaminants and the concern with a growing "drug culture" have forcefully brought this subject to the public's attention.

This volume contains papers presented during the February 1970 meeting of this study group. Included are several papers dealing with the use of animals to determine behavioral effects, human cooperation as affected by alcohol, and the effects of drug–drug and drug–food interactions on performance in man. Twelve papers and the subsequent discussion are presented.

Staff Review ■

Pharmacognosy and Phytochemistry. Edited by H. WAGNER and L. HORHAMMER. Springer-Verlag, 175 Fifth Ave., New York, NY 10010, 1971. vii + 386 pp. 17.5 × 25.5 cm. Price $13.20.

This volume represents the proceedings of the First International Congress on Pharmacognosy and Phytochemistry held in Munich, Germany, in November 1970. Because the investigation of medicinal plants has become multidisciplinary in approach, one of the goals of this meeting was to establish a basis for better coordination and cooperation. Most branches of research appear to have been represented at the Congress. This volume should be a useful reference source for scientists interested in natural substances.

Staff Review ■

Methods of Biochemical Analysis, Vol. 19. Edited by D. GLICK. Wiley, 605 Third Ave., New York, NY 10016, 1971. vii + 632 pp. 15.5 × 32.5 cm. Price $21.00.

This series has proved a valuable reference source for researchers in various fields. Topics covered in this volume are Isoelectric Focusing in pH Gradients—A Technique for Fractionation and Characterization of Ampholytes; Mass Spectrometry in the Determination of Structure of Certain Natural Products Containing Sugars; The Determination of Carbohydrate in Biological Materials by Gas–Liquid Chromatography; Activation Analysis of the Biological Trace Elements; and Polarography of Proteins, Analytical Principles and Applications in Biological and Clinical Chemistry.

Staff Review ■

Probit Analysis, 3rd ed. By D. J. FINNEY. Cambridge University Press, 32 E. 57th St., New York, NY 10022, 1971. xv + 333 pp. 14.5 × 22 cm. Price $18.50.

This book concerns the statistical aspects of dose–response experiments in which the response is quantal. It includes the planning of the experiment as well as the analysis of the results. This is the first new edition since 1952 and, while containing much of the information of previous editions, it has been completely rewritten and reorganized. One new chapter on the structure and use of a computer program has been added.

Staff Review ■

NOTICES

Infrared Analysis of Essential Oils. By J. BELLANTO and A. HIDALGO. Heyden & Son Ltd., Spectrum House, Alderton Crescent, London NW4, England, 1971. 154 pp. 23 × 33 cm. Price $28.00.

Encyclopedia of Industrial Chemical Analysis, Vol. 11, Corn Products to Drycleaning Agents. Edited by F. D. SNELL and L. S. ETTRE. Wiley, New York, NY 10016, 1971. xiv × 617 pp. 19 × 26 cm.

Principles of Physical Chemistry for Biology and Pharmacy, Second (SI) Edition. By L. SAUNDERS. Oxford University Press, 200 Madison Ave., New York, NY 10016, 1971. xi + 420 pp. 16 × 24 cm. Price $14.50.

Rodd's Chemistry of Carbon Compounds, Second Edition, Vol. II, Part E–Steroids. Edited by S. COFFEY. American Elsevier Publishing Co., 52 Vanderbilt Ave., New York, NY 10017, 1971. xv + 289 pp. 16 × 23 cm. Price $20.00.

British Pharmacopoeia 1968. Addendum 1971. Published on the recommendation of the Medicines Commission. The Pharmaceutical Press, 17 Bloomsbury Square, London WC1, England, 1971. xvii + 142 pp. 14 × 22 cm. Price $11.00.

Pharmacological Experiments on Intact Preparations. By the Staff of the Department of Pharmacology, University of Edinburgh, and L. J. MCLEOD. Williams & Wilkins, Baltimore, MD 21202 (American Agents), 1970. vii + 113 pp. 19 × 25 cm. Price $6.75.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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REVIEW ARTICLE

Chemistry and Pharmacology of Marijuana

JOHN L. NEUMEYER and RICHARD A. SHAGOURY

Keyphrases ☐ Marijuana—chemistry and pharmacology review ☐ Tetrahydrocannabinol—chemistry and pharmacology review ☐ Psychotomimetic agents—marijuana, review ☐ Cannabinoids—chemistry and pharmacology review

CONTENTS

NOMENCLATURE AND NUMBERING SYSTEM OF CANNABINOIDS 1435
CHEMICAL RESEARCH ON CANNABIS 1435
Chemistry of the Natural Cannabinoids 1436
Isolation and Structure Elucidation of Naturally Occurring Cannabinoids 1437
Synthesis of Naturally Occurring and Structurally Modified Cannabinoids 1439
Structure–Activity Relationships in Cannabinoids 1443
BIOGENESIS 1447
METABOLISM AND DISPOSITION 1447
Synthesis of Labeled Cannabinoids 1447
Elimination and Distribution of Tetrahydrocannabinols 1448
QUALITATIVE AND QUANTITATIVE ANALYSIS OF CANNABIS CONSTITUENTS 1449
PHARMACOLOGY OF CANNABINOIDS 1450
Animal Pharmacology 1450
Human Pharmacology 1452
Quantitation of Dose in Relation to Clinical Phenomena 1454
Summary of Pharmacological Effects in Man 1454

"Cannabis is used throughout the world for diverse purposes and has a long history characterized by usefulness, euphoria or evil, depending on one's point of view. To the agriculturist cannabis is a fiber crop; to the physician of a century ago it was a valuable medicine; to the physician of today it is an enigma; to the user, a euphoriant; to the police, a menace; to the traffickers, a source of profitable danger; to the convict or parolee and his family, a source of sorrow." (1).

As in so many other areas of drug research, real progress can be achieved in the understanding of the pharmacology and biogenesis of a naturally occurring drug only when the chemistry has been well established and the researcher has at his disposal pure compounds of known composition and stereochemistry. The last decade produced the necessary know-how in the chemistry of the Cannabis constituents so that chemists could devise practical and novel synthetic schemes to provide the pharmacologists with such materials. The precise biological effects of this intoxicant, known to man for several centuries, still remain to be elucidated; no doubt the next decade will yield such information.

Although the current world literature on Cannabis numbers some 2000 publications, few of these papers meet the criteria of modern scientific investigation. The purposes of this review are to provide the pharmaceutical scientist with current information relevant to his areas of specialization and to review the recent literature pertaining to this subject through December 1970.

The plant *Cannibis sativa* L. (family Cannabinaceae), the source of marijuana, is a tall annual weed. It sometimes reaches a height of 4.57 m. (15 ft.), depending on the part of the world where it is grown. It grows in almost any waste or fertile area. The weed is dioecious; that is, the male reproductive parts (stamens) are on one plant and the female parts (pistils) are on another. The male plants usually grow taller than the female plants (Fig. 1). The chemical substances responsible for the euphoric effect of marijuana are found primarily in a sticky resin that covers the female flowers and adjacent leaves. Although this resin is the prime



Figure 1—*Hemp plant* (C. sativa). *The active substances in the drug are contained principally in a sticky yellow resin that covers the flower clusters and top leaves of the ripe plant. (Reprinted, with permission, from "Marihuana" by L. Grinspoon (8), copyright December 1969, by Scientific American, Inc., all rights reserved.)*

source of the euphoric principles, other parts of the female as well as the male plants contain these same active principles (2, 3). This was shown by recent investigations, which uncovered one of the psychotomimetic substances, Δ^9-tetrahydrocannabinol, in the male plant (4). Other parts of the plant, namely, the seed, seed oil, seed oil cake, and fiber (hemp), are important and useful economic products.

Of the many preparations derived from the plant, marijuana (more commonly referred to as "pot" or "grass") is undoubtedly the most common one, at least in the United States. It is prepared simply by drying the flowering tops of the plants and is probably a mixture of male and female flowers in most cases.

The most potent of the plant preparations is hashish or charas. These preparations are the unadulterated resins from the flowering tops of cultivated female plants. The bhang of India is prepared from uncultivated female plants by cutting the tops of the plants and boiling or steeping them in water or milk. This decoction is either drunk or dried and smoked, and its potency is nearly equivalent to that of marijuana. Ganja, also of Indian origin, is prepared in the same way as bhang, except that only tops from very carefully selected cultivated female plants are used. This selectivity for plants of high resin content results in a preparation that is more potent than marijuana. Bhang, ganja, and other preparations such as Moroccan kif, Brazilian macohna, and South African dagga (all nearly equivalent to marijuana) are terms seldom encountered in the United States, since most of the *C. sativa* used here is in the form of dried flowering tops, that is, marijuana (5, 6) (Table I). It is estimated that 2–3 million people throughout the world use a variety of Cannabis preparations (7, 8).

Table II shows the tetrahydrocannabinol potency of a variety of plants grown in Mississippi from seeds obtained in different locations (2). Table I lists some of the Cannabis preparations and their composition.

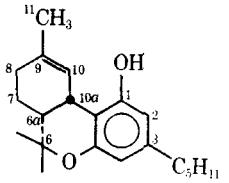
The plant *C. sativa* can vary widely in its chemical constituents, depending on the geography of the source material, the age of the harvested sample, the storage conditions, and other factors (10, 11).

NOMENCLATURE AND NUMBERING SYSTEM OF CANNABINOIDS

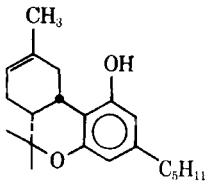
The term "cannabinoid" will be used in this report to refer to the group of compounds typical of, and present in, *C. sativa* as well as to their analogs and transformation products. This definition is adopted from Mechoulam and Gaoni (11), who originally proposed it in 1967.

At least four different numbering systems have been used in publications relating to the cannabinoids. A few research groups (7, 11–13) regarded the cannabinoids as substituted monoterpenes. The advantage of the monoterpene numbering system lies in the relationship of the cannabinoids to their biogenetic terpene precursor without the necessity of changing the numbering of specific carbon atoms. This system has disadvantages also (*i.e.*, in the monoterpene numbering system, the 3′—OH group in Δ^1-tetrahydrocannabinol becomes 2′—OH in cannabidiol). The dibenzopyran numbering system utilizes the formal chemical rules for the numbering of pyran-type compounds and was adopted by the National Institute of Mental Health (NIMH) (14) and the earlier publications of Adams (15). The formal (Chemical Abstracts) numbering system will be used throughout this report for those compounds containing the carbon skeleton corresponding to that of dibenzopyran. Both systems are shown here.

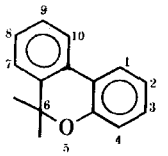
formal dibenzopyran numbering (used in this review)



Δ^9-*trans*-tetrahydrocannabinol

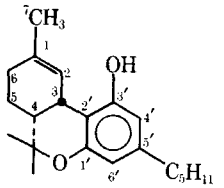


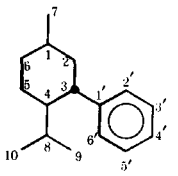
Δ^8-*trans*-tetrahydrocannabinol



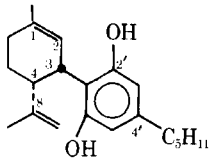
dibenzopyran numbering

monoterpenoid numbering (used in this review for terpenelike compounds only, *i.e.*, cannabidiol)





Δ′-*trans*-tetrahydrocannabinol



cannabidiol

CHEMICAL RESEARCH ON CANNABIS

It is beyond the scope of this review to present and discuss fully the investigations on the chemistry of Cannabis constituents reported over a period of 120 years. Rather, the present review attempts to summarize the pertinent chemistry relevant to an understanding of the biological effects of Cannabis and to review the chemistry of Cannabis that has appeared since the last review (7). Much of the older work was surveyed by Blatt (16); Adams (15) summarized his own work, and Todd (17) reviewed the field up to 1946. Since that time the chemistry of hashish was reviewed by Mechoulam and Gaoni (11), Korte *et al.* (18), Downing (19), two Ciba Foundation Symposia in 1965 (20) and in 1969 (21), and several shorter reviews (22–25).

Table I—Names for Common Preparations (9)

Name	Composition
Hashish Charas (India)	Pure resin from tops of female hemp plants; most potent material
Ganja (India)	Flowering tops of specially cultivated female plants; highly potent; used in smoking mixtures, beverages, and sweets
Bhang (India, Middle East)	Dried, uncultivated, mature, female plants; used in smoking mixtures and beverages
Marijuana (United States and Europe) Kif (Morocco) Dagga (South Africa) Macohna (Brazil)	Entire plant with variable proportions of leaves and flowering tops; used as smoking mixtures and as beverages; potency varies greatly; names such as "Panama red" and "Acapulco gold" describe highly potent preparations

Chemistry of the Natural Cannabinoids—In 1857, the Smith brothers (26) of Edinburgh showed that the physiologically active principle of Cannabis was contained in the alkali-insoluble, high boiling portion of the hemp resin, which was obtained by alcoholic extraction of the plant. No significant progress was made until 1897 when three Cambridge chemists, Wood, Spivey, and Easterfield (27), after effecting considerable purification of the resin through distillation, were able to isolate a crystalline acetate from the acetylated resin. Upon hydrolysis, the acetate yielded a phenolic compound which they called cannabinol.

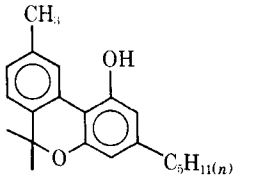
About 30 years later, Cahn (28) began work on the subject through his studies on cannabinolactone, a degradation product of cannabinol. As a result, he correctly deduced the structure of cannabinolactone, which was confirmed by Bergel and Vogel (29) by synthesis. These results led to a partial structure for cannabinol, which was not clarified until the 1940's when Cahn's colleagues in Britain and Adams and coworkers in America reopened the investigation. At this time, Adams *et al.* (30) isolated a new substance, cannabidiol, from marijuana; the same substance was isolated by Jacob and Todd (31) from Egyptian hashish. Both cannabinol and cannabidiol lacked hashish activity when tested on rabbits, so it was concluded that the active material remained in the noncrystalline portion of the esterified resin.

The locations of the hydroxyl groups in cannabidiol, which were determined from its pyrolysis products with pyridine hydrochloride, together with the fact that cannabinol also is present in hemp, made it certain that cannabinol was correctly represented by I (17).

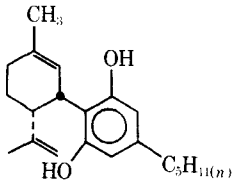
Table II—Δ^9-Tetrahydrocannabinol Potency of Marijuana Plants Grown in Mississippi from Various Seed Sources (2)

Seed Source	Percent Δ^9-Tetrahydrocannabinol
France	0.085
Italy	0.041–0.068
Mexico:	
Male plants	1.47
Female plants	1.31
Sweden	0.021
Turkey	0.05–0.40

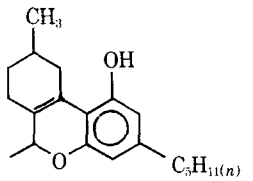
This structure was quickly confirmed by two independent syntheses of cannabinol by Adams *et al.* (32) and Ghosh *et al.* (33). Later, the structure of cannabidiol was clarified and shown to correspond to II (34, 35), although the position of the double bond in the ring was not known with certainty until 1963 (36).



cannabinol
I



cannabidiol
II



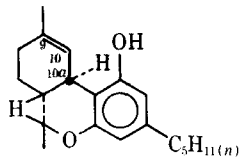
$\Delta^{6a,10a}$-tetrahydrocannabinol

IIIa

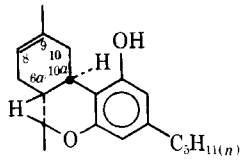
The synthetic route used by the former authors (29) produced an intermediate tetrahydrocannabinol, $\Delta^{6a,10a}$-tetrahydrocannabinol (IIIa), which was found to exhibit a high degree of marijuanalike activity in animals and in man. Concurrently, Adams and Baker (37) also established a synthetic route for IIIa, which was almost identical to the one reported by Ghosh *et al.* (33). It was also shown by Adams *et al.* (38) that cannabidiol (II) can be cyclized under acidic conditions to yield a mixture of optically and physiologically active tetrahydrocannabinols. In 1942, another group, working at the U. S. Bureau of Narcotics, succeeded in isolating a highly purified active fraction from the resin (39). Although no definite structure was offered for this substance, it was assumed to be a tetrahydrocannabinol isomer.

The discovery of the activity of $\Delta^{6a,10a}$-tetrahydrocannabinol led to the synthesis and pharmacological examination of a wide variety of related analogs in the hope of elucidating the relationship between chemical constitution and hashish activity. Some of these compounds were found to be many times more active than the semisynthetic tetrahydrocannabinol isomers. The results of these studies are discussed in detail later in this review. In spite of the progress achieved in the area of synthesis, the active natural constituents were not obtained pure; consequently, their structures were not fully elucidated.

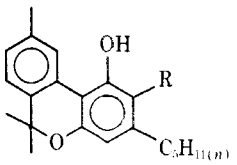
Knowledge of Cannabis has developed rapidly over the past 10 years as a result of intensive chemical investigations, which have considerably clarified its rather complex chemistry. This renewed interest is probably the combined result of its rapidly increasing use among the young, a reevaluation of its possible use as a medicinal, and a greater refinement of instrumental and chemical techniques. A host of new substances has been isolated from the resin (hashish), most of which have now been successfully characterized and synthesized. Among those substances isolated are



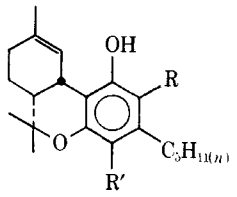
Δ^9-*trans*-tetrahydrocannabinol
IV



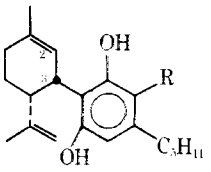
Δ^8-*trans*-tetrahydrocannabinol
V



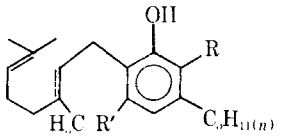
I: R = H, cannabinol
VI: R = COOH, cannabinolic acid



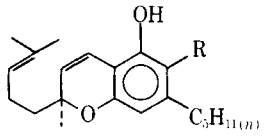
VII*a*: R = COOH, R′ = H
Δ^9-tetrahydrocannabinol acid A
VII*b*: R = H, R′ = COOH
Δ^9-tetrahydrocannabinol acid B



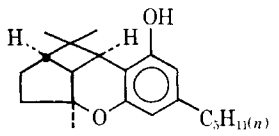
II: R = H, cannabidiol
VIII: R = COOH, cannabidiolic acid



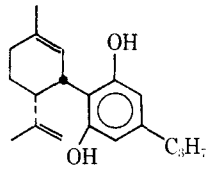
IX*a*: R = H, R′ = OH, cannabigerol
IX*b*: R = COOH, R′ = OH, cannabigerolic acid
IX*c*: R = H, R′ = OCH_3, cannabigerol methyl ether



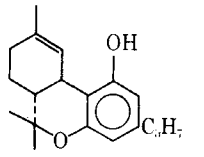
X*a*: R = H, cannabichromene
X*b*: R = COOH, cannabichromenic acid



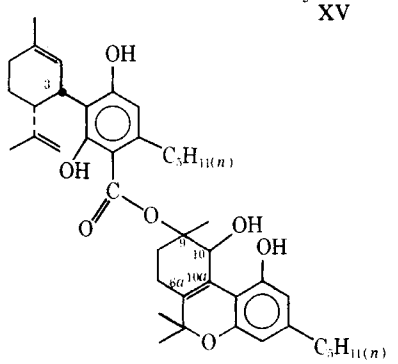
cannabicyclol (cannabipinol)
XI



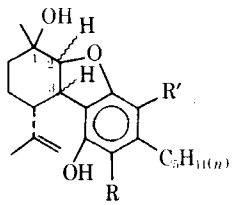
cannabidivarin
XII



n-propyl analog of Δ^9-tetrahydrocannabinol
XV



cannabitriol ester of cannabidiolic acid
XIII



XIV*a*: R = COOH, R′ = H, cannabielsoic acid A
XIV*b*: R = H, R′ = COOH, cannabielsoic acid B

structures of *C. sativa* cannabinoids

(−)-Δ^9-*trans*-tetrahydrocannabinol (IV), the generally accepted psychoactive principle, whose structure and stereochemistry have now been fully elucidated.

Isolation and Structure Elucidation of Naturally Occurring Cannabinoids—Column chromatography has been, without doubt, the major technique used in effecting the isolation of the naturally occurring cannabinoids. Usually an extract is made of the flowering tops or resin of the plant with petroleum ether. By repeated extraction with this solvent, all the active ingredients in the crude resin can be dissolved. The remaining inactive materials contain mostly phenolic polymers, small amounts of cannabielsoic acids, which can be extracted by benzene. The petroleum ether extract is separated into acidic and neutral fractions, both of which, on column chromatography, yield numerous cannabinoids and some unidentified sesqui- and triterpenes (7, 11). Adsorption chromatography (40), partition chromatography (40), preparative TLC (13), and countercurrent distribution (41) have been used with success by some workers, and it is not unusual to find two of these methods used together. The procedure used by Mechoulam and Gaoni (11) typifies the flexibility of column chromatography to effect separation of the cannabinoids:

> *"A hexane extract of hashish was separated into acidic and neutral fractions. The latter was chromatographed on Florisil or acid-washed alumina. The following identified compounds were eluted in order of increasing polarity: cannabidiol (II) (eluted with 5% ether in pentane), Δ^9-THC (IV), cannabinol (I), cannabichromene (Xa) and cannabigerol (IXa) (eluted with 15% ether in pentane). Repeated chromatography was needed to effect full separation. When the cannabidiol fractions were chromatographed on acid-washed alumina containing 12% silver nitrate, another component, cannabicyclol (XI), could be separated. Cannabichromene (Xa) and cannabigerol (IXa) could likewise be separated by the silver nitrate–alumina column. Cannabidiol, m.p. 66–7°, cannabicyclol, m.p. 152–3° and cannabigerol, m.p. 51–3°, were crystallized directly, while Δ^9-THC and cannabichromene were further purified by the preparation of crystalline derivatives and hydrolysis to the parent compound. Chromatography of the esterified acidic fraction yieldedcann abigerolic (IXb), cannabinolic (VI), and cannabidiolic (VIII) acids (as esters). The column chromatographic separations were monitored by thin-layer chromatography."*

The compounds isolated from the active neutral fraction are shown in Table III. A number of other workers described various methods of isolation and detection of Cannabis substituents (10, 42–48).

Earlier in this report, the structural elucidations of cannabinol (I) and cannabidiol (II) were briefly discussed. The structure of cannabinol was well established by two independent syntheses; the structure of cannabidiol was certain, except for the position of the double bond in the ring. The correct position of this double bond was not deduced until 1963 when NMR measurements showed the presence of only one olefinic proton in the compound (36). Additional support for this assignment was obtained from the NMR spectra of tetrahydrocannabidiol and its monoepoxide (11).

The relative stereochemistry of the two asymmetric centers, C_3 and C_4, in cannabidiol was deduced to be *trans* from an analysis of the coupling constants of the protons at these centers and by degradative evidence (36).

Cannabidiolic acid was first assigned Structure VIII (49, 50) (except for the position of the double bond), mainly on the basis of its conversion to cannabidiol by decarboxylation. The position of the carboxyl group was established by its IR and NMR spectra. The peak at 1698 cm.$^{-1}$ in the IR was assigned to an aromatic carboxyl group; in the NMR spectrum, only one aromatic proton was observed (51).

Since cannabigerol (IX*a*) has two more hydrogens than cannabidiol (II) but the same number of double bonds, it was concluded that cannabigerol possesses one ring less than cannabidiol. Its optical inactivity suggested that the two asymmetric centers present in cannabidiol are absent in its structure. The NMR spectrum indicates that the aromatic protons are identical, that the two protons at C-8 are strongly deshielded and split by a single adjacent proton, and that an isopropylidene group is present. The fact that the UV spectrum is identical with that of cannabidiol indicates that the double bonds are unconjugated, either to each other or to the aromatic ring (52, 53). The structure of cannabigerol was corroborated by synthesis (52). Cannabigerol monomethyl ether (IX*c*) was also identified (54) from the benzene extract of hemp and characterized by its physical properties, NMR spectrum, and synthesis from cannabigerol.

The structure of Δ^9-*trans*-tetrahydrocannabinol (IV) was elucidated in 1964 by Mechoulam and Gaoni (55). The carbon skeleton was determined by dehydrogenation to cannabinol, and the position of the double bond and the stereochemistry at the asymmetric centers were determined from NMR measurements and by synthesis studies. NMR studies showed that the olefinic C_{10} proton of IV is strongly deshielded compared to the C_2 proton in cannabidiol (II). On the other hand, the C_3 proton of II is more strongly deshielded than the C_{10a} proton of IV. This effect is the result of the orientation of the aromatic ring with respect to the C_2 and C_3 protons of II and the C_{10} and C_{10a} protons of IV and could only be observed if the double bond were in the Δ^9-position in IV. The Δ^8-isomer (V) was first prepared semisynthetically by Adams *et al.* (34), and further chemical and spectroscopic evidence (13, 56) confirmed the structure and established the *trans* stereochemistry at C_{6a} and C_{10a}. Conversion of cannabidiol (II) into Δ^8-*trans*-tetrahydrocannabinol (V) confirmed the suggested structure, and subsequent synthesis corroborated the stereochemical assignments (12). The absolute configuration of Δ^8-tetrahydrocannabinol and of Δ^9-tetrahydrocannabinol at the chiral centers, C_{6a} and C_{10a}, is R (57). The key to this assignment is the degradative correlation of cannabidiol (II) to D(−)-menthane carboxylic acid, m.p. 65–66°,$[\alpha]_D$ −44°, derived from D(−)-menthol, whose absolute configuration is known.

A Δ^9-tetrahydrocannabinol homolog, in which the side chain is C_3H_7 (instead of C_5H_{11}) (XV), was recently found in a sample of Pakistani hashish. It is one-fourth as active as Δ^9-tetrahydrocannabinol in producing a cataleptic effect in mice (58).

The structures of cannabinolic acid (VI), cannabigerolic acid (IX*b*), and tetrahydrocannabinol acid A (VII*a*) (59, 60) were determined by comparison of their NMR spectra with those of the corresponding neutral compounds (I, IX*a*, and IV) and, in the cases of VI and IX*b*, by actual conversion to the corresponding neutral compounds (I and IX*a*) by decarboxylation. In the acids, VI and VII*a*, where the two aromatic positions are not equivalent, the position of the carboxyl group was shown to be adjacent to the free phenolic group on the basis of strong deshielding of the phenolic proton caused by hydrogen bonding (11). An isomeric Δ^9-tetrahydrocannabinol acid B (VII*b*) was also isolated and characterized (61).

The structure of cannabichromene (X*a*) was suggested in 1966 (62, 63) on the basis of the UV spectrum showing double-bond conjugation with an aromatic ring, while the NMR spectrum indicated that two of the olefinic protons are not flanked by additional hydrogen atoms, an isopropylidene grouping is present, and one of the methyl groups is alpha to an oxygen while two others are olefinic. The structure of cannabichromene (X*a*) was corroborated by comparison of its hydrogenated product with the hydrogenated product resulting from the cyclization of cannabigerol (IX*a*) (62). The structure of *dl*-cannabichromene (X*a*) was confirmed by Mechoulam *et al.* (64) [by the chloranil dehydrogenation of cannabigerol (IX*a*)] and by others

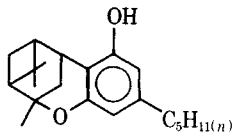
Table III—Constituents in Hashish, R_f Values, and Retention Times (VPC) of Some Natural Neutral Cannabinoids (7, 25)

	Yields[a]	R_f[b]	Retention Time[c]
Cannabicyclol (XI)	0.11	0.62	4 min., 33 sec.
Cannabidiol (II)	3.74 (1.4) (2.5)	0.58	5 min., 40 sec.
Δ^8-Tetrahydrocannabinol (V)	Not detected	0.57	7 min., 10 sec.
Δ^9-Tetrahydrocannabinol (IV)	3.30 (1.4) (3.4)	0.51	7 min., 52 sec.
Cannabinol (I)	1.30 (0.3) (1.2)	0.47	10 min., 12 sec.
Cannabichromene (X*a*)	0.19	0.43	5 min., 35 sec.
Cannabigerol (IX*a*)	0.30	0.42	9 min., 20 sec.

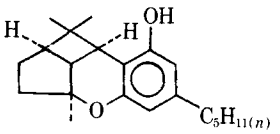
[a] As percent of hashish; determined by VPC. The numbers in parentheses are from two partial analyses of different batches. [b] Chromatoplates of silica gel. Elution with petroleum ether (b.p. 40–60°) and ether in a ratio of 4:1. [c] Column 2% OV-17 on Gas-Chromosorb Q: N_2 flow, 30 ml./min.; column temperature, 235°.

(65, 66). It is assumed (25) that naturally occurring cannabichromene is racemic, as in the related cannabichromenic acid (X*b*). This lack of optical activity points out that either cannabichromene is an artifact formed by a nonenzymatic oxidation of cannabigerol or that the intermediate formed by enzymatic oxidation is a symmetrical species. The lack of optical activity could also be due to the isolation procedure (25) involved in this compound. Pure cannabichromene shows no activity in the dog ataxia or monkey behavioral test in doses up to 10 mg./kg. (67). Cannabichromenic acid has been isolated from the benzene extract of hemp and shown to have Structure X*b* by NMR and by its ready conversion to cannabichromene (X*a*) with heat (68).

The structure of cannabicyclol (XI) was firmly established *via* an X-ray study of dibromocannabicyclol (69). The original formulation suggested for cannabicyclol (XVI) (11, 63) was revised to XI on the basis of its formation from cannabichromene (X*a*) by several chemical methods (69–72) and the X-ray study (69).



XVI

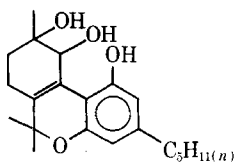


XI

Two tricyclic dihydrobenzofuran cannabinoids, cannabielsoic acid A (XIV*a*) and cannabielsoic acid B (XIV*b*), were isolated and characterized from the benzene extract of hashish in 0.08 and 0.04% yields, respectively (73). Compound XIV*a* was synthesized by an intramolecular photooxidative cyclization of cannabidiolic acid (VIII). The stereochemistry at C-2–C-3 was not confirmed but is probably *cis*.

Cannabidivarin (XII), a homolog of cannabidiol (II) in which the side chain on the phenyl ring is *n*-propyl (instead of *n*-pentyl), was also isolated from hashish. The structure and absolute configuration of cannabidivarin were determined by spectroscopic methods and confirmed by synthesis (74).

From the petroleum ether extract of hashish, the ester (XIII)[1] of cannabidiolic acid with tetrahydrocannabitriol was also isolated in small quantities (75). The alcohol moiety, tetrahydrocannabitriol (XVII), has not yet been isolated; it will in time, no doubt, also be found as a constituent of hashish.

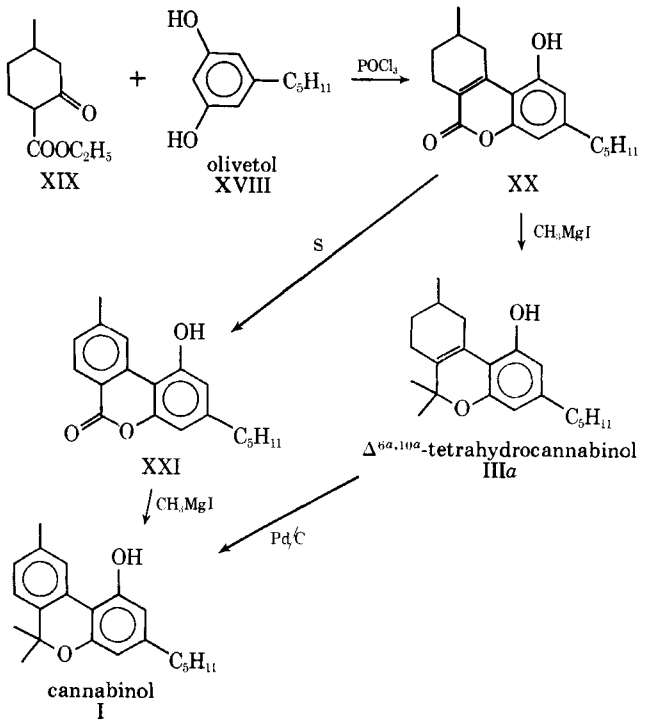


$\Delta^{6a,10a}$-tetrahydrocannabitriol
XVII

Other materials including noncannabinoid terpenes (76), nitrogen compounds (77), sugars (78), and phenolic compounds (77, 79) were isolated or detected in *C. sativa*.

Synthesis of Naturally Occurring and Structurally Modified Cannabinoids—Almost all of the noncarboxylic, naturally occurring cannabinoids that have been described were prepared synthetically or semisynthetically in the laboratory. These are cannabinol (I), cannabidiol (II), Δ^9-*trans*-tetrahydrocannabinol (IV), Δ^8-*trans*-tetrahydrocannabinol (V) (the corresponding *cis*-isomer of Δ^9-*trans*-tetrahydrocannabinol was also synthesized), cannabigerol (IX*a*), cannabichromene (X*a*), and cannabicyclol (XI). The syntheses of these compounds will be outlined.

In 1940, the biologically inactive cannabinol was the first cannabinoid to be synthesized by two groups working independently. One of the routes, followed by Adams and Baker (80), involved the condensation of ethyl 4-methyl-2-oxocyclohexanecarboxylate (XIX) with olivetol (XVIII) in the presence of phosphorus oxychloride. The resulting dibenzopyrone (XX) was dehydrogenated with sulfur and then treated with excess methylmagnesium iodide, yielding the desired product, cannabinol (I) (Scheme I).

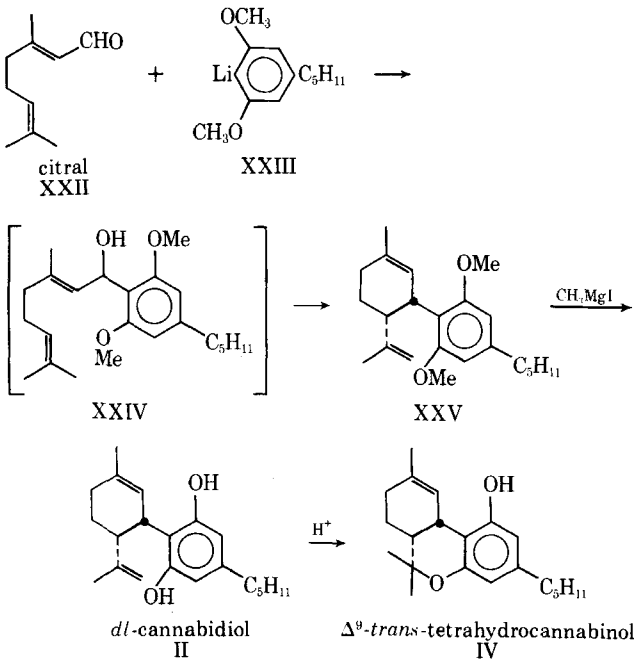


Scheme I—Synthesis of dl-$\Delta^{6a,10a}$-*tetrahydrocannabinol and of cannabinol*

Ghosh *et al.* (33, 81) made use of a similar route. They also produced the dibenzopyrone (XX), but they chose to treat it with methylmagnesium iodide before dehydrogenation. This latter route was of particular interest because the intermediate tetrahydrocannabinol (III*a*) was found to possess marijuanalike activity in animals and in man. Compound III*a* was also produced by Adams and Baker (37), who prepared a large series of $\Delta^{6a,10a}$-tetrahydrocannabinol homologs which will be discussed subsequently. Both groups described other routes for the synthesis of cannabinol (32, 82).

[1] The numbering system for this compound utilizes the dibenzopyran numbering system for the alcohol portion of the ester and the monoterpene numbering system for cannabidiolic acid.

The first total synthesis of *dl*-cannabidiol (II) and of *dl*-Δ^9-*trans*-tetrahydrocannabinol (IV), the major psychoactive principle of marijuana, was reported in 1965 by Mechoulam and Gaoni (83). Both of these cannabinoids were produced from the same reaction sequence (Scheme II). Citral (XXII) and the lithium



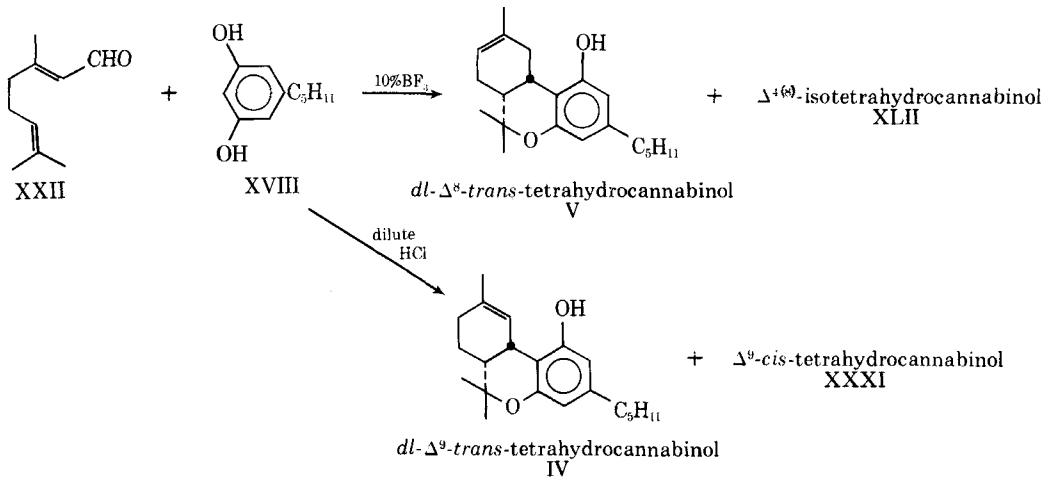
Scheme II—Synthesis of dl-*cannabidiol and of* dl-Δ^9-trans-*tetrahydrocannabinol*

salt of olivetol dimethyl ether (XXIII) were allowed to react at room temperature for 15 min.; the unresolved reaction mixture, presumably containing XXIV, was treated with *p*-toluenesulfonyl chloride in pyridine. The resulting cannabidiol dimethyl ether (XXV) was treated with excess methylmagnesium iodide, producing *dl*-cannabidiol (II). The diol was easily converted to *dl*-Δ^9-*trans*-tetrahydrocannabinol (IV) by treatment with 0.05% HCl in ethanol for 2 hr. The overall yield of IV was 2%. This synthesis was of little practical value since the yield was low, it was nonstereospecific, and it gave a racemic mixture. Numerous other investigators have since improved or modified this route or accomplished entirely new syntheses (47, 55, 84). Petrzilka *et al.* (85) reported a stereospecific synthesis of (−)-cannabidiol (II) in 25% yield by the condensation of (+)-*trans*- or (+)-*cis*-*p*-mentha-2,8-dien-1-ol (XXVIII) with olivetol (XVIII) in the presence of *N,N*-dimethylformamide dineopentylacetal (Scheme IVB).

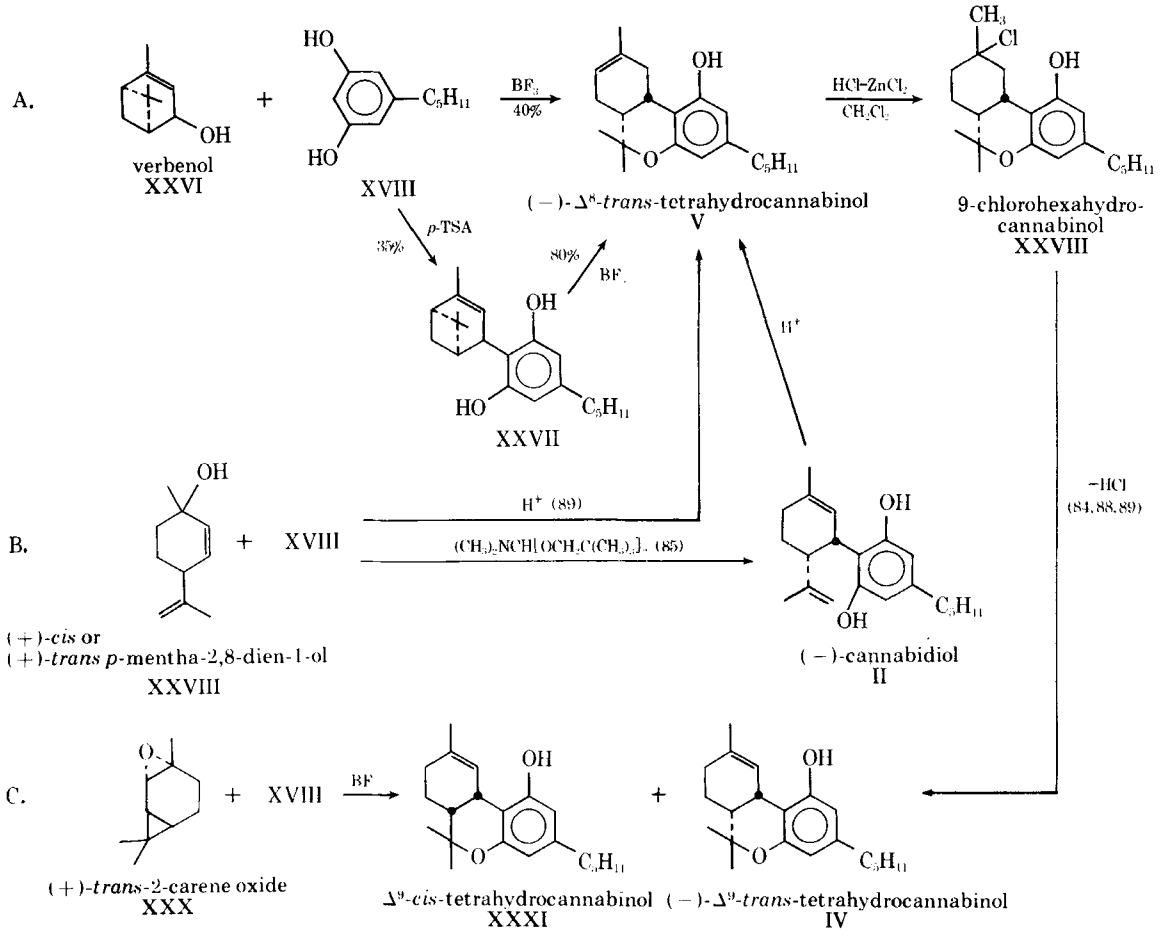
Taylor *et al.* (12) developed a facile one-step synthesis of *dl*-Δ^8- and *dl*-Δ^9-tetrahydrocannabinols. This procedure produces, in addition to the two *dl*-*trans*-tetrahydrocannabinol isomers, Δ^9-*cis*-tetrahydrocannabinol (XXXI) and $\Delta^{4(8)}$-isotetrahydrocannabinol (XLII), which are not found as naturally occurring cannabinoids (Scheme III). Citral (XXII) was reacted with olivetol (XVIII) in the presence of boron trifluoride and gave a mixture from which, after liquid column chromatography and vapor-phase chromatography, the *trans*-isomer (V) was recovered in 20% yield. The Δ^9-*cis*-isomer was formed by this condensation only under milder conditions (using very dilute HCl instead of boron trifluoride etherate).

dl-Δ^8-*trans*-Tetrahydrocannabinol (V) was also synthesized by Taylor and Strojny (86) *via* a Diels–Alder reaction. Fahrenholtz *et al.* (84) reported the synthesis of *dl*-Δ^9-*trans*-tetrahydrocannabinol and *dl*-Δ^8-*trans*-tetrahydrocannabinol, as well as of three unnatural isomers, *dl*-$\Delta^{9(11)}$-*trans*-tetrahydrocannabinol (LX), *dl*-$\Delta^{9(11)}$-*cis*-tetrahydrocannabinol, and *dl*-Δ^9-*cis*-tetrahydrocannabinol (LX). Their method involved a 10-step synthesis, in which the initial step was a von Pechman condensation of olivetol (XVIII) and diethyl α-acetoglutarate (LXIII). This method was employed by Nilsson *et al.* (87) for the synthesis of Δ^8- and Δ^9-tetrahydrocannabinol-^{14}C, which will be discussed later.

Three stereospecific syntheses of the psychoactive tetrahydrocannabinol isomers were reported (Scheme IV). Mechoulam *et al.* (88) synthesized (−)-Δ^9-*trans*-tetrahydrocannabinol (IV) and (−)-Δ^8-*trans*-tetrahydrocannabinol by the condensation of (−)-verbenol (XXVI) with olivetol (XVIII) in the presence of *p*-



Scheme III—Synthesis of dl-Δ^8-*tetrahydrocannabinol and* dl-Δ^9-*tetrahydrocannabinol*



Scheme IV—Synthesis of optically active (−)-Δ⁸-trans-*tetrahydrocannabinol*, (−)-Δ⁹-trans-*tetrahydrocannabinol, and* (−)-*cannabidiol*

toluenesulfonic acid (*p*-TSA). The pinene derivative (XXVII) was treated with boron trifluoride etherate at room temperature, producing (−)-Δ⁸-*trans*-tetrahydrocannabinol (V). The tetrahydrocannabinol (V) could also be prepared directly by treating the reactants with boron trifluoride etherate. (−)-Δ⁹-*trans*-Tetrahydrocannabinol (IV) was prepared by treating V with a dry hydrochloric acid–zinc chloride mixture, followed by reaction of the halogenated product (XXVIII) with sodium hydride in tetrahydrofuran. The overall yield was 19% (Scheme IVA).

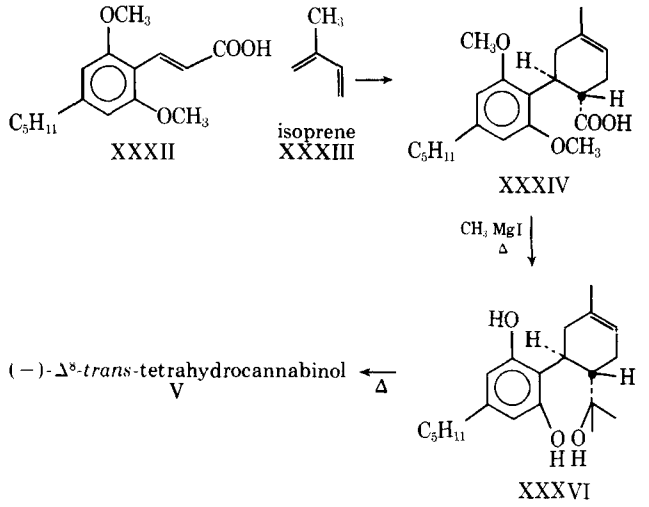
Petrzilka *et al.* (85, 89) also accomplished a stereospecific synthesis of these two psychoactive tetrahydrocannabinol isomers in a process that involves the condensation of olivetol with either (+)-*cis*- or (+)-*trans*-*p*-mentha-2,8-dien-1-ol (XXVIII) in the presence of acids (Scheme IVB). The (−)-Δ⁸-tetrahydrocannabinol (V) thus formed was converted to (−)-Δ⁹-tetrahydrocannabinol (IV) by the addition and elimination of hydrogen chloride in a manner similar to that of Mechoulam *et al.* (88). Potassium *tert*-amylate accomplished the dehydrogenation in benzene solution. *N,N*-Dimethylformamide dineopentylacetal was found to mediate the direct formation of (−)-cannabidiol (II) from XXVIII and XVIII (85).

Razdan and Handrick (90) reported an entry into the cannabinoids *via* carane derivatives; their one-step stereospecific synthesis leads to (−)-Δ⁸-*trans*-tetrahydrocannabinol (V) and (−)-Δ⁹-*trans*-tetrahydrocannabinol. Their method involves the interaction of (+)-*trans*-2-carene oxide (XXX) with an equimolar quantity of olivetol (XVIII) in the presence of *p*-toluenesulfonic acid to yield 23% of the Δ⁸-tetrahydrocannabinol isomer (V), 7% of Δ⁹-tetrahydrocannabinol (IV), and a complex mixture of other products. By modifying the molar ratio of the reactants or by employing 1% boron trifluoride etherate in methylene chloride, 28% of (−)-Δ⁹-*trans*-tetrahydrocannabinol (IV), Δ⁹-*cis*-tetrahydrocannabinol (XXXI), and a mixture of other products were obtained (Scheme IVC).

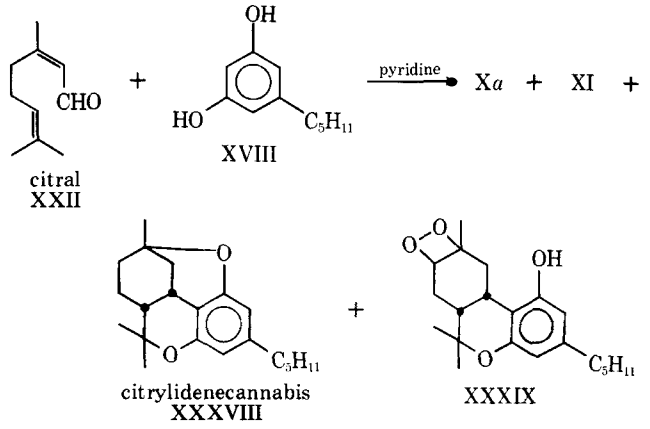
Jen *et al.* (91) reported a total synthesis of (−)-Δ⁸-*trans*-tetrahydrocannabinol (V), which involves the optical resolution of a racemic intermediate (XXXIV) (Scheme V). Fusion of the resulting (−)-XXXIV with methylmagnesium iodide gave the triol (XXXVI), which, on distillation and filtration in benzene through neutral Al_2O_3, gave a gum from XXXIV. Pure (−)-Δ⁸-*trans*-tetrahydrocannabinol was separated from the gum by preparative GLC.

Δ⁹- and Δ⁸-Tetrahydrocannabinol incorporating ^{14}C, tritium, and deuterium (87, 92–96) were synthesized for metabolism studies. Methods for these syntheses will be discussed in the section dealing with metabolism studies.

Cannabigerol (IX*a*) was synthesized by Mechoulam and Gaoni (52) by heating geraniol (XXXVII) with



Scheme V—Synthesis of (−)-Δ⁸-trans-tetrahydrocannabinol involving resolution of an intermediate (91)



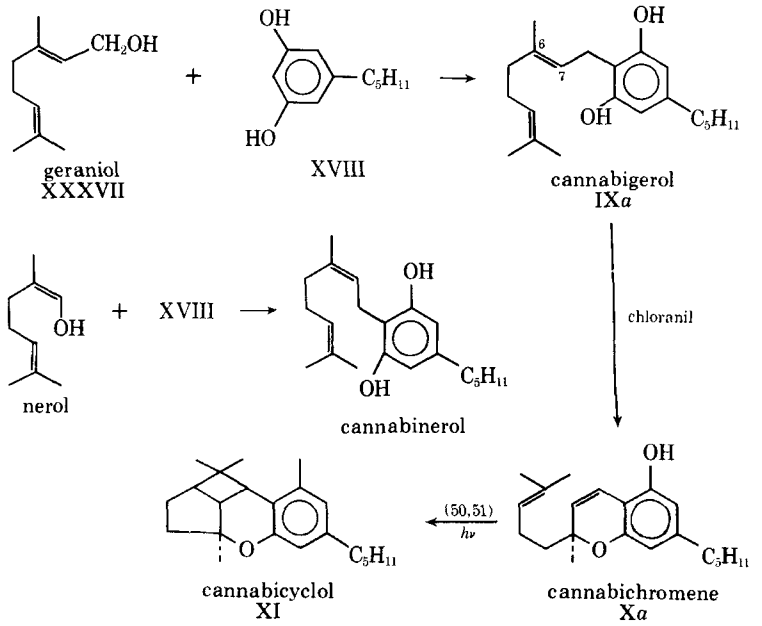
Scheme VII—Pyridine-catalyzed condensation products of olivetol with citral

olivetol (XVIII) in methylene chloride in the presence of *p*-toluenesulfonic acid at 20° (Scheme VI). The *cis*-isomer, cannabinerol, was similarly prepared in 39% yield from nerol and olivetol, confirming the geometry of the C_6—C_7 bond in cannabigerol (53).

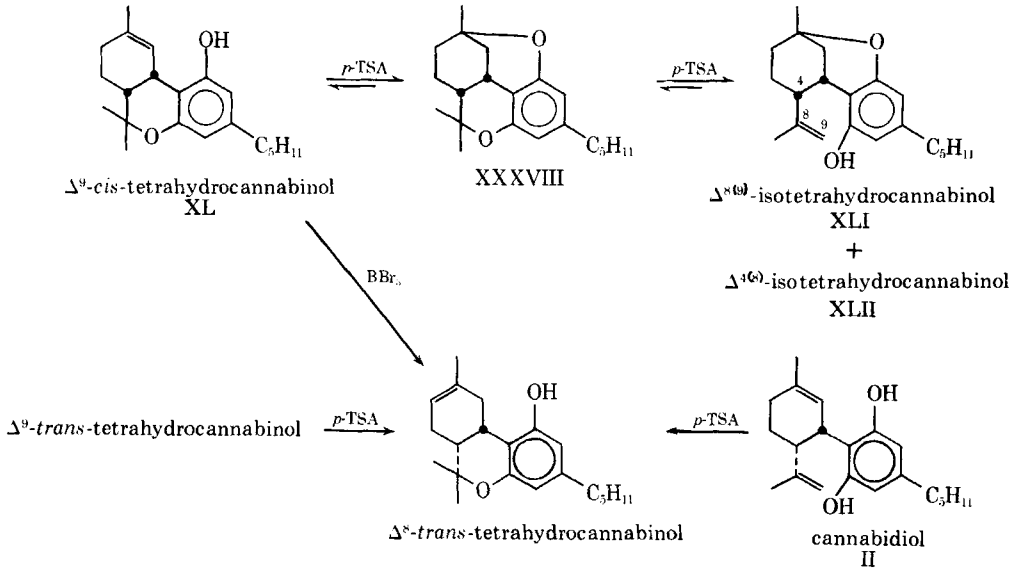
Mechoulam *et al.* (64) also prepared *dl*-cannabichromene (X*a*) by the dehydrogenation of cannabigerol (IX*a*) (in 45% yield) with chloranil (tetrachloroquinone) (Scheme VI). Cardillo *et al.* (97) synthesized *dl*-cannabichromene from geranyl bromide.

Crombie *et al.* (65, 72) prepared cannabicyclol (XI) from cannabichromene (X*a*) by photochemical means. Kane and Razdan (66) synthesized *dl*-cannabicyclol (XI) and *dl*-cannabichromene (X*a*) by heating equimolar quantities of citral (XXII) with olivetol (XVIII) in pyridine (Scheme VII). A third component isolated from this reaction mixture was the tetracyclic ether (XXXVIII), which had previously been obtained by the treatment of cannabigerol with chloranil (64). Razdan and Kane (98) subsequently reported the isolation of still another compound, a cyclic peroxide (XXXIX), from this pyridine-catalyzed reaction.

The isolation of XXXVIII in this reaction mixture and the observation that similar compounds were formed by varying the structure of the phenolic reactant (replacing olivetol with orcinol) led these authors to conclude that the reaction between substituted resorcinols and citral (XXII) in the presence of pyridine to form tetracyclic ethers of type XXXVIII is a general one. Such tetracyclic ethers will form isotetrahydrocannabinol derivatives of types XLI and XLII by reaction with *p*-toluenesulfonic acid (64, 66, 99, 100). An example for the interconversion of Δ⁹-*cis*-tetrahydrocannabinol (XL) to Δ⁸-*trans*-tetrahydrocannabinol was reported (101), and it was further shown that XL, XXXVIII, XLI, and XLII are interconvertible (71) (Scheme VIII). On the basis of this equilibrium and other results, Razdan and Zitko (71) proposed a mechanism of such acid-catalyzed transformations in cannabinoids. These authors concluded that in cannabinoids that have one of the oxygens tied



Scheme VI—Synthesis of cannabigerol, cannabichromene, and cannabicyclol



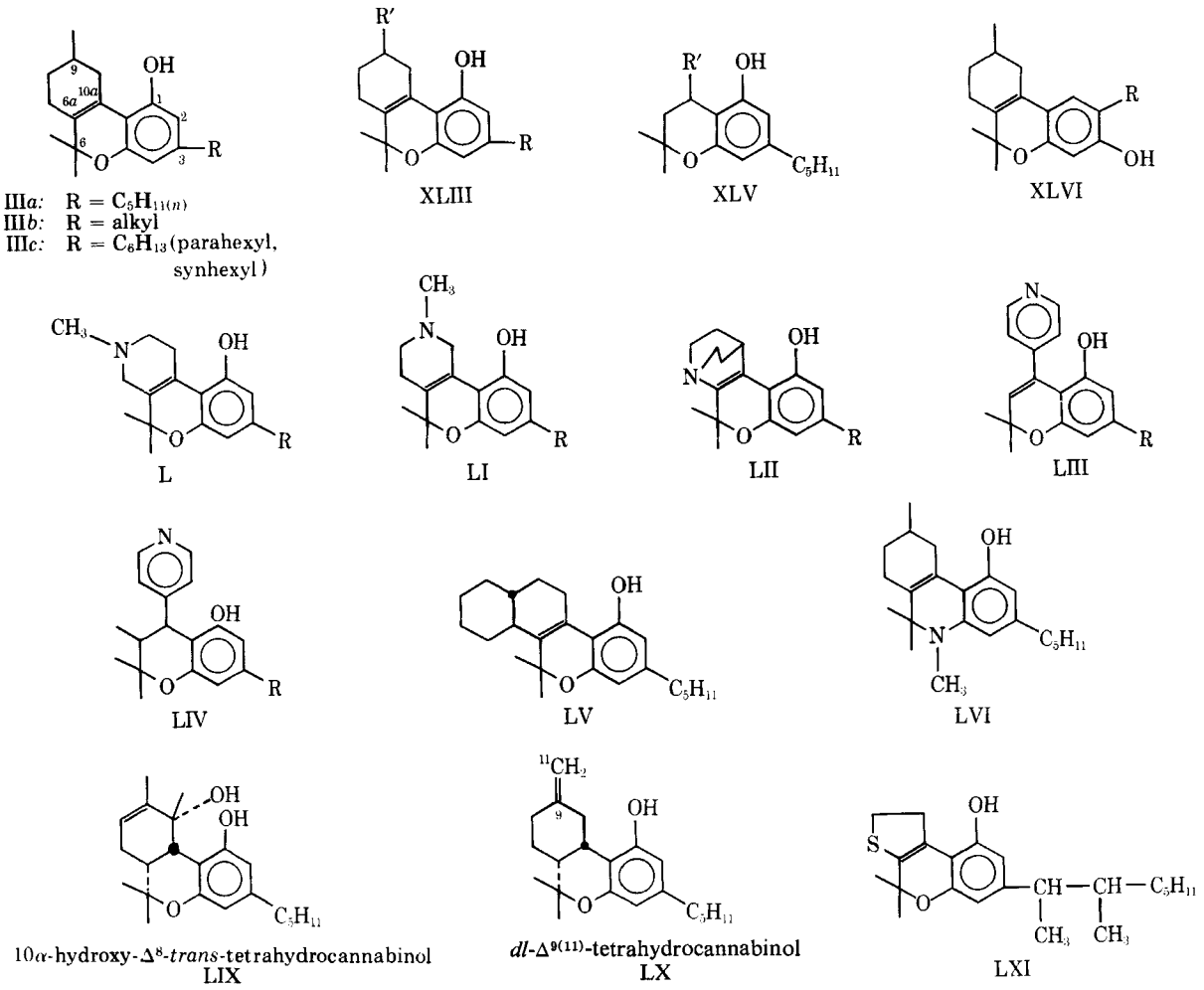
Scheme VIII—Acid-catalyzed transformations of Δ⁹-trans-*tetrahydrocannabinol*, Δ⁸-trans-*tetrahydrocannabinol*, *and* Δ⁹-cis-*tetrahydrocannabinol*

up in the form of a pyran ring, acid catalysts such as *p*-toluenesulfonic acid, which can protonate a double bond, affect transformation with retention of stereochemistry at the ring junction. However, nonprotonic acid catalysts such as BBr_3 or BF_3 effect interconversions with inversion at the ring junction to the more thermodynamically stable *trans*-form.

A general method developed for the synthesis of cannabinoid acids, utilizing methylmagnesium carbonate, was applied to the synthesis of Δ⁹-tetrahydrocannabinol acid A (VII*a*), cannabidiolic acid (VIII), and cannabigerolic acid (IX*b*) (102).

Structure–Activity Relationships in Cannabinoids—As mentioned earlier in this review, a compound, Δ⁶ᵃ,¹⁰ᵃ-tetrahydrocannabinol (III*a*), possessing marijuanalike activity but not occurring as a constituent of Cannabis, was prepared in 1940 by Ghosh *et al.* (33) and by Adams and Baker (37), independently of each other. The biological activity of this compound led Adams and others (83, 85, 86, 103, 104) to prepare a large series of closely related tetrahydrocannabinols (III*b*). The general method of synthesis employed by Adams *et al.* (105–109) involved ethyl 4-methyl-2-oxocyclohexanecarboxylate (XIX) (Scheme I), with an appropriate 5-alkylresorcinol in the presence of phosphorus oxychloride. Further conversion of the resulting pyrone with excess methylmagnesium iodide yielded the desired 3-alkyl-6*a*,10*a*-tetrahydrocannabinol (III*b*) (105). Loewe (110) showed that two major properties of Cannabis—the ability to block the blink reflex in rabbits [Gayer test (111)] and the ability to cause ataxia in the dog—were in part combined in the same molecule and in part embodied in different structures. Pharmacological data on the early cannabinoids were generally limited to either the Gayer test or the dog ataxia test. Of the tetrahydrocannabinols prepared in the 1940's, the most active compound on the basis of the dog ataxia and Gayer tests proved to be the one with a dimethylheptyl side chain [III*b*, R = $CH(CH_3)CH(CH_3)C_5H_{11}$]. Adams *et al.* (112) subsequently also synthesized the optically active *d*- and *l*-forms of Δ⁶ᵃ,¹⁰ᵃ-tetrahydrocannabinol (III*a*); they found that the *d*-form of III*a* had about 40% of the activity of the racemic III*a* and that the *l*-form had four to five times the physiological potency of the *d*-form. These optically active isomers were prepared by treating olivetol with the *d*- and *l*-forms of ethyl 4-methyl-2-oxocyclohexanecarboxylate. The potency of the dimethylheptyl analog [III*b*, R = $CH(CH_3)$-$CH(CH_3)C_5H_{11}$] led Aaron and Ferguson (113) to prepare the eight possible isomers of this compound. Their method, the same as used by Adams and Baker (80) (Scheme I), involves the resolution of the appropriate resorcinol into four isomers and their subsequent treatment with the (+)- and (−)-forms of ethyl 4-methyl-2-oxocyclohexanecarboxylate (XIX). The biological activity of these isomers has not yet been reported. One isomer of III, in which R is hexyl (III*c*, parahexyl, synhexyl, pyrahexyl), was studied in man (114) and found to have behavioral effects similar to those of the natural cannabinoids (115).

Other compounds were synthesized by Adams *et al.* (105, 116); the position of the methyl group in the cyclohexene ring was altered (XLIII) or more than one methyl group was added. This research group (117) also prepared a few compounds in which the 6-position of III*a* was substituted by higher alkyl groups. This was accomplished by reacting the appropriate Grignard reagent with the pyrone (XX) formed in the first step of the general reaction scheme. A more drastic alteration of the basic cannabinol structure, which eliminated the cyclohexane ring of III*a*, was achieved by Adams *et al.* (118) in a synthesis of XLV (R′ = CH_3). Ethyl acetoacetate was condensed with olivetol in benzene, using phosphorus oxychloride as the catalyst. The resulting coumarin was treated with methylmagnesium iodide, yielding the bicyclic product XLV (R′ = CH_3) (118). The 3-butyl derivative of XLV (R′ = C_4H_9) also was prepared by this method. Most of these compounds prepared by Adams *et al.* (118) were active in the dog ataxia test.



synthetic analogs of cannabinol

The pharmacological tests using rabbits and dogs measure only two aspects of the pharmacology of these substances. So far there is no evidence that the powerful psychotomimetic effects of *C. sativa* and its synthetic analogs in man have any relationship to the results of these tests in animals. The following conclusions can be drawn on the basis of the activity of these series compounds (III, XLIII, XLV, and XLVI).

1. In the series of compounds (105) with unbranched side chains, activity increases with an increase in the length of the carbon chain, reaching a maximum with the *n*-hexyl homolog and falling off with the higher homologs.

2. In the secondary series of compounds (106) (III*b*, R = *sec*-alkyl), in all except one case, the secondary groups produce molecules more active than the isomeric normal groups, and the activity increases with an increase in chain length. The *sec*-octyl homolog is higher in potency than the natural tetrahydrocannabinol isolated at that time.

3. Additional branching and lengthening of the *sec*-alkyl chain (107) in III*b* increase the potency to the point where the dimethylheptyl homolog is about 70 times more potent than is natural tetrahydrocannabinol.

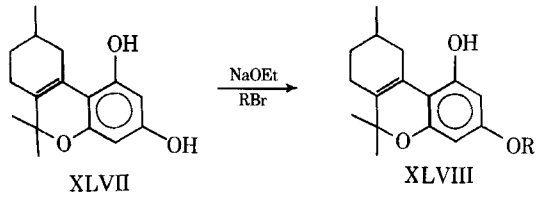
4. More potent compounds result from side-chain branching (R) close to the benzene ring rather than in more distant positions (108).

5. Further branching or extension beyond a seven-carbon chain appears to decrease activity, but, because of the longer duration of action of these higher homologs, it is impossible to determine where the peak of activity occurs in the alkyl series (108, 109).

6. Structural changes in the pyran and/or in the cyclohexene ring result in a decrease in activity.

Using essentially the method established by Adams (105–109), Avison *et al.* (119) and Taylor *et al.* (120) synthesized positional isomers (XLVI) with varying alkyl side chains on the aromatic ring. In some instances, such compounds showed marijuanalike and analgesic activity (119).

Bergel *et al.* (121) reported the synthesis (Scheme IX) of four derivatives (XLVIII), in which the *n*-amyl group in III is replaced by *n*-alkoxy groups. These were prepared by treating the dihydroxy pyran (XLVII) with the appropriate alkyl bromide. These workers also prepared a water-soluble derivative, the disodium tetrahydrocannabinyl phosphate, which showed no hashish activity in the Gayer test. Of the alkoxy derivatives (XLVIII), only the *n*-hexyloxy derivative (R = C_6H_{13}) appeared to show activity in doses between 10 and 20 mg./kg. by the Gayer test. The activity was only feeble, however. None of the other compounds showed any activity in doses up to 20 mg./kg.



XLVII XLVIII

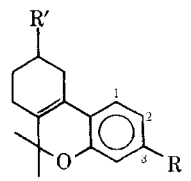
R = C_4H_9; C_5H_{11}; C_6H_{13}; C_7H_{15}

Scheme IX

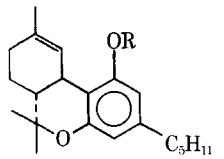
By a similar method (121), Alles *et al.* (122) synthesized two series of alkoxy and acyloxy derivatives (XLIII). They are distinguished from Bergel's compounds by the absence of the 1-hydroxy group and, in another series, the additional absence of the 9-methyl group (R′ = H). Neither of these two series of pyrans exhibited any significant degree of marijuana activity in dogs or rabbits.

Razdan (123) and Howes (124) prepared and tested water-soluble derivatives of Δ^9-tetrahydrocannabinol (XLIX*a* and *b*). The ether (XLIX*a*) was found to have a different profile than Δ^9-tetrahydrocannabinol, whereas the ester (XLIX*b*) showed similar properties to Δ^9-tetrahydrocannabinol (IV), although the compound was less active. The activity of the ester (XLIX*b*) was probably due to the *in vivo* hydrolysis to the phenolic cannabinoid (IV).

A number of nitrogen analogs of tetrahydrocannabinol were prepared and evaluated for their marijuanalike activity. Anker and Cook (125) synthesized L and its dihydro derivative in 1946, and they reported it to have no analgesic activity. No mention was made of other CNS effects of these compounds. In contrast to these earlier findings, Pars *et al.* (126) reported, in 1966, the synthesis of the isomeric nitrogen analog (LI) and its dihydro derivative; they found these compounds to be active on the CNS and to produce ataxia and motor deficits in mice, cats, dogs, and monkeys. Homologs of this azatetrahydrocannabinol, in which the side-chain R was varied, were also prepared by substituting 4-carbethoxy-*N*-methyl-3-piperidone for ethyl 4-methyl-2-oxocyclohexanecarboxylate in the general method for the synthesis of $\Delta^{6a,10a}$-tetrahydrocannabinol (III*a*) described in Scheme I. The activity of these nitrogen analogs encouraged these workers to prepare other aza-derivatives [LII (127), LIII (128), and LIV (128)], a sulfur analog [LXI (129)], and a "steroidal" analog



XLIII



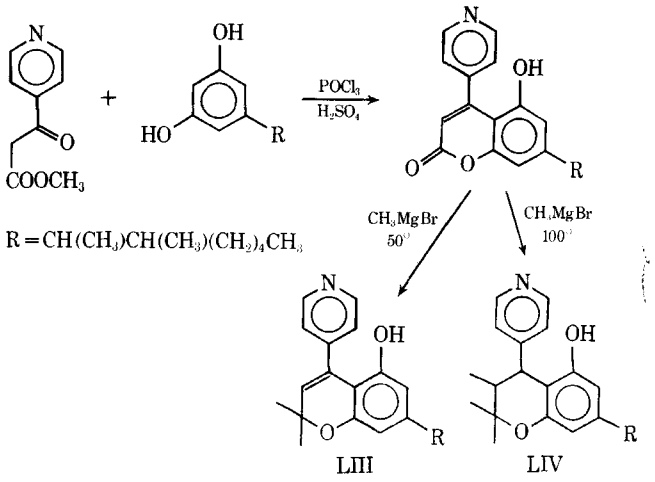
XLIX

R = CH_3; C_5H_{11}; OC_2H_5; $OCCH_3$; OCC_3H_7 (O)

R′ = H, CH_3

a) R = $-(CH_2)_2\overset{H}{\overset{+}{N}}(CH_2CH_3)_2Cl^-$

b) R = $C(CH_2)_3\overset{H}{\overset{+}{N}}(CH_2CH_3)_2Cl^-$ (O)



R = $CH(CH_3)CH(CH_3)(CH_2)_4CH_3$

LIII LIV

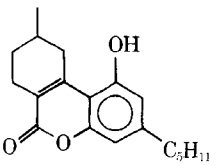
Scheme X—Grignard addition to 4-pyridylcoumarins (128)

[LV (130)]. These were all active on the CNS, except the "steroidal" analog (LV) (129, 131, 132).

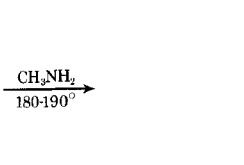
The effects of the aza-analog (LI) [R = $CH(CH_3)$-$CH(CH_3)C_5H_{11}$] were qualitatively similar to those of the sulfur analog (LXI) also containing a dimethylheptyl side chain (129). These investigators speculated that the introduction of a heterocyclic atom into the molecule enhances passage through the blood–brain barrier. Adams (16) previously reported that the tetrahydrocannabinol analogs (III) containing a dimethylheptyl side chain were considerably more active than those with the *n*-C_5H_{11} chain (110, 133). This may explain the activity of these heterocyclic analogs as compared with Δ^8-tetrahydrocannabinol (V) and Δ^9-tetrahydrocannabinol (IV).

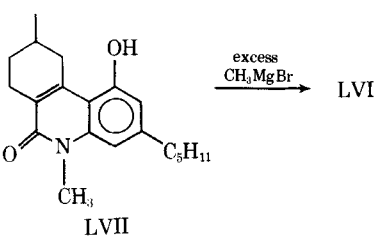
The synthesis of LIII and LIV involves a Grignard reaction with the 4-pyridylcoumarin prepared from methyl 3-oxo-3-(4-pyridyl)propionate and 5-(1,2-dimethylheptyl)resorcinol. The Grignard addition resulted in the usual 1,2-addition to give LIII at 50°, as well as the unexpected addition of a third mole of Grignard reagent to the double bond of the chromene to yield the 2,2,3-trisubstituted chroman (LIV) (128) (Scheme X).

A 5-azatetrahydrocannabinol analog was prepared in 1968 but was found to lack any CNS activity (134). The dibenzopyrone (XX), prepared by the method of



XX





LVII

Scheme XI

Adams and Baker (80) (Scheme I), was treated with methylamine to give the amide (LVII), which was converted to the 5-azatetrahydrocannabinol analog (LVI) with excess methylmagnesium bromide (Scheme XI).

The two isomeric 10-hydroxy-Δ^8-*trans*-tetrahydrocannabinol analogs were prepared and evaluated in the monkey (7). 10α-Hydroxy-Δ^8-*trans*-tetrahydrocannabinol (LIX) is almost as active as Δ^8-tetrahydrocannabinol (V), but the isomeric 10β-hydroxy-Δ^8-tetrahydrocannabinol is not active at 0.5 mg./kg.

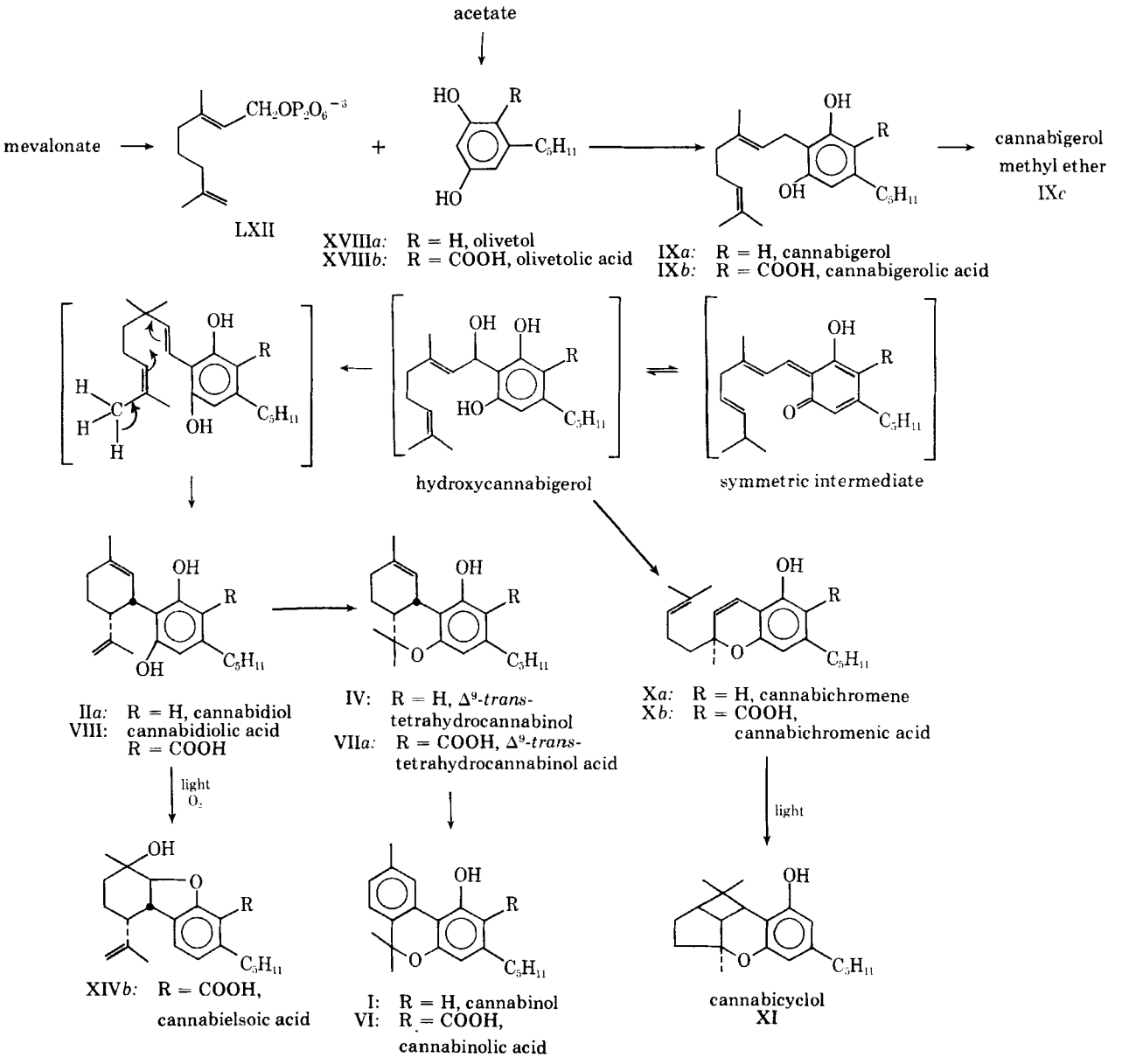
Three unnatural isomers of tetrahydrocannabinol were synthesized by Fahrenholtz *et al.* (84) in the course of their synthesis of *dl*-Δ^9-*trans*-tetrahydrocannabinol and *dl*-Δ^8-*trans*-tetrahydrocannabinol, referred to earlier. One of these isomers, $\Delta^{9(11)}$-*trans*-tetrahydrocannabinol (LX), was reported to be inactive (7). $\Delta^{8(9)}$-Isotetrahydrocannabinol (XLI), the synthesis of which has been discussed (Scheme VIII), was reported to be physiologically active by Razdan and Pars (131) at 10 mg./kg. in the mouse, and it was found to be inactive by Edery and Grunfeld (135) when tested in the monkey.

The structure–activity relationships in the cannabinoids can be summarized as follows:

1. In the $\Delta^{6a,10a}$-tetrahydrocannabinol series (III*b*), changes in the side chain of the aromatic ring can bring a considerable increase in activity, the most active compound being the dimethylheptyl compound. Synhexyl (III*b*, R = C_6H_{13}) shows strong activity orally (115), whereas $\Delta^{6a,10a}$-tetrahydrocannabinol (III*a*) is inactive up to 400 mcg./kg. (smoking) (136).

2. The major psychotomimetically active compound present in hashish is (−)-Δ^9-*trans*-tetrahydrocannabinol (67, 136–138). This compound is active on smoking and on ingestion (115, 136).

3. As with $\Delta^{6a,10a}$-tetrahydrocannabinol (III*a*), changes in the side chain of the natural cannabinoids cause a sharp increase in activity in the rhesus monkey. The most active compound is Δ^9-tetrahydrocannabinol, in which the side chain is dimethylheptyl (7).



Scheme XII—Postulated biogenesis of cannabinoids

4. Acetylation of the free hydroxyl in Δ^9-tetrahydrocannabinol (IV) or Δ^8-tetrahydrocannabinol (V) reduces the activity (7). The diethylaminobutyrate ester hydrochloride (XLIX*b*) is water soluble; it shows similar activity to Δ^9-tetrahydrocannabinol, although it is less active (123, 124).

5. Substitution on the aromatic ring of Δ^8-tetrahydrocannabinol (V) with a methyl group retains activity, while substitution with a carbomethoxy or hydroxyl group eliminates it (7).

6. α-Hydroxylation of Δ^8-tetrahydrocannabinol at the 10-position results in a compound almost as active as Δ^8-tetrahydrocannabinol, whereas the isomeric β-hydroxy compound is inactive in the monkey (7).

7. 11-OH-Δ^9-Tetrahydrocannabinol (LIV), a minor metabolite of Δ^9-tetrahydrocannabinol in man (139), is equally as active as Δ^9-tetrahydrocannabinol in animals.

8. Substitution of a hetero atom into the cyclohexene ring (LI, LXI) produces analogs that are qualitatively similar to the natural tetrahydrocannabinols when evaluated in animals (129).

9. Relationships of structure to activity of the many compounds synthesized to date will be clarified only when these compounds are studied in man.

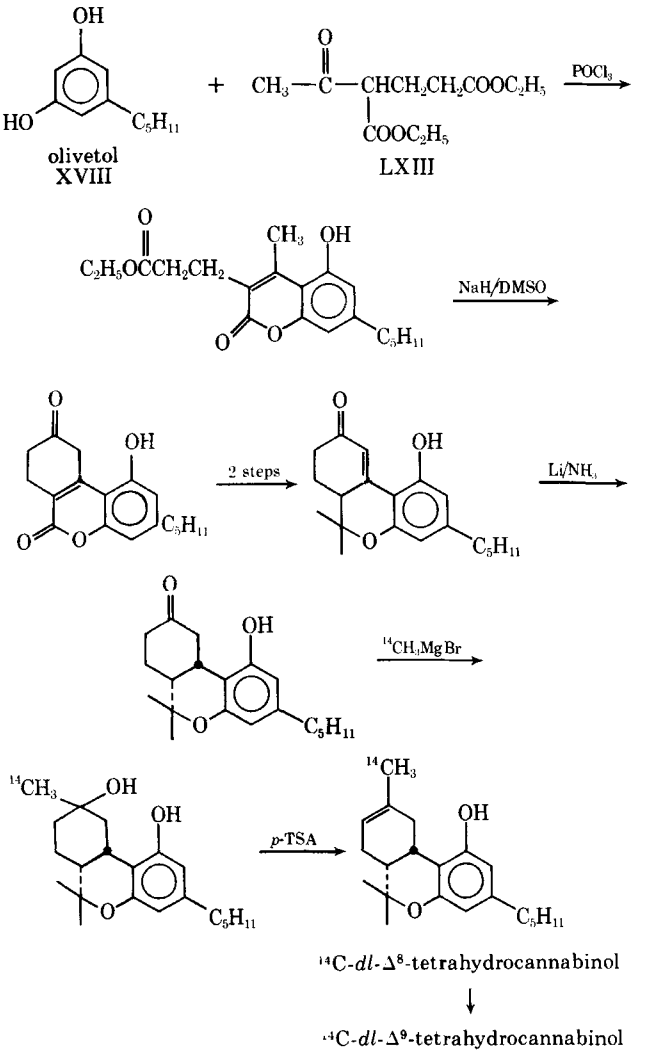
BIOGENESIS

Mechoulam (7) and Mechoulam and Gaoni (11) suggested a biogenetic scheme for the generation of cannabinoids. It was based upon a postulation, offered by Todd (17) and Sinonsen and Todd (140), in 1942, that the cannabinoids originated in the plant from a condensation of a terpene derivative with olivetol. Since definitive labeling experiments are lacking, the scheme proposed by Mechoulam (Scheme XII) seems to account for the various Cannabis constituents that have been found to date. It is suggested that geranyl pyrophosphate (LXII) (Scheme XII) condenses with olivetolic acid (XVIII*b*) [or olivetol (XVIII*a*)], resulting in the formation of cannabigerolic acid (IX*b*) [or cannabigerol (IX*a*)]. Since a direct cyclization of cannabigerol to cannabidiol (II*a*) is not possible (cannabidiol is in a higher state of oxidation than cannabigerol), an oxidized intermediate that can cyclize to either cannabidiol (II*a*) or cannabichromene (X*a*) was postulated. Cannabichromene (X*a*), cannabichromenic acid (X*b*), and cannabicyclol (XI) so far have been found optically inactive. Mechoulam indicated that either the cyclization leading to these compounds passes through a symmetric intermediate or that their formation involves a nonenzymatic process.

It is still debated whether the neutral cannabinoids are authentic natural products or whether these compounds are artifacts formed by decarboxylation of the corresponding cannabinoid (21). The formation of Δ^8-tetrahydrocannabinol can be readily rationalized by the isomerization of Δ^9-tetrahydrocannabinol by acids (Scheme VIII). The Δ^9-tetrahydrocannabinol isomer in the presence of air is slowly oxidized to cannabinol and compounds of higher molecular weight through oxidative phenolic coupling (7).

METABOLISM AND DISPOSITION

Synthesis of Labeled Cannabinoids—Metabolic studies on Cannabis constituents were hampered in the past by the lack of labeled compounds necessary to investigate the metabolic fate of these drugs. The recent progress in the development of synthetic methods applicable to the synthesis of labeled compounds has made such studies possible. Miras (20), in 1965, produced small amounts of tetrahydrocannabinol-^{14}C by growing Cannabis plants in $^{14}CO_2$. When the labeled Δ^9-tetrahydrocannabinol that was isolated was administered intraperitoneally to a rat, 68% of the radioactivity was eliminated in the feces and 12% *via* the urine within 5 days (20). More recently, Burstein and Mechoulam (92) prepared the Δ^8-tetrahydrocannabinol isomer specifically labeled at the C-10 position by the isomerization of Δ^9-tetrahydrocannabinol with tritiated *p*-toluenesulfonic acid. Facile deuteration and tritiation of both Δ^8-tetrahydrocannabinol and Δ^9-tetrahydrocannabinol by the use of labeled trifluoroacetic acid were also reported (95). Agurell *et al.* (93, 96) prepared Δ^9-tetrahydrocannabinol with high specific activities by treating Δ^9-tetrahydrocannabinol with tritiated water in the presence of phosphoric acid. These authors pointed out that, although Δ^9-tetrahydrocannabinol-^{3}H is stable in biological systems, gas chromatographic analysis of metabolic products can result in the exchange of the



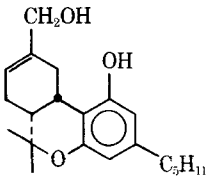
Scheme XIII—Total synthesis of ^{14}*C-dl-*Δ^8*-tetrahydrocannabinol and* ^{14}*C-dl-*Δ^9*-tetrahydrocannabinol (85) by the procedure of Fahrenholtz (84)*

label in the chromatograph, complicating the structural elucidation of the metabolites.

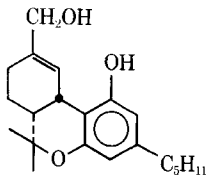
The synthesis of Δ^9- and Δ^8-tetrahydrocannabinol specifically labeled with ^{14}C at C-11 was accomplished by Nilsson *et al.* (87), essentially by the process developed by Fahrenholtz *et al.* (84). The synthetic sequence is shown in Scheme XIII. The label was introduced by the use of $^{14}CH_3Br$ in the final Grignard reaction.

Elimination and Distribution of Tetrahydrocannabinols—*Δ^9-Tetrahydrocannabinol*—The metabolism and distribution of Δ^9-tetrahydrocannabinol-^{14}C were studied in animals and man (93, 139, 141–144). Agurell *et al.* (93) showed that intravenously injected Δ^9-tetrahydrocannabinol-^3H was eliminated very slowly by the rat. About half of the administered drug remained in the body after 1 week, about 80% of the drug being excreted in the feces and the remainder being eliminated as metabolites in the urine. The elimination of Δ^9-tetrahydrocannabinol-^3H in the rabbit differs greatly from that in the rat. In the rabbit, the major elimination was *via* the kidneys, about 30% of the administered drug being excreted in the first 24 hr., in contrast to only a few percent excreted in the rat (141, 142). Nilsson *et al.* (142) identified one of the metabolites of Δ^9-tetrahydrocannabinol by an *in vitro* metabolism of Δ^9-tetrahydrocannabinol-^{14}C with rabbit liver as 11-hydroxy-Δ^9-tetrahydrocannabinol (LXIV). This metabolite was identified by the chemical conversion of LXIV to cannabinol (I) with *p*-toluenesulfonic acid and by mass and NMR spectrometry.

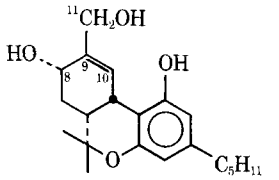
Working independently of the Nilsson group (142), Wall *et al.* (143) identified several *in vitro* metabolites from Δ^9-tetrahydrocannabinol-^3H from the 10,000-g supernatant prepared from male rat liver homogenate. These workers identified four *in vitro* oxidation products of Δ^9-tetrahydrocannabinol. After extraction and chromatography, Δ^9-tetrahydrocannabinol (IV), 11-hydroxy-Δ^9-tetrahydrocannabinol (LXIV), 8,11-dihydroxy-Δ^9-tetrahydrocannabinol (LXVI), and 11-acetoxy-Δ^9-



11-hydroxy-Δ^8-*trans*-tetrahydrocannabinol
LXV



11-hydroxy-Δ^9-*trans*-tetrahydrocannabinol
LXIV



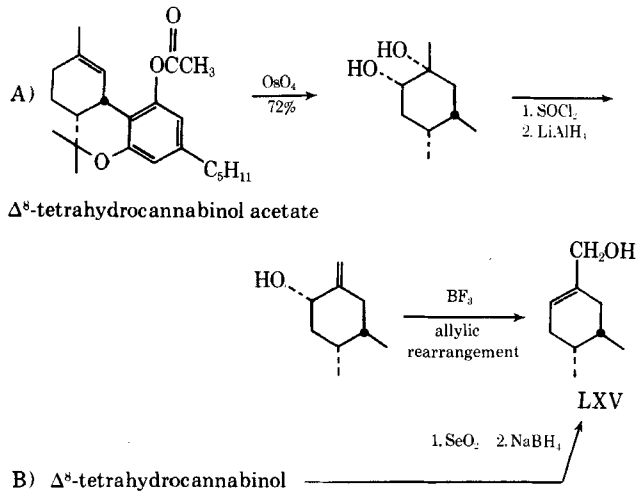
8,11-dihydroxy-Δ^9-*trans*-tetrahydrocannabinol
LXVI

identified metabolites of tetrahydrocannabinol

tetrahydrocannabinol, which is an artifact formed from LXIV during the extraction procedure, were identified. Preliminary behavioral and neuropharmacological examination of these metabolites indicated that 11-hydroxy-Δ^9-tetrahydrocannabinol (LXIV) was at least equipotent to Δ^9-tetrahydrocannabinol, whereas the dihydroxy metabolite (LXVI) was inactive. Subsequent to these earlier studies, this group found that the 11-hydroxy metabolites of Δ^8- and Δ^9-tetrahydrocannabinol are more active than the parent compounds (*viz.*, IV and V) when administered to mice by either the intravenous or intracerebral route; Christensen *et al.* (143) speculated that the 11-hydroxy metabolites may be the active forms of tetrahydrocannabinol in the mouse. A significant study, carried out at the National Institute of Mental Health (NIMH) (139), in which naive human volunteers were administered intravenously with 0.5 mg. of Δ^9-tetrahydrocannabinol-^{14}C, showed that its metabolites appear within 10 min. after administration and persist along with the precursor compound. These workers showed that 30% of the administered radioactivity was excreted in the urine and that less than 1% was unchanged Δ^9-tetrahydrocannabinol. This finding is significant for the development of assay procedures for the detection of Δ^9-tetrahydrocannabinol *via* urine samples. These results also indicated that less than 1% of the urinary radioactivity was unchanged Δ^9-tetrahydrocannabinol and that 11-hydroxytetrahydrocannabinol-^{14}C did not appear to account for more than a small percentage of the metabolites. Eighty percent of the metabolites still remained uncharacterized. About 50% of the radioactivity administered as Δ^9-tetrahydrocannabinol-^{14}C was recovered in feces, of which about 20% was the 11-hydroxytetrahydrocannabinol (LXIV) metabolite. Thus, after intravenous administration of Δ^9-tetrahydrocannabinol-^{14}C, the concentration in plasma rapidly declines, with a half-life of about 30 min.; two-thirds of the total radioactivity excreted in the urine is present during the 1st day. During this initial phase, the plasma contains higher concentrations of metabolites of Δ^9-tetrahydrocannabinol than of the parent drug.

In vitro studies (145) indicated that Δ^9-tetrahydrocannabinol was found to be 80–95% associated with lipoproteins. Lemberger *et al.* (139), in the NIMH studies in humans, pointed out that since Δ^9-tetrahydrocannabinol is a nonpolar compound, it may accumulate in fat or other tissue, such as lung, which has an affinity for drugs. Since the usual route of administration of marijuana is *via* inhalation, these findings are significant. Studies in animals confirmed that higher levels of Δ^9-tetrahydrocannabinol were present in the lungs than in other tissues (146, 147). The finding that Δ^9-tetrahydrocannabinol and its metabolites persists in humans for long periods indicates that the drug and its metabolites may accumulate in tissue when administered repeatedly.

Sofia and Barry (148) contend that the central depressant effect of Δ^9-tetrahydrocannabinol is attributable primarily to the parent compound rather than to the 11-hydroxy metabolites. Kubena and Barry (149) found that prolongation of barbital sleeping time in mice by Δ^9-tetrahydrocannabinol was enhanced when its hydroxylation to the 11-hydroxy metabolite was blocked by the microsomal enzyme inhibitor, SKF525A (β-diethylaminoethyldiphenyl propylacetate hydrochloride).



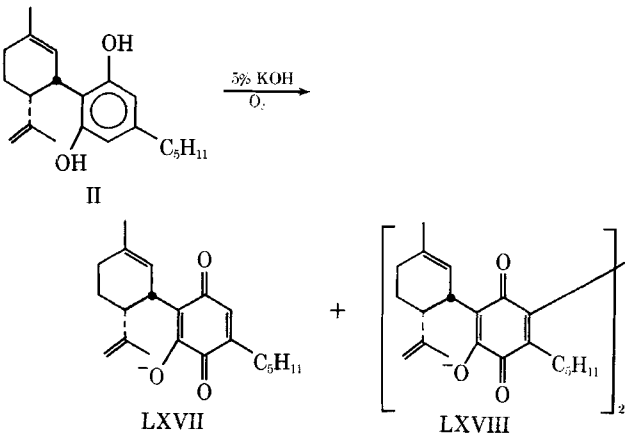
Scheme XIV—Synthesis of 11-hydroxy-Δ⁸-trans-tetrahydrocannabinol (65)

Δ⁸-Tetrahydrocannabinol—The metabolism of Δ⁸-tetrahydrocannabinol was studied in rabbits (150) and in rats (151). Δ⁸-Tetrahydrocannabinol-^{3}H, administered intravenously to rabbits, indicated that several water-soluble substances containing tritium were present. Treatment of the mixture with 0.1 *N* perchloric acid gave one major compound (150), which could also be obtained by incubation of Δ⁸-tetrahydrocannabinol-^{3}H with a homogenate of rabbit liver (152). This compound was identified as 11-hydroxy-Δ⁸-tetrahydrocannabinol (LXV) by mass spectrometry and chemical transformation to cannabinol (I) and was confirmed by synthesis (152, 153) (Scheme XIV). Synthetic 11-hydroxy-Δ⁸-tetrahydrocannabinol, when administered to monkeys (154), was active at approximately the same dose levels as Δ⁸-tetrahydrocannabinol. A synthesis of LXV in two steps, by oxidation of Δ⁸-tetrahydrocannabinol with selenium dioxide and reduction of the product with sodium borohydride (Scheme XIVB), was also reported (151). A modification of this procedure (153) involves the selenium dioxide oxidation of Δ⁸-tetrahydrocannabinol acetate, yielding 11-hydroxy-Δ⁸-tetrahydrocannabinol diacetate. This method was similarly applied to the synthesis of LXV in 1% yield from Δ⁸-tetrahydrocannabinol. Foltz *et al.* (151), in studies with Δ⁸-tetrahydrocannabinol-^{14}C, found approximately 13% of the radioactivity in the liver 30 min. after injection into rats. The major metabolite produced *in vivo* was LXV, which was also formed by hepatic microsomes *in vitro*. These studies confirmed that the behavioral effects of this metabolite (LXV) in rats were similar to those imparted by Δ⁸- and Δ⁹-tetrahydrocannabinol.

QUALITATIVE AND QUANTITATIVE ANALYSIS OF CANNABIS CONSTITUENTS

The analysis of cannabinoids received considerable attention by numerous groups. Beam (155), in 1911, reported that hashish extracts give a deep-purple color with a 5% ethanolic potassium hydroxide solution. Because of the importance of Cannabis identification for legal purposes, this test was subjected to numerous studies regarding its specificity, reliability, and sensitivity (10). Out of 120 plant species examined, only two, *Rosmarinus officinalis* and *Salvia officinalis*, give a weakly positive reaction; out of 48 pure substances of vegetable origin, consisting of such diverse chemical types as monoterpenes, sesquiterpenes, and aromatic compounds, only juglone, which is a quinone, developed a color close to that of the Beam test.

Curiously, the identification of hashish is based on the presence of the physiologically inactive constituents, cannabidiol (II) and cannabigerol (IX*a*), and their corresponding acids. The active constituent in Cannabis, Δ⁹-tetrahydrocannabinol, gives a negative Beam test. Mechoulam *et al.* (156) clarified the chemical basis for the Beam test (Scheme XV). Under the reaction condi-



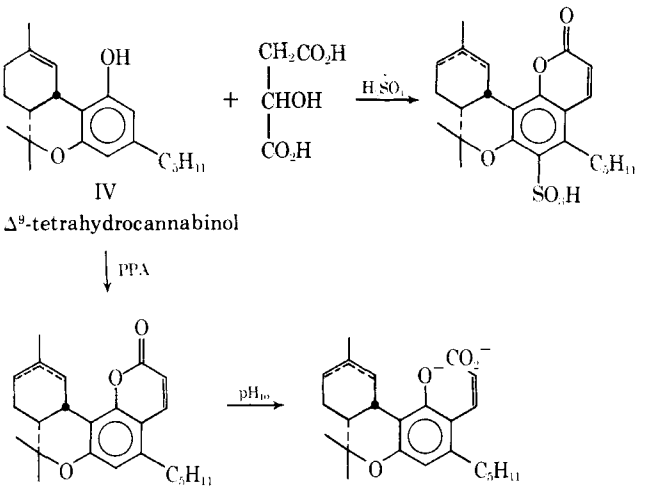
Scheme XV—Chemical basis for Beam test

tions of this test, cannabidiol (II) is oxidized to the monomeric quinone (LXVII) and the dimeric quinone (LXVIII). The violet color is due to the anions of these hydroxyquinones. Oxidation of Δ⁸-tetrahydrocannabinol with *m*-chloroperbenzoic acid similarly produces the quinone (LXVII). TLC is now used more widely for qualitative analysis. Korte and Sieper (46) employed silica gel impregnated with dimethylformamide and cyclohexane as the solvent. Numerous other solvent systems have been used, the R_f values being affected by the solvent (25, 43–45, 157). Mechoulam (7) and Gaoni and Mechoulam (25) reported a system in which chromatoplates of silica gel were eluted with petroleum ether (b.p. 40–60°)–ether in a ratio of 8:2, and the plates were sprayed with potassium permanganate solution. The R_f values of the major neutral cannabinoids are tabulated in Table III (7, 25).

VPC was extensively employed for the analysis of the cannabinoids (158, 159). The columns in use today are SE-30 (159–161), XE 60 (45), Carbowax 20M (162), OV-17 (163), and 2% OV-17 on Chromosorb Q at 235° (25). The retention times of the major natural cannabinoids are also tabulated in Table III. Mechoulam (7) pointed out that all the cannabinoid acids undergo decarboxylation at the high temperatures employed for VPC. Thus, a VPC analysis will give all the tetrahydrocannabinol available to a smoker in a certain sample. A methanol extraction is necessary to remove the tetrahydrocannabinol acids. Hydrocarbon extraction of the plant does not remove tetrahydrocannabinol acids. Preliminary treatment of the fresh plant by heating at 110° for 15 min. was effective for the decarboxylation of the tetrahydrocannabinol acids.

This method was utilized by Kimura and Okamota (209) for determining the tetrahydrocannabinol content in Cannabis. When exact determination of the cannabinoid content is required, decarboxylation can be prevented by esterification.

There is, at present, no generally accepted method useful for the detection and quantitation of the constituents of marijuana in mammalian materials. Bullock *et al.* (164) developed an analytical method based on the fluorescence produced with certain Cannabis constituents by condensation with malic acid (Scheme XVI



Scheme XVI—Chemical basis for fluorimetric method of analysis of cannabinoids (164)

and Table IV). This method was found useful for quantitating Δ^9-tetrahydrocannabinol in plasma samples containing at least 0.6 mcg. tetrahydrocannabinol in 2 ml.; given a 10-ml. sample, 0.05 mcg./ml. has been quantitated. The doses used in evaluating the method in the dog are, however, at least 20-fold greater than might be encountered with marijuana users on a milligrams per kilogram basis. In a study using blood samples obtained 20 min. after the subjects smoked a 400-mg. marijuana cigarette (0.9% tetrahydrocannabinol), no Δ^9-tetrahydrocannabinol was detected with two subjects. Blood levels of Δ^9-tetrahydrocannabinol must have been significantly below 0.05 mcg./ml. (165). This method has not been developed sufficiently to establish the amount of tetrahydrocannabinol metabolites present in plasma. By using a TLC separation prior to derivatization with malic acid, 1 mcg. of the 11-hydroxy metabolite (LXIV) was quantitated in dog and monkey feces (165). As pointed out by Lemberger *et al.* (139), little, if any, unchanged Δ^9-tetrahydrocannabinol may be excreted in the urine or feces.

Numerous detection methods for marijuana constituents (166–168), including smoke condensate (169, 170), were recently reported.

PHARMACOLOGY OF CANNABINOIDS

Animal Pharmacology—The early pharmacologic studies were carried out with extracts of Cannabis, such as "red oil," containing varying quantities of cannabinoids (7, 10, 11). Thus, much of the earlier data needed to be repeated when pure and standardized marijuana preparations became available.

Table IV—Excitation and Emission Maxima of Fluorescent Species Derived from Certain Cannabinoids with Malic Acid (164)

Compound	Excitation, nm.	Emission, nm.
Δ^9-Tetrahydrocannabinol (IV)	290	360
	380	470
Δ^8-Tetrahydrocannabinol (V)	290	360
	380	470
Cannabinol (I)	290	360
	370	460
Cannabidiol (II)	290	360
	370	460

Gershon (171) recently summarized the pharmacology of the cannabinoids in laboratory animals. Other significant reviews relating to the pharmacology of marijuana in animals appeared over the last 5 years (20, 21, 58). Recent investigations with pure Δ^9-tetrahydrocannabinol and with Δ^8-tetrahydrocannabinol in a variety of animal species corroborated, in general, the earlier findings of the psychopharmacological activity of marijuana extract (67, 154, 172). Scheckel *et al.* (138) studied the effects of *dl*-Δ^9-tetrahydrocannabinol (IV) and *dl*-Δ^8-tetrahydrocannabinol (V) in monkeys (intraperitoneally). They found that both isomers profoundly affect the behavior of monkeys, causing stimulation, depression, apparent hallucinations, and loss of ability or motivation to perform complex tasks. Studies with the synthetic levorotatory (−)-Δ^9-tetrahydrocannabinol (IV) and (−)-Δ^8-tetrahydrocannabinol (V) indicated that these isomers have the same type of activity as their racemates but are more potent. There was also a tendency for both drugs to increase tolerance to shock, but no general sedative or analgesic effects were observed. Mechoulam *et al.* (67) observed that, except for Δ^9-tetrahydrocannabinol, no other major active compounds were present in analyzed samples of hashish when administered intravenously to adult rhesus monkeys. The presence of only very minor quantities of Δ^8-tetrahydrocannabinol in these samples seems not to contribute appreciably to the biological activity of Cannabis. Such studies have not been carried out in humans, however.

Bicher and Mechoulam (173), working with these same two compounds, observed that 8 mg./kg. i.v. of either isomer in rabbits caused restlessness and increased motor activity and awareness. Also observed in most animals were a significantly increased cortical activity, a significantly lowered cortical arousal response threshold, and a prolonged length of ECoG arousal. These findings are contradistinct to morphine sulfate, which produced a decrease in frequency of ECoG waves and elevated the threshold of arousal response. The analgesic properties of both Δ^9- and Δ^8-tetrahydrocannabinol on mice and rabbits were found to be similar, 20 mg./kg. of the compounds being equivalent to 10 mg./kg. of morphine sulfate. LD_{50}'s of both compounds were greater than 1000 mg./kg. In the anesthetized dog, both isomers were shown by Razdan *et al.* (174) to potentiate epinephrine and norepinephrine in all parameters.

Marijuana extract, which consists of a mixture of both Δ^8- and Δ^9-tetrahydrocannabinol and other compounds, was reported (175) to prolong significantly the hexobarbital sleeping time and to increase significantly the amphetamine-induced activity in the mouse. Marijuana extract was found to be completely absent of hypnotic action in mice, rabbits, guinea pigs, cats, and dogs (176). There is some evidence that natural tetrahydrocannabinol possesses anticonvulsant activity (177).

Gill *et al.* (58) claimed that there are at least six pharmacologically effective components of Cannabis. Cannabis resin, given to mice, lowers body temperatures by up to 8°, produces analgesia and catalepsy, and prolongs pentobarbital sleeping time. Doses required for these effects ranged from 25 to 200 mg./kg., depending on the route of injection (58). In preliminary toxicity tests, the LD_{50} for a single dose lay between 1000 and 5000 mg./kg. when injected intraperitoneally at various dose levels. For doses repeated daily, it lay between 500 and 1000 mg./kg.

Other studies with marijuana extract indicated that, in the mouse, the duration of effects on body temperature and spontaneous activity correlates generally well with changes in brain amines (178). Striking aggressive behavior was shown to develop in starved rats treated with marijuana extract (179).

The parenteral administration of (−)-Δ^9-*trans*-tetrahydrocannabinol to pigeons demonstrated the behavioral tolerance to this drug (180). This procedure was used previously to demonstrate the behavioral tolerance to narcotics. These experiments showed not only that a marked tolerance develops to the behavioral effects of (−)-Δ^9-*trans*-tetrahydrocannabinol but also that the rate, pattern, and degree of tolerance development resemble, in some respect, those seen with narcotics. Previous failures to demonstate development of tolerance to the effects of marijuana in man and animals (137, 181, 182) may have been related to the route of administration, the purity of the drug, or the species examined.

A few studies were made on the teratogenic and mutagenic effects of Cannabis, only one of which was carried out on human subjects. In the studies carried out with marijuana extracts on cultured rat leucocytes, with rat embryonic cells in tissue culture, and with rat embryos whose mothers were treated with Cannabis resin, no chromosomal abnormalities were indicated and only slight mitotic inhibition was found (183). With pregnant rats injected intraperitoneally, incidences of fetal malformations, resorption of fetuses, and retarded development in the treated animals were significantly higher than in control groups (184). In contrast to these findings with marijuana extract, Borgen and Davis (185) found no teratogenic effects in rats injected with Δ^9-tetrahydrocannabinol in dosages up to 200 mg./kg. given throughout gestation.

The other naturally occurring cannabinoids were subjected to various pharmacological investigations. Cannabinol (I) was found devoid of any specific activity (30, 176, 186). Cannabidiol (II) demonstrated some antibiotic activity *in vitro* (11), but it was found devoid of hypnotic (176) as well as psychotomimetic activity (30), although it was shown to potentiate barbiturates (187). Recently, II was reported to cause severe motor deficit and ataxia in rabbits (10 mg./kg. i.v.) (181). Cannabigerol (IX*a*) also demonstrated antibiotic activity *in vitro* (11) but was devoid of psychotomimetic activity (154, 172). Cannabidiolic acid (VIII) and cannabichromene (X*a*) (67, 136, 154, 172) both were found ineffective as psychotomimetics; however, VIII demonstrated sedative and potent *in vitro* antibiotic activity (188), while X*a* produced ataxia and sedation in the dog (11) and loss of neuromuscular coordination in mice (15–30 mg./kg. s.c.) (174). Another cannabinoid possessing antibiotic activity is cannabigerolic acid (IX*b*). This compound's psychotomimetic activity has not been reported.

Natural cannabinoids whose biological activity has not been reported or who are inactive are cannabitriol ester of cannabidiolic acid (XIII), cannabinolic acid (VI), cannabigerol monomethyl ether (IX*c*), cannabidivarin (XII), cannabichromenic acid (X*a*), and cannabicyclol (XI).

Of the synthetic cannabinoids, parahexyl (III*c*) demonstrated the same order of activity as marijuana extract or chlorpromazine in suppressing the isolation-induced aggressive behavior in mice, the effective doses of all three being in the range of 3–5 mg./kg. i.p. (189). Parahexyl (III*c*) was shown to: (*a*) prolong hexobarbital sleeping time in mice (175), (*b*) have anticonvulsant activity in mice (177), and (*c*) lack hypnotic activity in five animal species (176). Loewe (176) demonstrated that a mixture of hexahydrocannabinols is active in the dog ataxia test.

$\Delta^{6a,10a}$-Tetrahydrocannabinol (III*a*) has actions in common with parahexyl and natural tetrahydrocannabinol; that is, it prolongs hexobarbital sleeping time and increases amphetamine-induced activity in the mouse (intraperitoneally) (175).

The pharmacological effects of the two most potent nonnatural cannabinoids synthesized to date, the dimethylheptylpyran [III*b*, $R=CH(CH_3)CH(CH_3)C_5H_{11}$] and the methyloctylpyran [III*b*, $R = CH(CH_3)C_7H_{15}$], were studied on cats and mice (190). Dagirmanjian and Boyd (190) reported that, in anesthetized cats, intravenous dimethylheptylpyran lowered blood pressure, depressed respiration, relaxed the gut, and depressed two polysynaptic reflexes, the knee jerk and the ipsilateral flexion reflex. The methyloctylpyran derivative produced similar effects to those of the dimethylheptylpyran derivative, but it was shorter acting and showed stimulatory action on the flexion reflex.

In mice, the barbiturate sleeping time was prolonged and amphetamine-induced activity was increased significantly by both drugs. The reported (190) 7-day LD_{50}'s in mice were 27 mg./kg. (17.5–41.6, 95% confidence level) for dimethylheptylpyran and 5.0 mg./kg. (3.5–7.0, 95% confidence level) for methyloctylpyran. The potencies in dogs and cats were not in the same order as the toxicities in mice. Boyd and Meritt (191) also reported that dimethylheptylpyran, in a dose of 0.2 mg./kg. i.v., increased the threshold for EEG arousal in the cat with acute preparations and increased the threshold for behavioral as well as EEG arousal with chronic preparations. A dose of 2.0 mg./kg. i.v. of thiopental was necessary to

produce similar results. Arousal was induced by electrical stimulation of the reticular formation.

Human Pharmacology—The knowledge of the pharmacologic effects of marijuana in humans is based chiefly on five important studies. The most comprehensive of these, the "La Guardia Report," was carried out by the New York Academy of Science in 1939 (182). However, it was not until 1967, when pure synthetic Δ^9-tetrahydrocannabinol became available, that the studies of Isbell *et al.* (136) in man were possible. Weil and his associates (137, 192, 193) studied "naive" and marijuana users and their responses to smoking marijuana. Hollister *et al.* (115) studied the clinical effects of Δ^9-tetrahydrocannabinol and synhexyl (IIIc) in man and found the effects quite similar to those observed with smoking marijuana. With high oral doses of Δ^9-tetrahydrocannabinol, these authors found disintegration of sequential thought which was related to impaired immediate memory (194).

Hollister *et al.* (115) also reported the biochemical effects in man from oral doses of Δ^9-tetrahydrocannabinol. These workers found:

1. The free fatty acids in the plasma remained unchanged (LSD elevated free fatty acids in plasma).

2. Glucose concentrations in the blood were unchanged.

3. Creatinine and phosphorus clearance were temporarily decreased.

Other studies with marijuana or pure synthetic derivatives were reported by Williams *et al.* (195), Clark and Nakashima (196), Pillard (197), Lemberger *et al.* (139), and the U. S. Army Chemical Research and Development Laboratories (211).

The significant findings of Isbell *et al.* (136) in humans, with pure (−)-Δ^9-*trans*-tetrahydrocannabinol rather than the crude extract, showed:

1. Regardless of the route of administration, (−)-Δ^9-*trans*-tetrahydrocannabinol (IV) caused no significant changes in pupillary size, respiratory rate, blood pressure, or threshold for elicitation of the knee jerk reflex.

2. Pulse rates were consistently elevated, and infection of the conjunctivae developed after large doses; 120 mcg./kg. orally and 50 mcg./kg. by smoking were recognized by patients as being similar to marijuana. [Other studies (115) showed that a range of 341–946 mcg./kg. orally produced similar marijuanalike effects (Table V)].

Table V—Dose in Relation to Physiological Effects in Man

Compound	Dose, mg. Oral	Dose, mg. Smoked[b]	Physiological Effects	Reference
Δ^9-Tetrahydrocannabinol[a]	9[c] 36[c]	2[c] 7.5[c]	Oral dose showed slow onset of action and longer duration. Smoking produced more rapid onset of action (seconds) but effects were briefer. Lower dose produced increased pulse rate, changes in mood, usually euphoric. Highest dose produced distortion in visual and auditory perception, depersonalization, and hallucinations resembling LSD.	136
Δ^9-Tetrahydrocannabinol[a]		2.25 9	Increased heart rate and reddening of the whites of the eyes. No changes in blood sugar. Marijuana-naive persons demonstrated impaired performance on simple intellectual and psychomotor tests which were dose related. Physiological and psychological effects reached maximum intensity within 0.5 hr. of inhalation and were completely dissipated within 3 hr.	137
Δ^9-Tetrahydrocannabinol[a]	20 40 60	6	Increased pulse rate; blood pressure fell slightly; at higher doses, orthostatic hypotension was observed. Conjunctival reddening was also constantly observed. Euphoria, sleepiness, and deep sleep at higher doses. Many psychotomimetic symptoms similar to LSD, mescaline, or psilocybin. Total food intake as well as hunger and appetite was increased.	115
Δ^9-Tetrahydrocannabinol[a]	50[c]		Impaired performance in complex reaction time, digit code memory, time estimation, hand steadiness, and reading comprehension. Suggested impairment of rapid decision making and short-term memory.	196
Δ^9-Tetrahydrocannabinol[a]		5	Impaired performance on pursuit meter and on performance tests.	202
$\Delta^{6a,10a}$-Tetrahydrocannabinol (III*a*)		7.5[c]	Activity similar to effects produced with Δ^9-tetrahydrocannabinol but considerably less potent.	115
		15[c]	No effects from smoking (400 mcg./kg.).	136
$\Delta^{6a,10a}$-Tetrahydrocannabinol (synhexyl, IIIc)	50–200	7.5	Activity like those with Δ^9-tetrahydrocannabinol but considerably less potent. Showed slower onset of symptoms, these being delayed by about 60 min. more than with Δ^9-tetrahydrocannabinol. The duration of action of synhexyl, however, was prolonged as compared to Δ^9-tetrahydrocannabinol.	115
$\Delta^{6a,10a}$-Tetrahydrocannabinol (III*b*, R = dimethylheptyl)	0.5–1.0 1.5–3.0 3.5–4.0		Fatigue, thirst, headache. Postural hypotension with temporary blurring or actual loss of vision on standing. Marked psychomotor retardation. Subjects stayed in bed and were unwilling or incapable of assuming an erect position. Sluggishness, inability to concentrate, and dimness and blurring of vision persisted for as much as 48 hr. Pulse rate increased when postural hypotension occurred but showed little or no change at higher dose levels.	210

[a] Δ^9-Tetrahydrocannabinol (IV) was extracted from hashish or marijuana and assayed for its tetrahydrocannabinol content or prepared synthetically. [b] In the compilation of these comparative doses, it was assumed that 50% of the active constituents introduced into the cigarette or occurring naturally in marijuana or hashish were destroyed by smoking. In some cases, dose was recalculated to indicate dose available to user. [c] All doses reported in micrograms per kilogram were converted to dose/75-kg. man if weight of man was not indicated.

3. Marijuana effects of (−)-Δ^9-*trans*-tetrahydrocannabinol (IV) are dose related.

4. The drug is about three times more potent when smoked than when ingested.

5. $\Delta^{6a,10a}$-Tetrahydrocannabinol (III*a*) produced no effects in doses up to 400 mcg./kg. by smoking [Hollister *et al.* (115) reported marijuanalike effects at half this dose], and synthetic cannabidiol dimethyl ether and cannabichromene produced no effects in doses up to 2.5 mg./kg. orally.

6. The effects of smoked Δ^9-*trans*-tetrahydrocannabinol (75–225 mcg./kg.) were compared with those of LSD given intramuscularly in doses of 0.5–1.5 mcg./kg. Subjective effects between the two drugs were not readily distinguished, but objective differences were marked.

Weil's (137, 192, 193) pioneering studies investigated the effects of marijuana in a formal double-blind experiment, and they are claimed to be the first attempts to collect basic clinical and psychological information on the drug by observing its effects on marijuana-naive human subjects in a laboratory setting. From these studies (137, 192, 193), these investigators drew the following conclusions:

1. Persons naive to marijuana do not have strong subjective experiences, even after high doses of the drug.

2. Marijuana-naive persons demonstrate impaired performance on simple intellectual and psychomotor tests after smoking the drug. Regular users who get "high" show a much less marked degree of impairment on the same tests; in some cases, they even improve.

3. Smoking marijuana increases the heart rate moderately and dilates the conjunctival blood vessels but causes no change in the respiratory rate, the pupil size, or the blood sugar levels.

4. Physiological and psychological effects reach maximum intensity within 0.5 hr. of inhalation, are diminished after 1 hr., and are completely dissipated after 3 hr.

5. Moderate marijuana smokers differ little in personality from those who do not smoke the drug, but heavy smokers of marijuana are rather less conventional.

6. Marijuana smokers tend to drink little alcohol.

A number of studies reported the effects of smoking on the components of marijuana. A report in 1964 indicated that smoking has no effect on the active constituent of marijuana (presumably Δ^9- and Δ^8-tetrahydrocannabinol), although 40% of the resin constituents disappeared during smoking (198).

Da Silva (199) showed that the tetrahydrocannabinol constituents were still found in the smoke condensate from combustion of Cannabis, although cannabidiol, which was present in the crude drug, was absent from the smoke condensate. Further support of the thermal stability of the naturally occurring tetrahydrocannabinol was given by Claussen and Korte (200), but they claimed that about 98%, instead of 40%, of the cannabinoids present in marijuana was destroyed by the smoking process and that tetrahydrocannabinol carboxylic acid readily decarboxylated to form the active tetrahydrocannabinol. Mechoulam (7) supported this latter finding in stating that the cannabinoid acids are converted into the respective neutral compounds, rapidly when heated and slowly when stored. This is given as one of the reasons for the higher activity of marijuana following smoking as compared with ingestion (49, 50). Incomplete absorption of drug following ingestion may also be a reason for the higher activity by the inhalation route. The burning process destroys part of the natural tetrahydrocannabinol already present in the plant. These findings were also confirmed by Kimura and Okamota (209).

Lemberger *et al.* (139) pointed out that since Δ^9-tetrahydrocannabinol is found in the lungs *via* the inhalation route, a critical degree of tissue saturation must be attained before effective threshold levels of Δ^9-tetrahydrocannabinol can be achieved. This could explain, in part, the phenomenon of "reverse tolerance" seen in chronic users of marijuana.

Another recent study described a comparison of the effects of marijuana and alcohol on simulated driving performance (201). The authors concluded that subjects experiencing a "social marijuana high" demonstrated no significant differences in total errors as compared with control conditions, whereas the same subjects intoxicated with alcohol accumulated significantly more accelerator, brake, signal, speedometer, and total errors as compared with control conditions. Although these results indicate that smoking marijuana does not impair driving performance, it is difficult to extrapolate conclusions from simulated driving conditions to actual driving conditions.

A more recent study, using subjects that smoked either calibrated marijuana cigarettes (10 mg. Δ^9-tetrahydrocannabinol per cigarette) or placebo cigarettes, produced significant decrements in human motor and mental performance (202).

A study of 12 healthy marijuana smokers indicated a possible hepatotoxicity of Cannabis, because eight of the 12 subjects showed evidence of mild liver dysfunction (203).

Of the few studies on the effect of Cannabis on chromosomes, only one was carried out on human subjects. In this study, leucocyte cultures from four healthy adult subjects were treated with (−)-*trans*-Δ^8-tetrahydrocannabinol during the last 24 hr. of culturing. No more chromosomal aberrations were found in these specimens than those observed in cultures from normal, healthy persons (204). Unfortunately, tests with the Δ^9-isomer present in larger quantities have not been reported.

A few of the many published case histories of persons experiencing unusual psychological effects induced by smoking marijuana are worth noting. One report presents cases of six persons who experienced hallucinations of color design or marked changes in perception with their eyes open (205). Another report described adverse reactions experienced by 11 individuals in a student population. Their difficulties included panic, fear, gross confusion, depersonalization, disorientation, depression, and paranoia following the use of the drug (206). One author described clinical syndromes of acute toxic psychosis associated with Cannabis derivatives and environmental stress observed in 12 soldiers seen in Vietnam (189). These cases of perplexing psychotic

reactions usually cleared in 1–4 days, but a few lasted a week or longer. In all instances, this was the patient's first admitted exposure to marijuana; in each case, marked physical symptoms appeared soon after the subjects began to smoke (207). In another study, four individuals reported the recurrence, in the drug-free state, of visual or somatic sensations previously experienced during the marijuana reaction (208). The possibility of adulterants incorporated into the marijuana cannot be ruled out in any of these studies where the purity and source of the drug was not definitely established.

Quantitation of Dose in Relation to Clinical Phenomena—The availability of synthetic cannabinoids over the last 3 years and the accurate determination of the active constituents in marijuana have now made it possible to provide some indication of the dose–response relationship with oral and smoked marijuana. A comparison of these results with the synthetic cannabinoids such as $\Delta^{6a,10a}$-tetrahydrocannabinol (III*a*) and the side-chain homologs where the *n*-amyl group was substituted with an *n*-hexyl group (III*c*, synhexyl) or a dimethylheptyl group [III*b*, R = $CH(CH_3)CH(CH_3)C_5H_{11}$], some of which (*i.e.*, synhexyl) had previously been evaluated in man, was now possible.

When marijuana is smoked, the amount of the active component Δ^9-tetrahydrocannabinol delivered is still uncertain. For this review, it was assumed that an average cigarette consists of 500 mg. of marijuana, containing an average of 1% active ingredients. The smoking process would destroy 50% of the active constituents. (This is still a matter of controversy.) The dose delivered would thus be 2.5 mg. of Δ^9-tetrahydrocannabinol per cigarette. Similarly, doses reported in micrograms per kilogram have been converted to mg./75-kg. man. The results of the studies carried out at the U. S. Public Health Service in Lexington, Ky. (136), at the Veterans Administration Hospital at Palo Alto, Calif. (115), at Boston University (137), at the University of Utah (196), and at the U. S. Army Chemical Research and Development Laboratories, Edgewood, Md. (210), are summarized and tabulated in Table V. The following observations can be made.

1. Synthetic tetrahydrocannabinol and marijuana extracts are orally active, but doses equivalent in effect to those from smoking are about three times greater.

2. When smoked, tetrahydrocannabinol is rapidly absorbed and effects appear within seconds to minutes, lasting 2–3 hr. after a single cigarette. Oral doses delay onset of symptoms from 30 min. to 2 hr.

3. Synthetic tetrahydrocannabinol analogs (III*a*, III*b*, and III*c*) show similar physiological effects, although structure–activity relationships have not singled out any specific pharmacological effect, except possibly the hypotensive effect produced by the dimethylheptyl homolog (III*b*).

4. Both $\Delta^{6a,10a}$-tetrahydrocannabinol (III*a*) and synhexyl (III*c*) were considerably less potent than Δ^9-tetrahydrocannabinol both on oral administration and when smoked.

Summary of Pharmacological Effects in Man—NIMH summarized the present state of knowledge on the pharmacological effects of marijuana in September 1970 in a report to the Secretary of Health, Education and Welfare (211):

> *"Marijuana is not a narcotic. Neither is it primarily a stimulant, sedative, tranquilizer or hallucinogen, although it shares some properties with each of these. At the same time, it lacks many of their other properties. In small doses it produces stimulation followed by sedation. In high doses it acts as a hallucinogen and can produce subjective changes somewhat resembling those of small amounts of LSD. Unlike many drugs* (e.g., *opiates and amphetamines) which require increasingly higher dosages over time to produce the same effect, marijuana users frequently report that with repeated use smaller doses produce the original effects. The apparent rapid disappearance from the blood of such substances as Δ^9-THC and Δ^8-THC following their injection indicates that they are rapidly transformed into other compounds.*
>
> *"Marijuana shares with other psychoactive drugs the problem of adequately describing and defining its subjective effects. There is considerable variability in the way in which the different sensations produced are interpreted by the user. What appear to be similar subjective states may be quite differently interpreted by different users. The same effect may be pleasurable to one user and unpleasant, even frightening, to another. It may also be that those finding the experience predominantly pleasant are inclined to minimize or ignore the less pleasant effects.*
>
> *"Although the state of intoxication is frequently vivid as described by the user, an observer may see little change from a normal state. Mild states of intoxication often go completely undetected. The user's mood may be quite variable from being happy and gregarious to quiet and detached. At higher doses, speech may be slowed or slurred. Physiological changes are notably minimal. Increase in pulse rate and bloodshot eyes are the most obvious. Dry mouth and throat along with an increase in appetite are common. Other physiological effects often are inconsistent or not reproducible.*
>
> *"Generally, simple physical and psychological performances are not much affected in short-term, moderate dosages while more complicated physical and psychological performances may be impaired. This may be due to an effect of marijuana intoxication on immediate memory. There is, however, great variability between users and little is presently known about the effects of long-term, chronic use on performance. Subjectively, time distortion appears common, with users reporting that a minute appears more like several minutes passage of time. Other subjective effects range from pleasant relaxation to acute anxiety, loss of reality contact, hallucinations and panic. These latter reactions are less common and much more likely when unexpectedly large doses of active material are consumed.*
>
> *"As is true of other psychoactive drugs, much appears to depend on the expectations of the user and the circumstances under which he uses the drug (set and setting). The user's set, referring to his total psychological makeup, mood at time of use and personal expectations, makes a considerable difference in the effects experienced, especially in low to moderate dosages. The external conditions of use, or setting, also make a great deal of difference in the total experience. A user feeling emotionally secure in a pleasant setting free of fear of detection is likely to have a different experience from someone taking the drug under more anxiety-producing conditions. The more neutral setting of the research laboratory may produce still different experiences. It is generally conceded that the individual and group expectations involved in the typical circumstances of marijuana use are important although difficult-to-evaluate aspects of the drug reaction."*

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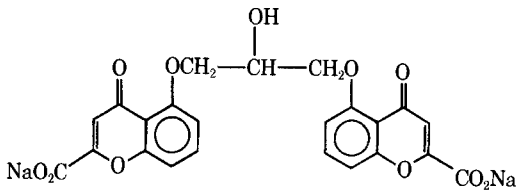
Solid-State Chemistry of Cromolyn Sodium (Disodium Cromoglycate)

J. S. G. COX*, G. D. WOODARD†, and W. C. McCRONE‡

Abstract □ A detailed crystallographic study was made to provide a better understanding and control of processing steps, purity control, and dosage formulations of cromolyn sodium. The compound, an odorless, white, hydrated crystalline powder, is the disodium salt of the dibasic acid: 1,3-bis(2-carboxychromon-5-yloxy)-2-hydroxypropane. No crystalline polymorphs were observed, but the crystals can quickly absorb (or lose) water as a continuous series of interstitial solid solutions. The amount of water absorbed or lost is proportional to the relative humidity of the environment of the crystals. At 90% R.H., the interstitial water approaches 24 wt. %, or nine molecules per molecule of cromolyn sodium. The absorbed water causes the lattice to expand reversibly, especially in the *b* crystallographic direction, and this results in remarkable changes in the X-ray diffraction pattern, density, and other physical properties. Above about 93% R.H., the compound absorbs more water than the crystals can hold. They then collapse to form one of two lyotropic mesophases, containing up to about 260 molecules of water per molecule of cromolyn sodium at 20° or about 10 wt. % cromolyn sodium.

Keyphrases □ Cromolyn sodium—crystallography □ Disodium cromoglycate—crystallography □ Aerosol dry powder—cromolyn sodium, crystallography □ Crystallography—cromolyn sodium (disodium cromoglycate)

Cromolyn sodium (I)[1] (also known as disodium cromoglycate) was recently introduced as a new pharmacological approach for the treatment of bronchial asthma (1). The compound, an odorless, white, hydrated crystalline powder, is the disodium salt of the dibasic acid: 1,3-bis(2-carboxychromon-5-yloxy)-2-hydroxypropane. Its crystals are, in a sense, an interstitial solid solution, with water the interstitial component. The unit cell expands to accommodate up to about nine molecules of water before collapsing to form liquid crystalline hydrates. Somewhat similar moisture pick-up was reported for zeolites (2), montmorillonite (3), rare earth oxalates (4, 5), zirconium oxalate (5), strychnine sulfate (5), hemoglobin (6), and another drug, cephaloridine (7).



chemical structure of cromolyn sodium

I

[1] Intal, Fisons Ltd., Pharmaceuticals Division, Loughborough, Leicestershire, England.

Cromolyn sodium crystals are, however, unique in their extent of reversible water absorption without collapse of the lattice.

No solid polymorph of cromolyn sodium has been observed, although there are mesophases (liquid crystalline forms) of the hydrate. There is a peritectic reaction, however, between the solid solution and the mesophase at 73°; hence, they have different compositions and are not polymorphs.

The equilibrium between water and cromolyn sodium is very rapid and reversible in the vapor state or in aqueous solutions. Even though all of the physical properties (density, lattice parameters, refractive indexes, *etc.*) vary with water content, the changes are least pronounced in the relative humidity (R.H.) range of about 30–70%. Crystals of cromolyn sodium contain from five to six molecules of water over this relative humidity range, and the physical properties are reasonably constant. Relative humidity values either higher than 70% or lower than 30% cause larger changes in the physical properties. Above 93%, the mesophase forms on the surface of the crystals and, given time and sufficient water, the entire sample is converted to the mesophase.

The objective of this study was to characterize cromolyn sodium crystals as an aid to trouble-free processing and optimum formulation of dosages. It was also felt that any information on the shape of the molecule (planar, linear, spherical, *etc.*) might be useful in connection with separate studies on its mode of action. This information was particularly desirable because the drug is administered as a dry powder aerosol.

EXPERIMENTAL

Determination of Moisture Content of Solid Solution—A constant-humidity chamber (Fig. 1) was designed and constructed to follow the weight of samples under known humidity conditions. The completely sealed system utilized a "quartz" spring balance in rapidly circulating air. Saturated aqueous solutions containing a solid excess of known inorganic salts were placed in the flask to maintain various constant, known, relative humidities.

The equipment was calibrated with balance weights. The extension of the spring was monitored by a traveling microscope fixed to the frame of the apparatus. Small marker flags on the wire suspending the aluminum foil pan were aligned with a cross-hair in the eyepiece of the microscope, and the height of the microscope was read on the scale. Changes in weight of 0.0001 g. could be measured with a sample weight of 0.1 g.

The sample was placed in the pan until the spring extension corresponded approximately to a weight of 0.1 g. The sample had been open to the laboratory atmosphere for a week before it was used. Simultaneously with the pan loading, an identical sample was

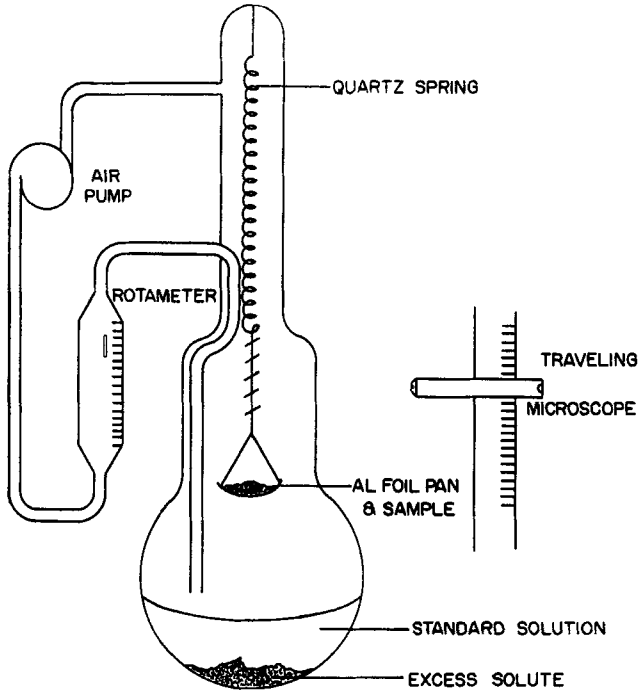


Figure 1—*Experimental arrangement for "quartz" spring studies.*

placed in a dry, tared, weighing bottle. This sample was heated at 150°, stoppered immediately on removal from the oven, and cooled over phosphorus pentoxide. This process was repeated until the sample showed constant weight. From these results, the moisture content was calculated for the sample on the quartz spring pan.

The aqueous solutions (containing excess solid solute) used to control the relative humidity in the apparatus are summarized in Table I. Phosphorus pentoxide was used to obtain 0% R.H.

After the proper aqueous solution was in position, the system was sealed and the air pump was started. Air blown at the surface of the solution caused ripples in the solution surface, thereby helping to keep it stirred. This method was preferred to bubbling the air through the solution because this procedure could give droplets of solution that might impinge on the pan and cause erroneous weight readings. The air was recirculated from the top of the column to the bottom flask at a rate of 3.6 l./min. To maintain the desired humidity, the apparatus was kept in a room thermostated at 20 ± 1°. The atmosphere rapidly reached equilibrium, judged by the rapid weight changes. Often, 80% of the final weight change occurred in the 1st hr. after the solutions were changed.

The air flow was stopped while readings were taken to avoid small disturbances in the pan position. Weight readings were taken every hour immediately after the solution was changed but at longer intervals as the rate of change decreased. Moisture contents were monitored with both increasing and decreasing humidity.

Melting-Point Data—Melting points were taken using a Mettler hot stage fitted to a polarizing microscope (Swift; Dick model). The samples were mounted in silicone oil to improve visibility and to help prevent chemical decomposition in air at the high temperatures involved.

Solubility Data—Cromolyn sodium is generally soluble in polar solvents and insoluble in nonpolar solvents. It forms solid solutions or lyotropic mesophases with water and discrete solvates with methanol, ethanol, propanol, diethylene glycol, ethylene glycol monomethylether, glycerol, propylene glycol, dipropylene glycol, dimethylformamide, and dimethyl sulfoxide.

Quantitative solubility data for cromolyn sodium in water only were determined in this study. Crystals were added to water at constant temperature until no more dissolved. The precise saturation point was then found by careful temperature adjustment. An aliquot sample of the saturated solution was then taken for gravimetric analysis. To be certain the solid residue was anhydrous, the solution was evaporated in a weighing bottle and dried at 150° for 12 hr. and to constant weight. The bottles were sealed at 150° and kept sealed during cooling and weighing.

Table I—Aqueous Solutions Used for Humidity Control

Saturated barium chloride	88
Saturated sodium acetate	76
Saturated calcium nitrate	51
Saturated calcium chloride	31
Sulfuric acid, 59.0 wt. %	20
Sulfuric acid, 67.2 wt. %	10

X-Ray Diffraction Data—Samples of cromolyn sodium were stored over saturated solutions of each salt listed in Table I for several months. These were then opened to the air and quickly sealed for X-ray diffraction. Later, X-ray diffraction data were redetermined by X-raying each sample in a humidity-controlled camera. A focusing vacuum X-ray diffraction camera[2] was modified to permit circulation of controlled humidity air. The humidity was controlled by circulating air of known relative humidity just as in the quartz spring experiment. The same solutions (Table I) were used, but the flow rate through the camera was increased to 5 l./min.

The sample was pressed into a 4-mm. hole, 0.25 mm. deep, in the center of a metal disk. Once this disk was fixed in the camera, the sample was exposed to the controlled-humidity atmosphere on both sides. A single sample was used for X-ray diffraction over the full range of 0–88% R.H.

In the first experiment, with phosphorus pentoxide in the chamber, the sample was stored under a continuously pumped vacuum for 1 week before X-raying. This procedure was repeated with the solutions; but above 10% R.H., the equilibrium period was reduced to overnight. The quartz spring experiments had shown that this was sufficient time for equilibrium. The humidity was increased successively throughout this series of experiments.

To index the X-ray powder diffraction data, it was necessary to obtain single crystal data. This, in turn, required growth of large, well-formed, single, clear crystals. These were obtained by slow addition of ethanol to a saturated aqueous solution in an atmosphere saturated with ethanol. Slow absorption of ethanol vapors by the aqueous solution soon precipitated elongated crystals which grew slowly for several days, some to a length of 1–2 cm. The best of these crystals were selected for X-ray diffraction as well as crystal optics and morphology.

Single crystal X-ray diffraction data (precession camera), taken at near 50% R.H., gave cell dimensions permitting indexing of the appropriate powder data. A computer program was designed to index the powder data taken at 51% R.H. The powder data taken

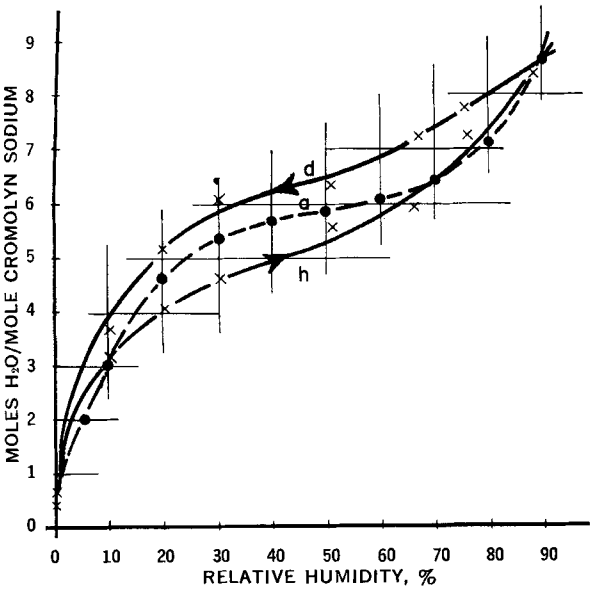


Figure 2—*Water content as a function of relative humidity during hydration (h), dehydration (d), and an average curve for all data determined (a).*

[2] Designed by Dr. James B. Nelson, McCrone Research Associates Ltd., London, England.

Table II—Powder X-Ray Diffraction Data as a Function of Relative Humidity (I/I_0 in Parentheses)

Reflection hkl	Relative Humidity, % 0	10	20	51	76	88
S	27.0(10)	27.6(2)	27.8(1)	28.0(<1)	—	—
010	14.0(5)	15.0(3)	15.5(3)	15.8(4)	15.8(2)	15.8(2)
S	13.5(4)	13.8(1)	13.9(<1)	—	—	—
100	10.8(8)	11.1(6)	11.2(6)	11.2(2)	11.2(3)	11.2(4)
S	9.7(8)	9.8(10)	9.8(6)	9.9(4)	9.9(7)	9.9(8)
S	9.0(1)	9.2(6)	9.4(3)	9.4(3)	—	—
110	8.6(3)	8.9(6)	9.0(6)	9.0(3)	9.0(2)	9.0(2)
S	8.2(<1)	—	—	8.5(<1)	8.5(<1)	—
020	7.05(3)	7.55(8)	7.7(10)	7.90(5)	7.90(1)	7.90(1)
S	6.65(2)	6.60(1)	6.60(2)	6.90(<1)	—	7.15(2)
120	5.85(<1)	6.20(1)	6.35(3)	6.40(1)	6.40(1)	—
S	5.55(<1)	—	5.70(1)	5.80(1)	—	—
200	5.35(<1)	5.50(2)	5.50(1)	5.55(1)	5.60(<1)	—
S	5.15(<1)	5.25(<1)	5.25(4)	5.25(3)	5.30(3)	5.30(1)
S	4.84(<1)	4.90(1)	4.90(<1)	4.90(<1)	—	—
S	4.48(<1)	4.60(<1)	4.65(<1)	4.70(1)	—	—
220	—	4.50(1)	4.55(1)	4.60(1)	4.60(1)	4.60(1)
S	3.95(1)	3.98(<1)	3.98(1)	4.20(1)	4.25(<1)	—
S	3.82(3)	3.88(3)	—	—	—	—
S	3.68(1)	—	—	—	—	—
230	—	3.73(5)	—	3.80(1)	3.80(1)	3.80(6)
300	3.61(1)	3.73(5)	3.75(6)	—	3.70(10)	3.72(8)
$10\bar{1}$	3.61(1)	3.65(3)	3.65(6)	3.69(10)	3.70(10)	3.72(8)
040	3.54(<1)	3.73(5)	3.88(5)	3.90(5)	3.92(4)	3.94(4)
$11\bar{1}$	3.48(<1)	3.52(5)	3.55(6)	3.60(1)	3.60(1)	3.63(<1)
310	3.48(<1)	3.65(3)	3.65(6)	—	3.60(1)	3.63(<1)
$20\bar{1}$	3.44(<1)	3.52(5)	3.55(6)	—	3.60(1)	—
001	3.37(<1)	3.40(3)	—	3.42(5)	—	—
140	3.37(<1)	3.52(5)	3.65(6)	3.69(10)	3.70(10)	3.72(8)
011	3.31(1)	3.34(4)	3.34(6)	3.34(4)	—	—
$21\bar{1}$	3.31(1)	3.40(3)	—	3.42(5)	—	—
$12\bar{1}$	3.22(1)	3.23(4)	3.25(5)	3.34(4)	3.35(3)	3.35(3)
320	3.22(1)	3.34(4)	3.34(6)	3.34(4)	3.35(3)	3.35(3)
$22\bar{1}$	3.10(<1)	3.17(2)	3.17(<1)	3.25(2)	3.25(6)	3.25(6)
021	3.05(<1)	3.10(<1)	3.11(2)	3.17(2)	3.17(<1)	3.17(<1)
$30\bar{1}$	3.05(<1)	3.10(<1)	3.11(2)	3.12(2)	—	—
050	—	—	3.11(2)	3.17(2)	3.17(<1)	3.17(<1)
240	2.95(<1)	3.10(<1)	3.17(<1)	3.25(2)	3.25(6)	3.25(6)

over the full humidity range were then indexed, and the values of *a*, *b*, and *c* were refined by successive iterations.

RESULTS AND DISCUSSION

Physical Properties—The properties of cromolyn sodium vary considerably with water content. The water content of the solid solution varies from zero to more than 24 wt. % corresponding to a range from zero to more than nine molecules of water per molecule of cromolyn sodium. The crystals exchange water freely with the atmosphere or with aqueous solutions. The rate-controlling step in the hydration–dehydration is diffusion of water through air (or aqueous solution) to the crystal. Crystals equilibrated in air at any given relative humidity show a corresponding water content. Undissolved crystals in excess, in any given aqueous solution, show an equilibrium water content corresponding to the relative humidity of air in equilibrium with that solution. If, however, the relative humidity exceeds 93%, the crystals collapse to form a mesophase.

Dehydration is easily effected by heat or desiccants. Rapid dehydration of the solid solution produces cloudy crystals, in contrast to the clearer crystals produced by slower changes in water content. Both clear and cloudy crystals show similar physical properties, although the clear crystals are able to hold somewhat more water before collapsing to the mesophase. Both clear and cloudy crystals are stable up to at least 93% R.H.

Moisture Content of Solid Solution—The importance of moisture content on structure and flow properties made it desirable to know the equilibrium moisture content for various relative humidities. It was also desirable to obtain a measure of the rate at which crystals gain and lose water when the humidity is changed. In early experiments, 1 week was allowed for equilibrium, but this was obviously unnecessary and was reduced to 24 hr. except at 0% R.H. over phosphorus pentoxide. The residual water came off slowly near 0%, and constant weight was not attained for 96 hr.

Figure 2 shows the change in water content as the relative humidity was increased and decreased for the same sample. This hysteresis curve would probably approach the average curve also shown in Fig. 2 if more time had been allowed for equilibrium at each relative humidity.

The shape of the hydration curve shows that, although there is a region of slow increase of moisture content with increasing humidity, there are no sharp plateaus corresponding to fixed hydrates. It is impossible to remove the final traces of water using phosphorus pentoxide. Oven-drying similar samples at 150° always removes slightly more water.

When the humidity of the air is altered, the rate of change of crystal moisture content is remarkably fast. Certainly the rate would be faster if the air humidity could be changed instantaneously, but the rate of moisture pick-up is still limited by the rate at which the air equilibrates.

The results of these and similar experiments permit plotting of an average equilibrium moisture content of cromolyn sodium crystals in air of known relative humidity (Fig. 2).

Melting-Point Data—Cromolyn sodium crystals are nearly always highly stressed, as indicated by the twisted and distorted crystals and by the behavior during dissolution in solvents on a microscope slide. Many needle crystals are distorted into C and S shapes, and many dissolve at greatly different rates in different portions of the same crystal. These indicate crystal lattice stress. In a given sample, some of the cromolyn sodium crystals, presumably somewhat stress relieved by physical cracking, melt at 264°, whereas the highly distorted and stressed crystals melt at 258°.

Solubility Data—The solubility data are incorporated in the composition diagram discussed later.

X-Ray Diffraction Data—The characterization of cromolyn sodium by X-ray diffraction would have been easier if the unusual behavior of these crystals with water vapor had been understood earlier. Early work in this laboratory indicated that crystals of cromolyn sodium only very slowly gained or lost water from the

Table III—Lattice Parameters for Cromolyn Sodium (Angstroms)

Relative Humidity	Cell Edge		
	a	*b*	*c*
0	11.5	14.0	3.58
10	11.8	15.0	3.62
20	11.9	15.5	3.64
30	11.9	15.7	3.66
40	11.9	15.7	3.67
51	11.9	15.8	3.68
76	11.9	15.8	3.70
88	11.9	15.8	3.70

atmosphere. Thus, it was assumed that crystals equilibrated over a period of several weeks could be X-rayed at ambient relative humidities without significant change in water content.

Although the crystals were exposed to ambient humidities for less than 1 min., they changed remarkably rapidly in water content. Fortunately, differences were found and, at the time, they seemed large. Later, the quartz spring experiments showed that equilibration of cromolyn sodium crystals with the atmosphere was very rapid. Earlier experiments had measured, in a sense, the rate of diffusion of water molecules to the crystals. The crystals absorbed these molecules rapidly; the absorption rate was limited by the rate of diffusion or circulation of water molecules to the crystal surfaces.

It is necessary now to discuss the fact that the powder patterns show a large number of lines that cannot be indexed with the *a*, *b*, and *c* derived from the single crystal data. Some lines are also sometimes unaccountably broad and may even disappear when the relative humidity is changed. Furthermore, some lines have spacings larger than *a*, *b*, or *c*. It seemed clear that a larger unit cell must be related to moisture absorption by the lattice. To explain (and index these lines), an expanded lattice was postulated. It was assumed that the water molecules absorbed by the lattice in layers parallel to 010 (thereby expanding principally the *b* lattice dimension) did not add precisely the same number of water molecules between each pair of unit cells. The *b* dimensions of successive cells might vary as the number of associated water molecules varied. Yet the total number of molecules of water per 5, 7, or so molecules of drug might well be

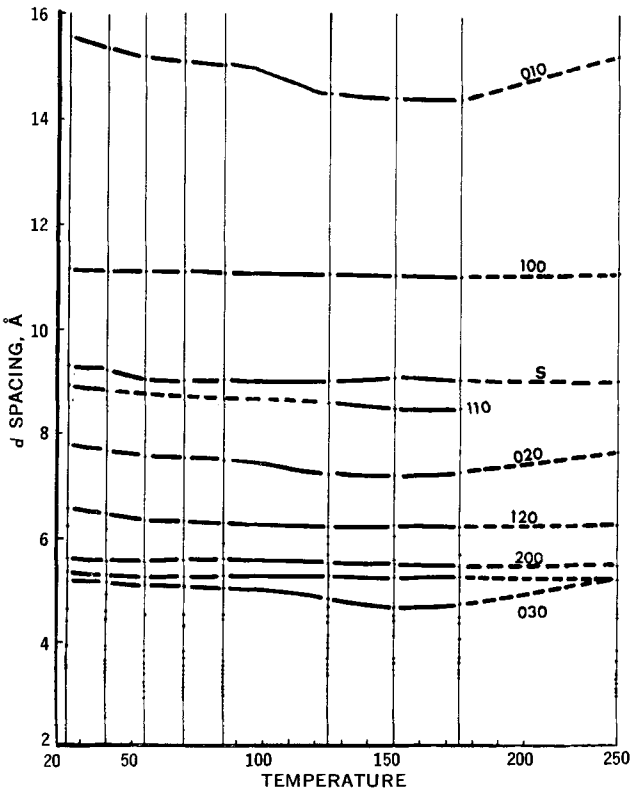


Figure 3—*Powder X-ray diffraction data during successive stages of heating (and recooling) of cromolyn sodium.*

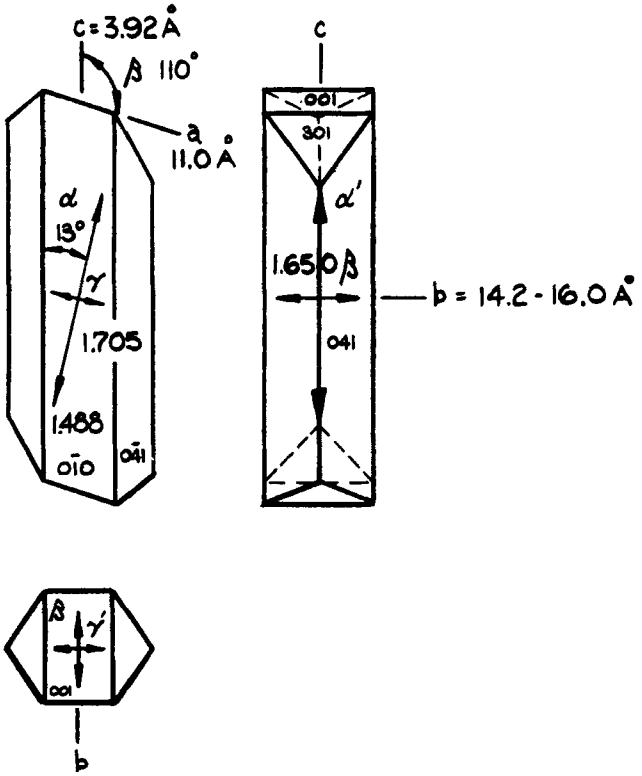


Figure 4—*Orthographic projection for crystals containing about five molecules of water per molecule of cromolyn sodium.*

constant. Then, in effect, the *b* dimension becomes 5, 7, or so times the value for the simplest cell containing one molecule of drug.

This expanded lattice hypothesis was tested by programming the computer to calculate all possible 0*k*0 diffraction lines for $k = 0$–36 and all values of *b* from *n*(14.0), *n*(14.2), *n*(14.4)...*n*(15.8), where $n = 1$–12. The lines, previously unindexed, were now easily indexed, usually with data for a new value of *b* equal to 7–9(14.0–15.8). The value of *b* was chosen in each case corresponding to the relative humidity during that diffraction run. These expanded lattice lines are marked S in Table II.

The expanded lattice lines tend to disappear for crystals equilibrated above 70% R.H. Small crystals show fewer expanded lattice lines than larger ones, presumably because diffusion of water molecules can be more easily equilibrated when distances are shorter. Small crystals, as a rule, show few, if any, expanded lattice lines, whereas large crystals often show more expanded lattice lines than parent lattice lines. One can conclude that large crystals must be hydrated or dehydrated slowly to avoid expanded *b*-axis spacings.

It is difficult to be sure of the indexing of all lines in the various powder patterns because of the multiple effects on spacings and intensities. Additional, though minor, variations occur in X-ray patterns for crystals of the same water content. This is probably due to the previous history of the sample and shows up predominantly in the expanded lattice lines.

The *b* crystallographic spacing expands continuously as the relative humidity increases (Table III). The change is most rapid below 20% R.H.; the unit cell dimensions are nearly constant above about 40% R.H. This is fortunate since the X-ray powder patterns

Table IV—Refractive Indexes of Cromolyn Sodium as a Function of Relative Humidity

Relative Humidity, %	Refractive Indexes, $n_D^{25°}$			Optic Axial Angle, $2V$
	α	β	γ	
4	1.488	1.68	1.705	40°
40	1.488	1.66	1.705	52°
90	1.488	1.615	1.705	76°

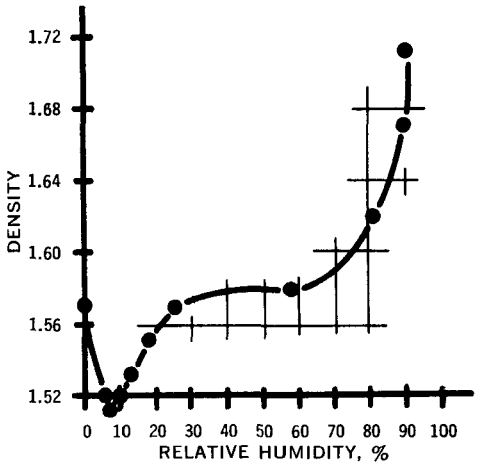


Figure 5—*Density of cromolyn sodium* versus *relative humidity.*

and densities are more nearly constant over the normal humidity range of 20–70%.

The effect of heat on the lattice dimensions was determined by installing a heating stage on an X-ray diffractometer. Powder diffraction data were taken at 45-min. intervals at successive temperatures of 25, 40, 55, 70, 85, 125, 175, and back to 25° (Fig. 3). It was difficult to index the shorter spacings because of the counterinfluence of lattice expansion on heating. The lines indexed show, however, that the crystals lose water gradually as the temperature rises, although more rapidly up to 40° and near 100°.

Crystal Morphology—Crystals of cromolyn sodium are not very well formed in laboratory or plant production. This is not, of course, unusual for a complex organic molecule. Most crystals are flat lath shapes, although a very few better formed rods can usually be found. Figure 4 is a somewhat idealized drawing, consistent with the observed X-ray data, optics, and morphology although, strictly speaking, the crystals must be hemisymmetric.

Crystal Optics—The optical properties vary considerably as a function of water content. The average crystal, containing about

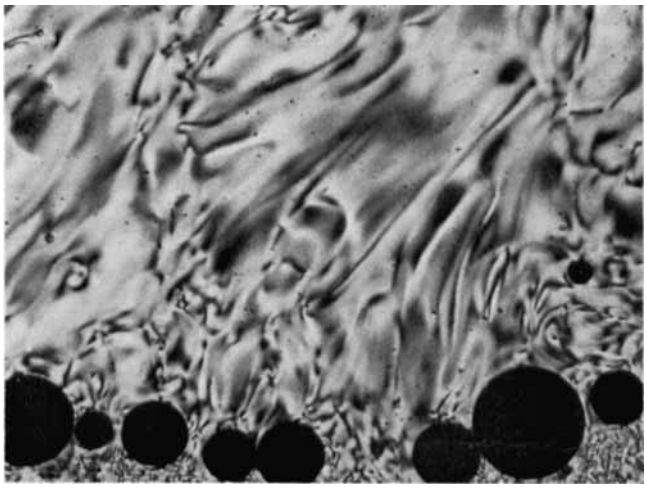


Figure 7—*A 20:80 cromolyn sodium–water mixture at room temperature showing the nematic mesophase; crossed polars, 100×.*

five molecules of water, is monoclinic with β parallel to b and α inclined to c, the direction of elongation, by 13° and lying in the obtuse β angle. The refractive indexes α, β, and γ are 1.488, 1.650, and 1.705, respectively, with $2V = 60°$ (−) and little or no dispersion.

The refractive indexes, especially β, vary with the degree of hydration. To measure these variations, a glove box was provided with a polarizing microscope and provision for varying the relative humidity. Although it was impossible to achieve a relative humidity lower than about 4%, measurements were made at that level as well as 40 and 90%. A few crystals on a microscope slide were allowed to equilibrate for a few minutes within the glove box before each measurement of refractive index. These measurements (Table IV) were made on several individual crystals by immersion methods.

Crystal Density—The density can be calculated from the weight and volume of the unit cell. The unit cell varies, however, in both weight and volume as the water content of either air or aqueous

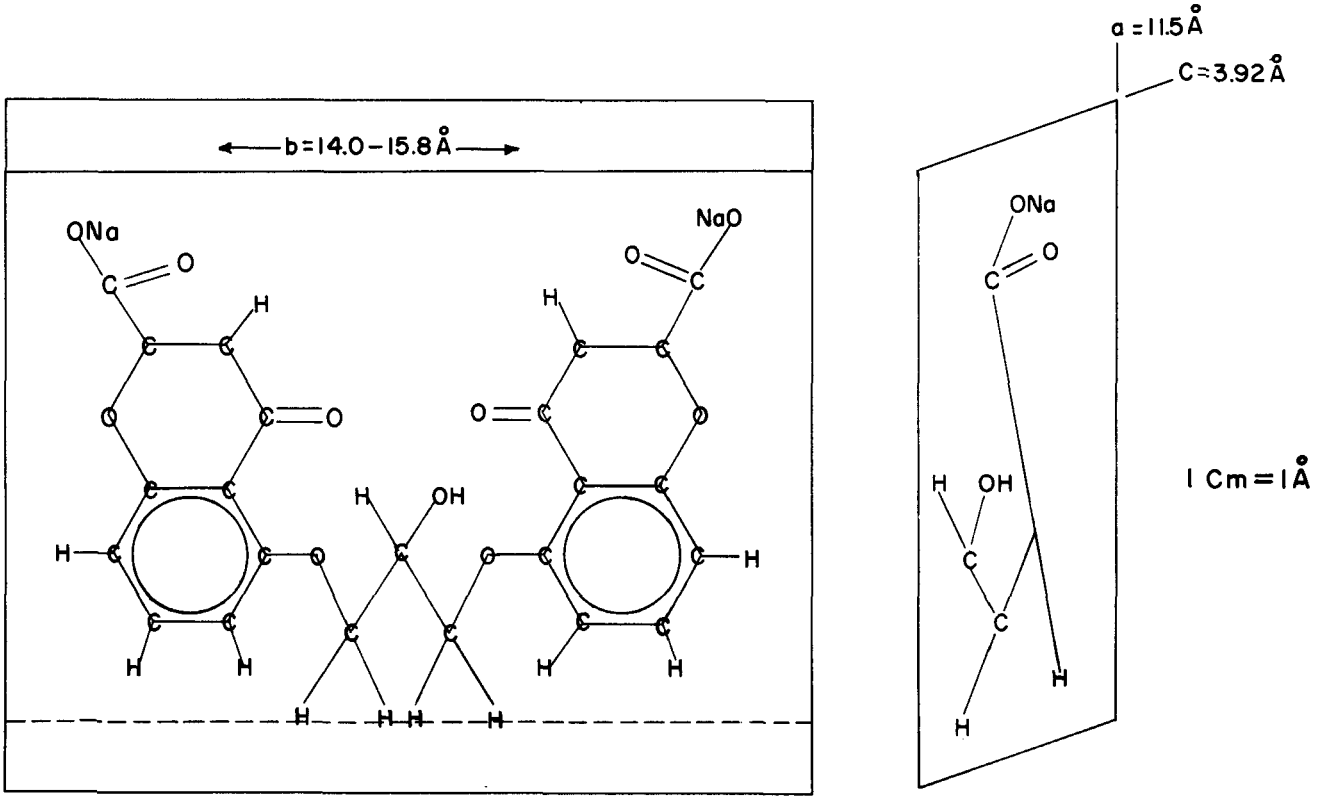


Figure 6—*Probable approximate crystal structure for cromolyn sodium and its solid solution with water.*

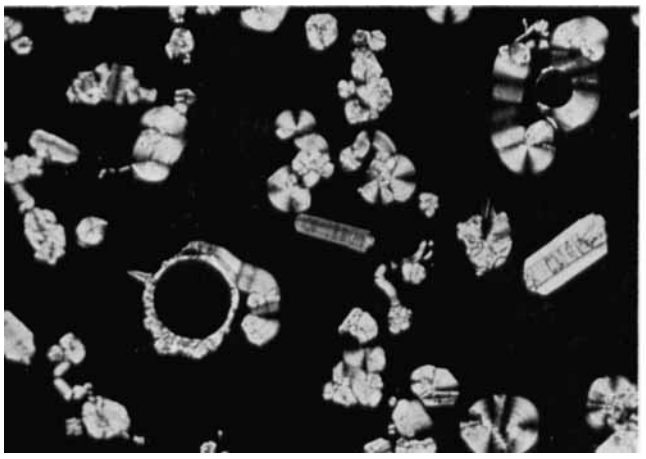


Figure 8—*Spherulites and bâtonets in a 20:80 cromolyn sodium–water mixture cooled to 50° from 55°; crossed polars, 100×.*

suspensions varies. The experimental lattice dimensions and water content, as a function of relative humidity, are used for this calculation. Experimentally, on the other hand, one would not be sure the flotation or displacement medium was not affecting directly measured density values.

The information in Table III is used to calculate the density of any sample as a function of relative humidity. The appropriate formulas are:

$$\text{density} = \frac{\text{weight of unit cell}}{\text{volume of unit cell}} \qquad \text{(Eq. 1)}$$

$$\text{density} = \frac{\text{mol. wt.} \times n \times 1.6604 \times 10^{-24}}{a \times b \times c \times \sin\beta} \qquad \text{(Eq. 2)}$$

where:

n = number of formula weights per unit cell
1.6604×10^{-24} = mass in grams of 1 unit of atomic weight
a, b, and c = dimensions of the unit cell expressed in cm.
β = crystallographic angle between the a and c axes

There is only one molecule per unit cell; hence, $n = 1$. This is apparent if only because densities higher than 1.5–1.7 by factors of 2, 4, or 8 (corresponding to $n = 2$, 4, or 8) are absurd for this compound. Several experimental density values, moreover, fall in the 1.5–1.6 range. The calculated density data are given in Fig. 5.

The unusual shape of the density–relative humidity relationship in Fig. 5 is a consequence of the relative rates of moisture pick-up and expansion of the lattice as the humidity increases. When the rate of expansion is high relative to the rate of moisture absorption, the density goes down (0–10%). When the rate of expansion decreases and the rate of moisture absorption is high, the density rises rapidly (60–90%). From about 25 to 60% R.H., both rates are correspondingly slow and the density changes only slightly.

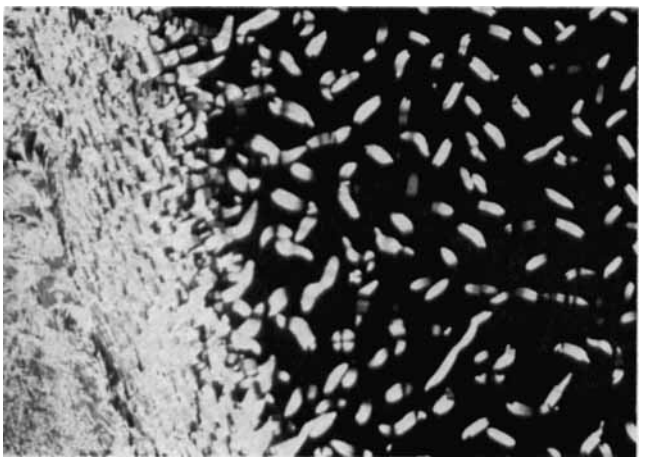


Figure 9—*The middle mesophase of cromolyn sodium crystallizing from water on cooling to 45°; crossed polars, 100×.*

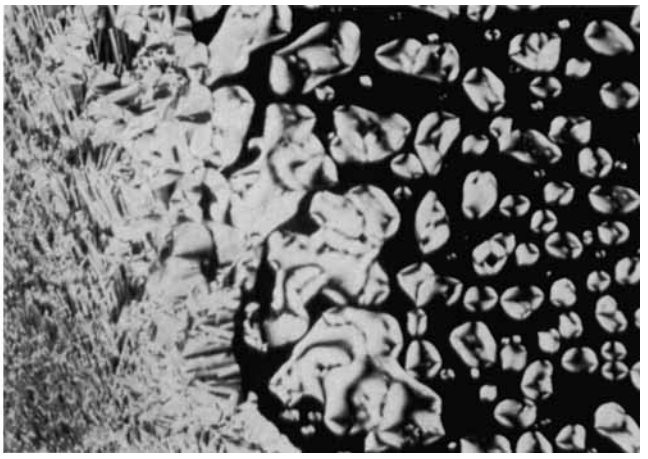


Figure 10—*Same field of view as Fig. 9 after transformation of the middle to the nematic mesophase. The temperature here is 41°; crossed polars, 100×.*

Crystal Structure—A fortunate set of circumstances made possible the prediction of a likely crystal structure for anhydrous cromolyn sodium. These circumstances are:

1. There is only one molecule per unit cell and every molecule must be oriented similarly within each unit cell.
2. One lattice spacing, c, is only 3.92 Å. Since the molecule must be able to fit entirely within a unit cell, it must lie in a planar configuration. Furthermore, for the same reason, the plane of all molecules in the crystal must lie more or less parallel to the a–b plane and perpendicular to c.
3. The refractive indexes and extinction angles confirm this arrangement of the molecule and, in addition, show the plane of the molecule lying about 13° from c in the obtuse β angle.
4. Both morphology and optics of the crystals, taken with the fact that there is one molecule per unit cell, show that the molecule has a plane of symmetry and this plane lies in the a–c crystallographic plane.

Study of molecular models suggests that there are only two possible structures, with the one shown in Fig. 6 most likely. The alternate possibility involves flipping the molecule over like a pancake. One of these two arrangements is most likely a close approximation to the crystal structure. Much more single crystal X-ray work under carefully controlled zero humidity conditions will be necessary to define precisely the position of each atom in the unit cell.

Water–Cromolyn Sodium Composition Diagram—Every known solid cromolyn sodium crystal can be characterized as an interstitial solid solution containing from zero to about nine molecules of water per molecule of drug, *i.e.*, 0–24 wt. % water.

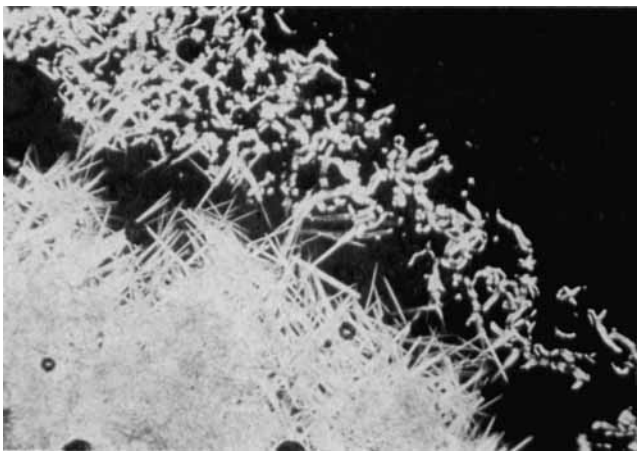


Figure 11—*The peritectic reaction between cromolyn sodium–water solid solution and the middle mesophase taking place at 73°. The needles at the top are the mesophase; crossed polars, 100×.*

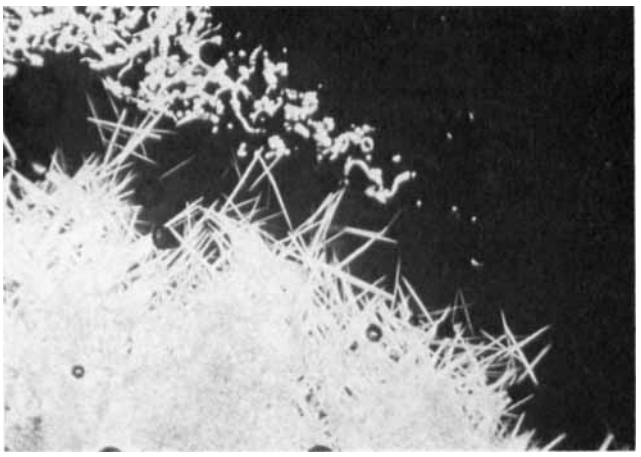


Figure 12—*Same field of view as Fig. 11 as the peritectic reaction continues at 73°, giving more and more of the solid solution; crossed polars, 100×.*

The water–cromolyn sodium composition diagram is based on microscopical observations and solubility data. The entire diagram was determined, at least semiquantitatively, by hot-stage microscopy. To complete the diagram, a solubility curve was determined and known mixtures were observed microscopically during heating.

If excess solid cromolyn sodium is placed in a drop of water between slide and coverslip at room temperature, a nematic lyotropic mesophase immediately forms. This mesophase shows a dynamic sinuous texture. Its appearance between crossed polars is shown in Fig. 7. On heating to about 42°, the appearance changes reversibly (the change is incongruent, *i.e.*, a peritectic). This change to a middle "M" phase is best observed in an aqueous mixture of 20–25 wt. % cromolyn sodium. If this mixture is heated above 42°, the "M" mesophase slowly dissolves. If the temperature is then slowly lowered (about 2°/min.) after holding for a minute or two at about 50°, excellent batonnets, spherulites, and fibers of "M" appear (Fig. 8). As the temperature continues to fall, the transition to the nematic mesophase is easily recognized at about 42°. At this temperature the more highly organized "M" mesophase collapses to the flowing sinuous nematic mesophase (Figs. 9 and 10).

Raising the temperature again reverses the transition at about 42°. Further increase in temperature to about 73° results in a second transition, this time to the solid solution, a crystalline phase (Figs. 11 and 12). This, too, is a reversible peritectic-type transition. A uniform mixture of about 15% cromolyn sodium shows the nematic phase (Fig. 11) at room temperature. At the edges of such a coverslip preparation, where water is evaporating at room temperature, one can observe the characteristic transition to the middle mesophase (Fig. 13). Careful study of this boundary between the nematic and

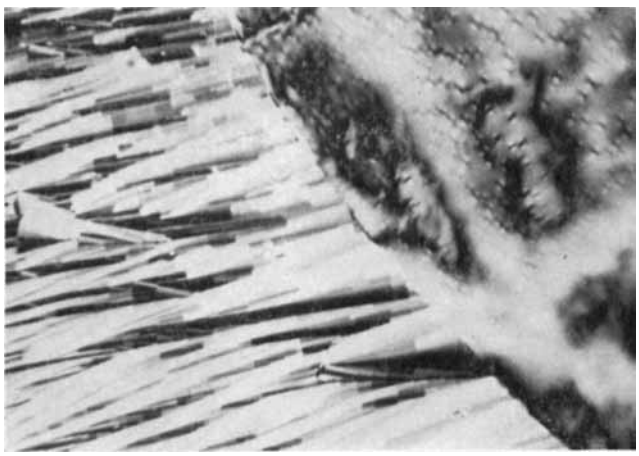


Figure 13—*A coverslip preparation of the nematic mesophase (right) slowly drying at the edge of the preparation to the give the middle mesophase (left); crossed polars, 100×.*

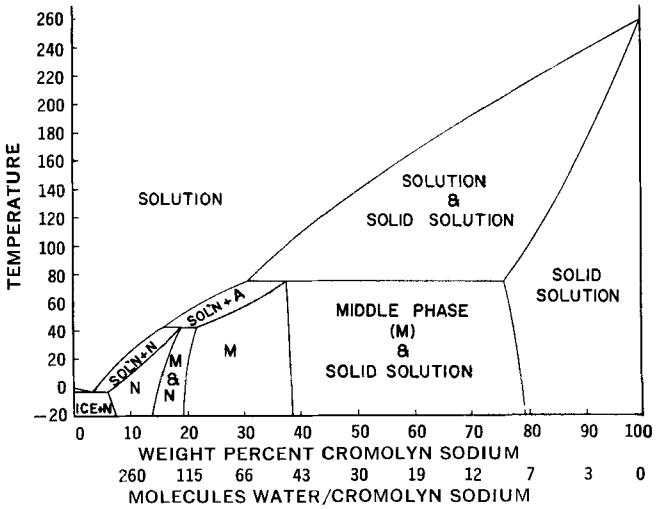


Figure 14—*The cromolyn sodium–water composition diagram.*

middle mesophases shows that the two mesophases are stable together over a short range of water content. This is best observed, as the stage is rotated, with crossed polars. The extinction pattern characteristic of the nematic mesophase extends into the region where the middle mesophase is growing.

These data define the composition diagram qualitatively. To make the diagram more quantitative, the boundary between aqueous solution and aqueous suspension of excess solute, the solubility curve, was determined. The eutectic temperature between water and the nematic mesophase was then measured using the Mettler hot stage. To measure this subambient temperature, the stage was chilled to below −20° using liquid nitrogen. The digital readout and programmed heating controls on the hot stage function from −20 to +300°; hence, temperatures can be measured over that range. A composition gradient preparation was made between slide and coverslip by placing a drop of water on one side and solid cromolyn sodium on the other. On contact, the nematic mesophase immediately formed. On placing this preparation in the precooled stage, all phases—water, eutectic, and cromolyn sodium—froze. Heating at 2°/min. gave a corrected melting point for the eutectic of −2.7°. The composition of the eutectic, 5 wt. % cromolyn sodium, is based on extrapolation of the solubility data to the eutectic temperature line.

To complete the diagram, a number of known mixtures were prepared. These were heated, thoroughly mixed, cooled, and kept sealed. A small portion of each was then placed between the slide and coverslip and observed carefully during heating in the Mettler hot stage. Any phase changes occurring during heating could be observed for each concentration. These changes included: (*a*) either mesophase giving a mixture of mesophase plus solution, (*b*) transition of nematic to middle mesophase, and (*c*) transition of middle mesophase to solid solution. The completed diagram (Fig. 14) is based on careful study of these prepared mixtures. Mixtures were prepared at 5 wt. % intervals, and the placing of the phase boundaries is believed accurate to within at least 2–3%.

The behavior of lyotropic mesophases is quite complex (8–10) and cromolyn sodium is no exception. More complete details will be published separately.

CONCLUSION

Cromolyn sodium crystallizes with a single crystal lattice; no crystalline polymorphs were observed. These crystals can quickly absorb up to 24 wt. % water as a continuous series of interstitial solid solutions. The hydrated crystals containing up to nine molecules of water per molecule of cromolyn sodium can then quickly lose water, with no significant change in the molecular arrangement.

The percentage of water in the lattice is proportional to the relative humidity of the surrounding atmosphere. Similar changes in water content are induced by surrounding the crystals with an aqueous solution. The water content of the crystals is then related to the relative humidity of the solution in which the crystals are immersed.

This absorbed water, amounting to as much as 24 wt. % at about 90% R.H., results in reversible expansion of the lattice, especially along the *b* crystallographic direction. This, in turn, causes remarkable changes in X-ray diffraction patterns, density, and other physical properties.

A relative humidity higher than about 93% causes cromolyn sodium to absorb more water than the crystals can hold. They then collapse to form one of two lyotropic mesophases, containing from about 45 to as much as about 140 molecules of water per molecule of cromolyn sodium at 20°.

These data should be very helpful to plant processing and formulation experts. With this background, production and research personnel can be more sure that they have the best processing and storage procedures as well as the best dosage form. Problems arising in the plant or during storage and use are also more quickly solved. No important drug should be marketed without a careful study of its solid-state chemistry.

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Quantitative Analysis of Microgram Quantities of Pilocarpine in Aqueous Solution

A. REPTA and T. HIGUCHI

Abstract □ Aqueous solutions containing microgram quantities of pilocarpine and pilocarpic acid were assayed quantitatively, using a kinetic method based on the catalytic activity of the imidazole portion of the alkaloids on the hydrolysis of 2,4-dinitrophenyl acetate. The results were accurate to better than ±5% over a range of concentration of 6–60 mcg. of pilocarpine/ml. The effects of temperature, salt concentration, and pH were studied. Procedures for analysis of mixtures of pilocarpine and pilocarpic acid are given. Also a method is described for the analysis of small volume samples containing less than 1 mcg. of pilocarpine.

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The kinetic approach employed arose from the recognition of the substituted imidazole portion of the pilocarpine molecule as a potential catalyst for the hydrolysis of esters such as the nitrophenyl acetates. The strong catalytic effects of imidazole and some of its derivatives were shown previously (3–8).

While the employment of the kinetics of both catalyzed and uncatalyzed reactions in quantitative analysis is well known (9–11), most of the methods used involve inorganic species and/or enzymes. The procedure described here represents one of the first successful applications of kinetic analysis to relatively simple organic molecules.

EXPERIMENTAL

Equipment and Chemicals—An Orion model 801 pH meter was used for pH determinations. Cary models 14 and 15 spectrophotometers were used for absorption measurements. Titrations were carried out using a Scientific Industries model 200 ultra-buret. Small volumes of liquid were measured using Hamilton syringes. Graduated 5-ml., glass-stoppered, tapered-tip, Pyrex centrifuge

This absorbed water, amounting to as much as 24 wt. % at about 90% R.H., results in reversible expansion of the lattice, especially along the *b* crystallographic direction. This, in turn, causes remarkable changes in X-ray diffraction patterns, density, and other physical properties.

A relative humidity higher than about 93% causes cromolyn sodium to absorb more water than the crystals can hold. They then collapse to form one of two lyotropic mesophases, containing from about 45 to as much as about 140 molecules of water per molecule of cromolyn sodium at 20°.

These data should be very helpful to plant processing and formulation experts. With this background, production and research personnel can be more sure that they have the best processing and storage procedures as well as the best dosage form. Problems arising in the plant or during storage and use are also more quickly solved. No important drug should be marketed without a careful study of its solid-state chemistry.

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EXPERIMENTAL

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tubes were used as reaction vessels in the assay procedure. Temperatures were controlled to ±0.1° for all studies by use of a thermostated water bath. Pilocarpine nitrate USP[1] and isopilocarpine nitrate[2] were used. The water used was distilled from acid permanganate solution in an all-glass apparatus. All other chemicals were of analytical grade. Calculations were carried out using the Olivetti Programma 101.

Methods—*A. Determination of Catalytic Effects of Pilocarpine, Pilocarpic Acid, Isopilocarpic Acid, and Isopilocarpine on Hydrolysis of 2,4-Dinitrophenyl Acetate in 5.5 ml. of Solution*—Borate buffers were prepared by addition of 0.0625 *M* sodium tetraborate solution to 0.25 *M* boric acid solution to yield the desired pH. The buffer system used in these studies consisted of 5 ml. of borate and 40 ml. boric acid stock solution. The resulting buffer solution had pH 7.45; but when the ester and sample solution were added, the final pH was 8.2 and this pH remained constant throughout the reaction.

Sample solutions containing from zero to nearly 50 mcg. of alkaloidal material/ml. of aqueous solution were prepared by dilution of a stock solution of known concentration. The preparation of the stock solutions of pilocarpic and isopilocarpic acid is discussed in Part D. The dilutions of the intact alkaloid were made just prior to their use to avoid the problem of hydrolysis which may occur in dilute solutions. The substrate solution consisted of the ester 2,4-dinitrophenyl acetate dissolved in acetonitrile, which had previously been dried over a Linde 4-A molecular sieve. The concentration was about 140 mcg. of 2,4-dinitrophenyl acetate/ml. of solution. All solutions were placed in a water bath at 25° for at least 0.5 hr. prior to use. After equilibration, 4 ml. of one of the pilocarpine solutions was pipeted into a 5-ml. glass-stoppered centrifuge tube[3], which had been temperature-equilibrated in the water bath. Then 1 ml. of buffer and 0.5 ml. of the ester solution were added. The contents were mixed and transferred to a stoppered 1-cm. quartz cell, and the absorbance changes were followed at 357 nm. The temperature of the sample compartment of the instrument was maintained at 25°.

In the early stages of this work, the increase in absorbance with time was followed continuously for more than 40% of the reaction. The sample was then allowed to stand until the reaction was complete. The limiting absorbance (A_∞) was then determined, and the observed rate constant was determined by plotting log ($A_\infty - A_t$) *versus* time in the usual manner. When the reaction appeared to have a half-life of more than 20 min., the absorbance was measured at suitable time intervals and the sample was replaced in the water bath when measurements were not being made. The absorbances and corresponding times were again plotted as described. In the terminal phases of the study and especially in checking the analytical method, only three absorbance readings were made. They were made: (*a*) immediately following addition of the ester, (*b*) at some time during the reaction, and (*c*) after the completion of the reaction. The absorbance and corresponding times were then used in the integrated first-order rate law, and the observed rate constant was calculated. It was found that all three of the described procedures for following the absorbance changes yielded results that were in good agreement.

B. Determination of Catalytic Effects of Pilocarpine in 2,4-Dinitrophenyl Acetate in 110 µl. of Solution—Those studies involving only small volumes of sample solution were carried out using the same buffer system already described. The 2,4-dinitrophenyl acetate solution used contained about 1.25 mg. of ester/ml. of acetonitrile solution. Solutions containing 0–15 mcg. of pilocarpine nitrate/ml. were prepared in 4 parts water and 1 part borate buffer. A volume of 100 µl. of this solution was placed in a centrifuge tube. To this was added 10 µl. of the ester solution, and the time of addition (t_0) was recorded. At some point during the reaction, the sample was diluted by the addition of 3 ml. of a solution containing 1 part buffer and 4 parts water. The time of dilution (t_d) was recorded. Part of the diluted sample was immediately transferred to a 5-cm. cell, and the absorbance at 357 nm. was recorded. The times at which the absorbance was first recorded (t_r) were noted, and the small change in absorbance was followed for 1–2 min. Use of this data in estimating the absorbance value (A_d) corresponding to the time of dilution (t_d) is discussed later.

The initial absorbance (A_0) was determined as follows: The ester solution was added to 3.1 ml. of the diluted buffer, and the time of addition (t_0) was recorded. The resulting solution was transferred to the 5-cm. cell, and the time at which the absorbance was first recorded (t_r) was noted. The small absorbance changes were recorded for several minutes after the initial reading. The initial absorbance (A_0) was estimated by a linear extrapolation of the recorded curve for a distance corresponding to the time that elapsed ($t_r - t_0$) from addition of the ester until the initial recorded value of the absorbance. The absorbance (A_d) corresponding to the time of dilution of the reaction mixture was estimated in a similar manner, using the elapsed time and recorded curve of each sample. The limiting absorbance (A_∞) for a series of samples was obtained by diluting each of several 110-µl. samples with 3 ml. of 0.1 *N* sodium hydroxide and measuring absorbance at 357 nm. Values obtained in this fashion agreed within ±0.5% with equilibrium measurements made on regular samples.

C. Partitioning of Pilocarpine between Chloroform and Aqueous Bicarbonate Solutions—The partition coefficient of pilocarpine between chloroform and 0.15 *M* aqueous potassium bicarbonate solution was found to be about 40. The procedure consisted of preparing solutions of known concentration of pilocarpine nitrate in water. Some exact volume (V_0) of chloroform and some exact volume (V_a) of aqueous pilocarpine nitrate solution of known concentration (C_p) were placed in a separator. A quantity of solid anhydrous potassium bicarbonate sufficient to make the aqueous volume 0.15 *M* in bicarbonate was then added to the separator. The contents were shaken vigorously and allowed to separate. The chloroform layer was drained off into a suitable stoppered centrifuge tube. To avoid the problem associated with the incomplete recovery of the chloroform from the separator, some volume (V_t) of the separated chloroform layer was pipeted into another tube. (For example, if 5 ml. of aqueous solution and 5 ml. of chloroform were used in the separator, after extraction the chloroform layer was separated and 4 ml. was pipeted into another container. V_t refers to the 4 ml. which, in this example, represented 80% of the total pilocarpine in the 5 ml. of chloroform used.) The measured volume of chloroform was then evaporated to dryness in an oil bath at about 65°. The residue was dissolved in 5 ml. of glacial acetic acid and then titrated with 0.01 *N* perchloric acid in glacial acetic acid, using 2 drops of 0.2% *p*-naphtholbenzein in glacial acetic acid as the indicator. The amount of pilocarpine, in moles, found in the titrated sample was multiplied by V_0/V_t. The value thus obtained was Q_0, the moles found in the total chloroform layer. The quantity of pilocarpine nitrate in the original aqueous solution was $V_aC_p = Q_t$ and represented the total moles of pilocarpine in the system. Thus, $Q_t - Q_0 = Q_a$, the moles of pilocarpine in the aqueous layer after extraction. The extraction coefficient can then be calculated from:

$$K_{CHCl_3/aqueous} = \frac{C_0}{C_a} = \frac{Q_0 V_a}{Q_a V_0} \qquad \text{(Eq. 1)}$$

D. Preparation of Pilocarpic Acid and Isopilocarpic Acid Solutions—Solutions of pilocarpic acid were prepared by pipeting 2 ml. of a 1% aqueous pilocarpine nitrate solution into a 100-ml. flask and adding 5 ml. of 0.1 *N* aqueous sodium hydroxide. The resulting solution was allowed to stand for 2–4 hr. to assure complete hydrolysis. Then 5 ml. of 0.1 *N* aqueous hydrochloric acid was added[4], and the solution was immediately brought to volume with water at 25°. Solutions of the desired concentration of pilocarpic acid were then prepared by dilution with water. The pilocarpic acid solutions were used on the day they were prepared. An identical procedure utilizing isopilocarpine nitrate was used for preparing isopilocarpic acid.

E. Analysis of Aqueous Solutions Containing Pilocarpine and Pilocarpic Acid—Solutions containing known concentrations of pilocarpine and pilocarpic acid were prepared by admixture of stock solutions. A portion of each of the resulting solutions was treated as described in Part A, and the observed rate constant, $k'_{obs.}$, was calculated. Another portion, consisting of 5 ml., was extracted with 5

[1] Mallinckrodt.
[2] Aldrich Chemical Co.
[3] These tubes were found to be suitable reaction vessels due to their shape, ease of handling, and the graduate markings which served as a rapid check to ensure that correct volumes of the various fluids had been added.

[4] The pH of the solution after addition of the hydrochloric acid was not allowed to fall below 4.0 in order to avoid any significant cyclization of pilocarpic acid to pilocarpine.

ml. of chloroform as described in Part D. Four milliliters of the chloroform extract was evaporated to dryness, and the residue was dissolved in 4 ml. of water. This solution was then treated as described in Part A, and the observed rate constant, $k_{obs.}$, was determined. It was approximated that extraction of pilocarpine into chloroform was complete; thus the difference between the observed rate constants was related to the concentration of pilocarpic acid, as shown in Eq. 8.

F. Temperature Dependence of Hydrolysis of 2,4-Dinitrophenyl Acetate—The observed rate constant for the hydrolysis of 2,4-dinitrophenyl acetate was carried out at 15, 25, and 35°, using the procedure described in Part A. One sample solution contained 15.4 mcg. of pilocarpine/ml. while the other contained no pilocarpine.

G. Effects of Sodium Chloride—Aqueous solutions containing 30 mcg. of pilocarpine/ml. were prepared. The 2,4-dinitrophenyl acetate solution was the same as used in Part A. Borate buffers of varying compositions were used in order that the final pH of the reaction mixture was 8.2. As the sodium chloride concentration was increased, the ratio of volumes of boric acid to borate solution was increased from 5:40 for the solution containing no sodium chloride to 12:40 for the 5% NaCl solution to 16.5:40 for the solutions containing 10% sodium chloride.

H. Determination of Catalytic Effects of Pilocarpine on Hydrolysis of 2,4-Dinitrophenyl Acetate at pH 7.9 and 8.5—The procedure used was the same as that described in Part A, except for the pH of the borate buffers used. Borate buffers prepared by mixing 40 ml. of the boric acid and 10 ml. of the borate stock solution had a pH of 7.82. After addition of the ester and sample solutions, the pH immediately rose to a value of 8.50. When 47 ml. of boric acid solution was mixed with 3 ml. of borate stock solution, the pH = 7.24; the pH of the solution obtained after addition of ester and sample was 7.90.

RESULTS AND DISCUSSION

Theoretical Considerations—The catalyzed hydrolysis of various esters by imidazole and imidazole derivatives was extensively studied (3–8). It was shown in the case of activated esters, such as the nitrophenyl acetates, that the catalysis at relatively neutral pH is due to nucleophilic attack of the free form of imidazole on the carbonyl carbon of the ester (4–6). The *N*-acyl imidazole thus produced then undergoes solvolysis to regenerate the imidazole. The present study was based on the predictions that pilocarpine, which contains a substituted imidazole, would also be an effective catalyst for the hydrolysis of certain esters, and the extent of catalysis would be a measure of the pilocarpine concentration. Based on the large body of data available for the hydrolysis of *p*-nitrophenyl acetate, it would ordinarily have been the substrate of choice in the present investigation. However, the half-life of that ester is measured in days in near neutral aqueous solution while the half-life of 2,4-dinitrophenyl acetate (DNPA) is of the order of hours under similar conditions (4). Thus, 2,4-dinitrophenyl acetate was chosen so that the assays could be done in a convenient time period. The hydrolysis of 2,4-dinitrophenyl acetate was monitored by observing the increasing absorption at 357 nm. resulting from production of the 2,4-dinitrophenoxide ion. The rate of hydrolysis of 2,4-dinitrophenyl acetate in borate-buffered solutions was found to be first order in the ester. The observed rate constant was found to be a linear function of the pilocarpine concentration, [*P*], at a given pH (Fig. 1). An acceptable rate expression for the reaction is shown in Eq. 2:

$$\frac{-d(\text{DNPA})}{dt} = (k_{H_2O} + k_{OH}[OH] + k_B[B] + k_p[P])\,[\text{DNPA}] \qquad \text{(Eq. 2)}$$

where k_{H_2O} is the pseudo-first-order rate constant for water hydrolysis, k_{OH} is the specific second-order rate constant for the hydroxide-ion-catalyzed reaction, and k_B and k_p are overall second-order rate constants for the buffer and the pilocarpine-catalyzed reaction, respectively. With any one given set of reaction conditions, such as in Fig. 1, the values of k_{H_2O}, k_{OH} [OH], and $k_B[B]$ became constant and Eq. 2 simplified to Eq. 3:

$$\frac{-d[\text{DNPA}]}{dt} = (k_0 + k_p[P])\,[\text{DNPA}] \qquad \text{(Eq. 3)}$$

where $k_0 = k_{H_2O} + k_B[B] + k_{OH}[OH]$. Thus, the observed rate constant for hydrolysis of 2,4-dinitrophenyl acetate under specified conditions may be expressed as:

$$k_{obs.} = k_0 + k_p[P] \qquad \text{(Eq. 4)}$$

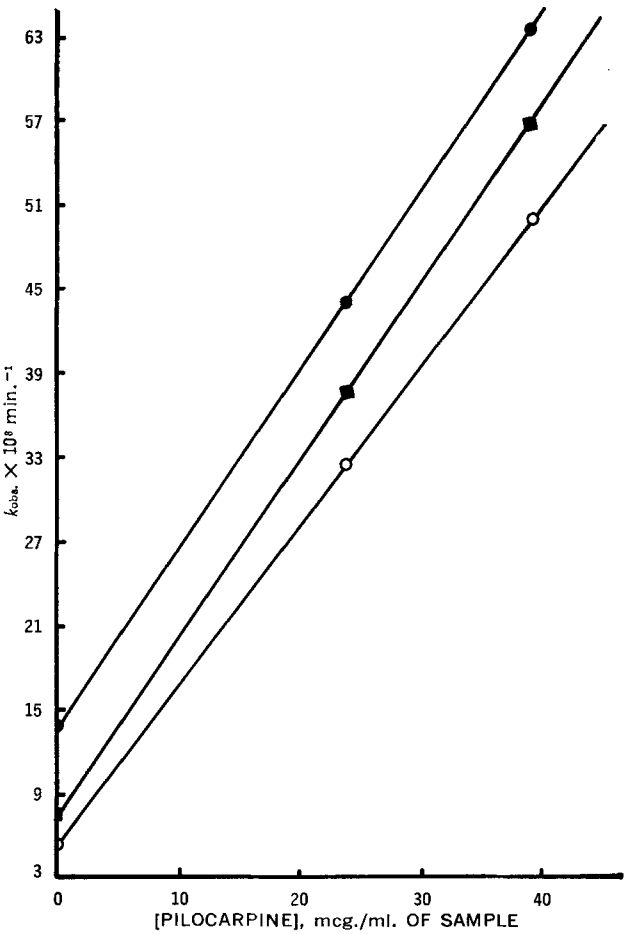

Figure 1—*Effects of pH and pilocarpine concentration on the observed rate of hydrolysis of 2,4-dinitrophenyl acetate in borate buffer solution at 25°. Key: ●, pH 8.5; ◆, pH 8.2; and ○, pH 7.9.*

The sensitivity of an assay procedure for pilocarpine based on Eq. 4 is obviously a function of the difference between k_0 and $k_{obs.}$ and the accuracy and reproducibility of their determination. For a given level of variation in reproducibility, the larger k_p is relative to k_0, the more sensitive will be the method. Thus, the ratio of k_p/k_0 should be maximized to maximize the sensitivity (10). To the end that k_0 be kept small, the buffer system chosen was borate, which apparently is a poor catalyst for ester hydrolysis. Also the buffer concentration employed was only that necessary to give suitable buffer capacity over the range of pilocarpine and ester concentrations employed.

Effects of pH—The effect of pH on the observed rate constant for 2,4-dinitrophenyl acetate hydrolysis is shown in Fig. 1. The k_p/k_0 values decrease from about 0.28 at pH 7.9 to 0.18 at pH 8.2 to 0.09 at pH 8.5. The system with pH 8.2 was chosen for further study and the development of the analytical procedure. The choice of these conditions represented a compromise between sensitivity and ease of application of the conditions to a routine analytical procedure, since at pH 8.2 the uncatalyzed reaction had a half-life of about 95 min., as compared to about 2.5 hr. at pH 7.9. In addition, the buffer capacity of the system at pH 7.9 was barely sufficient to maintain a constant pH.

Kinetic Analytical Procedure—Figure 2 shows the linearity of the dependence of $k_{obs.}$ on [*P*] at pH = 8.2. The least-squares best fit calculation yielded $k_0 = 7.2 \times 10^{-3}$ min.$^{-1}$ and $k_p = 1.28 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$. Variations in repeated measurements were found to be less than ±5%. The experimental method used for the analytical procedure and for the determination of the rate dependence of 2,4-dinitrophenyl acetate hydrolysis on [*P*] consisted of mixing

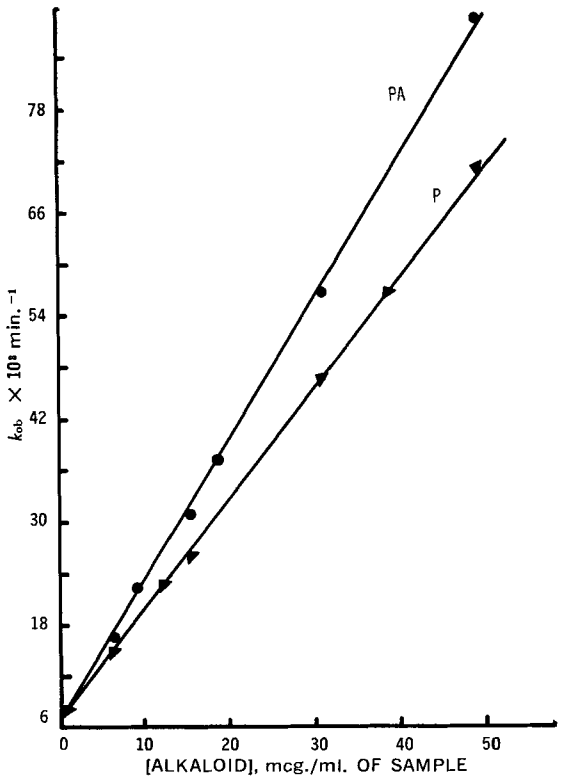

Figure 2—$k_{obs.}$ *for the hydrolysis of 2,4-dinitrophenyl acetate in borate buffer, pH 8.2, at 25° as a function of the concentration of the alkaloid pilocarpine* (P) *and pilocarpic acid* (PA) *obtained by hydrolysis of pilocarpine. Intercept* = k_0 = *7.2* × 10^{-3} *min.*$^{-1}$; *slope* (PA) = k_{PA} = *1.63* × 10^{-3} *ml./mcg.-min.; and slope* (P) = k_p = *1.28* × 10^{-3} *ml./mcg.-min.*

4 ml. of the sample solution containing pilocarpine as the nitrate salt, 1 ml. of 0.25 *M* pH 7.46 borate buffer, and 0.5 ml. of 2,4-dinitrophenyl acetate in acetonitrile. The addition of the sample and ester solution resulted in a rise in the pH to a value of 8.2, which remained constant throughout the reaction.

Several approaches were used in obtaining the absorbance–time data necessary for the calculation of the observed rate constant and [*P*]. The most convenient method consisted of making only three measurements at various times: an initial absorbance (A_0) at the beginning of the reaction (time = t_0), an absorbance (A_t) at a suitable time (t) during the reaction, and finally the limiting absorbance (A_∞) at some time (t_∞) after the reaction is complete. Using that data, $k_{obs.}$ was calculated according to Eq. 5:

$$k_{obs.} = \frac{2.303}{t - t_0} \log \left(\frac{A_\infty - A_t}{A_\infty - A_0} \right) \qquad \text{(Eq. 5)}$$

This method yielded results completely comparable to those obtained by a continuous recording of the absorbance changes. In addition, it had the advantage of greater efficiency with respect to instrument and personnel time required for each analysis.

Table I—Results of Kinetic Determination of Pilocarpine in Aqueous Solution

—Pilocarpine, mcg./ml.— Added	Found (mean)	Number of Determinations	Range of Results, mcg./ml.
46.1	46.8	2	44.4–48.4
43.0[a]	42.0	4	41.3–43.2
36.9	35.9	2	35.9–36.0
30.7[a]	30.3	11	29.3–31.7
18.4	18.3	3	18.2–18.6
12.3	12.2	2	12.1–12.3
6.1	6.4	2	6.3–6.4

[a] Values are the result of pooled data from runs on different days using different stock solutions.

Table II—Effects of Temperature on k_0, k_p, and k_p/k_0

Temperature	k_0, × 10^3 min.$^{-1}$	k_p, × 10^3 min.$^{-1}$	k_p/k_0
15°	2.7	0.77	0.28
25°	7.2	1.28	0.18
35°	19.5	1.88	0.01

Results of several analyses are shown in Table I. In all cases, the procedures used gave accuracy and precision of ±5%. All results are expressed as micrograms per milliliter of pilocarpine in the sample solution.

Effects of Temperature—The effect of temperature on the $k_{obs.}$ for the hydrolysis of 2,4-dintrophenyl acetate in the presence and absence of pilocarpine was investigated. It was expected that the pilocarpine-catalyzed rate would exhibit a lesser temperature dependence than the spontaneous rate. The observed rates of hydrolysis of 2,4-dinitrophenyl acetate was studied at 15, 25, and 35° in samples that differed only in that one series of solutions contained 15.4 mcg. of pilocarpine/ml. of sample while the other series contained no pilocarpine.

An Arrhenius plot of the data obtained showed a marked difference between the apparent activation energies of k_0 and k_p, the former being about 19.7 kcal./mole and the latter only 8.6 kcal./mole. This value of k_p compares favorably with the value of 8.4 kcal./mole obtained by Bruice and Benkovic (8) for the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate. This observed difference in the temperature dependence of k_0 and k_p is important from the standpoint of the sensitivity of the analytical procedure. As previously discussed, the larger the ratio k_p/k_0, the greater is the sensitivity of the method. Table II contains the k_0, k_p, and k_0/k_p values obtained at the various temperatures. Inspection of this data suggests that, all other considerations being equal, a threefold increase in sensitivity can be realized by carrying out the kinetic analysis at 15° rather than 35°.

Effects of Sodium Chloride—Kinetic analyses of pilocarpine in normal saline solution were found to give significantly higher values when compared to solutions containing little or no inorganic salts. To determine the cause of these high values, two series of solutions containing 0, 5, and 10 w/v % sodium chloride were prepared. One series contained 30 mcg. of pilocarpine/ml. of sample while the other had no pilocarpine. Each of these solutions was then diluted

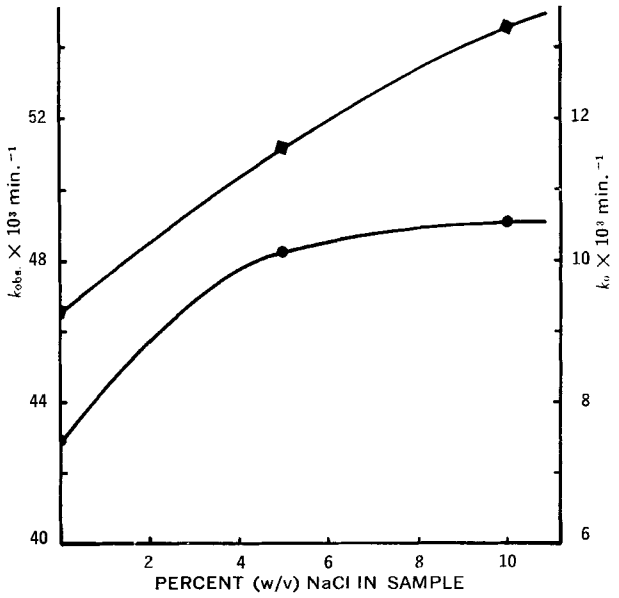

Figure 3—*Effect of sodium chloride concentration in the sample on* $k_{obs.}$ (◆) *and* k_0 (●) *at 25°. The scale on the left axis pertains to* $k_{obs.}$, *while that on the right axis pertains to* k_0.

Table III—Results of Kinetic Determination of Pilocarpine and Pilocarpic Acid in Solutions Containing Both Alkaloids

Sample[a]	Pilocarpine, mcg./ml. Added	Pilocarpine, mcg./ml. Found	Pilocarpic Acid[b], mcg./ml. Added	Pilocarpic Acid[b], mcg./ml. Found	Percent of Theoretical Found: Pilocarpine	Percent of Theoretical Found: Pilocarpic Acid
A	30.8	30.8	15.4	15.7	100.0	101.9
B	24.7	24.4	10.8	11.3	98.8	104.6

[a] Each sample was done in duplicate, and mean values were used. [b] In terms of pilocarpine hydrolyzed.

according to the normal procedure with a 0.25 *M* borate buffer of a suitable pH such that, upon dilution with the sample and addition of the ester solution, the final pH was 8.20–8.25 for all. The rate of hydrolysis of 2,4-dinitrophenyl acetate was then followed at 25° under the described conditions. Figure 3 shows that while increasing concentrations of sodium chloride increase both $k_{obs.}$ and k_0, the effects become less pronounced at higher salt concentrations. Since the relative magnitudes of the observed changes in k_0 and $k_{obs.}$ were similar, there would be no advantage realized, relative to sensitivity, by the addition of sodium chloride to the solution to be analyzed.

From these data, it can be seen that the 0.9% sodium chloride concentration of normal saline would be expected to have produced a maximum increase in $k_{obs.}$ of less than 1×10^{-3} min.$^{-1}$. The significantly larger deviation, which prompted this part of the study, could not be explained on the basis of salt catalysis. During these studies, it was noted, however, that relatively low concentrations of sodium chloride significantly increased the pH of the borate buffers; this effect was apparently responsible for the increased rates observed. These studies on salt effects did, however, point out the necessity of standardizing the reaction by use of standard solutions that are quite similar to those to be assayed.

Degradation Products of Pilocarpine—The products of the degradation of pilocarpine in aqueous solution have been suggested to be isopilocarpine (12, 13), isopilocarpic acid (12, 14, 15), and pilocarpic acid (14–16). The catalytic effects of each of these compounds on the hydrolysis of 2,4-dinitrophenyl acetate was investigated. The acids were prepared by alkaline hydrolysis of the appropriate parent alkaloids. The catalytic constants obtained for isopilocarpic acid[5], pilocarpic acid[5], and isopilocarpine were $k_{IA} = 1.59 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, $k_{PA} = 1.63 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, and $k_I = 1.27 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, respectively. The linear relationship between $k_{obs.}$ and $[PA]$ is shown in Fig. 2. When these values are compared with $k_p = 1.28 \times 10^{-3}$ ml. mcg.$^{-1}$ min.$^{-1}$, it is apparent that the catalytic effects of isopilocarpine and pilocarpine are the same and the catalytic effects of the acids are the same but greater than those of the intact alkaloids. The larger catalysis exhibited by the acids is believed to be due to a field effect, since they are charged species under the conditions used. Since pilocarpine and its most common degradation product catalyze the hydrolysis of 2,4-dinitrophenyl acetate, the described kinetic method may be used to assay quantitatively for any of these substances. However, it is necessary to separate the various species and, at present, there is no method by which this can be done.

The problem is simplified considerably if two conditions are assumed: (*a*) degradation of pilocarpine results in the production of pilocarpic and isopilocarpic acid only, and (*b*) the method need only distinguish between the intact pilocarpine and the therapeutically inactive acids[6]. The first assumption appears valid on the basis of recent work that showed the rate of disappearance of pilocarpine in neutral to alkaline aqueous solutions to be about the same as the rate of appearance of the acids (15, 16). The second assumption is also realistic, since only pilocarpine has been shown to have any therapeutic activity. Acceptance of these two conditions simplifies the problem to one of simply separating pilocarpine from the acids.

[5] Concentrations of the acids are calculated on the basis that 1 mcg. of the parent alkaloid is hydrolyzed to 1 mcg. of the respective acid.

[6] The significantly different rates of closure of pilocarpic acid and isopilocarpic acid to pilocarpine and isopilocarpine, respectively, were reported (15). This information may be the basis for a procedure for the analysis of mixtures of the two substances.

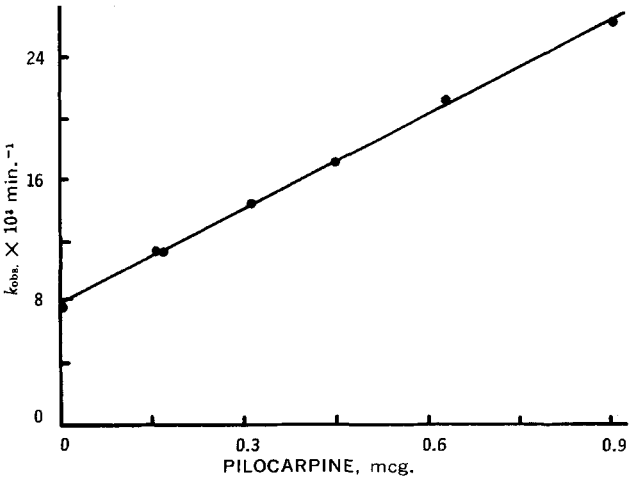

Figure 4—*Relationship between* $k_{obs.}$ *at 25° and amount of pilocarpine in 100 µl. of sample; total volume of reaction mixture is 110 µl.; buffered with borate at pH 8.2.*

Extraction Studies—Since pilocarpine is freely soluble in chloroform and pilocarpic acid and isopilocarpic acid would be in zwitterionic or anionic form at neutral to alkaline pH, and thus unlikely to be soluble in chloroform, extraction of aqueous solutions with chloroform was studied. Aqueous solutions of pilocarpine nitrate were made slightly alkaline (pH 8.5) by addition of solid anhydrous potassium bicarbonate at a concentration of 0.15 *M*. Immediately, the aqueous solution was extracted with a known volume of chloroform and the chloroform layer separated. The pilocarpine concentration in the chloroform extract was assayed both by nonaqueous titration and by the kinetic method. The results from the volumetric assays showed that 97.6% or more of the pilocarpine was extracted into chloroform when equal volumes of chloroform and aqueous solution were used. Results based on the kinetic assay indicated nearly 100% extraction under the same conditions. Based on these findings, the calculated partition coefficient[7] was $K_{o/a} \geq 40$. Similar studies on pilocarpic acid showed no detectable extraction of the pilocarpate species into the chloroform layer.

Determination of Pilocarpine and Pilocarpic Acid—Based on the results of the extraction studies, a routine was developed whereby the kinetic method could be used to determine the composition of aqueous solution containing both pilocarpine and pilocarpic acid. Solutions containing pilocarpine and pilocarpic acid were prepared by admixture of known volumes and concentrations of pilocarpine nitrate solution and pilocarpic acid solution. The observed rate constant for the hydrolysis of 2,4-dinitrophenyl acetate under normal conditions was determined. In addition, a volume of the solution containing both species was extracted with chloroform as previously discussed. A known fraction of the chloroform solution was warmed to remove all chloroform. The residue was dissolved in water, and the observed rate constant for the hydrolysis of 2,4-dinitrophenyl acetate was determined for these solutions by the kinetic procedure. From the observed rate constants and the calibrated curves in Fig. 2, the concentrations of pilocarpine and pilocarpic acid were determined according to the following equations.

For solutions containing pilocarpine (P) and pilocarpic acid (PA):

$$k'_{obs.} = k_0 + k_p[P] + k_{PA}[PA] \qquad \text{(Eq. 6)}$$

and for solutions from the chloroform extract:

$$k_{obs.} = k_0 + k_p[P] \qquad \text{(Eq. 7)}$$

$[P]$ in the solutions from the chloroform extract can be obtained directly from Fig. 2. The total pilocarpine in the original solution can then be calculated on the basis that virtually all pilocarpine is extracted. Subtracting Eq. 7 from Eq. 6 gives:

$$k'_{obs.} - k_{obs.} = k_{PA}[PA] \qquad \text{(Eq. 8)}$$

[7] $K_{o/a}$ = concentration of pilocarpine in chloroform/concentration of pilocarpine in 0.15 *M* aqueous potassium bicarbonate solution.

The left side of Eq. 8 is that part of $k'_{obs.}$ due to the catalytic hydrolysis of 2,4-dinitrophenyl acetate by pilocarpic acid; thus, $[PA]$ can be calculated from the catalytic constant for pilocarpic acid obtained from Fig. 2. Some results obtained by the method are found in Table III.

Effects of Other Nucleophiles—While no experimental attempt was made in this study to determine the effect of other nucleophiles on the rate of hydrolysis of 2,4-dinitrophenyl acetate, a substantial amount of data is available in the literature on the effects of nucleophiles on the hydrolysis of *p*-nitrophenyl acetate (8, 17). Since a free energy relationship exists between the rates of alkaline hydrolysis and imidazole-catalyzed hydrolysis of *p*-nitrophenyl and 2,4-dinitrophenyl acetate (4), the same rank and magnitude of difference in nucleophilic catalysis between imidazole and other nucleophilic substances could be expected.

In addition, since the catalytic effects of imidazole and pilocarpine are about the same, the results of Jencks and Carriuolo (17) on *p*-nitrophenyl acetate could be expected to apply in the present case. This suggests that the commonly found nucleophiles (such as carboxylate, diphosphate, and even aniline) in concentrations 10 times as great as the pilocarpine would not significantly affect the results obtained with the kinetic method. On the other hand, great care must be taken to exclude peroxides, hydrazine, hydroxylamines, and other such potential nucleophiles; even in trace concentrations, they would markedly alter the rate of the reaction and, therefore, the analytical results.

During this study, reagent grade dioxane, without further purification, was used once as a solvent for 2,4-dinitrophenyl acetate. When the solution was added to the borate-buffered sample containing no pilocarpine, the reaction went to completion in less than 3 min. This tremendous catalysis was attributed to the presence of some peroxides or peroxy acids in the dioxane, which is in qualitative agreement with published information (17).

Method for Microanalysis—If a sample containing less than 1–2 mcg. of pilocarpine is to be analyzed, the previously described method cannot be employed directly since the degree of variation in k_0 alone could exceed the catalytic effect produced by the alkaloid in the volume of solution used. However, since the method is dependent on concentration rather than amount, it should be possible to work with sufficiently small volumes of solution such that the concentration would be large enough to obtain meaningful results. For instance, if 10 ml. of solution contained 1 mcg. of pilocarpine, the normal analytical procedure could not be expected to detect this concentration. If, however, that solution is extracted with chloroform, as previously discussed, evaporated to dryness, and dissolved in 0.1 ml. of a suitable buffer system, the concentration would then be 10 mcg./ml., well within the capabilities of the kinetic procedure. Obviously, this same reasoning holds for solution volumes of less than 1 ml. having a concentration of 3–4 or more mcg. of pilocarpine/ml.

The application of the kinetic method to submicrogram quantities of pilocarpine in small volumes of solution was studied. Known amounts of pilocarpine as the nitrate salt were dissolved in a dilute buffer solution comprised of 4 parts water and 1 part 0.25 *M*, pH 7.46, borate buffer. One hundred microliters of this solution was then mixed with 10 μl. of acetonitrile solution containing 1.25 mg. of 2,4-dinitrophenyl acetate/ml. At some time, t_d, the solution was diluted with dilute buffer, and the absorbance corresponding to the time of dilution was suitably estimated. The initial absorbance (A_0) and the final absorbance (A_∞) were determined by appropriate methods, and the observed rate constants were calculated (Fig. 4). A linear dependence between $k_{obs.}$ and weight of pilocarpine was observed. Again, variation in values obtained was less than $\pm 5\%$.

On the basis of these data, it appears the procedure should be suitable for the analysis of quantities of pilocarpine as low as 100–150 ng. The intercept obtained in these studies is about 5–10% higher than that obtained with the method employing larger volumes. The value of the slopes by both methods are nearly identical when calculated in terms of the same units and concentrations. Whether or not the difference in intercepts is real or due to experimental error is not certain.

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In Vitro Dynamic Dialysis Technique to Determine Solute–Protein Binding Interactions

PETER C. FARRELL, ROBERT P. POPOVICH, and ALBERT L. BABB

Abstract □ A novel technique to evaluate solute–protein binding characteristics is presented with some preliminary data. The technique is based upon a dual closed-loop dialysis system using radioactive tracers. The method permits easy determination of binding interactions and membrane permeabilities at a series of temperatures and, thereby, readily enables evaluation of enthalpy and entropy effects of binding interactions. At present, the technique is being used to study protein interactions of various waste metabolites, drugs, and essential amino acids and hormones.

Keyphrases □ Solute–protein binding—determination, *in vitro* dialysis □ Protein–solute binding—determination, *in vitro* dialysis □ Dialysis, closed loop—solute–protein binding levels, hemodialysis removal rates □ Hemodialysis removal rates—dynamic closed-loop dialysis

Present techniques to determine solute–protein interactions can be divided into three categories: (*a*) direct visualization of the solute–protein complex, (*b*) electrostatic methods, and (*c*) indirect or substractive methods.

A complete review of the more common analytical procedures comprising these categories is provided by Steinhardt and Reynolds (1). Each technique has limitations; the choice of method, to a large extent, depends upon the system to be investigated.

For both direct methods and electrostatic procedures, there can be problems in distinguishing solute–protein interactions from system noise. These difficulties can be caused by a number of factors but are caused primarily by equipment sensitivity and solvent artifacts.

The subtractive methods are also plagued with certain artifacts. For example, methods such as equilibrium dialysis, which employ semipermeable membranes, have the inherent complications of osmotic and Donnan effects. Probably the major problem with indirect observations of binding is that if the binding is small, there is an inherently large error, because the concentration of bound species depends upon the difference between two large numbers, at least one of which is subject to error.

The *in vitro* dynamic method presented here does not purport to overcome all difficulties associated with the determination of ligand–protein interactions, but it provides certain advantages over presently available methods as well as accurately determining the degree of binding. The major advantages of this technique are:

1. It permits rapid evaluation of solute-binding characteristics.
2. Measurements can be readily performed at a series of temperatures to evaluate the enthalpy and entropy effects associated with binding interactions.
3. Simultaneous measurements of membrane permeability to various solutes are provided.
4. The technique is completely analogous to hemodialysis and permits evaluation of blood solute removal rates as well as quantifying the effect of binding interactions on dialyzer performance.

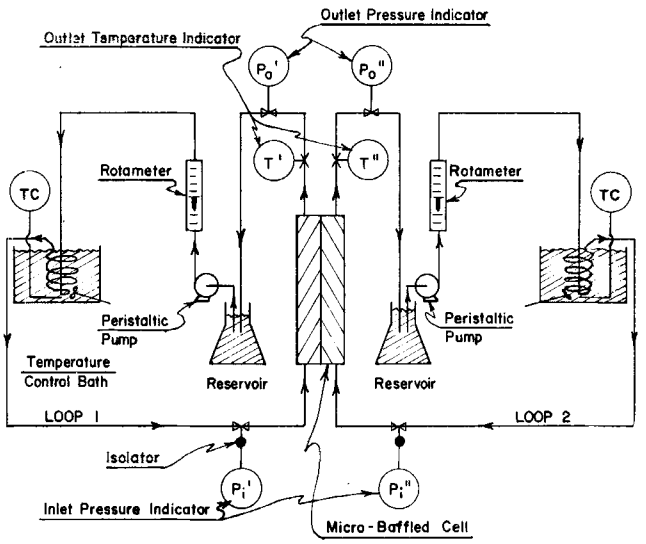

Figure 1—*Dual closed-loop dialysis system for* in vitro *binding studies.*

MATHEMATICAL MODEL

The proposed dynamic technique to determine solute–protein binding interactions is a kinetic method involving retardation of solute removal by reduction of solute activity as the result of binding. The membrane used in this study is regenerated cellulose[1], which is essentially impermeable to serum albumin and other plasma proteins (2). Consequently, any solute bound to plasma proteins becomes nondiffusable, resulting in a measurable decrease in the effective concentration driving force for molecular transfer.

A solute, exhibiting a binding affinity to proteins, exists as a free species (C_f) and a nondiffusing bound species (C_b). By assuming reversible equilibrium, the free–bound exchange can be written as:

$$\frac{\partial C_b}{\partial t} = \xi C_f - \eta C_b \qquad \text{(Eq. 1)}$$

where ξ and η are first-order association and dissociation constants.

If the membrane permeability (P_m) is the limiting resistance to mass transfer, then the membrane permeation condition is represented as:

$$\frac{dN}{dt} = -P_m A(C_{f_1} - C_{f_2}) \qquad \text{(Eq. 2)}$$

where N = mass of solute transferred, and A = effective membrane area.

If there is a significant fluid-film mass transfer resistance on either side of the membrane, then, in Eq. 2, P_m should be the overall mass transfer coefficient, h_o, defined by:

$$R\Sigma = \frac{1}{h_o} = R_m + R_{f_1} + R_{f_2} \qquad \text{(Eq. 3)}$$

[1] Cuprophan, PT 150.

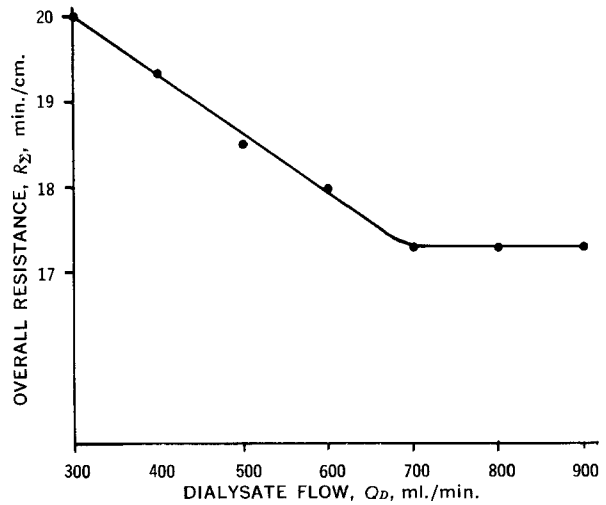

Figure 2—*Urea permeability as a function of flow rate, injection grade saline at 37°.*

where $P_m = 1/R_m$, and R_{f1} and R_{f2} are the resistances due to solute–solvent interactions on either side of the membrane.

From Eq. 1, under equilibrium conditions, $\partial C_b/\partial t$ is negligible in comparison with either term on the right-hand side. To a good approximation, $\xi C_f \simeq \eta C_b$, or:

$$\rho = \xi/\eta = C_b/C_f \qquad \text{(Eq. 4)}$$

where ρ is now referred to as the solute–protein binding coefficient.

The condition of equilibrium assumes that the time constants for binding and debinding are small compared to the smallest time constant in the system. There is a paucity of data on the kinetics of binding, but available data (3) indicate that this assumption is reasonable.

If μ refers to the plasma or human serum albumin volume fraction exclusive of proteins, it can easily be derived from Eqs. 4 and 2 that the membrane permeability can be computed for the following cases (4).

Case 1—Protein fraction in both loops 1 and 2 (Fig. 1):

$$P_m = -\frac{1}{At}\left[\frac{\mu_1\mu_2V_1V_2(1+\rho_1)(1+\rho_2)}{\mu_1V_1(1+\rho_1)+\mu_2V_2(1+\rho_2)}\right]\ln\left[\frac{C_1-\psi C_1}{C_1-\psi C_2^0}\right] \qquad \text{(Eq. 5)}$$

where $\psi = [\mu_1(1+\rho_1)]/[\mu_2(1+\rho_2)]$.

Case 2—Loop 1 contains dissolved protein (Fig. 1):

$$P_m = -\frac{1}{At}\left[\frac{\mu_1(1+\rho_1)V_1V_2}{\mu_1(1+\rho_1)V_1+V_2}\right]\ln\left[\frac{C_1-\mu_1(1+\rho_1)C_2}{C_1^0-\mu_1(1+\rho_1)C_2^0}\right] \qquad \text{(Eq. 6)}$$

Case 3—No excluded volume or protein fraction ($\mu_1 = \mu_2 = 1$ and $\rho_1 = \rho_2 = 0$):

$$P_m = -\frac{1}{At}\left(\frac{V_1 \cdot V_2}{V_1+V_2}\right)\ln\left[\frac{C_1-C_2}{C_1^0-C_2^0}\right] \qquad \text{(Eq. 7)}$$

The last equation represents the usual equation for calculating membrane solute permeability for a point dialyzer.

Equation 6 does not yield an explicit solution for the solute–protein binding coefficient ρ. Thus, the binding coefficient must be determined using an iterative technique. A digital computer program using regression analysis was written for this purpose (4). The experimental approach to determine the binding coefficient is as follows.

First, a statistically significant P_m is computed from Eq. 7 for a nonbinding environment such as saline. A separate run is then conducted from a potential solute-binding medium such as human serum albumin or plasma. Allowance is made for protein exclusion (μ_1 in Eq. 6). If the calculated P_m from Eq. 6 differs statistically from that previously obtained by Eq. 7, then binding is indicated. The correct value for ρ is then determined by increasing its value systematically from zero until (by successive iterations) the computed P_m in Eq. 6 equals the value of P_m computed from Eq. 7. When the equality is satisfied, the correct value of the binding coefficient is established. The concentrations C_1 and C_2 in Eqs. 5–7 refer to total concentrations in loops 1 and 2, respectively. Hence, in the case of Eqs. 5 and 6, C_1 and C_2 refer to the sum of free and bound solute concentrations. The other variables in the equations are well defined, with the exception of the solute–protein binding coefficient.

In using this technique, it is not mandatory that P_m be the membrane resistance solely, as long as identical experimental conditions are used for each solute in the different environments. However, an accurate assessment of solute permeability requires that fluid-film resistance be accounted for.

There are three approaches to obtain a true membrane permeability:

1. A correlation can be developed for the fluid-film mass transfer resistance so that this resistance can be subtracted from the overall resistance, as in Eq. 3.
2. A Wilson plot analogous to the heat transfer computation of Wilson (5) can be developed. This technique was used in mass transfer studies by Leonard and Bluemle (6).
3. Experiments can be run under such conditions that the turbulent boundary layer contributes negligibly to overall resistance.

The first approach depends upon developing a correlation of the form:

$$N_{SH} = aN_{Re}{}^bN_{Sc}{}^{1/3} \qquad \text{(Eq. 8)}$$

where the Sherwood and Reynolds numbers are based upon a characteristic length of the dialysate cell, such as channel height or axial length.

This approach was used very successfully by several researchers (7–10) for Leonard–Bluemle dialyzers, where the characteristic length is the membrane or cell diameter, and N_{Re} is the impeller Reynolds number. Smith *et al.* (7) characterized this type of dialyzer and developed a method whereby a true membrane permeability could be accurately determined.

The second approach is rather tedious and requires multiple runs to obtain a single permeability measurement. The third technique was used by Babb and his coworkers (11, 12); in the latter case, although the fluid-film resistance is not zero, its overall contribution can be made so low as to have a negligible effect on overall resistance, provided suitable experimental precautions are taken (4). This technique cannot be used as easily in Leonard–Bluemle dialyzers because of problems associated with membrane flutter (7), but this does not represent a serious problem for high flow in a rectangular dialyzer since transmembrane pressures are balanced and constant. This latter technique was chosen; as can be seen from Fig. 2, the overall resistance for urea transfer decreases to a minimum at a dialysate flow rate of Q_D = 700 ml./min. Figure 3 shows a similar plot for sucrose, where the overall resistance levels off at 600 ml./min. All experiments were conducted at Q_D = 800–900 ml./min. to ensure a negligible dialysate film resistance.

EXPERIMENTAL

A schematic diagram of the apparatus is shown in Fig. 1. The dialyzer used is a microbaffled unit, with a specially designed plastic innerlay containing machined baffles to induce turbulence. The average plate to membrane height is 1 mil. The present design is a modification of the B-G cell (11) and circumvents the problem of membrane puncture by nickel foam, degraded by fluid corrosion.

The apparatus consists of two separate loops with volumes of 170 ± 1 ml. Each loop contains a peristaltic pump, rotameters with needle valves to regulate pressure, temperature and pressure indicators, and a temperature-control bath able to control within ±0.05°[2].

In conducting runs, there are two possible approaches to sampling: (*a*) aliquots can be removed from the reservoir and replaced with a known concentration of solute (normally zero), or (*b*) aliquots can be taken without volume replacement such that the overall effect is negligible on the overall system volume.

[2] Equipment for use in such experiments is manufactured by Physio-Control Corp., Seattle, Wash., under license to the University of Washington.

Table I—Membrane Resistance and Permeability for Solutes Diffusing through Regenerated Cellulose[a] in Sterile, Isotonic Saline at 37°

Compound	Molecular Weight	Membrane Resistance $\pm\sigma_r$, min./cm.	Permeability, $\times 10^4$ cm./sec.
Urea[b]	60.1	16.8 ± 0.65	9.91
Guanidine	59.1	15.2 ± 0.65	10.94
Methylguanidine	73	18.3 ± 0.17	9.09
Creatinine	113.1	29.7 ± 0.53	5.62
Creatine	131.1	36.4 ± 0.39	4.58
Ur c acid[c]	168.1	36.5 ± 0.33	4.56
Lithium urate[d]	185.9	42.5 ± 0.63	3.92
Sucrose	342.3	85.5 ± 1.9	1.95
Adenosine triphosphate[e], 5.9 mg. %	507	1422.3 ± 52.9	0.12
Adenosine triphosphate, 5.9 mg. %	507	252.4 ± 5.3	0.66
Vitamin B_{12}[f]	1355	360.0 ± 5.7	0.46

[a] Cuprophan PT 150. [b] Range was 15.9–17.3 min./cm. [c] Solute concentration was 1–5 mg. %; range was 34.8–37.9 min./cm. [d] Solute concentration was 2–20 mg. %; range was 40.4–44.1 min./cm. [e] In deionized water; shows Donnan potential influence. [f] Range was 353.6–363.5 min./cm.

The first approach keeps an accurate V_1 and V_2, but there is the problem of lag time and the necessity for mass balance correction. The alternative approach results in a systematic volume error which depends upon aliquot size and number of samples. This latter approach is used. Eight to ten samples of 250 or 500 λ are taken over the course of a run. As the first and last samples do not involve volume considerations, the total volume error ranges from 0.9% to a maximum of 2.3%, with a typical error of less than 1%.

Concentration measurements are determined by liquid scintillation counting using radioactively tagged solutes (mostly ^{14}C and ^{3}H). This technique yields exceedingly accurate results. Counting efficiencies of greater than 90% for saline, human serum albumin, and plasma, and mass balance errors of better than 1.0% over 60-min. experimental runs are consistently obtainable. This assumes that there is no plating out of solute in either loop.

The scintillant cocktails are based upon the Beckman solubilizers (BBS-3 for plasma, human serum albumin, and saline; BBS-2 for blood samples), using Beckman's TLA as the fluor in reagent grade toluene. The scintillation counters used are Beckman's manual laboratory research counter (β-Mate II) and the Packard Tri-Carb Unit (model 3375). Eppendorf samplers are used for aliquot sampling at time intervals dependent upon the molecular weight of the transferring solute.

The main artifacts to be anticipated in using this technique are: (*a*) unaccounted for fluid-film mass transfer resistance, (*b*) osmotic effects, (*c*) ultrafiltration effects, (*d*) Donnan membrane potential effects, and (*e*) protein adsorption on the membrane.

The first point was already discussed. Furthermore, membrane permeability data obtained for urea, creatinine, and sucrose are in substantial agreement with published data (13).

Osmotic flow effects contribute negligibly to overall mass transfer characteristics. Estimates of the magnitude of flows indicate that its influence is insignificant (9) at physiological concentrations.

Ultrafiltration is accounted for by regulating backpressure on the exit streams of the dialysate cell. This procedure ensures that transmembrane pressures are balanced. Donnan effects, as shown by Smith *et al.* (7) and for adenosine triphosphate in this study (Table I), can be significant. However, for an isotonic saline environment, the influence of the Donnan potential is so small as to present a negligible source of error.

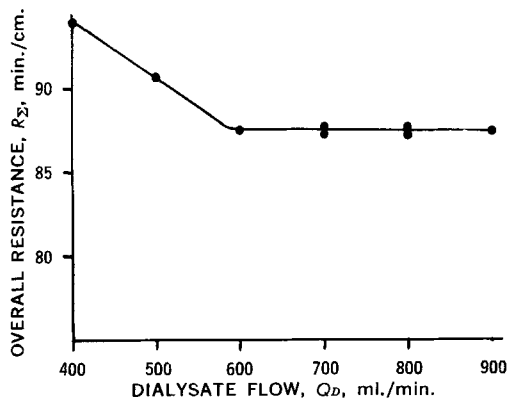

Figure 3—*Sucrose permeability as a function of flow rate, injection grade saline at 37°.*

Under normal hemodialysis conditions (laminar blood flow), it is reasonable to postulate that protein adsorption on the membrane may influence solute transfer rates. However, a recent study by Evans and Lines (14) indicated that possible thrombocytic, leucocytic, and proteinaceous deposition has a minimal effect on the blood clearance of urea and creatinine in multiple runs on a Kiil hemodialyzer. At the shear rates involved in this study, minimal interference from protein deposition would be expected. This is confirmed in this work for nonbinding solutes and in data reported by Colton *et al.* (2).

The membrane resistance shown in Tables I and II for those solutes not exhibiting binding (creatinine, creatine, vitamin B_{12}, and sucrose) are, within the limits of experimental error, the same for saline, human serum albumin, and plasma environments. Colton *et al.* (2), in studying the transport characteristics of blood-soaked cellulose membranes, reported only a 5% increase in membrane resistance for vitamin B_{12}[3] (mol. wt. = 1355) due to possible protein adsorption. These data are also within experimental error and are not significantly different from a normal saline environment.

RESULTS

Membrane permeability and resistance data for solutes of direct interest to hemodialysis studies are presented in Table I for diffusion through regenerated cellulose[1] at 37°. These data are for transfer from sterile, isotonic saline to the same solution on the other side of the membrane. With the exception of adenosine triphosphate, the membrane resistance increases approximately linearly with molecular weight, suggesting that the pore size of regenerated cellulose is considerably greater than the molecular size of vitamin B_{12}. The latter molecule closely approximates a sphere, with a diffusing area of about 223 $Å^2$ (13). In the case of adenosine triphosphate, there is a suggestion that molecular diffusion may not be occurring independently but that molecules may be associated in pairs. On the basis of molecular weight, a membrane resistance of about 130 min./cm. would be expected for adenosine triphosphate instead of the 252 min./cm. obtained. The higher resistance value for adenosine triphosphate in deionized water clearly shows the influence of the Donnan membrane potential due to the residual carboxylic groups of the cellulose.

Table II shows data on those solutes exhibiting marginal or negligible binding interactions with albumin and other plasma proteins. These data were confirmed independently by conventional ultrafiltration techniques. Table III presents data on the binding levels of two different forms of uric acid: lithium urate and virgin uric acid. The binding capacity of these two forms of uric acid differ markedly, and this fact caused considerable confusion in the

[3] Vitamin B_{12} (or one of its coenzyme forms) is known to bind reversibly at very low concentrations *in vivo* (15). This effect would be too small to be realized at 1 mg. % solute concentration.

Table II—Permeability and Resistance Data for Diffusion from Human Serum Albumin and Normal Human Plasma to Sterile, Isotonic Saline at 37° through Regenerated Cellulose[a]

Compound[b]	Medium	Apparent Membrane Resistance $\pm\sigma_r$, min./cm.	Actual Membrane Resistance $\pm\sigma_r$, min./cm.	Solute–Protein Binding Coefficient $\left(\frac{\text{g. bound/ml. plasma}}{\text{g. free/ml. plasma}}\right)$	Binding Level, %
Urea					
(100)	Human serum albumin[c] (50 g./l.)	18.0 ± 0.51	17.4 ± 0.46	0.015	1.5
(50)	Human serum albumin (50 g./l.)	15.9 ± 0.62	15.9 ± 0.62	—	—
(100)	Heparinized plasma	18.5 ± 0.40	17.3 ± 0.43	0.035	3.4
Creatinine					
(5)	Human serum albumin (50 g./l.)	30.3 ± 0.59	30.3 ± 0.59	—	—
(5)	Heparinized plasma	30.7 ± 0.73	30.7 ± 0.73	—	—
Guanidine					
(5)	Human serum albumin (50 g./l.)	22.2 ± 0.59	15.1 ± 0.65	0.195	16.3
(12)	Human serum albumin (50 g./l.)	20.1 ± 0.37	15.2 ± 0.18	0.120	10.7
Methylguanidine					
(6)	Human serum albumin (50 g./l.)	24.7 ± 0.8	18.5 ± 0.36	0.135	11.9
(6)	Heparinized plasma	26.7 ± 0.84	18.4 ± 0.25	0.225	18.4
Sucrose (20)	Human serum albumin (50 g./l.)	88.1 ± 1.53	88.1 ± 1.53	—	—
Vitamin B_{12}					
(1)	Human serum albumin (50 g./l.)	378.6 ± 5.9	378.6 ± 5.9	—	—
(1)	Citrated plasma	369.7 ± 6.4	369.7 ± 6.4	—	—
Creatine (5)	Human serum albumin (50 g./l.)	35.4 ± 0.32	35.4 ± 0.32	—	—
Adenosine triphosphate (5.9)	Human serum albumin (50 g./l.)	250.3 ± 4.9	250.3 ± 4.9	—	—

[a] Cuprophan PT 150. [b] Solute concentration (mg. %) in parentheses. [c] Albumisol (sterile, isoosmotic, pH ~7.0); Merck Sharp & Dohme.

literature (16–19). A standard technique to obtain high aqueous concentrations of the normally insoluble uric acid is to dissolve the acid with a solution of lithium carbonate at a concentration of 0.6 g./l. (20). This form of uric acid has been used in binding determinations in both plasma and human serum albumin and has resulted in considerable confusion in the reported binding levels of uric acid in normal human plasma.

From the data in Table I, it is observed that virgin uric acid has a considerably lower membrane resistance in saline solution at 37° (36.5 min./cm.) in comparison with lithium urate (42.5 min./cm.). The reduced permeability of the uricacid dissolved in lithium carbonate suggests possible chelating of lithium cations with the urate moiety. This form of complexing was observed by Albert (21) for divalent metal cations interacting with uric acid and other purines.

Table III shows that lithium urate has a considerably higher binding propensity for both human serum albumin and human plasma in comparison with virgin uric acid. For lithium urate in human serum albumin, the binding levels ranged from 20 to 40% whereas virgin uric acid had binding levels between 3 and 5%. In heparinized plasma (A + donors), the binding levels ranged from 40 to 55% whereas uric acid registered binding levels of about 5–8%. Representative data are given in Tables III and IV.

The mechanism by which the lithium urate binds is somewhat obscure, although Klotz and Loh Ming (22) established that both monovalent and divalent cations can form a bridge between protein and a normally nonbinding solute. However, this phenomenon has caused considerable confusion regarding reported binding data for uric acid to plasma proteins. Alvsaker (16–18) obtained comparable

Table III—Binding Levels and Membrane Resistance Data for Urate-Ion Diffusion through Regenerated Cellulose[a] from Human Serum Albumin and Plasma at 37°

Compound[b]	Medium	Apparent Membrane Resistance $\pm\sigma_r$, min./cm.	Actual Membrane Resistance $\pm\sigma_r$, min./cm.	Solute–Protein Binding Coefficient $\left(\frac{\text{g. bound/ml. plasma}}{\text{g. free/ml. plasma}}\right)$	Binding Level, %
Lithium urate (5)	Human serum albumin[c]	77.0 ± 1.92	42.2 ± 0.71	0.615	38.2
Lithium urate (10)	Human serum albumin	74.0 ± 1.48	42.1 ± 1.00	0.580	36.6
Lithium urate (10)	Heparinized plasma	111.2 ± 1.87	42.2 ± 0.8	1.205	55.0
Lithium urate (15)	Heparinized plasma	100.9 ± 2.00	42.1 ± 1.3	1.035	51.0
Lithium urate (10)	Citrated[d] plasma	62.9 ± 1.58	42.3 ± 1.37	0.350	26.0
Uric acid (1)	Human serum albumin	38.4 ± 0.36	36.7 ± 0.35	0.035	3.4
Uric acid (5)	Human serum albumin	39.1 ± 0.74	36.5 ± 0.41	0.050	4.8
Uric[d] acid (5)	Citrated plasma	38.6 ± 0.33	36.5 ± 0.40	0.035	3.4
Uric acid (10)	Heparinized plasma	42.0 ± 0.81	36.6 ± 0.68	0.090	8.8

[a] Cuprophan PT 150. [b] Solute concentration (mg. %) in parentheses. [c] Albumisol (50 g./l. sterile, isoosmotic); Merck Sharp & Dohme. [d] Demonstrates that citric acid interferes with urate-ion binding. This was later confirmed by ultrafiltration data (4).

Table IV—Ultrafiltration[a] Data for Solute Binding at 25°

Compound[b]	Medium	Original[c] Concentration (c.p.m.)	Filtrate Concentration	Binding Level, Percent Bound/Total
Urea (25)	Human serum albumin	57,390	58,568	1.6
Urea (25)	Heparinized plasma	65,804	67,730	3.5
Creatinine (5)	Human serum albumin	61,991	64,959	—
Creatinine (5)	Heparinized plasma	64,808	69,517	—
Lithium urate (5)	Human serum albumin	34,682	23,290	35.3
Lithium urate (5)	Heparinized plasma	34,674	18,029	51.2
Uric acid (5)	Human serum albumin	30,153	30,050	3.2
Uric acid (5)	Heparinized plasma	28,412	28,010	7.3

[a] Amicon Centri-flow membranes. [b] Solute concentration (mg. %) in parentheses. [c] Uncorrected for protein fraction volume exclusion.

binding data for both human serum albumin and plasma for uric acid predissolved in lithium carbonate, and Sheikh and Moller (19) reported the much lower binding levels obtained in this study for virgin uric acid. This issue was discussed in more detail elsewhere (23).

Table IV shows representative binding data, obtained by conventional ultrafiltration methods at 25°, which corroborate the *in vitro* closed-loop dialysis data.

The data presented here indicate that the binding levels of the major waste metabolites are small enough to be physiologically unimportant in terms of their influence on hemodialyzer performance. Also, the viability of using a dual closed-loop dialysis system to examine solute–protein binding phenomena is demonstrated. The standard deviations for those solutes exhibiting binding (Tables II and III) are comparable with equivalent data presented in Table I. Since membrane resistance data for binding solutes are obtained by an iterative procedure (4), it is encouraging that the standard deviations are so acceptable. Moreover, the fact that the dialysis technique yields binding levels equivalent to the ultrafiltration data (essentially an equilibrium method) suggests that binding equilibration for the solutes studied here is rapid. This lends support to the observations of Andreoli *et al.* (3).

Future work with this technique will center on the binding interactions of drugs and hormones that are reported to exhibit high degrees of reversible binding. For example, testosterone binding levels of 95% were reported by Hudson *et al.* (24) for normal males.

This technique not only provides estimates of the removal rates of various solutes during hemodialysis but also provides accurate determinations of solute–protein binding levels. Furthermore, because of the ease with which runs can be conducted at various temperatures, this technique can be used to calculate the variation of solute–protein binding coefficient as a function of temperature. The van't Hoff and Gibbs–Helmholtz relationships then enable enthalpy and entropy effects to be evaluated (4).

In summary, the dynamic closed-loop dialysis technique provides data of clinical importance and interest in permitting computation of: (*a*) solute removal rates for hemodialysis studies, (*b*) solute–protein binding levels, and (*c*) associated thermodynamic properties. This technique could prove useful as a clinical and research adjunct for both pharmacological and endocrinological investigations.

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Radioassay of Cortisol and Corticosterone by a Modified Competitive Protein-Binding Method

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Abstract □ A competitive protein-binding radioassay using adsorption was evaluated for the individual determination of cortisol and corticosterone. The competitive protein-binding assay using fuller's earth to separate bound and free cortisol was tested and found to offer no specificity for cortisol in favor of corticosterone. The introduction of a carbon tetrachloride/water partitioning procedure to separate corticosterone and cortisol made possible the individual determination of corticosterone and cortisol using the competitive protein-binding radioassay. The modified competitive protein-binding radioassay was evaluated as to precision, accuracy, and specificity; normal values of cortisol and corticosterone in human plasma were determined.

Keyphrases □ Cortisol, corticosterone in human plasma—determination, competitive protein-binding radioassay □ Radioassay, protein-binding—determination of cortisol, corticosterone in plasma □ Transcortin binding—cortisol–corticosterone separation, determination

The determination of cortisol and corticosterone in blood plasma is useful for evaluating the functional capacity of the adrenal cortex and for determining the amount of biologically active corticoid available to the tissues. This information is useful for clinical and investigational work. Murphy *et al.* (1) developed a method for the determination of plasma corticoids using the specific corticosteroid-binding globulin, transcortin. They named the method competitive protein-binding analysis (2). The method utilizes the rather specific binding properties of transcortin, which binds the steroids cortisol, corticosterone, and 11-deoxycortisol. Radioactive corticoid is added to a solution of diluted plasma, where the chief protein exerting steroid-binding effect is transcortin, and allowed to equilibrate. This forms the assay reagent.

Since the binding of the steroid is of a reversible nature, the addition of nonradioactive corticoid to the transcortin solution causes a change in the equilibrium such that the specific activity of the transcortin is decreased. If protein-bound and free steroid can be separated, this change in specific activity of the transcortin can be assessed. A standard curve can be prepared by plotting the activity of the transcortin *versus* the weight of steroid for a series of known weight standards. This can be utilized to determine the quantity of corticoid in an unknown sample from the activity of the transcortin after equilibration with the unknown and removal of the free steroid, providing the unknown does not itself contain any transcortin.

The need for separation has led to several modifications in the method. The separation of protein-bound and free steroid was achieved initially by equilibrium dialysis (1), later by gel filtration (3), and most recently by competitive adsorption (4). These modifications were claimed to have increased the speed, sensitivity, and specificity of the method. In this study, the method of Murphy (4) was tested and some further modifications were made, chiefly to improve specificity.

EXPERIMENTAL

Materials—All radioactive steroid preparations[1] were stored at −15° prior to dilution. The radiochemical purity of the radioactive steroids was checked by TLC and autoradiography. One spot was detected for each compound, indicating that no decomposition had occurred. The steroids used were cortisol-1,2-^{3}H (2 and 32 c./mmole) and corticosterone-1,2-^{3}H (1 and 39.1 c./mmole). The higher specific activity compounds were used in the preparation of the transcortin isotope solution; those of lower specific activity were used in the adsorption and recovery experiments. The transcortin isotope solution was prepared by diluting the desired quantity of plasma with distilled water and then adding the tritium-labeled steroid in a small quantity of ethanol. The solution was then brought to volume with distilled water. Nonradioactive cortisol and corticosterone were obtained commercially[2]. The cortisol was purified by recrystallization from aqueous methanol. Stock solutions of the steroids in redistilled ethanol were made up to a concentration of 10 mcg./ml. and stored at 0°. Working standards were prepared as required in 10-ml. quantities at the concentration of 10 ng./0.1 ml. (ng. = 10^{-9} g.).

Fuller's earth, technical[3], and Florisil[4], 80–100 mesh, were used. The adsorbents were measured in two Plexiglas spoons with capacities of 10.32 ± 1.02 and 59.14 ± 1.58 (*SD*) mg. for fuller's earth, and of 8.38 ± 0.81 and 41.80 ± 0.51 (*SD*) mg. for Florisil. In some cases, weighing of adsorbent was employed.

The scintillation fluid used was Bray's solution (naphthalene, 60 g.; diphenyloxazol, 4 g.; 1,4-di[2(5-phenyloxazolyl)]benzene, 0.2 g.; methanol, 100 ml.; ethylene glycol, 20 ml.; and dioxane sufficient to make 1 l.). Counting was done using a Picker Nuclear Liquimat 110 or 220 liquid scintillation counter equipped with ^{137}Cs external standard. Quench correction was done by the external standard count method for the Liquimat 110 and the channel ratio of the external standard method for the Liquimat 220. A digital PDP-8L computer was used in conjunction with the Liquimat 220 for quench correction. A program that computed the quench-corrected activity in disintegrations per minute and the reciprocal of activity in milliseconds per disintegration was used.

Plasma samples for analysis were collected in tubes containing ethylenediaminetetraacetic acid as anticoagulant. The samples were centrifuged at once at 2000 r.p.m. The plasma was collected and stored at −15° until analysis was performed. Steroid-free serum was obtained by dialyzing serum, which had been diluted 1 in 10 with physiological saline, against physiological saline. The saline was changed frequently during the 66.5-hr. dialysis period. After dialysis, the serum was freeze-dried and reconstituted to its original strength.

Methods—*Determination of Adsorption of Free Steroids*—For the determination of adsorption of free steroids, a measured quantity of adsorbent was added to 1 ml. of a solution of tritiated steroid which had been cooled to at least 10° for 15 min. The tubes were shaken for 2 min. on a horizontal mechanical agitator, cooled for 10 min. in an ice bath, and centrifuged. The supernatant, containing the unadsorbed steroid, was removed with a Pasteur pipet. One-half milliliter of the solution was added to 10 ml. of Bray's solution,

[1] Amersham-Searle, Des Plaines, Ill.
[2] British Drug Houses, Toronto, Ontario, and Nutritional Biochemicals Corp., Cleveland, Ohio, respectively.
[3] Fisher Scientific Co., Pittsburgh, Pa.
[4] Floridin Co., Tallahassee, Fla.

and the sample was counted. From the difference between initial activity and the activity remaining after treatment with adsorbent, it was possible to calculate the percentage adsorption of steroid. This method is essentially that described by Murphy (4).

Determination of Protein-Bound Steroid—To determine the percentage of steroid bound to protein, paired solutions (1 ml.) of tritiated steroids, one with and one without protein, were prepared. The tubes were warmed to 45° for 5 min. to promote rapid equilibration of the steroid with the protein. The samples were then cooled to 0° for 10 min. A measured quantity of adsorbent was added, and the tubes were shaken for 2 min. The tubes were returned to the ice bath for 15 min., then centrifuged, and returned to the ice bath; finally, the supernatant was removed. One-half milliliter of this supernatant was added to 10 ml. of Bray's solution, and the sample was counted. The samples without protein contained unadsorbed steroid in the supernatant, while the samples containing protein had unadsorbed steroid plus protein-bound steroid in the supernatant. The difference in the percentage of original activity remaining in the protein and nonprotein samples represents the percentage of steroid bound to protein.

Preparation of Standard Curves and Assay of Samples—Standards were prepared in duplicate in the desired range; usually, 10 standards were used for each determination of the standard curve. One milliliter of transcortin-isotope solution was added to each of the evaporated standards and samples. These were mixed and heated to 45° for 5 min. After cooling in an ice bath for 15 min., the adsorbent was added. The mixture was shaken for 2 min. on a horizontal agitator, cooled 10 min., and then centrifuged. The supernatant was collected and recentrifuged if the adsorbent was fuller's earth. One-half milliliter of the supernatant was added to 10 ml. of Bray's solution, and the samples were counted twice for a sufficient time to give 1% counting error with 2 σ statistics. A plot of milliseconds per disintegration *versus* nanograms of steroid in the standard was prepared, or a linear regression equation (5) describing this relationship was computed. The graph or equation was used to determine the quantity of steroid in the sample from the reciprocal of activity of the sample.

Preparation of Plasma Samples for Assay—Removal of transcortin from the plasma samples was achieved by alcohol precipitation. Two-tenths of a milliliter of plasma was diluted with 0.1 ml. of water; then 1 ml. of 95% alcohol was added, and the sample was mixed for 2 min. After centrifugation the supernatant was collected. Another milliliter of alcohol was added to the precipitate, and the mixing and centrifugation were repeated. The combined alcoholic supernatants were evaporated at reduced pressure in a freeze-drying apparatus.

To separate cortisol and corticosterone, the residue from the alcohol supernatant was dissolved in 2 ml. of water and partitioned against 20 ml. of carbon tetrachloride by mechanical mixing for 2 min. The corticosterone favors the carbon tetrachloride, while the cortisol remains in the water. The carbon tetrachloride was backwashed with 2 ml. of water, and the original water was backwashed with 20 ml. of carbon tetrachloride. The backwash phases were discarded. The water was evaporated in a freeze-drying apparatus; the carbon tetrachloride was evaporated at 45° in a stream of air. The samples were then ready for assay, as described previously.

RESULTS

Adsorption of Free Steroid—Experiments to determine the adsorption of free cortisol by fuller's earth were conducted using concentrations of cortisol from 1 to 40 ng./ml. and weights of fuller's earth from 10.3 to 118.3 mg. For cortisol concentrations of 5–40 ng./ml. and fuller's earth in weights of 41.3–118.3 mg., adsorption of free cortisol was constant at 85.9 ± 4.4% (n = 22). Decreasing either adsorbent or steroid weight below these ranges gave less adsorption. The adsorption of free corticosterone by Florisil was studied using concentrations of 1.4–36 ng./ml. of corticosterone and 8.4–120 mg. of Florisil. Percentage adsorption was constant for all concentrations of corticosterone tested. For 8.4 and 10 mg. of Florisil, adsorption was 81.6 ± 3.7% (n = 16); for 16.8–120 mg. of Florisil, adsorption was 88.2 ± 2.4% (n = 36).

Separation of Bound and Free Steroid—Using 5% human plasma as the source of transcortin, the percentage of protein-bound cortisol was determined when weights of fuller's earth from 10.3 to 118.3 mg. were used to separate bound and free cortisol (Table I).

Table I—Influence of Fuller's Earth on Protein-Bound ^{3}H-Cortisol[a]

Cortisol Weights, ng.	Weight of Fuller's Earth, mg. 10.3	20.6	41.3	69.5	90.1	118.3
10.4	36.1[b]	45.6	43.0	37.6	34.0	21.8
13.5	34.2	41.2	45.0	42.0	30.9	18.0
22.0	12.4	25.4	20.6	—	18.5	13.5

[a] Source of transcortin was 5% human plasma; concentrations of cortisol were 10.4, 13.5, and 22 ng./ml. [b] Expressed as percent protein-bound; each value of percent bound is the average of two determinations, two at each steroid concentration; values of percent protein-bound cortisol which are underlined are not significantly different at the 5% level by Duncan's multiple-range test (5).

Three concentrations of cortisol were used (10.4, 13.5, and 22 ng./ml.); however, the trend of adsorbent effect on protein binding was the same for all concentrations of steroid, although the percentage of protein-bound steroid decreased as the concentration of cortisol increased due to increased displacement of radioactive steroid from transcortin.

The percentage of protein-bound steroid was constant for weights of 10.3–69.5 mg. of fuller's earth and decreased at 118.3 mg. The decrease at 118.3 mg. indicates that at higher weights of adsorbent, some protein-bound steroid is probably adsorbed after dissociation of the complex. For corticosterone (0.88 ng./ml.) using 0.5% mouse plasma as the transcortin source, the percentage of protein-bound steroid was determined using 8.4–125.4 mg. of Florisil (Table II). Above 20 mg. Florisil, there was very little protein binding; 8.4 and 10 mg. gave substantial binding but were different from each other in the percentage bound.

To determine how much adsorbent to use for separation of bound and free steroid in the assay procedure, the results of the adsorption and protein-binding experiments were considered. An optimum amount of adsorbent was described as that which gave maximum removal of free steroid but also maximum protein binding, thus giving greatest sensitivity. Since a range of weights fulfilled these criteria for fuller's earth, a weight (59.1 mg.) in the middle of the range was chosen so that slight errors in measuring the adsorbent would have little influence on the assay. For Florisil, only the second part of the criteria was met in choosing 8.4 mg. as the weight.

Standard Curves—For cortisol, a standard curve was prepared using 5% human plasma as the transcortin source, ^{3}H-cortisol (4.65–11.05 μc./100 ml.) as the labeling agent, and 59.1 mg. fuller's earth as the adsorbent. The range employed was 0–40 ng. For ease of comparison, all data were corrected to 6 μc./100 ml. The standard curve which resulted when milliseconds per disintegration was plotted *versus* nanograms of cortisol was described by the equation $Y = 0.0381X + 1.203$, where Y = milliseconds per disintegration and X = nanograms. The standard deviation of the slope of this line, where 68 values were used to determine it, was 0.00213 (6). The standard deviation of an X value estimated using this equation was determined as 4.57, where the true value of X = 10 ng. and duplicate estimates are made (6).

For corticosterone, mouse plasma and human plasma were tried as sources of transcortin, both ^{3}H-cortisol and ^{3}H-corticosterone were used as labeling agents, and both Florisil (8.4 and 41.8 mg.) and fuller's earth (59.1 mg.) were used as adsorbents. Table III shows the data on the standard curves obtained using the various combinations of transcortin, labeling agent, and adsorbent for cortisol and corticosterone.

Table II—Influence of Florisil on Protein-Bound ^{3}H-Corticosterone[a]

Weight of Florisil, mg.	8.4	10	20	41.8	83.6	125.4
Percent protein-bound corticosterone	46.6	30.9	13.6	9.0	8.1	5.4

[a] Source of transcortin was 0.5% mouse plasma; concentration of corticosterone was 0.88 ng./ml.; each value of percent protein-bound corticosterone is the average of two determinations; values of percent bound which are underlined are not significantly different at 5% level by Duncan's multiple-range test.

Table III—Standard Curves

System	Steroid	Transcortin Source	Labeling Agent	Adsorbent	Equation	Range, ng.	n[a]	s_b[b]	cv_b[b]	s_x[c]
1	Corticosterone	0.5% Mouse plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0619X + 1.19$	0–5	6	0.0061	9.9	0.42
2	Corticosterone	0.5% Mouse plasma	^{3}H-Corticosterone	Florisil, 41.8 mg.	No slope	0–40	10	—	—	—
3	Corticosterone	3% Mouse plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0079X + 0.994$	0–20	10	0.0011	14	2.5
4	Corticosterone	1% Human plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0732X + 1.11$	0–10	39	0.010	14	1.5
5	Corticosterone	1% Human plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0378X + 0.914$[d]	0.5–2.5	6	0.0029	7.7	0.13
6	Corticosterone	1% Human plasma	^{3}H-Corticosterone	Fuller's earth, 59.1 mg.	$Y = 0.374X + 1.69$	0–5	8	0.016	4.3	0.18
7	Corticosterone	1% Human plasma	^{3}H-Cortisol	Fuller's earth, 59.1 mg.	$Y = 0.0678X + 1.31$	0–10	10	0.0036	5.3	0.48
8	Corticosterone	2.5% Human plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0267X + 1.03$	0–20	18	0.0025	9.4	3.0
9	Cortisol	5% Human plasma	^{3}H-Cortisol	Fuller's earth, 59.1 mg.	$Y = 0.0381X + 1.20$	0–40	68	0.0021	5.5	4.57

[a] Number of values used in determining the regression equation. [b] s_b = standard deviation of slope, b (6); cv_b = coefficient of variability for slope = $(s_b/b) \times 100$. [c] s_x = standard deviation of X where duplicate estimates of X are made using the regression equation (6). For corticosterone, X = 2 ng.; for cortisol, X = 10 ng. [d] In this case, steroid standards were added to steroid-free plasma.

Specificity—Specificity for cortisol or corticosterone individually was tested by assaying ethanol samples, containing known amounts of cortisol and/or corticosterone, and plasma samples, with and without additional amounts of cortisol and corticosterone. When plasma was used, it was necessary initially to precipitate proteins from the plasma with alcohol as described previously. Since initial experiments (Table IV) indicated that there was no specificity for either cortisol or corticosterone using various combinations of adsorbent and plasma, physical separation of the two steroids was needed to improve the specificity. Results of the specificity studies are shown in Table IV.

The 5% human plasma system, using 59.1 mg. of fuller's earth as the adsorbent, did not give specificity for cortisol; corticosterone was detected to the extent of 1.81 ± 1.08 mcg./mcg. of corticosterone in the sample. To improve the specificity for cortisol, carbon tetrachloride/water partitioning was employed. The water phase was assayed for cortisol. Comparison of sets of samples containing plasma only and the same plasma with additional amounts of corticosterone was done to see if interference of corticosterone was still detectable. In most cases, the added corticosterone did not significantly elevate the estimate of cortisol.

Assay of corticosterone using 1% human plasma and either 8.4 mg. Florisil or 59.1 mg. fuller's earth as the adsorbent did not provide a system specific for corticosterone; cortisol was detected to an approximately equal extent as corticosterone. Application of carbon tetrachloride/water partitioning to recover the corticosterone in the carbon tetrachloride resulted in some difficulties. When assay of the carbon tetrachloride residue for corticosterone was done using System 4, 6, or 7, negative amounts of steroid were detected; that is, the value of milliseconds per disintegration for the plasma samples fell below the zero point of the standard curve. This elevated activity in these samples could be due to a decrease in adsorption of steroid or an increase in protein binding. Ethanol precipitation of protein did not give complete deproteinization, as shown by a positive ninhydrin test. When the ethanol supernatant was treated with an excess of ammonium sulfate and filtered, the supernatant was free of protein. This ethanol supernatant was evaporated and partitioned as described previously. The carbon tetrachloride was

Table IV—Specificity of Competitive Protein-Binding Radioassay

Assay System	Sample	Number of Samples	Carbon Tetrachloride/Water Partition Step Used	Added Steroid, mcg./100 ml. Cortisol	Added Steroid, mcg./100 ml. Corticosterone	"Found" Steroid, mcg./100 ml. Cortisol	"Found" Steroid, mcg./100 ml. Corticosterone	Interference[a]
5% Human plasma, ^{3}H-cortisol, 59.1 mg. fuller's earth	Ethanol[b] solution of steroid	2	No	10	1	13.06 ± 0.59	—	3.06 ± 0.59
		2	No	10	5	15.54 ± 0.27	—	1.11 ± 0.06
		2	No	10	10	20.66 ± 1.78	—	1.07 ± 0.18
		2	No	10	20	30.83 ± 2.34	—	1.04 ± 0.11
		2	No	0	1	3.33 ± 1.78	—	3.33 ± 1.78
		2	No	0	5	8.96 ± 1.48	—	1.79 ± 0.30
		2	No	0	10	12.53 ± 0.84	—	1.25 ± 0.08
		2	Yes	10	0	8.12 ± 1.33		
		2	Yes	10	1	8.73 ± 2.18	—	No[d]
		2	Yes	10	10	9.54 ± 1.80		
		1	Yes	0	10	2.13	—	0.21
		7	Yes	0	0	9.11 ± 2.96		
		6	Yes	0	10	10.91 ± 2.32	—	No[e]
		8	Yes	0	0	10.75 ± 1.98		
		7	Yes	0	10	13.85 ± 1.97	—	0.31[f]
	Plasma[c]	4	Yes	0	0	12.03 ± 2.04		
		4	Yes	0	10	10.54 ± 1.67	—	No[g]
		3	Yes	0	0	11.94 ± 2.36		
		2	Yes	0	5	16.82 ± 3.10	—	No[h]
1% Human plasma, ^{3}H-corticosterone, 8.4 mg. Florisil	Ethanol[b] solution of steroid	2	No	10	0	—	7.27 ± 0.45	0.73 ± 0.04
1% Human plasma, ^{3}H-corticosterone, 59.1 mg. fuller's earth	Ethanol[b] solution of steroid	2	No	5	0	—	4.97 ± 0.35	1.00 ± 0.06
5% Human plasma, ^{3}H-cortisol, 59.1 mg. fuller's earth	Plasma[b]	8	Yes	0	0	—	10.96 ± 3.86	No[i]
5% Human plasma, ^{3}H-cortisol, 59.1 mg. fuller's earth	Plasma[b]	4	Yes	10	0	—	11.97 ± 5.87	

[a] Interference determined by subtracting the known concentration (mcg./100 ml.) of desired steroid from "found" concentration (mcg./100 ml.) of steroid and dividing the difference so obtained by the concentration of interfering steroid known to be in the system. [b] Recovery correction by yield determination. [c] Recovery correction by internal standard method. [d] $F < 1$; interference not significant. [e] $t = 1.20$; interference not significant, where t is Student's t. [f] $t = 3.30$; interference significant ($p < 0.01$). [g] $t = 1.13$; interference not significant. [h] $t = 2.03$; interference not significant. [i] $t = 0.36$; interference not significant.

Table V—Separation of Corticosterone and Cortisol by Carbon Tetrachloride/Water Partitioning[a]

Replicate	Water Phase		Carbon Tetrachloride Phase	
	Cortisol Recovery	Corticosterone Crossover	Corticosterone Recovery	Cortisol Crossover
1	74.1 ± 2.5[c]	15.6 ± 1.2[b]	48.5 ± 4.3[c]	6.3 ± 0.6[c]
2	74.1 ± 1.0[c]	6.9 ± 0.3[c]	76.2 ± 2.4[c]	7.5 ± 0.4[c]
3	—	8.6 ± 0.3[c]	62.0 ± 1.8[c]	—
4	—	12.6 ± 1.2[c]	35.4 ± 2.4[c]	—

[a] Results expressed as average percent ± standard deviation. [b] Number of samples equals 3. [c] Number of samples equals 4.

assayed using System 4, but negative values were still obtained. Therefore, crossover of sample protein to the assay step did not explain the failure of the assay. Evaporation of the carbon tetrachloride in a stream of nitrogen rather than air also did not improve the results. Systems 6 and 9, when applied to the carbon tetrachloride residue, gave positive values. System 9 gave adequate specificity for corticosterone after carbon tetrachloride/water partitioning and assay of the carbon tetrachloride residue. Specificity using System 6 was not confirmed.

Recovery—Recovery of cortisol and corticosterone from plasma after ethanol precipitation was checked using ^{3}H-steroids. The recovery was determined as 69.2 ± 11.8% (n = 12) for cortisol and 89.2 ± 7.3% (n = 12) for corticosterone. Recovery was reproducible on two different days.

Recovery of ^{3}H-cortisol and ^{3}H-corticosterone from aqueous solutions was determined after carbon tetrachloride/water partitioning (Table V). Replicates were performed on different days. The recovery of cortisol was reproducible from day to day [74.1 ± 1.8% (n = 8)], and crossover of cortisol into carbon tetrachloride was 6.9 ± 0.8% (n = 8). The recovery of corticosterone was reproducible on any given day but varied from day to day. For this reason, daily recovery indicators were needed in the total assay procedure. Two approaches were employed.

The first was recovery correction by isotope yield determination. With each batch of samples, two yield determination standards were included. One contained a measured amount of cortisol-1,2-^{3}H in 0.2 ml. of plasma, while the other contained corticosterone-1,2-^{3}H in the same volume of plasma. These standards were taken through the procedure of protein precipitation and partitioning in parallel with the other samples. The standards were processed no further except for counting of radioactivity. Each standard yielded two counting samples, one for recovery and one for crossover. The fractional recovery and crossover were calculated by comparison to the initial radioactivity added. Because the normal concentration of

Table VI—Comparison of Fluorometric, Double-Isotope Analysis, and Competitive Protein-Binding Radioassay Methods

Reference	SD[a]	Precision Range[a]	n	Normal Values, mcg./100 ml.[b] (n) Cortisol	Corticosterone	Comments
				Fluorometric		
16	—	—	—	9.8	0.2	—
17	1.7	10–20	24	21.96 ± 4.76[c]	—	8–9 a.m.
18	0.55	10–20	6	11 ± 4.4 (36)	—	10–12 a.m.
19	—	—	—	10.9 ± 0.55 (20)	3 ± 0.2 (20)	—
20	—	—	—	6–15	0.7–2.9	—
21	—	—	—	11.71 ± 4.31 (48)	—	Male, age 16–78
	—	—	—	13.01 ± 4.27 (33)	—	Female, age 12–69
	—	—	—	12.24 ± 4.34 (81)	—	Both sexes, all values 7:30 a.m.
22	—	—	—	9.2 ± 1.5 (30)	1.2 (9)	8–9 a.m.
23	—	—	—	11 ± 1	2 ± 0.5	—
24	0.80	10–19.9	39	9.6 ± 3.0 (33)	—	8–9:30 a.m.
	0.21	0–0.9	36	—	0.6 ± 0.6 (19)	8–9:30 a.m.
25	0.1–0.2	—	—	—	—	—
26	0.2	10–20	33	21.4 ± 5.6[c] (18)	—	7 a.m.
	—	—	—	13.0 ± 3.3[c] (18)	—	9 a.m.
27	—	—	—	15 (20)	1.1 ± 0.3 (20)	—
28	—	—	—	18 ± 4.3 (30)	—	—
29	—	—	—	19.1 ± 3.1[c] (18)	—	—
30	0.082	15–35	44	—	—	—
31	2.12	10–20	9	9.6 ± 2.7 (27)	—	9:30 a.m.
	0.34	1–2	9	—	0.4 ± 0.2 (27)	9:30 a.m.
32	1	—	—	—	—	—
33	2.1–2.2	—	16	13.8	5.2	—
34	1.2	10–20	75	14.6 ± 3.7 (50)	—	8 a.m.
				Double-Isotope Analysis		
35	—	—	—	10.5 (159)	2.5 (19)	8–10 a.m.
36	—	—	—	10.27 ± 3.1 (29)	0.56 ± 0.21 (28)	a.m.
	—	—	—	6.77 ± 3.13 (14)	0.34 ± 0.1 (13)	p.m.
37	—	—	—	11.8 ± 4.3 (25)	0.34 ± 0.25 (25)	9–10 a.m.
38	0–19	—	10	9.8 (17)	—	—
	0.03	—	11	—	0.66 (29)	—
				Competitive Protein-Binding Radioassay		
8	4.9	0–100	167	—	—	—
14	—	—	—	15.7 ± 4.6 (26)	—	8 a.m.
	—	—	—	8.2 ± 2.3 (25)	—	2 p.m.
	—	—	—	6.4 ± 2.7 (12)	—	8 p.m.
1	2.0	10–20	18	15.2 ± 5.1[c] (12)	—	Male, 9 a.m.
	—	—	—	17.6 ± 4.3[c] (12)	—	Female, 9 a.m.
3	1.2	10–20	20	—	—	—
4	2.0	10–20	20	8–24[c]	—	—
	—	—	—	6–18	4	9 a.m.
10	1.2	10–20	12	15[c]	—	8 a.m.
Present study	2.65	9–22	39	11.71 ± 1.50 (3)	—	a.m.
Present study	2.52	4–8	13	—	1.84 (1)	a.m.

[a] In mcg./100 ml.; if standard deviation given for several concentration ranges, the standard deviation for normal range is quoted. [b] ± Standard deviation. [c] Normal values apply to corticosteroids, not cortisol only.

cortisol in human plasma is much higher than that of corticosterone, crossover is less important in the determination of cortisol. In the case of corticosterone, correction for crossover is important to avoid overestimating the corticosterone concentration of the sample. To do this, the value of cortisol (corrected for recovery) is multiplied by the fraction crossover of cortisol into carbon tetrachloride. This figure is the contribution of cortisol to the estimate of corticosterone, so it is subtracted from the recovery-corrected amount of corticosterone to give the corticosterone (corrected) concentration. In physiological states where the ratio of cortisol to corticosterone is reversed, this procedure would be used to find the cortisol (corrected) concentration in the sample.

The second approach to recovery correction was the internal standard method. In this procedure, a sample containing a known amount of added cortisol (usually 10 ng./0.1 ml.) and another containing a known amount of added corticosterone are prepared, as well as a sample of the plasma containing no added steroid. These three samples are processed in parallel with the other samples. From the difference between the amount of steroid detected in the plasma-only sample and the sample with added steroid, the recovery can be determined.

In both cases, the recovery-corrected amount of steroid was calculated by dividing the uncorrected weight of detected steroid by the fractional recovery factor. The results are expressed in ng./0.1 ml. of plasma and are numerically equal to the concentration in mcg./100 ml. of plasma.

Precision—The precision of the total method was estimated by calculation of the standard deviation of replicate determination on aliquots of several plasma samples. System 9 was used to assay both cortisol and corticosterone. For cortisol in the range 9–22 mcg./100 ml., the standard deviation was 2.65 (n = 39). For corticosterone in the range 4–8 mcg./100 ml., the standard deviation was 2.53 (n = 13).

Accuracy—The accuracy of the method was determined using aliquots of four different plasma samples. The concentration of cortisol in each sample was determined; then 10 mcg./100 ml. of cortisol was added, and the concentration was again determined. Several replicate determinations were made in each case. When the internal standard method of recovery correction was used, the additional increments detected were 8.24, 9.12, 11.83, and 12.45 mcg./100 ml. compared to 8.40, 9.02, 15.94, and 4.57 mcg./100 ml. using the yield determination method of recovery correction. It was judged that the internal standard method gave more accurate results.

Normal Values—Several morning (10:00 a.m. to 12:30 noon) blood samples were drawn from a normal male subject to determine normal estimates of corticoids by the total method. Cortisol concentration, determined using System 9, was 11.71 ± 1.50 mcg./100 ml. (n = 3, recovery correction by internal standard method). When the method was applied to pooled human serum, the cortisol concentration was found to be 13.79 mcg./100 ml. (recovery correction by yield determination method). Using System 9, corticosterone concentration (12:30 noon) was determined to be 4.96 mcg./100 ml. (recovery correction by yield determination method). With System 5, the corticosterone concentration (10:15 a.m.) was determined as 1.84 mcg./100 ml. In this case, no recovery correction was needed since all samples and standards were processed in parallel through all the steps of protein precipitation, partitioning, and assay.

DISCUSSION

The results of the present study did not confirm the observation of Murphy (4) that the use of fuller's earth as an adsorbent for separation of bound and free steroid produced a system with increased specificity for cortisol. Using fuller's earth as the adsorbent, corticosterone was detected to an equal or greater extent than cortisol. When Florisil was the adsorbent, corticosterone was detected to a greater extent than cortisol. It seems reasonable that this effect would be observed since the measurement of steroid is dependent only on the decrease in activity observed. The adsorbent role is to remove free steroid after the equilibration of the sample steroid with transcortin. The effect of the adsorbent on the assay is dependent only on the form of radioactive steroid used, since adsorbent removal of other steroids goes undetected. The source of the protein used determines specificity. Since human transcortin has approximately equal affinity for cortisol and corticosterone (4), both are detected and there is no specificity for one or the other. ^{3}H-Cortisol on the transcortin is displaced by any steroid with equal or greater affinity for the transcortin, and the free ^{3}H-cortisol is removed from the solution by adsorption, depending on the affinity of the adsorbent for cortisol. The different adsorbents affect the slope of the standard curves and thus the sensitivity and precision of the assay, but they do not affect the specificity of the assay.

After carbon tetrachloride/water partitioning, specificity was improved so that cortisol could be determined in the presence of 5–10 times the normal concentration of corticosterone. The interference of corticosterone detected in some cases probably was due to the variable recovery of corticosterone into carbon tetrachloride. Corticosterone could be determined in the carbon tetrachloride phase without interference from cortisol. The carbon tetrachloride phase contains other steroids than corticosterone. It takes up 11-deoxycortisol just as avidly as corticosterone (partition coefficient water/carbon tetrachloride: corticosterone, 0.75; 11-deoxycortisol, 0.74) (7). Bowman and DeLuna (8) reported that 80% of 11-deoxycortisol is removed by extraction of plasma with 10 volumes of carbon tetrachloride. The normal levels of 11-deoxycortisol are low (0–2 mcg./100 ml.) (9) but may be significant when compared to the low levels of corticosterone in plasma (1 mcg./100 ml.). 11-Deoxycortisol is detected quite strongly in a competitive protein-binding type of assay (4, 10); therefore, the "corticosterone" value would be inflated by the presence of 11-deoxycortisol. This would be especially important in cases of pharmacological blockage of 11-hydroxylation with metyrapone[5], where levels of 11-deoxycortisol rise sharply.

Rudd and Black (11) showed that pregnanetriol and pregnanetriolone are present in the carbon tetrachloride phase. The blood levels of these two steroids are normally low, but they may be elevated in adrenal hyperplasia where the 21-hydroxylation is reduced. These two compounds would not likely be detected by competitive protein binding (10). Other steroids that would favor the carbon tetrachloride phase, judged from partition coefficient data (7), are testosterone, progesterone, and androstenedione. The normal levels of these steroids are 0.5 mcg./100 ml. for testosterone, 0.17 mcg./100 ml. for androstenedione, and less than 0.8 mcg./100 ml. for progesterone in males and less than 2 mcg./100 ml. in nonpregnant females (4, 12, 13). These levels are significant relative to corticosterone concentration. The two androgens would not interfere in a competitive protein-binding assay; however, progesterone would inflate the values of corticosterone estimated from the carbon tetrachloride phase (4). Recently, Kolanowski and Pizarro (14) published data showing that testosterone is strongly detected in a competitive protein-binding assay, so it may pose an interference problem.

The standard deviation of replicate determinations of cortisol in plasma samples where the concentration of cortisol was in the range 9–22 mcg./100 ml. was 2.65. This is comparable to precision of other assays reported in the literature (Table VI).

Normal morning concentrations of cortisol were found to be 11.71 mcg./100 ml. For corticosterone, using System 9, the morning concentration was found to be 4.96 mcg./100 ml.; using System 5, the level of corticosterone was determined as 1.84 mcg./100 ml. For cortisol, the value compares favorably with values from more specific double-isotope dilution methods and also values determined by fluorometric and competitive protein-binding methods (Table VI). The value for corticosterone using System 9 seems high compared to other reported values, although Van der Vies (33), using a a fluorometric method, reported a similar value. Both the present method and the Van der Vies method employed carbon tetrachloride/water partitioning to separate cortisol and corticosterone. Possibly some common interference affected both values. The high value of corticosterone detected with System 9 may be related to the better detection of corticosterone noted in the specificity study. Corticosterone may have greater affinity for the protein binding sites under these conditions. This is in keeping with the "polarity rule" (15). Two different sites might be involved in the binding of cortisol and corticosterone.

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Removal of Ferrous Ions by *N,N'*-Ethylenediaminetetraacetic Acid in Microbiological Assay of Pyridoxine

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Abstract □ The application of a general microbiological method for the determination of pyridoxine was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A modification was made to the method by using *N,N'*-ethylenediaminetetraacetic acid to sequester Fe^{+2} before acid hydrolysis of the preparations. By using this modification, 100% of the pyridoxine can be detected compared with 75 or 77% for 20:1 or 12:1 ratios of Fe^{+2}–pyridoxine, respectively.

Keyphrases □ Pyridoxine, multivitamin combinations—bioassay, ferrous-ion removal with *N,N'*-ethylenediaminetetraacetic acid □ Multivitamin combinations—pyridoxine bioassay □ *N,N'*-Ethylenediaminetetraacetic acid—ferrous-ion complexing, pyridoxine bioassay

In 1943, Atkin *et al.* (1) reported a microbiological method which employed a yeast, *Saccharomyces carlsbergensis*, to determine pyridoxine. Other studies (2–5) further supported this method. In natural products and pharmaceutical preparations, pyridoxine exists partly as the combined form which must be hydrolyzed prior to assay. The method was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A combination of Fe^{+2} with multivitamins is generally recommended for the treatment of iron-deficiency anemia (6). The interference of Fe^{+2} might be prevented by the addition of a compound such as *N,N'*-

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Removal of Ferrous Ions by *N,N'*-Ethylenediaminetetraacetic Acid in Microbiological Assay of Pyridoxine

JUH-HUEI HUANG

Abstract □ The application of a general microbiological method for the determination of pyridoxine was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A modification was made to the method by using *N,N'*-ethylenediaminetetraacetic acid to sequester Fe^{+2} before acid hydrolysis of the preparations. By using this modification, 100% of the pyridoxine can be detected compared with 75 or 77% for 20:1 or 12:1 ratios of Fe^{+2}–pyridoxine, respectively.

Keyphrases □ Pyridoxine, multivitamin combinations—bioassay, ferrous-ion removal with *N,N'*-ethylenediaminetetraacetic acid □ Multivitamin combinations—pyridoxine bioassay □ *N,N'*-Ethylenediaminetetraacetic acid—ferrous-ion complexing, pyridoxine bioassay

In 1943, Atkin *et al.* (1) reported a microbiological method which employed a yeast, *Saccharomyces carlsbergensis*, to determine pyridoxine. Other studies (2–5) further supported this method. In natural products and pharmaceutical preparations, pyridoxine exists partly as the combined form which must be hydrolyzed prior to assay. The method was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A combination of Fe^{+2} with multivitamins is generally recommended for the treatment of iron-deficiency anemia (6). The interference of Fe^{+2} might be prevented by the addition of a compound such as *N,N'*-

Table I—Composition of Assay Medium

Dextrose	100.0 g.
KH_2PO_4	1.10 g.
KCl	0.85 g.
$CaCl_2$	0.25 g.
$MgSO_4 \cdot 7H_2O$	0.25 g.
$FeCl_3$ solution (1%)	0.50 ml.
$MnSO_4$ solution (0.1%)	0.50 ml.
Potassium citrate	10.00 g.
Citric acid	2.00 g.
Acid hydrolysate of casein[a]	10.00 g.
Thiamine hydrochloride solution (10 mcg. thiamine/ml.)	50 ml.
Inositol solution (1.0 mg./ml.)	50 ml.
Biotin solution (0.8 mcg./ml.)	20 ml.
Riboflavin solution (100 mcg./ml.)	10 ml.
Ca pantothenate solution (200 mcg./ml.)	25 ml.
Nicotinic acid solution (200 mcg./ml.)	25 ml.
$(HN_4)_2 HPO_4$	2.00 g.
L-Asparagine	1.50 g.
Agar	20.00 g.
Water to make	1000 ml.

[a] Sheffield Hy-case, Sheffield Chemical, Division of National Dairy Products Corp.

ethylenediaminetetraacetic acid, which is known to form a stable complex with Fe^{+2} (7) and to remove undesired metals from living organisms (8, 9).

EXPERIMENTAL

Organism—*Saccharomyces carlsbergensis*, Fleishman 4228 (ATCC 9080), was used throughout these studies. It was incubated for 48 hr. at 30° on modified Sabouraud dextrose agar. One loopful of this culture was transferred to a flask containing 100 ml. of sterilized yeast dextrose broth. The inoculated flask was incubated for 20 hr. at 30° on a mechanical shaker. The cells were harvested and centrifuged under sterile conditions and then resuspended in 100 ml. of sterilized saline solution. This suspension was stored at 4° and used as the stock inoculum for up to 1 month.

Assay Medium—The assay medium was prepared according to the formula shown in Table I.

Plate Preparation—On the day of assay, 100 ml. of freshly prepared and cooled (48°) assay medium was inoculated with 3 ml. of stock inoculum suspension. Eleven milliliters of inoculated assay medium was applied to each petri plate and allowed to solidify. The plates were covered and preincubated at 30° for 4 hr.

Stock Standard Solution and Standard Solution of Pyridoxine—For the stock standard solution, 25.13 mg. of 99.48% pyridoxine hydrochloride standard was weighed into a 250-ml. volumetric flask and made up to volume with distilled water; this stock solution had a potency of 100 mcg. pyridoxine hydrochloride/ml. The solution was stored in the refrigerator and used within 30 days. On the day of assay, a series of dilutions of the stock standard solution was prepared to contain 1.0, 0.5, 0.25, and 0.125 mcg. of pyridoxine activity/ml. The 0.5-mcg. standard was used as the reference solution.

Sample Preparation—An aliquot of sample, expected to contain approximately 2.0 mg. of pyridoxine hydrochloride, was transferred to a 250-ml. conical flask, and 100 ml. of 1 *N* HCl was added. The flask was covered with an aluminum foil cap and autoclaved at 15 lb. pressure for 60 min. The solution was cooled to room temperature, adjusted to pH 6.8 with 2 *N* NaOH, transferred to a 100-ml. volumetric flask, and made up to volume with distilled water. This solution containing approximately 0.5 mcg. pyridoxine hydrochloride/ml. was then filtered.

Table III—Effect of Component in Sample A on Stability of Pyridoxine to Acid Hydrolysis

Component	Weight of Component in 2 mg. Pyridoxine	Average Pyridoxine Concentration, mcg.	Recovery, %
Blank	—	2063	100
Ascorbic acid	750 mg.	2046	99.2
Benzoic acid	25 mg.	2080	100.8
Ferrous sulfate	525 mg.	1490	74.5
Glucose	10 g.	2050	99.4
Menthol	1.9 mg.	2053	99.5
Niacinamide	8.25 mg.	2065	100.1
D-Pantothenol	4.5 mg.	2016	97.7
Riboflavin	5.25 mg.	2003	97.1
Thiamine	6.75 mg.	2036	98.7
Sodium saccharin	2.5 mg.	2020	97.9
Sucrose	1.25 g.	2017	97.8
Cyanocobalamin	49.4 mcg.	2061	99.9

Assay Procedure—The pyridoxine content of each preparation was determined by a cylinder plate method. Six stainless steel cylinders were equally spaced on each agar plate, 2.8 cm. from the center. Three plates were used for each point in the standard series. On each of these plates, three cylinders were filled with the 0.5-mcg. reference solution; the remaining three at opposite positions were filled with a standard solution. Three plates were also used for each sample. Three of the cylinders on each of these plates were filled with the 0.5-mcg. reference solution and three with the sample solution. The filled plates were incubated at 30° for 18 hr. All determinations were run in duplicate and repeated six times. All chemicals were either USP, NF, or ACS grade.

Calculation of Results—The resultant zones of exhibition were read on a Fisher Lilly zone reader and recorded. Averages of the readings of each point for each set of three plates and all readings of the 0.5-mcg./ml. reference solution were calculated. The average diameter for the reference solution was used to correct the average obtained for each point in the standard series and the samples. The corrected values of the standard diameters were substituted in the following formulas to calculate the line of best fit:

$$l = \frac{7a + 4b + c - 2d}{10} \qquad \text{(Eq. 1)}$$

$$h = \frac{7d + 4c + b - 2a}{10} \qquad \text{(Eq. 2)}$$

where l is the calculated diameter (in mm.) of the lowest concentration (0.125 mcg./ml.); h is the calculated diameter of the highest concentration (1.0 mcg./ml.); and a, b, c, and d denote the corrected diameters for each concentration from the lowest to the highest in that order. The values l and h were plotted on one-cycle semilogarithmic graph paper, with the concentration in micrograms per milliliter on the logarithmic scale and the zone diameters on the arithmetic scale. The two points were joined by a straight line.

The potency of each sample could be read from the line or, preferably, calculated according to the following equation (10):

$$U = \text{antilog}\left[\frac{\log H - \log L}{h - l}(u - l) + \log L\right] \qquad \text{(Eq. 3)}$$

Table II—Pyridoxine Stability against Acid Hydrolysis

	Autoclaved Time, min.					
	0	10	20	40	60	80
Pure pyridoxine, mcg.	5000	5015	4975	5003	5020	4982
Recovery, %	100	100.3	99.5	100.1	100.4	99.6
Sample A pyridoxine, mcg.	5150	5037	4913	4160	3725	3240
Recovery, %	100	97.8	95.4	83.2	74.5	64.8

Table IV—Effect of Mineral Ions on Stability of Pyridoxine to Acid Hydrolysis (100 mg. Ion/5 mg. Pyridoxine Solution)

Component	Average Pyridoxine Concentration, mcg.	Recovery, %
Blank	5000	100
Ferrous sulfate	3730	74.6
Copper sulfate	4892	97.8
Magnesium sulfate	4934	98.7
Manganese oxide	4848	97.0
Zinc sulfate	4964	99.3
Cobalt chloride	4848	97.0
Potassium iodide	4822	96.4
Sodium citrate	4916	98.32
Calcium sulfate	5006	100.1

Table V—Effect of Amount of Fe^{+2} on Pyridoxine Stability to Acid Hydrolysis

Pyridoxine, mg.	2.0	2.0	2.0	2.0	2.0
Fe^{+2}, mg.	0.0	10.0	20.0	40.0	60.0
Fe–pyridoxine ratio	0:1	5:1	10:1	20:1	30:1
Average pyridoxine concentration, mg.	2.000	1.666	1.617	1.492	1.428
Recovery, %	100	83.3	80.86	74.60	71.40

where U is potency of sample solution in final dilution, u is diameter of exhibited zone of sample solution, log H is logarithm of highest concentration of standard, h is diameter of exhibited zone of highest standard, log L is logarithm of lowest concentration of standard, and l is diameter of exhibited zone of lowest standard.

Statistical Analysis of Results—Reproducibility of the method and the significance of the results obtained by the *N,N'*-ethylenediaminetetraacetic acid modification were calculated from the following equations:

$$\text{standard deviation } (SD) = \sqrt{\frac{\Sigma(\bar{X} - x_1)^2}{n}} \qquad \text{(Eq. 4)}$$

$$\text{coefficient of variation } (CV) = SD \times \frac{100}{\bar{X}} \qquad \text{(Eq. 5)}$$

$$\text{standard error } (S\bar{X}) = \sqrt{\frac{SD}{K}} \qquad \text{(Eq. 6)}$$

$$\text{Student's } t = \frac{\bar{X} - u}{S\bar{X}} \qquad \text{(Eq. 7)}$$

where $\bar{X}$ is mean of potency, x_1 is potency of each test, K is number of treatments, n is degrees of freedom $= K - 1$, and u is theoretical potency.

The theoretical t value of 2.776 was derived from statistical tables for Biological, Agricultural, and Medical Research (11) at the 5% probability level ($p = 0.05$) and the degrees of freedom $n = 5 - 1 = 4$ for five replicates of results.

Determination of Effect of Component Contained in Sample A and Various Mineral Ions on Stability of Pyridoxine to Acid Hydrolysis—The stability of pure pyridoxine solutions and multivitamin Sample A solution was tested by the described procedure with variations in the time of autoclaving. Studies of the factors affecting pyridoxine were made by the addition of each component in the multivitamin Sample A. Various mineral ions were also added, and their effect on pyridoxine assay was shown.

Determination of Effect of *N,N'*-Ethylenediaminetetraacetic Acid on Pyridoxine Stability in the Presence of Fe^{+2} during Acid Hydrolysis—Various quantities of *N,N'*-ethylenediaminetetraacetic acid disodium salt were added to solutions containing Fe^{+2}–pyridoxine in the ratio 20:1 before acid hydrolysis, and the effect on the assay of pyridoxine was determined.

Determination of Reproducibility and Precision of Pyridoxine Assay with and without *N,N'*-Ethylenediaminetetraacetic Acid Treatment—Four multivitamin samples were tested by the general method and the *N,N'*-ethylenediaminetetraacetic acid modified method, and the precision of the results was determined.

RESULTS AND DISCUSSION

In the stability studies, only 74.5% of pyridoxine was found in the multivitamin Sample A, whereas no decrease in pyridoxine alone was observed under the same conditions (Table II). This indicates that Sample A contains some factor that decomposed pyridoxine during acid hydrolysis in the autoclave.

The addition of individual components of the multivitamin mixture showed that only ferrous sulfate had an appreciable effect on the recovery of pyridoxine (Table III). In comparing the mineral ions, 100 mg. cation/5 mg. pyridoxine was used; the results showed that there was no appreciable effect apart from Fe^{+2} (Table IV). It is clear that the major factor affecting the stability of pyridoxine under acid hydrolysis is Fe^{+2}. The quantity of Fe^{+2} affecting the stability of pyridoxine was further investigated, and the degradation was increased by increasing the Fe^{+2} (Table V). The percentage recovery fell from 83.3 to 71.4% for solutions with Fe^{+2}–pyridoxine ratios of 5:1 to 30:1, respectively.

The interference of Fe^{+2} was eliminated by the use of *N,N'*-ethylenediaminetetraacetic acid as a sequestering agent (Table VI). It was necessary to add 10 g. *N,N'*-ethylenediaminetetraacetic acid to a sample containing 100 mg. Fe^{+2} to eliminate interference; this quantity of *N,N'*-ethylenediaminetetraacetic acid was also effective in the multivitamin Sample A.

There was no significant difference between the two methods in the reproducibility of results as expressed by the coefficient of variation (Table VII). The recovery of pyridoxine in samples that did not contain Fe^{+2} was fairly close to 100% by both methods; but in Samples A and B, only 74.85 and 77.23% of pyridoxine were detected, respectively, by the general method compared with complete recovery by the *N,N'*-ethylenediaminetetraacetic acid method.

The t tests showed that there was a significant reduction in the recovery of pyridoxine only in samples containing Fe^{+2} to which *N,N'*-ethylenediaminetetraacetic acid had not been added.

The following procedure is, therefore, proposed for the determination of pyridoxine in pharmaceutical preparations as well as in natural products that may or may not contain Fe^{+2}. The sample

Table VI—Effect of *N,N'*-Ethylenediaminetetraacetic Acid–2Na on Stability of Pyridoxine in the Presence of Fe^{+2} to Acid Hydrolysis

	1	2	3	4	5	6	7	8	9
Pyridoxine, mg.	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Fe^{+2}, mg.	0	100	100	100	100	100	100	100	100
Fe–pyridoxine ratio	—	20:1	20:1	20:1	20·1	20:1	20:1	20:1	20:1
N,N'-Ethylenediaminetetraacetic acid, g.	0	0	1	2	3	4	6	8	10
A: Pure Pyridoxine Solution									
Average pyridoxine, mg.	5.000	3.378	3.491	4.037	4.264	4.440	4.643	4.754	4.994
Recovery, %	100[a]	67.56	69.82	80.74	85.28	88.80	92.86	95.08	99.99
B: Multivitamin Product A									
Average pyridoxine, mg.	2.022	1.507	—	—	—	—	1.884	1.985	2.016
Recovery, %	100[a]	74.53	—	—	—	—	93.18	98.17	99.70

[a] No heat treatment.

Table VII—Pyridoxine Products Assayed by *N,N'*-Ethylenediaminetetraacetic Acid Modified Method and General Method

	Sample A		Sample B		Sample C		Sample D	
Pyridoxine content, mg.	0.200		2.78		0.200		1.75	
Fe^{+2} content, mg.	4.00		33.3		0.0		0.0	
Fe^{+2}–pyridoxine ratio	20:1		12:1		0		0	
N,N'-Ethylenediaminetetraacetic acid–2Na added, g.	0	10	0	10	0	10	0	10
Assay performed								
1	0.1509	0.2051	2.216	2.820	0.2014	0.2180	1.775	1.775
2	0.1495	0.2034	2.095	2.846	0.2047	0.2035	1.781	1.798
3	0.1504	0.1977	2.185	2.910	0.2058	0.2118	1.787	1.745
4	0.1484	0.2028	2.156	2.799	0.2152	0.2185	1.703	1.752
5	0.1491	0.2015	2.131	2.843	0.2119	0.2158	1.746	1.703
Average ($\bar{X}$), mg.	0.14966	0.2021	2.157	2.844	0.2078	0.2135	1.756	1.755
Recovery, %	74.85	101.05	77.23	102.30	103.90	106.75	100.46	100.29
SD	0.001119	0.002779	0.04686	0.0417	0.0056	0.0062	0.0347	0.03557
$\bar{X} \pm 2SD$	0.14966± 0.002238	0.2021± 0.00556	2.157± 0.09372	2.844± 0.0834	0.2078± 0.0112	0.1135± 0.0124	1.758± 0.0694	1.755± 0.07114
Coefficient of variation, %	0.9418	1.375	1.726	1.47	2.69	2.90	1.97	2.02678
$S\bar{X}$	0.01496	2.02358	0.09681	0.09133	0.03347	0.03522	0.08332	0.08445
Theoretical *t* ($df = 4$)	2.776	2.776	2.776	2.776	2.776	2.776	2.776	2.776
t test	3.362	0.8902	6.4360	0.70076	0.2330	0.38330	0.09602	0.0592

containing approximately 2.0 mg. of pyridoxine is transferred to a 250-ml. conical flask. Then 100 ml. of 1 *N* HCl and 10 g. *N,N'*-ethylenediaminetetraacetic acid disodium salt are added and stirred until homogeneous. The flask is covered with an aluminum foil cap and autoclaved for 60 min. at 15 lb. pressure. The solution is cooled, adjusted to pH 6.8 with 2 *N* NaOH, transferred to a 1000-ml. volumetric flask, made to volume with distilled water, and then filtered. The filtrate is diluted with distilled water to the concentration that contains approximately 0.5 mcg. pyridoxine/ml. The bioassay is then carried out according to the general procedure.

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Isolation and Characterization of Some Solid Phases of Fluprednisolone

JOHN K. HALEBLIAN*, ROBERT T. KODA, and JOHN A. BILES

Abstract □ Seven different solid phases of fluprednisolone were isolated. Six were crystalline and one was amorphous. Of the crystalline phases, three were anhydrous, two were monohydrates, and one was a *tert*-butylamine disolvate. The phases were characterized using IR spectroscopy, X-ray powder diffraction, melting points, and densities. The heats of fusion were determined using differential scanning calorimetry. Heats of solution in water for three phases were also determined.

Keyphrases □ Fluprednisolone, solid phases—isolation, characterization □ Polymorphism—fluprednisolone □ IR spectrophotometry—identification, fluprednisolone solid phase □ X-ray powder diffraction—identification, fluprednisolone solid phase □ Differential scanning calorimetry—identification, fluprednisolone solid phase

Many drug molecules have been found to exist in more than one crystalline form. These crystalline modifications may have sufficient differences in their physical and thermodynamic properties that stability and biological availability may be affected. The importance of polymorphism on the therapeutic effectiveness of a drug is well recognized and previously was reported in the literature (1, 2).

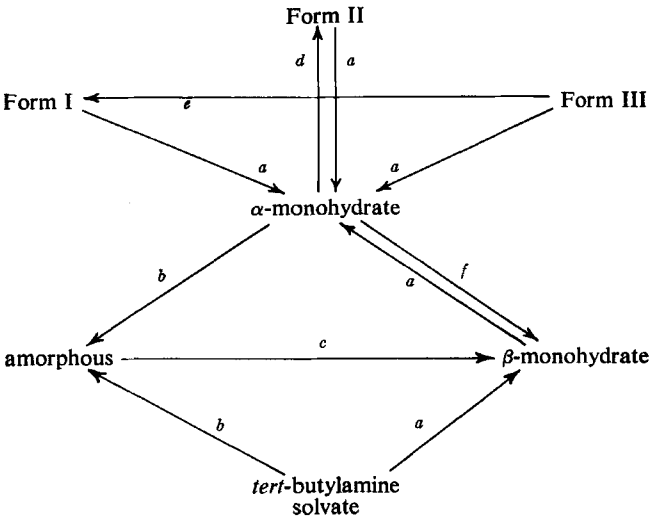
Fluprednisolone[1], 6α-fluoro-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione, was selected as the drug for study since there was evidence that this drug could be isolated in several crystalline phases[2]. This report describes the methods used in the isolation and characterization of some crystalline modifications of fluprednisolone.

EXPERIMENTAL

All X-ray powder diffractions were run on a spectrometer[3] adapted for X-ray diffraction, using copper Kα radiation[4] with a 1° beam slit and a 0.2° detector slit. All IR spectra were run as mineral oil mulls between sodium chloride windows on a spectrophotometer[5].

Heats of fusion were determined on a differential scanning calorimeter[6], using high temperature operation with a scan speed of 10°/min. Melting points were determined using either the differential scanning calorimeter or a microscope equipped with a micro hot-stage apparatus (Kofler).

The solubility of each crystalline phase was determined in water and *n*-octanol at temperatures between 0 and 60°. An excess of each form of the drug was added to the solvent in a 120-ml. bottle. The stoppered bottle was placed in an apparatus (Wruble) and was allowed to reach equilibrium at constant temperature (±0.1°). Equilibrium was considered established when the concentration of the steroid in solution remained constant for a 48-hr. period. Samples were withdrawn using a preheated syringe and filtered through an adapter (Sweeney), using a 0.22-μ Millipore filter. Aqueous solutions were assayed using a spectrophotometer (Beckman-Gilford) at 241.5 nm. Solutions of the steroid in *n*-octanol were assayed using a spectrophotometer (Beckman DB) at 241.5 nm.



a = suspension in water
b = 138° under vacuum (2 mm. Hg)
c = pressure
d = 180° for 5 min.
e = standing in air
f = aqueous suspension at 80° and seeded with β-monohydrate

Scheme I—Phase interconversions of the various polymorphs of fluprednisolone

The amount of solvent bound to the various crystalline phases of fluprednisolone was determined by either the Karl-Fischer method (for hydrates), weight loss studies, or the determination of molar absorbancies.

Densities of the crystalline phases were determined by the method of Bernal and Crowfoot (3). A few crystals of the steroid were suspended in mineral oil. Carbon tetrachloride was carefully added to the mineral oil–steroid mixture until a stable suspension of the crystalline phase existed, even after prolonged centrifugation. At this point, the density of the crystalline material was assumed to be identical to the mineral oil–carbon tetrachloride mixture. The density of the liquid phase was determined using a pycnometer.

Isolation of Phases—Fluprednisolone was crystallized from solutions of various solvents under different conditions. For example, Form I was isolated using the following procedures: (*a*) evaporation of a solution of fluprednisolone in ethyl acetate at room temperature and collection of the columnar crystals adhering to the sides of the beaker, (*b*) evaporation of an acetone solution of fluprednisolone on a hot plate, and (*c*) crystallization by cooling a hot saturated solution of fluprednisolone in anhydrous methanol in a sealed flask. Form II was obtained by heating the α-monohydrate at 180° for 5 min. on the stage of a melting-point apparatus (Fisher-Johns). Cooling of a hot saturated solution of fluprednisolone in acetone in a closed flask gave Form III. After crystallization occurred, the supernatant layer was decanted and the crystals were allowed to air dry at room temperature. If, on the other hand, an acetone solution of fluprednisolone was allowed to evaporate at room temperature in an open system, a mixture of Form III and the β-monohydrate was obtained. The anhydrous nature of Forms I, II, and III was verified by: (*a*) heating each of the phases in a fluid[7] and observing for the absence of effervescence, (*b*) absence of

[1] Marketed as Alphadrol by The Upjohn Co., Kalamazoo, Mich.
[2] Preliminary investigation by J. A. Biles.
[3] General Electric XRD-5.
[4] λ = 1.54051 Å.
[5] Perkin-Elmer Infracord, model 137.
[6] Perkin-Elmer, model DSC-1B.
[7] 555, Dow Corning Corp., Midland, Mich.

Table I—The d Distances of I/I_1 Values of Various Crystalline Phases of Fluprednisolone Using Copper Kα Radiation

Form I		Form II		Form III	
d, Å	I/I_1	d, Å	I/I_1	d, Å	I/I_1
10.3940	11.67	8.8378	20.00	8.9624	38.76
9.0173	28.46	7.8584	18.18	8.4181	17.98
8.3784	39.05	7.1608	55.45	6.3203	39.55
7.1320	9.49	6.4114	48.18	6.1035	44.94
5.9604	33.94	6.0619	98.18	5.5004	100.00
5.4835	100.00	5.6041	100.00	5.0348	91.01
5.1510	12.04	5.2111	56.36	4.7036	19.10
4.7410	19.85	5.0348	38.18	4.5026	14.83
4.4577	20.80	4.4801	57.27	4.2168	21.91
4.2168	19.49	4.0367	34.54	4.0550	10.11
3.7666	10.22	3.9783	20.91	3.9309	4.49
3.5448	9.49	3.5587	24.54	3.6746	19.10
3.4112	11.68	3.4502	16.36	3.5309	12.92
3.0557	7.66	3.2348	18.18	3.4241	15.17
				3.2406	20.22
				3.0455	21.35

α-Monohydrate		β-Monohydrate		*tert*-Butylamine Solvate	
d, Å	I/I_1	d, Å	I/I_1	d, Å	I/I_1
9.9270	58.12	7.8584	50.76	15.3580	100.00
7.4305	31.11	7.1897	14.36	7.6220	7.69
6.4816	99.49	6.4022	35.90	6.8042	6.25
6.2317	68.38	5.6041	100.00	6.0209	16.34
5.6041	72.65	5.3358	49.74	5.2789	36.06
4.8834	100.00	4.9511	51.28	4.6962	8.65
4.6068	50.43	4.5950	4.10	4.3287	10.09
3.9481	24.78	4.3496	13.33	4.1486	8.65
3.7354	35.04	4.2069	10.25	4.0008	3.85
3.5378	31.62	4.0008	14.87	3.7903	5.96
3.2406	27.35	3.8885	21.54	3.5309	4.61
3.0660	14.53	3.7509	15.38		
		3.5657	30.76		
		3.3857	16.41		
		3.2406	14.36		
		3.1078	4.10		

weight loss when the phases were dried at 138° under vacuum, and (*c*) comparison of the molar absorbancy at 241.5 nm.

The α-monohydrate phase was obtained by the evaporation of a saturated solution of fluprednisolone in methanol at room temperature in an open system. It was subsequently found that this phase could also be obtained by the evaporation of saturated solutions of fluprednisolone in ethanol and *n*-propanol. The β-monohydrate was obtained by evaporating a saturated solution of the steroid in water to approximately 5% of the original volume. The solution was filtered while hot, and the crystals were air dried at room temperature. The degree of hydration for both the α- and β-monohydrate phases was determined by Karl-Fischer methods, weight loss studies, and a comparison of their molar absorbancies with that of anhydrous Form I.

The *tert*-butylamine solvate was obtained by the crystallization of fluprednisolone from a solution in *tert*-butylamine. A comparison of the molar absorbancy of this solvated form with anhydrous Form I indicated that two molecules of *tert*-butylamine were bound to each molecule of fluprednisolone.

The amorphous phase of fluprednisolone was obtained by the lyophilization of a solution of the steroid in *tert*-butanol, as suggested by Shell (4).

The proof of the chemical nature of each polymorph was established by converting each phase to Form I. This was accomplished by dissolving each polymorphic phase in acetone and evaporating the acetone solution to dryness on a hot plate. In each case, the phase obtained was Form I of fluprednisolone.

RESULTS AND DISCUSSION

Seven different phases of fluprednisolone were isolated. Three were anhydrous, three were solvates, and one was amorphous. The anhydrous phases were designated Forms I, II, and III, with Form I being the most stable at room temperature. The dimorphic monohydrates were designated α- and β-monohydrates. The third solvate was the *tert*-butylamine disolvate.

Phase transitions occurred under a variety of conditions. Al crystalline phases were converted to the α-monohydrate upon suspension in water. Thus, aqueous suspensions of Forms I, II, and III

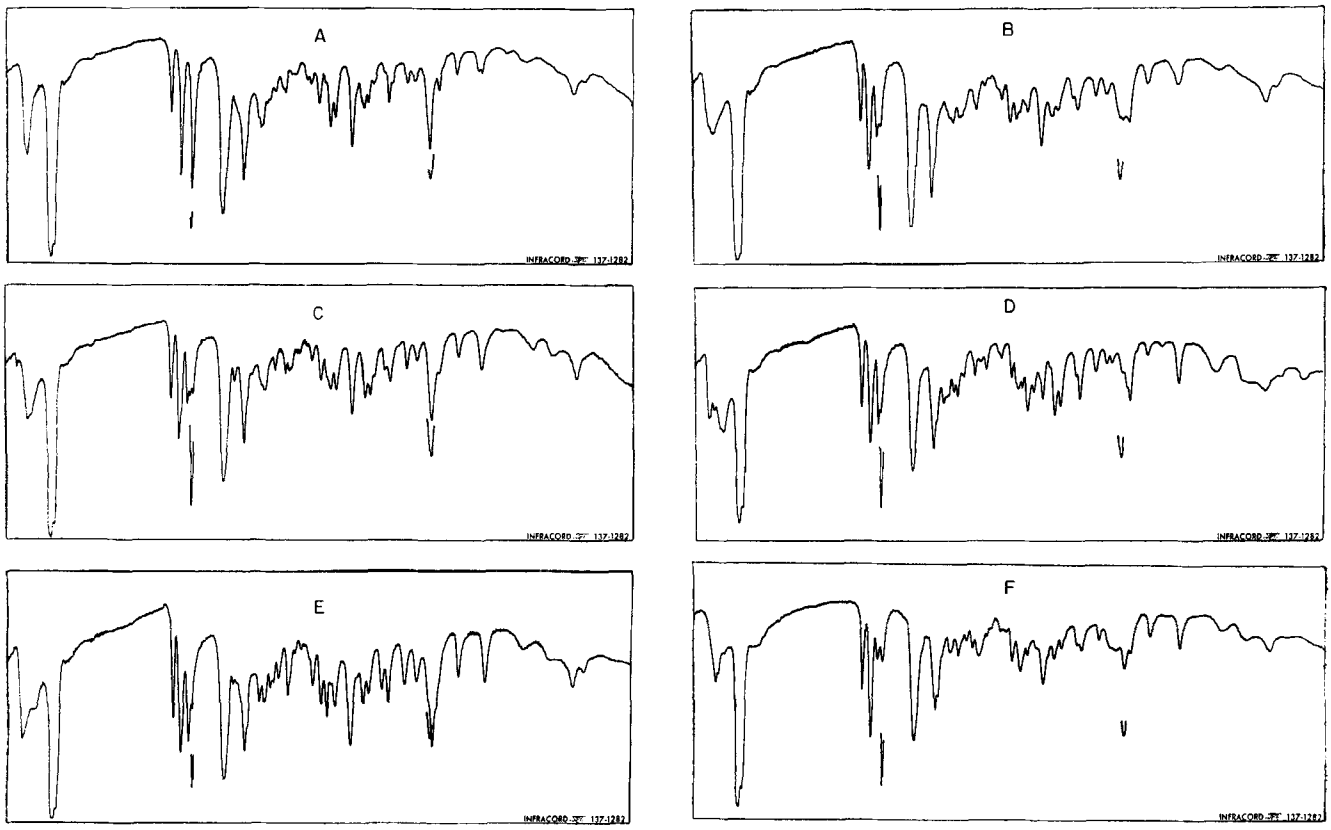


Figure 1—*IR spectra of the crystal modifications of fluprednisolone. Key: A, Form I; B, Form II; C, Form III; D, α-monohydrate; E, β-monohydrate; and F,* tert*-butylamine disolvate.*

Table II—Densities, Melting Points, and Heats of Fusion for Crystalline Modifications of Fluprednisolone

Crystalline Phase	Density	Melting Point—Micro Hot Stage	Melting Point—Scanning Calorimeter	Heat of Fusion, kcal./mole
Form I	1.318	199–200°	199°	4.616
Form II	1.350	193–194°	215°	5.245
Form III	1.388	208–210°	215°	5.476
α-Monohydrate	1.357	212°[a]	215°	5.245
β-Monohydrate	1.347	205°	219°	7.135
tert-Butylamine disolvate	1.177	105°	88°	5.689

[a] The α-monohydrate melted at 145°, resolidified at 157°, and finally melted at 212°.

and the β-monohydrate were transformed at various rates to the α-monohydrate. The *tert*-butylamine solvate in aqueous suspension, however, was initially converted to the β-monohydrate, which in turn was transformed into the α-monohydrate. Form III, in contact with air at room temperature, was slowly transformed to Form I.

The α-monohydrate and the *tert*-butylamine solvate were converted to an amorphous phase upon drying at 138° under vacuum (2 mm. Hg). The α-monohydrate was also converted to Form II when heated to 180° for 5 min. on the stage of a melting-point apparatus (Fisher-Johns). When an aqueous suspension of the α-monohydrate phase at 80° was seeded with a crystal of the β-monohydrate, the suspension was transformed into the β-monohydrate phase.

The amorphous phase was converted to the β-monohydrate when subjected to pressures of 7750 p.s.i. in a die having 50 mm.^{2} faces in a press (Carver). Compression of the amorphous phase was done without evacuation of the die. It is possible that atmospheric and adsorbed moisture could account for the formation of a hydrated phase from the anhydrous amorphous form.

A summary of phase interconversions is shown in Scheme I. The phases were identified using X-ray powder diffraction, IR spectroscopy, and differential scanning calorimetry. Partial conversions and mixtures could be detected by differential scanning calorimetry.

It was found that X-ray powder diffraction was the preferred method for the determination of various crystalline phases. The results of the X-ray powder diffraction patterns obtained on the XRD-5 spectrometer for six crystalline phases of fluprednisolone are shown in Table I. The *d* distances are given, and the relative intensities of each reflection were calculated as I/I_1 (I_1 being the most intense reflection).

The IR spectra for each crystalline polymorph are shown in Fig. 1. Although only small differences in IR spectra can be observed, these changes are of sufficient magnitude to be of value in phase identification. It is our feeling, however, that IR spectra are only useful in conjunction with other methods for the identification of phases.

The physical properties for each crystalline polymorph were studied. The densities, melting points, and heats of fusion are shown in Table II. The melting points for each phase were determined by using either a microscope equipped with a micro hot-stage apparatus

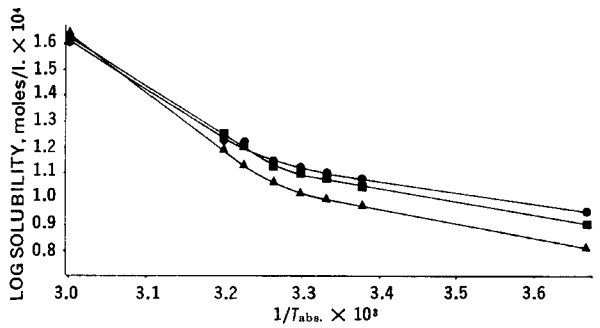


Figure 2—*Solubility of three phases of fluprednisolone in water as a function of temperature. Key:* ■, *Form I;* ▲, *α-monohydrate; and* ●, *β-monohydrate.*

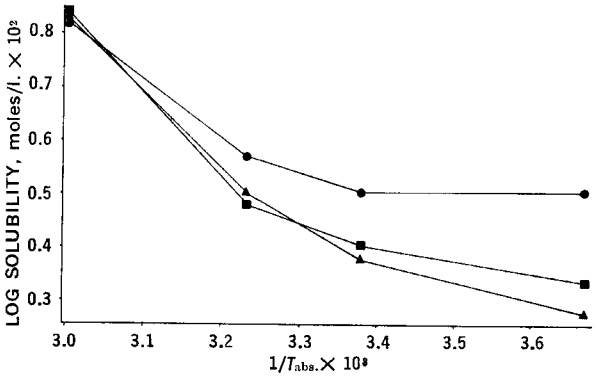


Figure 3—*Solubility of three phases of fluprednisolone in* n-*octanol as a function of temperature. Key:* ■, *Form I;* ▲, *α-monohydrate; and* ●, *β-monohydrate.*

(Kofler) or the differential scanning calorimeter. The heats of fusion were also determined on the differential scanning calorimeter using an indium standard. The differential scanning calorimetry thermogram for the α-monohydrate showed an endothermic peak at 130°, which was indicative of the loss of water of crystallization. This was followed by an exothermic peak at 150° where Form II precipitated from the supercooled melt after losing water. An exothermic peak followed at 215°, which was in agreement with the melting point of Form II. To confirm the findings, crystals were isolated after reaching the 150° peak. X-ray diffraction indicated that conversion of the α-monohydrate to Form II had occurred.

The equilibrium solubilities in water and *n*-octanol were determined for Form I, the α-monohydrate, and the β-monohydrate phases at temperatures varying between 0 and 60°. The plots of the logarithm of the molar solubility *versus* the reciprocal of the absolute temperature in water and *n*-octanol are presented in Figs. 2 and 3, respectively. The plots indicate that the slope of the best straight line through the points from 0 to 23° is significantly different from the slope of the line from 37 to 60°. This may be indicative of: (*a*) a crystalline phase change, or (*b*) alterations in the degree of association of fluprednisolone with solvent at various temperatures. It was previously found (Scheme I) that when Form I or the β-monohydrate was suspended in water at room temperature, phase transition occurred, with the formation of the α-monohydrate form. However, when the α-monohydrate was suspended in water above 37° and below 23°, phase transition was not observed.

Figure 4 presents the solubility data for the α- and β-monohydrate forms in water and *n*-octanol as the logarithm of the solubility ratio *versus* T^{-1}. From the slope of the best straight line drawn through the points, one may calculate the enthalpy of conversion ($\Delta H_{\alpha,\beta}$) of the β-form to the α-monohydrate form according to the method of Higuchi *et al.* (1). The transition temperature may be obtained by extrapolating the line to the point where the solubility ratio is unity ($s_\beta/s_\alpha = 1$). The transition temperature (T_t) at this point was 57.5°, and the enthalpy of conversion ($\Delta H_{\alpha,\beta}$) was 209 cal. mole^{-1}. Since the free energy of transition ($\Delta F_{\alpha,\beta}$) at the transition temperature is zero and $\Delta F_{\alpha,\beta} = \Delta H_{\alpha,\beta} - T\,\Delta S_{\alpha,\beta}$, the entropy of

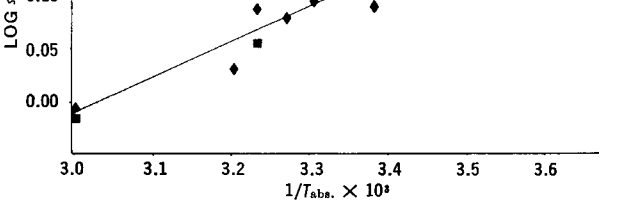


Figure 4—*Plot of the logarithm of the solubility ratios of the β-monohydrate to α-monohydrate phases of fluprednisolone as a function of temperature. Key:* ■, *in n-octanol; and* ◆, *in water.*

Table III—Heats of Solution and Fusion in Kilocalories per Mole for Three Polymorphic Forms of Fluprednisolone

Phase	ΔH_s (0–23°)	ΔH_s (37–57°)	ΔH_f[a]
Form I	0.510	2.106	4.616
α-Monohydrate	0.669	2.301	5.245
β-Monohydrate	0.469	2.092	7.135

[a] From differential scanning calorimetry studies.

transition ($\Delta S_{\alpha,\beta}$) for the β-monohydrate to α-monohydrate form may be calculated. By using this relationship, $\Delta S_{\alpha,\beta} = 0.632$ e.u. was obtained. It is difficult to extrapolate the results of the entropy changes of the β- to α-monohydrates to alterations in molecular arrangement in the crystal lattice. A larger $\Delta S_{\alpha,\beta}$ would indicate a greater degree of freedom of the molecules in the crystal lattice. The small $\Delta S_{\alpha,\beta}$ observed may represent minor alterations in bonding occurring with the interconversion of the α-form.

The heats of solution for Form I, the α-monohydrate, and the β-monohydrate were determined from 0 to 23° and from 37 to 57°. These values are compared with the heats of fusion determined from differential scanning calorimetry data and are shown in Table III. The difference in ΔH_s from 0 to 23° and from 37 to 57° from each phase was a constant value.

The β-monohydrate form exhibits 25% greater solubility than the α-monohydrate phase at room temperature. Although the differences in solubility are small, with a judicious choice of the proper polymorphic phase for use in pharmaceutical suspensions of fluprednisolone, physical stability of such preparations can be optimized.

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Comparison of Dissolution Rates of Different Crystalline Phases of Fluprednisolone by *In Vitro* and *In Vivo* Methods

JOHN K. HALEBLIAN*, ROBERT T. KODA, and JOHN A. BILES

Abstract □ The *in vitro* dissolution rates of six crystalline phases of fluprednisolone were determined and compared with *in vivo* dissolution rates from pellet implants in rats. The dissolution studies were correlated with animal weight loss and adrenal gland atrophy. Excellent correlations were obtained between the *in vitro* and *in vivo* dissolution rates; however, correlation with animal weight loss and adrenal gland atrophy was only fair.

Keyphrases □ Fluprednisolone, crystalline phases—dissolution rates comparison, *in vivo*, *in vitro* □ Dissolution rates, fluprednisolone phases—*in vivo*, *in vitro* comparison

It was demonstrated that factors such as the total surface area of the dosage form and the solubility of the drug in surrounding biological fluids have a profound influence on the rate of absorption from subcutaneous pellet implants (1–3). The site of implantation, animal activity, substances used in the preparation of the dosage form, and crystalline phase may also influence the absorption rate (4, 5).

The purpose of this study was to correlate the *in vitro* rates of dissolution of several solvated and nonsolvated phases of fluprednisolone[1] with *in vivo* rates of absorption from solid pellet implants and with two biological responses, namely, animal weight loss (6) and adrenal gland atrophy (7).

Six crystalline phases of fluprednisolone were selected for this study. Of these forms, three were anhydrous, two were monohydrates, and one was a *tert*-butylamine disolvate. The isolation and characterization of the polymorphic forms were previously described by Haleblian *et al.* (8).

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EXPERIMENTAL

Cylindrical pellets, 8 mm. in diameter, of six pure fluprednisolone phases were prepared by compression in a Carver press at 7750 p.s.i. for 15 sec. The *in vitro* dissolution rates of the pellets were determined by placing each pellet in a polyethylene holder which secured the pellet in the center of a 120-ml. bottle. Water was added to each bottle, and the bottles were placed in a Wruble apparatus. For each separate run of the experiment, either the solvent, the temperature, or the velocity of agitation of the Wruble apparatus was modified. At specific intervals of time, samples of solution were withdrawn from each bottle using a preheated syringe; the solution was passed through a Millipore filter (0.22 μ) using a Swinney adapter. Each sample was assayed spectrophotometrically at 241.5 nm. in a Beckman DU or Beckman DB spectrophotometer.

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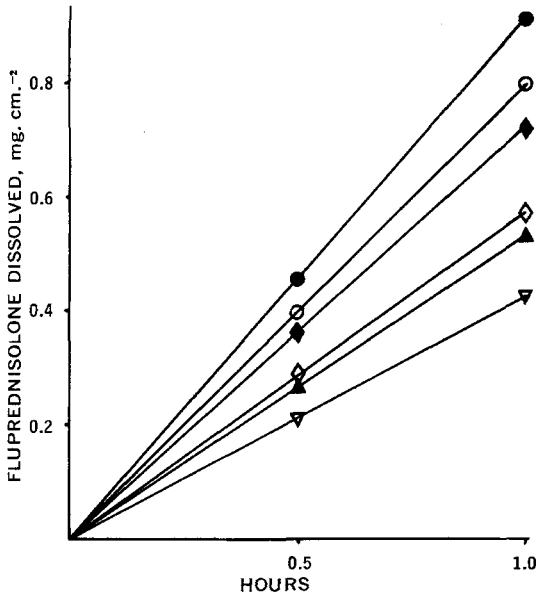

Figure 1—*Dissolution of various phases of fluprednisolone in water at 23°. Key:* ●, *Form I;* ◇, *Form II;* ○, *Form III;* ▽, *α-monohydrate;* ▲, *β-monohydrate; and* ◆, tert-*butylamine disolvate.*

veloped by Ballard and Nelson (3). One to three pellets of pure fluprednisolone phases were implanted subcutaneously in the abdominal region of rats. Sprague-Dawley rats of either sex, weighing between 225 and 430 g., were used in the implantation studies. The animals were anesthetized with ether, and a ventral midline incision was made. The subcutaneous connective tissue lateral to the incision was teased apart, and the pellets were implanted. The incision was closed by suturing after implantation. Five to eight animals were used in each test group. Four additional rats were used as unoperated controls. A third group of four rats were implanted with pellets of pure cholesterol. The test animals were allowed food and water *ad libitum.*

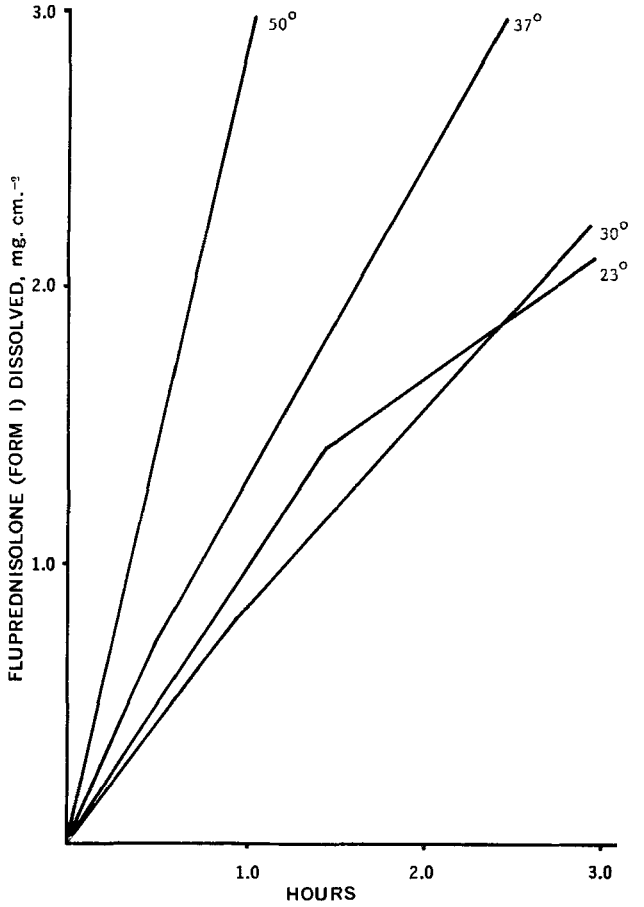

Figure 2—*Dissolution of Form I in water at various temperatures.*

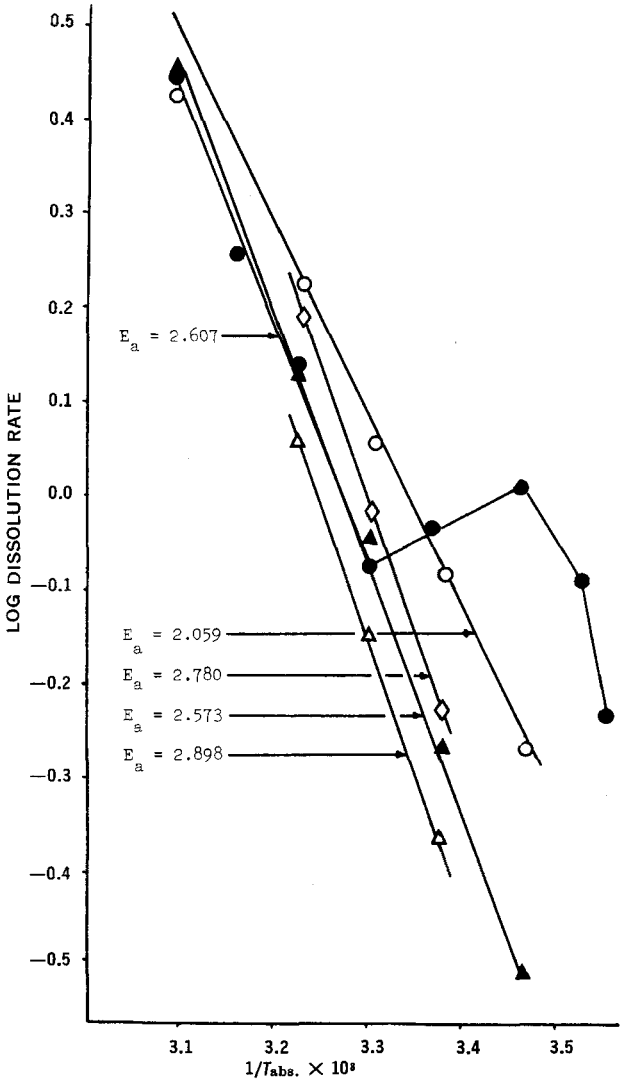

Figure 3—*Dissolution rates of various phases of fluprednisolone in water as a function of temperature. Key:* ●, *Form I;* ◇, *Form II;* ○, *Form III;* △, *α-monohydrate; and* ▲, *β-monohydrate.*

After 72 hr., the pellets of fluprednisolone were removed, cleaned of extraneous organic material, dried, and weighed. The average surface area of the pellets was also determined. The mean absorption rate per surface area of pellet was calculated for each crystalline phase.

The biological response to the absorbed drug was observed by determining its effect on animal weight loss and adrenal gland atrophy. The animals were weighed daily, and the percent loss in body weight was calculated. At the end of 72 hr., the pellets of fluprednisolone were removed, the animal was sacrificed, and the adrenal glands were removed. The whole gland was freed of surrounding lipid and connective tissue, and its wet weight was determined. The adrenal gland atrophy was compared to standard adrenal gland weights (9). From these data, the weight loss of adrenal gland per animal weight per hour was calculated.

RESULTS AND DISCUSSION

The dissolution rates of six phases of fluprednisolone in water were determined using the Wruble apparatus at 6 r.p.m. The results at 23° are presented in Fig. 1 as milligrams of fluprednisolone dissolved per square centimeter of surface area *versus* time. Over the initial

Table I—Adrenal Gland Weight Loss Produced by Various Crystalline Phases of Fluprednisolone

Phase	Number of Animals	Expected Mean[a] Adrenal Weight, mg.	Adrenal Gland Weight at End of Experiment, mg.	Adrenal Gland Weight Loss, mg.	Hours	Gram Atrophy per Gram Rat Weight per Hour × 10^{-7}
Form I	5	38.18 (36.9–39.5)	24.49 (23.05–25.95)	13.69 (12.75–14.85)	71.70	8.05 ± 0.26
Form II	7	44.96 (41.7–48.0)	28.41 (27.60–33.20)	12.10 (12.15–14.80)	71.35	6.57 ± 0.17
Form III	6	43.38 (39.3–45.8)	28.61 (26.55–30.40)	14.76 (12.75–16.85)	72.37	7.09 ± 0.12
β-Monohydrate	8	43.97 (42.0–45.8)	30.96 (29.05–33.25)	13.00 (10.95–14.35)	72.65	6.11 ± 0.19
α-Monohydrate	8	45.21 (43.0–48.4)	33.08 (29.85–36.20)	12.12 (9.60–14.90)	71.83	5.52 ± 0.27
tert-Butylamine disolvate	6	52.83 (50.9–57.31)	33.33 (29.95–39.15)	19.50 (11.75–26.15)	81.75	5.85 ± 0.57

[a] Reference 9.

Table II—Summary of *In Vitro* and *In Vivo* Dissolution Rates, Animal Weight Loss, and Adrenal Gland Weight Loss

Phase	*In Vitro* Dissolution Rate at 23°[a]	*In Vitro* Dissolution Rate at 37°, mg. $hr.^{-1}$ $cm.^{-2}$	*In Vivo* Dissolution Rate, mg. $hr.^{-1}$ $cm.^{-2}$	Percent Body Weight Loss (72 hr.)	Adrenal Gland Weight Loss[a] (72 hr.)
Form I	0.9167 ± 0.0129	1.3517 ± 0.0037	0.237 ± 0.010	17.0	8.05 ± 0.26
Form II	0.5714 ± 0.0079	1.5216 ± 0.0046	0.186 ± 0.004	12.6	6.57 ± 0.17
Form III	0.8038 ± 0.0063	1.6596 ± 0.0067	0.209 ± 0.011	11.2	7.09 ± 0.12
β-Monohydrate	0.5273 ± 0.0020	1.3312 ± 0.0041	0.162 ± 0.004	15.8	6.11 ± 0.19
α-Monohydrate	0.4096 ± 0.0038	1.1366 ± 0.0037	0.147 ± 0.005	14.7	5.52 ± 0.27
tert-Butylamine disolvate	0.7403 ± 0.0056	1.6363 ± 0.0071	0.204 ± 0.009	12.9	5.85 ± 0.57

[a] Adrenal atrophy per rat weight per hour × 10^{-7}.

60-min. period, the dissolution of each of the phases followed apparent zero-order kinetics.

Several phase transitions were observed during the dissolution studies. The *tert*-butylamine disolvate was rapidly converted to the β-monohydrate form, and Form I was converted to the α-monohydrate. The dissolution behavior of Form I in water at various temperatures is presented in Fig. 2. The rate of dissolution at 50° appears to be more rapid than the rate of transition. However, at 23, 30, and 37°, the transition rate of Form I to the α-monohydrate may be of the same order as the dissolution rate of Form I. It was previously reported (8) that the heat of solution of Form I from 0 to 23° differs from that determined at 37–57°. This difference in heat

Table III—Equations Correlating *In Vitro* and *In Vivo* Dissolution Rates[a] with Biological Effects

Equation	n	r	$s_{x \cdot y}$	Equation No.
In vitro dissolution rate at 23° and *in vivo* dissolution from subcutaneous pellet implants:				
$y = 0.169x + 0.079$	6	0.983	0.007	(1)
where x = *in vitro* dissolution rate at 23°, and y = *in vivo* dissolution rate				
In vitro dissolution rate at 37° and *in vivo* dissolution from subcutaneous pellet implants:				
$y = 0.119x + 0.009$	5	0.991	0.004	(2)
where x = *in vitro* dissolution rate at 37°, and y = *in vivo* dissolution rate				
Percent body weight loss and *in vitro* dissolution rate at 23°:				
$y = -8.936x + 18.894$	5	0.790	1.2848	(3)
where x = *in vitro* dissolution rate at 23°, and y = percent body weight loss				
Percent body weight loss and *in vitro* dissolution rate at 37°:				
$y = -6.674x + 23.166$	5	0.814	1.218	(4)
where x = *in vitro* dissolution rate at 37°, and y = percent body weight loss				
Adrenal gland weight loss and *in vitro* dissolution rate at 23°:				
$y = 4.519x + 3.1749$	5	0.976	0.2416	(5)
where x = *in vitro* dissolution rate at 23°, and y = adrenal gland weight loss				
Adrenal gland weight loss and *in vitro* dissolution rate at 37°:				
$y = 1.895x + 3.466$	5	0.681	0.5212	(6)
where x = *in vitro* dissolution rate at 37°, and y = adrenal gland weight loss				
In vivo dissolution rate and adrenal gland weight loss:				
$y = 0.030x + 0.004$	6	0.8379	0.0201	(7)
where x = adrenal gland weight loss, and y = *in vivo* dissolution rate				

[a] mg. $hr.^{-1}$ $cm.^{-2}$.

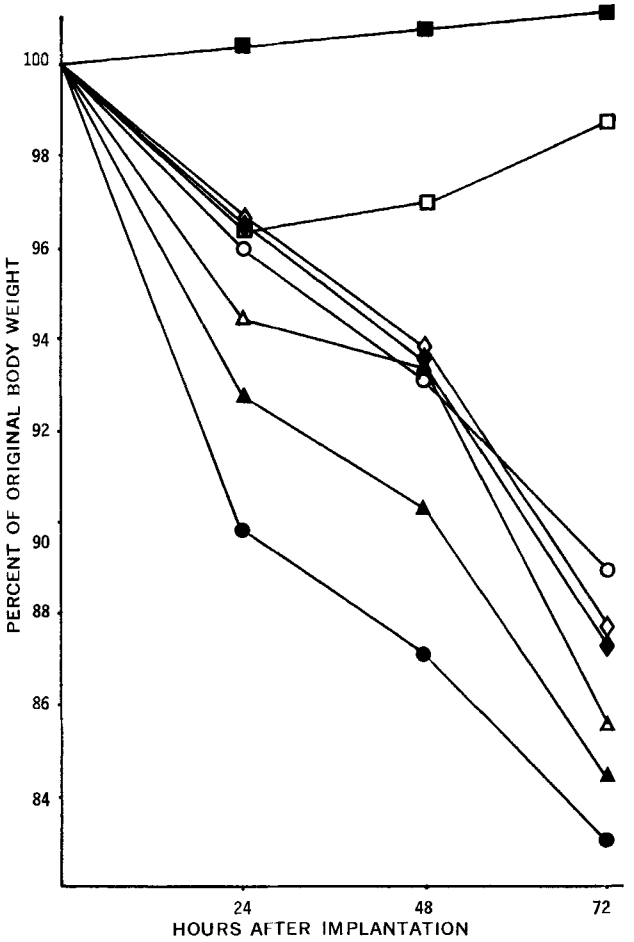

Figure 4—*Percent of original body weight remaining after fluprednisolone implantation. Key:* ■, *unoperated control;* □, *sham-operated control;* ●, *Form I;* ◇, *Form II;* ○, *Form III;* △, *α-monohydrate;* ▲, *β-monohydrate; and* ◆, tert-*butylamine disolvate.*

of solution was found to be due to a phase transition of Form I to the α-monohydrate.

The activation energy (E_a) for the dissolution process for each phase was determined by plotting the log of the dissolution rate against the reciprocal of the absolute temperature. Figure 3 indicates that the activation energy for the dissolution of Form II, Form III, α-monohydrate, and β-monohydrate is constant from 8 to 50°. However, the dissolution rate of Form I was observed to increase from 8 to 15°. At temperatures from 15 to 37°, the rate of conversion of Form I to the α-monohydrate appears to be significant enough to alter the dissolution rate; at temperatures above 37°, the dissolution process is more rapid than the phase interconversion process.

The *in vivo* dissolution rate of various phases of fluprednisolone pellets were determined in rats, using the implantation technique developed by Ballard and Nelson (3). The specific dissolution rate ($\bar{R}/\bar{A}$) was calculated by determining the weight of steroid dissolved per hour ($\bar{R}$) per average surface area of pellet ($\bar{A}$).

The biological effects of the absorbed steroid from pellet implants were determined by following animal weight loss and adrenal gland atrophy. A plot of percent of original body weight remaining *versus* time is shown in Fig. 4. The unoperated controls showed a consistent weight gain, while the sham-operated controls displayed an initial weight loss with a rapid recovery. This initial weight loss was probably due to surgical trauma. The animals that were implanted with pellets of the various phases of fluprednisolone showed a progressive weight loss.

In addition to body weight loss studies, adrenal gland atrophy was observed. At the end of the 72-hr. test period, each animal was sacrificed and the wet weight of the adrenal gland was determined (Table I). No attempt was made to relate the adrenal gland atrophy to any of the specific layers of the adrenal gland.

A summary of the *in vitro* and *in vivo* dissolution rates, animal weight loss studies, and adrenal gland atrophy is presented in Table II. Since three of the phases were solvated, pellet weight loss was converted to an equivalent fluprednisolone weight loss.

Equations correlating the *in vitro* and *in vivo* dissolution rates with animal weight loss and adrenal gland atrophy were determined by the method of least squares and are summarized in Table III, where n = number of data points, $s_{x \cdot y}$ = standard error of estimate, and r = correlation coefficient. From Eqs. 1 and 2, the *in vitro* dissolution rates at 23 and 37° correlate well with the *in vivo* dissolution from subcutaneous pellet implants. This finding supports the work of Hamlin *et al.* (10) who found that *in vitro* dissolution rates determined at low agitation intensities correlated well with *in vivo* dissolution rates from subcutaneous pellet implants. The *in vitro* dissolution rate of Form I at 37° was not included in the regression analysis since it was previously shown that Form I undergoes a phase transition in contact with water to the α-monohydrate form at temperatures below 50°. The *in vivo* dissolution of Form I, therefore, does not reflect the true dissolution rate of the pure phase but is a hybrid rate determined by the relative ratio of Form I and its transformation product (the α-monohydrate) present.

Equations 3 and 4 show the relationship between body weight loss and *in vitro* dissolution at 23 and 37°. As before, Form I was omitted in the calculations because of phase transition. Correlation of body weight and *in vitro* dissolution was only fair (Eq. 3, r = 0.790; Eq. 4, r = 0.814). This finding is not surprising since animal weight loss is not a valid measure of a specific pharmacologic response.

Equations 5 and 6 correlate adrenal gland weight loss and *in vitro* dissolution rates. At 23°, the rate of dissolution gave excellent correlations (r = 0.976) with adrenal gland weight loss, while the *in vitro* dissolution at 37° correlated poorly (r = 0.681). This result may be due to the rapid transition of Form I to the α-monohydrate form at 37°.

In vivo dissolution rate correlated only fairly with adrenal gland atrophy (Eq. 7, r = 0.8379). It is possible that phase transitions may have occurred at the pellet surface in the presence of fluids at the site of implantation.

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Determination of Therapeutic Blood Levels of Methamphetamine and Pentobarbital by GC

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Abstract □ A GC method is presented for the assay of methamphetamine and pentobarbital in blood following oral administration of the combination. The trichloroacetamide derivative of methamphetamine has higher electron-capture sensitivity and is more easily chromatographed than previously reported derivatives. Details concerning the extraction procedure, derivative formation, percent recoveries of both drugs, and blood level data are given.

Keyphrases □ Methamphetamine—GLC analysis, blood □ Pentobarbital—GLC analysis, blood □ GLC—analysis, methamphetamine and pentobarbital in blood

Various methods have been used for the determination of amphetamines (1–5) and barbiturates (6–10) from body fluids. Most of the more sensitive methods utilized GLC equipped with flame-ionization detectors. These methods have proved useful for therapeutic doses of barbiturates (11–15) but lack the necessary sensitivity required to monitor blood levels over a 24-hr. period from a small single dose of methamphetamine (15–18). Inquiries concerning the bioavailability of certain products led to the development of an analytical technique for the determination of either methamphetamine alone or in combination with a barbiturate.

The advent of the electron-capture detector (ECD), with its high sensitivity toward halogenated compounds, led to the formation of suitable derivatives by some workers (19–22). Bruce and Maynard (23) described a method based on the formation of heptafluorobutyramide from some secondary (*i.e.*, methamphetamine) and primary amines. Noonan *et al.* (24) used the trichloroacetamide derivative for the determination of amphetamine. Wilkinson (25) showed the pentafluorobenzamide of amphetamine to be highly sensitive, with excellent possibilities for monitoring blood levels.

Results obtained from different derivatives in this laboratory indicate that the trichloroacetamide is the derivative of choice for methamphetamine. The high order of sensitivity: trichloroacetamide > heptafluorobutyramide > pentaflurobenzamide, indicates its desirability from this standpoint. In addition, its lower volatility compared to heptafluorobutyramide negates

Table I—Percent Recovery of Methamphetamine[a]

Total Nanograms Added	Trial 1, %	Trial 2, %	Mean Recovery, %
	Urine, 1 ml.		
0	0	0	0
25	91.6	92.5	92.1
50	93.5	93.1	93.2
125	90.7	91.2	91.0
250	92.3	93.8	93.1
500	96.7	95.1	95.9
750	90.9	96.2	93.6
1000	95.2	95.7	95.5
1500	98.1	97.8	98.0
2000	94.4	93.2	93.8
	Blood, 15 ml.[b]		
0	0	0	0
25	67.3	65.9	66.1
50	66.1	68.2	67.2
100	65.2	68.7	67.0
200	68.1	72.1	70.1
400	71.4	71.3	71.4
800	66.0	72.4	69.3
1200	69.7	67.5	68.6
1600	66.0	72.3	69.2
2000	73.2	68.8	70.1

[a] Trial values were determined by: peak height spiked sample/peak height pure derivative. [b] The lower recoveries from blood could be due to erythrocyte binding. However, preliminary studies indicated no significant difference in percent recoveries when plasma or whole blood was used.

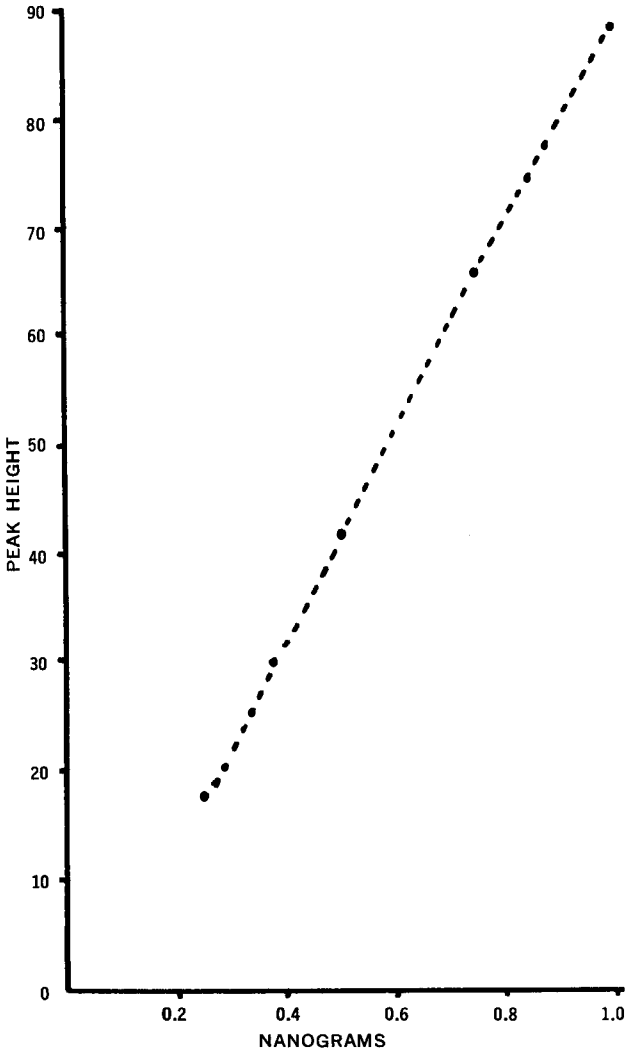

Figure 1—*Standard curve of methamphetamine trichloroacetamide, 3×10^{-9} amp.*

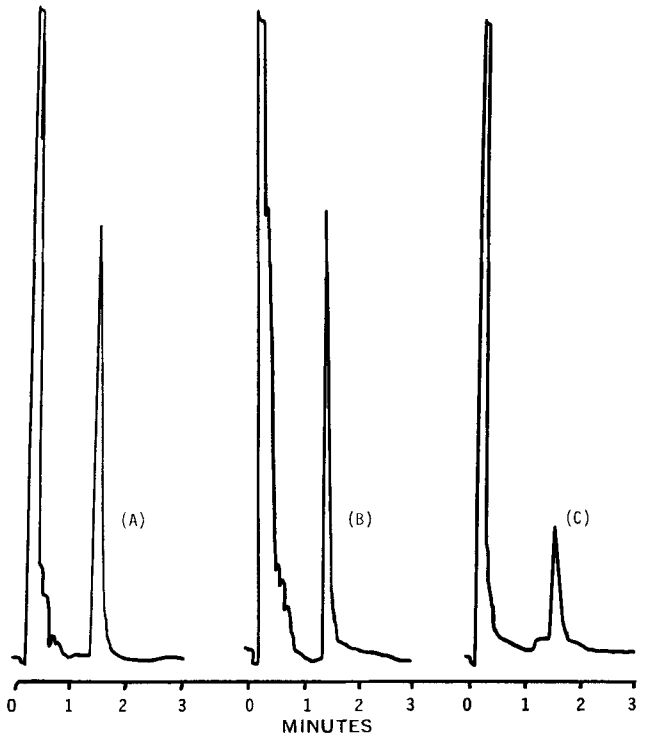

Figure 2—*Chromatographic responses of different methamphetamine derivatives. Key: (A), trichloroacetamide, 0.5 ng.; (B), heptafluorobutyramide, 2.0 ng.; and (C), pentafluorobenzamide, 2.0 ng.*

the need for temperature programming during successive blood determinations.

Samples containing barbiturates in addition to methamphetamine were selectively extracted; the barbiturate was determined by GLC, using a flame-ionization detector, with a method similar to that of Kazyak and Knoblock (15).

EXPERIMENTAL

Reagents—The following were used: trichloroacetyl chloride[1], redistilled, 17.8-cm. (7-in.) column; pyridine[2], distilled over CaH_2, 50.8-cm. (20-in.) column; and hexane[3], 1000 ml. washed with concentrated H_2SO_4 (6 × 50 ml.), 7 *N* NaOH (1 × 50 ml.), and H_2O (2 × 50 ml.), and distilled over CaH_2, 50.8-cm. (20-in.) column.

Apparatus—The following were used: gas chromatograph[4], chart speed 2 min./in.; electrometer, sensitivity range 3 × 10^{-6} to 10^{-12} amp.; flame-ionization detector, with air flow 600–700 ml./min. and hydrogen 60–70 ml./min.; and ECD, with 150 mc. tritium on titanium foil.

Procedures—*Preparation of Blood Sample*—The blood samples were collected in tubes containing a balanced mixture of potassium oxalate and ammonium oxalate[5]. The plasma layer of centrifuged whole blood (10 ml.) was removed and placed in a screw-cap tube (25 × 150 mm.) fitted with a Teflon liner. To this fraction was added distilled H_2O (10 ml.), 10% sodium tungstate (5 ml.), and 1.3 *N* H_2SO_4 (6 ml.). The mixture was allowed to stand for 10 min., followed by the addition of ether (25 ml.), and then briskly shaken and centrifuged. The ether extract was removed and placed in another tube, and the aqueous acid extract was placed in a 50-ml. centrifuge tube. The residue in the screw-cap tube was again treated with 1.3 *N* H_2SO_4 (10 ml.) and ether (25 ml.) and shaken. The ether and acid washings were combined with their respective extracts.

[1] Eastman.
[2] Purified grade, Matheson, Coleman and Bell.
[3] Nanograde, Mallinckrodt.
[4] Packard model 7301, equipped with Honeywell Class 19 recorders.
[5] Vacutainer specimen tube (7 ml.), containing 6 mg. ammonium and 4 mg. potassium oxalate, Becton Dickinson 4761.

Table II—Percent Recovery of Pentobarbital from Blood[a]

Micrograms per Milliliter	Trial 1, %	Trial 2, %	Mean Recovery, %
0	0	0	0
0.10	88.6	89.9	89.3
0.20	90.1	92.7	91.4
0.30	93.6	97.5	95.6
0.50	94.0	92.9	93.5
1.00	96.7	98.4	97.6

[a] Trial values were determined by: peak height spiked sample/standard $CHCl_3$ solution of pentobarbital.

Barbiturate Assay—The ether extract was dried (anhydrous Na_2SO_4, about 1 g.) and filtered through a pledget of glass wool, with the Na_2SO_4 being rinsed with ether (2 × 5 ml.). The combined rinsings and extract were evaporated to dryness under a stream of nitrogen. The evaporated extract was redissolved in chloroform (0.10 ml.), and 5 μl. was injected into a GC fitted with a flame-ionization detector. The column was glass [1.8 m. (6 ft.) long × 0.63 cm. (0.25 in.) o.d.], packed with acid-washed Chromosorb W[6], silylated with dimethylchlorosilane, and having a 5% SE-30 liquid load.

The instrument's operating conditions were as follows: inlet port, 220°; column, 195°; detector, 275°[7]; and a gas flow (nitrogen), 90 ml./min. Retention time for pentobarbital was 4.0 min. Since some high boiling compounds are extracted by the procedure, the system was temperature programmed after each injection. The



Figure 3—*Chromatogram of methamphetamine trichloroacetamide, 25 pg.*

[6] Hewlett-Packard.
[7] Detector temperature was maintained at 275° to avoid condensation of high boiling materials during temperature programming.

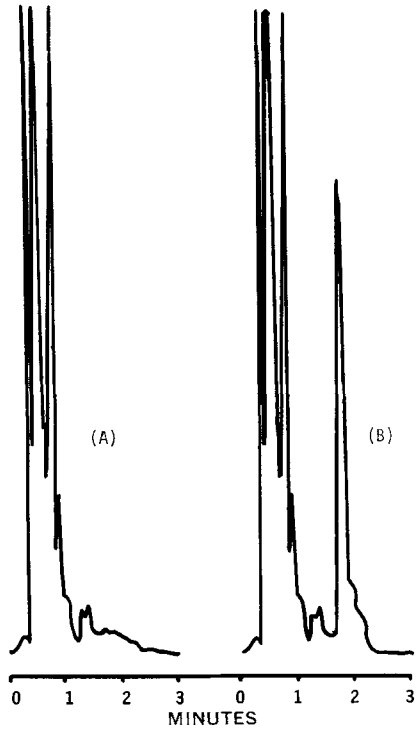

Figure 4—*Chromatograms of trichloroacetylated human plasma extracts. Key: (A), before administration; and (B), 2 hr. after administration of methamphetamine hydrochloride, 12.5 mg.*

initial hold of 195° was 5 min., with a programmed rate of 5°/min. to 275° and a final hold of 5 min.

Methamphetamine—The combined aqueous layers removed in the extraction procedure were made alkaline (about pH 14)[8] by the addition of 7 *N* NaOH (5 ml.) and extracted with hexane (3 × 25 ml.). The hexane extracts were combined and dried over NaOH pellets and decanted, and the pellets were rinsed with hexane (2 × 10 ml.). The combined rinsings and extract were evaporated under a stream of nitrogen to 1–2 ml. To this evaporated extract was added the derivatizing mixture[9] (0.1 ml.). The reaction was gently agitated

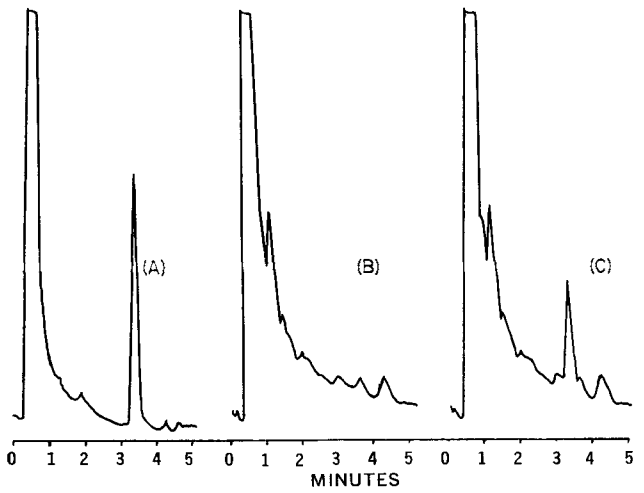

Figure 5—*Chromatograms of: (A), pentobarbital, 0.5 mcg. standard; (B), blank plasma; and (C), 6-hr. blood sample.*

[8] This pH gave cleaner chromatograms than those resulting from extractions at lower pH values. This may be due to the elimination of biogenic phenolic amines by sodium salt formations.

[9] Derivatizing mixture: To a solution of redistilled pyridine (1.580 g., 0.020 mole) in hexane (15 ml.) was added a solution of redistilled trichloroacetyl chloride (1.818 g., 0.010 mole) in hexane (30 ml.). The mixture was gently agitated and stoppered.

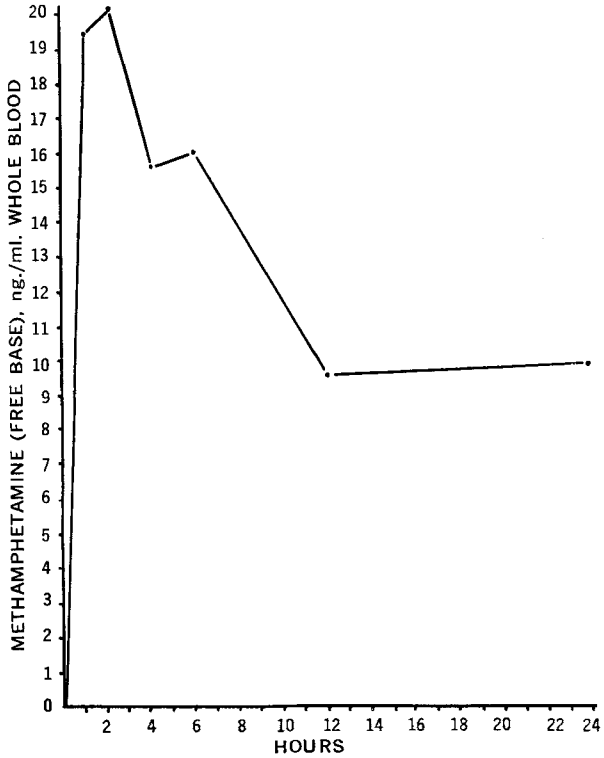

Figure 6—*Average blood level of 10 patients, each of whom received an oral liquid dose of 12.5 mg. methamphetamine hydrochloride.*

and allowed to stand at room temperature for 10 min.; distilled H_2O (1.0 ml.) was added, and the mixture was vigorously shaken. The sample was centrifuged, and the water layer was removed with a Pasteur pipet fitted with a 5-ml. syringe. In a similar manner, the following washings were completed: 10% HCl (1 ml.), 0.7 *N* NaOH (1 ml.), and H_2O (2 × 1 ml.).

The hexane extract was dried (anhydrous Na_2SO_4, about 0.25 g.) and transferred to a test tube (10 ml. graduated in 0.1 ml.). The Na_2SO_4 was rinsed with hexane (2 × 0.5 ml.), and the combined rinsings and extract were evaporated to 0.4 ml. under a stream of nitrogen. Quantities of 3–4 μl. of this solution were injected into a GC fitted with an ECD. The column was glass [1.2 m. (4 ft.) long by 0.63 cm. (0.25 in.) o.d.], packed with a 3% load of OV-1[10] on 80–100 mesh, Gas Chrom Q[10]. Retention time for the methamphetamine derivative was 1.5 min., with column, inlet port, and detector temperatures of 185° and a gas flow (nitrogen) of 105 ml./min. Confirmation of the trichloroacetamide derivative was accomplished by synthesizing a larger quantity under similar conditions and characterization by IR and elemental analysis. Gas chromatograms of the reference standard were identical under similar conditions to those of the submicro samples.

Methamphetamine Trichloroacetamide[11]—To a stirred solution of pyridine (7.91 g., 0.110 mole) and desoxyephedrine (7.46 g., 0.050 mole) in hexane (75 ml.) was added a solution of trichloroacetyl chloride (9.98 g., 0.055 mole) in hexane (25 ml.) over 10 min. A mild exothermic reaction occurred, accompanied by the precipitation of pyridine hydrochloride. The mixture was stirred for 3.5 hr. at room temperature and filtered, and the filtrate was washed with H_2O (1 × 25 ml.), 10% HCl (1 × 25 ml.), 0.7 *N* NaOH (1 × 25 ml.), and H_2O (2 × 25 ml.). The organic layer was dried (anhydrous Na_2SO_4), filtered, and evaporated under vacuum. The clear, yellow residue was distilled to give 12.6 g. (86%) of a colorless oil, b.p. 131–132°/0.09 mm.

Anal.—Calc. for $C_{12}H_{14}Cl_3NO$: C, 49.19; H, 4.91; N, 4.79. Found: C, 48.92; H, 4.79; N, 4.75.

[10] Applied Science Labs.

[11] Analyses were performed by Baron Consulting Co., Orange, CT 06477

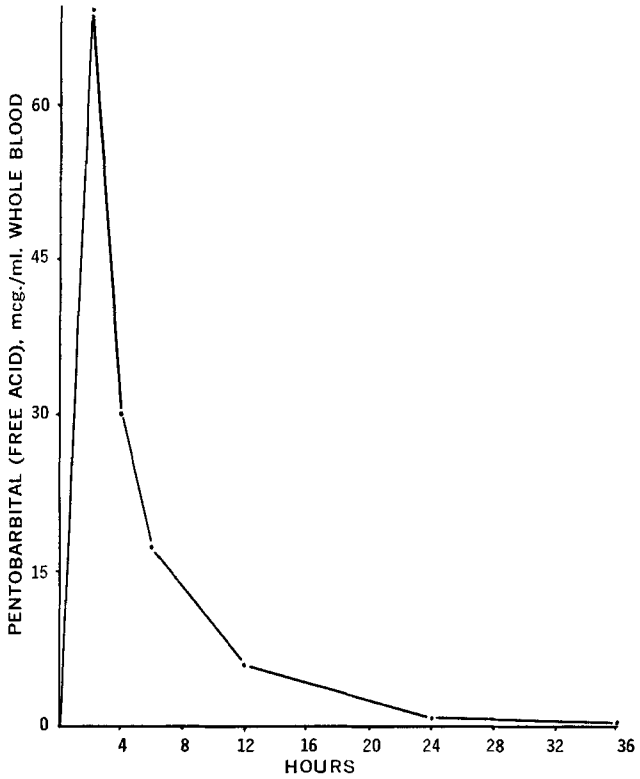

Figure 7—*Average blood level of 10 patients, each of whom received an oral liquid dose of 50 mg. pentobarbital (free acid).*

RESULTS AND DISCUSSION

Recoveries of methamphetamine from samples spiked with methamphetamine hydrochloride are given in Table I. The average percent recoveries for urine and blood, 18 trials each, were 94.0 ± 4.1% (range) and 68.4 ± 4.4%, respectively. Noonan *et al.* (24) reported approximately 50% recovery of amphetamine from both water and plasma based on the trichloroacetamide. Differences in recoveries between the two methods may be due in part to the use of a proton acceptor (pyridine). One might expect lower extraction recoveries from plasma (protein binding) than water. However, amines extracted from plasma could act as proton acceptors and facilitate higher reaction yields. The combination of both factors may explain the similar percent recoveries of amphetamine by Noonan *et al.* (24).

Electron-capture responses to the pure methamphetamine trichloroacetamide (Fig. 1) were linear over a 0–1-ng. range, as were responses from the derivatized urine and plasma extracts. Chromatograms obtained from different derivatives of methamphetamine (Fig. 2) illustrate the trichloroacetamide's greater sensitivity in relation to heptafluorobutyramide and pentafluorobenzamide, with 25 pg. of the trichloroacetamide easily discernible (Fig. 3). Efforts to reconcile these results, which deviate from some existing theories concerning the electron-capturing ability of various derivatives, will not be attempted in this paper. However, preliminary results indicate that electron-capture response is more difficult to predict than was previously thought.

Pentobarbital recoveries (Table II), 93.4 ± 4.8%, were in agreement with Ander's Method B (14), 92.0 ± 2.4%. Biological materials interfering with pentobarbital were not encountered. However, high boiling compounds were extracted, and temperature programming was necessary. Before the initiation of the procedure and after programming, it was essential to saturate the column by injecting relatively high concentrations of pentobarbital until constant peak heights were observed (usually, after programming, two or three injections of 0.5 mcg. were satisfactory).

Quantification of plasma levels for both methamphetamine and pentobarbital were based on peak height determinations. Blank blood samples were spiked with known amounts of each drug and carried through the entire procedure. Standard curves were run at the beginning and end of each day, with the mean values used for calculations. Chromatograms of methamphetamine from blood (Fig. 4) are practically free from extraneous peaks. Those of pentobarbital (Fig. 5) are of lesser quantity but quite acceptable and readily quantitated.

Ten female subjects were used to determine blood levels of both drugs. Each subject received a single oral liquid dose, containing 12.5 mg. methamphetamine hydrochloride and 50 mg. pentobarbital (free acid). Blood samples were taken at 1, 2, 4, 6, 12, and 24 hr. Average blood levels for methamphetamine and pentobarbital are given in Figs. 6 and 7, respectively. Methamphetamine levels were considerably lower than those reported by Bruce and Maynard (23) but in general agreement with Lebish's *et al.* (18) 1-and 2-hr. results[12].

The method described in the article was extremely sensitive for methamphetamine. Shortened work-up, usually accompanied by partial loss of chromatographic definition, can be accomplished by decreasing the number of washings after derivatization to a base and water wash. Failure to obtain consistently clean chromatograms was traceable to impure solvents, reagents, nitrogen (used for evaporating), and non-Teflon-lined caps. This method has been adapted for urinary methamphetamine metabolism and excretion studies and will be presented in a future publication.

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[12] One- and two-hour results were the only levels reported.

Dissolution of Slightly Soluble Powders under Sink Conditions III: Transport of Drug Solution across Screens and Membrane Barriers

ISMAT ULLAH* and DONALD E. CADWALLADER†

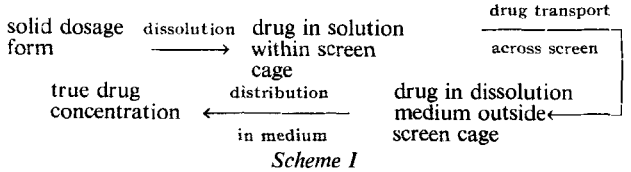
Abstract □ Transport studies were carried out with a three-compartment apparatus developed for dissolution studies of slightly soluble powders under sink conditions. Effects of different pore-size screens and membrane barriers, propeller placement, and rate of agitation were investigated. Experiments were also carried out to study the effect of temperature and the presence of polysorbate 80 in the dissolution medium. Screens and barriers proved to be potential hindrances to the transport of drug molecules in solution. Increases in the barrier pore size and rate of agitation decreased the time for development of equilibrium across the barriers. The presence of an additional impeller, above and close to the barrier, was found to be much more effective than rate of agitation alone. An increase in the temperature of the system also lowered the equilibrium time, while the presence of polysorbate 80 in the dissolution medium increased the time for equilibration.

Keyphrases □ Powders, slightly soluble—dissolution, sink conditions □ Dissolution, slightly soluble powders—sink conditions □ Drug transport, screens, membrane barriers—effect of impeller, agitation, temperature, polysorbate 80

The development of an apparatus to study the dissolution kinetics of slightly soluble powders under sink conditions was the subject of a prior report from this laboratory (1), and dissolution studies were carried out on salicylic acid and griseofulvin powders (1, 2). The dissolution apparatus was designed on the assumption that if a screen or membrane barrier was placed in the dissolution medium below the interface and the sample under study was introduced below this barrier, then, if flotation occurred, the sample would not come in contact with the organic phase. Assemblies recently were reported (3, 4) which utilize screens in their construction, and USP XVIII (5) and NF XIII (6) recommended one such assembly for dissolution testing.

In dissolution studies using such assemblies, the drug first dissolves from the dosage form into the surrounding dissolution medium which is caged by the screen. The drug molecules then pass from the caged area into the remaining bulk of dissolution medium. The dissolution of the dosage form is determined by testing the drug concentration in the medium outside the screen. The sequence of steps involved in such dissolution studies is shown in Scheme I.

To detect any dissolution from the dosage form, the drug molecules must cross the screen after dissolution takes place in the immediate surrounding medium. The screens could possibly create hindrances to the



Scheme I

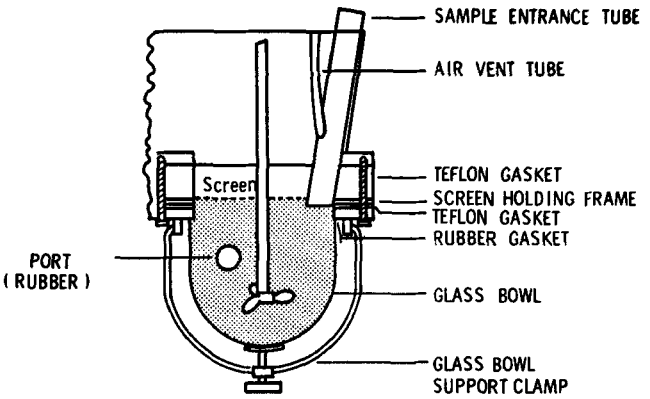


Figure 1—*Apparatus for transport studies (taken in part from Fig. 1,* Reference 1).

passage of drug molecules across them. The presence of such screens in dissolution assemblies could, therefore, be rate limiting if the drug transport problems are not solved properly. Preliminary studies using the earlier reported apparatus (1) showed that screens do, in fact, create a barrier to the transport of drug across them. Recently there was a report (7) about the reproducibility problem in using the USP apparatus (5). The problems were attributed to the slight bends in the shafts which result in additional vibrations. These vibrations could possibly affect the transport phase more than the dissolution phase in the dissolution scheme.

Any factors that would affect the transport of drug molecules through the screens could possibly affect the resultant dissolution rate. Factors such as screen mesh size, number and type of propellers, rate of agitation, temperature, and presence of surfactant in the dissolution medium could be quite critical for drug transport in the dissolution assemblies.

This report is concerned with the experiments conducted to investigate such factors in the three-compartment apparatus reported earlier (1, 2) and to demonstrate how the transport problem is circumvented by mechanical modifications of the dissolution apparatus which allow equilibrium to be established rapidly.

EXPERIMENTAL

Chemicals and Materials—The salicylic acid was USP grade[1]; polysorbate 80 also was used[2]. The other chemicals were reagent or certified ACS grade.

The various screens and filter membranes used in this study are listed in Table I.

Apparatus and Studies—The dissolution apparatus was described in detail in a previous paper (1). The transport study assembly con-

[1] Obtained from Fisher Scientific Co.
[2] Atlas Chemical Co.

Table I—List of Screens and Filter Membranes Used in Transport Studies

Mesh Size or Membrane Code	Pore Size, μ	Wire Diameter, μ	Material	Type
160[a]	96.50	63.50	Stainless steel	Plain
180[a]	83.80	58.42	Stainless steel	Plain
200[a]	68.60	58.42	Stainless steel	Twilled
Acropor AN-3000[b]	3.00	—	Acryl polyvinylchloride	Nylon cloth, reinforced
Acropor AN-800[b]	0.80	—	Acryl polyvinylchloride	Nylon cloth, reinforced
Acropor AN-450[b]	0.45	—	Acryl polyvinylchloride	Nylon cloth, reinforced

[a] Cambridge Wire Cloth Co. [b] Gelman Instrument Co.

sisted of the right-hand portion of the dissolution apparatus (Fig. 1). One or two propellers were attached to the stirrer shaft. A three-blade propeller, 3.8 cm. in diameter, was positioned 2.2 cm. from the bottom of the glass bowl in Compartment A. A three-blade turbine impeller, 6.0 cm. in diameter, was attached 0.2 cm. above the barrier.

The apparatus was immersed in a constant-temperature bath adjusted to 37.0°. A 455.0-ml. volume of 7.24×10^{-3} M (0.1%) salicylic acid solution in distilled water (previously equilibrated at 37.0°) was gently poured into Compartment A through the sample entrance tube with the help of a long-stem, 500-ml. separator[3]. This volume was just enough to fill the openings of the screen or filter. A 145.0-ml. volume of distilled water (previously equilibrated at 37.0°) was poured on the top of the screen or membrane using a liquid diffusion apparatus (Fig. 2).

The liquid diffusion apparatus consisted of a 1.2-cm. gas dispersion tube with fritted cylinder. The upper portion was formed into a funnel; the bottom, but not the sides, of the porous portion of the tube was flame sealed. The use of this apparatus prevented any appreciable disturbance of the solution beneath the barrier. The stirrer, previously set at the desired speed, was started, and 1.0-ml samples were removed from the solution above the screen at appropriate intervals. The samples were diluted with distilled water and assayed spectrophotometrically at 297 nm. using a Beckman DU-2 spectrophotometer. A volume of distilled water (37.0°) equal to the sample volume was added immediately after each sampling. From the concentration of salicylic acid in the samples, the percent of equilibrium concentration (5.49×10^{-3} M) was calculated as a function of time.

Using this procedure, the effects of propeller(s) and their placement, agitation, screen or membrane pore size, and a surfactant (polysorbate 80) were studied. Several experiments were also carried out at 25.0°.

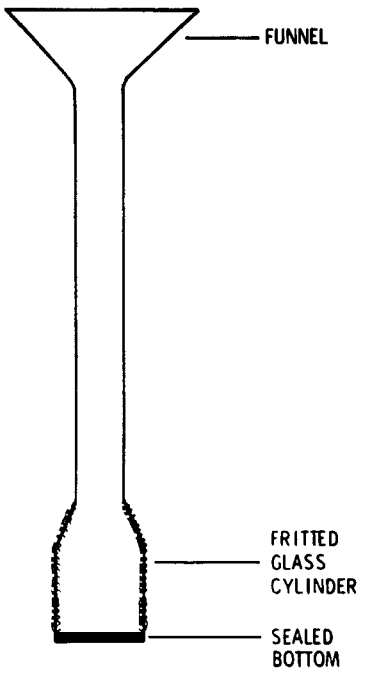


Figure 2—*Liquid diffusion apparatus.*

[3] The separator had a 20.0-cm. long stem which, when introduced through the sample entrance tube, would rest on the bottom of the glass bowl. There was no formation of foam below the screen when the dissolution medium was introduced in this manner.

RESULTS AND DISCUSSION

Effect of Barriers on Development of Equilibrium—Equilibrium studies with salicylic acid solutions (Fig. 3) showed that the screens and membranes, if placed in the dissolution medium, create a substantial barrier to the transport of drugs through the medium. The pore size of these barriers seems to have a direct relationship to the time needed for the required equilibrium to be established. Data in Fig. 4 show a log linear relationship between pore size and t_{30} (time for 30% equilibrium); however, there does not appear to be a

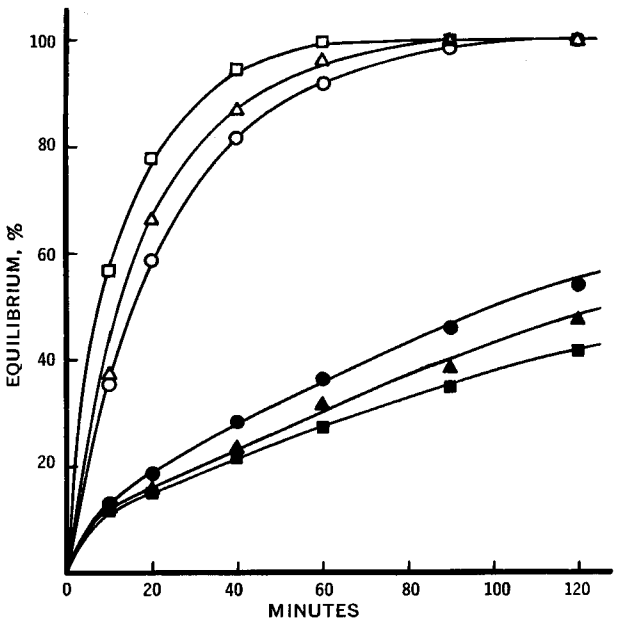


Figure 3—*Effect of screen mesh size or membrane pore diameter on the development of an equilibrium of salicylic acid solution at 55 r.p.m. and 37°. Key: □, 160 mesh; △, 180 mesh; ○, 200 mesh; ●, 3.0 μ; ▲, 0.8 μ; and ■, 0.45 μ.*

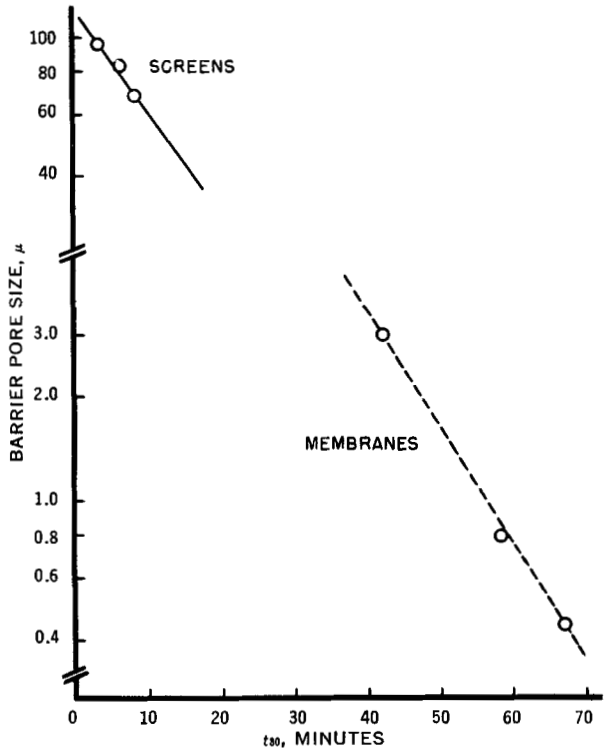


Figure 4—*Plots of logarithm of barrier pore size* versus *logarithm of* t_{30} *for the development of an equilibrium of salicylic acid solution at 55 r.p.m. and 37°. The* t_{30} *values were obtained from Fig. 3.*

direct linear relationship between the data obtained for the screens and the membrane barriers (*i.e.*, the lines when extrapolated do not meet). The slope for the screen curve was slightly less than the slope for the membrane barrier curve. These discrepancies could be due to relatively different amounts of open areas in screens and membrane barriers. The t_{30} values were used since the slow equilibrium times with the membrane barriers did not allow higher t values, common to both screens and membrane barriers, to be obtained.

Effect of Agitation on Development of Equilibrium—Agitation also seems to be a rate-limiting factor for the development of equilibrium. As expected, the equilibrium time for salicylic acid decreased with an increase in the rate of agitation (Fig. 5). This result was probably due to the increased circulation of the solution through the barrier pores under an increased rate of agitation. Figure 5 shows that it takes more than 1 hr. for equilibrium to be achieved across a 200-mesh screen at 25° even at 100 r.p.m. Under these conditions, it apparently would not be possible to develop a rapid equilibrium using high rates of agitation for these diffusion-controlled dissolution studies.

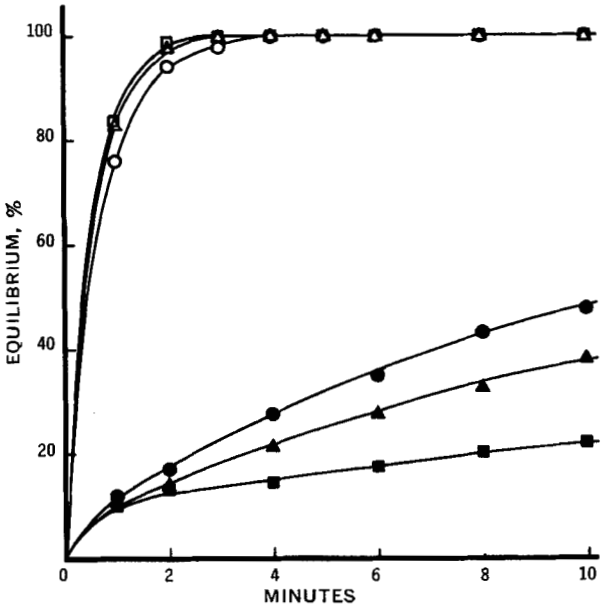


Figure 6—*Effect of an additional top impeller on the development of an equilibrium of salicylic acid solution across different barriers at 55 r.p.m. and 37°. Key: □, 160 mesh; △, 180 mesh; ○, 200 mesh; ●, 3.0 μ; ▲, 0.8 μ; and ■, 0.45 μ.*

Effect of Additional Top Impeller on Development of Equilibrium—The use of an additional impeller attached to the stirrer shaft above and close to the barriers caused equilibrium to be established rapidly (Fig. 6). With the screens used, which had relatively large pore sizes, it was possible to develop equilibrium in a reasonably short period (less than 4 min.). Although the development of equilibrium was

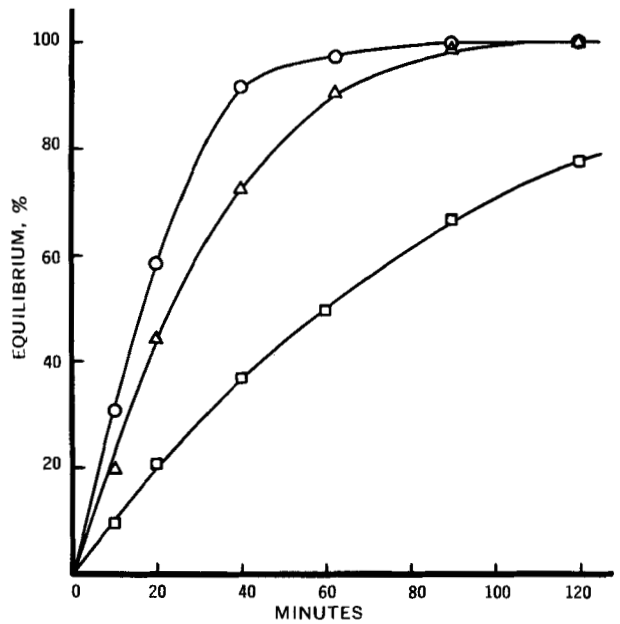


Figure 5—*Effect of agitation on the development of equilibrium of salicylic acid solution across a 200-mesh screen at 25°. Key: ○, 100 r.p.m.; △, 75 r.p.m.; and □, 55 r.p.m.*

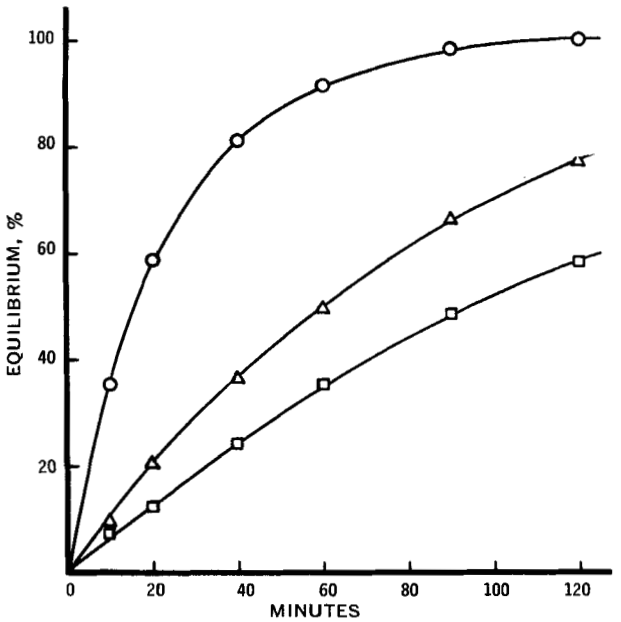


Figure 7—*Effect of temperature and polysorbate 80 on the development of an equilibrium of salicylic acid solution across a 200-mesh screen at 55 r.p.m. Key: ○, without polysorbate 80 at 37°; △, without polysorbate 80 at 25°; and □, with polysorbate 80 (0.05%) at 25°.*

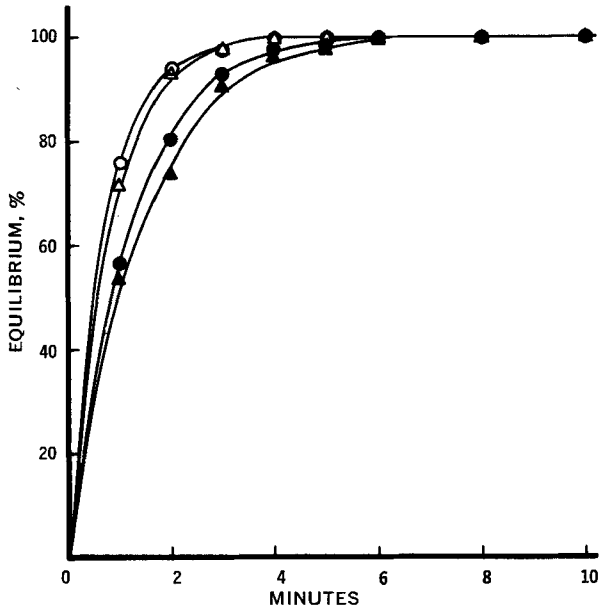


Figure 8—*Effect of temperature and polysorbate 80 (0.05%) on the development of an equilibrium of salicylic acid across a 200-mesh screen using an additional top impeller at 55 r.p.m. Key:* ○, *without polysorbate 80 at 37°;* △, *with polysorbate 80 at 37°;* ●, *without polysorbate 80 at 25°; and* ▲, *with polysorbate 80 at 25°.*

very rapid using screen barriers, the effect of barrier pore size was observed, with the 200-mesh screen effecting a slightly longer equilibrium time than the smaller mesh number screens. Although salicylic acid solution transport was also faster than it was without the additional impeller in the case of membrane barriers, equilibrium was not established rapidly; therefore, these membrane barriers would not be suitable for dissolution studies. Even a 3.0-μ pore-size membrane allowed only about 48% equilibrium in 10 min. and would be a rate-limiting factor in dissolution studies. It appears that a 200-mesh screen would be an appropriate barrier for further dissolution studies, using an additional top impeller.

The very rapid equilibrium in the presence of an additional top impeller must be due to the creation of an increased upward circulation of the solution, which overcomes hindrance created by the barrier to the movement of the drug solution across the screen openings.

Effect of Temperature and Polysorbate 80 on Development of Equilibrium—Temperature seems to have a substantial effect on the development of equilibrium (Fig. 7). Equilibrium time decreases with an increase in temperature. This result might be due to several factors. With an increase in temperature, there is increased molecular motion in the solution and greater probability for the drug solution to pass through the barrier pores and result in a faster development of equilibrium. As the temperature increases, the viscosity of the solution decreases, allowing easier circulation of the solution through the barrier pores. Since some heat of activation is also required to cross the barriers, an increase in temperature could facilitate the transport of the drug molecules across the barriers.

The presence of polysorbate 80 in the solution shows an adverse effect on the development of equilibrium (Fig. 7). This might be due to micelle formation of the surfactant. These micelles could create hindrance to the free molecular motion and result in an increased equilibrium time. Another factor could be due to the possible entrapment of the drug molecules in the micelles, which decreases the kinetic displacement of the molecules and, therefore, decreases the effective diffusion rate of these molecules.

The effect of temperature on the development of equilibrium can be seen even in the presence of the additional top impeller (Fig. 8). However, the transport rate of salicylic acid solution is not appreciably affected by the polysorbate 80 when the top impeller is used.

SUMMARY

1. The presence of barriers in the dissolution medium was found to be rate limiting in the establishment of a drug equilibrium between phases. The pore size of these barriers was established as the rate-limiting factor, and a linear relationship was shown between t_{30} for equilibrium and the barrier pore size.

2. An additional impeller was used above the barrier to develop rapid equilibrium. It was demonstrated that a 200-mesh screen is quite suitable as a barrier when this additional top impeller is used.

3. Agitation, temperature, and polysorbate 80 were found to be rate limiting for the development of an equilibrium. However, it was shown that in the presence of an additional top impeller, the presence of polysorbate 80 (0.05%) in the dissolution medium did not appreciably affect the equilibrium time.

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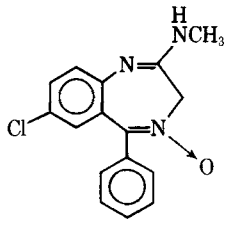
Biological Half-Life of Chlordiazepoxide and Its Metabolite, Demoxepam, in Man

MORTON A. SCHWARTZ, EDWARD POSTMA, and ZANE GAUT

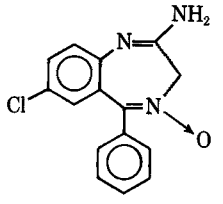
Abstract □ In a crossover study, single 20-mg. oral doses of chlordiazepoxide and one of its metabolites, demoxepam, were administered to six subjects. After chlordiazepoxide administration, the maximum plasma levels of intact drug were approximately 1 mcg./ml. and the half-life of plasma chlordiazepoxide ranged from 6.6 to 28 hr. In general, these half-lives were shorter and more variable than the range previously reported. In addition, a second metabolite, desmethylchlordiazepoxide, was found to reach maximum plasma levels of 0.14–0.46 mcg./ml. Demoxepam was not detected in the plasma of any subject after the single dose of chlordiazepoxide. The absorption of chlordiazepoxide was estimated to be 81% in the one subject given both an oral and an intravenous dose of the drug. After demoxepam administration, a redistribution of drug was apparent from the discontinuous fall-off curves. The eventual elimination of demoxepam from the plasma was relatively slow, with a range in half-life of 14–95 hr. In each subject, the half-life of demoxepam was markedly longer than that of chlordiazepoxide.

Keyphrases □ Chlordiazepoxide, metabolites—plasma levels, half-life, man □ Demoxepam—chlordiazepoxide metabolite, half-life, man □ Desmethylchlordiazepoxide—chlordiazepoxide metabolite, half-life, man □ Plasma levels, half-life—chlordiazepoxide, metabolites, man

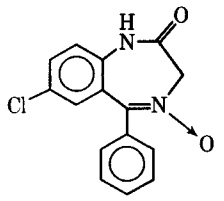
In 1963, Koechlin and D'Arconte (1) published a fluorometric assay for plasma chlordiazepoxide[1] and demoxepam[2]. They reported that the half-life of chlordiazepoxide in three human subjects ranged from 20 to 24 hr. and that demoxepam was a plasma metabolite of chlordiazepoxide in both man and dog. This biotransformation of chlordiazepoxide was confirmed in subsequent studies in which labeled demoxepam was shown to be a urinary metabolite of ^{14}C-chlordiazepoxide in both species (2). A subsequent investigation (3) of the *in vitro* metabolism of chlordiazepoxide resulted in the identification of another metabolite, the *N*-desmethyl derivative of chlordiazepoxide (desmethylchlordiazepoxide)[3], which could not be distinguished from chlordiazepoxide by the fluorometric assay. A separate specific fluorometric assay for desmethylchlordiazepoxide was incorporated into the Koechlin and D'Arconte assay to yield a method that provided for the direct measurement of plasma demoxepam and desmethylchlordiazepoxide and the differential assay of plasma chlordiazepoxide. Using this procedure, it was found (3) that desmethylchlordiazepoxide, in addition to demoxepam, was present in plasma following the intravenous administration of chlordiazepoxide hydrochloride to man.



chlordiazepoxide



desmethylchlordiazepoxide



demoxepam

One purpose of the present study was to redetermine the rate of chlordiazepoxide elimination in man, using the method that differentiates between chlordiazepoxide and desmethylchlordiazepoxide. In addition, since demoxepam is excreted in the urine in appreciable amounts after chlordiazepoxide administration (2), it was of interest to compare the rates of elimination in man of orally administered chlordiazepoxide and demoxepam. A crossover design was used to obtain a valid comparison of both rates.

EXPERIMENTAL

Design—Six healthy volunteers, normal with respect to hemoglobin, hematocrit, red blood cell count, white blood cell count, differential, serum glutamic-pyruvic transaminase, alkaline phosphatase, and urinalysis, participated in this study. They reported that they had taken no medication at all for 2 weeks prior to the study and that no chronic drug administration had occurred for the preceding 2 months. Each subject was given a 20-mg. oral dose of chlordiazepoxide and demoxepam, with a 1-week interval between doses. Information pertinent to the subjects, drugs, and the crossover design of treatment is presented in Table I.

Ten milliliters of oxalated blood was drawn just prior to the administration of each dose and 1, 2, 4, 6, 8, 12, 24, 48, and 72 hr. later. Plasma obtained on centrifugation was immediately frozen and stored at −7° until analyzed.

One additional experiment was performed approximately 6 months after the completion of the crossover study. Subject 6 (whose weight was unchanged from that shown in Table I) was given 20 mg. of chlordiazepoxide intravenously; 22.4 mg. of chlordiazepoxide hydrochloride sterile powder, Lot No. 206-11228, dissolved in 2.2 ml. of physiological saline was injected. Heparinized blood (10 ml.) was drawn at 0, 3, 6, 10, 20, and 30 min. and at 1, 2, 3, 5, 8, 24, 48, and 72 hr. Plasma was separated, frozen, and stored prior to analysis.

Plasma Assays—Duplicate 2-ml. aliquots of each plasma obtained after demoxepam administration were analyzed for dem-

[1] Chlordiazepoxide, 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide, is the active ingredient in the trademarked product Librium of Hoffmann–La Roche Inc., Nutley, N. J.

[2] Demoxepam, which was previously designated Ro 5-2092 or "lactam," is 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide.

[3] Desmethylchlordiazepoxide, which was previously designated Ro 5-0883/1 or metabolite D-M, is 2-amino-7-chloro-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide.

Table I—Data Concerning the Subjects and Administration of Chlordiazepoxide and Demoxepam

Subject[a]			Administration of Drugs[b]	
Number	Sex	Weight, kg.	First Drug	Second Drug
1	M	85.5	Chlordiazepoxide	Demoxepam
2	F	67.7	Demoxepam	Chlordiazepoxide
3	F	59.1	Demoxepam	Chlordiazepoxide
4	F	61.8	Chlordiazepoxide	Demoxepam
5	F	61.8	Chlordiazepoxide	Demoxepam
6	M	68.6	Demoxepam	Chlordiazepoxide

[a] The subjects ranged in age from 25 to 43 years. Subjects 1–5 were Caucasians; Subject 6 was a Negro. [b] Chlordiazepoxide was given as two Libritabs (Lot Item 7603), each of which contained 10 mg. of drug (as the free base). Demoxepam was administered in two tablets, Lot No. C-7187 (C-7189)-01, each of which contained 10 mg. of the compound.

oxepam by the fluorometric assay of Koechlin and D'Arconte (1). The specificity of this method was tested by determining the fluorescence produced by two phenolic metabolites of demoxepam recently found in the dog (4). Neither of these metabolites, the 9-hydroxy derivative of demoxepam nor the 5-(4-hydroxyphenyl) derivative, produced any fluorescence. Since these two close analogs did not interfere, the assay was considered specific for demoxepam.

Following chlordiazepoxide administration, duplicate 2-ml. plasma samples were analyzed for chlordiazepoxide, desmethylchlordiazepoxide, and demoxepam by the combined fluorometric procedures already described (3). The fluorescence was measured in a spectrofluorometer (Farrand) containing a new lamp (Xenon) which was over twice as sensitive as the lamp previously used. This resulted in a two- to threefold increase in sensitivity and a new sensitivity limit of approximately 0.1 mcg./ml. of plasma for each of the three benzodiazepines. The difficulties formerly encountered (3) in measuring low levels of these compounds were thereby considerably reduced.

RESULTS AND DISCUSSION

The plasma level data obtained after the oral administration of chlordiazepoxide and demoxepam to the six subjects are shown in Fig. 1 as a semilog plot of plasma levels *versus* time. The administered chlordiazepoxide appeared rapidly in the plasma of each subject and rose quickly to peak levels which then declined at an apparent first-order rate. The chlordiazepoxide metabolite, desmethylchlordiazepoxide, was readily determined in each subject's plasma and reached maximum levels between 8 and 24 hr. The other metabolite, demoxepam, did not reach measurable levels in any chlordiazepoxide-treated subject.

Following administration of demoxepam, the plasma levels of intact drug did not follow the pattern of an uninterrupted rise and decline as was observed with chlordiazepoxide. In every subject except Subject 3, an obvious plateau or trough in the fall-off curve was evident near the time at which the maximum plasma level of demoxepam was seen. This finding suggested that, in addition to the standard processes of absorption, distribution, and elimination, a redistribution of drug occurred. Apparently, the initial relatively rapid disappearance of plasma demoxepam was stopped or reversed by the reentrance of demoxepam into the plasma from some endogenous depot (such as adipose tissue) or from the GI tract (enterohepatic circulation). In Subject 3, the plateau in plasma levels of drug was not obvious because it was masked by the relatively slow disappearance of demoxepam from the plasma.

The curves of Fig. 1 also allow for a comparison of the elimination of the administered chlordiazepoxide and demoxepam. It is evident that in each subject, regardless of the order in which the drugs were given, chlordiazepoxide was eliminated much faster than was demoxepam.

In Table II, the elimination rates, determined by the method of least squares, are expressed in terms of both the rate constant (β) and the half-life. The peak plasma levels of approximately 1 mcg./ml. of chlordiazepoxide declined with rate constants which ranged from 0.025 to 0.105 $hr.^{-1}$; *i.e.*, the half-lives of plasma chlordiazepoxide ranged from a high value of 28 hr. to a low of 6.6 hr. If Subject 3 is excluded, this range is considerably shortened, to half-lives of 6.6–15 hr. The levels of the metabolite, desmethylchlordiazepoxide, reached 0.3–0.5 mcg./ml. in all subjects except Subject 3. Both the slower elimination of plasma chlordiazepoxide (half-life of 28 hr.) and the lower levels of desmethylchlordiazepoxide (0.14 mcg./ml.) in Subject 3 are indicative of an appreciably slower rate of chlordiazepoxide metabolism than that shown by the other five subjects.

It is evident that the previously reported (1) half-life of 20–24 hr. for chlordiazepoxide in man was an overestimation, resulting from the combination of two factors: (*a*) the assay used was not specific

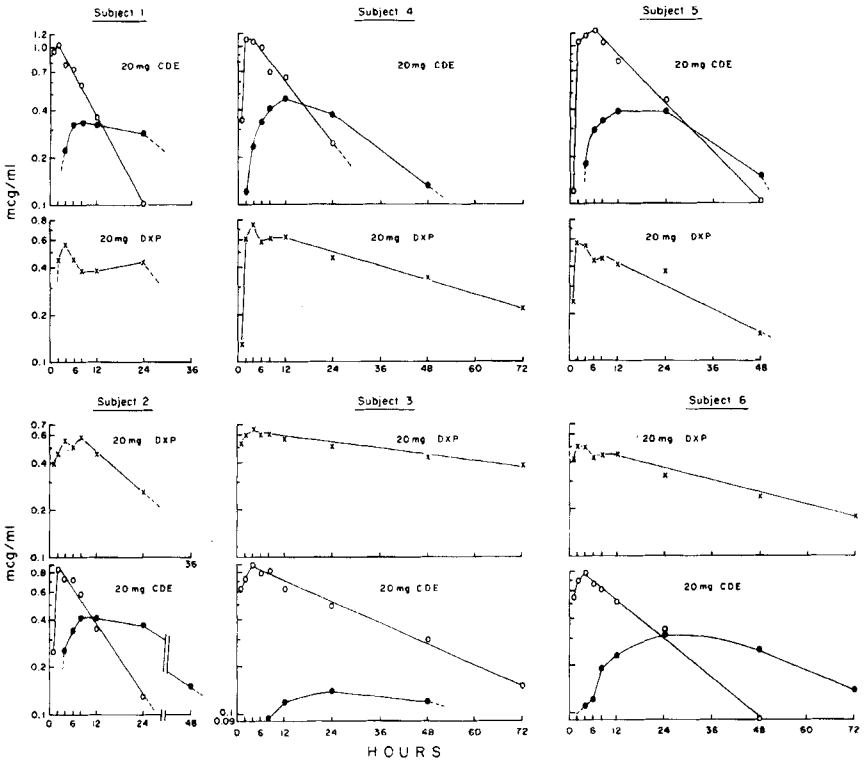


Figure 1—*Plasma levels of chlordiazepoxide and its metabolite, desmethylchlordiazepoxide, after a 20-mg. oral dose of chlordiazepoxide, and plasma levels of demoxepam after a 20-mg. oral dose of demoxepam. Subjects 1, 4, and 5 (top row) received chlordiazepoxide (designated CDE) first; Subjects 2, 3, and 6 (bottom row) received demoxepam (designated DXP) first. Key:* ○, *chlordiazepoxide level;* ●, *desmethylchlordiazepoxide level; and* ×, *demoxepam level. Broken line indicates that plasma concentrations were rising from or dropping to nonmeasurable levels.*

Table II—Plasma Concentrations of Drug (and Metabolite) following Oral Administration of Chlordiazepoxide and Demoxepam to Each of Six Subjects

Subject Number	Dose: 20 mg. p.o. of Chlordiazepoxide[a] — Plasma Chlordiazepoxide — Peak Level, mcg./ml. at hr.[b]	Elimination Rate[c] — β, hr.$^{-1}$	Half-Life, hr.	Plasma Desmethylchlordiazepoxide Peak Level, mcg./ml. at hr.[b]	Dose: 20 mg. p.o. of Demoxepam — Plasma Demoxepam — Peak Level, mcg./ml. at hr.[b]	Elimination Rate — β, hr.$^{-1}$	Half-Life, hr.	Ratio of the Rates of Elimination (Chlordiazepoxide β)/(Demoxepam β)
1	1.02 at 2	0.105	6.6	0.33 at 8	0.56 at 8	n.m.[d]	n.m.	n.m.
2	0.84 at 2	0.088	7.9	0.41 at 8–12	0.58 at 8	0.050	14	1.8
3	0.89 at 4	0.025	28	0.14 at 24	0.64 at 4	0.0073	95	3.4
4	1.10 at 2	0.073	9.5	0.46 at 12	0.74 at 4	0.017	42	4.3
5	1.24 at 6	0.059	12	0.38 at 12–24	0.56 at 2	0.026	27	2.3
6	0.78 at 4	0.047	15	0.31 at 24	0.50 at 2	0.015	46	3.1

[a] Following the chlordiazepoxide dose, plasma was analyzed for intact drug and two metabolites, desmethylchlordiazepoxide and demoxepam. Measurable levels of desmethylchlordiazepoxide, but not of demoxepam, were found in each subject. [b] The hour shown is the time at which the peak level was observed. [c] The elimination rate constant, β, and half-life are related: $\beta = 0.693/\text{half-life}$. [d] n.m. = not measurable.

but measured the combined plasma levels of chlordiazepoxide and desmethylchlordiazepoxide, and (*b*) plasma desmethylchlordiazepoxide rose to appreciable levels with time. The present study demonstrates that the half-life of chlordiazepoxide is not only shorter than previously reported but is also more variable. This variability may be genetically controlled, as was reported for antipyrine (5) and phenylbutazone (6).

The peak plasma levels of orally administered demoxepam, seen after 2–8 hr., varied within a relatively narrow range of 0.50–0.72 mcg./ml. (Table II). The half-life, however, was much more variable and ranged from 14 to 95 hr. Subject 3 was consistent in exhibiting the slowest elimination of both chlordiazepoxide and demoxepam. From the rates of elimination of chlordiazepoxide and demoxepam in the same subject (last column of Table II), it is clear that the metabolism of demoxepam was 2–4 times slower than that of chlordiazepoxide.

The intravenous administration of chlordiazepoxide to one subject provided data that allowed for a detailed pharmacokinetic analysis of its disposition. The plasma levels of chlordiazepoxide and desmethylchlordiazepoxide obtained after both routes of chlordiazepoxide administration to Subject 6 are compared in Fig. 2. The plasma levels of chlordiazepoxide found after intravenous administration apparently declined biexponentially and were fitted by means of an "N-LIN" computer program (7) to the biexponential equation:

$$C_t = Ae^{-\alpha t} + Be^{\beta - t} \qquad \text{(Eq. 1)}$$

in which C_t is the plasma level at time t, A is the zero-time intercept and α the rate constant of the fast disposition rate, and B and β are the corresponding parameters of the slow disposition rate.

The fast disposition rate constant, α, was found to be 11.5 hr.$^{-1}$, which corresponds to a half-life of only 3.6 min. The slow disposition rate constant, β, of 0.0488 hr.$^{-1}$ corresponds to a half-life of 14 hr., which agrees with the half-life of 15 hr. obtained when chlordiazepoxide was given orally to this subject (Table II). The volume of distribution of the central compartment (V_p) of a two-compartment open-system model (8) was calculated from

$$V_p = \text{dose}/(A + B) \qquad \text{(Eq. 2)}$$

to be 3.3 l. (dose = 20 mg. chlordiazepoxide, A = 5.0 mg./l., and B = 1.1 mg./l.). This volume, which is equivalent to only 4.8% of the body weight of Subject 6, approximates the plasma volume. The total volume of distribution was 25% of the body weight, regardless of whether calculated as described by Riegelman *et al.* (8) or Gibaldi *et al.* (9).

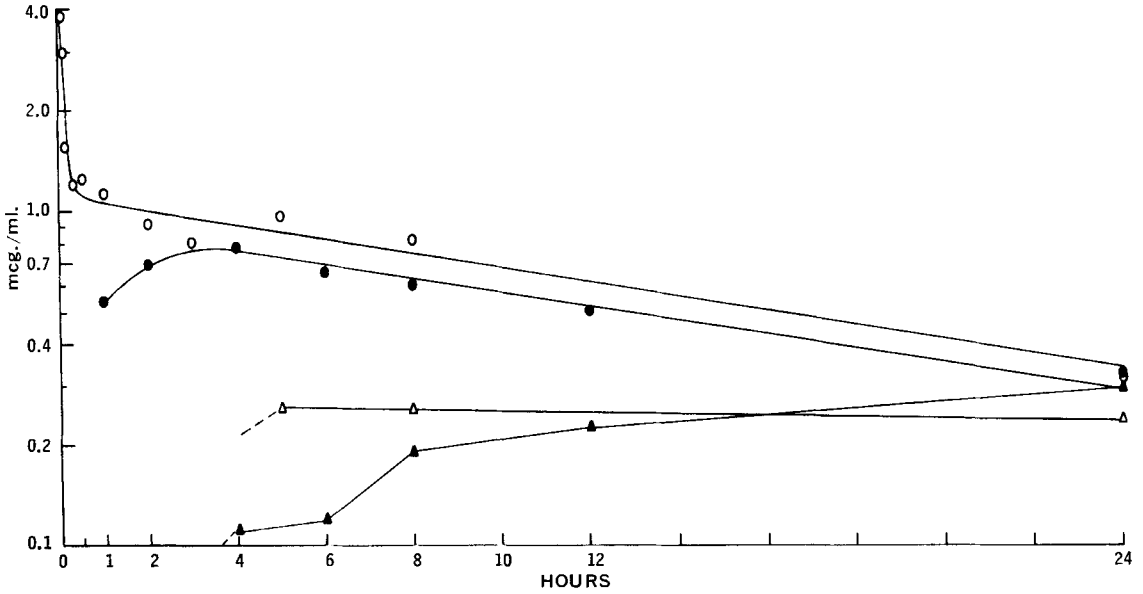


Figure 2—*Plasma levels of chlordiazepoxide and desmethylchlordiazepoxide after intravenous and oral administration of chlordiazepoxide to Subject 6. Key: intravenous chlordiazepoxide: plasma chlordiazepoxide* (O) *and desmethylchlordiazepoxide* (△). *Both chlordiazepoxide and desmethylchlordiazepoxide were not present in measurable amounts after 24 hr. Oral chlordiazepoxide: plasma chlordiazepoxide* (●) *and desmethylchlordiazepoxide* (▲); *these are the same curves shown in Fig. 1.*

The extent of chlordiazepoxide absorption was estimated by comparing the area subtended by the chlordiazepoxide plasma levels found after oral administration to the corresponding area seen after intravenous drug administration. This estimate indicated that 81% of the orally administered chlordiazepoxide was absorbed by Subject 6. In addition, it suggests that therapeutic doses of chlordiazepoxide are well absorbed in man.

Plasma levels of desmethylchlordiazepoxide were first measurable 5 hr. after intravenous chlordiazepoxide was given (Fig. 2). At this time, 0.26 mcg./ml. was seen; little decline was evident for the next 19 hr. Measurable levels of demoxepam were found only after 48 and 72 hr. and were close to the sensitivity limit of 0.14 mcg./ml.

Since the oral administration of ^{14}C-chlordiazepoxide to two human subjects resulted in the urinary excretion of only negligible amounts of intact drug (2), it is evident that chlordiazepoxide in man is eliminated almost entirely by biotransformation. The appearance of plasma desmethylchlordiazepoxide and the absence of plasma demoxepam in the present study support the contention (3) that desmethylchlordiazepoxide is an intermediate in the biotransformation of chlordiazepoxide to demoxepam. This pathway was recently demonstrated in the dog by Kaplan *et al.* (7). Their pharmacokinetic evaluation of chlordiazepoxide disposition revealed that the drug was eliminated by essentially quantitative biotransformation to desmethylchlordiazepoxide, which was then itself extensively biotransformed with approximately one-half going to demoxepam.

The accumulation of plasma levels of desmethylchlordiazepoxide and demoxepam in a human subject administered chlordiazepoxide chronically was previously reported (3). This finding is also supported by those presented here which showed that chlordiazepoxide administration led to persistent plasma levels of desmethylchlordiazepoxide and that demoxepam was eliminated at a considerably slower rate than was chlordiazepoxide. Since demoxepam was reported (10) to have antianxiety activity in man, this metabolite may play a significant role in contributing to the activity of chlordiazepoxide.

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Keyphrases □ *Paxillus atrotomentosus*, *P. panuoides*—pigment comparison □ Atromentin, *Paxillus* carpophores—isolation, identification □ Atromentic acid, *Paxillus* cultures—isolation, identification □ Xerocomic acid, *Paxillus* cultures—isolation, identification □ Tetronic acids—*P. atrotomentosus*, *P. panuoides* □ TLC—isolation, tetronic acids

Paxillus panuoides (Fr.) Fr. and *P. atrotomentosus* (Batsch) Fr. are lignicolous mushrooms, frequently found around old conifer stumps (1). In addition, the smaller, sessile *P. panuoides* may be found on sawdust piles or on timbers of mines, cellars, and similar places where it is a active wood destroyer. In the latter areas, carpophore formation can occur in the semidarkness after the soft yellow mycelium causes extensive decay and a vivid yellow discoloration of the wood (2). Similarly diffusible yellow pigments, which accumulate in surface cultures of *P. atrotomentosus*, were shown to include atromentic and xerocomic acids (3), diphenyl-substituted tetronic acid derivatives. Knowledge of pigments in carpophores of *P. atrotomentosus* and *P. panuoides* is restricted to the isolation of atromentin from the former species (4).

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The desirability of comparative studies of *P. atrotomentosus* and *P. panuoides* was suggested by the distinctive metabolic capabilities noted previously for carpophores and vegetative mycelium of *P. atrotomen-*

Table I—Pigments Detected by TLC Examination of Extracts of Carpophores and Culture Mixtures of *P. atrotomentosus* and *P. panuoides*

Pigment	*P. atrotomentosus*				*P. panuoides*			
	Carpophore		Culture		Carpophore		Culture	
	A[a]	B	A	C	A	B	A	C
Atromentin	+	+	−	−	+	+	−	−
Atromentic acid	−	−	+	+	−	−	+	+
Thelephoric acid	+	+	−	−	−	−	−	−
Xerocomic acid	−	−	+	+	−	−	+	+
Unidentified purple pigment	R_f 0.10[b]	R_f 0.71	−	−	R_f 0.10[b]	R_f 0.71	−	−
Unidentified red pigment	−	−	R_f 0.25	R_f 0.37	−	−	R_f 0.25	R_f 0.37

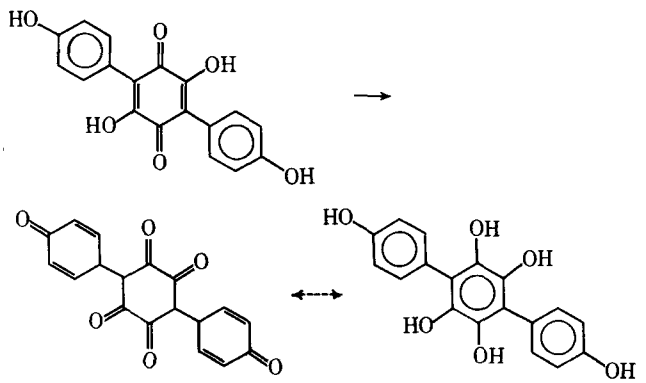
[a] System A: silica gel G thin layer, ether saturated with concentrated HCl; System B: polyamide thin layer, methanol–chloroform–H_2O–glacial acetic acid (54:36:6:1); System C: silica gel G thin layer, methyl ethyl ketone–H_2O–formic acid (250:25:1). [b] Evanescent.

tosus and the common lignicolous habitat and other gross properties shared by the two species. Terphenylquinone and tetronic acid derivatives, presumably metabolically related fungal constituents, were selected for the initial comparative studies to make the best utilization of existing knowledge. Studies were also undertaken to determine various properties of selected tetronic acids and to develop methods for their manipulation. The latter studies focused on the properties and manipulations that could have application in subsequent biologic studies and included a quantitative spectrophotometric procedure, pKa values, and UV and fluorescent spectra of the compounds at different stages of ionic dissociation.

EXPERIMENTAL

Source of Carpophores and Cultures—The carpophores of *P. panuoides* used were collected near McCleary, Washington; the fruiting bodies of *P. atrotomentosus* were obtained from Bainbridge Island, Washington. Both collections[1] were made in the autumn of 1968. The material was dried at 48° in a forced-air oven for 72 hr. The cultures[2] of *P. panuoides* and *P. atrotomentosus* were maintained routinely on cherry and 2% malt extract agar media.

Media and Cultural Conditions—Two media were used for surface cultivation of the *Paxillus* species. The cherry medium was prepared as previously described (3), and the 2% malt extract was acidified with HCl to pH 3.2 (2). Roux bottles (500 ml.), containing 75 ml. of the appropriate nutrient medium, were inoculated from a homogenate prepared from 14-day-old agar slant cultures of either *P. atrotomentosus* or *P. panuoides*. Cherry medium was inoculated with mycelium grown on cherry agar slants; growth from malt agar slants was used to inoculate the acidified malt extract medium.



Scheme I—Presumed heat and/or electron-bombardment-induced oxidation–reductions in the inlet system

The cultures were incubated at 20° for 50–60 days. Since deterioration of cultures was recognized as a potential problem in cultures grown in continuous or intense light, the cultures were incubated routinely in the dark. The aerial hyphae of *P. panuoides*, which had developed on both nutrient media at the time of harvest, were much flatter and thicker than the fluffy, droplet-covered aerial hyphae noted in the cultures of *P. atrotomentosus*. In all cases, the nutrient broth was yellow at this stage of development.

Chromatographic Studies—Several TLC procedures, previously used for separation and chromatographic monitoring of fungal terphenylquinones and pulvinic acid derivatives (3, 5), were selected for the initial evaluation of the extracts. Diethyl ether was used to extract the pigments from defatted carpophores, absolute methanol was employed with the freeze-dried culture mixtures, and authentic reference materials were used for chromatographic comparison. Table I summarizes the significant observations revealed by chromatographic examination of extracts of the carpophores and cultures of the two *Paxillus* species.

No chromatographic indication was observed for the presence of atromentin in extracts of the culture mixtures or for the occurrence of tetronic acids in the carpophores. The TLC system, utilizing silica gel G and HCl-saturated ether, was the best all-purpose system for the pigments in the extracts; it was used (preparative adsorbent layer) for separation of atromentic and xerocomic acids preparatory to quantitative determinations.

Extraction and Isolation of Atromentin—Samples (100 g.) of the dried mushrooms were reduced to a 40-mesh powder in a mill (Wiley). The powders were defatted with petroleum ether using a soxhlet apparatus. This procedure was followed by exhaustive extraction with diethyl ether, and these extracts were concentrated to dryness under reduced pressure at 35°. The samples of *P. atrotomentosus* and *P. panuoides* yielded 5.1 and 4.9 g., respectively, of diethyl ether-soluble extractives. A pigment with chromatographic properties corresponding to those of atromentin was obtained from the extracts by fractional crystallization using methanol and dioxane. The material was recrystallized three times from methanol–dioxane, and the respective yields of chromatographically pure pigment from the two species were 2.0 and 1.2 g.

Identification of Atromentin—The IR, UV, and mass spectra[3] of the pigment samples from both species were identical. The UV spectra ($\lambda_{max.}^{ethanol}$ 360 and 278 nm., $\lambda_{max.}^{dioxane}$ 385 and 268 nm., and $\lambda_{max.}^{pyridine}$ 550 and 360 nm.) agreed with those of authentic atromentin. IR spectra (KBr pellets) of the isolated samples and reference atromentin were indistinguishable, with absorption peaks at 3340, 2870, 1650, 1600, 1525, 1435, 1310, 1235, 1180, 1000, and 835 $cm.^{-1}$. Melting points of all atromentin samples were >300°.

High-resolution mass spectra of known atromentin and the samples isolated from the two *Paxillus* species exhibited base and parent ion peaks at *m/e* 324.0630 (calc. $C_{18}H_{12}O_6$, 324.0633). The mass spectrum of atromentin was observed to be complex; the fragments

[1] Identification was provided by Dr. D. E. Stuntz, Department of Botany, University of Washington, Seattle, Wash.

[2] Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

[3] IR and UV spectra were obtained with a Beckman IR spectrophotometer, model IR5A, and a Beckman UV spectrophotometer, model DB, Beckman Instruments, Inc., Fullerton, Calif. Mass spectra were determined with a Picker-AEI MS9 mass spectrometer, Picker Nuclear Division, White Plains, N. Y.; the mass spectrometer was used in conjunction with a Programmed Data Processor, model DPD-12, Digital Equipment Corp., Maynard, Mass., for studies of the fragmentation of atromentin.

Table II—Major Peaks in the High-Resolution Spectrum of Atromentin

Observed *m/e*	Calculated *m/e*	Formula	Relative Intensity, %
326.0790	326.0789	$C_{18}H_{14}O_6$	27
325.0710	325.0711	$C_{18}H_{13}O_6$	76
324.0630	324.0633	$C_{18}H_{12}O_6$	100
323.0470	323.0545	$C_{18}H_{11}O_6$	19
322.0430	322.0477	$C_{18}H_{10}O_6$	10
307.0590	307.0606	$C_{18}H_{11}O_5$	15
306.0540	306.0528	$C_{18}H_{10}O_5$	14
297.0730	297.0762	$C_{17}H_{13}O_5$	27
296.0700	296.0684	$C_{17}H_{12}O_5$	43
295.0610	295.0606	$C_{17}H_{11}O_5$	21
279.0630	279.0657	$C_{17}H_{11}O_4$	17
278.0540	278.0578	$C_{17}H_{10}O_4$	15
268.0710	268.0735	$C_{16}H_{12}O_4$	12
267.0640	267.0657	$C_{16}H_{11}O_4$	13
251.0660	251.0708	$C_{16}H_{11}O_3$	13
250.0590	250.0630	$C_{16}H_{10}O_3$	16
240.0730	240.0786	$C_{15}H_{12}O_3$	14
239.0690	239.0708	$C_{15}H_{11}O_3$	27
230.0190	230.0214	$C_{12}H_6O_5$	13
224.0800	224.0837	$C_{15}H_{12}O_3$	10
223.0770	223.0758	$C_{15}H_{11}O_2$	34
222.0680	222.0680	$C_{15}H_{10}O_2$	17
221.0620	221.0602	$C_{15}H_9O_2$	18
211.0760	211.0758	$C_{14}H_{11}O_2$	12
210.0670	210.0682	$C_{14}H_{10}O_2$	11
202.0260	202.0054	$C_{11}H_6O_4$	13
194.0720	194.0731	$C_{14}H_{10}O$	11
190.0210	190.0418	$C_{10}H_6O_4$	16
189.0180	189.0340	$C_{10}H_5O_4$	41
174.0310	174.0315	$C_{10}H_6O_3$	15
163.0380	163.0394	$C_9H_7O_3$	21
162.0290	162.0315	$C_9H_6O_3$	25
161.0330	161.0338	$C_9H_5O_3$	27
147.0080	147.0151	$C_8H_3O_3$	17
146.0090	146.0073	$C_8H_2O_3$	21
145.0000	144.9995	C_8HO_3	38
139.0330	139.0394	$C_7H_7O_3$	11
135.0450	135.0444	$C_8H_7O_2$	29
134.0380	134.0366	$C_8H_6O_2$	43
133.0290	133.0288	$C_8H_5O_2$	28
125.0320	125.0237	$C_6H_5O_3$	14
121.0280	121.0288	$C_7H_5O_2$	27
118.0400	118.0417	C_8H_6O	21
117.0320	117.0439	C_8H_5O	10
107.0480	107.0495	C_7H_7O	31
106.0410	106.0417	C_7H_6O	26
105.0330	105.0339	C_7H_5O	36
97.0361	97.0388	$C_5H_5O_2$	13
94.0408	94.0417	C_6H_6O	18
89.0424	89.0390	C_7H_5	15
82.0360	82.0317	C_5H_6O	10
78.0510	78.0468	C_6H_6	17
77.0432	77.0390	C_6H_5	24
76.0337	76.0372	C_6H_4	12

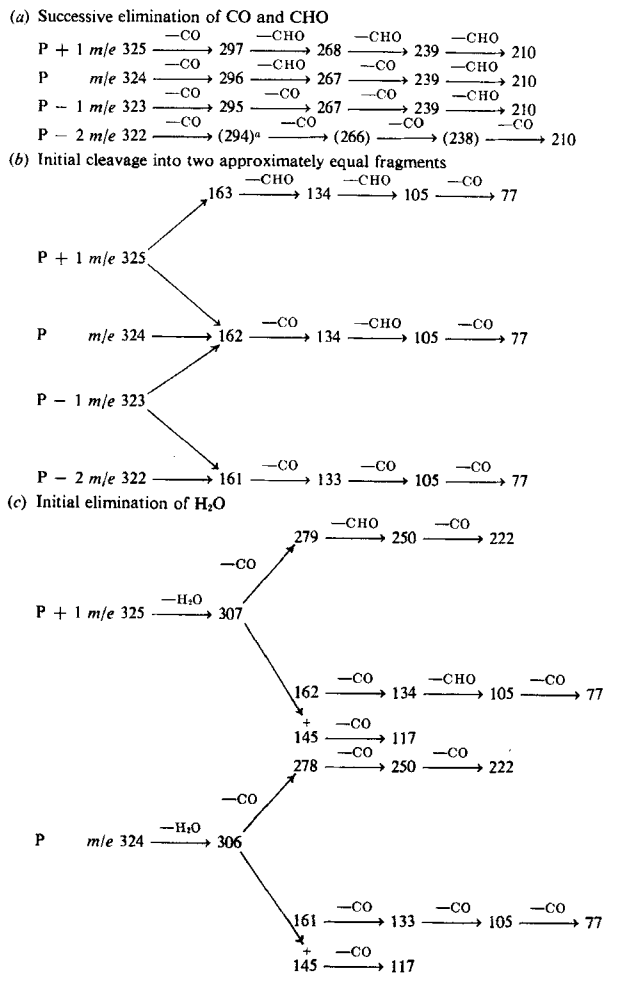
with a relative intensity >10% of base are listed in Table II. Possible fragmentation patterns are best visualized by assuming the initial reactions, when the sample is placed in the source at the high temperature (240°) required to obtain an adequate vapor pressure, involve heat and/or electron-bombardment-induced oxidation–reductions to give a series of compounds (Scheme I). Comparatively strong P + 1 and P + 2 ion peaks were observed, a phenomenon typical of *p*-benzoquinones (6). Correlation of the probable initial molecular forms and the observed major fragmentation peaks (Table II) suggests the involvement of at least three basic fragmentation pathways: (*a*) successive elimination of CO and CHO fragments, (*b*) cleavage into two equal or nearly equal fragments and then successive elimination of CO and CHO fragments, and (*c*) elimination of water and then successive elimination of CO and CHO fragments or cleavage into two large fragments prior to elimination of CO and CHO fragments. Table III summarizes some suggested major fragmentation pathways. It may be particularly significant that the pathways originating with the P − 2 ion peak parallel those reported for atromentic acid lactone (7).

Isolation and Identification of Thelephoric Acid—A small quantity of a black pigment was observed on the walls of the flask used for the diethyl ether extraction of carpophores of *P. atrotomentosus*, and 7 mg. of black crystalline material was obtained. The melting point was >300°, and the chromatographic properties of the pigment corresponded to those of thelephoric acid. The UV spectra ($\lambda_{max.}^{pyridine}$ 311 and 495 nm. and $\lambda_{max.}^{pyridine\text{-}H_2O}$ 338 and 540 nm.) agreed with those previously reported for thelephoric acid (5). The IR spectra of the black pigment and authentic thelephoric acid were identical, with absorption peaks at 3378, 2941, 1634, 1520, 1471, 1429, 1265, 1036, 863, 800, and 698 $cm.^{-1}$.

Isolation and Purification of Pigments from Cultures—Mixtures of the mycelium and nutrient broth from the two *Paxillus* species were freeze dried, and the dried samples were extracted exhaustively by repeated blending with cold absolute methanol. The alcoholic extracts were concentrated to dryness under reduced pressure at 35°, and the pigments were separated using a previously developed (3) dry-column chromatographic procedure with a silica gel adsorbent and ether saturated with concentrated HCl as the solvent system. The pigment bands corresponding to atromentic and xerocomic acids were eluted from the adsorbent with the acidified ether mixture, the solvent was removed, and the purified material was used for confirmation of identity.

Atromentic and xerocomic acids were recovered from culture mixtures of *P. atrotomentosus* and *P. panuoides* grown on both cherry and malt extract media. Xerocomic acid was the major pigment in all cases; tentative indications suggested that cultures of *P. panuoides* contained lower concentrations of the two tetronic acids, an indication that was confirmed by subsequent quantitative

Table III—Apparent Major Mass Spectral Fragmentation Pathways of Atromentin



[a] The relative intensities of fragments in parentheses were < 10% of base.

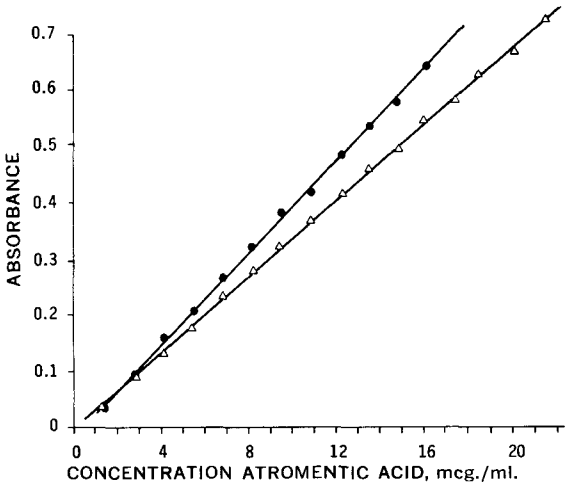


Figure 1—*Quantitation of atromentic acid. Key:* ●, *0.01* M $NaHCO_3$, *364 nm.; and* △, *distilled water, 378 nm.*

determinations. The unidentified red pigment remained at or near the origin in the dry-column procedure, and methanol was required to elute it from the silica gel.

Identification of Atromentic and Xerocomic Acids—IR, UV, and mass spectra were used to establish the identity of the isolated pigment samples with chromatographic properties corresponding to xerocomic acid. The limited availability of material corresponding to atromentic acid necessitated the use of only UV and mass spectra for identification of this pigment. The UV spectra [$\lambda_{max.}^{ethanol}$ 408 and 260 nm., $\lambda_{max.}^{water}$ 378 and 256 nm., and $\lambda_{max.}^{0.01\ M\ NaHCO_3}$ 620, 362, 320 (sh.), and 230 nm.] were identical for the xerocomic acid samples from both *Paxillus* species and were in agreement with those previously published for this tetronic acid (3). The atromentic acid samples from all of the cultures had identical UV spectra [$\lambda_{max.}^{ethanol}$ 390 and 257 nm., $\lambda_{max.}^{water}$ 375 and 258 nm., and $\lambda_{max.}^{0.01\ M\ NaHCO_3}$ 360 (sh.), 320, and 238 nm.], and these spectra were consistent with those known for atromentic acid (3). The IR spectra (KBr pellets) of authentic xerocomic acid and samples obtained from the cultures were indistinguishable, with absorption peaks at 3190, 2882, 2558, 1739, 1675, 1600, and 1513 $cm.^{-1}$.

Xerocomic acid samples from the cultures were observed to give high-resolution mass spectral parent ion peaks at *m/e* 338.0430, as anticipated for xerocomic acid lactone (calc. $C_{18}H_{10}O_7$, 338.0426); the fragmentation pattern, with ion peaks at *m/e* 310, 282, 226, 177, 161, 149, 133, 121, and 105, further confirmed the identity of these samples (3, 7). The corresponding data with the atromentic acid samples were parent ion peaks at *m/e* 322.0470 (calc. $C_{18}H_{10}O_6$, 322.0477) and fragmentation ions at *m/e* 294, 266, 238, 210, 161, 133, and 105, as anticipated (3, 7).

Effect of Heat on Xerocomic Acid—Xerocomic acid was observed to sublime at 242° to give dark-red needles. Agarwal and Seshadri

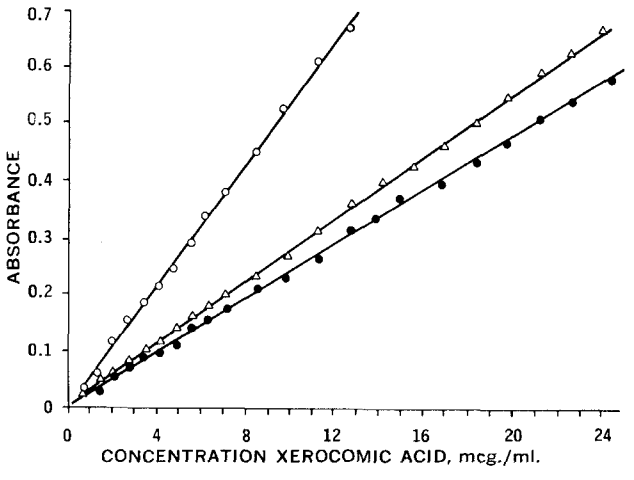


Figure 2—*Quantitation of xerocomic acid. Key:* ○, *0.01* M $NaHCO_3$, *364 nm.;* △, *distilled water, 378 nm.; and* ●, *0.01* M $NaHCO_3$, *600 nm.*

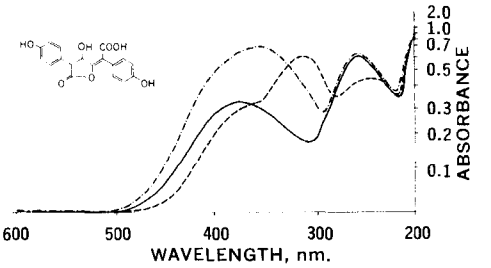


Figure 3—*Atromentic acid (9.08 mcg./ml.) spectra for pH 2.1 through 5.4* (—), *pH 8.5 through 9.3* (- - -), *and pH 10.8 through 11.9* (·-·); *analytical wavelength 316 nm.; pKa 6.39 ± 0.04.*

(8) reported the elimination of methanol and the formation of pulvinic acid lactone upon heating pulvinic acid to 192°. Thus, dehydration of xerocomic acid during sublimation was considered possible, and the properties of the red needles support such a change.

Mass spectral examination of the red needles revealed a high-resolution parent ion peak at *m/e* 338.0470, the parent ion peak of xerocomic acid lactone; this observation precluded the possibility of major pyrolytic fragmentation of the xerocomic acid molecule. The observed UV spectrum ($\lambda_{max.}^{ethanol}$ 438, 320, and 251 nm.) of the red material was distinct from the spectrum of xerocomic acid in ethanol, but it was similar to the UV spectrum ($\lambda_{max.}^{ethanol}$ 410, 312, and 240 nm.) of reference atromentic acid lactone. Absorption peaks in the IR spectrum (KBr pellet) of the red needles at 3448, 3279, 2924, 1815, 1779, 1653, 1600, 1511, 1445, and 1361 $cm.^{-1}$ were nearly identical to those observed with atromentic acid lactone, an observation that suggested similar characteristic functional groups. Also, the red material migrated farther than xerocomic acid in TLC System C (approximately R_f 0.70 and 0.50, respectively), a relationship that is known for other pulvinic acid derivatives and their lactones (3).

Quantitation of Atromentic and Xerocomic Acids—The UV spectra of atromentic and xerocomic acids in water and 0.01 *M* $NaHCO_3$ suggested the possibility of using a spectrophotometric procedure for quantitation of these tetronic acids. Preliminary studies with solutions of pure compounds revealed that satisfactory results could be obtained with both pigments in glass-distilled water measured at 378 nm. and in 0.01 *M* $NaHCO_3$ measured at 364 nm. and with xerocomic acid in the basic solution measured at 600 nm. Standard stock solutions (approximately 0.2 mg./ml.) of reference atromentic and xerocomic acids were prepared in glass-distilled water, and accurately measured volumes of these solutions were transferred to sufficient volumes of either distilled water or 0.01 *M* $NaHCO_3$ solution to give a 3-ml. total volume for each sample. The absorbances of solutions containing a range of approximately 1–28 mcg./ml. of the acids were measured, using water or appropriately diluted $NaHCO_3$ solutions as blanks. Figures 1 and 2 show standard curves for the concentration ranges, permitting feasible quantitative determinations under the various conditions; reproducibility among triplicate determinations at these concentrations was ±3%.

Table IV—Quantitative Data on Atromentic and Xerocomic Acids in 50-Day-Old Cultures of *P. atrotomentosus* and *P. panuoides*

Culture Mixture	Weight of Freeze-Dried Sample[a], g.	Atromentic Acid, mg./g.	Xerocomic Acid, mg./g.
P. atrotomentosus			
Cherry medium	11.37	0.18	39.5
Malt extract medium	8.02	0.01	47.4
P. panuoides			
Cherry medium	12.68	0.30	10.7
Malt extract medium	10.11	0.25	7.0

[a] Total solids representing mycelium and nutrient broth from 10 Roux culture flasks.

Table V—Fluorescent Properties of Atromentic, Pulvinic, and Xerocomic Acids

Tetronic Acid	Methanol: Excitation, nm.	Methanol: Emission, nm.	Methanol: Relative Intensity[a]	0.01 *M* $NaHCO_3$: Excitation, nm.	0.01 *M* $NaHCO_3$: Emission, nm.	0.01 *M* $NaHCO_3$: Relative Intensity
Nonirradiated						
Pulvinic acid	450	535	+	425	530	++++
Atromentic acid	480	550	+	450	535	++
Xerocomic acid	450	575	+	400	470	++
Irradiated						
Pulvinic acid	450	535	+++	425	530	++++
Atromentic acid	400	470	+++	400	470	++
		535	++		530	++
Xerocomic acid	350	470	++++	400	470	+
	425	510	++			

[a] Key to relative intensities: +, fluorescence ratio (ratio of intensity of emission peak to intensity of excitation peak) approximately 0.01; ++, fluorescence ratio >0.01 and <0.5; +++, fluorescence ratio >0.5 and <1.0; and ++++, fluorescence ratio >1.0.

Atromentic and xerocomic acids were separated, using either preparative TLC or the dry-column procedure, from known aliquots of extracts of the various culture mixtures, and the concentrations of the two acids were determined spectrophotometrically. Table IV lists the quantitative observations obtained with cultures of the two *Paxillus* species harvested after a 50-day incubation period.

The distinctive blue chromophore, formed in base by tetronic acids with a catechol function (6, 7), also suggested the possibility for quantitative determinations of mixtures of atromentic and xerocomic acids by making measurements at two wavelengths. Experimental observations revealed that such quantitative determinations were feasible in solution mixtures containing a total concentration of the two acids between 5 and 20 mcg./ml. and a minimum xerocomic acid concentration of 2.5 mcg./ml. The xerocomic acid in the mixture is determined from the absorbance in base at 600 nm. Absorbance of the same solution or a comparable solution in distilled water is measured at 364 or 378 nm., allowance is made for the amount of absorbance that can be attributed to the previously determined concentration of xerocomic acid, and the balance of the absorbance is assumed to represent the atromentic acid in the mixture. Ideally, it is preferable to make the measurements at both wavelengths with the same basic solution, but the steep slope of the standard curve for xerocomic acid in base at 364 nm. introduces practical limitations in mixtures containing a large xerocomic acid component.

Ionization Constants of Atromentic, Pulvinic, and Xerocomic Acids—Spectrophotometric procedures can be utilized to determine ionization constants for compounds characterized by ionic species with distinctive UV and visible spectra. The UV spectra observed for atromentic and xerocomic acids in water and $NaHCO_3$ solutions suggested the possibility of using this procedure with the pulvinic acid derivatives. A series of 0.01 *M* buffers (pH 2.1–11.9) was prepared in carbonate-free, glass-distilled water, according to the procedure of Albert and Serjeant (9); stock solutions of atromentic and xerocomic acids were prepared in the glass-distilled water, and a stock solution of pulvinic acid was prepared in absolute ethanol. Small volumes (0.15 ml.) of the stock solutions were added to sufficient volumes of buffer to give 3 ml. of buffer solutions, which contained approximately 10 mcg./ml. of a tetronic acid. The spectra were obtained at 24° with a recording spectrophotometer, and analytical wavelengths were selected for each compound. Figures 3–5 illustrate the representative spectra. After the analytical wavelength and the approximate pKa value were determined, spectra with buffer systems at increments of 0.2 pH unit were used to calculate the exact pKa value. The experimentally determined pKa values were 6.39 ± 0.04 (316 nm.) for atromentic acid, 6.86 ± 0.05 (296 nm.) for pulvinic acid, and 6.25 ± 0.05 (316 nm.) for xerocomic acid.

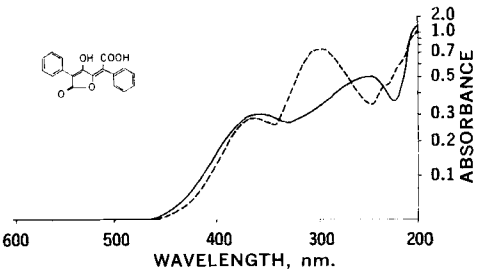


Figure 4—*Pulvinic acid (9.43 mcg./ml.) spectra for pH 2.1 through 6.0 (—) and pH 8.5 through 11.9 (- - -); analytical wavelength 296 nm.; pKa 6.86 ± 0.05.*

The spectra (Figs. 3 and 5) suggested that it should be possible to determine the ionization constants for the monohydroxyphenyl portions of both atromentic and xerocomic acids at analytical wavelengths in the 355–380-nm. region with mildly alkaline buffers (approximately pH 9). However, presumably the measurement of more than two ionic species under these conditions interfered with the determination of precise pKa values. The blue ionic species with xerocomic acid, which was attributed to the dihydroxyphenyl portion of the molecule and which could be measured at 600 nm. over the buffer range pH 6.3–11.9, faded rapidly above pH 8.5; thus, pKa values were unobtainable.

Fluorescent Properties of Atromentic, Pulvinic, and Xerocomic Acids—Light-induced degradative changes were noted previously with tetronic acid-containing cultures of *P. atrotomentosus* (3), and radiant energy was known to produce fluorescent photoproducts with atromentin (5). Studies were undertaken to determine the fluorescent properties of the tetronic acids at different stages of ionic dissociation, both before and after exposure to shortwave UV light. Stock solutions in spectrograde methanol were prepared, containing 1 mg./50 ml. and 10 mg./50 ml. of atromentic, pulvinic, and xerocomic acids. A portion of each solution was placed in a quartz cell and irradiated[4] for 18 hr. The fluorescent properties[5] of the dilute methanol solutions and of $NaHCO_3$ solutions (prepared by adding 0.3 ml. of the concentrated methanolic solutions to 2.7 ml. of 0.01 *M* $NaHCO_3$) are recorded in Table V.

CONCLUSIONS

The pattern of pigment distribution in carpophores and cultures of *P. atrotomentosus* and *P. panuoides* was observed to be identical, except for the presence of thelephoric acid in carpophores of *P. atrotomentosus*. Atromentin was isolated and identified from the

[4] Exposed at approximately 20 cm. to a Hanovia high pressure quartz mercury vapor lamp, model 30600, with a complete spectrum from 1849 to 4000 Å; Hanovia Chemical and Manufacturing Co., Newark, N. J.

[5] Baird Atomic Fluorispec, model SF-1, Baird Atomic Inc., Cambridge, Mass.

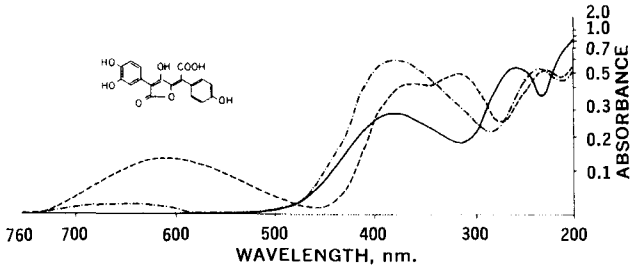


Figure 5—*Xerocomic acid (10.17 mcg./ml.) spectra for pH 2.1 through 5.2 (—), pH 8.5 through 9.3 (- - -), and pH 11.9 (·-·). Analytical wavelength 316 nm.; pKa 6.25 ± 0.05.*

carpophores of both species, and atromentic and xerocomic acids were isolated and identified from the culture mixtures. The tetronic acids were produced equally well when the fungi were grown on a cherry medium and an acidified malt extract medium; the utility of the latter medium was particularly significant for the fermentative production of these compounds, since it offered the advantage of circumventing the isolation problems caused by the anthocyanin pigments in the cherry decoction. Xerocomic acid was the major tetronic acid found in the cultures of both species; the ratios of xerocomic acid to atromentic acid were greater than 200 and approximately 36 for cultures of *P. atrotomentosus* and *P. panuoides*, respectively. An unidentified purple pigment was present in carpophores of both species, and the culture mixtures contained an unidentified red pigment. The observed distribution of pigments in the two *Paxillus* species further confirmed the distinctive metabolic capabilities of carpophores and vegetative mycelium which had been detected initially with *P. atrotomentosus* (3), but any contribution of the two unidentified pigments toward clarifying these metabolic capabilities must await further characterization of the pigments.

The quantitative spectrophotometric studies revealed feasible procedures for determination of atromentic and xerocomic acids in low concentrations (2–24 mcg./ml.) and in mixtures of certain compositions. Application of such quantitative methods, in conjunction with observations on UV and fluorescent properties and on molecular response to electron bombardment, may be useful in future studies to clarify the biologic involvement of tetronic acids and to establish the interrelationship between terphenylquinones and diphenyl-substituted tetronic acids.

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Drug Transfer across Rat Intestinal Musculature after Edetic Acid Treatment

RAMCHANDRA K. NAYAK and LESLIE Z. BENET

Abstract □ The columnar epithelium of the *in vitro* rat intestine was separated from the intestinal musculature by treatment with ice-cold edetic acid solution. The *in vitro* absorption kinetics through the denuded intestinal musculature for salicylate ion and acetanilide were followed in pH 7.4 sodium phosphate and potassium phosphate buffers, as well as in an isotonic sodium chloride solution where the effect of rinsing with Mg^{+2} and Ca^{+2} was also studied. Differences in directional transfer rates, as noted in previous studies with the untreated intestinal membrane, were not found. The transfer rates through the muscle layer were found to be at least twice as high as those found in the untreated membrane. As noted previously, nonionized acetanilide transfer rates were still approximately twice those seen for salicylate ion.

Keyphrases □ Drug-transfer rates, *in vitro*—effect of intestinal musculature, rat □ Membrane permeability coefficients—effect of edetic acid treatment □ Absorption kinetics, *in vitro*—salicylate and acetanilide through denuded intestinal musculature, rat □ Edetic acid—treatment of rat intestinal musculature, effect on drug transfer

Recent studies in this laboratory pointed out that drug ions exhibit directional permeability coefficients for passage across the rat intestine *in vitro* (1, 2), with mucosal to serosal transfer occurring at faster rates than serosal to mucosal transfer. It was proposed that the difference in directional permeability coefficients

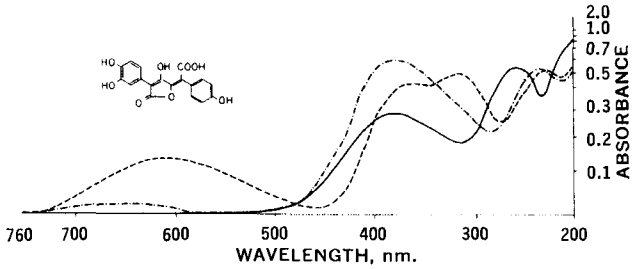

Figure 5—*Xerocomic acid (10.17 mcg./ml.) spectra for pH 2.1 through 5.2 (—), pH 8.5 through 9.3 (- - -), and pH 11.9 (·-·). Analytical wavelength 316 nm.; pKa 6.25 ± 0.05.*

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Drug Transfer across Rat Intestinal Musculature after Edetic Acid Treatment

RAMCHANDRA K. NAYAK and LESLIE Z. BENET

Abstract □ The columnar epithelium of the *in vitro* rat intestine was separated from the intestinal musculature by treatment with ice-cold edetic acid solution. The *in vitro* absorption kinetics through the denuded intestinal musculature for salicylate ion and acetanilide were followed in pH 7.4 sodium phosphate and potassium phosphate buffers, as well as in an isotonic sodium chloride solution where the effect of rinsing with Mg^{+2} and Ca^{+2} was also studied. Differences in directional transfer rates, as noted in previous studies with the untreated intestinal membrane, were not found. The transfer rates through the muscle layer were found to be at least twice as high as those found in the untreated membrane. As noted previously, nonionized acetanilide transfer rates were still approximately twice those seen for salicylate ion.

Keyphrases □ Drug-transfer rates, *in vitro*—effect of intestinal musculature, rat □ Membrane permeability coefficients—effect of edetic acid treatment □ Absorption kinetics, *in vitro*—salicylate and acetanilide through denuded intestinal musculature, rat □ Edetic acid—treatment of rat intestinal musculature, effect on drug transfer

Recent studies in this laboratory pointed out that drug ions exhibit directional permeability coefficients for passage across the rat intestine *in vitro* (1, 2), with mucosal to serosal transfer occurring at faster rates than serosal to mucosal transfer. It was proposed that the difference in directional permeability coefficients

might be related to a sodium-ion coupled transport, since when sodium ion was completely replaced by potassium in phosphate buffer solutions, the difference in permeability coefficients tended to disappear (1). However, more recently, Benet *et al.* (2) pointed out that salicylate ion also exhibited different directional flux rates in a tromethamine buffer; they suggested that the results seen previously (1) with sodium and potassium phosphate buffers might be better explained in terms of the effects of phosphate on the intestinal mucosa. Nonionized drugs did not show a difference in directional permeability coefficients in either sodium or potassium phosphate buffers.

One difficulty in extrapolating *in vitro* intestinal flux data to the *in vivo* transport of drugs is the fact that the *in vitro* fluxes are measured through the columnar epithelium *plus* the underlying connective and muscle tissue. Therefore, the rate-limiting step for *in vitro* transport might well be the intestinal musculature, a barrier that would not be encountered in *in vivo* transport. The present study was undertaken to determine the effect of the intestinal musculature on the *in vitro* transmural flux of drugs.

Two drugs, salicylate ion and nonionized acetanilide, were studied with respect to their transfer rates across the *in vitro* rat intestine in both directions. The intestine was pretreated with ethylenediaminetetraacetic acid, edetic acid, by the method of Berlin and Hawkins (3), to slough off the mucosal epithelium, leaving the underlying muscle layers intact for absorption studies.

EXPERIMENTAL

Materials—The following USP, NF, or reagent grade chemicals were used: salicylic acid, acetanilide, monobasic sodium phosphate anhydrous, monobasic potassium phosphate anhydrous, dibasic potassium phosphate anhydrous, sodium chloride, ethylenediaminetetraacetic acid, sodium hydroxide, potassium hydroxide, magnesium chloride hexahydrate, calcium chloride dihydrate, and a 100% oxygen gas.

Procedure—The pH 7.4 sodium phosphate buffers and the potassium phosphate buffers were prepared as previously described (1). Isotonic sodium chloride solution at pH 6.6 was prepared in the usual manner. The pH values of the solutions used were routinely checked at the beginning and end of each experiment on a research pH meter[1].

Male Sprague-Dawley rats, weighing 220–250 g., were fasted for 24 hr. prior to the experiment, but water was allowed *ad libitum*. The rats were rendered unconscious with carbon dioxide in an airtight chamber and were then sacrificed by a sharp blow at the base of the skull. The small intestine (from the pyloric junction to about the middle of the jejunum) was removed at once *via* a midline abdominal incision and flushed with 40 ml. of the buffer (warmed) to be used in the absorption study. The first 15-cm. segment was discarded and the next 2–12.5-cm. segments (stretched length using a 20-g. weight) were everted.

The segments of the gut were kept for 45 min. in a refrigerator in an ice-cold 0.1 *M* edetic acid solution of the appropriate buffer. At the end of this period, the segments of the gut were taken out and rinsed with buffer. The disrupted epithelium was then removed by gently moving a finger back and forth over the length of the everted intestinal segment. In alternating studies, the proximal or distal segments were reeverted. The denuded gut segments were then used to study the *in vitro* absorption kinetics using perfusion devices and techniques previously described (1, 2), where each intestinal segment was used for four consecutive experiments with the solutions changed after each run. At appropriate intervals, 0.1-ml. samples were removed from the inner chamber for assay. In the experiments to study the possibility of Mg^{+2} and Ca^{+2} ions reversing the effects of edetic acid treatment, the intestinal segments were rinsed in $MgCl_2$ and $CaCl_2$ solutions (both 0.1 *M* and at pH 6.6) for 5 min. each after the usual treatment with edetic acid.

For salicylate-ion transfer, the time for the experiment was 2 hr. and 20 min. (four 35-min. transport studies with each segment). For acetanilide transfer, however, absorption measurements were made during the following time periods: initial rate, 0–15 min.; rate 2, 35–50 min.; rate 3, 70–85 min.; and rate 4, 125–140 min. Fifteen-minute transfer experiments were necessary in the acetanilide studies to correspond with requirements described previously (1) so that transfer would appear to follow unidirectional zero-order kinetics.

A histological examination was also made to compare an untreated everted intestinal segment with the: (*a*) one kept in ice-cold isotonic NaCl solution (pH 6.6) for 45 min., (*b*) one kept in ice-cold 0.1 *M* edetic acid solution (pH 6.6) for 45 min., and (*c*) one kept in $MgCl_2$ and $CaCl_2$ (both 0.1 *M* and at pH 6.6) solutions after edetic acid treatment. The tissue was fixed in formalin solution, and the sections were stained with hematoxylin and eosin.

Assay Procedures—The 0.1-ml. samples were diluted and assayed as follows. Acetanilide was diluted with 2.0 ml. of the appropriate buffer, and the concentration was determined by UV measurement at 239 nm. on a spectrophotometer[2]; salicylic acid was diluted with 5.0 ml. of 0.1 *N* sodium hydroxide or potassium hydroxide, and the drug concentration was determined on a spectrophotofluorometer[3] at an excitation wavelength of 300 nm. and an emission wavelength of 408 nm. (uncorrected). The choice of diluent depended upon the presence or absence of sodium ion in the environment. Standard curves for each drug were made using five known concentrations of the drug in the appropriate diluent. Blank values were sufficiently low at all times during the sampling period and could be ignored. As in previous experiments (1, 2), no significant difference in transfer rates was observed between the first and second segments of the intestine.

Plots were made of the concentration in the inner chamber *versus* time. Rates of transfer, intercepts, and ratios were obtained by the use of an unweighted least-squares program executed on a desk calculator[4]. By using the everted and noneverted data, the following calculations were made: A, the average everted and noneverted rates and the everted/noneverted ratios for all determinations on a particular drug in a particular buffer; B, the average initial rates (everted and noneverted) and ratios (everted/noneverted), *i.e.*, the first rate measured on each intestinal segment; C, the average increase in rates over the time of the experiment as a percent of the initial rate, *i.e.*, (4th rate/1st rate) × 100; D, the average *X*-intercept or lag time observed with initial rate measurements; and E, the average total amount (milligrams) of a drug transferred across the gut during the time for the experiment (four runs). Differences between everted and noneverted values were tested at 95% or greater confidence levels using Student's test for paired data.

RESULTS AND DISCUSSION

The summary of the data is presented in Table I for salicylate ion and in Table II for nonionized acetanilide according to the format used previously (2). The level of significance for each of the five averages (A–E) used to compare transfer through everted and noneverted segments is also reported in Tables I and II. No significant differences between everted and noneverted transfer rates (at 95% or greater confidence levels) were observed for acetanilide in all three isotonic solutions or for salicylate ion in the isotonic solutions containing sodium. Previous studies (1, 2) showed that significant differences in directional permeability coefficients existed for ionized drugs in the presence of sodium ion.

The values of directional permeability coefficients reported in Tables I and II for both the salicylate ion and the nonionized acetanilide are at least twice as high as those obtained earlier with the untreated gut (2). Previous studies (3–14) reported changes in membrane permeability after treatment with, or in the presence of, edetic acid. In all of these studies, the transfer rates for different

[1] Beckman.

[2] Beckman DB-G.

[3] Aminco-Bowman.

[4] Hewlett-Packard model 9100A.

Table I—Summary of Transfer Data ± Standard Deviation for Salicylate Ion in Three Isotonic Solutions through Rat Intestinal Tissue from Which the Mucosa Was Removed by Treatment with Edetic Acid

	Number	Everted	Noneverted	Ratio	Level of Significance, Everted *versus* Noneverted
		Sodium Phosphate pH 7.4 Buffer			
A[a]	4	4.27 ± 0.74	4.47 ± 0.40	0.96 ± 0.14	N.S.[b]
B	4	3.93 ± 0.50	4.35 ± 0.54	0.92 ± 0.17	N.S.
C	4	117 ± 8	108 ± 19		N.S.
D	4	1.18 ± 0.87	1.21 ± 0.61		N.S.
E	4	8.96 ± 1.61	9.41 ± 1.01	0.95 ± 0.15	N.S.
		Potassium Phosphate pH 7.4 Buffer			
A	4	4.01 ± 0.43	5.33 ± 0.77	0.76 ± 0.12	$p < 0.05$
B	4	3.94 ± 0.42	5.45 ± 0.74	0.74 ± 0.15	$p < 0.05$
C	4	105 ± 8	97 ± 7		N.S.
D	4	1.65 ± 1.06	1.80 ± 1.25		N.S.
E	4	8.35 ± 0.94	11.00 ± 1.51	0.77 ± 0.10	$p < 0.05$
		Isotonic Sodium Chloride pH 6.6			
A	4	4.13 ± 0.78	4.76 ± 1.01	0.88 ± 0.12	N.S.
B	4	3.26 ± 0.80	4.15 ± 1.12	0.81 ± 0.18	N.S.
C	4	153 ± 21	134 ± 16		N.S.
D	4	2.89 ± 0.84	2.16 ± 1.48		N.S.
E	4	8.38 ± 1.51	9.92 ± 2.06	0.86 ± 0.13	N.S.
		Isotonic Sodium Chloride pH 6.6[c]			
A	4	4.92 ± 0.67	5.62 ± 0.63	0.88 ± 0.09	N.S.
B	4	4.58 ± 0.56	5.45 ± 0.94	0.85 ± 0.15	N.S.
C	4	107 ± 28	104 ± 32		N.S.
D	4	6.08 ± 2.46	4.70 ± 1.40		N.S.
E	4	9.34 ± 1.08	11.01 ± 0.65	0.85 ± 0.07	$p < 0.05$

[a] A, average rates—all runs, mcg./ml./min.; B, average initial rates, mcg./ml./min.; C, average rate increase, 4th rate/initial rate as %; D, average lag time for initial runs, min.; and E, average total amounts, mg. [b] Not significant. [c] Intestinal segments rinsed with 0.1 *M* $MgCl_2$ and 0.1 *M* $CaCl_2$, both at pH 6.6.

substances increased except in one case (8), where the uptake of cyanocobalamin was found to be markedly diminished. In a study on the intestinal absorption of pralidoxime ion, 2.5 m*M* edetic acid did not affect the transfer rates of the drug (15).

The effect of edetic acid on intestinal permeability has been related to its removal of bivalent ions from the structure of the membrane (7) and to the loss of adhesiveness of cells (8) due to disruption of the intercellular epithelial bridges. The relationship between membrane permeability and the depletion of bivalent ions from the membrane by chelation with edetic acid is supported by many observations. Feldman and Gibaldi (13) reported a significant increase in the salicylate transfer rate after incubation of the gut in 25 m*M* edetic acid. The effects were greater at pH 7.4 than at pH 6.0, which may be related to increased binding capacity of edetic acid for bivalent ions with increasing pH. The increase in phenol red absorption from the rat intestine observed by Tidball (7), after edetic acid exposure, could be reversed by rinsing the intestine with $CaCl_2$ or $MgCl_2$. It was also pointed out that the calcium salt of edetic acid had no effect on the permeability of the rat intestinal epithelium to phenol red. This was also found to be true in the absorption of heparin (11). These data postulate that edetic acid alters the aqueous permeability of intestinal epithelium by depleting Mg and Ca.

The alteration of permeability after treatment with edetic acid was previously thought to be independent of any mechanical damage to the cell (9) but, more recently, it was shown (10), with the help of electron micrographs, that treatment with edetic acid causes a reversible alteration in the fine structure of the tissue. In this laboratory, the histological examinations of the intestinal segments treated with 0.1 *M* edetic acid revealed that the epithelial layer of the mucosa was completely stripped off, with about 25% of basal cells of the mucosa still remaining. Edema was observed in muscularis mucosa. This edema was absent in the section of the gut rinsed with $MgCl_2$ and $CaCl_2$ solutions after edetic acid treatment. The muscle layer or the serosa was found to be intact after edetic acid treatment when compared with an untreated section.

The present findings, high transfer rates through the muscle layer and no difference in directional transport, are in accord with observations made earlier (4, 6) that the selective and absorptive elements are epithelial in the absorption of various drugs. A more conclusive statement must await further work being carried out in this laboratory where the intestinal musculature will be removed by surgical techniques and transport across the short-circuited epithelial membrane will be followed using radioactive tracers.

The values in Row C of Tables I and II give the average changes in rates observed during each 2-hr. and 20-min. transport experiment. No changes in transport rates were observed, except for the case where salicylate ion was placed in the unbuffered 0.9% sodium chloride solution. Therefore, except for this one unexplained case, the intestinal musculature was able to maintain a relatively constant transfer rate over the time of the experiment, and the transfer rates observed here might be considered the maximum values possible when transport through the *in vitro* intestine is considered.

As was noted in the previous untreated transport studies (2), the nonionized acetanilide yielded lag times (Row D) that were not significantly different than zero (at 95% confidence levels) for all three of the solutions studied. The lag times observed for salicylate ion were significantly different than zero, even though the lag time values found for transport through the intestinal musculature were one-half to one-third those found previously in the intact intestinal segment. As would be expected, from the values reported in Row C, there was little difference between the average initial rates and the average rates for all runs, as reported in Rows B and A. Likewise, since there was no significant difference in directional transport rates, the ratios found using the total amounts transferred (Row E) were almost identical to those found for the average rates (Row A).

As can be seen in Table I, significant differences were found between the everted and noneverted transport rates of salicylate ion in the potassium phosphate buffer. In this buffer, the everted transfer rates were similar to those found in the sodium phosphate and sodium chloride solutions. However, there was a marked increase in the rates observed through the noneverted segments. This was in contrast to the results found for the intact intestinal segments (1), where similar transfer rates were observed across the noneverted intestinal segment in both potassium and sodium phosphate buffers and a slight decrease in the everted transfer rate was found in going from the sodium to potassium phosphate buffers. At the present time, we can offer no explanation for this larger noneverted rate found in the edetic acid-treated segments. However, the potassium phosphate buffer is not causing a time-dependent degradation of the membrane, since the values in Row C are very close to 100%.

Table II—Summary of Transfer Data ± Standard Deviation for Acetanilide in Three Isotonic Solutions through Rat Intestinal Tissue from Which the Mucosa Was Removed by Treatment with Edetic Acid

	Number	Everted	Noneverted	Ratio	Level of Significance, Everted *versus* Noneverted
		Sodium Phosphate pH 7.4 Buffer			
A[a]	4	8.94 ± 0.55	9.47 ± 1.27	0.95 ± 0.09	N.S.[b]
B	4	8.94 ± 1.00	9.73 ± 0.89	0.92 ± 0.07	N.S.
C	4	103 ± 8	93 ± 6		N.S.
D	4	0.43 ± 0.27	0.21 ± 0.52		N.S.
E	4	8.31 ± 0.48	8.42 ± 0.69	0.99 ± 0.07	N.S.
		Potassium Phosphate pH 7.4 Buffer			
A	4	8.82 ± 0.64	8.72 ± 0.68	1.02 ± 0.11	N.S.
B	4	8.80 ± 1.74	8.83 ± 0.69	0.99 ± 0.13	N.S.
C	4	101 ± 19	100 ± 14		N.S.
D	4	0.54 ± 0.58	−0.38 ± 0.83		N.S.
E	4	8.01 ± 0.61	7.98 ± 0.57	1.01 ± 0.11	N.S.
		Isotonic Sodium Chloride pH 6.6			
A	4	8.94 ± 1.29	9.19 ± 0.52	0.97 ± 0.14	N.S.
B	4	8.34 ± 1.87	8.70 ± 0.46	0.97 ± 0.25	N.S.
C	4	112 ± 13	113 ± 9		N.S.
D	4	1.67 ± 2.29	1.71 ± 1.96		N.S.
E	4	8.06 ± 1.38	8.21 ± 0.82	0.98 ± 0.09	N.S,
		Isotonic Sodium Chloride pH 6.6[c]			
A	4	8.16 ± 0.22	8.35 ± 1.07	0.98 ± 0.12	N.S.
B	4	7.73 ± 1.25	8.18 ± 0.98	0.96 ± 0.22	N.S.
C	4	111 ± 17	108 ± 16		N.S.
D	4	0.77 ± 0.43	0.57 ± 0.55		N.S.
E	4	6.87 ± 0.96	7.00 ± 0.80	0.98 ± 0.08	N.S.

[a] See *Footnote a*, Table I. [b] Not significant. [c] See *Footnote c*, Table I.

When the segments were rinsed with $MgCl_2$ and $CaCl_2$ following edetic acid treatment, there was no significant change in acetanilide transport through membranes bathed in isotonic sodium chloride solutions. (See Table II, isotonic sodium chloride values with and without divalent-ion rinsing.) However, initial everted transfer rates of salicylate, as well as the lag times for both everted and noneverted transport, increased significantly (95% confidence levels) following the divalent cation rinses. At the same time, there was no significant change in average rates—all runs—and in total amounts transferred. As was noted earlier in the histological preparations, the muscularis mucosa showed edema following edetic acid treatment, but this edema was absent after the divalent cation rinse. Following the reasoning presented in the previous publication (2), the differences in lag time for salicylate might be explained on the basis of a size change in pores or intracellular channels. The size change could be influenced by the presence or absence of edema. These results in the muscle layer are in contrast to those recently reported by Mayersohn *et al.* (16) for intact intestinal sacs, where transport rates decreased as a result of increased water uptake by the segment. However, this difference may be explained in terms of the rate-limiting step being the epithelial membrane rather than the muscle layer. As previously noted, the acetanilide rates and lag times are unaffected by the presence or lack of edema; thus, this drug would appear to be absorbed through a different route.

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Absorption, Tissue Distribution, and Excretion of Bunolol-^{14}C by Dogs

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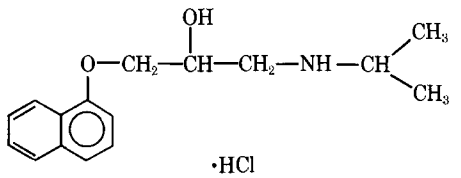
Abstract □ Encapsulated bunolol-^{14}C was absorbed quickly by dogs. The peak blood level 1 hr. after treatment corresponded to 4.6% of the dose or 5.1 mcg./ml. Within 72 hr., 54–75% of the radioactivity was excreted in the urine and 15–17% in the feces. At 72 hr., the highest tissue levels were only 0.6–1.4% of the ^{14}C dose in the GI tract and 0.6–1.1% in the liver. The radioactivity in the 0–24-hr. pooled urine included four unconjugated (37%) and six conjugated acids (5%), three unconjugated (24%) and five conjugated bases (11%), and 24% ether-insoluble metabolites. A radioactive unconjugated basic compound was purified from the 0–24-hr. urine. The compound, comprising 0.7% of the urinary ^{14}C, migrated as bunolol on TLC. Further identification was made by GLC; retention times for the acetates and trifluoroacetates prepared from authentic and urinary bunolol were identical. Bunolol glucuronide was not found in the 0–24-hr. urine.

Keyphrases □ Bunolol-^{14}C, metabolites—absorption, excretion, tissue distribution, dogs □ Absorption kinetics—bunolol-^{14}C, dogs □ Excretion kinetics—bunolol-^{14}C, dogs □ Urinary drug metabolism—bunolol-^{14}C, dogs

Bunolol {*dl*-5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride} was shown to be the most active analog among 30 compounds displaying β-adrenergic blocking activity (1). Following intravenous administration to barbiturate-anesthetized dogs, bunolol was estimated to be 2–3 times more potent than propranolol in antagonizing the isoproterenol-induced changes in heart rate, blood pressure, contractile force, and tachycardia (2, 3). Furthermore, bunolol displayed a greater separation between the β-adrenergic blocking and myocardial depressant doses than did propranolol (2). A study in anesthetized vagotomized dogs challenged with isoproterenol showed bunolol to be 20 times more potent than propranolol after oral administration (4). On the other hand, bunolol was found to be less active than propranolol against ouabain-induced arrhythmia in dogs (5). Bunolol and propranolol were equipotent in blocking isoproterenol protection of histamine-induced bronchoconstriction (6).



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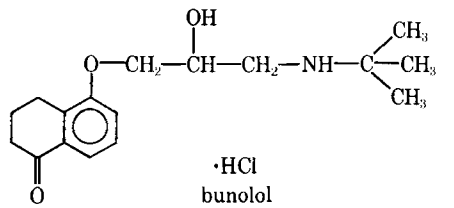

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bunolol

The purpose of the present study was to investigate the absorption, excretion, and tissue distribution of bunolol in the dog after oral administration. In addition, efforts were made to estimate quantitatively the unaltered drug and to determine the number and types of drug metabolites.

Table I—Blood Levels of Total ^{14}C after Oral Administration of Bunolol-^{14}C to Dogs

Dog Number	Radioactive Dose in Blood, mcg./ml. 30 min.	1 hr.	2 hr.	4 hr.	6 hr.	24 hr.	48 hr.
1	5.0	5.2	3.2	2.0	1.0	0.05	0.08
2	1.0	6.1	4.1	1.7	1.5	0.2	0.08
3	5.3	4.4	3.0	1.6	0.7	0.2	0.1
Mean	3.8	5.2	3.4	1.8	1.1	0.15	0.09

MATERIALS AND METHODS

Radioactive Bunolol—Radioactive bunolol was synthesized with ^{14}C in position 1 (carbonyl-C) of the saturated ring (7). The preparation was 99.0% pure, both chemically and radiochemically, as judged by TLC; it had a specific radioactivity of 4.90 mc./g.

Protocol—Twenty-milligram portions of bunolol-^{14}C were weighed into three gelatin capsules, and enough bunolol was added to provide 10-mg./kg. doses. The capsules were administered to female beagles weighing: Dog 1, 11.5 kg.; Dog 2, 11.5 kg.; and Dog 3, 13.0 kg. The dogs had free access to food and water.

Blood Levels and Radioactivity—Blood samples (about 2 ml.) were withdrawn from each dog at the following time intervals after treatment: 30 min. and 1, 2, 4, 6, 24, 48, and 72 hr. Duplicate aliquots of each blood specimen (1 ml. each) were diluted with distilled water (100-fold for the first five samples, 50-fold for the 24- and 48-hr. specimens, and 10-fold for the 72-hr. specimens) and stored overnight under refrigeration. These lysed blood mixtures were counted for radioactivity with a liquid scintillation spectrometer[1]. One milliliter of each urine collection was diluted with 18 ml. of scintillation solution and counted directly. The counting efficiency was determined by the external standardization method. The scintillation solution consisted of 7.0 g. 2,5-diphenyloxazole, 0.3 g. dimethyl 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene, and 100 g. naphthalene in 1.0 l. reagent grade dioxane.

TLC—Chromatograms for analytical purposes were run on 5 × 20-cm. glass plates coated with 250 μ of silica gel G bound with calcium sulfate[2]. For preparative efforts, 20 × 20-cm. plates were used. Before sample application, the plates were developed in methanol, dried in air, and heated in an oven at 140° for at least 30 min. One-dimensional chromatograms were developed using the following solvents: 1, chloroform–glacial acetic acid–methanol (15:1:4); 2, butanol–29.6% ammonia–water (4:1:3, upper phase); and 3, butanol–glacial acetic acid–ether–water (9:6:3:1).

[1] Packard Tri-Carb model 3320.
[2] Analtech.

Table II—Excretion of ^{14}C after Oral Administration of Bunolol-^{14}C to Dogs

Dog	Radioactive Dose in Urine, %				Radioactive Dose in Feces[a], %			
	0–24 hr.	24–48 hr.	48–72 hr.	0–72 hr.	0–24 hr.	24–48 hr.	48–72 hr.	0–72 hr.
1	49.5	2.9	1.4	53.8	No feces	14.7	2.5	17.2
2	65.0	2.2	1.5	68.7	14.4	1.0	1.2	16.6
3	69.1	4.8	1.4	75.3	7.8	4.9	2.1	14.8
Mean				65.9				16.2

[a] Almost half of the labeled end-products were not extracted and were measured by digesting the stool with hydroxide of hyamine 10-X.

The chromatograms were scanned for radioactivity with a radiochromatogram scanner[3]. The area under each peak was determined with a compensating polar planimeter[4]. Estimates of relative peak area were used to calculate the amounts of bunolol metabolized. To measure the ^{14}C on 20 × 20-cm. thin-layer plates, the plates were cut into 5-cm. strips and scanned.

GLC—The samples were injected into a gas chromatograph[5] fitted with dual columns and a differential flame-ionization detector. The columns were 1.8 m. × 0.31 cm. (6 ft. × 0.125 in.) [0.26 cm. (0.105 in.) i.d.] stainless steel, each packed with 3.45 g. of 3% OV-1 on 80–100 mesh Chromosorb W (HP). The columns were operated at 198° for assay of the acetate and at 173° for assay of the trifluoroacetate. The injector temperature was 278°; the helium carrier gas flow rate was 19 ml./min. for the sample column and 38 ml./min. for the reference column.

RESULTS

Blood Levels—The data presented in Table I indicate that bunolol was absorbed rapidly. The maximum blood concentration was observed after 1 hr. and corresponded to a mean of 4.6% of the dose in the systemic circulation. Subsequently, the blood ^{14}C level decreased gradually, and radioactivity was measurable after 48 hr. but not after 72 hr.

Urinary Excretion—All excreted urine was collected from each dog over the periods from 0 to 24 hr., from 24 to 48 hr., and from 48 to 72 hr. Radioactivity measurements (Table II) showed that most of the drug and its metabolites (49.5–69.1%) were excreted during the first 24 hr.; only small amounts (1.4–4.8%) were excreted in successive 24-hr. intervals. The cumulative urinary excretion reached 53.8–75.4% of the dose by 72 hr.

Fecal Excretion—The complete fecal output was collected at 24-hr. intervals for 3 days. Each collection was extracted repeatedly with 75% dioxane, and aliquots of the residues were digested by shaking overnight at 37° with hydroxide of hyamine 10-X[6]. The extracts and the digestion mixtures were counted for radioactivity, and the total value for each collection is listed in Table II. Despite the failure of Dog 1 to defecate during the 1st day, there was close agreement in the total fecal elimination of ^{14}C by the three dogs (14.8–17.2% of the dose in 72 hr.).

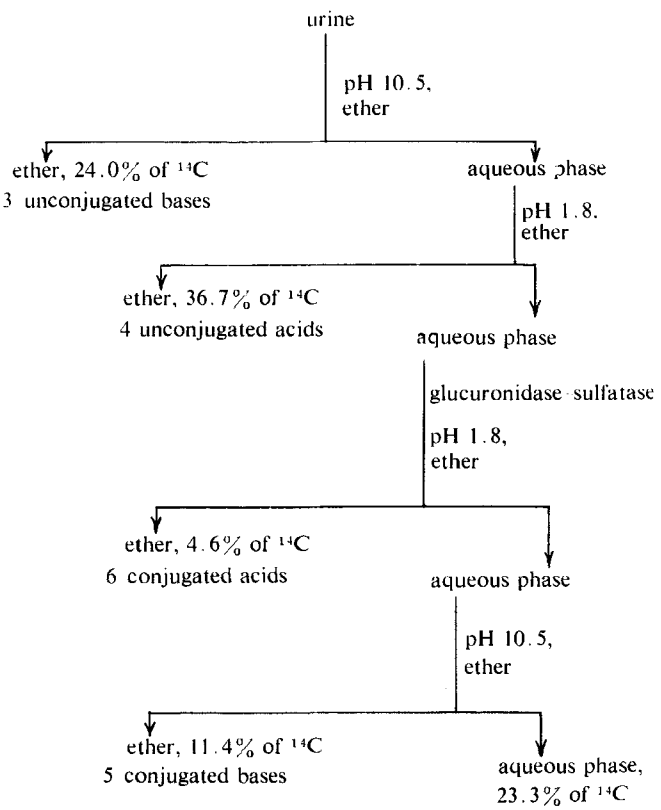
Tissue Distribution—Each dog was sacrificed 72 hr. after drug administration. The brain, heart, entire GI tract and its contents, kidneys, spleen, and liver were excised from each animal. Also removed were portions of fat (87–157 g.) and of muscle (102–123 g.). All of these tissues were homogenized and extracted thoroughly with 75% dioxane before portions of the residues were digested with hyamine. The quantities of radioactivity found were greatest in the GI tract and liver (Table III), possibly indicating prolonged enterohepatic circulation of drug metabolites.

Ether Fractionation of Urinary Metabolites—A 1-ml. aliquot of pooled urine collected from the three dogs from 0–24 hr. was adjusted to pH 10.5 and extracted three times with 4 ml. of ether. The aqueous layer was adjusted to pH 1.8 and again extracted three times with ether. The pH of the aqueous phase was adjusted to 6.5, and traces of ether were removed by aeration. A preparation containing 7500 Fishman units of β-glucuronidase and 8000 Whitehead units of aryl sulfatase[7] in 50 μl. was added and incubated at 37°. Two hours later, an additional 50 μl. of this enzyme preparation was added. After a total of 45 hr. of incubation, ether extractions were performed at pH 1.8 and 10.5. The corresponding ether extracts were pooled and assayed for ^{14}C. Practically all of the urinary ^{14}C was recovered (Scheme I).

The consideration of neutral structures was set aside, and the urine fractions were designated as unconjugated acids (37%), unconjugated bases (24%), conjugated acids (5%), and conjugated bases (11%). Approximately 23% of the urinary ^{14}C fell into none of these categories (Scheme I).

Number of Urinary Bunolol Metabolites—Ether extracts, obtained as described, were subjected to TLC in Solvent 1. As noted in Scheme I, radioscanning revealed the presence of three unconjugated and five conjugated bases and of four unconjugated and six conjugated acids. The elucidation of the structure of two of the unconjugated acids is the subject of another report (8), and an investigation of the structures of basic metabolites is in progress.

Purification of Bunolol from Dog Urine—The plan followed is shown in Scheme II. The starting material was 56 ml. of the urine collected from 0 to 24 hr. after treating three dogs with ^{14}C-bunolol. To remove unconjugated acids, the urine was extracted five times with two volumes of ether after pH adjustment to 1.8. To collect the bases for study, the urine was brought to pH 10.5 and extracted thoroughly with ether. The ether phase obtained by extraction of the alkalinized urine contained 24.6% of the ^{14}C. Following evapo-



Scheme I—Fractionation and TLC of drug metabolites in dog urine after bunolol-^{14}C administration

[3] Packard model 7201.
[4] Keuffel and Esser.
[5] Model 1670, HCL Scientific Inc., Rockford, Ill.
[6] Packard Instrument Co.
[7] Calbiochem.

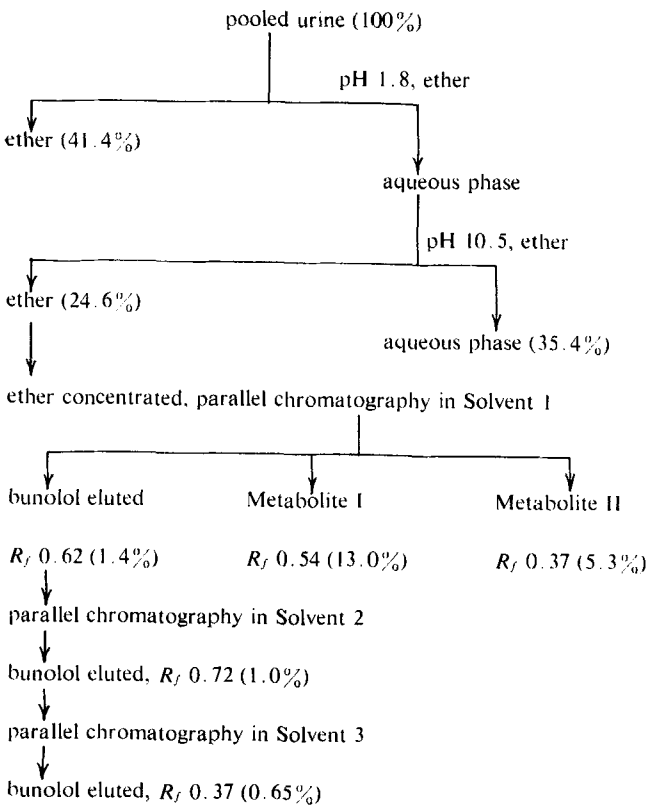
Table III—^{14}C Tissue Levels 72 Hr. after Oral Administration of Bunolol-^{14}C to Dogs

Tissue	Percent of Radioactive Dose		
	Dog 1	Dog 2	Dog 3
Brain	0.02	0.03	0.02
Fat[a]	0.02	0.03	0.01
GI tract, including contents	1.31	0.57	1.35
Heart	0.81	0.12	0.19
Kidney	0.03	0.46	0.03
Liver	0.55	1.13	0.76
Muscle[b]	0.18	0.11	0.02
Spleen	0.01	0.17	0.02
Total	2.95	2.62	2.40

[a] Samples assayed weighed 122 g. from Dog 1, 157 g. from Dog 2, and 87 g. from Dog 3. [b] Samples assayed weighed 123 g. from Dogs 1 and 2 and 102 g. from Dog 3.

ration to dryness, a few drops of methanol were added to obtain a clear solution which was streaked onto the central 12 cm. of five 20 × 20-cm. TLC plates.

Aliquots of a solution of authentic bunolol were spotted in the free areas flanking the urine extract. The plates were developed in Solvent 1, dried in air, and inspected under UV light to locate the reference bunolol. The band between the two bunolol spots was scraped from each plate. The scrapings from the five plates were pooled, and the radioactivity was extracted six times with 4-ml. portions of methanol. The pooled eluate represented 1.4% of the urinary ^{14}C. The concentrate was streaked as before onto a 20 × 20-cm. TLC plate. Synthetic bunolol was spotted in the free areas on both sides of the band, and the plate was developed in Solvent 2. Again the band that migrated parallel with bunolol was collected by scraping the plate and eluting with methanol. The eluate, now representing 1.0% of the urinary ^{14}C, was submitted to parallel chromatography in Solvent 3 as described previously. Elution yielded 0.65% of the urinary ^{14}C. When an aliquot of the final eluate was chromatographed in Solvent 1, the major radioactive peak migrated as bunolol and represented 73% of the ^{14}C in the



Scheme II—Purification of bunolol by solvent extraction and TLC

Table IV—Retention Times of Reference Bunolol and Bunolol Isolated from Dog Urine

Preparation	Retention Time, min.	
	Acetate (198°)[a]	Trifluoroacetate (173°)[a]
Reference bunolol	9.5[b]	11.2 (1.2)[c]
Urinary isolate	9.4 (1.7)[c]	11.1 (0.8)[c]

[a] Column temperature. [b] Average of 10 measurements; amounts injected ranged from 0.75 to 3.0 mcg. [c] Value in parentheses denotes amount (mcg.) injected.

sample. A slower migrating peak, possibly Metabolite I, was also present. Metabolite I, the major radioactive component in the ether extract (*cf.*, Scheme II) prepared from alkalinized urine, is as yet unidentified.

Identification of Bunolol in Urine—An entirely different technique was used to confirm the indication from TLC in three solvents that the urinary compound was bunolol. GLC was applied to two derivatives of bunolol. These derivatives were bunolol acetate and trifluoroacetate.

Bunolol acetate was synthesized and measured by injecting a solution of bunolol in acetic anhydride. Assay of the urinary bunolol was performed by injecting 3 μl. of a mixture containing the residue from the third chromatographic purification step, 20 μl. of acetic anhydride, and 10 mcg. of *p*-terphenyl, which served as internal standard, into the sample column. Into the reference column was injected 3 μl. of acetic anhydride 5 sec. earlier; this step stabilized the baseline.

The values of the retention time obtained with reference and urinary bunolol are in close agreement (Table IV). The amount of bunolol, represented by the area under the peak, was determined by chromatographing standard amounts of bunolol, ranging from 0.75 to 3.0 mcg./injection. A calibration curve was prepared by plotting the ratio of areas of bunolol to *p*-terphenyl. The retention time of *p*-terphenyl was 4 min. Duplicate measurements of the urinary sample indicated that 1.88 and 1.52 mcg. of urinary bunolol each were injected. The average value corresponded to 68 mcg. of bunolol eluted after the third chromatographic purification. Based on measurements of ^{14}C eluted and a purity of 73%, the amount of urinary bunolol at that purification state was 64 mcg. This correction is believed to be valid because it was found that Metabolite I would not produce peaks (0.8 mcg./injection) when injected in acetic anhydride solution.

Bunolol trifluoroacetate was evaluated by a different procedure. From a standard solution of bunolol, aliquots were transferred to 0.5-ml. conical reaction flasks equipped with Teflon-lined screw caps[8]. The solvent was evaporated to leave residues ranging from 20 to 200 mcg. of bunolol. A 50-μl. aliquot of trifluoroacetic anhydride (25% in methylene chloride)[9] was added to each residue. The flasks were capped quickly, and the reaction was allowed to proceed at room temperature for at least 40 min. Three-microliter aliquots of the various standards were injected onto the column. The calibration curve obtained indicated that the formation of the trifluoroacetate may serve as a sensitive and reproducible assay of bunolol. A suitable internal standard has not been found yet for this assay. The peak area relationship to bunolol concentration was selected over the peak height relationship because the experimental points fell closer to a straight line. The peak area of the urinary sample indicated that 0.78 mcg. of bunolol was injected. Assay of the sample for ^{14}C suggested 1.0 mcg. of bunolol, a value that was in reasonable agreement considering the volatility of trifluoroacetic anhydride.

A disadvantage of this method is the variability in the retention time. The values for the retention time increased markedly when smaller amounts of bunolol were employed. This difficulty was reduced by using an amount of reference bunolol of similar magnitude as that of the unknown. The retention times thus observed were 11 min. (Table IV). It is suggested that the observed variability of retention time in this assay is related to the adsorption of bunolol trifluoroacetate by the column.

[8] Kontes.
[9] Regis Chemical Co.

Recovery of Bunolol after TLC—To calculate the amounts of bunolol excreted in the urine, it was necessary to measure the recovery of bunolol after TLC. Therefore, varying amounts of bunolol-^{14}C, ranging from 2.5 to 50 mcg., were chromatographed. After scraping and elution, the amounts of ^{14}C were measured. Recoveries ranged from 81.6 to 95.5%. The extent of recovery appears to be unrelated to the amounts of bunolol employed. Based on an average recovery of 87.2%, and assuming identical recovery irrespective of the solvent, the corrected value for the amount of urinary bunolol is 0.71% of the urinary ^{14}C.

Presence of Conjugated Bunolol in Dog Urine—A 10-ml. sample of pooled urine was brought to pH 1.8 and extracted exhaustively with ether to remove unconjugated acids. The pH was raised to 10.5, and ether extractions were performed to remove unconjugated bases. The aqueous phase was then neutralized, freed of ether by aeration, and mixed with 10 μl. of an enzyme solution containing 1100 Fishman units of glucuronidase and 580 Fishman units of aryl sulfatase. After this mixture was incubated at 37° for 3.5 hr., an additional 10 μl. of the same enzyme solution was added, and the incubation was continued overnight. The incubation mixture was adjusted to pH 1.8 and extracted with ether to remove acidic aglycones. Then the pH was raised to 10.5 for ether extraction to collect the basic aglycones which might include bunolol. This latter extract was processed by TLC as described for the purification of unconjugated bunolol, but the radioactivity eluted after the third chromatographic purification corresponded to less than 0.2% of the urinary ^{14}C and did not permit identification.

DISCUSSION

Dogs quickly absorbed bunolol and established high blood levels of the drug and its metabolites. The present findings are consistent with an earlier observation of good oral absorption in a study of the cardiovascular pharmacology of bunolol (3). The blood concentration of bunolol and its metabolites reached a maximum within 1 hr. and slowly decreased to trace levels at 24 hr. Corresponding with this observation was the urinary excretion of most of the radioactivity within the first 24 hr. after treatment. These observations are comparable to the results obtained after ^{14}C-propranolol administration to dogs (9), although the bunolol dose was 10 times higher.

In terms of both intact drug and total radioactivity, the propranolol blood levels were maximal at 1 hr., and the drug was excreted completely in 48 hr. Following ^{14}C-bunolol administration, 16.2% of the radioactivity was passed into the feces in 72 hr. while 65.9% was excreted into the urine. Although no effort was made to recover radioactivity from the dog carcass, an additional 2.6% of the dose was found in target tissues, bringing the total mean recovery to 84.7%.

The sustained defecation of radioactivity from this readily absorbed drug and the relatively high levels of ^{14}C found in the GI tract and liver at sacrifice suggest extensive enterohepatic circulation of bunolol and its metabolites. Organs other than the liver and GI tract contained little radioactivity 72 hr. after bunolol administration. While 0.1–0.8% of the ^{14}C was detected in the heart, small amounts were also found in the brain (0.02–0.03%). Generally, the present tissue values for dogs are of an order of magnitude comparable to data reported in mice 24 and 96 hr. after administration of pronethalol (10).

Twenty-four hours after dosing dogs with bunolol-^{14}C (10 mg./kg. p.o.), 61% of the radioactivity was found in the urine. Approximately three-fourths of this radioactivity was distributed among 18 compounds, classified as unconjugated acids and bases and free acids and bases. The present study shows that only 0.7% of the urinary ^{14}C was excreted unchanged. The extensive and varied biotransformation of bunolol is reminiscent of the situation with pronethalol (10). Values of pronethalol content in urine of mouse, rat, and rabbit were 1.5, 0.3, and 0.7% of free pronethalol, whereas the values of pronethalol glucuronide were 1.7, 0.6, and 1.5%, respectively.

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Bunolol Metabolism by Dogs: Isolation and Identification of Two Acidic Metabolites

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Abstract □ The urine collected from three dogs for 24 hr. after oral administration of bunolol-^{14}C contained 61.2% of the administered radioactivity. An acidic metabolite, representing 16% of the urinary ^{14}C, was purified by ether extraction and preparative TLC. The methyl ester of the metabolite was prepared and submitted for analysis by mass spectrometry. The spectrum indicated the compound to be the methyl ester of 3-[(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)-oxy]lactic acid, and this compound was synthesized from 5-hydroxytetralone. The identity of the esterified urinary metabolite and the synthetic compound was established by mass spectrometry. A second unconjugated acid constituted 7% of the urinary ^{14}C. Its identity was established as [(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)-oxy]acetic acid by synthesis and comparative TLC. The 2,4-dinitrophenylhydrazones and the methyl esters of the metabolite and the synthetic material were prepared; comparison of their R_f values confirmed the identity of the metabolite.

Keyphrases □ Bunolol, metabolites—isolation, identification, dog urine □ β-(5-Oxytetralonyl)lactic acid—isolation, identification, bunolol metabolite, dogs □ (5-Oxytetralonyl)acetic acid—isolation, identification, bunolol metabolite, dogs □ TLC—isolation, identification □ Mass spectroscopy—identification

A new, highly potent, β-adrenergic blocking agent, bunolol {*dl*-5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride}, was metabolized extensively following oral administration to dogs (1). Urine collected for 24 hr. following bunolol-^{14}C administration contained only 0.7% of the urinary radioactivity as the unaltered drug. An experimental survey of the different classes of bunolol metabolites indicated the presence of as many as 18 urinary metabolites. Among these metabolites were three unconjugated and five conjugated bases and four unconjugated and six conjugated acids. The present report describes the isolation and identification of two acidic metabolites of bunolol from dog urine; β-(5-oxytetralonyl)lactic acid and (5-oxytetralonyl)acetic acid.

MATERIALS AND METHODS

^{14}C-Labeled Bunolol—Bunolol was synthesized with ^{14}C in position 1 (carbonyl-C) of the saturated ring (2). The preparation was 99.0% pure, both chemically and radiochemically, as judged by TLC; it had a specific activity of 4.90 mc./g.

Radioactivity Counting—Quantitative assays for ^{14}C were performed using a liquid scintillation spectrometer[1]. The external standardization method was used for quench corrections.

TLC—Chromatograms for analytical purposes were run on 5 × 20-cm. glass plates coated with 250 μ of silica gel G bound with calcium sulfate. For preparative efforts, 20 × 20-cm. plates were used. One-dimensional chromatograms were developed using the following solvents: 1, *n*-butanol–29.6% ammonia–water (4:1:3, upper phase); 2, *n*-butanol–glacial acetic acid–ether–water (9:6:3:1); 3, *n*-butanol–glacial acetic acid–ether–water (6:3:9:1); 4, methanol–29.6% ammonia–water (12:1:7); 5, methanol–ethyl acetate (1:4); 6, heptane–chloroform–ethanol (1:3:1); 7, chloroform–glacial acetic acid–methanol (15:1:4); and 8, chloroform–glacial acetic acid–methanol (54:1:5). TLC plates were scanned for radioactivity with a radiochromatogram scanner[2]. The area under each peak was determined with a compensating polar planimeter[3]. In this manner, R_f values and relative amounts of each metabolite were determined.

Mass Spectrometry Studies—A mass spectrometer[4] with a direct insertion probe was used. The source temperature was 140–160°, the ionizing energy was 70 ev., and the trap current was 100 μamp. Exact masses were determined by peak matching.

Synthesis of Metabolites—The synthesis of Metabolite V was carried out in an unambiguous manner from 5-hydroxytetralone, sodium hydroxide, and β-chlorolactic acid, utilizing a general method reported previously (3). The synthesis of Metabolite VI was performed similarly, using α-bromoacetic acid in place of β-chlorolactic acid. Mass spectra were taken of these compounds.

Preparation of Methyl Esters of Urinary and Synthetic Metabolites—The procedure of Metcalfe and Schmitz (4) was applied to portions of the urinary and synthetic metabolites. Samples of the metabolites, purified by preparative TLC (40 mcg. to 10 mg.), were dissolved in 0.5 ml. of methanol and combined with 1 ml. of a 14% solution of boron trifluoride in methanol[5]. After boiling for 5 min., the methanol was allowed to evaporate to approximately 0.2 ml., and 5 ml. of distilled water was added to the reaction mixture. The methylated compounds were extracted three times with ether. The ether was evaporated and, for identification by TLC, the residues were dissolved in 0.2–1 ml. of methanol.

Preparation of 2,4-Dinitrophenylhydrazone of Metabolite VI—Aliquots of urinary and synthetic Metabolite VI were diluted to 0.5 ml. with ethanol. A slight excess of a solution of 2,4-dinitrophenylhydrazine in ethanol and 20 μl. of 12 *N* HCl were added. Each mixture was boiled for 30 min. in a water bath with occasional ethanol addition to compensate for evaporative losses. Then aliquots of the reaction mixture were chromatographed in parallel with the 2,4-dinitrophenylhydrazones of the synthetic compounds.

Protocol—Twenty-milligram portions of bunolol-^{14}C were weighed into three gelatin capsules, and enough carrier bunolol was added to provide 10-mg./kg. doses. The capsules were administered to female beagles weighing: Dog 1, 11.5 kg.; Dog 2, 11.5 kg.; and Dog 3, 13.0 kg. The dogs had free access to food and water. Urine collected from 0 to 24 hr. from each dog was kept frozen until needed for investigation. The 0–24-hr. urine samples from the three dogs were pooled before investigation and were identical with those used to determine the extent of bunolol excretion (1).

Purification and Esterification of Metabolite V—To obtain Metabolite V, a 390-ml. aliquot of urine, containing 94-mg. equivalents of bunolol, was adjusted to pH 1.8 and extracted seven times with one-half volumes of ether. The concentrated ether extract was chromatographed using Solvent 7. A band, 26 mm. wide at R_f 0.66, contained most of the ^{14}C; it was scraped from the plate and eluted with methanol. The concentrate was rechromatographed using Solvent 1. The 17-mm. radioactive band at R_f 0.30 was scraped and eluted similarly. The concentrated eluate was again chromatographed in Solvent 7. The radioactive band (16 mm. wide, R_f 0.57) was scraped from the plates. An aliquot of the eluted metabolite was methylated by the boron trifluoride method (4). The methyl ester was chromatographed using Solvent 1. A radioactive band (16 mm. wide, R_f 0.73) was scraped off the plates.

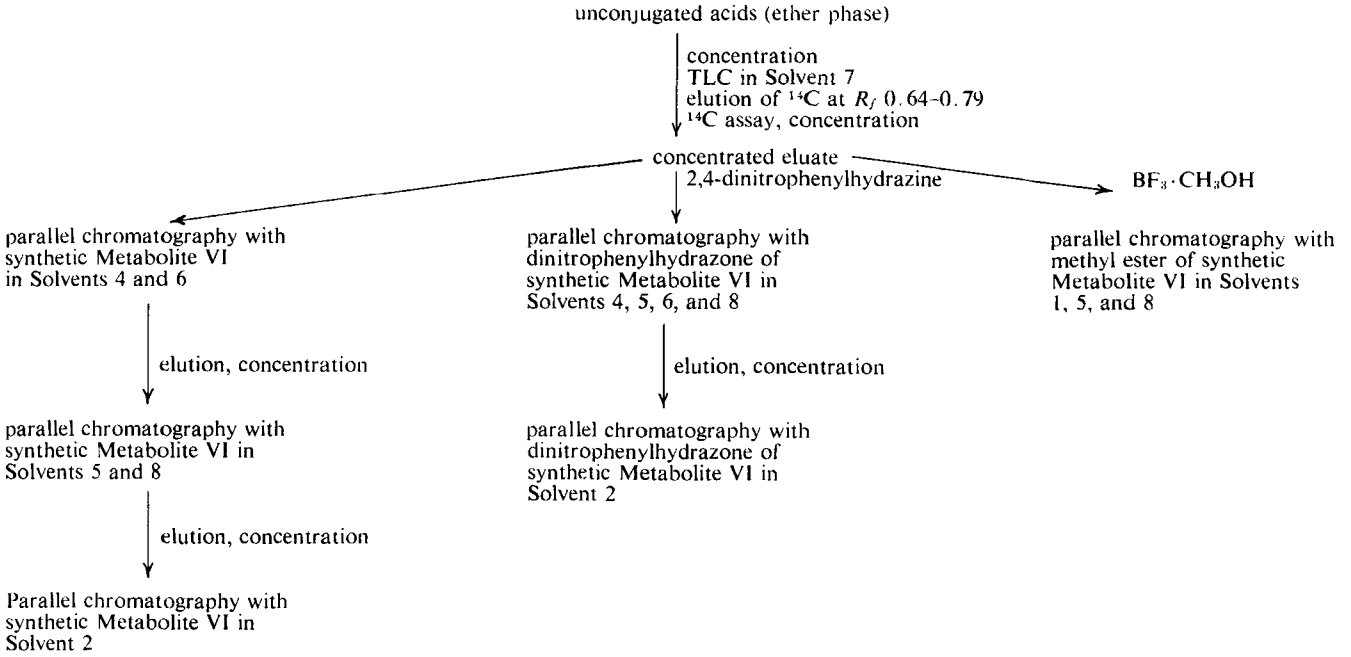
[1] Packard Tri-Carb model 3320.

[2] Packard model 7201.

[3] Keuffel and Esser.

[4] AEI model 902.

[5] Applied Science Laboratories, State College, Pa.



Scheme I—Chromatographic steps used to identify Metabolite VI in dog urine. Parallel chromatography refers to running two preparations side by side on the same plate. Parallel chromatography (as distinguished from cochromatography in which spotting is superimposed) permitted elution of only the radioactive material at the R_f of interest.

The radioactivity was eluted (total 3.77×10^6 d.p.m.), and the eluate was reduced to dryness. After drying *in vacuo* for 3 days, the sample weighed 3.0 mg. UV and mass spectra were taken on this preparation.

Quantity of Metabolite V in Dog Urine—A 56-ml. aliquot of the pooled urine was adjusted to pH 1.8 and extracted with ether. The ether phase contained 41.4% of the urinary radioactivity. An aliquot of the concentrated extract, containing 21,000 d.p.m., was chromatographed in Solvent 7. Radioactivity migrating in parallel to the synthetic compound was eluted and found to represent 41.8% of the ^{14}C in the ether extract. The eluate was concentrated and rechromatographed in Solvent 1. Most of the radioactivity (90.9%) moved parallel to the synthetic compound. The radioactivity was again eluted and rechromatographed in Solvent 2.

Search for Conjugated Metabolite V—For this purpose, the acidified urine after ether extraction was adjusted to 10.5. After extraction with ether to remove unconjugated bases, the aqueous phase was aerated to remove traces of ether. After adjusting the pH to 6.6, 0.46 ml. of a preparation containing 44,000 Fishman units of β-glucuronidase and 23,000 Fishman units of aryl sulfatase[6] was added. Following 16 hr. of incubation, an additional 0.46 ml. of the enzyme preparation was added, and the incubation was continued for 7 hr. Then the pH was adjusted to 1.8, and the solution was extracted three times with two volumes of ether. The ether was evaporated and the residue was taken up in methanol. This fraction represented 4.4% of the urinary ^{14}C. Aliquots of this sample were streaked onto 20 × 20-cm. TLC plates, which were developed previously in methanol and heated in an oven at 140° for 1–2 hr. After development in Solvent 7 and drying, a 5-cm. strip was cut from each plate and the ^{14}C was located by radioscanning. A radioactive band, R_f 0.54, representing 55% of the ^{14}C due to the conjugated acids, was scraped from the plate and eluted with methanol. Preparative TLC was applied to the eluate using Solvent 1. A radioactive band, R_f 0.24, represented 35% of the ^{14}C present on the plate. Chromatography of the eluate from this purification step with synthetic Metabolite V in Solvents 7 and 3 indicated that 74 and 73% of the ^{14}C, respectively, migrated as one peak parallel to the synthetic compound. This metabolite corresponded to 0.6% of the urinary radioactivity. The metabolite thus isolated was chromatographed in four solvents side by side with synthetic Metabolite V.

[6] Calbiochem.

Isolation of Metabolite VI from Urine—The chromatographic procedure used to isolate Metabolite VI is outlined in Scheme I. A 1-ml. aliquot from the urine pool used for the purification of Metabolite V was adjusted to pH 10.5 and extracted three times with 4 ml. of ether. The aqueous layer was adjusted to pH 1.8 and again extracted three times with ether. The ether extract was concentrated, and aliquots were chromatographed in Solvent 7. One radioactive peak, R_f 0.7, was noted to be similar to that of a reference compound, [(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)oxy]acetic acid. The chromatography of the ether extract was, therefore, carried out side by side with this synthetic compound, designated tentatively as synthetic Metabolite VI. The radioactive peak, corresponding to that of synthetic Metabolite VI (R_f 0.64–0.79), was scraped off the plate and eluted. The concentrate of this eluate was rechromatographed in parallel with the synthetic Metabolite VI in Solvents 4 and 6. Both chromatograms yielded single radioactive peaks which chromatographed side by side with synthetic Metabolite VI. The radioactive peaks were eluted from both chromatograms. The pooled eluate was concentrated and rechromatographed in parallel with synthetic Metabolite VI in Solvents 5 and 8. Most of the radioactivity migrated the same distance as the synthetic Metabolite VI. The elution of the tagged metabolite was repeated, and the pooled concentrate was rechromatographed in Solvent 2. Only one radioactive peak was found.

Quantity of Metabolite VI in Dog Urine—To estimate the quantity of urinary Metabolite VI, an aliquot of the pooled urine was acidified and extracted with ether. The ether phase was the same sample as that employed for estimating the quantities of urinary Metab-

Table I—Purification Scheme for Metabolite V

Purification Step	Recovery of Radioactivity, d.p.m. × 10^6
Unfractionated urine	171.28
Ether extract (pH 1.8)	50.91
Eluate from TLC, Solvent 7	28.74
Eluate from TLC, Solvent 1	19.18
Eluate from TLC, Solvent 7	8.47
Esterified sample: eluate from TLC, Solvent 1	5.89[a]

[a] Corrected for aliquot taken at previous step.

Table II—Molar Absorptivities of the Methyl Ester of Metabolite V and Bunolol (Free Base)

Compound	Molar Absorptivity[a] (ϵ) at 221 nm.	253 nm.	310 nm.
Metabolite V methyl ester	35,700	10,900	2,600
Bunolol	25,800	9,400	2,500

[a] Solutions employed were 0.06 m*M* in methanol.

Table III—R_f Values of Metabolite V and the Synthetic Compound

Solvent	Metabolite V[a]	Synthetic Compound[b]	Methyl Esters: Metabolite V[a]	Methyl Esters: Synthetic Compound[b]
1	0.32	0.28	0.76	0.76
5	0.02	0.11	0.73	0.75
7	0.50	0.51	0.89	0.87

[a] Detection by radioscanning. [b] Detection by fluorescence under UV ight.

olite V. The volume was reduced to near dryness, and a few drops of methanol were added. The relative amount of Metabolite VI was determined by repeatedly chromatographing this sample side by side with the synthetic sample, with subsequent determination of the relative peak area and elution.

RESULTS

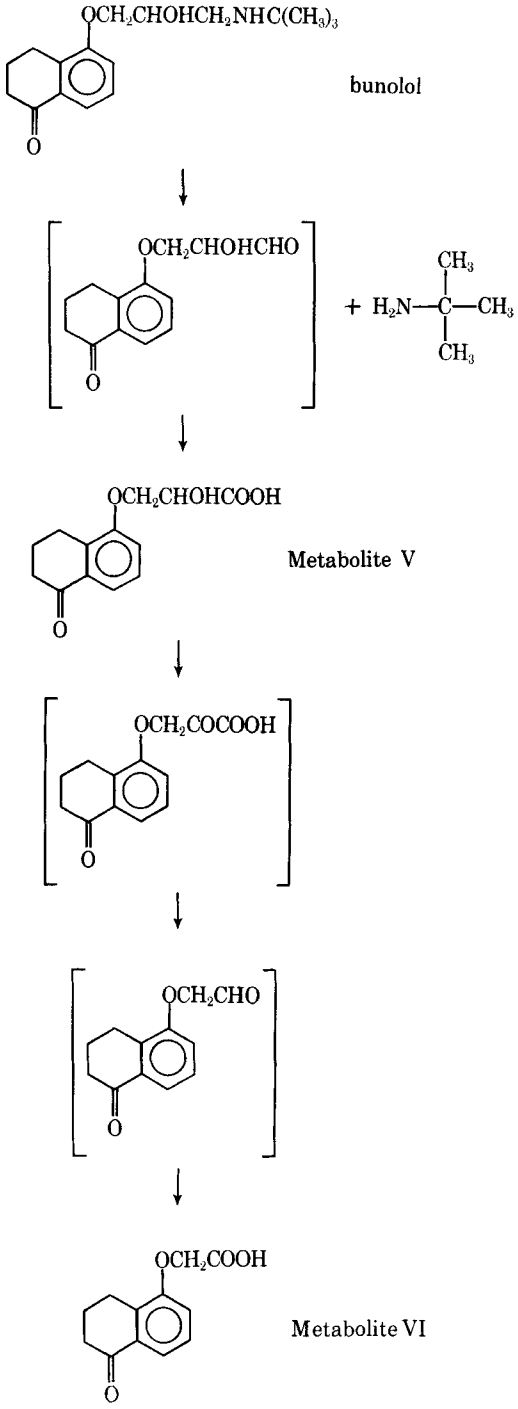
Isolation and Identification of Metabolite V—Table I shows the recoveries at various stages of the isolation of esterified Metabolite V from the urine of dogs treated with bunolol. From the specific radioactivity of bunolol (528,000 d.p.m./μmole), the 3.0 mg. of isolated methyl ester was judged to be approximately 64% pure. When a sample of the free acid was rechromatographed to the point of radiochemical purity, it was found to represent 15.7% of the urinary radioactivity.

The method used to purify Metabolite V was based upon its acidic character, a point confirmed by synthesizing the methyl ester of the metabolite. As indicated in Table II, the UV absorption of the methyl ester was not greatly different from the spectrum of bunolol; this observation suggested that the tetralone ring system was intact.

The mass spectrum of the methyl ester of Metabolite V showed the presence of peaks at *m/e* 264 and 161. The *m/e* 161 peak represented the 5-oxytetralone fragment. The precise mass of the derivatized metabolite was *m/e* 264.101. The presence of the 5-oxytetralone moiety and the methyl ester group suggested that the urinary isolate was β-(5-oxytetralonyl)lactic acid methyl ester ($C_{14}H_{16}O_5$), which has a calculated precise mass of 264.100. After this lactate was synthesized, its mass spectrum was taken and found to exhibit peaks at *m/e* 161 and 264, which were in the same intensity ratio as for esterified urinary Metabolite V. The synthetic compound showed fewer peaks than the urinary isolate, indicating contamination of the latter. The precise mass of the molecular ion of the synthetic ester was *m/e* 264.098. The agreement of the values for the molecular ions of the urinary and the synthetic samples indicates their identity. This identification was confirmed by parallel chromatography of Metabolite V and the synthetic lactic acid and of the methyl esters of both (Table III).

Table IV—R_f Values of an Acid Metabolite Isolated from the Fraction Containing the Conjugated Acid Metabolites; R_f Values of the Metabolite and Its Methyl Ester are Compared with Those of Synthetic Metabolite V and Its Methyl Ester

Solvent	Urinary Metabolite	Synthetic Metabolite V	Methyl Ester: Urinary Metabolite	Methyl Ester: Synthetic Metabolite V
1	0.26	0.30	0.81	0.77
3	0.74	0.71	—	—
5	0	0.08	0.85	0.83
7	0.40	0.43	0.89	0.85



Scheme II

The effort to establish the presence of a glucuronide of Metabolite V in dog urine ended equivocally. Although the urinary aglycone and its methyl ester migrated as their synthetic counterparts in several TLC systems (Table IV), the recovery was so low (0.6% of the urinary ^{14}C) that it might represent unconjugated Metabolite V attributable to incomplete extraction with ether.

Isolation and Identification of Metabolite VI—Metabolite VI was found in the acidic fraction, and its carboxylic character was confirmed by esterification. The presence of an active carbonyl group in the molecule was evident from 2,4-dinitrophenylhydrazone formation. The metabolite was identified as [(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)oxy]acetic acid on the basis of comparative TLC with the synthetic compound and by comparative TLC of its methyl

Table V—R_f Values of Urinary and Synthetic Metabolite VI and the Corresponding 2,4-Dinitrophenylhydrazones and Methyl Esters

Solvent	Urinary Metabolite VI[a]	Synthetic Metabolite VI[b]	2,4-Dinitrophenylhydrazones: Urinary Metabolite VI[a]	2,4-Dinitrophenylhydrazones: Synthetic Metabolite VI[c]	Methyl Esters: Urinary Metabolite VI[a]	Methyl Esters: Synthetic Metabolite VI[b]
1	—	—	—	—	0.75	0.72
2	0.90	0.90	0.97	0.96	—	—
4	0.91	0.92	0.05	0.0	—	—
5	0.25	0.22	0.86	0.83	0.75	0.75
6	0.04	0.04	0.78	0.84	—	—
8	0.47	0.51	0.95	0.94	0.85	0.82

[a] Detection by radioscanning. [b] Detection by UV fluorescence or iodine vapors. [c] Detection by its yellow color.

ester and 2,4-dinitrophenylhydrazone with the corresponding derivatives of the synthetic acid (Table V).

Radioactivity, migrating in parallel with synthetic Metabolite VI in Solvent 7, was eluted and found to represent 17.1% of the radioactivity in the ether extract. The eluate was concentrated and rechromatographed in Solvent 1. Only one radioactive peak was noted. After elution, rechromatography in Solvent 2 indicated that the metabolite was radiochemically pure. Urinary Metabolite VI thus represents 7.1% of the urinary radioactivity.

Search for Conjugated Metabolite VI—A thorough examination disclosed no Metabolite VI in the ether fraction containing the aglycones of conjugated acidic metabolites.

DISCUSSION

The two unconjugated acid metabolites found in dog urine bear a close structural relationship. It appears likely that Metabolites V and VI are linked by a common pathway, as shown in Scheme II.

The initial attack upon bunolol is considered to be oxidative dealkylation of the side chain with the formation of tertiary butylamine and a lactic aldehyde. Oxidation of the aldehyde intermediate would yield Metabolite V. We are postulating that Metabolite VI may be formed by three subsequent reactions: (*a*) oxidation of the secondary alcohol to carbonyl, (*b*) decarboxylation of the α-keto acid to an aldehyde, and (*c*) oxidation of the aldehyde to a carboxyl group.

Metabolism studies with propranolol and pronethalol also revealed the formation of carboxylic end-products resulting from oxidative attacks upon the side chains. Hydroxy acids were the principal urinary metabolites from both of these β-adrenergic blocking agents. Propranolol was converted into a naphthoxylactic acid analogous to bunolol Metabolite V in man (5, 6) and in four rodent species (6). Similarly, pronethalol yielded 2-naphthylglycollic acid in man and three rodent species (7). Subsequent oxidative decarboxylation shortened the side chain of pronethalol (7) in the manner observed to produce Metabolite VI from bunolol, and this observation leads us to consider that the preceding sequence of bunolol degradation observed in the dog may occur in other animal species and in man.

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Effects of Dwell Time, Volume of Dialysis Fluid, and Added Accelerators on Peritoneal Dialysis of Urea

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Abstract □ The effects of variables of procedure in peritoneal dialysis on the urea clearances obtained were evaluated by means of a full factorial experiment. Three levels of each variable (dwell time, dialysis rate constant *via* additives in the solutions, and dialysis fluid volume) were used in all combinations. Results were subjected to an analysis of variance, which showed each variable to have a significant effect on clearance. Only one two-factor interaction was found to be significant. The applicability of basic kinetic equations for prediction of urea clearances in peritoneal dialysis was demonstrated by computing clearances for the same conditions as used in the experiment.

Keyphrases □ Peritoneal dialysis of urea—effects of dwell time, volume of dialysis fluid, added accelerators, statistical analysis □ Urea—peritoneal dialysis, effects of variables in procedure, statistical analysis □ Dialysis, peritoneal—statistical analysis of effects of procedure variables

Peritoneal dialysis is an effective method used to remove endogenous poisons and to treat symptoms of uremia. The procedures used, however, vary with the different clinics and clinicians, and there seems to be little substantial evidence by which one might select the best operating conditions.

Some details of the dialysis procedure have been examined and specific recommendations have been made. Fine *et al.* (1) studied continuous dialysis, varying the rate of flow of dialysis fluid on the individual patient, and concluded that maximum clearance was obtained at a flow rate of 40–60 ml./min. Boen (2) studied the clearance of urea as a function of the rate of inflow of dialysis fluid and obtained a maximum clearance at an inflow of 3.5 l./hr. (58 ml./min.). Similar maxima were obtained at approximately the same flow rates for several other substances, including creatinine, uric acid, potassium, magnesium, phosphate, and sulfate. Boen (2) recognized that the apparent fall in clearance above 3.5 l./hr. might be an artifact due to a small number of measurements. Miller *et al.* (3) compared several techniques for peritoneal dialysis, including one procedure with a 10-min. period for the introduction of the fluid, zero dwell time, and a 15-min. drainage period. This procedure gave no greater clearance than the control method with a 25-min. dwell time. Other techniques, with inflows up to 100 ml./min., gave only modest improvements. Gross and McDonald (4) found the temperature of the dialysis fluid to be important; increases of 35% in urea clearance were obtained by warming the fluid to 37°.

The general tendency has been to use a volume of dialysis fluid of 2 l. without question, presumably because this amount is convenient and not large enough to cause patient discomfort. The time the fluid is allowed to remain in the abdomen varies with the clinician, usually from 30 min. to 1 hr., although shorter periods are sometimes encountered.

The question of choice of operating variables is of particular concern when substances are evaluated for acceleration of dialysis. For example, a substance that accelerates diffusion across the membrane would be expected to give the same clearance as the control if the dwell times were long enough for the establishment of equilibrium. Conversely, the shorter the dwell time, the more effective the accelerator might appear to be.

In selecting the volume of dialysis fluid to be used for each exchange, it would appear that the larger the volume the greater would be the clearance, since dialysis across the membrane is a function of concentrations in the two compartments and a larger volume would yield a larger amount.

Typical unanswered questions arise: Is it better to use a small volume of fluid and exchange it more rapidly or to use a large volume with longer dwell time? Is there an optimum time–volume ratio? Would there be a significant advantage in using a more rapid inflow and outflow of dialysis fluid?

Many questions concerning techniques involve more than one factor and represent, in statistical terms, questions about two-factor interactions. As a first attempt to answer such questions, a factorial experiment was run on the removal of urea by peritoneal dialysis. The full factorial design enables one to evaluate both the main effects of variables and the interactions of two factors at a selected number of levels.

It was thought also to be of value to determine whether the process of dialysis might be predicted by mathematical expressions; to this end, the same experiments were "run" on the computer.

EXPERIMENTAL

Selection of Variables—Three levels of each variable were selected to cover as broad a range as possible. Volumes of dialysis fluid were: V_1, 35 ml./kg.; V_2, 65 ml./kg.; and V_3, 100 ml./kg. Dwell times were: D_1, 0 min.; D_2, 30 min.; and D_3, 60 min. Accelerators were selected on the basis of previously evaluated rate constants (5); they were: K_1, control fluid consisting of dextrose (1.5%), sodium lactate (0.5%), sodium chloride (0.56%), calcium chloride (0.026%), and magnesium chloride hexahydrate (0.015%) in distilled water, with an apparent dialysis constant of 0.01 min.$^{-1}$; K_2, control fluid with 0.01% cetyltrimethylammonium chloride added, with an apparent constant of 0.026 min.$^{-1}$; and K_3, control fluid with 0.01% dioctyl sodium sulfosuccinate added, with an apparent constant of 0.041 min.$^{-1}$. All combinations of three levels of the three variables, V, D, and K, were tested, comprising a full factorial experiment of 3^3 or 27 experiments.

Intermittent Dialysis Procedure—Healthy, mature male rabbits were used, with no general anesthesia but with a 50-mg. intramuscular dose of chlorpromazine hydrochloride 1 hr. prior to the experiment. The animal was weighed, a urinary catheter was inserted, and 400 mg./kg. of ^{14}C-tagged urea was injected intravenously. This injection consisted of 4.7 mg. of ^{14}C-urea (specific activity 4.65 mc./mmole) plus 2 g. urea and 0.9 g. benzyl alcohol in sufficient water for injection to make 100 ml. One hour

Table I—Observed Clearances with Variations in Fluid Volume, Dwell Time, and Added Accelerators[a]

Set 1 Conditions	Set 1 Clearance, ml./min./kg.	Set 2 Conditions	Set 2 Clearance, ml./min./kg.	Set 3 Conditions	Set 3 Clearance, ml./min./kg.
$V_2D_2K_2$	1.188(0.873)	$V_2D_3K_2$	0.562(0.591)	$V_3D_2K_2$	1.417(1.343)
$V_3D_3K_2$	1.108(0.910)	$V_1D_2K_2$	0.594(0.470)	$V_3D_3K_3$	1.211(0.944)
$V_3D_2K_1$	0.684(0.736)	$V_2D_2K_1$	0.644(0.478)	$V_2D_3K_1$	0.455(0.417)
$V_2D_3K_3$	0.771(0.613)	$V_3D_2K_3$	1.596(1.552)	$V_2D_2K_3$	1.031(1.009)
$V_1D_3K_1$	0.300(0.224)	$V_3D_3K_1$	0.635(0.642)	$V_2D_1K_2$	1.533(0.869)
$V_1D_2K_3$	0.613(0.543)	$V_1D_3K_3$	0.362(0.330)	$V_1D_2K_1$	0.397(0.257)
$V_2D_1K_1$	0.532(0.350)	$V_2D_1K_3$	1.900(1.305)	$V_1D_1K_3$	1.102(0.703)
$V_3D_1K_3$	1.613(2.007)	$V_3D_1K_2$	1.756(1.338)	$V_3D_1K_1$	0.517(0.538)
$V_1D_1K_2$	0.770(0.468)	$V_1D_1K_1$	0.393(0.188)	$V_1D_3K_2$	0.453(0.318)
		Averages			
V_1	0.554(0.389)	D_1	1.124(0.863)	K_1	0.506(0.426)
V_2	0.957(0.723)	D_2	0.907(0.807)	K_2	1.042(0.798)
V_3	1.171(1.112)	D_3	0.651(0.554)	K_3	1.133(1.001)

[a] Computer-predicted values in parentheses.

was allowed for distribution of the exogenous urea throughout the body fluids; then the first dialysis fluid was introduced into the peritoneum *via* a previously inserted pediatric size peritoneal catheter. The dialysis fluid was injected in a few seconds by use of 50-ml. syringes filled in advance. The fluid was allowed to remain in the peritoneum for the prescribed dwell time; then drainage was started, allowing the fluid to drain through a 1-m. length of plastic tubing which hung down from the operating table into a graduated cylinder. In all cases, the drainage time was 10 min. Blood samples were taken from the marginal ear vein at the time of introduction of each dose of peritoneal fluid and at the end of the experiment. The volume of dialysate returned was noted in each instance, the fluid was mixed thoroughly, and a sample was taken for measurement. Repeated dialyses were performed over approximately 3 hr., thus utilizing 18 exchanges for 0-min., 5 for 30-min., and 3 for 60-min. dwell times.

Measurement of ^{14}C in Samples—One-half-milliliter samples of plasma and 1-ml. samples of dialysate were pipeted into counting vials, 10 ml. of counting fluid was added directly, and the vials were counted on a liquid scintillation counter, using the channels ratio method for quench corrections. The counting fluid consisted of a mixture of 2,5-diphenyloxazole, 4 g./l., and 1,4-bis-2(5-phenyloxazolyl)benzene, 0.3 g./l., in a solvent of 2 parts toluene and 1 part Triton X-100[1]. Results were calculated in terms of concentration, micrograms per milliliter, of exogenous urea in the sample.

Treatment of Data—Clearance values were selected to express results, since these values are customarily used in the clinic. For each exchange of dialysis fluid, the urea clearance was calculated by the well-known formula:

$$\text{clearance (ml./min.)} = \frac{UV}{P} \qquad \text{(Eq. 1)}$$

where U is the concentration of urea in the dialysate, V is the volume of dialysate collected divided by the cycle time (*i.e.*, the dwell time plus drainage time), and P is the average plasma concentration (mean of samples at beginning and end of the cycle). For each experiment, the clearances for individual cycles were averaged and divided by the weight of the animal to obtain values of milliliters per minute per kilogram. The clearances would be expected to vary with animal weight since the volume of dialysis fluid was determined on basis of weight.

Computer Predictions—Computer calculations used the equations of Rescigno and Segre (6) for plasma and dialysate concentrations at a given time:

$$X_1 = \frac{X_0}{m_2 - m_1}\left[(k_2 - m_1)e^{-m_1 t} + (m_2 - k_2)e^{-m_2 t}\right] \qquad \text{(Eq. 2)}$$

$$X_2 = \frac{X_0 k_1}{m_2 - m_1}\left(e^{-m_1 t} - e^{-m_2 t}\right) \qquad \text{(Eq. 3)}$$

where X_1 is the plasma level at time t, X_0 is the initial plasma level at the beginning of dialysis, $k_1 = k_2$ = dialysis rate constant, m_1 and m_2 are the roots of the auxiliary equation, and X_2 is the dialysate concentration. For this purpose, an average plasma level of 450 mcg./ml. was used as X_0, k_1 was taken as equal to k_2 since free diffusion is assumed in both directions, and an average value of excretion constant from previous experiments (0.0002 min.$^{-1}$) was taken as k_3. The values of m_1 and m_2 were calculated from the equations:

$$m_1 = \frac{q - \sqrt{q^2 - 4k_2k_3}}{2} \qquad \text{(Eq. 4)}$$

$$m_2 = \frac{q + \sqrt{q^2 - 4k_2k_3}}{2} \qquad \text{(Eq. 5)}$$

where $q = k_1 + k_2 + k_3$.

First the plasma level at the beginning and end of each cycle was computed. Then the dialysate concentration at the end of each minute of drainage was calculated, and this value was multiplied by one-tenth the dialysis fluid volume to predict the amount of urea removed each minute of drainage. These values were accumulated over the 10-min. period to get the total amount removed. Finally, the clearance was calculated. The process could be repeated over a number of exchanges, but a single cycle gave the same result. The computer program assumed perfect drainage at a constant rate of one-tenth the volume per minute and instantaneous injection of peritoneal fluid. Changes in the urinary excretion constant over a broad range had little effect on predicted dialysis clearance values.

RESULTS

The average clearance values observed and those predicted by the equations are presented in Table I; an analysis of variance of the observed data is shown in Table II.

The experiments demonstrated that shorter dwell times significantly increased the clearance values, zero dwell time giving nearly double the average clearances obtained with 60-min. dwell periods.

The volume of dialysis fluid used in each exchange also had a pronounced effect, with the highest volume giving nearly double the

Table II—Analysis of Variances, Observed Clearances

Source	Sum Squares	*df*	Mean Squares	*F*
Volume, V	1,767,314	2	883,657	39.30[a]
Dwell time, D	1,010,060	2	505,030	22.46[a]
Accelerator, K	2,065,639	2	1,032,820	45.94[a]
$V \times D$	166,154	4	41,539	1.85
$V \times K$	318,820	4	79,705	3.54
$D \times K$	511,204	4	127,801	5.68[b]
Subtotal	5,839,191	18		
Total	6,019,065	26		
Residual	179,874	8	22,484	
Estimate of standard error = 0.150 ml./min./kg.				

[a] Significant at 1% level. [b] Significant at 5% level.

[1] Palmetto Chemical and Supply Co.

clearance of the lowest. The highest volume would, of course, be impractical for man, requiring 6 l. for a 60-kg. man. Whether any increase in volume over the 2 l. commonly used would be practical remains for the clinician to determine, but any significant increase would be useful.

The added accelerators were quite effective in increasing average clearances, with the intermediate giving double the clearance of controls. The fact that accelerators were effective regardless of dwell time and fluid volume points to the potential value of such agents. Since dwell time can be reduced no further and the volume of dialysis fluid cannot be increased beyond certain limits due to patient discomfort, the use of accelerators may be the most promising approach to the improvement of clearances.

The effects of D, V, and K were not linear; in each case, the difference was greater between the least effective and intermediate levels than between the intermediate and most effective levels.

With a few exceptions, the clearances observed followed the trends predicted by the application of the equations. The exceptions were chiefly failure to predict as high a clearance as was observed with zero dwell time. This result is thought to be due to the time required to introduce the dialysis fluid and start drainage, thus allowing for slightly more extended drainage times than intended. Also, since there was no control over the rate of outflow of the fluid, some errors would result from the assumption of constant drainage rate.

In a majority of instances, the equations predicted clearances lower than those observed. A better fit of the equations to the data could, no doubt, be made, but this was not the intent of the calculations; rather, it was the purpose to use K values from previous data and see how well the equations would predict clearances under a variety of conditions. Some difficulties of the experimental technique make it impractical to have actual conditions follow the assumptions required for the computations, thus making it prudent to utilize predictive equations for estimating relative values or trends rather than attempt to obtain accurate values for a given set of conditions.

It was surprising to find little evidence of interactions between factors, although it was pleasing to find such clear main effects. When the experiment was planned, it was thought that an accelerator might be useful only with short dwell times, which would appear as a $D \times K$ interaction. This effect was, indeed, detected but was significant only at the 5% level. The interpretation of this interaction is that the accelerator effect was greater at short dwell times (at $D = 0$ min., $K_2/K_1 = 3$; at $D = 60$ min., $K_2/K_1 = 2$), although the accelerator effect was significant at all dwell times. Since there were no duplicates of individual combinations of factors in this experiment, the three-factor interaction cannot be evaluated.

DISCUSSION

It was of interest to examine, by means of equations, the puzzling finding of Boen (2) that a maximum clearance is attained at dialysate flow rates of 3.5 l./min. For the lower flow rates, Boen used a 10-35-15 schedule, where the first number indicates the time in minutes for introduction of fluid, the second indicates the dwell time, and the third is the drainage time. For the higher flows, he apparently used a 10-5-15 schedule, thus having two exchanges of fluid in an hour.

By using Eqs. 2 and 3 and k's from the animal data as before, the concentrations in dialysate during introduction of fluid were calculated and the overall concentration upon complete introduction was estimated. This value was then referred to calculated concentrations which would have been obtained had the entire volume been introduced instantaneously; the corresponding time was taken as the adjusted time by which to calculate later dialysate concentrations. It was found that the 10-min. introduction period at a constant rate of inflow was equivalent to a 6-min. period if all fluid has been introduced at once. By using these adjusted time values, the amount removed in the dialysate was calculated as before.

By using 60 ml./kg. of dialysis fluid and assuming a 60-kg. man, the clearance predicted with the 10-35-15 schedule was 22.6 ml./min.; with the 10-5-15 schedule, it was 21.2 ml./min. These relative values agree with the observations of Boen (2) and demonstrate that under these conditions the clearance may be expected to decrease in spite of increased flow of dialysis fluid. It also shows that the reason for the decrease at higher flow rates is the greater amount of time involved in introducing and draining the fluid.

If the dialysis fluid can be injected in 1 min. and drained in 10, giving a 0-20-10 schedule, the predicted clearance would be 30 ml./min.; for a 0-50-10 schedule, it would be 25 ml./min.; for a 0-0-10 schedule, it would be 15 ml./min. Thus, even with instantaneous introduction of fluid, a maximum would be found due to the finite time required for drainage. By use of this type of calculation, it should be possible to estimate optimum operating conditions for whatever introduction times or drainage times one might find convenient.

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Polycarboxylic Acid Ion-Exchange Resin Adsorbates for Taste Coverage in Chewable Tablets

SAUL BORODKIN and DEAN P. SUNDBERG

Abstract □ High potency adsorbates of methapyrilene, dextromethorphan, ephedrine, and pseudoephedrine were prepared by column procedures using a polymethacrylic acid ion-exchange resin. Taste evaluation of the adsorbates showed a significant reduction in bitterness of the drugs. Coating the adsorbate particles with a 4:1 ethylcellulose–hydroxypropylmethylcellulose mixture reduced the bitterness further. Taste coverage was maintained after incorporation of the coated adsorbate into chewable tablets. *In vitro* release-rate studies showed immediate and complete drug elution from uncoated adsorbates. Release from coated adsorbates varied with the extent of coating. *In vivo* drug availability was demonstrated by LD_{50} tests in mice and rats and by a urinary excretion crossover study in humans.

Keyphrases □ Tablets, chewable—improved palatability, coating-dependent release rates □ Taste coverage, chewable tablets—ion-exchange resins □ Bioavailability—chewable tablets, coating effect □ Timed-release tablets—coated drug–carboxylic ion-exchange resin absorbates □ Ion-exchange resins, polycarboxylic acid—taste coverage, chewable tablets

The adsorption of bitter drugs onto synthetic ion-exchange resins to achieve taste coverage has been well documented (1–7). With amine-containing drugs, polysulfonic acid resins are more efficient than polycarboxylic acid exchangers for this purpose since their lower pKa (approximately 2 *versus* 5–6) yields a stronger drug–resin bonding. However, this stronger bonding results in a slower *in vivo* elution of drug from the polysulfonic acid resins, a principle that has been utilized in sustained-release dosage forms (8–10). In addition, the exchange capacities of the latter resins are usually lower than those of the polycarboxylic acid exchangers (4–5 *versus* greater than 10 meq./g.), with the result that a lower quantity of drug may be adsorbed.

Previous studies in this laboratory on the interaction of amine drugs with polycarboxylic acid ion-exchange resins (11) indicated that these resins may be quite useful in taste coverage. These studies indicated that saliva, with an average pH of 6.7 and a cation concentration of 40 meq./l. (12), would only elute a limited percentage of drug from a polycarboxylic acid resin adsorbate. However, rapid and quantitative elution would occur as soon as the adsorbate is exposed to the low pH of the stomach. These studies also revealed that drugs containing tertiary amine groups give much higher selectivity coefficients than those with primary, secondary, or quaternary amines. Thus, taste coverage should be most efficient with tertiary amine drugs.

The particle coating of polycarboxylic acid ion-exchange resin adsorbates was also considered as a method for achieving taste coverage. Two recent patents described related procedures. In one (13), anion-exchange resins without drugs were coated for palatability; the other (14) dealt with coating of cation-exchange resin adsorbates for slow drug release. In such a method, the resin could be considered primarily a carrier for the drug in the coating step. Although this process would be more complex and expensive than direct coating of crystals, there could be several advantages:

1. The taste coverage ability of the uncoated adsorbate could be additive to that of the coating.
2. Polymer coatings could adhere more effectively to ion-exchange resin particles than to crystals.
3. The desired narrow particle-size range could be obtained by proper selection of the resin.
4. The resin particles could be more compact and have less surface area than most crystals.
5. The adsorbate could be less sensitive than pure drug to explosivity due to static charge.
6. The extremely rapid elution rate in acid could make the drug available more rapidly in the stomach.
7. The water adsorptive and swelling properties of the polycarboxylic acid ion-exchange resins could hasten tablet disintegration and thus provide drug availability without chewing.

This paper describes taste coverage studies with drug adsorbates made with a polymethacrylic acid ion-exchange resin. Four bitter drugs were selected for this evaluation. They included two with tertiary amine groups (methapyrilene and dextromethorphan) and two with secondary amines (ephedrine and pseudoephedrine).

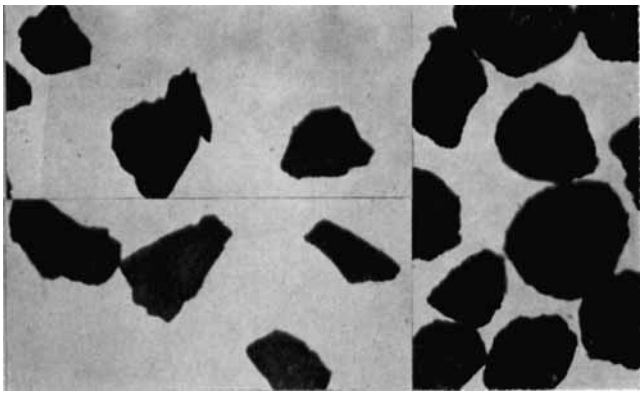

Figure 1—*Comparison of coated and uncoated pseudoephedrine adsorbate particles. Photograph on left shows uncoated adsorbate while the right photograph shows adsorbate with 31.0% coating.*

EXPERIMENTAL

Resin—In all studies, a polymethacrylic acid ion-exchange resin with a particle-size range of 72–147 μ, a crosslinkage of 4–5% divinylbenzene, and an exchange capacity of greater than 10 meq./g., was used.

Adsorbate Preparation—Approximately 400 g. of resin was slurried 30 min. in 4 l. of 1 *N* NaOH. The slurry was then transferred to a 10.2-cm. (4-in.) diameter, 1.5-m. (5-ft.) glass column equipped with a coarse fritted-glass disk at the bottom. The resin was then

Table I—Drug–Resin Adsorbate Preparation

Drug	Salt Used	Resin Cycle	Grams Drug Used per Gram Resin	Influent Concentration, mg./ml.	Yield[a], %	Adsorbate Potency[b], mg./g.
Methapyrilene	HCl	Na	1.12	50	99.9	496
Dextromethorphan	HBr	Na	1.44	20	98.4	522
Ephedrine	Base	H	1.89	50	96.3	645
Pseudoephedrine	HCl	Na	3.97	50	37.9	552

[a] Yield was drug adsorbed divided by drug added and determined from total drug in effluent. [b] Adsorbate potency is milligrams drug base per gram of dried adsorbate.

Table II—Bitterness Evaluations for Ephedrine Derivatives

Form[a]	Dosage[b]	Coating[c], %	Bitterness Level[d] after 10 sec.	1 min.	2 min.	5 min.	10 min.	15 min.
U	P	0	>3	>3	3	2½	1½	½
UA	P	0	2	1½–2	1½	½–1	)(	0
CA	P	23.4	)(	½	½	)(	0	0
CA	P	34.2	)(–½	½	)(	)(	0	0
CA	P	38.6	)(	)(	0	0	0	0
CA	P	42.0	0	)(	0	0	0	0
UA	T	0	1–1½	1½	1	)(–½	0	0
CA	T	23.4	½	½	½	)(	)(	0
CA	T	34.2	)(–½	)(	)(	)(	0	0
CA	T	38.6	)(–½	½	½	)(	0	0
CA	T	42.0	0	0	0	0	0	0

[a] U = drug base; UA = uncoated adsorbate; CA = coated adsorbate. [b] P = particles; T = chewable tablets. [c] Percent of coated adsorbate weight. [d] 3 = strong bitterness; 2 = moderate bitterness; 1 = slight bitterness;)(= threshold.

backwashed 1 hr. with water using a peristaltic pump. Four liters of 1 *N* NaOH was then pumped downflow at a rate of 4 l./hr., followed by 5 l. of water, 8 l. of 1 *N* HCl, 5 l. of water, 8 l. of 1 *N* NaOH, and 5 l. of water. The calculated amount of drug that would yield an adsorbate containing 50–60% drug was dissolved in water and pumped downflow at 4 l./hr. followed by 5 l. of water. Excess liquid was drained from the resin bed by suction, and the wet adsorbate was transferred to glass pans. It was then dried in a vacuum oven at 50° to constant weight.

Coating Procedure—The dextromethorphan, ephedrine, and pseudoephedrine adsorbates were coated to several different coating levels by a procedure similar to that described by Coletta and Rubin (15), using an air suspension coater. Based on their results, a 4:1 mixture of ethylcellulose–hydroxypropylmethylcellulose was selected as the coating material and used in all cases. Figure 1 compares coated adsorbate particles with uncoated adsorbate to emphasize the efficiency of the coating. The percentage coating for each sample was determined by assaying for drug before and after coating. Generally, the values obtained corresponded to those calculated from the amount of coating material added.

Tableting Procedure—A calculated weight of adsorbate, containing 11.67 g. of dextromethorphan, 20.48 g. of ephedrine, 21.94 g. of methapyrilene, or 49.15 g. of pseudoephedrine, was blended 20 min. in a twin-shell V blender with 7 g. of magnesium stearate and enough pregranulated mixture to make 700 g. The pregranulation was made by massing a mixture of 700 g. of mannitol, 292 g. of sucrose, and 8.0 g. of cyclamate sodium with alcohol, granulating through a 6-mesh screen, drying at 50°, and screening through a 16-mesh screen. The drug blend was compressed on a 16-station rotary compressing machine[1] to give approximately 1000 tablets weighing 700 mg. each.

Release-Rate Studies—Drug release from uncoated adsorbates was determined by adding 100 mg. of adsorbate to a 25-ml. test tube, followed by 10 ml. of 0.08 *N* HCl. The mixture was immediately stirred on a mixer[2] for the specified time and then rapidly filtered. Eleven different time periods, from 5 to 600 sec., were used with each adsorbate. The filtrates were then assayed by UV spectroscopy (11).

Drug release at pH 6.7 was determined by adding 500 mg. of adsorbate to a test tube, followed by 10 ml. of a pH 6.7 phosphate buffer (0.04 *N* in sodium ion). The mixture was immediately stirred on a mixer[2] for 1 min., rapidly filtered, and assayed.

Release from coated adsorbates and tablets was determined by adding 3.00 g. of adsorbate or eight gently crushed tablets to a 500-ml. conical flask suspended from a wrist-action shaker[3] into a 37° water bath. Three hundred milliliters of either 0.08 *N* HCl or simulated intestinal fluid USP (pancreatin omitted) was added, and the flask was shaken for 24 hr. Thirteen 5-ml. samples of solution were removed periodically from 5 min. to 7 hr., and a final sample was obtained at 24 hr. These were assayed by UV spectroscopy after the required dilutions.

Taste Evaluation—All taste evaluations were made by a trained flavor profile panel (16), using at least seven members for each sample. A time–intensity method (17) was used, in which a sample equivalent to a normal dose was held in the mouth (chewed in the case of tablets) for 10 sec. Bitterness levels were recorded immediately

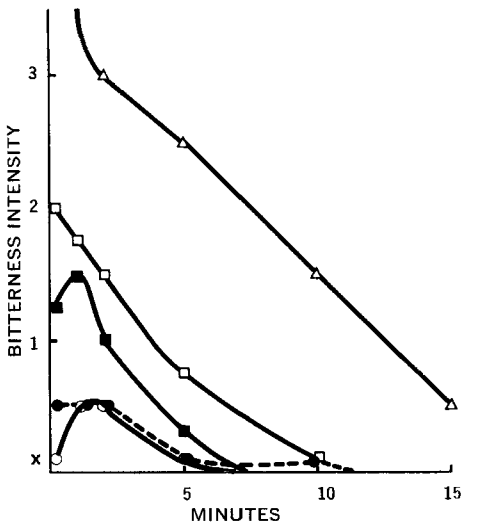

Figure 2—*Bitterness intensity curves for various ephedrine dosages. Key: △, ephedrine base; □, uncoated adsorbate; ■, uncoated adsorbate tablets; ○, 23.4% coated adsorbate; and ●, chewable tablets containing 23.4% coated adsorbate.*

[1] Stokes B-2, F. J. Stokes Corp., Philadelphia, Pa.
[2] Vortex Genie, Scientific Products, Evanston, Ill.
[3] Burrell Corp., Pittsburgh, Pa.

Table III—Bitterness Evaluations of Dextromethorphan Derivatives

Form[a]	Dosage[b]	Coating[c], %	Bitterness Level[d] after 10 sec.	1 min.	2 min.	5 min.	10 min.	15 min.
HBr	P	0	>3	>3	2½	1½	1	½
UA	P	0	2	2	1½	½	)(	0
CA	P	25.4	½	)(–½	)(	)(	0	0
CA	P	34.6	)(–½	)(	)(	0	0	0
CA	P	40.6	)(	)(	0	0	0	0
UA	T	0	1½	1½	1½–2	1½	1	)(
CA	T	25.4	)(–½	½	)(	)(	)(	0
CA	T	34.6	)(	)(–½	)(–½	)(	0	0
CA	T	40.6	)(	)(	)(	)(	0	0

[a] UA = uncoated adsorbate; CA = coated adsorbate. [b] P = particles; T = chewable tablets. [c] Percent of coated adsorbate weight. [d] 3 = strong bitterness; 2 = moderate bitterness; 1 = slight bitterness;)(= threshold.

Table IV—Bitterness Evaluations of Pseudoephedrine Derivatives

Form[a]	Dosage[b]	Coating[c], %	Bitterness Level[d] after 10 sec.	1 min.	2 min.	5 min.	10 min.	15 min.
HCl	P	0	>3	>3	>3	2	1	1
UA	P	0	2	2½	2	1½	—	—
CA	P	21.0	1	1½	1	½	)(	0
CA	P	25.4	)(	½	)(	)(	0	0
CA	P	31.0	)(	)(	0	0	0	0
CA	T	25.4	½	1½	1½–2	1½–2	½–1	)(

[a] UA = uncoated adsorbate; CA = coated adsorbate. [b] P = particles; T = chewable tablets. [c] Percent of coated adsorbate weight. [d] 3 = strong bitterness; 2 = moderate bitterness; 1 = slight bitterness;)(= threshold.

and then at 1, 2, 5, 10, and 15 min. Bitterness intensity values were based on a 0–3 scale, with 3 being strong bitterness (0.20% caffeine solution), 2 being moderate (0.10% caffeine), 1 being slight (0.05% caffeine), and being threshold (0.001%).

Determination of LD_{50}—Groups of either 10 female Swiss-Webster mice, 16–24 g. in weight, or six Sprague-Dawley male weanling rats, 40–70 g., were given various doses of the drugs suspended or dissolved (1–10%) in a 0.5% methylcellulose solution. The animals were observed for at least 7 days. The LD_{50} and associated confidence limits were calculated according to the method of Litchfield and Wilcoxon (18).

Urinary Excretion Study—Eight human subjects were used for this study. Four subjects were administered single 60-mg. doses of pseudoephedrine base in capsules; the other four received coated pseudoephedrine adsorbate (60 mg. drug) incorporated into chewable tablets. Urine samples were collected at 0–2, 2–4, 4–8, 8–12, and 12–24 hr. One week later, the subjects were crossed over and the sequence was repeated. The urine samples were assayed by GLC (19).

RESULTS AND DISCUSSION

Selection of the resin was based primarily on its particle-size range. Attempts to use resins with particles smaller than 72 μ presented considerable difficulty in the column operations. Although particles larger than 147 μ function better in column operations, their large size makes them too gritty for incorporation into chewable tablets.

A column procedure was found necessary to obtain adsorbates with the high drug concentrations desired. Batch operations gave both lower potency adsorbates and lower yields. These findings are consistent with the selectivity coefficients obtained for these drugs in an earlier study with carboxylic acid ion-exchange resins (11). Table I summarizes the results obtained in the preparation of the drug–resin adsorbates by column procedures.

The adsorption technique for each drug was adjusted to allow for variations in the physical constants of the drugs. The most efficient loading procedure would be to use the drug base and the resin on the acid cycle. However, only ephedrine base had sufficient solubility for such a procedure. In using soluble salts of the other three drugs, it was necessary to put the resin in the base form. If left in the acid form, the acid generated by adsorption would rapidly elute part of the drug and reduce yields. Nearly quantitative yields were obtained with dextromethorphan and methapyrilene. However, to get the desired high potency with pseudoephedrine, a large excess of drug was necessary. This resulted in a substantially reduced yield. The differences in adsorptive characteristics between these drugs can be attributed to the greater affinity of the resin for tertiary amines than secondary amines. At pH 5.5, the selectivity coefficients obtained for methapyrilene and dextromethorphan were, respectively, 24 and 43 times greater than that obtained for pseudoephedrine (11). Since ephedrine has a selectivity coefficient similar to pseudoephedrine, it too would have given poor yields if a drug salt and sodium cycle

Table V—Bitterness Evaluation of Methapyrilene Derivatives

Form	Dosage	Bitterness Level after 10 sec.[a]
HCl salt	Particles	>3
Uncoated adsorbate	Particles	1
HCl salt	Tablets	>3
Uncoated adsorbate	Tablets	2

[a] 3 = strong bitterness; 2 = moderate bitterness; 1 = slight bitterness.

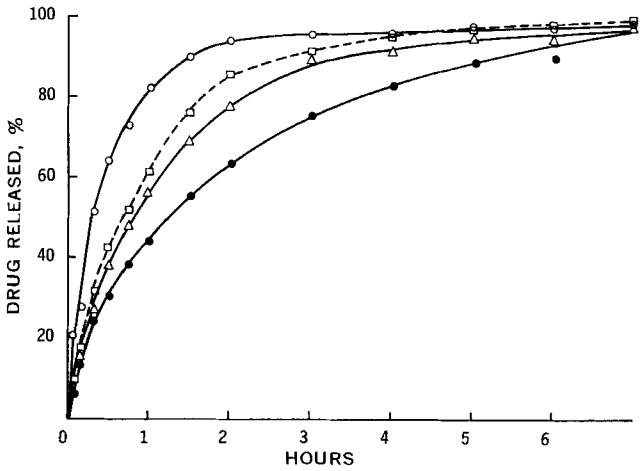

Figure 3—*Release of ephedrine from coated adsorbates in 0.08* N *HCl. Key:* ○, *23.4% coating;* □, *34.2% coating;* △, *38.6% coating; and* ●, *42.0% coating.*

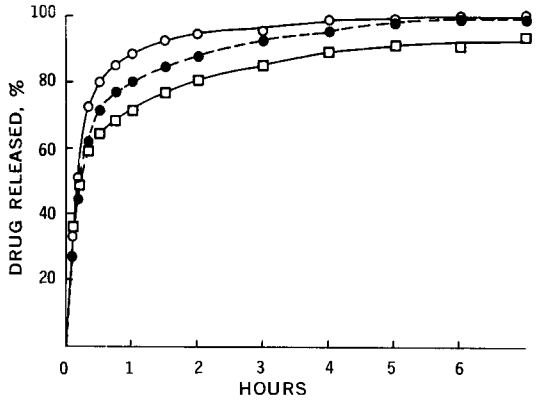

Figure 4—*Release of dextromethorphan from coated adsorbates in 0.08* N *HCl. Key:* ○, *25.4% coating;* ●, *34.6% coating; and* □, *40.6% coating.*

resin were used. Generally, the drug was added to the resin column as a 5% solution. However, the lower solubility of dextromethorphan hydrobromide would allow this concentration only by maintaining the solution temperature above 50°. It was found more convenient to reduce the influent concentration to 2% and accept the large volume increase.

Taste evaluations for the pure drugs, adsorbates, and coated adsorbates are shown in Tables II–V. In all samples except methapyrilene, time–intensity values were obtained to include aftertaste in the evaluation. Figure 2 graphically shows a comparison of the bitterness levels obtained from various ephedrine forms. In all cases, the pure drugs were extremely bitter. Adsorption onto the resin reduced the bitterness to some extent. This finding is consistent with the expectation that saliva would only elute a limited percentage of drug from the uncoated adsorbate. Table VI shows the percentages eluted from 500 mg. of adsorbate by 10 ml. of pH 6.7 (0.04 *M*) buffer in 1 min. These percentages were only slightly affected by exposure times above 30 sec., indicating that these numbers are close to equilibrium values. The larger percentages obtained with ephedrine and pseudoephedrine again emphasize the stronger bonding between the resin and tertiary amines.

Particle coating the adsorbates gave further bitterness reduction, with the extent of this reduction being dependent on the coating level. In all cases, a coating level of about 25% appeared to be sufficient to reduce bitterness to less than 1 (slight bitterness) on the intensity scale. Incorporation of the adsorbates into chewable tablets generally had little effect on the taste coverage. However, in some cases the bitterness levels were increased. This result can be attributed to rupture of the coating either during tablet compression or chewing. Incorporation of flavoring or sweetening agents into the tablet formula would be beneficial in counteracting this effect.

Availability of drug from the adsorbates was demonstrated in a number of *in vitro* experiments. Table VI shows the drug-release characteristics from uncoated adsorbates in several different solu-

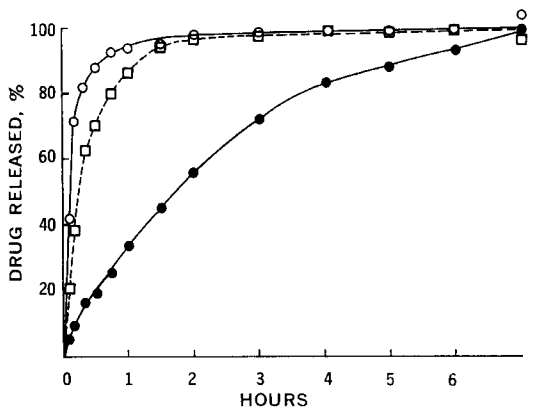

Figure 5—*Release of pseudoephedrine from coated adsorbates in 0.08* N *HCl. Key:* ○, *21.0% coating;* □, *25.4% coating; and* ●, *31.0% coating.*

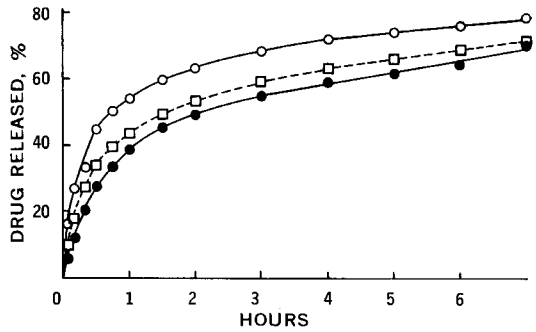

Figure 6—*Release of ephedrine from coated adsorbates in simulated intestinal fluid. Key:* ○, *23.4% coating;* □, *34.2% coating; and* ●, *42.0% coating.*

tions. In 0.08 *N* HCl, the elution half-lives were all below 15 sec. while 90% of the drugs were eluted in 0.8–2.2 min. Essentially complete drug release occurred within 15 min. Completion was demonstrated by eluting no additional drug when the resin particles were filtered and reslurried with fresh acid solution. This rapid and complete drug elution in 0.08 *N* HCl is due to the high pKa of the polymethacrylic acid ion-exchange resins. Assuming a pKa similar to the 4.85 reported by Gustafson (20) for this type of resin, only 0.022% of the potential anionic sites would be dissociated at pH 1.2, and less than 0.05% of the drug in the adsorbates would remain ionically bonded.

Table VI also shows the equilibrium distribution of drug in simulated intestinal fluid using 10 mg. of adsorbate/ml. of solution. Although the mixtures were allowed 24 hr. for equilibration, the reactions were again very rapid and essentially complete within 15–30 min. The equilibrium values varied with the drug, with greater than 90% eluted from the two secondary amine adsorbates and less than half from the tertiary amine adsorbates. Although drug availability might be affected by incomplete elution in the intestine, such a possibility appears remote. *In vivo* equilibrium percentages would be substantially higher than those shown in Table VI, since adsorbate concentrations in the body should be well below the 10 mg./ml. used to obtain the listed values. Lower adsorbate concentrations would result in an increase in the number of eluting cations per unit adsorbate weight and, thus, a shift in equilibrium. In addition, the rapid equilibration rates would cause continuous elution of drug from the resin to maintain equilibrium as the drug in solution is absorbed into the blood.

Drug release from the adsorbates was slowed considerably by the introduction of coating. Figures 3–5 show the release from coated adsorbates in 0.08 *N* HCl. The curves demonstrate that the release rate from a given adsorbate is governed primarily by the coating level. However, a comparison of the figures indicates that the drug used also has an effect; identical coating levels on different adsorbates will not necessarily yield similar release rates. This may be due to variability in physical constants of the drugs (solubility, diffusivity, selectivity coefficient, *etc.*) or simply the effect of the drug

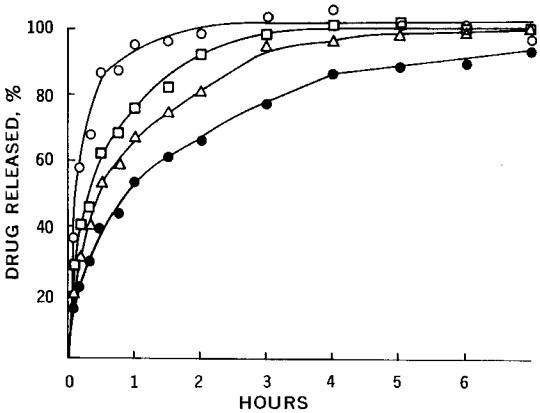

Figure 7—*Release of ephedrine from crushed tablets made from coated adsorbates in 0.08* N *HCl. Key:* ○, *23.4% coating;* □, *34.2% coating;* △, *38.6% coating; and* ●, *42.0% coating.*

Table VI—Release of Drug from Uncoated Adsorbates in pH 6.7 Buffer, 0.08 *N* HCl, and Simulated Intestinal Fluid

Drug	Percent Released after 1 min. in pH 6.7 Buffer	$t_{1/2}$ in 0.08 *N* HCl, min.	$t_{90\%}$ in 0.08 *N* HCl, min.	Percent Released after 24 hr. in Simulated Intestinal Fluid
Ephedrine	26.7	0.2	1.0	98.1
Dextromethorphan	4.89	0.1	0.8	30.4
Pseudoephedrine	32.1	0.1	1.1	92.5
Methapyrilene	3.43	0.2	2.2	45.5

on coating efficiency. As in the case of uncoated adsorbates, drug release in simulated intestinal fluid proceeded to equilibrium rather than completion. The equilibrium percentages for all coated adsorbates were similar to the values shown in Table VI for uncoated adsorbates. In addition to incomplete elution, the release rates for attaining equilibrium were slower. This is exemplified in Fig. 6 with coated ephedrine adsorbates. Although equilibrium for these samples was close to complete elution, the percentages released at each time interval were substantially less in simulated intestinal fluid than in 0.08 *N* HCl. This may have been due to a lower drug concentration of the solution within the coated resin particles caused by either decreased drug solubility or lower elution efficiency at pH 7.5 *versus* pH 1.2.

Drug release from ground tablets formulated from the coated adsorbates was slightly faster than that obtained from coated adsorbate particles. Figure 7 shows release curves from ephedrine tablets formulated from coated adsorbate. Since the rate increases obtained from tablets (Fig. 7 *versus* Fig. 3) are most pronounced in the early stages, it is most likely that coating rupture of some particles occurs during tablet compression or grinding. This effect would also be obtained when these tablets are chewed.

Acute oral toxicity tests in mice and rats were used to determine *in vivo* availability of drug from the resin adsorbates (Table VII). The high LD_{50} of the pure resin indicated that its contribution to toxicity should be minimal. The small differences in the LD_{50} values between drug salts and uncoated adsorbates (within confidence limits) implies that resin adsorption does not affect drug availability. Similarly, pseudoephedrine was available from crushed tablets made from coated adsorbate to the same extent as the drug base. However, the LD_{50} for coated pseudoephedrine adsorbate particles was substantially increased. This suggests a reduction of the peak blood level concentration, although not necessarily a decrease in total availability.

Total availability of drug from coated adsorbate was demonstrated in a crossover urinary excretion study in humans. Figure 8 compares the cumulative recovery of drug from pseudoephedrine base capsules and coated drug adsorbate formulated into chewable tablets. The results show slower, but complete, availability of drug from the coated adsorbate. Since the 31% coated pseudoephedrine

Table VII—Comparison of LD_{50} Results between Pure Drugs and Drug Adsorbates

Sample	Animals	LD_{50}, mg./kg.	95% Confidence Limits, mg./kg.
Resin	Mice	>10,000	—
Pseudoephedrine hydrochloride	Mice	371	275–530
Uncoated pseudoephedrine adsorbate	Mice	500	431–580
31% Coated pseudoephedrine adsorbate	Mice	910	812–1020
Pseudoephedrine base	Rats	660	537–812
Coated pseudoephedrine adsorbate (tablets)	Rats	680	577–803
Dextromethorphan hydrobromide	Mice	250	223–280
Uncoated dextromethorphan adsorbate	Mice	210	190–234

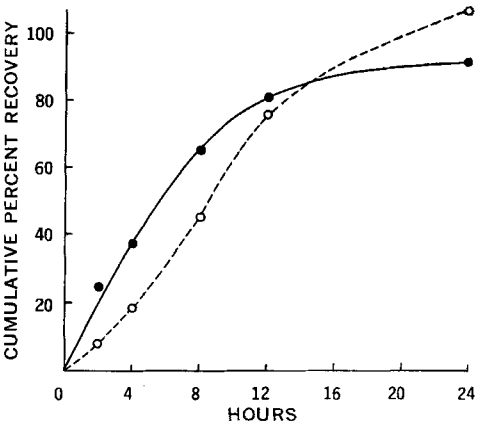

Figure 8—*Urinary excretion study of pseudoephedrine dosages administered to humans. Key:* ●, *pseudoephedrine base capsules; and* ○, *tablets containing 31% coated pseudoephedrine adsorbate.*

adsorbate used in this test was among the slowest releasing samples tested (Fig. 5), it probably represents maximum *in vivo* availability delay. Coated adsorbates with faster *in vitro* release rates should give more rapid and similarly complete availability.

Examination of the urinary excretion curves along with the *in vitro* results indicates that the coated drug–carboxylic acid ion-exchange resin adsorbates might be useful for sustained release as well as taste coverage. Whereas the use of sulfonic acid ion-exchange resins for this purpose appears to be primarily dependent on equilibrium phenomena, release from the coated adsorbates is primarily dependent on the coating level. Thus, desired drug release rates should be attainable by control of the coating level.

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Solubilization of Benzoic Acid Derivatives by Nonionic Surfactants: Location of Solubilizates in Hydrocarbon Core of Micelles and Polyoxyethylene Mantle

PASUPATI MUKERJEE

Abstract □ The micelles that nonionic surfactants containing polyoxyethylene head groups form in aqueous solution have a hydrocarbon core surrounded by a mantle, which can be described as a dense solution of polyoxyethylenes. Solubilization, or the uptake of organic molecules in such micelles, may involve two broadly defined loci: (*a*) the hydrocarbon core along with its interface, and (*b*) the polyoxyethylene mantle. It is shown that the solubilization of many benzoic acid derivatives and phenolic preservatives in such micelles can be described in terms of the two loci of solubilization. The relative importance of the two loci can be derived in an approximate fashion from solubilization data in systems where the polyoxyethylene chain length is varied on the basis of two simplifying assumptions. The distribution of the solubilized species between the two loci is related to the chemical structure of the solubilizate, and it is important in understanding the physical and chemical reactivities of the solubilized species.

Keyphrases □ Benzoic acid derivatives—solubilization in nonionic surfactant micelles, distribution □ Solubilization, benzoic acid derivatives—distribution in nonionic micelles □ Surfactant distribution loci—benzoic acid solubilizates

The solubilization of many benzoic acid derivatives and other solubilizates by the micelles of nonionic surfactants of the polyoxyethylene type has been the subject of a considerable amount of research in recent years (1, 2). One main interest has been the preservative action of some solubilizates in the presence of the nonionic surfactants. It is likely that only the free solubilizate molecules in the intermicellar fluid are primarily active as preservatives, whereas the molecules associated with the surfactant micelles are inactive. Therefore, the overall distribution coefficient of the solubilizate between the micelles and the intermicellar fluid is the quantity of primary interest in this connection.

For a better understanding of the interesting phenomenon of solubilization itself, there are two questions of some importance. The first concerns the variation of the overall distribution coefficient with solubilizate concentration in the micelle and the nature of the nonideality effects displayed by the distribution coefficients. This problem is discussed in another paper (3).

The second problem, the subject of the present paper, concerns the locus of solubilization and the relative importance of the hydrophobic core of the micelle and the hydrophilic exterior (mantle) as the seat of solubilizing action. Solubilization in the hydrocarbon core of the micelle is, of course, well established as a general phenomenon (4). For amphipathic, polar–nonpolar-type solubilizates, this solubilization in the micelle core may involve specific orientations. The hydrophilic part of the solubilizate molecule may be exposed to energetically favorable aqueous environments at the surface of the hydrocarbon core of the micelle, whereas the hydrophobic part may be buried inside the hydrocarbon core. Such statements can only be made for the average position and orientation of the solubilizate molecule in the micelle, because individual solubilizate molecules may be capable of assuming a variety of positions and orientations (5). Recent work (6, 7) suggested that even benzene and some of its nonpolar derivatives, which are only slightly hydrophilic when compared to saturated hydrocarbons, are located primarily at or near the hydrocarbon–water interface of the micelles.

For the purposes of the present discussion, we shall not distinguish between the location of the solubilized species strictly inside the hydrocarbon core of the micelle or partly at the interface of the hydrocarbon core and the surrounding aqueous media, since both types of location are made possible by the presence of the hydrocarbon core. Except for possible electrostatic effects at the micelle surface, solubilization of this type need not be qualitatively different for micelles composed of ionic or zwitterionic monomers and those containing polyoxyethylene-type head groups. For the latter, however, a different kind of solubilization is possible. Considerable evidence exists of weak interactions between long-chain nonmicellar polyoxyethylenes (polyethylene glycols) and many organic molecules as well as electrolytes in aqueous solution (8, 9). Thus, unlike most ionic and zwitterionic micelles, the polyoxyethylene-type nonionic micelles can, in principle, solubilize (bind, "complex," interact with) many organic substances in their outer layers or mantles, which can be looked upon as concentrated solutions of oxyethylene groups. These mantles, in many micelles, are comparable in dimension to the hydrocarbon core and often occupy much bigger volumes than the hydrocarbon core does.

Qualitative suggestions that solubilization of partially polar solubilizates in polyoxyethylene-type micelles may involve either the polyoxyethylene layer or the micelle core, including its interface, have been made by various investigators, and there has been some controversy in the literature as to the importance of either type of solubilization (1, 2, 10–12). The purpose of this paper is to show how, on the basis of two simplifying assumptions, the relative effectiveness of the two loci of solubilization may be evaluated in an approximate fashion from solubilization data obtained with surfactants of different polyoxyethylene chain lengths attached to the same hydrocarbon chain, and how the distribution of the solubilized molecules between the two loci of solubilization, the core and the mantle, is correlated with the chemical structure of the solu-

Table I—Distribution of Solubilizate between Core and Mantle of Polyoxyethylene Stearate Micelles

Solubilizate	r^a	a^b, eq./eq.	b^c, eq./eq.	Y^d	a'^e
Benzoic acid	0.996	0.016_9	0.98_2	0.69	—
p-Hydroxybenzoic acid	0.838	0.050_8	0.37_7	5.4	0.029
o-Hydroxybenzoic acid	0.986	0.015_1	0.98_5	0.61	0.0070
p-Aminobenzoic acid	0.870	0.025_9	0.47_4	2.2	—
Ethyl *p*-aminobenzoate	0.934	0.011_9	0.56_5	0.84	—
Butyl *p*-aminobenzoate	0.999	0.004_2	1.01_9	0.16	—
Methyl *p*-hydroxybenzoate	0.974	0.014_6	0.72_4	0.81	0.0073
Propyl *p*-hydroxybenzoate	0.991	0.006_9	0.76_3	0.36	0.0013_5

[a] Correlation coefficient using Eq. 2. [b] Amount solubilized in the mantle per equivalent of ethylene oxide. [c] Amount solubilized in the core per equivalent of stearyl group. [d] Ratio of the amount in the mantle and the amount in the core calculated for stearyl $(EO)_{40}$ micelles. [e] Binding in polyethylene glycol 4000 in equivalents per equivalent of oxyethylene group.

bilizate. Some applications of the findings in understanding chemical reactivities of solubilized molecules will also be indicated.

DISTRIBUTION OF SOLUBILIZATE BETWEEN CORE AND MANTLE

If micellar solubilization data are available from solubility studies (13) for a series of surfactants of the general formula $R(EO)_nOH$, where R is the alkyl chain moiety, EO stands for oxyethylene ($—OCH_2CH_2—$), and n, the number of EO groups, is varied, the distribution of the solubilizate between the micelle core composed of R groups and the mantle composed of the EO groups can be derived on the basis of two simplifying assumptions: (*a*) the amount solubilized in the core is proportional to the number of equivalents of R present in the micellar form, and (*b*) the amount solubilized (bound) to the mantle is proportional to the number of equivalents of EO group present. Suitable experimental data are available from the study of Goodhart and Martin (13). These authors measured the solubilities of eight benzoic acid derivatives at 25° in polyoxyethylene stearates[1] (so that R represents the stearyl group, $C_{17}H_{35}CO—$). The ionization of the solubilizate was suppressed by using 0.005 *N* hydrochloric acid. Goodhart and Martin (13) used five surfactants in which n varied from 20 to 100[2]. They expressed their micellar solubilities, *i.e.*, the solubility in excess of the saturation solubility in the absence of micelles, as equivalent of solubilizate per equivalent of EO group. According to the assumptions already stated, the amount solubilized, in equivalents per liter of solution, S', will be given by the simple equation:

$$S' = aC_{EO} + bC_R \qquad \text{(Eq. 1)}$$

where C_{EO} and C_R are the concentrations, in equivalents per liter, of EO groups and R, respectively; and a and b are proportionality constants. On dividing by C_{EO}, one obtains:

$$\frac{S'}{C_{EO}} = a + b\frac{C_R}{C_{EO}} \qquad \text{(Eq. 2)}$$

so that if S'/C_{EO}, in equivalents per equivalent, is plotted against the inverse of the EO/R mole ratio of the surfactant, a linear curve should be obtained, with the intercept a representing the solubilization in the mantle (equivalents of solubilizate/equivalent of EO group) and the slope b representing the solubilization in the core (equivalents of solubilizate/equivalent of R group).

Figures 1 and 2 show the plots of the data according to Eq. 2 for the eight systems investigated by Goodhart and Martin (13). Table I shows the values of a and b obtained by least-squaring procedures and the correlation coefficient, r, for linear regression. Considering the uncertainties in the composition of the commercial surfactants, the correlation coefficients are satisfactory, except for the strongly bipolar *p*-hydroxybenzoic acid and *p*-aminobenzoic acid, both of which show some curvature. This curvature is probably due to the breakdown of assumption (*b*). Although *p*-hydroxybenzoic acid appears to show the lowest correlation coefficient, the mean deviation of the experimental data from the best straight line is only about 5%.

STRUCTURAL CORRELATION

The satisfactory representation of the experimental data for all eight systems studied by Goodhart and Martin (13) suggests that the two assumptions used to derive Eq. 2 are reasonably valid for this system. The a and b values of Table I can now be used to calculate the distribution of the solubilized material between the core and the mantle of the micelles for any particular surfactant composition. Table I gives some examples of the calculated ratio, Y, of the amount in the mantle and the amount in the core for a typical surfactant $R(EO)_{40}OH$. The value of Y varies over a wide range, depending upon the structure of the solubilizate.

The values of a and b, and the values of Y calculated therefrom, show excellent qualitative correlations with the structure of the solubilizate as regards the distribution of the polar moiety in the molecule and the size of the nonpolar moiety. Thus, benzoic acid, which has a polar group at one end only and is amphipathic in the same sense as the surfactant molecules themselves, has a low value of a and high value of b. *o*-Hydroxybenzoic acid, which shows intramolecular hydrogen bonding (14), behaves very similarly to benzoic acid. *p*-Hydroxybenzoic and *p*-aminobenzoic acids, however, have polar groups at both ends of the molecule. As is to be expected, their solubility in the micelle core is lower than that of benzoic acid, while the binding to the polyoxyethylene mantle is more pronounced, presumably because this binding with the oxyethylene groups involves hydrogen bonding in part, and bipolar molecules of this kind have two hydrogen bond donor functions for attachment, unlike benzoic or *o*-hydroxybenzoic acid. As the acid group of either *p*-hydroxy or *p*-amino acid is esterified, however, it loses its hydrogen bond donor activity and becomes more hydrophobic. As a result, the binding to the polyoxyethylene mantle is reduced, whereas the solubility in the core becomes higher. Both the

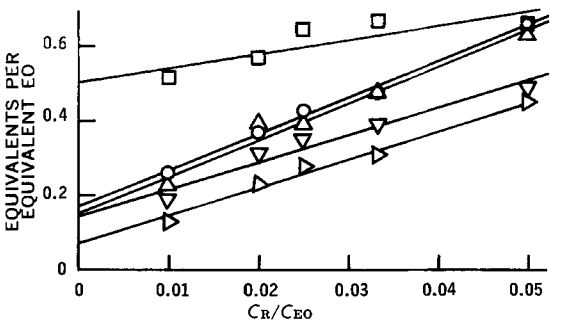

Figure 1—*Micellar solubilization in polyoxyethylene stearates. The number of equivalents solubilized per equivalent of oxyethylene group is plotted as the ordinate against the stearate–ethylene oxide mole ratio for the surfactants. Key:* □, p-*hydroxybenzoic acid;* ○, *benzoic acid;* △, o-*hydroxybenzoic acid;* ▽, *methyl* p-*hydroxybenzoate; and* ▷, *propyl* p-*hydroxybenzoate.*

[1] Commercially available as the Myrj class of surfactants.

[2] The ethylene oxide–stearate mole ratios used here for calculations are 20:1 for Myrj 49, 30:1 for Myrj 51, 40:1 for Myrj 52, 50:1 for Myrj 53, and 100:1 for Myrj 59. These composition figures were obtained from Dr. Paul Becher, Atlas Chemical Industries, Wilmington, Del. They differ slightly from the values used by Goodhart and Martin (13), whose solubility data were recalculated for the present compositions.

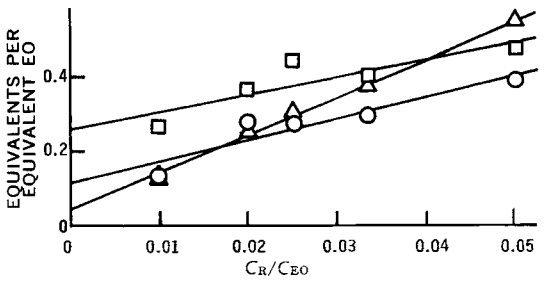

Figure 2—*Micellar solubilization in polyoxyethylene stearates. The number of equivalents solubilized per equivalent of oxyethylene group is plotted as the ordinate against the stearate–ethylene oxide mole ratio for the surfactants. Key:* □, p-*aminobenzoic acid;* ○, *ethyl* p-*aminobenzoate; and* △, *butyl* p-*aminobenzoate.*

increase in b and the decrease in a depend upon the size of the hydrophobic group used for esterification.

The structural correlations discussed here point out the great importance of the distribution of polar groups in a solubilizate molecule in micellar solubilization.

It is interesting to compare the values of a for the micellar systems with the corresponding values of the solubilities of the benzoic acid derivatives in polyethylene glycol itself. For this purpose, the latter quantity, termed a' and defined as equivalents bound per equivalent of EO group, was calculated from some data in the literature (15, 16) for four of the eight substances in Table I. These data are also given in Table I. The data were obtained from solubility studies in relatively dilute solutions of polyethylene glycol 4000 at 30°. This difference in temperature is not of great significance. The solubility of the two acids was determined in the presence of 0.003 N H_2SO_4 to suppress dissociation (15). The a and a' values are similar in order of magnitude, although the a' values are uniformly lower, by a factor of about 2 for three compounds and a factor of about 5 for propyl p-hydroxybenzoate. The a value derived for the latter is very low and, therefore, uncertain.

The comparison of a and a' values indicates that the ability of the polyoxyethylene mantle of the micelles to bind such molecules is substantially higher than that of polyoxyethylene glycol 4000 at low concentrations. Considering the structure of the micellar interface in detail, this effect does not seem unreasonable. The binding of p-hydroxybenzoic acid to polyethylene glycol was found to increase with the molecular weight of the glycol (17), presumably because multiple-site interactions are involved in these weak associations. Polyethylene glycol exists approximately as a random coil (18). In comparison, because of the geometrical restraints imposed by the micelle core, the oxyethylene groups near the micelle surface are forced to be in close proximity, a factor that leads to the head group self-interaction effect, previously analyzed, which reduces the stability of the micelles (19). The polyoxyethylene groups at the micelle surface are expected, therefore, to have much higher local concentrations of oxyethylene groups than polyethylene glycols, and the solubilization (binding) in the surface layer is likely to be facilitated by multiple interactions with neighboring chain elements.

The agreement of a and a' in their order of magnitude provides some additional support for this simple analysis and the relative importance of both loci of solubilization in polyoxyethylene-type micelles. For propyl p-hydroxybenzoate, for example, the experimental value of S'/C_{EO} is as high as 0.0448 for polyoxyethylene stearate 49. If the solubilization is ascribed entirely to the polyoxyethylene mantle, as is sometimes done, the value of a will exceed a' by a factor of more than 30. On the other hand, ascribing the micellar solubilization entirely to solubilization in the core, *i.e.*, assuming an a value of zero, is clearly inconsistent with the a' values in Table I.

The numerical values of a and b in Table I are obviously dependent upon the applicability of the two simplifying assumptions mentioned previously. Although the correlation coefficients and the description of the data (Table I) are fairly satisfactory, possibilities of compensating effects cannot be ruled out. In particular, the crowding of oxyethylene groups at the micelle surface as compared to polyethylene glycol, the factor mentioned in explaining why a values are likely to be higher than a' values, is expected to be a function of the ethylene oxide chain length. Because of the radial nature of the distribution of the EO groups around a spherical micelle, as the EO number becomes higher, the volume available to the EO groups increases nonlinearly. This effect should reduce the effective value of a as the EO chain length is increased and may be responsible, in part, for the curvature exhibited in Figs. 1 and 2, particularly by p-hydroxybenzoic and p-aminobenzoic acids.

APPLICATION TO OTHER SYSTEMS

The two loci of solubilization in polyoxyethylene-type micelles, the core and the mantle, differ greatly in the nature of the interactions the solubilized molecules display and the nature of the local environment around the solubilized molecules. The physical and chemical properties of the solubilized species thus depend, to a great extent, on its distribution between the core and the mantle.

Thakkar and Hall (20) and Bjaastad and Hall (21) investigated the effective polarities of the environments of solubilized testosterone, 2-heptanone, and D-camphor in polyoxyethylene-type surfactant micelles, using changes in absorption spectra. In such studies, the molecules solubilized in the mantle, if any, are likely to behave differently from those solubilized in the core. Similarly, the chemical reactions of solubilized species depend to a great extent on where they are located. Riegelman (22), for example, studied the effect of solubilization in micelles of $C_{16}H_{33}(EO)_nOH$ on the hydrolysis of benzocaine in sodium hydroxide solutions. The measured half-lives can be interpreted in terms of pseudo-first-order rate constants for the hydrolysis of free benzocaine in the intermicellar fluid and that of the solubilized benzocaine in terms of a distribution model (23). The rate constant for benzocaine solubilized in $C_{16}H_{33}(EO)_{14}OH$ is estimated to be about 22% of that of the free benzocaine (23). The structure of benzocaine, which is the ethyl ester of p-aminobenzoic acid, makes it unlikely that the molecules solubilized in the core of the micelle will be very accessible to attack by OH^- ions on the ester linkage, which is likely to be largely buried in the hydrocarbon core, resulting in steric protection. Experimental evidence for this can be derived from the half-life data of benzocaine hydrolysis in sodium dodecyl sulfate and cetyl trimethylammonium bromide solutions (22), both of which show much better protection against hydrolysis than $C_{16}H_{33}(EO)_{14}OH$.

With sodium dodecyl sulfate, this protective action can, in part, be ascribed to the fact that the negatively charged micelles repel the OH^- ions. However, with cetyl trimethylammonium bromide, the positively charged micelle should increase the reaction rate because OH^- ions concentrate at the positively charged micelle surface (23). In fact, a pronounced reduction of the rate is observed for the cationic system, indicating excellent steric protection (23). For the nonionic micelles, however, the previous considerations of this paper suggest that a substantial fraction of the solubilized benzocaine will be located in the mantle, where it is accessible to OH^- attack. From the data in Table I, the calculated fraction of solubilized molecules that reside in the mantle for stearyl $(EO)_{14}OH$ is 23%. The hydrolytic reactions were conducted in unsaturated systems, whereas the values in Table I were obtained for saturated solutions; cetyl ether and stearate esters are likely to show small differences in a and b values. Nevertheless, the similarity in the order of magnitude of the calculated fraction of benzocaine in the mantle for stearyl $(EO)_{14}OH$ (0.23) and the fractional decrease in the rate constant of benzocaine solubilized in cetyl $(EO)_{14}OH$ (0.22) as compared to free benzocaine suggests that the two-state model of solubilization in polyoxyethylene-type micelles should be of use in understanding chemical reactions of solubilized species.

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Keyphrases □ Benzoic acid derivatives—solubilization in nonionic surfactants, thermodynamic nonideality □ Thermodynamics, nonideal solubilization—benzoic acid derivatives in nonionic surfactants □ Solubilization, benzoic acid derivatives—in nonionic surfactants □ Micelles—effect of solubilizates on monomer–micelle equilibrium

Several early investigators (1–3), exploring the nature of the monomer–micelle equilibrium in micelle-forming surfactant solutions, made it clear that although micelles may form as a result of the association of many monomers, the laws of chemical equilibria governing association–dissociation reactions could still be applied to monomer–micelle equilibria. Indeed, the existence of a narrow range of concentrations, called the CMC, below which micelles are undetectable and above which nearly all additional surfactant solute forms additional micelles, and also the sharpness of the changes observed at the CMC could be related to the values of the equilibrium constants associated with the monomer–micelle equilibrium and the number of the monomers and counterions involved. More recently, many investigators have preferred a phase-separation model for micelle formation (see *Reference 4* for a survey of the literature). This model leads to a considerable simplification of the thermodynamic treatment of monomer–micelle equilibria. However, a substantial body of observations, summarized recently (4, 5), does not agree with this model; it has been stressed that the older mass-action model is to be preferred (4). Thus, a surfactant solution, below and above the CMC, can be looked upon as a two-component one-phase system.

When a solubilizate is now added to the system, it must, acting as a third component, affect the monomer–micelle equilibrium (6–8). Many experimental and theoretical studies indicate that the solubilizate does indeed affect the CMC and the molecular weights of micelles (7–11). Nevertheless, the analysis of many mixed surfactant systems, particularly mixtures of homologs involving only variations in hydrocarbon chain lengths, has suggested that the mixed micelle can be considered an ideal solution of its constituents (10). In other words, the mixed micelle can be treated in terms of a separate phase. Similarly, the solubilization of nonmicelle-forming species frequently has been treated in terms of a distribution of the solubilizate between the micelles and the nonmicellar fluid, treating the micelles as a separate phase (12). Thus, although the two-phase model seems to be inappropriate in describing monomer–micelle equilibria, it appears to be useful for solubilizing systems.

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The purposes of the present paper are to provide a simple rationale for this apparent contradiction and to

show why the distribution model based on the two-phase description of the system is frequently adequate. Based on this analysis, some nonidealities of solubilized systems are explored. It is shown, in particular, that the solubilization of preservatives in nonionic surfactants of the polyoxyethylene types can be described in terms of nonideality of mixing in the micelle phase and that the site-binding approximation of the Langmuir type, involving partial saturation of sites (13, 14), is probably unjustified.

DISTRIBUTION MODEL FOR SOLUBILIZATION

The recent elegant formulation of the thermodynamics of small systems by Hill (15) and its application to micellar systems by Hall and Pethica (16) greatly clarified the nature of the equilibria in micellar systems. The approach of thermodynamics of small systems seems to be particularly useful for describing solubilization (15, 16). The following discussion is based on this approach.

If a three-component system is considered where Component 1 is the solvent, Component 2 is the micelle-forming surfactant, and Component 3 is the solubilizate, the following equations can be written for the chemical potentials of the unmicellized Component 2, assumed to be monomeric, and Component 3 in free solution (16):

$$\mu_2^s = \mu_2^{os}(T,P) + kT \ln x_2^s\gamma_2^s \quad \text{(Eq. 1)}$$

$$\mu_3^s = \mu_3^{os}(T,P) + kT \ln x_3^s\gamma_3^s \quad \text{(Eq. 2)}$$

where μ = chemical potential, superscript s = free solution state, o = standard value, k = Boltzmann constant, T = absolute temperature, P = pressure, x = mole fraction, and γ = activity coefficient. For the same components in the micelle, one has:

$$\mu_2^m = \mu_2^{om}(T,P,\epsilon_m) + kT \ln x_2^m\gamma_2^m \quad \text{(Eq. 3)}$$

$$\mu_3^m = \mu_3^{om}(T,P,\epsilon_m) + kT \ln x_3^m\gamma_3^m \quad \text{(Eq. 4)}$$

where x_2^m and x_3^m = mole fractions in the micelle, and γ_2^m and γ_3^m = corresponding activity coefficients. Unlike the usual thermodynamics of large systems, in small system thermodynamics the standard μ^{om} values must be defined for constant$T$, P and also the so-called "subdivision potential" of the small system, ϵ_m (15, 16); ϵ_m is defined as:

$$\epsilon_m = -kT \ln x_m \quad \text{(Eq. 5)}$$

where x_m is the mole fraction of the micelle.

The monomer–micelle equilibrium and, therefore, the CMC in the presence of the solubilizate will, of course, be determined by the equation:

$$\mu_2^s = \mu_2^m \quad \text{(Eq. 6)}$$

Because of the dependence of μ_2^{om} on ϵ_m, Eq. 6, in the absence of a solubilizate (*i.e.*, in solutions of a pure surfactant) does not lead to the prediction of an infinitely sharp CMC or a constancy of the monomer activity above the CMC (16). These predictions, which are contrary to experience (4), have been the major objections against the earlier two-phase models for micelle formation based on macroscopic thermodynamics (4).

The usual operational definition of the CMC (4), where a small fraction of the order of 2% of the surfactant is micellized, corresponds effectively to a constant value of ϵ_m or x_m at a constant temperature in solubilizing systems. Equations 6 and 3 clearly show that any substance being solubilized by the micelle will affect the CMC through x_2^m and γ_2^m.

If Component 3, the solubilized component, is considered, one has at equilibrium:

$$\mu_3^s = \mu_3^m \quad \text{(Eq. 7)}$$

From Eqs. 2 and 4, one has:

$$(\mu_3^{om} - \mu_3^{os}) = -kT \ln (x_3^m\gamma_3^m/x_3^s\gamma_3^s) = -kT \ln K_3 \quad \text{(Eq. 8)}$$

where K_3 is the distribution coefficient of Component 3 between micelles and the free solution:

$$K_3 = \frac{x_3^m\gamma_3^m}{x_3^s\gamma_3^s} \quad \text{(Eq. 9)}$$

Unlike the distribution coefficients between bulk phases, K_3 is not only a function of T,P but also ϵ_m, and, therefore, the micellar concentration x_m. This dependence was derived by Hall and Pethica (6) to be:

$$-\left(\frac{\partial \ln K_3}{\partial \ln x_m}\right)_{T,P} = \frac{1}{\bar{N}_3^o} \quad \text{(Eq. 10)}$$

where $\bar{N}_3^o$ is the average value of the number of "micellized" solubilizate molecules of Component 3 in its standard state. If the interactions between the monomers and the solubilized component *in the micelles* can be considered to be ideal over the whole composition range $0 < x_3^m < 1$, then the standard state for Component 3 is a small system of pure 3 at the same T, P and ϵ_m as those of the mixed micellar system composed of monomers and the solubilized species. For nonmicelle-forming solubilized species, this "ideal" value for $\bar{N}_3^o$ when Component 3 forms its own "micelles" should be infinity; therefore, K_3 should be independent of x_m, and the distribution model should hold. When the solubilizate is a solid, the whole composition range may not be experimentally accessible, even though the mixing is ideal. Most solubilized systems are expected to be nonideal. It is unlikely, however, that the nonideality effects would alter the distribution model itself drastically. If the experimentally obtained ratio, x_3^m/x_3^s, can be shown to be independent of x_m, it would provide good evidence that K_3 is independent of x_m and can be handled as a bulk-phase distribution constant.

In the application of the distribution equation to practical situations, the equivalent concentration of micellized surfactant is needed for the calculation of x_3^m. This is usually calculated on the assumption that the surfactant monomer concentration remains constant above the CMC, its value being that of the CMC. This assumption is usually satisfactory when micellar degrees of association are high (16, 17). However, the CMC itself is affected by the presence of the solubilizate to a variable degree, depending upon $x_2^m\gamma_2^m$ (Eq. 3). In solubilizing systems where x_3^s is allowed to vary, x_3^m is substantial, and the total surfactant concentration is such that changes in the CMC are significant; this factor may be important in calculating x_m. However, in many solubilization experiments, particularly when long-chain nonionic surfactants are used, the CMC itself may be low enough to be negligible. In many solubilization experiments performed at saturation concentrations of the solubilizate, *i.e.*, an effectively constant value of $x_3^s\gamma_3^s$, the amount solubilized increases linearly with the total concentration of the surfactant above the CMC of the mixed system as determined from the solubilization experiment itself, even though the CMC of the mixed system may be substantially different from the CMC in the absence of the solubilizate (18). Such systems are consistent, therefore, with the distribution equilibrium with a constant x_3^m and γ_3^m and with a constant value of the CMC of the mixed system. However, systems are known [for example, the solubilization of some slightly soluble dyes in dodecylamine hydrochloride (19–21)] where the linear behavior is not maintained at high concentrations of micelles, suggesting changes in γ_3^m.

When x_3^s is very small, as in the systems to be discussed here, γ_3^s may be considered to be unity. The distribution coefficients of the solubilizate between the micelles and the nonmicellar solutions may, therefore, be redefined as:

$$K_D' = \frac{xf}{c} \quad \text{(Eq. 11)}$$

and:

$$K_D = \frac{x\gamma}{c} \quad \text{(Eq. 12)}$$

where, after dropping all the subscripts and superscripts, x = mole fraction of the solubilized component *in the micelle; c*, the molar concentration of the solubilizate in the solution, is proportional to x_3^s; γ = activity coefficient of the solubilized component in the micelle, defined for the infinite dilution standard state, so that $\gamma \to 1$ as

$x \rightarrow 0$; and f = activity coefficient defined with respect to the pure component as the standard state. For ideal mixtures, f is unity.

HENRY'S LAW BEHAVIOR

As is well known for bulk solutions (22), irrespective of the nature of the nonideality, in dilute solutions (*i.e.*, when $x \rightarrow 0$) $\gamma \rightarrow 1$ and $f \rightarrow$ constant. Therefore, x should be proportional to c. Such behavior, akin to Henry's law for gas solubilities, was reported for the solubility of propylene in potassium oleate solutions (12).

A corollary of this result is that for two solubilizates of similar structures, a and b, and similar kinds of interactions with micelles, the solubilities in different micelles may be related, particularly if the solubility is low. In other words, for different micelles the changes in f for a and b may be similar so that the saturation solubilities, x_a and x_b, may be proportional to each other. Such proportionalities were observed for the solubilities of several solubilizates in potassium myristate and potassium laurate (12). Figure 1 shows an example of the correlation of the solubilizing power of various micellar systems (expressed as the mole fraction of the solubilizate in the micellized surfactant at saturation) over a wide range for two solubilizate dyes, dimethylaminoazobenzene and Orange OT, the data being taken from the extensive work of Kolthoff and his coworkers (19–21). Such correlations may have some predictive value. The rough linearity of this correlation is presumably due to the facts that the solubilizing powers of micellar systems and liquid hydrocarbons of similar chain lengths are similar for these two dyes (19–21) and the value of x is low, so that the variation in f with x is small. For *trans*-azobenzene and naphthalene, for which x is considerably higher, the correlation is no longer linear (Fig. 1), suggesting that changes in f are no longer parallel, and changes in γ are significant because x is not small.

NONIDEALITY OF SOLUBILIZATION OF PRESERVATIVES IN NONIONIC SURFACTANTS

The solubilization of benzoic acid and various phenolic preservatives in polyoxyethylene-type surfactant micelles was investigated by a variety of techniques in unsaturated solutions (13, 14). The apparent distribution coefficient, *i.e.*, the amount solubilized per equivalent of surfactant at a particular equilibrium concentration of the free solubilizate, is usually found to be independent of the surfactant concentration (*i.e.*, x_m), providing experimental evidence that K_3 in Eq. 9 is a true constant independent of x_m. However, when the data are plotted as x *versus* c, pronounced deviations from Henry's law behavior are observed. Patel and Kostenbauder (13), who studied the solubilization of methyl *p*-hydroxybenzoate in polysorbate 80[1], assumed that solubilization occurs in the polyoxyethylene layer and used a Langmuir-type site binding picture for solubilization to explain their data. Donbrow *et al.* (14) arrived at different conclusions about the locus of solubilization but also used the Langmuir-type analysis. In another paper (23), it was shown that solubilized preservatives are associated in part with the hydrocarbon core of the nonionic micelles and in part with their polyoxyethylene mantle, the distribution between these two loci depending to a great extent on the molecular structure of the preservatives. These considerations suggest that the major part, about 70%, of the solubilized methyl *p*-hydroxybenzoate should be associated with the core of the polysorbate 80 micelles.

Although some evidence was recently obtained about *some* solid-like character of the micelle core (24), a variety of other evidence suggests that the micelle core is essentially fluid in nature. The description of the solubilization in terms of binding to fixed sites, an essential ingredient of the Langmuir model, does not, therefore, seem to be physically plausible. On the other hand, the concentrations of the preservatives in the micelles are often high, with a mole fraction as high as 0.5, so that substantial nonidealities are expected, particularly in view of the asymmetric nature of methyl *p*-hydroxybenzoate. It seems reasonable, therefore, to attempt to explore if the solubilization behavior can be interpreted in terms of nonideality of mixing in the micelle phase.

[1] Tween 80.

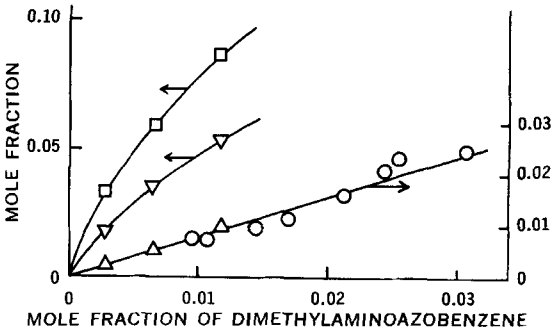

Figure 1—*Correlation of solubilizing powers of different surfactants for different solubilizates. Key:* ○, *Orange OT, in potassium laurate and dodecylamine hydrochloride solutions, at various concentrations of electrolyte and surfactant, 50°;* △, ▽, *and* □ *are all in potassium caprate, potassium laurate, and potassium myristate solutions (in order of increasing mole fraction) at 30°,* △, *Orange OT;* ▽, *trans-azobenzene; and* □, *naphthalene.*

Since the numerical data were not available, the distribution data for a typical system (methyl *p*-hydroxybenzoate in polysorbate 80) were obtained from the Langmuir-type equation:

$$\frac{\text{grams of polysorbate 80}}{\text{moles of methyl } p\text{-hydroxybenzoate}} = 2.5 \times 10^2 + \frac{11.6}{c} \quad \text{(Eq. 13)}$$

and were found to fit the data by Patel and Kostenbauder (13), where c = molar concentration of free methyl *p*-hydroxybenzoate. The numerical constants were derived from the straight-line plot of the authors, and "synthetic" distribution data were generated from the equation in the range of 0.0025–0.01 mole/l. for c, the range covered by the authors. The "synthetic" data are thus in exact agreement with the Langmuir-type equation. These data were then converted to mole fractions of methyl *p*-hydroxybenzoate in the micelle by using the estimated molecular weight of 1400 for the surfactant. No attempt was made to treat the fraction solubilized in the mantle separately; that is, the nonidealities were assumed to be similar for the solubilizate molecules in the two loci. Since roughly 70% of the solubilization is expected to be in the micelle core, reasonable corrections for the fraction in the mantle do not change the qualitative conclusions.

REGULAR SOLUTION APPROACH

The simplest type of nonideality of mixing is displayed by the so-called regular solutions (22) for which the activity coefficient f of the solubilized component in the micelle can be expressed as:

$$\ln f = (1 - x)^2 \omega / RT \quad \text{(Eq. 14)}$$

where ω = an interaction energy parameter, R = molar gas con-

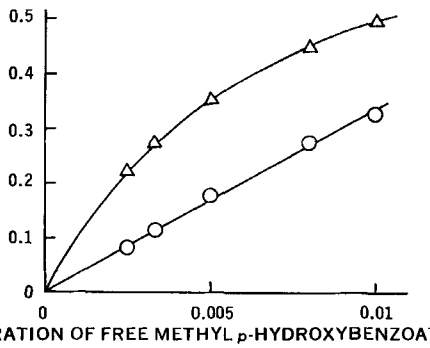

Figure 2—*Distribution behavior of methyl* p*-hydroxybenzoate in polysorbate 80 micelles. Key:* △, *mole fraction* x *of methyl* p*-hydroxybenzoate in micelles; and* ○, xf, *the activity coefficient* f *calculated by using regular solution theory.*

stant, and T = absolute temperature. For such solutions, as $x \rightarrow 0$, $\rightarrow \exp(\omega/RT)$. For the surfactant component, the activity coefficient f' is given by:

$$\ln f' = x^2\omega/RT \qquad \text{(Eq. 15)}$$

so that $f' \rightarrow 1$ as $x \rightarrow 0$.

In Fig. 2, a plot of x *versus* c shows the pronounced deviation from Henry's law at high x values. By using the activity coefficient from the regular solution theory, with $\omega/RT = -1.64$, the product xf, however, varies linearly with c within an average variation of about 2.5%, which is of the order of the experimental error. The distribution law (Eq. 11) is thus obeyed. The solubilization data are thus in good accord with a simple picture of nonideality. It should be stressed that the regular solution theory uses two parameters to relate the x *versus* c data, namely, K_D' and ω/RT, just as the Langmuir-type analysis does.

The success of the regular solution approach suggests that even fairly severe nonidealities in solubilized systems may be amenable to analysis in terms of the distribution law.

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Bis-Quaternary Ammonium Compounds: Derivatives and Congeners of Bicyclo[2.2.2]octane

JOSEPH G. CANNON*, KIM WENN YANG*, MELITA RODRIGUEZ†‡, and JOSEPH P. BUCKLEY†

Abstract □ A series of rigid congeners of hexamethonium was prepared, based upon bicyclo[2.2.2]octane, in which the interquaternary distance is varied and in which this distance is known with some degree of certainty. Data on hypotensive activities, taken as an index of ganglionic blocking potency, are consistent with earlier proposals that an interquaternary distance of 6.5–7.5 Å is optimum for polyalkylene bis-quaternary ganglionic blocking agents.

Keyphrases □ Hexamethonium congeners—bicyclo[2.2.2]octane, hypotensive activity □ Bicyclo[2.2.2]octane derivatives—interquaternary distances correlated with hypotensive effects □ Hypotensive activity—bis-quaternary ammonium compounds of bicyclo[2.2.2]octane □ Bis-quaternary ammonium compounds—interquaternary distance correlated with hypotensive effects

There has been controversy in the literature for many years concerning the validity of the "two-point attachment" hypothesis of Barlow and Ing (1) for explaining the ganglionic blocking mechanism of bis-quaternary ammonium salts, typified by hexamethonium. The attractive theory that the length of the most active bis-quaternary polyalkylene molecule corresponds to, or even is a measure of, an interreceptor distance is complicated by the fact that these flexible compounds can assume an infinite number of conformations and, consequently, their "molecular length" cannot be defined precisely. Gill (2) concluded that ganglionic blocking molecules must possess a "range" of interquaternary distances between the limits of 6 and 7.8 Å. Biel and DiPierro (3) introduced a triple bond into a series of C_5 and C_6 bis-quaternary compounds and found that these derivatives (in which the rigid carbon–carbon triple bond limits somewhat the flexibility of the carbon chain and forces it into a more extended form) were more potent than their saturated parent compounds in producing hypotension, which was considered to be at least in part due to an action at autonomic ganglia. This effect of the acetylenic link on blood pressure was verified in somewhat similar C_5 and C_6 bis-quaternary polyalkylene systems by Neumeyer *et al.* (4). They reported that when the triple bond was reduced to a *cis*-olefinic group, hypotensive activity diminished, but that reduction to a *trans*-olefin increased activity above that observed for the parent saturated molecule. It was concluded that the

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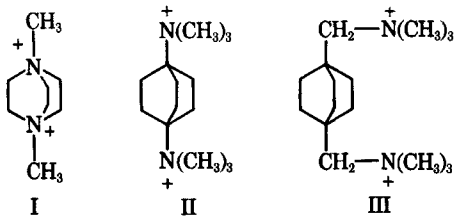

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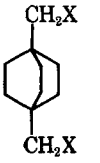
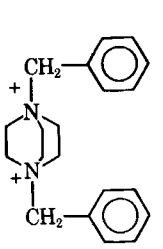
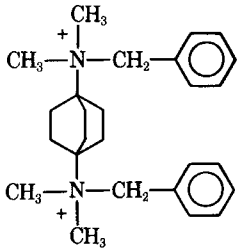


IX: X = Cl
X: X = Br



XI



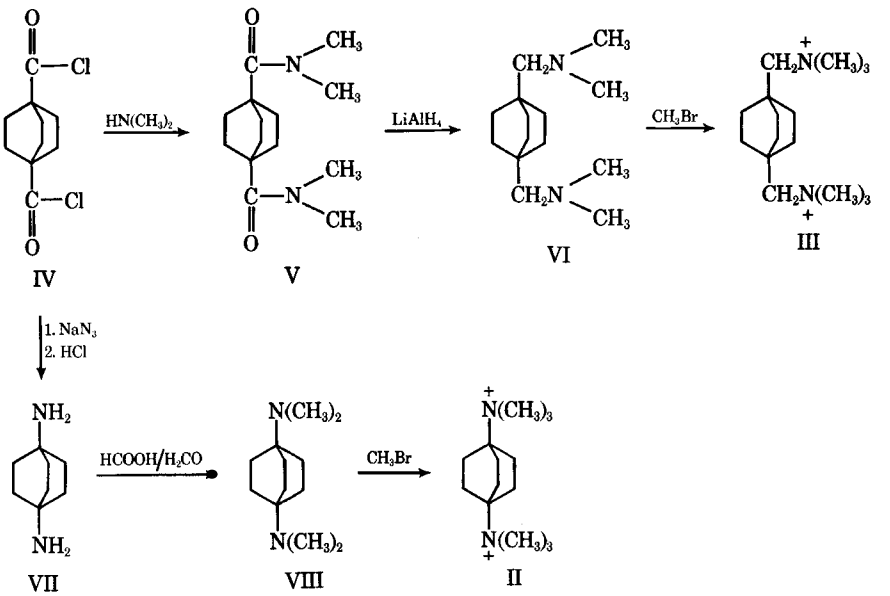
XII

geometry of the *cis*-double bond, in which the polyalkylene chain is folded back over itself, is detrimental to hypotensive effect, whereas the imposition of a more extended conformation upon the chain (by acetylenic or *trans*-olefinic groups) tends to enhance hypotensive activity. Neumeyer (5) concluded that maximal hypotensive activity is manifested when the distance between the two quaternary centers is restricted between 6.5 and 7.5 Å.

A fundamental problem common to all of these compounds is that, despite the introduction of some degree of rigidity into the systems, a considerable amount of flexibility yet remains in the chains joining the quaternary heads and, hence, the interquaternary distances at *in vivo* receptors cannot be precisely defined. In addition, many of the moieties introduced into bis-quaternary compounds to impart rigidity and some degree of conformational integrity [benzene rings (6), acetylenic links (3, 4), and cyclopropane rings (7)] contain π-electrons or possess some degree of unsaturated character. These moieties might well influence physical and chemical properties of the molecule to the extent that they could affect biological activity. The bicyclo[2.2.2]octane system represents a rigid, relatively nonstrained system, lacking a π-electron cloud and having no unsaturated character. 1,4-Diazabicyclo[2.2.2]octane has a similar character, and it was the purpose of the work described here to utilize these two ring systems as the method of imparting a greater rigidity to bis-quaternary systems related to hexamethonium, such that the interquaternary distances could be determined with some degree of confidence and so that attempts could be made to correlate these distances with hypotensive effects.

Specifically, three compounds, I, II, and III, seemed of interest. Inspection of Dreiding models revealed that the interquaternary distances in the diazabicyclooctane derivative (I) and in the 1,4-bis-quaternary ammonium bicyclo[2.2.2]octane system (II) are fixed and rigidly held, being approximately 2.60 and 6.12 Å, respectively. The bis-1,4-(trimethylammoniummethyl)bicyclo[2.2.2]octane system (III) possesses somewhat more flexibility; the range of interquaternary distances in the two conformational extremes of this molecule is between 6.5 and 7.7 Å. Thus, it might be predicted that, on the basis of Gill's (2) and Neumeyer's (5) proposals, Compound III would exhibit the greatest hypotensive effect; Compound II, being on the borderline of optimal interquaternary distance, might show some hypotensive effect; and Compound I should be inert.

The commercial availability of 1,4-diazabicyclo-[2.2.2]octane made possible the facile preparation of Compound I.



Scheme I—Preparation of Diaminobicyclo[2.2.2]octane Derivatives

Compounds II and III were prepared (Scheme I) from bicyclo[2.2.2]octane-1,4-dicarbonyl chloride (IV), which was prepared by a combination of literature methods. An alternate route to the diamide (V) involved aminolysis of diethyl bicyclo[2.2.2]octane-1,4-dicarboxylate (XV) with dimethylamine in the presence of *n*-butyllithium; this procedure is described in a separate article (8).

Attempts to prepare Compound III by alkylation of trimethylamine with 1,4-dichloromethylbicyclo[2.2.2]-octane (IX) or with its dibromo analog (X) were unsuccessful; the starting halogen systems were inert toward trimethylamine at steam bath temperatures. The bicyclo[2.2.2]octane ring system exhibits many of the chemical properties of a neopentyl group. It was reported (9) that neopentyl halides are extremely unreactive toward many reagents in bimolecular reactions. Holtz and Stock (10) showed that 1-tosyloxymethylbicyclo[2.2.2]octane has neopentyl character and reacts in bimolecular reactions only with powerful nucleophiles.

1,4-Diazabicyclo[2.2.2]octane was diquaternized with a bulky group, benzyl bromide, as was 1,4-bis-(dimethylamino)bicyclo[2.2.2]octane (VIII); these bis-quaternary compounds (XI and XII) were submitted for biological evaluation.

DISCUSSION

Hypotensive effects are summarized in Table I. Compounds I, II, and III exhibited relative potencies consistent with the predictions based upon the interquaternary distances cited previously. Compound III, having an interquaternary distance range of 6.5–7.7 Å, was the only compound in the series effective at 1 mg./kg. Insofar as hypotensive effect is a measure of ganglionic blocking potency, these data support the proposal that optimal interquaternary distance for ganglionic blockade is in the region 6.5–7.5 Å. The low order of activity of XI and XII can be ascribed, at least in part, to their possessing interquaternary distances below the optimal range. Compound VI, the tertiary amine analog of the most active bis-quaternary, III, exhibited moderate hypotensive effects which may merit further investigation.

Table I—Hypotensive Effects of Bis-Quaternary Compounds in Anesthetized Rats[a]

Compound Number	Number of Rats	Decrease in Mean Blood Pressure, mm. Hg —Dose Levels, mg./kg.— 1.0	2.5	5.0	7.5	10.0
I	4	—	2.0	10.0	14.0	29.0
II	4	—	20.2	40.2	52.7	72.0
III	4	16	36.0	85.0	—[b]	—
XI	4	—	13.1	19.2	20.2	25.0
XII	4	—	4.0	13.2	17.2	46.2
VI[c]	4	—	3.0	10.0	25.0	53.0
Mecamylamine	6	—	—	34.0	—	—
Hexamethonium	8	—	—	35.0	—	—

[a] Urethan, 1.2 g./kg. i.p. [b] All rats died with this dose. [c] Ditertiary amine analog of III.

EXPERIMENTAL[1]

Diethyl 2,5-Diketobicyclo[2.2.2]octane-1,4-dicarboxylate (XIII)—This compound was prepared in 45% yield by the method of Holtz and Stock (11), m.p. 112° [lit. (11) m.p. 111–112°].

Bis-Ethylenethioketal of Diethyl 2,5-Diketobicyclo[2.2.2]octane-1,4-dicarboxylate (XIV)—This compound was prepared from XIII in 95% yield by the method of Roberts *et al.* (12), m.p. 90.5–92° [lit. (12) m.p. 91.8–92.7°].

Diethyl Bicyclo[2.2.2]octane-1,4-dicarboxylate (XV)—This compound was prepared in 82% yield by Raney nickel desulfurization of XIV, as described by Roberts *et al.* (12), b.p. 90–105° (0.01 mm.), n_D^{26} 1.4660 [lit. (12) b.p. 113–115° (0.3 mm.), n_D^{25} 1.4667].

Bicyclo[2.2.2]octane-1,4-dicarbonyl Chloride (IV)—This compound was prepared in 89% yield from XV by the procedure of Kauer *et al.* (13), m.p. 96–98° [lit. (13) m.p. 97.5–98.5°].

1,4-Diaminobicyclo[2.2.2]octane (VII)—This compound was prepared from IV by the method of Kauer *et al.* (13), m.p. 67–68.5° [lit. (13) m.p. 67.5–68.5°].

1,4-Bis(dimethylamino)bicyclo[2.2.2]octane (VIII)—This procedure was adapted from a method of Baltzly and Buck (14). A mixture of 15.0 g. (0.07 mole) of VII, 70 ml. of 37% formaldehyde solution, and 0.2 ml. of 90% formic acid was heated in a bomb at 135–145° for 24 hr. The resulting clear, colorless solution was treated with 10 ml. of concentrated HCl and evaporated to dryness under reduced pressure from a water bath. The solid residue was suspended in 100 ml. of $CHCl_3$ and shaken with 50 ml. of H_2O containing 10 g. of KOH. The organic layer was separated, dried (Na_2SO_4), filtered, and evaporated to leave a white solid, which was recrystallized twice from H_2O to give 11.8 g. (86%) of product, m.p. 47–48°.

Anal.—Calc. for $C_{12}H_{24}N_2$: C, 73.41; H, 12.32; N, 14.22. Found: C, 73.24; H, 12.21; N, 13.99.

A picrate salt was recrystallized from EtOH, m.p. 240.6° dec.

Anal.—Calc. for $C_{24}H_{30}N_8O_4$: C, 44.04; H, 4.62; N, 17.12. Found: C, 44.28; H, 4.80; N, 16.76.

1,4-Bis(hydroxymethyl)bicyclo[2.2.2]octane (XVI)—Compound XV (21 g., 0.083 mole) was added dropwise to a refluxing suspension of 9 g. (0.24 mole) of $LiAlH_4$ in 200 ml. of purified tetrahydrofuran, and the resulting mixture was refluxed for 10 hr. The excess $LiAlH_4$ was decomposed with 10 ml. of H_2O, and the resulting mixture was filtered; the solid on the filter was washed with three 50-ml. portions of Et_2O which were added to the clear filtrate. This solution was dried (Na_2SO_4) and filtered; removal of the volatiles from the filtrate resulted in a viscous, liquid residue which solidified upon standing and was recrystallized from benzene; yield, 10.5 g. (72%); m.p. 108.2° [lit. (11) m.p. 107–108°].

1,4-Bis(chloromethyl)bicyclo[2.2.2]octane (IX)—Thionyl chloride (15 ml., 25 g., 0.21 mole) was added dropwise to a solution of 5.4 g. (0.032 mole) of XVI in 10 ml. of dry pyridine. Then the reaction mixture was refluxed for 12 hr. Volatiles were removed under reduced pressure on a steam bath, and the residue was extracted with Et_2O. After evaporation of the ethereal extract, the liquid residue was distilled, b.p. 74–77° (0.1 mm.), to afford 3.6 g. (53%) of material, n_D^{28} 1.4619.

Anal.—Calc. for $C_{10}H_{16}Cl_2$: C, 57.98; H, 7.79; Cl, 34.23. Found: C, 57.82; H, 7.82; Cl, 34.23.

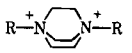
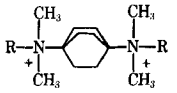
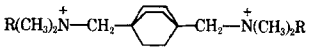
1,4-Bis(bromomethyl)bicyclo[2.2.2]octane (X)—Phosphorus tribromide (10 ml., 28.5 g., 0.106 mole) was added dropwise to 5 g. (0.030 mole) of XVI, and the reaction mixture was refluxed for 10 hr. The excess phosphorus tribromide was decomposed with H_2O; a large amount of tar separated. This mixture was extracted with $CHCl_3$; the extract was dried (Na_2SO_4) and filtered, and the filtrate was taken to dryness on a steam bath. The dark-brown viscous residue was chromatographed on a 20 × 2-cm. neutral alumina column and was eluted with $CHCl_3$. The residue from evaporation of the eluate was distilled at 110–120° (1.0 mm.) to yield 1.2 g. (13%) of product, n_D^{30} 1.5573.

Anal.—Calc. for $C_{10}H_{16}Br_2$: C, 40.57; H, 5.41; Br, 53.99. Found: C, 40.69; H, 5.78; Br, 53.24.

***N,N,N',N'*-Tetramethylbicyclo[2.2.2]octane-1,4-dicarboxamide (V)**—To 6.0 g. (0.02 mole) of IV in 40 ml. of anhydrous acetone, cooled in an ice water bath, was added with stirring an excess of anhydrous dimethylamine. The reaction mixture was filtered, and the solid that collected on the filter was washed with a small volume of anhydrous acetone which was added to the filtrate. The combined organic solutions were evaporated, and the residue was recrystallized from EtOH–H_2O, m.p. 161–162°; yield, 6 g. (92%); IR (KBr) 1605 $cm.^{-1}$ (amide C=O).

[1] All boiling points are uncorrected; melting points were determined with a Du Pont 900 differential thermal analyzer and/or in open glass capillaries in a Thomas-Hoover Uni-Melt apparatus and are corrected. Elemental analyses were performed by Hufmann Laboratories, Wheatridge, Colo., and Galbraith Laboratories, Knoxville, Tenn. IR spectra were recorded on Beckman IR5-A and IR-10 instruments.

Table II—Bis-Quaternary Ammonium Compounds

Compound Number	R	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
						
I	CH_3[a]	300–301°[b]	75	$C_8H_{18}Br_2N_2$	C, 31.81 H, 6.01 N, 9.27	C, 31.88 H, 6.04 N, 9.61
XI	$CH_2C_6H_5$	274–275°[c]	55	$C_{20}H_{26}Br_2N_2$	C, 52.88 H, 5.77 N, 6.17	C, 52.72 H, 6.08 N, 6.25
						
II	CH_3	Above 300°[b]	52	$C_{14}H_{30}Br_2N_2$	C, 43.54 H, 7.83 N, 7.25	C, 43.54 H, 7.84 N, 6.89
XII	$CH_2C_6H_5$	280–281°[c]	46	$C_{26}H_{38}Br_2N_2$	C, 57.94 H, 7.11 N, 5.20	C, 57.82 H, 7.13 N, 4.89
						
III	CH_3	Above 300°[b]	64	$C_{16}H_{34}Br_2N_2$	C, 46.39 H, 8.27 N, 6.77	C, 46.58 H, 8.25 N, 6.87

[a] 1,4-Diazabicyclo[2.2.2]octane obtained from Aldrich Chemical Co. [b] From methanol. [c] From ethanol.

Anal.—Calc. for $C_{14}H_{24}N_2O_2$: C, 66.60; H, 9.59; N, 11.10. Found: C, 66.58; H, 9.45; N, 11.17.

1,4-Bis(dimethylaminomethyl)bicyclo[2.2.2]octane (VI)—A solution of 6.3 g. (0.025 mole) of the diamide (V) in 30 ml. of purified tetrahydrofuran was added dropwise to a suspension of 5 g. of $LiAlH_4$ in 50 ml. of purified tetrahydrofuran, and the resulting mixture was refluxed for 2 hr. Excess $LiAlH_4$ was decomposed with 5 ml. of H_2O, and the resulting mixture was filtered. The solid on the filter was washed with three 50-ml. portions of Et_2O which were added to the filtrate. This combined solution was dried (Na_2SO_4), filtered, and evaporated to afford a white solid residue, which was recrystallized from EtOH to yield 5.0 g. (89%) of product, m.p. 60–61°.

Anal.—Calc. for $C_{14}H_{28}N_2$: C, 74.91; H, 12.57; N, 12.51. Found: C, 74.91; H, 12.56; N, 12.24.

Bis-Quaternary Compounds—The appropriate diamine (0.02 mole) and an excess of methyl bromide or benzyl bromide in 10–15 ml. of 2-propanol were heated in a sealed tube on a steam bath for 4 hr. The reaction mixture was cooled, and the solid that separated was collected on a filter and recrystallized (Table II).

Pharmacology—Male Wistar rats, weighing between 200 and 250 g., were divided into six groups of four rats each. The animals were anesthetized with urethan, 1.2 g./kg. i.p., and secured to an operating board, ventral side up. A midline incision was made into the neck area, and the trachea was catheterized to permit free breathing. One of the carotid arteries was cannulated with a polyethylene catheter connected to a Statham pressure transducer, using the system filled with heparinized saline (5000 units/100 ml.). Blood pressure was recorded on a polygraph, and the femoral vein was catheterized for administration of the compounds. Each of the six groups of rats was treated with one of the compounds, using a dose schedule of 1.0, 2.5, 5.0, 7.5, and 10.0 mg./kg. with a minimum period of 10 min. between doses.

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2,4-Dinitrophenylhydrazine: A Sensitive Quantitative Reagent for Determining Microgram Quantities of Prednisone

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Abstract □ 2,4-Dinitrophenylhydrazine is suggested as a quantitative colorimetric reagent for the determination of microgram quantities of prednisone. The sensitivity of this reagent is compared with that of other steroid colorimetric reagents such as blue tetrazolium and isonicotinic acid hydrazide. Extraction procedures are presented which isolate prednisone from formulations containing salicylamide, acetaminophen, aspirin, and ascorbic acid, where the ratio of analgesics to steroid is 300 to 1. 2,4-Dinitrophenylhydrazine quantitatively determines prednisone in concentrations as low as 1.26×10^{-6} *M*.

Keyphrases □ 2,4-Dinitrophenylhydrazine—colorimetric reagent, analysis of prednisone □ Colorimetry—analysis, prednisone with 2,4-dinitrophenylhydrazine □ Prednisone—separation from analgesic mixtures, colorimetric analysis

Pharmaceuticals containing steroids in combination with aspirin, sodium salicylate, salicylamide, acetaminophen, ascorbic acid, and aluminum hydroxide gel are currently being marketed by pharmaceutical manufacturers. These steroids are usually the adrenocortical steroids (cortisone or derivatives of cortisone), of which prednisone is the most prevalent in these combinations. The ratio of the steroid to the analgesic component is usually in the order of 1 to 300.

The USP XVIII assay for prednisone tablets (1) cannot be used to assay these combinations without some modification of the extraction technique. The USP XVIII extraction procedure will not separate prednisone from these components, and the blue tetrazolium reagent (2) will not develop the characteristic steroid color unless the prednisone is completely separated. It was observed in this laboratory that salicylamide inhibits the development of this color (3). Graham *et al.* (4) found that a salicylamide–hydrocortisone ratio of 60:1 reduces the absorbance of the blue tetrazolium color by 2%.

Other commonly used reactions for steroid analysis are the Porter–Silber reaction (5, 6) and the Umberger reaction (7). The Porter–Silber reaction uses phenylhydrazine–sulfuric acid as a colorimetric reagent which is specific for the 17,21-dihydroxy-20-keto side chain of corticosteroids. The Umberger reaction uses isonicotinic acid hydrazide, which reacts with Δ^4-3-ketosteroids, as a colorimetric reagent.

2,4-Dinitrophenylhydrazine is a common reagent used extensively in the determination of aldehydes and ketones. Mason *et al.* (8) found that cortisone forms a C-3 monohydrazone with 2,4-dinitrophenylhydrazine in the cold. The quantitative isolation and determination of progesterone as the 2,4-dinitrophenylhydrazone are given in the NF XIII monograph for progesterone injections (9). Testosterone forms a 2,4-dinitrophenylhydrazone, but a quantitative isolation and gravimetric determination are not possible (10).

Lappin and Clark (11) used 2,4-dinitrophenylhydrazine as a colorimetric reagent in the determination of trace amounts of carbonyl compounds. They enhanced the sensitivity of the reagent by adding a solution of sodium or potassium hydroxide to an alcoholic solution of the 2,4-dinitrophenylhydrazone, producing a very intense wine-red color.

Gornall and MacDonald (12) quantitatively determined corticosteroids in extracts of urine, blood plasma, and tissues, using 2,4-dinitrophenylhydrazine. The concentration of these ketosteroids in the final extracts of these substances was in the milligram or microgram range. This low concentration prevented precipitation of the steroid hydrazone; however, the addition of alkali to the alcoholic hydrazone solution produced a wine-red color having a maximum absorbance between 425 and 495 nm., depending upon the specific ketosteroid being determined. The lowest concentration of steroid quantitatively determined by Gornall and MacDonald (12) was 6.38 μmoles/l. (cortisone) or 6.38×10^{-6} *M*.

The work of Gornall and MacDonald (12) suggested that their techniques could be applied to the assay of prednisone in tablets containing 1 mg. or less of prednisone and 350 mg. or more of salicylamide, acetaminophen, or aspirin. To eliminate possible interference by these analgesic components, the prednisone should be isolated before the formation of the 2,4-dinitrophenylhydrazone. Aspirin may be separated by extraction with 1 *N* sodium bicarbonate, and salicylamide and acetaminophen may be separated by extraction with aqueous alkaline solutions (pH 10 or more) or with acidic ferric chloride. Alkaline solutions of pH 10 or more should not be used because of the possibility of the degradation of the steroid.

In the present study, prednisone was extracted from the tablet excipients with methanol. The methanol extract was concentrated to a residue, which was dissolved in either sodium bicarbonate or acidic ferric chloride solutions and then extracted with chloroform.

Table I—Composition of Samples

Ratio	Synthetic Mixture	Commercial Tablet A (Colored, Uncoated)	Commercial Tablet B (White, Uncoated)	Commercial Tablet C (Colored, Enteric Coated)
	Prednisone	Prednisone	Prednisone	Prednisone
	Salicylamide	Salicylamide	—	Salicylamide
	Aspirin	Acetaminophen	Aspirin	—
	Ascorbic acid	Ascorbic acid	Ascorbic acid	Ascorbic acid
	Starch	—	Aluminum hydroxide gel	—
	Magnesium stearate	—	—	—
	Sunset yellow dye	—	—	—
Salicylamide–prednisone	465:1	250:1	—	300:0.75
Aspirin–prednisone	462:1	250:1	325:0.75	—
Ascorbic acid–prednisone	35:1	25:1	20:0.75	50:0.75

To compare the sensitivities of three colorimetric reagents that could be used for the determinative step, aliquots of the same chloroform solution of the isolated prednisone were treated with the following: (*a*) blue tetrazolium, the USP XVIII steroid reagent (2); (*b*) isonicotinic acid hydrazide, Umberger's reagent (7); and (*c*) 2,4-dinitrophenylhydrazine, as used by Gornall and MacDonald (12).

The samples used in this study are shown in Table I.

EXPERIMENTAL

Apparatus—A recording UV visible spectrophotometer with 1-cm. cells was used.

Reagents—*Methyl Alcohol*—Use analytical reagent grade, acetone free.

Acidic Ferric Chloride—Mix 12 ml. of 60% $FeCl_3 \cdot 6H_2O$ with 48 ml. of 0.05 *N* hydrochloric acid.

Sodium Bicarbonate—Use 1 *N* solution.

Chloroform—Use analytical reagent grade.

Acid–Methanol (1:3)—Mix 1 volume HCl (C.P. reagent, sp. gr. 1.18) with 3 volumes of methyl alcohol. Prepare once a week.

Acid–Methanol (1:7)—Mix 1 volume of hydrochloric acid with 7 volumes of methyl alcohol. Prepare once a week.

2,4-Dinitrophenylhydrazine—Dissolve 100 mg. of 2,4-dinitrophenylhydrazine in 100 ml. of acid–methanol (1:3) (may be kept 1 week if stored in the cold).

Sodium Hydroxide—Protect prepared solution (4.0 *N*) from carbon dioxide to prevent formation of sodium carbonate, which would precipitate during the final dilution with methanol.

Blue Tetrazolium—Dissolve 50 mg. of blue tetrazolium in 10 ml. of 95% ethyl alcohol. Heating on steam bath facilitates solution of the blue tetrazolium. Filter solution if not clear.

Tetramethylammonium Hydroxide—Dilute 1 ml. of 10% aqueous tetramethylammonium hydroxide to 10 ml. with 95% ethyl alcohol.

Isonicotinic Acid Hydrazide—Dissolve 100 mg. of isonicotinic acid hydrazide in about 100 ml. of methanol, add 1 ml. of acid–methanol (1:7), and dilute to 200 ml. with methanol.

Standard Solution—Use prednisone USP reference standard, 0.01 mg./ml.

Procedure—*Sample Preparation*—Transfer a weighed amount of the powdered sample, equivalent to about 0.75–1 mg. of prednisone, to a 50-ml. conical flask. Add 30 ml. of methanol, place a small funnel in the flask (to act as a small condenser), and keep near the boiling point while stirring for 30 min., preferably with a magnetic stirrer. Allow to cool to room temperature, and filter into a 100-ml. volumetric flask. Wash the conical flask with five 10-ml. portions of methanol, pouring each portion through the filter. Dilute the combined filtrate and washings to 100 ml.; mix well.

Extraction—Samples containing salicylamide and/or acetaminophen and ascorbic acid: Concentrate a 50-ml. aliquot of the sample preparation to a residue on the steam bath, using a small air current. In each of three 60-ml. separators, place 5 ml. of acidic ferric chloride solution. Add 15 ml. of water each to Separators 2 and 3. Add 15 ml. of water to the sample residue, mix well to dissolve all water-soluble material, and add to Separator 1. Wash the residue beaker with 20 ml. of chloroform, and add to Separator 1. Shake for 1 min. and allow layers to separate. Draw off the chloroform layer from Separator 1 into Separator 2, and shake for 1 min. Then add the separated chloroform layer in Separator 2 to Separator 3 and shake. Salicylamide and acetaminophen should be completely removed from the chloroform layer in Separator 3; if the aqueous layer is

Table II—Determination of Prednisone in Synthetic and Commercial Samples with Three Reagents

Sample	Declaration, mg./Tablet	DNPH[a] Found, mg./Tablet	DNPH[a] Percent of Declaration	Blue Tetrazolium Found, mg./Tablet	Blue Tetrazolium Percent of Declaration	INH[b] Found, mg./Tablet	INH[b] Percent of Declaration
Synthetic mixture	1.016[c]	1.016	100.0	1.064	104.7	0.998	98.2
	1.016	1.016	100.0	1.064	104.7	0.998	98.2
	1.016	0.982	96.6	1.038	102.2	0.954	93.9
Av.	1.016	1.005	98.9	1.055	103.9	0.983	96.8
Commercial Tablet A	1.000	0.91	91.0	0.89	89.0	0.88	88.0
	1.000	0.97	97.0	0.92	92.0	0.92	92.0
Av.	1.000	0.94	94.0	0.905	90.5	0.90	90.0
Commercial Tablet B	0.750	0.74	98.7	0.79	105.3	0.69	92.0
	0.750	0.75	100.0	0.74	98.7	0.68	90.7
Av.	0.750	0.745	99.4	0.765	102.0	0.685	91.4
Commercial Tablet C	0.750	0.70	93.3	0.71	94.7	0.62	82.7
	0.750	0.70	93.3	0.75	100.0	0.63	84.0
Av.	0.750	0.70	93.3	0.73	97.4	0.625	83.4

[a] 2,4-Dinitrophenylhydrazine reagent. [b] Isonicotinic acid hydrazide reagent. [c] Synthetic mixture, mg./1 g.

Table III—Recovery Determinations of Prednisone in Combinations

Sample	DNPH[a] Added, mg.	DNPH[a] Found, mg.	DNPH[a] Percent of Recovery	Blue Tetrazolium Added, mg.	Blue Tetrazolium Found, mg.	Blue Tetrazolium Percent of recovery	INH[b] Added, mg.	INH[b] Found, mg.	INH[b] Percent of Recovery
Standard prednisone	2.12	2.01	94.8	2.12	2.04	96.2	2.12	1.99	93.9
	0.530	0.539	101.7	0.530	0.537	101.3	0.530	0.501	94.5
	0.530	0.526	99.2	0.530	0.509	96.0	0.530	0.508	95.8
Av.			98.6			97.8			94.7
Standard prednisone + Tablet A	1.164	1.156	99.3	1.165	1.110	95.3	1.164	1.093	93.9
	1.250	1.292	103.4	1.250	1.236	98.9	1.250	1.220	97.6
Av.			101.4			97.1			95.8
Standard prednisone + Tablet B	1.034	1.012	97.9	1.034	1.058	102.3	1.034	0.982	95.0
	1.044	1.081	103.5	1.044	1.054	100.9	1.044	1.031	98.8
Av.			100.7			101.6			96.9
Standard prednisone + Tablet C	1.150	1.152	100.2	1.150	1.246	108.3	1.150	1.129	98.2
	1.048	1.063	101.4	1.048	1.181	112.7	1.048	1.030	98.3
Av.			100.8			110.5			98.2

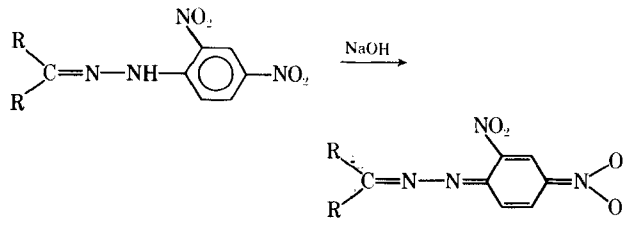
[a] 2,4-Dinitrophenylhydrazine reagent. [b] Isonicotinic acid hydrazide reagent.

purple (indicating the presence of salicylamide or acetaminophen), extract the chloroform layer from Separator 3 with another portion of water and acidic ferric chloride solution. Repeat the extraction procedure, using three 20-ml. portions of chloroform to wash out the residue beaker and passing each portion successively through Separators 2 and 3. Wash the combined chloroform extracts with 20 ml. of water. Concentrate the washed extracts to about 25 ml., transfer quantitatively to a 50-ml. volumetric flask, and dilute to volume with chloroform.

Samples containing aspirin and ascorbic acid: Concentrate a 50-ml. aliquot of the sample preparation to a residue on the steam bath. Add 20 ml. of 1 *N* sodium bicarbonate to the sample residue, mix well to dissolve all water-soluble material, and place in a separator. Wash the residue beaker with 20 ml. of chloroform, and add the wash to the separator. Shake for 1 min.; allow layers to separate. Draw off the chloroform layer into a second separator containing 20 ml. of 1 *N* sodium bicarbonate, and shake for 1 min. Repeat the extraction procedure, using three 20-ml. portions of chloroform to wash out the residue beaker and passing each portion successively through both separators. Wash the combined chloroform extracts with 20 ml. of water. Concentrate the washed extracts to about 25 ml., transfer quantitatively to a 50-ml. volumetric flask, and dilute to volume with chloroform.

Synthetic mixture: Use the combined extraction procedure for samples containing salicylamide and/or acetaminophen and samples containing only aspirin.

Color Development—*2,4-Dinitrophenylhydrazine*—Evaporate to dryness an aliquot of the chloroform solution of extracted prednisone equivalent to about 100 mcg. of prednisone. Avoid excessive heating of the dry residue. Dissolve the residue in 1 ml. of methanol, and transfer to a small (5-ml.) glass-stoppered tube. Quantitatively rinse the residue beaker with four 1-ml. portions of methanol, and add to the small tube. Carefully concentrate the methanol solution in the tube to about 1 ml. Wash down the sides of the tube with 1 ml. of methanol, and add 2.0 ml. (pipet) of 2,4-dinitrophenylhydrazine reagent. Loosely stopper the tube, and heat for 2 hr. at 60° (a constant-temperature heating block is extremely useful for this purpose). Cool to room temperature, and transfer quantitatively to a 25-ml. volumetric flask containing 2.0 ml. (pipet) of 4.0 *N* sodium hydroxide and 10 ml. of methanol.



Scheme I

Mix well, dilute to volume with methanol, and allow to stand for 30 min.

Prepare a reagent blank by carrying a mixture of 2.0 ml. of methanol and 2.0 ml. of 2,4-dinitrophenylhydrazine reagent through the color development.

Evaporate a 10-ml. aliquot of the standard prednisone solution to about 1 ml., transfer quantitatively to a small glass-stoppered tube, and treat like the sample.

Scan the sample and standard solutions between 750 and 400 nm., using the reagent blank as the reference. The maximum absorbance occurs at about 480 nm.

Blue Tetrazolium—Evaporate to dryness, in separate 25-ml. glass-stoppered conical flasks, an aliquot of the chloroform solution of extracted prednisone equivalent to about 100 mcg. of prednisone and a 10-ml. aliquot of the standard prednisone solution. Add 10 ml. of 95% ethyl alcohol to each of the residue flasks and to a blank flask. Add 2.0 ml. of blue tetrazolium reagent to each flask, mix well, and then add 2.0 ml. of the diluted tetramethylammonium hydroxide solution. Mix well; allow to stand 90 min. in the dark. Scan the sample and standard solutions between 750 and 400 nm., using the reagent blank as the reference. The maximum absorbance occurs at about 525 nm.

Isonicotinic Acid Hydrazide—Evaporate to dryness, in separate 25-ml. glass-stoppered conical flasks, an aliquot of the chloroform solution of extracted prednisone equivalent to about 100 mcg. of prednisone and a 10-ml. aliquot of the standard prednisone solution. Add 10 ml. of the isonicotinic acid hydrazide reagent to each of the residue flasks and to a blank flask. Swirl residue flasks to ensure complete solution of the residues, and allow to stand at room temperature for 2 hr. Scan the sample and standard solutions between 600 and 300 nm., using the reagent blank as a reference. The maximum absorbance occurs at about 410 nm.

RESULTS AND DISCUSSION

The results of the prednisone determinations in which the three colorimetric reagents were used are tabulated in Table II. The average prednisone values obtained with 2,4-dinitrophenylhydrazine ranged from 93.3 to 99.4% of the known or declared amount of prednisone; the range was 90.5–103.9% with blue tetrazolium and 83.4–96.8% with isonicotinic acid hydrazide. A comparison of these values indicates the accuracy and precision of the assays performed with 2,4-dinitrophenylhydrazine as the colorimetric reagent.

Recovery determinations for sample solutions spiked with known weights of prednisone and for solutions of standard prednisone (a procedural standard) carried through the entire assay procedure are tabulated in Table III. The average percentage recoveries from the procedural standard were 98.6% (2,4-dinitrophenylhydrazine), 97.8% (blue tetrazolium), and 94.7% (isonicotinic acid hydrazide); from the three spiked commercial samples, they were 100.9, 103.1, and 96.9%, respectively.

When the hydrazone reaction mixture is mixed with methanolic alkali, a black color immediately develops; on standing, this color changes to wine-red (sample and standard tubes) or to light yellow or tan (reagent blank). The wine-red color of the steroid hydrazone is stable, and its wavelength of maximum absorbance differs from that of the yellow or tan color produced by the excess reagent. Nevertheless, a reagent blank is advisable because it will reduce or eliminate background absorption over the range scanned.

For assay purposes, a standard curve is not essential. No error is encountered in the comparison of the absorbances of the sample and standard solutions, provided the concentrations of these solutions are approximately equal. However, to establish or demonstrate the linearity of absorbance and concentration at the microgram level, a standard curve was prepared. This curve shows linearity over a range of 0.45–5 mcg. of prednisone/ml., and it passes through the origin.

Interferences may be encountered from any compound containing carbonyl groups. These interferences may be eliminated by the proper extraction technique in the isolation of the prednisone. The samples used in this study contained ascorbic acid, which forms a hydrazone with the 2,4-dinitrophenylhydrazine reagent. However, in isolating prednisone from the other active ingredients, ascorbic acid is removed and is not present in the final chloroform solution of the extracted prednisone.

The behavior of the 2,4-dinitrophenylhydrazones of steroids in alkaline solution is not clearly established. However, Gornall and MacDonald (12) and others (11, 13, 14) suggested that the very intense wine-red color is due to the formation of the resonating quinoidal ion I (Scheme I).

Each carbonyl produces one quinoidal ion; therefore, the formation of more than one quinoidal ion per molecule of steroid should greatly increase the molar absorptivity of the steroid hydrazone. For cortisone, Gornall and MacDonald (12) reported a molar absorptivity of 43,600 at 475 nm. The average molar absorptivity of the prednisone hydrazone calculated from the standard absorbances obtained for the standard curve was 51,646 at 480 nm.

Gornall and MacDonald (12) found that the C-3 and C-20 carbonyls of cortisone are completely converted to hydrazones and its C-11 carbonyl is partially converted on heating 90 min. at 59°. These workers stated that the 2,4-dinitrophenylhydrazone of a carbonyl group that has reacted completely appeared to have a molar absorptivity in the vicinity of 20,000. By using the average calculated molar absorptivity of 51,646, and 20,000 as representative of a fully reacted carbonyl group, it appears that the C-3 and C-20 carbonyls of prednisone are completely converted, while its C-11 carbonyl, like the C-11 carbonyl of cortisone, is partially converted to the hydrazone.

The complete reaction of the C-3 and C-20 carbonyls of prednisone suggests that an accurate quantitative determination with 2,4-dinitrophenylhydrazine depends on the absence of any degradation products of prednisone in the sample being analyzed. The most common type of prednisone degradation is the oxidation of the C-17 side chain. This results in the loss of the C-20 carbonyl from the prednisone molecule and the formation of a complex mixture of organic acids together with neutral compounds such as aldehydes and ketones (4). This method of prednisone isolation will not separate prednisone and any neutral decomposition products (aldehydes or ketones); therefore, the 2,4-dinitrophenylhydrazone color formed with a degraded sample will not be truly representative of prednisone. TLC may be used to determine the condition of the extracted prednisone.

A portion of the synthetic mixture was heated on the steam bath in the presence of alkali and air. This procedure resulted in the degradation of prednisone at the C-17 side chain. Assay results of this decomposed prednisone extract are tabulated in Table IV. The 2,4-dinitrophenylhydrazine and blue tetrazolium results indicate average values of 78.5 and 60.9%, respectively, of the true prednisone value of the mixture. The isonicotinic acid hydrazide results agree with those in Table II and suggest an intact prednisone molecule. However, the low results with 2,4-dinitrophenylhydrazine and blue tetrazolium, which depend on the intact C-17 side chain, definitely indicate the degraded state of the prednisone molecule. Quantitative agreement between the assay results obtained with all three colorimetric reagents (Table II) indicates a completely intact (not decomposed or degraded) prednisone molecule.

Use of a sample weight containing 0.75–1 mg. of prednisone prevents the use of column partition chromatography to isolate the prednisone. The amount of salicylamide, acetaminophen, or aspirin in this sample weight would overload the partition chromatography column normally used. However, by reducing the sample size by a factor of 20, column partition chromatography can be used (instead of separator extraction) for the isolation of the steroid. This would provide from 37 to 50 mcg. of prednisone for the formation of the 2,4-dinitrophenylhydrazone. The concentration of the hydrazone solution used for absorbance measurement would be 37–50 mcg./25 ml. or 1.48–2 mcg./ml. This concentration is in the linear range of 1–5 mcg./ml. (Fig. 1).

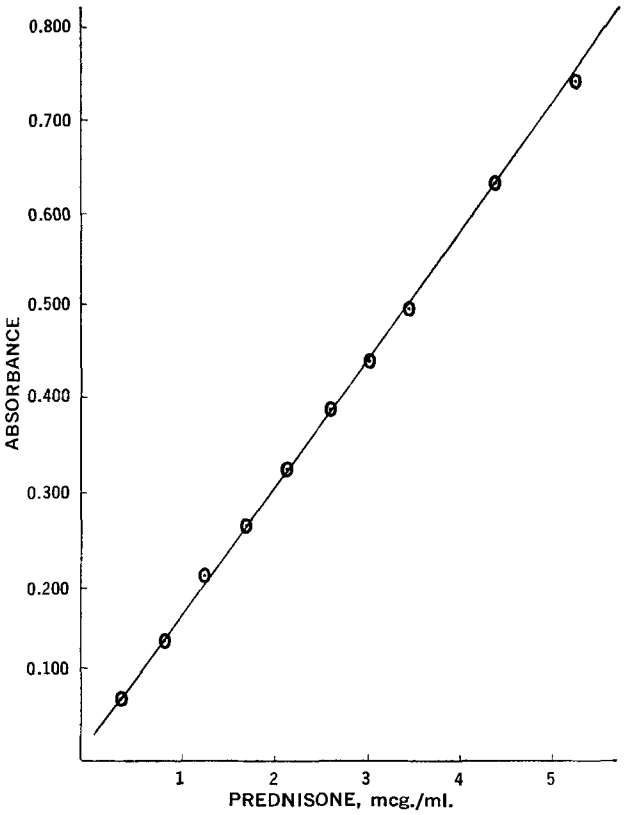


Figure 1—*Standard curve for prednisone with 2,4-dinitrophenylhydrazine reagent.*

CONCLUSIONS

2,4-Dinitrophenylhydrazine may be used as a colorimetric reagent for the quantitative determination of prednisone.

2,4-Dinitrophenylhydrazine is very sensitive for microgram quantities of prednisone. It is more sensitive than the commonly used steroid reagents, blue tetrazolium and isonicotinic acid hydrazide.

To compare the sensitivities of the three colorimetric reagents, prednisone was quantitatively determined in concentration of: (*a*) 1.17×10^{-5} *M*, 2,4-dinitrophenylhydrazine; (*b*) 2.03×10^{-5} *M*, blue tetrazolium; and (*c*) 2.89×10^{-5} *M*, isonicotinic acid hydrazide. The lowest concentration of prednisone determined with 2,4-dinitrophenylhydrazine and showing linearity with absorbance was 0.45 mcg./ml. or 1.26×10^{-6} *M* (1.26 μmoles).

Table IV—Prednisone Determinations Indicating Degradation of Prednisone in a Synthetic Mixture Containing 1.04 mg./g.

DNPH[a]		Blue Tetrazolium		INH[b]	
Found, mg./Tablet	Percent of Theory	Found, mg./Tablet	Percent of Theory	Found, mg./Tablet	Percent of Theory
0.83	79.8	0.60	57.7	0.96	92.3
0.77	74.0	0.55	52.9	0.97	93.3
0.85	81.7	0.75	72.1	1.01	97.1
Av. 0.817	78.5	0.63	60.9	0.98	94.2

[a] 2,4-Dinitrophenylhydrazine reagent. [b] Isonicotinic acid hydrazide reagent.

The experiments showed that microgram quantities of other ketosteroids might be similarly assayed.

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Keyphrases □ 3-Hydroxy-$\Delta^{1,5}$-diene steroids—reaction with aluminum chloride in benzene–nitrobenzene, colorimetric analysis □ Steroids—reaction with aluminum chloride in benzene–nitrobenzene, colorimetric analysis for 3-hydroxy-$\Delta^{1,5}$-diene group □ Colorimetry—analysis, 3-hydroxy-$\Delta^{1,5}$-diene steroids

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The analysis of 17α-methyl-1,5-androstadiene-3β,-17β-diol (I) by Kato's procedure (1, 2) was tried but without success; the mixture turned an olive-green color and had a very poorly defined absorption maximum.

Tauber (3) and Lange *et al.* (4) reported color formation for many steroids in chloroform when reacted with 70% perchloric acid. Lange *et al.* (4) reported that colorless, well-forme dcrystals of sterol–perchloric acid were precipitated when sterols containing the 3-hydroxy-5-ene grouping were dissolved in chloroform and then treated with a stoichiometric amount of perchloric acid. When an excess of perchloric acid was used, the crystals redissolved and characteristic colors were produced. Alteration of either of the two essential groups in the sterol resulted in failure to give a precipitate, but color formation still took place.

Early work on the color formation of cholesterol when treated with sulfuric acid–acetic anhydride (Liebermann–Burchard reaction) (5, 6) and zinc chloride–acetyl chloride (Tschugaeff reaction) (7) has formed the basis for more recent work. The latter papers deal with colors formed with unsaturated steroids when treated with an acid–iron reagent (8–10).

When the steroid (I) was tested in these systems, it gave a positive color reaction with all of them. Since such a large variety of steroids give positive color reactions with these systems, it was desirable to try to obtain a reaction that would be specific for the reactive grouping of I.

In the course of the Kato reaction, it was noted that I produced a satisfactory red color by merely allowing aluminum chloride in nitrobenzene to react with the steroid at room temperature. The utilization of this color as an analytical procedure for I is reported here.

EXPERIMENTAL

Apparatus—The absorption curves were obtained using a spectrophotometer[1] with 1-cm. cells.

Reagents and Solvents—The following were used: aluminum chloride, anhydrous[2]; benzene, analytical reagent, thiophene free[2]; and nitrobenzene[3], m.p. 5–6°. The aluminum chloride and benzene were used without further purification. Solutions of aluminum chloride in nitrobenzene became increasingly dark (in the absence of added steroid) as the nitrobenzene aged. To minimize this effect conveniently, the nitrobenzene was washed twice with 0.5 *N* HCl and then three times with 0.5 *N* NaOH, dried with anhydrous

[1] Carey model 15.
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Table I—Effect of Nitrobenzene–Benzene on Absorbance

Nitrobenzene–Benzene[a]	5:1	5:3	5:5	5:10	5:15
Absorbance[b] (495 nm.)	0.748	0.710	0.676	0.588	0.565

[a] Milliliters of benzene added to 5 ml. nitrobenzene. Dilution to 25 ml. with benzene was made 15 min. after mixing two solvents. [b] Steroid (I) concentration 0.696 mg./25 ml. final dilution.

sodium sulfate, and filtered. Blanks prepared using this nitrobenzene remained satisfactorily light colored during the analysis.

Aluminum Chloride Solution—Dissolve 7 g. aluminum chloride in 100 ml. nitrobenzene.

Steroid Solution—Dissolve 20–150 mg. 17α-methyl-1,5-androstadiene-3β,17β-diol in 100 ml. benzene.

Procedure—In a 25-ml. volumetric flask, add 2 ml. steroid solution and 3 ml. benzene. Add 5 ml. aluminum chloride solution, mix by swirling, and allow to stand at room temperature for 15 min. Add benzene to the mark and mix well.

A blank is prepared in the same manner, with the substitution of 2 ml. benzene for the steroid solution.

Measure the absorbance, at 495 nm., of the sample *versus* the blank. All samples should be read within 30 min. after the final dilution is made.

DISCUSSION

The rate of color formation was found to be greatest when the reaction was run in neat nitrobenzene. However, the analytical absorption maximum at 495 nm. decreased in intensity somewhat rapidly with time and was partially obscured by a second maximum at 450 nm.

Other solvents were tried, but the red color failed to form. In benzene or toluene, a red precipitate was obtained instead of a red solution. A combination of nitrobenzene and benzene, however, proved satisfactory; the undesirable peak at 450 nm. was strongly suppressed and the color stability substantially improved. The color was rapidly discharged when alcohol, chloroform, or carbon tetrachloride was added to the system.

The initial ratio of nitrobenzene to benzene used (see *Experimental* section) and the time allowed for color development prior to final dilution had an effect on color intensity and stability.

Effect of Ratio of Nitrobenzene to Benzene—The color intensity varied inversely with increasing amounts of benzene relative to nitrobenzene. The data in Table I were obtained by adding varying amounts of benzene to a fixed volume of a solution of aluminum chloride in nitrobenzene at a constant steroid concentration.

The 5:5 ratio was found to be convenient in the analyses performed, using pharmaceutical preparations containing the steroid.

The intensity of the color reached a maximum approximately 15 min. after the two solvents were mixed and showed good stability up to 1 hr. after final dilution.

The absorbance followed a linear relationship with concentration and had a molar absorptivity of 8300 (a = 27.0).

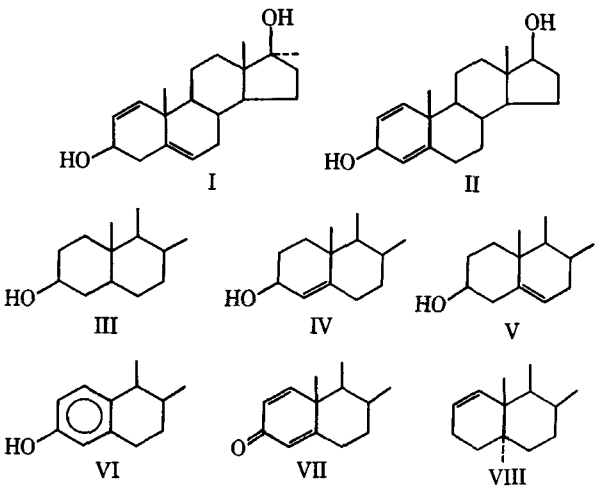
Other Reagents and Solvents—Nitrobenzene solutions of acetic acid, trichloroacetic acid, ferric chloride, tin tetrachloride, and zinc chloride were prepared. Only tin tetrachloride produced a red color with the steroid.

Tin tetrachloride was then dissolved in acetonitrile, benzene, dimethyl sulfoxide, nitrobenzene, and 1-nitropropane. Only in nitrobenzene and nitropropane was the color produced.

The intensity of the color produced in the tin tetrachloride–nitropropane–steroid system was only about one-fifth as intense as that in the aluminum chloride–nitrobenzene–steroid system. Since the stability of the color was also poorer in the former system, there appeared to be no advantage in using it over the latter one.

Other Compounds—The reaction, performed as described in this paper, appears to be specific for the 3-hydroxy-$\Delta^{1,5}$-diene grouping of I. The only other material tried that gave a positive reaction was $\Delta^{1,4}$-androstadiene-3β,17β-diol (II). However, the color would only form in neat nitrobenzene, and a much larger amount of steroid was necessary to obtain an intensity comparable to that of I.

Other compounds that gave no color by this procedure were (shown in partial structure): cholestan-3β-ol (III), Δ^4-androstene-3α,11β,17β-triol-11-acetate (IV), 25-hydroxycholesterol (V), estradiol (VI), betamethasone (VII), Δ^1-cholestene (VIII), ethyl morphine, phenol, and allyl alcohol.



Investigation of Reaction—To try to obtain some mechanistic information, the reaction was run on a larger scale by two methods.

Method 1—Compound I, 400 mg., was dissolved in nitrobenzene, and a solution of $AlCl_3$ in nitrobenzene was added to it. After 5 min. at room temperature, water was added. The nitrobenzene phase was then separated, dried with anhydrous Na_2SO_4, and filtered; the filtrate was evaporated under N_2 to a brown, oily residue. The residue was then triturated with methanol and a tan powder, slightly soluble in methanol, remained; yield = 150 mg.

Method 2—Compound I, 500 mg., was dissolved in 500 ml. benzene, and 3 g. $AlCl_3$ was added to it. The mixture was stirred for 4 hr. at room temperature and then handled as in Method 1. The methanol-insoluble residue was also obtained; yield = 200 mg.

The residual powders were subjected to various spectrometric analyses and to elemental analysis. The elemental analysis gave essentially identical results for the two procedures: C (87.6%), H (9.50%), Cl (0.9%), N (0.0%), O (3.5%). The starting material, $C_{20}H_{30}O_2$, has: C (79.5%), H (9.90%), O (10.6%). Since identical results were obtained by both methods, the incorporation of a solvent molecule into the methanol-insoluble material may be disregarded.

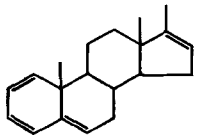
Replicate runs were made, and the elemental analysis was obtained for each run. The C and H results were essentially constant, but the results for oxygen varied from a low of 1.1% to a high of 4.3%. This variability made the selection of a possible empirical formula impossible.

The IR spectrum, run as a mineral oil mull and as a carbon tetrachloride solution, showed the material to be essentially a hydrocarbon; there was no carbonyl and very little OH absorption. Bands assignable to conjugated double bonds and possibly to an aromatic system were clearly present.

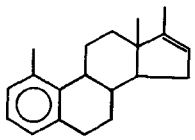
The NMR spectrum gave very poor resolution and was useless in structure elucidation.

The visible and UV spectra in isooctane were obtained. A complex curve resulted, which showed a maximum at 480 nm. and shoulders at 450 and 420 nm. in the visible region. The UV gave shoulders at 290 and 260 nm. and a maximum at 234 nm. (a = 45.0 at 234 nm.). The starting material, I, has no absorption above 210 nm. The UV data, therefore, support the IR spectrum in that a conjugated system was generated by the reaction.

Both GLC, using 3% OV-1, and TLC, on silica gel, revealed that the powder is a multicomponent material.



IX (m/e 266)



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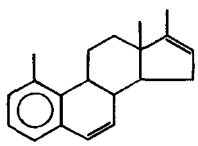

XI (m/e 264)

In the mass spectrum, the highest mass ion was found at 615 and the base peak at 532 *m/e*. Although the material fragmented into a great many ions, the highest concentration of them clustered into two groups: one ranged from 530 to 536 and the other from 262 to 270 *m/e*. Since the starting material has a molecular weight of 302, the possibility that the ion at 615 *m/e* is the parent ion of a dimer of the starting material may be ruled out. The ion could possibly arise, however, from the fragmentation of a trimer formed from an intermediate generated during the reaction. The ions in the groups at *m/e* 530–536 and 262–270 could then be due to dimers and monomers, respectively, fragmenting from the trimers or present in the mixture obtained from the reaction.

Of the many structures possible, three monomeric ones that can be written for the ions and that appear to be consistent with the various spectral data and with the starting material are shown in IX, X, and XI.

Structures such as X and XI can arise from a rearrangement similar to that given for the dienol–benzene rearrangement (11).

It seems, therefore, that the initial reaction is a Friedel–Crafts reaction on the allylic alcohol group of I with the formation of an allyl carbonium ion. This carbonium ion can be stabilized not only by the excess aluminum chloride but also by the nitrobenzene (12, 13). The stabilized carbonium-ion complex, conjugated with one or more double bonds, results in the red color observed in the analytical reaction. Red oils were reported previously in reactions of aluminum chloride, or bromide, with aromatic hydrocarbons (14). When the complex is destroyed by the addition of water, polymeric material is formed by attack of the carbonium ions on the various double bonds initially present in the molecule or generated during the reaction.

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Keyphrases □ Oxytetracycline—separation, analysis from degradation products, urea-impregnated chromatographic paper □ Paper chromatography—separation, analysis, oxytetracycline □ Urea complexes—separation, analysis of oxytetracycline □ UV spectrophotometry—identification, oxytetracycline

The microbiological methods widely used for the determination of tetracycline antibiotics are not satisfactory, since some degradation products of these antibiotics proved to have antibacterial activity (1, 2). In view of the findings that relate a reversible fanconi-type syndrome to the ingestion of degraded tetracycline capsules (3–7), interest has developed in the analytical determination of tetracycline antibiotics in the presence of their degradation products.

Novelli *et al.* (8) pointed out the need of applying experimental correction factors, which are different for each component, in the spectrophotometric determination of tetracycline and its degradation products. This is necessary to make the results coincide with the theories and with the microbiological determinations.

A review of the literature dealing with the quantitative paper chromatographic determination of oxytetracycline revealed that there are only scanty publications concerning this aspect.

Selzer and Wright (9) separated different tetracyclines and their epimers on a paper chromatogram, previously impregnated with McIlvaine's buffer, pH 3.5. Sztaricskai (10) described a circular paper chromatographic method for determining oxytetracycline in the presence of its acidic decomposition products, using Whatman No. 1 chromatographic paper previously impregnated with phosphate buffer, pH 3.0, and a butanol–acetic acid–water (4:1:5) solvent system overnight. This method was claimed to be useful for estimating oxytetracycline in intermediary products and animal nutrients, but it was not reported to be applicable for the determination of oxytetracycline in pharmaceutical preparations. On applying this method to a sample of degraded oxytetracycline, we were unable to detect the fluorescent zones under UV lamp according to the description of the author; furthermore, no sharp separation of the degradation products was observed, and the experiment was time consuming (overnight running).

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Higuchi and Bolton (11) reported that urea formed a complex with oxytetracycline and increased its solubility in buffer solution, pH 5.0. Based on this finding, it seemed valuable to use urea solution as an immobile phase for establishing a paper chromatographic method which allows separation and subsequent determination of oxytetracycline in the presence of its degradation products. The purpose of the present study is to establish an accurate procedure for the determination of oxytetracycline in different pharmaceutical preparations in the presence of its degradation products.

EXPERIMENTAL[1]

Separation of Oxytetracycline from Its Degradation Products—*Preparation of Chromatogram*—A series of chromatographic papers was impregnated with McIlvaine's buffer, pH 5.0. Other series of papers were impregnated with the same buffer containing 10% w/v of urea. The papers were then blotted between sheets of absorbent papers.

Solvents—A mixture of chloroform–ethyl acetate–pyridine (1:1:1), was used with papers impregnated with the buffer alone; the same solvent, previously saturated with urea, also was used with the papers impregnated with the buffer containing urea.

Method—Ten microliters from each freshly prepared methanolic solution, containing 1 mg./ml. of oxytetracycline base (international standard), and 10 μl. of the same solution after 1 month of storage at room temperature (34°) were separately spotted on each series of papers. The chromatograms were then partially dried in air; while still damp, they were subjected to ascending running by being hung in the chromatographic jars, each containing 100 ml. of the corresponding solvent. When the solvent front reached about 22 cm. above the starting line, the papers were removed and dried in air. They were then exposed to ammonia vapor for 5–15 sec., and the separated spots were identified under UV light. The oxytetracycline and its degradation products separated on the chromatogram were identified by analogy to the Chas. Pfizer test procedure (12). The mean R_f values of these components were calculated and are presented in Figs. 1 and 2.

Quantitative Analysis along a Horizontal Line—Two hundred microliters of a freshly prepared methanolic solution of oxytetracycline base was spotted along a horizontal line (10 cm.) on the chromatographic paper, previously impregnated with 10% w/v urea in McIlvaine's buffer, pH 5.0. Ten microliters of freshly prepared oxytetracycline (international standard) solution in methanol was spotted at the same level of the previously applied line at a distance 2.5 cm. away from the edge of paper. The chromatogram was then transferred to the chromatographic jar containing the corresponding, previously mentioned solvent. When the solvent front reached about 22 cm. above the starting line, the chromatogram was removed and dried in air. It was then exposed to ammonia vapor for the specified time and examined under UV light. The horizontal area at the level of the standard spot (yellow fluorescence) was cut off and then eluted with 20 ml. of dilute HCl (pH 1.8). The absorbance of oxytetracycline present in the eluate was determined spectrophotometrically at 352 nm. for 1 hr. against oxytetracycline base (international standard), which was chromatographed and eluted in the same manner. A blank for spectrophotometric determination was prepared by eluting an equal area from a chromatographic paper similarly treated without antibiotic. A series of chromatographic determinations was conducted on oxytetracycline (international standard) to calculate the percent recovery of the antibiotic (Table I).

Chromatographic Determination of Oxytetracycline in Different Pharmaceutical Preparations—The previously mentioned method was applied to determine the oxytetracycline content of capsules, tablets, injections, suspensions, pediatric drops, and ointments. The procedure adopted for preparing the sample before applying on the chromatographic paper was varied according to the nature of the dosage form used.

Capsules and Tablets—An exact weight, corresponding to 100 mg. oxytetracycline, from the pooled contents of five capsules or five powdered tablets was dissolved in a sufficient amount of methanol, and the volume was completed to 100 ml. The resulting turbid solution was centrifuged before being applied onto the chromatographic paper, and then 200 μl. of the methanolic clear solution was measured. The procedure was completed as previously described.

Suspensions and Pediatric Drops—An exact volume of the well-shaken sample, corresponding to 25 mg. oxytetracycline, was measured and diluted to 25 ml. with methanol in a volumetric flask. The resulting turbid solution was then centrifuged prior to applica-

Table I—Percent Recovery of Oxytetracycline (International Standard) after Chromatography

Number of Experiment	Oxytetracycline in 0.2 ml. Methanolic Solution, mcg.	Oxytetracycline Found after Chromatography, mcg.	Deviation	
			Micrograms	Percent w/v
1	200.0	197	−3	−1.5
2	200.0	192	−8	−4.0
3	200.0	193	−7	−3.5
4	200.0	195	−5	−2.5
5	200.0	194	−6	−3.0
6	200.0	195	−5	−2.5
7	200.0	192	−8	−4.0
		Mean =	−6	−3.0

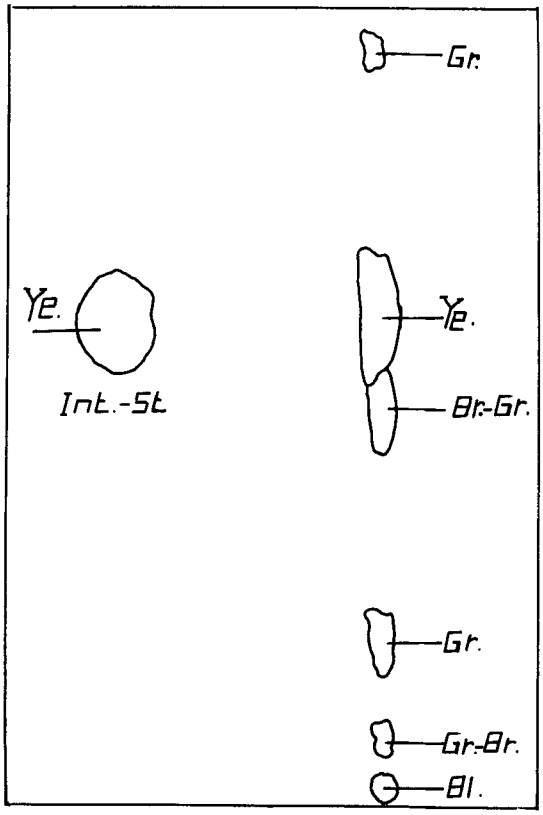

Figure 1—*Chromatogram of degraded oxytetracycline base on paper impregnated with buffer, pH 5.0. Key: Gr. (green) = α- or β-apoterramycin, R_f 0.95 or 0.2; Ye. (yellow) = oxytetracycline, R_f 0.61; Br.-Gr. (brownish-green) = terrinilide, R_f 0.49; Gr.-Br. (greenish-brown) = unknown, R_f 0.08; Bl. (blue) = 6β-deoxyoxytetracycline, R_f 0.02; and Int.-St. = international standard of oxytetracycline base dihydrate, R_f 0.61.*

[1] The following apparatus and reagents were used: (*a*) spectrophotometer, Carl Zeiss Jena; (*b*) UV lamp, HPW-125W, Typ. 57202 E/7, Philips; (*c*) chromatographic jars (20 × 25 × 35 cm.), with ground-glass lid; (*d*) Whatman No. 1 chromatographic papers (17.5 × 28 cm.); (*e*) methanol, chloroform, ethyl acetate, pyridine, urea, phosphoric acid, and hydrochloric acid, all analytical grade; (*f*) oxytetracycline base dihydrate USP XVII; and (*g*) international standard of oxytetracycline base dihydrate (900 I.U./mg.), provided by the World Health Organization.

Table II—Stated and Found Potency Values of Oxytetracycline in Different Pharmaceutical Preparations

Dosage Form	Stated Potency	Found Potency (Average of Four Determinations)	Standard Deviation
Capsules:			
No. 1	250 mg./capsule	250.22 mg./capsule	±3.44
No. 2	250 mg./capsule	248.28 mg./capsule	±3.15
Tablet	250 mg./tablet	219.45 mg./tablet	±2.38
Suspension	125 mg./5 ml.	115.10 mg./5 ml.	±0.55
Pediatric drops:			
No. 1	100 mg./ml.	97.04 mg./ml.	±0.87
No. 2	100 mg./ml.	100.70 mg./ml.	±0.46
Injections:			
No. 1	100 mg./2 ml.	100.25 mg./2 ml.	±1.08
No. 2	250 mg./2 ml.	260.77 mg./2 ml.	±1.46
Ointments:			
No. 1	30 mg./g.	30.23 mg./g.	±0.27
No. 2	5 mg./g.	4.02 mg./g.	±0.02
No. 3 (ointment base composed of 4% polysorbate in vaseline)	30 mg./g.	29.47 mg./g.	±0.01

tion. In the case of colored suspensions of calcium dioxytetracycline pediatric drops, the dye interfered with the chromatographic separation and determination. The dye was removed by centrifugation of an accurately measured volume of the supension, corresponding to 200 mg. oxytetracycline (as calcium salt), and subsequent washing of the precipitate with successive 5-ml. portions of distilled water. The precipitate, which was partially soluble in methanol, was made completely soluble by dissolving it in 10% w/v phosphoric acid (10 ml.) and completed up to 200 ml. with methanol. Then, 200 μl. of the solution was applied directly onto the chromatographic paper, and the assay was completed as described.

Injections—A measured volume (0.2 ml.), corresponding to 5 mg. oxytetracycline, was diluted to 5 ml. with methanol; 200 μl. was applied directly onto the chromatographic paper.

Ointments—An exact weight of the ointment, corresponding to 30 mg. oxytetracycline, was treated with the least amount (6 ml.) of petroleum ether (b.p. 60–80°). This solvent was chosen because the antibiotic is almost insoluble in it (13). The insoluble antibiotic was extracted by shaking in a separator with three, exactly measured, successive 5-ml. portions of 0.1 *N* HCl. The combined antibiotic–acidic solutions were then diluted with a further 15 ml. of methanol; 200 μl. was applied onto the chromatographic paper.

In the presence of a nonionic surface-active agent[2], the difficulty produced by emulsion formation, during extraction of the antibiotic, was overcome by centrifugation of the formed emulsion.

Hydrocortisone acetate and polymyxin B sulfate were found not to interfere with the chromatographic separation. The results obtained are presented in Table II. Each potency reported in the table represents the average of four determinations.

The oxytetracycline contents of oxytetracycline suspension and pediatric drops No. 1 were determined by the prescribed method after 14 days of storage at room temperature and then after a further 5 days subsequent to the latter period at 50°.

RESULTS AND DISCUSSION

Results obtained by impregnating the chromatographic paper with McIlvaine's buffer, having different pH values, revealed that pH 5.0 was the most suitable for the separation of oxytetracycline from its degradation products (Fig. 1).

Figure 2 shows the effect of urea on the R_f values of the antibiotic and its degradation products.

Figures 1 and 2 reveal that impregnation of the chromatographic paper with 10% w/v urea solution in McIlvaine's buffer, pH 5.0, gave a sharp and complete separation of oxytetracycline from its degradation products. On comparing the R_f value of each component in the presence and absence of urea (Figs. 1 and 2), it was found that the use of urea resulted in the lowering of the R_f values of the separated components. This lowering of the R_f values and the sharp separation were parallel with the results obtained by using

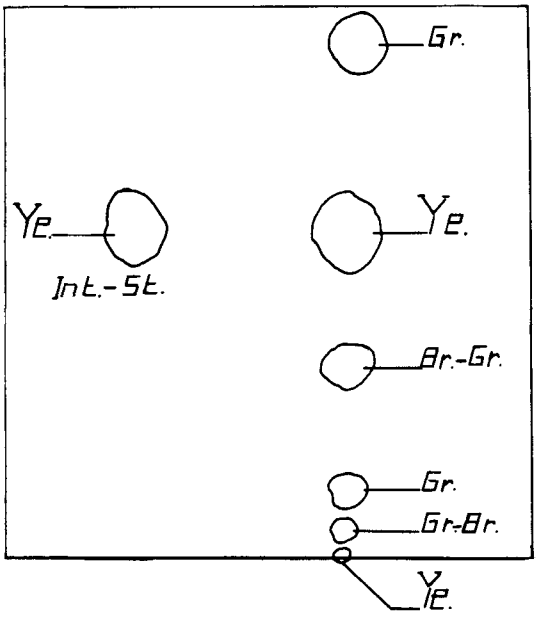

Figure 2—*Chromatogram of degraded oxytetracycline base on paper impregnated with buffer, pH 5.0, containing 10% w/v urea. Key: Gr. (green) = α- or β-apoterramycin, R_f 0.76 or 0.1; Ye. (yellow) = oxytetracycline or epiterramycin, R_f 0.48 or 0.01; Br.-Gr. (brownish-green) = terrinilide, R_f 0.28; Gr.-Br. (greenish-brown) = unknown, R_f 0.04; and Int.-St. = international standard of oxytetracycline base dihydrate, R_f 0.48.*

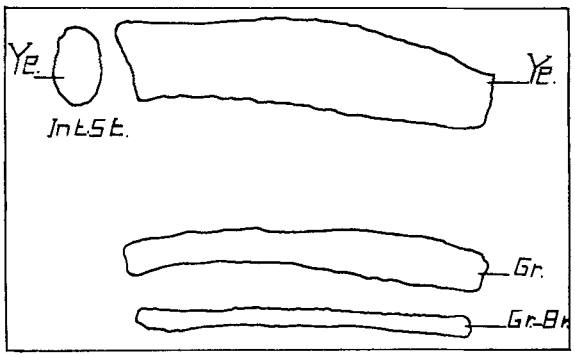

Figure 3—*Chromatogram of oxytetracycline base sample X_1 using horizontal line technique. Key: Ye. (yellow) = oxytetracycline; Gr. (green) = α- or β-apoterramycin; and Gr.-Br. (greenish-brown) = unknown.*

[2] Tween 20.

Table III—Microbiological and Paper Chromatographic Determinations of Oxytetracycline

Sample	Oxytetracycline Content, mcg./mg.		Standard Deviation (Chromatographic)
	Microbiological Method	Chromatographic Method	
X_1	820.0	790	±0.09
X_2	900.0	859	±2.88

edetic acid in the separation of tetracycline antibiotics (14). The influence of urea can be explained on the basis that urea forms a complex with the antibiotic on the chromatographic paper impregnated with buffer solution at pH 5.0 (11). The formation of oxytetracycline–urea complex on the paper chromatogram was confirmed by the authors by observing the shift toward the visible range of the spectrum in the maximum absorption of the antibiotic eluate, using McIlvaine's buffer, from the chromatogram previously impregnated with urea solution.

It was found that the application of more than 20 mcg. of the antibiotic per spot resulted in incomplete separation. To achieve a good quantitative separation, it would be necessary to apply at least 200 mcg. of the antibiotic on the chromatographic paper, using the horizontal line technique (Fig. 3), to provide a final concentration of about 10 mcg./ml. in the antibiotic eluate.

The percent recovery of oxytetracycline from the chromatogram (Table I) showed that the accuracy of the quantitative determination of this antibiotic by the prescribed method is adequate for its application in pharmaceutical preparations. The data indicated also that the antibiotic was not decomposed during the running time, as shown by the absence of degradation spots on the chromatogram of oxytetracycline (international standard). The stability of the antibiotic in the eluting solution (dilute HCl, pH 1.8) during the time needed for the spectrophotometric determination (1 hr.) was indicated by the absence of degradation spots on rechromatographing the acid eluate of the antibiotic. A further confirmation was carried out by observing the unchanged absorbances of the antibiotic acid eluate during 1 hr. The recovery losses presented in Table I were eliminated to some extent by using a reference standard similarly treated for each determination.

Table II clearly shows that the found potencies of the different dosage forms tested were nearly similar to the stated potencies, except with the oxytetracycline tablet and suspension. In the latter preparations, the number and the area of zones representing the degradation products of oxytetracycline were more than in the other dosage forms tested. Accordingly, the present method can be efficiently applied for determining oxytetracycline in pharmaceutical preparations.

The use of methanol with dilute hydrochloric acid in dissolving calcium dioxytetracycline, present in pediatric drops No. 2, prior to application on the chromatographic paper interfered with chromatographic separation. This unexpected observation may be attributed to the possible formation of a calcium chloride–oxytetracycline complex in the presence of hydrochloric acid (11), which in turn prevents the formation of oxytetracycline–urea complex on the chromatogram. This difficulty was overcome by using dilute orthophosphoric acid instead of hydrochloric acid.

Comparing the present method with the official microbiological technique, two different samples of oxytetracycline base dihydrate (X_1 and X_2), obtained from different manufacturers, were determined by both methods. The microbiological determination was carried out by using the cup plate method and *Bacillus* (var. mycoides, ATCC No. 9643) as a test organism (15). The results obtained are compiled in Table III. The values in the table are the averages of four determinations. From the table, it is obvious that there was an agreement between the prescribed method and the official microbiological procedure within 5% limits.

The present method proved to be useful in following the stability of oxytetracycline in different pharmaceutical dosage forms. It is obvious that oxytetracycline suspension is less stable than the pediatric drops (Table IV).

SUMMARY

1. The separation of oxytetracycline from its degradation products was quantitatively effected, using chromatographic paper previously impregnated by urea solution at pH 5.0.

2. The formation of an oxytetracycline–urea complex on the paper chromatogram was confirmed by observing the shift in the maximum absorption of oxytetracycline.

3. A quantitative paper chromatographic method is presented for the determination of oxytetracycline in bulk and in pharmaceutical dosage forms after separation from its degradation products.

4. The agreement of the presented method with the microbiological assay was discussed.

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Table IV—Stability of Oxytetracycline Suspension and Pediatric Drops

Dosage Form	Initial Concentration	Percent Remainder of Oxytetracycline[a]			
		After 14 Days at Room Temperature (34°)	Standard Deviation, %	After 14 Days at Room Temperature (34°) and 5 Days at 50°	Standard Deviation, %
Suspension	115.10 mg./5 ml.	82.97	±1.12	17.34	±0.32
Pediatric drops	97.04 mg./ml.	94.12	±0.64	69.29	±1.19

[a] Average of four determinations.

Modified Iodimetric Determination of Echothiophate Iodide

CHARLES WARNER, FRANK DiBERNARDO, ARTHUR BYLOW, ANWAR HUSSAIN*, and B. T. KHO

Abstract □ Kinetic and chemical data are used to increase the precision and specificity of the previously described iodimetric method for echothiophate iodide. Hydrolysis is effected at pH 12.0 to ensure quantitative cleavage of the S—P bond of the echothiophate ion while minimizing interference by probable impurities. The product of hydrolysis—thiocholine—is determined by iodimetric titration under acidic conditions. The coefficient of variation for the raw material and the smallest dosage form is less than 1.0%.

Keyphrases □ Echothiophate iodide—analysis, iodimetric titration of thiocholine □ Iodimetric titration—thiocholine, echothiophate iodide analysis □ Thiocholine, iodimetric titration—analysis, echothiophate iodide □ Coulometry, standardization—iodine titrant, echothiophate iodide analysis

Echothiophate iodide[1] (I), a long-acting cholinesterase inhibitor for the control of glaucoma, was analyzed by two different procedures (1, 2). These methods differ in detail but are based upon the same fundamental chemistry (Scheme I) (3). The thiocholine iodide (II), the product of the alkaline hydrolysis (Step 1), is determined by titration with an oxidant (Step 3). This titer is stoichiometrically related to the quantity of I that has been hydrolyzed.

This report describes the reexamination of Steps 1 and 3 to establish the reagents and conditions for optimum specificity and accuracy. The fundamental kinetic studies by Hussain *et al.* (3) of the chemistry of I in aqueous solutions provided the theoretical data needed to improve the specificity of Step 1. Coulometric techniques were used to develop a standardization procedure for the dilute iodine titrant, which is very unstable. A TLC procedure (3) may be used to detect the presence of impurities.

EXPERIMENTAL

Reagents—The following were used: echothiophate iodide[2]; arsenic trioxide solution, 0.006 *N*, prepared from NBS primary standard as described in USP (2) and diluted to proper volume; iodine titrant, 0.004 *N*, 0.1 *N* iodine[3], diluted to proper titer with distilled water and standardized by titration of 0.006 *N* arsenic trioxide in pH 7 phosphate buffer (2); phosphate buffer, pH 12.0, prepared by dissolving 54.4 g. anhydrous dibasic sodium phosphate in 1 l. of distilled water and adjusting to pH 12.0 with 50% aqueous sodium hydroxide.

Deoxygenated distilled water was used throughout; it was prepared by boiling and cooling under a stream of nitrogen.

Apparatus—All iodimetric titrations were performed potentiometrically with a combination Pt–Ag/AgCl electrode. A Heath Malmstadt-Enke pH and millivolt recording electrometer was synchronized with a Sargent model C constant-rate buret (Catalog No. S-11120-10). A Beckman model 40495 high-alkalinity pH electrode was calibrated with a pH 12.45 buffer and then used to monitor the pH 12.0 buffer.

Coulometric titrations were performed with a Sargent coulometric current source S-30974. The generating and counter electrodes were fabricated from 18-gauge platinum wire. The general procedure was described previously (4). The iodine titrant was generated at 12 microequivalents/min. Potentiometric end-point detection was used.

Titration of Echothiophate Iodide—All reagents, which were not prepared with deoxygenated distilled water, were deoxygenated with a stream of nitrogen.

A sample of echothiophate iodide, weighing 125 mg., was dissolved in distilled water and diluted to 100 ml. A 10.0-ml. aliquot of this solution was added to the titration vessel along with 30 ml. distilled water and 10 ml. pH 12.0 buffer. A stream of nitrogen was directed into the titration vessel and onto the liquid during transfer. The headspace of the titration vessel was flushed with nitrogen and capped to reduce the amount of oxygen in the solution. [*Note:* A number of experiments indicated that exclusion of oxygen is not a critical step, but it was deemed advisable because the thiocholine is oxidized by oxygen under basic conditions (5).] After 20 min. at 25 ± 3°, the reaction was quenched by the addition of 2 ml. glacial acetic acid. The solution was titrated with 0.004 *N* iodine to an endpoint as indicated by the potential change of the combination Pt–Ag/AgCl electrode.

The blank titration was carried out exactly as already described, except the glacial acetic acid was added first, followed immediately by the pH 12.0 buffer. The 20-min. hydrolysis step was omitted and the sample was titrated immediately. This titer corrected for the amount of free thiol present in the sample. Because the normality of the iodine solution is altered by dust, stopcock grease, and light, it is advisable to restandardize periodically during a series of determinations.

Table I—Titration of Possible Impurities of Echothiophate Iodide

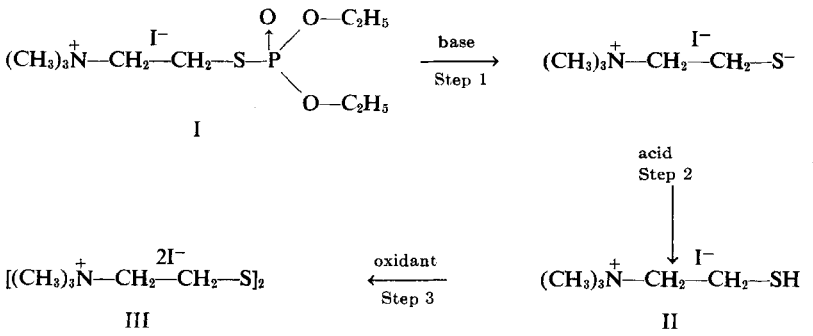
Compound	Percent Titration[a,b] This Method	USP XVII	USP XVIII
$[(CH_3)_3\overset{+}{N}—CH_2—CH_2—S]_2$ $2I^-$ (III)	8	90	57
$(CH_3)_3\overset{+}{N}—CH_2—CH_2—S—\overset{O}{\overset{\uparrow}{P}}(O—C_2H_5)(O—H)$ I^- (IV)	—	—	—
$(CH_3)_2N—CH_2—CH_2—S—\overset{O}{\overset{\uparrow}{P}}(O—C_2H_5)_2$ (V)	35	88	90

[a] Relative to an equal weight of echothiophate iodide as 100%. [b] The hydrolytic procedure for the raw material was used in each case. [c] No hydrolysis detected.

RESULTS AND DISCUSSION

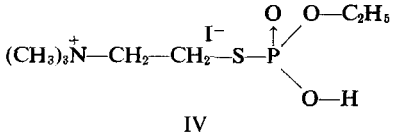
Hydrolysis of S—P Bond—Previous work (3) established that the hydrolysis of I in aqueous media within pH range 10–12 proceeds exclusively by cleavage of the S—P bond to yield II. At successively lower pH's, a greater and greater proportion of I hydrolyzes with

[1] Phospholine iodide, Ayerst Laboratories.
[2] Prepared by Dr. Gordon Myers, Ayerst Research Laboratories, Montreal, Canada.
[3] Anachemia Limited, Champlain, N. Y.



Scheme I

cleavage of the C—O bond to produce (2-mercaptoethyl)trimethylammonium iodide *O*-ethyl phosphorothioic acid (IV). This sub-



IV

stance will not, under the conditions of the analysis, hydrolyze further to yield II (3). Thus, to ensure quantitative generation of II, the solution must be more alkaline than pH 9.

The necessity of careful pH control is clearly apparent from previous work (3). At pH 12.0, the half-life of echothiophate is 1.1 min.; therefore, the reaction is essentially complete in the 20-min. reaction time required by the procedure described in this report. A reduction in pH of only 1 unit increases the half-life to 10 min.; therefore, after 20 min., only 75% of I is converted to thiocholine. The present method requires a phosphate buffer, in contrast to the previously published procedures (1, 2) which depend upon the addition of sodium hydroxide for pH adjustment. These methods provide a sufficiently basic solution by the addition of a large excess of sodium hydroxide. The result is that while I is completely hydrolyzed, there is a loss of specificity (Table I). The results given in Table I illustrate that the method described in this paper minimizes the interference by probable impurities, *e.g.*, III, IV, and V.

TLC—The presence of excessive amounts of III, IV, and V, which would cause an unacceptably high level of interference, can be detected by TLC (3).

Statistical Evaluation of the Method—A collaborative study, involving three different laboratories with a total of five workers, was performed. Each analyst randomly selected 20 vials from a common stock, pooled the samples, and made one, and only one, determination per day. The same sample of raw material was analyzed at each laboratory. To test interlaboratory agreement, Laboratories 1 and 2 analyzed the same solution of raw material which was made up daily. Each laboratory was limited to one vial and one raw material assay per day.

Each analyst prepared his own reagents and calibrated his own instrumentation; however, all drew from a common supply of oxidimetric standard, which had been standardized by coulometry.

Because there were no significant differences in the standard deviations obtained by the five analysts, the results were pooled according to the following relationship (6):

$$(\Sigma n - m)s^2 = \Sigma\Sigma(x - \bar{x})^2 \qquad \text{(Eq. 1)}$$

where m equals the number of collaborators and n equals the total number of determinations. The other symbols have the usual significance. The resulting standard deviation and the overall mean were used to calculate the percent coefficients of variation (Table II).

The results given in Table I prove that the new procedure is more specific than the USP XVIII method. Careful attention to standardization of reagents is necessary to achieve the precision noted in this work.

Table II—Collaborative Study of the Echothiophate Iodide Method

Sample	Laboratory 1	Laboratory 2	Laboratory 3	Percent Coefficient of Variation
Vial[a], mg./vial	1.68	1.69	1.65	0.78[b]
Raw material, mg./g.	96.74	97.05	96.07	0.81[c]

[a] Lyophilized Product No. 1062, Batch No. 1NI, input 1.65 mg./vial. [b] Based upon 21 determinations. [c] Based upon 23 determinations.

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Neutron Activation Analysis of Halogens in Drugs

MICHEL MARGOSIS, JAMES T. TANNER, and JOSEPH P. F. LAMBERT*

Abstract □ A rapid, efficient, and highly specific method is presented for the determination of total chlorine, bromine, or iodine in various drugs. The halogen content is determined by nondestructive neutron activation and may be used for the presumptive assay of halogen-containing drugs in various dosage forms. The relative freedom from laboratory contamination, the independence from matrix effect, and the rapidity and specificity of analysis make this technique well suited for the determination of major elements in such complex substances as drugs. The method involves subjecting samples to thermal neutron bombardment and correlating the measured activity from the induced radionuclides of samples with that of standards. The radionuclides of interest in this study are ^{38}Cl, ^{80}Br, ^{82}Br, and ^{128}I, which are produced by a neutron reaction (n,γ) in a nuclear research reactor.

Keyphrases □ Neutron activation analysis—halogens in drugs □ Radionuclides, halogen—neutron activation analysis, drugs □ Halogens—neutron activation analysis, drugs

Organic pharmaceutical products containing halogens are frequently assayed by decomposition with an alkali or Schöniger oxygen-flask combustion and subsequent titration of the ionic halide species (1–3). This technique is destructive, tends to be slow and tedious, and is unsuitable for substances combined in intractable oleaginous formulations, particularly when present in microquantities, or for substances containing large amounts and varieties of active and inactive components.

Neutron activation analysis (NAA) is considered most useful for trace analysis because of its unique combination of high sensitivity and specificity and its freedom from contamination by laboratory reagents. To attain high sensitivity, however, radiochemical separation techniques are often required. The chief advantage of NAA for major elemental constituents in pharmaceutical chemicals is its nondestructive nature; *i.e.*, it does not require any physicochemical preparative steps. Consequently, it is rapid, simple, and much less prone to losses; nevertheless, it retains a high degree of specificity, primarily because the technique is normally unaffected by the complex organic matrix of many pharmaceutical products. Thus, NAA is most useful when the preservation of the chemical integrity of the sample is of particular concern, *e.g.*, in forensic or regulatory cases.

Basically, the method involves irradiating a particular product with thermal neutrons and recording and measuring the resulting induced radioactivity with a γ-ray spectrometer. Since the nuclear parameters of each element are highly specific and well known, the reaction with thermal neutrons is predictable (4). The qualitative and quantitative analyses of that element in the product are then derived from the emitted γ-rays obtained as a spectrum.

Table I summarizes the nuclear properties of the halogen elements of interest in this study. The bombardment of these elements with thermal neutrons results in a reaction (n,γ), which is favored by a relatively high cross-section-producing radionuclides (5).

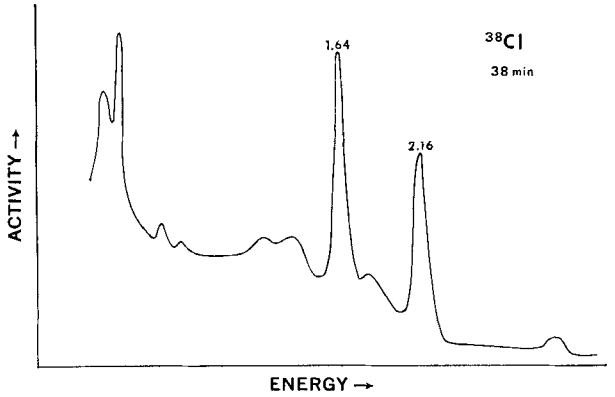

Figure 1—*Typical γ-ray spectrum of ^{38}Cl.*

This study shows that NAA can be used to determine the chlorine, bromine, or iodine content of several pharmaceutical chemicals and that it is useful as a presumptive assay method for various dosage forms.

EXPERIMENTAL

Irradiation Containers—Solid or liquid samples and standards were placed in separate 1-ml. snap-cap polyethylene vials which were then sealed into individual polyethylene bags. The vials had been previously analyzed in this laboratory for their halogen content and were found to contain less than microgram amounts of halogens; these amounts are insignificant compared to the level being determined.

Standards—Standard chlorine, bromine, and iodine solutions were prepared by dissolving accurately weighed samples of reagent grade ammonium chloride, sodium bromide, and potassium iodide in distilled water. Aliquots of each were transferred to the vials. Prior to assay, the concentrations of halide in the samples and standards were ascertained to be at about the same level.

Samples—Milligram quantities of bulk compounds (accurately weighed), 1 ml. of liquid samples or dilutions of them, 1 g. or less of ointments or oils, and tablets or capsules were packaged in the vials and analyzed without further treatment. The bulk chemicals used were readily available from commercial sources (USP grades) and were analyzed as received without drying or any other treatment.

Apparatus—A 1-Mw. nuclear research reactor[1] (swimming pool type) provided the neutrons for sample irradiations. An exposure tube flux of about 10^{13} n/cm.^{2}/sec. was used for samples containing less than about 1 mg. of halogen, and a pneumatic tube (rabbit) flux of about 10^{12} n/cm.^{2}/sec. was used for samples containing more than about 1 mg. of halogen. γ-Ray spectra were obtained with the apparatus previously described (6).

Procedure—Samples were packed individually with a standard into a rabbit and fired to the terminus of the reactor's pneumatic tube facility. When using the exposure tube, three samples and a standard were loaded into an irradiation bucket and lowered into the irradiation position. Irradiation times, ranging from 10 to 120

[1] At the Naval Research Laboratory, Washington, D. C.

Table I—Pertinent Nuclear Properties of Halogens for Activation with Thermal Neutrons

Stable Nuclide	Abundance, %	Cross-Section, barns	Radionuclide Formed	Half-Life, $t_{1/2}$	Major γ-Rays, Mev.
^{35}Cl	75.4	0.005	^{36}Cl	3×10^5 years	—
^{37}Cl	24.6	0.56	^{38}Cl	37.3 min.	1.64, 2.16
^{79}Br	50.5	8.5	^{80}Br	17.6 min.	0.511, 0.618
^{81}Br	49.5	3.1	^{82}Br	35.6 hr.	0.55, 0.78, 1.04, 1.32
^{127}I	100	6.4	^{128}I	25 min.	0.445, 0.525

Table II—Determination of Chlorine in Various Drugs

Sample	Chlorine Found by NAA Method — Percent Actual	Chlorine Found by NAA Method — Percent of Theory	Percent of Drug by Official Method
Chloramphenicol	21.85	99.7	99.3
Chloramphenicol palmitate	13.25	104.9	102.0
Chloramphenicol sodium succinate	15.28	95.9	98.0
Chlortetracycline bisulfate	6.117	99.3	100.5
Demethylchlortetracycline base	7.705	100.9	101.3
Griseofulvin	9.98	99.3	98.4
Sodium cloxacillin monohydrate	6.84	91.9[a]	95.4
Sodium dicloxacillin monohydrate	13.97	100.6	99.1[c]
Chlorobutanol (hydrous)	56.3	93.9[b]	—
Chlorothen citrate	7.23	99.4	—
Carbinoxamine maleate	8.80	101	—
Chlorpheniramine maleate	9.03	99.5	—
Aluminum chlorhydroxyallantoinate	11.4	101	—
Chloranil	56.8	98.2	—

[a] Uncorrected for interference of ^{24}Na (see text). [b] Uncorrected for water content (6% maximum). [c] Based on Schöniger flask combustion method.

sec., were varied to control the total radioactivity produced in the sample.

Samples and accompanying standards were then counted at specific decay times at a suitable distance from the detector to prevent any changes of the pulse height analyzer (PHA) calibration due to activity levels. The photopeak was analyzed by the method of Covell (7). This procedure yielded a practical activity measurement, including the statistical variation associated with radioactive decay.

RESULTS

The γ-ray spectrum of ^{38}Cl shown in Fig. 1 exhibits two major peaks. The peak at 2.16 Mev., generally freer from interferences, was used in this analysis. The chlorine content of several commercial grade pharmaceutical chemicals and the assays of several chlorine-containing drugs in dosage forms, as determined by NAA, are shown in Tables II and III. A large spectral interference from ^{24}Na was observed in sodium cloxacillin monohydrate; this may account for the difference listed in Table II. Other substances containing a lower sodium content relative to chlorine, such as chloramphenicol sodium succinate or sodium dicloxacillin monohydrate, did not exhibit this interference to any noticeable extent.

The neutron activation method was especially useful when applied to veterinary drug formulations used to treat mastitis (6). These products contain varying mixtures of antibiotics, steroids, and sulfonamides in oils and often incorporate a variety of additives such as chlorobutanol as a preservative. However, no convenient analytical method is available for determining the chlorine-containing component in these formulations. In the absence of other chlorine-containing substances, nondestructive NAA was shown to be well suited for control or regulatory purposes. NAA of over two dozen such products from different manufacturers gave results averaging better than 95%, with a coefficient of variation of less than 6%. Although results for chlorine content of these samples could not be confirmed by another method, these results may be considered presumptive substantiation that the method works well with this type of product.

Results shown in Table III for various dosage forms of chloramphenicol, griseofulvin, sodium dicloxacillin, aluminum chlorhydroxide, oxytetracycline hydrochloride, and other oil suspensions generally indicate good correlation between chlorine content as determined by NAA and drug content as found by official methods (8). Because individual capsules or tablets from a given lot were tested by NAA whereas other pooled samples were tested by the official method, some disparity is likely. In some cases, NAA yielded higher values than the official methods. These values are attributable to extraneous sources of chlorine such as colorants or inks used in printing identifying markings.

Two lots of griseofulvin and one of chloramphenicol, which yielded more erratic results, were subjected to closer scrutiny (Table IV). A sampling of each was irradiated repetitively eight

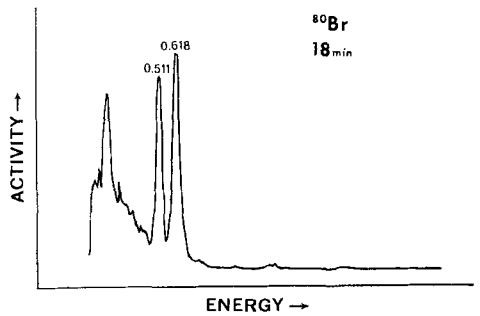

Figure 2—*Typical γ-ray spectrum of ^{80}Br.*

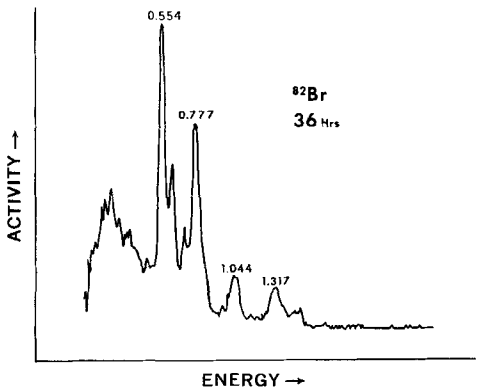

Figure 3—*Typical γ-ray spectrum of ^{82}Br.*

Table III—Determination of Chlorine for Assay in Various Drugs in Dosage Forms

Sample	Type	Chlorine Found by NAA[a] — Milligram Drug	Chlorine Found by NAA[a] — Percent Label Claim	Chlorine Found, Percent Label Claim by Official Method
Chloramphenicol	Soft gelatin capsule	280/capsule	112	98.8[b]
Chloramphenicol	Ointment	10.1/g.	101	97.2[b]
Chloramphenicol	Capsule, Lot 1	257/capsule	102.7	102.8[c]
Chloramphenicol	Capsule, Lot 2	260/capsule	104	105.6[c]
Chloramphenicol	Otic solution, Lot 1	5.79/ml.	116	118[b]
Chloramphenicol	Otic solution, Lot 2	6.28/ml.	126	121[b]
Chloramphenicol palmitate	Suspension	31.4/ml.	100	105.3[b]
Chloramphenicol sodium succinate	Injection	77.0/ml.	106	104.3[c]
Griseofulvin	Capsule, Lot 1	136/capsule	108.8	106.8[c]
Griseofulvin	Capsule, Lot 2	263/capsule	105	100.5[c]
Griseofulvin	Tablet, Lot 1	261/tablet	104	100.6[c]
Griseofulvin	Tablet, Lot 2	500/tablet	99.9	102[c]
Dicloxacillin sodium	Capsule, Lot 1	117/capsule	93.4	100.8[d]
Dicloxacillin sodium	Capsule, Lot 2	125/capsule	99.8	96.0–101.1
Dicloxacillin sodium	Capsule, Lot 3	241/capsule	96.2	98.4[d]
Aluminum chlorhydroxide	Solution, Lot 1	3.52[e]	110	—
Aluminum chlorhydroxide	Solution, Lot 2	3.63[e]	113	—
Aluminum chlorhydroxide	Solution, Lot 3	3.32[e]	109	—
Oxytetracycline hydrochloride	Otic ointment, Lot 1	0.618	107	—
Oxytetracycline hydrochloride	Otic ointment, Lot 2	0.604	104	—
Chlorobutanol	Oil suspension, Lot 1	1.87%	99.5	—
Chlorobutanol	Oil suspension, Lot 2	4.92%	98.4	—
Chlorobutanol	Oil suspension, Lot 3	5.54%	98.8	—

[a] Calculated from theoretical chlorine content of the drug; based on label claim. [b] By microbiological assay. [c] By UV spectrophotometry. [d] By iodometric assay. [e] Calculated as percent chlorine.

times on different days with an accompanying standard. The results of this study show a coefficient of variation of less than 6%.

The γ-ray spectrum of the 17.6-min. ^{80}Br nuclide (Fig. 2) shows the two major photopeaks used for analysis. Results obtained from bromine-containing drugs based on this nuclide are shown in Table V. Figure 3 shows the γ-ray spectrum of the 35.6-hr. ^{82}Br, and results based on this nuclide are listed in Table VI. Analyses based on the two different radionuclides were used mainly for verification. The longer-lived nuclide appeared to yield more precise data, probably due to less background interference from a longer decay time as well as the use of longer counting times to improve statistics.

Although the analyses of these bromine-containing drugs were not confirmed by other methods, the results obtained from NAA are believed to be reliable. The high values obtained for brompheniramine maleate based on both radionuclides and for homatropine methylbromide based on ^{82}Br indicate the probable presence of bromine-containing impurities or degradation products.

The γ-ray spectrum of ^{128}I (Fig. 4) shows the intense γ-ray photopeak at 0.44 Mev. used to analyze iodine-containing samples. Tables VII and VIII show results of analyses made at the milligram and microgram levels of iodine, respectively. The iodine content of several of these samples was also determined by the Schöniger flask combustion technique, but the results appeared to be generally lower than those obtained from NAA. The high results obtained by both methods with one batch of iodochlorhydroxyquin can be

Table IV—Study of Precision of Method for Chlorine[a]

	Chlorine Found, mg. — Griseofulvin Tablets, 500 mg.	Griseofulvin Capsule, 125 mg.	Chloramphenicol Soft Capsule, 250 mg.
	49.92	14.14	59.89
	52.39	13.55	57.99
	48.07	13.46	64.69
	52.50	13.40	56.67
	50.43	14.08	66.09
	48.74	14.57	60.97
	48.13	12.92	62.30
	51.45	13.20	64.75
Average	50.20	13.67	61.67
Calculated chlorine content from label claim, mg.	50.25	12.56	54.86
Percent label claim based on chlorine found	99.9	108.8	112.4
SD	1.80	0.549	3.40
Coefficient of variation, %	3.59	4.02	5.51
95% confidence limit	±3.00%	±3.36%	±4.60%

[a] One sample of each was irradiated eight different times on different days and compared each time with a standard ammonium chloride solution irradiated at the same time.

Table V—Determination of Bromine in Various Drugs Based on ^{80}Br

Sample	Type	Bromine Found, Percent of Label Claim or Theory		
		Percent of Theory	at 0.51 kev.	at 0.63 kev.
Homatropine methylbromide	Bulk	21.58	104.9	111.9
Brompheniramine maleate	Bulk	18.36	119.1	117.9
Hydroxyamphetamine hydrobromide	Bulk	34.42	100.5	97.8
Brompheniramine maleate	Tablet	—	103.6	100.2
Hydroxyamphetamine hydrobromide	Spray	—	101.0	102.4

ascribed to degradation, since the material was noticeably discolored.

Because of the relatively high content of iodine in bulk chemicals and in macrodosage forms, these samples were irradiated in the pneumatic system. Each exposure was comprised of groups of two sets placed on top of each other in the rabbit, each set consisting of one sample and one standard. Since each set was in a different location in the rabbit and thus at a different distance from the neutron source, it was exposed to a different flux, as noted by an activity differential of about 25% (Table IX). However, replications of these irradiations on 12 separate aliquots resulted in a coefficient of variation of less than 3%, due mostly to the short time of irradiation. This reproducibility allows for a reliable correction factor for flux variation, if necessary.

DISCUSSION

Three of the generated radionuclides of halogens have characteristic half-lives of less than 1 hr. Significantly, this situation allows several analyses to be performed within that time interval. In the case of bromine, the existence of another longer-lived radionuclide permits qualitative and quantitative corroboration on the same sample after complete decay of the shorter-lived radionuclide. Although the assay is not as rapid, the usefulness of NAA can thus be extended to include pharmaceuticals containing major amounts of other elements that would normally dominate and, hence, interfere with the accumulated γ-ray spectrum immediately after neutron exposure.

Selection of Test Conditions—The choice of irradiation position, duration, cooling-wait time, and counting geometry was manipulated to maintain a sufficiently low dead-time, generally less than 20%. Securing a low dead-time is necessary for obtaining reliable counts. A lower dead-time allows the gain of the analyzer to remain constant and the γ-ray photopeaks to remain undistorted. The shorter-lived nuclides of ^{38}Cl, ^{80}Br, and ^{128}I were counted for 1 or 2 live min. at a distance of 10–15 cm. from the detector, whereas the ^{82}Br samples were counted for 40 min. in the well detector to gain maximum sensitivity from the detector.

Quantitative analysis based on induced radionuclides of relatively short half-lives requires an adjustment of the activity measurement. A decay factor, calculated according to the standard radioactive decay law, was therefore used to correct all measurements to the same time equivalent, usually the end of neutron exposure.

Table VI—Determination of Bromine in Various Drugs Based on ^{82}Br

Sample	Type	Bromine Content, Theory or Label Claim	Bromine Found, Percent of Theory or Label Claim	
			at 1.04 Mev.	at 0.77 Mev.
Acetyl carbromal	Bulk	28.63%	94.9	95.8
Anisotropine methylbromide	Bulk	22.05%	98.4	98.3
Bromindione	Bulk	26.53%	101.5	104.7
Dexbrompheniramine	Bulk	18.36%	96.1	97.9
Dextromethorphan hydrobromide	Bulk	21.58%	103.7	103.2
Homatropine methylbromide	Bulk	21.58%	113.2	113.6
Merbromine	Bulk	19.86%	97.6	99.8
Pipobroman	Bulk	44.88%	92.1	94.2
Scopolamine hydrobromide	Bulk	20.79%	96.5	97.6
Tetrabromophenolphthalein ethyl ester	Bulk	45.7%	104.7	103.7
5-Bromosalicylamide	Bulk	27.35%	107.5	106.2
4,5-Dibromosalicylamide	Bulk	43.07%	102.1	101.7
Tribromsalan	Bulk	53.28%	91.6	91.9
Tetrabromosalicylamide	Bulk	60.44%	91.7	92.6
Bromisovalum	Bulk	35.82%	97.2	98.1
Brompheniramine maleate	Bulk	18.36%	114.9	110.4
Homatropine methylbromide	Tablet	5.825 mg./5 tablets	95.2	97.9
Homatropine methylbromide	Pediatric solution	0.281 mg./ml.	97.7	94.6
Dextromethorphan hydrobromide	Syrup	0.647 mg./ml.	95.8	91.7
Sulfobromophthalein sodium	Injection	19.1 mg./ml.	104.8	101.0
Homatropine methylbromide	Capsule	0.279 mg./capsule	109.8	105.4
Pipenzolate bromide	Tablet	0.923 mg./tablet	95.5	95.5
Hydroxyamphetamine hydrobromide	Spray	1.721 mg./ml.	102.6	102.7
Hydroxyamphetamine hydrobromide	Solution	3.442 mg./ml.	96.6	101.8

Table VII—Determination of Iodine in Various Drugs

Sample	Type	Iodine Content, Theory or Label Claim	Iodine Found: Percent of Theory or Label Claim	Iodine Found: Percent by Chemical Method
Bismuth oxyiodide	Bulk	36.06%	102.5	—
Erythrosine (FD&C Red No. 3)	Bulk	57.70%	95.5[a]	—
Idoxuridine	Bulk	35.84%	97.5	—
Iodohydroxyquin, Lot 1	Bulk	41.54%	123.8[b]	113[c]
Iodohydroxyquin, Lot 2	Bulk	41.54%	99.9[d]	92[c]
2-Iodobenzoic acid	Bulk	51.17%	102.3	—
Potassium iodide	Bulk	76.45%	99.5	—
Sodium dextrothyroxine	Bulk	63.54%	92.7[e]	—
Sodium diatrizoate	Bulk	59.87%	101.0	—
Sodium iothalamate	Bulk	59.87%	95.5	—
Sodium methiodal	Bulk	52.04%	95.0	—
Meglumine diatrizoate, Lot 1	Injection	15.0%	101.5	98.1[f]
Meglumine diatrizoate, Lot 2	Injection	15.0%	99.7	99.9[f]
Meglumine diatrizoate, Lot 3	Injection	15.0%	99.0	99.9[f]
Meglumine diatrizoate, Lot 4	Injection	15.0%	98.5	97.4[f]
Meglumine diatrizoate, Lot 5	Injection	37.0%	99.9	—
Meglumine diatrizoate, Lot 6	Injection	40.0%	100.1	—
Meglumine iodipamide	Injection	26.0%	100.2	—
Iodochlorhydroxyquin, Lot 1	Ointment	12.5 mg./g.	98.8	101[f]
Iodochlorhydroxyquin, Lot 2	Ointment	12.5 mg./g.	94.5	—
Iodochlorhydroxyquin, Lot 3	Ointment	12.5 mg./g.	96.9	—
Iodochlorhydroxyquin, Lot 1	Lotion	12.5 mg./ml.	104.6	103[f]
Iodochlorhydroxyquin, Lot 2	Lotion	12.5 mg./ml.	103.8	—
Iodochlorhydroxyquin, Lot 3	Lotion	12.5 mg./ml.	103.6	—
Penicillin G diethylaminoethyl hydroiodide, Lot 1	Injection	34.0 mg./ml.	98.9	—
Penicillin G diethylaminoethyl hydroiodide, Lot 2	Injection	34.0 mg./ml.	94.3	—
Sodium iodide	Injection	84.66 mg./ml.	97.5	—

[a] Corrected. These samples normally contain 7% volatiles, 90% tetraiodo, 5% triiodo, and 0–1% diiodo analogs, and about 2% NaCl. [b] Sample was discolored. [c] By Schöniger flask combustion and UV absorption; single assays. [d] Different source, fresh sample. [e] As is, or 98.8% on dried basis. [f] Results submitted by manufacturer.

Factors affecting activity such as variability in the neutron flux are minimized by treating the sample and standard identically so that activity and halogen content are directly related.

Elimination of Interferences—In cases where the half-life of the radionuclide is relatively long, correction due to radioactive decay becomes negligible and may be omitted. Thus, no such correction was used when quantitating bromine based on ^{82}Br. The sample was allowed to decay for about a week to minimize the effect of such radionuclides as ^{24}Na.

Additive interferences from accompanying transmutation reactions with epithermal and fast neutrons, producing ^{38}Cl from Ar, K, or S, or ^{80}Br and/or ^{82}Br from Kr, Rb, U, or Se, or ^{128}I from Xe, are negligible. The quantity of these elements, if present in these products, is very limited. The probability of the reactions is extremely small because of the relative abundance of these elements, the minute cross-section of these reactions, and the short irradiation times.

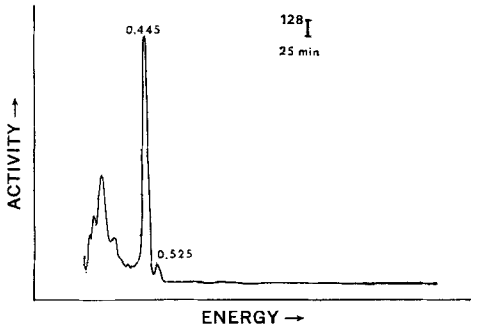

Figure 4—*Typical γ-ray spectrum of ^{128}I.*

Spectral interferences attributed to extraneous radionuclides may be minimized by several techniques. One method employs a lithium-drifted germanium [Ge(Li)] detector; another allows the interfering radionuclides of shorter half-lives to decay first. The use of a Ge(Li) detector is desirable because of its highly discriminate resolution. However, its inherent lower efficiency, roughly 5% relative to sodium iodide, can be a major drawback. Interfering radionuclides having a relatively shorter lifetime than the one of interest are allowed to decay to a negligible level before taking the activity measurements. The use of a differential count, *i.e.*, the difference in the γ-ray spectra for two time intervals, is used to determine the effect of spectral and background interferences. This technique proves particularly useful for lower concentration levels. These techniques of quantitation are performed to ensure the sensitivity and specificity of the NAA method.

Precision—Precision involved in nondestructive NAA is largely determined by weighing the samples and standards and by the net count. Since relatively large sample sizes were used in these studies, the net count had the greatest effect on precision. The irradiation and measurement of activity were controlled to obtain a net average count of better than 50,000 and a standard deviation of better

Table VIII—Determination of Iodine in Thyroid Compounds[a]

Sample	Iodine Content, Theory or Label Claim	Iodine Found	Percent of Theory or Label Claim
T4 (from TLC)	65.34%	67.1%	102.7
T3 standard solution 1	58.48%	73.94%	126.4
T4 standard solution 1	65.35%	69.26%	106.0
T3 (from TLC)	58.48%	57.19%	97.8
T3 standard solution 2	58.48%	61.88%	105.8
T4 standard solution 2	65.34%	67.50%	103.3
Thyroid tablet, 1 gr.	130 mcg./tablet	161.3 mcg./tablet	124
Sodium L-thyroxine, 0.1 mg.	57.1 mcg./tablet	64.2 mcg./tablet	112
Sodium D-thyroxine, 2 mg.	1271 mcg./tablet	1204 mcg./tablet	94.7
Sodium D-thyroxine, 4 mg.	2542 mcg./tablet	2134 mcg./tablet	83.9
Thyroid powder, Lot 1	0.529%	0.597%	113
Thyroid powder, Lot 2	0.0672%	0.0704%	105
Thyroid powder, Lot 3	0.20%	0.225%	113
Thyroid powder, Lot 4	0.225%	0.242%	108
Thyroid powder, Lot 5	0.35%	0.445%	127
Thyroid powder, Lot 6	0.38%	0.418%	110
Thyroid powder, Lot 7	0.49%	0.433%	90.4
Thyroid powder, Lot 8	0.84%	0.785%	93.5
Thyroid powder, Lot 9	0.85%	0.924%	109

[a] Values on thyroid powder all obtained by Schöniger flask combustion and UV absorption.

than 1%. The lowest count obtained was about 2000, which gave a standard deviation of about 5%. Systematic errors associated with other variables are minimized by treating samples and standards together during irradiation and counting them identically with respect to geometry. Uncontrollable variations may arise from nonhomogeneity of samples, particularly solids.

NAA measures the elemental content regardless of the physicochemical state of the sample. It does not necessarily relate the element to the compound nor to the biological activity of the drug, which is of paramount concern. The technique offers the advantages of increased elemental specificity, rapidity, nondestructiveness, and simplicity. Thus, with the ever increasing accessibility of neutron sources and computer interfacing to perform calculations, NAA can readily be employed as a reliable assay method for bulk pharmaceutical chemicals and as a rapid screening method in the presumptive assay of a multitude of drugs.

Table IX—Study of Precision in Iodine Measurement[a]

Standard	Rabbit Top, A_0	Standard	Rabbit Bottom, A_0
1	110,128	2	143,769
3	110,783	4	143,952
5	115,885	6	149,846
7	112,248	8	148,736
9	113,943	10	145,808
11	116,282	12	151,169
13	118,157	14	150,320
15	115,927	16	145,145
17	110,259	18	144,545
19	113,838	20	142,246
21	108,441	22	143,869
23	115,404	24	149,248
Average	113,441		146,554
SD	3,035		3,093
Coefficient of variation, %	2.67		2.11
95% Confidence limit	1.70%		1.34%

[a] Twelve different irradiations of two standard solutions (24 different aliquots) in each rabbit, showing a flux differential of about 25% from the top to the bottom.

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Automation of NF Method I–USP Dissolution-Rate Test

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Abstract □ The construction and operation of an automated dissolution-rate system are described. The system incorporates the NF XIII Method I–USP XVIII dissolution apparatus, using stirring basket assemblies and 1000-ml. resin flasks. Six tests are conducted simultaneously, using a commercially available spectrophotometer capable of monitoring seven separate cells at programmed intervals and a six-channel pump. Data are presented, demonstrating the application of the system using UV analysis.

Keyphrases □ Dissolution-rate testing—automated, multiple system □ UV spectrophotometry—monitoring, automated dissolution-rate testing □ Tablets, dissolution-rate testing—automated, multiple system

Dissolution-rate measurements are used extensively in the pharmaceutical industry to help control manufacturing processes and to detect changes in production conditions. They also are used to evaluate changes in ingredient properties, packaging materials, and storage conditions with respect to drug release from solid dosage forms. A multitude of methods for dissolution-rate testing have been employed, all having the following essential features: a dissolution vessel, a dissolution fluid, a means of agitation, a means of sampling or transporting the dissolution fluid, and an analytical procedure to measure the dissolved drug.

To reduce manpower or to produce more dissolution-rate data per unit time, some methods have been automated to varying degrees (1–9). Among the first to recognize the need for automated instrumentation to provide sufficient amounts of dissolution-rate data were Schroeter and Wagner (1). They used the USP disintegration apparatus to provide agitation and circulated the filtered sample of test fluid through a flow cell, with UV absorbances recorded on a strip chart. In the dissolution-rate testing apparatus of Pernarowski *et al.* (8), agitation was achieved by rotating a 10-mesh wire basket containing the solid dosage form. Additional agitation was gained by the attachment of stirring blades to the top of the basket. The design of this basket assembly was modified by replacing the 10-mesh screen with 40 mesh and removing the stirring blades, and it was incorporated into the dissolution-rate apparatus of NF XIII Method I (9) and USP XVIII (10).

The increased need for dissolution-rate testing in these laboratories prompted a survey of procedures applicable to automation. The NF–USP method was selected because of its flexibility and its apparent widespread acceptance by the pharmaceutical industry. Following the assembly of a single unit, the need for additional dissolution-rate data prompted the construction of a system capable of multiple testing. The mating of commercial modules and the construction of new units to give the capability of monitoring dissolution rates in six separate vessels, using UV analysis, are the subjects of this report.

EXPERIMENTAL

Equipment—The assembled system is shown in Fig. 1. The commercially available units consist of the following:

1. Beckman Kintrac VII[1]. This spectrophotometric system is capable of monitoring seven separate cells, six test solutions and one reference, at programmed intervals. Recorder chart values of absorbance readings are linearized electronically, thereby permitting expansion or attenuation, depending on the dissolution characteristics of the sample. The absorbance of each cell, corresponding to a particular flask, is recorded on a strip chart and represents percentage dissolution of a tablet or capsule at recorded time periods.
2. Pump, six channel[2]. The original plunger gaskets were replaced with Teflon–asbestos cord packing.
3. Water baths, three: (*a*) 95 l.[3]—holds the six dissolution vessels while dissolution rates are determined; (*b*) 20 l.[4]—maintains a second set of six dissolution flasks with fluid at the proper temperature; and (*c*) 8 l.[5]—circulates water to a separate 11-l. insulated reservoir; the water is brought to the proper temperature for use in the preparation of dissolution media.
4. Flow cells, 10, 1.0, and 0.5 mm.[6].

The constructed units consist of the following:

1. A rack for the 95-l. water bath holds and positions the six, 1000-ml. dissolution flasks in a straight line, 14.6 cm. apart, at their centers. A coil spring is located under each flask to raise it above the top surface of the rack for ease of loading and unloading. The flasks are pressed against the springs by the weight of the stirring unit when it descends into position.
2. A manifold plate covers the dissolution flasks and provides inlet and outlet connections for sampling the six flasks. Inlet and outlet tubes consist of 4.8-mm. o.d., 0.5-mm. wall, stainless steel tubing, with 60-mesh stainless steel cylindrical screen filtering devices attached to the outlet tubes. The manifold is correctly positioned relative to the dissolution flasks by pins extending through holes in the manifold. The manifold, with inlet and outlet tubing attached, can be picked up manually, brought forward, and placed in a tank for cleaning of lines and for standardization between tests.
3. A vertically movable platform supports the six NF–USP dissolution baskets and their drive train. The platform is raised and lowered by a pair of worm gear screw jacks[7] positioned at the ends of the platform and mounted on the table top. So that the jacks rise in unison, each is driven by a timing belt from a single, totally enclosed, 0.33-hp., ball-bearing motor[8]. Microswitches, located above

[1] Beckman Instruments, Fullerton, Calif.
[2] Hughes, Marton Equipment Inc., Beverly, Mass.
[3] Precision Scientific Co., Chicago, Ill.
[4] Blue M Electric Co., Blue Island, Chicago, Ill.
[5] Tamson model P29, Witt Sales, Cleveland, Ohio.
[6] Hellma, Jamaica, N. Y.
[7] Model WJ62-18, Joyce, Crulland Co., Dayton, Ohio.
[8] Reliance Electric Co., Cleveland, Ohio.

and below the table top, stop the units at the ends of travel. Due to the momentum developed when moving the platform, it was necessary to apply a brake[9] on the motor shaft when stopping. As safety measures, additional microswitches were installed at both ends of travel of the movable platform and on a dual idler running on the two belts powering the screw jacks. If either belt breaks, the microswitch causes the main power supply to be disconnected and the motor brake to be applied.

4. A common motor assembly drives the six stirring baskets[10], which are connected in line with flexible couplings[11]. The vertical output shaft of each drive is provided with a bushing to hold the shaft of the dissolution basket. A 0.05-hp. motor[12] drives the angle gears by means of a timing belt. Direct current power is supplied to the motor by a speed controller[13]. The speed is infinitely variable from 0 to 325 r.p.m. Visual readout of the basket rotation is provided by a tachometer generator[14], mounted on the drive motor, and a speed dial in revolutions per minute, located on the main control panel.

5. Polyethylene tubing of approximately 3.2 mm. is used for all flow lines, except for 3.2-mm. Tygon tubing[15] attached to exit and entrance ports of the flow cells. Fifteen-gauge stainless steel tubing, bent at a 45° angle and embedded in a plastic block, connects the Tygon and polyethylene tubing (Fig. 1). The polyethylene tubing is fully inserted into the 4.8-mm. stainless steel tubing of the common manifold, which extends into the dissolution vessels, at a level approximating the midpoint of the stirring basket assembly. Insertion of the polyethylene tubing into the entrance and exit tubes of the manifold facilitates isolation of individual flow lines for cleaning and testing purposes. Hold-up volume of the flow system is also reduced. All lines are of equal length polyethylene and Tygon tubing and have equal hold-up volume.

6. Two emergency stops are provided for the operator, one at the control panel and one on the vertically moving head. Stainless steel guarding covers all electrical and moving parts.

7. A special table to hold the constructed units and the 95-l. water bath was assembled from welded 6.4 × 6.4-cm., 11-gauge steel tubing, providing a rigid, vibration-free mounting surface.

Operating Procedure—Prior to determining dissolution rates of solid dosage units, the six flow cells are corrected for their absorbances against an air blank while fluid is pumped through them. Appropriate standards are pumped through the flow cells, with exit lines leading to waste. Span adjustments of the spectrophotometer are made so that desired values for the standard are recorded on the chart. Lines are rinsed and placed in the dissolution vessels containing the specified volumes of dissolution fluid, pumping is continued, and a zero baseline for all flow cells is verified. Tablets or capsules are placed in the basket assemblies, which are automatically lowered into the flasks. Basket rotation automatically begins when the baskets are 2 cm. from the bottom of the flasks. The absorbance of each solution is determined at approximately 1-min. intervals.

Interpretation of Results—The percent of tablet dissolved within the desired time period is interpolated from the chart. Each series of six bar graphs represents absorbance readings for the six dissolution vessels. As an aid in determining dissolution rates as a function of time, a template was constructed so that only the desired absorbance recordings on the strip chart could be viewed. Openings were made in a translucent overlay at regular intervals, thereby exposing only absorbance recordings from a single flow cell.

RESULTS AND DISCUSSION

With the multiple testing system, six dosage units can be tested simultaneously. Selection of this number was geared to the capabilities of the commercial spectrophotometric system and to the fact that the NF–USP specifications are expressed in units of six tablets or capsules.

[9] Model 50603, Able Manufacturing Co., River Edge, N. J.
[10] Six model R-3100 type 3-way "Anglegear" units, having 1:1 ratios, Airborne Accessories Corp., Hillsdale, N. J.
[11] Model A-060 couplings, Lovejoy Flexible Coupling Co., Chicago, Ill.
[12] Type NSH-33R, Bodine Electric Co., Chicago, Ill.
[13] Model SL-32, Minarik Electric Co., Los Angeles, Calif.
[14] Bodine Electric Co., Chicago, Ill.
[15] Manifold Tubing, Technicon Corp., Tarrytown, N. Y.

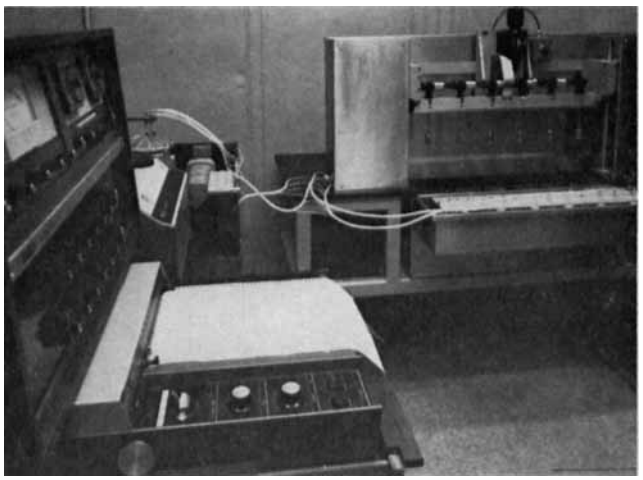

Figure 1—*Instruments comprising the automated six-unit dissolution-rate apparatus.*

The entire flow system (flow lines, pump heads, and flow cells) is flushed in approximately 0.5 min. Absorbance plateaus for standards are recorded in this period.

The dwell time of each flow cell is optimized so that satisfactory absorbance readings are recorded and subsequent ones are made as rapidly as possible. Routinely, a dwell time of approximately 7 sec., giving identical recordings for continuous or intermittent sampling, is used. After a reading is made for the last cell, the cell compartment stops, reverses the direction of rotation, and returns to the reference position for a new series of readings. Any of the cell positions can be bypassed, read continuously, or read at programmed intervals. The latter function is particularly useful in determining dissolution rates for sustained-release products.

Initially, rotation of the basket assemblies were checked frequently by other means than the tachometer generator. Slow rotation speeds were checked by counting revolutions. A strobe light was used for determining revolutions per minute in excess of 75 r.p.m.

Two modifications were made in the Beckman Kintrac VII system which aided reliable, long-term dissolution-rate testing: (*a*) the electronic system was altered so that the chart drive could be stopped

Table I—Dissolution Rates for Steroid Tablets and Antibiotic Capsules Using the Mechanized Six-Unit Dissolution-Rate Testing System (NF–USP Method)

Lot Number	$T_{20\%}$[a] Average of Six Tablets	$T_{20\%}$[a] Slowest Tablet	$T_{40\%}$ Average of Six Tablets	$T_{40\%}$ Slowest Tablet	$T_{60\%}$ Average of Six Tablets	$T_{60\%}$ Slowest Tablet
			Prednisolone, 5 mg./C.T.			
1	0.7	1.0	1.3	1.6	1.9	2.2
2	1.2	1.7	2.0	2.7	2.9	3.8
3	1.2	1.8	2.1	2.8	2.9	3.9
4	1.6	2.6	2.6	4.2	3.9	6.7
5	3.2	5.1	5.0	7.2	7.0	9.7
6	0.8	0.9	1.2	1.4	1.8	2.2
7	1.3	4.4	2.3	7.9	3.4	11
8	1.0	1.5	1.8	2.7	3.0	4.3
9	1.4	2.2	2.5	3.9	3.8	5.6
10	2.3	7.0	4.6	15	7.0	24
			Tetracycline Hydrochloride, 250 mg./cap.			
1	3.4	3.7	4.1	4.4	5.0	5.3
2	3.6	4.3	4.4	5.3	5.4	6.5
3	4.1	5.6	5.3	7.2	6.4	8.1
4	3.6	4.4	4.6	5.3	5.9	6.9
5	3.3	4.3	4.4	5.8	5.6	6.7
6	3.6	4.8	4.9	6.3	5.9	6.9
7	3.4	4.3	3.9	5.1	5.2	5.9
8	3.2	3.7	4.3	5.2	5.6	6.9
9	2.6	3.1	3.8	4.6	5.0	5.6
10	2.5	2.8	3.4	4.1	4.7	5.3

[a] T = time in minutes.

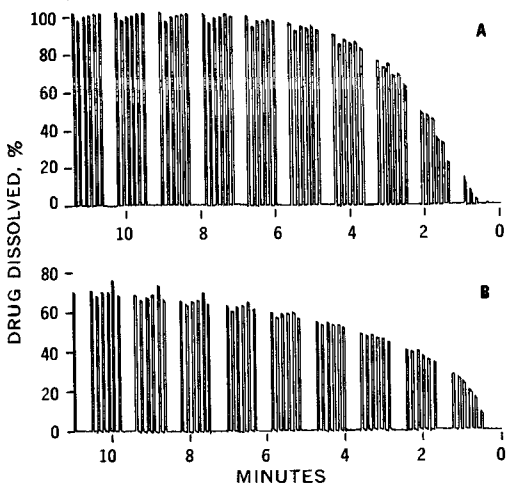

Figure 2—*Dissolution profiles for tablets showing: (A) little tablet-to-tablet variation, with rapid tablet dissolution and plateauing of responses; and (B) little tablet-to-tablet variation but with somewhat slow tablet dissolution and gradual plateauing of responses.*

in between bar graph recordings at programmed intervals (for example, for readings made only once each hour for sustained-release formulations); and (*b*) a reliable recorder pen was constructed from a 23-gauge stainless steel tube. The tube was rolled shut, and a capillary opening was made. The constructed pen was connected to the original ink supply line and inserted into a specially constructed aluminum mount, machined to fit into the original pen holder.

The automated dissolution-rate apparatus was used extensively in testing steroid and antidiabetic tablets and antibiotic capsules. It was used almost daily for the last 18 months. Recorded instrumental drift was negligible in routine testing of tablet and capsule dissolution rates. For long periods of continuous operation (for example, with sustained-release formulations), recorded drift can be corrected by adjusting the baseline by using the "off zero" of the reference absorbance. (Drift never exceeded 2% of full chart for 8 hr. of continuous operation.) Among the various steroid tablets tested were hydrocortisone, methylprednisolone, prednisone, and prednisolone. Water was the dissolution fluid, the cell lightpath was 10 mm., the wavelength was at absorbance maxima (approximately 246 nm.), and the basket rotation was 100 r.p.m. Tolbutamide tablets were tested using the tromethamine buffer at a pH of 7.6. The cell lightpath was 0.5 mm., the wavelength was 226 nm., and the basket rotated at 150 r.p.m. Tetracycline hydrochloride capsules were tested in 0.1 *N* hydrochloric acid, with basket rotation

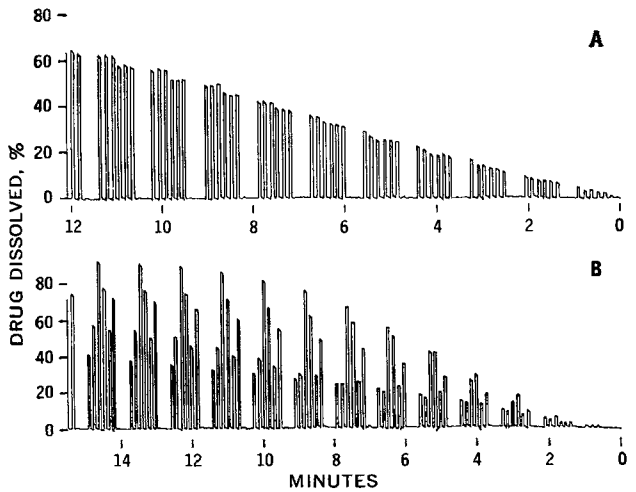

Figure 3—*Dissolution profiles for tablets showing: (A) dissolution of active ingredients at a relatively slow, uniform rate; and (B) considerable tablet-to-tablet variation and relatively slow dissolution.*

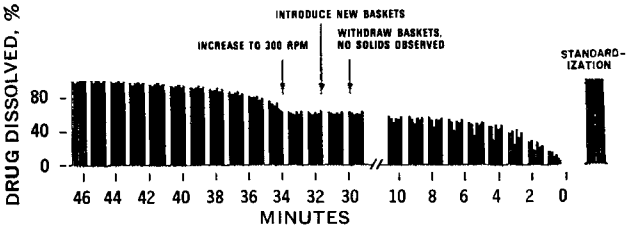

Figure 4—*Dissolution profiles suggesting complete dissolution at approximately 60% of label. Increased stirring rate (increased agitation) caused an additional 40% of drug to dissolve.*

at 150 r.p.m. The cell lightpath was 1.0 mm., and a wavelength of 268 nm. was used.

Table I gives individual data for prednisolone tablets and tetracycline hydrochloride capsules from 10 lots of each. The table gives the average for the six tablets and the slowest dissolving tablet. Data for the slowest tablet are also given since slow dissolving tablets —not the averages—determine passage or rejection of lots by NF and USP criteria. Requirements are that the lot passes if not more than two of 12 tablets fail to meet the test limits.

Figures 2 and 3 give recorder tracings of dissolution profiles as examples of four lots of differently dissolving tablets from commercial sources. Figure 2 gives data for rapidly and uniformly dissolving tablets (A) and for uniform, initially faster, but eventually slower dissolving tablets (B) than those of the first group. Figure 3 gives the results for uniformly dissolving tablets, which dissolved at a constant rate (A), and nonuniformly dissolving tablets (B).

Use of the automated unit has provided opportunities for observations which are considered pertinent. The rigidly constructed, mechanized basket assembly was nearly free of vibration as comparted to a single unit constructed with Fisher Frame. Vibration that was visually nonperceptible was rated as severe by a commercial vibration analyzer[16]. Vibration with the single unit had an enhancing effect on the dissolution rate of particular tablets when stirred at low rates. The effect of vibration was largely eliminated when the rotation speed was increased (11).

Occasionally, tablet fragments passing through the 40-mesh baskets remained on the bottom of the vessels, undisturbed by the agitation provided. Figure 4 shows portions of dissolution profiles obtained for methylprednisolone tablets in which this condition was observed. At a stirring rate of 100 r.p.m., the dissolution profiles plateaued, indicating a drug content of approximately 60% of label. Although the baskets were observed to be entirely free of tablet remnants, material from disintegrated tablets could be seen lying undisturbed on the vessel bottoms. When the rotation speed was increased to 300 r.p.m., thereby increasing agitation, the absorbance values increased sharply, reaching new plateaus corresponding to 100% of label.

The experimental data with the NF–USP basket assembly also tend to confirm the findings and conclusions of Lin *et al.* (12). They noted that dissolution material occasionally clogged the screen of the 40-mesh basket. On the other hand, when they employed an 8-mesh basket, large drug particles and their aggregates accumulated at the bottom of the dissolution flask. When using the 40-mesh basket, clogging of the screen was experienced with some tablets as rapid disintegration occurred; yet with other tablets, particles passed through the screen and settled at the bottom of the vessels. It is possible that a larger mesh size, perhaps 20, and an additional means of agitation should be provided.

SUMMARY AND CONCLUSIONS

A detailed description is given of the construction and operation of a dissolution-rate system utilizing the NF–USP apparatus. The system incorporates the stirring basket assembly and 1000-ml. resin flask of NF–USP, a six-channel pump, and a spectrophotometer capable of monitoring seven separate cells at programmed intervals. Data are presented, demonstrating the application of the system for capsules and tablets.

[16] International Research and Development Co., Columbus, Ohio.

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Data for Fig. 4 were supplied by M. L. Knuth, Control Division, The Upjohn Co.

Dry Powder Aerosols I: A New Powder Inhalation Device

J. H. BELL, P. S. HARTLEY, and J. S. G. COX

Abstract □ A new portable device is described for administration of powdered drugs by inhalation. Powder contained in a standard hard gelatin capsule is induced to flow from the capsule into the air stream by a vibratory feed mechanism arising from a rotor driven by the inhaled air. The relationship between air flow rate, vibratory cycle, and powder emission was investigated, and the results indicate a good degree of coordination of dose administration with inhalation into the inspired air stream. The ability of the device to dispense size-graded fractions of lactose, in the range of 4–400 μ, was investigated. Poor flow of the finest powders was demonstrated and can be overcome by use of a coarse flow aid.

Keyphrases □ Aerosol powder drugs—portable inhaler □ Inhaler, portable—aerosol powder drugs

Aerosol therapy constitutes a major part of the therapeutic program of many patients with airways disease. It is not generally realized that, with the pressurized aerosol or other portable inhalers, the administration of medication requires coordination of activation with the inspiratory cycle of respiration if variation in the quantity and site of drug deposition in the airways is to be minimized. The introduction of medication into the inhaled air near the end of inspiration confines drug entry and deposition to the upper respiratory tract. Many of the finer drug particles in the inhaled air would have insufficient time to deposit by sedimentation in the large diameter airways of this region before being swept out by the exhaled air flow. In contrast, drug introduced at the beginning of deep inspiration could be carried far into the lungs by the inhaled air, increasing the opportunity for fine-particle deposition in the narrow airways of the deep lung and markedly altering the pattern of drug distribution. An ideal inhalation device would ensure administration of inhalant reproducibly and conveniently with minimal patient effort, cooperation, or need for coordination.

The depth and degree of penetration of particulate matter into the respiratory tract and its subsequent deposition are an integrated effect of independent variables concerned with the characteristics of the aerosol cloud and the respiratory cycle (1). In the laboratory, experimental aerosols varying in such properties as particle size and degree of polydispersion can be prepared by selection of a suitable aerosol generator, and the quantity administered to a patient can be accurately controlled by timing the period of inhalation. To achieve reproducible particle deposition, control must be achieved over the physiological events of rate and volume of inspiration, since these play a role in determining the sizes and areas of deposition of particles within the airways. These factors are significant for particles larger than 8–10 μ, which can gain entry to the upper airways during oral inhalation when the protection afforded by the nasal cavities is bypassed. These particles can represent a major fraction of the weight of drug administered in a pharmaceutical inhalant.

Control of the respiratory cycle in man is readily attained in the laboratory by use of a spirometer and metronome, which provide the subject with an immediate indication of breathing depth and rate (2). In clinical practice, however, it is difficult if not impossible to train many patients to inhale to a specific pattern and simultaneously to coordinate dose administration at a particular point in the inhalation cycle. Consequently, the amount and site of drug deposition in in the airways and lungs may be variable, notwithstanding high precision of dose metering by the adminis-

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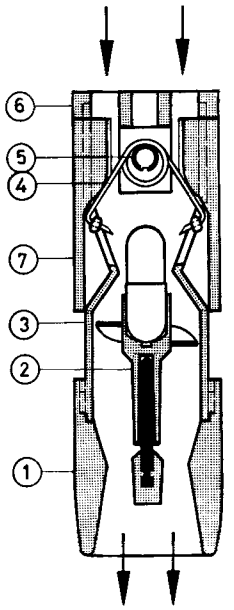

Figure 1—*The inhaler. The arrows indicate the direction of air flow during inhalation. Key: 1, mouthpiece; 2, tapered bearing; 3, casing; 4, piercing needle; 5, support and pivot for piercing needle; and 6, end cap. Scale: 1:1 approximately.*

tration device. This problem was recently studied by McIlreath *et al.* (3), using a new type of breath-actuated pressurized aerosol in which bronchodilator response in asthmatic children was used elegantly as an index of the depth of aerosol penetration. The results clearly showed the necessity for proper use of pressurized aerosols to achieve optimal response.

Administration of certain drugs by inhalation may be impractical using available portable devices such as nebulizers or pressurized aerosols. The output of solution nebulizers is limited by the physicochemical characteristics of drug solubility and solution viscosity; the pressurized aerosol has limitations in the quantity of inhalant that can be dispensed before problems such as mechanical obstruction of valve orifices occur. The preparation of stable inhalant solutions or suspensions is complicated by the lack of adequate toxicity data for the usual range of formulation aids such as surfactants and suspending agents. The direct use of dry powders in inhalation devices offers an alternative approach to drug administration but involves problems of powder fluidization, flow, and dispersion, many of which require further research (4, 5).

The recent introduction of the drug cromolyn sodium[1] (6, 7), which is administered by inhalation using a novel device, provided an opportunity to study some of these problems. In this study, attention was concentrated on the degree of coordination of dose administration with inhalation into the inspired air stream, together with the general characteristics of this particular device.

EXPERIMENTAL

The Inhaler—The arrangement is shown in Fig. 1. Essentially, a small open tube contains a plastic rotor, which revolves at high speed when air is drawn through it. Powdered drug, contained in a standard hard gelatin capsule, is inserted in a holder on the rotor. The rotor is mounted in the inhaler body by means of a loose-fitting, plastic, tapered bearing on a stainless steel shaft, the latter being inserted axially at the mouthpiece end. When air is inhaled *via* the mouthpiece, the turbovibratory action of the rotor causes the powdered drug to be dispensed into the inspired air through two diametrically opposed perforations in the capsule wall. The perforations are made immediately before inhalation by the operation of the simple piercing mechanism shown in the diagram.

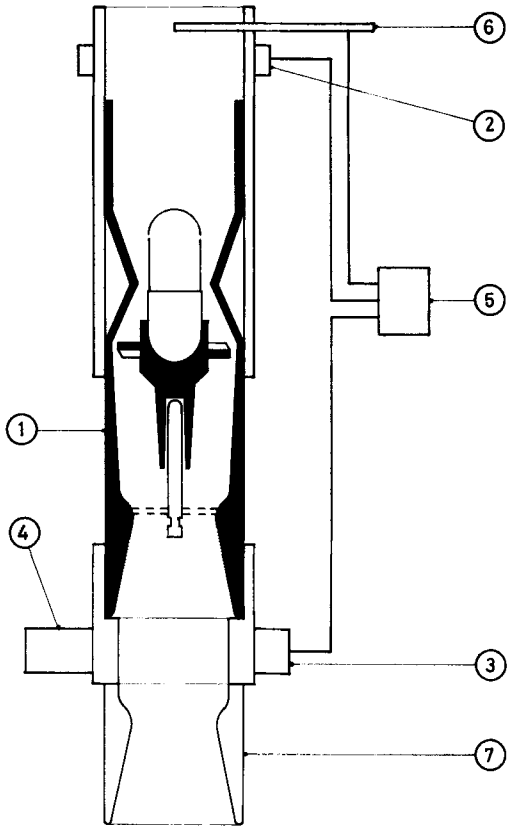

Figure 2—*Instrumentation of inhaler. Key: 1, inhaler; 2, strain gauge attached to close-fitting sleeve; 3, photoelectric cell; 4, light source; 5, multichannel recorder; 6, hot wire anemometer; and 7, mouthpiece.*

The powder is fluidized within the capsule by vibrations induced by the characteristic motion of the rotor bearing during rotation. In a normal lubricated bearing, a sliding contact would be established; in the inhaler, the relative dimensions of the rotor and shaft, together with the absence of lubricant, produce a rolling contact. This induces a whirling motion in the rotor, which is translated into a high frequency vibration of the capsule walls. The onset of this whirling causes powder to be conveyed along the capsule walls to the exit holes, irrespective of the orientation of the inhaler.

Instrumented Inhaler—The passage of air through the inhaler (Spinhaler) initiates vibrations in the rotor, felt throughout the body of the device, together with discharge of powder from the capsule. These events were individually monitored, using an instrumented inhaler shown in Fig. 2. A constant-temperature hot wire anemometer[2] mounted at the air entry port was used to detect the rapid changes in air flow rate encountered during inhalation. Since the particular motions of the rotor prevented direct mounting of a vibration sensor, a semiconductor strain gauge[3] was bonded indirectly onto the inhaler casing and served to detect vibrations transmitted *via* the stainless steel shaft. A photoelectric cell[4], mounted across the mouthpiece, detected powder discharge by 90° light scattering. Output from the three sensors was recorded simultaneously on a three-track high speed UV recorder[5]. The instrumented inhaler was operated by inserting a capsule [with holes drilled pre

[1] Intal, Fisons, (di)sodium cromoglycate.

[2] C.T.A. type 55DO5, probe type 55A, Disa, Harrow, Middlesex, England.
[3] PIXI model 8101, Endevco Corp., Royston, Herts, England.
[4] Photo duo-diode, type BPY 69, Mullard, England.
[5] Type SE 2000, S. E. Laboratories, Feltham, Middlesex, England.

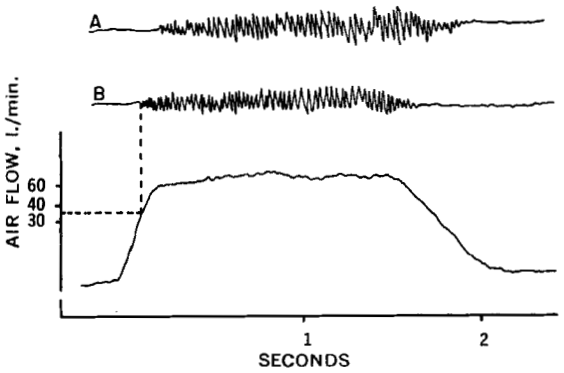

Figure 3—*Example of chart recording showing sequence of events occurring during inspiration through the inhaler. Key: A, powder discharge; and B, rotor vibration.*

viously at 0.07-cm. (0.028-in.) diameter] in the holder, starting the recorder, and then inhaling through the tube. A typical trace is shown in Fig. 3. Actual air flow rates were obtained by calibration of the hot wire anemometer against a flowmeter[6] in a separate experiment.

Effect of Powder Particle Size on Discharge of Powder from the Capsule—The flow of powders is affected by a number of factors such as particle size and size distribution, particle shape and density, surface roughness, hardness, moisture content, and bulk density (4). The influence of particle size is probably the most important; over a given size range, however, differences may be found between materials due to varying contributions from these other effects. It was of interest to examine the behavior of powders of varying properties in the inhaler. In the first instance, differing particle size was used to achieve this variation. The test powder used was lactose BP from which eight narrow size fractions were prepared using a centrifugal classifier[7], jet sieve[8], and test sieves[9] covering the range in steps from 4 to 400 μ. Capsules were partially filled at a standard volume, which gave mean fill weights ranging from 45 to 60 mg. for the

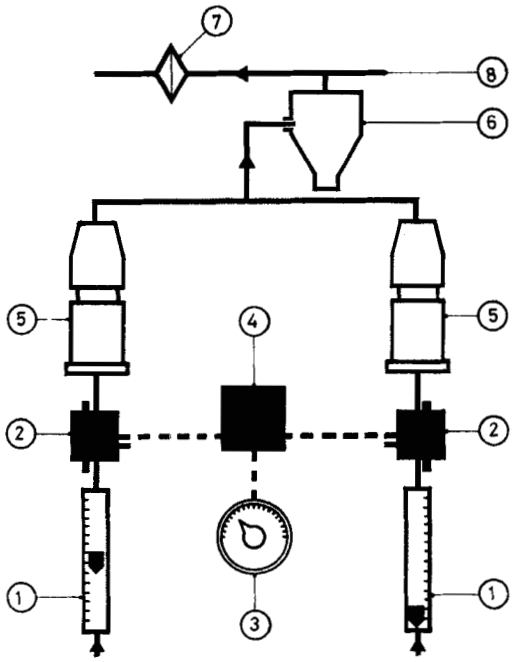

Figure 4—*Test rig used to evaluate powder flow from inhaler capsule. Key: 1, flowmeters; 2, solenoid valves; 3, timer; 4, changeover relay; 5, inhalers; 6, cyclone; 7, filter; and 8, air bleed.*

[6] G. A. Platon Ltd., Basingstoke, Hants, England.
[7] Bahco Ltd., Bahco House, London, E.C.1, England.
[8] Type 200, Alpine (Machinery) Ltd., Uxbridge, Middlesex, England.
[9] British Standard Institute, Specification No. 410.

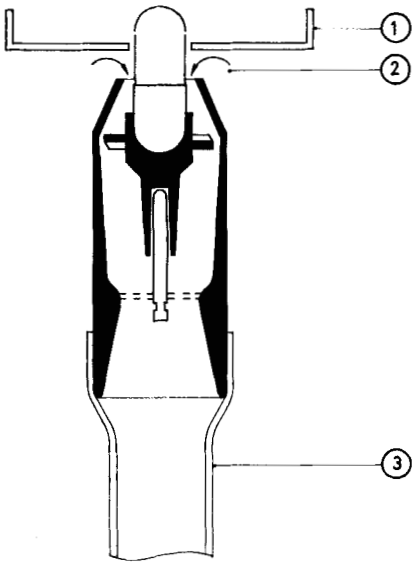

Figure 5—*Arrangement used to collect powder emerging directly from inhaler capsule. Key: 1, powder collection tray; 2, air flow lines; and 3, connection to vacuum line.*

various powder fractions using a Tevopharm Cap. III[10] semiautomatic filling machine.

A test rig was constructed to provide a reproducible air flow through the inhaler (Fig. 4). Air was drawn through an inhaler, at rates that could be varied by adjusting an air leak, and measured by means of a flowmeter. An exactly similar circuit was arranged parallel to the test circuit so that air flow rates could be accurately adjusted. The test circuit was brought into operation for a predetermined period by means of a timer[11] energizing solenoid valves[12]. Powder aerosol in the air from the test circuit was removed

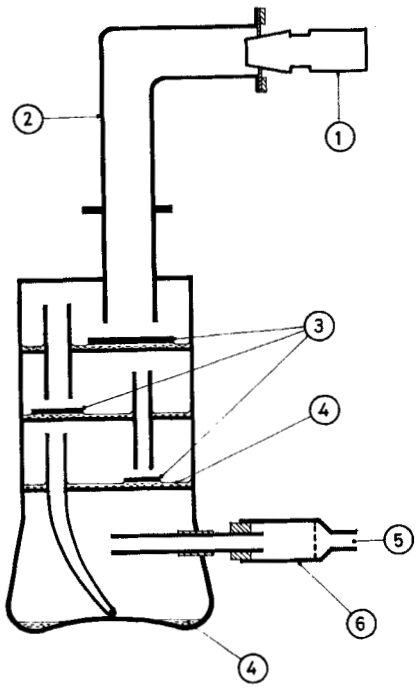

Figure 6—*Multistage liquid impinger. Key: 1, inhaler; 2, simulated "throat"; 3, impaction stages; 4, impingement liquid; 5, to vacuum; and 6, filter.*

[10] Tevopharm-Schiedam N.V., Holland.
[11] Type 144, Crouzet (England) Ltd., Thanet House, Brentford, Middlesex, England.
[12] Type 4801, Burkert Contromatic Ltd., South Ascot, Berks, England.

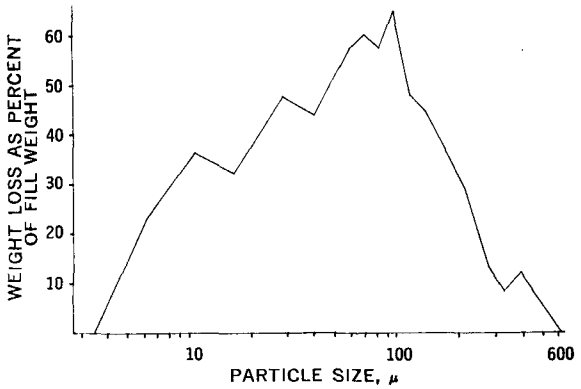

Figure 7—*Loss of powder from the inhaler capsule under standard air flow conditions as mean particle size of powder action was changed.*

by a cyclone, backed by a filter placed immediately before a vacuum pump. Holes were drilled at 0.07-cm. (0.028-in.) diameter in filled No. 2 hard gelatin capsules and "flash" removed with a scalpel blade. The weight of powder lost in each experiment was determined by weighing 100 filled capsules, representing each size fraction, before and after exposure to the air flow. An air flow rate of 60 l./min. and an air flow period of 2.0 sec. were used.

Assessment of Powder Dispersion—The inhaler has two distinct actions: (*a*) the transfer of the powder from the capsule into the airstream, and (*b*) the breakup and dispersion of the powder cloud into fine particulate material suitable for inhalation. Examination of powder emerging from the capsule inside the inhaler was carried out using the arrangement shown in Fig. 5, the inhaler capsule being exposed by cutting away part of the casing. Suction applied at the mouthpiece caused the capsule to spin and transport powder to the punctured holes. The powder particles emerging directly from the capsule were collected on a tray coated with a thin film of polyethylene glycol 300 as an adhesive; size analysis was carried out by microscope counting[13].

Particle-size analysis of the whole cloud produced after the particles pass through the inhaler may be carried out using the cascade impaction technique described by May (8) and widely used in aerosol studies. Commercially available instruments based on this technique are usually designed for the analysis of dilute aerosols and are unsuitable for concentrated powder aerosols such as those generated by the inhaler. A more suitable instrument for analysis is the multistage liquid impinger (9) (Fig. 6), in which the impaction surfaces consist of sintered-glass disks immersed in liquid, backed up by a liquid swirl impinger to trap very fine particles. A 90° glass bend, 2.5 cm. in diameter, coated with polyethylene glycol 300 as adhesive, was fitted prior to the first stage to simulate impaction conditions in the throat, An additional impaction stage and filter separated the powder into six fractions. The air flow rate was set at 60 l./min. by adjustment of a vacuum pump. The inhaler with drilled capsule in place was inserted, the pump was switched on, and

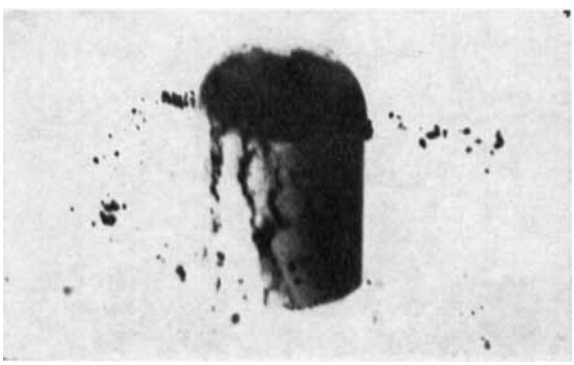

Figure 8—*Frames from high-speed film showing large agglomerates of drug and lactose emerging from inhaler capsule prior to dispersion.*

[13] British Standard Institute, Specification No. 3406: Part 4, 1963.

Table I—Analysis of Inhaler Cloud by Multistage Liquid Impinger

Percent by Weight of Drug Retained on					
"Throat"	1	2	3	4	Filter
68.9	7.2	4.2	7.0	11.3	1.4

the flow of air was maintained for 30 sec. Water was used as the impingement liquid. Each stage was carefully washed out and diluted appropriately with phosphate buffer at pH 7.4. The cromolyn sodium content was determined spectrophotometrically at 326 nm. using absorptivity (*a*) 16.3.

RESULTS AND DISCUSSION

Instrumented Inhaler—The traces obtained when air was inhaled through the inhaler were of the form illustrated in Fig. 3. The inhaler air flow rate rapidly built up to a maximum, which was maintained for most of the inhalation period, followed by a gradual decay. Vibrations in the rotor began at flow rates of 35–40 l./min. and ceased when the inhalation rate fell below this value. Powder emission followed almost exactly the occurrence of the vibrations. The short time interval between the detection of vibration and powder discharge was attributed to the time required for powder transport from the capsule to the location of the photoelectric cell across the mouthpiece of the inhaler. Powder was only discharged during inhalation through the device, which thus automatically coordinated dispensing of the drug with the inhalation of air.

Effect of Particle Size on Powder Flow from the Capsule—The graph of Fig. 7 shows the variation in lactose powder loss from the inhaler capsule under standard air flow conditions as the mean particle size of the powder fraction was changed. Maximum powder flow was obtained with powder particles in the size range of 70–100 μ, with marked reductions occurring with the finer and coarser samples. Examination of capsules containing the lactose powder less than 10 μ in size revealed extensive coating of the internal wall of the hard gelatin capsule. Poor flow of fine powder particles is a well-documented occurrence (10); the vibratory motion of the capsule probably was inadequate to overcome the interparticulate and adhesive forces between the finer particles and the gelatin. In the case of very coarse particles, such forces would be unlikely to play any part. A possible explanation for the low rates of flow found is that intermittent blocking occurred during passage of the particles through the drilled holes.

The poor flow exhibited by the finest powders is of significance in formulation since these particle sizes are important in inhalation

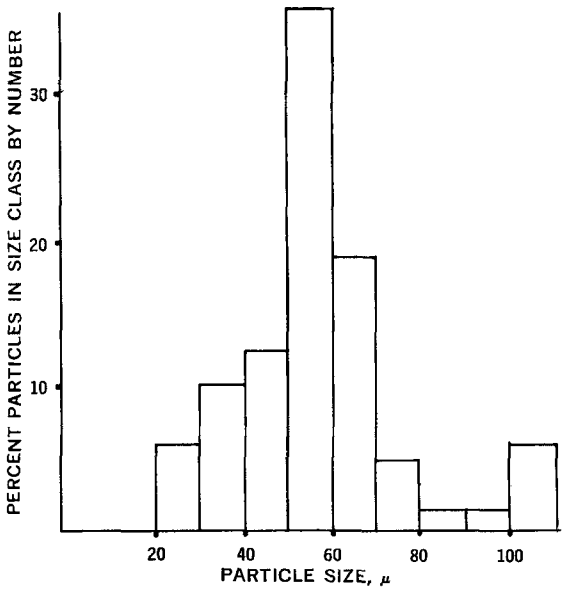

Figure 9—*Particle-size distribution of agglomerates emerging directly from inhaler capsule.*

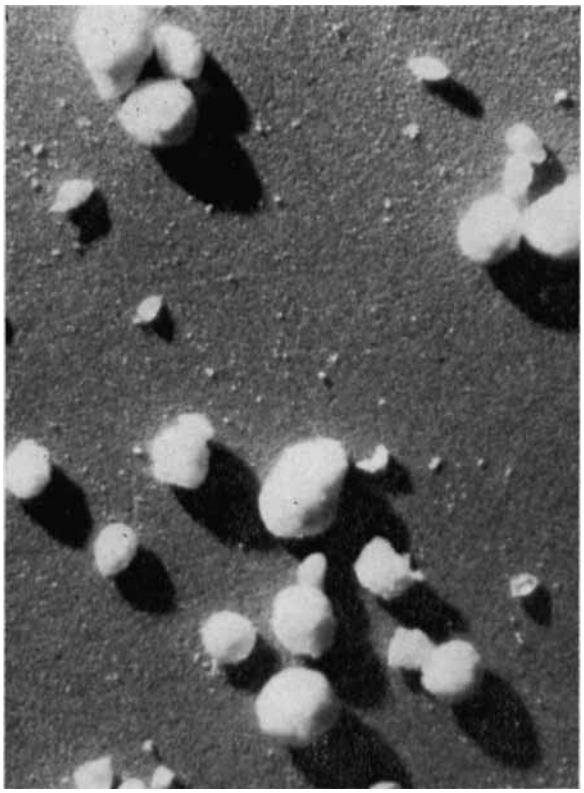

Figure 10—*Photomicrograph of larger agglomerates of drug and lactose which emerge directly from the inhaler capsule prior to dispersion. The rounded shape is characteristic.*

therapy. Means were sought to overcome this problem, the simplest being to mix the very fine particles with a suitable flow aid. The use of a proportion of particles in a coarser size range has been found to be effective. When powder mixtures of this type are prepared, any degree of flow can be produced according to the proportion of free-flowing diluent selected. For example, cromolyn sodium, after grinding in a micronizer, typically has a mass median diameter of 2.6 μ with a geometric standard deviation of 1.4 measured by the Coulter counter. The material is cohesive, exhibits the poor flow properties characteristic of powders in this particle-size range, and will not empty alone from an inhaler capsule. When mixed with coarse lactose, containing 70% by weight of particles between 30 and 60 μ in size, the flow properties improve significantly; preparations can then be formulated which are acceptable in routine management of patients with airways disease.

Powder Dispersion—Examination of the material emerging directly from the capsule showed decreasing amounts of aggregation of the particles as the mean size of the fractions increased. This finding was not unexpected considering the known properties of powders (4, 10). When mixtures of fine and coarse particles were used, examination of the material emerging from the capsule revealed that the fine particles were formed into large agglomerates, either alone or around the coarser flow aid. High speed photography confirmed this effect (Fig. 8). A particle-size distribution for the agglomerates from one batch of a 1:1 mixture of coarse lactose and micronized cromolyn sodium is shown in Fig. 9, which reveals an absence of fine particles less than 20 μ. The turbulent airstream created in the inhaler by a patient's deep inspiration causes many of these agglomerates of drug and lactose to break up, producing fine particles which pass with the inhaled air into the respiratory tract (Figs. 10 and 11). A typical analysis, by the multistage impinger, of the cloud generated by the inhaler is given in Table I. The stages of the impinger represent increasing fineness of particles and have increasing possibility of penetration into the respiratory tract, the 50% effective cut-off sizes (11) being approximately 20 μ for the "throat" and 9.8, 5.5, 3.1, and 0.46 μ for successive stages. About 25% by weight of the cromolyn sodium particles are less than 6 μ in size and, hence, likely to penetrate into the deeper airways (9).

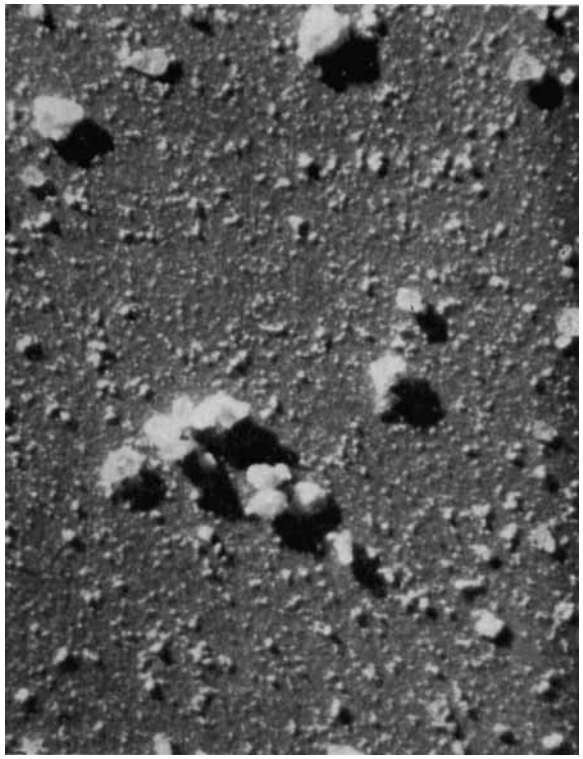

Figure 11—*Photomicrograph of powder after dispersion by the inhaler. The large particles are lactose with some adherent fine drug particles.*

The ability of the inhaler to disperse very fine powders is limited ultimately by the energy available from the patient's inhalation effort. Further work in the design of inhalers of this type and in the properties of powders for inhalation should lead to increased knowledge of the mechanisms of powder dispersion in turbulent airstreams and improvement in the efficiency of such devices.

CONCLUSION

The new inhaler achieves coordination of inspiratory effort and inhalant administration by a mechanism involving a rotor, driven by the inhaled air, avoiding the use of propellants such as liquified fluoroalkane gases. The drug is presented in convenient unit dosage form, and the total dose administered can be controlled by the capsule fill weight. Release of medicament occurs early in the inhalation period and continues while adequate air flow into the lungs is maintained, providing distribution of inhalant over the surface of the airways. The preparation of powders with the requisite flow and dispersion properties suitable for use in devices of this type represents a challenge in pharmaceutical technology. The characteristics of the inhaler will prove of interest in the administration of other drugs *via* the inhalation route.

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Automated *In Vitro* Dissolution-Rate Technique for Acidic and Basic Drugs

ASHOK C. SHAH

Abstract □ A continuous, automated, potentiometric titration technique for studying *in vitro* dissolution rates of acidic and basic type drugs from their compressed tablet formulations is described. The application of this technique was demonstrated by evaluating dissolution rates of sulfa- and sulfonylurea tablets, representing acidic drugs, and an antibiotic drug tablet formulation, representing a basic drug. The results obtained by the titration assay were confirmed by other analytical methods. Tablet excipients (lactose, sucrose, starch, magnesium stearate, polyvinylpyrrolidone, calcium phosphate, fumed silica, talc, and microcrystalline cellulose) present in these formulations did not interfere with the titration assay. The titration technique was utilized also in measuring release rates from drug pellets having constant surface area.

Keyphrases □ Tablets, *in vitro* dissolution rates—determination, automated potentiometric titration method □ Potentiometric titration, automated—determination, *in vitro* tablet dissolution rates

An automated method for determining *in vitro* dissolution rates of solid dosage forms was developed by Schroeter and his coworkers (1, 2). The essential features of this method were the circulation of the dissolution fluid through a spectrophotometer flowcell and the automatic recording of the change in absorbance as a function of time. Dissolution-rate determinations using this principle were reported in several studies (3–6), and this technique is still probably widely employed throughout the pharmaceutical field. The automated spectrophotometric method, however, cannot be applied to many solid dosage forms for a number of reasons, including the lack of characteristic absorbance by the drug molecule, interference by other tablet ingredients in the spectral assay, and the use of dissolution fluid containing pepsin and pancreatin enzymes. In such instances, these studies have to be conducted by withdrawal of dissolution fluid samples at several time intervals and their subsequent individual sample analysis to determine the drug concentration.

The present study was initiated to develop a convenient *in vitro* dissolution-rate procedure for the enteric coated tablet formulations of a weak basic drug. The spectrophotometric method proved to be unapplicable in this case because of the reasons mentioned earlier. Hence, a continuous titration technique was developed for the automated dissolution-rate evaluation of this product. According to this technique, the drug, as it dissolves in solution, is rapidly titrated by the addition of the required amount of titrant liquid to maintain a set constant pH of the dissolution media. This is monitored by a pH-stat titrator instrument. From the amount of titrant added as a function of time, which is recorded on a chart, the dissolution rate of the drug is estimated. A similar technique was previously employed in evaluating dissolution and reaction rates of antacid compounds (7, 8). In the present report, the general application of the titration technique as an automated dissolution method for the tablet formulation of acidic or basic drugs is evaluated. Also, the use of this technique in studying dissolution rates from a constant-surface pellet of a drug was investigated.

EXPERIMENTAL

Test Samples and Reagents—Two different 250-mg. compressed tablet formulations of an antibiotic drug and their corresponding enteric coated tablets, 500-mg. compressed tablets of a sulfa drug, and 500-mg. compressed tablets of sulfonylurea were used as samples. The excipients present in these formulations were lactose, starch, sucrose, polyvinylpyrrolidone, calcium phosphate, fumed silica, talc, magnesium stearate, and microcrystalline cellulose. The quantity of excipients present in these formulations constituted approximately 10–50% of the total tablet weight.

Hydrochloric acid and sodium hydroxide titrant solutions of known normality were prepared in carbonate-free distilled water. Pancreatin NF, polysorbate 80 USP, and other chemicals (all analytical grade) were used.

Instrumentation—The instrumental setup shown in Fig. 1 was employed in most of the experiments. It consisted of a pH-stat automatic recording titrator[1] (A), titration buret (B), dissolution test apparatus (C), a pulsating-type pump[2] (D), and a Beckman model DBG recording spectrophotometer (E). With this setup, the dissolution rates could be determined simultaneously by the automated titration technique as well as by the automated spectrophotometric analysis. Both these techniques were employed in the

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Figure 1—*Instrumental setup for the automated determination of dissolution rates. Key: A, pH-stat titrator; B, titration buret; C, dissolution test apparatus; D, peristaltic pump; and E, spectrophotometer.*

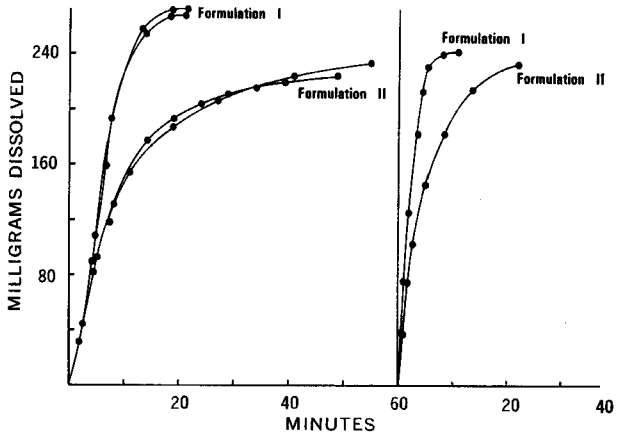

Figure 3—*Dissolution rates of the compressed tablet formulations of an antibiotic drug determined by the automated titration technique using the rotating basket apparatus (left) and the USP disintegration apparatus (right).*

evaluation of sulfonylurea tablets; for all other dissolution experiments, only parts A, B, and C were used.

A schematic diagram of the dissolution test apparatus used in the present study is shown in Fig. 2. It consisted of a thermostated beaker (A) with a stainless steel rotating basket assembly (C), held in the center about 2 cm. from the bottom of the beaker and attached to a constant-speed stirrer motor (E). The rotating basket design was essentially similar to that employed in the NF method I dissolution apparatus (9) but was further modified by placing two 1-cm. long stainless steel wire loop impeller blades (D) on two opposite sides of the basket. By this modification, it was possible to prevent formation of any stagnant layers of particles due to particles settling at the bottom of the beaker. Agitation of the dissolution liquid at a constant speed was achieved by rotation of the basket assembly at a predetermined revolutions per minute. A calomel and a glass electrode (F) were immersed into the liquid, and their terminals were connected to the pH-stat titrator unit. A fine tip Teflon tube (D) immersed into the liquid served as a buret through which titrant solution was added.

In one dissolution experiment, a USP disintegration apparatus was employed instead of the apparatus shown in Fig. 2. The electrodes and the Teflon tip buret were held in two separate cylindrical compartments of the basket.

Dissolution Procedure—The glass and calomel electrodes were calibrated with the standard buffer solution, and the pH-stat instrument was set to a desired end-point titration pH corresponding to a value at which the dissolution experiment was to be performed.

The apparatus shown in Fig. 2 was employed for the evaluation of all tablet samples. A tablet sample was placed in the basket (C); then the basket was immersed into a measured volume of unbuffered dissolution fluid equilibrated at 37° in the thermostated beaker (A). The liquid was stirred by rotating the basket at a predetermined revolutions per minute. The rate of dissolution was followed by titration of the drug as it dissolved by means of automatic intermitent additions of small volumes of titrant liquid through the Teflon tip buret (D) into the dissolution media. The dissolution rates were estimated from the rate of consumption of titrant liquid, the pH of the media, the pKa of the drug, and the normality of the titrant liquid.

Experimental Conditions—The dissolution rates of antibiotic tablets were determined in 170 ml. of simulated intestinal fluid at pH 7.5 with 100-r.p.m. stirring. The composition of simulated intestinal fluid USP was modified by replacing sodium phosphate with an equimolar quantity of sodium succinate in order to eliminate any buffer effect at pH 7.5 due to phosphate species but still maintain electrolyte concentration with the succinate ions. Dissolution studies for sulfa tablets and the powder sulfa drug were performed in 300 ml. of water at pH 7.5 with 300-r.p.m. stirring. For sulfonylurea tablets, conditions employed were 750 ml. of water at pH 7.5 with 300-r.p.m. stirring. In addition, compressed tablet samples of the antibiotic drug were evaluated using the USP disintegration apparatus. In this case, experiments were performed by following dissolution rates of three tablets in 750 ml. of simulated intestinal fluid at pH 7.5 and 37°. Since the dissolved drug existed

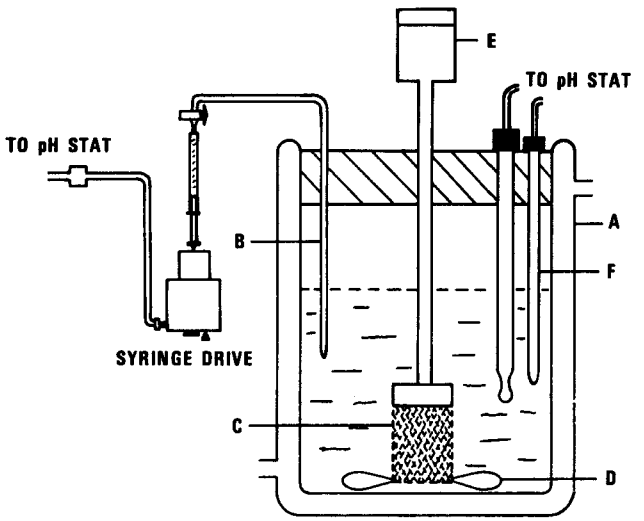

Figure 2—*Dissolution test apparatus. Key: A, thermostated beaker; B, titration buret; C, rotating basket assembly; D, wire loop impeller blades; E, constant-speed stirrer motor; and F, electrodes.*

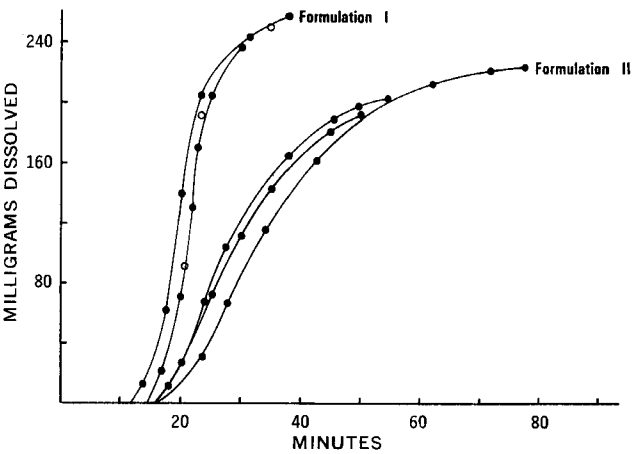

Figure 4—*Dissolution rates of the enteric coated antibiotic tablet formulations determined by the automated titration technique. The open circles represent the microbiological assay results.*

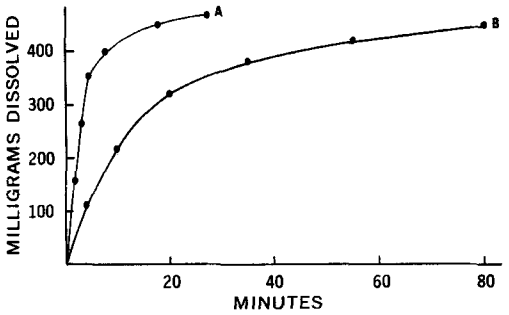

Figure 5—*Dissolution-rate profile of a sulfa drug determined by the automated titration technique. Key: A, powder drug, 500 mg.; and B, compressed tablet, 500 mg.*

in solution mostly in the ionized state, the solubility was not a problem in these experiments.

Dissolution rates from constant-surface pellets were determined in 170 ml. of water and also in 170 ml. of aqueous 0.5% polysorbate 80 solution at pH 7.5, 37°, with 300-r.p.m. stirring. The apparatus shown in Fig. 2 was used for the constant-surface pellet studies, except that the rotating basket assembly (A) was replaced with a stirrer having three 1.27-cm. (0.5-in.) impeller blades. The pellet samples were prepared by compressing a weighed amount of powder drug at 400 lb. force on a hydraulic press in a 1.27-cm. (0.5-in.) diameter stainless steel die. The pellet, held inside the die, was suspended in the apparatus at a particular height so that one flat surface of the pellet was exposed to the dissolution media.

RESULTS AND DISCUSSION

Dissolution rates determined by the continuous titration technique for the two different 250-mg. compressed tablet formulations of the weak basic antibiotic drug are represented in Fig. 3. The curves shown in the left portion of Fig. 3 were obtained when these tablets were tested in the rotating basket-type apparatus, while the curves shown in the right portion of Fig. 3 were obtained with the USP disintegration apparatus. It is evident from these results that, in both experiments, Formulation I showed a relatively rapid rate of dissolution as compared to Formulation II. It is important to notice that the total amount of drug dissolved, calculated on the basis of the amount of titrant acid consumed, was found to be more than 96% of the theoretical amount of drug present in these tablets. This finding suggests that the excipient present in the tablets did not significantly interfere with the titration assay of this drug.

The dissolution-rate results for the two enteric coated tablet formulations of the antibiotic are shown in Fig. 4. In this case, the initial lag time observed in the dissolution curve was due to the time required for the dissolution of the enteric coating. An acidic ingredient present in the enteric coating during initial dissolution time caused a slight decrease in the pH of the media. Therefore, during the dissolution of the coat, the pH had to be adjusted by the manual addition of a few drops of dilute sodium hydroxide solution. This adjustment, however, did not influence the tablet dissolution-rate results. The curves shown in Fig. 4 for each enteric coated tablet formulation were obtained upon repeated runs with the fresh samples. The titration assay results were further confirmed by the microbiological assay of the dissolution fluid samples collected at specific time intervals during a dissolution experiment with the enteric coated tablet. These results, shown as open circles in Fig. 4, are in good agreement with the titration assay results.

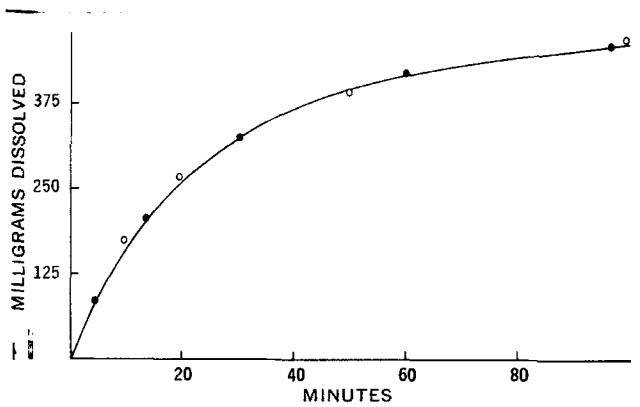

Figure 6—*Dissolution-rate profile of the sulfonylurea tablets determined by automated titration technique (smooth line with closed circles) and by automated spectrophotometric analysis (open circles).*

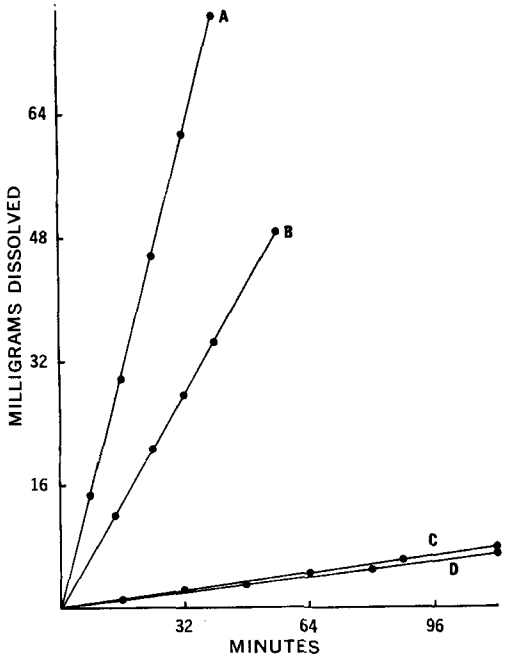

Figure 7—*Constant-surface pellet dissolution rates of two polymorphic forms of a drug determined by automated titration method. Key: A, Form I in 0.5% polysorbate 80; B, Form I in water; C, Form II in 0.5% polysorbate 80; and D, Form II in water.*

The dissolution-rate profiles for the 500-mg. weak acidic sulfa drug tablet and for 500 mg. of pure sulfa drug powder, obtained by the automated titration technique, are shown in Fig. 5. As evident from these results, the powder drug dissolved at a faster rate than the tablet. In both cases, however, the total amount of titrant consumed upon complete dissolution was equivalent to the theoretical amount needed to neutralize more than 95% of the drug, indicating thereby that the titration technique can be satisfactorily applied for the dissolution studies of this tablet.

The automated titration technique with the simultaneous automated spectrophotometric analysis technique was employed in the dissolution-rate determination of a sulfonylurea tablet. The results of this study, shown in Fig. 6, also suggest that either technique is suitable for the dissolution-rate determination of this tablet.

The drug-release rates from constant-surface pellets of acidic or basic drugs can be conveniently determined by the automated titration technique. Constant-surface pellet dissolution-rate measurements were previously studied to investigate the dissolution characteristics of crystalline states of a drug and certain other factors, like wettability and pellet porosity, that would influence the drug release rates (10, 11). The application of automated titration techniques in these types of studies is illustrated by evaluating dissolution characteristics of the two polymorphic forms of a basic drug. The results obtained for the two crystal forms of the drug in water and in 0.5% polysorbate 80 medium are shown in Fig. 7. A linear relationship between the amount dissolved as a function of time was observed in all experiments. Polymorphic Form II dissolved at a much slower rate in comparison with Form I, and both of these drug forms showed enhanced rates in the surfactant solution. The detail study of this system will be the subject of a separate communication.

The examples presented in this study suggested the applicability of the titration method in studying *in vitro* dissolution of acidic or basic drugs. However, as in the case of any other analyt-

ical technique employed for dissolution studies, one must verify that the excipients present in the formulations indeed do not interfere with the titration assay. The titration method cannot be employed in studying dissolution of capsule formulations, since the acidic nature of gelatin capsules causes overlap with the titration assay of the drug. In the automated spectrophotometric method, the dissolution fluid has to be filtered and then externally circulated through a photometric cell. Therefore, clogging of the filter screen and the lag time involved between the actual dissolution of the drug and its spectrophotometric measurement are the obvious problems encountered with this method. In the titration technique, however, these problems are nonexistent because the dissolution fluid does not have to be filtered or externally circulated.

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Keyphrases □ Naloxone, metabolites—determination in human urine □ 7,8-Dihydro-14-hydroxynormorphinone, naloxone, and *N*-allyl-7,8-dihydro-14-hydroxynormorphine—isolated and identified as metabolites of naloxone in human urine □ Spectrofluorimetry—identification, naloxone metabolites □ TLC—identification, naloxone metabolites

Naloxone (*N*-allyl-7,8-dihydro-14-hydroxynormorphinone) is a potent narcotic antagonist in laboratory animals (1, 2) and in man (3). Fujimoto's (4) isolation of the *N*-allyl-7,8-dihydro-14-hydroxynormorphine-3-glucuronide metabolite of naloxone in chicken urine aroused speculation concerning its occurrence as a human metabolite. In rabbit (4) and in man (5), naloxone glucuronide has been reported as the sole metabolite of naloxone.

In the present study, an isolation procedure developed by Fujimoto and his coworkers (4–6) was used to separate the metabolites of naloxone. These were identified as the glucuronide of naloxone, 7,8-dihydro-14-hydroxynormorphinone (EN-3169), and *N*-allyl-7,8-dihydro-14-hydroxynormorphine (EN-2265).

EXPERIMENTAL

Approximately 2 l. of urine from a pooled 2-week urine collection was obtained from a patient. The urine was preserved under toluene and refrigerated. The patient was participating in a study of naloxone in the treatment of opiate dependence and was receiving between 1.0 and 1.8 g. of naloxone in single, daily oral doses during the 2-week collection period.

Column Chromatography—Metabolites were isolated from urine on a resin column[1] (5). Prior to column chromatography, 10 ml. of urine was adjusted to pH 5.3 with acetic acid and incubated overnight at 37° with 0.2 ml. of glusulase[2], an extract of *Helix pomatia* intestine which contains glucuronidase and sulfatase activity. The glusulase-treated urine was applied to a 2.5 × 10-cm. column of the resin. This process was followed by 50 ml. of distilled water and then 300 ml. of absolute methanol. The urine and water effluents were discarded. After discarding the first 10 ml. of methanol effluent, 250 ml. was collected. The methanol eluate was evaporated to dryness on a rotary evaporator[3] and redissolved in 0.2 ml. of methanol.

TLC—Five microliters of the concentrated methanol eluates was applied to a precoated silica gel TLC plate[4]. Controls and standards treated in a manner identical with that already described were also applied to the plate. Plates were developed in two solvent systems: A, chloroform–methanol–acetic acid (100:60:2 v/v); and B, chloroform–dioxane–ethyl acetate–concentrated ammonia (25:60:10:2.5 v/v). The solvents were used singly for developing

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ical technique employed for dissolution studies, one must verify that the excipients present in the formulations indeed do not interfere with the titration assay. The titration method cannot be employed in studying dissolution of capsule formulations, since the acidic nature of gelatin capsules causes overlap with the titration assay of the drug. In the automated spectrophotometric method, the dissolution fluid has to be filtered and then externally circulated through a photometric cell. Therefore, clogging of the filter screen and the lag time involved between the actual dissolution of the drug and its spectrophotometric measurement are the obvious problems encountered with this method. In the titration technique, however, these problems are nonexistent because the dissolution fluid does not have to be filtered or externally circulated.

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Table I—R_f Values of Naloxone and Its Metabolites

Compound	R_f in Solvent System A[a]	R_f in Solvent System B[b]
Naloxone	0.65	0.35
EN-2265	0.40	0.22
EN-3169	0.29	0.04

[a] Solvent A: chloroform–methanol–acetic acid (100:60:2 v/v). [b] Solvent B: chloroform–dioxane–ethyl acetate–concentrated ammonia (25:60:10:2.5 v/v).

in one direction and in combination (Solvent A followed by Solvent B) for two-dimensional TLC.

Spray Reagents—Detection of the metabolites on TLC plates was achieved with iodoplatinate spray reagent (7), ferri-ferrocyanide reagent (8), and Dragendorff reagent oversprayed with 5% aqueous sodium nitrite (9). Spraying with iodoplatinate resulted in characteristic blue to purple spots on a pink background. The Dragendorff–nitrite spray revealed the metabolites as brown spots on a yellow background, and the ferri-ferrocyanide reagent oxidized suitable substrates to fluorescent derivatives which were observed as blue spots on a purple background under UV light.

Spectrofluorimetry—A spectrophotofluorometer[5] was used to obtain the fluorescence excitation and emission spectra of EN-2265 after reaction with ferri-ferrocyanide reagent (10). Pure EN-2265 was oxidized to "pseudo-EN-2265," 2,2′-bi(*N*-allyl-7,8-dihydro-14-hydroxynormorphine), with ferri-ferrocyanide reagent, and the spectra were obtained for comparison with material eluted from TLC plates after spraying with ferri-ferrocyanide spray reagent. The eluting agent was 1 ml. of 0.1 *M* sodium pyrophosphate adjusted to pH 8.5 with 1 *N* hydrochloric acid. The eluate was transferred to a microcell for fluorometric scanning.

RESULTS

TLC of the glusulase-hydrolyzed urine sample in the two solvent systems, both singly and two dimensionally, resulted in the detection of three spots. These spots were visualized with both iodoplatinate and Dragendorff–nitrite reagents and coincided with naloxone, EN-2265 (the reduced naloxone), and EN-3169 (the dealkylated naloxone). The spots coinciding with EN-2265 and standard EN-2265 were the only ones that fluoresced under UV light after being sprayed with ferri-ferrocyanide reagent. Without glusulase hydrolysis, iodoplatinate reagent revealed only one spot near the origin. This spot, undetectable with the other spray reagents, probably consists of the conjugates of naloxone and its metabolites. The R_f values of naloxone and its metabolites are presented in Table I.

Two-dimensional TLC was necessary to obtain adequate separation of EN-2265 from other substances for spectrofluorimetry. The fluorescent spot obtained after two-dimensional TLC and spraying with ferri-ferrocyanide was eluted. The fluorescence excitation and emission spectra of the metabolite were identical with those of pure EN-2265, which has an excitation maximum of 330 nm. and an emission maximum of 430 nm. (both maxima uncorrected).

DISCUSSION

Evidence has been presented for the occurrence of several conjugated metabolites of naloxone in the urine of a human subject. The substances were isolated from urine, after hydrolysis with glusulase, and identified by TLC as naloxone, 7,8-dihydro-14-hydroxynormorphinone (EN-3169), and *N*-allyl-7,8-dihydro-14-hydroxynormorphine (EN-2265). The identification of the latter compound was verified by its fluorescence emission and excitation spectra. A free phenolic hydroxy group in the 3-position and a free 6-hydroxyl group are necessary for formation of a fluorescent derivative when oxidizing with ferri-ferrocyanide reagent.

The requirement for hydrolysis before the free bases are detectable and the well-documented conversion of similar drugs to glucuronides indicate that these conjugated metabolites are normally present as glucuronides. Fujimoto (4) reported the presence of EN-2265 and of naloxone glucuronides in the urine of chickens given naloxone orally. Naloxone 3-glucuronide was also isolated from human urine (5). Since glusulase contains sulfatase as well as glucuronidase, the possibility of the occurrence of sulfate ether conjugates exists, but this has not been demonstrated.

Nalorphine and structurally analogous drugs are enzymatically *N*-dealkylated by liver microsomes (11, 12). The *N*-dealkylated metabolite of naloxone, EN-3169, was identified in human urine, in this study, by comparison with a standard on TLC.

Although Fujimoto (5) did not rule out the formation of EN-2265 from naloxone in man, he reported detecting nothing corresponding to EN-2265 after TLC of human urine extracts. To correlate data from this laboratory with his, Fujimoto supplied us with the glucuronides isolated from the urine of rabbits and chickens which had been treated orally with naloxone. These glucuronides were hydrolyzed and chromatographed as described in the *Experimental* section. While Fujimoto and Haarsted (6) reported the formation of EN-2265 in chickens but not in rabbits, we found it to be present in the glucuronides of both species. Although EN-2265 occurs in rabbits at a much lower concentration than in chickens, it was detectable with Dragendorff–nitrite and ferri-ferrocyanide sprays.

These findings indicate that EN-2265 is a metabolite of naloxone in rabbit, chicken, and man, but that its lower concentration in rabbit and man requires more sensitive detection methods than iodoplatinate spray reagent. The use of glusulase rather than acid hydrolysis and the TLC methodology provided the necessary sensitivity.

[5] Baird-Atomic, model SF-1.

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Increased Dissolution Rates of Water-Insoluble Cardiac Glycosides and Steroids *via* Solid Dispersions in Polyethylene Glycol 6000

WIN LOUNG CHIOU* and SIDNEY RIEGELMAN

Abstract □ Dissolution rates of water-insoluble drugs such as digitoxin, 17-methyltestosterone, hydrocortisone acetate, and prednisolone acetate were markedly increased when dispersed in polyethylene glycol 6000. In addition, a supersaturation of each drug could be quickly attained when an excess amount of drug was studied. The increased dissolution rate was believed to be attributed to the molecular and/or the colloidal dispersion of the drug in the matrix of polyethylene glycol 6000.

Keyphrases □ Dissolution rates—digitoxin, 17-methyltestosterone, hydrocortisone acetate, prednisolone acetate solid dispersions □ Polyethylene glycol 6000 dispersions—increased dissolution rates of water-insoluble glycosides, steroids □ Digitoxin–polyethylene glycol 6000 solid dispersion—dissolution rate □ 17-Methyltestosterone–polyethylene glycol 6000 solid dispersion—dissolution rate □ Prednisolone acetate–polyethylene glycol 6000 solid dispersion—dissolution rate □ Hydrocortisone acetate–polyethylene glycol 6000 solid dispersion—dissolution rate □ Absorption, oral, fast release—polyethylene glycol–insoluble drug dispersions

From the consideration of effects of the molecular size, viscosity, and supercooling of polyethylene glycol polymers on the formation of fast-release solid dispersions, Chiou and Riegelman (1) recently predicted that these water-soluble polymers should be able to serve as universal carriers in increasing dissolution rates and oral absorption of poorly water-soluble or water-insoluble drugs. Although the exact physical nature has not been fully studied, the *in vitro* dissolution rate of griseofulvin, an insoluble antifungal antibiotic, was shown to increase markedly when solid dispersed in polyethylene glycol 4000, 6000, and 20,000 (1). More encouragingly, the absorption rates and total availability of griseofulvin dispersed in polyethylene glycol 6000 after oral administration to dogs (2) and man (3) were much faster and higher as compared to commercially available, micronized products. The dissolution characteristics of four additional, insoluble drugs dispersed in polyethylene glycol 6000 are discussed in this communication to substantiate further the contention concerning the universality of utilization of polyethylene glycol polymers as matrix carriers.

EXPERIMENTAL

Materials—The following were used: hydrocortisone acetate, reagent grade[1]; 17-methyltestosterone, reagent grade[2]; prednisolone acetate, reagent grade[3]; digitoxin, USP grade, microcrystalline[4]; and polyethylene glycol 6000[5]. Except for polyethylene glycol 6000, powders of the drugs all passed through a 200-mesh sieve.

Preparations of Solid Dispersion—Physical mixtures of polyethylene glycol 6000 and insoluble drugs were heated with stirring in an oil bath until completely melted. Clear and colorless melts were then poured onto a stainless steel plate and allowed to solidify by natural cooling. After several days of standing in the desiccator, solid masses were powdered, and an 80–200-mesh fraction was then collected for the dissolution-rate studies.

Table I—Twenty, Fifty, and Seventy Percent Dissolution Times for Selected Drugs in Various Physical Forms in Half-Saturation Dissolution Test

Preparations	T_{20}, min.	T_{50}, min.	T_{70}, min.
Pure prednisolone acetate[a]	8.0	45.0	—
Fused mixture of prednisolone[a] acetate–polyethylene glycol 6000 (5:95 w/w)	≪1.0	≪1.0	~0.6
Pure 17-methyltestosterone	2.0	12.0	28.0
Fused mixture of 17-methyltestosterone–polyethylene glycol 6000 (5:95 w/w)	≪1.0	≪1.0	~0.6
Pure hydrocortisone acetate	20.0	—	—
Fused mixture of hydrocortisone acetate–polyethylene glycol (5:95 w/w)	≪1.0	≪1.0	1.5
Pure microcrystalline digitoxin	15.0	80.0	—
Fused mixture of digitoxin–polyethylene glycol 6000 (2:98 w/w)	≪1.0	≪1.0	0.3–0.5

[a] This test system utilized only 30% saturation.

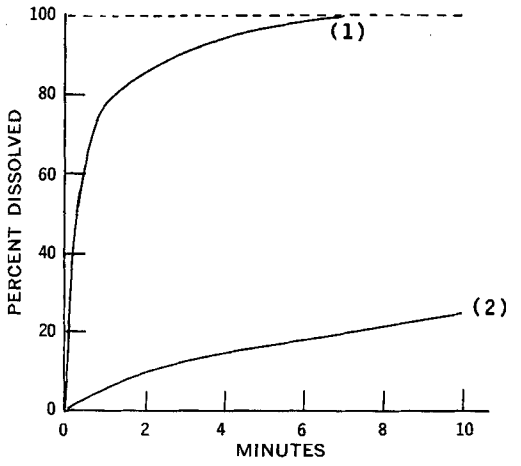

Figure 1—*Dissolution-rate studies of prednisolone acetate, 2.5 mg./500 ml. water, at 25°. Key: (1), 5% prednisolone acetate–polyethylene glycol 6000; and (2), pure prednisolone acetate powder.*

[1] Merck Sharpe & Dohme Research Laboratory, West Point, Pa. (lot No. 09C0017).
[2] J. T. Baker Chemical Co., Phillipsburg, N. J. (lot No. 5-4900).
[3] The Upjohn Co., Kalamazoo, Mich. (lot No. U-5955).
[4] C. G. Boehringer and Suthne G.b.H., Mannheim, Germany (lot No. 65618).
[5] Carbowax 6000, Union Carbide Co.

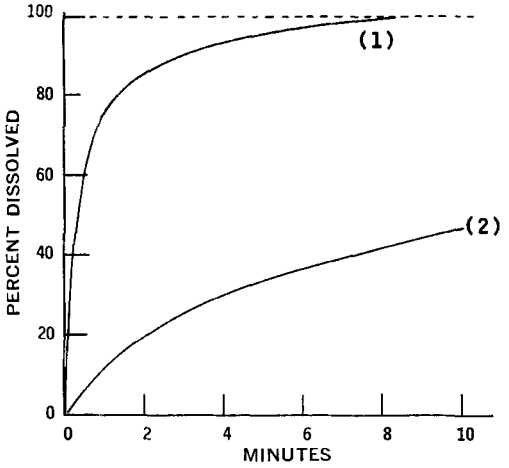

Figure 2—*Dissolution-rate studies of 17-methyltestosterone, 8 mg./500 ml. water, at 25°. Key: (1), 5% 17-methyltestosterone–polyethylene glycol 6000; and (2), pure powder.*

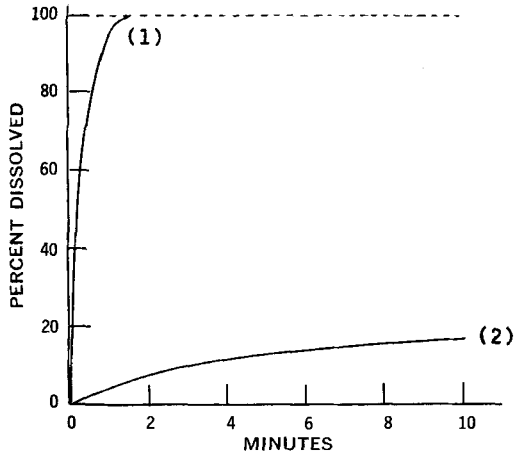

Figure 4—*Dissolution-rate studies of digitoxin. 3.5 mg./500 ml. water, at 25°. Key: (1), 2% digitoxin–polyethylene glycol 6000; and (2), microcrystalline pure powder.*

Stability Studies of Fused Mixtures—To test whether the drugs were decomposed by the fusion process, UV spectral and TLC characteristics of the pure and the processed samples were studied. In the UV study, the spectra of the pure and the dispersed drugs in the 1% ethanol solutions were scanned from 340 to 200 nm. by the recording Beckman DB spectrophotometer. Ethanol solutions of pure and dispersed drugs were spotted on the thin-layer plate and developed in the solvent system of chloroform–ether–methanol (6:3:1 v/v). The plates were sprayed with 20% sulfuric acid and heated at 110° for 1 hr.

Dissolution-Rate Studies—Dissolution rates of the pure and dispersed drugs were studied in 500 ml. distilled water at room temperature. The apparatus and the procedure were similar to those previously described (1). Two different amounts of drugs were used, one equivalent to 50% (30% in the case of prednisolone acetate) the amount that dissolves in 500 ml. water and one equivalent to three times (1.8 times in the case of prednisolone acetate) the saturated solubility if all dissolves. The solubility of each drug in 100 ml. of water at 25° is as follows: 1.0 mg. for hydrocortisone acetate (4), 3.2 mg. for methyltestosterone (4), 1.4 mg. for digitoxin (estimated in this laboratory), and 1.7 mg. for prednisolone acetate (estimated in this laboratory).

The wavelengths used for measuring optical absorbance in the dissolution-rate studies were as follows: 250 nm. for the hydrocortisone acetate, 17-methyltestosterone, and prednisolone acetate, and 220 nm. for digitoxin. In the study of the higher concentration of 17-methyltestosterone, 215 nm. (minimum absorbance) was used. The concentration of drugs was calculated according to established Beer's law plots. The presence of the carrrier in this study did not affect the assays. All studies were run at least in duplicate. The dissolution characteristics of the solid dispersions were reproducible, with only negligible variation between runs. A slightly larger but relatively insignificant variation was seen between runs of the pure compounds.

RESULTS AND DISCUSSION

Stability Studies—UV spectra and molar extinction coefficients of the pure and processed drugs were identical within experimental error, indicating the lack of decomposition; a single spot with the same R_f value was found for both the pure and processed compounds on the thin-layer plates. However, the sample of the processed digitoxin showed two additional spots, one slightly darker and one lighter than the spot of pure digitoxin. This result probably indicates some decomposition of digitoxin after the fusion process, although it could not be detected by the UV spectra. Digitoxin might be protected from decomposition by lowering the fusion temperature (170° used in this study), decreasing the proportion of digitoxin in the solid dispersion, or using the solvent method. Further investigation of this aspect should be undertaken.

Dissolution-Rate Studies—Table I and Figs. 1–4 summarize the strikingly enhanced dissolution rates obtained by the dispersion systems when the half-saturation dissolution test was utilized. The time required to dissolve 50% (designated by T_{50}) of the four insoluble drugs was much less than 1 min., and the time required to dissolve 70% (designated by T_{70}) was also less than 1 min. with the exception of hydrocortisone acetate (1.5 min.). Furthermore, all the drugs dissolved completely in less than 8 min.

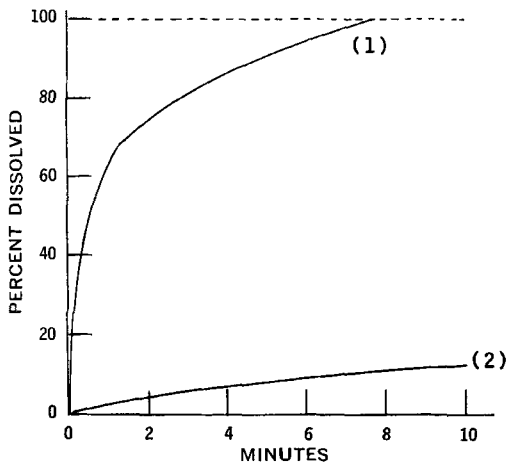

Figure 3—*Dissolution-rate studies of hydrocortisone acetate, 2.5 mg./500 ml. water, at 25°. Key: (1), 5% hydrocortisone acetate–polyethylene glycol 6000; and (2) pure powder.*

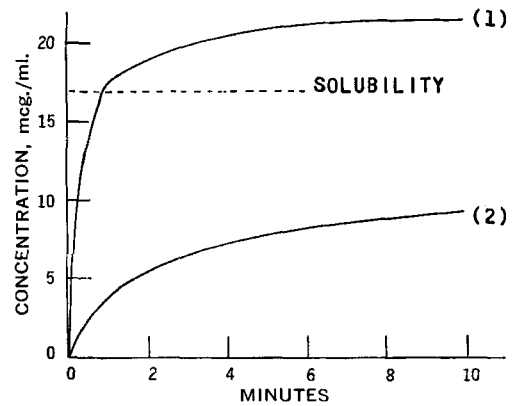

Figure 5—*Dissolution-rate studies of prednisolone acetate, 15 mg.-500 ml. water, at 25°. Key: (1), 5% prednisolone acetate–polyethylene glycol 6000; and (2), pure powder.*

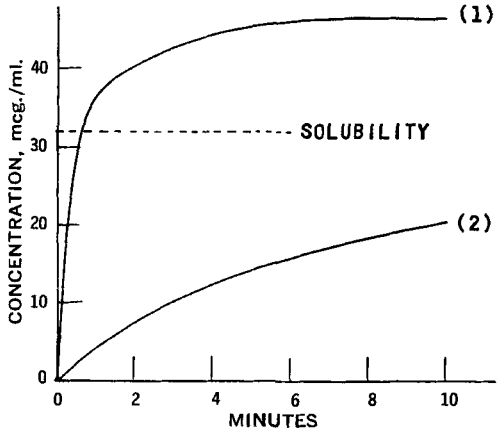

Figure 6—*Dissolution-rate studies of 17-methyltestosterone, 48 mg./500 ml. water, at 25°. Key: (1), 5% 17-methyltestosterone–polyethylene glycol 6000; and (2), pure powder.*

The dissolution from the conventional or microcrystalline (digitoxin only) powders was much slower. T_{20} ranged from 2 to 20 min., and T_{50} ranged from 12 to 80 min. When the supersaturated dissolution test method was utilized, all drugs in the dispersed systems reached supersaturation in less than 1 min. (Figs. 5–8). The fast dissolution and the attainment of supersaturation of all four drugs dispersed in polyethylene glycol 6000 are similar to what were previously reported for griseofulvin (1). Although the exact physical nature of these dispersed systems was not investigated in this preliminary study, it is believed that the reduction of the particle size of the drug to the molecular and/or colloidal level is the primary contributing factor for these striking phenomena. This belief is based on the theoretical considerations of: (*a*) molecular size difference between the polymer and the drug, and (*b*) supercooling and viscous effect of the drug–polyethylene glycol melt (1). Other possible contributing factors, such as solubilization due to the formation of soluble complexes with the polymer and the polymorphic form of the drug pres-

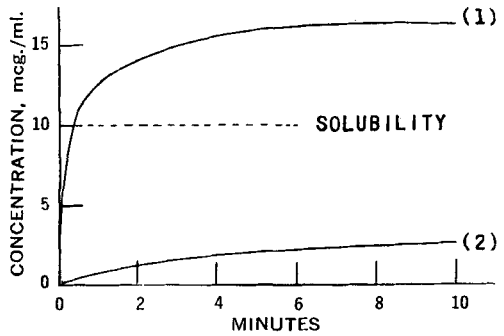

Figure 7—*Dissolution-rate studies of hydrocortisone acetate, 15 mg./500 ml. water, at 25°. Key: (1), 5% hydrocortisone acetate–polyethylene glycol 6000; and (2), pure powder.*

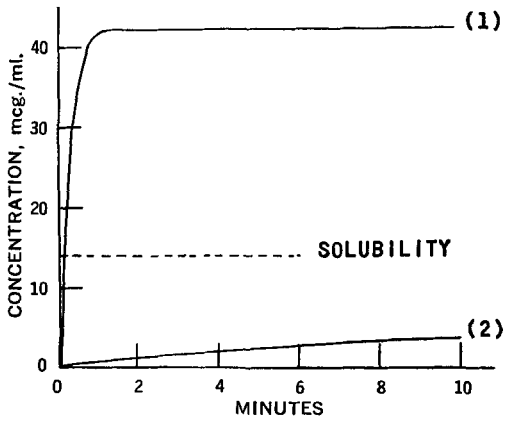

Figure 8—*Dissolution-rate studies of digitoxin, 21 mg./500 ml. water, at 25°. Key: (1), 2% digitoxin–polyethylene glycol 6000; and (2), pure microcrystalline powder.*

ent in the dispersion form, remain to be explored. The dissolution study for the fused pure drugs was not performed because of their decomposition after melting. Their melting points are much higher than the temperatures used to prepare the drug–polyethylene glycol melts.

From evidence in this and previous studies, one can justify the universality of the polyethylene glycol polymers to serve as fast-release carriers for poorly soluble drugs. Although griseofulvin has been shown to increase absorption in both man and dogs by dispersing in polyethylene glycol 6000, it appears that this type of system may have application to enhance absorption of poorly soluble drugs that can be incorporated into these systems without decomposition.

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Microcrystallization Methods for Aspirin, Mebutamate, and Quinine Sulfate

A. AFFONSO and V. R. NAIK

Abstract □ A technique to obtain microcrystals of some drugs was investigated. As high as 80–90% of the crystals obtained by this method had dimensions below 10 μ. The process is only applicable to drugs that are more soluble in glycerin than in water. It consists of diluting, with ice water, a hot saturated solution of the drug in glycerin while stirring at high speed and applying external cooling. Yields and economics of the process are also mentioned. The microcrystalline drugs (aspirin, mebutamate, and quinine sulfate) were found to possess increased solubilities which seemed to be due to the particle size rather than polymorphism or any other change. When administered orally to experimental animals, microcrystalline aspirin attained quicker and higher serum levels than did the raw form.

Keyphrases □ Microcrystallization methods—aspirin, mebutamate, quinine sulfate □ Microcrystalline drugs—method of preparing aspirin, mebutamate, quinine sulfate, effect of particle size □ Aspirin—preparation of microcrystalline form, effect on serum levels □ Mebutamate—preparation of microcrystalline form □ Quinine sulfate—preparation of microcrystalline form

Microcrystallization of sparingly soluble drugs was tried by previous researchers (1–5). The purpose of this study was to examine whether the crystallization of some drugs that are more soluble in glycerin than in water could be controlled by dilution with ice water, external cooling, and high speed stirring. Although these factors which influence crystallization are well known, study of the glycerin–water system proved to be ideal because glycerin is cheap and comparatively innocuous and the whole process can be adapted for largescale production. The problem of cooling large quantities of liquid is not significant, because the saturated solution of drug in glycerin at 80° is diluted with ice water at 3° and the external cooling needed has only to bring the resulting temperature of 40° to about 5°.

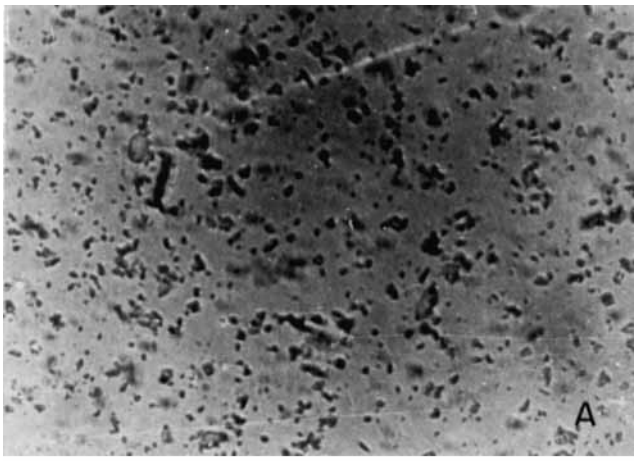
The drugs chosen for the study included aspirin, mebutamate, and quinine sulfate. The microcrystalline products obtained had interesting properties such as higher solubility in water while the melting points corresponded to chemically pure forms. They did not seem to be polymorphs or to have undergone any other change in the process. When microcrystalline aspirin, for example, was given orally to rabbits, the serum level concentration rose quicker and higher than with the raw drugs. Another point of interest is that the glycerin could be reused for subsequent batches without affecting the efficiency of the process.

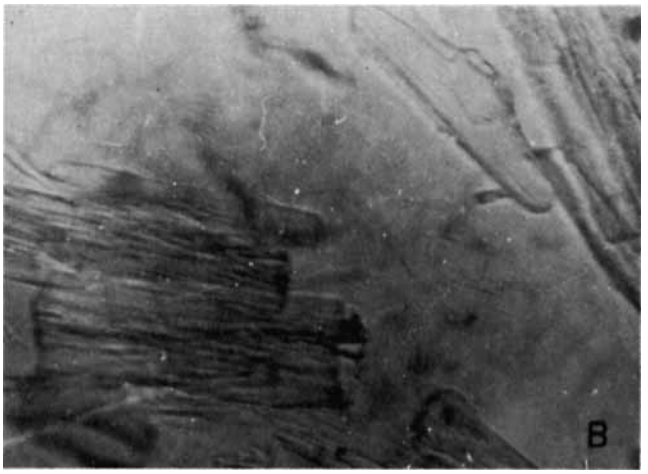


Figure 1—*Setup for microcrystallization.*

Table I—Yield, Percentage Occurrence, and Solubility of Microcrystalline Aspirin

	Weight Taken, g.	Weight Obtained, g.	Yield, %	Particle Size, μ	Occurrence, %	Solubility in Water at 29°, % w/v	
						Of Raw Aspirin	Of Microcrystalline Aspirin
Processed with fresh glycerin	50	41.3	82.6	1–4	99–100	0.359	0.571
Processed with recovered glycerin containing 8.7 g. of aspirin	50, including weight in glycerin	41.2	82.4	1.5–4	99–100		0.569





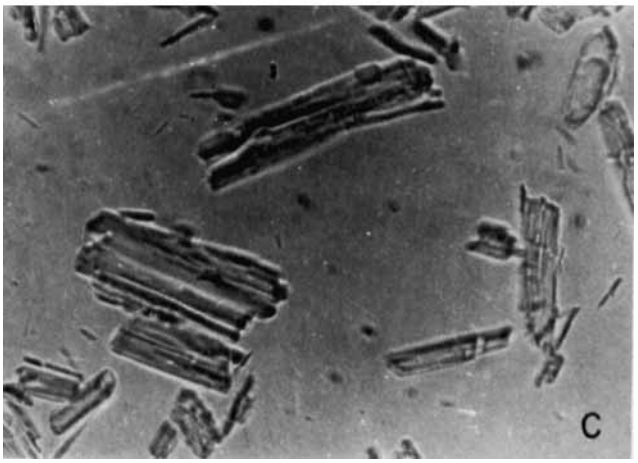


Figure 2—*(A) Microcrystallized aspirin seen in a random field (× 450). (B) Raw form of aspirin. (C) Commercial sample of micronized aspirin seen in a random field (×450).*

EXPERIMENTAL

Materials—Commercial aspirin (average crystal size 1–2 mm.), mebutamate (average crystal size 150 μ), and quinine sulfate (average crystal size 130 μ), conforming to pharmacopeial standards, were used for this study. Pharmacopeial grade glycerin was used for the process.

Apparatus—The experimental setup consisted of a stainless steel jacketed vessel (2 l.) fitted with a high speed stirrer (5000 r.p.m.). Figure 1 shows the arrangement for adding ice water at a fast rate.

Methods—The following methods were arrived at after studying the effects on particle size with various amounts of each drug, various amounts of glycerin, various amounts of ice water, and various temperatures. Only the conditions giving the best results are reported.

1. Aspirin (50 g.) was dissolved in 1125 ml. of glycerin at 80° to obtain a saturated solution. The clear solution was transferred to the stainless steel vessel. Stirring and external cooling were started immediately. This procedure was followed by the quick addition of ice water at 3°. Stirring was continued until the temperature dropped to 5° (7–10 min.).

The slurry of microcrystals was filtered under vacuum through grade 44 filter paper. The filtration was slow but could be accelerated by the addition of ice water at 3°. The product was washed with ice water, suction filtered, and dried in an air circulation drier.

The filtrate (2 l.) contained 8.7 g. of aspirin; it was vacuum distilled to remove about 96% (maximum) of the added water. It could then be reused for fresh batches.

2. Mebutamate (52.5 g.) was dissolved in 1125 ml. of glycerin at 80° to obtain a clear saturated solution. The process was repeated as described for aspirin. The filtrate (2 l.) contained 7.9 g. of mebutamate.

3. Quinine sulfate (63 g.) was dissolved in 1125 ml. of glycerin at 80° to obtain a clear saturated solution. The process was followed as described for aspirin. The filtrate (2 l.) contained 8 g. of quinine sulfate.

RESULTS

Tables I–III show the results of particle-size occurrence, solubility, and yields for aspirin, mebutamate, and quinine sulfate,

Table II—Yield, Percentage Occurrence, and Solubility of Microcrystalline Mebutamate

	Weight taken, g.	Weight Obtained, g.	Yield, %	Particle Size, μ	Occurrence, %	Solubility in Water at 29°, % w/v	
						Of Raw Mebutamate	Of Microcrystalline Mebutamate
Processed with fresh glycerin	52.5	44.6	84.9	1.5–6	99–100	0.549	0.675
Processed with recovered glycerin containing 7.9 g. of mebutamate	52.48, including weight in glycerin	44.58	84.96	1.5–7	99–100		0.669

Table III—Yield, Percentage Occurrence, and Solubility of Microcrystalline Quinine Sulfate

	Weight taken, g.	Weight Obtained, g.	Yield, %	Particle Size, μ	Occurrence, %	Solubility in Water at 29°, % w/v: Of Raw Quinine Sulfate	Solubility in Water at 29°, % w/v: Of Microcrystalline Quinine Sulfate
Processed with fresh glycerin	63	55	87.3	1–10	88	0.258	0.543
Processed with recovered glycerin containing 8 g. of quinine sulfate	62.8, including weight in glycerin	54.8	87.2	1–10	80		0.540

respectively. Table IV shows the solubility and percent w/v of the drugs in 75:25 glycerin–water mixtures, these mixtures being the ones from which the microcrystals were obtained. Table V shows the melting points and optical rotations of microcrystalline drugs and of corresponding chemically pure forms. Figure 1 shows the setup for obtaining the microcrystals. Figure 2 shows photomicrographs of microcrystals of aspirin, its raw form, and a commercial sample of micronized aspirin. Figure 3 shows the salicylate serum levels of rabbits receiving orally microcrystalline aspirin and its raw form.

DISCUSSION

From Tables I–III, it can be seen that the microcrystallization process described is quite successful from both yield and quality viewpoints. As high as 80–90% of the crystals had dimensions below 10 μ. Any mechanical process of micronization could hardly be expected to give such values in one operation. Figure 2 illustrates the fact that a commercial brand of micronized aspirin does contain a large percentage of coarse crystals. The microcrystals obtained have increased solubilities, and it can be said that blood serum levels are higher than those obtained after the administration of raw forms. The process could be adapted for largescale production of microcrystals of drugs that are more soluble in glycerin than in water.

Table IV—Solubility of Drugs in 75:25 Glycerin–Water Mixtures at Two Temperatures

Drug	Solubility at 29°	Percent w/v at 3°
Aspirin	0.459	0.238
Mebutamate	0.804	0.421
Quinine sulfate	0.465	0.229

Table V—Melting Points and Optical Rotations of Raw and Microcrystallized Drugs

Drug	Melting Point: Raw Form	Melting Point: Microcrystalline Form	Optical Rotation
Aspirin	136°	138°	—
Mebutamate	77°	78°	—
Quinine sulfate	—	—	$[\alpha]_D^{25}$ −220° for both forms

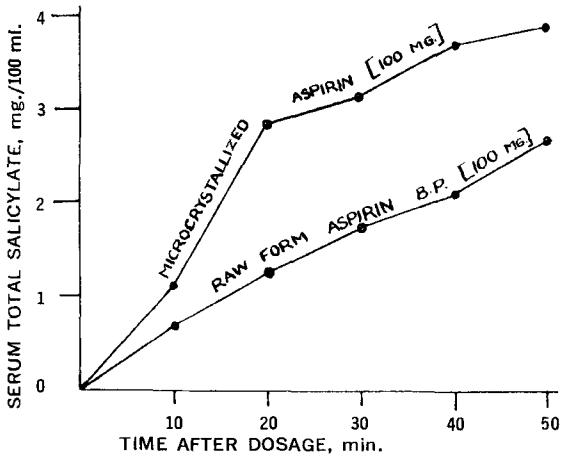


Figure 3—*Salicylate serum level in rabbits receiving both forms of aspirin orally.*

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Dissolution Rates under Sink Conditions

PAUL J. NIEBERGALL, EDWIN T. SUGITA, and ROGER L. SCHNAARE

Abstract □ Equations are given for the dissolution of a drug into an aqueous phase overlayered with a lipid phase, in which back transfer from the lipid phase is assumed possible. Consideration of both the presence and absence of back transfer, assuming in each that the amount of drug in the aqueous phase either approaches saturation or remains negligible, results in four equations for following the amount of drug in the lipid phase *versus* time. Three of the four equations are identical in form, predicting that after a brief lag time, a plot of the amount of drug in the lipid phase *versus* time will become linear. However, only in the instance for which no back transfer occurs and for which the buildup of drug in the aqueous phase is negligible (Case IV) is the slope of the line equal to the zero-order dissolution-rate constant, k_0. In the other cases, the slope represents a fraction of k_0, depending upon the relative rates of dissolution, partitioning, and back transfer. Thus, it becomes important to be completely certain that the conditions under which dissolution studies into a "perfect sink" are carried out do actually meet the requirements of Case IV.

Keyphrases □ Dissolution rates under sink conditions—equations presented considering back transfer □ Drug dissolution—equations developed considering back transfer □ Sink conditions, dissolution rates—role of back transfer from lipid phase, equations presented

The concept of dissolution of a solid into an aqueous phase followed by partitioning into a lipid phase, presumably acting as a perfect sink, was investigated (1, 2) and found to be applicable to dissolution-rate and dissolution–partitioning-rate studies. In both of these reports, it was assumed that the lipid phase acted as a perfect sink, and no transfer of the drug from the lipid phase back into the aqueous phase could occur. However, the data of Khalil and Martin (3) for systems containing similar phases to those used previously (1, 2) indicate that back transfer from the lipid phase could easily occur. It was decided, therefore, to investigate all of the possible conditions that could exist when a solid dissolves into an aqueous phase overlayered with an organic phase.

THEORY

The dissolution of a solid into an aqueous phase with subsequent partitioning into an immiscible organic phase may be represented by the following kinetic model:

$$A \xrightarrow{k_1} B \underset{k_3}{\overset{k_2}{\rightleftharpoons}} C \qquad \text{(Eq. 1)}$$

in which A represents the amount of drug in the solid form, B represents the drug dissolved in the aqueous phase, and C represents the amount of drug in the lipid phase; k_1, k_2, and k_3 are first-order rate constants.

The following differential equations can be used to describe the system:

$$-dA/dt = k_1(W_s - B) = k_0 - k_1B \qquad \text{(Eq. 2)}$$

$$dB/dt = k_1(W_s - B) - k_2B + k_3C = k_0 - (k_1 + k_2)B + k_3C \qquad \text{(Eq. 3)}$$

$$dC/dt = k_2B - k_3C \qquad \text{(Eq. 4)}$$

Table I—Definition of the Constants α, β, and γ of Eq. 7 for Cases II, III, and IV: $C = \alpha t - \beta(1 - e^{-\gamma t})$

Case	α	β	γ
II	$\frac{k_0k_2}{(k_2 + k_3)}$	$\frac{k_0k_2}{(k_2 + k_3)^2}$	$k_2 + k_3$
III	$\frac{k_0k_2}{(k_1 + k_2)}$	$\frac{k_0k_2}{(k_1 + k_2)^2}$	$k_1 + k_2$
IV	k_0	k_0/k_2	k_2

in which W_s represents the amount of drug needed to saturate the given volume of the aqueous phase, and k_0 is the zero-order dissolution-rate constant. These equations were integrated under the following four sets of conditions, using Laplace transforms:

Case I: $k_3 \sim k_2$ and $B \sim W_s$ (back transfer occurs)
Case II: $k_3 \sim k_2$ and $B \ll W_s$ (back transfer occurs)
Case III: $k_3 = 0$ and $B \sim W_s$ (no back transfer)
Case IV: $k_3 = 0$ and $B \ll W_s$ (no back transfer)

The resulting equation for the appearance of drug in the lipid phase for Case I is:

$$C = \frac{k_0k_2}{k_1k_3} + \frac{k_0k_2}{m_1(m_1 - m_2)}e^{-m_1t} - \frac{k_0k_2}{m_2(m_1 - m_2)}e^{-m_2t} \qquad \text{(Eq. 5)}$$

in which:

$$m_1 = -1/2\left[-(k_1 + k_2 + k_3) + \sqrt{(k_1 + k_2 + k_3)^2 - 4k_1k_3}\right] \qquad \text{(Eq. 6a)}$$

$$m_2 = -1/2\left[-(k_1 + k_2 + k_3) - \sqrt{(k_1 + k_2 + k_3)^2 - 4k_1k_3}\right] \qquad \text{(Eq. 6b)}$$

A plot of C *versus* time would be constantly curving until equilibrium between the aqueous and lipid phases had been attained. The equilibrium amount of drug in the lipid phase at this point, C_∞, would be equal to k_0k_2/k_1k_3 or W_sk_2/k_3. Use of the feathering or backward projection technique on Eq. 5 would then enable m_1 and m_2 to be evaluated. The sum of $m_1 + m_2$ gives the quantity $(k_1 + k_2 + k_3)$, while the product of m_1m_2 gives k_1k_3. However, unless either k_2 or k_3 is known from an independent partitioning study in which the drug in solution is followed kinetically as it transfers between the two phases, the zero-order rate constant, k_0, cannot be evaluated from Eq. 5. The nonlinearity in a plot of C *versus* time for Case I distinguishes it from the other three cases, all of which show linear segments when C is plotted *versus* time.

Cases II, III, and IV are interesting in that they can all be represented by the following general equation:

$$C = \alpha t - \beta(1 - e^{-\gamma t}) \qquad \text{(Eq. 7)}$$

in which α, β, and γ are constants which are defined for each case in Table I. The importance of Eq. 7 is that it illustrates the fact that a plot of C *versus* time for these three cases would be identical in shape, becoming linear when $e^{-\gamma t}$ becomes much less than 1. However, only in Case IV would the slope of the linear portion of the plot be equal to the zero-order dissolution-rate constant, k_0. In the other cases, the slope would represent only a fraction of k_0, depending upon the relative rates of dissolution, partitioning, and back transfer. Furthermore, for Cases II and III, the zero-order dissolution-rate constant cannot be obtained from plots of Eq. 7. Either k_2 or k_3 must be obtained from independent partitioning-rate studies. Since these three cases (Cases II, III, and IV) cannot be distinguished from each other by a plot of C *versus* time, the lipid phase must be chosen with great care to ensure that perfect sink

conditions do actually exist. For this to happen, the partitioning rate constant, k_2, must be appreciably greater than both k_1 and k_3.

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Hepatic Injury Caused by *N*-γ-Phenylpropyl-*N*-benzyloxyacetamide: A Light and Electron Microscopic Study

K. KOVACS, B. D. GARG, and J. D. KHANDEKAR*

Abstract □ W-1372 (*N*-γ-phenylpropyl-*N*-benzyloxyacetamide), a hypolipemic and antiatherosclerotic compound, given orally to rats in corn oil induces fatty degeneration of the hepatocytes. Electron microscopically, it was possible to note accumulation of lipid droplets and liposomes, progressive dilatation, disorganization and degranulation of the rough-surfaced endoplasmic reticulum, and injury of the mitochondria as well as distension of the sacs of the Golgi apparatus with disappearance of their vacuolar content. Possibly, a defect in hepatocellular lipoprotein synthesis or release might be responsible for this lipid accumulation.

Keyphrases □ *N*-γ-Phenylpropyl-*N*-benzyloxyacetamide—hepatic injury, detection by light and electron microscopic study □ Hepatic lipid accumulation—induced by W-1372, detected by electron microscopy □ Electron microscopy—detection of liver damage induced by *N*-γ-phenylpropyl-*N*-benzyloxyacetamide

N-γ-Phenylpropyl-*N*-benzyloxyacetamide (W-1372) lowers the blood level of cholesterol, phospholipids, and triglycerides (1, 2). In addition, it reduces the extent of aorta atherosclerosis in squirrel monkeys and rabbits fed on a cholesterol and fat-rich diet (1–3). The light and electron microscopic changes found in the liver of rats treated with this hypolipemic and antiatherosclerotic compound are described here.

EXPERIMENTAL

Materials and Methods—Twenty-four female ARS/Sprague-Dawley rats[1], with a mean initial body weight of 95 g. and maintained *ad libitum* on Purina laboratory chow[2] and tap water, were divided into four equal groups, one of which served as untreated controls. The second group received 1 ml. of corn oil, by stomach tube, twice daily for 3 days. The animals of Groups 3 and 4 were given 5 or 10 mg. of W-1372 (Wallace), respectively, in 1 ml. of corn oil twice daily *per os*, also for 3 days. All treated rats were killed without anesthesia, by destruction of the medulla oblongata, 16 hr. after the last gavage. The untreated animals were sacrificed at the same time.

For light microscopic examination, fresh liver tissue was fixed in alcohol formol or neutral formaldehyde and embedded in paraffin. Sections (4–8 μ thick) were cut and stained with hematoxylin–phloxine or by the Periodic Acid Schiff (PAS) technique. Frozen sections were also cut and stained with Oil red O or Sudan black B.

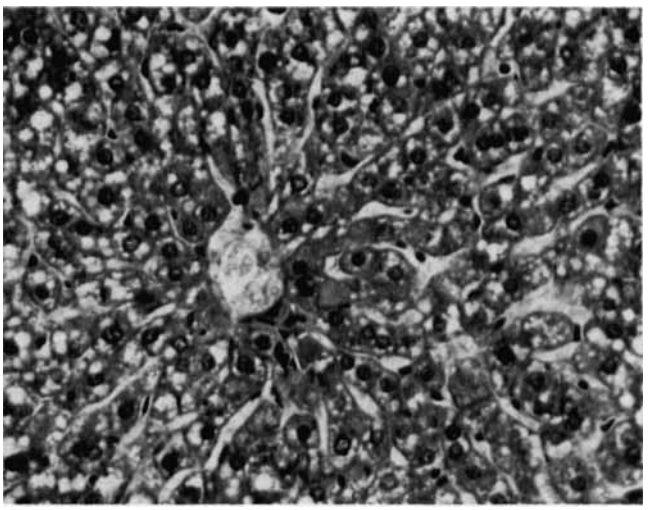

Figure 1—*Liver of a rat treated with 10 mg. of W-1372 for 3 days. Numerous lipid droplets are seen in the cytoplasm; hematoxylin–phloxine.* (×300)

Material for electron microscopic examination was obtained from three rats per group by excising a small portion of tissue from the left lateral lobe of the liver and placing it in Millonig's osmium fixative where it was minced into tiny cubes and kept for 1 hr. at 4°. The specimens were then dehydrated in graded ethanol and embedded in Epon resin. Sections (0.5 μ thick) were cut on a microtome[3], stained with toluidine blue, and examined under a light microscope. Ultrathin sections (approximately 50 nm.) were cut from selected midzonal areas, stained with uranyl acetate and Reynolds' lead citrate, and examined under an electron microscope[4].

RESULTS

The livers of rats treated with 10 mg. of W-1372 were enlarged, smooth-surfaced with somewhat rounded edges, pale brownish-yellow, and more fragile than those of the controls. A mild yellow discoloration was also noticeable after treatment with 5 mg. of W-1372, but other gross changes were not evident. No alterations were observed in the livers of animals that received corn oil alone.

[1] Madison, Wis.
[2] Ralston Purina Co. of Canada.
[3] Porter-Blum MT-2.
[4] Carl Zeiss EM 9A.

conditions do actually exist. For this to happen, the partitioning rate constant, k_2, must be appreciably greater than both k_1 and k_3.

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K. KOVACS, B. D. GARG, and J. D. KHANDEKAR*

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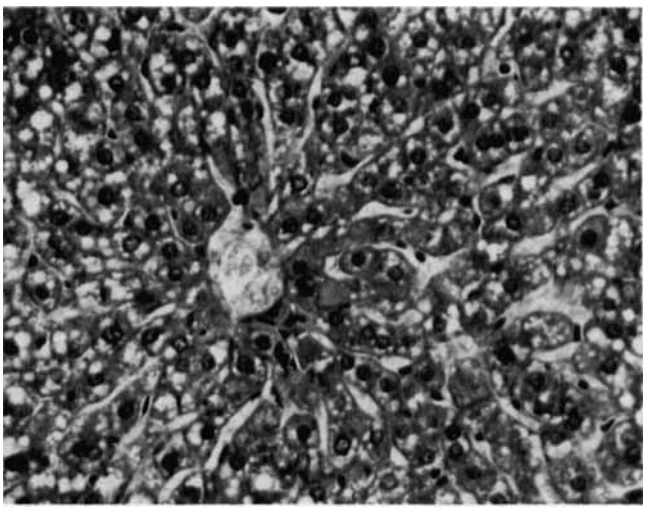

Figure 1—*Liver of a rat treated with 10 mg. of W-1372 for 3 days. Numerous lipid droplets are seen in the cytoplasm; hematoxylin–phloxine.* (×300)

Material for electron microscopic examination was obtained from three rats per group by excising a small portion of tissue from the left lateral lobe of the liver and placing it in Millonig's osmium fixative where it was minced into tiny cubes and kept for 1 hr. at 4°. The specimens were then dehydrated in graded ethanol and embedded in Epon resin. Sections (0.5 μ thick) were cut on a microtome[3], stained with toluidine blue, and examined under a light microscope. Ultrathin sections (approximately 50 nm.) were cut from selected midzonal areas, stained with uranyl acetate and Reynolds' lead citrate, and examined under an electron microscope[4].

RESULTS

The livers of rats treated with 10 mg. of W-1372 were enlarged, smooth-surfaced with somewhat rounded edges, pale brownish-yellow, and more fragile than those of the controls. A mild yellow discoloration was also noticeable after treatment with 5 mg. of W-1372, but other gross changes were not evident. No alterations were observed in the livers of animals that received corn oil alone.

[1] Madison, Wis.
[2] Ralston Purina Co. of Canada.
[3] Porter-Blum MT-2.
[4] Carl Zeiss EM 9A.

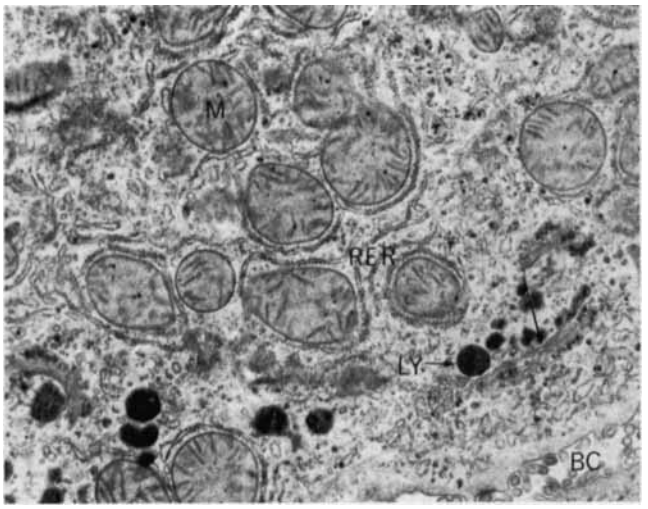

Figure 2—*Liver electron micrograph of control rat treated with corn oil alone showing characteristic features. Key: M, mitochondrion; RER, rough-surfaced endoplasmic reticulum; arrow, Golgi apparatus; LY, lysosome; and BC, bile canaliculus.* (×*13,400*)

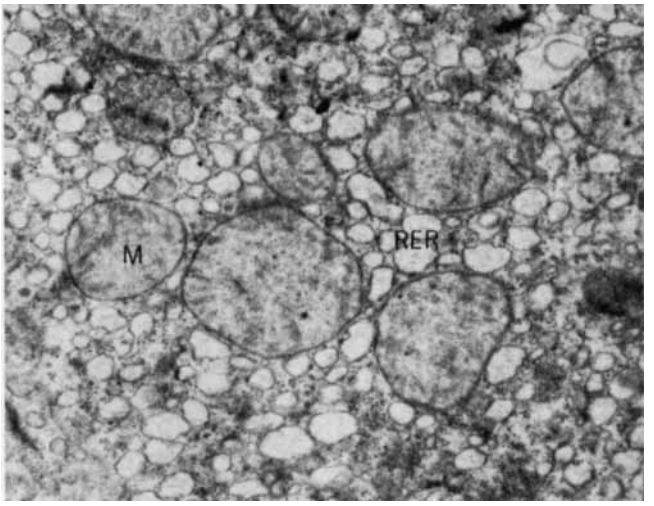

Figure 3—*Liver electron micrograph of a rat treated with 10 mg. of W-1372 for 3 days. The rough-surfaced endoplasmic reticulum (RER) is markedly distended and disorganized. The mitochondrial cristae are diminished in size and number.* (×*19,300*)

Under the light microscope, extensive lipid accumulation was seen in the hepatocytes of rats given 10 mg. of W-1372 (Fig. 1). Lipid droplets were uniformly distributed throughout the liver lobule with the exception of the hepatocytes adjacent to the central vein, which appeared to be unaffected. The droplets varied in size: in some cells, only small droplets were present; in others, they coalesced, occupied a larger portion of the cytoplasm, and displaced the nucleus eccentrically. Frozen sections stained with Oil red O or Sudan black B confirmed that the intracytoplasmic vacuoles represented lipid material.

There was a slight decrease in the amount of glycogen granules, as revealed by the PAS technique. The sinusoids were somewhat compressed by the swollen, vacuolated liver cells, but the original architecture of the liver was not distorted. Neither necrosis, inflammation, nor connective tissue proliferation was present; in experiments in which larger amounts of W-1372 were given, however, disseminated foci or necrosis was also seen[5]. Only slight lipid accumulation was found in the livers of rats treated with 5 mg. of W-1372. There were no morphologic abnormalities in animals given corn oil alone.

Under the electron microscope, many moderately electron-dense lipid droplets were seen in the cytoplasm of rats treated with 10 mg. of W-1372. These droplets varied in size and number in the individual liver cells.

The endoplasmic reticulum membranes exhibited progressive swelling, dilatation, and disorganization as compared with the controls (Figs. 2 and 3). The number of membrane-attached ribosomes was decreased. Large cisternae were formed, the walls of which were lined with dilated and partially degranulated endoplasmic reticulum membranes. Their lumina were filled with a moderately osmiophilic amorphous material. These structures corresponded to the so-called liposomes described by others (4–6). At some places, the changes were markedly advanced; the endoplasmic reticulum membranes were very irregular in shape and size, disrupted, and aggregated into almost unrecognizable masses.

The mitochondria were slightly swollen, but their double-limiting membranes were usually well preserved. The matrical density and number of intramitochondrial electron-dense granules were somewhat decreased. The cristae were diminished in size and number. Occasionally, they almost completely disappeared, endowing the intramitochondrial compartment with a homogeneous appearance.

The Golgi apparatus, which normally consists of from two to five closely parallel arrays of flattened sacs and numerous small spherical vesicles filled with a finely granular, moderately electron-dense material, also exhibited conspicuous abnormalities (Fig. 4). The sacs were markedly distended. The vesicles were dilated and, in several places, appeared empty.

[5] H. Selye and F. Lefebvre, to be published.

Milder electron microscopic changes were present in the hepatocytes of rats given 5 mg. of W-1372. No fine structural alterations were found in the livers of animals treated with corn oil alone.

DISCUSSION

Previous publications dealing with the various effects of W-1372 provide very few indications of its hepatotoxicity. Berger *et al.* (1, 2) found that this compound causes reversible liver enlargement in rats and an increase in total hepatic lipids, cholesterol, phospholipids, and triglycerides, but histopathologic abnormalities were not described.

Hepatocellular fat accumulation is the result of an imbalance between the rate of lipid synthesis and breakdown as well as uptake and elimination. In fact, it is a fundamental nonspecific reaction of the hepatocytes to various types of injury. It occurs quite commonly in man and can be induced easily in experimental animals in many ways such as: (*a*) administration of carbon tetrachloride, ethionine, ethanol, phosphorus, or puromycin; (*b*) orotic acid feeding; or (*c*) choline-deficient diets (4, 7, 8). It seems justifiable, on the basis of the present findings, to add to this list another compound that is also capable of inducing fatty degeneration of the hepatocytes: W-1372.

The structural lesions, including the electron microscopic changes found in the livers of rats treated with W-1372, are similar

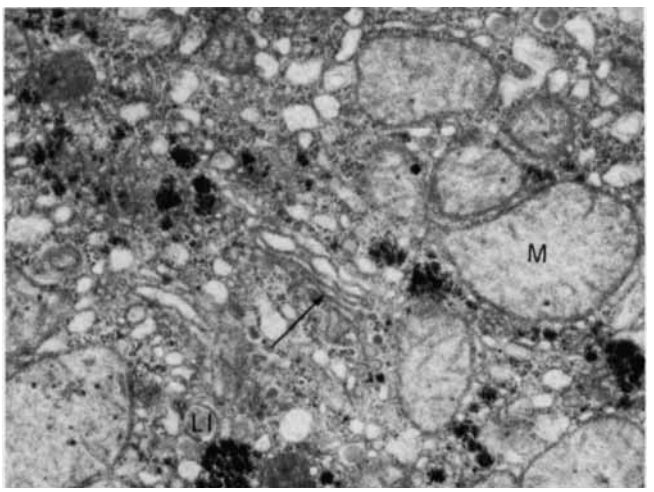

Figure 4—*Liver electron micrograph of a rat treated with 10 mg. of W-1372 for 3 days. The sacs of the Golgi apparatus (arrow) are dilated and appear to be empty. Liposomes (LI) are also seen.* (×*20,000*)

to those that occur in the course of hepatic injury such as carbon tetrachloride or ethionine poisoning (9–11). It was demonstrated that these toxicants produce fatty livers by interfering with hepatocellular lipoprotein synthesis or release (4). Furthermore, it was shown that the presence of numerous liposomes and the alterations in the Golgi apparatus, consisting of saccular dilatation and disappearance of their vacuolar content (also seen in the present experiments), are electron microscopic signs indicative of the failure of lipoprotein secretion (4, 6, 8, 12). Further investigations are, however, required to verify whether a similar mechanism is also responsible for the fatty degeneration caused by W-1372.

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Characteristics of a Highly Purified Pyrogenic Lipopolysaccharide

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Abstract □ A highly purified lipopolysaccharide from *Klebsiella pneumoniae* was examined for qualitative chemical composition and, in a collaborative study, for pyrogenic activity. The principal constituents determined after hydrolysis were galactose and mannose, together with unidentified fatty acids. A dose of 0.001 mcg./kg. administered to groups of eight rabbits resulted in a high percentage of positive pyrogenic responses as defined by USP XVIII.

Keyphrases □ Lipopolysaccharide, pyrogens—isolation, purification, characterization, activity □ Pyrogenic lipopolysaccharide from *Klebsiella pneumoniae*—isolation, purification, and characterization, pyrogenic activity tested □ Bacterial pyrogens—isolation and purification of lipopolysaccharide from *Klebsiella pneumoniae*, determination of constituents and pyrogen activity

In a continuing effort to find a bacterial pyrogen that might be generally acceptable as a pyrogen control, scientists of the National Center for Antibiotics Analysis (NCAA) recently undertook to purify a previously known lipopolysaccharide from *Klebsiella pneumoniae* (ATCC 12833). Details of the methods used for earlier extracts of the organism were reviewed by Selzer (1).

In the latest study, a highly purified material was obtained. This paper reports on some qualitative chemical characteristics of the substance and presents the results of a collaborative study to determine its pyrogenic activity in rabbits.

EXPERIMENTAL

Isolation and Purification—The *K. pneumoniae* was maintained on slants containing 10 ml. of soybean–casein digest agar. For seeding purposes, a new slant was inoculated and incubated at 32–35° for 24 hr. With 3 ml. of sterile soybean–casein digest broth (USP XVIII), the growth was washed from the slant into a 38 × 200-mm. tube containing 100 ml. of the same broth. The broth tube was incubated at 32–35° for 24 hr. Approximately 4 ml. of the broth culture was transferred to each of 25 Roux bottles containing 300 ml. of soybean–casein digest agar with 0.25% dextrose. The bacterial suspension was spread over the surface with the aid of sterile glass beads. The Roux bottles were incubated at 32–35° for 3 days. The resulting growth was then washed from each Roux bottle with 20 ml. of sterile pyrogen-free distilled water. All washings were collected in a sterile, 1000-ml., screw-cap conical flask. One hundred milliliters of 37% formaldehyde was then added, and the mixture was allowed to stand overnight at room temperature. On the next day, the cells were centrifuged, washed with water to remove extracellular material, and freeze dried.

The basic purification procedure was that of Westphal and associates, as modified and described by Selzer (1).

Chemical Analysis—The lipid fraction of the purified material, analyzed by GC, appeared to contain several fatty acids, which cannot be identified at the present time. The carbohydrate moiety was hydrolyzed under various conditions, and the simple sugars were detected by descending paper chromatography.

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Chemical Analysis—The lipid fraction of the purified material, analyzed by GC, appeared to contain several fatty acids, which cannot be identified at the present time. The carbohydrate moiety was hydrolyzed under various conditions, and the simple sugars were detected by descending paper chromatography.

The following solvent systems were used: I, 1-propanol–ethyl acetate–acetic acid–water (30:35:15:20); II, 1-propanol–ethyl acetate–pyridine–water (30:35:15:20); III, 1-propanol–ethyl acetate–pyridine–acetic acid–water (30:40:10:5:15); IV, 1-propanol–water (85:15); and V, 1-propanol–ethyl acetate–water (60:25:15) with filter paper[1].

The sprays for the neutral sugars were *p*-anisidine phosphate and aniline phosphate; for the amino sugar, ninhydrin and the Elson-Morgan reagent were used; and for 2-keto-3-deoxy octonate (2), thiobarbituric acid (3) was used.

The conditions of hydrolysis varied with the different sugars. Short periods of hydrolysis (5–15 min.) were used in attempts to detect dideoxyhexoses (4). Since the thiobarbituric acid spray is used to detect the dideoxyhexoses as well as 2-keto-3-deoxy-octonate, these chromatograms were checked for both substances.

To detect the neutral and amino sugars, the lipopolysaccharide was refluxed with 0.4 *N* sulfuric acid for 1 hr. The solid lipid material that appeared on cooling was extracted with hexane, evaporated under nitrogen, and analyzed by GC. The aqueous solution was hydrolyzed for 4 hr. more, yielding a brown solution, which was clarified with Darco S51, neutralized with $BaCO_3$, filtered, and freeze dried. The dried material was dissolved in a minimum of water, and several microliters were spotted on the paper for chromatography.

The amino sugars were more readily ascertained by means of HCl hydrolysis. The lipopolysaccharide was refluxed with 0.4 *N* HCl for 1 hr., the lipid was removed, and the hydrolysis was continued for 5 hr. longer. The brown solution was clarified with Darco S51, neutralized with Dowex-3, and freeze dried. The dried material was treated as described previously for chromatography.

Heptose (usually glycero-manno-heptose), present in many lipopolysaccharides, was checked colorimetrically by the Osborn modification (5) of the cysteine–H_2SO_4 reaction.

The purified pyrogen was stored in a desiccator. Prior to testing for pyrogenic activity, a determination of loss on drying in the vacuum oven was made. Uptake of moisture on exposure was also determined.

Molecular weight was determined by ultracentrifugation. An elemental analysis was also made.

Pyrogenic Activity[2]—Each participating laboratory was furnished a sample of the preparation and a uniform protocol for testing. The sample was to be vacuum dried, cooled under desiccation, and weighed. A solution containing 100 mcg. of sample/ml. of sterile pyrogen-free water was prepared first. An aliquot was then diluted to 1.0 mcg./ml. with the same diluent. An aliquot from the second solution was further diluted to 0.01 mcg./ml.; from this last solution, the test solutions listed in Table I were prepared. The basic test procedure was that described in USP XVII (p. 863)[3], except that 32 rabbits were to be injected at the same hour, 8 for each dose level, with 1.0 ml./kg. administered by rapid intravenous injection. Upon completion of the test, each laboratory was requested to send their results to NCAA for summarization and analysis.

RESULTS

Chemical Analyses—Short-period hydrolysis of the polysaccharide fraction failed to expose any dideoxyhexoses when chromatograms of the hydrolysate were sprayed with thiobarbituric acid. 2-Keto-3-deoxyoctonate, however, was detected on all of these chromatograms.

After H_2SO_4 hydrolysis, the principal constituent was found to be galactose, followed closely by mannose. Traces of some amino sugars were also present.

[1] Whatman No. 1, Whatman No. 2, and S & S 589 Blue Ribbon.

[2] The following 12 laboratories currently engaged in pyrogen testing participated in the collaborative study: Abbott Laboratories, N. Chicago, Ill.; Belgian Pharmaceutical Association, Brussels, Belgium; Bristol Laboratories, Syracuse, N. Y.; Commonwealth Serum Laboratories, Parkville, Victoria, Australia; Connaught Medical Research Laboratories, Willowdale, Ontario, Canada; Lederle Laboratories, Pearl River, N. Y.; Eli Lilly and Co., Indianapolis, Ind.; Merck Sharp & Dohme, West Point, Pa.; National Center for Antibiotics Analysis, Food and Drug Administration, Washington, D. C.; Chas. Pfizer and Co., Inc., Brooklyn, N. Y.; E. R. Squibb and Sons, Inc., New Brunswick, N. J.; and Wyeth Laboratories, Inc., West Chester, Pa.

[3] USP XVIII is equally applicable.

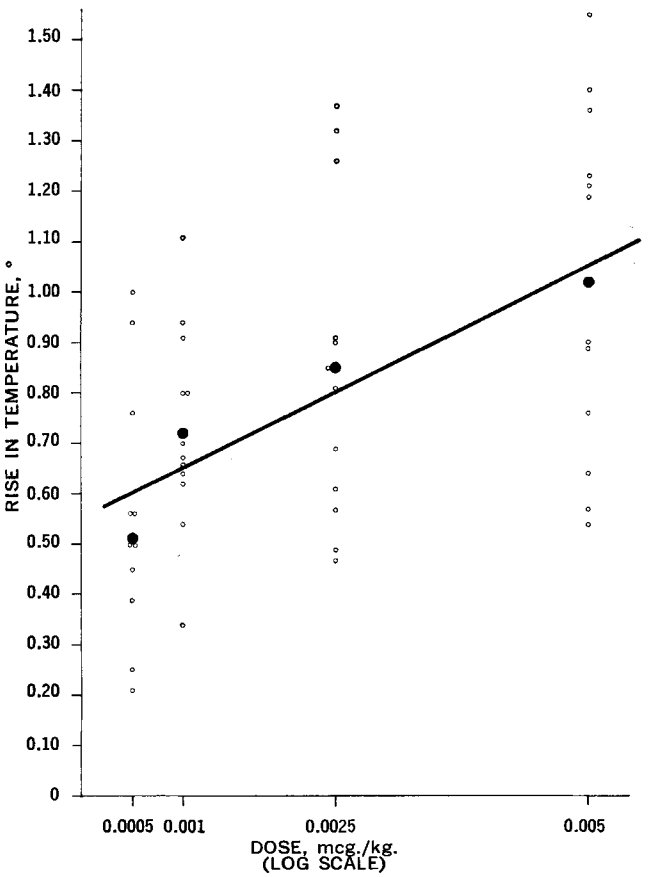

Figure 1—*Distribution of the average temperature rise obtained by each laboratory at each dose level. Key: ● = average for each dose.*

After HCl hydrolysis, galactose and mannose were, again in that order, the major constituents. Glucosamine was also detected.

Heptose was not detected colorimetrically. Paper chromatography was not used inasmuch as the large amount of galactose present would have interfered with the determination.

In summary, the carbohydrate components of the lipopolysaccharide appear to be galactose, mannose, 2-keto-3-deoxy-octonate, and glucosamine, in descending order of magnitude.

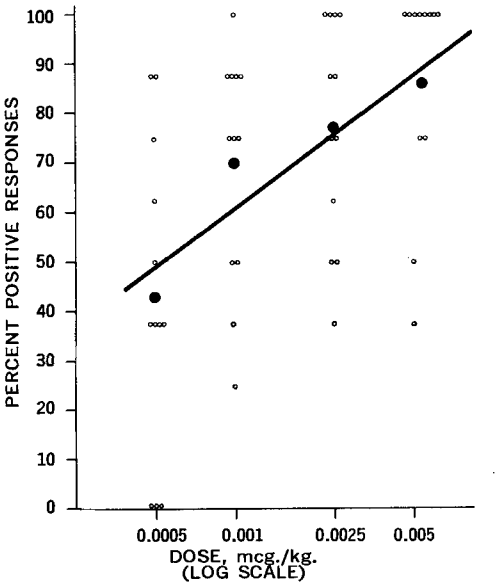

Figure 2—*Distribution of the percent of rabbits showing pyrogenic rises for each laboratory at each dose level. Key: ●, average for each dose.*

Table I—Results of Collaborative Assay[a] of NCAA Pyrogen, 1969

Laboratory	Test Doses: 0.0005 mcg./kg. Number of Rises	Sum	Average Rise	0.001 mcg./kg. Number of Rises	Sum	Average Rise	0.0025 mcg./kg. Number of Rises	Sum	Average Rise	0.005 mcg./kg. Number of Rises	Sum	Average Rise
A	5/8	4.5°	0.56°	7/8	5.6°	0.70°	8/8	7.4°	0.91°	8/8	11.2°	1.40°
B	6/8	6.1°	0.76°	7/8	7.5°	0.94°	7/8	10.1°	1.26°	8/8	12.4°	1.55°
C	3/8	3.1°	0.39°	6/8	5.3°	0.66°	6/8	4.9°	0.61°	6/8	5.1°	0.64°
D	3/8	4.00°	0.50°	4/8	5.35°	0.67°	5/8	6.50°	0.81°	8/8	9.95°	1.23°
E	0/8	1.65°	0.21°	4/8	5.00°	0.62°	3/8	3.75°	0.47°	4/8	4.30°	0.54°
F	7/8	7.5°	0.94°	7/8	7.3°	0.91°	8/8	11.0°	1.37°	8/8	9.5°	1.19°
G	3/8	3.6°	0.45°	7/8	6.4°	0.80°	7/7	6.3° (7.2°)	0.90°	7/7	6.2° (7.1°)	0.89°
H	4/8	4.5°	0.56°	6/8	6.4°	0.80°	7/8	6.8°	0.85°	8/8	9.7°	1.21°
J	7/8	8.1°	1.00°	8/8	9.0°	1.11°	8/8	10.6°	1.32°	8/8	10.9°	1.36°
K	3/8	4.0°	0.50°	6/8	5.1°	0.64°	6/8	5.5°	0.69°	8/8	7.2°	0.90°
L	0/8	2.0°	0.25°	2/8	2.7°	0.34°	4/8	4.6°	0.57°	6/8	6.1°	0.76°
M	0/8	−1.0°	−0.12°	3/8	4.3°	0.54°	4/8	3.9°	0.49°	3/8	4.6°	0.57°
Average		4.08°	0.51°		5.82°	0.72°		6.85°	0.85°		8.17°	1.02°
Number of pyrogenic rises		41/96			67/96			73/95			82/95	
Percent		43			70			77			86	
Range of temperature changes		−0.8–+1.6°			−0.1–+1.6°			−0.4–+2.0°			−0.3–+2.2°	

[a] *Departures from the protocol:* Laboratory A used three unrestrained rabbits at 0.0025 mcg./kg. and eight at 0.005 mcg./kg. Manual rectal thermometers were used as well as electronic sensors. Laboratory B did two complete determinations. The one tabulated utilized two weighings of the pyrogen for preparation of the dilutions. Laboratory G, for lack of space, used only seven rabbits for the two higher doses. The figures in parentheses are extrapolations of the sums to eight rabbits. In two of Laboratory K's test groups, the control temperature variation between the rabbits in each group was considerably more than the 1° permitted by the USP.

In the vacuum oven, the purified pyrogen lost approximately 2.9% moisture. On exposure, it proved moderately hygroscopic, stabilizing as the moisture content reached almost 12%.

The molecular weight was 4.77×10^6. The elemental analysis was as follows: C, 45.45%; H, 6.13%; N, 1.15%; and P, 0.065%.

Collaborative Pyrogenic Evaluation—A summary of the findings of each laboratory is given in Table I. Although response to the pyrogen varied considerably, as recorded by the different laboratories, the overall average temperature rise increased with increasing dosage. Likewise, the number and percent of rabbits showing pyrogenic rises increased as the dosage increased. (According to USP criteria, a pyrogenic rise is an elevation of 0.6° above the initial, or control, temperature reading.)

The latitude and correlation of the temperature rises, averaged for each laboratory, are better visualized in the distribution graph (Fig. 1). The upward bias at the successively higher dose levels is readily apparent. The slope of the regression line is $0.01 \pm SE = 0.0025$, with the intercept at 0.55°. Thus, a positive slope is indicated, which is significantly different from zero at a probability level of $p < 0.001$.

Figure 2 is similarly constructed to show the distribution of the percent of rabbits giving positive responses for each laboratory at each dose. The slope of the regression line of the mean percentages is 28% per log dose (base 5). It is significant at $p < 0.001$.

DISCUSSION

A quantitative analysis of the chemical components of the *Klebsiella* pyrogen was not made. The estimated qualitative composition, however, agrees well with that given by Berger *et al.* (6) in a summary of determinations by investigators for other known pyrogens. The large percentage of galactose present is intriguing in view of the abundance of glucose found in polysaccharides in general.

The wide variation in results obtained by the 12 laboratories in the collaborative assay emphasizes the reasons preventing general adoption of a pyrogen standard. In Table I, for example, Laboratory J and Laboratory M represent extremes of responsiveness as measured both by the number of rabbits having pyrogenic rises and by the average rise at each dose level; J displayed the greatest sensitivity, M the least, and the spread is considerable.

One aim of the study was to establish the test dose that reasonably could be expected to produce a minimal pyrogenic response as defined by the USP. This dose is 0.001 mcg./kg.; 10 of the 12 laboratories reported a sufficient number of rabbits responding (four or more out of eight), and 11 of the 12 reported a sufficiently high sum of the temperature rises (more than 3.7°).

The highest dose used (0.005 mcg./kg.) was expected, on the basis of preliminary trials, to give a very high percentage of individual positive responses. Eight laboratories did, in fact, report 100% response, yet the total pyrogenic rises occurred in only 86% of the rabbits injected. To test the sensitivity of individual animals, a higher dose of this material might have to be used.

Other characteristics of this highly potent material are presently under study.

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Sewarine, a New Phenolic Akuammicine Alkaloid from *Rhazya stricta*

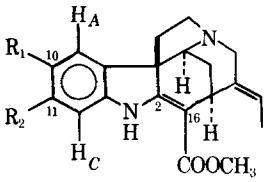
YUSUF AHMAD*, P. W. Le QUESNE†, and N. NEUSS‡

Abstract □ Sewarine, a phenolic alkaloid from *Rhazya stricta*, is assigned the structure 10-hydroxyakuammicine on the basis of chemical reactions and spectral data. Sewarine is the second phenolic akuammicine alkaloid to be isolated from natural sources. NMR and mass spectral data are discussed in detail.

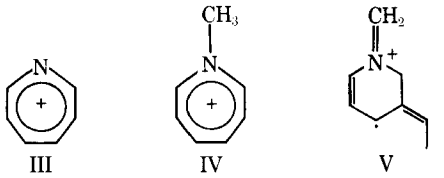
Keyphrases □ Sewarine, a phenolic akuammicine alkaloid from *Rhazya stricta*—identification, structure elucidation by NMR and mass spectroscopy □ Akuammicine alkaloids—elucidation of sewarine structure by mass spectroscopy and NMR □ *Rhazya stricta* alkaloids—identification and structure elucidation of sewarine

The use of local herbs and plants for medicinal purposes in Pakistan today is widespread, and the traditional *unani* system of medicine mainly relies on these for medicinal agents. Among the plants of the *unani* system, the *Rauwolfia*, *Swertia*, and *Cannabis* species have aroused international interest on account of their physiologically active constituents. *Rhazya stricta* (Apocynaceae) is used in the *unani* system for antitumor treatment; previous investigations showed it to be a rich source of indole alkaloids. So far, (−)-quebrachamine (1), akuammidine (rhazine) (2), rhazidine (3–5), (+)-1,2-dehydroaspidospermidine (6), (±)- and (+)-vincadifformine (6), aspidospermidine (7), eburnamonine (7), eburnamenine (7), and vincamidine (strictamine) (8) and the biogenetically important secamines (9), secodines (10), and strictosidine (11) have been isolated. As part of the continuing investigations of the phenolic alkaloids of this plant, a new alkaloid, sewarine, was obtained (12). In a recent preliminary report (13), the derivation of Structure I, 10-hydroxyakuammicine, for sewarine was outlined; the evidence leading to this assignment is now augmented and given in full.

Sewarine has the molecular formula $C_{20}H_{22}N_2O_3$ from microanalyses of the free base and a number of salts (12) and from high-resolution mass spectrometry (M^+ at *m/e* 338.162; calc. for $C_{20}H_{22}N_2O_3$, 338.163). Sewarine is insoluble in almost all organic solvents but can be purified *via* the hydrochloride salt. The UV spectrum of the hydrochloride shows $\lambda_{max.}^{MeOH}$ 230, 311, and 340 nm. (ε 10,800, 14,400, and 11,900), which

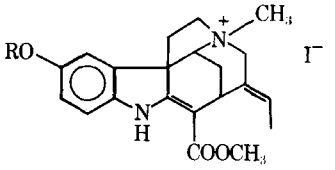


I: $R_1 = OH$, $R_2 = H$
II: $R_1 = R_2 = H$
VIII: $R_1 = H$, $R_2 = OH$
XIX: $R_1 = H$, $R_2 = OCH_3$

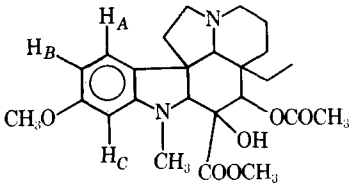


III IV V

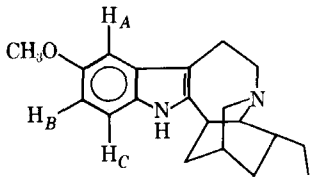
is very similar to that of akuammicine (II) and indicates the presence of the 2-methyleneindoline chromophore. The additional conjugated carbomethoxy group is inferred from the IR spectrum ($\nu_{max.}^{mineral\ oil}$ 1675 cm.$^{-1}$) and NMR spectrum (3H singlet, τ^{CD_3OD} 6.31), and the latter spectrum also shows the presence of an ethylidene group (1H, quartet, τ 4.20, J = 6 Hz., and 3H, doublet, τ 8.60, J = 6 Hz.). These functional groups are characteristically found in akuammicine alkaloids, and the assignment of sewarine to this group is strengthened by the mass spectral fragmentation pattern, which shows pronounced groups of peaks at *m/e* 92, 107, and 121 (92.052: calc. for C_6H_6N, 92.050; 107.073: calc. for C_7H_9N, 107.073; 121.089: calc. for $C_8H_{11}N$, 121.089). These empirical compositions are in accord with Structures III, IV, and V, respectively, assigned to ions at these values in the mass spectrum of akuammicine (14). The strikingly high negative specific rotation of sewarine is also characteristic of the akuammicine skeleton with absolute configuration as shown in Structures I and II (15–18). These data indicate that sewarine is a hydroxyakuammicine alkaloid.



VI: R = H
VII: R = CH_3



IX



X

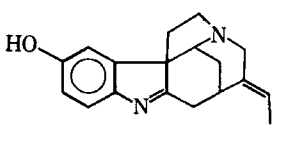
Table I—Chemical Shifts of Aromatic Protons (τ Units)

	H_A	J_{AB}, Hz.	H_B	J_{BC}, Hz.	H_C	Solvent
Vindoline	3.09	8	3.70	2	3.92	$CDCl_3$
Ibogaine	3.05	2	3.25	9	2.92	$CDCl_3$
Sewarine hydrochloride	3.02	2	3.30	7	3.17	CD_3OD

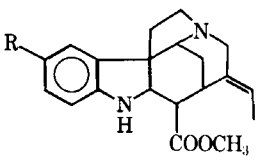
The unusual solubility properties of sewarine led to the use of CD_3OD and the alkaloid hydrochloride for NMR spectroscopy. The spectrum shows only three aromatic protons, suggesting that the hydroxyl group is phenolic. Confirmation of this was obtained in several ways. First, the UV spectrum of sewarine undergoes a pronounced bathochromic shift in alkaline solution. Second, the methiodide (VI) (12) of sewarine revealed, on electrometric titration, an acidic pKa′ of 12.1 (phenolic —OH). Third, sewarine, on methylation with methyl iodide–sodium methoxide, gave an *O,N*-dimethyl quaternary iodide (VII), whose NMR spectrum in CD_3OD showed characteristic phenolic methyl ether (τ 6.17) and quaternary N—CH_3 (τ 6.35) (19) singlets, respectively.

The location of the phenolic hydroxyl group at C-10 on the akuammicine skeleton is revealed by spectral measurements. First, the NMR spectrum of sewarine shows the aromatic protons to be in a 1,2,4-relationship, thus limiting the possible structures for sewarine to 10- or 11-hydroxyakuammicine (I or VIII, respectively). Comparison of the sewarine spectrum with those of vindoline (IX) and ibogaine (X) (Table I) clearly indicates a strong similarity between the sewarine (I: $R_2 = H_B$) and ibogaine (X) spectra. These data are also in accord with those and for other hydroxylated indole derivatives (20, 21). Corroboration of this assignment is also obtained from UV spectral data. The magnitude of the bathochromic shift of the UV spectrum of sewarine in alkaline solution [to $\lambda_{max.}^{MeOH}$ 324 and 363 nm. (ϵ 12,200 and 9500)] is much closer to that measured for *p*-hydroxyaniline [$\lambda_{max.}^{EtOH,\ neutral}$ 232 and 300 nm. (ϵ 7450 and 2320) → $\lambda_{max.}^{EtOH,\ alkaline}$ 248 and 314 nm. (ϵ 10,800 and 2240)] than to that for *m*-hydroxyaniline [$\lambda_{max.}^{EtOH,\ neutral}$ 234 and 285 nm. (ϵ 6130 and 2160) → $\lambda_{max.}^{EtOH,\ alkaline}$ 240 and 293 nm. (ϵ 7000 and 3090)].

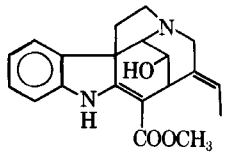
Further chemical work on sewarine also confirms its similarity to akuammicine. On heating with hydrochloric acid in a sealed tube, sewarine gives a descarbomethoxy derivative, which is assigned Structure XI. The NMR spectrum of this compound lacks the 3H singlet of the carbomethoxy group of sewarine, and the benzylic and allylic regions of the spectrum are complex. Treatment of sewarine with sodium borohydride in aqueous acid solution gives a dihydro derivative. This compound is somewhat unstable in the air, reverting readily to sewarine, but its spectral characteristics clearly show it to be the 2,16-dihydro compound (XII). In the IR spectrum, the ester carbonyl peak falls at 1735 $cm.^{-1}$, which shows that the α,β-unsaturated ester has been reduced. In the NMR spectrum, the signals from the aromatic, carbomethoxy, and ethylidene groups are clearly seen, with a new 1H multiplet at τ 5.90, which is assigned to the new 2-proton.



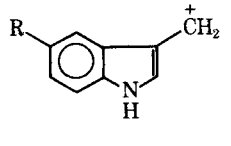
XI



XII: R = OH
XVI: R = H



XIII

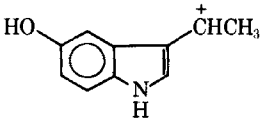


XIV: R = OH
XV: R = H

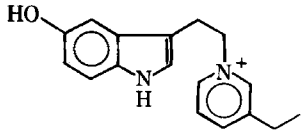
High-resolution mass spectrometry of sewarine and its derivatives supports these structural assignments. First, sewarine, although a hydroxyakuammicine, does not lose water or OH on electron impact. This behavior is in contrast to that of hydroxyakuammicine alkaloids with aliphatic hydroxy groups; mossambine (XIII) (22), for example, gives a peak at *m/e* 321 ascribable to the loss of OH from the bridging carbon. The contrasting behavior of sewarine further confirms its phenolic character. Besides giving the characteristic fragments III, IV, and V on electron impact, sewarine gives an ion at *m/e* 146.064 (calc. for C_9H_8NO, 146.061), which is assigned Structure XIV. Another characteristic mass spectral fragmentation of akuammicine involves the loss of the carbomethoxy group, and analogous behavior is seen for sewarine, in whose spectrum peaks at *m/e* 278.146 (calc. for $C_{18}H_{18}N_2O$, 278.142) and *m/e* 279.151 (calc. for $C_{18}H_{19}N_2O$, 279.150) are prominent.

2,16-Dihydrosewarine (XII) gives a mass spectrum closely analogous to that of 2,16-dihydroakuammicine (XVI) (14), except for the displacement by 16 mass units of characteristic fragments incorporating the indole nucleus at *m/e* 144 and 251 in XVI to 160 and 267, respectively, in XII. These ions from XII are assigned Structures XVII (160.081: calc. for $C_{10}H_{10}NO$, 160.076, and 161.084; calc. for $C_{10}H_{11}NO$, 161.084) and XVIII (267.149: calc. for $C_{17}H_{19}N_2O$, 267.150). Other fragments at *m/e* 130, 139, and 194, arising from the aliphatic portions of the molecule, are common to the spectra of both alkaloids and were discussed for akuammicine itself (14).

The data accumulated show that sewarine is 10-hydroxyakuammicine. This alkaloid is only the second phenolic akuammicine derivative to have been isolated from natural sources; recently, the 11-hydroxyakuammicine structure (VIII) was assigned to the alkaloid vinervine, which occurs with its methyl ether vinervinine (XIX) in *Vinca erecta* (23, 24). The investigation of the phenolic alkaloids of *Rhazya stricta* is continuing, and further work in this area will be reported.



XVII



XVIII

EXPERIMENTAL[1]

Sewarine (I)—Sewarine was obtained as small crystals: m.p. 245° dec.; UV $\lambda_{max.}^{MeOH}$ 220, 311, and 340 nm. (ϵ 10,800, 14,400, and 11,900); IR $\nu_{max.}^{mineral\ oil}$ 3280 (N—H), 1675 (α,β-unsaturated ester >C=O), 1600 (exocyclic acrylic ester intramolecularly H-bondable to the indole —NH), 1572 cm.$^{-1}$ (aromatic —C=C—); mass spectrum *m/e* 338 (M^+) (66), 323 (7), 279 (18), 278 (18), 222 (15), 160 (3), 146 (4), 139 (5), 121 (100), 107 (8), 92 (5). Sewarine hydrochloride had m.p. 210° dec.; $[\alpha]_D^{32}$ −724° (c 0.1 in EtOH); one basic pKa′ of 7.7 (electrometric titration in 66% dimethylformamide; apparent mol. wt. 370 (calc. for $C_{20}H_{22}N_2O_3 \cdot HCl$, 374); NMR given in text. Precipitation of sewarine from the solution of its hydrochloride at pH 12 prevented the determination of the acidic pKa′ of its phenolic function (see sewarine *N*-methiodide).

***O*-Methylsewarine *N*-Methiodide (VII)**—A mixture of sewarine (338 mg.) and sodium methoxide (108 mg.) in dry methanol (20 ml.) was heated under reflux with methyl iodide (1 ml.) for 3 hr. Removal of solvents under reduced pressure gave a solid which, on recrystallization from the minimum quantity of methanol, gave, in almost quantitative yield, pale-yellow prismatic needles of VII: m.p. 243–244° dec.; $[\alpha]_D^{25}$ −414° (c 0.106 in MeOH); NMR τ^{CD_3OD} 6.17 (3H, singlet, phenolic —OCH_3), 6.35 (3H, singlet, quaternary —NCH_3).

Anal.—Calc. for $C_{22}H_{27}IN_2O_3$: C, 53.43; H, 5.51; N, 5.67. Found: C, 53.55; H, 5.49; N, 5.67.

When sodium methoxide was omitted from this procedure, sewarine *N*-methiodide (VI) was obtained, identical with material previously prepared (6); in addition, $[\alpha]_D^{25}$ −434° (c 0.086 in MeOH); NMR τ^{CD_3OD} 6.55 (3H, singlet, quaternary N—CH_3; one acidic pKa′ of 12.1 by electrometric titration in 66% dimethylformamide; apparent mol. wt. 512 (calc. for $C_{21}H_{25}IN_2O_3 \cdot CH_3OH$, 512).

The insolubility of the free base, sewarine, in the usual organic solvents led to its being recovered unchanged after 1 week of contact with ethereal diazomethane in an attempted *O*-methylation reaction.

Descarbomethoxysewarine (XI)—Sewarine (500 mg.) in 20% aqueous hydrochloric acid (25 ml.) was heated (N_2 atmosphere) in a sealed tube in a steam bath for 16 hr. The brown solution was decolorized (Norit), cooled, and basified with aqueous ammonia. The resulting white precipitate (300 mg.) was dried and crystallized from methanol to give XI as pale-yellow long prismatic needles: m.p. 205–206° dec. (on rapid heating to ~200°); $[\alpha]_D^{25}$ −210° (c 0.278 in MeOH); NMR τ ~ 2.8–3.4 (3H, multiplet, aromatic protons), 4.50 (1H, quartet, J = 6 Hz., vinyl H of ethylidene group), 8.21 (3H, doublet, J = 6 Hz., CH_3 of ethylidene group); mass spectrum *m/e* 280 (M^+) (100), 265 (15), 251 (32), 250 (35), 249 (45), 198 (13), 197 (26), 196 (32), 175 (27), 174 (71), 160 (14), 159 (19), 140 (10), 122 (25), 121 (65), 120 (16), 108 (14), 107 (11), 94 (11), 93 (43), 92 (9), 80 (7). The analytical sample, recrystallized from methanol, lost 9.98% of its weight, equivalent to 1 CH_3OH of crystallization, when dried over P_2O_5 at 100°.

Anal.—Calc. for $C_{18}H_{20}N_2O$: C, 77.11; H, 7.19; N, 9.99. Found: C, 76.76; H, 7.00; N, 10.23.

2,16-Dihydrosewarine (XII)—Sewarine (300 mg.), dissolved in 10% aqueous hydrochloric acid (50 ml.), was treated with sodium borohydride (400 mg.) at 0–10° during 90 min. with vigorous stirring. After 30 min. extra stirring, the solution, maintained below 10°, was basified with aqueous ammonia, and the resulting suspension was extracted with ether (5 × 50 ml.). The dried ($MgSO_4$) ether layer, on evaporation, gave a solid (165 mg.) which, on crystallization from ether, gave XII as pale-yellow prisms: m.p. (on vacuum-dried sample) 202° dec. (rapid heating to ~190°); $[\alpha]_D^{25}$ −112° (c 0.190 in MeOH); $\nu_{max.}^{mineral\ oil}$ 1735 cm.$^{-1}$ (saturated ester —C=O; τ^{CDCl_3} ~3.5 (3H, multiplet, aromatic protons), 4.75 (1H, quartet, J = 7 Hz., vinyl H of ethylidene group), 5.90 (1H, multiplet, C—2H), 6.18 (3H, singlet, —$COOCH_3$), 8.42 (3H, doublet, J = 7 Hz., CH_3 of ethylidene group), mass spectrum *m/e* 341 (9), 340 (M^+) (44), 322 (5), 321 (13), 309 (5), 281 (4), 268 (4), 267 (16), 195 (12), 194 (100), 182 (8), 160 (29), 159 (16), 146 (23), 144 (5), 140 (8), 139 (36), 134 (5), 130 (5), 122 (6), 121 (5), 108 (8), 107 (9), 92 (5), 90 (6).

Anal.—Calc. for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.66; H, 7.05; N, 8.27.

[1] Analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich. Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Optical rotations were measured in a 0.1-dm. cell with a Bendix-Ericsson automatic polarimeter, and NMR spectra were measured with Varian A-60 or HA-100 spectrometers. Mass spectra were taken with A.E.I. MS-902 or Consolidated Electrodynamics CEC-21-110 instruments.

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Stoichiometric Balances and Stability Constants of Amine–Bromthymol Blue Complexes

V. DAS GUPTA

Abstract □ The stoichiometric balances and absolute stability constants of some amines (atropine, chlorpheniramine and its maleate salt, dextromethorphan hydrobromide, methapyrilene hydrochloride, and quinine and its hydrochloride salt) were determined, using chloroform as the solvent. In chloroform, bromthymol blue was unable to replace the acid part of the amine salts. Nevertheless, the addition of water appears to help this replacement by separating the charges. The absolute stability constant values (between 2.5×10^4 and 6.0×10^{10}) indicate that the compounds formed by reacting amines with bromthymol blue are complexes in nature rather than the salts.

Keyphrases □ Amine–bromthymol blue complexes—stoichiometric balances, stability constants □ Bromthymol blue–amine complexes—stoichiometric balances, stability constants □ Stability constants—amine–bromthymol blue complexes, in chloroform

Several methods have been published for the quantitative determination of amines and quaternary ammonium ions, in which the acid dyes form color compounds extractable from the aqueous solution with organic solvents. The color in the organic solvent is measured spectrophotometrically. The work in biology was summarized by Axelrod (1), and a number of applications to pharmaceutical products were summarized by Higuchi and Bodin (2).

Gupta and Cadwallader (3) reported the stoichiometric balance of the thiamine–bromthymol blue complex. Schill (4), Gupta *et al.* (5), and Hull and Biles (6) reported the partition coefficients or equilibrium constants of these complexes between the aqueous phase and the organic solvent. In the published literature, there is little information available regarding the stoichiometric balance, the nature of the reaction, and the stability constants of these complexes. The purpose of this research was to investigate these three properties of some amine–bromthymol blue complexes. The amines tested were: atropine, chlorpheniramine and its maleate salt, dextromethorphan hydrobromide, methapyrilene hydrochloride, and quinine and its hydrochloride salt.

EXPERIMENTAL

Reagents—All chemicals and reagents used were either USP, NF, or ACS grade. Bromthymol blue was purchased[1] and used without further purification.

Preparation of Solutions—Solutions of amines and bromthymol blue (2×10^{-4} *M*) in chloroform were prepared using the simple solution method.

Determination of Stoichiometric Balance—Job's method (7), of continuous variation was used to determine the stoichiometric balance of dye to amines. Ten (10.0-), 8.0-, 6.0-, 4.0-, and 2.0-ml. quantities of the dye solution were placed in 25-ml. volumetric flasks. Two (2.0-), 4.0-, 6.0-, 8.0-, and 10.0-ml. quantities of the amine solution were mixed with the dye solution, respectively, and enough chloroform was added to bring it to volume. The absorbance value of each solution was measured at 420 nm. (using a Bausch & Lomb Spectronic 20). In each case, a blank was prepared by substituting pure chloroform for the amine solution. The results are presented in Table I. The stoichiometric balances of amines to dye were determined either by plotting absorbance values against the mole fraction of the dye or intuitively from the maximum absorbance value. For a constant total concentration of dye and amine, the complex is at its greatest concentration (indicated by maximum absorbance value) at a point where the dye and the amine are combined in the ratio in which they occur in the complex. The results are presented in Table II. Samples of curves are presented in Fig. 1.

Effect of Water on Stoichiometric Balances—Separate experiments were carried out on dextromethorphan hydrobromide and methapyrilene hydrochloride solutions to investigate the effect of water on the stoichiometric balances. A 10.0-ml. quantity of the dye solution in chloroform was mixed with 2.0 ml. of the amine salt solution in chloroform, and enough chloroform was added to bring the volume to 25.0 ml. After recording the absorbance value, 4.0 ml. of distilled water was added, and the mixture was shaken for 1 min. in a separator. The chloroform layer was collected and centrifuged at high speed for 5 min.

The absorbance of the clear solution was measured against a blank prepared by substituting pure chloroform for the amine salt solution. The changes in the absorbance values are presented in Table I, and the changes in stoichiometric balances are presented in Table II.

[1] Eastman Organic Chemicals.

Table I—Absorbance Values of Various Amine–Dye Complexes[a]

Dye–Amine Ratio	Absorbance Value of the Amine–Dye Complex When the Amine Is						
	At	Ch	ChM	DeH	MeH	Qu	QuH
5:1	0.289	0.53	0.288	0.016[b]	0.173[c]	0.558	0.283
2:1	0.566	0.942	0.499	0.018	0.289	0.931	0.496
1:1	0.832	0.792	0.597	0.01	0.339	0.877	0.592
2:3	0.579	0.532	0.475	0.012	0.274	0.588	0.458
1:2	0.573	0.532	0.493	0.012	0.323	0.588	0.506
1:5	0.287	0.266	0.282	0.012	0.216	0.305	0.281

[a] At = atropine–dye complex; Ch = chlorpheniramine–dye complex; ChM = chlorpheniramine maleate–dye complex; DeH = dextromethorphan hydrobromide–dye complex; MeH = methapyrilene hydrochloride–dye complex; Qu = quinine–dye complex; and QuH = quinine hydrochloride–dye complex. [b] The absorbance reading was 0.228 after treatment with water. [c] The absorbance reading was 0.385 after treatment with water.

Table II—Stoichiometric Balances of Various Amine–Dye Complexes[a]

Amine–Dye Complex	Stoichiometric Balance (Dye–Amine)
At	1:1
Ch	2:1
ChM	1:1
DeH	No reaction[b]
MeH	1:1[c]
Qu	2:1
QuH	1:1

[a] For the abbreviations used, see Table I, *Footnote a.* [b] The stoichiometric balance was 1:1 after treatment with water since the absorbance value increased from almost zero to 0.228 (Table I). [c] The stoichiometric balance was 2:1 after treatment with water since the absorbance value increased from 0.173 to 0.385 (Table I).

Table III—Stability Constant Values for Various Amine–Dye Complexes[a]

Dye–Amine Ratio	Stability Constant Value When the Complex Is						
	At	Ch	ChM	DeH	MeH	Qu	QuH
5:1	—[b]	5.4×10^{9}	—[b]	—[c]	2.2×10^{4}	2.2×10^{10}	—[b]
2:1	1.6×10^{6}	3.9×10^{10}	1.9×10^{5}	—[c]	2.1×10^{4}	3.0×10^{10}	2.0×10^{5}
1:1	1.4×10^{7}	6.6×10^{10}	1.6×10^{5}	—[c]	2.3×10^{4}	—[d]	1.6×10^{5}
1:2	4.9×10^{6}	6.3×10^{10}	1.8×10^{5}	—[c]	2.8×10^{4}	—[d]	2.5×10^{5}
1:5	—[b]	9.2×10^{10}	—	—[c]	2.4×10^{4}	—[d]	—[b]
2:3	9.8×10^{6}	9.3×10^{10}	2.1×10^{5}	—[c]	2.8×10^{4}	—[d]	2.0×10^{5}
Average values	6.9×10^{6}	6.0×10^{10}	1.9×10^{5}	—	2.5×10^{4}	2.6×10^{10}	2.0×10^{5}

[a] For the abbreviations used, see Table I, *Footnote a.* [b] An average value of these readings was assumed to represent 1.6×10^{-6} *M* concentration of the complex (see text). [c] No reaction. [d] It was not possible to calculate this value, since the free dye concentration turned out to be slightly below zero. This is probably an experimental error due to the assumption made under *Footnote b.*

Determination of Stability Constants—To determine the stability constants, an average of the six absorbance values of the atropine, chlorpheniramine maleate, and quinine hydrochloride complexes (Table I) when the amine–dye ratios were 1:5 and 5:1 was assumed to represent a 1.6×10^{-6} *M* concentration of the complex (the same as that of the amine or the dye, whichever is at a lower concentration). This assumption appears reasonable, since the concentration of either the amine or the dye is five times higher than the other component, and the stoichiometric balance for these complexes is 1:1 (Table II). If the stoichiometric balance was 1:2 (amine–dye), such as in chlorpheniramine and quinine, the same average absorbance value was assumed to represent only 0.8×10^{-6} *M* concentration of the complex, since the dye part is responsible for the absorbance. This assumption facilitated the determination of the concentration of the complex from the absorbance values of all other mixtures. The concentrations of either the free amine or its salt and the dye were calculated by difference. Stability constants were computed using two different formulas, depending upon the stoichiometric balance. For 1:1 complexes:

$$K = \frac{(C_c)}{(C_a)(C_d)} \qquad \text{(Eq. 1)}$$

and for 2:1 (dye–amine) complexes:

$$K = \frac{(C_c)}{(C_a)(C_d)^2} \qquad \text{(Eq. 2)}$$

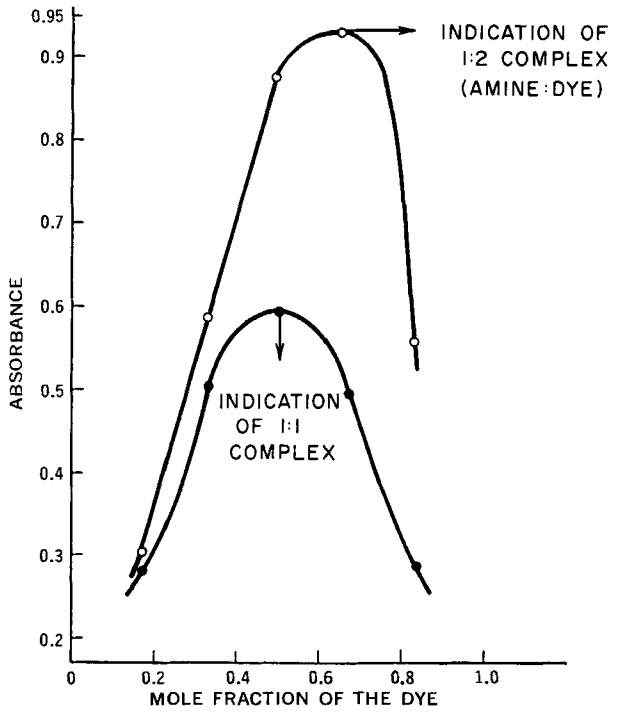

Figure 1—*Plot of absorbance values against mole fraction of the dye. Key:* ○, *quinine–dye complex; and* ●, *quinine hydrochloride–dye complex.*

where (C_c) is the concentration of the complex in moles per liter, (C_a) is the concentration of the amine or its salt in moles per liter, and (C_d) is the concentration of the dye in moles per liter. The results are presented in Table III.

DISCUSSION AND CONCLUSIONS

Stoichiometric Balances and Nature of Reaction—It is obvious that the stoichiometric balance in chloroform varies depending on whether the amine is free or in the form of its salt (Table II). For example, the stoichiometric balance of chlorpheniramine maleate or quinine hydrochloride is 1:1, while it is 2:1 (dye–amine) for free chlorpheniramine base or free quinine base. It appears, therefore, that in the case of chlorpheniramine maleate and quinine hydrochloride, only one nitrogen is occupied by the acidic group and the second nitrogen is free to react with the acidic dye to form a complex. The stoichiometric balance of free atropine base was determined to be 1:1 (Table II), since there is only one nitrogen in the base. It was not possible to conduct studies on easily available atropine salts, such as sulfate and hydrochloride, due to their insolubility in chloroform.

A salt (hydrobromide) of dextromethorphan (one nitrogen) failed to show the formation of any complex in chloroform (Table II), since the only nitrogen available to react with the dye was occupied by a hydrobromic acid group. Nevertheless, treatment with water (see *Experimental*) separated the hydrobromic acid group from dextromethorphan and the dye was able to form a 1:1 complex with the amine, as evidenced by an increase in the absorbance value from almost zero to 0.228. An absorbance value of 0.228 was not high enough to indicate complete extraction of the dextromethorphan–dye complex with chloroform, since a value of 0.285 would represent complete extraction (Table III, *Footnote b*). This was expected since some of the complex stayed in water. The concentration of the complex remaining in the aqueous phase depends upon its pH, as reported earlier (5).

Methapyrilene hydrochloride showed a stoichiometric balance of 1:1 (Table II). By assuming that one nitrogen was occupied by hydrochloric acid and the other two were free, a stoichiometric balance of 2:1 (dye–amine) would have been predicted. It appears, therefore, that one of the free nitrogens in methapyrilene hydrochloride is too weak to react. Even the other one appears to be weakly reactive, since the absorbance value of the complex was lower (Table I) as compared with other amine salts, such as chlorpheniramine maleate and quinine hydrochloride. On treating the methapyrilene hydrochloride–dye complex with water, the dye was able to replace the hydrochloric acid (as explained for dextromethorphan hydrobromide), as evidenced by an increase in the absorbance value from 0.173 to 0.385 (Table I). This increase indicated that the stoichiometric balance changed from 1:1 to 2:1 (dye–amine).

Stability Constants—The absolute stability constant values of various complexes were determined to be within the range of from 2.5×10^{4} to 6.0×10^{10} (Table III). This information may be helpful in deciding the controversial point regarding the nature of the compounds formed when amines react with acidic dyes such as bromthymol blue. Apparently, these compounds are complexes in nature rather than the salts.

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FRANK J. VILLANI, CLAIRE A. ELLIS, and THOMAS A. MANN

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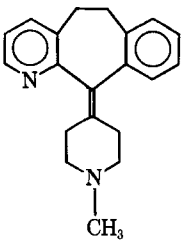
Keyphrases □ 4-Azaketones and derivatives—synthesis, screened for biological activity □ 10,11-Dihydro-5*H*-dibenzo[*a,d*]cycloheptene and related compounds—synthesis of 4-azaketones and derivatives

Compounds I and II have shown very potent antianaphylactic and antihistaminic activities in laboratory animals[1] and in man (1–4). It was of interest to homologate the seven-membered ring to prepare the corresponding amino derivatives from the benzopyridocyclooctanones (III and IV). This report summarizes attempts in this area.

Ketone III was prepared by the intramolecular cyclization of 3-(γ-phenylpropyl)picolinic acid or its corresponding nitrile in a large excess of polyphosphoric acid. Reaction of III with the Grignard reagent, prepared from 1-methyl-4-chloropiperidine, gave the expected tertiary carbinol (V). Reductive alkylation (5), using the disodio derivative of III and 1-methyl-4-chloropiperidine, resulted in a cleaner product in excellent yield.



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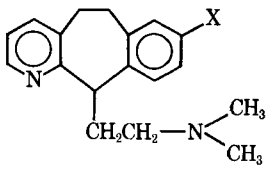

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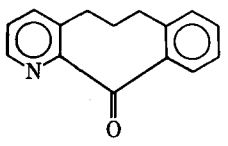
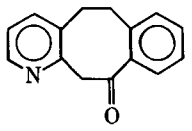


III



IV

All attempts at the dehydration of V to introduce the exocyclic double bond as in I failed, and the azaindenoindene derivative (VI) was isolated. The structure of VI was established by the usual physical measurements. Of special importance was the weak absorbance in the UV spectrum ($\epsilon_{270\ nm.}$ 6060) compared to that of Compound I ($\epsilon_{239\ nm.}$ 11,000, $\epsilon_{267\ nm.}$ 6100, and $\epsilon_{275\ nm.}$ 5900).

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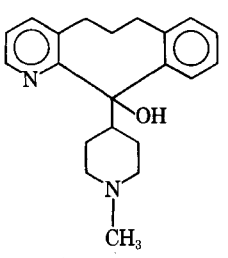
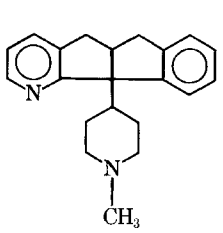


V



VI

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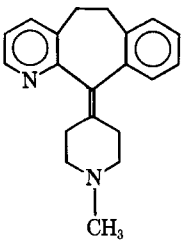

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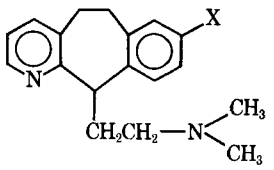

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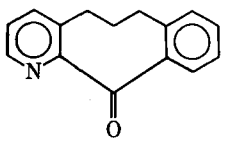
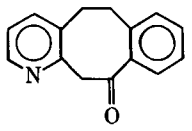


III



IV

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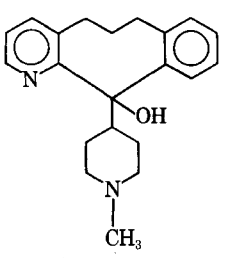
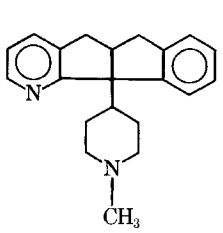


V



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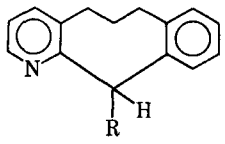
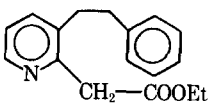


VII*a*: R = H
VII*b*: R = $(CH_2)_2N(CH_3)_2$
VII*c*: R = $(CH_2)_3N(CH_3)_2$



VIII

substituted derivatives (VII*b* and VII*c*).

To prepare ketone IV, the lithio derivative of 2-methyl-3-phenethylpyridine was carbonated and converted into the ester VIII. Cyclization of VIII resulted in the isolation of IV, although in low yield due to the ease of decarboxylation of the intermediate pyridyl acetic acid. Considerable amounts of 2-methyl-3-phenethylpyridine were formed during this reaction. Grignard addition to IV was not successful because this compound exists primarily in the enol form.

Reduction of IV under Wolff-Kishner conditions gave the deoxygenated derivative, which resisted all alkylation attempts.

The compounds were screened for antianaphylactic, antihistamine, and antitetrabenazine activity in laboratory animals. The compounds were surprisingly inactive in the antianaphylactic screen at a dose of 10 mg./kg. orally. Antihistaminic potency (*in vitro*) was of a very low order, with a PD_{50} greater than 80 mcg./l. Compound VII*c* showed an ED_{50} of approximately 20 mg./kg. orally in mice against tetrabenazine-induced ptosis, but it was of insufficient potency to warrant further investigation.

EXPERIMENTAL

β-Phenethyl-3-pyridyl Ketone—In a typical preparation, 3-cyanopyridine (20.8 g.) in ether (150 ml.) was added dropwise to a Grignard reagent prepared from β-phenethylbromide (42.5 g.) and magnesium (5.6 g.) in ether (1 l.). The reaction mixture, containing a yellow precipitate, was stirred under reflux (steam bath) for 16 hr. Concentrated hydrochloric acid (200 ml.) was added, and stirring was continued for an additional 6 hr. at room temperature. The ether layer was removed, and the acid solution was heated under reflux with stirring for an additional 4 hr. After cooling, the solution was basified with NH_4OH and extracted with chloroform. The solvent was removed and the product distilled, b.p. 161–170°/1 mm. (yield 20.6 g., 48%), n_D^{26} 1.5814. A small sample was crystallized for analysis from ether–petroleum ether (b.p. 30–60°), m.p. 31–33°.

Anal.—Calc. for $C_{14}H_{13}NO$: C, 79.59; H, 6.20; N, 6.63. Found: C, 79.48; H, 6.36; N, 6.66.

A small sample was converted to the hydrochloride, which was recrystallized from ethanol–ether and had a melting point of 136–138°.

Anal.—Calc. for $C_{14}H_{13}NO \cdot HCl$: C, 67.87; H, 5.70; N, 5.66. Found: C, 67.70; H, 5.80; N, 5.65.

3-(γ-Phenylpropyl)pyridine—A mixture of the preceding ketone (189 g.), diethylene glycol (1.2 l.), NaOH (77.5 g.), and 85% hydrazine hydrate (78 ml.) was heated in a flask equipped with a downward condenser until the internal temperature reached 240° and was maintained at this temperature for an additional 4 hr. The cooled reaction mixture was extracted thoroughly (five times) with ether, the extracts were washed with water (five times), the solvent was removed, and the product was distilled to give 152 g. (86%) of a colorless liquid boiling at 130–134°/2 mm., n_D^{26} 1.5610.

3-(γ-Phenylpropyl)pyridine-1-oxide—Hydrogen peroxide (30%, 100 ml.) was added to a cold solution of the 3-(γ-phenylpropyl)-pyridine (166 g.) in glacial acetic acid (250 ml.). The mixture was heated to 60° and so maintained for 20 hr.; then it was poured into ice water (2 l.). The solution was basified (NH_4OH) and extracted with chloroform. The chloroform was removed *in vacuo* on the steam bath, and the residue (171 g., 93%) was recrystallized from benzene–hexane, m.p. 36–37°.

Anal.—Calc. for $C_{14}H_{15}NO \cdot \frac{1}{2} H_2O$: C, 75.65; H, 7.26; N, 6.30. Found: C, 75.41; H, 7.38; N, 6.08.

2-Cyano-3-(γ-phenylpropyl)pyridine—Dimethyl sulfate (64.5 g). was added dropwise to the *N*-oxide (108.5 g.), keeping the temperature between 80 and 85° (6). The mixture was heated on the steam bath for 3 hr., and 200 ml. of water was added. This solution was added under nitrogen and at 0° to a solution of sodium cyanide (76 g.) in water (215 ml.). Stirring was continued at 0° for 4 hr., and the mixture was permitted to warm to room temperature overnight. The product was extracted with chloroform and washed with water, the solvent was removed, and the residue was distilled at 1 mm. The major fraction (b.p. 165–185°/1 mm.) was redistilled to give 64.5 g. (60%) of an oil boiling at 170–175°/1 mm., n_D^{27} 1.5685. This fraction soon solidified, and a small sample was recrystallized from benzene–hexane, m.p. 56–59°.

Anal.—Calc. for $C_{15}H_{14}N_2$: C, 81.05; H, 6.35; N, 12.60. Found: C, 81.15; H, 6.59; N, 12.66.

The other fractions consisted of the 4- and 6-cyano compounds and were discarded.

3-(γ-Phenylpropyl)picolinic Acid—The nitrile (40.5 g.), sodium hydroxide (40.5 g.), ethanol (300 ml.), and water (75 ml.) were heated under reflux for 30 hr. The solvents were removed *in vacuo*, and the residue was dissolved in water and neutralized with acetic acid. The clear solution was extracted thoroughly with chloroform, and the solvent was removed. The solid residue was dissolved in 15% sodium bicarbonate solution, neutralized with acetic acid, and extracted with chloroform. After removal of the solvent, the residue was triturated with isopropyl ether and air dried to give 38.5 g. (87.5%) of the acid, m.p. 36–39°. The analytical sample was recrystallized from isopropyl ether and had a melting point of 39–40°.

Anal.—Calc. for $C_{15}H_{15}NO_2$: C, 74.66; H, 6.27; N, 5.81. Found: C, 74.09; H, 6.33; N, 5.95.

6,7 - Benzo - 12*H* - 5,6,7,12 - tetrahydrocycloocta[1,2*b*]pyridine-12-one (III)—*Method 1*—3-Phenethylpicolinic acid (50 g.) and polyphosphoric acid (2.5 kg.) were heated with stirring to 190–200° for 3 hr. and poured into ice water. The solution was neutralized with sodium hydroxide (50% solution) and extracted with ether. The ether extracts were concentrated on the steam bath, and the residue was triturated with petroleum ether. The product was recrystallized from benzene–petroleum ether to give 34 g. (73%) of a white solid, m.p. 157–159°.

Anal.—Calc. for $C_{14}H_{13}NO$: C, 79.59; H, 6.20; N, 6.63. Found: C, 80.16; H, 6.24; N, 6.43.

Method 2—A mixture of nitrile V*b* (42.5 g.) and polyphosphoric acid (2.1 kg.) was heated with stirring at 230–235° for 4 hr., poured into ice water, and processed as in Method 1. A crude product, 27 g. (62.4%), was obtained, m.p. 153–155°.

12 - Hydroxy - (1 - methyl - 4 - piperidyl) - 6,7 - benzo - 12*H*-5,6,7,12-tetrahydrocycloocta[1,2*b*]pyridine (V)—*Grignard Method*—Under nitrogen, the Grignard reagent was prepared from 1-methyl-4-chloropiperidine (13.2 g.) and magnesium (2.4 g.) in tetrahydrofuran (100 ml.), using 1,2-dibromoethane (0.5 ml.) as an initiator. At 10°, ketone III (7 g.) in tetrahydrofuran (250 ml.) was added dropwise, and the mixture was stirred for 45 min. The solvent was removed *in vacuo* on the steam bath; ether (300 ml.) was added, followed by a saturated solution of ammonium chloride. The organic layer was separated and the aqueous layer extracted ($CHCl_3$); the combined extracts concentrated to a residue which resisted crystallization. The material was chromatographed on alumina (250 g.), using 15% ethyl acetate–benzene as the eluant. Seven fractions of 750 ml. each were collected, with the major product being in Fractions 5 and 6. The solvents were removed, and the residues were recrystallized twice from isopropyl ether to give 2 g. (20%) of product, m.p. 120–121°.

Anal.—Calc. for $C_{21}H_{26}N_2O$: C, 78.22; H, 8.13; N, 8.69. Found: C, 78.15; H, 8.20; N, 8.88.

Reductive Alkylation Method—Sodium (2.7 g.) was dissolved in anhydrous ammonia (about 200 ml.). After 20 min., ketone III (13 g.) in tetrahydrofuran (100 ml.) was added dropwise, and stirring was continued for an additional 10 min. 4-Chloro-1-methylpiperidine (7.8 g.) in 25 ml. of tetrahydrofuran was added and the mixture was stirred overnight. Ammonium chloride (5 g.) was added portionwise, and the excess ammonia was allowed

to evaporate. Water (100 ml.) and benzene (100 ml.) were added and the product was extracted with benzene. The benzene solution was evaporated *in vacuo*, and the residue was recrystallized from isopropyl ether. There was obtained 14.2 g. (76%) of V having a melting point of 119–122°.

4 - Aza - 4*b* - (1 - methyl - 4 - piperidyl) - 9,10 - dihydroindeno-[1,2*a*]indene (VI)—Carbinol V (13 g.) and polyphosphoric acid (750 ml.) were heated with stirring at 160° for 18 hr. The warm mixture was poured into ice, made basic with sodium hydroxide, and extracted with chloroform. The residue, after removal of solvent, would not crystallize readily; it was converted to the hydrochloride salt, which was recrystallized twice from ethanol–ether, m.p. 220–224°. The amine base was liberated from the hydrochloride (NH_4OH), and the product was extracted with chloroform. After removal of the solvent, a benzene solution of the residue was chromatographed on 500 g. of alumina, using ether as the eluant and collecting fractions of 300 ml. each. The major portion of the product was eluted in the first five fractions. After removal of the solvent, the residue was recrystallized from petroleum ether, m.p. 95–97° ($\epsilon_{270\ nm.}$ 6060; *m/e* 304).

Anal.—Calc. for $C_{21}H_{24}N_2$: C, 82.85; H, 7.95; N, 9.20. Found: C, 83.00; H, 7.88; N, 9.20.

6,7 - Benzo - 12*H* - 5,6,7,12 - tetrahydrocycloocta[1,2*b*]pyridine (VII*a*)—By using the same procedure as for VI, a mixture of ketone III (44.6 g.), sodium hydroxide (17.5 g.), 99–100% hydrazine hydrate (14.8 g.), water (17.5 ml.), and diethylene glycol (400 ml.) was heated to 240° for 2 hr. and processed as described. It gave 30.5 g. (73%) of product, m.p. 76–78°, from petroleum ether.

Anal.—Calc. for $C_{15}H_{15}N$: C, 86.08; H, 7.22; N, 6.69. Found: C, 86.20; H, 7.24; N, 6.58.

General Alkylation Procedure: Preparation of VII*b* and VII*c*— Potassium metal (2 g.) was added in small portions to liquid ammonia (about 600 ml.), using ferric oxide as the catalyst. After 2 hr., a solution of VII*a* (8.4 g.) in toluene (40 ml.) was added dropwise, followed in 45 min. by the dimethylaminoalkyl chloride (0.045 mole) in toluene (25 ml.). Stirring was continued for an additional 2.5 hr., toluene (150 ml.) was added dropwise, and the ammonia was allowed to evaporate. The reaction mixture was then heated on the steam bath for 6 hr. Water (10 ml.) was added, and the reaction mixture was extracted with chloroform. The solvents were removed, and the residue was dissolved in benzene. Water (10 ml.) was added, and the pH was brought to 4.5–5 by the addition of 1 *N* HCl. The benzene layer, containing unreacted azahydrocarbon, was discarded; the aqueous layer was basified with NH_4OH and extracted with chloroform. The chloroform was removed, and the residue was distilled.

*VII*b—Boiling point 160–167°/1 mm.; yield 27%; n_D^{27} 1.5835. This product soon crystallized and was recrystallized from petroleum ether, m.p. 87–90°.

Anal.—Calc. for $C_{19}H_{24}N_2$: C, 81.38, H, 8.63; N, 9.99. Found: C, 81.51; H, 9.43; N, 9.95.

*VII*b *Maleate*—This compound, m.p. 149–151°, was recrystallized from isopropyl acetate.

Anal.—Calc. for $C_{19}H_{24}N_2 \cdot C_4H_4O_4 \cdot H_2O$: C, 66.64; H, 7.30; N, 6.76. Found: C, 66.27; H, 7.46; N, 6.85.

*VII*c—This compound was obtained (54%) as an amber-colored oil, b.p. 205–210°/1.5 mm.; n_D^{27} 1.5712.

Anal.—Calc. for $C_{20}H_{26}N_2$: C, 81.58; H, 8.90, N, 9.52. Found: C, 81.51; H, 9.00; N, 9.90.

*VII*c *Maleate*—This compound, m.p. 164–166°, was recrystallized from isopropanol.

Anal.—Calc. for $C_{20}H_{26}N_2 \cdot C_4H_4O_4$: C, 70.22; H, 7.37; N, 6.82. Found: C, 70.07; H, 7.32; N, 7.23.

Ethyl-3-(β-phenethyl)-2-pyridylacetate—Phenyllithium was prepared from bromobenzene (15.7 g.) and lithium (1.4 g.) in ether (100 ml.). 2-Methyl-3-phenethylpyridine (19.7 g.) in an equal volume of ether was added dropwise and stirred for 30 min. at room temperature. The dark-red solution was poured onto powdered dry ice (75–100 g.), and the dry ice was permitted to evaporate. Ethanol (75 ml.) was added, and the mixture was saturated with anhydrous hydrogen chloride and allowed to stand overnight at room temperature. The solvents were removed *in vacuo* on the steam bath. Water was added to the residue, and the mixture was extracted with chloroform. The chloroform extracts were washed with dilute sodium bicarbonate solution, the solvent was removed, and the residue was distilled. The fraction, b.p. 140–172°/2 mm. (11 g.), was chromatographed on silica gel (350 g.), using finally 50% ether–50% pentane as the eluting agent and collecting fractions of about 200 ml. The solvent was removed from the combined fractions (Fractions 12–20), and the residue was distilled, b.p. 165–170°/1.5 mm.; yield 6.5 g. (24%).

Anal.—Calc. for $C_{17}H_{19}NO_2$: C, 75.81, H, 7.11, N, 5.20. Found: C, 75.70; H, 7.20; N, 5.47.

5,6 - Benzo - 5,6,11,12 - tetrahydrocycloocta[1,2*b*]pyridine - 11-one (IV)—The preceding ester (6.5 g.) and polyphosphoric acid (350 g.) were heated with stirring at 155–160° for 1.5 hr. The warm mixture was poured into ice, basified with ammonia, and extracted with chloroform. The chloroform extracts were washed with water, the solvent was removed, and the residue was recrystallized several times from hexane; yield 1.2 g. (24.5%), m.p. 122–123°.

Anal.—Calc. for $C_{15}H_{13}NO$: C, 80.69; H, 5.87; N, 6.27. Found: C, 80.59; H, 5.92; N, 6.29.

5,6 - Benzo - 5,6,11,12 - tetrahydrocycloocta[1,2*b*]pyridine (IX)—Ketone IV (5.6 g.), 85% hydrazine hydrate (5.3 g.), diethylene glycol (50 ml.), and potassium hydroxide (7.4 g.) were heated at 240° for 3.5 hr., and the product was isolated as previously described to give 2.6 g. (50%) of product, m.p. 86–87°.

Anal.—Calc. for $C_{15}H_{15}N$: C, 86.08; H, 7.22; N, 6.69. Found: C, 85.95; H, 7.30; N, 6.78.

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Synthesis of ^{14}C-Labeled Bunolol

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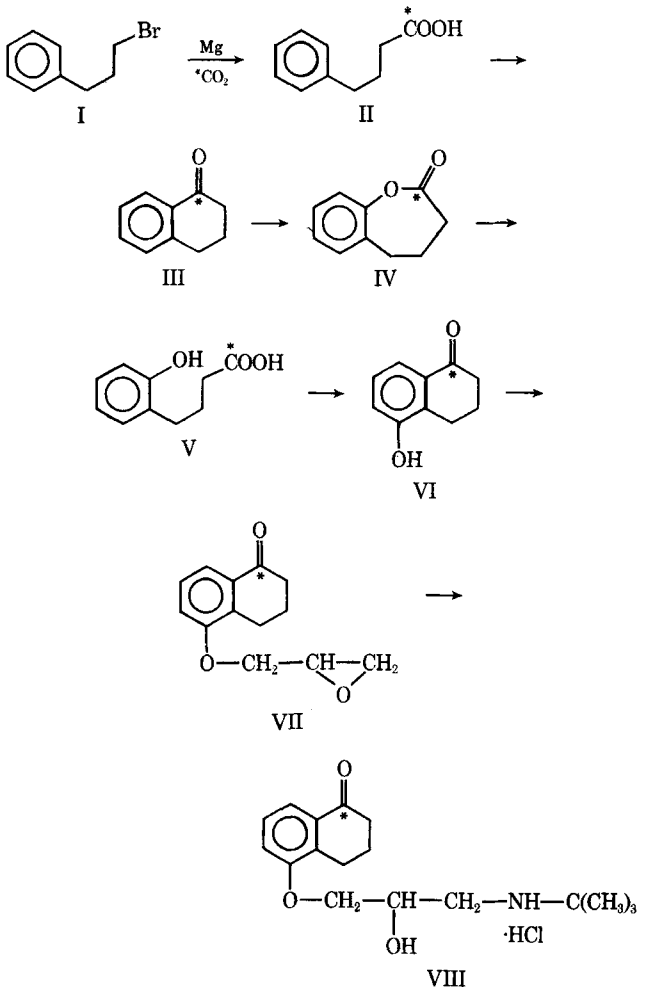
Abstract □ Ring-labeled bunolol, *dl*-5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone-1-^{14}C, was synthesized in several steps by initially allowing 3-phenylpropylmagnesium bromide to react with $^{14}CO_2$. The resulting 4-phenylbutyric-1-^{14}C acid was cyclized to α-tetralone-1-^{14}C. The ring was then oxidized and opened to 4-(2-hydroxyphenyl)butyric-1-^{14}C acid, which, when recyclized, gave 5-hydroxytetralone-1-^{14}C The 5-hydroxytetralone-1-^{14}C was allowed to react, sequentially, with epichlorohydrin, *tert*-butylamine, and gaseous hydrogen chloride to give bunolol hydrochloride. After purification, a number of physical tests established the chemical and radiochemical purity of this material.

Keyphrases □ Bunolol, ^{14}C-labeled—synthesis □ *dl*-5-[3-(*tert*-Butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone-1-^{14}C (bunolol)—synthesis

Bunolol hydrochloride, *dl*-5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride (VIII), is a new β-adrenergic blocking agent whose cardiovascular pharmacology was reported by Robson *et al.* (1). The drug was labeled with ^{14}C to facilitate determining its absorption and excretion rates, blood levels, tissue distribution, and biotransformation (2). The synthesis, shown in Scheme I, was designed to incorporate a ^{14}C atom into position 1 of the naphthalenone ring to assure retention of the label during metabolism.

The preparation of 4-phenylbutyric-1-^{14}C acid (II) was modified from Rupe and Proske's (3) procedure to allow for the handling of radioactive materials in conventional apparatus as described by Collins (4). The cyclization to α-tetralone-1-^{14}C (III) and its oxidation, ring opening, and recyclization to 5-hydroxy-α-tetralone-1-^{14}C (VI) were adapted to allow for the handling of small quantities from the procedures of Pattison and McMillan (5), which were based on the work of Schroeter (6). The subsequent conversions were modified from those of Schwender *et al.* (7).

The overall chemical yield was 7.3%, while the radiochemical conversion was 6.4%. The physical constants, which established that VIII was 99% chemically and radiochemically pure, are included in the *Experimental* section.



Scheme I

EXPERIMENTAL

4-Phenylbutyric-1-^{14}C Acid (II)—Three flame-dried, three-necked, round-bottom flasks were connected in series, with equilibrated addition funnels in the first two flasks, a $CaSO_4$ drying tube between the second and third flasks, and two reflux condensers and a rubber septum fitted to the third flask.

Into the first flask, 0.7 g. Ba-$^{14}CO_3$ (100.8 mc., specific activity 145 mc./g.)[1] was added, and 30 ml. of 5 *N* perchloric acid (previously degassed) was added to the addition funnel. Into the second flask, 4.24 g. (25 mmoles total) of $BaCO_3$ was added, and 220 ml. of 5 *N* perchloric acid (previously degassed) was added to the addition funnel. To the third flask, 4.98 g. (25 mmoles) of 3-phenylpropyl bromide and 0.7 g. (28.8 mmoles) of ether-washed magnesium turnings were added. The entire system was flushed with nitrogen gas and then kept at atmospheric pressure. To the third flask, 250 ml. of anhydrous ether, dried over Safe-Na[2], was injected through the septum using a hypodermic syringe. The reaction was magnetically stirred and allowed to reflux gently for 2 hr. During this time, the magnesium turnings reacted completely. This flask was cooled to −45° using a mixture of 150 parts carbon tetrachloride and 20 parts chloroform over dry ice (4). The pressure was reduced with an oil pump to about 12 mm., as measured with a mercury-filled manometer, and the system was closed.

The perchloric acid was allowed to drip slowly onto the magnetically stirred Ba-$^{14}CO_3$. When all the $^{14}CO_2$ was liberated, the unlabeled $BaCO_3$ was decomposed. The liberation of CO_2 under these conditions was quantitative, and the uptake was rapid. The progress of the reaction was readily followed by the initial rise and then drop in pressure observed after each addition. After all of the CO_2 was liberated, the reaction was allowed to stir at −45° for an additional 30 min., and the cooling bath was removed. When room temperature was attained, a pump[3] was used to transfer the un-

[1] Mallinckrodt/Nuclear, St. Louis, Mo.

[2] J. T. Baker Chemical Co., Phillipsburg, N. J.

[3] Toepler pump, Roder Instrument Co., Los Altos, CA 94022

reacted $^{14}CO_2$ into a flask containing a magnetically stirred, filtered solution of 16.8 g. of $Ba(OH)_2 \cdot 8H_2O$ in 300 ml. of distilled water.

The third flask, containing the Grignard complex, was cooled to −20°; 50 ml. of 6 *N* aqueous hydrochloric acid was added slowly. The phases were separated, and the aqueous phase was extracted with one 50-ml. portion of ether. The ether phases were combined and extracted with six 50-ml. portions of 1 *N* aqueous sodium carbonate. The combined aqueous phases were washed with 50 ml. of ether and then acidified by adding 75 ml. of 6 *N* aqueous hydrochloric acid. This was extracted with four 75-ml. portions of ether, which were combined, dried ($MgSO_4$), and filtered; the solvent was removed to give 1.50 g of 4-phenylbutyric-1-^{14}C acid.

The flask containing the trapped Ba-$^{14}CO_3$ was refluxed for 5–10 min. to coagulate the precipitate; it was then cooled and filtered. After drying, the recovered Ba-$^{14}CO_3$ weighed 2.5 g. (58.4% recovery), which was recycled in the above reaction, making the necessary molar adjustments. This procedure gave an additional 1.21 g. of 4-phenylbutyric-1-^{14}C acid, or a total of 2.71 g. (66%) plus the recovery of 0.3 g. of Ba-$^{14}CO_3$.

The 4-phenylbutyric-1-^{14}C acid had a specific activity[4] of 4.51 mc./mmole (theory = 4.31 mc./mmole) and was sufficiently pure to prepare III.

α-Tetralone-1-^{14}C (III)—A mixture of 2.91 g. (49.7 mmoles) of sodium chloride and 14.65 g. (109.7 mmoles) of aluminum trichloride, after flushing with nitrogen, was mechanically stirred, and the flask was introduced into a preheated oil bath at 100°. The oil bath temperature was raised to about 170° over 30 min. During this time, a dark-amber melt resulted. The oil bath was removed, but the reaction was allowed to stir until the internal temperature dropped to 110°. The 2.71 g. (16.5 mmoles) of 4-phenylbutyric-1-^{14}C acid was added in one portion, the stirring was continued, and the flask was reimmersed into the oil bath at about 145°. The temperature of the oil bath was raised to 200° over 30 min. and kept at this temperature for an additional 15 min. The black mass was cooled in an ice bath, and 33 ml. of benzene was added. The complex was decomposed by the portionwise addition of 73.9 g. of ice and 7.39 ml. of concentrated hydrochloric acid. After about 45 min. of stirring, no solids remained.

The phases were separated, and the aqueous phase was extracted with six 15-ml. portions of benzene. The benzene extracts were combined, protected from light, and dried overnight with magnesium sulfate and about 1 g. of powdered sodium carbonate. The solids were filtered, and the solvent was removed. The residual brown oil was distilled; the fraction boiling at 133°/15 mm. was collected. The α-tetralone-1-^{14}C, a pale-yellow oil, weighed 1.46 g. (61%) and was used to prepare V.

4-(2-Hydroxyphenyl)butyric-1-^{14}C Acid (V) (8)—To a magnetically stirred solution of 1.46 g. (10 mmoles) of α-tetralone-1-^{14}C in 7 ml. methanol, containing 0.54 ml (30 mmoles) of water cooled to −12° in a bath of *tert*-amyl alcohol and dry ice, was added a slurry of 4.50 g. (15 mmoles) of finely ground potassium persulfate in 3.33 ml. (60 mmoles) of concentrated sulfuric acid. The persulfate–sulfuric acid slurry was magnetically stirred and added portionwise over about 1 hr., keeping the internal temperature of the reaction between 0 and 5°. After the addition was complete, the bath was replaced with an ice bath, and the reaction was allowed to stir an additional 2 hr. at 0–5°.

The dark mass was poured over 10 g. of ice and extracted with three 25-ml. portions of chloroform. The organic phases were combined and extracted with three 10-ml. portions of 1 *N* aqueous sodium hydroxide. The combined basic extracts were allowed to reflux for 15 min., cooled, and acidified with 10 ml. of 6 *N* hydrochloric acid. This mixture was extracted with three 25-ml. portions of chloroform after the addition of 10 g. of sodium chloride. The organic phases were combined, dried ($MgSO_4$), and filtered; then the solvent was removed. The residual oil solidified when scratched to give 1.2 g. (67%) of 4-(2-hydroxyphenyl)butyric-1-^{14}C acid, which was used to prepare VI.

5-Hydroxy-α-tetralone-1-^{14}C (VI)—The cyclization of 1.2 g. (6.7 mmoles) of 4-(2-hydroxyphenyl)butyric-1-^{14}C acid was accomplished in the same manner as described for the cyclization of II to yield III. A mixture of 1.17 g. (20 mmoles) of sodium chloride and 5.93 g. (44.5 mmoles) of aluminum trichloride was used.

The reaction was allowed to decompose by slowly adding 45 g. of ice and 4.5 ml. of concentrated hydrochloric acid to the dark mass. It was necessary to break up some of the solids mechanically. After stirring for 45 min., the insolubles were filtered. The cake was triturated with 10 ml. of water, refiltered, and washed with two 5-ml. portions of water. The solid weighed 1.09 g. (100%) after drying in a pistol for 4 hr. at 61° at a pressure of 0.1 mm. The crude 5-hydroxy-α-tetralone-1-^{14}C was used to prepare VII.

5-(2,3-Epoxypropoxy)-3,4-dihydro-1(2*H*)-naphthalenone-1-^{14}C (VII)—To a solution of 1.09 g. (6.7 mmoles) of 5-hydroxy-α-tetralone-1-^{14}C in 10.9 ml. of absolute ethanol and 1.35 ml. of 6 *N* sodium hydroxide was added 9.29 g. (100 mmoles) of epichlorohydrin in one portion. This mixture was vigorously stirred at room temperature for 24 hr. The reaction was filtered, the insolubles were washed with three 2-ml. portions of ethanol, and the solvents were removed from the combined filtrates.

The brown residue was partitioned between 16.5 ml. of water and 16.5 ml. of chloroform. The aqueous phase was extracted with an additional three 16.5-ml. portions of chloroform. The combined organic phases were washed with two 7-ml. portions of water, dried ($MgSO_4$), and filtered; then the solvent was removed. The brown oil was further extracted with ten 10-ml. portions of anhydrous ether. The solvent was removed from the combined ether phases, and the residue was distilled. That fraction boiling at 132–134°/0.1 mm. weighed 0.74 g. (51%); it was a pale-yellow oil. This 5-(2,3-epoxypropoxy)-3,4-dihydro-1(2*H*)-naphthalenone-1-^{14}C was used to prepare VIII.

***dl*-5-[3-(*tert*-Butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone-1-^{14}C Hydrochloride (VIII)**—To a solution of 0.74 g. (3.4 mmoles) of VII in 7.4 ml. of ethanol was added 1.24 g. (17 mmoles) of *tert*-butylamine, and the solution was allowed to reflux for 45 min. The solvent was removed, and the residue solidified when scratched. The solid was triturated with 8 ml. of cold cyclohexane. The off-white solid, when filtered, gave 0.7 g. (71%) of the free base of VIII.

This was allowed to dissolve in 3.5 ml. of chloroform and was converted into the hydrochloride salt by allowing gaseous hydrogen chloride to bubble through the magnetically stirred solution for about 15 min. The addition of 3.5 ml. of anhydrous ether precipitated the salt, which, after filtering, was triturated with 6 ml. of cold cyclohexane. The solid was recrystallized by dissolving in 1.4 ml. boiling absolute ethanol, which was filtered; two portions of 0.35 ml. of anhydrous ether were added to the filtrate. The precipitate, when filtered, gave 0.60 g. (53.8%) of VIII. The specific activity[4] was determined to be 4.43 mc./mmole (13.5 mc./g.). This product was diluted by allowing a mixture of 0.6 g. of synthesized VIII and 1.0 g. of authentic VIII to recrystallize from 16 ml. of boiling absolute ethanol, to which was added two 4-ml. portions of anhydrous ether. The precipitate, when filtered, gave 1.3 g. of VIII which melted at 225–227°.

A UV spectrum was determined in ethanol and exhibited maxima at 221 and 254 nm., identical to an authentic sample. The absorptivity at these maxima was 72.8 and 25.9, respectively, which was 99.6% of the reference standard. An IR spectrum was determined as a mull and exhibited maxima only at the same frequencies as an authentic sample. TLC on a glass plate coated with 250 μ of silica gel GF, spotted with 200 mcg. of material and irrigated with a mixture of 9 parts chloroform (saturated with ammonium hydroxide) and 1 part methanol[6], showed the main UV-absorbing spot at an R_f of about 0.55 (identical with an authentic sample) and traces at about 0.83 (about 0.5%) and at the origin (about 0.25%). When scanned for radioactivity[6], the main spot contained 99.0% of the radioactivity on the plate, while the faster moving spot contained 0.75% and the spot on the origin contained 0.25%. This faster moving contaminant was identified as either *dl*-5,5′-[3,3′-(*tert*-butylamino)bis(2-hydroxypropoxy)]-bis[3,4-dihydro-1(2*H*)-naphthalenone]hydrochloride or the analogous *meso*-compound[7].

A gas chromatogram[5], using a 1.82-m. (6-ft.) glass column packed with 4% OV-17 on 80/100 mesh Gas Chrom Q (operated at 188°

[4] A model 3310 Packard Tri-Carb liquid scintillation spectrometer, equipped with automatic external standardization, was used. The cocktail was composed of 7.0 g. 2,5-diphenyloxazole, 0.3 g. 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene, and 100 g. naphthalene dissolved in 1 l. 1,4-dioxane.

[5] This system was developed by A. D. Lewis and M. Goodenough of these laboratories.

[6] A model 7200 Packard radiochromatogram scanner was used.

[7] This compound was synthesized by R. Novack of these laboratories.

with a helium gas flow rate of 55 ml./min.) and equipped with a flame detector, gave a single peak with an R_t of 34 min.

The specific activity was determined[4] to be 1.61 mc./mmole (4.90 mc./g.).

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Abstract □ A method is presented for the determination of *in vitro* dissolution rates for anagestone acetate, which yields results representative of *in vivo* bioavailability in animal studies. This procedure appeared to be satisfactory in preliminary screening of tablet formulations of anagestone acetate.

Keyphrases □ Anagestone acetate—formulation effects of progestational proliferation, rabbit uterus, oral administration □ Dissolution rates, *in vitro*, anagestone acetate—correlated to *in vivo* bioavailability, rabbits □ Formulation, effect on progestational proliferation, rabbit uterus—preliminary tablet screening of anagestone acetate

Even though tablet disintegration time is widely used, it has inherent defects which limit its usefulness in screening tablet formulations. Various reviews (1, 2) emphasized that the length of time required for a tablet to disintegrate *in vitro* cannot be taken as a direct indication of the time required for *in vivo* availability. Much of the literature suggests that better correlations are obtained using *in vitro* dissolution-rate studies; however, no single dissolution test can be applied to all drugs. The possibility that a single dissolution test can be applied to drugs having similar physicochemical properties remains to be established.

In an effort to develop tablet formulations containing anagestone acetate[1] that would allow ease of formulation and yet ensure maximum bioavailability, a correlation has been utilized between dissolution-rate studies and a physiological test in rabbits depending upon the drug's presence *in vivo* at its site of action. This paper reports the results of these studies for tablets prepared by varying the manner in which the drug was added to the tablet formulations.

EXPERIMENTAL

Test Products—Three different formulations were studied initially. Formulation A was prepared by incorporating anagestone acetate into the tablet formulation as a solution in methylene chloride. Formulation B was prepared by adding micronized anagestone acetate to the tablet formulation in the dry form. Formulation C was prepared by incorporating anagestone acetate powder (30 mesh) into the tablet formulation in the dry form.

Two additional test formulations, D and E, were prepared in the same manner as that described for Formulation A. Formulation D was identical to Formulation A in all respects. Formulation E differed from Formulation D in that it contained 10% starch, whereas Formulation D contained 22% starch.

All the tablet formulations contained the same amount of anagestone acetate (2 mg.), and each tablet weighed 100 mg.

The weight, hardness, and thickness of these formulations were essentially the same, and the disintegration times (with disks) ranged from 4 to 6 min. for all five formulations.

Dissolution Apparatus—An assembly similar to that described by Levy and Hayes (3) was used in the dissolution-rate studies. The apparatus was modified slightly by placing a coarse screen[2] 2.54 cm. (1 in.) above the bottom of the 400-ml. Griffen beaker. A four-blade 5-cm. diameter metal stirrer, attached to a stirring motor affording precise speed control, was used. Due to the very low aqueous solubility of anagestone acetate, a dissolution medium of 25% *tert*-butanol in water was employed to improve the "sink" conditions of the system. Three hundred milliliters of this dissolution medium was placed in the beaker, and the system was equilibrated at 37°. The stirrer was immersed in the dissolution medium to

[1] Anagestone acetate, a progestational agent, is 6α-methyl-4-pregnen-17-ol-20-one acetate, supplied by the Organic Chemistry Division of Ortho Research Foundation.

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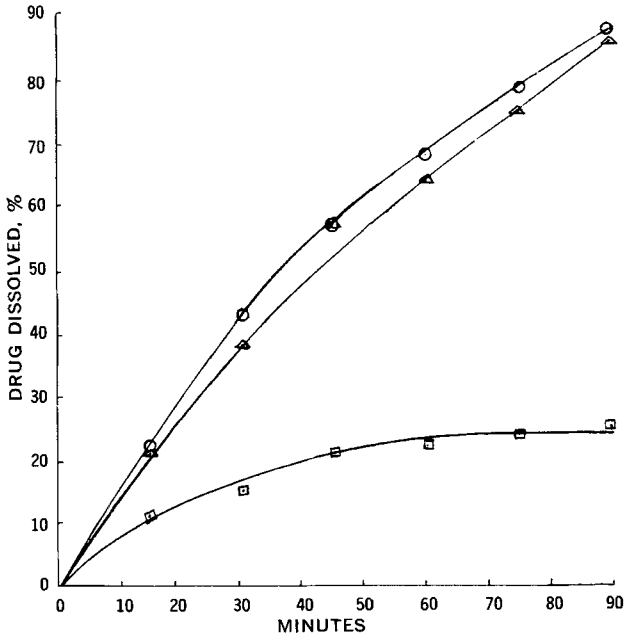

Figure 1—*Dissolution of anagestone acetate from tablets. Key:* ○, *Formulation A;* △, *Formulation B; and* □, *Formulation C.*

a depth of 25 mm., carefully centered, and rotated at 45 r.p.m. Six tablets of the test drug were placed in the beaker; 1 ml. of the fluid was withdrawn at specified intervals for analysis, using a pipet wound with glass wool at the tip, and replaced with 1 ml. of fresh fluid preheated to 37°.

Method of Drug Analysis—The dissolution samples were analyzed by GC (4)[3]. A 0.9-m. (3-ft.) column with 3% diethylene glycol succinate was used isothermally at 215°. Dissolution aliquots were injected directly into the chromatograph, and measurements were made using an internal standard technique.

Test of *In Vivo* Availability in Rabbits—A physiological test specific for the detection of *in vivo* progestational activity of the administered steroid was utilized to estimate the bioavailability of anagestone acetate when administered orally in the test formulations. The progestational steroids caused proliferation of the uterine endometrium; the bioassay, using the McPhail method (5), involving the induction of uterine endometrial changes in estrogen-primed immature rabbits, was used to evaluate relative potency of various formulations. This test is the most satisfactory and meaningful technique for evaluation of progestational activity, and can be used for quantitative comparisons (6). The standard error of this procedure is about ±20%.

The drug was administered over 5 days to nonfasting rabbits. Five animals were treated at each of three dose levels. The animals were sacrificed at the end of the test, and the histological examination was performed on the uterus. The uterine sections were graded for the degree of progestational proliferation according to McPhail (5). These data were not subjected to statistical analysis.

RESULTS AND DISCUSSION

Figure 1 illustrates that the dissolution rate of Formulation C was slower than that of the other formulations and that Formulations A and B had virtually identical dissolution rates in the system employed. From these data, it can be concluded that the particle size of the drug affected dissolution rate. Since both Formulations A and B exhibited similar dissolution profiles and Formulation A had the easier manufacturing process, it was tentatively decided that the addition of drug to the tablet formulation as a solution in methylene chloride (Method A) was the procedure of choice.

The three formulations studied eventually were judged unsuitable for manufacture, since they tended to produce a soft tablet. It was found that reducing the starch concentration in Formulation A from 22 to 10% (Formulation E) produced a tablet of the desired hardness and yet the dissolution profile remained identical to the one shown for Formulation A.

The five test formulations were subjected to *in vivo* evaluation in rabbits. Table I summarizes the endometrial response (McPhail index) for the test products at three dosage levels. The four formulations (A, B, D, and E) that exhibited similar rapid dissolution rates also exhibited similar *in vivo* activity, whereas virtually no activity was observed when the slower dissolving Formulation C was administered. This finding correlates well with the observed rate of dissolution for this formulation.

The observed correlation of dissolution patterns wth biologic (endometrial) response (in rabbits) seems to suggest that dissolution rates determined in a 25% *tert*-butanol–water system might be used as a guide in the development of tablet formulations for anagestone acetate. Additional work is in progress to determine the applicability of the procedure for related steroidal compounds.

Table I—Progestational Proliferation of Rabbit Uterus after Oral Administration

Formulation	Dose Level, mg.	Endometrial Response, McPhail index (5)
A	0.05	0.6
	0.10	1.8
	0.20	2.1
B	0.05	0.3
	0.10	1.1
	0.20	2.6
C	0.05	0
	0.10	0.1
D	0.05	0.6
	0.10	2.7
	0.20	3.5
E	0.05	0.0
	0.10	2.7
	0.20	3.0

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[3] An F&M model 5750, equipped with dual-flame detector system, was utilized.

Use of General Partial Fraction Theorem for Obtaining Inverse Laplace Transforms in Pharmacokinetic Analysis

Keyphrases □ Pharmacokinetic theory—inverse Laplace transforms, use of general partial fractions □ Laplace transforms, inverse—use of general partial fraction theorem

Sir:

Many mathematical methods have been employed to obtain solutions to pharmacokinetic models. Among these are the classical methods of solving differential equations and using Laplace transforms, eigenvectors, and eigenvalues. During the past few years, the use of Laplace transforms has proved to be the simplest method when dealing with linear models. Although many models have been described using the Laplace derivation, the very simple general methods (1) for solving these derivations have been overlooked by most workers. This communication discusses the general partial fraction theorem, shown in Eq. 1, and illustrates how it is used to obtain inverse transforms in pharmacokinetic analysis.

If the quotient of two polynomials, $P_{(s)}/Q_{(s)}$, is such that $Q_{(s)}$ has a higher degree and contains the factor $(s - \lambda_i)$, which is not repeated, then:

$$L^{-1}\left\{\frac{P_{(s)}}{Q_{(s)}}\right\} = \sum_{i=1}^{m} \frac{P(\lambda_i)}{Q'(\lambda_i)} e^{\lambda_i t} = \sum_{i=1}^{n} \frac{P(\lambda_i)}{Q_i(\lambda_i)} e^{\lambda_i t} \quad \text{(Eq. 1)}$$

where:

λ_i's = roots of the polynomial $Q_{(s)}$
$Q'(\lambda_i)$ = derivative of $Q_{(s)}$ with the roots substituted for s
$Q_i(\lambda_i)$ = value of the denominator when λ_i is substituted for all the s terms except for the term originally containing λ_i, this term being omitted
s = standard notation used in Laplace operations

The first sum in Eq. 1 is often called Heaviside's expansion (1).

Application of this technique to compartmental analysis is shown with respect to the two-compartment model for a drug and its metabolite in Scheme I. If k_{24} is assumed to be zero, the Laplace transforms for the amount of drug in Compartment 1 (x_1) following an intravenous bolus injection of the drug is described by Eq. 2:

$$x_1 = \frac{(s + k_{21})D}{(s + \alpha)(s + \beta)} = \frac{P_{(s)}}{Q_{(s)}} \quad \text{(Eq. 2)}$$

where:

D = dose injected
$\alpha\beta = k_{21}k_{13}$
$\alpha + \beta = k_{12} + k_{21} + k_{13}$

Equation 2 may easily be derived using determinants (2), and the solution may be found in any inverse Laplace transform table. Thus, the general methods can be appropriately demonstrated for this familiar equation.

Heaviside's Expansion (First Sum in Eq. 1)—The roots of the polynominal, $Q_{(s)}$, obtained from Eq. 2 are $\lambda_1 = -\alpha$ and $\lambda_2 = -\beta$. Now the first derivative of $Q_{(s)}$ is given by: $Q_{(s)}' = 2s + \alpha + \beta$. By solving for the amount of drug in Compartment 1 (X_1) using Heaviside's expansion:

$$X_1 = \sum_{i=1}^{2} \frac{P(\lambda_i)}{Q'(\lambda_i)} e^{\lambda_i t} = \left\{\frac{D(k_{21} - \alpha)}{-2\alpha + \alpha + \beta}\right\} e^{-\alpha t} + \left\{\frac{D(k_{21} - \beta)}{-2\beta + \alpha + \beta}\right\} e^{-\beta t} \quad \text{(Eq. 3)}$$

which reduces to the familiar answer:

$$X_1 = \frac{(k_{21} - \alpha)D}{(\beta - \alpha)} e^{-\alpha t} + \frac{(k_{21} - \beta)D}{(\alpha - \beta)} e^{-\beta t} \quad \text{(Eq. 4)}$$

General Partial Fraction Theorem (Second Sum in Eq. 1)—The roots of the polynomial, $Q_{(s)}$, are $\lambda_1 = -\alpha$ and $\lambda_2 = -\beta$. The term $Q_i(\lambda_i)$ may be defined as follows. When:

$$i = 1 \qquad Q_i(\lambda_i) = (\lambda_1 + \beta) = (\beta - \alpha) \quad \text{(Eq. 5a)}$$

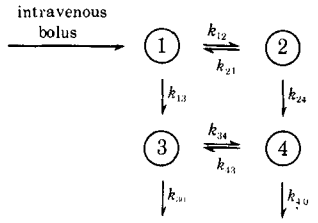
$$i = 2 \qquad Q_i(\lambda_i) = (\lambda_2 + \alpha) = (\alpha - \beta) \quad \text{(Eq. 5b)}$$

Therefore, applying the general partial fraction equation, we obtain:

$$X_1 = \sum_{i=1}^{n} \frac{P(\lambda_i)}{Q_i(\lambda_i)} e^{\lambda_i t} = \frac{D(k_{21} - \alpha)}{(\beta - \alpha)} e^{-\alpha t} + \frac{D(k_{21} - \beta)}{(\alpha - \beta)} e^{-\beta} \quad \text{(Eq. 6)}$$

The answer obtained in Eq. 6 using the general partial fraction equation is exactly that obtained in Eq. 4 using Heaviside's expansion, but the step requiring the taking of the derivative of the denominator is eliminated. The advantage of the one-step general partial fraction method might be more readily seen in the model presented by Rowland *et al.* (3) to describe the distribution of aspirin and salicylic acid following a bolus injection of aspirin as shown in Scheme I. The Laplace transform of the equation describing the amount of salicylic acid in the plasma, x_3, following a bolus injection of aspirin is given by:

$$x_3 = \frac{k_{13}(s + E_2)(s + E_4)D + A_{13}D}{(s + \alpha)(s + \beta)(s + \gamma)(s + \delta)} \quad \text{(Eq. 7)}$$



Scheme I—Compartmental model describing the distribution and elimination of a drug injected into Compartment 1. Elimination proceeds from both the central and peripheral compartments, with a metabolite, 3, undergoing analogous disposition.

where:

$E_2 = k_{21} + k_{24}$; $E_4 = k_{43} + k_{40}$; $A_{13} = k_{12}k_{24}k_{40}$

α,β = fast and slow disposition constants describing an intravenous bolus injection of aspirin

γ,δ = fast and slow disposition constants describing an intravenous bolus injection of salicylic acid (3)

A general simplified method for deriving the Laplace transform given in Eq. 7 will be published (4). At present the equation can be derived using determinants, but this derivation is not necessary to demonstrate the method of obtaining inverse Laplace transforms and it is not included here. Since there are no repeated $(s - \lambda_i)$ factors in the denominator of Eq. 7 and since the degree of s is higher in the denominator than in the numerator, the general partial fraction theorem may be used to carry out a one-step solution for the amount of drug in Compartment 3:

$$X_3 = \frac{k_{13}(E_2 - \alpha)(E_4 - \alpha)D + A_{13}D}{(\beta - \alpha)(\gamma - \alpha)(\delta - \alpha)} e^{-\alpha t} + \frac{k_{13}(E_2 - \beta)(E_4 - \beta)D + A_{13}D}{(\alpha - \beta)(\gamma - \beta)(\delta - \beta)} e^{-\beta t} + \frac{k_{13}(E_2 - \gamma)(E_4 - \gamma)D + A_{13}D}{(\alpha - \gamma)(\beta - \gamma)(\delta - \gamma)} e^{-\gamma t} + \frac{k_{13}(E_2 - \delta)(E_4 - \delta)D + A_{13}D}{(\alpha - \delta)(\beta - \delta)(\gamma - \delta)} e^{-\delta t} \quad \text{(Eq. 8)}$$

Let us review the procedure. When the factor $(s + \alpha)$ is omitted from the denominator (that is, when the root $\lambda_1 = -\alpha$ is used), all values of s in Eq. 7 are substituted by $-\alpha$ and this root appears in the exponential term $(e^{-\alpha t})$. Next, the factor $(s + \beta)$ is omitted when the root $\lambda_2 = -\beta$, *etc.* In practice, an easy way to carry out the taking of the anti-Laplace is to cover the factors in the denominator one by one with your finger while substituting the root of the covered factor for all the remaining s terms. If a single s term appears in the denominator, as when zero-order infusion equations are derived, the root for this factor is zero.

The method presented in this work is very easily used, even with complicated Laplace transform equations, so that the investigator may immediately write down the anti-Laplace without taking any derivatives and without breaking down the equation into parts that may reasonably be found in a table. A future publication (4) will contain a more extensive coverage of the method, including ways to solve anti-Laplace operations when the stated conditions of higher degree and nonrepeating factors in the denominator are not met.

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Are There Spherical Micelles?

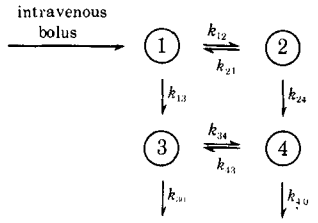
Keyphrases ☐ Micelles—nonspherical shape surfactants ☐ Surfactants—formation of nonspherical micelles

Sir:

While many shapes have been postulated for micelles in relatively concentrated aqueous surfactant solutions, it is commonly agreed that the small micelles formed at low concentrations (up to a low multiple of the CMC) are spherical (1–8). The generally accepted model for the spherical micelles is that the hydrocarbon chains of the surfactant molecules are randomly arranged in the interior of the micelle, forming a spherical core or oil droplet, and that the hydrophilic headgroups form a concentric external spherical shell surrounding the hydrocarbon core. The headgroups are hydrated and shield the hydrocarbon chains from contact with water. The core is in a liquid-like state of disorder, and the hydrocarbon chains interact with each other by van der Waals forces (1). Despite computations (4, 9) showing that spherical micelles can exist only within a limited range of aggregation numbers, interpretation of viscosity and diffusion measurements of micelles (10–12) are commonly based on a spherical shape.

The combination of simple geometric considerations with the experimentally determined micellar sizes indicates that it is unlikely that surfactants having a single normal alkyl chain as their hydrocarbon moiety form spherical micelles.

Geometric Considerations—The following parameters refer to the core of the micelle formed by the hydrocar-



Scheme I—Compartmental model describing the distribution and elimination of a drug injected into Compartment 1. Elimination proceeds from both the central and peripheral compartments, with a metabolite, 3, undergoing analogous disposition.

where:

$E_2 = k_{21} + k_{24}$; $E_4 = k_{43} + k_{40}$; $A_{13} = k_{12}k_{24}k_{40}$

α,β = fast and slow disposition constants describing an intravenous bolus injection of aspirin

γ,δ = fast and slow disposition constants describing an intravenous bolus injection of salicylic acid (3)

A general simplified method for deriving the Laplace transform given in Eq. 7 will be published (4). At present the equation can be derived using determinants, but this derivation is not necessary to demonstrate the method of obtaining inverse Laplace transforms and it is not included here. Since there are no repeated $(s - \lambda_i)$ factors in the denominator of Eq. 7 and since the degree of s is higher in the denominator than in the numerator, the general partial fraction theorem may be used to carry out a one-step solution for the amount of drug in Compartment 3:

$$X_3 = \frac{k_{13}(E_2 - \alpha)(E_4 - \alpha)D + A_{13}D}{(\beta - \alpha)(\gamma - \alpha)(\delta - \alpha)} e^{-\alpha t} + \frac{k_{13}(E_2 - \beta)(E_4 - \beta)D + A_{13}D}{(\alpha - \beta)(\gamma - \beta)(\delta - \beta)} e^{-\beta t} + \frac{k_{13}(E_2 - \gamma)(E_4 - \gamma)D + A_{13}D}{(\alpha - \gamma)(\beta - \gamma)(\delta - \gamma)} e^{-\gamma t} + \frac{k_{13}(E_2 - \delta)(E_4 - \delta)D + A_{13}D}{(\alpha - \delta)(\beta - \delta)(\gamma - \delta)} e^{-\delta t} \quad \text{(Eq. 8)}$$

Let us review the procedure. When the factor $(s + \alpha)$ is omitted from the denominator (that is, when the root $\lambda_1 = -\alpha$ is used), all values of s in Eq. 7 are substituted by $-\alpha$ and this root appears in the exponential term $(e^{-\alpha t})$. Next, the factor $(s + \beta)$ is omitted when the root $\lambda_2 = -\beta$, *etc.* In practice, an easy way to carry out the taking of the anti-Laplace is to cover the factors in the denominator one by one with your finger while substituting the root of the covered factor for all the remaining s terms. If a single s term appears in the denominator, as when zero-order infusion equations are derived, the root for this factor is zero.

The method presented in this work is very easily used, even with complicated Laplace transform equations, so that the investigator may immediately write down the anti-Laplace without taking any derivatives and without breaking down the equation into parts that may reasonably be found in a table. A future publication (4) will contain a more extensive coverage of the method, including ways to solve anti-Laplace operations when the stated conditions of higher degree and nonrepeating factors in the denominator are not met.

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Are There Spherical Micelles?

Keyphrases ☐ Micelles—nonspherical shape surfactants ☐ Surfactants—formation of nonspherical micelles

Sir:

While many shapes have been postulated for micelles in relatively concentrated aqueous surfactant solutions, it is commonly agreed that the small micelles formed at low concentrations (up to a low multiple of the CMC) are spherical (1–8). The generally accepted model for the spherical micelles is that the hydrocarbon chains of the surfactant molecules are randomly arranged in the interior of the micelle, forming a spherical core or oil droplet, and that the hydrophilic headgroups form a concentric external spherical shell surrounding the hydrocarbon core. The headgroups are hydrated and shield the hydrocarbon chains from contact with water. The core is in a liquid-like state of disorder, and the hydrocarbon chains interact with each other by van der Waals forces (1). Despite computations (4, 9) showing that spherical micelles can exist only within a limited range of aggregation numbers, interpretation of viscosity and diffusion measurements of micelles (10–12) are commonly based on a spherical shape.

The combination of simple geometric considerations with the experimentally determined micellar sizes indicates that it is unlikely that surfactants having a single normal alkyl chain as their hydrocarbon moiety form spherical micelles.

Geometric Considerations—The following parameters refer to the core of the micelle formed by the hydrocar-

bon chains of the surfactants, assuming it to be spherical: m = molecular weight of the hydrocarbon moiety of a single surfactant molecule, and v = its volume; s = surface area per hydrocarbon chain on the sphere delimiting the hydrocarbon core; M = molecular weight of the hydrocarbon core of one micelle, V = its volume, S = its surface area, R = its radius, and d = its specific gravity; z = aggregation number or number of surfactant molecules per micelle; and N = Avogadro's number.

Expressing the dimensions in Angstrom units results in:

$$V = 10^{24}zm/dN \qquad \text{(Eq. 1)}$$

and:

$$V = zv = (4\pi R^3)/3 \qquad \text{(Eq. 2)}$$

Combining Eqs. 1 and 2 results in:

$$R = 0.7346\,(zm/d)^{1/3} \qquad \text{(Eq. 3)}$$

If the hydrocarbon moiety is a normal alkyl chain containing n carbon atoms:

$$m = 14.03n + 1.01 \cong 14.03n \qquad \text{(Eq. 4)}$$

Substituting in Eq. 3 yields:

$$R = 1.772\,(zn/d)^{1/3} \qquad \text{(Eq. 5)}$$

For the hydrocarbon core of the micelle to be a sphere of uniform density without a hole in the center, R cannot exceed the extended length of the hydrocarbon chain. This length is $1.27n$ Å, neglecting the length of the C—H bond of the terminal methyl group but including the length of the bond between the carbon atom at the other chain end and the nitrogen, oxygen, or sulfur atom of the headgroup. From Eq. 5:

$$1.772\,(zn/d)^{1/3} \leqslant 1.27n \qquad \text{(Eq. 6)}$$

$$z \leqslant 0.368dn^2 \qquad \text{(Eq. 7)}$$

The values for the specific gravity of the hydrocarbon cores are estimated from the effective molar volumes at 25° of the methyl group (33.3 cm.^{3}) and the methylene group (16.2 cm.^{3}). These volumes were calculated from the densities of liquid n-alkanes (13). The specific gravities were used to calculate the maximum values for z consistent with spherical micelles by means of Eq. 7 (Table I).

Comparison with Experimental Micellar Sizes—Figure 1 shows the experimentally determined aggregation numbers for micelles of surfactants which have a single normal alkyl chain as their hydrocarbon moiety. The values for ionic (6, 7) and nonionic (14, 15) surfactants refer to dilute surfactant solutions in water, with no added electrolytes. The size of ionic micelles is often increased by the addition of salts as the surfactant solubility is lowered (7). Only those nonionic surfactants with cloud points at least 40° above the temperatures of the micellar molecular weight determinations are included, because the size of nonionic micelles increases rapidly with temperature once the temperature approaches the point of macroscopic phase separation (14, 15).

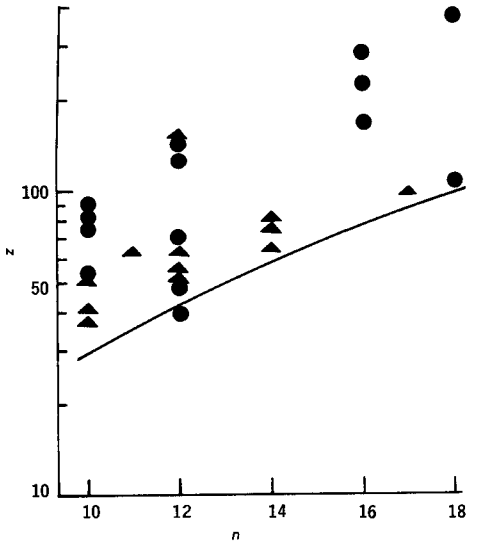

Figure 1—*Aggregation numbers,* z, *of surfactants* versus *the number,* n, *of carbon atoms in the normal alkyl moiety. Key:* ▲, *ionic surfactants; and* ●, *nonionic surfactants. Points above the curve refer to nonspherical micelles.*

Light scattering was frequently employed to determine z. In the case of ionic surfactants without swamping electrolyte, the reported values (7) are probably low (16). Ultracentrifugation, diffusion with viscosity measurements, and osmotic pressure measurements were also employed (6, 7, 14, 15). Only those ultracentrifugation data obtained without supporting electrolyte are included in Fig. 1.

The maximum z values consistent with spherical micelles, calculated according to Eq. 7, are shown in Fig. 1 together with the experimentally observed z values. The observed aggregation numbers are generally larger, indicating that the corresponding micelles should not be spherical. If these micelles were spherical, some of the polar headgroups would have to be buried inside the hydrocarbon core, out of contact with water, which is energetically unfavorable. Alternatively, there would be a hole in the center of the micelle, which is worse.

Further Geometric Considerations—Another way to consider this problem is to calculate the value of s for a spherical micelle:

$$s = S/z = (4\pi R^2)/z \qquad \text{(Eq. 8)}$$

Combining with Eq. 5 gives:

$$s = 39.46(n^2/d^2z)^{1/3} \qquad \text{(Eq. 9}$$

Combining with Eq. 7 gives:

$$55.08/d \leqslant s \qquad \text{(Eq. 10)}$$

The values of the specific gravities of Table I indicate that the area per hydrocarbon chain on the perimeter of the oil droplet exceeds 67–70 Å^2, which is over twice the limiting area per molecule of sodium dodecyl sulfate at the air–water interface. Therefore, in spherical anionic micelles, the polar headgroups shield the hydrocarbon cores only incompletely. This results in an extensive hydrocarbon–water interface, which is energetically unfavorable.

Table I—Maximum Values for the Aggregation Number of Micelles of *n*-Alkyl Surfactants Consistent with Spherical Shape

n	$d_{25°/25°}$[a]	$z_{max.}$[b]
10	0.791	29
12	0.802	42
14	0.811	58
16	0.818	77
18	0.823	98

[a] Of $CH_3(CH_2)_{n-1}$. [b] From Eq. 7.

Effect of Hydrocarbon Compressibility—If micelles have radii greater than the extended hydrocarbon chain length, spherical shape implies that some of the polar headgroups are buried inside the hydrocarbon core or that there is a hole in the center of the micelle, provided that the specific gravity of the core is that listed in Table I for atmospheric pressures. The possibility is now examined that compression of the oil droplet which constitutes the hydrocarbon core, resulting from its small radius of curvature, is capable of closing that hole.

According to the Laplace equation, the pressure within the spherical oil droplet exceeds the external pressure by a difference:

$$\Delta P = 2\gamma/R \qquad \text{(Eq. 11)}$$

where γ is the interfacial tension. The compressibility coefficient is defined as:

$$\beta = -(\Delta V/\Delta P)/V_0 \qquad \text{(Eq. 12)}$$

For surfactants derived from *n*-dodecane, $R_{max.} = (12)(1.27) = 15.24$ Å $= 15.24 \times 10^{-8}$ cm. By setting the oil–water interfacial tension at 50 dynes/cm., $\Delta P = 6.56 \times 10^8$ dynes/cm.[2] according to Eq. 11. For higher *n*-alkanes at low pressure, β is of the order of 100×10^{-6} atm.$^{-1}$ or 10^{-10} cm.[2]/dyne (13). By Eq. 12, $\Delta V/V_0 = -0.0656$; *i.e.*, the volume decreased and the specific gravity increased by 6.56%. The higher specific gravity is (0.802)(1.0656) = 0.855. By Eq. 7, the maximum value of z is now 45. This still falls short of the observed association numbers (7, 14, 15).

These estimates represent the upper limit. Actual compressions are certainly smaller because of the following reasons:

1. In selecting the values of γ and β, the fact that the outer ends of the hydrocarbon chains are connected to hydrophilic groups was neglected.

2. The Laplace equation was derived for the situation where R is large compared to the thickness of the interfacial layer (17). In the present situation, this is not true, so that ΔP is probably smaller.

3. The repulsion between ionized headgroups (18) or the crowding of polyoxyethylene chains reduces the core density somewhat, so that the specific gravity values of Table I are slightly high.

The conclusion for the surfactants examined, namely, those which have a single normal hydrocarbon chain for hydrophobic moiety, is the following: With very few exceptions, experimentally determined aggregation numbers of small micelles are too high to be consistent with spherical shape.

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Structure and Antimalarial Activity o Aminoalcohols and 2-(*p*-Chlorophenyl)-2-(4-piperidyl)tetrahydrofuran

Keyphrases □ Aminoalcohols—structure–antimalarial activity relationships □ 2-(*p*-Chlorophenyl)-2-(4-piperidyl)tetrahydrofuran—structure–antimalarial activity relationships □ Antimalarial agents—structure–activity relationships of aminoalcohols and 2-(*p*-chlorophenyl)-2-(4-piperidyl)tetrahydrofuran

Sir:

A series of substituted tetrahydrofuran derivatives synthesized by Marxer (1, 2) showed some interesting antimalarial activity (3, 4). The most active member of this series is 2-(*p*-chlorophenyl)-2-(4-piperidyl)tetra-

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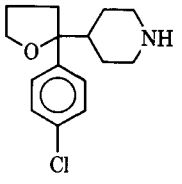

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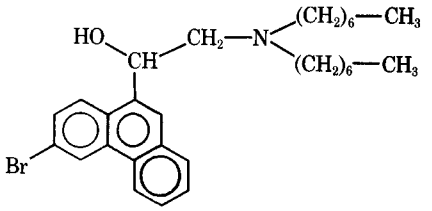

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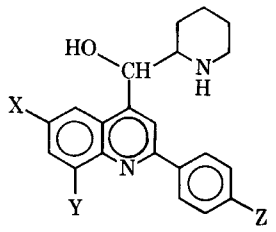


III

hydrofuran (I). Compound I has a marked antimalarial action against blood-induced infection with *Plasmodium berghei berghei* N strain in mice. It is also active against strains of *P. berghei* resistant to chloroquine, mepacrine, primaquine, cycloquanil, and dapsone, as well as against infections with *P. berghei yoelii* (Nigeria) in mice.

A preliminary structure *versus* antimalarial activity relationship study (4) of compounds of this series disclosed that: (*a*) the antimalarial activity is decreased when the piperidyl nitrogen atom is substituted, (*b*) change of the position of the piperidyl nitrogen from 4 to 2 or 3 decreases the activity, (*c*) no activity is found when the piperidine ring is replaced by a pyridine ring, and (*d*) decrease in activity results when the chlorine atom on the benzene ring is replaced by hydrogen, methyl, or methoxy groups.

The structure–activity pattern of compounds of Type I bears a close resemblance to that of another series of antimalarials—the aminoalcohols (5) [for example, compounds of Type II (6) and compounds of Type III (7, 8)]. Each series of compounds is composed of three parts: a planar (aromatic or heteroaromatic) ring, an oxygen-containing portion, and a nonplanar nitrogen-containing portion.

Examination and comparison of molecular models of these compounds revealed that the distances between the oxygen atom, the nitrogen atom, and the aromatic ring (the ring to which the side-chain substituent is attached) are approximately the same in both series under certain conformations. By using Dreiding molecular models (9–11), when the center ring of II or the pyridine ring of III is placed directly over the benzene ring of I and the carbon atoms attached to the rings are superimposed on one another, the oxygen and the nitrogen atoms of either II or III can be turned along their axes in such a way that they can be superimposed on the oxygen and the nitrogen atoms of I, respectively. The same relationship naturally exists when the phenanthrene ring or the quinoline ring of II or III is replaced with other planar aromatic or heteroaromatic ring systems. It is possible that compounds of both series, although displaying antimalarial activity of a different nature, might bind to the same pertinent receptor sites of certain biopolymers of malarial parasites. In this regard, it would seem that, since all of these compounds contain planar ring systems as their components, the antimalarials may intercalate with the parasite deoxyribonucleic acid double helix. Intercalation with deoxyribonucleic acid by other groups of antimalarials [chloroquine and its analogs (12–24) and primaquine and its analogs (25, 26)] is well known and has been investigated in detail.

A close study of Dreiding (9–11) and Briegleb–Stuart (27, 28) molecular models of these compounds revealed that the oxygen and the nitrogen atoms are in close proximity to each other in these molecules and that they are linked by hydrogen bonding (the H-atom can be contributed by either O or N) to form a five-membered ring, as shown in IV*a* or IV*b* (n = 1). The interatomic distance between the oxygen atom and the nitrogen atom and the distance of each atom to the center of the aromatic ring (to which the side chain is attached) were measured and is presented in Scheme I.

From the aforementioned information, it can be readily visualized that:

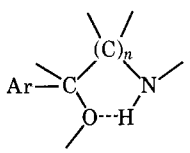
1. Antimalarial activity is abolished when the piperidine ring on the side chain of Compound I or III is replaced by a planar pyridine ring (7, 8, 29).

2. In the case of Compound III, the interatomic distance between N and O remains the same when these two atoms are chemically bound through a —CH_2— linkage. Larger substituents on the nitrogen atom sterically distort the shape of the original five-membered ring, which may affect the antimalarial activity. The nitrogen atom of Compound II is not affected by this restriction since it is attached to free-rotating aliphatic chains.

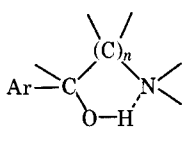
3. Insertion of a —CH_2— linkage between the carbinol carbon and the nitrogen atom transforms the five-membered ring into a six-membered ring (IV, n = 2). However, the interatomic distance between N and O remains approximately the same, which does not affect the antimalarial activity[1].

4. Reduction of the planar aromatic or heteroaromatic ring markedly affects the antimalarial activity (30, 31) since intercalation with deoxyribonucleic acid would be less probable with nonplanar rings.

5. π-Deficient heterocyclic ring systems such as quinoline or electron-withdrawing groups (CF_3, Br,

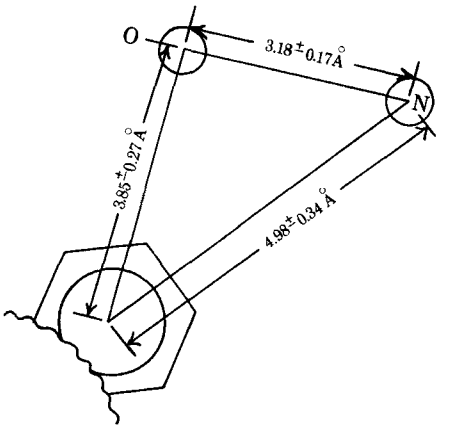


IV*a*



IV*b*

[1] Unpublished data from Midwest Research Institute laboratory.



Scheme I

Cl, *etc.*) substituted on the aromatic rings may act as acceptors in the formation of charge-transfer complexes with deoxyribonucleic acid; hence, they contribute to antimalarial activity.

The preceding account, which may also be intercorrelated with quinine (32, 33) and related cinchona alkaloids, is subject to confirmation or correction by molecular orbital calculations and other studies. The general approach may be regarded as a working hypothesis in studying the mode of action of certain antimalarials and in designing more effective agents for malarial chemotherapy.

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INDEX TO AUTHORS
INDEX TO SUBJECTS

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REVIEW ARTICLE

Aspects of Nitrate Ester Metabolism

M. H. LITCHFIELD

Keyphrases ☐ Nitrate esters—review ☐ Glycerol trinitrate—metabolism, pharmacology ☐ Pentaerythritol tetranitrate—metabolism, pharmacology ☐ Ethylene, propylene glycol dinitrates—metabolism, pharmacology ☐ Vasodilators—nitrate esters ☐ Metabolism—nitrate esters

Nitrate esters are those compounds formed by the nitration of alcohols and contain the chemical grouping —C—O—NO_2, with the nitrogen linked to the carbon atom *via* an oxygen atom. The best known ester is glycerol trinitrate (I)[1], which is representative of nitrates of polyhydric alcohols widely used in medicine and as explosives since the mid-nineteenth century. In medicine, they are used as drugs in the treatment of angina pectoris; in this context, they are still of primary importance, even though it is now over 100 years since Brunton described their pharmacological effects (1). The principal uses of nitrate esters in the explosives industry are in the manufacture of dynamite, which contains I as one of its main ingredients together with ethylene glycol dinitrate (II) when antifreeze properties are required. Personnel engaged in the manufacture of these explosives can be exposed to the vasodilator actions of the esters which can enter the body *via* the lungs or skin.

In view of the widespread therapeutic use of nitrate esters and the hazards arising from occupational exposure, they have been subjected to detailed pharmacological investigations in man and in animals. Several reviews on this subject appeared in recent years (2–9). Although their metabolism has been studied for nearly 100 years, no review has been devoted entirely to the subject. Two reviews (10, 11) in the mid-1940's included some discussion of metabolism as it affected toxicity and pharmacology.

Studies of the metabolism of nitrate esters developed originally from the efforts of earlier investigators to establish a relationship between their breakdown *in vivo* and the physiological effects produced. Particular interest centered on the formation of inorganic nitrite, since the physiological responses from nitrate esters, such as the ability to lower blood pressure, are very similar to those of the nitrite ion. As early as 1883, Hay (12) showed that when I was incubated with cat blood for 30–40 min., the ester was completely broken down and inorganic nitrite formed. Although his analytical method for showing this result was rather crude, subsequent studies confirmed this basic action of blood and other tissues on nitrate esters. The metabolism in blood was shown to occur almost wholly in the red cell, since separated plasma showed no power of breakdown of I while the erythrocytes had a greater activity than whole blood (13). Pentaerythritol tetranitrate (III) and erythritol tetranitrate (IV) also liberate nitrite when incubated with blood, and they produce this entity in blood when administered orally to dogs (14). The rates of formation of nitrite differ markedly, however, being slowest with III and fastest with IV.

It is evident that inorganic nitrite produced from the breakdown of nitrate esters is itself metabolized, as was indicated by very early studies. Leech (15) and Cash and Dunstan (16) showed that after an injection of sodium nitrite into animals, a large proportion appeared as

[1] Roman numerals in parentheses refer to list of nitrate esters in Table I.

Table I—Nitrate Esters

I	Glycerol trinitrate
II	Ethylene glycol dinitrate
III	Pentaerythritol tetranitrate
IV	Erythritol tetranitrate
V	Mannitol hexanitrate
VI	1,2-Glycerol dinitrate
VII	1,3-Glycerol dinitrate
VIII	Glycerol mononitrate
IX	Pentaerythritol[a]
X	Pentaerythritol mononitrate
XI	Pentaerythritol dinitrate
XII	Pentaerythritol trinitrate
XIII	Ethylene glycol mononitrate
XIV	Propylene glycol 1,2-dinitrate
XV	Propylene glycol 2-mononitrate
XVI	Propylene glycol 1-mononitrate
XVII	Isosorbide dinitrate
XVIII	2-Isosorbide mononitrate
XIX	5-Isosorbide mononitrate

[a] Not a nitrate.

nitrate in the urine. Oxidation also takes place in animal tissue homogenates where the disappearance of added nitrite is balanced by the appearance of nitrate (17).

The first investigations concerned solely with the mode of breakdown of nitrate esters without reference to pharmacological action were carried out by Oberst and Snyder (18) in 1948. They measured the rate of breakdown of I and mannitol hexanitrate (V) in liver homogenates by following the nitrite produced with time. The reaction was shown to be dependent upon hydrogen-ion concentration, with maximum activity at pH 8.4. In later studies, Heppel and Hilmoe (19) found that I reacted with reduced glutathione to liberate inorganic nitrite and that the reaction was catalyzed by a hog liver enzyme. These investigations were the forerunners of the much more detailed studies of the reaction mechanisms and metabolism of nitrate esters that have taken place in recent years.

METABOLISM

Glycerol Trinitrate (I)—TLC analysis of ether extracts from the reaction of I in liver homogenates and from the urine of rats dosed with I demonstrated the formation of 1,2- and 1,3-glycerol dinitrates (VI and VII) (20). *In vivo*, the course of metabolism can be followed by analyzing at timed intervals the pooled urine from rats dosed with I. After 6 hr., I is present along with larger amounts of the two dinitrates and a trace of glycerol mononitrate (VIII). After 24 hr., I is almost absent and the dinitrates are the major metabolites; after 30 hr., excretion appears almost complete since none of the nitrates can be detected. The formation of the more water-soluble dinitrates from I is another example of a classical metabolic reaction whereby a relatively lipid-soluble compound is converted to more polar derivatives.

In glutathione–liver enzyme-catalyzed reactions, 90% of the nitrate groups enzymically degraded in the I molecule can be accounted for by the appearance of VI, VII, and inorganic nitrite. From these studies, it seemed that the metabolism of I did not proceed significantly beyond the removal of one nitrate group, since VIII was detected only in trace amounts and glycerol not at all. It was possible that since nitrate assays were used for the detection of metabolites on TLC plates, a completely denitrated product such as glycerol would be overlooked. In fact, more recent studies (21) showed that glycerol is a product of the *in vivo* breakdown of I. After oral administration to rats, the ester is quickly absorbed and rapidly degraded to carbon dioxide. Combined radiochemical and TLC methods demonstrate that over half the I is absorbed from the GI tract within 30 min. of dosing and that maximum tissue levels of ^{14}C are attained in the same time. Within 4 hr., approximately equal amounts (20%) of ^{14}C are exhaled as CO_2 in the expired air and appear as metabolites in the urine. No unchanged I is detected, and it is apparent that degradation to CO_2 must proceed *via* denitration to glycerol, the principal urinary metabolite.

The extraction of tissues reveals an accumulation of nonextractable material as the breakdown proceeds, and it appears that glycerol enters normal metabolic processes leading to the deposition of ^{14}C-glycogen and, possibly, lipids. This observation was followed up with studies on the incorporation of ^{14}C from ^{14}C-I into rat liver glycogen, lipid, protein, and RNA (22). These were assayed 1 hr. after ^{14}C-I administration, when nonextractable material was shown to be maximal. Levels of liver lipid, glycogen, protein, RNA, and DNA are unaffected, but radioactivity is present in all components. The results of this study indicate that glycerol produced from I metabolism enters a variety of normal anabolic processes.

Part of the I metabolism takes place in blood since incubation with rat serum denitrates 76% of the ester in 1 hr. (23). Twice as much VII is produced as VI, a finding that is the reverse of *in vivo* results in urine (21). The pH maximum for the reaction is 7.8, and at 37° the activity is only 70% of that in the optimum temperature range at 50–57°. In rat plasma, I is bound to protein to the extent of 60%, as is VI, while VII shows only 35% binding. Therefore, the binding could be attributable mainly to adjacent nitrate groups (24).

Pentaerythritol Tetranitrate (III)—Cass *et al.* (25) predicted that III would denitrate during metabolism to give products containing one to three nitrate groupings. This prediction was amply confirmed by the extensive studies of Di Carlo and his coworkers on the metabolism of this compound. They realized that many previous studies on the comparative pharmacology and metabolism of nitrate esters relied only upon the measurement of liberated inorganic nitrate or nitrite and that little attempt had been made to find what other metabolites were formed. Using ^{14}C-III and TLC and radioscanning (26), they separated and determined III and the metabolites pentaerythritol (IX), pentaerythritol mononitrate (X), pentaerythritol dinitrate (XI), and pentaerythritol trinitrate (XII) formed during reactions *in vitro* and *in vivo*. Compound III is denitrated in human blood *in vitro* (27) mainly to XI and XII, with small amounts of IX and X. The reaction takes place mainly in the red cells, with 62% III denitration in 1 hr., although some degradation occurs in plasma (18% in 1 hr.). The denitration occurs stepwise, with III being converted to the trinitrate faster than XI is formed from the latter. Compound III is strongly bound to plasma protein, and XII less so, while IX, X, and XI are not bound to any degree. The binding of III (85%) is much stronger than I (60%).

Single oral doses of 10 mg. III/kg. body weight were used in metabolic studies on mice and rats (28–30). Within 4 hr. of dosing, ^{14}C reaches the maximal level in blood, principally in the form of IX, X, and XI. Blood clearance is rapid due to excretion and uptake into organs, where ^{14}C levels are higher than in blood after 1 hr. Compounds IX and X are the major metabolites in the tissues, showing that degradation is further advanced than in blood. Excretion of breakdown products *via* the lungs is negligible, amounting to less than 0.3% of the total dose in both mice and rats. In the 24-hr. urine, 36% of the dose is excreted mainly as IX from mice, and 25–30% is excreted from rats in the form of IX and X in the ratio of 5:3. The urinary excretion after the initial 24 hr. is very slow, less than 60% of the dose being accounted for in 4 days from mice. The absorption and excretion of III in mice were compared directly with those of its parent alcohol IX. The former, as was shown, is absorbed slowly and is excreted as IX; the latter is absorbed quickly, and most of it is excreted unchanged within 24 hr.

In man (31) the urinary excretion of metabolites after oral administration of ^{14}C-III is similar to that found in the rat. Compounds IX and X are the principal metabolites, together with a small amount of XI. The overall recovery of ^{14}C in the 24-hr. urine is approximately 36% of the original dose. In a study of patients with a known history of coronary artery disease, it was shown that there was no difference compared with a control group in the metabolism of III regarding blood levels attained and amounts of metabolites excreted after oral doses of the ester.

An estimation of the rate of absorption of III after oral administration was made from studies where the radiolabeled ester was placed directly into the ligated stomach or intestines of starved rats (32). The biggest absorption overall takes place from the large intestine, 12.9% in 4 hr., and the smallest from the stomach, 3.7% in 4 hr. The faster rate of absorption from the large intestine is ascribed to the fact that III is readily degraded to XII by bacterial flora. Furthermore, since this ester is more water and lipid soluble than the highly insoluble III, it is able to pass more easily through the intestinal wall. Scarcely any hydrolysis of III occurs in the stomach or small intestine. Compound III is absorbed much more slowly from the gut than is I, which shows up to 100% absorption in 6 hr. (33).

The apparently greater absorption of XII indicated in these studies led to an interest in the possibility of its use as an antianginal agent in its own right. Some absorption and metabolic studies were carried out in the rat, dog, and man (34–36) in conjunction with its clinical evaluation. In all three species, the trinitrate is more rapidly absorbed than III. In the rat, the trinitrate behaves similarly to I, with 60% of the ^{14}C dose absorbed from the GI tract in 4 hr. compared with 28% after III administration. Dog and man show similarities, with a fast urinary excretion of the ^{14}C label after oral XII administration, reaching 88% in the dog and 91.5% in man after 48 hr., the principal metabolites being IX and X. These two metabolites are the main breakdown products in the rat, but some unchanged trinitrate is also found in urine. The slower rate of metabolism in the rat is indicated by the significant levels of XII present in blood 4 hr. after administration, whereas the unchanged ester is absent after 1 hr. in dog and in man.

Studies on the biotransformation of III in heart subcellular fractions (37) are especially relevant since this organ is associated with the pharmacological action of nitrate esters. Nuclear, mitochondrial, postmitochondrial, microsomal, and postmicrosomal fractions of rat heart are all active in degrading III. The tri- and dinitrates are the principal metabolites after an 18-hr. incubation of the fractions at 37°, with 10 mcg. ^{14}C-III, nicotinamide, and a $NADPH_2$-generating system in KCl phosphate buffer at pH 7.4. Similarly, incubation of III with homogenates of parenchymal or reticuloendothelial cells of mouse liver shows degradation of the ester by both fractions, although neither produces complete conversion by removal of all four nitrate groupings (38). It is claimed that the results of this study differ from other investigations which showed that the parenchymal and reticuloendothelial fractions play specific and quite different roles in metabolism of compounds by the liver.

Ethylene and Propylene Glycol Dinitrates (II and XIV)—The metabolism of these two compounds is very similar and may be reviewed together. Administration of II to rabbits leads to maximal concentrations in the blood 1–2 hr. after injection, while inorganic nitrate produced by metabolism reaches its maximum at 4–5 hr. (39). The surprising finding of this study is that inorganic nitrite could not be detected in the blood in contrast to its detection by nearly all other workers after *in vitro* and *in vivo* reactions of nitrate esters.

Clark and Litchfield (40) presented a comprehensive picture of the fate of II in the rat, with measurement of blood levels after injection, together with complementary excretion and *in vitro* studies. Compound II quickly disappears from the bloodstream, and inorganic nitrite and nitrate are formed. Ethylene glycol mononitrate (XIII) is also formed; it seems probable that in the first instance II breaks down to the mononitrate and inorganic nitrite, the latter being rapidly oxidized to nitrate, thus accounting for its own disappearance from the bloodstream and its absence from urine. Inorganic nitrate persists for a longer period in the bloodstream and, since it is stable, it is removed by the normal excretory processes. It does, in fact, form the major metabolite in the urine, accounting for nearly 60% of the original dose of II within 24 hr.

Compound XIII is also rapidly metabolized *in vivo;* after injection in rats, only a trace is excreted unchanged. During the metabolism, a small amount of inorganic nitrite is present in blood, presumably as an intermediate in the formation of inorganic nitrate which is again the major metabolite in urine. Ethylene glycol is a urinary metabolite from the *in vivo* breakdown of II and XIII, showing that complete denitration is achieved for both esters. Propylene glycol 1,2-dinitrate (XIV) is similarly metabolized in the rat (41) to give inorganic nitrite and nitrate in blood, with the latter a major metabolite in the urine. This ester, however, gives rise to two mononitrates due to partial denitration; since less than 0.5% of either is excreted in urine, it is assumed that they can be rapidly metabolized in the same way as

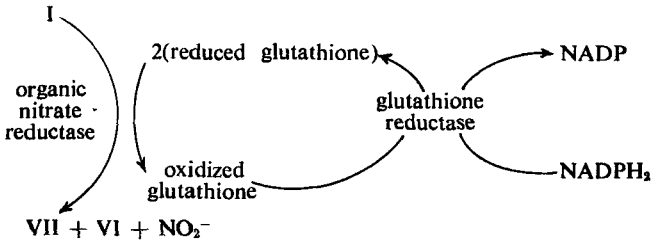
XIII. Propylene glycol 2-mononitrate (XV) predominates in the blood, and it seems likely that there is a reaction favoring the formation of this isomer.

The initial breakdown of the dinitrates to mononitrates and nitrite can be simulated in blood *in vitro*. The reaction is shown to occur entirely in the red cells, the plasma having minimal activity. Compound XIII is stable under these conditions, and it is possible to account for all the breakdown products of II *in vitro* after a 2.5-hr. incubation at 37°. Compound XIV gives rise to propylene glycol 1-mononitrate (XVI) and XV as well as inorganic nitrite. The 2-isomer again predominates as it does *in vivo;* and since both isomers are stable in blood *in vitro*, there must be a reaction favoring the formation of the former.

The metabolism of II and XIV in the rat is similar to that of I and III, since they denitrate to give mononitrates and a parent alcohol, and none of the original molecule is excreted unchanged in the urine. The fate of the carbon skeleton of II may be assessed by collation of the above results with those from the ^{14}C studies in mice (42). Excretion is rapid, 68% of the ^{14}C appearing in the first 24 hr., mainly in the urine and expired air. Volatile organic compounds account for only 2% of the activity in the expired air, the main breakdown product by this route, CO_2, amounting to 23% of the original activity. Compound II is, therefore, degraded *via* XIII to ethylene glycol, and the latter is then excreted partly unchanged in the urine and partly by complete breakdown to CO_2 in expired air. The terminal excretion and degradation of ethylene glycol is consistent with the observations of Gessner *et al.* (43).

Miscellaneous Nitrate Esters—The esters already reviewed have been the most studied because of their widespread clinical or commercial uses. Other nitrate esters have been investigated less thoroughly and usually only in comparison with the esters already discussed. In one such study with I and V, both esters were shown to form less than one nitrite ion per molecule in an enzymic reaction with reduced glutathione in rat liver mitochondrial suspensions (44). This observation substantiated previous results (18) that only 15% of the expected nitrite was produced from the reaction between V and tissue homogenates. The comparison of *in vitro* breakdown was investigated further by Needleman and Hunter (45) with incubations at 37°, employing 0.5 m*M* V, 3.1 m*M* IV, and 8.8 m*M* isosorbide dinitrate (XVII) with reduced glutathione and liver-soluble enzyme. TLC analysis of extracts shows that the three esters are degraded within 4 hr., with V and IV producing four nitrated metabolites and XVII producing one.

Similar results are found *in vivo* after intraperitoneal injection of 10 mg. of each nitrate in rats. Compound V is excreted unchanged with traces of three metabolites, while a small amount of intact IV is excreted with four clearly defined nitrated metabolites. Compound XVII is excreted primarily as one metabolite with traces of the parent compound and a second metabolite. None of the metabolites could be positively identified. Compound XVII was the subject of further studies *in vivo* in man and in the dog (46), where the orally administered ester did not appear in the 24-hr. urine. Only 1% of the dose



Scheme I—Diagram of the I transformation reaction coupled with the oxidized glutathione reductase assay system (reprinted from Reference 45)

appeared as the mononitrates, 2-isosorbide mononitrate (XVIII) or 5-isosorbide mononitrate (XIX), and it seems apparent that stepwise denitration led to a completely denitrated product.

In dogs, 1-chloro-2,3-propanediol dinitrate after oral administration is not detectable in the 24-hr. urine, but two metabolites are shown to be present by TLC on silica gel (47). These are the glucuronides, probably of the two mononitrates, formed by the partial denitration of the ester to give a free hydroxyl group available for conjugation with glucuronic acid.

MECHANISM AND SITES OF REACTION

Earlier studies showed that nitrate esters break down in tissues and that a reductive process is involved (12, 13, 18, 19), while the liberated inorganic nitrite is converted to nitrate by an oxidative reaction (15–17). One early attempt to postulate a mode of breakdown was by Krantz *et al.* (48), who suggested a reduction of the nitrate esters to the corresponding nitrite esters as the first step in metabolism, followed by hydrolysis to liberate inorganic nitrite. However, they discounted this theory after further studies, and it was left to Oberst and Snyder (18) to resurrect it as a result of their investigations with liver homogenates. The breakdown of the esters cannot be accounted for by hydrolysis to inorganic nitrate followed by reduction to nitrite, since it was shown that the latter reaction did not occur in the system investigated.

Needleman and Hunter (45), expanding upon the studies of Heppel and Hilmoe (19), showed that the reaction of nitrate esters with glutathione in the presence of the enzyme organic nitrate reductase is coupled with another enzymic reaction involving $NADPH_2$ to maintain a pool of glutathione in its reduced form. They presented the scheme shown in Scheme I; by showing that nitrite formation equaled $NADPH_2$ consumption, they were able to use the measurement of $NADPH_2$ disappearance to establish an assay system to determine the relative rates of enzymic activity with various nitrate esters as substrates. Under these conditions, V was shown to be the most actively transformed, followed by IV and then I. Compounds III, VI, and VII were shown to be transformed at comparatively very slow rates. The evidence suggests that reduced glutathione acts as a hydrogen donor for the reduction of nitrate esters, with two molecules of glutathione reacting with one of ester to liberate one molecule of inorganic nitrite. Williams (49) suggested that the overall reaction for a nitrate group may take place as follows:

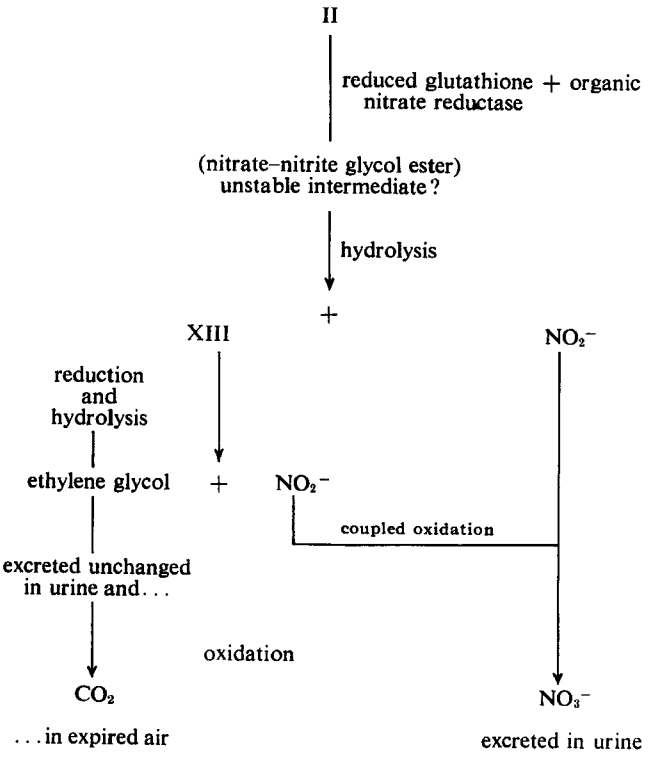
$$RO{\cdot}NO_2 \xrightarrow{+\,2H} RO{\cdot}NO \xrightarrow{H_2O} ROH + NO_2^-$$

The assumption, which was made by others (19), that an organic nitrite grouping exists before hydrolysis may be difficult to prove due to the short half-life involved, since it is known that compounds such as glycerol trinitrite decompose spontaneously in aqueous media (50). Efforts to detect a nitrite ester during studies on the metabolism of II were unsuccessful (40).

The enzyme responsible for the catalysis of the breakdown of nitrate esters was initially named "nitrite-forming enzyme" by Heppel and Hilmoe (19) and subsequently as "glycerol trinitrate metabolizing enzyme" by Needleman and Krantz (20) and then as organic nitrate reductase (45). This enzyme that catalyzes the glutathione–organic nitrate reaction was characterized (19, 20) and is listed by the Enzyme Commission under E.C. No. 1.8.6.1. as glutathione:polyolnitrate oxidoreductase (51). This enzyme is quite different from the nitroreductases, which act only under anaerobic conditions, and has been distinguished from the conjugating enzyme glutathione-*S*-alkyltransferase (20, 52). It is inconceivable, therefore, as was suggested (53), that glutathione may form conjugates with nitrate esters resulting in metabolites of the mercapturic acid type.

Organic nitrate reductase is widely distributed in mammalian tissues, but the location of the enzyme has been the subject of some debate. Studies with rat liver (44) and heart (37) showed the mitochondria to be most active in degrading organic nitrates, whereas in another study (20) the greatest activity was allocated to the 100,000×*g* soluble fraction of rat liver homogenate, with the mitochondria the least active. These differences may reflect differences in preparation of homogenate fractions and conditions of assay. Since the glutathione–organic nitrate reaction is linked to another enzymic reaction involving glutathione reductase (Scheme I), the activity of the latter enzyme presumably plays an important part in determining the activity of organic nitrate reductase. Glutathione reductase is present largely in liver and kidney and is most active in the 20,000×*g* supernatant fraction of rat liver homogenate but is almost wholly absent in mitochondria (54). The absence of a reduced glutathione-regenerating system in an assay for organic nitrate reductase could lead to the recording of low enzyme activity. The liver organic nitrate reductase system requirement for $NADPH_2$ and reduced glutathione does not always appear necessary for the reaction in other tissues. Degradation of III in heart subcellular fractions does not correlate with their glutathione content (37). Recently, it was shown that rat serum does not require $NADPH_2$ or reduced glutathione to degrade I (23). It is probable that two or more enzymes can be involved in reactions catalyzed by organic nitrate reductase. Fractions from column chromatography[2] of liver extracts from several species show two distinct peaks of enzyme activity (55), and their relative specific activities toward I, IV, and V as substrates are quite different. The possibility of two or more enzymes is also reflected by the difference between the rapid *in vivo* metabolism of XIII and its stability *in vitro* compared with the rapid breakdown of II in both systems (40).

[2] DEAE-Sephadex.



Scheme II—Metabolism of II in the rat

The oxidation of inorganic nitrite to nitrate in tissue homogenates is dependent upon the catalase content of the homogenate (17). Thus, kidney homogenate with about one-quarter of the catalase content of liver homogenate has one-quarter of the latter's oxidative capacity, and serial additions of catalase to a homogenate proportionately increase the oxidation rate of nitrite. It was demonstrated that the reaction is comparable to that shown by Keilin and Hartree (56) for the oxidation of alcohols and is catalyzed through a D-amino acid oxidase or xanthine oxidase system linked with catalase. Hydrogen peroxide formed by the oxidation is utilized by the catalase for the coupled oxidation of nitrite. The enzymic reduction of inorganic nitrate to nitrite in animal tissue is possible under anaerobic conditions (57), but aerobically this is extremely small compared with nitrite oxidation. The present picture of the reactions and possible mechanisms involved in the metabolism of a nitrate ester is depicted in Scheme II, which shows diagrammatically the breakdown of the II molecule in the rat.

ANALYTICAL CONSIDERATIONS

The determination of nitrate esters and partially denitrated breakdown products in biological materials may be classified under the headings colorimetric, TLC, and GLC analyses. In each case, the ester is extracted from the aqueous medium by an organic solvent and, after an intermediate step if necessary, submitted to the appropriate technique for its determination.

The most common procedure for colorimetric analysis of nitrate esters involves, after extraction, alkaline hydrolysis and colorimetric measurement of liberated

nitrite. This basic procedure has been used by investigators for at least the last 40 years (58–61). Ethyl ether is the solvent generally employed for extraction, and the efficiency of removal is 100% in some cases. The next steps, hydrolysis and colorimetric determination, may be most conveniently carried out on the ether extract directly (59, 61). Hydrolysis does not liberate a constant percentage of nitrite from all esters, but each ester liberates a constant proportion under standard conditions of time, temperature, and alkalinity. By varying the alkalinity, it is possible to carry out a differential hydrolysis to distinguish between two esters (60). The nitrite liberated by the hydrolysis step is measured colorimetrically by a diazotization and coupling reaction, sulfanilic acid or sulfanilimide being used as the initial agent and 1-naphthylamine or *N*-(1-naphthyl)-ethylenediamine as the coupling agent. The overall procedure provides a relatively sensitive method for those esters readily hydrolyzed, II and I being detectable at the 0.5-mcg./ml. level.

The colorimetric methods are virtually nonspecific, apart from a few cases where differential hydrolysis can be applied. This is not a disadvantage if only one ester is present, but cases arise during metabolism when mixtures of esters are present due to partial denitration of the parent nitrate ester. Chromatographic methods overcome the latter difficulty, provide adequate separation, and, at the same time, maintain sensitivity of detection. Di Carlo *et al.* (26) developed a TLC separation for III and its four metabolites, which they used as a basic procedure throughout their studies on the metabolism of III. The products of metabolism are removed from urine or tissue by extraction with ether, methanol, or dioxane. After concentration, aliquots are spotted onto 0.25-mm. silica gel G plates, and the chromatograms are developed using a toluene–ethyl acetate–butanol–water (10:5:2:2) solvent system (31). The nitrates are detected on the plates by spraying with 1% diphenylamine and subjecting to UV radiation for a few minutes with the appearance of blue spots (62). By utilizing ^{14}C-labeled III, the ester and its four metabolites can be identified and measured after TLC by a combination of radioscanning and nitrate assay. Similar methods were applied to the determination of I and the di- and mononitrate metabolites (63). TLC followed by identification with diphenylamine spray reagent has been used for urinary analysis in metabolism studies on I (20), XVII (46), and 1-chloro-2,3-propanediol dinitrate (47).

In 1960, Evered and Pollard (64) showed that nitrate esters could be separated on a GLC column, and this technique has been applied to the determination of II, XIII, XIV, XVI, XV, XVII, and I in urine and tissues (61, 65–67). Extraction procedures are simple, involving shaking the sample for a few minutes with an organic solvent and injecting microliter quantities of the extract onto a column with a 5 or 10% silicone stationary phase at a temperature between 100 and 140°. The electron-capture detector is normally used for measuring the separated components, since it is much more sensitive and selective for nitrate esters than the flame-ionization detector. It is possible to measure 0.01 p.p.m. nitrate ester in a sample by this technique, which means that the metabolism of minute doses of ester can be followed.

Inorganic nitrite and nitrate arising from the metabolism of nitrate esters can be determined by a variety of methods. Crandall (13) used the Griess–Ilosvay colorimetric procedure to measure nitrite produced by I breakdown in perfusates. Stieglitz and Palmer (68) made a thorough study of the application of this method for the determination of trace amounts of nitrite in blood. The deproteinized sample is analyzed using 1-naphthylamine and amino-G acid for color development. To attain the sensitivity needed for the measurement of the very low normal blood nitrite concentrations (about 1 mcg./100 ml.), a sample volume of 8 ml. is required. Apart from the introduction of *N*-(1-naphthyl)ethylenediamine as a faster coupling agent (69), the basic method described here has been used with only minor modifications to the present day by investigators studying the appearance of nitrite during metabolic reactions of nitrate esters (18, 19, 25, 44, 70, 71).

Although the determination of inorganic nitrite in blood, urine, and tissue homogenates has proved to be straightforward, the measurement of inorganic nitrate has not. This is due basically to the inherent difficulties of the colorimetric determination of small amounts of nitrate ion, the methods being prone to interference and lacking specificity. The methods can be divided into direct and indirect procedures, the latter depending upon the reduction of nitrate to nitrite prior to colorimetric analysis. The direct methods involve *m*-xylenol (17), phenoldisulfonic acid (72), or brucine (59) as reagents. Among reducing agents for the indirect method are zinc dust in the presence of ammonium hydroxide and a manganous chloride catalyst (73, 74) and the novel use of bacterial nitratase (75).

Automated methods are especially useful for the rapid analysis of large numbers of samples arising from metabolism studies or clinical trials. A method was described using the Technicon AutoAnalyser (76), whereby the dialysis unit separates nitrite and nitrate from blood protein and eliminates any preliminary manual deproteinization. Nitrite in a sample is first measured by a diazotization and coupling reaction, while nitrate is reduced to nitrite by a zinc column incorporated into the system prior to color formation. The recovery of added anion to blood is reproducible and almost 100%.

There should be no delay in the determination of nitrate esters and metabolites on samples submitted for analysis, since further breakdown or other changes can take place rapidly *in vitro* (61, 76).

METABOLISM AND PHARMACOLOGICAL EFFECTS

Hay (77), in 1883, recognized that the therapeutic action of I bore a strong resemblance to that of inorganic nitrites, and he suggested that the mode of action of this ester arose from its breakdown to nitrite. This assumption was soon discounted (78) on the grounds that the amount of nitrite so produced was not enough to account for the potent vasodilating power of I; another theory was put forward that the physiological action of this ester is due to the intact molecule (79). The latter observations were endorsed by later work in this field, where the administration of minute doses of 60 mcg./kg.

IV or I injected intravenously in dogs produced a marked decrease in blood pressure with no increase of blood nitrite above control values (70). Similarly, after the oral administration of III to man, the levels of blood inorganic nitrite do not correlate with physiological responses, although the interesting suggestion arising from this study is that blood nitrite levels may not reflect the intracellular concentrations where the pharmacological effects are initiated (25). It is possible that the nitrate ester acts as a "carrier" and releases nitrite intracellularly. In studies with II in the rat (40), the time course of metabolism manifest by XIII and inorganic nitrite and nitrate production was compared with recordings of blood pressure, which is depressed very rapidly and almost maximally after II injection and before any significant breakdown of the molecule can have occurred. The delayed return of the blood pressure to normal can be attributed only in some small part to the metabolites XIII and inorganic nitrite. The blood pressure response to XIV and its metabolites is very similar (41).

Attempts to link the action of nitrate esters at the cellular level with observed pharmacological effects still occupies the attention of several investigators. It was established (80) that there is a relationship between the ability of nitrate esters to stimulate mitochondrial respiration, their rate of enzyme reaction with glutathione, and their oil–water solubility. The more water-soluble nitrates, such as VIII, show little or no reaction with reduced glutathione and have no effect on mitochondrial respiration in contrast to the more lipid-soluble esters which have much greater activity in both respects. It was also shown that those esters more rapidly denitrated in the glutathione-dependent reductase reaction are the more potent vasodilators (81) and that lipid solubility is not such a good index of vasodepressor activity when the molecular configuration is such that the reaction is blocked, as with long-chain derivatives of I.

It is unlikely that the ATP–ATP-ase reaction is the target enzyme system of nitrate esters, since the correlation of vasodilator action with ATP-ase inhibition obtained on rabbit aorta could not be repeated in the uterus or skeletal muscle (82) nor in other studies (83) with coronary ATP-ase in dogs. Sodium nitrite and some nitrate esters are uncouplers of oxidative phosphorylation in rat liver mitochondria (84), with I and IV being much less effective than V. These differences are not due to differences in rates of metabolism, since the enzyme-catalyzed reaction with glutathione in mitochondrial suspensions is greater with I than with the other two nitrates (44). Since I is the more potent of these three vasodilators, any relationship between vasodilation and uncoupling of aerobic phosphorylation remains questionable.

Compounds I, II, and XIV are monoamine oxidase inhibitors (85–87), but their metabolites have no such effect.

Contradictory evidence was obtained from the limited number of studies carried out concerning the relationship between metabolism and the onset of tolerance to the repeated administration of nitrate esters. Crandall (13), using *in vitro* assays, showed that blood from dogs made tolerant to II degrades nitrate esters more slowly than controls, while Hasegawa and Sato (39), with a small number of experiments in rabbits, indicated that the metabolism of II increased in rate after repeated administration of the ester. In more detailed studies with II (88) in the rat, it was shown that the metabolism of this ester, manifest by blood levels and urinary excretion of metabolites, is unaltered after daily administration for 2–8 weeks. It was suggested that the tolerance that develops to the cardiovascular effects of these esters may be due to the changing influence of a physiological compensatory mechanism rather than to any change in the pattern of metabolism.

The production of methemoglobin due to the action of nitrate esters on blood has received limited attention. The lack of interest is shown by the fact that Bodansky (89), in a comprehensive review of compounds forming methemoglobin, did not mention nitrate esters. This effect was noted toward the end of the last century (12, 90), but it was not until the mid-1930's, when spectrophotometric methods sensitive to less than 1% methemoglobin were developed (91, 92), that the way was opened for more precise studies. Experiments with cats (93) showed II to be 10–20 times more effective than XIII and 4 times as effective as I in producing methemoglobin. By taking into account the ratio of ester given to the amount of methemoglobin formed, it was concluded that the reaction is molecular and not catalytic, as is the case with aromatic nitro compounds. The injection of an LD_{50} of XIV in the rat brings about almost complete conversion of hemoglobin to methemoglobin, and the cause of death can be due simply to anoxia brought about by the destruction of the oxygen-carrying power of the blood (41).

CONCLUDING DISCUSSION

Early studies indicated that nitrate esters break down rapidly in contact with animal tissue, with the production of inorganic nitrite. The introduction in recent years of more sophisticated methods of analysis, such as TLC and GLC, and the use of radiolabeling have facilitated the detection and determination of metabolites in addition to inorganic nitrite and nitrate. Together with automated procedures for the latter, these methods have ensured the attainment of a more comprehensive picture of the progress of metabolism by enabling several parameters to be measured collectively and quickly.

Although the production of inorganic nitrite has been universally demonstrated, Hasegawa and Sato (39, 94) claimed that none is detected after the injection of large doses of II in the rabbit. Their main evidence for the absence of nitrite devolves upon observations of the type of methemoglobin produced in the blood *in vivo* compared with that *in vitro* where II and nitrite are shown to form methemoglobins with different absorption maxima at 500 and 540 nm., respectively. It is doubtful if this criterion can be applied *in vivo*, bearing in mind the number of parameters affecting methemoglobin formation and reduction (95).

Detailed *in vivo* metabolic studies of I, III, II, and XIV show that the parent molecules are denitrated rapidly and do not appear in significant quantities in rat urine after 24 hr. Originally, it was thought that I was

resistant to further degradation after the removal of one nitrate group (20). However, recent evidence (21) shows I to have the same pattern of breakdown as the other esters, which is denitration stepwise to the parent alcohol. This process is slowest with III where the pentaerythritol so formed does not break down to carbon dioxide, as do the other alcohols, but is excreted unchanged.

In vitro, I, II, and XIV lose only one nitrate group, the reaction stopping at this point since VI and VII or mononitrates so formed are relatively stable under the experimental conditions. In contrast, III is degraded as far as XI (37), although this apparent difference may be due to the length of time the reactions were studied. In whole blood *in vitro*, III loses only one nitrate group after 2 hr. of incubation, which compares with II breakdown (40); it is probable that under strictly comparable conditions, the breakdown of the four nitrate esters *in vitro* is very similar. There is contradictory evidence concerning the fraction of blood most active in degrading nitrate esters. Several studies showed that all the activity resides in the red cells, with little or no reaction in plasma (13, 40, 41). Other investigations, however, indicate appreciable breakdown of I in rat serum and plasma (23, 24), and it was shown that III is significantly denitrated in human plasma, although to a lesser extent than in red cells (27).

Although radiochemical studies with nitrate esters can account for the fate of ^{14}C during metabolism, only 60% could be recovered in studies to account quantitatively for the fate of the nitrate groupings (40, 41). It is possible that —OH groups formed after denitration can conjugate with glucuronic acid and remove partially denitrated products by this route, although it seems that this is only a minor pathway (47). It is conceivable that ester nitrate nitrogen may be eliminated by pathways such as reduction to ammonia or elemental nitrogen, and exploratory studies using the heavy isotope ^{15}N can be envisaged.

It has been established that the metabolism of nitrate esters proceeds *via* an enzyme-catalyzed reaction with reduced glutathione, the latter acting as a hydrogen donor for the reductive process. The enzyme, organic nitrate reductase, is present in many tissues, although its subcellular location was the subject of some controversy (20, 37, 44). Needleman and Hunter (45), by establishing an assay system containing liver organic nitrate reductase and glutathione coupled with another involving $NADPH_2$ and glutathione reductase to maintain glutathione in a reduced form, were able to relate the low enzymic transformation of VI and VII with their relative stability *in vivo*. Such relationships do not necessarily hold for other nitrate esters since III and XIII, which have little or no activity *in vitro*, are both actively transformed *in vivo* (29, 40). These differences suggest that more than one reaction may be involved in their metabolism. Recently, it was suggested that rat serum contains an enzyme which will catalyze nitrate ester reduction in the absence of glutathione (23), and other evidence shows the presence of two organic nitrate reductases in liver (55).

According to current theory, the enzyme-catalyzed reduction of nitrate esters proceeds *via* the formation of a nitrate–nitrite ester, the nitrite moiety hydrolyzing to form an —OH group and releasing inorganic nitrite. The reaction proceeds on consecutive nitrate groupings until full denitration has occurred. Although this has not been substantiated experimentally, no other feasible suggestion has been put forward.

The quest for the link between metabolism and pharmacological action of nitrate esters is still the subject of many studies. All the evidence suggests that the direct action of the intact molecule is responsible for effects such as vasodilatation, and the only effect of metabolism seems to be to terminate such action. The onset of tolerance to these esters does not seem to arise from an altered metabolism (88). It is apparent that the oil–water partition coefficient (2, 80, 96, 97) plays a part in differentiating the pharmacological potency of different nitrate esters, and it is evident that the more water-soluble metabolites are not as effective as the parent ester. The more prolonged therapeutic action of III compared with I is attributed in part to its binding to nonenzymic protein molecules and demonstrates another factor contributing to differences in pharmacological action (98).

Despite the impressive evidence for the direct action of the intact molecule, it is still possible that metabolism occurring intracellularly may present a different picture from that shown by circulating blood levels. Inorganic nitrite liberated in a cell from a lipid-soluble nitrate ester may have a much more potent effect than when it is administered alone and is unable to enter cells to the same extent. Similarly, the question remains whether methemoglobin is produced by the intact molecule or by inorganic nitrite released by denitration.

The study of the metabolism of nitrate esters has slowly evolved during the last 80–90 years in a rather uncoordinated fashion. Recent investigations added greatly to the sum of knowledge and clarified many points, but they also raised new problems of interpretation of the facts. Apparent differences between the metabolism of individual esters can be resolved only by a comprehensive study of their breakdown under comparable routes of administration, dose levels, times of study, and analytical procedures and by taking into account such parameters as oil–water partition coefficients and the effects of protein binding.

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Spectropolarimetric and Circular Dichroism Methods for Determining the Activity of Penicillin

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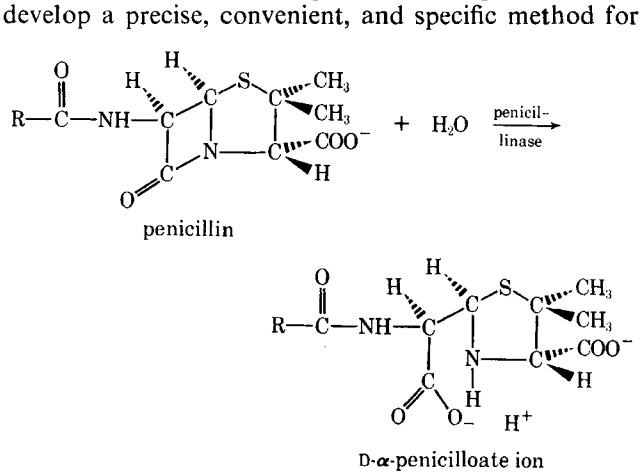
Abstract □ Experimental and mathematical analysis of an analytical spectropolarimetric method for determination of penicillins shows that this approach can yield precisions of the order of one part per thousand or better. The method, based on optical rotatory changes elicited by enzymatic catalysis, is sufficiently sensitive to detect losses in drug activity occurring in solution at room temperature in periods of less than 1 hr. Applications of the procedure to both penicillins sensitive and resistant to penicillinase catalysis and their mixtures are presented.

Keyphrases □ Penicillin, activity—determination using spectropolarimetry and circular dichroism □ Spectropolarimetry—determination, penicillin activity □ Circular dichroism—determination, penicillin activity

An interesting polarimetric assay procedure specific for penicillins susceptible to penicillinase catalysis was proposed by Levy (1) in 1951. This method, in essence, related the penicillin concentration to the changes in optical rotation of an aqueous solution of penicillin observed after hydrolysis of the β-lactam ring by penicillinase (Scheme I). The change in rotation was determined on a custom-built recording polarimeter (2), which used a mercury vapor lamp and two filters to isolate the mercury line (5460 Å). The polarimetric assay procedure never became too popular since most measurements were made in the visible region where the sensitivity was low. In addition, the change in rotation due to the mutarotation of the penicilloate ion was relatively large. The selection of a proper end-point was never determined, thus reducing the precision of this assay procedure.

The specific aim of the present investigation was to develop a precise, convenient, and specific method for the analyses of penicillins based on the use of a more sensitive, commercially available recording spectropolarimeter. Any changes in optical activity resulting from the addition of penicillinase, a specific enzyme for the hydrolysis of the β-lactam ring, to an aqueous solution of penicillin is a direct result of the action of this enzyme on the penicillin. The presence of other optically active materials has no effect on the assay, since the assay relates the change in optical activity arising from the cleavage of the β-lactam ring by this specific enzyme to the penicillin concentration.

These studies included application of the method to the analyses of both nonresistant and resistant classes of penicillin. The structural formulas of those studied and the names used in this paper are given in Table I.



Scheme I

PRESENT ASSAY METHODS

Several chemical methods are available for the analysis of penicillin (3). Among these, the iodometric and hydroxamic acid methods are the two most widely used. Results from these two chemical methods are in good agreement with microbiological data for a relatively pure substance, but poor agreement was obtained with samples containing relatively large amounts of degraded penicillin.

The principle of the iodometric method (3–5) is that the penicilloate ion takes up iodine, while there is no consumption of iodine by the intact penicillin molecule. This method also can be made relatively specific by utilizing enzymatic cleavage of the β-lactam ring. This method is not, however, applicable to penicillins, such as cloxacillin, that possess unsaturation in their side chain, since the side chain takes up iodine regardless of whether the β-lactam ring is intact (4). The iodine consumption appears to depend upon pH and iodine concentration even for systems where it is used (3). The stoichiometry of the reaction is not known exactly, but it appears that 8–9 atoms of iodine are taken up by each molecule of benzylpenicilloate (4). Because of this variation, the iodine equivalence must be determined by analyzing samples of pure penicillin under the same assay conditions to be used in assaying the unknown samples. A blank estimate also must be made.

In the hydroxamic acid method, hydroxylamine reacts with the β-lactam ring of penicillin at pH 7 to form a hydroxamic acid (3–5). Addition of ferric ions to the hydroxamic acid solution produces a color which is stable for about 5 min. (4). Different penicillins produce different color intensities (4). Hydroxylamine reacts only with the intact β-lactam ring and not with penicilloate. On the other hand, hydroxylamine reacts with other compounds, such as esters, that contain a carbonyl group.

EXPERIMENTAL

Apparatus—Measurements of optical rotation and circular dichroism (CD) were made on a spectropolarimeter and with a CD accessory[1]. It was found that a 5-cm. cell could be removed,

[1] Cary 60 recording spectropolarimeter and Cary model 6001 circular dichroism accessory.

shaken, and replaced without causing a significant variation in the spectropolarimetric reading. Because this was not true for the 1-cm. cell, the 1-cm. cell was securely fastened to the cell holder with tape; the cell holder, with the cell in it, was then shaken.

Chemicals and Reagents—*Bacillus cereus* penicillinase solutions[2] were prepared by dissolving one vial (800,000 units/vial) in 10 ml. of distilled water for the penicillinase-sensitive penicillin and one vial in 0.5 ml. of distilled water for the penicillinase-resistant penicillin. The penicillins used were potassium benzylpenicillin[3], phenoxymethylpenicillin acid[4], ampicillin B[5], and sodium cloxacillin[6].

Potassium benzylpenicillin[7] and sodium cloxacillin[8] were twice recrystallized by two different methods. In the first method, the penicillin salt was dissolved in a minimal amount of water and was precipitated by slowly adding approximately 20 volumes of 1-butanol (6, 7). The precipitate was then washed with ethyl acetate. In the second method, the penicillin salt was dissolved in a minimal amount of methanol and was precipitated by slowly adding approximately 5 volumes of ethyl acetate (6, 7). The precipitate was then washed with ethyl acetate.

Commercial grade ampicillin was once recrystallized by dissolving it in a minimal amount of 1 *N* hydrochloric acid at 70° and was precipitated by adding 2 *N* sodium hydroxide (8) at 70°. The precipitate was washed with boiling ethyl acetate.

Reagent grade potassium phosphate, monobasic and dibasic, sodium acetate, acetic acid, sodium carbonate, sodium hydroxide, and hydrochloric acid were used to prepare the buffer solutions.

Procedure for Penicillinase-Sensitive Penicillin Using Optical Rotation—An aqueous solution of the penicillin to be studied was prepared by rapidly dissolving 0.10–0.15 g., accurately weighed, of penicillin in 500 ml. of 0.1 *M* phosphate buffer solution, pH 7.0. A 5-cm. cell was filled with this aqueous penicillin solution and placed in the spectropolarimeter. The assay was carried out at a constant wavelength of 255 nm. (or 247 nm.), with a spectral bandwidth of 40 Å (slitwidth of 2.4 mm.), a chart speed of 25 sec./division, and usually a full range scale of 0.4 deg. The pen center was set at zero, and the pen was adjusted with the manual zero and zero suppression to the 1.0 line on the chart paper.

With the filled cell in place, the rotation was recorded for 1–2 min. Then 50 μl. of penicillinase solution (80,000 units/ml.) was rapidly injected into the cell, which was shaken and then replaced, and the recording was reinitiated.

Since spontaneous degradation of penicillin begins as soon as an aqueous solution is prepared, the time between the preparation of the solution and the assay was recorded. The timing was started as soon as the penicillin was dissolved and recorded when the cell was shaken. A kinetic curve of the rapid drop in rotation *versus* time was recorded (Fig. 1). When the pen reached zero on the chart paper, the pen center was switched from zero to 1.0 so that the change in rotation covered twice the width of the chart paper. The pen continued to drop rapidly until all the penicillin was degraded by the penicillinase. This was followed by a slow reduction in the rotation of the solution.

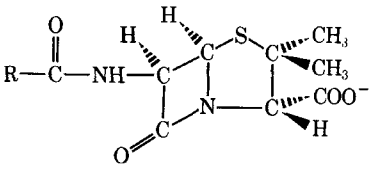
After each assay, the cells were filled with 3 *N* nitric acid and allowed to stand for a few minutes to inactivate the residual enzyme.

Procedure for Penicillinase-Sensitive Penicillin Using CD—For this situation, a wavelength of 231 nm. was used. This required that a smaller concentration of penicillin and a full range of 0.2 deg. be used. Approximately 3.5 mg., accurately weighed, of benzylpenicillin was dissolved in 50 ml. of buffer solution. Except for these modifications, the procedure described for the penicillinase-sensitive system using optical rotation was followed.

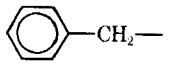
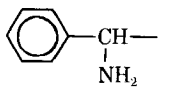
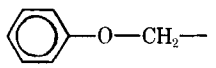
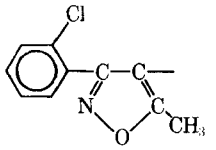
Procedure for Penicillinase-Resistant Penicillins Using Optical Rotation—For these samples, somewhat greater concentrations of both penicillin and penicillinase were used along with a reduction in the pathlength. Approximately 50–60 mg., accurately weighed, of the penicillin to be studied was dissolved in 100 ml. of 0.1 *M* phosphate buffer solution, pH 7.0, and a 1-cm. cell, which was affixed in a permanent position to the cell holder, was filled with this solution. Fifty microliters of penicillinase solution (1,600,000 units/ml.) was injected into the cell. Except for these modifications, the procedure described for the penicillinase-sensitive system using optical rotation was followed.

Procedure for Assaying a Mixture of a Penicillinase-Sensitive and a Penicillinase-Resistant Penicillin—Approximately 0.085–0.090 g. of the mixture, accurately weighed, was dissolved in 100 ml. of 0.1 *M* phosphate buffer solution, pH 7.0. A 1-cm. cell, securely fastened to the cell holder, was filled with this solution, and a baseline for the rotation of the active mixture was run on the spectropolarimeter. Ten microliters of penicillinase solution (80,000 units/ml.) was rapidly injected into the solution in the cell, the cell and cell holder were shaken and then replaced, and the recording was reinitiated. A kinetic curve of the rapid drop in rotaton *versus* time was re-

Table I—Structure of Penicillins Used in This Study



general formula for penicillin

Side Chain (R)	Penicillin
	Benzylpenicillin
	Ampicillin
	Phenoxymethylpenicillin
	Cloxacillin

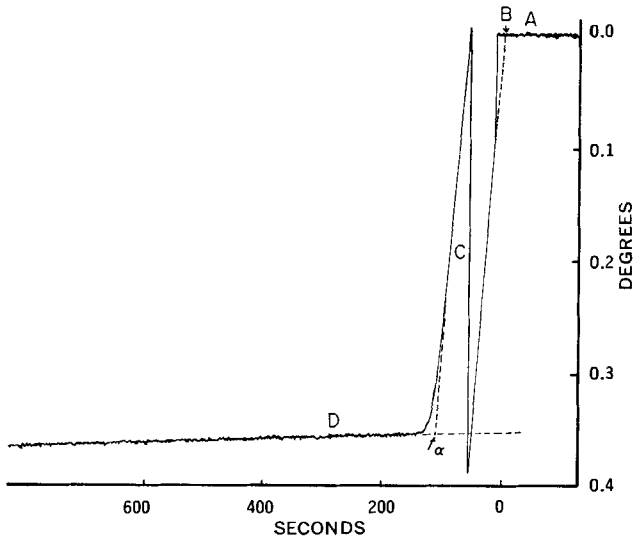


Figure 1—*Typical polarimetric assay recording of benzylpenicillin, with the extrapolated lines showing how the proper end-point was selected.*

[2] Donated by Riker Laboratories, Northridge, Calif., Neutrapen injectable.
[3] Wyeth's house standard, 1591 units/mg., control No. W620248-S1.
[4] Wyeth's house standard, 1664 units/mg., control No. E16-1.
[5] Wyeth's reference standard, 961 mcg./mg., lot No. C-10351. Wyeth samples were donated by Wyeth Laboratories.
[6] Ayerst reference standard, 905 mcg./mg. as the free acid, lot No. BRL-1621, and commercial grade, both furnished by Ayerst Laboratories.
[7] Purchased from Cal-Biochem.
[8] Ayerst commercial grade.

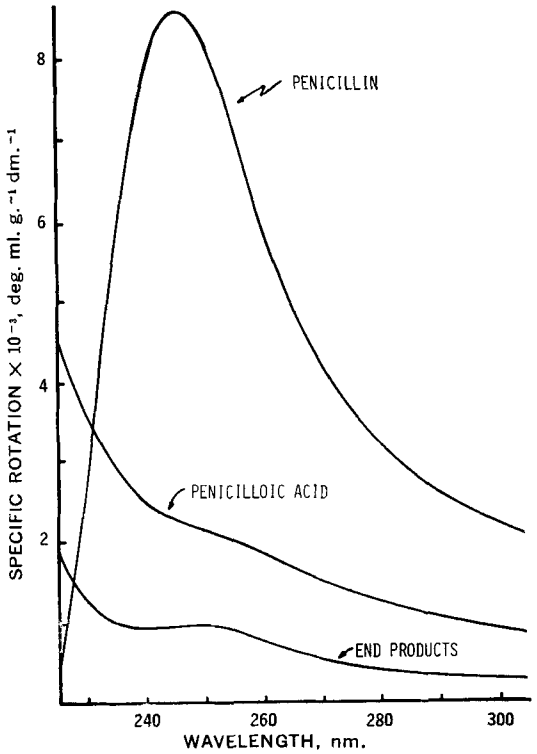


Figure 2—*ORD spectra of benzylpenicillin,* D-α-*benzylpenicilloate, and the final equilibrated mixture in a 0.1* M *phosphate buffer solution, pH 7.00.*

corded. This curve represented the degradation of the penicillinase-sensitive penicillin by penicillinase.

For the determination of the amount of penicillinase-resistant penicillin, the special 1-cm. cell was filled with a fresh sample of the above solution and a baseline of the active penicillins was run. Then 50 μl. of penicillinase solution (1,600,000 units/ml.) was

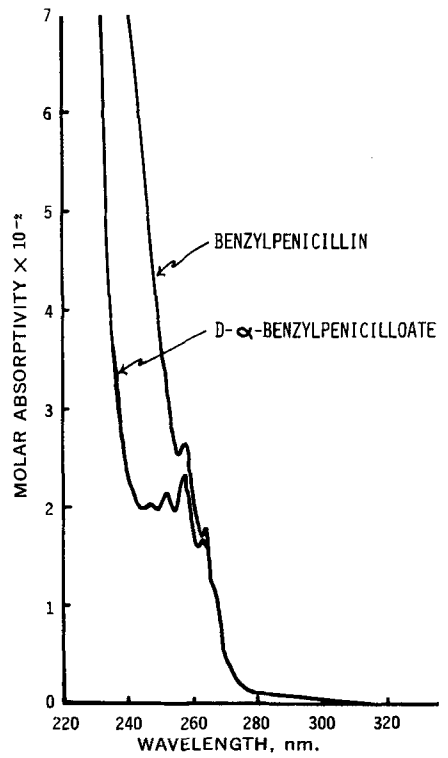


Figure 3—*UV spectra of benzylpenicillin and* D-α-*benzylpenicilloate ion in 0.1* M *phosphate buffer solution, pH 7.00.*

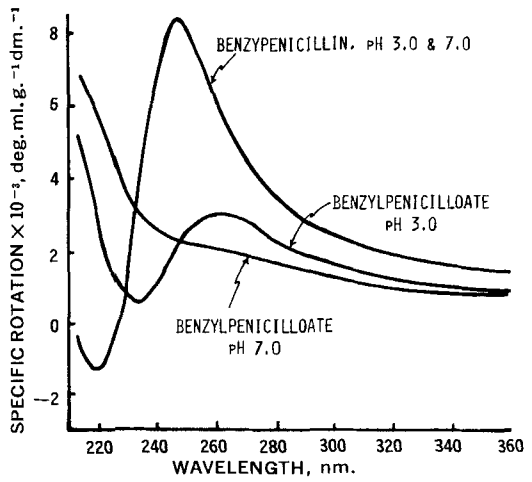


Figure 4—*ORD spectra of benzylpenicillin and its penicilloic acid derivative at pH 3.00 and 7.00.*

injected, and the recording was reinitiated. The observed curve represented the degradation of both the penicillinase-sensitive penicillin and the penicillinase-resistant penicillin.

RESULTS AND DISCUSSION

Typical Polarimetric Assay Curve—Figure 1 shows a typical polarimetric recording obtained at 255 nm. as a function of time for a sample of benzylpenicillin following the addition of penicillinase. Segment A represents the optical rotatory activity of the penicillin prior to the addition of the enzyme. At Point B, penicillinase solution was injected, producing the observed rapid linear decrease in rotation (Segment C) corresponding to the enzymatic cleavage of the β-lactam ring to form D-α-benzylpenicilloate. This was followed by a very slow decrease in rotation seen in the terminal phase (Segment D) due to the mutarotation of D-α-benzylpenicilloate (9). This observed behavior is typical of all penicillinase-sensitive penicillins.

Optical Rotary Dispersion (ORD) and UV Spectra for Benzylpenicillin and Its Degraded Products—ORD and UV spectra of benzylpenicillin and its degraded products were recorded in order to select an appropriate wavelength for the assay. The ORD spectra for benzylpenicillin, D-α-benzylpenicilloate, and the final equilibrated mixture of the degraded product are shown in Fig. 2.

The optical activities of penicillins in the UV and visible regions are largely due to the $n-\pi^*$ transition (10) of the carbonyl group in the β-lactam ring. It can be seen from the CD spectrum of benzylpenicillin (Fig. 11) that this transition has its maximum at 231 nm. When penicillins are degraded by penicillinase, the β-lactam ring is cleaved, thus causing a large reduction in the optical rotation in the UV and visible regions. The degraded product, D-α-benzylpenicilloate ion, then undergoes mutarotation giving the final ORD spectrum of the equilibrated mixture.

Figure 2 shows that the maximum change in optical rotation, arising from the cleavage of the β-lactam ring, occurs at the maximum in the ORD spectrum of benzylpenicillin, 247 nm. In this region, the change in optical rotation ascribable to the mutarotation is relatively small compared to the change in optical rotation due to the cleavage reaction. In the visible region, however, the magnitude of the mutarotation is almost the same as the magnitude of the cleavage reaction. However, the rate of change for the mutarotation step is much slower. This means that the relative magnitude of the terminal change in Fig. 1 would be much greater in the visible region.

Figure 3 shows the UV spectrum for benzylpenicillin and its degraded products. There is a rapid increase in absorption below 255 nm., somewhat limiting the concentration of penicillin that can be used in the assay. For this reason, the assays were carried out at both 247 and 255 nm.

Effect of pH on ORD Spectra of Benzylpenicillin and Its Degraded Products and on Change in Specific Rotation—Figure 4 shows the ORD spectra of benzylpenicillin and its penicilloic acid derivative at pH 7.00 and 3.00. The ORD spectra of the intact

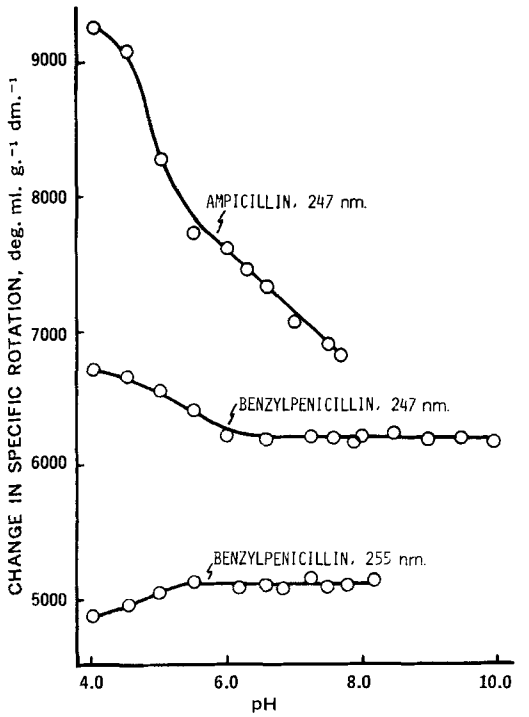


Figure 5—*Effect of pH on the change in specific rotation for benzylpenicillin at 247 and 255 nm. and for ampicillin at 247 nm. due to the catalytic hydrolysis of the β-lactam ring by penicillinase.*

penicillin at these two pH's are essentially the same, while there is a significant difference in the spectra of benzylpenicilloate. At pH 7.00, the carboxyl group formed by the cleavage of the β-lactam ring (pKa = 4.7) (11, 12) is essentially unprotonated; at pH 3.00, this carboxyl group exists mainly in its protonated form. When this carboxyl group is protonated, there is a positive cotton effect around 243 nm., while the unprotonated species has a positive plain ORD curve in this region.

Figure 5 shows the pH profiles for the change in specific rotation due to the hydrolysis of the β-lactam ring for benzylpenicillin at 247 and 255 nm. and for ampicillin at 247 nm.

Selection of End-Point—To convert the results from a polarimetric curve (Fig. 1) to an accurate quantitative measure of the amount of penicillin contained in the original sample, the penicillin concentration must be related directly to the decrease in the optical rotatory properties of the solution produced by the action of the enzyme. Levy (1) simply used the intersection of an extrapolated line from the initial rapid apparent zero-order cleavage reaction and an extrapolated line from the slower mutarotation. However, this point is dependent on the amount of enzyme used, particularly in the visible region where the relative change in the mutarotation step is large. In this section, the problem is analyzed theoretically to yield the best way of estimating the theoretical ΔR equivalent to an instantaneous cleavage reaction.

The overall rate of the catalyzed cleavage reaction obeys the classical Michaelis-Menten mechanism for enzymatic systems (13):

$$\underset{(S)}{\text{penicillin}} + \underset{(E)}{\text{penicillinase}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(ES)}{\text{enzyme–substrate complex}}$$

$$\underset{(ES)}{\text{enzyme–substrate complex}} \xrightarrow{k_2} \underset{(P)}{\text{penicilloate}} + \underset{(E)}{\text{penicillinase}}$$

$$\underset{(P)}{\text{penicilloate}} \xrightarrow[\text{(mutarotation)}]{k_3} \underset{(P')}{\text{diastereoisomers}}$$

It can readily be shown that the rate of degradation in these systems is given by (14):

$$\frac{-d[S]}{dt} = \frac{k_2 E_T [S]}{K_m + [S]} \qquad \text{(Eq. 1)}$$

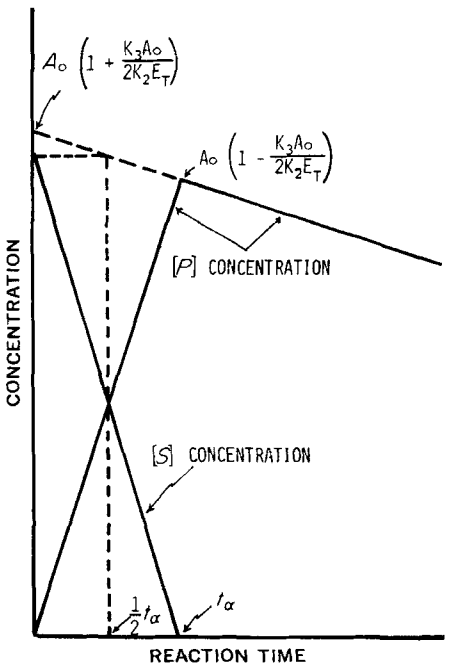


Figure 6—*Schematic drawing of the penicillin and its penicilloic acid derivative concentrations as a function of time for a penicillinase-sensitive penicillin.*

where [S] is the concentration of the penicillin, K_m is the Michaelis constant, k_2 is the rate constant for the breakdown of the penicillin–enzyme complex, and E_T is the total concentration of the enzyme. Since K_m for the penicillinase-sensitive penicillins is always very small, it can be neglected in the denominator except at very low substrate concentrations. Thus, at higher residual penicillin concentrations, a zero-order kinetic equation is obtained: $(-d[S]/dt) = k_2E_T$.

For Penicillinase-Sensitive Penicillin—Schematic plots of penicillin and penicilloic acid concentrations *versus* time for a penicillin–penicillinase system having a low K_m value are shown in Fig. 6. To obtain a precise measurement for the change in rotation due only to the cleavage of the β-lactam ring by penicillinase, a point must be selected on the extrapolated mutarotation curve of the penicilloic acid which represents the concentration of penicilloic acid equivalent to the initial concentration of penicillin and which has not undergone any mutarotation. In the following derivation, it is shown that this time can be approximated at $^1/_2t_\alpha$, where t_α is the hypothetical time required to complete the cleavage on the assumption that the reaction is strictly zero order.

In the strictly zero-order range, the kinetic rate equation for the disappearance of [S] can be approximated by: $S_0 - [S] = k_2E_Tt$, the integration of Eq. 1. When $t = t_\alpha$, $[S] = 0$; therefore:

$$t_\alpha = \frac{S_0}{k_2E_T} \qquad \text{(Eq. 2)}$$

Since the secondary change in rotation is presumably pseudo-first order, the differential rate equation describing [P] between $t = 0$ and $t = t_\alpha$ is:

$$\frac{d[P]}{dt} = k_2E_T - k_3[P] \qquad \text{(Eq. 3)}$$

Upon integrating and solving for [P]:

$$[P] = \frac{k_2E_T}{k_3}(1 - e^{-k_3t}) \qquad \text{(Eq. 4)}$$

By expanding the exponential part in a power series and evaluating the expression at t_α, the following equation is obtained:

$$[P]_{t=t_\alpha} = S_0 - \frac{k_3S_0^2}{2k_2E_T} + \ldots \qquad \text{(Eq. 5)}$$

For $t > t_\alpha$, after all the penicillin has been degraded to penicilloic

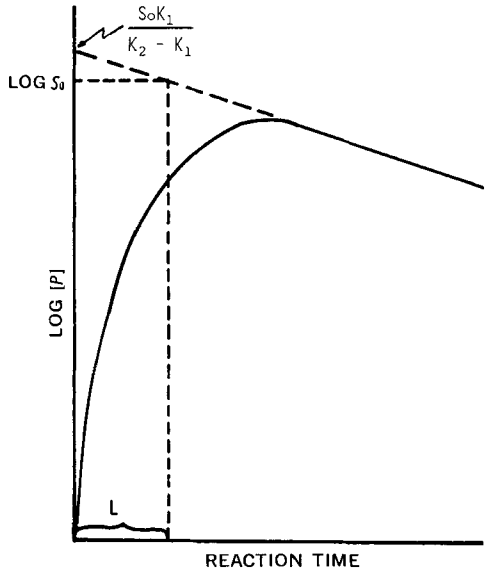


Figure 7—*Schematic drawing of the logarithm of the penicilloate-ion concentration* versus *reaction time for a penicillinase-resistant penicillin.*

acid, the secondary change in rotation is described by:

$$\frac{d[P]}{dt} = -k_3[P] \quad \text{or} \quad [P] = Ce^{-k_3t} \qquad \text{(Eq. 6)}$$

The integration constant, C, can be evaluated by equating Eqs. 4 and 6 at t_α:

$$Ce^{-k_3t_\alpha} = \frac{k_2E_T}{k_3}(1 - e^{-k_3t_\alpha}) \qquad \text{(Eq. 7)}$$

By solving for C, expanding the exponential in a power series, and substituting the expression for t_α (Eq. 2), Eq. 8 is obtained:

$$C = S_0 + \frac{k_3S_0^2}{2k_2E_T} + \ldots \qquad \text{(Eq. 8)}$$

The integration constant, C, represents the hypothetical concentration of $[P]$ required at $t = 0$ for a hypothetical system in which there is only P going to P' (*i.e.*, no S going to P) to give the same concentration changes for $t > t_\alpha$ in the above $S \rightarrow P \rightarrow P'$ system.

Now the problem is to select some time on the extrapolated P curve that represents the concentration of P equivalent to S_0. This point can be approximated by using Eq. 6, with a power series expansion of the exponential, and Eq. 8. Or it can be more easily seen graphically by extrapolating the right-hand portion of the mutarotation curve back to zero time. This point does not yield a concentration value corresponding to the initial penicillin concentration but is too high by the amount $(S_0^2k_3/2k_2E_T)$ (Eq. 8). On the other hand, the intersection of the initial zero-order cleavage

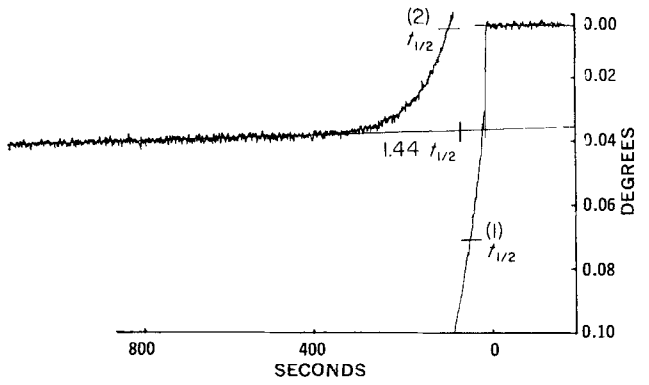


Figure 8—*Typical polarimetric assay recording with the extrapolated mutarotation line for cloxacillin in a 0.1* M *phosphate buffer solution, pH 7.00.*

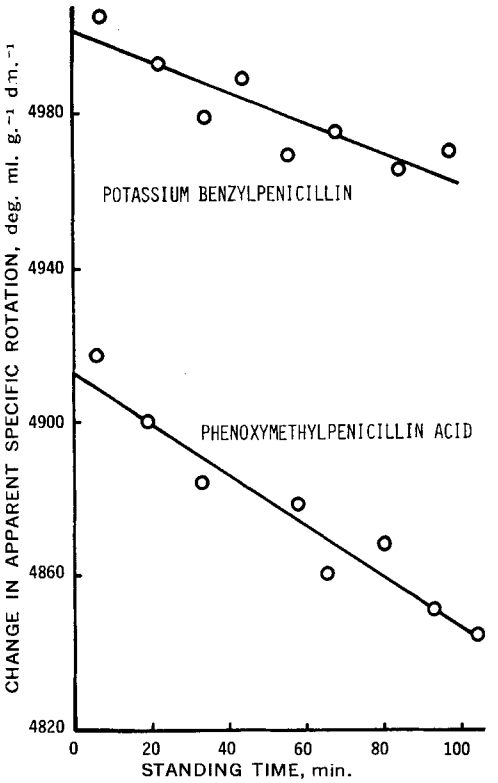


Figure 9—*Plot showing the decrease in the assay values for benzylpenicillin and phenoxymethylpenicillin as a function of the time the solutions were standing at room temperature prior to assaying.*

reaction and the slow mutarotation step occurring at t_α give a concentration value too low by the same amount (Eq. 5). On this basis, it is proposed that the optical rotational value of penicilloate that can be correlated with the initial penicillin concentration occurs at $^1/_2t_\alpha$ on the optical activity–time plot (Fig. 1) for penicillins that cleave largely through zero-order kinetics. The change in optical rotation related to the penicillin concentration was simply obtained by subtracting the value for the rotation at $^1/_2t_\alpha$ on the extrapolated mutarotation line from the initial rotation for the intact penicillin.

For Penicillinase-Resistant Penicillin—Since these penicillins did not follow the conditions already stated but instead exhibited pseudo-first-order kinetics with respect to the penicillin for the cleavage reaction, a different approach must be used. In Fig. 7, a behavior of this type is evident in a polarimetric plot obtained during the cleavage of cloxacillin in the presence of *Bacillus penicillinase.*

In these instances, there are two consecutive first-order reactions:

$$S \xrightarrow{k_1} P \xrightarrow{k_2} P'$$

where k_1 is the pseudo-first-order rate constant for the catalytic hydrolysis of the β-lactam ring, and k_2 is the first-order rate constant for the mutarotation.

The concentration of D-α-penicilloate, P, is given by (15):

$$[P] = \frac{S_0k_1}{k_2 - k_1}(e^{-k_1t} - e^{-k_2t}) \qquad \text{(Eq. 9)}$$

Since $k_1 >>> k_2$, e^{-k_1t}, at sufficiently large time, is very small compared to e^{-k_2t} and can be neglected. Under these conditions:

$$[P] = \frac{S_0k_1}{k_2 - k_1}e^{-k_2t} \qquad \text{(Eq. 10)}$$

and a plot of log $[P]$ against t is linear. If this linear portion of the curve is extrapolated back (Fig. 7), it passes through a point where log $[P]$ = log S_0. The time at which log $[P]$ = log S_0, L, is related to the rate constants by:

$$1 = \frac{k_1}{k_2 - k_1}e^{-k_2L} \qquad \text{(Eq. 11)}$$

Table II—Apparent Change in Specific Rotation for Some Penicillinase-Sensitive Penicillins Due to Catalytic Hydrolysis of β-Lactam Ring by Penicillinase

Penicillin	Concentration, mcg./ml.	Number of[a] Assays	$[\Delta\alpha]^{25°}_{255\ nm.}$[b]	Standard Deviation[c] from Line
Potassium benzylpenicillin				
Commercial house standard,	320.14	8	5003.3 ± 6.0	8.3
1591 units/ml.	279.71	8	5029.2 ± 6.1	7.7
	197.40	8	5020.6 ± 6.3	17.6
	260.35	8	5038.2 ± 5.8	7.2
	313.00	8	5000.8 ± 6.0	7.4
	56.90	8	5058.0 ± 5.8	14.6
	316.99	9	4999.8 ± 5.5	6.9
Recrystallized from methanol	305.20	3	5035.5 ± 8.5	11.4
with ethyl acetate	242.18	5	5061.4 ± 7.6	7.6
Recrystallized from water with	240.28	9	5061.7 ± 5.2	8.0
n-butanol	283.30	5	5101.5 ± 6.8	16.8
	276.60	10	5009.2 ± 5.2	9.7
Ampicillin B				
Commercial reference standard,	264.60	8	5688.0 ± 6.1	8.4
961 mcg./mg.	264.60	8	5665.7 ± 5.9	12.4
	255.13	3	5692.2 ± 9.9	13.9
	251.94	5	5670.0 ± 9.3	7.3
	222.00	14	5622.9 ± 4.3	20.8
Recrystallized from water	263.18	8	5533.1 ± 5.6	27.7
	263.18	8	5591.4 ± 5.3	27.3
Phenoxymethylpenicillin acid				
Commercial house standard,	297.71	8	4904.9 ± 5.8	5.8
1664 units/mg.	297.56	9	4901.3 ± 5.7	6.2
	290.47	8	4911.7 ± 5.6	7.2
	308.01	5	4906.5 ± 16.4	8.1
	303.31	8	4912.6 ± 5.7	6.8
	291.15	8	4919.5 ± 5.9	7.3
	301.78	8	4926.3 ± 7.1	12.1

[a] The number of times the solution was assayed. [b] The change in specific rotation at zero standing time was obtained from the intercept of a regression line through a plot of $[\Delta\alpha]^{25°}_{255\ nm.}$ against the time the solution was standing before being assayed (Fig. 9). These values are not corrected for purity or dilution. The standard error in the intercept (*Reference 13*) was also recorded. [c] This value represents the deviation of the experimental points for the regression line (Fig. 9).

If this identity is expanded in a power series:

$$k_1 - k_2 = k_1(1 - k_2L + \ldots) \qquad \text{(Eq. 12)}$$

and all terms greater than the linear term are neglected, the following expression is obtained:

$$L = \frac{1}{k_1} = \frac{(1/0.693)}{t_{1/2}} = 1.44t_{1/2} \qquad \text{(Eq. 13)}$$

where $t_{1/2}$ is the first half-life of the reaction ($S \rightarrow P$).

Thus, in Fig. 8, the value of rotational change that would be affected only by the initial penicillin concentration and independent of other variables in the system would occur only at the indicated point. Since the zero time is not known, the time between the first two half-lives was determined, multiplied by 0.44, and added to the first half-life time to obtain the proper end-point.

Correction for Spontaneous Degradation of Samples—Another important factor, which must be considered in the development of any really precise analytical method for these relatively unstable antibiotics, is the significant spontaneous degradation that takes place as the sample stands. The loss is usually negligible for dry samples but becomes significant in aqueous solutions. Figure 9 shows a typical plot for the enzyme-induced change in apparent specific rotation at room temperature and pH 7.0 with standing time for a 0.1 *M* phosphate buffer solution of benzylpenicillin and phenoxymethylpenicillin. In this study, assays were carried out on benzylpenicillin, ampicillin, phenoxymethylpenicillin, and cloxacillin. At room temperature and under the conditions generally employed, losses of approximately 1% were observed in 170 min. for benzylpenicillin and cloxacillin and in 90 min. for ampicillin and phenoxymethylpenicillin. The intercept represents the change in apparent specific rotation for the original dry sample before any spontaneous degradation of an aqueous solution occurs. Because of the much greater precision of the proposed method, this approach is necessary to take full advantage of its capability. With conventional methods, the losses in sample activities on standing are essentially ignored, even though the time involved may be considerable.

Precision and Accuracy of Assay Procedure—The precision is a measure of the deviation of the individual results from their mean value (16). Here, standard deviation is used to express the precision of the assay. To determine the precision of this procedure, assays were carried out several times on the same solution. Since the change in apparent optical rotation decreased with the time the sample was standing prior to assay, due to spontaneous degradation of the penicillin (Fig. 9), the standard deviation from a mean value cannot be used. Instead, the standard deviation from a regression line is used as a measure of the precision. The values given in the last column in Tables II and III represent the standard deviation of the experimental points from the regression line and, therefore, are measures of the precision for a single measurement.

Table III—Apparent Change in Specific Rotation at Zero Standing Time for a Pencillinase-Resistant Penicillin Due to Catalytic Hydrolysis of β-Lactum Ring by Penicillinase

Sodium Cloxacillin	Concentration, mcg./ml.	Number of Assays	$[\Delta\alpha]^{25°}_{255\ nm.}$[b]	Standard Deviation[c] from Line
Commercial grade	503.65	12	2681.8 ± 8.8	15.9
Commercial reference standard, 905 mcg./mg. as free acid	496.85	5	2799.0 ± 13.2	5.0
	503.70	5	2808.9 ± 13.2	14.5
Recrystallized from water with *n*-butanol	608.25	9	2877.1 ± 9.6	14.5
Recrystallized from methanol with ethyl acetate	678.45	3	2792.7 ± 16.0	2.7

[a,b,c] See corresponding footnotes in Table II.

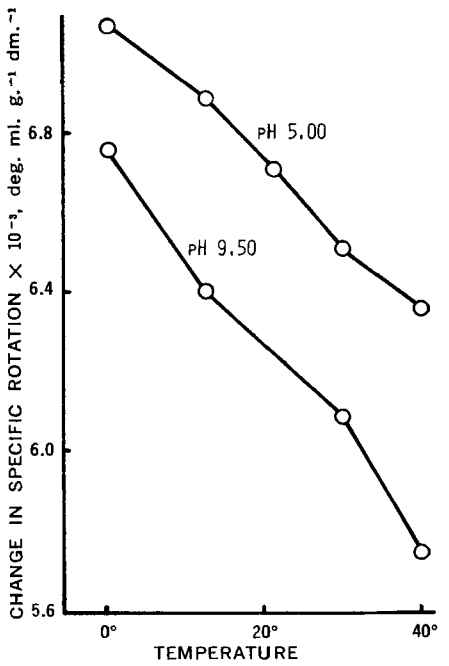


Figure 10—*Plot showing the effect of temperature on the change in specific rotation at 247 nm. due to the catalytic hydrolysis of the β-lactam ring in benzylpenicillin by penicillinase at pH 5.00 and 9.50.*

The joint estimate of standard deviation[9] of the change in apparent specific rotation for the experimental points from their regression lines for the penicillinase-sensitive penicillin was 13.3 deg. ml. g.$^{-1}$ dm.$^{-1}$, and it was 14.2 deg. ml. g.$^{-1}$ dm.$^{-1}$ for the penicillinase-resistant penicillin. This represents a precision of 0.27 and 0.50%, respectively. The instruction manual for the spectropolarimeter (17) claims a precision of 0.2% full-range scale [25.4 cm. (10 in.)] for a single reading when not limited by noise. Most of this error can be attributed to chart printing error. Since two readings and the switching of the pen center are required in this assay procedure, a precision much greater than 0.3% should not be expected if readings are taken off the chart.

Thus, the precision offered by this polarimetric method appears to be far superior to any of the presently available assay procedures. The hydroxylamine and iodometric methods do not give precisions greater than 1–2%, whereas the microbiological methods are at best semiquantitative procedures. In this polarimetric assay procedure, a precision of at least 0.3% was obtained. A far greater precision probably could be obtained by introducing some modifications into the method to improve the accuracy of the readout. Intrinsically, the method appears to be capable of readily yielding precisions of the order of a few parts per 10,000 since polarimetric readings can discern differences of a few tenths of a millidegree.

These polarimetric assay procedures are based on relating the intercept value, obtained from a linear plot of the change in apparent specific rotation against standing time for the aqueous solution, to the corresponding values for an absolute pure compound. Thus, the accuracy for these assay procedures depends on the determination of the absolute changes in specific rotation expected for the various penicillins. The intercept value of the regression lines along with the standard error in determining the intercept are given in Tables II and III for nonresistant and resistant penicillins, respectively. The values accepted in this investigation for the absolute change in specific rotation for the pure penicillins used in this study are given in Table IV. They were obtained by correcting the intercept values (Tables II and III) for the purity of the dry sample and averaging them. The value for benzylpenicillin appears to be very close to the true value. The value accepted here was the overall average for all the assays on the benzylpenicillin recrystallized both from water with 1-butanol and from methanol with ethyl acetate. This value was slightly larger than the average value obtained from the commercial reference standard[3]. The values for ampicillin[5], phenoxymethylpenicillin acid[4], and sodium cloxacillin[6] were obtained from a single reference standard, so the accuracy of these values are dependent on the labeled value of their respective reference samples and the purity at the time of assay. These values are probably quite accurate but not as dependable as the value given to potassium benzylpenicillin.

Table V shows a comparison of the spectropolarimetric penicillinase assay procedure to microbiological and iodometric assay procedures. Ampicillin samples, whose microbiological assays were in good agreement with the chemical assays, were received[10] along with the microbiological and chemical assay data. The values from the spectropolarimetric penicillinase method were obtained approximately 1 month after the microbiological and chemical assays.

For any real precise assay method, there is another important source of error: the sampling error. Several lots of the penicillin with varying purity are mixed together, so the batch may not be completely homogeneous in purity. Since only very small samples (usually less than 150 mg. to prepare a 500-ml. solution) are used, the sampling error can become significant. This sampling error could account for some of the variation shown in Table V.

Application of Optical Procedure for Assaying a Mixture of Penicillinase-Sensitive Penicillin and Penicillinase-Resistant Penicillin—The results from assaying mixtures of ampicillin (a nonresistant penicillin) and cloxacillin (a resistant penicillin) by optical rotation at 255 nm. are given in Table VI. In this table, a comparison was made of the concentration of the penicillins used to prepare the solutions and the activity of the penicillins determined by the assay. The activities of the penicillins used for preparing the mixtures were separately determined from the optical rotatory assay procedure for each respective penicillin.

The mixtures were prepared from known amounts of each penicillin. The ampicillin concentration was assayed by injecting 10 μl. of penicillinase solution (80,000 units/ml.) into the mixture. The selection of an end-point was obtained by adjusting the point at zero time (*i.e.*, the time at which the penicillinase solution was injected) on the extrapolated slower decreasing terminal phase. A small correction was made by extrapolating from the end-point at zero time to $^1/_2t_\alpha$ on a line with a slope obtained by averaging the slopes for the mutarotation phase from the assays of ampicillin alone. This correction was only an increase of 0.3–0.4% over the assayed value for ampicillin obtained at zero time.

The concentration of cloxacillin in the mixture was determined by subtracting the change in optical rotation determined for ampicillin from the total change in optical rotation for both penicillins. The total change in optical rotation was obtained by injecting 50 μl. of penicillinase solution (1,600,000 units/ml.) into the mixture. The selection of an end-point for the total change in rotation was essentially the same as the procedure described previously for penicillinase-resistant penicillins.

From Table IV, it can be seen that the change in specific rotation for ampicillin is about twice the change in specific rotation for

Table IV—Values for Change in Specific Rotation at 255 nm. Assigned to Pure Penicillin

Penicillin	$[\Delta\alpha]^{25°}_{255\ nm.}$[a], deg. ml. g.$^{-1}$ dm.$^{-1}$
Potassium benzylpenicillin	5053.9
Ampicillin (anhydrous acid)	5897.8
Penicillin V acid	5006.3
Sodium cloxacillin	2956.3

[a] The values given here are averages of the change in apparent specific rotation given in Tables I and II corrected for purity but not for the dilution caused by injecting 0.05 ml. of a penicillinase solution into 16 ml. of the penicillinase-sensitive penicillin solution or 0.05 ml. of a penicillinase solution injected into 3.2 ml. of the penicillinase-resistant penicillin solution.

[9] $$\sigma = \sqrt{\frac{\Sigma_i(n_i - 2)s_i^2}{\Sigma_i(n_i - 2)}}$$

where n_i is the number of experimental points used to determine the regression line, and s_i is the standard deviation of the points from their regression line. The summation is over all the regression lines to be combined.

[10] From Ayerst Laboratories, Rouses Point, N. Y.

Table V—A Comparison of Penicillinase–Spectropolarimetric Method to Microbiological and Iodometric Methods for Analyzing Activity for Several Samples of Ampicillin

Ampicillin Sample	Microbiological Assay[a] —Commercial Laboratory— Number 1	Number 2	Iodometric Method[a] ——Commercial Laboratory—— Number 3	Number 1	Number 4	Penicillinase–Spectropolarimetric Method		
Trihydrate	840	848	850	824	—	814	810	—
Trihydrate	843	746	855	839	—	819	812	824
Trihydrate	860	848	863	835	—	824	818	—
Trihydrate	841	848	860	840	—	818	813	—
Trihydrate	846	840	858	—	—	830	827	—
Trihydrate	821	826	847	836	—	823	823	—
Anhydrous	956	—	976	950	961	957	957	—

[a] Data furnished by Ayerst Laboratories, Rouses Point, N. Y.

cloxacillin. Therefore, any error in determining the ampicillin concentration would have twice the effect on the determination of the cloxacillin concentration. For example, if the ampicillin concentration was underestimated by 0.5%, then the cloxacillin concentration would be overestimated by 1%.

General Comments on These Assay Procedures—No effect on the change in apparent specific rotation due to the hydrolysis of the β-lactam ring by penicillinase was found when the ionic strength of the penicillin solution was changed from 0.005 to 1.0 *M*. Figure 10 shows the effect of temperature on the change in apparent specific rotation at 247 nm. due to the catalytic hydrolysis of the β-lactam ring in benzylpenicillin by penicillinase at pH 5.00 and 9.50.

The cell compartment in the recording spectropolarimeter has an operating temperature of 27°, while the room temperature was kept at 25°. Thus, there may have been a small change in rotation due to temperature change. The time required for the cleavage reaction to go to completion was approximately 2 min. Usually less than 5 min. elapsed from the time the solution was placed in the cell compartment until the completion of the cleavage reaction. Sometimes there was a slight decrease in the baseline for the intact penicillin due to temperature change. Later, when a temperature bath was used to bring the buffer solution to 27° prior to the preparation of the penicillin solution, this slight decrease in the baseline was almost completely eliminated.

At a constant pH and temperature, it was found that the spectropolarimetric penicillinase assay method for benzylpenicillin follows a Beer's-type law from a concentration of 1×10^{-5} to 1.3×10^{-3} *M*. At lower concentrations, there was greater deviation on both sides of the line since a small full range (0.02 deg.) was used, thus increasing the noise. A concentration of 2×10^{-6} *M* for benzylpenicillin and ampicillin was close to the smallest concentration that could be detected.

The presence of any other optically active material, such as sugars, does not affect the assay since the assay procedure relates the penicillin concentration to the changes in optical rotation caused by the cleavage of the β-lactam ring by penicillinase. The enzyme is very specific for the β-lactam ring, so the presence of degraded penicillin does not affect the assay. This was checked by assaying several samples of ampicillin which were subjected to degradation study at room temperature.

Sodium benzylpenicillin tablets, 250,000 or 400,000 units, were dissolved in 1 l. of the buffer solution. Without filtering, this solution with the suspended starch from the tablet was assayed at 255 nm. in a 5-cm. cell with a full range of 0.2 and 0.4 deg., respectively. The degradation curves were a little noisy due to the scattering of the light by the suspended starch, but an accurate assay could be made. When the solution was filtered, there was some loss of activity.

Since the change in specific rotation for ampicillin has a greater variation with pH than benzylpenicillin (Fig. 5), it is conceivable that a mixture of these two penicillins could be assayed by making measurements at two pH's, *e.g.*, at pH 7.50 where the difference between the change in specific rotation for these two penicillins is small and at pH 4.00 where this difference is significant. From these two readings, it should be possible to calculate the concentrations of both penicillins in the mixture.

Penicillin with UV absorption too great in the range of the present optical rotation measurement (247 or 255 nm.) can be assayed at a

Table VI—Assays of Mixtures of Ampicillin and Cloxacillin

Penicillin[a]	Weighed Concentration, mcg./ml.	Activity[b], mcg./ml.	Change in Rotation, deg.	Activity Measured, mcg./ml.	Percent Activity Assayed
Cloxacillin	342.00	332.7	0.0983	332.5	99.9
Ampicillin	405.00	381.9	0.2204	373.6	97.8
Cloxacillin	386.25	375.8	0.1116	377.5	100.4
Ampicillin	541.45	510.6	0.2940	498.5	97.6
Cloxacillin	380.20	359.3	0.1062	359.2	100.0
Ampicillin	453.70	427.8	0.2474	419.5	98.0
Cloxacillin	234.10	221.2	0.0639	216.1	97.7
Ampicillin	503.55	474.8	0.2777	470.8	99.2
Cloxacillin	438.45	414.3	0.1320	446.5	107.8
Ampicillin	189.45	178.7	0.1043	176.8	99.0
Cloxacillin	275.25	260.1	0.0796	269.4	103.6
Ampicillin	386.85	364.8	0.2108	357.4	98.0
Cloxacillin	297.45	281.1	0.0681	230.3	82.0
Ampicillin	365.45	344.6	0.1990	337.4	97.9
Cloxacillin	301.65	285.1	0.0896	303.1	106.3
Ampicillin	593.85	560.0	0.3256	552.1	98.6

[a] The ampicillin sample used in the mixtures was recrystallized from water at 70° (*Reference 8*). A purity of 94.3% was obtained by comparing the average change in specific rotation given in Table I to the change in specific rotation for a pure compound given in Table III. The first two mixtures contained sodium cloxacillin, which was recrystallized from water with *n*-butanol, and a purity of 97.3% was assigned by comparing the results in Table II to the value given in Table III. In the remaining six mixtures, the sodium cloxacillin was recrystallized from methanol with ethyl acetate, and a purity of 94.5% was assigned by comparing the results in Table II to the absolute value given in Table III. [b] Activity equals weighed concentration times the purity.

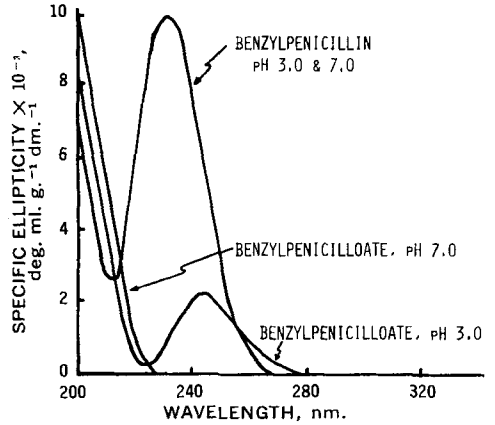


Figure 11—*CD spectra of benzylpenicillin and its penicilloic acid derivative at pH 3.0 and 7.0.*

longer wavelength, but this increases the relative magnitude of the mutarotation; hence, the selection of a proper end-point becomes more critical.

Results from Assaying Benzylpenicillin by CD—A CD phenomenon is associated with any medium that exhibits optical rotation. The CD spectra for benzylpenicillin and its penicilloic acid derivative at pH 7.00 and 3.00 are shown in Fig. 11. The CD spectra for the intact penicillin at these two pH's are essentially the same, showing a maximum around 231 nm., which corresponds to the cotton effect for benzylpenicillin in Fig. 4. However, there is a significant difference in the spectra of benzylpenicilloate at these two pH's. At pH 3.00, where the carboxyl group formed by the cleavage of the β-lactam ring is mostly protonated, a positive CD spectrum is observed between 225 and 260 nm., with a maximum at 243 nm. corresponding to the positive cotton effect in the ORD spectrum in Fig. 4. At pH 7.00, where this carboxyl group is mostly unprotonated, there is no CD spectrum for benzylpenicilloate in this region and, hence, no cotton effect is observed in the ORD spectrum. Therefore, for pH's above 5.50, the selection of an end-point was greatly simplified by the fact that the mutarotation step was not observed in the assay curves. The final reading was constant, and the change in the apparent specific ellipticity was obtained by subtracting the final reading from the initial reading. Below pH 5.50, the mutarotation could be observed with CD but not to the extent observed with optical rotation; thus the end-pcint of $^1/_2t_\alpha$ should be used. Figure 12 shows how the change in specific ellipticity at 231 nm. for benzylpenicillin varies with pH at 27°.

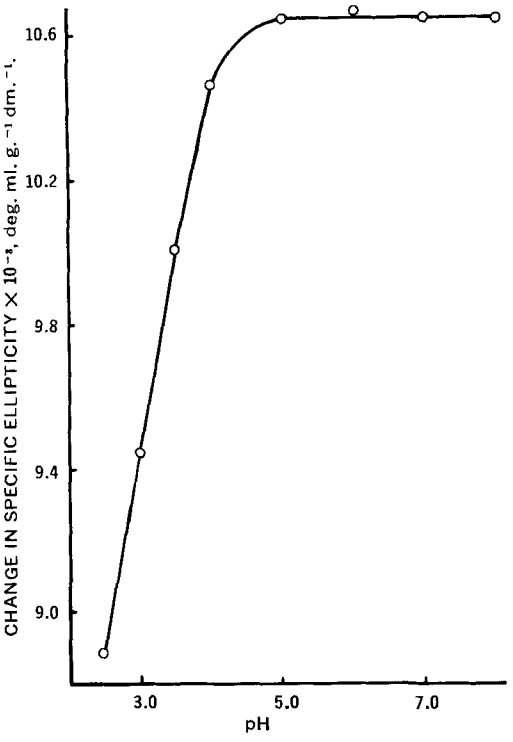


Figure 12—*Plot showing the effect of pH on the change in specific ellipticity at 231 nm. due to the catalytic hydrolysis of the β-lactam ring in benzylpenicillin by penicillinase.*

CD appears to be the method of choice when not limited by UV absorption at 231 nm. Since most of the penicillins used today have much greater UV absorption then benzylpenicillin, this procedure is only applicable to a few penicillins.

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Solubility Analysis of Multicomponent Systems Capable of Interacting in Solution

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Abstract □ The results obtained in this study show that individual solubility profiles may be of no value for predicting the equilibrium solubility of mixtures of drugs, particularly when the drugs are capable of interacting in solution to form a very insoluble complex.

Keyphrases □ Mixed solvent system—effect on drug solubility, drug–drug interactions □ Solubility analysis—mixed solvent system–mixtures of drugs, difficulties encountered in using individual solubility profiles □ Drug solubility—multicomponent systems capable of interacting in solution, deviation from individual solubility profiles □ Complexes, drug—in mixed solvent systems, effect on solubility

Many reports in the literature concern the use of mixed solvent systems to solubilize various compounds. Moore (1) attempted a semiempirical approach by trying to manipulate the dielectric constant of cosolvent blends to dissolve given amounts of drugs. This approach was the subject of several theoretical and practical studies (2–4). Other investigations of pharmaceutical interest noted that multiple peak solubilities may exist for a drug as a function of the solvent composition or dielectric constant (5–7).

Although the equilibrium solubilities of a number of drugs as a function of various solvent mixtures have been studied, no information is available concerning the effect of solvent composition on the solubility of multicomponent systems in which drug–drug interactions or complexation are likely to occur. Such information would be of interest since many liquid pharmaceuticals are formulations of more than one drug and since mixed solvents are frequently required in these preparations because of the limited water solubility of the solutes.

The two model compounds, theophylline and phenobarbital, were chosen for this study because they are known to form a complex in solution (8). Solubility profiles in several solvent systems were determined for each drug, for mixtures of the drugs, and for the known complex. These data were used to evaluate whether solubility profiles of the individual drugs can be utilized to predict the effect of solvent on the equilibrium solubility of binary solutes when complexation occurs between the two drugs.

EXPERIMENTAL

Materials—The following were used: chloroform A.R.; alcohol USP; sulfuric acid, reagent grade; sodium hydroxide pellets A.R.; dibasic (anhydrous) sodium phosphate A.R.,; propylene glycol USP; glycerin USP; Carbowax 400[1]; theophylline NF (anhydrous), recrystallized from water and dried at 105° for 4 hr., m.p. 270–274°; and phenobarbital USP (powder), recrystallized from ethanol and dried at 105° for 2 hr., m.p. 174–178°.

A pH 11.0 phosphate buffer was prepared by dissolving 28.4 g. of anhydrous dibasic sodium phosphate in 2000 ml. of distilled water and adjusting to pH 11.0 with 1.0 *N* sodium hydroxide.

Diluted sulfuric acid was prepared by adding 57 ml. of sulfuric acid to about 100 ml. of distilled water, cooling to room temperature, and diluting to 1000 ml. with distilled water.

The solvents were prepared on a percent volume basis with the organic component added by buret.

The theophylline–phenobarbital (2:1) complex, prepared as previously described (8), melted at 249–251°.

Anal.—Calc. for $C_{26}H_{28}N_{10}O_7$: C, 52.7; H, 4.73; N, 23.6. Found: C, 52.63; H, 5.12; N, 23.85.

The 1 *N* sodium hydroxide was prepared by dissolving 40 g. of sodium hydroxide pellets A.R. in sufficient distilled water to make 1 l. Standardization was against potassium acid phthalate.

Equipment—The samples were maintained at 25.0 ± 0.5° by immersion in a water bath shaker[2,3]. A spectrophotometer[4] was used for analysis of the solutes. A pH meter[5] was used in the buffer preparation.

Equilibration—An excess of theophylline, or phenobarbital, or the previously isolated complex was added to 10 ml. of the solvent mixture contained in a screw-top vial. The vial was closed, using a Parafilm[6] liner in the cap, and sealed with several turns of electrical tape. Equilibration was obtained by shaking in a constant-temperature bath at 25.0 ± 0.5° for at least 36 hr. The samples were then removed, and an appropriate volume of the supernatant liquid was withdrawn from each vial by pipet and prepared for assay. The pipets were fitted with a glass wool prefilter to exclude undissolved solids.

Mixtures of theophylline and phenobarbital were equilibrated under conditions identical to those already described. In each solvent system, the amount of theophylline added was in excess of its equilibrium solubility. The total amount of phenobarbital added to these systems was such that: (*a*) all the theophylline present (in solution plus excess solid) would be complexed, and (*b*) the equilibrium solubility of phenobarbital would be exceeded in all the solvent concentrations examined.

Assay Procedure—*Individual Solubilities*—Filtered aliquots of the solutions containing either theophylline or phenobarbital were diluted with pH 11.0 phosphate buffer and analyzed spectrophotometrically *versus* buffer solution in 1-cm. silica cells at 275 and 240.5 nm., respectively. The concentration of theophylline or phenobarbital in the sample was calculated using an *a* (1%, 1 cm.) for theophylline of 635.7 and for phenobarbital of 417.5.

Solubility of Previously Isolated Complex—Filtered aliquots from solutions containing excess complex were diluted with pH 11.0 buffer and analyzed spectrophotometrically at 275 and 240.5 nm. in 1-cm. silica cells. The concentration of phenobarbital and theophylline in the original solution was calculated by solving the simultaneous equation for each component. The *a* (1%, 1 cm.) of theophylline at 240.5 nm. is 217.1; for phenobarbital at 275 nm., it is 24.7. The *a* (1%, 1 cm.) of theophylline and phenobarbital at 275 and 240.5 nm., respectively, was already given.

Solubilities in Mixture—Filtered 2-ml. aliquots were transferred to a separator containing 1 *N* sodium hydroxide to decompose the complex into its components (8). This solution was acidified with concentrated sulfuric acid, and diluted sulfuric acid was added. The acidic phase was extracted with chloroform; the chloroform layers were combined in a second separator and back-extracted with diluted sulfuric acid. The acidic phases were combined and

[1] Union Carbide Corp., New York, N. Y.

[2] Eberbach Corp., Ann Arbor, Mich.
[3] New Brunswick Scientific Co., New Brunswick, N. J.
[4] Model 240, Gilford Instrument Laboratories, Inc., Oberlin, Ohio.
[5] Radiometer-type PHM 26, The London Co., Westlake, Ohio.
[6] American Can Co., Neenah, Wis.

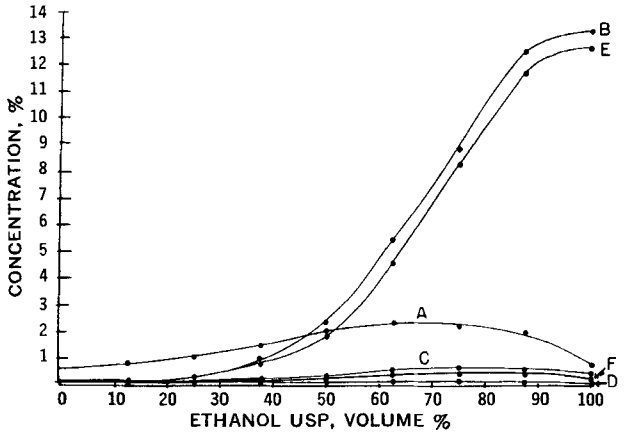

Figure 1—*Solubility profiles for single solutions of theophylline and phenobarbital, mixtures of the two compounds, and the previously isolated theophylline–phenobarbital complex in alcohol USP. Key: A, theophylline alone; B, phenobarbital alone; C, theophylline from previously isolated complex; D, theophylline from mixture; E, phenobarbital from mixture; and F, phenobarbital from previously isolated complex.*

made to a known volume. The absorbance was determined at 265.5 nm. *versus* diluted sulfuric acid in 1-cm. silica cells.

The chloroform layer was extracted with pH 11.0 buffer, and the aqueous extracts were combined and made to a known volume with buffer solution. The absorbance was determined at 240.5 nm. *versus* pH 11.0 buffer in 1-cm. silica cells.

Calculation of the theophylline and phenobarbital in the respective solutions was as follows: $Cu = Au/(A/c)$, where Au is the absorbance of the sample solution, Cu is its concentration, and A/c is the constant ratio of absorbance to concentration found with standard solutions at the same wavelength as the sample and under the same experimental conditions.

Preparation of Standards—Standard solutions were prepared which contained the following concentrations of theophylline and phenobarbital:

	Theophylline, mg./ml.	Phenobarbital, mg./ml.
Standard I	0.5	1.8
Standard II	1.0	12.0
Standard III	1.5	30.0
Standard IV	1.7	60.0
Standard V	1.3	120.0

Two milliliters of each standard solution was extracted and assayed as described under the assay procedure for the mixture. Absorbance and concentration values from the appropriate standard solutions were used as described previously to calculate the theophylline and phenobarbital in the mixture.

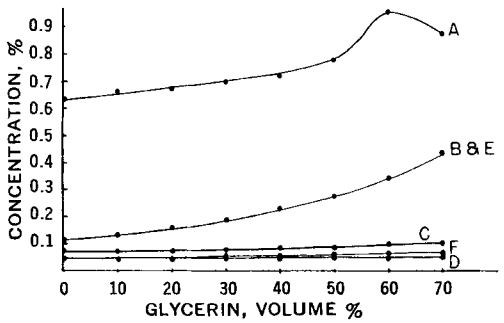

Figure 2—*Solubility profiles for single solutions of theophylline and phenobarbital, mixtures of the two compounds, and the previously isolated theophylline–phenobarbital complex in glycerin. Key: A, theophylline alone; B, phenobarbital alone; C, theophylline from the previously isolated complex; D, theophylline from the mixture; E, phenobarbital from the mixture; and F, phenobarbital from the previously isolated complex.*

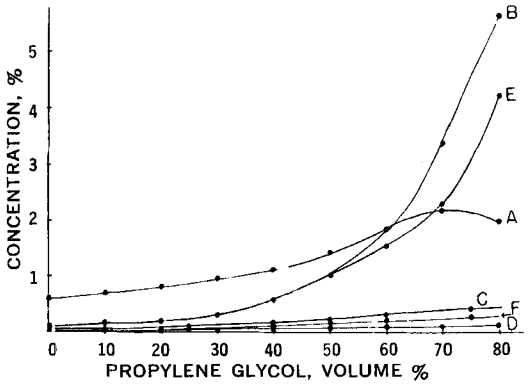

Figure 3—*Solubility profiles for single solutions of theophylline and phenobarbital, mixtures of the two compounds, and the previously isolated theophylline–phenobarbital complex in propylene glycol. Key: A, theophylline alone; B, phenobarbital alone; C, theophylline from the previously isolated complex; D, theophylline from the mixture; E, phenobarbital from the mixture; and F, phenobarbital from the previously isolated complex.*

The reported results are averages of at least duplicate runs on each material.

RESULTS AND DISCUSSION

Figures 1–4 show the equilibrium solubility profiles for single solutions of theophylline and phenobarbital, mixtures of the two compounds, and the previously isolated theophylline–phenobarbital complex in various aqueous organic solvent blends. Although the occurrence of multiple solubility maxima has been reported for certain compounds when increments of change in solvent composition or dielectric constant are small (5–7), the increments of organic solvent used in these experiments were too large to show whether multiple peak solubilities existed for these systems.

Since physical mixtures of theophylline and phenobarbital complex in solution (8), this complex was prepared, isolated, and equilibrated in each solvent blend, and the equilibrium solubilities were determined. Although the complex (T_2P) exists in solution as an equilibrium mixture of free (T,P) and interacting (T_2P) components, the absorption curve of the diluted sample was found to be a composite of the individual curves of the drugs in solution, and the total solubility of each component, regardless of molecular state, could be quantitated.

Only the solubility isotherms in ethanol–water are discussed in detail since these results are generally applicable to the other solvent systems studied.

The equilibrium solubilities of individual single solutions of theophylline and phenobarbital in hydroalcoholic blends are shown in Fig. 1. Examination of this figure shows that the solubility of phenobarbital (1-B) increases with increasing alcohol concentration, reaching maximum solubility in alcohol USP. Theophylline (1-A), on the other hand, reaches maximum solubility at about 60–65% by volume, alcohol USP, and further addition of alcohol results in a gradual decrease in solubility.

Also shown in Fig. 1 are the solubility isotherms for theophylline (1-C) and phenobarbital (1-F) obtained from equilibration of the previously prepared complex, where the concentration of theophylline and phenobarbital represents both free and complexed drug in solution. The equilibrium concentration of theophylline obtained from the dissolution of the known complex is very low compared with the concentration obtained when pure theophylline is equilibrated alone. Furthermore, although the solvent polarity has a significant effect on the solubility of the pure drugs, this effect is minimal with respect to the known complex. This finding is somewhat surprising since the theophylline–phenobarbital complex would be expected to be less polar than pure theophylline or phenobarbital. As a result, it would be reasonable to assume that the solubility of the known complex would increase significantly in solvents of lower polarity.

The data presented in the figures for the systems containing physical mixtures of theophylline and phenobarbital show that these solubility isotherms are complex and indicate that the individual

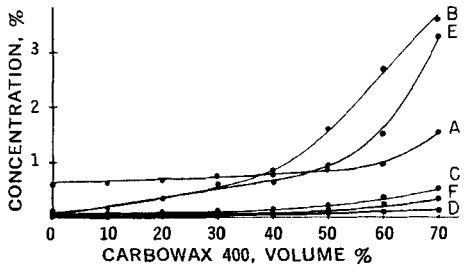

Figure 4—*Solubility profiles for single solutions of theophylline and phenobarbital, mixtures of the two compounds, and the previously isolated theophylline–phenobarbital complex in Carbowax 400. Key: A, theophylline alone; B, phenobarbital alone; C, theophylline from the previously isolated complex; D, theophylline from the mixture; E, phenobarbital from the mixture; and F, phenobarbital from the previously isolated complex.*

solubility profiles cannot be used to predict the equilibrium solubility of mixed components in systems where drug–drug interactions occur. In regard to the equilibration of the mixed components, it should be noted again that in each solvent system the amount of theophylline added was in excess of its equilibrium solubility. The total amount of phenobarbital added to these systems was such that: (*a*) all the theophylline present (in solution plus excess solid) would be complexed, and (*b*) the equilibrium solubility of phenobarbital would be exceeded in all of the solvent concentrations examined.

The solubility isotherm for theophylline obtained from equilibration of the mixtures in hydroalcoholic solvents is shown in Fig. 1-D. Since theophylline and phenobarbital do interact in solution to form a complex, the total amount of theophylline present in solution in the mixture samples is due primarily to the solubility of this complex. Therefore, it is interesting to compare this equilibrium theophylline concentration to the theophylline concentration present in systems containing only the isolated complex. Such a comparison shows that the concentrations in the two systems are significantly different. The concentration of theophylline in the systems containing only the isolated complex is higher and probably represents an apparent solubility due to the dissociated and undissociated complex. The lower solubility of theophylline in the mixture samples is probably the result of a decreased dissociation of the complex due to the excess phenobarbital present in solution. Therefore, the solubility of theophylline from the mixtures may represent the intrinsic solubility of the undissociated complex. The difference in solubility of theophylline obtained from equilibration of the known complex and the mixtures may be due to the fraction of the complex that dissociates in the former samples.

The equilibrium solubility of phenobarbital in the mixture samples is also shown in Fig. 1-E. Its solubility is lower than that obtained for the pure drug, which was unexpected since the mixture samples did contain excess phenobarbital. The factors responsible for this difference are not known, but several mechanisms may be operative. For example, although the complex solubility is very low, salting-out effects cannot be overlooked. Or, in the presence of excess phenobarbital, the possibility exists of changing stoichiometric ratios of the complex, as seen in Higuchi and Lach's studies (9). Or, perhaps, multiple equilibria may result in a multiordering.

Although all of the mixture samples contained excess phenobarbital, as previously discussed, it would be reasonable to assume that the reverse situation, systems containing enough theophylline to complex all the phenobarbital plus an amount in excess of its equilibrium solubility, would result in comparable solubility profiles.

From the results of this study, it would be expected that individual solubility profiles may be of no value in predicting the equilibrium solubility of mixtures of drugs, particularly when the drugs are capable of interacting to form a very insoluble complex. This can be illustrated by examination of the solubilities in 25% v/v alcohol USP. The equilibrium solubility of single solutions of theophylline and phenobarbital in this solvent is approximately 11 and 3 mg./ml., respectively. When a mixture of these compounds was equilibrated in the same solvent system, the apparent solubility of the complex which formed, calculated on the basis of theophylline in solution, was approximately 1 mg./ml. Furthermore, attempts to predict the solubility of this mixture, which contained excess phenobarbital, on the basis of the solubility of the known complex was also of little value, since the apparent solubility of a previously prepared complex in the same solvent system was found to be approximately 2.3 mg./ml. Therefore, the preparation of a formulation in which the solutes are totally soluble may present considerable difficulty, because the single drugs may be present in a concentration less than their saturation solubility but, because of the insoluble nature of the complex which may form, a precipitate results. The use of dielectric constants to predict solubility would probably, according to these preliminary data, lead to erroneous conclusions.

While this study reports results for saturated systems, similar results were obtained using more practical concentrations. For example, a 40% v/v alcohol USP solution containing 13 mg./ml. of theophylline and 0.8 mg./ml. of phenobarbital yielded a precipitate upon standing for a few hours. This occurred in spite of the fact that that individual components were soluble at this concentration level.

As previously mentioned, the results of this investigation were discussed only in regard to the hydroalcoholic systems. The results from the other solvent systems were qualitatively similar to the hydroalcoholic blends and, for this reason, were not discussed. The solubility studies were terminated in these blends at approximately 70–80% organic solvent by volume because the mixture samples became so viscous and contained so much precipitate that a clear solution could not be obtained for analysis.

Although only mixtures of two drugs were used in this study, the situation would probably become even more complicated if additional components are included that could also undergo complexation.

Further work is in progress on other multicomponent-containing systems where drug–drug interaction does not occur and in systems where such interactions occur, resulting in an increase in the solubility of the complex.

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Toxicological and Pharmacological Properties of Sea Pansy, *Renilla mulleri*

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Abstract □ *Renilla mulleri* specimens, collected from the Mississippi Gulf Coast, were homogenized and centrifuged, and the supernatant liquid was lyophilized. Mice injected with 100–250 mg./kg. i.p. of the lyophilized extract dissolved in phosphate buffer (pH 6.5) became lethargic, and most of them died within 24 hr. The LD_{50} in mice was 160 mg./kg. A dose of 10–20 mg./kg. i.v. produced a fall in blood pressure and a distinct bradycardia in anesthetized cats. These effects were not abolished by either bilateral vagotomy, atropinization, or adrenergic blockade. At higher doses, the extract produced irregularities of heart actions. In isolated rabbit heart preparations, the extract produced a decrease in the rate and amplitude of cardiac contractions and the coronary outflow. The extract had a relaxant effect on isolated rabbit duodenum, guinea pig ileum, and rat uterus. The effect of the extract on the blood pressure, heart, and isolated smooth muscles is due to a direct myotropic effect.

Keyphrases □ *Renilla mulleri*—toxicology, pharmacology □ Sea pansy—toxicology, pharmacology □ Toxicology—*Renilla mulleri* (sea pansy)

The class *Anthozoa* is comprised of two subclasses (1): the *Alcyonaria*, which includes the soft corals, sea fans, sea pens, and sea pansies; and *Zoantharia*, which includes the sea anemones and true corals.

A sea pansy colony consists of a flattened, kidney-shaped rachis, on the upper surface of which the polyps are born (2). The short-stemmed sea pansy, *Renilla mulleri*, is common in the northern Gulf of Mexico and extends southward to Brazil (3). There are no known published data on the biotoxicity of *Alcyonarians* (4). Therefore, this study was undertaken to investigate the toxicity of the sea pansy (*R. mulleri*) obtained from the Gulf of Mexico.

MATERIALS AND METHOD

R. mulleri[1] specimens were found to be abundant around Cat Island, Ocean Springs, Miss. They were taken at a salinity and temperature range of 24.9–30.0 p.p.t. and 15.0–24.9°, respectively. Twenty specimens, with total weight of 120 g., were homogenized in a chilled homogenizer for 10 min. The homogenate was centrifuged for 10 min. at 9000 r.p.m., and the supernatant liquid (light red in color) was lyophilized. The lyophilized material (dark red) was dissolved either in distilled water or 0.1 *M* phosphate buffer (pH 6.5) for pharmacological studies.

Toxicity Studies—General acute toxicity and various toxic manifestations were studied on male albino mice. Mice weighing 15–20 g. were randomly grouped with five animals in each group. They were administered intraperitoneally with the aqueous solution of the lyophilized material and housed in individual cages provided with food and water. The toxic symptoms and behavioral changes exhibited by the animals were observed for 48 hr. The LD_{50} was estimated according to the method of Horn (5).

Pharmacological Studies—*Locomotor Activity*—Albino mice[2] were used for motor activity studies. An automatic timer provided a 12-hr. dark, 12-hr. light schedule in the room housing the animals; air conditioning maintained room temperature between 22 and 23° (72 and 76°F). All mice were allowed to adapt to these environmental conditions for a minimum of 7 days before being used in the experiment.

Measurement of spontaneous locomotor activity was recorded by means of three photocell activity cages (actometers)[3]. Each actometer was covered with a sound-attenuating box. Electromechanical counters registered an activity count each time the animal traversed one-sixth the circumference of the runway. By means of a stopwatch and the counter, counts were taken every 15 min. for 2 hr. The statistical evaluation of the data in this study involved use of one-factor analysis of variance (6).

Blood Pressure and Respiration—The effects of the toxin on blood pressure, heart, and respiration were studied in anesthesized cats. Overnight fasted cats (2.5–3.5 kg.) of both sexes were anesthetized with 35–40 mg./kg. sodium pentobarbital intraperitoneally. The right common carotid artery was exposed at the neck region and cannulated with a polyethylene cannula, which was connected to a previously calibrated (linear core) pressure transducer[4] for the blood pressure recording. Respiration was recorded from chest electrodes, which were connected to an impedance pneumograph[4]. While recording respiration, the same electrodes were connected to an ECG preamplifier for obtaining standard Lead II electrocardiograms. The ECG preamplifier was calibrated in such a manner that each deflection represented 1 mv. in amplitude and 10 mm. in height. The pressure transducer, pneumograph, and ECG preamplifier were connected to a physiograph[5] by standard transducer cables for recording. The ECG patterns were interpreted according to the description of Burch and Winsor (7) and Friedman (8).

All injections were made through the right femoral vein, which was exposed and cannulated with a venous polyethylene cannula. The cholinergic activity of the toxin was studied in bilaterally vagotomized and completely atropinized animals; *e.g.*, both vagi were cut at the cervical level and the animals were injected with atropine (3 mg./kg. i.v.). Complete parasympathetic blockade was ensured when no response was obtained with a test dose of acetylcholine (5 mcg./kg. i.v.).

Autonomic ganglia were blocked by the administration of hexamethonium bromide (5 mg./kg. i.v.) in three successive doses. The animals were injected with phenoxybenzamine (3 mg./kg.) for complete blockade of peripheral sympathetic α-receptors, which was ensured by observing reversal of the action of epinephrine (5 mcg./kg. i.v.) on the blood pressure. β-Receptors were blocked by dichloroisoproterenol (4 mg./kg. i.v.). For determining the action on the baroreceptors of the carotid sinus, carotid occlusion was performed three times for 30 sec., before and after the administration of the toxin.

Isolated Heart Preparation—The effect of the toxin on isolated rabbit heart was studied to investigate the effects on the cardiac muscle, using Langendroff's technique as modified by Anderson and Craver (9). The chest was opened and the heart removed. It was placed into an oxygenated Locke solution and gently squeezed to remove blood from the aorta. The aorta was freed from its attachment to the pulmonary artery, and a cannula was tied to it. After the cannula was fixed in place, a hook with thread was fixed on the tip of the ventricle and the heart perfused. The thread was passed through a pulley and attached to a myograph[6], which was connected to the physiograph by standard transducer cables for recording. A constant pressure was maintained throughout the perfusion.

[1] Identified by Dr. Gordon Gunter, Gulf Coast Research Laboratory, Ocean Springs, Miss.
[2] Southern Animal Farm, Prattville, Ala.
[3] Model R064, Woodard Research Corp., Herndon, Va.
[4] E & M Instrument Co., Inc., Houston, Tex.
[5] Model DMP-4A, NARCO.
[6] Model B-655, E & M Instrument Co., Inc., Houston, Tex.

Table I—Effects of *R. mulleri* Toxin on Locomotor Activity

Minutes	Activity Counts/15 min.[a] I (20 mg./kg.) Control	I (20 mg./kg.) *Renilla*	II (40 mg./kg.) Control	II (40 mg./kg.) *Renilla*
15	453.6 ± 82.6	178.5 ± 35.7[b]	592.1 ± 83.8	206.6 ± 42.7[c]
30	317.1 ± 75.4	83.1 ± 36.6[b]	343.0 ± 41.0	135.0 ± 42.9[c]
45	269.8 ± 45.6	81.8 ± 33.6[b]	256.6 ± 24.4	62.0 ± 20.6[c]
60	321.6 ± 70.8	72.8 ± 17.7[b]	202.3 ± 17.7	78.3 ± 25.7[c]
75	201.3 ± 38.6	71.3 ± 15.6[b]	166.8 ± 18.1	65.6 ± 18.2[c]
90	221.5 ± 31.0	55.3 ± 11.0[c]	169.6 ± 21.5	50.0 ± 23.4[c]
105	213.5 ± 60.7	50.3 ± 12.3[b]	158.6 ± 25.8	26.1 ± 17.4[c]
120	203.0 ± 32.9	31.1 ± 7.9[c]	197.3 ± 14.0	16.0 ± 3.3[c,d]
Total	2266.5 ± 30.0	622.5 ± 119.7[c]	2080.0 ± 153.8	639.8 ± 146.3[c]

[a] Each value represents the mean ± the standard error for a group of six mice. [b] $p < 0.01$ when compared with the control group. [c] $p < 0.05$ when compared with the control group. [d] $p < 0.05$ when 20-mg./kg. dose was compared with 40-mg./kg. dose.

Isolated Hind Quarter of the Rat—The action of the toxin on the blood vessels was recorded on the hind-quarter preparation of albino rats (10). The animals were sacrificed and eviscerated. A cannula was tied in the abdominal aorta, and the body wall and vertebral column were cut above the point of cannulation.

The hind quarter of the rat was then laid on a piece of muslin attached to a wire, which rested on a glass funnel. A marriotte bottle full of Ringer's solution was connected by a rubber tubing to the cannula. The vessels of the rat were perfused until the perfusate was free from blood. The outflow from the vessels passed through the muslin and the funnel dropwise and was measured by counting the drops per minute. All injections were made directly into the connecting rubber tubing.

Isolated Smooth Muscle—Rabbits weighing 1–2 kg. and guinea pigs weighing 300–500 g. of both sexes were used. The animals were sacrificed, and the intestines were exposed. An actively contracting loop of ileum (guinea pig) or duodenum (rabbit), 4–6 cm. in length, was selected and cut. This section was suspended in a 25-ml. bath containing Tyrode's (guinea pig ileum) or Ringer's solution (rabbit duodenum). One end of the loop was tied to the lower hook of the holder and the other end was attached to the myograph. The preparation was suspended in an organ bath of Tyrode's solution, which was maintained at 37° and oxygenated with 95% O_2 and 5% CO_2.

RESULTS

Toxicity Studies—Mice injected with various doses of the toxin became lethargic within a few minutes. The animals remained motionless and later developed respiratory distress; some died within 30 min. The LD_{50} was estimated to be 160 mg./kg.

Pharmacological Studies—*Locomotor Activity*—As shown in Table I, *Renilla* toxin reduced the motor activity of mice. The absorption of the toxin seemed to be rapid, as was indicated by the significant reduction in motor activity in the first 15 min. Both 20 and 40 mg./kg. significantly reduced the motor activity; however, there was no significant difference between the two doses except at the 120-min. interval.

Effect on Blood Pressure and Respiration of the Cat—Five experiments were carried out on anesthetized cats with different doses of toxin, ranging from 20 to 40 mg./kg. A 20–60 mm. Hg decrease in the arterial blood pressure was observed (Fig. 1A). There was a marked initial fall in the blood pressure, which then stabilized at a level of 10–20 mm. Hg below the baseline. A single dose produced a persistent fall in blood pressure which lasted for 7 hr. The decrease in the blood pressure showed a consistent dose–response relationship. Slowing of the pulse rate was a consistent observation, occurring within 5 sec. after the injection of the toxin (Fig. 1A). All of the doses caused a marked change in respiratory rate, which remained above the normal level throughout the experiment (Fig. 1B).

The fall in blood pressure was not modified by bilateral vagotomy, atropinization, ganglionic blockade, sympathetic blockade, or treatment with antihistaminic agents. A similar blood pressure response to carotid occlusion was obtained before and after the administration of the toxin.

Effect on Electrocardiogram of the Cat—With a 20-mg./kg. dose, there was an increase in the P-R interval, R wave amplitude, and S-T segment, and there was an inversion of the T wave (Fig. 1D). When a 30-mg./kg. dose was used, there was a further increase in the R wave amplitude and P-R interval, depression in the S-T segment, and deep inversion of the T wave (Fig. 1E). This was followed by a 1:3 complete heart block with 40 mg./kg. (Fig. 1F).

Effect on Isolated Rabbit Heart—The control values (five observations) for coronary flow rate (2.8 ± 0.4 ml./min.), heart rate (101 ± 7 beats/min.,), and amplitude of cardiac contractions (21.0 ± 0.5 mm. deflection) were consistent throughout the experiment. The injection of the toxin was made into the coronary perfusion

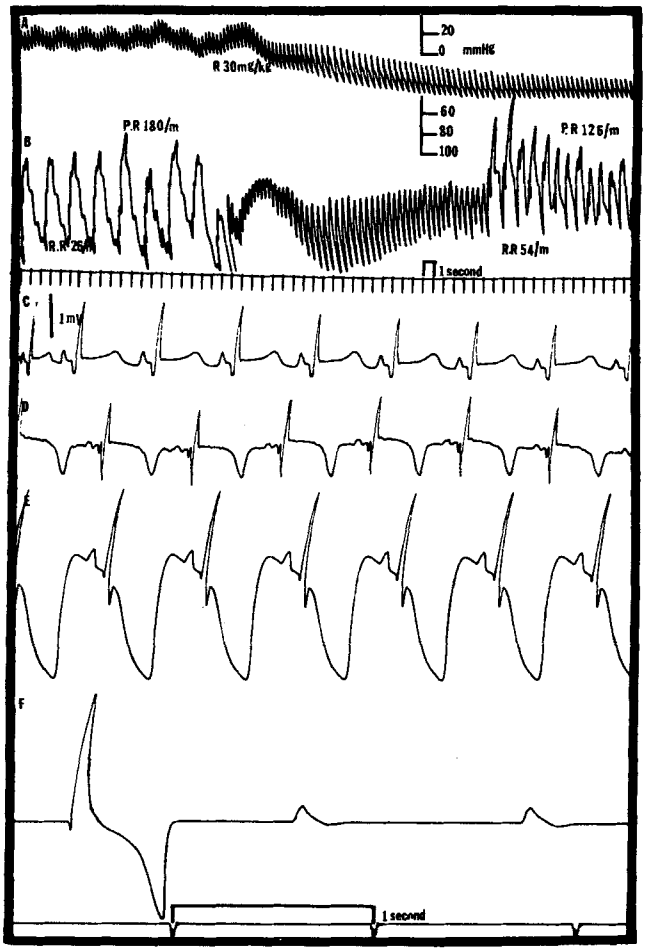

Figure 1—*Effect of* R. mulleri *toxin on: A, blood pressure*; B, respiration; C, ECG control; D, E, F, various changes in ECG at the dose levels of 20, 30, and 40 mg./kg. of the toxin. Key: R =* R. mulleri *toxin, PR = pulse rate, RR = respiration rate, and mv. = millivolt. *No initial blood pressure is shown on the scale; instead the scale is divided in such a manner that initial blood pressure reads as 0.00 mm. Hg. The pressure transducer was calibrated for a 100-mm. Hg rise or fall in the blood pressure, which is shown on the scale.*

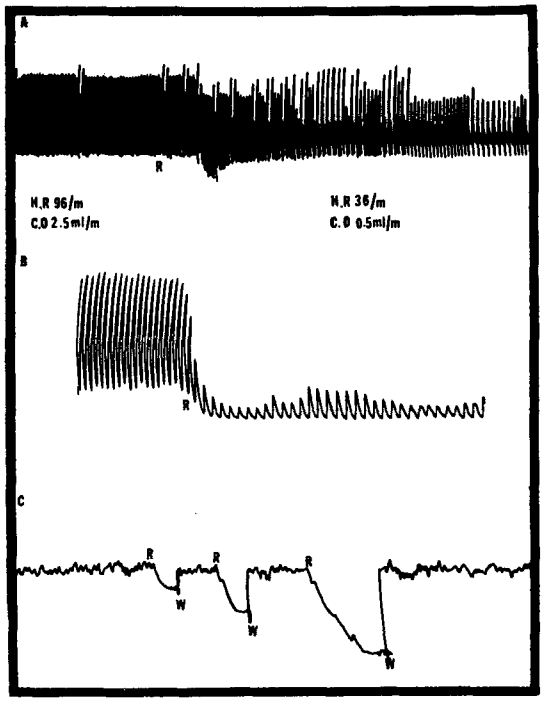

Figure 2—*Effects of sea pansy* (R. mulleri) *extract on the isolated rabbit heart, isolated rabbit duodenum, and isolated guinea pig ileum. Key: A = effect of 2-mg. dose of the toxin injected into the perfusion fluid of the isolated heart preparation; B = effect of 200 mcg./ml. of the toxin on the isolated rabbit duodenum; C = effect of 100, 200, and 300 mcg./ml. of the toxin on the isolated guinea pig ileum; R* = R. mulleri *extract; HR = heart rate; CO = coronary outflow; /m = per minute; and W = wash.*

circuit. With a dose of 1 mg., there was an immediate decrease in the coronary outflow and heart rate to 2.1 ± 0.2 ml./min. and 72 ± 6 beats/min., respectively; however, there was no change in the amplitude. The effects persisted over 10 min. and gradually returned to normal.

A 2-mg. dose had a more persistent effect, lasting for more than 30 min. (Fig. 2A). The changes in the coronary outflow and heart rate from control were 2.7 ± 0.3 ml./min. and 38 ± 4 beats/min., respectively. The amplitude fluctuated without any regular pattern, probably because of some irregular beats of the heart at this dose level. When a 3-mg. dose was used, there were drastic changes in all three parameters, resulting in irregular ventricular contractions and decreased amplitude; ultimately the heart stopped within 10 min.

Effect on Isolated Hind Quarter of the Rat—Two doses of the toxin, 2 and 4 mg., injected directly into the perfusate caused a significant vasodilation. The increase in the number of drops from control (12 ± 2 drops/min.) was 19 ± 3 and 27 ± 2 drops/min., respectively. The effect of the 4-mg. dose lasted for more than 30 min.

Effect on Isolated Rabbit Duodenum—The toxin in the 200-mcg./ml. dose reduced the pendular movements and relaxed the rabbit duodenum. The effect was so persistent (Fig. 2B) that repeated washings could not bring the intestine strip back to the baseline.

Effect on Isolated Guinea Pig Ileum—The toxin at doses of 100, 200, and 300 mcg./ml. relaxed the guinea pig ileum (Fig. 2C). Doses higher than 300 mcg./ml. relaxed the muscle to such an extent that repeated washings could not bring it back to normal.

DISCUSSION

The *Renilla* toxin was found to be toxic to mice, having an LD_{50} of 160 mg./kg. In gross behavioral observation and motor activity studies, the toxin appeared to have CNS depressant action.

The toxin at various dose levels produced a persistent fall in blood pressure. This effect was not blocked by cholinergic, adrenergic, and ganglionic blockers, nor could antihistaminic agents abolish the effect. The speculation that the fall in the blood pressure produced by the toxin was due partially to a direct effect on the peripheral blood vessels was confirmed by the vasodilatory effect observed in the studies on isolated hind quarter of the rat.

ECG recordings from cats injected with the toxin showed prolongation of the P-R interval, which was due to a slow transmission of impulses from the A-V node. A prolonged Q-R-S complex suggested a depression of the rate of ventricular depolarization, and a depressed T wave indicated cardiac ischemia. Higher doses depressed the Q-R-S complex and further inverted the T wave. This was followed by 1:2 and 1:3 complete atrioventricular block, which was due to extremely poor conduction of impulses from the atrium into the ventricle.

On the isolated heart preparation, the toxin in smaller doses reduced the heart rate, amplitude of contraction, and coronary outflow. At higher doses, the toxin caused irregularities of cardiac contractions. The fall in the blood pressure seems to be attributable to two factors: (*a*) decreased cardiac output caused by the effect of the toxin on the heart, depressing contractility, excitability, and automaticity; and (*b*) vasodilation, as indicated by an increase in outflow in the rat's hind-leg preparation.

The toxin damaged the heart at higher doses, as indicated in both *in vivo* and *in vitro* experiments. It appeared that the toxin acted both on the conduction system and cardiac muscle, as indicated by cardiac ischemia and complete heart block.

The toxin relaxed rabbit duodenum and guinea pig ileum, but no effect on the rat uterus or frog skeletal muscle was indicated.

SUMMARY

R. mulleri (sea pansy) was toxic to mice, having an LD_{50} of 160 mg./kg. The toxin reduced the motor activity in mice. It produced a persistent fall in the blood pressure, had a vasodilatory effect on the isolated hind quarter of the rat, and caused relaxation of extravascular smooth muscles. The toxin caused cardiac ischemia and 1:2 and 1:3 complete atrioventricular block in intact animals, as indicated by ECG recordings. The fall in blood pressure could be attributed to two factors: (*a*) decreased cardiac output caused by the effect of the toxin on the heart, depressing contractibility, excitability, and automaticity; and (*b*) vasodilation, as indicated by increased outflow in the rat hind-quarter perfusion test.

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Numerical Solution of Nonlinear Pharmacokinetic Equations: Effects of Plasma Protein Binding on Drug Distribution and Elimination

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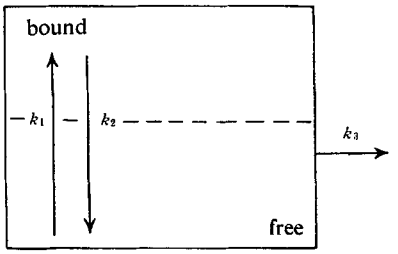
Abstract □ Numerical methods are described for the solution of the differential equations arising from nonlinear binding of drugs to plasma proteins, assuming one- and two-compartment pharmacokinetic models. These numerical methods should be of general utility in studying multicompartment models. The application of these methods to several systems, both hypothetical and real, suggested that binding of drugs to plasma protein should cause detectable nonlinearity in the log *C versus t* plot only if doses are sufficiently high to approach saturation of binding sites or if the number of binding sites in plasma is small. The effect of competition for binding sites in plasma on drug concentrations in tissues was studied by assuming a two-compartment model. It appears that, unless the tissue distribution volume is quite small, competition for binding sites would not be expected to have a large effect.

Keyphrases □ Pharmacokinetics—quantitation of nonlinear equations, effect of plasma protein binding on drug distribution and elimination □ Drug–plasma protein binding—quantitation of nonlinear equations □ Binding site competition—drug–plasma, two-compartment model

It is commonly accepted that binding of drugs to plasma proteins plays an important role in the pharmacokinetics and pharmacology of tightly bound substances. Classes of compounds for which protein binding is considered significant include the penicillins (1), sulfonamides (2), anticoagulants (3, 4), nonsteroid anti-inflammatory agents (5), several compounds of interest in cancer chemotherapy (6, 7), and certain endogenous hormones such as thyroxine (8) and the corticosteroids (9). In particular, a great deal of emphasis has been laid on the possibility of adverse drug reactions arising from competition for plasma protein binding sites. Brodie (10) called attention to several cases of drug toxicity where changes in binding may be implicated. Among these, the interactions of warfarin and several sulfonamides with phenylbutazone (3, 4, 11) and the observation of sulfonamide-related kernicterus in the newborn (12–15) are particularly noteworthy.

Despite the long-standing interest in this problem, quantitative consideration of the implications of protein binding in pharmacokinetics has been limited. The lack of closed solutions to the nonlinear differential equations generated by a rigorous statement of the problem has led workers in the field either to adopt highly limited simplifying assumptions or to pursue indirect solutions. The most obvious simplification is to assume a linear relationship between bound and free drug. However, some of the most interesting cases involve drug levels that approach or exceed the binding capacity of the plasma proteins, where such a relationship will not hold. Krüger-Thiemer *et al.* (16) presented some more sophisticated derivations of expressions relating plasma binding to pharmacokinetics, but they found it necessary to assume either a one-compartment model or instantaneous distribution of drug between two compartments. Even in the simple two-compartment case, the solution was presented in an inverse form which does not allow calculation of drug concentrations at specified times. Martin (17, 18) discussed some implications of plasma binding equilibria on drug distribution but he did not treat the kinetic problems in detail.



Scheme I

This article reports a method of attack on the problem which employs approximate numerical integration methods to generate solutions to nonlinear pharmacokinetic equations. In principle, the method outlined for the two-compartment case should be applicable to any multicompartment pharmacokinetic model.

The format adopted for presentation of the results and conclusions is as follows. First, the theoretical basis and assumptions used to calculate concentration–time curves, assuming one- and two-compartment models with binding, are presented. Next, the behavior of the one-compartment model with binding is explored, using a hypothetical drug with certain defined properties. This model is also used to study the pharmacokinetic properties that might be expected if the drug is bound to an easily saturable specific carrier protein. Finally, the behavior of the two-compartment model with binding is explored. With this model, the problem of potential changes in pharmacokinetic and tissue levels resulting from displacement of drug from plasma binding sites is studied using data from the literature.

THEORETICAL

The fundamental assumptions used in this treatment are:

1. Binding occurs only to plasma proteins and follows simple saturation kinetics. The binding process can be described by a single macroscopic dissociation constant.
2. Binding equilibrium is achieved virtually instantaneously with respect to distribution and elimination.
3. Aside from binding, all other processes (*i.e.*, distribution and elimination) are linear.
4. Distribution and elimination processes operate only on free drug.

One-Compartment Model—In Scheme I, let C_b = concentration of bound drug, C_f = concentration of free drug, C_t = total con-

centration of drug, P = total concentration (free plus bound) of protein binding sites, and $K_d = k_2/k_1$, *i.e.*, the dissociation constant of the drug–protein complex.

Since binding follows simple saturation kinetics, the concentration of bound drug can be described by Eq. 16 of Klotz (19). In the notation above:

$$\frac{C_b}{P} = \frac{(1/K_d)C_f}{1 + (1/K_d)C_f} \qquad \text{(Eq. 1)}$$

By rearranging terms:

$$C_b = \frac{PC_f}{K_d + C_f} = C_t - C_f \qquad \text{(Eq. 2)}$$

If $C_f \ll K_d$, Eq. 2 reduces approximately to:

$$C_b = \frac{PC_f}{K_d} \qquad \text{(Eq. 3)}$$

Thus, at sufficiently low values of C_f, there is approximately a linear relationship between bound and free drug. Rearrangement of Eq. 2 yields a quadratic expression in C_f, whose positive root is:

$$C_f = {}^1/_2[-(P + K_d - C_t) + \sqrt{(P + K_d - C_t)^2 + 4K_dC_t}] \qquad \text{(Eq. 4)}$$

Since transfer processes operate only on free drug:

$$\frac{dC_t}{dt} = -k_3C_f = -\frac{k_3}{2} \times [-(P + K_d - C_t) + \sqrt{(P + K_d - C_t)^2 + 4K_dC_t}] \qquad \text{(Eq. 5)}$$

While no closed solution of Eq. 5 is obtained, numerical solutions are simple and convenient. Given a zero-time value of C_t and a time interval, τ, the fourth-order Runge-Kutta approximation is used (20) to calculate a value of C_t at $t = \tau$. The procedure is then repeated starting with this calculated C_t to obtain a value at $t = 2\tau$; in this way, an entire concentration–time curve may be constructed. At each time point, Eq. 4 may be used to calculate C_f from C_t.

Two-Compartment Model—The terms in Scheme II are as already defined, except that V_1 and V_2 are the distribution volumes of the inner and outer compartments, respectively. C_2 is the drug concentration (presumed all free) in the outer compartment, and C_t, C_f, and C_b refer to the inner compartment. If $X_i (i = 2, f, t \ldots)$ is the total amount of drug in a given state and, therefore, $C_i = X_i/V_1$ or V_2, one may write:

$$\frac{dX_t}{dt} = k_{21}X_2 - k_{12}X_f - k_3X_f \qquad \text{(Eq. 6)}$$

$$\frac{dX_2}{dt} = k_{12}X_f - k_{21}X_2 \qquad \text{(Eq. 7)}$$

With the further simplifying assumption (21) that:

$$k_{21} = \frac{V_1}{V_2}k_{12} \qquad \text{(Eq. 8)}$$

the concentration changes in the inner and outer compartments are then given by:

$$\frac{dC_t}{dt} = k_{12}(C_2 - C_f) - k_3C_f \qquad \text{(Eq. 9)}$$

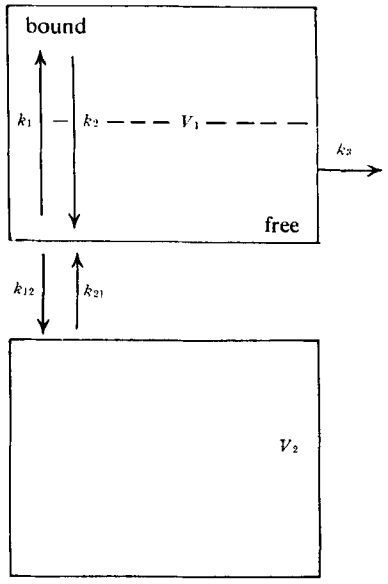
$$\frac{dC_2}{dt} = k_{12}\frac{V_1}{V_2}(C_f - C_2) \qquad \text{(Eq. 10)}$$

Equations 4, 9, and 10 in the three unknowns, C_t, C_f, and C_2, do not yield a closed solution but, once again, numerical methods are available. A solution by successive approximations was chosen, making use of the relationships:

$$\frac{dC_f}{dt} = \frac{dC_t}{dt}\left(1 + \frac{C_t - P + K_d}{\sqrt{(P + K_d - C_t)^2 + 4K_dC_t}}\right) \qquad \text{(Eq. 11)}$$

$$\frac{d^2C_t}{dt^2} = k_1\left(\frac{dC_2}{dt} - \frac{dC_f}{dt}\right) - k_2\frac{dC_f}{dt} \qquad \text{(Eq. 12)}$$

$$\frac{d^2C_2}{dt^2} = k_1\frac{V_1}{V_2}\left(\frac{dC_f}{dt} - \frac{dC_2}{dt}\right) \qquad \text{(Eq. 13)}$$



Scheme II

To start the solution, the first and second derivatives of the three unknowns are calculated at $t = 0$, using Eqs. 9–13 and the initial values $C_t(0) = C_0$ and $C_2(0) = 0$, where C_0 is the administered dose divided by V_1. Taking a time interval τ, C_t and C_2 are estimated at $t = \tau$ by:

$$C(\tau) = C(0) + \tau\frac{dc}{dt} + \frac{\tau^2}{2}\frac{d^2C}{dt^2} \qquad \text{(Eq. 14)}$$

and, similarly, at $t = \tau/2$. These initial estimates are refined by the method of successive approximations (20), evaluating the integrals by Simpson's rule. The final values at $t = \tau$ are then used to obtain values at $t = 2\tau$ by the application of Simpson's rule alone, and the changes of C_t, C_f, and C_2 with time are thus constructed point by point.

FORTRAN programs were written for the Honeywell time-sharing computer system to calculate concentration–time curves, assuming either a one- or two-compartment model, from input values for the model parameters. Several convenient features were incorporated into the programs, notably the capability of handling pharmacokinetic data expressed on a weight basis along with binding data expressed on a molar basis, and a provision for altering the binding parameters at any specified time along the curve to mimic the effect of competition for binding sites.

The one-compartment calculations are quite straightforward, but the two-compartment calculations involve considerable difficulty, chiefly because calculated concentration values tend to oscillate, instead of behaving monotonically, with time. Even with small values of τ and stringent criteria of convergence (currently, 1 part in 10^6 is being used), calculated concentration values begin to oscillate with time after a comparatively short distance along the concentration curve. More sophisticated methods of numerical integration probably would minimize this tendency but would require considerably more computer time.

RESULTS AND CONCLUSIONS FOR A ONE-COMPARTMENT MODEL WITH BINDING

To generate theoretical curves for the one-compartment model, the case of a hypothetical drug with the following properties was considered first:

1. The drug has a molecular weight of 150.
2. Drug binds only to serum albumin, whose concentration is 4.4% and whose molecular weight is 67,000.
3. At sufficiently low drug concentrations, the drug is 89% bound.
4. The distribution volume is 50 ml./kg.
5. Free drug has a half-life of 30 min.

Concentration–time curves for a wide range of drug doses were

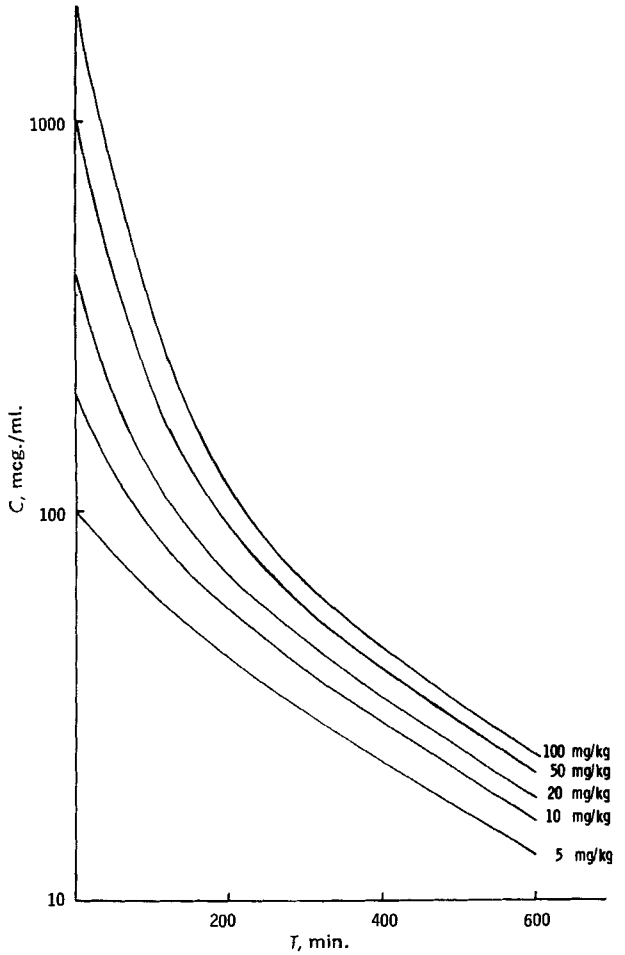


Figure 1—*Calculated concentration–time curves in plasma of a hypothetical drug of mol. wt. 150, 89% bound to serum albumin at low concentrations, with one binding site per albumin molecule. Doses are shown with each curve, and other assumptions are given in the text.*

calculated assuming one binding site (Fig. 1) or 10 binding sites (Fig. 2) per molecule of serum albumin.

At sufficiently low doses, the relationship between bound and free drug is linear and there is an apparent first-order elimination of drug. The rate constant of this elimination process is simply the rate constant for free drug multiplied by the ratio of free drug to total drug:

$$k_{app.} = k_3 \frac{C_f}{C_t} \qquad \text{(Eq. 15)}$$

Regardless of the size of the initial dose, the concentration–time curve eventually approaches this simple exponential decay as the drug concentration falls. However, at high initial doses, there is a phase of more rapid elimination due to the presence of drug beyond the binding capacity of the serum albumin.

With the present assumptions, it appears that substantial curvature of log concentration–time plots is seen only if initial doses are large or if the number of binding sites on the protein is small, permitting drug to exceed the binding capacity of the protein. It seems reasonable to expect that blood levels of many drugs will fall in the region where the binding relationship is linear (Eq. 3), at least in the absence of competitive binding by another species.

It is not uncommon to interpret curved log concentration–time plots as indicative of a multicompartment distribution of drug. While the equations derived when binding is not linear are not multiexponential in form, it is of interest to determine how closely the calculated curves for such a situation can be fitted by a multiexponential function. Figure 3 shows the result of an attempt to fit the 100-mg./kg. curve of Fig. 2 to the expression derived by assuming a two-compartment model without binding, using an unweighted least-squares method similar to that of Wagner and Metzler (22). The individual points are those from which the curve of Fig. 2 was constructed, calculated by the nonlinear binding program, while the smooth curve is the least-squares fit to the biexponential, with the kinetic parameters as shown. In an experimental situation, the noise inherent in the data would most likely mask the small systematic deviation, giving the appearance of an even closer fit. Several experiments of this type revealed no simple relationship between the binding parameters used to generate the points of Fig. 3 and the parameters calculated from a least-squares fit to the standard two-compartment model, except that the fitted inner compartment volume was found to be the true distribution volume. In any case, extreme caution is indicated in applying multicompartment models to systems where the binding characteristics are unknown, since nonlinear binding of drug to protein may mimic multicompartment distribution quite closely.

Although, in the case of drugs that bind to serum albumin, binding sites may be present in excess, in several systems a drug is bound to a specific carrier protein present in low concentration in the plasma. Concentration–time curves for two such compounds—aldosterone and thyroxine—were calculated, using literature values for the binding parameters and assuming doses within a reasonable pharmacologic range. To simplify calculations, it was assumed that aldosterone is bound only to transcortin and that thyroxine is bound only to thyroxine-binding globulin, ignoring binding to other plasma proteins. A half-life of 30 min. is arbitrarily assumed for free drug in both cases. These calculations are an attempt to model only one facet of the pharmacokinetics of these substances. Such important features as true elimination rates and rates of endogenous synthesis are not considered here; hence, the calculated curves are best used

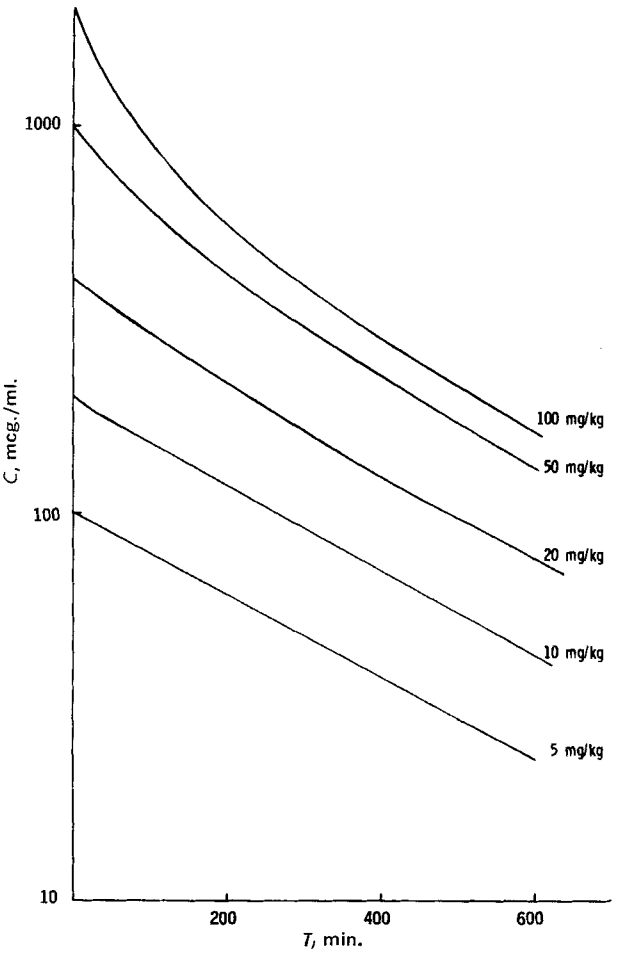


Figure 2—*Calculated concentration–time curves in plasma of the drug illustrated in Fig. 1, assuming 10 binding sites per albumin molecule. Doses are shown with each curve, and other assumptions are given in the text.*

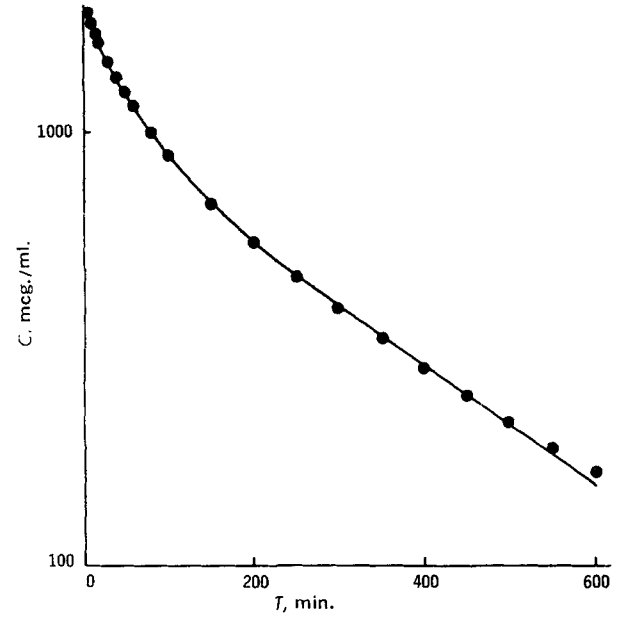


Figure 3—*Fitting of the 100-mg./kg. curve of Fig. 2 to a two-compartment model without binding. Points are taken from Fig. 2; the smooth curve is the least-squares fit to the two-compartment model. Fitted values of the apparent constants are: distribution half-time = 120 min., elimination half-time = 130 min., inner compartment volume = 50 ml./kg., and outer compartment volume = 25 ml./kg.*

merely to point out some possible features of the systems to which plasma protein binding may contribute.

For aldosterone, the assumed values (9) are:

1. Transcortin concentration = 2.5 mg./100 ml.
2. Transcortin molecular weight = 45,000.
3. Association constant for single binding site = 6.5×10^6.
4. Aldosterone molecular weight = 360.

Calculated concentration–time curves for aldosterone doses of 0.02–0.2 mg. are shown in Fig. 4. While a slight curvature is discernible at the high doses, experimental data would be expected to fall almost on a straight line. For all practical purposes, despite the limited amount of transcortin present in plasma, aldosterone can be expected to fall within the range where the binding relationship is linear at reasonable dose levels.

Thyroxine, on the other hand, appears to exceed the binding capacity of thyroxine-binding globulin at high doses. The assumed values (8) are:

1. Thyroxine-binding globulin concentration = 2 mg./100 ml.
2. Thyroxine-binding globulin molecular weight = 50,000.
3. Association constant for single binding site = 5×10^9 (*i.e.*, thyroxine 99% bound in the range where the binding relationship is a linear one).
4. Thyroxine molecular weight = 777.

Curves for doses of 0.2 and 2 mg. are shown in Fig. 5. The higher dose shows extremely sharp curvature in the concentration–time curve; in fact, unless measurements are made at very early times, it would be quite easy to miss the nonlinear portion of the curve. If only the linear portion is seen, extrapolated zero-time concentrations would be far lower than the true initial concentration of thyroxine, and the distribution volume would appear to increase with increasing dose. It must be reemphasized that, because of the arbitrary choice of an elimination half-time for aldosterone and thyroxine, the curves in Figs. 4 and 5 could not be expected to fit the true situations exactly. However, the general shape of these curves should indicate some points of interest and significance in the expected pharmacokinetics of these substances.

RESULTS AND CONCLUSIONS FOR A TWO-COMPARTMENT MODEL WITH BINDING

The effect of plasma protein binding on the distribution of drugs into tissues has been the subject of much consideration. In qualitative terms, it was often noted that an increase in the proportion of free drug in plasma results in more rapid elimination and in a rise

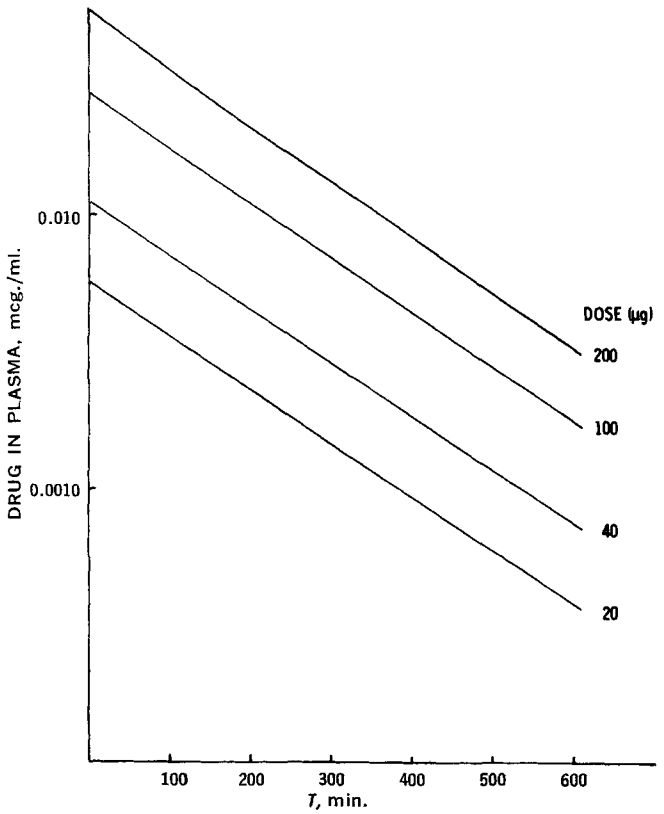


Figure 4—*Calculated concentration–time curves in plasma of aldosterone, using the assumed values given in the text. Doses are shown with each curve.*

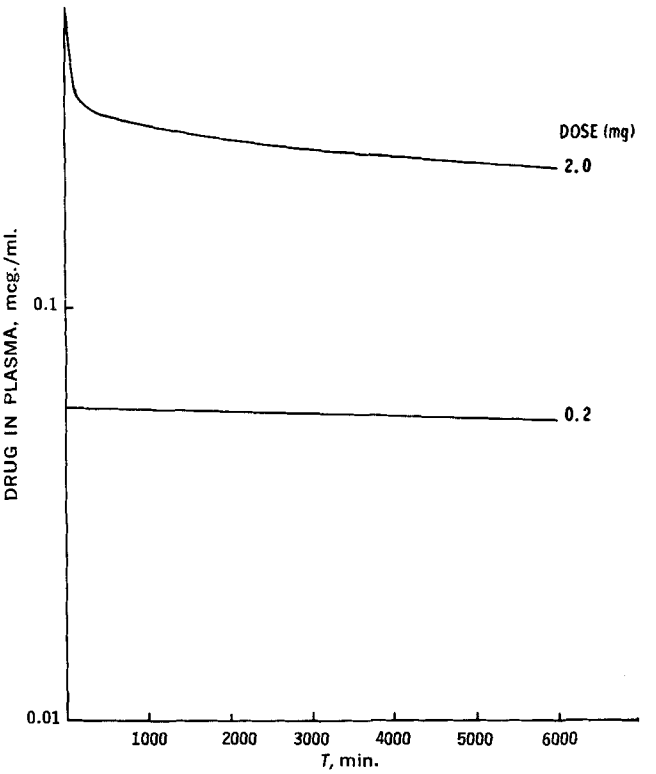


Figure 5—*Calculated concentration–time curves in plasma of thyroxine, using the assumed values given in the text. Doses are shown with each curve.*

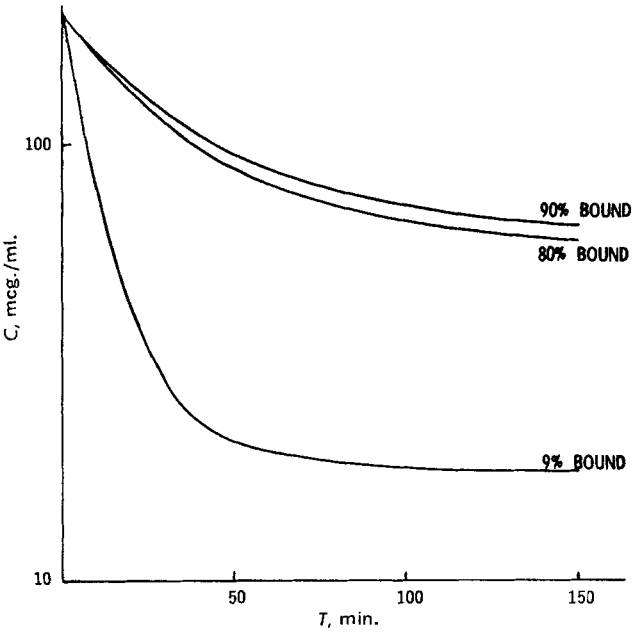


Figure 6—*Calculated concentration–time curves in plasma of a 10-mg./kg. dose of hypothetical drug of mol. wt. 150 in the two-compartment system. The inner distribution volume is 50 ml./kg.; the outer is 600 ml./kg. The proportion of binding in the linear phase is shown with each curve.*

in drug concentration in tissues (10, 17, 23). A decrease in the proportion of bound drug from 90 to 80%, for example, doubles the plasma concentration of free drug. Thus, it was suggested (10) that even fairly small competitive effects could be extremely important in changing drug levels in tissues.

This mechanism of changing tissue levels of drug, however, may actually be limited to a relatively small number of special cases. The crucial variable is the ratio of tissue distribution volume to plasma volume—in the notation used for the two-compartment case, V_2/V_1. If V_2/V_1 is large, the total amount of drug liberated from plasma proteins, even with relatively large shifts in binding, is insufficient to cause a large concentration change in the tissue volume into which it is diluted.

This point is illustrated using the hypothetical drug of mol. wt. 150 whose behavior in the one-compartment system was already described. In addition to the previous assumptions, a distribution half-time of 5 min. and an outer compartment volume of 600 ml./kg., a reasonable estimate for nonplasma body water, were assumed. The dose is 10 mg./kg., and there are 10 binding sites per serum albumin molecule. The computer program used to calculate concentration–time curves from the two-compartment model does not incorporate the equations for binding competition. Therefore, three sets of independent calculations were carried out, each assuming a different binding constant, to show the effect of changing a system in which drug is 90% bound in the region where the linear binding relationship of Eq. 3 holds to one in which drug is 80 or 9% bound.

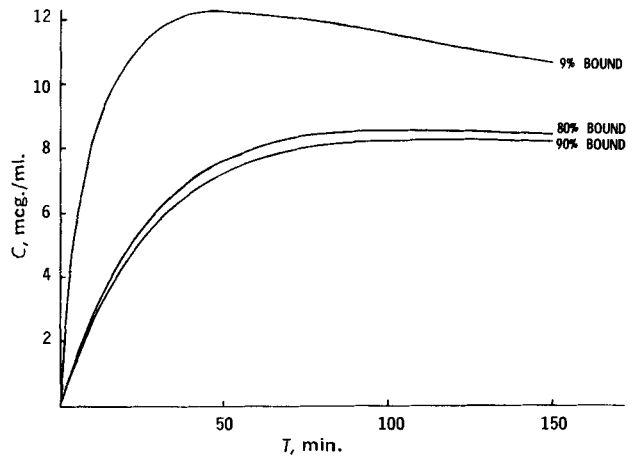


Figure 7—*Calculated concentration–time curves in the tissue compartment for a 10-mg./kg. dose of a hypothetical drug of mol. wt. 150, corresponding to the plasma curve of Fig. 6.*

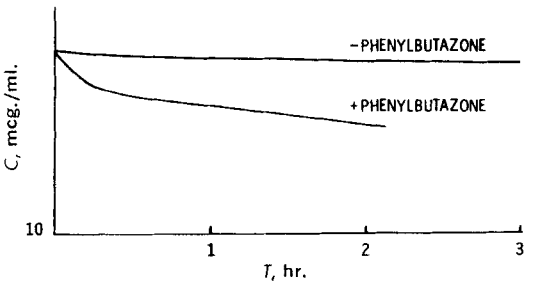


Figure 8—*Calculated concentration–time curves in plasma of warfarin in the presence and absence of phenylbutazone, using the assumptions presented in the text.*

The concentration–time curves for plasma are shown in Fig. 6. Considerable curvature is noticeable at a 10-mg./kg. dose of this hypothetical drug because of the phase corresponding to distribution into tissue. A doubling of free drug concentration, as the proportion of binding is dropped from 90 to 80%, produces very little change in the plasma kinetics, while a more dramatic shift to 9% binding greatly increases the rate of disappearance from plasma. The situation calculated for the tissue compartment is shown in Fig. 7. Because of the large value of V_2/V_1, even the shift from 90 to 9% binding produces less than a doubling of tissue concentration (except at very early times), while the increase due to the shift from 90 to 80% bound is very slight. Thus, if a drug is redistributed into body water, it seems unlikely that binding competition, no matter how effective, could cause large increases in tissue concentration.

Nonetheless, there are several examples in the literature where displacement of bound drug is thought to have major effects. One of the best known is the case of warfarin and phenylbutazone (3, 4). Administration of phenylbutazone decreases the half-life of warfarin but potentiates its anticoagulant activity. O'Reilly and Levy (4) showed that the effect of phenylbutazone is on the relationship between the synthesis of prothrombin complex activity and total concentration of warfarin in plasma. They concluded that phenylbutazone increases the proportion of free warfarin in plasma, making it more available to its pharmacologic receptor site.

The quantitative basis of this effect may be explored using the data of Aggeler *et al.* (3). Extrapolation of the warfarin concentration–time curves in plasma yields an estimated total distribution volume of only 100 ml./kg. If one assumes that warfarin can be fitted to a two-compartment model with inner and outer compartment volumes both equal to 50 ml./kg., it becomes apparent that the dilution factor due to V_2/V_1 is quite small.

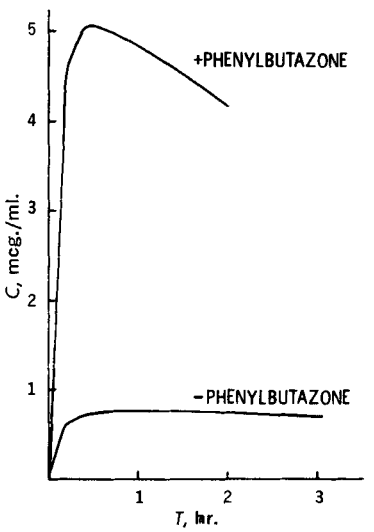


Figure 9—*Calculated concentration – time curves in the tissue compartment of warfarin in the presence and absence of phenylbutazone, corresponding to the plasma curves of Fig. 8.*

Calculations of predicted warfarin concentration–time curves were carried out using the following values (3):

1. Association constant for single binding site = 6.65×10^4.
2. Association constant in the presence of phenylbutazone = 7×10^3.
3. Distribution half-time = 5 min.
4. Inner and outer compartment volumes = 50 ml./kg.
5. Elimination half-time of total drug in the region where the binding relationship is linear = 44.8 hr.
6. Initial dose = 1.5 mg./kg.
7. Warfarin molecular weight = 308.

Because of the strong tendency of the successive approximation procedure to generate concentrations that become nonmonotonic with time, the calculated concentration–time curves (Figs. 8 and 9) are of only very short duration, barely sufficient, in fact, to illustrate the elimination of drug from either plasma or tissues. Even so, the effect of phenylbutazone on the pharmacokinetics of warfarin is quite apparent. The half-life of warfarin in plasma (Fig. 8) is somewhat decreased in the presence of phenylbutazone, but the most dramatic effect is on the warfarin concentration in the outer compartment (Fig. 9). It seems clear that the low value of V_2/V_1 is sufficient to give rise to a large increase in tissue concentration of warfarin in the presence of phenylbutazone.

Other special cases in which binding competition is of great importance may arise from other circumstances. The sulfonamide-related kernicterus of the newborn (12–15) appears to be due to binding competition between sulfonamides and endogenous bilirubin. The data on this system seem not to be amenable to simple pharmacokinetic analysis at the present time. Josephson and Furst (15) report, for example, that bilirubin appears to be more tightly bound to serum albumin than sulfamethoxypyridazine or sulfamoxole, yet the sulfa drugs appear to displace bilirubin in competitive binding experiments while bilirubin does not displace the sulfa drugs. Unless the apparent contradictions in these data are resolved by direct determination of binding constants, it will not be possible to model the bilirubin–sulfonamide system. Even if V_2/V_1 is quite large for bilirubin, the continuous endogenous synthesis of the compound could still give rise to a large increase in tissue bilirubin concentration by titrating the patient, so to speak, up to a new steady-state level. This titration effect, which would not be possible with a single dose of an exogenous drug, may be responsible for the severity of the sulfonamide–bilirubin interaction.

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Urinary Excretion of Ephedrine in Man without pH Control following Oral Administration of Three Commercial Ephedrine Sulfate Preparations

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Abstract □ In a feasibility study, 25-mg. single oral doses of ephedrine sulfate, in the form of a commercial syrup and two commercial capsules, were administered in crossover fashion to three nonsmoking male subjects. Urinary pH was not controlled, but adequate urinary flow rates were maintained by regulated water intake. Urinary excretion of ephedrine was measured over a 48-hr. period by a GC method. There were no significant differences in average amounts of ephedrine excreted during any of the 11 sampling intervals, in average peak excretion rates, nor in average times of occurrence of the peak excretion rate. There were insufficient subjects used in this preliminary study to form definite conclusions about differences between or among dosage forms. The urinary excretion data were adequately described by the two-compartment open model with first-order absorption and lag time. The average elimination half-life of ephedrine was 5.99 hr. estimated from the β-values obtained by exponential fitting. Average urinary pH was 6.30 (95 of the 99 samples fell within 5.32 to 7.28). The data provided little, if any, evidence of significant relationships between deviations of observed from theoretical excretion rates of ephedrine and urinary pH, urinary excretion rates of ephedrine and urinary pH, or excretion rates of ephedrine and urinary flow rates.

Keyphrases □ Excretion rates, urinary—ephedrine sulfate, without pH control □ Ephedrine sulfate—oral commercial preparations, urinary excretion rates without pH control, man □ GLC—determination, ephedrine sulfate in urine

It was suggested that the study of the kinetics of basic drugs in humans could give rise to invalid conclusions unless reabsorption of drug and/or metabolite in the kidney tubules is absent or negligible (1). Acidification of the urine by oral administration of ammonium chloride during such a study is used to obtain a urinary pH of 4–5. Theoretically, the greater concentration of ionized species of drug at this pH would reduce reabsorption compared to that at higher urinary pH values. This argument was applied to such drugs as the ephedrines, amphetamines, and chlorpheniramine (1–4). Controlled acidification of urine, although linearizing logarithmic excretion-rate values with respect to time, may bring about a situation somewhat removed from that obtained under normal clinical conditions not only in the GI tract but also in the kidneys. Furthermore, kinetic constants derived from such a study could be quite different from those obtained under more normal clinical conditions.

This problem was investigated during a recent preliminary study on the relative bioavailability of some commercial preparations of ephedrine sulfate. This feasibility study was to determine whether absorption of ephedrine sulfate from commercial capsules is different than from a solution (syrup) and whether the absorption of the drug from some commercial capsules is dissolution rate controlled. During this study, urinary pH was not controlled. High urinary flow rates were obtained, however, by providing subjects with controlled amounts of water. Unchanged drug in urine was measured by a GC assay, different from that of Beckett and Wilkinson (2).

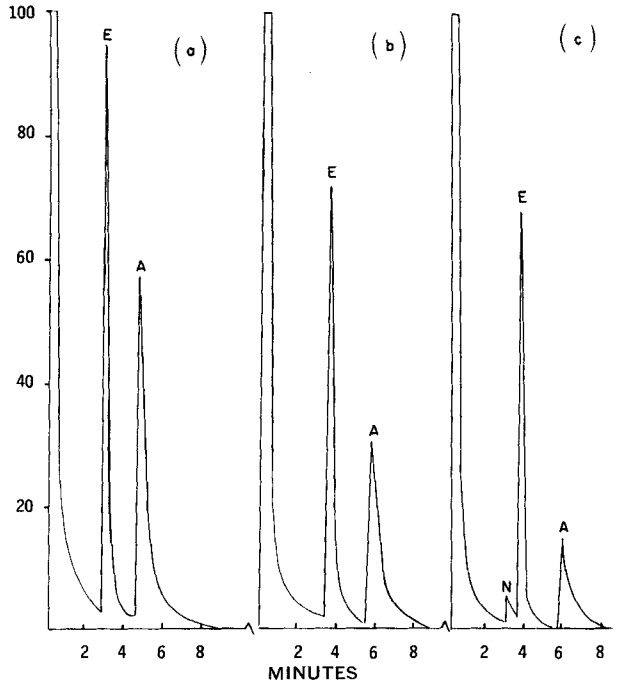


Figure 1—*Examples of gas chromatograms. (a) Gas chromatogram obtained from an extract of blank urine spiked with ephedrine sulfate (12 mcg./ml.) and 4-aminoacetophenone (5 mcg./ml.). (b) Gas chromatogram obtained by assay of a 0.5–1.0-hr. urine sample of Subject 1. (c) Gas chromatogram obtained by assay of a 6–9-hr. urine sample of Subject 1. Key: E, ephedrine peak; A, 4-aminoacetophenone peak; and N, norephedrine peak.*

EXPERIMENTAL

Reagents—The following were used: standard—ephedrine sulfate USP[1]; internal standard—4′-aminoacetophenone[2]; ether for anesthesia[3]; 5 *N* NaOH solution; sodium hydroxide T.S. USP; 2 *N* HCl solution; anhydrous Na_2SO_4, granular[1]; and Silyl-8[4].

Dosage Forms—One solution of ephedrine sulfate as the syrup and two commercial brands of capsules, hereafter called Treatments A, B, and C[5], were employed.

[1] Mallinckrodt Chemical Works.
[2] Eastman Organic Chemicals, No. 631.
[3] E. R. Squibb & Sons, No. 4084.
[4] Pierce Chemical Co.
[5] A = ephedrine sulfate syrup NF XII, Eli Lilly & Co., Control No. 3JC75A; B = ephedrine sulfate capsules USP XVII, 25 mg. (3/8 gr.), Eli Lilly & Co., Control No. 3DA76A; and C = ephedrine sulfate capsules, 3/8 gr. (25 mg.), American Pharmaceutical Co., Control No. 9R12876.

Table I—Amounts of Ephedrine[a] Excreted in Urine during Each Sampling Interval (A) and Urinary pH (B)

Time Interval, hr.	Treatment A, Subject 1	Treatment A, Subject 2	Treatment A, Subject 3	Treatment B, Subject 1	Treatment B, Subject 2	Treatment B, Subject 3	Treatment C, Subject 1	Treatment C, Subject 2	Treatment C, Subject 3
0–0.5									
A	0.172	0.562	1.94	0.144	0.021	0.056	0.085	0.074	0.188
B	6.73	5.20	5.65	5.56	6.52	6.61	6.25	5.21	5.71
0.5–1.0									
A	1.47	2.02	2.70	1.40	0.483	1.12	1.17	3.06	1.87
B	6.82	6.23	6.41	6.46	6.72	6.53	6.17	5.89	6.32
1.0–1.5									
A	0.653	1.60	1.44	1.04	0.917	1.54	1.48	2.69	1.79
B	7.08	6.40	6.49	6.51	6.83	6.52	6.54	6.12	6.15
1.5–2.0									
A	0.793	1.36	1.46	0.849	1.12	1.07	1.14	1.49	1.31
B	7.08	6.33	6.50	6.48	6.79	6.10	6.59	6.38	6.13
2.0–2.5									
A	0.556	1.33	1.03	0.773	0.503	0.746	0.990	1.06	1.24
B	7.82	6.00	6.46	6.48	6.82	6.96	6.64	6.60	6.43
2.5–3.0									
A	0.557	0.918	1.27	0.455	0.642	0.759	0.731	1.15	1.50
B	7.06	6.43	6.43	6.48	6.75	6.75	6.65	6.65	5.89
3–6									
A	2.57	3.22	3.17	4.22	3.52	4.24	4.68	4.50	2.40
B	6.92	6.04	5.68	6.42	6.82	5.45	6.04	6.68	5.86
6–9									
A	1.30	2.52	2.37	0.557	3.38	2.70	2.97	1.97	4.17
B	5.60	6.87	6.22	5.26	6.57	6.41	5.50	7.01	5.81
9–12									
A	2.49	1.76	2.07	1.94	2.88	3.01	2.62	1.97	1.44
B	6.27	5.73	5.43	6.17	5.51	6.42	5.73	5.70	6.67
12–24									
A	2.94	2.22	2.29	3.75	3.44	3.27	2.50	2.91	3.61
B	6.77	6.22	6.34	6.01	6.60	7.02	5.57	5.71	5.85
24–48									
A	0.950	0.780	0.492	0.599	0.766	0.898	0.627	0.492	0.595
B	5.86	5.77	6.13	6.38	5.77	6.98	5.88	6.34	6.55

[a] Expressed as milligrams of ephedrine sulfate.

Apparatus—All final estimations were done on a gas chromatograph[6] with a flame-ionization detector and a recorder (Mosely) with a chart integrator (disc).

The 1.83-m. (6-ft.) GC column had an internal diameter of 4 mm. and was packed with 3% OV-1 on Gas-Chrom Q, 100–200 mesh. The column was conditioned overnight between runs at 180° with minimum nitrogen flow. It was silanized with 10 μl. of Silyl-8 after each run. Operating conditions were: oven temperature, 140°; injection block temperature, 140°; and detector temperature, 230°. Gas flow rates were: air, 300 ml./min. at 20 lb./sq. in.; hydrogen, 35 ml./min. at 10 lb./sq. in.; and nitrogen, 60 ml./min. at 20 lb./sq. in.

Extractions were done on a vertical rotary mixer, 31 r.p.m., adapted to hold 15-ml. centrifuge tubes. All centrifugations were done in a centrifuge (Adams Dynac) at maximum speed (2460 r.p.m., 1280 r.c.f.).

Assay Procedure—Urine (5 ml.), water (1 ml.), and 2 *N* HCl (0.5 ml.) were combined in a 15-ml. centrifuge tube, fitted with a Teflon-lined screw cap, and mixed by circular rotation for 5 min. The mixture was extracted with 2 × 2 ml. of ether for 5 min. and centrifuged for 10 min.; then the ether layers were discarded. To the urine were then added 5 *N* NaOH (0.5 ml.) and exactly 0.5 ml. of a 0.05-mg./ml. solution of internal standard in water. The mixture was extracted with 3 × 2 ml. of ether. The extracts were combined in a 15-ml. centrifuge tube.

Anhydrous sodium sulfate (600 mg. or 0.4 ml. soft packed in a finely tapered centrifuge tube) was added to the ether extracts, and the tube was agitated vigorously on a vortex mixer for 1 min. After centrifugation, the ether layer was decanted off into a finely tapered centrifuge tube and the volume was reduced to approximately 50 μl. under a gentle stream of nitrogen at room temperature. Approximately 4–5 μl. was injected onto the column.

Although some peak tailing was observed with this system, it was not sufficient to interfere with peak area measurement. Ephedrine, norephedrine (the principal urinary metabolite of ephedrine), and internal standard had retention times of approximately 3.9, 3.6, and 5.8 min., respectively. The norephedrine peak could be clearly seen at the later urinary collections. No attempt was made in this study, however, to quantitate it. This peak did not interfere with peak area measurement of ephedrine. Typical chromatograms are shown in Fig. 1.

Quantitation was achieved by use of the relationship:

$$\text{concentration of ephedrine sulfate} = \frac{E/A}{0.128} \qquad \text{(Eq. 1)}$$

where E = peak area due to ephedrine, A = peak area due to the internal standard, and 0.128 = a constant obtained by dividing E/A values by urinary concentration of ephedrine sulfate in spiked urine during assay standardization.

Two spiked samples were carried through with each assay batch as a check on assay reproducibility.

Assay Standardization—Five-milliliter lots of blank urine from a nonsmoking male were spiked with 0.1, 0.2, 0.4, and 0.6 ml. of a 0.1-mg./ml. solution of ephedrine sulfate in water to give urine concentrations of 2, 4, 8, and 12 mcg./ml. The water volume thus added was deducted from the 1.0 ml. of water added at the start of the assay. The assay of the spiked urine was carried out exactly as already described, and the required constants were obtained.

Assay of Dosage Forms—The two lots of capsules and the syrup were assayed for ephedrine sulfate content by official assay procedures (5, 6). To determine capsule-to-capsule variation within a batch, individual capsules were also assayed by a modification of the USP method for ephedrine sulfate capsules. The contents of one capsule were carried through the USP assay with suitable adjustments of solvent volumes. The 0.1 *N* NaOH and H_2SO_4 solutions used in the original assay were each diluted 10 times to enable similar titration volumes to be measured for the smaller amount of ephedrine sulfate present.

Study Conditions—Three nonsmoking male subjects, age 27–32 years and weighing between 68.1 and 77.2 kg. (150 and 170 lb.), with normal vital signs, were used. All subjects had received no known enzyme-inducing agents for 30 days and no medication at

[6] Hewlett-Packard F&M model 402.

all for 7 days preceding the study, and they received none during the study.

Treatments consisted of one 25-mg. or $^3/_8$-gr. capsule or a volume of syrup containing 25 mg. of ephedrine sulfate (6.25 ml., 8.14 gr.). Capsules were swallowed whole with 6 fl. oz. of water. Syrup was weighed into a suitable container, and total dosage was ensured by rinsing all syrup from the container with water during administration.

Subjects were fasted overnight (from 10 p.m.) and for 4 hr. postdosing. The dose was administered at about 8 a.m., and urine was collected quantitatively in polyethylene bottles containing 0.5 ml. of toluene in the intervals 0–0.5, 0.5–1, 1–1.5, 1.5–2, 2–2.5, 2.5–3, 3–6, 6–9, 9–12, 12–24, and 24–48 hr. following treatment. To ensure adequate urine flow, 6 fl. oz. of water was taken orally at −1.5, −1, −0.5, 0, 0.5, 1, 1.5, 2, 2.5, 3, 6, and 9 hr. on each treatment day.

The volume and pH of each urine sample at room temperature were recorded. A small aliquot was then retained and quick frozen until just prior to the assay. Treatments were separated by a 7-day period. The following three-phase crossover dosage schedule was used:

	Treatment—Week		
Subject	I	II	III
1	A	B	C
2	B	C	A
3	C	A	B

RESULTS

Assay Standardization—The $E/(A \times$ concentration) ratio obtained from nine sets of urine spiked with ephedrine sulfate at 2.0–12.0 mcg./ml. on 9 different days (35 measurements) averaged 0.128 ± 0.0196 (one standard deviation). The ratios obtained from the different concentrations, 2.0, 4.0, 8.0, and 12.0 mcg./ml., were 0.129 ± 0.024, 0.123 ± 0.015, 0.124 ± 0.018, and 0.136 ± 0.021, respectively.

The ratios obtained from urines spiked at a concentration of 8.0 mcg./ml. and assayed on the same days as the subjects' urines (16 measurements) averaged 0.137 ± 0.017. This mean did not differ significantly from 0.128 ± 0.0196 ($t = 1.63$, $df = 49$, $0.2 > p > 0.1$); therefore, the figure 0.128 was used as the denominator in the equation to obtain the urinary concentration of ephedrine.

Dosage Form Assay—Four official assays of ephedrine sulfate syrup (Treatment A) yielded a concentration of 25.1 ± 0.43 mg. of drug/6.25 ml. of syrup. Capsules C yielded 25.1 and 25.4 ± 1.5 mg./capsule in two official and five single-capsule assays, respectively. Capsules B yielded 24.1 ± 0.15 and 24.1 ± 0.37 mg./capsule in three official and four single-capsule assays, respectively. Potencies obtained from the official assays were used as the basis of all absorption and recovery estimations. The very small standard deviations obtained in the single-capsule assays enabled the doses to be stated accurately.

Urinary Excretion of Ephedrine—The amounts of ephedrine sulfate excreted in urine and the urinary pH values are given in Table I.

A typical excretion rate, urinary pH, and urinary output profile are shown in Fig. 2. Only small deviations from this pattern were observed throughout the study by subjects receiving all three dosage forms. The average excretion rates of ephedrine sulfate are summarized in Fig. 3. Although lower excretion rates are apparent after Treatment B compared to those after Treatments A and C during the 1–3-hr. period, analysis of variance for crossover design showed no significant differences among treatment average amounts of ephedrine sulfate excreted in any time interval or in the total 48-hr. period, either expressed as milligrams or as percent of the administered dose.

There were also no significant differences among either peak ephedrine excretion rates or among times of occurrence of the peak excretion rate. There were no significant differences among elimination half-lives of ephedrine, obtained either by plotting terminal logarithmic excretion rates *versus* time or from exponential fitting. These data are summarized in Table II.

The individual subject and treatment average cumulative amounts of ephedrine sulfate excreted as a function of time were fit by the two-compartment model with first-order absorption and lag time.

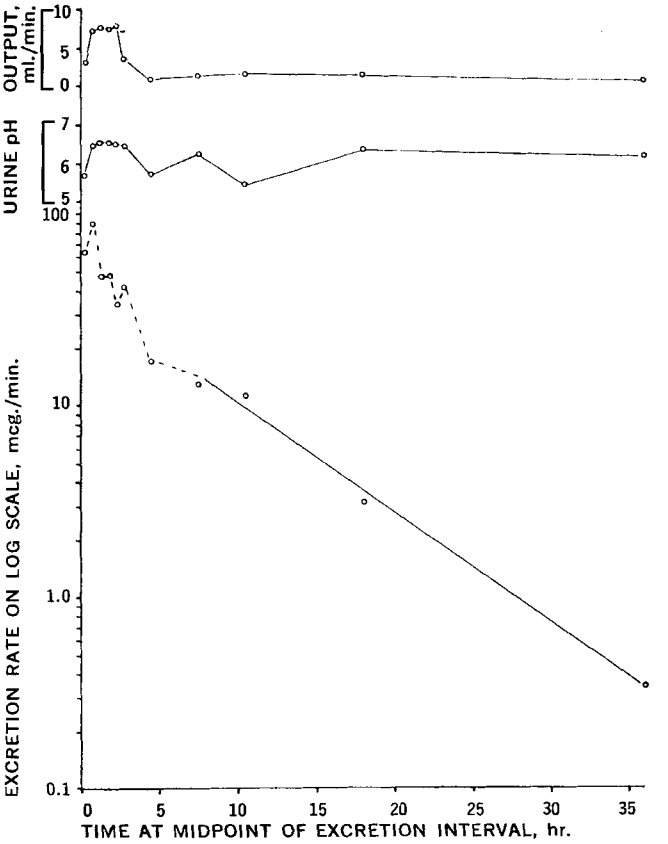
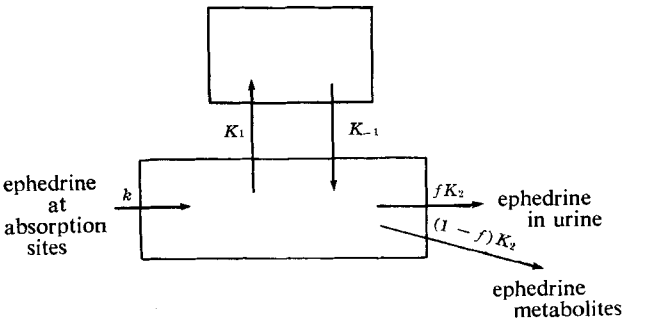


Figure 2—*Semilogarithmic plot of urinary excretion rate of ephedrine and plots of urinary pH and urinary output against midpoints of the excretion intervals for Subject 3 following Treatment A.*

By using Eqs. 2 and 3 (7), in conformity with the model shown in Scheme I, the data were fitted with the program NONLIN[7] on an IBM 360/67 computer.



Scheme I

$$A_u = A_u^{\infty}\left[1 - kK_2\left\{\left(\frac{K_{-1}-\alpha}{\alpha(k-\alpha)(\beta-\alpha)}\right)e^{-\alpha(t-t_0)} + \left(\frac{K_{-1}-\beta}{\beta(k-\beta)(\alpha-\beta)}\right)e^{-\beta(t-t_0)} + \left(\frac{K_{-1}-k}{k(\alpha-k)(\beta-k)}\right)e^{-k(t-t_0)}\right\}\right] \quad \text{(Eq. 2)}$$

where $\alpha > \beta$ and:

$$\alpha,\beta = {}^1/_2[(K_1 + K_{-1} + K_2) \pm \sqrt{(K_1 + K_{-1} + K_2)^2 - 4K_{-1}K_2}] \quad \text{(Eq. 3)}$$

$K_2 = fK_2 + (1 - f)K_2$ = overall rate constant for elimination of ephedrine

[7] The program NONLIN was supplied by Dr. C. M. Metzler, The Upjohn Co., Kalamazoo, Mich.

Table II—Summary of Average Cumulative Milligrams of Ephedrine Sulfate Excreted, Peak Excretion Rates, Times of Occurrence of Peak Excretion Rates, Half-Lives Obtained from Terminal Data and from Exponential Fitting, and Results of Tests of Significance by Analysis of Variance for Crossover Design

Parameter	Treatment Averages A	B	C	Statistics[a]
Milligrams of ephedrine sulfate excreted at				
0–0.5 hr.	0.890	0.074	0.116	a
0.5–1 hr.	2.06	1.00	2.03	b
1–1.5 hr.	1.23	1.17	2.00	b
1.5–2 hr.	1.20	1.01	1.32	b
2–2.5 hr.	0.972	0.674	1.10	a
2.5–3 hr.	0.915	0.619	1.13	c
3–6 hr.	2.99	3.99	3.86	b
6–9 hr.	2.06	2.21	3.03	a
9–12 hr.	2.11	2.61	2.01	a
12–24 hr.	2.49	3.49	3.00	b
24–48 hr.	0.717	0.754	0.550	a
Total (0–48 hr.)	17.6	17.6	20.1	b
Percent of assay dose excreted in 48 hr.	70.1	72.2	80.2	c
Peak excretion rate, mcg./min.	68.7	45.2	71.2	b
Time of occurrence of peak excretion rate	0.75	1.25	0.92	a
Half-life, hr. (terminal rate)	6.12	5.51	5.33	a
Half-life, hr. (exponential fit)	5.99	6.71	5.28	a

[a] a = not significant ($p > 0.25$), b = not significant ($0.25 > p > 0.10$), and c = not significant ($0.10 > p > 0.05$), according to analysis of variance for crossover design.

f = fraction of ephedrine reaching Compartment 1 that is excreted in the urine

In the initial computer fitting, six parameters (k, K_1, K_{-1}, K_2, A_u^∞, and t_0) were estimated. Results of these initial fits indicated very small lag times, t_0, for data obtained after Treatments A and C, and estimates of A_u^∞ were the same or only slightly larger (range 0–0.5 mg.) than the corresponding observed cumulative amounts excreted in 48 hr. For data observed after Treatment B, the estimated t_0 values were large enough that they should not be ignored and, again, the estimates of A_u^∞ were nearly the same as the corresponding observed cumulative amounts excreted in 48 hr. (range from −0.7 to 0.4 mg.). The goodness of fit of observed data to the theoretical cumulative excretion curve derived from the model is demonstrated by the closeness of r^2 and Corr. to 1.00 and the lack of areas of poor fit. In these initial fittings, although values of r^2 and Corr. ranged from 0.997 to 1.00, the standard deviations of the estimated parameters were quite large since so many parameters were being estimated.

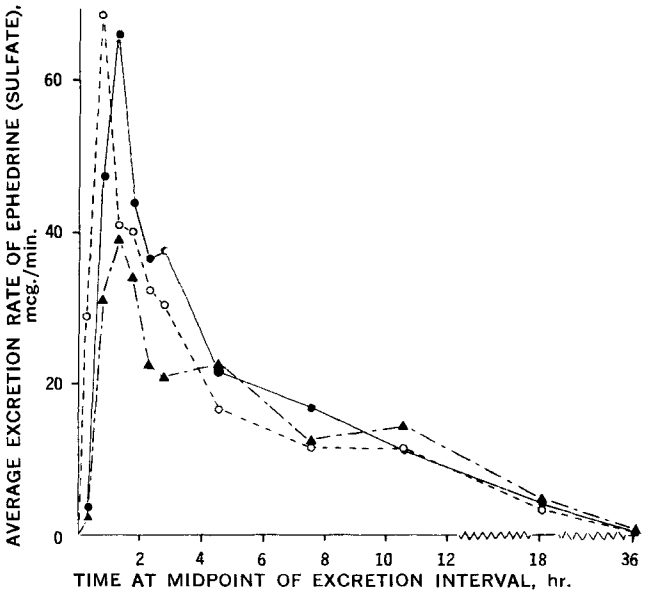


Figure 3—*Average excretion rate profiles for Treatments A, B, and C. Key:* O- - -O, *Treatment A;* ▲— - —▲, *treatment B; and* ●——●, *Treatment C.*

In the final computer fitting, four parameters (k, K_1, K_{-1}, and K_2) were estimated from data obtained after Treatments A and C. For these fittings, the lag time, t_0, was made equal to zero, and the A_u^∞ value obtained from the initial fitting was used as a constant in each case. For data obtained after Treatment B, five parameters (k, K_1, K_{-1}, K_2, and t_0) were estimated; again, the A_u^∞ value obtained from the initial fitting was used as a constant in each case. The parameters estimated and their standard deviations are given in Table III. The reduction in the number of parameters estimated significantly reduced the magnitude of the standard deviations. However, the standard deviations derived from urinary data with this model are still larger than those usually seen when plasma concentration data are fit to the same model. Table III also lists the values of A_u^∞ employed, the measures of fit, r^2, and Corr., the calculated values of α and β, and the $T_{1/2}$ calculated from β (*i.e.*, $t_{1/2} = 0.693/\beta$). The parameters and standard deviations shown in Table III were derived from individual subject data and would not be improved by utilizing a larger panel of subjects. Figure 4

Table III—Estimated Parameters (Their Standard Deviations) and Some Secondary Parameters Resulting from Nonlinear Least-Squares Fitting of Cumulative Amounts of Ephedrine Sulfate Excreted as a Function of Time (Two-Compartment Open Model with First-Order Absorption and Lag Time)

Parameters	Treatment A 1	2	3	Treatment B 1	2	3	Treatment C 1	2	3
k (hr.$^{-1}$)	2.75 (4.10)[a]	1.21 (6.66)	4.11 (1.70)	0.643 (1.09)	1.81 (7.31)	2.18 (17.5)	1.31 (1.57)	0.926 (1.15)	4.13 (9.28)
K_1 (hr.$^{-1}$)	0.370 (0.410)	0.796 (0.268)	0.594 (0.149)	1.41 (1.75)	0.178 (0.834)	0.238 (1.84)	0.285 (0.434)	1.51 (1.00)	0.609 (0.916)
K_{-1} (hr.$^{-1}$)	0.514 (0.134)	0.406 (0.101)	0.421 (0.021)	0.308 (0.357)	0.576 (0.529)	0.473 (0.703)	0.794 (0.918)	0.398 (0.332)	0.648 (0.181)
K_2 (hr.$^{-1}$)	0.175 (0.106)	0.503 (0.226)	0.414 (0.064)	0.714 (1.52)	0.142 (0.189)	0.190 (0.482)	0.193 (0.157)	0.943 (1.32)	0.263 (0.215)
t_0 (hr.)	0.0[b]	0.0[b]	0.0[b]	0.119 (0.125)	0.480 (0.858)	0.452 (1.53)	0.0[b]	0.0[b]	0.0[b]
A_u^∞ (milligrams)	14.7[b]	18.3[b]	20.3[b]	16.0[b]	18.1[b]	18.7[b]	19.5[b]	21.3[b]	20.3[b]
r^2[c]	0.999	1.00	1.00	0.998	0.998	0.996	0.999	0.999	0.997
Corr.[d]	0.998	1.00	1.00	0.997	0.998	0.999	0.999	0.998	0.997
Secondary parameters									
α (hr.$^{-1}$)	0.996	1.57	1.29	2.34	0.792	0.786	1.14	2.72	1.40
β (hr.$^{-1}$)	0.0929	0.130	0.135	0.0942	0.103	0.114	0.135	0.138	0.122
$T_{1/2}$ (hr.)	7.46	5.35	5.15	7.35	6.71	6.08	5.14	5.01	5.69

[a] Standard deviation. [b] Held constant during fitting. [c] $r^2 = (\Sigma obs.^2 - \Sigma dev.^2)/\Sigma obs.^2$. [d] Corr. is the correlation coefficient for the regression of predicted and observed cumulative amounts of ephedrine excreted.

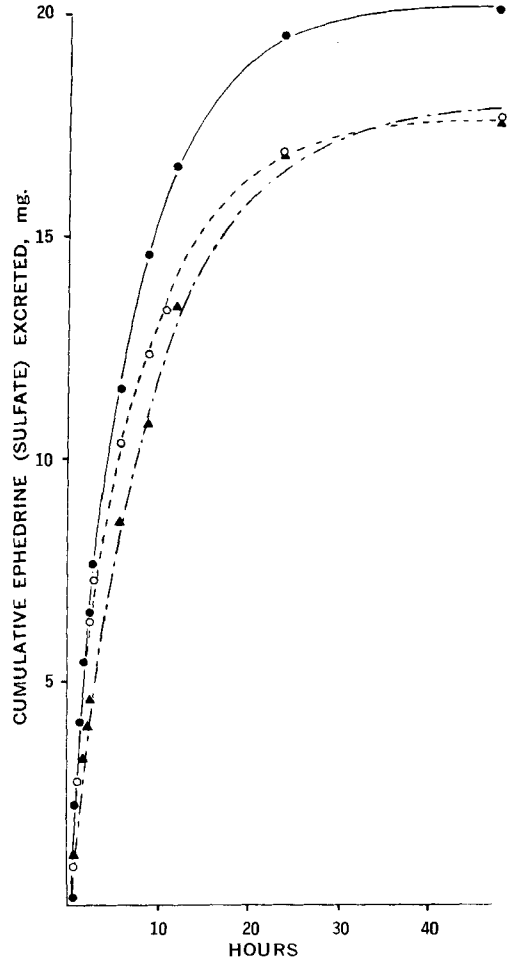


Figure 4—*Average cumulative amounts of ephedrine (as sulfate) excreted in the urine as a function of time. The lines drawn through the points are the theoretical curves based on nonlinear least-squares fitting to Eqs. 2 and 3. Key: O- - - -O, Treatment A; ▲— - —▲, Treatment B; and ●——●, Treatment C.*

shows the fits to the average data points for each treatment; these fits are similar to those obtained with the individual subject's data.

The overall average elimination half-life of ephedrine was 5.65 hr. (range 4.94–7.17 hr.), obtained by plotting terminal values of the logarithm of the excretion rate *versus* time, and 5.99 hr. (range 5.01–7.46 hr.), obtained by exponential fitting to the two-compartment open model. These half-lives appear more representative of those applicable to the clinical use of the drug than the lower values reported by Wilkinson and Beckett (1) obtained under conditions of controlled acidic urinary pH.

Correlation coefficients between deviations of actual from theoretical urinary excretion rates of ephedrine and urinary pH are given in Table IV. Three sets of correlation coefficients based upon two different mathematical models were calculated. The coefficients in Columns 1 and 2 of Table IV are based upon theoretical excretion rates calculated with Eq. 2; coefficients were calculated for the β-phase only and also using the entire data. The correlation coefficients in Column 3 of Table IV are based on deviations of observed and theoretical rates where the latter were obtained with Eq. 4 using terminal excretion rate data only:

$$\ln Y = \ln Y_0 - k_{el} \cdot t \qquad \text{(Eq. 4)}$$

In Eq. 4, Y is the theoretical excretion rate at time t, Y_0 is the rate obtained by extrapolating the least-squares excretion rate line back to time zero, and k_{el} is the slope of the straight line.

By employing Eq. 2, four negative and five positive correlations, all nonsignificant, were obtained over the whole excretion time. From the β-phase data, four positive and five negative correlations, all nonsignificant, were obtained. From Eq. 4, five positive and two negative nonsignificant correlations and two significant negative correlations were obtained. Significant correlations also could not be obtained between excretion rates of ephedrine sulfate and urinary flow rates, nor between urinary excretion rates of ephedrine *per se* and urinary pH.

Table IV—Correlation Coefficients between Deviations[a] of Observed and Theoretical Urinary Excretion Rates of Ephedrine and Urinary pH

		Correlation Coefficients		
		Equation 2		
Subject	Treatment	β-Phase	Whole Period	Equation 4
1	A	+0.292	−0.023	+0.302
1	B	+0.830	+0.452	−0.310
1	C	+0.543	−0.095	+0.325
2	A	+0.003	+0.562	+0.383
2	B	−0.563	−0.324	−0.883
2	C	−0.589	+0.303	+0.188
3	A	−0.163	+0.018	−0.927
3	B	−0.283	−0.223	+0.124
3	C	−0.082	+0.296	−0.244

[a] Deviations above theoretical lines were considered positive, and those below were considered negative.

DISCUSSION

In such a small preliminary study of the type performed, there are really insufficient data to make conclusions about differences between or among dosage forms. A larger panel of subjects would be required. However, the data collected were analyzed by appropriate analyses of variance for crossover design, and no significant differences were found. Previous pharmacokinetic studies were done with ephedrine solutions and, to the authors' knowledge, the present study is the first to be performed in man using capsule dosage forms.

Wilkinson and Beckett (4) evaluated their data according to the one-compartment open model and obtained excellent fits. The data in the present study were evaluated with the two-compartment open model with first-order absorption, and excellent fits were also obtained. Such equivocal results are not surprising in light of the report of Wagner and Metzler (8). In fitting to the two-compartment open model with first-order absorption, the standard deviations of the estimated parameters (Table III) are quite large, as might be expected from urinary excretion data.

The ratio β/K_2 gives the fraction of the total amount of drug in the body that is in the inner compartment during the β-phase; this value averaged 0.431 with a standard deviation of 0.226.

There is no doubt at all that alteration in urinary pH affects the urinary excretion of ephedrine. In the studies of Wilkinson and Beckett (1), under conditions of controlled acidic urine (pH about 5), a mean overall elimination $t_{1/2}$ value of 3.03 hr. was reported. In these studies with uncontrolled urinary pH, the mean urinary pH value was 6.30 with a standard deviation of 0.49; 95 out of the 99 urine samples fell within the pH range 5.32–7.28. The average overall elimination $t_{1/2}$ value was 5.66 hr. when estimated by a procedure similar to the one used by Wilkinson and Beckett (1). However, the lack of significant correlations between ephedrine excretion rates and urinary pH and the deviations of actual from theoretical urinary excretion rates and urinary pH obtained in the authors' studies are of interest. The pKa of ephedrine is 9.58 (9). In the urinary pH range encountered in these studies, the percent of the ionized drug in the urine varied from 99.99 to 99.51%. In such a narrow range of urinary pH and percent ionized drug, it is not too surprising that no significant correlations were obtained, particularly in light of analytical error, possible differences in recorded pH of urine and actual tubular pH, and the method of correlation, which employed small differences in predicted and observed rates (both of which are subject to error) with the observed pH (which was relatively constant).

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Total Synthesis of (±)-Bisnorargemonine

CHUNG-HSIUNG CHEN*, T. O. SOINE†, and KUO-HSIUNG LEE‡

Abstract □ (±)-Bisnorargemonine (I) was synthesized by two alternate but similar routes, differing only in the relative positions of the methoxy and benzyloxy groups on the aromatic rings of the 1-benzylisoquinoline-derived moieties. Both the standard Bischler–Napieralski procedure and the Pictet–Gams modification were employed in the construction of the needed isoquinoline intermediates. Conversion of the latter to the methiodide followed by partial reduction to the *N*-methyl-1,2-dihydro form and, finally, acid-catalyzed cyclization provided (±)-I, which was identical with natural (−)-I except for melting point and optical rotatory power.

Keyphrases □ (±)-Bisnorargemonine—synthesis, structure identification □ Alkaloids, from benzylisoquinolines—synthesis, structure identification, (±)-bisnorargemonine □ UV spectrophotometry—structure, identification, (±)-bisnorargemonine □ NMR spectroscopy—structure, identification, (±)-bisnorargemonine

Bisnorargemonine, an alkaloid, was first isolated by Kier and Soine (1) from *Argemone munita* subsp. *rotundata* (Rydb) G. B. Ownb. It was shown to have a pavine-type ring system by diazomethylation to argemonine (II) (2) which had earlier been established (3, 4) as the (−)-rotatory form of known (±)-*N*-methylpavine (5). Furthermore, based on its unique NMR spectrum, Structure I had been postulated for natural bisnorargemonine (6).

The purposes of this study were to substantiate the suggested structure by an unequivocal synthesis and to provide a practical synthetic method for preparing the alkaloid for other related studies.

The successful synthesis of (±)-norargemonine (III) by Lee and Soine (7) provided a model procedure which could, presumably, be followed in the synthesis of (±)-I reported here. Scheme I[1] shows that the final step in the synthesis of III is by way of the acid-catalyzed ring closure of IV*c* through a C_4-protonated immonium intermediate (5) to provide the pavine-type skeleton. It is apparent that, following the same scheme, two alternate routes lead to the same racemic target compound, *i.e.*, IV*a* → I*a* and IV*b* → I*b*, I*a* and I*b* being identical. The first route, IV*a* → (±)-I, has a methoxyl group directing cyclization and was the subject of a previous communication (8). The alternate route, IV*b* → (±)-I, with a benzyloxy group directing cyclization, also was achieved but, considering the strong acid medium used in cyclization, it is very likely that ether cleavage occurred prior to cyclization and that the ensuing phenolic hydroxyl group was the actual directing group. Since the original communication (8) was of necessity brief, both synthetic schemes are described in detail here.

[1] Ph = phenyl in this and subsequent schemes.

DISCUSSION

The construction of the isoquinolines, XI*a* and XI*b*, was the key in the synthetic scheme to the target compound, (±)-I (Scheme II). One common way of achieving this objective has been catalytic dehydrogenation of the corresponding 1,2,3,4-tetrahydroisoquinolines as employed in the synthesis of III (7). Unfortunately, dehydrogenation was always accompanied by cleavage of the benzyloxy groups, and subsequent benzylation was necessary to resynthesize the benzyl ether. Exploration of the Pictet–Gams modification of the Bischler–Napieralski procedure (9) for the synthesis of XI*a* and XI*b* provided a more convenient route since the dehydrogenation step was eliminated.

As outlined in Scheme II, the β-methoxy-β-phenylethylamines (VII*a* and VII*b*) were prepared from the corresponding β-nitrostyrenes (V*a* and V*b*) by addition of sodium methoxide and subsequent reduction of the adducts (VI*a* and VI*b*) with lithium aluminum hydride, as described by Rosenmund *et al.* (10). The acids (VIII*a* and VIII*b*) were synthesized from *O*-benzylvanillin and *O*-benzylisovanillin according to the procedures of Douglas and Gulland (11) and Robinson and Sugasawa (12), respectively. Prior to condensation with the amines, VIII*a* and VIII*b* were converted to their acid chloride forms, IX*a* and IX*b*.

The amides, X*a* and X*b*, were readily obtained by interaction of VII*a* with IX*a* and of VII*b* with IX*b*, respectively. Compounds X*a* and X*b* were subsequently cyclized in one step with phosphorus oxychloride to XI*a* and XI*b*, respectively. The NMR spectral

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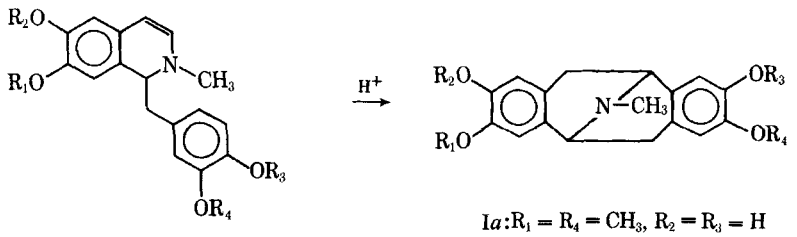
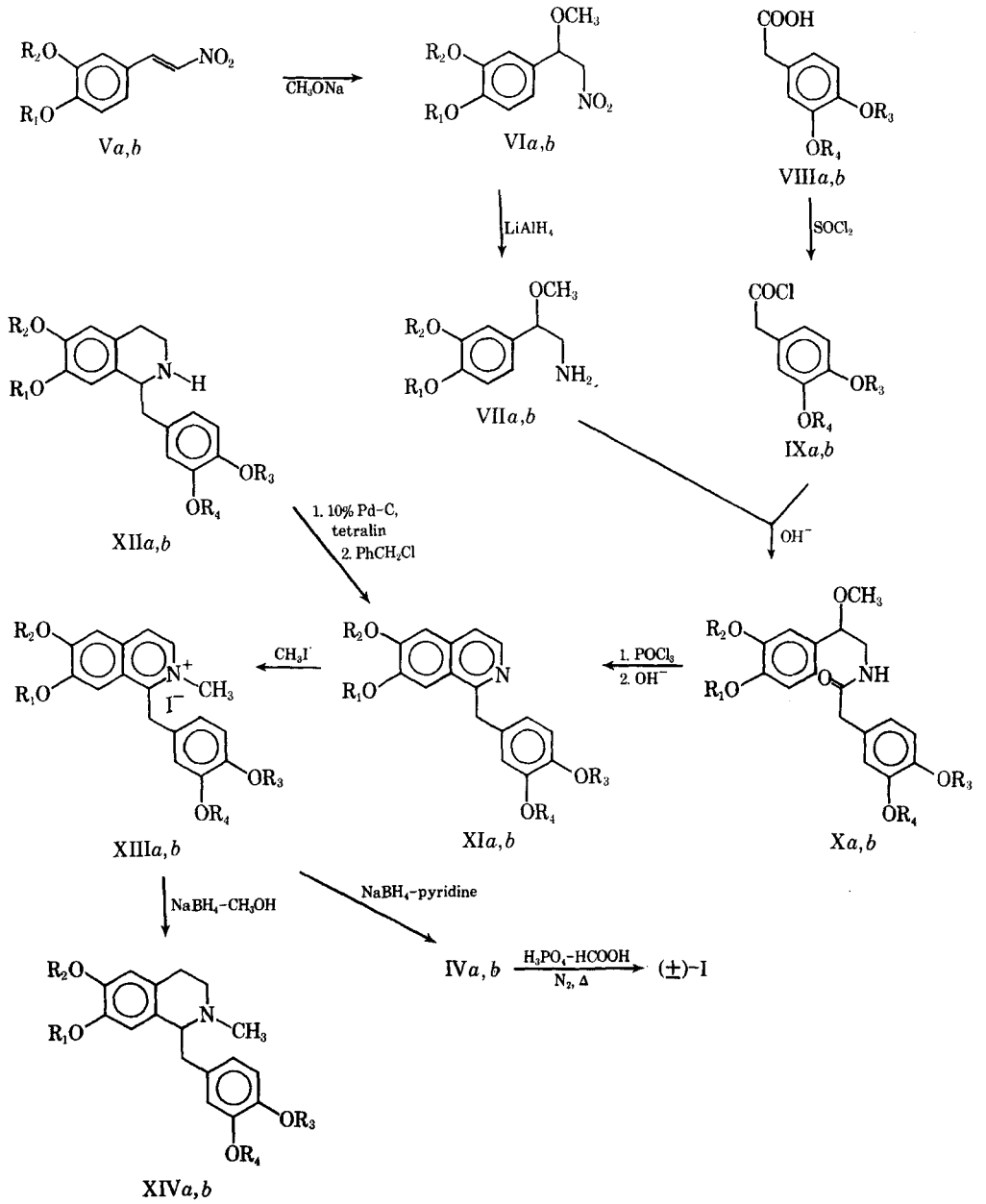


IV*a*: $R_1 = R_4 = CH_3$, $R_2 = R_3 = CH_2Ph$
IV*b*: $R_1 = R_4 = CH_2Ph$, $R_2 = R_3 = CH_3$
IV*c*: $R_1 = CH_2Ph$, $R_2 = R_3 = R_4 = CH_3$

I*a*: $R_1 = R_4 = CH_3$, $R_2 = R_3 = H$
I*b*: $R_1 = R_4 = H$, $R_2 = R_3 = CH_3$
III: $R_1 = H$, $R_2 = R_3 = R_4 = CH_3$
II: $R_1 = R_2 = R_3 = R_4 = CH_3$

Scheme I

data for X*a* and X*b* as well as XI*a* and XI*b* are recorded in Table I. The loss of the β-OCH_3 singlets in the isoquinolines is clearly evident, and the singlet for the methylene group in the phenylacetyl moiety shifted downfield by about 1 p.p.m. when deshielded by attachment to the isoquinoline ring at the 1-position. The UV spectra for XI*a* and XI*b* (see *Experimental*) were also in keeping with those expected for 1-benzylisoquinoline chromophores (7). Compounds XI*a* and XI*b* could also be obtained, as previously mentioned, by catalytic dehydrogenation of the tetrahydroisoquinolines, XII*a* and XII*b*, synthesized according to Tomita and Kunitomo (13, 14). The products of dehydrogenation were a mixture of phenolic isoquinolines, which were not purified but were



Scheme II—For each compound shown: a, $R_1 = R_4 = CH_3$, $R_2 = R_3 = CH_2Ph$; *and* b, $R_1 = R_4 = CH_2Ph$, $R_2 = R_3 = CH_3$

Table I[a]—NMR Data for X*a*, X*b*, XI*a*, XI*b*, XIII*a*, XIII*b*, IV*a*, and IV*b*

	OCH_3	OCH_2Ph	$-\overset{O}{\overset{\|}{C}}-CH_2$	$\beta-OCH_3$
X*a*	6.17 (s, 6H)	4.89 (s, 4H)	6.57	6.93
X*b*	6.13 (s, 6H)	4.86 (s, 4H)	6.55	6.82

	OCH_3[b]				OCH_2Ph[b]				C_1-CH_2	C_3-H
	C_6	C_7	C_3'	C_4'	C_6	C_7	C_3'	C_4'		
XI*a*	—	6.16	6.25	—	4.81	—	—	4.96	5.49	1.71 (d, J = 5.8 Hz.)
XI*b*	6.00	—	—	6.18	—	4.87	4.98	—	5.57	1.63 (d, J = 5.7 Hz.)

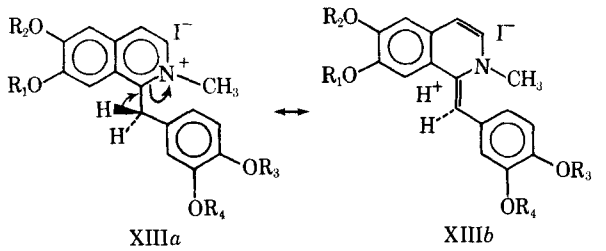
	OCH_3				OCH_2Ph				C_1-CH_2	$\overset{+}{N}-CH_3$	C_3-H and C_4-H *AB* quartet
	C_6	C_7	C_3'	C_4'	C_6	C_7	C_3'	C_4'			
XIII*a*	—	6.05	6.18	—	4.62	—	—	4.97	4.96[c]	5.55	1.39, 1.81 (J = 7 Hz.)
XIII*b*	5.85	—	—	6.18	—	4.72	4.97	—	5.05	5.65[d]	1.34, 1.69 (J = 6.8 Hz.)

	OCH_3[e]				OCH_2Ph[e]				C_1-H	$N-CH_3$	C_3-H (d of d) and C_4-H (d)	C_8-H
	C_6	C_7	C_3'	C_4'	C_6	C_7	C_3'	C_4'				
IV*a*	—	6.40	6.27	—	4.81	—	—	4.81	5.50 (t)	7.19 (s)	3.86, 4.55 ($J_{3,4}$ = 7.3 Hz.; $J_{1,3}$ = 1.2 Hz.)	3.72 (s)
IV*b*	6.24	—	—	6.24	—	5.06	4.91	—	5.52 (t)	7.25 (s)	3.89, 4.56 ($J_{3,4}$ = 7 Hz.; $J_{1,3}$ = 1.5 Hz.)	3.64 (s)

[a] For X*a*, X*b*, XI*a*, XI*b*, XIII*a*, and XIII*b*, τ values in $CDCl_3$ are given, while τ values in pyridine-d_5 are listed for IV*a* and IV*b*. [b] These assignments were made by comparison with model compounds reported by Brochmann-Hanssen and Hirai (18). [c] An approximate value; signal obscured by $C_4'-OCH_2Ph$. [d] This signal disappeared by making a deuterated methiodide salt (see *Experimental*). [e] These assignments were based on the observation by Barton *et al.* (15) that a substitution at the C_7-position is shielded due to the benzyl group at the C_1-position.

rebenzylated to afford XI*a* and XI*b*. Some loss of product is unavoidable due to simultaneous quaternization. The isoquinolines, XI*a* and XI*b*, were identical by both synthetic routes. Compounds XII*a* and XII*b* were converted to their hydrochlorides and were found to agree with the reported melting-point values (13, 14). This independent route used for the synthesis of XI*a* and XI*b* also served to confirm the previously assigned structures from the cyclization of amides X*a* and X*b*.

Conversion of XI*a* and XI*b* to the methiodides XIII*a* and XIII*b*, respectively, followed by sodium borohydride reduction in pyridine by the method of Barton *et al.* (15), provided the desired *N*-methyl-1,2-dihydroisoquinolines (IV*a* and IV*b*). The NMR data for XIII*a* and XIII*b* as well as for IV*a* and IV*b* are recorded in Table I. Characteristic N^+-CH_3 singlets for XIII*a* and XIII*b* were observed, but the most interesting effect of quaternization was on the $-CH_2$ protons attached to the 1-position. As compared to XI*a* and XI*b*, the signals for the C_1-CH_2 groups in XIII*a* and XIII*b* were shifted downfield by about 0.5 p.p.m. and appeared as broadened lines. This finding can be rationalized by the following resonance contribution causing electron withdrawal from the CH_2 protons to the isoquinoline ring system. Furthermore, this hyper-



XIII*a* XIII*b*

conjugative effect increases the bond order of C_1-CH_2, restricting free rotation around the bond. A further effect of quaternization is noted in the downfield shift of the signals for OCH_3 and OCH_2Ph at C_6 and C_7, whereas those at C_3' and C_4' are unaffected.

The structural assignments for XIII*a* and XIII*b* were confirmed further by reduction with sodium borohydride in aqueous methanol to give the *N*-methyl-1,2,3,4-tetrahydroisoquinolines, XIV*a* and XIV*b*, respectively, which corresponded in physical properties to the identical compounds reported by Kunitomo (16).

Compounds IV*a* and IV*b* showed characteristic UV absorptions for the 1,2-dihydroisoquinoline chromophore, and the structural assignments were further substantiated by the NMR spectra (Table I). The signals for $>N-CH_3$ were shifted upfield by more than 1.5 p.p.m. as compared to $\overset{+}{N}-CH_3$ in XIII*a* and XIII*b*. The C_3-H and C_4-H formed an *AB* quartet (J = 7 Hz.), with further splitting of the C_3-H by C_1-H (J = about 1.5 Hz.), which appeared as a broadened triplet around 5.5 p.p.m., indicating the long-range coupling between C_1-H and C_3-H as shown by Barton *et al.* (15).

The final step in the synthesis, acid-catalyzed cyclization of IV*a* → (±)-I and IV*b* → (±)-I, was carried out as described by Battersby and Binks (5), except care was taken to perform it under a nitrogen atmosphere. The reaction mixture was analyzed by combined TLC and column chromatography (CC), using natural I as the reference compound. The purified (±)-I's from both routes were identical to one another in their TLC, UV, IR, NMR, GLC, and mass spectral (MS) properties. They were also identical to natural I, except that (±)-I obtained from IV*a* showed a line-broadening effect in its NMR spectrum (dimethyl sulfoxide-d_6) taken at a 40° temperature in a microcell. Raising the probe temperature to 60 and 70° progressively sharpened the lines, and the resulting spectra were then virtually identical with those of natural I taken at 40° in a conventional NMR tube. The most likely explanation for the line broadening observed is a decreased resolution in the microcell, which was improved at higher temperatures since such line broadening was much less evident with the product obtained from IV*b* when measured in the usual NMR tube.

The yield of (±)-I from IV*a* was 1.5% of theory, whereas that from IV*b* was 21% of theory, suggesting that, at least in this case, the phenolic group is more favored as a directing group for the cyclization.

EXPERIMENTAL[2]

1-Methoxy-1-(3-benzyloxy-4-methoxyphenyl)-2-nitroethane (VI*a*)—3-Benzyloxy-4-methoxy-β-nitrostyrene (V*a*) was prepared by condensation of *O*-benzylisovanillin with nitromethane according to the method reported by Gairaud and Lappin (17). Addition of sodium methoxide to V*a* by the method of Rosenmund *et al.* (10) gave VI*a* in 65% yield, a pale-yellow powder from ether, m.p. 100–102°. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 1551 and 1376 (strong, NO_2). NMR (τ, in $CDCl_3$): 6.82 (s, 3H, β-OCH_3), 6.11 (s, 3H, C_4-OCH_3), and 4.83 (s, 2H, C_3-OCH_2Ph).

[2] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. UV spectra were taken in ethanol solutions with a Cary model 14 spectrophotometer. IR spectra were determined in mineral oil or KBr with a Perkin-Elmer 237B grating IR spectrophotometer. NMR spectra were measured with a Varian Associates model A-60D nuclear magnetic resonance spectrometer, using tetramethylsilane as an internal standard, and are recorded in Table I or in the following descriptions. Mass spectra were taken with a Hitachi Perkin-Elmer RMU-6D mass spectrometer. GLC was performed on a Perkin-Elmer 900 instrument equipped with dual flame-ionization detectors. Elemental analyses were performed by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, and by M-H-W Laboratories, Garden City, Mich.

Anal.—Calc. for $C_{17}H_{19}NO_5$: C, 64.35; H, 6.03; N, 4.41. Found: C, 64.35; H, 5.80; N, 4.17.

1-Methoxy-1-(4-benzyloxy-3-methoxyphenyl)-2-nitroethane (VI*b*)—4-Benzyloxy-3-methoxy-β-nitrostyrene (V*b*), prepared from *O*-benzylvanillin and nitromethane, was reacted with sodium methoxide as described previously to afford VI*b* in 86% yield, a pale-yellow powder from methanol, m.p. 102–103.5°. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 1540 and 1374 (strong, NO_2). NMR (τ, in $CDCl_3$): 6.73 (s, 3H, β-OCH_3), 6.09 (s, 3H, C_3-OCH_3), and 4.83 (s, 2H, C_4-OCH_2Ph).

Anal.—Calc. for $C_{17}H_{19}NO_5$: C, 64.35; H, 6.03; N, 4.41. Found: C, 64.50; H, 5.89; N, 4.25.

β-Methoxy-β-(3-benzyloxy-4-methoxyphenyl)ethylamine (VII*a*)—To an ice-cooled and stirred suspension of 1.52 g. (0.040 mole) of lithium aluminum hydride in 100 ml. of anhydrous ether was added dropwise a solution of 3.17 g. (0.010 mole) of VI*a* in 60 ml. of anhydrous tetrahydrofuran. This reaction mixture was refluxed for 3 hr. After being cooled, the resulting complex was decomposed by successive addition of wet ether, water, and a saturated solution of potassium sodium tartrate. The gel which formed was removed by filtration and washed with ether. The ether layer was separated from the filtrate, and the aqueous layer was extracted twice with fresh ether. The combined ethereal solution was washed with water, dried (anhydrous $MgSO_4$), and evaporated under vacuum to yield a pale-brown oil (VII*a*). IR $\nu_{max.}^{L.f.}$ cm.$^{-1}$: 3361 (NH_2). NMR (τ in $CDCl_3$): 8.59 (s, 2H, NH_2), 6.77 (s, 3H, β-OCH_3), 6.09 (s, 3H, C_4-OCH_3), and 4.78 (s, 2H, C_3-OCH_2Ph). Compound VII*a* was converted to the oxalate salt by mixing it in an ethereal solution with a calculated equivalent amount of oxalic acid in methanol. The white precipitate (3.29 g., 99% of theory) was collected and crystallized from methanol as feathers, m.p. 157–161°.

Anal.—Calc. for $C_{36}H_{44}N_2O_{10}$: C, 65.05; H, 6.67; N, 4.21. Found: C, 64.86; H, 6.87; N, 4.26.

β-Methoxy-β-(4-benzyloxy-3-methoxyphenyl)ethylamine (VII*b*)—Lithium aluminum hydride reduction of VI*b* was carried out as described previously to give VII*b* as a pale-brown oil in virtually quantitative yield. IR $\nu_{max.}^{L.f.}$ cm.$^{-1}$: 3361 (NH_2). NMR (τ in $CDCl_3$): 8.12 (s, 2H, NH_2), 6.73 (s, 3H, β-OCH_3), 6.10 (s, 3H, C_3-OCH_3), and 4.85 (s, 2H, C_4-OCH_2Ph). The oxalate salt of VII*b*, prepared as described previously for VII*a*, provided featherlike white crystals from methanol, m.p. 179–184°.

Anal.—Calc. for $C_{36}H_{44}N_2O_{10}$: C, 65.05; H, 6.67; N, 4.21. Found: C, 65.15; H, 6.85; N, 4.02.

***N*-(4′-Benzyloxy-3′-methoxyphenylacetyl)-β-methoxy-β-(3-benzyloxy-4-methoxyphenyl)ethylamine (X*a*)**—4-Benzyloxy-3-methoxyphenylacetic acid (VIII*a*) was prepared by the method of Douglas and Gulland (11). This acid (1.36 g., 0.005 mole) was refluxed with 2.5 ml. (0.035 mole) of thionyl chloride in 25 ml. of chloroform for 2 hr. The reaction mixture was evaporated, and the excess thionyl chloride was removed by repeated addition and evaporation of anhydrous toluene. The oily residue of acid chloride (IX*a*) was dissolved in 30 ml. of dry benzene; the resulting solution was added dropwise, with stirring and cooling, to a mixture of 30 ml. of 10% NaOH and 30 ml. benzene solution containing VII*a* (1.66 g., 0.005 mole of amine) prepared from its oxalate salt. After the addition, the reaction mixture was stirred for 1 hr. at 0°. The benzene layer was separated, and the aqueous layer was extracted twice with fresh benzene. The combined benzene extract was washed successively with 5% hydrochloric acid and water and dried over anhydrous potassium carbonate. Removal of benzene under vacuum gave a yellow oil, which was crystallized in ethanol to give 1.6 g. (59%) of X*a* as white fine needles, m.p. 96.5–98.5°. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 3271 (associated >N—H) and 1643 (C=O).

Anal.—Calc. for $C_{33}H_{35}NO_6$: C, 73.18; H, 6.51; N, 2.59. Found: C, 73.43; H, 6.51; N, 2.44.

***N*-(3′-Benzyloxy-4′-methoxyphenylacetyl)-β-methoxy-β-(4-benzyloxy-3-methoxyphenyl)ethylamine (X*b*)**—3-Benzyloxy-4-methoxyphenylacetic acid (VIII*b*) was prepared according to the method described by Robinson and Sugasawa (12). This acid was converted into acid chloride (IX*b*) and then condensed with the amine (VII*b*) as described in the preparation of X*a*. The reaction product after workup was crystallized from ethanol to give X*b* (72%) as a white powder, m.p. 112–114°. IR $\nu_{max.}^{mineral\ oil}$ cm.$^{-1}$: 3333 (associated >N—H) and 1648 (C=O).

Anal.—Calc. for $C_{33}H_{35}NO_6$: C, 73.18; H, 6.51; N, 2.59. Found: C, 72.97; H, 6.60; N, 2.39.

1-(3′-Methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxyisoquinoline (XI*a*)—*Method A: Cyclization of the Amide, X*a—A mixture of 0.37 g. (0.0007 mole) of X*a* and 0.9 ml. (0.01 mole) of phosphorus oxychloride in 6 ml. of toluene was refluxed for 1.5 hr. The reaction mixture was evaporated under vacuum to give a dark-brown oily residue. This residue was then taken up in chloroform and shaken with dilute ammonium hydroxide solution. The chloroform layer was separated, dried (anhydrous K_2CO_3), and evaporated under vacuum to yield a dark-brown oil. The oil was crystallized from benzene as a white residue and recrystallized from the same solvent to afford 0.13 g. (38%) of XI*a*, m.p. 146–147°. UV $\lambda_{max.}^{EtOH}$ nm. (log ε): 240 (5.37), 270 (4.10, shoulder), 280 (4.11), 315 (3.88), and 328 (3.92).

Anal.—Calc. for $C_{32}H_{29}NO_4$: C, 78.18; H, 5.95; N, 2.85. Found: C, 77.91; H, 5.97; N, 2.66.

*Method B: Dehydrogenation of 1,2,3,4-Tetrahydroisoquinoline, XII*a—Compound XII*a* was synthesized according to Tomita and Kunitomo (14). A mixture of 4.6 g. (0.0094 mole) of XII*a* and 1 g. of 10% palladium-on-charcoal in 50 ml. of tetralin was stirred and heated under an atmosphere of nitrogen for 1.5 hr. This was followed by another addition of 1 g. of catalyst and heating for another 30 min. The reaction mixture was filtered immediately to remove the catalyst; the filtrate, after being cooled, was diluted with excess anhydrous ether to produce a yellow solid. The solid was collected by filtration and washed well with anhydrous ether, yield 1.8 g. TLC analysis (alumina, chloroform and 95% ethanol in 20:1 ratio) showed that the solid was a mixture of more than three components. This mixture (1.8 g.) was then mixed with 1.6 g. of benzyl chloride and 0.9 g. of anhydrous potassium carbonate in 30 ml. of anhydrous methanol and refluxed for 3 hr. The reaction mixture was filtered immediately to remove potassium carbonate. Removal of methanol under vacuum gave a dark-brown residue, which was further purified by passing it through a neutral alumina[3] (70 g.) column using chloroform as the eluting solvent. The first 150 ml. of eluant was evaporated to give an oil which crystallized from a benzene–ether mixture. This was recrystallized from benzene to yield 0.63 g. (14% from XII*a*) of XI*a*, m.p. 146–147°. The IR spectrum was identical with XI*a* prepared from X*a*.

1-(3′-Benzyloxy-4′-methoxybenzyl)-6-methoxy-7-benzyloxyisoquinoline (XI*b*)—*Method A: Cyclization of the Amide, X*b—Following the same procedure as in the synthesis of XI*a* from X*a*, the amide X*b* was cyclized by treating with phosphorus oxychloride in refluxing toluene to give XI*b* in 49% yield. The product was crystallized from benzene as fine white needles, m.p. 169–171°. UV $\lambda_{max.}^{EtOH}$ nm. (log ε): 240 (4.62), 270 (3.59, shoulder), 279 (3.59), 314 (3.29), and 328 (3.39).

Anal.—Calc. for $C_{32}H_{29}NO_4$: C, 78.18; H, 5.95; N, 2.85. Found: C, 78.21; H, 6.09; N, 2.83.

*Method B: Dehydrogenation of 1,2,3,4-Tetrahydroisoquinoline, XII*b—Compound XII*b* was prepared by the procedure described by Tomita and Kunitomo (13). By following the same procedure already described, XII*b* was dehydrogenated and subsequently benzylated to give XI*b* in 38% yield. The IR spectrum was identical with the sample prepared from X*b*.

1-(3′-Methoxy-4′-benzyloxybenzyl)-2-methyl-6-benzyloxy-7-methoxyisoquinoline Iodide (XIII*a*)—A solution of 0.10 g. of XI*a* in 2 ml. of dimethylformamide and 1 ml. methyl iodide was refluxed for 1.5 hr. This was followed by addition of 1 ml. of methyl iodide and continuous heating for another 30 min. After being cooled, the reaction mixture was evaporated; the resulting solution was diluted with enough anhydrous ether to cause complete crystallization. The crystals deposited were filtered off and washed well with anhydrous ether. Recrystallization from an acetone–methanol mixture gave 0.12 g. (91%) of XIII*a* as yellow needles, m.p. 174–176.5°. UV $\lambda_{max.}^{EtOH}$ nm. (log ε): 258 (4.74), 286 (4.03, shoulder), and 320 (4.18).

Anal.—Calc. for $C_{33}H_{32}INO_4$: C, 62.56; H, 5.09; N, 2.21. Found: C, 62.27; H, 5.03; N, 1.92.

1-(3′-Benzyloxy-4′-methoxybenzyl)-2-methyl-6-methyl-7-benzyloxyisoquinoline Iodide (XIII*b*)—By the same procedure already described, XIII*b* was prepared from XI*b*. The crude product was recrystallized from the methanol–acetone mixture to give fine yellow needles in almost quantitative yield, m.p. 200–202°. UV $\lambda_{max.}^{EtOH}$ nm. (log ε): 259 (4.84), 286 (3.82 shoulder), and 318 (4.04).

[3] Woelm, activity grade I.

Anal.—Calc. for $C_{33}H_{39}INO_4$: C, 62.56; H, 5.09; N, 2.21. Found: C, 62.43; H, 5.12; N, 2.20.

To assign the signal for the N^+—CH_3 group, iodomethane-d_3 was used to prepare XIII*b* in the *N*-methyl-d_3 form. The singlet at 5.56 τ (Table I) was missing from the spectrum of the deuterated species as compared with that of the undeuterated one[4].

1-(3′-Methoxy-4′-benzyloxybenzyl)-2-methyl-6-benzyloxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline (XIV*a*)—Sodium borohydride (0.40 g.) was added, portionwise, into a suspension of 0.203 g. (0.00032 mole) of XIII*a* in 40 ml. of methanol and 6 drops of water. Following the addition, the mixture was refluxed for 1 hr. The reaction mixture was evaporated, and the resulting residue was dissolved in water (50 ml.). The aqueous solution was extracted with ether (6 × 50 ml.); the combined ethereal extract was dried (anhydrous K_2CO_3) and evaporated under vacuum to give a pale-brown oil. This oil crystallized from 95% ethanol to give 0.136 g. of XIV*a* as fine colorless needles, m.p. 89–92° [lit. (16) m.p. 91–93°].

1-(3′-Benzyloxy-4′-methoxybenzyl)-2-methyl-6-methoxy-7-benzyloxy-1,2,3,4-tetrahydroisoquinoline (XIV*b*)—The methiodide XIII*b* (0.11 g., 0.00017 mole) was reduced with sodium borohydride as described for XIII*a*. The oily product crystallized from 90% ethanol to give XIV*b* (96%) as fine colorless needles, m.p. 88.5–89.5° [lit. (16) m.p. 89.5–90.5°].

1-(3′-Methoxy-4′-benzyloxybenzyl)-2-methyl-6-benzyloxy-7-methoxy-1,2-dihydroisoquinoline (IV*a*)—The methiodide XIII*a* (0.100 g., 0.0016 mole) was added, portionwise, to a suspension of 0.025 g. of sodium borohydride in 4 ml. of pyridine. The mixture was shaken until the methiodide dissolved completely. Another 0.020 g. of sodium borohydride was then added, and the mixture was shaken for another 5 min. The reaction mixture was then taken up in ether and shaken with water (30 ml.); the aqueous layer was extracted twice with fresh ether. The combined ethereal extract was dried (anhydrous K_2CO_3), and evaporated to remove ether. Traces of pyridine remaining in the residue were removed by applying high vacuum at room temperature. The oily residue was then dissolved in a few milliliters of ethanol and cooled in a refrigerator to yield 0.046 g. (57%) of IV*a* as fine colorless needles. Recrystallization from the same solvent gave m.p. 64–67°. This compound decomposed slowly upon standing in the air. UV $\lambda_{max.}^{EtOH}$ nm. (log ϵ): 255 (3.58, shoulder), 285 (3.25), and 334 (3.54).

Anal.—Calc. for $C_{34}H_{33}NO_4$: C, 78.08; H, 6.55; N, 2.76. Found: C, 78.32; H, 6.52; N, 2.50.

1-(3′-Benzyloxy-4′-methoxybenzyl)-2-methyl-6-methoxy-7-benzyloxy-1,2-dihydroisoquinoline (IV*b*)—Sodium borohydride reduction of 0.300 g. (0.00048 mole) of the methiodide, XIII*b*, in the same manner as described for VI*a* gave an oily product which crystallized from methanol to yield IV*b* (57%). Recrystallization from ethanol gave colorless rosettes, m.p. 93–97°. UV $\lambda_{max.}^{EtOH}$ nm. (log ϵ): 256 (3.89, shoulder), 285 (3.58), and 336 (3.96).

Anal.—Calc. for $C_{33}H_{33}NO_4$: C, 78.08; H, 6.55; N, 2.76. Found: C, 78.16; H, 6.64; N, 2.66.

Cyclization of IV*a* to (±)-Bisnorargemonine (I)—A mixture of 1.50 g. (0.0030 mole) of IV*a* in 3 ml. of 85.6% phosphoric acid and 7.5 ml. of 90.8% formic acid was heated for 4 hr. at 120° under an atmosphere of nitrogen. The reaction mixture, after being cooled, was diluted with water and extracted twice with chloroform (2 × 50 ml.). The aqueous layer was then made alkaline with dilute ammonia (10%) and again extracted with chloroform (6 × 100 ml.). The combined chloroform extract was then dried (anhydrous K_2CO_3) and evaporated under vacuum to yield a brown residue (about 1 g.). TLC analysis on silica gel [benzene–methanol (3:2)] indicated more than three components in this mixture, one of which corresponded to natural I with R_f 0.50. This mixture was further fractionated by passing it through a silica gel column[5] (20 g., 60–200 mesh), with benzene–methanol (3:2) as the eluant. Fractions 10–20 (10 ml. per fraction) showed spots corresponding to R_f 0.50. All these fractions were combined and evaporated under vacuum to give a greenish-brown residue. This residue was then dissolved in a small amount of chloroform, and crystallization occurred upon standing at room temperature. The crystals collected were gelatinous but, on drying under vacuum at 100° for 24 hr., gave 0.015 g. (1.5% from IV*a*) of a white powder, m.p. 231.5–233.5°. The IR (KBr) and UV spectra were identical in every aspect with those of natural I. Attempts were made to analyze this synthetic material by GLC. A silyl derivative was prepared by mixing 1 mg. of the powder in 5 ml. anhydrous ether with 0.5 ml. of trimethylsilyl chloride, 0.5 ml. of hexamethyldisilazane, and a few drops of pyridine. The supernatant from the mixture was analyzed on an OV-1 column (3% on Chromosorb W, at 215° column temperature, with nitrogen as the carrier gas at 30 ml./min.) and gave a single peak with retention of 11.4 min. A silyl derivative of natural I was similarly prepared and gave, under the same conditions, an identical retention time.

The NMR spectrum (in dimethyl sulfoxide-d_6) of this synthetic material taken in a microcell at 40° showed line broadening. Raising the probe temperature to 60 and 70° improved the line shape, and the spectra obtained were identical with those of natural I taken in the same solvent.

Cyclization of IV*b* to (±)-Bisnorargemonine (I)—A mixture of 1.3 g. (0.0025 mole) of IV*b* in 2.6 ml. of 85.6% phosphoric acid and 6.5 ml. of 97% formic acid was heated, and the reaction mixture was worked up as described previously to afford a reddish-brown oil (about 1 g.). This oil was fractionated on a silica gel column (70 g.), with benzene–methanol (10:1) as the eluting solvent. Fractions 20–40 (20 ml. per fraction) were combined and evaporated to give a pale-brown residue. This residue was crystallized in chloroform to afford 0.18 g. (21% from VI*b*) of a white powder, m.p. 226–227°. The IR, MS, UV, and GLC behaviors were identical with those of natural I and synthetic (±)-I from the IV*a* route. The NMR spectrum taken at 40° in a normal cell showed a much less line-broadening effect and was virtually identical with the spectrum of natural I.

[4] This led to the discovery of an erroneous assignment in a previous paper by Lee and Soine (7). For Compound XV cited therein, the singlet at 5.54 τ should be assigned to the N^+—CH_3 group, while the singlet at 5.02 τ should be assigned to the C_1—CH_2 group.

[5] Baker Analyst No. 3405.

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Drug Absorption V: Influence of Food on Oral Absorption of Phenobarbital in Rats

S. KOJIMA*, R. B. SMITH, and J. T. DOLUISIO

Abstract □ This paper reports the effect of food on the oral absorption of phenobarbital in the rat. The presence of food was found to lower significantly the serum levels of intact drug and to reduce dramatically (and at low doses, 100 mg./kg., to eliminate) sleeping times induced by phenobarbital. The amounts of drug remaining unabsorbed in the stomach and intestines were determined in fasted and nonfasted rats. Greater than 90% of the amount unabsorbed was found in the stomach in both test conditions, but the amounts unabsorbed were greater in the nonfasted rats. *In situ* studies indicate that phenobarbital is primarily absorbed from the intestines. Thus, it is concluded that the presence of food decreased the pharmacological activity of phenobarbital by decreasing the rate of absorption and that this decreased absorption rate is due primarily to slowed gastric emptying. Since the drug is rapidly absorbed in the intestine, the extent of absorption is the same in the nonfasted rat as in the fasted rat.

Keyphrases □ Absorption kinetics—oral phenobarbital, effect of food, rats □ Phenobarbital—effect of food on oral absorption, rats □ Barbiturate absorption—effect of food, rats

A number of investigations have been concerned with the absorption characteristics of barbiturates. For example, Kakemi *et al.* (1, 2) studied the *in situ* absorption of various barbiturates from the rat stomach and intestine and the effect of pH, Sjogren *et al.* (3) studied absorption from various dosage forms, and Bush *et al.* (4) studied the serum level profiles for ultrashort-acting barbiturates. The effect of concomitant administration of alcohol on oral administration was also studied (5). Even though many of the factors affecting oral absorption have been characterized, little work has been done on the effect of food. The paucity of reports on the effect of food is not limited to barbiturates but extends to most drugs. The effect of food on barbiturate absorption is of particular interest, since barbiturates are in widespread use as sedatives and hypnotics in situations where food consumption is not controlled.

The results of the studies done on the effect of food showed that the presence of food in the GI tract influences significantly the oral absorption of drugs. In a previous paper (6), the authors reported that the rate of dicloxacillin absorption in man had been markedly decreased (approximately 200%) by food, but the extent of dicloxacillin ultimately absorbed decreased by a small percentage (14%). MacDonald *et al.* (7) found that food delayed absorption but did not decrease the amount of four sulfonamides eventually absorbed. Bush *et al.* (4) found that food delayed onset and significantly increased the duration of hypnosis for thiopental in a single subject who broke protocol by eating a cheese sandwich. The effect of food may result from hindered diffusion to the mucosal absorption surface, decreased dissolution rate of solid dosage forms, or delayed stomach emptying for drugs absorbed primarily in the intestine.

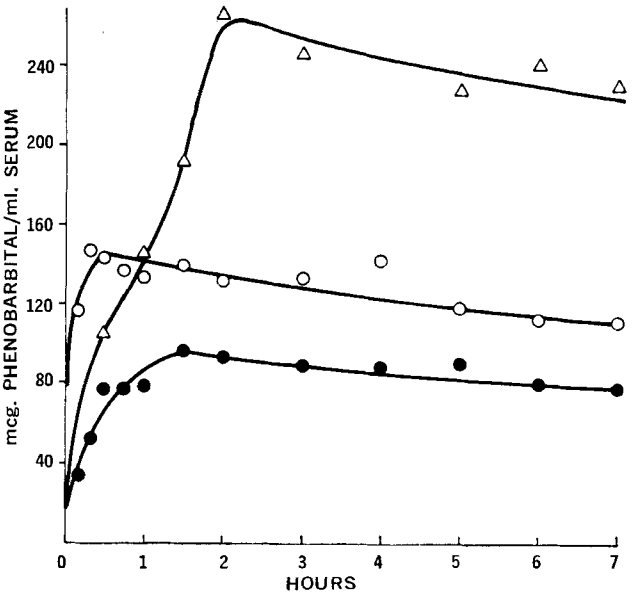


Figure 1—*Serum levels of phenobarbital following oral administration of phenobarbital in fasted and nonfasted rats. Key:* ○, *fasted, 100 mg./kg.;* ●, *nonfasted, 100 mg./kg.; and* △, *nonfasted, repeated dosing of two 150-mg./kg. doses 1 hr. apart.*

The purpose of the present investigation was to study the influence of food on the absorption rate, extent of absorption, and pharmacological action of phenobarbital when administered in aqueous solution to the rat. It was expected that food would delay and/or decrease the apparent rate of absorption of phenobarbital, consequently delaying onset and perhaps increasing duration of hypnosis. If this were the case, a human subject that consumed food before taking a hypnotic dose of barbiturate may experience an abnormal delay in the onset of action and take more drug, with the consequent hazard of toxicity.

This paper reports the animal results obtained thus far. A future publication will deal with human studies on the effect of food on barbiturate absorption.

EXPERIMENTAL

Reagents and Equipment—All chemicals were reagent grade except phenobarbital USP and mephobarbital NF XII. A gas chromatograph[1] and a scintillation counter[2] were used.

Test Animals—Male Sprague-Dawley albino rats, weighing between 200 and 490 g., were used. The rats were fasted 48 hr. prior to experimentation, but drinking water was allowed *ad libitum*. The rats were kept in cages having wide mesh floors to minimize

[1] Varian Aerograph Series 1700.
[2] Packard Tri Carb model 3375.

Table I—Onset and Duration of Action of Phenobarbital following Oral Administration with and without Food

	Number of Rats	Dose, mg./kg.	Onset of Action, min.	Duration of Action, min.
Fasting (p.o.)	11	100	28 ± 7.2	227 ± 23.6
Nonfasting (p.o.)	10	100	—[a]	—[a]
	12	300	76 ± 35.7	>540
	12	300[b]	79 ± 11.4	>600

[a] All rats did not lose the righting reflex. [b] Repeated dosing of two 150-mg./kg. doses 1 hr. apart.

coprophagy. For studies on the effect of food, the rats were allowed food[3] for about 16 hr. after fasting 48 hr. The rats consumed about 0.08–0.1 g. food/g. body weight.

***In Situ* Rat Model**—The procedure for studying drug transfer kinetics in the *in situ* rat model was described previously (8).

Drug Administration—Phenobarbital solutions administered to the rats were freshly prepared by the addition of 1.1 equivalents of sodium hydroxide to the acid. The solutions were of such concentration that the rats received 0.01 ml. orally/g. body weight. The oral administration was carried out by use of a stomach catheter.

Duration of Action of Phenobarbital—The duration of action (hypnosis) of phenobarbital was defined as the time between the loss of the righting reflex after administration of the drug and the time the animal regained the righting reflex.

Experimental Procedures—The rats were sacrificed periodically after administration of phenobarbital. The serum was collected immediately after the animals were sacrificed. The contents of the gut (stomach and intestines) were washed out using 15–20 ml. of water, and the washings were centrifuged.

To determine the initial passage of solution into the duodenum in fasted rats, 0.01 ml./g. body weight of a solution containing ^{14}C-polyethyleneglycol as a nonabsorbable marker was administered. After administration, the rats were immediately sacrificed by decapitation and opened by midline incision. The stomach was tied off at the pyloric junction, and then both the stomach and intestines were removed. This process required from 2 to 3 min. to complete. The contents of both the stomach and intestines were washed into separate Kimex tubes with 5–10 ml. of water and centrifuged. Samples of the supernatant were then analyzed by scintillation counting.

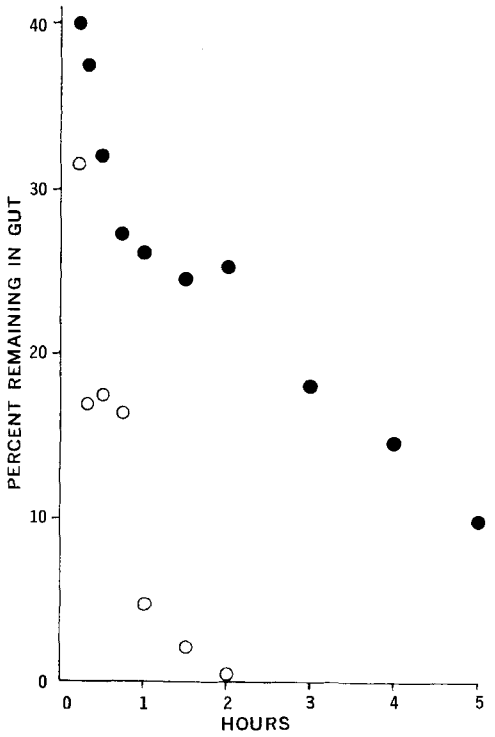


Figure 2—*Percent of phenobarbital remaining in the gut (stomach and intestines) following oral administration of phenobarbital in fasted and nonfasted rats. Key: ○, fasted, 100 mg./kg.; and ●, nonfasted, 100 mg./kg.*

[3] Purina Lab Chow.

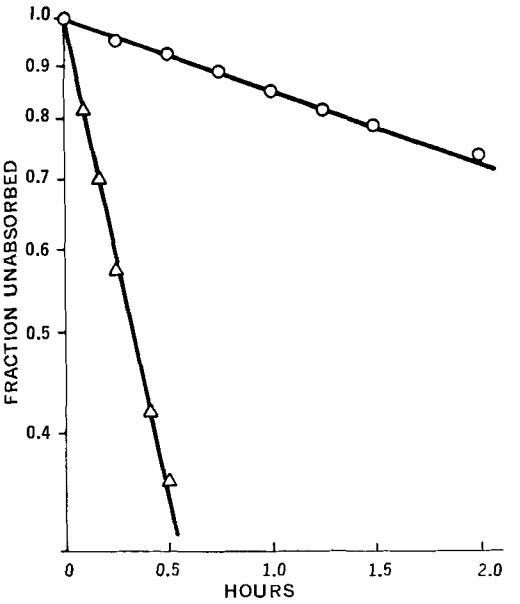


Figure 3—*Semilogarithmic plots of the disappearance of phenobarbital from the rat stomach and intestine* in situ. *Average values for three rats. Key: ○, stomach, pH 3.0; and △, intestine, pH 6.0.*

Analytical Procedures—To 1.0 ml. of serum or gut sample in a 15-ml. Kimex tube was added 1.0 ml. of 0.2 *M* phosphate buffer (pH 5.85). The mixture was extracted with 6, 6, and 2 ml., successively, of ethyl ether by shaking on a mixer for 2 min. The combined extract was evaporated at about 45°. The resulting residue was dissolved in an adequate volume of 2.5 mg. % mephobarbital–chloroform solution and submitted to GLC.

Conditions of GLC—A gas chromatograph equipped with a hydrogen flame-ionization detector was used. The carrier gas was nitrogen. The column was 1.8 m. (6 ft.) × 0.63 cm. (0.25 in.) (o.d.) glass, containing a packing of 3% PPE-20 on 100–120 mesh Supelcoport.

The calibration curve for phenobarbital was prepared as follows. The sample solutions (5–80 mcg./ml.) were prepared by dissolving different amounts of phenobarbital in a chloroform solution of mephobarbital (2.5 mg.%) as the internal standard. At the fixed range and attenuation of the instrument, 2 μl. of sample solution was injected into the gas chromatograph. The peak area was determined by triangulation. The calibration curve was obtained by plotting the concentration of phenobarbital against the peak area ratio of phenobarbital to mephobarbital.

RESULTS AND DISCUSSION

The onset and duration of hypnotic action of phenobarbital for doses of 100 mg./kg. body weight in fasted and nonfasted rats are shown in Table I. Food had a significant effect on the hypnotic action of phenobarbital. For the 100-mg./kg. dose, the fasted rats had an average onset time of 28 min. and an average duration of 227 min.; however, the same dose in nonfasted rats produced no hypnotic effect, *i.e.*, not a single rat lost the righting reflex.

The serum levels of phenobarbital at various times following oral administration of this same dose are shown in Fig. 1. The percent remaining in the gut at various times was also found for this dose and is shown in Fig. 2. The serum level curves demonstrated that the presence of food concomitant to oral administration of phenobarbital delayed the attainment and decreased the magnitude

Table II—Percent Phenobarbital Remaining in the Stomach, Intestines, and Cecum following Oral Administration

	Hours					
	0.16	0.5	1.0	2.0	3.0	5.0
100 mg./kg. Fasted						
Stomach	28.3	7.7	13.0	2.0	0.7	0.1
Intestines	1.2	2.6	0.9	0.3	0.4	0.3
Cecum	0.2	0.2	0.2	0.2	0.3	0.3
Total	29.6	10.6	14.0	2.5	1.3	0.7
100 mg./kg. Nonfasted						
Stomach	42.3	40.5	24.0	15.5	18.0	16.5
Intestines	0.2	0.3	0.5	0.4	0.4	0.4
Cecum	0.1	0.2	0.4	0.7	1.2	1.1
Total	42.5	41.1	24.9	16.6	19.6	18.0

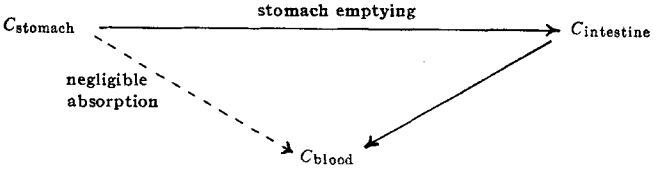
of the maximum serum level. It is evident from the plots of percent remaining to be absorbed that the presence of food decreased the rate of absorption. For the 100-mg./kg. dose, the percent remaining to be absorbed at 1 hr. was 4.5% in fasted rats as compared to 26% in nonfasted rats. These data indicate that the presence of food decreases the rate of absorption and, consequently, the prolonged absorption alters the serum level profile. If a minimum concentration in serum necessary to elicit a response is assumed, these data can be correlated to the hypnotic action described earlier. The presence of food delays attainment of this minimum serum level and, hence, delays onset; depending on the relationship between the maximum level obtained and the minimum effective level, the duration of action may be increased or decreased.

The absorption of phenobarbital from the rat stomach and intestine was studied using an *in situ* technique (8). The fractions unabsorbed for the stomach and intestine are plotted in Fig. 3. It is apparent that the rate of absorption is much greater in the intestine, $t_{1/2}$ = 19 min. for intestinal absorption (pH 6.0) and $t_{1/2}$ = 255 min. for gastric absorption (pH 3.0). This difference in absorption rates was also observed by Kakemi *et al.* (1, 2). This result suggests that phenobarbital absorption is similar to the absorption of other weak acids reported previously (9). In the previous investigation, the conclusion was that the small intestine may be the main site of absorption for both weak acids and bases, possibly due to the large surface area, blood supply, and longer residence time. Since negligible absorption may occur in the stomach, the effect of food may have resulted from delayed stomach emptying and, hence, a delay before the drug reaches the primary absorption site—the small intestine.

To test this hypothesis, the determination of the percent of drug remaining was repeated; however, in this experiment the percents in the stomach, small intestine, and large intestine (including cecum) were determined separately (Table II). As can be seen from Table II, greater than 90% of the drug remaining to be absorbed at any given time was contained in the stomach in both fasted and nonfasted rats. Virtually no drug reached the large intestine. This finding not only supports the hypothesis but also suggests that since intestinal absorption is efficient, the presence of food will not significantly decrease the extent of phenobarbital absorption unless degradation occurs in the gastric fluid.

It is also possible that the apparent difference in gastric emptying time between fasted and nonfasted rats was due to rapid movement of solution through the stomach during dosing rather than following drug administration. The solution may immediately pass into the duodenum in fasted animals due to the volume of the drug solution, whereas the presence of food may inhibit this passage. This possibility was tested by administering a solution containing a nonabsorbable marker, ^{14}C-polyethyleneglycol, and determining the percentage of solution that had passed into the duodenum immediately (within 3 min.) after intubation in fasted rats. It was found that an average of 10.4% (a range of from 5.6 to 18.3%) was passed into the duodenum in eight rats. These data indicate that the initial emptying in fasted rats will not account for the difference in stomach emptying between fasted and nonfasted rats.

Further studies were carried out to measure the onset and duration of action and the serum level in nonfasted rats when 150 mg./kg. of phenobarbital was administered orally and then a second



Scheme I

150-mg./kg. dose was administered 1 hr. later (Fig. 1 and Table I). The time of onset of action was 79 min., that is, about 20 min. after the administration of the second dose. The duration of action was greater than 10 hr.; seven of the 12 rats studied slept for over 29 hr. and three rats died. When a single dose of 300 mg./kg. of phenobarbital was administered orally to nonfasted rats, the onset and duration of action were approximately the same as for those of the repeated dosing (Table I). The deviation of onset of action was greater for the single 300-mg./kg. dose than for the repeated dosing, and one rat did not lose the righting reflex. As shown in Fig. 1, the serum levels in the repeated-dosing case increased greatly after the administration of the second dose, reaching a maximum in about 1 hr. after the second dose, *i.e.*, 2 hr. after the first dose. These data suggest that the absorption of the second dose is faster and the rapid rise in the 1st hr. after the second dose reflects the faster absorption of the second dose along with residual absorption of the first dose. More importantly, however, these data indicate that in man if a second dose is self-administered, there is a significant hazard of intoxication due to a delayed onset of action.

This study demonstrated the effect of food on the oral absorption of phenobarbital. It was shown that absorption was negligible from the stomach and, consequently, that the primary site for absorption was the small intestine. The presence of food altered absorption, primarily by delaying stomach emptying and hence increasing the time for the drug to reach its primary absorption site. Since the intestine may be the primary absorption site for a great number of drugs (9), this example may represent a model for the effect of food in general on absorption (Scheme I). Thus, if intestinal absorption is very fast, the rate of stomach emptying may become rate limiting in the absorption process in the presence of food. The increased time that the drug is in the stomach may also reduce the amount of drug eventually absorbed for drugs that are unstable in gastric fluid.

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Binding of 5,5-Diphenylhydantoin and Its Major Metabolite to Human and Rat Plasma Proteins

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Abstract □ Equilibrium dialysis was used to determine the degree of plasma protein binding of both 5,5-diphenylhydantoin and its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, in man and in the rat. Binding studies show that diphenylhydantoin is extensively bound to human and rat plasma proteins and that the amount of diphenylhydantoin bound per gram protein, at equivalent free diphenylhydantoin concentrations, is greater in human plasma than in rat plasma. Since the rat has higher (≈1.6-fold) free diphenylhydantoin levels at equivalent total plasma drug levels, correlation of free diphenylhydantoin levels in these species with the *in vivo* incidence of drug-induced gingival hyperplasia was not possible. Dialysis studies show that the *para*-hydroxy metabolite is strongly plasma protein bound and that the amount of metabolite bound per gram protein, at equivalent free metabolite concentrations, is greater in rat plasma than in human plasma. Since a given total metabolite plasma concentration results in higher (≈1.4-fold) free levels in human plasma than in rat plasma, differences in plasma protein binding of the metabolite between these species *in vivo* may contribute to an explanation for the species selectivity of diphenylhydantoin-induced gingival hyperplasia.

Key phrases □ 5,5-Diphenylhydantoin, metabolite—plasma protein binding, rats, man □ 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin—plasma protein binding, rats, man □ Plasma protein binding—5,5-diphenylhydantoin and metabolite, rats, man □ Equilibrium dialysis—drug–plasma protein binding □ Gingival hyperplasia—effect of drug–plasma protein binding

The antiepileptic, 5,5-diphenylhydantoin, has been shown by several investigators to be partially bound to plasma proteins (1–12). The possibility exists that species differences in the degree of plasma protein binding of diphenylhydantoin may account for species-specific drug responses.

Chronic administration of diphenylhydantoin can produce a high incidence (mean approximately 35%) of gingival hyperplasia in man (10, 13, 14). However, it has not yet been possible to produce this effect experimentally in the rat and most other laboratory species (10, 15–17). Consequently, it has been difficult to study the etiology of the adverse reaction to diphenylhydantoin, and the mechanism involved remains obscure. Differences in free plasma levels of diphenylhydantoin or of its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, that result from species differences in plasma protein binding may help to explain the species selectivity of this adverse drug reaction.

The equilibrium dialysis studies reported here determined the degree of plasma protein binding of both diphenylhydantoin and its *para*-hydroxy metabolite in the human and the rat. The results of these studies provide a comparison of free plasma drug and of free drug–metabolite concentrations between these species. The possible influence of drug binding on the development of gingival hyperplasia in these species is discussed.

Table I—Total Plasma Protein Values for Diphenylhydantoin Binding Studies

Plasma Source		Total Protein[a,b], mg./ml.
Species	Sex	
Human GC	Male	84.6 ± 1.0
Human JW	Male	94.0 ± 1.1
Human JO	Female	83.2 ± 1.5
Human KS	Female	85.2 ± 0.8
Rat	Male	79.5 ± 1.4
Rat	Female	85.0 ± 1.2

[a] Mean of eight determinations with standard deviation of the mean. [b] Two-tailed Student *t* test shows significant difference ($p < 0.05$) for: human *versus* rat, male rat *versus* female rat, and human JW *versus* other human samples.

EXPERIMENTAL

Materials—*Chemicals*—ACS reagent grade chemicals or the equivalent obtained from standard sources were employed in these studies. The following hydantoin derivatives were used: 5,5-diphenylhydantoin[1], 5-(*p*-hydroxyphenyl)-5-phenylhydantoin[2], 5-(*m*-hydroxyphenyl)-5-phenylhydantoin[2], and 5,5-diphenylhydantoin-4-^{14}C (10.9 mc./mmole)[3].

Animals—Male and female Holtzman rats, weighing between 200 and 270 g., were employed. Rats were maintained on a complete diet[4] and water *ad libitum* for at least 1 week prior to use.

Human Volunteers—Adult Caucasian male and female humans[5], between the ages of 21 and 29 years, volunteered for these studies. The participants abstained from alcohol, coffee, tea, nicotine, and all drugs for 24 hr. preceding the blood sampling.

Methods—*Diphenylhydantoin Colorimetric Assay*—Diphenylhydantoin was determined in aliquots of plasma and of diffusate obtained from equilibrium dialysis experiments by means of the colorimetric method of Dill *et al.* (18). The sodium diphenylhydantoin concentration was calculated using the extinction coefficient determined from diphenylhydantoin standards carried through the procedure simultaneously with unknown samples.

Diphenylhydantoin Radioisotope Measurement—Aqueous diphenylhydantoin-4-^{14}C aliquots (1.0–3.0 ml.) from dialysis studies were adjusted to 3.0 ml. with distilled water and mixed with 10.0 ml. of scintillator solution[6] to form an emulsion gel stable at 5°. ^{14}Carbon was counted by liquid scintillation spectrometry. The diphenylhydantoin sodium concentration was calculated using the specific activity of ^{14}carbon determined by counting diphenylhydantoin-4-^{14}C samples of known drug concentration.

para-*Hydroxy Metabolite GLC Assay*—The *p*-hydroxy metabolite was quantitated in plasma and diffusate aliquots by means of the GLC method of Chang and Glazko (19) as modified by Conard *et al.* (10, 20). The modification permits extraction of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin and its internal standard, 5-(*m*-hydroxyphenyl)-5-phenylhydantoin, without extraction of plasma constituents which cause interference peaks. Metabolite quantitation was carried out using an internal standard, digital integrator area count of peaks, and predetermined relative weight responses (metabolite/internal standard) from standard samples carried through the extraction and assay procedure.

[1] Dilantin, Parke, Davis and Co., Ann Arbor, Mich.
[2] Parke, Davis and Co., Ann Arbor, Mich.
[3] Schwarz BioResearch, Inc., Orangeburg, N. Y.
[4] Rockland Teklad Mouse-Rat Pellet Complete Diet.
[5] Employees of the American Dental Association, Research Institute.
[6] Insta-Gel, Packard Instrument Co., Inc., Downers Grove, Ill.

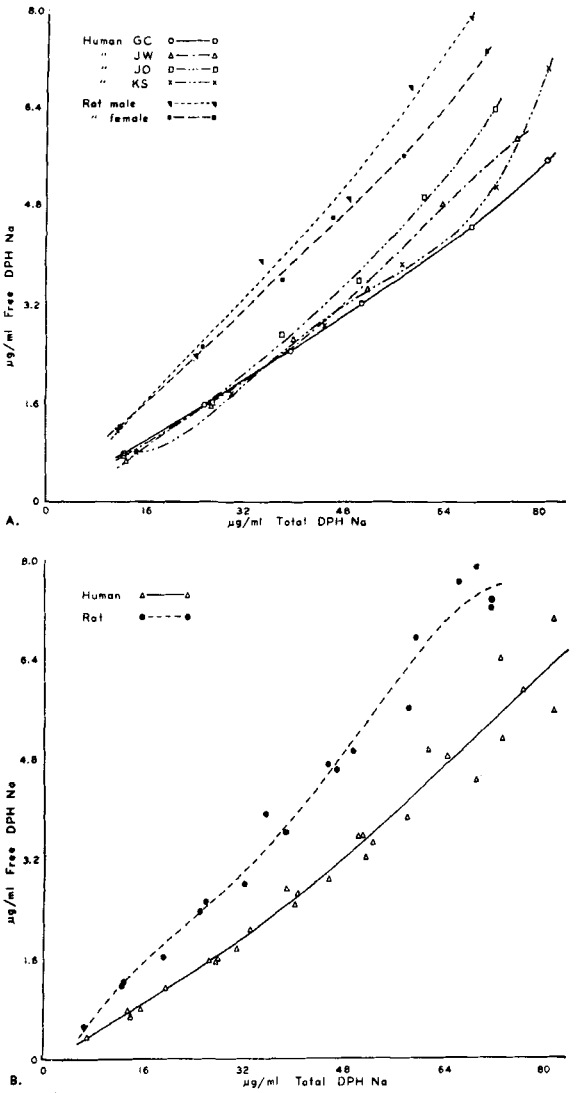

Figure 1—*Sodium 5,5-diphenylhydantoin (DPH Na) binding to six plasma sources as determined by the colorimetric assay. Key: A, data from individual plasma samples; and B, data grouped according to species of plasma. Each point represents the mean binding values from one dialysis experiment; each line drawn is a least-squares curve, computer fitted using a fourth-order polynomial.*

Protein Determination—The colorimetric protein assay of Lowry *et al.* (21) was utilized to determine total plasma protein concentration. Protein was quantitated using a standard curve prepared daily with known amounts of crystalline bovine plasma albumin[7]. The percent albumin in plasma preparations was determined by cellulose acetate electrophoresis and zone measurement by densitometry. Electrophoresis of plasma was run in barbital buffer, μ = 0.075, pH 8.6, at 250 constant volts. Densitometry was performed on the cellulose acetate membrane after trichloroacetic acid fixation and staining[8].

Equilibrium Dialysis Method—Binding of diphenylhydantoin and of the *p*-hydroxy metabolite to plasma proteins was determined *in vitro* by equilibrium dialysis. Studies utilized undiluted human and rat plasma freshly prepared daily from heparinized blood. Human plasma studies employed plasma from individuals, while the rat plasma studies utilized pooled plasma from 8–12 animals of one sex. The amounts of diphenylhydantoin added to the dialysis system were chosen to result in plasma diphenylhydantoin concentrations after equilibrium equivalent to *in vivo* levels ranging from low to very toxic. A similar concentration range was employed for the metabolite of diphenylhydantoin. Plasma samples of either 5 or 10 ml. were placed in size 20 dialysis tubing[9] and dialyzed against either 25 or 50 ml. of diffusate, respectively. The diffusate consisted of modified Locke's physiological salt solution buffered to pH 7.4 with 0.04 *M* phosphate, as described by Eik-Nes *et al.* (22), and 1.0 ml. drug solution. The dialysis system was maintained at 4° with continuous stirring. After 24 hr., sufficient time for equilibration, duplicate aliquots of each fraction were removed for drug extraction and assay or for radiodrug assay. The concentration of drug (diphenylhydantoin or metabolite) determined in the diffusate fraction represents the free drug level, while the concentration of drug (diphenylhydantoin or metabolite) measured in the plasma fraction represents the total drug level, a sum of free and bound drug. The change in pH observed during equilibration was nominal (<0.2 unit) and constant between dialysis experiments.

RESULTS AND DISCUSSION

Diphenylhydantoin Binding to Plasma—Figure 1A permits a comparison of free diphenylhydantoin levels between the various plasma sources; the bottom four lines represent data from individual human plasma samples of both sexes and the top two lines represent data from rat plasma of each sex. These data indicate

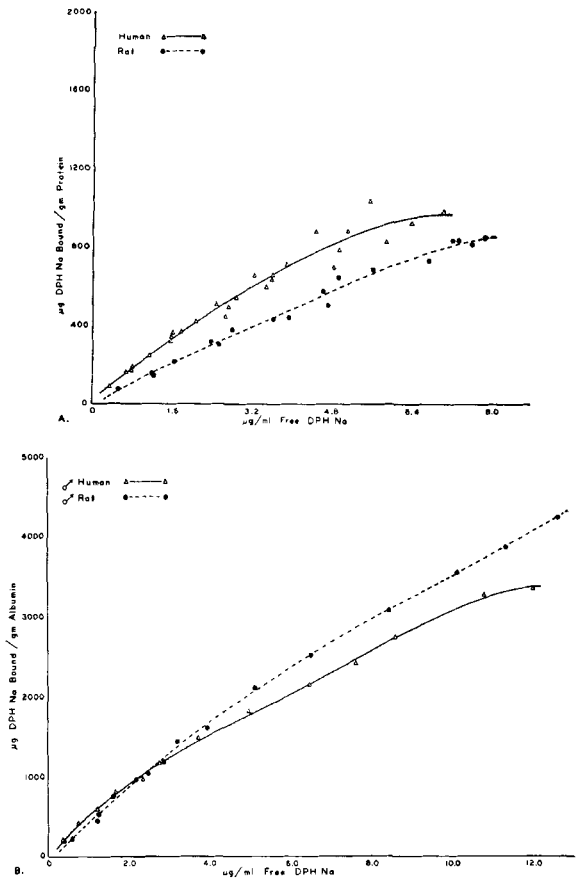

Figure 2—*Sodium 5,5-diphenylhydantoin (DPH Na) binding to human and rat plasma. Key: A, binding per gram total protein as determined by the colorimetric assay; and B, binding per gram albumin as determined by the radiodrug assay. Each point represents the mean binding values from one dialysis experiment; each line drawn is a least-squares curve, computer fitted using a fourth-order polynomial.*

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[8] With Ponceau S.
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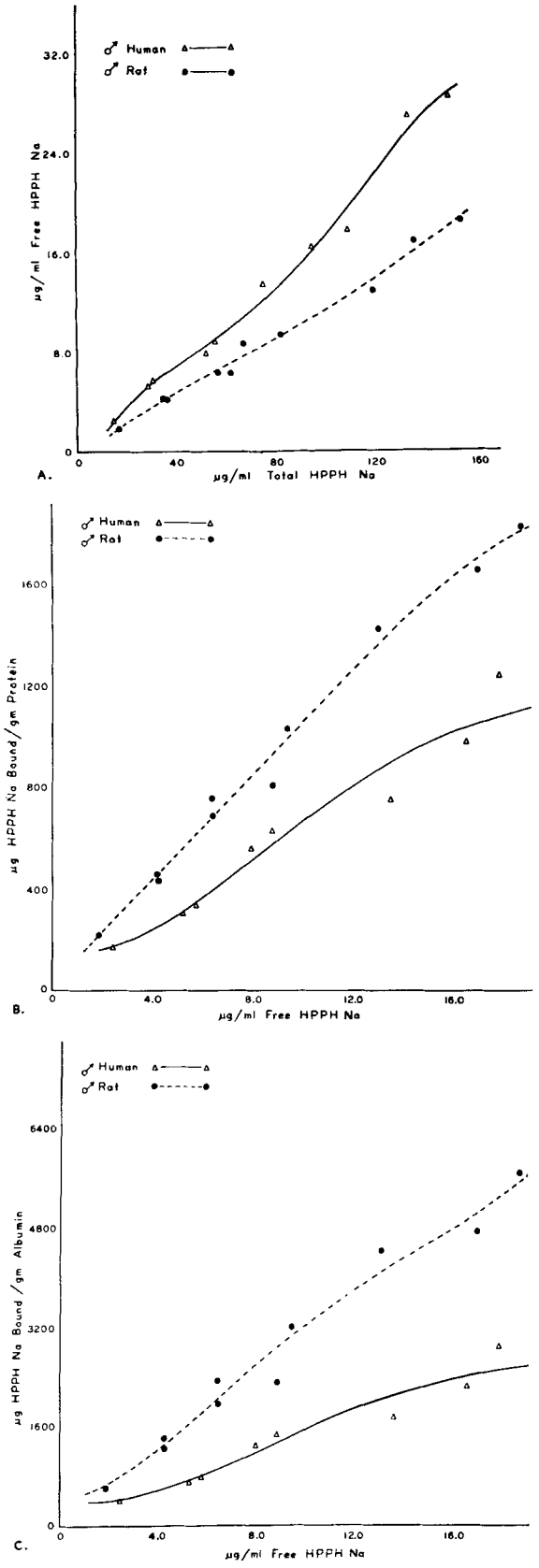

Figure 3—*Sodium 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH Na) binding to human and rat plasma as determined by the GLC assay. Key: A, data from each species of plasma; B, binding per gram total protein; and C, binding per gram albumin. Each point represents the mean binding values from one dialysis experiment; each line drawn is a least-squares curve, computer fitted using a fourth-order polynomial.*

that diphenylhydantoin is extensively bound to plasma proteins of both man and rat, but the degree of binding differs between the rat and individual humans. Since the concentration of free diphenylhydantoin in human plasma is lower than that in rat plasma at all equivalent total diphenylhydantoin levels studied (5–80 mcg./ml.), grouping of the dialysis data according to species appears justified. Grouping of the data in this manner (Fig. 1B) permits a direct comparison of diphenylhydantoin binding between species. It can be seen that at an intermediate total plasma diphenylhydantoin level, such as 40.0 mcg./ml., which has been encountered during therapeutic use of diphenylhydantoin, the free drug level in rat plasma is 3.9 mcg./ml., while that in human plasma is 2.5 mcg./ml. At this total drug level, the rat/human free drug ratio is 1.6; this value shows that a species difference exists in the binding of diphenylhydantoin to plasma proteins.

When these data are expressed as fraction sodium diphenylhydantoin bound *versus* total plasma drug level, it becomes apparent that the bound drug fraction decreases with increasing total diphenylhydantoin level. The fraction bound in human plasma throughout the total plasma drug concentration range employed is 0.95–0.92, while in rat plasma the fraction bound is 0.91–0.89.

The species difference in diphenylhydantoin binding in plasma shown here results in higher free drug levels in rat plasma than in human plasma at any given total diphenylhydantoin plasma level. These results, obtained by equilibrium dialysis, should be indicative of the degree of *in vivo* drug binding to plasma proteins in man and in the rat. Therefore, differences in drug binding between these two species apparently do exist and may contribute to species differences in pharmacological and toxicological responses. However, this species difference observed in free plasma diphenylhydantoin levels *in vitro* does not appear to contribute to the *in vivo* failure of diphenylhydantoin to induce gingival hyperplasia in the rat (10).

Diphenylhydantoin–Plasma Protein Binding—Total protein concentration values for the six sources of plasma utilized are summarized in Table I. Statistical analysis shows that significant differences exist between species of plasma and between sexes of rat plasma (*Footnote b*, Table I). Human total protein values are within the normal range (23); rat total protein values are consistent for the strain. These total protein values were utilized in subsequent calculations of the amount of diphenylhydantoin bound per gram total protein. Data expressed in this manner are corrected for drug binding differences which result from differences in plasma protein concentration between plasma sources. In Fig. 2A, it can be seen that human plasma proteins adsorb more drug per gram protein over the free diphenylhydantoin levels studied than do the rat plasma proteins. This difference between species in diphenylhydantoin binding is similar to that observed in Fig. 1A.

Diphenylhydantoin-^{14}C–Plasma Protein Binding—Since species differences in total protein do not account for differences in diphenylhydantoin binding to plasma proteins, the influence of species differences in albumin levels on binding was evaluated in additional studies. Diphenylhydantoin-^{14}C was employed in these studies to improve analytical sensitivity and precision. A significant difference exists in the albumin fraction between the two species of plasma (*Footnote c*, Table II). The albumin values shown in Table IIA are means of the values used to calculate the amount of diphenylhydantoin bound per gram albumin in these studies. The diphenylhydantoin-^{14}C dialysis binding data, when plotted similarly to Fig. 2A, are in good agreement and substantiate the species difference in diphenylhydantoin binding to human and rat plasma proteins.

However, when the ^{14}carbon binding data are plotted as micrograms of sodium diphenylhydantoin adsorbed per gram albumin *versus* free sodium diphenylhydantoin concentration to correct for species differences in albumin concentration (Fig. 2B), the species difference in diphenylhydantoin binding observed previously (Fig. 2A) is diminished at low free drug levels. In addition, the species difference in diphenylhydantoin binding is reversed at high free drug levels. A comparison of these data with the data presented in Fig. 2A shows that the observed species difference in diphenylhydantoin binding to human and rat plasma proteins can be accounted for in part by the differences in plasma albumin concentration.

***para*-Hydroxy Metabolite Binding to Plasma**—In view of the conclusion that species differences in diphenylhydantoin–plasma protein binding do not appear to correlate with the species-specific

Table II—Total Protein and Albumin Values for Plasma Preparations

Plasma Source: Species	Plasma Source: Sex	Number of Preparations[a]	Total Protein[b,c], mg./ml.	Percent Albumin[b,c]	Albumin[b,c], mg./ml.
A. Values from Diphenylhydantoin-^{14}C Binding Studies					
Human GC	Male	7	87.2 ± 0.6	42.9 ± 0.3	37.4 ± 0.3
Rat	Male	3	77.7 ± 1.6	32.0 ± 1.0	24.8 ± 0.2
B. Values from *para*-Hydroxy Metabolite Binding Studies					
Human GC	Male	3	86.9 ± 0.3	42.9 ± 0.2	37.2 ± 0.3
Rat	Male	2	79.0 ± 2.2	33.5 ± 1.4	26.4 ± 0.4

[a] Eight determinations per plasma preparation. [b] Mean value with standard deviation of the mean. [c] Two-tailed Student *t* test shows significant difference ($p < 0.01$) between species.

induction of gingival hyperplasia, the binding of the major diphenylhydantoin metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, to plasma proteins was studied by equilibrium dialysis. Figure 3A permits a comparison of free metabolite levels between species of plasma. These data indicate that the metabolite is strongly bound to proteins of both species of plasma. Free metabolite levels in the human plasma are higher than those in the rat plasma throughout the total plasma metabolite range employed (10–160 mcg./ml.). At an intermediate total plasma metabolite level, such as 80 mcg./ml., the free metabolite level in human plasma is 13.0 mcg./ml., while that in rat plasma is 9.0 mcg./ml. At this total metabolite level, the human/rat free drug ratio is 1.4; this value indicates that the binding of the metabolite also differs in these two species and that this binding difference varies markedly from that found to exist for the parent drug.

When these data are expressed as fraction metabolite bound *versus* total plasma metabolite level, it becomes apparent that the bound fraction decreases with increasing total metabolite level. The fraction bound throughout the total plasma metabolite concentration range employed is 0.89–0.88 in rat plasma, while in human plasma the fraction bound is 0.84–0.81. A comparison of these data with those obtained for diphenylhydantoin indicates that in rat plasma the fraction bound is decreased slightly for the metabolite, which is a more polar molecule than is the parent drug, while in human plasma the fraction bound is greatly decreased for the metabolite relative to the parent drug.

This species difference in the metabolite binding to plasma proteins results in higher free *para*-hydroxy metabolite levels in human plasma than in rat plasma at any given total metabolite level. Consequently, this difference observed in free plasma metabolite concentrations between species *in vitro* may contribute to an explanation for the *in vivo* species selectivity of diphenylhydantoin-induced gingival hyperplasia.

***para*-Hydroxy Metabolite–Plasma Protein Binding**—Total protein, percent albumin, and albumin concentration values for the plasma utilized in diphenylhydantoin metabolite dialysis binding studies are summarized in Table IIB. These values are means of the values used to calculate the amount of metabolite bound per gram total protein and the amount of metabolite bound per gram albumin. In Fig. 3B, it can be seen that rat plasma proteins adsorb more metabolite per gram total protein at free metabolite levels, ranging from 1.0 to 20.0 mcg./ml., than do the human plasma proteins. This difference in metabolite binding between species is similar to that observed in Fig. 3A. When these binding data are corrected for differences in albumin levels (Fig. 3C), the species difference previously observed in the binding of the metabolite is increased.

CONCLUSION

The interaction of drugs with plasma proteins is of both theoretical and practical importance; drug–plasma protein binding modifies drug distribution in the body and, therefore, can influence the dose–response relationship, the rate of drug elimination, and the pharmacological and toxicological actions of a drug (24–27). Species differences in plasma protein binding consequently could account for species-specific adverse drug reactions.

Plasma protein binding of diphenylhydantoin and of its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, was determined using equilibrium dialysis of undiluted human and rat plasma samples to compare the degree of *in vitro* protein binding between these species. This comparison was made to determine whether species differences in free plasma drug or drug–metabolite levels correlate with *in vivo* species differences in pharmacological and toxicological responses.

Data reported here show that diphenylhydantoin is extensively bound to proteins in human and rat plasma and that human plasma proteins bind a greater amount of diphenylhydantoin *in vitro* at all total plasma drug concentrations studied than do rat plasma proteins. This difference in drug binding between species results in lower free diphenylhydantoin levels in the human than in the rat at equivalent total drug plasma levels. Therefore, it appears that plasma protein binding differences between these species do not contribute to the *in vivo* failure of diphenylhydantoin to induce gingival hyperplasia in the rat.

Species differences in plasma albumin concentration suggest that this species difference in diphenylhydantoin binding may be explained in part by species differences in albumin levels.

It also has been shown that the major diphenylhydantoin metabolite is strongly protein bound in human and rat plasma and that rat plasma proteins bind the metabolite to a greater extent *in vitro* at all total plasma concentrations studied than do human plasma proteins. This binding difference results in higher free metabolite levels in the human than in the rat at equivalent total plasma levels; this situation is the opposite of that found for diphenylhydantoin. These free metabolite levels observed *in vitro* correlate with the *in vivo* incidence of diphenylhydantoin-induced gingival hyperplasia in man and rat. In the event that *in vivo* differences in the binding of the metabolite to proteins parallel *in vitro* binding differences between these species, plasma protein binding of the metabolite may contribute to an explanation for the species selectivity of the drug-induced pathology.

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Effects of Dietary Components on GI Absorption of Acetaminophen Tablets in Man

JAMES M. JAFFE, JOHN L. COLAIZZI*, and HERBERT BARRY, III†

Abstract □ Various high carbohydrate, high protein, and high lipid test meals were administered concurrently with acetaminophen tablets to human subjects to study the effects of foods on GI absorption of this drug. Balanced test meals and a fasting condition were also employed. An indication of the rate and extent of drug absorption was obtained by measuring urinary excretion at 1.5-hr. intervals over a 9-hr. period. The initial rate of excretion of acetaminophen and its metabolites was significantly reduced with a majority of the carbohydrate test meals. High protein, high lipid, or balanced meals appeared to have no statistically significant effect, while the fasting condition showed only a trend toward initially higher excretion values. The cumulative amounts of total acetaminophen and metabolites excreted in the urine at the end of 9 hr. showed little difference among test meals. The apparent inhibition of acetaminophen absorption by carbohydrate test meals could be partially attributed to an interaction with pectin in some cases.

Keyphrases □ Absorption kinetics, GI—acetaminophen tablets, effect of foods, man □ Acetaminophen tablets—effect of foods on GI absorption, man □ Dietary considerations—GI absorption of acetaminophen tablets in man □ Foods—effect of high carbohydrate, high protein, high lipid test meals on GI absorption of acetaminophen tablets, man

It is generally recognized that administration of drugs by the oral route a short time before or after a meal may alter absorption of the drug from the GI tract. However, few definitive studies have correlated specific dietary components with effects on drug absorption. Generally, meals have been reported to retard absorption. Wood (1) indicated that with five different commercial aspirin preparations tested in humans, half-lives of absorption were more than doubled by the nonfasting condition. Hirsch and Finland (2) demonstrated that similar doses of erythromycin stearate and erythromycin propionate administered orally after breakfast resulted in significantly lowered blood levels when compared with equal doses given before breakfast. In the same study, however, blood levels achieved with orally administered triacetyloleandomycin did not vary significantly when given either before or after breakfast.

Peterson and Finland (3) showed that sulfadiazine administered orally after the morning meal was absorbed more slowly, but more completely, than when administered to fasting subjects. Kirby *et al.* (4) and Rosenblatt *et al.* (5) showed that foods, especially those containing significant amounts of divalent metal ions, can inhibit absorption of tetracyclines, at least partially by a chelation mechanism. Reduction of absorption efficiency by food was also shown for orally administered lincomycin (6) and penicillin (7).

Contrary to these studies, others demonstrated that a meal preceding administration of a drug may enhance its absorption from the GI tract. Levy and Jusko showed that absorption of riboflavin (8) and riboflavin-5′-phosphate (9) was enhanced when administered orally after a standard breakfast of cornflakes and milk, and they attributed these effects to decreased intestinal transit rates. Crounse (10) demonstrated that the absorption of orally administered griseofulvin was doubled when given after a high lipid breakfast (consisting of bacon, eggs, cream, and butter) compared with high protein or high carbohydrate meals or even compared with the fasting state. More recently, Kabasakalian *et al.* (11) indicated that a high fat meal followed by griseofulvin administration at breakfast increased drug absorption,

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Table I—Summary of Components Utilized in Test Meal Series I

Component	Food Used	Amount of Food Used, g.	Grams of Each Component in Amount of Food Used[a] C	P	L	Percent Daily Requirement[a] C	P	L
High protein	Tuna	178.0	0	50.0	1.4	0	52.6	1.3
High lipid	Pecans	75.3	9.5	6.9	55.0	3.2	7.3	52.4
High carbohydrate	Jelly	115.4	75.0	0.2	0	25.4	0.2	0
	Crackers	105.6	75.0	9.7	12.5	25.4	10.2	11.9

[a] C = carbohydrate, P = protein, and L = lipid.

Table II—Summary of Components Utilized in Test Meal Series II

Component	Food Used	Amount of Food Used, g.	Grams of Each Component in Amount of Food Used[a] C	P	L	Percent Daily Requirement[a] C	P	L
High protein	Turkey	152.0	0	50.0	5.9	0	52.6	11.9
High lipid	Butter	61.1	0.2	0.4	55.0	0.07	0.4	52.4
	Bread	23.0	11.2	11.3	0.54	3.8	2.3	0.5
High carbohydrate	Dates	199.0	150.0	4.4	1.2	50.8	4.6	1.2

[a] C = carbohydrate, P = protein, and L = lipid.

Table III—Summary of Components Utilized in Test Meal Series III

Component	Food Used	Amount of Food Used, g.	Grams of Each Component in Amount of Food Used[a] C	P	L	Percent Daily Requirement[a] C	P	L
High carbohydrate	Marshmallows	198.5	150	3.0	0	50.0	3.2	0
High carbohydrate	Crackers	211.2	150	19.4	25.0	50.8	20.4	23.8
High carbohydrate	Jelly	230.8	150	0.4	0	58.8	0.4	0

[a] C = carbohydrate, P = protein, and L = lipid.

but this effect was not found when the high fat meal followed by griseofulvin administration was at supper.

The present study reports the effects of some high carbohydrate, high protein, and high lipid test meals on the absorption of orally administered acetaminophen tablets in humans. The objective of this investigation was to determine whether effects of food on the absorption of this drug are specific with respect to the particular dietary component.

EXPERIMENTAL

Subjects—The subjects employed in this study were normal, adult, healthy, male Caucasians whose ages ranged from 20 to 31 years (average age 25 years) and whose weights ranged from 59 to 86 kg. (average weight 76 kg.).

Test Meals—The food given as test meals in Series I, II, and III consisted of one-half of the normal daily requirement for each component needed by an average 25-year-old male (12). For the balanced meal, each of the three components was one-third of the high component test meal. This information is summarized in Tables I–III. The pectin test conditions employed (Series IV) were based on the 0.4% of pectin contained in grape jelly[1] used in Series I. For comparison, a higher pectin test was also employed containing 10 times the amount of pectin (4%) present in the other pectin test. The pectin was administered as a jelly prepared by dispersing it in the 270 ml. of water which the subjects normally ingested at the initial time period. A major consideration in the selection of the specific foods necessary to provide the required components in Series I, II, and III was the relationship of that component to other components contained in the same food. To be considered acceptable, a food high in one component (*e.g.*, carbohydrate) could not contain any more than 20% of another component (protein or lipid). Another consideration was that the food be palatable with a minimum of discomfort for the subjects ingesting it.

Procedure for Subjects—The experimental subjects were required to fast after the evening meal prior to the morning that the test meal was to be administered. However, they were permitted to take tea, coffee, soda, or water. They were also asked to get a normal night's sleep. Upon arriving at the laboratory, the subjects ingested the test meal with 270 ml. of water. The time required for consuming the meals was approximately 10 min. Immediately upon completion of the meal, each subject took one 325-mg. acetaminophen tablet[2]. The subjects were then required to provide a total void every 1.5 hr. for a total of 9 hr. (six voids). Each participant was also required to drink 180 ml. of water after each void to assure an adequate volume of urine.

The subjects were instructed to refrain from eating or drinking food or beverage of any kind (except the specified amounts of water) for 5 hr. after administration of the test meal and drug. After this time, the subjects were permitted to resume their normal diets. The subjects repeated this sequence each week until all test meals of the series were taken.

Sequence of Tests—In each of the four test series, a group of subjects was tested with each of the different test meals or test conditions in the series, with a usual and minimal interval of 1 week between successive tests. Different subjects were assigned the test meals or conditions in different sequences in order to eliminate possible sequential effects. The series of tests were conducted with an interval of 1 month between Series I and II and less than 1 month between Series II and III. The interval between Series III and IV was about 10 months, and the entire testing procedure was completed within a span of 1.5 years.

Five subjects were common to each of the first two test meal series, three additional subjects were tested in Series I only, and three in Series II only. Series III was tested with six subjects (four of whom had been previously tested in both Series I and II and two of whom had been previously tested in Series II only). Series IV also involved six subjects (three of whom had been previously tested in Series I, II, and III and one of whom had been previously tested in Series II and III only).

Analytical—The analytical technique utilized for the determination of acetaminophen in urine was based on a procedure employed by Ballard (13) and adapted from the method of Welch and Conney (14). The method involves the acid hydrolysis of acetamino-

[1] Welch Foods, Inc., Westfield, NY 17487

[2] Tylenol tablets, supplied by McNeil Laboratories, Inc., Fort Washington, PA 19034

Table IV—Cumulative Amount of Acetaminophen (Milligrams) Excreted at Each Time Interval for Series I, II, III, and IV

	Hours					
	1.5	3	4.5	6	7.5	9
Test Series I						
Carbohydrate	10.2	44.1	102.2	143.8	181.2	198.5
Protein	37.0	89.0	137.5	173.0	196.5	214.8
Lipid	35.4	89.8	138.6	178.7	207.5	226.9
Balanced	28.0	84.0	137.4	173.4	195.9	213.1
Fast	70.2	132.7	161.8	199.3	229.3	247.5
Test Series II						
Carbohydrate	11.8	49.4	105.9	154.1	189.2	209.4
Protein	14.7	54.5	99.4	130.9	155.4	173.9
Lipid	23.2	68.0	108.9	144.7	173.1	188.4
Balanced	25.8	75.2	118.8	149.7	168.4	180.2
Fast	30.8	79.6	131.6	165.3	195.2	214.1
Test Series III (All Carbohydrate)						
Crackers	16.3	59.0	112.1	150.0	179.0	201.0
Jelly	9.2	49.2	122.5	174.5	209.4	233.2
Marshmallows	28.7	81.7	149.4	195.5	224.6	247.7
Test Series IV						
Fast	26.8	55.6	92.4	121.7	144.8	160.6
Low pectin	27.5	72.8	117.9	142.8	166.0	183.1
High pectin	13.5	57.2	103.3	144.6	172.7	198.3

phen and its conjugates present in the urine to *p*-aminophenol. This compound was then coupled with phenol in the presence of hypochlorite to form an indophenol dye, the concentration of which could be determined spectrophotometrically.

Statistical Analysis of Data—The data were expressed in terms of milligrams acetaminophen excreted, both at each successive 90-min. interval after ingestion and cumulatively to the last collection at 9 hr. The statistical reliability of the differences among test meals and conditions, time intervals, and test series was tested by analysis of variance with the aid of a program (BMD 02V) used on the IBM. 7090 computer (15). The residual term consisted of the pooled interactions of subjects with each of the other variables and combinations of variables included in the analysis. Separate analyses were performed for the five subjects tested in both Series I and II, for the six additional subjects tested in one but not the other of these two series, for the six subjects tested in Series III, and for the six subjects tested in Series IV.

RESULTS

Table IV shows the mean cumulative excretion of acetaminophen at each 1.5-hr. interval for the different test series. Effects of particular test meals or conditions on the rate of excretion are indicated by the noncumulative amounts excreted at each time interval portrayed in the figures.

Figure 1 shows a comparison among three test conditions (carbohydrate, fasting, and balanced) for the five subjects tested in both Series I and II. The carbohydrate test conditions decreased the amount of acetaminophen excreted initially, followed by a compensatory increase at later time intervals. An analysis of variance on the five subjects who participated in Series I and II provided a test of the difference between the carbohydrate and the balanced test meal conditions, which is the most comparable condition. This analysis showed a reliable interaction between test meals and times ($F = 9.39$, $df = 5/80$, $p < 0.001$), signifying a different excretion–time profile for the two test meals. Use of the linear trend for the six time periods results in an even higher F value ($F = 24.24$, $df = 1/80$, $p < 0.001$). Figure 1 shows that the amount of excretion after the carbohydrate test meals tended to be depressed in the earlier time periods and elevated in the later ones. The lower excretion values with carbohydrates than with the balanced test meals were statistically reliable during the first 1.5 hr. ($F = 9.26$, $df = 1/12$, $p < 0.05$) and at the 3-hr. time period ($F = 7.08$, $df = 1/12$, $p < 0.05$), whereas carbohydrate was reliably higher at 4.5 hr. ($F = 5.70$, $df = 1/12$, $p < 0.05$) and at 6 hr. ($F = 8.28$, $df = 1/12$, $p < 0.05$).

The data in Table IV indicate very little difference in acetaminophen excretion values at each time interval among the protein, lipid, balanced, and fasting conditions. An analysis of variance applied to these four conditions, omitting carbohydrate, showed no statistically significant differences. Although Fig. 1 shows that the fasting conditions resulted in elevated levels throughout the entire span of the test, there was no reliable difference among the five test meal conditions in cumulative excretion at the end of 9 hr.

Since the results obtained with carbohydrate differed from those obtained with the other test conditions in Series I and II, three different carbohydrate test conditions were compared with each other in Series III. Figure 2 shows sizable differences among them in temporal pattern of acetaminophen excretion. The interaction between the three test foods and six time intervals was statistically reliable ($F = 2.55$, $df = 10/75$, $p < 0.05$). There was also a statistically significant difference among the three test foods at the 1.5-hr. interval ($F = 5.17$, $df = 1/10$, $p < 0.05$), the lowest amount of acetaminophen being excreted with the jelly and the highest with the marshmallows. The crackers resulted in intermediate scores. The values obtained for the jelly treatment corresponded rather closely to the 1.5-hr. interval obtained for earlier carbohydrate results (crackers and jelly in Series I and dates in Series II). However, as with the carbohydrate test meals in Series I and II, the jelly treat-

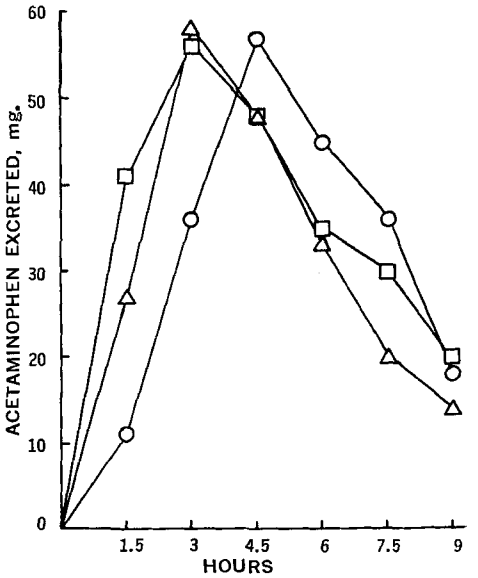

Figure 1—*Mean amount of acetaminophen excreted in the urine during each time interval for carbohydrate, balanced, and fasting conditions for the five subjects common to Series I and II (noncumulative). Key: □, fasting; △, balanced; and ○, carbohydrate.*

ment in Series III was only temporarily lower than the others. A compensatory increase for the jelly test meal began at 4.5 hr., at which time there was a reliable difference among the three treatments ($F = 7.43$, $df = 2/10$, $p < 0.05$). At the end of 9 hr., a statistically significant difference in cumulative acetaminophen excretion was found among the three carbohydrate test meals ($F = 6.22$, $df = 2/10$, $p < 0.05$). Table IV shows that the cumulative excretion was lowest with the crackers and highest with the marshmallows.

Since pectin was a component of the two carbohydrate test meals in Series I and II, and also of the jelly treatment in Series III which decreased acetaminophen excretion at the earliest time interval (1.5 hr.), low and high test conditions were compared with a fasting condition in Series IV. Figure 3 shows a comparison among the low pectin, high pectin, and fasting conditions. A statistically reliable interaction of test conditions with the linear function for the six time periods was found, comparing the high pectin test with the fasting condition ($F = 4.87$, $df = 1/50$, $p < 0.05$) and also with the low pectin test condition ($F = 7.28$, $df = 1/50$, $p < 0.01$). Figure 3 shows that after the high pectin treatment, the acetaminophen excretion was depressed in the initial time period and elevated in later time periods. Comparisons in particular time periods showed that the high pectin test condition differed reliably from the low pectin at 1.5 hr. ($F = 11.97$, $df = 1/5$, $p < 0.05$) and at 6 hr. ($F = 12.69$, $df = 1/5$, $p < 0.05$) and from the fasting condition at 6 hr. ($F = 10.73$, $df = 1/5$, $p < 0.05$).

DISCUSSION

The statistical analyses of the results of the four different phases of this study reveal that certain specific dietary components can significantly alter the absorption pattern of orally administered acetaminophen, while others result in only little or no difference from what is observed when the drug is administered in the fasting condition. These conclusions are based on the values obtained for the amounts of acetaminophen or its metabolites excreted in the urine of the subjects studied over 9 hr. The rate of urinary drug excretion is a function of the blood level of the drug and of its metabolites at any given time and, thus, serves as an indication of absorption.

The acetaminophen excretion averages obtained from Series I and II showed that there was significantly less total drug and metabolite excretion during the first 1.5-hr. interval with the high carbohydrate test meals, consisting of the crackers and jelly test meals (Series I) and also with the test meal of dates (Series II). In general, the other dietary components (protein and lipid) had little apparent effect on initial absorption of acetaminophen. Although the administration of all of the test meals resulted in lowered average values for the amount of total acetaminophen and metabolites excreted as compared with the fasted subjects, the trend was not entirely consistent.

In the third phase of the experiment, the comparison among three carbohydrate test meals showed that the ability to retard the initial absorption of acetaminophen does not appear to be common to all carbohydrate substances. The results of this portion of the study, in comparison with the findings obtained with the other series, showed that the absorption with marshmallows is more similar to the pattern found with the noncarbohydrate conditions. Both the jelly and the crackers test meals hindered absorption initially, jelly to the lesser extent, whereas the marshmallow test meal appeared to have little or no effect on acetaminophen absorption.

These findings indicate that certain carbohydrate substances (jelly, dates, and, to a lesser degree, crackers), when eaten in substantial amounts, initially inhibit the GI absorption of orally administered acetaminophen. The reduction of the amount of acetaminophen excreted averaged from 47 to 68% compared with lipid, protein, or balanced test meals during the first 1.5-hr. interval, and it averaged from approximately 22 to 41% over the initial 3-hr. period. Compared with the fasting condition, the reduction in the amount of acetaminophen excreted after the carbohydrate meals averaged from 60 to 76% over the first 1.5-hr. interval and from 33 to 53% over the initial 3-hr. period. These values indicate that the delayed absorption could probably be of clinical significance when analgesia is required.

A noteworthy fact is that the carbohydrate with the greatest inhibiting effect, jelly, contains a significant amount of pectin, whereas the carbohydrate with no inhibitory effect, marshmallows, contains little or no pectin. The carbohydrate test meals used in

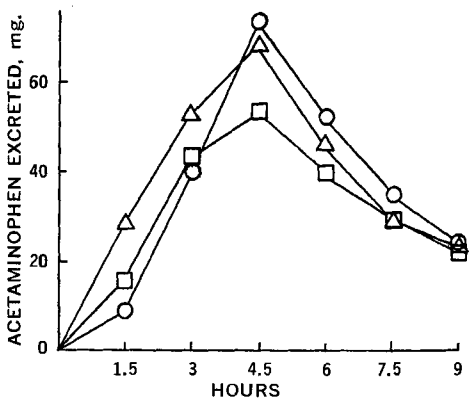

Figure 2—*Mean amount of acetaminophen excreted during each time interval for diets in Series III for the six subjects (noncumulative). Key: □, crackers; ○, jelly; and △, marshmallows.*

Series I (crackers and jelly) and probably also in Series II (dates) also contained pectin. A slightly greater inhibitory effect with Series I than with Series II might be due to a probably greater pectin content in the jelly–crackers test than in the test meal of dates. The crackers alone, in Series III, contain no pectin but differ from the other test meals in that they provide a large amount of starch, have minimal water content, are relatively bulky, absorb gastric fluids, and are not readily soluble in or miscible with the aqueous fluids of the GI tract. The marshmallows differ from the crackers in nearly all of these aspects.

This inhibitory effect of carbohydrate in Series I, II, and III persisted for 1–3 hr., followed in general by significantly higher acetaminophen excretion than with the other dietary conditions at these later time intervals. This trend also was seen in Series IV for the high pectin test but was slightly short of statistical significance at the 5% level. In general, the cumulative excretion values achieved with the subjects on jelly, dates, or jelly and crackers test meals were similar to the other test conditions at the intervals beyond 3 hr., although the actual amounts of drug excreted during the later intervals were greater with the carbohydrate test meals. The carbohydrates appear to cause merely a delay in absorption, extending it over a longer time period, rather than permanently inhibiting absorption.

In addition to the apparent lack of effect of marshmallows on acetaminophen absorption, the other noncarbohydrate conditions (protein, lipid, and balanced) cause only minimal and generally statistically insignificant effects on absorption as compared with the fasting condition. These findings are contrary to the common opinion that the presence of food in the GI tract generally reduces the rate or extent of drug absorption (16). A more realistic point of view, which has likewise been expressed as a generality, is that: "dietary

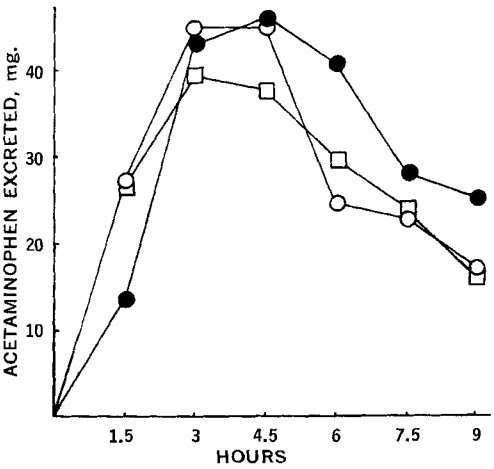

Figure 3—*Mean amount of acetaminophen excreted during each time interval for test conditions in Series IV for the six subjects (noncumulative). Key: □, fasting; ○, low pectin; and ●, high pectin.*

effects on drug absorption are quite variable and frequently depend on the physicochemical properties of the drug and the mechanism by which it is absorbed" (16). The results of the present study suggest that an inhibitory effect of food on drug absorption is limited to certain food components, to certain test conditions, or to certain types of subjects.

The present data give evidence that acetaminophen is capable of absorption from both the stomach and the intestine. This finding is in agreement with the work of Weikel and Lish (17) who found that, in rats, acetaminophen is absorbed from both the stomach and all portions of the small intestine and that it is relatively insensitive to changes in gastric emptying rates. The high carbohydrate test meals, which tended to retard acetaminophen absorption, would actually be expected to reduce the rate of gastric emptying less than the other test meals (18, 19), whereas the high lipid test meals, which markedly reduced the rate of gastric emptying, showed little or no tendency to retard acetaminophen absorption. Greater effects of the gastric emptying rate might be found with a drug such as aspirin, which is absorbed mainly from the stomach and the proximal small intestine (17).

The apparent depression of the initial absorption rates of acetaminophen by certain carbohydrates employed in this study cannot be explained by any effect of the monosaccharide or disaccharide sugar content alone, such as by competition for absorption sites or by viscosity effects, because this effect was not observed with the marshmallow high carbohydrate test meal. The depression of initial absorption rates found with all of the other high carbohydrate test meals could possibly be attributed to the pectin content in these substances. The presence of pectin in jelly and the likely presence of pectin or pectic substances in dates could reduce the absorption of acetaminophen by increasing viscosity and, thereby, possibly retarding both the dissolution rate of acetaminophen and the diffusion of dissolved acetaminophen throughout the gastric fluids and to the membrane absorptive sites. Pectin is known to act as an adsorbant and protectant in the GI tract and is used clinically as an antidiarrheal preparation for the purpose of adsorbing toxins, bacteria, and other noxious materials. Being polymeric in nature, pectin could also act as a complexing agent for drugs like acetaminophen in addition to possibly retarding absorption by adsorption. If the pectin content of jelly and dates is acting to retard absorption of acetaminophen by complexation or adsorption mechanisms, the process must be reversible since only the initial rate of absorption is retarded and not the ultimate amount of drug absorbed.

Series IV gave evidence that pectin alone can inhibit the rate of acetaminophen absorption. The high pectin test condition reduced the rate of absorption initially, as did the carbohydrate meals containing pectin in Series I–III. However, the amount of pectin contained in the jelly and dates test meals of Series I–III is probably more similar to the low pectin test condition of Series IV, which showed no reliable difference from the fasting condition. Thus, while the pectin content of these foodstuffs might have been a factor in retarding initial acetaminophen absorption, some other characteristics of the jelly and dates must have been involved.

Several factors might explain the reduction in initial acetaminophen absorption by crackers. The dry, bulky, and water-absorptive nature of crackers could decrease or delay absorption by decreasing the amount of biological fluid available in the GI tract and, thereby, reducing the dissolution rate of the drug, especially when administered as a solid dosage form (20). This could be a limiting factor in the absorption process since acetaminophen is considered to be only a very slightly soluble drug (21) for which dissolution would be rate limiting (22). Alteration of gastric emptying rate and intestinal transit time by bulk effects could also affect the absorption of acetaminophen since the bulk volume of the meal was greater for the crackers than for any of the other test meals. A tendency to complex with certain drugs was reported for starches, especially those drugs with amino or nitro groups (23). Since crackers are high in starch content and acetaminophen has an aminophenol structure, absorption might possibly be inhibited by a complex formation of this type.

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Normal and Promoted GI Absorption of Water-Soluble Substances II: Absorption of Vitamin B_{12} from Ligated Stomach and Intact Intestine of the Rat

W. W. DAVIS and C. J. KREUTLER

Abstract □ Both normal and promoted absorption of vitamin B_{12} in the doubly ligated stomach and the intact GI tract of the rat were investigated using ^{57}Co-labeled cyanocobalamin. Very little absorption of vitamin B_{12} occurs normally from the ligated stomach of the rat. Much more absorption is seen from the intact intestine. Both gastric and intestinal absorption are markedly increased by addition of a nonionic surface-active agent, polyoxyethylene-20-oleyl ether, to the aqueous solution of the vitamin. The effect of *ad libitum* feeding upon either normal or promoted absorption is minor. At high vitamin B_{12} levels, somewhat more normal absorption occurs in fasted than in fed animals. The results reported here are interpreted to be similar to the effects of surface-active absorption promoters on the absorption of other water-soluble substances.

Keyphrases □ Vitamin B_{12}—stomach and GI absorption, surfactant effect, rats □ Cyanocobalamin-^{57}Co—stomach and GI absorption, surfactant effect, rats □ Absorption, stomach and GI—vitamin B_{12}, rats

The normal occurrence of vitamin B_{12} in the food of all animals is small, and no active transport system is recognized for its absorption. The absorption from normal food sources would be inadequate but for the occurrence and special role of intrinsic factor. This relationship was extensively reviewed by Castle (1).

In the human, the condition known as pernicious anemia is one in which inadequate intrinsic factor is secreted in the stomach to permit the absorption of physiologically adequate amounts of vitamin B_{12}. Adequate oral therapy may be provided either by supplementing the intrinsic factor generally with supplementary vitamin B_{12} (2) or by administering large amounts of vitamin B_{12} without supplementing intrinsic factor (3).

In the rat, as in man, intrinsic factor is secreted in the stomach, binds vitamin B_{12} in the stomach and the intestine, and, as a complex in the intestine, is somehow responsible for improved efficiency of the passive absorption of the vitamin from dilute solutions (1). The quantity of intrinsic factor produced in the stomach is normally sufficient to allow the absorption from the food of physiologically adequate amounts of vitamin B_{12}.

Glass (4) reviewed a variety of extragastric factors that influence the absorption of vitamin B_{12} and discussed their possible modes of action. Okuda *et al.* (5) administered a number of surfactants in undiluted form to rats and observed an increase in absorption of vitamin B_{12}. Their experimental evidence supported their suggestion that increased absorption was due to delayed intestinal transit of the highly viscous mixture, a mechanism devoid of therapeutic interest. Preliminary experience in this laboratory employing dilute surface-active absorption promoters (6) indicated that the efficiency of absorption of vitamin B_{12} in test animals might be increased by the use of dilute solutions of selected surface-active agents, acting either in the stomach or in the intact intestine by inducing a reversible hyperabsorptive state of the GI mucosa.

EXPERIMENTAL

Male rats of the CFE strain[1], weighing 150–275 g., were used. The rats were caged individually and fasted for 15–20 hr. before beginning the experiment.

Rats with Ligated Stomach—The animal was anesthetized with ether, and the abdominal area was exposed through a midline incision. A pyloric ligation was performed, and the 2-ml. aqueous test solution was introduced into the stomach by a stomach tube prior to esophageal ligation. Care was taken not to occlude blood vessels unnecessarily. The abdominal incision was closed with wound clamps and coated with collodion. A gauze dressing was applied and the animal was placed in a holding cage where it recovered from the anesthesia within 5 min. Two hours after dosing, the animals were sacrificed and their liver and kidneys were excised for assay.

Intact Rats—Intact rats were lightly anesthetized with ether and were given 2 ml. of the aqueous test solution by stomach tube. These animals were held for 2, 6, 16, or 24 hr. before they were sacrificed, and their liver and kidneys were excised for assay. After 2 hr. postdosing, food[2] and water were provided *ad libitum* to some animals, while other animals were fasted throughout the 24-hr. experiment.

Preparation of Oral Dose—The 2-ml. aqueous test solution administered to the rats contained 0.4 $\mu c.$ ^{57}Co-vitamin B_{12}[3] in pH 7.0 isosmolar sodium phosphate buffer. Additional unlabeled vitamin B_{12} was added so that the standard solutions contained 400, 2000, or 6000 ng. of the vitamin. The nonionic surfactant, polyoxyethylene-20-oleyl ether[4], when administered, was present at a 1% concentration in the 2-ml. dose.

Assay of Tissue Radioactivity—The excised livers and kidneys were placed in wheaton vials for γ-counting. The radioactivity was determined using an autogamma spectrometer[5]. Computations of the vitamin B_{12} content of the tissues were made, and the results were expressed in nanograms. The total uptake by liver and kidneys was also expressed as percent of the administered dose and referred to as the "efficiency of absorption."

RESULTS

Absorption from Ligated Stomach—Results of the experiments with gastric ligated animals are presented in Table I. In the absence of an absorption promoter, the absorption of the drug was extremely limited. When the absorption promoter was present, much more absorption occurred. Both with and without promoter, the increasing dose of the vitamin (400, 2000, and 6000 ng./animal) resulted in an approximately proportionate increase in absorption at the higher concentrations. Less than proportionate absorption occurred at the lowest dose level.

[1] Carworth Farms, New York City, Rockland County, N. Y.
[2] Purina Laboratory Chow.
[3] Supplied by Merck and Co., Inc., Rahway, N. J.
[4] Brij-98, Atlas Chemical Industries, Wilmington, Del.
[5] Model 410A, Packard Instrument Co., Inc., LaGrange, Ill.

Table I—Normal and Polyoxyethylene-20-oleyl Ether-Promoted Absorption of Vitamin B_{12} in Ligated Rat Stomach[a]

Treatment Group	Sacrifice Time after Dosing, hr.	Weight, g. Mean	Weight, g. Range	Liver and Kidney Uptake of Vitamin B_{12}: Nanograms of Vitamin B_{12} Liver, Mean	Kidney, Mean	Total Mean	Total Range	Percent of Dose, Total Mean
400-ng. dose								
Control[b] (vitamin B_{12} only)	2	207	2	0.30	0.17	0.5	0.7	0.1
Promoted[c] (with 1% promoter)	2	196	13	1.60	1.08	2.7	0.4	0.7
2000-ng. dose								
Control[b] (vitamin B_{12} only)	2	259	14	2.18	0.85	3.0	1.5	0.2
Promoted[c] (with 1% promoter)	2	248	5	25.03	19.41	44.4	2.3	2.2
6000-ng. dose								
Control[b] (vitamin B_{12} only)	2	184	3	8.23	3.53	11.8	21.5	0.2
Promoted[c] (with 1% promoter)	2	190	4	73.49	62.29	135.8	34.9	2.3

[a] Values are recovered vitamin B_{12} calculated from the means of total radioactivity recovered. Each treatment group consisted of two animals. [b] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 400, 2000, or 6000 ng. vitamin B_{12} in pH 7.0 isosmolar sodium phosphate buffer. [c] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 400, 2000, or 6000 ng. vitamin B_{12} and 1% polyoxyethylene-20-oleyl ether in pH 7.0 isosmolar sodium phosphate buffer.

Absorption from Intact GI Tract—Experiments were performed in intact rats with the same three dose levels of vitamin B_{12}. The accumulation of absorbed radioactivity in the organs of the intact animals was followed over periods of up to 24 hr. The influence of *ad libitum* food and water was examined.

Three tables present the data: Table II for 400 ng., Table III for 2000 ng., and Table IV for 6000 ng. The data presented allow several comparisons of single parameter effects. First, the presence of promoter produced an increase over normal absorption. There are 18 such direct comparisons of the averages of pairs of animals. Except for one of these comparisons (Table II, Footnote *d*), all showed greater absorption and accumulation when polyoxyethylene-20-oleyl ether was added.

At 2 hr., the accumulation in the liver was greater than in the kidneys. At later times, the accumulation in the kidneys was greater than in the liver. A minor exception appears (Table III) for the 24-hr. fasted normal data.

As the dose of vitamin B_{12} was increased, the percent of the dose absorbed by the normal intrinsic factor-assisted process diminished. This decrease is seen in 11 of 12 comparisons. This decrease in efficiency of normal absorption was most prominent in progressing from the lowest dose to the intermediate dose. With promoter present, absorption approached a constant efficiency as the concentration of vitamin B_{12} increased (Tables II–V). This efficiency was lower than in the intrinsic factor-assisted process at the lowest concentration of the vitamin. With high doses of the vitamin, the limiting efficiency of absorption in the presence of promoter was much higher than in the absence of promoter.

Table VI presents the influence of promoter as ratios of efficiency in the presence *versus* the absence of promoter. At low concentrations of the vitamin, where the normal intrinsic factor-assisted absorption was most efficient, the addition of promoter added little further efficiency. The average ratios for promoted *versus* normal absorption was only 1.5 and 1.6 for fasted and fed animals, respectively. At high concentrations of the vitamin, this ratio was higher, averaging 2.0 and 4.1 for the fasted and fed animals, respectively.

The effect of feeding *versus* fasting is shown clearly in Table V. Efficiency of normal absorption at the low concentration of vitamin B_{12} was approximately equal in fasted and fed animals. Efficiency of normal absorption at the high concentration was lower in the presence of food. When promoter was present, no prominent difference in absorption was observed between fed and fasted animals, either at low or high concentrations of the vitamin.

DISCUSSION

Reynell *et al.* (7) found little or no absorption of vitamin B_{12} in the stomach of the rat. Okuda (8) found no absorption from a dose of 50 ng. of vitamin B_{12} in the pyloric-ligated stomach of the rat. The data presented here show that 0.12% of a dose of 400 ng. of vitamin B_{12} is absorbed normally from the doubly ligated stomach of the rat after 2 hr. When the dose of vitamin is increased to 6000 ng., the percent of the dose absorbed normally is somewhat higher—

Table II—Normal and Polyoxyethylene-20-oleyl Ether-Promoted Absorption of Vitamin B_{12} (400 ng.) in Intact Rats[a]

Treatment Group	Sacrifice Time after Dosing, hr.	Weight, g. Mean	Weight, g. Range	Liver and Kidney Uptake of Vitamin B_{12}: Nanograms of Vitamin B_{12} Liver, Mean	Kidney, Mean	Total Mean	Total Range	Percent of Dose, Total Mean
Fasted animals								
Control[b] (vitamin B_{12} only)	2	192	26	1.07	0.37	1.4	1.5	0.4
	6	233	60	3.87	7.04	10.9	2.8	2.7
	24	170	6	5.99	8.68	14.7	0.5	3.7
Promoted[b] (with 1% promoter)	2	222	13	2.38	1.29	3.7	4.3	0.9
	6	169	7	7.15	7.26	14.4	2.2	3.6
	24	164	1	9.97	15.31	25.3	1.0	6.3
Fed animals								
Control[b] (vitamin B_{12} only)	6	171	1	2.58	5.92	8.5	3.4	2.1
	16	176	11	3.68	8.21	11.9	1.2	3.0
	24	217	18	4.90	11.57	16.5	0.1	4.1
Promoted[b] (with 1% promoter)	6	157	11	3.71	10.59	14.3	4.8	3.6
	16	180	3	5.65	13.54	19.2	4.4	4.8
	24	209	49	3.48	10.44	(13.9)[d]	3.4	(3.5)[d]

[a] Values are recovered vitamin B_{12} calculated from the means of total radioactivity recovered. Each treatment group consisted of two animals. [b] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 400 ng. vitamin B_{12} in pH 7.0 isosmolar sodium phosphate buffer. [c] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 400 ng. vitamin B_{12} and 1% polyoxyethylene-20-oleyl ether in pH 7.0 isosmolar sodium phosphate buffer. [d] These figures resulted from combination of data from two animals, giving total recoveries in 12.20 and 15.64 ng. vitamin B_{12}. Several comparisons indicate these figures to be inconsistent with related data. They were accordingly ignored in all comparisons and summary generalizations of data in the text.

Table III—Normal and Polyoxyethylene-20-oleyl Ether-Promoted Absorption of Vitamin B_{12} (2000 ng.) in Intact Rats[a]

Treatment Group	Sacrifice Time after Dosing, hr.	Weight, g. Mean	Weight, g. Range	Liver and Kidney Uptake of Vitamin B_{12}: Nanograms of Vitamin B_{12} Liver, Mean	Kidney, Mean	Total Mean	Total Range	Percent of Dose, Total Mean
Fasted animals								
Control[b] (vitamin B_{12} only)	2	170	10	3.72	0.87	4.6	1.0	0.2
	6	208	4	9.63	16.07	25.7	5.3	1.3
	24	165	13	18.51	17.65	36.2	9.0	1.8
Promoted[c] (with 1% promoter)	2	250	0	8.41	4.31	12.7	5.5	0.6
	6	239	58	12.93	23.65	36.6	11.3	1.8
	24	165	18	34.80	47.64	82.4	0.1	4.1
Fed animals								
Control[b] (vitamin B_{12} only)	6	167	0	6.00	10.13	16.1	4.2	0.8
	16	179	3	11.31	27.01	38.3	7.1	1.9
	24	242	72	9.56	32.57	42.1	23.5	2.1
Promoted[c] (with 1% promoter)	6	164	9	13.27	22.93	36.2	1.6	1.8
	16	169	5	19.49	51.95	71.4	17.5	3.6
	24	231	58	26.55	96.26	122.8	35.2	6.1

[a] Values are recovered vitamin B_{12} calculated from the means of total radioactivity recovered. Each treatment group consisted of two animals. [b] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 2000 ng. vitamin B_{12} in pH 7.0 isosmolar sodium phosphate buffer. [c] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 2000 ng. vitamin B_{12} and 1% polyoxyethylene-20-oleyl ether in pH 7.0 isosmolar sodium phosphate buffer.

Table IV—Normal and Polyoxyethylene-20-oleyl Ether-Promoted Absorption of Vitamin B_{12} (6000 ng.) in Intact Rats[a]

Treatment Group	Sacrifice Time after Dosing, hr.	Weight, g. Mean	Weight, g. Range	Liver and Kidney Uptake of Vitamin B_{12}: Nanograms of Vitamin B_{12} Liver, Mean	Kidney, Mean	Total Mean	Total Range	Percent of Dose, Total Mean
Fasted animals								
Control[b] (vitamin B_{12} only)	2	233	23	6.06	3.47	9.5	1.5	0.2
	6	204	16	43.34	55.90	99.2	68.9	1.7
	24	169	0	48.64	54.76	103.4	31.4	1.7
Promoted[c] (with 1% promoter)	2	237	22	40.88	27.71	68.6	13.4	1.1
	6	226	4	44.00	117.31	161.3	2.3	2.7
	24	167	15	100.28	145.85	246.1	1.6	4.1
Fed animals								
Control[b] (vitamin B_{12} only)	6	167	9	12.69	19.21	31.9	12.6	0.5
	16	180	9	16.14	35.92	52.0	27.4	0.9
	24	214	30	13.53	35.27	48.8	19.3	0.8
Promoted[c] (with 1% promoter)	6	169	12	36.55	61.46	98.0	32.0	1.6
	16	178	4	54.10	142.79	196.9	21.3	3.3
	24	237	54	57.71	201.59	259.3	138.5	4.3

[a] Values are recovered vitamin B_{12} calculated from the means of total radioactivity recovered. Each treatment group consisted of two animals. [b] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 6000 ng. vitamin B_{12} in pH 7.0 isosmolar sodium phosphate buffer. [c] The 2-ml. dose contained 0.4 μc. ^{57}Co–vitamin B_{12} with a total of 6000 ng. vitamin B_{12} and 1% polyoxyethylene-20-oleyl ether in pH 7.0 isosmolar sodium phosphate buffer.

viz., 0.20%. This observation appears consistent with the proposal that normal passive absorption from the stomach is proportional to the concentration of free uncomplexed vitamin B_{12}. The fraction of unbound vitamin was very small in the experiments of Okuda (8), in which only 50 ng. was used; this fraction was larger in the present experiment in which 400 ng. was used, and it approached substantially all free vitamin in the 6000-ng. experiments. Efficiency of promoted gastric absorption also increases with the fraction of uncomplexed vitamin. With increasing dose and fraction in the free form, the efficiency increases to about 2.3% of the dose.

In the intact rat, the experimental data on normal absorption of the vitamin are consistent with the following interpretation. The intrinsic factor–vitamin complex is formed in the stomach to the limit of occurrence of intrinsic factor. This is less than enough to complex all of the 400-ng. dose, so that part of the 400-ng. dose is free. The complexed portion is strongly adsorbed on the intestinal wall, and the vitamin is more efficiently absorbed from this adsorbed complex than from the noncomplexed, nonadsorbed portion. When the total dose of vitamin is increased without increasing the quantity of intrinsic factor, the contribution of the intrinsic factor-assisted process becomes negligible, and the overall efficiency of absorption falls toward a characteristic value. In the absence of promoter, this value is roughly 1.7% of the dose in the fasted animal or 0.8% of the dose in the fed animal over 24 hr.

Administration of promoter with the vitamin in the intact rat does not affect formation of the vitamin–intrinsic factor complex. Again, high efficiency of absorption is seen at a low concentration of vitamin where the intrinsic factor-assisted process is maximally effective. The increased efficiency of absorption of the relatively small amount of uncomplexed vitamin in the presence of promoter results in an overall high efficiency of absorption.

Table V—Efficiency of Normal and Promoted Absorption Expressed as Percent of Dose Absorbed

Organ	Hours	Vitamin B_{12} Administered, ng. 400	2000	6000
Stomach (normal)	2	0.1	0.2	0.2
Stomach (promoted)	2	0.7	2.2	2.3
Intestine (normal)	2	0.4	0.2	0.2
Intestine (promoted)	2	0.9	0.6	1.1
Intestine, fasted (normal)	6	2.7	1.3	1.7
Intestine, fasted (promoted)	6	3.6	1.8	2.7
Intestine, fed (normal)	6	2.1	0.8	0.5
Intestine, fed (promoted)	6	3.6	1.8	1.6
Intestine, fasted (normal)	24	3.7	1.8	1.7
Intestine, fasted (promoted)	24	6.3	4.1	4.1
Intestine, fed (normal)	24	4.1	2.1	0.8
Intestine, fed (promoted)	24	(−)	6.1	4.3

Table VI—Ratios of Polyoxyethylene-20-oleyl Ether-Promoted Absorption to Normal Absorption Efficiencies in the Rat

Time of Sacrifice	Vitamin B_{12} Dose, ng. 400	2000	6000
Ligated stomach animals			
2 hr.	5.6	14.8	11.3
Fasted intact animals			
2 hr.[a]	3.3	2.8	7.1
6 hr.	1.3	1.4	1.6
24 hr.	1.7	2.3	2.4
Average	1.5	1.8	2.0
Fed intact animals			
6 hr.	1.7	2.2	3.3
16 hr.	1.6	1.9	3.8
24 hr.	—	2.9	5.3
Average	1.6	2.3	4.1

[a] Excluded from averages.

At a higher concentration of the vitamin, the intrinsic factor-assisted absorption contributes little and the efficiency of absorption falls and approaches a value characteristic of the uncomplexed vitamin in the "hyperabsorptive" intestine. This value is above 4.0% of the dose absorbed whether the animal is fed or fasted.

Previous work in these laboratories demonstrated a hyperabsorptive state of the dog stomach which resulted specifically from a surfactant-organ interaction (6). This hyperabsorptive state subsequently returned to a normal absorptive state after removal of the promoter. Unpublished studies in these laboratories also demonstrated similarly hyperabsorptive states of segments of the GI tract of dogs, rats, and chickens. The present study indicates that the ligated stomach and the intact GI tract of the rat becomes hyperabsorptive for uncomplexed vitamin B_{12} under the influence of polyoxyethylene-20-oleyl ether.

It can be concluded from Tables II–IV that the absorption in intact animals continued beyond 16 hr., because the fraction of the dose absorbed continued to increase between 16 and 24 hr. Roughly one-half of the total absorption occurred during the first 6 hr. A check of fecal and urinary radioactivity indicated that at 6 hr. very little excretion had occurred, while by 16 hr. most of the unabsorbed radioactivity had passed out of the GI tract.

The ratios of efficiency of promoted *versus* normal absorption in the intact intestine (Table VI) vary with dose and time of sacrifice. The highest ratios are for the fed animals at high dose and for the animals sacrificed at 2 hr. The 2-hr. results may well include some of the influence of the high ratio of promoted *versus* normal absorption characteristic of the stomach. It was concluded that the effect of the absorption promoter, polyoxyethylene-20-oleyl ether, is to render the stomach and intestine hyperabsorptive, improving the efficiency of absorption of only the uncomplexed portion of the vitamin. The ability of the endogenous intrinsic factor to enhance the absorption of small doses of vitamin B_{12} is not impaired by the presence of polyoxyethylene-20-oleyl ether.

It is not intended in this paper to consider or to imply the therapeutic usefulness of promoted absorption in vitamin B_{12} therapy. Data from clinical reports were cited because much of the knowledge of vitamin B_{12} absorption was obtained from clinical experience. The surprising effectiveness of polyoxyethylene-20-oleyl ether in promoting absorption of vitamin B_{12} of molecular weight 1355 suggests that passive absorption mechanisms may extend to substances of even higher molecular weights.

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Keyphrases □ Nornuciferines—*in vitro* metabolic and photolytic conversions to lysicamine □ Lysicamine—metabolic and photolytic conversion from nornuciferines, identification □ Aporphine biotransformations—nornuciferine metabolism □ TLC fluorimetry, reverse—analysis, nornuciferines □ Photolysis—conversion of nornuciferines to lysicamine □ TLC—identification, lysicamine □ Mass spectroscopy—identification, lysicamine

The metabolism of apomorphine was studied by Kaul *et al.* (1–4). Besides these investigations, however, no studies have appeared relative to the biological fate of aporphine alkaloids. This is somewhat surprising since aporphines occur widely in the plant kingdom (5, 6). In addition, naturally derived aporphines, as well as many synthetic analogs, possess interesting biological activities (6–10). Of particular significance are reports

Table VI—Ratios of Polyoxyethylene-20-oleyl Ether-Promoted Absorption to Normal Absorption Efficiencies in the Rat

Time of Sacrifice	Vitamin B_{12} Dose, ng. 400	2000	6000
Ligated stomach animals			
2 hr.	5.6	14.8	11.3
Fasted intact animals			
2 hr.[a]	3.3	2.8	7.1
6 hr.	1.3	1.4	1.6
24 hr.	1.7	2.3	2.4
Average	1.5	1.8	2.0
Fed intact animals			
6 hr.	1.7	2.2	3.3
16 hr.	1.6	1.9	3.8
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Average	1.6	2.3	4.1

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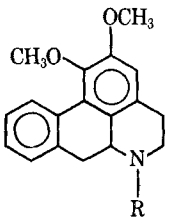
that apomorphine and its congeners may be useful in the treatment of Parkinsonism (11–13) and chronic manganism (13). Evidence of the dopinergic activity of aporphines (14–18) and use of apomorphine as an experimental probe of the CNS (19) underline the importance of further elucidation of aporphine metabolism.

A systematic study of aporphine biotransformations was commenced in these laboratories with an investigation of the *in vitro* metabolism of the nuciferine analogs, I–VIII, by rat, rabbit, and guinea pig liver microsomes. Results of these investigations are described in this report.



Compound	R
I	—H
II	—CH_3
III	—CH_2CH_3
IV	—$CH_2CH_2CH_3$
V	—CH_2— 
VI	—$CH_2CH{=}CH_2$
VII	—$CH_2C{\equiv}CH$
VIII	—$CH_2C_6H_5$

EXPERIMENTAL[1]

Disodium glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and the sodium salt of nicotinamide adenine dinucleotide phosphate were used as purchased[2]. The nornuciferine derivatives employed were described previously (20).

1,2-Dimethoxydibenzo[*de*,*g*]quinoline-7-one (Lysicamine)—This compound was prepared according to Tomita *et al.* (21) and purified by preparative TLC using Solvent System 1, m.p. 208–209° dec. [lit. (21) m.p. 208–210° dec.].

TLC—Silica gel GF_{254} layers (250 μ), prepared by a method described previously (20) or obtained commercially[3], were used throughout. Plates scored into 1–2-cm. channels were developed 10 cm. in the following solvent systems: 1, chloroform–methanol (85:15); 2, chloroform–methanol–acetic acid (8:1:1); 3, cyclohexane–chloroform–piperidine (8:1:1); 4, benzene–piperidine (9:1); 5, benzene–ethyl acetate–piperidine (6:3:1); and 6, chloroform–acetic acid (3:1). Detection was *via* quenching of 254-nm. radiation or by fluorescence in 320–400-nm. radiation.

Reverse TLC Fluorimetry—Silica gel GF_{254} plates (scored into 2-cm. channels), spotted with samples containing 1.0–5.0 mcg. of nornuciferine, were developed (10 cm.) in Solvent System 1. Plates were scanned in the direction of the solvent development. The fluorescence quench mode was employed with the following conditions: $\lambda_{exc.}$, 254 nm.; span, 300; gain, ×100; neutral density filter, 3.00; slit width, 1 mm., length, 0.65 cm.; scanning rate, 3.2 cm./min.; and recorder speed, 1.9 cm./min. Area measurements were obtained with the formula, $A = h \cdot w_{1/2}$, where A = area, h = peak height, and $w_{1/2}$ = width at half-height.

GC—The conditions reported previously were employed (20).

Liver Preparations and Incubations—Livers from exsanguinated rats (Holtzman males), rabbits (New Zealand males)[4], and guinea pigs (Albino males) were washed with 0.25 *M* sucrose solution and homogenized[5] (at 0°) with four volumes of the latter.

Homogenates were centrifuged at $g\times10{,}000$ for 30 min. The $g\times10{,}000$ supernatant (1 ml. ≡ 1 g. liver) was used as such or centrifuged further at $g\times100{,}000$ (for 1.5 hr.), depositing microsomes which were suspended in 0.25 *M* sucrose solution (1 ml. ≡ 1.25 g. liver).

In the standard incubation procedure, 1 μmole of nornuciferine derivative (hydrochloride salt) was mixed with 2.5 ml. of $g\times10{,}000$ supernatant or microsomal preparation, 1 ml. of 0.1 *M* tromethamine–HCl buffer (pH 7.6), 1 μmole of nicotinamide adenine dinucleotide phosphate, 5 μmoles of disodium glucose 6-phosphate, and 2.0 units of glucose 6-phosphate dehydrogenase; the total volume of the incubation mixture was 4.3 ml. Control mixtures were prepared in the same way but with a liver preparation which had been heated on a steam bath for 2 min. A second blank was prepared without substrate. Mixtures were incubated in a shaker-type water bath at 37.0 ± 0.1° in the air and in the absence of light. The effect of magnesium ion on enzyme activity was studied with magnesium chloride (5 mmoles) added to the incubation mixture prior to the addition of the microsomal preparation. Optimum pH was determined with 0.1 *M* tromethamine–HCl buffers of pH 6.0, 6.5, 7.0, 7.4, 7.6, and 8.0. For rate studies, the total volume of the incubation mixture was 12.9 ml., and 2.1-ml. aliquots were removed at appropriate time intervals.

Extraction of Incubation Mixtures—After heating on a steam bath for 2 min., the incubation mixtures were cooled and then extracted with three 2-ml. portions of 25% isoamyl alcohol in *n*-heptane. The extracts were combined, washed twice with water, dried over anhydrous sodium sulfate, and evaporated to dryness with a current of cold air in the absence of light. The residue was dissolved in 0.3 ml. of chloroform for TLC.

Photolytic Conversion of Nornuciferines to Lysicamine—Nornuciferines (1 μmole) were dissolved in 1-ml. portions of chloroform. The solutions were exposed to sunlight for 4 hr. at room temperature and then subjected to TLC. The effect of solvent variation on lysicamine formation was studied with solutions in ethanol, benzene, and *tert*-butyl alcohol. The effect of light on the conversion of lysicamine was further studied by bubbling air through chloroform and *tert*-butyl alcohol solutions of nornuciferine (and its hydrochloride salt) in the dark.

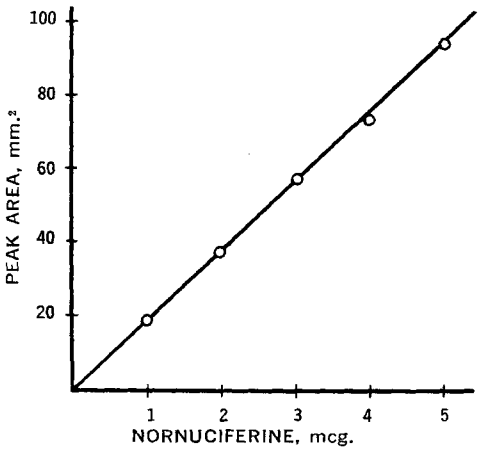


Figure 1—*Standard curve for reverse TLC fluorimetry of nornuciferine.*

RESULTS AND DISCUSSION

Quantitation of Nornuciferine—Initial metabolism experiments revealed the need for a sensitive quantitative method for nornuciferine (I). The GC method previously described (20) was suitable for mixtures of I and the *N*-alkylnornuciferines (II–VIII) where the former was present in significant proportion. However, where I composed 5% or less of the aporphine mixture, quantitation was difficult since the retention times of I and some of its derivatives (*e.g.*, II and III) were close, leading to insufficient resolutions.

[1] Melting points were obtained with a Thomas-Hoover apparatus and are corrected. IR spectra were recorded on Beckman IR-5A and 10 instruments. UV and visible spectra were obtained with Beckman DK-2 and Gilford-240 spectrophotometers. GC was performed with a Hewlett-Packard 5750B gas chromatograph equipped with dual flame-ionization detectors. Reverse TLC fluorimetry was accomplished with a Nester-Faust Uniscan 900 scanner connected to a Texas Instruments, model PS01W6A, 1-mv. recorder.

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

[3] Analtech, Newark, Del.

[4] Pel-Freez Biologicals, Rogers, Ark.

[5] Virtis-45 homogenizer.

Table I—Reverse TLC Fluorimetry of Nornuciferine

Amount Developed, mcg.	Amount Found, mcg.	Percent Recovery
1	1.01	101.0
1	0.98	98.0
1	0.97	97.0
2	1.90	95.0
2	2.04	102.0
2	1.95	97.5
3	2.90	96.6
3	2.94	98.0
4	3.82	95.5
4	3.88	97.0
5	4.78	95.6
5	4.87	97.4
5	4.84	96.8

Mean = 97.5 (n = 13)
Relative standard deviation = ±1.9%

The use of a quantitative TLC technique seemed attractive since excellent resolution of I from II–VIII could be achieved through TLC (20). The procedure of reverse TLC fluorimetry was readily employed and provided the requisite sensitivity. Figure 1 shows a standard curve for quantitating I following development in Solvent System 1. Consistently linear relationships were obtained over the range of 1–5 mcg. Curves embracing quantities beyond this range tended to become hyperbolic (22).

In a typical experiment, two amounts of I were developed, and peak areas derived from these were used in calculating the quantity of I in samples developed on the same plate. Thus, up to eight samples could be analyzed per plate (20 cm. wide), with a resulting analysis time of approximately 10 min./sample. Results of experiments designed to evaluate accuracy and precision of the method described are given in Table I. Overall, the recovery values are good while the precision is better than those reported with similar techniques (23, 24).

***In Vitro* Metabolism**—Extracts obtained from incubation mixtures of nuciferine (II) with rat and rabbit liver preparations (g× 10,000 supernatant) were submitted to TLC in Solvent Systems 2, 3, and 4. Compound I was observed along with an unknown metabolite referred to here as I*a*. The formation of I and I*a* was estimated to be less than 1%. The degree of conversion was not increased by using the microsomal (g×100,000) preparation or by addition of magnesium chloride (25). Compounds III and IV showed even less conversion to I, while *N*-dealkylation of V through VIII could not be detected. The absence of I*a* following microsomal incubation of III and IV seemed to be related to low *N*-dealkylation activity. Indeed, when I was incubated with rat and rabbit liver preparations, relatively greater quantities of I*a* were formed.

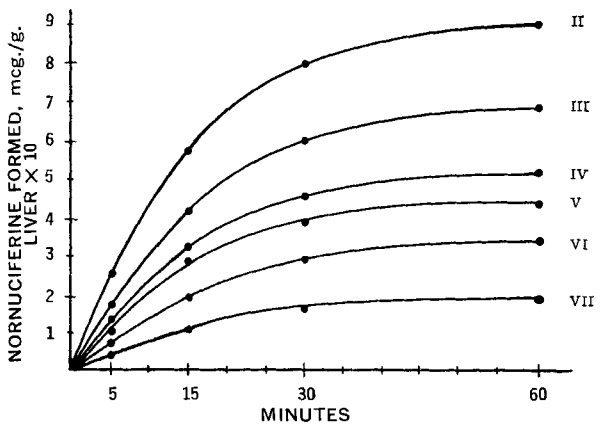
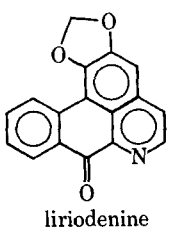
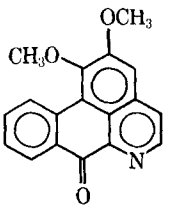


Figure 2—N-*Dealkylation of Compounds II–VII by guinea pig microsomes. Each point represents an average of from three to four determinations.*

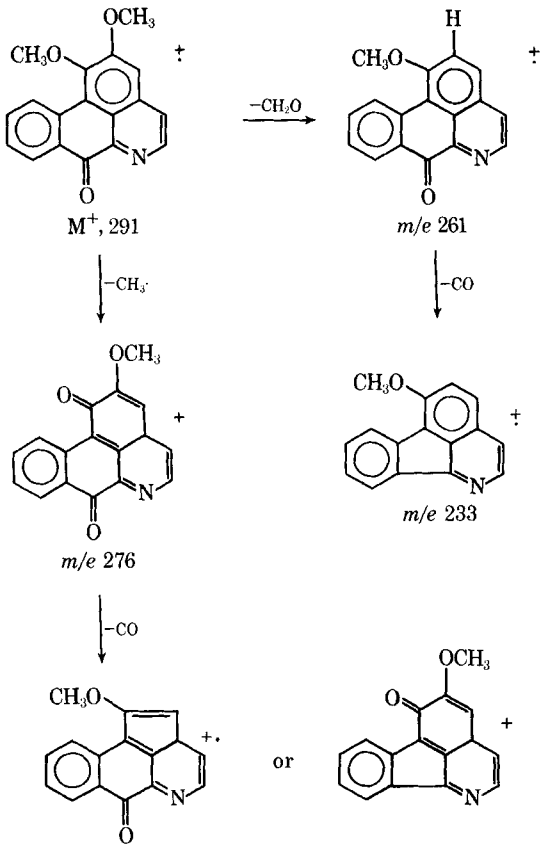

liriodenine


lysicamine

In contrast to these results, guinea pig liver microsomal preparations showed considerably enhanced *N*-dealkylation activity. When studied over the pH range of 6.0–8.0, optimum activity was found between 7.4 and 7.6. Interestingly, I*a* was not observed as a metabolite with this species.

The greater *N*-dealkylation activity observed with guinea pig microsomes permitted study of relative rates. The results of this investigation are depicted in Fig. 2. In every instance, the reaction was linear for 15–20 min., with sharp departure from linearity after 30 min. The deviation from linearity was not due to limiting amounts of substrate or known cofactors (26–28). The *N*-dealkylation rates of II–V were inversely related to the size and complexity of *N*-alkyl groups (29). The *N*-dealkylation rates of Compounds VI–VII showed decided differences from that of II, while *N*-dealkylation of VIII was too low to be quantitated. These results may be a function of decreases in lipid solubilities of Compounds VI–VIII because of their more polar *N*-alkyl groups. McMahon (30) noted that there is a good correlation between lipid solubilities and *N*-dealkylation rates. That is, as lipid solubility decreases, the rate of *N*-dealkylation decreases. This was observed and quantitated by Hansch *et al.* (31) and Lien and Hansch (32) for a number of microsomal transformations and appears to be related to the fact that microsomal enzymes are coated with a lipid layer. Thus, lipid solubility can be a rate-limiting factor because of its effect on substrate availability at enzyme sites.

Other than Compound I*a* (*vide infra*), no other metabolites were observed following incubation of Compounds I–VIII with rat,



Scheme I

Table II—TLC and Spectral Data of Ia and Lysicamine

System	TLC $R_f \times 10^2$ Ia	TLC $R_f \times 10^2$ Lysicamine	UV/Visible[a] $\lambda_{max.}$, nm. (ϵ) Ia	UV/Visible[a] $\lambda_{max.}$, nm. (ϵ) Lysicamine	IR[b] ν C=O, cm.$^{-1}$ Ia	IR[b] ν C=O, cm.$^{-1}$ Lysicamine	Mass Spectra[c] m/e (Relative Abundance) Ia	Mass Spectra[c] m/e (Relative Abundance) Lysicamine
1	65	65	235 (26,900)	235 (29,510)	1662	1668	291 (100)	291 (100)
2	50	50	270 (22,100)	270 (25,700)	—	—	276 (4)	276 (2)
3	8	8	307 (5,700)	307 (5,750)	—	—	261 (1)	261 (1)
4	27	27	400 (7,300)	400 (8,700)	—	—	248 (57)	248 (41)
5	37	37	453 (3,200)[d]	453 (3,800)[d]	—	—	233 (7)	233 (5)
6	15	15	—	—	—	—	—	—

[a] Ethanol solutions. [b] Spectra were nearly superimposable; solid films. [c] See Scheme I for structural assignments; peaks at m/e less than 233 were of relatively low abundance and showed no differences in position. [d] Visible band after addition of one drop of 0.1 N HCl.

rabbit, or guinea pig liver preparations. Of particular interest was the apparent absence of *O*-dealkylation, which was an anticipated biotransformation (20).

Identification of Metabolite Ia—TLC of extracts obtained after incubation of I and II with rat and rabbit liver preparations revealed the presence of a yellow-colored compound (Ia) with similar mobility to I and II in neutral and acidic solvent systems (1, 2, and 6) but lower mobility in basic solvent systems (3–5).

During these investigations, it was observed that chloroform and chloroform–methanol solutions of Compounds I–VIII, but especially I, developed a yellow color when exposed to light. Following TLC examination, it was shown that the yellow component of these solutions possessed identical mobility to Ia. Photochemically produced Ia was purified and isolated by preparative TLC. A UV-visible spectrum of Ia showed four bands (in ethanol): $\lambda_{max.}$ = 235, 270, 307, and 400 nm. The 400-nm. band did not shift in alkali (ethanolic sodium hydroxide) but did shift to 453 nm. when Ia was treated with hydrochloric acid.

Compound Ia showed no OH or NH stretching bands in the IR but did possess a band at 1662 cm.$^{-1}$, which could be indicative of a highly conjugated carbonyl function. A search of the literature revealed that these data were very close to those of the naturally occurring oxoaporphines, liriodenine (33, 34), and lysicamine (35).

Spectral and TLC comparison of Ia with an authentic sample of lysicamine (21) showed that the two were identical (Table II). Mass spectra[6] of Ia and lysicamine contained three principal peaks, as indicated in Table II; ionization and fragmentations giving rise to these peaks are proposed in Scheme I. The fragmentations depicted are well documented (36, 37), while equivalent peaks were reported for several liriodenine analogs (38, 39).

Cava and Dalton (40) reported that liriodenine could be produced in 30% yield by passage of air through a *tert*-butyl alcohol solution of anonaine, the methylenedioxy homolog of I. *tert*-Butyl alcohol solutions of I were similarly treated to yield lysicamine; however, in the absence of light, no conversion was detected by TLC. The photolytic conversion of I to lysicamine was most efficient in chloroform, although it also occurred in ethanol and benzene. Compounds II–VIII could also be photolytically converted to lysicamine, but to a much lesser extent, and other products were detected by TLC. Under laboratory fluorescent lighting, chloroform solutions of I were shown (by visible spectroscopy) to be quantitatively converted to lysicamine in 7 days. Attempts were made to standardize the lighting requirement, for instance, by using mercury vapor lamps with principal emission at 254 nm. or 320–400 nm. These conditions, however, gave mixtures of reaction products.

Since lysicamine could arise as an artifact and not an actual metabolite of I, experiments were designed to investigate this possibility. Colorimetric analysis of Ia in the presence of I or II was possible since the latter do not absorb at 400 nm. Thus, extracts obtained from incubations of I and II with rat liver preparations were analyzed using blanks obtained from I and II incubated with denatured enzyme. Following TLC identification, Ia was quantitated. A 1.20% conversion of I to Ia and a 0.50% conversion of II to Ia were observed. These data tend to confirm the proposed intermediacy of I in the formation of Ia from II.

[6] The authors are indebted to Dr. William L. Budde and the Purdue University Mass Spectrometry Center for these determinations.

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Dialysis and Circular Dichroism Study of the Binding of Sulfaethidole to Crystalline and Fraction V Bovine Serum Albumin

H. B. KOSTENBAUDER*, M. J. JAWAD*, J. H. PERRIN†, and V. AVERHART†

Abstract □ Sulfaethidole was found to exhibit similar binding to both crystalline and Fraction V bovine serum albumin. Binding of sulfaethidole to both crystalline and Fraction V bovine serum albumin results in considerable induced optical activity, with no evidence of change in the α-helical structure. Although equilibrium dialysis studies indicate one primary site and three secondary binding sites for sulfaethidole, only the binding at the primary site is detectable from circular dichroism studies.

Keyphrases □ Sulfaethidole binding to crystalline and Fraction V bovine serum albumin—studied by dialysis and circular dichroism □ Dialysis—sulfaethidole binding to bovine serum albumin, determination □ Circular dichroism—sulfaethidole binding to bovine serum albumin, determination

It was recently noted that sulfaethidole exhibits similar binding to both crystalline and Fraction V bovine serum albumin, while salicylate ion shows markedly lower binding to Fraction V than to crystalline bovine serum albumin (1). It was further observed that the addition of 10 mg.% sulfaethidole to 4.55% bovine serum albumin dispaced salicylate from binding to crystalline bovine serum albumin but markedly enhanced the binding of salicylate to Fraction V bovine serum albumin. It was suggested that sulfaethidole might induce a subtle conformational change in Fraction V bovine serum albumin; studies were, therefore, undertaken to determine if such conformational change could be detected by circular dichroism (CD) measurements.

EXPERIMENTAL[1]

Dialysis Procedure—Ten milliliters of a 5.00% w/v serum albumin (7.25×10^{-4} *M*) solution in 0.054 *M* phosphate buffer, pH 7.4, adjusted with sodium chloride to be isotonic, was placed in dialysis bags made by knotting the ends of the dialysis tubing. Bags were placed in 120-ml., wide-mouth, screw-capped bottles containing 10 ml. of buffered sulfaethidole solution, and the containers were agitated for 12 hr. at 37°. Prior to analysis, the volumes of inside and outside solutions were carefully measured, and protein concentrations were corrected for volume change (final protein concentration was 4.55% w/v). Total sulfaethidole concentrations in the protein compartment at equilibrium ranged from approximately 3.2 to 73 mg.% (1.1×10^{-4}–25.7×10^{-4} *M*). Buffer solutions and glassware were sterilized, and the aseptic technique was used in preparing solutions and dialysis bags. Dialysis tubing was boiled in three changes of distilled water and finally in buffer solution.

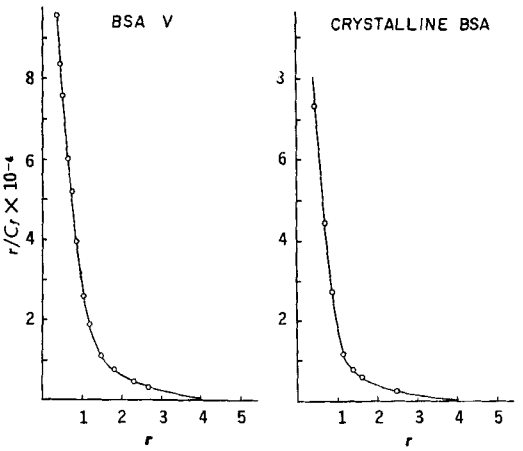

Figure 1—*Scatchard plot for binding of sulfaethidole by bovine serum albumin in 0.054* M *phosphate buffer, pH 7.4, 37°. For bovine serum albumin V, the solid line corresponds to* $n_1 = 1$, $K_1 = 1.5 \times 10^5$, $n_2 = 3$, *and* $K_2 = 1.6 \times 10^3$. *For crystalline bovine serum albumin, the solid line corresponds to* $n_1 = 1$, $K_1 = 1.2 \times 10^6$, $n_2 = 3$, *and* $K_2 = 1.0 \times 10^3$.

Analytical Procedures—Sulfaethidole concentration was determined for both inside and outside solutions following equilibrium, utilizing the Bratton–Marshall (2) procedure. Inside and outside solutions from a sample containing no sulfaethidole were utilized as blanks. Absorbances were determined at 535 nm. using a spectrophotometer[2].

[1] Visking dialysis tubing, 27/32, Union Carbide Corp., Chicago, Ill.; Fraction V bovine serum albumin, Armour Pharmaceutical Co., Chicago, Ill.; and crystalline bovine serum albumin, Nutritional Biochemicals Corp., Cleveland, Ohio, were used as received from the supplier. Sulfaethidole [N^1-(5-ethyl-1,3,4-thiadiazol-2-yl)sulfanilamide] was recrystallized from water, m.p. 185–186°; all other chemicals were reagent grade.

[2] Cary 15, Cary Instruments, Monrovia, Calif.

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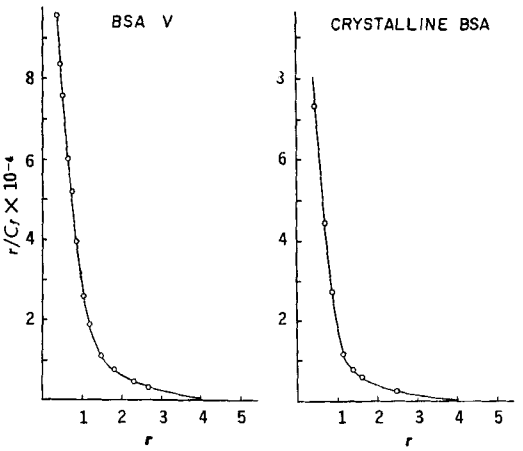

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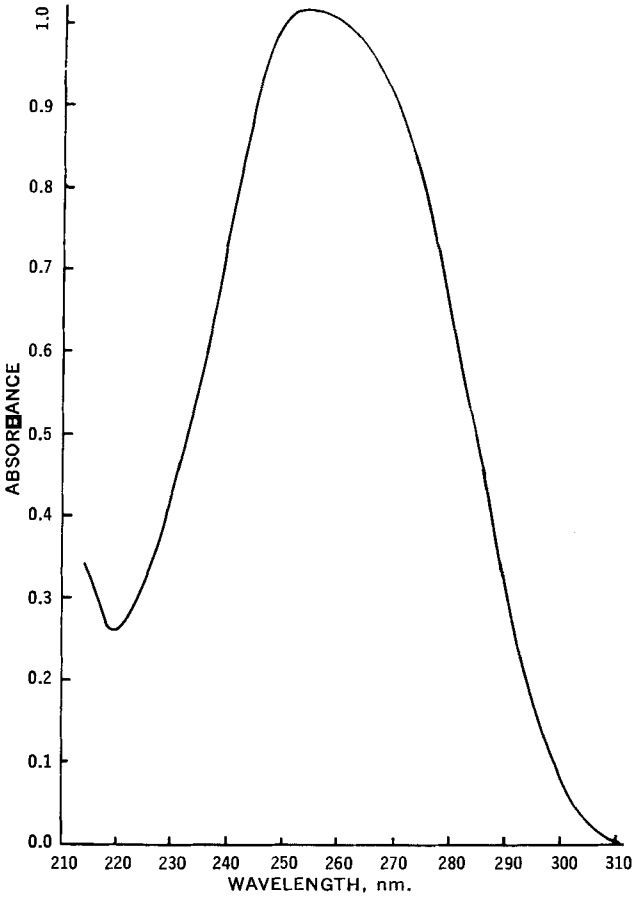

Figure 2—*UV curve for 5.6 × 10⁻⁵ M sulfaethidole in 0.054 M phosphate buffer, pH 7.4, pathlength 1 cm.*

CD Measurements—The CD spectra of the optically clear solutions were obtained in 2-, 5-, 10-, or 20-mm. cells at 25°, using the 6002 attachment to a spectropolarimeter[3]. The dynode voltage was never allowed to exceed 0.35, and the signal-to-noise ratio was always greater than 50 to 1. The UV curve was obtained in a spectrophotometer[2].

RESULTS

Protein Binding—The concentration of sulfaethidole found in the nonprotein-containing compartment at equilibrium was taken as the concentration of free drug, and the concentration of sulfaethidole determined in the protein compartment was taken as free plus bound drug. Scatchard plots (Fig. 1) were constructed, assuming a molecular weight of 69,000 for the serum albumin. Analysis of the Scatchard plots (3) indicated that both Fraction V and crystalline bovine serum albumin exhibited one primary binding site (n_1) and probably three secondary binding sites (n_2). All data points were in agreement (better than ± 3%) with computer-generated Scatchard plots for the model $r = (n_1K_1C_f)/(1 + K_1C_f) + (n_2K_2C_f)/(1 + K_2C_f)$, if $n_1 = 1$ and $n_2 = 3$, and if for crystalline bovine serum albumin, $K_1 = 1.2 \times 10^5$ and $K_2 = 1.0 \times 10^3$, and for Fraction V bovine serum albumin, $K_1 = 1.5 \times 10^5$ and $K_2 = 1.6 \times 10^3$. No other integer values for n_1 and n_2 provide an acceptable fit of the data for the concentration range investigated.

CD—In the pH 7.4 buffers of these investigations, bovine serum albumin V and crystalline bovine serum albumin showed similar CD spectra down to 200 nm., and the spectra obtained were those associated with the α-helical form. Addition of sulfaethidole did not alter the spectra of either below 250 nm. Additions of salicylate and mixtures of salicylate and sulfaethidole also caused no deviations from the spectra associated wth the α-helical conformation.

[3] Cary 60.

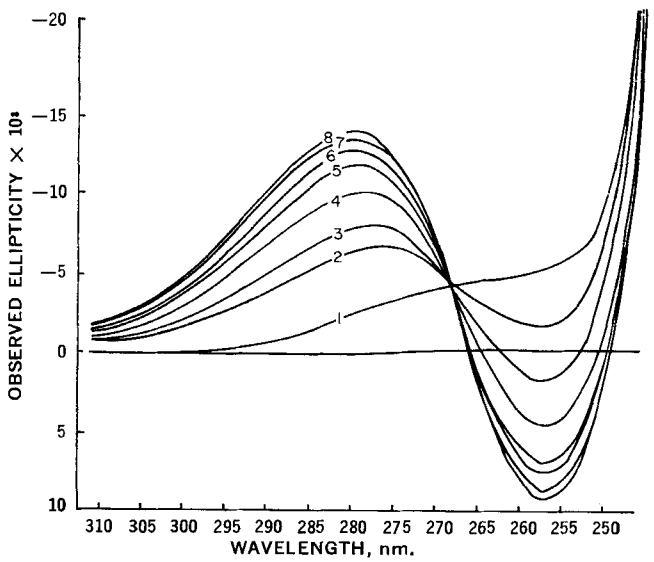

Figure 3—*CD curves for the binding of sulfaethidole to bovine serum albumin V, using 2-mm. cells, at the following D/P ratios: 1 = no drug, 2 = 0.29, 3 = 0.49, 4 = 0.78, 5 = 1.17, 6 = 1.46, 7 = 1.9, and 8 = 2.4.*

However, sulfaethidole alone showed considerable induced optical activity at higher wavelengths with both bovine serum albumin V and crystalline bovine serum albumin. Because of the large absorbances at the wavelengths of investigation, the effect of salicylate on these induced activities could not be determined; however, salicylate alone did not show any measurable induced activity.

The UV curve of sulfaethidole in buffer (Fig. 2) clearly results from at least two overlapping electronic excitations within the sulfaethidole molecule and gives a peak at 254 nm. These electronic transitions result in two induced CD peaks on binding to both albumins, a positive peak at approximately 257 nm. and a negative peak at approximately 279 nm. (Fig. 3).

The curves in Fig. 3 are those obtained with bovine serum albumin V; crystalline bovine serum albumin gave, within experimental error, identical curves. Figure 3 shows the optical activity induced at wavelengths above 250 nm. for various drug/protein (D/P) ratios, as well as the small contribution of the bovine serum albumin V at these wavelengths.

For these investigations of induced optical activity, the protein concentrations were fixed at 1.45×10^{-5} mole/l. as in previous studies (4), and drug-to-protein ratios of up to 10 to 1 (Figs. 3–5) were used. In Figs. 4 and 5, the induced ellipticities at both observed peaks are plotted at various D/P ratios for the two fractions, the

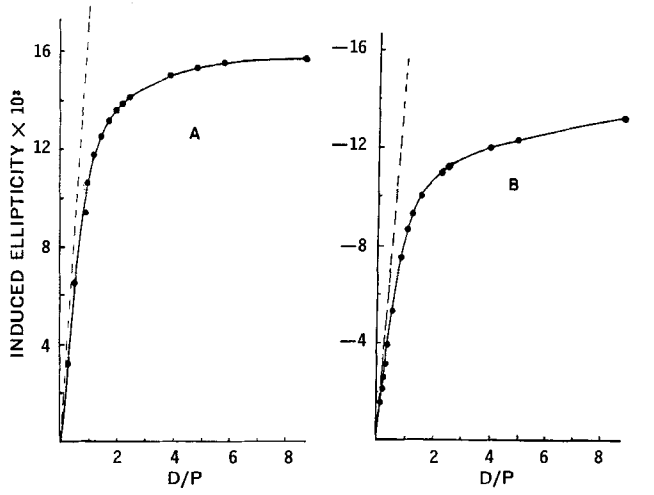

Figure 4—*Bovine serum albumin V—plot of induced ellipticity in 5-mm. cells for various D/P ratios. Bovine serum albumin concentration = 1.45 × 10⁻⁵ M. Key: A, 256.8 nm.; and B, 278.8 nm.*

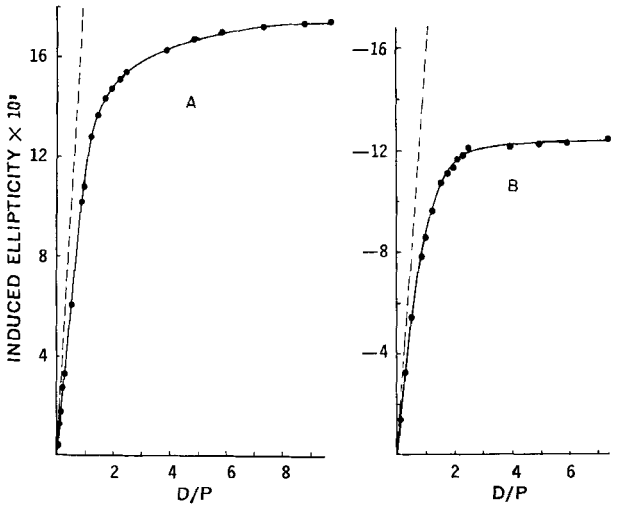

Figure 5—*Crystalline bovine serum albumin—plot of induced ellipticity in 5-mm. cells for various D/P ratios. Bovine serum albumin concentration = 1.45 × 10⁻⁵ M. Key: A, 256.8 nm.; and B, 278.8 nm.*

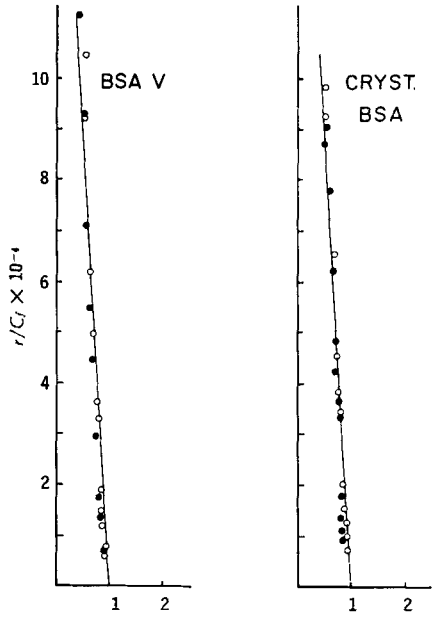

Figure 6—*Scatchard plots from CD data at 25°. Key: ●, data for 256.8 nm.; and ○, data for 278.8 nm. The solid line is the least-squares line; for bovine serum albumin V, it corresponds to* n = *1 and* K = *2.2 × 10⁵; for crystalline bovine serum albumin, it corresponds to* n = *1 and* K = *2.1 × 10⁵.*

induced ellipticity being defined as the observed ellipticity minus the small fixed contribution of the protein at the same wavelength. Figures 4 and 5 indicate a remarkable similarity in the optical activity induced by the two bovine serum albumins. The magnitudes of the induced activities for similar D/P concentrations at both the positive and negative peaks are similar for both albumins.

These data were analyzed according to the method of Rosen (5), in which it is assumed that the tangent to the curve as drug concentration approaches zero can be used to indicate the concentration of bound drug at all drug concentrations. The tangent is indicated as a dotted line in Figs. 4 and 5. By utilizing this procedure, concentrations of free and bound drug were calculated from the CD data; these data are presented as Scatchard plots in Fig. 6. Binding data from the CD and dialysis studies are not in agreement. While the dialysis data illustrated in Fig. 1 show one primary site and three secondary sites, binding to only the primary site is detectable from the CD study. Although the dialysis studies were conducted at 37° and the CD studies at 25°, the affinity constants obtained from the CD studies are in reasonable agreement with the values for the primary site affinity constants obtained from the dialysis study. Least-squares analysis of the CD binding data give an affinity constant of 2.1×10^5 for crystalline bovine serum albumin and 2.2×10^5 for Fraction V bovine serum albumin.

Although there is an indeterminate error associated with constructing a tangent and, therefore, in affinity constants calculated by this method, no tangent that might be constructed provides data indicative of other than a single binding site. It is, therefore, suggested that although sulfaethidole clearly exhibits both primary and secondary binding sites, only the binding at the primary site may be detected by CD studies. CD studies provided no evidence for the secondary interactions, even though such studies were extended to higher drug-to-protein ratios than were used in the dialysis studies. The concentrations of albumins used in the CD experiments were one-fiftieth of those used in the dialysis experiments, a situation necessitated by the high UV absorption of the protein and drug and the possibility of turbidity when higher concentrations of albumin are used. The two peaks in the induced CD curves probably result from excitations within both the sulfanilamide and the thiadiazole portions of the sulfaethidole molecule, and the similarity of the shapes of the curves for both the positive and negative deviations in Figs. 4 and 5 suggests a nonspecific nature of the binding as far as the sulfaethidole molecule is concerned. The rigid nature of the complex necessary for induced optical activity in drug–protein interactions in aqueous systems (4, 6, 7) seems to indicate a predominantly hydrophobic phenomenon.

The results from both the CD and dialysis studies suggest very little difference in the binding of sulfaethidole to bovine serum albumin V or crystalline bovine serum albumin. The CD data indicate little or no change in conformation of the α-helical proteins on binding of the sulfaethidole; however, any overall minor, but locally significant, conformational changes in the protein which may result on binding would probably not be observed by the CD technique.

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Biosynthesis of Deuterated Benzylpenicillins II: Isolation and Characterization of a Highly Deuterated Benzylpenicillin

BRUCE C. CARLSTEDT*†, HENRY L. CRESPI, MARTIN I. BLAKE†, and JOSEPH J. KATZ

Abstract □ By using a modified replacement culture technique in combination with deuterated substrates, highly deuterated benzylpenicillin was isolated from the broth and characterized by PMR spectroscopy. Analysis of the spectra revealed an average replacement of 89% of the protons (^1H) with deuterium (^2H) atoms. Complete replacement by ^2H is observed in the phenylacetyl group, the C-3 position of the thiazolidine ring, and the C-6 position of the β-lactam ring. Partial substitution (64%) is noted in the C-5 position of the β-lactam ring and in the methyl groups (77%) at the C-2 position of the thiazolidine ring. The methyl groups of highly deuterated benzylpenicillin are all preformed in the seed inoculum, since there appears to be no *de novo* biosynthesis of the methyl groups during fermentation.

Keyphrases □ Benzylpenicillin, deuterated—biosynthesis, isolation, PMR characterization □ Deuteration, benzylpenicillin—*P. chrysogenum* cultures, ^2H-substrates □ *Penicillium chrysogenum* cultures—benzylpenicillin deuteration □ Biosynthesis, deuterated benzylpenicillin—*P. chrysogenum* cultures, ^2H-substrates □ PMR spectroscopy—analysis, structure, deuterated benzylpenicillin

In a previous paper (1), the participation of solvent deuterium oxide in the biosynthesis of benzylpenicillin was reported. *Penicillium chrysogenum* (Wisconsin strain 53-414) was cultured in a defined medium containing glucose, acetate, lactate, and phenylacetic acid as carbon sources and 99.8% deuterium oxide as solvent. Partially deuterated benzylpenicillin was isolated from the culture and the extent of solvent participation in the biosynthesis was determined by analysis of the PMR spectra. Complete incorporation of deuterium in the C-3 and C-6 positions and partial incorporation at the C-5 position were observed.

In the present study, a highly deuterated benzylpenicillin, produced by fermentation in the presence of ^2H-substrates, was isolated from the broth and characterized by PMR spectroscopy.

EXPERIMENTAL

Preparation of Slants—*P. chrysogenum*, Wisconsin strain 53-414, was utilized for this study and maintained on slants as described earlier (1).

Preparation of Seed Culture—The seed culture medium was prepared according to the formula shown in Table I and involved two separate steps. The pH of Part A was adjusted to 6.9 with 25% KOH prior to the addition of $CaCO_3$. Forty milliliters of Part A was transferred to each of a series of 250-ml. conical flasks; the flasks were plugged with cotton and autoclaved. Part B of the medium containing acetate and ^2H-pyruvate was prepared separately; the solution was adjusted to pH 6.9 with concentrated HCl and added by millipore filtration to the sterile flasks containing Part A of the medium. The millipore filter was 25 mm. in diameter with a pore size of 0.22 μ.

The sodium ^2H-pyruvate used in Part B of the seed culture medium was prepared by exchanging ^1H-pyruvate with D_2O, based on a procedure of Rose (2). Sodium ^1H-pyruvate was dissolved in D_2O in a 1:8.85 ratio; the solution was filtered and heated in an autoclave at 140° for 20 min. The solvent was removed by flash evaporation at 40° under vacuum, and the residue was dissolved in sufficient D_2O to yield a 9% solution, based on the original weight of sodium ^1H-pyruvate. This solution was then heated in an autoclave for 20 min. at 140° to ensure complete exchange. This ^2H-pyruvate was used for the preparation of Part B of the seed culture medium and was added (0.4 g./100 ml. seed culture medium) after 57, 69, and 81 hr. of growth, respectively.

Table I—Composition of Seed Culture Medium

	Amount
Part A:	
Dextrose	6.00 g.
Lactose	1.00 g.
Standard salt mixture	10.00 ml.
Water to make	80.00 ml.
$CaCO_3$	1.5 g.
Part B:	
NH_4OH	0.447 g.
Acetic acid	0.273 g.
^2H-Pyruvate sodium	0.565 g.
Water to make	20.00 ml.
Standard salt mixture:	
KH_2PO_4	3.00 g.
$MgSO_4 \cdot 7H_2O$	0.25 g.
$FeSO_4 \cdot 7H_2O$	0.10 g.
$CuSO_4$	0.005 g.
$ZnSO_4 \cdot 7H_2O$	0.02 g.
$MnSO_4 \cdot H_2O$	0.02 g.
$CaCl_2$	0.05 g.
Water to make	100.0 ml.

The resulting inoculum, consisting of pellets ranging from 1 to 3 mm. in diameter, was recovered aseptically by collection on filter paper in a büchner funnel. The pad was rinsed once with sterile distilled water, rinsed twice with sterile D_2O, and stored in a sterile container.

Deuterated Culture Techniques—The modified replacement technique described earlier (1) was used for culturing *P. chrysogenum* in D_2O. The D_2O nutrient medium shown in Table II was used in this study and differed from the medium used previously (1) in that sodium pyruvate was substituted for lactic acid. The D_2O nutrient medium was adjusted to an apparent[1] pH of 6.9 (3) with concentrated HCl; 50 ml. was transferred into a series of 250-ml. conical flasks, which were plugged with cotton and autoclaved. One milliliter of vitamin mixture was added to each flask, and the culturing technique reported earlier for inoculation and fermentation was followed.

^1H-Phenylacetic acid or ^2H-phenylacetic acid in D_2O was neutralized with 2.5% KOH in D_2O. The solutions were diluted with D_2O to give a final concentration of 8.34 or 8.84 mg./ml., respectively, and were autoclaved in multiple-dose vials. Daily addition of 1.5 ml. dextrose in D_2O (8.3%) and 0.5 ml. ^1H- or ^2H-phenylacetic acid solution was accomplished with a sterile syringe and needle by injection into the culture flask through the cotton plug.

Culture Monitoring Techniques—All cultures were monitored for penicillin titer, pH values, and deuterium content by procedures already described (1).

Extraction Procedure—*Isolation and Identification of Benzylpenicillin*—Scheme I shows a flowchart for the extraction, purification, and isolation of the highly deuterated benzylpenicillin. The procedures and methods of the extraction and purification through the preparation of the *N*-ethylpiperidine salt of benzylpenicillin

[1] The apparent pH of a D_2O solution is that pH observed with a pH meter. The pD of a solution is the apparent pH plus 0.4 unit.

Table II—Composition of D_2O Nutrient Culture Medium and Vitamin Mixture

Ingredient	Amount
D_2O Nutrient Culture Medium	
Dextrose	1.00 g.
Standard salt mixture[a]	10.00 ml.
NH_4OH	0.447 g.
Acetic acid	0.273 g.
Sodium pyruvate	0.565 g.
D_2O to make	100.00 ml.
Vitamin Mixture	
Biotin	0.005 g.
Inositol	0.005 g.
Ca pantothenate	0.005 g.
Pyridoxine hydrochloride	0.005 g.
Thiamine hydrochloride	0.005 g.
D_2O to make	100.00 ml.

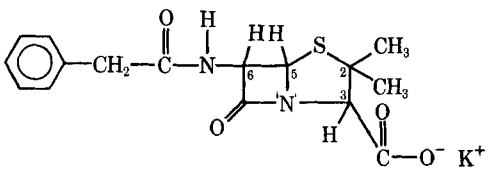
[a] Prepared as in Table I but with D_2O as the solvent.

were described, and the steps involved are indicated in the flowchart. The conversion of the *N*-ethylpiperidylbenzylpenicillin to the potassium salt of benzylpenicillin was accomplished by ion-exchange chromatography. Three grams of a resin[2] was soaked in distilled water for 1 hr. and placed in a small column (0.9-cm. i.d.) over a fiber glass plug. The resin, in the H^+ form, was converted to the potassium form by washing the column with 20 ml. of 1 *M* KOH at a flow rate of 10 drops/min. The column was then washed with water until the eluate was neutral as recorded on the pH meter. The *N*-ethylpiperidine salt was dissolved in 1 ml. distilled water and added to the column. The column was eluted with distilled water at a flow rate of 10 drops/min. The first 25 ml. was collected and lyophilized, and the residue was stored in a vacuum desiccator. A second volume of 5 ml. was collected but showed no residue on lyophilization.

Identification of Benzylpenicillin—IR and PMR spectroscopy were used for identification of benzylpenicillin and determination of deuterium uptake. The PMR spectra were obtained on a spectrometer[3] (probe temperature, 31°) after dissolving the sample in 0.01 *M* phosphate D_2O buffer (apparent pH between 6.5 and 7.5) or in deuterochloroform. The lock material was hexamethyldisiloxane, internally for deuterochloroform solutions and as an external capillary for D_2O solutions.

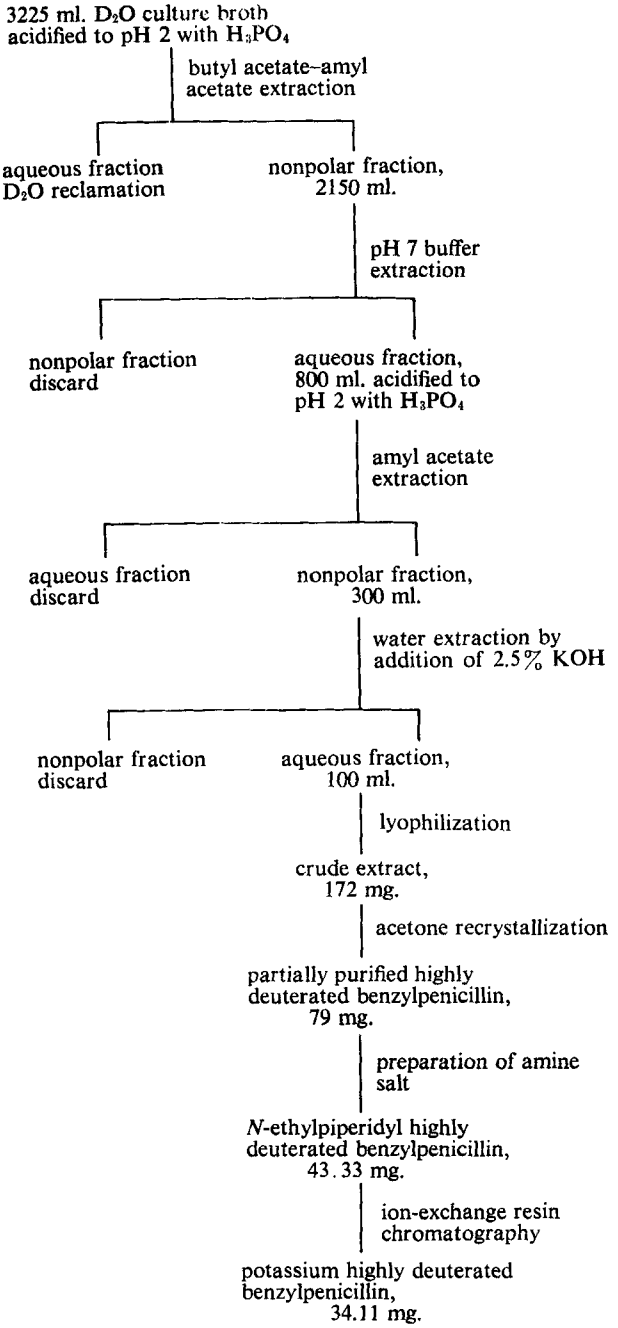
RESULTS AND DISCUSSION

A partially deuterated benzylpenicillin, III (Table III), isolated from a fermentation broth in which all the components of the culture medium except solvent D_2O were in the protio form, showed that deuterium atoms from the solvent were incorporated into benzylpenicillin during biosynthesis (1). The inclusion of ^2H-substrates in the culture medium is necessary if additional deuterium atoms are to be incorporated into this molecule. Nona *et al.* (4) and Mrtek *et al.* (5) utilized ^2H-substrates to produce deuterated griseofulvin and ergot alkaloids, respectively. The objectives of the present study were: (*a*) to examine the biosynthetic processes in both the seed inoculum and the D_2O nutrient culture medium by incorporating ^2H-pyruvate into the nutrient solutions, and (*b*) to produce, isolate, and characterize a benzylpenicillin containing a high enrichment of deuterium in nonexchangeable positions in the molecule.



potassium benzylpenicillin
I

[2] Biorad AG 50W-X8.
[3] Varian HA 100.



Scheme I —Synopsis of Extraction, Purification, and Isolation Procedure for Highly Deuterated Benzylpenicillin

Biosynthesis of Benzylpenicillin and Expected Isotopic Composition—Benzylpenicillin containing deuterium atoms in the benzyl side chain has been produced by biosynthetic and synthetic means. Behrens *et al.* (6) synthesized deuterophenylacetyl-^{15}N-DL-valine and found the deuterophenylacetyl moiety was incorporated into the benzylpenicillin molecule during biosynthesis. Laskar and Mrtek (7) synthesized deuterobenzyl-d_7-penicillin from 6-aminopenicillanic acid and ^2H-phenylacetic acid. Since phenylacetic acid is a precursor for benzylpencillin (8), inclusion of ^2H-phenylacetic acid in the fermentation medium should oblige the mold to biosynthesize a benzylpenicillin which is fully deuterated in the benzyl side chain.

Previous work demonstrated that in nutrient D_2O solutions (protio substrates) the incorporation of deuterium is almost complete at the C-3 position (Structure I) of the thiazolidine ring and the C-6 position of the β-lactam ring. A partial incorporation (approximately 67%) of deuterium at the C-5 position was also

Table III—Chemical Shifts (δ, p.p.m.) of Protio and Deuterated Benzylpenicillins[a,b]

	Compound	Side-Chain Phenyl	Amide —NH—	C-5 Proton	C-6 Proton	C-3 Proton	Side-Chain $-CH_2-$	Thiazolidine $(CH_3)_2$
Solvent[c]: 0.01 *M* phosphate D_2O buffer, apparent pH 6.5–7.5								
I	K Benzylpenicillin[d]	7.38		5.53d (4 Hz.)	5.43d (4 Hz.)	4.23	3.68	1.56, 1.49
II	K pd-Benzylpenicillin	7.36		5.51	—	4.22[e]	3.67	1.56, 1.48
III	K pd-Benzylpenicillin	7.36		5.50	—	4.22[e]	3.68	1.54, 1.46
V	K pd-Benzylpenicillin	7.43		5.51	5.42[e]	4.18[e]	3.62	1.44, 1.36
VIII	K Benzylpenicillin	7.34		5.51d (4 Hz.)	5.41d (4 Hz.)	4.22	3.65	1.55, 1.48
IX	K hd-Benzylpenicillin	—		5.51	—	—	—	1.57, 1.49
Solvent[f]: $CDCl_3$								
IV	NEP[g] pd-Benzylpenicillin	7.38	6.28	5.63	—	4.36[e]	3.70	1.63, 1.53
VI	NEP hd-Benzylpenicillin	7.30[e]	6.30	5.58	—	4.29[e]	3.65[e]	1.58, 1.48
VII	Benzylpenicillinic acid[h]	7.34	6.35d (8.5 Hz.)	5.52	5.70d (4 Hz.) 5.49d (4 Hz.)	4.38	3.67	1.52, 1.45

[a] d denotes doublet; *J* values in Hz. [b] pd denotes partially deuterated; hd denotes highly deuterated. [c] Referred to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt. [d] Assignments from (1). [e] Very small peak. [f] Referred to tetramethylsilane. [g] *N*-Ethylpiperidyl. [h] Green *et al.* (15).

observed. The methyl groups on the thiazolidine ring remained free of deuterium. It was shown that penicillin is biosynthesized from L-cysteine (9) and valine (10). In the biosynthesis of valine (11), two molecules of pyruvate are condensed to form the methyl groups of valine, which is in turn incorporated into penicillin (12) to give the methyl groups on the thiazolidine ring. If deuterated pyruvate is utilized by the mold, then the methyl groups of penicillin should be fully deuterated under our experimental conditions.

Preparation and Utilization of ^{2}H-Pyruvate by *P. chrysogenum* —Rose (2) described a technique for tritiation of pyruvate by heating sodium pyruvate in tritiated water to achieve exchange of the protons of the methyl groups with ^{3}H of the solvent, A modification of this procedure, as described under *Experimental*, yielded ^{2}H-pyruvate by heating sodium ^{1}H-pyruvate dissolved in D_2O.

Fermentation of *P. chrysogenum* 53-414 in a D_2O nutrient culture medium containing ^{2}H-pyruvate (Table II) should yield benzylpenicillin with deuterium incorporated in the methyl groups. Chemical shifts for partially deuterated benzylpenicillin II (Table III) obtained from such a fermentation showed a spectrum very similar to that of the partially deuterated benzylpenicillin III (Table III) obtained from the solvent participation study (1). These results indicate that ^{2}H-pyruvate from the fermentation medium is not utilized for the biosynthesis of benzylpenicillin in D_2O nutrient cultures. When ^{2}H-pyruvate was used in the fermentation medium, the PMR spectrum of the material purified through the acetone recrystallization step exhibited fewer and smaller peaks in the upfield region of the spectrum (due to impurities) than did corresponding samples from fermentations using lactate.

Halliday and Arnstein (13) developed a replacement technique for the study of benzylpenicillin biosynthesis by a washed mycelium technique and observed that only oxygen and phenylacetic acid were necessary for the continued biosynthesis of benzylpenicillin under the conditions studied. They concluded that intracellular substances that are not lost in the washing procedure are readily available for penicillin biosynthesis. Since a modified replacement technique (1) was utilized for the present study, it was felt that incorporation of ^{2}H-components into the seed culture medium might prove useful. Reformulation of the seed culture medium used earlier was necessary, and the formula shown in Table I proved suitable.

Preliminary experiments indicated that not only was ^{2}H-pyruvate necessary in the seed culture medium for deuterium incorporation in the C-2 methyl groups but that greatest ^{2}H incorporation occurred when additional ^{2}H-pyruvate was added to the seed culture medium near the end of the seed inoculum growth. A fermentation was conducted in which the ^{2}H-pyruvate was added to the seed culture medium and ^{1}H-phenylacetic acid was added to the D_2O nutrient culture medium. A normal fermentation showed an antibiotic titer of approximately 73 units/ml. The partially deuterated benzylpenicillin V (Table III) was extracted from the broth, and the *N*-ethylpiperidine salt was prepared. Chemical shifts for this compound (IV) are shown in Table III, and the PMR spectrum is shown in Fig. 1.

The resonance for the amide proton (*g*) represents a single proton; thus, the area beneath this peak is the area equivalent to one proton. Deuterium incorporation at the C-5 position was found to be 64%, an amount similar to that reported earlier. The expected value for the ratio of the area for the amide proton to the area for the phenyl protons is 1:5; the experimental ratio was 1:5.1. The expected ratio for the area of phenyl protons to the area for side-chain methylene protons is 5:2, but the experimental value was 5:1.5, indicative of exchange of the methylene protons with deuterium from the solvent during the fermentation. The *N*-ethylpiperidine salt was converted back to the potassium salt by ion exchange. The chemical shifts for partially deuterated benzylpenicillin V are recorded in Table III, and the PMR spectrum is shown in Fig. 2. The ratio of the area for the phenyl protons to the area for the methyl groups on the thiazolidine ring would be expected to be 5:6, but the experimental value was 5:1.45, indicating that 76% of the protons normally expected to be present in the methyl groups had been replaced by deuterium from the ^{2}H-pyruvate in the seed inoculum.

The resonances for the partially deuterated methyl groups seen in Fig. 2 are sharp peaks and are not the usual broad peaks associated with a partially deuterium-substituted functional group. The resonances are presumably not due to a mixture of $-CH_3$, $-CH_2D$, and $-CHD_2$ but probably result from methyl groups on the thiazolidine ring that are either all $-CH_3$ or all $-CD_3$. These methyl groups arise from the methyl groups of pyruvate, which is

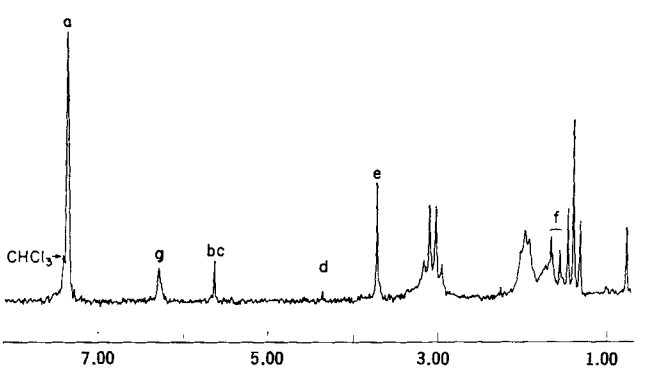


Figure 1—*PMR spectrum of* N-*ethylpiperidyl partially deuterated benzylpenicillin IV in* $CDCl_3$. *Key: a, side-chain phenyl; b, C-5 proton; c, C-6 proton; d, C-3 proton; e, side-chain methylene; f, thiazolidine dimethyl; and g, amide proton.*

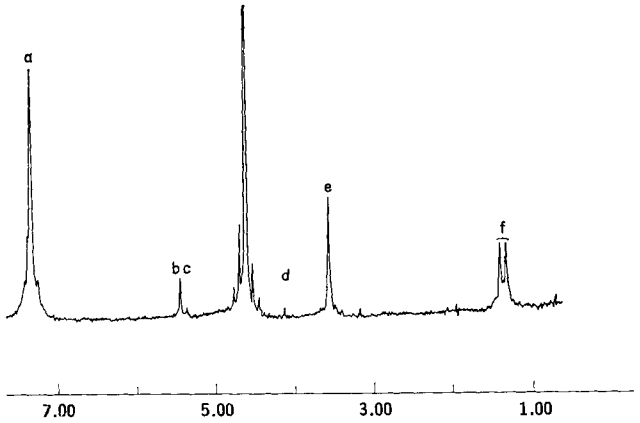


Figure 2—*PMR spectrum of potassium partially deuterated benzylpenicillin V in D_2O buffer. Key: see Fig. 1.*

present from the pyruvate added to the seed inoculum or which arises from the metabolism of lactose and dextrose in the seed inoculum. The pyruvate generated from the sugars would yield methyl groups containing predominantly protons, and even though large amounts of ^{2}H-pyruvate are added to the seed inoculum, utilization of some of the ^{1}H-pyruvate would be expected.

Other points of deuteration were observed (C-3, C-5, and C-6 positions) and were similar in isotopic composition to those observed in the earlier solvent participation study (1).

Highly Deuterated Benzylpenicillin—The information gathered from the described experiments was utilized for the production of a highly deuterated benzylpenicillin. A fermentation was conducted utilizing the ^{2}H-pyruvate additions to the seed culture medium and ^{2}H-phenylacetic acid additions to the D_2O nutrient culture medium. The fermentation proceeded normally, except that the initial drop in apparent pH was not as great as previously noted but rose to a plateau as expected. The cultures were harvested at 120 hr.; the extraction, purification, and isolation procedures shown in Scheme I were performed. An overall yield of 34.1 mg. antibiotic was attained. The IR spectrum of the isolated material exhibited the three carbonyl absorption maxima at 1763, 1662, and 1608 cm.$^{-1}$, which were in accord with expected values (14).

Chemical shifts for the *N*-ethylpiperidine salt (VI) are shown in Table III. The PMR spectrum was normal, except for the conspicuous absence of side-chain phenyl (7.30 p.p.m.) proton and methylene (4.29 p.p.m.) proton resonances because of deuteration

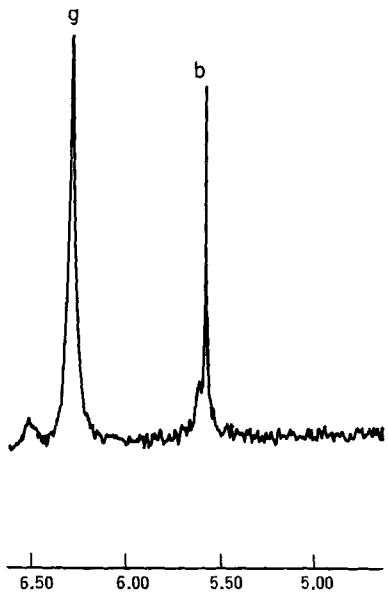


Figure 3—*PMR spectrum (5.0–6.5 p.p.m.) of N-ethylpiperidyl highly deuterated benzylpenicillin in $CDCl_3$. Key: see Fig. 1.*

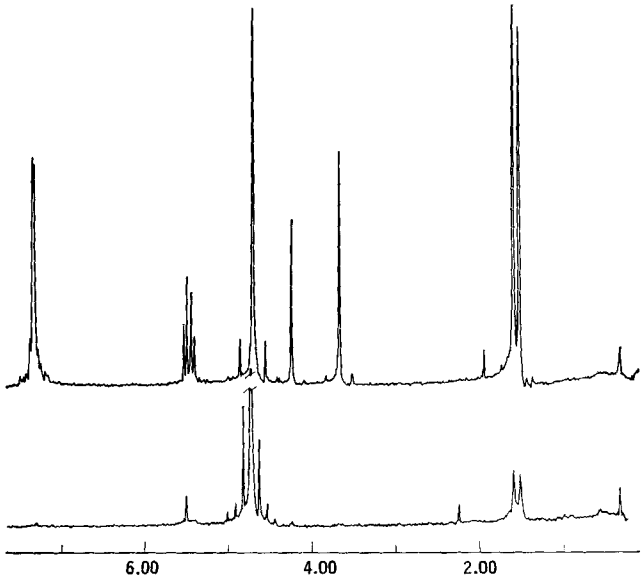


Figure 4—*PMR spectrum of equimolar solutions of potassium ^{1}H-benzylpenicillin VIII (upper) and potassium highly deuterated benzylpenicillin IX (lower) in D_2O buffer.*

at these sites. Figure 3 shows the resonances for the amide proton (6.30 p.p.m.) and protons at the C-5 position (5.58 p.p.m.). Chemical shifts for benzylpenicillinic acid (VII) dissolved in $CDCl_3$ are given in Table III. Green *et al.* (15) assigned the resonance at 5.52 p.p.m. to the C-5 proton, the two doublets centered at 5.70 and 5.49 p.p.m. to the C-6 proton (which is coupled to the C-5 proton and the amide proton), and the doublet at 6.35 p.p.m. to the amide proton. However, these workers (16) later revised the assignments. The single resonance assigned to the C-5 proton, which would normally be expected to be a doublet because of coupling to the C-6 proton, had been called an anomalous result. By use of a spectrometer with greater resolution, a different compound (*para*-dimethylaminophenyl ester of benzylpenicillin), and other solvents, it was shown that the C-6 proton does yield a normal double doublet and the C-5 proton does yield a doublet as expected. These workers attributed the anomalous pattern in $CDCl_3$ to chance equivalence of the chemical shifts for the β-lactam protons. The lack of splitting observed in Fig. 3 for the amide resonance again is evidence that the protons responsible for the 5.58 p.p.m. peak reside at the C-5 position and not the C-6 position (1). The ratio of the areas beneath the amide and C-5 resonances was 1:0.36 and indicated that deuteration at the C-5 position was 64%.

Biosynthesis of Deuterated Benzylpenicillins Based on PMR Spectra—Figure 4 shows the PMR spectra of equimolar concentrations of protio potassium benzylpenicillin (upper) and highly deuterated potassium benzylpenicillin (lower); the chemical shifts for these compounds (VIII and IX, respectively) are given in Table III. The ratio of the areas beneath the methyl resonances for the upper and lower spectra is 1:0.227, respectively, and indicates that deuterium replacement in the methyl groups is of the order of 77%. This result is compatible with the 76% deuterium incorporation observed with previous compounds. Deuterium replacement in the highly deuterated benzylpenicillin was estimated to be, on

Table IV—Deuterium Incorporation in Highly Deuterated Benzylpencillin

Group	Number of Protons	Percent Incorporation
Side-chain phenyl	5	99
C-5 Proton	1	64
C-6 Proton	1	90–95
C-3 Proton	1	90–95
Side-chain methylene	2	99
Thiazolidine methyls	6	77
Amide proton	1	99

the average, approximately 89% (Table IV) based on the deuterium replacement observed in preliminary work and on a comparison of the spectra in Fig. 4.

These results can be used to study the site of penicillin biosynthetic processes in D_2O nutrient culture fermentations. It is probable that parts of the biosynthesis occur in both the seed inoculum and the fermentation, because the seed culture ^2H-pyruvate was shown to be essential for deuterium incorporation in the methyl groups of the penicillin, and antibiotic titers were only observed in the fermentation medium. Deuterium from the methyl groups of ^2H-pyruvate in the seed culture medium was incorporated into the methyl groups of valine and, hence, into the methyl groups of penicillin. Washing the mycelium by the modified replacement technique apparently does not remove the components involved in the valine biosynthesis, which are probably intracellular substances.

Preliminary experiments showed that ^2H-pyruvate in the fermentation medium was not utilized for penicillin biosynthesis. It is apparent that 23% of the methyl groups arise from valine derived from ^1H-sugars in the H_2O seed culture medium and that 77% of the methyl groups are deuterated and are derived from ^2H-pyruvate added to the seed culture medium, with no back exchange with H_2O. The α-proton of valine is incorporated into the C-3 position of the benzylpenicillin molecule, but replacement of the C-3 proton by a solvent proton (or deuterium atom) occurs during ring closure of penicillin biosynthesis (12). The biosynthesis of the amino acid cysteine, which is utilized for the β-lactam portion of the molecule, probably takes place almost wholly in the D_2O nutrient culture medium, as evidenced by the sites of deuteration in the benzylpenicillin molecule. Arnstein and Crawhill (12) concluded that the α-proton and one of the β-protons of cysteine are retained during penicillin biosynthesis.

Observations made in this study indicate that full deuteration occurs at the C-6 position and approximately two-thirds deuterium replacement occurs at the C-5 position, *i.e.*, the α-position and β-position of cysteine, respectively. If the cysteine was biosynthesized in the seed culture medium and the α-proton was retained during the biosynthesis of benzylpenicillin, then no deuterium would be expected at the C-6 position. Full deuteration of this position is observed, however. Also, if one of the β-protons of the cysteine is retained during penicillin biosynthesis, then no deuterium would be anticipated at the C-5 position of penicillin. However, a peak is observed for the C-5 position, indicating the presence of protons, but the area represents only about one-third proton when compared to an amide proton in PMR spectra (Fig. 3). Exchange of β-protons with deuterium from the solvent must occur at some point of the cysteine biosynthesis since two-thirds replacement of the protons of the β-position is observed. Thus, the major portion of cysteine biosynthesis would probably take place in the D_2O fermentation medium, although the precursors (*e.g.*, 3-phosphoglycerate) may be retained from the seed inoculum. The components for the valine biosynthesis, *i.e.*, pyruvate, apparently originate almost totally in the seed inoculum, but formation into the valine may take place in either the seed culture or the fermentation.

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Drug Permeation through Thin-Model Membranes III: Correlations between *In Vitro* Transfer, *In Vivo* Absorption, and Physicochemical Parameters of Substituted Benzoic Acids

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Abstract □ Comparative transfer-rate studies employing a series of benzoic acid analogs were conducted across a polymeric model biomembrane. Statistical correlations were sought between the determined *in vitro* transfer-rate constants and *in vivo* absorption data reported in the literature. Relationships with certain solubility-related parameters were also investigated. The *in vitro* transfer-rate constants were compared to *in vivo* absorption data reported in the literature for buccal and intestinal absorption. Significant correlations were obtained, indicating the strong association that exists between these two variables. The *in vitro* transfer rates of the benzoic acid derivatives were also shown to be statistically related to the reciprocal water solubility and the log partition coefficient of the substituent group on benzoic acid. No correlation was found between drug permeation, as expressed by the *in vitro* transfer-rate constant, and the molecular weight or volume of the compound, confirming the dominant role that solubility of the penetrating species plays in controlling permeation through this membrane system.

Keyphrases □ Benzoic acids, membrane permeation—correlations between *in vitro* transfer, *in vivo* absorption, physicochemical parameters □ Membrane permeation, benzoic acids—*in vitro* transfer, *in vivo* absorption, physicochemical parameters □ Biomembrane model, polymeric—*in vitro–in vivo* drug-absorption correlations □ Transfer rates, *in vitro*, benzoic acids—*in vivo* absorption and physicochemical correlations

Some correlations involving physicochemical parameters with either *in vitro* or *in vivo* absorption data have been reported in the literature. The absorption rates of barbituric acid derivatives from the rat stomach and small intestine were correlated with their respective partition coefficients (1, 2). Similar experiments were carried out using a series of sulfonamides (3,4). Correlations pertaining to *in vitro* transfer rates and partition coefficients appeared more recently. Thus, the apparent diffusion constants of various barbitals through silicone rubber sheeting were correlated to their partition coefficients between chloroform and pH 4.7 acetate buffer (5). These authors observed no significant correlation between the apparent diffusion constants and drug solubility, or its reciprocal, in pH 4.7 acetate buffer.

Recently, Bates *et al.* (6) examined the *in vitro* transport of both free and complexed drug species and qualitatively correlated it to the corresponding gastric absorption data derived by Goto *et al.* (7).

The present study was designed to demonstrate the utility of a polymeric biomembrane in establishing *in vitro–in vivo* correlations for passive drug absorption. This biomembrane, consisting of natural membrane components contained within an ethylcellulose polymer matrix, was described previously (8, 9). Accordingly, a comparative study of the *in vitro* transfer rates of various benzoic acid derivatives through the model biomembrane was undertaken. Subsequently, statistical correlations of these data were sought with *in vivo* absorption data reported in the literature and physicochemical parameters such as substituent partition coefficients, solubility, and molecular size.

Table I—Absorption Wavelength Used in Spectrophotometric Concentration Determinations of Benzoic Acid Derivatives

Compound[a]	Absorption Wavelength in pH 1.2 Buffer, nm.	Absorption Wavelength in Water, nm.	Absorption Wavelength in Water Saturated with 1-Octanol, nm.
BA[b]	274	—	270
2-Hydroxy-BA[c]	303	—	—
3-Hydroxy-BA	298	—	—
4-Hydroxy-BA	256	—	—
2,4-Dihydroxy-BA	258	248	250
2,5-Dihydroxy-BA	238	230	230
2-Methoxy-BA	238	284	280
2-Methyl-BA	233	—	224
3-Methyl-BA	236	—	—
4-Methyl-BA	242	—	—
2,4-Dimethyl-BA	244	238	—
2,5-Dimethyl-BA	238	280	—
2-Nitro-BA	264	—	268

[a] Eastman Chemicals, unless otherwise stated. [b] BA = benzoic acid. [c] Baker and Adamson.

Table II—Accumulated Data on Methyl-Substituted Benzoic Acids

Acid	*In Vitro* Transfer-Rate Constant, k_d, hr.$^{-1}$	*In Vivo* Buccal Absorption-Rate Constant, ml. min.$^{-1}$[a]	Log k_{pc} of Substituent Group[b]	Solubility at 25°, g. 100 ml.$^{-1}$
BA[c]	0.093	6.5	0.0	0.35[d]
4-Methyl-BA	0.148	9.6	0.32	0.035[d]
2-Methyl-BA	0.154	9.3	0.36	0.12[d]
3-Methyl-BA	0.182	8.9	0.45	0.098[d]
2,5-Dimethyl-BA	0.248	12.7	0.76	0.018[e]
2,4-Dimethyl-BA	0.288	10.6	0.90	0.016[e]

[a] From Beckett and Moffatt (11). [b] Derived from data of Beckett and Moffat (11) using *n*-heptane–0.1 *N* HCl partition coefficients. [c] BA = benzoic acid. [d] From Seidell (12). [e] Determined in this study.

EXPERIMENTAL

Materials—The materials used were reported previously (9), with the exception of the benzoic acid derivatives, which were used as received from the manufacturers (Table I).

General Experimental Protocol—The procedures for membrane formation and permeation studies using dialysis cells were published previously (8).

Comparative Transfer Studies—Benzoic acid and 12 mono- or disubstituent derivatives were used (Table I). In all cases, transfer of these compounds took place from 0.1 *N* HCl (pH 1.2) into pH

Table III—Accumulated Data on Hydroxy- and Methoxy-Substituted Benzoic Acids

Acid	*In Vitro* Transfer-Rate Constants ($hr.^{-1}$) $\times 10^4$, pH 6.0	*In Vivo* Intestinal Absorption Rate Constant, $min.^{-1}$ [a]	Log k_{pc} of Substituent Group	Solubility at 25°, g. 100 $ml.^{-1}$
BA[b]	14.4	0.92	0.0[c]	0.35[d]
2,5-Dihydroxy-BA	0.088	0.01	−0.13[c]	2.2[e]
2,4-Dihydroxy-BA	0.212	0.07	0.19[c]	0.60[e]
3-Hydroxy-BA	0.963	0.10	−0.38[f]	1.1[d]
4-Hydroxy-BA	1.23	0.19	−0.30[f]	0.64[d]
2-Hydroxy-BA	1.39	0.66	0.41[g]	0.22[d]
2-Methoxy-BA	1.44	0.56	−0.28[c]	0.42[e]

[a] Data from Nogami *et al.* (14). [b] BA = benzoic acid. [c] Octanol–water partition coefficients determined in this study. [d] From Seidell (12). [e] Determined in this study. [f] Octanol–water partition coefficients from Fujita *et al.* (10). [g] Octanol–water partition coefficients from Lien (15).

7.4 buffer. Two-millimolar concentrations were used, except where the aqueous solubility was less than this amount. The concentrations of benzoic acid derivative remaining in the pH 1.2 phase were determined at known time intervals, using predetermined calibration curves, at the wavelengths shown in Table I. In most cases, the duration of the experimental run exceeded one half-life of the test substance; all runs were carried out in triplicate. In all cases, except one, the experimentally observed rate constants were regarded as the true first-order disappearance-rate constants, since 98% or greater of the compound was undissociated. The determined rate constant for the exception, 2-nitrobenzoic acid, was corrected accordingly.

Partition Studies—Mutually saturated solutions of octanol and distilled water were used. Five milliliters of equilibrated octanol was added to 5 ml. of equilibrated water containing the drug at a concentration of 100 mg./l. The mixture was agitated at 25° for 36 hr., a time found to be adequate for establishing equilibrium conditions. The extent of partitioning was established by spectrophotometric analysis (Table I) of the aqueous phase, using the equilibrated water as the reference. The drug in the octanol phase was determined by difference, ignoring association. The true partition coefficient was computed, taking into account the degree of dissociation of the compounds, using the method of Fujita *et al.* (10). The pH of the aqueous phase was determined at equilibrium. The true partition coefficient was shown not to be significantly influenced by concentration. All determinations were carried out in duplicate; the results were averaged and expressed in terms of the partition coefficient of the substituent group so as to conform to the literature values used.

Equilibrium Solubility Studies—Equilibrium aqueous solubilities of five benzoic acid derivatives were determined at 25°. An excess of drug, together with approximately 100 ml. of distilled water, was placed in a flask. The sealed flasks were continuously agitated in a water bath, and samples were removed by means of a pipet fitted with a filter plug. In all cases, equilibrium was attained within 48 hr. Appropriate volumetric dilutions were performed on the equilibrated samples, and the concentrations were determined by spectrophotometric analysis. Duplicates were run, and the results were averaged.

RESULTS

General—As noted previously, pH 1.2 was chosen for Compartment A to facilitate transport, since only one of the weak acids examined was more than 2% ionized at this pH. Since the model membrane was shown previously (9) to comply with the pH-partition hypothesis, the *in vitro* rate constants obtained at pH 1.2 were corrected to the pH conditions used in the *in vivo* studies.

Methyl-Substituted Benzoic Acids—Beckett and Moffat (11) studied the buccal absorption of methyl-substituted benzoic acids. Although a pH of 4.0 was employed in their *in vivo* study, the reported rate constants were for the transfer of unionized drug; consequently, no correction of the observed *in vitro* rates are required. The true first-order *in vitro* disappearance-rate constants of the methyl-substituted benzoic acids are listed in Table II, together with the buccal absorption-rate constants reported by Beckett and Moffat (11). Table II also lists the aqueous solubilities of these compounds, together with the log partition coefficients of the substituent groups. The log partition coefficient of the substituent was used to draw attention to the effect of the various groups and is obtained by subtracting the log value of benzoic acid from that of the various derivatives. This approach was demonstrated by Fujita *et al.* (10) for octanol–water partition coefficients. Furthermore, Collander (13) showed that partition coefficients derived in one organic solvent may be related to those obtained from a second organic solvent.

Hydroxy- and Methoxy-Substituted Benzoic Acids—In the *in vivo* intestinal perfusion studies reported by Nogami *et al.* (14), the initial perfusion fluids were adjusted to pH 6.0. The apparent first-order *in vitro* rate constants at this pH are presented in Table III, together with the observed *in vivo* intestinal absorption coefficients. Table III also contains the aqueous solubilities of these compounds and the log values of the substituent group partition coefficients based on the octanol–water system. These latter are Hansch substituent constants (16).

DISCUSSION

***In Vitro–In Vivo* Correlations**—Tables II and III present the *in vitro* transfer data and the *in vivo* absorption data for the methyl-substituted and the hydroxy- and methoxy-substituted benzoic acids, respectively. Statistical *in vitro–in vivo* correlations were attempted with these groups of compounds. The results are contained in Table IV.

Correlation of the buccal absorption-rate constants, which reflect only unionized drug transfer, to the true first-order disappearance-rate constants at the 95% confidence level shows that all correlations are real. At the more stringent 99% confidence level, however, there is no real correlation. In the case of the intestinal absorption-rate coefficients determined by Nogami *et al.* (14), two of the four combinations used showed a real correlation at the 95% confidence level.

Table IV—Correlations between *In Vitro* Transfer-Rate Constants and *In Vivo* Absorption Data Reported in Literature

Type of Benzoic Acid Derivative	*Y versus X*	Regression Coefficient ± 95% Confidence Interval	Correlation Coefficient (Significance Level — Greater Than)
Methyl-substituted (Table II)	$\sqrt{k_{1.2}}$[a] *versus* $\sqrt{k_{buc}}$[b]	0.201 ± 0.153	0.88 (95%)
	$k_{1.2}$ *versus* $\sqrt{k_{buc}}$	0.166 ± 0.142	0.85 (95%)
	$\sqrt{k_{1.2}}$ *versus* k_{buc}	0.0347 ± 0.0307	0.84 (95%)
	$k_{1.2}$ *versus* k_{buc}	0.0287 ± 0.0279	0.82 (95%)
Hydroxy- and methoxy-substituted (Table III)	$\sqrt{k_{6.0}}$[c] *versus* k_{int}[d]	0.0274 ± 0.0209	0.83 (98%)
	$\sqrt{k_{6.0}}$ *versus* $\sqrt{k_{int}}$	0.0283 ± 0.0257	0.79 (95%)
	$k_{6.0}$ *versus* k_{int}	0.0011 ± 0.0011[e]	0.76 (95%)
	$k_{6.0}$ *versus* $\sqrt{k_{int}}$	0.0011 ± 0.0013[e]	0.68 (90%)

[a] True first-order disappearance-rate constant (pH 1.2). [b] Buccal absorption constant from Beckett and Moffat (11). [c] Apparent first-order disappearance-rate constant (pH 6.0). [d] Intestinal absorption coefficient from Nogami *et al.* (14). [e] Correlation is not real.

Table V—Correlations between *In Vitro* Transfer-Rate Constants and Solubility-Related Parameters

Type of Benzoic Acid Derivative	X^a	Regression Coefficient ± 95% Confidence Interval	Correlation Coefficient (Significance Level — Greater Than)
Methyl-substituted (Table II)	Log k_{pc} of substituent	0.219 ± 0.030	0.99 (99%)
	Water solubility^{-1}	0.0025 ± 0.0017	0.90 (98%)
	Log water solubility^{-1}	0.119 ± 0.092	0.87 (95%)
Hydroxy- and methoxy-substituted (Table III)	Log k_{pc} of substituent	0.141 ± 0.140	0.76 (95%)
	Water solubility^{-1}	0.0353 ± 0.0210	0.89 (99%)
	Log water solubility^{-1}	0.117 ± 0.129[b]	0.72 (90%)
ortho-Substituted[c]	Log k_{pc} of substituent	0.195 ± 0.189	0.89 (95%)
	Water solubility^{-1}	0.0210 ± 0.0242[b]	0.85 (90%)
	Log water solubility^{-1}	0.208 ± 0.181	0.90 (95%)

[a] *Y*-axis in all cases was *in vitro* true first-order disappearance-rate constant. [b] Correlation is not real. [c] Data from benzoic acid, 2-hydroxybenzoic acid, 2-methoxybenzoic acid, 2-methylbenzoic acid, and 2-nitrobenzoic acid ($k_d' = 0.0155$; log $k_{pc} = 0.04$; $S = 0.74$ g. 100 ml.$^{-1}$).

***In Vitro* Physicochemical Parameter Correlations**—Certain physicochemical parameters of the compounds under investigation are contained in Tables II and III. Correlations between the true first-order rate constants and solubility expressions were sought by the use of three parameters: the log partition coefficient of the substituent group, the reciprocal water solubility, and the log of the reciprocal water solubility. The use of the first parameter places emphasis on the solubility properties of the substituent groups and was used before in relating to *in vivo* absorption data (11, 17). The reciprocal of the water solubility should be a reflection of the lipoidal solubility of the compound; the log value was used to parallel the log partition coefficient.

As may be seen from Table II, increased lipid solubility with increasing methylation is suggested by the log partition coefficients of the substituent groups and the aqueous solubility values. This increase in lipid solubility is reflected in the true first-order disappearance-rate constants. It also appears that the degree of substitution, and not the position of substitution, plays the dominant role. The hydroxy and methoxy analogs (Table III) produced substantially smaller true first-order disappearance-rate constants when compared to the methyl analogs. This was anticipated and verified by the higher water solubility exhibited upon the addition of these hydrophilic groups onto the parent molecule. The one exception was salicylic acid. Its unique lipid solubility may be in part attributed to its potential for intramolecular hydrogen-bond formation. The accelerated rate of transfer of the 2,4-dihydroxy analog may also be predicted from its lower water solubility and higher partition coefficient. The benzoic acid derivatives are assumed to undergo total dimerization in *n*-heptane (used with the methyl derivatives); dimerization will not occur in octanol (used with the hydroxy and methoxy derivatives).

The results of the statistical evaluations are contained in Table V. Seven of the nine correlations attempted were real at the 95% confidence level. In two of the three series, the reciprocal water solubility correlated better than the logarithm of this parameter, pointing to the utility of this simple property in predicting membrane permeation.

Attempts also were made to establish a correlation between the *in vitro* transfer rates and the reciprocal of the square root of the molecular weight and the molecular volume. The latter was calculated according to the method and data of Bondi (18). However, in no instance was a real correlation at the 95% confidence level found, indicating that the permeation of the compounds studied is independent of molecular size and that the real influence is that of solubility.

When the results of the statistical correlations presented in Tables IV and V are examined together, several points become apparent. First, solubility plays a dominant role in determining the *in vitro* transfer rates across this membrane. Second, significant relationships do exist between these *in vitro* transfer rates and the *in vivo* absorption data in the literature. This suggests that the thin polymeric model biomembrane, whose mechanism of transfer is based on solubility, adequately mimics the passive diffusion functionality of the biological membrane. Third, although it would be desirable to have but one form of data presentation result in correlations for all types of *in vivo* data, this is not the case in the present study. This may be a reflection of the route of absorption and may also arise from the diverse experimental protocols employed in the *in vivo* investigations whose data were used in this work.

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GC Determination of Tolazamide in Plasma

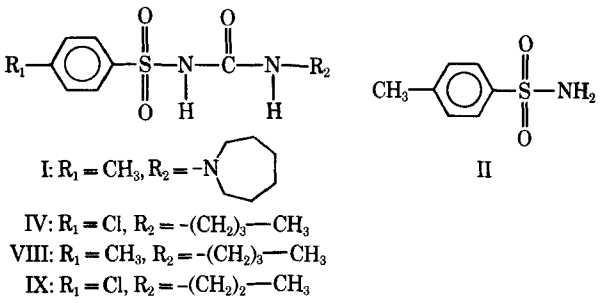
J. A. F. WICKRAMASINGHE* and S. R. SHAW

Abstract □ A highly specific, sensitive, GC method for the determination of tolazamide is described. The lower limit of detection sensitivity was 0.048 mcg. tolazamide "on column" or 0.7 mcg. tolazamide/ml. plasma. The chromatographic species was identified as *p*-toluenesulfonamide by GC–mass spectrometry. The method is based upon quantification of the sulfonamide. The utility of the procedure was demonstrated by its application to the determination of tolazamide in the plasma of drug-treated guinea pigs.

Keyphrases □ Tolazamide—GLC analysis, guinea pig plasma □ GLC—analysis, tolazamide in plasma

The sulfonylureas are widely used as oral hypoglycemic agents in the therapy of diabetes. Studies on the duration of drug action necessitate highly sensitive and specific analytical methodology for the determination of blood levels of the circulating drug. The need for such methodology becomes particularly important in the blood level determination of the more potent antidiabetic agents, *e.g.*, tolazamide[1] (I), encountered in biological samples at relatively lower concentrations. Most analytical procedures described in the literature (1–3) are colorimetric or spectrophotometric methods which lack high sensitivity and/or specificity. A GC method for the determination of tolbutamide and chlorpropamide was reported recently (4) (see *Results and Discussion* section).

The present article describes a specific and sensitive GC method for the determination of tolazamide (I).



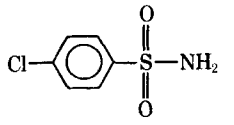
I: $R_1 = CH_3$, $R_2 = -N$

II

IV: $R_1 = Cl$, $R_2 = -(CH_2)_3-CH_3$

VIII: $R_1 = CH_3$, $R_2 = -(CH_2)_3-CH_3$

IX: $R_1 = Cl$, $R_2 = -(CH_2)_2-CH_3$



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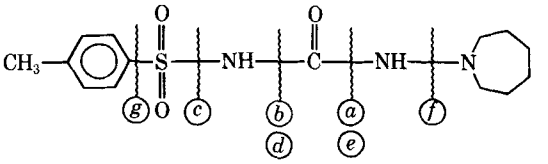

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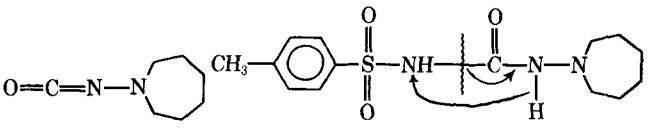

VI VII

EXPERIMENTAL

Biological—*Animals*—Male, Kuiper strain, albino guinea pigs (600–700 g. body weight) were used in the study. The animals were housed in holding cages. The guinea pigs were fasted for 16 hr. prior to dosing.

Dosing—Tolazamide was suspended in the sterile vehicle No. 100[2] and administered orally as a single dose of 15 mg./kg. body weight. Control animals received appropriate volumes (about 0.5 ml.) of the diluent by the same route. All animals were fasted for an additional 4 hr. after dosing. Food was then returned. The animals had access to water *ad libitum* at all times.

Blood and Urine Collection—Blood samples were drawn by heart puncture from the mildly anesthetized (ether) guinea pigs at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 14, 24, 36, and 48 hr. One guinea pig per time interval was sacrificed. The blood samples, collected in heparinized tubes, were centrifuged for 10 min. at 2000 r.p.m. The plasma was harvested and stored at −18°.

Preparation and Extraction of Plasma Standards and Specimens from Dosed Animals—*Chloroform Standards*—Aliquots of a standard solution of tolazamide in chloroform containing 1.0, 2.0, 3.0, 4.0, 5.0, 7.0, 9.0, 10.0, and 12.0 mcg. were placed in glass-stoppered, 15-ml. centrifuge tubes. Aliquots (0.1 ml.) of the internal standard solution, containing 12 mcg. of IV[3], were added to each tube. All of the samples were evaporated to dryness under a gentle stream of nitrogen. Each sample was then reconstituted in 20 μl. of chloroform and analyzed by GC in the manner described later.

Guinea Pig Plasma Standards—Aliquots of a standard solution of tolazamide in chloroform containing 1.0, 2.0, 3.0, 4.0, 5.0, 7.0, 9.0, 10.0, and 12.0 mcg. were placed in glass-stoppered, 15-ml. centrifuge tubes. The samples were evaporated to dryness under a gentle stream of nitrogen. One-milliliter aliquots of control guinea pig plasma were added to each centrifuge tube and mixed well using a Vortex mixer. A control plasma blank was prepared by adding 1 ml. plasma to a 15-ml. centrifuge tube and processing it in the same manner as the other samples. The pH of all samples were adjusted to 2.5 using 1 *N* HCl and extracted with chloroform (5 ml./sample). Emulsions which formed were broken up by centrifugation at 1000 r.p.m. A measured volume of the chloroform extract (about 80%) from each extraction was pipeted out into fresh centrifuge tubes and evaporated to dryness under nitrogen. The residues were subjected to TLC in the manner described later.

Plasma Specimens from Dosed Guinea Pigs—One-milliliter aliquots of the plasma samples were placed in 15-ml. centrifuge tubes. The pH of each sample was adjusted to 2.5 using 1 *N* HCl, and the samples were processed in the same manner as the plasma standards.

TLC of the Guinea Pig Plasma Extracts—TLC was carried out on precoated TLC plates of silica gel F-254 of 250-μ thickness[4]. The plasma extracts were subjected to TLC in a solvent system composed of chloroform–methanol–formic acid (98.5:1.0:0.5). Reference spots of authentic tolazamide were used. The tolazamide zones

[1] 1-(Hexahydroazepin-1-yl)-3-*p*-toluenesulfonylurea.

[2] Vehicle No. 100 contains per milliliter: sodium carboxymethylcellulose (5 mg.), polysorbate 80 (4 mg.), sodium chloride (9 mg.), and benzyl alcohol (9 mg.) in distilled water. Supplied by The Upjohn Co., Kalamazoo, Mich.

[3] 1-(*n*-Butyl)-3-*p*-chlorobenzenesulfonylurea.

[4] Merck, Brinkmann Instruments, Inc., Westbury, N. Y.

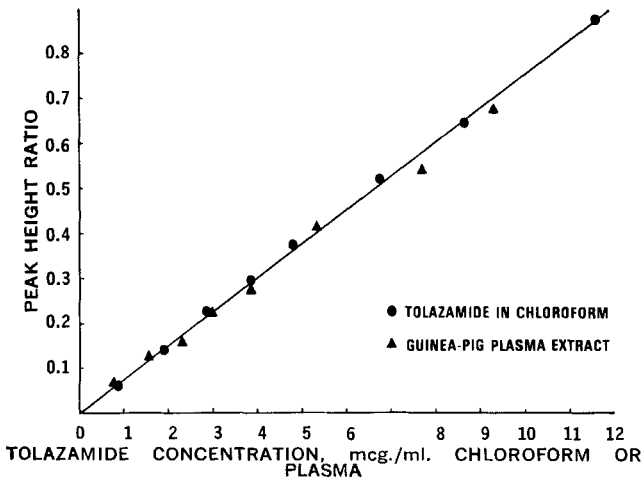

Figure 1—*Standard curves for tolazamide extracted from guinea pig plasma and in chloroform.*

were located by examination of the chromatograms using a UV lamp (254 nm.). The zones were removed separately and extracted out with methanol (2 × 1 ml.). To each extract was added 0.1 ml. of the internal standard solution containing 12 mcg. of IV, and the total was evaporated to dryness under nitrogen. The residue was reconstituted in 20 μl. of chloroform and analyzed by GC.

GC—GC was carried out on a gas chromatograph[5] equipped with a flame-ionization detector. Glass columns (U-shaped, 70 cm. × 3 mm.) packed with 0.5% Carbowax 20M on 80–100-mesh Chromosorb G were utilized throughout the study. Helium was used as the carrier gas at a flow rate of 80 ml./min. Oxygen and hydrogen flow rates were adjusted to give maximum response. The column oven was operated isothermally at 190°, the flash heater at 236°, and the detector at 220°. Under these conditions, the *p*-toluenesulfonamide fragment (II) and the *p*-chlorobenzenesulfonamide fragment (III) had retention times of ~2 and ~3.2 min., respectively. Aliquots (~1 μl.) of the TLC extracts of standard and dosed plasma specimens were analyzed by GC.

Internal Standard—A solution of IV in chloroform (0.12 mg./ml.) was employed as the internal standard for GC.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to II (resulting from the thermal fragmentation of I) by the height of the peak due to III (resulting from the thermal fragmentation of IV). Standard curves for the recovery of tolazamide from guinea pig plasma were constructed by plotting the concentration of tolazamide (in micrograms per milliliter of plasma) against the peak height ratios. A typical standard curve is presented in Fig. 1.

Mass Spectrometry—Mass spectra were recorded in a high-resolution mass spectrometer[6].

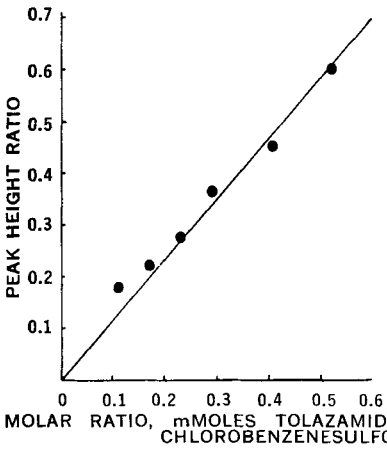

Figure 2—*Peak height ratio of tolazamide/1-(n-butyl)-3-p-chlorobenzenesulfonylurea, expressed as a function of concentration of tolazamide.*

[5] F&M model 402.
[6] Consolidated Electrodynamics Corp., model 21-110.

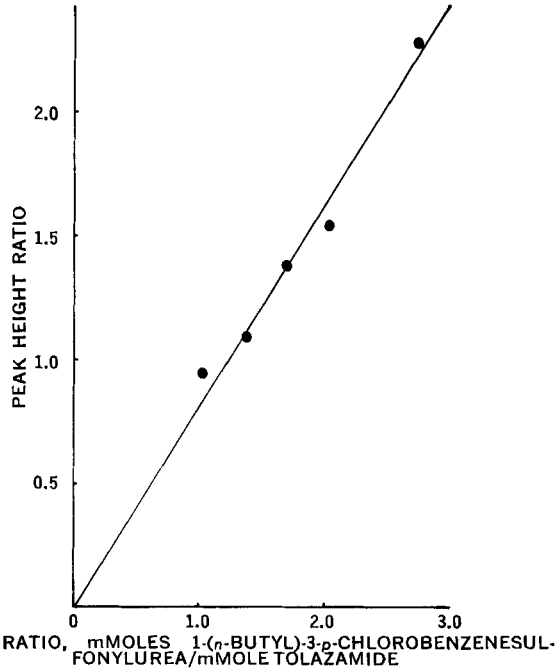

Figure 3—*Peak height ratio of 1-(n-butyl)-3-p-chlorobenzenesulfonylurea/tolazamide expressed as a function of concentration of 1-(n-butyl)-3-p-chlorobenzenesulfonylurea.*

Mass Spectrum of Tolazamide—At Low Temperature—A spectrum of tolazamide was recorded using the direct probe at 145° (70 ev.). This spectrum showed a molecular ion at *m/e* 311 and prominent peaks at: (*a*) 197 (M-114), (*b*) 171 (M-140), (*c*) 155 (M-156), (*d*) 140 (M-171), (*e*) 113 (M-198), (*f*) 98 (M-213), and (*g*) 91 (M-220), base peak; corresponding to a fragmentation of the type indicated in V.

At High Temperature—A spectrum of tolazamide was recorded using the heated inlet system at 220° (70 ev.). The spectrum showed no molecular ion. Prominent peaks were present at *m/e* 171 (M-140), 155 (M-156), and 140 (M-171).

GC–Mass Spectrometry—GC–mass spectra were recorded on an LKB-9000 gas chromatograph–mass spectrometer. The column packing utilized for the GC analysis was 3% OV-17 on Gas-Chrom Q (80–120 mesh). The column was maintained at a temperature of 200° and the flash heater at 220°. Helium was used as the carrier gas at a flow rate of ~45 ml./min.

Tolazamide—Three GC peaks were observed. The first GC peak was of low retention time, ~0.2 min. The mass spectrum of this material showed a prominent peak at *m/e* 140, which may be attributed to the isocyanate fragment (VI). The mass spectrum also showed peaks at *m/e* 112 (M-28) and 84 (M-56). The second GC peak was the most intense and had a retention time of ~1 min. The mass spectrum of this material showed a molecular ion at *m/e* 171 corresponding to *p*-toluenesulfonamide (II). The spectrum also showed prominent peaks at *m/e* 155 (M-16), 139 (M-32), and 123 (M-48). The third GC peak was of low intensity and rather diffuse. It had a retention time of ~6 min. The mass spectrum showed peaks at *m/e* 256, 171, 156, and 139. The nature of this material is obscure.

1-(n-Butyl)-3-p-chlorobenzenesulfonylurea (IV)—Only one major GC peak was observed under the conditions employed. This peak had a retention time of ~2 min. The mass spectrum of this material showed a molecular ion at *m/e* 191, which may be attributed to *p*-chlorobenzenesulfonamide (III). The spectrum showed other prominent peaks at *m/e* 175 (M-16), 127 (M-64), and 111 (M-80).

RESULTS AND DISCUSSION

GC of tolazamide using column packings of 1% SE-30 on 80–100-mesh Gas-Chrom Q (column temperature, 200°; flash heater, 275°) or 1% OV-17 on 80–100-mesh Gas-Chrom Q (column temperature, 150°; flash heater, 192°) revealed a major sharp peak of ~1-min. retention time and a minor peak of very low retention time (~0.2

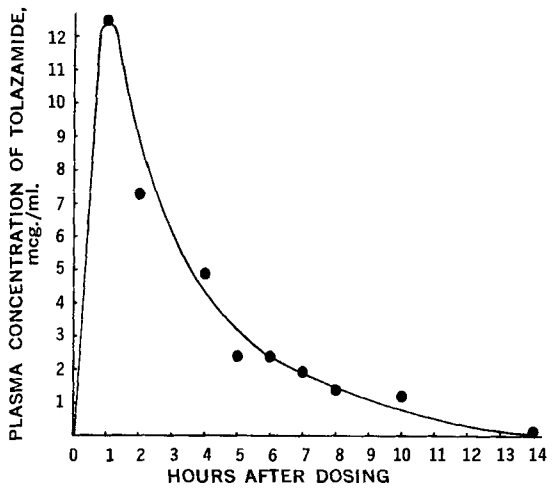

Figure 4—*Plasma concentrations of tolazamide* versus *time in guinea pigs after single-dose oral administration of 15 mg./kg. body weight.*

min.) poorly resolved from the solvent. These GC peaks were investigated on a gas chromatograph–mass spectrometer analyzer unit, using a column packed with OV-17 (3%) on Gas-Chrom Q at a column temperature of 200° and a flash heater temperature of 220°. A mass spectrum of the major GC peak showed a molecular ion at *m/e* 171 and a fragmentation pattern (see *Materials and Methods* section) which indicated that the material corresponding to the peak was *p*-toluenesulfonamide (II). A mass spectrum of the minor GC peak of low retention time showed a molecular ion at *m/e* 140 and a fragmentation pattern (see *Materials and Methods* section) which indicated that this peak was due to the isocyanate (VI). The two fragments, II and VI, obviously resulted from the thermal degradation of tolazamide (see VII).

The thermal fragmentation property of tolazamide was clearly demonstrated by mass spectrometry of the sulfonylurea at low and high temperatures. A mass spectrum of tolazamide, recorded on the high-resolution mass spectrometer at 145° using the direct probe, showed a distinct molecular ion at *m/e* 311 and fragments derived from it by further fragmentation (see *Materials and Methods* section) in the manner indicated in V.

On the other hand, a mass spectrum of tolazamide, recorded using the heated inlet system at 220°, showed no molecular ion. The fragmentation pattern was markedly different. Prominent peaks were observed at *m/e* 171 (base peak), corresponding to a toluenesulfonamide fragment, and at *m/e* 140, corresponding to the isocyanate fragment (see *Materials and Methods* section). Most of the other peaks observed could be attributed to further fragmentation of these ions.

To improve the GC resolution of the *p*-toluenesulfonamide from the solvent to facilitate working at higher sensitivities, several column packings were examined. The best results were obtained with a column packed with 0.5% Carbowax 20M on Chromosorb G (80–100 mesh). Such a column packing was employed by Liliedahl (5) for the separation and determination of *o*- and *p*-toluenesulfonamides.

Prior to examining the consistency of the GC thermal fragmentation of tolazamide, it was necessary to select a satisfactory internal standard. Several structurally related sulfonylurea compounds were screened. Compound IV was deemed to be the most satisfactory. GC–mass spectrometric analysis under the conditions already described showed only one major GC peak. A mass spectrum of the material corresponding to this peak showed a molecular ion at *m/e* 191 and a fragmentation pattern (see *Materials and Methods* section) which indicated that it was indeed III. The latter is obviously derived from IV by a thermal process analogous to that by which II is produced from tolazamide (see VII). The isocyanate fragment is probably lost with the solvent or it may undergo further degradation. The TLC mobility of IV was found to be identical with that of tolazamide on silica gel in the solvent system of chloroform–methanol–formic acid (98.5:1:0.5). The thermal fragmentation of tolazamide (I) and the internal standard (IV)

Table I—Recovery of Tolazamide Added to Guinea Pig Plasma as Measured by GC Assay

Tolazamide Added, mcg./ml.	Tolazamide Found, mcg./ml.	Percent Recovery
0.771	0.867	112
1.542	1.660	107
2.314	2.082	90
3.085	3.024	100.5
3.856	3.631	94.2
5.398	5.512	102.1
7.712	7.166	92.9
9.254	9.005	97.3

[a] Mean ± standard deviation = 99.6 ± 7.64%.

to yield *p*-toluenesulfonamide (II) and *p*-chlorobenzenesulfonamide (III), respectively, as measured by GC response was found to be linear over the concentration ranges examined—*viz.*, that of ~0.1 to ~0.9 mcg. tolazamide and ~0.6 to ~3.2 mcg. IV "on column," respectively. The concentrations, expressed as molar ratios, were plotted *versus* the peak height in Figs. 2 and 3. The "on column" lower limit of detection sensitivity was 0.048 mcg. tolazamide.

A study was conducted to determine the extent of fragmentation of tolazamide (I) and the internal standard (IV) under the GC conditions employed. A plot of the ratios of the peak heights of the fragmentation products, II and III, expressed as a function of the corresponding molar ratios, was assumed to represent 100% fragmentation of I and IV. From a comparison of the slope of this plot with the slope of a plot of the ratios of peak heights of I and III *versus* the corresponding molar ratios, the fragmentation of I was estimated to be 30%. Similarly, the fragmentation of the internal standard (IV) was estimated to be 20% from a comparison of the slope of a plot of the peak height ratios of II and the internal standard (IV) expressed as a function of the corresponding molar ratios, with the slope of the plot representing 100% fragmentation of I and IV.

Determination of Tolazamide in Guinea Pig Plasma—The procedure for the determination of tolazamide in guinea pig plasma consists of: (*a*) chloroform extraction of the acidified plasma samples (pH 2.5), (*b*) TLC of the chloroform extract, and (*c*) analysis of the recovered tolazamide by GC using a flame-ionization detector. With this procedure, tolazamide "spiked" plasma standards were extracted, and a standard curve for the recovery of tolazamide from guinea pig plasma was constructed (Fig. 1). The recovery of tolazamide from guinea pig plasma under these conditions was quite satisfactory (average recovery of 99.6%; standard deviation ±7.64%), as may be seen from Fig. 1 and Table I. The lower level of detection sensitivity for tolazamide in guinea pig plasma was 0.70 mcg./ml. (plasma blank is equivalent to ~0.6 mcg. tolazamide/ml.).

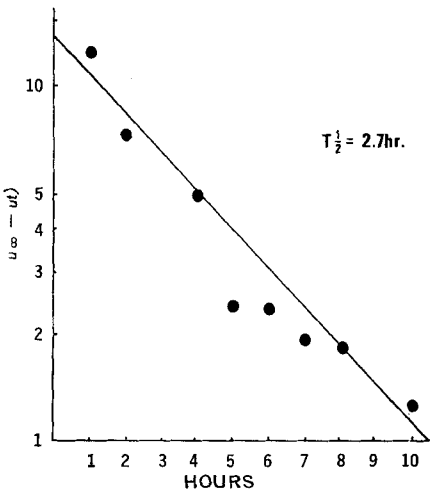

Figure 5—*Plasma drug disappearance half-life of tolazamide in guinea pigs after single-dose (15 mg./kg. body weight) oral administration of tolazamide.*

Specimens of plasma from tolazamide-treated guinea pigs (single oral dose of 15 mg. drug/kg. body weight) were extracted and analyzed in the same manner as described for the plasma standards. The concentration of tolazamide was determined from the standard curve. A peak plasma tolazamide concentration of 12.5 mcg./ml. was observed at 1 hr. after oral drug administration, indicating rapid drug absorption. A plot of the plasma concentrations of tolazamide (expressed in micrograms per milliliter) *versus* time (in hours) is presented in Fig. 4. By using the data between 1 and 14 hr., the plasma drug disappearance half-life was graphically estimated to be 2.7 hr. (Fig. 5).

During the course of this work, Sabih and Sabih (4) published a GC method for the determination of tolbutamide (VIII) and chlorpropamide (IX). These authors claimed to have analyzed the intact methyl derivatives of these drugs by GC. However, they did not establish the identity of the chromatographic species either by collecting a sample of the effluent or by GC–mass spectrometry. Considering the high operating temperatures reported (4) (flash heater at 330° and column temperature at 210°), the thermal fragmentation of the methyl derivatives cannot be excluded.

The present GC method described for the determination of tolazamide (I) in guinea pig plasma is better than the previously reported methods (1–3, 5) since: (*a*) it has greater detection sensitivity [the lower limit being 0.7 mcg. tolazamide/ml. plasma rather than 20 mcg./ml. plasma (2) or ~3 mcg./ml. plasma (3)], and (*b*) it has greater specificity on account of the preliminary TLC purification step which is capable of resolving intact tolazamide from its metabolites as well as from plasma contaminants. The method could be conveniently adapted for determination of other related sulfonylureas in pharmaceutical preparations as well as a variety of biological specimens.

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Opium Alkaloids X: Biosynthesis of 1-Benzylisoquinolines

E. BROCHMANN-HANSSEN*, C.-C. FU, A. Y. LEUNG†, and G. ZANATI

Abstract □ Radioactively labeled norreticuline was incorporated into papaverine in *Papaver somniferum* to an extent of about 5% without prior demethylation to norlaudanosoline. 1,2-Dehydronorreticuline does not participate in the biosynthesis of this alkaloid. (+)-Laudanosine is derived from (+)-reticuline *via* (+)-laudanidine, while (+)-codamine plays only a minor role. Radiodilution studies indicated that tetrahydropapaverine and papaveroline 6,3′,4′-trimethyl ether (pacodine) occur in the opium poppy. Based on the results of a series of tracer experiments, a scheme was proposed for the biosynthesis of benzylisoquinoline alkaloids in *P. somniferum*.

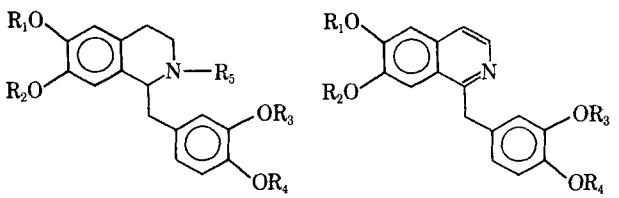
Keyphrases □ 1-Benzylisoquinolines—proposed biosynthesis in *P. somniferum* □ Biosynthesis—1-benzylisoquinolines in *P. somniferum*, proposed □ *Papaver somniferum*—proposed biosynthesis of 1-benzylisoquinolines □ Radioactive labeling—norreticuline, biosynthesis in *P. somniferum* □ Opium alkaloids—proposed biosynthesis of 1-benzylisoquinolines

The benzylisoquinoline alkaloids are widely distributed in the *Papaveraceae* family, occurring most extensively as the 1,2,3,4-tetrahydro derivatives. Four alkaloids of this general structure have been isolated from the opium poppy or from its dried latex, namely, codamine (I), laudanidine (II), laudanosine (III), and reticuline (IV). Two of these are present in both enantiomeric forms (II and IV) with an excess of one of the optical isomers [(−)-laudanidine and (+)-reticuline]. Norreticuline (V) has not yet been isolated but was shown to be an effective precursor of morphine and codeine (1).

The oxidized, fully aromatic benzylisoquinolines are not as common; two such alkaloids, papaverine (VIII) and palaudine (IX), were isolated from opium (2).

PREVIOUS WORK

In 1910, Winterstein and Trier (3) proposed that the benzylisoquinoline system is derived biosynthetically from two units of 3,4-dihydroxyphenylalanine (dopa) which, by decarboxylation and oxidative deamination, were believed to give rise to 3,4-dihydroxyphenethylamine (dopamine) and 3,4-dihydroxyphenylacetaldehyde, respectively. Formation of a Schiff's base and ring closure would give the substance known as norlaudanosoline (tetrahydropapaveroline) (VI). Modern theories (4) consider the reacting units to be



I: $R_1 = R_3 = R_4 = R_5 = CH_3$, $R_2 = H$
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IV: $R_1 = R_4 = R_5 = CH_3$, $R_2 = R_3 = H$
V: $R_1 = R_4 = CH_3$, $R_2 = R_3 = R_5 = H$
VI: $R_1 = R_2 = R_3 = R_4 = R_5 = H$
VII: $R_1 = R_2 = R_3 = R_4 = CH_3$, $R_5 = H$
VIII: $R_1 = R_2 = R_3 = R_4 = CH_3$
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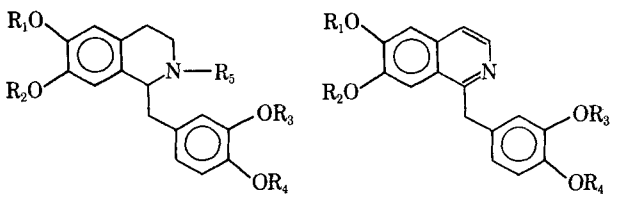

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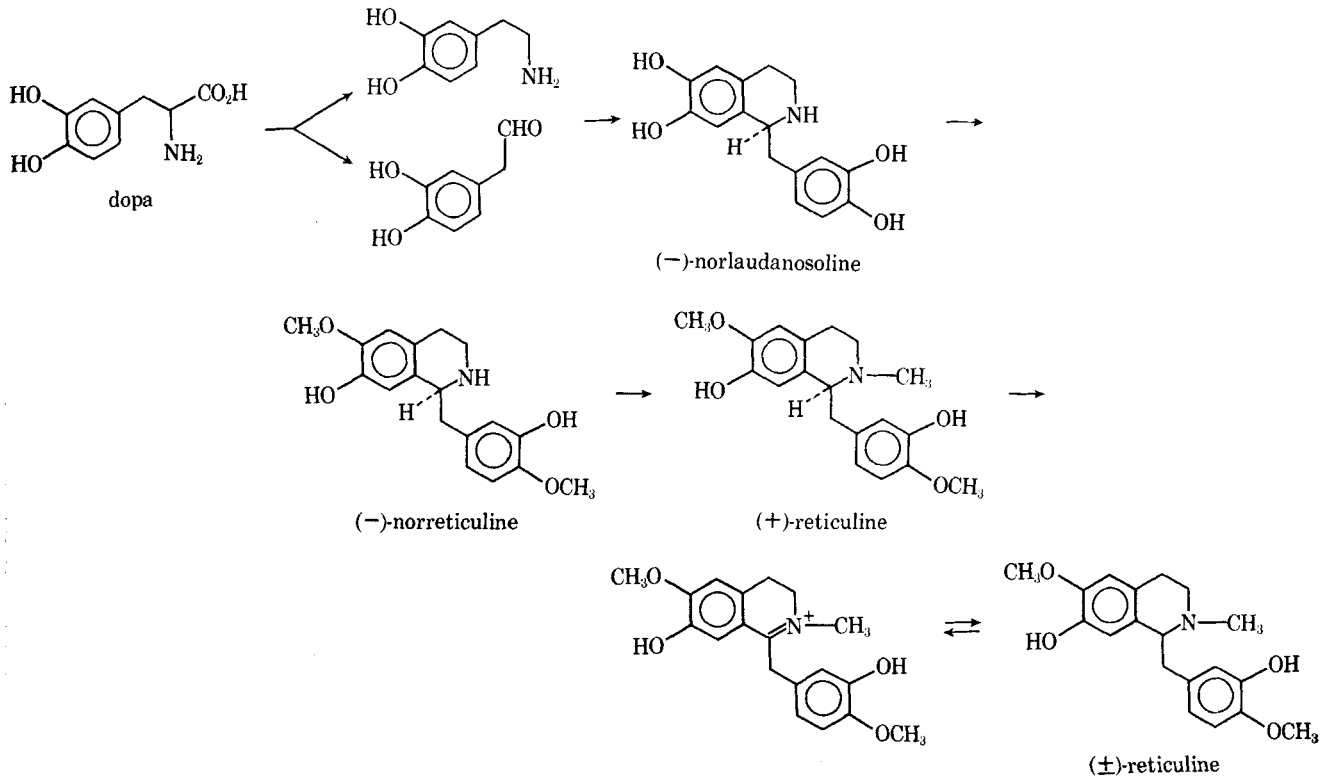


Scheme I—Proposed biosynthesis of (±)-reticuline

dopamine and 3,4-dihydroxyphenylpyruvic acid. There is considerable evidence that norlaudanosoline is the primary substance from which the other benzylisoquinolines are derived by dehydrogenation and/or methylation. Biosynthetic methylations do not usually occur at random but in a definite sequence. Furthermore, they show a great deal of stereoselectivity. Tracer experiments suggested that natural norlaudanosoline in the opium poppy is the levorotatory isomer (5). It undergoes *O*-methylation to (−)-norreticuline, which is further methylated to (+)-reticuline. (+)-Reticuline is capable of undergoing racemization *via* an oxidation–reduction system, which presumably involves 1,2-dehydroreticuline (5) (Scheme I). The levorotatory isomer thus produced is utilized for biosynthesis of the hydrophenanthrene alkaloids. (+)-Reticuline, on the other hand, is required for the biosynthesis of aporphines, protoberberines, protopines, and phthalide-isoquinolines in the opium poppy. (+)-Norlaudanosoline, which on methylation would produce (−)-reticuline directly, cannot serve as a precursor of this important alkaloid (5).

Papaverine—Earlier experiments showed that tyrosine (a precursor of dopa) and norlaudanosoline are effectively incorporated into papaverine (1, 6, 7). However, nothing is known with regard to the sequence of the methylation steps and the dehydrogenation necessary to transform norlaudanosoline to papaverine. There is also a question as to the mechanism of dehydrogenation. It might proceed stepwise or by a concerted process. Battersby *et al.* (8) showed that 1,2-dehydronorreticuline can serve as a precursor of morphine.

Incorporation of this compound into papaverine would give some measure of support to the possibility of a stepwise dehydrogenation. To study these questions, feeding experiments were performed with radioactively labeled norreticuline and 1,2-dehydronorreticuline. Papaverine was isolated and purified to constant radioactivity, and the position of the label was ascertained by controlled degradation.

Laudanosine—In general, biosynthetic reactions are stereospecific and, as a rule, only one of the optical isomers is formed. Reticuline is the only alkaloid in the opium poppy that has been shown to undergo racemization in the plant. This process serves a very important biosynthetic function as a prerequisite for the production of morphine alkaloids. Since laudanidine exists in the plant in both enantiomeric forms, one may surmise that these are derived directly from the respective isomers of reticuline by methylation of the phenolic hydroxyl group in the 7-position. The steric relationship of this hydroxyl group to the basic nitrogen is not affected by the configuration of the asymmetric center at C-1, and both isomers may be expected to fit the active site of the enzyme. Methylation of the hydroxyl function in the 3′-position is more stereoselective. It appears that only the L-structures have the proper configuration to fit the enzyme surface to give (+)-codamine and (+)-laudanosine. It was of interest to determine whether (+)-codamine, (+)-laudanidine, or both can serve as precursors of laudanosine. To this end, radioactively labeled (±)-codamine and (±)-laudanidine (laudanine) were administered to separate batches of *Papaver somniferum*. (±)-Laudanosine was added as a "cold" carrier during the extraction, isolated, purified to constant radioactivity, and subjected to controlled degradation.

RESULTS AND DISCUSSION

The names and amounts of labeled precursors and the results of the feeding experiments are given in Table I. Administration of (±)-norreticuline-(3-^{14}C) (XI) gave good incorporation into papaverine (XIX). Controlled degradation was performed by *N*-methylation, reduction to laudanosine with sodium borohydride, Hoffmann degradation, and ozonolysis, as described by Battersby and Harper (6). The carbon atom in the 3-position was recovered as formaldehyde, which was purified as the dimedone derivative. Since practically all radioactivity (93%) resided in this position, incorporation of norreticuline into papaverine had not involved any randomization of the label. This experiment did not prove that norreticuline was an obligatory precursor of papaverine.

It was conceivable for norreticuline to undergo demethylation to norlaudanosoline in the plant before being dehydrogenated. The experiment was, therefore, repeated with (±)-norreticuline-(6-*O*-$^{14}CH_3$). The Indra variety of *P. somniferum* used in 1969 proved to be free of papaverine, and no radioactivity could be detected in the alkaloid isolated after addition of the "cold" carrier. The Noordster variety is rich in papaverine, and addition of carrier is not necessary. When norreticuline-(6-*O*-$^{14}CH_3$) was administered to these plants, good incorporation of radioactivity into papaverine was obtained. A Zeisel *O*-demethylation recovered all radioactivity as triethylmethylammonium iodide. 1,2-Dehydronorreticuline-(3-^{14}C) was not incorporated into papaverine and is, therefore, not an intermediate in a stepwise dehydrogenation to the aromatic

Table I—Results of Feeding Labeled Precursors to *Papaver somniferum*

Precursor	Amount Administered, μc.	Number of Plants	Year	Variety	Alkaloid Added as Carrier	Alkaloid Isolated	Incorporation, %	Degradation, Percent of Radioactivity Retained
(±)-Reticuline-(3-^{14}C)	45	9	1966	Noordster	None	Papaverine	0.07	—
1,2-Dehydronorreticuline-(3-^{14}C)	11.5	5	1967	Noordster	None	Papaverine	0.00034	—
(±)-Norreticuline-(3-^{14}C)	14.5	3	1967	Noordster	None	Papaverine	1.47	93
(±)-Norreticuline-(6-*O*-$^{14}CH_3$)	49.2	10	1969	Indra	Papaverine	Papaverine	0.0008	—
(±)-Norreticuline-(6-*O*-$^{14}CH_3$)	19.7	7	1970	Noordster	None	Papaverine	4.95	102
(±)-Codamine-(3-^{14}C)	11	4	1967	Noordster	(±)-Laudanosine	(±)-Laudanosine	0.036	—
(±)-Laudanidine-(*N*-$^{14}CH_3$)	44.3	7	1969	Indra	(±)-Laudanosine	(±)-Laudanosine	0.155	99
(±)-Laudanidine-(*N*-$^{14}CH_3$)	43.4	7	1970	Noordster	(±)-Laudanosine	(±)-Laudanosine	2.032	104
(±)-Norlaudanosoline-(3-^{14}C)	11.2	10	1970	Noordster	(±)-Tetrahydropapaverine	(±)-Tetrahydropapaverine	0.868	—
(±)-Norreticuline-(6-*O*-$^{14}CH_3$)	19.7	7	1970	Noordster	Pacodine	Pacodine	0.017	—

benzylisoquinolines, nor is it reduced to norreticuline in the plant. The incorporation of 1,2-dehydronorreticuline into morphine observed by Battersby *et al.* (8) must involve *N*-methylation and reduction to reticuline, in that order.

The slight, but still significant, incorporation of reticuline into papaverine is indicative of *N*-demethylation. Biosynthetic *N*-demethylation of alkaloids does not appear to occur to any great extent in the way that *O*-demethylations do. However, it was reported by Barton *et al.* (9) for a quaternary alkaloid (tembetarine = *N*-methylreticuline). Whether the *N*-demethylation observed in this feeding experiment with reticuline takes place before or after aromatization is still subject to question. By the reverse isotope dilution technique, it was found that (±)-norlaudanosoline-(3-^{14}C) (X) gave good incorporation of radioactivity into tetrahydropapaverine (XIV), and (±)-norreticuline-(6-*O*-$^{14}CH_3$) (XI) conferred a small but measurable amount of radioactivity to papaveroline 6,3′,4′-trimethyl ether (pacodine) (XVIII). The amounts of these alkaloids remaining after purification to constant radioactivity were too small to permit determination of the position of the label, and the experiments will be repeated. Although tetrahydropapaverine might be an intermediate between norreticuline and papaverine, it is more likely that aromatization occurs at the norreticuline stage. The presence of palaudine (XVII) and pacodine (XVIII) lends support to this view. Both alkaloids are present in low concentrations, indicative of a fast turnover, presumably by methylation to papaverine. Work is in progress to detect the hypothetical papaveroline 6,4′-dimethyl ether and to determine its effectiveness as a precursor of papaverine.

(±)-Laudanidine-(*N*-$^{14}CH_3$) (XIII) was incorporated well into laudanosine (XVI). Selective demethylation showed that all radioactivity resided in the *N*-methyl group. (±)-Codamine (XV) gave only a low degree of incorporation. Therefore, (+)-laudanidine may be looked upon as the immediate precursor of laudanosine, while the codamine route represents only a minor pathway. This also tends to explain the presence of an excess of (−)-laudanidine, which appears to be an end-product in the biosynthesis.

Based on the experimental results discussed here, the proposed biosynthetic pathways of the benzylisoquinoline alkaloids are summarized in Scheme II.

EXPERIMENTAL

The methods used for the determination of radioactivity, cultivation of plants, and administration of labeled precursors were described in a previous publication (10).

Synthesis of Labeled Precursors—Several practice runs were always made with nonradioactive materials prior to the synthesis of the labeled compounds. The intermediates were characterized by TLC, GLC, NMR, IR spectroscopy, and melting points. The final products were compared with authentic natural alkaloids when available.

1,2-Dehydronorreticuline-(3-^{14}C) Hydrochloride—1-(3-Benzyloxy-4-methoxybenzyl)-3,4-dihydro-6-methoxy-7-benzyloxy-[3-^{14}C]-isoquinoline hydrochloride (20 mg.), prepared as described earlier (10), was dissolved in 0.5 ml. of ethanol and 0.5 ml. of concentrated hydrochloric acid. The solution was refluxed for 1 hr. and evaporated to dryness. The residue was crystallized from a mixture of ethanol and ether to give 8.2 mg. of 1,2-dehydronorreticuline-(3-^{14}C) hydrochloride, m.p. 208–210°.

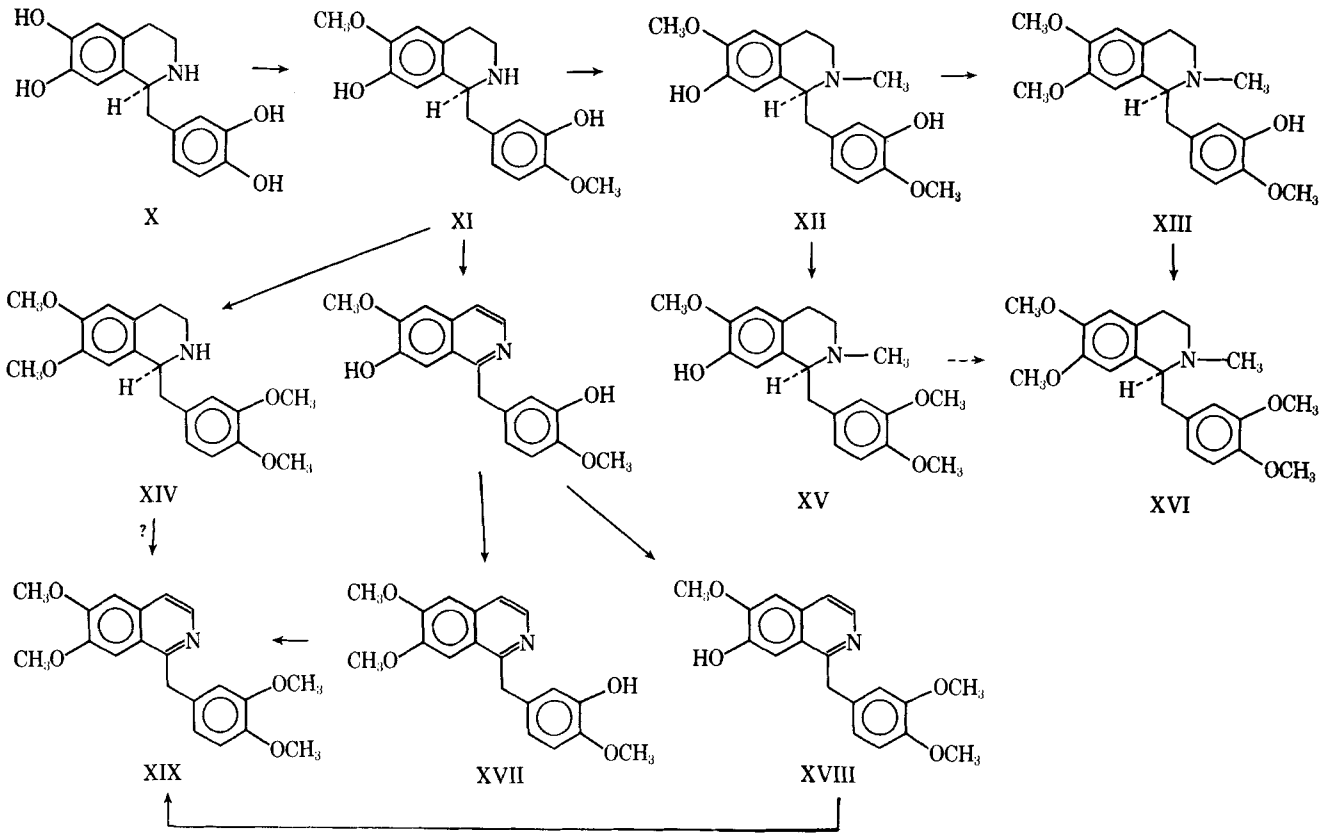
Anal.—Calc. for $C_{18}H_{20}ClNO_4$: C, 61.80; H, 5.72; N, 4.00. Found: C, 61.27; H, 5.61; N, 3.96.

Specific activity = 0.48 mc./mmole.

(±)-Norreticuline-(3-^{14}C) Hydrochloride—1-(3-Benzyloxy-4-methoxybenzyl)-3,4-dihydro-6-methoxy-7-benzyloxy-[3-^{14}C]-isoquinoline hydrochloride (21 mg.) was dissolved in methanol (2 ml.), and 30 mg. of sodium borohydride was added in portions. After about 12 hr. of stirring, the reaction mixture was evaporated to dryness, and 20 ml. of water and 0.5 ml. of 2 *N* sodium hydroxide were added. The solution was extracted with chloroform, and the combined chloroform extracts were evaporated to give a residue of *O,O*-dibenzylnorreticuline-(3-^{14}C). It was dissolved in 0.5 ml. of ethanol and 0.5 ml. of concentrated hydrochloric acid, refluxed for 1 hr., and evaporated to dryness. The residue was crystallized from a mixture of ethanol and ether. The substance was identified by TLC and GLC with (±)-norreticuline prepared previously (10) with nonradioactive materials.

(±)-Norreticuline-(6-O-$^{14}CH_3$) Hydrochloride—3-Hydroxy-4-benzyloxybenzaldehyde was prepared by refluxing 3,4-dihydroxybenzaldehyde (4 g.), benzyl chloride (3.5 ml.), and anhydrous potassium carbonate (15 g.) in 150 ml. of acetone for 20 hr. The reaction mixture was chromatographed on a column of silica gel (Woelm, activity II) with benzene. The fractions containing 3-hydroxy-4-benzyloxybenzaldehyde were concentrated under reduced pressure to yield pale-yellow needles, which were recrystallized from benzene; yield 1.64 g.; m.p. 119–123° [lit. (11) m.p. 122°, from ethanol]. The compound was further identified by methylation with methyl iodide and potassium carbonate in acetone to give 3-methoxy-4-benzyloxybenzaldehyde, m.p. 65°. It showed no melting-point depression when mixed with benzylvanillin but showed a 10–12° depression when mixed with benzylisovanillin.

3-Hydroxy-4-methoxybenzaldehyde (620 mg.) was suspended in 10 ml. of anhydrous methanol; a solution of sodium methoxide, prepared from 60 mg. of metallic sodium and 10 ml. of absolute methanol, was added dropwise with stirring. The resulting solution was evaporated to dryness under reduced pressure, and the residue



Scheme II—Proposed biosynthetic pathways of benzylisoquinolines in Papaver somniferum

was suspended in 8 ml. of dry, redistilled dimethylformamide. Radioactive iodomethane (141 mg., 2 mc.) was distilled into the suspension through a vacuum manifold system. The reaction vessel was sealed, and the mixture was allowed to stand at room temperature for 20 hr. It was then heated in a water bath at about 65° for 24 hr. with occasional shaking. The seal was broken, and an excess of iodomethane (4 ml.) was added. The container was sealed again, heated at 65° for 3 hr., and allowed to stand overnight at room temperature. The reaction mixture was transferred quantitatively to a separator with the aid of 5 ml. of benzene and 70 ml. of 0.1 *N* sodium hydroxide and shaken vigorously. The aqueous layer was extracted with 4 × 5 ml. of benzene, and the combined benzene extracts were evaporated to dryness. The oily residue was crystallized from isopropyl ether to give 363 mg. of 3-methoxy-(^{14}C)-4-benzyloxybenzaldehyde, m.p. 65°. The aldehyde was reacted with nitromethane to give 3-methoxy-(^{14}C)-4-benzyloxynitrostyrene and reduced with lithium aluminum hydride to 3-methoxy-(^{14}C)-4-benzyloxyphenethylamine, and the synthesis was continued as described previously (10). The substance was identical by TLC and GLC with synthetic nonradioactive (±)-norreticuline. Specific activity = 1.02 mc./mmole.

(±)-*Codamine-(3-^{14}C)*—The synthesis of codamine-(3-^{14}C) was similar to that of reticuline-(3-^{14}C) (10), 3,4-dimethoxyphenylacetic acid being used instead of 3-benzyloxy-4-methoxyphenylacetic acid. The synthetic compound was identical with natural codamine isolated from opium (12) by TLC, GLC, and IR spectroscopy. Specific activity = 0.51 mc./mmole.

(±)-*Norlaudanosoline-(3-^{14}C) Hydrochloride*—The synthesis was carried out as described by Battersby *et al.* (1). The product had a melting point of 278–281° [lit. (1) m.p. 278–281°] and a specific activity of 0.36 mc./mmole.

(±)-*Laudanidine-(N-$^{14}CH_3$)*—1-(3-Benzyloxy-4-methoxybenzyl)-3,4-dihydro-6,7-dimethoxyisoquinoline hydrochloride (467 mg.), prepared as described by Deulofeu (13), was suspended in water; nitrogen was bubbled through the suspension, and sodium bicarbonate was added to pH about 8–9. The mixture was extracted with ether (4 × 20 ml.), and the combined ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 5 ml. of benzene and transferred to a methylation vessel, 1 cm. in diameter and 10 cm. long. It was connected to a vacuum manifold system through which radioactive iodomethane-(^{14}C) (141 mg., 2 mc.) was distilled into the benzene solution. The reaction vessel was sealed off and left at room temperature overnight. An oily film appeared on the wall of the tube and turned crystalline. After 4 days, an excess of nonradioactive iodomethane (4 ml.) was added, and the mixture was allowed to stand at room temperature for 24 hr. The crystals were collected and suspended in 30 ml. of absolute methanol, and 1 g. of sodium borohydride was added in small portions and with stirring. The yellow suspension turned colorless. After 12 hr. at room temperature, the solution was acidified with 5 ml. of 2 *N* hydrochloric acid and refluxed for 1 hr. Water (50 ml.) was added, and the solution was extracted with ether. The aqueous layer was basified with ammonium hydroxide to pH 9 and extracted repeatedly with ether. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness, and the residue crystallized from a mixture of ethanol and chloroform.The crystalline compound was identical with natural laudanine isolated from opium by TLC, GLC, and IR spectroscopy. Specific activity = 1.53 mc./mmole.

(±)-*Reticuline-(3-^{14}C)*—The synthesis was described in a previous communication (10).

Alkaloids for Carrier Dilution—(±)-*Laudanosine*—This compound was obtained commercially[1].

(±)-*Tetrahydropapaverine (Norlaudanosine)*—This compound was synthesized as described by Battersby *et al.* (1). The hydroiodide salt melted at 255–256° [lit. (1) 256°].

Papaveroline 6,3',4'-Trimethyl Ether (Pacodine)—The synthesis was carried out as described by Brochmann-Hanssen and Hirai (2).

Extraction and Separation of Alkaloids—The extraction of the plant material and the isolation of the total alkaloids followed the procedure described earlier (10).

Papaverine and Pacodine—The acidic, aqueous solution containing the total alkaloids was extracted with chloroform (4 × 300 ml.), and the combined chloroform extracts were evaporated to dryness.

[1] Mallinckrodt Chemical Works.

The residue was dissolved in a minimum amount of chloroform and chromatographed on a column of silica gel (Woelm, activity I) with a mixture of chloroform and ethanol (98:2). The fractions containing papaverine were combined and evaporated to dryness. The residue was further purified by preparative TLC on silica gel, with benzene–ethanol (4:1) as the developing solvent. The band containing papaverine was scraped off and extracted with warm methanol. Evaporation of the methanol gave a pale-brown residue, which was crystallized from ether to give papaverine, m.p. 146–147°, of constant radioactivity.

After extraction of papaverine, the acidic, aqueous layer was basified with ammonium hydroxide to pH 8–9 and extracted with chloroform. Evaporation of chloroform gave a residue, which was chromatographed on a column of silica gel (Woelm, activity IV) with chloroform. The fractions containing pacodine were combined and evaporated to dryness. The residue was crystallized from methanol to give pacodine of constant radioactivity.

Laudanosine—The acidic, aqueous solution of the total alkaloids was made alkaline with 10% sodium hydroxide to pH 14 and extracted with ether (8 × 100 ml.). The combined ether extracts were washed with water (3 × 10 ml.), dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was chromatographed on a column of neutral aluminum oxide (Woelm, activity IV) with a mixture of chloroform and isopropyl ether (1:1). The fractions containing laudanosine were combined and evaporated to dryness. The residue was crystallized from ether to give laudanosine of constant radioactivity, m.p. 115–116° [lit. (14) m.p. 114–115°].

Tetrahydropapaverine—The acidic, aqueous solution containing the total alkaloids was extracted with chloroform (3 × 200 ml.). The aqueous layer was basified with sodium hydroxide to pH 14 and extracted with a mixture of chloroform and isopropyl alcohol (3:1). The extract was evaporated to dryness; the residue was dissolved in a minimum amount of chloroform, transferred to a column of neutral aluminum oxide (60 g.), and eluted with chloroform–methanol (98:2). The fractions containing tetrahydropapaverine were combined and evaporated to dryness. The residue was subjected to preparative TLC on silica gel with chloroform–methanol (9:1). The band corresponding to tetrahydropapaverine was extracted with methanol. Evaporation of the solvent gave a residue which was dissolved in ether. Addition of hydriodic acid caused precipitation of the hydroiodide, which was recrystallized from water to constant radioactivity, m.p. 255–256° [lit. (1) m.p. 256°].

Degradation of Papaverine—Papaverine (33 mg.), isolated from the plants to which (±)-norreticuline-(3-^{14}C) had been administered, was reacted with iodomethane to give the corresponding methiodide, which was reduced with sodium borohydride in methanol to (±)-laudanosine. This compound was quaternized with iodomethane and subjected to Hoffmann degradation to give a mixture of *cis*- and *trans*-laudanosine methines. Methylation of the mixture with iodomethane gave a yellow residue of *cis*- and *trans*-laudanosine methine methiodide (6). The methiodide was suspended in 5 ml. of water; freshly prepared silver oxide (from 150 mg. of silver nitrate) was added, and the mixture was shaken for 1.5 hr. and filtered. The filtrate was made strongly alkaline with 1 g. of potassium hydroxide, refluxed for 2 hr., cooled, and extracted with chloroform (3 × 20 ml.). The combined chloroform extracts were evaporated to dryness under reduced pressure. The pale-yellow residue (11 mg.) was dissolved in 3 ml. of ethyl acetate, and the solution was cooled in a dry ice–acetone bath. Ozone was passed into it for 20 min., followed by oxygen for 5 min., and the reaction mixture was evaporated to dryness. To the residue were added 8 ml. of water, 90 mg. of zinc dust, and 5 mg. of silver nitrate. The mixture was refluxed for 0.5 hr. and then distilled into a solution of 60 mg. of dimedone in 1.2 ml. of ethanol and 3 ml. of water. When one-half of the volume had distilled over, an equal volume of water was added to the flask and the distillation was continued. Long needles of the formaldehyde–dimedone derivative formed in the receiving flask; they were collected, washed thoroughly with cold distilled water, and crystallized from a mixture of methanol and water to constant radioactivity.

Papaverine, isolated from plants to which (±)-norreticuline-(6-*O*-$^{14}CH_3$) had been administered, was subjected to Zeisel *O*-demethylation as follows. Papaverine (10 mg.), 50 mg. of ammonium iodide, 500 mg. of phenol, 5 drops of chloroauric acid (0.1 g. in 3.5 ml. of water), and 2 ml. of hydriodic acid (freshly distilled from red phosphorus) were placed in the flask of a modified Clark alkoxy apparatus. The mixture was allowed to stand at room temperature for 20 min. in a current of nitrogen and then was heated gradually to 180° in a bath of Wood's metal. The nitrogen gas carrying liberated methyl iodide passed through a trap containing a mixture of 5% sodium thiosulfate and 5% cadmium sulfate (1:1) into a solution of 10% triethylamine in ethanol (5 ml.). The receiving vessel was immersed in a bath of dry ice and acetone. The temperature of the Wood's metal bath was maintained at 180–195° for 1 hr. The triethylamine solution was allowed to stand overnight at room temperature before it was evaporated to dryness. The residue of triethylmethylammonium iodide (28 mg.) was crystallized from ethanol and ether to constant radioactivity.

Degradation of Laudanosine—Laudanosine, isolated from the plants to which (±)-laudanidine-(*N*-$^{14}CH_3$) had been administered, was first *O*-demethylated as described previously. When the reaction was complete—as indicated by formation of the theoretical amount of triethylmethylammonium iodide—the triethylamine solution in the receiving vessel was replaced; the temperature of the Wood's metal bath was raised slowly to 360° where it was maintained for 1 hr. for *N*-demethylation. After 8 hr. at room temperature, the triethylamine solution was evaporated to dryness and the residue was crystallized to constant radioactivity.

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Potential Aldosterone Antagonists: Spiro-[androstane-17-yl-5′-oxazolidine] Derivatives and Their NMR Spectra

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Abstract □ The synthesis of some spiro-[androstane-17-yl-5′-oxazolidine] derivatives and their NMR spectra are described. Aldosterone antagonistic activity of some of the synthesized compounds is reported.

Keyphrases □ Spiro-[androstane-17-yl-5′-oxazolidine] derivatives—synthesis, NMR characterization, potential aldosterone antagonists □ Oxazolidine, steroidal derivatives—synthesis, NMR characterization, potential aldosterone antagonists □ Aldosterone antagonists, potential—spiro-[androstane-17-yl-5′-oxazolidine] derivatives □ NMR spectroscopy—analysis, structure, spiro-[androstane-17-yl-5′-oxazolidine] derivatives

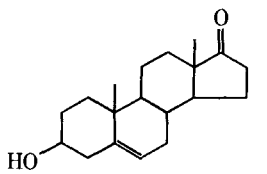
In the search for drugs to counteract the clinical potassium excreting–sodium retaining effects of aldosterone, several investigators (1–4) reported the preparation of C_{17} spiro compounds. So far, however, attention has been focused only upon synthesis of spirolactones or spirolactams. Accordingly, the authors undertook the preparation of compounds having both oxygen and nitrogen heteroatoms in the ring appended at C_{17}. Oxazolidine derivatives of this type were synthesized (5, 6) before but not for examination as potential antialdosterone agents.

The present study examines the effects upon the NMR spectra of a number of steroidal 17-oxazolidines caused by a change in substituents at either the nitrogen atom or the carbon atom situated between the oxygen and nitrogen of the oxazolidine ring.

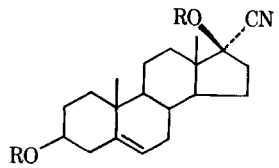
DISCUSSION

The cyanohydrin of the 17-carbonyl group (I) was prepared according to the modified method of Ercoli and De Ruggieri (7), giving mainly the 17α-cyano-17β-ol (II). Monitoring the reaction by TLC failed to detect any trace of the β-cyano isomer. The melting point of the crude cyanohydrin varied from batch to batch, but it could be raised to a constant value by exhaustive washing of the preparation with boiling methanol. The melting-point depression was due to the incomplete conversion of the ketone (I) to its cyanohydrin; this finding is in contrast to the observation reported earlier (7). The configuration assigned to Compound II was based on its conversions to the known (6) diacetate (III) and oxazolidine (IV). The diacetate (III) was prepared earlier by Heusser *et al.* (6), but a different method was used in the present study; *p*-toluenesulfonic acid was substituted for pyridine and refluxing was for 30 min. so that a purer product could be obtained in a shorter time. Again, monitoring of the reaction by TLC detected only a single isomer.

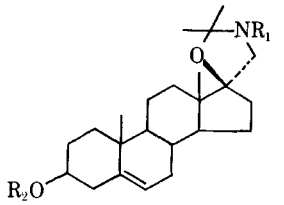
When Compound VI, prepared according to the method of Heusser *et al.* (6), was treated with lithium aluminum hydride, Compound VIII was obtained. A cyclic structure (5), rather than the open structure of VIII*a* proposed by earlier workers (6), was determined previously for this product (5) and now confirmed by examination of its NMR spectrum. The NMR spectrum of Com-



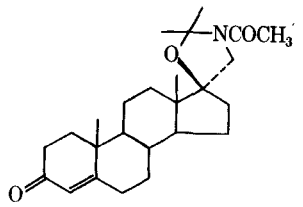
I



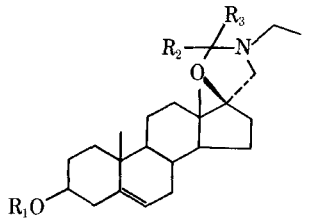
II: R = H
III: R = $COCH_3$



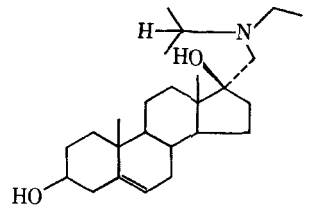
IV: $R_1 = R_2 = H$
V: $R_1 = COCH_3$, $R_2 = H$
VI: $R_1 = R_2 = COCH_3$



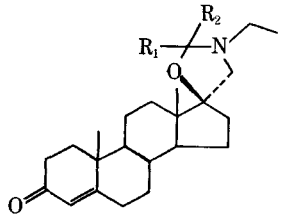
VII



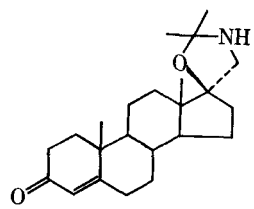
VIII: $R_1 = H$, $R_2 = R_3 = CH_3$
IX: $R_1 = COCH_3$, $R_2 = R_3 = CH_3$
XVII: $R_1 = R_2 = R_3 = H$



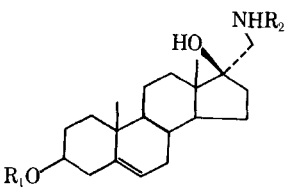
VIII*a*



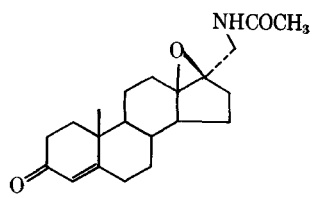
X: $R_1 = R_2 = CH_3$
XVIII: $R_1 = R_2 = H$



XI



XII: $R_1 = R_2 = H$
XIII: $R_1 = H$, $R_2 = COCH_3$
XIV: $R_1 = R_2 = COCH_3$
XV: $R_1 = H$, $R_2 = NHCH_2CH_3$



XV

Table I—NMR Spectral Data: Chemical Shifts (δ, p.p.m.) and Coupling Constants (Hz.) of Protons of the Compounds

Compound	$C_{18}(\delta)$ Calc.	$C_{18}(\delta)$ Obs.	$C_{19}(\delta)$ Calc.	$C_{19}(\delta)$ Obs.	C_3	C_4	C_6	C_4'	N_3'	C_2'
IV	0.734	0.86	1.030	1.000	3.45	—	5.32	2.72 3.27 J = 12 Hz.	—	1.135 1.357
XI	0.770	0.90	1.210	1.170	—	4.67	—	2.73 3.26 J = 12 Hz.	—	1.315 1.350
V	0.734	0.90	1.030	1.010	3.45	—	5.32	3.20 3.68 J = 10 Hz.	2.02	1.555
VI	0.734	0.90	1.040	1.020	4.65	—	5.34	3.21 3.69 J = 10 Hz.	2.02	1.555
VII	0.770	0.930	1.210	1.170	—	4.68	—	3.21 3.67 J = 10 Hz.	2.02	1.55
VIII	0.734	0.850	1.030	1.000	3.45	—	5.32	2.37 3.05 J = 9.0 Hz.	1.06 CH_2 nonequivalent	1.120 1.160
X	0.770	0.880	1.210	1.165	—	5.66	—	2.37 3.05 J = 9.0 Hz.	1.06	1.115 1.165
XIV	0.770	0.880	1.040	1.020	4.55 1.98 2.00	—	5.32	3.20 3.42 J_{AX} = 7 J_{BX} = 4 J_{AB} = 14	1.98 2.00	—
XVI	0.770	0.900	1.030	1.020	3.45	—	5.32	2.42 2.80 J = 12 Hz.	1.08 2.65 J = 7 Hz.	—
XVII	0.734	0.900	1.030	1.000	3.45	—	5.32	2.43 2.93 J = 10 Hz.	1.08 2.49 J = 7 Hz.	4.13 4.20 J = 3 Hz.
XVIII	0.770	0.930	1.210	1.170	—	5.68	—	2.43 2.93 J = 10 Hz.	1.08 2.49 J = 7 Hz.	4.17

pound VIII*a* would be expected to show methyl resonances split by 5–7 Hz. due to the isopropyl group present in the molecule. However, in the NMR spectrum of the present sample, the gem-dimethyl grouping caused two resonances that were magnetic field dependent; the spacing between these signals increased from 2.0 Hz. at 60 MHz. to 3.5 Hz. at 100 MHz. Furthermore, when the NMR sample solution was shaken with D_2O, an area corresponding to only one hydroxyl proton was seen to have undergone exchange; the NMR spectrum of the product obtained after acetylation of VIII showed a single acetyl peak and no hydroxyl proton capable of exchange with D_2O. Compound VIII was also synthesized by the action of acetone on 17α-ethylaminomethyl-5-androsten-3β,17β-diol (XVI) in the presence of a trace of acetic acid. The compounds obtained by these two synthetic procedures were identical according to melting-point determination, TLC, and IR and NMR spectroscopy. Finally, the IR spectrum of the α,β-unsaturated ketone (X), prepared by Oppenauer oxidation of VIII, showed no hydroxyl peak. These data are inconsistent with any alternatives to the proposed structure of VIII.

Oxazolidine (IV) was converted to its diacetate (VI) by the action of acetic anhydride and pyridine; VI was, in turn, saponified to its 3-hydroxy analog (V) with sodium carbonate solution. Oppenauer oxidation of V gave the α,β-unsaturated ketone VII.

Acetylation of Compound XII occurred on nitrogen and at the 3-hydroxyl position to yield XIV; the substitution pattern was established by elemental analysis, NMR spectroscopy [signals at δ 2.00 and 1.98 (3-OAc and NHAc, respectively)], and IR spectroscopy (3-OAc, 1735 cm.$^{-1}$; —NHAc, 1643 cm.$^{-1}$). Earlier work with related compounds showed that 17-OAc absorption in the IR occurs at 1752 cm.$^{-1}$, which was absent in this case. This is predictable since the 17-hydroxyl group is sterically hindered, favoring acetylation at the 3-hydroxyl position and at the amino group in Compound XII.

Selective hydrolysis of XIV yielded hydroxy Compound XIII, which, on Oppenauer oxidation, yielded the α,β-unsaturated ketone XV. Lithium aluminum hydride reduction of XIV yielded XVI, which, by treatment with formaldehyde and a catalytic amount of acetic acid, yielded XVII. Oppenauer oxidation of XVII gave the corresponding α,β-unsaturated ketone (XVIII).

The preparation of Compounds VII and IX was reported previously (6).

NMR DISCUSSION[1]

It is well known (8) that the resonance frequencies of C_{18} and C_{19} methyl group protons in NMR spectra provide a very sensitive probe for observing the nature and orientation of substituent groups in the steroidal skeleton. Also, the concept of additivity (9–13) of chemical shifts for these angular methyl groups due to diamagnetic shielding effects of various functions has been well established. However, the additivity rule does not hold (14, 15) in certain cases where there is a conformational change in a steroid ring or rings or an alteration in relative positions of two functional groups caused by a dipole interaction between them. The compounds examined in this study exhibit substitution at C_3, C_4, C_5, and C_{17}. The diamagnetic shielding effects upon C_{18} and C_{19} methyl protons due to the first three can be readily calculated according to the additivity rule (10). The following discussion will assay the effects of substitution at C_{17}.

C_{18} Methyl Proton—The signal of this angular methyl group occurs in the NMR spectrum of 5-androsten-3β-ol and 4-androsten-3-one at δ 0.734 and 0.770, respectively. In the present compounds, which have a spiro-oxazolidine ring at C_{17}, the chemical shift of the C_{18} methyl groups ranges between δ 0.850 and 0.900 for alcohols and between 0.880 and 0.930 for ketones. Since the change in chemical shifts of 0.03–0.04 p.p.m. between alcohols and ketones is quite consistent with the additivity rule, attention may be limited to the C_{18} methyl resonance of alcohols.

The diamagnetic shielding of the oxazolidine ring upon the C_{18} methyl protons may be studied by comparing the chemical shifts of

[1] The NMR spectral data were obtained by running 5–6% solutions of the compounds on a Varian HR-100 NMR spectrometer.

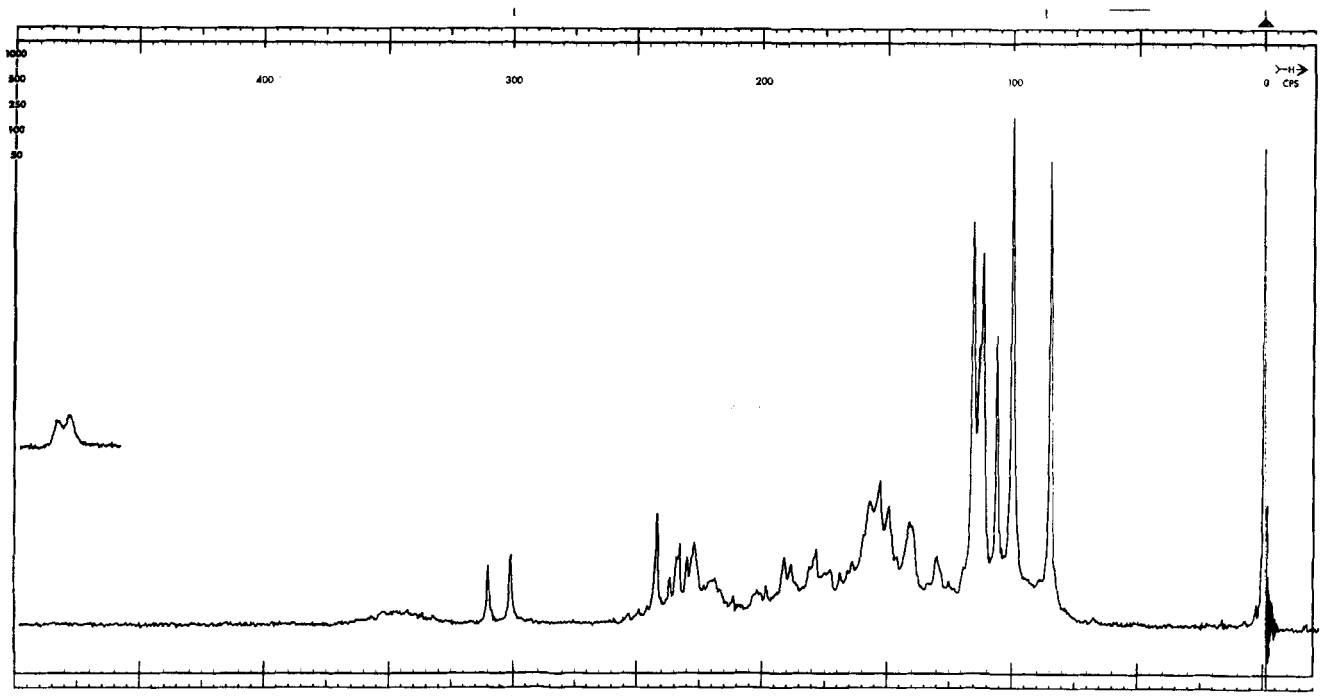


Figure 1—*NMR spectrum of spiro-[5-androsten-3β-ol-17-yl-5'-(N-ethyl-2',2'-dimethyloxazolidine)] (VIII).*

the C_{18} methyl group in Compounds XVI and XVII. In XVI, the C_{18} methyl group would experience effects due to the 3β-hydroxyl, Δ5, 17β-hydroxyl, and 17α-ethylaminomethyl functions. The calculated chemical shift, using the additivity value for the first three groups, would be δ 0.770 p.p.m. for the C_{18} methyl resonance. The observed value for this angular methyl group in XVI is δ 0.900. Thus, the remaining downfield shift of 0.13 p.p.m. is attributable to the 17α-ethylaminomethyl function. It is known that 17β-C_2H_5, 17β-C_8H_{17}, and 17β-C_9H_{19} groups produce *upfield* shifts of 0.140, 0.050, and 0.033 p.p.m., respectively, in C_{18} methyl resonances. Therefore, the downfield shift of 0.13 p.p.m. must be caused by an inductive effect, transmitted through space, of the nitrogen atom present in the side chain. The C_{18} methyl resonance in the spectrum of XVII also occurs at δ 0.90, an observation that is not too surprising, since the orientation of the C_{17} substituents in relation to the C_{18} methyl groups in the two compounds is quite similar. The C_{18} methyl resonance of VIII occurs at δ 0.850, 0.050 p.p.m. upfield compared to the corresponding signal of XVII. This effect may be attributable to introduction of the gem-dimethyl group at C_2'. The substitution of an acetyl group in place of the ethyl function in V produces partial double-bond character of the nitrogen–acetyl bond and its greater electron-withdrawing power, causing the C_{18} methyl signal to appear at δ 0.900. In Compound IV, the C_{18} peak appears at δ 0.860, which means that hydrogen substitution at the nitrogen atom in place of the ethyl group produces very little change in the C_{18} methyl resonance.

C_{19} Methyl Protons—The chemical shifts of this tertiary methyl in the spectrum of 5-androsten-3β-ol and 4-androsten-3-one are δ 1.030 and 1.210, respectively. In the present compounds, having a spiro-oxazolidine ring at C_{17}, the C_{19} methyl resonance occurs near δ 1.00 for alcohols and at δ 1.170 for ketones. Since the change in the chemical shift of the C_{19} methyl group of from 0.170 to 0.180 p.p.m. in passing from alcohols to ketones is also consistent with the additivity rule, only the C_{19} methyl resonances of alcohols will be discussed.

The diamagnetic shielding effect of the oxazolidine ring upon the C_{19} methyl protons may be studied similarly by comparing the chemical shifts of the C_{19} methyl resonances of XVI and XVII. The C_{19} methyl group in XVI may be affected by four functional units: the hydroxyl 3β-, Δ5, 17β-hydroxy, and 17α-ethylaminomethyl groups. Unlike the C_{18} methyl resonance, the C_{19} methyl signal experiences no effect from the 17β-hydroxyl group. Hence, the total predicted value for the C_{19} methyl resonance of XVI is 1.030 p.p.m., the same as in 5-androsten-3β-ol; the observed chemical shift of the C_{19} methyl signal in XVI is δ 1.02. Therefore, the contribution due to the 17α-ethylaminomethyl group is very slight. In XVII, the observed shift is δ 1.00. Therefore, the slight upfield shift of the C_{19} methyl resonance must be due to the entire oxazolidine ring or to the ring nitrogen. This effect is somewhat similar to the upfield shift (0.020 p.p.m.) produced upon the C_{19} methyl resonance by a C_8H_{17} or C_9H_{19} group attached at the 17β-position.

NMR of Protons Associated with Oxazolidine Ring Protons at C_4'—The chemical shifts and coupling constants of these two protons are influenced by the character of the nitrogen atom in the ring and, to a much lesser extent, by the substituent at C_2'. Since the two methylene protons see the nitrogen atom differently (one is closer to the lone pair of electrons on the nitrogen atom than the other), these protons are magnetically as well as chemically nonequivalent. Naturally, the proton closer to the lone pair of electrons is less shielded, and its signal appears at lower field. In IV, these two protons resonate at δ 2.720 and 3.270 (Δδ 0.550), while in VIII the methylene hydrogens appear at δ 2.370 and 3.050 (Δδ 0.680). The upfield shift for the two protons in the latter compound can be explained by the presence of an ethyl group on the nitrogen atom; this alkyl group, in turn, contributes a different degree of shielding to the two methylene hydrogens. The chemical shift of the two methylene protons at C_4' in V is to a much lower field (δ 3.200 and 3.680) than the two earlier compounds. As mentioned in the section on C_{18} methyl resonances, the nitrogen atom in V assumes certain sp^2 character, and the π-electron redistribution therein causes a downfield shift in the methylene signals.

The effect caused by the substituent at C_2' can be observed by comparing the chemical shifts of the two methylene protons in VIII and XVII, where the protons in question resonate at δ 2.370 and 3.05 and 2.43 and 2.93, respectively. This effect is somewhat indirect. The elimination of the gem-dimethyl group at C_2' increases the inversional freedom of the amine nitrogen and, thereby, changes the character of the ethyl group. In VIII, the methylene protons of the ethyl group are nonequivalent because the bulky gem-dimethyl group hinders rapid interconversion of the nitrogen position; in XVII, the gem-dimethyl function is replaced by hydrogen atoms which permit rapid flipping of the amine nitrogen and, consequently, cause equivalence of the methylene protons of the ethyl group (Table I and Figs. 1 and 2).

The coupling constants of the two methylene protons attached to C_4' also vary due to the change in the character of the nitrogen atom (Table I).

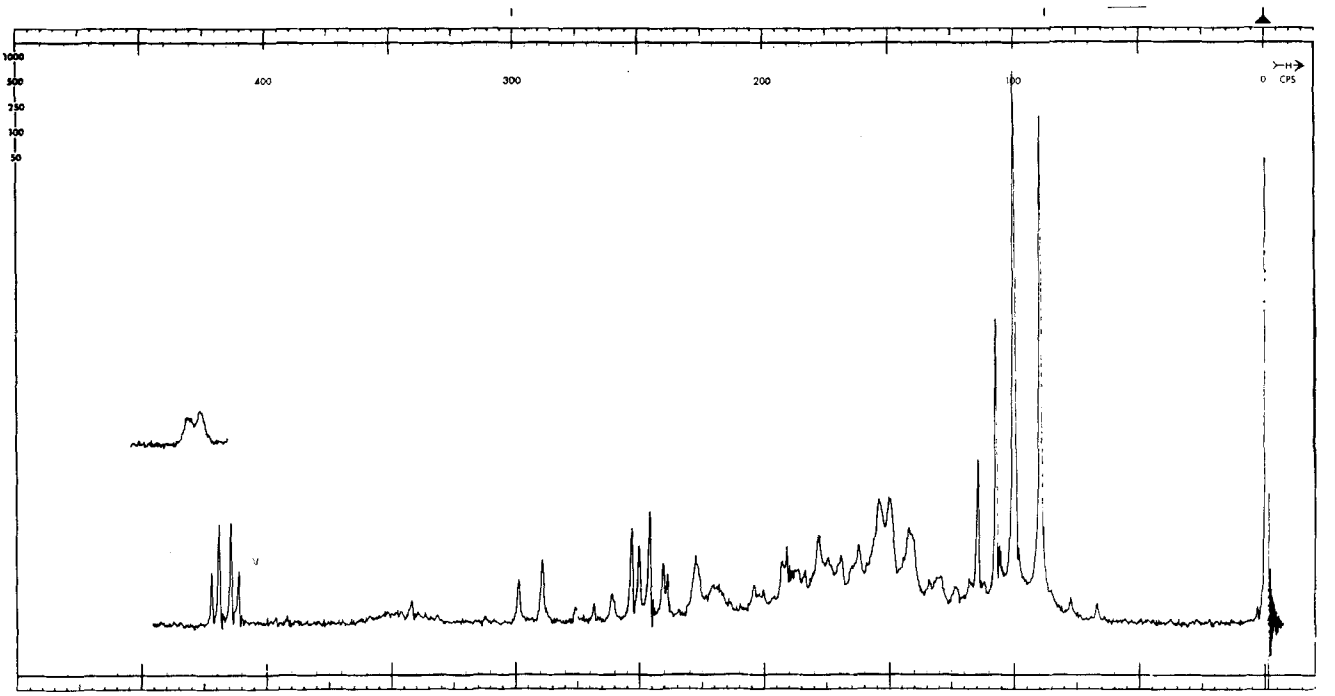


Figure 2—*NMR spectrum of spiro-[5-androsten-3β-ol-17-yl-5′-(N-ethyloxazolidine)] (XVII).*

Protons at N_3′—Aliphatic NH protons usually occur at δ 1–2 and are often not discernible from the other high field signals in the spectrum unless the NMR sample solution is heated or shaken with D_2O. (In the latter case, an area corresponding to the amine hydrogen disappears from the spectrum.) The *N*-acetyl group hydrogens of the compounds reported here appear at the characteristic position, δ 2.00. The nonequivalence of the methylene protons of the ethyl groups on the nitrogen atoms in VIII and XVII was already discussed.

Methyl Protons at C_2′—The methyl protons of the gem-dimethyl group exhibit two signals in IV at δ 1.315 and 1.360 for the same reason as in the case of methylene protons at C_4′. Since these gem-dimethyl protons are one more σ-bond away than the methylene protons at C_4′, the effect of the nitrogen is much less pronounced; consequently, the chemical shift difference between these gem-dimethyl signals is much smaller. In VIII, these methyl resonances appear at higher fields, δ 1.120 and 1.160, which are also consistent with the introduction of an ethyl group on the nitrogen atom. However, in VII the resonance due to gem-dimethyl protons appears as a singlet at δ 1.550. This may be explained by the fact that the nature of the nitrogen atom here is no longer the same as in the two earlier compounds but possesses a certain double-bond character, which makes the N⋯C(—O) bond planar. In this configuration, the *N*-acetyl group bisects the gem-dimethyl groups, thereby providing them with almost equal shielding.

In XVII, the methylene protons at C_2′ appear at δ 4.130 and 4.200 as an *AB* quartet, having a geminal coupling constant of 3 Hz. The magnitude of this coupling constant is quite in harmony with similar values reported in the literature (16). However, these protons appear as a singlet at δ 4.170 in the 3-oxo-Δ4-derivative of V, an observation that would suggest some type of long-range effect caused by the addition of the two substituents to the steroid skeleton.

Compounds VII, X, XI, XIII, and XVII were tested for aldosterone antagonistic activity (Table II) in the adrenalectomized rat. The dose of each compound was 800 mcg./rat. None of the compounds displayed appreciable antagonistic activity.

EXPERIMENTAL

17α-Cyano-5-androstene-3β,17β-diol (II)—To 15.84 g. of 5-androsten-3β-ol-17-one (I) was added 22 ml. of freshly prepared acetone cyanohydrin (17). The mixture was warmed at 50° for 5 min. and then allowed to remain at room temperature for 2 hr. The product was washed with petroleum ether (30–60°) and then with boiling methanol and finally was dried *in vacuo* to yield 16 g., m.p.[2] 206°, $\nu_{max.}^{KBr}$ 2260 cm.$^{-1}$ (17α-C≡N).

Anal.—Calc. for $C_{20}H_{29}NO_2$: C, 76.20; H, 9.26; N, 4.44. Found: C, 76.84; H, 9.43; N, 4.76.

17α-Cyano-5-androstene-3β,17β-diol Diacetate (III)—To 2 g. cyanohydrin (II) was added 68 mg. of *p*-toluenesulfonic acid and 16 ml. of acetic anhydride. The mixture was heated at water bath temperature for 30 min. and then cooled over an ice bath and diluted with cold water. The precipitate was collected by filtration, washed with water, and recrystallized from chloroform–methanol, yielding 1.6 g. as white prisms, m.p. 204.5–205.5° [lit. (6) m.p. 203–206° dec.], $\nu_{max.}^{KBr}$ 1725 cm.$^{-1}$ (3-OAc) and 1752 cm.$^{-1}$ (17-OAc). The NMR spectrum showed two acetate peaks at δ 3.39 and 3.52.

Anal.—Calc. for $C_{24}H_{35}NO_4$: C, 71.79; H, 8.79; N, 3.49. Found: C, 71.90; H, 8.58; N, 3.61.

Spiro-[5-androsten-3β-ol-17-yl-5′-(2′,2′-dimethyloxazolidine)] (IV)—This compound was prepared according to the method of Heusser *et al.* (6), m.p. 199–201° [lit. (6) m.p. 189–190° dec.], $\nu_{max.}^{KBr}$ 1370 cm.$^{-1}$ split peak (gem-dimethyl). The NMR spectrum showed two methyl singlets at δ 1.315 and 1.357 (2′,2′-gem-dimethyl) and a pair of doublets at δ 2.73 and δ 3.26 (J = 12 Hz.; 4′—CH_2—).

Anal.—Calc. for $C_{23}H_{37}NO_2$: C, 76.83; H, 10.36; N, 3.89. Found: C, 76.61; H, 10.41; N, 3.69.

Spiro-[5-androsten-3β-ol-17-yl-5′-(*N*-acetyl-2′,2′-dimethyloxazolidine)] (V)—To a solution of 1 g. of *N*-acetyl oxazolidine acetate (VI) in 30 ml. methanol was added 8 ml. of a 6% solution of sodium carbonate. The mixture was stirred for 2 hr., poured into 100 ml. water, filtered, dried, and crystallized from methanol, yielding 0.7 g. prisms, m.p. 231–233° [lit. (6) m.p. 230–232° dec.], $\nu_{max.}^{KBr}$ 3450 cm.$^{-1}$.

Anal.—Calc. for $C_{25}H_{39}NO_3$: C, 74.77; H, 9.79; N, 3.61. Found: C, 75.00; H, 9.97; N, 3.80.

Spiro-[5-androsten-3β-ol-yl-5′-(*N*-acetyl-2′,2′-dimethyloxazolidine)] Acetate (VI)—One gram of oxazolidine (IV) was dissolved in a mixture of 50 ml. pyridine and 20 ml. acetic anhydride. The mixture was stirred overnight at room temperature and poured into ice-cold water. The precipitate was collected by filtration, dried, and recrystallized from acetone, yielding 0.7 g. of white needles, m.p. 162–163° [lit. (6) m.p. 162–163° dec.], $\nu_{max.}^{KBr}$ 1720 cm.$^{-1}$ (3-OAc) and 1643 cm.$^{-1}$ (—NHAc). The NMR spectrum showed two methyl singlets at δ 4.65 and 2.02 [3—O—C(=O)—CH_3 and 3′—N—C(=O)—CH_3].

[2] When the product was washed with further quantities of methanol, the melting point of the product was raised to 227–228°.

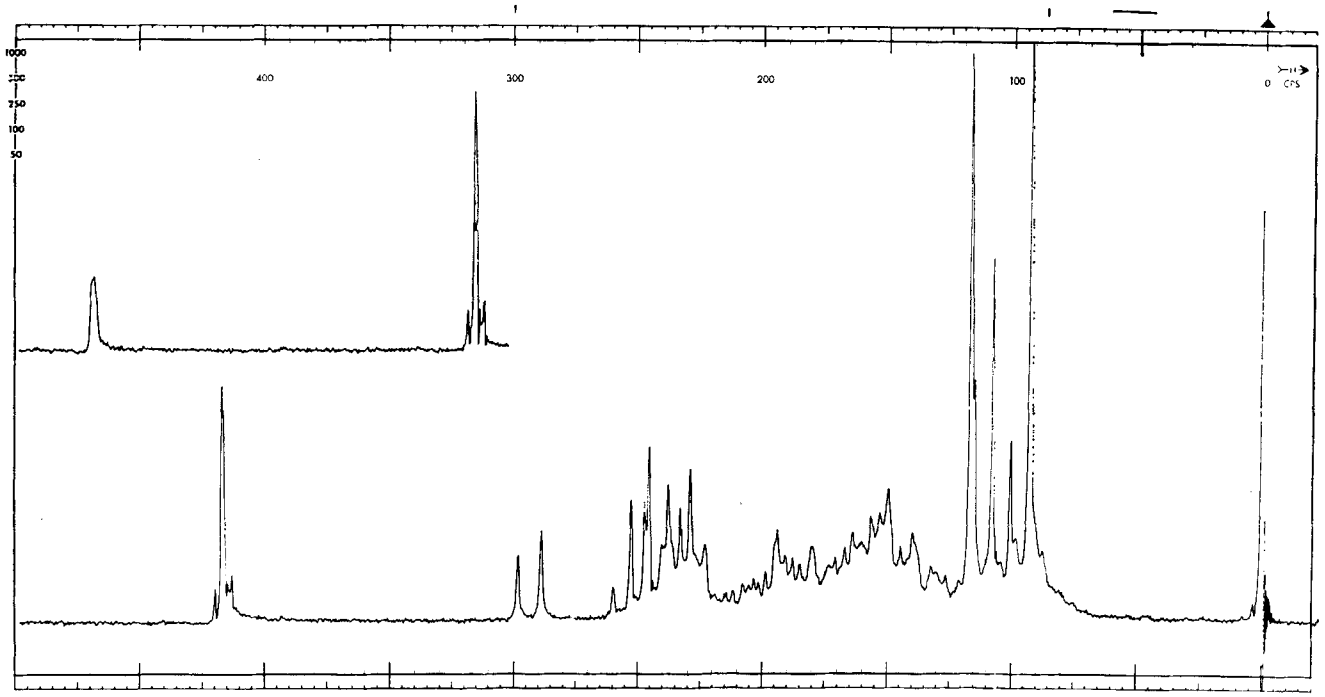


Figure 3—*NMR spectrum of spiro-[4-androsten-3-one-17-yl-2′-(N-ethyloxazolidine)] (XVIII).*

Spiro-[4-androsten-3-one-17-yl-5′-(*N*-acetamido-2′,2′-dimethyloxazolidine)] (VII)—A solution of 500 mg. of *N*-acetyloxazolidine (V) in 5 ml. of cyclohexanone and 20 ml. of toluene was azeotroped under a Dean-Stark head for 1 hr. A solution of 500 mg. of aluminum-*tert*-butoxide in toluene was added, and the mixture was refluxed for 8 hr. The reaction mixture was cooled to room temperature, treated with 3 ml. of water, filtered, and concentrated under reduced pressure and finally under high vacuum. Addition of 10 ml. of *n*-hexane yielded 350 mg. of white needles. Recrystallization from acetone–hexane gave the analytical sample, m.p. 221–223° [lit. (6) m.p. 212–214° dec.], $\nu_{max.}^{KBr}$ 1665 cm.$^{-1}$.

Anal.—Calc. for $C_{25}H_{37}NO_3$: C, 75.15; H, 9.93; N, 3.51. Found: C, 75.16; H, 9.49; N, 3.54.

Spiro-[5-androsten-3β-ol-17-yl-5′-(*N*-ethyl-2′,2′-dimethyloxazolidine)] (VIII)—A solution of 500 mg. of *N*-acetyloxazolidine acetate (VI) in a mixture of 10 ml. benzene and 20 ml. anhydrous ether was added dropwise, with constant stirring, to a suspension of 500 mg. of lithium aluminum hydride in 30 ml. anhydrous ether. After addition was complete, the reaction mixture was stirred for 30 min. and refluxed for an additional 30 min. The slurry was decomposed with ether–water and filtered; evaporation of the solvent under vacuum produced a residue which was mixed with the dried precipitate and extracted with *n*-hexane for 72 hr. Evaporation of the solvent under vacuum and crystallization from acetone yielded 400 mg. of white crystals, m.p. 145–146°. It was also possible to synthesize this compound in the following way: 250 mg. of the amine (XVI) was extracted with 20 ml. acetone containing 0.005 ml. of acetic acid for 72 hr. Filtration and removal of the solvent gave a solid which was recrystallized from acetone, m.p. 143–145°. The NMR spectrum showed two methyl singlet peaks at δ 2.37 and 3.05 (2′,2′-gem-dimethyl) and a triplet δ 1.06 (J = 7 Hz., 4′—N—CH_2).

Anal.—Calc. for $C_{25}H_{41}NO_2$: C, 77.47; H, 10.66; N, 3.61. Found: C, 77.27; H, 10.56; N, 3.61.

Spiro-[4-androsten-3-one-17-yl-5′-(*N*-ethyl-2′,2′-dimethyloxazolidine)] (X)—A 500-mg. sample of *N*-ethyldimethyloxazolidine (VIII) was dried by azeotropic distillation with 25 ml. of benzene under a Dean-Stark head. After distillation of 5 ml. of benzene, a solution of 500 mg. of aluminum-*tert*-butoxide in benzene and 10 ml. of absolute acetone was added. The reaction mixture was refluxed 24 hr., cooled to room temperature, diluted with 3 ml. of distilled water, and filtered. The filtrate was concentrated *in vacuo*, and the residue was chromatographed on grade I neutral alumina[3]. Elution with *n*-hexane gave the crude α,β-unsaturated ketone X which, after crystallization from acetone, yielded 240 mg. of white needles, m.p. 117–119°, $\nu_{max.}^{KBr}$ 1670 cm.$^{-1}$. The NMR spectrum showed a vinyl proton peak at δ 5.66.

Anal.—Calc. for $C_{25}H_{39}NO_2$: C, 77.87; H, 10.19; N, 3.63. Found: C, 77.57; H, 10.63; N, 3.74.

Spiro-[4-androsten-3-one-17-yl-5′-(2′,2′-dimethyloxazolidine)] (XI)—A solution of 0.5 g. of 3β-ol-17-yl-dimethyloxazolidine (IV) in 19 ml. of toluene was refluxed under a Dean-Stark head for 1 hr. A solution of 0.53 mg. aluminum-*tert*-butoxide in 10 ml. of toluene and 6 ml. of absolute acetone was added and refluxed for 37 hr. The reaction mixture was cooled to room temperature, and 1 ml. of distilled water was added. Filtration and removal of the solvent under reduced pressure gave a residue which was triturated with 5 ml. of *n*-hexane, yielding white crystals. The product was chromatographed over grade II neutral alumina[3]. Elution with *n*-hexane–benzene (9:1) gave the desired product, which was recrystallized from acetone, yielding 100 mg. of white needles, m.p. 179–180° [lit. (6) m.p. 179–180° dec.], $\nu_{max.}^{KBr}$ 1670 cm.$^{-1}$, $\lambda_{max.}$ 241 nm. The NMR spectrum showed a vinyl proton peak at δ 5.66.

[3] E. Merck.

Table II—Aldosterone Antagonistic Activity of the Compounds Tested[a]

Compound		Percent Block of a 12-mcg. Deoxycorticosterone Acetate[b] Dose
Aldactone		95
VII	Spiro-[4-androsten-3-one-17-yl-5′-(*N*-acetamido-2′,2′-dimethyloxazolidine)]	24
X	Spiro-[4-androsten-3-one-17-yl-5′-(*N*-ethyl-2′,2′-dimethyloxazolidine)]	22
XI	Spiro-[4-androsten-3-one-17-yl-5′-(2′,2′-dimethyloxazolidine)]	15
XIII	17α-Acetamidomethyl-5-androsten-3β,17β-diol	0
XVII	Spiro-[5-androsten-3β-ol-17-yl-5′-(*N*-ethyloxazolidine)]	13

[a] Results are expressed in terms of the percent block of a 12-mcg. dose of deoxycorticosterone acetate by a dose of 800 mcg./rat. [b] Doca.

Anal.—Calc. for $C_{23}H_{35}NO_2$: C, 77.26; H, 9.87; N, 3.92. Found: C, 77.22; H, 10.08; N, 3.83.

17α-Aminomethyl-5-androstene-3β,17β-diol (XII)—This compound was prepared according to the modified method of Heusser *et al.* (6). Oxazolidine (IV) (100 mg.) was dissolved in 0.5 ml. of acetic acid and diluted with 10 ml. of water. The amine was precipitated out by the addition of 15% sodium hydroxide and collected by filtration. The dried residue was extracted with methanol–acetonitrile (1:1) for 72 hr. to yield 70 mg. of white needles, m.p. 222–223° [lit. (6) m.p. 222–223° dec.].

17α-Acetamidomethyl-5-androstene-3β,17β-diol (XIII)—To a solution of 0.5 g. of XIV in 15 ml. methanol was added 8 ml. of 6% sodium carbonate. The mixture was stirred for 2 hr. and poured in water. The precipitate was collected by filtration, dried, and recrystallized from methanol–acetonitrile to yield 0.3 g. of white platelets, m.p. 240.5–241.5°.

Anal.—Calc. for $C_{22}H_{35}NO_3$: C, 73.09; H, 9.76; N, 3.87. Found: C, 73.29; H, 9.90; N, 3.90.

17α-Acetamidomethyl-5-androstene-3β,17β-diol, 3-Acetate (XIV)—A solution of 0.5 g. of XII in 28 ml. pyridine and 12 ml. acetic anhydride was stirred overnight. The solution was poured into cold water, filtered, and crystallized from methanol, yielding 0.45 g. of crystalline XIV, m.p. 227–228°, $\nu_{max.}^{KBr}$ 1643 cm.$^{-1}$ (—NHAc), 1730 cm.$^{-1}$

Anal.—Calc. for $C_{24}H_{37}NO_4$: C, 71.43; H, 9.24; N, 3.47. Found: C, 71.18; H, 9.11; N, 3.29.

17α-Acetamidomethyl-17β-hydroxy-4-androsten-3-one (XV)—A solution of 500 mg. of XIII in 5 ml. of cyclohexanone and 100 ml. of toluene was refluxed under a Dean-Stark head for 1 hr. A toluene solution containing 500 mg. of aluminum-*tert*-butoxide was added, and reflux was resumed for 12 hr. The reaction mixture was cooled to room temperature, and 3 ml. of water was added; filtration and removal of the solvent under reduced pressure and finally under high vacuum gave a crude α,β-unsaturated ketone which was precipitated by the addition of *n*-hexane and collected by filtration. Chromatography on grade IV neutral alumina[3] and elution with benzene and benzene–chloroform (9:1) gave the desired product. The analytical sample was obtained by crystallization from acetone–*n*-hexane, yielding 100 mg., m.p. 199–201°, $\nu_{max.}^{KBr}$ 1670 cm.$^{-1}$.

Anal.—Calc. for $C_{22}H_{33}NO_3$: C, 73.50; H, 9.25; N, 3.90. Found: C, 73.45; H, 9.17; N, 3.95.

17α-Ethylaminomethyl-5-androstene-3β,17β-diol (XVI)—A solution of 400 mg. of XIV in 40 ml. of tetrahydrofuran was added dropwise, with constant stirring, to a suspension of 500 mg. of lithium aluminum hydride in 40 ml. of tetrahydrofuran. After addition was complete, the reaction mixture was refluxed for 24 hr. and then decomposed with tetrahydrofuran–water and filtered. After most of the tetrahydrofuran was distilled off *in vacuo*, the crude amine was precipitated out by the addition of 6% NaOH solution. The residues were combined and extracted for 72 hr. with petroleum ether (30–60°), yielding 250 mg. of prisms. Recrystallization from petroleum ether afforded the analytical sample, m.p. 128–129°. The NMR spectrum showed a 3-proton triplet signal at δ 1.08 (—$NHCH_2$—CH_3).

Anal.—Calc. for $C_{22}H_{37}NO_2$: C, 76.03; H, 10.73; N, 4.03. Found: C, 76.01; H, 10.79; N, 3.89.

Spiro-[5-androsten-3β-ol-17-yl-5′-(*N*-ethyloxazolidine)] (XVII)—To a solution of 200 mg. of *N*-ethylamine (XVI) in 10 ml. methanol was added 0.047 ml. of 37% formaldehyde solution and 0.01 ml. of glacial acetic acid. The reaction mixture was stirred at room temperature for 15 min. and then refluxed for 30 min. The mixture was cooled on an ice bath and treated with 6 ml. of distilled water and 6 ml. of 6% sodium hydroxide solution. The precipitated ethyloxazolidine was collected by filtration, dried, and recrystallized from methanol–acetonitrile (1:4), yielding 150 mg. of white needles, m.p. 155–157°. The NMR spectrum showed a pair of doublets at δ 4.13; 4.20 (J = 3Hz.; 2′-CH_2).

Anal.—Calc. for $C_{23}H_{37}NO_2$: C, 76.83; H, 10.37; N, 3.90. Found: C, 76.64; H, 10.50; N, 4.10.

Spiro-[4-androsten-3-one-17-yl-2′-(*N*-ethyloxazolidine)] (XVIII)—A solution of 500 mg. of *N*-ethyloxazolidine (XVII) in 25 ml. of benzene was refluxed under a Dean-Stark head. After 5 ml. of benzene was distilled out, a solution of 500 mg. of aluminum-*tert*-butoxide in benzene and 6 ml. of absolute acetone was added. The reaction mixture was refluxed for 24 hr., cooled to room temperature, diluted with 3 ml. of distilled water, and filtered. Concentration of the filtrate *in vacuo* and chromatography of the residue on grade I neutral alumina[3], using *n*-hexane as the eluant, gave the α,β-unsaturated ketone. Crystallization from *n*-hexane yielded 250 mg. of white needles, m.p. 120–121°, $\nu_{max.}^{KBr}$ 1670 cm.$^{-1}$. The NMR spectrum showed a vinyl proton peak at δ 5.68 (Fig. 3).

Anal.—Calc. for $C_{23}H_{35}NO_2$: C, 77.27; H, 9.87; N, 3.92. Found: C, 77.08; H, 9.90; N, 3.91.

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Influence of Formulating Factors on Drug Safety of Timed-Release Nitroglycerin Tablets

W. A. RITSCHEL

Abstract □ A peroral sandwich model tablet with timed release, containing proxyphylline and nitroglycerin, was developed. One layer of the tablet constituted the rapidly disintegrating and dissolving initial phase; the other, the depot phase, was in the form of an insoluble, indigestible skeleton. Amount of drugs in the tablet and their liberation rate constants were based on pharmacokinetic calculations. Upon chewing and masticating a depot tablet, the retard action usually is lost due to an increase of the surface. This problem would not be serious with proxyphylline, which is relatively nontoxic, but could be dangerous or fatal in the case of nitroglycerin. To obtain safety with this product, proxyphylline of the depot phase was incorporated into the pores of an indigestible plastic skeleton of the matrix, and nitroglycerin was dissolved in the plastic particles of the matrix. *In vitro* and *in vivo* experiments proved the loss of depot action for both drugs upon mastication of the tablet, yet the nitroglycerin release was slow enough not to cause unwanted side effects.

Keyphrases □ Nitroglycerin—with proxyphylline, formulating timed-release tablets, absorption □ Timed-release tablets—nitroglycerin and proxyphylline □ Bioavailability—timed-release nitroglycerin tablets □ Drug safety—skeleton matrix depot tablets, nitroglycerin □ Depot tablets—*in vitro* drug availability

In a previous study (1, 2), a proxyphylline peroral dosage form, a two-layer tablet with timed release, was developed. Based on pharmacokinetic calculations (Table I), the biopharmaceutical development of the dosage form was done and verified *in vivo* in man using blood level curves (3, 4).

The purpose of the present research work was to incorporate the short-acting nitroglycerin into this existing preparation. The peroral absorption of nitroglycerin has been and is still questioned. Salter (5) stated that nitroglycerin is not decomposed by the gastric juice, but its rate of absorption is slow. Sollman (6) mentioned that nitroglycerin is more potent when orally (buccally) administered instead of perorally, since by the latter route nitroglycerin is absorbed into the portal circulation and then destroyed by the liver. That nitroglycerin is also perorally absorbed—at least in the rabbit—was shown by Turner (7), Lorenzetti *et al.* (8), and Bogaert *et al.* (9). A study of the bioavailability of nitroglycerin upon peroral administration *versus* buccal administration was, therefore, included in this research.

Table I—Pharmacokinetics of Proxyphylline in a Timed-Release Tablet into Which Nitroglycerin Was to Be Incorporated

Biological half-life	$T_{1/2} = 4.3$ (hr.)
Absorption rate constant	$K_A = 1.3$ (hr.$^{-1}$)
Elimination rate constant	$K_{EL} = 0.163$ (hr.$^{-1}$)
Time to reach peak	$T_P = 2.5$ (hr.)
Therapeutic concentration to maintain for 12 hr.	$B_D = 0.8$ (mg./100 ml.)
Single dose producing desired blood level	$D_B = 0.48$ (g.)
Liberation constant from depot phase	$k_R^1 = 0.4$ (hr.$^{-1}$)
Equation for plasma concentration	$C = 11.5 \cdot e^{-0.163T} - 12.5 \cdot e^{-1.3T}$
Percent absorbed relative to the amount ultimately absorbed	$\frac{A_T}{A}, 100 = CT + K_{EL}TC_{DT}$ Percent after 0.25 hr. = 35.6 0.5 hr. = 45.5 1.0 hr. = 74.4 1.5 hr. = 100.0

Maintenance dose:

$$D_M = \frac{K_{EL} \cdot B_D}{k_R^1} = 0.312 \text{ (g.)}$$

Initial dose:

$$D_I = D_B - D_M \cdot (k_R^1 \cdot T_P) = 0.177 \text{ (g.)}$$

Total dose per tablet:

$$W = D_B - D_M \cdot (k_R^1 \cdot T_P) + \frac{K_{EL} \cdot B_D}{k_R^1} = 0.489 \text{ (g.)}$$

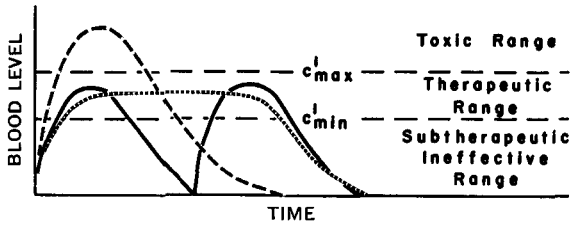

Figure 1—*Schematic blood level curves upon peroral administration. Key: ——, normal single dose; - - -, double single dose; and . . ., dose with timed release.*

In the development of a drug product, one must also consider the safety of the drug. If a drug has a short biological half-life, a dosage form with a matrix that slowly releases the drug is logical. But higher doses have to be given than are usually given in a single dose. If the high dose would be given instantaneously, it could result in toxic blood levels (Fig. 1).

Upon chewing or masticating the previously developed proxyphylline timed-release tablets, the depot effect was lost due to an increase in surface area. This problem is not serious with proxyphylline, which is relatively nontoxic, but it could be dangerous or even fatal with a high dose of nitroglycerin.

EXPERIMENTAL

Materials—Proxyphylline[1] (β-hydroxypropyltheophylline) and nitroglycerin solution[2], 0.1% ethanol, were used as supplied. Ethylcellulose powder[3] of different viscosity grades, 10, 20, and 45 cps., was sieve screened for different particle-size ranges with mean particle sizes of 0.5 and 1.0 mm., respectively. Polyvinyl acetate[4] and a polyamide[5] were screened for different particle-size ranges, and a polyacrylate[6] was sieve screened as mentioned previously. Organic solvents for granulation were of laboratory grade.

[1] Siegfried AG., Zofingen, Switzerland.
[2] Sprengstoff AG., Dottikon, Switzerland.
[3] Ethocel, Dow Chemical Co., Midland, Mich.
[4] Hoechst AG., Frankfurt/Main, West Germany.
[5] Ultramid, BASF, Ludwigshafen, West Germany.
[6] Eudragit Retard, Röhm and Haas, Darmstadt, West Germany.

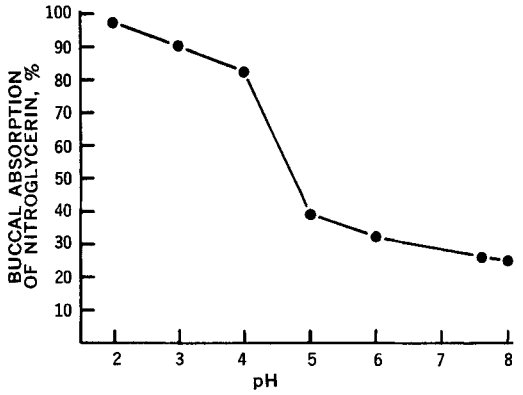

Figure 2—*Buccal absorption of nitroglycerin.*

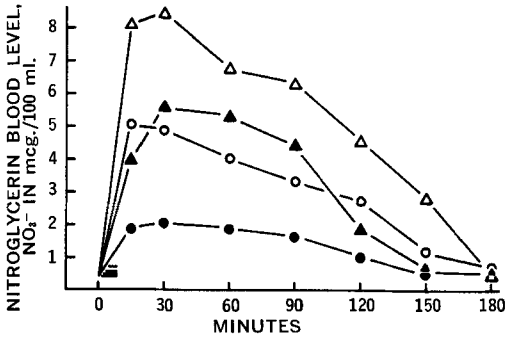

Figure 3—*Numeric plot of nitroglycerin blood levels. Key: △, 1.6 mg. buccally; ▲, 1.6 mg. perorally; ○, 0.8 mg. buccally; and ●, 0.8 mg. perorally.*

Methods—The buccal absorption test was carried out as described by Beckett and Triggs (10), with a slight modification. A buffer solution of the same pH as the test solution was used for rinsing the oral cavity prior to the test and immediately after use of the test solution. The rinsing solution after the test was combined with the expelled test solution.

The spectrophotometric determination of proxyphylline and nitroglycerin (as NO_3^-) in *in vitro* testing and in biological material was described elsewhere (11).

In the present study, the nitroglycerin was first incorporated with the proxyphylline in a matrix tablet by mixing proxyphylline powder of a size $<125\ \mu$ with an alcoholic solution of nitroglycerin. Upon the evaporation of the alcohol, the drug was again passed through a screen of $125\ \mu$. The drug mixture was then mixed with plastic particles between 0.5 and 1 mm. in size, using either polyvinyl chloride or ethylcellulose or the polyamide[5] or polyvinyl acetate, or the polyacrylate[6]. The drug–plastic mixture was then compressed into biconvex tablets of 11-mm. diameter. In *in vitro* evaluation of both proxyphylline and nitroglycerin, a timed release was found from all preparations testing the intact tablets in artificial GI fluid. When the tablets were mechanically divided into two or four parts or powdered, both drugs were released at much faster rates with the increased surface area. As mentioned previously, this problem would not be dangerous with proxyphylline but it would be with nitroglycerin.

To circumvent the fast release of nitroglycerin from accidently masticated or chewed tablets, a technological procedure was sought to act as a safety valve. The following method has been successful. Nitroglycerin, dissolved in an organic solvent [alcohol–acetone (1:1)], was used for impregnation of plastic granules[2] which must be at least partly soluble in the organic solvent employed. After evaporation of the solvent, the plastic–nitroglycerin mass was screened out to particle sizes of 0.5–1 mm. These nitroglycerin–plastic particles were then mixed with proxyphylline and compressed into tablets. For the final preparation, a two-layer tablet was chosen. The first layer, being the initial phase, contained 0.2 mg. of nitroglycerin and 180 mg. of proxyphylline. The drugs were liberated from this initial layer within 5 min. in *in vitro* testing. The depot phase constituted a matrix in which the nitroglycerin (5 mg.) was dissolved in the form of a solid-solid solution in the plastic material[3] and contained 310 mg. proxyphylline (mean particle size $d' = 125\ \mu$) in the pores of the matrix. *In vitro* drug liberation from the timed-release dosage form was determined, using both the half-change method (12) and the modified half-change method (13), adding enzymes (pepsin and pancreatin) in powder form at hourly intervals to the artificial GI fluids.

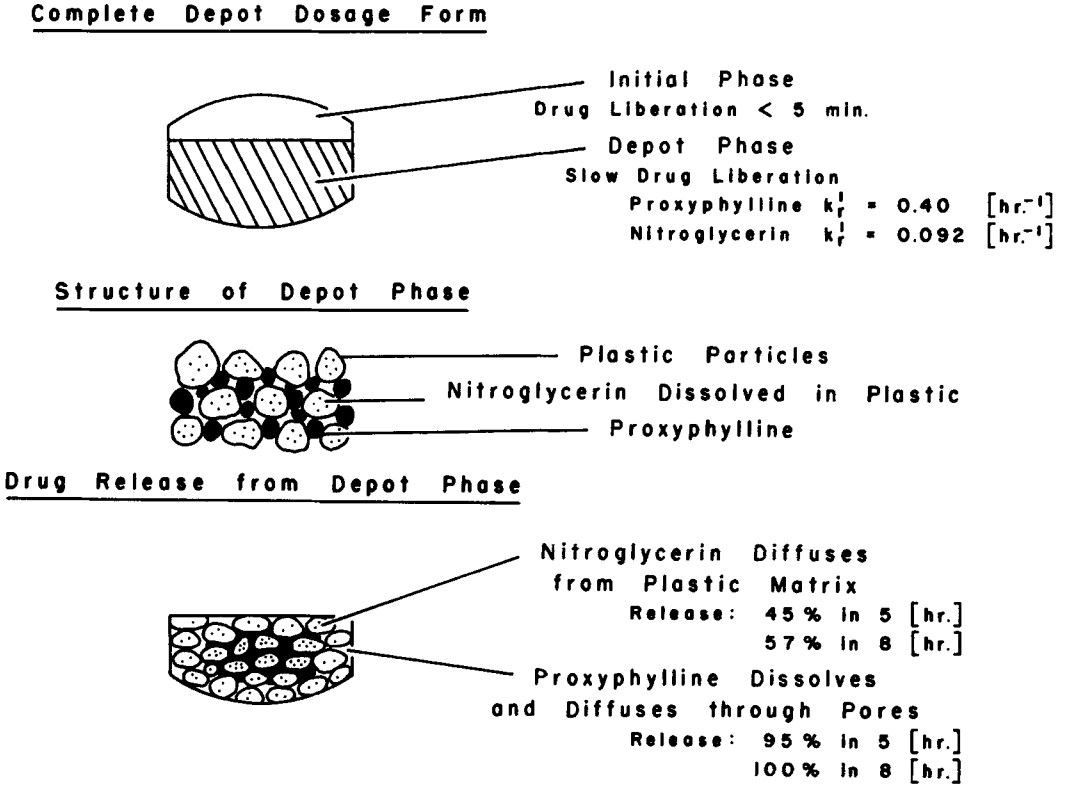

Figure 4—*Diagrammatic structure of the peroral sandwich tablet with timed release and its release characteristics.*

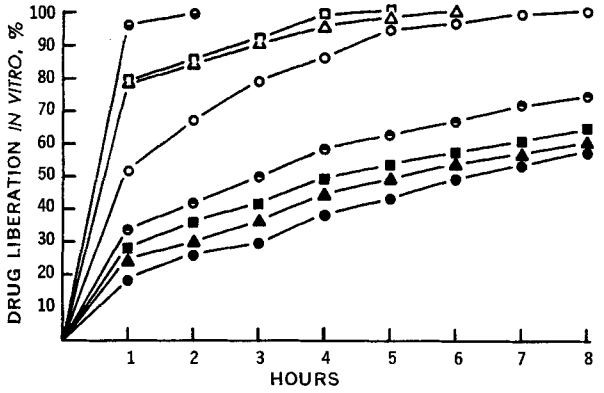

Figure 5—In vitro *drug liberation. Key: Proxyphylline:* ○, *intact tablet;* △, *cut into two parts;* □, *cut into four parts; and* ◒, *powdered. Nitroglycerin:* ●, *intact tablet;* ▲, *cut in.to two parts;* ■, *cut into four parts; and* ◓, *powdered.*

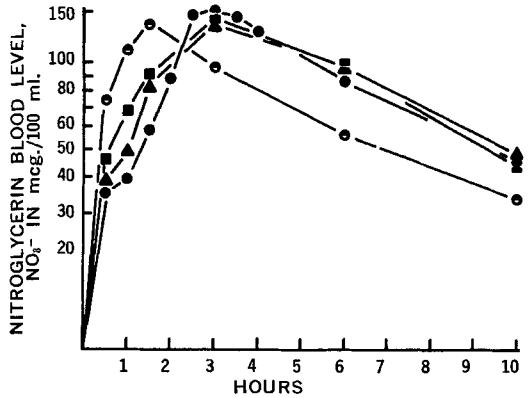

Figure 6—*Nitroglycerin blood level after peroral administration. Key:* ●, *intact tablet;* ▲, *cut into two parts,* ■, *cut into four parts; and* ◓, *powdered and masticated.*

RESULTS AND DISCUSSION

Nitroglycerin Peroral Bioavailability—By using the buccal absorption test, it was found that absorption of nitroglycerin decreased with increasing pH (Fig. 2). From this observation, absorption of nitroglycerin could be anticipated from the acidic stomach.

To study the bioavailability upon peroral administration, nitroglycerin capsules[7] of 0.8 and 1.6 mg. were administered buccally and perorally to three volunteers. The blood levels were determined over 3 hr. and plotted on numeric graph paper under consideration of the blank values (Fig. 3). The areas under the blood level curves (AUC) were determined, and the bioavailability was calculated in percentage according to Eq. 1:

$$\% \text{ bioavailability} = \frac{\text{AUC perorally}}{\text{AUC buccally}} \cdot 100 \qquad \text{(Eq. 1)}$$

For the smaller and higher doses, 35.99 and 54.52%, respectively, were obtained. From this experiment, it was obvious that nitroglycerin is perorally absorbed, although the amount absorbed is less than buccally.

The use of three volunteers allows only a limited evaluation. But if the bioavailability upon peroral administration is only 50%, it is highly questionable whether a potent and toxic drug such as nitroglycerin should be used perorally. Erratic absorption efficiencies could minimize the range between safe and toxic. Furthermore, there seems to be no correlation between hypotensive action of certain aliphatic nitric acid esters and the amount of nitrate and nitrite ions in plasma (14). Onset and action in small doses of organic nitrates is observed to be as rapid as inorganic nitrate, and the clinical effects do not correlate with an elevation of blood nitrate (15). The study described here is, therefore, primarily of academic interest and has to be considered as exemplary of the type of studies that should be conducted for formulating timed-release medications.

***In Vitro* Release Studies**—*In vitro*, the proxyphylline was released from the depot matrix by the apparent first-order rate of $k_r^1 = 0.40\ [h^{-1}]$, and nitroglycerin was released by apparent first-order kinetics of $k_r^1 = 0.092\ [h^{-1}]$. The proxyphylline, incorporated into the pores of the matrix, dissolves as soon as the artificial stomach fluid enters the pores; 95% is released within 5 hr. Nitroglycerin, dissolved in the plastic matrix, must diffuse through the plastic material into the artificial stomach fluid; 45% is released within 5 hr. (Fig. 4).

As was found in a previous clinical study of proxyphylline tablets with timed release (3, 4), the depot effect was lost on mastication. Therefore, drug release was tested *in vitro*. As shown in Fig. 5, the nitroglycerin timed-release pattern was still maintained but the timed release of proxyphylline was certainly lost when tablets were subdivided. Thus, the nitroglycerin should have prolonged activity. This hypothesis was finally verified by *in vivo* experimentation. Figure 6 represents the mean curves from three subjects.

Incorporation of nitroglycerin into plastic granules in the form of a solid-solid solution results in retarded drug release, whereby the main liberation rate-limiting step apparently is the diffusion of nitroglycerin from the plastic granules. The actual surface area is certainly of great influence too but to a lesser extent. *In vivo*, it was found that the actual peak height does not increase upon mastication of the nitroglycerin depot dosage form, but the timed release is markedly reduced. Yet the release is slow enough not to cause unwanted side effects.

The example of nitroglycerin–proxyphylline tablets with timed-release shows that it is possible to incorporate two different drugs with different biological half-lives into one dosage form. Emphasis is given to biopharmaceutical evaluation for drug safety.

[7] Nitrolingual, 0.0008 g., Pohl-Boskamp, Hohenlockstedt, West Germany.

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Comparative Pharmacokinetics of Chlorphenesin Carbamate and Methocarbamol in Man

ARLINGTON A. FORIST and RAY W. JUDY

Abstract □ Serum drug levels following oral administration of large single doses of chlorphenesin carbamate and methocarbamol were fitted to a one-compartment open model. The appearance of each drug was described by a short lag time followed by a rapid first-order absorption and attainment of maximum levels. No statistically significant differences were found between estimates of mean lag times (t_0, 0.5 and 0.2 hr.), half-lives for absorption ($t_{1/2}$, 0.4 and 0.6 hr), and times for attainment of maximum serum concentrations (t_m, 1.9 and 1.4 hr.) for chlorphenesin carbamate and methocarbamol, respectively. Chlorphenesin carbamate was distributed throughout a significantly larger relative volume (1.27 *versus* 0.48 l./kg.) and yielded a correspondingly smaller maximum serum concentration (15.3 *versus* 29.8 mcg./ml., 2-g. dose). The mean biological half-life for chlorphenesin carbamate, 3.14 hr., was significantly greater than the value of 1.20 hr. obtained for methocarbamol.

Keyphrases □ Chlorphenesin carbamate—pharmacokinetic parameters, compared to methocarbamol, man □ Methocarbamol—pharmacokinetic parameters, compared to chlorphenesin carbamate, man □ Pharmacokinetic parameters—comparative, chlorphenesin carbamate and methocarbamol, man □ Plasma levels—comparative, chlorphenesin carbamate and methocarbamol, man

Chlorphenesin carbamate [3-(*p*-chlorophenoxy)-1,2-propanediol-1-carbamate][1] is a well-tolerated, orally active agent effective in the treatment of skeletal muscle trauma and inflammation (1–5). The pharmacology (6), metabolism (7–10), and analytical characterization (11–13) of this drug were reported. As a centrally active muscle relaxant, chlorphenesin carbamate produced a longer duration of effect than mephenesin carbamate [3-(*o*-tolyloxy)-1,2-propanediol-1-carbamate] in rats and mice (6).

Clinical estimation of duration of effect is difficult. Determination of the persistence of drug in the circulation, the resultant of rates of absorption, distribution, and elimination, provides an alternative approach assuming a correlation between biological response and circulating drug concentrations. Such a correlation was shown for structurally similar compounds in the cat (14). Accordingly, the present study was conducted to characterize the pharmacokinetics of chlorphenesin carbamate in man. Earlier investigations in man with mephenesin carbamate (15) and methocarbamol [3-(*o*-methoxyphenoxy)-1,2-propanediol-1-carbamate] (16) indicated higher and more prolonged plasma levels for the latter. However, no determination of pharmacokinetic parameters was possible from the data presented. Therefore, to permit a direct comparison with chlorphenesin carbamate, methocarbamol was included in this study.

[1] Maolate is the registered trademark of The Upjohn Co. for chlorphenesin carbamate.

Table I—Design of Comparative Study

Week	Subject: Chlorphenesin Carbamate	Subject: Methocarbamol
1	Fe, Sz	Mo, Pr
2	Ma, Mi	Br, Jo
3	Mo, Pr	Fe, Sz
4	Br, Jo	Ma, Mi

EXPERIMENTAL

Pilot Study (I)—To test the analytical method and provide preliminary data on pharmacokinetic parameters, a pilot study was conducted with chlorphenesin carbamate in two normal adult male volunteers (Re, 70.5 kg., and Mi, 90.9 kg.). Each was fasted from 6:00 p.m. of the previous day until 11:00 p.m. of the test day; a regular diet was permitted thereafter. At 8:00 a.m. of the test day, Subject Re received 3 g. and Subject Mi received 2 g. of chlorphenesin carbamate orally as 500-mg. compressed tablets. Water was consumed at the rate of 8 oz. every 2 hr. for the first 12 hr. and *ad libitum* thereafter. Serum samples were collected at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 24 hr. and frozen until analyzed.

Comparative Study (II)—Eight normal adult male volunteers, weighing 52.3–95.5 kg. (mean 76.5 kg.), received 2-g. oral doses of chlorphenesin carbamate and of methocarbamol as 400-mg. compressed tablets according to the schedule shown in Table I. Subjects were treated and samples were collected as in the pilot study.

Serum Analyses—Drug concentrations were determined by the following modification of the procedure of Morgan *et al.* (17). Two milliliters each of serum and 0.2 *N* sodium hydroxide were mixed in a 32-ml. glass-stoppered centrifuge tube, held 30 min. in a boiling water bath, rapidly cooled to room temperature, and vigorously shaken with 25 ml. of chloroform. Phases were separated by centrifugation for 5 min. at 2000 r.p.m., the aqueous phase was discarded, and 20 ml. of the chloroform extract was transferred to a 50-ml. round-bottom flask and carefully evaporated to dryness at atmospheric pressure in a stream of N_2. Care must be taken at this point to avoid sublimation of chlorphenesin or guaiacol glyceryl ether. One milliliter of 0.005 *M* periodic acid in 1 *M* sulfuric acid was added to the residue, oxidation was allowed to proceed for 1 hr. at room temperature, and 0.5 ml. of 0.1 *M* sodium arsenite was added. After at least 15 min., 4 ml. of chromotropic acid reagent (1.25 g. in 20 ml. of water carefully diluted to 200 ml. with 12.5 *M* sulfuric acid) was added. After thorough mixing, the solution was transferred to a glass-stoppered tube and held in a boiling water bath for 30 min.; it was then rapidly cooled.

Absorbance was determined at 570 nm. *versus* a reagent blank. Results for each subject were corrected for the pretreatment sample (serum blank) for the same subject. Calculations were based on standard curves obtained by the analysis of serum samples containing known amounts of added chlorphenesin carbamate or methocarbamol. A coefficient of variation of 5% or less was observed in the analysis of standards. Mean serum blanks were equivalent to approximately 3.5 and 2.0 mcg./ml. of chlorphenesin carbamate and methocarbamol, respectively.

Treatment of Data—Serum drug levels for each subject and each drug were fitted to a one-compartment open model, using the nonlinear least-squares program of Metzler (18) written for an IBM 360/30 computer. The pharmacokinetic parameters generated for chlorphenesin carbamate and methocarbamol were compared by the two-sided sign test (19). Subject Mo failed to exhibit measurable

Table II—Serum Drug Levels in Human Subjects following Oral Administration

	Serum Levels, mcg./ml.							
	Study I				Study II			
	Chlorphenesin Carbamate						Methocarbamol	
	Subject Re[a]		Subject Mi[b]					
Hours	Obs.	Calc.[c]	Obs.	Calc.[c]	Obs.	Calc.[c]	Obs.	Calc.[c]
1	10.2	10.5	10.7	10.6	8.3	8.3	25.8	26.2
2	19.3	19.4	17.0	17.6	14.9	14.7	25.5	23.9
3	21.4	20.2	18.1	17.5	13.3	13.7	14.5	15.4
4	17.7	18.5	17.2	15.4	12.5	11.6	8.6	8.8
5	16.4	16.2	11.5	13.0	8.8	9.5	3.5	4.8
6	13.8	14.0	9.4	10.7	7.6	7.7	4.5	2.6
8	9.8	10.2	7.7	7.2	4.9	5.1	2.2	0.7
10	7.4	7.4	4.4	4.8	3.8	3.3	—[e]	—
12	5.3	5.4	4.1	3.2	—[d]	—	—	—
15	3.7	3.3	—[d]	—	—	—	—	—

[a] 3-g. dose. [b] 2-g. dose. [c] Calculated from nonlinear least-squares fit of observed concentrations to Eq. 1. [d] Values <3.5 mcg./ml. on terminal portion were rejected. [e] Values <2.0 mcg./ml. on terminal portion were rejected.

serum drug levels following methocarbamol administration and was not included in the comparison.

RESULTS AND DISCUSSION

Analytical Method—Serum drug levels were determined by modifications of the procedure of Morgan *et al.* (17). The method involves: (*a*) alkaline hydrolysis of the carbamates in diluted serum to yield the corresponding glycols (chlorphenesin and guaiacol glyceryl ether), (*b*) chloroform extraction of the glycols, (*c*) periodate oxidation of the glycols, and (*d*) spectrophotometric determination of the resulting formaldehyde by reaction with chromotropic acid. Conditions were standardized for chlorphenesin carbamate and applied directly to methocarbamol.

Substitution of the periodic acid reagent for the bicarbonate-buffered reagent described by earlier workers (17, 20) provided greater manipulative simplicity with no increase in reaction time; oxidation of chlorphenesin was complete within 1 hr. Chloroform extraction of chlorphenesin was quantitative and unaffected by changes in the aqueous volume or the presence of serum proteins. Alkaline hydrolysis of chlorphenesin carbamate under the conditions of Morgan *et al.* (17) gave a maximum yield of chlorphenesin in simple aqueous solutions within 20 min. However, when serum was treated similarly (1 ml. plus 0.1 ml. 1 *N* sodium hydroxide), the mixture gelled during heating, making subsequent extraction impossible. Greater dilution of serum proteins at the same alkalinity (*vide supra*) provided a satisfactory system; production of chlorphenesin was maximal within 30 min. and was not affected by the presence of serum components. The overall yield was 85%, comparing favorably with the reported values of 60% for mephenesin and 80% for guaiacol glyceryl ether from mephenesin carbamate and methocarbamol, respectively (17).

Buhler (7, 8) showed that the major metabolite of chlorphenesin carbamate in man is the *O*-glucuronide; small amounts of *p*-chlorophenoxylactic acid, *p*-chlorophenoxyacetic acid, and *p*-chlorophenol were also found. Bruce *et al.* (21) recently reported that the major metabolites of methocarbamol in man are glucuronide and sulfate conjugates of the drug, 3-(*o*-hydroxyphenoxy)-1,2-propanediol-1-carbamate, and 3-(*p*-hydroxy-*o*-methoxyphenoxy)-1,2-propanediol-1-carbamate. None of these compounds responded in the present analytical procedure. The method is, therefore, specific for the drugs in the presence of their metabolites.

Pharmacokinetics—Serum levels observed for chlorphenesin carbamate in Study I (Table II) indicated that the analytical method possessed adequate sensitivity at the 2-g. oral dose, that the sampling schedule defined the appearance and disappearance of drug in the serum, and that the biological half-life was about 4 hr. Computer fitting showed these data to be consistent with a one-compartment open model such that serum drug concentration, C, at any time, t, could be calculated from:

$$C = \frac{FD}{V}\left(\frac{k}{k-K}\right)[e^{-K(t-t_0)} - e^{-k(t-t_0)}] \qquad \text{(Eq. 1)}$$

where F = fraction of the dose absorbed, D = dose, V = volume of distribution, k = first-order rate constant for absorption, K = first-order rate constant for elimination, and t_0 = lag time before absorption.

Pharmacokinetic parameters obtained from the nonlinear least-squares fit (18) of these data are shown in Table III. In addition to the constants of Eq. 1, half-lives for absorption ($t_{1/2}$) and elimination ($T_{1/2}$), maximum concentration (C_m), time of maximum concentration (t_m), and relative volume of distribution (V_r) have been included. Values for the two subjects were in good agreement. Excellent agreement between observed and calculated serum drug levels (Table II), obtained from Eq. 1 and the constants of Table III, supported the choice of the pharmacokinetic model.

Observed serum levels of chlorphenesin carbamate and methocarbamol in Study II were fitted to the same model. Representative data (Subject Mi) are shown in Table II. Results were similar to those from Study I, except for a more rapid achievement of maximum levels. With chlorphenesin carbamate, observed maximum levels occurred at 2 hr. in six subjects and at 3 hr. in one. With methocarbamol, observed maximum levels occurred at 1 hr. in four subjects, at 2 hr. in two, and at 3 hr. in one. As a consequence, estimations of the parameters of absorption (t_0, k, and $t_{1/2}$) are subject to considerable uncertainty and must be treated as approximations.

Comparative calculated pharmacokinetic parameters for the seven subjects are shown in Table IV, along with mean values and tests for significance based on the two-sided sign test. The appearance of each drug in the serum could be characterized by a short lag time followed by a rapid first-order absorption and attainment of maximum levels. No statistically significant differences were found in estimates of t_0, k, $t_{1/2}$, and t_m for the two drugs. These observations are consistent with rapid dissolution (22) and high aqueous and lipid solubilities (7, 23). Absorption from the upper intestine is indicated.

Comparisons of drug distribution provide a different picture. Highly significant differences in maximum serum concentrations (C_m) and in relative volumes of distribution (V_r) were found. Oral absorption of chlorphenesin carbamate was essentially complete; over 85% of the dose was excreted in the urine as the major metab-

Table III—Pharmacokinetic Parameters of Chlorphenesin Carbamate, Study I

Parameter[a]	Subject (Dose) Re (3 g.)	Mi (2 g.)
t_0, hr.	0.54 (0.03)	0.48 (0.07)
k, hr.$^{-1}$	1.04 (0.11)	1.04 (0.29)
K, hr.$^{-1}$	0.161 (0.011)	0.203 (0.039)
FD/V, mcg./ml.	28.7 (1.22)	26.7 (3.51)
$t_{1/2}$, hr.	0.66	0.66
t_m, hr.	2.66	2.43
C_m, mcg./ml.	20.4	18.0
$T_{1/2}$, hr.	4.30	3.42
V_r, l./kg.[b]	1.49	0.82

[a] Values in parentheses are standard deviations. [b] Maximum relative volume of distribution assuming $F = 1$.

Table IV—Comparative Pharmacokinetic Parameters

Subject	Drug[a]	t_0, hr.	k, hr.$^{-1}$	$t_{1/2}$, hr.	t_m, hr.	C_m, mcg./ml.	K, hr.$^{-1}$	$T_{1/2}$, hr.	V_r[b], l./kg.
Fe	CC	0.7	2.4	0.29	1.8	21.7	0.211	3.29	1.40
	M	0	1.0	0.67	1.2	43.3	0.764	0.91	0.38
Sz	CC	0.7	1.6	0.44	2.1	11.5	0.270	2.57	1.48
	M	0	7.9	0.09	0.3	42.8	0.667	1.04	0.46
Ma	CC	0.1	0.9	0.76	2.4	16.6	0.164	4.23	1.30
	M	0.2	0.6	1.08	2.2	26.2	0.390	1.78	0.56
Mi	CC	0.6	1.5	0.46	2.2	14.8	0.209	3.31	1.08
	M	0.2	1.2	0.58	1.3	27.4	0.673	1.03	0.38
Pr	CC	0.3	1.6	0.44	1.7	14.7	0.253	2.74	1.21
	M	0.2	1.3	0.54	1.2	22.2	0.743	0.93	0.53
Br	CC	0.4	3.2	0.22	1.3	14.6	0.196	3.54	1.19
	M	0.8	1.4	0.51	2.0	22.8	0.436	1.59	0.54
Jo	CC	0.4	1.6	0.45	1.8	13.4	0.299	2.32	1.26
	M	0.3	1.1	0.65	1.5	23.6	0.618	1.12	0.50
Mean	CC	0.5	1.8	0.44	1.9	15.3	0.229	3.14	1.27
	M	0.2	2.1	0.59	1.4	29.8	0.613	1.20	0.48
p		>0.1	>0.1	>0.1	>0.1	<0.016	<0.016	<0.016	<0.016

[a] CC = chlorphenesin carbamate, M = methocarbamol. [b] Maximum relative volume of distribution.

olite within 24 hr. Therefore, it may be concluded that the observed differences reflect extensive distribution of chlorphenesin carbamate relative to methocarbamol. A similar pattern is seen in the comparative plasma levels of mephenesin carbamate and methocarbamol reported by Huf *et al.* (16).

The mean biological half-life of 3.14 hr. for chlorphenesin carbamate was highly significantly different from the 1.20 hr. for methocarbamol. In the former case, formation of the *O*-glucuronide represents the only important route of drug elimination and is the rate-limiting process measured (7). With methocarbamol, Bruce *et al.* (21) showed that conjugates of the drug, of 3-(*o*-hydroxyphenoxy)-1,2-propanediol-1-carbamate, and of 3-(*p*-hydroxy-*o*-methoxyphenoxy)-1,2-propanediol-1-carbamate are the major metabolites excreted in human urine along with small amounts of unidentified materials. Thus, at least three simultaneous processes of metabolism (conjugation, *O*-demethylation, and hydroxylation) contribute to the shorter half-life of methocarbamol. The longer half-life for chlorphenesin carbamate is consistant with a longer duration of effect.

The one-compartment open model represents an over simplification of the actual biological system as do all models. However, this model was consistent with the data as shown in Table II, and was considered adequate for the purposes of this study. Maass *et al.* (24) recently showed that plasma levels of mephenesin in the dog can be described by the same model. Similarly, Bruce *et al.* (21) invoked this model in determining the half-life for total methocarbamol-related materials (radioactivity) in man.

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Outline of Details for Microbiological Assays of Antibiotics: Second Revision

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Abstract □ Pertinent data are presented for the performance of 83 different microbiological assays for antibiotics. These include the official methods for all antibiotics approved for human use in the United States plus methods for 10 other antibiotics. For analysts already familiar with basic procedures, these data will serve as a ready guide for the performance of assays.

Keyphrases □ Microbiological assays—guide to antibiotics □ Antibiotics—outline of 83 microbiological assays

An outline of details of microbiological assays for antibiotics was published by Kirshbaum and Arret (1) in 1959. A second outline, revising and updating the original version, was published by the same authors (2) in 1967. Since new antibiotics continue to be discovered, requiring new assay methods, and since existing methods have been revised and modified, a new outline reflecting the latest available microbiological assays is now needed.

The 1967 outline gave details for 57 different official microbiological assays; the present outline gives details for 83 different assays, including 10 that are nonofficial. As in the previous two publications, pertinent information is tabulated in a condensed form for ready reference by the analyst who performs microbiological assays of antibiotics. Detailed directions for performing the assays are not given, since the tabulations are intended for analysts already familiar with the basic assay techniques.

Since the last outline appeared (2), the Federal Register published condensations of microbiological assays in which the outline approach to details of assays is followed to some extent. These were codified in the Code of Federal Regulations (3). For sample preparation methods for individual drugs, one must refer to the specific monograph (4).

In 1955, Grove and Randall (5) published their laboratory manual which received worldwide acceptance as an authoritative guide for antibiotic assay and analysis. They used a sequential numbering system to identify assay culture media. Since commercial manufacturers of culture media now use these numbers to identify their media, we, as well as the Code of Federal Regulations, have uniformly continued this numbering system to avoid confusion in specifying the proper culture media.

The test organisms used for the various assays and their methods of preparation are listed in Table I. The media and buffers are listed in Tables II and III, respectively. Pertinent information for cylinder plate assays and for turbidimetric assays is given in Tables IV and V, respectively.

Table I—Test Organisms for Various Assays

Test Organism		Method of Preparation
A	*Staphylococcus aureus* (ATCC 6538P)	1
B	*Sarcina subflava* (ATCC 7468)	1
C	*Sarcina lutea* (ATCC 9341)	1
D	*Staphylococcus epidermidis* (ATCC 12228)	1
E	*Saccharomyces cerevisiae* (ATCC 9763)	6[a]
F	*Bordetella bronchiseptica* (ATCC 4617)	1
G	*Bacillus cereus* var. *mycoides* (ATCC 11778)	3
H	*Bacillus subtilis* (ATCC 6633)	2
I	*Klebsiella pneumoniae* (ATCC 10031)	1
J	*Escherichia coli* (ATCC 10536)	1
K	*Streptococcus faecalis* (ATCC 10541)	5
L	*Micrococcus flavus* (ATCC 10240)	1
M	*Microsporum gypseum* (ATCC 14683)	4
N	*S. lutea*, resistant to dihydrostreptomycin (ATCC 9341a)	1
O	*S. aureus*, resistant to novobiocin (ATCC 12692)	1
P	*S. aureus*, re istant to dihydrostreptomycin (ATCC 6538-DR)	1
R	*S. subflava*, resistant to dihydrostreptomycin (ATCC 7468/d)	1
T	*S. cerevisiae* (ATCC 2601)	6
U	*M. flavus*, resistant to neomycin (ATCC 14452)	1
V	*M. flavus*, resistant to dihydrostreptomycin (ATCC 10240A)	1
W	*Pseudomonas pyocyanea* (ATCC 25619)	1

[a] For candicidin assay, use Method 1.

PREPARATION OF MICROBIAL SUSPENSIONS

Several different methods are used for the preparation of microbial suspensions.

Method 1—Maintain the test organism on slants of Medium 1 and transfer to a fresh slant once a week. Using 3 ml. of saline T.S. USP, wash the organism from the agar slant (which has been incubated for 24 hr. at 32–37°) onto a large agar surface of Medium 1[1], such as that provided by a Roux bottle containing 250 ml. of agar. Incubate for 24 hr. at 32–37°. Wash the resulting growth from the nutrient surface, using 50 ml. of sterile saline T.S. USP Store the test organism under refrigeration.

[1] For the preparation and maintenance of the antibiotic-resistant organisms listed in Table I, prepare Medium 1 to contain the appropriate antibiotic as described in Sections 25, 27, 28, and 33 of Table II.

Table II—Media

Medium	Ingredients	Grams
1	Peptone	6.0
	Pancreatic digest of casein	4.0
	Yeast extract	3.0
	Beef extract	1.5
	Dextrose	1.0
	Agar	15.0
	Distilled water to make 1000 ml.	
	pH 6.55 ± 0.05 after sterilization	
2	Same as Medium 1, except omit pancreatic digest of casein and dextrose	
3	Peptone	5.0
	Yeast extract	1.5
	Beef extract	1.5
	Sodium chloride	3.5
	Dextrose	1.0
	Monobasic potassium phosphate	1.32
	Dibasic potassium phosphate	3.68
	Distilled water to make 1000 ml.	
	pH 7.0 ± 0.05 after sterilization	
5	Same as Medium 2, except adjust pH so that it is 7.9 ± 0.1 after sterilization	
8	Same as Medium 2, except adjust pH so that it is 5.90 ± 0.05 after sterilization	
9	Pancreatic digest of casein	17.0
	Papaic digest of soybean	3.0
	Sodium chloride	5.0
	Dibasic potassium phosphate	2.5
	Dextrose	2.5
	Agar	20.0
	Distilled water to make 1000 ml.	
	pH 7.25 ± 0.05 after sterilization	
10	Same as Medium 9, except use 12 g. instead of 20 g. of agar. Boil to dissolve ingredients; then add 10 ml. of polysorbate 80	
11	Same as Medium 1, except adjust pH so that it is 7.9 ± 0.1 after sterilization	
13	Peptone	10.0
	Dextrose	20.0
	Distilled water to make 1000 ml.	
	pH 5.65 ± 0.05 after sterilization	
18	Same as Medium 11, except boil to dissolve ingredients; then add 20 ml. of polysorbate 80	
19	Peptone	9.4
	Yeast extract	4.7
	Beef extract	2.4
	Sodium chloride	10.0
	Dextrose	10.0
	Agar	23.5
	Distilled water to make 1000 ml.	
	pH 6.1 ± 0.1 after sterilization	
20	Dextrose	40.0
	Peptone	10.0
	Chloramphenicol (activity)	0.05
	Agar	15.0
	Distilled water to make 1000 ml.	
	pH 5.65 ± 0.05 after sterilization	
21	Dissolve 10 mg. cycloheximide/ml. of distilled water and sterilize by filtration through a membrane filter having a porosity of 0.22 μ. Aseptically, add 20 ml. sterile solution/l. Medium 20 which has been autoclaved and cooled to 50°	
22	Dextrose	40.0
	Peptone	10.0
	Distilled water to make 1000 ml.	
	pH 5.65 ± 0.5 after sterilization	
25	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile dihydrostreptomycin sulfate solution to give a final concentration of 1000 mcg. dihydrostreptomycin activity/ml. of solution. Sterile dihydrostreptomycin sulfate solution is prepared by filtering a solution containing 10 mg. dihydrostreptomycin/ml. distilled water through a membrane filter of 0.22-μ porosity	
27	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile neomycin sulfate solution to give a final concentration of 100 mcg. neomycin activity/ml. medium. Sterile neomycin sulfate solution is prepared by filtering a solution containing 10 mg. neomycin/ml. distilled water through a membrane filter of 0.22-μ porosity	
28	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile dihydrostreptomycin sulfate solution to give a final concentration of 500 mcg. dihydrostreptomycin activity/ml. medium. Sterile dihydrostreptomycin sulfate solution is prepared by filtering a solution containing 10 mg. dihydrostreptomycin/ml. distilled water through a membrane filter of 0.22-μ porosity	
29	Beef extract	6.0
	Peptone	10.0
	Agar	15.0
	Distilled water to make 1000 ml.	
	pH 7.9 ± 0.1 after sterilization	
32	Same as Medium 1, except add 300 mg. hydrated manganese sulfate ($MnSO_4H_2O$)/l. of medium	
33	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile sodium novobiocin solution to give a final concentration of 10 mcg. novobiocin activity/ml. medium. Sterile sodium novobiocin solution is prepared by filtering a solution containing 2.5 mg. novobiocin/ml. distilled water through a membrane filter of 0.22-μ porosity	

Method 2—Proceed as described in Method 1, but standardize the suspension as follows. Centrifuge and decant the supernatant liquid. Resuspend the sediment with 50–70 ml. of sterile saline USP T.S., and heat the suspension for 30 min. at 70°. Store the spore suspension under refrigeration.

Table III—Buffers

	Buffer	Ingredients	Amount
I	1% phosphate buffer, pH 6.0 ± 0.05	Dibasic potassium phosphate	2 g.
		Monobasic potassium phosphate	8 g.
		Distilled water to make	1000 ml.
III	0.1 *M* phosphate buffer, pH 7.9 ± 0.1	Dibasic potassium phosphate	16.73 g.
		Monobasic potassium phosphate	0.523 g.
		Distilled water to make	1000 ml.
IV	0.1 *M* monopotassium phosphate buffer, pH 4.5 ± 0.05	Monobasic potassium phosphate	13.6 g.
		Distilled water to make	1000 ml.
VI	10% phosphate buffer, pH 6.0 ± 0.05	Same as I, except use 10 times as much of each salt	
X	0.2 *M* phosphate buffer, pH 10.5 ± 0.1	Dibasic potassium phosphate	35 g.
		10 *N* potassium hydroxide	2 ml.
		Distilled water to make	1000 ml.
XIII	0.01 *N* methanolic hydrochloric acid	1.0 *N* hydrochloric acid	10 ml.
		Methyl alcohol to make	1000 ml.
XV	80% isopropyl alcohol solution	Isopropyl alcohol	800 ml.
		Distilled water to make	1000 ml.
XVI	1% sodium carbonate	Sodium carbonate	10 g.
		Distilled water to make	1000 ml.

Table IV—Plate Diffusion Assays

Antibiotic	Test Organism	Suggested Milliliters of Suspension per 100 ml. of Media	Optimal Zone size (mm.) Produced by Reference Concentration[a]	Medium—Base	Medium—Seed	Initial Solution of Standard	Diluent for Further Dilutions	Final Concentration for Standard Response Line, units or mcg./ml.	Incubation Temperature
Amphomycin	U	0.5	12–14	2	1	100 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Amphotericin B	E	0.2	16–18	None	19[b]	1000 mcg./ml. in dimethyl sulfoxide	X	0.64, 0.8, *1.0*, 1.25, 1.56	30°
Ampicillin	C	1.0	18–20	11	11	100 mcg./ml. in distilled water	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35°
Bacitracin	B or R[c]	0.2	16–18	2	1	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Bacitracin	L or V[d]	0.2	17–19	2	1	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Carbenicillin	W	0.05	16–18	9	10	1000 mcg./ml. in I	I	64, 80, *100*, 125, 156	37°
Cephalexin	A	0.05	18–20	2	1	1000 mcg./ml. in I	I	12.8, 16.0, *20.0*, 25.0, 31.2	37°
Cephaloglycin	A	0.2	18–20	2	1	100 mcg./ml. in distilled water	IV	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Cephaloridine	A	0.3	19–21	2	1	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Cephalothin	A	0.05	14–16	2	1	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Chloramphenicol	C	2.0	17–19	1	1	10,000 mcg./ml. in ethanol	I	32, 40, *50*, 62.5, 78.1	32–35°
Chlortetracycline	G	0.4	17–19	8	8	1000 mcg./ml. in 0.01 *N* HCl	IV	0.064, 0.08, *0.1*, 0.125, 0.156	30°
Clindamycin	C	1.5	18–20	11	11	1000 mcg./ml. in distilled water	III	0.64, 0.80, *1.0*, 1.25, 1.56	37°
Cloxacillin	A	0.2	18–20	2	1	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Sodium colistimethate	F	0.04	13–15	9	10	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Colistin	F	0.04	15–17	9	10	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Cycloserine	A	0.04	14–16	2[e]	1	1000 mcg./ml. in distilled water	I	32, 40, *50*, 62.5, 78.1	30°
Dactinomycin	H	0.02	16–18	5[e]	5	10,000 mcg./ml. in methanol	III	0.5, 0.71, *1.0*, 1.41, 2.0	37°
Demeclocycline	G	0.4	17–19	8	8	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.080, *0.10*, 0.125, 0.156	30°
Dicloxacillin	A	0.2	18–20	2	1	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Dihydrostreptomycin	H	0.05	14–16	5	5	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Doxycycline	G	0.4	18–20	8	8	1000 mcg./ml. in 0.1 *N* HCl	III	0.064, 0.080, *0.10*, 0.125, 0.156	30°
Erythromycin	C	1.0	17–19	11	11	10,000 mcg./ml. in methanol	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Gentamicin	D	0.03	15–17	11	11	1000 mcg./ml. in III	III	0.064, 0.08, *0.1*, 0.125, 0.156	37°
Griseofulvin	M	1.0	16–18	20	21	1000 mcg./ml. in dimethylformamide[f]	III	3.2, 4.0, *5.0*, 6.25, 7.81 (48 hr.)	30°
Hetacillin	C	0.5	15–17	11	11	1000 mcg./ml. in III	III	0.064, 0.080, *0.10*, 0.125, 0.156	32°
Kanamycin	A	0.01	16–18	11	11	1000 mcg./ml. in III	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Kanamycin B	H	0.05	14–16	5	5	1000 mcg./ml. in III	III	0.64, 0.80, *1.0*, 1.25, 1.56	37°
Lincomycin	C	1.0	16–18	11	11	1000 mcg./ml. in distilled water	III	1.28, 1.6, *2.0*, 2.5, 3.12	37°
Leucomycin[g]	C	1.0	18–20	None	19[b]	10,000 mcg./ml. in ethanol, 1000 mcg./ml. in I	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Methacycline[g]	G	0.4	17–19	8	8	1000 mcg./ml. in XIII	IV	0.064, 0.080, *0.10*, 0.125, 0.156	30°
Methicillin	A	0.2	14–16	2	1	1000 mcg./ml. in I	I	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Mithromycin	A	0.1	15–17	8[e]	8	100 mcg./ml. in distilled water	I	0.5, 0.71, *1.0*, 1.41, 2.0	32°

(*Continued*)

Table IV—(*Continued*)

Antibiotic	Test Organism	Suggested Milliliters of Suspension per 100 ml. of Media	Optimal Zone size (mm.) Produced by Reference Concentration[a]	—Medium— Base	Seed	Initial Solution of Standard	Diluent for Further Dilutions	Final Concentration for Standard Response Line, units or mcg./ml.	Incubation Temperature
Nafcillin	A	0.2	14–16	2	1	1000 mcg./ml. in I	I	1.28, 1.6, *2.0*, 2.5, 3.12	32–35°
Neomycin	A	0.04	16–18	11	11	1000 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Neomycin	D	0.2	16–18	11	11	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Novobiocin	D	2.0	16–18	2	1	10,000 mcg./ml. in ethanol, 1000 mcg./ml. in III	VI	0.32, 0.4, *0.5*, 0.625, 0.781	35°
Nystatin[h]	T	1.0	16–18	None	19[b]	1000 mcg./ml. in dimethylformamide[f]	VI	12.8, 16, *20*, 25, 31.2	30°
Oleandomycin	D	0.2	16–18	11	11	10,000 mcg./ml. in ethanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	37°
Oxacillin	A	0.1	18–20	2	1	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Oxytetracycline	G	0.4	18–20	8	8	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30°
Paromomycin	D	0.2	19–21	11	11	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Paromomycin	H	0.05	16–18	29	29	1000 mcg./ml. in III	III	1.28, 1.56, *2.00*, 2.50, 3.12	37°
Penicillin G	A	0.2	17–19	2	1	1000 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Phenethicillin	C	0.4	17–19	11	11	1000 units/ml. in distilled water	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35°
Phenoxymethylpenicillin	A	0.2	17–19	2	1	1000 units/ml. in methanol	I	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Polymyxin	F	0.04	15–17	9	10	20,000 units/ml. in distilled water	VI	6.4, 8.0, *10.0*, 12.5, 15.6	37°
Puromycin[g]	C	0.1	16–18	2	1	1000 mcg./ml. in III	III	0.64, 0.80, 1.0, 1.25, 1.56	30°
Rifampin	H	0.1	15–17	2	2	1000 mcg./ml. in methanol	I	3.2, 4.0, *5.0*, *6.25*, *7.81*	30°
Ristocetin[g]	G	0.4	15–17	8	8	1000 mcg./ml. in I	I[i]	6.4, 8.0, *10.0*, 12.5, 15.6	30°
Rolitetracycline	G	0.4	17–19	8	8	1000 mcg./ml. in methanol	IV	0.64, 0.80, *1.0*, 1.25, 1.56	30°
Streptomycin	H	0.05	14–16	5	5	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Tennecitin[g]	E	0.3	17–19	None	19[b]	4000 mcg./ml. in dimethyl sulfoxide	I	0.64, 0.80, *1.0*, 1.25, 1.56	37°
Tetracycline	G	0.4	17–19	8	8	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30°
Thiostrepton[g]	A	0.1	14–16	None	19[b]	1000 mcg./ml. in newly adjusted dimethylformamide (pH 6.5–7.0)	III	0.64, 0.80, *1.0*, 1.25, 1.56	32–35°
Troleandomycin	D	0.2	15–17	18	18	1000 mcg./ml. in XV	X	9.6, 12, *15*, 18.8, 23.4	37°
Tylosin[g]	C	0.5	14–16	11	11	10,000 mcg./ml. in methanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	32°
Tylosin[g]	N	1.0	14–16	11	11	10,000 mcg./ml. in III	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Vancomycin	G	0.2	16–18	8[e]	8	400 mcg./ml. in distilled water	IV	6.4, 8.0, *10.0*, 12.5, 15.6	30°
Viomycin	H	0.05	17–19	5[e]	5	1000 mcg./ml. in distilled water	III	32, 40, *50*, 62.5, 78.1	37°

[a] Zone sizes listed here should be construed as guides only rather than as absolute values. [b] No base layer is used. Add 8 ml. of inoculated seed layer only. [c] Test Organism R is used for products containing bacitracin and dihydrostreptomycin. [d] Test Organism V is used for products containing bacitracin and dihydrostreptomycin. [e] Use 10 ml. instead of 21 ml. [f] Solution is further diluted in solvent to 20 times the final concentration of each concentration of the standard curve so that each final dilution is 1 + 19 with indicated buffer. [g] Not official methods. [h] Use low actinic glassware. [i] Containing 0.1% $NaNO_2$.

Method 3—Use Method 2, but heat shock for 30 min. at 70° prior to centrifugation and wash the spore suspension three times with 25–50 ml. of sterile distilled water each time. Reconstitute with 50–70 ml. of sterile distilled water. Store the spore suspension under refrigeration.

Method 4—Grow the organisms for 6–8 weeks at 25° in several 3-l., wide-mouth conical flasks, each containing 200 ml. of Medium 22. Check the growth for sporulation. When sporulation is 80% or greater, harvest the spores on the mycelial layer with a sterile spatula or other convenient instrument. The spores are on top of the

floating mat. Place harvested spore material in 50 ml. of saline. Store the spore suspension under refrigeration.

Method 5—Maintain the test organisms in 100-ml. portions of nutrient broth—Medium 3. For the test, prepare a fresh subculture by transferring a loopful of the stock culture to 100 ml. of the same nutrient broth and incubate for 16–18 hr. at 37°. Store this broth culture under refrigeration.

Method 6—Use Method 1, but incubate the slants at 30° for 24 hr. and incubate the Roux bottles at 30° for 48 hr.

DETERMINATION OF INOCULUM TO BE USED

For many years, official methods in the Federal Regulations gave instructions for standardizing microbial suspensions by spectrophotometric measurement of light transmission or absorbance. A uniform method for this measurement, described by Kirshbaum *et al.* (6), was incorporated into the Regulations. However, the great number and variety of microbial assays make such an approach to standardization confusing; the point of diminishing returns in giving details probably has been reached. If suspensions are prepared as described in Methods 1–6, growth characteristics are sufficiently uniform so that the inoculum for assays can be adequately determined by test trials without any other adjunctive standardization steps.

For Plate Assays—After the suspension is prepared, add a different volume of it (*e.g.*, 1.0, 2.0, and 3.0 ml., respectively) to each of several different flasks containing 100 ml. of the agar specified for the seed layer in Table IV. Use the volume listed under "Suggested Milliliters of Suspension . . ." in Table IV as a guide. Using the

Table V—Turbidimetric Assays

Antibiotic	Test Organism	Suggested Milliliters of Suspension per 100 ml. of Media	Medium	Initial Solution of Standard	Diluent for Further Dilutions	Final Concentration for Standard Response Line, units or mcg./ml.	Incubation Temperature[a]
Candicidin	E	0.2	13	1000 mcg./ml. in dimethyl sulfoxide	Distilled water	0.03, 0.042, *0.06*, 0.084, 0.12	25°
Capreomycin	I	0.1	3	1000 mcg./ml. in distilled water	Distilled water	64, 80, *100*, 125, 156	37°
Chloramphenicol	J	0.1	3	10,000 mcg./ml. in ethanol	I	2.0, 2.24, *2.5*, 2.79, 3.12	37°
Chlortetracycline	A	0.1	3	1000 mcg./ml. in 0.01 *N* HCl	IV	0.038, 0.048, *0.060*, 0.075, 0.094	37°
Cycloserine	A	0.1	3	1000 mcg./ml. in distilled water	Distilled water	32.0, 40.0, *50.0*, 62.5, 78.1	37°
Demeclocycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.080, *0.10*, 0.125, 0.156	37°
Dihydrostreptomycin	I	0.1	3	1000 mcg./ml. in distilled water	Distilled water	24, 26.8, *30*, 33.5, 37.5	37°
Doxycycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.08, *0.10*, 0.125, 0.156	37°
Sodium fusidate[b]	A	0.25	3	1000 mcg./ml. in 1% sodium carbonate XVI	Distilled water	80, 89, *100*, 112, 125	37°
Gramicidin	K	1.0	3	1000 mcg./ml. in 95% ethanol USP	95% ethanol USP	0.028, 0.034, *0.040*,[c] 0.048, 0.057	37°
Methacycline	A	0.1	3	1000 mcg./ml. in XIII	IV	0.032, 0.040, *0.050*, 0.062, 0.078	37°
Minocycline	A	0.2	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.032, 0.040, *0.050*, 0.062, 0.078	37°
Oleandomycin	A	0.1	3	1000 mcg./ml. in III	III	2.00, 2.24, *2.5*, 2.80, 3.12	37°
Oxytetracycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.160, 0.200, *0.250*, 0.312, 0.390	37°
Ristocetin[b]	A	0.3	3	1000 mcg./ml. in I	I	9.0, 11.6, *15.0*, 19.4, 25.0	37°
Rolitetracycline	A	0.1	3	1000 mcg./ml. in methanol	IV	0.160, 0.200, *0.250*, 0.312, 0.390	37°
Soframycin[b]	A	0.2	3	500 mcg./ml. in distilled water	Distilled water	1.92, 2.40, *3.0*, 3.75, 4.65	37°
Spectinomycin	J	0.02	3	1000 mcg./ml. in III	III	12.8, 16.0, *20.0*, 25.0, 31.2	37°
Streptomycin	I	0.1	3	1000 mcg./ml. in distilled water	Distilled water	24, 26.8, *30*, 33.5, 37.5	37°
Tetracycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.160, 0.200, *0.250*, 0.312, 0.390	37°
Troleandomycin	I	0.05	3	1000 mcg./ml. in XV	I	16.0, 20.0, *25*, 31.2, 39.0	37°
Tyrothricin	K	1.0	3	1000 mcg./ml. in 95% ethanol USP	95% ethanol USP	0.14, 0.17, *0.20*, 0.24, 0.285[c]	37°
Viomycin	I	0.05	3	5000 mcg./ml. in distilled water	Distilled water	64, 80, *100*, 125, 156	37°

[a] Overnight incubation is recommended for the candicidin assay. An incubation period of 3 hr. is recommended for assays using Test Organisms I and K. An incubation period of 4 hr. is recommended for assays using Test Organisms A. [b] Not official methods. [c] One-tenth milliliter of each solution is added to each tube.

several inocula prepared in this manner, prepare inoculated plates as described for the specific antibiotic assay. Fill each cylinder with the reference concentration of the antibiotic and then incubate the plates. After incubation, examine and measure the zones of inhibition produced on the plates. The volume of suspension that produces the optimum zones of inhibition with respect to both clarity and diameter determines the inoculum to be used for the assay. Table IV gives optimal zone sizes that might be expected for each assay.

For Turbidimetric Assays—Proceed as described for plate assays, but instead of agar, add varying amounts of the suspension to 100 ml. of the broth described for the assay in Table V, using the volume described under "Suggested Milliliters of Suspension . . ." as a guide. Using the several inocula prepared in this manner, proceed as described for the specific antibiotic assay, but run only the high and low concentrations of the standard response line. After incubation, read the absorbances of the appropriate tubes. Determine which inoculum produces the best response between the low and high antibiotic concentrations, and use this inoculum for the assay.

DETAILS OF ASSAY PROCEDURES

For those assays to which the uniform procedure does not apply, reference should be made to the appropriate section of the Code of Federal Regulations (3).

In Table IV, under the column headed "Medium," there are the subheadings "Base" and "Seed." Unless otherwise specified, 21 ml. of base agar and 4 ml. of seed agar should be used for each plate. For turbidimetric assays in Table V, 9 ml. of inoculated broth should be added to each tube containing 1 ml. of sample, unless otherwise specified.

The column marked "Initial Solution of Standard" indicates the method for dissolving the reference standard to be used for the assay. Unless a concentration of 1000 mcg. or units per milliliter is indicated, the solution should immediately be further diluted with the indicated buffer to obtain a stock solution of convenient concentration.

The entries under "Final Concentration for Standard Response Line" are self-explanatory. The italicized concentration in each series is the reference concentration. Official FDA working standards are supplied to laboratories receiving antibiotic certification services. For other laboratories, reference standards are supplied by the USP or NF for some antibiotics listed in those compendia

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Determination of Sodium *p*-Aminosalicylate in the Presence of *m*-Aminophenol

MARTIN I. BLAKE, KENNETH MAKRIS, and JAMES HUNT

Abstract □ The *p*-aminosalicylic acid content in mixtures containing *m*-aminophenol is determined by a modification of the official assay procedure. The *m*-aminophenol is removed by passing a solution of the mixture in dimethylformamide through a column containing a strong cation-exchange resin. The eluate is then treated according to the official method.

Keyphrases □ *p*-Aminosalicylic acid—determination in *m*-aminophenol mixtures, ion-exchange chromatography □ Ion-exchange chromatography—determination of *p*-aminosalicylic acid in *m*-aminophenol mixtures

The official procedure for determining *p*-aminosalicylic acid, its salts and dosage forms, is based on the diazotization reaction involving aromatic amines. In USP XVII, an external indicator was used; while in USP XVIII, the end-point is detected potentiometrically. *m*-Aminophenol, the major breakdown product of *p*-aminosalicylic acid, if present, is also diazotized and constitutes an interference in the official assay procedure. However, the *m*-aminophenol content is determined by spectrophotometric analysis.

A variety of techniques have been proposed for the determination of *p*-aminosalicylic acid and its salts. These were reviewed by Lach and Cohen (1) and were referred to in an earlier paper (2). In the latter paper, a nonaqueous differentiating titration procedure was proposed for analyzing mixtures of *p*-aminosalicylic acid and *m*-aminophenol. Titrations were conducted potentiometrically, with sodium methoxide as the titrant and dimethylformamide as the titration solvent. Titrations were also performed visually, using thymol blue as the indicator. The presence of *m*-aminophenol does not interfere with the end-point detection for *p*-aminosalicylic acid. In a subsequent paper (3), a procedure was described for the separation and determination of mixtures containing *p*-aminosalicylic acid and *m*-aminophenol. Separation was effected by use of a strong cation-exchange resin. *m*-Aminophenol was

several inocula prepared in this manner, prepare inoculated plates as described for the specific antibiotic assay. Fill each cylinder with the reference concentration of the antibiotic and then incubate the plates. After incubation, examine and measure the zones of inhibition produced on the plates. The volume of suspension that produces the optimum zones of inhibition with respect to both clarity and diameter determines the inoculum to be used for the assay. Table IV gives optimal zone sizes that might be expected for each assay.

For Turbidimetric Assays—Proceed as described for plate assays, but instead of agar, add varying amounts of the suspension to 100 ml. of the broth described for the assay in Table V, using the volume described under "Suggested Milliliters of Suspension . . ." as a guide. Using the several inocula prepared in this manner, proceed as described for the specific antibiotic assay, but run only the high and low concentrations of the standard response line. After incubation, read the absorbances of the appropriate tubes. Determine which inoculum produces the best response between the low and high antibiotic concentrations, and use this inoculum for the assay.

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The column marked "Initial Solution of Standard" indicates the method for dissolving the reference standard to be used for the assay. Unless a concentration of 1000 mcg. or units per milliliter is indicated, the solution should immediately be further diluted with the indicated buffer to obtain a stock solution of convenient concentration.

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MARTIN I. BLAKE, KENNETH MAKRIS, and JAMES HUNT

Abstract □ The *p*-aminosalicylic acid content in mixtures containing *m*-aminophenol is determined by a modification of the official assay procedure. The *m*-aminophenol is removed by passing a solution of the mixture in dimethylformamide through a column containing a strong cation-exchange resin. The eluate is then treated according to the official method.

Keyphrases □ *p*-Aminosalicylic acid—determination in *m*-aminophenol mixtures, ion-exchange chromatography □ Ion-exchange chromatography—determination of *p*-aminosalicylic acid in *m*-aminophenol mixtures

The official procedure for determining *p*-aminosalicylic acid, its salts and dosage forms, is based on the diazotization reaction involving aromatic amines. In USP XVII, an external indicator was used; while in USP XVIII, the end-point is detected potentiometrically. *m*-Aminophenol, the major breakdown product of *p*-aminosalicylic acid, if present, is also diazotized and constitutes an interference in the official assay procedure. However, the *m*-aminophenol content is determined by spectrophotometric analysis.

A variety of techniques have been proposed for the determination of *p*-aminosalicylic acid and its salts. These were reviewed by Lach and Cohen (1) and were referred to in an earlier paper (2). In the latter paper, a nonaqueous differentiating titration procedure was proposed for analyzing mixtures of *p*-aminosalicylic acid and *m*-aminophenol. Titrations were conducted potentiometrically, with sodium methoxide as the titrant and dimethylformamide as the titration solvent. Titrations were also performed visually, using thymol blue as the indicator. The presence of *m*-aminophenol does not interfere with the end-point detection for *p*-aminosalicylic acid. In a subsequent paper (3), a procedure was described for the separation and determination of mixtures containing *p*-aminosalicylic acid and *m*-aminophenol. Separation was effected by use of a strong cation-exchange resin. *m*-Aminophenol was

retained by the column, while *p*-aminosalicylic acid appeared in the eluate. Both components were recovered and analyzed by nonaqueous titration.

In the present study, a modification of the official assay procedure is proposed for determining the *p*-aminosalicylic acid content in mixtures containing *m*-aminophenol. The *m*-aminophenol is removed by passing a solution of the mixture in dimethylformamide through a column containing a strong cation-exchange resin. The eluate is then treated by the standard diazotization procedure.

EXPERIMENTAL

Apparatus—All titrations were performed potentiometrically with a titrimeter[1] equipped with a sleeve-type calomel and platinum electrode system. A 50-ml. buret (1 cm. i.d.) served as the chromatographic column.

Reagents and Chemicals—A strong cation-exchange resin[2] (200–400 mesh), *m*-aminophenol, sodium *p*-aminosalicylate, and dimethylformamide were obtained from commercial sources and were the best quality available. The *m*-aminophenol was further purified by recrystallizing from hot water. All other chemicals and solvents employed in this study were reagent grade and were used without further purification. A sodium nitrite solution (0.1 *M*) was prepared and standardized according to USP XVIII (4).

Column Preparation—The cation-exchange resin column was prepared, regenerated, and conditioned to dimethylformamide as described earlier (5, 6). Prior to being used for a run, the column was washed with 25 ml. of dimethylformamide. The height of the column was 6.5 cm., and the flow rate for all separations was 0.5 ml./min.

Assay Procedure—Stock solutions containing sodium *p*-aminosalicylate and *m*-aminophenol were prepared by dissolving appropriate amounts of the powders, accurately weighed, in 50 ml. of dimethylformamide. The composition of the stock solutions corresponded to the 10 mixtures listed in Table I. A 10-ml. aliquot of the stock solution was transferred by pipet to the resin column. When the solution disappeared below the top layer of the resin, additional dimethylformamide was added to the column until 50 ml. of eluate was collected in a 100-ml. calibrated glass-stoppered cylinder. Distilled water was added to the 100-ml. mark. The cylinder was stoppered and shaken thoroughly to assure the formation of a homogeneous solution. The contents of the cylinder were transferred quantitatively to a 200-ml. beaker. The solution was analyzed for aminosalicylate content by the diazotization procedure described for sodium aminosalicylate in USP XVIII (4), starting with: "..., and add 25 ml. of glacial acetic acid...." A blank run was conducted for each stock solution, and the necessary corrections were made to the actual runs. The blank determination usually consumed less than 0.30 ml. of sodium nitrite solution.

Aliquots of the stock solutions were also analyzed by the official procedure directly. The chromatographic separation step was omitted in these runs.

DISCUSSION

In aqueous solution, *p*-aminosalicylic acid and its salts break down rapidly and impart a dark-brown coloration to the solution, the intensity of the color increasing with time. The primary degradation product is *m*-aminophenol, which is unstable and is readily oxidized, forming a series of complex polymeric products which eventually precipitate from the solution.

As already noted, *m*-aminophenol undergoes the diazotization reaction and interferes with the official assay procedure. Ion-exchange chromatography has been explored as a possible technique for separating the *m*-aminophenol from the *p*-aminosalicylic acid. In an earlier paper (5), a weak cation-exchange resin[3] was used to convert sodium *p*-aminosalicylate to *p*-aminosalicylic acid, thus permitting the differentiating titration of *m*-aminophenol and *p*-aminosalicylic acid. Both compounds are apparently too weakly basic to be retained by a carboxylic acid-type resin. When a strong cation-exchange resin[2] was employed, the *m*-aminophenol was held back by the resin, and the *p*-aminosalicylic acid appeared in the eluate. This technique was utilized in the present study to remove *m*-aminophenol from mixtures containing both *m*-aminophenol and *p*-aminosalicylic acid. The eluate was then analyzed by the official procedure for *p*-aminosalicylic acid content.

[1] Fisher, model 35.
[2] Dowex 50-X8 (or equivalent).
[3] Amberlite IRC-50

Table I—Data for Analysis of Sodium *p*-Aminosalicylate in the Presence of *m*-Aminophenol

Sodium *p*-Aminosalicylic Acid Taken[a], mg.	*m*-Aminophenol Added, mg.	Sodium *p*-Aminosalicylic Acid Recovered[b], %
0.0	50.0	0.0
50.0	50.0	96.2
60.0	40.0	96.4
70.0	30.0	98.5
80.0	20.0	97.5
90.0	10.0	95.5
100.0	0.0	99.7
100.0	100.0	98.7
120.0	80.0	100.8
140.0	60.0	98.3
160.0	40.0	100.8
180.0	20.0	97.5

[a] Contained in a 10-ml. aliquot of stock solution. [b] Average of duplicate runs on each aliquot from same stock solution.

In preliminary studies, it was found that separation of *m*-aminophenol from *p*-aminosalicylic acid could only be achieved if the ion-exchange procedure was performed under nonaqueous conditions, as described in this paper. Although the diazotization mixture contained 50% dimethylformamide, the dimethylformamide apparently did not interfere in the diazotization reaction. This is reflected in the low blank readings (0.30 ml.). When 50% dimethylformamide was used as the eluting solution, separation of *m*-aminophenol from *p*-aminosalicylic acid was not successful. It appears that in an aqueous medium, dimethylformamide imparts sufficient basicity to the solution to effect displacement of the *m*-aminophenol from the resin sites. However, when the resin is conditioned to dimethylformamide and if it is the eluting solvent, the *m*-aminophenol is strongly held by the acidic sites on the resin and separation is possible. Table I shows analysis data for a series of 10 synthetic mixtures of sodium *p*-aminosalicylate and *m*-aminophenol. The data indicate that separation of the *p*-aminosalicylic acid from *m*-aminophenol was successful. Quantitative recoveries for *p*-aminosalicylic acid are reported.

When aliquots of the stock solutions were analyzed by the official procedure (not chromatographed), quantitative recovery for the total of *p*-aminosalicylic acid and *m*-aminophenol was obtained.

The proposed procedure was developed as an assay method for studying the kinetics of the decomposition of *p*-aminosalicylic acid and its salts in liquid dosage forms. It is also applicable as a general assay method for *p*-aminosalicylic acid and its salts, free and in dosage forms. The need for obtaining the amount of *m*-aminophenol present in order to determine the *p*-aminosalicylic acid content is obviated.

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Colorimetric Determination of Acetyl Sulfisoxazole in the Presence of Its Hydrolysis Products, Sulfisoxazole and Acetic Acid

JOSEPH A. FELDMAN and VISHNU U. PATEL

Abstract □ The official assay for acetyl sulfisoxazole, the diazotization method, does not differentiate between acetyl sulfisoxazole and its main hydrolysis product, sulfisoxazole. A spectrophotometric method, involving the formation of ferric acetohydroxamate, was developed, and conditions necessary for reproducible reaction were developed. The method provides a relatively simple and rapid means by which acetyl sulfisoxazole is quantitatively determined in the presence of its main hydrolysis products, sulfisoxazole and acetic acid.

Keyphrases □ Acetyl sulfisoxazole—colorimetric determination in the presence of sulfisoxazole □ Ferric acetohydroxamate—formation in the colorimetric determination of acetyl sulfisoxazole □ Colorimetry—determination, acetyl sulfisoxazole

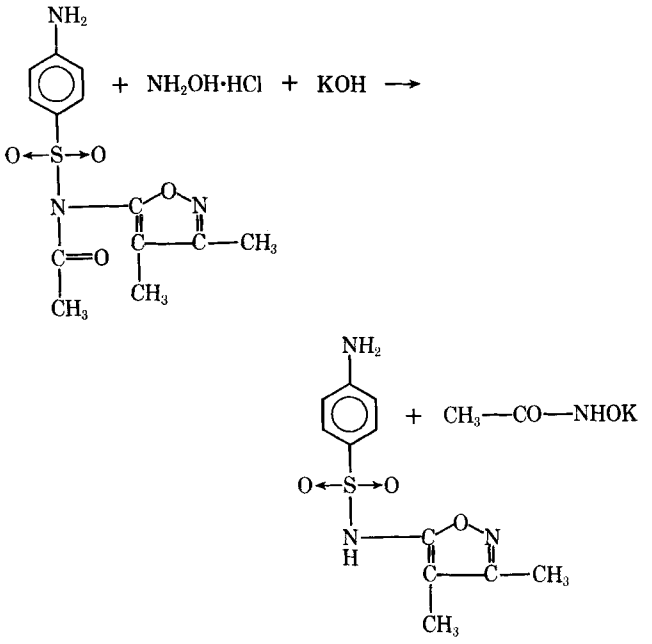
Acetyl sulfisoxazole NF is a white crystalline powder, m.p. 192–195°. Acetyl sulfisoxazole is practically insoluble in water, slightly soluble in alcohol, and sparingly soluble in chloroform. Chemically, it is N^1-acetyl-N^1-(3,4-dimethyl-5-isoxazolyl)sulfanilamide, and it is prepared by the acetylation of a solution or suspension of the alkali metal salt of N^1-(3,4-dimethyl-5-isoxazolyl)sulfanilamide (sulfisoxazole) (1, 2).

The analytical procedure presently official in NF XIII (3) is based on the diazotization of the primary amino group attached to the aromatic ring and the determination of the end-point electrometrically. Since this functional group is present in both sulfisoxazole and acetyl sulfisoxazole, this method of analysis cannot be used to differentiate between the two sulfonamides.

Acetyl sulfisoxazole has been analyzed by diazotization, X-ray diffractometry of the oral suspension (4), and the Bratton and Marshall method as adapted by Flake *et al.* (5) and Hagler *et al.* (6). Neither the cited procedures nor the official method allow for the direct quantitative analysis of acetyl sulfisoxazole in the presence of sulfisoxazole. Bican-Fister and Kajganovic (7) reported the application of TLC to the separation of sulfonamide mixtures, but this method is useful qualitatively rather than quantitatively.

The most common colorimetric method for the analysis of sulfonamides is the method of Bratton and Marshall. This method involves the diazotization of sulfonamide with sodium nitrite in dilute acid, followed by decomposition of the excess nitrite with sulfamic acid and coupling of the diazo compound with *N*-(1-naphthyl)ethylenediamine. The important disadvantage of this method and other colorimetric methods reported in the literature (8–12) is that the absorption frequencies and molar absorbance coefficients produced with acetyl sulfisoxazole and sulfisoxazole are identical. Therefore, these methods are not differential.

In an attempt to find a specific analysis that would be both quantitative and differential between acetyl sulfisoxazole and sulfisoxazole, the method of Feigl *et al.* (13) was selected. By this method, carboxylic acid derivatives (acid anhydrides, acid halides, and esters) are converted to corresponding hydroxamic acid salts by allowing them to react with hydroxylamine hydrochloride in an alkaline medium. The hydroxamic acid salt is then allowed to react with ferric chloride in the presence of dilute acid to produce a red-violet ferric hydroxamate. Soloway and Lipschitz (14) reported that amides can be converted into hydroxamic acid salts with hydroxylamine. The hydroxaminolysis of amides and the formation of colored complexes of the hydroxamic acids so derived with ferric ion afford convenient means of determining amides in the presence of their amino compounds and acid constituents. A procedure for the quantitative analysis of sodium sulfacetamide, which is the sodium salt of N^1-acetylsulfanilamide, involves the formation of ferric acetohydroxamate and measurement of the absorbance at the 540-nm. wavelength, as reported by Schleider *et al.* (15). This method permits the determination of intact sulfacetamide in the presence of its hydrolysis products, sulfanilamide and sodium acetate.



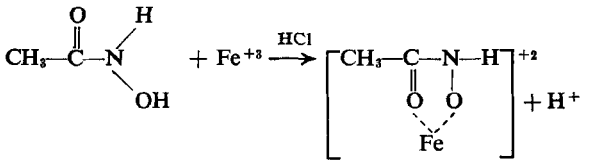
Scheme I

Acetyl sulfisoxazole, a sulfonamide acetylated at the N^1-position, could conceivably react with hydroxylamine hydrochloride in alkaline medium to form the acetohydroxamic acid salt, as shown by Scheme I. The acetohydroxamic acid salt produced could then react with ferric ion in acidic medium (pH 0.5–1.5)

Table I—Effect of Temperature on Color Produced (Absorbance)

Acetyl Sulfisoxazole, mg.	At Ice Bath Temperature	At Room Temperature	At Boiling Water Bath Temperature
0.0	0.030	0.030	0.030
1.0	0.375	0.370	0.370
2.0	0.690	0.685	0.700

to produce the colored ferric hydroxamate according to Scheme II (16).



Scheme II

EXPERIMENTAL

Apparatus—The following were used: a colorimetric spectrophotometer[1], and one set (12) of standard matched test tube cells, i.d. 11.67 mm[2].

Reagents—The following were used: acetyl sulfisoxazole[3], sulfisoxazole[3], acetic acid A.R., sodium acetate A.R., potassium hydroxide A.R., hydroxylamine hydrochloride[2], isopropanol A.R., methanol A.R., ferric chloride A.R., hydrochloric acid A.R., and distilled water.

Solutions—The following were used: saturated hydroxylamine hydrochloride solution (prepared by dissolving 83 g. in 100 ml. of distilled water), 10.5 *M* potassium hydroxide, 4.1 *M* hydrochloric acid solution (50% v/v), isopropanol (33.3% v/v), 0.37 *M* ferric chloride solution in 0.1 *M* hydrochloric acid, and 0.2% w/v methanolic solution of acetyl sulfisoxazole. The solutions were stored under refrigeration and allowed to come to room temperature prior to use.

The proposed method was applied to solutions containing acetyl sulfisoxazole, sulfisoxazole, acetic acid, and sodium acetate; it was found that the color was produced exclusively by acetyl sulfisoxazole. The effects of temperature, quantity of reagents, and reaction time for optimum color formation and rate of color fading were determined.

Since acetyl sulfisoxazole is practically insoluble in water, a methanolic solution of acetyl sulfisoxazole was used. Transfer 0–1-ml. portions of the methanolic solution of acetyl sulfisoxazole into test tubes, and add sufficient quantity of methanol to adjust the volume to 1 ml. To each tube, add 3 ml. of the isopropanol solution, followed by the addition of 1 ml. of the hydroxylamine hydrochloride solution and 1 ml. of the potassium hydroxide solution. Mix the solutions, and allow the reaction to proceed for the specified time interval. Then add 2 ml. of the hydrochloric acid solution. Check the pH of the resulting solution to ascertain whether the pH is between 0.5 and 1.5. If the pH is higher than 1.5, adjust by addition of one or two drops of concentrated hydrochloric acid. Add 1 ml. of the ferric chloride solution and shake. Measure the absorbance of the solution immediately at a wavelength of 540 nm.

The absorbance was plotted as a function of concentration of acetyl sulfisoxazole. A straight-line relationship was observed for a 0–2-mg. concentration of acetyl sulfisoxazole, indicating adherence to Beer's law over this range.

DISCUSSION

In the studies of the rate of reaction of acetyl sulfisoxazole with hydroxylamine hydrochloride, the instantaneous reaction was observed at room temperature. The minimum amounts of reagents necessary for the maximum color production in the systems containing 2 mg. or less of acetyl sulfisoxazole were determined. The Lambert–Beer curve was plotted, and the results were reproducible.

[1] Spectronic-20, Bausch & Lomb, Inc.
[2] Fisher Scientific Co.
[3] Hoffmann–La Roche, Inc.

Table II—Effect of Reaction Time on Color Produced[a]

Sample	Minutes	Absorbance
1	0.0	0.520
2	15	0.515
3	30	0.520
4	45	0.530
5	60	0.525
6	75	0.515

[a] A blank for each sample gave 0.000 absorbance.

Effect of Temperature Applied after Addition of Hydroxylamine Hydrochloride on Absorbance—A series of triplicate test tubes, containing 0.0, 0.5, and 1.0 ml. of the acetyl sulfisoxazole solution, was prepared. To each tube, isopropanol solution, hydroxylamine hydrochloride solution, and potassium hydroxide solution were added as described previously. One set of tubes was allowed to remain at room temperature for 1 hr.; the other two sets were kept at ice bath and boiling water bath temperatures, respectively, for 1 hr. The contents of each tube were then brought to room temperature and acidified with hydrochloric acid solution; the ferric chloride solution was added as previously described. The absorbance of the solutions was measured at 540 nm. (Table I).

Effect of Varying Length of Time after Addition of Hydroxylamine on Absorbance—A sample of 0.75 ml. of 0.2% acetyl sulfisoxazole solution was placed into each of six test tubes. The isopropanol solution, hydroxylamine hydrochloride solution, and potassium hydroxide solution were added as described previously. The tubes were set aside at room temperature for 15, 30, 45, 60, and 75 min., respectively. As each sample was removed, hydrochloric acid and ferric chloride solutions were added as described. Blanks, corresponding to each time interval, were also prepared. The absorbances were then determined at a wavelength of 540 nm. (Table II).

Effect of Various Concentrations of Acetyl Sulfisoxazole on Absorbance—Samples of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 ml. of the 0.2% methanolic solution of acetyl sulfisoxazole, containing the equivalent of 0–2 mg. of acetyl sulfisoxazole, respectively, were placed in test tubes, and sufficient methanol was then added to each tube to adjust the volume to a total of 1 ml. Three milliliters of the isopropanol solution, 1 ml. of the hydroxylamine hydrochloride solution, and 1 ml. of potassium hydroxide solution were then added in the stated order to the sample. To each tube, 2 ml. of 4 *M* hydrochloric acid solution was added, and the pH was checked. Then 1 ml. of ferric chloride solution (0.3 *M* in 0.1 *N* HCl) was added, and the absorbance was determined at 540 nm. Three analyses were performed upon each of the concentrations under identical conditions of time and temperature (Table III).

Rate of Color Fading of Ferric Complex—One-milliliter samples of a methanolic solution of acetyl sulfisoxazole, containing 1.50 mg. of acetyl sulfisoxazole/ml., were used to produce the reference color in the manner described previously. The colored solutions produced were allowed to stand at room temperature, and the absorbance of samples of this solution was measured at intervals for a total of 90 min. elapsed time (Table IV).

Effects of Altering Reaction Conditions on Absorbance—The following results were observed when isolated changes in the previously stated procedure were investigated.

Table III—Effect of Concentration of Acetyl Sulfisoxazole on Absorbance[a]

Acetyl Sulfisoxazole, mg.	Absorbance (Average of Three Runs)
0.0	0.000
0.2	0.080
0.4	0.105
0.6	0.235
0.8	0.315
1.0	0.405
1.2	0.470
1.4	0.560
1.6	0.645
1.8	0.710
2.0	0.790

[a] Reproducibility of individual values was within the limitation of the spectrophotometer employed.

Table IV—Rate of Color Fading of Ferric Complex[a]

Minutes	Absorbance
0	0.560
1	0.560
2	0.550
3	0.550
4	0.545
5	0.545
6	0.545
7	0.540
8	0.540
9	0.540
10	0.540
11	0.535
12	0.530
13	0.530
14	0.520
15	0.520
30	0.500
45	0.480
60	0.465
75	0.450
90	0.440

[a] A blank for each sample was adjusted for 0.000 absorbance.

When the isopropanol solution was replaced with distilled water, the absorbance value for a 1-mg. sample of acetyl sulfisoxazole decreased from 0.405 to 0.28.

When the potassium hydroxide solution was replaced with distilled water, the absorbance value for a 1-mg. sample of acetyl sulfisoxazole decreased from 0.400 to 0.010, thereby showing essentially no reaction.

When the order of addition of the hydroxylamine hydrochloride solution and the potassium hydroxide solution was reversed, the absorbance value for a 1-mg. sample of acetyl sulfisoxazole decreased from 0.405 to 0.015.

When the amounts of the hydroxylamine hydrochloride solution, potassium hydroxide solution, and ferric chloride solution were increased, the absorbance values for a 1-mg. sample of acetyl sulfisoxazole were identical within experimental variation.

Quantitative Determination of Acetyl Sulfisoxazole in the Presence of Its Hydrolysis Products—The results shown in Table V were observed when the stated procedure was applied to acetyl sulfisoxazole alone and in an admixture containing its hydrolysis products. The values reported represent the average value of four determinations. The absorbance value of 0.470 for 1.5 mg. acetyl sulfisoxazole/10 ml. compares favorably to the absorbance value of 0.520 for 1.5 mg. acetyl sulfisoxazole/9 ml. volume.

CONCLUSIONS

A procedure allowing the quantitative analysis of acetyl sulfisoxazole was developed. The conditions necessary for optimum and reproducible results were established.

The procedure consists of diluting a 1-ml. sample of a methanolic solution of acetyl sulfisoxazole with 3 ml. of aqueous isopropanol solution (33.3% v/v). To this assay preparation, 1 ml. of saturated hydroxylamine hydrochloride solution (83 g. in 100 ml.) and 1 ml. of potassium hydroxide solution (10.5 *M*) are added and mixed. The reaction is instantaneous at room temperature. Subsequently, 2 ml. of hydrochloric acid solution (4.1 *M*) is added to acidify the solution to a pH between 0.5 and 1.5; then 1 ml. of ferric chloride solution (0.37 *M* in 0.1 *N* HCl) is added, and the solution is shaken. The absorbance of each sample is then determined at a wavelength of 540 nm. A straight-line relationship passing through the origin was observed in the plot of absorbance as a function of the initial concentration of acetyl sulfisoxazole over a range from 0 to 2 mg. of acetyl sulfisoxazole/4 ml. of assay preparation.

Table V—Quantitative Determination of Acetyl Sulfisoxazole in the Presence of Its Hydrolysis Products

Concentration/10 ml. Total Volume	Absorbance
Acetyl sulfisoxazole, 1.5 mg.	0.475
Acetyl sulfisoxazole, 1.5 mg., plus sulfisoxazole, 5 mg., and acetic acid, 3–4 mg.	0.470
Acetyl sulfisoxazole, 1.5 mg., plus sulfisoxazole, 5 mg., and sodium acetate, 4.95 mg.	0.470

SUMMARY

Known methods of analysis were examined for application to the quantitative determination of acetyl sulfisoxazole in the presence of sulfisoxazole. The official diazotization method does not differentiate between these two sulfonamides. Therefore, a spectrophotometric (colorimetric) method, involving the formation of ferric acetohydroxamate, was developed and employed. The effects of temperature, reaction time, quantity of reagents, and concentration of acetyl sulfisoxazole on the development of color were examined. The stated method provides for a relatively simple and rapid means by which the concentration of acetyl sulfisoxazole is quantitatively determined in the presence of its main hydrolysis product, sulfisoxazole.

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IR Determination of Trace Quantities of Dicyandiamide and Cyanamide in Guanidine Sulfate

TIBOR URBANYI and ARMIN WALTER*

Abstract □ A sensitive quantitative IR absorption method was developed for the determination of dicyandiamide as an impurity in a pharmaceutical preparation. This method is suitable for the determination of dicyandiamide in very low concentrations in insoluble guanidines and for the quantitative detection of dicyandiamide in many other compounds. The method is based on the determination of the nitrile stretching vibration in the region of 2200 cm.$^{-1}$. Although dicyandiamide forms as a by-product in an alkaline solution from the excess of cyanamide used in the reaction, the proposed method utilizes the separation of cyanamide and dicyandiamide by a simple extraction procedure, using methylene chloride and 2-ethoxyethanol as solvents. The sensitivity of the method was improved by using an internal standard technique. The superiority of this method over existing methods is discussed, along with the importance and usefulness of IR absorption, particularly in everyday pharmaceutical analysis.

Keyphrases □ Dicyandiamide—separation, IR analysis, particularly in guanidine sulfate □ Cyanamide—separation, IR analysis, particularly in guanidine sulfate □ Guanidine sulfate—separation, analysis of dicyandiamide and cyanamide □ IR spectrophotometry—analysis, trace dicyandiamide and cyanamide

Cyanamide, although a classical raw material for organic synthesis (1), was formerly prepared either in small quantity in a laboratory (2) or in commercial quantity for captive use. Its tendency to react with itself, predominantly to form dimers, was a barrier to commercial development until recently. Efforts to circumvent this tendency have succeeded, and the stabilized anhydrous monomer (3) and a concentrated aqueous solution have become available commercially. Tonnage agricultural and metallurgical applications have been described (4).

In the pharmaceutical industry, cyanamide is particularly useful in the synthesis of guanidine derivatives (5), but dimerization should be eliminated or, if unavoidable, minimized. In either case, analytical controls to assure drug purity are needed.

On reviewing the literature data of the determination of dicyandiamide and cyanamide, it was found that the methods can be classified into several categories: titrimetric, polarographic, colorimetric, gravimetric, and chromatographic.

To the authors' knowledge, only two IR spectrophotometric (6, 7) dicyandiamide determination procedures are reported in the literature. The measurement of the absorption in the nitrile region is based on the utilization of an internal standard in paraffin oil or in a KBr pellet. The authors claim that the method is fairly sensitive, but the particle size of the sample, especially in oil, must be critically controlled. Titrimetric procedures are commonly used for the quantitative determination of dicyandiamide (8) as well as for cyanamide (9). However, reducing agents and aminoguanidine should be removed before the silver nitrate titration. The authors pointed out that the speed of operation is critical. The polarographic method (10) for the determination of dicyandiamide is complicated and time consuming. Colorimetric methods (11) are based on the reaction with pentacyanoammoniumferrate, which yields a red complex with cyanamides. Gravimetric determinations are often used (12, 13); dicyandiamide is hydrolyzed and allowed to react with picric acid to form guanylurea picrate. This method suffers from the typical experimental problems that arise with the gravimetric technique. Chromatographic methods were reported which deal with the separation and detection of cyanamide and its derivatives (14, 15). However, both papers only gave data for the quantitative determination of urea and not for cyanamide and dicyandiamide. Quantitatively, as many as 16 cyanamide derivatives may be separated by this technique.

Since the industrial application of cyanamide has grown rapidly, it was obvious that a suitable analytical method was needed for its determination. It was found that unreacted cyanamide undergoes dimerization to dicyandiamide if the reaction mixture is slightly alkaline. However, in a process where the reaction mixture is neutral or slightly acidic, dimerization does not occur and cyanamide remains as an impurity.

This paper describes an IR absorption method which can be used routinely, primarily for the quantitative determination of dicyandiamide. Cyanamide, another possible impurity, can be determined in the parent compound with an additional extraction. The method is based on the measurement of the C≡N stretching vibration in the region of 2200 cm.$^{-1}$.

EXPERIMENTAL

A recording spectrophotometer[1], equipped with 0.1-mm. sodium chloride cells, was used to record the spectra. Methylene chloride and 2-ethoxyethanol[2] were the solvents.

Preparation of Stock Solutions—Solutions containing 1 mg./ml. cyanamide in methylene chloride and dicyandiamide in 2-ethoxyethanol were prepared.

Preparation of Standard Solutions—Pipet 4, 6, 8, and 10 ml. of stock solutions into separate 10-ml. volumetric flasks, and dilute to volume with the appropriate solvents.

Preparation of Sample Solutions—Accurately weigh 1 g. of sample (guanidine compound) into a centrifuge tube, and extract several times with a small portion of methylene chloride. Transfer the solution onto a sintered-glass funnel, and filter it into a 10-ml. volumetric flask. This solution is for the cyanamide assay. Extract the remaining dry powder further with several small portions of 2-ethoxyethanol, and collect the filtrate in another 10-ml. volumetric flask. This solution is for the determination of dicyandiamide.

[1] Perkin-Elmer IR 621.
[2] Cellosolve.

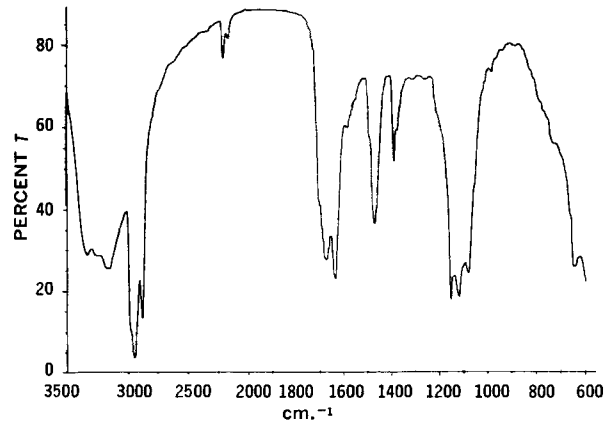

Figure 1—*IR absorption spectrum of the crude guanidine sulfate in a mineral oil mull.*

Procedure—Transfer the standard and sample solutions in turn to 0.1-mm. sodium chloride cells, and place the solutions into the spectrophotometer. To obtain reliable absorption values, the instrument is set at 3× ordinate scale and the speed, attenuation, and slit program are adjusted according to the instrument manual specification. The instrument is set to scan from 2550 to 2000 cm.$^{-1}$, using the appropriate solvents as blanks in the reference cell. By using the regular baseline technique, the absorbances at the maxima are calculated. The absorbance values of the standard solutions are plotted against the weight of the standards to obtain the calibration curve. The amount of impurities in the sample solutions is determined from the calibration curve in a similar manner.

The absorption caused by the stretching vibration of the triple bond of nitriles occurs at 2250 cm.$^{-1}$ for cyanamide and 2200 cm.$^{-1}$ for the dicyandiamide.

DISCUSSION

The IR absorption technique is seldom used for the quantitative determination of small amounts of impurities because of the lack of sensitivity. This technique is superior to other methods because of its specificity, but it can be applied only for quantitative determination of compounds that have strongly absorbing vibration groups. Cyanamide and dicyandiamide, the latter being the more probable contaminant, have a fairly strong absorption in a selective region which can be considered for quantitative determination.

Because the solubilities of cyanamide and dicyandiamide are quite different, the selection of proper transparent solvents for their separation was critical. Polar solvents were not transparent in the region of interest; they also dissolved the parent compound, which was undesirable because of potential interference. Nonpolar solvents are transparent in the region of interest, but the solubility of both cyanamide and dicyandiamide is negligible. Methylene chloride was found to be a suitable solvent for cyanamide because dicyandiamide

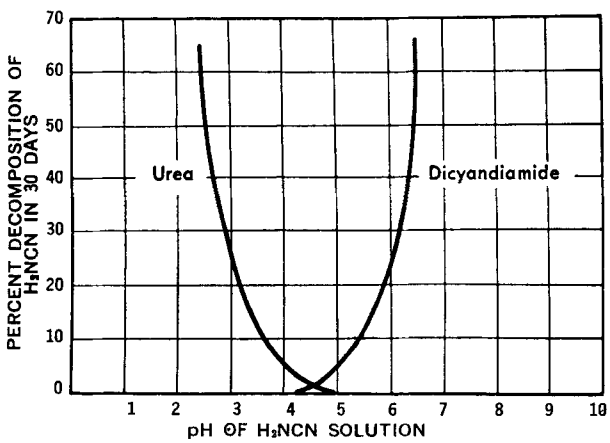

Figure 2—*Stability of cyanamide in aqueous solution.*

Table I—Reproducibility of Standard Absorbance Values

Percent Concentration	Cyanamide: Average[a] Absorbance	Cyanamide: Percent Standard Deviation	Dicyandiamide: Average[a] Absorbance	Dicyandiamide: Percent Standard Deviation
0.04	0.0510	0.62	0.0347	0.64
0.06	0.0785	0.65	0.0524	0.95
0.08	0.1027	1.32	0.0706	0.79
0.10	0.1278	0.54	0.0876	0.79

[a] Average of five determinations.

was completely insoluble and the solubility of the guanethidine compound was negligible. 2-Ethoxyethanol was found to be a suitable solvent for dicyandiamide, and the product had a low solubility. Although the cyanamide and dicyandiamide were both soluble in 2-ethoxyethanol, their separate determinations in a single solvent were impossible because of spectral interferences due to close absorption.

The IR spectrum in a mineral oil mull of the crude guanidine sulfate containing dicyandiamide as an impurity can be seen in Fig. 1. The weak absorption band observed at 2200 cm.$^{-1}$ is characteristic of the C≡N stretching frequency because of dicyandiamide remaining in the sample before recrystallization. The disappearance of this characteristic nitrile absorption band in a mineral oil mull after recrystallization is indicative of a higher quality compound; however, it does not mean that the impurity has been completely removed. The proposed method is sensitive enough to detect any remaining impurities which, in such cases, is below 0.5%.

The guanidine sulfate is basic enough to dimerize any cyanamide present to dicyandiamide. Although cyanamide is fairly stable in the solid state and in solution, in both cases it dimerizes rapidly to dicyandiamide in the presence of traces of alkali or heat. The stability of cyanamide in an aqueous solution can be seen in Fig. 2. To prove the dimerization of cyanamide, the following test was performed. A mole-to-mole portion of cyanamide dissolved in methylene chloride was mixed with basic guanidine sulfate, and the mixture was stirred overnight at room temperature. After the methylene chloride was completely removed, the solid sample was subjected first to methylene chloride and then to 2-ethoxyethanol extraction. The extraction with methylene chloride showed no evidence of the presence of cyanamide, while dicyandiamide was quantitatively recovered in the 2-ethoxyethanol extract. This test was controlled by TLC, and the absence of cyanamide and the presence of dicyandiamide were noted.

A similar test was carried out to demonstrate that the dimerization does not occur if the reaction mixture is neutral or slightly acidic. The guanidine monosulfate (molar base-to-acid ratio of 1:1) was mixed with a methylene chloride solution of cyanamide. The mixture was treated as before, and finally the dry powder was subjected to methylene chloride and 2-ethoxyethanol extraction. As expected, only the unchanged cyanamide was recovered. Tests were performed to exclude the possible formation of an impurity from the starting material and dicyandiamide. These experiments indicated that no interaction occurred between the two compounds under reaction conditions similar to those used in the process.

Table II—Separation of Cyanamide and Dicyandiamide by Extraction Procedure

	Cyanamide[a]	Dicyandiamide[a]
Average absorbance	0.0515	0.0549
Percent standard deviation	0.91	1.07
Percent recovery	100.0	101.0

[a] Average of five determinations.

Table III—Recovery of Dicyandiamide in Synthetic Mixture

Average absorbance[a]	0.0393
Percent standard deviation	0.57
Percent recovery	101.5

[a] Average of three determinations.

Table IV—Determination of Dicyandiamide in Crude Sample

Sample	Percent Dicyandiamide[a]
A	0.10
B	0.48
C	0.30

[a] Average of duplicate determinations.

Linear calibration curves, which follow Beer's law, were obtained for cyanamide and dicyandiamide using the pure substances in the appropriate solvents. The reproducibility of the absorbance values with consecutive determinations can be seen in Table I. Experimental data, however, indicate that the standard calibration curves are reproducible and linear over a wide range. Table II summarizes the data obtained for a synthetic mixture of cyanamide and dicyandiamide (4:6). These data indicate that satisfactory separation was achieved for these components using the proposed extraction procedure. The recovery of dicyandiamide, the only contaminant resulting from the preparation of guanidine sulfate, was further investigated utilizing synthetic mixtures. To a dicyandiamide-free sample of guanidine sulfate, a known amount of dicyandiamide was added; after proper mixing, it was extracted with 2-ethoxyethanol. The results of the recovery of dicyandiamide can be seen in Table III for consecutive determinations.

Fairly accurate results were obtained when several crude samples were analyzed for dicyandiamide when the amount of contamination was over 0.2%. The results of these tests are summarized in Table IV. However, the results in Table V indicate that purified samples usually have less than 0.2% of dicyandiamide. In the latter case, detection of the dicyandiamide, or in special cases the cyanamide, can be appreciably improved with a simple technique (16), which offers significantly greater sensitivity than the more conventional techniques. This technique is based on the fact that upon addition of a known amount of the impurity to the sample, the absorption of the impurity can be significantly increased if the detection of very small amounts is desired. If the amount of impurity added is substracted from the total absorbance value, the net amount of impurity found in the sample results. Figure 3 illustrates the application of the modified technique. This technique was successfully applied for dicyandiamide detection in the guanidine sulfate where the concentration of this component was as low as 0.015%.

SUMMARY

IR absorption, as employed in this study, was found to be a suitable technique for the quantitative determination of dicyandiamide as an impurity in guanidine sulfate. The proposed method can be used for the separation of cyanamide from the dicyandiamide with a simple extraction procedure. Both compounds are considered to be moderately toxic; therefore, a sensitive method is required for their determination in pharmaceuticals. The method presented is superior to procedures reported in the literature because of its increased sensitivity and specificity. The limit of detection of the cyanamides was improved by a simple technique where the sensitivity of the detection is critical. The formation of dicyandiamide during the process is explained, and the accuracy of the method is discussed.

Table V—Determination of Dicyandiamide in Recrystallized Samples by Modified Technique

Sample	Dicyandiamide Added, mg.	Total[a] Dicyandiamide Found, mg.	Percent Impurity
A	5.0	5.25	0.025
B	5.0	5.00	0.000
C	5.0	5.25	0.025
D	5.0	5.15	0.015

[a] Average of duplicate determinations.

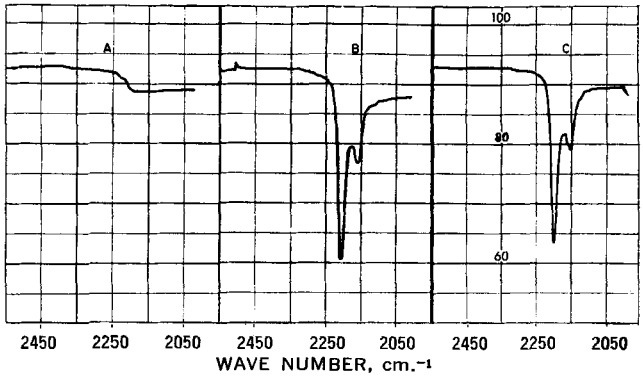

Figure 3—*IR absorption spectrum of: A, sample containing less than 0.1% dicyandiamide with regular method; B, same sample as in A with added 5.0 mg. dicyandiamide; and C, standard solution containing 5.0 mg. dicyandiamide.*

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Polarographic Determination of Ethacrynic Acid

EDWARD M. COHEN

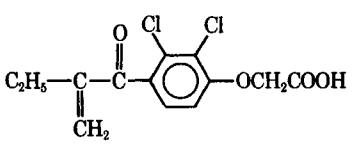
Abstract □ Polarography was applied to the analysis of ethacrynic acid in the presence of its principal degradation product, a dimer. The application of the polarographic assay to dosage forms is discussed, and some stability data are given.

Keyphrases □ Ethacrynic acid—polarographic assay, dosage forms □ Polarography—analysis, ethacrynic acid

Ethacrynic acid (I)[1] is a potent saluretic agent, chemically unrelated to previous diuretic agents. The chemical synthesis and biological properties of ethacrynic acid and a number of its homologs and analogs are given elsewhere (1). These studies established that the α,β-unsaturated aryl ketone moiety was essential for optimum biological activity. Any alteration of this grouping, such as saturation of the double bond or reduction of the ketone to the corresponding alcohol, resulted in a marked diminution in diuretic activity. The α,β-unsaturated ketone group is quite reactive chemically. A number of agents such as sodium bisulfite, mercaptans, and bromine add rapidly across the double bond. In addition, ethacrynic acid undergoes a Diels-Alder type of condensation to produce a dimer (II) (2).

Dimerization occurs under a variety of conditions and is the principal degradative pathway for the compound in pharmaceutical dosage forms. This finding led to a search for assay procedures that would distinguish between intact ethacrynic acid and the dimer. The usual bromination assay proved unsuitable, since the reactive double bond of the dimer takes up bromine quantitatively. The UV absorbances of ethacrynic acid ($\lambda_{max.}$ 271 nm., $A^{1\%}_{1\ cm.}$ 117 in CH_3OH) and the dimer ($\lambda_{max.}$ 261 nm., $A^{1\%}_{1\ cm.}$ 127) were not sufficiently different to distinguish between the compounds in a stability-indicating assay procedure.

A procedure was devised for direct measurement of II in the presence of ethacrynic acid based on the reaction of ethacrynic acid with excess sodium bisulfite (by addition across the double bond in neutral aqueous solution) to form a water-soluble nonextractable adduct while II remained unreacted (3). Acidification of the neutral solution, followed by extraction with benzene, removed II, leaving the polar ethacrynic acid–bisulfite adduct in the acidic aqueous layer. The separated dimer was then



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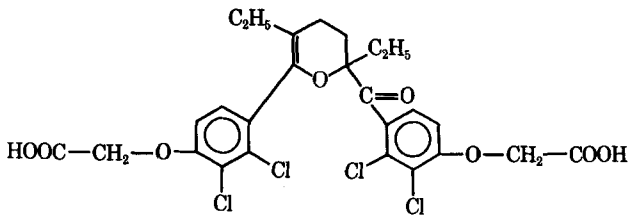

II

measured by UV analysis. Other compounds, which either do not react with sodium bisulfite or are extractable under these conditions, accompany the dimer in the benzene layer.

On the assumption that ethacrynic acid would be reducible at the dropping mercury electrode (DME), polarography was tried as an assay procedure. Preliminary experiments revealed that it gave well-defined polarographic steps which could be used to assay for intact ethacrynic acid in the presence of II. The polarographic assay procedure developed was successfully applied to the analysis of the solid, as well as its pharmaceutical dosage forms, and some of the data obtained are given in this publication.

EXPERIMENTAL

Equipment and Reagents[2]—Polarographic measurements were carried out with an electrochemograph[3]. A standard H-cell was used containing a saturated calomel electrode (SCE) separated from the sample compartment by an agar plug (3% agar dispersion prepared with saturated KCl solution) and a fritted-glass diaphragm. The DME was employed at a drop time of 2.5 sec., and at an ethacrynic acid concentration of 0.2 mg./ml. in the supporting electrolyte described below, a wave height of 4.2 μamp. was obtained for the ethacrynic acid step.

A 0.50 *M*, pH 8.0, phosphate buffer, used as part of the supporting electrolyte, was prepared as follows. Dissolve 68.1 g. of KH_2PO_4 in about 400 ml. of purified water. Add a sufficient quantity of 1.0 *N* sodium hydroxide to adjust the pH to 8.0 and dilute to 1000 ml. with purified water. Other components of the supporting electrolyte were Emulphor EL-620[4] (maximum suppressor), used as a 0.1% stock solution in purified water, and alcohol USP. All chemicals employed were the best grade available and were not purified further.

Polarographic Procedure—For ethacrynic acid tablets, accurately weigh a portion of finely powdered tablet sample, equivalent to about 100 mg. of ethacrynic acid, into a 50-ml. volumetric flask. Add about 35 ml. of alcohol and mechanically agitate the flask for about 30 min.; then dilute to the mark with alcohol. Centrifuge a portion of the dispersion and pipet 5 ml. of the clear supernatant into a second 50-ml. volumetric flask containing 25 ml. of phosphate buffer and 1 ml. of the Emulphor EL-620 solution. Dilute to the mark with purified water and mix well. Transfer a portion of this solution to the H-cell and purge with nitrogen. At the end of the nitrogen purge, insert the DME, direct the nitrogen stream over the top of the solutions, and record the polarogram from −1.0 v.

[1] Edecrin, Merck Sharp & Dohme, West Point, Pa.

[2] The ethacrynic acid, dimer, and other compounds discussed here were synthesized and characterized at the Merck Sharp & Dohme Research Laboratories, West Point, Pa., and Rahway, N. J.

[3] Leeds and Northrup model E.

[4] Antara Chemicals, Division of GAF, New York, N. Y.

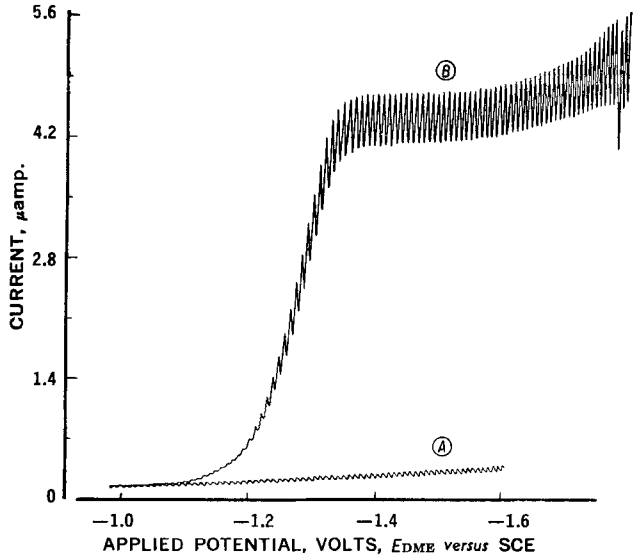

Figure 1—*Ethacrynic acid polarogram in pH 8.0 phosphate buffer containing 10% alcohol. Key: A, blank; and B, 0.2 mg./ml. of ethacrynic acid.*

versus the SCE to −1.8 v. *versus* the SCE. Prepare both a blank solution and a standard solution (0.2 mg. ethacrynic acid/ml.), using a standard reference lot of ethacrynic acid, in the same supporting electrolyte as employed for the test sample. Obtain the polarograms for the blank and standard solutions in the same way.

For sodium ethacrynate for injection, reconstitute a vial of 1 ml. of purified water and dilute the contents to 25 ml. with alcohol. Pipet 5 ml. of the reconstituted solution into a 50-ml. volumetric flask and proceed as described for the tablet assay starting with: "containing 25 ml. of phosphate buffer. . . ."

Calculations—Take the net step height of the recorded polarograms as the difference between the step height of the standard or sample solution at −1.45 v. *versus* the SCE and the height exhibited by the blank solution at −1.45 v. *versus* the SCE. Use the average of the recorded pen excursions for computing the step height if damping is employed:

$$\text{mg. found} = \frac{S_1}{S_2} \times \text{mg. standard} \times \text{dilution factor} \quad \text{(Eq. 1)}$$

where mg. found = milligrams of ethacrynic acid found in portion of sample taken for analysis, S_1 = net step height of sample solution, S_2 = net step height of standard solution, and mg. standard = milligrams of standard taken for analysis.

RESULTS AND DISCUSSION

General Polarographic Behavior—Ethacrynic acid exhibits a well-defined polarographic step at the DME in pH 8.0 buffer containing 10% alcohol, with an $E_{1/2}$ of −1.28 v. *versus* the SCE (Fig. 1). The $E_{1/2}$ is pH dependent, shifting to more negative potential with increasing pH. At a pH below 7, the single wave is split into two whose total height is about the same height as the single wave exhibited at a higher pH. A second, more negative, less well-defined step is also observed for ethacrynic acid at pH 8.0, with an $E_{1/2}$ of −1.75 v. The height of the second step is about one-tenth the height of the first step. The dimer (II) gives a poorly defined polarographic step, with an $E_{1/2}$ of −1.68 v. *versus* the SCE, which does not interfere with the first ethacrynic acid step (Fig. 2).

The height of the first ethacrynic acid step is proportional to the concentration over a fairly wide range. No deviation from linearity for a calibration curve was noted for solutions up to about 2×10^{-3} m*M* in ethacrynic acid.

Specificity of Polarographic Assay—As previously indicated, II does not interfere with the ethacrynic acid step (Fig. 2). Dihydroethacrynic acid, the bisulfite–ethacrynic acid adduct, the cysteine–ethacrynic acid adduct, and several other ethacrynic acid reaction products, in which the double bond of the α,β-unsaturated ketone

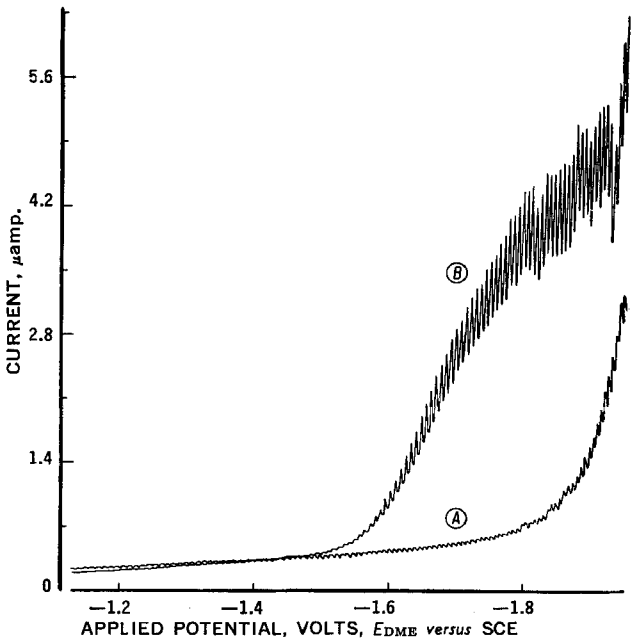

Figure 2—*Dimer (II) polarogram in pH 8.0 phosphate buffer containing 10% alcohol. Key: A, blank; and B, 0.2 mg./ml. of the dimer (II).*

moiety was "saturated," likewise did not interfere with the ethacrynic acid step.

Precision Studies—The relative standard deviation for the mean of duplicates for both ethacrynic acid tablet assays and sodium ethacrynate intravenous assays was within ±2% based on 6 months of experience with the assays in several laboratories.

Interference Studies—None of the excipients employed in the ethacrynic acid formulations was found to interfere with the assay at the ethacrynic acid dilution normally used (final concentration of 0.2 mg./ml.).

Stability of Ethacrynic Acid in Dosage Forms—No significant amount of degradation was noted for ethacrynic acid in both dosage forms stored for up to 36 months at room temperature. Some loss of intact ethacrynic acid was noted by polarography in samples stored at 37° for 30 months. The thermal instability of crystalline ethacrynic acid at elevated temperatures was demonstrated by heating the compound alone at 105° for 44 hr. About 30% intact ethacrynic acid was found (Fig. 3). By way of contrast, crystalline ethacrynic acid samples that had been stored in screw-capped, glass bottles at

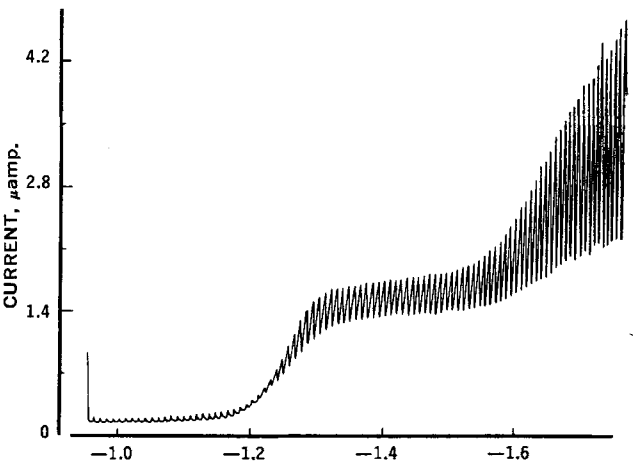

Figure 3—*Ethacrynic acid polarogram after storage of solid for 44 hr. at 105°. Wave height shown is about 30% of expected for intact ethacrynic acid.*

room temperature for almost 4 years did not exhibit any degradation.

Stability of Ethacrynic Acid in Solution—A solution of sodium ethacrynate (pH 6.8, 20 mg./ml.) showed no loss of intact ethacrynic acid after 3 days at room temperature. Losses were noted at elevated temperatures, however, indicating that sterile solutions of ethacrynic acid should be prepared by suitable filtration rather than by autoclaving.

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TECHNICAL ARTICLES

Effects of Moisture in Compaction of Particulate Material

JOHN E. REES* and EDWARD SHOTTON

Abstract □ By using an ideal particulate system of crystalline sodium chloride and three liquids—water, decahydronaphthalene, and light liquid paraffin—the role of moisture in the compaction process was investigated. Interrelated effects of liquid at the die wall boundary and within the compact are discussed. Although differences in the behavior of the three liquids could be attributed partly to the viscosity difference, water apparently exerted a boundary lubricant effect in addition to hydrodynamic properties. Despite its lower viscosity, water was, therefore, a more effective lubricant than light liquid paraffin. In general, decahydronaphthalene adversely affected the compaction process. Cumulative changes in the difference between applied and transmitted force during consecutive compressions can be used to assess the lubricant efficiency of materials.

Keyphrases □ Compaction process—role of moisture, ideal particulate system □ Compression changes—lubricant efficiency assessment, ideal particulate system □ Water as lubricant—with sodium chloride, role in compaction process □ Die wall effects—studied using ideal particulate system of sodium chloride and water, decahydronaphthalene, or paraffin

During compaction of particulate material, the proportion of applied force transmitted to the die wall is influenced by several factors including: (*a*) the radial component of the applied force, and (*b*) the effective area of contact and coefficient of friction at the die wall. Since interparticulate lubrication increases the ratio of radial stress to axial stress (1), application of lubricant to the die is more effective than the addition of lubricant to a powder before compression (2). However, lubrication of both the die wall and interparticulate junctions produces a net decrease in the die wall reaction (1), because reducing the coefficient of interparticulate friction usually has little effect on die wall friction compared with a decrease in the coefficient of friction at the die wall.

A reduction in friction may be produced by: (*a*) hydrodynamic lubricants, forming a finite layer of low shear strength which prevents contact between adjacent surfaces; and (*b*) boundary lubricants which reduce adhesive forces between the surfaces.

The high viscosity of a boundary lubricant may produce an excessive resistance to shear (3). Therefore, a medium viscosity is often preferable, although the resistance to contact between sliding surfaces is less. Whereas values of shear strength were not directly related to lubricant efficiency, Lewis (4) found that lubricants with a lower melting point were more efficient, possibly due to the formation of a film on the die. Strickland *et al.* (5) showed that if more than 1% stearic acid was present in a sulfathiazole granulation, the semisolid material itself resisted compression. For a liquid lubricant such as mineral oil, however, increasing the concentration to 4% produced a continual decrease in the required compression force.

Numerous authors (6–8) commented on the effects of moisture during compaction. Anomalous compression results, attributed by Train (9) to electrostatic effects, were eliminated by humidifying the material. Wolff *et al.* (10) observed that a nonlubricated granulation could not be compressed when the moisture content was greater than 4%, although the results of Stephenson (11) and Shotton and Rees (8) showed that die wall friction may be reduced in the presence of moisture.

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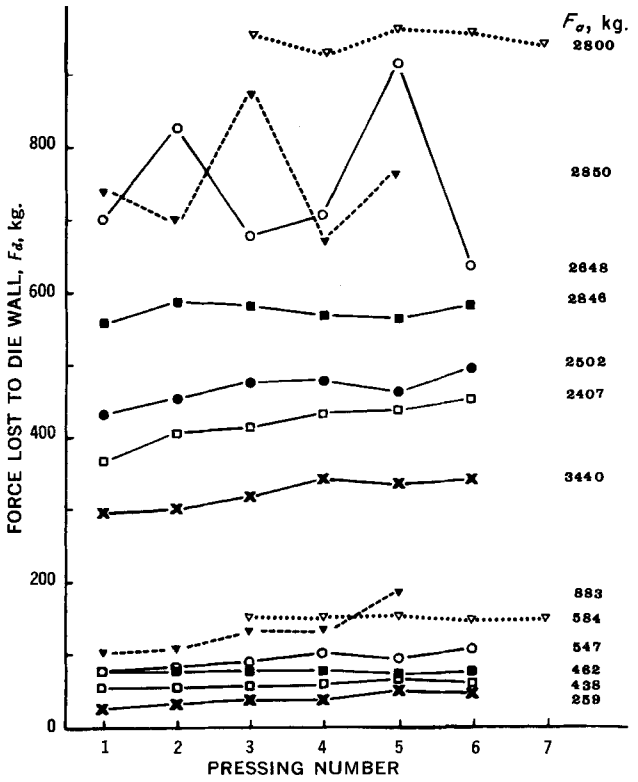

Figure 1—*Effect of moisture on the changes in* F_d *with pressing number. Dried material: conditioned die* (▽), *and clean die* (▼). *Moisture content: 0.02%* (○), *0.16%* (■), *0.34%* (●), *0.55%* (□), *and 10%* (×).

EXPERIMENTAL

Selection and Characterization of Materials—A pharmaceutical tablet is usually a complex aggregate of particles of active substance and several excipients. The compression behavior of such a system depends on the interrelated properties of the constituents; therefore, it is preferable to use a simple system to study the compaction process.

Sodium chloride BP was selected as a crystalline material that forms a coherent compact by direct compression. The crystals were screened to obtain 30–40-, 60–80-, and 80–100-mesh fractions, corresponding to mean particle sizes of approximately 500, 220, and 160 μ. Adhering fines were then removed using an air-jet sieve[1] with a 100-mesh screen. The density (2.17 g. cm.$^{-3}$) was determined pycnometrically, and the compression weight was then calculated as sufficient material (982 mg.) to produce a plane-faced, cylindrical compact of 1.2-cm. (about $^{15}/_{32}$-in.) diameter and 0.4-cm. (about $^{5}/_{32}$-in.) theoretical thickness at zero porosity.

Since the compaction behavior might be influenced by solvent properties of a liquid additive, it was decided to compare the effects of moisture and a nonsolvent. It was difficult experimentally to minimize evaporation of volatile liquids other than water. Therefore, a liquid of low volatility and high flash point, immiscible with water and a nonsolvent for sodium chloride, was required. Light liquid paraffin BPC was selected, but its viscosity (31.2 cps.) is appreciably higher than that of the moisture present as a saturated aqueous solution of sodium chloride (approximately 1.8 cps.) on the crystal surfaces. Thus, experiments were also made with decahydronaphthalene[2], which possesses fairly low volatility and a viscosity of 2.3 cps.

A reduction in particle size by the solvent action of water decreases the weight of solid in each compression sample. In most experiments, 30–40-mesh material was used; but to assess the possible effects of a decrease in particle size by dissolution, 60–80- and 80–100-mesh fractions of dried material were studied. The effect of reducing the weight of 30–40-mesh material to 928 mg. also was investigated.

Addition of Moisture to Particulate Material—Exposing the material to relative humidities of 60–90% produced a suitable range of moisture contents. Individual samples of dried sodium chloride, sufficient to produce single compacts, were stored in uncapped glass vials above appropriate saturated salt solutions. When the required moisture content was reached, each vial was sealed with a polyethylene closure, and compression experiments were completed with a minimum delay.

Moisture Determination—The moisture content of representative samples was determined using the remote weighing assembly of a gram electrobalance[3] installed in a vacuum chamber with an observation panel. A humidified sample was weighed and then dried for 1 hr. in an oven at 110° and 10 mm. Hg. Without cooling, the sample was replaced on the electrobalance, and the vacuum chamber was rapidly evacuated to 10 mm. Hg. After 15 min., air was allowed to enter the chamber through a drying tube before the dried sample was weighed.

Addition of Nonsolvents to Particulate Material—A uniform distribution of 0.1–10.0% decahydronaphthalene or light liquid paraffin was obtained by adding the calculated weight of liquid to a bulk quantity of sodium chloride in a cylindrical container, followed by standardized agitation. The container was shaken manually in a three-dimensional movement with simultaneous rotation about its axis[4]. Uniformity in weight of selected compacts from which the liquid was removed by chloroform extraction indicated that replicate samples of the compressed material were identical. Any loss of liquid during compaction could also be confirmed using this solvent extraction technique.

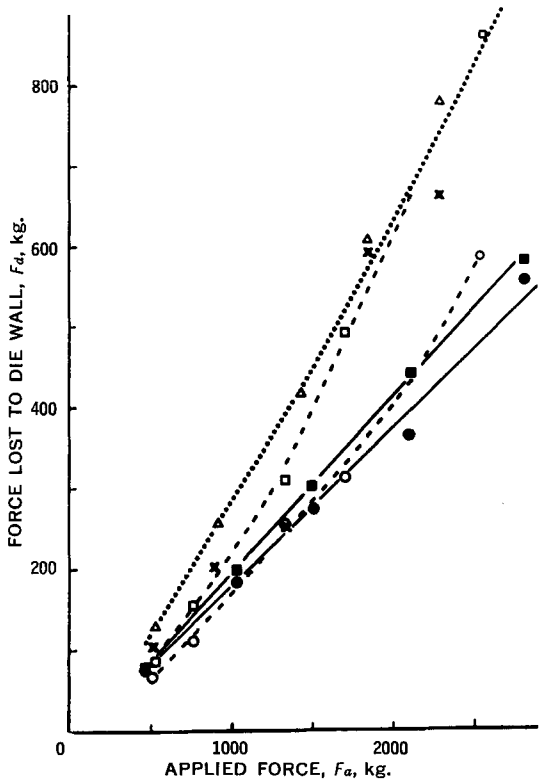

Figure 2—*The relations between applied force and* F_d *during the first and sixth pressings in the presence of a low concentration of liquid. Liquid content: 0.16% moisture: 1st* (–●–) *and 6th* (–■–), *0.1% light liquid paraffin: 1st* (- -○- -) *and 6th* (- -□- -), *0.1% decahydronaphthalene: 1st* (×) *and 6th* (△). *Dried material in a conditioned die* (...).

[1] Alpine Air Jet Sieve, model 200, Alpine, Augsburg, West Germany.
[2] Dekalin MAR (microanalytical reagent grade), Hopkin & Williams Ltd., Chadwell Heath, England. It consisted of 36% *trans*-isomer and 64% *cis*-isomer.
[3] Cahn Instrument Co., Paramount, Calif.
[4] As in a Turbula mixer, Willy A. Bachofen, Basle, Switzerland.

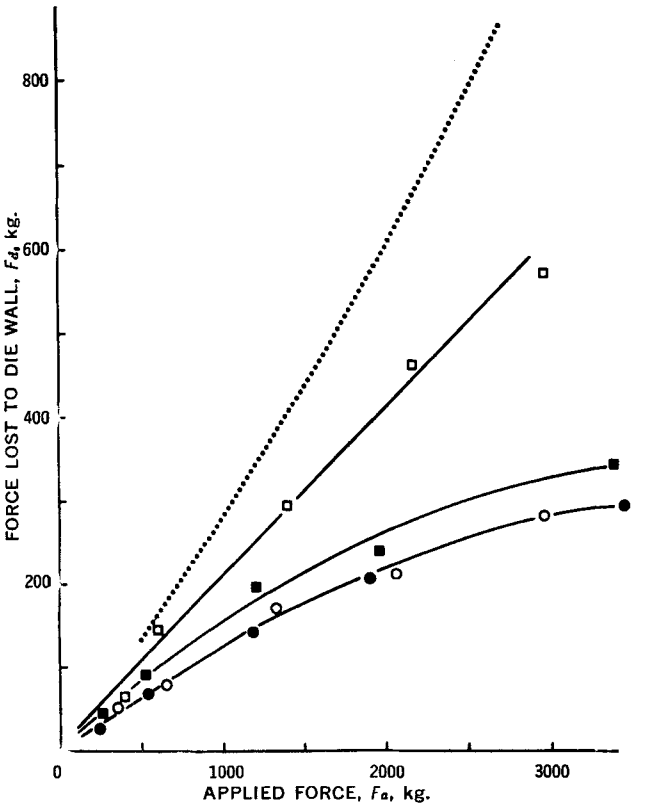

Figure 3—*The relations between applied force and* F_d *during the first and sixth pressings in the presence of 10% moisture: 1st* (●) *and 6th* (■), *and 10% light liquid paraffin: 1st* (○) *and 6th* (□). *Dried material in a conditioned die* (...).

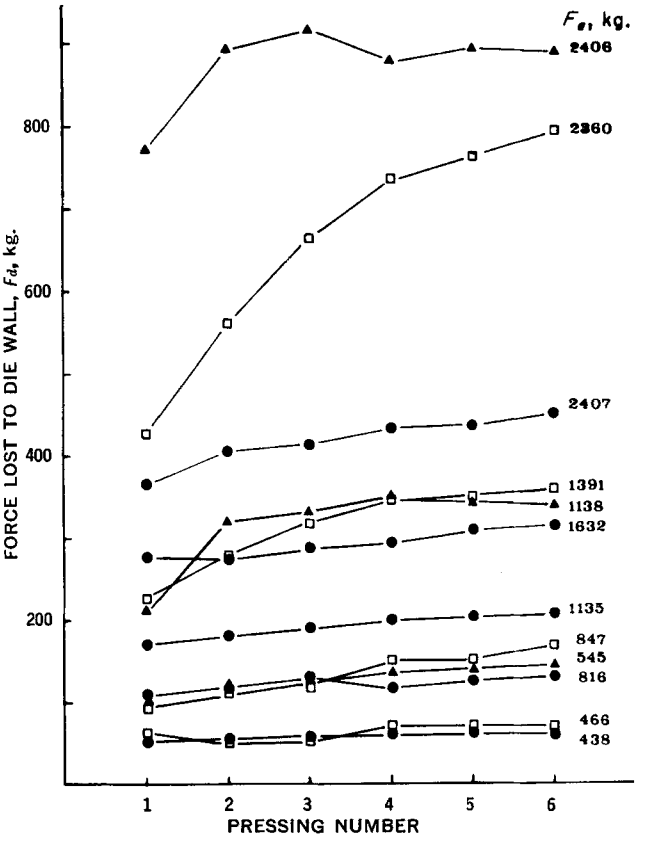

Figure 4—*Dependence of* F_d *on the number of pressings in the presence of 0.55% moisture* (●), *0.5% light liquid paraffin* (□), *and 0.5% decahydronaphthalene* (▲).

Compression Equipment and Measurement of Compaction Forces—Plane-faced punches[5] of 1.2-cm. (about $^{15}/_{32}$-in.) diameter were used with a single-punch eccentric tablet press[6]. The upper punch and the lower punch holder were instrumented (12) using 0.31-cm. (0.125-in.), linear foil resistance strain gauges[7], which were coated with a polysulfide rubber compound[8] to protect them from atmospheric humidity. Details of the techniques for calibration of the strain gauge circuits were described elsewhere (12). The applied loads, corresponding to a 0.1% resistance change in the gauge circuits, were 1027 kg. for the upper punch and 3151 kg. for the lower punch holder.

The strain gauge circuits were connected to separate channels of a carrier amplifier[9] and a six-channel recorder[10] with pencil-type moving-coil galvanometers[11]. Galvanometer deflections were recorded on UV-sensitive paper[12], and the measurements were converted to force data using the calibration values already quoted. The difference, F_d, between the force applied by the upper punch, F_a, and the force transmitted to the lower punch, F_b, during a compression cycle is a function of the force lost to the die wall due to friction.

Compression Technique—To prevent rapid loss of moisture from humidified sodium chloride samples, it was necessary to establish an elevated ambient relative humidity in equilibrium with the particular samples being compressed. Similarly, to avoid moisture uptake during the compression of dried sodium chloride and samples containing light liquid paraffin or decahydronaphthalene, the ambient conditions were controlled at 20% relative humidity. It was not possible to study the compression characteristics of samples containing more than 2% decahydronaphthalene because of rapid loss by evaporation.

Six compacts were prepared at each of five compression settings of the tablet press, the punches and die being cleaned before studies at a different pressure.

For sodium chloride containing no liquid, the weighed samples were dried for 1 hr. at 110° and stored over silica gel for 24 hr. before use. Compression behavior was investigated in a clean die and in a die conditioned by the previous compaction of material (8).

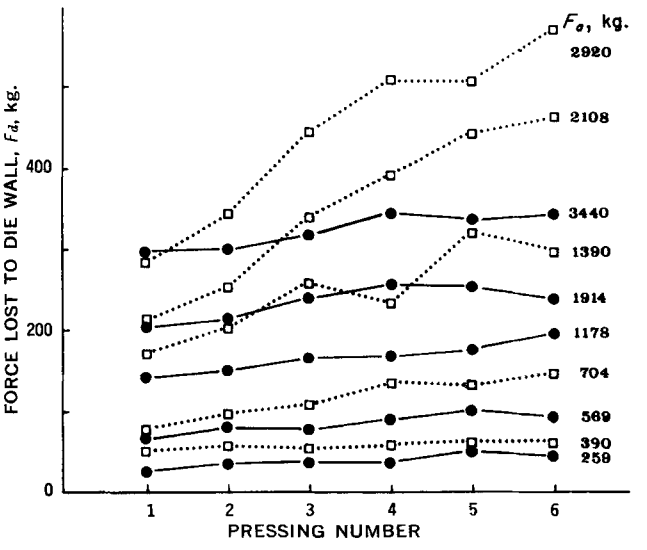

Figure 5—*Dependence of* F_d *on the number of pressings in the presence of 10% moisture* (●) *and 10% light liquid paraffin* (□).

[5] Lewin Engineering, London, England.
[6] W. Lehman & Co., London, England.
[7] Saunders Roe, East Cowes, Isle of Wight, England.
[8] PR 720, British Paints Ltd., London, England.
[9] Type 1070, New Electronic Products, London, England.
[10] Type 1050, New Electronic Products, London, England.
[11] Type BB, undamped frequency 250 c/s, New Electronic Products, London, England.
[12] Oscilloscript D, Gevaert, Mortsel, Belgium.

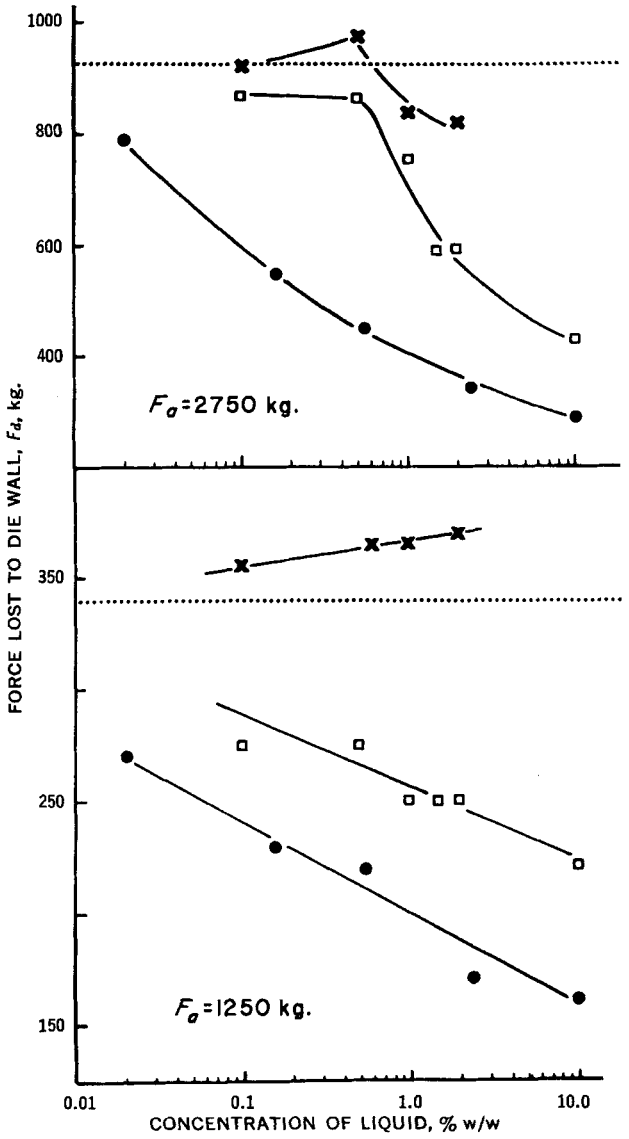

Figure 6—*Effect of liquid concentration on the force lost to the die wall at high (2750 kg.) and low (1250 kg.) applied force. Liquids: moisture* (●), *light liquid paraffin* (□), *and decahydronaphthalene* (×). *Dotted line represents the value of* F_d *for dried material pressed in a conditioned die at each applied force.*

Relative Volume of Compacts—Immediately after ejection, the dimensions of each compact were measured using a thickness gauge, and the weight was determined. These measurements, and the experimentally determined density of the crystalline material, were used to calculate the ratio of the observed volume of the compact to the volume of material present as a true solid.

RESULTS AND DISCUSSION

Figure 1 shows, for a series of moisture contents, the relation between the force lost to the die wall and the number of compacts pressed in a die at high and low applied force. Although 0.02% moisture reduced F_d, the fluctuating values showed that efficient lubrication could not be maintained during compaction at high pressure. Higher moisture contents further reduced F_d, and only small changes in the value of F_d occurred between the first and sixth compressions.

For material containing the same amount of water or light liquid paraffin, the values of F_d during the *first* compression at each pressure were almost equal (Figs. 2 and 3). However, during consecutive compressions with light liquid paraffin, the die wall force increased (Figs. 4 and 5); during the sixth compression with 0.1% light liquid paraffin at high pressure, F_d was the same as for dried material pressed in a conditioned die (Fig. 2). There was little evidence of a die wall lubricant effect of decahydronaphthalene even during the first compression (Figs. 2 and 4).

Despite the low viscosity of moisture, there was less cumulative contamination of the die wall with compressed material than in the presence of the other liquids, as shown by the relation between F_d and the number of consecutive compressions. This finding may be attributed to a boundary lubricant effect of moisture, which supplements the hydrodynamic effect shown by light liquid paraffin and decahydronaphthalene. If the lubricant film breaks down and the shear strength of bonds between the die and the compressed material exceeds that of the material itself, contamination of the die wall occurs. A reduction in the adhesive forces at the die wall by a boundary effect of moisture reduces this effect.

Figure 6 summarizes the effect of liquid concentration. Each coordinate represents the mean value for six compressions. Although the replicate values of F_d increased to different extents (Figs. 4 and 5), the graphs clearly indicate differences in the behavior of the three liquids. In all cases, moisture produced the lowest force lost to the die wall; with an increasing moisture content, there was a continual decrease in F_d. It was suspected that the increases in F_d with decahydronaphthalene (Fig. 6) were due to interparticulate lubrication, which increased the radial component of the applied force as reported for other systems (1, 13, 14). Although this almost certainly occurred with moisture and light liquid paraffin, the values of F_d were consistently less than for dried material since these two liquids are more effective die wall lubricants than decahydronaphthalene.

At 2750 kg. of applied force, samples containing more than 0.5% light liquid paraffin or decahydronaphthalene showed a decrease in F_d (Fig. 6), which was associated with expression of liquid to the die wall. Determination of the residual liquid after compression also showed that a higher viscosity retarded the expression of liquid during consolidation. This result increases the interparticulate lubricant effect of light liquid paraffin and may contribute to the higher values of F_d than for moisture. Air enclosed by liquid in the voids increases the force transmitted radially to the die but also facilitates expression of liquid. Furthermore, liquid that occupies a large proportion of the void space offers a hydrodynamic resistance to consolidation of a compact.

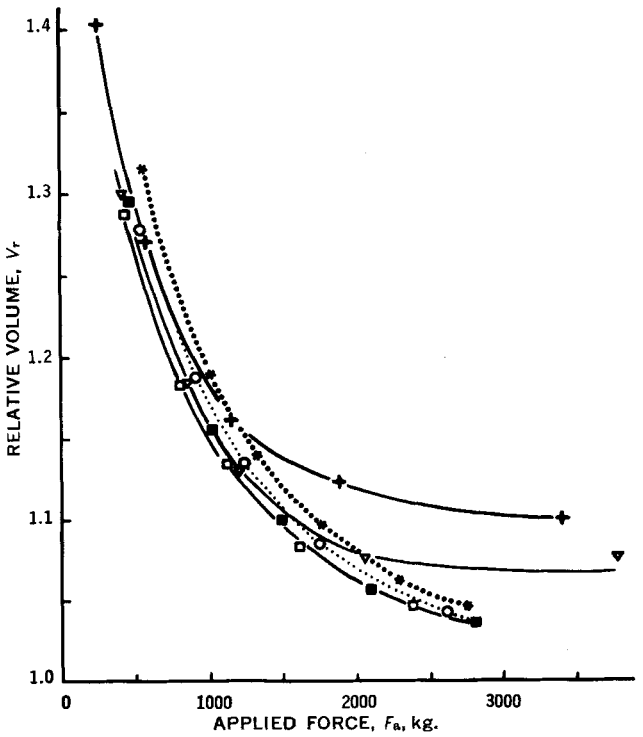

Figure 7—*Effect of applied force on the consolidation of compacts containing moisture. Moisture content: 0.02%* (···○···), *0.16%* (■), *0.55%* (□), *2.4%* (▽), *and 10%* (+). *Dried material in a conditioned die* (···*···).

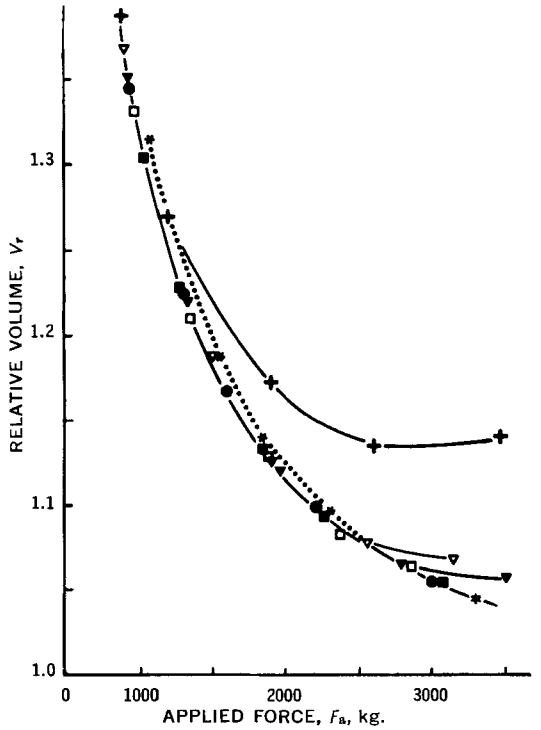

Figure 8—*Effect of applied force on the consolidation of compacts containing light liquid paraffin. Liquid concentration: 0.1% (■), 0.5% (□), 1.0% (●), 1.5% (▼), 2.0% (▽), and 10% (+). Dried material in a conditioned die (···*····).

Many of these conclusions are supported by the results shown in Figs. 7 and 8. Since moisture provided more effective lubrication, the results (Fig. 7) show clearly the effect of liquid concentration. Increasing the moisture content to 0.55% reduced the relative volume, but the hydrodynamic resistance to consolidation in the presence of 2.4 and 10% moisture reversed this trend, even at low applied pressure. The results for light liquid paraffin show similar effects (Fig. 8), but, in most cases, the relative volume was higher than with moisture. Negligible improvement in consolidation was observed with decahydronaphthalene at applied forces below 800 kg. At higher forces, the consolidation of material was restricted; above 2000 kg., a hydrodynamic resistance to consolidation was evident.

A small decrease in the thickness of a compact by a reduction in the compression weight from 982 to 928 mg. produced only small changes in the force lost to the die wall and the relative volume (15). Even 10% moisture dissolves less than 4% of the solid material, and lower concentrations have proportionately less effect. It was concluded that this effect of solvent action was negligible.

A reduction in particle size of about 60% from 30–40 to 60–80 mesh increased the force lost to the die wall and the relative volume of the compacts produced. However, since even with 10% moisture the reduction in mean linear dimension of particles will be only about 1%, size reduction by solvent action probably had no significant effect on the forces of compression or the relative volume of a compact.

Although moisture caused a rounding-off of many sodium chloride crystals due to preferential dissolution at the edges and corners, it is unlikely that this change in particle shape facilitated compaction. According to Lazarus and Lachman (16), rounded surfaces are characteristic of crystals of potassium chloride which cannot be readily compressed.

Huffine (17) considered that moisture increases the resistance to consolidation of particulate sodium chloride due to increases in the tensile strength and yield point of crystals when surface defects are removed by dissolution. However, there is some doubt (18) whether this conclusion is valid, and the present results show no evidence of this effect.

SUMMARY AND CONCLUSIONS

1. Moisture, light liquid paraffin, and decahydronaphthalene produce several interrelated effects during compaction of particulate sodium chloride, including: (*a*) die wall lubrication, (*b*) interparticulate lubrication, (*c*) hydrodynamic resistance to consolidation, and (*d*) expression of interstitial liquid to the die wall.

2. Increases in force lost to the die wall, F_d, during consecutive compressions represent cumulative contamination of the die wall. This criterion may be used to assess the lubricant efficiency of materials.

3. In the presence of moisture, the force lost to the die wall and the increases in this parameter with consecutive compressions were appreciably less than with light liquid paraffin or decahydronaphthalene. It is concluded that moisture possesses boundary lubricant properties in addition to the hydrodynamic effects shown by light liquid paraffin.

4. There was no evidence of an optimum lubricant concentration of moisture or light liquid paraffin. However, concentrations of liquid exceeding 0.5% caused a hydrodynamic resistance to consolidation of compacts.

5. With more than 0.5% liquid, F_d decreased as liquid was expressed to the die wall from within a compact.

6. Decahydronaphthalene has a negligible die wall lubricant effect. High values of F_d were attributed to an increase in the radial component of applied force by interparticulate lubrication.

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Timed-Release Tablets Employing Lipase–Lipid–Sulfamethizole Systems Prepared by Spray Congealing

KARAMAT A. JAVAID*, JULIAN H. FINCHER, and CHARLES W. HARTMAN†

Abstract □ *In vitro* dissolution profiles of some spray-congealed formulations of sulfamethizole, incorporated in a lipid–lipase matrix and compressed into tablets, are reported. The formulations employ a pancreatic lipase–glyceryl trilaurate and glyceryl tristearate system, with enzyme–substrate combinations serving as a release-controlling vehicle to produce a timed-release effect. The granules made by spray congealing were compressed into tablets, and *in vitro* dissolution tests were carried out in simulated fluids. The main portion of the drug was released through the hydrolysis of substrate by lipase, and the amount of drug released after 1.5 hr. was dependent upon the lipase activity measured in the form of percent hydrolysis of substrate. Effects of lipase accelerators such as calcium ions and glyceryl monostearate on lipase activity were evaluated in terms of drug release. Different concentrations (2, 5, and 8% w/w) of calcium carbonate increased the drug release. Lower concentrations (1, 2, and 5% w/w) of glyceryl monostearate increased while higher concentrations (7 and 10% w/w) decreased the drug release.

Keyphrases □ Timed-release tablets—spray-congealed lipase–lipid–sulfamethizole systems □ Lipase–lipid–drug system—effect of lipase accelerators on drug-release rates □ Drug-release rates—lipase–lipid–sulfamethizole systems □ Calcium—as lipase accelerator, effect on drug release from timed-release tablets □ Glyceryl monostearate—as lipase accelerator, effect on drug release from timed-release tablets

The physiological functions of the GI tract are the digestion and absorption of nutrient materials and medicaments. Many physiological factors may influence the release and absorption of drug from the GI tract; one such factor is the effect of digestive secretions of the alimentary canal. The overall problem in standardizing absorption from oral dosage forms involves overcoming the variability of the physiological, physical, and chemical properties of the GI tract. The most practical and successful methods of controlling intensity and duration of drug action depend on the control of release and absorption. Timed release of a drug can possibly be obtained by incorporating the drug in an enzyme–substrate matrix. Brewton (1) showed that triamcinolone release can be controlled from encapsulated granules employing lipase–lipid–drug combinations. Lybrand (2) demonstrated the effect of proteinase–protein–drug systems on the dissolution rates of sulfanilamide tablets.

The purposes of this investigation were: (*a*) to prepare a timed-release sulfamethizole tablet employing a spray-congealed lipase–lipid–drug system, and (*b*) to study the release of drug controlled by the digestion of substrate by lipase in the formulation.

Table I—Quantities, in Milligrams, of Ingredients in 200 mg. of Spray-Congealed Drug–Lipid Granules

Formulation	Mesh Size	Sulfamethizole	Glyceryl Tristearate	Glyceryl Trilaurate	Lipase
A	$^{23}/_{35}$	63.50	86.35	40.63	9.52
B	$^{23}/_{35}$	66.67	90.67	42.66	—
C	$^{35}/_{50}$	63.50	86.35	40.63	9.52
D	$^{35}/_{50}$	66.67	90.67	42.66	—

EXPERIMENTAL

Manufacture of Spray-Congealed Granules—The composition of lipase–lipid–drug granules made by spray congealing was as follows:

glyceryl trilaurate[1]	16.00 g.
glyceryl tristearate[2]	34.00 g.
sulfamethizole NF[3]	25.00 g.
crude lipase[4] (5% w/w of substrate and drug)	
calcium carbonate USP[5] (0, 2, 5, and 8% w/w)	
glyceryl monostearate[1] (0, 1, 2, 5, 7, and 10% w/w)	

The hot mixture of lipid materials, sulfamethizole (7.4 μ), lipase powder, and any additive (if used) was prepared in a glass beaker immersed in a boiling water bath. The slurry was then poured into a separator previously heated to 100° by a heating pad. The mixture was spray congealed in a laboratory spray dryer[6] at room temperature by running through a centrifugal wheel atomizer (5 cm. in diameter) preheated to 110° for 3 hr. in an oven. Compressed air at a pressure of 6 kg./cm.^{2} rotated the atomizer at about 35,000 r.p.m., and the rate of atomization by centrifugal disk atomizer was approximately 100 g./min. The moisture content of spray-congealed granules determined on a moisture balance[7] ranged between 0.3 and 0.8% w/w. The granules were sized into $^{23}/_{35}$ and $^{35}/_{50}$ mesh for dissolution-rate studies. The composition of the granule formulations studied is given in Table I.

Spray congealing was used in the formulation of lipase–lipid–drug granules to minimize the possible thermal inactivation of lipase. Bullock (3) and Wilkinson *et al.* (4) reported that various spray-dried enzymes can be prepared without loss of activity using a 120° air current. A lipase powder with 4.4% moisture was stable in the oily suspension when heated to 100° for 1 hr. (3). The inactivation of lipase depends not only on the moisture content of the powder but also on the freedom with which the moisture can escape. During the preparation of lipid–lipase–drug melt, the suspension was stirred to facilitate the escape of moisture. The moisture content of the lipid–lipase–drug granules prepared was below 0.9%. The lipase in lipid melt was stable since the final temperature of the melt was 98° after the 65 min. of heating required for its preparation.

Preparation of Tablets—The ingredients and the quantities used in the manufacture of the tablets are listed in Tables II and III. A $^{23}/_{50}$-mesh portion of granules was used to compress a 200-mg. tablet on a hand-operated tablet press[8]. Double-layered tablets, containing 152 mg. of drug–lipid and 48 mg. of initial-release granules, were also compressed. The initial-release granules were composed of 33.33% drug, 40% lactose, and 13.33% each of starch and sucrose. The amount of sulfamethizole in a 200-mg. tablet ranged between 57.97 and 66.67 mg., depending on different amounts of various additives. The weight of individual tablets was checked, and tablets were checked with a Stokes hardness tester to ensure that the hardness was between 6 and 7 kg.

[1] C. P. Hall Co.
[2] Sterotex HM, The Capital City Products Co.
[3] C. M. Bundy Co.
[4] Reheis Chemical Co.
[5] Fisher Scientific Co.
[6] Nerco Niro, Nichols Engineering and Research Corp.
[7] Ohaus, model 6000.
[8] Erweka tablet press, Type EKO.

Table II—Quantities, in Milligrams, of Ingredients in a 200-mg. Single-Layered Tablet Made with $^{23}/_{50}$-Mesh Spray-Congealed Drug–Lipid Granules

Formulation	Sulfamethizole	Glyceryl Tristearate	Glyceryl Trilaurate	Lipase	Calcium Carbonate	Glyceryl Monostearate
E	63.50	86.35	40.63	9.52	—	—
F	66.67	90.67	42.66	—	—	—
G	62.30	84.73	39.88	9.35	3.74	—
H	65.36	88.89	41.83	—	3.92	—
I	60.61	82.42	38.79	9.09	9.09	—
J	63.49	86.35	40.64	—	9.52	—
K	59.00	80.23	37.76	8.85	14.16	—
L	61.73	83.95	39.51	—	14.81	—
M	62.89	85.54	40.25	9.43	—	1.89
N	66.01	89.77	42.24	—	—	1.98
O	62.31	84.73	39.87	9.35	—	3.74
P	65.36	88.89	41.83	—	—	3.92
Q	60.61	82.42	38.79	9.09	—	9.09
R	63.49	86.35	40.64	—	—	9.52
S	59.52	80.95	38.10	8.93	—	12.50
T	62.31	84.73	39.88	—	—	13.08
U	57.97	78.84	37.10	8.70	—	17.39
V	60.61	82.42	38.79	—	—	18.18
W	61.16	83.18	39.15	9.17	3.67	3.67
X	64.10	87.18	41.02	—	3.85	3.85

Dissolution Studies—The rotating-bottle method, described by Souder and Ellenbogen (5), was used for testing the *in vitro* release of the drug. An accurately weighed amount of 200 mg. of drug–lipid granules or one 200-mg. tablet was placed in each of six bottles containing 75 ml. of dissolution medium. The bottles were rotated at 40 r.p.m. in a water bath maintained at 37 ± 1°. The dissolution media used were simulated gastric fluid (6) (pH 1.2) for the first 1.5 hr. and then simulated intestinal fluid (pH 8.3) (7) for up to 12.5 hr. One bottle was removed from the bath at 0.5-, 1.5-, 3-, 6-, 10-, and, occasionally, 12.5-hr. intervals, and a portion of the fluid was filtered.

A 0.5-ml. portion of filtrate was analyzed using a modified Bratton–Marshall (8) assay procedure for sulfonamides. The percent transmittance of the resulting solution was determined on a colorimeter[9] at 545 nm. and compared to a standard curve. Since tablets contained different amounts of drug, the release was calculated on the basis of percent drug released from the dosage form. Duplicate dissolution tests were conducted on two formulations prepared separately using the same formula, and an average percent drug release is used for reporting the results. The variation between the two release values from all the formulations containing lipase was less than ±9.2%, whereas the variation from formulations without lipase ranged up to ±10.2%. A formulation blank without lipase was tested simultaneously to account for the release due to leaching and surface dissolution in addition to the main release caused by the lipolytic digestion of substrate.

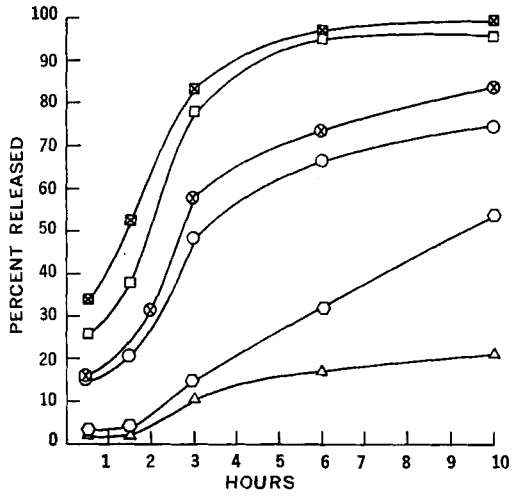

Figure 1—*Percent of sulfamethizole released from granules and tablets during* in vitro *dissolution. Key:* □, *Granules A;* ○, *Granules B;* ⊠, *Granules C;* ⊗, *Granules D;* ○, *Tablet E; and* △, *Tablet F.*

[9] Spectronic 20, Bausch & Lomb.

Determination of Percent Hydrolysis—During the dissolution-rate studies, a determination of lipid hydrolysis was made in simulated intestinal fluid (after the tablets were in the simulated gastric fluid for 1.5 hr.), using a modification of the Lazo-Wasem method (9) to establish a correlation between the amount of drug released and the percent hydrolysis. Determinations for preparations with and without lipase were carried out simultaneously to account for the free fatty acids present in the lipid substrate used in formulation. A 25-ml. portion of fluid was measured from the sample bottle at each interval. To this portion, 1 ml. of concentrated hydrochloric acid was added to destroy any lipase, if present. The solution was shaken with 50 ml. of benzene for 30 min. to extract fatty acids into the benzene layer. The flasks containing the mixtures were stored in a refrigerator to facilitate the separation of two layers. A 25-ml. portion of the upper benzene layer was titrated with 0.055 *N* NaOH solution in methanol, and percent hydrolysis was calculated as follows:

$$\text{percent hydrolysis} = \frac{\text{meq. substrate hydrolyzed} \times 100}{\text{meq. substrate used}} \quad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

A timed-release formulation was prepared by spray congealing lipase–lipid–drug combinations and compressing the granules into tablets. Lipase causes the controlled digestion and erosion of the substrate and subsequent release of the embedded drug particles.

To determine the effect of granule size on the rate of drug release, granules of $^{23}/_{35}$ and $^{35}/_{50}$ mesh were tested. Figure 1 shows that there was essentially no difference in the rate of release between Granules A and C and Granules B and D, except during the first 3 hr. where faster dissolution occurred in both formulations. The more rapid drug release in the first 3 hr. was due to the effect of leaching and surface dissolution of partially exposed and some free drug on the granules. The difference in the amount of drug released from formulations with and without lipase ranged approximately from 10 to 30% in both $^{23}/_{35}$- and $^{35}/_{50}$-mesh granules. The difference, which is relatively larger after 1.5 hr., is indicative of the release caused by the lipolytic digestion of substrate in alkaline pH. Further studies with granules were discontinued because the release from granules with lipase was only about 62% higher than the release from granules without lipase at the point of biggest difference; the release was 153% higher from tablets with lipase as compared to tablets without lipase. The difference in drug release from granules was probably due more to the effect of surface dissolution, while for tablets it was due more to the digestive effect of the lipase on the substrate.

Table III—Quantities, in Milligrams, of Ingredients in a 200-mg. Double-Layered Tablet Made with Spray-Congealed Drug–Lipid Granules and Initial-Release Granules

Formulation	Drug–Lipid Granules					Initial-Release Granules			
	Sulfamethizole	Glyceryl Tristearate	Glyceryl Trilaurate	Lipase	Glyceryl Monostearate	Sulfamethizole	Lactose	Starch	Sucrose
Y	47.35	64.40	30.31	7.10	2.84	16.00	19.20	6.40	6.40
YF	49.67	67.56	31.79	—	2.98	16.00	19.20	6.40	6.40
Z	46.06	62.64	29.48	6.91	6.91	16.00	19.20	6.40	6.40
ZF	48.25	65.63	30.88	—	7.24	16.00	19.20	6.40	6.40

Figure 1 shows that about 90% of the drug was released within 4 hr. from Granules A and C containing lipase. However, when the granules were compressed into tablets, the release was extended more than 10 hr. Figure 1 also shows the percent of sulfamethizole released from Tablets E and F made from $^{23}/_{50}$-mesh granules with and without lipase. The slow rate of drug release from tablets is due to the compaction, decreased porosity, and reduced surface area caused by the tableting of granules. A difference of about 33% drug release between Tablets E and F at 10-hr. periods demonstrates that lipolytic action resulting in the digestion of substrate in the tablet causes increased drug release.

It was reported in the literature that calcium ions accelerate the action of lipase on triglycerides (10). Wills (11) reported that calcium helps to stabilize the activity of lipase. Experiments were carried out to determine the effect of calcium ions on lipase activity in terms of drug release. As seen in Fig. 2, no increase in drug release was observed in acidic medium up to 1.5 hr. because lipase is not activated until it reaches alkaline pH. An increase in the percentage of sulfamethizole released from Tablets G, I, and K containing lipase and various amounts of calcium carbonate was noted in simulated intestinal fluid as compared to the release from Tablet E, whereas no increase was observed from Tablets H, J, and L containing various amounts of calcium carbonate but no lipase. Since the percent drug release from Tablets H, J, and L containing various concentrations of calcium carbonate was approximately the same, only one curve was drawn in Fig. 2 to represent the drug release from all of the blank formulations. Since the amount of sulfamethizole released after 1.5 hr. was primarily related to the rate of hydrolysis, the marked increase in the drug release demonstrated that calcium ions exert an accelerating effect on the rate of hydrolysis. This finding is in agreement with the results of Brewton (1) and Wills (11).

In vitro studies showed that glyceryl monostearate accelerates lipolysis by emulsifying the insoluble fats and oils in alkaline pH, thus exposing a larger surface area for lipase action (12). Experiments were carried out to determine the effectiveness of glyceryl monostearate as a lipase accelerator by studying the rate of drug release. Figure 3 shows that the release from Tablets M, O, and Q, containing 1, 2, and 5% glyceryl monostearate with lipase, respectively, increased in simulated intestinal fluid; the release from Tablets S and U, containing 7 and 10% glyceryl monostearate, respectively, decreased as compared to the release observed from Tablet E containing lipase but no glyceryl monostearate. The decrease in drug release at 7 and 10% concentrations of glyceryl monostearate may be due to the micellar solution entrapment of the drug. No increase in drug release was noted in the formulations containing lipase and various concentrations of glyceryl monostearate in acidic medium up to 1.5 hr. With 1–10% concentrations of glyceryl monostearate in Tablets N, P, R, T, and V without lipase, no increase was observed in the amount of drug released. Since the percent drug release from Tablets N, P, R, T, and V containing various concentrations of glyceryl monostearate was approximately the same, only one curve was drawn in Fig. 3 to represent the drug release from all of the blank formulations.

The possibility existed that differences in the amount of drug release from formulations with and without lipase might be due to some nonenzymatic effect of lipase *per se, i.e.*, solubility. However, heat-inactivated lipase and albumin substituted for lipase gave a release similar to the formulations containing no lipase.

Since the drug is released mainly through the hydrolysis of the substrate by lipase in addition to the release by surface dissolution and leaching, it was desirable to know some empirical relationship between the percent hydrolysis and the corresponding drug release. Figure 4 shows that the percent drug release was dependent upon the percent hydrolysis of the substrate in tablets.

Since the drug was not being released in sufficient quantity in the first 1.5 hr., an initial-release portion of the drug was incorporated by employing a double-layered tablet using a sugar and starch granulation. Figure 5 presents the drug release from formulations made from drug–lipid granules containing 2% glyceryl monostearate and initial release granules with 25% of the total drug in a tablet. In both Tablets Y and YF, with and without lipase, respectively, a release of about 28% was observed in the first 1.5 hr. due to the drug in the initial-release granules. After 10 hr. of the digestion period, 99.1% of the drug was released from Tablet Y containing lipase as compared to

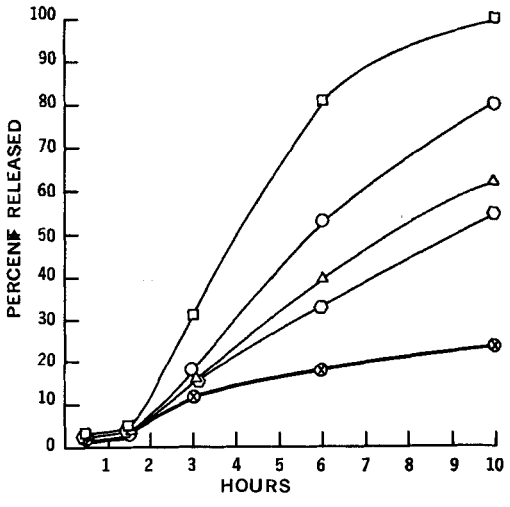

Figure 2—*Percent of sulfamethizole released from tablets containing various concentrations of calcium carbonate during* in vitro *dissolution. Key:* ○, *Tablet E;* △, *Tablet G, 2%;* ○, *Tablet I, 5%;* □, *Tablet K, 8%; and* ⊗, *Tablets H, J, and L, 2, 5, and 8%.*

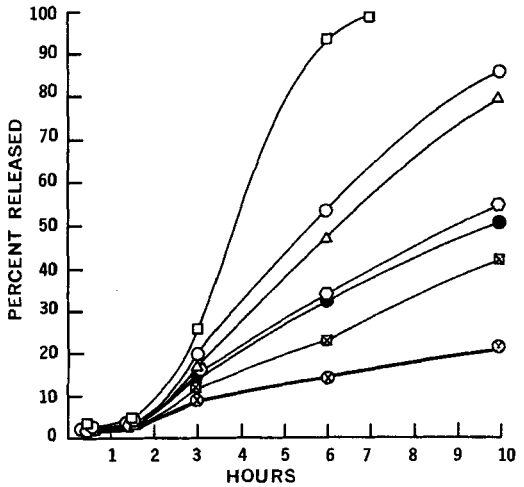

Figure 3—*Percent of sulfamethizole released from tablets containing various concentrations of glyceryl monostearate during* in vitro *dissolution. Key:* ○, *Tablet E;* □, *Tablet M, 1%;* ○, *Tablet O, 2%;* △, *Tablet Q, 5%;* ●, *Tablet S, 7%;* ⊠, *Tablet U, 10%; and* ⊗, *Tablets N, P, R, T, and V, 1, 2, 5, 7, and 10%.*

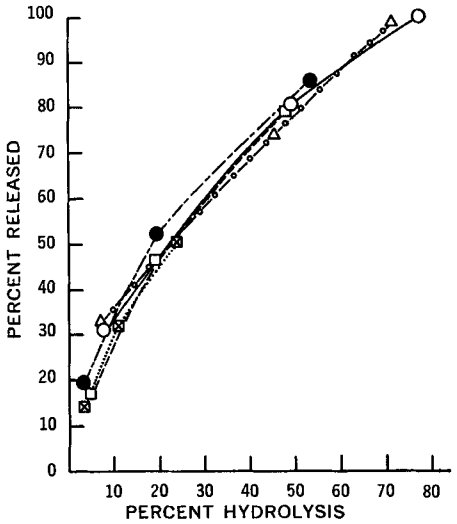

Figure 4—*Correlation of percent hydrolysis of substrate in tablets with percent of sulfamethizole released during* in vitro *dissolution at 3-, 6-, and 10-hr. periods. Key:* ○, *Tablet K, 8% calcium carbonate;* ●, *Tablet O, 2% glyceryl monostearate;* □, *Tablet Q, 5% glyceryl monostearate;* ⊠, *Tablet S, 7% glyceryl monostearate; and* △, *Tablet W, 2% calcium carbonate and 2% glyceryl monostearate.*

46.1% from Tablet YF without lipase. In previous experiments with different amounts of glyceryl monostearate, it was found that higher concentrations of glyceryl monostearate delayed the drug release. Based on this finding, the amount of glyceryl monostearate was increased to 5% in drug–lipid granules to extend the release to a 12-hr. period. The amount of drug released from Tablets Z and ZF, with and without lipase, respectively, was 98.9 and 47.4%, respectively, after a 12.5-hr. digestion period (Fig. 5).

From the present studies carried out on sulfamethizole–lipid–lipase preparations, it appears that adjustment of the lipase–lipid ratio and other lipase accelerators could be used to prepare timed-release dosage forms with desired release patterns.

SUMMARY AND CONCLUSIONS

1. *In vitro* dissolution rates of some timed-release, spray-congealed formulations of sulfamethizole, pancreatic lipase, lipid substrate, and certain lipase accelerators such as calcium carbonate and glyceryl monostearate were tested.

2. The release of the drug from the granules of $^{23}/_{35}$ and $^{35}/_{50}$ mesh was essentially the same except during the first 3 hr. where faster dissolution was observed. The granules were compressed into tablets so that the release was extended more than 10 hr.

3. The addition of various concentrations (2, 5, and 8%) of calcium carbonate to the drug–lipid granules progressively increased the drug release from tablets containing lipase, whereas no increase was noticed from tablets containing the same amount of calcium carbonate but no lipase.

4. Lower concentrations (1, 2, and 5%) of glyceryl monostearate together with lipase increased the drug release, while concentrations of 7 and 10% decreased the drug release as compared to the release from the tablets containing lipase only. The drug release from tablets containing various amounts of glyceryl monostearate without lipase was essentially the same as the release from the tablets without lipase (blank formulations).

5. The percent drug release was dependent upon the percent hydrolysis after 1.5 hr., during which time the release was due more to the effect of lipase than to surface dissolution and leaching.

6. With the incorporation of 25% drug in initial-release granules and 75% drug in drug–lipid granules containing 2% glyceryl monostearate, the release was extended up to 10 hr.

7. The drug release from tablets containing 25% drug in initial release granules and 75% in drug–lipid granules with 5% glyceryl monostearate was extended up to 12.5 hr.

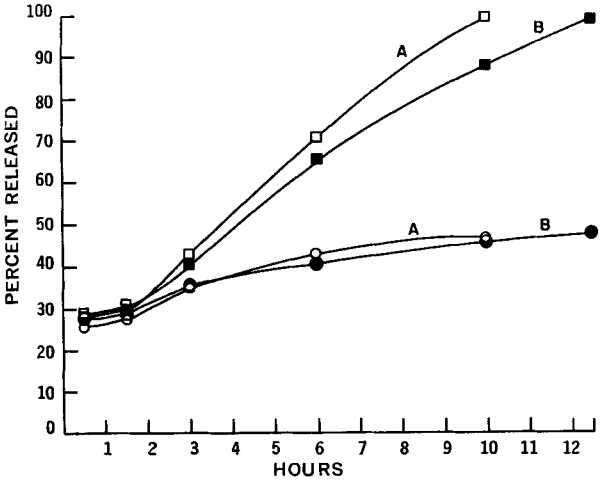

Figure 5—*Percent of sulfamethizole released from double-layered tablets made from drug–lipid granules containing: A, 2% glyceryl monostearate; and B, 5% glyceryl monostearate and initial release granules with 25% of sulfamethizole in a tablet during* in vitro *dissolution. Key:* □, *Tablet Y;* ■, *Tablet Z;* ○, *Tablet YF; and* ●, *Tablet ZF.*

8. The present studies showed that some additives such as calcium carbonate and glyceryl monostearate could affect the rate of drug release by changing the activity of lipase. By using a proper additive in appropriate concentration to control the digestion of the substrate by the lipase and by choosing the optimum lipase–lipid ratio, a timed-release system with a desired release pattern possibly could be formulated.

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Membrane Diffusion I: Design and Testing of a New Multifeatured Diffusion Cell

GORDON L. FLYNN and EDWIN W. SMITH

Abstract □ A new cell with a high diffusional area to diffusional volume ratio, precisely and positively controlled stirring planar with the plane of diffusion, variable speeds, facile assembly, provision for coupling compartments to flow cells or to an external reservoir, and maintenance of temperature by total immersion was designed, built, and checked for its functionality. Details of the shakedown experiments and design are presented.

Keyphrases □ Diffusion system—development of a vertical membrane equicompartment diffusion cell with high area to volume ratio □ Membrane transport, *in vitro*—development of a diffusion cell with high area to volume ratio

A perusal of the literature generates the observation that a formidable task facing the investigator concerned with *in vitro* membrane transport is the construction of a suitable diffusional system. Variables such as membrane integrity, area and thickness as well as temperature, compartmental concentrations, sampling, and stirring, must be provided for and/or controlled precisely. These must be accomplished without sacrifice of analytical sensitivity. Many cell prototypes can be found in the literature (1–12) and, considering that most were constructed for similar purposes, their diversity is remarkable. However, a rough classification based on gross similarity or dissimilarity of the donor and receptor compartment size and geometry is possible. Because of idiosyncrasies associated with membrane positioning, subclassification of the equicompartment systems according to membrane placement, *i.e.*, horizontal or vertical, is also convenient.

A relatively simple cell system to construct is a horizontal equicompartment unit. In such systems, the membrane is sandwiched between two cells stacked upon one another. The top compartment may be opened or sealed. Typical examples were described by Poulsen (1), Aguiar and Weiner (2), Scala *et al.* (3), Scheuplein (4), Tregear (5), and Haleblian *et al.* (6). The major problem with these systems is adequate and uniform control of stirring. Gravity allows for stirring of the lower compartment with a magnetic stirring device. Stirring of the upper compartment is solved either by neglecting agitation completely or by introducing a mechanical stirrer on a shaft. In any case, stirring in the respective compartments is dissimilar in kind and rate. Special concerns with these systems also include air bubble formation on the membrane undersurface, particularly at elevated temperatures, and the presence of a hydrostatic head. The latter can potentially distend the membrane. Other nonspecific problems common to all the diffusion cell types mentioned are awkward assembly and difficulty in obtaining a uniform seal on the membrane. Bolts are usually used to clamp the respective compartments together and these must be uniformly tightened.

A second type of system may be constructed by clamping a membrane over the end of a piece of tubing or bottle and immersing it in a large reservoir (beaker, tank, *etc.*). Examples were given by Garrett and Chemburkar (7), Kostenbauder *et al.* (8), and Most (9). Temperature is a bit easier to control relative to the first type of cell discussed because immersion is facilitated. Adequate stirring is more difficult to obtain. The assembly, air bubbles, and hydrostatic head may or may not be troublesome, depending on the overall design. With this type of system, sensitivity is generally compromised since the design precludes maximization of the diffusional area to compartmental volume ratio. In the case of Garrett and Chemburkar (7), this situation effectively masked the nonsteady-state portion of their diffusional curves and prevented assessment of lag times (10).

The third type of diffusional cell, including the present cell, is the vertical membrane equicompartment system. A complexity arising from turning the first type of cell on its side is that stirring in the plane of the membrane is most difficult to accomplish. *A priori*, it seems as if this would be required to minimize diffusional layer thicknesses and provide for diffusional layer uniformity. Thus, Shenouda and Mattocks (11) chose to use mechanical stirring perpendicular to the plane of diffusion. Stirring in the plane of diffusion has, on oc-

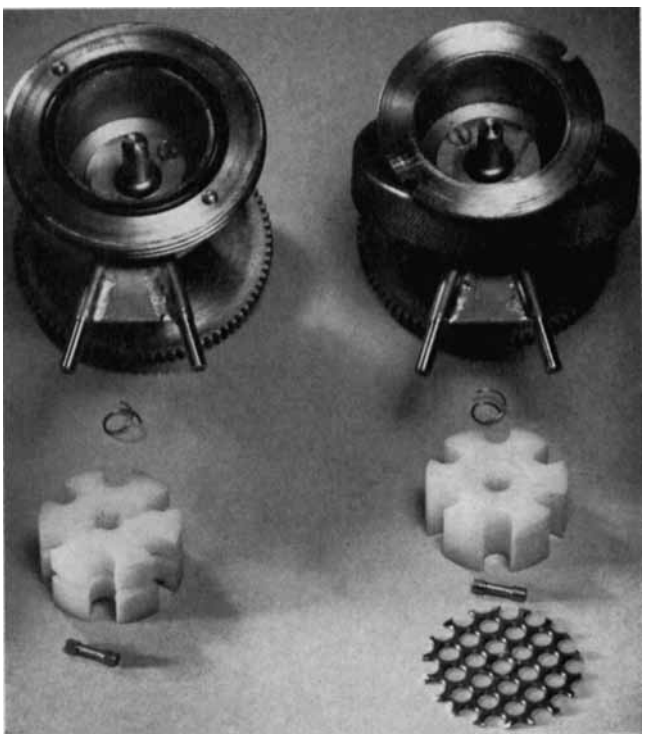

Figure 1—*Completely disassembled cell. (See text for description.)*

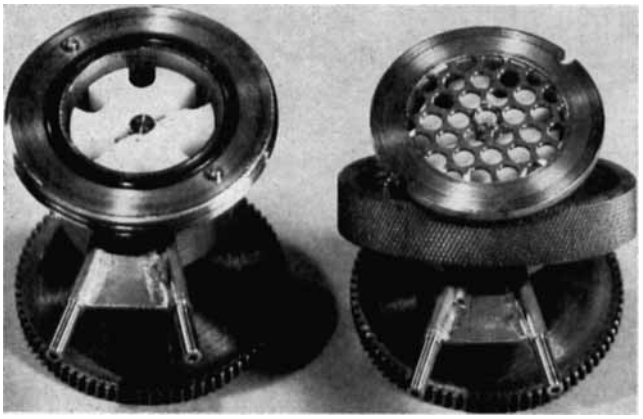

Figure 2—*Assembled cell halves.*

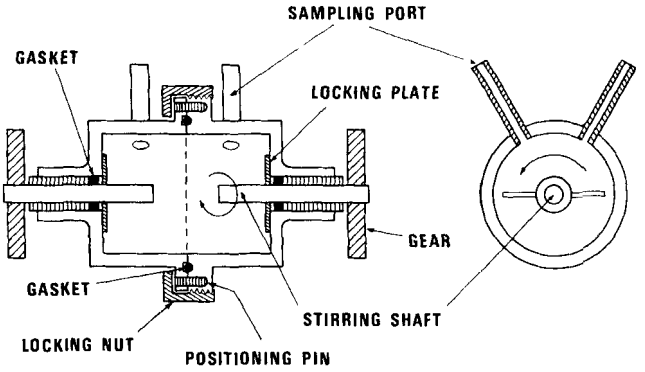

Figure 4—*Engineering drawing of diffusion cell.*

casion, been resolved. Misra *et al.* (12) and Olson *et al.* (13) published two ingenious designs in which magnetic stirrers are mounted on shafts protruding from the back walls of each cell half. Generally speaking, the advantages of this type of design are that cell immersion is feasible, air pocket formation is minimized, and the hydrostatic head is eliminated.

For reasons explicitly discussed in the paper by Olson *et al.* (13), the cell feature about which a diffusional experimenter should be most concerned is cell sensitivity. For any cell, sensitivity will be proportional to the diffusional area divided by the compartmental volume in which the appearance or disappearance of permeant is being followed. It has been shown (9, 10, 13) that nonsteady-state diffusion is fleeting for some practical systems and, without maximum sensitivity, may be indecipherable within the total diffusional curve. For these reasons, an attempt was made to construct a diffusional cell with a high area to volume ratio. At the same time, consideration was given to ease of assembly, temperature control by immersion, stirring planar with the diffusional plane, and other aforementioned factors. Since the care with which a cell is built and evaluated has much to do with the credibility of experimental conclusions, a rather complete analysis of cell performance is presented in this first membrane transport paper.

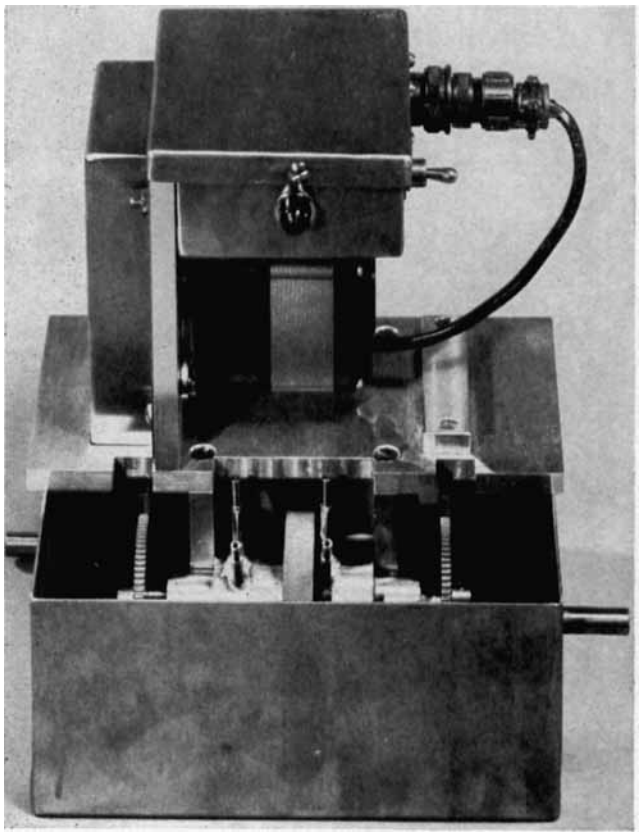

Figure 3—*Completely assembled cell.*

CELL DESCRIPTION

The features of the diffusion cell are best understood by examining Figs. 1–4. In Fig. 1, the cell is completely disassembled. The parts from the top of the picture to the bottom are the cell compartments, the stirring shaft O-ring seals, the springs for spring loading the stirrers, the Teflon stirrers, the stirrer locking pins, and the membrane supporting screen. The stirrer shafts inside the compartments are exposed. The O-ring seals fit tightly into a precisely machined die at the base of the stirring shaft, providing fluid-tight interfaces with the movable stirring shaft and the fixed compartment wall. These O-ring gaskets are securely positioned with a covering stainless steel template.

In Fig. 2, the respective cell halves are assembled. This picture shows the large brass nut (tilted with respect to the right compartment) and the threads at the rim of the left compartment. This provision for joining the cells assures that the fastened pressure will be uniform around the membrane circumference. The black ring in the flange of the left-hand compartment is a seated O-ring for sealing the membrane. Just outside this ring are two pins which are placed in the notches of the right-hand compartment during assembly to obtain reproducible positioning of the cell halves. At the base of each half cell is a gear attached externally to the stirring shaft. When the cells are fully assembled, these gears are connected through other gears to the stirring motor. The membrane support screen, which was alternately used or omitted, is in place in the right-hand compartment.

In Fig. 3, the completely assembled cell sits in the cell rack which also houses the motor. This whole assembly is in a small water bath. Constant temperature is maintained with an auxiliary Tamson force/suction circulating water bath. The cell is equipped with three interchangeable synchronous motors providing speeds of 30, 60, and 120 r.p.m.

Figure 4 provides details on relative dimensions and gives a contrasting perspective on cell design. The diffusional area is 10 $cm.^2$; with Teflon stirrers in place, the individual compartmental volumes are approximately 15 ml.

EXPERIMENTAL PROCEDURE

Sample Preparation—An excess of *p*-aminoacetophenone was placed in a 2-l. flask, and deionized water was added. This solution was stirred overnight and filtered. The concentration was determined to be 4.616 mg./ml. by UV spectrophotometry.

Table I—Compilation of Diffusional Steady-State Rates and Lag Times as a Function of Variables

Run Number	Membrane Thickness, μ	Stirring Speed, r.p.m.	Steady-State Rate, A/min.	Uncorrected Lag Time, min.	Screen[a]	General Comment
1	126.0	30	0.1177	N.R.[b]	−	—
2	126.0	30	0.1242	N.R.	−	Reversed direction through flow cell
3	126.0	30	0.1137	N.R.	−	Repeat of 2
4	126.0	30	0.1149	N.R.	−	Repeat of 2
5	126.0	30	0.1199	N.R.	−	Repeat of 2
6	126.0	30	0.1183	N.R.	−	Repeat of 2
7	126.0	30	0.1130	N.R.	−	Repeat of 2
8	126.0	30	0.1199	N.R.	−	Flow rate changed to 30 ml./min.[c]
9	126.0	30	0.1013	N.R.	+	Repeat of 8—with screen
10	126.0	30	0.1157	N.R.	+	Repeat of 8—with screen
11	126.0	30	0.1212	N.R.	+	Donor solution preheated to 37° -w/screen
12	126.0	30	0.1355	N.R.	+	Repeat of 11
13	126.0	30	0.1214	N.R.	+	Repeat of 11
14	126.0	60	0.1130	N.R.	−	Changed stirrer to 60 r.p.m.
15	126.0	60	0.1290	N.R.	−	Repeat of 14
16	126.0	60	0.1400	N.R.	−	Repeat of 14
17	481.6	60	0.0249	19.5	−	Changed to 0.051-cm. (0.019-in.) membrane
18	481.6	60	0.0238	19.3	−	Repeat of 17
19	481.6	60	0.0253	19.3	−	Repeat of 17
20	481.6	60	0.0244	N.R.	−	Repeat of 17
21	481.6	60	0.0244	N.R.	−	Repeat of 17
22	281.7	60	0.0440	9.75	−	Changed to 0.025-cm. (0.010-in.) membrane
23	126.0	60	0.1605	N.R.	−	Changed to used 0.013-cm. (0.00495-in.) membrane
24	126.0	60	0.1282	2.75	−	Changed to fresh 0.013-cm. (0.00495-in.) membrane
25	281.7	60	0.0426	10.00	−	Changed to 0.025-cm. (0.011-in.) membrane
26	281.7	60	0.0421	10.75	−	Repeat of 25
27	281.7	60	0.0483	9.25	−	Repeat of 25
28	281.7	60	0.0430	9.50	−	Repeat of 25
29	281.7	60	0.0449	9.25	−	Repeat of 25
30	281.7	60	0.0430	9.75	−	Repeat of 25
31	126.0	60	0.1515	N.R.	−	Changed to used 0.013-cm. (0.00495-in.) membrane
32	126.0	60	0.1554	2.00	−	Repeat of 31
33	126.0	60	0.1193	2.25	−	Changed to fresh 0.013-cm. (0.00495-in.) membrane
34	126.0	60	0.1253	2.10	−	Repeat of 33
35	126.0	60	0.1186	2.40	−	Repeat of 33
36	126.0	60	0.1173	2.30	−	Repeat of 33
37	481.6	60	0.0245	18.5	+	0.051-cm. (0.019-in.) membrane with screen
38	481.6	60	0.0246	15.8	+	Repeat of 37
39	481.6	60	0.0246	19.25	+	Repeat of 37
40	126.0	60	0.1211	1.73	+	0.025-cm. (0.011-in.) membrane with screen
41	126.0	60	0.1211	2.00	+	Repeat of 40
42	281.7	60	0.0440	8.75	+	0.013-cm. (0.00495-in.) membrane with screen
43	281.7	60	0.0440	9.50	+	Repeat of 42

[a] −, no screen; +, screen in place. [b] N.R. = not recorded. [c] Normal flow rate was 2.5 ml./min.

Preparation of Membranes—The membranes were cut from sheets of dimethylpolysiloxane, reportedly 0.013, 0.025, and 0.051 cm. (0.005, 0.010, and 0.020 in.) thick, respectively, with a die specially made for the diffusional cell. They were thoroughly rinsed and then allowed to equilibrate with water for 48 hr. or longer. No absorbance was observed from membranes treated in this manner.

Diffusion Studies—In each run the diffusion cell was assembled with a dimethylpolysiloxane membrane of the indicated thickness sandwiched between the compartments. The cell was then placed in the bath maintained at exactly 37°.

The receptor phase was connected by chemically resistant black tubing to a Technicon proportionating pump and through the pump to a 1-cm. flow cell mounted in a spectrophotometer[1]. Deionized water was drawn from a reservoir into and through the flow cell and to the empty receptor compartment by the action of the pump. Eventually the compartment filled, and the water was drawn through the remaining lines to and through the pump. When the cell and lines were thoroughly flushed, the loose ends were connected under the surface of the deionized water reservoir to prevent air from getting into the system as it was closed. After a moment to allow for temperature equilibration, the donor compartment was filled by pumping approximately 25 ml. of solution into it with a 30-ml. air-free syringe. The overflow was piped to a beaker and discarded. Then, by joining the two donor ports with a short piece of tubing, the donor compartment was sealed. All runs were at 37 ± 0.01°.

[1] Beckman DB.

EXPERIMENTAL RESULTS

Table I lists the information gathered from 43 runs. The variables manipulated from run to run are either cited in the comments column or tabulated. These variables include the stirring rate, membrane thickness, presence or absence of the membrane supporting screen, flow rate, direction of flow, and preheating of the donor phase. Also evaluated was the reuse of membranes. The steady-

Table II—Thickness of Dimethylpolysiloxane Nonreinforced Membranes

Measurement Number	Thickness, in. H-0574[a]	H-0640[a]	H-0596[a]
1	0.0049	0.0110	0.0187
2	0.0049	0.0110	0.0187
3	0.0047	0.0112	0.0188
4	0.0053	0.0110	0.0189
5	0.0043	0.0111	0.0192
6	0.0053	0.0113	0.0189
7	0.0055	0.0110	0.0192
8	0.0048	0.0109	0.0191
Average values			
Inches	0.00496	0.01109	0.01896
Centimeters	0.0126	0.0282	0.0482
Microns	126.0	282.0	482.0

[a] Dow Chemical Co.

state rate is reported as it was taken off the chart paper in units of absorbance per minute. Conversion to more conventional units will be discussed in subsequent papers (10, 16).

The reported thicknesses of the purchased dimethylpolysiloxane membranes[2] were, in ascending order, 0.013, 0.025, and 0.051 cm. (0.005, 0.010, and 0.020 in.). These were checked using a micrometer by the method of Garrett and Chemburkar (7). These data are reported in Table II. A check on these values was made by simultaneously measuring eight membrane thicknesses. The average thicknesses by this method were 0.012, 0.027, and 0.048 cm. (0.00479, 0.01091, and 0.01890 in.).

DISCUSSION

In general, the evaluation of the cell went smoothly. Reliable estimates of both the steady-state velocity and the lag time were possible. The lag time gives an assessment of the apparent diffusivity, D', from the equation of Daynes (14) and Barrer (15):

$$t_{lag} = \frac{h^2}{6D'} \qquad \text{(Eq. 1)}$$

where t_{lag} represents the lag time, and h is the membrane thickness. Most (9) showed that the diffusivity so obtained is not equivalent to the specific diffusion constant through pure polymer for dimethylpolysiloxane membranes due to physical adsorption on silica filler in the membranes. The full significance of this fact will be treated in a subsequent paper (16). It is worthy of note here that the high membrane area-to-receptor compartment volume made estimation of the lag time possible.

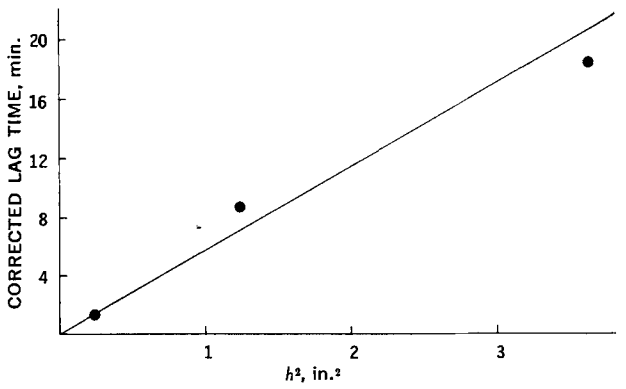

Figure 5—*Plot of average corrected lag time at a given membrane thickness* versus *the square of membrane thickness*, h^2. *Corrected lag times equal observed lag times minus 1.25 min. or the time required to pump diffusion cell contents into the spectrophotometer lightpath. The data are reasonably consistent with expected linear dependency.*

[2] Dow Chemical Co.

Table III—Use of the Membrane Support Screen

Thickness, μ	Steady-State Velocity, A/min. With	Without
126	0.1181	0.1211
282	0.0433	0.0440
482	0.0240	0.0246

These experiments were carried out specifically to determine the experimental sensitivity to stirring rate, preheating of the donor phase by varying the membrane thickness, reusing membranes, and placing the membrane support screen in the system. Reproducibility and the associated statistical parameters were also sought.

Varying Stirring Rate—A comparison of Runs 2–8 with Runs 14–16, 24, and 33–36 indicates that stirring rate is not critical above 30 r.p.m. The average for the former, which were run at 30 r.p.m., is 0.1173, while the latter 60-r.p.m. set is 0.1238. Both are within the 95% confidence limits for runs at 37° with a 126-μ membrane (see *Reproducibility*). Obviously, no benefit would be derived by going to 120 r.p.m.

Preheating Donor Phase—The average value for Runs 11–13, which were preheated, is 0.1260. These values can be compared directly with Runs 9, 10, 40, and 41 (average value of 0.1148). The former is just above the upper 95% confidence limit, 0.1256, while the latter is just below the lower limit, 0.1167. This finding suggests a significant increase in rate when preheating is performed. The actual increase, if real, is at best marginal. This result is not surprising, because the cell is made of a good heat-conducting material, stainless steel, and is itself preequilibrated to 37°.

Varying Membrane Thickness—As expected, the steady-state velocity decreased and the lag time increased as the membrane thickness increased. For a given membrane thickness, both the steady-state rate and lag time were reasonably reproducible. Based upon Eq. 1, a plot of lag time *versus* the membrane thickness squared should yield a straight line, and this was the observed case (Fig. 5). The lag times used in this plot were corrected for the time required for solution in the cell to be pumped into the spectrophotometer flow cell, 1.25 min., called the mechanical lag time. Therefore, despite physical adsorption on filler, lag times apparently show the same dependency on thickness (h^2) as is found in simpler membrane cases.

Reusing Membranes—Runs 23, 31, and 32 were performed with previously used membranes. Significantly higher steady-state rates were observed (average 0.1558 A/min.) relative to runs using fresh membranes (average 0.1211 A/min.). Our observations indicate that this result is not so much due to deterioration of the membrane but to the presence of noneluted *p*-aminoacetophenone in the membrane. This produced a "reservoir effect," making it difficult to assess both slope and intercept for a given run. This complication is specific to the experimental situation as the runs were only followed to an absorbance of 1.0.

Placing Support Screen in Diffusional Pathway—Table III summarizes the results of runs performed with and without the membrane supporting disk. Surprisingly, these rates are not significantly different, suggesting that the stirring was sufficiently forceful to sweep solvent under the support screen. Therefore, the membrane area available for diffusion was not appreciably altered.

Reproducibility—A statistical treatment of data from Runs 3–7, 9, 19–21, 29, 38, 40, and 41 produces a mean value for the 126-μ membrane of 0.1211, with 95% confidence limits of $0.1167 \leq x \leq 0.1254$. The standard sample error is 0.0020, and the percent coefficient of variation is 6.29%. Better precision was expected. However, the results are sufficiently reproducible to make conclusions drawn therefrom valid and more reproducible than those reported by Garrett and Chemburkar (7) using a similar system. Possible causes are variation in individual membrane thicknesses and/or variation in the receptor compartmental volume from run to run (air pockets). These factors will be checked in future experiments.

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Semiautomated, Simultaneous Assay of Thiamine, Riboflavin, Pyridoxine, and Niacinamide in Multivitamin Preparations

RHYS BRYANT, F. J. BURGER, R. L. HENRY, and F. B. TRENK

Abstract □ A semiautomated analytical system was developed for the simultaneous determination of thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), and niacinamide in multivitamin preparations. The automated analytical methods are similar in principle to the USP manual procedures.

Keyphrases □ Multivitamin preparations—semiautomated, simultaneous analysis □ Vitamin analysis—thiamine, riboflavin, pyridoxine, and niacinamide in multivitamin preparations

A semiautomated method was developed for the simultaneous analysis of four vitamins (niacinamide, pyridoxine, thiamine, and riboflavin) in multivitamin formulations; it is similar in principle to the USP (1) manual procedures. The method is a significant extension of the work by Khoury (2, 3) and Albright and Degner (4), who developed methods for simultaneous determination of two vitamins in multivitamin formulations.

EXPERIMENTAL

Equipment—The analytical train consisted of the following Technicon[1] modules: Liquid Sampler II, three proportioning pumps (model I), a dialyzer (model I), two colorimeters (model I) with 15-mm. flow cells, two Fluorometer I units, two double-pen recorders[2], and an autoclave[3].

Reagents—*Niacinamide Buffer (pH 6.8)*—Prepare by dissolving 206.5 g. sodium phosphate dibasic (A.R.) and 57.3 g. citric acid (A.R.) in 2 l. of distilled water. Before use, dilute 200 ml. buffer and 2 ml. polysorbate 80[4] to 1 l.

Pyridoxine Buffer—Prepare a 20% (w/v) sodium acetate (A.R.) solution (Solution A) and a second solution (Solution B) consisting of 470 g. ammonium chloride (A.R.) and 470 ml. ammonium hydroxide (A.R.) (58%) dissolved in water and diluted to 2 l. with water. Mix equal portions of Solutions A and B plus 4 ml. of polysorbate 80/l. before use.

Buffer (pH 4.0)—Prepare by dissolving 219 g. sodium phosphate dibasic (A.R.) and 258 g. citric acid (A.R.) in water and diluting to 2 l. with water. Before use, dilute 200 ml. buffer plus 10 g. potassium chloride (A.R.) to 1 l. with water.

Cyanogen Bromide[5]—Use 7% (w/v) in distilled water.

Sulfanilic Acid—Disperse 50 g. of sulfanilic acid[5] in approximately 800 ml. water. Add ammonium hydroxide (58%) slowly until dissolution of the sulfanilic acid is achieved. Adjust to pH 4.5 with concentrated hydrochloric acid (A.R.), and dilute to 1 l. with water.

N, *2,6-Trichloro-p-benzoquinoneimine Solution*—Dissolve 200 mg. of *N*,2,6-trichloro-*p*-benzoquinoneimine[5] in 500 ml. isopropyl alcohol.

Table I—Relative Standard Deviations of Three Types of Vitamin Preparations[a]

	Thiamine		Riboflavin		Pyridoxine		Niacinamide	
	Automated	Manual	Automated	Manual	Automated	Manual	Automated	Manual
Decavitamin drops	1.2	2.7	1.9	3.5	1.7	1.9	3.2	3.8
Vitamin premix for nutritional products	2.1	0.8	1.1	9.1	2.1	3.2	3.4	5.2
Decavitamin tablet granulations	2.1	1.2	2.0	3.4	0.6	4.9	4.2	5.1

[a] Values are given as percentages.

[1] AutoAnalyzer, Technicon, Tarrytown, NY 10591

[2] Model 67A-(TC)-2PHPH570-00.
[3] American Sterilizer Co., model 57CR.
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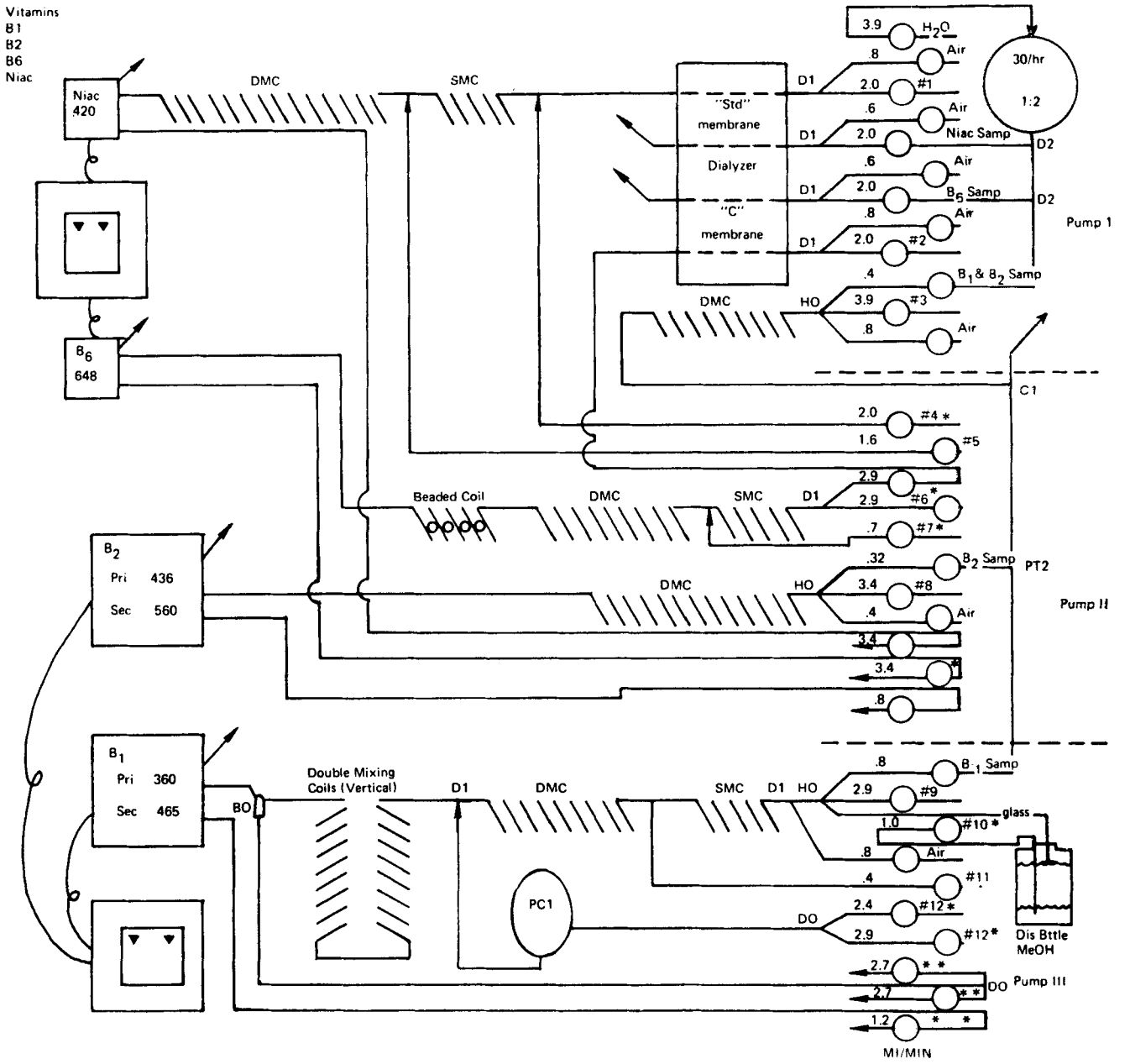

Figure 1—*Schematic diagram of automated system. Key: 1, niacin buffer; 2, pyridoxine buffer; 3, pH 4.0 buffer; 4, 7% cyanogen bromide; 5, 5% sulfanilic acid; 6, isopropyl alcohol; 7,* N,2,6-trichloro-p-benzoquinoneimine *solution; 8, pH 4.0 buffer; 9, 1% potassium chloride; 10, heavy mineral oil; 11, oxidizing reagent; 12, isobutyl alcohol; *, solvaflex pump tubing; and **, acidflex pump tubing. All others are standard pump tubing.*

Potassium Ferricyanide Oxidizing Reagent for Vitamin B_1—Prepare fresh daily 0.3% (w/v) solution of potassium ferricyanide in 10% (w/v) sodium hydroxide.

Isobutyl Alcohol (A.R.)—Saturate with water.

Methanol (A.R.), isopropyl alcohol (A.R.), and heavy mineral oil were also used.

Standards—A stock standard solution containing 0.200 mg./ml. each of thiamine USP, riboflavin USP, and pyridoxine USP and 2.000 mg./ml. of niacinamide USP was prepared by autoclaving the riboflavin in 0.02 *N* acetic acid, cooling, and adding the remaining vitamins, with subsequent dilution to volume with 0.02 *N* acetic acid. Autoclaving was necessary to solubilize riboflavin. This solution was stable for 4 weeks when stored at 5°.

A working standard was prepared daily by adding a 10.0-ml. aliquot of stock standard solution to a 200-ml. volumetric flask and autoclaving with 20 ml. of 0.1 *N* hydrochloric acid for 5 min. at 121°. The flask was subsequently cooled and diluted to volume with distilled water. Four additional levels of standard concentrations were prepared by diluting 10, 20, 30, and 40 ml. of working standard to 50 ml. with distilled water.

These resultant standard solutions were equivalent to 2, 4, 6, 8, and 10 mcg./ml. of vitamins B_1, B_2, and B_6 and 20, 40, 60, 80, and 100 mcg./ml. of niacinamide.

Sample Preparation—An accurately measured quantity of sample, equivalent to 1–5 mg. of vitamins B_1, B_2, and B_6 and 10–50 mg. of niacinamide, was placed in a 500-ml. volumetric flask with 50 ml. of 0.1 *N* hydrochloric acid, autoclaved for 5 min. at 121°, cooled, and diluted to volume with distilled water.

Procedure—A schematic diagram of the automated system is shown in Fig. 1. An aliquot of each sample was divided among the four subsystems for analysis of appropriate vitamins where respective transmittance or fluorescent intensities were recorded (Figs. 2 and 3).

DISCUSSION

Interference from dyes in the sample with both pyridoxine and niacinamide determinations necessitated the use of a dialyzer.

Table II—Comparison of Manual and Automated Analysis Results

Sample	Thiamine		Riboflavin	
	Automated	Manual	Automated	Manual
Vitamin premix				
A	7.50 mg./g.	7.33 mg./g.	3.70 mg./g.	3.75 mg./g.
B	3.22 mg./g.	3.13 mg./g.	4.79 mg./g.	4.77 mg./g.
C	3.66 mg./g.	3.58 mg./g.	6.56 mg./g.	6.50 mg./g.
Decavitamin granulation				
A	1.52 mg./470 mg.	1.54 mg./470 mg.	1.75 mg./470 mg.	1.63 mg./470 mg.
B	1.51 mg./470 mg.	1.55 mg./470 mg.	1.74 mg./470 mg.	1.70 mg./470 mg.
C	1.05 mg./500 mg.	1.03 mg./500 mg.	1.38 mg./500 mg.	1.50 mg./500 mg.
Decavitamin syrup	0.23 mg./ml.	0.22 mg./ml.	0.33 mg./ml.	0.33 mg./ml.
Decavitamin drop				
A	1.26 mg./0.6 ml.	1.23 mg./0.6 ml.	1.42 mg./0.6 ml.	1.43 mg./0.6 ml.
B	1.26 mg./0.6 ml.	1.27 mg./0.6 ml.	1.47 mg./0.6 ml.	1.45 mg./0.6 ml.
	Pyridoxine		Niacinamide	
	Automated	Manual	Automated	Manual
Vitamin premix				
A	7.44 mg./g.	7.46 mg./g.	85.7 mg./g.	89.2 mg./g.
B	1.50 mg./g.	1.53 mg./g.	29.1 mg./g.	28.3 mg./g.
C	1.72 mg./g.	1.77 mg./g.	40.2 mg./g.	41.4 mg./g.
Decavitamin granulation				
A	1.34 mg./470 mg.	1.35 mg./470 mg.	17.3 mg./470 mg.	15.9 mg./470 mg.
B	1.34 mg./470 mg.	1.31 mg./470 mg.	17.7 mg./470 mg.	15.6 mg./470 mg.
C	1.11 mg./500 mg.	1.12 mg./500 mg.	15.6 mg./500 mg.	15.7 mg./500 mg.
Decavitamin syrup	0.23 mg./ml.	0.22 mg./ml.	2.14 mg./ml.	2.07 mg./ml.
Decavitamin drop				
A	1.23 mg./0.6 ml.	1.19 mg./0.6 ml.	8.67 mg./0.6 ml.	8.52 mg./0.6 ml.
B	1.24 mg./0.6 ml.	1.21 mg./0.6 ml.	8.70 mg./0.6 ml.	8.76 mg./0.06 ml.

During sample preparation, niacinamide is partially hydrolyzed to niacin; but by treating standard and sample in a like manner, the degree of hydrolysis was found to be the same for standard and sample. The niacinamide buffer must be below pH 7 if ferrous ion is present to prevent ferrous hydroxide formation, which decreases the efficiency of the dialysis membrane.

The thiamine method of Khoury (3) was used with slight modification. Methanol was found to improve the partitioning of thiochrome from the aqueous phase to the isobutyl alcohol. Methanol, after contact with standard, solvaflex, or acidflex tubing, caused quenching of fluorescence. This necessitated addition of methanol by displacement with mineral oil.

Removal and inversion of the fluorometer door to remove water from the cell were used in lieu of flushing the lines with methanol as in Khoury's method (3). Addition of water to the isobutyl alcohol reagent prevented precipitation of potassium chloride at the point of introduction of isobutyl alcohol in the analytical train.

The natural fluorescence in a sample, which might interfere in riboflavin determination, can be measured by the addition of sodium

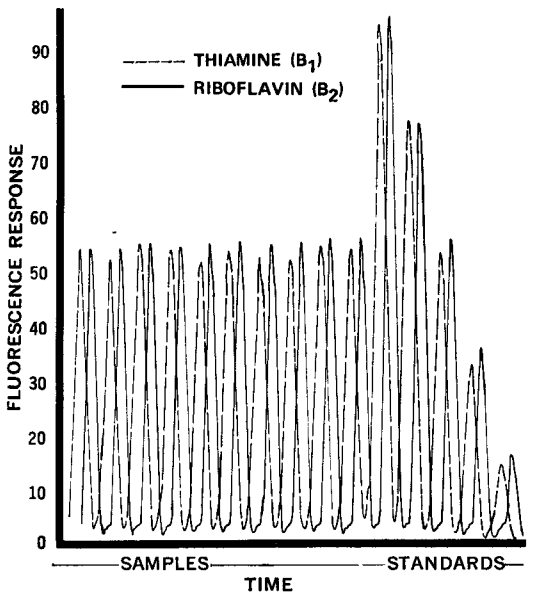

Figure 2—*Typical curves for vitamin B_1 and B_2 standards and samples.*

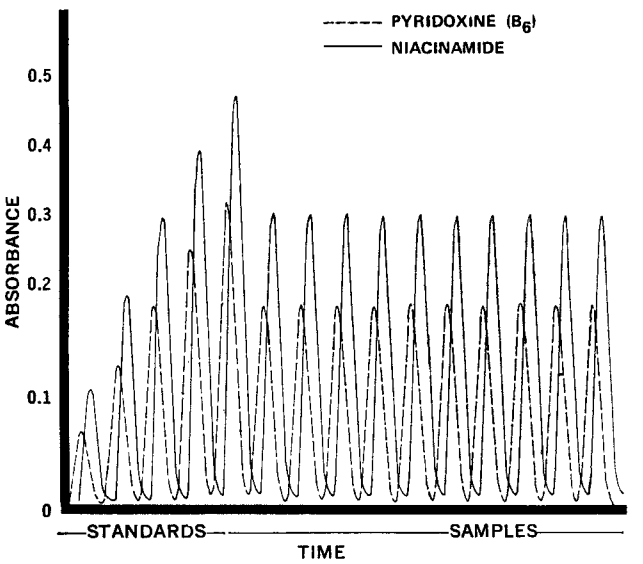

Figure 3—*Typical curves for vitamin B_6 and niacinamide standards and samples.*

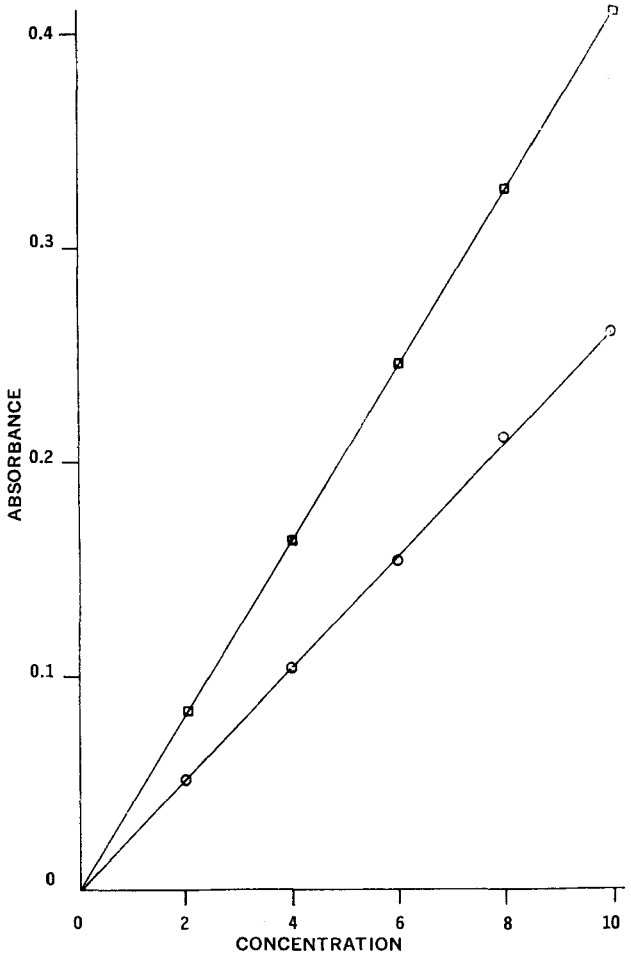

Figure 4—*Standard curves for pyridoxine and niacinamide. Key:* O, *pyridoxine (mcg./ml.); and* □, *niacinamide (mcg./ml. × 10).*

hydrosulfite to the sample after analysis. The sodium hydrosulfite reduces riboflavin to nonfluorescent leucoflavin. No measurable background fluorescence was observed for any of the samples assayed routinely.

The color reaction for pyridoxine is for *para*-unsubstituted phenols. A correction can be made by adding boric acid to complex selectively pyridoxine and reassaying the samples for interfering phenols. No measurable amount of interference was observed for any samples assayed routinely. If ascorbic acid is present, addition of copper chloride to the pyridoxine buffer is necessary to oxidize interfering ascorbic acid.

RESULTS

Standard Curves—The average response (transmittance or fluorescence) for each vitamin at five concentrations was determined. A plot of the five values for each vitamin gave a straight line (Figs. 4 and 5).

Reproducibility of Standards—The reproducibility of the method was checked by the analysis of 10 standards. The coefficient of variations observed were: 1.77% for thiamine, 1.17% for riboflavin, 0.52% for pyridoxine, and 0.85% for niacinamide.

Reproducibility of Sample Preparations—A decavitamin drop, a decavitamin granulation, and a vitamin powder mixture were assayed each day for 10 consecutive days by both the automated procedure and the manual USP XVII procedures. A comparison of the standard deviations calculated for both automated and manual assay results is shown in Table I. A further comparison of the automated and manual procedures is given in Table II for a variety of samples.

SUMMARY

An automated method for the simultaneous assay of four vitamins (thiamine, riboflavin, pyridoxine, and niacinamide) in multivitamin preparations was described. The automated system proved reliable and provided an approximate fivefold increase in assay capability.

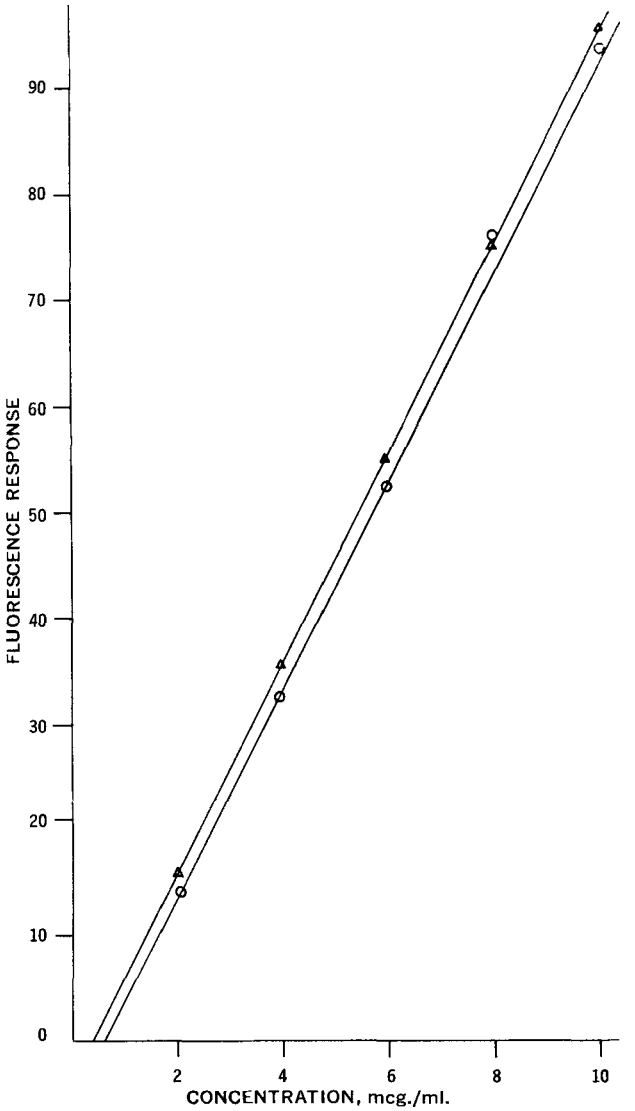

Figure 5—*Standard curves for vitamin* B_1 (O) *and vitamin* B_2 (Δ).

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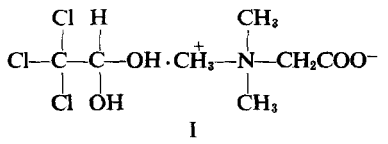
Automated Assay of Single Tablets of Chloral Betaine

RHYS BRYANT, F. J. BURGER, and F. B. TRENK

Abstract □ An automated analytical system was used to determine the amount of chloral hydrate in a single tablet. The intensity of a blue complex between chloral hydrate and quinaldine ethiodide in alkaline solution was a measure of its concentration in the tablet.

Keyphrases □ Chloral betaine tablets—automated colorimetric assay □ Chloral hydrate–quinaldine ethiodide complex—automated colorimetric assay of chloral betaine tablets □ Colorimetry—automated assay, chloral hydrate in chloral betaine tablets

Chloral betaine[1] (I) is a chemical complex of chloral hydrate and betaine. After ingestion, chloral betaine reduces to chloral hydrate, which is subsequently reduced to trichloroethanol, a potent hypnotic. Hence, it provides the recognized sedative and hypnotic effectiveness of chloral hydrate: induction of natural restful sleep, easy arousal, and minimal, if any, "hangover" (1).



I

Chloral betaine is usually assayed titrimetrically after basic hydrolysis (2). However, this procedure does not readily lend itself to automation. A colorimetric method for the determination of chloral hydrate (3) was found to be adaptable to automation.

Application of automated analyses to pharmaceutical preparations is well documented in the literature (4–6).

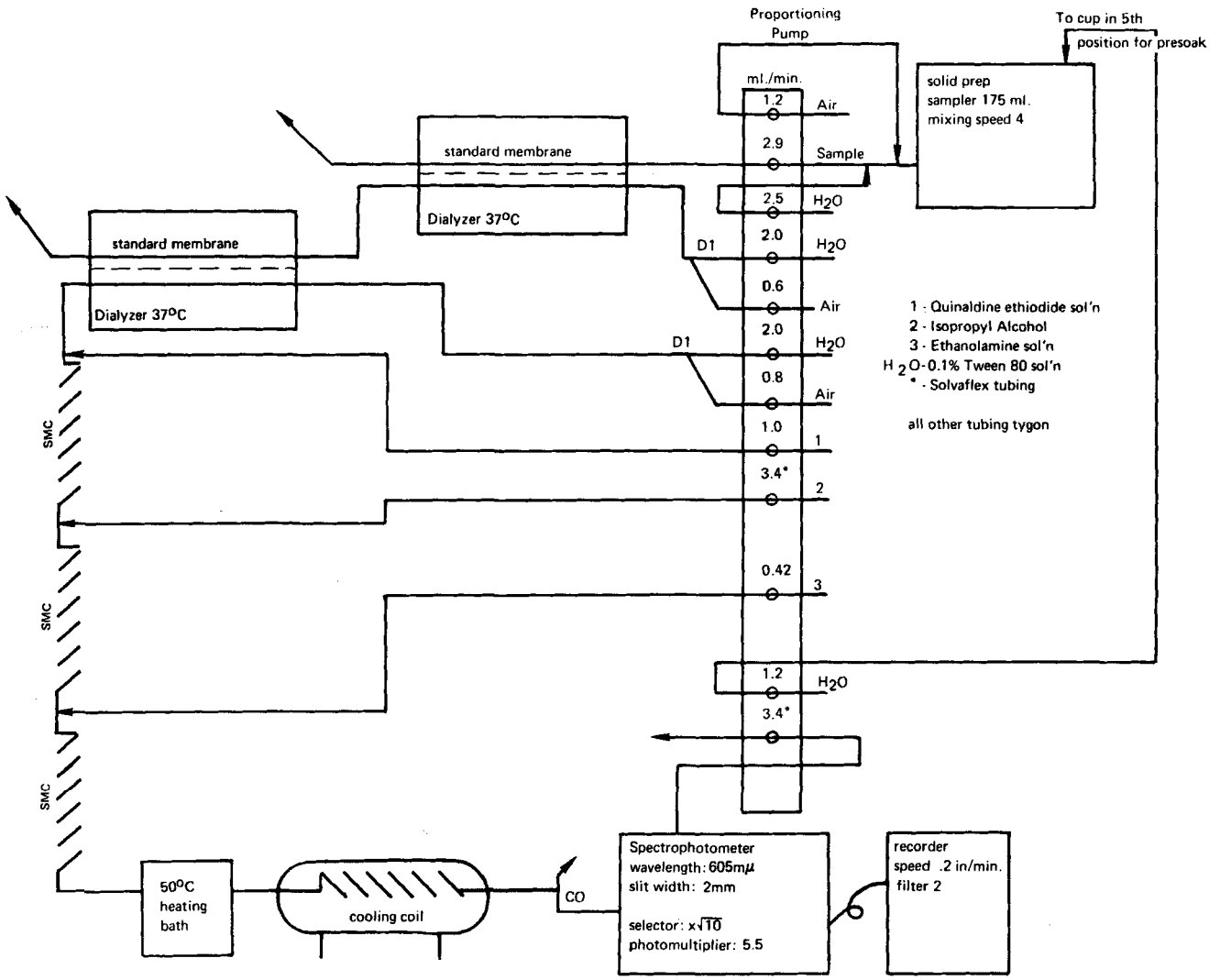


Figure 1—*Schematic diagram for chloral betaine, 500 mg., tablet analysis.*

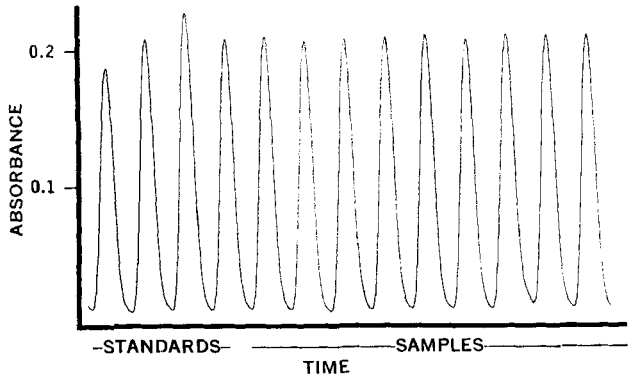


Figure 2—*Typical curves for chloral betaine standards and samples.*

The application of an automated colorimetric assay to single tablets of chloral betaine is now reported.

EXPERIMENTAL

Equipment—The analytical train consisted of the following Technicon[2] modules: (*a*) Solidprep sampler, (*b*) proportioning pump (model I), (*c*) dialyzer (model I), and (*d*) heating bath with 12.2-m. (40-ft.) coil, in conjunction with a spectrophotometer[3] using a microflow cell (9120 No. 5) and a recorder[4].

Reagents—The following were used: *Quinaldine Ethiodide Solution*—Quinaldine ethiodide[5] (1-ethylquinaldinium iodide) (0.75% w/v) in distilled water.

Ethanolamine Solution—Ethanolamine[6] (0.1 *M*) in distilled water.

Isopropyl Alcohol—Analytical reagent[7].

Water containing 0.10% polysorbate 80[8] was also used. Water was used as a diluent in the Solidprep sampler.

Standards—Aqueous solutions of chloral betaine were made up at three levels of concentration (78.1, 91.9, and 105.7 g./l.). Ten-milliliter aliquots of each solution were equivalent to 425, 500, and 575 mg. of chloral hydrate, respectively, when diluted with 175 ml. of water in the Solidprep sampler.

Samples—Production lots of tablets were used in this study. The theoretical content of each tablet was 870 mg. of chloral betaine (equivalent to 500 mg. of chloral hydrate).

Procedure—A schematic drawing of the automatic analyzer system is shown in Fig. 1. Each tablet was individually disintegrated in the presence of water. An aliquot of the resulting suspension was doubly dialyzed to remove extraneous solid matter and to dilute the sample further. The dialyzate was then reacted with quinaldine ethiodide and ethanolamine solutions and subsequently passed through the spectrophotometer where the intensity of the color at 605 nm. was measured and recorded (Fig. 2).

Tablets were sampled at a rate of 20/hr.

The standard solutions were analyzed several times during the day to check for any variation in absorbance. No significant variation was observed.

[1] Beta-Chlor, Mead Johnson Laboratories, Division of Mead Johnson and Co.
[2] Technicon Corp., Tarrytown, N. Y.
[3] Hitachi Perkin-Elmer model 139.
[4] Sargent model TR-05.
[5] Eastman Kodak.
[6] Matheson Coleman and Bell.
[7] Mallinckrodt.
[8] Tween 80, Atlas Chemical Industries, Inc., Wilmington, Del.

Table I—Statistical Results for Chloral Betaine Tablets

Lot	Mean, mg. Chloral Hydrate per Tablet	Coefficient of Variation, %
1	493	1.2
2	502	1.7
3	490	2.4
4	509	1.3
5	512	2.0
6	506	1.7
7	508	1.6
8	499	1.2

Calculation—The average absorbance for each standard was plotted *versus* tablet equivalent to give a standard curve. Reference to the standard curve for each sample yielded the milligrams of chloral hydrate per tablet.

DISCUSSION

Preliminary data indicated incomplete disintegration of the tablet in the Solidprep sampler due to tablet hardness. This problem was overcome by the addition of water to the sample cup at position five on the sampler tray, thus allowing for a 12-min. presoak in 3.6 ml. of water before sampling.

By using the described analytical system, the linearity of absorbance values obtained for standard solutions was satisfactory from 0 to 160% of declared tablet potency. Above 160%, negative deviations from the standard curve were observed, indicating the possibility of limiting concentrations of one or both colorimetric reagents.

RESULTS

Standard Curve—The average absorbance for each of the three standards was determined. A plot of the values gave a straight line in accordance with Beer's law.

Reproducibility of Standards—The reproducibility of the method was checked by the analysis of 30 standards (three levels of concentration). The coefficient of variation ranged from 1.1 to 1.7%.

Statistical Results—The results of eight lots of 50 tablets were evaluated. The data are presented in Table I.

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Synthesis and Anti-Inflammatory Activity of Fluorinated Benzamides

WILLIAM S. DORSEY and WILLIAM D. EASTERLY, Jr.

Abstract □ Six fluorine-containing *N*-substituted benzamides were synthesized, and their physical constants were determined. Their acute toxicity was determined, and LD_{50} values are given. The compounds were screened for anti-inflammatory activity using a modification of the rat-paw edema method. None of the compounds showed activity comparable to hydrocortisone acetate or indomethacin. However, two of the compounds showed sufficient activity to warrant further study.

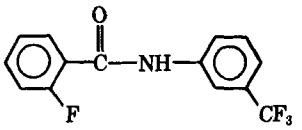
Keyphrases □ Benzamides, fluorinated—synthesized as potential anti-inflammatory agents, physical constants determined, screened using rat-paw edema test □ Anti-inflammatory agents, potential—synthesis and pharmacological evaluation of six fluorine-containing *N*-substituted benzamides

The increased recognition of the widespread and diversified nature of the inflammatory diseases, together with the empirical manner of treatment, recently has stimulated more research in this area. Some research has resulted in the development of new, nonsteroidal drugs, several of which have shown significant therapeutic activity. Indomethacin (1, 2), mefenamic acid (3), and flufenamic acid (4) are typical examples.

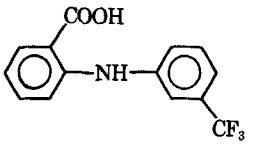
The compounds synthesized in this investigation were screened for anti-inflammatory activity because of their structural similarity to flufenamic acid and because they all contain the trifluoromethyl group which has been associated with anti-inflammatory activity, as shown by Rorig and Wagner (5). The similarity in structure may be seen when one of the prepared compounds is compared with flufenamic acid. The work of Rorig and Wagner (5) indicated that certain 4-trifluoromethyl pyrimidines are quite effective in alleviating the heat, swelling, and rubor characteristics of the inflammatory response to tissue injury.

EXPERIMENTAL

Synthesis—All of the compounds were prepared by a modification of the procedure used by Chase and Weller (6). Equimolar quantities of the appropriate amine[1] and acid chloride[2] were reacted together at room temperature in a mixture of chloroform and pyridine. The amine and an equimolar portion of pyridine were placed in the chloroform, and the acid chloride was added dropwise with stirring. When the initial exothermic reaction subsided, the mixture was refluxed for 30 min. The resulting amide was separated from the reaction mixture by filtration and then recrystallized from $CHCl_3$, ethanol, or aqueous ethanol, depending upon its solubility. The product was then dried in an oven at 40–50° and assayed.



N-(3-trifluoromethylphenyl)-2-fluorobenzamide



flufenamic acid

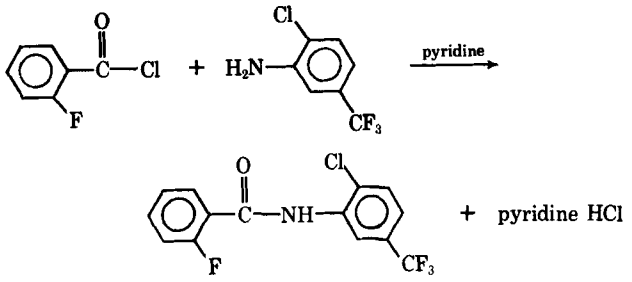
The preparation of *N*-(2-chloro-5-trifluoromethylphenyl)-2-fluorobenzamide represents the general reaction (Scheme I) involved in the preparation of the compounds reported in this paper.

The analytical results for the compounds are given in Table I[3].

Acute Toxicity Studies—The approximate LD_{50} of each compound was determined, using basically the method of Deichman and LeBlanc (7). This method consisted of injecting intraperitoneally four albino rats with the test compound suspended in 1% methylcellulose solution. The dosage to be administered to each of the four animals was based on data from Jordan and Easterly (8), in which *N*-(3-trifluoromethylphenyl)-2,4-dichlorobenzamide, an analog of the compounds prepared in this study, showed an LD_{50} greater than 7.0 g./kg. body weight. Two animals were given doses above 7.0 g./kg., and two animals were given doses below 7.0 g./kg. After 24 hr., the animals were observed and any fatalities were recorded. Two additional doses were subsequently given at dosage levels in between the closest fatal and nonfatal doses. This procedure allowed for a more exact determination of the approximate LD_{50} values.

Results are shown in Table II.

Anti-Inflammatory Studies—The method used to test the compounds for anti-inflammatory activity was a modification of that described by Winter *et al.* (9). Each compound tested was suspended



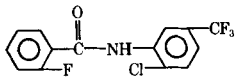
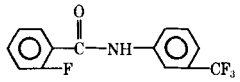
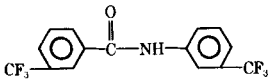
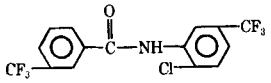
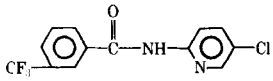
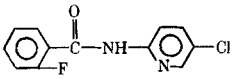
Scheme I

[1] Obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

[2] Obtained from Matheson Chemical Co., Inc., Norwood, Ohio; Pierce Chemical Co., Rockwood, Ill.; and K & K Laboratories, Inc., Plainview, N. Y.

[3] Nitrogen assays were by the Kjeldahl method and halogen determinations were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Table I—*N*-Substituted Benzamides

Compound Substituent Group	Structure of Compound	Melting Point[a]	Analysis, %—Calc.	Found
N-(2-Chloro-5-trifluoromethylphenyl)-2-fluoro-		98–100°	Cl 11.18 F 23.94 N 4.41	11.10 24.08 4.52
N-(3-Trifluoromethylphenyl)-2-fluoro-		96–98°	Cl — F 26.95 N 4.94	— 26.66 4.99
N-(3-Trifluoromethylphenyl)-3-trifluoromethyl-		122–124°	Cl — F 34.23 N 4.20	— 33.04 4.12
N-(2-Chloro-5-trifluoromethylphenyl)-3-trifluoromethyl-		123–125°	Cl 9.67 F 31.02 N 3.81	9.81 31.35 3.81
N-(4-Chloro-2-pyridyl)-3-trifluoromethyl-		115–117°	Cl 11.81 F 18.96 N 9.32	11.90 19.37 9.25
N-(4-Chloro-2-pyridyl)-2-fluoro-		105–108°	Cl 14.17 F 7.58 N 11.18	13.92 7.75 11.05

[a] All melting points were taken on a Thomas-Hoover capillary melting-point apparatus.

in a 1% methylcellulose solution and administered orally by stomach tube to each of six albino male Holzman rats, weighing approximately 250–300 g. each. To ensure equal hydration in all animals, the suspensions were prepared to contain the desired dosage in a 5.0-ml. volume.

One hour after feeding of the compound, 0.05 ml. of a 1% suspension of carrageenin in sterile saline was injected into the plantar region of the hind paw of each animal. The volume of the paw was determined immediately and again after 3 hr.

Foot volume was measured by immersing the paw into mercury contained in a Pyrex U-tube of approximately 30 mm. diameter. The paw was immersed to a previously marked ink line at the level of the lateral malleolus. The mercury in the left column of the U-tube was in contact with isopropyl alcohol containing a small amount of the dye, eosin Y. The interface of the two immiscible liquids was at the midpoint of a bulb, of approximately 85 mm. diameter, connected at the top with a capillary tube graduated in millimeters. The mercury displaced by the immersed paw displaced a proportionate amount of the lighter alcohol, the level of which extended into the calibrated capillary tube. Volume was measured by reading the difference in millimeters of the colored liquid in the capillary tube before and after immersion and converting this millimeter reading into milliliters. Immersion of an object of 1 ml. in volume caused a displacement of 28.4 mm. in the level of the liquid in the capillary tube.

The paws appeared to reach a condition of peak edema in about 3 hr.; therefore, another volume determination was performed at that time. The increase in volume was calculated by comparing the paw volume measurements at injection time and 3 hr. later. Control animals received only tap water, and another group of animals received only 1% methylcellulose. Results are given in Table III.

Table II—Acute Toxicity

Compound	LD_{50}, g./kg.
Benzamide, *N*-(2-chloro-5-trifluoromethylphenyl)-2-fluoro-	>15.7
Benzamide, *N*-(3-trifluoromethylphenyl)-2-fluoro-	4.50
Benzamide, *N*-(3-trifluoromethylphenyl)-3-trifluoromethyl-	>12.5
Benzamide, *N*-(2-chloro-5-trifluoromethylphenyl)-3-trifluoromethyl-	>15.1
Benzamide, *N*-(4-chloro-2-pyridyl)-3-trifluoromethyl-	13.70
Benzamide, *N*-(4-chloro-2-pyridyl)-2-fluoro-	4.80

RESULTS AND DISCUSSION

None of the compounds tested showed edema-inhibiting activity comparable to hydrocortisone acetate or indomethacin. Two of the compounds, the *N*-(4-chloro-2-pyridyl)-2-fluorobenzamide and the *N*-(2-chloro-5-trifluoromethylphenyl)-2-fluorobenzamide, were significantly more active than the others and may warrant further testing.

It would appear that the fluorine atom in the *ortho*-position of the benzamide ring may be a necessary structural component for the anti-inflammatory activity. Furthermore, it would appear that the *N*-chloropyridylbenzamides have slightly more anti-inflammatory (antiedema) activity than those without this chemical group. More compounds in this series would have to be prepared and tested, however, before definitive structure–activity relationships could be established.

Results were calculated as a percentage increase rather than as a total increase in the volume of the paw before and 3 hr. after in-

Table III—Anti-Inflammatory Studies

Compound	Dose, mg./kg.	Mean Percent Increase in Paw Volume (±*SEM*)	Percent Inhibition of Edema (Mean)[a]
Controls			
Tap water	5.0 ml.	35.50 ± 4.29	0
Methylcellulose, 1%	5.0 ml.	37.94 ± 1.28	−7.34[b]
Hydrocortisone[c] acetate	20	2.08 ± 0.65	93.40
Indomethacin[d]	10	14.35 ± 2.71	59.60
Benzamide, *N*-(2-chloro-5-trifluoromethylphenyl)-2-fluoro-	500	27.65 ± 8.49	21.59
Benzamide, *N*-(3-trifluoromethylphenyl)-2-fluoro-	500	33.28 ± 3.77	5.85
Benzamide, *N*-(3-trifluoromethylphenyl)-3-trifluoromethyl-	500	37.39 ± 7.18	−5.78
Benzamide, *N*-(2-chloro-5-trifluoromethylphenyl)-3-trifluoromethyl-	500	35.58 ± 5.95	−0.65
Benzamide, *N*-(4-chloro-2-pyridyl)-3-trifluoromethyl-	500	32.09 ± 5.94	9.64
Benzamide, *N*-(4-chloro-2-pyridyl)-2-fluoro-	500	23.35 ± 3.31	34.00

[a] Data were calculated on the basis that the controls showed no inhibition. [b] Negative values indicate a decrease in inhibition (*i.e.*, increased edema). [c] Produced by Roussel Corp., New York, N. Y. [d] Supplied by Merck Sharp & Dohme Research Lab., Rahway, N. J.

jection of the phlogistic agent. This was done to offset any possible error caused by the difference in the size of the animals used. The standard error of the mean (*SEM*) was calculated according to the formula given by Burn *et al.* (10):

$$s\bar{y} = \sqrt{\frac{S(y - \bar{y})^2}{N(N-1)}} \qquad \text{(Eq. 1)}$$

where $s\bar{y}$ = *SEM*, $\bar{y}$ = mean value, y = any single value, N = total number of animals used in the assay, and S = sum.

The method and techniques developed for the anti-inflammatory screening carried out in this investigation proved to be quite satisfactory. The method was simple and quick, and the apparatus was sufficiently sensitive for the determination of therapeutic levels of antiedema activity. Results with the hydrocortisone acetate and indomethacin, the control compounds, compared favorably with the results of others using antiedema assay procedures (2). Carrageenin seemed to possess a distinct advantage as a phlogistic agent because it produced an edema effectively controlled by the single, oral, nontoxic doses of the known anti-inflammatory agents used in the studies.

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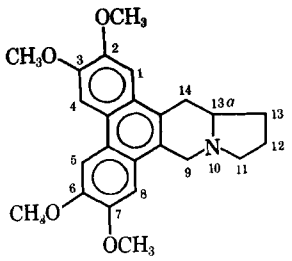
Alkaloids of Tylophora III: New Alkaloids of *Tylophora indica* (Burm) Merrill and *Tylophora dalzellii* Hook. f.

KOPPAKA V. RAO*, RICHARD A. WILSON, and BERNICE CUMMINGS

Abstract □ During a reexamination of the alkaloidal constituents of *Tylophora indica*, three new alkaloids (designated A, B, and C) were isolated. Analytical and spectral data indicate that these alkaloids are related to tylophorine and tylophorinine, which are also present in the plant. Alkaloid B is shown to be a desmethyltylophorine, and Alkaloid C is shown to be a desmethyltylophorinine. *T. dalzellii* has a relatively low alkaloid content, and desmethyltylophorinine is the major constituent. A brief description of the antileukemic activity of desmethyltylophorinine is presented.

Keyphrases □ *Tylophora indica*—isolation of three new alkaloids □ *Tylophora dalzellii*—desmethyltylophorinine determined as major constituent □ Desmethyltylophorinine—isolated and identified from *Tylophora indica* and *Tylophora dalzellii* □ Desmethyltylophorine—isolated and identified from *Tylophora indica*

In a recent publication, isolation of six new alkaloids from *Tylophora crebriflora*, together with the known members tylocrebrine, tylophorine, and septicine, was described (1). Structures for the new members were proposed based on the dibenzo[*f,h*]pyrrolo[1,2*b*]isoquinoline skeleton with four or five oxygen-bearing substituents (2). Interest in these alkaloids arose because of the antitumor activity shown by some members of this group. In this connection, it seemed worthwhile to reexamine the alkaloidal constituents of the related species *T. indica* (Burm) Merrill. A related species, *T. dalzellii* Hook. f., had not been examined, so a brief study of it also was undertaken.



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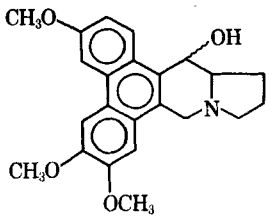

II

DISCUSSION

From *T. indica*, Govindachari *et al.* (3) isolated two alkaloids tylophorine and tylophorinine. They assigned Structures I and II for these compounds, respectively, and later recorded their syntheses (4–7).

The crude alkaloid from *T. indica*[1] was isolated by the following steps: extraction with 0.5% methanolic acetic acid, concentration,

[1] The plant material used was received from India. It was identified and a voucher specimen was preserved at Chas. Pfizer & Co., Inc., Maywood, N. J.

jection of the phlogistic agent. This was done to offset any possible error caused by the difference in the size of the animals used. The standard error of the mean (*SEM*) was calculated according to the formula given by Burn *et al.* (10):

$$s\bar{y} = \sqrt{\frac{S(y - \bar{y})^2}{N(N-1)}} \qquad \text{(Eq. 1)}$$

where $s\bar{y}$ = *SEM*, $\bar{y}$ = mean value, y = any single value, N = total number of animals used in the assay, and S = sum.

The method and techniques developed for the anti-inflammatory screening carried out in this investigation proved to be quite satisfactory. The method was simple and quick, and the apparatus was sufficiently sensitive for the determination of therapeutic levels of antiedema activity. Results with the hydrocortisone acetate and indomethacin, the control compounds, compared favorably with the results of others using antiedema assay procedures (2). Carrageenin seemed to possess a distinct advantage as a phlogistic agent because it produced an edema effectively controlled by the single, oral, nontoxic doses of the known anti-inflammatory agents used in the studies.

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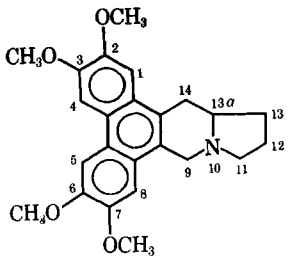
Alkaloids of Tylophora III: New Alkaloids of *Tylophora indica* (Burm) Merrill and *Tylophora dalzellii* Hook. f.

KOPPAKA V. RAO*, RICHARD A. WILSON, and BERNICE CUMMINGS

Abstract □ During a reexamination of the alkaloidal constituents of *Tylophora indica*, three new alkaloids (designated A, B, and C) were isolated. Analytical and spectral data indicate that these alkaloids are related to tylophorine and tylophorinine, which are also present in the plant. Alkaloid B is shown to be a desmethyltylophorine, and Alkaloid C is shown to be a desmethyltylophorinine. *T. dalzellii* has a relatively low alkaloid content, and desmethyltylophorinine is the major constituent. A brief description of the antileukemic activity of desmethyltylophorinine is presented.

Keyphrases □ *Tylophora indica*—isolation of three new alkaloids □ *Tylophora dalzellii*—desmethyltylophorinine determined as major constituent □ Desmethyltylophorinine—isolated and identified from *Tylophora indica* and *Tylophora dalzellii* □ Desmethyltylophorine—isolated and identified from *Tylophora indica*

In a recent publication, isolation of six new alkaloids from *Tylophora crebriflora*, together with the known members tylocrebrine, tylophorine, and septicine, was described (1). Structures for the new members were proposed based on the dibenzo[*f,h*]pyrrolo[1,2*b*]isoquinoline skeleton with four or five oxygen-bearing substituents (2). Interest in these alkaloids arose because of the antitumor activity shown by some members of this group. In this connection, it seemed worthwhile to reexamine the alkaloidal constituents of the related species *T. indica* (Burm) Merrill. A related species, *T. dalzellii* Hook. f., had not been examined, so a brief study of it also was undertaken.



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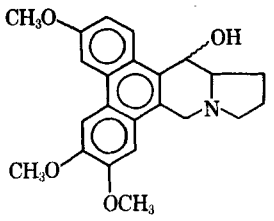

II

DISCUSSION

From *T. indica*, Govindachari *et al.* (3) isolated two alkaloids tylophorine and tylophorinine. They assigned Structures I and II for these compounds, respectively, and later recorded their syntheses (4–7).

The crude alkaloid from *T. indica*[1] was isolated by the following steps: extraction with 0.5% methanolic acetic acid, concentration,

[1] The plant material used was received from India. It was identified and a voucher specimen was preserved at Chas. Pfizer & Co., Inc., Maywood, N. J.

Table I—Properties of the Alkaloids of *T. indica*

	Alkaloid A		Alkaloid B		Alkaloid C	
Melting point	250–252° dec.		235–237° dec.		253–255° dec.	
Analysis	$C_{22}H_{23}NO_5 \cdot H_2O$		$C_{23}H_{25}NO_4$		$C_{22}H_{23}NO_4 \cdot {}^1/_2H_2O$	
	Calc.	Found	Calc.	Found	Calc.	Found
C	66.15	66.89	72.80	72.48	70.61	70.65
H	6.31	6.45	6.64	6.45	6.46	6.31
N	3.50	3.45	3.68	3.56	3.74	3.76
OCH_3	15.52 (2)	15.28	24.51 (3)	23.95	16.56 (2)	16.85
Molecular weight, *m/e*[a]	381		379		365	
UV spectrum	$\lambda_{max.}$	log *E*	$\lambda_{max.}$	log *E*	$\lambda_{max.}$	log *E*
	290	4.48	290	4.30	288	4.32
	257	4.70	257	4.80	258	4.65
Paper chromatographic behavior, R_f: formamide[b]–chloroform	0.2		0.5		0.1	

[a] Mass spectra were obtained on a Hitachi–Perkin-Elmer single-focusing instrument with 70 ev. as the ionizing voltage. [b] Whatman No. 1 filter paper moistened with a 30% solution of formamide in acetone, blotted, and developed with chloroform saturated with formamide, after the samples are spotted. Tylophorine and tylophorinine show R_f values of 0.7 and 0.3, respectively.

partition of the concentrate between 0.1 *N* HCl and ethyl acetate, extraction of the aqueous layer with chloroform at pH 9, concentration of the extract, and crystallization from methanol. It was freed from most of the tylophorine in the form of its slightly soluble hydrochloride. The mother liquors were then subjected to countercurrent distribution in the following system: 3% aqueous acetic acid–chloroform–*n*-butanol (3:2:1). The presence of five alkaloids was revealed, two of which were identified as tylophorine and tylophorinine. The remaining three appeared to be new and were designated as Alkaloids A, B, and C in the order of increasing polarity in the distribution. Together, these three alkaloids represented approximately 10–15% of the total alkaloid content; of the three, Alkaloid C was the major component. The analytical and physical data are given in Table I.

Alkaloid A has two phenolic groups and two methoxyls. The general similarity of its UV and IR spectra with those of tylophorine suggests that the substitution pattern of tylophorine may be present, with the fifth oxygen presumably being located at 14, in analogy with other members of this group.

Alkaloid B has three methoxyls. Its UV spectrum is very similar to that of tylophorine but shifts in basic solution, thus suggesting the presence of a phenolic hydroxyl. Methylation with diazomethane yields tylophorine. Alkaloid B is thus desmethyltylophorine, although the position of the phenolic group cannot be located unequivocally at this time.

Alkaloid C has two methoxyls, one phenolic hydroxyl, and one benzylic hydroxyl. It forms a diacetate, m.p. 225–227°.

Table II—NMR Spectra of Acetates of Tylophorinine and Alkaloid C

Proton	Tylophorinine	Alkaloid C	Remarks
1	τ 2.32, 2.17	2.35, 2.20	*AB* pattern
2	2.79, 2.94	2.79, 2.94	*AB* pattern, split (J = 2)
4	2.24	2.35	Split (J = 2)
5	2.24	1.77	Singlet
8	2.97	2.97	Singlet

Table III—Antileukemic Activity of Desmethyltylophorinine[a]

Dose, mg./kg.	Change in Body Weight	Increase in Survival Time, %
12	−2.2	135
8	−1.5	149
6	+0.2	145
4	+1.3	138
2.7	+2.3	123

[a] The author is indebted to Dr. T. J. McBride of the John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, N. J., for the data in this table. The assay was carried out according to the protocols given in Cancer Chemotherapy Report No. 25, Dec. 1962. An increase in survival rate of 125% or higher is considered as positive activity.

Anal.—Calc. for $C_{26}H_{27}NO_6$: C, 69.47; H, 6.05; N, 3.11. Found: C, 69.05; H, 6.22; N, 3.25.

Its IR spectrum (bands at 1725 and 1760 cm.$^{-1}$) and NMR spectrum (3H peaks at τ 7.87 and 7.49) support the presence of an alcoholic and a phenolic acetyl group, respectively. Methylation with diazomethane leads to tylophorinine. Because of the low solubility in organic solvents, satisfactory NMR spectra could not be obtained. A comparison of the NMR spectra of the acetates of Alkaloid C and of tylophorinine is shown in Table II.

The spectra suggest that the phenolic hydroxyl is at either 3 or 6. A choice between the two is not possible from these data, and further work is planned.

Unlike the related species, *T. crebriflora* and *T. indica*, *T. dalzellii* is rather low in its alkaloid content. After routine extraction, the crude alkaloid was subjected to countercurrent distribution in the system already described. One major peak represented nearly all of the alkaloid. It was crystallized from chloroform–methanol, m.p. 253–255°.

Anal.—Calc. for $C_{22}H_{23}NO_4 \cdot H_2O$: C, 68.91; H, 6.57; N, 3.65; OCH_3 (2), 16.20. Found: C, 68.64; H, 6.37; N, 3.37; OCH_3, 15.60. Its melting point and analytical, spectral, and chromatographic data showed that it was identical with Alkaloid C of *T. indica*, desmethyltylophorinine.

Of the five alkaloids of *T. indica*, only Alkaloid C showed significant activity in murine leukemia (L-1210 system) (Table III).

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Occurrence of Atromentin and Thelephoric Acid in Cultures of *Clitocybe subilludens*

G. SULLIVAN, R. D. GARRETT, and R. F. LENEHAN

Abstract □ Atromentin was found to be present in both 30- and 45-day-old cultures of *Clitocybe subilludens* Murr., whereas thelephoric acid was present in the 30-, 45-, and 60-day-old cultures. These cultures were grown on 6% malt extract agar under continuous light conditions at 29° for 15, 30, 45, and 60 days. Cultures grown in the absence of light failed to produce detectable quantities of either terphenylquinone. Examination of all cultures for diphenyl-substituted tetronic acids failed to demonstrate detectable amounts. It is postulated from these observations that atromentin may act as a precursor for thelephoric acid, since the disappearance of the former coincides with the appearance of the latter terphenylquinone.

Keyphrases □ *Clitocybe subilludens*—occurrence of atromentin and thelephoric acid in cultures □ Atromentin—occurrence in 30- and 45-day-old *Clitocybe subilludens* cultures □ Thelephoric acid—occurrence in 30-, 45-, and 60-day-old *Clitocybe subilludens* cultures

The production of terphenylquinones and tetronic acids by saprophytic cultures of fungi has been established. Divekar *et al.* (1) reported the isolation of volucrisporin from cultures of the hyphomycete *Volucrispora aurantiaca* Haskins. Phlebiarubrone, an orthoquinone produced by *Phlebia strigosozonata* (Schw.) Lloyd, also was detected in cultures of this basidiomycete (2). Gaylord *et al.* (3) were unable to detect atromentin in cultures of *Paxillus atrotomentosus* (Batsch) Fr., an agaric which produces this terphenylquinone under natural growing conditions. However, two diphenyl-substituted tetronic acids, xerocomic and atromentic acids, were isolated from the vegetative mycelia by these investigators.

Since carpophores of *Clitocybe subilludens* Murr. are known to contain atromentin (4), it appeared desirable to examine surface cultures of this basidiomycete for the presence of terphenylquinones and diphenyl-substituted tetronic acids.

EXPERIMENTAL

Origin and Maintenance of Culture—Carpophores of *C. subilludens*[1] were collected in Austin, Tex., in the autumn of 1967. Isolates[2] were obtained from these mushrooms and maintained on 6% malt extract agar slants in a humidified incubator at 29°.

Incubation of Surface Cultures—Sixteen 2800-ml. Fernbach flasks, each containing 200 ml. of sterile 6% malt extract agar, were aseptically inoculated with equal aliquots of a homogeneous suspension of *C. subilludens*. The suspension was prepared from one of the stock slants, sterile water, and a sterilized microblender. One group (Group A) of eight flasks was incubated at 29° under continuous light exposure. The remaining eight flasks (Group B) were incubated at 29° in continuous darkness. At incubation periods of 15, 30, 45, and 60 days, respectively, the mycelium of one flask from Group A and one flask from Group B was harvested and extracted according to Procedure I. Concurrently, the mycelium of a second flask from each of Groups A and B was harvested and extracted according to Procedure II.

Table I—Dry Weights of Harvested Mycelial Mats

Incubation Period, Days	Dry Weight, g. Group A	Dry Weight, g. Group B
15	0.6793	0.4618
30	0.7430	0.4981
45	1.6050	0.4098
60	3.9400	0.5242

Extraction Procedure I—Each harvested mycelial mat was dried at 45° for 48 hr., ground to a 20-mesh powder with a Wiley mill, weighed, placed in a soxhlet apparatus, and sequentially extracted with *n*-hexane (48 hr.), ether (24 hr.), chloroform (24 hr.), a benzene–methanol azeotrope[3] (24 hr.), methanol (2 hr.), acetone (36 hr.), and ethanol (48 hr.). Each extract was evaporated to a dry residue, weighed, and analyzed by TLC.

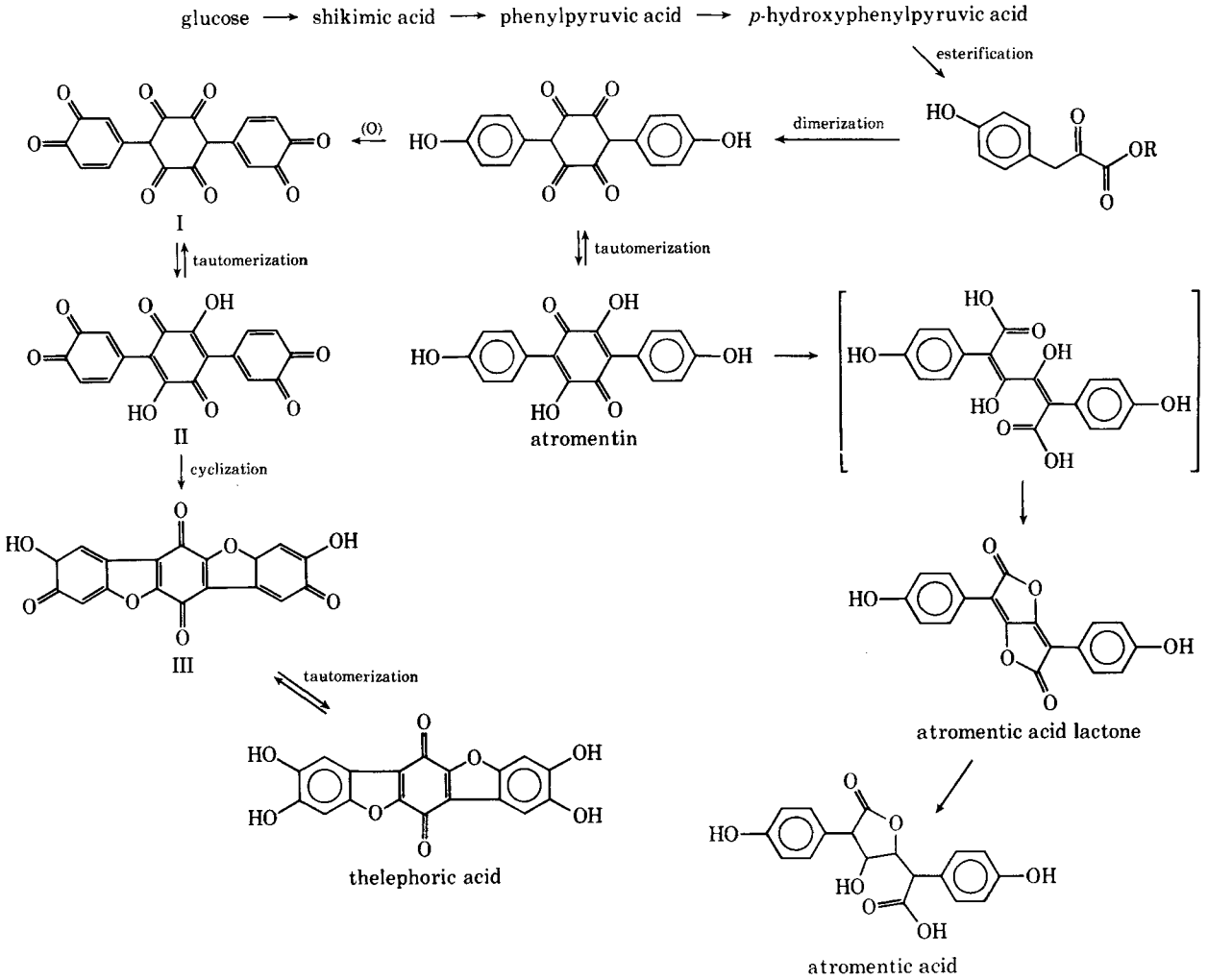
Extraction Procedure II—This procedure was carried out under conditions of subdued light to minimize possible photodegradation of tetronic acids. An aqueous slurry of each harvested mycelial mat was prepared with a microblender, placed into a 250-ml. separator, and extracted with successive 100-ml. portions of ether and ethyl acetate. Each organic extract was concentrated and analyzed by TLC. No evidence for the presence of tetronic acid derivatives was noted.

Chromatographic Analysis—Silica gel G[4] plates, prepared according to Stahl (5), were employed for each TLC system. Longwave UV light and iodine vapors were used for visualization. Authentic samples[5] of tetronic acids (atromentic and pulvinic), atromentin, and thelephoric acid were used as reference markers on each TLC plate. These reference compounds were found to have R_f values of 0.87, 0.86, 0.70, and 0.44, respectively, when methanol–0.30 molal oxalic acid dihydrate in methanol–formic acid (100:2:0.1) was used as the mobile phase. When benzene–ethyl formate–formic acid (13:5:4) was employed as the mobile phase, R_f values of 0.50, 0.78, 0.45, and 0.00, respectively, were obtained.

Isolation and Identification of Atromentin—The residue obtained from the ether extract (Procedure I) of the 30-day-old culture grown in continuous light was selected, since TLC analysis indicated the presence of a significant amount of material whose TLC characteristics were identical with authentic atromentin. The residue was shaken with ether, filtered, evaporated to a dry residue, and shaken with warm chloroform. The resulting suspension was filtered, and the residue was washed with ethanol and filtered. Evaporation of the ethanolic filtrate to dryness produced a brownish solid which was washed with water and dried. The dried brownish solid was subjected to a pyridine–water color reaction test (6); it was observed to sublime and decompose without melting above 280°; $\nu_{max.}^{KBr}$ 3448, 3350, 1650, 1515, 1450, 1110, 999, and 834 cm.$^{-1}$; $\lambda_{max.}^{dioxane}$ 268 and 385 nm.; $\lambda_{max.}^{MeOH}$ 276 and 360 nm. All data obtained by these analyses were identical to those of reference atromentin.

Isolation and Identification of Thelephoric Acid—TLC analysis indicated that a significant amount of material of R_f value identical to that of reference thelephoric acid was present in the acetone fraction (Procedure I) of the 60-day-old culture grown in continuous light. This solid residue was washed twice with ether and twice with methanol. Black crystals were obtained after filtration. The black crystalline compound gave a positive pyridine–water color reaction

[1] Previously identified by Dr. H. E. Bigelow, Department of Botany, University of Massachusetts.
[2] Voucher specimen, American Type Culture Collection No. 22516.
[3] Benzene–methanol (60.5:39.5).
[4] Brinkmann Instruments, Inc., Westbury, N. Y.
[5] Obtained from Dr. L. R. Brady, College of Pharmacy, University of Washington.



Scheme I—Biogenetic Scheme

test; it did not melt below 350°; $\nu_{max.}^{KBr}$ 3200, 1645, 1525, 1273, 1038, and 802 cm.$^{-1}$; $\lambda_{max.}^{EtOH}$ 264, 305, and 485 nm.; $\lambda_{max.}^{dioxane}$ 263, 305, and 450 nm. All these data were identical to those of reference thelephoric acid. In addition, the tetraacetate derivative of the isolated thelephoric acid was prepared according to the method described by Lounasmaa (7). This derivative exhibited m.p. 330–335°; $\nu_{max.}^{KBr}$ 1780, 1680, 1530, 1366, 1262, 1200, 1154, 1124, 1033, and 1015 cm.$^{-1}$; $\lambda_{max.}^{dioxane}$ 263, 283, and 360 nm. These data were identical to those reported in the literature for thelephoric acid tetraacetate (8).

RESULTS AND DISCUSSION

The weights of the dried mycelial mats obtained after 15-, 30-, 45-, and 60-day-incubation periods under conditions of continuous light exposure (Group A) and continuous dark exposure (Group B) are shown in Table I.

Chromatographic analysis of all residues obtained by soxhlet extraction of the *C. subilludens* mycelial mats from 15-, 30-, 45-, and 60-day-incubation periods, under continuous exposure to light, indicated atromentin to be present only in the ether fractions from the 30- and 45-day-old cultures. Thelephoric acid was initially detected in the acetone and ethanol fractions from the 30-day-old cultures. All remaining soxhlet fractions (except hexane) from the 45- and 60-day-old cultures were found to contain increasing amounts of thelephoric acid. Therefore, the concentration of thelephoric acid was noted to increase with time; the greatest amount of this terphenylquinone was detected in the ethanol fraction of the 60-day-old culture. Tetronic acids were not detected in any fraction.

No terphenylquinones were detected in any extract obtained from cultures grown in the absence of light (Group B). In addition, no tetronic acids were detected in any extract obtained from cultures from either Group A or B.

It is apparent from these results that light plays a major role in both continued mycelial growth and the production of terphenylquinones in cultures of *C. subilludens*.

Read *et al.* (9) demonstrated the incorporation of ^{14}C-labeled shikimic acid, phenylalanine, phenyllactic acid, and *m*-tyrosine into the terphenylquinone pigment, volucrisporin. From these observations, it was postulated that atromentin, leucomelone, polyporic acid, and probably thelephoric acid would be derived from appropriately substituted phenylpropanoid compounds by a similar pathway. The incorporation of labeled phenylalanine into the terphenyl ring system of phlebiarubrone (2) further substantiated the existence of this biogenetic pathway. Tracer experiments with phenylalanine, phenyllactic acid, and polyporic acid, which were readily incorporated into pulvinic acid lactone and calycin in the lichen *Pseudocyphellaric croccata* (10), established a definite relationship between terphenylquinones and diphenyl-substituted tetronic acids. A particularly interesting observation was made by Gaylord *et al.* (3). These investigators found that cultures of *P. atrotomentosus* produced detectable amounts of xerocomic and atromentic acids, whereas the mature carpophore yielded only atromentin.

The occurrence of atromentin and thelephoric acid and the apparent absence of tetronic acid derivatives in cultures of *C. subilludens* do not contradict the possible interrelationships of these compounds, since the biogenetic control mechanism may be inoperable or nonexistent in cultures of this basidiomycete. The simultaneous disappearance of atromentin with the appearance of thelephoric acid suggests that atromentin may act as a precursor to thelephoric acid.

Scheme I represents a possible biosynthetic relationship which

may exist between terphenylquinones and diphenyl-substituted tetronic acid. Although Read *et al.* (9) suggested that hydroxylation of the phenylpropanoid compound occurs prior to the formation of the terphenyl skeleton, it appears reasonable that hydroxylation could occur after dimerization. If atromentin is a precursor to thelephoric acid, this reaction must take place after terphenyl formation. It appears that Compounds I, II, and III are plausible biosynthetic intermediates in the conversion of atromentin to thelephoric acid. Tracer studies are indicated to establish the biogenetic relationship between atromentin and thelephoric acid as they exist in cultures of *C. subilludens*.

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Isolation and Identification of Constituents from *Cudrania javanensis*

JOSEPH E. KNAPP and PAUL L. SCHIFF, Jr.

Abstract □ Osajaxanthone, vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, and (−)-(*S*)-stachydrine were isolated from extracts of the bark of the tropical shrub *Cudrania javanensis* Trécul (Moraceae).

Keyphrases □ *Cudrania javanensis*—isolation and identification of osajaxanthone, vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, (−)-(*S*)-stachydrine □ Column chromatography—separation of *Cudrania javanensis* constituents

Cudrania javanensis Trécul (Moraceae) is a climbing shrub or small tree of tropical Asia, Australia, and Polynesia (1). Extracts of the plant have been used to dye cotton yellow (2). The plant is commonly called cockspur thorn in Australia, where it has been suspected of poisoning stock (3). The absence of knowledge of the constituents of *Cudrania* species prompted us to undertake a phytochemical investigation of this plant. The isolation and identification of osajaxanthone (I), vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, and (−)-(*S*)-stachydrine (II) are reported here.

The plant material was moistened with dilute ammonium hydroxide and extracted by percolation to exhaustion with ethanol. The extract residue was partitioned between dilute hydrochloric acid and ether. The ether solution was extracted with dilute potassium hydroxide, washed with water, and set aside. The alkaline solution was acidified and reextracted with ether. Chromatography of this ether extract over silicic acid–Celite (4:1) afforded osajaxanthone (I), vanillic acid, monomethyl fumarate, and *p*-hydroxybenzoic acid. Osajaxanthone was first isolated from the root bark of the Osage Orange [*Maclura pomifera* (Moraceae)] (4) and subsequently from the trunk wood of Brazilian *Kielmeyera corymbosa* (Guttiferae) (5) and the heartwood of Malaysian *Calophyllum scriblitifolium* (Guttiferae) (6). Closely related xanthones from *Maclura* species have been reported to be toxic to goldfish and mosquito larvae (4). The presence of this xanthone may account for the use of *Cudrania* extracts as yellow dyes.

The dilute hydrochloric acid extract was basified with ammonium hydroxide and extracted successively with ether and chloroform. The organic extracts were combined and set aside. The remaining alkaline solution was acidified, and a quaternary alkaloid fraction was collected by precipitation with ammonium reineckate. Treatment of the reineckate complex with an anion-exchange resin in the chloride form afforded a quaternary alkaloid chloride fraction which, upon chromatography over neutral alumina, yielded (−)-(*S*)-stachydrine (II), characterized as the hydrochloride salt.

EXPERIMENTAL[1]

Plant Material—*C. javanensis* (Moraceae) bark[2] was received from Australia and identified at the point of collection. The bark was air-dried and ground to a No. 4 mesh powder.

[1] Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. The UV spectra were obtained on a Perkin-Elmer model 202 recording spectrophotometer, and the IR spectra were determined on a Perkin-Elmer model 257 spectrometer or a Beckman model IR-8 spectrometer in KBr pellets. NMR spectra were recorded in $CDCl_3$ solution on a Bruker Scientific, Inc., model B-90 instrument equipped with a time-averaging computer, with $(CH_3)_4Si$ as the internal standard and chemical shifts reported in δ (p.p.m.) units. Mass spectra were taken with a LKB-9000 mass spectrometer. Optical rotations were measured in a Rudolph polarimeter. All reagents were analytical reagent grade, unless otherwise noted.

[2] Obtained from the Meer Corp., New York, N. Y. (Lot. No. MS 1871).

may exist between terphenylquinones and diphenyl-substituted tetronic acid. Although Read *et al.* (9) suggested that hydroxylation of the phenylpropanoid compound occurs prior to the formation of the terphenyl skeleton, it appears reasonable that hydroxylation could occur after dimerization. If atromentin is a precursor to thelephoric acid, this reaction must take place after terphenyl formation. It appears that Compounds I, II, and III are plausible biosynthetic intermediates in the conversion of atromentin to thelephoric acid. Tracer studies are indicated to establish the biogenetic relationship between atromentin and thelephoric acid as they exist in cultures of *C. subilludens*.

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Isolation and Identification of Constituents from *Cudrania javanensis*

JOSEPH E. KNAPP and PAUL L. SCHIFF, Jr.

Abstract □ Osajaxanthone, vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, and (−)-(*S*)-stachydrine were isolated from extracts of the bark of the tropical shrub *Cudrania javanensis* Trécul (Moraceae).

Keyphrases □ *Cudrania javanensis*—isolation and identification of osajaxanthone, vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, (−)-(*S*)-stachydrine □ Column chromatography—separation of *Cudrania javanensis* constituents

Cudrania javanensis Trécul (Moraceae) is a climbing shrub or small tree of tropical Asia, Australia, and Polynesia (1). Extracts of the plant have been used to dye cotton yellow (2). The plant is commonly called cockspur thorn in Australia, where it has been suspected of poisoning stock (3). The absence of knowledge of the constituents of *Cudrania* species prompted us to undertake a phytochemical investigation of this plant. The isolation and identification of osajaxanthone (I), vanillic acid, monomethyl fumarate, *p*-hydroxybenzoic acid, and (−)-(*S*)-stachydrine (II) are reported here.

The plant material was moistened with dilute ammonium hydroxide and extracted by percolation to exhaustion with ethanol. The extract residue was partitioned between dilute hydrochloric acid and ether. The ether solution was extracted with dilute potassium hydroxide, washed with water, and set aside. The alkaline solution was acidified and reextracted with ether. Chromatography of this ether extract over silicic acid–Celite (4:1) afforded osajaxanthone (I), vanillic acid, monomethyl fumarate, and *p*-hydroxybenzoic acid. Osajaxanthone was first isolated from the root bark of the Osage Orange [*Maclura pomifera* (Moraceae)] (4) and subsequently from the trunk wood of Brazilian *Kielmeyera corymbosa* (Guttiferae) (5) and the heartwood of Malaysian *Calophyllum scriblitifolium* (Guttiferae) (6). Closely related xanthones from *Maclura* species have been reported to be toxic to goldfish and mosquito larvae (4). The presence of this xanthone may account for the use of *Cudrania* extracts as yellow dyes.

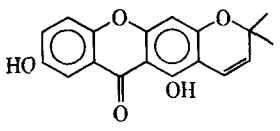
The dilute hydrochloric acid extract was basified with ammonium hydroxide and extracted successively with ether and chloroform. The organic extracts were combined and set aside. The remaining alkaline solution was acidified, and a quaternary alkaloid fraction was collected by precipitation with ammonium reineckate. Treatment of the reineckate complex with an anion-exchange resin in the chloride form afforded a quaternary alkaloid chloride fraction which, upon chromatography over neutral alumina, yielded (−)-(*S*)-stachydrine (II), characterized as the hydrochloride salt.

EXPERIMENTAL[1]

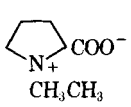
Plant Material—*C. javanensis* (Moraceae) bark[2] was received from Australia and identified at the point of collection. The bark was air-dried and ground to a No. 4 mesh powder.

[1] Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. The UV spectra were obtained on a Perkin-Elmer model 202 recording spectrophotometer, and the IR spectra were determined on a Perkin-Elmer model 257 spectrometer or a Beckman model IR-8 spectrometer in KBr pellets. NMR spectra were recorded in $CDCl_3$ solution on a Bruker Scientific, Inc., model B-90 instrument equipped with a time-averaging computer, with $(CH_3)_4Si$ as the internal standard and chemical shifts reported in δ (p.p.m.) units. Mass spectra were taken with a LKB-9000 mass spectrometer. Optical rotations were measured in a Rudolph polarimeter. All reagents were analytical reagent grade, unless otherwise noted.

[2] Obtained from the Meer Corp., New York, N. Y. (Lot. No. MS 1871).



I



II

Extraction and Initial Fractionation—The dried ground bark (3.25 kg.) was moistened with ammonium hydroxide–water (1:10) and extracted by percolation to exhaustion with ethanol. The dark syrupy residue (335 g.) remaining after removal of the alcohol under reduced pressure at 40° was suspended in 2 l. of ether and extracted initially with 3 × 1-l. portions of 5% hydrochloric acid and subsequently with 3 × 1-l. portions of 1% potassium hydroxide. The combined potassium hydroxide fraction was made acidic to pH 2–3 with hydrochloric acid while cooling and was extracted with 3 × 3-l. portions of ether. The resulting dried (anhydrous sodium sulfate) ether fraction, on evaporation under reduced pressure, yielded an oily residue (46.9 g.) of crude acidic substances. The ether layer remaining after extraction with potassium hydroxide solution was washed with water and dried (anhydrous sodium sulfate), and the solvent was removed under reduced pressure to afford a dark oily residue (76.7 g.) of neutral substances.

The combined hydrochloric acid extract was made alkaline (pH 8–9) with ammonium hydroxide and extracted successively with 3 × 2-l. portions of ether and 3 × 2-l. portions of chloroform. The ether and chloroform extracts were combined and dried (anhydrous sodium sulfate), and the solvent was removed under reduced pressure to yield a crude alkaloidal oil (4.2 g.).

The alkaline solution was reacidified to pH 3 with hydrochloric acid and treated with a 2% ammonium reineckate solution until precipitation was complete. The precipitate was filtered by suction, washed with ether, and air-dried to afford a crude quaternary alkaloid reineckate fraction (6.54 g.).

Isolation of (−)-(*S*)-Stachydrine (II)—The quaternary alkaloid reineckate fraction was suspended in 150 ml. of 50% aqueous acetone and stirred for 5 hr. with 20 ml. of Dowex-1-X4 (Cl) resin[3]. The resin was removed by filtration and washed with 50 ml. of 50% aqueous acetone. The filtrate and the washing were combined and evaporated to dryness to afford a crude quaternary alkaloid chloride residue (6.12 g.). Chromatography of this residue over neutral alumina[4] with methanol as an eluent gave a dark syrup (1.29 g.); on treatment with several milliliters of methanol and enough concentrated hydrochloric acid to afford a distinctly acidic solution, it yielded white plates of (−)-(*S*)-stachydrine hydrochloride (II) (120 mg.), m.p. 227–229° dec. [lit. (17) 222° dec.], $[\alpha]_D^{28}$ −25.0 (c 0.515, H_2O) {lit. (7) $[\alpha]_D^{22}$ −27.5 [c 2, H_2O]}, identified by comparison (IR, mixed melting point) with an authentic sample. Stachydrine was prepared by treatment of an aqueous solution of (−)-(*S*)-proline[5] with silver oxide, methanol, and methyl iodide as described by Patchett and Witkop (8) for betonicine [(−)-4-hydroxystachydrine]. Stachydrine hydrochloride, m.p. 228–228.5° dec., $[\alpha]_D^{28}$ −27.0 (c 1.0, H_2O), was prepared by treating an ethereal methanolic solution of the synthetic stachydrine with dry hydrogen chloride gas.

Chromatography of Acidic Fraction—A silicic acid[6]–Celite[7] (4:1) column was packed with 230 g. of the adsorbent and poured as a slurry in benzene. An 18-g. aliquot of the acidic fraction was dissolved in benzene and applied to the column; elution was begun with 1-l. portions of benzene.

Isolation of Osajaxanthone (I)—Evaporation of the fifth benzene fraction and recrystallization of the residue (489 mg.) from hexane–chloroform afforded bright-yellow needles of osajaxanthone (I) (18.6 mg.), m.p. 255–257° dec.; $\lambda_{max.}^{MeOH}$ 206 nm. (log ε 4.04), 232 (4.10), 2.39 (4.10), 248 (4.08), 284 (4.46), 338 (3.73), and 378 (3.49), with a bathochromic shift in methanolic potassium hydroxide and aluminum chloride solutions but no change in methanolic sodium acetate; $\nu_{max.}$ 1645 cm.$^{-1}$ (xanthone C=O). The NMR spectrum showed the characteristic signals of a 2,2-dimethylchrom-3-ene system (9), with a sharp singlet at δ 1.57 for the gem-dimethyl group and a pair of doublets centered at δ 5.61 and 6.69 (J = 10.8 Hz.) for the two *cis*-olefinic protons. The mass spectrum showed a molecular ion peak at *m/e* 310 for $C_{18}H_{14}O_5$ and a base peak at *m/e* 295, corresponding to the loss of methyl group from the dimethylpyran ring, leaving a resonance-stabilized pyrilium cation (6). Identification was by comparison of the UV, IR, and mass spectra with an authentic sample and by a mixed melting-point determination.

Isolation of Vanillic Acid—Evaporation of the seventh benzene fraction and recrystallization of the residue (828 mg.) from hexane–chloroform afforded white needles of vanillic acid (14.6 mg.), m.p. 204–206°; $\lambda_{max.}^{MeOH}$ 206 nm. (log ε 4.24), 216 (4.25), 257 (3.98), and 291 (3.67); UV, IR, and mixed melting-point data were identical with an authentic sample[8].

Isolation of Monomethyl Fumarate—Evaporation of the eighth column fraction and recrystallization of the residue (311 mg.) from hexane–chloroform afforded white needles of monomethyl fumarate (30 mg.), m.p. 144–145°; $\lambda_{max.}^{MeOH}$ 208 nm. (log ε 4.28); $\nu_{max.}$ 1718 (C=O, α,β-unsaturated ester), 1685 (C=O, α,β-unsaturated acid), and 1630 cm.$^{-1}$ (C=C, α,β-unsaturated system); UV, IR, mass spectra, and mixed melting-point data were identical with an authentic sample prepared by partial hydrolysis of dimethyl fumarate according to Erlenmeyer and Schoenauer (10).

Isolation of *p*-Hydroxybenzoic Acid—After further elution of the column with benzene–chloroform mixtures and chloroform, elution with 2% methanol in chloroform yielded a crystalline residue (2.17 g.), which was recrystallized from chloroform–methanol to afford white needles of *p*-hydroxybenzoic acid (310 mg.), m.p. 295–296° dec.; $\lambda_{max.}^{MeOH}$ 208 nm. (log ε 4.21) and 255 (4.09); UV, IR, and mixed melting-point data were identical with an authentic sample[8].

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[3] Dow Chemical Co., Midland, Mich.
[4] Woelm, activity grade V, Alupharm Chemicals, New Orleans, La.
[5] Nutritional Biochemicals, Cleveland, Ohio.
[6] Mallinckrodt Chemical Works, St. Louis, Mo.
[7] Supplied as "Celite Analytical Filter Aid" by Johns-Manville Co., New York, N. Y.
[8] Eastman Organic Chemicals, Rochester, N. Y.

Monolayers of Polyvinylpyrrolidone Copolymers

JOEL L. ZATZ and BEVERLY KNOWLES

Abstract □ The surface properties of graft copolymers of polyvinylpyrrolidone and vinyl acetate were investigated. The vinyl acetate grafts anchor the copolymer to the surface. Compression forces the vinylpyrrolidone residues beneath the surface, imparting considerable flexibility and accounting for the high compressibility and low cohesion observed.

Keyphrases □ Polyvinylpyrrolidone and vinyl acetate copolymers—surface properties of monolayers □ Copolymers, graft—surface properties of polyvinylpyrrolidone and vinyl acetate monolayers □ Surface properties—polyvinylpyrrolidone and vinyl acetate copolymer monolayers

Surface activity is an important attribute of many polymers used in the pharmaceutical industry. Such properties as surface rheology, adhesive strength, and orientation at an interface may be determining factors in certain applications. These properties are conveniently studied by spreading polymers as monolayers at the surface. Using this technique, Jarvis (1) was able to relate the antifoaming activity of some silicones with their low surface viscosity. Glazer (2) investigated the effect of polymer structure on adhesion of some ethoxylin resins. Monolayer experiments suggested that some polymers stabilize emulsions by forming an interfacial barrier to coalescence (3). In addition, monolayer studies provided insight into the molecular properties of polymers used as pharmaceutical coatings (4–7). The behavior of monolayers of some cellulose derivatives was correlated with the ability of these materials to function as enteric coatings (6). The effect of pH on monolayers of cellulose acetate phthalate was investigated (7).

Most surface work on polymers of pharmaceutical interest has dealt with linear molecules. However, for certain applications, graft and block copolymers offer advantages. Homopolymers, which are not usually compatible, may be combined in the same molecule in such a manner that, for the most part, the properties of each component are retained. This type of polymer often finds application as a dispersing agent or surfactant or as a film former. In this communication, experiments on monolayers of graft copolymers of polyvinylpyrrolidone and vinyl acetate are described.

EXPERIMENTAL

Copolymers of polyvinylpyrrolidone and vinyl acetate[1] were purified by precipitation from solution in isopropanol, using *n*-hexane as the nonsolvent. The polymer mass was washed repeatedly with *n*-hexane and then dried *in vacuo* at 50° to constant weight. The relative content of vinylpyrrolidone was determined by nitrogen analysis. The composition of the purified materials is summarized in Table I.

Water was deionized and distilled from an all-glass still. Organic liquids (reagent grade) were found to be free of surface-active impurities (8). Inorganic materials (reagent grade) were not further purified. The trough of the surface balance was made of Teflon. Surface pressure was determined by the Wilhelmy plate method, using a roughened platinum plate. The temperature of the subphase was maintained at 25 ± 0.1° by a Lauda K-2 thermostat, except as otherwise indicated. Water was used as the subphase. The copolymers were spread from solution in isopropanol–benzene, using an Agla micrometer syringe.

[1] Polyvinylpyrrolidone/vinyl acetate copolymers, GAF Corp.

Table I—Composition of Copolymers

Copolymer Designation	Total Monomers, % w/w	Vinylpyrrolidone Content, % w/w	Vinylpyrrolidone Content, % Vinylpyrrolidone Residues
335	<0.01	29.2	24.2
535	<0.01	48.6	42.3
735	<0.01	61.2	55.0

RESULTS AND DISCUSSION

Monolayer Stability—Force–area isotherms for the polyvinylpyrrolidone copolymers studied are given in Fig. 1. At large values of surface area (over about 2 m.²/mg.), compression results in a relatively small increase in surface pressure. Copolymer segments are dilute in the surface, and reduction of available surface area forces water molecules into the subphase. As the surface area is further reduced, copolymer segments are brought into contact with one another, and compression causes the surface pressure to rise more steeply. In the region of close contact, the π–A isotherms are linear. All three curves exhibit a sharp rise in surface pressure at an area of about 0.2 m.²/mg. Monolayer collapse occurs at a surface area below 0.04 m.²/mg. This area is quite small in comparison to the collapse area of most polymer monolayers previously studied.

The copolymers under investigation consist of a "backbone" of polyvinylpyrrolidone onto which vinyl acetate monomers are grafted. Each molecule can be thought of as comprising several polyvinyl acetate strands bound to a single long unit of polyvinylpyrrolidone. It was shown that polyvinyl acetate may be spread as a monolayer on water (9, 10). However, it was found that polyvinylpyrrolidone does not form a stable monolayer, apparently because of the attraction of the vinylpyrrolidone residues for the subphase

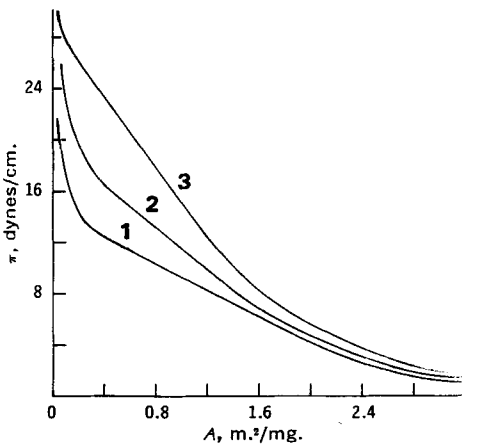

Figure 1—π–A *curves for polyvinylpyrrolidone copolymers. Key: 1, 735; 2, 535; and 3, 335.*

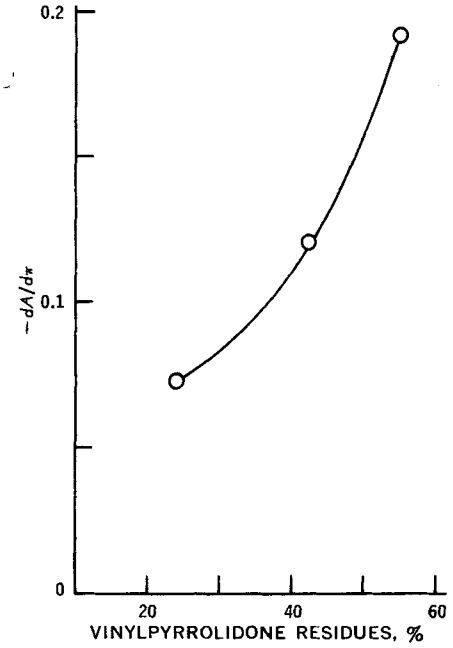

Figure 2—*Effect of vinylpyrrolidone content on monolayer compressibility.*

(11). Because of their polyvinylpyrrolidone content, the copolymers are somewhat water soluble. (One gram of the 735 copolymer, which has the highest polyvinylpyrrolidone content, dissolves in about 100 ml. of water.) This information, coupled with the extremely small area at collapse, promotes the suspicion that either spreading of the monolayer is not complete or that some material is lost from the surface during an experiment (9, 12). However, experimental evidence demonstrates that spreading is indeed complete and that dissolution of copolymer from the surface into subphase does not occur:

1. The π–A curves obtained when the copolymers were spread from isopropanol–benzene solution were identical to those in which methylene chloride was the spreading solvent. Also, in some experiments, the concentration of copolymer in the surface was increased by spreading additional material rather than by compression. Both techniques yielded the same result. The copolymers were, therefore, completely spread (9, 12).

2. If the monolayers were compressed, expanded, and then recompressed, the initial π–A curve was reproduced. Surface pressure readings were independent of the rate of compression and of the period of time allowed to elapse after spreading before compression was begun. In addition, surface pressure readings were found to be

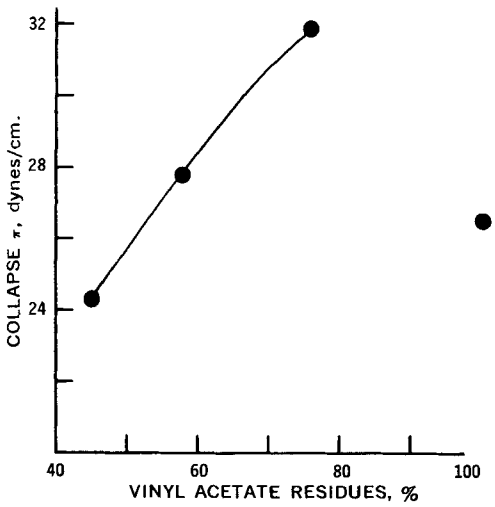

Figure 3—*Influence of vinyl acetate content on monolayer collapse pressure.*

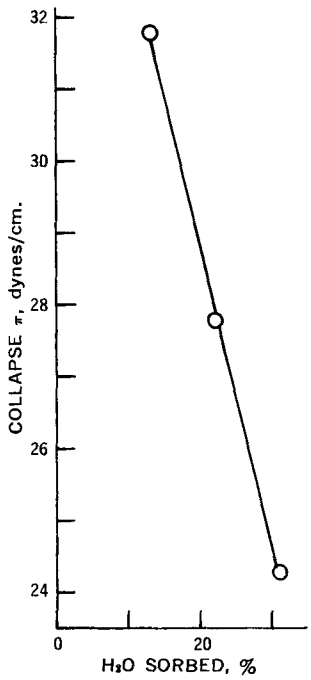

Figure 4—*Relationship between monolayer collapse pressure and moisture sorption (at 80% relative humidity) in free films of polyvinylpyrrolidone copolymers.*

stable when the monolayers were allowed to stand under compression at constant area for up to 30 min. These results indicate that dissolution of copolymer from the surface did not occur during an experiment.

Cohesion at the Interface—Gaines (13) defined a polymer monolayer as a system in which each residue of the macromolecule is adsorbed at the interface. When the residues are closely packed, compression causes changes in orientation which allow the segments to be accommodated at the interface. In this state, cohesion between hydrocarbon portions of polymers is often apparent (4) and many substances form two-dimensional gels (9).

Qualitative surface viscosity measurements (14) revealed that gel formation at the surface did not occur with any of the polyvinylpyrrolidone copolymers. The monolayers remained fluid up to collapse. Other experiments were performed in which the temperature of the subphase was changed to 15 and 35°, respectively. The temperature dependence of surface pressure was found to be negligible for all of the copolymers. The enthalpy of spreading was, therefore, very close to zero (15). This finding argues against the existence of significant interactions between neighboring copolymer segments and is consistent with the rheological data. Another consideration is the small surface areas to which the copolymer monolayers may be compressed, thus making inclusion of all copolymer residues in the surface impossible. It appears that these monolayers do not conform to Gaines' definition.

Surface Structure of Polyvinylpyrrolidone Copolymers—When a large area is available to the copolymer molecules, it is likely that both the vinylpyrrolidone and vinyl acetate residues occupy the interface. As the area available to the molecules in the surface is reduced, some of the polymer segments leave the surface. It is possible that partial collapse occurs, resulting in the formation of an "overfilm" (9). However, it appears more probable that the attraction of water molecules for the vinylpyrrolidone segments causes them to penetrate into the subphase. This arrangement imparts considerable flexibility to compression to the copolymers and makes them more highly compressible than if all of the segments were constrained to remain at the surface.

In Fig. 2, $-dA/d\pi$, a measure of monolayer compressibility (16), is plotted as a function of vinylpyrrolidone content. The increase in compressibility as a function of the percentage of vinylpyrrolidone residues is consistent with the notion that compression results in their submersion into the subphase. The ability of the copolymers to respond to compression by forcing a portion of the molecule into an adjacent bulk phase explains how the monolayers can be compressed to very small areas prior to collapse and accounts for the fact that the monolayers are fluid and exhibit little cohesion. The polyvinyl acetate grafts serve to anchor the copolymers to the surface and are largely responsible for stability of the monolayers.

Figure 3 shows the relationship between collapse pressure and vinyl acetate content. The collapse pressure of pure polyvinyl acetate (10) is included for comparison. Increasing the vinyl acetate content results in higher collapse pressure. It is apparent that some interaction occurs between vinylpyrrolidone and vinyl acetate residues, since the collapse pressure of the 335 copolymer is higher than that of pure polyvinyl acetate.

Relation between Monolayers and Free Films—Previous work showed that certain properties of monolayers may be related to those of free films (5, 6). Collapse pressure in a group of related polymers is a measure of "hydrophobicity." A similar relation was found with the materials studied here. As shown in Fig. 4, moisture sorption in free films is inversely proportional to monolayer collapse pressure.

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Influence of Drug Particle Size after Intramuscular Dosage of Phenobarbital to Dogs

L. G. MILLER and J. H. FINCHER

Abstract □ Phenobarbital suspensions containing drug particles of different size ranges were separately administered, intramuscularly, to beagle dogs. The particle size of the dose affected the blood drug level curves. A comparison of the areas under the curves with the respective particle sizes indicated that particle size influenced the biological availability of phenobarbital. Data obtained suggest that by controlling the drug particle size, one may be able to regulate the duration of action of phenobarbital.

Keyphrases □ Particle size, drugs—effect on intramuscular administration of phenobarbital, beagle dogs □ Phenobarbital, intramuscular administration—effect of particle size on blood levels and bioavailability, beagle dogs □ Blood levels, dogs—effect of phenobarbital particle size after intramuscular administration □ Bioavailability—effect of phenobarbital particle size, intramuscular administration, beagle dogs

Within the last 15 years, it has become increasingly apparent that the biological availability of drugs can be altered considerably by their dosage forms and their physical properties. Particle-size influences on drug availability constitute an important part of pharmaceutical technology (1). Most reported studies to ascertain the influence of drug particle size were conducted with oral dosage forms, and only a few of these studies treated its effect in parenteral forms (2). Drugs in the GI tract are subjected to varying degrees of agitation and fluid composition; however, at intramuscular injection sites the agitation intensity is very low and the fluid composition should remain relatively constant. The intramuscular route may, therefore, be more useful in studying the influence of drug particle size. Reported herein are preliminary studies to determine the effects of drug particle size by intramuscular injection.

EXPERIMENTAL

Phenobarbital USP and sodium phenobarbital USP were utilized in these studies. The purities of the two drugs, based on dry weight, were determined by USP methods to be approximately 100% for the phenobarbital crystals and 92% for the sodium phenobarbital granules.

Twenty-five healthy, male, beagle dogs of approximately the same age and weighing between 10 and 15 kg. were the test subjects. From this group, three sets of eight were selected and alternated so that the same animal was not used for two successive experiments. The dogs were fasted for 18 hr. prior to use, but free intake of water was allowed. Intravenous injections were given in the cephalic vein of one foreleg, while the intramuscular injections were given in the gluteus medius; blood samples were drawn from the cephalic vein of the foreleg not used for the intravenous injection.

The particles to be studied were prepared by precipitating free phenobarbital from aqueous solutions of sodium phenobarbital with a 0.5 *N* hydrochloric acid solution. This procedure was done in an alcohol–dry ice bath at −10° under varying degrees of agitation and precipitation rates. A gross analysis of the crystals was made using a microscope, and the particle-size distribution was determined with a Coulter counter[1] (Fig. 1).

[1] Model B, Coulter Electronics Industrial Division, Chicago, IL 60614

Figure 3 shows the relationship between collapse pressure and vinyl acetate content. The collapse pressure of pure polyvinyl acetate (10) is included for comparison. Increasing the vinyl acetate content results in higher collapse pressure. It is apparent that some interaction occurs between vinylpyrrolidone and vinyl acetate residues, since the collapse pressure of the 335 copolymer is higher than that of pure polyvinyl acetate.

Relation between Monolayers and Free Films—Previous work showed that certain properties of monolayers may be related to those of free films (5, 6). Collapse pressure in a group of related polymers is a measure of "hydrophobicity." A similar relation was found with the materials studied here. As shown in Fig. 4, moisture sorption in free films is inversely proportional to monolayer collapse pressure.

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Influence of Drug Particle Size after Intramuscular Dosage of Phenobarbital to Dogs

L. G. MILLER and J. H. FINCHER

Abstract □ Phenobarbital suspensions containing drug particles of different size ranges were separately administered, intramuscularly, to beagle dogs. The particle size of the dose affected the blood drug level curves. A comparison of the areas under the curves with the respective particle sizes indicated that particle size influenced the biological availability of phenobarbital. Data obtained suggest that by controlling the drug particle size, one may be able to regulate the duration of action of phenobarbital.

Keyphrases □ Particle size, drugs—effect on intramuscular administration of phenobarbital, beagle dogs □ Phenobarbital, intramuscular administration—effect of particle size on blood levels and bioavailability, beagle dogs □ Blood levels, dogs—effect of phenobarbital particle size after intramuscular administration □ Bioavailability—effect of phenobarbital particle size, intramuscular administration, beagle dogs

Within the last 15 years, it has become increasingly apparent that the biological availability of drugs can be altered considerably by their dosage forms and their physical properties. Particle-size influences on drug availability constitute an important part of pharmaceutical technology (1). Most reported studies to ascertain the influence of drug particle size were conducted with oral dosage forms, and only a few of these studies treated its effect in parenteral forms (2). Drugs in the GI tract are subjected to varying degrees of agitation and fluid composition; however, at intramuscular injection sites the agitation intensity is very low and the fluid composition should remain relatively constant. The intramuscular route may, therefore, be more useful in studying the influence of drug particle size. Reported herein are preliminary studies to determine the effects of drug particle size by intramuscular injection.

EXPERIMENTAL

Phenobarbital USP and sodium phenobarbital USP were utilized in these studies. The purities of the two drugs, based on dry weight, were determined by USP methods to be approximately 100% for the phenobarbital crystals and 92% for the sodium phenobarbital granules.

Twenty-five healthy, male, beagle dogs of approximately the same age and weighing between 10 and 15 kg. were the test subjects. From this group, three sets of eight were selected and alternated so that the same animal was not used for two successive experiments. The dogs were fasted for 18 hr. prior to use, but free intake of water was allowed. Intravenous injections were given in the cephalic vein of one foreleg, while the intramuscular injections were given in the gluteus medius; blood samples were drawn from the cephalic vein of the foreleg not used for the intravenous injection.

The particles to be studied were prepared by precipitating free phenobarbital from aqueous solutions of sodium phenobarbital with a 0.5 *N* hydrochloric acid solution. This procedure was done in an alcohol–dry ice bath at −10° under varying degrees of agitation and precipitation rates. A gross analysis of the crystals was made using a microscope, and the particle-size distribution was determined with a Coulter counter[1] (Fig. 1).

[1] Model B, Coulter Electronics Industrial Division, Chicago, IL 60614

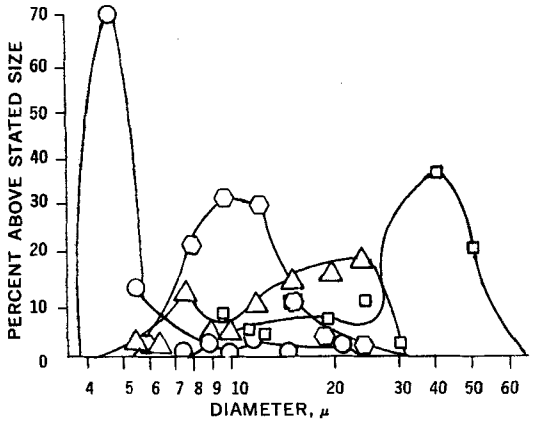

Figure 1—*Differential particle-size distributions of phenobarbital as analyzed by the Coulter counter. Key: ○, Sample P-4, mean diameter 6.63 μ; □, Sample P-5, mean diameter 29.96 μ; △, Sample P-6, mean diameter 17.16 μ; and ⬡, Sample P-7, mean diameter 10.68 μ.*

The parenteral solutions were made by dissolving an amount of sodium phenobarbital equivalent to 500 mg. of the free acid in a 5-ml. disposable syringe with 3 ml. of distilled water. The solutions were not made until just prior to the time of injection to reduce the degree of drug degradation.

Parenteral suspensions were prepared by weighing 500 mg. of the recrystallized phenobarbital sample, mixing it with 1% Marasperse CB[2], and placing it in a 5-ml. syringe. Distilled water was drawn into the syringe, and the suspension was shaken by hand until it was as uniform as possible. Parenteral suspensions were administered intramuscularly through a 16- or 18-gauge needle.

Sample assays were performed using a variation of Goldbaum's UV spectrophotometric method (3). The values were derived from a standard curve plotted from known concentrations of phenobarbital in 0.5 *N* sodium hydroxide assayed *versus* a sodium hydroxide blank in a grating spectrophotometer[3].

Each milliliter of a blood sample was placed in 0.2 ml. of a sodium citrate solution (4 g./100 ml.). Blood samples were subjected to a chloroform and sodium hydroxide extraction as follows. One- and two-tenths milliliter of blood (citrated) was pipeted into a 60-ml. separator containing 30 ml. of reagent chloroform. The separator was shaken by hand for 1 min., and the phases were allowed to separate for 1 min. A 25-ml. fraction of the chloroform was drawn through a dry filter paper into a graduate and then placed in a second separator containing 5.0 ml. of 0.5 *N* sodium hydroxide. After 1 min. of shaking and 1 min. of settling, the chloroform portion was drawn from the separator and discarded. The aqueous solution phase was decanted into a 5-ml. centrifuge tube and centrifuged for 1 min. to separate the remaining chloroform fraction. A 2.5-ml. sample of the clear supernatant was pipeted into a clean, dry cell[4] and analyzed against the sodium hydroxide blank. Several blood blanks were assayed by this procedure, and an average differential was used to determine the unknown concentration less the background.

Mean blood levels were determined after separate administrations of intravenous and intramuscular solutions, and the data were used as controls to ascertain the extent and rate of availability from suspensions. Blood samples were collected at intervals of 30 min. and 1, 3, 6, 9, 24, and 30 hr. Suspensions having mean particle diameters of 6.6, 10.68, 17.16, and 29.96 μ were given intramuscularly, and blood samples were collected in the same manner as described previously. The blood samples were kept cold by either ice coolers or refrigeration until the time of the assay.

To determine if the injected particle had any adverse effects on the muscle tissue or if tissue encapsulation had occurred, the site of injection was marked on one dog and examined by surgical procedures 72 hr. after administration of the larger particle suspension. The attending veterinarian conducted this examination, and his report was that the tissue at the site of injection remained normal and gave no indication of encapsulation (4).

[2] American Can Co., Chemical Products Sales, Neenah, Wis.
[3] Beckman DB-G, Beckman Instruments, Inc., Fullerton, CA 80566
[4] Beckman DU.

RESULTS AND DISCUSSION

The individual blood levels obtained after intramuscular administration of the various phenobarbital particle distributions are reported in Table I. Also reported in Table I are mean blood levels and the estimated standard deviation. The mean blood levels were plotted as a function of time (Fig. 2) for ease of comparison. It is obvious from Fig. 2 that the rates of availability of all intramuscular suspensions were slower than that of the intramuscular solution, and it is also apparent that the larger particle suspensions were available to a lesser extent than the smaller particle suspensions in a 30-hr. interval.

The areas under the blood level curves are a function of the biological availability of the total dose from each drug system (5). If the area is calculated for a definite time interval and if the blood concentration curve is an inexact differential depending upon absorption rates of the particles, then a comparison of the areas with the respective particle sizes is a comparative measure of the absorption rates of the particles.

By assuming that this hypothesis holds true, an examination of the 30-hr. data reveals that as the particle size increases the area under the blood level curve decreases (Table II). A semilog plot of the percent of area under the intravenous blood level curve as a function of particle diameter in microns yielded a linear expression.

Since surface area is proportional to diameter[2] (d^2) and since a linear plot of relative availability (as indicated by percent of intravenous dose) *versus* d^2 was not linear, the possibility that relative availability would be a direct function of surface area of the sample was not evident. Due to the nature of the dosage unit in the injection site, one would expect the availability to be a function of

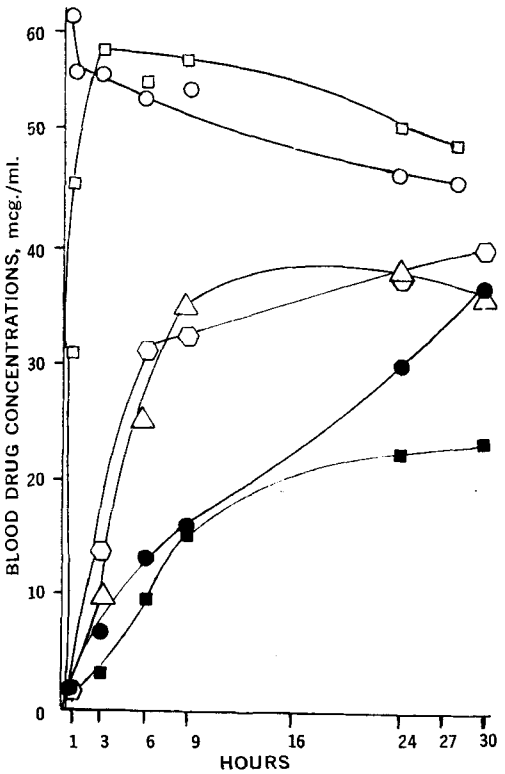

Figure 2—*Mean blood levels of phenobarbital in dogs as a function of time in hours after administration. Key: ○, intravenous solution of sodium phenobarbital equivalent to 500 mg. of free acid; □, intramuscular solution of sodium phenobarbital equivalent to 500 mg. of free acid; △, intramuscular suspension of Sample P-4 (6.63 μ); ■, intramuscular suspension of sample P-5 (29.96 μ); ●, intramuscular suspension of Sample P-6 (17.16 μ); and ⬡, intramuscular suspension of Sample P-7 (10.68 μ), each suspension containing 500 mg. of the free acid.*

Table I—Individual Blood Levels (Micrograms per Milliliter) Obtained at the Stated Times after Intramuscular Administration of the Various Phenobarbital Particle Distributions[a]

Sample	Trial	Hours: 1	3	6	9	24	27	30
		Blood Levels, mcg./ml.						
P-4 (6.63 μ)	1	6.0	26.4	38.4	58.4	49.8	58.8	48.3
	2	0.0	7.2	24.0	36.0	43.2	66.0	24.0
	3	6.6	20.4	53.4	54.0	52.8	46.8	53.4
	4	0.0	4.6	12.0	18.6	33.0	29.4	36.6
	5	0.0	0.0	15.6	31.8	28.2	33.6	33.8
	6	0.0	0.0	11.8	18.0	27.0	27.6	27.6
	Mean blood level	2.1	9.8	25.9	36.1	39.0	43.7	37.3
	SD	±3.25	±11.1	±16.8	±17.15	±11.14	±16.12	±11.53
P-5 (29.96 μ)	1	9.6	5.4	10.8	15.6	31.8	46.5	12.0
	2	3.0	6.0	11.4	27.0	16.2	33.0	24.6
	3	0.0	2.4	11.4	18.0	30.6	27.6	37.2
	4	0.0	4.6	11.6	17.6	26.4	27.2	35.1
	5	0.0	0.0	4.8	7.2	13.2	12.0	11.4
	6	0.0	0.0	9.0	8.4	16.8	22.2	22.8
	Mean blood level	2.1	3.1	9.8	15.6	22.5	28.1	23.8
	SD	±3.86	±2.67	±2.65	±7.24	±8.07	±11.45	±10.95
P-6 (17.16 μ)	1	3.0	9.6	18.0	13.2	33.6	40.8	47.4
	2	3.0	7.4	9.6	18.0	42.3	54.6	37.2
	3	0.0	4.2	9.6	11.6	16.2	15.6	22.5
	4	0.0	6.8	16.2	21.6	31.2	33.6	43.2
	Mean blood level	1.5	7.0	13.3	16.1	30.8	36.1	37.6
	SD	±1.73	±2.24	±4.39	±4.56	±10.86	±16.24	±10.90
P-7 (10.68 μ)	1	4.8	18.6	35.4	39.0	49.2	45.0	51.6
	2	6.0	31.2	55.2	46.8	43.2	40.2	46.5
	3	0.0	3.6	16.8	15.0	30.6	31.2	34.2
	4	0.0	12.0	35.1	37.5	37.2	45.6	41.1
	5	0.0	18.0	39.0	43.2	45.0	49.2	44.6
	6	0.0	0.0	14.4	16.2	28.8	31.8	28.2
	Mean blood level	1.8	13.9	32.6	32.9	39.0	40.5	41.0
	SD	±2.81	±11.36	±15.13	±13.82	±8.19	±7.54	±8.55

Sample	Trial	Hours: 0.25	0.5	1	3	6	9	24	30
		Blood Levels, mcg./ml.							
Intramuscular solution (control)	1	—	39.6	50.5	70.8	61.2	63.0	—	—
	2	—	32.4	47.4	39.6	37.8	46.8	—	—
	3	—	31.2	42.0	64.8	62.9	61.2	—	—
	4	—	37.0	45.7	66.0	63.7	68.3	—	—
	5	—	37.2	46.3	60.0	60.6	61.2	58.2	52.8
	6	—	21.0	37.8	45.6	51.0	49.8	45.6	59.3
	7	—	24.0	45.6	63.0	57.6	60.0	52.2	71.4
	Mean	—	31.8	45.0	58.5	55.7	57.7	52.0	50.2
	SD	—	±7.01	±4.07	±11.50	±9.29	±9.29	±6.31	±16.4
Intravenous solution (control)	1	67.2	63.5	61.2	61.0	62.3	55.4	—	—
	2	68.0	87.0	90.0	63.6	69.0	64.1	—	—
	3	64.0	59.4	55.8	56.3	50.4	61.2	49.2	53.4
	4	49.8	48.0	47.4	49.8	39.5	37.2	39.6	38.3
	5	55.7	50.2	28.8	55.7	49.8	58.2	53.3	50.2
	Mean	60.9	61.6	56.6	55.5	54.2	55.2	47.4	47.3
	SD	±7.75	±15.36	±22.32	±8.66	±11.66	±10.58	±7.14	±7.94

[a] Dose 500 mg. suspended with the aid of Marasperse CB (1% of solid weight).

Table II—Comparison of Relative Areas under the Mean Blood Level Curves between 0 and 30 hr. and Percent of Intravenous Dose after Administration of 500 mg. of Phenobarbital Acid or Its Equivalent of the Sodium Salt to Beagle Dogs[a]

	Intravenous Solution	Intramuscular Solution	Sample Description: Phenobarbital Suspensions Injected Intramuscularly — Sample P-4 (Mean Diameter, 6.63 μ)	Sample P-5 (Mean Diameter, 29.96 μ)	Sample P-6 (Mean Diameter, 17.16 μ)	Sample P-7 (Mean Diameter, 10.68 μ)
Relative areas under mean curve	1563.2(5)	1587.6(6)	983.0(6)	507.2(6)	649.8(4)	952.8(6)
Percent of intravenous dose	100	101	62.9	32.4	41.5	60.9

[a] Numbers in parentheses indicate the number of dogs used to make the blood level curve.

something less than the absolute surface area of the injected mass. The apparent surface area of the injected mass is not known.

The possibility of controlling blood levels of phenobarbital for an extended period of time by regulation of particle size and size distribution of a single intramuscular dose is evident from these data. There was much variability in the individual blood level data obtained with each particle suspension during the first 3–6 hr., but it was not attributed to the dissolution and release pattern from the injected dose. This variability was probably due to a lower concentration of the drug in the body of the dog and its rapid distribution to the various tissues, leaving very little drug in the blood at the time of sampling. Based on the mean intravenous blood level estimate, the "apparent volume of distribution" was approximately 8.8 l.; since the blood volume in dogs weighing between 10 and 15 kg. is about 7% (700–1050 ml.) of body weight, the greater part of the drug must be in other tissues of distribution. As more drug is absorbed from the intramuscular suspensions, the relative standard error becomes smaller.

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Spectrofluorometric Assay of Apomorphine in Brain Tissue

W. KENT VAN TYLE and A. M. BURKMAN

Abstract □ A fluorometric technique has been developed that accurately and reliably measures submicrogram quantities of apomorphine in brain tissue. The assay involves an immiscible solvent extraction procedure which is highly efficient and makes possible the detection of apomorphine in concentrations of 0.1 mcg./g. tissue. The fluorometric assay is about 40 times more sensitive than the spectrophotometric method currently in use.

Keyphrases □ Apomorphine—spectrofluorometric analysis of submicrogram quantities in brain □ Spectrofluorometry—analysis of submicrogram quantities of apomorphine in brain

The increasing interest in apomorphine and related aporphines as centrally active behavioral and emetic stimulants has generated many questions concerning the CNS distribution of these compounds (1, 2). Using a spectrophotometric assay, Kaul *et al.* (3–7) studied apomorphine organ distribution and metabolism but were unable to estimate reliably the CNS levels due to the insufficient sensitivity of their method. Although a variety of techniques have been devised for the compound's determination (8, 9), none provides the sensitivity required to determine submicrogram quantities of apomorphine in small volumes of biological material.

The observation that crystalline apomorphine emits a bluish fluorescence under UV light and the acknowledged fact that fluorometry can be an extremely sensitive assay technique suggested the possibility of using a fluorometric method for apomorphine determination.

This report describes the development of such a procedure for estimating microquantities of apomorphine.

EXPERIMENTAL

Spectral Characteristics and Effect of Solvent—The output of the spectrophotofluorometer[1] was fed simultaneously into a photomultiplier microphotometer and an X-Y recorder[2]. A fused quartz cell (10.5 × 10.5 × 46 mm.) was used for all samples, with instrument slit arrangement number 5.

[1] An Aminco-Bowman spectrophotofluorometer equipped with a 1P21 potted photomultiplier tube assembly was used for all fluorescence measurements.

[2] Spectrophotofluorometer, microphotometer, and X-Y recorder were manufactured by American Instrument Co., Inc., Silver Spring, Maryland.

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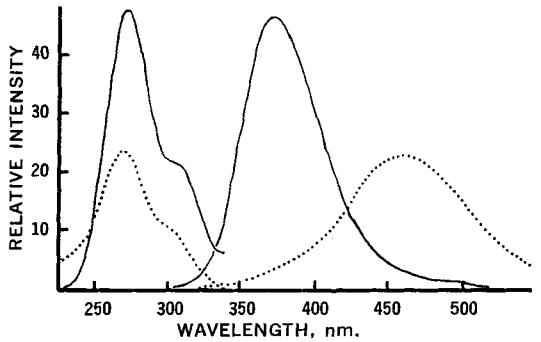

Figure 1—*Fluorescence spectra of apomorphine in ethyl acetate (0.33 mcg./ml. maximum, solid lines) and water (1 mcg./ml., dotted lines).*

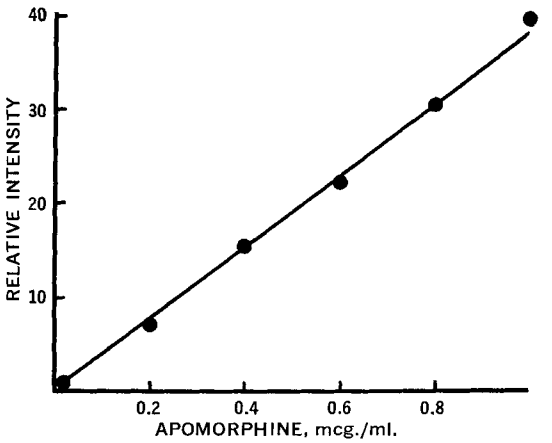

Figure 2—*Linear relation of fluorescence intensity to concentration of apomorphine in the extracted aqueous standard.*

The spectral characteristics of apomorphine were determined in both neutral distilled water and ethyl acetate[3], according to the method described by Udenfriend *et al.* (10). The aqueous solution contained 1 mcg. of apomorphine base/ml., and the ethyl acetate solution was prepared by extracting 5 ml. of aqueous solution with 15 ml. of ethyl acetate[4].

Effect of Concentration—The relation of fluorescence intensity to concentration of apomorphine base was determined by preparing serial dilutions of a 1.0-mcg./ml. aqueous apomorphine solution. The range of concentrations investigated was 0.01–1.0 mcg./ml. water. A 5-ml. aliquot of the aqueous standard was extracted for 2–3 min. with 15 ml. of ethyl acetate, and the fluorescence of the ethyl acetate extract was measured using the excitation and emission maxima for apomorphine fluorescence in this solvent (270/370 nm.).

Extraction Procedure for Brain—A modification of the extraction procedure suggested by Kaul *et al.* (4) for the estimation of apomorphine in biological materials was employed in this investigation. Not more than 1 g. of tissue to be assayed was homogenized in 3–4 ml. of 0.1 *N* HCl and 1 ml. of ethyl acetate in a homogenizer[5] equipped with Pyrex microhomogenization flasks. The acid homogenate was quantitatively transferred to a volumetric flask, and sufficient 0.1 *N* HCl was added to make a total volume of 10 ml. A 5-ml. aliquot of the acid homogenate was transferred to a 50-ml. glass-stoppered centrifuge tube and was shaken for 2–3 min. with 5 ml. of ethyl acetate The sample was centrifuged, the ethyl acetate was aspirated and discarded, and the previous wash step was repeated with another 5-ml. portion of ethyl acetate which was similarly discarded. The ethyl acetate washes significantly reduced the fluorescence of tissue blanks. Under acidic conditions the protonated apomorphine remained in the aqueous phase (pKa 8.9). Following aspiration of the second ethyl acetate wash, the pH of the homogenate was adjusted to 7.0 ± 0.5 by the addition of solid sodium bicarbonate; the pH was verified on the pH meter[6] equipped with a combination electrode[7]. The apomorphine present in the sample was extracted by shaking with 15 ml. of ethyl acetate for 2–3 min., the mixture was centrifuged, and the organic phase was withdrawn with a Pasteur pipet. Fluorescence intensity of the ethyl acetate extract was determined using the 270/370-nm. wavelengths.

Appraisal of Method—To determine the ability of the assay procedure to quantify apomorphine accurately in the presence of potentially interfering biological materials, addition–recovery experiments were performed with acidified pigeon brain homogenates. Two whole pigeon brains were homogenized in sufficient 0.1 *N* HCl to make 125 ml. of a stock homogenate containing approximately 30 mg. of tissue/ml. The sample was prepared by diluting 1.0 ml. of neutral apomorphine HCl solution containing 100 mcg. of base/ml. with sufficient acidified brain homogenate to make 100 ml. of sample. Five 5-ml. aliquots of the sample solution were assayed according to the described procedure along with a 5-ml. aliquot of the brain homogenate containing no apomorphine. The tissue blank thus determined was subtracted from the sample fluorescence intensity.

Excitation and emission spectra were obtained on assayed samples of mouse and pigeon brain following intravenous apomorphine administration to ascertain whether any naturally occurring fluorescent materials were being carried through the extraction to influence the total fluorescence intensity attributed to apomorphine. Male albino mice and adult domestic pigeons received apomorphine HCl in isotonic saline in doses of 10 and 1 mg./kg., respectively. The spectra thus obtained were compared with those of authentic apomorphine in ethyl acetate.

RESULTS AND DISCUSSION

The spectral characteristics for the fluorescence of apomorphine in neutral distilled water and ethyl acetate are presented in Fig. 1. The primary excitation peak for apomorphine was found to be 270 nm. in both solvents. A lesser excitation peak appeared in both solvents at 305 nm. The position of the emission peak, however, was solvent dependent and was 460 nm. in distilled water and 370 nm. in ethyl acetate. The fluorescence intensities of the emission spectra of solvent blanks were in the range of 0.2–0.3 unit. All wavelength determinations reported are observed values and were not corrected for instrumental variation. The intensity of apomorphine fluorescence was also solvent dependent, and it was found that the compound's fluorescence in ethyl acetate was about seven times that seen in water.

The virtues of ethyl acetate as an extraction solvent for apomorphine have been amply documented (4, 9) so that examination of this solvent's suitablity as a fluorescence vehicle seemed appropriate. However, since water might also represent an acceptable fluorescence medium, it was necessary to assess its relative utility as well. The decision to compare these solvents at a neutral pH was influenced by two observations. Preliminary studies on the pH-dependent character of apomorphine fluorescence in water revealed that intensity plateaus to a maximum value over the pH range of 3–8. Determinations in neutral water ensured optimum sensitivity. A pH of 7 was particularly appropriate inasmuch as Kaul *et al.* (4) established that ethyl acetate "effects complete extraction of the base from an aqueous solvent at pH 6–8. . . ."

Ethyl acetate was ultimately adopted as the fluorescence medium because of the greater assay sensitivity associated with its use.

As indicated in Fig. 2, the relation of fluorescence intensity to apomorphine concentration in ethyl acetate is linear over the range of concentrations investigated. It should be reiterated that a 5-ml. sample of apomorphine standard was extracted with 15 ml. of ethyl acetate and the fluorescence of this ethyl acetate extract was determined. Therefore, the concentrations indicated in Fig. 2 refer to those of the aqueous standard from which the apomorphine was extracted.

Addition–recovery experiments and spectral analyses of brain extracts revealed that the extraction procedure is highly efficient at removing apomorphine from brain tissue with minimal contamination of interfering fluorescent materials of biological origin. The percent recovery ± standard error of added apomorphine to

[3] Spectral quality ethyl acetate (Matheson, Coleman and Bell, Norwood, Ohio) was used in all procedures requiring this solvent.
[4] If virtually all of the apomorphine originally in the water solution were partitioned into ethyl acetate, the maximum attainable concentration of apomorphine base in ethyl acetate would be 0.33 mcg./ml.
[5] Virtis "45."
[6] Corning model 12.
[7] Corning Semimicro.

pigeon brain homogenate was found to be 98.2 ± 1.0. Likewise, fluorescence spectra of extracts from brains of mice and pigeons which had been administered apomorphine systemically were identical to spectra obtained for authentic apomorphine in ethyl acetate.

Although several procedures were reported for the quantitative determination of apomorphine, none possesses adequate sensitivity for studies of the compound's CNS distribution. Kaul *et al.* (4) reported that the spectrophotometric assay was unable to detect the presence of apomorphine in tissue homogenates in concentrations less than 4 mcg./g. Preliminary investigations of the compound's CNS distribution in mice using the fluorometric assay reported here indicate that concentrations of apomorphine as low as 0.1 mcg./g. brain tissue can be readily determined. As a result of the increased sensitivity of this fluorometric procedure, studies of apomorphine disposition in brain are now being productively pursued.

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Synthesis and Biological Evaluation of Some 4-Arylazopyrazoles

W. U. MALIK, H. G. GARG*, and VEENA ARORA

Abstract □ A variety of derivatives of N^1-(4-methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles and N^1-hippuryl-4-arylazo-3,5-dimethylpyrazoles were prepared. Preliminary biological data also are described.

Keyphrases □ 4-Arylazopyrazoles—synthesis and biological evaluation as antimicrobial agents □ Antimicrobial agents, potential—synthesis and biological evaluation of 4-arylazopyrazoles □ N^1-(4-Methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazole derivatives—synthesis and biological evaluation as antimicrobial agents □ N^1-Hippuryl-4-arylazo-3,5-dimethylpyrazole derivatives—synthesis and biological evaluation as antimicrobial agents

The interest in the synthesis and biological evaluation of pyrazole derivatives has been renewed by the fact that 1-carbamoyl-3-methyl-4-(2-chloro-4-nitrophenylhydrazono)-2-pyrazolin-4,5-dione possesses anti-*Trichinella spiralis* activity (1).

This report includes the syntheses of N^1-(4-methoxyphenylsulfinyl) - 3,5 - dimethyl - 4 - arylazo-, N^1 - hippuryl-3,5-dimethyl-4-arylazo-, and N^1-hippuryl-3-methyl-4-arylazo-5-phenylpyrazoles. The preparation of others was recently described (2, 3).

The syntheses of N^1-(4-methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles and N^1-hippuryl-4-arylazo-3,5-dimethylpyrazoles were achieved by the condensation of 2,3,4-pentanetrione-3-arylhydrazones (4) with 4-methoxyphenylsulfinylhydrazine (5) and hippurylhydrazine (6), respectively. N^1-Hippuryl-4-arylazo-3-methyl-5-phenylpyrazoles were similarly prepared from 1-phenyl-2-arylhydrazono-1,2,3-butanetrione (7) and hippurylhydrazine (6).

BIOLOGICAL RESULTS

4-Arylhydrazono-1-carbamoyl-3-methyl-2-pyrazolin-5-ones (2) and 3,5-dimethyl-4-arylazo-5-phenyl-N^1-carbamoylpyrazoles (3) were tested for antimicrobial activity (8) against *Staphylococcus aureus* (No. 20390), *Klebsiella pneumoniae* (No. 1200), *Pseudomonas aeruginosa* (No. 1320), *Escherichia coli* (No. 12140), *Trichophyton mentagraphytes* (No. 17410), *Candida albicans* (No. 3470), and *Mycobacterium tuberculosis* (No. H37RV). They were found inactive.

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The IR spectra of representatives of all the pyrazoles showed characteristic bands of —C=C—N=N in the range 1480–1540 cm.$^{-1}$, aryl C=C in the range 1580–1670 cm.$^{-1}$, substituted phenyl in the range 690–730 cm.$^{-1}$, and —NH in the range 2700–3400 cm.$^{-1}$ (broad). UV spectra of the representatives showed $\lambda_{max.}^{EtOH}$ between 237–246 and 327–347 nm. These data are summarized in Table I.

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Table I—Spectral Data of N^1-(4-Methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles and N^1-Hippuryl-4-arylazo-3,5-dimethylpyrazoles

Number	IR, cm.$^{-1}$ —C=C—N=N—	IR, cm.$^{-1}$ Aryl C=C	IR, cm.$^{-1}$ Substituted Phenyl	IR, cm.$^{-1}$ —NH	IR, cm.$^{-1}$ —NO_2	UV, μ $\lambda_{max.}^{EtOH}$	
1[a]	1540	1600	715, 690	3400	1375	246	347
2[b]*	1505	1630	730, 690	3100	—	244	243
3[c]	1520	1670	720	2850	—	241	346
4[b]†	1500	1610	720, 730	3000	—	240	335
5[b]‡	1500	1580	720	2850	—	239	331

[a] Table IV, No. 2. [b] Table II: *No. 4, † No. 5, and ‡ No. 2. [c] Table III, No. 1.

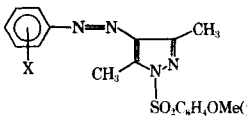

Table II—Characteristics of N^1-(4-Methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles

Number	X	Yield, %	Melting Point	Color[a]	Formula	Analysis, % Calc.	Found
1	H	70	114°	BrYSN	$C_{18}H_{18}N_4O_3S$	C 58.37	58.42
						H 4.86	5.00
						N 15.13	15.02
						S 8.64	8.53
2	2-MeO	72	193°	GYSP	$C_{19}H_{20}N_4O_4S$	N 14.00	13.82
						S 8.00	8.21
3	4-MeO	70	162°	YFlN	$C_{19}H_{20}N_4O_4S$	N 14.00	13.92
						S 8.00	8.13
4	2,4-Cl_2	65	197°	GSp	$C_{18}H_{16}Cl_2N_4O_3S$	Cl 16.17	16.09
						S 7.28	7.22
5	3,4-Me_2	60	180°	YSN	$C_{20}H_{22}N_4O_3S$	N 14.07	14.03
						S 8.04	7.91
6	4-Cl-2,5-$(MeO)_2$	65	240° dec.	MSSp	$C_{20}H_{21}ClN_4O_5S$	Cl 8.50	8.44
						S 6.88	6.82
7	5-Cl-2,4-$(MeO)_2$	55	250° dec.	DYN	$C_{20}H_{21}ClN_4O_5S$	Cl 8.50	8.43
						S 6.98	6.83

[a] B, brown; Br, bright; D, dark; F, fibers; Fl, fluffy; G, golden; M, mustard; N, needles; O, orange; P, plates; S, shiny; Sp, specks; and Y, yellow.

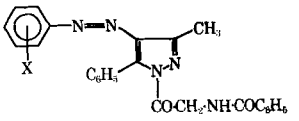

Table III—Characteristics of N^1-Hippuryl-3,5-dimethyl-4-arylazopyrazoles

Number	X	Yield, %	Melting Point	Color[a]	Formula	Analysis, % Calc.	Found
1	2-Me	70	200°	GSSp	$C_{21}H_{21}N_5O_2$	C 67.20	66.52
						H 5.60	5.43
						N 18.66	18.54
2	2-MeO	65	223° dec.	SGYN	$C_{21}H_{21}N_5O_3$	N 17.45	17.82
3	4-MeO	72	160–162°	BYFlN	$C_{21}H_{21}N_5O_3$	N 17.45	17.81
4	4-Cl-2,5-$(MeO)_2$	55	230°	MYSSp	$C_{22}H_{22}ClN_5O_4$	Cl 7.54	7.62
5	5-Cl-2,4-$(MeO)_2$	65	202°	DYSSp	$C_{22}H_{22}ClN_5O_4$	Cl 7.54	7.73

[a] See *Footnote* [a] of Table II.

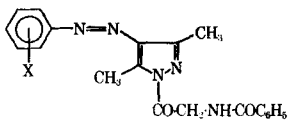

Table IV—Characteristics of N^1-Hippuryl-3-methyl-4-arylazo-5-phenylpyrazoles

Number	X	Yield, %	Melting Point	Color[a]	Formula	Analysis, % Calc.	Found
1	4-NO_2	55	177°	OSN	$C_{25}H_{20}N_6O_4$	C 64.10	64.01
						H 4.27	4.22
						N 17.90	17.83
2	3-NO_2	60	175°	GYSp	$C_{25}H_{20}N_6O_4$	N 17.90	17.72
3	2-MeO	70	118°	YOSp	$C_{26}H_{23}N_5O_3$	N 17.62	17.33
4	4-MeO	60	158°	DYSN	$C_{26}H_{23}N_5O_3$	N 17.62	17.24

[a] See *Footnote* [a] of Table II.

trione-3-arylhydrazone (0.005 mole) dissolved in an alcohol–acetic acid mixture. The resultant solution was boiled under reflux for several hours and then cooled. The reaction mixture was diluted with water, and the crystals which separated were collected and purified by recrystallization from ethanol. Characteristics of N^1-(4-methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles are listed in Table II.

N^1-Hippuryl-3,5-dimethyl-4-arylazopyrazoles—These compounds were obtained from hippurylhydrazine (0.005 mole) and 3-arylhydrazono-2,3,4-pentanetrione (0.005 mole) by the same procedure as was adapted for N^1-(4-methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles. The yields and physical constants of these pyrazoles are listed in Table III.

N^1-Hippuryl-3-methyl-4-arylazo-5-phenylpyrazoles—Treatment of 2-arylhydrazono-1-phenyl-1,2,3-butanetriones with hippurylhydrazine under conditions similar to those used in other cases gave the pyrazole derivatives listed in Table IV.

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Classification of Nicotine Block at Neuromuscular Junction

S. D. COHEN*, B. A. NICHOLAS†, and H. J. JENKINS

Abstract □ The effect of nicotine on the electrical threshold of the neuromuscular junction in a rat sciatic-gastrocnemius preparation was studied and compared with the effects of a true curarizing agent and those of a pseudocurarizing agent on the threshold of the like structure of the same preparation. Low doses of both nicotine and succinylcholine chloride caused a decrease in the predrug electrical threshold level of the neuromuscular junction, while high doses of these drugs caused an elevation of the predrug threshold level. Both low and high doses of dimethyl tubocurarine chloride, on the other hand, caused an elevation in this threshold level. Eserine salicylate enhanced the early blockades caused by nicotine and succinylcholine chloride but opposed the late blockades caused by these same drugs. Eserine salicylate opposed both the early and the late stages of a neuromuscular blockade brought about by dimethyl tubocurarine chloride. Nicotine and succinylcholine chloride induced a spastic paralysis in chicks, whereas dimethyl tubocurarine chloride induced a flaccid paralysis in other chicks of the same age and weight. On the basis of these studies, nicotine is classified as a neuromuscular blocking agent of the pseudocurare type which does not exert its effect through acetylcholine release.

Keyphrases □ Nicotine—effect on electrical threshold, neuromuscular junction, rat sciatic-gastrocnemius preparation, classified as neuromuscular blocking agent of pseudocurare type □ Neuromuscular blockage—nicotine effects studied, compared to other agents, classified, using rat sciatic-gastrocnemius preparation

Nicotine is thought to bring about a stimulation of ganglionic and related receptors, causing a depolarization of the postsynaptic membrane and a transient response. If the dose is adequate, it is thought to block somehow these same receptors as a result of, first, a prolonged depolarization and, later, a stabilized polarization phase.

The block of nicotine at the ganglion was considered by Pelikan (1) to result from an ability to prevent the release of acetylcholine at this site and not from its ability to compete with acetylcholine for the site receptors. On the other hand, Lundberg and Thesleff (2) recognized the ability on the part of nicotine *per se* to stimulate at the ganglionic receptor sites, but they stated that there is no complete correlation between the ganglion blocking action of nicotine and its prolonged ganglion cell depolarization because the blocking action outlasts the depolarization. The work of Paton and Perry (3) supported the view that the ganglionic block of nicotine has a second phase which occurs after the membrane becomes repolarized.

Langley (4), in 1909, discovered an antagonism by curare of nicotine's effect at the neural region of the amphibian skeletal muscle fiber. Thesleff (5), in 1955, found that the neuromuscular blockades of nicotine, succinylcholine, decamethonium, and acetylcholine develop during the depolarization of the muscle sole plate and continue after the transmembrane potential of this structure is spontaneously restored, without removal of the depolarizing agent. He concluded that the neuromuscular blocking action of these drugs is the result of a decrease in the sensitivity of the endplate to the neuronal transmitter.

Beani and Bianchi (6, 7), on the basis of their work in the guinea pig phrenic nerve-diaphragm preparation, classified nicotine as a stimulatory neuromuscular blocking agent. However, they agreed with Thesleff that its competitive block of cholinergic receptors at the

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Classification of Nicotine Block at Neuromuscular Junction

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Abstract □ The effect of nicotine on the electrical threshold of the neuromuscular junction in a rat sciatic-gastrocnemius preparation was studied and compared with the effects of a true curarizing agent and those of a pseudocurarizing agent on the threshold of the like structure of the same preparation. Low doses of both nicotine and succinylcholine chloride caused a decrease in the predrug electrical threshold level of the neuromuscular junction, while high doses of these drugs caused an elevation of the predrug threshold level. Both low and high doses of dimethyl tubocurarine chloride, on the other hand, caused an elevation in this threshold level. Eserine salicylate enhanced the early blockades caused by nicotine and succinylcholine chloride but opposed the late blockades caused by these same drugs. Eserine salicylate opposed both the early and the late stages of a neuromuscular blockade brought about by dimethyl tubocurarine chloride. Nicotine and succinylcholine chloride induced a spastic paralysis in chicks, whereas dimethyl tubocurarine chloride induced a flaccid paralysis in other chicks of the same age and weight. On the basis of these studies, nicotine is classified as a neuromuscular blocking agent of the pseudocurare type which does not exert its effect through acetylcholine release.

Keyphrases □ Nicotine—effect on electrical threshold, neuromuscular junction, rat sciatic-gastrocnemius preparation, classified as neuromuscular blocking agent of pseudocurare type □ Neuromuscular blockage—nicotine effects studied, compared to other agents, classified, using rat sciatic-gastrocnemius preparation

Nicotine is thought to bring about a stimulation of ganglionic and related receptors, causing a depolarization of the postsynaptic membrane and a transient response. If the dose is adequate, it is thought to block somehow these same receptors as a result of, first, a prolonged depolarization and, later, a stabilized polarization phase.

The block of nicotine at the ganglion was considered by Pelikan (1) to result from an ability to prevent the release of acetylcholine at this site and not from its ability to compete with acetylcholine for the site receptors. On the other hand, Lundberg and Thesleff (2) recognized the ability on the part of nicotine *per se* to stimulate at the ganglionic receptor sites, but they stated that there is no complete correlation between the ganglion blocking action of nicotine and its prolonged ganglion cell depolarization because the blocking action outlasts the depolarization. The work of Paton and Perry (3) supported the view that the ganglionic block of nicotine has a second phase which occurs after the membrane becomes repolarized.

Langley (4), in 1909, discovered an antagonism by curare of nicotine's effect at the neural region of the amphibian skeletal muscle fiber. Thesleff (5), in 1955, found that the neuromuscular blockades of nicotine, succinylcholine, decamethonium, and acetylcholine develop during the depolarization of the muscle sole plate and continue after the transmembrane potential of this structure is spontaneously restored, without removal of the depolarizing agent. He concluded that the neuromuscular blocking action of these drugs is the result of a decrease in the sensitivity of the endplate to the neuronal transmitter.

Beani and Bianchi (6, 7), on the basis of their work in the guinea pig phrenic nerve-diaphragm preparation, classified nicotine as a stimulatory neuromuscular blocking agent. However, they agreed with Thesleff that its competitive block of cholinergic receptors at the

Table I—Effect of Low and High Doses of Neuromuscular Blocking Agents on Electrical Thresholds of Rat Sciatic-Gastrocnemius Preparations

	Change in Threshold[a], %		
	Nicotine	Decamethonium	Succinylcholine
Low dose	−39 ± 2[b]	+7166 ± 3244	−35 ± 14
High dose	+189 ± 38[c]	+7347 ± 4639	+255 ± 73

[a] Calculated as the difference between thresholds before and 15 min. after administration of blocking agent, expressed as percent of threshold before first injection. [b] A minus (−) sign indicates that threshold decreased after the drug. [c] A plus (+) sign indicates that threshold increased after the drug. Each value represents the mean ±*SEM* of three to five experiments.

neuromuscular junction is its major inhibitory mechanism with respect to skeletal muscle contraction.

The study reported here represents an attempt to determine, on the basis of the relative effectiveness of antidotal antagonism of nicotine, succinylcholine, and curare blocks, the proper classification of the neuromuscular blockade of nicotine. It also represents an attempt to determine whether the effect of nicotine at the neuromuscular junction is a direct or an indirect effect, the latter achieved through the inhibition of acetylcholinesterase.

EXPERIMENTAL

Adult female rats, weighing between 175 and 250 g., were anesthetized with a solution containing 50 mg. chloralose and 500 mg. urethan/ml. at a dose of 1.5 ml./kg. body weight, administered intraperitoneally. The trachea was exposed and cannulated with a 5.08-cm. (2-in.) piece of tubing (PE 205). Through a sidearm of the tubing, the animal was allowed to respire unassisted until such time as this should be impossible; then the sidearm was closed and the respiratory pump was turned on.

The left external jugular vein was exposed and cannulated with tubing (PE 50) attached at its opposite end to a saline-filled syringe. All drugs were administered through the sidearm of this tubing and were flushed into the animal with 0.2 ml. of saline. The dorsal muscles of the right hind leg were exposed and the gastrocnemius was isolated, a ligature being placed around the Achilles tendon. The tendon was then cut distal to the ligature, and the muscle was attached by way of a pulley to a lever weighted with 5 g. Contraction of the muscle exerted an upward pull on the short (attached) end of the lever, moving the opposite end downward on the fulcrum. Affixed to the end moving down was a short piece of wire. If the muscle contraction were adequate, this wire made contact with a brass plate 2 mm. beneath, closing the circuit in which the lever was incorporated and lighting a bulb. Extreme care was taken during the preparation to ensure an uninterrupted blood supply to the gastrocnemius. At the end of each individual determination, the muscle was cut in half. If no observable bleeding occurred, results of the determination were discarded.

The sciatic nerve was exposed close to its point of emergence from the vertebral column. After the nerve was sectioned, its peripheral end was secured to shielded electrodes (HA 305), and single shocks from a stimulator (AEL 104 A) were applied to the nerve at the rate of one every 10 sec. Only the intensity of these shocks was varied. A liquid petrolatum coating kept the nerve from drying out. Special care was taken to immobilize the hind leg so that gastrocnemius contractions would move only the attached muscle lever. The muscle was kept moist and warm with normal saline (37–38°) dripping from a buret positioned above.

Threshold voltage was specified as that which was just adequate when applied to the muscle through the sciatic nerve to elicit a contraction response sufficient to move the tip of the attached lever downward a distance of 2 mm. At the end of this excursion, the lever tip made contact with the brass plate, closing the circuit and lighting the bulb to indicate attainment of the threshold response. Threshold voltage was determined by serial increases in the intensity of single induction shocks applied to the preparation.

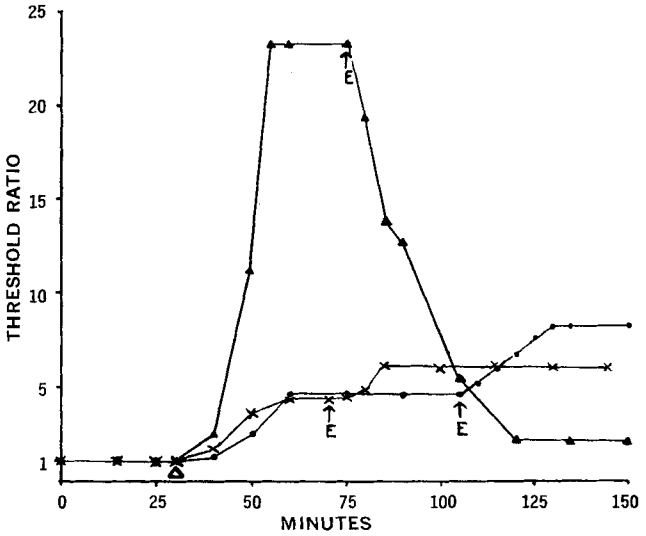

Figure 1—*Effect of eserine salicylate (E) on the change produced in the electrical threshold of the rat sciatic-gastrocnemius preparation by nicotine (·—·), dimethyl tubocurarine chloride (——), and succinylcholine chloride (×—×). The open triangle (△) indicates the first injection of the blocking agent. Each line represents the results of one experiment. Doses of eserine were the same. The threshold ratio is the electrical threshold (in volts) at any given time divided by the electrical threshold at zero time.*

Threshold voltage was determined every 5 min. Drugs were not administered unless the same threshold voltage was obtained for six successive determinations (30 min.). Low and high cumulative doses were employed.

At preestablished "low" doses, nicotine, dimethyl tubocurarine chloride, and succinylcholine chloride (0.100, 0.010, and 0.001%, respectively) showed some neuromuscular blocking activity but did not affect respiration. "High" doses of these agents (1.000, 0.100, or 0.010%, respectively) were sufficient to inhibit spontaneous respiration while exerting marked neuromuscular blocking activity. All solutions were given intravenously in an injection volume of 1.0 ml./kg. Three to five animals were used for each experimental condition tested.

A dose of blocking agent was administered every 5 min. until an inhibition of neuromuscular transmission was evidenced in an elevation in threshold voltage. The new threshold voltage was then determined every 15 min. thereafter for a period of not less than 2 hr. All doses of blocking agent were submaximal; that is, the inhibition of transmission could be overcome with an electrical stimulus of sufficient intensity.

In the first portion of the study related to nicotine's blocking mechanism, the rectus abdominis muscle of the frog was suspended in a 50-ml. bath chamber containing modified Ringer solution (100 ml. Ringer diluted to 140 ml. with water) through which oxygen was bubbled. The responses of rectus muscles during a series of 90-sec. contacts with nicotine (10^{-3} mm.) (8) after exposure to the acetylcholinesterase of horse serum for 15 min. were compared with

Table II—Effect of Eserine Salicylate on Early and Late Phases of Neuromuscular Blockades Produced by Nicotine, Dimethyl Tubocurarine, and Succinylcholine

Phase of Blockade	Change in Threshold[a], %		
	Nicotine	Dimethyl Tubocurarine	Succinylcholine
Early (<120 min.)	+35 ± 22[b]	−79 ± 3	+28 ± 7
Late (>120 min.)	−62 ± 1[c]	−78 ± 7	−56 ± 1

[a] Calculated as the difference between thresholds before and 15 min. after administration of drug, expressed as percent of threshold before first injection. [b] A plus (+) sign indicates enhancement of the neuromuscular blockade. [c] A minus (−) sign indicates antagonism of the neuromuscular blockade. Each value represents the mean ±*SEM* of three to five experiments.

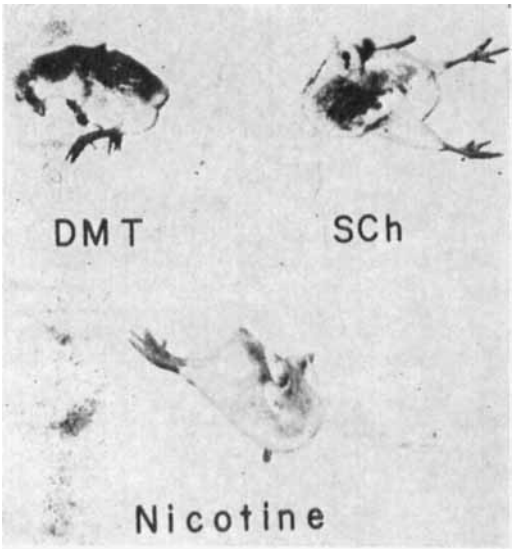

Figure 2—*Effect of intravenous injection of dimethyl tubocurarine chloride (DMT), succinylcholine chloride (SCh), and nicotine on 4–7-day-old chicks.*

the responses prior to exposure. These responses to nicotine were then compared with the responses to eserine following exposure of the latter to acetylcholinesterase. In the second portion of the study, the rabbit duodenal strip was suspended in oxygenated (95% O_2, 5% CO_2) Ringer solution (0.1% dextrose added) in the inner chamber of a constant-temperature bath kept at approximately 37°. The effect of eserine added to the chamber was recorded, and the strip was washed with a fresh dextrose–Ringer solution and allowed to return to its previous tone. Then the effect of an ensuing 12–15-min. suspension of the strip in regular (dextrose-free) Ringer was recorded. At the conclusion of this period, the effect of eserine added to the chamber was recorded during a 3-min. exposure and compared with that of nicotine added to the chamber a short time later.

RESULTS AND DISCUSSION

Low doses of nicotine and succinylcholine repeatedly diminished the threshold voltage of this rat sciatic-gastrocnemius preparation while high doses repeatedly elevated this threshold voltage. Any effective dose of dimethyl tubocurarine, on the other hand, only elevated threshold voltage (Table I).

In the early phase (within 1.5–2 hr. after injection) of the nicotine and succinylcholine blocks, eserine repeatedly elevated threshold voltage, enhancing these blocks (Fig. 1); it repeatedly lowered the threshold voltage of the late phase (more than 2 hr. after injection) of the nicotine and succinylcholine blocks, opposing these blocks.

In both early (Fig. 1) and late phases of the dimethyl tubocurarine block, eserine repeatedly lowered the threshold voltage (Table II).

According to Buttle and Zaimis (9), the intravenous injection of depolarizing blockers into young chicks, 4–7 days old, causes a rigid extension of the head and legs. Intravenous injection of polarizing blockers, on the other hand, causes a flaccid paralysis of the head and legs. Nicotine and succinylcholine injected intravenously into such chicks produced a spastic extensor paralysis of the neck and legs, whereas dimethyl tubocurarine produced a flaccid paralysis of the neck and legs (Fig. 2).

A number of investigators believe that the effect of nicotine is achieved both centrally and peripherally as a result of acetylcholine release. Accordingly, an attempt was made to determine whether the neuromuscular blocking action of nicotine is dependent upon the release and/or maintenance of acetylcholine. The availability of acetylcholine was diminished to the point of no response to eserine (in this study) in the frog rectus abdominis muscle by exposure of the tissue to acetylcholinesterase (of horse serum). In the isolated rabbit ileum, acetylcholine was depleted after immersion in a dextrose-free Ringer solution as determined by the absence of response to eserine (10). In both preparations, nicotine exerted a strong stimulant effect shortly after the absence of response to eserine.

According to the results presented, nicotine, unlike the so-called pseudocurares, does not turn its attention from acetylcholinesterase receptors to the cholinergic receptors of the neuromuscular junction during its skeletal muscle blocking action. Rather, nicotine seems to exert a direct effect throughout, first bringing about a depolarization of the muscle sole plate through union with the cholinergic receptors of the neuromuscular junction, then somehow stabilizing these same receptors (after repolarization of the muscle fiber membrane). Nicotine's depolarization of the muscle sole plate through union with its cholinergic receptors is responsible initially for its threshold-lowering effect at the neuromuscular junction and shortly thereafter for the first or depolarizing phase of its blocking action. The subsequent stabilization of the same muscle sole plate receptors is responsible for the second or polarizing phase of its blocking action.

Nicotine, although its effect pattern simulates that of the pseudocurare agents, gives evidence of belonging in a class of neuromuscular blocking agents by itself, by virtue of the fact it achieves that pattern throughout by acting directly on the receptors of the neuromuscular junction.

SUMMARY

1. Like succinylcholine, nicotine first diminishes and later elevates threshold voltage of the neuromuscular junction. Dimethyl tubocurarine only elevates threshold voltage of the neuromuscular junction.

2. During the early phase of nicotine's threshold elevation, eserine contributes to the elevation; during the late phase of nicotine's threshold elevation, eserine antagonizes the elevation. Eserine has the same qualitative, if not quantitative, effect on early and late phases of succinylcholine threshold elevation. On the other hand, eserine antagonizes both the early and late phases of dimethyl tubocurarine's threshold elevation. Nicotine and succinylcholine produce a spastic paralysis of the head and legs of the young chick; dimethyl tubocurarine produces a flaccid paralysis of these members.

3. The action of nicotine at the neuromuscular junction appears to be direct rather than indirect through the release and/or maintenance of acetylcholine.

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Antagonism of Norepinephrine Responses of Isolated Rabbit Aorta by Aliphatic Sulfur-Containing Amines

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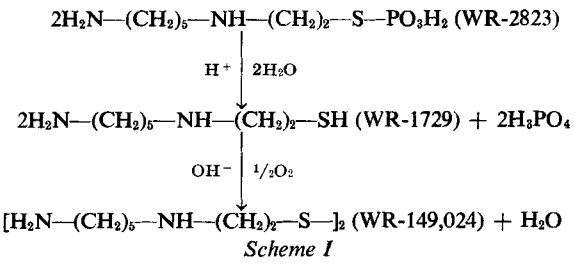
Abstract □ *S*-[2-(5-Aminopentyl)aminoethyl]phosphorothioic acid (WR-2823), *S*-[2-(5-aminopentyl)aminoethane]thiol (WR-1729), and 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane (WR-149,024) were tested on the isolated rabbit aorta for effects to antagonize the responses to norepinephrine. The relative potency of these agents to antagonize responses to norepinephrine was WR-149,024 > WR-1729 > WR-2823. The pA_2 plot for WR-149,024 against norepinephrine gave a value of 5.47, with a slope not significantly different from −1.0. WR-149,024 did not antagonize responses to angiotensin or histamine. These findings are consistent with the hypotheses that WR-149,024 is the active form of this series of agents and that these chemicals antagonize norepinephrine responses by competitive inhibition at the α-adrenergic receptors.

Keyphrases □ Sulfur-containing aliphatic amines—α-adrenergic blockage, antagonism of norepinephrine responses, rabbit aorta □ α-Adrenergic blockage—sulfur-containing aliphatic amines, antagonism of norepinephrine responses, rabbit aorta □ Norepinephrine responses—antagonism by sulfur-containing aliphatic amines, rabbit aorta

S-[2-(5-Aminopentyl)aminoethyl]phosphorothioic acid (WR-2823) has α-adrenergic blocking properties *in vivo* as judged by the effect of the agent on the cardiovascular responses to sympathomimetic amines in several species (1). We speculated at that time that this action might be due to the hydrolysis product of WR-2823, namely, *S*-[2-(5-aminopentyl)aminoethane]thiol (WR-1729). The latter compound is oxidized in the presence of oxygen to a symmetrical disulfide, 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane (WR-149,024). These three compounds are related as shown in Scheme I.



Scheme I

All three compounds have α-adrenergic blocking properties on the cardiovascular responses to sympathomimetic amines in the anesthetized rat (2). The present study was done to verify the α-adrenergic blocking properties of these agents using an isolated vascular smooth muscle and to quantitate the anti-adrenergic potency of the members of this series.

EXPERIMENTAL

WR-2823 occurred as a mixture containing 75% WR-2823, 11% water, 7% WR-149,024, and 7% inorganic phosphate (3). WR-1729 and WR-149,024 occurred as the di- and tetrahydrochlorides, respectively[1]. All materials were white granular powders with faint-to-moderate mercaptan odors; they were very soluble in water.

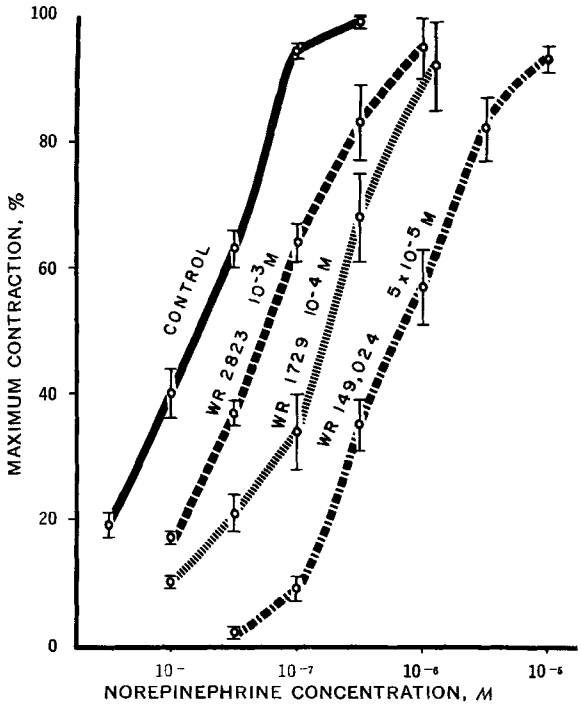


Figure 1—*Effect of WR-2823, WR-1729, and WR-149,024 on the concentration–response curve of norepinephrine on the isolated rabbit aorta. Each point represents the mean and* SEM (n = 4). *Control response is the composite of the preexposure responses. The responses after a 1-hr. exposure to each chemical were derived from separate experiments.*

Norepinephrine[2] was diluted in 0.9% saline containing 0.1% sodium bisulfite so that when added in volumes of 1 ml. in a 130-ml. muscle bath, appropriate concentrations were obtained. Histamine dihydrochloride and angiotensin amide[3] were similarly dissolved and diluted in distilled water.

New Zealand white rabbits of either sex, weighing 2.5–3.5 kg., were used. Four spiral strips of thoracic aorta from each rabbit were suspended simultaneously in an isolated muscle bath containing a balanced salt solution at 37.5°, according to the method of Furchgott (4). After a 4-hr. equilibration period at 2-g. resting tension, cumulative concentration–response curves to norepinephrine were run. The bath was drained and filled with fresh bathing solution three times; the test chemicals were then added as solutions in distilled water to the bath at various concentrations for various time periods. With the chemicals still in the bath, cumulative concentration–response curves to norepinephrine were repeated. The ED_{50} was estimated before and after the test drug was added. The $\log_{10}$ of the ratio of these two estimates (after:before) was assessed as a function of time and concentration to compare the potency of the drugs. The pA_2 for WR-149,024 was estimated by the method of Schild (5) using a 90-min. contact time. The effect of WR-149,024 at

[1] All chemicals were synthesized by Dr. W. Gannon, Regis Chemical Co., U. S. Army Contract No. DA-49-193-MD-2590.
[2] Levophed.
[3] Hypertensin.

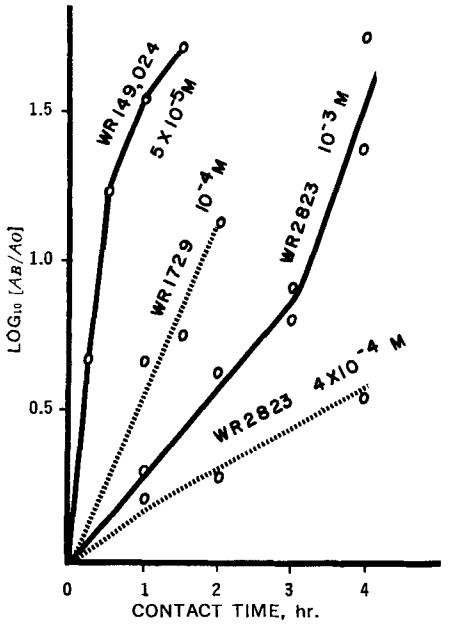


Figure 2—*Time course for the onset of the antagonism of the response of the isolated aortic strip of rabbits to norepinephrine by WR-2823, WR-1729, and WR-149,024. Key:* A_B = *concentration of norepinephrine required to give 50% maximum response in the presence of the antagonist, and* A_O = *concentration of norepinephrine required to give 50% maximum response in the absence of antagonist. Each ratio represents the results of a paired experiment from four strips.*

5×10^{-5} *M* for a 90-min. contact time on the responses of the aortic strips to histamine and angiotensin was similarly evaluated.

RESULTS

The effects of 1-hr. exposure to WR-2823, WR-1729, and WR-149,024 on the response of the isolated rabbit aorta to norepinephrine are shown in Fig. 1. Each chemical caused a parallel shift of the concentration–response curve to the right. The time course for the onset of antiadrenergic effects of WR-2823, WR-1729, and WR-149,024 is shown in Fig. 2. The onset of action and the potency of WR-149,024 were greater than those of the other two chemicals of this series. WR-1729 had a faster onset of action and greater potency than WR-2823.

The plot of the pA_2 estimate for WR-149,024 at 90-min. contact time is shown in Fig. 3. The pA_2 estimated from the least-squares

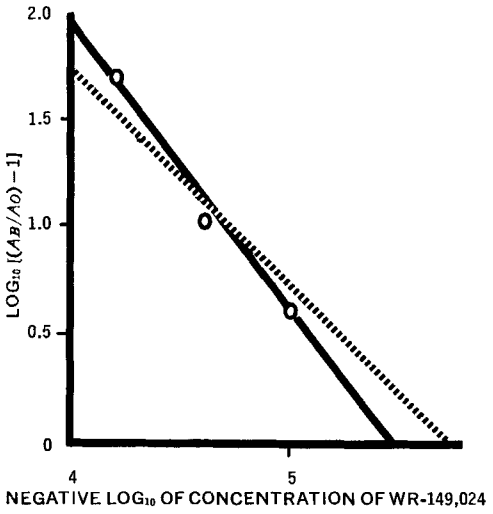


Figure 3—*Plot for the estimate of the* pA_2 *for WR-149,024 using a 90-min. contact time against the responses to norepinephrine. (For explanation of symbols, see Fig. 2 and text.) Key: —, slope of −1.31; and - - - -, slope of −1.0.*

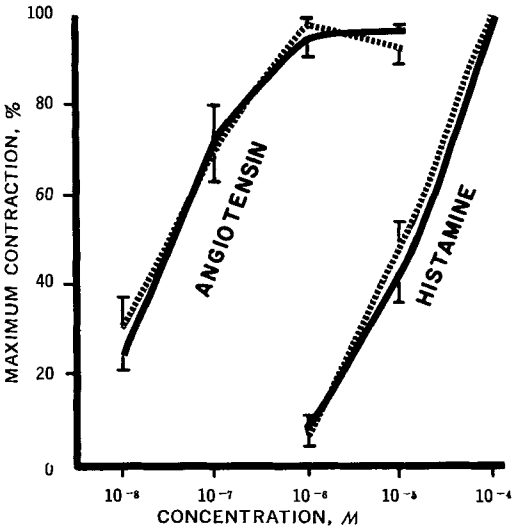


Figure 4—*Effect of WR-149,024 on the responses of isolated aortic strips to histamine or angiotensin. Each point represents the mean and* SEM (n = 4). *Key: —, control responses; and - - -, responses after a 90-min. contact with* 5×10^{-5} M *WR-149,024 in the bath.*

slope of −1.31 is 5.47; the pA_2 estimated from a slope of −1.0 is 5.73.

Responses to histamine and angiotensin were unchanged following a 90-min. exposure to 5×10^{-5} *M* WR-149,024 (Fig. 4).

DISCUSSION

The order of potency of antagonism of norepinephrine by this series of compounds is WR-2823 < WR-1729 < WR-149,024. The order of the rate of onset of antagonism is the same, with WR-2823 reaching its maximum potency most slowly, WR-149,024 reaching its maximum potency most rapidly, and WR-1729 being intermediate in rate of onset. There are two possible explanations for these two observations. These findings could represent differences in diffusion rates of the chemicals to the receptor sites, or they may be due to a conversion of the phosphorothioate to the thiol and the oxidation of the thiol to the disulfide, with this latter compound being the actual active form of this series. These experiments do not rule out either explanation, but the observation that WR-2823 had not reached equilibrium with the receptors even at 4 hr. would certainly represent an unusually slow diffusion rate. At this time, the explanation involving chemical hydrolysis is favored.

WR-149,024, the most potent member of the series, does not antagonize the responses to angiotensin or histamine at a concentration and contact time that depress the responses to norepinephrine by about 70-fold. This finding shows that the antagonism is specific for adrenergic mechanisms. The plot of the pA_2 estimate gives a line whose slope, within experimental error, is not different from −1.0. These data do not rule out other explanations, but they are consistent with the concept that WR-149,024 acts to block adrenergic mechanisms at the level of the α-adrenergic receptors as an equilibrium antagonist.

WR-2823 and WR-149,024 were shown to have efficacy in the prevention of mortality from shock due to hemorrhage, endotoxin, or anaphylaxis in several species (6–11). These chemicals are being proposed for clinical trials for therapy of endotoxin or hemorrhagic shock. If the efficacy of these compounds in this proposed use is dependent upon their ability to block peripheral adrenergic responses, the disulfide should clearly be superior to the phosphorothioate owing to its greater potency and more rapid onset of action.

All commonly used α-adrenergic blocking agents have cyclic components. The possibility that this series of aliphatic amines has α-adrenergic blocking properties raises a question concerning the requirement for aromatic configuration for α-adrenergic blockade.

These experiments provide no insight into the possible role of the sulfur components of these chemicals with respect to interaction with the adrenergic receptors. Nevertheless, they raise the interesting possibility that these agents may act by forming a mixed disulfide with the receptors or with some other cellular component at an "allosteric site."

CONCLUSION

These findings are consistent with the hypotheses that these aliphatic amines act to antagonize adrenergic responses by competitive inhibition of the α-adrenergic receptors and that the active form is 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane. If these hypotheses are correct, they suggest new ideas concerning the need for aromatic structural components for α-adrenergic receptor blocking drugs.

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VICTOR F. SMOLEN* and ROLLAND I. POUST†

Abstract □ Electrical potentials developed at a human epidermal surface–aqueous solution boundary in response to varying concentrations of sodium and potassium ions were measured *in vivo*, both in the presence and absence of hexachlorophene in the solutions. The results provide further evidence for a previously postulated mechanism of hexachlorophene interaction with epidermal colloids which implicated allosterically effected changes in the electron densities of ionogenic groups vicinally located to hexachlorophene interaction sites.

Keyphrases □ Hexachlorophene—role in changes in electrical response specificity for sodium and potassium ions, human finger epidermis, mechanism of action □ Epidermis, electrical response specificity—hexachlorophene-induced changes and its mechanism of action

In a recent article concerning the interaction of hexachlorophene with human finger epidermis (1), a molecular mechanism of interaction was postulated whereby unionized hexachlorophene molecules and/or anions hydrogen bonded to peptide linkages of the epidermal proteins to effect changes in the net fixed-charge density on the surface. An inductive effect, which allosterically altered the dissociation constants of ionogenic side groups located vicinally to hexachlorophene interaction sites, was implicated as operative in the observed phenomena. Ling (2) pointed out that such changes in proton-ionization constants of ionogenic groups can also induce an altered selectivity of the ionogenic groups for associating specific monovalent cations.

In accordance with Ling (2) and Eisenman (3), the electrical response of cation-selective electrodes to specific ions is dependent upon the anionic field strength of the groups fixed to the electrically responding surface; the field strength underlies the association affinity of the surface groups for specific cations and determines the rank order of ion selectivity manifested in the electrical response behavior of the surface. Therefore, provided the magnitude of the effect is sufficient for detection, the interaction of hexachlorophene with the epidermal surface colloids may be expected to alter inductively the rank order of their selective electrical response to cations in solution.

EXPERIMENTAL

Electrical potentials were measured, utilizing the previously described experimental arrangement (1), for solutions containing HCl, NaCl, and KCl ranging in concentration from 1.0 to 10^{-5} *M*, both in the absence and presence of saturated concentrations of hexachlorophene. The previously described (1) pretreatment of fingers was employed, with the exception that prehydration was carried out in double-distilled water to effect a removal of ions from the epidermal surface. The reference solution was 1.0 *M* KCl in all instances. Measurements were initiated at both high and low concentrations and were recorded only after the potentials remained constant for a minimum of 30 sec. All NaCl and KCl solutions had pH values above 5.75. Six replications were performed on a 25-year-old male volunteer.

RESULTS AND DISCUSSION

The mean values of electrical potentials observed when the measurements were initiated at high and low ion concentrations are plotted as a function of the negative logarithm of the cation concentration in Figs. 1 and 2. The initial linear portions of the curves represent a least-squares regression analysis fit to the data. The relative specificities of the epidermal proteins for interaction with the ions were computed using the following equation as presented

CONCLUSION

These findings are consistent with the hypotheses that these aliphatic amines act to antagonize adrenergic responses by competitive inhibition of the α-adrenergic receptors and that the active form is 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane. If these hypotheses are correct, they suggest new ideas concerning the need for aromatic structural components for α-adrenergic receptor blocking drugs.

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RESULTS AND DISCUSSION

The mean values of electrical potentials observed when the measurements were initiated at high and low ion concentrations are plotted as a function of the negative logarithm of the cation concentration in Figs. 1 and 2. The initial linear portions of the curves represent a least-squares regression analysis fit to the data. The relative specificities of the epidermal proteins for interaction with the ions were computed using the following equation as presented

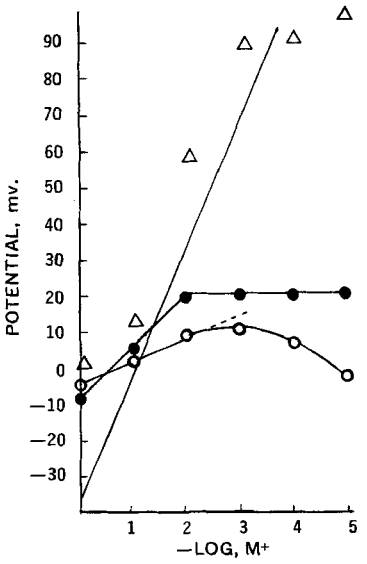

Figure 1—*Electrical response of human finger epidermal surface to hydrogen* (△), *sodium* (●), *and potassium* (○) *ions in the absence of hexachlorophene.*

by Eisenman (3) for glass ion-selective electrodes and electrically responding surfaces in general (4):

$$V_I - V_H = \frac{RT}{F} \ln K_{HI} \qquad \text{(Eq. 1)}$$

where V_I and V_H are the observed potentials (least-squares intercepts) for the experimental and reference (H^+) ions, respectively; R is the gas constant; T is the absolute temperature; and F is the Faraday constant. The symbol K_{HI} is an empirical constant describing the relative selectivity of the protein to the experimental cation *versus* the reference hydrogen cation. The value of K_{HI} in the presence and absence of hexachlorophene was calculated for each cation. The results of these calculations are summarized in Table I; it appears that in the absence of hexachlorophene, potassium ion is more selectively interacted while sodium ion is preferred when hexachlorophene is present. A paired *t*-test comparison of the two curves revealed the reversal between sodium and potassium specificities as statistically different at $p < 0.05$.

These results indicate a hexachlorophene-induced change in the specificity of the anionic surface groups interacting with the cations. As mentioned earlier, Ling (2) stated that shifts in dissociation constants of ionogenic groups may give rise to alterations in ion specificity. These shifts, described in terms of changes in *c*-value, can change an anionic site's preference for certain cations and can be brought about by the inductive effect. The *c*-value is a parameter

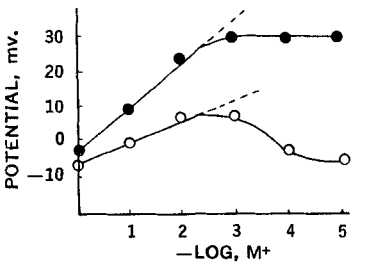

Figure 2—*Electrical response of human finger epidermal surface to sodium* (●) *and potassium* (○) *ions in the presence of hexachlorophene.*

Table I—Summary of the Relative Electrical Response Selectivity of the Human Epidermal Surface to Sodium and Potassium Ions in the Absence and Presence of Hexachlorophene

Cation	K_{HI}[a] Hexachlorophene Absent	K_{HI}[a] Hexachlorophene Present
Na^+	2.78	3.74
K^+	3.36	3.33

[a] The differences are statistically significant at the 95% confidence level.

expressing the electron density of the anionic oxyacid group and underlies the differences in pK values of ionogenic groups. Thus, the inductive effect, which was previously postulated for hexachlorophene interaction, is capable of altering the ion specificity of the epidermal proteins.

Exchange resins (5) having weakly acidic carboxyl groups (high pK) selectively adsorb sodium ion in preference to potassium ion, whereas strongly acidic sulfonic groups (low pK) selectively adsorb potassium ion over sodium ion. Therefore, if hexachlorophene, through an inductive effect, does raise the pK values of the titrable groups in the region above 5.6 as was postulated (1), the observed reversal in the affinity of the epidermal protein to sodium and potassium ions could be expected.

SUMMARY AND CONCLUSIONS

The selectivity of the proteins comprising the epidermal surface to sodium and potassium ions was studied, both in the presence and absence of hexachlorophene. It was found that a reversal in the affinity of ionogenic groups to sodium and potassium ion occurred; it is attributable to a shift in the pK values of the cation-interacting ionogenic groups as a result of hexachlorophene interaction producing an inductive change in their electron densities. These results constitute predictive evidence and lend additional credence to the previously postulated (1) mechanism of hexachlorophene interaction with epidermal protein. In addition, the presently described experiment exemplifies the manner in which the electrometric study of specific ion–tissue surface interactions may be utilized to provide mechanistic insight into the nature of the processes involved in drug–tissue binding.

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New Schistosomicidal and Carcinostatic Agents of the Thiaxanthone Type (I)

I. NABIH and A. ZAYED

Abstract □ A new class of compounds, structurally related to axanthones and including the naphthalene system in its reduced tetrahydro form (tetralin), was synthesized and examined for activity in the areas of schistosomiasis and cancer. While 4-(β-diethylaminoethylamino)-1,2-cyclohexenothiaxanthone hydrochloride showed pronounced activity in both areas, its isomer, 2-(β-diethylaminoethylamino)-3,4-cyclohexenothiaxanthone hydrochloride, was completely inactive in schistosomiasis but still active against biologically inoculated tumors.

Keyphrases □ Thiaxanthone derivatives—synthesis as potential schistosomicidal and carcinostatic agents □ 4(or 2)-(β-Diethylaminoethylamino)-1,2(or 3,4)-cyclohexenothiaxanthone hydrochloride—synthesis as potential schistosomicidal and carcinostatic agents

Thiaxanthone derivatives are considered the first nonmetallic organic compounds found to be biologically active for the oral treatment of schistosomiasis (1).

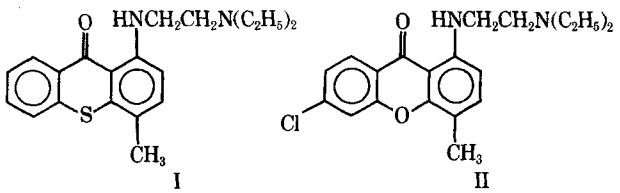
DISCUSSION

The most interesting representatives in this group relative to structure–activity relationships are 1-(β-diethylaminoethylamino)-4-methylthiaxanthone[1] (I) and its oxygen isostere, 1-(β-diethylaminoethylamino)-4-methyl-6-chloroxanthone[2] (II).

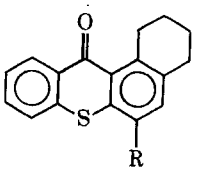
Studies showed that the structural feature necessary for the biological activity within this group is a dialkylaminoalkylamino side chain *para* to a methyl on the aromatic ring (2).

This class of compounds has received increasing interest, since they exert strong carcinostatic activity (3, 4). The most promising member in this group, I, is rather poorly tolerated by the human and produces toxic effects during treatment.

These considerations suggested the synthesis of new compounds of the thiaxanthone type, containing a methylene group in a cyclic six-membered, saturated ring system *para* to an amino side chain, thus incorporating the naphthalene system in its reduced tetrahydro form. This structure type was suggested because tetrahydronaphthalene tends to fix molecular oxygen under mild conditions to form the peroxide (5, 6). It also was biologically demonstrated that molecular oxygen is directly incorporated in the naphthalene metabolism through an oxygenation process (7, 8). Such oxygen carriers may affect glycolysis in the parasite, which seeks its main source of energy for living and reproduction through this metabolic process. Some involved enzymes, such as phosphoglyceraldehyde dehydrogenase and phosphofructokinase, contain sulfhydryl groups essential to their operation. The oxidation of these groups through the presence of oxygen may result in decreased enzymatic activity and, hence, diminished glycolysis.



[1] Lucanthone or Miracil D, Farbenfabriken Bayer AG, Leverkusen, West Germany.
[2] Miracil C, Farbenfabriken Bayer AG, Leverkusen, West Germany.



III: R = —Cl
IV: R = —$NHCOCH_3$
V: R = —NH_2
VI: R = —$NHCH_2CH_2N(C_2H_5)_2$

Another reason for including tetrahydronaphthalene in these structures is that they may biologically undergo oxidation or dehydrogenation to quinoids of the naphthoquinone type, and it is already established that naphthoquinones can highly reduce the rate of glycolysis in schistosomes (9).

Upon condensation of thiosalicylic acid with 6-chlorotetralin in the presence of concentrated sulfuric acid, 4-chloro-1,2-cyclohexenothiaxanthone (III) was obtained.

6-Chlorotetralin was prepared, as well as its 5-chloro isomer, through direct chlorination of tetralin in the presence of iodine as a catalyst (10). The separation of each was effected through sulfonation, by which only 5-chlorotetralin was sulfonated.

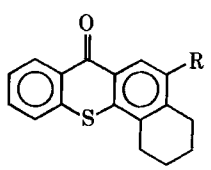
Replacement of the 4-chloro atom in III by the diethylaminoethylamino side chain failed to occur under different experimental conditions. An alternative route was adopted where 6-acetaminotetralin (11, 12) was the component in the condensation reaction with thiosalicylic acid. This tetralin derivative was prepared through nitration of tetralin, and a mixture of 5- and 6-nitrotetralins was formed (13). The latter was isolated through fractional distillation, reduced by iron and hydrochloric acid (14), and acetylated to give 6-acetaminotetralin. Its condensation with thiosalicylic acid in the presence of concentrated sulfuric acid gave 4-acetamino-1,2-cyclohexenothiaxanthone (IV), which, upon hydrolysis, gave 4-amino-1,2-cyclohexenothiaxanthone (V).

Diazotization of V and treatment with cuprous chloride gave a chloro product identical to III.

Treatment of the amino compound, V, with 2-diethylaminoethyl chloride gave 4-(β-diethylaminoethylamino)-1,2-cyclohexenothiaxanthone (VI).

Similarly, 5-acetaminotetralin was prepared through acetylation of the corresponding amine. Its condensation with thiosalicylic acid in the presence of concentrated sulfuric acid gave 2-acetamino-3,4-cyclohexenothiaxanthone (VII), which, upon hydrolysis, yielded 2-amino-3,4-cyclohexenothiaxanthone (VIII). Upon heating VIII with 2-diethylaminoethyl chloride, 2-(β-diethylaminoethylamino)-3,4-cyclohexenothiaxanthone (IX) was obtained.

Diazotization of VIII and treatment with cuprous chloride gave 2-chloro-3,4-cyclohexenothiaxanthone (X).



VII: R = —$NHCOCH_3$
VIII: R = —NH_2
IX: R = —$NHCH_2CH_2N(C_2H_5)_2$
X: R = —Cl

Biological Screening—*Schistosomiasis*—A group of mice infected with *Schistosoma mansoni* and showing viable ova in their stools were given oral doses of 60 mg./kg. of the hydrochloride of VI for 12 consecutive days.

Another infected group was similarly treated with the hydrochloride of IX at the same dosage level for the same period.

A third infected group was given no treatment and served as the control.

One week after treatment, collected stools from the groups were examined for ova excretion; the examination was continued for 6 weeks at 1-week intervals. The animals were sacrified 8 weeks thereafter, and the liver and mesenteric venules were examined for the presence of dead and living worms.

In the first group, treated with the hydrochloride of VI, there was a gradual decrease in ova excretion until none was found. Only dead worms were found in the liver.

In the second group, treated with the hydrochloride of IX, no depression in ova count was observed. Living worms were distributed in the liver and mesenteric venules.

In the third group, the control, no remarkable change in the ova count was observed. Living worms were found in the liver and in mesenteric venules.

From these findings, it could be concluded that VI is biologically active in experimental schistosomiasis, while its isomer (IX) is devoid of such activity.

Tumor Experiments—Two groups of mice, inoculated with 10^6 tumor cells of Ehrlich ascites carcinoma, were given 80 mg./kg. i.p. of both IV and VII hydrochlorides in aqueous solutions for 5 consecutive days.

Cell counts and weight observations after inoculation showed that both compounds possessed an inhibitory effect on tumor growth (Fig. 1).

Detailed data, methods, and techniques used for biological screening will be fully described in a separate report.

EXPERIMENTAL[3]

6-Chlorotetralin—This compound was prepared as described by Schroeter (10).

4-Chloro-1,2-cyclohexenothiaxanthone (III)—A mixture of 20 g. (0.13 mole) of thiosalicylic acid, 21.6 g. (0.13 mole) of 6-chlorotetralin, and 30 ml. of concentrated sulfuric acid was shaken at room temperature for 25 hr.; it was then cooled and poured over ice water. The product formed was collected and thoroughly washed with an ammonia solution and then water. After drying, it weighed 27 g. (70%). Upon recrystallization from acetone, it melted at 100–101°.

Anal.—Calc. for $C_{17}H_{13}ClOS$: C, 67.88, H, 4.36; S, 10.66. Found: C, 68.25; H, 4.58; S, 10.39.

4-Acetamino-1,2-cyclohexenothiaxanthone (IV)—To a mixture of 4 g. (0.026 mole) of thiosalicylic acid and 75 ml. of concentrated sulfuric acid was added portionwise, with shaking, 5 g. (0.026 mole) of 6-acetaminotetralin. Shaking continued at room temperature for 20 hr. Then the mixture was cooled and poured over ice water. The precipitate formed was collected and washed in succession with water, aqueous ammonium hydroxide, and water. After drying, the product weighed 6.7 g. (82%). Upon recrystallization from benzene, it melted at 230–231°.

Anal.—Calc. for $C_{19}H_{17}NO_2S$: C, 70.56; H, 5.3; S, 9.89. Found: C, 70.00; H, 5.49; S, 9.90.

4-Amino-1,2-cyclohexenothiaxanthone (V)—To a solution of 5 g. of II in 100 ml. of ethanol was added 30 ml. of hydrochloric acid (37%). This was refluxed for 5 hr. and then cooled. The precipitate formed was isolated and boiled with NaOH (50%) for 10 min. The product was filtered and washed with water to give 4.2 g. of III after drying. Upon recrystallization from ethanol, it melted at 185–186°.

Anal.—Calc. for $C_{17}H_{15}NOS$: C, 72.56; H, 5.37; N, 4.94; S, 11.39. Found: C, 72.53; H, 5.66; N, 4.79; S, 11.41.

[3] Melting points are uncorrected and were taken in open capillary tubes, using a Gallenkamp melting-point apparatus. Microanalyses were performed by the Microanalytical Laboratory, National Research Centre, Cairo, U.A.R., and the Spang Microanalytical Laboratory, Ann Arbor, Mich. IR spectra were determined on a U.R. 10 Carl Zeiss-Jena IR, using KBr pellets.

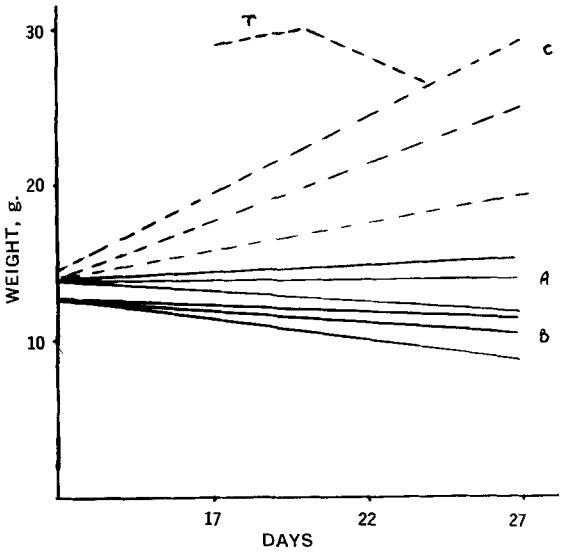


Figure 1—*Effect of treatment with Compound VI hydrochloride (A) and IX hydrochloride (B) on growth of mice tumors of the Ehrlich ascites carcinoma type. Each mouse was implanted with 10^6 tumor cells. C = control. T illustrates effect of treatment by A on a control mouse after 20 days of inoculation.*

4 - (β - Diethylaminoethylamino) - 1,2 - cyclohexenothiaxanthone (VI)—A mixture of 1 g. of III and 0.5 ml. of 2-diethylaminoethyl chloride was heated under reflux at 170° for 3 hr.; after cooling, the product was boiled with NaOH (50%) for 10 min. After decantation of the alkali, the residue was recrystallized from ethanol to give 1.2 g. (90%) of IV, m.p. 95–96°. IR: 1630 (C=O), 3310 (—NH—), 680 (—S—), and 3017 $cm.^{-1}$ (cyclohexyl).

Anal.—Calc. for $C_{23}H_{28}N_2OS$: C, 72.59; H, 7.41; N, 7.36; S, 8.43. Found: C, 72.44; H, 7.27; N, 7.34; S, 8.51.

The hydrochloride was prepared by dissolving VI in dry ether and passing a stream of dry HCl through the solution. The yellow product was filtered, washed with dry ether, and dried to give a hydrochloride, m.p. 150–155°.

4-Chloro-1,2-cyclohexenothiaxanthone (III)—To a cold mixture of 40 ml. of hydrochloric acid (37%), 10 ml. of acetic acid, and 2 g. of V was added a solution of 1 g. of sodium nitrite in 50 ml. water. This mixture was poured over a solution of 1 g. of cuprous chloride in 50 ml. of hydrochloric acid (37%). The product was filtered to give 1.3 g. After recrystallization from acetone, it melted at 100–101° (identical to that obtained through the direct condensation of 6-chlorotetralin with thiosalicylic acid, with no depression in a mixed melting-point determination).

2-Acetamino-3,4-cyclohexenothiaxanthone (VII)—A mixture of 4 g. (0.026 mole) of thiosalicylic acid, 5 g. (0.026 mole) of 5-acetaminotetralin, and 80 ml. of concentrated sulfuric acid was shaken at room temperature for 30 hr., stirred at 60° for 2 hr., and then poured after cooling over ice water. The precipitate formed was filtered and washed with water, ammonia solution, and water. After drying, it gave 6 g. (70%) of VII. Upon recrystallization from benzene, it melted at 298–300°.

Anal.—Calc. for $C_{19}H_{17}NO_2S$: C, 70.56; H, 5.3; N, 4.32, S, 9.89. Found: C, 70.57; H, 5.16; N, 4.05; S, 9.80.

2-Amino-3,4-cyclohexenothiaxanthone (VIII)—A mixture of 5 g. of VII, 125 ml. of absolute ethanol, and 30 ml. of hydrochloric acid (37%) was refluxed for 10 hr. and then cooled. The hydrochloride formed was filtered and dried; it melted at 285–287°. This was boiled with 50% sodium hydroxide for about 15 min. and then filtered, washed with water, and dried to give 4.2 g. (96%) of the free amine VIII. It was recrystallized from ethanol as yellow needles, m.p. 239–240°.

Anal.—Calc. for $C_{17}H_{15}NOS$: C, 72.56, H, 5.37; N, 4.94; S, 11.39. Found: C, 72.86; H, 5.64; N, 5.23; S, 11.52.

2 - (β - Diethylaminoethylamino) - 3,4 - cyclohexenothiaxanthone (IX)—A mixture of 1 g. of VIII and 0.5 ml. of 2-diethylaminoethyl chloride was heated under reflux at 180° for 7 hr. The mixture was boiled with a few milliliters of 50% sodium hydroxide and then

washed with water. Recrystallization of the residue from dilute ethanol gave 1.3 g. (94%) of IX, m.p. 114–115°. IR: 1620 cm.$^{-1}$ (C=O), 3340 cm.$^{-1}$ (—NH—), 700 cm.$^{-1}$ (—S—), and 3017 cm.$^{-1}$ (cyclohexyl).

Anal.—Calc. for $C_{23}H_{28}N_2OS$: C, 72.59; H, 7.41; N, 7.36; S, 8.43. Found: C, 72.53; H, 7.25; N, 7.28; S, 8.55.

The hydrochloride was similarly prepared as described for IV, m.p. 169–170°.

2-Chloro-3,4-cyclohexenothiaxanthone (X)—Diazotization of VIII and treatment of the diazonium solution with cuprous chloride, as described for III, gave X. After recrystallization from ethanol, it melted at 186–187°.

Anal.—Calc. for $C_{17}H_{13}ClOS$: C, 67.88; H, 4.36; S, 10.66. Found: C, 68.41; H, 4.67; S, 10.73.

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Preparation and Activity of β-Substituted Acetylcholine Iodides

GEORGE H. COCOLAS*‡, ELLEN C. ROBINSON*§, WILLIAM L. DEWEY†, and THEODORE C. SPAULDING†‖

Abstract □ The enantiometers of (−)- and (+)-acetyl β-ethylcholine iodide were prepared following the resolution of 1-dimethylamino-2-butanol from (+)-tartaric acid and (+)-bromocamphorsulfonic acid for the (−)- and (+)-isomers, respectively. The absolute configuration of the (−)-enantiomer was determined by the synthesis of (−)-acetyl β-ethylcholine iodide from R(−)-2-hydroxybutyric acid. The optically active acetyl β-phenylcholine iodides were prepared from the optically active mandelic acids. These enantiomers show that the R(−)-isomers have greater affinity for the acetylcholinesterase receptor and that the S(+)-isomers are more potent agonists on guinea pig ileum.

Keyphrases □ Acetylcholine iodides, β-substituted—preparation, activity □ Acetyl β-ethylcholine iodide, enantiomers—preparation, absolute configuration, activity

It was shown previously (1, 2) that acetylcholinesterase and the smooth muscle of guinea pig ileum each reacts best with only one of the enantiomers of acetyl β-methylcholine. Cocolas *et al.* (3) also pointed out that the enantiomers of acetyl β-methylcholine likely assume a conformation in which the interface between them and the corresponding susceptible receptor area, during interaction, is the same as that for acetylcholine; that is, the facet of the choline fragment facing the receptor is the side that resembles acetylcholine.

We sought to extend the study of stereochemical requirements at the acetylcholinesterase and the smooth muscle receptor in guinea pig ileum by examining the activity of β-ethyl and β-phenyl acetylcholines. Some investigators (1, 4, 5) assumed that S(+)-acetyl β-methylcholine has better affinity for acetylcholinesterase than the R(−)-enantiomer. It might be expected that acetylcholinesterase would produce a greater disparity between the action of the enantiomers of β-ethyl- and β-phenyl-substituted acetylcholines, since the increase in bulk would produce a more remote possibility of similar accommodation of the enantiomeric pairs. An examination of the enzymatic activity of each optical isomer of β-ethyl and β-phenyl acetylcholine iodide was undertaken to test the hypothesis of enantiomeric stereoselectivity of the acetylcholinesterase receptor area. The potency of these enantiomeric pairs on guinea pig ileum was also investigated as a measure of their biologic activity on the muscarinic receptor site.

EXPERIMENTAL[1]

1-Dimethylamino-2-butanol—To a solution of 50 g. 1-nitro-2-butanol (6) in 50 ml. 90% formic acid and 125 ml. formalin (40%)

[1] Melting points were taken on a Mel-Temp apparatus and are uncorrected. Specific rotations were taken on a Cary 60 spectropolarimeter. The IR spectra were run on a Perkin-Elmer model 257 spectrophotometer. Microanalyses were carried out by M-H-W Laboratories, Garden City, Mich.

washed with water. Recrystallization of the residue from dilute ethanol gave 1.3 g. (94%) of IX, m.p. 114–115°. IR: 1620 cm.$^{-1}$ (C=O), 3340 cm.$^{-1}$ (—NH—), 700 cm.$^{-1}$ (—S—), and 3017 cm.$^{-1}$ (cyclohexyl).

Anal.—Calc. for $C_{23}H_{28}N_2OS$: C, 72.59; H, 7.41; N, 7.36; S, 8.43. Found: C, 72.53; H, 7.25; N, 7.28; S, 8.55.

The hydrochloride was similarly prepared as described for IV, m.p. 169–170°.

2-Chloro-3,4-cyclohexenothiaxanthone (X)—Diazotization of VIII and treatment of the diazonium solution with cuprous chloride, as described for III, gave X. After recrystallization from ethanol, it melted at 186–187°.

Anal.—Calc. for $C_{17}H_{13}ClOS$: C, 67.88; H, 4.36; S, 10.66. Found: C, 68.41; H, 4.67; S, 10.73.

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EXPERIMENTAL[1]

1-Dimethylamino-2-butanol—To a solution of 50 g. 1-nitro-2-butanol (6) in 50 ml. 90% formic acid and 125 ml. formalin (40%)

[1] Melting points were taken on a Mel-Temp apparatus and are uncorrected. Specific rotations were taken on a Cary 60 spectropolarimeter. The IR spectra were run on a Perkin-Elmer model 257 spectrophotometer. Microanalyses were carried out by M-H-W Laboratories, Garden City, Mich.

was added 2.0 g. 10% palladium–charcoal catalyst. The mixture was shaken in an atmosphere of hydrogen at an initial pressure of 75 p.s.i.g. on a Parr apparatus until the theoretical amount of hydrogen was taken up (24–36 hr.). The catalyst was removed, 75 ml. concentrated HCl solution was added, and the solvent was removed on a rotary evaporator with the aid of a hot water bath. The residue was dissolved in 100 ml. water and made alkaline with NaOH solution, and the product was extracted with ether. The ethereal solution was dried over anhydrous Na_2SO_4 and distilled, yielding 47.0 g. of a fraction, b.p. 138° [lit. (7) b.p. 142–143°]; n_D^{25} 1.4125; $\nu_{max.}^{CHCl_3}$ 3465 cm.$^{-1}$, broad (OH).

Anal.—Calc. for $C_6H_{15}NO$: C, 61.50; H, 12.90. Found: C, 61.28; H, 13.19.

Resolution of (+)- and (−)-1-Dimethylamino-2-butanol—The methods of Major and Bonnett (8) were used to separate the enantiomers.

(−)-1-Dimethylamino-2-butanol—The salt prepared from 40 g. racemic 1-dimethylamino-2-butanol and 52.5 g. (+)-tartaric acid was recrystallized 16 times from 96% ethanol, using 6 ml. solvent for each gram of salt and finally collecting 14.0 g. of a diastereoisomeric salt which showed no change in rotation from the previous recrystallization; $[\alpha]_{589.3\ nm.}^{25°}$ +0.5° (c 10.0, H_2O). The free base was obtained by dissolving the salt in a minimum amount of water, making the solution alkaline with 20% NaOH, and separating the optically active 1-dimethylamino-2-butanol. The product was dried over anhydrous Na_2SO_4 and distilled, b.p. 143–144°; n_D^{25} 1.4245; $[\alpha]_{589.3\ nm.}^{25°}$ −16.8° (c 1.0, 1 *N* HCl).

Anal.—Calc. for C, 61.50; H, 12.90. Found: C, 61.38; H, 12.89.

(+)-1-Dimethylamino-2-butanol—The ammonium salt (161 g.) of (+)-bromocamphorsulfonic acid (9) in 1 l. water was passed through a Dowex-50W resin (H^+ form), and the eluate was concentrated to a faint-yellow viscous liquid of (+)-bromocamphorsulfonic acid. The acid was mixed with 300 ml. ethanol and 61.0 g. racemic 1-dimethylamino-2-butanol, and the solution was stirred until homogeneous. The solution was neutral. The ethanol was removed on a rotary evaporator under reduced pressure, and the residue was treated with 500 ml. ethyl acetate. Then 204 g. of crystals was collected; $[\alpha]_{589.3\ nm.}^{25°}$ +69.0° (c 1.0, H_2O). The solid was recrystallized three times using 6 ml. of a 10:1 mixture of EtOAc–EtOH to each gram of solid, at which time a constant rotation for the two recrystallizations was observed; $[\alpha]_{589.3\ nm.}^{25°}$ +74.0° (c 1.0, H_2O). The free base was obtained as described for the (−)-isomer. (+)-1-Dimethylamino-2-butanol was distilled, b.p. 141–142°; n_D^{25} 1.4241; $[\alpha]_{589.3\ nm.}^{25°}$ +17.0° (c 1.0, 1 *N* HCl).

Anal.—Calc. for C, 61.50; H, 12.90. Found: C, 61.33; H, 13.12.

(−)-Acetyl β-Ethylcholine Iodide—(−)-1-Dimethylamino-2-butanol (200 mg.) was mixed with 2 ml. 2-propanol and 1.0 g. acetic anhydride. After 25 min., 1.0 g. methyl iodide was added, and the solution was allowed to remain 5 min. more at room temperature. Anhydrous ether was slowly added to initiate precipitation. The collected product (235 mg.) was recrystallized from 2-propanol, m.p. 181–182°; $[\alpha]_{589.3\ nm.}^{25°}$ −19.6° (c 1.0, H_2O); $\nu_{max.}^{KBr}$ 1740 cm.$^{-1}$ (C=O).

Anal.—Calc. for $C_9H_{20}INO_2$: C, 35.77; H, 6.99. Found: C, 35.69; H, 7.08.

(+)-Acetyl β-Ethylcholine Iodide—This compound was prepared according to the method already described. The product melted at 182–183°; $[\alpha]_{589.3\ nm.}^{25°}$ +19.8° (c 1.0, H_2O); $\nu_{max.}^{KBr}$ 1740 cm.$^{-1}$ (C=O).

Anal.—Calc. for $C_9H_{20}INO_2$: C, 35.77; H, 6.99. Found: C, 35.67; H, 6.78.

Resolution of R(−)-2-Hydroxybutyric Acid—2-Hydroxybutyric acid (1) was resolved with (−)-morphine, according to the method of Levene and Haller (10). The barium salt had a specific rotation of +17.5 at 589.3 nm. The free acid was a very hygroscopic, low melting solid and was purified by distillation, b.p. 85°/0.4 mm. Hg; $\nu_{max.}^{mull}$ 1730 cm.$^{-1}$ (C=O).

Anal.—Calc. for $C_4H_8O_3$: C, 46.60; H, 7.74. Found: C, 45.85; H, 8.20.

Determination of Absolute Configuration of (−)-Acetyl β-Ethylcholine Iodide R(+)-2-Acetoxybutyric Acid—R(−)-2-Hydroxybutyric acid (9.0 g.) was mixed with 20 g. of acetyl chloride at room temperature. When evolution of hydrochloric acid subsided, the excess acetyl chloride was removed by distillation and the product was distilled, b.p. 86°/0.2 mm. Hg; wt. 10.8 g.; $[\alpha]_{589.3\ nm.}^{25°}$ +41.0° (c 1.0, CH_3OH); $\nu_{max.}^{mull}$ 1725 cm.$^{-1}$ (ester C=O).

Anal.—Calc. for $C_6H_{10}O_4$: C, 49.33; H, 6.90. Found: C, 49.21, H, 7.07.

R(+)-*N,N*-Dimethyl-2-acetoxybutyramide—R(+)-2-Acetoxybutyric acid (10.8 g.) was mixed with 50 g. of thionyl chloride, and the mixture was refluxed for 30 min. The excess thionyl chloride was removed in a rotary evaporator, and the residue was dissolved in 200 ml. anhydrous Et_2O. This solution was added dropwise to an anhydrous ethereal solution containing 10 g. of dimethylamine. The precipitate was removed, and the filtrate was distilled to give 11.0 g. of product, b.p. 81°/0.4 mm. Hg; n_D^{25} 1.4490; $[\alpha]_{589.3\ nm.}^{25°}$ +4.87° (c 3.2, CH_3OH); $\nu_{max.}^{KBr}$ 1735 cm.$^{-1}$ (ester C=O), 1650 cm.$^{-1}$ (amide C=O).

Anal.—Calc. for $C_8H_{15}NO_3$: C, 55.43; H, 8.66. Found: C, 55.32; H, 8.90.

R(−)-1-Dimethylamino-2-butanol—To a suspension of 7.5 g. $LiAlH_4$ in 200 ml. anhydrous ether was added 11.0 g. of R(+)-*N,N*-dimethyl-2-acetoxybutyramide in 100 ml. ether. The reaction was refluxed for 3 hr., cooled, and treated with 50 ml. of a 40% potassium and sodium tartrate solution. The ether was filtered from the precipitate, dried over anhydrous Na_2SO_4, and distilled to yield 4.0 g. of the optically active aminoalcohol, b.p. 145°; n_D^{25} 1.4242; $[\alpha]_{589.3\ nm.}^{25°}$ −17.0° (c 2.5, 1 *N* HCl).

Anal.—Calc. for $C_6H_{15}NO$: C, 61.50; H, 6.99. Found: C, 61.42; H, 7.12.

R(−)-β-Ethylcholine Iodide—An ethereal solution of the optically active aminoalcohol was mixed with methyl iodide to precipitate the product. R(−)-β-Ethylcholine iodide was recrystallized from 2-propanol, m.p. 159–160°; $[\alpha]_{589.3\ nm.}^{25°}$ −17.5° (c 1.0, H_2O); $\nu_{max.}^{KBr}$ 3375 cm.$^{-1}$ (OH).

Anal.—Calc. for $C_7H_{18}INO$: C, 31.01; H, 6.69. Found: C, 31.28; H, 6.98.

R(−)-Acetyl β-Ethylcholine Iodide—This compound was prepared from R(−)-1-dimethylamino-2-butanol in the same manner as described for the resolved 1-dimethylamino-2-butanol, m.p. 182–183°; $[\alpha]_{589.3\ nm.}^{25°}$ −19.6° (c 1.0, H_2O). The IR spectrum was identical with that of the R(−)-isomer obtained from resolution with (+)-tartaric acid.

Anal.—Calc. for $C_9H_{20}INO_2$: C, 35.77; H, 6.99. Found: C, 35.99; H, 6.74.

R(−)-*N,N*-Dimethyl-*O*-acetylmandelamide—Ten grams of R(−)-mandelic acid[2] was treated with 30 g. of acetyl chloride for 30 min., at which time all the mandelic acid had dissolved and no more hydrochloric acid was being evolved. The excess acetyl chloride was removed under reduced pressure in a rotary evaporator, and the residue was mixed with 30 g. $SOCl_2$ and refluxed for 45 min. The excess $SOCl_2$ was removed by distillation, and the residue was dissolved in 100 ml. anhydrous ether. The ethereal solution was added dropwise to a cooled solution of 10 g. dimethylamine in dry ether. The dimethylamine hydrochloride was removed by filtration, and the filtrate was evaporated to give a solid residue of the crude product, 12.5 g., m.p. 90–92°. The crystals were purified by recrystallization from cyclohexane, m.p. 97–98°; $[\alpha]_{589.3\ nm.}^{25°}$ −189° (c 0.4, CH_3OH); $\nu_{max.}^{KBr}$ 1735 cm.$^{-1}$ (ester C=O), 1650 cm.$^{-1}$ (amide C=O).

Anal.—Calc. for $C_{13}H_{15}NO_3$: C, 65.14; H, 6.84. Found: C, 65.03; H, 6.99.

R(−)-*N,N*-Dimethyl-1-phenylethanolamine—A solution of 5.0 g. of R(−)-*N,N*-dimethyl-*O*-acetylmandelamide in 60 ml. tetrahydrofuran was added dropwise to a suspension of 5.9 g. $LiAlH_4$ in 100 ml. tetrahydrofuran, and the mixture was then refluxed for 4 hr. The excess $LiAlH_4$ was destroyed by ethyl acetate, and 50 ml. of 40% potassium and sodium tartrate solution was added. The ether solution was filtered from the precipitate, and the filtrate was dried over Na_2SO_4 (anhydrous) and distilled to give 3.0 g. of product, b.p. 58–60°/0.15 mm. Hg; n_D^{25} 1.5164; $[\alpha]_{589.3\ nm.}^{25°}$ −62.5° (c 1.0, CH_3OH); $\nu_{max.}^{mull}$ 3430 cm.$^{-1}$, broad (OH).

Anal.—Calc. for $C_{10}H_{15}NO$: C, 72.69; H, 9.15. Found: C, 72.48; H, 9.38.

R(−)-Acetyl β-Phenylcholine Iodide—This compound was prepared in a manner similar to that described for (−)-acetyl β-ethylcholine iodide. The product was recrystallized from a methanol–ether mixture, m.p. 195–196.5°; $[\alpha]_{589.3\ nm.}^{25°}$ −65.0° (c 1.0, H_2O).

[2] Aldrich Chemical Co., Inc.

Table I—Muscarinic and Enzymologic Activity of β-Substituted Acetylcholine Iodides

Iodide	Muscarinic Activity: Guinea Pig Ileum Potency of Acetylcholine	Muscarinic Activity: 95% Confidence Limits	Acetylcholinesterase Activity[a]: Hydrolysis, %	Acetylcholinesterase Activity[a]: K_m 10^{-4}	Acetylcholinesterase Activity[a]: K_i[b] 10^{-4}
Acetylcholine[c]	1.00	—	100	1.37	—
Acetyl β-methylcholine					
S(+)	0.802[d]	0.661–0.973[e]	19	—	No inhibition
RS	0.622	0.456–0.848[e]	11	—	—
R(−)	0.090	0.063–0.129[e]	0	—	5.5
Acetyl β-ethylcholine					
S(+)	0.0121	0.0081–0.018[f]	39.0	1.55	No inhibition
RS	0.0067	0.0051–0.0087[f]	28.0	—	—
R(−)	0.00028	0.00019–0.00041[f]	0	—	3.1
Acetyl β-phenylcholine					
S(+)	0.001	0.0007–0.0013[f]	11.0	—	No inhibition
RS	Inactive	—	8.0	—	—
R(−)	0.00001	0.000004–0.0000123[f]	0	—	6.3

[a] *Electrophorus electricus* acetylcholinesterase, Worthington Biochemical Corp., Freehold, N. J. [b] Physostigmine sulfate under similar conditions gave a K_i value of 7.74×10^{-8}. All inhibitors were competitive. [c] Used perchlorate salt for acetylcholinesterase study and bromide salt for guinea pig ileum study. [d] Reference 3. [e] Mean λ value for these assays is 0.123. [f] Mean λ value for these assays is 0.198.

Anal.—Calc. for $C_{13}H_{20}INO_2$: C, 44.71; H, 5.77. Found: C, 44.49; H, 5.72.

S(+)-*N*,*N*-Dimethyl-*O*-acetylmandelamide—This compound was prepared from S(+)-mandelic acid as described previously, m.p. 98–99°; $[\alpha]^{25^\circ}_{589.3\ nm.}$ +192° (c 0.4, CH_3OH).

Anal.—Calc. for $C_{13}H_{15}NO_3$: C, 65.14; H, 6.84. Found: C, 65.16; H, 6.85.

S(+)-*N*,*N*-Dimethyl-1-phenylethanolamine—This compound was prepared from the S(+)-mandelamide derivative as described previously, b.p. 58–60°/0.15 mm. Hg; $[\alpha]^{25^\circ}_{589.3\ nm.}$ +64.8° (c 2.2, CH_3OH); n^{25}_D 1.5171; $\nu^{mull}_{max.}$ 3430 cm.$^{-1}$, broad (OH).

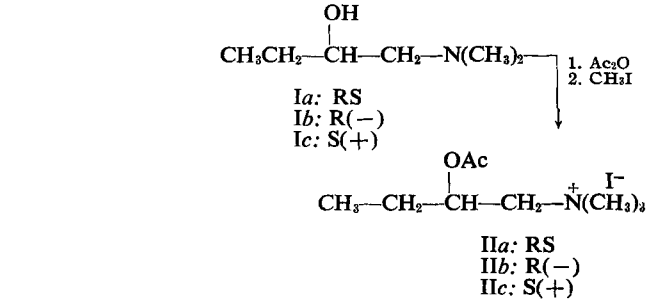
Anal.—Calc. for $C_{10}H_{15}NO_2$: C, 72.69; H, 9.15. Found: C, 72.89; H, 9.44.

S(+)-Acetyl β-Phenylcholine Iodide—This compound was prepared from S(+)-*N*,*N*-dimethyl-1-phenylethanolamine as described previously, m.p. 195–196.5°; $[\alpha]^{25^\circ}_{589.3\ nm.}$ +66.5° (c 1.0, H_2O).

Anal.—Calc. for $C_{13}H_{20}INO_2$: C, 44.71; H, 5.77. Found: C, 44.47; H, 5.72.

Pharmacology—The muscarinic potency of each compound in relation to acetylcholine was determined by a bioassay on the isolated guinea pig ileum. The ileum was suspended in Krebs–Henseleit solution, aerated with 95% O_2–5% CO_2, and maintained at 37°. Acetylcholine and the unknown drug were tested at minimal, medium, and submaximal concentrations as determined prior to the actual assay. The compounds were run on separate days using different guinea pigs for each experiment. A 3 × 3 bioassay was used for the determinations.

Enzymology—Enzyme-catalyzed hydrolysis of the compounds and inhibition of acetylcholine perchlorate hydrolysis by eel acetylcholinesterase were determined at pH 7.5 by titration of the liberated acetic acid with NaOH solution (0.0005 *N*) using a Sargent pH-Stat. Concentrations of substrate varying from 1.0 to 5.0 × 10^{-4} *M* were used in a medium consisting of 0.02 *M* $MgCl_2$ and 0.1 *M* NaCl, and 0.25 unit of eel acetylcholinesterase for the kinetic determinations. The enzyme was diluted just prior to use by a solution mixture of 0.02 *M* $MgCl_2$, 0.1 *M* NaCl, and 0.005% bovine serum albumin. Inhibitor concentrations were 3.0 × 10^{-4} *M*. The reaction rates were measured at 25° for 5 min. The rate was linear. A graphic plot of *S*/*V* *versus* *S* provided K_m and K_i values. Comparisons of hydrolysis rates were carried out at a 5.0 × 10^{-4} *M* substrate concentration over the same reaction period as already described.

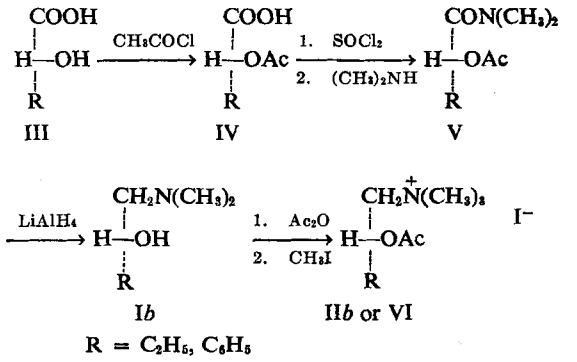


Scheme I

RESULTS AND DISCUSSION

Racemic 1-dimethylamino-2-butanol (I*a*) was prepared from 1-nitro-2-butanol. The (+)- and (−)-isomers of 1-dimethylamino-2-butanol (I*b* and I*c*, respectively) were resolved with (+)-tartaric acid and (+)-3-bromocamphorsulfonic acid, respectively, according to the methods of Major and Bonnett (8) for resolving 2-dimethylamino-1-propanol. The racemic and optically active aminoalcohols (I*a*, I*b*, and I*c*) were converted to the corresponding acetyl β-ethylcholine iodides (II*a*, II*b*, and II*c*) (Scheme I).

(−)-Acetyl β-ethylcholine (I*b*) was related to R(−)-2-hydroxybutyric acid (III: R = C_2H_5) by the stereospecific route shown in Scheme II. The assignment of the configuration of R(−)-2-hydroxybutyric acid was established by Levene and Haller (10) relating it to R(−)-lactic acid. Compound III was obtained by resolution of the racemic acid through its morphine salt. Compound III was acetylated to give R(+)-2-acetoxybutyric acid (IV: R = C_2H_5) which, when treated with thionyl chloride and then dimethylamine, gave R(+)-*N*,*N*-dimethyl-2-acetoxybutyramide (V: R = C_2H_5). Lithium aluminum hydride reduction of V gave R(−)-1-dimethylamino-2-butanol (I*b*), which was acetylated and treated with methyl iodide to give R(−)-acetyl β-ethylcholine iodide (II*b*); this compound was identical to the quaternary acetate obtained from the resolution of 1-dimethylamino-2-butanol with (+)-tartaric acid. (+)-1-Dimethylamino-2-butanol (I*c*), having the



Scheme II

same optical rotation but in the opposite direction, was assigned the S-configuration. It follows then that (+)-acetyl β-ethylcholine iodide (IIc) be assigned the S-configuration. The optically active R(−)-aminoalcohol was also used to prepare the quaternary alcohol derivative.

The synthesis of the enantiomers of acetyl β-phenylcholine (VI: R = C_6H_5) was carried out from the corresponding optically active mandelic acids, as described in Scheme II; *i.e.*, R = C_6H_5. S(+)-and R(−)-Mandelic acids gave S(+)-and R(−)-acetyl β-phenylcholine, respectively.

The results of the experiments in which the potency of the enantiomers of acetyl β-ethylcholine and acetyl β-phenylcholine iodide were determined in relation to acetylcholine bromide on guinea pig ileum are shown in Table I. The S(+)-acetyl β-ethylcholine and S(+)-acetyl β-phenylcholine enantiomers showed 44 and 100 times, respectively, greater muscarinic potency than their corresponding optical antipodes. This finding is consistent with the stereoselectivity by muscarinic receptors for S(+)-muscarine (11) and S(+)-acetyl β-methylcholine (1, 3) over their enantiomers. Although racemic acetyl β-phenylcholine did not give measurable results at the concentration levels used in the 3 × 3 assay, a very large concentration (1000 mcg./ml.) caused a 44-mm. contraction of guinea pig ileum. A 1000-mcg./ml. dose of R(−)-acetyl β-phenylcholine produced only a 27-mm. contraction on the same tissue. In all cases, the chemicals were found not to interfere with subsequent exposure of the tissue to acetylcholine.

Each enantiomer was examined as a substrate and inhibitor of acetylcholinesterase. The results of these studies are also presented in Table I. While none of the enantiomers showed any significant substrate activity during the 5-min. interval, both R(−)-acetyl β-ethylcholine and R(−)-acetyl β-phenylcholine were competitive inhibitors of acetylcholinesterase. The competitive inhibition can be interpreted as receptor affinity by these molecules (see Eqs. 1–5). The dissociation constant, K_s, for acetylcholine and eel acetylcholinesterase at pH 7.5 and 25° is 2.6×10^{-4} (12). The K_I values of 3.1×10^{-4} and 6.3×10^{-4} determined for the β-ethyl and β-phenyl compounds, respectively, indicate a favorable comparison of affinity for these inhibitors with acetylcholine for the active site on acetylcholinesterase. Both the S(+)-β-ethyl and S(+)-β-phenyl enantiomers showed some substrate activity, but no inhibition of acetylcholinesterase was observed. As expected, the racemic compounds had lower hydrolysis rates than the corresponding S(+)-isomers.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \qquad \text{(Eq. 1)}$$

$$K_s = \frac{k_{-1}}{k_{+1}} \qquad \text{(Eq. 2)}$$

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \qquad \text{(Eq. 3)}$$

$$K_I = \frac{k_{-1}}{k_{+1}} \qquad \text{(Eq. 4)}$$

$$\text{affinity} = \frac{k_{+1}}{k_{-1}} \qquad \text{(Eq. 5)}$$

A comparison of the enzymatic and muscarinic activity of the enantiomers of acetyl β-ethylcholine and acetyl β-phenylcholine shows that the muscarinic receptor of guinea pig ileum and the receptor area of eel acetylcholinesterase each best accommodates one isomer of an enantiomeric pair. The area between the esteratic and anionic sites of the two receptor areas differs such that the muscarinic site accommodates the S-configuration and the enzyme receptor accommodates the R-configuration of β-substituted acetylcholines, as described previously by Cocolas *et al.* (3).

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Antimicrobial Properties of Natural Phenols and Related Compounds II: Cinnamylated Phenols and Their Hydrogenation Products

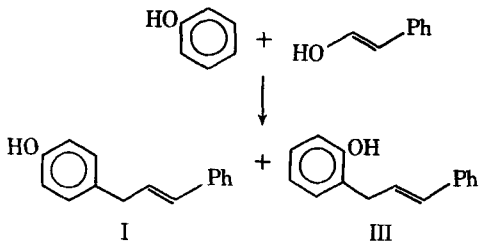
L. JURD, K. L. STEVENS, A. D. KING, Jr., and K. MIHARA*

Abstract □ A variety of nuclear alkylated *C*-cinnamylphenols were synthesized and tested for antimicrobial activity. These compounds are particularly effective against Gram-positive bacteria.

Keyphrases □ Cinnamylphenols and hydrogenation products—synthesized as possible antimicrobial agents, screened □ Antimicrobial agents, potential—synthesis of cinnamylphenols and hydrogenation products, pharmacological screening □ Phenols, cinnamylated and hydrogenation products—synthesized as possible antimicrobial agents, screened

It was recently reported (1) that obtusastyrene, a phenolic constituent of *Dalbergia sisoo* (2), is a potent, natural fungicide, the presence of it and a number of related compounds possibly being responsible for the high resistance of the heartwood of this species to fungal attack. Obtusastyrene completely inhibits the growth *in vitro* of a variety of Gram-positive bacteria, yeasts, and molds; in this respect, its activity compares favorably with that of a number of synthetic phenolic preservatives in current use.

The structure of obtusastyrene was established as *trans*-4-cinnamylphenol. Since, with the exception of obtusastyrene, the antibacterial and antifungal properties of *C*-cinnamylated phenols had not been examined previously, a variety of nuclear alkylated analogs of obtusastyrene was synthesized and screened *in vitro* for their effects on the growth of microorganisms.



Scheme I

MATERIALS AND METHODS

Cinnamylation of Phenols: General Procedure—Obtusastyrene (I) is easily synthesized in quantity by the acid-catalyzed condensation of cinnamyl alcohol with phenol in aqueous solutions (3, 4). The isomeric 2-cinnamylphenol (III) is formed as a minor product in this reaction (Scheme I).

o-, *m*-, and *p*-Cresol, thymol, carvacrol, and other 4-alkylphenols were cinnamylated under similar conditions to yield the condensation products listed in Table I. Separation of pure products was effected by distillation and, where applicable, by repeated crystallization. Identity of all products was confirmed by elemental analyses and by determination of their NMR spectra. In this connection, it is noteworthy that the NMR spectra of the 1,3-substituted propene portion of the cinnamylated phenols may be used to estab-

Table I—Cinnamylated Phenols and Their Hydrogenation Products

Reactant Phenol	Compound	Product: Identity	Molecular Formula	Boiling Point	Melting Point
Phenol	I	4-Cinnamylphenol (obtusastyrene)	$C_{15}H_{14}O$	169°/0.4 mm.	64°
	II	Dihydro-4-cinnamylphenol	$C_{15}H_{16}O$	—	63–64°
	III	2-Cinnamylphenol	—	—	57°
	IV	Dihydro-2-cinnamylphenol	—	—	—
*p*Cresol	V	2-Cinnamyl-4-methylphenol	$C_{16}H_{16}O$	170°/0.4 mm.	57°
	VI	Dihydro-2-cinnamyl-4-methylphenol	$C_{16}H_{18}O$	155°/1.0 mm.	—
o-Cresol	VII	4-Cinnamyl-2-methylphenol	$C_{16}H_{16}O$	—	71°
	VIII	Dihydro-4-cinnamyl-2-methylphenol	$C_{16}H_{18}O$	165°/0.2 mm.	—
	IX	2-Cinnamyl-6-methylphenol	$C_{16}H_{16}O$	147–148°/0.2 mm.	—
m-Cresol	X	4-Cinnamyl-3-methylphenol + 2-cinnamyl-5-methylphenol	$C_{16}H_{16}O$	150–170°/0.05 mm.	—
4-Ethylphenol	XI	2-Cinnamyl-4-ethylphenol	$C_{17}H_{18}O$	—	70–70.5°
	XII	Dihydro-2-cinnamyl-4-ethylphenol	$C_{17}H_{20}O$	—	29–30°
4-*n*-Propylphenol	XIII	2-Cinnamyl-4-*n*-propylphenol	$C_{18}H_{20}O$	—	38–40°
	XIV	Dihydro-2-cinnamyl-4-*n*-propylphenol	$C_{18}H_{22}O$	—	—
4-Isopropylphenol	XV	2-Cinnamyl-4-isopropylphenol	$C_{18}H_{20}O$	—	59–62°
	XVI	Dihydro-2-cinnamyl-4-isopropylphenol	$C_{18}H_{22}O$	—	—
2,4-Dimethylphenol	XVII	2-Cinnamyl-4,6-dimethylphenol	$C_{17}H_{18}O$	187°/1.0 mm.	—
Thymol	XVIII	4-Cinnamyl-3-methyl-6-isopropylphenol	$C_{19}H_{22}O$	175°/0.03 mm.	—
	XIX	Dihydro-4-cinnamyl-3-methyl-6-isopropylphenol	$C_{19}H_{24}O$	170°/0.05 mm.	—
	XX	2-Cinnamyl-3-methyl-6-isopropylphenol	$C_{19}H_{22}O$	175°/0.03 mm.	—
Carvacrol	XXI	4-Cinnamyl-2-methyl-5-isopropylphenol	$C_{19}H_{22}O$	185°/0.05 mm.	—
4-Methoxyphenol	XXII	2-Cinnamyl-4-methoxyphenol	$C_{16}H_{16}O_2$	213°/2.4 mm.	90°
	XXIII	Dihydro-2-cinnamyl-4-methoxyphenol	$C_{16}H_{18}O_2$	—	66°

Table II—Effects of Cinnamylated Phenols and Their Dihydro Derivatives on Growth of Bacteria and Fungi[a]

	Bacteria									Fungi													
Compound	*Bacillus cereus* 2006	*Sarcina lutea*	*Staphylococcus aureus* SG8A	*Streptococcus lactis*	*Acaligenes faecalis* B170	*Escherichia coli* ML30	*Pseudomonas aeruginosa* III	*Salmonella typhimurium* Tm1	*Serratia marcescens*	*Zygosaccharomyces japonicus* C124	*Candida tropicalis* C147	*Pichia chodatii*/var. fermentans C238	*Hansenula anomala*	*Saccharomyces cerevisiae*/var. ellipsoideus Y44	*Torula utilis* NRRL Y660	*Aspergillus flavus* NRRL 3145	*Aspergillus niger* A-7705	*Penicillium chrysogenum* 52	*Rhizopus senti* NRRL 2868	*Botrytis cinerea* NRRL 3492	*Byssochlamys fulva* NRRL 3493	*Alternaria* sp.
I	25	25	25	25	50–100	50–100	–	–	–	12	12	25	50	25	50	100	50	50	6	25–50	25	25
II	25	25–50	25–50	25	200	200	200	200	200	25	25	50	50	25	25	200	50–100	25	50	12–25	50	25
III	12	25	25	25	100	100	–	–	–	12	12–25	50	50	25	50	100	100	25–50	50	12–25	25	25
IV	12	25	25	12	–	–	–	–	–	12	12	50	25	12	25	50	50	25	25	12	25	25
V	12	12	12	12	–	–	–	–	–	12	25	>200	50	25	>200	>200	>200	100	>200	25	25	100
VI	12	12	12	12	–	–	–	–	–	12	12	–	25		12	>200	>200	200	100	>200	>200	100
VII	12	12	12	12	–	–	–	–	–	12	25	200	100	25	100	>200	>200	50	50	100	25	50–100
VIII	12	12	12	12	–	–	–	–	–	12	12	>200	100	12	>200	200	>200	25	50	25	25–50	25–50
IX	12	12	12	12	–	–	–	–	–	25	25	–	–	25	>200	>200	>200	>200	>200	200	>200	>200
X	12	25	25	12	–	–	–	–	–	12	12	200	–	>200	200	200	100	50	50	100	100	100
XI	12	12	12	12	–	–	–	–	–	12	12	–	–		–	–	>200	200	100	200	200	200
XII	12	12	12	12	–	–	–	–	–	12–25	12–25	–	–	50	100	–	–	>200	–	100–200	>200	>200
XIII	12	12	12	12	–	–	–	–	–	>200	50–100	–	–	–	–	>200	>200	–	>200	–	–	–
XIV	25	12	12	12	–	–	–	–	–	200	200	–	–	–	–	–	–	–	–	–	–	–
XV	12	12	12	12	–	–	–	–	–	25–50		–	–	–	–	–	–	–	200	200	–	–
XVI	12	25	12–25	12	–	–	–	–	–	50	12	–	–	–	–	>200	>200	–	>200	100	–	–
XVII	12		12	12	–	–	–	–	–	12	12	–	–	–	–	–	–	–	–	–	–	–
XVIII	±	–	+	+	–	–	–	–	–	±	–	–	–	–	–	–	–	–	–	+	–	±
XIX	+	+	+		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
XX	±	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–	±	+	+
XXI	+	+	+	++	–	–	–	–	–													
XXII	25	25	25	25	–	–	–	–	–	25	50	>200	100	50	>200	–	–	>200	–	>200	50	100–200
XXIII	13	12	25–50	25	–	–	–	–	–	25	25	–	50	25	200	–	–	>200	100	200	200	200
															–	200		–	–			

[a] Numbers refer to approximate minimal inhibitory concentrations in micrograms/milliliter. >200 = growth not inhibited with 200 mg./ml. (highest concentration tested). + = complete inhibition of growth with 500 mcg./ml. These compounds were not tested at lower concentrations. – = no growth inhibition with 500 mcg./ml. ± = slight growth with 500 mcg./ml.

lish the location of the cinnamyl group relative to the phenolic hydroxyl group. Thus, it was found that the methylene doublet (J = 5 Hz.) occurs in the region δ 3.40–3.44 when it is *para* to the hydroxyl (as in obtusastyrene, I) and in the region δ 3.46–3.57 when it is *ortho* to the hydroxyl (as in Compounds III and V). Only one exception to this generalization was noted—*viz.*, in Compound XXI the methylene doublet occurs at δ 3.50. The position of the low multiplet due to the vinyl proton adjacent to the allylic methylene group is also characteristic. The methine proton in those compounds in which the cinnamyl group is *para* to the hydroxyl occurs in the region δ 6.16–6.24, while in the *ortho*-cinnamyl phenols it occurs between δ 6.25 and 6.30.

The cinnamylation of *o*-cresol illustrates the general reaction procedure: a solution of *o*-cresol (108 g.) and cinnamyl alcohol (68 g.) in formic acid (200 ml.) and water (80 ml.) was heated on a steam bath for 30 min. Distillation of the oily product, which separated upon addition of water (800 ml.), gave a fraction, b.p. 163–168°/0.2 mm. (83 g.). This fraction was dissolved in ether (60 ml.) and slowly diluted at boiling with Skellysolve F (600 ml.). Colorless needles, m.p. 71°, separated on cooling. Recrystallized from ether–Skellysolve F, pure 4-cinnamyl-2-methylphenol (7) was obtained as colorless prismatic needles, m.p. 71° [lit. (5) m.p. 71–72°].

Anal.—Calc. for $C_{16}H_{16}O$: C, 85.7; H, 7.19. Found: C, 85.6; H, 7.27.

Chromatography of the ether–Skellysolve F filtrate showed the presence of residual 4-cinnamyl-2-methylphenol and a second minor reaction product. The ether–Skellysolve F solution was extracted with 10% aqueous KOH (3 × 100 ml.), which preferentially removes the 4-cinnamyl compound, until chromatography of the ether–Skellysolve F solution showed the presence of only the minor product. Evaporation and distillation of the solution then gave 2-cinnamyl-6-methylphenol (IX) as a colorless oil, b.p. 147–148°/0.2 mm. (4.1 g.).

Anal.—Calc. for $C_{16}H_{16}O$: C, 85.7; H, 7.19. Found: C, 85.4; H, 7.24.

Hydrogenation of Cinnamylphenols—The *trans*-cinnamylphenols (10 g.) in tetrahydrofuran (50 ml.) were converted into the saturated dihydro derivatives by hydrogenation at room temperature and approximately 30 lb. pressure in the presence of a 5% palladium–carbon catalyst. The dihydro derivatives were purified by distillation under reduced pressure or by crystallization.

Antimicrobial Bioassay—All compounds were initially tested at a concentration of 200 or 500 p.p.m. (w/v). Plates were prepared by adding a measured amount of the phenol in acetone solution to 10 ml. of sterilized medium, mixing thoroughly, pouring into 60 × 15-mm. plastic petri dishes, and allowing the gel to set and age overnight. The plates were then inoculated with the test organisms. In the case of bacteria and yeasts, the inoculation was done by the Lederberg and Lederberg (6) replica plating technique, applying nine bacteria or seven yeasts on each plate. In the case of molds, drops of homogenized culture were placed on the surface of the plates, applying three or four molds per plate. The media used were: plate count agar (Difco), pH 7.0, for bacteria; and potato dextrose agar (Difco), pH 5.6, for yeasts and molds. Inoculated control plates were also prepared with media containing the largest amount of acetone (0.80 ml.) used in the preparation of the test plates. We have determined that acetone has a minimal influence on the growth of organisms in this procedure. The plates were incubated at 28° for 1–5 days and evaluated by comparison with the controls.

RESULTS AND DISCUSSION

The *trans*-cinnamylphenols and their dihydro derivatives were screened initially at the high concentration (500 mcg./ml.) for activity against 22 representative microorganisms by the Lederberg and Lederberg (6) replica plating technique. The minimal concentrations of active compounds required for complete inhibition of microbial growth were then determined (Table II). A number of di-*C*-cinnamylphenols, as well as various derivatives (acetates, benzoates, and methyl ethers) of the mono-*C*-cinnamylphenols, showed no activity at high concentrations and are not included in the table.

As indicated in Table II, the antimicrobial activities of 2-cinnamylphenol (III) and its dihydro derivative (IV) are comparable with those of obtusastyrene (I) and dihydroobtusastyrene (II). All four compounds strongly inhibit the growth of Gram-positive bacteria (*Bacillus cereus*, *Sarcina lutea*, *Staphylococcus aureus*, and *Streptococcus lactis*) at concentrations of about 25 mcg./ml. and of 11 species of yeasts and molds at concentrations of 12–50 mcg./ml. Higher concentrations (50–100 mcg./ml.) were required for inhibition of growth of *Aspergillus* species. These compounds are generally ineffective against Gram-negative bacteria, although at higher concentrations (200 mcg./ml.) dihydroobtusastyrene (II) completely inhibits the growth of all five species of Gram-negative bacteria examined.

In considering the effect of nuclear alkylation on the antimicrobial properties of the cinnamylphenols (I and III) and their dihydro derivatives, it is apparent from Table II that the introduction of methyl, ethyl, and propyl groups (Compounds V–XVII) enhances the activity of these compounds against Gram-positive bacteria (growth inhibition of all four species occurs with 12 mcg./ml. or less). However, with the exception of their inhibitory effect on the yeasts *Zygosaccharomyces japonicus* and *Candida tropicalis*, nuclear alkylation markedly lowers their activity against fungi.

Studies in progress indicate that the action of the cinnamylcresols (Compounds V and VII) is bactericidal, and it is rapidly exerted against *S. aureus* and other Gram-positive organisms.

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Monitoring Tablet Dissolution with a Sodium Ion-Selective Glass Electrode

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Abstract □ The usefulness of the sodium ion-selective electrode (SIE) in measuring the disintegration and dissolution of tablets containing sodium salicylate was evaluated. This potentiometric measurement of sodium appearance in solution was found to agree with spectrophotometric assays for salicylate and sodium in solution. The SIE employed in this manner provides an accurate, inexpensive, and readily automated methodology for following tablet disintegration and dissolution.

Keyphrases □ Sodium ion-selective glass electrode—monitoring tablet dissolution □ Dissolution, monitoring tablets—use of sodium ion-selective electrode □ Potentiometric measurement of sodium—monitoring tablet dissolution

In recent years, a number of ion-selective electrodes were introduced and are now commercially available. These electrodes make it possible to measure the concentration of sodium, potassium, calcium, and other ions potentiometrically (1). These electrodes have many possible applications in pharmaceutics which have not been explored. One application is the monitoring of tablet dissolution when the tablet contains an ion to which the electrode will respond selectively. The advantages of a potentiometric measurement over the commonly employed spectrophotometric methods are twofold. First, the electrode may be placed in the dissolution media where it continuously measures ion concentration as a function of time. This eliminates the need for periodic sample collection or circulation of the sample for measurement outside the dissolution chamber. Second, the potentiometric method presents an alternative analytical technique when the spectrophotometric methods are limited by interferences or the drug does not absorb in the visible or UV spectrum. In this article, the utility of a sodium ion-selective electrode (SIE) in following the dissolution of tablets containing sodium salicylate is evaluated.

APPARATUS

The tablet dissolution apparatus employed was the same as described by Levy and Hayes (2). Measurement of sodium-ion activity was accomplished with a sodium-ion electrode[1], with a saturated calomel electrode[2] as reference. The electrode potential was measured with a pH meter[3] operated in the expanded millivolt position, with the signal relayed to a recorder[4]. All dissolution apparatus and calibrating solutions were maintained at 37 ± 1° by a water bath[5].

EXPERIMENTAL

The SIE was employed in its operable pH range of 7–10, and a 10^{-3} *M* sodium-ion concentration was included as a blank in all of the buffered solutions. In this manner, the pH was always at least four units higher than the pNa, as recommended by the manufacturer (3). The SIE was routinely calibrated before and after each tablet dissolution study; the potential drift was found to be insignificant. After the initial calibration, the electrode was stabilized in the dissolution media, a tablet was added, and the sodium concentration was followed as a function of time.

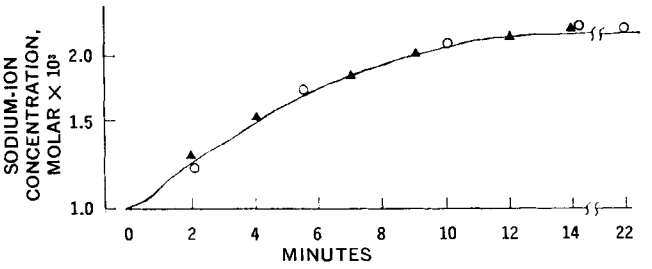

Figure 1—*Electrode response during tablet dissolution* (——). *Key:* ▲, *spectrophotometric salicylate assay; and* ○, *atomic absorption assay for* Na^+; *37 ± 1°, pH = 9.0.*

The reliability of the SIE in measuring the appearance of sodium ions in solution was checked three ways. Simulated tablet dissolutions were run by pumping concentrated sodium salicylate solutions (0.5–1.0 *M*) into the dissolution apparatus under conditions identical to the tablet dissolution studies, and the electrode response was recorded. These solutions were pumped at precisely determined rates (0.3–1 ml./min.) by a peristaltic pump[6] through 17-gauge Teflon tubing to the position in the apparatus where a tablet would have been placed. Two independent analytical techniques were used to evaluate the response of the SIE during actual tablet dissolution studies. For each technique, 1-ml. samples were drawn from the dissolution media in the proximity of the SIE by pipeting through a pledget of glass wool. Suitable dilutions of these samples were assayed for total sodium concentration using an atomic absorption spectrophotometer[7]. The salicylate concentration of the samples was determined at pH 9 using a spectrophotometer[8] at 296 nm.

All sodium salicylate tablets employed in this study were prepared in these laboratories from the same batch numbers of raw materials and under identical tableting conditions. Each tablet contained approximately 50 mg. sodium salicylate, 100 mg. lactose, 345 mg. cornstarch, and 5 mg. stearic acid.

RESULTS AND DISCUSSION

In all work using the SIE, it is necessary to calibrate the electrode in the range of sodium-ion concentration expected from the tablet dissolution. Since the manufacturer recommends a pH range of 7–10 and a pH at least four units greater then the highest pNa (2), a base concentration of sodium was established at 1×10^{-3} *M*. All work was done in this pH range and at a sodium concentration between 1×10^{-3} and 3×10^{-3} *M*. The electrode was found to respond linearly with concentration in this range, with no drift from the established calibration during the maximum time necessary for a tablet dissolution. It was also found that when changing the pH of the system, minimum electrode response time and maximum stability could be ensured if the electrode was immersed overnight in the medium to be used.

[1] Beckman No. 39278.
[2] Coleman No. 3-152.
[3] Corning model 12.
[4] Varian G 1000.
[5] Blue M model MW 1120.
[6] Harvard model 1201.
[7] Perkin-Elmer 290 B.
[8] Beckman DU.

Table I—Time–Dissolution Comparison for Sodium Salicylate Tablets by Three Methods[a]

Minutes	Concentration, m*M*		
	SIE	Atomic Absorption	UV Spectrophotometer
2	0.300	0.300	0.350
4	0.580	0.600	0.625
7	0.850	0.860	0.830
10	0.95	1.00	1.15
14	1.25	1.30	1.34
22	1.20	1.40	1.37

[a] All assays carried out with phosphate buffer at pH 9 and at 37°.

The equilibration time of electrode response to changes in sodium-ion concentration was determined by pumping a concentrated sodium salicylate solution into the beaker under conditions of tablet dissolution. It was found that the electrode-recording system follows the change in sodium concentration even at rates more than twice those observed during actual tablet dissolution.

To control extraneous sodium content, the initial tablet dissolutions were carried out using compressed tablets manufactured in these laboratories. Identical tablet dissolutions were recorded in systems buffered at pH 7.5 and 9.0, and no significant change in drug release was noted.

Figure 1 shows the change in electrode potential for the dissolution of a sodium salicylate tablet. Since the tableting procedure introduces some inherent variation in the individual characteristics of each tablet, it was necessary to ascertain that the recorded variation was indeed due to the tablet and not to the analytical method. This was accomplished by correlation with results from atomic absorption spectrophotometry and UV spectrophotometric analysis. Table I shows the values obtained by each of the three assay methods. Note the agreement of molar concentrations determined by the SIE system and the atomic absorption method. Both of these methods assay for sodium ions. The UV spectrophotometric method assays for salicylate content and, within experimental variation, confirms the sodium-ion data.

In summary, the SIE with attached recording devices provides a means of measuring tablet dissolution that is accurate and relatively inexpensive. It also provides a continuous direct readout of drug appearance without the disadvantages incurred by repeated sampling and was shown to be as reproducible and accurate as either of the spectrophotometric methods used.

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Synthesis and Pharmacological Evaluation of 1,4-Dihydro-2*H*-3,1-benzoxazin-2-one Derivatives

FRANCO De MARCHI, GIAN FRANCO TAMAGNONE, and MARIO V. TORRIELLI

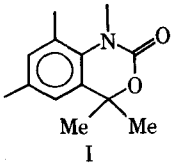
Abstract □ Some 1,4-dihydro-2*H*-3,1-benzoxazin-2-one derivatives were synthesized and tested for pharmacological activity. 4-Phenyl-6-chloro-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 4) exhibited anticonvulsant activity against chemically and electrically induced seizures and low acute toxicity in mice. Structure–activity relationships are discussed.

Keyphrases □ 1,4-Dihydro-2*H*-3,1-benzoxazin-2-one derivatives—synthesis as possible anticonvulsants, screened for pharmacological activity, structure–activity relationships □ Anticonvulsants, potential—synthesis and pharmacological screening of 1,4-dihydro-2*H*-3,1-benzoxazin-2-one derivatives, structure–activity relationships □ 3,1-Benzoxazin-2-one derivatives—synthesis and pharmacological screening as possible anticonvulsants, structure–activity relationships

3,1-Benzoxazin-2-ones are relatively new potential therapeutic agents. A number of derivatives of Structure I were recently reported by Bernardi *et al.* (1) to have anticonvulsant activity. A series of structurally related 1,3-benzoxazin-2-ones was described by the same authors (2), among which 2,3-dihydro-4*H*-1,3-benzoxazin-2-one-3-acetamide (II) showed antireserpine activity (3–5). Some 4-phenyl-substituted-3,1-benzoxazin-2-ones were also synthesized (6, 7), but apparently no pharmacological data were reported. The present paper deals with the synthesis and pharmacological evaluation of the 1,4-dihydro-2*H*-3,1-benzoxazin-2-one derivatives listed in Table I.

CHEMISTRY

N-Alkylation of the known 1,4-dihydro-2*H*-3,1-benzoxazin-2-one with 2-bromoacetamide in the presence of sodium hydride gave Compound 1. Benzoxazinones 2, 4, and 8 were more conveniently obtained by sodium borohydride reductive cyclization of the corresponding 2-trichloroacetamidobenzophenones (8) than through basic cyclization of 2-trichloroacetamidobenzhydrols (8, 9) or by reaction of 2-aminobenzhydrols with phosgene (6, 7). *N*-Methylation of Compounds 2, 4, and 8 with methyl iodide in the presence of sodium hydride gave benzoxazinones 3, 5, and 9, respectively. Similarly, Compounds 6 and 7 were obtained from Compound 4 by alkylation with 2-bromoacetamide or ethyl bromoacetate.



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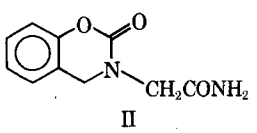

II

Table I—Time–Dissolution Comparison for Sodium Salicylate Tablets by Three Methods[a]

Minutes	Concentration, m*M*		
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2	0.300	0.300	0.350
4	0.580	0.600	0.625
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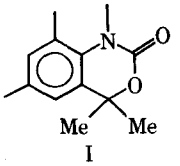
CHEMISTRY

N-Alkylation of the known 1,4-dihydro-2*H*-3,1-benzoxazin-2-one with 2-bromoacetamide in the presence of sodium hydride gave Compound 1. Benzoxazinones 2, 4, and 8 were more conveniently obtained by sodium borohydride reductive cyclization of the corresponding 2-trichloroacetamidobenzophenones (8) than through basic cyclization of 2-trichloroacetamidobenzhydrols (8, 9) or by reaction of 2-aminobenzhydrols with phosgene (6, 7). *N*-Methylation of Compounds 2, 4, and 8 with methyl iodide in the presence of sodium hydride gave benzoxazinones 3, 5, and 9, respectively. Similarly, Compounds 6 and 7 were obtained from Compound 4 by alkylation with 2-bromoacetamide or ethyl bromoacetate.



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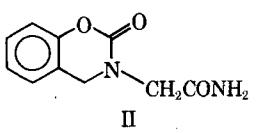

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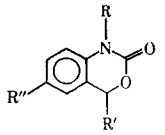

Table I—1,4-Dihydro-2*H*-3,1-benzoxazin-2-ones

Compound	R	R′	R″	Crystallization Solvent[a]	Yield, %[b]	Melting Point[c]	Formula	Analysis, %[d]	Calc.	Found
1	CH_2CONH_2	H	H	A	60	218–220°	$C_{10}H_{10}N_2O_3$	C	58.25	58.03
								H	4.89	4.86
								N	13.58	13.32
2	H	C_6H_5	H	B	63	179–181° dec.	$C_{14}H_{11}NO_2$	C	74.65	74.72
								H	4.92	4.85
								N	6.22	6.13
3	CH_3	C_6H_5	H	C	62	134–136°	$C_{15}H_{13}NO_2$	C	75.30	75.25
								H	5.48	5.61
								N	5.85	5.77
4	H	C_6H_5	Cl	D	65	191–193° dec.	$C_{14}H_{10}ClNO_2$		—	—
5	CH_3	C_6H_5	Cl	D	60	185–187° dec.	$C_{15}H_{12}ClNO_2$		—	—
6	CH_2CONH_2	C_6H_5	Cl	E–F	58	191–193° dec.	$C_{16}H_{13}ClN_2O_3$	C	60.67	60.84
								H	4.14	4.34
								Cl	11.18	11.60
								N	8.84	8.51
7	$CH_2COOC_2H_5$	C_6H_5	Cl	B–G	60	129–130°	$C_{18}H_{16}ClNO_4$	C	62.52	62.61
								H	4.66	4.65
								Cl	10.25	10.35
								N	4.05	3.74
8	H	C_6H_5	NO_2	H–I	53	225–227° dec.	$C_{14}H_{10}N_2O_4$		—	—
9	CH_3	C_6H_5	NO_2	J	63	165–167°	$C_{15}H_{12}N_2O_4$	C	63.38	63.68
								H	4.26	4.41
								N	9.86	9.76

[a] A, methanol; B, benzene; C, isopropyl alcohol; D, ethanol; E, water; F, acetone; G, cyclohexane; H, dioxane; I, hexane; and J, toluene. [b] Yields are of pure products. [c] Melting points were taken in capillary tubes sealed at one end with a partial immersion thermometer and are uncorrected. [d] Where no analytical data are reported, the synthesis of compounds was described elsewhere (8, 9).

PHARMACOLOGY

The experiments were performed on mice (male albino Swiss) or rats (male Wistar); animals were always treated intraperitoneally with benzoxazinones suspended in olive oil–5% acacia gum (1:1) (2.0 ml./100 g. body weight). The following dose levels were chosen on a logarithmic scale: 50, 100, and 200 mg./kg. Behavioral observations were carried out in mice according to the general screening procedure described by Irwin (10). The antagonism of the derivatives against chemically and electrically induced seizures in mice was studied. Strychnine (3 mg./kg. i.p.) and pentylenetetrazole (80 mg./kg. i.p.) were employed as chemical agonists. The activity of the tested compounds was evaluated as antagonism against strychnine death or inhibition of the clonic convulsions induced by pentylenetetrazole.

The maximal electroshock test was performed by an a.c. constant-current stimulator. A 150-v. current of 0.2-sec. duration was employed to deliver the shock. Prevention of tonic limb extension was considered as an index of anticonvulsant activity. The benzoxazinones were given 60 min. before the agonists. The ED_{50} was determined according to Miller and Tainter (11). Motor activity was evaluated in mice with a modified Dews apparatus (12). The animals were treated 30 min. before the recording, and motility was registered continuously for 7 hr. Analgesic and anti-inflammatory activities were evaluated by the hot-plate method (13, 14) (mice) and on carrageenin-induced edema in the rat hind paw (15). The acute toxicity was evaluated in mice, the observation being prolonged for 48 hr. The obtained results are summarized in Table II.

Benzoxazinones 2–5, 8, and 9 induced a moderate depression of the CNS, without influencing the rectal temperature in mice. The other derivatives were inactive. Tested for anticonvulsant properties, Compounds 2 and 4 antagonized both chemically and electrically induced seizures, the most active being Compound 4. Benzoxazinone 3 showed a detectable anticonvulsant activity against electroshock only. Compounds 4, 5, 8, and 9 slightly decreased spontaneous motor activity. The depression of motility was most pronounced with Compound 4. None showed analgesic or anti-inflammatory properties. Furthermore, a low acute toxicity of benzoxazinones resulted after intraperitoneal administration.

As regards the structure–anticonvulsant activity relationships, the last seems to be linked to the structure of 4-phenyl-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 2); 6-substitution with a chlorine atom (Compound 4) enhanced the activity, while its replacement with a nitro group (Compound 8) abolished it. *N*-Alkylation is detrimental for anticonvulsant action, because *N*-methyl derivatives 5 and 9, as well as the acetamide 6 and the ethyl ester 7, were practically ineffective against chemically or electrically induced convulsions; Compound 3 showed antielectroshock activity only. Moreover, in these experiments, Compound 1, the isomer of 1,3-benzoxazin-2-one (II) previously described (3–5), seemed to be devoid of any antireserpine activity, which is a peculiar feature of II.

Table II—Pharmacological Activities of 1,4-Dihydro-2*H*-3,1-benzoxazin-2-ones

Compound	Depression of CNS	Anticonvulsant Activity, ED_{50}, mg./kg. i.p. Antistrychnine	Anti-pentylenetetrazole	Antimax-shock	Motor Activity, mg./kg. i.p.	Acute Toxicity, LD_{50}, mg./kg. i.p.
1	I[a]	I	>200	I	I	>800
2	++[b]	140	105	135	I	>800
3	+[b]	>200	>200	143	I	>800
4	++	80	78	52	100↓[c]	>800
5	+	>200	>200	200	200↓	>800
6	I	>200	>200	200	I	>800
7	I	I	I	I	I	>800
8	+	I	>200	200	200↓	>800
9	+	I	>200	200	200↓	>800

[a] I = inactive. [b] ++ or + = more or less severe depression. [c] ↓ = decreased motility.

EXPERIMENTAL

Crystallization solvents, yields, melting points, and analyses of the benzoxazinones 1–9 are reported in Table I. IR spectra were recorded on a spectrophotometer[1]. Each analytical sample gave a single spot on TLC (silica gel G); the solvent systems used were: benzene–methanol (95:5 and 4:1), and chloroform–ethyl acetate–diethylamine (7:1:1 and 7:2:1).

1,4-Dihydro-2*H*-3,1-benzoxazin-2-one-1-acetamide (Compound 1)—To 6.0 g. (0.125 mole) of sodium hydride (50% mineral oil dispersion) in 600 ml. of anhydrous dimethylformamide, 18.6 g. (0.125 mole) of 1,4-dihydro-2*H*-3,1-benzoxazin-2-one (8) was added; the mixture was stirred 1.5 hr. at room temperature until a clear solution was obtained. After adding 20.0 g. (0.145 mole) of 2-bromoacetamide and stirring for 2 hr. at room temperature, the reaction mixture was extracted with hexane to remove the mineral oil, evaporated to dryness under reduced pressure, diluted with 1000 ml. of chloroform, washed with water, and dried (Na_2SO_4). Evaporation of the solvents gave crude Compound 1, which was purified by crystallization; IR (mineral oil) 3405, 3315, 3220 (NH_2), 1705 (NHCOO), and 1670 cm.$^{-1}$ ($CONH_2$).

2-Trichloroacetamidobenzophenone—To a stirred solution of 59.2 g. (0.30 mole) of 2-aminobenzophenone and 37.4 g. (0.37 mole) of triethylamine in 800 ml. of anhydrous benzene, 67.3 g. (0.37 mole) of trichloroacetyl chloride was added dropwise; the resulting solution was refluxed for 45 min. The reaction mixture was cooled; washed with 0.5 *N* NaOH, 0.5 *N* HCl, and water; and dried ($CaCl_2$). After removal of the solvent *in vacuo*, 88 5 g. (86%) of product was obtained and employed without further purification for cyclization to Compound 2. A pure sample, m.p. 99–101°, was obtained by crystallization from methanol; IR (mineral oil) 3275 (NH), 1718 ($NHCOCCl_3$), and 1640 cm.$^{-1}$ (CO).

Anal.—Calc. for $C_{15}H_{10}Cl_3NO_2$: C, 52.60; H, 2.94; Cl, 31.03; N, 4.09. Found: C, 52.72; H, 3.01; Cl, 31.11; N, 4.05

4-Phenyl-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 2)—To a stirred solution of 82.1 g. (0.24 mole) of 2-trichloroacetamidobenzophenone in 330 ml. of anhydrous dioxane, 11.4 g. (0.30 mole) of sodium borohydride was added; the suspension was stirred at room temperature for 6 hr. The reaction mixture was cooled to 0°, slightly acidified with 1 *N* HCl, and diluted with water to 2000 ml. The separated benzoxazinone, Compound 2, was washed with water, dried, and crystallized; IR (mineral oil) 3145, 3090 (NH), and 1703 cm.$^{-1}$ (CO).

4-Phenyl-6-chloro-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 4) and 4-Phenyl-6-nitro-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 8)—The products were synthesized substantially as described for Compound 2; a detailed description of their preparation was reported elsewhere (8).

4-Phenyl-1-methyl-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 3)—To a stirred solution of 20.0 g. (0.09 mole) of Compound 2 in 55 ml. of anhydrous dimethylformamide, 4.8 g. (0.10 mole) of sodium hydride (50% mineral oil dispersion) and 20.1 g. (0.14 mole) of methyl iodide were slowly added at 30–40°. After 45 min. at reflux, the reaction mixture was extracted twice with 100 ml. of warm isopropyl ether–hexane (1:1) and finally with 200 ml. of warm isopropyl ether–acetone (4:1). The solid which separated from isopropyl ether–hexane extracts and the residue obtained after evaporation of the solvents of the final extraction were combined and crystallized; IR (mineral oil) 1698 cm.$^{-1}$ (CO).

[1] Perkin-Elmer model 257.

4-Phenyl-6-chloro-1-methyl-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 5) and 4-Phenyl-6-nitro-1-methyl-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (Compound 9)—The method of synthesis was substantially the same as described for Compound 3; a detailed description of the preparation of Compound 5 was reported elsewhere (9).

4-Phenyl-6-chloro-1,4-dihydro-2*H*-3,1-benzoxazin-2-one-1-acetamide (Compound 6)—The product was synthesized substantially as was Compound 1, starting from benzoxazinone 4.

4-Phenyl-6-chloro-1,4-dihydro-2*H*-3,1-benzoxazin-2-one-1-acetic Acid Ethylester (Compound 7)—A solution of 41.4 g. (0.16 mole) of Compound 4 in 120 ml. of anhydrous dimethylformamide was treated with 8.6 g. (0.18 mole) of sodium hydride (50% mineral oil dispersion) and, dropwise, with 44.0 g. (0.26 mole) of ethyl bromoacetate. After 45 min. at reflux and extraction with hexane to remove the mineral oil, 300 ml. of chloroform and 150 ml. of water were added; the organic layer was separated, dried, and concentrated *in vacuo*. The oily residue, by treatment with ether, gave crude Compound 7, which was purified by crystallization; IR (mineral oil), 1752 (COOEt) and 1723 cm.$^{-1}$ (NHCOO).

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New Compounds: Symmetrically Substituted Tetraalkyl Methanes

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Abstract □ The syntheses of tetrakis(3-hydroxypropyl)methane, tetrakis(3-bromopropyl)methane, tetrakis(3-cyanopropyl)methane, and tetrakis(3-carboxypropyl)methane are described. An abnormal conversion of tetrakis(β-bromoethyl)methane to tris(β-iodoethyl)-β-bromoethylmethane is discussed.

Keyphrases □ Tetraalkyl methanes, symmetrically substituted—synthesis □ Tetrakis(3-hydroxypropyl)methane, tetrakis(3-bromopropyl)methane, tetrakis(3-cyanopropyl)methane, and tetrakis(3-carboxypropyl)methane—synthesis

During an investigation on polyquaternary salts, it was of interest to prepare and study the reactions of some symmetrically substituted tetraalkyl methanes. An examination of the literature (1) shows that there are only a few examples of these relatively simple compounds, mainly derivatives of pentaerythritol and compounds such as I. This series has been extended to the next higher homologs, II.

The synthetic route proceeded by the reduction of I (X = $COOC_2H_5$) to the corresponding tetraol (II, X = OH), which was then converted to the tetrabromide (II, X = Br). Exchange of the tetrabromide by tetranitrile (II, X = CN) followed by hydrolysis gave methane tetrabutyric acid (II, X = COOH).

An interesting observation was made in the attempted preparation of the corresponding tetraiodo compound from I (X = Br). By refluxing the tetrabromide with an excess of sodium iodide for various periods of time, only three bromine atoms were replaced. This is in contrast with pentaerythritol tetrabromide, which readily gave the tetraiodo compound (2). Attempts to convert the pentaerythritol tetrabromide to the tetranitrile have been unsuccessful over several years. However, the conversion of I (X = Br) to the tetranitrile proceeds in almost quantitative yield. In addition, the exchange of bromide in I (X = Br) for triethylamine at 90° for 24 hr. yielded only starting bromide.

EXPERIMENTAL[1]

Tetrakis(3-hydroxypropyl)methane (II, X = OH)—A solution of 10 g. (0.024 mole) of tetrakis(β-carboethoxyethyl)methane (1) in 100 ml. of ether was added dropwise to a solution of 10 g. of lithium aluminum hydride dissolved in 1 l. of ether, and the mixture was stirred for 24 hr. at room temperature. After decomposition of the reaction mixture with 40 ml. of water and filtering, the filter cake was extracted three times with boiling absolute alcohol. All filtrates were combined and evaporated. The solid, 4.2 g. (70.4%), when recrystallized from absolute alcohol and then from a methanol–ether mixture, gave m.p. 164–165°. An additional crystallization from water did not change the melting point.

$C(CH_2CH_2X)_4$ I

$C(CH_2CH_2CH_2X)_4$ II

X = OH, Br, CN, COOH

Anal.—Calc. for $C_{13}H_{28}O_4$: C, 62.87; H, 11.36. Found: C, 62.71; H, 11.35.

Tetrakis(3-bromopropyl)methane (II, X = Br)—To a mixture of the above polyol, 4.1 g. (0.0165 mole), and 100 ml. of 48% HBr was slowly added, with cooling, 50 ml. of sulfuric acid. The solution was heated on a steam bath overnight, diluted with water, and filtered, 8 g. (97%), m.p. 72–74°. The crude product was recrystallized from ethyl acetate–petroleum ether, m.p. 73–74°.

Anal.—Calc. for $C_{13}H_{24}Br_4$: C, 31.23; H, 4.84; Br, 63.93. Found: C, 31.23; H, 4.72; Br, 63.75.

Tetrakis(3-cyanopropyl)methane (II, X = CN)—A mixture of 7 g. (0.014 mole) of the tetrabromide, 150 ml. of acetonitrile, 20 ml. of water, and 15 g. of potassium cyanide was refluxed for 8 hr. The reaction mixture was separated from a small amount of salt layer and stripped of solvent. The residue was recrystallized from ethanol–acetonitrile, 3.7 g. (93%), m.p. 169–170°.

Anal.—Calc. for $C_{17}H_{24}N_4$: C, 71.79; H, 8.51; N, 19.70. Found: C, 72.02; H, 8.45; N, 19.58.

Tetrakis(3-carboxypropyl)methane (II, X = COOH)—The tetranitrile, 3.5 g. (0.0123 mole), was hydrolyzed with 130 ml. of concentrated HCl. As the reaction proceeded, the solution became clear; after continued refluxing, the product precipitated. After 8 hr. the solid was filtered, 3.5 g. (79%), and recrystallized from ethanol (500 ml.), m.p. 241–242°.

Anal.—Calc. for $C_{17}H_{28}O_8$: C, 56.66; H, 7.83. Found: C, 56.82; H, 8.05.

Tris(β-iodoethyl)-β-bromoethylmethane—A solution of 6 g. (0.0132 mole) of tetrakis(β-bromoethyl)methane dissolved in 600 ml. of acetonitrile was treated at reflux with 12 g. of sodium iodide dissolved in a minimal amount of water. The mixture was refluxed for 4 hr., filtered hot, diluted with 200 ml. of water, and allowed to crystallize. The crude product, 6.4 g. (82.9%), was twice recrystallized from ethyl acetate, m.p. 214–216°.

Anal.—Calc. for $C_9H_{16}BrI_3$: C, 18.05; H, 2.75; Br, 13.68; I, 65.09. Found: C, 18.35; H, 2.82; Br, 13.57; I, 65.41.

[1] Microanalyses were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. All melting points were obtained with a Thomas-Hoover capillary-type melting-point apparatus and are corrected. All compounds corresponded in structure to their IR spectra.

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Marijuana: Some Pharmacological Studies

Keyphrases ☐ Marijuana, effects—6-week pharmacological and toxicity study, rats ☐ Δ^9-Tetrahydrocannabinol, effects—6-week pharmacological and toxicity study, rats

Sir:

l-Δ^9-Tetrahydrocannabinol is the major psychoactive component of marijuana. Interest has developed in examining its effects in animals for the possible implications in humans. We wish to report studies on the pharmacology and toxicity of Δ^9-tetrahydrocannabinol in rats following repeated inhalation.

Δ^9-Tetrahydrocannabinol (10 mg.), prepared from the Δ^8-isomer as described in the literature (1), was dissolved in light petroleum ether (b.p. 30–60°) and injected into a half-length cigarette. The petroleum ether was allowed to evaporate, and the cigarettes were then mounted on wire at the bottom of large vacuum desiccators. Male Sprague-Dawley rats, 200–250 g., in groups of three were exposed to the smoke of Δ^9-tetrahydrocannabinol in the closed container every day for 10 min. excluding weekends for 6 weeks. A previous experiment using the same method with tritiated Δ^9-tetrahydrocannabinol had shown the retention of radioactivity in animals for 7 days after one exposure to the smoke (2).

Cardiovascular effects were evaluated by the measurement of blood pressure from the caudal artery with a physiograph. CNS activities were determined as the change in motor activity using an activity cage[1] and the time the animals were able to remain on a rotating rod. All pharmacological tests (six animals per group) were performed twice a week at least 18 hr. after an inhalation period to avoid the peak action of Δ^9-tetrahydrocannabinol (usually observable around 2–4 hr. after administration of the compound). Two control groups were used for comparison: one group inhaled the smoke from cigarettes without Δ^9-tetrahydrocannabinol and the other group were nonsmoking animals. The body weights of the three groups were also recorded weekly.

Animals were sacrificed at the end of the 6th week. Adrenals, brain, heart, kidneys, liver, lungs, testes, and spleen were carefully dissected and weighed to the milligram. All values were analyzed statistically using the Student *t* test. Slices of brain, kidney, liver, and lung were immediately fixed with 2% glutaraldehyde for histological studies. Plasma samples were obtained from the blood for determination of glutamic oxaloacetic transaminase and urea nitrogen.

[1] Metro Scientific Co.

A significant fall in blood pressure below that of both the cigarette control and nonsmoking animals was observed in rats that had repeatedly inhaled the smoke containing Δ^9-tetrahydrocannabinol for 5 and 6 weeks: 5th week, blood pressure (mm. Hg) 131.7 ± 6.06 (nonsmoking controls, A), 127.4 ± 4.18 (cigarette controls, B), 116.7 ± 9.83 (Δ^9-tetrahydrocannabinol-treated, C), no significant difference between A and B but a significant difference between B and C ($p < 0.05$) and A and C ($p < 0.01$); 6th week, 127.5 ± 2.74 (A), 124.2 ± 8.01 (B), 115.0 ± 6.32 (C), no significant difference between A and B, significant difference between B and C ($p < 0.10$) and A and C ($p < 0.001$). No significant change was noted before the 5th week.

Repeated inhalation of Δ-^{9}tetrahydrocannabinol resulted in a slightly increased motor activity of the animals at the final week of study. The increase was over both the cigarette control and nonsmoking groups ($p < 0.10$). The two control groups did not differ in motor activity. Although cigarette smoke impaired the animals' performance on the rotarod, no difference was observed between the cigarette- and Δ^9-tetrahydrocannabinol-treated animals.

When compared with the two groups of control animals, no weight variation in rats was detected during the 6 weeks of Δ^9-tetrahydrocannabinol inhalation. Nor did any variation occur in the weights of individual organs examined. Gross histological studies did not reveal any morphological changes due to the smoke containing Δ^9-tetrahydrocannabinol. Results from plasma glutamic oxaloacetic transaminase and urea nitrogen tests indicated no impairment of liver or kidney function. All these findings were obtained from only a 6-week study, and the results might be different if the length of exposure to Δ^9-tetrahydrocannabinol smoke was longer.

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Drug Concentration in Goldfish at the Pharmacologic End-Point

Keyphrases ☐ Goldfish, overturn—relationship between bathing fluid and body drug concentrations ☐ Drug concentration, goldfish—overturn as a function of bathing fluid concentration ☐ Critical drug concentration—4-aminoantipyrine-induced overturn, goldfish ☐ 4-Aminoantipyrine—goldfish overturn as a function of bathing fluid concentration

Sir:

Recently, a two-compartment reversible model describing the occurrence of a pharmacologic effect in goldfish as a function of drug concentration in the bathing solution was examined (1). The mathematical relationships derived from this model and the experimental results demonstrate that a critical concentration is necessary in the fish's body before a pharmacologic response will occur. This concentration was assumed to be constant, regardless of the drug concentration in the bathing solution. The purpose of the present study was to determine the amount of drug in the fish's body at the time of occurrence of the pharmacologic effect as a function of drug concentration in the bathing solution.

Goldfish, weighing 2.5–3.7 g. (mean 3.1 g.), were obtained locally, and the time for 4-aminoantipyrine-induced overturn was determined (1). When overturn occurred (or in one group of experiments after 24 hr.), the fish were immediately transferred to 1 l. of distilled water for exactly 2 min. This procedure was necessary to remove any drug solution adhering to the fish's body and to expel any fluids retained in the fish's mouth. After this 2-min. rinse, the fish were frozen and assayed as previously described (2). Drug recovery determinations were carried out by homogenizing the fish with either 0.05 or 0.1 mg. of drug and assaying (2). Five fish were used at each level, and an average recovery of 98% was obtained.

The reciprocal time–drug concentration plot is shown in Fig. 1. As predicted by the proposed model (1), curvature occurs at the lower concentrations and dilute solutions of 4-aminoantipyrine (5 mg.% and less) do not cause overturn (Table I). No difference in 4-aminoantipyrine content could be detected in the goldfish at any concentration causing overturn. Goldfish exposed to 5 mg.% 4-aminoantipyrine solutions for 24 hr. without overturn had a significantly lower body content of drug than those fish that overturned.

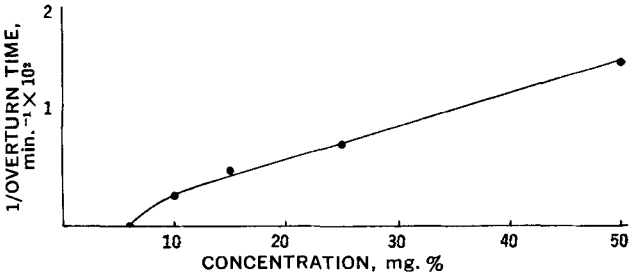

Figure 1—*4-Aminoantipyrine-induced overturn of goldfish as a function of concentration. (Data are from Table I.)*

The results of these experiments demonstrate that a critical concentration in the fish's body is necessary to induce a pharmacologic effect. This concentration appears to be independent of drug concentration in the bathing fluid over the range studied.

Table I—4-Aminoantipyrine Content in Goldfish at Overturn

Bathing Solution Concentration, mg. %	Number of Fish	Mean Overturn Time, min. ± *SD*[a]	Mean Drug Content, mg. Drug/g. Fish Tissue ± *SD*[b]
50	8	68.0 ± 1.8	0.071 ± 0.012
25	8	134.5 ± 11.1	0.066 ± 0.012
15	8	198.9 ± 25.1	0.068 ± 0.013
10	7	370.9 ± 30.3	0.065 ± 0.012
5	8	∞[c]	0.046 ± 0.005

[a] All significantly different from each other by Student's *t* test, $p < 0.05$. [b] 5 mg. % significantly different from all others by Student's *t* test, $p < 0.05$. No difference between 50, 25, 15, and 10 mg. %. [c] Greater than 24 hr.

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Formation of Coordinate Covalent Complex between Lithium and 8-Quinolinol

Keyphrases ☐ Lithium–8-quinolinol coordinate covalent complex—formation, fluorescence ☐ 8-Quinolinol–lithium coordinate covalent complex—formation, fluorescence ☐ Fluorometry—formation, analysis lithium–8-quinolinol complex

Sir:

Lithium ion reacts with 8-quinolinol to form a species whose fluorescence has been observed in water and in chloroform, and it has been suggested to represent the basis for a fluorometric determination of lithium (1). The fluorescence of the reaction product of lithium and 8-quinolinol has recently been under study in this laboratory. Our findings indicate that in water the lower limit of detection of lithium, by fluorometry, with 8-quinolinol ($\sim 5 \times 10^{-4}$ *M*) is not particularly impressive nor conducive to the employment of fluorometry as an analytical method of choice for lithium. However, the fact that a fluorescent product forms at all in the

reaction of Li^+ and 8-quinolinol is rather surprising and has some significance as far as the coordination chemistry of alkali metal ions in general and the neuropharmacology of lithium (2) in particular are concerned.

8-Quinolinol in dilute acid, neutral, and dilute basic aqueous solutions (those of concern here and in biological systems) is nonfluorescent. However, the excited cation and excited anion of 8-quinolinol show green fluorescence in concentrated acid (3) and concentrated base (4) solutions, even though the ground-state cation predominates below pH 5 and the ground-state anion is present above pH 10. In the pH range of 5–10, the predominant ground-state species derived from 8-quinolinol is the neutral phenolic species. The observation of fluorescence from 8-quinolinol only in concentrated acid or base solutions has been shown to be due to the attainment of prototropic equilibrium in the lowest excited singlet state between the cation and the zwitterion derived from 8-quinolinol (pK* −7) (3) and between the zwitterion and the anion (pK* 16) (4). Consequently, excitation of 8-quinolinol in neutral, dilute acid, or dilute base solutions results in the formation of the nonfluorescent excited zwitterion, no matter which ground-state species is excited. Accordingly, in the presence of alkali metal ions such as Na^+, K^+, Rb^+, and Cs^+ which, by virtue of their low charges and large ionic radii are very weakly polarizing and do not tend to form complexes with soft Lewis bases such as 8-quinolinol, the ligand exists in near neutral solutions predominantly as the free neutral species, and no fluorescence is observed from these solutions. However, solutions containing 8-quinolinol and Li_2CO_3 show the same fluorescence as those of commercial samples of lithium–8-quinolinolate in water ($\bar{\nu}_{max.}$ 2.08 $\mu m.^{-1}$).

Chelates of 8-quinolinol with polyvalent nontransition metal ions (*e.g.*, Mg^{+2} and Zn^{+2}) are well known for their green fluorescence (5). The fluorescences of these compounds are explicable in terms of the qualitative similarity in the electronic configuration of the chelated 8-quinolinolate to that of the protonated 8-quinolinol (the metal ion polarizes both the phenolic group and the heterocyclic nitrogen atom positively). Since ligand-exchange kinetics are generally slow compared with the kinetics of prototropic reactions, the excited chelate fluoresces, even in near neutral aqueous solutions, before it can dissociate in the lowest excited singlet state. Moreover, the electronic absorption spectra of the nontransition metal chelates of 8-quinolinol show low frequency absorption maxima at frequencies below 3 $\mu m.^{-1}$ in neutral aqueous and chloroform solutions, while the ^1La band of 8-quinolinol (the neutral species) appears at 3.20 $\mu m.^{-1}$ in both of these solvents. The ^1La band of the protonated 8-quinolinol appears at 2.79 $\mu m.^{-1}$.

The sodium, potassium, rubidium, and cesium salts of 8-quinolinol in water, buffered to pH 7.00, all show their long wavelength absorption maxima at 3.20 $\mu m.^{-1}$. (Actually the term "salts" is used loosely here; at pH 7.00, the 8-quinolinol is almost exclusively present as the neutral free ligand.) However, the lithium salt shows its long wavelength maximum at 2.87 $\mu m.^{-1}$. Consequently, it is proposed that while Na^+, K^+, Rb^+, and Cs^+ all form predominantly ionic salts with the 8-quinolinolate anion, in which the anion is free to hydrolyze in water, the compound formed between lithium and 8-quinolinolate anion is largely covalent, thereby accounting for its fluorescence and the large red shift of the 8-quinolinol absorption spectrum in neutral aqueous solutions. In support of this conclusion, continuous variation plots of the Li^+ to 8-quinolinol mole ratio *versus* fluorescence intensity and absorbance of the long wavelength absorption band of the complex, respectively, were carried out at pH 12.0 (to assure complete dissociation of the free ligand to the anion). These studies indicate that the complex has a 1:1 stoichiometry. Presumably, the remaining two coordination sites of the sp^3 hybridized Li^+ ion are hydrated.

The formation of a coordinate covalent complex involving Li^+ can be rationalized on the basis of the small ionic radius of lithium, imparting it with polarizing ability greater than that of a typical alkali metal ion. The small radii of first row elements have been employed to explain frequent deviations from "normal" periodic behavior. These results may be of some interest in analytical and inorganic chemistry. However, the implications are farther reaching in the area of neuropharmacology. It has been known for some time that lithium carbonate and other lithium salts have been effective in the pacification of manic psychotic behavior (2). The mechanism of sedation is not understood, although it has been proposed that Li^+ alters the metabolism of norepinephrine and catecholamine (6). If Li^+ was to occur exclusively as an ionic species *in vivo*, it could only cross the lipoprotein neural cell membrane by active transport processes, since a charged species, being lipophobic, could not cross by diffusional means. If, however, Li^+ was present to some extent as an uncharged chelate species (*e.g.*, with an amino acid) lipophilicity, as evidenced by the extractability of lithium–8-quinolinolate into chloroform, and thus diffusional membrane permeability come into the realm of possibility. Whether lithium acts at the outer neural surface, crosses the neural membrane by active transport or by diffusional transport, is conceivably of importance in interpreting the nature of its sedative action.

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Drug Concentration in Goldfish at the Pharmacologic End-Point

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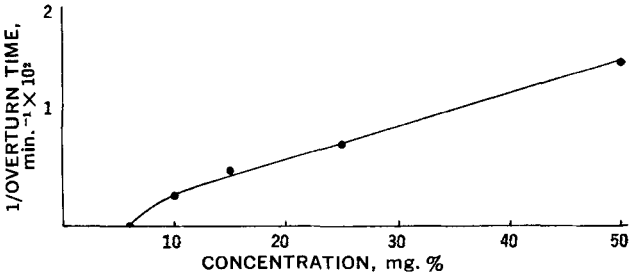

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8-Quinolinol in dilute acid, neutral, and dilute basic aqueous solutions (those of concern here and in biological systems) is nonfluorescent. However, the excited cation and excited anion of 8-quinolinol show green fluorescence in concentrated acid (3) and concentrated base (4) solutions, even though the ground-state cation predominates below pH 5 and the ground-state anion is present above pH 10. In the pH range of 5–10, the predominant ground-state species derived from 8-quinolinol is the neutral phenolic species. The observation of fluorescence from 8-quinolinol only in concentrated acid or base solutions has been shown to be due to the attainment of prototropic equilibrium in the lowest excited singlet state between the cation and the zwitterion derived from 8-quinolinol (pK* −7) (3) and between the zwitterion and the anion (pK* 16) (4). Consequently, excitation of 8-quinolinol in neutral, dilute acid, or dilute base solutions results in the formation of the nonfluorescent excited zwitterion, no matter which ground-state species is excited. Accordingly, in the presence of alkali metal ions such as Na^+, K^+, Rb^+, and Cs^+ which, by virtue of their low charges and large ionic radii are very weakly polarizing and do not tend to form complexes with soft Lewis bases such as 8-quinolinol, the ligand exists in near neutral solutions predominantly as the free neutral species, and no fluorescence is observed from these solutions. However, solutions containing 8-quinolinol and Li_2CO_3 show the same fluorescence as those of commercial samples of lithium–8-quinolinolate in water ($\bar{\nu}_{max.}$ 2.08 $\mu m.^{-1}$).

Chelates of 8-quinolinol with polyvalent nontransition metal ions (*e.g.*, Mg^{+2} and Zn^{+2}) are well known for their green fluorescence (5). The fluorescences of these compounds are explicable in terms of the qualitative similarity in the electronic configuration of the chelated 8-quinolinolate to that of the protonated 8-quinolinol (the metal ion polarizes both the phenolic group and the heterocyclic nitrogen atom positively). Since ligand-exchange kinetics are generally slow compared with the kinetics of prototropic reactions, the excited chelate fluoresces, even in near neutral aqueous solutions, before it can dissociate in the lowest excited singlet state. Moreover, the electronic absorption spectra of the nontransition metal chelates of 8-quinolinol show low frequency absorption maxima at frequencies below 3 $\mu m.^{-1}$ in neutral aqueous and chloroform solutions, while the ^1La band of 8-quinolinol (the neutral species) appears at 3.20 $\mu m.^{-1}$ in both of these solvents. The ^1La band of the protonated 8-quinolinol appears at 2.79 $\mu m.^{-1}$.

The sodium, potassium, rubidium, and cesium salts of 8-quinolinol in water, buffered to pH 7.00, all show their long wavelength absorption maxima at 3.20 $\mu m.^{-1}$. (Actually the term "salts" is used loosely here; at pH 7.00, the 8-quinolinol is almost exclusively present as the neutral free ligand.) However, the lithium salt shows its long wavelength maximum at 2.87 $\mu m.^{-1}$. Consequently, it is proposed that while Na^+, K^+, Rb^+, and Cs^+ all form predominantly ionic salts with the 8-quinolinolate anion, in which the anion is free to hydrolyze in water, the compound formed between lithium and 8-quinolinolate anion is largely covalent, thereby accounting for its fluorescence and the large red shift of the 8-quinolinol absorption spectrum in neutral aqueous solutions. In support of this conclusion, continuous variation plots of the Li^+ to 8-quinolinol mole ratio *versus* fluorescence intensity and absorbance of the long wavelength absorption band of the complex, respectively, were carried out at pH 12.0 (to assure complete dissociation of the free ligand to the anion). These studies indicate that the complex has a 1:1 stoichiometry. Presumably, the remaining two coordination sites of the sp^3 hybridized Li^+ ion are hydrated.

The formation of a coordinate covalent complex involving Li^+ can be rationalized on the basis of the small ionic radius of lithium, imparting it with polarizing ability greater than that of a typical alkali metal ion. The small radii of first row elements have been employed to explain frequent deviations from "normal" periodic behavior. These results may be of some interest in analytical and inorganic chemistry. However, the implications are farther reaching in the area of neuropharmacology. It has been known for some time that lithium carbonate and other lithium salts have been effective in the pacification of manic psychotic behavior (2). The mechanism of sedation is not understood, although it has been proposed that Li^+ alters the metabolism of norepinephrine and catecholamine (6). If Li^+ was to occur exclusively as an ionic species *in vivo*, it could only cross the lipoprotein neural cell membrane by active transport processes, since a charged species, being lipophobic, could not cross by diffusional means. If, however, Li^+ was present to some extent as an uncharged chelate species (*e.g.*, with an amino acid) lipophilicity, as evidenced by the extractability of lithium–8-quinolinolate into chloroform, and thus diffusional membrane permeability come into the realm of possibility. Whether lithium acts at the outer neural surface, crosses the neural membrane by active transport or by diffusional transport, is conceivably of importance in interpreting the nature of its sedative action.

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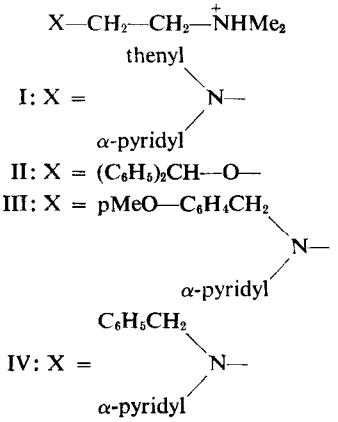
Solution Conformations of Antihistamines

Keyphrases ☐ Antihistamines—activity–conformation relationships ☐ Conformation, analysis—related to antihistamine activity ☐ Stereospecificity—antihistamine activity–*trans*-conformation relationships ☐ PMR spectroscopy—structure, conformation, antihistamines

Sir:

On the basis of similarities in the crystal structures of histamine and the antihistamine methapyrilene hydrochloride[1] (I), it was concluded that for $XCH_2CH_2N^+HMe_2$ compounds to show antihistaminic activity, it is essential that N^+ and X be in a *trans*-conformation (1). Many antihistamines have this general formula, in which the substituent X may be >N, —O, or saturated C, with a variety of groups attached.



Some support for this suggestion follows from the crystal structure of the physiologically inactive amino acid histidine, which has a *gauche*-arrangement of the NH_3^+ group and the imidazole ring (1, 2).

However, a number of assumptions are interposed between these structural observations and the deduction of a meaningful structure–activity relationship. For example, it is assumed that: (*a*) the receptors for the antihistamine and histamine are identical (3), and (*b*) the receptor-bound conformation of the drug is the same as that of the drug in solution or in the solid (3, 4). There is also the assumption, often unverified, that the solid-state conformation of the flexible $XCCN^+$ chain is preserved when the crystal lattice is destroyed in aqueous solution or *in vivo*. Indeed, it was recently shown (5) that the histamine univalent cation in D_2O does not have the *trans*-conformation found in the solid (6) but rather has approximately equal proportions of the *trans*-conformer and of each of the two *gauche*-conformers. In this communication, I present evidence, obtained from proton magnetic resonance (PMR) spectra, on the solution conformations of a number of antihistamines.

The PMR spectra were obtained at 60 and 100 MHz. (probe temperatures of 39 and 30°, respectively) on D_2O solutions of the salts of the antihistamines (approximately 40% by weight). The one free base studied was run as a 1:1 (by volume) solution in $CDCl_3$. The conformational information comes from an analysis of the *AA'BB'* system of the 1,2-disubstituted ethane fragment (7, 8).

For the $XCH_2—CH_2N^+$ chain, three conformations with respect to the C—C bond are possible: one with the substituents X and N^+ in a *trans*-disposition and two in which they are *gauche*. With the assumptions of 60° dihedral angles and only two vicinal proton–proton coupling constants, J_t and J_g, for a particular compound, Abraham and Pachler (8) showed that the rotationally averaged vicinal coupling constants, J_{AB} and $J_{AB'}$, are given by:

$$J_{AB} = n_t J_g + \tfrac{1}{2}(1 - n_t)(J_g + J_t) \quad \text{(Eq. 1)}$$

and:

$$J_{AB'} = n_t J_t + (1 - n_t) J_g \quad \text{(Eq. 2)}$$

where n_t is the proportion of *trans*-conformer. The *AA'BB'* spectral parameters, N and L, are defined by:

$$N = J_{AB} + J_{AB'} \quad \text{(Eq. 3)}$$

$$L = J_{AB} - J_{AB'} \quad \text{(Eq. 4)}$$

and it can be shown that:

$$N = \tfrac{1}{2}[J_t + 3J_g + n_t(J_t - J_g)] \quad \text{(Eq. 5)}$$

and:

$$L = (\tfrac{1}{2} - \tfrac{3}{2}n_t)(J_t - J_g) \quad \text{(Eq. 6)}$$

Furthermore, the parameter combination of $1/2N + 1/6L$ is independent of the proportions of the conformers present and is equal to the average of the vicinal coupling constants, since a little algebra shows that:

$$\tfrac{1}{2}N + \tfrac{1}{6}L = \tfrac{1}{3}(2J_{AB} + J_{AB'}) = J_{av} = \tfrac{1}{3}(J_t + 2J_g) \quad \text{(Eq. 7)}$$

The expressions for N and L show that, since $J_t - J_g$ is positive (9), the smaller the value of N the smaller the proportion of *trans*-conformer; and if L is positive, then n_t is less than 1/3 and the *gauche*-conformers are more stable. The sign of L cannot be determined from the spectrum; but in favorable cases, it can be obtained by using Abraham and Pachler's (8) correlation: $1/2N + 1/6L = 18.0 - 0.8\Sigma E$, where ΣE is the sum of the Huggins electronegativities (10) for the six atoms directly bonded to the C—C fragment.

Diphenhydramine hydrochloride (II)[2], an antihistamine of the dimethylaminoethyl ether type, gives a spectrum whose parameters have the following values (in Hz.): N, 10.1; L, ±3.5; K and M, which are, respectively, the sum and difference of the geminal coupling constants, −29.8 and ±2.1. The correlation between the average vicinal coupling constants and the substituent electronegativities, gives a calculated J_{av} of 5.7 Hz., whereas the experimental average is 5.6 Hz. ($L > 0$) or 4.5 Hz. ($L < 0$). I conclude that L is positive and, hence, that the *gauche*-forms are more stable and predominate in solution. This finding is in keeping with the anticholinergic properties of this substance (11) and the known *gauche*-$OCCN^+$ skeleton of acetylcholine in solution (12).

[1] Methapyrilene (I) is 2-[(2-dimethylaminoethyl)-2-thenylamino]-pyridine.

[2] Diphenhydramine (II) is 2-diphenylmethoxy-*N*,*N*-dimethylethylamine.

The three other antihistamines studied, pyrilamine maleate (III)[3], tripelennamine hydrochloride (IV)[4], and methapyrilene hydrochloride (I), are ethylenediamine derivatives. Their salts gave spectra which suggested that $L = 0$. This value is not expected, for it corresponds to equal proportions of all three conformers. Since the departure from $L = 0$ that can be recognized depends in part on the value of M, an attempt was made to estimate this parameter for the NCH_2CH_2N system by studying pyrilamine free base. Its spectrum showed $N = 14.6$ and $L = \pm 3.6$ with $M = \pm 2.0$ Hz.; the electronegativity relationship yields 6.1 Hz. for J_{av}, which is to be compared with the experimental value of 7.9 Hz. ($L > 0$) and 6.7 Hz. ($L < 0$). Thus, L is negative, *i.e.*, n_t is greater than 1/3 so that the *trans*-form is favored in solution and is more stable than the *gauche*-forms.

The N values for the salts, the species present at physiological pH, are 12.1 Hz. (pyrilamine), 12.0 Hz. (tripelennamine), and 11.8 Hz. (methapyrilene). By assuming an upper limit of $M = 2$ Hz. for these molecules, the observed spectra are consistent with L values between -1 and 1 Hz. If only the *trans*-form were present in solution, N and L values of approximately 16 and -6 Hz., respectively, would be expected (13, 14). Although there are experimental and theoretical uncertainties in deducing precise conformer proportions from the coupling constants, it is quite clear that the decreases in N and L, compared with pyrilamine free base, are accompanied by a decrease in the proportion of the *trans*-conformer. Attempts to allow for possible electronegativity effects of a substituent on the proton couplings only reinforce this decrease. Thus, in contrast to methapyrilene in the solid, these three closely related ethylenediamine antihistamines are not exclusively in the *trans*-conformation in solution; in fact, conformationally, they seem very similar to the histamine cation itself.

Although none of the salts studied here is exclusively in the *trans*-form in solution, the ethylenediamine derivatives are among the most active antihistamines. These $NCCN^+$ types have pA_2 values approximately 1 unit higher than the $OCCN^+$ type (15). While it is probably an oversimplification to ascribe all of this difference in activity to conformational preference, the order of decreasing activity is also the order of decreasing N values and, hence, decreasing proportion of the *trans*-conformer. This observation is consistent with the stereospecificity observed in antihistamines of the allylamine type (16), where UV spectra indicate that the more active geometric isomer has the basic N atom and the α-pyridyl ring in a *trans*-arrangement.

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I thank J. L. Little and R. I. Willing for recording spectra and the following for generously providing samples: Eli Lilly and Co. (methapyrilene hydrochloride), Parke, Davis & Co. (diphenhydramine hydrochloride), May & Baker (Australia) (pyrilamine maleate), and Ciba Co. (tripelennamine hydrochloride).

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BOOKS

REVIEWS

Handbook of Experimental Pharmacology, Volume 28, Part 1, Concepts in Biochemical Pharmacology, Edited by B. B. BRODIE and J. R. GILLETTE. Springer-Verlag New York, Inc., 175 Fifth Ave., New York, NY 10010, 1971. xvi + 471 pp., 16.5 × 25 cm. Price $50.40.

This volume continues the high tradition of the Handbook series for breadth of coverage and criticality in the review of specialized aspects of pharmacology.

Part 1 of Volume 28 presents a detailed critique of current information on drug absorption, transport, and disposition in tissues and drug disposition *via* several excretory routes. Part 2 will cover the topics of microsomal and nonmicrosomal biotransformation of drugs as well as bioanalytical methodology for the study of drug metabolism and the isolation and identification of drug metabolites. When complete, this volume will likely become the standard reference work on all aspects of drug absorption, transport, and disposition.

The book is divided into three sections. Section One is entitled Routes of Drug Administration and contains chapters on Absorp-

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tion of Drugs from the Gastrointestinal Tract (L. S. Schanker); Buccal Absorption of Drugs (A. H. Beckett and R. D. Hossie); Subcutaneous and Intramuscular Injection of Drugs (J. Schou); Absorption, Distribution and Excretion of Gaseous Anesthetics (H. Rackow); and Absorption of Drugs through the Skin (M. Katz and B. J. Poulsen). Particularly impressive is the fact that the editors have included and encouraged discussion in depth of aspects of drug absorption by routes other than oral administration. The chapters by Katz and Poulsen on drug absorption through the skin, by Greene on aerosols, and by Rackow on gaseous anesthetics are especially timely and well done.

Section Two covers Sites of Drug Transport and Disposition and includes chapters presenting a theoretical description of drug–protein interactions (W. Settle, S. Hegeman, and R. M. Featherstone); Physical Methods for Studying Drug–Protein Binding (C. F. Chignell); Effects of Binding to Plasma Proteins on the Distribution, Activity and Elimination of Drugs (P. Keen); Competition between Drugs and Normal Substrates for Plasma and Tissue Binding Sites (H. M. Solomon); drug entry into the central nervous system (D. P. Rall), bone (H. M. Foreman), adipose tissue (L.C. Mark), and placental transfer of drugs (M. Finster and L. C. Mark). The concluding chapters in this section covering autoradiography in experimental pharmacology (L. J. Roth) and drug accumulation at sympathetic nerve endings (I. J. Kopin) present information which has been extensively reviewed elsewhere and which is, in any case, out-of-place in the context of this volume. Section Two logically leads the reader from a discussion of the underlying physical and chemical forces involved in drug–protein interactions to a description of methodology which can be employed to identify and quantify such interactions and thence to an exposition of their significance to the activity of drugs and to the action of endogenous substances such as hormones.

Section Three: Sites of Drug Excretion surveys the role of the kidney (I. M. Weiner), bile (R. L. Smith), and milk (F. Rasmussen) as systems or vehicles for drug excretion. The book is concluded with a pertinent chapter of toxicological interest on Extracorporeal and Peritoneal Dialysis of Drugs by G. E. Schreiner, J. F. Maher, W. P. Argy, and L. Siegel.

The major strengths of this work are three in number. First, the organization is highly logical and proceeds from the development of the theoretic base for each topic to a discussion of the significance of that topic to drug action in the intact organism. Secondly, each chapter is written by an authority in the field in whose laboratory much of the early work on the development and significance of the subject matter was undertaken. This provides a degree of scope and criticality otherwise unobtainable. Thirdly, the editors have wisely chosen the topics to be covered under the broad heading of "biochemical pharmacology"; their choice leads to a structure which may yet convey some meaning to this frequently misapplied and misunderstood phrase.

"Concepts in Biochemical Pharmacology" should be made available to graduate students in pharmacology, medicinal chemistry, and biopharmaceutics as a reference work and to the research worker in these disciplines looking for an overview of the underlying principles and processes by which drugs are absorbed, translocated in the body, and excreted. The price of this volume should not deter its wide utilization.

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AMA Drug Evaluations 1971. Evaluated by the AMA Council on Drugs. American Medical Association, Chicago, IL 60610, xxviii + 1040 pp. 28 × 21 cm. Price $15.00.

One of the most frequently encountered problems in the area of drugs is to find a reliable, complete, and reasonably up-to-date source for drug information. The publication of "AMA Drug Evaluations 1971" is a significant effort toward filling that void. The AMA has attempted to bring together in a single source essential therapeutic information about the most commonly used drugs.

The 1040-page guide includes more than 1200 single entity drugs and mixtures, describing effectiveness, safety, reactions, and dosage. Evaluative statements based on opinions of the AMA Council on Drugs are also included. Whenever possible, statements comparing relative effectiveness and safety are given. The following information is given for each drug: appraisal, action and uses, limitations and precautions, adverse reactions, dosage, route of administration, preparations, available sizes and strengths, known sources of supply, and commercial name or names by which the drug is sold in the United States.

The book is divided into two major sections. The first of these is subdivided into 90 therapeutic classifications. Each chapter contains an introductory statement that discusses the overall therapeutic category, followed by descriptions and evaluations of all drugs in the class—old or new, single entity or combination.

Drugs marketed in the last ten years make up the second section, the New Drugs Section. In this section, drugs are listed alphabetically by generic name; more detailed information is provided for these newer drugs.

A somewhat unusual, but most helpful, feature of the book is the inclusion of three indexes. The Indications Index uses diseases, symptoms, and other indications as the main headings. The second index uses various adverse reactions as the main headings, and the third index utilizes nonproprietary (generic) and trade names of drugs as the entry terms.

This book should prove to be an indispensable reference source for those in need of reliable and concise drug information. Although this book was four years in the works, it was well worth the wait.

Staff Review ■

NOTICES

The Pineal Gland. A Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and J. KNIGHT. Churchill Livingstone, London, England, 1971. xi + 401 pp. 16 × 24 cm.

The Family and Its Future. A Ciba Foundation Symposium. Edited by K. ELLIOTT. J. & A. Churchill, 104 Gloucester Place, London, England, 1970. x + 230 pp. 15.5 × 23.5 cm.

Hormones and the Immune Response. Ciba Foundation Group No. 36. Edited by G. E. W. WOLSTENHOLME and J. KNIGHT. J. & A. Churchill, 104 Gloucester Place, London, England, 1970. ix + 172 pp. 12.5 × 18.5 cm.

Hypertrophic Obstructive Cardiomyopathy. Ciba Foundation Study Group No. 37. Edited by G. E. W. WOLSTENHOLME and M. O'CONNOR. J. & A. CHURCHILL, 104 Gloucester Place, London, England, 1971. ix + 220 pp. 13 × 19 cm.

Handbook of Drug Interactions. By GERALD SWIDLER. Wiley-Interscience, New York, NY 10016, 1971. vi + 384 pp. 15.5 × 23.5 cm. Price $15.

Pyrogens and Fever. A Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and J. BIRCH. Churchill Livingstone, London, England, 1971. xi + 232 pp. 16 × 24 cm.

Teamwork for World Health. A Ciba Foundation Symposium. Edited by G. WOLSTENHOLME and M. O'CONNOR. J. & A. Churchill, 104 Gloucester Place, London, England, 1971. xiii + 242 pp. 16 × 24 cm.

Identifizierung Von Arzneistoffen. By H. AUTERHOFF and K.-A. KOVAR. Wissenschaftliche, Verlagsgesellschaft mbH, 7000 Stuttgart 1, Postfach 40, Germany, 1971. 143 pp. 21 × 29 cm. Price DM 27.50. (*German*)

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Reviewed by William F. Bousquet
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INDEX TO AUTHORS
INDEX TO SUBJECTS

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Journal of Pharmaceutical Sciences

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REVIEW ARTICLE

Peritoneal Dialysis: A Review

ALBERT M. MATTOCKS and EMAD A. EL-BASSIOUNI

Keyphrases ☐ Peritoneal dialysis—review ☐ Renal failure—applications of peritoneal dialysis ☐ Poisons, endogenous and exogenous—removal by peritoneal dialysis ☐ Barbiturate poisoning—treatment with peritoneal dialysis ☐ Drug poisoning—treatment with peritoneal dialysis

Peritoneal dialysis has become a commonly practiced technique for the treatment of both acute and chronic kidney failure and for removal of dialyzable exogenous and endogenous poisons. With the increased availability of medical services today and the further increases expected in the future, peritoneal dialysis likely will find ever widening use, particularly for elderly patients.

The amounts of sterile fluid required for peritoneal dialysis are quite large, the average procedure utilizing 40–60 l. for a single patient. Along with this fluid, special administration sets, catheters, trocars, syringes, and auxiliary drugs and services are needed which the pharmacist may be called upon to supply. Thus, the pharmacist should be knowledgeable as to the process of peritoneal dialysis and the drugs, supplies, and services it requires, and he should be prepared to assist in the selection or adjustment of fluids to fit the needs of a particular patient.

For this country to achieve the goal of extending medical services to all in need of them, it seems likely that peritoneal dialysis will have to be commonly conducted in the home by the patient himself. This situation will place a new burden on the pharmacist to supply the needs of the patients in his locality, to advise the patients on many associated problems, and to consult with the physicians on the care of the patients.

For the research pharmacist, peritoneal dialysis presents a special challenge. At present the process is relatively simple, and little has been done to develop more sophisticated agents to increase the safety of the patient, increase clearances of given toxins, or promote the dialysis of drugs and poisons now considered "nondialyzable." Thus, there exists in this field a great opportunity for the pharmaceutical scientist to apply his special knowledge and skill for the benefit of a large number of seriously ill persons.

HISTORICAL DEVELOPMENT OF PERITONEAL DIALYSIS

Wegner (1) is credited with the first experiments on peritoneal lavage. In 1877, he studied the fall in temperature produced by introducing cold saline solution into the peritoneum; in the course of this work, he noted that hypertonic dextrose or glycerin was increased in volume by body water. In 1894, Starling and Tubby (2) studied the transference of substances from solution in the peritoneal cavity to the blood and in the reverse direction. They demonstrated that transport from the peritoneum *via* the lymph must be so slow as to be negligible, and they concluded that passage must occur directly to the blood. They showed that hypertonic solutions would increase in volume when instilled into the peritoneum and that hypotonic solutions would decrease in volume. Indigo carmine and methylene blue were observed to be readily absorbed from the peritoneum. Starling and Tubby (2) found that absorption of serum from the peritoneum was extremely slow, while absorption of an isotonic crystalloid solution might be fairly rapid. In their search for an explanation, they failed to recognize the tendency of the

individual species in solution to attain independent equilibrium with the body fluids.

Cunningham (3), in 1920, examined the absorption of dextrose from a 10% solution in an effort to evaluate intraperitoneal injection as a means for administering large volumes of fluid. His studies on rats showed complete absorption of the fluid within a little more than 12 hr. He observed some changes in mesothelial cells in the diaphragm, spleen, and omentum with animals given repeated daily administrations, but these changes were rapidly reversible and no permanent or threatening effects were observed.

Putnam (4), in 1922, demonstrated the properties of the peritoneal membrane remarkably well. He injected, intraperitoneally, water and saline solutions with an added dye, trypan blue, and analyzed the peritoneal fluid at intervals of time, measuring chloride, urea, dextrose, dye, protein, and total fluid volume. He found chloride and urea to equilibrate between blood and peritoneal fluid in 2–3 hr. and the volume of fluid to decrease continuously to nearly zero in 9 hr. The relative dye concentration followed the total fluid volume quite closely. Putnam (4) also observed that several dyes (Congo red, alizarin, acid fuchsin, and trypan blue), when injected intravenously, did not appear in peritoneal fluid. This was due, most likely, to binding of the dyes to serum proteins, which was not recognized at the time. Putnam (4) also showed that a number of other organic compounds and dyes pass the peritoneal membrane, including salicylate, ferrocyanide, phenolsulfonphthalein, and iodide. He concluded that, except for the anomaly observed with dyes as previously mentioned, the peritoneal membrane behaves much like a simple colloidal membrane as used in the laboratory.

A number of other workers (5) demonstrated in similar fashion the permeability of the peritoneal membrane to crystalloids and water.

Ganter (6), in 1923, made the first evaluation of peritoneal dialysis for the treatment of uremia. He used rabbits and guinea pigs with ligated ureters and dialyzed with small volumes (50 ml.) of physiological saline. He allowed the fluid to remain in the peritoneum for 2–4 hr., at which time the nonprotein nitrogen in the dialysate was approximately equal to that of the blood. The amount of fluid recovered was low, 10–30 ml. Ganter (6) reported moderate clinical improvement in animals so treated. It is now known that the volumes of fluid used in Ganter's experiments were too small for rapid removal of endogenous poisons, and the composition of the fluid was such that fairly rapid absorption would occur, thus reducing the effectiveness of the dialysis.

Bliss *et al.* (7), in 1932, treated nephrectomized and kidney-functionless dogs with balanced salt solutions, using 750 ml. of fluid for each exchange, a 10-min. dwell time, and up to 20 exchanges of fluid. Their results were dramatic as to the removal of nonprotein nitrogen. Animals survived up to 16 days. These authors identified phosphate, creatinine, urochrome, urea, sulfates, chlorides, and traces of protein in the return fluid.

Abbott and Shea (8) gave considerable thought to the conditions for which peritoneal dialysis should be utilized, to the types of fluid most likely to be successful, to the volumes of fluid to be used, and to the method by which the fluid should be exchanged. They compared 5% dextrose, Ringer's solution, Hartmann's solution, and a Solution A (a balanced salt solution with bicarbonate, phosphate, magnesium, and dextrose). They concluded that Ringer's solution is not suitable for peritoneal lavage since it reduces blood bicarbonate, and they suggested that the use of added bicarbonate in the fluid is desirable. Furthermore, they found the intermittent dialysis procedure to be more practical than a continuous flow method.

Wear *et al.* (9) also recognized the value of Hartmann's solution for removal of nonprotein nitrogen and the need to prevent acidosis. Haam and Fine (10) observed similar removal of nonprotein nitrogen with intermittent dialysis.

Seligman *et al.* (11), in 1946, studied the treatment of nephrectomized dogs with continuous flow dialysis and found that the uremic animal could be successfully managed. They used fluids containing concentrations of electrolytes matched to normal extracellular fluid.

Grollman *et al.* (12) resolved several of the problems in treating uremia by peritoneal dialysis. They treated nephrectomized dogs and later human patients by more frequent dialysis but with fewer exchanges. They restricted the dietary intake so as to avoid excess inorganic salts and protein, and they adjusted the dialysis fluids as required for the individual. These modifications, along with careful attention to avoid infection or pyrogenic reaction and blood transfusion when severe anemia threatened, enabled them to keep dogs alive much longer than had previous workers. This research demonstrated most clearly the benefit of peritoneal dialysis in the treatment of uremia. Intermittent dialysis has since been employed successfully in many hospitals and homes (13–26).

THE PERITONEAL MEMBRANE

For a substance to pass from the blood to the fluid in the peritoneum, it must cross the peritoneal membrane. The anatomical structure of this membrane has been well described (27–30), and it would be of immense value to know the mechanism of transport across this membrane along with the factors affecting it. But knowledge of this function is quite limited. The total area of the membrane is said to be approximately 22,000 $cm.^2$ (31). Of this surface, it would appear that the mesentery must be the most effective portion, since it probably represents the largest fraction of the total area, it is richly supplied with blood vessels, and it is probably in better contact with the peritoneal fluid than other portions of the membrane. Early work of Starling and Tubby (2) showed that transport *via* lymph is negligible in most instances. Thus, it appears that passive diffusion across the peritoneal membrane remains the basic process by which substances equilibrate between peritoneal fluid and blood.

Gosselin and Berndt (32–35) measured the permeability of the isolated mesentery to radioactive rubidium

and phosphorus and were able to estimate a porosity for the membrane. They also postulated that the mesentery may function in transport as three separate layers. Knochel *et al.* (36) postulated that the mesentery may act as a classical lipid membrane and allow passage only of undissociated molecules with relatively high oil/water partition coefficients. Shenouda and Mattocks (37) measured transport in both directions and concluded that ionization appeared to have little effect on the rate of passage across the mesentery. Later, Penzotti (38) tested compounds known to accelerate peritoneal dialysis *in vivo* and found that they had no effect on dialysis across the isolated membrane. This finding led him to question the validity of results obtained with the isolated mesentery. His results might also be explained on the basis that transport across the mesentery is not the limiting factor in peritoneal dialysis, but some other step in the process, such as passage across the capillary membrane, may control the rate of passage from blood to dialysate.

McLean *et al.* (39) found that tris(hydroxymethyl)-aminomethane increased the rate of diffusion of urea into the peritoneum; since urea is nonionized in the medium, they concluded that the effect must be an alteration in membrane permeability. Henderson (40–42) noted that hypertonic solutions enhanced the transfer of urea into the peritoneum and, at first, concluded that the effect persisted even though the hypertonic fluid was replaced by isotonic medium. This led him to believe that alteration in membrane permeability had occurred. These findings indicate that methods of improving rates of transfer across the peritoneal membrane may be possible so that more effective peritoneal dialysis might be accomplished.

PERITONEAL DIALYSIS TECHNIQUES AND PROCEDURES

As a result of the experience of many workers, a relatively simple general procedure for conducting peritoneal dialysis has evolved. The intermittent method is generally preferred because of its simplicity, ease of maintaining a closed sterile system, and the lack of a need for complicated equipment. A precise detailed description of the technique was presented by Maxwell *et al.* (43). Briefly, it consists of inserting a sterile catheter into the peritoneum through a small incision in the midline of the abdomen about one-third of the way from the umbilicus to the pubic bone. The catheter is placed in such a position that it is not entangled with the omentum or mesentery, and a natural pathway is sought where little resistance is encountered. In some instances, placement of the catheter is made easier by injection of an initial volume of 1–2 l. of fluid by hypodermic syringe prior to insertion of the trocar and catheter. This procedure enables one to avoid the complications of entanglement with the membrane and aids in avoiding puncture of the intestines.

Once the catheter is inserted, care is taken to protect the skin and abdominal muscle in the region of insertion from introduction of bacteria through movement of the catheter caused by manipulation or normal movement of the abdomen in breathing. This is achieved by generous use of topical antiseptics and coverage with a sterile gauze pad. The aseptic technique must be used in connecting the solution tubing, and new sterile tubing is commonly used with each new exchange. The usual practice is to instill 2 l. of peritoneal dialysis fluid for each exchange, allowing inflow and outflow by gravity. When the fluid has remained in the peritoneum for the prescribed dwell time, usually 15–60 min., the fluid bottles are lowered below the patient level and the dialysate is collected in the original bottles without opening the system. In this procedure, it is important to keep the tubing filled with fluid so as to establish a syphoning action for draining without delay. Amounts of fluid returned approximate those introduced, except where highly hypertonic or hypotonic fluids are used. In this stage of the process, it is important to prevent contamination of the fluids in the return bottles and tubing; otherwise, bacteria may find their way back to the peritoneum to cause infection. This is achieved by use of bacterial filters through which air is vented or by use of collapsible plastic bags as fluid containers. Recycling by this procedure is continued with new supplies of sterile fluid until the desired effect is attained (urea nitrogen, creatinine, or other indicator substance reaches acceptable level). Others have also described the general methods for conducting intermittent dialysis (44, 45).

Askari *et al.* (46) described in detail a typical continuous peritoneal dialysis procedure. Two tubes or catheters are placed in the peritoneum, one on each side of the abdomen, with the inflow and outflow tips so placed as to minimize direct flow from one to the other (shortcircuiting). Fluid is introduced and drained by gravity in a continuous flow of about 2 l./hr. for an adult. This procedure has the advantage of having no loss of dialysis time required for drainage. Theoretically, it should give more rapid dialysis due to maintenance of lower concentrations of endogenous poisons in the dialysate and a higher diffusion gradient. These advantages are often not realized, however, due to direct channeling and relatively poor exposure of the fresh fluid to the mesentery.

Shinaberger *et al.* (47) combined the use of continuous dialysis with an extracorporeal dialyzer. The fluid in the peritoneum was cycled through a twin-coil dialyzer where endogenous poisons were dialyzed through the cellophane membrane into an electrolyte bath made with tap water. Efficient dialysis was reported utilizing high flow rates, with a minimum loss of protein from the patient and the need for only 4–6 l. of sterile dialysis fluid for the complete treatment. In comparing several different methods for dialysis, Miller *et al.* (48) found no significant advantage to the combined dialysis method over rapid flow with continuous dialysis. Apparently, these workers did not consider the advantage of needing much less sterile fluid, which may be especially important in home dialysis.

The efficiency of dialysis is generally expressed in terms of clearances, these being independent of blood level and thus comparable from one dialysis to another. Clearances are calculated by the well-known equation:

$$C = UV/P \qquad \text{(Eq. 1)}$$

where C is the clearance in milliliters per minute, U is the concentration of the species being followed (usually urea or creatinine) in the return fluid, V is the flow rate of dialysis fluid in milliliters per minute (total volume of fluid returned divided by total time involved in the exchange), and P is the average plasma concentration in the same units as U (usually the average of samples taken at the beginning and end of an exchange).

Several variations in procedure or technique may significantly affect the efficiency of peritoneal dialysis. Gross and McDonald (49) found that dialysis fluid warmed to 37° gave 35% better clearances than fluid introduced at 20°. Although most workers reported using fluids that were warmed to body temperature, the findings of Gross and McDonald serve to caution against laxity in controlling this factor.

Several workers examined the effect of flow rate of dialysis fluid on clearance. The volume of fluid used for each exchange was not varied greatly, presumably to avoid overdistention of the abdomen and discomfort to the patient. Thus, increases in flow rates were achieved chiefly by shortening the dwell time of the fluid in the abdomen. This makes comparison difficult, since during the period of introduction and drainage of the fluid the full volume is not within the peritoneum, and the time used for introduction and drainage may vary from one worker to the next.

Boen (50) compared urea clearances to flow rates for 268 exchanges (22 dialyses in 16 patients). His rates ranged from about 250 ml./hr. to 5 l./hr. He found an increase in clearance with flow rate up to a maximum average clearance of 28.5 ml./min. at a flow rate of 3.5 l./hr. and then a moderate decrease in clearance at higher flow rates. Boen (50) recognized this decrease as having questionable significance, since averages at the higher rates represented relatively few tests.

Gross and McDonald (49) compared clearances for flow rates from 1.1 to 8.5 l./hr. without observing a maximum. Tenckhoff *et al.* (51) also found continued increase in clearance with increased flow rates. Later, Penzotti and Mattocks (52) pointed out that the maximum reported by Boen (50) could be predicted mathematically when one considers the effect of drainage time on the flow rate.

Several mechanical devices have been developed to minimize the attendant time in peritoneal dialysis, to make home dialysis more practical, and to increase flow rates for greater efficiency.

An automatic cycling machine was developed by Boen *et al.* (53) in 1962. A pump transfers fluid from a large carboy to an elevated 2-l. bottle, and a time-controlled stopcock is opened to allow gravity flow of the fluid into the peritoneum. The bottle is then refilled for the next exchange, and a small vacuum pump creates a negative pressure on the receiving carboy. At the end of the dwell time, a stopcock is automatically opened to direct flow from the peritoneum into the receiving carboy. Thus, the whole cycle is operated by a timer which can be preset to the cycle desired. A typical setting was 5 min. input, 30 min. dwell, and 10 min. drain. Experience with a similar device was reported by Sicilia *et al.* (54). McDonald (55) used a recycling machine which contained a bacterial filter to sterilize the fluid as it was used. This enabled him to use nonsterile water for the dialysis fluid, with considerable savings in cost and effort. He recognized the possibility of failing to remove pyrogenic substances with the filter but encountered no difficulty in his tests. Tenckhoff *et al.* (56) and Lasker *et al.* (57) reported successful use of recycling machines for home dialysis.

Schwid and Vidt (58) made some inexpensive, simple, and quite useful modifications for automatically stopping the outflow at the desired volume, thus preventing the introduction of air into the peritoneum. Miller *et al.* (48) compared several methods of dialysis, including the Shinaberger method, and found the most increase in clearance with rapid intermittent exchanges but no increase with extracorporeal dialysis.

Several workers reported the use of indwelling buttons or openings and indwelling catheters which facilitate the introduction of peritoneal dialysis fluid. Boen *et al.* (53) designed a fitting which was implanted in the abdominal wall. This served as a tube through which the peritoneal catheter could be readily inserted and avoided the need for repeated puncture. A silicone rubber cannula for implantation was described in 1964 (59). It is implanted so as to act as a suspended perforated coil in the peritoneal cavity. The cannula is protected by a cap and dressing when not in use. Palmer and McDonnell (26) reported on the successful use of this cannula over several years. Tenckhoff and Curtis (60) reported on the long-term use of two types of implanted silastic catheters which proved particularly useful for home dialysis. Barry *et al.* (61) also reported on the use of an indwelling catheter with which they had several failures. Malette *et al.* (62) described the design and use of a peritoneal access button which is implanted beneath the skin and thus might reduce the incidence of infection. Although there have been problems with these devices, the implanted catheter is promising and seems to be a necessity if a large number of patients are to be treated at home by peritoneal dialysis.

The irrigating solutions used in peritoneal dialysis vary but generally contain electrolytes in concentrations approaching those of extracellular fluid: Na, 140 meq./l.; Ca, 3–3.5 meq./l.; Mg, 1.5 meq./l.; Cl, 101–104 meq./l.; and lactate, bicarbonate, or acetate, 40–45 meq./l. Potassium may be added as plasma levels fall during dialysis or it may be present in the solution at minimum level, 2.5–3.5 meq./l. Dextrose is added to increase the osmotic pressure to match or slightly exceed the osmolarity of the uremic extracellular fluid, the usual concentration being 1.5–2.0%. Much higher levels of dextrose are used when treating edema, usually 7–10%. Often the composition of the fluid is modified to suit the individual patient (63).

The use of lactate instead of bicarbonate has made the preparation of commercial solutions feasible, preventing precipitation of calcium and allowing autoclaving without loss of fixed base. More recently, acetate was used, having the advantages of being less susceptible to bacterial growth (64) and of forming less easily caramelized solutions (65).

The presence of minerals hastens the caramelization of dextrose on autoclaving, and bisulfite is often used

to retard oxidation. High concentrations of bisulfite, as have been used with 7% dextrose solutions, may constitute a toxic hazard (66, 67). Substantial caramelization not only causes undesirable discoloration but also appears to cause toxic effects (64). The caramelization problem becomes much more difficult with large containers, since heating time for sterilization is greatly increased to allow for heat conduction throughout the body of fluid. Thus, this becomes a major difficulty in supplying the large volumes of fluid for home use and the automatic cycling machines. At present, most workers use commercial solutions available in 1- and 2-l. containers.

Yutuc *et al.* (68) reported on the use of sorbitol in place of dextrose for peritoneal dialysis fluids. Sorbitol is stable to autoclaving in the fluid and would eliminate the difficulty in handling large volumes. Yutuc *et al.* (68) observed no untoward reactions in their tests and found the plasma levels of sorbitol to remain at low levels. Apparently, due to slower absorption from the peritoneum, the sorbitol enables more water to be removed than does dextrose. Sorbitol should be used with caution since it may be quite slowly metabolized and, in the absence of urinary function, its plasma level might become sufficiently high to cause dehydration of the tissues.

Gjessing (69) added amino acids to the peritoneal dialysis fluid and found that this substantially reduced the loss of serum protein and amino acids in patients who were regularly dialyzed. This procedure promises to offer a simple way to avoid one side effect commonly encountered in chronic patients.

KINETICS OF PERITONEAL DIALYSIS

Several mathematical relationships have been used to describe the process of peritoneal dialysis for the purpose of interpreting results and for predicting results for a given set of conditions. Boen (70) calculated ratios of clearances for various substances to the clearance of urea, thus obtaining relative diffusion rates across the peritoneal membrane. Such calculations on one set of data gave the following ratios (×100): potassium, 90; phosphate, 62; creatinine, 61; uric acid, 62; calcium, 51; magnesium, 47; and indican, 40. These values are useful in evaluating the efficiency of dialysis, in considering the practicality of removal of a new substance by dialysis, and in predicting the time needed for a given dialysis.

Henderson and Nolph (42) derived an equation for calculating dialysance based on principles of passive diffusion. They showed that the dialysance ratio of inulin to urea, D_i/D_u, should be essentially constant if the membrane areas for permeation of the two substances are not changed. They also noted that a change in the volume of distribution of a substance would not significantly affect the dialysance ratio. With these equations, they examined data obtained by using hypertonic solutions followed by isotonic solutions in the course of peritoneal dialysis. They found that the hypertonic solution accelerated dialysis and the effect persisted even though followed by isotonic fluids. The hypertonic solution caused a change in dialysance ratio, indicating a true effect on the membrane. These workers postulated several possible mechanisms for this effect and thought the most likely one to be a change in membrane pore structure.

Penzotti and Mattocks (71) used a two-compartment model to describe the peritoneal dialysis process for urea and found that the data fit well to the corresponding equations of Rescigno and Segre (72). The pseudo-first-order constants were then used to compare dialysis rates with different fluids. In later work, Penzotti and Mattocks (52) applied these equations and constants to intermittent dialysis, assuming a constant rate of drainage. Predicted values for clearances were generally somewhat lower than observed values, although the agreement was reasonable and relative values and trends were in good agreement.

CLINICAL APPLICATIONS OF PERITONEAL DIALYSIS

Acute Renal Failure—The first marked success with peritoneal dialysis was the treatment of acute renal failure. This was to be expected, since techniques and procedures suitable for long-term treatment evolved with experience. Boen (73) summarized the indications for dialysis as hyperkalemia, gross overhydration, severe acidosis, or marked clinical deterioration; he stated that long-term peritoneal dialysis in chronic renal failure was not yet justified. He summarized his results from patients with acute renal failure.

Hager and Merrill (74), in 1963, took the attitude that the patient should be spared the clinical manifestations of uremia and that dialysis should be started early enough to maintain the blood chemistry as nearly normal as possible. They pointed to several advantages of peritoneal dialysis over hemodialysis in such cases, including the availability of equipment, the shorter time to institute dialysis, and the applicability to a larger spectrum of patients. They felt that the slower clearance with peritoneal dialysis may be a significant advantage since it allows time for equilibrium of extracellular and intracellular fluids. In their report, they cautioned against the use of lactate-containing fluids where liver failure may be present. A summary of their procedures and experiences is included in their report.

Norman *et al.* (75) reported their experiences with patients having open-heart surgery. They recommended early use of peritoneal dialysis for these patients once definite symptoms of renal failure are observed.

Schwartz *et al.* (76), in 1966, reported on the success in treatment of acute renal failure with peritoneal dialysis over 6 years at Walter Reed Army Medical Center. They noted that successful dialysis with acute renal failure encouraged clinicians to consider peritoneal dialysis for other conditions characterized by homeostatic imbalance.

In some hypercatabolic conditions, the accumulation of tissue breakdown products is so rapid that peritoneal dialysis has been said to be too slow to lower the blood levels sufficiently (77–79), and hemodialysis is recommended. Cameron *et al.* (80), on the basis of their experience, disagreed with this conclusion. In such conditions, the use of peritoneal dialysis still is in question (81).

In 1962, Etteldorf *et al.* (82) reported on the use of peritoneal dialysis for acute renal failure in children. In one case, they repeated dialysis 85 times during a 33-day period. They found the procedure to be both practical and effective. Since then, Lloyd-Still and Atwell (83) reported on treatment of infants with acute renal failure for whom hemodialysis was difficult. Manley and Collipp (84) and Lugo *et al.* (85) later reported on treatment of neonates with peritoneal dialysis. Both groups considered peritoneal dialysis to be a potential lifesaving procedure in acute renal failure of the newborn.

Among the many other applications of peritoneal dialysis to acute renal failure, an especially interesting one is the treatment of patients who suffer renal failure as a result of falciparum malaria. Several workers reported success with this application (86, 87).

Chronic Renal Failure—Long-term treatment of chronic uremia by peritoneal dialysis has been encouraging but not completely satisfactory. Boen *et al.* (53), in 1962, had a patient who could no longer be maintained on hemodialysis due to a clotting problem at the site of cannulation. They used an implanted catheter and automatic cycling machine and reported satisfactory results with two to three treatments per week for 12 weeks.

Pateras *et al.* (88), in the same year, summarized results with 30 chronic uremia patients. Their results were encouraging, although they did not report the period of time over which the patients were maintained on peritoneal dialysis.

Stevens *et al.* (89) reported on 54 cases of chronic uremia treated by peritoneal dialysis between 1961 and 1963. All patients demonstrated biochemical improvement, and all but six showed clinical improvement. Fifteen of the patients survived longer than 3 months from the time of first dialysis, and seven survived more than 6 months. These workers suggested that those patients with a urine volume of 1 l./day prior to initial dialysis appear to have the best chance of prolonged survival on peritoneal dialysis and that this may be a criterion for selection of chronic patients for treatment.

Vertes *et al.* (90) treated 11 geriatric patients with peritoneal dialysis, noting that most or all of these patients would probably not be considered eligible for hemodialysis. Of these patients, five had an acute exacerbation of previously benign renal failure which was controlled with peritoneal dialysis for a short time; they were then discharged under dietary restrictions. Four patients had advanced chronic renal failure and were maintained satisfactorily by peritoneal dialysis to the time of the report. Two patients required dialysis for edema associated with chronic renal failure and these responded well. These workers suggested that peritoneal dialysis be tried more frequently on the geriatric uremia patient.

Palmer and McDonnell (26) reported on treatment of seven patients over 50 years of age who had end-stage chronic renal failure and who could no longer be maintained with conservative management. They used the "intramural cannula," a silicone rubber catheter, which was implanted in the abdomen. Six of the patients were maintained in a satisfactory state from 6 months to 3.5 years at the time of the report.

Abella *et al.* (91) treated six patients with terminal renal failure and found the implanted peritoneal button of Merrill *et al.* (92) to be unsatisfactory. They used repeated puncture for peritoneal dialysis. Most of their patients were later given transplants. The greatest difficulty with these patients during peritoneal dialysis was peritoneal infection.

Goldsmith *et al.* (93) found peritoneal dialysis to serve well in maintaining seven of eight patients during their wait for renal transplantation.

In 1968, Edelbaum *et al.* (94) reported on the use of peritoneal dialysis in acute episodes of chronic renal disease. They recognized the need for peritoneal dialysis when patients cannot be subjected to hemodialysis, but their experience with repeated peritoneal dialysis was poor. Problems consisted primarily of peritoneal infection, limited biochemical control, and loss of protein.

Numerous others published reports and summaries on the use of peritoneal dialysis for both acute and chronic uremia (95–129). Sufficient experience has now been gained that it is an accepted procedure and is used in practically all medical centers. One of the most interesting developments has been the use of peritoneal dialysis in the home. The work of Tenckhoff and Curtis (60) gives special encouragement to those looking forward to management of all patients who will benefit by peritoneal dialysis.

Removal of Endogenous Poisons—Peritoneal dialysis has been used not only for removal of the endogenous toxins accumulated in the uremic patient but also for other diseased conditions in which endotoxins accumulate.

A case of hyperammonaemia due to ornithine transcarbamilase deficiency in an infant was reported by Herrin and McCredie (130). Immediately before dialysis, blood ammonia was 1970 mcg. %, and hourly cycles of intermittent peritoneal dialysis were instituted. The ammonia level fell to 800 mcg. % after 4 hr. and to 640 mcg. % 12 hr. later, with regain of consciousness. Dialysis was continued for 36 hr., with reduction of blood ammonia to 330 mcg. %.

A deeply comatose, moribund patient with homologous serum hepatitis was also treated with peritoneal dialysis (131). Blood ammonia decreased from 560 to 294 mcg. % after 24 hr. of dialysis. Clinical improvement following peritoneal dialysis in another case was reported (132), blood ammonia being lowered from 800 to 100 mcg. %. Dialysate concentrations of ammonia ranged from 200 to 100 mcg. % in this case. Krebs and Flynn (133) treated a patient with acute hepatitis who developed hepatic coma. Exchange transfusion was used and, to prevent accumulation of ammonia resulting from use of 7-day-old banked blood, peritoneal dialysis was instituted concurrently. Gradual and steady clinical improvement was noted, with striking reduction of serum bilirubin, although no data were presented on ammonia levels.

The removal of both direct-reacting and indirect-reacting bilirubin *via* peritoneal dialysis was studied (134). Minimal amounts of bilirubin (0.5 mg. %), all

direct reacting, were found in the dialysate with standard dialyzing fluid. The addition of albumin to the dialysate gave a progressive increase in removal of both direct- and indirect-reacting fractions of bilirubin. Despite removal of 747 mg. of bilirubin by dialysis, the patient's serum concentration had not decreased at the end of dialysis. Hobolth and Devantier (135) dialyzed a 4-day-old jaundiced newborn using an albumin-containing dialysis fluid. Their results were in agreement with those of Grollman and Odell (134) on indirect-reacting bilirubin. In both cases, serum half-life was 57.9 hr. and the removal rate was 1.2% of the total body bilirubin per hour. In an experimental study on Gunn rats (136), the addition of 3.5% human serum albumin to the dialysis fluid resulted in removal of over half the bilirubin of circulating blood. Replacement of albumin by various concentrations of dextran, polyvinylpyrrolidone, or lipid proved ineffective in extracting bilirubin.

Hyperuricemia-complicated leukemia has been treated with peritoneal dialysis (137–140). Up to 4.5 g. of uric acid was removed in 29 hr. of dialysis (137). A peritoneal urate clearance, ranging from 4.3 to 13.0 ml./min., was reported by Barry *et al.* (138). Maher *et al.* (140) treated a leukemic patient in whom mannitol had failed to produce diuresis. They administered allopurinol to block further urate synthesis and instituted peritoneal dialysis. The urate blood level was decreased sharply.

Using dogs as experimental animals, Knochel and Mason (141) found that alkalinization of the dialysate with tris(hydroxymethyl)aminomethane or acidification of plasma by induced respiratory acidosis increased urate concentration in the peritoneal fluid. This finding suggested that the peritoneum has a preferential permeability to nonionized uric acid. Urate diffusion across the membrane was not affected by lactate or pyrazinamide, both agents being known to block renal excretion, and this was indicative that transport across the peritoneal membrane of urate is not active transport.

Oxalate has also been removed by peritoneal dialysis. Zarembski *et al.* (142) used continuous dialysis on a patient with primary hyperoxaluria and renal failure. The peritoneal clearance of oxalate was found to be 5.1–6.6 ml./min. Despite the removal of 43 mg./day, the plasma oxalate remained elevated, probably due to the rate of endogenous production exceeding the rate of dialysis.

Peritoneal dialysis was used to treat acute pancreatitis, with the rationalization that the damaged pancreas may liberate toxic proteolytic enzymes and vasoactive peptides into the peritoneal cavity and that these might be removed by peritoneal lavage to reduce the complications of shock and visceral damage (143). Rasmussen (144) used hypothermic peritoneal dialysis in treating experimental hemorrhagic pancreatitis and reported that this technique markedly decreased the amount of hemorrhage and necrosis within the pancreas as well as the rise in serum enzymes. Favorable results also were reported with peritoneal dialysis alone (145, 146) or with added proteinase inhibitor (143).

Removal of Drugs and Exogenous Poisons—A wide variety of drugs and industrial chemicals are involved in accidental poisonings and attempted suicides, and either peritoneal dialysis or hemodialysis is often used in an attempt to remove these agents. In general, hemodialysis offers more rapid removal, which is of prime importance in such cases, but often an artificial kidney is not available or the patient's condition does not allow its use. Excellent summaries of dialysis in the treatment of poisonings were published by Maher and Schreiner (147) and Schreiner (148).

Sedatives and Tranquilizers—In the broad group of sedatives and tranquilizers, the barbiturates are the most commonly met in poisonings, especially in attempted suicides. Of these, pentobarbital is the most frequent agent. In 1951, Muirhead (149) suggested the use of peritoneal dialysis for barbiturate intoxication; since then, it has been studied by several workers. Lackey *et al.* (150) used isotonic and hypertonic dialysis fluids in dogs and found that not more than 6.9% of the dose was removed in a 3-hr. period and that the hypertonic fluid was no better than isotonic. Bloomer (151) studied the removal of pentobarbital and concluded that the amount removed by peritoneal dialysis was so low, about one-fourth that removed by metabolism, that he questioned its value. Berman and Vogelsang (152) studied the effect of albumin added to the dialysis fluid and obtained clearances 2–3 times that with standard fluid. Bourne (153) also tested fluid with human serum albumin added and found that the preservative in the albumin interfered with the barbiturate assay. Later, Bourne *et al.* (154) repeated these tests, using radioactive pentobarbital, and found albumin to increase the amount removed by only 30%. This result was partially due to low returns of dialysate because of coagulation of the albumin in the peritoneum, and a full return would have yielded an increase of about 75%.

Recently, Kudla *et al.* (155) studied peritoneal dialysis of pentobarbital in rabbits and found that 0.25% of *N*-myristyl-β-aminopropionic acid added to dialysis fluid gave clearances about twice that of control fluid. A case of attempted suicide with pentobarbital was treated by Valtin (156). Peritoneal dialysis was conducted for 14 hr. The initial and final serum concentrations were 3.6 and 2.2 mg. %, respectively. Dialysis removed 182 mg. of the drug. The estimated dose taken was 3–4 g. Lash *et al.* (157) utilized peritoneal dialysis for both removal of drug and "core" rewarming. The patient was saved. Approximately 237 mg. of drug was removed, and the dose taken was 3 g. From reports to date, it is still dubious that peritoneal dialysis removes sufficient pentobarbital to have a significant effect upon the patient's condition.

With the other barbiturates, the rate of removal by peritoneal dialysis is said to be related to their rate of diffusion, degree of protein binding, and degree of ionization (158). Campion and North (158) tested the addition of albumin and tris(hydroxymethyl)aminomethane to the dialysis fluids. The albumin served to bind the barbiturates within the peritoneum, while the tris(hydroxymethyl)aminomethane caused ionization of them in the peritoneal fluid, both effects reducing the effective concentration within the dialysate and

thus increasing the concentration gradient and rate of diffusion. They obtained clearances for three barbiturates with control and 6% albumin solutions, respectively, as follows: for amobarbital, 6.2 and 10.2 ml./min.; for secobarbital, 5.7 and 9.7 ml./min.; and for phenobarbital, 4.8 and 6.3 ml./min. Knochel *et al.* (36) previously found tris(hydroxymethyl)-aminomethane to remove phenobarbital at twice the rate of control fluid; later, Knochel and Barry (159) demonstrated removal of secobarbital 10 times as fast with tris(hydroxymethyl)aminomethane as with control fluid. Berman and Vogelsang (152) found addition of albumin to remove secobarbital 1.5 times as fast as the control. Kudla *et al.* (155) found 0.25% *N*-myristyl-β-aminopropionic acid to give clearances for phenobarbital of about 1.8 times the control; for amobarbital and butabarbital, clearances were about twice those for the control.

Rosenbaum and Mandanas (160) used a recirculation technique with anion-exchange resin and tested dialysis with lipid and albumin fluids for removal of phenobarbital. They found 5% albumin to be the most effective agent. Setter *et al.* (161) obtained clearances for secobarbital of 4 ml./min. with control fluid and 6 ml./min. with 2.5% albumin. Cohen (14) used peritoneal dialysis for removal of amobarbital in a poisoning case. With an initial blood level of 1.65 mg. %, he removed 129 mg. The amount absorbed by the patient was unknown. In a patient who had ingested 1.3 g. secobarbital and 3.5 g. butabarbital, urinary clearance was poor and peritoneal dialysis cleared 5–10 times as much as did the kidneys (162). Whiting *et al.* (163) were able to remove 2.24 g. of phenobarbital by two courses of dialysis, 36 hr. each, from a patient who had probably ingested 10 g. of the drug. From the literature reports, it appears that peritoneal dialysis is an effective means for removal of phenobarbital, it is somewhat effective clinically with butabarbital and amobarbital, and its clinical value with secobarbital and pentobarbital is questionable.

Glutethimide has been involved in a number of poisonings, and overdosage is particularly difficult to manage (164). Peritoneal dialysis was used by Frey (165) in treatment of a patient who had ingested 15 g. of the drug. Thirty-two exchanges, one every 2 hr., were performed. The amount of glutethimide recovered in the dialysate was 705 mg. (4.7% of the dose) which, though small, was 5 times as much as was excreted by the kidneys. Both Frey (165) and Barbour (166) recommended peritoneal dialysis in spite of the low concentrations removed in dialysate. The rapidity with which peritoneal dialysis can be started and the fact that it can be maintained constantly led them to feel that it was advantageous. McDonald *et al.* (167), in 1963, did not recommend peritoneal dialysis as adequate treatment for glutethimide intoxication. DeMyttenaere *et al.* (168) used forced diuresis, hemodialysis, and peritoneal dialysis concurrently on a case of glutethimide poisoning and recommended that use of the three procedures together be considered. Shinaberger *et al.* (169) developed a procedure for using 15% cottonseed oil emulsion as the dialysis fluid and tested it on dogs. With the lipid emulsion, they were able to increase the extraction of glutethimide more than fivefold.

Diphenylhydantoin is one of the most widely used anticonvulsants. Although there are relatively few reported cases of severe intoxication with it, early and rapid reduction in blood and tissue concentrations of diphenylhydantoin after large overdoses is believed to be of great urgency to avoid permanent damage to the nervous system (170, 171). Tenckhoff *et al.* (170) dialyzed a child who had been in a coma for 3 days following the ingestion of about 2.8 g. of the drug. Blood levels decreased from 11.2 to 0.75 mg. % in the first 16 hr. of dialysis and to 0.35 mg. % after 24 more hr. of peritoneal dialysis. Another case of acute toxicity was successfully treated with peritoneal dialysis by Andia *et al.* (172). The dialysate attained a concentration equal to two-thirds that of the plasma in 1 hr. Effective peritoneal dialysis was reported by Blair *et al.* (171) in a case of mixed diphenylhydantoin and primidone poisoning, with significant reduction in blood levels of both drugs. In experimental studies on rabbits, Kudla *et al.* (155) found that dialysis fluid with 0.25% *N*-myristyl-β-aminopropionic acid gave twice the peritoneal clearance obtained with a control fluid.

There is evidence for the beneficial use of peritoneal dialysis in the treatment of meprobamate intoxication. Hardy *et al.* (173) reported the successful treatment of a case of attempted suicide after ingestion of 32 g. of meprobamate as well as amobarbital and secobarbital. Dyment *et al.* (174) treated a case of meprobamate poisoning by peritoneal dialysis and reported a peritoneal clearance of 3.5 ml./min. In another patient who ingested 20 g. of meprobamate, Mouton *et al.* (175) reported recovery of 1375 mg. in the dialysate and a decrease in blood level from more than 10 to 0.8 mg. %. Removal of more than 1 g. in the dialysate and 900 mg. in the urine was reported by Castelle and Sode (176). In an experimental study to evaluate the potential of peritoneal dialysis, Maddock and Bloomer (177) used Ringer's lactate solution in a dog. The peritoneal concentration of meprobamate reached about 50% of the plasma concentration or about 60% of the concentration of diffusible meprobamate in 60 min.

Treatment of ethchlorvynol poisoning has been successfully carried out with peritoneal dialysis, although there are conflicting reports on the value of added albumin in the dialysis fluid. Schultz *et al.* (178) found that the addition of albumin significantly increased the removal rate of ethchlorvynol, but Teehan *et al.* (179) were unable to confirm this finding. Exchanges containing albumin gave only slight increases in removal which they attributed to a small increase in drainage volume due to the oncotic effect of added protein. In their study the concentration of the drug was the same in dialysates with and without albumin. They reported a peritoneal clearance of 18.5 ml./min. A combination of peritoneal dialysis and exchange transfusion was used in treating a child with acute ethchlorvynol intoxication (180).

Neuromuscular blockage by gallamine was promptly reversed by peritoneal dialysis in a patient with renal failure who received the drug during a surgical operation (181). A case of methaqualone poisoning was

treated by Proudfoot *et al.* (182) by alternate peritoneal dialysis and hemodialysis. Peritoneal clearance of methaqualone was estimated to be 7.5 ml./min. Paraldehyde intoxication was treated by peritoneal dialysis (183); metabolic acidosis associated with paraldehyde poisoning was properly corrected, but blood levels and clearances of the drug were not reported. Schreiner (148), in his extensive review, stated that the phenothiazines are not dialyzable. He cited a number of publications in which neither hemodialysis nor peritoneal dialysis was successful in removing these drugs.

Analgesics—Numerous cases of salicylate toxicity are encountered because of accidental ingestion, therapeutic overdosage, and suicidal attempt. The ensuing alterations in body chemistry represent a complex and serious metabolic disturbance. The initial respiratory alkalosis, as a result of hyperventilation due to stimulation of the respiratory center, is soon followed by metabolic acidosis. The complexity of such disturbance is greatest in infants and small children (184, 185).

Salicylate is readily removed by peritoneal dialysis, and clinical improvement in intoxicated patients following dialysis is well documented (166, 186–188). Cohen (14) reported recovery of 4.1 g. of salicylate in the dialysis fluid in a case of aspirin poisoning. In another case (189), the blood salicylate level was reduced from 78 mg. % by peritoneal dialysis, with removal of 427 mg. in the dialysate.

Success in accelerating salicylate dialysis *via* substances added to the dialysis fluid has been achieved. Nahas *et al.* (190, 191) used tris(hydroxymethyl)-aminomethane and bicarbonate with the aim of maintaining a maximum concentration gradient through high dialysate pH. Their experimental work with dogs gave 1.3 and 1.6 times as much removal with tris-(hydroxymethyl)aminomethane and bicarbonate, respectively, as with control fluid. The addition of albumin to the dialysis fluid also enhanced the removal of salicylate by virtue of the high affinity of salicylate for protein, especially at low concentrations. Etteldorf *et al.* (192) used 5% albumin solutions and pooled serum on dogs to remove about 1.5 times as much salicylate as with the control. Similar results were reported by James *et al.* (193). Three times as much salicylate was removed by the combined administration of bicarbonate and peritoneal dialysis with 5% albumin as by urine alkalinization alone (194). Etchart (195) recovered 3 g. of salicylate from 12 l. of dialysate during 16 hr. of peritoneal dialysis. Favorable clinical results and removal rates were also reported with 5% albumin solutions (196, 197). Mattocks (198) could accelerate salicylate clearance in rabbits by nearly 5 times by using a solution containing 1% anthranilic acid and 0.25% *N*-myristyl-β-aminopropionic acid. It was thought that the local unbinding effect of anthranilic acid on salicylate, both in plasma and on membrane proteins, might play a role in this acceleration. With 0.25% *N*-myristyl-β-aminopropionic acid alone added to the control fluid, peritoneal clearance was more than twice that of the control fluid.

Methyl salicylate poisoning also has been treated with peritoneal dialysis (14, 199, 200). Kloss and Boeckman (201) lowered the blood salicylate level in an infant poisoned with methyl salicylate from 118 mg. % after 24 hr. of dialysis, and 176 mg. of salicylate was removed in the peritoneal fluid.

Acute poisoning after ingestion of propoxyphene is rare. Karliner (202) listed 15 cases from the literature, including a fatal case he had treated with 122 exchanges of peritoneal fluid. Initial blood level was 0.97 mcg./ml., and the dialysate concentration ranged from 0.24 to 0.03 mcg./ml.

Peritoneal dialysis was used to treat a patient with acute acetaminophen poisoning after ingestion of 24.4 g. (203). The initial blood level of the drug was 10.2 mcg./ml., and 10.2 mg. was removed in the dialysate in 6 hr.

Antidepressants—Wallace *et al.* (204) reported the successful treatment of a 9.08-kg. (20-lb.) child who had typical symptoms of amphetamine toxicity and had ingested an unknown number of tablets containing 15 mg. methamphetamine and 65 mg. phenobarbital each. Because of difficulties in hemodialyzing a small child and the promptness with which peritoneal dialysis could be started, peritoneal dialysis was chosen for treatment. After dialysis for 5 hr., the symptoms of toxicity abated. A total of 67 mg. of methamphetamine and 140 mg. of phenobarbital was removed in the dialysate. In an experimental study, Zalis *et al.* (205) administered lethal doses of amphetamine to dogs and treated the test group of 13 animals with peritoneal dialysis over 6 hr. Eighty-five percent of the animals showed no signs of toxicity, and 69% survived. The authors noted that the recovery of unchanged amphetamine in the dialysate was so low, 2.3%, that it did not reflect the value of the treatment. They concluded that peritoneal dialysis appears to be an effective form of therapy for amphetamine poisoning.

Sunshine and Yaffe (206) reported a case of amitryptyline poisoning in a 15-month-old child where peritoneal dialysis was used for 16 hr., removing only 0.15% of the ingested drug. They suggested that firm protein binding, rapid entry into and fixation to body tissues, and poor water solubility of the drug accounted for its poor removal.

Lipkin and Kushnick (207) successfully treated a child with pargyline poisoning by using peritoneal dialysis. Intermittent exchange was continued for 24 hr., at which time the patient was much improved. Amounts of drug extracted by dialysis were not measured.

Alcohols—Setter *et al.* (208) summarized results with removal of methanol by hemodialysis and peritoneal dialysis. They pointed out that with extremely high levels of methanol, hemodialysis should be used to reduce the level as rapidly as possible with the hope of avoiding visual impairment. In dogs dialyzed with 1.5 and 7% glucose fluids, average clearances were 24 and 31 ml./min., respectively, using peritoneal dialysis. Stinebaugh (209), in 1960, reported on clinical use of peritoneal dialysis in methanol intoxication. Initial blood levels of 180 and 200 mg. % in two patients were reduced to zero after 18 and 12 hr. of dialysis. A third patient, with an initial blood level of 532 mg. %, died. In the two patients who recovered, vision was left impaired. Wenzl *et al.* (210) reported the recovery of a 10-week-old infant with severe

methanol poisoning by treatment with peritoneal dialysis and intravenous bicarbonate. During an outbreak of methanol poisoning in Kentucky, Kane *et al.* (211) treated four cases with peritoneal dialysis, three of whom survived. Peritoneal clearances were 5–10 times that obtained with forced diuresis. For ethanol poisoning in a 4-year-old child, Dickerman *et al.* (212) used peritoneal dialysis and increased the removal of alcohol more than twofold over the expected rate without dialysis.

Heavy Metals—Mehbod (213) combined the use of calcium sodium edetate and peritoneal dialysis in the treatment of acute lead poisoning. He found that intravenous edetate greatly increased the amount of lead removed in the dialysate in three of four patients. Even with edetate, considerably more lead was cleared by dialysis than by the kidneys. Batson and Peterson (214) reported the use of peritoneal dialysis along with dimercaprol for mercury poisoning and recommended the combined procedure. Covey (215) used edetate along with peritoneal dialysis for the recovery of a child with ferrous sulfate poisoning. Lavender and Bell (216) found peritoneal dialysis to remove so little iron in a case of iron intoxication in an adult that its use was of doubtful value.

Miscellaneous Drugs and Poisons—Cocco and Pazourek (217) treated a 3-year-old child, who had swallowed 2304 mg. of isoniazid, with peritoneal dialysis for 72 hr., recovering 52% of the dose in the return fluid.

Gurr (218) used peritoneal dialysis after hemodialysis in the treatment of a patient with eucalyptus oil poisoning. The value of peritoneal dialysis in this case was not clear.

Yeh *et al.* (219) found that peritoneal dialysis and hemodialysis removed about the same amounts of α-methyldopa (60%) in patients using this drug.

Successful removal of boric acid in poisoning cases can be effected by peritoneal dialysis (20, 220–222). Segar (220) reported on three newborns who were severely poisoned through error by using boric acid solutions in their feeding mixture. Two survived. Marked clinical improvement was noted following 2 days of peritoneal dialysis, and dialysis removed 3.5 and 2.2 g. of boric acid in the two surviving cases Wong *et al.* (221) also reported favorable results with peritoneal dialysis for boric acid intoxication. Baliah *et al.* (222) treated an infant with boric acid poisoning, with the serum concentration dropping from 43 to 30 mg. % after 10 hr. of dialysis and to 15 mg. % after 34 hr. The amount removed in the dialysate was more than 752 mg.

The efficiency of peritoneal dialysis for the removal of quinine is controversial. Markham *et al.* (223) successfully dialyzed a patient who had ingested 3.84 g. of quinine sulfate. Over 16 hr., dialysis removed 415 mg., associated with a decline of plasma level from 9.2 to 5.25 mg./l. McKenzie *et al.* (224) also described cases of severe quinine poisoning. In one patient, dialysis for 24 hr. removed 640 mg., but blood levels were not available. In the second patient, 24 hr. of dialysis was accompanied by a decrease in plasma level from 9.2 to 5 mg./l. Other workers (87, 225–227) reported peritoneal dialysis to remove insignificant amounts of quinine in humans and in dogs. Poor peritoneal clearance was thought to be due to high protein binding, 70% (228). Quinine extraction after alkaline buffering of the dialysis fluid with tris(hydroxymethyl)aminomethane to pH 10 was one-fourth as great as with commercial fluid, pH 5.0 (225). This finding leads one to expect that the ionized form of quinine may be dialyzed more rapidly; if so, the variation in results reported may have been due to pH conditions of the individual dialysis.

Ackerman *et al.* (229) studied the dialysis of digoxin in 14 patients and reported a peritoneal clearance of 8 ml./min. and a serum half-life of 88 hr.

Graw and Clarke (230) reported a case of mixed chlorpropamide and salicylate toxicity. Peritoneal dialysis removed an insignificant amount of chlorpropamide, even with the addition of albumin.

Schmitt *et al.* (231) studied the clearance of bromide in dogs and reported a peritoneal clearance of 13.7 ml./min. as compared to 0.4 ml./min. for spontaneous urinary clearance.

A patient who took 40 g. of sodium chlorate was successfully managed by peritoneal dialysis (232). Pringle and Smith (233) also mentioned a case where the patient recovered after 14 days of dialysis.

Peritoneal dialysis was demonstrated to be effective in clearing chromium in a case of potassium dichromate toxicity, with a drop in serum level from 590 to 90 mcg. % after 84 hr. (234).

Thomas (235) treated a patient who had ingested 150 ml. of Lysol (50% cresols in soap) with peritoneal dialysis for 23 hr. The first exchange was found to contain 10 mg. free phenol. The initial total phenol serum level was 950 mcg. %.

Atkins *et al.* (236) treated cycloserine poisoning with peritoneal dialysis and obtained good reduction in plasma levels and removal of toxic symptoms after 21 hr. Measurements of amount in dialysate were felt to be inaccurate due to decomposition of the drug in the fluid and failure to account for metabolites.

Swick *et al.* (237) used peritoneal dialysis in colistin intoxication in a patient where renal failure had occurred. Dialysis effectively removed the drug and restored the kidney function.

Morton (238) treated a 15-month-old child for tetracycline intoxication and demonstrated efficient removal of the drug in the dialysate. She pointed to the value of peritoneal dialysis, particularly in the period of oliguria.

PROBLEMS OF ELECTROLYTE BALANCE

The problems and complications of electrolyte balance in peritoneal dialysis are numerous and complex. Only a few of these, which are peculiar to peritoneal dialysis, can be included in this review. In general, problems have stemmed from: (*a*) failure to take into account that each chemical species will seek equilibrium without regard to the other substances present; (*b*) failure to adjust concentrations to allow for volume changes in dialysis fluid, especially when it is hypertonic; (*c*) failure to account for changes in binding of

certain electrolytes with change in pH; and (*d*) removal of certain electrolytes from extracellular fluid so rapidly that equilibrium between intracellular and extracellular fluids cannot be maintained. These "failures" are not the result of negligence but are due to the fact that the dynamics of the substances under these conditions and their interrelationships are not fully understood. A number of these complications, as well as other hazards of peritoneal dialysis, were discussed by Maher and Schreiner (239), Ribot *et al.* (240), Matalon *et al.* (241), and Stewart *et al.* (242).

Maher and Schreiner (239) described the dialysis disequilibrium syndrome which results from overly rapid removal of urea from extracellular fluid. This creates an osmotic gradient between extracellular fluid and intracellular fluid which may result in cerebral edema. They also discussed the problem encountered with hypertonic solutions in patients with carbohydrate intolerance. Glucose is added to make the solution hypertonic so as to remove large amounts of water. Water moves into the fluid faster than glucose is absorbed, and the glucose is expected to be metabolized rapidly enough to keep the blood sugar low. The patient who cannot metabolize the glucose rapidly may attain extremely high glucose blood levels, which may lead to cellular dehydration.

In the use of hypertonic dialysis solutions, the movement of water is generally faster than that of electrolytes; rapid exchanges of such fluid may remove a disproportionate amount of water, causing concentration of the extracellular electrolytes. This was the finding of Nolph *et al.* (243) with sodium, and it explained the frequent occurrence of hypernatremia with the use of hypertonic dialysis fluids. They suggested the adjustment of sodium concentrations in hypertonic fluids by a simple factor which allows for the sieving coefficient of the ion.

Swales (244) measured sodium absorption in a number of dialyzed patients. He found that the usual dialysis fluid, with 140 meq./l. of sodium, may greatly increase the plasma sodium level of the hyponatremic patient and thus may result in pulmonary edema and heart failure. Swales (244) suggested that this be avoided either by use of low sodium fluid for the hyponatremic patient or by use of fluids sufficiently hypertonic to assure withdrawal of large volumes of fluid from the patient.

The uremic patient on digitalis therapy may have a digitalis level above normal to adjust for the depressant effect of high potassium levels. Upon dialysis with low potassium fluids, the depressant effect may be removed and the patient endangered by digitalis toxicity. Maher and Schreiner (239) encountered nine such episodes.

Hypocalcemia is a common finding in chronic uremia patients, even though calcium is included in the dialysis fluid. Garrett and Cuddihee (245) measured calcium diffusion and concluded that, on the average, 13% of instilled calcium is absorbed across the peritoneal membrane. The absorption was found to be a function of the fluid loss during dialysis. Atkinson *et al.* (246) made radiological measurements of the mineral content of bone of patients on maintenance dialysis, and their results showed a gradual loss of bone with time.

COMPLICATIONS AND HAZARDS OF PERITONEAL DIALYSIS

Peritoneal Infection—In early studies of peritoneal dialysis, infection of the peritoneum was recognized as a major hazard (247, 248); it still is a problem, especially in chronic cases (243). Frank *et al.* (248), in 1946, suggested that the chief sources of contamination were improper preparation of fluid and apparatus, introduction of bacteria *via* the skin at the site of puncture, and introduction of contaminated air into the catheter. These sources have been removed by development of present-day commercial catheters, fluids, and administration sets and by better preparation and protection of the puncture area. The addition of other substances to the dialysis fluid at the bedside undoubtedly results in some contamination, as has been the case with intravenous fluids, but the development of intravenous additive programs should improve this situation.

In spite of the improved techniques and equipment, reports in recent years still showed frequent occurrence of peritoneal infection (56, 57, 249–251). Some of the practices of handling administration sets were faulty, such as using the tubing for repeated dialysis over a period of time long enough to develop significant growths from slight contamination in the tubing, allowing the outflow tube to be in contact with collected fluid, and using nonsterile containers for collection of outflow fluid. Mowbray (252) and Cohen and Percival (25) demonstrated the reduction in infection which might be attained *via* improvements in technique.

It was found that the return fluid is commonly contaminated with bacteria; Schweinburg *et al.* (253) demonstrated that bacteria can migrate across the intestine, which may explain the frequent finding of contaminated effluent in the absence of any indication of peritonitis.

A number of workers used antibiotics prophylactically in peritoneal dialysis, often by adding them directly to the dialysis fluid (85, 93, 94, 115, 244, 250). Others felt that this was unnecessary and even irrational, since the antibiotic being used may not be effective against the organism involved (60, 254).

The present attitude with regard to antibiotics appears to be not to use them prophylactically but to institute therapy promptly should evidence of infection be found. Numerous articles have appeared regarding the effectiveness of antibiotics in peritonitis when administered therapeutically in the dialysis fluid (255–265). Although there is variation between drugs and between infections, the general attitude appears to be that antibiotics should be used intraperitoneally for local effect and intramuscularly (when appropriate) for systemic effect when septicemia is present.

Loss of Protein—Occurrence of protein in dialysate was noted early; and Boen (266) found 0.5–1.0 g./l., representing a loss of about 40 g. in a 48-hr. dialysis period. The distribution of proteins was roughly equivalent to that of serum.

Berlyne *et al.* (267) measured protein losses in 12 patients. Volumes of dialysis fluid varied from 14 to 79 l. and protein concentration in return fluid ranged from 0.68 to 4.49 g./l. Total protein losses for individual patients ranged from 10 to 207 g., which led to a mea-

surable fall in serum protein. They recommended that plasma protein levels be followed in peritoneal dialysis patients and that replacement therapy be instituted as needed.

Strauch *et al.* (268) studied the effect of variables of procedure in peritoneal dialysis upon the loss of protein. They observed that longer periods of stay of fluid in the abdomen resulted in higher protein loss, that increased osmolarity of the fluid gave higher loss, and that the loss of protein was greatest in the first exchange, dropping rapidly during the following five periods and then remaining practically constant. Their findings were interpreted as representing a constant rate of transfer from blood into dialysate, thus yielding the higher concentration in the first exchange with highest concentration gradient and highest concentration with longest dwell time. They pointed out that the loss in patients with peritonitis may reach 300 g.; in such cases, the loss cannot be made up by dietary means. They recommended recovery of proteins between dialyses and reinfusion, use of fluids with as low an osmolarity as possible, use of as short a cycle time as possible, and intravenous replacement of protein for patients in negative nitrogen balance.

In another report, Berlyne *et al.* (269) measured amino acid losses, as well as protein losses, and found a wide spectrum of amino acids present in the dialysate. Average losses of amino acids were 2.96 g. for an average of 27 l. of dialysate. The protein losses were 10–40 g., but there seemed to be no direct correlation between protein and amino acid losses. They pointed out that the loss of amino acids causes the hypoproteinemia to be more persistent. They recommended dietary or parenteral replacement of amino acids as well as proteins.

Gordon and Rubini (270) studied losses in 38 patients. The average protein loss in dialysis was about 72 g., with a wide variation of 27–248 g. lost during a 24-hr. dialysis. The wide variations could not be attributed to osmolarity of fluid, infection, plasma protein concentration, or clinical state of the patient. Protein losses tended to be consistent with the same patient in repeated dialysis. In successive exchanges, the concentration was usually higher in the first. Protein concentration increased with increased osmolarity (for a given patient). Sampling of a single exchange showed the protein to reach a concentration maximum in about 20 min.

Lindner and Tenckhoff (271) ran protein balance studies on patients who had been on peritoneal dialysis at home for 6 months or longer. They found nitrogen losses greater than intake on dialysis days, but this situation was more than compensated for during nondialysis days. The protein losses during dialysis were lower than had been previously observed when repeated puncture was used or than were usually reported in the literature; the authors suggested that the atraumatic nature of dialysis with implanted catheters, as used in this study, may explain this finding. Their data showed that long-term peritoneal dialysis can maintain the patient active and well to a degree comparable to that with hemodialysis.

An interesting observation was recorded by Gutch (272), who found that silastic catheters appeared to cause lower protein loss than those made of other materials. No mechanism for this finding is yet presented.

Pulmonary Complications—Pulmonary complications are occasionally an adverse side effect of peritoneal dialysis. Berlyne *et al.* (273) included pneumonia, atelectasis, purulent bronchitis, and pleural effusion in this category. They stated that distention of the peritoneal cavity with dialysis fluid causes elevation of the diaphragm, with reduction in vital capacity. For the uremic patient with lowered resistance, infection of the collapsed segments may occur and further infection may result from aspiration of retained bronchial secretions due to inhibition of coughing. They compared patients with a 2-hr. dialysis cycle to those with a 45-min. cycle which used a 5–7-min. drainage facilitated by vacuum. They also varied dialysis fluid volume. With the slow cycle, 2-l. dialysis, six of eight patients developed pulmonary complications. With the rapid cycle, 2-l. dialysis, three of seven patients had pulmonary problems; with the rapid cycle, 1-l. dialysis, only one of nine patients had a pulmonary complication. These workers recommended reduction of the amount of dialysis fluid for each exchange from 2 to 1 l., shorter cycles for exchange, reduction of overall dialysis time, and use of a sitting position during dialysis.

Swartz *et al.* (274) studied conditions leading to pulmonary edema during peritoneal dialysis. They found that in most patients in supine position and in all patients in sitting position, the instillation of peritoneal fluid led to an immediate fall in cardiac output. This fall was reversed by drainage of the fluid. They suggested this effect may be due to a decrease in lung volume and vascular bed due to mechanical factors or by release of pressor amines causing pulmonary vasoconstriction. They recommended that, upon recognition of pulmonary edema, the dialysis fluid be drained immediately. They further stated that dialysis can usually be restarted using smaller volumes of fluid without difficulty.

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RESEARCH ARTICLES

Molecular Interactions and Solubility of Polar Nonelectrolytes in Nonpolar Solvents

HO-LEUNG FUNG* and TAKERU HIGUCHI

Abstract □ An attempt was made to describe quantitatively the deviations from ideal solubility of nonelectrolytes in nonpolar solvents as a result of three contributions: (*a*) entropic, (*b*) regular solution, and (*c*) specific interactions. The relative magnitudes of these contributions were theoretically estimated. Experimentally, it was found that for the systems studied involving poorly soluble polar solutes in a series of hydrocarbon solvents, the entropic correction term appeared to account for deviation from ideal solubility behavior. Regular solution correction appeared to be unnecessary for these systems. Specific interactions were found to be the dominant factors in determining solubilities of polar solutes in solvents possessing acid–base characters. Solvate association constants between various solutes and chloroform or ether were determined by the solubility technique. The good agreement between solubility data and solvate equilibria equations for a wide variety of chemically different solutes and the two interacting solvents seems to indicate support for the stoichiometric solvate species treatment of solubility.

Keyphrases □ Solubility, nonelectrolytes, polar—in nonpolar solvents, consideration of entropic–enthalpic–specific interactions □ Nonelectrolytes, polar, solubility in nonpolar solvents—consideration of entropic, enthalpic, and specific interactions □ Solvation—polar organic solvents, stoichiometric species approach

The ability to predict thermodynamic activity coefficients of drug molecules in any given environment would be of immense value in considerations of mechanisms of drug delivery. The magnitudes of these coefficients are largely determined by the nature and extent of molecular interactions between the dissolved drug and its surrounding solvent molecules. The various types of interactions present in different environments are often reflected in the macroscopic solubility of the drug in these media. A careful analysis of the various factors and their relative contributions that determine the solubility would help toward a better understanding of molecular interactions in solution.

The solubility of nonpolar nonelectrolytes in nonpolar solvents can often be explained on the basis of cohesive energy differences between the components (1). The same regular solution treatment, however, has now been extended to systems involving polar nonelectrolytes as well as polar solvents such as dimethylacetamide, propylene glycol (2), nitrobenzene, and carbon disulfide (3). Recently, Higuchi *et al.* (4) pointed out that although straight-line correlations can sometimes be obtained for irregular systems using the solubility parameter (5), correlation with regular solution theory in these cases goes well beyond its original intentions. Instead, the data can be better explained on the basis of specific solvation (6).

In this paper, data are presented on the solubility of a variety of nonelectrolytes such as acetanilide, carbazole, picric acid, salicylic acid, and phthalic anhydride in organic solvents ranging from the completely nonpolar hydrocarbons through more polar solvents such as chloroform, diethyl ether, and acetic acid. The results are analyzed to determine quantitatively the relative contribution of "physical" *versus* "chemical" interactions toward solubility in these systems. The nature of specific solvation involving polar organic solvents is also discussed.

EXPERIMENTAL

Reagents—All reagents were of reagent grade. Cyclohexane was distilled over phosphorus pentoxide. *n*-Hexane (ACS) was used without further purification. Isooctane (2,2,4-trimethylpentane) was distilled over sodium. Dodecane was passed through a column of silica gel. *n*-Heptane, decane, and hexadecane were purified by passing the solvent through a column of silica gel and then distilling over sodium under reduced pressure. Diethyl ether, tetrahydrofuran, and *n*-propyl ether were distilled over lithium aluminum hydride. Chloroform was washed with distilled water five to six

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The ability to predict thermodynamic activity coefficients of drug molecules in any given environment would be of immense value in considerations of mechanisms of drug delivery. The magnitudes of these coefficients are largely determined by the nature and extent of molecular interactions between the dissolved drug and its surrounding solvent molecules. The various types of interactions present in different environments are often reflected in the macroscopic solubility of the drug in these media. A careful analysis of the various factors and their relative contributions that determine the solubility would help toward a better understanding of molecular interactions in solution.

The solubility of nonpolar nonelectrolytes in nonpolar solvents can often be explained on the basis of cohesive energy differences between the components (1). The same regular solution treatment, however, has now been extended to systems involving polar nonelectrolytes as well as polar solvents such as dimethylacetamide, propylene glycol (2), nitrobenzene, and carbon disulfide (3). Recently, Higuchi *et al.* (4) pointed out that although straight-line correlations can sometimes be obtained for irregular systems using the solubility parameter (5), correlation with regular solution theory in these cases goes well beyond its original intentions. Instead, the data can be better explained on the basis of specific solvation (6).

In this paper, data are presented on the solubility of a variety of nonelectrolytes such as acetanilide, carbazole, picric acid, salicylic acid, and phthalic anhydride in organic solvents ranging from the completely nonpolar hydrocarbons through more polar solvents such as chloroform, diethyl ether, and acetic acid. The results are analyzed to determine quantitatively the relative contribution of "physical" *versus* "chemical" interactions toward solubility in these systems. The nature of specific solvation involving polar organic solvents is also discussed.

EXPERIMENTAL

Reagents—All reagents were of reagent grade. Cyclohexane was distilled over phosphorus pentoxide. *n*-Hexane (ACS) was used without further purification. Isooctane (2,2,4-trimethylpentane) was distilled over sodium. Dodecane was passed through a column of silica gel. *n*-Heptane, decane, and hexadecane were purified by passing the solvent through a column of silica gel and then distilling over sodium under reduced pressure. Diethyl ether, tetrahydrofuran, and *n*-propyl ether were distilled over lithium aluminum hydride. Chloroform was washed with distilled water five to six

Table I—Recrystallizing Solvents of Various Solids and Their Melting Points

Solid	Recrystallizing Solvent	Melting Point—Observed	Melting Point—Literature[a]
Acetanilide	Aqueous ethanol	113–114°	113–114°
Benzophenone	Petroleum ether	48–49°	48.1°
p-Benzoquinone	Petroleum ether	114–116°	115–117°
Carbazole	Aqueous ethanol	242–243°	247° (245°[b])
Phenyl benzoate	Ethanol	69–69.5°	71°
Phthalic anhydride	Chloroform–cyclohexane	130–131°	130.8°
Picric acid	Chloroform	121–122°	122–123°
Salicylic acid	Hot water	156–158.5°	159°
Succinic anhydride	Acetic anhydride	119–120°	119.6°

[a] "Handbook of Chemistry and Physics," 48th ed., Chemical Rubber Co., Cleveland, Ohio, 1967. [b] "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N. J., 1968.

times, dried over powdered calcium chloride overnight, filtered, and distilled over phosphorus pentoxide. Glacial acetic acid was dried over molecular sieves[1]. Pyridine was dried by refluxing with solid KOH, followed by fractional distillation. *tert*-Butyl alcohol was dried over $MgSO_4$, filtered, distilled, and stored over molecular sieves. Methanol-free acetone was dried with molecular sieves.

Solids were recrystallized from the solvents indicated in Table I.

Solubility Studies—An amount of solid at least five times in excess of its solubility was allowed to equilibrate with the solvent or solvent mixture in a stoppered and sealed volumetric flask, which was continuously shaken on a wrist-action shaker[2] in a thermostated bath (25.0°) for at least 24 hr. An aliquot of the saturated solution was pipeted out, and the concentration of the solute was determined by the gravimetric, titrimetric, or spectrophotometric (UV) method.

Gravimetric Determination—The aliquot of saturated solution was quantitatively transferred to a preweighed aluminum pan. The solvent was allowed to evaporate until a control sample showed that evaporation was completed. The molar solubility was then calculated from the solid residue left. The method was used for cases in which the solubility of solute was quite large, *e.g.*, benzophenone and phenyl benzoate in all solvents studied, and phthalic anhydride in strongly interacting solvents.

Titrimetric Method—Phthalic and succinic anhydride solubilities in moderately interactive solvents such as diethyl ether, di-*n*-propyl ether, glacial acetic acid, and *tert*-butyl alcohol were determined by nonaqueous titration with morpholine, as described by Siggia (7).

Spectrophotometric (UV) Method—For systems in which the saturated solubility was very small and the solutes exhibited a UV peak with large enough molar absorptivity, the aliquot was diluted accurately from 10 to 100 times in either chloroform, ether, or cyclohexane; the concentrations were determined by UV. Table II shows the diluent solvent used, the wavelength at which UV measurements were made, and the molar absorptivities at these wavelengths. The solutes in these solutions were found to obey Beer's law in the concentration ranges used[3].

Determination of Solvate Formation Constants—The solvate formation constants are defined by:

$$K_{ij} = \frac{(A_iS_j)}{(A)_0^i(S)^j} \qquad \text{(Eq. 1)}$$

for the equilibrium $iA + jS \overset{K_{ij}}{\rightleftharpoons} A_iS_j$ in a system which is comprised of the solute A, inert solvent I, and interacting solvent S. A_0 is the molar solubility of the solute in I. The solutions studied are usually dilute, so that i is usually unity.

It can be readily shown that:

$$(A)_{obs.} = (A)_0 + \sum K_{ij}(A)_0^i(S)^j \qquad \text{(Eq. 2)}$$

where $(A)_{obs.}$ = the observed solubility of A in the solvent mixture.

If only the first-order term is important, $K_{1:1}$ can be obtained from the slope of the plot $(A)_{obs.} - (A)_0$ *versus* (S), as shown in Eq. 3.

$$(A)_{obs.} - (A)_0 = K_{1:1}(A)_0(S) \qquad \text{(Eq. 3)}$$

If both first- and second-order interactions are important, the constants can be obtained from the slope and intercept of a plot from Eq. 4:

$$\frac{(A)_{obs.} - (A)_0}{(S)} = K_{1:1}(A)_0 + K_{1:2}(A)_0(S) \qquad \text{(Eq. 4)}$$

If the third-order term is also significant, $K_{1:1}$ can be estimated from the increase in solubility at low interactive solvent concentrations, at which region Eq. 3 is approximately obeyed. The higher order terms can then be obtained by the following relationship:

$$\left[\frac{(A)_{obs.} - (A)_0}{(S)} - K_{1:1}(A)_0\right]\Big/(S) = K_{1:2} + K_{1:3}(S) \qquad \text{(Eq. 5)}$$

RESULTS AND DISCUSSION

Entropic and Cohesive Energy Contributions for Inert Solvents—Tables III and IV show, respectively, the mole fraction and molar solubilities of acetanilide, carbazole, picric acid, salicylic acid, and phthalic anhydride in inert hydrocarbon solvents and two slightly polar solvents possessing acid–base characters—*viz.*, chloroform and ether. The mole fraction solubilities were obtained by assuming sufficient dilute solutions such that the total number of moles present was equal to the number of moles of solvent. In Table V, the dielectric constant, dipole moment, molar volume, and solubility parameter of the solvents are listed. Several general features are apparent from these results:

1. For inert hydrocarbon solvents, the solubility generally appeared to increase with an increasing molar volume of the solvent. However, the differences in solubility in these solvents were quite small.

2. The *molar* solubilities of the solutes in inert hydrocarbon solvents appeared to be identical, within experimental error, and seemed to be independent of the molar volume and solubility parameter of the solvent.

3. For the solutes studied, the solubilities were much higher in chloroform and ether. The relative magnitudes of solubilities in these two solvents seemed to be very dependent on the nature of the solute.

These observations may be rationalized by considering the relative contributions of nonspecific (physical) and specific (chemical) interactions toward the observed solubility in various solvents.

For hydrocarbons, specific solute–solvent interaction is absent. The solubility of a poorly soluble solute in these solvents can be broken down into just entropic (athermal) and enthalpic (regular solution) contributions. The Flory–Huggins expression (8, 9) for size correction can be adopted (10, 11) to calculate the statistical contribution to the infinite dilution solute activity coefficient ($\gamma_2^{ath.}$):

$$\ln \gamma_2^{ath.} = \ln \frac{1}{r} + \left(1 - \frac{1}{r}\right) \qquad \text{(Eq. 6)}$$

where r is the ratio of the molar volume of the solvent to that of the solute, V_1/V_2. Where the solute is only very slightly soluble, $\gamma_2^{ath.}$ is approximately inversely proportional to its saturated solubility, if

Table II—UV Conditions Used for Determination of Solubilities of Various Solutes in Inert Solvents

Solute	Diluent Solvent	Wavelength, nm.	Molar Absorptivity, M^{-1} cm.$^{-1}$
Acetanilide	Cyclohexane	238.5	1.4093×10^4
Carbazole	Ether	292.5	1.873×10^4
Phthalic anhydride	Chloroform	300	2.175×10^4
Picric acid	Chloroform	336	4.816×10^3
Salicylic acid	Chloroform	306	4.170×10^3

[1] Linde 4A.
[2] Burrell.
[3] The instruments used were Cary 14, 15, and 16 spectrophotometers.

Table III—Mole Fraction Solubilities of Several Polar Substances in Nonpolar Solvents

Solvent	Solute: Acetanilide	Carbazole	Picric Acid	Salicylic Acid	Phthalic Anhydride
Cyclohexane	8.1×10^{-5}	1.8×10^{-4}	4.4×10^{-5}	4.3×10^{-4}	6.9×10^{-4}
n-Hexane	—	1.7×10^{-4}	3.9×10^{-5}	4.8×10^{-4}	6.5×10^{-4}
n-Heptane	1.4×10^{-4}	2.3×10^{-4}	4.8×10^{-5}	4.8×10^{-4}	7.2×10^{-4}
Isooctane	1.2×10^{-4}	1.8×10^{-4}	4.1×10^{-5}	3.8×10^{-4}	7.0×10^{-4}
Decane	1.8×10^{-4}	2.9×10^{-4}	8.2×10^{-5}	6.2×10^{-4}	9.6×10^{-4}
Dodecane	1.8×10^{-4}	3.2×10^{-4}	7.5×10^{-5}	6.4×10^{-4}	10.9×10^{-4}
Hexadecane	2.9×10^{-4}	5.9×10^{-4}	13.5×10^{-5}	10.4×10^{-4}	16.2×10^{-4}
Chloroform	1.8×10^{-1}[a]	3.7×10^{-3}	4.4×10^{-3}[b]	1.3×10^{-2}[a]	6.3×10^{-2}
Diethyl ether	1.9×10^{-2}[a]	1.3×10^{-2}	6.4×10^{-3}[b]	2.5×10^{-1}[c]	1.2×10^{-2}

[a] 30°, estimated from values from A. Seidell, "Solubilities of Organic Compounds," vol. II, 3rd ed., D. Van Nostrand, New York, N. Y., 1941. [b] 20°, from same source as in [a]. [c] Estimated from "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N. J., 1968.

Table IV—Molar Solubilities of Several Polar Substances in Nonpolar Solvents

Solvent	Solute: Acetanilide	Carbazole	Picric Acid	Salicylic Acid	Phthalic Anhydride
Cyclohexane	7.5×10^{-4}	1.7×10^{-3}	4.1×10^{-4}	4.0×10^{-3}	6.4×10^{-3}
n-Hexane	—	1.3×10^{-3}	3.0×10^{-4}	3.4×10^{-3}	5.0×10^{-3}
n-Heptane	9.3×10^{-4}	1.6×10^{-3}	3.3×10^{-4}	3.3×10^{-3}	4.9×10^{-3}
Isooctane	9.3×10^{-4}	1.1×10^{-3}	2.5×10^{-4}	2.3×10^{-3}	4.22×10^{-3}
Decane	9.3×10^{-4}	1.5×10^{-3}	4.2×10^{-4}	3.2×10^{-3}	4.9×10^{-3}
Dodecane	7.8×10^{-4}	1.4×10^{-3}	3.3×10^{-4}	2.8×10^{-3}	4.8×10^{-3}
Hexadecane	8.3×10^{-4}	1.7×10^{-3}	3.9×10^{-4}	3.0×10^{-3}	4.7×10^{-3}
Chloroform	2.23[a]	4.5×10^{-2}	5.5×10^{-2}[b]	1.6×10^{-1}[a]	6.3×10^{-2}
Diethyl ether	1.5×10^{-1}[a]	1.25×10^{-1}	6.1×10^{-2}[b]	2.4[c]	1.2×10^{-2}

[a] 30°, estimated from values from A. Seidell, "Solubilities of Organic Compounds," vol. II, 3rd ed., D. Van Nostrand, New York, N. Y., 1941. [b] 20°, from same source as in [a]. [c] Estimated from "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N. J., 1968.

the entropic factor is mainly responsible for deviation from ideality. Thus, the solute solubility in any solvent (s) compared with that in a reference solvent (c = cyclohexane) can be theoretically estimated from the molar volumes of the solute and solvents:

$$\ln \frac{x_c^{\text{ath.}}}{x_s} = \ln \frac{\gamma_s}{\gamma_c} = \ln \frac{V_c}{V_s} + V_2 \left(\frac{1}{V_c} - \frac{1}{V_s} \right) \qquad \text{(Eq. 7)}$$

For a solid solute, V_2 represents the molar volume of the supercooled solid. Accurate values of V_2 for the solutes studied are unavailable. However, for the purpose of comparison, and based on published results of V_2 for organic solutes (2, 12), an approximate value of 100 cm. (3) can be assigned to V_2 for salicylic acid and phthalic anhydride. In Table VI, the quantities log $(x_c/x_s)_{\text{obs.}}$ of these two solutes are shown, where $(x_c/x_s)_{\text{obs.}}$ represents the ratio of observed mole fraction solubilities in cyclohexane and solvent, respectively. In the last two columns, values of log $(x_c/x_s)_{\text{obs.}}$ − log $(x_c/x_s)^{\text{ath.}}$ for the two solutes are listed.

For the hydrocarbon solvents, it is apparent that the athermal correction can be used to account for almost all of the differences in solubility of these two solutes because log $(x_c/x_s)_{\text{obs.}}$ − log $(x_c/x_s)^{\text{ath.}}$ values are, within experimental error, nearly zero for both solutes.

The apparent independence on all parameters of molar solute solubilities in hydrocarbons (Table IV) appears to be consistent with the above conclusion. It appears that the molar volume correction is compensated by the choice of molarity as the unit. Kuntz *et al.* (13) discussed the importance in the choice of concentration units in determining the properties of weak complexes by the Benesi–Hildebrand equation. Drawing data from the literature, they found that molarity seemed to be a better unit for describing these systems. The authors' result also indicates that desirability of using molarity as the unit when solvent effects on specific interactions are studied, since the secondary effect due to molar volume differences in the inert solvents seems to be well compensated.

The enthalpic contribution can also be estimated for these systems. The solubility parameters for a series of sulfonamides were found to lie in a range of 11–17 (2), and most organic solutes are believed to have δ_2 in the same range. The contribution to solubility from regular solution can be estimated (1), and Table VII shows the predicted solubility ratios, x_s/x_c, for solutes having a solubility parameter from 11 to 17 in the solvents studied. If the estimated range of the solubility parameter for common organic solutes is correct, then the regular solution theory predicts that: (*a*) among the hydrocarbons, solubility in cyclohexane will be the highest; (*b*) solubility of a solute in heptane will be identical to that in ether, since both solvents have the same solubility parameter, and (*c*) solubility in

Table V—Physical Properties of Solvents Used

Solvent	D[a]	μ[b]	V_{298}[c]	δ[d]
Cyclohexane	2.02	0	109	8.2
n-Hexane	2.0	0	132	7.3
n-Heptane	—	0	148	7.4
Isooctane	1.94	0	166	6.9
Decane	2.0	0	192[e]	7.8[f]
Dodecane	2.0	0	222[e]	7.9[f]
Hexadecane	—	0	294	8.0
Chloroform	4.8	1.02	81	9.2
Diethyl ether	4.34	1.15	105	7.4

[a] Dielectric constant, values obtained from "Handbook of Chemistry and Physics," 48th ed., Chemical Rubber Co., Cleveland, Ohio, 1967. [b] Dipole moments, from same source as in [a]. [c] Molar volume at 25° (1). [d] Solubility parameter (1). [e] Estimated by assuming 15 cm.^{3}/CH_2 unit in alkanes. [f] Estimated from heat of vaporization.

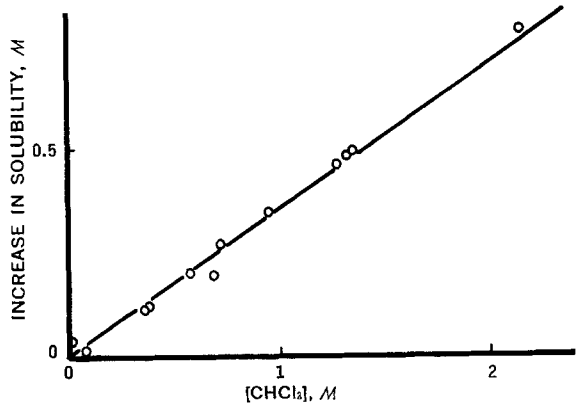


Figure 1—*Increase in solubility of phenyl benzoate* versus $[CHCl_3]$ *in chloroform–cyclohexane solvent mixtures at 25° (solubility in cyclohexane = 0.393* M).

Table VI—Comparison of Athermal Contribution with Observed Solubilities of Phthalic Anhydride and Salicylic Acid[a]

Solvent S	$\log\left(\frac{x_c}{x_s}\right)^{ath.}$	$\log\left(\frac{x_c}{x_s}\right)_{obs.}$ Phthalic Anhydride	$\log\left(\frac{x_c}{x_s}\right)_{obs.}$ Salicylic Acid	$\log\left(\frac{x_c}{x_s}\right)_{obs.}$ Phthalic Anhydride	$\log\left(\frac{x_c}{x_s}\right)^{ath.}$ Salicylic Acid
n-Hexane	−0.013	0.0261	−0.010	0.0391	0.003
n-Heptane	−0.028	−0.018	−0.048	0.010	−0.020
Isooctane	−0.046	−0.006	−0.0539	0.040	0.008
Decane	−0.074	−0.143	−0.159	−0.069	−0.085
Dodecane	−0.106	−0.199	−0.173	−0.093	−0.067
Hexadecane	−0.18	−0.374	−0.383	−0.194	−0.203
Chloroform	−0.009	−1.960	−1.480	−1.951	−1.471
Diethyl ether	+0.0009	−1.240	−2.764	−1.239	−2.765

[a] Symbols are defined in the text.

chloroform will at most be 3–4 times higher than that in cyclohexane. Examination of Table III shows all these predictions to be incorrect: solubility in cyclohexane for all solutes is generally the lowest among hydrocarbons, and solubility in ether and chloroform is much higher than predicted. It then appears that regular solution treatment to solubility in noninteractive hydrocarbons is at most marginally valid, whereas application to interactive solvents such as chloroform and ether is completely unsuccessful.

Specific Interaction and Solubility—As pointed out earlier, the solubilities of the solutes studied were much higher in chloroform and ether. These enhancements were too much in excess to be explained either by the molar volume of the solvent (Table VI) or by its solubility parameter (Table VII). The data also indicated that the measured solubility was very dependent on the specific chemical nature of both the solute and solvent.

Further evidence of specific interaction is indicated on examining the solubility of phthalic anhydride in a series of slightly polar and polar solvents (Table VIII). There appeared to be no simple relationship between the solubility and the physical properties such as dielectric constant, dipole moment, molar volume, and solubility parameter of the solvent. For example, the solubility in pyridine was higher than that in acetone, although the latter had a higher dielectric constant and dipole moment; the solubility was at least five times higher in chloroform than in ether, in spite of similar dielectric constants and dipole moments of the two solvents. Information on specific solvation in slightly polar organic solvents can be obtained from solubility data in mixed solvents comprised of an interactive component, such as chloroform (an acid) or ether (a base), and an inert solvent such as cyclohexane. These solvent mixtures have comparatively small excess free energy of mixing, and large increases in solubility on addition of the interactive solvent would be a result from specific solvation.

Table VII—Predicted Relative Mole Fraction Solubility in Various Solvents (Reference Solvent: Cyclohexane) for Varying Solubility Parameter Value of the Solute

Solubility Parameter of Solute	x_s/x_c *n*-Hexane ($\delta_1 = 7.3$)	Heptane Ether ($\delta_1 = 7.4$)	Isooctane ($\delta_1 = 6.9$)	Chloroform ($\delta_1 = 9.2$)
11	0.651	0.687	0.518	1.402
12	0.517	0.611	0.428	1.623
13	0.500	0.543	0.357	1.879
14	0.438	0.483	0.292	2.176
15	0.384	0.430	0.242	2.250
16	0.337	0.382	0.200	2.917
17	0.295	0.340	0.165	3.378

Table VIII—Solubility of Phthalic Anhydride in Solvents with Nonzero Dipole Moments at 25°

Solvent	D[a]	μ[b]	V_{298}[c]	δ[d]	Solubility, M	Solubility, Mole Fraction
Diethyl ether	4.34	1.15	105	7.4	0.116	0.012
Di-*n*-propyl ether	3.4	1.3	138[e]	6.9[h]	0.04	0.0055
Chloroform	4.8	1.02	81	9.2	0.78	0.063
tert-Butyl alcohol	10.9[f]	1.0[g]	95[e]	10.6	0.04	0.0038
Acetic acid	6.15	1.74	57[e]	14.4[i]	0.27	0.015
Tetrahydrofuran	7.4	1.7	81[e]	8.9[i]	1.2	0.097
Pyridine	12.3	2.2	81	10.7	2.9	0.23
Acetone	20.7	2.89	74[e]	10.0[h]	1.15	0.085

[a,b,c,d] Same as in Table V. [e] Estimated, V = molecular weight/density. [f] 30°. [g] From Footnote [d], Table V. [h] J. D. Crowley, G. S. Teague, Jr., and J. W. Lowe, Jr., *J. Paint Technol.*, **38**, 272(1966). [i] Estimated from heat of vaporization.

Interaction with Chloroform—The hydrogen-donating nature of chloroform was well documented by Pimentel and McClellan (14). Several studies involving enhancement of solubility in chloroform as evidence of chloroform–base association were reported (15–17), but quantitative determinations of the association constants were not made. These constants are usually determined through NMR studies, and 1:1 association constants with chloroform were obtained for some organophosphorus compounds and long-chain tertiary amines (18), piperidine (19), benzene (20), aromatic hydrocarbons, acetonitrile and dimethyl sulfoxide (21), and amines (22, 23). In all these systems, both chloroform and base concentrations were usually low, and the assumption of a stoichiometric 1:1 complex seemed to satisfy the experimental observations. The authors wished to ascertain whether solubility studies in chloroform can also be rationalized in terms of stoichiometric complexes.

The validity of this stoichiometric solvate species approach can be tested by measuring the solubility of the solute in a series of solvent mixtures consisting of different chloroform concentrations in cyclo-

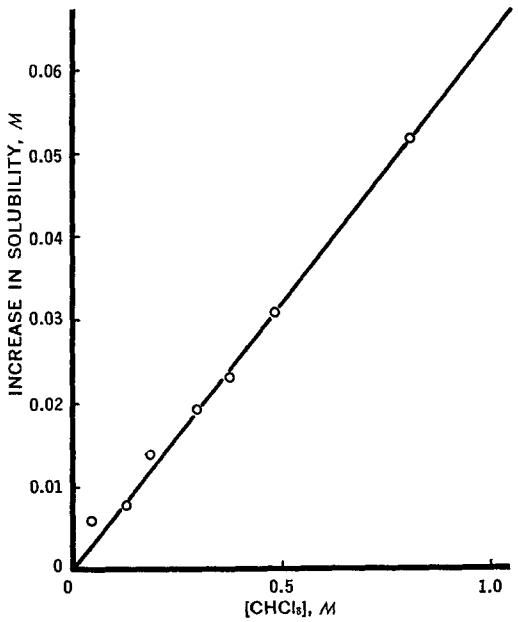


Figure 2—*Increase in solubility of benzoquinone* versus [$CHCl_3$] *in chloroform–cyclohexane solvent mixtures at 25° (solubility of benzoquinone in cyclohexane = 5.65 × 10⁻² M).*

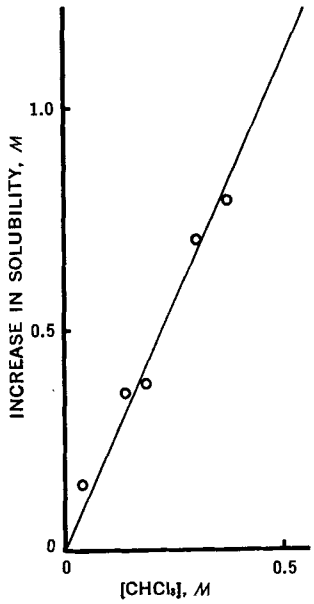


Figure 3—*Increase in solubility of benzophenone* versus *[$CHCl_3$] in chloroform–cyclohexane solvent mixtures at 25° (solubility of benzophenone in cyclohexane = 2.13* M).

hexane. The increase in solubility due to the interactive solvent is plotted against the concentration of chloroform according to Eq. 3.

Figures 1–3 show these plots for the solutes phenyl benzoate, benzoquinone, and benzophenone, respectively. In all these cases, the increase in solubility is linearly related to chloroform concentration, indicating that stoichiometric 1:1 chloroform–solute complexes are formed in these systems. It is also apparent that the presence of a small amount of the interactive solvent increases the solubility far beyond that which can be attributed to entropic and/or cohesive energy corrections.

The solubility of phthalic anhydride for the whole mole fraction range in chloroform–cyclohexane solvent mixtures can also be rationalized in terms of stoichiometric solvates. For high concentrations of chloroform, the second- and third-order solvates have to be considered (Eq. 5). Figure 4 shows the solubility of the anhydride in the whole range of chloroform–cyclohexane solvent mixtures. The 1:1 complex constant can be approximated by the straight line

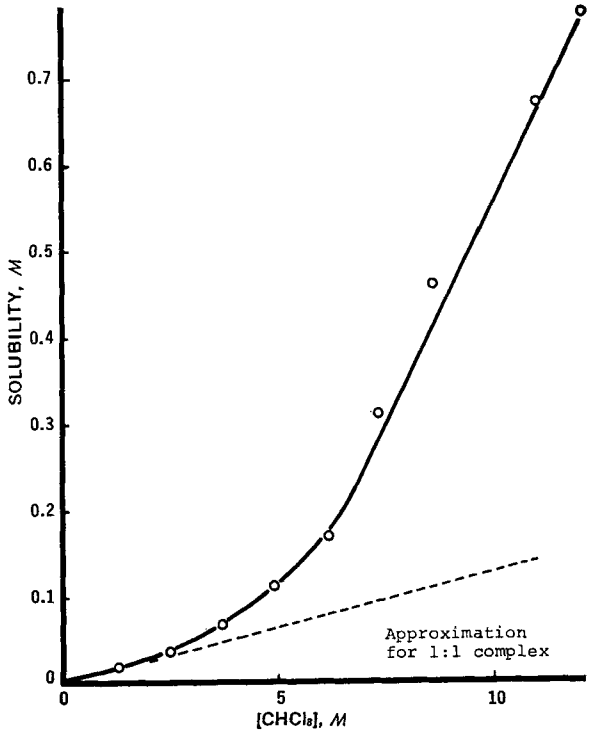


Figure 4—*Solubility of phthalic anhydride* versus *[$CHCl_3$] in chloroform–cyclohexane solvent mixtures at 25°.*

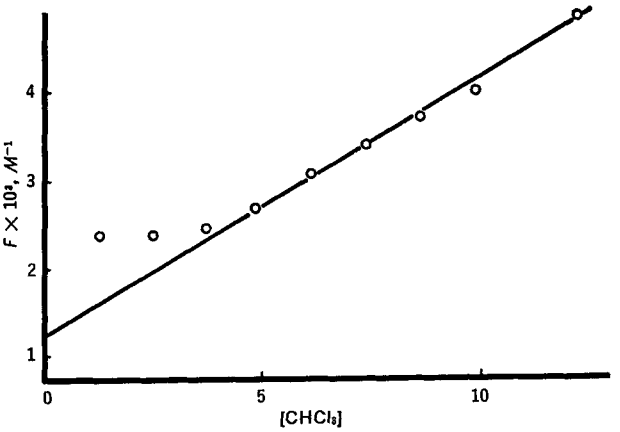


Figure 5—*Determination of* $K_{1:2}$ *and* $K_{1:3}$ *for phthalic anhydride–chloroform complex formation.*

$$F = \frac{\Delta solubility/[CHCl_3] - K_{1:1}[anhydride]_0}{[CHCl_3]}$$

(*See* Experimental *section for derivation.*)

drawn for low chloroform concentrations. By using this approximation, the relationship represented by Eq. 5 is shown in Fig. 5, from which $K_{1:2}$ and $K_{1:3}$ can be obtained. Table IX lists the solvate constants obtained.

The 1:1 interaction constants are all in the neighborhood of 1 M^{-1} and are similar to or greater than similar interaction constants found for amines (unpublished data from this laboratory), which are usually slightly less than 1 M^{-1}. By NMR, the interaction constant between chloroform and ether was found to be 1.52 M^{-1}. Thus, in a nonpolar solvent such as cyclohexane, the basicity of amines appears to be similar to those of ketone, ester, or anhydride groupings, whereas ether is a stronger base toward chloroform than all these groups. These results seem to substantiate further the contention that pKa values in water are not satisfactory measures of acidity or basicity in nonpolar solvents. Similar conclusions were reached in studies of hhdrogen-bonded complex formation of various bases with *p*-fluorophenol in carbon tetrachloride (24). These findings about acidity and basicity in nonpolar solvents must be taken into account when reaction mechanisms in these solvents are considered.

Interaction with Ether—Specific solvate formations are also indicated from solubility studies in ether–cyclohexane mixtures. Figures 6 and 7 show the plots of solubility *versus* ether concentration in ether–cyclohexane solvent mixtures for picric acid and carbazole, respectively. For the range of solvent compositions studied (up to 0.6 *M* in ether), the increase in solubility of both solutes was found to be linearly related to the concentration of ether; therefore, 1:1 solvate complexes may be postulated in both cases. Positive deviation from linearity at higher ether concentrations, as is seen for the carbazole–ether system in Fig. 7, may be attributed to formation of higher order complexes. The interactions between ether and phthalic anhydride and between ether and succinic anhydride were also

Table IX—Solvate Equilibrium Constants of Various Solutes with Chloroform at 25° Obtained from Solubility Method

Solute	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-2})	$K_{1:3}$ (M^{-3})
Phenyl benzoate	0.89	—	—
Benzoquinone	1.06	—	—
Benzophenone	1.01	—	—
Phthalic anhydride	0.61	0.214	0.043

Table X—Solvate Equilibrium Constants of Various Solutes with Ether at 25° Obtained from Solubility Method

Solute	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-2})
Carbazole	3.53	—
Picric acid	2.73	—
Phthalic anhydride	0.606	0.10
Succinic anhydride	0	0.392

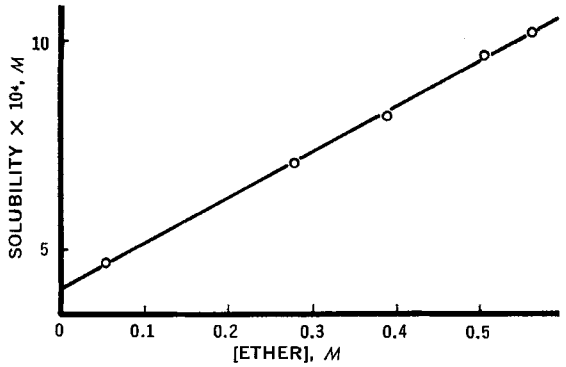


Figure 6—*Solubility of picric acid* versus [*ether*] *in ether–cyclohexane solvent mixtures at 25°.*

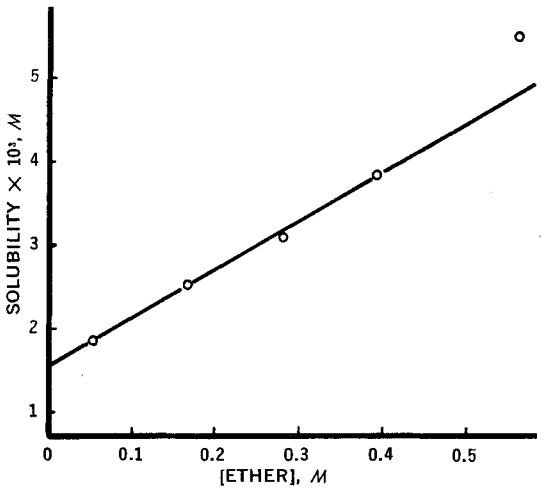


Figure 7—*Solubility of carbazole* versus [*ether*] *in ether–cyclohexane mixtures at 25°.*

studied. Data were collected for the whole mole fraction range of solvent composition. In both cases, the solubility increases were quantitatively explained by Eq. 4, *i.e.*, through the formation of both 1:1 and 1:2 solute–solvent complexes. Table X lists the ether solvate constants obtained for these solutes.

The interaction mechanisms between ether and solutes seem to involve hydrogen bonding in the cases of picric acid and carbazole (presumably through I and II, respectively), whereas strong charge-transfer interactions are suspected between the carbonyls in the anhydrides and the ether oxygens. Carbazole has a stronger binding tendency toward ether than picric acid, although the latter is a stronger acid in aqueous solution. This may be a result of reversal of the acidity scale in nonaqueous solution, due perhaps to steric interference in picric acid or to some interaction between the ether molecule with the aromatic nucleus in carbazole.

CONCLUSIONS

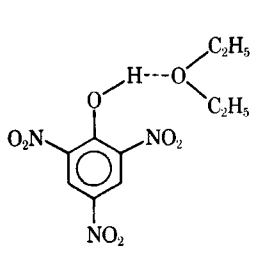
The results in this study indicated that:

1. The solubilities of the polar nonelectrolytes studied were much higher in "interactive" solvents, such as chloroform and ether, than in inert hydrocarbons, although the dielectric constants of these solvents differed only slightly.

2. In a series of inert hydrocarbon solvents, the variations in solubility of polar nonelectrolytes studied appeared to be mainly due to the molar volume differences of the inert solvents. Correction for cohesive energy differences, as formulated by the regular solution theory, appeared to be unnecessary for these systems.

3. The solubility of phthalic anhydride in a range of moderately polar nonaqueous solvents appeared to be unrelated to the dielectric constant, polarity, solubility parameter, or molar volume of the solvent.

4. The enhancement of solubility of nonelectrolytes in chloroform and ether seemed to be rationalized best in terms of specific solute–solvent interactions. Nonspecific interactions were found to contribute insignificantly in these solvents. Solubility data obtained from mixed solvents appeared to be consistent with the proposal of specific solvation involving stepwise formation of solute–solvent complexes in solution.



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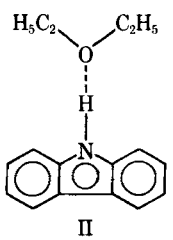

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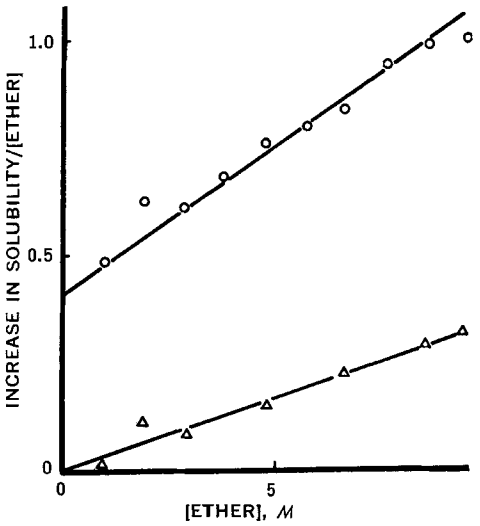

Figure 8—*Determination of solvate equilibrium constants between ether and phthalic anhydride* (○) *amd ether and succinic anhydride* (△) *from solubility data of the anhydrides in ether–cyclohexane solvent mixtures at 25°.*

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G. L. FLYNN and T. J. ROSEMAN

Abstract □ Dimethylpolysiloxane is an isotropic polymer which provides an excellent nonpolar barrier for diffusional studies. Commercial polymer contains appreciable siliceous filler to enhance mechanical properties. Diffusion through pure polymer may be characterized as Fickian, but diffusion through heterogeneous polymer is complex due to adsorption on the active filler. Two solutions integrating Fick's second law with special cases of the Langmuir adsorption isotherm are known. The first solution, Case I, is for the region where adsorption is directly proportional to the local concentration of permeant; Case II applies when the mass of penetrant adsorbed per unit of filler is fixed. The effect of filler in the dimethylpolysiloxane membrane is particularized with respect to these equations. Specifically, lag time relationships indicated that adsorption was linear over the greatest part of the concentration range employed for the specific penetrants, *p*-aminoacetophenone and ethyl *p*-aminobenzoate. These and literature data afforded a complete characterization of the membrane diffusional system with respect to the benzoate ester. Concepts here are general and a similar approach can be used to investigate other heterogeneous barriers, living or synthetic. Attention is particularly drawn to the influence of specific binding on the passage of chemicals through cellular membranes and the skin and to the application of these principles in the design of drug delivery systems.

Keyphrases □ Dimethylpolysiloxane barriers, heterogeneous—influence of physical adsorption on molecular flux, equations □ Diffusion, membrane—physical adsorption influence on molecular flux, heterogeneous dimethylpolysiloxane barriers, equations □ Adsorption, physical—influence on molecular flux, lag time relationships, dimethylpolysiloxane heterogeneous barriers □ Membrane diffusion—physical adsorption effects on flux, using heterogeneous dimethylpolysiloxane barriers

The present study deals in part with membranes containing a dispersed silica phase in a continuum of dimethylpolysiloxane polymer. Holliday (1) suggested that the following three criteria are necessary to define a heterogeneous, two-phased polymeric media: (*a*) the geometry, concentration, distribution, orientation, *etc.*, of the dispersed phase; (*b*) the composition or state of matter of the dispersed phase; and (*c*) the composition and state of matter of the continuous phase. In the current study, emphasis is also drawn to the character of the dispersed phase surface; hence, a fourth criteria for characterization is: (*d*) the extent, composition, and properties of the dispersed phase surface or dispersed phase–continuous phase interface.

The dimethylpolysiloxane membranes employed are presumed to contain between 20 and 30% fumed silica filler. The dispersed phase has a large and active surface per unit volume, is randomly oriented, may be considered to be heterogeneous in size (within limits), and is impervious to the diffusing species.

The polymer forming the continuum is an elastomeric silicone rubber which, by itself, has little mechanical strength. It forms an isotropic medium around the filler. It exists above its glass transition point and is without crystallinity at the moderate temperatures of the experiments. Previous investigators (2–5), studying diffusion through dimethylpolysiloxane barriers, found Fick's first law strictly obeyed while Fick's second law was also applicable in limited cases with pure polymer. These membranes have little or no tendency to imbibe water. This noncellulosic quality makes them attractive as membranes for simulation of the drug-transport phenomenon. Fillers, typically silicas or graphites, are incorporated into rubbers to provide mechanical strength. The improved properties result from strong particle–polymer physical interactions, the polymer likely having multiple-site attachment on the particulate surface (6). This implies that significant portions of the dispersed phase surface are occupied by polymer and are unavail-

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able for adsorption of additional species. Due to the translational kinetic energy of polymer segments, the available surface at the microscopic level will be in a state of flux, although the total available adsorbing surface at the isothermal condition will be statistically constant.

In the present studies, it is assumed *a priori* that the continuum is little affected by the presence of the filler and that diffusion through the external phase will be unchanged qualitatively and quantitatively on the molecular level. These assumptions are not meant to discount the gross effects of reduced volume available for diffusion and increased averaged diffusional pathlength or tortuosity, τ. Stationary-state transport would be expected to be directly proportional to the continuous phase volume fraction, V_1, and inversely proportional to τ (7). The latter factor also exerts influence on nonsteady-state diffusion.

In the case of fumed silica in dimethylpolysiloxane, another factor is operative in altering and lengthening nonstationary-state barrier penetration; this factor is physical adsorption on the filler surface (3). At any plane in the membrane perpendicular to the vector of diffusion, a transitory equilibrium between diffusant, polymer, and available siliceous surface must be established, partially at the expense of further penetration. Lag times are lengthened, and typical treatment of the diffusional curve generates values for diffusion constants or diffusivities that are significantly distorted. For complex adsorption isotherms, simultaneous solution of Fick's laws with adsorption phenomena are generally not tractable (7). However, Finger *et al.* (8) and Higuchi and Higuchi (7) provided two solutions for special cases of Langmuirian adsorption. They designated as Case I the situation where adsorption is strictly linear with concentration and as Case II the situation where adsorption is constant per unit filler. These solutions are for the extremes of the isotherm. The present studies constitute application and extension of these concepts using heterogeneous dimethylpolysiloxane barriers.

EXPERIMENTAL

Materials—Dimethylpolysiloxane membranes[1] were cut to size for the diffusion cell with a specially made die and washed carefully with deionized water. These membranes were then stored under distilled water until an experiment was initiated, at which time they were again thoroughly rinsed and placed in the diffusion cell. In each complete experiment, the membranes received identical treatment; but from one total experiment to another, storage times in water varied.

Fillerless silicone polymer[2] membranes were prepared in these laboratories. Dimethylpolysiloxane was polymerized between two metal plates separated by 0.051-cm. spacers. Circular membranes were cut as previously described and thoroughly rinsed prior to insertion in the diffusion cell.

The permeants, *p*-aminoacetophenone[3] and ethyl *p*-aminobenzoate[4], were obtained in a highly refined state and were used as received. Deionized water was the sole solvent used in preparing the permeating solutions and the receptor phase.

Equipment—A specially designed diffusion cell, with horizontal placement of compartments and stirring planar with the plane of diffusion, was employed. Particulars concerning the operation of this cell were recounted previously (9). Appearance of diffusant in the receptor compartment was monitored continuously by a spectrophotometer[5] equipped with a flow cell and a 12.7-cm. (5-in.) recorder. Circulation to the flow cell was accomplished by either a proportionating pump[6] or a 23-r.p.m. pump[7], providing flow rates of approximately 2 and 15 ml./min., respectively. The latter was also used in connection with an external donor reservoir in some experiments. Concentrations of diffusing species in the donor phase were determined on a recording spectrophotometer[8].

Procedures—Donor phase solutions were prepared in either of two ways depending on the experimental design. In runs where no external donor reservior was used, excess permeant was equilibrated with water at 37° in a shaker bath. These solutions, including some excess solid, were then transferred *via* a 30-ml. syringe as rapidly as possible to the donor compartment of the diffusion cell, also maintained at 37°. Overflow was piped through a tube to a beaker and discarded.

For runs at concentrations less than saturation, a reservoir or 300-ml. capacity external-jacketed cell was employed. A weighed amount of permeant was placed in the external cell, and either 150 or 300 ml. of deionized water was added, depending on the desired concentration. This solution was stirred with a magnetic stirring bar until all the drug was in solution and the temperature reached 37°. Approximately 18 ml. of the reservoir solution was placed in the donor compartment by syringe, the reservoir was connected to the diffusion cell ports, and its contents were circulated through the cell at 15 ml./min., allowing for a complete change in donor solvent approximately each minute.

In all cases, penetrant, essentially as the free base, was collected in neutral deionized water. Analyses of concentrations on the receptor side indicated that the receptor concentration never exceeded 10% of the most dilute donor concentration and was generally in the 0.1–1% range of that in the applied phase. Therefore, sink conditions existed. Excess drug in the donor compartment or the presence of the external reservoir at subsaturation assured essentially constant donor concentration for a given run.

Calculations—Lag times were obtained by extrapolation of the steady-state portion of the diffusional curves to the time axis. Distances along this axis were measured to the nearest $^1/_{64}$ in. Chart speeds were either 2.54 cm. (1 in.)/min. or 0.25 cm. (0.1 in.)/min., affording estimation of lag times to the nearest second or 10 sec., respectively. An averaged mechanical lag time, the time for the cell contents to be pumped into the lightpath of the spectrophotometer, was determined for each pumping velocity and subtracted from the observed gross value. Mechanical lag times were 15.5 sec. at 15 ml./min. and 1.25 min. at 2 ml./min.

Steady-state slopes were obtained graphically in units of absorbance per minute. These were converted to total steady-state flux, Q_T, in milligrams per second by Eq. 1, using independently determined molar absorptivities, a_m, of 16,800 and 17,200 for *p*-aminoacetophenone and ethyl *p*-aminobenzoate, respectively, a receptor cell volume of 17.8 ml., and the appropriate molecular weight, *MW*:

$$Q_T = \frac{(A/\text{min.}) \times 0.0178 \times MW}{a_m \times 60} = \text{mg./sec.} \qquad \text{(Eq. 1)}$$

The apparent permeability coefficient, P', was calculated from:

$$P' = Q_T \times \frac{h}{AC^0} \qquad \text{(Eq. 2)}$$

where h is the membrane thickness, A is cross-sectional area (10 cm.^{2}), and C^0 represents the concentration in milligrams per milliliter in the applied phase.

Apparent diffusivities were obtained from the lag time relationship of Daynes (10) and Barrer (11):

$$D' = \frac{h^2}{6t_L} \qquad \text{(Eq. 3)}$$

where D' represents the apparent diffusivity, and t_L is the corrected

[1] Silastic sheeting, Dow Corning, Midland, Mich. Dow Corning Medical Products Bulletin 14-184.
[2] Gift from Dow Corning, Midland, Mich.
[3] Eastman Organic Chemicals.
[4] Aldrich Chemical Co.
[5] Beckman DB.
[6] Technicon Autoanalyzer, model 1.
[7] New Brunswick Scientific, model PA-23.
[8] Cary 11.

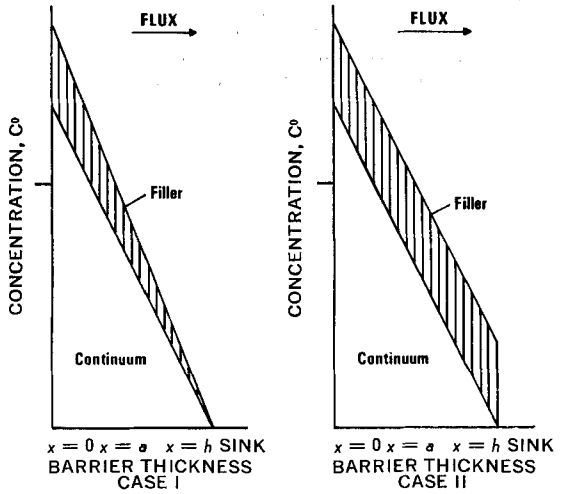

Figure 1—*Plot showing developing concentration gradients in heterogeneous barriers governed by Case I and Case II behaviors, respectively. The crosshatched area represents sorbed permeant; the open area represents the concentration gradient in the continuum. For a given permeant and polymer, the gradient in the polymer would be independent of filler amount and sorption mechanism in the ideal case.*

extrapolated lag time. Apparent partition coefficients, represented by $k_{app.}$, were obtained from the ratio of P' to D'. Steady-state fluxes per unit area, Q, are used in the tables and graphs, and these are exactly an order of magnitude less than the total steady-state flux due to the 10-cm.^{2} membrane cross-sectional area.

THEORETICAL

Diffusion of a molecule through a liquid in the absence of any mixing force requires the formation of a molecular-sized void or hole in the liquid. The energy for formation of a hole is considerable and has been equated with the energy expended in evaporating a molecule of solvent (12). It has been reasoned that a solute molecule occupies each hole formed to which it has access because it would be oscillating 10^{12}–10^{13} times a second and have many kinetic thrusts in the direction of any given hole during the hole's lifetime (13). Thus, the movement of mass through a liquid is dependent on the frequency of hole formation of sufficient size to accommodate the diffusing species. For large molecules relative to the solvent, several solvent molecules must be simultaneously displaced to form occupiable voids. This is the basis for the molecular radius dependency of the diffusion coefficient.

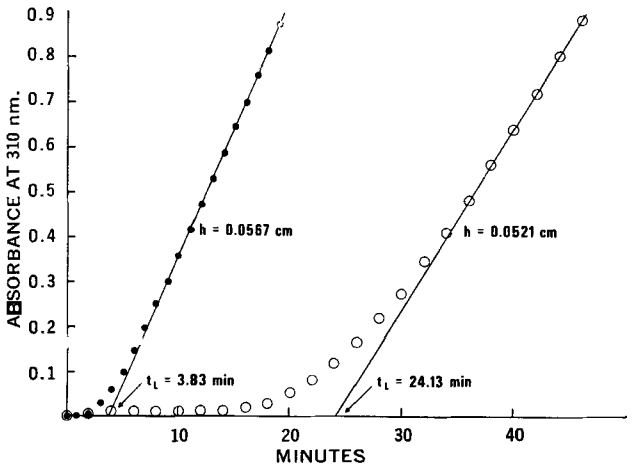

Figure 2—*Diffusional curves as a function of time for the appearance of* p*-aminoacetophenone through pure dimethylpolysiloxane membrane* (●) *and membrane containing filler* (○). *Runs were at 37°. Due to a holdup time in the flow cell lines, the true lag time is 15.5 sec. less than the indicated intercepts.*

Molecular flux through continuous polymer is analogous to flux through liquids. In this case, molecular voids are formed by the random oscillation of polymeric units (14). Hydrogen and other small molecules may require only monomeric unit displacements. Larger diffusional species need cooperative oscillations, possibly involving polymer segments containing tens of units. Obviously, the degree of interaction between polymer chains is a major determinant for the mobility of segments, the ease of hole formation, and, thus, the diffusivity of molecular species. Two factors seriously complicate this simple diffusional model for many polymers: polymer crystallinity and the presence of large percentages of imbibed solvent. Crystallinity introduces regions of very low diffusivity relative to the diffusivity in the surrounding amorphous mass. At a minimum, solvent tends to facilitate the oscillation of polymeric segments by reducing chain interactions (plasticization) and changing permeability. In the extreme, solvent may actually become the diffusional medium itself. Because silicone elastomer is above its glass transition point (point of appearance of crystallinity) at ambient temperature and solvent (water) is denied access by the polymer's physical–chemical nature, these complexities are absent in the present case and this description of diffusion is applicable.

The presence of filler in dimethylpolysiloxane barriers severely complicates interpretation of diffusional data. Most (3), using membranes similar to those employed here, demonstrated that lag times could be increased about 15-fold by as little as 25% by weight silica filler. Changes in stationary-state flux were proportional to the increase in filler content from 0 to 25%. If the assumption that diffusion through the continuum is unaffected by the presence of the filler is strictly true, then the attrition in flux with increasing filler content is attributable only to tortuosity and decreased external phase volume fraction. It is obvious from Most's data (3) that decreasing the volume fraction, V_1, accounts for most of the lost flux and that tortuosity cannot be appreciably greater than 1 (Table IV). For membranes containing inert filler, lag times obtained from typical diffusion curves would be independent of either V_1 or V_2, the respective volume fractions, but dependent on the tortuosity squared as the effective membrane thickness, H, would be equal to τh and the Daynes (10) and Barrer (11) lag time equation would become:

$$t_L = \frac{H^2}{6D} = \frac{\tau^2 h^2}{6D} \qquad \text{(Eq. 4)}$$

A tortuosity of about 4 would be required to produce the expanded lag times observed by Most (3), and this is totally inconsistent with the steady-state data. Identical phenomena were observed in the present studies.

Sorption by the filler provides the only explanation consistent with all facets of the known diffusional behavior. For silica filler, this role is limited to a surface interaction. Although filler phenomena have been widely studied in the rubber industry because of their practical importance there, the only successful attempts to quantitate the actual involvement of active filler were made by Higuchi and Higuchi (7) and by Finger *et al.* (8). These investigators derived equations for the influence of Langmuir adsorption on the movement of penetrant through a heterogeneous barrier. The Langmuir (15) isotherm is:

$$\frac{x}{m} = \frac{abC}{1 + bC} \qquad \text{(Eq. 5)}$$

where x/m is the amount of material adsorbed per unit weight of filler, a and b are constants, and the C is concentration of the equilibrium phase. Lag time equations have been derived for the cases where $1 \gg bC$ and $x/m \simeq abC$ (I) and where $bC \gg 1$ and $x/m \simeq a$ (II). The derived expressions are:

$$\text{Case I} \qquad t_L = \frac{h^2}{6D}(V_1 + KV_2) \qquad \text{(Eq. 6)}$$

$$\text{Case II} \qquad t_L = \frac{h^2}{D}\left(\frac{1}{4} + \frac{K'V_2}{2k_p C^0 D}\right) \qquad \text{(Eq. 7)}$$

Previously undefined terms are K and K', which are constants related to the filler adsorptive capacity; C^0, which is the concentration in the applied (donor) phase; and k_p, which is the true membrane continuum/water (solvent) partition coefficient. A significant modification of the Case I equation is to be advanced in this report.

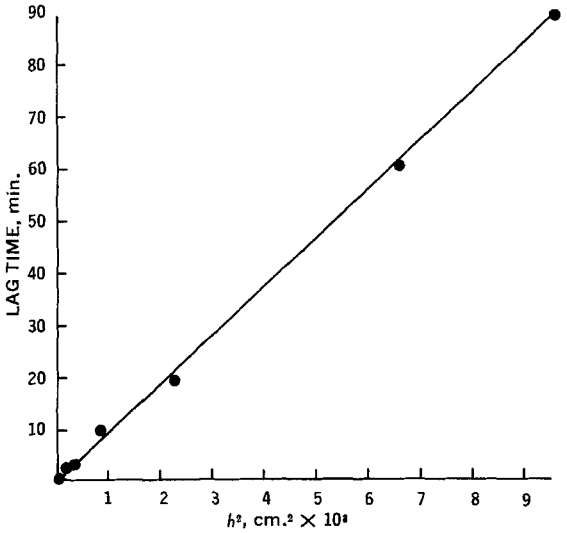

Figure 3—*Plot of lag time,* t_L, versus *the square of membrane thickness,* h^2. *Good correlation is evident for membranes ranging in thickness from* 7.6×10^{-3} *cm. (76 μ) to* 9.80×10^{-2} *cm. (980 μ).*

The mechanisms implicit in these equations may be differentiated by the donor phase concentration dependency in Case II. Total and partial concentration gradients for the two cases in a hypothetical barrier are depicted in Fig. 1. These are arbitrarily drawn to a point $x = a < h$, where x is the distance perpendicular to the diffusional plane. Since a, the point of furthest penetration, is less than h, these are drawn for $t < t_L$. The total area, open and shaded, reflects the total barrier concentration. The shaded area is that portion of the total gradient attributable to adsorption, while the open area represents the gradient in the continuum. It can be seen that at every plane in the barrier up to point a, adsorption is fixed in Case II but proportional to C_x in Case I. Thus, in Case I the concentration at the furthermost point of penetration is zero. This will be true for all $a < h$ and also for $a = h$ when there is a receptor sink. In Case II, a finite concentration entirely due to adsorption exists at a. Finger *et al.* (8) observed Case II behavior for a system in which silica was dispersed in white petrolatum. The present studies, on the other hand, are concerned more with relationships evolving from Case I. The limit as $K \to 0$ for the literature Case I expression is:

$$\lim_{K \to 0} t_L = \lim_{K \to 0} \left[\frac{h^2}{6D} (V_1 + KV_2) \right] = \frac{h^2 V_1}{6D} \qquad \text{(Eq. 8)}$$

implying a volume fraction dependency even for inert filler. It is easy to construct models where V_1 is small; there is no tortuosity to complicate issues and significantly shortened lag times are predicted, *i.e.*, a membrane containing a high volume fraction of inert cylindrical rods oriented perpendicular to the diffusional plane. Facilitation of diffusion leading to reduced lag times is not possible in such circumstances because the lag time would be insensitive to both concentration and area available for diffusion. Actually, the lag time obtained in this example should be exactly equal to that for pure membrane. Thus, Eq. 6 is not strictly applicable, particularly at low values of K.

The Higuchi and Higuchi (7) ratio for Case I lag times focuses on the amount of penetrant in filled membrane relative to fillerless barrier. Total concentration *per se* does not influence the lag time, but the amount of penetrant lost to the adsorbing surface relative to that diffusing in the continuum does. When comparing fillerless and filled membrane, this may be represented as the ratio of the concentration gradients. The respective gradients at any $x = a \leq h$ would be:

$$\text{fillerless} = \frac{C_i - C_a}{a} \qquad \text{(Eq. 9)}$$

$$\text{filled} = \frac{(C_i + BC_i) - (C_a + BC_a)}{a} \qquad \text{(Eq. 10)}$$

where B is a constant proportional to the amount of filler and its ability to adsorb penetrant, C_i is the membrane concentration at the donor membrane interface, and C_a is the membrane concentration at point a; B may be expressed as KV_2, where K is the sorptive capacity and V_2 is the filler volume fraction. For the conditions of a receptor sink, C_a will be zero for all a and the ratio of filled to fillerless gradient will be equal to $1 + KV_2$. Based on this reasoning, the Case I lag time expression is:

$$t_L = \frac{h^2}{6D} (1 + KV_2) \qquad \text{(Eq. 11)}$$

Equation 11 correctly predicts a lag time dependency on only h and D when $K \to 0$ and is used exclusively in all further analysis. Equations 6 and 11 are essentially identical for large values of K. In addition, to be strictly accurate, h may need modification to take into account increased diffusional distance resulting from a nonlinear diffusional pathway (tortuosity).

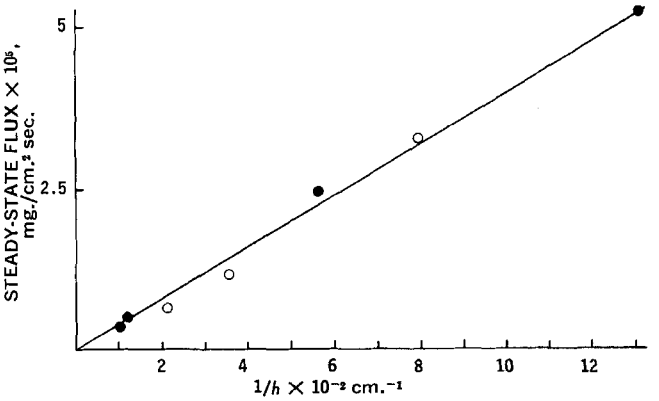

Figure 4—*Plot of steady-state flux,* Q, *against the reciprocal of membrane thickness,* 1/h, *for saturated dose solutions at 37°. The open data points* (○) *indicate nonreinforced membranes; the solid circles* (●) *indicate nylon-reinforced barriers. The nylon obviously had little effect on the permeation velocity.*

EXPERIMENTAL RESULTS

Absorbance changes in the receptor compartment of the diffusion cell accompanying the passage of *p*-aminoacetophenone through a pure dimethylpolysiloxane membrane, 0.0567 cm. in thickness, and a commercially available silica-filled membrane, 0.0521 cm. in thickness, are shown in Fig. 2. The thicknesses were determined at the cessation of each run by the method of Garrett and Chemburkar (2) and represent an average of 10 individual measurements, each at a different location on the membrane surface. It is immediately apparent from Fig. 2 that the lag time was lengthened in the presence of the filler without a correspondingly large decrease in the stationary-state flux. Lag times require correction by 15.5 sec. in this case and were actually 214 and 1430 sec. for fillerless and filled membranes, respectively, in these runs. Data from runs using homogeneous membranes furnish valid values for the specific diffusivity, D, as D' in Eq. 3 becomes D. The average value of D obtained in this manner for *p*-aminoacetophenone was 2.44×10^{-6} cm.²/sec. at 37°. The average value for the permeability coefficient for pure membrane was 7.83×10^{-8} cm.²/sec. The true dimethylpolysiloxane/water partition coefficient for the *p*-aminoacetophenone is calculable from the ratio of P to D and is 3.21×10^{-2}. Furthermore, since saturated aqueous solutions were introduced into the donor compartment, the product of the partition coefficient and the independently determined aqueous solubility yields the dimethylpolysiloxane solubility: 0.317 mg./ml. This latter calculation is valid only if the thermodynamic activity at the membrane–solution interface is the same as in the bulk of the donor solution; *i.e.*, there is no concentration drop across a diffusion layer. Detailed proof that this requisite was met will be provided in a future article. Because the permeability coefficient, P, for fillerless membranes is equal to the product of the specific diffusivity and partition coefficient, Dk_p, but the apparent permeability coefficient, P', for heterogeneous membranes must be adjusted for filler volume fraction and tortuosity:

$$P' = Dk_p \frac{(1 - V_2)}{\tau} = Dk_p \frac{V_1}{\tau} \qquad \text{(Eq. 12)}$$

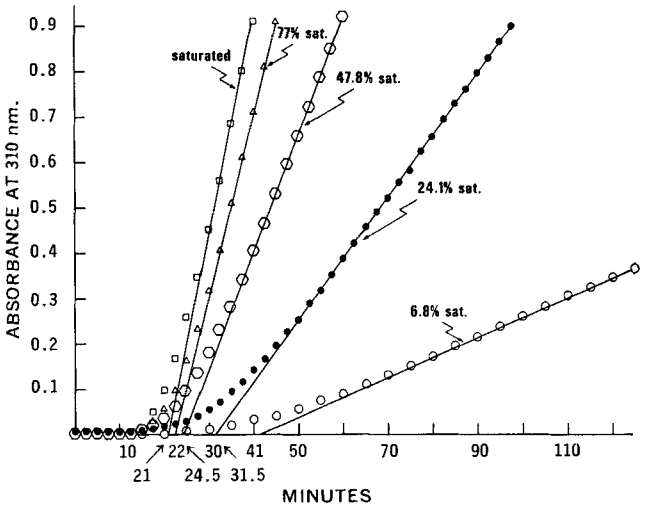

Figure 5—*Absorbance changes at 310 nm. accompanying the diffusion of* p-*aminoacetophenone through dimethylpolysiloxane membranes. The respective curves are for applied phase concentrations of: 9.89 mg./ml. (saturation),* □*; 7.68 mg./ml.,* △*; 4.72 mg./ml.,* ○*; 2.38 mg./ml.,* ●*; and 0.67 mg./ml.,* ○.

it is possible to estimate the ratio of V_1/τ for filled membranes. The average experimental value for *p*-aminoacetophenone was found to be 6.1×10^{-8} cm.²/sec.; P'/P is therefore about 0.78. The permeability was only reduced by roughly 20% by the presence of the filler, while the lag time was extended by a factor of about 7 (Fig. 2).

As a check on the system, a plot of lag time *versus* h^2 for membranes of different thickness should yield a straight line (Eqs. 7 and 11). This is shown in Fig. 3 for *p*-aminoacetophenones, where lag time data for the permeation of a series of membranes varying in thickness from 7.6×10^{-3} to 9.8×10^{-2} cm. are presented. Some of these membranes were reinforced with a nylon netting. Sorption by the netting was assumed to be negligible and, therefore, its presence was not expected to influence lag times (Eq. 11). This appeared to be the case. It was, of course, assumed that the silica/dimethylpolysiloxane ratio was unchanged. Lag times were observed to increase as immersion in water was increased. In the cases of long exposures, a slippery, rinsable film formed on the membrane surface, suggesting some material was leached from within. Because of this variation, membranes deliberately received uniform water treatment and were stored overnight under water and rinsed thoroughly prior to making each run.

In Fig. 4, stationary-state fluxes from the same diffusional runs are plotted against the reciprocal of membrane thickness, $1/h$. A linear relationship was observed despite the presence of nylon reinforcement in several of the barriers, suggesting that the volume fraction occupied by the netting is small and may be discounted. The linearity of steady-state flux to $1/h$ is to be expected only when the total barrier to diffusion exists within the membrane itself. Otherwise, significant deviations would be experienced as thickness is reduced due to relatively increased diffusion layer contributions to the diffusional resistance. However, no significant deviations were experienced. This is attributable to the small solvent/membrane partition coefficient and a greater diffusion coefficient in water relative to the dimethylpolysiloxane barrier.

The two simultaneous solutions of Fick's law and special cases of Langmuir adsorption as expressed in lag times have been given (Eqs. 7 and 11). These two cases can be differentiated by concentration dependency. Therefore, studies were initiated to determine the concentration dependency for *p*-aminoacetophenone and also ethyl *p*-aminobenzoate. In Fig. 5, plots of the raw diffusional curves for *p*-aminoacetophenone are presented for runs at 310 nm., 37°, and varied applied phase concentration ranging from about 7% saturation to saturation, using 0.0476-cm. membranes. It is readily seen that lag times increased with declining concentration. While lag times doubled between the extremes of concentration, the lag time at about 50% saturation was barely distinguishable from that at saturation. The diffusion profile data for all concentrations employed are summarized in Table I. When these data are plotted as lag time *versus* percent saturation (Fig. 6) and compared with the theoretical curves for Cases I and II, it is apparent that although

Table I—Concentration Dependency Data for Permeation of *p*-Aminoacetophenone through 0.0476-cm. Dimethylpolysiloxane Membranes at 37°

Run	Concentration, mg./ml.	Percent Saturation	Corrected Lag Time, min.	Steady-State Flux × 10^5, mg./cm.²/sec.	$D' \times 10^7$, Apparent Diffusivity, cm.²/sec.
1	9.89	100	19.75	1.245	3.19
2	9.73	98.2	22.5	1.130	2.80
3	9.40	95.1	21.25	1.092	2.96
4	7.93	80.2	21.75	0.898	2.89
5	7.06	71.4	19.75	0.964	3.18
6	6.77	68.4	23.25	0.803	2.71
7	5.43	54.9	24.25	0.676	2.60
8	4.70	47.6	23.55	0.689	2.67
9	4.07	41.2	22.25	0.530	2.83
10	4.07	41.2	23.0	0.654	2.74
11	2.67	26.9	29.75	0.396	2.12
12	2.45	24.8	27.5	0.383	2.29
13	2.38	24.1	30.75	0.367	2.05
14	1.33	13.5	35.0	0.216	1.80
15	1.27	12.9	32.5	0.238	1.94
16	0.667	6.7	40.0	0.118	1.57
17	0.350	3.5	40.0	0.062	1.57

Table II—Concentration Dependency Data for Permeation of Ethyl *p*-Aminobenzoate through 0.0476-cm. Dimethylpolysiloxane Membranes at 37°

Run	Concentration, mg./ml.	Percent Saturation	Corrected Lag Time, min.	Steady-State Flux × 10^5, mg./cm.²/sec.	$D' \times 10^7$, Apparent Diffusivity, cm.²/sec.
1	1.735	100	9.8	2.42	6.15
2	1.735	100	10.0	2.23	6.03
3	1.60	92.3	10.3	2.11	5.85
4	1.47	84.7	10.5	2.04	5.74
5	1.33	76.9	9.7	1.98	6.21
6	1.20	69.3	10.8	1.77	5.58
7	1.07	61.6	11.0	1.53	5.48
8	0.935	53.9	10.8	1.51	5.58
9	0.800	46.2	12.0	1.28	5.02
10	0.668	38.5	12.6	0.994	4.78
11	0.533	30.7	12.3	0.847	4.90
12	0.400	23.1	14.4	0.603	4.18
13	0.267	15.4	15.9	0.464	3.79
14	0.133	7.69	17.5	0.198	3.44

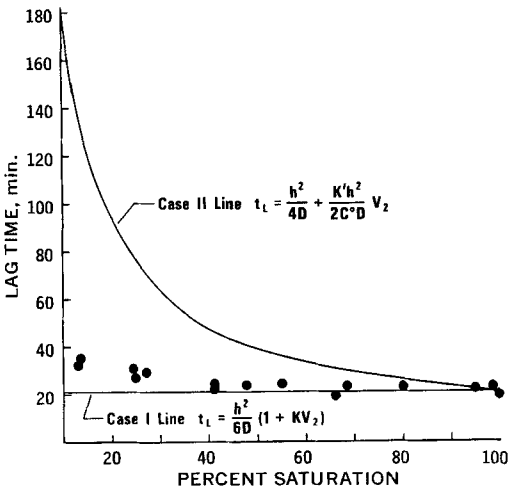

Figure 6—*Plot showing lag time as a function of applied phase concentration for* p-*aminoacetophenone at 37° using 0.0476-cm. membranes. Theoretical lines representing Case I and Case II behavior are superimposed on the plot. These lines were calculated assuming the saturation lag time value, 21.3 min., to be exact.*

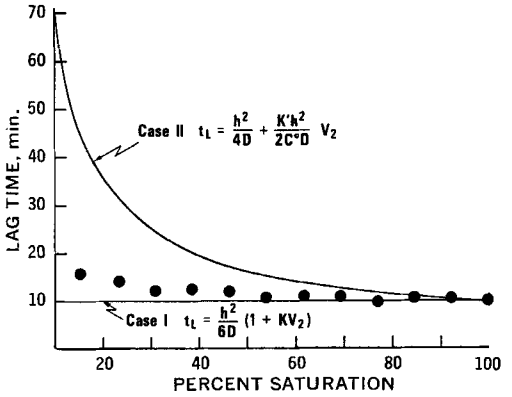

Figure 7—*Plot showing lag time as a function of applied phase concentration for ethyl* p*-aminobenzoate at 37° using 0.0476-cm. membranes. In this case the theoretical lines for Case I and Case II behavior are calculated assuming the 10-min. saturation lag time to be exact;* D *used in these calculations was 2.67 × 10^{-6} cm.² sec.*

Case I is not strictly followed over the entire concentration range, Case II is clearly not applicable. Actually, Case I is a reasonable approximation of the observed diffusional behavior, particularly above 40% saturation. The theoretical curves are based on a 21.3-min. lag time at saturation, an average value for the membranes as processed, and this value is assumed to be exact.

To compare these data with literature data for ethyl *p*-aminobenzoate (3), a similar concentration dependency was initiated for this permeant. The results are summarized in Table II. In Fig. 7, the corrected lag times are plotted against the percent saturation. As in the previous situation, Case I dependency was observed, at least to concentrations less than half-saturation. At the lowest concentrations, lag times deviated from strict Case I behavior. Case II is again completely inconsistent with the experimental data. In this plot, the theoretical curves are drawn assuming the saturation lag time of 10 min. to be correct. Runs were at 37°, using 0.0476-cm. membranes.

It is expected that processes involved in adsorption in the stationary state will be in facile equilibrium with diffusional processes and that steady-state flux for a given membrane thickness will be unaffected by adsorption and only reflect the relative concentration in the applied phase. As seen in Fig. 8 for *p*-aminoacetophenone and in Fig. 9 for ethyl *p*-aminobenzoate, this appeared to be strictly true only at low concentration. At the higher concentrations employed, there were significant deviations from linearity for the ketone and some slighter curvatures for the less water-soluble ester. The deviations are in the right direction to be attributed to the appearance of significant solute–solute interactions. If the total solubility reflects some self-interaction, then activity coefficients would decrease as saturation is approached and account for curvature in either case.

DISCUSSION

Fusion of intermediate Langmuir adsorption behavior or more complex isotherms with Fick's law is not generally manageable. Fortunately, in the present studies the experimental data reasonably fit a relationship for which adsorption is linear with concentration. Under this situation, the Case I adsorption model allows extrapolation of data as a function of filler concentration and adsorptive capacity. Finger *et al.* (8) and Higuchi (16) provided the nonstationary-state relationships governing the permeation of filled systems considered under Case I. When the partition isotherm is linear, Fick's second law need only be modified to account for the amount of permeant adsorbed relative to the concentration in the continuous phase. This factor is directly proportional to the ratio of total concentration gradients in filled and pure systems and leads to the lag time expression given in Eq. 11.

The concentration independence of lag time in Case I has already been stressed, but several other features of this relationship are worthy of note. Lag times are related to the square of the barrier thickness as is the case for homogeneous barriers and Case II as well. Thus, if the simple lag time relationship is applied to the heterogeneous barrier with active filler, an apparent diffusivity is

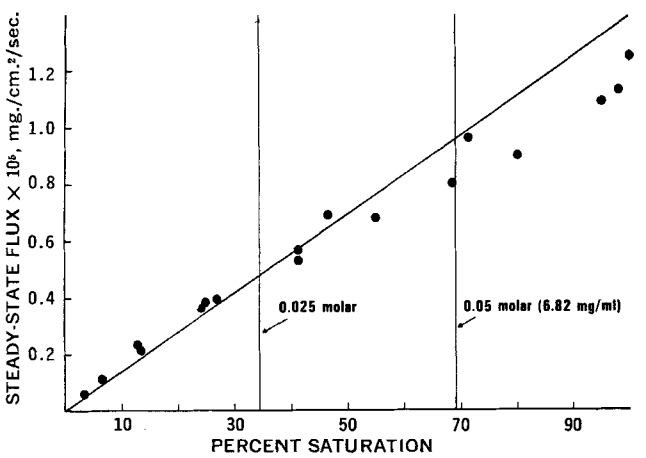

Figure 8—*Plot showing the influence of applied phase concentration of* p*-aminoacetophenone on steady-state flux at 37°. Theoretically, a linear dependency is expected, but there are obvious deviations at high concentrations. Membranes were 0.0476 cm. thick.*

obtained which is independent of membrane thickness for a given filler at a given concentration. The relationship of the apparent diffusivity, D', to more fundamental parameters is given in Eqs. 13 and 14:

$$t_L = \frac{h^2}{6D'} = \frac{h^2}{6D} + \frac{Kh^2}{6D} V_2 \qquad \text{(Eq. 13)}$$

$$\frac{1}{D'} = \frac{1}{D} + \frac{K}{D} V_2 \qquad \text{(Eq. 14)}$$

It can be seen from Eq. 13 that a plot of lag time divided by h^2 against filler volume fraction, V_2, should yield a straight line with an intercept of $1/6D$ and a slope of $K/6D$. Theoretically, one can determine the true diffusion coefficient with membranes of different filler fractions by this method. Similarly, a plot of $1/D'$ *versus* V_2 yields a line with an intercept at $1/D$ and a slope of K/D, again providing estimation of both K and D. The solution of Eq. 14 for D' results in:

$$D' = \frac{D}{1 + KV_2} \qquad \text{(Eq. 15)}$$

Hence, D' is hyperbolically related to V_2.

Two methods are used for estimating diffusivities in polymers: the lag time method in which they are directly calculated and steady-state permeability coupled with equilibrium partition co-

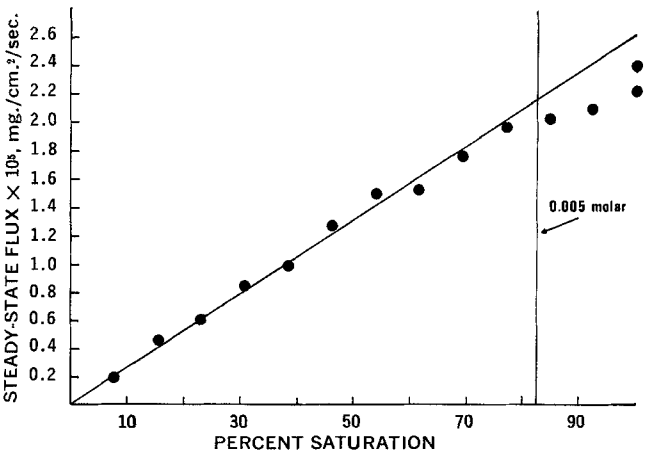

Figure 9—*Steady-state flux of ethyl* p*-aminobenzoate at 37° through 0.0476-cm. membranes as a function of applied phase concentration. Slight, but likely, real deviation from linearity is experienced near saturation concentration. Despite markedly decreased aqueous solubility, the flux is roughly twice that observed for* p*-aminoacetophenone at equivalent percent saturation.*

Table III—Lag Times for the Steady-State Line as a Function of Membrane Type and Adsorption Mechanism

Membrane Type, Adsorption Mechanism	Lag Time Relationship
Homogeneous	$h^2/6D$
Heterogeneous, Case I adsorption	$\frac{\tau^2 h^2}{6D}(1 + KV_2)$
Heterogeneous, Case II adsorption	$\frac{\tau^2 h^2}{D}\left(\frac{1}{4} + \frac{K'}{2k_pC^0}V_2\right)$
Heterogeneous, multiple but independent Case I or Case II adsorption sites	$\frac{\tau^2 h^2}{D}\left(\sum_n\left[\frac{1}{6} + (Kn)V_2\right] + \sum_m\left[\frac{1}{4} + \frac{K'm}{2k_pC^0}V_2\right]\right)$

efficient measurement. Barrer and Chio (17) showed that these methods yield essentially the same results with pure polymers. However, for a heterogeneous barrier, active filler, and Case I sorption, results would not be in agreement by the two methods. Total concentration in the membrane at equilibrium, C_T, may be expressed as:

$$C_T = C_mV_1 + KC_mV_2 \qquad \text{(Eq. 16)}$$

where C_m is the concentration in the external polymeric membrane phase. The equilibrium apparent partition coefficient would be:

$$k_{eq.} = \frac{C_m(V_1 + KV_2)}{C_s} = k_p(V_1 + KV_2) \qquad \text{(Eq. 17)}$$

where C_s is the equilibrium concentration in the bathing solvent. Obviously, C_m/C_s is the true partition coefficient, k_p. The apparent equilibrium diffusivity, $D'_{eq.}$ (the ratio of the apparent permeability constant to the apparent partition coefficient, $k_{eq.}$), is:

$$D'_{eq.} = \frac{P'}{k_{eq.}} = \frac{DV_1}{\tau(V_1 + KV_2)} \qquad \text{(Eq. 18)}$$

which is not equivalent to Eq. 15. Both equations yield $D' = D$ as V_2 approaches zero. If KV_2 is large relative to 1, apparent diffusivities differ by the factor V_1/τ. This factor is the difference between the true and the apparent permeability coefficients. If V_1/τ is not large, the methods should give comparable results. It is interesting and supportive that differences observed by VanAmerongen (18) using heterogeneous barriers are generally in the predicted direction; that is, the equilibrium diffusivity is generally smaller than that obtained using the lag time technique.

The general equation for the steady-state or linear portion of the typical diffusion curve with the boundary conditions previously specified may be expressed as:

$$Q_T = \frac{ADk_pC^0V_1}{\tau h}[t - t_L] \qquad \text{(Eq. 19)}$$

where the lag time, t_L, may be obtained from Table III for the various resolvable situations. The values of both V_1 and τ are equal to 1 in the homogeneous case. A hypothetical example of a Case I

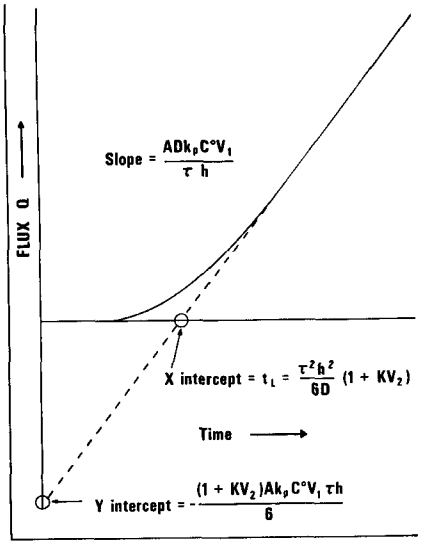

Figure 10—*Theoretical flux curve showing the mathematical values of the intercepts and slope in a diffusional experiment governed by Case I adsorption.*

curve is given in Fig. 10. The equations for slope and intercepts contain five generally independently determinable variables (V_1, V_2, A, h, and C^0) and four unknowns (D, k_p, τ, and K). If τ is not too different from 1, not an unusual situation, estimates of the other unknown parameters may be obtained from a single diffusional run. Otherwise, either D or k_p has to be determined independently. Therefore, in contrast to the opinion of Most (3), the heterogeneous membrane may be characterized diffusionally if the adsorption mechanism is known and is tractable.

In the present case, filler and polymer volume fractions were unknown because commercial polymer was used and this information was not provided. A rough estimate is possible using the permeability coefficient ratio for pure and filled polymer: 0.78 (see *Experimental*). If τ is about 1.1 (see the following paragraph), then V_1 would be about 0.86 and V_2 would be about 0.14. These volume fractions translate to a weight percentage for the filler phase of 27.4, using densities for dimethylpolysiloxane and silica of 0.97 and 2.20, respectively. The weight percent of filler by independent density measurement was 20.6 and thus was only in fair agreement with the steady-state diffusion value.

A rigorous test of the applicability of Eqs. 11 and 13–15 is possible using literature data. Most's (3) recent publication on the passage of ethyl *p*-aminobenzoate through dimethylpolysiloxane barriers containing differing amounts of silica filler is particularly helpful. These data, slightly reworked, are summarized in Table IV. Both V_1 and V_2 were calculated from the weight percentages. The ratio of transmission rates, which are in actuality thickness-averaged steady-state fluxes, provided estimates of τ/V_1, and the product of V_1 times this ratio generated τ. Densities of 0.97 and 2.2 were again used for polymer and filler. Equation 13 predicts that a plot of lag time divided by h^2 against V_2 will be linear. The computer-processed line using this relationship for these data has a correlation coefficient of 0.946 and gives values for D of 8.01×10^{-7} cm.^{2}/sec. and K of 49. Most (3) plotted apparent diffusivity, D', against percent filler

Table IV—Summary of Most's Data (3) on Ethyl *p*-Aminobenzoate Permeation of Dimethylpolysiloxane Membrane, Including Estimates of Volume Fractions and Tortuosity

Weight Percent Filler	V_1	V_2	h, cm.	Transmission Rate $\times 10^5$, μmoles/cm./sec.	Lag Time $\times 10^{-3}$, sec.	$D' \times 10^6$, cm.^{2}/sec.	Relative Transmission Rate, τ/V_1	τ
0	1	0	0.128	1.055	1.50	1.78	1	1
12.5	0.941	0.059	0.139	0.973	13.2	0.242	1.08	1.02
15	0.928	0.072	0.197	0.917	44.9	0.144	1.15	1.07
15	0.928	0.072	0.134(0.144)[a]	0.917	23.8	0.147	1.15	1.07
25	0.872	0.128	0.207	0.834	63.2	0.114	1.27	1.11
25	0.872	0.128	0.128	0.834	22.5	0.119	1.27	1.11

[a] Value required to give reported diffusivity.

Table V—Membrane Solubility, Maximum Rate, Specific Diffusivity, and Partition Coefficient for Ethyl *p*-Aminobenzoate and *p*-Aminoacetophenone at 25 and 37°

Compound	$T°$	Specific Diffusivity, cm.²/sec.	Solubility in Dimethylpolysiloxane[a], mg./ml.	k_p	Maximum Transmission Rate[b]	C_s(Water), mg./ml.
Ethyl *p*-aminobenzoate	27	1.78×10^{-6}	0.83	0.97[d]	1.48×10^{-6}	0.866
	37	(2.67×10^{-6})[c]	1.68			1.74
p-Aminoacetophenone	27	1.63×10^{-6}[c]				
	37	2.44×10^{-6}	0.317	0.0321[e]	7.74×10^{-7}	9.89

[a] Independently determined aqueous solubility, C_s, times k_p. [b] $D \times k_p \times [C_s$. [c] Using a literature E_a of 7.55 kcal./mole for *p*-aminoacetophe none (2). [d] Equilibrium method, Most (3). [e] Lag time method.

which is directly proportional to V_2, and obtained a hyperbolic curve. This is predicted by Eq. 15. If $1/D'$ is plotted against V_2, a straight line is obtained (Fig. 11). The reciprocal of the intercept gives D and was 8.23×10^{-7} cm.²/sec. The value for K obtained with the apparent diffusivity plot was 50.9. The line drawn through the points in Fig. 11 has a correlation coefficient of 0.953. The tortuosity used in a previous calculation was drawn from these data. Weight percent by the relative permeability coefficient method was close to the highest weight percent used by Most (3); therefore, a τ of 1.1 was chosen. Higuchi and Higuchi (7) suggested that tortuosities for uniform spheres will grow linearly up to the point of close packing or a V_2 of 0.77. At this point, τ will be 1.5. The values of τ obtained from Most's data (3) are in good agreement with this hypothesis (Fig. 12). It will be noticed that the arbitrary choice of the unitless parameter, volume fraction, as the measure of mass of adsorbent present makes the value of K unitless also.

The magnitude of K and the resulting product of KV_2 determines the filler effect on the lag time. The K value of 50 for ethyl *p*-aminobenzoate means that a filler volume fraction of 0.02 (about 4% filler by weight) will produce distortions in lag time relative to pure membrane in excess of 100%; 40% filler leads to an order of magnitude increase in lag time.

A comparison of some specific parameters of the ethyl *p*-aminobenzoate and *p*-aminoacetophenone data is given in Table V. Data for two temperatures are given because Most (3) worked at 27° and the present studies were at 37°. Experimental partition coefficients, which are assumed to be relatively temperature insensitive, for the two permeants differ by a factor of 25. However, the specific diffusivities for the two compounds at 27° are virtually identical, which attests to their similar molecular volumes and chemical natures. The 27° value for *p*-aminoacetophenone was calculated using a reported value for the diffusional activation energy of 7.55 kcal./mole (2).

Conceptual application of the present studies extends far beyond specific consideration of dimethylpolysiloxone films. For instance, a rapid method of determining solubility for pure membranes or materials which can be formed into a thick film was demonstrated. This method depends on the thermodynamic activity of the diffusant in the saturated applied phase being equivalent to the activity at the surfacemost layer of the membrane. Both would be equal to the thermodynamic activity of the crystalline solid. The product of the partition coefficient obtained in the diffusional run and the applied phase concentration yields the polymer solubility. This method is particularly valuable for partition coefficients less than one because equilibrium measurement of concentration changes in the applied phase results in small differences in relatively large numbers.

The high specific diffusivities in dimethylpolysiloxane polymer relative to other polymeric materials suggest several practical applications. Based on present theories, hole formation must occur with great frequency, implying that the polymer has great segmental mobility. Use of dimethylpolysiloxane membranes in separation is thus possible; because the material does not imbibe water, these separations may be unique relative to cellulosic barriers. One such specialized application is in the Llewellen and Littlejohn (19) separator, which is the Varian interface between gas chromatograph and mass spectrophotometer. Enrichment of the desired organic at the expense of carrier gas approaches several orders of magnitude per membrane passage (several are used in series).

The use of dimethylpolysiloxane as the polymeric vehicle for drug-containing vaginal rings and implants is also dependent on good diffusant mobility. Kincl *et al.* (20) found that permeability of progesterone through a representative variety of polymeric membranes was 100–1000 times less than through dimethylpolysiloxane. While diffusivity is not the sole factor governing permeability, it is likely the major determinant in this situation. Drug release from implants of materials with diffusivities two to three orders of magnitude less than dimethylpolysiloxane would be extremely slow. For many agents, biological activity would be precluded because effective tissue levels would be neither reached nor maintained.

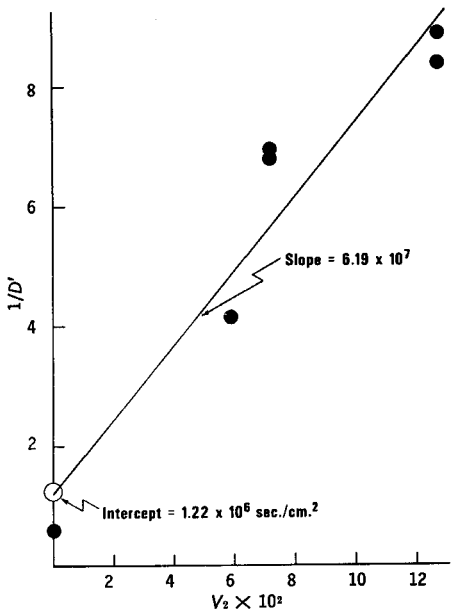

Figure 11—*Fit of Most's data (3) to Case I behavior [the reciprocal of apparent diffusivity, 1/D', should be linear with* V_2 *(Eq. 11)]. The correlation coefficient for the statistical line drawn through the points is 0.953.*

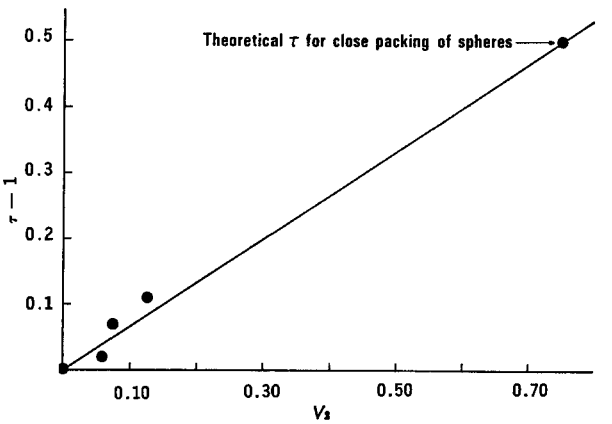

Figure 12—*Plot of* $\tau - 1$ versus V_2 *for Most's ethyl* p*-aminobenzoate data (3) showing that the estimated tortuosities are consistent with theory.*

Understanding of biological membranes is furthered by knowledge of the workings of simpler barriers. The present studies indicate possible effects of specific binding on the nonsteady-state processes governing membrane permeability. From the standpoint of biological membranes, protein binding would have effects analogous to those observed for physical adsorption in the dimethylpolysiloxane films. Both phenomena are dependent on the same basic interaction factors, *i.e.*, hydrogen bonding, hydrophobic bonding, van der Waal's forces, *etc.*, and the simplest cases are mathematically equivalent. Since protein is a major component of living membranes and interacts strongly with a myriad of compound types, one may assume that its presence influences preequilibrium processes involved in transport and distribution of drug and metabolites on the cellular level. However, considering cellular dimensions, it is difficult to ascribe a significance to this relative to all other distribution operations. When macroscopic living barriers such as the skin (stratum corneum), mucosal tissues, and cornea are considered, specific binding is potentially activity determining. The stratum corneum, which is principally protein (keratin and cellular wall remnants), has been reported to be extremely impenetrable relative to other equivalently dense synthetic barriers. Reported diffusivities run to 10^{-13} cm.^{2}/sec. (21). However, diffusivities have been determined using methods that give grossly exaggerated lag times or equilibrium partition coefficients if protein binding is great, either of which directly affect and decrease estimated values of D. Based on these considerations, one can better understand why Scheuplein *et al.* (21) argued that polar molecules permeate slowly through skin not because they are rigorously excluded from the skin but because they are so firmly bound within it.

GLOSSARY OF TERMS

A = area
C^0 = applied phase concentration
C_i = concentration in membrane continuum at donor/membrane interface
C_a = concentration in membrane continuum at point $a \leq h$
C_m = concentration in membrane continuum at equilibrium
C_T = total concentration in heterogeneous membrane, equilibrium condition
D = specific diffusivity, pure membrane
D' = apparent diffusivity, heterogeneous barrier, lag time method
$D_{eq.}$ = apparent diffusivity, heterogeneous barrier, equilibrium method
h = membrane thickness
H = effective diffusional membrane thickness, heterogeneous barrier
K = adsorption constant, Case I
K' = adsorption constant, Case II
k_p = true membrane/solvent partition coefficient
$k_{app.}$ = apparent partition coefficient, heterogeneous barrier, lag time method
$k_{eq.}$ = apparent partition coefficient, heterogeneous barrier, equilibrium method
P = permeability coefficient, pure membrane
P' = permeability coefficient, heterogeneous barrier
Q = flux per unit area
Q_T = total flux
t_L = lag time
V_1 = volume fraction continuous phase, heterogeneous barrier
V_2 = volume fraction filler
τ = tortuosity

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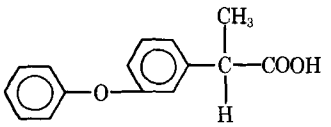
Physiological Disposition of Fenoprofen in Man I: Pharmacokinetic Comparison of Calcium and Sodium Salts Administered Orally

ALAN RUBIN, BRUCE E. RODDA, PATRICIA WARRICK, ANTHONY RIDOLFO, and CHARLES M. GRUBER

Abstract □ Because orally administered *dl*-2-(3-phenoxyphenyl)-propionic acid, fenoprofen, may be useful for maintenance anti-inflammatory/analgesic therapy in man, evaluations were begun of certain pharmacokinetic parameters related to the absorption and disposition of this compound. This study compares these parameters for two salts of fenoprofen. A two-compartment open model was used to analyze plasma concentration data; the model accurately described the plasma levels following oral administration of sodium and calcium fenoprofen. A one-compartment model also provided reasonably accurate descriptions and was used to simulate plasma concentrations in multiple-dose situations from single-dose data. Under the conditions of the study, fenoprofen, administered orally as the sodium or calcium salt, was readily absorbed from the GI tract; however, absorption after the calcium salt was delayed slightly compared to the sodium salt. The bioavailability, distribution, and elimination of fenoprofen appeared to be independent of the salt form of the drug.

Keyphrases □ *dl*-2-(3-Phenoxyphenyl)propionic acid—absorption, distribution, calcium and sodium salts bioavailability, man □ Fenoprofen—absorption, distribution, calcium and sodium salts bioavailability, man □ Absorption kinetics, GI—*dl*-2-(3-phenoxyphenyl)propionic acid (fenoprofen), man □ Bioavailability—calcium and sodium salts of fenoprofen, man □ Plasma levels—fenoprofen, man

Northover (1) showed that some aryl- and alkyl-substituted phenoxyacetic acids exhibited anti-inflammatory properties. One such compound, *dl*-2-(3-phenoxyphenyl)propionic acid, or fenoprofen, exhibited anti-inflammatory and analgesic activities in experimental animals (2) and in man (3–5). The sodium and calcium salts of fenoprofen investigated in the present study are crystalline dihydrates. The chemical structure of fenoprofen is shown here.



fenoprofen

This publication is the first in a series designed to provide basic information about the physiological disposition of fenoprofen in man.

Initially, fenoprofen as the sodium salt was investigated as an anti-inflammatory analgesic. The calcium salt was included later because it was apparently more stable that was the sodium salt, which becomes amorphous and darkened in specific pharmaceutical formulations. Thus, as part of initial studies to describe the pharmacodynamics of fenoprofen in man, it was necessary to compare certain pharmacokinetic parameters of both salts of fenoprofen. This particular study was designed to answer the following questions:

1. What can be learned about the absorption and physiological disposition of fenoprofen in man from the analysis of plasma concentration data after oral administration?
2. How do the sodium and calcium salts of fenoprofen compare pharmacokinetically?

It was anticipated that such information would be useful in developing rational dosage regimens for fenoprofen in man, as well as providing a basic understanding of the physiological disposition of the drug.

The results indicate that fenoprofen, administered as either the sodium or calcium salt, is readily absorbed from the GI tract. The bioavailability, distribution, and elimination of fenoprofen were similar for the two salts, although absorption after the calcium salt was delayed slightly relative to the sodium salt.

EXPERIMENTAL

Subjects—The four subjects[1] were adult males between the ages of 21 and 30 years, ranging in weight from 64.9 to 94.4 kg. (143 to 208 lb.) and in height from 1.72 to 1.91 m. (5 ft. 8 in. to 6 ft. 3 in.). Three subjects were Caucasian; one, J.W., was Negro. Informed consent was obtained from each subject prior to participation in the study. The subjects underwent routine clinical and diagnostic tests with no clinical evidence of illness.

Medication—Each subject ingested a single oral dose of fenoprofen sodium on one occasion and of fenoprofen calcium on another. Two subjects were given the sodium salt initially and the calcium salt 1 week later; the remaining two subjects received the salts in the opposite order. Each dose was equivalent to 250 mg. fenoprofen and was swallowed with at least 180 ml. of water. Fenoprofen sodium was formulated in two capsules, one containing 50 mg. acid equivalent plus 570 mg. cornstarch USP and the other containing 200 mg. acid equivalent plus 382 mg. starch. Fenoprofen calcium was formulated in a single capsule containing 250 mg. acid equivalent plus 222 mg. starch. The medications were administered at 6:00 a.m. after an overnight fast; food was withheld for an additional 6 hr. after administration. No other medication was administered.

After dosing, the men were unrestricted as to movement or position. Smoking and water consumption were permitted. During the tests the subjects were carefully observed. No adverse effects were evident.

Fenoprofen in Plasma—The concentration of fenoprofen in 1-ml. aliquots of plasma was assayed using the GLC method of Nash *et al.* (6). Briefly, this assay involves hexane extraction of fenoprofen from the protein-free supernate of plasma, separation of contaminants by reextraction of fenoprofen into base, acidification, and reextraction of fenoprofen into hexane. After evaporation of the hexane, the fenoprofen content is measured quantitatively as the trimethylsilyl ester. This assay is quite reproducible and is accurate to about 0.4 mcg. fenoprofen/ml. plasma. Blank plasma samples exhibit apparent fenoprofen concentrations of 0.2 mcg./ml. All data are expressed in terms of the free acid.

[1] Four volunteers from the Indiana Reformatory at Pendleton were admitted to the research ward of the Lilly Laboratory for Clinical Research in Indianapolis.

Pharmacokinetics—A preliminary study of fenoprofen, designed to obtain pharmacokinetic information after intravenous administration, indicated that consideration of the body as a single compartment might not adequately describe the plasma kinetics of fenoprofen. Therefore, a two-compartment open model was used to describe the fenoprofen kinetics (7). This model is represented schematically in Scheme I where:

$C_t^{(1)}$ = concentration of fenoprofen in the central (or "plasma") compartment at any time, t
V_1 = apparent volume of central compartment
$C_t^{(2)}$ = concentration of fenoprofen in the peripheral (or "tissue") compartment at any time, t
V_2 = apparent volume of the peripheral compartment
k_{ab} = first-order absorption rate constant
k_{12} = first-order rate constant representing diffusion from the central to the peripheral compartment
k_{21} = first-order rate constant representing diffusion from the peripheral to the central compartment
k_d = first-order rate constant representing disappearance of fenoprofen from the central compartment by metabolism and excretion

This model is based on the following assumptions:

1. A dose (D) of fenoprofen is administered orally, and a fraction (f) of this dose is absorbed at a first-order rate (represented by k_{ab}) following an initial lag time (t_0). The lag time may be a function of dissolution, gastric emptying, *etc.*
2. Once absorbed, free fenoprofen disappears from the central compartment *via* three first-order processes: excretion, metabolism, and diffusion into the peripheral compartment. Because the plasma was analyzed for fenoprofen and not for circulating metabolite, metabolism cannot be distinguished from excretion in the overall disappearance of the drug from plasma. However, studies in progress should allow such distinctions.
3. Diffusion of fenoprofen from the peripheral to the central compartment follows first-order kinetics.

Under the stated assumptions of the two-compartment open model, the following equation describes the concentration of fenoprofen in the central compartment at any time $t > t_0$:

$$C_t^{(1)} = \frac{k_{ab} fD}{V_1}\left[\frac{(k_{21}-\alpha)e^{-\alpha(t-t_0)}}{(k_{ab}-\alpha)(\beta-\alpha)} + \frac{(k_{21}-\beta)e^{-\beta(t-t_0)}}{(k_{ab}-\beta)(\alpha-\beta)} + \frac{(k_{21}-k_{ab})e^{-k_{ab}(t-t_0)}}{(\alpha-k_{ab})(\beta-k_{ab})}\right] \quad \text{(Eq. 1)}$$

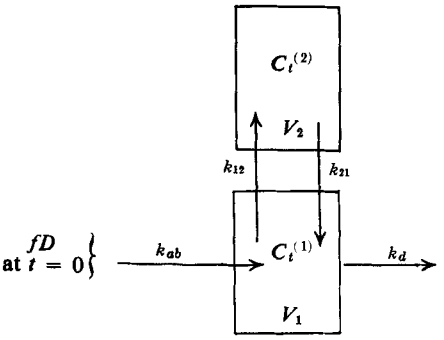
where:

$$\alpha = \tfrac{1}{2}\left[k_{12}+k_{21}+k_d+\sqrt{(k_{12}+k_{21}+k_d)^2-4k_{21}k_d}\right]$$

and:

$$\beta = \tfrac{1}{2}\left[k_{12}+k_{21}+k_d-\sqrt{(k_{12}+k_{21}+k_d)^2-4k_{21}k_d}\right]$$

Initial estimates of parameters in Eq. 1 were derived for each subject–salt pair using an exponential stripping procedure. These approximations were then refined using nonlinear least-squares estimation techniques. Final parameter estimates are presented in Table I.



Scheme I—Two-compartment open model, first-order absorption

RESULTS AND DISCUSSION

Plasma concentrations of fenoprofen were predicted using Eq. 1 and the appropriate experimentally determined constants of Table I. The observed concentrations and those described by the two-compartment model are displayed in Fig. 1. The similarity between actual and predicted concentrations suggests that the parameter estimates are satisfactory under this model. When the sodium salt was given to the four subjects, the characteristic concentration pattern showed a maximum between 0 and 1 hr., as compared with 0.5 and 2 hr. for the calcium salt. At acidic pH, the calcium salt has a slower dissolution rate than the sodium salt; this may explain the delayed appearance of fenoprofen in the plasma following ingestion of the calcium salt.

The observed peak concentration for the sodium salt had a mean of 32 mcg. fenoprofen/ml. plasma (range 18–45); the corresponding value for the calcium salt was 27 (range 23–31) mcg./ml. The actual peak concentrations and the times of their occurrence ($t'_{max.}$) were not observed because of inadequate sampling during this period. However, $t'_{max.}$ values were estimated using the two-compartment model (Table I). Twelve hours after dosage with either salt, the mean concentration had decreased to about 1.5 (range 0.8–2.4) mcg./ml.

The experimental design precluded a precise estimate of the percent of dose absorbed. Although a two-compartment open model was proposed for the kinetic description of fenoprofen, inadequate sampling at certain times effectively reduced the model to a single compartment. Riegelman *et al.* (8) demonstrated that, in such a situation, estimates of V_1 are too large; thus fD/V_1 in this study (35 mg./l.) could be considered a lower bound, and the true value could be much greater. If V_1 were as small as the volume of circulating plasma, about 3.2 l., then the fraction of the dose of fenoprofen appearing unchanged in the plasma following oral administration would be at least 45%. Studies are in progress that include intravenous dosing to allow more confident estimation of this fraction. Among factors affecting the rate and extent of absorption of drugs in solution are their dissociation constants and lipid solubilities of the unionized forms (9–11). In addition, in the case of administration of solid dosage forms, absorption is likely to be dissolution-rate limited.

The absorbed fenoprofen appears to be confined to a single compartment which acts as if it were instantaneously equilibrating with

Table I—Parameter Estimates, Two-Compartment Open Model

Treatment	Subject	Body Weight, kg.	fD/V_1, mg./l.	k_{ab}, min.$^{-1}$	k_{12}, min.$^{-1}$	k_{21}, min.$^{-1}$	k_d, min.$^{-1}$	t_0[a], min.	$t'_{max.}$[b], min.
Fenoprofen calcium	J.H.	95	26.5	0.65	<0.001	<0.001	0.005	27	35
	W.J.	65	36.4	0.03	<0.001	<0.001	0.005	29	101
	R.L.	75	31.0	0.39	<0.001	<0.001	0.006	21	32
	J.W.	65	39.1	0.02	<0.001	<0.001	0.005	15	107
Fenoprofen sodium	J.H.	95	41.3	0.32	<0.001	<0.001	0.011	10	21
	W.J.	65	39.7	0.90	<0.001	<0.001	0.006	10	16
	R.L.	75	20.4	0.06	<0.001	<0.001	0.003	14	67
	J.W.	65	30.5	0.07	<0.001	<0.001	0.004	15	58

[a] t_0 = predicted time lag between drug administration and appearance of measurable amount of fenoprofen in plasma. [b] $t'_{max.}$ = predicted time period to attain peak plasma concentration of fenoprofen.

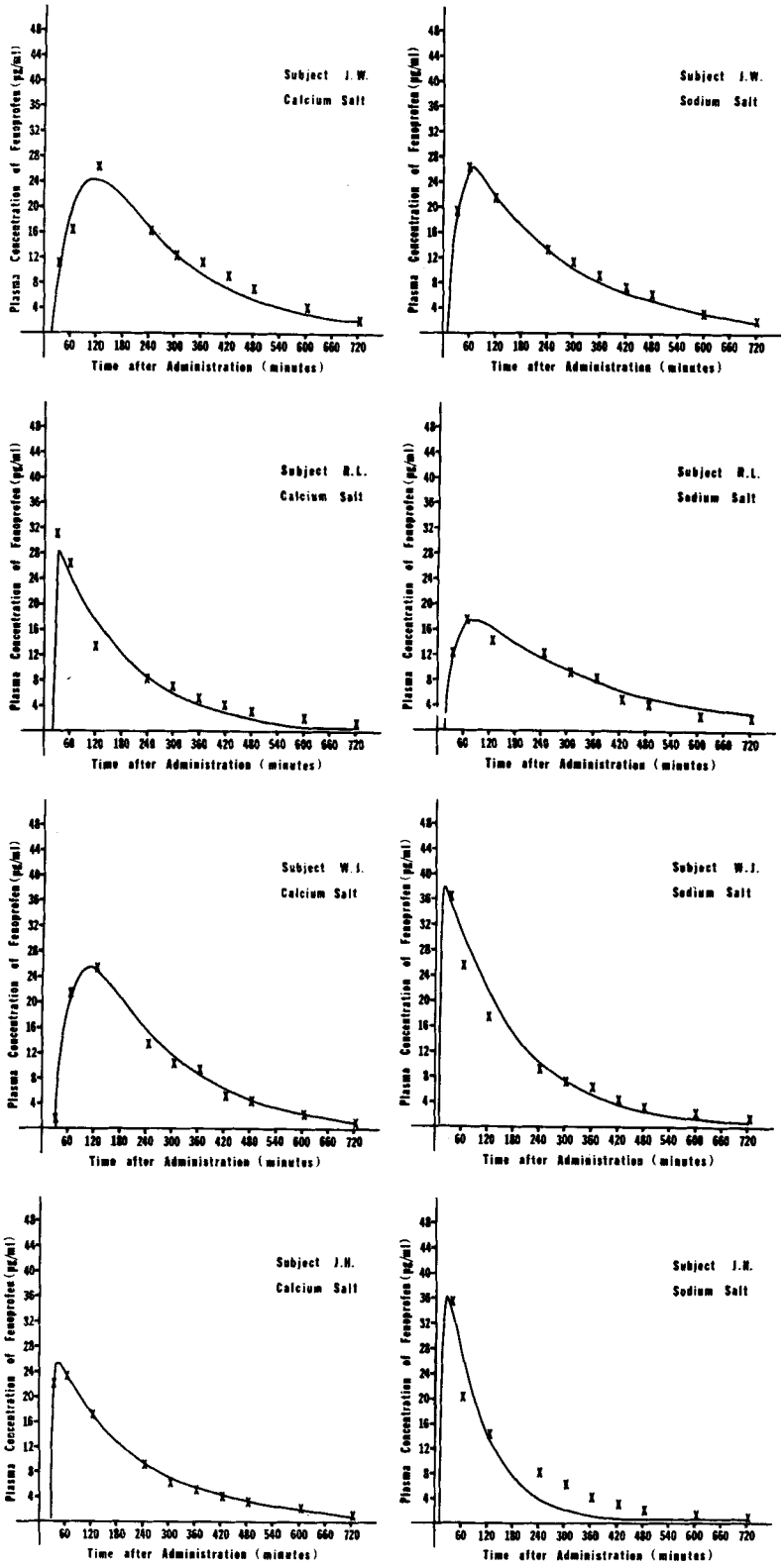


Figure 1—*Fenoprofen was administered orally to four human subjects as the calcium and sodium salts. Each curve describes the concentrations predicted by a two-compartment open-model system; the plotted (×) values represent concentrations determined experimentally.*

the plasma. This could be related to a high degree of binding of fenoprofen to plasma proteins. By using equilibrium dialysis techniques, it was observed that such binding does occur over a wide pH range (5.4–9.4). At concentrations of 40 mcg. fenoprofen/ml. and 4.9 mg. human serum albumin/ml. (*i.e.*, only one-eighth the concentration of albumin present in normal plasma), 99% of fenoprofen was bound to albumin at pH 7.4. Moreover, not only was the binding to protein extensive, but the apparent affinity between the drug and albumin favored the binding process ($K_{assoc.}$ = 30,000).

In other studies, where radioactive plasma samples from subjects who had received ^{14}C-fenoprofen were ultrafiltered, less than 1% of the radioactivity was present in the ultrafiltrate, and over 99% remained in the retentate. Almost all of this radioactivity was accounted for as unchanged fenoprofen.

Distribution of fenoprofen is in accord with a two-compartment model (7, 12), but the distribution into a second compartment appears to be minimal. Table I contains the first-order constants, representing the rates of diffusion of fenoprofen from the central to

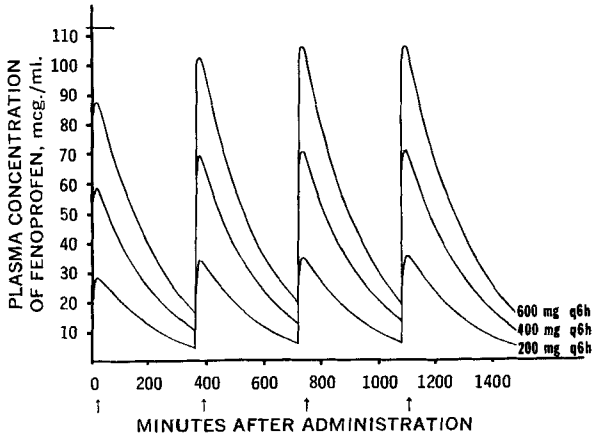


Figure 2—*Hypothetical plasma concentrations of fenoprofen in subjects treated orally with 200, 400, or 600 mg. The dosage is administered every 6 hr., as indicated by the arrows. (See text for assumptions.)*

the peripheral compartment (k_{12}) and the rates from the peripheral to the central compartment (k_{21}). Although these constants are <0.001 (*i.e.*, <0.1%/min.), individual values vary considerably below this limit so that little confidence can be placed in them. Because V_2 (the apparent volume of distribution of the peripheral compartment) depends on the ratio of k_{12} and k_{21}, which are regarded as unreliable, estimates of V_2 would be meaningless. Moreover, if as much as 0.1% of the concentration of fenoprofen in the central compartment were to enter the peripheral compartment per minute, then the effect of this compartment on the system would be minimal. This minimal effect of the peripheral compartment was the same for the two salts[2]. Thus, it appears that the physiological disposition of orally administered fenoprofen could be adequately described by a one-compartment open model in this study.

Figure 1 reflects a general agreement between the experimental and predicted concentrations. In several cases, however, an underestimation of the concentration occurred from 2 to 6 hr. following administration[3]. This underestimation could be explained by an enterohepatic recirculation of fenoprofen, which would be expected to produce a secondary rise in plasma concentration. An enterohepatic recirculation was shown to occur in rats (13), but its occurrence in man is, as yet, unverified.

Fenoprofen concentrations in plasma were predicted for multiple-dose situations. Because the information obtained in this study did not allow adequate description of parameters associated with a peripheral compartment, these predictions were based on a one-compartment open model which assumes that: fenoprofen, upon oral administration, is completely absorbed by a first-order rate process, is distributed uniformly within a single compartment, and is eliminated by a first-order rate process. The parameters of this model were based upon the following representative values: k_{ab} = 0.15 min.$^{-1}$; k_d = 0.005 min.$^{-1}$; f/V = 0.14 l.$^{-1}$; a dosing interval = 6 hr.; and dosages = 200, 400, or 600 mg. of fenoprofen. The kinetic parameters are assumed to be independent of dose in the range under consideration, and single- and multiple-dose plasma levels are considered comparable. Figure 2 depicts the plasma concentrations predicted under these conditions.

Pharmacokinetic calculations (14) indicated that after the second dose, greater than 95% of the equilibrium plasma concentration would be achieved. To reach the equilibrium state at once, a reasonable loading dose would be 1.2 times the maintenance dose (provided, of course, this higher dose was compatible with the pharmacologic effects of the drug).

When a drug is administered repeatedly, $t'_{max.}$ decreases until equilibrium is established. For fenoprofen, however, equilibrium is reached so quickly after the initial dose that clinically this delay is merely a curiosity.

The maximum plasma concentrations to be expected at equilibrium with this regimen at doses of 200, 400, and 600 mg. are 36, 71, and 107 mcg./ml., respectively; corresponding minimum values are 6, 12, and 20 mcg./ml. Thus, the fluctuation ratio ($C_{max.}/C_{min.}$) ranges between 5 and 6. Until sufficient clinical experience is gained with fenoprofen concerning a possible relationship between efficacy and plasma concentrations, the applicability of these predictions to clinical practice must be considered only as theoretical guidelines. However, one additional volunteer (K.H.) was dosed orally every 6 hr. with 500 mg. of fenoprofen as the sodium salt. After 108 doses, the estimated f/V, k_{ab}, and k_d values were virtually identical to the corresponding values obtained after a single dose. Also, the values of the pharmacokinetic parameters agreed quite well with those obtained in this study in which 250 mg. of fenoprofen was used. Thus, in this subject, the kinetics of fenoprofen appeared to be dose independent (in this range), and single-dose data accurately predicted multiple-dose plasma concentrations.

Considerable agreement exists between sodium and calcium fenoprofen with respect to the parameters of the model. Absorption-rate constants, diffusion-rate constants, bioavailabilities, disappearance-rate constants, and half-lives (about 2.5 hr.) were similar for each salt of fenoprofen. Although differentiation between the two salts is uncertain with only four subjects, absorption of fenoprofen administered as the sodium salt began earlier in each subject than absorption following ingestion of the calcium salt. However, there was no consistent indication with respect to k_{ab} values that one salt was absorbed faster than the other. Furthermore, the data are consistent with a conclusion of equivalent availability of fenoprofen from the two salts. By assuming that the apparent volume of the central compartment remains constant for each individual, the parameter fD/V_1 is a measure of the amount of fenoprofen appearing in the central compartment (availability). Subjects J.H. and W.J. exhibited greater availability of fenoprofen from the sodium salt, whereas the reverse was true for Subjects R.L. and J.W. These results would be expected if the two salts were identical; therefore, no additional statistical tests were appropriate.

One subject (W.J.), who had received the sodium salt, exhibited peak concentrations of fenoprofen immediately after a lag time of 10 min. This nearly instantaneous peak negated the estimation of a meaningful value for his absorption parameter, k_{ab}. However, because this value was needed to predict the plasma concentration curve and to provide reasonable estimates of other pharmacokinetic parameters, a k_{ab} value of 0.90 min.$^{-1}$ was selected arbitrarily. Thus, it was assumed that 90% of the available dose was absorbed per minute.

These results indicate that fenoprofen administered as either the calcium or sodium salt is readily absorbed from the GI tract. Absorption of fenoprofen after administration of the calcium salt was delayed slightly relative to the sodium salt. Bioavailability, distribution, and elimination were similar for the two salts.

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[2] Intravenous and oral data from studies in progress, designed to describe more precisely the fenoprofen kinetics, indicate the existence of a nontrivial peripheral compartment.

[3] This underestimation may not appear noteworthy from observation of Fig. 1. However, the "raw" data do indicate a frequent underestimation between 2 and 6 hr. (which is lost in the photographic reduction of individual graphs for reproduction).

(14) J. G. Wagner, "Pharmacokinetics," J. M. Richards Laboratory, Grosse Point Park, Mich., 1969, p. 80.

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Prediction of Stability in Pharmaceutical Preparations XVI: Kinetics of Hydrolysis of Canrenone and Lactonization of Canrenoic Acid

EDWARD R. GARRETT and CHONG MIN WON

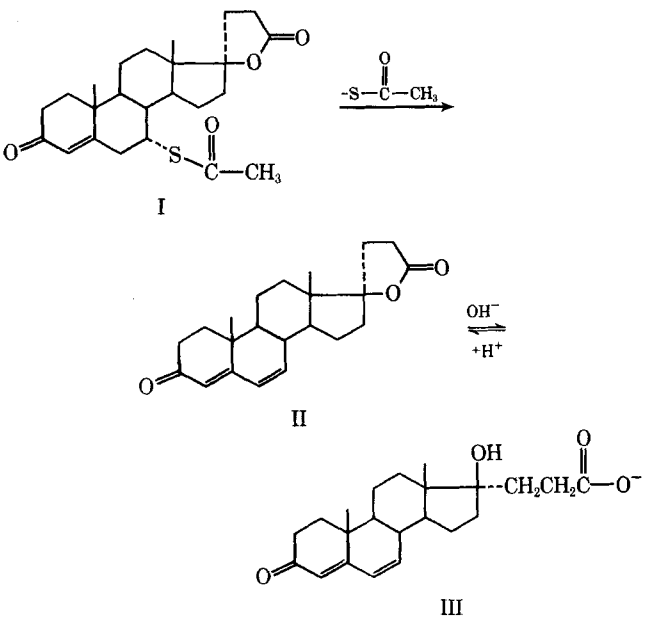
Abstract □ The kinetics of the hydrolysis of the lactone canrenone, 3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl)propionic acid γ-lactone, and the lactonization of its corresponding canrenoic acid salt, potassium 3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl)propionate, were studied by spectrophotometrically analyzing the chloroform-extracted lactone from canrenoic acid solution. The log *k*–pH profiles in the pH range of 1–12 in various buffer solutions at 25.0, 37.5, 45.0, 60.0, 70.0, and 79.9° show that the kinetics of lactonization include hydrogen-ion attack on the undissociated canrenoic acid molecule and a pH-independent closure of the canrenoate anion. In addition to the hydroxide-ion-catalyzed hydrolysis of the lactone, general base-catalyzed hydrolysis in carbonate buffer solution was observed and attributed to carbonate-dianion attack. The rate constants, equilibrium constants, pKa′ values, solubilities, and Arrhenius' parameters were obtained. Maximum concentrations of canrenoic acid salt to maintain elegant pharmaceutical preparations are given as a function of pH.

Keyphrases □ Canrenone, canrenoic acid—hydrolysis–lactonization kinetics, pH effect, appropriate pharmaceutical preparations □ Pharmacokinetics—canrenone hydrolysis, canrenoic acid lactonization, pH effect □ Hydrolysis—canrenone □ Lactonization—canrenoic acid □ UV spectrophotometry—analysis, canrenone and canrenoic acid

Spironolactone (I), 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)propionic acid γ-lactone, has been widely used in the treatment of edema that has not responded properly to treatment with conventional diuretics (1). Spironolactone exhibits a specific antagonism to the tendency of the adrenal steroid aldosterone to increase the reabsorption of sodium by reversibly competing with aldosterone at the receptor sites and thus modifying the sodium-retaining electrolyte excretion pattern, which is said to be the mechanism responsible for the production and maintenance of edema (2–4).

Canrenone (II), aldadiene [3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl)propionic acid γ-lactone], may be the conjugated diene steroid found in the plasma after the oral administration of spironolactone (5). It may be formed by the elimination of the thioacetate group of spironolactone and may possess similar biological properties as an aldosterone antagonist (Scheme I). As is common with γ-lactones, canrenone (II) is stable in acid and hydrolyzes in alkali to the corresponding canrenoic acid salt (III), potassium 3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl)propionate. This study was undertaken to determine the quantitative transformations of canrenone in aqueous solution as a function of pH to supply the basic information necessary for preparing stable aqueous pharmaceutical preparations of canrenone and canrenoic acid.

In addition, it is important to know the ease of the acid–lactone transformation to anticipate the species that may appear in the body under GI and other physiological conditions. As yet, it has not been clarified whether the lactone or the acid, or a metabolite derived from one or the other, is the pharmacologically active compound. The two species also might show different tendencies for protein binding and, hence, influence the distribution pattern of the drug in the



Scheme I

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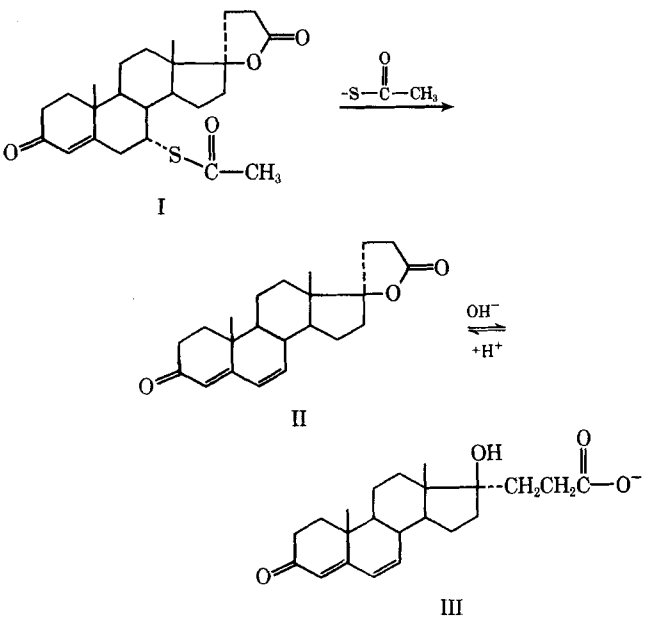


Scheme I

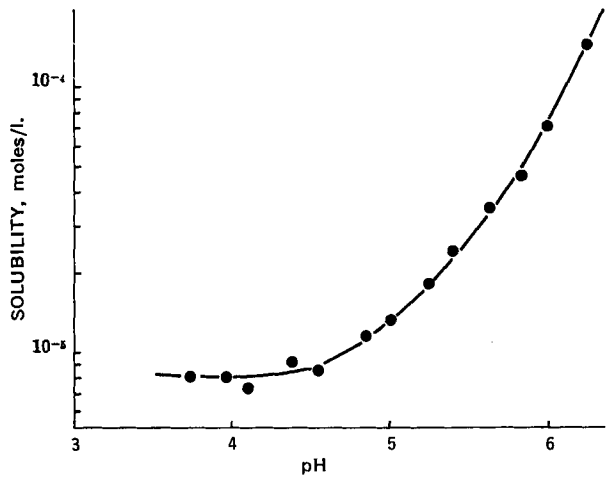


Figure 1—*Semilogarithmic plot of the molar solubility of canrenoic acid as a function of pH at 25.0°.*

body. Thus, this study on the interconversion of lactone and acid can serve as background for biopharmaceutical and pharmacokinetic studies of canrenone and canrenoic acid and as a basis for optimal design of appropriate dosage forms.

EXPERIMENTAL

Materials and Reagents—Canrenone (II), 3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl)propionic acid γ-lactone, and the potassium salt of canrenoic acid, potassium canrenoate (III), potassium 3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl)propionate, were used in this study[1]. All other chemicals were of reagent grade.

Kinetic Procedures—A filtered aqueous solution of canrenone (about 9×10^{-6} *M*) was used as a stock solution for the kinetic studies on lactone hydrolysis. An aqueous stock solution of potassium canrenoate (about 4×10^{-3} *M*) was used for the kinetic studies on lactonization. The kinetic studies of hydrolysis and lactonization of canrenone and canrenoate anion, respectively, were made on final solutions in the concentration range of $1.0\text{–}3.6 \times 10^{-5}$ *M*.

For slow reactions, *i.e.*, half-lives in days, 5–20 ml. of stock buffer solutions and appropriate amounts of 1 *M* KCl to maintain an ionic strength of 0.1 (0.16 in phosphate buffer) were added to appropriately diluted volumes of the stock solutions. The first

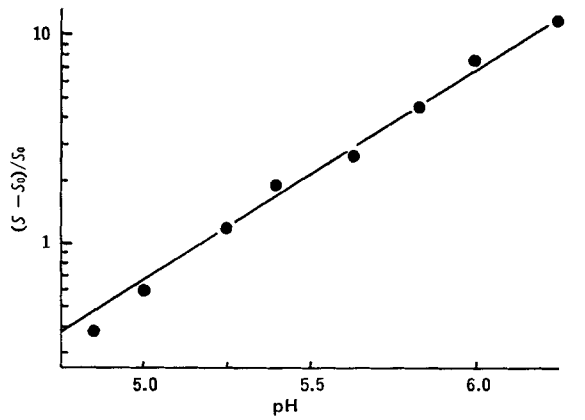


Figure 2—*Semilogarithmic plot of the solubility function* $(S - S_0)/S_0$ *against pH at 25.0° in accordance with* $\log(S - S_0)/S_0 = pH - pKa'$, *where* S *is the total molar solubility of canrenoic acid and its anion at a given pH, and* S_0 *is the intrinsic solubility of the undissociated canrenoic acid. The slope is unity, and the intercept permits the calculation of pKa' as 5.20.*

analyses of lactone concentration were made after the reacting solutions equilibrated in the temperature bath.

For the fast reactions, *i.e.*, half-lives in hours or minutes, 99.0 ml. of appropriately diluted stock solutions of the reacting substances, adjusted to appropriate ionic strengths, was preheated in the temperature bath prior to the addition of 1.0 ml. of the acid or alkali used to accelerate the reaction. The bath temperatures were maintained within ±0.1° for 79.9° and within ±0.05° for all other temperatures.

The compositions of the buffer solutions at the various temperatures studied are given in Table I. Samples of the reacting solutions were taken at various times, cooled to room temperature, and analyzed for the lactone. The fast reactions were followed for 5–6 half-lives and the slow ones for at least 2–3 half-lives. The concentration of the lactone at infinite time was obtained after the reaction went 10 half-lives.

Assay Procedure—Cooled aliquots (4.00 ml.) of the reacting solutions were put into a 30-ml. separator, which contained 5.00 ml. of chloroform and 3.00 ml. of carbonate buffer ($[CO_3^{-2}] = 0.15$ *M*, $[HCO_3^-] = 0.05$ *M*), pH 10.4. The funnel was shaken vigorously for 1 min., the chloroform layer was separated, and the absorbance of the extracted lactone was read at 280 nm. with a slit width of 0.2 mm. against a buffer-saturated chloroform blank in a spectrophotometer[2]. Within the significance of the spectral assay, all of the lactone was extracted into the chloroform layer under these conditions, and none could be spectrophotometrically observed in the aqueous layer. However, all of the potassium canrenoate remained in the aqueous layer under these conditions, and none could be spectrophotometrically observed in the chloroform layer.

Determination of Equilibrium Constants—The equilibrium constants were determined at different pH values at an ionic strength of 0.1 by analyzing the concentrations of the lactone and the acid in the sample after the reaction reached an equilibrium. Measurements also were made after the rate studies confirmed the achievement of this equilibrium. The assay procedure was used to determine the lactone concentration. The total acid and lactone concentration was obtained by partitioning both acid and lactone into 5.00 ml. of the chloroform layer by shaking 4.00 ml. of the sample solution together with the chloroform and 3.00 ml. of formate buffer ([HCOOH] = 0.16 *M*, [HCOONa] = 0.04 *M*), pH 3.0. The absorbance of the chloroform layer was read against a buffer-saturated chloroform blank at 280 nm. with a slit width of 0.2 mm. The molar absorptivity (ϵ = 29,000) at the wavelength of maximum absorption (280 nm.) of the lactone canrenone and canrenoic acid in the chloroform was the same. The acid concentration was then calculated from the observed difference of the two assay procedures.

pKa' Determination—The pKa' (apparent pKa) of canrenoic acid (III) was determined by measuring the solubilities of the acid as a function of pH. A series of acetic acid–acetate, monohydrophosphate–dihydrophosphate solutions, ionic strength 0.1, ranging in pH values from 3.7 to 6.2, was prepared. Potassium canrenoate solution (1.00 ml. of 2.8×10^{-2} *M*) was added to 99.0 ml. of each buffer solution. This addition produced a precipitate of canrenoic acid whose solubility was exceeded at these pH values. The solutions were shaken at 25.0° in a mechanical shaker. After the solutions equilibrated, they were filtered. After adjustment of an aliquot with carbonate buffer to pH 10.4, as previously described for the lactone assay procedure, the resultant canrenoic acid solution was extracted with chloroform to remove the lactone; the separated buffered aqueous layers were spectrophotometrically assayed at $\lambda_{max.}$ 294 nm. (ϵ = 25,000) at pH 10.4 for the sum of the canrenoic acid and its anion concentrations in the original solution at the various pH values. From these values, the total solubility of canrenoic acid and its anion could be calculated at the several pH values. The pKa' values at higher temperatures were determined from similar solubility determinations at the two pH values of 4.0 and 5.2 (acetate buffer). The intermittently shaken solutions were maintained in a constant-temperature water bath.

RESULTS AND DISCUSSION

Estimation of pKa'—The low solubility of canrenoic acid (III) in water necessitated the use of the solubility method for the

[1] Obtained from G. D. Searle & Co. as SC-9376 and SC-14266' respectively.

[2] Beckman DU.

Table I—Apparent First-Order Rate Constants, k^a, in sec.$^{-1}$ for the Achievement of Equilibrated Lactonization of Canrenoic Acid and Hydrolysis of Canrenone as a Function of pH[b] at Various Temperatures

Medium[c]	25.0° pH	25.0° k	37.5° pH	37.5° k	45.0° pH	45.0° k
[HCl]						
0.100	1.10	6.91×10^{-3}	—	—	—	—
0.070	1.23	4.77×10^{-3}	—	—	—	—
0.050	1.38	3.55×10^{-3}	—	—	—	—
0.040	1.47	2.80×10^{-3}	—	—	—	—
0.010	2.04	7.45×10^{-4}	2.04	1.62×10^{-3}	2.04	3.44×10^{-3}
[HCOOH] [NaOH]						
0.050 0.010	3.05	1.01×10^{-4}[d]	3.00	2.00×10^{-4}[l]	3.00	4.46×10^{-4}[l]
[CH_3COOH][CH_3COONa]						
0.040 0.010	4.00	9.98×10^{-6}[e]	4.00	2.65×10^{-5}[m]	4.00	4.43×10^{-5}[u]
0.010 0.040	5.23	1.08×10^{-7}[f]	—	—	5.15	4.11×10^{-6}[v]
[Na_2HPO_4] [KH_2PO_4]						
0.005 0.020	6.28	1.13×10^{-7}[g]	—	—	—	—
0.016 0.004	—	—	7.42	5.94×10^{-7}[n]	—	—
0.018 0.002	—	—	7.75	6.91×10^{-7}[o]	—	—
[H_3BO_3] [NaOH]						
0.010 0.003	8.80	7.73×10^{-7}[h]	—	—	8.70	8.71×10^{-6}[w]
[Na_2CO_3] [$NaHCO_3$]						
0.0020 0.0040	9.60	6.93×10^{-6}[i]	9.65	2.50×10^{-5}[p]	9.60	4.80×10^{-5}[x]
0.0035 0.0045	—	—	10.00	5.20×10^{-5}[q]	—	—
0.0060 0.0060	—	—	—	—	10.05	1.23×10^{-4}[y]
0.0045 0.0035	—	—	10.15	7.70×10^{-5}[r]	—	—
0.0053 0.0027	10.25	2.25×10^{-5}[j]	10.30	9.10×10^{-5}[s]	—	—
[NH_2CH_2COOH][NaOH]						
0.0104 0.0096	10.65	4.30×10^{-5}[k]	—	—	—	—
[NaOH]						
0.005	11.6	5.43×10^{-4}	—	—	—	—
0.010	12.0	1.20×10^{-3}	—	—	—	—
0.015	12.1	1.89×10^{-3}	—	—	—	—
0.020	12.2	2.35×10^{-3}	—	—	—	—
[CH_3COOH][CH_3COONa]						
0.040 0.010	—	—	4.00	1.89×10^{-4}[z]	—	—
0.010 0.015	—	—	4.80	3.28×10^{-5}	—	—
0.010 0.040	5.20	5.72×10^{-6}	5.20	1.10×10^{-5}[a']	5.20	2.90×10^{-5}[a']
[Na_2HPO_4] [KH_2PO_4]						
0.005 0.020	—	—	6.20	1.05×10^{-5}[b']	6.20	2.75×10^{-5}[f']
0.006 0.004	7.00	4.68×10^{-6}	7.00	9.60×10^{-6}	7.00	2.81×10^{-5}[g']
0.016 0.004	7.40	6.86×10^{-6}	7.40	1.39×10^{-5}[c']	7.40	3.91×10^{-5}
0.018 0.002	7.80	9.14×10^{-6}	7.80	2.05×10^{-5}[d']	7.80	6.56×10^{-5}
[H_3BO_3] [NaOH]						
0.010 0.003	—	—	8.70	8.75×10^{-5}	—	—

[a] The rate constants are the sums of the hydrolysis and the lactonization rate constants. They are the values obtained by extrapolation to zero buffer concentration whenever a buffer catalysis was observed. [b] The pH values in HCl and NaOH were calculated from pH = $-\log \gamma_{HCl}$[HCl] and pH = pKw − pOH, where pOH = $-\log \gamma_{NaOH}$[NaOH] where the activity coefficients, γ_{HCl} and γ_{NaOH}, and pKw values were obtained from the literature (10). All other pH values were measured by a Radiometer pH meter equipped with a Corning semimicro combination electrode (temperature range $-5 \sim 100°$). The pH meter was standardized with standard buffers (Sargent-Welch) of pH 4.0, 7.0, and 10.0 at the same temperature. [c] KCl was added to maintain the ionic strength, μ, at 0.1 (0.16 in phosphate buffer). [d] The 10^4k for multiples of the stated buffer concentration were: 1×, 0.98; 2×, 0.96; 3×, 1.10; and 4×, 1.00. [e] The 10^5k for multiples of the stated buffer concentration were: 1×, 1.07; 2×, 1.07; 3×, 1.14; and 4×, 1.19. [f] The 10^7k for multiples of the stated buffer concentration were: 1×, 1.13; 2×, 1.46; 3×, 1.68; and 4×, 1.92. [g] The 10^7k for multiples of the stated buffer concentration were: 1×, 1.32; 2×, 1.38; 3×, 1.48; and 4×, 1.57. [h] The 10^7k for multiples of the stated buffer concentration were: 1×, 7.73; 2×, 7.73; 3×, 7.73; and 4×, 7.74. [i] The 10^6k for multiples of the stated buffer concentration were: 1×, 7.14; 1.5×, 7.33; 3.5×, 7.90; and 5.0×, 8.04. [j] The 10^5k for multiples of the stated buffer concentration were: 0.75×, 2.32; 1.13×, 2.68; 2.64×, 3.02; and 3.77×, 3.19. [k] The 10^5k for multiples of the stated buffer concentration were: 1×, 4.77; 2×, 6.17; 3×, 7.00; and 4×, 7.45. [l] The 10^4k for multiples of the stated buffer concentration were: 2×, 2.15; 3×, 1.95; and 4×, 1.91. [m] The 10^5k for multiples of the stated buffer concentration were: 1×, 2.84; 2×, 3.03; 3×, 3.34; and 4×, 3.23. [n] The 10^7k for multiples of the stated buffer concentration were: 1×, 5.94; 1.5×, 5.94; 2.0×, 5.94; and 2.5×, 5.94. [o] The 10^7k for multiples of the stated buffer concentration were: 1×, 6.91; 1.5×, 6.91; 2.0×, 6.91; and 2.5×, 6.91. [p] The 10^5k for multiples of the stated buffer concentration were: 1×, 2.57; 1.5×, 2.92; 3.5×, 3.42; and 5.0×, 3.50. [q] The 10^5k for multiples of the stated buffer concentration were: 1×, 5.48; 2×, 5.97; 3×, 6.17; and 4×, 6.66. [r] The 10^5k for multiples of the stated buffer concentration were: 1×, 7.63; 2×, 8.44; 3×, 9.29; and 4×, 9.59. [s] The 10^5k for multiples of the stated buffer concentration were: 1×, 9.44; 2×, 10.3; 3×, 11.0; and 4×, 11.1. [t] The 10^4k for multiples of the stated buffer concentration were: 1×, 4.46; 2×, 4.46; 3×, 4.46; and 4×, 4.46. [u] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 4.61; 1.5×, 5.22; 2.0×, 5.49; and 2.5×, 5.57. [v] The 10^6k for multiples of the stated buffer concentration were: 1×, 4.11; 2×, 4.11; 3×, 4.11; and 4×, 4.11. [w] The 10^6k for multiples of the stated buffer concentration were: 1×, 8.61; 2×, 8.97; 3×, 8.38; and 4×, 8.90. [x] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 5.20; 1.5×, 5.43; 3.5×, 6.14; and 5.0×, 6.95. [y] The 10^4k for multiples of the stated buffer concentration were: 1×, 1.23; 2×, 1.23; 3×, 1.23; and 4×, 1.23. [z] The 10^4k for multiples of the stated buffer concentration were: 1.0×, 2.14; 1.5×, 2.26; 2.0×, 2.46; and 2.5×, 2.59. [a'] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 1.39; 1.5×, 1.39; 2.0×, 1.54; and 2.5×, 1.51. [b'] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 1.15; 1.5×, 1.30; 2.0×, 1.38; and 2.5×, 1.39. [c'] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 1.34; 1.5×, 1.28; 2.0×, 1.47; and 2.5×, 1.47. [d'] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 2.38; 1.5×, 2.38; 2.0×, 2.62; and 2.5×, 2.71. [e'] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 2.82; 1.5×, 2.78; 2.0×, 3.27; and 2.5×, 2.84. [f'] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 2.65; 1.5×, 2.66; 2.0×, 2.99; and 2.5×, 3.10. [g'] The 10^5k for multiples of the stated buffer concentration were: 1.0×, 2.84; 1.5×, 2.84; 2.0×, 2.81; and 2.5×, 2.77.

determination of the ionization constant. If S_0 is the intrinsic solubility of the undissociated acid, [HA], the ionization constant:

$$K_a' = [H^+][A^-]/[HA] \quad \text{(Eq. 1)}$$

can be expressed in terms of the solubilities (6) as:

$$K_a' = [H^+](S - S_0)/S_0 \quad \text{(Eq. 2)}$$

$$\log [(S - S_0)/S_0] = \text{pH} - \text{pKa}' \quad \text{(Eq. 3)}$$

where S is the total solubility of the undissociated canrenoic acid in equilibrium with its anion at a given pH.

When the logarithm of $(S - S_0)/S_0$ is plotted against pH, a straight line is produced with a slope of unity and the negative intercept of pKa'. The analytical procedure was presumed to remove any lactone formed during the equilibrium process, and lactonization was presumed to be a slower process than the equilibration between the solid canrenoic acid and its saturated solution. The solid phase of the equilibrated mixture was confirmed to be canrenoic acid by

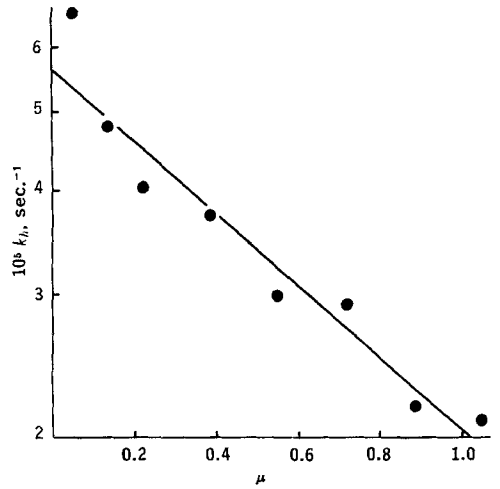


Figure 3—*Semilogarithmic plot of the apparent first-order rate constants in the hydrolysis of canrenone as a function of ionic strength, μ, in carbonate buffer (pH 10.5) at 25.0°.*

the fact that it was instantaneously soluble in mildly alkaline aqueous solution.

As would be expected from the relations of Eqs. 2 and 3, the solubility approaches the constant value S_0 as the pH decreases (Fig. 1). The plot of log $[(S - S_0)/S_0]$ *versus* pH is shown in Fig. 2. The intercept estimates a pKa′ value of 5.20 at 25.0° (Eq. 3). No pKa′ dependence on temperature was observed within experimental error. The experimental pKa′ values at the various temperatures are: 25.0°, 5.20; 37.5°, 5.21; 45.0°, 5.25; and 60.0°, 5.02.

Effect of Ionic Strength on Hydrolysis Rate—The hydrolysis of canrenone in carbonate buffer of pH 10.5 was tested for a salt effect with KCl up to ionic strengths of 1.25. A negative salt effect was observed (Fig. 3) since the logarithms of the hydrolysis rate constant significantly and linearly decreased with ionic strength. At higher ionic strengths (7, 8), the activity coefficient of an ion in aqueous solution at 25° adheres to:

$$\log \gamma_i = -0.509 z_i^2 \sqrt{\mu} + b_i \mu \qquad \text{(Eq. 4)}$$

where z_i is the charge on species i. It can be shown by transition-

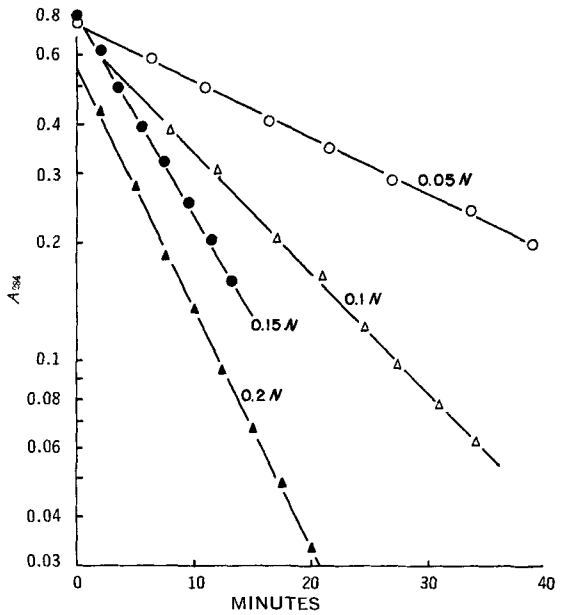


Figure 4—*Typical first-order plots for hydrolysis of canrenone in various NaOH concentrations at 25.0°. The absorbance values, A, of the unreacted lactone were measured at 284 nm. in the chloroform extract.*

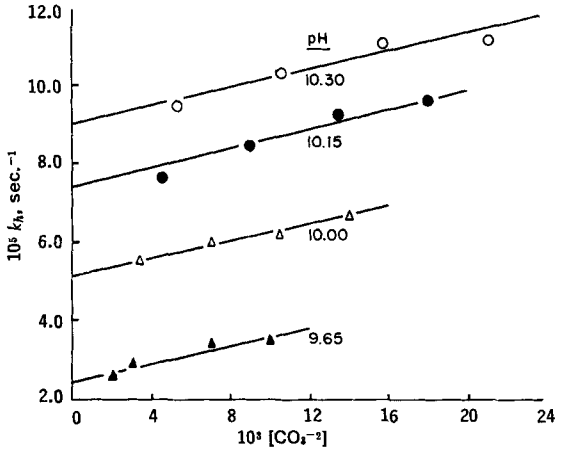


Figure 5—*Apparent first-order rate constants in hydrolysis of canrenone in carbonate buffer at 37.5° as a function of pH and the concentration of buffer anion, [CO_3^{-2}].*

state theory (9) that:

$$\log k = \log k_0 + (b_0 + b_i - b\ddagger)\mu \qquad \text{(Eq. 5)}$$

where b_i is the coefficient of ionic strength for hydroxide ion, b_0 is for the lactone, and $b\ddagger$ is for the activated complex, when k_0 is the rate constant at zero ionic strength. The conformity of the data of Fig. 3 to Eq. 5 indicates that the ionic concentration has a greater effect on the activated complex formed from hydroxide ion and lactone than it does on the two separated species. Of course, the possibility does exist that the activity of the lactone itself is decreased with increased ionic strength.

Rate Constants—The apparent first-order rate constants (Table I) were estimated from the slopes of appropriately plotted experimental data in accordance with:

$$\log |A_{eq} - A| = -kt/2.303 + \log |A_{eq} - A_0| \qquad \text{(Eq. 6)}$$

or by the method of least squares using the Wang 700 calculator. There was no significant difference between the statistical and graphical methods. The A_{eq} values were the final absorbances of the reaction, the A_0 values were the initial readings, and the absorbance values A were read at any given time t. At any constant pH, semilogarithmic plots in accordance with Eq. 6 increased or decreased linearly with time to provide confidence in k as an apparent first-order rate constant. Typical first-order plots for various NaOH concentrations are given in Fig. 4.

The separation of the apparent first-order lactonization (k_l) and hydrolysis (k_h) rate constants was achieved with the use of the equilibrium constants, since the apparent first-order rate constant for the achievement of an equilibrium is the sum of the forward and backward rate constants (Eq. 7) and the equilibrium constant is the quotient (Eq. 8):

$$k = k_h + k_l \qquad \text{(Eq. 7)}$$

$$K = k_h/k_l \qquad \text{(Eq. 8)}$$

Thus, k_h and k_l can be obtained from the experimental k and K values obtained at a given pH value.

General Acid–Base Catalysis—The rate constants were independent of buffer concentration at constant pH and ionic strength for formate and borate buffers and gave no evidence of general acid–base catalysis. The buffer catalysis by acetate and phosphate buffers was insignificant. However, significant buffer catalysis was observed with carbonate and glycine buffers in the hydrolysis of canrenone. The rate constants for the reactions studied in different buffer concentrations are given in the footnotes of Table I.

The possible catalytic contributions to the apparent first-order rate constant in the carbonate buffer region where only complete hydrolysis of the lactone was observed, on the assumption of no water attack, may be expressed by:

$$k_h = k_{OH}[OH^-] + k_{HCO_3}[HCO_3^-] + k_{CO_3}[CO_3^{-2}] \qquad \text{(Eq. 9)}$$

where k_h is the apparent first-order rate constant for the hydrolysis in the alkaline region, and k_{OH}, k_{HCO_3}, and k_{CO_3} are the respective catalytic rate constants. Since, when K_{HCO_3} is the dissociation constant of HCO_3^-:

$$[HCO_3^-]/[CO_3^{-2}] = [H^+]/K_{HCO_3} \quad \text{(Eq. 10)}$$

$$k_h = k_{OH}[OH^-] + (k_{HCO_3}[H^+]/K_{HCO_3} + k_{CO_3})[CO_3^{-2}] \quad \text{(Eq. 11)}$$

or:

$$k_h = k_{OH}[OH^-] + (k_{HCO_3} + k_{CO_3}K_{HCO_3}/[H^+])[HCO_3^-] \quad \text{(Eq. 12)}$$

the plots of k_h against $[HCO_3^-]$ or $[CO_3^{-2}]$ at constant $[H^+]$ should be linear and of positive slopes with intercept values of ($k_{OH}[OH^-]$). When the slopes, S_1, of Eq. 11:

$$S_1 = k_{HCO_3}[H^+]/K_{HCO_3} + k_{CO_3} \quad \text{(Eq. 13)}$$

are plotted against the hydrogen-ion activity, the resultant slope is k_{HCO_3}/K_{HCO_3} and the resultant intercept is k_{CO_3}. Similarly, from the slopes, S_2, of Eq. 12 against $1/[H^+]$:

$$S_2 = k_{HCO_3} + k_{CO_3}K_{HCO_3}/[H^+] \quad \text{(Eq. 14)}$$

k_{HCO_3} and k_{CO_3} can be evaluated separately when K_{HCO_3} is known.

The apparent rate constant, k_h, was plotted against $[CO_3^{-2}]$ (Fig. 5) and $[HCO_3^-]$ (Fig. 6). The fact that the slopes (S_1) in Fig. 5 are independent of pH within experimental error indicates the nonvariance of ($k_{HCO_3}[H^+]/K_{HCO_3} + k_{CO_3}$) with $[H^+]$ and, thus, the lack of a catalytic rate constant, k_{HCO_3}, in carbonate buffer-catalyzed hydrolysis of the lactone. The catalytic rate constant, k_{CO_3}, was 1.1×10^{-3} sec.$^{-1}$ at 37.5°. The identical value of k_{CO_3} can be obtained from the slope of plots of the slopes (S_2) of Fig. 6 against $1/[H^+]$ in accordance with Eq. 12. The intercept of zero confirms the fact that $k_{HCO_3} \sim 0$.

The intercepts of the plots in Figs. 5 and 6 vary and are consistent with hydroxyl-ion catalysis; *i.e.*, the intercepts ($k_{OH}[OH^-]$) *versus* $[OH^-]$ give a linear plot of slope k_{OH}. The line passes through zero and confirms the fact that there is no water hydrolysis. The k_{OH} value estimated by such a method was 0.21 sec.$^{-1}$ at 37.5°. The rate constants were extrapolated to zero buffer concentrations, and the determined $k_{OH}[OH^-]$ values were used in the log k–pH profiles.

Specific Acid–Base Catalysis—Plots of the apparent first-order rate constants against HCl and NaOH (Fig. 7) in strongly acidic and alkaline regions were linear and with zero intercept in accordance with:

$$k_l = k_{HCl}[HCl] \quad \text{(Eq. 15)}$$

$$k_h = k_{NaOH}[NaOH] \quad \text{(Eq. 16)}$$

In these highly acidic regions, all canrenoic acid was converted to the lactone canrenone within the precision of the experimental method. In these highly alkaline regions, all of the lactone canrenone was converted to the canrenoic acid within the precision of the experimental method. Both lines (Fig. 7) had zero intercepts to indicate the lack of water reaction.

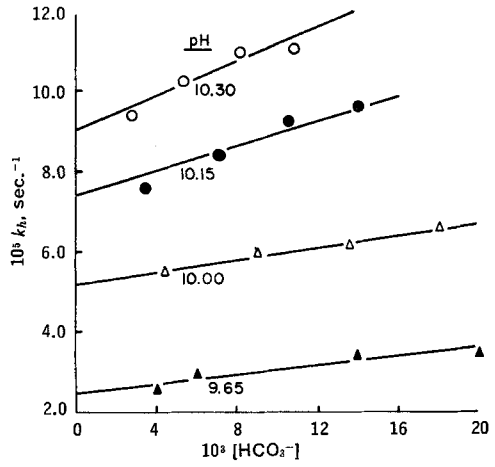


Figure 6—*Apparent first-order rate constants in hydrolysis of canrenone in carbonate buffer at 37.5° as a function of pH and the concentration of buffer acid, [HCO_3^-].*

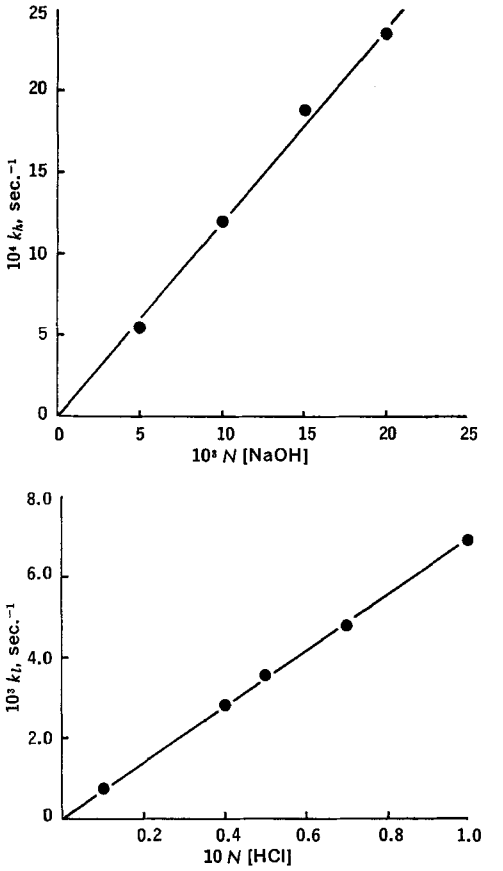


Figure 7—*Apparent first-order rate constant dependence on [NaOH] for hydrolysis of canrenone (top) and on [HCl] for lactonization of canrenoic acid (bottom) at 25.0°.*

Equilibrium Constants—The equilibrium constants at any pH value can be defined as the ratio of apparent first-order hydrolysis

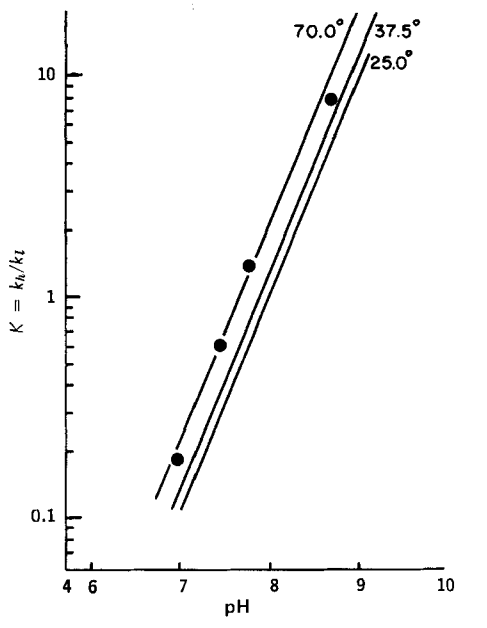


Figure 8—*Semilogarithmic plot of equilibrium constants, K ($= k_h/k_l$), as a function of pH. The drawn lines at 25.0 and 37.5° were calculated from the microscopic rate constants with the use of Arrhenius plots.*

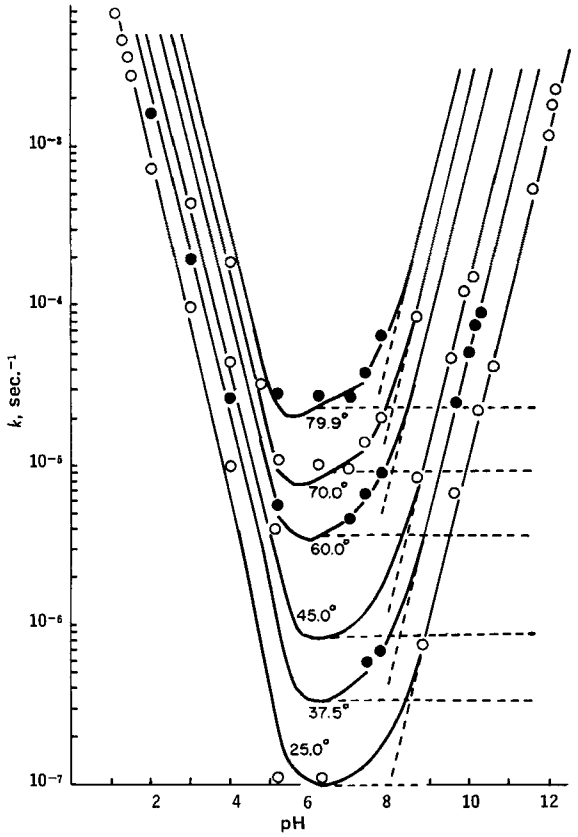


Figure 9—*Log* k–*pH profile for the lactonization of canrenoic acid and hydrolysis of canrenone at 25.0, 37.5, 45.0, 60.0, 70.0, and 79.9°. The drawn lines were calculated from the derived microscopic rate constants. The dashed lines were the separated log* k–*pH profiles for lactonization and hydrolysis.*

and lactonization constants. Consequently, it is a function of hydrogen- and hydroxyl-ion activity. The experimental equilibrium constants ($K = k_h/k_l$) at 70.0° are plotted semilogarithmically in Fig. 8. Experimental values of K obtained at 79.9° were not significantly different.

Log *k*–pH Profile—The log k–pH profiles were constructed from the first-order rate constants (Table I) and pH values at 25.0, 37.5, 45.0, 60.0, 70.0, and 79.9°. The sum of the rate constants of the lactonization and the hydrolysis as a function of pH are plotted in Fig. 9. The separated log k–pH profiles for lactonization and hydrolysis are shown in this figure by the dashed lines. The pH values of the strongly acidic or strongly alkaline solutions were calculated from:

$$\text{pH} = -\log \gamma_{\text{HCl}}[\text{HCl}] \qquad \text{(Eq. 17)}$$

$$\text{pH} = \text{pKw} - \text{pOH} = \text{pKw} + \log \gamma_{\text{NaOH}}[\text{NaOH}] \qquad \text{(Eq. 18)}$$

where pKw and the activity coefficients, γ_{HCl} and γ_{NaOH}, were obtained from the data in the literature (10).

The logarithmic transformations of Eqs. 15 and 16 are:

$$\log k_l = \log k_{\text{H}} - \text{pH} \qquad \text{(Eq. 19)}$$

$$\log k_h = \log k_{\text{OH}} - \text{pKw} + \text{pH} \qquad \text{(Eq. 20)}$$

where the specific bimolecular rate constants, k_{H} and k_{OH}, are now defined with respect to hydrogen- and hydroxide-ion activities rather than their respective molarities, as were the k_{HCl} and k_{NaOH} of Eqs. 19 and 20. The separated k_l and k_h values were calculated on the basis of the observed overall first-order rate constants of change (Table I and Fig. 9) and the determined equilibrium constants (Fig. 8). The bases for this separation were given previously in Eqs. 7 and 8.

The log k_h decreases with decreased pH with slope of unity in accordance with Eq. 20 (Fig. 9). The apparent lactonization rate constant conforms to the dependency of Eq. 21 below a pH of 3

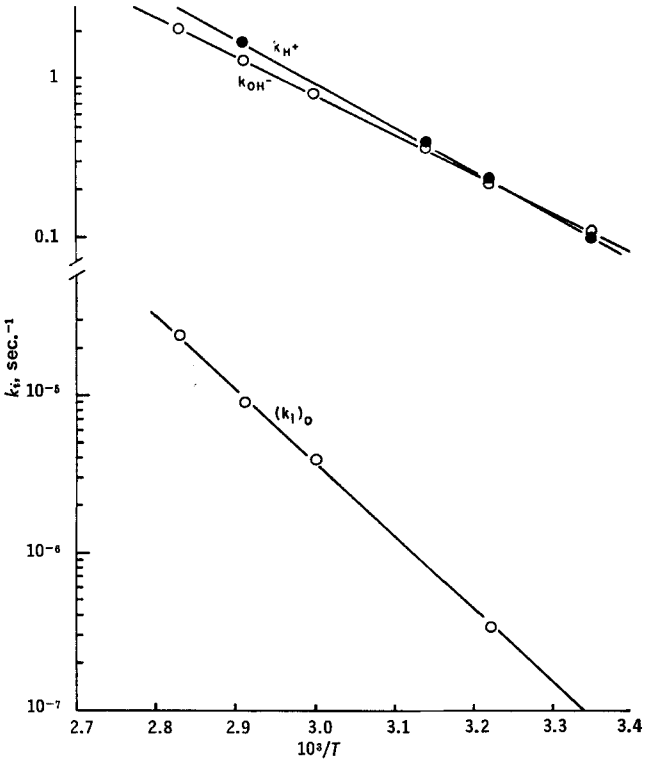


Figure 10—*Arrhenius plots of the microscopic rate constants for lactonization of canrenoic acid,* k_H *and* $(k_l)_0$, *and hydrolysis of canrenone,* k_{OH}.

where essentially all the acid molecules are undissociated. The apparent first-order lactonization rate constant tends to be independent of pH as the fraction of dissociated acid present increases (as pH tends to exceed the pKa′). This indicates a pH-independent lactonization of the anion. Hence, the overall lactonization can be formulated as:

$$k_l = k_{\text{H}}[\text{H}^+]f_{HA} + (k_l)_0 f_A = k_{\text{H}}[\text{H}^+]^2/([\text{H}^+] + K_a) + (k_l)_0 K_a'/([\text{H}^+] + K_a') \qquad \text{(Eq. 21)}$$

where f_{HA} and f_A are the fractions of undissociated and dissociated acids, respectively (11), and $(k_l)_0$ is the rate constant for a pH-independent lactonization of the anion. The solid drawn lines in the log k–pH profile (Fig. 9) were derived from the sums of the first-order rates constants of Eqs. 20 and 21.

The fact that a plot of log K *versus* pH (Fig. 8) is linear and of positive slope of unity in the pH regions where the amounts of canrenone and canrenoic acid assayed were significant indicates that:

$$K = k_h/k_l = k_{\text{OH}}[\text{OH}^-]/(k_l)_0 \qquad \text{(Eq. 22)}$$

Table II—Microscopic Rate Constants[a], Arrhenius Parameters[b], and Entropies of Activation[c] for Hydrolysis of Canrenone and Lactonization of Canrenoic Acid

Temperature	k_{H}, sec.$^{-1}$	k_{OH}, sec.$^{-1}$	$10^8(k_l)_0$, sec.$^{-1}$
25.0°	0.10	0.11	—
37.5°	0.24	0.21	34.0
45.0°	0.40	0.36	—
60.0°	—	0.80	400
70.0°	1.70	1.32	900
79.9°	—	2.12	2400
E_a, kcal.	12.84	11.42	21.23
log A	8.40	7.38	8.50
$\Delta S^{\ddagger}$, e.u.	−20.0	−24.7	−19.5

[a] Where $k_h = k_{\text{OH}}[\text{OH}^-]$, $k_l = k_{\text{H}}[\text{H}^+]f_{HA} + (k_l)_0 f_A$. [b] Where $\log k = -E_a/2.303RT + \log A$. [c] Where $\Delta S^{\ddagger}/R = 2.303\,[\log A - \log(kT/h)] - 1$.

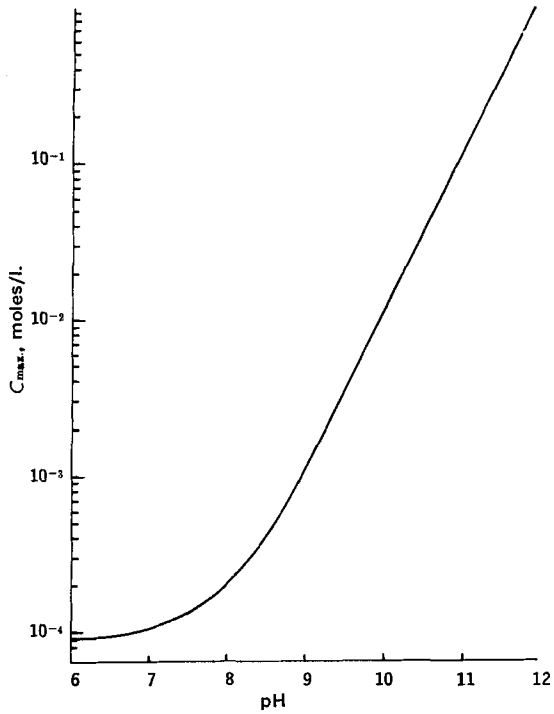


Figure 11—*Semilogarithmic plot of the maximum molar concentration, $C_{max.}$, of stabilizable potassium canrenoate in aqueous preparations as a function of pH at 25.0°.*

so that:

$$\log K = \log [k_{OH}/(k_l)_0] - pKw + pH \qquad \text{(Eq. 23)}$$

where the intercept $\log [k_{OH}/(k_l)_0] - pKw = 7.96$ at 25.0°. Thus, in the pH region where significant amounts of both species are present in equilibrium, the hydrolysis rate must be hydroxide ion dependent and the lactonization rate must be pH independent.

If this was not the case and if hydrolysis was $[OH^-]$ dependent and lactonization was $[H^+]$ dependent:

$$K = k_h/k_l = k_{OH}[OH^-]/k_H[H^+] \qquad \text{(Eq. 24)}$$

It would follow that:

$$\log K = \log (k_{OH}K_w/k_H) + 2pH \qquad \text{(Eq. 25)}$$

and the plot of Fig. 8 would be linear with positive slope of 2, which is not the case.

If lactonization of undissociated acid was pH dependent and that of dissociated acid was pH independent, and hydrolysis was hydrogen-ion as well as hydroxyl-ion catalyzed, then:

$$K = k_h/k_l = (k_H'[H^+] + k_{OH}[OH^-])/\{k_H[H^+]f_{HA} + (k_l)_0 f_A\} \qquad \text{(Eq. 26)}$$

Thus, it would follow that at high alkalinity when $[H^+]$ is negligible, the log K is the function of pH as given in Eq. 23 and justified by the plot of Fig. 8. This would also be true in the neutral pH region of Fig. 8 if $k_H[H^+]f_{HA}$ and $k_H'[H^+]$ were negligible. At higher acidity, *i.e.*, where $[H^+] \gg [OH^-]$, it would follow that $K = k_H'/k_H$ so that the equilibrium constant would tend to become invariant with pH at low pH values and the plot of log K *versus* pH of Fig. 8 would tend to flatten at lower pH values. This could not be concluded within the experimental error. Although the acid-catalyzed hydrolysis of γ-lactones was observed in the hydrolysis of γ-butyrolactone (12), γ-substituted γ-lactones (13), and pilocarpine (14), one could not conclude that it was significant in the case of canrenone. At least the acid-catalyzed hydrolysis rate cannot be any more than 5% as large as the acid-catalyzed lactonization rate, *i.e.*, the equilibrium constant in highly acidic solution cannot be greater than 0.05.

Microscopic Rate Constants—The values of the microscopic rate constants, k_H and k_{OH}, at all temperatures were estimated directly from the best fit of the log k–pH profile (Fig. 9) in low and high pH regions, respectively. The bimolecular rate constants of pH-independent lactonization of the dissociated acid were estimated from the best fit of the profile in the pH 7–8 region where essentially all the acid is in the dissociated form. The microscopic rate constants at different temperatures are given in Table II. The solid lines in the log k–pH profile (Fig. 9) were calculated from the k_i values in Table II.

Rate Dependency on Temperature—The Arrhenius equation gives the quantitative relation of rate constants and temperature. The logarithmic form of the equation is:

$$\log k = -E_a/2.303RT + \log A \qquad \text{(Eq. 27)}$$

where E_a is the activation energy, and A is the frequency factor. The Arrhenius plots of the logarithms of the various catalytic rate constants are shown in Fig. 10 for: k_{OH}, the specific base catalytic hydrolysis rate constant; k_H, the specific acid catalytic lactonization rate constant; and $(k_l)_0$, the pH-independent lactonization rate constant of the dissociated acid. The Arrhenius parameters were calculated from the plots in accordance with Eq. 27. The values of E_a, log A, and $\Delta S‡$ (entropy of activation) are included in Table II.

Since the rate of achievement of an equilibrium in the neutral pH regions at lower temperatures is an extremely slow process, the equilibrium constants at lower temperatures were calculated from the ratio of Eqs. 20 and 21. The microscopic rate constants given in these equations were based on the values of the constants estimated from the use of their Arrhenius parameters (Eq. 27 and Table II). Similar plots of such calculated equilibrium constants as a function of pH are given in Fig. 8 for 25.0 and 37.5°.

Mechanism for Hydrolysis of Canrenone and Lactonization of Canrenoic Acid—Long and Friedman (15), by using ^{18}O-labeled water, showed that acyl oxygen scission of a γ-lactone occurs in both the acid- and base-catalyzed reactions and that the hydrolysis passes through an intermediate with enough lifetime to allow

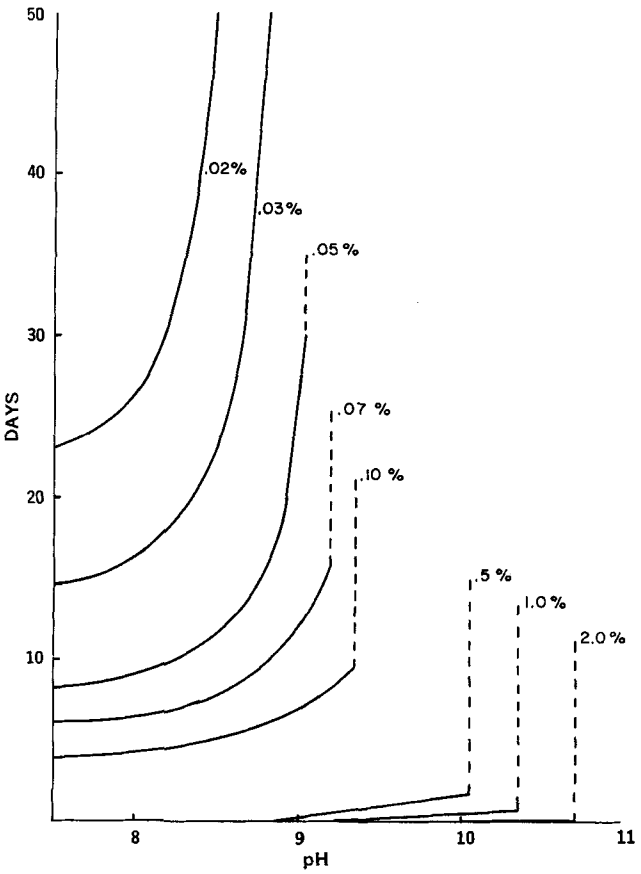
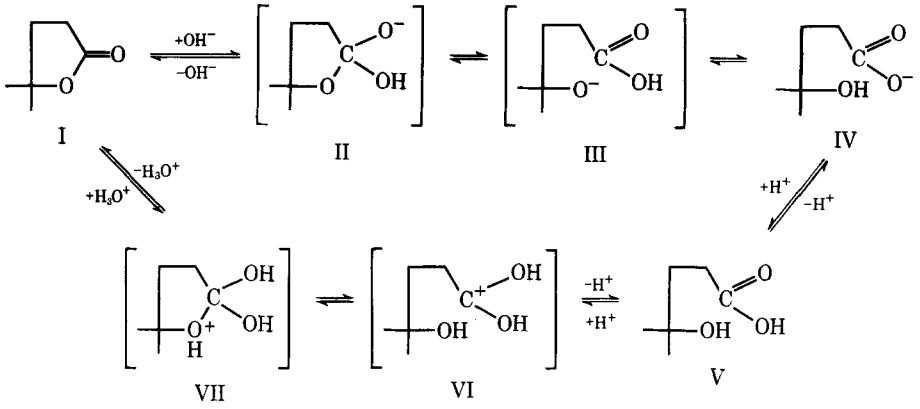


Figure 12—*Time required to start forming precipitates of canrenone in aqueous preparations of potassium canrenoate as a function of pH and concentration of potassium canrenoate at 25.0°.*



Scheme II

appreciable oxygen exchange, such as that found in the hydrolysis of simple esters. Long *et al.* (12) also studied the kinetics of the acid-catalyzed hydrolysis of γ-butyrolactone in strongly acidic solutions and found that the rate of hydrolysis is proportional to the concentration of hydrogen ion, but the rate of the lactonization is proportional to h_0 (Hammett's acidity function). This indicated that the activated complex consisted not only of a molecule of lactone and hydrogen ion but also of a water molecule [Hammett–Zucker hypothesis (16)].

Scheme II is consistent with these mechanisms for lactonization and hydrolysis of γ-lactones. The sequence I → IV explains the base-catalyzed hydrolysis of canrenone and is consistent with a rate dependency of the apparent first-order rate constant on $k_{OH}[OH^-]$. It is also consistent with the pH-independent lactonization of the canrenoic acid monoanion, which may be initiated by an intramolecular general base attack on the alcoholic group (IV → III) (possibly mediated by a molecule of water), which then results in the intramolecular nucleophilic attack of the resultant alcoholate anion on the carbonyl carbon to produce the common activated complex, II.

The sequence V → VI → VII → I explains the acid-catalyzed lactonization of undissociated canrenoic acid and is consistent with a rate dependency of the apparent first-order rate constant on $k_H[H^+]f_{HA}$. The possible reverse reaction, the acid-catalyzed hydrolysis of canrenone, does not appear to be large enough to affect significantly the equilibrium for all practical purposes. This was also true for the acid-catalyzed hydrolysis of pilocarpine (14), although the acid-catalyzed hydrolysis of γ-butyrolactone was significant (12). Bruice and Pandit (17) showed that alkyl substitutions on the carbons of structures amenable to intramolecular reaction also favor the cyclization.

Prediction of Stability—The pH-independent lactonization occurs in the alkaline region at all pH values, as shown in the log k–pH profile (Fig. 9). The extent of its formation is a function of its equilibrium with the hydroxide-ion-catalyzed hydrolysis. The lactone canrenone precipitates when the concentration of the lactone in equilibrium at a given pH value exceeds its solubility. Thus, the pharmaceutical stability of the canrenoic acid salt is a function of the solubility of the lactone and the pH-dependent thermodynamic equilibrium of the reversible lactonization and hydrolysis processes. The solubilities of the lactone, canrenone (S_L), in water were found to be: 25.0°, 9.4×10^{-5} *M*; 37.5°, 7.79×10^{-4} *M*; and 60.0°, 2.43×10^{-3} *M*. Substitution of the ratio of canrenoate anion (at pH values much higher than 5.2 where the acid concentration is negligible) to canrenone concentrations for the equilibrium constant K in Eq. 23 results in:

$$\log K = \log([\text{canrenoate}]/[\text{canrenone}]) = \log[(C_{max.} - S_L)/S_L] = \log[(C_{max.} - 9.4 \times 10^{-5})/9.4 \times 10^{-5}] = \text{pH} - 7.96 \quad \text{(Eq. 28)}$$

where $C_{max.}$ in moles/l. is the maximum concentration of canrenoic acid salt that can be prepared at a given pH in solution at 25.0° and yet will not give an ultimate precipitate of equilibrated canrenone. A plot of $C_{max.}$ calculated from Eq. 28 for various pH values is given in Fig. 11 and clearly shows the maximum concentrations of canrenoate solution that can be prepared at any specific pH value to maintain a true solution at 25.0°.

The time required to reach that point where the lactone will precipitate from a canrenoic acid salt solution is a function of the concentration of the canrenoic acid salt as well as the pH, since both the rate and equilibrium constants are pH dependent. The lactone concentration, L_{eq}, in moles/l. at equilibrium may be expressed by:

$$L_{eq} = C_0/(1 + K) \quad \text{(Eq. 29)}$$

where C_0 is the initial concentration in moles/l. of the canrenoic acid salt and $K = (C_0 - L_{eq})/L_{eq}$. The apparent first-order transformation of canrenoate anion to the lactone, canrenone, at a constant pH may be expressed by:

$$\log(C - C_{eq}) = \log(C_0 - C_{eq}) - kt/2.303 \quad \text{(Eq. 30)}$$

where C is the concentration of nonlactonized canrenoic acid or anion at any time t, and k is the apparent first-order rate constant (Table I) to achieve an equilibrium. If the concentration, C, of canrenoate is limited so that precipitates of the lactone cannot occur, then:

$$C = C_0 - S_L \quad \text{(Eq. 31)}$$

and, from Eq. 29:

$$C_{eq} = KL_{eq} = KC_0/(1 + K) \quad \text{(Eq. 32)}$$

Thus, substitution of Eqs. 31 and 32 into Eq. 30 and subsequent rearrangement result in

$$t_{S_L} = (2.303/k)\log[C_0/\{C_0 - S_L(1 + K)\}] \quad \text{(Eq. 33)}$$

and the time, t_{S_L}, when the lactone, canrenone, of solubility S_L precipitates from a solution of C_0 (moles/l.) concentration may be calculated at any temperature when the apparent first-order rate constant, k (Table I), for the achievement of the equilibrium and the equilibrium constant, K (Fig. 8), are known. These times, t_{S_L}, required for precipitation of the lactone for various concentrations of the canrenoic acid salt are given in Fig. 12. Even a dilute solution of potassium canrenoate (0.02%) at pH 8.0 gives a precipitate in less than a month. Thus, if it is still desirable to have a soluble preparation of potassium canrenoate, either adjuncts should be added to increase the hydrolysis rate of the lactone without affecting the rate of lactonization or mixed solvents should be considered so that a small amount of lactone in the equilibrium will be completely solubilized.

Biopharmaceutical Aspects of Spironolactone, Canrenone, and Canrenoic Acid—Gantt *et al.* (18) reported originally that the addition of polysorbate 80 enhanced the absorption of spironolactone. Subsequently, they revised (19) their conclusion to state that the addition of polysorbate 80 does not contribute directly to the previously observed increased absorption. This revision was due to their observation that the smaller particle size of spironolactone was preserved when the drug was encapsulated with the surfactant; thus they attributed the improved absorption to the

particle size rather than to the addition of the surfactant. The smaller particle-sized spironolactone was as well absorbed as the one with polysorbate 80. Their observation was supported by several clinical and blood level studies (20–22). Venning (23) argued against this dependence of absorption on particle size by claiming that blood level studies demonstrated that conventional tablets with an average particle size of 1 μ were as poorly absorbed as those of 10 μ. He concluded that "absorption of spironolactone is dependent upon some more complex function of the physical state within the finished tablets." The fact of the matter was that these two sets of tablets had different formulation factors. However, dissolution studies by Levy (24) showed that the dissolution rates were faster with the more readily absorbed new tablet formulations than with the old ones. He assigned this discrepancy to the dissolution rate-limited absorption of spironolactone.

If canrenoic acid salt were administered orally, the drug would be transformed readily to its undissociated form and undoubtedly precipitate as such in the stomach. Lactonization would be anticipated to occur prior to absorption at the pH of the stomach (pH 1) since the half-life of the lactonization is only 30 sec. The overall absorption process should be limited by the rate of dissolution and the intrinsic solubility of the canrenoic acid (5.7 mcg./ml. at 37.5°). Certainly, the oral administration of the canrenoic acid salt may give more rapid dissolution (25), wherein it would be precipitated as a fine, readily redissolvable particle. Thus, this could serve as an alternative method of orally administering the lactone. In the less acidic conditions of the intestine, canrenoic acid would be more soluble and the absorption process would be facilitated if the dissolution is rate determining. Since the rate of lactonization is much slower at pH 5.3 at the absorption surface of the intestine (half-life 12 days), the major portion of the drug would be absorbed as the acid species rather than as the lactone. Once the drug is absorbed, either as canrenoic acid or as canrenone, it should remain as it is in the body fluids (hydrolysis or lactonization is slow at pH 7.4 with a half-life of 24 days, provided that enzymic processes do not catalyze lactonization or hydrolysis). Therefore, the rate of transfer of the drug from the stomach to the intestine may not only determine the overall rate of absorption but may also determine the ratio of the acid and the lactone absorbed. These predictions are based on the assumption that the drug transforms with the same rate in the body fluids as in aqueous solution. A recent study of plasma levels of potassium canrenoate in man by Karim *et al.* (26) showed that the plasma levels of canrenoic acid *and* canrenone were about equal 3 hr. after intravenous administration of potassium canrenoate. This finding indicates that there are enzymatic processes and, hence, an *in vivo* equilibrium is probably established much faster.

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Enamine Prodrugs

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Abstract □ Five enamine derivatives of phenylpropanolamine were prepared in a search for novel amine prodrug derivatives. The compounds hydrolyzed *in vitro* at vastly different rates in phosphate buffer at pH 7.4, but the rates were not accelerated by dilute plasma. As expected, these enamines hydrolyzed faster as the pH was lowered. Two compounds were chosen for pharmacologic tests. Acid hydrolysis data suggested that Compound III would hydrolyze *in vivo* to give phenylpropanolamine but that Compound IV would not. Compound III was less toxic and also less potent in the pressor and antitussive tests than phenylpropanolamine (molar basis), giving further support to the suggestion that III was converted *in vivo* to phenylpropanolamine. In contrast, IV was more toxic than phenylpropanolamine (molar basis) but was not active in the pressor test. Thus, IV did not behave as a prodrug of phenylpropanolamine. While the authors did not pharmacologically evaluate Compounds I and II, which hydrolyze faster than III, it is predicted that they would have been more potent than III in these tests. Thus, it has been shown that enamines are potentially useful prodrug derivatives of amines.

Keyphrases □ Phenylpropanolamine, enamine derivatives—amine prodrugs, pharmacological evaluation □ Enamine derivatives of phenylpropanolamine—prodrugs, pharmacological evaluation □ Amine prodrugs—enamine derivatives of phenylpropanolamine □ Prodrugs, amine—enamine derivatives of phenylpropanolamine

There are surprisingly few types of amine prodrug derivatives. Amides (1, 2), Schiff bases (3), and Mannich bases (4) are perhaps the best known. Enamines, or α,β-unsaturated amines, are used extensively as intermediates and as protective groups in selective organic reactions (5–7). Some have been tested as potential medicinal agents (8, 9) but, apparently, none has been evaluated as a prodrug. Enamine structural types vary widely (5), as do their hydrolysis rates (10). The enamine made from pyrrolidine and cholestan-3-one hydrolyzes readily to cholestan-3-one when heated at reflux for 5 min. in 95% ethanol, but the related enamine of stigmastadienone must be heated at reflux for 4 hr. in a sodium acetate–acetic acid buffered solution to regenerate the ketone. Heyl and Herr (10) attributed these hydrolysis differences to the degree of unsaturation. The mechanism for enamine hydrolysis was described recently (11, 12).

For this study, several enamines were made from phenylpropanolamine in a search for derivatives that would hydrolyze at significantly different rates.

EXPERIMENTAL

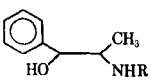
Chemistry—Melting points were determined in capillary tubes on a Thomas-Hoover apparatus and are uncorrected. Reported yields are of pure compounds; most reactions were run once.

Intermediates—Phenylpropanolamine base was purchased, as were the other reactants for Compounds I–V. The five are *N,N*-diethylacetoacetamide, *tert*-butyl acetoacetate, ethyl acetoacetate, diethyl ethoxymethylenemalonate, and diethyl acetonedicarboxylate, respectively.

General Procedure—Molar equivalents of phenylpropanolamine and the other reactants were refluxed in dry benzene for about 2 hr. until the calculated amount of water was collected in a Dean-Stark apparatus; Compound IV produced ethanol instead of water as a by-product. Most of the benzene was removed *in vacuo*, and hexane was added. The cooled solutions provided the products, but two did not crystallize. They were distilled *in vacuo*.

***In Vitro* Hydrolysis Rates**—Half-lives for hydrolysis of the compounds in 0.1 *M* pH 7.4 phosphate buffer, with and without 2% human, 2% dog, or 2% mouse plasma, were determined by spectrophotometric (13) or pH Stat (14) methods as previously described. HCl (0.01 *N*) was the titrant.

Table I—Phenylpropanolamine Enamines



Compound	R	Formula	Molecular Weight	Yield (Pure), %	Melting Point	IR[a] OH,NH	IR[a] C=O	IR[a] C=C	Analysis, % Calc.	Found
I	C(CH3)=CH—CON(C_2H_5)$_2$	$C_{17}H_{26}N_2O_2$	290.4	54	86–87°	3.05	6.20	6.45	C 70.31	70.38
									H 9.02	9.11
									N 9.65	9.81
II	C(CH3)=CH—$CO_2C(CH_3)_3$	$C_{17}H_{25}NO_3$	291.4	27	Oil	2.91	6.10	6.23[b]	C 70.07	69.72
									H 8.65	8.76
									N 4.81	4.83
III	C(CH3)=CH—$CO_2C_2H_5$	$C_{15}H_{21}NO_3$	263.3	96	92.5–93.5°	2.95	6.17	6.25	C 68.42	68.27
									H 8.04	7.96
									N 5.32	5.33
IV	CH=C—($CO_2C_2H_5$)$_2$	$C_{17}H_{23}NO_5$	321.4	48	91–92°	2.92	5.95 6.06	6.25	C 63.53	63.95
									H 7.21	7.25
									N 4.36	4.57
V	C(CH2—$CO_2C_2H_5$)=CH—$CO_2C_2H_5$	$C_{18}H_{25}NO_5$	335.4	41	Oil	2.96	5.81 6.10	6.26[b]	C 64.46	64.45
									H 7.51	7.69
									N 4.18	4.08

[a] Wavelengths in microns; mineral oil mulls unless stated otherwise. [b] Neat.

Table II—Hydrolysis Half-Lives for Phenylpropanolamine Enamines (37°)[a]

Compound	Half-Lives in pH 7.4 Phosphate Buffer —with and without 2% v/v Human Plasma— Plasma, min.	Buffer, min.	Half-Lives in pH 7.4 Phosphate Buffer —with Other Plasmas— Plasma	$t_{1/2}$, min.	—Half-Lives at Other pH's— Buffer pH	$t_{1/2}$, min.
I	3.4[b]	4.0	2% Mouse	3.0	6	1.1
					6.4	1.5
					7	2.5
					8.4	4.6
II	10.25	9.75	2% Dog	10	6.4	4.0
		9.35[c]	2% Mouse	8.5	8.4	11.5
					9.5	36.6[c]
III	40	36	2% Mouse	32	5	0.6[d], 0.6[c]
					6	5
					8	35
IV	Not hydrolyzed	Not hydrolyzed	2% Mouse	Not hydrolyzed	4	Not cleaved
					5.5	32
V	>160	160	—	—	6	72

[a] Values are single determinations based on phenylpropanolamine appearance. [b] Values determined on pH Stat (with 0.01 *N* HCl as titrant) except where indicated. [c] UV determination. [d] $t_{1/2}$ with 0.5% pepsin at pH 5, 0.6 min.

Table III—Acute Oral Toxicity in Mice

Compound	Dose, mg./kg. as Base	Mortality, %	LD_{50} and 95% Fieller Confidence Limits, mg./kg. as Base	LD_{50}, moles/kg.
Phenylpropanol-amine hydrochloride	750	0	1443 (1185–1853)	9.5×10^{-3}
	1100	20		
	1520	60		
	2350	100		
III	1000	0	5105 (3243–[a])	19×10^{-3}
	1587	10		
	2519	20		
	5038	50		
IV	1000	10	2227 (1596–3145)	6.9×10^{-3}
	1587	20		
	2519	70		
	3998	80		
Controls	—	0	—	—

[a] Because of the nature of the data, an upper confidence limit could not be calculated.

Acute Toxicity in Mice—Male mice[1], weighing 15–25 g., were divided into groups of 10 and dosed orally with vehicle or with drug suspended or dissolved in 0.5% tragacanth. Dose volumes varied from about 10 to 40 ml./kg. After being dosed, animals were observed at intervals for several hours for overt effects and fatalities. Survivors were housed in groups according to compound and dose level and were checked once daily for 7 days to determine whether additional fatalities occurred. Studies were terminated on the 7th day postdrug, and LD_{50}'s and potency ratios were calculated according to the minimum logit chi square method of Berkson (15).

Pressor Activity in Unanesthetized Rats—Sexually mature male and female rats[2] were used. Animals were anesthetized with ether, and the right carotid arteries were cannulated with a piece of heparin-filled polyethylene tubing. After the animals recovered from the ether, blood pressure was recorded by means of a transducer complex[3] attached to a direct writing optical oscillograph[3]. Control readings were taken until blood pressure and heart rate stabilized. Test compounds were administered orally as suspensions or solutions in 0.5% tragacanth at a dose volume of 10 ml./kg. Control animals received 10 ml./kg. of vehicle. Blood pressure recordings were taken at 5, 15, 30, 60, 90, and 120 min. postdrug. At least two animals received vehicle and served as controls in each study, and three or four animals were used at each dose level of test compound.

Antitussive Activity—Antitussive activity was evaluated in dogs according to the method described by Tedeschi *et al.* (16). Four sexually mature mongrel dogs, weighing between 9 and 15 kg., were used. Drugs were administered orally in gelatin capsules. Animals were tested prior to drug administration to establish control values and then at hourly intervals until the cough response returned to control level.

[1] Carworth Farms.
[2] Charles River.
[3] Sanborn.

RESULTS AND DISCUSSION

Synthesis and Physical Properties—Synthesis did not present any unusual problems, but two compounds resisted all attempts at crystallization. They were obtained as pure oils upon distillation. Molecular weights, yields, melting points, elemental analyses, and IR assignments are shown in Table I.

***In Vitro* Hydrolysis Behavior**—Hydrolysis half-life data are summarized in Table II. The enamines cleaved at different rates in phosphate buffer at pH 7.4: I and II hydrolyzed rapidly, III hydrolyzed slowly, V hydrolyzed very slowly, and IV did not hydrolyze. Hydrolysis was not accelerated by adding dilute plasma to

Table IV—Pressor Activity in Unanesthetized Rats

Compound	Dose, mg./kg. as Base	Maximum Effect, % Increase over Predrug Control Level
Phenylpropanolamine hydrochloride	50	27
III	218	10
	436	32
	871	24
IV	500	No effect
	1000	No effect

Table V—Cough Inhibition in Dogs

Compound	Dose, mg./kg. as Base	Mean Average and Inhibition, hr. Postdrug 1	2	3	4	5	6	7	Maximum Mean Percent Inhibition	ED_{50} and 95% Fieller Confidence Limits, mg./kg.	Molar ED_{50}
Phenylpropanolamine hydrochloride	5	21	16	28	5	—	—	—	37		
	10	35	47	55	46	35	12	—	57	7.8 (4.8–11.8)	5.2×10^{-5}
	15	52	64	64	56	51	30	—	70		
III	10	15	18	15	18	4	—	—	21		
	26	30	39	35	43	36	18	—	48	25.8 (17.8–38.7)	9.8×10^{-5}
	52	23	45	64	54	53	57	47	74		

the buffer. As expected, the compounds generally cleaved faster as the pH was lowered. Two of the compounds, III and IV, were chosen for pharmacologic tests. The acid hydrolysis data suggested that III would hydrolyze *in vivo* to give phenylpropanolamine but that IV would not hydrolyze.

Acute Toxicity—The acute oral toxicity data are recorded in Table III. On a weight basis, phenylpropanolamine is 3 times more toxic than III and 1.5 times more toxic than IV. But LD_{50}'s should be compared in moles per kilogram because 1 mole of each prodrug will release 1 mole of phenylpropanolamine regardless of the molecular weight of the prodrug. Thus, when toxicities are compared on this realistic basis, IV is slightly more toxic than phenylpropanolamine while phenylpropanolamine is twice as toxic as III. This suggests that the true molar toxicity of intact IV is greater than that of phenylpropanolamine and that III is not absorbed and converted rapidly to phenylpropanolamine because, if it were, the molar toxicities of III and phenylpropanolamine would be equivalent. These data do not, however, rule out the possibility that IV is cleaved to phenylpropanloamine.

Pressor Activity—Pressor activity data are cited in Table IV. The oral administration of 50 mg./kg. of phenylpropanolamine elicited a pressor response that reached a peak at about 5 min. postdrug and returned to control level by 60 min. postdrug. Oral administration of III at doses of 218, 436, and 871 mg./kg. elicited pressor responses accompanied by compensatory bradycardias. The maximum responses to the two higher doses were about equal in magnitude, but the duration of the pressor response was greater with the highest dose. The time of peak effect was about 15 min. postdrug with III, and all three doses produced pressor responses that remained above predrug control levels for at least 120 min. postdrug. No pressor response was elicited with doses of 500 and 1000 mg./kg. of IV. Thus, III showed the expected pressor activity of a phenylpropanolamine prodrug. Because IV was inactive, it was presumed that it was not cleaved and, therefore, that IV is not a prodrug of phenylpropanolamine.

Antitussive Activity—Compound IV was not tested because it did not show pressor activity. Compound III was active in the antitussive test. It was about one-half as potent as phenylpropanolamine on a molar basis. Equipotent doses of these compounds produced the same incidence of peripheral vasodilation, mydriasis, and piloerection. Thus, by these test parameters, III appears to be converted to phenylpropanolamine after oral dosing in dogs.

CONCLUSION

A series of phenylpropanolamine enamines were found to hydrolyze at significantly different rates in phosphate buffer at pH 7.4. Hydrolysis was not accelerated when dilute plasma was added to the system. As expected, the compounds cleaved faster as the pH of the test system was lowered.

Two compounds were chosen for pharmacologic tests. From hydrolysis data, it was expected that III would hydrolyze *in vivo* to give phenylpropanolamine but that IV would not hydrolyze. Acute toxicity data in mice suggest that the conversion of III to phenylpropanolamine is not rapid because III has only about one-half the molar toxicity of phenylpropanolamine. Tests for pressor activity in rats showed that III produced pressor activity, presumably after conversion to phenylpropanolamine, but was less potent than phenylpropanolamine. These data support the suggestion that the conversion of III to phenylpropanolamine is not rapid. Because IV was inactive in this test, it was concluded that it was not hydrolyzed *in vivo* to phenylpropanolamine and, therefore, that it was not a prodrug of phenylpropanolamine; it was not studied further.

In the antitussive test in dogs, III was about one-half as potent on a molar basis as phenylpropanolamine. Thus, these data also support the suggestion that the conversion of III to phenylpropanolamine is not rapid. The data from three tests in three species suggest that III is indeed hydrolyzed *in vivo* to phenylpropanolamine. In contrast, the pressor activity data suggest that IV is not hydrolyzed *in vivo* to phenylpropanolamine.

In summary, enamines are potentially useful prodrug derivatives.

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Drug Transport V: Mechanism of Potassium-Ion Inhibition of Passive Transfer of Solutes across Everted Rat Intestine

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Abstract □ In previous reports, it was shown that the inclusion of certain cations and sugars in the drug–buffer solution, or other alteration in the basic buffer solution, that results in net tissue fluid uptake also produces a marked reduction of passive solute transfer across the everted rat intestine. Since the extent of inhibition of intestinal transfer under any given condition varied considerably from one drug to another, it was decided to examine the effect of potassium ion (K^+), a particularly pronounced inhibitor, on the intestinal transfer of a series of solute molecules of widely different permeability characteristics. The quantitative replacement of Na^+ by K^+ in a Krebs bicarbonate buffer resulted in some degree of transfer inhibition of every compound studied. An inverse relationship was found between the degree of transfer inhibition and permeability. The intestinal transfer of poorly permeable compounds such as bromthymol blue or eosine blue was virtually abolished in the presence of K^+. A high degree of correlation was noted between percent inhibition and molecular weight of solute molecules, suggesting that the most important parameter influencing the extent of inhibition of transfer is the molecular size of the compound. A mechanism is proposed whereby polar compounds are assumed to traverse the isolated, everted rat intestine *via* intercellular channels existing between adjacent mucosal epithelium cells. When the intestine is exposed to buffer solutions causing tissue fluid uptake, a portion of the fluid penetrates the mucosal cell, causing swelling of the cell and producing a narrowing of the apical portion of the intercellular channel.

Keyphrases □ Drug transport, everted rat intestine—potassium-ion inhibition □ Potassium ion—mechanism of passive-transport inhibition □ Tissue fluid uptake relationship—drug transport

Previous reports in this series showed that various cations present in a drug–buffer solution can have a marked influence on passive solute transfer across the everted rat intestine. In the presence of potassium (1, 2), there is a significant reduction in the mucosal-to-serosal transfer of riboflavin, salicylate, and sulfanilamide. Other cations, including NH_4^+, guanidine$^+$, and Li^+, produce varying degrees of transfer inhibition of these molecules. Several recent literature reports supported these observatons on the influence of K^+ on passive solute transfer. Caldwell *et al.* (3, 4) showed that the passive transfer of digoxin and ouabain across the everted rat intestine is significantly reduced in the presence of a K^+ buffer. In addition, there was direct verification of the authors' observations on the influence of K^+ on salicylate transfer by Benet *et al.* (5).

Inclusion of certain other components in the drug–buffer solution or alteration in the basic buffer solution produces the following effects on transfer: (*a*) glucose and xylose reduce solute transfer, whereas the inclusion of mannitol has no effect (6); and (*b*) a hypotonic buffer solution significantly decreases solute transfer, while a hypertonic buffer exerts no influence on transfer (7).

A common characteristic of all buffer components causing an inhibition of solute transfer is their influence on tissue fluid uptake. There is a direct, quantitative relationship between the percent inhibition of transfer and the extent of tissue fluid uptake under these varying conditions (6). However, the extent of inhibition of transfer produced by any given buffer component varies from one drug to another. Thus, consistently, the transfer of riboflavin is inhibited to a significantly greater extent than is the transfer of salicylate or sulfanilamide. Since these differences cannot be attributed to the buffer component, they must be related to the permeability characteristics of the molecule.

Accordingly, it was decided to examine the effect of K^+ on the intestinal transfer of a series of solute molecules of widely different permeability characteristics and to attempt to correlate the biologic parameters with appropriate properties of the molecules. It was anticipated that this approach would prove useful in understanding the mechanism(s) of passive drug transfer across the isolated intestine.

EXPERIMENTAL

Intestinal Transfer Studies—Intestinal transfer rates were determined using a modification of the method of Crane and Wilson (8). Preparation of the everted rat intestine and determination of transfer rate were discussed in a previous report (2). The following briefly summarizes the procedure and notes any departures from the usual methods.

Male, Sprague-Dawley rats, weighing about 250 g., were fasted 20–24 hr. prior to the experiment. Water was allowed *ad libitum*. After severing the intestine at the pyloric junction, the first 15 cm. of proximal intestine was discarded and the following 20 cm. was divided into two 10-cm. segments after eversion. The proximal 10-cm. segment was designated Segment 1. The normal buffer solution (Na^+ control) was a modified Krebs bicarbonate buffer[1], pH 7.4. The K^+ buffer was prepared by quantitatively replacing the sodium salts of the control buffer with the corresponding potassium salts. In each experiment, 2 ml. of buffer was placed inside the sac (serosal solution); the entire preparation was placed into approximately 100 ml. of buffer solution containing the drug (mucosal solution), which was continually gassed with O_2–CO_2 (95:5 v/v). The drugs studied are listed in Table I.

In any given experiment, the mucosal and serosal solutions were identical in composition except for the presence of the drug in the mucosal solution. Usually, the serosal compartment was sampled every 10 min. during the entire 2-hr. experiment. The entire serosal volume was removed at the sampling time. Two milliliters of buffer solution was then introduced into the serosal compartment as a rinse, immediately removed, and added to the previous sample. Finally, another 2-ml. portion of buffer was placed into the serosal compartment and withdrawn at the next sampling interval. In

[1] KCl, 5 m*M*; KH_2PO_4, 1 m*M*; $NaHCO_3$, 26 m*M*; and NaCl, 122 m*M*.

Table I—Compounds Selected for Intestinal Transfer Studies

Compound	Molecular Weight	pKa	Mucosal Concentration, mg./ml.	Assay Procedure
Aniline (A)	93.1	4.7	0.1	*Reference 9* (see text)
Antipyrine (AP)	188.2	1.4	0.5	*Reference 10* (see text)
Benzocaine (*p*-ethylaminobenzoate) (B)	165.2	2.8	0.1	*Reference 9*
Bromthymol blue (BTB)	624	—	1.0	In 0.1 *N* NaOH at 620 nm.
Eosine blue (EB)	624	—	0.5	In 0.1 *N* NaOH at 520 nm.
Methyl orange (MO)	327	—	0.25	In 0.1 *N* HCl at 510 nm.
2-Pyridine aldoxime methiodide (PAM)	264	—	0.4	In 0.1 *N* NaOH at 335 nm.
Riboflavin (R)	376	10.2	0.02	*Reference 11*
Salicylamide (SAM)	137	8.3	1.0	*Reference 12* as modified in *Reference 13*
Salicylate (SA)	137	3.0	2.0	*Reference 12* as modified in *Reference 14*
Sulfanilamide (S)	172	10.4	0.1	*Reference 9*

two cases, eosine blue and bromthymol blue, the compounds traversed the intestinal preparation so slowly that the serosal solution was sampled only at 60 and 120 min. Each serosal sample was assayed for drug content using the methods noted in Table I.

Aniline concentration was determined using a previously described method (9) with the following modification. It was found that the rate of coupling to produce a stable color was much too slow using the concentration of reagent originally suggested. Consequently, the concentration of Marshall's reagent [*N*-(1-naphthyl)ethylenediamine·2HCl] was increased to 500 mg./ml. This modification produced a stable color, which was read spectrophotometrically after a 2-hr. waiting period. The increased concentration of reagent had no effect on the maximal absorbance reading or on the time for stable color development for a compound (sulfanilamide) which is readily assayed using the usual reagent concentration. Antipyrine was also determined according to a previous report (10), but absorbance readings were made exactly 20 min. after the addition of $NaNO_2$. The initial concentration of the respective compounds in the mucosal solution, as noted in Table I, remained essentially constant throughout the experiment due to the large volume of the mucosal solution. Moreover, due to frequent sampling of the serosal compartment, in all but two cases the serosal drug concentration never exceeded 13% of the mucosal concentration. The maximum serosal concentrations for aniline and salicylamide were 20 and 30%, respectively, of the mucosal drug concentration.

Comparison of the permeability characteristics of the various solutes was based on the mucosal-to-serosal clearance of each compound over the 1st hr. of study. Clearance (milliliters per hour) was calculated by dividing the cumulative amount of solute transferred in 1 hr. in the Na^+ control buffer by the mucosal solute concentration. The effect of K^+ on intestinal transfer was quantified in terms of the percent inhibition of transfer over a 1-hr. period:

$$\% \text{ inhibition} = 100(1 - \frac{\text{1 hr. cumulative transfer } K^+}{\text{1 hr. cumulative transfer in } Na^+}) \quad \text{(Eq. 1)}$$

Determination of Extracellular Tissue Volume—The extracellular volume of intestinal tissue was determined in various buffer solutions. The marker used was ^{14}C-carboxylinulin[2] (specific activity 2.0 mc./g., 97% pure). The inulin was used as obtained from the supplier. Levi (15) reported that inulin space measurements using inulin from this manufacturer provides consistently good, reproducible estimates of extracellular volume. The inulin was dissolved in distilled water, and dilutions made from this stock were taken to prepare various tagged buffer solutions. Snyder's scintillation fluid (16) was used (20 ml./scintillation vial) for all samples. The composition of this fluid is:

0.3000 g.	1,4-bis[2-(5-phenyloxazolyl)]benzene[3]
7.0000 g.	2,5-diphenyloxazole[3]
100.0 g.	naphthalene[3]
	dioxane to make 1 l.

All materials other than dioxane were of scintillation grade.

The rat intestine was everted as described previously, and the first 15 cm. was discarded. Five or six 5-cm. segments were prepared from each intestine and made into sacs (17). A ligature was tightly tied around one end of the segment, and a long blunt needle attached to a 1-ml. syringe was introduced into the open end of the segment. Another ligature was loosely tied around the needle, and 1 ml. of the appropriate buffer soluton (*i.e.*, the same solution as the mucosal buffer), previously warmed at 37°, was placed into the segment. The ligature about the needle was then tightened and the needle was withdrawn. All sacs were then placed into a beaker containing the tagged inulin buffer solution at 37°, which was gassed with an O_2–CO_2 mixture (95:5 v/v).

Extracellular space measurements were made under two experimental conditions. The tagged inulin was present initially only in the mucosal solution, or it was present in the mucosal solution as well as in the 1 ml. of serosal solution in the sac. The sacs were removed after 60 min. incubation in the mucosal solution and gently, but thoroughly, rinsed with normal saline. The sac was cut open at one end and the serosal fluid was permitted to run out. Three snips of tissue (about 100 mg. each) from each segment were then placed into individual, previously tared scintillation vials and the vials were immediately capped. Several samples (0.1 ml.) of the mucosal inulin buffer solution were also taken and placed into vials containing scintillation fluid. Each tared vial was then weighed to determine the final wet weight of tissue. One milliliter of solubilizer[4] was added to each vial, and the vials were placed into a water bath maintained at 45–50° to hasten the digestion process. After the tissue was in solution (generally after 3–4 hr.), the vials were cooled to room temperature and scintillation fluid was added. All samples were counted for 10 min. at 10,000 c.p.m. in a liquid scintillation spectrometer[5].

Quenching of radiation due to the presence of tissue was corrected for by use of an internal standard (^{14}C-toluene). Generally, there was a 12% loss of counting efficiency due to tissue quenching. If samples were read within a day of preparation, unusually high counting rates were obtained, apparently due to an interaction between the solubilizer[4] and the scintillation fluid. After preparing blank vials (*i.e.*, tissue + solubilizer + scintillation fluid

[2] Lot. No. 511-021, New England Nuclear Corp., Boston, Mass.

[3] Amersham-Searle Co., Arlington Heights, Ill.

[4] NCS solubilizer, Amersham-Searle Co., Lots F-7230 and F-7290.

[5] Packard Tri-Carb, model 2002, Packard Instrument Co., Downers Grove, Ill.

Table II—Clearance and K^+ Inhibition of Transfer

Compound	Clearance, ml./hr.[a]	Percent Inhibition of Transfer in K^+[b]
Bromthymol blue	0.016[c] (46)	86[c]
Digoxin	0.100[d]	67[d]
Eosine blue	0.126 (43)	91
Methyl orange	0.380 (12)	75
Riboflavin	0.540 (12)	56
Sulfanilamide	0.570 (14)	34
Pyridine aldoxime methiodide	0.720 (11)	52
Salicylate	0.800 (18)	27
Antipyrine	1.720 (4)	24
Benzocaine	3.240 (6)	13
Salicylamide	3.420 (4)	16
Aniline	4.600 (4)	6

[a] Based upon cumulative amount transferred across Segment 1 after 1 hr. in the Na^+ control buffer. Represents the average of five experiments (four for sulfanilamide). Parenthetic values denote the coefficient of variation expressed as a percentage. [b] Based upon cumulative amount transferred across Segment 1 after 1 hr. in the K^+ buffer. Represents the average of five experiments (four for sulfanilamide). [c] Based upon cumulative amount transferred after 2 hr. [d] Calculated from *Reference 4.*

with no added radioactivity), it was found that if samples were read 3 days after preparation, this background counting rate was reduced to a sufficiently low level to be subtracted from all samples. Since this background rate represents at most 1% of the activity present in the sample, there is little error introduced after correcting for this level of activity. As standard procedure, therefore, all samples were counted 3 days after preparation.

Extracellular tissue volume was calculated from the following relationship:

$$\text{extracellular space (ml./g.)} = \frac{\text{c.p.m./g. tissue}}{\text{c.p.m./ml. mucosal inulin solution}} \quad \text{(Eq. 2)}$$

RESULTS

Solute Properties and K^+ Inhibition of Transfer—Table II lists clearance values for each compound based upon the 1-hr. cumulative transfer in the Na^+ control buffer, and the percent inhibition of transfer in the K^+ buffer based on the 1-hr. transfer data. There appears to be a distinct relationship between the clearance value and the extent of inhibition of transfer in the presence of the K^+ buffer; as the clearance value increases (*i.e.*, as permeability increases), the percent inhibition of transfer decreases. Thus, bromthymol blue and eosine blue, which have the smallest clearance values, are inhibited to the greatest extent. The most permeable compounds, benzocaine, aniline, and salicylamide, are inhibited the least. Since these three compounds exhibit the greatest permeability, it is possible that the rather small, constant level of inhibition of transfer produced by K^+ is not a true measure of the extent of inhibition. That is, since these compounds readily traverse the membrane, the rate-limiting step in the overall transfer process may be the rate of movement of the molecule through the mucosal solution to the mucosal surface (*i.e.*, bulk diffusion rate-limited transfer). If this were true, then the rate of appearance of the drug in the serosal solution would be a measure of diffusion through the mucosal solution and not transfer across the membrane. Such a situation could conceivably account for the low level of effect of K^+ on the transfer of these permeable compounds. If, in fact, the overall transfer process was bulk diffusion rate limited, then the transfer rate should be sensitive to stirring effects in the mucosal solution. This possibility was examined by providing rapid stirring in the mucosal solution by means of a small magnetic stirrer. The transfer rates of both aniline and salicylamide were independent of stirring rate; as a result, it may be concluded that transfer across the membrane is not rate limited by bulk diffusion.

Figure 1 illustrates the inverse relationship between the extent of inhibition of transfer and clearance. The clearance of digoxin

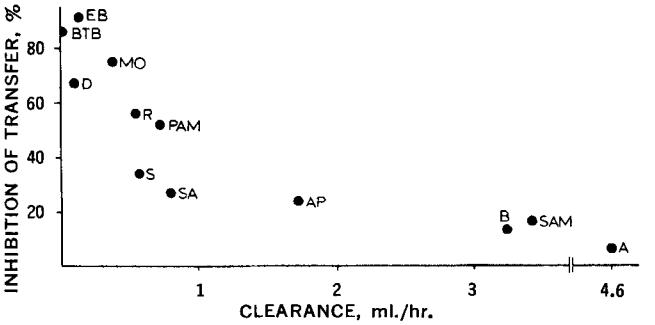

Figure 1—*Relationship between percent inhibition of transfer after 1 hr. in the K^+ buffer and the clearance after 1 hr. in the Na^+ control buffer. Each point represents the average of five experiments (four for sulfanilamide) using Segment 1. Key: see Table I.*

and the percent inhibition of transfer of digoxin in the presence of K^+ were calculated from data in a recent literature report (4). While the degree of inhibition produced by K^+ is clearly related to the permeability of the compound, it was desirable to develop this relationship further.

Figures 2 and 3 are log–log plots of percent inhibition of transfer and of clearance in the Na^+ buffer, respectively, as a function of molecular weight. A least-squares line was fitted to all points in each graph. In both cases, a highly significant relationship is observed. The plot of percent inhibition *versus* molecular weight yields a correlation coefficient of 0.884 and indicates that the larger the molecule the greater the extent of inhibition of transfer. The plot of clearance in the Na^+ buffer *versus* molecular weight also yields a high correlation coefficient (0.880) and suggests that, at least for the compounds studied, clearance decreases with increasing molecular weight.

Extracellular Space of Intestinal Tissue in the Presence of Various Buffer Solutions—The relationship between tissue fluid uptake and the inhibition of transfer by various buffer components was developed previously (2, 6). This relationship suggests that the fluid uptake produced by a given inhibitor may be responsible for the alteration of the transfer process. Since fluid uptake by the intestinal tissue may localize either within cells or in extracellular space, an attempt was made to determine where this fluid localizes in the tissue by measuring extracellular space.

Initially, attempts were made to determine extracellular space by placing closed everted intestinal sacs into a tagged inulin solution (inulin present only in the mucosal solution). Although a value of approximately 22% was obtained for this space in the Na^+ control

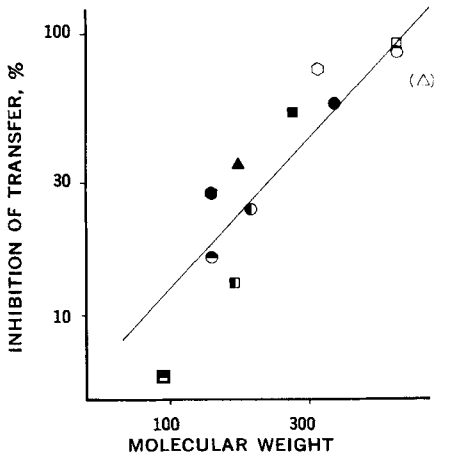

Figure 2—*Relationship between percent inhibition of transfer after 1 hr. in the K^+ buffer and the molecular weight. Values for percent inhibition are the average of five experiments (four for sulfanilamide) using Segment 1. A least-squares regression line was drawn through all points. Key: ●, salicylate; ■, 2-pyridine aldoxime methiodide; □, eosine blue; ○, methyl orange; ●, riboflavin; ▲, sulfanilamide; ◐, antipyrine; ○, bromthymol blue; ▬, aniline; ◓, salicylamide; ◧, benzocaine; and △, digoxin.*

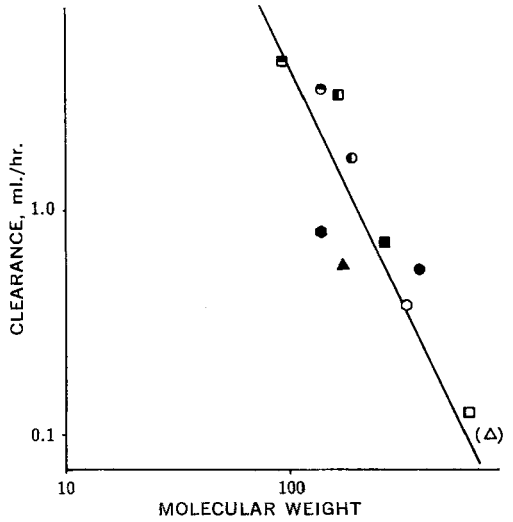

Figure 3—*Relationship between the 1-hr. clearance in the Na^+ control buffer and the molecular weight. Clearance values represent the average of five experiments (four for sulfanilamide) using Segment 1. A least-squares regression line was drawn through all points. Key: same as Fig. 2.*

buffer, which is consistent with certain literature reports (18, 19), the great variability precluded a meaningful comparison of extracellular space values under different conditions. As a result, inulin was placed into both mucosal and serosal solutions. This improved the reproducibility of the results, as shown in Table III. Although the value of 49% obtained in the Na^+ control buffer seems unusually large, a recent literature report (19) suggested that this value is reasonable.

Preliminary examination of the extracellular space data based on final tissue weight suggests a clear relationship between extracellular space and inhibition. The last four buffers listed in Table III (250 m*M* glucose, hypotonic, K^+, and 25 m*M* glucose) markedly inhibit the transfer of various polar compounds and significantly decrease extracellular space. On the other hand, tromethamine, mannitol, and hypertonic buffers have little effect on transfer and are either without effect on or actually increase extracellular space. However, there is some controversy as to whether extracellular space values should be calculated on the basis of final tissue weights (19). Considering the equation used to calculate extracellular space, it is clear that any factor which tends to increase or decrease the weight of the tissue will give an apparent change in extracellular space regardless of where the fluid is localized. Since it has been found that all buffer solutions which inhibit solute transfer also produce an increase in tissue weight, the value for tissue weight will be larger than the control. This results in an apparent decrease in extracellular space.

Table III—Total Extracellular Space of Rat Intestinal Tissue in the Presence of Various Buffer Solutions

Buffer Solution[a]	Percent Extracellular Space Based upon Final Tissue Weight[b]	Percent Extracellular Space Based upon Initial Tissue Weight[c]
Na^+ control	49 ± 6 (14)	52
Hypertonic	65 ± 4 (15)	61
Tromethamine	54 ± 6 (15)	56
Mannitol	44 ± 3 (15)	45
250 m*M* glucose	41 ± 4 (15)	47
Hypotonic	40 ± 5 (15)	44
K^+	34 ± 4 (15)	40
25 m*M* glucose	33 ± 3 (15)	40

[a] As reported in *References 2, 6,* and *7.* [b] Represents the average ± standard deviation of the mean. The number of tissue pieces used to determine extracellular space are given in parentheses. [c] Represents the experimentally measured extracellular space of the control corrected for tissue changes due to fluid uptake in the various buffer solutions.

Furthermore, the decrease in space will be related to the extent of fluid uptake. When one corrects the extracellular space values shown in Table III for water uptake, *i.e.*, when one calculates extracellular space based on initial rather than final tissue weights, it can be seen that there is little change in extracellular space with different experimental conditions. A recent report by Jackson and Cassidy (20) also demonstrated no relationship between tissue fluid uptake by the rat intestine and extracellular space and is consistent with the present findings.

DISCUSSION

From the previous reports in this series (2, 6, 7), it seems clear that some parameter related to tissue fluid uptake is responsible for the observed decrease in solute transfer in the presence of various buffer solution components. A plausible mechanism that would rationalize previous experimental observations as well as the apparent dependency of the magnitude of transfer inhibition on the size of the solute must take into account the route of movement of the compound across the membrane. With respect to solute movement across multicellular membranes, one may consider at least three possible routes: (*a*) movement *via* a lipid route (*i.e.*, through the cells *via* the lipid membranes), (*b*) transport through aqueous-filled channels existing between adjacent cells (*i.e.*, intercellular spaces), and (*c*) transport *via* pores existing within the cell membrane in contact with the mucosal bathing solution. The least probable route would be the latter. It is doubtful that there would be discontinuities in the cell membrane of sufficient size to accommodate compounds of a molecular weight in excess of 100 (21).

With respect to solute translocation *via* a lipid route, one would expect that this pathway would be limited solely to compounds having sufficient nonpolar characteristics. In the present study, it was found that those compounds which could be considered lipid soluble are also the most permeable molecules. It would seem most plausible that these compounds traverse the intestine essentially by penetration across the cell membrane, and it is the transfer of these compounds which is least inhibited by K^+. This observation, as well as several other experimental findings, suggests that K^+ and the other inhibitors are preferentially affecting another route of solute movement across the intestine which is distinct from the lipid pathway.

One possibility, therefore, to explain the results of the inhibition of solute transfer is to assume that polar compounds penetrate the isolated, everted rat intestine *via* an intercellular route. The mechanistic explanation for inhibition of transfer would be based upon the following reasoning. Under normal conditions (*i.e.*, in the presence of the Na^+ control buffer), polar molecules penetrate the isolated intestine by movement along intercellular channels existing between adjacent mucosal cells, whereas nonpolar molecules would primarily use a lipid route. When the intestine is placed into a buffer solution causing tissue fluid uptake, a portion of the fluid penetrates the mucosal cell, causing swelling of the cell. This increase in cell volume produces an expansion of adjacent cells, resulting in a narrowing of the intercellular channels and decreasing the effective diameter of these channels.

Apparently inconsistent with this possibility is the observation that there is no substantial decrease in the extracellular space in the presence of those materials causing a reduction in solute transfer. This finding may not be inconsistent, however, based upon the morphology of the intestinal epithelium. The intercellular channels have an appearance very much like a funnel. At the luminal surface of the intestine, the intercellular spaces are rather long and narrow and gradually funnel-out approximately two-thirds the way down the epithelial cell. Beyond that point, these channels are very large and this region probably represents virtually all of the mucosal extracellular volume. Several studies with rat intestine illustrated that the basal portion of the intercellular channels can be widely distended. Tomasini and Dobbins (22) observed distention of these channels using the electron microscope, and Dobbins (23) showed these spaces to be approximately 200 Å wide. Palay and Karlin (24) reported average distances of 95–120 Å, with some distances as great as 50–200 nm. separating adjacent mucosal cells. Thus, these channels beyond their narrow regions should be able to accommodate relatively large molecules. As a result, any narrowing of the intercellular channels at their closest points would have little

effect on the total extracellular space measured experimentally, although it may have a profound effect on the relative width of these narrow channels. Support for such a mechanism includes the following experimental observations:

1. Those materials whose transfer is not dependent upon use of intercellular channels should be affected little, if at all, by buffer components producing a decrease in transfer of polar solutes. In fact, the observation has been made that those compounds which are lipid soluble and readily permeable and whose transfer most likely involves movement *via* a lipid route are affected the least by K^+ (6–16% inhibition).

2. All buffer components that result in an inhibition of transfer increase tissue fluid uptake (2, 6, 7). A portion of this fluid uptake produces cellular swelling in the rat intestine (20, 25, 26). Other investigators showed that fluid uptake in a variety of different tissues produces cellular swelling at the expense of extracellular spaces (27–30), although this has not been shown to be the case for intestinal tissue both in the present and in a previous literature report (20). In addition, those materials that have little effect on transfer do not increase tissue fluid uptake.

3. It would be expected that if the effective diameter of narrow intercellular spaces was decreased due to cellular expansion, the larger the molecule the greater would be the inhibition of transfer. Such a relationship exists between molecular weight and inhibition. In addition, under control conditions there is an inverse relationship between molecular weight and clearance, indicating a size dependence for transfer consistent with restricted movement through rather narrow channels.

There are several conflicting reports in the literature with respect to the accessibility of intercellular channels at the luminal membrane surface. Several investigators (23, 24, 31, 32) feel that the tight junction at the luminal border between adjacent epithelial cells presents a real barrier to solute accessibility to the intercellular channels. This conclusion is primarily based upon electron microscopic examination of intestinal tissue. Clarkson (33) developed equations to analyze salt and water transport across the isolated rat ileum, and he concluded that there are at least two distinct pathways for the movement of these materials: one having a large diameter (compared to the hydrated ionic radii of Na^+, K^+, and Cl^-) located extracellularly and permitting passive transport of salt and water, and the other having a small diameter, located intracellularly and associated with active transport processes. The author also suggested that epithelial cells extruded from the luminal surface leave "gaps" which partially determine the passive permeability of the intestinal tissue, although these "gaps" may be transient and occupy a small fraction of the total mucosal surface area.

In vivo measurements of the rat ileum (34) based upon mannitol diffusion indicate the presence of aqueous channels having radii in the order of 30–40 Å, whereas the equivalent mean pore radius of the epithelial cell membrane is only 4 Å (35). More recently, Loehry *et al.* (36) studied the permeability of the rabbit small intestine *in situ* by measuring the plasma-to-lumen and lumen-to-plasma clearances of various water-soluble molecules over the 60–33,000 molecular weight range and of ^{125}I-labeled polyvinylpyrrolidone molecules over the 8000–80,000 molecular weight range. The authors reported that all substances studied pass through the small intestinal wall and that an inverse relationship exists between permeability and molecular weight identical to that found in the present study. Moreover, the permeability pattern is virtually identical whether diffusion occurs from plasma to lumen or from lumen to plasma. These findings require one to postulate the existence of aqueous channels with much greater diameters than those proposed for the epithelial cell membranes. It would appear, based on the present finding as well as on several literature reports, that an aqueous intercellular route across the mucosal epithelium plays a very significant role in the intestinal transport of polar molecules both in isolated systems and *in vivo*.

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Inhibited Dissolution of Drug Crystals by Certified Water-Soluble Dyes III

J. PICCOLO* and R. TAWASHI

Abstract □ Results of dissolution-rate studies on diethylstilbestrol microcrystals in the presence of low concentrations of various certified water-soluble dyes are presented. Under the conditions described, marked and different degrees of inhibition were observed. Information on the dissolution inhibition process was obtained by the study of the dissolution-rate dependence on chemical group, class, and concentration of the dye. The data obtained show that cationic dyes were more reactive in lower concentration than anionic dyes and suggest higher adsorption intensity of the cationic dye molecules at the primary dissolution sources on the crystal surface.

Keyphrases □ Dissolution rates—diethylstilbestrol microcrystals' inhibition by water-soluble dyes □ Dyes, water soluble—drug crystal dissolution, inhibition □ Diethylstilbestrol microcrystals—dissolution rates, dye inhibition □ Crystals, diethylstilbestrol—dissolution rates, dye inhibition

In previous articles (1–3), the inhibitory effect of low concentrations of FD&C Blue No. 1 on the dissolution rate of crystalline drugs was established. The effect of the degree of undersaturation and the dependence of dissolution rate on the inhibitor concentrations of powder systems were also investigated. The data were explained in the light of preferential adsorption of the dye molecules on the primary dissolution sources on the crystal surface.

The purposes of this publication are to: (*a*) determine the effect of other certified water-soluble dyes on the dissolution rate, (*b*) study the influence of the dye concentration on the dissolution rate, and (*c*) correlate the results obtained with the previously suggested mechanism and with the chemical nature of the dyes.

EXPERIMENTAL

Materials—The dyes used in this study were FD&C Blue No. 1[1], FD&C Red No. 2[1], FD&C Red No. 3[1], FD&C Violet No. 1[1], D&C Green No. 5[1], Ext. D&C Blue No. 1[2] (Methylene Blue), and Malachite Green[3]. Diethylstilbestrol[4] was selected as a model substance for poorly water-soluble drugs. The dissolution media was 0.90% NaCl solution[5].

Dissolution-Rate Studies—All dissolution experiments were conducted using the Coulter counter technique (3–6). A suspension of diethylstilbestrol was prepared by adding 1 ml. of ethyl alcohol, containing 10 mg. of drug, to 150 ml. of 0.90% NaCl solution. Magnetic stirring was maintained for 2 hr. The suspended crystals were then sonified for 2 hr. (3).

The dissolution rate of the diethylstilbestrol microcrystals was determined using the Coulter counter equipped with a 30-μ aperture tube. The data were plotted as previously described (3), and the rate of change of radius (dr/dt) was determined for the powder system in the absence and in the presence of the dyes.

[1] Rose & Laflamme Limited, Montreal, Canada.
[2] J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] The British Drug Houses (Canada) Ltd., Toronto, Canada.
[4] Matheson, Coleman & Bell Inc., East Rutherford, N. J.
[5] Normal saline for injection, Abbott Laboratories, Montreal, Canada.

Table I—Effect of 10 mcg./ml. of Various Water-Soluble Dyes on the Dissolution Rate of Diethylstilbestrol[a]

Dye	Dissolution in 0.90% NaCl Containing Dye—Dissolution Rate, cm./sec.
D&C Green No. 5	4.40×10^{-8}
FD&C Red No. 2	2.72×10^{-8}
FD&C Red No. 3	2.64×10^{-8}
FD&C Blue No. 1	2.40×10^{-8}
Malachite Green	1.80×10^{-8}
Ext. D&C Blue No. 1	1.60×10^{-8}
FD&C Violet No. 1	0.64×10^{-8}

[a] Dissolution in 0.90% NaCl = 5.67×10^{-8} cm./sec.

RESULTS AND DISCUSSION

Table I shows the effect of the various water-soluble dyes on the dissolution rate of diethylstilbestrol crystalline powder in normal saline. It permits a comparison between the dissolution rates obtained with these dyes at a concentration as low as 10 mcg./ml. and the dissolution rate in normal saline. Quantitatively, the magnitude of the inhibitory effect of these dyes at the same concentration varied considerably.

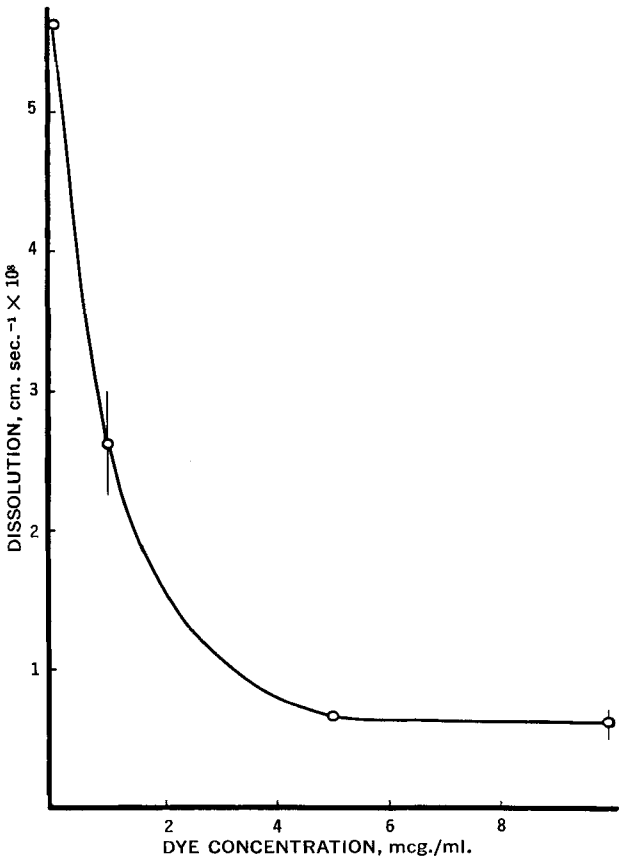

Figure 1—*Effect of FD&C Violet No. 1 concentration on the dissolution rate of diethylstilbestrol crystalline powder.*

Table II—Effect of Various Concentrations of Water-Soluble Dyes on the Dissolution Rate of Diethylstilbestrol

Dye Concentration, mcg./ml.	Dissolution Rate, cm./sec. × 10^5				
	FD&C Violet No. 1	FD&C Red No. 3	D&C Green No. 5	Ext. D&C Blue No. 1	Malachite Green
0	5.67	5.67	5.67	5.67	5.67
0.1	—	—	—	2.40	3.50
0.5	—	—	—	—	2.33
1.0	2.63	—	—	1.79	1.80
5.0	0.67	2.80	4.80	—	—
10.0	0.64	2.64	4.40	1.60	1.80
20.0	—	1.90	3.39	—	—
50.0	—	1.60	3.10	—	—
100.0	—	—	2.11	—	—

The dissolution-rate dependence on inhibitor concentration was systematically determined. The dissolution rates of diethylstilbestrol microcrystals in 0.90% NaCl solution containing various concentrations of five water-soluble dyes are shown in Table II. These data were plotted as dissolution rate *versus* inhibitor concentration (Figs. 1–5). An important property common to all of these dyes is that the curves obtained tend to stabilize after a certain concentration of dye is reached. This fact is consistent with previous findings using a different model substance (sulfaguanidine) and a different method for measuring the dissolution rate (single crystal technique).

Another observation associated with inhibited dissolution by dyes is the concentration level at which the dye activity starts. Unlike other dyes, Ext. D&C Blue No. 1 and Malachite Green showed an outstanding activity on the dissolution rate of diethylstilbestrol at extremely low concentrations. Figures 4 and 5 demonstrate the marked decrease in dissolution rate in the presence of a dye concentration as small as 0.1 mcg./ml. Furthermore, stabilization in the dissolution rate occurs after a concentration of only 1 mcg./ml. of these two dyes is reached.

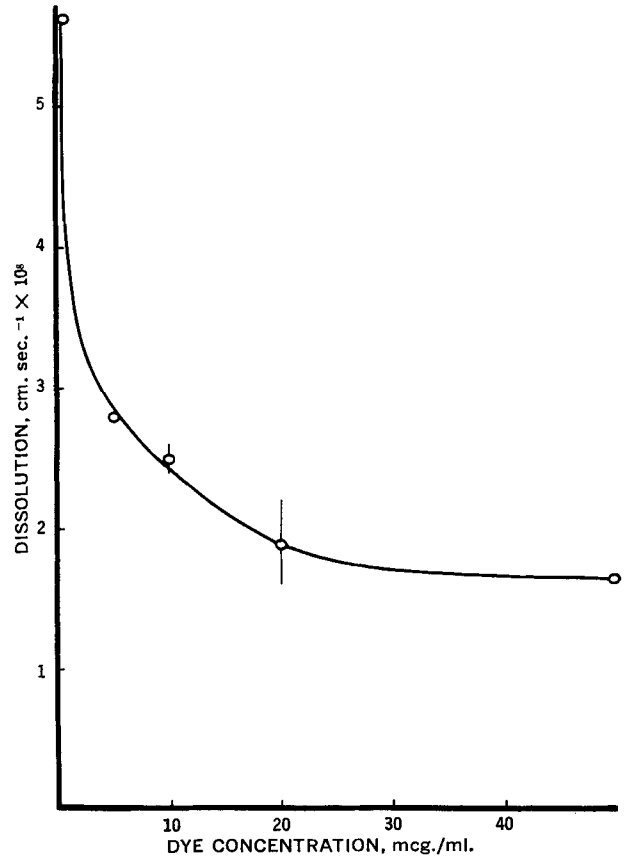

Figure 2—*Effect of FD&C Red No. 3 concentration on the dissolution rate of diethylstilbestrol crystalline powder.*

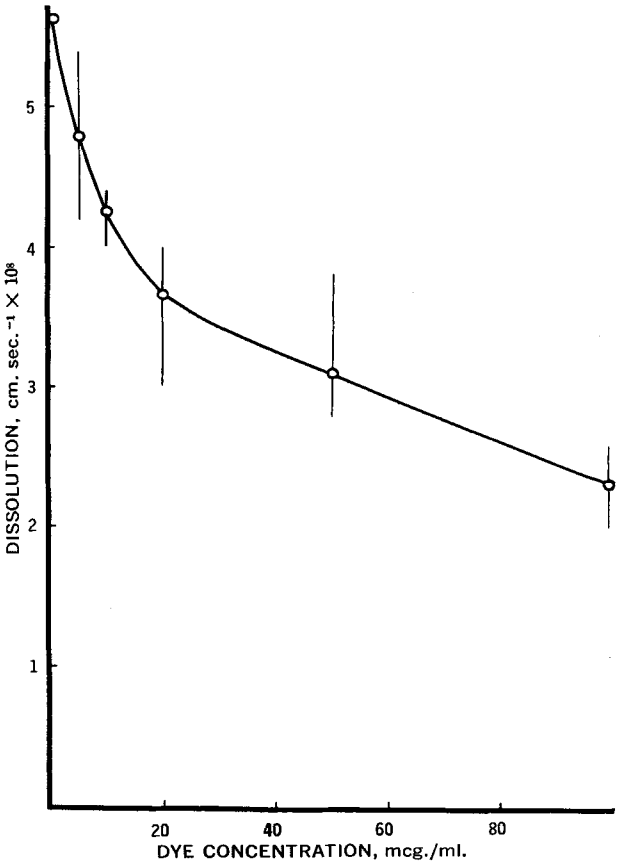

Figure 3—*Effect of D&C Green No. 5 concentration on the dissolution rate of diethylstilbestrol crystalline powder.*

In a previous report (3) on the dissolution inhibition of diethylstilbestrol by FD&C Blue No. 1, results were discussed on the basis of preferred adsorption of the dye molecules on the primary dissolution sources of the crystal surface. A correlation for this situation was then suggested by applying the relationship between dissolution rate and dye concentration (3). The influence of FD&C Blue No. 1 on the dissolution rate could be correlated satisfactorily in terms of a Langmuir adsorption isotherm. If the dissolution rate is considered as a simple function of the dye concentration in solution, the Langmuir adsorption isotherm can be written:

$$R = R_0 \left(1 - \frac{KbC}{1 + bC}\right) \qquad \text{(Eq. 1)}$$

where R = dissolution rate with dye, R_0 = dissolution rate without dye, K = fraction of surface covered when the surface is "saturated" with dye, C = dye concentration in bulk solution, and b = Langmuir isotherm constant.

The same correlation was applied to the present results, using the data in Figs. 1–5. The K and b values obtained for the different dyes investigated by a method previously described (3), along with their chemical group and class, are given in Table III.

Table III—Adsorption and Dissolution Inhibition Constants for Various Water-Soluble Dyes on Diethylstilbestrol

Dye	Chemical Group	Class	K	b
FD&C Blue No. 1[a]	Triphenylmethane	Anionic	0.77	4.79
FD&C Violet No. 1	Triphenylmethane	Anionic	0.97	1.42
FD&C Red No. 3	Fluorescein	Anionic	0.77	0.29
D&C Green No. 5	Quinone	Anionic	0.74	0.05
Ext. D&C Blue No. 1	Thiazine	Cationic	0.73	13.76
Malachite Green	Triphenylmethane	Cationic	0.69	20.70

[a] For FD&C Blue No. 1, see *Reference 3*.

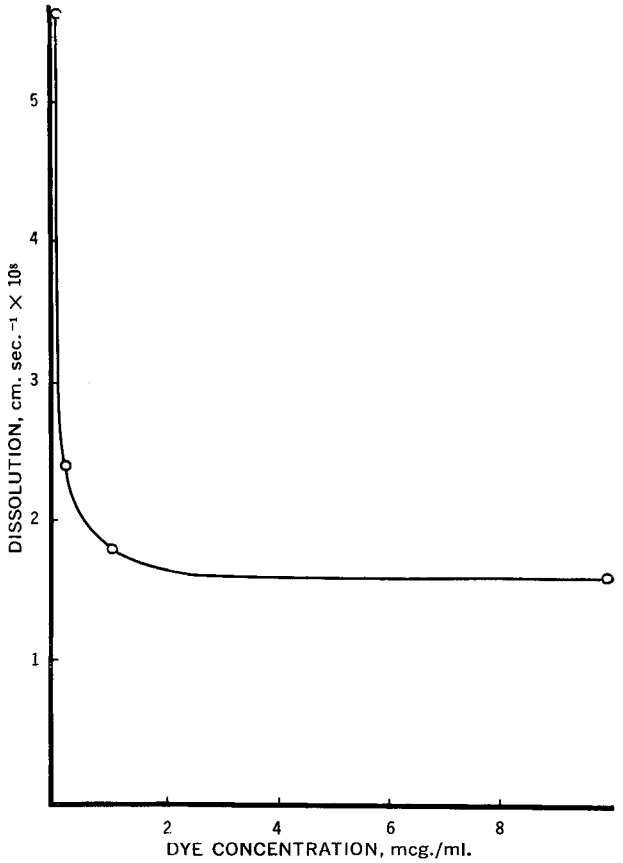

Figure 4—*Effect of Methylene Blue concentration on the dissolution rate of diethylstilbestrol crystalline powder.*

The *b* values are indicative of adsorption intensity, and *K* is a measure of the saturation capacity of the surface for the inhibitor. From these results, it is apparent that the *K* values are smaller than 1.0 for all of the investigated dyes. This fact supports a selective adsorption mechanism rather than complete coverage of the crystal surfaces and is consistent with previous findings (1, 3).

Table III also shows that the *b* values are much greater for cationic dyes than for anionic dyes. This agrees with the results reported by Michaels and Colville (7) in their work on growth inhibition of adipic acid crystals by surfactants. The authors determined the *b* values for anionic and cationic surfactants and found that these values were greater for cationic surface-active agents.

The outstanding activity of these cationic dyes is illustrated in Figs. 4 and 5. The high *b* values obtained indicate that the effect of these dyes can be attributed to a higher adsorption intensity. Quantitative characterization of this behavior in the near future will provide information on growth and dissolution inhibition of drug crystals.

In summary, the results of this investigation established that: (*a*) a relatively large number of the existing certified water-soluble dyes

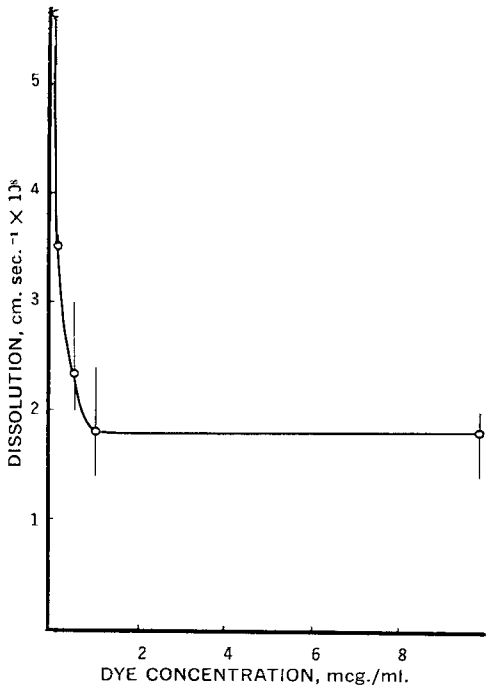

Figure 5—*Effect of Malachite Green concentration on the dissolution rate of diethylstilbestrol crystalline powder.*

exert a significant inhibition on the dissolution rate of diethylstilbestrol, and (*b*) cationic dyes are more reactive in lower concentrations than are anionic dyes.

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Binding Study of *p*-Hydroxybenzoic Acid Esters to Bovine Serum Albumin by Fluorescent Probe Technique

H. W. JUN, R. T. MAYER*, C. M. HIMEL†, and L. A. LUZZI

Abstract □ *N-n*-Butyl[(5-dimethylamino)-1-naphthalene]sulfonamide is a fluorescent probe which has minimal fluorescence in water but fluoresces strongly when bound to protein. The spectral property of this probe was used for an indirect measurement of the binding of *p*-hydroxybenzoic acid esters (parabens) to bovine serum albumin. The method is capable of providing simple, sensitive, and rapid determination of binding and the nature of binding. The data indicate that the mechanism leading to the binding of parabens to bovine serum albumin could be of a hydrophobic nature. The aromatic ring rather than the aliphatic side chain appears to be the primary binding site of the parabens when competing with this probe. Quantum yield information for the sulfonamide is discussed.

Keyphrases □ Drug–plasma protein binding—determination, fluorescent probe technique □ *N-n*-Butyl[(5-dimethylamino)-1-naphthalene]sulfonamide—as a fluorescent probe, parabens–bovine serum albumin binding □ Parabens–bovine serum albumin binding—determination, fluorescent probe technique □ Bovine serum albumin–parabens binding—determination, fluorescent probe technique □ Fluorescent probe technique—parabens–bovine serum albumin binding determination □ NMR spectroscopy—determination, parabens–bovine serum albumin binding

The binding of drugs by plasma proteins has been recognized as an important factor in drug availability, drug efficacy, and drug transport for many years (1). Various experimental techniques have been employed to study drug–protein interactions (2), with the majority of them directed at the elucidation of the extent of binding. In spite of extensive studies in this area, little information has been generated concerning the nature of binding sites and the mechanisms responsible for binding. One technique recently employed to examine the conformational nature of drug–protein interactions is nuclear magnetic relaxation measurements (3, 4). Fluorescence spectroscopy also has been employed to study binding phenomena, but it has been used primarily to examine enzyme–substrate interactions (5, 6).

The fluorescence techniques described in this study make use of a fluorescent probe as an indicator of binding. Fluorescent probes are defined (7) as fluorescent compounds whose spectral properties (such as fluorescence excitation, emission, and quantum yield) are affected by the physical and chemical environment of the probe molecules. Since the concept of a fluorescent probe was first introduced by Weber (8) in 1952, various experimenters (5, 6, 9) studied the applications of this technique in biochemical research related to binding.

Fluorescent probe techniques are capable of providing data similar to what could be obtained through dialysis studies without the relevant problems of membrane binding, Donnan effects, and time consumption. In addition, it may be possible to estimate the nature of binding and binding sites of the complex from the chemical structure and spectral properties of fluorescent probes.

The purpose of the present study was to examine the potential of the fluorescent probe techniques in the area of drug–protein interactions. Specifically, an attempt was made to establish the usefulness of the techniques for elucidating the nature of binding of parabens and bovine serum albumin. It was also thought that NMR data would be helpful in substantiating fluorescence findings.

EXPERIMENTAL

Apparatus—All absorption and fluorescence spectra were run on a Spectro 210[1]. This instrument was fully described in a previous publication (10). The pH measurements were obtained with a pH meter[2] calibrated using standard buffer solutions.

Chemicals and Solvents—The fluorescence probe, *N-n*-butyl-[(5-dimethylamino)-1-naphthalene]sulfonamide, was prepared in this laboratory[3]. Synthesis of this compound and other pertinent data concerning it will be presented elsewhere. Methyl-*p*-hydroxybenzoate[4] and ethyl-, propyl-, and *n*-butyl-*p*-hydroxybenzoates[5] were used. Water was double distilled from glass. Methanol was spectroscopic grade, and all other chemicals were reagent grade or of special purity. Bovine serum albumin was purchased[6]. Concentrations of the protein were determined by measuring the absorbance at 280 nm. and using $a = 0.66$ ($E^{1\%}_{1\,cm.} = 6.6$); the molecular weight of 66,000 was used to determine molar concentrations (11).

Methods—Fluorescence intensities of the bound sulfonamide were measured at two protein concentrations (4.0×10^{-6} and 1.0×10^{-4} *M*) in appropriate buffers. Two milliliters of each protein concentration was titrated with successive additions of 2 μl. of 1×10^{-3} *M* probe in methanol. Methanol did not affect the binding of the probe in the concentration range used. Binding of the parabens was determined by titrating a mixture of the drug and protein with the probe solution, and calculations were based on comparison to albumin–sulfonamide data.

Fluorescence titrations were performed manually with Hamilton microsyringes. All binding data were determined at an emission wavelength of 515 nm. Excitation and emission bandwidths were set at 100 Å. Temperatures in the fluorescence well of the fluorometer were controlled to 25 ± 0.1°, except in those experiments where temperature was a variable. The inner filter effect was minimized by keeping absorbance low at excitation wavelengths (less than 0.05 *A*). Irradiation periods for protein solutions were maintained for less than 5 min. to minimize photodecomposition of the tryptophan and histidine residues within the protein. Quantum yield determinations were made using the following equation given by Turner (12):

$$\phi_u = \phi_s \frac{a_u A_s d_s \lambda_s}{a_s A_u d_u \lambda_u} \qquad \text{(Eq. 1)}$$

where:

a = emission peak area corrected for solvent blank
A = absorbance at excitation wavelength
d = dilution factor in dilution of sample used for optical density measurements to concentration used in fluorescence measurements

[1] G. K. Turner and Associates, Palo Alto, Calif.
[2] Radiometer model 26, The London Co., Westlake, Ohio.
[3] By the method described by C. M. Himel *et al.* in data as yet unpublished from these laboratories.
[4] Fisher Scientific Co.
[5] Eastman Organic Chemicals.
[6] Nutritional Biochemicals Corp.

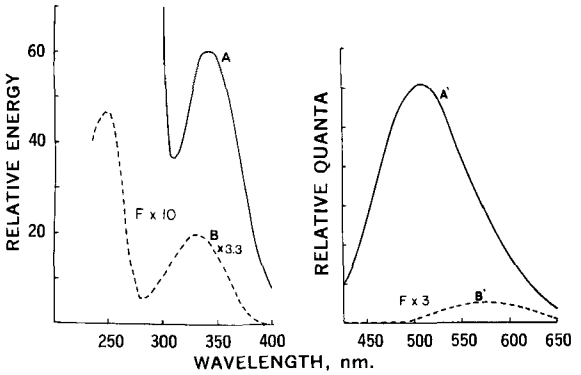

Figure 1—*Excitation and emission spectra of 1 × 10⁻⁶ M probe in the presence and absence of bovine serum albumin. Curves A and A' are the excitation and emission curves of probe in pH 7.2 tromethamine buffer solution of 8 × 10⁻⁵ M albumin. Curves B and B' are the excitation and emission curves of probe in the absence of albumin. Curve B is at a sensitivity about 3.3 times greater than B', A, and A'.*

λ = excitation wavelength
ϕ = quantum yield
s, u = subscripts denoting standard (s) and unknown (u)

Although quantum yield can be obtained by absolute methods (13), it is more convenient to use comparative methods in which compounds of known quantum yield are used as reference standards. Fluorescence measurements for quantum yield studies were made in 3-mm. quartz microcells[7] with special holders to limit inner filter effects. 5-Dimethylamino-1-naphthalenesulfonic acid was used as the reference standard, taking its quantum yield to be 0.36 at an excitation wavelength of 320 nm. (14). NMR data were obtained as described in a previous report (4). For the NMR studies, solutions were prepared by dissolving the parabens in 50% v/v polyethylene glycol 400 and water.

TREATMENT OF DATA

The fluorescence enhancement of the probe, upon addition to bovine serum albumin, was determined, and these data were used to calculate the binding constants for the albumin–probe complex. The fraction of bound probe was calculated from the following equation (5):

$$X = \frac{(I_0/I_f) - 1}{(I_b/I_f) - 1} \qquad \text{(Eq. 2)}$$

where I_0 and I_f refer to the fluorescence intensities of a given concentration of probe in solutions of low protein concentration and in solutions without protein, respectively. I_b refers to the fluorescence intensity of the same concentration of probe in solutions of high protein concentration; I_b then gives the fluorescence intensity of the probe in the presence of excess binding sites. After the value X is found for each point along the titration curve, the Scatchard equation (15) may be applied to determine the association constant of the probe:

$$\bar{V}/A = nK_a - \bar{V}K_a \qquad \text{(Eq. 3)}$$

where $\bar{V}$ is the number of moles of bound probe per mole of protein, A is the concentration of free probe, n is the number of binding sites on the protein, and K_a is the association constant of probe. The $\bar{V}$ is determined by multiplying the value for X by the ratio of the total probe concentration to the total protein concentration in solution. When $\bar{V}/A$ is plotted against $\bar{V}$, a straight line is obtained with a slope equal to $-K_a$. The ordinate and abscissa intercepts of this line give nK_a and n, respectively. The Scatchard equation assumes that the intrinsic association constant for all binding sites are equal.

When compounds such as the parabens were added to albumin solutions prior to titrations with probe, a decrease in fluorescence of the probe–albumin complex was observed due to competition for binding sites. When Scatchard plots were made from the binding data taken for the sulfonamide in the presence of the parabens, straight lines were obtained with decreased slopes but with identical abscissa intercepts, indicating competition between the probe and parabens for the same binding sites. The decrease in probe binding and the subsequent decrease in fluorescence of the probe–albumin complex can be used to calculate the binding constant of the parabens. Klotz *et al.* (16) derived equations describing simple competition between two ligand molecules for identical protein binding sites:

$$K_b = \frac{n(P_t)K_a(A) - K_a(A)(PA) - (PA)}{(B_t)K_a(A) - (P_t)K_a(A) + K_a(A)(PA) + (PA)} \times \frac{K_a(A)}{(PA)} \qquad \text{(Eq. 4)}$$

where:

K_b = association constant for competitor
K_a = association constant for probe
A = concentration of free probe
PA = concentration of bound probe
n = number of binding sites
P_t = total concentration of protein
B_t = total concentration of competitor

RESULTS AND DISCUSSION

Examination of the binding of parabens to bovine serum albumin was carried out with the use of fluorescent probe techniques. Since the fluorescent probe undergoes changes in fluorescence properties as a result of binding to the albumin, it was useful as an indication of interactions. Figure 1 shows fluorescence excitation and emission spectra of the probe in the presence and absence of bovine serum albumin. The fluorescence intensity of the probe (1×10^{-6} M) in aqueous solution was not significant (Curve B'); but when the albumin was added, the intensity was greatly enhanced, with a 60-nm. hypsochromic shift in wavelength of the emission maximum (Curve A'). The emission wavelength for Curve A was 515 nm., and the excitation wavelength for Curve A' was 340 nm. The emission wavelength for Curve B was 575 nm., and the excitation wavelength for B' was 335 nm. Excitation and emission bandwidths were fixed at 2.5 and 10.0 nm., respectively, for all curves. The areas under the emission curves are proportional to the quantum yields.

The quantum yield (0.057) of probe in aqueous solution increased approximately 12 times to 0.7 in the presence of albumin. These changes were due to alterations in the environment of the sulfonamide (17, 18). This is strong evidence of the binding between probe and protein. The high quantum yield of the probe may also be taken as an indication of a hydrophobic interaction, since quantum yield of similar molecules varies with the polarity of the environment[8]

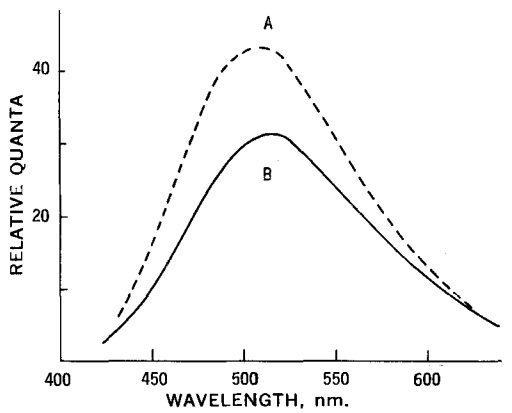

Figure 2—*Corrected emission spectra of the probe–albumin complex in the absence of a binding site competitor (- - -) and in the presence of 1 × 10⁻⁴ M methylparaben (——). Albumin concentration is 1.62 × 10⁻⁵ M in pH 7.2 tromethamine buffer. Excitation wavelength is at 335 nm.; emission and excitation bandwidths are set at 10.0 and 2.5 nm., respectively.*

[7] American Instrument Co.

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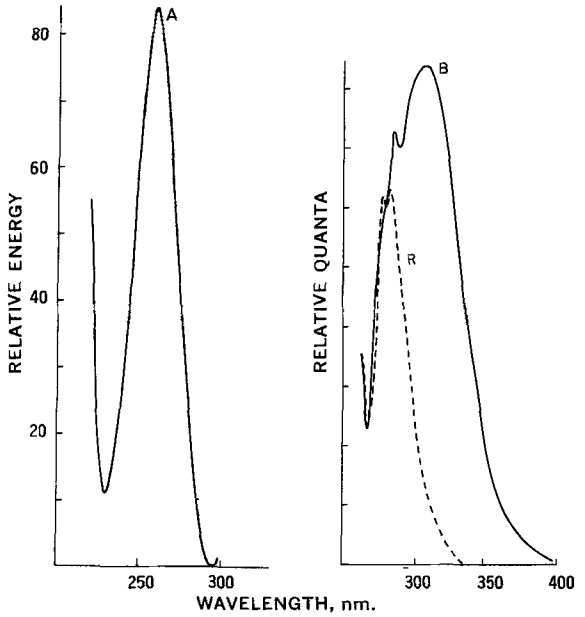

Figure 3—*Corrected excitation (A) and emission (B) spectra of a 6.6 × 10⁻⁶ M solution of* n*-butylparaben. Curve R is the Raman scatter peak for methanol at an excitation wavelength of 260 nm. Curve A excitation is at 260 nm. Curve B emission is at 305 nm. Excitation and emission bandwidths are fixed at 10.0 and 2.5 nm., respectively, at 25°.*

(9, 17). As an example, wavelength shifts and increases in quantum yield are seen when this probe is dissolved in less polar solvents such as ethanol and *n*-butyl alcohol; consequently, its emission properties can be equated to the polarity of the environment of the molecule. The large increase in quantum yield in the presence of albumin makes this probe useful to the study because interference from unbound probe material is low. In addition to these observations, the increase in binding constant for the probe from 1.618×10^5 M^{-1} at 15° to 1.810×10^5 M^{-1} at 25° to 2.115×10^5 M^{-1} at 35° and the increase in binding sites from one at 0.05 *M* KCl to two at 1.0 *M* KCl concentrations are further evidence that the nature of the complex is indeed hydrophobic. Structurally similar probes were reported to bind at hydrophobic binding sites of proteins (9).

Figure 2 shows the fluorescence emission spectra of the complex in the absence (Curve A) and in the presence (Curve B) of competitively binding methylparaben. A decrease in fluorescence of the complex was due to competition between the probe and methylparaben for the same or adjacent binding sites. The difference in fluorescence intensities with and without paraben is related to the binding of the drug and is used to calculate the binding constants for the paraben–protein complex (see *Treatment of Data*).

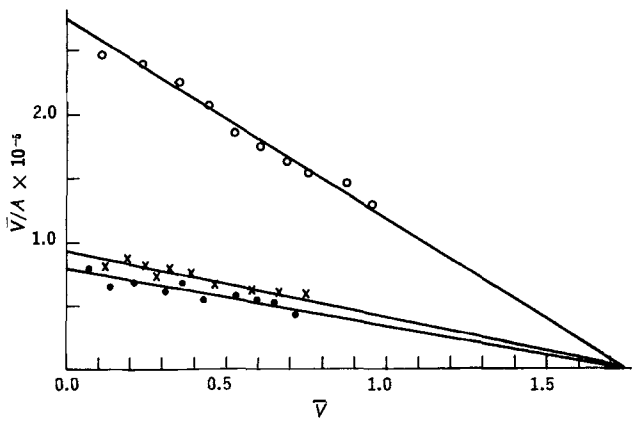

Figure 4—*Scatchard plots for probe–albumin complex (○) in the presence of methylparaben (●) and in the presence of ethylparaben (×) at 15°.*

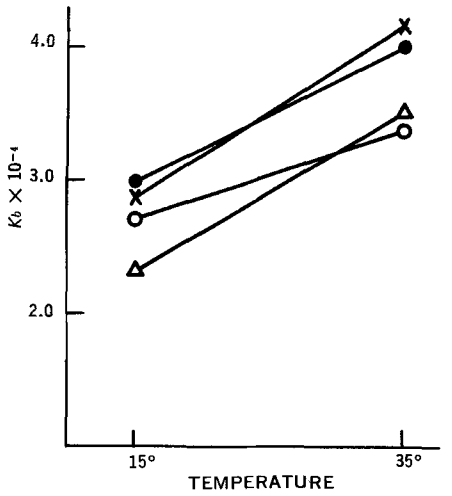

Figure 5—*Effect of temperature on the binding of parabens by bovine serum albumin. Key: ×, methylparaben; △, ethylparaben; ○, propylparaben; and ●,* n*-butylparaben.*

Figure 3 shows the excitation and emission spectra of *n*-butylparaben. The Raman scatter peak (*R*) for methanol at an excitation wavelength of 260 nm. is also shown. This figure is included since the fluorescence of paraben in methanol may prove to be useful for quantitative determination of parabens. Methyl-, ethyl-, and propylparabens also fluoresce in methanol, but their spectra are not shown because of the great similarity to that of *n*-butylparaben. The excitation and emission maxima are 260 and 300 nm. for methylparaben and 260 and 302.5 nm. for ethyl- and propylparabens, respectively.

Figure 4 consists of Scatchard plots utilized to calculate the binding constant of the probe and to show the decrease in the binding of this compound when in competition with methyl- and ethylparabens. Identical abscissa intercepts and decreased slopes indicated competition between the probe and methyl- and ethylparabens for the binding sites.

From the finding that the sulfonamide binds at the hydrophobic binding site of the albumin, and since the parabens were shown to compete with the sulfonamide for the binding sites, it is possible to rationalize that the binding of parabens to the albumin is also of a hydrophobic nature. Furthermore, a competition for the binding site between paraben and probe would not be observed if parabens did not bind at the same binding sites as did the probe. A hydrophobic interaction is considered to exist when two or more nonpolar groups come into contact with each other, thereby decreasing the extent of interaction with the surrounding water. Higuchi and Lach (19) indicated a possible role of hydrophobic interactions for binding of these types of drugs to macromolecules, and various other authors (20–22) discussed the contribution of hydrophobic interactions to drug–protein complex formation.

Corollary experiments were carried out to obtain additional information concerning the nature of binding in the paraben–albumin complex. Figure 5 shows the effect of temperature on paraben–albumin binding. The binding appeared to increase with an in-

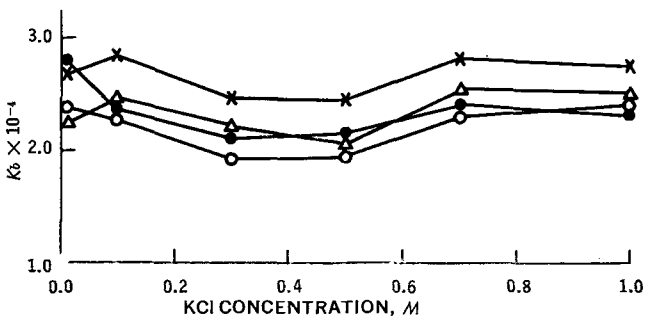

Figure 6—*Effect of ionic strength on the binding of parabens by bovine serum albumin at 25°. Key: ×, methylparaben; △, ethylparaben; ○, propylparaben; and ●,* n*-butylparaben.*

Table I—Thermodynamic Parameters for Bovine Serum Albumin–Paraben Binding at pH 7.2

	Temperature	Binding Constant, $M^{-1} \times 10^{-4}$	$\Delta H°$, kcal./mole	$\Delta G°$, kcal./mole	$\Delta S°$, e.u.	$T\Delta S°$, kcal./mole
Sulfonamide–albumin	15°	16.18	2.362	−0.275	9.16	2.637
	35°	21.15	2.362	−0.458	9.16	2.820
Methylparaben	15°	2.881	3.322	−0.605	13.6	3.917
	35°	4.199	3.322	−0.878	13.6	4.189
Ethylparaben	15°	2.330	3.639	−0.685	14.3	4.118
	35°	3.521	3.639	−0.771	14.3	4.404
Propylparaben	15°	2.745	2.182	−0.577	9.58	2.765
	35°	3.513	2.182	−0.769	9.58	2.957
n-Butylparaben	15°	2.978	2.679	−0.625	11.5	3.312
	35°	4.037	2.679	−0.854	11.5	3.542

Table II—Comparison of the Relaxation Rate[a] Ratios of Bound[b] over Free Drug[c] at Various pH Values at Constant Temperature

	Methylparaben		Ethylparaben		Propylparaben		*n*-Butylparaben	
pH	Phenyl Proton	Side-Chain[d] Methyl Proton	Phenyl Proton	Side-Chain Methyl Proton	Phenyl Proton	Side-Chain Methyl Proton	Phenyl Proton	Side-Chain Methyl Proton
4.5	1.75	—	1.94	1.22	1.87	1.18	1.93	1.31
7.6	2.38	—	2.36	1.68	2.30	1.52	2.41	1.56
8.8	2.06	—	2.22	1.77	2.18	1.40	2.28	1.88
9.5	2.02	—	2.13	1.85	2.04	1.32	1.96	1.74

[a] NMR spectra were obtained with Varian Associates HA-100 (see *References 4* and *5*). [b] With 1% bovine serum albumin. [c] In the absence of bovine serum albumin. [d] Was not able to measure due to overlap.

crease in temperature from 15 to 35° for all parabens. It is known that hydrophobic interactions tend to increase as temperature increases within the range studied (23). This endothermic nature of the binding suggests that hydrophobic interactions play a major role in the formation of the paraben–albumin complex.

Thermodynamic data obtained using the probe technique are shown in Table I. Since the enthalpy change is smaller than the term $T\Delta S$ for the binding of paraben to albumin, the binding may be due largely to the entropy effect, which is a typical thermodynamic observation for hydrophobic interactions (23, 24). In other words, the paraben–albumin binding was enhanced with a positive entropy term through the destruction of the "iceberg" surrounding paraben molecules. The term iceberg was introduced by Frank and Evans (25) to describe the icelike structure of water surrounding hydrophobic molecules.

Figure 6 shows the effect of ionic strength on paraben–albumin binding. Binding was not influenced by ionic strength resulting from concentrations of 0.05 to 1.0 *M* KCl. Since the energy of electrostatic interactions varies inversely with the dielectric constant for the solution (26), an electrostatic interaction between paraben and albumin would have been decreased by addition of salt. However, no influence of salt concentration on the binding was found, thus limiting the possibility of electrostatic interactions as a primary binding mechanism.

Hydrophobic binding between parabens and albumin can take place by virtue of interaction between the hydrocarbon groups on the side chain (27) of the paraben with hydrophobic sites of bovine suerm albumin. On the other hand, the aromatic ring system (28) of the parabens might also be involved in hydrophobic binding. Since NMR techniques for the elucidation of binding sites of drug molecules are well established (3, 4), this method was used to find the preferential binding sites of the small molecules. The NMR results, as shown in Table II, indicate that the aromatic portion of the parabens rather than the aliphatic side chain was primarily involved in the binding. It can be seen from this table that the NMR peaks of the phenyl protons of parabens were broadened to a greater extent as a result of the interaction with bovine serum albumin than were those for the side-chain protons under the identical experimental conditions.

The finding that the binding constants for methyl-, ethyl-, propyl-, and *n*-butylparabens are of the same order of magnitude (Table I) indicates that the contribution from the side chain of parabens may not be significant for the paraben–albumin interaction. This, however, is not in agreement with a previous study (29) using dialysis techniques, in which the binding constants of parabens were found to differ. The discrepancies in the binding constants determined in the two studies may be due to the fact that dialysis techniques measure more binding sites for the parabens, whereas the fluorescent probe techniques detect only those sites to which the probe molecules may bind. It was found that the probe binds to only one or two sites and, consequently, the competition could be observed only for these sites. In this case, the aromatic ring system of the paraben was involved in the binding, thus partially explaining the different binding constants obtained from the experimentation reported here as compared with dialysis studies. Continuing studies are in progress to clarify this point.

SUMMARY

Although recent studies (30, 31) indicated that probably more than one mechanism is responsible for a particular complex, this study showed, through several avenues, that hydrophobic interactions are primarily responsible for the paraben–albumin complex. These studies included: (*a*) the demonstration of competition between the parabens and *N*-*n*-butyl[(5-dimethylamino)-1-naphthalene]sulfonamide, which were found to bind at hydrophobic binding sites of the protein; (*b*) thermodynamic data indicating that the binding process between parabens and albumin was in concert with those described for hydrophobic interactions; (*c*) the paraben–albumin interaction which was not weakened by an increase in salt concentration; and (*d*) NMR data showing that the aromatic ring was primarily involved in binding.

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Microbiologic Determination of Drug Partitioning I: Gelatin–Acacia Complex Coacervate System

SADEGH JAVIDAN, RIAZ-UL HAQUE*, and ROBERT G. MRTEK†

Abstract □ An experimental method was developed for the determination of the extent of the partitioning of phenylmercuric nitrate in coacervate systems, as measured by the effect of the antimicrobial drug on the growth of *Escherichia coli*. Bacterial growth is utilized to investigate changes in the coacervate partition coefficient as a function of drug concentration. The method is sensitive for microgram quantities of this antimicrobial agent and is useful in drug partitioning at levels found in physiologic systems.

Keyphrases □ Drug partitioning—microbiological determination, gelatin–acacia coacervate system □ Phenylmercuric nitrate—microbiological determination of partitioning in coacervate systems □ Antimicrobial agents—determination of drug partitioning using coacervate systems □ Coacervates, complex—drug partitioning of antimicrobial agents □ Gelatin–acacia complex coacervate system—microbiological determination of drug partitioning

The term coacervation has been used to describe the salting out of certain types of lyophilic colloids into liquid droplets (1). Kruyt and Bundenberg de Jong (2) introduced the word in 1929 to characterize the flocculation or separation of liquids from solution when at least one separated phase contained a colloidal solute. The term has since been subdivided into simple coacervation and complex coacervation; complex coacervation is mainly dependent on the charges on the molecules, while simple coacervation results from phase separation due to the interaction of nonionized entities of macromolecules (1, 3). The mechanism for the coacervation process was studied by Kruyt (4), Bundenberg de Jong (5, 6), Voorn (7), Cohen and Vassiliades (8), Takruri (1), and others.

Coacervates generally contain a large amount of water; when these systems separate, the more dense coacervate settles to the bottom and contains about 99% of the colloidal solute. The upper layer is termed the "equilibrium liquid" or "dissolved coacervate layer" (2) and is in equilibrium with the coacervated phase. Water in the coacervate layer is structurally different from regular (liquid) water or from water in equilibrium with the coacervate phase. The state of aggregation of water molecules in coacervates was compared to the state of water in living systems. It was postulated (1) that the difference in aggregation states between water in coacervates and in the equilibrium layer is an important determinant in the distribution of electrolytes in these systems.

Takruri (1) studied the high uptake of organic molecules in complex coacervate systems and compared them to similar systems existing in nature. Several researchers (1, 9–12) suggested that these systems may serve as models for the study of absorption and distribution characteristics of drugs.

The purpose of this study was to utilize complex coacervates containing gelatin and acacia as a model system whereby drug partitioning of antimicrobial agents could be determined. Concentrations of phenylmercuric nitrate below the minimum inhibitory con-

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Table I—Components Used to Study the Partitioning of Phenylmercuric Nitrate in Gelatin–Acacia Complex Coacervates

Tube	A–G Stock Solution, ml.	0.1 N HNO_3, ml.	Milliliters Phenylmercuric Nitrate, 600 mcg./ml.	Sterile Distilled Water, ml.	Abbreviation of the System[a]	Coacervate, ml.
A	4	1.5	0	24.5	C_0	2
B	4	1.5	n[b]	q.s. 30 ml.	C_+	2
C	4	0	0	26	N_0	0
D	4	0	n[b]	q.s. 30 ml.	N_+	0

[a] C_0 = coacervate system, no drug; C_+ = coacervate system with drug; N_0 = noncoacervate system, no drug; and N_+ = noncoacervate system with drug. [b] 0.2, 0.4, 0.8, 1.6, 3.2, 4.8, 6.4, or 12.8 ml. of phenylmercuric nitrate. (Only one concentration of drug was used in each experiment.)

centration (MIC) were used to measure the inhibitory effect on the growth of *Escherichia coli* by means of the Coulter counter. Garrett and Miller (13) applied microbial kinetics in the prediction and quantification of antimicrobial agents with the use of the Coulter counter.

In this investigation, an antimicrobial drug was incorporated into coacervating and noncoacervating systems. Partitioning between equilibrium and coacervate phases was allowed to occur in the system. After separation of the phases, an aliquot of equilibrium liquid was withdrawn and incubated with organisms. The amount of antimicrobial agent in the equilibrium layer was presumed to be directly related to the amount of drug that had partitioned into the coacervate phase. By comparing the inhibitory effect of this equilibrium liquid with an aliquot of noncoacervate systems, an estimate of the amount of drug incorporated into the coacervate phase may be offered.

EXPERIMENTAL

Organism and Reagents—*E. coli* B/r (ATCC 23227) was used as the test organism throughout this investigation. This strain (UV and X-ray resistant) of *E. coli* is uniform in size, with the majority of a rapidly growing cell population distributed between 0.98 and 1.23 μ in diameter. Furthermore, this strain does not stick to the orifice of the Coulter counter aperture tube.

The lyophilized pellet was reconstituted, and organisms were transferred to an agar slant. The slant was incubated at 37° for 16 hr. to allow growth and then stored in the refrigerator at 5° for up to 2 weeks before subcultures were made.

Phenylmercuric nitrate[1] was the antimicrobial agent used in the partitioning studies. No. 1 granulated acacia USP[2] and Bacto gelatin[3] were used as colloidal solutes in the coacervate system. Nitric acid[4] and hydrochloric acid, analytical grade[5], were used to form coacervates. The 0.1 N solutions of these acids were filtered through a 0.22-μ millipore filter[6] and stored in brown bottles. Formaldehyde solution USP[7] was used to arrest the growth of the organisms.

Media—Thirty-seven grams of the dried Difco E.C. medium was rehydrated in 1 l. of cold, sterile, distilled water and stirred at room temperature until a clear solution was achieved. This solution was filtered through 2- and 0.22-μ millipore filters to remove all cellular and particulate debris. Filtered media were distributed in batches of 18 ml. among 125-ml. conical flasks and autoclaved at 15 lb. pressure (121°) for 15 min.

Preparation of Coacervate Systems—*Stock Solution*—A modification of the method used by Takruri (1) was adopted. The concentration of gelatin was changed from 5 to 4.325% w/v. The procedure was followed by dissolving 24 g. acacia in about 150 ml. hot distilled water in a 250-ml. beaker. In another 250-ml. beaker, 17.3 g. gelatin was dispersed in 150 ml. hot distilled water. Stirring was maintained throughout the process until complete dissolution was achieved. The hot solutions were strained separately through gauze and combined in a large beaker.

The total volume was adjusted to 400 ml. by the addition of warm distilled water. The final concentrations of gelatin and acacia in this solution were 4.325 and 6% w/v, respectively. This solution, labeled as A–G stock solution, was distributed in 35-ml. batches among several 60-ml. bottles. After autoclaving for 15 min., the bottles of A–G stock solution were stored at 5° until used.

Formation of Coacervate—Prior to the formation of the coacervate systems, samples of A–G stock solution were removed from the refrigerator and allowed to come to room temperature. Then, under a bacteriological hood equipped with UV light, 2 ml. of the stock solution was pipeted into each of several 15-ml. sterilized, graduated, centrifuge glass tubes; 0.8 ml. of 0.1 N HCl (or HNO_3) was added to the stock solution from a 1-ml. pipet, and the volume was adjusted to 15 ml. using sterile distilled water. The instantaneous appearance of cloudiness after adding the distilled water indicated the coacervation process. The final concentrations of acacia and gelatin in the coacervate system were 0.8 and 0.576% w/v, respectively. The tubes were capped and shaken vigorously by hand until complete mixing of the components was achieved. Then the tubes remained undisturbed in a cabinet at 37° for 24 hr. for complete separation of the layers in the coacervate system. In some experiments, when rapid separation of the coacervate layer from the equilibrium layer was desired, centrifugation at 1200×g in a centrifuge[8] for 10 min. was performed instead. At the end of the 24-hr. standing time (or after centrifugation), the volume of coacervate at the bottom of the graduated centrifuge tube was measured visually.

Noncoacervate systems were prepared by the same procedure used for forming coacervates except the acid was eliminated.

Effect of Heat of Sterilization on Coacervation—To determine optimum conditions for the preparation of large volume coacervate phases, the effect of heat of sterilization on the formation of coacervates was determined by autoclaving the various components at 121° for 15 min. The final volume of the coacervate layer was measured after 10 min. of centrifugation at 1200×g. For this purpose, several different coacervate systems were used. Gelatin and acacia were separately autoclaved, sterilized in combination, or not sterilized. Additionally, studies were made on systems that had been sterilized by autoclaving after the coacervation process was completed. For all of these experiments, 0.8 ml. 0.1 N HCl was used to adjust the pH for coacervation, and 2 ml. of A–G stock solution was used. The total volume of each system was 15 ml.

Effect of Different Acids on Coacervation—Two milliliters of A–G stock solution was placed in each of four 15-ml. centrifuge tubes. Then 0.8 ml. 0.1 N HCl, HNO_3, H_2SO_4, or CH_3COOH was added to each tube. The volume was adjusted to 15 ml. by the addition of distilled water. The tubes were capped and shaken vigorously by hand until all components were mixed. Then after centrifugation at 1200×g for 10 min., the volume of the coacervate layer was recorded and the pH of the equilibrium layer was measured with a pH meter[9]. The effect of different acids on coacervation was also determined in the presence of 6 ml. stock solution of phenylmercuric nitrate in each tube.

Effect of Drug Concentration on Coacervation—A stock solution of phenylmercuric nitrate (100 ml.) was prepared by dissolving

[1] Eastman grade, Eastman Organic Chemicals.
[2] Penick Co.
[3] Difco Laboratories, Detroit, Mich.
[4] Baker analyzed reagent.
[5] E. I. duPont de Nemours & Co.
[6] Millipore Corp.
[7] J. T. Baker Chemical Co.
[8] International, model HN.
[9] Sargent model DR.

Table II—Effect of Heat of Sterilization[a] on the Volume of Gelatin–Acacia Complex Coacervate

Coacervate System[b]	Volume of Coacervate, ml.
Gelatin and acacia separately sterilized	1.0
Sterilized A–G stock solution	1.0
Nonsterilized A–G stock solution	1.3
Coacervate formed from A–G stock solution by addition of acid and then sterilized	0

[a] 121° for 15 min. [b] Total volume of the system is 15 ml.

60 mg. of the drug in 100 ml. sterile distilled water at room temperature with the aid of a magnetic stirrer. The solution was filtered once through a 0.22-μ millipore filter and stored at room temperature in a brown bottle until used. This stock solution contained 600 mcg./ml. phenylmercuric nitrate and was prepared fresh every 3 weeks.

Into eight 45-ml. graduated centrifuge tubes were placed 0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 ml. of phenylmercuric nitrate stock solution, respectively. To each tube were added 4 ml. of A–G stock solution and 1.5 ml. 0.1 N HNO_3. Hydrochloric acid could not be used in these experiments, because it reacted with phenylmercuric nitrate and formed a white precipitate of phenylmercuric chloride which is less soluble in water. The volume of each tube was adjusted to 30 ml. by addition of sterile distilled water. After the tubes were capped and the caps were secured to the neck of the centrifuge tubes with rubber bands, they were shaken horizontally in a 5-gal. reciprocating water bath shaker[10] at 37° for 6 hr.

Then the capped tubes were stored undisturbed in a cabinet at 37° for 24 hr. The volume of the coacervate layer was recorded at the end of this period. The sequence of the addition of different components such as drug, acid, A–G stock solution, and water or the duration of the shaking period was altered to study any possible effect of those factors on the final volume of coacervate. The shaking period was tested for 0.5, 1, 2, 3, 4, 5, and 6 hr.

Partitioning of Phenylmercuric Nitrate in Gelatin–Acacia Complex Coacervate—In each experiment, four 45-ml. centrifuge tubes were identified as A, B, C, and D. Tubes A and B were used to form coacervating systems; in Tubes C and D, noncoacervating systems were prepared. The amounts of different components pipeted in each tube are shown in Table I. From the tests run in the previous section, the specific sequence of the addition was determined to be immaterial.

The four tubes were glass stoppered. The stopper was attached to the neck of the tube with rubber bands and placed horizontally in a water bath shaker for 6 hr. as previously described. During this period, the partitioning of drug was completed. Afterward the tubes were removed and placed undisturbed at 37° for 24 hr.

Growth Conditions—A separate slant of *E. coli* B/r was used for each experiment. Material was scraped off and inoculated into a 125-ml. flask containing 18 ml. E. C. medium. The culture was allowed to grow without agitation at 37° for 16 hr. From this culture, a loopful was inoculated into 18 ml. of fresh broth in a 125-ml. loosely capped flask. The new culture broth was allowed to grow for 6 hr. in the water bath shaker (37°). At the end of 6 hr., 0.1 ml. of the growing culture was removed and the total number of organisms was determined using a Coulter counter[11]. This procedure required approximately 7–10 min. The growing culture was appropriately diluted with fresh E. C. medium to contain 10^3 organisms/ml. of the culture.

One milliliter of this diluted inoculum was added to each of 12 replicate flasks containing 18 ml. of fresh broth. The cultures were placed in the water bath shaker (37°) for 90 min. before incorporating the drug samples. At the end of this period, 1 ml. of noncoacervate system (Tubes C and D, Table I) or 1 ml. of the upper equilibrium phase of the coacervate system (Tubes A and B, Table I) was added to each of the three replicate cultures. The flasks containing the drugs were permitted to grow an additional 4.5 hr. on the shaker.

[10] Eberbach Corp., Ann Arbor, Mich.
[11] Model B, Coulter Electronic Co., Hialeah, Fla.

Table III—Effect of Different Acids on the Volume of Gelatin–Acacia Complex Coacervate in the Presence or Absence of Phenylmercuric Nitrate

0.8 ml. 0.1 N Acid	In Presence of Phenylmercuric Nitrate		In Absence of Phenylmercuric Nitrate	
	pH	Coacervate, ml.	pH	Coacervate, ml.
HCl	—	—[a]	3.67	1.0
HNO_3	3.65	1.0	3.70	1.0
H_2SO_4	3.80	0.9	3.81	0.8
CH_3COOH	4.20	0.5	4.25	0.4

[a] Insoluble phenylmercuric chloride precipitated.

Viable Count—At the end of 4.5 hr. of incubation, 0.5 ml. of each culture was withdrawn into sterilized flasks and appropriately diluted with fresh medium so that 60–100 colonies/plate would result. One milliliter of this diluted solution was pipeted onto each of three replicate agar plates. The plates were incubated at 37° for 48 hr. before counts were made. The colonies were counted with a colony counter[12].

Total Count Method—After withdrawing samples for viable counts, one drop of formaldehyde solution was added to each of the 12 flasks to kill the organisms. Then the content of each flask was diluted to 100 ml. with filtered, sterile, normal saline. One-tenth milliliter was diluted to 100 ml. (1000×) using Isoton[13] so that the total organism count would not exceed the coincidence limit at the following setting on the Coulter counter (model B): $A = I = 1/2$, gain = 88, $T_L = 5.2$, $T_U = \infty$, $k = 0.185$, $t = 5$ sec., and tube orifice = 30 μ. This setting registers as counts all organisms above 0.78 μ in diameter without significant interference from the background noise. The mean of four total counts was obtained for each sample.

To relate the total number of organisms in each flask to the number of organisms inoculated (here 10^3), two parameters are defined: "reproductive coefficient" (R.C.) and "reproductive index" (R.I.):

$$\text{R.C.} = \frac{\text{total number of organisms in each flask}}{\text{total number of organisms inoculated}} \quad \text{(Eq. 1)}$$

$$\text{R.I.} = \frac{\text{R.C. in presence of drug}}{\text{R.C. in absence of drug}} \times 100 \quad \text{(Eq. 2)}$$

Coincidence of Total and Viable Counts of Organisms—Into nine 15-ml. centrifuge tubes were placed 0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6, and 6.4 ml. phenylmercuric nitrate stock solution (600 mcg./ml.), respectively. Then, 2 ml. A–G stock solution was added to each tube and the volume was adjusted to 15 ml. by addition of sterile distilled water. The tubes were capped, shaken by hand, and then stored at 37° for 24 hr. Since no acid was added to these tubes, no coacervation occurred.

After 24 hr., 1 ml. from each tube was pipeted into each of three replicate 19-ml. cultures, growing in 125-ml. flasks inoculated 90 min. prior to the incorporation of drug. After 4.5 hr., viable and total counts were made as described previously.

RESULTS AND DISCUSSION

The effect of the heat of sterilization on the volume of coacervate layer is shown in Table II. The conditions of sterilization reduced the volume of coacervation when compared to the nonsterile system. The presence of acid in the system totally destroyed the coacervate. This was probably due to the hydrolysis of the colloidal solutes composing the coacervate layer in the presence of acid.

The effect of different acids on the volume of coacervate is shown in Table III. This table indicates that the effect of the drug on the pH and, consequently, on the volume of coacervate is negligible. Hydrochloric acid could not be used in the presence of phenylmercuric nitrate because these two components reacted and formed the highly insoluble phenylmercuric chloride which precipitated at the bottom of the tubes.

[12] Cenco, Central Scientific Co.
[13] Coulter Electronic Co., Hialeah, Fla.

Table IV—Coacervation Volume in Gelatin–Acacia System Due to Fermentation of Glucose by *E. coli*

Glucose in System, %	Volume of Coacervate Formed, ml.—Days 1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
1	0	0	0.1	0.1	0.12	0.14	0.15	0.2	0.3	0.3
2	0.1	0.2	0.2	0.4	0.5	0.7	0.75	0.8	0.82	0.85
3	0.5	0.8	1.0	1.1	1.3	1.5	1.6	1.75	1.9	1.9
4	1.0	1.2	1.5	1.75	1.85	2.0	2.1	2.2	2.5	2.5
5	1.1	1.5	1.6	1.8	2.0	2.2	2.4	2.4	2.4	2.4

The importance of pH in biological systems using coacervates can be illustrated by the continuously changing volume of the coacervate phase when *E. coli* is incubated in the system. Over a 10-day period, the acidic metabolites produced from glucose fermentation by *E. coli* sufficiently reduced the pH of the system to produce a measurable increase in the coacervation phase (Table IV).

When different amounts of phenylmercuric nitrate were added to coacervating systems, no difference in the volume of coacervate occurred (Table I). The ratio of the volume of coacervate to the volume of A–G stock solution was consistently 1:2. In forming coacervates, the sequence of the addition of different components, as well as the length of the shaking period (for partitioning of the drug in coacervate), had no effect on the volume of the coacervate layer. These results suggest that the presence of partitioning of phenylmercuric nitrate in the experimental system had no measurable effect on the volume of the coacervate phase.

Since pH is a critical factor in the determination of the total volume of the coacervate phase, the absence of a volume effect attributable to the addition of phenylmercuric nitrate provides a rapid means of visually determining that the drug did not alter the pH of the system.

The partitioning studies of phenylmercuric nitrate in coacervate systems are based on the assumption that if phenylmercuric nitrate partitions uniformly, then the concentration of drug in the equilibrium layer of a coacervate system must be the same as in the noncoacervate system. Consequently, the same aliquots of each system should inhibit the growth of *E. coli* to the same extent. From Fig. 1 and Table V, it is evident that the distribution of phenylmercuric nitrate in the coacervate system is not uniform. This is apparent

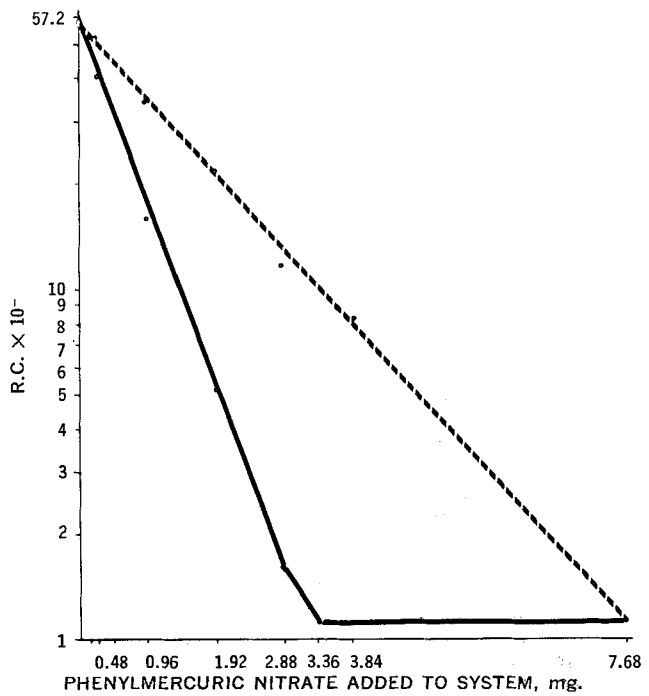

Figure 1—*Effect of concentration of phenylmercuric nitrate on R.C. of* E. coli *B/r. Key: - - -, coacervate system; and ——, noncoacervate system.*

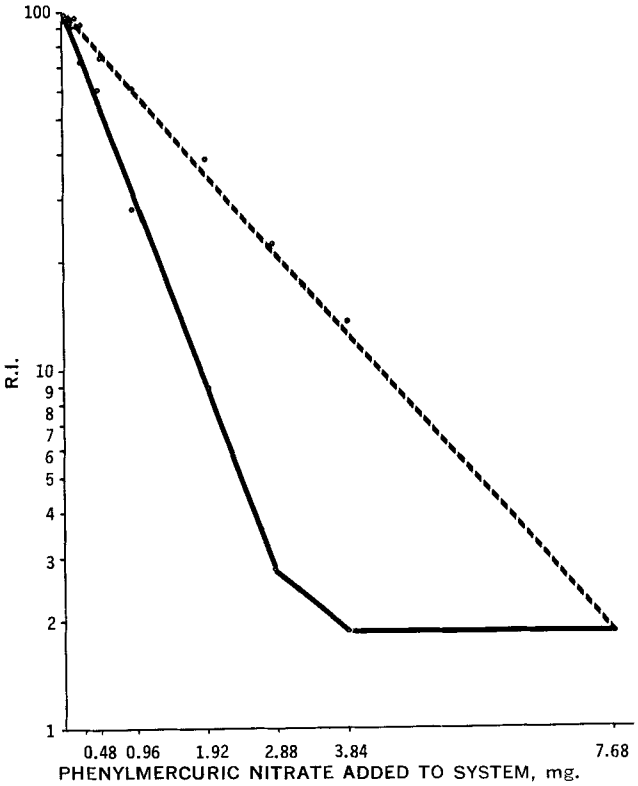

Figure 2—*Effect of concentration of phenylmercuric nitrate on R.I. of* E. coli *B/r. Key: - - -, coacervate system; and ——, noncoacervate system.*

when Columns 4 and 5 in Table V are compared. The difference in the slopes of the lines in Fig. 1 is related to the partitioning of phenylmercuric nitrate in the coacervate layer and the equilibrium liquid.

Aliquots taken from tubes N_0 or C_0 (see Table I for abbreviations) resulted in maximum microbial growth (R.I. = 100), as shown in Fig. 2. The gradual increase of drug decreased R.C. and R.I. until these parameters remained constant with increasing amounts of drug, when complete bacterial growth inhibition occurred. In the absence of drug, R.C. for the noncoacervate system was slightly larger than the corresponding value for its equilibrium layer in the coacervate system (Table V). This difference is attributed to the presence of larger amounts of acacia in the noncoacervate aliquot than in the aliquot of equilibrium layer. When R.C.'s were converted into an R.I., this difference was eliminated since the cell growth at each concentration of drug was divided by the cellular growth at zero drug concentration in the same system (Fig. 2). The calculation of R.I. uses N_0 and C_0 as growth standards in the presence of drug. In Table V, constant R.C. values of under 16 at high phenylmercuric nitrate concentration may be interpreted as the R.C. for the 90 min.

Table V—Microbial Growth of *E. coli* B/r in the Presence of Phenylmercuric Nitrate in Gelatin–Acacia Complex Coacervate and Noncoacervate Systems

Milligrams Drug Added to 30-ml. Systems (1)	R.C. Noncoacervate System (2)	R.C. Coacervate System (3)	R.I. Noncoacervate System (4)	R.I. Coacervate System (5)
0	572	558	100	100
0.120	520	531	90.91	95.16
0.24	410	519	71.68	93.01
0.48	342	416	59.79	74.55
0.96	161	340	28.15	60.93
1.92	52	215	9.09	38.53
2.88	16	125	2.79	22.40
3.84	11	80	1.92	14.33
7.68	11	11	1.92	1.97

Table VI—Comparison of Total and Viable Counts of *E. coli* Growth in Cultures Treated with Phenylmercuric Nitrate

Phenylmercuric Nitrate Added to System, mg. (1)	Phenylmercuric Nitrate Concentration, mcg./ml. In Noncoacervate System (2)	Phenylmercuric Nitrate Concentration, mcg./ml. In Culture Media (3)	R.C. Total Count (4)	R.C. Colony Count (5)	R.I. Total Count (6)	R.I. Colony Count (7)
0	0	0	580	569	100	100
0.48	32	1.6	342	338	58.96	59.40
0.96	64	3.2	161	156	27.76	27.41
1.44	96	4.8	100	93	17.24	16.34
1.92	128	6.4	52	49	8.96	8.61
2.40	160	8.0	28	25	4.83	4.39
2.88	192	9.6	16	6	2.76	1.05
3.36	224	11.2	11	0	1.90	0

of growth prior to the addition of large amounts of phenylmercuric nitrate.

Coincidence in total (Coulter count) and viable (colony count) numbers of *E. coli*/ml. in drug-free and phenylmercuric nitrate-treated cultures is evident for all concentrations of drug up to 10 mcg./ml. (Table VI). Viable counts at the end of 90 min. confirmed these figures. As can be seen from Table VI, Column 7, at concentrations in excess of 160 mcg./ml. phenylmercuric nitrate, the requirements for the calculated parameters to be valid were not met. The R.I. is totally based on live, albeit partially inhibited, cells. At concentrations above 160 mcg./ml. phenylmercuric nitrate, there were no viable cells as determined by colony counts of virtually zero.

Additional studies are now in progress to quantify the partitioning of various drugs and to determine the effect of total drug concentration in the system on the apparent partition coefficient by applying this method.

SUMMARY AND CONCLUSIONS

1. Gelatin–acacia complex coacervate was utilized as a model to study the partitioning of phenylmercuric nitrate.

2. It was shown that phenylmercuric nitrate did not affect the coacervation process; therefore, during its partitioning, the volume of the coacervate layer was constant.

3. To apply microbial growth to the investigation of drug distribution in the coacervate system, two parameters were defined:

$$\text{reproductive coefficient (R.C.)} = \frac{\text{total number of organisms in each flask}}{\text{total number of organisms inoculated}}$$

$$\text{reproductive index (R.I.)} = \frac{\text{R.C. in presence of drug}}{\text{R.C. in absence of drug}} \times 100$$

4. R.C. and R.I. provide an estimation of the partitioning characteristic of the drug in a coacervate system.

5. This method is valid at R.I. values higher than 25 when applied for phenylmercuric nitrate.

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Influence of HLB Values of Surfactants on Ephedrine Release from Emulsified Liquid Systems

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Abstract □ This study treated primarily the release characteristics of ephedrine from liquid emulsion systems as they were affected by the hydrophilic–lipophilic nature of the emulsifier present. Polysorbate 60 and sorbitan monostearate were varied by weight percent to produce emulsifiers having different hydrophilic–lipophilic balance (HLB) values. The experiments utilized a dialysis technique and consisted of seven controls plus the study of the emulsified systems throughout an HLB range of 4.7–14.9. The data indicated that the HLB value of the surfactants affected the release rate of ephedrine from an emulsified system, with the largest release rates occurring in the upper HLB range. The release of ephedrine from the oil phase was shown to be the rate-limiting step in its release from the emulsion. Surfactants did not impair the release of ephedrine from an oil system (control), whereas an inhibitory effect was noted in the water system (control). The data also indicated than an interfacial barrier was present in the emulsion and that ephedrine was released at two different rates from each of the various systems; the initial rate was greater than the second.

Keyphrases □ HLB values, surfactants—influence on ephedrine release characteristics from emulsified liquid systems, dialysis technique □ Surfactants, HLB values—effect on ephedrine release rates, oil and water emulsified systems □ Ephedrine release rates—effect of surfactant HLB values, oil and water emulsified systems, dialysis technique □ Hydrophilic–lipophilic balance—effect of polysorbate 60 and sorbitan monostearate values on ephedrine release rates in emulsified systems

The fact that emulsions are used as vehicles for medicinal agents is well established (1), and many investigators have studied the release of various drugs from liquid and semisolid systems (2–6). During most of these studies, the composition of the emulsion was considered to be an important factor affecting drug release. One important component known to influence drug release is the surfactant, and the hydrophilic–lipophilic balance (HLB) is considered to be one of the most important physical properties affecting release of drugs from vehicles (7–9). The results of most studies involving HLB and its effects have been elusive and inconclusive. Micellar solutions (10) and interfacial barriers (11) have been considered also as important factors influencing drug release.

The objective of this research was to determine the influence of the HLB value of the surfactant on the release rate of ephedrine from emulsified, liquid systems.

EXPERIMENTAL

The emulsifiers were used as received from the manufacturer since they were felt to be representative of those used in industry or in a pharmacy. An HLB range of about 5–15 was chosen, and the emulsifiers were picked accordingly. Polysorbate 60[1] with an HLB of 14.9 and sorbitan monostearate[2] with an HLB of 4.7 were considered satisfactory, since the only difference between the two molecules was the weight percent of polyoxyethylene groupings. This procedure allowed evaluation of emulsions and their parameters at HLB values of 4.7, 6.0, 7.0, . . . , 14.0, and 14.9. The drug, ephedrine[3], was selected since it was known to be soluble in both oil and water and could be assayed easily using spectrophotometric methods.

To ensure uniformity, all experimentation was performed on a weight basis with the drug supplying 1%, the surfactants 5%, and the paraffin oil[4] and water each 47%.

Phase I: Preliminary Procedures—*Preparation of Drug and Surfactants*—The moisture content of each surfactant was determined by using moisture balance[5]. The hydrous ephedrine was dried in a desiccator over concentrated sulfuric acid for a minimum of 72 hr. to remove the water of hydration.

Determination of HLB Values—The concentration of the surfactant required to obtain the desired HLB value was calculated by simple allegation, and then the correction factor was applied for the moisture contained in the surfactants. The average moisture was 3 and 2.5% for the polysorbate 60 and the sorbitan monostearate, respectively.

Assay Procedures—Aqueous solutions of ephedrine were assayed using a spectrophotometer[6] at 257 nm.

Dialysis Procedure—Two dialysis cells[7] (flowthrough type), each with a capacity of 10 ml., were used.

Standard dialysis membranes[8], having an average pore diameter of 4.8 nm., were soaked in distilled water at least 24 hr. for hydration purposes prior to use for dialysis. A thin coating of stopcock grease was applied to the inner walls of the cells before fitting the membrane to ensure there was no leakage from the compartments.

In the initial preparation, after the membrane was placed in position, the cells were weighed and exactly 10 g. of solution was introduced into each cell, thereby eliminating such factors as entrapment of air and viscosity. The weight of the sink was 100 g.; since the sink was water, the volume approximated 100 ml.

Two cells were arranged in series and immersed in a constant-temperature bath maintained at 37.5° by a power unit[9] and heater circulator. Connected to the inlet side of the dialysis cells by latex rubber tubing was a pump[10], which was used to maintain a constant flow rate of 60 ml./min. through the dialysis cells. This flow rate was maintained throughout all steps of the experimentation.

A rubber tube from the outlet side of the dialysis cells emptied into a 500-ml. round-bottom flask, which was used as the reservoir sink. This was also immersed in the constant-temperature bath and was used for the purpose of collecting samples. The samples were collected at definite time intervals, their UV absorbance was determined, and they were immediately returned to the sink, thereby negating the use of correction and/or dilution factors due to the loss of drug or volume of liquid. Connections were made to the inlet side of the pump from the reservoir, thereby completing a full circle and allowing the flow of liquid.

Phase II: Control Study—*Surfactant Dialysis from Water to Aqueous Sink*—To determine whether the surfactants would permeate the membrane, a separate surfactant–water solution or dispersion was prepared with each surfactant by introducing 5 g. of the surfactant into 95 g. of water, heating to 65° with stirring, and then cooling to 40°. Each surfactant–water solution or dispersion was dialyzed twice according to the previously described procedure.

[1] Tween 60, Atlas Chemical Corp., Wilmington, Del.
[2] Arlacel 60, Atlas Chemical Corp., Wilmington, Del.
[3] Ephedrine NF (hydrous), Merck and Co., Inc., Rahway, N. J.
[4] Paraffin oil USP, Fisher Scientific Co., Fair Lawn, N. J.
[5] Ohaus Scale Corp., Union, N. J.
[6] Model DB-G, Beckman Instruments, Inc., Fullerton, Calif.
[7] The Chemical Rubber Co., Cleveland, Ohio.
[8] Oxford Laboratories, San Mateo, Calif.
[9] Thermonitor, E. H. Sargent & Co., Cleveland, Ohio.
[10] Zero-Max Kinetic Clamp, Sigmamotor, Inc., Middleport, N. Y.

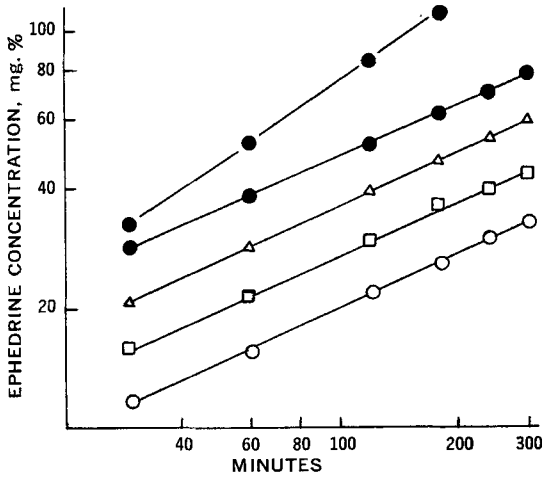

Figure 1—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 4.7. Each point on the figure represents the mean of two separate dialyses. Key: ●, ephedrine + water; △, ephedrine + water + surfactant; ●, ephedrine + oil; □, ephedrine + oil + surfactant; and ○, ephedrine emulsion.*

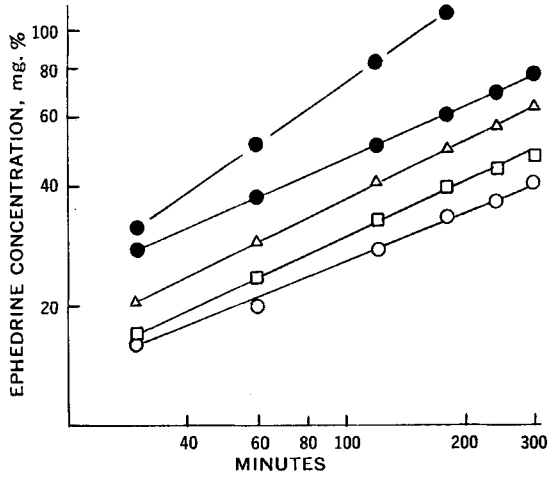

Figure 3—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 7.0. Each point on the figure represents the mean of two separate dialyses. Key: ●, ephedrine + water; △, ephedrine + water + surfactant; ●, ephedrine + oil; □, ephedrine + oil + surfactant; and ○, ephedrine emulsion.*

The surface tension of the sink was checked at the beginning of the run and at intervals throughout a total dialysis time of 13–17 hr. Surface-tension measurements were performed using a tensiometer[11]. Since the surface tension of the sink did not change significantly during the extended dialyses, it was concluded that sorbitan ester[12] and polysorbate 20 did not dialyze.

Phase III: Control Study—*Dialysis of Ephedrine from Water to Aqueous Sink*—One gram of ephedrine was dissolved in 99 g. of water, heated to 65° with stirring, and then cooled to 40°. Dialysis, sampling, and assay were performed according to previously described procedures.

Phase IV: Control Study—*Dialysis of Ephedrine from Oil to Aqueous Sink*—One gram of ephedrine was dissolved in 99 g. of oil, heated to 65° with stirring, and then cooled to 40°. The dialysis procedure was performed and analyzed as in Phase III.

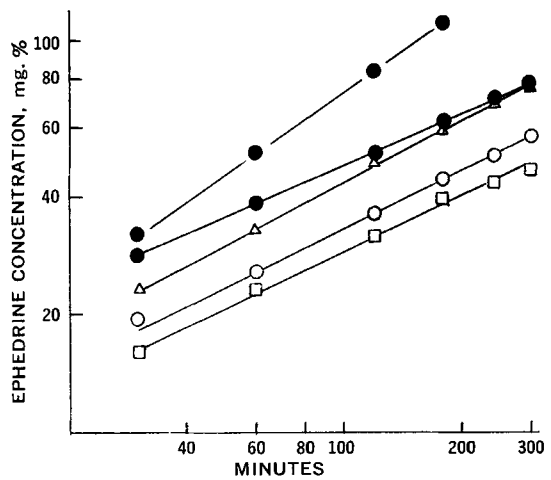

Figure 2—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 6.0. Each point on the figure represents the mean of two separate dialyses. Key: ●, ephedrine + water; △, ephedrine + water + surfactant; ●, ephedrine + oil; □, ephedrine + oil + surfactant; and ○, ephedrine emulsion.*

[11] Cenco DuNouy, Central Scientific Co., Chicago, Ill.
[12] Span 20.

Phase V: Control Study—*Dialysis of Ephedrine from Surfactant–Water Systems at Each HLB*—For each HLB value, each surfactant was weighed in the proper ratio to effect the desired HLB value. The water and drug were weighed and added in sufficient amounts to bring the total weight of the mixture to 100 g. (5 g. surfactant–1 g. drug–94 g. water). This mixture was heated to 65° with stirring on a hot plate-magnetic stirrer and cooled to 40°. The sorbitan monostearate was not soluble but dispersed readily with stirring. Dialysis, sampling, and assay were performed as previously described.

Phase VI: Control Study—*Dialysis of Ephedrine from Surfactant–Oil Systems at Each HLB*—For each HLB value, the surfactant, drug, and oil were combined in a weight ratio of 5:1:94 in an analogous manner to Phase V. The mixture was heated with stirring to 60° and cooled to 40°. Stirring was necessary to disperse the insoluble polysorbate 60. Dialysis, sampling, and assay were performed as previously described.

Phase VII: Control Study—*Distribution Coefficients at Each HLB Value*—The distribution apparatus was assembled using a 150-ml. beaker with a 7.62-cm. (3-in.) piece of glass tubing (8 mm. in diameter) glued to the inside wall. This tubing extended to within 1.27 cm. (0.5 in.) of the bottom. The apparatus permitted sampling

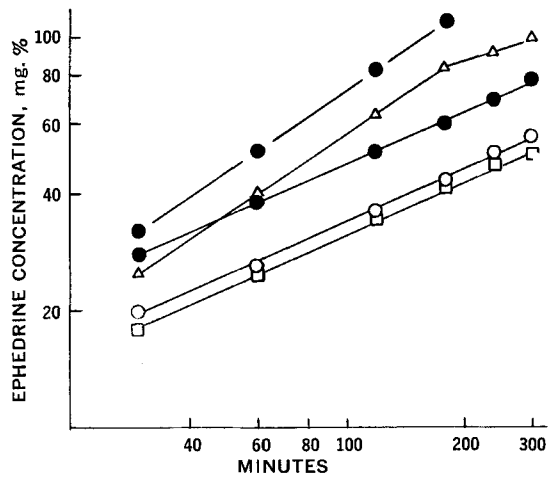

Figure 4—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 8.0. Each point on the figure represents the mean of two separate dialyses. Key: ●, ephedrine + water; △, ephedrine + water + surfactant; ●, ephedrine + oil; □, ephedrine + oil + surfactant; and ○, ephedrine emulsion.*

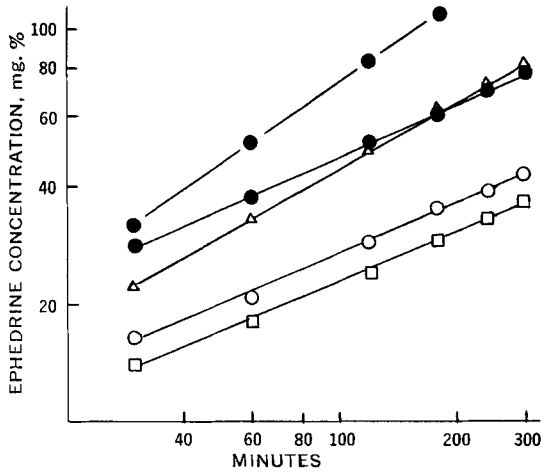

Figure 5—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 9.0. Each point on the figure represents the mean of two separate dialyses. Key:* ●, *ephedrine + water;* △, *ephedrine + water + surfactant;* ●, *ephedrine + oil;* □, *ephedrine + oil + surfactant; and* ○, *ephedrine emulsion.*

of the aqueous phase (lower phase) without disturbance to the liquid interface and allowed the interfacial area to remain constant in all studies.

The water and oil phases for the determination of the distribution coefficients were prepared exactly as those for emulsions. The water phase (water–surfactant–drug) was poured into the distribution apparatus; then the oil phase (oil–surfactant) was carefully placed on the surface of the water phase. The beaker was gently placed in a constant-temperature bath without disturbing the interface between the two liquids. At definite intervals, samples were drawn from the water phase and analyzed for the drug content. The sample was collected about 6 mm. (0.25 in.) from the bottom of the beaker in every case, because this was felt to give a more representative sample than from the tube.

To determine the amount of ephedrine in the oil phase, a 1-g. sample of the oil was drawn, diluted with 20 ml. of 0.1 *N* HCl, and shaken gently for 30 min. On acidifying the oil, the ephedrine should become completely water soluble due to the formation of ephedrine hydrochloride. After shaking, the sample was allowed to stand for 10 min. so the oil would separate. A small amount of emulsification occurred during this phase. The sample to be analyzed was drawn from the lower portion of the liquid (acid solution) and

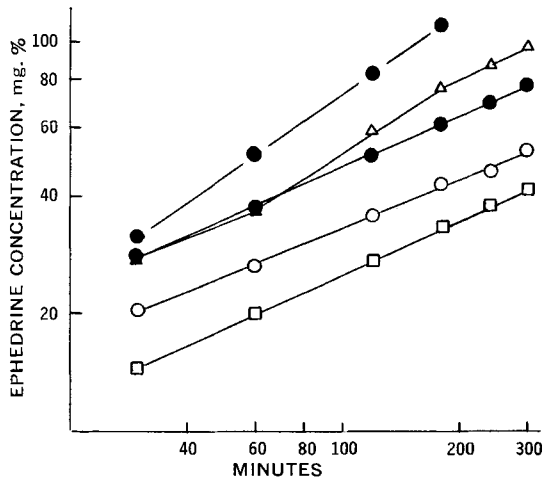

Figure 6—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 10.0. Each point on the figure represents the mean of two separate dialyses. Key:* ●, *ephedrine + water;* △, *ephedrine + water + surfactant;* ●, *ephedrine + oil;* □, *ephedrine + oil + surfactant; and* ○, *ephedrine emulsion.*

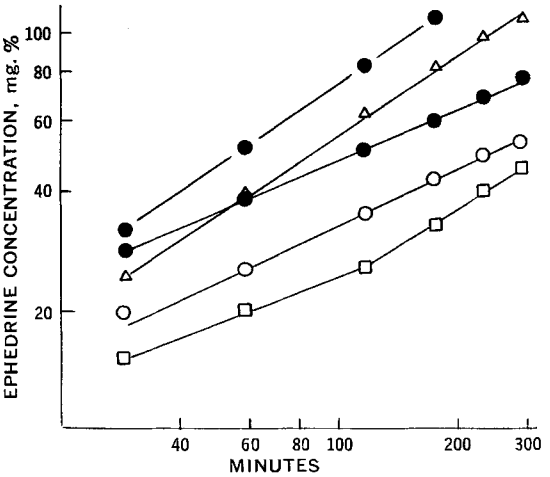

Figure 7—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid sytems in which the emulsifier had an HLB value of 11.0. Each point on the figure represents the mean of two separate dialyses. Key:* ●, *ephedrine + water;* △, *ephedrine + water + surfactant;* ●, *ephedrine + oil;* □, *ephedrine + oil + surfactant; and* ○, *ephedrine emulsion.*

passed through a filter system[13] utilizing a filter of 0.8-μ pore diameter. The sample was then analyzed spectrophotometrically at 257 nm. For this assay procedure, a new standard curve was prepared using standards of ephedrine in 0.1 *N* HCl.

An attempt was made to sample the interface between the oil and water using a syringe. The needle of the syringe was inserted through the oil phase (top layer), and samples were drawn at the interface of the two liquids. Much spontaneous emulsification had occurred at the time of sampling, and it was impossible to determine the exact position of the interface. The layer of spontaneous emulsification varied from about 1 to 8 mm., becoming increasingly greater as the HLB value was lowered. Analysis procedures were the same as reported for the oil samples.

Phase VIII: Emulsion Study—The surfactants, previously calculated for the desired HLB value, were weighed in separate 100-ml. beakers. One gram of ephedrine, dissolved in 47 g. of water, was added to the water-soluble surfactant and heated with stirring to 65°. Forty-seven grams of oil was added to the beaker with the oil-soluble surfactant and heated with stirring to 60°. The water phase was poured into a previously warmed glass container of a blender.

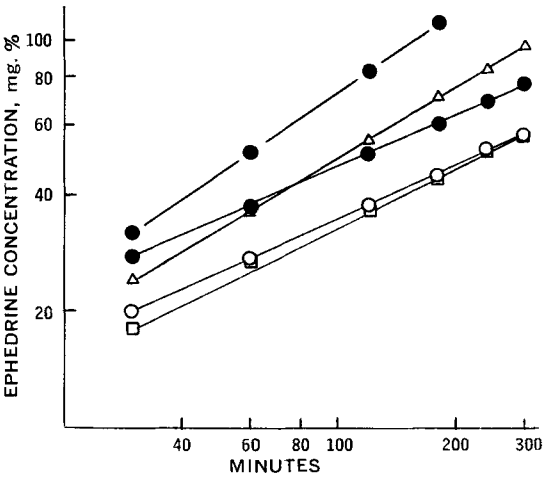

Figure 8—*Results of dilayses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 12.0. Each point on the figure represents the mean of two separate dialyses. Key:* ●, *ephedrine + water;* △, *ephedrine + water + surfactant;* ●, *ephedrine + oil;* □, *ephedrine + oil + surfactant; and* ○, *ephedrine emulsion.*

[13] Millipore Corp., Bedford, Mass.

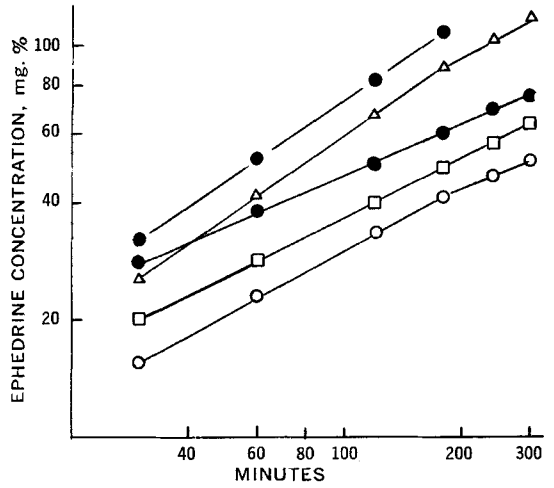

Figure 9—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 13.0. Each point on the figure represents the mean of two separate dialyses. Key:* ●, *ephedrine + water;* △, *ephedrine + water + surfactant;* ●, *ephedrine + oil;* □, *ephedrine + oil + surfactant; and* ○, *ephedrine emulsion.*

The blender was adjusted to the lowest speed, and the oil phase was rapidly poured into the water phase. An emulsion immediately formed, and the blender ran exactly 30 sec. The contents were transferred to a 250-ml. beaker and allowed to equilibrate for 1 hr. At the end of 1 hr., the contents were gently swirled by hand and transferred to dialysis cells. Dialysis procedures were initiated according to standardized procedures previously described.

The emulsion was tested as to type, o/w or w/o, by dilution techniques; all emulsions were found to be o/w.

RESULTS AND DISCUSSION

The results of Phase I, preliminary preparations, and Phase II, surfactant dialyses, were discussed previously in the *Experimental* section. It is well to reiterate that in Phase II the surfactants did not dialyze.

The results of the dialyses in Phases III–VI and VIII are reported in Figs. 1–11, and the data fit a log–log-type function with respect to time and concentration. In these plots, the control curves representing the dialyses of ephedrine from water and ephedrine from oil were repeated each time for ease of comparison. Thus, the

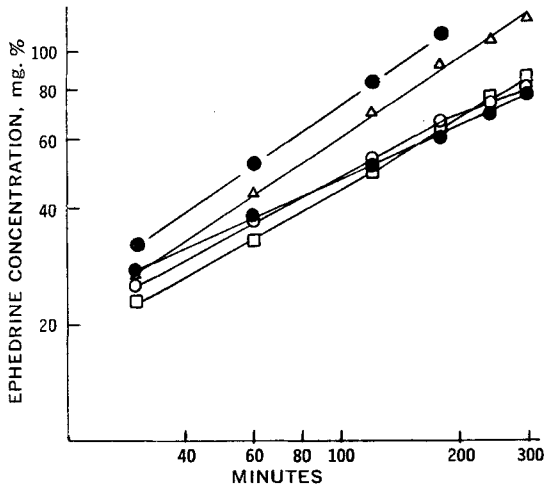

Figure 10—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 14.0. Each point on the figure represents the mean of two separate dialyses. Key:* ●, *ephedrine + water;* △, *ephedrine + water + surfactant;* ●, *ephedrine + oil;* □, *ephedrine + oil + surfactant; and* ○, *ephedrine emulsion.*

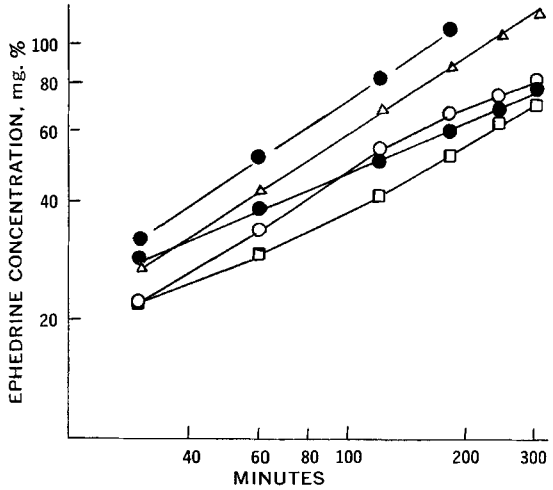

Figure 11—*Results of dialyses of ephedrine from mineral oil in water emulsified, liquid systems in which the emulsifier had an HLB value of 14.9. Each point on the figure represents the mean of two separate dialyses. Key:* ●, *ephedrine + water;* △, *ephedrine + water + surfactant;* ●, *ephedrine + oil;* □, *ephedrine + oil + surfactant; and* ○, *ephedrine emulsion.*

equations for the the curves had the same general formula:

$$y = bt^n \qquad \text{(Eq. 1}a\text{)}$$

$$\log y = \log b + n \log t \qquad \text{(Eq. 1}b\text{)}$$

where y = concentration of drug in milligrams percent, b = intercept, n = slope of the line, and t = time in minutes.

Since Figs. 1–11 cannot contain the 0% point, they exemplify the secondary or "steady-state" release pattern. The initial release rate was much faster and represents an equilibration period brought about by an initial surface release and/or a rapid diffusion of the drug from the aqueous phase until such release is controlled by either diffusion from the oil phase or diffusion through the interfacial area. The intercepts (not shown as such) of the log–log plots in Figs. 1–11 are indicative of the initial release pattern.

From the n values (Table I), some observations may be made about the secondary release rates from the various systems.

Drug Release from Water *versus* Drug Release from Oil—The release rate of ephedrine from the water (n = 0.725, antilog 5.31) was much greater than its release rate from the oil (n = 0.446, antilog 2.79). This was attributed to a slower diffusion rate of ephedrine through the more viscous oil (Table I).

Drug–Oil (n = 0.446, Antilog 2.79) *versus* Drug–Surfactant–Oil Release at Different HLB Values—Only HLB values 8 and 9 were below the control value of 0.446, and those were only slightly below. All others were above or considerably above the control value. This indicated that the surfactants in oil did not hinder the release rate of the drug; in most cases, as the HLB value approached

Table I—Calculated Slopes (n) of Ephedrine Concentration *versus* Time Plots Shown in Figs. 1–11[a]

	Ephedrine Emulsion		Ephedrine–Oil–Surfactant		Ephedrine–Water–Surfactant	
HLB	n	Antilog	n	Antilog	n	Antilog
4.7	0.435	2.72	0.447	2.80	0.472	2.96
6.0	0.496	3.13	0.460	2.88	0.535	3.43
7.0	0.427	2.67	0.472	2.96	0.493	3.11
8.0	0.435	2.84	0.440	2.75	0.673	4.71
9.0	0.418	2.62	0.442	2.77	0.562	3.64
10.0	0.423	2.65	0.453	2.84	0.640	4.37
11.0	0.473	2.97	0.446	2.79	0.667	4.64
12.0	0.457	2.86	0.497	3.14	0.610	4.07
13.0	0.547	3.53	0.507	3.17	0.704	5.06
14.0	0.548	3.53	0.600	3.98	0.677	4.75
14.9	0.610	4.07	0.598	3.96	0.660	4.57

[a] n_w = 0.725, antilog 5.31 (ephedrine–water). n_o = 0.446, antilog 2.79 (ephedrine–oil).

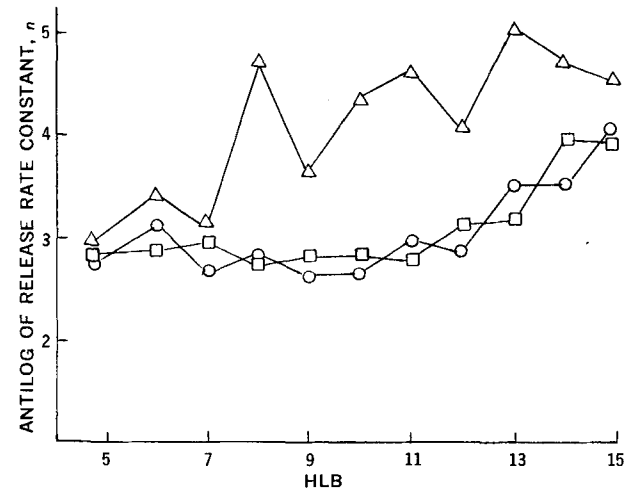

Figure 12—*Ephedrine dialysis rate constant as a function of HLB value of surfactant in various systems. Key:* ○, *ephedrine emulsion;* □, *ephedrine–oil–surfactant; and* △, *ephedrine–water–surfactant.*

14.9, the surfactant seemed to enhance the release of the drug from the oil (Table I).

Drug–Water (n = 0.725, Antilog 5.31) *versus* Drug–Surfactant–Water Release at Different HLB Values—In no case did the drug release from the drug–surfactant–water mixtures exceed the control value of 0.725, antilog 5.31, which indicated that the drug was interacting to a certain degree with the surfactants in water. This impaired release probably was due to a physical trapping of the drug in the micelles. As the HLB values increased from 4.7 to 14.9, the n values became larger, showing a greater release rate. This indicated that increasing the weight percent of polyoxyethylene groups tended to enhance the release rate of the drug (Table I).

Ephedrine Emulsion *versus* Controls at Different HLB Values—Figure 12 is a plot of the n values of all systems *versus* the HLB value of the surfactant in the separate systems. For each HLB value, no n value of the emulsified system was as large as the n value of the ephedrine–water control (n = 0.725, antilog 5.31) or the ephedrine–surfactant–water control. This again indicated that the emulsified system inhibited the release rate of the drug over the control values already described. However, for the control value of drug–oil (n = 0.446, antilog 2.79), the emulsified system did not show this inhibitory effect and, in fact, seemed to enhance the release rate of the drug over the drug–oil control at many HLB values.

Essentially, a zero slope was obtained with the n values of the emulsion and ephedrine–oil–surfactant control until an HLB value

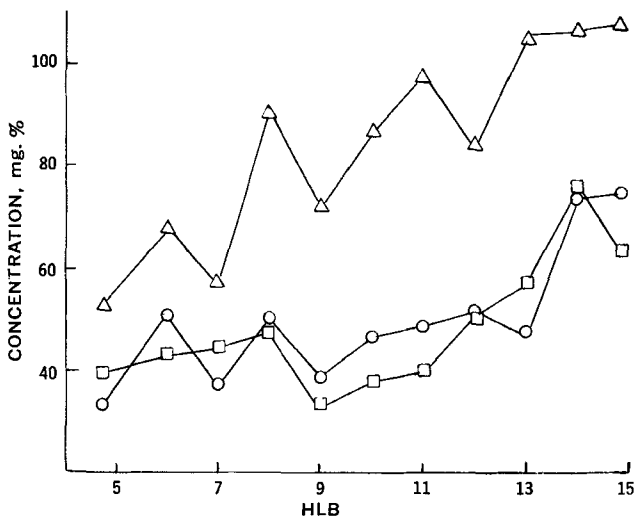

Figure 13—*Ephedrine concentration after 240 min. of dialysis as a function of HLB values of surfactant in various systems. Key:* △, *ephedrine–water–surfactant;* ○, *ephedrine emulsion; and* □, *ephedrine–oil–surfactant.*

of 12 was reached where the slope became positive. It was interesting to note the intertwining effect of the two values. At HLB 9–10, this was suddenly reversed, which could indicate the formation of the most stable emulsion.

From the close proximity of the curves and the intertwining effect of the release rates of the ephedrine–surfactant–oil and the emulsion systems at the various HLB values, it was concluded that the release rate from the oil system was the controlling factor in the release of the drug from the emulsified system.

The release of the drug from the water system was faster than the oil system at every comparable HLB value. Thus, the drug release from the oil was the rate-limiting factor.

Previously, it was shown that the surfactants did not retard the release of the drug from oil systems. Consequently, it would be expected from the control values that the rate of drug release from the emulsion would be the same or greater than the surfactant–oil system. However, in the emulsified system, at HLB values of 4.7, 7.0, 9.0, 10.0, 12.0, and 14.0, a lower rate of drug release was obtained than from the surfactant–oil system (Table I). This behavior was strongly indicative of the existence of an interfacial barrier between the oil globules and the water, which could be due to the arrangement and packing of the surfactants at the interface.

The concentration of ephedrine (milligrams percent) attained in the sink in a time interval of 240 min. reflects the net result of both release patterns during the time period in question (Fig. 13). The differences in the general shapes of Fig. 13 from Fig. 12 reflect the influence of the initial release rate on the total amount of drug dialyzed. The shape of the ephedrine–water–surfactant curves in Figs. 12 and 13 are the same except at HLB values of 13 and 14.9. The reasons for these apparent differences are not known. The absence of an oil–water interface in the ephedrine–surfactant–water system, the similarity of the curves, and the dissimilarity of the analogous emulsion curves further substantiate the possibility that transport of the drug through the interface in an emulsion system can be inhibited. This inhibition is apparently a function of the HLB value of the surfactant.

The fit of the data observed in the secondary or true rate of release to a log–log-type function (Freundlich isotherm type) indicates that a surface adsorption–evaporation process is occurring.

Inflection points were noted in all curves in Fig. 12 involving slope as a function of HLB. It is possible that at these points the shape of the micelle changes. Beecher (12) proposed that the micellar properties of nonionic surface-active agents could best be interpreted in terms of a rod-shaped micelle for the more lipophilic agents and a spherical micelle for the more hydrophilic agents. A change in shape would produce a change in the surface area of the micelle. Consequently, if the drug interacted with the micelle, the availability of the drug for dialysis would be changed.

Microscopic examination of the emulsions with the help of a micrometer eyepiece revealed that globules in all emulsions were essentially the same size; thus, change in the surface area of the droplets was not a factor in the fluctuations. The consistency of two separate dialyses conducted at each HLB value indicates these fluctuating points to be real differences and not random error in the experiments.

The distribution coefficients were attempted in accordance with the procedures previously described. Many problems became apparent in this study. In an effort to keep all parameters constant, no change was made in the concentration of the surfactants, drug, or phase volumes. The interfacial area between the oil and water was much less than when emulsified. Consequently, the water-soluble surfactant, polysorbate 60, settled out of solution and interfered with the analysis procedures from the water phase. The interference became appreciable around an HLB value of 10. This was not noticed at the lower HLB values; however, since the interference did become appreciable, all data must be regarded as less than exact. In an effort to salvage some of the experimentation, it was decided to sample the oil on the last set of distribution coefficients. This was described in the experimental procedures. Below an HLB value of 10, there was a greater loss of ephedrine from the water solution than was recovered in the oil solution. Above this value, recovery seemed more complete. This indicates that a significant amount of the drug was collecting at the interface, thereby indicating an interfacial barrier as postulated previously. Samples collected at the interface indicated a higher concentration of drug than would be expected, lending further support to the postulation. A control study indicated that the ephedrine

was stable over a 7-day period and that results were not influenced by degradation.

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Normal and Promoted GI Absorption of Water-Soluble Substances III: Absorption of Antibiotics from Stomach and Intestine of the Rat

C. J. KREUTLER and W. W. DAVIS

Abstract □ Absorption of soluble antibiotics was studied in the doubly ligated stomach of the rat, in the ligated small intestine, and in the intact GI tract. When surface-active absorption promoters are administered with the antibiotic in the ligated stomach, promoted absorption occurs but the onset is slow. When the drug–promoter combination is put in the ligated intestine, blood levels of the drug are elevated to severalfold the normal levels and the rise is extremely rapid. Absorption promoters exert a smaller influence in the intact GI tract. Their effectiveness is confined to the first 30 min., after which approximately normal levels of drug are seen. Emptying of the liquid dose from the stomach at 6–8 min. was followed by an immediate rapid rise of blood levels characteristic of the rapid response of the intestine to promoters. Accordingly, the blood level *versus* time curves obtained when promoters are employed in the intact rat are interpreted to be a result of a rapid but transient promoted absorption in the duodenum–small intestine, with little contribution from promoted gastric absorption.

Keyphrases □ Surfactants—role in enhancing antibiotic absorption, stomach, small intestine, and GI tract compared, rats, toxicity □ Antibiotics, soluble—comparison of doubly ligated stomach, ligated small intestine, and intact GI tract absorption, rats, effect of surfactants □ Absorption, soluble antibiotics—role of surfactants in doubly ligated stomach, ligated small intestine, intact GI tract, rats □ Surface-active absorption promoters—influence on water-soluble antibiotics in stomach and intestine, rats

Absorption of normally nonabsorbed or poorly absorbed water-soluble drugs from a Thomas gastric fundic pouch of the dog is greatly increased by certain surface-active agents (1). Nonionic, anionic, cationic, and zwitterionic surface-active agents were shown to promote the absorption of several types of antibiotics. Further work demonstrated similar promoted absorption in other segments and in the intact GI tract of the dog.

Another study demonstrated a greatly increased absorption of vitamin B_{12} from the stomach and the intact GI tract of the rat when an absorption promoter was added (2). The present study was initiated to characterize the response of the rat to absorption promoters and, particularly, to distinguish the response of the stomach and the intestine.

EXPERIMENTAL

Handling of Animals—Male Wistar and CFE strain rats, weighing 150–300 g., were fasted in individual cages with wide-mesh screen floors for approximately 15–20 hr. prior to operation and dosing. Blood samples were obtained by amputating the tip of the tail. Enough blood was collected to fill the capillarity capacity of from three to six 0.63-cm. (0.25-in.) diameter filter pads. The filter pads were placed directly on agar plates and were assayed for microbiological activity of the drug by standard disk-plate procedures[1].

Surgical Procedure—The animal was anesthetized with ether, and the stomach was exposed through a midline incision in the abdominal area. A ligation was placed at the pyloric sphincter. Aqueous drug solution was administered by stomach tube, after which an esophageal ligation was made at the cardiac sphincter, care being taken not to occlude blood vessels. The abdominal incision was then closed with wound clamps. After the rat recovered from the anesthesia in a restraining cage, it was conscious throughout the remainder of the experiment. Blood samples were periodi-

[1] Cephalothin and penicillin V were assayed against *Bacillus subtilis* (ATCC 6633). Cephaloridine was assayed against *Sarcina lutea* (PC1-1001-FDA).

was stable over a 7-day period and that results were not influenced by degradation.

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Normal and Promoted GI Absorption of Water-Soluble Substances III: Absorption of Antibiotics from Stomach and Intestine of the Rat

C. J. KREUTLER and W. W. DAVIS

Abstract □ Absorption of soluble antibiotics was studied in the doubly ligated stomach of the rat, in the ligated small intestine, and in the intact GI tract. When surface-active absorption promoters are administered with the antibiotic in the ligated stomach, promoted absorption occurs but the onset is slow. When the drug–promoter combination is put in the ligated intestine, blood levels of the drug are elevated to severalfold the normal levels and the rise is extremely rapid. Absorption promoters exert a smaller influence in the intact GI tract. Their effectiveness is confined to the first 30 min., after which approximately normal levels of drug are seen. Emptying of the liquid dose from the stomach at 6–8 min. was followed by an immediate rapid rise of blood levels characteristic of the rapid response of the intestine to promoters. Accordingly, the blood level *versus* time curves obtained when promoters are employed in the intact rat are interpreted to be a result of a rapid but transient promoted absorption in the duodenum–small intestine, with little contribution from promoted gastric absorption.

Keyphrases □ Surfactants—role in enhancing antibiotic absorption, stomach, small intestine, and GI tract compared, rats, toxicity □ Antibiotics, soluble—comparison of doubly ligated stomach, ligated small intestine, and intact GI tract absorption, rats, effect of surfactants □ Absorption, soluble antibiotics—role of surfactants in doubly ligated stomach, ligated small intestine, intact GI tract, rats □ Surface-active absorption promoters—influence on water-soluble antibiotics in stomach and intestine, rats

Absorption of normally nonabsorbed or poorly absorbed water-soluble drugs from a Thomas gastric fundic pouch of the dog is greatly increased by certain surface-active agents (1). Nonionic, anionic, cationic, and zwitterionic surface-active agents were shown to promote the absorption of several types of antibiotics. Further work demonstrated similar promoted absorption in other segments and in the intact GI tract of the dog.

Another study demonstrated a greatly increased absorption of vitamin B_{12} from the stomach and the intact GI tract of the rat when an absorption promoter was added (2). The present study was initiated to characterize the response of the rat to absorption promoters and, particularly, to distinguish the response of the stomach and the intestine.

EXPERIMENTAL

Handling of Animals—Male Wistar and CFE strain rats, weighing 150–300 g., were fasted in individual cages with wide-mesh screen floors for approximately 15–20 hr. prior to operation and dosing. Blood samples were obtained by amputating the tip of the tail. Enough blood was collected to fill the capillarity capacity of from three to six 0.63-cm. (0.25-in.) diameter filter pads. The filter pads were placed directly on agar plates and were assayed for microbiological activity of the drug by standard disk-plate procedures[1].

Surgical Procedure—The animal was anesthetized with ether, and the stomach was exposed through a midline incision in the abdominal area. A ligation was placed at the pyloric sphincter. Aqueous drug solution was administered by stomach tube, after which an esophageal ligation was made at the cardiac sphincter, care being taken not to occlude blood vessels. The abdominal incision was then closed with wound clamps. After the rat recovered from the anesthesia in a restraining cage, it was conscious throughout the remainder of the experiment. Blood samples were periodi-

[1] Cephalothin and penicillin V were assayed against *Bacillus subtilis* (ATCC 6633). Cephaloridine was assayed against *Sarcina lutea* (PC1-1001-FDA).

Table I—Effect of Various Promoters on Absorption of Cephaloridine from the Doubly Ligated Rat Stomach

Promoter	Number of Animals	Time of Peak Blood Level, min.	Peak Blood Level and Standard Error, mcg./ml.[a]
Control			
No promoter	9	120	0.38 ± 0.17
Nonionic			
Polyoxyethylene-20-cetyl ether[b,c]	3	70	2.47 ± 0.28
Polyoxyethylene-20-stearyl ether[b,d]	3	120	2.39 ± 0.13
Polyoxyethylene-20-oleyl ether	9	120	1.88 ± 0.09
Polyoxyethylene-24-cholesterol ether[e]	3	70	4.87 ± 1.75
Surfonic LF-16[f]	3	60	2.61 ± 0.30
Surfonic LF-17[f]	3	70	1.84 ± 0.52
Anionic			
Sodium lauryl sulfate	4	60	3.80 ± 0.79
Cationic			
Cetyltrimethylammonium bromide[g]	2	120	3.20 ± 0.40
Cetylpyridinium bromide	2	120	1.60 ± 0.65

[a] Values are the mean of highest antibiotic value observed during 120 min. following administration to a rat of 1.5% antibiotic at 125 mg./kg. with and without 0.75% promoter at 62.5 mg./kg. in pH 7.0 isosmolar sodium phosphate buffer, except as noted. [b] Atlas Chemical Industries, Inc., Wilmington, Del. [c] Brij-58. [d] Brij-78. [e] Solulan C-24, American Cholesterol Products, Inc., Edison, N. J. [f] Modified primary alcohol–ethylene oxide adducts, Jefferson Chemical Co., Inc., Houston, Tex. [g] Concentration 0.91%.

cally taken from the tail. Absorption was followed for approximately 2 hr., after which time the rat was sacrificed, the stomach was excised, and the contents were examined. If any coprophagy occurred, the data from such animals were rejected.

For the intestinal ligation, the rat was anesthetized with ether. The intestinal area was exposed with the aid of a metal hook through an abdominal incision. Ligations were made at the pyloric sphincter and at the ileal end of the small intestine immediately adjacent to the cecum. The aqueous test solution was introduced into the small intestine using a 27-gauge needle inserted into the duodenum distal to the pyloric ligation. A third ligation was then made around the duodenum distal to the point of entry of the needle so as to prevent any leakage of the test solution from the puncture. The operation was then completed by closure of the abdominal cavity with wound clamps as previously described. The same holding and bleeding procedures were employed as for stomach ligation experiments.

To dose the intact rat, the animal was lightly anesthetized with ether, and the drug solution was introduced into the stomach through a stomach tube. The animal was placed in a restraining cage and was held there for the duration of the experiment while periodic blood samples were taken.

Preparation of Oral Dose—Drugs were dissolved in pH 7.0 isosmolar sodium phosphate buffer or in pH 3.7 isosmolar sodium citrate–sodium phosphate buffer. The administered dose was usually 125 mg./kg. antibiotic, with or without 62.5 mg./kg. surface-active agent. The volume of the administered oral dose varied in proportion to the weight of the animal. The concentrations of the two components in the dose were 1.5 and 0.75%, respectively. If other compositions were employed, they are noted.

RESULTS

Absorption from Ligated Stomach—Initial experiments were conducted in which a single (pyloric) ligation only was performed. In these experiments, little or no absorption of antibiotic was seen even when a promoter was added. The accumulation of large volumes of acidic fluid was observed. Excessive gastric acid secretion is known to be detrimental to promoted absorption in the dog. When the rats were predosed orally 1 hr. before the experiment with an anticholinergic compound[2], the gastric fluid volume remained low, and the pH remained within the range of 5–7 during the subsequent 2-hr. study. High antibiotic blood levels were achieved following this procedure, with promoters included with the antibiotic.

In 1966, Brodie and Knapp (4) showed that ligating the esophagus and the pylorus of a rat markedly decreased the volume and the titratable acidity of the gastric contents. With this procedure, the absorption promoter, polyoxyethylene-20-oleyl ether[3], enhanced the absorption of cephaloridine[4]. Good volume and pH control were also observed without employing an anticholinergic predose. This technique, without an anticholinergic predose, was thereafter employed for studying the gastric absorption.

The promoted absorption of cephaloridine under these conditions is dose responsive. Absorption is also promoted by some other, but not all, nonionic, anionic, and cationic surface-active agents (Table I). The absorption of cephaloridine is dose responsive also to its own concentration when a constant concentration of absorption promoter is employed.

A 2% solution of sodium cephalothin[5] in pH 7.0 isosmolar phosphate buffer, with and without polyoxyethylene-20-oleyl ether, was administered in the doubly ligated stomach. The peak blood levels of this antibiotic were increased by a factor of approximately two by the inclusion of this promoter.

The gastric absorption of potassium penicillin V[6] was examined in a pH 3.7 isosmolar sodium citrate–phosphate buffer and a pH 7.0 isosmolar sodium phosphate buffer. The efficiency of normal absorption at the lower pH value was much greater than at pH 7.0, approximately 3.5-fold. The resulting blood levels at pH 3.7 were approximately proportional to its concentration. The addition of the promoter with potassium penicillin V at pH 3.7 caused only a small increase in absorption.

Absorption from Ligated Duodenum and Small Intestine—Cephaloridine or cephalothin in pH 7.0 isosmolar sodium phosphate buffer, with and without promoter, was introduced into the doubly ligated duodenum and small intestine of fasted rats. The data in Table II clearly demonstrate that this agent effectively increased the absorption of these antibiotics from this segment. The rapidity with which blood levels rose when mixtures of antibiotic and promoter were placed in the segment may be contrasted with the much slower rate of rise of blood levels from the same dose placed in the ligated stomach. When the duodenum and small intestine were ligated only at the cecum, the resulting blood levels from either normal or promoted absorption were lower than when the segment was doubly ligated.

Absorption from Intact GI Tract—For correlation with absorption experiments in which promoters are employed in the intact animal, a series of experiments on stomach emptying was conducted. To determine the approximate time of emptying of a liquid dose from a fasted intact stomach, a solution containing 0.15% phenol red, 1.5% cephaloridine, and 0.75% polyoxyethylene-20-oleyl ether was given orally through a stomach tube to lightly anesthetized rats. This was the same dosing procedure later used in intact animal experiments. Phenol red reportedly is not absorbed from the rat stomach (7) or from the intestine (8). At various times the animals were reanesthetized, an abdominal incision was made, and

[2] α- and β-DL-(1-Methyl-3-pyrrolidinyl)-α-phenyl-α-(2-thienyl)glycolate methyl bromide (3), administered at 20 mg./kg.

[3] Brij-98, Atlas Chemical Industries, Inc., Wilmington, Del.

[4] 7-[α-(2-Thiophene)acetamido]-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine, Loridine, Lilly (5).

[5] Sodium salt of 7-[α-(2-thiophene)acetamido]-cephalosporanic acid, Keflin, Lilly (6).

[6] Potassium salt of phenoxymethyl penicillin V-cillin K, Lilly.

Table II—Normal and Promoted Absorption from Doubly Ligated Duodenum–Small Intestine

Drug Combination	Number of Animals	Minimum Detectable Levels, mcg./ml.	Whole Blood Levels (mcg./ml.) and Standard Errors (Time Postdosing, min.) 20	40	60	80	120	180
Cephaloridine only	5	0.25	0.41 ±0.13	0.95 ±0.19	1.90 ±0.35	2.03 ±0.37	1.66 ±0.46	1.54 ±0.38
Cephaloridine with polyoxyethylene-20-oleyl ether	5	0.25	3.44 ±0.72	9.36 ±1.14	10.52 ±0.62	8.36 ±1.71	3.58 ±0.64	0.95 ±0.18
Cephalothin only	4	0.10	0.17 ±0.11	0.34 ±0.04	0.40 ±0.05	0.35 ±0.04	0.18 ±0.01	—
Cephalothin with polyoxyethylene-20-oleyl ether	5	0.10	1.39 ±0.10	5.90 ±0.69	5.96 ±0.97	2.25 ±0.37	0.76 ±0.20	—

Table III—Normal and Promoted Absorption from Intact Rat GI Tract

Drug Combination	Number of Animals	Minimum Detectable Levels, mcg./ml.	Whole Blood Levels (mcg./ml.) and Standard Errors (Time Postdosing, min.) 20	40	60	90	120	180
Cephaloridin only	15	0.25	0.33 ±0.09	0.82 ±0.23	1.12 ±0.28	1.35 ±0.30	0.79 ±0.17	0.45 ±0.12
Cephaloridine with polyoxyethylene-20-oleyl ether	11	0.25	1.16 ±0.37	1.10 ±0.28	1.04 ±0.21	0.93 ±0.13	0.79 ±0.17	0.57 ±0.12
Cephalothin only	4	0.10	0.21 ±0.02	0.33 ±0.06	0.33 ±0.05	0.34 ±0.10	—	—
Cephalothin with polyoxyethylene-20-oleyl ether	5	0.10	0.50 ±0.06	0.30 ±0.04	0.25 ±0.06	0.17 ±0.05	—	—

a clamp was quickly placed at the pyloric sphincter. Simultaneously with these ligations, a blood sample was taken. Additional clamps were also placed at various locations throughout the GI tract. The stomach and small intestine were then excised and separated. The fluid contents of the segments were emptied and flushed thoroughly with neutral buffer. These solutions were appropriately diluted with more buffer and centrifuged to remove any particulate matter. The optical density of each phenol red solution was determined at 550 nm. employing a spectrophotometer[7]. During the interval between 8 and 9 min. after dosing, approximately 80% of the administered dose passed into the small intestine[8]. This experiment and the corresponding blood levels of antibiotic are shown in Fig. 1. The appearance of the antibiotic in the blood correlated remarkably well with the time of emptying into the duodenum.

To follow the normal and promoted absorption of antibiotics from the intact GI tract of the rat, cephaloridine or cephalothin in pH 7.0 isosmolar sodium phosphate buffer, with and without promoter, was given *via* a stomach tube. In the blood samples taken at 20 min., antibiotic levels with absorption promoters were increased as much as three times over those observed without promoter. By 40–60 min., this difference disappeared. Averaged data are shown in Table III.

A second series of intact animals were orally dosed with antibiotic alone or with the promoter–drug combination as in the foregoing experiment, but they were additionally predosed with polyoxyethylene-20-oleyl ether 20 min. before the antibiotic administration. The early blood levels were then generally further elevated by factors averaging 1.6 times the levels without predosing. However, none of these levels was equal to the results seen in doubly ligated duodenum–small intestine animals.

Toxicity Effects of Absorption Promoters—Histological examinations were made of organs taken from the rat after treatment with this surface-active agent. The tissues were slightly damaged in all ligated specimens. However, identical effects were found in ligated tissues similarly exposed to saline solution or to drug solution without the surfactant. This observed slight damage is, therefore, attributed to the manipulation of the organ or to the ligation procedure.

Previous studies in these laboratories (1) indicated that no irreversible functional alteration of the absorbing tissues in the Thomas canine fundic pouch was attributable to promoters, since continued use in individual animals did not alter the absorption characteristic of the gastric tissue. In the present study, promoters were administered to rats over 2 weeks. Rats were placed in individual cages and given 0.75% solutions of sodium lauryl sulfate, polyoxyethylene-24-cholesterol ether, or polyoxyethylene-20-oleyl ether as their only source of drinking water for a 2-week period. The animals were given Purina Rat Chow and were free to eat and drink *ad libitum*. The food and water intake, weight loss or gain, and the general physical appearance of the animals were followed daily and compared to control animals given plain water and food. After 2 weeks, one-half of the animals in each category were returned to plain water and food and were observed for a 4-week period. One-half of the rats were fasted overnight and thereafter were employed for absorption studies.

These absorption experiments evidenced the ability of the animals to exhibit the typical promoted absorption after the continued administration of promoters. Daily water and food consumption and weight gain were greatly depressed when sodium lauryl sulfate solution was given (Table IV). These rats showed adverse physical changes during the test period. Among the animals placed on water and a normal diet after the 2-week test, the most notable change occurred among the sodium lauryl sulfate-treated rats. Their physical appearance improved immediately, and their food intake and growth rate far surpassed that of the others.

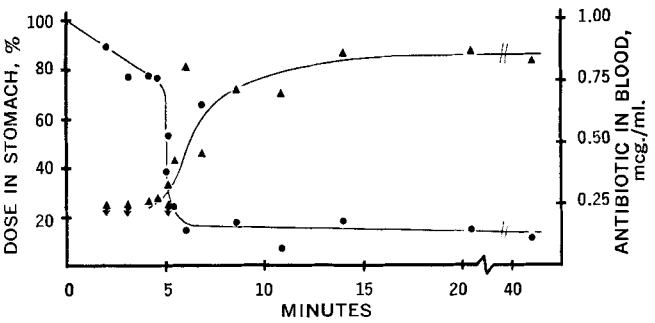

Figure 1—*Time course of emptying of a liquid dose of cephaloridine in the presence of polyoxyethylene-20-oleyl ether from the intact rat stomach (●) and of appearance of antibiotic in the blood (▲). Each point represents one animal from which one value for emptying and one for blood level were obtained.*

[7] Beckman model DU.

[8] Recovery of phenol red from the stomach and intestine averaged 103.6% in the 14 animals employed in this experiment. This value indicates that no great loss of dye from stomach or intestine occurred by promoted absorption.

Table IV—Food and Water Consumption and Weight Gain during 2-Week Administration of Promoters

Drinking Solution	Number of Animals	Initial Weight, g.	Intake, g.		Weight Gain, g.			
			Water	Food	Test Period	During Recovery 1 week	During Recovery 4 weeks	Overall
Control	2	165.5	349.0	245.0	80.0	—[a]	—[a]	—[a]
Sodium lauryl sulfate	2	164.5	267.2	191.2	14.7	—[a]	—[a]	—[a]
Polyoxyethylene-20-oleyl ether	2	159.0	341.6	236.7	74.2	—[a]	—[a]	—[a]
Polyoxyethylene-24-cholesterol ether	2	169.0	320.2	236.2	59.2	—[a]	—[a]	—[a]
Control	2	264.7	361.5	269.7	49.2	26.0	56.0	105.2
Sodium lauryl sulfate	2	257.5	262.0	182.5	2.0	72.0	133.0	135.0
Polyoxyethylene-20-oleyl ether	2	259.7	292.5	241.2	32.5	25.2	49.2	81.7
Polyoxyethylene-24-cholesterol ether	2	260.7	304.0	244.7	35.2	23.0	66.0	101.2

[a] Animals used for absorption experiments.

DISCUSSION

Previous investigators examined the absorption of acidic and basic organic compounds from the rat stomach and the rat duodenum and small intestine (7, 8). The stomach is often a significant site of absorption. Similar studies were done in other animals and in man, demonstrating the capabilities of the stomach in all species to absorb by passive diffusion a variety of acidic and weakly basic drugs (9). Such data are consistent with the hypothesis that the gastric or intestinal mucosa, acting as a lipoidal barrier, is selectively permeable to the more lipid-soluble unionized form of drugs, either free acid or free base.

In the present experiments in rats, the normal gastric absorption of potassium penicillin V was much more rapid when the drug was administered at pH 3.7 in sodium citrate–phosphate buffer than when administered at neutral pH in phosphate buffer. This finding is in agreement with expectations. At pH 3.7, approximately 10% of the antibiotic is in the nonionized free acid form, whereas a negligible fraction is in the free acid form at pH 7.0.

All other absorption studies reported here, either in the stomach or intestine, were done in a pH 7.0 isosmolar sodium phosphate buffer system, at which pH the carboxyl groups of the antibiotics, for all practical purposes, are totally dissociated. The normal absorption of cephaloridine (a zwitterion at pH 7.0), and of cephalothin (a fully ionized acid salt) is also extremely limited from the neutral stomach. These results are entirely expected, considering their negligible lipid solubility at pH 7.0.

The present results, concerned with the effect of absorption promoters on gastric absorption at pH 7.0, do not fall within the scope of the Schanker hypothesis which proposes correlation of absorption and lipid solubility. The hypothesis is useful in studying weak organic acids to allow understanding of relative absorbability of different drugs in the stomach or other GI segment as determined greatly by local pH. The dissociation constant determines the relative availability of the more lipid-soluble unionized form at the local pH. The use of buffer at neutral pH explicitly avoids the occurrence of the lipid-soluble form of the drugs.

It has been suggested that the mechanism of influence of surface-active absorption promoters, effective as they are under conditions of negligible lipid solubility of the drug, is to render the membrane a less effective lipid barrier to the passive diffusion of such ionized drugs. An earlier report from these laboratories showed that the surface-active absorption promoters act by a colloidal influence upon the absorptive organ and that the effect is rapidly reversible (1). The present work presents no additional information regarding mechanism or reversibility but appears consistent with the earlier conclusions.

In routine procedures employed here, the first blood samples were taken approximately 20 min. after dosing. Before this time, as seen from the experiment on stomach emptying in intact rats, most of the dose had emptied into the small intestine and a rapid rise in blood levels had already begun. Accordingly, the initial levels seen in these experiments on normal and promoted absorption in the intact animal should be attributed to intestinal rather than to gastric absorption. This initial rise in blood levels is rapid, as it is in ligated duodenum–small intestine, even though blood levels never rise to the high values seen in the isolated ligated duodenum–small intestine experiments. This rapid rise in blood levels contrasts with the slow rise when the drug–promoter mixture is administered in the ligated stomach, lending further evidence that the duodenum–small intestine is the responsible segment in the intact animal experiments.

The promoter appears to impose a very fleeting hyperabsorptive state on the duodenum–small intestine and then loses its effectiveness to elevate absorption. This may be due to rapid emptying of the stomach followed by dilution of the dose in the duodenum, or to the subsequent rapid passage of a liquid dose out of the more absorptive upper part of the small intestine. The comparatively poor results in the intact animals also raise the interesting question of possible specific incompatibilities of polyoxyethylene-20-oleyl ether with intestinal secretions in the intact GI tract.

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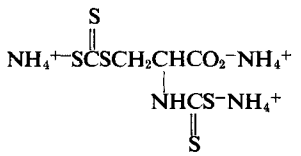
Metabolism of the Radioprotective Agent Triammonium 2-Dithiocarbamyl-3-dithiocarbonylthiopropanoate in the Mouse

WILLIAM O. FOYE, RITA S. F. CHU, KIRIT A. SHAH, and WILLIAM H. PARSONS

Abstract □ To determine whether one or both of the sulfur-containing functions of the dithiocarbamate trithiocarbonate (I) of cysteine is responsible for its radioprotective ability, ^{35}S-I was synthesized and its metabolism was studied in mice. The major metabolite was the dithiocarbamate of cystine, which was found in the liver, kidney, and spleen. The protein-bound component was also found in these tissues. Other metabolites found were intact I and possibly cystine in the liver and sulfate in the kidney and spleen. ^{35}S-I was rapidly eliminated, and radioactive compounds which appeared in the urine included the dithiocarbamate, unchanged I, sulfate, and an unidentified substance in small amount.

Keyphrases □ Triammonium 2-dithiocarbamyl-3-dithiocarbonylthiopropanoate, radiolabeled—metabolism studied, metabolites determined, mice □ Radioprotective agents—metabolism, metabolic products of triammonium 2-dithiocarbamyl-3-dithiocarbonylthiopropanoate determined, mice □ Radiolabeling—synthesis of triammonium ^{35}S-2-dithiocarbamyl-3-dithiocarbonylthiopropanoate for metabolism study

Appreciable radiation protection in mice, of a higher degree than that conferred by cysteine, has been found for the product (I) of carbon disulfide condensation of cysteine, the dithiocarbamate trithiocarbonate (1). To determine whether one or both of these sulfur-containing groups survives *in vivo*, and may thus be responsible for the radiation protection, a study of the metabolic products of I in mice was carried out. Metabolism studies of other dithiocarbamates showed that this function remains intact *in vivo* (2); little is known of the stability of the trithiocarbonate function to animal metabolism.



I

Distribution and excretion studies with other radiation-protective agents, notably the amino thiols, revealed sizable amounts of these substances bound to protein (3) within a very short time. Excretion is also rapid, 30% of the injected radioactivity of the S-labeled 2-mercaptoethylamine appearing in the urine of dogs in 6 hr. (4). Taurine and sulfate appear to be the major metabolites of 2-mercaptoethylamine (5), with both the thiol and disulfide appearing in the urine.

To determine the distribution of I in such radiosensitive tissues as the liver, kidney, and spleen, it was first necessary to synthesize the compound using ^{35}S-carbon disulfide.

Table I—R_f Values of Sulfur-Containing Compounds in *n*-Butanol–Pyridine–Water (5:3:5)

Compound	R_f	95% Confidence Level
Cystine	0.16	0.02
Cysteic acid	0.27	0.04
Taurine	0.42	0.02
Taurocholic acid	0.74	0.04
Thiourea	0.65	0.01
Sodium sulfate	0.19	0.04
Sodium thiosulfate	0.21	0.04
Potassium thiocyanate	0.71	0.03
Cysteine	0.67	0.03
	0.17	0.04
I	0.79	0.01

EXPERIMENTAL

Triammonium ^{35}S-2-Dithiocarbamyl-3-dithiocarbonylthiopropanoate—^{35}S-Carbon disulfide was obtained[1] and had a radioactivity of 10 mc. ml.$^{-1}$ at the time of use. The ^{35}S-carbon disulfide (1 ml.) was cooled prior to use, in an ice bath, and a small piece of iron rod was placed in the top of the container and held away from the glass seal by a magnet. The container with the ^{35}S-carbon disulfide was attached to a three-necked flask which was evacuated to 20 mm. pressure; the magnet was withdrawn, allowing the rod to break the glass seal, and the ^{35}S-carbon disulfide slowly distilled into the ice-cooled flask and condensed. A solution of L-cysteine (0.968 g.) in 3 ml. of concentrated ammonium hydroxide and 5 ml. of water was added dropwise, and the reaction mixture was stirred for 1 hr. at ice bath temperature. Absolute ethanol (50 ml.) was added, giving a bright-yellow precipitate, which was isolated, washed with cold ethanol, and dried *in vacuo*. A 49% yield was obtained of material having a specific activity of 1.25 mc./mmole.

Tetrasodium Bis(2-dithiocarbamyl-3-thiopropanoate)—Cystine (5 g., 0.02 mole) was dissolved in 25 ml. (0.03 mole) of 5% sodium hydroxide solution, and 20 ml. (0.33 mole) of carbon disulfide was added slowly with stirring at room temperature. After it was stirred for 4 hr., the mixture was allowed to evaporate at room temperature. A 50% yield of yellow solid was obtained, which was washed well with ethanol and dried ($CaCl_2$) and did not give a melting point; R_f (butanol–pyridine–water, 5:3:5) 0.57; $\lambda_{max.}^{H_2O}$ 270 nm. (log $E_{max.}$ = 3.20); $\nu_{max.}^{KBr}$ 1275, 1050, and 945 cm.$^{-1}$.

Anal.—Calc. for $C_8H_8N_2Na_4O_4S_6$: C, 20.00; H, 2.56; N, 5.83. Found: C, 20.02; H, 2.91; N, 5.87.

Paper Chromatography—Paper chromatography was carried out in an ascending system using Whatman No. 1 paper. Samples of known quantity (50–100 mcg./10 λ) were applied to a 25 × 21-cm. sheet and dried by hot air. After being dried, the sheet was rolled into a cylinder and placed in a jar for developing; it was developed at 4° for 24 hr. The solvent system was composed of *n*-butanol, pyridine, and water (5:3:5) (Table I).

Determination of I Stability *In Vitro*—Compound I (100 mcg./10 λ) was dissolved in phosphate buffer at pH values of 6.1, 7.4, and 8.4. The solutions were chromatographed immediately on formation and after standing at room temperature for 1 hr. (Table II). Compounds known to be end-products of cysteine and sulfur metabolism were also chromatographed, and R_f values were determined (Table I). To locate the spots, the chromatogram sheet was cut into strips, each of which was treated with one of the following reagents by dipping: ninhydrin–collidine reagent (6), Grote's reagent (7), *N*-ethylmaleimide–KOH (8), ammoniacal $CuCl_2$–hydroxylamine (9), phosphomolybdic acid (10), $BaCl_2$–$KMnO_4$ (11), and $Fe(NO_3)_3$ (12).

[1] New England Nuclear Corp., Boston, Mass.

Table II—Stability of I *In Vitro*: R_f Values of Breakdown Products

pH	R_f	95% Confidence Level
6.1 and 7.4	0.59	0.04
	0.48	0.02
	0.36	0.04
	0.16	0.02
8.4	0.81	0.03
	0.64	0.08
	0.47	0.03
	0.39	0.01
	0.23	0.01

Table III—Distribution of Radioactivity in Mice

Hours	Percent of Administered Radioactivity—Liver	Kidney	Spleen	Urine	Feces
0.5	17.6 (5.8)[a]	4.7 (1.9)	1.1 (0.6)	53.9 (8.8)	— —
1	9.4 (1.4)	3.3 (1.1)	0.7 (0.3)	51.2 (5.1)	— —
2	9.4 (3.3)	1.4 (0.7)	0.8 (0.7)	52.3 (7.1)	6.3 (3.5)

[a] Numbers in parentheses are standard deviations.

Areas on the I strips giving positive ninhydrin–collidine reagent and Grote's reagent reactions were eluted with 90% ethanol and with 0.1 *N* alcoholic KOH. UV spectra of the eluates were obtained with a spectrophotometer[2].

Determination of I Stability *In Vivo*—The distribution and excretion of ^{35}S-labeled I were investigated at three time intervals after a given dose of compound: at 0.5, 1, and 2 hr. Male Swiss mice (Webster strain) were used; a group of five was used for each determination, and 20–30 were used for each experiment.

^{35}S-I was dissolved in water immediately before use. The mice were given a dose of 10 mg. (0.2 ml.) i.p. of labeled compound by injection and were put into a metabolism cage equipped to separate urine and feces. Food and water were not restricted during the experiment. Urine samples for the first two intervals were collected directly after the mice were sacrificed; all other excreta were collected from the cage.

After the animals were killed at the designated times, the livers, kidneys, and spleens were removed and kept frozen until used. Individual organs from five mice were pooled, and the pooled tissues were homogenized with a small amount of ice-cold distilled water (liver with 4 ml., kidneys with 1 ml., and spleen with 0.5 ml.). The homogenates were centrifuged at 10,000 to 15,000 r.p.m. in a refrigerated centrifuge. Urine was diluted to 1.5 ml. with cold distilled water, and feces were extracted with 0.5 ml. Ten lambdas of each preparation was chromatographed. After the chromatogram was developed, it was treated with ninhydrin–collidine reagent. It was then cut into strips 25 × 2.5 cm., and the strips were scanned for radioactivity using a Scanogram II[3].

RESULTS AND DISCUSSION

The I molecule bears two sulfur-containing functions, R—S—C(=S)—S$^-$ and R—NH—C(=S)—S$^-$, both containing C(=S) and S$^-$. They are, therefore, not distinguishable by chemical color reagents, but the UV absorption spectra of a sufficient number of examples of each have been recorded to allow these functions to be distinguished. Janssen (13) assigned peaks (in water) at 253 and 279 nm. to N—C(=S)—S$^-$ and peaks at 303 and 333 nm. to S—C(=S)—S$^-$. Also, using 95% ethanol, Foye *et al.* (14) found trithiocarbonate absorption peaks at 303 and 333 nm. and the dithiocarbamate absorption peaks at 255–257 and 290–295 nm. with a much weaker peak at 342–345 nm.

[2] Beckman model DU.
[3] Baird-Atomic Instrument Co.

***In Vitro* Determinations**—Dissolution of I in acidic medium resulted in immediate decomposition with evolution of gas (detected by lead acetate paper). At pH 7.4, gas was not detected for 0.5 hr.; at pH 8.4, no sign of decomposition was evident for 1 hr. UV absorption of the freshly prepared solutions showed peaks at 255, 292, and 333 nm., characteristic of both dithiocarbamate and trithiocarbonate groups. After 1 hr., the spectra of the solutions at pH 6.1 and 7.4 showed a broad band at 253–275 nm. For the solution at pH 8.4, a sharper peak at 268 nm. appeared. These peaks are indicative of a dithiocarbamate but not a trithiocarbonate group. It was apparent that the trithiocarbonate group had suffered decomposition.

Paper chromatograms of I at these three pH values were quite similar (Table II). A spot at R_f 0.59, positive to ninhydrin–collidine reagent, *N*-ethylmaleimide–KOH reagent, $CuCl_2$–hydroxylamine reagent, and Grote's reagent, indicative of both C=S and S=H, was eluted with 90% ethanol and showed a broad peak in the UV at 256–274 nm. This indicated a dithiocarbamate with loss of the trithiocarbonate group. Two spots with R_f values of 0.48 and 0.36 were positive to ninhydrin–collidine reagent but to none of the other reagents; these spots did not appear in the *in vivo* measurements and were not identified. Another spot at R_f 0.16, positive to Grote's reagent, agrees with the R_f value of cystine.

The chromatogram of the I solution at pH 8.4 showed small differences in R_f values from those of solutions at pH 6.1 and 7.4 and revealed an additional spot at R_f 0.81, which was positive to ninhydrin–collidine reagent, $CuCl_2$–hydroxylamine reagent, and Grote's reagent. UV absorption of the eluate of this spot was found at 254 and 332 nm., characteristic of both the dithiocarbamate and trithiocarbonate groups; it was considered to be due to intact I. On the scans of ^{35}S-I after chromatographing solutions at pH 7.4 and 8.1, two radioactive peaks were found at R_f 0.59 and 0.79, with more of the radioactivity in the peak at R_f 0.79, particularly at pH 8.1. These peaks, on the basis of the UV absorption of the corresponding I solutions, represent the dithiocarbamate and intact I, respectively.

Synthesis of Dithiocarbamate—Attempted synthesis of the dithiocarbamate of cysteine led either to I or unreacted cysteine. Attempts to isolate a hydrolysis product of I containing the dithiocarbamate but not the trithiocarbonate group gave solutions from which sulfur was slowly deposited. Reaction of cystine with carbon disulfide, however, gave the bis-dithiocarbamate of cystine, which gave an R_f value (0.57) approximating both that of the hydrolysis product and one of the metabolic products. UV absorption at 270 nm. was characteristic of a dithiocarbamate, as was IR absorption at 1275, 1050, and 945 cm.$^{-1}$.

Distribution *In Vivo*—Thirty minutes after the injection of ^{35}S-I, 18% of the administered radioactivity was found in the liver (Table III). Scans of the chromatograms for radioactivity showed four peaks with R_f values of 0.79, 0.59, 0.15, and 0.0; the highest

Table IV—Radioactive Components in Mouse Liver: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks—R_f 0.79	R_f 0.59	R_f 0.15	R_f 0.0
0.5	25.6 (6.6)[a]	39.7 (17.9)	10.2 (4.8)	26.1 (17.8)
1	32.6 (3.5)	33.9 (10.5)	5.1 (1.0)	29.8 (5.4)
2	— —	67.8 (3.0)	— —	26.4 (8.7)

[a] Numbers in parentheses are standard deviations.

Table V—Radioactive Components in Mouse Kidney: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks—R_f 0.59	R_f 0.20	R_f 0.0
0.5	29.7 (5.5)[a]	36.2 (5.8)	30.3 (8.8)
1	30.8 (8.1)	45.6 (9.8)	23.8 (7.4)

[a] Numbers in parentheses are standard deviations.

level of radioactivity (40%) was associated with the peak at 0.59 (Table IV). The peak at 0.59 (measured at the center) corresponds quite well to the spots of R_f 0.59 for a dithiocarbamate for the *in vitro* experiments of both I and ^{35}S-I and to R_f 0.57 for the bis-dithiocarbamate of cystine.

The component at R_f 0.79 appears to be intact I, which showed the same R_f value *in vitro*. The substance of R_f 0.15 is most likely the disulfide cystine, since cystine had an R_f of 0.16 in the *in vitro* system. However, for cystine to show radioactivity, sulfur exchange between the ^{35}S-CS_2 and the thiol group of cysteine would be required.

Radioactivity was found remaining at the origin of the chromatograms of all tissue homogenates. Although it was not possible to determine the chemical nature of this component, it is most likely protein bound. Stromme (15), for instance, found diethyldithiocarbamate bound to plasma and liver proteins through mixed disulfide formation.

In regard to the possibility of conjugation products, Stromme (15) found that diethyldithiocarbamate is excreted to a small extent as the *S*-glucuronide in rats, along with sulfate, unchanged dithiocarbamate, and CS_2. However, Kaslander (16) found very little of this dithiocarbamate conjugated in humans (0.75%). Other conjugates of dimethyldithiocarbamate, including the alanine, α-aminobutyric acid, and β-glucoside derivatives, were found as metabolic products from plant tissues and microorganisms (17). These conjugates had low R_f values (in propanol–H_2O) over a range (0.26–0.39) in which no radioactivity was found in our determinations.

After 1 hr., the level of radioactivity in the liver had decreased by about 50%, and it remained the same at 2 hr. (Table III). Similar storage and excretion behavior has been noted for other radioprotective thiols. After 1 hr. (Table IV), the amount of intact I appeared to increase somewhat, but the amount, if considered within the limits of the standard deviation, probably was comparable to that at 0.5 hr. After 2 hr., no I was evident. The amount of disulfide also diminished, and at 1 hr. it was only about 5% of the measured amount. After 2 hr., only dithiocarbamate and the protein-bound component remained in the liver homogenate.

The amount of radioactivity in the kidneys (Table V) was much less than in the liver, less than 5% of the administered dose being present after 0.5 hr. This level declined to 1.4% after 2 hr. Kidney homogenates showed the presence of three components: dithiocarbamate, a substance of R_f 0.20 attributable to sulfate (inorganic sulfate showed an R_f 0.19 *in vitro*), and protein-bound compound. Here, the amount of radioactivity was fairly evenly distributed among the three compounds, that due to sulfate increasing with time. The amount of dithiocarbamate stayed the same during the time interval observed, while the amount of protein-bound component diminished.

Only 1% of the injected radioactivity was found in the spleen; this level diminished slightly with time (Table VI). Similarly to the kidney homogenate, no disulfide was visible in the spleen homogenate, but dithiocarbamate and the protein-bound component were found along with sulfate at R_f 0.20. The sulfate amount again increased with time; the amount of dithiocarbamate was relatively constant, and that of the protein-bound component diminished slightly.

Excretion—Compound I was metabolized and excreted rather rapidly. Over 50% of the administered radioactivity appeared in the urine at each time interval (Table III). Radioactivity was found in the feces after 2 hr.; no attempt was made to determine loss through respiration, where any carbon disulfide would be expected to appear. Carbon disulfide has been found in the expired air of rats after administration of diethyldithiocarbamate or disulfiram[4] (15, 18).

Four radioactive peaks were found in the urine: one corresponding to dithiocarbamate, one to sulfate, one to I, and one to a new component at R_f 0.92 (Table VII). The amount of the latter substance was relatively small, and its identity was not established.

In the first 0.5 hr. after injection, the radioactive component excreted in the largest amount was intact I, with dithiocarbamate and sulfate excreted in lesser amounts. The amount of I diminished rapidly, whereas the other two compounds increased in quantity. After 2 hr., sulfate accounted for 43% of the radioactivity excreted and dithiocarbamate 38%, while I had declined to 13%.

[4] Antabuse, Ayerst Labs.

Table VI—Radioactive Components in Mouse Spleen: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks		
	R_f 0.59	R_f 0.20	R_f 0.0
0.5	29.4 (5.3)[a]	26.4 (4.6)	39.0 (8.9)
1	30.7 (5.2)	39.0 (11.9)	36.0 (10.7)

[a] Numbers in parentheses are standard deviations.

Table VII—Radioactive Components in Mouse Urine: Percent Radioactivity after Injection of ^{35}S-I

Hours	Percent Radioactivity in Peaks			
	R_f 0.92	R_f 0.80	R_f 0.59	R_f 0.20
0.5	—	36.2 (8.5)[a]	31.9 (6.9)	28.4 (2.4)
1	8.4 (4.0)	16.7 (12.9)	36.2 (4.9)	37.4 (5.4)
2	7.9 (3.5)	13.3 (6.5)	38.3 (3.6)	42.6 (5.8)

[a] Numbers in parentheses are standard deviations.

About 6% of the administered radioactivity was found in the fecal extract after 2 hr. Radioactive peaks corresponded to dithiocarbamate and sulfate, which were present in roughly equal amounts.

It can be seen that the most widely distributed metabolite of I in mice is the dithiocarbamate of cystine, found in the liver, kidney, and spleen. Protein-bound components were also present in these tissues; other metabolites found were unchanged I and possibly cystine in the liver and sulfate in the kidney and spleen. The metabolism and excretion of I were quite rapid, over 50% of the injected radioactivity appearing in the urine at each time interval measured. Radioactive compounds which appeared in the urine include the dithiocarbamate, intact I, sulfate, and a small amount of unidentified substance of a higher R_f value than any of the metabolic products and considerably higher than that of intact I.

The dithiocarbamate, because of its more general distribution and the fact that a variety of dithiocarbamates have been found radioprotective (19), may contribute significantly to the radiation protection exerted by I. Unchanged I could very well contribute to the protection, since it has thiol groups capable of hydrogen atom-exchange reactions (20) as well as does the dithiocarbamate. Since irradiation of the animals is generally done 15–30 min. after administration of the candidate protecvtie agent in antiradiation screening, it appears from the results here that the dithiocarbamate is already well distributed in radiosensitive organs by this time, and intact I is present in some tissues. Although cysteine itself is radiation protective (21), it shows a much lower order of protection than does I; and since none or practically none of it was found in the mouse experiments, it can be concluded that cysteine is not contributing to the radioprotection.

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C. L. HUANG* and G. N. MIR†

Abstract □ The aqueous extract of the hypobranchial glands of *Thais haemastoma*, administered intravenously to anesthetized cats at doses equivalent to 60–90 mg. of fresh gland/kg. body weight, produced an increase in the blood pressure accompanied by tachycardia. These effects were partially blocked by hexamethonium bromide but were not modified by atropine or adrenergic blockade. In the isolated rabbit heart preparation, the extract caused an increase in the heart rate, coronary outflow, and amplitude of cardiac contractions. The extract produced marked contractions in the guinea pig ileum, rabbit duodenum, and rat uterus, which were blocked by atropine and hexamethonium bromide, and induced contractions in the frog *rectus abdominis* muscle, which were abolished by *d*-tubocurarine. It also blocked the conductions in the frog nerve–muscle preparations. It appeared that two active components were present in the hypobranchial gland extract; one produced a direct stimulatory effect on the blood pressure and heart actions and the other acted as a neuromuscular blocking agent of depolarizing type. The LD_{50} in mice was found to be 215 mg./kg.

Keyphrases □ *Thais haemastoma*—pharmacological properties of hypobranchial gland, toxicity □ Hypobranchial glands, *Thais haemastoma*—pharmacology, toxicity □ Toxicity—hypobranchial gland of *Thais haemastoma*

Thais haemastoma is a sea snail which belongs to a family of *Muricidae*. *Thais* usually feeds on oysters, and with its sharp rasplike device called the radula, it can drill through an oystershell in a few hours. Because of its carnivorous eating habit, *Thais* often presents a considerable economic threat to oyster farming. The drilling seems to be aided by acids or enzymes secreted from its salivary gland or tongue, but these secretions are not necessary for drilling. Apparently, *Thais* injects a poison into a molluscan prey at the moment it opens the shell. The poison is considered to be located in the hypobranchial gland or purple gland, which is part of the skin and appears as a conspicuous, folded glandular structure on the roof of the mantle cavity. The gland appears as three narrow, elongated parallel bands or zones oriented in an anteroposterior direction (1). Fisher (2) claimed that it functions as a genital organ, a viewpoint also held by Jullien (3, 4). Erspamer and Glassar (5) suggested that a neurotoxin, murexine, is used by the gastropods for food procurement and as a defense mechanism; however, no further information supports this speculation.

A review of the literature revealed no pharmacological study on this marine animal. Therefore, this study was undertaken to investigate the toxicity and pharmacological properties of the hypobranchial gland of *Thais haemastoma* (Clench), obtained from the Gulf of Mexico.

EXPERIMENTAL

Materials—One hundred and twenty specimens were collected near the Biloxi bridge, Biloxi, Miss., and the surrounding areas where the oysterbeds exist. Most *Thais* were taken by dredges and some in trawls. The salinity and temperature varied from 24.9 to 30.0 p.p.t. and from 15.0 to 24.9°, respectively.

The hypobranchial glands from 30 animals were weighed (10 g.) and homogenized in a blender (Waring) for 5 min., using distilled water as a solvent. The homogenate was centrifuged at 9000 r.p.m. for 5 min., and the yellowish supernatant liquid was used for pharmacological investigations. The doses used were expressed in terms of the fresh gland weight.

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Table I—Effect of Hypobranchial Gland Extract of *Thais haemastoma* on Locomotor Activity of Mice (Activity Counts/15 Min.)[a]

Minutes	75 mg./kg. Control	75 mg./kg. Hypobranchial Gland	150 mg./kg. Control	150 mg./kg. Hypobranchial Gland
15	451.3 ± 95.3	482.6 ± 178.0	482.8 ± 119.1	123.8 ± 60.5[b]
30	376.3 ± 56.3	318.8 ± 145.3	457.3 ± 153.3	77.3 ± 25.7[b]
45	305.0 ± 81.7	191.6 ± 89.5	370.1 ± 77.0	46.1 ± 5.2[c]
60	229.6 ± 50.1	116.0 ± 39.4	328.1 ± 54.9	34.6 ± 9.9[c]
75	220.0 ± 37.3	65.0 ± 29.6[b]	312.0 ± 86.1	20.0 ± 5.6[b]
90	104.5 ± 20.3	23.8 ± 5.3[c]	291.0 ± 89.9	43.3 ± 9.9[b]
105	125.1 ± 16.3	42.8 ± 13.9[c]	211.1 ± 33.3	19.3 ± 5.3[c]
120	176.0 ± 23.8	45.0 ± 11.8[c]	158.6 ± 11.6	20.8 ± 5.1[c]
Total	2003.1 ± 278.1	1285.0 ± 368.4	2608.3 ± 590.8	385.5 ± 60.6[c]

[a] Each value represents the mean ± standard error for a group of six mice. [b] $p < 0.05$ when compared with the control. [c] $p < 0.01$ when compared with the control.

glands and housed in individual cages provided with food and water. The toxic symptoms and behavioral changes were observed for 48 hr. The LD_{50} was estimated according to the method of Horn (6).

Pharmacological Studies—*Locomotor Activity*—Albino mice[1] were used for motor activity studies. An automatic timer provided the 8 a.m.–8 p.m. light schedule, and the air conditioning maintained the temperature between 22 and 24° in the room housing the animals. All mice were allowed a minimum of 7 days to adapt to these environmental conditions.

Measurement of the spontaneous locomotor activity was recorded by means of three photocell activity cages (actometers)[2], consisting of a circular runway 9 cm. wide and 20 cm. deep. Six photocells were located in the outside wall 3 cm. above the floor, and the entire actometer was covered with a sound-attenuating box. Electromechanical counters registered an activity count each time the animal traversed one-sixth the circumference of the runway. By means of a stopwatch and the counter, counts were taken every 15 min. for 2 hr. The statistical evaluation of the data in this study involved use of one-factor analysis of variance (7).

Blood Pressure and Respiration—The effects of the toxin on blood pressure, respiration, and heart were studied in anesthetized cats. Overnight fasted cats (2.5–3.5 kg.) of both sexes were anesthetized with 35–40 mg./kg. i.p. of sodium pentobarbital. The right common carotid was exposed at the neck region and cannulated with a polyethylene cannula, which was connected to a previously calibrated (linear core) pressure transducer[3] for a blood pressure recording. Respiration was recorded from chest electrodes, which were connected to an impedance pneumograph[3]. While recording respiration, the same electrodes were connected to an ECG preamplifier for obtaining standard Lead II electrocardiograms. The ECG preamplifier was calibrated in such a manner that each deflection represented 1 mv. in amplitude and 10 mm. in height. The pressure transducer, pneumograph, and ECG preamplifier were connected to a physiograph[4] with standard transducer cables for recording. The ECG recordings were interpreted according to the description of Burch and Winsor (8) and Friedman (9).

All injections were made through the right femoral vein, which was exposed and cannulated with a venous polyethylene cannula. The cholinergic activity of the toxin was studied in bilaterally vagotomized and completely atropinized animals; *i.e.*, both vagi were cut at the cervical level and the animals were injected with atropine (3 mg./kg. i.v.), and complete parasympathetic blockade was ensured when no response was obtained with a test dose of acetylcholine (5 mcg./kg. i.v.). Autonomic ganglia were blocked by the administration of hexamethonium bromide (5 mg./kg. i.v.) in three successive doses. The animals were injected with phenoxybenzamine (3 mg./kg.) for a complete blockade of the peripheral sympathetic α-receptors, which was ensured by observing reversal of the action of epinephrine (5 mcg./kg. i.v.) on the blood pressure. β-Receptors were blocked by dichloroisoproterenol (4 mg./kg. i.v.). To determine the action on the baroreceptors of the carotid sinus, carotid occlusion was performed three times for 30 sec., both before and after the administration of the toxin.

Isolated Heart Preparation—The effect of the toxin on the isolated rabbit heart was studied by Langedorff's technique, as modified by Anderson and Craver (10). The chest was opened and the heart was removed. It was placed into an aerated Locke's solution and gently squeezed to remove the blood from the aorta. The aorta was freed from its attachment to the pulmonary artery, and a cannula was tied to it. A hook with thread was fastened on the tip of the ventricle, and the heart was perfused. The thread was passed through a pulley and attached to a myograph[5], which was connected to the physiograph[4] by standard transducer cables for recording. A constant pressure was maintained throughout the perfusion.

Isolated Hind Quarter of the Rat—The action of the hypobranchial gland extract on the blood vessels was studied with the hind-quarter preparation of albino rats (11). The animals were sacrificed and eviscerated. A cannula was tied to the abdominal aorta, and the body wall and vertebral column were cut above the point of cannulation. The hind quarter of the rat was placed on a circular muslin attached to a wire resting on a glass funnel. A marriotte bottle containing Ringer's solution was connected to the cannula by a rubber tubing, and the preparation was perfused until the perfusage was free from blood. The outflow from the vessels passed through the muslin into the funnel and was measured dropwise. A constant pressure was maintained throughout the perfusion, and all injections were made directly into the connecting rubber tubing.

Isolated Smooth Muscle—Rabbits, 1–2 kg., and guinea pigs, 300–500 g., of both sexes were used. The animals were sacrificed and the intestines exposed. An actively contracting loop of ileum (guinea pig) or duodenum (rabbit) of 4–6 cm. in length was selected and cut. This was suspended in a 25-ml. bath containing Tyrode's (guinea pig ileum) or Ringer's solution (rabbit duodenum). One end of the loop was tied to the hook on a holder and the other end attached to the myograph. The preparation was suspended in an organ bath with Tyrode's solution, which was maintained at 37° and aerated with 95% O_2–5% CO_2.

Isolated Uterus Preparation—Young female rats, weighing 150–200 g., were used. Estrus was induced by injecting 0.1 mg./kg. s.c. of stilbestrol 24 hr. prior to the experiment. The animal was sacrificed, and both horns of the uterus were isolated. The horns were transferred to a dish containing warm DeJalon's solution (NaCl, 9.0; KCl, 0.42; glucose, 0.5; $NaHCO_3$, 0.5; and $CaCl_2$, 0.03 g./l.), and each horn was cut longitudinally into two strips. The threads were tied to the end of the strip (about 2 cm. in length) and mounted in an organ bath (25 ml.) containing DeJalon's solution, which was maintained at 30–37°, pH 7.4, and aerated with 95% O_2–5% CO_2. The preparation was left in the bath for 30 min. to equilibrate.

Frog Rectus Abdominis *Muscle Preparation*—The frog *rectus abdominis* was obtained from *Rana pipiens* according to the method of Burn (11). The frog was decapitated and pithed. The muscle was dissected from the pelvic girdle to its insertion in the cartilage of the pectoral girdle. Threads were attached to both ends of the muscle, and an initial weight of 0.5–1.0 g. was applied on it. The preparation was kept at room temperature (25°) in frog Ringer's solution, which was aerated with 95% O_2–5% CO_2. An interval of about 30 min. was allowed for the preparation to stabilize.

[1] Southern Animal Farms, Prattville, Ala.
[2] Model R064, Woodard Research Corp., Herndon, Va.
[3] E & M Instrument Co., Inc., Houston, Tex.
[4] NARCO, model DMP-4A.
[5] Model B-655, E & M Instrument Co., Inc., Houston, Tex.

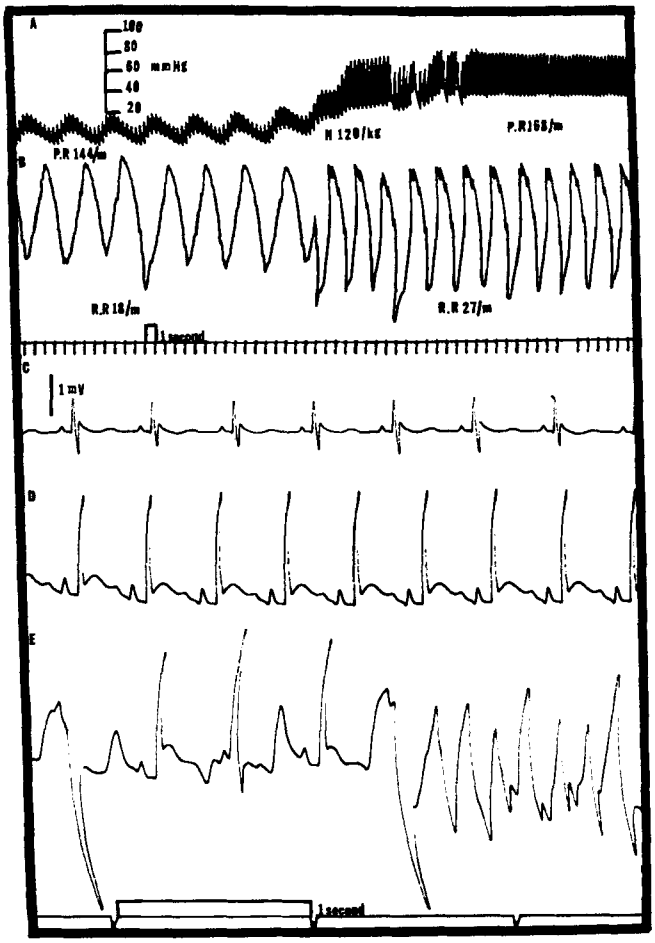

Figure 1—*Effect of hypobranchial gland extract of* Thais haemastoma *on: A, blood pressure*; B, respiration; C, ECG (control); D, ECG at dose level of 100 mg./kg.; and E, ECG at dose level of 150 mg./kg. H = hypobranchial gland extract, PR = pulse rate, RR = respiratory rate, m = minute, and mv = millivolt. (* No initial blood pressure is shown on the scale; instead the scale is divided in such a manner that initial blood pressure reads as 0.00 mm. Hg. The pressure transducer was calibrated for a 100-mm. Hg rise or fall in the blood pressure, which is shown on the scale.)*

Nerve–Muscle Preparation—The frog was decapitated, pithed, and pinned prone on a frog board. The *gastrocnemius* was isolated at the tendon, and a thread was fastened around it. The tendon was cut free from its attachment to the heel and the muscle connected to the myograph with a thread. The skin on the back of the animal above the urostyle was dissected. The sciatic nerve was exposed, and a sleeve electrode connected to the stimulator cable was placed around it. Both single and continuous stimuli of 2-msec. duration at 25 v. were applied.

RESULTS

Toxicity Studies—With doses of 25–100 mg./kg., there was an initial increase in the muscular tension, followed by a relaxation of varied duration depending upon the dose. Doses higher than 100 mg./kg. produced an immediate muscular relaxation. With lethal doses, mild convulsions were noted in some animals before death. The LD_{50} in mice was found to be 215 mg./kg.

Pharmacological Properties—*Locomotor Activity*—As shown in Table I, 75 mg./kg. of the toxin did not produce a significant change in the motor activity for the first 75 min. However, there was a significant reduction in the motor activity after this period. With 150 mg./kg., significant changes in the motor activity were observed within the first 15 min. and continued for the next 2 hr.

Effect on Blood Pressure and Respiration of the Cat—Intravenous injection of the toxin in graded doses from 30 to 50 mg./kg. produced little effect on the blood pressure, pulse rate, and respiration in anesthetized cats. Doses from 60 to 90 mg./kg. showed a transient rise in the blood pressure (20–40 mm. Hg), with a slight increase in the heart rate and respiratory rate. Larger doses of the substance, from 100 to 150 mg./kg., produced an immediate and persistent rise (30–50 mm. Hg) in the blood pressure (Fig. 1), accompanied by an increase in the pulse rate and respiratory rate which lasted for approximately 30 min. The pressor effect was not blocked either by atropinization or bilateral vagotomy, except by hexamethonium bromide which was able to antagonize partially the effect on the blood pressure.

Effect on ECG of the Cat—With doses from 100 to 150 mg./kg., there was an increase in the amplitude of the P wave and R wave and an increase in the P-T interval (Fig. 1D) followed by an irregular ventricular beat. Before the animal died, the characteristic pattern of the ventricular beat was lost, and most of the beats presented a rapid sequence of broad oscillation, on top of which a rudimentary Q-R-S complex is indicated by notching (Fig. 1E). Dichloroisoproterenol, a β-adrenergic blocker, reduced the effect of the toxin on the heart, particularly when irregular ventricular rhythms appeared.

Effect on Isolated Rabbit Heart– The control values (10 observations) for coronary flow rate (2.3 ± 0.4 ml./min.), heart rate (65 ± 8 beats/min.), and amplitude of cardiac contractions (25 ± 5 mm. deflection) were consistent throughout the experiment. The injection of various doses of toxin into the coronary perfusion circuit brought about changes in all three parameters. Four milligrams injected into the perfusion fluid (Fig. 2A) produced an immediate increase in the coronary flow rate (3.5 ± 0.3 ml./min.), heart rate (135 ± 5 beats/min.), and amplitude of cardiac contractions (30 ± 3 mm. deflection). The effect lasted for 5 min. or more.

When 8 mg. of the toxin was injected into the perfusion fluid, stimulatory effects were noted in all three parameters. Initially, several irregular beats were observed (Fig. 2B) for the first 10–15 sec. but, thereafter, the heart became regular and continued beating

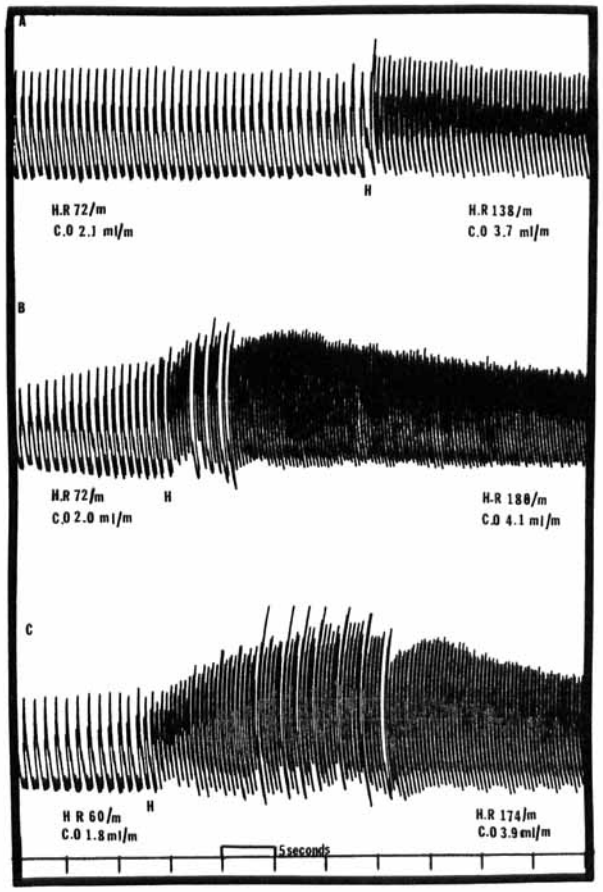

Figure 2—*Effect of hypobranchial gland extract of* Thais haemastoma *on isolated rabbit heart. Key: A, 4 mg.; B, 8 mg.; and C, 12 mg. H = hypobranchial gland extract, HR = heart rate, CO = coronary outflow, and m = minute.*

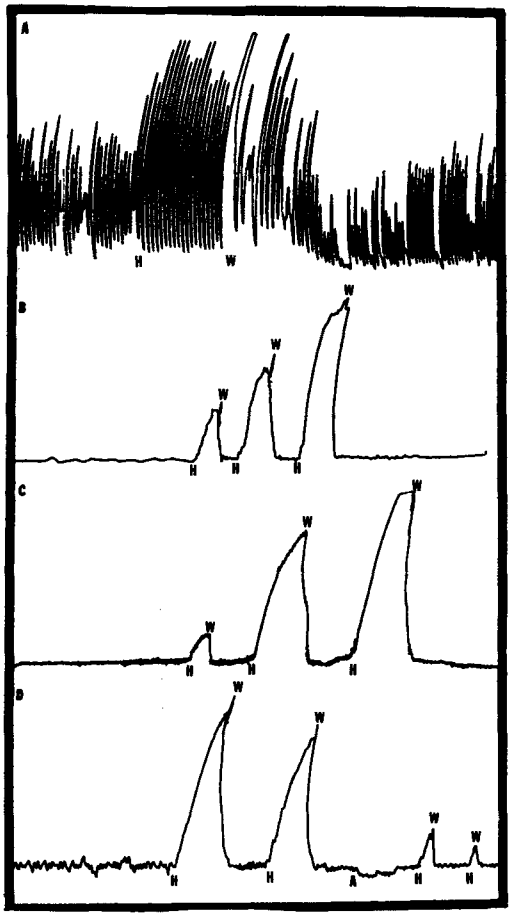

Figure 3—*Effect of hypobranchial gland extract of* Thais haemastoma *on: A, isolated rabbit duodenum (300 mcg./ml.); B, guinea pig ileum (200, 400, and 600 mcg./ml.); C, rat uterus (200, 400, and 600 mcg./ml.); and D, guinea pig ileum (showing blockade of the effect of the toxin, 300 mcg./ml., by atropine sulfate, 0.1 mcg./ml.). H = hypobranchial gland extract, W = wash, and A = atropine sulfate.*

normally for about 10 min. The values for the three parameters were: coronary outflow, 4.0 ± 0.2 ml./min.; heart rate, 185 ± 5 beats/min.; and amplitude of cardiac contractions, 32 ± 2 mm. deflection.

Immediately following the injection of a 12-mg. dose, the heart started beating irregularly (Fig. 2C), which lasted for more than 40 sec. The values for the three parameters were: coronary outflow, 4 ± 0.2 ml./min.; heart rate, 180 ± 5 beats/min.; and amplitude of cardiac contractions, 32 ± 2 mm. deflection. The effect of this dose lasted for 15 min. or longer; then all three parameters declined slowly and ultimately returned to the control levels.

Effect on Isolated Rabbit Duodenum—The isolated loop of the rabbit duodenum responded to 300 mcg./ml. of the toxin (Fig. 3A) with contractions. The high doses of the toxin produced an intense spasm of the intestinal loop, followed by rhythmic movements of increasing amplitude. The stimulatory effect of the duodenum loop lasted for more than 15 min. and ceased abruptly after washing with physiological saline solution.

Effect on Guinea Pig Ileum—Like the rabbit duodenum, guinea pig ileum responded to the toxin with contractions. The threshold concentration was 200–600 mcg./ml., with a good dose–response relationship (Fig. 3B). Atropine sulfate in doses (0.1 mcg./ml.) that partially abolished the effect of acetylcholine (0.1–0.2 mcg./ml.) also abolished the effect of the toxin (Fig. 3D). Hexamethonium bromide completely abolished the effect of the toxin on the guinea pig ileum.

Effect on Isolated Uterine Muscle—The toxin caused contractions of the uterine strip. The threshold concentration was 200–600 mcg./ml., and a good dose–response relationship was obtained (Fig. 3C). Atropine sulfate partially blocked the effect of the toxin on the uterine muscle.

Effect on Isolated Frog Rectus Abdominis *Muscle*—When added to the fluid bath containing the *rectus abdominis*, the toxin produced contractions with a slower onset of time than that of acetylcholine but with a comparable amplitude. The threshold dose was 300 mcg./ml. This effect was blocked by 16 mcg./ml. of *d*-tubocurarine (Fig. 4A).

Effect on Frog Nerve–Muscle Preparation—The toxin (20 mg.) injected into the dorsal lymph sac of the frog produced a complete neuromuscular blockage (Fig. 4B) characterized by the loss of contractions and the action potentials when the motor nerve was stimulated.

DISCUSSION

Hypobranchial gland toxin of *Thais haemastoma* was found to be toxic to mice, having an LD_{50} of 215 mg./kg. In gross observations and motor activity studies, the toxin produced muscular relaxation of varied duration depending upon the dose.

The toxin at various dose levels produced a hypertensive effect. The conclusion that the hypertensive effect produced by the toxin was due partially to autonomic ganglionic stimulation was indicated by the partial blockage of the rise in the arterial blood pressure by hexamethonium bromide.

The ECG recordings from the cats injected with the toxin showed an increase in the P wave amplitude, P-T interval, and R wave amplitude. With higher doses, ventricular beats of various types were observed, which seemed to emanate from different ventricular centers. However, no distinct evidence of auricular activity was noted and, finally, the heart went into ventricular fibrillation. Evidence indicated that the cardiac arrhythmia was partially blocked by β-adrenergic blocking agents, suggesting that part of the effects of the toxin on the heart may be mediated through β-receptors.

The effects on the isolated heart preparation indicated that the toxin was a powerful cardiac stimulant. All three parameters (heart rate, coronary outflow, and amplitude of cardiac contractions) were increased.

The effects on the ECG and isolated heart preparation suggested that the toxin acted directly on β-receptors of the myocardium, cells of the pacemaker, and the conduction system. Cardiac contractions were more powerful, cardiac output was enhanced, and the work of the heart was markedly increased by the toxin. The action of the toxin in inducing arrhythmias and, to some extent, in inducing cardiac automaticity was antagonized by β-blocking agents.

The rise in the blood pressure may be due to two factors: (*a*) autonomic ganglion stimulation, and (*b*) increased cardiac output observed in the isolated heart preparation.

The toxin produced contractions in the rabbit duodenum, guinea pig ileum, and rat uterus. A constant dose–response relationship

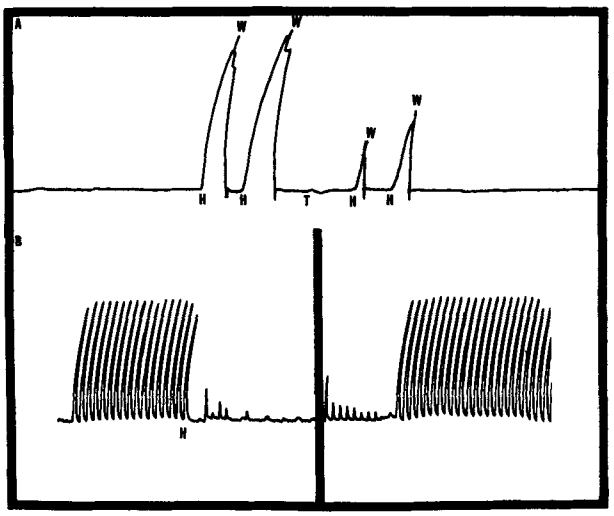

Figure 4—*Effect of hypobranchial gland extract of* Thais haemastoma *on: A, frog* rectus abdominis *muscle (30 mcg./ml.); and B, frog nerve–muscle preparation (20 mg. injected into the dorsal lymph sac). H = hypobranchial gland extract, W = wash, and T =* d*-tubocurarine.*

was noted. Both atropine sulfate and hexamethonium bromide reduced the spasmogenic effect of the toxin, suggesting that the toxin had a nicotinic effect. The muscarinic action, if any existed, was very weak.

On the isolated skeletal muscle, the toxin produced contractions which were blocked by *d*-tubocurarine. The contracture response in the skeletal muscle and the blockage of the transmission of nerve impulses in the frog nerve–muscle preparation suggested that the toxin was a depolarizing blocking agent.

The results of the experiments on the isolated tissues were in accord with the findings of Erspamer and Glasser (5) and Quilliam (12) on murexine obtained from *Murex trunculus*. The *Thais* toxin produced a blockage in the vertebrate neuromuscular transmission similar to that produced by murexine. The blockades produced by murexine and *Thais* toxin were preceded by a muscular fasciculation which was antagonized by *d*-tubocurarine.

Erspamer and Glasser (5) observed that murexine caused a biphasic response in blood pressure, namely, a transient fall followed by a rise in the blood pressure which was abolished by hexamethonium bromide. On the isolated rabbit heart preparation, murexine in smaller doses had little effect, but higher doses showed depression of the heart actions. Unlike murexine, the toxin from the hypobranchial gland of *Thais haemastoma* did not exhibit an initial fall in the blood pressure but rather produced a sustained rise which was partially antagonized by hexamethonium bromide. The observation that the toxin produced a simultaneous increase in the heart rate, coronary outflow, and force of contraction was in accord with earlier observations of Roaf and Nierenstein (13), in which they suggested that hypobranchial glands of *Purpura lapillus* had a physiological activity similar to that of adrenaline.

These observations suggested that the toxin of the hypobranchial gland of *Thais haemastoma* contained two principles: (*a*) murexine, which was shown by the effect of the toxin on isolated smooth and skeletal muscles and its neuromuscular blocking properties, and (*b*) another substance, which acted as a cardiotonic agent as indicated by the effect of the toxin on the blood pressure, intact heart, and isolated heart preparations.

SUMMARY

Hypobranchial gland of *Thais haemastoma* produced a yellowish viscid slime. The slime was found to be toxic to mice, having an LD_{50} of 215 mg./kg. The toxin produced muscular relaxation of varied duration in mice. The toxin produced a hypertensive effect in anesthetized cats. This effect seemed to be partially due to ganglionic stimulation, as indicated by a partial blockage by hexamethonium bromide. The rest of the hypertensive effect could be attributed to an increased cardiac output, observed in both intact and isolated hearts. The effects on the ECG and isolated heart suggested that the toxin acted directly on β-receptors of the myocardium, cells of the pacemaker, and conduction system. Cardiac contractions were more powerful, cardiac output was enhanced, and the work of the heart was markedly increased by the toxin. The action of the toxin in inducing arrhythmias and, to some extent, in inducing cardiac automaticity was antagonized by a β-blocking agent. The toxin produced contractions in various smooth muscles, which were blocked by hexamethonium bromide, suggesting that the toxin had a nicotinic action. The toxin produced contractions in the isolated skeletal muscles which were blocked by *d*-tubocurarine. The toxin blocked conduction in the frog nerve–muscle preparation, suggesting that the toxin is a neuromuscular blocking agent of the depolarizing type.

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Nonaqueous Titration of Barbiturates in Tetramethylurea

C. H. HSIAO, H. J. RHODES, and M. I. BLAKE

Abstract □ Tetramethylurea was evaluated as a solvent for the nonaqueous titration of barbiturates and several of their dosage forms and combinations. Tetrabutylammonium hydroxide and sodium methoxide in benzene–methanol were used as titrants. Titrations were performed visually and potentiometrically with a titrimeter equipped with a glass–calomel electrode system. Comparison was made with the solvent dimethylformamide.

Keyphrases □ Barbiturates—nonaqueous titration in tetramethylurea □ Tetramethylurea, solvent evaluation—nonaqueous titration of barbiturates □ Titration, nonaqueous—barbiturates in tetramethylurea

The physical properties of tetramethylurea were described by Luttringhaus and Dirksen (1), who noted that tetramethylurea is an excellent solvent for a wide variety of organic compounds. Salts and polar compounds, in general, show a lower solubility in this solvent. It is stable, inert, and practically odorless; it has a low toxicity and is commercially available at moderate cost. It can be prepared in a high state of purity and essentially anhydrous. Tetramethylurea has not been studied to any great extent for its use as a nonaqueous titration solvent.

Culp and Caruso (2) titrated potentiometrically several phenols and carboxylic acids using tetrabutylammonium hydroxide as the titrant. The authors concluded that tetramethylurea is a useful solvent for titrating very weak to strong acids. Differentiation of benzoic acid and phenol was unsuccessful. In a subsequent paper, these authors (3) found thymol blue, phenolphthalein, and azo violet as useful indicators for the visual titration of acids in tetramethylurea. Azo violet proved the most useful for very weak acids.

In the present study, tetramethylurea was compared with dimethylformamide as a solvent for the titration of a series of barbiturates and a number of their dosage forms using tetrabutylammonium hydroxide as the titrant.

EXPERIMENTAL

Apparatus—Titrations were performed visually or potentiometrically with a titrimeter[1] equipped with microattachment accessories including a microcalomel[2] and glass[3] electrode system.

A 10-ml. buret, graduated in 0.02-ml. increments and equipped with a Teflon stopcock and a Teflon delivery tip tapered to a small bore, was used to deliver the titrants. Titrations were performed in a 10-ml. beaker, and stirring was effected with a magnetic stirrer using a 1-cm. length nail encased in Teflon as the stirring bar.

[1] Fisher model 35.
[2] Fisher No. 13-639-79.
[3] Fisher No. 13-639-77.

Reagents—Tetramethylurea was obtained commercially[4]. Sodium methoxide, 0.1 *N*, in benzene–methanol (10:1) was prepared and standardized as described earlier (4). Tetrabutylammonium hydroxide, 0.1 *N*, was prepared and standardized as reported by Cundiff and Markunas (5) or was purchased[5] as a 25% solution in methanol. The titrant solution was prepared by diluting 25 ml. to 200 ml. with dry benzene and was then standardized. The barbiturates and their dosage forms were obtained from commercial sources. Thymol blue indicator solution was 0.3% in anhydrous methanol, and phenolphthalein indicator was 1.0% in anhydrous methanol. Other chemicals and all solvents used in this study were reagent grade and were employed without further purification.

General Procedure for Free Barbiturates—About 50 mg. of each barbiturate, accurately weighed into a 10-ml. beaker, was dissolved in 5 ml. of tetramethylurea or dimethylformamide. The solution, magnetically stirred, was titrated potentiometrically with 0.1 *N* tetrabutylammonium hydroxide or 0.1 *N* sodium methoxide. During the titration, the tip of the buret was immersed into the titration solution. The end-point in the titration curve was determined from the inflection in the titration curve obtained by plotting volume (milliliters) of titrant added *versus* millivolt readings. Typical titration curves are shown in Fig. 1, and analysis data are recorded in Table I. The feasibility of a visual titration was investigated by adding two drops of thymol blue or phenolphthalein indicator solution to the titration solution prior to a potentiometric titration. The appropriate color change at the end-point in the titration was determined by comparing the color change with the graphic end-point. For all visual titrations, a blank determination was conducted and the necessary corrections were made.

Analysis of Barbiturate Tablets—Twenty tablets, accurately weighed, were triturated to a fine powder. An aliquot of the powder

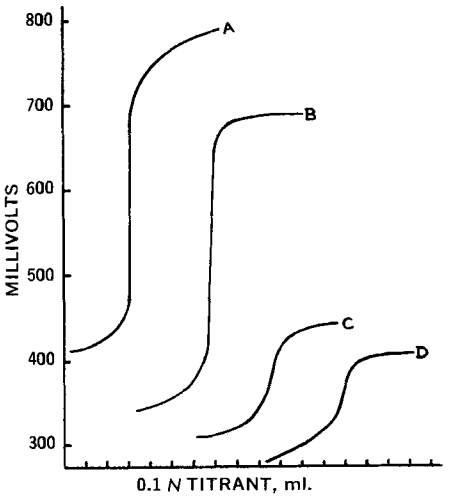

Figure 1—*Typical titration curves for free barbiturates. Curves A and B represent titrations in tetramethylurea and dimethylformamide, respectively, using tetrabutylammonium hydroxide as the titrant. Curves C and D represent titrations in tetramethylurea and dimethylformamide, respectively, using sodium methoxide as the titrant.*

[4] Ott Chemical Co., Muskegon, Mich.
[5] Eastman.

Table I—Analysis Data for Free Barbiturates

Powder	Recovery, %: TBAH[a] TMU[c]	TBAH[a] DMF[d]	SMO[b] TMU	SMO[b] DMF
Amobarbital	101.27 ± 0.41[e]	102.82 ± 0.20	96.03 ± 1.14	99.16 ± 0.33
Barbital	100.99 ± 0.33	100.49 ± 0.41	98.69 ± 0.10	99.35 ± 0.47
Hexobarbital	97.96 ± 1.69	98.60 ± 1.27	97.13 ± 0.23	98.61 ± 0.11
Phenobarbital	100.50 ± 0.16	100.30 ± 0.31	99.41 ± 0.39	99.25 ± 0.90
5-Allyl-5-ethylbarbituric acid	100.21 ± 1.08	102.73 ± 0.60	97.43 ± 0.11	98.87 ± 0.07
5-Ethylbarbituric acid	99.69 ± 0.13	99.25 ± 0.84	97.77 ± 0.43	98.69 ± 0.23
Ethylbenzylbarbituric acid	102.67 ± 0.28	102.11 ± 0.53	95.30 ± 0.41	98.44 ± 1.49
Ethylisoamylthiobarbituric acid	100.76 ± 0.11	101.94 ± 0.36	95.16 ± 0.50	95.06 ± 0.58
2-Hydroxypropyl-*n*-hexyl-thiobarbituric acid	102.21 ± 0.45	97.53 ± 2.14	98.77 ± 0.47	96.30 ± 0.06
5-Isopropylbarbituric acid	100.10 ± 0.38	99.67 ± 0.80	99.29 ± 0.31	99.17 ± 0.04

[a] Tetrabutylammonium hydroxide. [b] Sodium methoxide [c] Tetramethylurea. [d] *N,N*-Dimethylformamide. [e] Standard deviation is based on at least three determinations.

mass equivalent to about 50 mg. of barbiturate was accurately weighed into a 10-ml. beaker and dissolved in 5 ml. of tetramethylurea or dimethylformamide. The solution, magnetically stirred, was titrated potentiometrically with 0.1 *N* tetrabutylammonium hydroxide. The feasibility of a visual titration was explored. The data are recorded in Table II.

Extent of Interference by Water—The extent to which water interferes with the determination and detection of the potentiometric and visual end-points, respectively, was studied by titrating phenobarbital in tetramethylurea and dimethylformamide containing increasing percentages of water. Two series of stock solutions were prepared, the first containing 1–10% of water (v/v) in tetramethylurea, and the second containing 1–60% water (v/v) in dimethylformamide. About 50 mg. of phenobarbital, accurately weighed, was dissolved in 5 ml. of the stock solution in a 10-ml. beaker, and the solution was titrated visually or potentiometrically with 0.1 *N* tetrabutylammonium hydroxide as described previously. Typical titration curves are shown in Fig. 2.

Sensitivity of Procedure—The sensitivity of the titration procedures in tetramethylurea and dimethylformamide was evaluated by titrating decreasing sample weights of phenobarbital with 0.01 *N* tetrabutylammonium hydroxide. The 0.01 *N* tetrabutylammonium hydroxide was prepared by diluting 0.1 *N* tetrabutylammonium hydroxide with benzene–methanol (10:1) and standardizing the solution against primary standard benzoic acid. A series of stock solutions was prepared which contained 1–10 mg. of phenobarbital/5 ml. of solution. A 5-ml. aliquot of each solution was transferred by pipet to a 10-ml. beaker and was titrated visually or potentiometrically as described previously.

RESULTS AND DISCUSSION

Since tetramethylurea is an excellent solvent for most classes of organic compounds and is completely miscible with water, and since it is not reactive, it seems to be a solvent worth investigating as a titration medium. To date, little interest has been shown in tetramethylurea for this purpose, as is reflected in the dearth of publications appearing in the literature.

A series of barbiturates and typical dosage forms were studied here because they are weak organic acids of pharmaceutical importance and have been the subject of numerous studies involving titration in nonaqueous solvents. These were noted earlier (6). Sodium methoxide and tetrabutylammonium hydroxide were used as titrants, and comparisons were made between tetramethylurea and dimethylformamide as titration solvents. The similarity in the potentiometric titration curves is shown in Fig. 1. While millivolt changes and the overall inflection in the titration curves are essentially the same for both solvents, tetrabutylammonium hydroxide produces significantly greater millivolt changes at the end-point than does sodium methoxide. Analysis data shown in Table I indicate quantitative recoveries for eight barbiturates in the four systems studied. While the data were calculated on the basis of the potentiometric end-points, satisfactory end-points were also obtainable with thymol blue indicator. Color changes (yellow to blue) were equally sensitive in both dimethylformamide and tetramethylurea. The phenolphthalein end-point was less satisfactory and appeared consistently prior to the graphic end-point. Thymol blue indicator permitted detection of the end-point with a single drop of titrant addition in either solvent with both titrants.

A series of tablet dosage forms containing barbiturates was analyzed by potentiometric titration using tetrabutylammonium hydroxide as titrant and tetramethylurea and dimethylformamide as the titration solvent. The analysis data are recorded in Table II. Essentially the same results were obtained in either solvent. A high recovery is reported for hexobarbital tablets in both solvents. Since the tablets were not pretreated to isolate the barbiturate from the excipients in the tablet, other acidic components in the formulation may have interfered in the titration. If this was the case, the pKa value for the interfering acid must have approximated that of the barbituric acid, since only one inflection in the titration curve was noted. On the other hand, the high recovery may indicate a high barbiturate content in the tablet. The analysis data for all other dosage forms appear reasonable and within acceptable limits of barbiturate content.

The dosage forms were titrated only with tetrabutylammonium hydroxide because it produced greater millivolt changes at the end-

Table II—Analysis Data for Barbiturate Dosage Forms

Tablet	Dosage Size, mg.	Recovery, %: TMU[a]–TBAH[b]	DMF[c]–TBAH
Amobarbital	100	96.66 ± 0.39[d]	96.24 ± 0.49
Barbital	325	100.33 ± 1.28	100.58 ± 0.99
Hexobarbital	250	106.75 ± 0.71	107.12 ± 0.16
Mephobarbital	32	104.45 ± 0.09	103.82 ± 0.25
Mephobarbital	100	104.88 ± 0.65	101.82 ± 0.20
Mephobarbital	200	100.95 ± 0.32	101.00 ± 0.55
Phenobarbital	15	94.74 ± 0.44	95.53 ± 0.18
Phenobarbital	30	99.35 ± 0.99	99.15 ± 0.17
Phenobarbital	60	97.74 ± 0.17	99.19 ± 0.30
Phenobarbital	100	99.73 ± 0.66	99.30 ± 0.43

[a] Tetramethylurea. [b] Tetrabutylammonium hydroxide. [c] *N,N*-Dimethylformamide. [d] Standard deviation is based on at least three determinations.

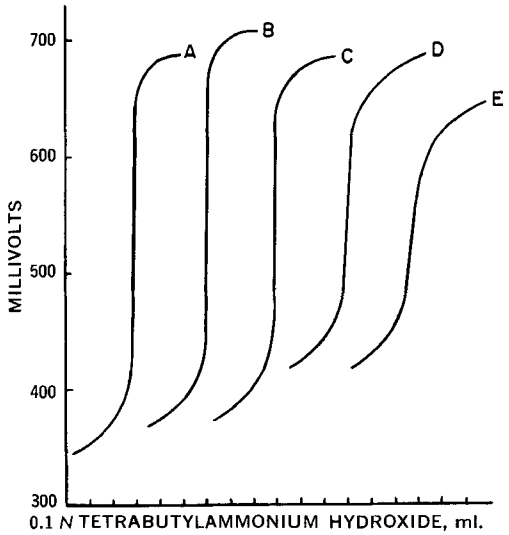

Figure 2—*Effect of water on the titration of phenobarbital in dimethylformamide. Curves A, B, C, D, and E represent 0, 5, 10, 30, and 60% (v/v) of water in dimethylformamide, respectively. The curves have been displaced to show the effect of water on the nature of the titration curve. The actual end-point was not affected.*

point than did sodium methoxide. Preliminary studies with the latter indicated that there was considerable drifting of the titrimeter scale needle as the end-point was approached and, in general, the titration was less satisfactory. Visual titration of the dosage forms was unsuccessful because of interference of the excipients with detection of the end-point color change due to the turbidity of the solution.

The presence of water did not apparently interfere in the titration when dimethylformamide was the solvent. A series of titration curves is shown in Fig. 2 where phenobarbital is titrated in dimethylformamide containing increasing concentrations of water. When the water content was 30% or higher (Curves D and E), the titration mixture separated into two layers[6] which became emulsified through rapid stirring. This did not interfere with the potentiometric titration. When significant amounts of water were present (20% or more), the visual end-point became obscure and visual titration was not possible. At water concentrations over 60%, titrations were not possible because of the insolubility of the barbiturate in the solvent mixture.

In tetramethylurea, water concentrations as low as 1% caused considerable drifting in the titrimeter needle as the end-point was approached and did not permit a satisfactory potentiometric titration. Because of the difficulty in end-point detection in the presence of water, percent recoveries were not determined. Visual titrations did not produce reproducible end-points in the presence of water. It is apparent that tetramethylurea must be anhydrous when used as a solvent for nonaqueous titrations.

[6] The upper layer is apparently benzene from the titrant solvent being thrown out by the water in the titration mixture.

Table III—Sensitivity of Potentiometric Titration Procedure for Phenobarbital

Phenobarbital Weight Taken, mg.	Recovery, % in DMF[a]	Recovery, % in TMU[b]
50[c]	100.50 ± 0.16[d]	100.30 ± 0.31
10	104.93 ± 0.25	103.66 ± 0.22
5	102.93 ± 0.25	103.95 ± 1.90
1	99.84 ± 1.01	98.29 ± 2.24

[a] *N,N*-Dimethylformamide. [b] Tetramethylurea. [c] 0.1 *N* titrant used for this sample size. [d] Average deviation is based on at least three determinations.

The sensitivity of potentiometric titrations in tetramethylurea and dimethylformamide was studied by titrating phenobarbital over the concentration range of 1–50 mg., using 0.01 *M* tetrabutylammonium hydroxide as titrant. Recovery data are shown in Table III. At low concentrations of phenobarbital, titrations appear to be more successful in dimethylformamide than in tetramethylurea. Visual end-points were not readily detectable in either system.

Tetramethylurea appears to be a useful solvent for titrating weak acids, free and combined in tablet dosage forms. Further studies will be required to determine whether it is as widely applicable as dimethylformamide, dimethyl sulfoxide, and methyl isobutyl ketone as a nonaqueous titration solvent. Such studies are currently in progress.

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Colorimetric Assay Procedure for Dissolution Studies of Meprobamate Formulations

JOHN W. POOLE, GEORGE M. IRWIN, and STEPHEN YOUNG

Abstract □ A colorimetric analytical procedure applicable to N—H-containing compounds was employed in dissolution studies of meprobamate formulations. The method, which can be applied directly to the aqueous dissolution sample, gave results comparable to the official GC analytical procedure.

Keyphrases □ Meprobamate—colorimetric analysis for dissolution studies, compared to compendial methods □ Dissolution studies, meprobamate—development of applicable colorimetric method □ Colorimetry—meprobamate analysis in dissolution studies, compared to compendial methods

The dissolution test for meprobamate tablets in USP XVIII (1) specifies a GC procedure for analysis of drug in solution. This procedure requires extracting the active compound from the aqueous dissolution sample before analytical measurement. A colorimetric method that can be applied directly to the aqueous dissolution sample was employed in these laboratories. This method provided results consistent with those obtained using the compendia-recommended GC procedure. The colorimetric method utilized in this work was first reported by Ellis and Hetzel (2) for the determination of meprobamate in urine and was modified by Zappala and Simpson (3) for the determination of panthenol in multivitamin preparations.

EXPERIMENTAL

Dissolution—The dissolution of meprobamate from an experimental lot of meprobamate tablets was determined by the method described in USP XVIII (1). The aqueous samples obtained were analyzed for meprobamate in solution by both GC and colorimetric procedures.

Reagents—*Borate Buffer, 0.05* M—Dissolve 3.1 g. of reagent grade boric acid and 3.7 g. of potassium chloride in about 900 ml. of water. Adjust to pH 10.5 with 2 *N* NaOH and dilute to 1 l. with water.

Chlorinating Solution—Dilute a 5.25% sodium hypochlorite solution (Clorox) (1:30) with 0.05 *M* borate buffer. Prepare this solution daily and protect from light.

Acidified Phenol Solution—Use 0.5% phenol in 0.1 *M* hydrochloric acid.

Potassium Iodide Solution—Use 1.0% potassium iodide in distilled water. Prepare this solution daily and protect from light.

Colorimetric Procedure—Place 1 ml. of an aqueous solution containing 0.025–0.25 mg. meprobamate/ml. in a 50-ml. volumetric flask. Add 2 ml. of chlorinating solution, taking care to deliver the reagent directly into the solution without getting any on the neck of the flask. Swirl the solution, stopper the flask, and allow it to stand for 20 min. at room temperature. Add 2 ml. acidified phenol reagent, rinsing down the neck of the flask. Again, swirl the solution, stopper the flask and allow it to stand for 5 min. Add 1 ml. potassium iodide solution, and swirl the flask. After 15 sec., dilute the sample to the mark with absolute ethanol. Wait 15 min. and determine the absorbance at 358 nm. on a suitable spectrophotometer. A blank of 1.0 ml. of distilled water taken through the same procedure serves as reference.

Table I—Percent of Meprobamate in Solution at 30 Min. from Six Individual Dosage Units of an Experimental Tablet Formulation Determined by Two Methods

	Percent of Labeled Meprobamate in Solution	
	GC	Colorimetric
	84	88
	85	92
	94	88
	82	86
	88	90
	91	88
Average	87.3	88.7
Coefficient of variation	5.2	2.3

RESULTS AND DISCUSSION

The procedure as described was shown to adhere to Beer's law over the range of 0.025–0.25 mg. meprobamate. The precision of the analytical method was tested on a series of 10 replicate samples containing 0.25 mg. meprobamate/ml., and the coefficient of variation found was 1.54%.

The dissolution of meprobamate from a series of experimental and trade formulations of this compound employing a variety of common tablet excipients was evaluated by this procedure. In every case, the method was satisfactory and no analytical interference was observed. However, only one dosage unit was utilized in these studies with not less than 500 ml. of solvent. Therefore, the excipient concentration in the analytical sample was always small, with 0.07 mg./ml. being the maximum concentration attained.

It is obvious that interference will be encountered with products incorporating N—H-containing substances, either as excipients or active components. In such instances, appropriate separation techniques must be employed prior to analysis.

A dissolution test was completed on an experimental lot of tablets on which both the compendia-recommended GC and colorimetric analytical procedures were used (Table I). The average percent in solution for the GC procedure was 87.3%, with a coefficient of variation of 5.2%; the colorimetric method gave an average percent in solution of 88.7%, with a coefficient of variation of 2.3%.

Whereas the GC method requires an extraction step, with subsequent evaporation and redilution of the sample, the colorimetric assay has an advantage in that the aqueous sample can be analyzed directly.

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Coulometric Titration of *N*-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide Hydrochloride

S. OLIVERI-VIGH*, J. J. DONAHUE, J. E. HEVERAN†, and B. Z. SENKOWSKI

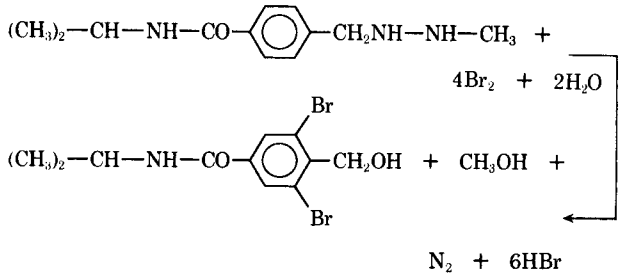
Abstract □ A coulometric titration for the determination of *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride, procarbazine hydrochloride, is described. The method is specific and is based on the quantitative oxidation of procarbazine hydrochloride by internally generated iodine to the corresponding "azo" compound. The standard deviation for the method was ±0.2 and ±0.3%, while the precision *ts* at the 95% confidence level was ±0.6 and ±0.7% for the drug substance and capsules, respectively.

Keyphrases □ *N*-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride—analysis, coulometric titration □ Procarbazine hydrochloride—analysis, coulometric titration □ Iodine—oxidation of procarbazine hydrochloride, coulometric titration □ Coulometry—analysis, procarbazine hydrochloride

Methods presently available for the determination of *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride, procarbazine hydrochloride, in capsules[1] include a polarographic determination based on the oxidation of the "hydrazo" group, —N(—)—N(—)—, to the "azo" group, —N=N—[2], and a coulometric titration using internally generated bromine (1).

The polarographic procedure, while specific for the active hydrazo group, requires comparison to a reference standard for the drug substance and preliminary extraction of the active drug substance from the excipients for the dosage form. The coulometric procedure of Beral and Stoicescu (1), while less time consuming, is not specific, since electrochemically generated bromine not only oxidizes the —N(—)—N(—)— bond but also substitutes into the phenyl ring as indicated in Scheme I. Therefore, most intermediates and decomposition products are titratable with coulometrically generated bromine.

Since iodine can theoretically oxidize the hydrazo moiety to the azo group without substituting in the phenyl ring, a coulometric titration with internally generated iodine should be a more specific method for the determination of procarbazine hydrochloride in both the drug substance and the dosage form.



Scheme I

[1] Matulane, Hoffmann-La Roche Inc., Nutley, NJ 07110
[2] Quality Control Procedure, Hoffmann-La Roche Inc., Nutley, NJ 07110

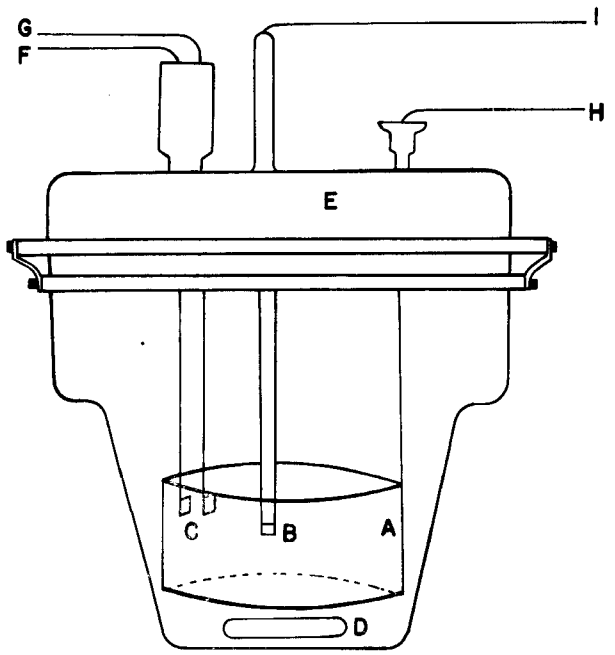


Figure 1—*Coulometric titration cell diagram. Key: A, platinum generating electrode; B, platinum reference electrode; C, platinum indicating electrodes; D, stirring bar; E, fitted top; F, to Ref position on the Orion pH meter; G, to KF position on the Orion pH meter; H, to positive terminal of constant-current source; and I, to negative terminal of constant-current source.*

EXPERIMENTAL

Reagents and Solutions—Buffered 0.5 *M* potassium iodide solution was prepared by dissolving approximately 80 g. of reagent grade potassium iodide[3] and approximately 13 g. of sodium bicarbonate[4] in demineralized water and diluting to 1 l. The pH of this solution was 8.4 ± 0.1.

Apparatus—The titrant-generating system consisted of a coulometric current source[5] in conjunction with two platinum electrodes. A platinum wire inserted in a buffered 0.5 *M* potassium iodide salt bridge was used as the cathode.

The indicating system consisted of two polarized platinum flag electrodes. A constant current of 10 μamp. was imposed on these electrodes using a digital pH meter[6] in the KF mode. The readout from the digital pH meter was recorded on an *x-y* recorder[7]. A titration vessel[8] with cover[9] was used as the cell. The arrangement of electrodes in the cell and a block circuit diagram for the combined system are given in Figs. 1 and 2, respectively. All cell connections were made as shown (Fig. 2). The current source was set to give a current of 96.5 mamp. ± 0.1%. The *x*-axis of the recorder was set at 10 sec./cm.

Procedure—The contents of 20 capsules were ground, and the equivalent of approximately 40 mg. of procarbazine hydrochloride

[3] Mallinckrodt Chemical Works.
[4] Certified ACS, Fisher Scientific Co.
[5] Sargent-Welch, model IV.
[6] Orion Research, model 801.
[7] Moseley Autograph, model 7001 AM.
[8] Metrohm EA-875-50.
[9] Metrohm EA-874.

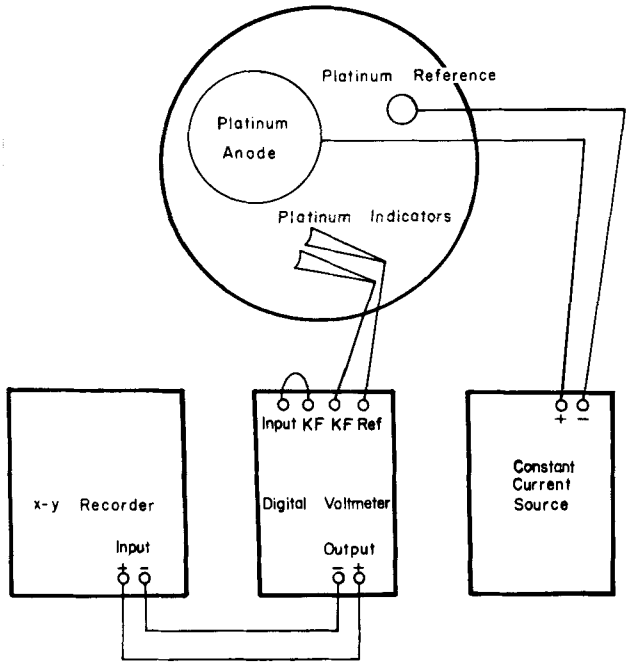


Figure 2—*Block circuit diagram for coulometric titration apparatus.*

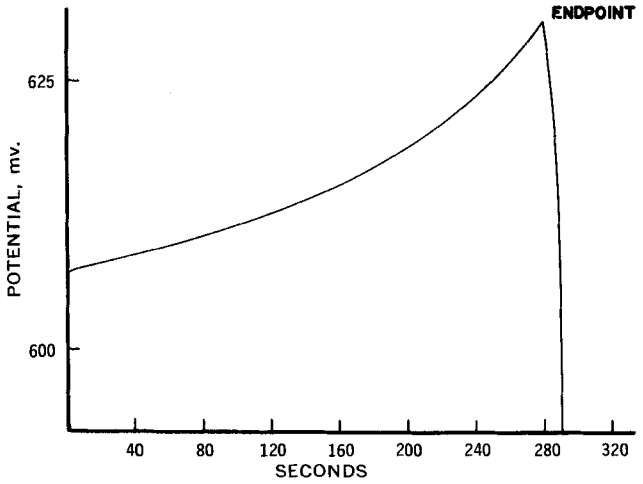


Figure 3—*Typical titration curve for procarbazine hydrochloride.*

was weighed accurately and transferred to the cell. Potassium iodide solution, approximately 140 ml., was added to the cell to cover the electrodes completely. The resulting solution was stirred using a magnetic stirrer. After the power supply of the current source had warmed for 5 min., the titration was initiated by simultaneously turning the cell current switch to On and the recorder to Start. After the end-point was reached, as noted by the peak readout potential (Fig. 3), the current switch on the current source and the recorder were turned off.

Calculations—The milligrams of procarbazine as the base was calculated from the following equation:

$$\text{mg./capsule} = \frac{(\text{number of seconds to end-point})(0.1289)(0.8585) \times (\text{average capsule fill, mg.})}{\text{weight of sample (mg.)}} \quad \text{(Eq. 1)}$$

where 0.1289 = [(96.5 mamp.) (257.8 mg./mmole)]/[(2 electrons/mmole) (96,500 coulombs/equivalent)], and 0.8585 = procarbazine hydrochloride to procarbazine base conversion factor.

RESULTS AND DISCUSSION

Determination of Procarbazine Hydrochloride Drug Substance—Initial assays were performed on 40-mg. samples of the drug substance. The average value for five determinations was 100.2%, with a standard deviation of ±0.2%. The precision *ts* for 4 degrees of freedom at the 95% confidence level was ±0.6% (Table I).

Table I—Determination of Procarbazine Hydrochloride Drug Substance

Milligrams Taken	Milligrams Found	Percent Assay
39.58	39.54	99.90
40.97	41.03	100.1
41.51	41.66	100.4
40.48	40.66	100.4
40.14	40.32	100.5
Average		100.2
Standard deviation		±0.2
Precision[a]		±0.6

[a] *ts* for 4 degrees of freedom at the 95% confidence level.

Determination of Procarbazine Hydrochloride in Dosage Forms—Determination of procarbazine hydrochloride in dosage forms was also evaluated. The average for eight determinations was 51.31 mg./capsule (102.6% of claim, based on 50 mg.). The standard deviation was ±0.17 mg./capsule or ±0.3%, and the precision *ts* for 7 degrees of freedom at the 95% confidence level was ±0.42 mg./capsule or 0.7% (Table II).

Titration Product—The nature of the product formed in the titration was investigated. The product was monitored polarographically at different stages of the titration. The diffusion current was a linear function of the generating time over the range 0–500 sec. The half-wave potential for this product, −1.08 v. *versus* Ag/AgCl, compared favorably with the half-wave potential for the azo compound [*N*-isopropyl-α-(2-methylazo)-*p*-toluamide], which was −1.02 v. The titration product obtained, using the present method, is apparently different than that obtained using classical iodometric procedures. Attempts to titrate procarbazine hydrochloride directly with 0.1 *N* iodine indicated that even after 4 equivalents of iodine was consumed, an equivalence point was not obtained. This observation emphasized the definite advantage of coulometric titrations in preventing the localization of excess titrant during analysis. Localization of excess titrant, which usually occurs during classical titrations, may lead to unfavorable stoichiometric conditions, resulting in undesirable side reactions.

Effect of Other Possible Impurities—To check for interference from possible impurities such as 4-formylbenzoic acid isopropyl amide (amide) and *N*-isopropyl-α-(methylhydrazone)-*p*-toluamide (hydrazone), procarbazine hydrochloride was titrated coulometrically in the presence of each of these compounds. A mixture of 90% procarbazine hydrochloride, 5% amide [R—C(=O)—NHR'], and 5% hydrazone (RCH=N—NHR') assayed at 90% procarbazine hydrochloride.

Table II—Determination of Procarbazine Hydrochloride in Dosage Form

Milligrams per Capsule Found	Percent Claim[a]
51.38	102.8
51.57	103.1
51.15	102.3
51.31	102.6
51.01	102.0
51.42	102.8
51.30	102.6
51.34	102.7
Average 51.31	102.6
Standard deviation ±0.17	±0.3
Precision[b] ±0.42	±0.7

[a] Based upon 50 mg./capsule. [b] *ts* for 7 degrees of freedom at the 95% confidence level.

These data indicated that quantitative conversion of procarbazine hydrochloride to the azo compound occurred during the titration. No evident substitution of iodine into the phenyl ring resulted, therefore eliminating aromatic substitution as a source of interference.

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Analytical Applications of *p*-Nitrosobenzoic Acid I: Specific Colorimetric Determination of *m*-Aminophenol in *p*-Aminosalicylic Acid and Sodium *p*-Aminosalicylate

I. K. SHIH

Abstract □ *p*-Nitrosobenzoic acid was used in the colorimetric analysis of *m*-aminophenol. It reacted with *m*-aminophenol in sodium bicarbonate solution, producing an intensified orange–yellow color. The reaction product was isolated and characterized as 3-hydroxyazobenzene-4′-carboxylic acid. The color produced in sodium bicarbonate was stable for at least 24 hr. and exhibited a maximum absorption peak at 440 nm. Beer's law was obeyed over a wide range of *m*-aminophenol concentrations (3.2–16.0 mcg./ml.). By applying low temperature control, the reaction specificity of *m*-aminophenol with *p*-nitrosobenzoic acid can be achieved. By using this color reaction, a new method for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate was developed. The method is accurate, precise, sensitive, simple, and specific. Samples of *p*-aminosalicylic acid or sodium *p*-aminosalicylate containing as little as 0.01% of *m*-aminophenol can be accurately determined. In the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, this color reaction of amino compounds was inhibited.

Keyphrases □ *m*-Aminophenol—colorimetric analysis (*p*-nitrosobenzoic acid) in *p*-aminosalicylic acid and sodium *p*-aminosalicylate □ *p*-Nitrosobenzoic acid—colorimetric analysis, *m*-aminophenol □ 3-Hydroxyazobenzene-4′-carboxylic acid—formation, colorimetric analysis of *m*-aminophenol with *p*-nitrosobenzoic acid □ *p*-Aminosalicylic acid, sodium *p*-aminosalicylate—colorimetric determination of *m*-aminophenol □ Colorimetry—analysis, *m*-aminophenol

In 1904, the chemical synthesis of *p*-nitrosobenzoic acid was reported by Alway (1), but further study on this compound has not appeared in the literature. The photodegradation reactions of chloramphenicol were investigated recently, and *p*-nitrosobenzoic acid was recognized as a possible intermediate in the photodegradation reactions (2). *p*-Nitrosobenzoic acid is a stable compound, decomposing at around 250° (1, 2). It possesses two potential functional groups: carboxylic acid and nitroso. The unique physicochemical properties of this compound were applied in this study to the analytical problem of *m*-aminophenol determination in *p*-aminosalicylic acid and sodium *p*-aminosalicylate.

Both the USP (3) and BP (4) require a limit test for *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate, but official methods are tedious. Although numerous analytical procedures for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate were proposed, the reported methods (5–11) were not reliable because of poor specificity. The complexity of this problem is reflected by Tatjana's work (11). He pointed out that due to the structural similarity of *m*-aminophenol and *p*-aminosalicylic acid, it was impossible to find a reagent that would react with *m*-aminophenol but not with *p*-aminosalicylic acid.

The present study was undertaken to solve this controversial *m*-aminophenol analysis by using *p*-nitrosobenzoic acid as a specific colorimetric reagent able to distinguish amino compounds of different chemical structures. Condensation between nitroso and amino compounds is a known reaction, giving rise to azo compounds (1, 12, 13). Experiments were designed to study the color reaction of *p*-nitrosobenzoic acid with a variety of amino compounds in sodium bicarbonate solution. By using the highly sensitive and specific color reaction of *m*-aminophenol with *p*-nitrosobenzoic acid, a simple and reliable method for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate was developed. *p*-Nitrosobenzoic acid was used for the first time as a potential analytical reagent.

EXPERIMENTAL

Apparatus—Recording spectrophotometers[1] were used.

Chemicals—*p*-Nitrosobenzoic acid was photosynthesized in this laboratory (2). The following chemicals were either reagent or USP grade: *m*-aminophenol[2], *o*-aminophenol[2], *p*-aminophenol[2], *p*-nitroaniline[2], sodium *p*-aminosalicylate USP[3], *p*-aminobenzoic acid[4], *p*-aminosulfonic acid[4], sodium salicylate[4], isoniazid[5], benzo-

[1] Bausch & Lomb 505 and Beckman IR-8.
[2] Eastman Organic Chemicals.
[3] May & Baker.
[4] Fisher.
[5] Hoffmann–La Roche.

These data indicated that quantitative conversion of procarbazine hydrochloride to the azo compound occurred during the titration. No evident substitution of iodine into the phenyl ring resulted, therefore eliminating aromatic substitution as a source of interference.

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Analytical Applications of *p*-Nitrosobenzoic Acid I: Specific Colorimetric Determination of *m*-Aminophenol in *p*-Aminosalicylic Acid and Sodium *p*-Aminosalicylate

I. K. SHIH

Abstract □ *p*-Nitrosobenzoic acid was used in the colorimetric analysis of *m*-aminophenol. It reacted with *m*-aminophenol in sodium bicarbonate solution, producing an intensified orange–yellow color. The reaction product was isolated and characterized as 3-hydroxyazobenzene-4′-carboxylic acid. The color produced in sodium bicarbonate was stable for at least 24 hr. and exhibited a maximum absorption peak at 440 nm. Beer's law was obeyed over a wide range of *m*-aminophenol concentrations (3.2–16.0 mcg./ml.). By applying low temperature control, the reaction specificity of *m*-aminophenol with *p*-nitrosobenzoic acid can be achieved. By using this color reaction, a new method for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate was developed. The method is accurate, precise, sensitive, simple, and specific. Samples of *p*-aminosalicylic acid or sodium *p*-aminosalicylate containing as little as 0.01% of *m*-aminophenol can be accurately determined. In the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, this color reaction of amino compounds was inhibited.

Keyphrases □ *m*-Aminophenol—colorimetric analysis (*p*-nitrosobenzoic acid) in *p*-aminosalicylic acid and sodium *p*-aminosalicylate □ *p*-Nitrosobenzoic acid—colorimetric analysis, *m*-aminophenol □ 3-Hydroxyazobenzene-4′-carboxylic acid—formation, colorimetric analysis of *m*-aminophenol with *p*-nitrosobenzoic acid □ *p*-Aminosalicylic acid, sodium *p*-aminosalicylate—colorimetric determination of *m*-aminophenol □ Colorimetry—analysis, *m*-aminophenol

In 1904, the chemical synthesis of *p*-nitrosobenzoic acid was reported by Alway (1), but further study on this compound has not appeared in the literature. The photodegradation reactions of chloramphenicol were investigated recently, and *p*-nitrosobenzoic acid was recognized as a possible intermediate in the photodegradation reactions (2). *p*-Nitrosobenzoic acid is a stable compound, decomposing at around 250° (1, 2). It possesses two potential functional groups: carboxylic acid and nitroso. The unique physicochemical properties of this compound were applied in this study to the analytical problem of *m*-aminophenol determination in *p*-aminosalicylic acid and sodium *p*-aminosalicylate.

Both the USP (3) and BP (4) require a limit test for *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate, but official methods are tedious. Although numerous analytical procedures for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate were proposed, the reported methods (5–11) were not reliable because of poor specificity. The complexity of this problem is reflected by Tatjana's work (11). He pointed out that due to the structural similarity of *m*-aminophenol and *p*-aminosalicylic acid, it was impossible to find a reagent that would react with *m*-aminophenol but not with *p*-aminosalicylic acid.

The present study was undertaken to solve this controversial *m*-aminophenol analysis by using *p*-nitrosobenzoic acid as a specific colorimetric reagent able to distinguish amino compounds of different chemical structures. Condensation between nitroso and amino compounds is a known reaction, giving rise to azo compounds (1, 12, 13). Experiments were designed to study the color reaction of *p*-nitrosobenzoic acid with a variety of amino compounds in sodium bicarbonate solution. By using the highly sensitive and specific color reaction of *m*-aminophenol with *p*-nitrosobenzoic acid, a simple and reliable method for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate was developed. *p*-Nitrosobenzoic acid was used for the first time as a potential analytical reagent.

EXPERIMENTAL

Apparatus—Recording spectrophotometers[1] were used.

Chemicals—*p*-Nitrosobenzoic acid was photosynthesized in this laboratory (2). The following chemicals were either reagent or USP grade: *m*-aminophenol[2], *o*-aminophenol[2], *p*-aminophenol[2], *p*-nitroaniline[2], sodium *p*-aminosalicylate USP[3], *p*-aminobenzoic acid[4], *p*-aminosulfonic acid[4], sodium salicylate[4], isoniazid[5], benzo-

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[2] Eastman Organic Chemicals.
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[4] Fisher.
[5] Hoffmann–La Roche.

Table I—Stability of *p*-Nitrosobenzoic Acid Reagent

Weeks	Molar Absorptivities—$\lambda_{max.}$, 313 nm.	$\lambda_{max.}$, 292 nm.	$\lambda_{max.}$, 223 nm.
Fresh solution	1.24×10^4	9.33×10^3	6.61×10^3
1	1.24×10^4	9.34×10^3	6.62×10^3
2	1.24×10^4	9.33×10^3	6.60×10^3
3	1.25×10^4	9.33×10^3	6.61×10^3
4	1.24×10^4	9.32×10^3	6.60×10^3
5	1.24×10^4	9.32×10^3	6.62×10^3

caine[6], sodium sulfathiazole[6], sulfisoxazole[6], sulfanilamide[6], sodium bicarbonate USP[7], ethyl acetate[4], and ethanol[4].

Preparation of *p*-Nitrosobenzoic Acid Reagent—Thirty milligrams of *p*-nitrosobenzoic acid was dissolved in 200 ml. of 1% sodium bicarbonate solution. This reagent preparation can be kept at room temperature for 5 weeks without significant deterioration.

Preparation of *m*-Aminophenol Standard Solution—*m*-Aminophenol was recrystallized from 70% hot alcohol, m.p. 123°. Forty milligrams of *m*-aminophenol was dissolved in 500 ml. of distilled water. Thus, each milliliter contained 80 mcg. of *m*-aminophenol. A freshly prepared standard solution was used.

Stability Study of *p*-Nitrosobenzoic Acid Reagent—A reagent solution containing 7.5 mcg./ml. of *p*-nitrosobenzoic acid was prepared in 1% sodium bicarbonate solution. The stability of the reagent was evaluated at room temperature by recording its absorption spectrum in a range from 210 to 360 nm. over an extended period (Table I).

Reaction of *m*-Aminophenol with *p*-Nitrosobenzoic Acid and Stability—Four milliliters of standard *m*-aminophenol solution was pipeted into a 25-ml. volumetric flask containing 10 ml. of water and 5 ml. of 5% sodium bicarbonate solution. An aliquot of 5 ml. of *p*-nitrosobenzoic acid reagent was transferred to the flask. The solution was then brought to volume with water and mixed thoroughly. Reaction was allowed to take place at room temperature (about 25°). A second solution was similarly prepared, and the flask was immersed in an ice bath maintained at 0–4°. Absorption spectra of the color solutions were recorded every 30 min. in a range from 380 to 530 nm. against a reagent blank. Absorption spectra of solutions were also checked 24 hr. after color development (Table II). To obtain the maximum accuracy of this method as later applied to the determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate, color reaction, unless otherwise specified, was carried out at 0–4°.

Synthesis of 3-Hydroxyazobenzene-4′-carboxylic Acid—Fifty milligrams of *p*-nitrosobenzoic acid and 100 mg. of *m*-aminophenol were dissolved in 50 ml. of 1% sodium bicarbonate solution. After standing 3 hr. at room temperature, the reaction mixture was transferred to a separator and extracted with ten 30-ml. portions of ethyl acetate to remove unreacted *m*-aminophenol. The aqueous solution was then acidified dropwise with dilute hydrochloric acid and extracted with three 50-ml. portions of ethyl acetate. Evaporation of the combined ethyl acetate extract under reduced pressure yielded 70 mg. of dark-colored product (91% yield), m.p. about 150° dec.; visible spectrum: $\epsilon = 4920$ ($\lambda_{max.}$ 440 nm. in 1% sodium bicarbonate solution), $\epsilon = 3640$ ($\lambda_{max.}$ 428 nm. in 0.1 *N* hydrochloric acid); IR spectrum (KBr): $\bar{\nu}$ 3448 cm.$^{-1}$ (OH), 1689 cm.$^{-1}$ (aryl CO_2H), 1580 cm.$^{-1}$ (aryl C=C), *etc.*

Anal.—Calc. for $C_{13}H_{10}N_2O_3$: C, 64.50; H, 4.14; N, 11.57. Found: C, 63.74; H, 4.40; N, 10.73.

Specificity of *p*-Nitrosobenzoic Acid Reagent—Several amino and phenolic compounds were tested with *p*-nitrosobenzoic acid reagent at three temperatures: 0–4, 25, and 100°. Samples were dissolved in 40 ml. of 1% sodium bicarbonate solution, and 10 ml. of reagent was added to each sample. Visual observations of color reaction are given in Table III.

Relationship between *m*-Aminophenol Concentration and Color Intensities—Standard *m*-aminophenol solutions (1–5 ml.) were pipeted into 25-ml. volumetric flasks containing 10 ml. of water, 5 ml. of 5% sodium bicarbonate solution, and 5 ml. of *p*-nitrosobenzoic acid reagent. Solutions were brought to volume with water and mixed thoroughly. The flasks were then immersed in an ice bath maintained at 0–4°. Absorbances of solutions were measured 3 hr. after color development against a reagent blank at 440 nm. For replicate study, six 4-ml. portions of standard *m*-aminophenol solutions were used (Table IV).

[6] British Drug Houses.
[7] Science Borealis.

Table II—Color Development and Color Stability

Hours	A^a	$\lambda_{max.}$, nm.	A^b	$\lambda_{max.}$, nm.
0.5	0.210	440	0.350	440
1	0.301	440	0.498	440
1.5	0.379	440	0.549	440
2	0.450	440	0.580	440
2.5	0.502	440	0.601	440
3	0.540	440	0.610	440
3.5	0.570	440	0.612	440
4	0.589	440	—	—
4.5	0.610	440	—	—
5	0.615	440	—	—
—	—	—	—	—
24	0.620	440	0.615	440

[a] Color was developed at 0–4°. [b] Color was developed at room temperature.

Interference Study—Four 50-mg. samples of sodium *p*-aminosalicylate were accurately weighed and transferred to 25-ml. volumetric flasks containing 10 ml. of water, 5 ml. of 5% sodium bicarbonate solution, and 4 ml. of standard *m*-aminophenol solution. Then 5 ml. of *p*-nitrosobenzoic acid reagent was pipeted into each sample. Solutions were brought to volume with water and mixed thoroughly. Sample flasks were then immersed in an ice bath maintained at 0–4°. After 3 hr. of color development, absorbances of sample solutions were concomitantly determined with a similarly treated standard *m*-aminophenol solution against a reagent blank at 440 nm. An interference study of isoniazid was also performed (Table V).

Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate Reference Material—Four 500-mg. samples of sodium *p*-aminosalicylate were accurately weighed and transferred to 25-ml. volumetric flasks. The color development procedure was the same as previously described. The USP method (3) was also used in this study (Table VI).

Quantitation of *m*-Aminophenol in *p*-Aminosalicylic Acid and Sodium *p*-Aminosalicylate Dosage Form—Twenty tablets were weighed and ground to a fine powder. An accurately weighed portion of powder, equivalent to about 500 mg. of *p*-aminosalicylic acid, was transferred to a 25-ml. volumetric flask. The color development procedure was the same as previously described. The results are given in Table VII.

Table III—Color Reactions of *p*-Nitrosobenzoic Acid with Amino and Phenolic Compounds

Compound	Reaction Conditions and Visual Observations[c]—0–4° (0–3 hr.)	25° (0–3 hr.)	100° (0–3 min.)
m-Aminophenol[a]	OY	OY	—
o-Aminophenol[b]	sY	Y	—
p-Aminophenol[b]	sP	P	—
Sodium *p*-aminosalicylate	vsY	sY	OY
p-Aminobenzoic acid	NR	NR	sY
Benzocaine	NR	NR	sY
p-Nitroaniline	NR	NR	NR
p-Aminosulfonic acid	NR	NR	sY
Sulfanilamide	NR	NR	OY
Sodium sulfathiazole	NR	NR	Y
Sulfisoxazole	NR	NR	NR
Sodium salicylate	NR	NR	NR
Phenol	NR	NR	NR
Isoniazid	sY	sY	sY

[a] Five milliliters of standard solution, equivalent to 0.40 mg. of *m*-aminophenol, was used. [b] Ten milligrams of sample was used; for all others, a quantity of 4 g. was used. [c] OY = orange-yellow, Y = yellow, P = purple, s = slight, vs = very slight, and NR = no color reaction.

Table IV—Relationship between *m*-Aminophenol Concentrations and Absorbances

Standard Solution, ml.	*A*, 440 nm.
1	0.135
2	0.272
3	0.409
4[a]	0.544, 0.544, 0.544
—	0.546, 0.550, 0.548
5	0.670

[a] Six 4-ml. samples of *m*-aminophenol standard solution were used for replicate study.

RESULTS AND DISCUSSION

Stability of *p*-Nitrosobenzoic Acid Reagent—The stability of the reagent was evaluated by recording its UV absorption spectrum over an extended period of time. Molar absorptivities remained unchanged (Table I), indicating that the reagent preparation, when stored at room temperature, could be kept for 5 weeks without significant deterioration.

Color Reaction of *m*-Aminophenol with *p*-Nitrosobenzoic Acid—*m*-Aminophenol was reacted with *p*-nitrosobenzoic acid in sodium bicarbonate solution, producing an intensified orange-yellow color with a maximum absorption peak at 440 nm. The concentration of sodium bicarbonate, varying from 1 to 10%, did not have significant effect on the color development. The color produced was stable for at least 24 hr. (Table II). The reaction product was isolated. Based on the analytical results and supported by the known amino–nitroso condensation reaction (1, 12, 13), the product was characterized as 3-hydroxyazobenzene-4′-carboxylic acid. The maximum absorption wavelength of this compound in the visible region was shifted from 440 nm. in 1% sodium bicarbonate solution (ϵ = 4920) to 428 nm. in 0.1 *N* hydrochloric acid (ϵ = 3640). Neither absorbances nor visible spectra of both sample solutions were changed over 24 hr.

Specificity of *p*-Nitrosobenzoic Acid—In contrast to *m*-aminophenol, amounts of phenolic and amino compounds, varying from 10 mg. to 4 g., were used to react with *p*-nitrosobenzoic acid in sodium bicarbonate solution at 0–4, 25, and 100° (Table III).

Sodium *p*-aminosalicylate, *p*-aminobenzoic acid, benzocaine, *p*-nitroaniline, *p*-aminosulfonic acid, sulfonamides, sodium salicylate, and phenol, when tested at concentrations 10,000 times that of *m*-aminophenol, failed to give a color reaction with the reagent. Trace amounts of *m*-aminophenol impurity in sodium *p*-aminosalicylate (3, 4) were responsible for the slight color observed. All samples except *p*-nitroaniline, sulfisoxazole, sodium salicylate, and phenol showed various degrees of color reaction when tested at 100°. These experimental results clearly indicated that in the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, this color reaction of amino compounds was inhibited under ordinary reaction conditions. Thus, neither sodium *p*-aminosalicylate nor isoniazid would interfere with *m*-aminophenol determination (Table V). Sodium salicylate and phenol did not react to the color test due to a lack of the amino functional group. Aminophenols reacted with the reagent, yielding different color species. According to visual observations, reactivities of the three aminophenols were approximately in the following decreasing order: $m > o > p$. Thus, this color reaction may be used as a specific identity test for those compounds.

Table V—Interference Study

Composition of Synthetic Mixture, mg. MAP[a]	PASNa[b]	INH[c]	Recovery of MAP, % PASNa	INH
0.320	50.0	—	99.9	—
0.320	50.0	—	99.9	—
0.320	50.0	—	100.1	—
0.320	50.0	—	100.2	—
0.320	—	5.0	—	100.2
0.320	—	5.0	—	100.4
0.320	—	5.0	—	100.6
0.320	—	5.0	—	100.1
Average			100.0	100.3
SD			±0.14	±0.22

[a] Four milliliters of standard solution, equivalent to 0.08 mg. of *m*-aminophenol (MAP), was used. [b] PASNa = sodium *p*-aminosalicylate. [c] INH = isoniazid.

Table VI—Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate Reference Material

m-Aminophenol Found, % Method A[a]	Method B[b]	USP Method[c]
0.012	0.042	0.01
0.012	0.042	n.r.[d]
0.012	0.042	n.r.
0.012	0.042	n.r.

[a] Color was developed at 0–4°. [b] Color was developed at room temperature. [c] A quantity of 690 mg. of sodium *p*-aminosalicylate was used according to the method. [d] n.r. = negative result.

Beer's Law and Replicate Study of *m*-Aminophenol—A linear relationship between absorbances and *m*-aminophenol concentrations was obtained (Table IV). Replicate studies showed good agreement of individual results (Table IV).

Experimental Conditions for Precise Determination of *m*-Aminophenol in Sodium *p*-Aminosalicylate and *p*-Aminosalicylic Acid—Since the method was primarily designed for quantitation of small quantities of *m*-aminophenol in sodium *p*-aminosalicylate and *p*-aminosalicylic acid, the control of the reaction temperature at 0–4° is essential to minimize possible degradation of *p*-aminosalicylic acid into *m*-aminophenol during color development. Since absorbances of sample solutions were concomitantly determined with the reference sample of *m*-aminophenol, 3 hr. of color development would be sufficient.

Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate—Sodium *p*-aminosalicylate USP was analyzed for the *m*-aminophenol content. Results of 0.012 and 0.042% was obtained when the color of samples was developed at 0–4 and 25°, respectively. Degradation of sodium *p*-aminosalicylate at higher temperature may account for this discrepancy. Thus, for precise *m*-aminophenol determination, low temperature control (0–4°) must be applied. The *m*-aminophenol content of sodium *p*-aminosalicylate was also determined by the USP method (3). The amount of *m*-aminophenol was calculated from the following equation: % *m*-aminophenol = $(A - 0.320)/1.16$, where *A* is the absorbance of sample solution measured at 420–435 nm. Three negative values and one positive result were obtained (Table VI). In view of the photometric error and poor reproducibility of the method, negative values probably resulted from the sample containing *m*-aminophenol in the neighborhood of 0.01%. In a qualitative sense, the official method was in agreement with the proposed method.

For routine control purpose of *m*-aminophenol, a simple procedure is suggested: Use a calculated amount of *m*-aminophenol, equivalent to the maximum tolerance as specified in the official monographs. Develop color from an accurately weighed sample of sodium *p*-aminosalicylate (or *p*-aminosalicylic acid) and *m*-aminophenol standard under the same conditions using *p*-nitrosobenzoic acid reagent. The color developed from the sample should not be deeper than that of the *m*-aminophenol standard.

Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate and *p*-Aminosalicylic Acid Dosage Form—Commercial products of sodium *p*-aminosalicylate and *p*-aminosalicylic acid tablets were

Table VII—Quantitation of *m*-Aminophenol in *p*-Aminosalicylate and Sodium *p*-Aminosalicylate Dosage Forms

	m-Aminophenol Found, %[a] Product A[b]	Product B[c]	Product C[d]
	0.018	0.041	0.030
	0.018	0.041	0.032
	0.018	0.041	0.031
	0.018	0.042	0.031
Average =	0.018	0.041	0.031

[a] Percent *m*-aminophenol was calculated on the basis of declared amount of *p*-aminosalicylate. [b] Effervescent *p*-aminosalicylate tablet. [c] Buffered *p*-aminosalicylate–isoniazid tablet. [d] Sugar-coated sodium *p*-aminosalicylate tablet.

analyzed for *m*-aminophenol content. The results are given in Table VII.

CONCLUSIONS

1. Condensation reaction between *p*-nitrosobenzoic acid and a variety of amino compounds in sodium bicarbonate solution was studied. *m*-Aminophenol reacted with *p*-nitrosobenzoic acid, producing an intensified, stable orange–yellow dye. The product was isolated and characterized as 3-hydroxyazobenzene-4′-carboxylic acid. In the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, the color reaction of amino compounds was inhibited. Thus, sodium *p*-aminosalicylate, *p*-aminobenzoic acid, benzocaine, *p*-aminosulfonic acid, sulfonamides, *p*-nitroaniline, *etc.*, when tested at concentrations 10,000 times that of *m*-aminophenol, failed to give the color reaction.

2. All aminophenols reacted with *p*-nitrosobenzoic acid, producing discernible colors. The relative rates of color development were approximately in the following decreasing order: $m > o > p$. This color reaction thus can be used for specific identity tests for the three isomeric aminophenols.

3. By using *p*-nitrosobenzoic acid as a specific color-producing agent for *m*-aminophenol, a sensitive and reliable method for the direct determination of *m*-aminophenol in sodium *p*-aminosalicylate or in *p*-aminosalicylic acid was developed. Samples of sodium *p*-aminosalicylate and *o*-aminosalicylic acid containing as little as 0.010% of *m*-aminophenol could be accurately determined.

4. *p*-Nitrosobenzoic acid was used for the first time as a potential colorimetric reagent. Other possible applications of this new reagent are worth investigating in the future.

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Elimination of Choline Interference in Microbiological Assay of Inositol in Pharmaceutical Products

LEWIS WALENROD and REUBEN LEVINTON

Abstract □ A method for the assay of inositol in the presence of choline is described which eliminates interference by the latter. By using *Schizosaccharomyces pombe* as the test organism, results comparable to those obtained with *Saccharomyces carlsbergensis* were obtained without the need for separation of the interfering compound.

Keyphrases □ Inositol—microbiological assay in the presence of choline without interference, using *Schizosaccharomyces pombe* as test organism □ *Schizosaccharomyces pombe*—used as test organism to eliminate interference in inositol analysis, compared to *Saccharomyces carlsbergensis* □ Choline—eliminated as interference in nositol microbiological assay

The most widely used method of assay for inositol in the United States, proposed by Atkins *et al.* (1) and Jurist and Foy (2), employs *Saccharomyces carlsbergensis* as the test organism. In pharmaceutical preparations, inositol and choline are often used in combination as lipotropic agents; choline produces significant inhibition of the response of *S. carlsbergensis* to inositol (3).

Various methods have been suggested to eliminate the effect of this interference. It was found that passing dilute aqueous solutions through columns of a cation exchanger[1] produced interference-free eluates. Taylor and McKibbin (3) compensated for the interference by adding choline to the standard tubes. However, both of these procedures involve additional manipulations which are time consuming as well as potential sources of error.

This paper describes a method, routinely used in these laboratories, which permits the direct assay of inositol in the presence of choline without the need for separative or compensatory processes. The assay is based on the method of Norris and Darbre (4).

EXPERIMENTAL

The test organism used was a strain of *Schizosaccharomyces pombe* (ATCC 16491)[2]. The yeast is maintained by monthly transfer on malt agar slants consisting of 10 g. malt extract (Difco), 0.2 g. yeast extract (Difco), and 1.8 g. agar/100 ml. distilled water. Slants are incubated at room temperature for 24 hr. and then stored under refrigeration.

[1] Folin Decalso, The Permutit Co., New York, N. Y..

[2] Obtained from F. W. Norris, Department of Biochemistry, University of Birmingham, Birmingham, England. It is currently available from the American Type Culture Collection, Rockville, MD 20852

analyzed for *m*-aminophenol content. The results are given in Table VII.

CONCLUSIONS

1. Condensation reaction between *p*-nitrosobenzoic acid and a variety of amino compounds in sodium bicarbonate solution was studied. *m*-Aminophenol reacted with *p*-nitrosobenzoic acid, producing an intensified, stable orange–yellow dye. The product was isolated and characterized as 3-hydroxyazobenzene-4′-carboxylic acid. In the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, the color reaction of amino compounds was inhibited. Thus, sodium *p*-aminosalicylate, *p*-aminobenzoic acid, benzocaine, *p*-aminosulfonic acid, sulfonamides, *p*-nitroaniline, *etc.*, when tested at concentrations 10,000 times that of *m*-aminophenol, failed to give the color reaction.

2. All aminophenols reacted with *p*-nitrosobenzoic acid, producing discernible colors. The relative rates of color development were approximately in the following decreasing order: $m > o > p$. This color reaction thus can be used for specific identity tests for the three isomeric aminophenols.

3. By using *p*-nitrosobenzoic acid as a specific color-producing agent for *m*-aminophenol, a sensitive and reliable method for the direct determination of *m*-aminophenol in sodium *p*-aminosalicylate or in *p*-aminosalicylic acid was developed. Samples of sodium *p*-aminosalicylate and *o*-aminosalicylic acid containing as little as 0.010% of *m*-aminophenol could be accurately determined.

4. *p*-Nitrosobenzoic acid was used for the first time as a potential colorimetric reagent. Other possible applications of this new reagent are worth investigating in the future.

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Elimination of Choline Interference in Microbiological Assay of Inositol in Pharmaceutical Products

LEWIS WALENROD and REUBEN LEVINTON

Abstract □ A method for the assay of inositol in the presence of choline is described which eliminates interference by the latter. By using *Schizosaccharomyces pombe* as the test organism, results comparable to those obtained with *Saccharomyces carlsbergensis* were obtained without the need for separation of the interfering compound.

Keyphrases □ Inositol—microbiological assay in the presence of choline without interference, using *Schizosaccharomyces pombe* as test organism □ *Schizosaccharomyces pombe*—used as test organism to eliminate interference in inositol analysis, compared to *Saccharomyces carlsbergensis* □ Choline—eliminated as interference in nositol microbiological assay

The most widely used method of assay for inositol in the United States, proposed by Atkins *et al.* (1) and Jurist and Foy (2), employs *Saccharomyces carlsbergensis* as the test organism. In pharmaceutical preparations, inositol and choline are often used in combination as lipotropic agents; choline produces significant inhibition of the response of *S. carlsbergensis* to inositol (3).

Various methods have been suggested to eliminate the effect of this interference. It was found that passing dilute aqueous solutions through columns of a cation exchanger[1] produced interference-free eluates. Taylor and McKibbin (3) compensated for the interference by adding choline to the standard tubes. However, both of these procedures involve additional manipulations which are time consuming as well as potential sources of error.

This paper describes a method, routinely used in these laboratories, which permits the direct assay of inositol in the presence of choline without the need for separative or compensatory processes. The assay is based on the method of Norris and Darbre (4).

EXPERIMENTAL

The test organism used was a strain of *Schizosaccharomyces pombe* (ATCC 16491)[2]. The yeast is maintained by monthly transfer on malt agar slants consisting of 10 g. malt extract (Difco), 0.2 g. yeast extract (Difco), and 1.8 g. agar/100 ml. distilled water. Slants are incubated at room temperature for 24 hr. and then stored under refrigeration.

[1] Folin Decalso, The Permutit Co., New York, N. Y..

[2] Obtained from F. W. Norris, Department of Biochemistry, University of Birmingham, Birmingham, England. It is currently available from the American Type Culture Collection, Rockville, MD 20852

Table I—Comparison of Recovery of Inositol from Pharmaceutical Products Using *S. carlsbergensis* and *S. pombe*

Sample	Label Claim, mg./Dose	Inositol Found[a]					
		S. carlsbergensis				*S. pombe*	
		Untreated		Treated			
		mg./Dose	%	mg./Dose	%	mg./Dose	%
Two-piece capsule[b]	83	53.8	64.8	87.5	105.4	85.4	102.9
Soft gelatin capsule[c]	10	6.13	61.3	9.85	98.5	9.20	92.0
Pet supplement (powder)[d]	60	36.9	61.5	64.5	107.5	61.2	102.0
Soft gelatin capsule[e]	40	19.9	49.8	42.0	105.0	40.4	100.8
Pet supplement (tablet)[d]	5	2.44	48.8	4.78	95.6	5.08	101.6
Pet supplement (granule)[d]	5	2.77	55.4	4.53	90.6	5.67	113.4

[a] All values reported are averages of not less than three separate assays. [b] Contains B-complex vitamins, liver, choline, methionine, and inositol. [c] Contains B-complex vitamins, liver, yeast, choline, and inositol. [d] Contains A, D, E, and B-complex vitamins, minerals, liver, yeast, alfalfa, soy, milk, choline, methionine, and inositol. [e] Contains B-complex vitamins, safflower oil, choline, methionine, and inositol.

Basal Medium[3]—The basal medium consisted of the following:

dextrose	40.0 g.	niacin	1.0 mg.
ammonium sulfate	4.0 g.	pyridoxine hydrochloride	1.0 mg.
potassium phosphate, monobasic	3.0 g.	thiamine hydrochloride	1.0 mg.
magnesium sulfate	0.5 g.	D-biotin	0.8 mg.
calcium chloride	0.5 g.	trace elements solution	2.0 ml.
potassium iodide	0.2 mg.	yeast extract	0.1 g.
calcium		lactate buffer	20 ml.
D-pantothenate	1.0 mg.	water	q.s. to 1000 ml.

The trace elements solution used in the basal medium consisted of the following:

boric acid	0.10 g.	ferrous sulfate	0.014 g.
zinc sulfate	0.027 g.	ammonium molybdate	0.019 g.
cupric sulfate	0.029 g.	water	q.s. to 1000 ml.
manganese sulfate	0.030 g.		

Preparation of the yeast extract supplement used in the basal medium as described by Norris and Darbre (4) was too involved for routine control analytical work. Investigation of commercially available yeast extracts indicated that the addition of 100 mg. BBL[4] yeast extract/l. of basal medium gave comparable results. Several other commercial yeast extracts produced very high blanks.

The lactate buffer used in the basal medium is prepared as follows. Concentrated sodium hydroxide solution is added to 250 ml. of syrupy lactic acid until, on diluting 1:100, a pH of 4.8 is obtained. This buffer is then diluted 1:500 for use.

Five to ten liters of basal medium are prepared at one time. The batch is divided into bottles containing approximately 400 ml. each and stored in the deep freeze. Under these conditions, it is stable for at least a year.

Preparation of Samples—Samples are dispersed in distilled water and allowed to stand for several minutes with occasional shaking. Heat may be applied to help solution of inositol from capsules, tablets, *etc.* Samples are diluted in distilled water to an estimated potency of 1 mcg./ml.

Preparation of Inoculum—Growth from a 24–48-hr. culture is transferred to 10 ml. of sterile physiological saline with the aid of a loop. After thorough mixing, the resultant suspension should read about 70% transmission against a saline blank in a colorimeter[5] equipped with a 620-nm. filter (equivalent to an absorbance of 0.078 in a spectrophotometer[6] using 1-cm. cells). If necessary, the concentration of the inoculum is adjusted with cells from another slant or by the addition of saline.

Assay Procedure—A standard curve is prepared in duplicate in 22 × 200-mm. test tubes covering the range of 0–5 mcg./ml. of inositol in 0.5-mcg. increments. Volumes of all tubes are brought to 5 ml. with distilled water, and 5 ml. of basal medium is added to each tube. An additional zero-mcg. tube is prepared for use as an uninoculated blank. Then 1-, 2-, 3-, and 4-ml. aliquots of sample are set up in duplicate. Water and medium are added as previously described. After capping, sample and standard tubes are sterilized in flowing steam for 5 min., cooled in a water bath, and inoculated.

After inoculation, the tubes are incubated for 72 hr. at room temperature on a rotary shaker. At the end of the incubation period, growth is stopped by steaming the run for 5 min.

The colorimeter is set at 100% transmission with the uninoculated blank, and all tubes are read. The averaged standard readings (as percent *T*) are plotted against concentration (in micrograms) on rectangular coordinate paper, and a smooth curve is drawn connecting the points. Sample tube concentrations are read from the curve. Values are converted to a potency per milliliter basis, averaged, and multiplied by the dilution factor to obtain sample potency.

[3] All salts calculated on anhydrous basis.
[4] Baltimore Biological Laboratories.
[5] Evelyn.
[6] Beckman DU.

RESULTS AND DISCUSSION

Table I presents the inositol values obtained on several multivitamin preparations, all of which contain both inositol and choline. Approximately 50–60% of the labeled inositol content was recovered using *S. carlsbergensis* when the choline was not removed. Prior removal of the choline resulted in recoveries approximating label claims.

The proposed method, using *S. pombe*, gave satisfactory inositol recovery without the use of any separative procedures or corrections, making it suitable for use in routine analytical work.

In addition, this organism is not inhibited by relatively high salt concentrations. In the analysis of natural products requiring hydrolysis (HCl), therefore, it is only necessary to neutralize the acid in the hydrolysate instead of removing it by distillation.

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Automated System for Analytical Microbiology III: Computer Interpolation of Potency

F. KAVANAGH

Abstract □ Highly accurate photometric assays for antibiotic substances, such as those obtainable with a system composed of automated components, require an accurate method of obtaining potencies of samples. The usual manual interpolation of sample potencies from a standard dose–response line may introduce an error of several percent. Errors can come from the use of an inappropriate dose–response line and from the inability of the person interpolating to differentiate potencies with a resolution of less than 1%. A new dose–response line for manual interpolation of antibiotic potencies is described. Interpolations by means of a digital computer from that equation and from the relation log *A versus C* were both shown to reduce errors considerably. In addition, the computer may be put on-line and provide a typed report as soon as the last tube in an assay is processed.

Keyphrases □ Microbiology, analytical—computer interpolation of potency with automated systems □ Dose–response interpolation in automated microbiological methods—computer interpolation of potency □ Automated microbiological methods—use of digital computer to minimize errors introduced by manual interpolation of potencies □ Potency determination, automated microbiological methods—computer interpolation of data □ Computer interpolation of potency data—use with automated microbiological methods

Highly accurate photometric assays for antibiotic substances, such as those that may be obtained with a system composed of automated components[1], require an accurate method of obtaining potencies of samples. Values of sample potencies obtained by interpolation from the standard curve by the usual manual means can be in error by several percent. Such an error is both too large and unnecessary. A new presentation of the dose–response line was developed to reduce errors of manual interpolation for many assay systems. Application of a digital computer to the problem effected further reduction in errors of interpolation.

Unlike certain chemical assays which are absolute, microbiological assays are comparative in that the response of a sample is compared with the responses of standards within the same test. Usually, responses of the standards are plotted on graph paper. Potency of a sample is then obtained by interpolating from the standard response curve to find the concentration of standard corresponding to the response of the sample. The assumptions and pitfalls of such procedures were discussed at length elsewhere (1).

Dose–response lines may vary in shape from straight to strongly curved. Whatever the shape, they are approximated in practice by straight lines drawn between adjacent calibration points. The size of the error in estimation of potency caused by use of such an approximated curve depends upon the curvature of the line and the distance of the entry point from a calibration point. In addition to this error of an arithmetical nature, an error is caused by the inability of the person interpolating to be certain of the location of a point on the line with an error of less than about 1% of potency. Although the turbidity can be measured to the nearest millivolt (1000 mv. = 100% *T*) and absorbance can be calculated with three-digit accuracy, the data cannot be plotted on graph paper with equal accuracy.

Three conventional dose–response lines used in antibiotic assaying and a new one were programmed for a computer and tested to determine which gave the smallest computational errors and the least bias.

Table I—Measured Turbidities of Standards and Samples—Monensin Assayed with *St. faecalis* at pH 6

Substance	Turbidities, mv., Sample Size 0.10 ml.		0.15 ml.	
Standard				
0	378	378	403	403
1	427	426	471	469
2	475	473	529	527
3	512	512	571	569
4	544	544	603	602
5	570	569	636	635
6	591	590	664	663
Sample				
1.5	451	451	501	500
2.5	495	492	551	550
3.5	529	528	590	587
4.5	559	557	621	618
5.5	581	580	651	651

ACCURATE ASSAY SYSTEM

The type of assay system in which computational errors or errors of interpolation as great as 1% may be significant will now be described. A system for performing highly accurate turbidimetric microbiological assays was introduced in 1970[2] by Kuzel and Kavanagh (2, 3). The system consists of three elements: an automatic diluter, an automatic reader, and an incubation bath. The difficult-to-do operations are mechanized so that the critical steps of measuring sample volume, broth volume, and turbidity may be performed with a relative standard deviation of 0.1% or less.

The philosophy of the design was to measure so accurately and to control temperature of incubation and critical operations so carefully that significant errors could occur only external to the system. The answers obtained from this system could then be free

[1] Such as the Autoturb System.

[2] By the Elanco Division of Eli Lilly and Co.

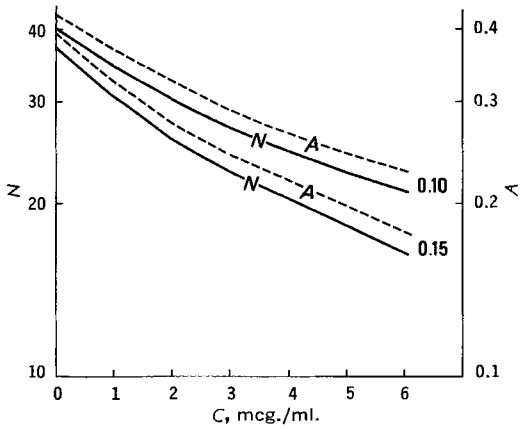

Figure 1—*Dose–response lines for monensin*–St. faecalis *assay for the two volumes of sample of 0.10 and 0.15 ml. Curves labeled* N *are for Eq. 1; curves labeled* A *are for Eq. 2. Only one of each pair of curves is shown.*

from operational bias and have a small relative error. In some assays, interpolation of potency from a standard curve seemed to be the main source of error. To reduce this last source of error to insignificance and to increase ease and speed of analysis, potencies are interpolated by a digital computer from the standard curve.

The automatic diluter prepares two sets of dilutions from each sample by means of four separate diluting channels arranged in pairs. Therefore, four responses are obtained for each sample or standard (no distinction is made in treatment of standards and samples). In the data reported here, one pair of samples received 1.5 times as much antibiotic as the other pair. Since four tubes are prepared for each sample, the data occur in sets of four (Table I). Each of the four entries for a sample represents a separate diluting channel. All standards and all samples pass once through each channel. The standards and samples pipeted by a channel are the assay unit. Potencies of samples in an assay unit are interpolated from the standard curve prepared from the standards in that unit. The advantage of this procedure is that the absolute volume pipeted by a channel need not be known because it is the same for standards and samples. What is important is that the volume be exactly ($\leq$0.1% error) the same for standards and samples. Apparently the automatic diluter achieves such accuracies.

When manual interpolation is used to obtain sample potencies in this system, the two responses from one sample size are averaged before interpolating from the standard curve. Thus, there are two standard curves, one for each size of sample. The curves usually are different because of a 0.5% difference in concentration of inoculum (1).

DOSE–RESPONSE LINES

At least 10 relations between concentration of antibiotic and the response obtained from the test have been used in antibiotic assaying. Of these, the one with a theoretical basis (4), a variant of it, and the new one just mentioned were investigated because usually these three are nearer to straight lines than the other seven. In addition, a relationship much used in official methods was included.

A theoretical relation between concentration, C, of the antibiotic and the response, N, obtained as concentration of bacteria

Table II—Potencies of the Five Samples of Table I Interpolated from Eq. 4

Putative Concentration	Concentration, mcg./ml., Interpolated from Eq. 4 — 0.10		0.15		Mean	*RSD*
1.50	1.483	1.515	1.493	1.510	1.500	1.00
2.50	2.526	2.471	2.503	2.527	2.506	1.04
3.50	3.516	3.485	3.575	3.526	3.526	1.06
4.50	4.564	4.507	4.523	4.462	4.514	0.93
5.50	5.512	5.512	5.512	5.548	5.521	0.33

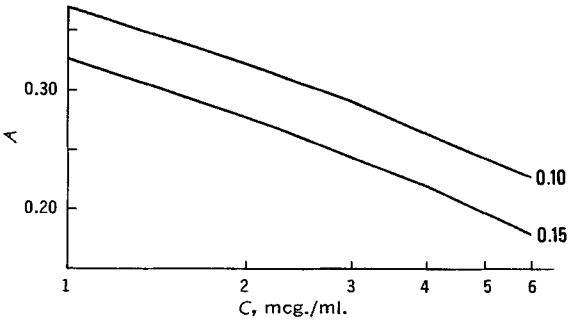

Figure 2—*Data of Fig. 1 plotted to relate absorbance and logarithm of the concentration.*

(1, 4) was shown to be of the form:

$$\log N = A - BC \qquad \text{(Eq. 1)}$$

where A and B are constants.

Values of N are not measured directly but are derived from the turbidity of the suspension by means of a calibration curve relating N to a corresponding absorbance. Accurate values of N can be obtained only by calculations which, however, are too extensive to do manually for a test involving 80 responses as in this automated system.

As a first approximation, N is proportional to absorbance, A. Substituting A for N in Eq. 1 gives Eq. 2:

$$\log A = E - FC \qquad \text{(Eq. 2)}$$

Accurate values of absorbance are easily computed from percent transmittance. Equations 1 and 2 are only approximately straight in actual practice. Therefore, a straight-line approximation of the equations will be slightly erroneous between the break points. This is illustrated by the curves in Fig. 1. Such straight-line approximations are employed in both manual and digital computer treatments. The commonly used relation, Eq. 3:

$$A \textit{ versus } \log C \qquad \text{(Eq. 3)}$$

is illustrated by Fig. 2.

A method of plotting and of computation possessing minimum inherent errors was needed. The method should use the response of the photometer (millivolts) to avoid the effort and errors of conversion. A new equation was devised to give a straight or nearly straight dose–response line when photometer response in millivolts was plotted against the concentration of antibiotic eliciting the response. The response, described by Eq. 4, is called an inverted logarithmic response (1) and is illustrated by Fig. 3. The equation of the line is of the form:

$$\log (D - V) = G - HC \qquad \text{(Eq. 4)}$$

where V is the photometer output in millivolts, C is the concentration of antibiotic, and G and H are constants for a particular line.

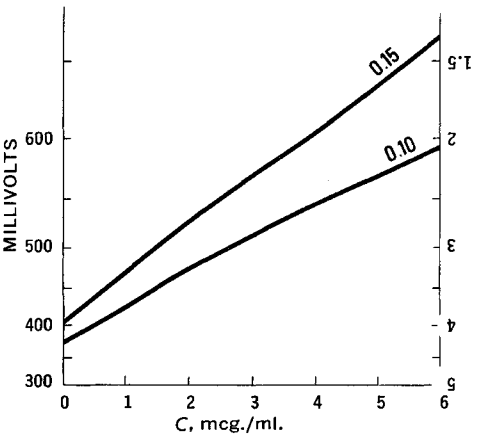

Figure 3—*Inverted logarithmic plot (Eq. 4) of the same data used in Fig. 1,* D = *800.*

Table III—Potencies and Relative Standard Deviations of the Five Samples in Table I Interpolated from Four Equations

Putative Concentration	Eq. 1		Eq. 2		Eq. 3		Eq. 4	
	Potency	*RSD*	Potency	*RSD*	Potency	*RSD*	Potency	*RSD*
1.5	1.536	1.04	1.516	1.01	1.430	1.06	1.500	1.00
2.5	2.536	1.05	2.519	1.05	2.474	1.09	2.506	1.03
3.5	3.550	1.08	3.537	1.08	3.507	1.12	3.526	1.06
4.5	4.538	0.85	4.526	0.88	4.504	0.87	4.514	0.93
5.5	5.544	0.41	5.533	0.38	5.516	0.41	5.521	0.33
ARE[a], %	1.17		0.72		0.64		0.38	
Bias	+		+		−		+	

[a] *ARE* is the average relative error as a percentage of the average potency. The errors were summed without regard to sign.

By shifting the starting point (changing value of D) of the curves up or down on the graph, the lines may go from concave to convex. At some intermediate starting point, the lines are straight or slightly sigmoid. Any starting point not at a major division of the semilogarithmic paper is very inconvenient except when a computer is employed. Therefore, as a practical matter, the starting point is selected to fall on the major division that gives a dose–response line nearest to a straight line over the region of principal interest. In many assays, the line is as straight as it can be plotted and drawn by hand. Interpolation of potencies from such a line is still subject to small errors because of the difficulty of estimating the value of C corresponding to a measured value of turbidity. Computer interpolation eliminates this last error.

An important advantage of Eq. 4 is the use of raw data; no transformation of photometer response is made as in Eqs. 1–3. This is an advantage in manual but not in computer interpolation. A value of D must be assigned before the equation can be used. A restriction is that D must always be greater than the largest response from the photometer. Usually, values of D between 800 and 1200 are used; it is 800 in Fig. 3. Had the 400-mv. point been put at 6 on the log scale, D would have been 1000. A change of 100 in the value of D has a trivial effect upon calculated potency for those assays to which the equation is applicable; *i.e.*, gives a nearly straight line. The value of D may be found by means of the computer program mentioned later or by plotting on semilog paper. If the latter procedure is followed, D is changed in value by steps of 100, and the points of the standard curve are plotted. The value of D that gives the lines nearest to straight in the region of interest is entered into the computer program.

Although Eq. 4 has no apparent theoretical basis, it does have the advantage of being more nearly straight than the others for many antibiotic assays.

If a line is mathematically straight, any deviation of the actual points from the line is presumed to be the result of an error. Upon this assumption, the "best" straight line through the points of a standard line may be found by means of a least-squares solution. A computer program was written to do this for Eq. 4. Values were assigned to D in steps of 10, the best straight line was found, the values of turbidity corresponding to each C were computed from the equation for the line, and the difference between computed and measured turbidity was found, squared, and summed. The value of D giving a minimum sum was then used in calculating the dose–response line from which potencies of samples were computed. This iterative computation of the best straight line could occupy the computer for as long as 75 sec.

When experience showed that most dose–response lines were not straight but slightly sigmoid, this approach to computation of dose–response lines was abandoned in favor of point-to-point interpolation. The computer programs to do this were written in Dartmouth Basic and run on a computer[3]. Basic was used because of the ease of programming. The programs in Basic were discarded at the end of this experiment. New programs were written in FORTRAN and assembly language to be used for routine data acquisition and for calculations by an on-line computer.

TEST OF EQUATIONS

The equations were tested with data from assays known to be capable of the precision required. These were: penicillin G–*Staphylococcus aureus*, erythromycin–*S. aureus*, and monensin–*Streptococcus faecalis* assays (5). Data from monensin and penicillin assays were selected to illustrate the point-to-point interpolation by computer from the four equations. Responses in the form of the millivolt output of the photometer, concentrations at the points of the standard curve, values of D, and the coefficients of the calibration equation needed to compute N were entered into the computer. A set of four potencies and their mean were obtained for each sample. The relative standard deviation (coefficient of variation) for each set of four potencies was printed with their mean.

The samples were of two kinds: standards prepared to have concentrations between points on the standard curve and concentrations used as points of the standard curve. Since the samples were prepared from the same stock solution as the standards, the assumption was made that the sample concentrations were as accurately known as the standards. Errors in preparing the samples and standards should be less than 1%.

DISCUSSION

The data for a standard curve and five samples are given in Table I. The readings of turbidities of the pairs of assay channels differ because the channels are not identical, as was shown by diluting dye solutions (3), and because of small errors.

Potencies for the samples of Table I are given in Table II. Potencies were interpolated by means of Eq. 4. Each of the first four entries on a line represents the potency obtained from the corresponding channel of the diluter. Potency was independent of sample size. Relative standard deviation (*RSD*, coefficient of variance) was used as a statistical measure of agreement within the set of four responses constituting an assay of a sample. It was a measure of the ability of the assay system (antibiotic, organism, and equipment) to perform reliably, but it was not a measure of the accuracy of the assay. The *RSD* of about 1% is quite satisfactory for such an assay system and is about the size of error caused by an uncertainty of 1 mv. in measuring turbidity. The digital voltmeter of the reader had a last place uncertainty of reading of 1 digit or 1 mv. The agreement among the four potencies obtained for a sample is typical of assays performed with this automated system when the assay system of organism and antibiotic is capable of high precision (1).

The data of Table I were processed with the aid of the four interpolation equations, and the results are shown in Table III. Each potency is the mean of the four responses obtained in assaying a sample. The potencies are not strongly dependent upon the equation used in the interpolation, because none of the dose–response lines is strongly curved (Figs. 1–3) and all may be approximated by straight-line segments. The values of *RSD* are about the same for all equations, because they are principally a measure of agreement among the four turbidities obtained for a sample. The equation used for interpolation had only a second-order effect upon *RSD*.

The errors caused by the approximations inherent in the interpolation expressions were different for the four equations. Equation 1 gave the largest error because the relation used to compute N from turbidity (millivolts) was itself only an approximation. Equation 2 was next in error. Equation 3 gave the third largest errors; the low bias at the 1.5 and 2.5 parts of the dose–response line was a principal cause of the large errors. At the higher part of the dose–response line, Eq. 3 was the best. It was rejected in favor of Eq. 4 because it was not as applicable over the whole range of the dose–response line. Equation 4 gave the least error over the entire range

[3] Hewlett-Packard model 2115.

Table IV—Assay of Nine Samples of Penicillin G with *S. aureus*[a]

Known Potency of Sample	Eq. 1		Eq. 2		Eq. 3		Eq. 4	
	Mean	*RSD*	Mean	*RSD*	Mean	*RSD*	Mean	*RSD*
2.50	2.574	2.1	2.534	2.1	2.484	2.1	2.504	2.0
3.00	3.099	1.3	3.090	1.2	3.079	1.0	3.083	1.1
3.33	3.366	2.5	3.341	2.5	3.310	2.4	3.322	2.4
3.33	3.383	1.9	3.358	1.9	3.326	1.8	3.338	1.9
3.00	2.979	0.5	2.976	0.6	2.971	0.7	2.973	0.6
3.00	3.127	1.3	3.115	1.2	3.101	1.1	3.106	1.1
2.50	2.602	1.3	2.562	1.3	2.512	1.4	2.532	1.3
2.00	2.000	0.7	2.000	0.7	1.998	0.8	1.998	0.7
1.50	1.505	1.5	1.505	1.4	1.504	1.2	1.504	1.3
ARE[b], %	2.00		1.61		1.09		1.05	

[a] Samples are listed in the order in which they occurred in the test. [b] *ARE* is the average relative error as a percentage of the average potency. The errors were summed without regard to sign.

of the assay, as shown by the size of the average relative error (*ARE*). A quite different assay, penicillin G by an *S. aureus* method, supported these conclusions (Table IV). The indication of bias given in the last line of Table III shows the direction of the error caused by the straight-line approximation. The errors were small even for the worst condition. Occasionally, Eq. 3 or the response *V versus C* will give the best straight line in an antibiotic assay. The response to use for growth-promoting substances is *N* or *A versus C*.

The best relation is the one giving a dose–response line nearest to straight over the region of interest. Although the inverted log response has been the most generally applicable for antibiotic assaying, other relations may be better under certain circumstances. The analyst who wishes to minimize computational errors must be prepared to use the form of dose–response line best for a particular assay.

When only the relative standard deviations of the four responses obtained for each sample are considered, there is little reason to choose one of the four equations relating response and concentration over the others. Those who believe the inherent error of a microbiological assay to be at least $\pm 10\%$ would consider the equations to be equivalent. Those analysts who judge the quality of an assay by the statistics of the responses constituting a potency would draw the same conclusion. However, a pharmaceutical preparation is sold on the basis of the quantity of active drug, not upon the statistics of the responses. Therefore, the equations to be used in computing potencies should be the ones giving minimum error.

The emphasis placed here upon achieving minimum error should not be taken as denigration of the statistics of response for a sample. Such statistics of this automated system provide the analyst a quick check of the operations of the equipment and of identity of standard and sample. The potencies obtained from each element of a pair of channels should be nearly identical when the equipment is operating correctly. The two potencies obtained from the two sets of channels will be nearly identical when sample and standard contain the same active drug. Any substantial difference indicates a difference in composition of standard and sample and a questionable assay.

In addition to reducing errors and increasing resolution of an assay, the computer can calculate all parts of an assay and be put on-line to provide a typed report as soon as the turbidity of the last tube in a test is measured.

To achieve the inherent accuracy of this automated system, a computer must be used to make the interpolations from a dose–response line selected to minimize computational errors.

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Semiautomated Fluorometric Determination of Rauwolfia Alkaloids

B. N. KABADI

Abstract □ A semiautomated nitrous acid fluorometric method for the determination of reserpine and rauwolfia alkaloids in unit dose is described. A simple, manual, alcoholic acid extraction method for the recovery of reserpine or reserpine–rescinnamine group alkaloids from a large number of tablets or powders is described. Following the chloroform extraction of the sample solution, the extract is washed with dilute hydrochloric acid and bicarbonate solutions and then reacted with nitrous acid to produce a yellowish-green fluorescence. The fluorogen is measured at an excitation wavelength maximum of 390 nm. and a fluorescent wavelength maximum of 510 nm. The automated system is designed to determine reserpine or rauwolfia alkaloids in the concentration range of 0.15–0.3 g. %. The rate of analysis of the samples is set at 20 an hour. Reserpidine, yohimbine, and bendroflumethiazide do not interfere in the reserpine or rauwolfia alkaloids determination.

Keyphrases □ Reserpine—semiautomated fluorometric analysis □ Rauwolfia alkaloids—semiautomated fluorometric analysis □ Nitrous acid fluorometry—analysis, reserpine and rauwolfia alkaloids □ Fluorometry—analysis, reserpine and rauwolfia alkaloids

Simple extraction and determination of highly potent active ingredients in low concentration by a precise chemical procedure from products containing several components, some of natural origin, and unit dose analysis requirements for homogeneity are some of the new challenges faced by the pharmaceutical industry today. Often, the official assay method is not applicable to unit doses containing a low concentration of a drug such as *Rauwolfia serpentina* root tablets. By using a preliminary, manual, alcoholic acid extraction procedure, the determination of the reserpine–rescinnamine group alkaloids in pharmaceutical products containing rauwolfia root powder was automated based on a sensitive, nitrous acid fluorometric method.

The lengthy official extraction method for the recovery of rauwolfia alkaloids from root powders or tablets was not practical for automation. However, the alcoholic sulfuric acid extraction procedure of Kunze *et al.* (1) was suitable for the manual operation. This procedure, with some modifications, was adopted in this work.

The analytical procedures published for the quantitative determination of rauwolfia alkaloids are either colorimetric (2, 3) or fluorometric (4, 5). The colorimetric procedures are not sensitive and specific for the determination of low concentration level drugs. The specific *p*-toluenesulfonic acid fluorometric method for reserpine of Jakovljevic *et al.* (4), which is claimed to be sensitive, did not give reproducible results with rauwolfia alkaloids because of interference from the decomposition products. However, the author's previously reported (6) nitrous acid fluorescent method for reserpine, with modifications, was satisfactory for the automated determination. This procedure was extended to the determination of reserpine in single tablets and in powders.

EXPERIMENTAL[1]

Reagents—*Sulfuric Acid Reagent*—Forty milliliters spectral grade sulfuric acid was added to 1 l. of anhydrous methanol.

Sodium Nitrite Reagent—Two grams sodium nitrite was dissolved in 50 ml. distilled water and diluted to 1 l. with methanol.

Hydrochloric Acid Reagent—Twenty milliliters concentrated HCl was diluted to 1 l. with distilled water.

Sodium Bicarbonate Reagent—Twenty grams sodium bicarbonate was dissolved in 1 l. distilled water.

0.5 N Sulfuric Acid Aqueous Reagent—Fifteen milliliters concentrated sulfuric acid was added to 1020 ml. water.

Chloroform Reagent—Reagent grade chloroform was saturated with distilled water.

Solvent—Two hundred milliliters 0.5 *N* sulfuric acid in distilled water was added to 800 ml. anhydrous methanol (ACS grade).

Standard Preparation—Fifty milligrams reserpine USP reference standard, previously dried at 60° under reduced pressure for 3 hr., was transferred to a 500-ml. volumetric flask, dissolved in a few drops of chloroform, and diluted to volume with methanol (stock solution).

Ten milliliters of the reference standard stock solution was transferred to a second 100-ml. volumetric flask and diluted to volume with solvent (dilute stock solution). Working standards were prepared by diluting the reference standard dilute stock solution as follows. A series of seven aliquots in 1-ml. increments, ranging from 7 to 13 ml., was pipeted into seven 50-ml. volumetric flasks. Each flask was adjusted to volume with solvent.

Sample Preparation—Due to the volatile and corrosive nature of the solvent and to the slow dissolution of the active material, the Solid Prep Sampler module was not utilized. The following manual method was developed for the preparation of the sample.

The tablets or the weighed aliquot powders were placed separately in a series of 120-ml., amber, wide mouth, screw-cap bottles containing exactly 50–100 ml. solvent. The samples were pulverized carefully for 50 sec. by inserting the Polytron probe into the bottle. After pulverization, the liquid adhering to the probe was drained by rotating the blades at low speed. The sample bottles were capped and left standing for 20 min.

At the end of the 20-min. interval, a second homogenization of the sample was carried out with the Polytron. This procedure gave consistent results due to the disruption of the imbibed root cells and release of the active constituents into the solvent. Heating or centrifugation of the sample for clarification was not necessary.

Assay Procedure—The flow system and the manifold settings are shown in Fig. 1.

Modifications in Manifold—*Positive Chloroform-Displacement Attachment*—Initially, Acidflex manifold tubing was utilized to introduce chloroform into the system. However, the life of the Acidflex tubing carrying the reagent chloroform was short, and the precision of the analysis was poor. This was due to the resistance offered to the flow of chloroform by the beaded extractor coil. Therefore, the reagent Acidflex tubing was substituted in its place by a positive chloroform-displacement system.

The displacement system consisted of a 2-l. ground-glass stoppered bottle filled with chloroform. The glass inlet and outlet

[1] The following equipment was used: Technicon liquid sampler (Autoanalyzer); Technicon proportioning pump; Technicon line recorder; Technicon Turner fluorometer, model 111 (Technicon Corp., Tarrytown, N. Y.); and a Polytron blender, model PT-20 (Brinkmann Instrument Corp., Westbury, N. Y.).

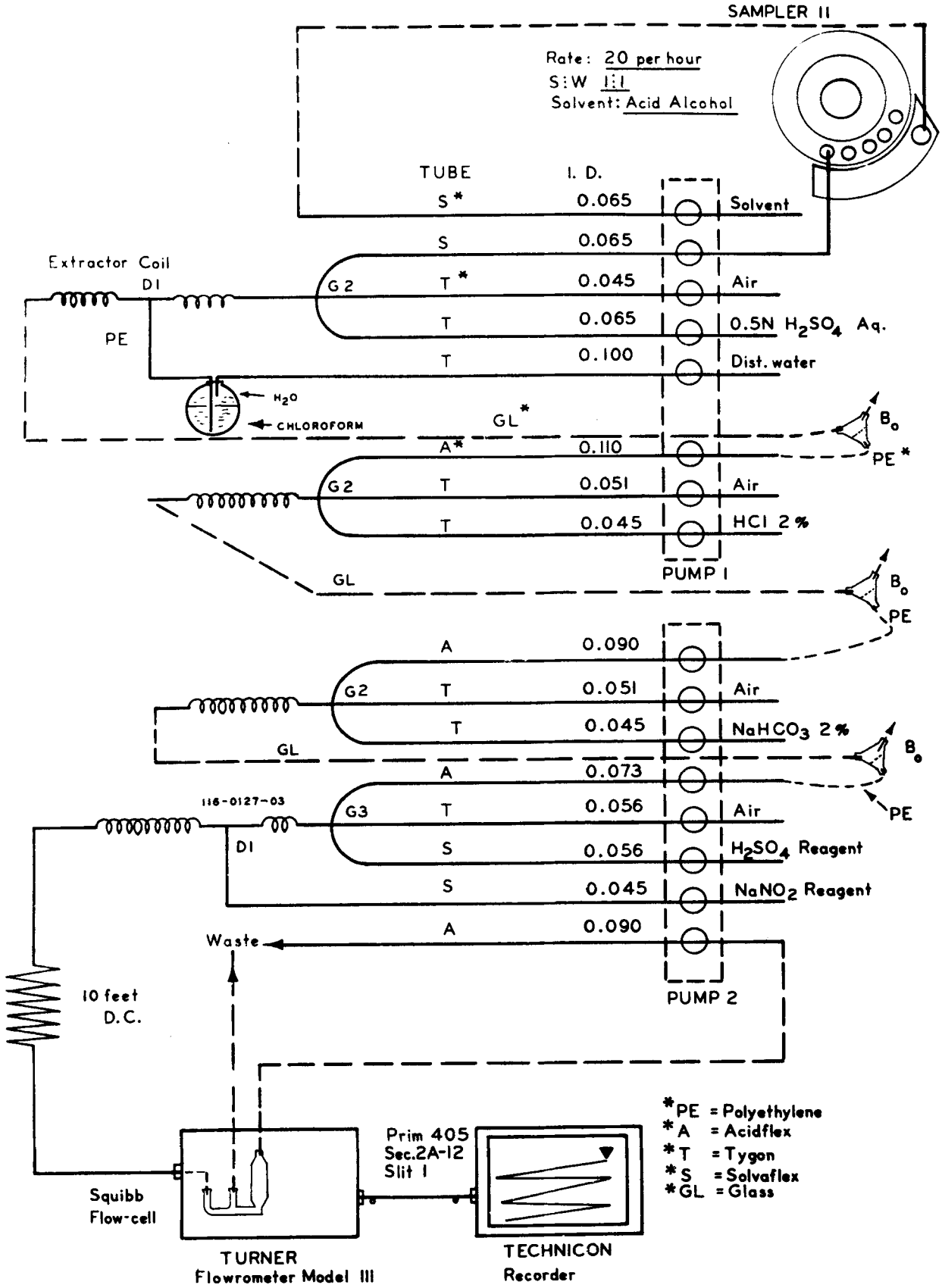

Figure 1—*Flow diagram for the determination of rauwolfia alkaloids.*

tubes were fused to the ground-glass stopper. The short inlet tube carrying the distilled water was connected to the pump through the heavy wall sleeving tubing. The long outlet tube, reaching to the bottom of the chloroform layer, was connected to the manifold through the polyethylene tubing (i.d. 0.034). Before the displacement bottle was connected to the manifold system, it was completely filled with chloroform, leaving no air pockets. To prevent entrapped air from entering through the heavy wall water inlet tubing into

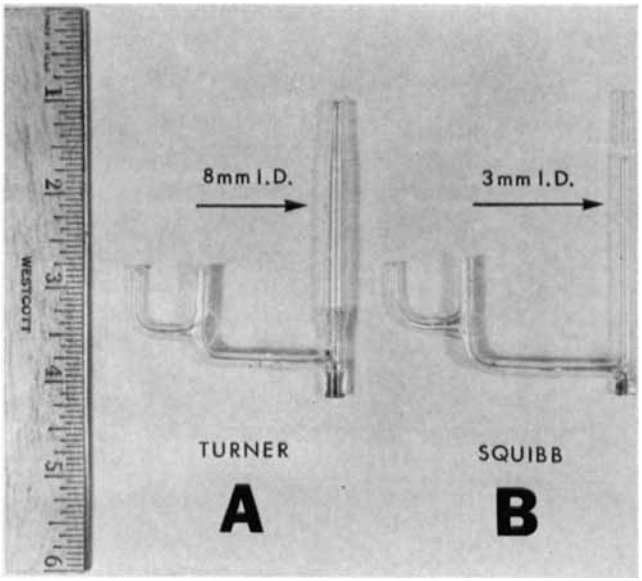

Figure 2—*Modified flow cell. Key: A, Turner flow cell; and B, Squibb flow cell.*

the bottle, this tubing was disconnected from the bottle at the beginning of pumping. After the tubing was completely filled with water, it was reconnected to the water inlet tube on the stopper of the displacement bottle. However, the polyethylene tubing carrying chloroform was initially connected directly both to the displacement bottle and to the manifold. This procedure ensured that no air bubbles were trapped in the reagent water, manifold water, or glass inlet tubes connecting the displacement bottle.

Modifications of Automated Chemistry Door of Fluorometer—The large flow cell of the fluorometer was originally held between two holders. It was attached to the sensitivity-boosting reflecting mirrors on the back and the large lens on the front. This flow cell was replaced by a homemade, small diameter, Pyrex glass flow cell (Fig. 2B). The mirrors, the lens, and the top holder were discarded. The new flow cell was held in position by its lower holder and was connected to the door through the Acidflex-inlet manifold, waste pick-up, and waste debubbler tubings.

RESULTS AND DISCUSSION

Homogenization by a Polytron blender, using an alcoholic sulfuric acid solvent system, was found to be an efficient and quick manual extraction method for solids and powders. No heat was generated during the blending operation. Chloroform could not be used as a solvent due to its low penetrating power into the root cells of rauwolfia samples (7).

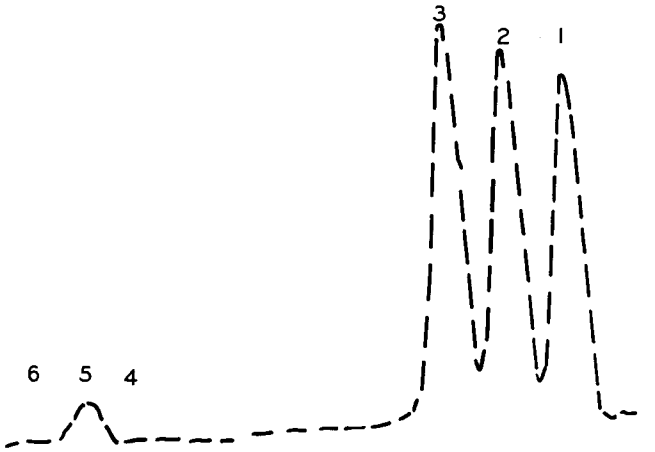

Figure 3—*Recordings from fluorometric analysis. Key: 1, reserpine USP; 2,* R. serpentina *root powder; 3, rescinnamine; 4, blank, reserpine USP; 5, blank,* R. serpentina *root powder; and 6, blank, rescinnamine.*

In the flow system (Fig. 1), the sample was aspirated and mixed with the aqueous sulfuric acid. This was necessary to prevent the formation of a homogeneous phase between the alcoholic sample and the chloroform on mixing. Chloroform was added to the diluted sample. The partitioning of the chloroform-soluble reserpine–rescinnamine alkaloids from the sample was accomplished in an extractor coil. As the liquids from the extractor emerged into the phase separator, the heavier chloroform phase was reaspirated continuously into the flow system. The chloroform extract was washed consecutively with dilute HCl and bicarbonate solutions in two separate double-mixing coils. This process removed the products of decomposition of reserpine–rescinnamine. The washed chloroform extract was then acidified and combined with nitrite reagent. The combined acid–nitrite/reserpine–rescinnamine solution was passed through a small delay coil to generate the optimum fluorescence. The recordings of this fluorescent solution were made at the excitation wavelength maximum of 390 nm. and an emission wavelength of 510 nm.

Specificity and Stability Studies—The semiautomated fluorometric method (6) for determining reserpine in tablets was not satisfactory for the determination of complex rauwolfia alkaloids. There was no interference by the excipients; however, there was considerable interference from the decomposition products of reserpine–rescinnamine. This interference was eliminated by the introduction of acid and base washings of the chloroform extracts of the sample.

The following experiments were carried out to demonstrate the specificity of the method for the analysis of reserpine–rescinnamine alkaloids.

Table I—Comparison of Manual and Automated Methods

Product Description	Drug Weight	Constituent Analyzed	Manual Method	Automated Results[a], mg./Sample	Manual Results, mg./Sample
Powdered *Rauwolfia serpentina* NF	Powder blend, 100 mg.	Reserpine–rescinnamine	NF	0.166 0.165	0.164 0.164
Tablet A[c]	Rauwolfia root powder, 100 mg.	Reserpine–rescinnamine	NF	0.164[b] 0.171[b] 0.170[b]	0.158 0.173 0.164
Tablet B[c,d]	Rauwolfia root powder, 50 mg.	Reserpine–rescinnamine	NF	0.088[b] 0.081[b] 0.075[b]	0.088 0.084 0.083
Tablet C[c]	Reserpine, 0.25 mg.	Reserpine	USP	0.240	0.233
Powder D[c]	Powder, 10 mg. Reserpine, 0.08 mg.	Reserpine	Squibb	0.088 0.087	0.084 0.085
Manufacturer X tablet	Rauwolfia root	Reserpine–rescinnamine	NF	0.170	0.167
Manufacturer Y tablet	Rauwolfia root powder, 100 mg.	Reserpine–rescinnamine	NF	0.173	0.169

[a] Different lots. [b] Average of 10 tablets (bulk of the tablets was the root powder; the tablet to tablet variation was small). [c] A = Squibb brand name Raudixin; B = Squibb brand name Rauzide; C = Squibb brand name Rau-sed; and D = Squibb brand name Orticalm. [d] Also contains 4 mg. bendroflumethiazide.

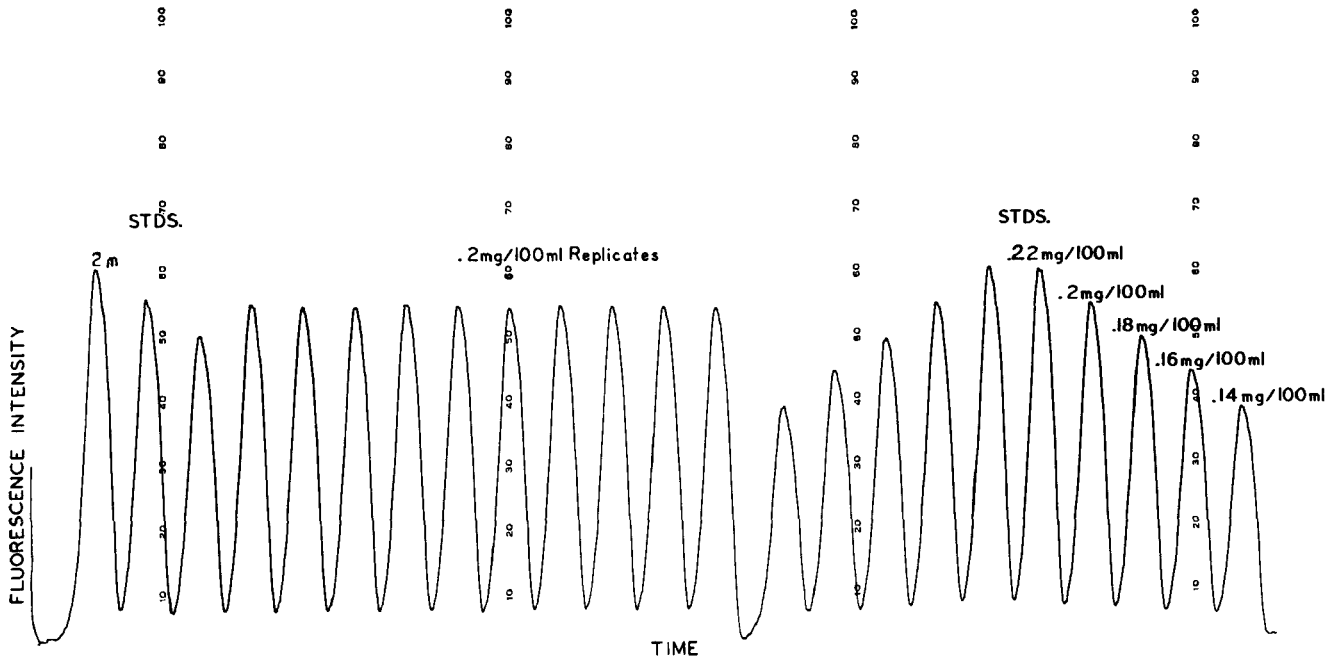

Figure 4—*Reproducibility and effect of concentration on analysis of reserpine standard.*

Figure 3 shows the results of the analysis of reserpine, rescinnamine, and rauwolfia root powder at 0.2-mg.% level for reserpine and rescinnamine and at 100-mg.% level for root powder. The recordings indicate that, at the same concentration level, reserpine has a lower fluorescence than rescinnamine (8).

Peaks 4, 5, and 6 of Fig. 3, corresponding to reserpine, rauwolfia root powder, and rescinnamine, were obtained with no sodium nitrite reagent added to the acidified chloroform extract. This is the normal blanking condition of the automated method. The appearance of no peaks from reserpine and rescinnamine (Peaks 4 and 6) and a definite small peak from the root powder under blanking conditions indicated interferences from nonalkaloidal fluorescent material. Therefore, blanking was necessary for the absolute determination of reserpine–rescinnamine group alkaloids. Generally, the blank values corresponded to 5–7% of the total value of the rauwolfia sample.

The related alkaloids, reserpidine and yohimbine (9), present in the *R. serpentina* species were analyzed using the automated system and were found not to interfere at the 5-mg.% level. It was reported (10) that alstonine, rauwolscine, sarpagine, and raunescine did not fluoresce when treated with nitrous acid. Bendroflumethiazide at the 8-mg.% level does not interfere in this automated analytical system.

Sensitivity—The sensitivity of the method increased due to the nonaqueous nature of the reaction system. Heat was not required to increase the sensitivity of the reaction, and this also prevented the evolution of nitrous acid gas from the reaction mixture. Adding excess $NaNO_2$ and water to the nitrite reagent increased the fluorescence; however, excess water and nitrite precipitated when the reagent was added to the chloroform. Increasing the concentration of sulfuric acid in the reagent proportionately increased the fluorescence. However, the excess acid produced noise in the recordings and affected the flexibility of the manifold tubing.

Precision and Accuracy—The precision of the method was determined as the variability obtained upon repeated analysis of the reserpine standard. A relative standard deviation of a 0.0002% reserpine standard or samples on replicate analysis was less than 1%. Recordings of the various concentrations, even though not at steady state and with the baseline not returning completely to its original starting position, showed that there was no carryover (Fig. 4).

The accuracy of the procedure was studied by comparing the automated method with the manual method. Both analyses were made on the same pooled powdered samples. The automated analytical results were in general agreement with the manual results (Table I).

SUMMARY

An automated nitrous acid fluorometric method for the determination of reserpine and rauwolfia alkaloids in unit doses was described.

A simple manual technique for the extraction of reserpine and rauwolfia alkaloids from the single tablets containing root powders was developed.

The effect of various interfering decomposition products of reserpine–rescinnamine on the analysis of rauwolfia alkaloids was discussed.

There was no interference from the excipients of tablets or of powder dosage forms or from reserpidine, yohimbine, and bendroflumethiazide.

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Effect of Particle Size and Speed of Compaction on Density Changes in Tablets of Crystalline and Spray-Dried Lactose

J. T. FELL* and J. M. NEWTON

Abstract □ Tablets were prepared from three particle-size fractions of crystalline and spray-dried lactose by the application of known pressures at a slow speed (upper punch speed 1 mm./min.) and a high speed (upper punch speed 600 mm./min.). The relative volumes and densities of the tablets were determined after ejection from the die and, for the slow speed, under pressure. Changes in volume with load were dependent on the particle size, on the speed of compaction, and on whether the volume was determined at pressure or after release of pressure. Calculation of the mechanism of densification by two different methods showed that particle rearrangement was greatest for the smallest size fraction both at slow and high speeds. Calculation of the yield pressure and the pressure necessary to fill the interparticle void space also was made.

Keyphrases □ Tableting, lactose—effect of particle size and speed of compaction on density □ Compaction—effect of particle size and speed of compaction on lactose tablet density □ Density, lactose tablets—effect of particle size and speed of compaction □ Lactose, crystalline and spray dried—tablet density changes related to particle size and compaction speed

The changes in density of powder beds which take place during the application of an applied load are of fundamental importance in the process of tableting. In addition to the characterization of tablet dimensions, various authors discussed the changes in terms of the mechanisms involved in the densification process (1–4). In all cases, the authors admitted to oversimplification of the problem. Nevertheless, conclusions have been drawn as to the effect of material characteristics and particle size on the densification process.

In terms of the effect of particle size, there appears to be some confusion. From work with sodium chloride, sucrose, and quartz, Huffine and Bonilla (1) considered that slippage was predominant to a higher pressure range and that the pressing modulus (defined as the ratio of log applied pressure to relative volume of the compact, where relative volume = volume of the compact/true volume of solid in compact) was low for finer particles. In contrast, Heckel (2) reported that for metal powders, densification due to particle rearrangement was greater for larger size particles. Hersey and Rees (3) found that volume changes were independent of particle size when crystalline lactose and spray-dried lactose were compacted at pressure greater than 49 MN./m.^{2}. Their work with sodium chloride indicated densification effects related to particle size, but no calculation of the relative mechanisms was made.

The final stages of compaction are related to the material rather than the particle geometry. Heckel (2) related the yield strength of metal powders to the linear portion of the curve of $\ln 1/(1 - D)$ (where D = apparent density) against pressure. Cooper and Eaton (4) supported this finding with studies of ceramic powders, the harder material requiring higher pressures to achieve densification.

Table I—Pressing Moduli for Particle-Size Fractions of Crystalline and Spray-Dried Lactose[a]

	Particle Size, μm.								
	0–32			75–104			150–210		
	a	b	c	a	b	c	a	b	c
Spray dried	2.70	2.94	4.51	3.22	3.83	3.07	3.66	3.93	3.31
Crystalline	2.81	3.01	2.82	3.11	3.76	3.07	3.88	3.88	3.31

[a] a = slow speed compaction, at pressure; b = slow speed compaction, after release of pressure; and c = fast compaction, after release of pressure.

In arriving at these conclusions, the methods of application of pressure have differed. In addition, the density of the compact has been assessed while under pressure (1, 4) and after the release of pressure (2, 3). The present study shows how these factors affect the assessment of the mechanisms of densification for different size fractions of lactose.

EXPERIMENTAL

Materials—The crystalline lactose[1] was reagent grade α-lactose monohydrate complying with BP standards. The spray-dried lactose[2] complied with USP standards. Both types of lactose were found by polarimetric observation and estimation of moisture content to be entirely α-lactose monohydrate. Particle-size fractions of less than 32, 75–104, and 150–210 μm. were obtained by means of an air-jet sieve[3]. A sufficient quantity of each size fraction was dried at 90° for 24 hr. and stored over silica gel until required for use.

Methods—*Compaction by Physical Testing Instrument*—A physical testing instrument[4] was modified to take a conventional 12.7-mm. flat-faced punch and die system, as described previously (5). In all cases, 0.5-g. samples of the previously dried powder were compressed. The die was lubricated with a 5% suspension of magnesium stearate in carbon tetrachloride. Five tablets were prepared at each applied pressure at a compression rate of 1.0 mm./min. When the desired pressure was attained, the crosshead was reversed at the same speed as the compaction process. The loss of pressure due to die wall friction was found to be less than 1.5%. Corrections were applied for the distortion of the punches under pressure.

Compaction by Instrumented Tablet Machine—A single-punch tablet machine[5] was instrumented using foil strain gauges[6]. The outputs from the bridges constructed on the upper punch and lower punch holders were fed into a signal conditioning unit[7]; from here, the amplified signal was recorded on a UV recording galvanometer[8], using moving coil galvanometers[9]. The instrumentation was cali-

[1] British Drug Houses Ltd., Poole, Dorset, England.
[2] McKesson Robbins, London, E.C. 4, England.
[3] Alpine Air-Jet, Lavino (London) Ltd., London, E.C. 2, England.
[4] Floor model, Instron Ltd., High Wycombe, Bucks, England.
[5] Type E3, Manesty Ltd., Speke, Liverpool 24, England.
[6] Type HF 26, Showa Sokki, Kenkyusho, Japan.
[7] Type MR 701, Data Acquisition Ltd., Stockport, Cheshire, England.
[8] Type 2005, S.E. Laboratories (Engineering) Ltd., Feltham, Middlesex, England.
[9] Type B, 450, S.E. Laboratories (Engineering) Ltd., Feltham, Middlesex, England.

Table II—Values of D_A, D_B, and D_0 Obtained as Described by Heckel (2)[a]

Density Determined	Particle-Size Fraction, μm.	Crystalline D_0	Crystalline D_A	Crystalline D_B	Spray Dried D_0	Spray Dried D_A	Spray Dried D_B
At pressure	0–32	0.322	0.728	0.406	0.285	0.703	0.418
	75–104	0.587	0.760	0.173	0.538	0.748	0.210
	180–210	0.618	0.764	0.146	0.527	0.754	0.227
Released after slow compaction	0–32	0.322	0.728	0.406	0.285	0.696	0.411
	75–104	0.587	0.761	0.174	0.538	0.754	0.218
	180–210	0.618	0.769	0.151	0.527	0.754	0.227
Released after fast compaction	0–32	0.322	0.773	0.451	0.285	0.668	0.383
	75–104	0.587	0.731	0.144	0.538	0.728	0.190
	180–210	0.618	0.711	0.997	0.527	0.741	0.214

[a] D_0 = initial apparent relative density of powder; D_A = densification due to die filling and particle rearrangement; and D_B = densification due to particle rearrangement.

brated using the physical testing instrument to enable both upper and lower punch forces to be determined. A 12.7-mm. diameter flat-faced punch and die system was used, being cleaned and then lubricated with a suspension of magnesium stearate in carbon tetrachloride between the preparation of each tablet. The tablets were prepared at a machine speed of 40 tablets/min. (corresponding to an upper punch speed of 600 mm./min.), the die being filled with 0.5 g. of the required powder. Five tablets were prepared at each machine setting.

Density Determinations—*True Powder Density*—This was determined with an air pycnometer[10]. The value for all samples was 1.546 g./ml. Confirmation of the absence of entrapped air within the spray-dried particles was provided by the method of Verhoog (6).

Apparent Density of Tablets—The weight of tablets was determined to ±0.0001 g. after compaction. The volume of the ejected tablet was determined from its dimension, determined to ±0.005 mm. with a micrometer. The volume of tablets at pressure was determined from the area of the tablet die and the height, determined as the difference between the initial height of the Instron crosshead as compaction commences and the crosshead height at the given applied pressure. The apparent density of the compact is the mass divided by the tablet volume.

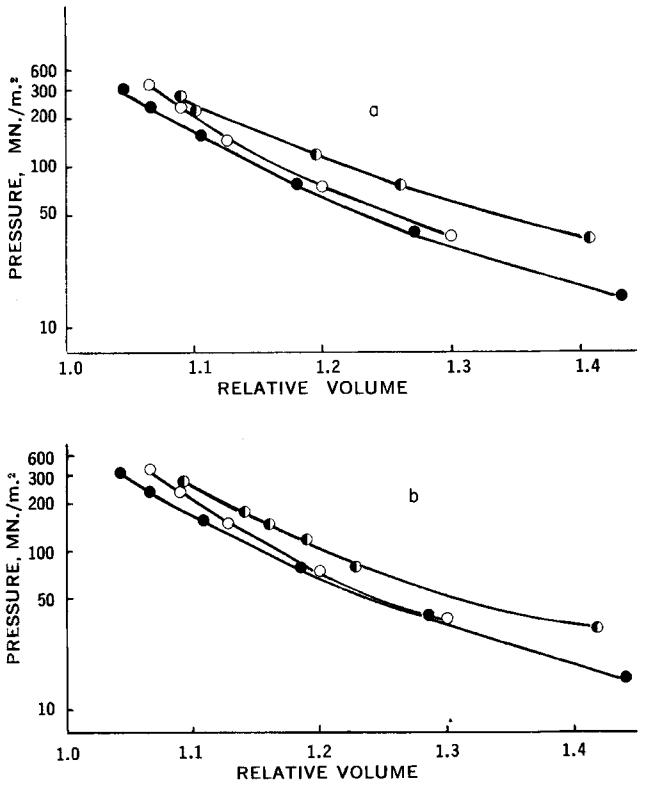

Figure 1—*Relative volumes of tablets compacted at known pressures, expressed by the method of Huffine and Bonilla (1). (a) = 75–104-μm. crystalline lactose. (b) = 75–104-μm. spray-dried lactose. Key: ●, relative volume determined at pressure, slow compaction; ○, relative volume determined after release of pressure, slow compaction; and ◐, relative volume determined after release of pressure, fast compaction.*

[10] Model 930, Beckman Instruments Ltd., Glenrothes, Fife, Scotland.

RESULTS AND DISCUSSION

The increase in density of a powder bed as a pressure is applied forms the basis of the preparation of tablets. The mechanisms of density changes which are considered to occur were reported by many authors and recently listed by Hersey and Rees (3). There is, however, little quantitative evidence of the relative magnitude of the various postulated mechanisms and of the influence of various factors on these mechanisms. The overall changes are readily observable in the present powders when the results are expressed in the manner used by Huffine and Bonilla (1) (Fig. 1). The pressing modulus obtained from such plots is given in Table I.

Whichever compaction system is considered, the pressing modulus is usually lower for finer particles, indicating a greater change in volume for a given pressure increase for these finer powders. Tablets prepared at slow speed have a higher pressing modulus for ejected tablets than for tablets at presssure. At low loads, the relative volumes of tablets, both under load and ejected, are similar because there is little chance of elastic recovery; whereas at high loads, elastic recovery is significant, leading to a higher relative volume for ejected tablets and different pressing moduli. While the effects of particle size are reduced by compaction at a higher speed, it is surprising that Hersey and Rees (3) reported that they were absent. For the two larger size fractions, a higher relative volume (lower relative density) is obtained when the powders are compacted at a faster rate, supporting the findings of Bada and Nagufuji (7).

Rather than consider actual changes in volume with pressure, information on the mechanism of densification can be obtained by

Table III—Values of Coefficients a_1 and a_2 Determined as Described by Cooper and Eaton (4)[a]

Particle-Size Fraction, μm.	Crystalline Lactose a_1	Crystalline Lactose $a_1 + a_2$	Spray-Dried Lactose a_1	Spray-Dried Lactose $a_1 + a_2$
0–32	0.66	1.00	0.60	1.00
75–104	0.62	1.00	0.72	1.00
180–210	0.59	1.00	0.61	1.00

[a] a_1 = fraction of theoretical compaction that would be achieved at infinite pressure for the filling of voids of the same dimensions as the particle; and $a_1 + a_2$ = total fraction of theoretical compaction that can be achieved by filling voids of the same and smaller dimensions than the particles.

Table IV—Yield Pressure and Pressure Necessary to Fill Voids within Compacts for Three Particle-Size Fractions of Crystalline and Spray-Dried Lactose

Compaction Conditions and Density Determination	Particle-Size Fraction, μm.	Crystalline Lactose			Spray-Dried Lactose		
		Yield Pressure Py, MN./m.²	Pressure to Fill Voids, MN./m.²		Yield Pressure Py, MN./m.²	Pressure to Fill Voids, MN./m.²	
			k_1	k_2		k_1	k_2
Slow compaction at pressure	0–32	164	2.7	42.4	162	2.4	32.7
	75–104	171	3.6	38.0	163	2.7	36.4
	150–210	139	2.7	28.6	127	2.6	23.6
Slow compaction, released	0–32	220	2.7	67.2	209	2.4	50.1
	75–104	222	3.6	71.0	355	2.7	75.8
	150–210	212	2.7	69.2	186	2.6	63.5
Fast compaction, released	0–32	179	—	—	135	—	—
	75–104	235	—	—	240	—	—
	150–210	204	—	—	179	—	—

expressing the results in the manner described by Heckel (2). The results do not obey the relationship:

$$\ln \frac{1}{(1 - D)} = KP + A \qquad \text{(Eq. 1)}$$

where D = relative density = apparent density of tablet/true density of powder, A and K are constants, and P = applied pressure. However, there is usually sufficient linear portion to allow extrapolation to calculate the density functions described by Heckel (2) (Fig. 2). The results given in Table II show clearly that for tablets compressed at slow speeds, the total densification due to filling of the die and particle rearrangement, D_A, increases as the size of the particle increases. This supports the findings of Heckel (2); but when the effect of die filling is taken into consideration, the present results are opposite to those of Heckel (2). That is, the present results show that densification due to particle rearrangement, D_B, decreases with a decrease in particle size. The reason for this difference stems from the values of D_0, the value for relative apparent density of the powder. The values given by Heckel (2) increase with a decrease in particle size. This is somewhat surprising, because it is generally considered (8) that bulk density decreases with a decrease in particle size.

Further support for the present findings is obtained by treating the results as described by Cooper and Eaton (4). (Only those tablets produced at slow compaction could, in fact, be treated by this procedure due to difficulties in obtaining tablets in the high porosity region on the tablet machine.) Tablet densities at low pressure are the same at pressure and when released; hence, values of a_1 are the same for both systems. This analysis is based on dividing the compaction process into two stages: (*a*) filling of holes of the same dimension of the particles by rearrangement, and (*b*) filling of holes of smaller dimensions than the particles by fragmentation and plastic flow. The relative magnitude of the dimensionless coefficients, a_1 and a_2, in the equation:

$$V^* = a_1 e^{-(k_1/P)} + a_2 e^{-(k_2/P)} \qquad \text{(Eq. 2)}$$

where V^* = fractional volume compaction[11], k_1 and k_2 are coefficients with units of pressure, and P = applied pressure, indicates the fraction of theoretical compaction that would be achieved at infinite pressure by each particular process. The results in Table III show that particle rearrangement a_1 would form a higher fraction of theoretical compaction for smaller particles than larger particles of crystalline lactose. The results are not conclusive for spray-dried lactose. Thus, the indications again support the concept that particle rearrangement is a more important feature of the compaction of small particles. The value of 1.0 obtained for a_1 and a_2 in all cases indicates that, theoretically, it should be possible to compact all the samples to zero porosity.

A further insight into the mechanism of compaction is provided by consideration of the constants K of Eq. 1 and k_1 and k_2 of Eq. 2. The former constant was related by Heckel (2) to the yield strength of the material and further related by Hersey and Rees (3) to the yield pressure, Py, by the equation:

$$K = \frac{1}{Py} \qquad \text{(Eq. 3)}$$

The values of Py for the different size fractions of different types of lactose are given in Table IV. Certain general trends are present. The values for yield pressure obtained when tablet density is measured at pressure are generally lower than those where tablet density is determined after ejection from the die. The density value at pressure also contains an elastic component which can give a false low value to the yield pressure. The 75–104-μm. size fraction has the highest yield pressure, irrespective of the method of application of load and measurement of density. The yield pressures for the samples of spray-dried lactose are generally lower than those for crystalline lactose. The lower value of yield pressure tends to favor plastic deformation during compaction. The values reported here are all lower than the average value of 401.7 MN./m.² reported by Hersey and Rees (3).

The values for k_1 and k_2 in Eq. 2 are related to the force necessary for the two stages of compaction reported by Cooper and Eaton (4). The values in Table IV confirm the findings of these workers in that the values for k_1, the pressure necessary to fill the interparticle void space of the same dimensions of the particle, are lower than the values for k_2, the pressure necessary to fill the interparticle void space of smaller dimensions than the particles. The values of k_1 and k_2 again support the findings for yield pressure that spray-dried lactose requires less pressure to fill voids than crystalline lactose. Due to elastic recovery, the values of k_1 and k_2 deter-

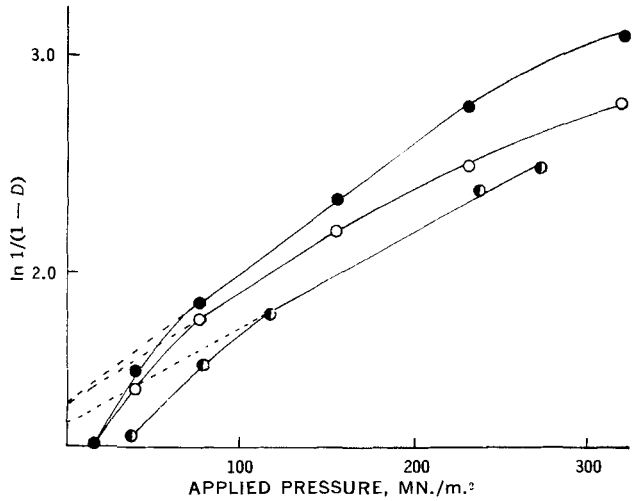

Figure 2—*Changes in tablet relative densities with applied pressure, expressed by the method of Heckel (2), for crystallized lactose of 75–104 μm. Key: ●, relative density determined at pressure, slow compaction; ○, relative density determined after release, slow compaction; and ◐, relative density determined after release, fast compaction.*

[11] $V^* = (V_0 - V)/(V_0 - V_\infty)$, where V_0 = volume of powder at zero pressure, V = volume of tablet at pressure P, and V_∞ = volume of tablet when all of the holes are filled, *i.e.*, the true volume of the solid present in the tablet.

mined from results with ejected tablets indicate that higher pressures are needed to fill the interparticulate void space than are in fact necessary. The effect of particle size is less clearly defined in the values of k_1 and k_2 but, again, there is the indication that the 75–104-μm. size fraction is the most resistant to deformation.

These results deal only with samples of lactose. When taken in conjunction with the previous work (1, 2, 4), the conclusion possibly could have application to other materials.

CONCLUSIONS

1. For tablet volumes determined at pressure, the smaller the original particle size, the greater is the relative volume at all applied pressures.

2. For tablet volumes determined after ejection from the die, the difference in relative volume between the two larger particle-size fractions is considerably reduced. The relative volumes for tablets prepared at slow compaction are lower than those of the two larger particle-size fractions, but the opposite is true for tablets prepared at the higher rate of compaction.

3. Calculation of the densification due to particle rearrangement by the method of Heckel (2) shows that the smallest size fraction undergoes the greatest rearrangement both at slow and high speed compaction. Particle rearrangement is generally greater for the spray-dried lactose.

4. Calculation of the densification due to particle rearrangement by the method of Cooper and Eaton (4) confirms this finding for crystalline lactose. The results for spray-dried lactose are, however, inconclusive.

5. The yield pressure calculated from the slope of the densification–pressure curves of Heckel (2) indicates that the middle size fraction has the highest value for both compaction systems and methods of measuring tablet density. The yield pressures are higher for tablets ejected from the die.

6. The yield pressure for spray-dried lactose is generally lower than for crystalline lactose.

7. The pressures necessary to fill the voids of smaller sizes than the particles, *i.e.*, those required to cause particle fracture and plastic flow, are always higher than the pressures necessary to fill the voids of the same dimensions as the particles, *i.e.*, to effect particle rearrangement.

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Batch Production of Pharmaceutical Granulations in a Fluidized Bed I: Effects of Process Variables on Physical Properties of Final Granulation

WILLIAM L. DAVIES and WALTER T. GLOOR, Jr.*

Abstract □ The investigation concerns the effects of process variables associated with the fluidized bed granulation technique on the physical properties of the final granulation. The process variables investigated include binder solution addition rate, air pressure to the binary nozzle, inlet air temperature during the granulation cycle, and binary nozzle position with respect to the fluidized solids. When the rate at which the aqueous binder solution added to the fluidized bed of powders was increased, the ability of the solution to wet and penetrate the solids was enhanced, resulting in: (*a*) a larger average granule size, (*b*) a less friable granulation, (*c*) a more fluid granulation, and (*d*) a decreased granulation bulkiness. Similar results, also traceable to enhanced binder solution efficiency, occurred with a decrease either in the binary nozzle air pressure or in the inlet air temperature during the granulation cycle. The position of the binary nozzle with respect to the fluidized powders had significant effects upon the average granule size and granule friability. The effects upon the granulation flow properties and bulkiness, however, were slight.

Keyphrases □ Granulation, fluidized bed—effects of process variables on granule and tablet physical properties □ Fluidized bed granulation—effects of process variables on granule and tablet physical properties

Although fluidization theory and techniques have been known for many years and have been discussed extensively in the literature (1–5), pharmaceutical applications in this area are relatively recent. Uses have been limited to the drying (6–9) and coating (10–16) of solids; there has been little investigation in the area of granulation.

Granulation in a fluidized bed for tableting purposes was first introduced by Wurster (11). He determined material loss from the column and the drug and moisture content of the final granulation. Theory, design, and operation of equipment for the continuous production of tablet granulations in a fluidized bed were presented by Scott *et al.* (17) and Rankell *et al.* (18).

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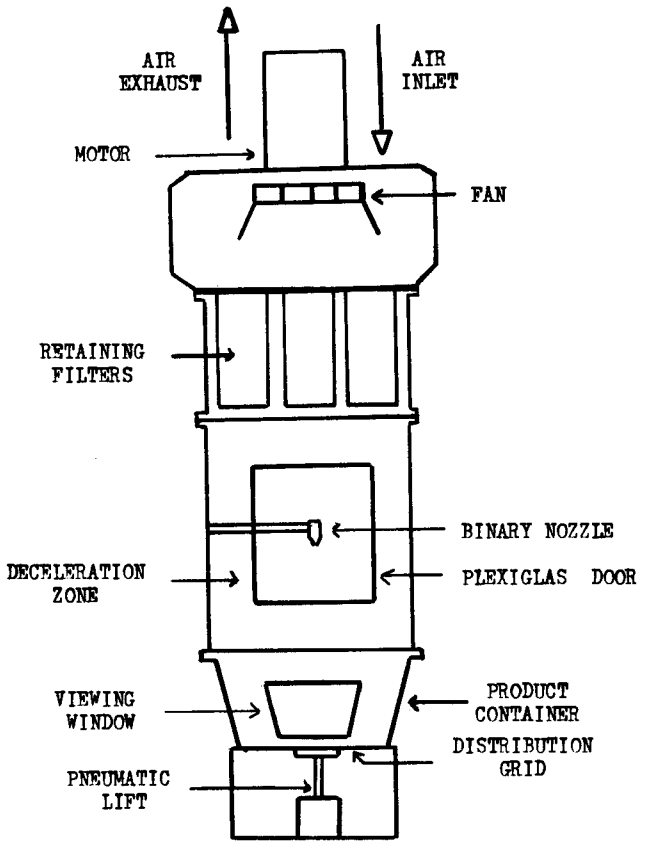

Figure 1—*Fluid bed spray granulator.*

In addition to material and energy balances and heat and mass transfer relationships, the authors investigated the performance of the continuous process with regard to entrainment loss, cyclone efficiency, granule compressibility, *etc.*

The intent of the present investigation was to determine the effects of process variables associated with the fluidized bed granulation technique on the physical properties of the final granulation.

EXPERIMENTAL

Materials and Formulation—The materials used were lactose USP[1], cornstarch[2], magnesium stearate USP[3], gelatin powder USP[4], and benzene[5].

The following formulation was used for all batch granulation operations:

	Percent w/w
Hydrous lactose USP	86.0–87.5
Cornstarch	10.0
Magnesium stearate USP	0.5
Gelatin USP	2.0–3.5
Distilled water	—

This classical formulation was chosen since each ingredient is widely accepted in pharmacy for the function it serves. To maintain a constant batch size of 10 kg. and to minimize effects on the physical properties of the granulations, the concentration of the major ingredient, lactose, was altered to compensate for the amount of dry binder in a particular batch. The gelatin binder solution was prepared by first thoroughly hydrating the gelatin powder in 2 kg. of distilled water for 10 min. The slurry, contained in a double boiler, was then gradually heated with stirring to 80°, the temperature at which the solution was used.

Table I—U. S. Standard Sieves and Sieve Openings

Sieve Number	Sieve Opening, μ
20	840
30	595
40	420
50	297
60	250
80	177

Equipment—A fluid bed spray granulator[6] (Fig. 1) was used in the preparation of all granulations. The granulator is a 10–25-kg. product capacity, stainless steel, combination fluid bed granulator and dryer. Ambient air is drawn through an inlet damper into the unit by means of a motor and fan located at the top of the granulator. The inlet air is prefiltered through fiberglass filters before passing over steam-heated coils for inlet air temperature control. A thermostat on the face of the unit regulates the steam feed to the coils. The filtered, temperature-controlled air is drawn up through a distribution grid and 60-mesh stainless steel screen at the bottom of the product container. In addition to product support, the grid and screen serve to distribute the fluidizing medium evenly over the cross section of the granulator. The diameter of the product container increases from 35.6 cm. (14 in.) at its base to 53.3 cm. (21 in.), the diameter of the expansion chamber. This chamber serves as a product deceleration zone.

A movable, binary atomization nozzle for liquid addition is centered in the expansion chamber. Spray from the nozzle is in a downward direction, countercurrent to the air flow. At the top of the expansion chamber, a nylon filter prevents fine particle entrainment losses as the air exits through an exhaust duct to the exterior of the building. For purposes of removing fines or low density powders from the filter surface, a pneumatically activated shaking device is attached to the filter frame. Air volume requirements for fluidization are controlled by the inlet and exit dampers. For air volume measurements, a pitot tube connected to a standard pressure gauge[7] is installed in the exhaust duct. Dry bulb thermometers are inserted below the distribution grid and just above the nylon filter for inlet and outlet air temperature measurements, respectively.

Granulation and Drying—The lactose and cornstarch were weighed directly in the product container using a scale[8]. The container was pneumatically locked in place in the granulator prior to initiation of the granulation cycle. The following machine settings were maintained throughout the study unless otherwise specified. The inlet air temperature was adjusted to 50° by the thermostat for the granulation cycle. The fluidizing air volume was maintained at approximately 350 cu. ft./min. by adjusting the inlet and exit dampers. The binary nozzle was centered in the expansion chamber 72.4 cm. (28.5 in.) above the distribution grid. An air pressure to the nozzle head of 1.5 kg./cm.^{2} was maintained for atomization of the binder solution. The air dome on the nozzle head was set in the number four position. (The dome setting regulates the diameter of the spray cone and is adjusted to prevent contact between the atomized binder solution and the walls of the granulator.)

The powders in the product container were mixed by fluidization for 1 min. prior to the addition of the binder solution. Because of the nature of particle movement in the fluidized bed, ideal mixing of powder blends is accomplished in this relatively short time (2). Upon completion of the mixing operation, the binder solution was pumped in an atomized form into the fluidized bed of powders by means of a calibrated metering pump and the binary spray nozzle system.

Since all binder solutions were aqueous and the total weight of water in each solution was constant, the total weight of solution was dependent upon the weight of solid binder employed for any one particular granulation. The degree of wetting imparted to the fluid-

[1] Hydrous powder, Mallinckrodt Chemical Works, St. Louis, Mo.
[2] Buffalo brand starch, Corn Industries, Cherry Hill, N. J.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Allied Chemical, Industrial Chemicals Division, Morristown, N. J.
[5] Thiophene free, certified ACS, Fisher Scientific Co., Fair Lawn, N. J.

[6] Glatt model WSG-15, Fa. W. Glatt, Haltingen, West Germany.
[7] Dwyer Instruments, Inc., Michigan City, Ind.
[8] Model 2131, Toledo Scale, Division of Reliance Electric Co., Toledo, Ohio.

Table II—Effects of Addition Rate of the Binder Solution on Physical Properties of the Granulation

Addition Rate, g. H_2O/min.	Average Granule Size, μ	Friability, %	Bulk Density, g./ml.	Granule Density, g./ml.	Porosity, %	Flow Rate, g./min.
85	240	15.6	0.41	1.484	72.37	154.1
100	256	11.3	0.44	1.489	70.45	167.0
115	263	8.6	0.46	1.489	69.11	172.4
130	271	8.4	0.46	1.489	69.11	179.6
145	278	6.9	0.48	1.493	67.85	182.9

ized powders was, therefore, dependent upon the addition rate of water rather than total binder solution. To keep the wetting rate constant for varying concentrations of binder solutions, the metering pump was adjusted to deliver 100 g. of water to the powdered bed per minute.

Once granulation of the powders was completed, the metering pump for binder addition was turned off; the drying cycle was initiated by elevating the inlet air temperature to 65° by means of the thermostat. The granulations were dried to an outlet dry bulb temperature of 49°. The exhaust dry bulb temperature is critical, since it is related to the product moisture content. The shaking device for the exhaust filter was activated several times during both the granulation and drying cycles to prevent accumulation of fine powders on the filter surface. Magnesium stearate, previously screened through a 60-mesh screen, was then distributed over the dried granules, and the granulations were fluidized for 1 min. to disperse the lubricant on the surface of the granules. The lubricated granulations were allowed to cool to room temperature in the product container prior to transfer to polyethylene bags for storage.

Physical Properties of Granulations—*Granule Size*—The average granule size was determined by sieve analysis. Six U. S. Standard sieves (Table I) were nested in descending order with respect to screen opening. Approximately 100 g. of granulation was placed on the top sieve, and the sieves were shaken for 5 min. at 800 vibrations/min. using a sieve shaker[9].

The average granule size on any sieve was determined in microns by averaging the size of the openings of the sieve through which the granules passed and the size of the openings of the sieve upon which the granules were retained. The weight retained on each tared sieve was converted to percent retention and multiplied by the average granule size on that sieve. The sum of these products divided by 100 (percent) yielded an average granule size. Sieve analyses were performed for each batch of granulation in triplicate, and reported results are average values.

Granule Friability—Granule friability was determined using a modification of the ASTM test for the friability of coal (19). A 100-g. sample of the 40/50- (passed/retained) mesh fraction of granulation was placed in a 1-l. jar and rotated in a shaker-mixer[10] for 30 min. at 100 r.p.m. The sample was then placed on the top sieve in a descending series of four U. S. Standard sieves (numbers 50, 60, 80, and 100). The sample was shaken for 5 min. as previously described. The size distribution was determined, and the percent friability of the sample was calculated (20). Percent friability determinations were made in triplicate and averaged. In no instance did an individual granulation range in friability by more than 0.5%.

Density—A loose bulk density measurement was made on all granulations. Thirty grams of a granulation was poured through a standard 12.7-cm. (5-in.) diameter, short-stem glass funnel into a 100-ml. graduated cylinder. The orifice of the funnel stem was aligned with the 100-ml. graduation on the cylinder. The volume occupied by the granulation was read to the nearest 0.5 ml., and the loose bulk density was calculated in grams per milliliter. The bulk densities recorded are averages of six determinations.

Granule density was determined by a method outlined by Bauer and Lewin (21). Approximately 3 g. of granulation was accurately weighed to the nearest 0.1 mg. and transferred to a 25-ml. pycnometer. Enough benzene was added to fill the pycnometer, and the total weight was accurately determined. All granule density determinations were made at 25°. Since this procedure is limited to solids heavier than, and insoluble in, the displacement medium, and to ensure that water would not interfere with the determinations, the benzene was dried with metallic sodium prior to use. Reported granule densities are averages of six determinations.

Porosity—Using the method of Martin (22), percent interparticulate porosity was calculated for each granulation from the bulk and granule density measurements obtained from the individual granulations:

$$\%e = 1 - \rho b/\rho g \qquad \text{(Eq. 1)}$$

where ρb is the bulk density and ρg is the granule density.

Flowability—Granule flowability was measured using a standard glass funnel of 100-mm. diameter, 60° bowl angle, with a 36-mm. stem length and a 6-mm. internal stem diameter. The funnel was equipped with a stopper in the stem orifice and was supported by a ring stand. Seventy-five grams of granulation was carefully poured into the funnel, the stopper was removed, and the flow rate of the granulation was determined to the nearest 0.3 sec. Reported flow rates are averages of six determinations.

All granules exceeding 1680 μ (12 mesh) were removed from the granulations prior to flowability studies to prevent interferences in granulation flow through the exit orifice. In no instance did this size granule exceed 0.1% of the total granulation. To minimize relative humidity effects on granulation flow, all granulations were allowed to equilibrate at 45 ± 5% relative humidity prior to determining flow rates under these humidity conditions.

Percent Loss on Drying—Percent loss on drying of each granulation was determined gravimetrically using a moisture balance[11]. All samples were dried on the balance to an equilibrium loss on drying.

RESULTS AND DISCUSSION

Since, in the preparation of pharmaceutical granulations, drying is one of the most critical of all operations, this stage was of primary importance in the operation of the fluid bed spray granulator. To minimize the effects of moisture on the physical properties of the granulations, it was desirable to dry all batches of granulations to a constant percent loss on drying value. Drying was conducted to an exhaust dry bulb temperature of 49° for all granulations. This temperature, in equilibrium with the fluidized solids temperature (9), yielded an indication of the dryness of the product. While this exhaust air temperature–moisture content relationship may vary with the granulation, depending upon its content and physical properties, all batches in this investigation differed only by the 2–3.5% solid binder concentration, and drying to an exhaust air temperature of 49° produced granulations with percent moisture loss on drying values of 2.10 ± 0.15.

The success of granule formation depended upon a number of operational variables associated with the fluid bed granulator. The most influential variables appear to include: (*a*) the addition rate of the binder solution, (*b*) the degree of atomization of the binder solution, (*c*) the temperature of the fluidizing medium during the granulation cycle, and (*d*) the position of the binary nozzle with respect to the fluidized solids. The effects of these factors on the physical properties of the resulting granulations were investigated.

The effects on granule formation of the rate of binder solution addition to the fluidized powders are depicted in Table II. Twenty-two hundred grams of a 9.1% w/w gelatin solution was introduced at varying rates to the fluidized batches of the standard lactose formulation, with resultant influences on the physical properties of the granulated products. An enlargement in the average granule size was noted with each increase in the rate of addition of the binder solution. This growth in granule size may be attributed to an increase in the penetration and wetting capabilities by the aqueous

[9] Fisher Wheeler, Fisher Scientific, Boston, Mass.
[10] Turbula, Willy A. Bachofen, Basel, Switzerland.
[11] Model 6000, Ohaus Corp., Union, N. J.

Table III—Effects of Inlet Air Temperature during the Granulation Cycle on Physical Properties of the Granulation

Inlet Air Temperature	Average Granule Size, μ	Friability, %	Bulk Density, g./ml.	Granule Density, g./ml.	Porosity, %	Flow Rate, g./min.
25 ± 2°	311	6.2	0.54	1.492	63.81	196.1
40 ± 2°	272	7.1	0.50	1.497	66.60	192.9
50 ± 2°	249	11.8	0.44	1.502	70.71	168.8
55 ± 2°	235	14.7	0.41	1.495	72.58	160.1

binder solution, which was brought about by the increased liquid flow through the atomization nozzle. Larger spray droplets of binder solution per unit of time resulted in an overall slower rate of evaporation of the solvent by the drying medium.

The friability test is based upon the assumption that weak granules will disintegrate when agitated in a shaker device, whereas strong granules will lose only their surface roughness. Results of the friability tests show decreased friability with increased rates of addition of the binder solution. Just as granule size may be increased by the greater wetting ability of the binder at higher rates of addition, less friable granules are formed by augmenting the wetting and penetration capabilities of the binder solution.

The bulk density of a granulation is primarily dependent upon particle size, particle-size distribution, particle shape, and cohesive forces (23, 24). Small granules have a low free-fall velocity and a high surface-to-mass ratio, a combination that reduces the amount of granule movement as the sample granulation is poured into a cylinder for bulk density measurement. In addition, the greater cohesiveness of small particles produces an arching or bridging of the granules within the cylinder. An increase in the average granule size reduces the particulate interactions due to a lower surface-to-mass ratio. The resultant decrease in granulation void spaces is manifested by a higher bulk density value. These effects were observed in bulk density and porosity determinations of the granulations prepared at varying rates of binder addition. However, it is felt that the bulk density increase and corresponding porosity decrease with an increase in the average granule size are not solely attributable to the free-fall velocity and surface effects of the granules. Instead, the primary effect is the greater degree of wetting of the powder bed by the increasing rates of addition of the binder solution. The greater wetting ability of the binder solution not only effects an increase in the average granule size but also produces a more dense granulation.

Porosity determinations do not include intraparticulate void spaces because the displacement medium, benzene, used in the determination of granule density does not penetrate the fine capillaries within the granules. Granule density was unaffected by varying the rate of binder addition. At the low concentration of binder (2% of the total formulation), changes in the granulated product were not sufficiently significant to alter the intraparticulate voidages. For a single formulation, a change in the intraparticulate pore spaces of the granules is needed to effect a change in granule density.

Several investigators demonstrated the principal factors influencing the fluidity of particulate solids (25–27). In this investigation of granulation orifice flow rates, the porosity of the granulation was of greatest importance. The granulation flow rate was inversely related to the porosity of the granulation in the granulation size ranges of this study. The observed increasing flow rate, associated with increasing average granule size, was due to the less porous larger bulk density granulations formed at higher rates of binder solution addition.

Table III relates the effects of the inlet air temperature during the granulation cycle on the physical properties of the granulated product. Twenty-two hundred grams of a 9.1% w/w gelatin binder solution was used for each batch. Higher inlet air temperatures during granule formation produced smaller, more friable granulations. The elevated temperatures of the fluidizing medium increased the rate of evaporation of the aqueous binder solvent, thereby lowering the extent of wetting and penetration of the fluidized bed of powders by the binder solution.

The fluidizing air temperature during granulation is of major importance to the success of the operation. From Table III, it can be concluded that a very high inlet air temperature during granulation would result in a spray-drying effect of the atomized binder solution with little or no granule formation. On the other hand, granulating at room temperature tends to cause premature agglomeration of the powders due to overwetting by the binder solution. This agglomeration or caking of the powders makes it difficult to maintain a fluidized state. The magnitude of this problem grows as binder concentration, rate of binder addition, and spray droplet size are increased.

The more porous granulations obtained at higher inlet air temperatures during the granulation cycle are due to the bulkiness of the products, which results from the larger quantities of unprocessed powders in the batches. The granulation process decreases the bulkiness of the powders by densifying them through wetting with the binder solution. This bulk density increase and increasing average granule size result not from increased binder adhesiveness but from an increased wetting of the fluidized powders by the binder solution. In the fluidized bed granulation technique, granule size may be controlled either through degree of wetting of the fluidized solids or through increased binder concentration (increased binder adhesiveness). Granule size enlargement due to the increased wetting capabilities of the binder solution results in a more dense granulation, while larger average size granulations obtained through increased binder concentration result in less dense granulations. As expected, the larger, more dense granulations formed at lower inlet air temperatures showed better flow properties.

Atomization of the binder solution occurs in the binary nozzle head, at which point the liquid binder and air converge to form fine droplets of aqueous binder. The degree of atomization of the binder solution is controlled by the proportion of air and liquid mixture in the nozzle head. Table IV illustrates the effects of increasing the proportion of air to liquid in the nozzle on the granulation properties. An increase in the air pressure from 0.5 to 2 kg./cm.^{2} brought about a steady decline in the average granule size of granulation batches produced with 2350 g. of a 14.9% w/w gelatin binder solution. The decrease in the average granule size, with its corresponding increase in granule friability, is attributed to the finer spray droplet formation of the binder solution as a result of increasing the proportion of air to liquid in the atomization process. This effect is comparable to that of decreasing the rate of liquid addition while maintaining a constant air pressure to the nozzle. The conclusions drawn for the size and friability properties of granulations manufactured by varying these operational processes are parallel and, therefore, are not reiterated here.

The decreased bulkiness of the granulation manufactured at 0.5 kg./cm.^{2} air pressure is attributed to a more thorough penetration of the binder solution into the powder bed. This enhanced binder solution penetration results from a slower evaporation rate of the binder solvent in its larger droplet size. The slight decrease in granule density with decreasing nozzle air pressure is due to the greater proportion of intragranular voidages associated with the increased average granule size.

Table IV—Effects of Air Pressure to the Binary Nozzle on Physical Properties of the Granulation

Air Pressure, kg./cm.^{2}	Average Granule Size, μ	Friability, %	Bulk Density, g./ml.	Granule Density, g./ml.	Porosity, %	Flow Rate, g./min.
0.5	438	6.9	0.43	1.453	70.41	116.4
1.0	354	8.4	0.38	1.469	74.13	110.6
1.5	327	9.6	0.37	1.475	74.91	114.0
2.0	292	11.7	0.37	1.474	74.90	119.6

Table V—Effects of Height of the Binary Nozzle above the Distribution Grid on Physical Properties of the Granulation

Nozzle Height above Grid, cm. (in.)	Average Granule Size, μ	Friability, %	Bulk Density, g./ml.	Granule Density, g./ml.	Porosity, %	Flow Rate, g./min.
81.91 (32.25)	286	12.2	0.36	1.484	75.74	115.1
72.39 (28.50)	292	11.7	0.37	1.474	74.90	119.6
62.86 (24.75)	313	10.9	0.36	1.472	75.54	118.6
53.34 (21.00)	344	8.3	0.37	1.468	74.80	116.1

The degree of atomization of the binder solution had little effect upon the density of the granulation until the air pressure to the nozzle was reduced to its lowest value. These results were observed at a single rate of binder addition and inlet air temperature and may, therefore, not apply to other liquid feeds or fluidizing temperatures.

Of greater interest, however, is the equality of granule flow rates of the four granulations. As expected, with the equal densities and relatively similar size distributions of the granulations produced at air pressures of 1.0, 1.5, and 2.0 kg./cm.^{2}, there were little differences in the weights of granule flow per unit time. However, a greater flow rate was expected with the more dense granulation produced at 0.5 kg./cm.^{2} air pressure. It is suspected that the lower than anticipated flow rate was caused by the increased proportion of 20-mesh granules in this granulation. These large granules interfere with the flow of granulation through the narrow orifice of the funnel stem. Zenz and Othmer (2) reported that, for uninterrupted flow, the internal diameter of the orifice should exceed 5–7 times the diameter of the largest granules. The ratio of the orifice diameter to the largest granule fraction in this granulation is 5 to 1.

The position of the binary nozzle within the fluid bed granulator is most easily described with respect to the height of the nozzle head above the air distribution grid at the base of the product container. Table V depicts the effects of the nozzle position on the physical properties of standard granulations manufactured from 2350 g. of a 14.9% w/w gelatin binder solution. The only significant effects of the nozzle site are on the average granule size and granule friability. The closer the nozzle is positioned to the fluidized bed of powders, the more efficiently will the atomized binder solution be able to facilitate the formation of granules. This increased efficiency of the binder, as the nozzle is lowered in position, is again due to its increased ability to wet and penetrate the fluidized solids.

In discussing the nozzle site, the temperature and velocity of the fluidizing medium, the solids density and batch size, and the volatility of the binder solvent must all be established. Positioning of the nozzle too near the fluidized solids may cause an interference in the spray pattern of the atomized binder solution or a possible obstruction within the nozzle head as a result of continuous impingement of the solid particles on the nozzle orifice. At the same time, a distant nozzle location results in a spray drying of the binder solution with little granule formation.

The nozzle height, particularly in its lowest position, produced no significant changes in the granulation densities or flow properties, a fact that is difficult to explain. Earlier results and discussions led to the anticipation of a greater bulk density value for the larger average granulation size produced at the lowest nozzle location. An examination of the individual sieve fractions of the granulations manufactured at various nozzle positions showed little difference in the quantity of fines present in the granulations. The increase in the average granule size, as the nozzle was lowered closer to the fluidized powders, resulted from a small but increasing number of large granules due to the proximity of the nozzle to the fluidized powder bed. However, lowering the nozzle without increasing the diameter of the spray cone resulted in an increase in the number of solid particles in the area beyond the conical pattern of the atomized binder solution. For this reason, there were approximately an equal number of fine granules in all batches. Since the fine component of a granulation is most influential with respect to its physical properties, there were little variations in the densities and flow properties of the granulations manufactured at various nozzle positions.

Having established the major operational influences and obvious capabilities of the fluid bed granulator, it was desirable to investigate further the merits of this single-stage granulation process. The effects of various granulating agents and concentrations on the physical properties of the resultant granulations and tablets compressed from these granulations will be the subject of subsequent reports.

CONCLUSIONS

In the fluid bed granulation process, granules are formed when the powders, wetted by the atomized binder solution, adhere to one another as contact is made in the heated fluidizing medium. The physical properties of granulations manufactured by this process are dependent upon both the individual formulations granulated and the various operational variables associated with the process.

When the rate at which the aqueous binder solution added to the fluidized bed of powders is increased, the ability of the solution to wet and penetrate the solids is enhanced, resulting in: (*a*) a larger average granule size, (*b*) a less friable granulation, (*c*) a more fluid granulation, and (*d*) a decreased granulation bulkiness. Similar results, also traceable to enhanced binder solution efficiency, occur with a decrease either in the binary nozzle air pressure or in the inlet air temperature during the granulation cycle.

The position of the binary nozzle with respect to the fluidized powders has significant effects upon the average granule size and granule friability. The effects upon the granulation flow properties and bulkiness, however, are slight. As the binary nozzle is lowered closer to the fluidized solids, binder efficiency is enhanced, resulting in an increase in the average granule size and a corresponding decrease in granule friability.

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Influence of Fluid Motion in Dissolution-Rate Determinations

S. BISAILLON* and R. TAWASHI

Abstract □ A method is presented for dissolution-rate studies based on three-dimensional movement of the dissolution cell around its axis. By using this agitating system, the solute flux from benzoic acid and iodine disks was studied at different flow velocities. The results obtained were compared with the rotating-disk method. The dependence of dissolution rate on the power of flow velocity and on the activation energy was determined experimentally. The Reynolds and Peclet numbers were calculated from the parameters of the experiments, and the values obtained were compared with theory for laminar and turbulent flow.

Keyphrases □ Dissolution rates—effect of fluid motion and activation energy, Turbula shaker mixer and rotating-disk methods compared □ Fluid motion—effect on dissolution rates □ Rotating-disk method—compared with Turbula shaker mixer method, influence of flow velocity and activation energy on dissolution rates □ Turbula shaker mixer—dissolution-rate determinations, compared to rotating-disk method

Dissolution is the rate-determining step of numerous mass-transfer processes such as catalysis, adsorption, extraction, and drug absorption. Modern hydrodynamic concepts such as theories of turbulent or laminar flow and boundary layer (1–4) are now being used in the solving of problems involved with systems of hydromechanical equations. Information on the effect of agitating intensity in dissolution test procedures is scarce and insufficient (5–8) for developing a routine control procedure. An unexpected variable (vibration effect) in the latest USP and NF rotating-basket dissolution tests was reported by Beyer and Smith (9).

The present publication deals with the effect of speed of agitation on the rate of solute transfer in geometrically similar systems. The dissolution behavior in a new agitating system based on a three-dimensional movement of the dissolution cell around its axis was tested experimentally and compared with data obtained from the rotating disk. The data obtained could be used to predict the quantitative performance of the agitating system and to find a better design for a dissolution test procedure. The choice of the agitating system described in this study is based on previous experience in solid mixing and in solubility determinations. Solid–solid mixing was improved remarkably by using this agitating system (10), and in solubility studies of poorly soluble drugs, the equilibrium was reached after a shorter period of time than with other conventional methods (11).

Table I—Equations for Evaluation of the Dimensionless Reynolds and Peclet Numbers

Reynolds' number	$= \frac{\text{momentum transfer by turbulent flow}}{\text{momentum transfer by molecular transport}}$
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	$N_{RE} = \frac{UL^2}{\nu} = \frac{UL^2\rho}{\eta} \quad : \quad N_{PE} = \frac{UL^2}{D}$

Table II—The Reynolds (N_{RE}) and Peclet (N_{PE}) Numbers as Calculated for the Turbula Shaker Mixer and for the Rotating Disk

Temperature	N_{RE} Rotating Disk	N_{RE} Turbula	N_{PE} Rotating Disk	N_{PE} Turbula
25°	200	27,000	0.20×10^6	27.7×10^6
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THEORETICAL CONCEPTS

The analysis of the behavior of flows by similarity theory is now widely applied. Even if geometrical similarity, identity of boundary conditions, and Reynolds' number are sufficient conditions for the similarity of two flows, only a few systems have been satisfactorily solved from their cylindrical convective diffusion equation.

Taking into consideration the streamlines at the surface of a rotating disk serving as the reaction site, Cochran (12) developed the cylindrical convective diffusion equation for the rotating disk, from which Levich (1, 13) came to the following expression:

$$R = kD^{2/3}\nu^{-1/6}\omega^{1/2}c_s \qquad \text{(Eq. 1)}$$

It can be rearranged to give:

$$K_T = kD^{2/3}\nu^{-1/6}\omega^{1/2} \qquad \text{(Eq. 2)}$$

The significance of each term in these equations is listed in the *Appendix* at the end of this article.

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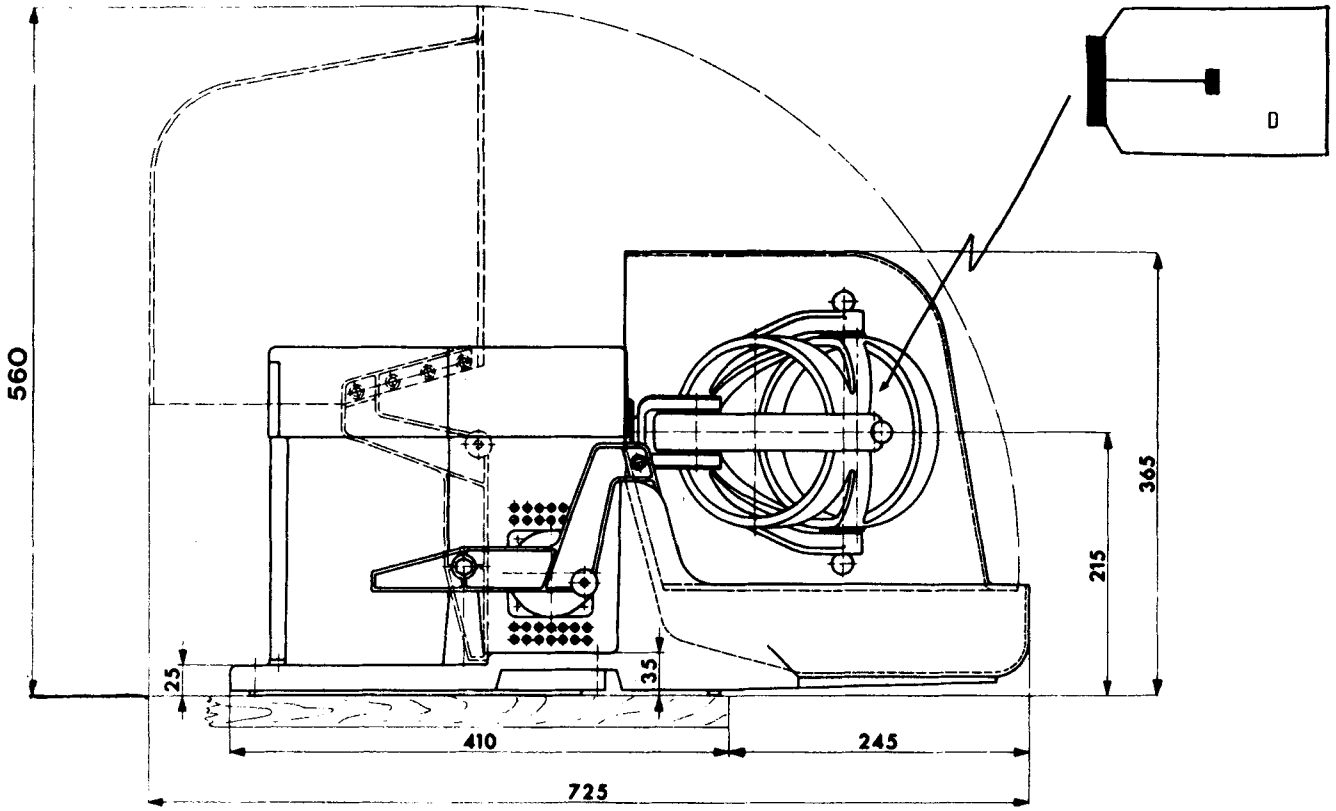

Figure 1—*Turbula shaker mixer (dimensions in millimeters). D is the dissolution cell.*

the speed of rotation, one is in the presence of unsteady chaotic motion in which the flow velocity fluctuates about some average value. Levich (1, 13) found that when the flow was laminar, the exponent attributed to the speed of rotation was 0.5; when one passes into turbulent regime, the exponent passes to unity.

Similarity theory, along with the analysis of variables in well-defined equations, often refers to dimensionless analysis. From the cylindrical convective diffusion equations, two dimensionless groups could be used in this respect. The Reynolds number represents the momentum transfer of the fluid by turbulence divided by molecular transport, and the Peclet number represents the ratio between mass transfer in the fluid by convective diffusion and molecular diffusion (Table I). In this table, U = speed of rotation in centimeters per second, L = diameter in centimeters, ν = kinematic viscosity where $\nu = \rho/\eta$ and ρ = density in grams per milliliters and η = viscosity in poises, and D = diffusion coefficient in square centimeters per second. Values of the Reynolds number, which indicate the passage from laminar to turbulent flow, are in the region of 10^4–10^5. No exact value can be given. Geometrical conditions, especially in the critical region, can contribute markedly to produce turbulence. The Peclet number for liquids, in which D is in the order of 10^{-5}–10^{-6}, is generally in the order of 10^3–10^5. Higher value show an improvement in mass transfer in the solution.

The activation energy of the transport process can also confirm the presence of laminar or turbulent flow in a system. The dependence of the dissolution-rate constant on the temperature is related to the activation energy of the process by:

$$K_T = Ae^{-E_a/T} \qquad \text{(Eq. 3)}$$

and:

$$\log K_T = \log A - E_a/T \qquad \text{(Eq. 4)}$$

A plot of log K_T *versus* $1/T$ gives a linear curve in which the slope represents the activation energy of the process. Nogami *et al.* (3)

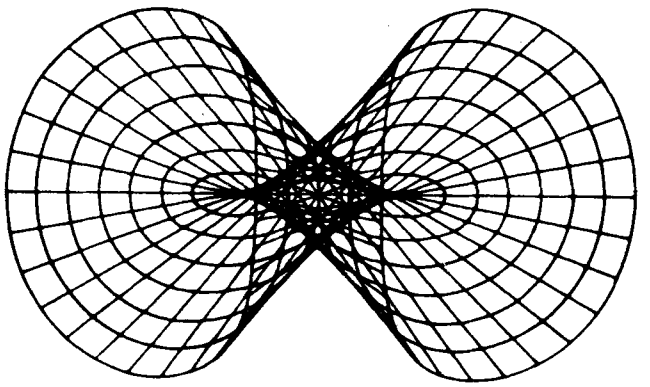

Figure 2—*Path of the motion traveled by equidistant points on the container axis (plane projection vertical to transmission shafts).*

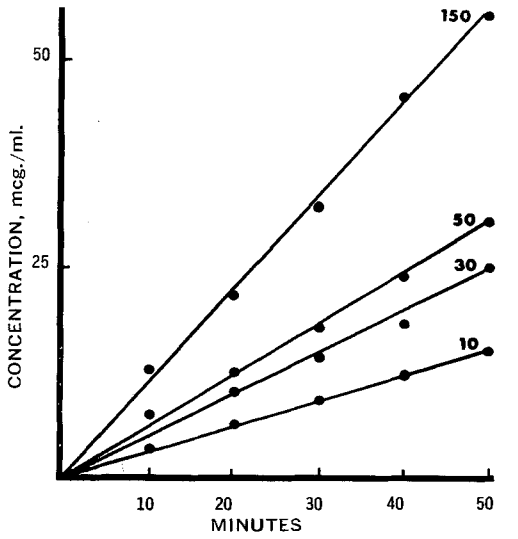

Figure 3—*Dissolution of benzoic acid by the rotating-disk method as a function of time at 37° and at different speeds of rotation.*

Table III—Study of the Solubility, Diffusion Coefficient, Dissolution Rate, and Dissolution-Rate Constant as a Function of Temperature

Temperature	Diffusion Coefficient, cm.²/sec.	Solubility (C_s), g./l.	Dissolution Rate (R), g./cm.²/sec. Rotating Disk	Dissolution Rate (R), g./cm.²/sec. Turbula	$K_T(R/C_s)$, cm./sec. Rotating Disk	$K_T(R/C_s)$, cm./sec. Turbula
20°	7.573×10^{-6}	2.75	—	6.20×10^{-6}	—	2.214×10^{-3}
30°	9.830×10^{-6}	3.74	3.38×10^{-6}	—	0.904×10^{-3}	—
37°	11.591×10^{-6}	4.43	4.52×10^{-6}	14.32×10^{-6}	1.020×10^{-3}	3.233×10^{-3}
45°	13.794×10^{-6}	5.62	6.50×10^{-6}	21.40×10^{-6}	1.157×10^{-3}	3.808×10^{-3}

reported values of 3.3–3.5 kcal./mole for the energy of activation in rotating-disk experiments in the laminar region, with a rotating velocity of 60–120 r.p.m. When the flow was in the turbulent region, values of 4.4–4.6 kcal./mole were reported by Levich (1). These considerations are increasingly important in examining the effect of fluid motion on dissolution rates and the effect of agitating intensity on other mass-transfer processes.

MATERIALS AND METHODS

Apparatus—The essential features of the agitating system used in this study are the flow pattern and agitating intensity provided by the shaker mixer (Turbula shaker mixer[1]). The model used in this work is presented in Fig. 1. The fluid flow produced by this shaker is based on the principle of "inversion kinematics" developed by Schatz (14). The mixing vessel, a 2-l. cylindrical container, is subjected to a rhythmically pulsating three-dimensional movement. This movement is an alternative acceleration and retardation in which the potential energy resulting from the braking phase passes directly into the generation of the subsequent acceleration. Figure 2 demonstrates the path of motion traveled by equidistant points on the axis of the mixing chamber.

Dissolution-Rate Studies—The materials investigated were benzoic acid and iodine. Both pure substances were compressed into a disk, 1.88 cm. in diameter, with a pressure of 50,000 p.s.i. The compressed disk was placed in a die at the center of the dissolution cell, and one of the flat surfaces was exposed to the dissolution medium.

When a given speed of rotation is imparted to the whole container, the drug disk rotates the same number of revolutions as the dissolution cell. The temperature of the dissolution medium was kept constant during the dissolution-rate studies by a specially designed, thermostatically controlled cabinet around the shaking system. The dissolution medium was 1500 ml. of 0.1 N HCl to assure sink conditions in the study of dissolution behavior of the test substances. The 2-l. dissolution cell, with the same amount of 0.1 N HCl, was used to study the dissolution of the test substances using the rotating-disk technique. In these experiments, temperature was regulated by a thermostatically controlled water bath, and the drug disk was rotated by a synchronized motor[2] at different speeds of rotation. The height of the centered drug disk from the bottom of the cell was the same in both the Turbula and in these experiments. Samples were withdrawn at different intervals of time, filtered, and analyzed spectrophotometrically[3].

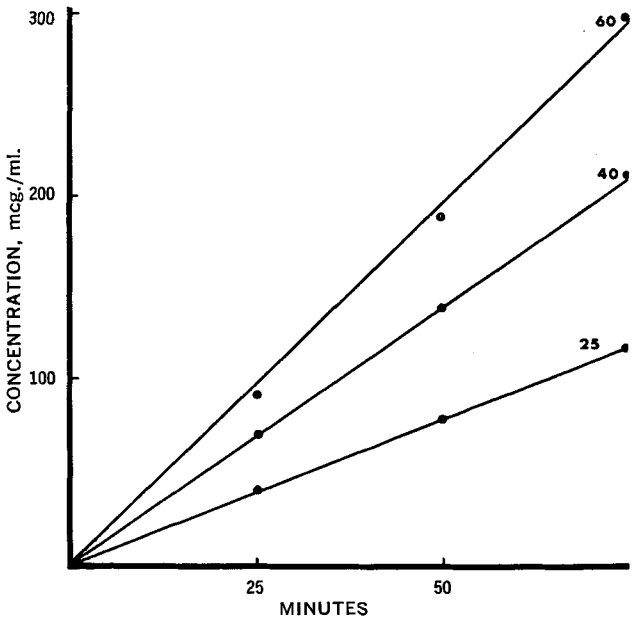

Figure 4—*Dissolution of benzoic acid in the Turbula shaker mixer as a function of time at 37° and at different speeds of rotation.*

RESULTS AND DISCUSSION

A study of the effect of speed of rotation on the dissolution behavior of a benzoic acid compressed disk showed a marked difference in the rates between the two methods (Figs. 3 and 4). This difference became more evident when the log dissolution rate was plotted against the speed of rotation for the two processes (Fig. 5). Experimental values, computed from the work of Emanuel and Olander (15) on the dissolution of benzoic acid by the rotating disk, showed good agreement with the present findings. The two curves were parallel but they did not superimpose because of the different temperature used in the two studies. The slopes of these curves were calculated, and the values obtained corresponded to the exponents of the angular velocity in the Levich equation, the revolutions per minute being directly proportional to the distance (in centimeters per second) traveled by the disk. A value of 0.5 was calculated for both rotating-disk curves, while for the Turbula a value of approximately 1 was obtained (1.035). The value obtained indicates the passage of the flow from laminar to turbulent This change in the exponent of the speed of rotation was verified with iodine (Fig. 6) and the same relation was obtained: the exponent of the flow velocity was close to unity (0.908).

The dimensionless Reynolds and Peclet numbers were determined for the two methods. In calculating both these numbers, the

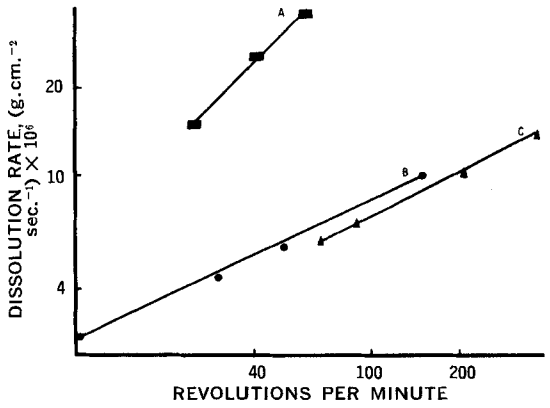

Figure 5—*Comparison of the rate of dissolution of benzoic acid as a function of speed of rotation. A is the dissolution rate in the Turbula; B is the dissolution rate from the rotating-disk method; C is the dissolution rate computed from* Reference 2.

[1] Manufactured by Willy A. Bachofen, Basel, Switzerland, and distributed by Chemical & Pharmaceutical Industry Co., Inc., New York, NY 10013

[2] Heavy duty synchronous motors, Hurst Manufacturing Corp., Princeton, Ind.

[3] Spectronic 600, Bausch & Lomb, Rochester, N. Y.

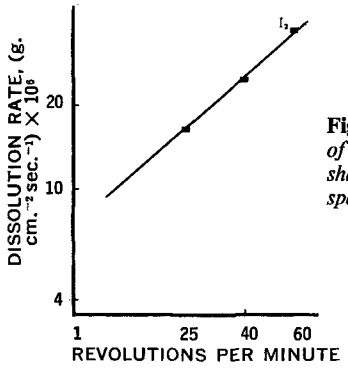

Figure 6—*Rate of dissolution of iodine at 37° in the Turbulo shaker mixer as a function of speed.*

L (signification already mentioned) used was 1.88 cm. for the rotating disk and 22 cm., the diagonal of the moving dissolution cell, for the Turbula shaker mixer. It is apparent from Table II that at two different temperatures, a remarkable difference exists in the two processes. The Peclet and Reynolds numbers were 135 times greater for the Turbula apparatus than in the rotating-disk system. For the Turbula shaker, the Reynolds number obtained was in the critical region of the passage from laminar to turbulent regime, although the speed of rotation was only 30 r.p.m. As mentioned previously, the geometrical conditions favored the creation of chaotic motions; they reinforced each other instead of damping the turbulent eddies.

The effect of temperature on the dissolution-rate constant was further studied for the two processes. The diffusion coefficients at various temperatures were calculated from molar volumes (16); the solubilities, dissolution rates, and dissolution-rate constants were determined experimentally as a function of temperature (Table III). From these parameters and from an Arrhenius plot (Fig. 7), the activation energy for the dissolution processes was determined. The values obtained were compared with values reported in the literature for the laminar and turbulent regimes (Table IV). For the rotating disk, there is a good agreement between the value obtained and that reported by Nogami *et al.* (3). In the Turbula shaker mixer, comparison demonstrates that even at low revolutions per minute, the dissolution mass transfer has an activation energy consistent with the turbulent flow regime as mentioned by Levich (1).

In summary, the computation of the solute flux rate from the surface of a disk in the Turbula shaker mixer was found to be proportional to a higher power of flow velocity (close to unity), which is consistent with turbulent flow This finding was supported

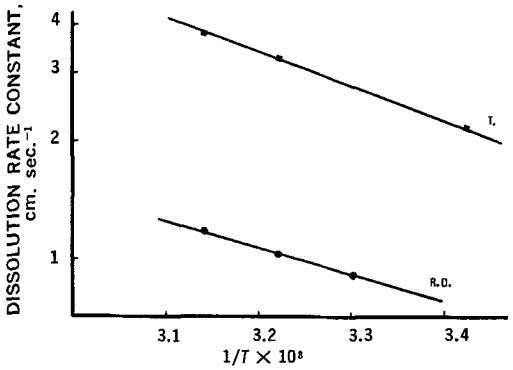

Figure 7—*Temperature dependence of the dissolution-rate constant of benzoic acid for the rotating disk (R.D.) and the Turbula shaker mixer (T).*

Table IV—Energy of Activation Computed from the Arrhenius Plot (Fig. 7) and Compared with Literature[a]

Activation Energy (E_A)			
Laminar Flow		Turbulent Flow	
E_A^1	E_A^N	E_A^2	E_A^L
cal./mole		cal./mole	
3475	3500	4419	4600

[a] E_A^N is the reported value by Nogami *et al.* (3); E_A^L is the reported value by Levich (1); E_A^1 is the value obtained for the rotating-disk method; E_A^2 is the value obtained in the Turbula shaker mixer.

by the determination of the energy of activation and by the calculation of the Reynolds and Peclet numbers.

With the improvement of the mixing conditions by energetic movement and random agitation of the fluid, the transfer of solute by random turbulent eddies remarkably increased the rate of dissolution. Hence, if the agitating system described is capable of providing a higher dissolution at low revolutions per minute, it may offer a convenient and practical way to increase the rate of other heterogeneous processes such as catalysis, extraction, adsorption, and crystallization.

APPENDIX

R = dissolution rate in g./cm.²/sec.
k = constant
D = diffusion coefficient in cm.²/sec.
ν = kinematic viscosity in stokes
ω = angular velocity in rad./sec.
c_s = solubility in g./l.
K_T = dissolution-rate constant in cm./sec.

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Apparent Enzyme Inhibition through Enzyme-Induction Studies as a Possible Mode of Action of Certain Cobalt Compounds

JOSEPH J. MITALA* and RONALD F. GAUTIERI

Abstract □ The results of this investigation show that cobaltous chloride and sodium cobaltinitrite may inhibit cortisone-induced cleft palate and methylcholanthrene tumorigenesis in albino mice *via* enzyme inhibition.

Keyphrases □ Cobaltous chloride—enzyme inhibition of cortisone-induced cleft palate and methylcholanthrene tumorigenesis, albino mice, mode of action □ Sodium cobaltinitrite—enzyme inhibition of cortisone-induced cleft palate and methylcholanthrene tumorigenesis, albino mice, mode of action □ Enzyme inhibition—cobalt compounds, possible mode of action

During the past decade, much enthusiasm has been generated concerning the modification, by chemical means, of the complex processes of carcinogenesis and cleft palate inception (1–4). Out of a myriad of investigations concerned with the blocking of these two different pathological processes, one finds that cobaltous chloride and sodium cobaltinitrite used alone in the proper amount and time are both successful in reducing the incidence of methylcholanthrene-induced tumors and cortisone-induced cleft palate (1, 3). In addition, by themselves, cobaltous chloride and sodium cobaltinitrite both were shown to cause a low incidence of cleft palate.

In an attempt to explain these findings, many theories have been presented. Orzechowski *et al.* (1), in 1964, suggested that sodium cobaltinitrite reduced the amount of available oxygen to cancerous tissue through the formation of methemoglobinemia and, hence, caused inhibition of tumor growth. In 1968 (4, 5), the "ionic hormonal precursor hypothesis" was postulated to explain inhibition of cleft palate on one hand and induction on the other. In this case, it was believed that cobalt was acting in fetal tissue as a hormone and, although when used together with cortisone it interfered with its action, when used alone it mimicked the action of the parent hormone. It was suggested at that time that possibly cobalt acts like cortisone in primordial or aquatic forms of life and could be considered, therefore, as an ionic hormone performing the task in fetal tissue that cortisone would normally accomplish in later life if exogenously administered.

Many studies have shown that drug-metabolizing enzymes in liver microsomes are increased when animals are treated with various drugs and carcinogens. This increase in activity correlates directly with an increase in the concentration of enzyme protein and is referred to as "enzyme induction" (6). Conney *et al.* (7) demonstrated that pretreatment of rats with phenobarbital stimulates liver microsomal enzymes, which metabolize zoxazolamine as well as aromatic polycyclic hydrocarbons (7).

In light of the fact that specific cobalt compounds reduce the number of methylcholanthrene-induced tumors, the possibility exists that these same compounds stimulate liver enzymes which may metabolize methylcholanthrene and, hence, not allow the formation of tumors.

It is well known that certain enzymes in the liver microsomes metabolize not only drugs but also a variety of normal body substrates, such as steroid hormones, at a given rate. If the metabolizing-enzyme concentration is increased, hormonal metabolism would be increased. Therefore, because it is known that certain inorganic cobalt compounds inhibit the inception of cortisone-induced cleft palate (3), the possibility also exists that the cobalt compounds stimulate liver enzymes which may metabolize the cortisone and prevent its action in fetal tissue.

Therefore, the purpose of this investigation was to ascertain whether enzyme induction is produced by cobaltous chloride and sodium cobaltinitrite which, if observed, could account for the apparent nulling of the carcinogenic action of methylcholanthrene and the teratogenic action of cortisone.

MATERIALS AND METHODS

Ninety-six CF-1 female mice, weighing between 14 and 40 g. and averaging 25 g., were used in this experiment. These animals were divided into eight groups of 12 mice each and further divided into subgroups of six mice each. Cages were provided for each subgroup.

The diet consisted of food[1] and tap water, both supplied *ad libitum*.

All mice in each subgroup were numbered by punching holes in their ears.

All solutions were prepared of such concentrations that no animal received more than 0.45 ml. at one time. The phenobarbital solutions were prepared on the Friday prior to pretreatment injections and stored at room temperature. At that time, a 0.6% solution of phenobarbital in a 0.9% sodium chloride solution was prepared.

The sodium hexobarbital solutions were prepared 1 day prior to their use and stored at room temperature at a concentration of 1.0%.

The zoxazolamine[2] was obtained in the powdered form. The solution was prepared 1 day prior to use by adding 3.6 ml. of 1 *N* HCl to 300 mg. of zoxazolamine, which had been weighed out on an analytical balance, and diluting to 15 ml. with a 0.9% sodium

[1] Purina Laboratory Chow.
[2] Supplied through the courtesy of McNeil Laboratories, Fort Washington, Pa.

Table I—Duration of Hexobarbital Sleeping Time and Zoxazolamine Paralysis

Pretreatment Group[a]	Treatment	Duration of Action, min.
1. Phenobarbital	Hexobarbital	23[b]
Saline	Hexobarbital	125
2. Cobaltous chloride (LD)	Hexobarbital	86[b]
Saline	Hexobarbital	67
3. Cobaltous chloride (HD)	Hexobarbital	80[b]
Saline	Hexobarbital	44
4. Sodium cobaltinitrite (LD)	Hexobarbital	40
Saline	Hexobarbital	46
5. Sodium cobaltinitrite (HD)	Hexobarbital	43
Saline	Hexobarbital	47
6. Phenobarbital	Zoxazolamine	11[b]
Saline	Zoxazolamine	72
7. Cobaltous chloride (LD)	Zoxazolamine	49
Saline	Zoxazolamine	46
8. Cobaltous chloride (HD)	Zoxazolamine	46
Saline	Zoxazolamine	51

[a] Number of animals per group = 12. LD = low-dose cobaltous chloride, 10 mg./kg., and sodium cobaltinitrite, 20 mg./kg. HD = high-dose cobaltous chloride, 20 mg./kg., and sodium cobaltinitrite, 40 mg./kg. [b] p value 0.05 significantly different from saline control using Student's t test.

chloride solution. This solution also was stored at room temperature.

The solutions were all prepared according to the procedures outlined by Conney *et al.* (7).

The saline used in this experiment was obtained from 30-ml. multiple-dose vials previously prepared in this laboratory. The following drugs were always freshly prepared just prior to their injection: cobaltous chloride, 0.1 and 0.2%; and sodium cobaltinitrite, 0.2 and 0.4%.

Pretreatment consisted of sodium phenobarbital, cobaltous chloride, or sodium cobaltinitrite administered to a subgroup. For each subgroup receiving one of the test drugs, an additional subgroup was injected with a volume of saline corresponding to the dosage volume of the test drug.

The pretreatment injections were administered intraperitoneally twice a day, in the morning between 8:15 and 9:30 a.m. and in the early afternoon between 12:10 and 1:50 p.m., for 4 consecutive days, Monday through Thursday. All of the mice were weighed[3] to the nearest gram prior to the injections.

The doses of phenobarbital, hexobarbital, and zoxazolamine were those used by Conney *et al.* (7). The doses of cobaltous chloride and sodium cobaltinitrite were those that had been found to give the most significant results (1, 3) in previous experiments in this laboratory. They were as follows:

Drug	Dose
phenobarbital	1.5 mg. in 0.25 ml. of 0.9% sodium chloride solution (fixed)
hexobarbital	125 mg./kg.
zoxazolamine	100 mg./kg.
cobaltous chloride	10 mg./kg. (low dose)
	20 mg./kg. (high dose)
sodium cobaltinitrite	20 mg./kg. (low dose)
	40 mg./kg. (high dose)

On the 5th day, Friday, all animals were injected intraperitoneally, between 8:53 a.m. and 1:51 p.m., with either sodium hexobarbital or zoxazolamine. At this time, each animal was caged individually to determine the duration of action of the drug administered.

None of the drugs administered throughout the experiment was observed to be lethal to the animals.

RESULTS AND DISCUSSION

The criterion used in this investigation to observe enzyme induction was derived from a study by Conney *et al.* (7). These workers demonstrated the enzyme-inducing effects of phenobarbital upon the rate of metabolism of hexobarbital and zoxazolamine. In both cases, the duration of action was measured by determining the length of time necessary for animals to regain their righting reflexes after an intraperitoneal injection. The observed responses were hypnosis with hexobarbital and paralysis with zoxazolamine. In their experiments, animals pretreated with phenobarbital showed a significant decrease in the duration of action of both hexobarbital and zoxazolamine. In the present investigation, these results were confirmed (Table I, Groups 1 and 6) before further experimentation was attempted.

Since it is known that cobalt, in some manner, antagonizes both the tumorigenic effects of methylcholanthrene and cleft palate production by cortisone, it was hypothesized that these antagonistic effects may be brought about by increasing the concentration of enzymes that metabolize methylcholanthrene as well as cortisone.

Although no significant effect was noted when zoxazolamine (Table I, Groups 7 and 8) was used in conjunction with cobalt pretreatment, the duration of the hexobarbital response (hypnosis) appeared to be contrary to the results anticipated. Administration of hexobarbital to animals previously treated with cobaltous chloride showed an increase in the duration of hypnosis (Table I, Groups 2 and 3). Therefore, if enzyme systems are responsible for the observed results, it seems that enzyme inhibition, rather than enzyme induction, occurred.

According to Conney (6), an increase in liver microsomal enzymes parallels an increase in the rate of drug metabolism, thereby demonstrating the effect of enzyme induction. With this fact in mind, it follows that the rate of drug metabolism depends upon the concentration of metabolizing enzymes. Should this rate be decreased, it may be postulated that the concentration of metabolizing enzymes also is decreased, *via* enzyme inhibition. The action of cobaltous chloride on hexobarbital clearly demonstrates this theory (Table I, Groups 2 and 3). However, the observed effect of the cobalt compounds upon the response to zoxazolamine was not as evident. Nevertheless, enzyme inhibition may be occurring in both cases, but the means of determining this were not observed *via* duration of paralysis with zoxazolamine.

If the genesis of cancer with methylcholanthrene involves the introduction of new enzyme systems, which promote tumor growth, into the parent cell, then tumor growth would also be inhibited if these enzymes are inhibited *via* "protein deletion" (8). Accordingly, if excess cortisone produces its cleft palate effect by acting in conjunction with enzymes present in palate tissue, then cleft palate inception may be inhibited by removing these necessary enzymes. Consequently, because it was shown that both cobaltous chloride and sodium cobaltinitrite reduce the incidence of methylcholanthrene-induced tumors as well as cortisone-induced cleft palate inception (1, 3), and because apparent enzyme inhibition was observed in this present investigation, it may be hypothesized that these cobalt compounds exert their actions against cancer inception and cleft palate induction through enzyme inhibition.

[3] On a Hanson Dietetic Scale, model 1440.

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Opium Alkaloids XI: Biosynthesis of Aporphines in *Papaver somniferum*

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Abstract □ When specifically labeled (±)-reticuline was administered to opium poppies, it was incorporated into isoboldine to an extent of about 0.08%. No incorporation of reticuline could be observed in magnoflorine. Since reticuline, isoboldine, and magnoflorine are known to exist in the opium poppy, it may be concluded that aporphines with a 1,2,9,10-substitution pattern (isoboldine type) can be biosynthesized by direct phenol coupling (*ortho–para*), while this may not be the case for aporphines with substituents in positions 1,2,10,11 (corytuberine type). Because of steric factors, these aporphines are more likely to be produced *via* a dienone intermediate (proaporphine) followed by a dienone–phenol rearrangement.

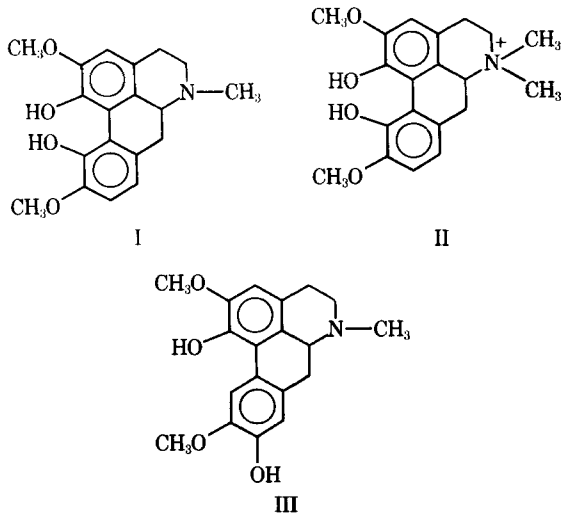
Keyphrases □ *Papaver somniferum*—biosynthesis of aporphines □ Aporphines—biosynthesis in *Papaver somniferum* □ (+)-Reticuline—incorporation into isoboldine, determination of aporphine biosynthetic pathways in *Papaver somniferum* □ Opium alkaloids—aporphine biosynthetic pathways in *Papaver somniferum*

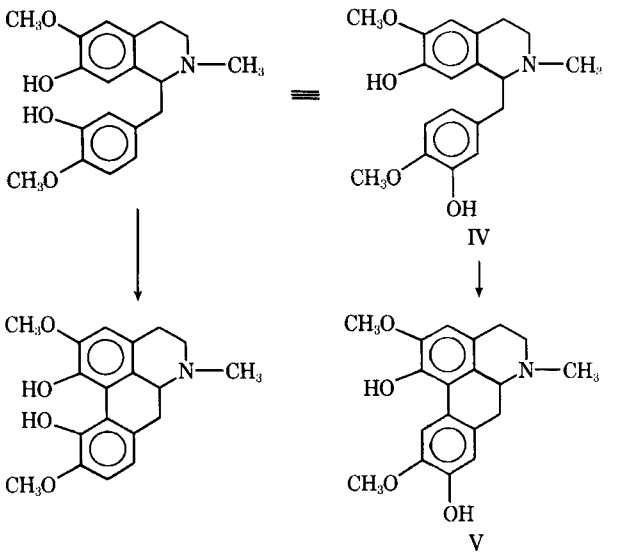
The aporphines have only recently joined the family of opium alkaloids. Nijland (1) reported the isolation of corytuberine (I) and magnoflorine (II) in 1965. Two years later, isoboldine (III) was added to the list (2). These alkaloids have the absolute configuration corresponding to the *S*-series, which relates them to (+)-reticuline, (+)-orientaline, and (−)-norlaudanosoline (2, 3).

In their famous paper on oxidative coupling of phenols, Barton and Cohen (4) proposed two biosynthetic pathways by which suitable, phenolic tetrahydroisoquinolines might be converted to aporphines, either by a direct coupling (Scheme I) or indirectly *via* an intermediate dienone which, in turn, could undergo rearrangements to a variety of aporphines (Scheme II). Although the biosynthetic pathways leading to aporphines are far from elucidated, there is today considerable evidence in support of both the direct coupling and the mechanisms that require the intermediate dienone. *In vitro* oxidation of reticuline (IV) by reagents that favor formation of free radicals has produced isoboldine (V) (5, 6). Even better yields were obtained when the nitrogen was quaternary (7, 8). However, never has a product been isolated from such reaction mixtures to indicate that a direct *ortho–ortho* coupling to aporphines takes place. Aporphines also have been synthesized by oxidative coupling to a dienone, reduction to the corresponding dienol, and rearrangement to the aporphine. The synthesis of isothebaine (IX) from orientaline (VI) *via* orientalinone (VII) was performed by Battersby and coworkers (9, 10) and is illustrated in Scheme III. Rearrangement of orientalinone without prior reduction has given rise to isocorytuberine (VIII) (11), an alkaloid not yet isolated from nature. Similar "biogenetic-type" syntheses of aporphines by dienone–phenol and dienol–benzene rearrangements were reported by several investigators (12–16).

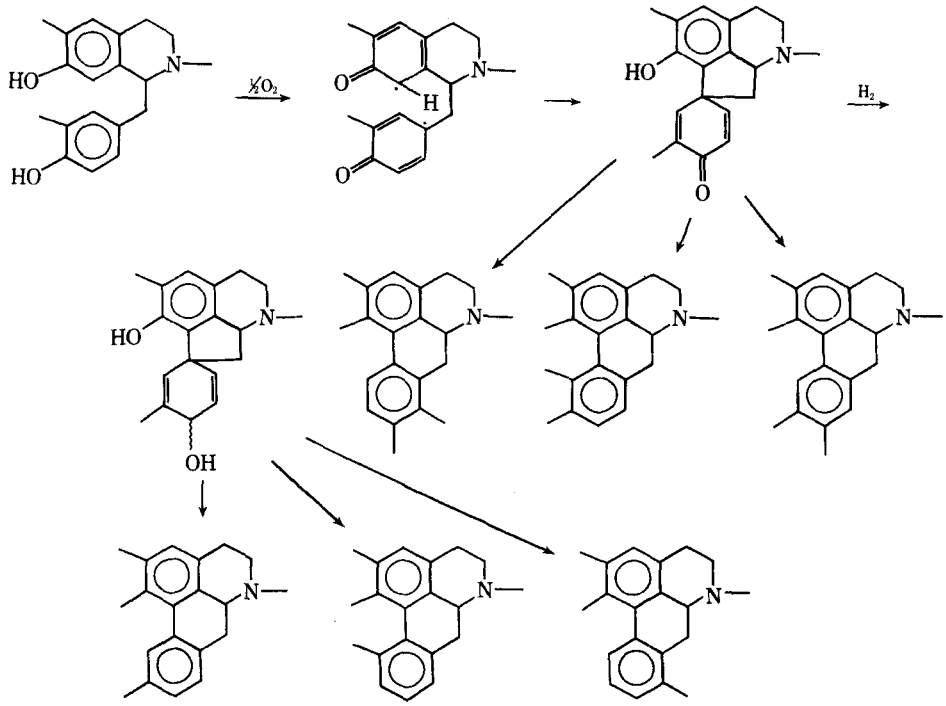
Orientalinone and related dienones are present in many plants and are generally referred to as proaporphines. Experimental evidence for the biosynthetic (*in vivo*) conversion of such compounds to aporphines by dienone–phenol and dienol–benzene rearrangements was furnished by Barton *et al.* (16), Haynes *et al.* (17), and Battersby *et al.* (18–20).

Blaschke (21, 22) reported good incorporation of reticuline (X) into bulbocapnine (XI) in *Corydalis cava* in a process that might be expected to involve corytuberine (XII) as an intermediate (Scheme IV). This represents the only experimental evidence in favor of a





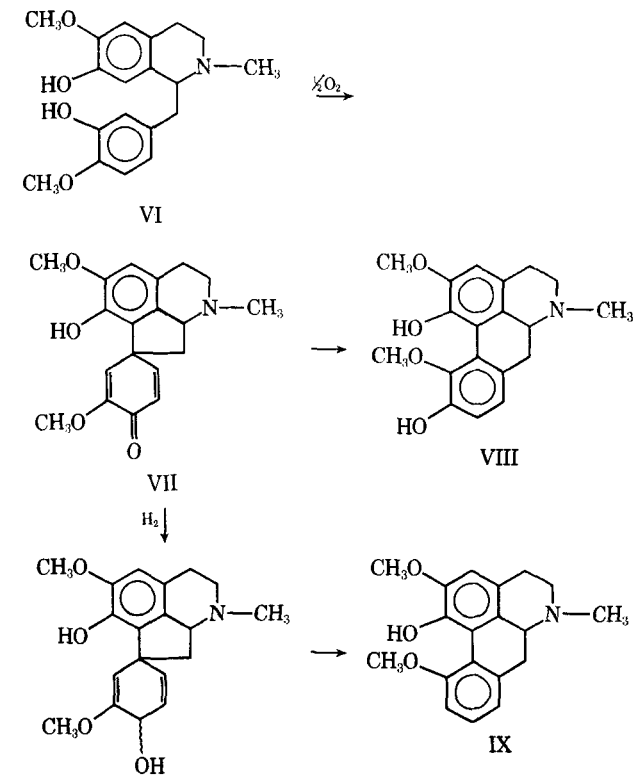
Scheme I—Proposed biosynthesis of aporphines by direct oxidative coupling of reticuline



Scheme II—Proposed biosynthetic pathways of aporphines via *a dienone intermediate*

direct *ortho–ortho* coupling in the biosynthesis of aporphines, although an *in vitro ortho–ortho* coupling to a dienone was achieved (11).

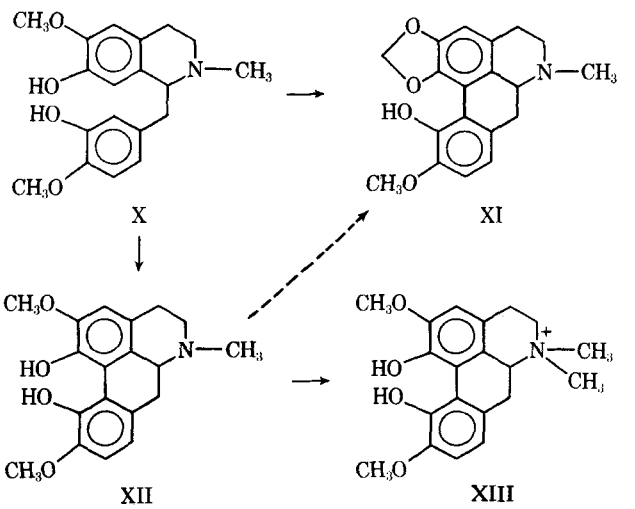
Since the opium poppy contains reticuline as well as diphenolic tetrasubstituted aporphines with both 1,2,9,10- and 1,2,10,11-substitution patterns, it seems to lend itself to an examination of aporphine biosynthesis. As a first step in this investigation, it was decided to study the possibility that reticuline might be the precursor of both types of aporphines by a direct oxidative coupling as illustrated in Scheme I. The amounts of aporphines present in the opium poppy are too small for direct isolation, and the reverse isotope dilution technique was used. Labeled reticuline was administered to the plants, and isoboldine and magnoflorine were added as "cold" carriers during the extraction. The alkaloids were isolated and purified to constant radioactivity.



Scheme III—Synthesis of isothebaine (IX) and isocorytuberine (VIII) from orientaline (VI) via *orientalinone (VII)*

RESULTS AND DISCUSSION

The amounts of labeled reticuline administered, the number and the variety of plants, and the results obtained are given in Table I. When (±)-reticuline-(3-^{14}C) was administered to opium poppies



Scheme IV—Proposed biosynthesis of bulbocapnine (XI), corytuberine (XII), and magnoflorine (XIII) from reticuline (X)

Table I—Radioactive Feeding Experiment with *Papaver somniferum* L.

Precursor	Amount Administered—μc.	Amount Administered—Number of Plants	Year	Variety	Alkaloid Added as Carrier	Incorporation, %	Degradation, Activity in Isolated Fragment, %
(±)-Reticuline-(3-^{14}C)	80.31	18	1968	Noordster	(±)-Isoboldine	0.073	—
(±)-Reticuline-(N-$^{14}CH_3$)	144.2	14	1969	Indra	(±)-Isoboldine	0.084	98.9
(±)-Reticuline-(N-$^{14}CH_3$)	144.2	14	1969	Indra	(+)-Magnoflorine iodide	0.001	—

(Noordster), it was incorporated into isoboldine to an extent of 0.073% [based on the amount of (±)-isoboldine added as cold carrier]. The amount of isoboldine remaining after purification to constant radioactivity was too small for controlled degradation and determination of the position of the label. The feeding experiment was, therelore, repeated with (±)-reticuline-(N-$^{14}CH_3$) (Indra), (±)-isoboldine, and (+)-magnoflorine being used as carriers. Again, isoboldine showed good incorporation of radioactivity (0.084%), whereas magnoflorine was inactive. The isolated and purified isoboldine was degraded by a selective Zeisel demethylation procedure. The *N*-methyl group contained all radioactivity (98.9%). This finding strongly suggests that isoboldine is biosynthesized by a direct oxidative *ortho–para* coupling of reticuline in the opium poppy. It is conceivable, however, that isoboldine may also be produced by an alternative pathway, involving orientalinone and a dienone–phenol rearrangement. Work is in progress to study this possibility.

The fact that reticuline was not incorporated into magnoflorine tends to indicate that 1,2,10,11-substituted aporphines may not be biosynthesized by a direct phenol coupling of reticuline in the opium poppy. This is in contrast to the results reported by Blaschke (21, 22) for bulbocapnine. From spectroscopic data as well as model considerations, it is evident that substituents in positions 1 and 11 cause considerable steric hindrance to coplanarity (3, 23–25), thereby making an *ortho–ortho* coupling of reticuline seem very difficult.

There is still a great deal of work to be done before the biosynthesis of aporphines can be fully understood. Based on the evidence available at present, one can only conclude that there are several pathways and mechanisms leading to natural aporphines. It is even possible that different plants may produce the same aporphine by different routes, depending on the precursors and enzyme systems available.

EXPERIMENTAL

The syntheses of (±)-reticuline-(3-^{14}C) and (±)-reticuline-(N-$^{14}CH_3$) and the methods for determination of radioactivity, cultivation of plants, and administration of labeled substances were described in a previous communication (26).

Alkaloids for Carrier Dilution—*Magnoflorine Iodide*—This was used as supplied[1].

(±)-Isoboldine—This was synthesized by a modified Pschorr ring closure reaction. 1-[3-(Benzyloxy-4-methoxy-6-aminobenzyl)]-6-methoxy - 7 - benzyloxy - *N* - methyl - 1,2,3,4 - tetrahydroisoquinoline (0.48 g.), prepared as described by Tomita and Kikkawa (27), was dissolved in a mixture of 1.5 ml. concentrated sulfuric acid, 15 ml. glacial acetic acid, and 15 ml. water. The solution was cooled in ice, and a solution of 0.12 g. of sodium nitrite in 4 ml. of water was added dropwise. After the addition was complete, the solution was stirred for 15 min. Concentrated sulfuric acid (20 ml.), 60 ml. of water, and 2.3 g. of zinc powder were added, and the mixture was refluxed for 1 hr. (28). The solution was filtered, and the filtrate was made basic with ammonia and extracted with chloroform. Evaporation of the chloroform gave a residue (256 mg.) which was purified by preparative TLC on silica gel with chloroform–methanol (9:1) and, finally, by crystallization from methanol, m.p. 122–123°. The product was compared with natural isoboldine by TLC on silica gel [chloroform–methanol (9:1), benzene–ethanol (8:2)], by GC of the free base and the trimethylsilyl derivative, and by NMR spectroscopy.

Extraction, Separation, and Purification of Alkaloids—The plants were extracted as described previously (26).

Magnoflorine—The aqueous acidic solution of total alkaloids was first extracted with chloroform to remove the weakly basic alkaloids, adjusted to pH 9 with ammonia, and then extracted with a mixture of chloroform and isopropyl alcohol (3:1). The aqueous phase was acidified with concentrated hydrochloric acid, and magnoflorine precipitated as the reineckate (29). The precipitate was washed with water, dried in a desiccator, and dissolved in acetone (200 ml.). A saturated solution of silver sulfate in water was added dropwise until no further precipitation occurred. The solution was filtered, and the filtrate concentrated to about 20 ml. The precipitate which formed during concentration was removed by filtration, and a saturated aqueous solution of potassium iodide was added. The solution was placed in a refrigerator to crystallize, and the crystals of magnoflorine iodide were recrystallized to constant radioactivity (9 d.p.m./mg.), m.p. 224–225° [lit. (29) 224–225°]. TLC on silica gel with a mixture of *n*-propanol, ammonium hydroxide, and water (4:1:1) gave the same R_f value as an authentic sample of magnoflorine iodide.

Isoboldine—The aqueous, acidic solution of total alkaloids from the plants to which (±)-isoboldine had been added as cold carrier was extracted with several portions of chloroform to remove the weakly basic alkaloids. The aqueous phase was basified to pH 8–9 with ammonia and extracted with chloroform. Evaporation of the chloroform left a dark residue, which was dissolved in 0.5 *N* hydrochloric acid. After addition of sodium hydroxide to pH 14, nonphenolic alkaloids were removed by extraction with chloroform. Ammonium chloride was added to bring the pH to about 9, and the solution was extracted with ether. Evaporation of the ether gave a dark-red residue, which was purified by column chromatography on silica gel with chloroform–methanol (97:3). The fractions containing isoboldine were combined and evaporated to dryness. The residue was dissolved in a small amount of ether, an excess of diazomethane in ether was added, and the mixture was set aside at room temperature for 2 days. After evaporation of the solvent, the residue was purified by preparative TLC on silica gel with chloroform–methanol (9:1). The resulting *O,O*-dimethylisoboldine (glaucine) was dissolved in ether (20 ml.), an excess of iodomethane was added, and the mixture was allowed to stand at room temperature for 12 hr. The crystals of glaucine methiodide were collected and recrystallized from a mixture of methanol and petroleum ether to constant radioactivity, 429 d.p.m./mg., corresponding to 634 d.p.m./mg. calculated on the basis of isoboldine.

The aqueous, acidic solution of total alkaloids from the plants to which (±)-isoboldine and magnoflorine had been added as cold carriers was first extracted as already described for magnoflorine. The chloroform–isopropyl alcohol extract was evaporated to dryness, and the residue was treated as described for isoboldine. The column fractions containing isoboldine were combined and evaporated to dryness, and the residue was crystallized from methanol to constant radioactivity, 900 d.p.m./mg., m.p. 122–123°. It was identified as isoboldine by TLC and GLC.

Degradation of Radioactive Isoboldine—Isoboldine isolated from the plants to which (±)-reticuline-(N-$^{14}CH_3$) had been administered was degraded by a selective Zeisel demethylation method as de-

[1] Supplied by Dr. Jack L. Beal.

scribed previously (30). The triethylmethylammonium iodide resulting from *N*-demethylation was crystallized from a mixture of ethanol and ether to constant radioactivity, 1128 d.p.m./mg.

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Liquid Crystalline Phases in Aerosol Formulations I: Phase Equilibria in Propellant Compositions

S. FRIBERG, L. RYDHAG, and G. JEDERSTRÖM*

Abstract □ Phase equilibria in a system of water, mixed emulsifiers, and liquid propellant are determined by visual observation of the number of phases after centrifugation. Three different liquid phases and one liquid crystalline phase are observed. A small increase in the amount of water gives rise to a complex phase pattern, which is explained by the interaction between the two emulsifiers and the water.

Keyphrases □ Propellant composition—determination of phase equilibria □ Phase equilibria, determination—water–emulsifier–propellant systems, by visual observation □ Emulsifier–water interaction—role in phase equilibria of water–emulsifier–propellant systems

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Later, Saunders (6) suggested that the stability of aerosol emulsions and foams could be related to "molecular complexes" forming multilayers of liquid crystalline character at the interface. The fact that surfactants, water, and amphiphilic substances can form liquid crystalline phases when mixed in certain proportions (7, 8) was cited as proof of the suggestion.

Because of this suggestion and the increased importance of aerosol packings, an investigation of the presence of liquid crystalline phases in systems of water, liquified chlorofluorocarbons, and one emulsifier was of interest. This publication is the first report on this investigation in which the emulsifier system of octanoic acid and 1-aminooctane was used. The system earlier gave well-defined results (9) in connection with emulsion stability.

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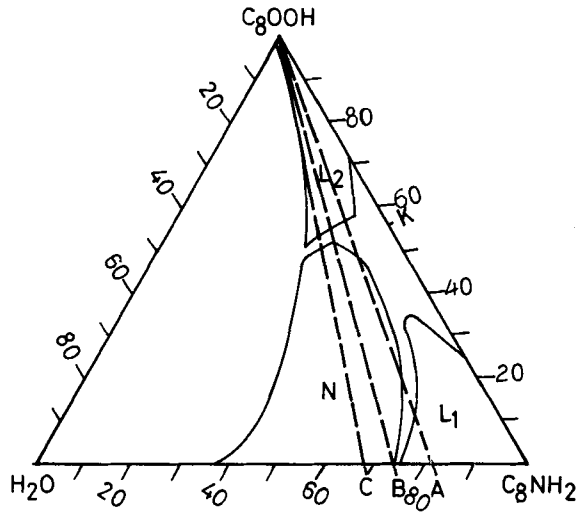

Figure 1—*Phase regions in the system of water–1-aminooctane–octanoic acid (H_2O–C_8NH_2–C_8OOH). Key: N = liquid crystalline phase, "neat phase"; L_1, L_2 = isotropic solutions; and K = equimolar solid compound, $C_8NH_3^+OOC_8^-$.*

EXPERIMENTAL

Materials—The water was twice distilled. 1-Aminooctane[1] (C_8NH_2) (GC > 99%), octanoic acid[2] (C_8OOH), and a propellant[3] were used.

Phase Equilibria—The acid, amine, and water were weighed into glass containers, which were sealed and cooled to −20°. The propellant ($C_2Cl_2F_4$) was then added; the pressurized packaging, the aerosol, was formed as the temperature slowly increased. The different phases in the aerosol were separated by ultracentrifugation at 8000×*g* (20°) during 2 hr. and identified by visual observation in a microscope with polarized light.

RESULTS

Figure 1 presents the water–emulsifier phase equilibria determined previously (10). It shows three liquid regions, one liquid crystalline region, and one solid phase. The liquid crystalline phase (N) has a lamellar structure. It forms two-phase regions: (*a*) with the amine solution (L_1) with the solid compound consisting of an equimolar ratio of amine and acid (K), and (*b*) with the acid solution (L_2). It (N) extends from the amine–water axis toward the acid corner, dissolving the acid to equimolar ratio by forming an ammonium salt within the liquid crystalline phase (5). The few percent excess acid are solubilized, resembling the process of solubilizing hydrocarbon (5). The amine solution solubilizes acid and water, and the acid solution behaves similarly toward water and amine.

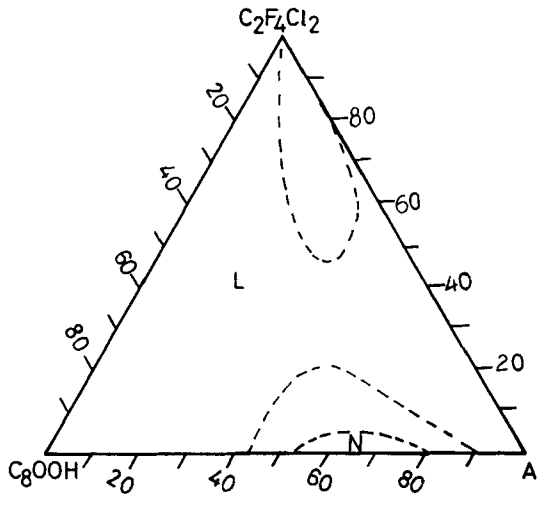

Figure 2—*Phase regions in the system of octanoic acid–propellant–water/amine [water (19%), 1-aminooctane (81%)] (C_8OOH–$C_2Cl_2F_4$–A). Key: L = isotropic solution; and N = liquid crystalline phase, "neat phase."*

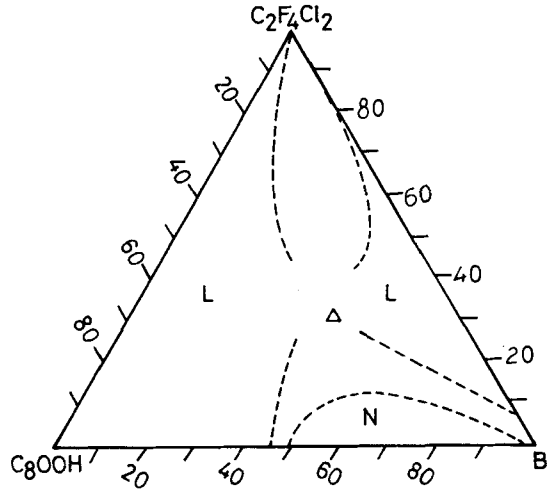

Figure 3—*Phase regions in the system of octanoic acid–propellant–water/amine [water (27%), 1-aminooctane (73%)] (C_8OOH–$C_2Cl_2F_4$–B). Key: L = isotropic solution; N = liquid crystalline phase, "neat phase"; and Δ = three-phase area indication.*

Three lines are drawn (A, B, and C) in the diagram; the phase regions in the systems formed by these lines and $C_2Cl_2F_4$ as the third component are given by Figs. 2–4.

The system (Fig. 2) containing least water (C_8OOH–A in Fig. 1) shows complete solubility of the propellant and the acid in each other. The solubility region extends from the C_8OOH–$C_2Cl_2F_4$ axis in a broad region to the amine/water–$C_2Cl_2F_4$ axis, where the components also are soluble in each other. This liquid area is in equilibrium with the $C_2Cl_2F_4$ by means of a two-phase area, starting at the same ratio of the acid to the water/amine solution, which forms the two-phase area at the base axis.

The liquid crystalline phase solubilizes a maximum of 6% $C_2Cl_2F_4$, being in equilibrium with the solution.

Increasing the water content gives rise to a similar diagram (Fig. 3). The solution region in this case does not form a continuous channel between the two-phase areas. There is a three-phase region in the center (marked by a small triangle but not determined exactly). The liquid crystalline phase reaches the corner representing the amine/water mixture.

After further increasing the water content, the phase diagram (Fig. 4) presents several phases. To the left, a liquid phase area is formed from the acid dissolving about 25% of the amine/water mixture. This liquid is mutually miscible with the $C_2Cl_2F_4$. The second liquid region reaches from the acid (amine/water) axis to about 50% of $C_2Cl_2F_4$. The third liquid region covers part of the $C_2Cl_2F_4$ (amine/water) axis, showing only limited solubility of the three components in each other. The liquid crystalline phase solubilizes up to 14% of the $C_2Cl_2F_4$ and forms a continuous region from the amine/water corner to 50% acid.

DISCUSSION

The results presented evidence of the solubilization of the propellant, $C_2Cl_2F_4$, into the different solutions and into the liquid crystalline phase of the water/1-aminooctane–octanoic acid system. They also demonstrated the factors that govern the association conditions giving rise to the different phases.

The minimum mutual solubility of the liquefied driving gas with the two liquids, octanoic acid and the amine/water solution, respectively, is found with the compositions of acid (water/amine), where the liquid crystalline phase exposes *maximum* capacity of $C_2Cl_2F_4$ solubilization. This is well demonstrated by a comparison of Figs. 2

[1] Fluka AG Chemische Fabrik, Switzerland.
[2] Kodak p.a., Eastman Distillation Products Industries.
[3] Frigen 114, Farbwerke Hoechst AG, West Germany.

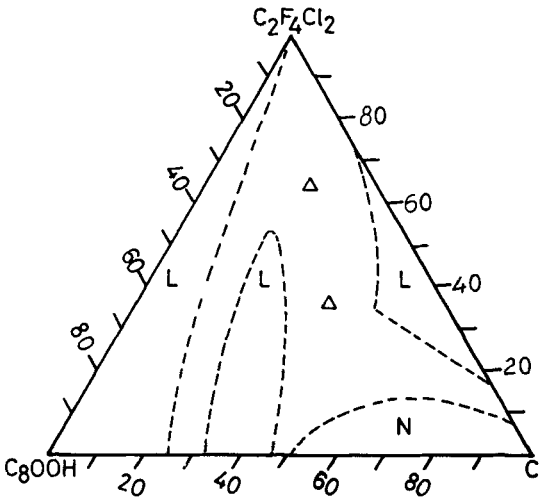

Figure 4—*Phase regions in the system of octanoic acid–propellant–water/amine [water (33%), 1-aminooctane (67%)](C_8OOH–$C_2Cl_2F_4$–C). Key: L = isotropic solution; N = liquid crystalline phase, "neat phase"; and Δ = three-phase area indication.*

and 3. The increase of water content from the composition with 40% acid in Fig. 2 to the corresponding one in Fig. 3 is only 5%. This change causes an enhanced solubilization of the propellant in the liquid crystalline phase from 4.5 to 11%, which is accompanied by the disappearance of the solubility region connecting the two axes meeting at the propellant corner. In Fig. 2, the minimum width of this region is about 20%.

To understand this behavior, it is necessary to consider two different factors which are operating when different association structures are formed in the system. According to the first factor, the amine and the acid react with each other in nonaqueous solutions to form an ion pair: $C_8NH_3^+C_8OO^-$. This compound is soluble to some extent in the acid, in the amine, and to about half the magnitude in a nonpolar substance, *p*-xylene. The compound is not soluble in water due to the influence of the hydrophobic hydrocarbon chains. The second factor is the pronounced interaction between the water and the amine, manifested in the formation of the liquid crystalline phase.

The liquid crystalline phase solubilizes octanoic acid to a few percent *in excess* of the equimolar ratio. This high solubility, about 10 times that of a hydrocarbon, arises from the ionization reaction described earlier. The reaction results in a liquid crystalline phase in which the hold of water is reduced from 65 to 25 wt. % when the ratio of acid to amine is raised from zero to one. Constancy is, on the other hand, found in the minimum content of amine, varying only between 30 and 40% by weight. This finding implies an exchange of water molecules in the immediate vicinity of an amine molecule to octanoic acid molecules, but it is not evidence of any similarity of interaction of acid/amine with water/amine. The exclusion of one compound when the other is present is dependent on the site where the interaction occurs.

These factors may be applied to the present results according to the following discussion. The complete mutual solubility of the acid and $C_2Cl_2F_4$ is understood in terms of two nonpolar substances molecularly dispersed in each other. The amine/water solution consists of dissolved or solubilized water in amine, which forms the "continuous" phase. The chlorofluoroethane shows complete mutual solubility with the amine, as expected.

When the acid and the amine/water solutions are mixed, the previously mentioned salt formation to an ammonium carboxylate takes place. The polar compound formed causes a reduced solubility in the liquefied driving gas. The weak dispersion forces from the $C_2Cl_2F_4$ do not give rise to an interaction sufficient to prevent a phase separation due to the strong reciprocal dipolar interaction of the ammonium carboxylate molecules. The reduction of solubility in the liquefied propellant is given a rational explanation in this manner.

When the relative amount of acid is increased, an increase of the mutual solubility is based on the strong hydrogen bonds formed between the acid and the ionized carboxylate group of the ammonium salt. The strongly attached acid molecules reduce the electrical field strength around the polar group and facilitate the solubility. This fact explains the more rapidly enhanced solubility when the acid is in excess compared to the behavior of the amine.

At low concentrations of the driving gas, a liquid crystalline phase is formed when the acid is added to the water/amine solution. Such a behavior is expected since the region of existence of the liquid crystalline phase involves less minimum water content in the liquid crystalline phase with an increasing amount of acid (Fig. 1).

When the water/amine ratio is raised, the stability of the liquid crystalline phase toward addition of a nonpolar substance is augmented. This fact is referred to the volume ratio of water and amine, which has an optimum where the layered structure is at maximum stability. A water content less than this value decreases the energy difference between the liquid crystalline phase and the amine liquid phase containing reversed micelles with water. A water content in excess of this value reduces the corresponding energy difference to an aqueous solution. The optimum ratio also includes the maximum interaction of water and amine, which leads to less tendency to form reversed micelles in the comparatively nonpolar $C_2Cl_2F_4$. Consequently, the solubility in the latter diminishes with increasing water content. Furthermore, at high water content, the strong hydrogen bonds between ionized carboxylic groups in the ammonium salt and the nonionized carboxylic acid molecules are influenced by the present water molecules reducing the bond strength. As a result, the reduction of aggregation of the acid to the polar compound gives rise to a reduced solubility of the association structures in $C_2Cl_2F_4$. A secondary effect arising from the same origin is the formation of micelles. Unpublished results give evidence of the existence of micelles at acid contents less than 30% counted on a total of water, amine, and acid (Fig. 1). The split of the solubility area to the left in Fig. 4 has to be referred to this fact.

With regard to these factors, the phase regions of the systems containing the driving gas are mainly related to the forces between the polar parts of the surface-active substances and the hydrophobic interaction with the water molecules. The phase equilibria in the system are responsible for the properties of the aerosol which is expelled; these relations will be treated in a following report.

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Identity of a Major Chromogen in *p*-Aminosalicylic Acid and Sodium *p*-Aminosalicylate Dosage Forms

I. K. SHIH

Abstract □ A major chromogen found in *p*-aminosalicylic acid and sodium *p*-aminosalicylate dosage forms was identified to be 3,3′-dihydroxyazoxybenzene. This compound could be produced by oxidation or sunlight irradiation of *m*-aminophenol, a common impurity of *p*-aminosalicylic acid and sodium *p*-aminosalicylate. Methods of synthesis for 3,3′-dihydroxyazoxybenzene are described.

Keyphrases □ *p*-Aminosalicylic acid, sodium *p*-aminosalicylate dosage form—identification of major chromogen, 3,3′-dihydroxyazoxybenzene □ 3,3′-Dihydroxyazoxybenzene—identification as a major chromogen in *p*-aminosalicylic acid, sodium *p*-aminosalicylate dosage form, synthesis, photosynthesis □ Sodium *p*-aminosalicylate, *p*-aminosalicylic acid dosage form—identification of major chromogen, 3,3′-dihydroxyazoxybenzene

Decarboxylation of *p*-aminosalicylic acid into *m*-aminophenol and carbon dioxide was extensively studied (1–4). It seems generally agreed that *m*-aminophenol did not have a significant role in the adverse reactions of *p*-aminosalicylic acid but that the chromogenic factors probably did[1].

Although discoloration of aged tablets of *p*-aminosalicylic acid and its sodium salt has been a common phenomenon, chromogenic substances have not been identified in the past. In this study, experiments were designed to determine the source of the major chromogen occurring in the commercially available tablets of *p*-aminosalicylic acid, *p*-aminosalicylic acid–isoniazid, and sodium *p*-aminosalicylate. The identity of a major chromogen is described in this report.

EXPERIMENTAL[2]

Materials—All chemicals and solvents were of reagent grade. The following were used: *m*-aminophenol[3]; hydrogen peroxide, 30% solution[4]; zinc dust[5]; ethyl acetate[5]; ethanol[5]; chloroform[5]; methylene dichloride[5]; acetone[5]; and Eastman chromagram sheet silica gel 6061.

Synthesis of 3,3′-Dihydroxyazoxybenzene—Three grams of *m*-aminophenol was dissolved in a mixture of 50 ml. of hydrogen peroxide and 100 ml. of distilled water. The reaction was allowed to take place overnight at room temperature (about 25°). The dark-colored crude product was then filtered and collected (yield 1.2 g.). The crude product was dissolved in 50 ml. of hot ethyl acetate and filtered. The filtrate was transferred into a chromatographic column (1.2 × 26 cm.) packed with a slurry of aluminum oxide containing 20 g. dispersed in 100 ml. of ethyl acetate. The column was eluted with the same solvent. A cherry-red eluate was obtained. Evaporation of the eluate under reduced pressure yielded a dark residue. Recrystallization of the product in 70% ethanol gave rosettelike crystals in dark cherry-red (yield 98 mg.). The product was insoluble in dilute mineral acids and sodium bicarbonate solution but soluble in sodium carbonate or fixed alkaline solutions (a typical solubility property of phenols). The compound could be decolorized by zinc dust reduction in dilute hydrochloric acid.

Table I—R_f Values of 3,3′-Dihydroxyazoxybenzene

Samples[a]	Solvent Systems[b] I	II	III	IV	V	VI
R	0.15	0.25	0.30	0.48	0.52	0.70
A	0.15	0.25	0.30	0.48	0.52	0.70
B	0.15	0.25	0.30	0.48	0.52	0.70
C	0.15	0.25	0.30	0.48	0.52	0.70
D	—	—	—	—	—	—

[a] R = 3,3′-dihydroxyazoxybenzene; A = effervescent *p*-aminosalicylic acid tablet; B = buffered *p*-aminosalicylic acid–isoniazid tablet; C = sugar-coated sodium *p*-aminosalicylate tablet; D = sodium *p*-aminosalicylate USP; three lots of each tablet were tested. [b] I = chloroform–ethyl acetate (5:1); II = chloroform–ethyl acetate (3:1); III = chloroform–ethyl acetate (2:1); IV = chloroform–ethyl acetate (1:1); V = methylene dichloride–acetone (6:1); and VI = methylene dichloride–acetone (2:1).

TLC studies of the compound in various solvent systems (Table I) showed only one distinct spot under visible or UV light, m.p. about 210° dec.; visible spectrum: $\lambda_{max.}$ 525 nm. (ethanol); IR spectrum (KBr): $\bar{\nu}$ (max.) 3250 cm.$^{-1}$ (aromatic C—H stretching), 1430, 1500, 1550 cm.$^{-1}$ (aromatic C=C in-plane vibrations), 1600 cm.$^{-1}$ (N=N stretching), 1280 cm.$^{-1}$ (N→O stretching), 1210, 1380 cm.$^{-1}$ (O—H bending), and 3620 cm.$^{-1}$ (O—H stretching).

Anal.—Calc. for $C_{12}H_{10}N_2O_3$: C, 62.60; H, 4.34; N, 12.16. Found: C, 62.85; H, 4.38; N, 12.06.

Photosynthesis of 3,3′-Dihydroxyazoxybenzene—Three grams of *m*-aminophenol was dissolved in 200 ml. of ethanol in a stoppered flask. The sample solution was then subjected to intermittent sunlight irradiation in a temperature range of 16–27°. After 96 hr. of irradiation, the sample solution was evaporated to dryness under reduced pressure. To remove unreacted *m*-aminophenol from the residue, the crude product was dissolved in 120 ml. of ethyl acetate and extracted with ten 50-ml. portions of dilute hydrochloric acid solution followed by three 50-ml. portions of water in a 250-ml. separator. The ethyl acetate layer was then transferred into a chromatographic column (1.2 × 26 cm.) packed with a slurry containing 20 g. of aluminum oxide dispersed in 100 ml. of ethyl acetate. The column was eluted with the same solvent. A cherry-red eluate was obtained, which was then evaporated to dryness under reduced pressure. Recrystallization of the product from 70% ethanol gave rosettelike dark cherry-red crystals (yield 52 mg.).

TLC studies of the product in various solvent systems (Table I) showed only one distinct spot when visualized under visible or UV light. Visible and IR spectra of the compound were identical to those of 3,3′-dihydroxyazoxybenzene obtained in the preceding synthesis.

Identification of 3,3′-Dihydroxyazoxybenzene in *p*-Aminosalicylic Acid and Sodium *p*-Aminosalicylate Dosage Forms—Tablets of effervescent *p*-aminosalicylic acid, buffered *p*-aminosalicylic acid–isoniazid, and sodium *p*-aminosalicylate were tested. Sample powder equivalent to 3–4 g. of *p*-aminosalicylic acid was dissolved in 100 ml. of water. The solution was filtered, and the filtrate was extracted with two 25-ml. portions of ethyl acetate. The combined ethyl acetate layer was washed with three 25-ml. portions of water. Evaporation of the ethyl acetate layer under reduced pressure yielded a brown residue. A similar procedure was also applied to the reference material of sodium *p*-aminosalicylate. Symbols A, B, C, and D were designated to represent residues obtained from effervescent *p*-aminosalicylic acid tablets, *p*-aminosalicylic acid–isoniazid tablets, sodium *p*-aminosalicylate tablets, and sodium *p*-aminosalicylate USP, respectively.

[1] Private communication from Dr. L. M. Lueck, Parke-Davis & Co., Detroit, Mich.
[2] A Bausch & Lomb 505 recording spectrophotometer and a Beckman IR-8 recording spectrophotometer were used.
[3] Eastman Organic Chemicals.
[4] Anachemia Chemicals Ltd.
[5] Fisher.

The residues were dissolved in a very small amount of methanol for TLC studies. An amount of 0.5 mcg. of 3,3′-dihydroxyazoxybenzene dissolved in methanol was used for spotting. For Samples A, B, C, and D, sufficient amounts to make the spot visually distinct were applied. Thin-layer plates were developed in various solvent systems, and the spots were located by visible or UV light. The results are given in Table I.

RESULTS AND DISCUSSION

Preliminary studies indicated that air oxidation of an aqueous solution of *m*-aminophenol was much faster than that of *p*-aminosalicylic acid and its sodium salt. It is thus postulated that some of the chromogens which occurred in aged *p*-aminosalicylic acid dosage forms might be contributed from oxidation of *m*-aminophenol, a common impurity of *p*-aminosalicylic acid and its sodium salt (5, 6).

To effect mild oxidation of *m*-aminophenol, hydrogen peroxide was used as the oxidizing agent in this study. The reaction conditions chosen were room temperature and about neutral pH. A dark cherry-red compound was isolated and identified as 3,3′-dihydroxyazoxybenzene. Sunlight irradiation of *m*-aminophenol in ethanol also yielded the same azoxy compound.

Commercially available tablets of *p*-aminosalicylic acid, *p*-aminosalicylic acid–isoniazid, and sodium *p*-aminosalicylate were tested for the presence of this specific chromogen. TLC studies of samples in various solvent systems clearly indicated that 3,3′-dihydroxyazoxybenzene is a common chromogen which occurred in those samples being tested (Table I), although this compound was not detected in the reference material of sodium *p*-aminosalicylate.

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Inhibition of Nicotinate Phosphoribosyl Transferase by Nonsteroidal Anti-Inflammatory Drugs: A Possible Mechanism of Action

Z. N. GAUT* and H. M. SOLOMON

Abstract □ When incubated with ^{14}C-nicotinic acid and phosphoryl-ribose-1-pyrophosphate, human platelet lysate incorporates radioactivity into nicotinic acid mononucleotide. Under these conditions, the apparent K_m of nicotinic acid for nicotinate phosphoribosyl transferase is 2.4×10^{-5} *M*. Along with 2-hydroxynicotinic acid ($K_i = 2.3 \times 10^{-4}$ *M*), the following nonsteroidal anti-inflammatory compounds competed reversibly with nicotinic acid for the enzyme: flufenamic acid ($K_i = 4.6 \times 10^{-5}$ *M*), mefenamic acid ($K_i = 7.6 \times 10^{-5}$ *M*), salicylic acid ($K_i = 1.6 \times 10^{-4}$ *M*), phenylbutazone ($K_i = 1.6 \times 10^{-4}$ *M*), and indomethacin ($K_i = 4.2 \times 10^{-4}$ *M*). Such inhibition may explain the decreases in nicotinamide adenine dinucleotide phosphate content of rat liver after administration of salicylate. Furthermore, suppression of nicotinamide adenine dinucleotide biosynthesis by anti-inflammatory drugs may restrain mucopolysaccharide biosynthesis and, thereby, reduce inflammatory responsiveness. Hence, this antagonism of niacin may be involved in the pharmacologic activity and, particularly, the toxicity of anti-inflammatory drugs.

Keyphrases □ Nicotinate phosphoribosyl transferase—inhibition by nonsteroidal anti-inflammatory drugs, mechanism of action, using radiolabeled nicotinic acid □ Human platelet lysate—evaluation of influence of nonsteroidal anti-inflammatory drugs on nicotinate phosphoribosyl transferase, mechanism of action □ Nicotinic acid, radiolabeled—used to evaluate influence of nonsteroidal anti-inflammatory drugs on nicotinate phosphoribosyl transferase

The human platelet incorporates 7-^{14}C-nicotinic acid into nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, and other compounds (1) consistent with intermediates of the pathway of Preiss and Handler (2, 3). Such incorporation is hindered by analogs of nicotinic acid, by salicylic acid, and by other anti-inflammatory drugs (4–6).

Data concerning the intact platelet suggested inhibition of nicotinamide adenine dinucleotide biosynthesis early in the pathway, probably at the step catalyzed by nicotinate phosphoribosyl transferase (5). To evaluate this possibility further, the influence of various nonsteroidal anti-inflammatory drugs on nicotinate phosphoribosyl transferase in human platelet lysate was studied.

METHODS

The following compounds were obtained from commercial sources: 7-^{14}C-nicotinic acid (sp. act. 59.1 mc./mmole)[1], phosphoryl-ribose-1-pyrophosphate[2], nicotinamide mononucleotide[3], salicylic

[1] Amersham Searle.
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The residues were dissolved in a very small amount of methanol for TLC studies. An amount of 0.5 mcg. of 3,3′-dihydroxyazoxybenzene dissolved in methanol was used for spotting. For Samples A, B, C, and D, sufficient amounts to make the spot visually distinct were applied. Thin-layer plates were developed in various solvent systems, and the spots were located by visible or UV light. The results are given in Table I.

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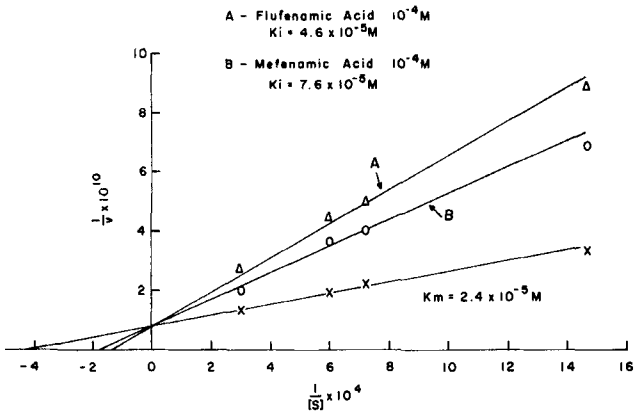

Figure 1—*Inhibition of nicotinate phosphoribosyl transferase in human platelet lysate by flufenamic and mefenamic acids. Velocity is expressed as moles nicotinic acid mononucleotide formed per milligram platelet per hour; substrate concentration is expressed as moles per liter. Results of representative experiments are shown.*

acid[4], phenylbutazone[5], indomethacin[6], and flufenamic and mefenamic acids[7]. 2-Hydroxynicotinic acid was synthesized[8]. 7-^{14}C-Nicotinic acid proved >98% pure on thin layers of cellulose in the solvent systems employed by Hagino *et al.* (7). Nicotinic acid and nicotinic acid mononucleotide exhibited the same R_f's on cellulose as were previously reported for paper chromatography (7). Nicotinic acid mononucleotide was prepared from nicotinamide mononucleotide by the method of Wagner (8).

Approximately 200 mg. (wet weight) of human platelets was lysed in 1.5 ml. of distilled water. Then 0.2-ml. aliquots were incubated in Ca^{+2}-free, Krebs–Ringer bicarbonate buffer modified to contain: Mg^{+2}, 3.3 m*M;* phosphorylribose-1-pyrophosphate, 1.5 m*M;* and various concentrations of 7-^{14}C-nicotinic acid. Solutions of anti-inflammatory agents were added to some incubates in parallel experiments. Incubations were carried out in total volumes of 0.5 ml. in a metabolic shaker under 95% O_2–5% CO_2 for 1 hr. at 37°. The reaction was stopped by immersing the tubes in solid CO_2–acetone mixture. Subsequently, "carrier" nicotinic acid and nicotinic acid mononucleotide were added to the incubates, and the compounds were separated on thin layers of cellulose in the solvent systems employed by Hagino *et al.* (7). More than 98% of the radioactivity migrated with these standard compounds.

RESULTS

7-^{14}C-Nicotinic acid incorporation into nicotinic acid mononucleotide was linear up to 3 hr. of incubation and proportional to substrate concentrations at 6.8, 14, 17, and 34 μM. Kinetics were evaluated according to the method of Lineweaver and Burke (9), in which velocity was expressed as moles of nicotinic acid mononucleotide formed per milligram platelet per hour (Fig. 1). The intercepts, for calculation of the apparent K_m and K_i's, were obtained by the method of least squares. The apparent K_m for nicotinic acid, with phosphorylribose-1-pyrophosphate and Mg^{+2} in excess, was 2.4×10^{-5} *M* and agreed closely with that of purified bovine liver nicotinate phosphoribosyl transferase (2.5×10^{-5} *M*) reported by Smith and Gholson (10). The formation of nicotinic acid mononucleotide was competitively inhibited by flufenamic acid ($K_i = 4.6 \times 10^{-5}$ *M*), mefenamic acid ($K_i = 7.6 \times 10^{-5}$ *M*), salicylic acid ($K_i = 1.6 \times 10^{-4}$ *M*), phenylbutazone ($K_i = 1.6 \times 10^{-4}$ *M*), 2-hydroxynicotinic acid ($K_i = 2.3 \times 10^{-4}$ *M*), and indomethacin ($K_i = 4.2 \times 10^{-4}$ *M*).

DISCUSSION

Platelet aggregation, a precursor of blood coagulation (11), is inhibited by various nonsteroidal anti-inflammatory drugs; hence, O'Brien (12) postulated a connection between inflammation and platelet function. The present study describes another common feature of these drugs in the platelet: inhibition of nicotinate phosphoribosyl transferase by reversible competition with nicotinic acid. Although anti-inflammatory agents inhibit the biosynthesis of nicotinamide adenine nucleotides, obligatory cofactors in a host of reactions supportive to metabolism in the platelet, the possible correlation between niacin antagonism and platelet aggregation remains undefined.

A more attractive hypothesis relates niacin antagonism by these drugs to their anti-inflammatory activity. Such drugs also inhibit the oxidation of uridine-5′-diphosphoglucose, competitively with nicotinamide adenine dinucleotide and noncompetitively with uridine-5′-diphosphoglucose (13). Therefore, Lee and Spencer (13) theorized that mucopolysaccharide biosynthesis is restrained and inflammatory responsiveness is reduced. It follows that inhibition of nicotinamide adenine dinucleotide biosynthesis by anti-inflammatory agents would exert the same restraint on this pathway since tissue concentrations of the cofactor would be diminished.

Imsande and Handler (14) suggested that the reaction catalyzed by nicotinate phosphoribosyl transferase was rate limiting for nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate formation. Therefore, such inhibition of this enzyme may explain the decreases in nicotinamide adenine dinucleotide phosphate content of rat liver after administration of salicylate, as reported by Slater and Sawyer (15).

The concentrations of nicotinic acid employed in the present study were greater than those in human serum and within the range of those in human whole blood (16); also, the apparent K_i's of the inhibitory drugs are within the range of therapeutic blood levels (17, 18). Hence, this antagonism of niacin at the cellular and enzyme level may be involved in the pharmacologic activity and, particularly, the toxicity of anti-inflammatory drugs.

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Photodegradation Products of Chloramphenicol in Aqueous Solution

I. K. SHIH

Abstract □ An aqueous solution of chloramphenicol was degraded upon exposure to sunlight, UV, and tungsten light. The major photodegradation products were hydrochloric acid, *p*-nitrobenzaldehyde, *p*-nitrobenzoic acid, 4,4′-azoxybenzoic acid, and *p*-aminophenyl-2-acetamido-1,3-propanediol. Photolysis of *p*-nitrobenzaldehyde in water gave exclusively one product, *p*-nitrosobenzoic acid, with 92% yield. The data obtained suggest that *p*-nitrosobenzoic acid may be an intermediate in the reaction mixture of chloramphenicol. Photolysis of chloramphenicol in ethanol and benzene was also similarly conducted. No degradation products were found. The chemistry of the photodegradation products suggests that chloramphenicol in water under the influence of light undergoes oxidation, reduction, and condensation reactions.

Keyphrases □ Chloramphenicol—six photodegradation products in aqueous solution isolated and identified □ Photodegradation products, chloramphenicol—isolated and identified, from water, ethanol, and benzene, using sunlight, UV, and tungsten light □ Light stability, chloramphenicol—using sunlight, UV, and tungsten light, in three solvents, six photodegradation products isolated and identified

The effect of light is often considered an important factor in drug stability. Nevertheless, there is only little information on the light stability of chloramphenicol (1). Photodegradation reactions of this antibiotic are practically unknown. The present study was undertaken to isolate and identify the major photolysis products of chloramphenicol. Experiments were conducted in solvents of different polarity (*i.e.*, water, ethanol, and benzene) using sunlight, UV, and tungsten light. Six photodegradation products are reported.

EXPERIMENTAL[1]

Materials—Phenylhydrazine hydrochloride, *p*-nitrobenzaldehyde, *p*-nitrobenzoic acid, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sodium nitrite, ammonium sulfamate, stannous chloride, zinc dust, aluminum oxide, and solvents were reagent grade (Fisher). Eastman chromagram sheet (6061, silica gel) was used. The reference material of chloramphenicol used in this study was white, crystalline chloramphenicol of high purity[2], m.p. 151° [lit. (2) m.p. 150–153°].

Photolysis of Chloramphenicol in Aqueous Solution—One liter of the sample solution (0.25%, pH 5.4) was prepared with distilled water in a stoppered glass flask and exposed to sunlight in a temperature range of 21–30°. As photolysis progressed, the sample solution slowly turned yellow, and orange-yellow precipitates were observed to form. After 96 hr. of irradiation, the solution was filtered (pH 1.9). The filtrate was designated as Fraction A, and the crude product was designated as Fraction B (yield 404 mg.). Sample solutions were also irradiated with UV and tungsten light in an air-conditioned room. The distance between light sources and samples was approximately 5 cm. The temperature of the sample solutions reached an equilibrium after 4 hr., *i.e.*, 28–29° (UV) and 39–40° (tungsten light). After 96 hr. of irradiation, the samples were analyzed. Major photodegradation products were found to be essentially the same as those identified from sunlight-treated sample. Control samples (0.25%) were prepared in low actinic flasks and treated in the same way as the samples. No degradation products were detected.

Photolysis of Chloramphenicol in Ethanol and Benzene—Samples were dissolved in ethanol and benzene (0.25%). Solutions were irradiated in the same way as previously described. No degradation products were detected.

Isolation and Identification of Major Components from Fraction A—*Hydrochloric Acid (Acidimetric and Volhard's Chloride Determination)*—Fifty milliliters of Fraction A was titrated with 0.1 *N* sodium hydroxide solution, using phenolphthalein T.S. as the indicator. A freshly prepared sample solution (0.25%) served as the blank. After correction for the blank, 6.00 ml. of titrant was consumed. The total yield of hydrogen-ion concentration in 1 l. of sample solution was thus found to be 12 milliequivalents. Fifty milliliters of Fraction A also was titrated with 0.05 *N* silver nitrate solution. After correction for the blank, 12.05 ml. of titrant was consumed. The total yield of chloride-ion concentration in 1 l. of sample solution was thus found to be 12.05 milliequivalents.

p-*Nitrobenzaldehyde*—Two hundred milliliters of Fraction A was distilled. Approximately 150 ml. of distillate was collected and then extracted with two 50-ml. portions of ether. Evaporation of the ether extract yielded 33 mg. of product. The product was recrystallized from hot water, m.p. 106° (uncorr.) [lit. (3) m p. 107°]. No depression of melting point with an authentic sample of *p*-nitrobenzaldehyde was observed. The IR spectrum (KBr) of the product was found to be essentially the same as that of *p*-nitrobenzaldehyde.

p-*Nitrobenzoic Acid*—Two hundred milliliters of Fraction A was made alkaline with 5 ml. of sodium hydroxide solution (10% w/v) and extracted with six 60-ml. portions of ether. The aqueous phase was then acidified with 10 ml. of dilute hydrochloric acid and extracted with two 50-ml. portions of ether. Evaporation of the ether extract yielded about 24 mg. of crude product. Sublimation at a temperature range of 210–220° under atmospheric pressure yielded a pure substance, m.p. 240° (uncorr.) [lit. (3) m.p. 241°]. No melting-point depression of the product with an authentic sample of *p*-nitrobenzoic acid was observed. The IR spectrum (KBr) of the product was found to be essentially the same as that of *p*-nitrobenzoic acid.

p-*Aminophenyl-2-acetamido-1,3-propanediol*—One hundred milliliters of chloramphenicol solution (0.25%) was mixed for 6 hr. with 0.15 g. of zinc dust at room temperature. The reaction mixture was then filtered. A clear filtrate was obtained which, on standing, turned yellow, exhibiting essentially the same UV spectrum as previously reported (4), *i.e.*, $\lambda_{max.}$ 237 nm. and $\lambda_{min.}$ 217 nm. This compound was not isolated and was assumed to be *p*-aminophenyl-2-acetamido-1,3-propanediol (4).

Approximately 20–30 μl. of Fraction A was spotted on a thin-layer plate along with 5 μl. (containing about 1 mcg.) of reference sample, which was synthesized as described previously. The thin-layer plate was developed in a solvent system of two parts methanol and one part chloroform. After development, the plate was sprayed consecutively with 0.1% sodium nitrite, 0.2% hydrochloric acid, 0.4% ammonium sulfamate, and 0.2% *N*-(1-naphthyl)ethylenediamine dihydrochloride aqueous solutions. One spot from Fraction A corresponded to the reference sample and showed a purple to pink color, R_f 0.75.

Isolation and Identification of Major Components from Fraction B—*4,4′-Azoxybenzoic Acid*—A sample of 150 mg. of Fraction B was dissolved in 5 ml. of ammonium hydroxide T.S. The sample solution was then transferred to an aluminum oxide column (2.4 × 64 cm.) packed with a slurry of aluminum oxide, 120 g. in methanol. The column was eluted with about 100 ml. of methanol and then

[1] The following equipment was used: Bausch & Lomb recording spectrophotometer, 505; Beckman IR-8; UV lamp (emitting light mainly at 365 nm., Fisher); and tungsten light (ordinary light bulb, 100 w.).

[2] Obtained from Parke-Davis.

with 50% methanol. Approximately 200 ml. of pale-yellow eluate was collected. Acidification of the eluate with glacial acetic acid gave a pale-yellow product (yield 120 mg.).

Purity of the product was tested as follows. Four milligrams of the sample was dissolved in 10 ml. of 0.1 *N* sodium hydroxide solution. Then 5 μl. of the sample solution was spotted on a thin-layer plate and developed in a solvent system of 10 parts methanol and 0.1 part glacial acetic acid. After development, the plate showed only one distinct spot when visualized under visible light or UV lamp, R_f 0.67.

The product was insoluble in alcohol, ether, and most organic solvents but was readily soluble in organic bases, sodium bicarbonate, and alkaline solution; m.p. about 240° dec.; UV spectrum: a = 62.5, $\lambda_{max.}$ 333 nm. (0.1 *N* sodium hydroxide); IR spectrum: $\bar{\nu}_{max.}^{KBr}$ 2648, 2538, 1672, 1592, 1433, 1416, 1284 cm.$^{-1}$, *etc.;* mass spectrum (isotopic analysis)—Calc. for $C_{14}H_{10}N_2O_5$: *m/e* 286 (100), 287 (16.3), 288 (2.2). Found: *m/e* 286 (100), 287 (16.5), 288 (2.2). The neutralization equivalent was: 144 (found), 143 (theory).

Anal.—Calc. for $C_{14}H_{10}N_2O_5$: C, 58.74; H, 3.49; N, 9.77. Found: C, 58.65; H, 3.50; N, 9.90.

Synthesis of 4,4′-Azoxybenzoic Acid—One gram of *p*-nitrobenzoic acid was dissolved in a mixture of 100 ml. ethanol and 10 ml. glacial acetic acid. Then 0.58 g. zinc dust was slowly added. The reaction mixture was gently refluxed for 1 hr. The product was filtered and washed with hot methanol (yield 0.2 g.).

The purity of the product was tested by using the same TLC technique as previously described. Only one spot was observed when visualized under visible or UV lamp, R_f 0.67; m.p. about 240° dec.; UV spectrum: a = 62.9, $\lambda_{max.}$ 333 nm. (0.1 *N* sodium hydroxide). The IR spectrum (KBr) of this synthetic substance was identical to that of the product isolated from the chloramphenicol irradiation study. The neutralization equivalent was: 141 (found), 143 (theory).

Anal.—Calc. for $C_{14}H_{10}N_2O_5$: C, 58.74; H, 3.49; N, 9.77. Found: C, 58.52; H, 3.64; N, 9.62.

p-*Nitrosobenzoic Acid*—Two grams of *p*-nitrobenzaldehyde was dissolved in 2 l. of hot water. The sample solution was then exposed to sunlight (21–30°) for 96 hr. The product was filtered and washed with hot water until the washings gave a negative test with phenylhydrazine T.S. A pale-yellow product was obtained (yield 1.84 g., 92% of theory).

The purity of the product was tested as follows. A sample of 4 mg. was dissolved in 10 ml. of 0.1 *N* sodium hydroxide solution. Then 5 μl. was spotted on a thin-layer plate and developed in a solvent system of 20 parts chloroform and 0.1 part glacial acetic acid. The plate showed only one distinct spot under a UV lamp, R_f 0.30. After spraying the plate consecutively with 0.1% stannous chloride (prepared in dilute hydrochloric acid), 0.1% sodium nitrite, 0.4% ammonium sulfamate, and 0.2% *N*-(1-naphthyl)-ethylenediamine dihydrochloride aqueous solutions, a purple-to-pink spot was developed. This color reaction is typical of aromatic nitro compounds. To differentiate this compound from *p*-nitrobenzoic acid, a specific color spot test was developed. After spraying the thin-layer plate with a 0.2% alkaline 2-naphthol solution (prepared in sodium hydroxide T.S.), a gray color spot was developed which, on standing, slowly turned pink. The limit of this test was about 0.5 mcg., and *p*-nitrobenzoic acid failed to give this test.

The product was not soluble in water or most organic solvents but was readily soluble in organic bases, sodium bicarbonate, and alkaline solutions; m.p. about 250° dec.; UV spectrum: a = 56.6, $\lambda_{max.}$ 288 nm. and a = 63.6, $\lambda_{max.}$ 313 nm. (0.1 *N* sodium hydroxide); IR spectrum (KBr): $\bar{\nu}_{max.}$ 2646, 2538, 1678, 1592, 1422, 1292, 1263 cm.$^{-1}$, *etc.;* NMR spectrum (solvent dimethyl sulfoxide), J = 9 Hz. (coupling constant of *ortho*-hydrogen). The neutralization equivalent was: 149 (found), 151 (theory).

Anal.—Calc. for $C_7H_5NO_3$: C, 55.60; H, 3.31; N, 9.27. Found: C, 54.99; H, 3.30; N, 9.42.

This compound was not detected in the photolysis mixture of chloramphenicol.

DISCUSSION

The photolysis of chloramphenicol was conducted in solvents of different polarities, *i.e.*, water, ethanol, and benzene. Sample solutions were exposed to sunlight, UV, and tungsten light. Control samples were prepared in low actinic flasks and treated in the same way as the samples. After 96 hr. of irradiation, samples were analyzed. Degradation products were present only in aqueous sample solutions. No products were detected either in control samples or samples prepared in ethanol and benzene. The evidence thus suggested that the degradation products, as subsequently described, were not present in the starting material of chloramphenicol but were produced as a result of a photochemical reaction. Preliminary tests, originally designed for other studies, indicated that chloramphenicol in hydrochloric acid (0.1 *N*), when heated in an oven at 97° for 72 hr., did not yield any products other than normal hydrolysates. It is, therefore, concluded that neither temperature nor high hydrogen-ion concentration would cause the observed photochemical reactions. The products described herein must be the photodegradation products.

The major photodegradation products of chloramphenicol were isolated and identified. They were *p*-nitrobenzaldehyde, *p*-nitrobenzoic acid, 4,4′-azoxybenzoic acid, and *p*-aminophenyl-2-acetamido-1,3-propanediol. Since one or two acids in addition to hydrochloric acid are present in the photolysis mixture of chloramphenicol, results obtained from hydrogen- and chloride-ion determination merely support the possibility that hydrochloric acid is a product of the reaction.

p-Nitrobenzaldehyde was detected after a few minutes of irradiation of an aqueous solution of chloramphenicol. For this reason, photolysis of *p*-nitrobenzaldehyde was performed. Sunlight irradiation of an aqueous *p*-nitrobenzaldehyde solution gave exclusively *p*-nitrosobenzoic acid with 92% yield. To distinguish this compound from *p*-nitrobenzoic acid, a specific color spot test using alkaline 2-naphthol solution was developed.

Condensation between nitroso and hydroxyamino compounds is a well-known reaction, giving rise to the azoxy compound (5). Although this reaction was facilitated in an alkaline condition, condensation may also proceed in an acid medium, as exemplified by the chemical synthesis of 4,4′-azoxybenzoic acid employed in this study. *p*-Nitrosobenzoic acid was not detected in the photolysis mixture of chloramphenicol. However, subsequent isolation and identification of 4,4′-azoxybenzoic acid provided indirect evidence for the possible intermediate formation of *p*-nitrosobenzoic acid in the irradiated chloramphenicol reaction system, although the photochemical mechanism was not ascertained in the present study.

Chemistry of the photodegradation products suggests that chloramphenicol in water under the influence of light undergoes oxidation, reduction, and condensation reactions.

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Chemical Constituents of *Cannabis sativa* L. Root

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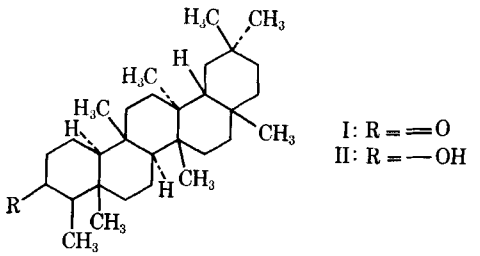
Abstract □ An extract of *Cannabis sativa* L. root yielded two pentacyclic triterpenes, friedelin and epifriedelanol, and *N*-(*p*-hydroxy-β-phenylethyl)-*p*-hydroxy-*trans*-cinnamamide. Structures of the triterpenes were confirmed by preparation of derivatives and comparison to authentic friedelin. Epifriedelanol was also oxidized to friedelin. The amide was confirmed by synthesis.

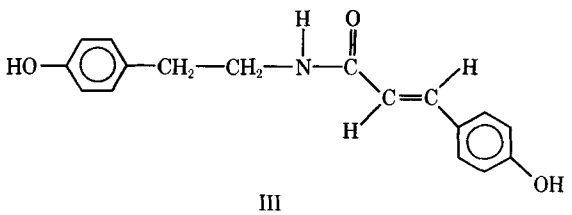
Keyphrases □ *Cannabis sativa* L.—isolation and identification of chemical constituents □ Friedelin—isolated and identified from *Cannabis sativa* □ Epifriedelanol—isolated and identified from *Cannabis sativa* □ *N*-(*p*-Hydroxy-β-phenylethyl)-β-hydroxy-*trans*-cinnamamide—isolated and identified from *Cannabis sativa*

Marijuana grown under a National Institute of Mental Health contract afforded a large quantity of root material. An ethanolic extract of *Cannabis sativa* L. roots yielded the pentacyclic triterpene ketone, friedelin (friedelan-3-one) (I), its corresponding β-alcohol, epifriedelanol (II), and *N*-(*p*-hydroxy-β-phenylethyl)-*p*-hydroxy-*trans*-cinnamamide (III). The triterpenes were characterized by preparation of derivatives and comparison with authentic friedelin[1].

Friedelin was first isolated from cork as a mixture with cerin by Chevreul (1). In 1899, Istrati and Ostrogovich (2) separated this mixture and obtained the pure triterpene. The structure of friedelin, however, was not determined until 1956 by Corey and Ursprung (3). Friedelin and epifriedelanol have been isolated from numerous plant species, including *Salix japonica* (Salicaceae) (4), *Photinia glabra* (Rosaceae) (5), Rhododendron species (Ericaceae) (6), and *Ceratopetalum apetalum* (Cunoniaceae) (7).



I: R = =O
II: R = —OH



III

[1] The reference samples were provided by Dr. Robert Stevenson, Department of Chemistry, Brandeis University, Waltham, Mass., and Dr. P. R. Jefferies and Dr. E. L. Ghisalberti, University of Western Australia, Nedlands, West Australia.

Table I—Mouse Tail-Flick Reaction Times[a]

Treatment	Premedication	20 Min.	60 Min.
Control, 50% dimethylsulfoxide	2.5	3.0	2.7
Amide:			
25 mg./kg. s.c.	2.2	3.1	2.8
50 mg./kg. s.c.	2.7	3.6	4.1
100 mg./kg. s.c.	2.7	6.4	4.8

[a] Six mice per dose. Values are in seconds.

The amide was characterized by synthesis. The only report of this compound was in 1968, when Rondest *et al.* (8) reported the isolation of this amide from the bark of *Evodia belahe* B. (Rutaceae). Analgesic activity of this amide was noted in a mouse behavioral test. To confirm this activity, the compound was run in a tail-flick test. The results are shown in Table I.

EXPERIMENTAL[2]

Plant Material—Roots of *C. sativa* L. (Cannabinaceae) were used in this study[3].

Extraction—Air-dried ground roots of *C. sativa* (4.7 kg.) were percolated with a total of 140 l. of ethanol. The extract was evaporated *in vacuo* at 40° to leave a brown syrup (155.6 g.). During the concentration, a white crystalline material (28 g.) precipitated. Recrystallization from benzene afforded white plates which, on TLC on silica gel G using a solvent system of chloroform–methanol (70:1) and visualization with a vanillin–sulfuric acid spray reagent (9), indicated the presence of two major spots, R_f 0.73 and 0.61.

Isolation of Triterpenes—Chromatography of the crystalline triterpene mixture (500 mg.) on a column of neutral Woelm alumina, grade I (45 g.), gave a fraction eluted with 50% benzene in petroleum ether (30–60°) which yielded friedelin (150 mg.). Recrystallization of the triterpene from benzene gave white needles (60 mg.), m.p. 252.5–254.5° [lit. (6) m.p. 248–252°]; $[\alpha]_D^{30}$ −23.9° (c 1.0, $CHCl_3$) [lit. (6) $[\alpha]_D^{29}$ −27.5° (c 0.34, $CHCl_3$)]. An IR spectrum showed characteristic signals at $\nu_{max.}$ 1710 cm.$^{-1}$ (carbonyl stretch), 2925 and 2870 cm.$^{-1}$ (C—H stretch), and 1380 cm.$^{-1}$ (C—H bend). The molecular formula, as determined by high-resolution mass spectrometry, was found to be $C_{30}H_{50}O$, with a molecular ion of 426.3848 (calc. 426.3861). The fragmentations of friedelin were in accordance with those described by Courtney and Shannon (10). There was no depression of melting point when mixed with authentic friedelin, and IR spectra were superimposable. Friedelin oxime was prepared according to Stevenson (11) to give white plates, m.p. 289.5–292° [lit. (11) m.p. 289–292°]; $[\alpha]_D^{30}$ +29.0° (c 0.34, $CHCl_3$). Friedelin-2,4-dinitrophenylhydrazone was prepared in the usual manner to give orange crystals, m.p. 292–294° dec. [lit. (6) m.p. 291° dec.]; $[\alpha]_D^{30}$ +24.6° (c 0.33, $CHCl_3$).

[2] Melting points were determined on a Thomas-Hoover Uni-melt melting-point apparatus and are corrected. IR spectra were run in KBr using a Perkin-Elmer 257. Optical rotations were determined on a Perkin-Elmer 141 polarimeter. Mass spectra were obtained by the Battelle Memorial Institute, Columbus, Ohio.

[3] Grown in the Marihuana Garden of the Department of Pharmacognosy, School of Pharmacy, University of Mississippi, during the summer of 1969. Voucher specimens are deposited in the Herbarium of the School of Pharmacy.

Further elution with additional 50% benzene in petroleum ether (30–60°) gave, on recrystallization from chloroform, white plates of epifriedelanol (100 mg.), m.p. 274.5–277° [lit. (4) m.p. 274°]; $[\alpha]_D^{31}$ +18.9° (c 1.0, $CHCl_3$) [lit. (3) $[\alpha]_D$ + 20.0° (c 0.44, $CHCl_3$)]. An IR spectrum showed characteristic signals at $\nu_{max.}$ 3490 $cm.^{-1}$ (O—H stretch), 2925 and 2870 $cm.^{-1}$ (C—H stretch), and 1365 $cm.^{-1}$ (C—H bend). The molecular formula, by mass spectrometry, was found to be $C_{30}H_{52}O$, with a molecular ion of 428.4014 (calc. 428.4018). The acetate (prepared with pyridine and acetic anhydride) gave, on recrystallization from benzene–ethanol, white plates, m.p. 285–286° [lit. (4) m.p. 289°]; $[\alpha]_D^{31}$ + 26.0° (c 0.25, $CHCl_3$) [lit. (4) $[\alpha]_D$ +24.7°]. The benzoate (prepared with benzoyl chloride and pyridine) separated from ethanol in plates, m.p. 250–251° [lit. (3) m.p. 252–254°]; $[\alpha]_D^{30}$ +31.2° (c 0.25, $CHCl_3$) [lit. (3) $[\alpha]_D$ +34.0° (c 0.52, $CHCl_3$)]. Oxidation of epifriedelanol (20 mg.) with chromic acid in acetic acid, according to Fieser (12), yielded white needles of friedelin (12 mg.), m.p. 258–259°, identical with an authentic sample by comparison of a mixed melting point and IR spectra.

Isolation of Amide—The ethanolic residue was dissolved in ether (1.25 l.) and shaken with 1% hydrochloric acid (3 × 1.25 l.). The ether layer was dried over anhydrous sodium sulfate and evaporated to yield a residue (26.8 g., Fraction A). The acid extract was basified with concentrated ammonia solution and extracted with chloroform (3 × 3.0 l.). The chloroform solution was dried over anhydrous sodium sulfate and evaporated to give a residue (4.7 g., Fraction B).

Fraction B was chromatographed over silicic acid[4]–diatomaceous earth[5] (4:1) (173 g.). Elution with 1% methanol–chloroform and recrystallization from chloroform–methanol yielded white microrosettes of amide (7.5 mg.), m.p. 252–254° dec. [lit. (8) m.p. 252–255°]; UV $\lambda_{max.}^{EtOH}$ 227 (log ϵ 4.1), 296 (log ϵ 4.1), and 312 nm. (log ϵ 4.1) [lit. (8) UV $\lambda_{max.}^{EtOH}$ *226* (log ϵ 4.2), *288* (log ϵ 4.1), and 312 nm. (log ϵ 4.0)]; UV $\lambda_{max.}^{EtOH+KOH}$ 243 (log ϵ 4.3), 312 (log ϵ 4.0), and 360 nm. (log ϵ 4.1) [lit. (8) UV $\lambda_{max.}^{EtOH+KOH}$ 229 (log ϵ 4.4), 312 (log ϵ 4.0), and 351 nm. (log ϵ 4.0)]. The IR spectrum showed major absorptions of $\nu_{max.}$ 3200 (broad, OH and NH stretch), 1650 (amide C=O stretch), 1590 and 1510 (aromatic C=C stretch), 1230 (phenolic C—O stretch), 980 (*trans*-disubstituted C=C bend), and 830 $cm.^{-1}$ (*para*-substituted aromatic C—H bend). The molecular formula, by mass spectrometry, was found to be $C_{17}H_{17}NO_2$, with a molecular ion of 283.1233 (calc. 283.1208). The fragmentations were in exact agreement with those described by Rondest *et al.* (8). The synthesis of *N*-(*p*-hydroxy-β-phenylethyl)β-hydroxy-*trans*-cinnamamide was carried out as described (8) from *p*-hydroxy-*trans*-cinnamic acid and tyramine in the presence of dicyclohexylcarbodiimide. The crystalline dicyclohexylurea was filtered from the reaction mixture, and the filtrate was chromatographed on silicic acid–diatomaceous earth (4:1). Elution with 4% methanol–chloroform and recrystallization from hexane–acetone afforded synthetic *N*-(*p*-hydroxy-β-phenylethyl)-*p*-hydroxy-*trans*-cinnamamide (618 mg.), m.p. 252–254° dec. The IR and UV spectra were superimposible with that of the isolate, and there was no depression of mixed melting point. TLC suggested that this amide is also present in Fraction A, the fraction in which one would have expected to find this substance because of its physical properties.

[4] Mallinckrodt.
[5] Celite, Johns-Manville Co.

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Contact Angles and Wettability of Human Skin

HANS SCHOTT

Abstract □ The contact angles of several liquids on human skin were measured before and after extraction of surface lipids with acetone. The critical surface tension of the skin was determined by extrapolating the cosines of the contact angles of the liquids as a function of their surface tensions to unity. The critical surface tensions for untreated and extracted skin were in the range of 22–30 dynes/cm. These low values are probably caused by reductions in contact angles resulting from furrows in the skin and by small amounts of air trapped at the furrows underneath the drops rather than by low free energy or polarity of the skin surface. Application of benzalkonium chloride and of a hand lotion reduced the contact angles of water on the skin.

Keyphrases □ Skin, human—measurement of contact angles of liquids before and after extraction of surface lipids □ Contact angles and wettability—liquids on human skin, critical surface tension determined □ Surface tension, critical—correlation to contact angles, human skin □ Wettability and contact angles—several liquids on human skin, critical surface tension determined

The surface properties of human skin are important in connection with the spreading of topically applied drugs and cosmetics. Measurements of the contact angles of various liquids were undertaken in view of the paucity of available data (1, 2).

THEORY

The spreading of a drop of liquid (L) placed on a solid horizontal surface (S) in air (A) is described by the equation:

$$\gamma_{SA} = \gamma_{SL} + \gamma_{LA} \cos\theta \qquad 0° \leq \theta < 180° \qquad \text{(Eq. 1)}$$

where γ represents interfacial tension, and θ is the contact angle measured in the liquid. This equation applies to the equilibrium condition, when the difference between the solid–air and solid–liquid interfacial tensions equals the adhesion tension. Strictly speaking, γ_{LA} refers to the interfacial tension between the liquid and its vapor (3).

Zisman (4) and his coworkers uncovered the linear relationship:

$$\cos\theta = a - b\gamma_{LA} \qquad \text{(Eq. 2)}$$

In the limit, $\cos\theta = 1$, and $\gamma_{LA} = \gamma_C$ equals the critical surface tension of the solid. Liquids with surface tensions equal to and smaller than γ_C will spread indefinitely on the solid surface, while liquids with $\gamma_{LA} > \gamma_C$ will make a finite contact angle (4). From Eq. 2:

$$\cos\theta = 1 + b(\gamma_C - \gamma_{LA}) \qquad \text{(Eq. 3)}$$

The critical surface tension has been identified with the surface tension of the solid (5, 6) but, more likely, it is somewhat smaller (7).

EXPERIMENTAL

Methods—Contact angles were measured by projecting the image of the drop against a vertically held piece of paper at a magnification of 20–30×, tracing the drop profile, drawing the tangent to the profile at the point where it intersects the baseline, and measuring the angle formed by the two. The light source, finger support, and lens were mounted on an optical bench.

Water drops were placed on the dorsal surface of the middle sections of the middle and ring fingers. The two joints of the fingers were bent to stretch the skin and smooth out its surface as much as possible. Additional measurements were made on the underside of the forearm. The drop profiles were traced within 3 sec. after placing the drops on the skin so that the advancing contact angles were measured. Due to the ridges and furrows and the nonuniformity of the skin, the hysteresis between the advancing and receding angles was considerable.

The hands and arms were those of females in the 20–28-year-old age group. In view of the large variation between individual subjects, more detailed work was done on a single subject. The mean of 64 measurements of the contact angles of water on the untreated skin of her two middle and ring fingers, made over a period of 3 months, was 87.8°, and the standard deviation of the mean was 1.3°.

This excellent reproducibility was obtained despite the lack of temperature control. Since the drop profiles were traced within 3 sec. of depositing the drops, and since the drop size was fairly uniform, the drops presumably warmed to approximately the same extent on contact with the skin. Neglecting the effect of temperature on γ_C, the difference between the contact angle of water on untreated skin between 18 and 37° of temperature is only about 3° according to Eq. 3.

The extensive measurements on a single subject were extended over 3 months to permit the skin to regenerate, between measurements, any lipids or other constituents removed by the drops of organic liquids in contact with it. The areas of the fingers and forearms used for contact angle measurements were not washed with soap for at least 20 hr. prior to the measurements to prevent precipitation of the hydrophobic lime soaps (8).

Surface tensions were measured at 19 ± 1° by means of a Wilhelmy balance equipped with a sand-blasted platinum blade.

Materials—The liquids chosen were doubly distilled water, reagent grade benzyl alcohol, absolute ethyl alcohol, and aqueous solutions of propylene glycol USP. The latter solutions bridge the gap between the surface tension of water (73 dynes/cm.) and of propylene glycol (36 dynes/cm.). This range is covered by few if any pure liquids of pharmaceutical importance.

RESULTS AND DISCUSSION

Contact Angles—For 13 subjects, the mean contact angles of pure water on the untreated skin of the fingers, averaged from six values measured on three drops, varied between 60 and 91°, with an overall mean value of 74°. Comparable reported values are 58° (2) and 75° (1). When lipids were removed from the skin by a 2-min. immersion in acetone followed by wiping dry with towels and drying in air, the range of contact angles for eight subjects was from 48 to 90°, with a mean of 64°. Lipid extraction reduced the contact angle of water in every instance, by amounts varying between 1 and 27°, with a mean reduction of 13°.

For the single subject, the contact angles of water were as follows: untreated skin of the fingers, 87.8 ± 1.3° (64 measurements); extracted skin of the fingers, 81.5 ± 2.0° (20 measurements); untreated skin of the forearms, 84.3 ± 1.3° (30 measurements); and extracted skin of the forearms, 91.8 ± 1.2° (30 measurements). For comparison, the contact angle of water on a defatted wool fiber, which also consists of keratin, was 85°. The effect of lipid extraction on the contact angles of 43% propylene glycol was less consistent: increases were observed for three subjects and reductions for four. In two additional instances, the difference was 1° or less.

The reported values (2) of the contact angles of water on solvent-extracted skin, 134° for acetone or 139° for hexane, are unusually high. For comparison, the contact angle of water on Teflon, one of the solids of lowest surface free energy known, is 108° (4) or 106° (9). One might attribute the high value reported to increased roughness of the extracted skin resulting from the removal of lipids, which leaves a somewhat porous surface. For contact angles greater than

Table I—Average Cosines of Contact Angles Obtained with Different Liquids on One Subject

Liquid: Composition	Surface Tension, dynes/cm.	Finger: Untreated Skin cos θ	Finger: Untreated Skin σ[a] (N)[a]	Finger: Acetone-Extracted Skin cos θ	Finger: Acetone-Extracted Skin σ[a] (N)[a]	Forearm: Untreated Skin cos θ	Forearm: Untreated Skin σ[a] (N)[a]	Forearm: Acetone-Extracted Skin cos θ	Forearm: Acetone-Extracted Skin σ[a] (N)[a]
Water	73.0	0.038	0.022(64)	0.148	0.035(20)	0.100	0.024(30)	−0.032	0.022(30)
11% PG[b]	61.8	0.373	0.025(24)	0.352	0.035(12)	0.287	0.040(22)	0.101	0.037(18)
43% PG	48.6	0.437	0.026(30)	0.419	0.020(16)	0.505	0.019(18)	0.427	0.030(18)
90% PG	39.8	0.658	0.031(12)	0.721	0.014(12)	0.776	0.011(16)	0.727	0.025(16)
Benzyl alcohol	40.9	0.790	0.016(12)	0.750	0.033(20)	0.827	0.015(14)	0.868	0.015(16)
Ethyl alcohol	23.0	1.000	—	1.000	—	1.000	—	1.000	—
Critical surface tension, dynes/cm.		24.7		22.2		29.4		30.6	

[a] Standard deviation of mean (number of angles measured). [b] Propylene glycol.

90°, increasing surface roughness increases the value of the apparent contact angle (3). However, an increase of the order of 70° would require a high degree of roughness, or a large amount of air pockets on the skin, unlikely to be present in viable human skin.

Critical Surface Tension—Cosines of the contact angles of different liquids on untreated and extracted skin of one subject are listed in Table I; they are plotted against surface tension in Fig. 1. The constants in Eq. 2, obtained from the linear dependence of cos θ on γ_{LA} by the method of least squares, have the following values: for untreated skin of the fingers, $a = 1.475$ and $b = 0.01924$; for acetone-extracted skin of the fingers, $a = 1.379$ and $b = 0.01707$; for untreated skin of the forearm, $a = 1.630$ and $b = 0.0214$; and for extracted skin of the forearm, $a = 1.798$ and $b = 0.0261$. The corresponding critical surface tensions are 24.7, 22.2, 29.4, and 30.6 dynes/cm., respectively. They are in fair agreement with the reported value of 26.8 dynes/cm. (2). Ethyl alcohol was not included in the calculations because it spread indefinitely on untreated and extracted skin.

These critical surface tensions are suspiciously low. By comparison, the γ_C values for smooth plastic surfaces are 16–18 dynes/cm. for Teflon (4, 5), 29 dynes/cm. for polypropylene, and 31 dynes/cm. for polyethylene (5). The presence of a dipole, in polyvinyl chloride, increases the critical surface tension to 39 dynes/cm. (5). For 66-nylon, the value is 46 dynes/cm. (10). These values are based on contact angles measured mostly with polar liquids capable of hydrogen bonding and are, therefore, directly comparable with the present data (11). Based on the nature and concentration of polar groups, the critical surface tension of the skin keratin should be at least as great as that of 66-nylon. The two polymers are similar in gross chemical composition inasmuch as both are rich in amide groups.

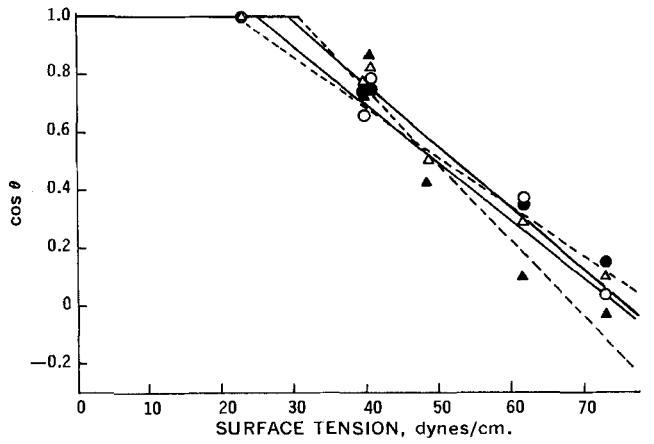

Figure 1—*Cosines of contact angles measured on one subject as a function of surface tension of the wetting liquids. Key: △, untreated forearm; ▲, acetone-extracted forearm; ○, untreated finger; ●, acetone-extracted finger; —— untreated skin; and - - -, acetone-extracted skin.*

Physical Factors Affecting Critical Surface Tension—It is unlikely that the critical surface tension of smooth skin would be smaller than that of solid hydrocarbons. Some factors which could affect the value of γ_C are discussed here.

1. Taking into account noncoherent surface roughness, *i.e.*, isolated elevations and depressions, would reduce γ_C further. If a roughness factor r is defined as the ratio between the actual area of the rough surface and the apparent or geometric area of the smooth surface (3):

$$r = \cos \theta_{app}/\cos \theta_{true} \geq 1 \qquad \text{(Eq. 4)}$$

Therefore, the cosines of the true contact angles, *i.e.*, the contact angles which would be observed on smooth skin, are smaller than the cosines of the corresponding apparent contact angles since the angles are smaller than 90°. Using the former in Fig. 1 would lower the lines, so their intercept with the horizontal line for cos $\theta = 1$, namely, γ_C, would become smaller. Even for the very low value of $r = 1.25$, γ_C drops from 23 to 11 dynes/cm.

2. Skin contains relatively deep furrows. If these furrows make an angle of inclination, ϕ, with the horizontal skin surface, the liquid front of the drop advancing perpendicularly to them has an apparent contact angle equal to the true contact angle plus ϕ. If $\phi = 15°$, $\theta_{true} = \theta_{app} - 15$. Subtracting 15° from the apparent contact angles measured with the five liquids on acetone-extracted fingers increases γ_C from 22 to 32 dynes/cm. For $\phi = 30°$, γ_C becomes 38 dynes/cm.

3. An alternative explanation is based on the assumption that the liquid drops trap pockets of air in the furrows of the skin surface as they advance. This process makes the skin a composite surface. A fraction, f_{SL}, of the area of the total apparent solid–liquid interface consists of true solid–liquid interface. The remainder, the fraction f_{LA}: consists of liquid–air interface. Neglecting hydrostatic pressure (12),

$$\cos \theta_{app} = f_{SL} \cos \theta_{true} - f_{LA} \qquad \text{(Eq. 5)}$$

Therefore, $\cos \theta_{true} > \cos \theta_{app}$, and the lines of Fig. 1 are displaced toward higher cosines if the measured contact angles are replaced by the true contact angles. This results in higher values for the true critical surface tension of skin, *i.e.*, for skin without adhering air pockets trapped beneath the liquid. If only 5% of the total apparent liquid–skin interface consisted of liquid–air interface for all five liquids, the true critical surface tension of the untreated skin would increase from 24.7 to 29.8 dynes/cm. according to Eq. 5.

Optical examination of drops of liquids on skin at low magnification with a grazing incident light beam showed the presence of minute air bubbles trapped beneath the drops, most of them attached to the furrows of the skin. This effect probably also accounts for the small difference in wettability of untreated and acetone-extracted skin.

The combination of Factors 2 and 3 seems adequate to account for the difference in critical surface tension between smooth 66-nylon and human skin, which amounts to about 19 dynes/cm.

Effect of Added Materials—When a commercial hand lotion was applied to the untreated skin of the fingers of one subject, followed by a 30-min. drying period, the contact angle of water decreased

from 87 ± 3° (16 measurements) to 52 ± 2° (24 measurements)[1]. Application of the hand lotion reduced the mean contact angles of water on seven other subjects by 20–32°, with an average decrease of 28°. The angles were always measured after a 30-min. drying period. The moisturizing effect of the lotion includes the skin surface, possibly because some portion of the lotion was not absorbed by the skin but remained on the surface.

In another experiment, hands were immersed in a 1:1000 benzalkonium chloride solution USP for 10 min. Excess solution was then rinsed off by holding the hands for 5 sec. under running tap water and for 1 sec. under running distilled water. The contact angles of water were measured after drying with towels and in air. On placing drops on the skin, the initial contact angles were near 90°. After about 1 sec., the water drops suddenly began to spread and the contact angles decreased. Evidently, benzalkonium chloride which had been physically adsorbed on the skin was desorbed and dissolved by the drop, reducing its interfacial tension against skin as well as its surface tension. The mean value of the contact angles was lowered from 86 ± 3 to 48 ± 3° (18 measurements).

When hands treated with benzalkonium chloride were washed by 5-min. immersions in running lukewarm tap water, followed by rinsing with distilled water and drying with towels and in air, the average contact angle of water from 24 measurements increased to 84 ± 3°. Within the precision of the measurements, this value does not differ significantly from the initial value of 86 ± 3°, indicating that most if not all of the benzalkonium chloride was removed by immersion in water. Apparently, its adsorption by the skin is largely physical and reversible. This conclusion is made with some reservation, because chemisorption of a monolayer of alkyltrimethylammonium halides by glass, with the headgroups oriented toward the surface and the hydrocarbon tails away from it, produced sizable increases in the contact angles of water (13). Since the contact angles of water on untreated skin are already high, chemisorbed benzalkonium cations might not increase them at all.

[1] The ± sign precedes the standard deviation of the mean.

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Physical Properties of Quaternary Ammonium Salts of Phenothiazine Derivatives

C. L. HUANG* and C. T. CHANG†

Abstract □ Physical constants, including melting points and IR and NMR spectra, and analytical data of the quaternary ammonium salts of nine clinically used phenothiazines are presented. Assignments for the spectral peaks were made and are discussed. These compounds were prepared in good yields, so the formation of quaternary ammonium salt may serve as a convenient method for the isolation and identification of these phenothiazines.

Keyphrases □ Phenothiazine derivatives, quaternary ammonium salts—synthesis, physical properties □ Quaternary ammonium salts of phenothiazine derivatives—synthesis, physical properties □ NMR spectroscopy—identity □ IR spectrophotometry—identity

Quaternary ammonium salts of phenothiazine neroleptics have been demonstrated to have a wide variety of biological activities. It was indicated in a previous study (1) that all of the quaternary ammonium salts of these compounds possess antimicrobial activities on Gram-positive and Gram-negative organisms and have a toxicity higher than the corresponding parent compound. Evidence was presented to show that the ^{14}C quaternary ammonium salts of phenothiazines were able to cross the blood–brain barrier to reach the brain tissues (2). The metabolic pathways of the quaternary ammonium salts of phenothiazines (mepazine, promethazine, promazine, chlorpromazine, triflupromazine, prochlorperazine, and trifluoperazine) are quite different from the corresponding hydrochloride salts; the quaternary ammonium salt is found predominantly in the feces (3) while the majority of the hydrochloride salt is excreted through the kidneys (4) and the lungs (5).

Although spectra–structure correlation of tertiary phenothiazines was reported (6), there is paucity of information concerning the physical properties of the quaternary ammonium salts of phenothiazines. The purpose of this paper is to present physical data, including melting points and UV, IR, and NMR spectra, of 11 quaternary ammonium compounds of nine clinically used phenothiazine derivatives: mepazine, promethazine, promazine, chlorpromazine, triflupromazine, prochlorperazine, trifluoperazine, perphenazine, and fluphenazine. Since all of these compounds form

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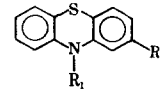
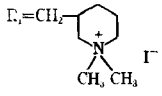
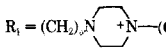
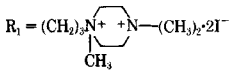


Table I—Physical Constants and Analytical Data of Quaternary Ammonium Salts of Phenothiazine Derivatives

Parent Compound	Quaternary Methiodide	IR, μ			Melting Point	Formula	Analysis, % Calc.		Found	NMR, τ
Mepazine	R = H 	3.45	7.80	9.60	298–301° dec.	$C_{20}H_{25}IN_2S$	C	53.09	53.21	2.70–3.10 (m, 8H, aromatic)
		6.25	7.95	10.00			H	5.53	5.45	6.13 (d, 2H)
		6.35	8.10	10.47			N	6.19	5.99	6.50–6.80 (m, 4H)
		6.80	8.53	10.75			S	7.08	7.06	6.90 (3, 3H)
		7.45	8.90	11.55						7.06 (3, 3H)
		7.62	9.10	13.10						7.80–8.00 (m, 1H)
										8.00–9.35 (m, 4H)
Promethazine	R = H $R_1 = CH_2CH(CH_3)\overset{+}{N}(CH_3)_3 \cdot I^-$	3.35	8.08	11.20	225–226° dec.	$C_{18}H_{23}IN_2S$	C	50.70	50.80	2.61–3.18 (m, 8H, aromatic)
		6.20	8.33	11.75			H	5.40	5.51	5.83 (d, 2H)
		6.35	8.85	12.05			N	6.57	6.68	6.59 (s, 9H)
		6.45	9.25	13.15			S	7.51	7.50	7.83 (m, H)
		6.97	9.70	13.40						8.42 (d, 3H, J = 6 Hz.)
		7.60	10.65	13.85						
Promazine	R = H $R_1 = (CH_2)_3\overset{+}{N}(CH_3)_3 \cdot I^-$	3.42	8.00	10.86	255–257°	$C_{18}H_{23}IN_2S$	C	50.70	50.80	2.75–3.13 (m, 8H, aromatic)
		6.28	8.35	11.62			H	5.40	5.50	6.07 (t, 2H, J = 7 Hz.)
		6.88	8.90	13.10			N	6.57	6.70	6.33–6.87 (m, 2H)
		7.49	9.63	13.68			S	7.51	7.49	6.92 (s, 9H)
		7.80	10.37							7.67–8.15 (m, 2H)
Chlorpromazine	R = Cl $R_1 = (CH_2)_3\overset{+}{N}(CH_3)_3 \cdot I^-$	3.33	8.03	10.35	145–147°	$C_{18}H_{22}ClIN_2S$	C	46.91	47.23	2.63–2.90 (m, 7H, aromatic)
		6.40	8.37	10.47			H	4.78	5.11	5.73–(t, 2H, J = 7 Hz.)
		6.87	8.67	11.35			N	6.08	5.96	6.00–6.35 (m, 2H)
		7.15	8.90	11.53			S	6.95	6.74	6.60 (s, 9H)
		7.60	9.10	12.50						7.17–7.82 (m, 2H)
		7.85	9.70	13.07						
Trifluprom azine	R = CF_3 $R_1 = (CH_2)_3\overset{+}{N}(CH_3)_3 \cdot I^-$	3.25	8.01	11.30	169–170°	$C_{19}H_{22}F_3IN_2S$	C	46.16	46.33	2.53–2.92 (m, 7H, aromatic)
		6.22	8.54	12.08			H	4.49	4.48	5.39 (t, 2H, J = 7 Hz.)
		6.82	8.90	13.08			N	5.67	5.71	6.25–6.53 (m, H)
		7.01	9.25	13.78			S	6.49	6.62	6.87(s, 9H)
		7.50	10.65							7.68–7.95 (m, 2H)
Prochlorperazine (I)	R = Cl 	3.43	8.38	11.05	165–167°	$C_{21}H_{27}ClIN_3S$	C	48.92	49.37	2.92–3.25 (m, 7H, aromatic)
		6.38	8.86	11.95			H	5.23	5.40	6.10 (t, 2H, J = 7 Hz.)
		6.85	9.02	12.50			N	8.13	8.17	6.50–6.75 (m, 4H)
		7.13	9.60	13.30			S	6.90	7.04	6.90 (s, 6H)
		7.43	9.90							7.20–7.65 (m, 6H)
		8.05	10.78							8.02–8.35 (m, 2H)
Prochlorperazine (II)	R = Cl 	9.30	9.23	11.86	224–225°	$C_{22}H_{30}ClI_2N_3S$	C	40.17	40.34	2.50–3.00 (m, 7H, aromatic)
		6.60	9.55	12.23			H	4.60	4.68	5.99 (s, 12H)
		7.10	10.13	12.58			N	6.39	6.24	6.51 (s, 6H)
		7.45	11.00	13.50			S	4.87	4.69	6.65 (s, 3H)
		8.40	11.16							7.65–8.00 (m, 2H)

crystalline quaternary ammonium salts in good yields (approximately 80%), quaternary ammonium salt formation may serve as a convenient method for the isolation of the parent compound.

EXPERIMENTAL[1]

Preparation of Quaternary Ammonium Salts—The hydrochloride salt (0.5 g.) of each phenothiazine derivative was dissolved in water (or in aqueous methanol in case of less soluble salts), and the solution was adjusted to pH > 10 by adding potassium carbonate solution. The oily precipitate which occurred was extracted several times with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate, and then the solvent was evaporated under reduced pressure to yield 0.45–0.46 g. of viscous oil. The free base of each compound obtained was dissolved in acetone, and methyl iodide (1.05 mole equivalents for the monomethiodides and more than 2.2 mole equivalents for the dimethiodides of piperazinyl derivatives) dissolved in acetone was added. The reaction was allowed to proceed at room temperature for 1 hr. (promazine, trifluoperazine, and perphenazine) to 18 hr. (triflupromazine, mepazine, promethazine, and chlorpromazine).

In some cases (mepazine, promazine, trifluoperazine, and perphenazine), the quaternary ammonium salt precipitated as such; in others (promethazine, chlorpromazine, triflupromazine, and prochlorperazine), addition of ether or benzene was necessary to precipitate the product. The crude product was collected and recrystallized from acetone or acetone–ether to yield the final product (80–85%). The physical constants, including melting points and UV and IR absorption peaks, and elemental analyses are shown in Table I.

DISCUSSION

IR Spectra—The IR spectra of quaternary ammonium salts are in general similar with those of the corresponding parent compounds (Table I). The IR spectra of quaternary ammonium salts derived from the 10-substituted phenothiazine have a strong peak in the region of 13.1–13.3 μ, which is assigned to the out-of-plane bending vibrations of 1,2-disubstituted benzene (7). In the case of quaternary ammonium salts derived from 2,10-disubstituted phenothiazines, besides a band at 13.1–13.3 μ (assignable to the out-of-plane bending vibration of 1,2-disubstitution of ring A), at least three more bands of varying intensity are present in the regions of 10.6–10.9, 11.3–11.6, and 12.3–12.5 μ, which are due to the substitution in position 2 of the phenothiazine ring system and to 1,2,4-trisubstitution of ring B. Besides the two characteristic bands at 6.3 and 6.4 μ, spectra typical of the aromatic ring system were observed in all compounds.

NMR Spectra—Table I shows the important NMR signals of these compounds. In all the compounds, the signals of the aromatic protons appeared as a multiplet in the range of 2.48–3.25 τ. In all the monomethiodide derivatives, the spectrum of the two protons on the carbon adjacent to the ring nitrogen was shown as a triplet in the region of 5.39–6.10 τ, with a coupling constant of 6–7 Hz. The signals of the methylene protons on the second carbon of the propyl group (except mepazine and promethazine) were recorded as a multiplet at 7.17–8.35 τ. The spectrum of the methylene protons on the first carbon of the propyl group of promazine, chlorpromazine, and triflupromazine appeared as a multiplet in the region of 6.00–6.87 τ. However, in the monomethiodides of prochlorperazine and trifluoperazine, these two protons combined with four protons on carbons at positions 3 and 5 of the piperazinyl ring system to form a multiplet in the region of 7.20–7.68 τ. The protons on two carbons attached to the nitrogen at position 1 of the piperazinyl ring system were shown at 6.50–6.53 τ as a multiplet. Those methyl protons on the quaternary nitrogen of all the monomethiodides appeared as a sharp singlet between 6.59 and 7.25 τ. In the mepazine methiodide, the doublet signal at 6.09 τ was due to the protons on the carbon at position 2, and the multiplet signal

[1] UV absorption spectra were taken in a Perkin-Elmer model 202 spectrophotometer. IR absorption spectra were recorded in a Perkin-Elmer Infracord model 137E, using KBr pellets as mulls. NMR spectra were taken in a 10–20% $(CD_3)_2SO$ or $(CD_3)_2CO$ solution in a Varian A-60 spectrometer, using tetramethylsilane as an internal standard.

in the range of 7.40–7.68 τ came from protons on the carbon at position 5 of the piperidyl ring system. Five protons from the carbons at positions 3, 4, and 5 formed a multiplet in the range of 8.07–8.83 τ. The single proton on the first carbon of the isopropyl group in promethazine methiodide had a multiplet at 7.83 τ, and the methyl protons on the side chain appeared as a doublet at 8.42 τ, with a coupling constant of 6 Hz.

In the dimethiodide derivatives of prochlorperazine and trifluoperazine, a broad peak observed at 5.99 and 6.08 τ, respectively, represented 12 protons, including four protons on the carbons at positions 1 and 3 of the propyl group and eight protons on the piperazinyl ring system. In these two compounds, the methyl protons on the nitrogen at position 4 of the piperazinyl ring system had a singlet at 6.65 and 6.75 τ, respectively. The dimethiodide of perphenazine and fluphenazine had a broad singletlike peak at 6.03 and 6.00 τ, respectively, representing 17 protons from the first and third carbons of the propyl, piperazinyl, and hydroxyethyl groups. The methyl protons adjacent to the nitrogen at position 1 of the piperazinyl ring system of perphenazine and fluphenazine showed a single peak at 6.72 and 6.67 τ, respectively, while the methyl protons adjacent to the nitrogen at position 4 exhibited a singlet signal at 6.60 and 6.52 τ, respectively.

In all cases, quaternization of the terminal nitrogen in the side chain of phenothiazine derivatives appeared to shift the spectrum of the methyl protons on the terminal nitrogen downfield by 0.58–1.24 τ. The presence of a substituent group of Cl and CF_3 on the ring system shifted the aromatic peaks downfield. This effect was more prominent with the presence of CF_3 in position 2 of the phenothiazine ring system.

SUMMARY

Physical properties, including melting points and IR and NMR spectral data, of quaternary ammonium salts of some important phenothiazine derivatives have been presented. All these compounds gave crystalline products with a good yield (80–85%), so formation of a quaternary ammonium salt may serve as a convenient method for the isolation and identification of these clinically used phenothiazines.

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Direct Measurement of Aspirin

SAUL L. KANTER and WILLIAM R. HORBALY

Abstract □ A fluorometric procedure for the direct measurement of aspirin in which interference due to salicylic acid and its conjugates was eliminated by reaction with ceric ammonium nitrate was modified, improving recovery of aspirin from 57 to 93%.

Keyphrases □ Aspirin—fluorometric determination, elimination of interference by salicylic acid □ Fluorometry—determination of aspirin without interference of salicylic acid

A direct determination of aspirin in human blood samples by fluorometry was described previously (1). In this method, an ethylene dichloride extract of salicylates is treated with an aqueous solution of ceric ammonium nitrate, which reacts with salicylic acid and its conjugates, leaving only aspirin in the organic phase. In this laboratory, it was found that the recovery of aspirin from an ethylene dichloride solution after treatment with an aqueous solution of ceric ammonium nitrate was only 57% and that the determination of aspirin was affected by the amount of salicylic acid present. For amounts of salicylic acid of 25, 100, and 200 mcg., the slopes of aspirin standardization curves varied 4.3, 8.6, and 18.9%, respectively, from the slope of an aspirin standardization curve without salicylic acid. The difference between each slope was statistically significant from the others. Improvement of recovery of aspirin to 93% was obtained by using 3 ml. of 0.01 *N* acetic acid and 0.05 ml. of ceric ammonium nitrate instead of 5.9 ml. of water and 0.10 ml. of ceric ammonium nitrate, respectively.

Although the determination of aspirin was still affected by the amount of salicylic acid present, the variation of the slopes of equivalent standardization curves was reduced to 0.7, 0.7, and 3.4%, respectively, from the slope of an aspirin standardization curve without salicylic acid. While the slope of the aspirin standardization curve containing 200 mcg. of salicylic acid differed statistically from the slopes of aspirin standardization curves containing 25 mcg. and zero salicylic acid, respectively, the slope of the aspirin standardiza-

in the range of 7.40–7.68 τ came from protons on the carbon at position 5 of the piperidyl ring system. Five protons from the carbons at positions 3, 4, and 5 formed a multiplet in the range of 8.07–8.83 τ. The single proton on the first carbon of the isopropyl group in promethazine methiodide had a multiplet at 7.83 τ, and the methyl protons on the side chain appeared as a doublet at 8.42 τ, with a coupling constant of 6 Hz.

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Although the determination of aspirin was still affected by the amount of salicylic acid present, the variation of the slopes of equivalent standardization curves was reduced to 0.7, 0.7, and 3.4%, respectively, from the slope of an aspirin standardization curve without salicylic acid. While the slope of the aspirin standardization curve containing 200 mcg. of salicylic acid differed statistically from the slopes of aspirin standardization curves containing 25 mcg. and zero salicylic acid, respectively, the slope of the aspirin standardiza-

tion curve containing 100 mcg. of salicylic acid and the others did not.

EXPERIMENTAL

Aliquots of aspirin in ethylene dichloride containing 2.5, 5.0, 10.0, 20.0, 30.0, and 40.0 mcg. as salicylic acid, respectively, were transferred to snap-cap bottles. The volumes of each were adjusted to 50 ml. with ethylene dichloride. Three milliliters of 0.01 *N* acetic acid and 0.05 ml. of an aqueous solution of 40% ceric ammonium nitrate were added. The bottles were capped and shaken on a reciprocal shaker at approximately 200 c.p.m. for 15 min. The contents of each bottle were transferred to 25 × 150-mm. screw-cap culture tubes. (The screw-cap culture tubes hold almost the entire volume.) The tubes were capped with Teflon-lined screw caps and centrifuged 10 min. at a relative centrifugal force (RCF) of approximately 1000 to separate the two phases.

The aqueous phase was removed by aspiration, and 30.0 ml. of the ethylene dichloride phase of each was transferred to other snap-cap bottles. Six-milliliter aliquots of 1% $NaHCO_3$ were added, and the bottles were capped and shaken at approximately 200 c.p.m. for 10 min. The contents of each bottle were transferred to clean 25 × 150-mm. screw-cap culture tubes and centrifuged 5 min. at approximately 1000 RCF. Four-milliliter aliquots of the $NaHCO_3$ (upper) phase were transferred to 19 × 150-mm. test tubes. One milliliter of 6 *N* NaOH was added and thoroughly mixed with the $NaHCO_3$. The fluorescence was measured after 10 min. using a fluorometer[1] with a general-purpose lamp and primary filters 7-54(2) and 34A, sandwich arrangement, and secondary filter 5-58, which are equivalent to 325 and 400 nm., respectively. This procedure was repeated three times, adding 25, 100, and 200 mcg. of salicylic acid in ethylene dichloride, respectively, before adjusting the volumes to 50 ml. with ethylene dichloride. Equivalent sets of aspirin solutions with and without added salicylic acid were prepared, treated with 5.9 ml. of water and 0.1 ml. of 40% ceric ammonium nitrate, and shaken at approximately 200 c.p.m. for 1 hr. as described by Cotty and Ederma (1). The procedure was continued as already described.

A final set of aspirin solutions in ethylene dichloride, equivalent to the two sets without added salicylic acid, was prepared as the control. Thirty-milliliter aliquots were transferred to other snap-cap bottles, 6.0 ml. of 1% $NaHCO_3$ were added to each, and the procedure was continued as described. Appropriate blanks were run.

The data in Fig. 1, recording the effect of adding salicylic acid on the recovery of aspirin by both the modified and the reference methods, show the significant reduction in the range of aspirin standardization curves from 18.9 to 3.4%, and the improvement in the recovery of aspirin from 57 to 93% of theoretical using the modification described by the authors.

A number of experiments, using different concentrations of acetic acid and shaking periods that varied from 1 to 60 min., were performed to determine the concentration of acetic acid at which: (*a*) most interference of salicylic acid was eliminated, (*b*) net recovery of aspirin was the same whether or not salicylic acid was present, and (*c*) the least amount of shaking time was required.

The data presented in Table I are a pertinent summary of these experiments in which 10 mcg. of aspirin was used to evaluate recovery of aspirin and 200 mcg. salicylic acid was used to evaluate efficiency of elimination of interference of salicylic acid. Both aspirin and salicylic acid were prepared in ethylene dichloride. Per group of three, one contained 10 mcg. aspirin and 200 mcg. salicylic acid, another contained 200 mcg. salicylic acid only, and the third contained 10 mcg. aspirin only. The first evaluated recovery of aspirin in the presence of salicylic acid, the second was a blank for the salicylic acid, and the third was a control for the aspirin. All were brought to a volume of 30 ml. with the appropriate amount of ethylene dichloride before treating with acetic acid and ceric ammonium nitrate. The data show that for a range of acetic acid from 0.005 to 0.015 *N*, the net results were the same. Therefore, 0.01 *N* was selected as optimum. At higher concentrations of acetic acid, 0.02 *N* for example, 30 min. of shaking time instead of 15 was required to obtain a minimum blank value.

Other data, not recorded, showed that with 3 ml. of 0.01 *N* acetic acid and 0.05 ml. of 40% ceric ammonium nitrate, shaking more than 15 min. did not further reduce the blank value due to salicylic acid but did continue to reduce the recovery of aspirin slightly but measurably. These data also showed that 25% of the 36% increase in recovery of aspirin was due to changing from water to 0.01 *N* acetic acid, and the remainder was due to the reduction of the volume from 6 to 3 ml.

[1] Turner, model 110.

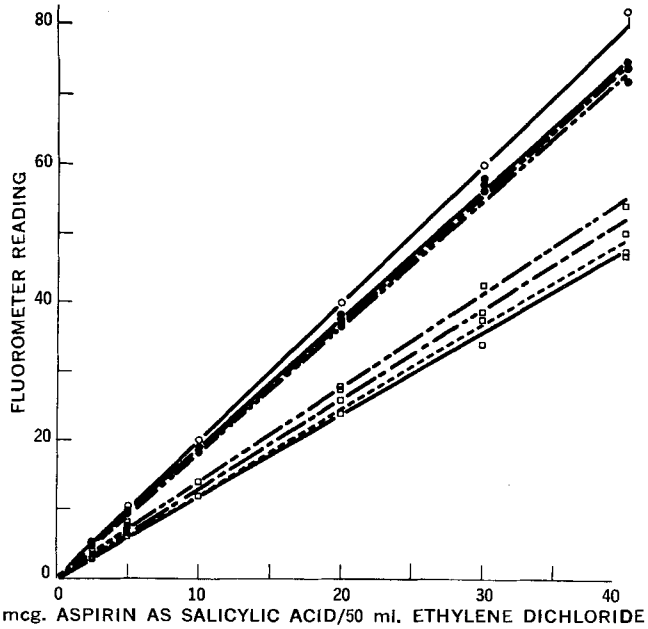

Figure 1—*Effect of ceric ammonium nitrate treatment on the recovery of aspirin. Key:* ○, *control not treated with ceric ammonium nitrate;* ●, *modified method, 3 ml. of 0.01* N *acetic acid and 0.05 ml. of 40% ceric ammonium nitrate;* □, *reference method (1), 5.9 ml. of water and 0.1 ml. of 40% ceric ammonium nitrate;* ——, *zero salicylic acid;* - - -, *with 25 mcg. salicylic acid;* — — —, *with 100 mcg. salicylic acid; and* - - — - -, *with 200 mcg. salicylic acid.*

DISCUSSION

In the method of Cotty and Ederma (1) showing recovery of aspirin added to pooled whole blood, no evaluation of the effect of ceric ammonium nitrate treatment on aspirin was presented. Observation shows that the "protection" against the effect of ceric ammonium nitrate attributed to acetylation of the phenolic group of salicylic acid is apparently not as complete as expected under the conditions described (1). Use of a very dilute solution of acetic acid

Table I—Effect of Acetic Acid Concentration on Recovery of Aspirin and Elimination of Interference of Salicylic Acid

Aspirin, mcg.	Salicylic Acid, mcg.	Acetic acid, 3 ml.	Fluorometric —Reading 3×[a]— Total	Net	Ceric Ammonium Nitrate Treatment[b], min.
10	200	0.005 *N*	34	30	15
0	200		4		
10	0		29.5	29	
10	200	0.01 *N*	34	29.5	15
0	200		4.5		
10	0		29	28.5	
10	200	0.015 *N*	33.5	28.5	15
0	200		5		
10	0		28.8	28.3	
10	200	0.02 *N*	34	29	30
0	200		5		
10	0		28.5	28	

[a] Fluorometric reading at the 3× slit. [b] Each contained 0.05 ml. of 40% ceric ammonium nitrate.

Table II—Statistical Analysis of Aspirin Standardization Curves Modified and *Reference 1* Methods

Salicylic Acid Added, mcg.	Slope: Average	Slope: Statistical	*SD*, %	*CV*[a], %	Ratio to Control, %
Control					
0	2.02	2.04	±0.020	±0.98	
Ceric Ammonium Nitrate Treatment According to Modified Method					
0	1.90	1.89	±0.019	±1.0	93
25	1.82	1.88	±0.019	±1.0	92
100	1.87	1.88	±0.026	±1.4	92
200	1.84	1.83	±0.034	±1.8	90
Ceric Ammonium Nitrate Treatment According to Reference Method					
0	1.16	1.16	±0.020	±1.70	57
25	1.24	1.21	±0.030	±2.50	59
100	1.32	1.26	±0.022	±1.75	61
200	1.38	1.38	±0.024	±1.74	68

[a] Coefficient of variation.

enhances this "protection" considerably. Premixing of ceric ammonium nitrate and acetic acid is not recommended. The effectiveness of the ceric ammonium nitrate deteriorates rapidly under these conditions. Because the original work with ceric salts and phenols used HNO_3 (2), the latter was also tried. With 2 *N* HNO_3, recovery was almost as good as with acetic acid, but net recovery of aspirin was not the same whether or not salicylic acid was present.

Precision was evaluated by comparing the averages of the coefficients of variation of each of the four standardization curves obtained by each method (Table II). Averages and standard deviations were 1.30 ± 0.38 and 1.92 ± 0.39% for the modified and reference methods, respectively, yielding a difference between averages that was statistically significant at the 1% level (3). Reliable values by both the modified and original methods require knowledge of the salicylic acid content because blank values and aspirin standardization curves vary with salicylic acid content. While an average blank obtained with 50 mcg. salicylic acid may be used for amounts of salicylic acid up to 100 mcg. for both methods, reducing accuracy only slightly, the modified method permits using the aspirin standardization curve (calibrated with 100 mcg. salicylic acid) for a range of 0–200 mcg. salicylic acid, which is a definite advantage over the original method which requires using aspirin standardization curves appropriate to the amount of salicylic acid present (Fig. 1) (4). The modification also improves sensitivity approximately 30%.

A modification for developing fluorescence with aspirin is also presented. At a NaOH concentration of 1.2 *N*, heat was not required to convert aspirin to salicylic acid, and the fluorescence of equivalent amounts of aspirin and salicylic acid was found to be equal.

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Reduced Side Effects following Administration of a New Anorectic Agent, 4′-Chloro-2-(ethylamino)propiophenone, in Timed-Release Form

EARL ROSEN, S. M. FREE, and GEORGE C. HEIL

Abstract □ Studies representing several parts of a development program for the anorexigenic SK&F 70948 are summarized. *In vitro* release rates and the results of an anorectic study in dogs demonstrated the timed-release characteristics of a 25:75 blend (25% nontimed-release pellets and 75% wax–lipid-coated pellets). The global efficacy, weight loss, and side-effect incidence of a 75- and 150-mg. timed-release formulation were studied in a multiinvestigator clinical trial; a parallel multiinvestigator clinical trial included 25- and 50-mg. t.i.d tablet regimens. In each comparison, the incidence of side effects seen with the timed-release formulation was significantly less than that seen with the t.i.d tablets ($p < 0.01$). However, the global efficacy and anorectic activity of the timed-release and t.i.d regimens did not differ significantly. These results suggested that the lower incidence of side effects following timed-release medication was related to the constant and sustained body levels of drug seen with the timed-release formulation.

Keyphrases □ 4′-Chloro-2-(ethylamino)propiophenone—reduction of side effects using timed-release form □ Timed-release dosage forms—reduction of side effects of 4′-chloro-2-(ethylamino)-propiophenone □ Anorectic agent, 4′-chloro-2-(ethylamino)propiophenone—reduction of side effects found using timed-release dosage form

During pharmaceutical development, one often attempts to modify a drug's activity in order to increase its usefulness in the treatment of disease. One such modification—prolongation of drug action—has been attempted over the years using a number of different methods with varying degrees of success. From studies comparing prolonged-action preparations with other dosage forms, it has become clear that each drug in-

Table II—Statistical Analysis of Aspirin Standardization Curves Modified and *Reference 1* Methods

Salicylic Acid Added, mcg.	Slope: Average	Slope: Statistical	*SD*, %	*CV*[a], %	Ratio to Control, %
Control					
0	2.02	2.04	±0.020	±0.98	
Ceric Ammonium Nitrate Treatment According to Modified Method					
0	1.90	1.89	±0.019	±1.0	93
25	1.82	1.88	±0.019	±1.0	92
100	1.87	1.88	±0.026	±1.4	92
200	1.84	1.83	±0.034	±1.8	90
Ceric Ammonium Nitrate Treatment According to Reference Method					
0	1.16	1.16	±0.020	±1.70	57
25	1.24	1.21	±0.030	±2.50	59
100	1.32	1.26	±0.022	±1.75	61
200	1.38	1.38	±0.024	±1.74	68

[a] Coefficient of variation.

enhances this "protection" considerably. Premixing of ceric ammonium nitrate and acetic acid is not recommended. The effectiveness of the ceric ammonium nitrate deteriorates rapidly under these conditions. Because the original work with ceric salts and phenols used HNO_3 (2), the latter was also tried. With 2 *N* HNO_3, recovery was almost as good as with acetic acid, but net recovery of aspirin was not the same whether or not salicylic acid was present.

Precision was evaluated by comparing the averages of the coefficients of variation of each of the four standardization curves obtained by each method (Table II). Averages and standard deviations were 1.30 ± 0.38 and 1.92 ± 0.39% for the modified and reference methods, respectively, yielding a difference between averages that was statistically significant at the 1% level (3). Reliable values by both the modified and original methods require knowledge of the salicylic acid content because blank values and aspirin standardization curves vary with salicylic acid content. While an average blank obtained with 50 mcg. salicylic acid may be used for amounts of salicylic acid up to 100 mcg. for both methods, reducing accuracy only slightly, the modified method permits using the aspirin standardization curve (calibrated with 100 mcg. salicylic acid) for a range of 0–200 mcg. salicylic acid, which is a definite advantage over the original method which requires using aspirin standardization curves appropriate to the amount of salicylic acid present (Fig. 1) (4). The modification also improves sensitivity approximately 30%.

A modification for developing fluorescence with aspirin is also presented. At a NaOH concentration of 1.2 *N*, heat was not required to convert aspirin to salicylic acid, and the fluorescence of equivalent amounts of aspirin and salicylic acid was found to be equal.

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Reduced Side Effects following Administration of a New Anorectic Agent, 4′-Chloro-2-(ethylamino)propiophenone, in Timed-Release Form

EARL ROSEN, S. M. FREE, and GEORGE C. HEIL

Abstract □ Studies representing several parts of a development program for the anorexigenic SK&F 70948 are summarized. *In vitro* release rates and the results of an anorectic study in dogs demonstrated the timed-release characteristics of a 25:75 blend (25% nontimed-release pellets and 75% wax–lipid-coated pellets). The global efficacy, weight loss, and side-effect incidence of a 75- and 150-mg. timed-release formulation were studied in a multiinvestigator clinical trial; a parallel multiinvestigator clinical trial included 25- and 50-mg. t.i.d tablet regimens. In each comparison, the incidence of side effects seen with the timed-release formulation was significantly less than that seen with the t.i.d tablets ($p < 0.01$). However, the global efficacy and anorectic activity of the timed-release and t.i.d regimens did not differ significantly. These results suggested that the lower incidence of side effects following timed-release medication was related to the constant and sustained body levels of drug seen with the timed-release formulation.

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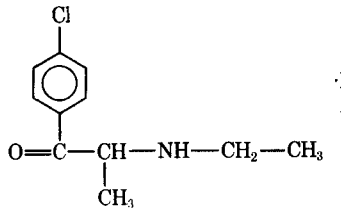
During pharmaceutical development, one often attempts to modify a drug's activity in order to increase its usefulness in the treatment of disease. One such modification—prolongation of drug action—has been attempted over the years using a number of different methods with varying degrees of success. From studies comparing prolonged-action preparations with other dosage forms, it has become clear that each drug in-



SK&F 70948

volved must be considered separately and that attention must be paid to such factors as the critical dose range, the speed and duration of action, and the reliability of the therapeutic response anticipated. Most important, such considerations must be related to the advantages and disadvantages of the dosage forms being evaluated. Perhaps the most important advantage provided by prolonged-action preparations is a reduction in the variation of the drug concentration in blood and tissues that is commonly associated with multiple-dose therapy. In theory, if high peak concentrations can be avoided, the incidence and/or severity of side effects may be reduced.

The formulation of anorectic agents in prolonged-action dosage forms has become widely accepted. One such agent, SK&F 70948, a propiophenone derivative, was evaluated in clinical studies designed to provide information on global efficacy, anorectic activity, and side effects produced at different dose levels with both timed-release and tablet dosage forms. One objective of these dose range studies was to compare the incidence of side effects associated with the two dose forms; this was based on the hypothesis that, for the same total dose, the timed-release form would demonstrate a lower incidence of side effects.

In this publication, *in vitro* release data for the timed-release formulations tested are reported and an animal model found predictive of the clinical results is described.

EXPERIMENTAL

***In Vitro* Release**—Nontimed-release medicated pellets were prepared as previously reported (1, 2) and made to assay from 32 to 74% SK&F 70948. A portion of these pellets was retained, and the remaining pellets were coated with approximately 4% paraffin wax and varying increments of glyceryl stearate to prolong drug release. The release rate was determined using the rotating apparatus described in NF XIII (3).

Accurately weighed 1.0 ± 0.1 g. riffled samples were placed in 90-ml. cylindrical bottles, and 60 ml. of simulated gastric fluid USP, T.S., preheated to 37°, was added. The bottles were then placed in the 44-r.p.m. rotating apparatus submerged in a constant-temperature bath maintained at 37°. At the end of 0.5 hr., one bottle was removed from the bath, the contents were filtered through a 40-mesh metal screen, and the residue was rinsed on the screen with 25 ml. of distilled water. At the end of 1.5 hr., all the remaining bottles were removed from the bath, and the contents of the individual bottles were filtered through 40-mesh metal screens. The residues were washed free of gastric fluid with 25 ml. of distilled water, and each sample of residual pellets was returned to its respective bottle. Sixty milliliters of modified intestinal fluid[1], preheated to 37°, was added, and the bottles were replaced in the bath. After 0.5, 3, 5.5, and 8.5 hr. of additional rotation, the individual bottles were removed, the pellets were collected on 40-mesh screens, and the residual SK&F 70948 was determined as described under *Assay*.

Assay—Residual pellets were placed in a mortar, ground with 0.1 *N* aqueous H_2SO_4, and transferred to a 200-ml. volumetric flask containing about 75 ml. of 0.1 *N* H_2SO_4. The flask was shaken for 0.5 hr. and diluted to volume with the same solvent. The contents were filtered through Whatman No. 1 filter paper, with the first 15 ml. of filtrate being discarded. A 5.0-ml. aliquot of the filtrate was transferred to a 250-ml. separator containing about 25 ml. of 0.1 *N* aqueous H_2SO_4 and extracted with 30 ml. of ethyl ether. The aqueous layer was drained into a 100-ml. volumetric flask. The ether layer was washed with 30 ml. of 0.1 *N* aqueous H_2SO_4, and the latter was drained into the flask. The same solvent was used to dilute to volume. This solution was further diluted to a final concentration of about 0.015 mg. of SK&F 70948/ml. using the same solvent.

The UV absorption spectrum was recorded on a suitable spectrophotometer from 300 to 230 nm. Absorbance at the maximum (about 262 nm.) was measured and substracted from the absorbance at 290 nm. This difference was denoted ΔA. Using a spectrophotometer[2], 1st gear, 1-cm. cells, a concentration of 0.01476 mg. of SK&F 70948 (base)/ml. gave a ΔA of 1 unit.

Anorectic Effect in Dogs—Eleven adult mongrel dogs (six females and five males), weighing 10–17 kg., were preselected from a large colony of dogs that consistently ate food upon presentation and were used in this test. Control feeding studies were conducted, and it was determined that each dog would consume small portions of food (approximately 50 g.) every 30 min. over a 10-hr. period. On the day before the test, the dogs received their normal daily food offering and 2 hr. later the food cups were removed so that no food was available for 18 hr. before drug administration. Water was available *ad libitum*. On the day of the test, the dogs received either: (*a*) 48 mg./kg. of SK&F 70948 in a timed-release blend (25:75), or (*b*) three doses of 16 mg./kg. of crystalline SK&F 70948 3 hr. apart (t.i.d.). The drugs were prepared in gelatin capsules and administered in the first offering of food. The last two doses of the t.i.d. treatment were administered orally, by hand, 3 and 6 hr. after the initial dosing. Food was presented every 30 min. for 9 hr., and each offering was left in the cage for 30 min. If uneaten, the food was removed and a fresh portion was then presented. Anorexia was defined as failure to consume all of the food over two successive 30-min. periods. One week later, the animals received the second drug

Table I—*In Vitro* Release

Formulations Tested *In Vivo*	Percent Drug Released at Specified Time, hr. 0.5	2	4.5	7	10
T/R[a] blend for dog anorexia study (48 mg./kg.)	21	53	69	79	—
75-mg. T/R blend for human study	28	50	75	89	—
150-mg. T/R blend for human study	29	54	70	79	86
300-mg. T/R blend for human study	32	62	74	79	89

[a] T/R stands for timed release.

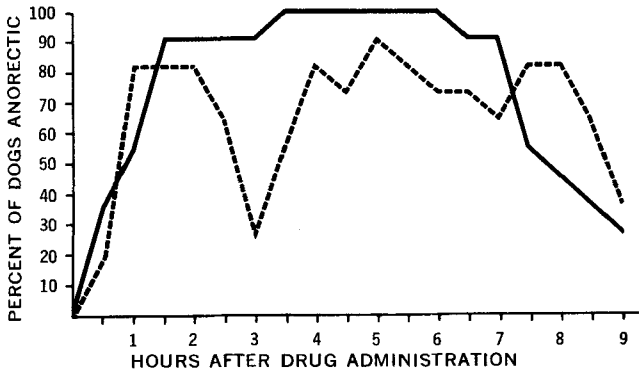


Figure 1—*Anorectic effect in dogs. Key: ——, SK&F 70948 timed-release blend, 48 mg./kg.; and - - -, SK&F 70948, 16 mg./kg. t.i.d. (at 0, 3, and 6 hr.).*

[1] Modified intestinal fluid: Dissolve 10 g. of pancreatin NF in about 700 ml. of pH 6.0 ± 0.1 phosphate buffer, and dilute to 1 l. with the same solvent.

[2] Cary model 11.

Table II—Clinical Results

	Timed-Release Formulations		
	75 mg.	150 mg.	300 mg.
Number of patients	109	112	61
Global efficacy (good and excellent results), %	30	48	50
Anorectic activity (weight loss in 8 weeks), lb.	5.2	7.4	9.3
Side effects (all), %	13	13	27
	T.I.D. Tablet		
	75 mg. (3 × 25)	150 mg. (3 × 50)	
Number of patients	125	128	
Global efficacy (good and excellent results), %	24	44	
Anorectic activity (weight loss in 8 weeks), lb.	3.6	6.1	
Side effects (all), %	28	36	

treatment so that each dog received both drug regimens in a crossover design.

Clinical Studies—In this phase of the development program, two multiinvestigator studies were carried out to measure global efficacy, anorectic activity, and side-effect incidence. These studies compared:

Study 1—75, 150, and 300 mg. of SK&F 70948 (base) in timed-release form and a standard drug.

Study 2—25- and 50-mg. tablets of SK&F 70948 (base) and a standard drug, all presented t.i.d.

Thirteen different investigators participated in each study. All followed parallel protocols where only the presentation frequency differed to accommodate the dosage form.

The patient description in the protocol indicated that patients were to be at least 10% overweight, as determined by actuarial scales, and willing to continue therapy for 8 weeks. In addition, all patients were to be treated with a 1000–2000 cal. diet and be given the same advice concerning fluid and salt restrictions and exercise programs. The timed-release form was to be taken 1 hr. before lunch; the tablets were to be taken midmorning, 2 hr. before the evening meal, and 2 hr. after the evening meal.

Each patient was interviewed and examined at 2-week intervals when the weight, blood pressure, and pulse were recorded, the anorectic effects were noted, and the presence of any side effects was marked on a case report form.

Metabolism, excretion, and biological availability studies also were conducted during this period on the plain drug and the 150-mg. timed-release form (4).

RESULTS AND DISCUSSION

Experience with the *in vitro* release patterns of preliminary formulations suggested a preparation providing 10 hr. of gradual release following a priming dose as a prototype for dog anorectic testing.

Table III—Statistical Analysis for Dose-Response Studies

Within Timed-Release Study
Global efficacy
75 mg. *versus* 150 or 300 mg.—$p < 0.05$
Anorectic activity (weight loss)
75 mg. *versus* 150 mg.—$p < 0.05$
150 mg. *versus* 300 mg.—$p < 0.05$
Side effects
75 or 150 mg. *versus* 300 mg.—$p < 0.05$
Within T.I.D. Study
Global efficacy
75 mg. *versus* 150 mg.—$p < 0.01$
Anorectic activity (weight loss)
75 mg. *versus* 150 mg.—$p < 0.05$
Side effects
75 mg. *versus* 150 mg.—N.S.

Table IV—Statistical Comparison of Clinical Findings

Global efficacy
75 mg. timed release *versus* 25 mg. t.i.d.—N.S.
150 mg. timed release *versus* 50 mg. t.i.d.—N.S.
Anorectic activity
75 mg. timed release *versus* 25 mg. t.i.d.—N.S.
150 mg. timed release *versus* 50 mg. t.i.d.—N.S.
Side effects
75 mg. timed release *versus* 25 mg. t.i.d.—$p < 0.01$
150 mg. timed release *versus* 50 mg. t.i.d.—$p < 0.01$

***In Vitro* Release**—When blends comprising 25% nontimed-release pellets and 75% wax–lipid-coated pellets were tested for *in vitro* release, the results shown in Table I were obtained. These *in vitro* data indicated that the goal had been achieved and that the formulation was suitable for *in vivo* testing.

Anorectic Effect in Dogs—Both the timed-release blend and the crystalline compound administered t.i.d. produced anorexia within 30–60 min. after administration (Fig. 1). With the timed-release blend, anorexia occurred in 91% of the dogs 1.5 hr. postdrug and in 100% of the dogs 3.5 hr. postdrug. The anorectic effect remained at the 91–100% level until 7 hr. after administration (a total of 6 hr.), when the effect began to diminish gradually. In contrast, the first dose of the t.i.d. treatment produced anorexia in 82% of the dogs in the 1st hr., falling to 27% by the 3rd hr. when the second dose was administered. One hour after the second administration (4th hr.), 82% of the dogs were again anorectic. By the 6th hr., when the third dose was administered, 73% of the dogs were still anorectic. No increase in anorectic activity was observed until 1.5 hr. after the third dose, when 82% of the dogs were affected. As with the timed-release blend treatment, the anorexia was markedly diminished by the end of the test (9th hr.). The time course of the anorexia (Fig. 1) indicates that the t.i.d. schedule produced an irregular pattern of anorectic activity while the timed-release blend produced a more sustained and consistent anorectic effect in a greater percentage of dogs. In view of these data, the timed-release preparation appeared to be a more efficient and more effective dosage form, producing more complete and sustained pharmacological effect than the t.i.d. dosage regimen.

After timed-release properties for the 25:75 blend in dogs were demonstrated successfully, the 75-, 150-, and 300-mg. formulations shown in Table I were prepared for human testing. The clinical results obtained with these regimens are summarized in Table II, and pertinent statistical comparisons are summarized in Table III.

Statistical analysis showed that a dose–response relationship did exist within each study, and this finding validated the methodology.

Because the two studies were designed in such a similar way, it was also possible to compare the results obtained in one study with those obtained in the other (Table IV).

Statistical analyses for global efficacy and side-effect incidence used the standard chi-square tests for 2 × 2 contingency tables. Weight loss averages were compared using one-sided Student's *t* tests.

In comparing these findings, it was readily apparent that the global efficacy and anorectic activity of the timed-release and t.i.d. regimens did not differ statistically, but the incidence of side effects seen after the administration of the timed-release form was significantly less than that seen after t.i.d. therapy.

One explanation for the reduced side-effect incidence can be found in the work done by Beckett and Hossie (4). These investigators studied the urinary excretion of SK&F 70948 and its metabolites following the administration of the 150-mg. timed-release form and a 50-mg. t.i.d. regimen. Their data showed that the "staircase" effect and spikes seen after the 4- and 8-hr. doses of the t.i.d. regimen were eliminated by the timed-release formulation, which maintained a steady level of unchanged drug for 16 hr. These findings led to the conclusion that the constant and sustained lower body levels of drug obtained with the timed-release formulation very likely accounted for its lower incidence of side effects.

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(3) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 882.
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New Compounds: Anils

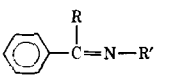
HEINO A. LUTS

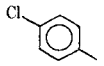
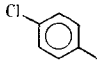
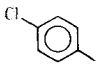
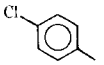
Abstract □ A series of new anils (ketimines) was synthesized by the condensation of a ketone with a desired amine.

Keyphrases □ Anils, potential antianxiety agents—synthesis by condensation of ketone with amines, preliminary screening □ Ketimines, potential antianxiety agents—synthesis, preliminary pharmacological screening □ Antianxiety agents—synthesis of new anils, preliminary screening

In search for new compounds with CNS activity, a series of anils was prepared for pharmacological evaluation (I) (Table I). These compounds were synthesized by the condensation of a ketone with a desired amine according to the procedure described in the literature (1). Preliminary pharmacological studies indicate only insignificant antianxiety properties. The complete results will be published later.

Table I—Anils



Number	R	R′	Formula	Boiling Point	Molecular Weight	n_D^{20}	Yield, %	Analysis, % Calc.	Found
I		—$CH_2CH_2N(CH_3)_2$	$C_{17}H_{19}ClN_2$	180–184°/0.6 mm.	286.78	1.5864	73	C 71.19 H 6.68 Cl 12.37 N 9.77	70.84 6.70 12.77 9.41
II		—$CH_2CH_2N(CH_2CH_3)_2$	$C_{19}H_{23}ClN_2$	160–164°/0.5 mm.	314.83	1.5735	57	C 72.48 H 7.36 Cl 11.26 N 8.89	72.39 7.44 11.50 8.68
III		—$CH_2CH_2CH_2N(CH_3)_2$	$C_{18}H_{21}ClN_2$	182–1840°/0.6 mm.	300.80	1.5790	73	C 71.87 H 7.03 Cl 11.78 N 9.31	71.63 7.03 11.69 9.41
IV		—$CH_2CH_2CH_2N(CH_2CH_3)_2$	$C_{20}H_{25}ClN_2$	164–176°/0.6 mm.	328.86	1.5678	58	C 73.04 H 7.66 Cl 10.77 N 8.51	72.76 7.59 11.01 8.33
V		—$CH_2CH_2N(CH_2CH_3)_2$	$C_{18}H_{23}N_3$	182–183°/0.3 mm.	280.37	1.5655	60	C 77.10 H 7.91 N 14.99	77.03 8.28 14.98
VI		—$CH_2CH_2CH_2N(CH_3)_2$	$C_{17}H_{21}N_3$	168–170°/0.3 mm.	267.35	1.5769	64	C 76.37 H 7.91 N 15.72	76.63 7.68 15.51
VII		—$CH_2CH_2CH_2N(CH_2CH_3)_2$	$C_{19}H_{25}H_3$	176–177°/0.3 mm.	295.41	1.5595	62	C 77.24 H 8.53 N 14.22	76.94 8.40 14.21
VIII		—$CH_2CH_2CH_2N(CH_3)_2$	$C_{16}H_{20}N_2S$	176–182°/0.6 mm.	272.39	1.5855	56	C 71.28 H 7.74 N 9.78	71.42 7.52 9.62
IX		—$CH_2CH_2N(CH_2CH_3)_2$	$C_{17}H_{22}N_2S$	172°/0.4 mm.	286.41	1.5836	55	C 70.54 H 7.40 N 10.28	70.37 7.24 9.98
X		—$CH_2CH_2CH_2N(CH_2CH_3)_2$	$C_{18}H_{24}N_2S$	182°/0.3 mm.	300.34	1.5714	53	C 71.98 H 8.05 N 9.33	71.94 8.22 9.19

(2) E. Rosen, T. Ellison, P. Tannenbaum, S. M. Free, and A. P. Crosley, Jr., *J. Pharm. Sci.*, **56**, 365(1967).
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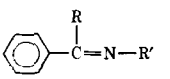
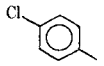
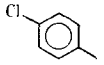
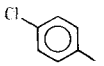
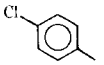
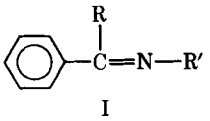


Number	R	R′	Formula	Boiling Point	Molecular Weight	n_D^{20}	Yield, %	Analysis, % Calc.	Found
I		—$CH_2CH_2N(CH_3)_2$	$C_{17}H_{19}ClN_2$	180–184°/0.6 mm.	286.78	1.5864	73	C 71.19 H 6.68 Cl 12.37 N 9.77	70.84 6.70 12.77 9.41
II		—$CH_2CH_2N(CH_2CH_3)_2$	$C_{19}H_{23}ClN_2$	160–164°/0.5 mm.	314.83	1.5735	57	C 72.48 H 7.36 Cl 11.26 N 8.89	72.39 7.44 11.50 8.68
III		—$CH_2CH_2CH_2N(CH_3)_2$	$C_{18}H_{21}ClN_2$	182–1840°/0.6 mm.	300.80	1.5790	73	C 71.87 H 7.03 Cl 11.78 N 9.31	71.63 7.03 11.69 9.41
IV		—$CH_2CH_2CH_2N(CH_2CH_3)_2$	$C_{20}H_{25}ClN_2$	164–176°/0.6 mm.	328.86	1.5678	58	C 73.04 H 7.66 Cl 10.77 N 8.51	72.76 7.59 11.01 8.33
V		—$CH_2CH_2N(CH_2CH_3)_2$	$C_{18}H_{23}N_3$	182–183°/0.3 mm.	280.37	1.5655	60	C 77.10 H 7.91 N 14.99	77.03 8.28 14.98
VI		—$CH_2CH_2CH_2N(CH_3)_2$	$C_{17}H_{21}N_3$	168–170°/0.3 mm.	267.35	1.5769	64	C 76.37 H 7.91 N 15.72	76.63 7.68 15.51
VII		—$CH_2CH_2CH_2N(CH_2CH_3)_2$	$C_{19}H_{25}H_3$	176–177°/0.3 mm.	295.41	1.5595	62	C 77.24 H 8.53 N 14.22	76.94 8.40 14.21
VIII		—$CH_2CH_2CH_2N(CH_3)_2$	$C_{16}H_{20}N_2S$	176–182°/0.6 mm.	272.39	1.5855	56	C 71.28 H 7.74 N 9.78	71.42 7.52 9.62
IX		—$CH_2CH_2N(CH_2CH_3)_2$	$C_{17}H_{22}N_2S$	172°/0.4 mm.	286.41	1.5836	55	C 70.54 H 7.40 N 10.28	70.37 7.24 9.98
X		—$CH_2CH_2CH_2N(CH_2CH_3)_2$	$C_{18}H_{24}N_2S$	182°/0.3 mm.	300.34	1.5714	53	C 71.98 H 8.05 N 9.33	71.94 8.22 9.19



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EXPERIMENTAL[1]

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Keyphrases □ Dimethyl *N,N'*-bis(2-amino-3-oxophenoxazine-4,6-dimethyl-1,9-dicarbonyl)-*dl*-dialaninate—synthesis □ Phenoxazine ring system analogs—synthesis of dimethyl *N,N'*-bis(2-amino-3-oxophenoxazine-4,6-dimethyl-1,9-dicarbonyl)-*dl*-dialaninate

The extreme potency of the antibiotic actinomycin has generated considerable interest in the chemistry and pharmacology of this natural product (1). Actinomycin has been shown to be a complex amide of a substituted phenoxazinedicarboxylic acid and a cyclic pentapeptide. Because of interest in heteroaroylamino acids (2), it seemed desirable to prepare some simple analogs of phenoxazine ring systems (3). Accordingly, dimethyl *N,N'*-bis(2-amino-3-oxophenoxazine-4,6-dimethyl-1,9-dicarbonyl)-*dl*-dialaninate (II) was synthesized by hydrogenation of *N*-(3-benzyloxy-4-methyl-2-nitrobenzoyl)-*dl*-alanine methyl ester (I), followed by oxidation with *p*-benzoquinone.

EXPERIMENTAL[1]

N-(3-Benzyloxy-4-methyl-2-nitrobenzoyl)-*dl*-alanine Methyl Ester (I)—3-Benzyloxy-4-methyl-2-nitrobenzoic acid (4), 5.75 g. (0.02 mole), was suspended in 20 ml. of dry benzene and heated under gentle reflux with thionyl chloride (12 ml.) for 1.5 hr. The resulting solution was concentrated under reduced pressure to remove the excess thionyl chloride, and the residual acid chloride was then redissolved in dry benzene (100 ml.). *dl*-Alanine methyl ester dihydrochloride, 3.52 g. (0.02 mole), was added, and the mixture was heated under reflux for 15 hr.; then any undissolved material was separated from the hot solution. The filtrate was diluted with *n*-hexane. The solids which separated on cooling were recrystallized from 95% ethanol to give 3.74 g. (50%) of *N*-(3-benzyloxy-4-methyl-2-nitrobenzoyl)-*dl*-alanine methyl ester (I), m.p. 148–150°; $\nu_{max.}$ 3380 (NH), 1740 (ester CO), 1660 (amide CO), 1530 and 1370 (NO_2), and 780 and 745 cm.$^{-1}$ (substituted benzene).

Anal.—Calc. for $C_{19}H_{20}N_2O_6$: C, 61.28; H, 5.41. Found: C, 61.44; H, 5.71.

Dimethyl *N,N'*-Bis(2-amino-3-oxophenoxazine-4,6-dimethyl-1,9-dicarbonyl)-*dl*-dialaninate (II)—*N*-(3-Benzyloxy-4-methyl-2-nitrobenzoyl)-*dl*-alanine methyl ester (I), 2.98 g. (0.008 mole), was dissolved in 200 ml. of hot ethyl acetate and hydrogenated over 1 g. of palladium-on-charcoal (5%) at room temperature for 19 hr. The catalyst was removed by filtration, and the colorless filtrate was evaporated in a rotary evaporator. The intermediate *o*-aminophenol was redissolved in absolute alcohol (100 ml.). A solution of 2.59 g. (0.024 mole) of *p*-benzoquinone in 50 ml. of absolute alcohol was added. The mixture, exposed to the atmosphere, was stirred for 1 hr., and the red solid (1.62 g., 80%) was separated by filtration. The analytical sample was obtained from chloroform by means of a soxhlet extractor to give bright-red crystals, m.p. > 260°; $\nu_{max.}$ 3400 and 3250 (NH, NH_2), 1730 and 1700 (ester and quinone CO), 1660 (amide CO), and 740 and 725 cm.$^{-1}$ (substituted benzene); $\lambda_{max.\ (nm.)}^{abs.\ EtOH}$ 238 (log ϵ 4.79), 421 (log ϵ 4.58), and 442 (log ϵ 4.59).

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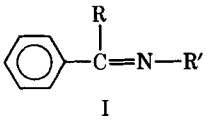

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COMMUNICATIONS

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Keyphrases □ *N*-Methyl-3-benzoyl-3-hydroxypiperidine—novel behavior, Von Braun demethylation □ *N*-Methylethanolamines, tertiary—Von Braun demethylation, novel reaction with cyanogen bromide □ Von Braun demethylation—novel reaction of tertiary *N*-methylethanolamines with cyanogen bromide

Sir:

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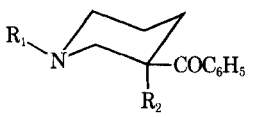
Under the usual reaction conditions, V (8 g.) afforded a benzene-insoluble, water-soluble plastic mass. The IR spectrum exhibited bands at 2218 (CN) and 1675 cm.$^{-1}$ (C=O, ketone) as well as a band at 3470 cm.$^{-1}$, characteristic of but weaker than the expected absorption for the OH group of V. The acid-washed, benzene-soluble fraction of the reaction mixture (<1 g.) exhibited bands at 2218 (CN) and 1680 cm.$^{-1}$ (C=O, ketone) but no band in the 3500-cm.$^{-1}$ region. This oil exhibited a positive Beilstein test for halogen. Following hydrolysis in concentrated HCl, water and exces acid were removed *in vacuo*. The residue was dissolved in water. The solution was washed with chloroform, alkalized with sodium carbonate, and extracted again with chloroform. Evaporation of the extract afforded



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II: $R_1 = CH_3$, $R_2 = O_2CCH_3$
III: $R_1 = CH_3$, $R_2 = OCH_3$
V: $R_1 = CH_3$, $R_2 = OH$
VI: $R_1 = CN$, $R_2 = OH$
XII: $R_1 = H$, $R_2 = OH$
XIII: $R_1 = CH_3$, $R_2 = OCN$
XIV: $R_1 = CH_3$, $R_2 = Br$
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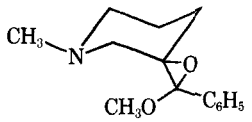
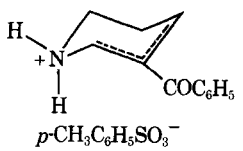
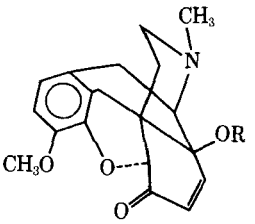


IV



p-$CH_3C_6H_5SO_3^-$

XI



VII: R = $OCCH_3$
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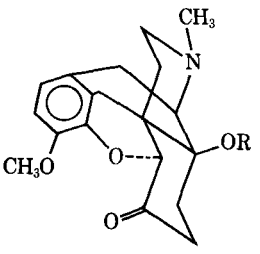

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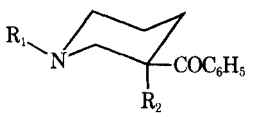

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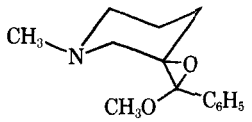
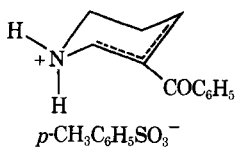
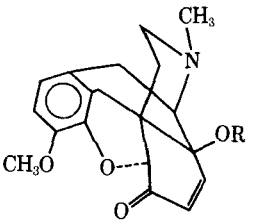


IV

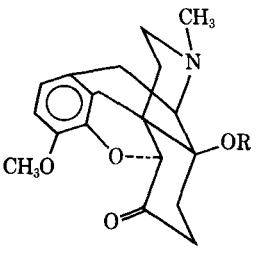


p-$CH_3C_6H_5SO_3^-$

XI



VII: R = $OCCH_3$
IX: R = H



VIII: R = $OCCH_3$
X: R = H

<0.5 g. of oil, which was dissolved in 20 ml. of ethanol and treated with a solution of 1.0 g. of *p*-toluenesulfonic acid in 10 ml. of ethanol. The resulting salt (<70 mg.) was either 4,5,6,1- or 1,2,5,6-tetrahydro-3-benzoylpiperidinium *p*-toluenesulfonate (XI), m.p. 105–106°; IR (mineral oil) 1685 cm.$^{-1}$ (C=O, $\alpha,\beta,\alpha',\beta'$-unsaturated ketone) and no band in the 3500-cm.$^{-1}$ region.

Anal.—Calc. for $C_{19}H_{21}NO_4S$: C, 63.49; H, 5.89; N, 3.90; S, 8.92. Found: C, 63.9; H, 6.62; N, 3.72; S, 8.82.

While acid may conceivably have been present in trace amounts in the benzene fraction of the reaction mixture, formation of XI from VI by acid catalysis is contrary to all chemical precedent and to all experience with Compounds V, VI, and XII (2, 3, 11–15). This, as well as the fact that the neutral, benzene-soluble oil contains bromine which must be organically bound, clearly requires functionalization of the C_3—OH group of V. Thus, in the presence of CNBr, formation of alkyl cyanate (XIII) must occur. Subsequent considerations clearly implicate this as the first step. Formation of the α-bromo ketone (XIV) from the α-cyanato ketone (XIII) is consistent with both the chemistry of alkyl cyanates (8–10) and the well-known conversion of I to V under *quasi*-Favorskii conditions. The latter reaction is now known to proceed (with retention of configuration) through internally assisted ion pairs (3, 11–15). There is no precedent to support participation of the corresponding cyanamide moiety of XV in cyanate–bromide exchange. In fact, quite the reverse is true (13, 15). This tends to rule out XV as a precursor to XVI and suggests that cyanate formation is the first step.

Predilection of CNBr for the hydroxyl group rather than the amine group in V is explicable on the basis of the following facts. The carbonyl (benzoyl) group has an acid-strengthening effect on the hydroxyl group (3, 4). The hydroxyl group, undoubtedly as a function of its (axial) stereochemistry, is totally hydrogen bonded; as a result, the nitrogen bears significant positive character (3, 4, *cf.*, 5). This rationale augurs well for the absence of measurable quantities of VI in the benzene-soluble fraction and requires that XIV is the precursor of XVI, the principal component of the benzene-soluble fraction. While olefin might have been generated at the levels of XIII–XVI, it may also have been formed from XVII during the workup.

In view of the fact that only one characterizable compound could be isolated from the reaction mixture, and in view of the interesting chemical and stereochemical aspects of this reaction, further investigations are now in progress using optically pure V.

(1) J. Von Braun, *Ber.*, **49**, 2624(1916).
(2) T. B. Zalucky, L. Malspeis, H. Patel, and G. Hite, *J. Pharm. Sci.*, **54**, 687(1965).
(3) E. E. Smissman and G. Hite, *J. Amer. Chem. Soc.*, **82**, 3375(1960).
(4) G. Hite, E. E. Smissman, and R. West, *ibid.*, **82**, 1207(1960).
(5) T. B. Zalucky and G. Hite, *J. Med. Chem.*, **3**, 615(1961).
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(8) K. A. Jensen and A. Holm, *Acta Chem. Scand.*, **18**, 826 (1964).
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(11) T. B. Zalucky, L. Malspeis, and G. Hite, *J. Org. Chem.*, **29**, 3143(1964).
(12) T. B. Zalucky, S. Marathe, L. Malspeis, and G. Hite, *ibid.*, **30**, 1324(1965).
(13) H. Patel and G. Hite, *ibid.*, **30**, 4337(1965).
(14) H. Patel and G. Hite, *J. Pharm. Sci.*, **56**, 1189(1967).
(15) H. Patel and G. Hite, *J. Org. Chem.*, **30**, 4336(1965).

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Efficient Deoxygenation of Water by Gas Permeation

Keyphrases ☐ Oxygen levels in water—technique for reduction, gas permeation ☐ Deoxygenation of water—by gas permeation ☐ Gas permeation—utilized for reducing oxygen levels in water ☐ Water—deoxygenation by gas permeation

Sir:

The oxidative processes which drug moieties undergo involve dissolved molecular oxygen, which is assumed to be a free radical propagated route (1, 2). Schou (3) noted that dissolved oxygen levels must reach a critical concentration before the chain reactions are initiated. In a consideration of the kinetic implications of oxidative degradation, it would appear worthwhile to investigate the possibility of developing a simple, rapid, and economical procedure to deoxygenate water used in the preparation of liquid pharmaceutical systems. Therefore, we recently made preliminary studies in the deoxygenation of solvent water utilizing a gas permeator[1] equipped with dacron fibers.

The operation of gas permeators is based on the principle of selective separation using the membrane walls of hollow fibers. The selectivity of the membrane permits removal of a particular component in the system, depending upon the type of fiber employed; the separation follows Fick's law of diffusion. The selection of the proper membrane fiber is based on solubility considerations, because the component to be removed should be more soluble than the water in the membrane.

Cole and Genetelli (4) recently demonstrated that laboratory models of gas permeators are efficient and prac-

[1] Permasep, DuPont.

<0.5 g. of oil, which was dissolved in 20 ml. of ethanol and treated with a solution of 1.0 g. of *p*-toluenesulfonic acid in 10 ml. of ethanol. The resulting salt (<70 mg.) was either 4,5,6,1- or 1,2,5,6-tetrahydro-3-benzoylpiperidinium *p*-toluenesulfonate (XI), m.p. 105–106°; IR (mineral oil) 1685 cm.$^{-1}$ (C=O, $\alpha,\beta,\alpha',\beta'$-unsaturated ketone) and no band in the 3500-cm.$^{-1}$ region.

Anal.—Calc. for $C_{19}H_{21}NO_4S$: C, 63.49; H, 5.89; N, 3.90; S, 8.92. Found: C, 63.9; H, 6.62; N, 3.72; S, 8.82.

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Efficient Deoxygenation of Water by Gas Permeation

Keyphrases ☐ Oxygen levels in water—technique for reduction, gas permeation ☐ Deoxygenation of water—by gas permeation ☐ Gas permeation—utilized for reducing oxygen levels in water ☐ Water—deoxygenation by gas permeation

Sir:

The oxidative processes which drug moieties undergo involve dissolved molecular oxygen, which is assumed to be a free radical propagated route (1, 2). Schou (3) noted that dissolved oxygen levels must reach a critical concentration before the chain reactions are initiated. In a consideration of the kinetic implications of oxidative degradation, it would appear worthwhile to investigate the possibility of developing a simple, rapid, and economical procedure to deoxygenate water used in the preparation of liquid pharmaceutical systems. Therefore, we recently made preliminary studies in the deoxygenation of solvent water utilizing a gas permeator[1] equipped with dacron fibers.

The operation of gas permeators is based on the principle of selective separation using the membrane walls of hollow fibers. The selectivity of the membrane permits removal of a particular component in the system, depending upon the type of fiber employed; the separation follows Fick's law of diffusion. The selection of the proper membrane fiber is based on solubility considerations, because the component to be removed should be more soluble than the water in the membrane.

Cole and Genetelli (4) recently demonstrated that laboratory models of gas permeators are efficient and prac-

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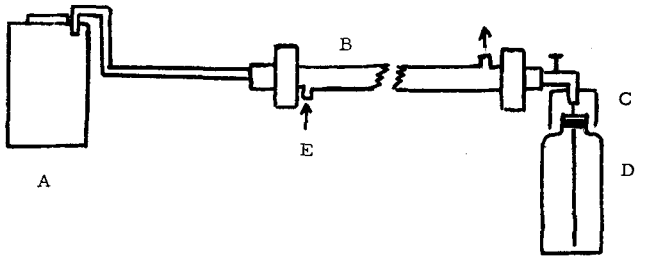

Figure 1—*Gas permeator system. Key: A, pressure tank; B, permeator; C, nitrogen hood; D, collection tank; and E, nitrogen flush.*

tical alternatives to air blowing as a method for reducing dissolved gas levels in waste water. This technique is especially applicable to removal of pollutants in waste water, since the concentrations of gases is relatively low and the pollutant is transferred across the membrane at a higher molar concentration.

The system is simple in both design and construction. It consists of a large number of hollow fibers of suitable composition (dacron, nylon, polypropylene, *etc.*) enclosed in a hollow, noncontaminating metal tube. The water to be treated is forced through the fibers; the dissolved gases permeate the fiber walls and are carried off by a vacuum or nitrogen flush. The overall flux required is small since the concentration of permeant is low. The only pressures involved are those required to compensate for hydraulic losses in the separator.

Our apparatus was slightly modified from that of Cole and Genetelli (4), since we were working with a standard-size permeator whose technical data are shown here:

permeator number	015-70-0264
serial number	DP-168
shell inner diameter	3.81 cm. (1.50 in.)
shell pressure rating	1500 psig.
fibers	Dacron
number of tubes	741,600
tube outside diameter	32.8 μ
tube inner diameter	16.5 μ
tube percent hollow	25.46%
number of leaking tubes	38
number of leaking tubes, %	0.005%

Deionized water in a pressure tank was forced through the permeator under 100 lb. of pressure using nitrogen gas. A nitrogen flush was employed to remove the permeant from around the fiber bundle. The deaerated solvent water was collected at a rate of 30 ml./min. under a nitrogen hood to prevent air contamination. After consideration of solubility characteristics of available fibers, a dacron fiber was utilized in the system; it was felt that this membrane would be most efficient in removal of dissolved oxygen. A basic diagram of the system is shown in Fig. 1.

Table I—Effect of Gas Permeation upon Dissolved Oxygen Levels and Resulting pH

Pass Number	Dissolved Oxygen, p.p.m. at 25°	pH
0 (oxygenated, normal conditions)	8.37	6.12
1	0.70	6.80
2	0.12	7.30

The efficiency of the gas permeator in reducing dissolved oxygen levels and the effect of multiple passes through the system were investigated by measuring dissolved oxygen levels at 25° with a dissolved oxygen analyzer[2] having a sensitivity of 0.01 p.p.m. O_2.

As seen in Table I, dissolved oxygen levels were reduced greater than 90% with a single pass through the permeator and were reduced more than 98% with a double pass. Decarbonation of the deionized water occurred simultaneously with deoxygenation, as evidenced by a change in the pH with each pass through the permeator. There may be some leaching of alkali from the column or impurities in the nitrogen gas, since the pH 7.30 is higher than expected.

This technique appears to be an efficient method of reducing dissolved oxygen levels in water. Its applicability for the protection of oxygen-sensitive pharmaceuticals will be reported in a future communication.

(1) L. Lachman, *Drug Cosmet. Ind.*, **102**, 36(1968).
(2) *Ibid.*, **102**, 146(1968).
(3) S. A. Schou, *Amer. J. Hosp. Pharm.*, **17**, 153(1960).
(4) C. A. Cole and E. S. Genetelli, *Environ. Sci. Technol.*, **4**, 514(1970).

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[2] Delta Scientific model 106.

REVIEWS

Molecular Properties of Drug Receptors. A Ciba Foundation Symposium. Edited by R. PORTER and M. O'CONNOR, J. and A. Churchill, London, England, 1970. ix + 298 pp., 15 × 23.5 cm.

This recent volume in the Ciba Symposia Series carries on in the tradition established by other volumes in this series: that is to say, it is excellently presented and makes extremely stimulating reading.

The book is divided into four major segments. The first, which has to do with pharmacological properties of receptors, contains chapters by W. D. M. Paton, S. Thesleff, S. E. Mayer, and P. G. Waser. All of these names are well known to people who have been involved in the area of autonomic receptors, and the papers presented are excellent summaries of recent work in that field. The next section deals primarily with the structure of active sites, and contains a review of crystallographic studies on the active site of lysozyme by Beddell, Moult, and Phillips, a paper on the nuclear magnetic resonance spectrum of protein binding sites by Jardetzky, and a paper on fluorescent probes of biological macromolecules by Stryer. This is followed by a section involving conformational changes in proteins which includes a paper by J. Wyman on the interaction of the subunits of hemoglobin and a paper on conformational transitions in membrane excitation by a group led by J. P. Changeux. The final section deals primarily with attempts at receptor isolation and includes a paper by Singer on affinity labeling, a paper by a group led by A. Karlin on acetylcholine receptors in electroplax, and, finally, a paper by A. S. V. Burgen on the nature of complex formation. Each paper is followed by a short discussion and there is a general discussion after each section.

As is generally the case in Ciba Symposia volumes, the papers are of uniformly high quality and present an excellent overview of recent work being done in the laboratories of the scientists involved. Any given paper will contain much that is familiar to workers in that particular field. However, the major advantage to a symposium of this type is the bringing together of a number of scientists working in closely related fields, and the stimulation provided by the interchange of ideas which takes place. The Chairman of this meeting, Dr. F. H. C. Crick, has done a masterful job in bridging the disciplines involved, and as he states in his opening remarks, "... people here come from very different disciplines. I would, therefore, appeal to people on both the pharmacological side and the protein side not just to talk to the people who already know the field, but to the opposite side. My only usefulness as Chairman is that I have got to the stage where I hardly know about either side. Consequently, up to a point, I can ask the silly questions, but I hope that everyone else will do so, too, if a speaker is using jargon about his own subject."

The discussions form the high point of the book for me and provide real insight into the thinking of some of the leaders of research in the drug receptor field. To this end the book is valuable not only as a reference for scientists interested in this area, but as an example of the intellectual process involved in research. As Sir Francis states in his final summary, "I seem to have summed up at intervals, so I won't make a grand summing up now. I have certainly been left with the impression that although there is a lot of hard work ahead, there are also very real grounds for optimism in many directions. There are many things which can be tried. I think many people will go on trying the things they were doing before, but perhaps they will do it in a wider context. One or two new ideas have come up which should help. Although before the meeting I knew to some extent the sort of problems involved, I can see them much more clearly now." This final sentence also expresses the opinion of this reviewer and I am sure will characterize the response of all readers of this volume. I recommend it highly.

Reviewed by Jules B. LaPidus
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NOTICES

A Documented Cross-Indexed Manual to Human Drug Interactions (Second Edition). By N. J. SAWYER, C. M. KING, and B. A. HELLUMS. University of Alabama, Birmingham, AL 35233, 1971. 133 pp. 21.5 × 28 cm. Price $5.00.

Antimicrobial Agents and Chemotherapy—1970. Edited by G. L. HOBBY. American Society for Microbiology, Bethesda, Md., 1971. xi + 545 pp. 18 × 26 cm. Price $15.00.

Flame Emission and Atomic Absorption Spectrometry. (Volume 2, Components and Techniques). Edited by J. A. DEAN and T. C. RAINS. Marcel Dekker, New York, NY 10016, 1971. xiv + 362 pp. 16 × 23.5 cm. Price $22.75.

6-Hydroxydopamine and Catecholamine Neurons. Edited by T. MALMFORS and H. THOENEN. American Elsevier, New York, NY 10017, 1971. 368 pp. 16 × 23.5 cm. Price $22.50.

Lidocaine in the Treatment of Ventricular Arrhythmias. Edited by D. B. SCOTT and D. G. JULIAN. Williams & Wilkins, Baltimore, MD 21202, 1971. xiii + 241 pp. 14.5 × 22.5 cm. Price $11.50.

British Pharmaceutical Codex 1968—Supplement 1971. Prepared in the Department of Pharmaceutical Sciences, Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, England, 1971. xxx + 144 pp. 14.5 × 23 cm. Price *f* 4-20.

Index Nominum 1970/71. Edited and published by La Societe Suisse de Pharmacie, Zurich, Switzerland, 1970. xxvi + 1159 pp. 15.5 × 22.5 cm.

Antibiotic and Chemotherapy. Third Edition. By L. P. GARROD and F. O'GRADY. Williams & Wilkins, Baltimore, MD 21202, 1971. ix + 499 pp. 14 × 22 cm. Price $16.25.

Pesticides in the Environment (Volume 1, Part I). Edited by R. WHITE-STEVENS. Marcel Dekker, New York, NY 10016, 1971. xv + 270 pp. 15.5 × 23.5 cm. Price $23.50.

ERRATA

In the article titled "Use of the Derjaguin, Landau, Verwey, and Overbeek Theory to Interpret Pharmaceutical Suspension Stability" (1), the following corrections should be made:

On page 523, the results for the sedimentation heights, H_u/H_o, %, for the sulfamerazine series in Fig. 3 were wrongly plotted. The correct figure appears below:

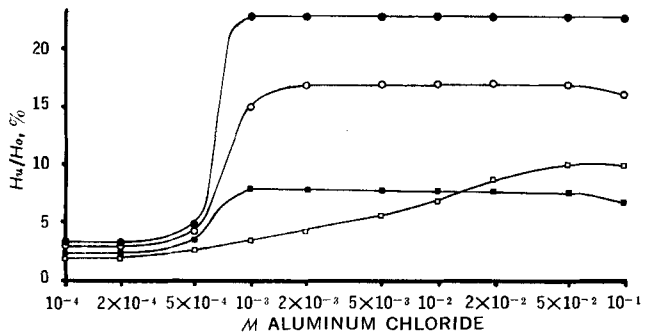

Figure 3—*Coagulation of several drugs in 10^{-3} M ammonium dioxyethylated dodecyl sulfate by aluminum chloride at pH 2.0. Key:* ■, *sulfamerazine;* □, *griseofulvin, coarse;* ○, *griseofulvin, fine; and* ●, *hydrocortisone.*

On page 523, Table II, the first two zeta potential values would be quoted more realistically to the nearest 0.5 mv., *i.e.*, −46.5 and −32.5 mv., in view of the relatively low precision of electrophoresis measurements.

On page 523, column 2, line 24 should read "ADDS" instead of "SDDS."

On page 524, Table III, the second figure in the column headed Suspension and Zeta Potential, mv., should be 32.7^2 instead of 3.27^2. A horizontal line should be drawn across Table III after the first set of values for zeta potential 4.5^4. The results below this line refer to a Hamaker constant, A, of 5×10^{-13}. The first two zeta potential values in Table III would be quoted more realistically to the nearest 0.5 mv., *i.e.*, −46.5 and −32.5 mv., in view of the relatively low precision of electrophoresis measurements.

On page 525, Table IV should appear as shown below:

Table IV—Rheological Examination of Griseofulvin Suspensions

Suspension	Shear Rate, sec.$^{-1}$	Viscosity, cps.
1	2620	2.7
	1310	2.7
	873	2.8
	436	2.6
2	2620	2.7
	1310	2.9
	873	2.8
	436	2.8
3	2620	4.0
	1310	4.7
	873	4.9
	436	7.0
4	2620	5.0
	1310	6.0
	873	6.9
	436	9.6

On page 525, Reference 3, the volume number should be 20 and the page number should be 204S.

(1) B. A. Matthews and C. T. Rhodes, *J. Pharm. Sci.*, **59,** 521 (1970).

In the article titled "Kinetics of the Decomposition of a Mannich Base" (1), the following correction should be made:

On page 1774, column 1, the fourth line should read "10^5 M^{-1} min.$^{-1}$" instead of "10^{-5} M^{-1} min.$^{-1}$."

(1) J. A. Mollica, J. B. Smith, I. M. Nunes, and H. K. Govan, *J. Pharm. Sci.*, **59,** 1770(1970).

In the article titled "Synthesis and Anticonvulsant Activity of Substituted 2-Thioquinazolin-4-ones I: Preliminary Studies" (1), the following corrections should be made:

On page 129, the first column of Table III should be changed from the published values of 1–8 to read as follows:

Compound Number (Table-Number)
I-1
I-2
I-3
II-1
I-4
I-5
II-2
II-3
Diphenylhydantoin
Phenobarbital
Normal saline

On page 129, the first column of Table IV should be changed from the published values of 1–8 to read as follows:

Compound Number (Table-Number)
I-1
I-2
I-3
II-1
I-4
I-5
II-2
II-3
Trimethadione
Normal saline

(1) A. C. Glasser, L. Diamond, and G. Combs, *J. Pharm. Sci.*, **60,** 127(1971).

In the article titled "Solubility Parameter of Selected Sulfonamides" (1), the following corrections should be made:

On page 239, column 2, the 34th line should read "$(1–1.8 \times 10_t^{-4})$ (11), where t" instead of "$(1–1.8 \times 10^{-4})$ (11), where it."

On page 240, column 1, Eq. 1 should read:

$$\delta = \left(\frac{H_v - RT}{V^1}\right)^{1/2}$$

(1) C. Sunwoo and H. Eisen, *J. Pharm. Sci.*, **60,** 238(1971).

In the article titled "Serum and Urine Levels of Ethchlorvynol in Man" (1), the following correction should be made:

On page 262, column 1, the exponential in the last term of Eq. 1 should be e^{-kt} instead of $e^{-\beta t}$.

(1) L. M. Cummins, Y. C. Martin, and E. E. Scherfling, *J. Pharm. Sci.*, **60,** 261(1971).

In the article titled "Determination of Therapeutic Blood Levels of Methamphetamine and Pentobarbital by GC" (1), the following correction should be made:

On page 1495, Fig. 7, the y-axis should read: 0.15, 0.30, 0.45, 0.60.

(1) R. C. Driscoll, F. S. Barr, B. J. Gragg, and G. W. Moore, *J. Pharm. Sci.*, **60,** 1492(1971).